HIV-1 INTEGRASE INHIBITOR MUTATIONS: ANALYSIS OF STRUCTURAL AND BIOCHEMICAL EFFECTS

BY

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Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy in Virology

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Preface

The experimental work described in this thesis was carried out in the following laboratories:

- The Hasso Plattner Research Laboratory at the HIV Pathogenesis Program, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, from February 2018 to October 2020 under the supervision of Prof Michelle Gordon.
- The Medical Biochemistry Laboratory, George Campbell Building (South entrance), Howard College Campus, Durban, from November 2019 to October 2020 under the supervision of Dr. Rene Khan.

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 tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

Signed

[redacted] 04 June 2021

N Mbhele (student) Date

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Prof M.L. Gordon (supervisor) Date

Signed

[redacted] 23 June 2021

Dr. R Khan (co-supervisor) Date

Declaration

I Nokuzola Mbhele declare that:

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2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other scientists' data, graphs, pictures, or other information unless specifically acknowledged as being sourced from other scientists'.

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Publication

Nokuzola Mbhele, Benjamin Chimukangara and Michelle Gordon (2021). HIV-1 integrase strand transfer inhibitors: A review of current drugs, recent advances and drug resistance. *International Journal of Antimicrobial Agents* DOI: 10.1016/j.ijantimicag.2021.106343

Author contributions: Prof Gordon and I conceived the review article idea. I wrote the manuscript and Dr Chimukangara contributed to the writing of the manuscript. Prof Gordon encouraged and supervised the writing. All authors contributed to the final manuscript.

Nokuzola Mbhele and Michelle Gordon. Structural effects of HIV-1 subtype C integrase mutations on the activity of integrase strand transfer inhibitors in South African patients. *Journal of Biomolecular Structure & Dynamics* DOI: 10.1080/07391102.2021.1972840

Author contributions: Prof Gordon devised the project and supervised it throughout. I planned and performed the experiments and analysed the data. I wrote the manuscript in consultation with Prof Gordon

Manuscript to be submitted

Nokuzola Mbhele, Rene Khan, and Michelle Gordon. Effect of HIV-1 subtype C integrase inhibitor mutations on the viral enzymatic activity.

Authors contributions: Prof Gordon devised the main conceptual idea. Dr Khan and I verified the methods. I carried out the experiments, overlooked by Dr Khan. I wrote the manuscript and Prof Gordon and Dr Khan provided critical feedback and helped shape the analysis and manuscript.

Dedication

To my wonderful family: o-Mbhele, o-Godide, o-Sompisi abaphemba ngamakhandamadoda abanye bephemba ngomlilo.

My dearest granny no-Mashimane abakhulu.

''Now to him who is able to do far more abundantly than all that we ask or think,

according to the power at work within us,

to him be glory in the church and in Christ Jesus throughout all generations,

forever and ever. Amen."

Ephesians 3: 20-21

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Abbreviations and Acronyms

3D	Three- dimensional
3TC	Lamivudine
5-CITEP	1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone
ABC	Abacavir
AMBER	Assisted model building with energy refinement
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
ART	Antiretroviral therapy
ATV	Atazanavir
ATLAS	Antiretroviral Therapy a*s* Long-Acting Suppression
AZT	Zidovudine
BREC	Biomedical Research Ethics *Co*mmi*ttee*
BSA	Bovine serum albumin
cART	Combination antiretroviral therapy
CCD	Catalytic core domain
CCR5	C-C chemokine receptor type five
CD4+	Cluster of differentiation 4
CDC	Center for Disease Control
copies/mL	Copies per millilitre
cPPT	Polypurine tract
CPU	Central processing
CTD	C-terminal domain
CyP A	Cyclophilin A
CRF	Circulating recombinant form
CXCR4	C-X-C chemokine type four receptor
DNA	Deoxyribonucleic acid
DKA	Diketo acid
dNTP	Deoxynucleoside triphosphate
DRC	Democratic Republic of Congo
DRV	Darunavir
DTG	Dolutegravir
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid

E_{ele}	Electrostatic
EFV	Efavirenz
ENV	Envelope
ESCRT	Endosomal sorting complexes required for transport
ETR	Etravirine
FDA	Food and drug administration
FLAIR	First Long-Acting Injectable Regimen
FTC	Emtricitabine
GAAF	Generalized Atomic Forcefield
gp	Glycoprotein
GMQE	Global model quality estimation
GPU	Graphic processing unit
HIV	Human immunodeficiency virus
HIV-1C	HIV-1 subtype C
INSTIs	Integrase strand transfer inhibitors
LA-CAB	Long-acting Cabotegravir
LEDGF/p75	Lens epithelium derived growth factor
LMICs	Low-and- middle-income countries
LPV	Lopinavir
LTR	Long terminal repeat
mg	Milligram
MHCII	Class II major histocompatibility complex
MM-GBSA	Molecular mechanics-generalized born surface area
MM/PBSA	Molecular Mechanics-Poisson Boltzmann surface area
$MnCl_2$	Manganese (II) chloride
MuT	Mutant
NaCl	Sodium chloride
Nef	Negative regulatory factor
NLS	Nuclear localization signal
NVE	Microcanonical ensemble
NPT	Isothermal-Isobaric ensemble
nm	Nanometer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors

NTD	N-terminal domain
NVT	Canonical ensemble
NVP	Nevirapine
nM	Nanomolar
ns	Nanosecond
p450 CYP3A	Cytochrome P450 3A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein databank
PI	Protease inhibitor
PIC	Pre-integration complexes
Pin1	Peptidyl-prolyl isomerases
Pol	Polymerase
PR	Protease
P-TEDb	Positive transcriptional elongation factor b
QMEAN	Qualitative model energy analysis
RAL	Raltegravir
Rev	Regulator of viral expression
RIN	Residue interaction network
RMSF	Root mean square fluctuation
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RPM	Revolutions per minute
RPV	Rilpivirine
RT	Reverse transcriptase
RTC	Reverse transcription complex
SASA	Solvent accessible surface area
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
Tat	Transactivating protein
TAMs	Thymidine analogue mutations
TAR	Transactivation response region

TDF	Tenofovir disoproxil fumarate
tRNA	Transfer ribonucleic acid
URFs	Unique recombinant forms
UGT1A1	UDP-glucuronosyltransferase 1A1
UNAIDS	The Joint United Nations Programme on HIV/AIDS
WT	Wild-Type
Vif	Viral infectivity factor
VL	Viral load
Vpr	Viral Protein R
Vpu	Viral Protein U
WHO	World Health Organization

Symbols

α	Alpha
β	Beta
Å	Angstrom
Cα	Alpha carbon
Cmax	Maximum plasma concentration
K_d	Dissociation constant
Kcal	Kilocalories
Kcal/ mol	Kilocalorie per mole
kDa	Kilo Daltons
IC50	Half-maximal inhibitory concentration
IC90	Ninety-percent maximal inhibitory concentration
µL	Microlitres

Abstract

Introduction

Combination antiretroviral therapy (cART), composed of drugs from different drug classes, is an effective HIV-1 treatment strategy. As part of cART, integrase strand transfer inhibitors (INSTIs) have become essential drugs and are now recommended for use in first-line, second-line, and subsequent HIV-1 treatment regimens. Though highly potent, the use of first-generation INSTIs Raltegravir and Elvitegravir still resulted in the development of integrase drug resistance mutations. Second-generation INSTIs Dolutegravir, Bictegravir, and Cabotegravir were developed to combat the emerging resistant virus strains to first-generation INSTIs and are considered some of the best antiretroviral drugs in HIV-1 treatment. Despite the fundamental changes and improved performance in second-generation INSTIs, they are not immune to drug resistance. This highlights the need to understand the molecular mechanisms of resistance to INSTIs. This thesis, through a combination of structural and biochemical methods, seeks to understand resistance development in South African HIV-1 subtype C (HIV-1C) viruses and the effect of resistance mutations on enzyme-substrate binding, DNA binding, and 3' processing.

Methods

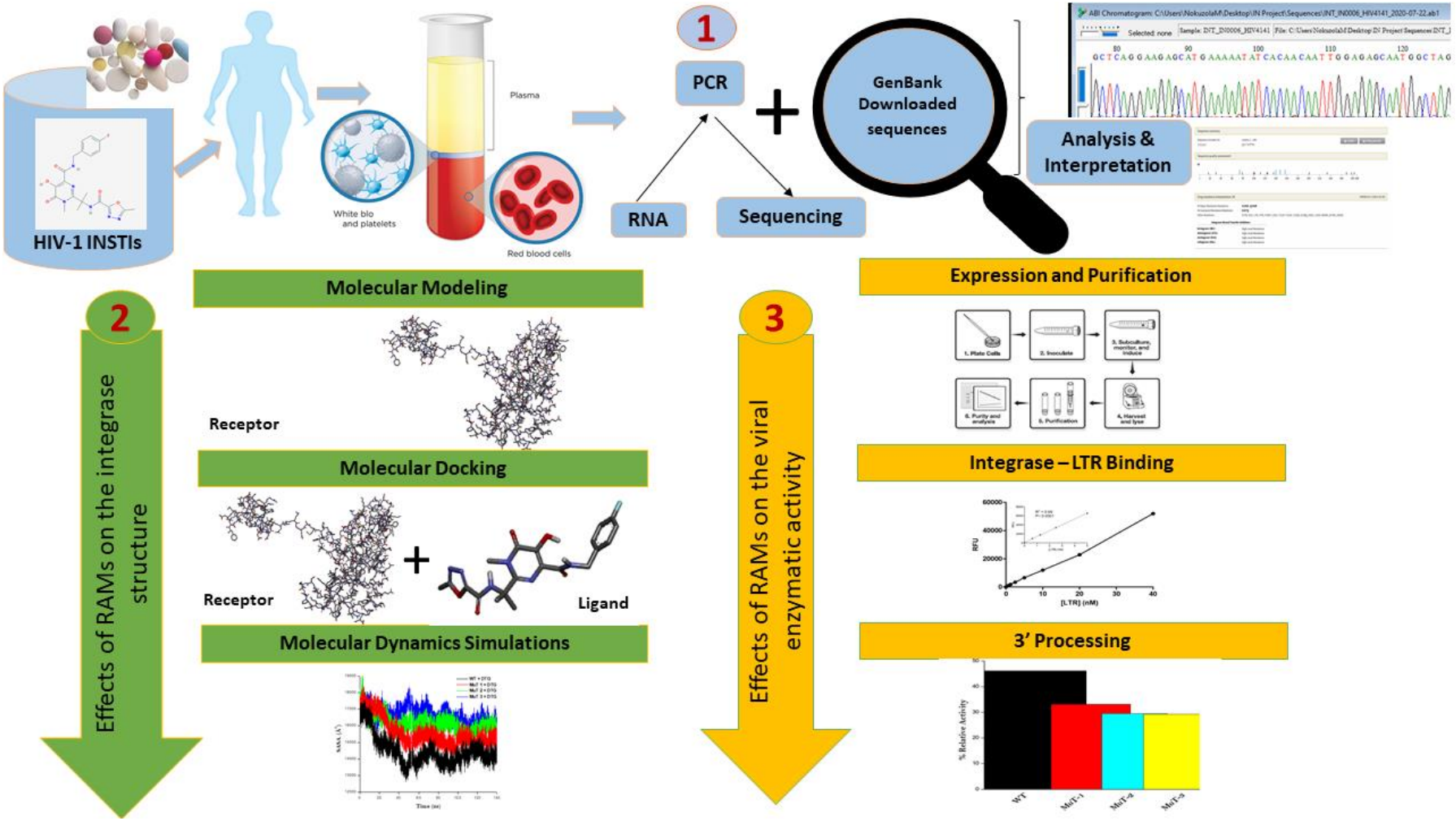
A total of 48 HIV-1C sequences were analyzed in this study, of which 7 had a virologic failure (i.e. plasma viral loads >1000 copies/mL) and 41 were INSTI naïve isolates (32 treatment-naïve South African HIV-1C integrase sequences downloaded from GenBank and 9 INSTI-naïve isolates amplified in our laboratory). Virologic failures were receiving at least 6 months of INSTI-based cART and presented at the King Edward VIII hospital, a 3rd line regimen referral hospital in Durban, South Africa. Viral RNA was extracted, and the integrase region was amplified and sequenced using Sanger sequencing. To investigate the effect of mutations on the integrase structure, wild-type and representative mutant isolates were modeled on the SWISS model online server and visualized in Chimera v1.13.1. Raltegravir, Elvitegravir, and Dolutegravir were docked into each of the structures using the AutoDock-Vina Plugin available on Chimera, and molecular dynamics simulations were conducted using the AMBER 18 package. Integrase biochemical assays were carried out using a wild-type protein and the 3 mutant recombinant proteins that were expressed and purified. Integrase - LTR binding and 3' processing assays were then performed.

Results

Only one of the 7 (14,28%) INSTI-treated isolates had major mutations (i.e., G140A and Q148R). In addition, this isolate harboured the E157Q minor mutation and previously identified polymorphisms. Interestingly, S119T & V151I, located near the integrase active site, were only found in INSTI failures. Structural analysis results showed a reduced binding affinity for the mutants, which was supported by their weaker hydrogen-bond interaction compared to the wild-type. Our findings showed that the G140A+Q148R double mutant had the strongest effect on the HIV-1C protein structure and binding of EVG and RAL with binding free energies of -12.49 and -11.45 kcal/mol for EVG and RAL, respectively, which are approximately three times lower than the wild-type binding energy. Biochemical assays performed with purified integrase showed a decrease in integrase-LTR binding for all mutants. The 3' processing activity was slightly decreased in the mutants compared to the wild-type protein; however, no appreciable differences were observed across the mutant isolates.

Conclusions

Changes near the highly conserved active site residues in HIV-1C integrase core domain and mutations in the 140's loop have a negative effect on *in vitro* integrase activity, suggesting that these changes impact viral integration. While they are still few reports of INSTI resistance-associated mutations (RAMs) in South Africa , identification of the G140A+Q148R double mutant for the first time in South African HIV-1 clinical samples, and the identification of S119T and V151I in INSTI-treated patients warrants further investigation. This data broadens the understanding of HIV-1C resistance against INSTIs and adds to the available knowledge of drug resistance mutations that guide therapeutic decisions.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CHAPTER 1

INTRODUCTION

“The discovery of HIV and the proof that it was the cause of AIDS were the first major scientific breakthroughs that provided a specific target for blood-screening tests and opened the doorway to the development of antiretroviral medications. “- Anthony Fauci

1. Introduction

1.1 Origin

The origin of the human immunodeficiency virus (HIV) is highly controversial with diverging and converging theories (Hirsch et al., 1989, Hooper, 1999, Gao et al., 1999, Korber et al., 2000, Sharp and Hahn, 2011, Marx et al., 2011). However, it is generally accepted that HIV originated from the Simian immunodeficiency virus (SIV), a species of retrovirus found in chimpanzees and monkeys (Hirsch et al., 1989, Gao et al., 1999). Evidence supporting this comes from a virus sample sequenced in 1959 (ZR59) (Jenum et al., 1988) from an African male patient in the Democratic Republic of Congo (DRC) (Zhu et al., 1998, Keele et al., 2006). The ZR59 HIV sample showed a close phylogenetic relationship with the SIV strain isolated from the chimpanzee, *Pan Troglodytes* (Keele et al., 2006).

1.2 Nomenclature and Phylogeny

HIV, from the family Retroviridae and genus Lentivirus, can be classified as HIV-1 and HIV-2. HIV-1 is responsible for the worldwide Acquired Immunodeficiency Syndrome (AIDS) epidemic, while HIV-2 presents clinical symptoms similar to HIV-1 (Wilkins et al., 1993) but is much less common and immunologically distinct (Clavel et al., 1986). HIV-1 includes four groups namely M, N, O and P, all with different geographical distribution (Burke, 1997, Plantier et al., 2009, Robertson et al., 2000, Vallari et al., 2011). The major group, M, can be further subdivided into ten subtypes i.e. A, B, C, D, F, G, H, J, K and the recently described subtype L, and about 58 circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs) (Désiré et al., 2018, Ndjoyi-Mbiguino et al., 2020, Yamaguchi et al., 2020); Figure 1.1.

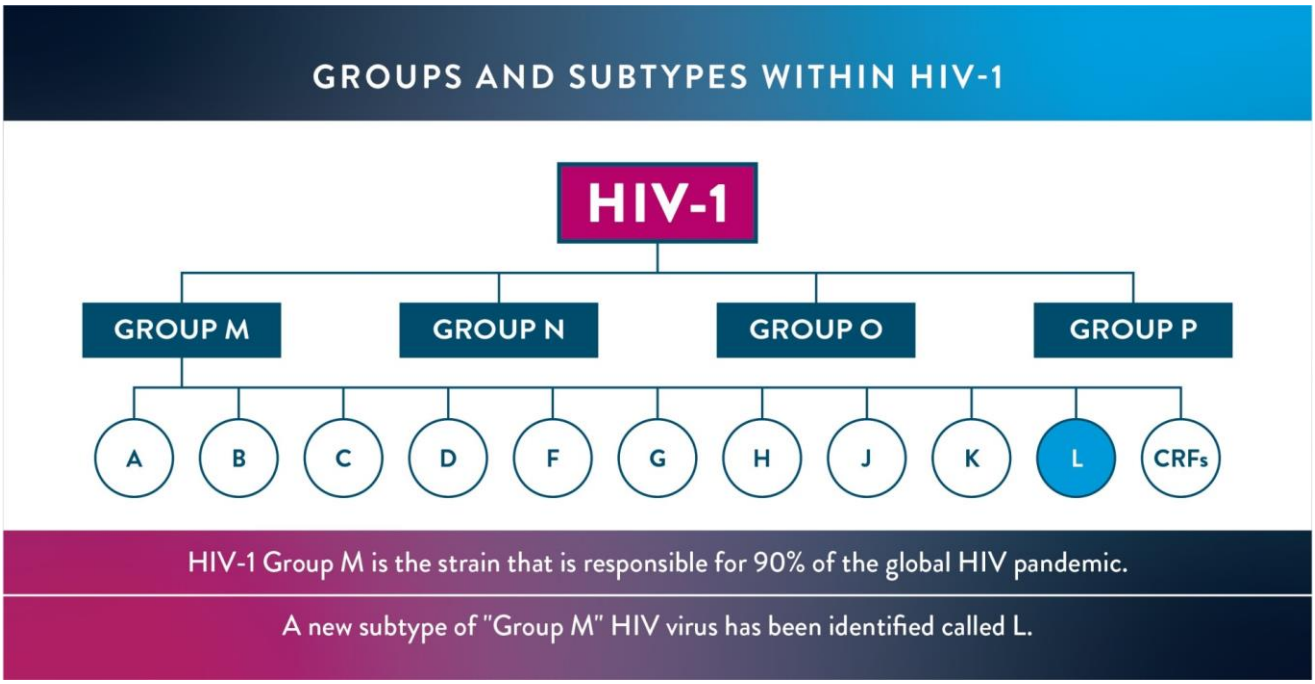


Figure 1.1 Groups and subtypes within HIV-1, subtype L is the latest addition to the viral subtypes classified under HIV-1 group M. Image adopted from (Abbott, 2019).

1.3 HIV-1 structure and genome organization

HIV-1 is roughly spherical and has a diameter of approximately 100-150 nm. Retroviruses are characterized by their storage of genetic information in ribonucleic acid (RNA) and therefore require a mechanism to translate RNA to deoxyribonucleic acid (DNA) which carries their host's genetic information. The HIV-1 genome comprises diploid full-length copies of single-stranded RNA stabilized by a ribonucleoprotein complex p7 (Coffin, 1992), and divided into nine genes encoding for three polyproteins, two regulatory proteins, and four accessory protein, shown in Figure 1.2 below. The HIV-1 polymerase (Pol), Gag, and envelope (Env) are classified as polyproteins (Costin, 2007); Pol encodes for protease (PR), reverse transcriptase (RT), and integrase. Gag is a precursor of four structural proteins, the viral capsid (p24), the nucleocapsid proteins p6 and p7, and the viral matrix. Envelope consists of two subunits namely the glycoprotein gp120 and the transmembrane glycoprotein gp41. The transactivator of transcription (Tat) and regulator of viral expression (Rev) are classified as regulatory proteins; and viral protein unique (Vpu), viral protein R (Vpr), viral infectivity factor (Vif), and negative regulatory factor (Nef) are classified as accessory proteins. The function of each of these proteins is outlined in Table 1.1.

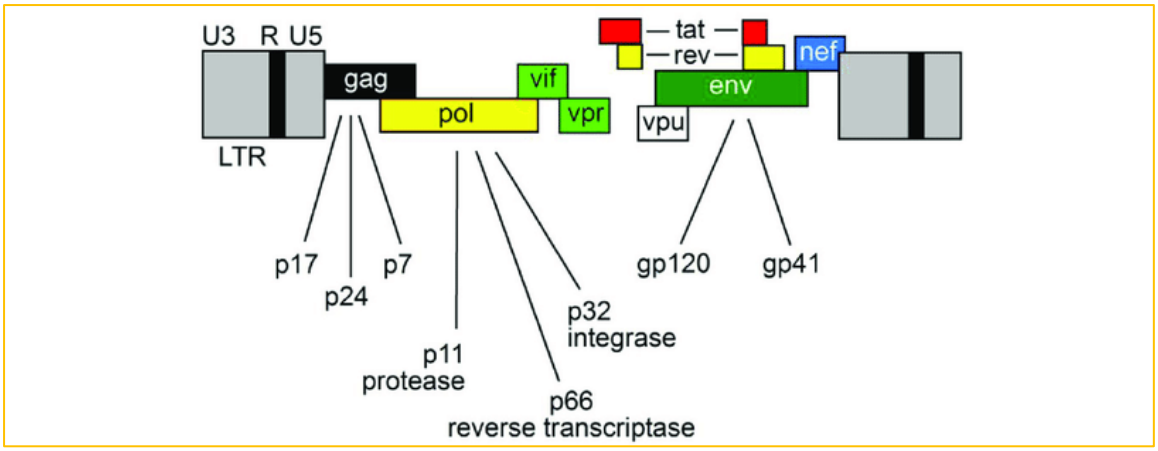


Figure 1.2 The HIV-1 genome. The Gag, Pol and Env genes are precursors for the viral proteins. Gag and Pol encode the viral core and the viral replication enzymes, respectively and are directly expressed from the viral promoter in the long terminal repeat (LTR). The Env gene encodes the envelope glycoprotein. HIV-1 accessory genes are also shown. The figure was obtained from (Felli et al., 2017).

Table 1.1 HIV proteins and their functions

	Genes	**Function**
Polyproteins	Gag (Matrix, Capsid and nucleocapsid)	Matrix – Binds RNA to the plasma membrane and incorporates the host envelope into virions during assembly Capsid - Forms the characteristic core capsid of the virion, which encloses the viral genome Nucleocapsid - Chaperones the conversion of the genomic RNA into viral DNA, and drives the selection and dimerization of the genomic RNA at the initial stage of viral particle assembly (Muriaux and Darlix, 2010)
	Env (gp41 and gp120)	gp41- Transmembrane component for the fusion of virus membrane to host cell membrane gp120- Surface component for CD4 receptor and co-receptor binding (Guttman and Lee, 2013)
	Gag-Pol (PR, RT and integrase)	PR- Sequential proteolytic processing of the Gag and Gag-Pol polyproteins into mature chains RT- Conversion of single-stranded viral RNA into double-stranded proviral DNA Integrase- Integration of the viral DNA into the host chromosomal DNA (Zhang et al., 2008, Delelis et al., 2008)
Regulatory proteins	Tat (transactivating protein)	Regulates transcription of genes that code for HIV-1 proteins (Watson and Edwards, 1999)
	Rev (regulator of viral expression)	Binds to unspliced RNA transcripts and targets them for passage out of the host cell nucleus (Rosen, 1991)

Accessory proteins	Nef (negative factor)	Regulates HIV replication, enhances viral infectivity	(Freund et al., 1994)
	Vif (viral infectivity factor)	Aids viral infectivity, role in virion assembly	(Rose et al., 2004)
	Vpr (viral protein R)	Aids virus replication	(Bukrinsky and Adzhubei, 1999)
	Vpu (viral protein U)	Aids virus budding and release from the host cell	(González, 2015)

1.4 HIV-1 life cycle

The basic steps of the HIV-1 replication cycle are viral entry, uncoating, reverse transcription, proviral integration, transcription & translation, assembly, budding & maturation as summarised in Figure 1.3 below.

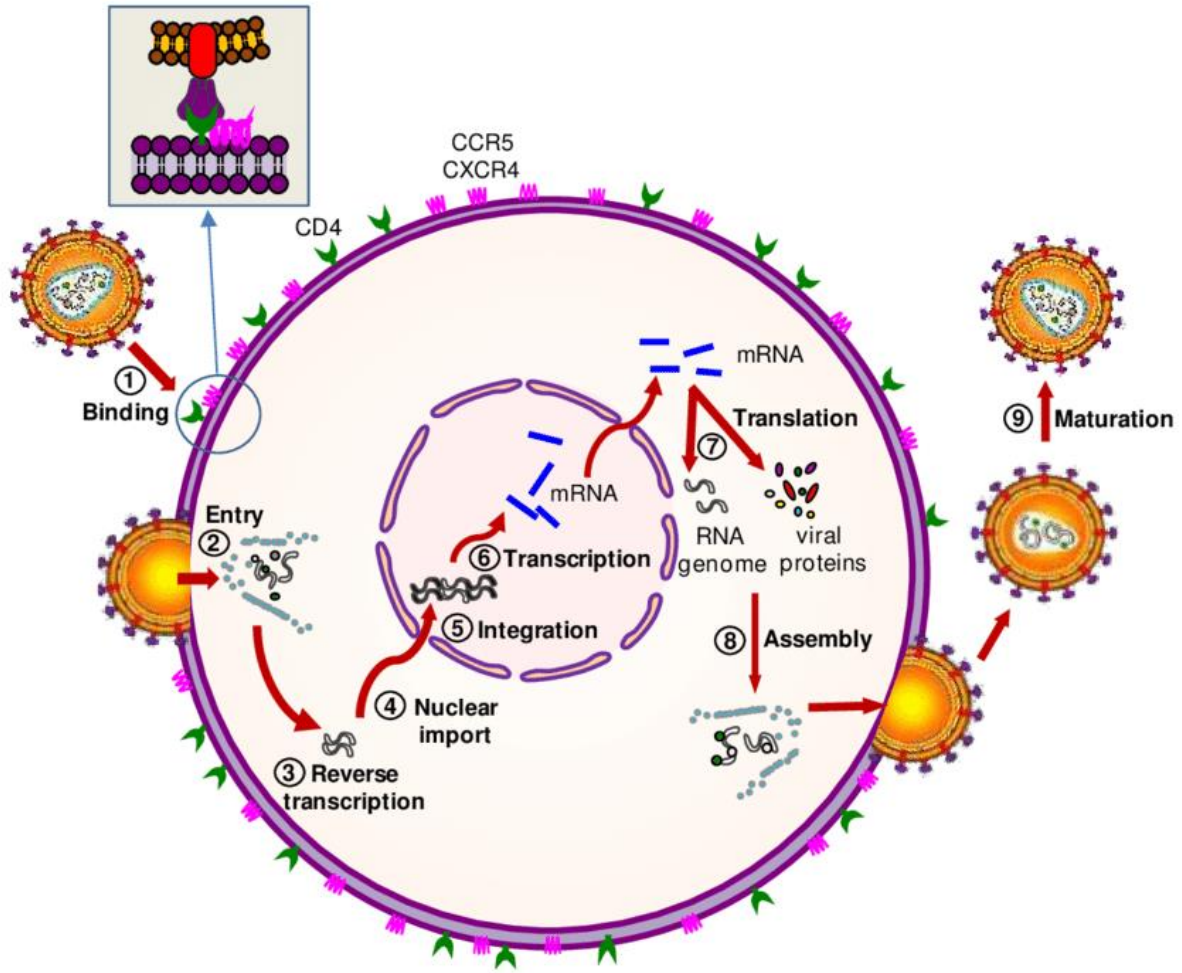


Figure 1.3 The basic steps of the HIV-1 replication cycle. Diagram from (Herrera-Carrillo and Berkhout, 2015) showing virus attachment to receptors and coreceptors followed by fusion with the cell membrane. Reverse transcriptase converts RNA to DNA; integration occurs with the host cell nucleus followed by transcription of viral RNAs by cellular transcription factors which are later translated into structural and viral proteins. The last events are budding and maturation.

1.4.1 Viral entry and tropism

HIV-1 entry into the host cell is initiated by the viral envelope binding onto the host cell's CD4+ T cell receptor. The receptor interacts directly with the class II major histocompatibility complex (MHC II) to form part of the T cell activating signals (Doyle and Strominger, 1987). The class II MHC complex is expressed in macrophages and activates naïve T cells; their function is to bind antigens derived from pathogens and display them on the cell surface to be recognized by appropriate T cells. The CD4 receptor contains four immunoglobulin domains (D1 to D4), the first domain D1 is the primary binding site for gp120 (Doms, 2000). The gp120-CD4 encounter is said to be the crucial trigger for gp120 dissociation and a series of refolding events in the gp41 fusion protein (Dalgleish et al., 1984), leading to fusion of viral and host cell membranes (Harrison, 2008). A pre-hairpin fusion intermediate state takes place followed by a fusogenic state then finally a post-fusion state that is an introduction to the gp41 native state. These sequential conformational changes are essential to the HIV-1 life cycle as they enable the virus core (p24) entry into the target cell cytoplasm.

The virus binds to specific host cell receptors such as the C-X-C chemokine type four receptor (CXCR4) and C-C chemokine receptor type five (CCR5) (Alkhatib et al., 1996, Choe et al., 1996, Deng et al., 1996, Feng et al., 1996, Vandekerckhove et al., 2011). Viruses are designated into two types depending on the receptor they bind onto; R5 variants bind the CCR5 chemokine receptor and X4 variants bind the CXCR4 chemokine receptor. During the early stages of HIV-1 infection, R5 viruses are common while X4 variants emerge later in the clinical disease progression state, and loss of CD4+ T-cells (Koot et al., 1993, Maas et al., 2000, Shepherd et al., 2008, Weiser et al., 2008). Variants R5X4 are capable of using both CXCR4 and CCR5 receptors, although one receptor is preferred over the other (Glushakova et al., 1999, Coakley et al., 2005).

1.4.2 Uncoating

HIV-1 is enclosed by a capsid shell that dissociates within the cell and influences the completion of reverse transcription of the viral genome, also known as uncoating. Uncoating accompanies the conversion of the reverse transcription complex (RTC) and the pre-integration complexes (PICs). Viral and cellular factors that are said to participate in uncoating include cyclophilin A (CyP A) (Javanbakht et al., 2007), integrase (Briones et al., 2010, Misumi et al., 2010), and peptidyl-prolyl isomerases (Pin1) (Misumi et al., 2010). Studies have shown that the cellular factor CyP A binds to capsid and aids the process of uncoating (Auewarakul et al., 2005, Warrilow et al., 2008, Aiken and AIDS, 2006). Capsid directly interacts with Pin 1 after entry into the host cell (Misumi et al., 2010). It has also been shown that HIV-1 is uncoated in a dysfunctional manner when Pin 1 is depleted (Misumi et al., 2010). Viral

integrase which is associated with PICs plays an important role in the uncoating process by promoting capsid core stability (Briones et al., 2010).

1.4.3 Reverse transcription

Reverse transcription is the process of transcribing single-stranded RNA into double-stranded DNA by the RNA-dependent-DNA polymerase enzyme, one of the key steps in the HIV-1 replication cycle. The reverse transcriptase (RT) enzyme was independently discovered by Howard Temin and David Baltimore in 1970. It is an asymmetric heterodimer with p66 and p51 subunits consisting of 560 and 440 amino acids, respectively, and is derived from the Gag-Pol precursor protein (Kohlstaedt and Steitz, 1992). The RT enzyme with the ribonuclease H (RNase H, 15 kDa) catalyses the selective hydrolysis of RNA from the RNA:DNA heteroduplex replication intermediate (Sluis-Cremer et al., 2004).

Reverse transcription begins when transfer RNA (tRNA), acquired from the host, binds to the primer binding site of the full-length RNA template, the enzyme RT recognizes tRNA-RNA complex and extends the primer from the 3'OH group towards the HIV-1 RNA 5'end by its RNA-dependent DNA polymerase activity (Barat et al., 1989, Isel et al., 1996, Götte et al., 1999, Sluis-Cremer and Tachedjian, 2008). The result of this process is the synthesis of the minus-strand U5 and the R sequences in a DNA-RNA hybrid. Two stretches of polypurine RNA segments, the polypurine tract (cPPT) and the 3' polypurine tract (3'PPT) are used as priming sites for plus-strand DNA synthesis initiation. The next event involves the plus-strand DNA being transferred to the 3' end of the minus strand, where complementary base pairing between the primer binding site sequences occurs. Synthesis of the plus strand takes place until the central termination signal is reached and at this point, a central DNA flap is created. Double-stranded DNA containing the LTRs is produced from the minus and plus-strands which is then transported to the nucleus via the PIC and then serves as a substrate for integration into the host cell by the enzyme integrase.

1.4.4 Proviral integration

Integrase catalyses the processing of the 3' end which removes a 3' dinucleotide from each end of the viral DNA and strand transfer resulting in the covalent linkage of the viral and host DN, Figure 1.4. The two reactions (3' processing and strand transfer) occur *in vivo* sequentially and are also energetically independent (Delelis et al., 2008). In the first reaction, 3' processing, cleavage occurs at the 3' end of a conserved CA dinucleotide and exposes the terminal 3'-OH group that is to be joined to the target DNA (Craigie, 2001, Delelis et al., 2008). A water molecule serves as a nucleophilic agent to conduct the endonucleolytic cleavage at the ends of the viral DNA (Engelman et al., 1991, Skinner et al., 2001).

In the strand transfer reaction, the cleaved DNA is inserted into the target DNA. It has been demonstrated that both reactions occur by a one-step transesterification mechanism of phosphodiester bonds by attacking nucleophiles (Engelman et al., 1991, Delelis et al., 2008). Both reactions can be reproduced *in vitro* using purified recombinant integrase, short duplex oligonucleotides that mimic the ends of the viral LTR U5 / U3, and a divalent metal ion Mn^{2+} or Mg^{2+} (Leh et al., 2000b, Leh et al., 2000a). HIV-1 PICs contain viral integrase, RT, Vpr, matrix, and lens epithelium derived growth factor (LEDGF/p75). Two distinct nuclear localization signals (NLS)-containing regions of the matrix protein enables PIC import to the nucleus. The Vpr also plays a role in directing the PIC to the nucleus, although it does not have NLS, it binds to a member of the importin alpha family. The importin alpha family is a class of proteins involved in protein transportation and binds to the NLS. The cellular factor LEDGF/p75 also contains NLS and has been shown to bind to integrase, therefore, driving the PIC translocation across the membrane (Cherepanov et al., 2003). The resulting integrated provirus serves as a template for viral production.

1.4.4.1 LEDGF/p75

The lens LEDGF/p75 is currently the most researched integrase interaction partner. It was identified by coimmunoprecipitation of HIV-1 complexes (Cherepanov et al., 2003) and confirmed by the Engelman and Benarous group as an integrase interactor using the yeast two-hybrid confirmation system (Busschots et al., 2005, Emiliani et al., 2005). This commonly expressed protein has been implicated as a key regulator of cellular survival meaning strong association with DNA, transcriptional activators, and other antiapoptotic proteins (Van Maele et al., 2006). Crystallography has shown that the integrase:LEDGF stoichiometry was likely to be 2:1, with several LEDGF residues shown to be critical for integrase-LEDGF interaction (Van Maele et al., 2006). A class of integrase inhibitors referred to as LEDGINS (also known as allosteric integrase inhibitors) were designed to prevent the integrase-LEDGF interaction (Kessl et al., 2012, Christ et al., 2010).

1.4.4.2 LEDGINS

The discovery of 2-(quinolin-3-yl) acetic acid derivatives by structure-based design approaches resulted in the inhibitors of integration termed ‘LEDGINs”. Their distinct pharmacophore features are a quinolone ring, a phenyl ring, a carboxylic acid, and a methoxy group at the α-position of the carboxylic acid (Sharma et al., 2014). LEDGINs are small molecules that bind in the LEDGF/p75 binding pocket of HIV integrase, blocking HIV-1 replication by allosterically interfering with integrase catalytic activity (Christ et al., 2010, Christ et al., 2012, De Luca et al., 2010). As a consequence, researchers targeted the LEDGF/p75 – integrase interaction as a potential inhibitor of replication (Christ et al.,

2012). By blocking LEDGF/p75 – integrase interaction and interfering with catalytic activity, LEDGINS can inhibit both strand transfer and 3’ processing (Christ and Debyser, 2015). Several LEDGINS at various stages of development have been reported (Choi et al., 2018, Christ et al., 2011, Christ and Debyser, 2015).

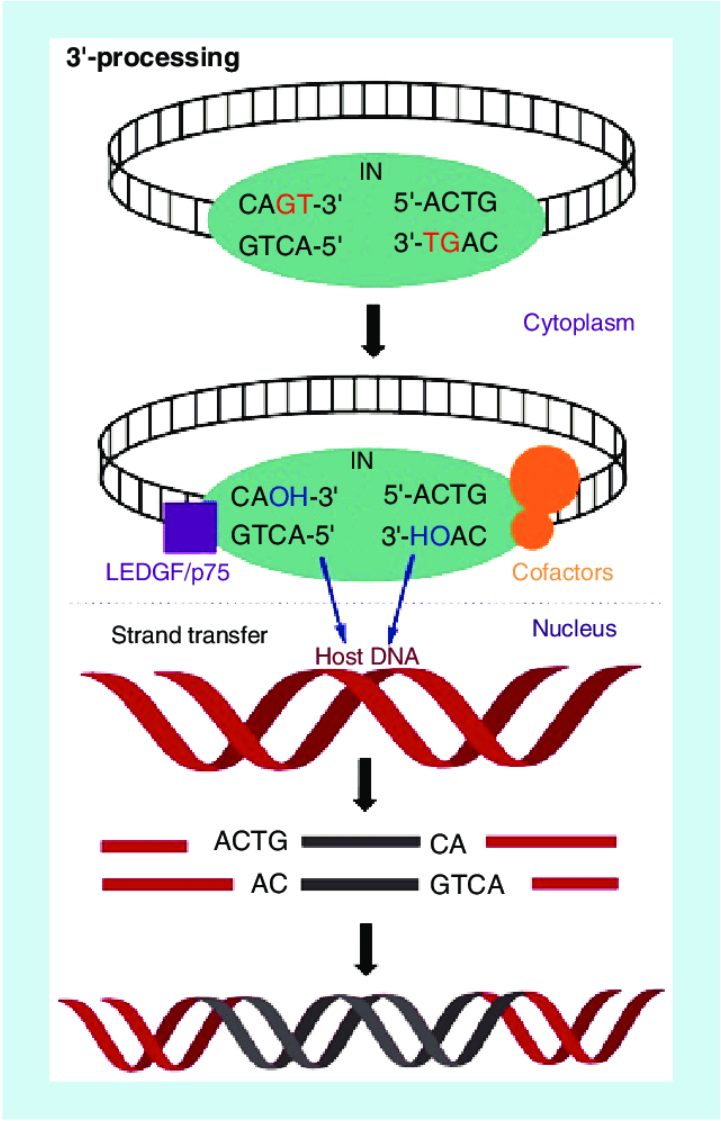


Figure 1.4 Schematic diagram of HIV-1 integrase activity adopted from (Choi et al., 2018). The first step of integration is 3’ processing, the removal of two nucleotides adjacent to a conserved CA from both viral cDNA 3’ends. Strand transfer is when the processed viral DNA is inserted into the host chromosomal DNA.

1.4.5 Transcription and Translation

Transcription is initiated in the TATA box sequence by the binding of enzyme RNA polymerase II to the LTR region of HIV-1. The newly integrated HIV-1 provirus acts as a template for viral RNA transcription and encodes viral proteins. The Tat protein interacts with the transactivation response region (TAR) leading to the recruitment of the positive transcriptional elongation factor b (P-TEDb) complex. The TAR and P-TEDb complex leads to the phosphorylation of the RNA polymerase II C-terminal domain stimulating the enzyme processes to produce full-length transcripts (Cherepanov et al., 2003). It has been shown that shorter transcripts are produced in the absence of Tat due to poor RNA polymerase II processivity.

A combination of unspliced, partially spliced and multiple spliced mRNAs are produced during the transcription process. The transcripts are ready to be translated by host ribosomes once they enter the cytoplasm. The partially spliced mRNA (5kb) is translated into Gag, Env, Vif, Vpu, Vpr, and Gag-Pol precursors in the cytoplasm whereas the small multiple spliced mRNA (1-2kb) are translated into Rev, Nef, and Tat proteins in the endoplasmic reticulum (Freed and genetics, 2001).

1.4.6 Assembly, budding, and maturation

There are three stages of virion morphogenesis: assembly, budding, and maturation (Sundquist and Kräusslich, 2012). The HIV-1 virion assembly takes place in the plasma membrane. The virion and essential components required for viral infectivity i.e. copies of the positive sense genomic viral RNA, cellular $tRNA^{Lys,3}$ molecules, the Gag and Env polyproteins, and the three viral enzymes PR, RT, and integrase are created (Sundquist and Kräusslich, 2012, Blood, 2016). Sequential events mediated by HIV-1 Gag polyprotein mediates the events in the virion assembly, these include binding the plasma membrane and making protein-protein interactions necessary to create the viral core (Balasubramaniam and Freed, 2011).

The next stage is the budding process where the virus crosses the plasma membrane and obtains its lipid envelope interspersed by the viral gp120/gp41 trimeric spikes; budding is mediated by endosomal sorting complexes required for transport (ESCRT) (Sundquist and Kräusslich, 2012, Checkley et al., 2011). The virion acquires its lipid envelope and Env protein spikes as it buds from the plasma membrane. As the immature virion buds, PR is activated and cleaves Gag into its virus constituent MA, central gag domain, nucleocapsid gag domain, and p6 proteins to form a mature and infectious virus (Sundquist and Kräusslich, 2012, Brown, 1997). HIV-1 maturation is essential for the released virus particles to become infectious and initiate a new round of infection (Wang et al., 2015).

1.5 Infection, transmission, and disease progression

Several viral and host factors determine the variability of HIV-1 infection outcome and rate of disease progression in infected individuals (Naif, 2013). The exact mechanism of how these factors contribute to the infection of CD4+ T cells and the persistence of R5 and X4 strains is still not well elucidated (Naif, 2013, Woodham et al., 2016, Ward et al., 2020). Infection with HIV-1 is initially asymptomatic, and this is followed by slight changes in the immune system. The initial immunosuppression stage spans up to three months until seroconversion, where HIV-1 antibodies can be detected. The development of symptoms from primary infection to advanced immunosuppression takes several years. The majority of patients progress to AIDS within ten years, whereas 5% of patients remain in the asymptomatic phase for ten years and above (Long -Term Non-Progressors) and approximately 15 % of patients take two to five years to progress to AIDS these are called Rapid Progressors (Kan, 2007). Infection is acquired across the mucosal membrane during sexual and perinatal transmission.

1.6 Genetic diversity and epidemiology

HIV-1 is characterized by a high degree of genetic variability within infected persons; subtype diversity is important for epidemiological purposes and is also pertinent in clinical settings (Abecasis et al., 2013). HIV-1 diversity has been attributed to the lack of proofreading mechanism by the RT enzyme causing high mutation and recombination rate (Hu and Hughes, 2012, Coffin and Swanstrom, 2013, Boltz et al., 2012, Maldarelli et al., 2013, Perelson et al., 1996). HIV-1 diversity has given rise to numerous subtypes and this genetic variation can influence the development of drug resistance and susceptibility to certain antiretroviral drugs. Some subtypes have distinct biological properties and sequence variability, studies have also suggested that different subtypes show different disease progression (Abecasis et al., 2005, Abecasis et al., 2009, Camacho et al., 2007, Brenner et al., 2003, Grossman et al., 2004, Palma et al., 2012). Subtype A is common in West & Central Africa and Russia, subtype B is common in America, Australia, Japan, and Europe, and subtype C is common in Southern and Eastern Africa and India.

Recombination between subtypes is another contributing factor to genetic diversity and plays an important role in the global pandemic, for example in the Republic of Congo approximately 27% of strains circulating in the country are recombinants (Kita et al., 2004, Bikandou et al., 2004). The distribution of HIV-1 subtypes and recombinants is concerning. Reports are indicating that subtype differences may exist with regards to integrase strand transfer inhibitor (INSTI) resistance development, a phenomenon known to exist for RT inhibitors (Bar-Magen et al., 2010a, Brenner et al., 2011, Loizidou et al., 2009). The prevalence of polymorphisms and resistance mutations in HIV-1 integrase has already been analyzed in a panel of different HIV-1 subtypes (Reigadas et al., 2013).

HIV-1 subtype B and C wild-type integrase enzymes have similar susceptibility to clinically validated INSTIs (Bar-Magen et al., 2009), however, the presence of resistance mutations may differentially affect susceptibility to various INSTIs (Bar-Magen et al., 2010a). For example, the well-documented INSTI resistance mutation Y143CHR which affects susceptibility to HIV-1B may not affect the susceptibility of the HIV-1 subtype C (HIV-1C) enzyme to DTG (Hightower et al., 2011).

HIV-1 epidemiology is largely dominated by non-B subtypes (Santos and Soares, 2010), so there is a concern that the majority of studies conducted on HIV-1 drug design to evaluate acquisition and impact of drug resistance mutations have been primarily performed for subtype B strains. Such concern is strengthened by the fact that more than two-thirds of the people living with HIV-1 are in African countries where subtype C is predominant. For example, out of the 38 million people living with HIV at the end of 2019, 20.7 million are from Eastern and Southern Africa (UNAIDS, 2020). Since the beginning of the epidemic, 76 million people have been infected with the virus and about 30 million people have died of HIV/AIDS (https://www.who.int). The Joint United Nations Programme on HIV/AIDS (UNAIDS) report on the global AIDS epidemic has estimated that from the 38 million (32.2 million – 37.2 million) people living with HIV at the end of 2019, approximately 1.7 million (1.2 million – 2.2 million) were new HIV infections (UNAIDS, 2020).

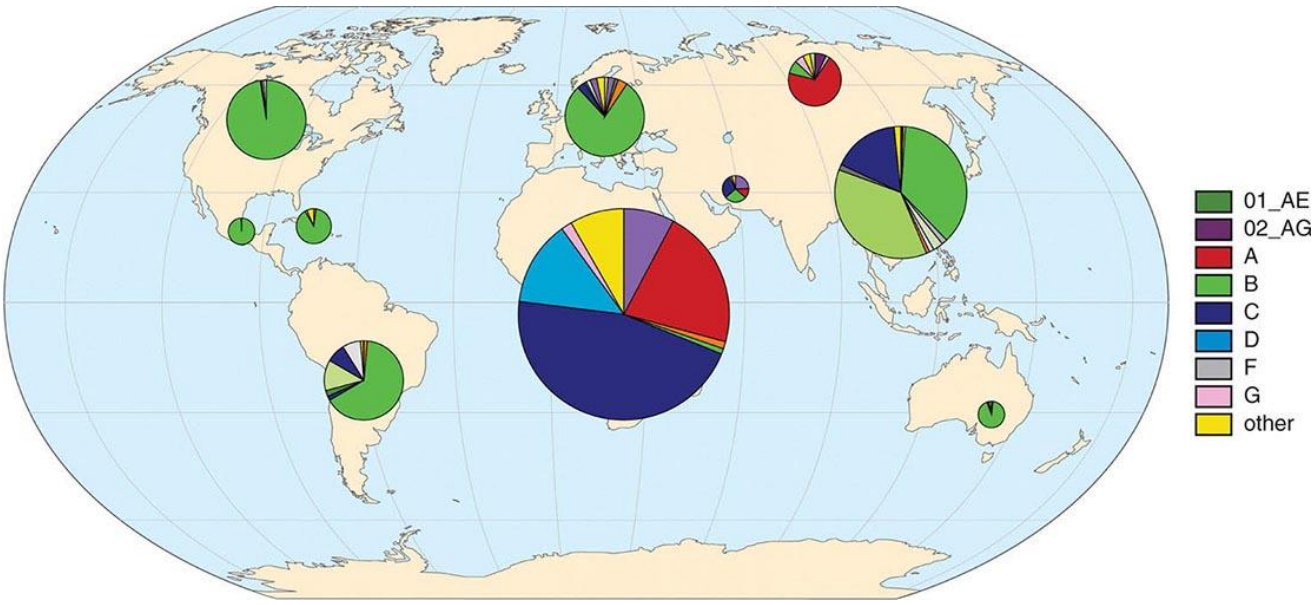


Figure 1.5 Geographical distribution of HIV-1 subtypes and CRF's. The most common subtype, C, accounting for approximately 50% of prevalent infections worldwide, also causing the majority of infections in sub-Saharan Africa. Figure adapted as-is from the Los Alamos National Laboratory HIV Sequence Database *www.hiv.lanl.gov/components/*

1.7 HIV-1 treatment

Currently, there are drugs that target the RT, PR, and integrase enzymes as well as CCR5 co-receptor binding and the fusion process. On the treatment front, the most notable advancement is the use of combination antiretroviral therapy (cART) commonly referred to as highly active antiretroviral therapy (HAART). The effectiveness of cART is due to the combination of different classes of drugs that have been observed to effectively delay the emergence of antiretroviral drug-resistant viral strains (Schulenburg and Le Roux, 2008). In most low- and middle-income countries (LMICs) the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) is standard for first-line cART. Two NRTIs and a protease inhibitor (PI) are included for second-line treatment. The newest class of approved antiretroviral drugs, (INSTIs), specifically inhibit strand transfer and block integration of the HIV-1 DNA into the cellular DNA (Arts and Hazuda, 2012). The World Health Organization (WHO) recommended the use of INSTIs in third-line cART regimens (WHO, 2016). Moreover, in South Africa Dolutegravir (DTG) (a second-generation INSTI drug) has been recommended in first-line treatment (Moorhouse et al., 2018). Important steps in the HIV-1 life cycle that are blocked by antiretroviral drugs are shown in Figure 1.6.

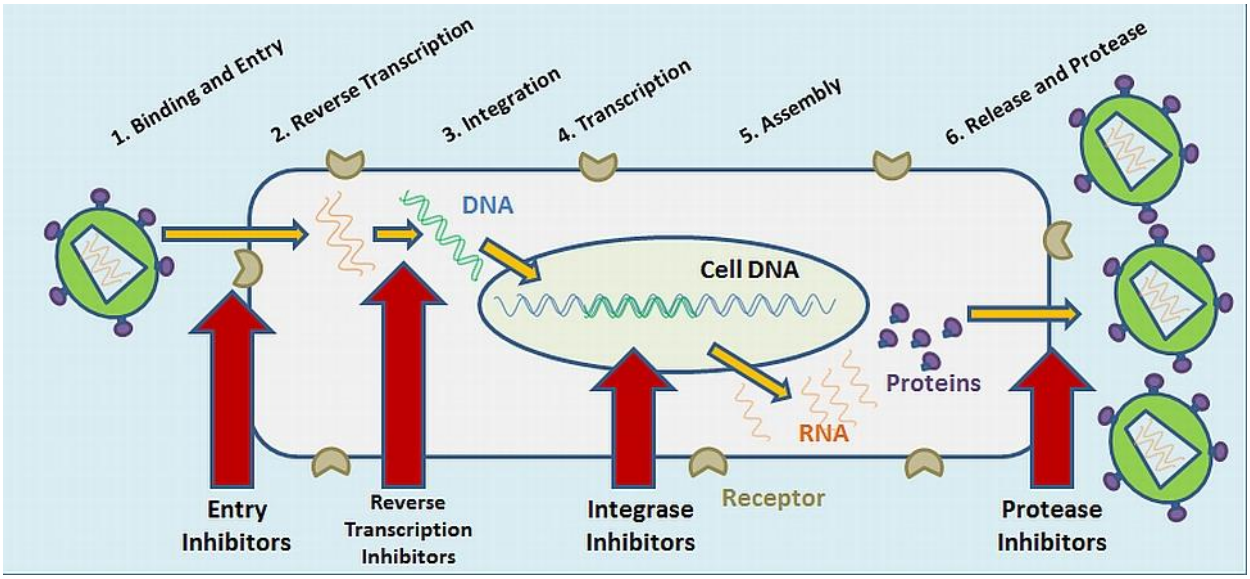


Figure 1.6 Diagram showing anti-drug target steps in the HIV-1 replication cycle. Diagram from https://www.bcm.edu/departments/molecular-virology/hiv-aids

Table 1.2 Classes of antiretroviral drugs and their mode of action

Drug Class	Abbreviation	Mechanism of action	Specific action
Entry inhibitors	-	Entry inhibition	Bind to viral gp41 / gp120 or host cell CD4+ or chemokine (CCR5) receptors (Wilen et al., 2012, Meintjes et al., 2014)
Nucleoside reverse transcriptase inhibitors	NRTIs	Inhibits reverse transcriptase	These analogues mimic the normal building blocks of DNA, preventing transcription of viral RNA to DNA (Meintjes et al., 2014)
Non-nucleoside reverse transcriptase inhibitors	NNRTIs	Inhibits reverse transcriptase	Alter the conformation of the catalytic site of reverse transcriptase and directly inhibit its action (Maga et al., 2010)
Protease inhibitors	PIs	Inhibits protease activity	Inhibit the final maturation stages of HIV replication, resulting in the formation of non-infective viral particles (Ghosh et al., 2016)
Integrase strand transfer inhibitors	INSTIs	Inhibits viral integration	Prevent the transfer of proviral DNA strands into the host chromosomal DNA (Pommier et al., 2005)

1.8 Historical development of HIV therapeutics targeting HIV integrase

Merck investigators and Shionogi scientists have been pioneer companies in the research and development of INSTIs. In the year 2000, Merck discovered diketo acids (DKA's) inhibitors after screening 250 000 compounds (Hazuda et al., 2000). These compounds were found to inhibit integrase strand transfer with significantly high potency (Hazuda et al., 2000, Reinke et al., 2002) and were referred to as INSTIs. DKA's bind to the HIV-1 integrase catalytic site in the presence of viral DNA LTRs (Espeseth et al., 2000) and also interact with the viral DNA 5' end (Marchand et al., 2002, Pais et al., 2002). The first integrase inhibitor to be co-crystalized with the integrase catalytic core (CCD) domain was a prototype DKA 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone (5-CITEP), discovered by Shionogi scientists (Figure 1.7 A).

Following 5-CITEP, Merck discovered a series of compounds: L-708,906, (Hazuda et al., 2000) L-731,988 (Hazuda et al., 2000, Marchand et al., 2002), 870,810 and L-870,812 (Figure 1.7 B, C, E & F; respectively), with the latter both derivatives of 8-hydroxy-[1,6]-naphthyridine-7-carboxamides (Zhuang et al., 2003, Hazuda et al., 2004). Both L-870,810 and L-870,812 were the first INSTI compounds to show anti-HIV activity in experimental animal models, however, long-term use resulted in liver and kidney toxicity thus halting them from further clinical development (Semenova et al., 2008, Nair and Okello, 2015). Subsequent variations of DKA's based on the 5-CITEP backbone led to the first clinically tested INSTI compound, i.e. S-1360 (Figure 1.7 D). The compound was developed by Shionogi

and initially had a good pharmacological and pharmacokinetic profile in animals, however, in human trials was found to be rapidly cleared through glucuronidation (Rosemond et al., 2004) and its development was curtailed. Further work by Merck investigators on lead compounds such as L-870,812 including pharmacokinetic profiles, physicochemical properties, and optimization for potency led to the discovery of the first INSTI to be approved by the Food and Drug Administration (FDA) for HIV-1 clinical trials, named Raltegravir (RAL) (Summa et al., 2006, Anker and Corales, 2008). Figure 1.7 summarises the development of INSTIs leading to the clinically relevant and promising compounds i.e. RAL, Elvitegravir (EVG), DTG, Bictegravir (BIC) and Cabotegravir (CAB) (Figure 1.7 G, H, I, J & K, respectively) .

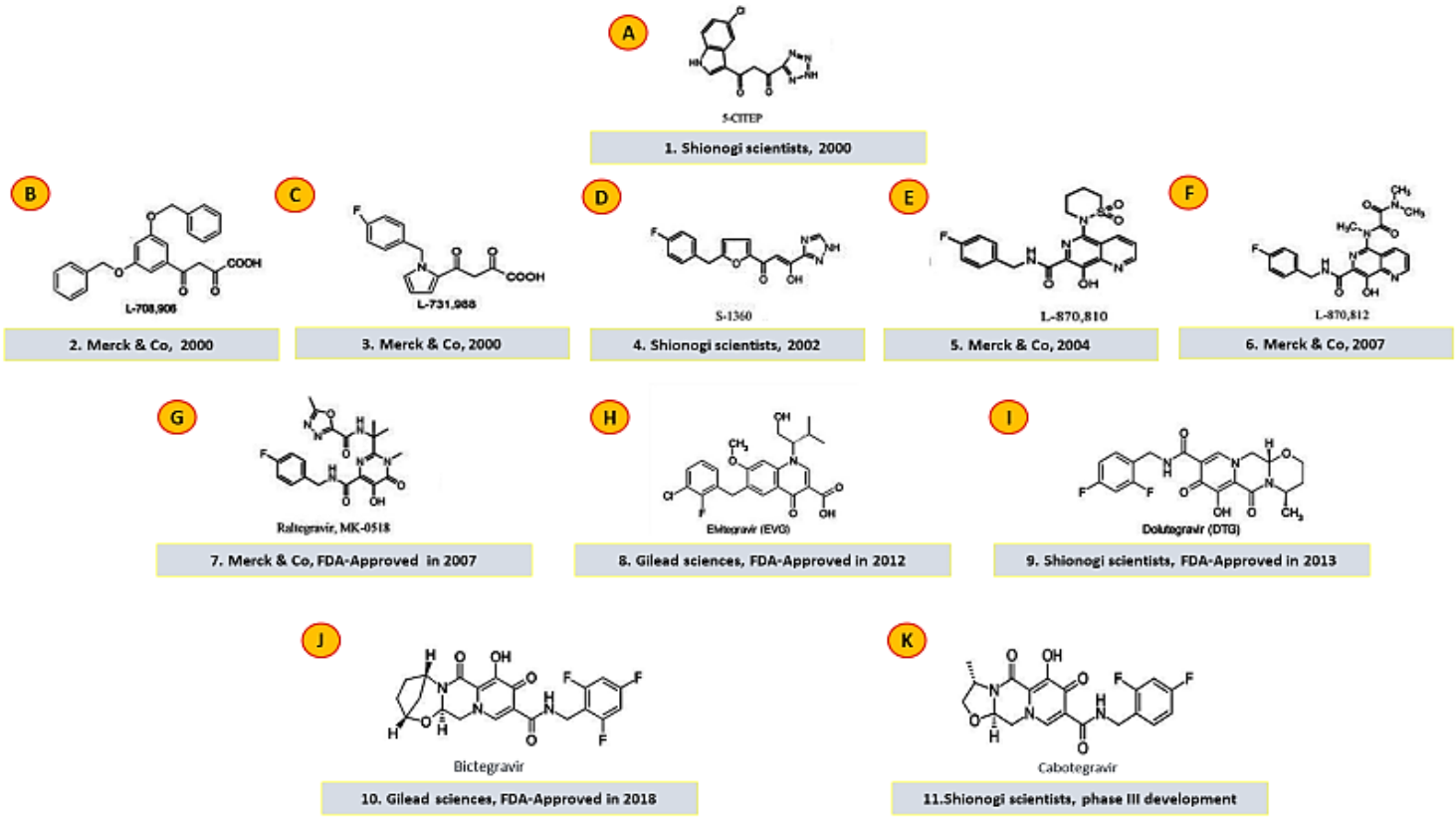


Figure 1.7 Integrase strand transfer inhibitors structural evolution. (A) 5-CITEP (McColl and Chen, 2010), (B) L-708,906 (Dubey et al., 2007), (C) L-731,988 (Dubey et al., 2007), (D) S-1360 (McColl and Chen, 2010), (E) L-870,810 (McColl and Chen, 2010), (F) L-870,812 (McColl and Chen, 2010), (G) Raltegravir (McColl and Chen, 2010), (H) Elvitegravir (Wainberg et al., 2016), (I) Dolutegravir (Wainberg et al., 2016), (J) Bictegravir (Hassounah et al., 2017) and (K) Cabotegravir (Hassounah et al., 2017).

Note: Images were adapted from different articles with references shown for each image.

1.9 Recently discontinued integrase inhibitors

As an inevitable consequence of drug development, some potential INSTI agents were discontinued before they could be approved for use in HIV-1 treatment. In 2009, Merck identified MK-2048, a DKA derivative with the potential to inhibit HIV-1 resistant variants generated to first-generation compounds (Bar-Magen et al., 2010b). Despite MK-2048 having a favorable resistance profile, it had an extremely low half-life of only 1-1.5 hours, and its efficacy was reduced more than eight-fold by the G118R and E138K mutations (Bar-Magen et al., 2010b). Therefore, its clinical development was stopped due to its poor pharmacokinetic profile. It however continues to be studied as a prototype second-generation INSTI (Seegulam and Ratner, 2011). BMS-707035, a structural and functional motif of L780,810 and RAL also possessed *in vitro* inhibiting activity similar to that of RAL and had reached the second stage of clinical trials. However, the emergence of resistant HIV-1 strains resulted in its clinical trial being discontinued (Al-Mawsawi et al., 2008). BI224436, a 3-quinoline acetic acid derivative, is a non-catalytic site integrase inhibitor that binds to the integrase allosteric pocket and blocks the strand transfer conformational changes (Fader et al., 2014), just like NNRTIs inhibit reverse transcription. It was thought to be a potent inhibitor of integrase as it targets the non-catalytic integrase domain, however, three mutations A128T, A128N, and L102F occurring in the conserved region of integrase reduced its binding affinity with integrase in HIV-1 infected patients, thus limiting its advancement as an integrase drug (Fenwick et al., 2014).

Table 1.3 Antiretroviral drugs currently available in South Africa

Drug	**Drug class**	**Notes / Monitoring required**
Tenofovir disoproxil fumarate (TDF)	NRTI	Can cause renal failure or renal-tubular wasting syndrome. Creatinine monitoring at regular intervals is recommended (Venter et al., 2018)
Lamivudine (3TC)	NRTI	None routinely required
Emtricitabine (FTC)	NRTI	None routinely required
Abacavir (ABC)	NRTI	Can cause a fatal hypersensitivity reaction in patients with HLA-B*5701. If feasible, this allele should be excluded before starting ABC, although it is very rare in people of African descent (Small et al., 2017)
Zidovudine (AZT)	NRTI	Should only be used in special circumstances as a first-line drug (WHO, 2018b)
Efavirenz (EFV)	NNRTI	Remains a good first-line ART option for patients who tolerate DTG poorly, or where DTG is contraindicated or declined (Nel et al., 2020)
Nevirapine (NVP)	NNRTI	NVP is no longer recommended for new patients because of its adverse side effect profile (Nel et al., 2020)
Rilpivirine (RPV)	NNRTI	RPV is another good first-line option, but it cannot be co-administered with RIF-based TB treatment and should not be started in patients with a VL > 100 000 copies/mL (Nel et al., 2020)
Etravirine (ETR)	NNRTI	ETR may be used as part of third-line therapy where appropriate but is not recommended as a first-line agent (Nel et al., 2020)
Lopinavir (LPV)	PI	Lopinavir is co-formulated with ritonavir; this regimen has greater gastrointestinal (GI) side effects than other PI combinations, and is associated with a worse metabolic profile (Chandwani and Shuter, 2008)
Atazanavir (ATV)	PI	Atazanavir and DRV offer a better side effect profile than LPV (Nel et al., 2020)
Darunavir (DRV)	PI	DRV has the highest barrier to resistance of any drug in this class (Nel et al., 2020)
Raltegravir (RAL)	INSTI	An excellent ART option but has a lower barrier to resistance than DTG
Dolutegravir (DTG)	INSTI	DTG is preferred to RAL because it has a higher barrier to resistance (Steegen et al., 2019)

1.10 HIV-1 drug resistance & prevalence in INSTIs

HIV-1 drug resistance is a major public concern and one of the most common causes of therapeutic failure, especially in LMICs with large treatment programmes like South Africa where over seven million people were living with HIV-1 in 2020 (www.unaids.org/en/regionscountries). As a result, South Africa has the largest treatment programme in the world with more than four million people on cART. While the successful rollout of cART is necessary to halt HIV replication, studies have suggested an increased prevalence of acquired drug resistance in treated individuals (Steegen et al., 2016, Manasa et al., 2016) and transmitted drug resistance in newly infected individuals (Phillips et al., 2014). Resistance mutations to the currently prescribed drugs are listed in Table 1.4. A study conducted between 2009 and 2016 in Canada revealed that the prevalence of INSTI resistance remains low compared to PIs and NNRTIs in treated patients (Kamelian et al., 2019). A Ugandan study reported 47% (24 of 51) INSTI resistance prevalence in RAL-based third-line failures and 4% in DTG-based regimen failures (Ndashimye et al., 2018). In South Africa, as from October 2018, 1084 patients were on INSTI-based cART; 650 patients on a DTG-containing regimen and 434 on a RAL-containing regimen [Third-line cART committee communication, May 2019 (Steegen et al., 2019)]. Few data exist on the prevalence of resistance to INSTIs, especially in LMICs.

1.11 Drug resistance testing

In South Africa, drug resistance testing is only limited to third-line failures due to limited resources (Moorhouse et al., 2018). Drug resistance tests are used to determine whether the HIV-1 strain infecting an individual has developed resistance to one or more antiretroviral drugs to be included in the treatment regimen. The viral gene is analysed for the presence of one or more mutations associated with cART. Two types are generally used i.e. genotypic and phenotypic drug resistance testing. Resistance genotyping identifies nucleotide substitutions associated with drug resistance in the gene of interest and is one of the earliest applications of gene sequencing for clinical purposes. Genotypic tests also provide insights into the potential for resistance to emerge (Shafer, 2002).

Viral phenotyping measures the response of a virus to a particular antiretroviral. They are used to analyse the effect of resistance mutations on drug susceptibility. Commonly, they involve inserting a particular gene of interest, e.g. integrase into a deleted vector to produce a recombinant virus. Cells are infected with mutant and wild-type viruses and subjected to varying concentrations of antiretrovirals. The concentration of the drug that inhibits viral replication by 50% (IC50) or 90% (IC90) relative to a control is calculated. To measure drug susceptibility, a fold-change between the wild-type and mutant virus is used (Sebastian and Faruki, 2004). Therefore, the phenotypic drug resistance tests

can detect viral production or the enzymatic activity affected by HIV-1 inhibitors *in vitro* (Petropoulos et al., 2000).

Table 1.4 Key antiretroviral drug resistance mutations reproduced from (Nel et al., 2020).

Drug	**Key mutations selected**
3TC or FTC	Selects for M184V, which compromises both 3TC and FTC and slightly impairs the activity of ABC but increases susceptibility to AZT and TDF
TDF	Selects for K65R, which compromises TDF and ABC but increases susceptibility to AZT. Tenofovir disoproxil fumarate also selects for K70E, which causes low-level resistance to TDF, ABC, and possibly 3TC/ FTC
ABC	Selects for L74V, which compromises ABC. May also select for K65R, which compromises TDF and ABC but increases susceptibility to AZT
AZT	Selects for TAMs, which may ultimately compromise all NRTIs
d4T	Selects for TAMs, which may ultimately compromise all NRTIs
EFV or NVP	Selects for K103N, which causes high-level resistance to EFV and NVP. Also selects for Y181C and other NNRTI mutations, which cause resistance to EFV, NVP, RPV, and ETR
RPV	Selects for several mutations, including E138K, which compromise its susceptibility
LPV	Selects for I47A, L76V and V82A/F/T/S
ATV	Selects for I50L, which causes high-level resistance to ATV but not to other PIs. Also selects for I84V and N88S
DRV	Selects for I54L/M
RAL	Selects for Q148H/K/R, Y143C, and N155H, which cause resistance to RAL and, in certain combinations, to DTG too
DTG	Very rarely selects for resistance in INSTI naïve provided that it is coupled with at least one other fully active drug. In patients with prior RAL exposure, mutations such as Q148H may cause decreased DTG susceptibility when combined with additional mutations

TAMs - Thymidine analogue mutations

1.12 Proteins and their structure hierarchy

Proteins are large macromolecules made up of linear chains of amino acids, they are fundamental components of all living cells (Widłak, 2013). Proteins catalyze a range of chemical reactions, provide structural rigidity to the cell, control gene function, control the flow of material through the membranes, and act as sensors (Lodish et al., 2000). The major characteristic that allows proteins to perform their diverse functions is their ability to specifically bind other molecules. One of the major areas of biological research today is how proteins, constructed from 20 amino acids, carry out all the diverse functions. Amino acids are the building blocks of proteins. All 20 amino acids are made up of a basic amino group ($-NH_2$), an acidic carboxyl group (−COOH), and an organic R group (or side chain) that is unique to each amino acid. The structures of the common amino acids are grouped into categories**.** Hydrophilic amino acids are with polar side chains; they are water-soluble and are prime contributors to the overall charge of the protein as well as its solubility. Hydrophobic amino acids are aliphatic and only slightly soluble in water. To make it easier to understand the complexity of the protein structure, early structural biochemists conceptually divided protein structures into four hierarchical levels of organization namely primary, secondary, tertiary, and quaternary protein structures; shown in Figure 1.8 below.

In brief, the primary protein structure is the simplest level, i.e. the linear arrangement or sequence of amino acids that constitute to the polypeptide chain. The final protein structure depends on this sequence (Lodish et al., 2000). The secondary structure is comprised of regions stabilized by hydrogen bonds between atoms in the polypeptide backbone. When hydrogen bonds form between atoms, the backbone folds into an α helix (spiral rod-like structure) or a β sheet (planar structure composed of β strands) (Banach et al., 2019). The next higher level is the tertiary structure, the three-dimensional (3D) shape of the protein with regions stabilized by side-chain interactions. The tertiary structure stabilizing forces are hydrophobic interactions between the non-polar side chains and sometimes disulfide bonds (Bayse and Pollard, 2019). These forces hold the α helices, β sheets, and random coils in a compact internal scaffold. Finally, a quaternary structure, which is a multimeric protein containing two or more polypeptide chains held together by a non-covalent bond. Not every protein has a quaternary structure (Bleiholder and Liu, 2019, Widłak, 2013).

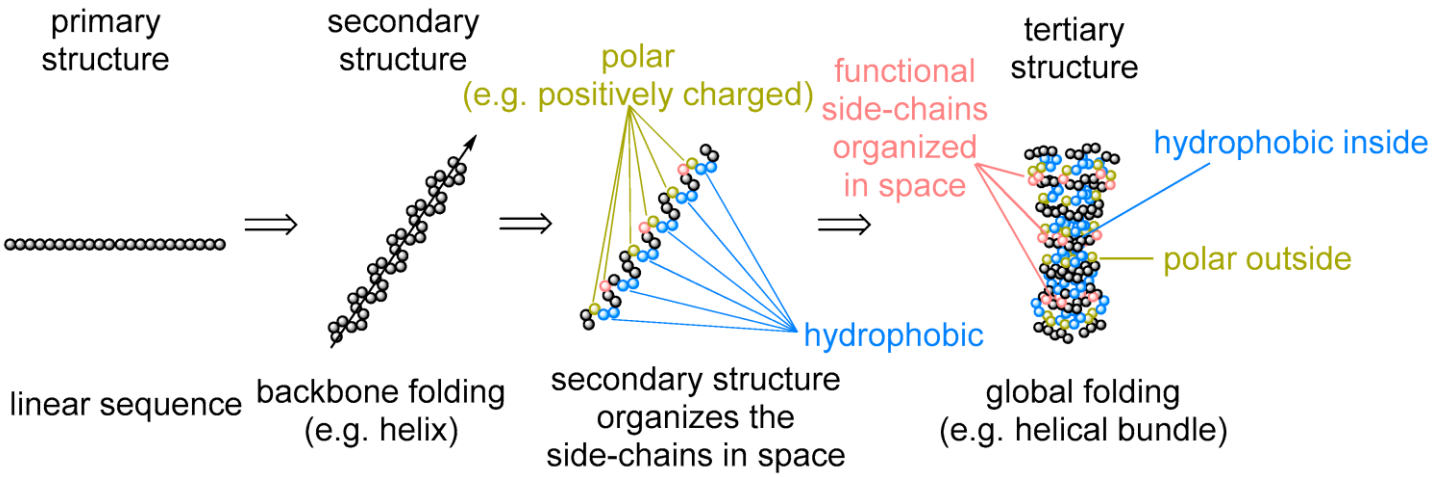


Figure 1.8 The different levels of protein structure are known as primary, secondary, tertiary, and quaternary structures. Figure adopted from (Kubyshkin and Budisa, 2019).

1.13 Sequence-structure relationship in proteins

Since Anfinsen demonstrated that bovine pancreatic ribonuclease could regain its native tertiary structure spontaneously after being exposed to a denaturant (Anfinsen, 1973), the prediction of protein from its amino acid sequence has been known to be a possibility. The past years have seen a wide range of methods for predicting both the structural and functional properties of proteins. Knowing the relationship between structure and function combined with the availability of crystallized structure of proteins has motivated the development of computational studies for predicting molecular functions using sequence and structure information (Kim et al., 2003, Lee et al., 2007, Pearson, 1996, Harrison et al., 2003). The first step towards studying protein function is predicting binding sites followed by determining the overall function of the protein in terms of biochemical function or its biological role. Successful methods that have predicted protein function have taken into account a wide range of biological data such as nucleotide sequence data, amino acids and protein structure (Sadowski and Jones, 2009, Eisenhaber, 2006).

1.14 Computational methods to study HIV- 1 drug resistance

Multiple computational methods are used to study HIV-1 drug resistance. The methods can be classified as ligand-based such as quantitative structure-activity relationship (QSAR); structure-based such as molecular docking and molecular dynamics, and universal methods such as 3D QSAR (differentiate between the methods), however, structure-based methods that rely on the 3D structure of the protein are the most frequently used.

1.14.1 Homology modeling

Homology modeling, also known as comparative modeling relies on the biological fact that two sequences sharing high similarity have a similar structure. In this method, the 3D structure is obtained in the following steps: (I) Identifying the proper template for a given protein using BLAST search, (II) sequence alignment, (III) correcting alignment to ensure the conserved residues are aligned, (IV) backbone generation, (V) loop modeling, (VI) modeling sidechain using rotamer libraries, and (VII) model validation by stereochemical evaluation using the residues in the allowed regions of the Ramachandran plot as well as favourable energies. SWISS-MODEL and MODELLER are widely used computational tools for predicting protein 3D structure in homology modeling. SWISS-MODEL is a web server for homology modeling where proteins models can be built and validated in a workspace (Bordoli et al., 2009), and MODELLER provide protein models by comparative modeling; it calculates nonhydrogen atoms to generate a model and can also be used for loop modeling and protein optimization (Fiser and Šali, 2003). PRIMO, PyMod, and MaxMod are other methods or servers used to model proteins.

1.14.2 Molecular docking

Molecular docking is used to predict the preferred orientation of the receptor to a ligand when bound to each other to form a stable complex. The predominant binding mode(s) of a ligand with a protein of known 3D structure is predicted. Two basic steps that are related to sampling methods and scoring schemes are involved in the docking process namely (I) prediction of the ligand conformation, its position and orientation, and (II) assessment of the binding affinity (Gu et al., 2014, Gromiha et al., 2019, Meng et al., 2011a, Goodford, 1985). Knowledge of the binding site location before the docking process increases docking efficiency. Information about these sites can be obtained by comparing the target protein with a family of proteins sharing similar functions or with proteins that have been co-crystallized with other ligands (Fan et al., 2009). In the absence of binding site knowledge, online servers such as GRID (Goodford, 1985, Kastenholz et al., 2000), POCKET (Levitt and Banaszak, 1992), SURFNET (Laskowski, 1995), PASS (Brady and Stouten, 2000), and MMC (Gu et al., 2014, Mezei, 2003) can also be utilized to identify putative active sites within proteins.

The two most common theories that describe the receptor-ligand interaction are the lock-and-key and the induced-fit. The lock-and-key theory was proposed by Fischer (Meng et al., 2011b), whereby the ligand fits into the receptor like a lock and key. In this model both the receptor and ligand are treated as a rigid body. The induced-fit theory by Koshland (Jiang and Kim, 1991, Hammes, 2002) states that

the protein's active site continually changes its shape with ligand interaction. Koshland, therefore, suggested that the ligand and receptor should be treated as flexible during docking.

Molecular docking is achieved through firstly, sampling conformations of the ligand in the protein active site and secondly, ranking these conformations via a scoring function (Meng et al., 2011b). The scoring functions serve to delineate the correct poses from incorrect poses. They involve estimating the binding affinity between protein and ligand and can be divided into force-field, empirical and knowledge-based (Kitchen et al., 2004). Table 1.5 lists some examples of scoring functions and the molecular docking software in which they are implemented (Ashtawy and Mahapatra, 2015).

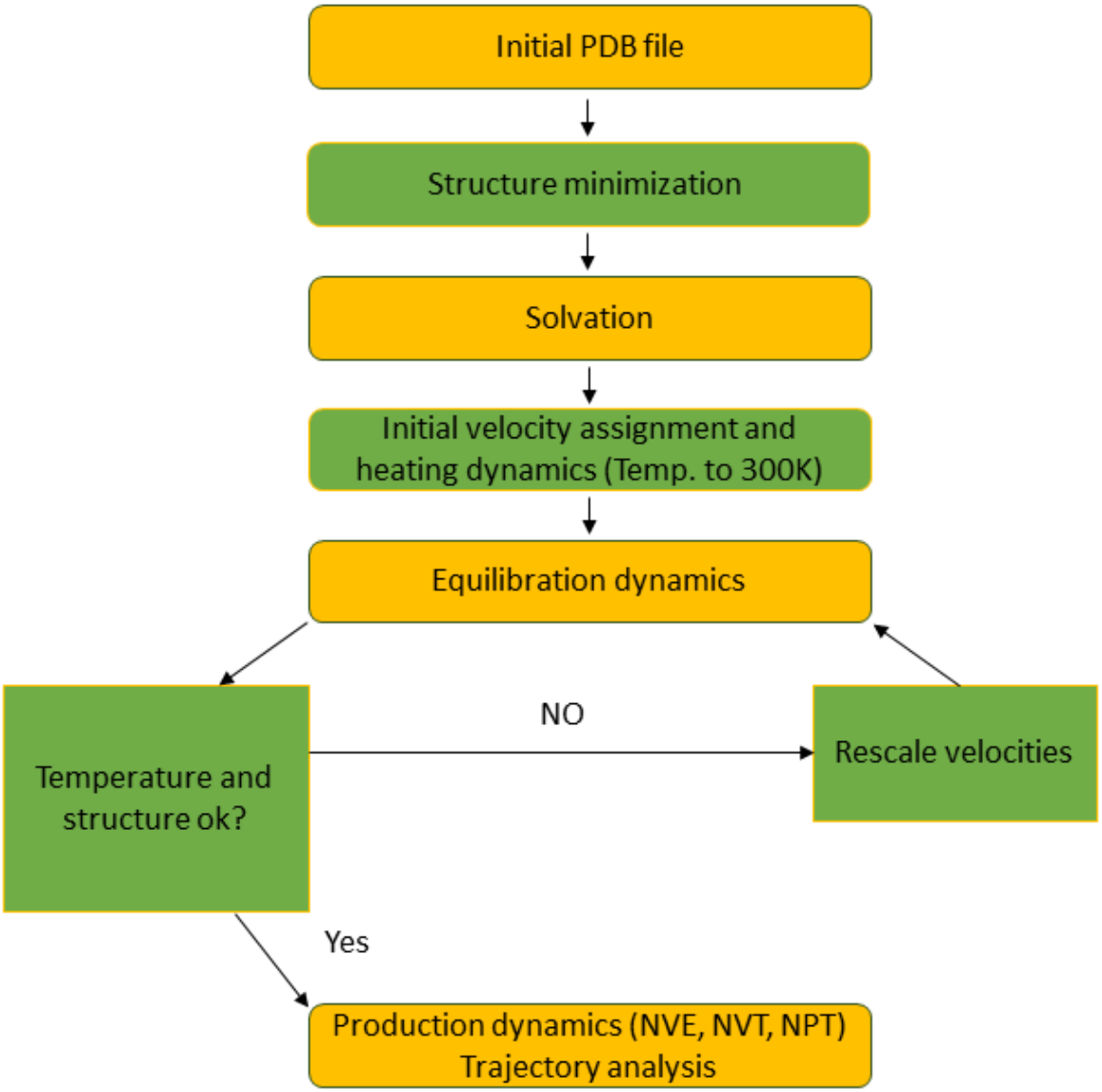
Table 1.5 List of the commonly used scoring functions in protein-ligand docking

Scoring Function	Software	Type of Scoring Function	Reference
Jain	Discovery Studio	Empirical	(Jain, 1996)
LigScore		Knowledge-based	(Krammer et al., 2005)
Ludi		Empirical	(Böhm, 1994)
PLP		Empirical	(Gehlhaar et al., 1995)
PMF		Knowledge-based	(Muegge, 2001)
ChemScore	SYBYL	Empirical	(Eldridge et al., 1997)
D-Score		Force Field based	(Ewing et al., 2001)
G-Score		Force Field based	(Jones et al., 1997)
F-Score		Empirical	(Tripos, 2001)
PMF-Score		Knowledge-based	(Muegge, 2001)
ASP	GOLD	Empirical	(Mooij et al., 2005)
Chem Score		Empirical	(Eldridge et al., 1997)
GoldScore		Forcefield	(Jones et al., 1997)
GlideScore	Glide	Empirical	(Friesner et al., 2004)
DrugScore	Standalone	Knowledge-based	(Velec et al., 2005)
X-Score		Empirical	(Wang et al., 2002)
Vina	Autodock	Empirical	(Morris et al., 1996)
		Force Field based	

1.14.3 Molecular dynamics

Molecular dynamics simulations, first developed in the late 70s (Hamelberg et al., 2004) are computer simulation methods that allow one to predict the time evolution of a system of interacting particles (atoms, molecules, granules, etc). They describe the patterns, strength, and properties of protein behaviour, drug-receptor interactions, and conformational changes a molecule or protein may undergo at various conditions. Molecular dynamics simulations are carried out to understand how molecules assemble in terms of their structure and their microscopic interactions. They give a route to understanding the properties of the system i.e. transport coefficients, time-dependent responses to perturbations, rheological properties, and spectra (Allen, 2004). They act as a bridge between theory and experiments, and between the microscopic and macroscopic world. Simulations are set up by heating molecules to a specific temperature to overcome potential energy barriers and achieve stable conformations at high temperatures. For a system of interest, the idea is to specify a set of initial conditions (initial positions & velocities of all particles in the system) and to solve a set of classical equations of motion for all particles in the system. The equations that govern the action of particles correspond to Sir Isaac Newton's second law of classical mechanics. The steps involved in protein simulations are summarised in Figure 1.9.

Force fields are a collection of equations that describe the dependency of the energy of a system on the coordinates of its particles. They represent bonded and non-bonded interactions. Bonded interactions are characterized by stretching of bonds, bending of valence angles, and rotation of dihedrals, where else, non-bonded interactions evaluate electrostatic data and dispersion (Vanommeslaeghe and Guvench, 2014). The molecular dynamics software includes force fields namely, Assisted Model Building with Energy Refinement (AMBER), Chemistry of HARvard Macromolecular Mechanics (CHARMM), and GROningen Machine for Chemical Simulations GROMACS.



NVE, Microcanonical ensemble; NVT, Canonical ensemble; NPT, Isothermal-Isobaric ensemble

Figure 1.9 Flow diagram depicting steps involved in molecular dynamics simulations of a protein

Since its introduction, molecular dynamics simulations have been widely used to study proteins in a variety of fields e.g. in chemistry and biochemistry: molecular structures, reactions, drug design, vibration relaxation and energy transfer, the structure of membranes, dynamics of large biomolecules, and protein folding; in statistical mechanics and physics: theory of liquids, properties of statistical ensembles, structure and properties of small clusters and phase transitions; and in material science: point, linear, and planar defects in crystals and their interactions, microscopic mechanisms of fracture, surface reconstruction, melting and faceting. The structure of HIV-1 integrase-inhibitor complexes has been investigated by computational modeling. Keseru and Kolossbury had performed flexible docking on 5-CITEP and L-731988 (Keserû and Kolossváry, 2001) to identify whether Merck inhibitors bind in the same mode. Molecular dynamics simulations have also been applied to native and mutated

integrase in the past years to investigate HIV-1 integrase DNA binding and to understand the role of drug resistant mutations on the conformational dynamics of the HIV-1 integrase core domain (Lins et al., 2000, Brigo et al., 2005, Lee et al., 2005). Recently, molecular dynamics simulations have been applied to investigate the effect of mutations on HIV-1C integrase (Chitongo et al., 2020).

1.15 The rationale for this thesis

Although initial reports have shown HIV-1C wild-type integrase to exhibit similar degrees of susceptibility to RAL and EVG as that of HIV-1B wild-type (Bar-Magen et al., 2010a, Brenner et al., 2011), data indicates that those integrase enzymes with known resistance mutations may differentially affect susceptibility to INSTIs in subtype B and C (Mesplède et al., 2015, Mikasi et al., 2021). While the majority of INSTI resistance research has been conducted using HIV-1 subtype B infected populations because of its predominance in developed countries (Wainberg and Brenner, 2012), data on HIV-1C (which is highly endemic in sub-Saharan Africa) is limited (HIV/AIDS, 2014b, de Waal et al., 2018). Subtype-specific polymorphisms in HIV-1C integrase have been shown to affect the protein structure (Quashie et al., 2015b), which could potentially influence the development of resistance to INSTIs (Chehadeh et al., 2017, Wainberg and Brenner, 2012, Low et al., 2009). The altered side-chain interactions and conformational changes induced by the mutations could impact enzyme functioning through altered DNA binding (Quashie et al., 2015a, Quashie et al., 2015b, Machado et al., 2019). It was therefore important to identify and determine the impact of subtype-specific polymorphisms and INSTI RAMs on HIV-1C integrase structure and functioning, especially in light of the expanding access to INSTI-based cART in LMICs.

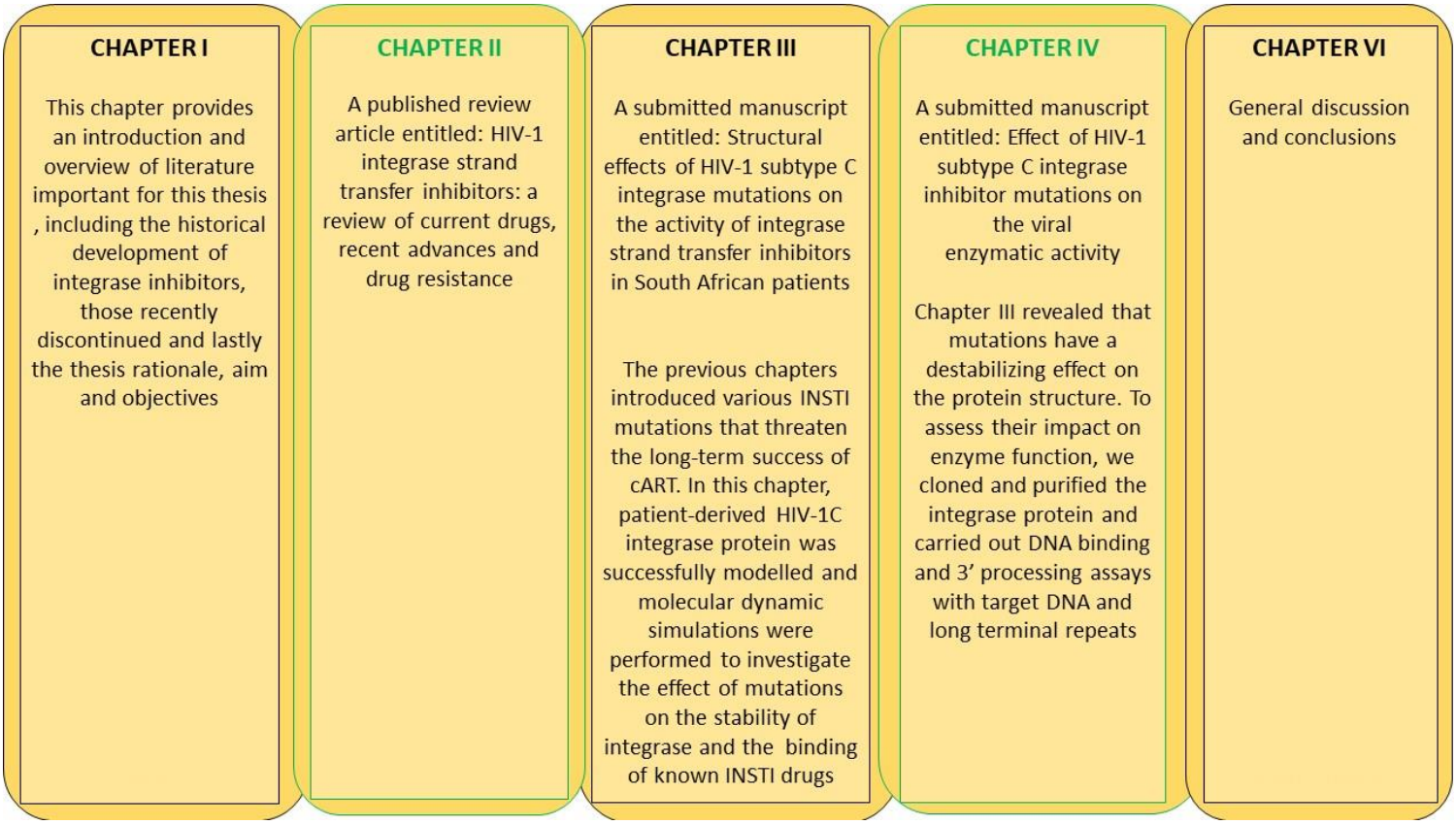
1.16 Study aim and objectives

The overall aim of the thesis was to determine the effect of HIV-1C RAMs on integrase protein structure and function. This was accomplished through fulfilling the following objectives:

- To genotypically characterize HIV-1C integrase gene in a South African context and identify RAMs associated with INSTIs
- To determine the effect of integrase RAMs on HIV-1C enzyme-substrate binding using computational methods
- To determine the effect of integrase RAMs in HIV-1C on retroviral DNA binding
- To determine the effect of RAMs on the integrase 3' processing activity

1.17 Thesis Outline

The chapters presented in this thesis are as follows:



CHAPTER TWO

HIV-1 INTEGRASE STRAND TRANSFER INHIBITORS: A REVIEW OF CURRENT DRUGS, RECENT ADVANCES AND DRUG RESISTANCE

Abstract

Antiretroviral therapy (ART) has been imperative in controlling the HIV epidemic. Most low- and middle-income countries (LMICs) have used nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) extensively in the treatment of HIV. However, integrase strand transfer inhibitors (INSTIs) are becoming more common. Since their identification as a promising therapeutic drug, significant progress has been made which has led to the approval of five INSTIs by the Food and Drug Administration, i.e. Dolutegravir (DTG), Raltegravir (RAL), Elvitegravir (EVG), Bictegravir (BIC), and Cabotegravir (CAB). INSTIs have shown to effectively halt HIV-1 replication and are commended for having a higher genetic barrier to resistance, in comparison to NRTIs and NNRTIs. More interestingly, DTG has shown a higher genetic barrier to resistance compared to RAL and EVG, and CAB is being used as the first long-acting agent in HIV-1 treatment. Considering the increasing interest in INSTIs for HIV-1 treatment, we focus our review on the retroviral integrase, development of INSTIs and their mode of action. We also discuss each of the INSTI drugs, including potential drug resistance and known side effects.

Keywords: HIV-1; Integrase inhibitor; Antiretroviral therapy

Background

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) was first reported in 1981 (Becken et al., 2019), and soon became a major epidemic and one of the greatest health challenges to humankind. Today, scientific and therapeutic progress against HIV has been remarkable. There has been a dramatic change in the life expectancy of people living with HIV, as new antiretroviral therapies are developed. Previously antiretroviral therapy (ART) was initiated based on clinical evidence of AIDS or a decrease in CD4 cell count. Recently life-long ART is initiated immediately after a patient's diagnosis, regardless of the CD4 cell count (i.e. test-and-treat) (Senthilingam, 2015), as supported by well-defined benefits of early ART initiation.

Antiretroviral regimens containing a combination of at least two, preferably three active drugs from two or more drug classes are recommended. The most common antiretroviral drugs used in low- and middle-income countries (LMICs), include reverse transcriptase, and protease inhibitors (PIs). Most first-line regimens use combinations of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), with PIs being used in second-line and integrase strand transfer inhibitors (INSTIs) in third-line regimens. However, INSTIs are becoming more common in first-line ART, with the World Health Organization (WHO) recommending the use of Dolutegravir (DTG) (i.e. an INSTI drug) in first-line treatment (WHO, 2016, Moorhouse et al., 2018).

This article reviews the development of INSTIs including those under late stages of clinical testing, their chemical structure, and mechanism of action. We discuss in detail the development of DTG and its use in sub-populations such as pregnant women and in people living with HIV-1 and tuberculosis (TB) co-infection. We also discuss the development of Raltegravir (RAL), Elvitegravir (EVG) and Bictegravir (BIC), as well as Cabotegravir (CAB), which was recently approved for use in HIV treatment by the US Food and Drug Administration (FDA) (Durham and Chahine, 2021, Voelker, 2021). We further describe potential drug resistance to the INSTIs and known side effects, paying particular attention to the adult population, highlighting differences in first- and second-generation structural features and provide a summary on the use of INSTIs.

HIV-1 Integrase

Chemical structure and INSTI mode of action

HIV integrase is a 32kDa protein encoded within the HIV *pol* gene together with the HIV reverse transcriptase and protease enzymes. Integrase is at the 3' end of the *pol* gene, and its generation occurs during virus maturation when HIV protease cleaves the *gag-pol* polyprotein. It is comprised of three structural domains, i.e. the amino-terminal domain (NTD), the catalytic core domain (CCD), and the carboxy-terminal domain (CTD) (Figure 1).

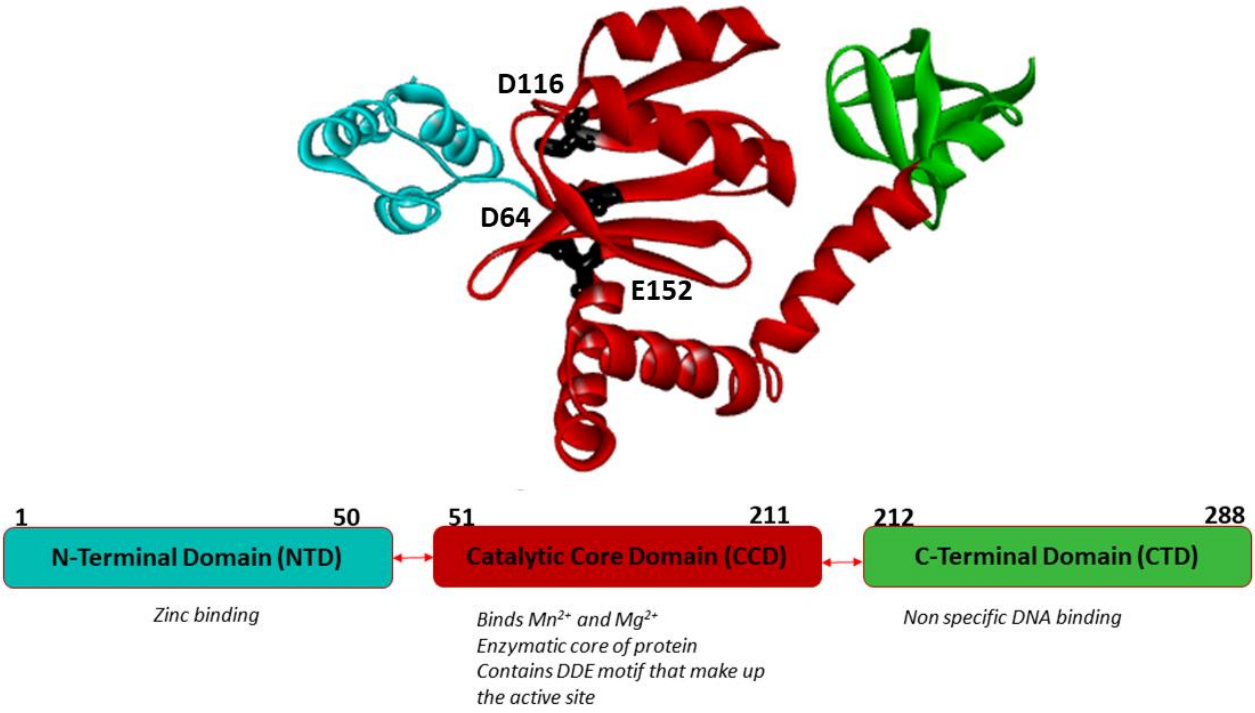


Figure 1 Structural domains of integrase.

Integrase functions as a dimer, and/or exists in an oligomeric state. The integrase inner and outer subunits have a highly dynamic interaction, an essential property for integrase function, and the dimer is stabilized through the additional interactions between the NTD monomers (McKee et al., 2008). The elucidation of retroviral structures by X-ray crystallography and single-particle cryogenic-electron microscopy (cryo-EM) has provided insight into the integration of retroviral integrase (Engelman and Cherepanov, 2017, Passos et al., 2017). Although the previously used prototype foamy virus (PFV) crystal structure was useful in providing an initial image of the retroviral intasome structure, it has limitations including that the HIV-1 integrase system behaves differently from PFV in terms of folding, recognition, and stability (Dayer, 2016). Recently, high-resolution simian immunodeficiency virus red-

capped mangabey ($SIV_{rcm)}$ and HIV intasome structures determined in the presence of bound second-generation inhibitors have been of great interest to further improve the structural basis of INSTIs development that will benefit future drug design (Cook et al., 2020). Moreover, the recently determined SIV_{rcm}/HIV structures revealed important differences in the active site that were previously concealed in structures of PFV intasomes (Cook et al., 2020).

Integrase is now better understood as a nucleotidyltransferase enzyme with two divalent metal cations at the active site. In the HIV replication cycle, integrase catalyses the insertion of the reverse-transcribed viral genome into the host cell (Ciuffi, 2016). The two distinct steps catalysed by integrase are 3' processing and strand transfer. The 3' end processing step removes a 3' dinucleotide from each viral DNA and strand transfer covalently links the viral and host DNA. To block viral integration, INSTIs bind to the metal cations, blocking the enzyme active site by inactivating the intasome and dislocating the 3' terminal nucleotide of the viral DNA (Kotova et al., 2010). INSTIs prevent formation of the covalent bond with host DNA, thus inhibiting the incorporation of viral DNA into the host genome.

HIV integrase strand transfer inhibitors development

Merck investigators and Shionogi scientists were pioneer companies in the research and development of INSTIs. Merck discovered diketo acids (DKA's) inhibitors after screening a series of compounds including L-708,906, (Hazuda et al., 2000), L-731,988 (Hazuda et al., 2000), 870,810 and L-870,812. The latter two compounds were both derivatives of 8-hydroxy-[1,6]-naphthyridine-7-carboxamides (Zhuang et al., 2003) and were found to inhibit integrase strand transfer with significantly high potency (Hazuda et al., 2000), resulting in the term INSTIs. DKA's bind to the HIV-1 integrase catalytic site in the presence of viral DNA long terminal repeats and also interact with the viral DNA 5' end (Marchand et al., 2002).

Subsequent variations of DKA's led to the first clinically tested INSTI compound, S-1360. The compound was developed by Shionogi, and initially had a good pharmacological and pharmacokinetic profile in animals, however, it was found to be rapidly cleared through glucuronidation in human trials (Rosemond et al., 2004) and its development was curtailed. Further work by Merck investigators on lead compounds such as L-870,812 including pharmacokinetic profiles, physicochemical properties and optimization for potency, led to the discovery of the first INSTI to be approved by the FDA for HIV-1 clinical trials, named RAL (Anker and Corales, 2008). To date, there are five INSTIs approved by the FDA; i.e. RAL, EVG, DTG, BIC and CAB, classified as first- and second-generation INSTIs. Supplemental Table S1 summarises the structural differences between the first and second-generation INSTIs.

FDA approved first-generation INSTIs

First-generation INSTIs include RAL and EVG, drugs that have a bicyclic central pharmacophore scaffold ring system responsible for metal chelation.

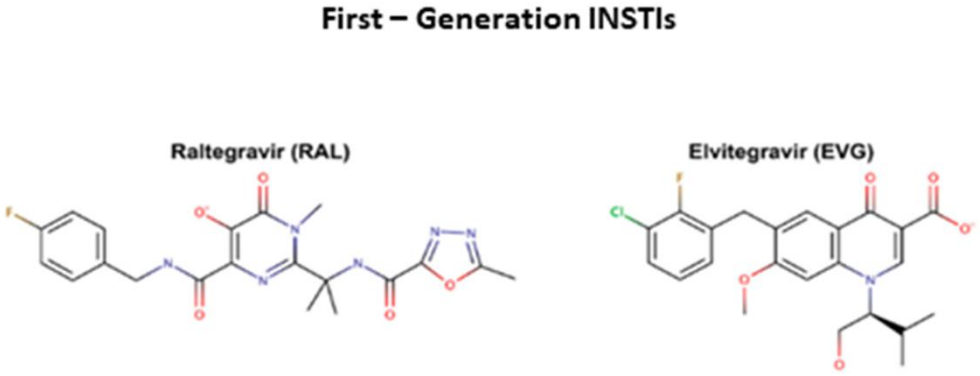


Figure 2 Chemical structures of first-generation INSTIs, RAL and EVG.

(This figure has been reproduced with permission from Lee et al., 2019 (Lee et al., 2019)).

Raltegravir

Raltegravir was the first-generation INSTI for HIV-1 treatment to be approved by the FDA (Summa et al., 2008). The terminal half-life of RAL is approximately 9 hours supporting the use of a twice-daily dosage. Within the first two days of dosing twice daily with RAL, a pharmacokinetic steady state is achieved (Temesgen and Siraj, 2008). When a single 400mg dose of RAL is administered, a delay is shown in the time to reach maximum plasma concentration (C_{max}), with a 34% decrease in C_{max} (Kassahun et al., 2006). One of the advantages of RAL is that no dosing adjustments are necessary for age, body mass index, gender and even for patients with kidney conditions (Temesgen and Siraj, 2008, Kassahun et al., 2006).

During clinical trials, a randomized non-inferiority placebo-controlled multi-centre 004 study, was conducted in HIV infected adults that are naïve to antiretroviral drugs with an HIV-1 RNA plasma level of ≥5000 copies/mL, or CD4 T cell count of ≥100 cells/mm^3 at screening. Four RAL doses (i.e. 100, 200, 400 and 600 mg) were compared to the placebo in the first part of the study conducted over 10 days in 35 patients (Markowitz et al., 2006). Following the 10-day study, all participants had similar antiviral response on all RAL groups, and this was significantly greater compared with placebo by day 10, with

at least half of the participants achieving an HIV RNA level of <400 copies/mL in each RAL dose group, with no serious adverse events reported (Markowitz et al., 2006).

The second part of the 004 study consisting of 198 participants, showed non-inferior safety and efficacy of RAL, when compared to Efavirenz (EFV), Lamivudine (3TC) and Tenofovir (TDF), among treatment naïve participants (Markowitz et al., 2006). At week 48, participants had >2.2 $\log_{10}$ decline in viral RNA, in both RAL and EFV arms. Also, an increase in CD4 T cell count was demonstrated in all arms, with mild to moderate adverse events across all treatment arms (Markowitz et al., 2006). A subsequent 005 study further assessing efficacy and safety of RAL among 178 HIV infected treatment-experienced participants with triple-class resistance (i.e. NRTI, NNRTI and PI resistance) showed better virological and immunological outcomes ($p<0.0001$) with RAL when compared to the placebo arm (Grinsztejn et al., 2007). BENCHMARK studies also evaluated the safety and efficacy of RAL in participants with triple-class resistance and showed superior efficacy with RAL compared to placebo arms at week 24 (Cooper, 2007, Steigbigel, 2007). These studies supported the use of RAL which was approved by the FDA for HIV treatment in 2007, and has mainly been used as a third-line ART drug, although this is bound to change with the introduction of DTG in first-line ART.

Resistance to RAL and reported side effects

Most patients that experience virological failure while on a RAL-containing regimen have been shown to fail treatment with mutations that confer resistance to RAL (Kassahun et al., 2007, Temesgen and Siraj, 2008). Major mutations R263K, N155H, Q148HKR, Y143RHC and F121Y have been identified in the integrase enzyme coding region. Also, accessory mutations such as L74M, E138AK, G140AS, E92Q, and T97A may be present in RAL virological failure but contribute modest resistance when not combined with major mutations (Temesgen and Siraj, 2008). These mutations, however, seem to reduce viral replication capacity (Kassahun et al., 2007). Reported RAL side effects including nausea, muscle pain, tenderness, weakness, occasional dizziness**,** severe skin reactions, allergic reactions, and liver problems, could contribute to inadequate treatment adherence, increasing the risk of developing drug resistance (National Institutes of Health, 2020).

Elvitegravir

Elvitegravir was developed by Gilead Sciences and was the second INSTI approved by the FDA for HIV treatment. EVG is a 4-quinolone-3-carbocyclic acid compound that has potent antiviral activity to various strains of HIV (Kobayashi et al., 2011). It is primarily metabolised by cytochrome p450 CYP3A. Co-administrating it with a strong CYP3A inhibitor such as Cobicistat or Ritonavir substantially boosts

its plasma concentration and prolongs its elimination half-life to approximately 9.5 hours, with once-daily administration at a 150mg dosage (Ramanathan et al., 2011).

During clinical trials, EVG's antiviral activity was evaluated in a 10-day phase 1b monotherapy trial. Forty participants infected with HIV-1 were grouped into five ritonavir dosages (200mg, 400mg and 800mg twice daily; 50 mg plus 100 mg once daily or 800mg also once a day). All participants receiving 400 mg EVG showed a viral load (VL) reduction of >1 $\log_{10}$ copies/mL, with half of the participants showing a VL reduction of >2 $\log_{10}$ copies/mL. Phase II clinical trials showed >1 $\log_{10}$ copies/mL VL reduction in over 90% of participants that were receiving 50 and 125 mg EVG plus ritonavir, and an increase in mean CD4 T cells compared to controls (Shimura and Kodama, 2009, Health). A phase III efficacy and safety study on ritonavir-boosted EVG (EVG/r) showed virologic suppression to <50 copies/mL at 48 weeks, with 150 mg EVG/r once daily (Health). Since its approval by the FDA for HIV treatment in 2014, EVG has not been used extensively in public ART programs in LMICs.

Resistance to EVG and reported side effects

Studies have reported emergence of the R263K, N155H, Q148HKR, S147G, F121Y, E92Q and T66I as major resistance mutations, as well as accessory mutations including T97A, E92G and T66AK from EVG treatment (Wohl et al., 2014, Clumeck et al., 2014). Notably, these mutations show relatively similar resistance patterns with RAL (i.e. cross-resistance). The most commonly reported EVG side effects include diarrhoea and immune reconstitution inflammatory syndrome (National Institutes of Health, 2020).

FDA approved second-generation INSTIs

Development of drug resistant mutations to first-generation INSTIs increased the need for new and improved antiviral INSTIs that have limited or no cross-resistance. Figure 3 shows the chemical structures of the second-generation INSTIs, and Supplemental Figure S1 shows their interaction with viral DNA.

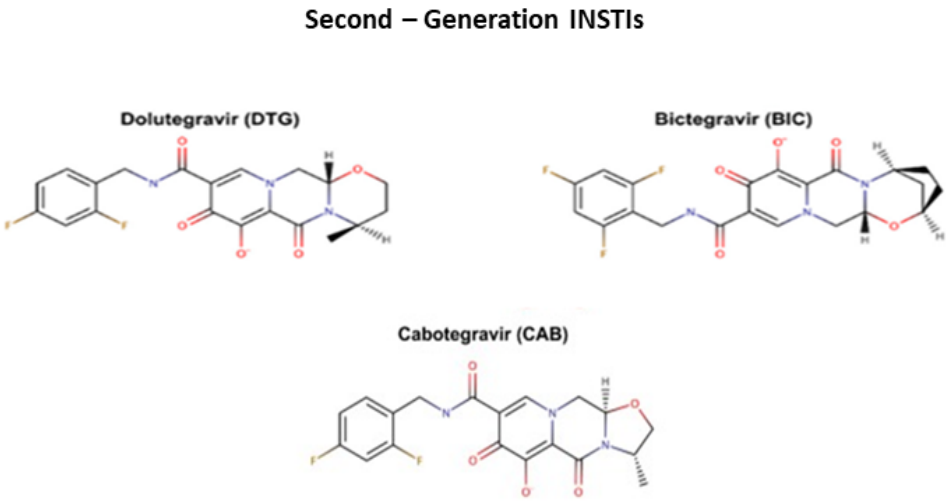


Figure 3 Chemical structures of second-generation INSTIs DTG, BIC and CAB, showing their structurally similar tri-cyclic central pharmacophores.

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Dolutegravir

Dolutegravir is a highly potent chiral compound that was developed through optimization of a series of carbamoyl pyridone analogs, and approved by the FDA for HIV treatment in 2013 (Kobayashi et al., 2011). The C-5 carboxamide on DTG renders it more structurally flexible, allowing DTG to be more embedded into the integrase active site hydrophobic pocket when compared to other INSTIs (Johns et al., 2013). DTG's notable advantage is its ability to maintain high potency against mutant HIV-1 strains that are resistant to RAL and EVG (Moorhouse et al., 2018). In the SPRING-1 study, DTG was shown to be effective and tolerable when given once daily to ART-naïve adults (without a pharmacokinetic booster), compared to EFV (Stellbrink et al., 2013). The SPRING-2 and SAILING studies assessing once daily 50mg dosage of DTG against twice daily 400mg RAL showed that DTG was non-inferior to RAL in ART-naïve and ART-experienced individuals, respectively (Raffi et al., 2013, Cahn et

al., 2013). In the VIKING study, using DTG 50mg twice daily showed greater activity in individuals with documented INSTIs resistance compared to a 50mg once daily dosage, with a significant proportion achieving VLs of <50 copies/mL at week 24 (Eron et al., 2012). These results showed that DTG may be warranted even in the presence of INSTI resistance with higher dosage (Eron et al., 2012), making DTG a potential drug in salvage regimens.

Following assessment of DTG benefits and risks, its lower cost, tolerability, as well as the increased number of ART-naïve people with NNRTI resistance, the WHO recommended its use as the preferred drug for first-line treatment (Moorhouse et al., 2018). As such, more countries in LMICs (including South Africa) are transitioning from the use of NNRTI-based first-line regimens to the one pill a day combination of TDF, 3TC and DTG (Zash et al., 2018, Dorward et al., 2018). In an effort to minimize the cost of ART and long-term adverse effects, a shift from the three-drug DTG-based regimen to a two drug (DTG + 3TC) regimen has been under investigation (Cento and Perno, 2020). The GEMINI phase I & II non-inferiority trials showed long term efficacy and safety of the dual drug regimen compared to the three drug DTG-based regimen in ART treatment-naïve adults (Cahn et al., 2020). Subsequently, in the phase III, randomized, non-inferiority TANGO study, DTG + 3TC demonstrated noninferiority in maintaining virologic suppression in patients switching from a tenofovir alafenamide (TAF)-based regimen versus those who remained on the TAF-based regimen (van Wyk et al., 2020).

Of special interest are women of childbearing age, due to the potential for neural tube birth defects when DTG is used at the time of conception or during the first three months of pregnancy (Dorward et al., 2018, WHO, 2018a). The WHO previously recommended effective contraception when DTG is used in such instances, as more data around its risks in pregnancy were still being investigated. The Tsepamo study presented at the 2020 AIDS conference, reported that the prevalence of neural tube defects in babies exposed to DTG is much lower than initially reported, resulting in the WHO recommending DTG for all adults, including women of childbearing potential (SAHCS, 2020).

However, there remain concerns in concurrent use of DTG-based ART and Rifampicin-based TB treatment because of potential drug-drug interactions. Rifampicin is a potent inducer of CYP3A and glucuronosyltransferase, UGT. Dolutegravir is a substrate of UGT1A1 and CYP3A, and so co-administration with Rifampicin potentially decreases DTG concentrations (Cottrell et al., 2013). In such cases DTG twice daily dosing has shown to be more effective (Dooley et al., 2019).

Resistance to DTG and reported side effects

Despite DTG's high genetic barrier to resistance, there is still potential for development of HIV drug resistance to the drug. The major mutations associated with DTG resistance are R263K, Q148HKR, and G118R, with some minor mutations (i.e. N155H, G140AS, E138AKT, T66K, E92Q, and F121Y) also being accessory to DTG resistance. Some of the risk factors associated with DTG resistance include poor treatment adherence, low CD4 count or high VL at the time of DTG initiation, and drug-drug interactions from co-treatment (Lübke et al., 2019). Besides the concern in birth defects, the most commonly reported side effects associated with DTG are nausea and occasional dizziness (Cid-Silva et al., 2017). Of late, there are also worrying side effects of DTG-associated liver problems and weight gain (Bourgi et al., 2019, Wang et al., 2018).

Bictegravir

Bictegravir, the most recent INSTI developed by Gilead Sciences (Lazerwith et al., 2016), differs from other INSTIs in that it contains a unique bridged bicyclic ring and a distinct benzyl tail (Tsiang et al., 2016). An in vitro study showed that BIC exhibits synergistic antiviral activity with Emtricitabine and Tenofovir alafenamide (FTC/TAF), as well as with Darunavir (Tsiang et al., 2016). BIC also displayed an improved resistance profile over RAL and EVG, comparable to that of DTG. A phase I study evaluating BIC antiviral activity, safety and pharmacokinetics showed BIC to be well tolerated, with good absorption and plasma half-life among drug naïve HIV-infected adults, following 10 days of once daily BIC therapy (Tsiang et al., 2016). Phase II studies comparing BIC to DTG for initial ART among 98 ART-naïve participants showed both drugs to be highly effective, safe and well-tolerated with no significant resistance observed (Sax et al., 2017). Following phase III trials using single-tablet regimen of BIC + FTC/ TAF and subsequent studies, BIC was approved by the FDA for HIV treatment in 2018 (Han et al., 2017a). These studies include a randomized controlled clinical trial of BIC co-formulated with FTC/TAF in a fixed-dose combination (BIC/FTC/TAF) versus DTG + FTC/TAF in treatment-naïve HIV-1 positive adults (Stellbrink et al., 2018). The single-tablet combination of BIC proved virologically non-inferior to the DTG combination after 96 weeks.

Resistance to BIC and reported side effects

The major BIC resistance mutations include R263K and Q148H, with accessory mutations G140S, E138K, T66K, E92Q, and G118R (Wensing et al., 2019). Notably, the R263K and Q148H mutations have been reported to cause cross-resistance across both first and second-generation INSTIs. The most commonly reported BIC side effects include diarrhoea, nausea and tiredness (National Institutes of Health, 2020).

Cabotegravir

Cabotegravir is a new integrase inhibitor developed by ViiV Healthcare, with a carbamoyl pyridone structure similar to DTG (Yoshinaga et al., 2015). It has a long half-life of approximately 40 hours as an oral formulation and 21-50 days as an injectable. Phase I/II clinical trials showed that CAB has the potential to be used for HIV-1 treatment as a long-acting drug, i.e. LA-CAB (Han et al., 2017b). In a phase I study, time profiles for LA-CAB absorption and plasma concentration were similar between intramuscular and subcutaneous (IM and SC) administration, with LA-CAB 80 mg IM dose achieving mean concentrations above protein adjusted IC_{90} and rectal & cervicovaginal tissue concentrations from less than 8 - 28% of corresponding plasma concentrations for 16 weeks (Spreen et al., 2014a). These results indicated that LA-CAB can be administered less frequently (potentially once a month) and still remain efficacious in prevention and treatment of HIV-1 infection (Han et al., 2017a).

A phase I study assessing the pharmacokinetics, safety and tolerability of CAB and rilpivirine (RPV) (Spreen et al., 2014b) in participants receiving an oral dose of 30 mg daily for 14 days showed that LA-CAB and RPV injections were well tolerated with minor side effects. Therapeutically relevant plasma concentrations for CAB and RPV suggest that LA-CAB and RPV dual therapy has potential for treatment of HIV-1 infection (Han et al., 2017a). In a phase II study, once daily dose of CAB (i.e. 5 or 30 mg) showed a 2.3 $\log_{10}$ copies/mL plasma viral decrease compared to the placebo, demonstrating that once daily doses of 5 or 30 mg CAB reduce plasma HIV-1 VL, and that CAB is well tolerated in both healthy and HIV-1 infected individuals (Spreen et al., 2013, Han et al., 2017a). Two phase III studies, the Long-Acting Injectable Regimen (FLAIR) study and ART as LA Suppression (ATLAS) study, showed high level of viral suppression with low discontinuations due to virologic failure following CAB/RPV LA injections (Fernandez and van Halsema, 2019). Based on these results, CAB/RPV LA regimen dosed once monthly was approved in Canada to replace current ART in clinically stable, virally suppressed adult patients (Markham, 2020). More recently, the once-a-month dosing of CAB/RPV (Cabenuva) has been approved by the FDA as the first complete LA injectable regimen for the treatment of HIV-1 in

virologically suppressed adults with no suspected resistance to CAB or RPV (Durham and Chahine, 2021, Voelker, 2021). In addition, FDA approved an oral lead-in of CAB (Vocabria) in combination with RPV (Edurant) to be taken for one month before starting treatment with Cabenuva (Durham and Chahine, 2021).

Resistance to CAB and reported side effects

Long-acting drugs such as CAB help to reduce events of non-adherence, thus limiting potential development of drug resistance. However, there are mutations already known to confer resistance to CAB. Again, major resistance mutations R263K, and Q148HKR have been reported, including G140R and G118R, and accessory mutations N155H, S153FY, G140ACS, E138AKT and T66K. The major mutations in CAB are similar to those that cause resistance to DTG, which raises concerns over the utility of CAB once people fail DTG-based ART with integrase resistance mutations (Wensing et al., 2019). The most commonly reported CAB side effects are fatigue, fever, headache and nausea (National Institutes of Health, 2020).

Drug profile summary of each INSTI

Table 1 briefly summarizes each of the INSTI drugs discussed in this review, including their advantages and disadvantages. The development of drug resistance remains an issue and highlights the need to continue developing new and improved drugs for HIV treatment.

Table 1 Summary comparison of INSTIs including their advantages and disadvantages

	RAL	**EVG**	**DTG**	**BIC**	**CAB**
FDA status	Approved in 2007	Approved in 2012	Approved in 2013	Approved in 2018	Approved in 2021
Generation	First	First	Second	Second	Second
Dosing	400mg, twice daily 600mg, once daily	150mg once daily + booster	50mg, once daily in INSTI-naïve patients 50mg, twice daily in INSTI-experienced patients	50mg BIC + FTC/TAF, once daily	LA-CAB, 80mg once a month Oral CAB, 30mg once daily
Half-life: $t_{1/2}$	~9 hours	~9.5 hours	~15 hours	~18 hours	LA CAB, ~21-50 days Oral CAB, 40 hours
Metabolism	UGT1A1	Cytochrome P450 CYP3A4, major UGT1A1/3, minor	Glucuronidation UGT1A1, major CYP3A4, minor	Cytochrome P450 CYP3A4 UGT1A1	UGT1A1, major UGT1A9, minor
Clinically significant drug interactions	Rifampicin Etravirine Tipranavir/ritonavir	Rifampicin Antacids Efavirenz Nevirapine	Rifampicin Efavirenz Nevirapine	Rifampicin Antacids	No data available
Food requirement	No	Yes	No	No	LA CAB, No Oral CAB, Yes
Use in pregnancy	Recommended	Not Recommended	Alternative preference	No data available	No data available
Use in HIV/TB co-infection	Dose adjustment is required	Not Recommended	Dose adjustment is required	Not recommended	No data available
Advantages	- INSTI with the longest track record of safety and efficacy	- Administer once daily	- Administer once daily - High genetic barrier to Resistance	- Administer once daily - Low rate of resistance	- Administer once daily and/or bimonthly with a longer half-life - Low rate of resistance
Disadvantages	- Resistance develops rapidly from virologic failure - Cross-resistance to EVG - Lower genetic barrier to resistance compared to DTG and BIC	- Resistance develops rapidly from virologic failure - Cross-resistance to RAL - Lower genetic barrier to resistance than DTG and BIC - Must be administered with a pharmacologic booster	- Separate dosing from antacid - Raises serum creatinine levels	- Raises serum creatinine levels - Insufficient data in pregnant women	- Injection intolerance - Insufficient data in pregnant women

Conclusions

The UNAIDS 90-90-90 goal aims for ending the AIDS epidemic by 2030 (HIV/AIDS, 2014a). Durable efficacy, tolerability and safety of HIV drugs play a pivotal role in achieving these ambitious goals. In addition, focus on simplified ART regimens is crucial in future clinical developments of HIV treatment. HIV integrase has been validated as a therapeutic target with INSTIs approved by the FDA and other agents already in clinical trials. The FDA approved INSTIs have been proven to be important for the treatment of HIV-1 infected people (Mesplède et al., 2014), with DTG being particularly shown to have a much higher genetic barrier to resistance.

One of the noticeable limitations of INSTIs in ART is potential cross-resistance due to mutations Q148H and R263K (Figure 4). These two mutations cause low to high-levels of resistance to all available INSTIs (Wensing et al., 2019). Treatment naïve individuals on RAL and EVG based therapy experience virological failure associated with mutations in the RT and/or integrase region (Mesplède and Wainberg, 2015), whereas no mutation in either RT or integrase has been found from treatment naïve individuals experiencing DTG failure. However, treatment-experienced INSTI-naïve individuals experience DTG failure associated with the emergence of R263K mutation showing that previous exposure to RAL and EVG can compromise the efficacy of DTG when used as a single antiretroviral agent.

	E	E	F		G	L	N	Q	R				T	Y
RAL	92	138	121		140	74	155	148	263				97	143
	Q	AK	Y		AS	M	H	HKR	K					HRS
	E		F				N	Q	R	S		T	T	
EVG	92		121				155	148	263	147		66	97	
	GQ		Y				H	HKR	K	G		AIK	A	
	E	E	F	G	G		N	Q	R			T		
DTG	92	138	121	118	140		155	148	263			66		
	Q	AKT	Y	R	AS		H	HKR	K			K		
		E		G	G		N	Q	R		S	T		
CAB		138		118	140		155	148	263		153	66		
		AKT		R	ACRS		H	HKR	K		FY	K		
	E	E		G	G			Q	R			T		
BIC	92	138		118	140			148	263			66		
	Q	K		R	S			H	K			K		

Figure 4 Mutations in the integrase enzyme associated with resistance to INSTIs. The vertical rectangle in red highlights cross-resistance from mutations at codons 148 and 263 of the enzyme.

Since R263K confers low-level resistance to DTG in tissue culture and is rarely selected *in vivo*, the introduction of the efficacious DTG in first-line ART provides hope for achieving lower population VLs and decreased transmissions. The structural differences of DTG (Table S1) do not only allow it to be

highly effective against HIV-1, but also more potent against integrase mutants that confer resistance to first-generation INSTIs i.e. RAL and EVG (Smith et al., 2018). Although the usefulness of most drugs is limited by the emergence of resistance mutations, there is a need to develop and test new INSTIs with no serious side effects, and drugs that can inhibit resistant viral strains that emerge under the selective pressure of current INSTIs.

Despite the advantages of BIC demonstrating improved antiviral potential against various INSTI resistant clinical isolates compared to DTG and demonstrating a slow rate of resistance mutations (Hassounah et al., 2017), Q148 and R263K/M50I mutations have been reported to reduce its effectiveness (Anstett et al., 2017). Long-acting injectable formulations of drugs with established oral efficacy such as CAB could provide more simplified options and potentially more convenient ART, which would subsequently reduce emergence of drug-resistant virus (Cihlar and Fordyce, 2016). However, we emphasize that despite the evidence of emerging resistance to INSTIs, the rates remain lower compared to other ART drug classes.

In summary, this review shows that INSTIs have a future role in novel therapies to achieve the UNAIDS 90-90-90 goal and need to be further explored. Their ability to suppress virus in people with multi-drug resistance strains of HIV-1 is encouraging. Their unique properties of offering minimal toxicity, high viral suppression, high antiviral activity, high genetic barrier for the second-generation INSTIs and less severe side effects make them an important drug class-backbone for future regimens. However, there is a need to develop new generations of INSTIs if the drug class is to be used for several years to come, as well as developing new drug class agents for HIV treatment.

CHAPTER THREE

STRUCTURAL EFFECTS OF HIV-1 SUBTYPE C INTEGRASE MUTATIONS ON THE ACTIVITY OF INTEGRASE STRAND TRANSFER INHIBITORS IN SOUTH AFRICAN PATIENTS

Link between the above chapters and three

The previous chapters introduced HIV-1integrase, its structure, and discussed INSTIs as well as their resistance mutations. We further highlighted the importance of INSTIs in cART. Three drugs of this class are currently available for use in the South African healthcare sector i.e. RAL, EVG, and DTG. In this chapter, we obtained HIV-1C whole blood samples from patients failing an INSTI-based cART to identify mutations and polymorphisms in order to examine how they affect the stability of integrase as well as the binding of the drugs to the protein. This was done by constructing a homology model of HIV-1C integrase and performing molecular docking and molecular dynamic simulations.

Abstract

HIV-1 integrase enzyme is responsible for the integration of viral DNA into the host genomic DNA. INSTIs are highly potent antiretroviral agents that inhibit this process, and are internationally approved for the treatment of both naïve and treated HIV-1 patients, however, their long-term efficacy is threatened by development of drug resistance strains resulting in resistance mutations. This work aimed to examine the effect of INSTI resistance-associated mutations (RAMs) and polymorphisms on the structure of HIV-1C integrase. Genetic analysis was performed on seven HIV-1C infected individuals with virologic failure after at least 6 months of INSTI-based antiretroviral therapy, presenting at the King Edward VIII hospital in Durban, South Africa. These were compared with sequences from 41 INSTI-naïve isolates. Integrase structures of selected isolates were modeled on the SWISS model online server. Molecular docking and dynamics simulations were also conducted using AutoDock-Vina and AMBER 18 force fields, respectively. Only one INSTI-treated isolate (14,28%) harboured major mutations (G140A and Q148R) as well as the E157Q minor mutation. Interestingly, S119T and V151I were only found in patients failing raltegravir (an INSTI drug). Molecular modeling and docking showed that RAMs and polymorphisms associated with INSTI-based therapy affect protein stability and this is supported by their weakened hydrogen-bond interactions compared to the wild-type. To the best of our knowledge, this is the first study to identify a double mutant in the 140's loop region (G140A+Q148R) from South African HIV-1C isolates and study its effects on RAL, EVG, and DTG binding.

Introduction

The HIV-1 Pol gene encodes for the enzyme integrase that catalyzes the chromosomal integration of newly synthesized double-stranded DNA into the host genomic DNA (Bessong and Nwobegahay, 2013). HIV-1 integrase is a 32 kilo Dalton (kDa) protein comprising 288 amino acids (Sachithanandham et al., 2016), a class of nucleotidyltransferase enzymes with three conserved spatially arranged carboxylates, and three structural domains namely the NTD, the CCD, and the CTD.

INSTIs have been shown to inhibit integrase by tightly binding to the viral DNA, displacing the DNA 3'-hydroxyl group from the integrase active site (Engelman, 2019), and chelating bound cations depending on the drug dose (Marchand et al., 2002). In countries like South Africa where the rates of HIV-1 infection are very high, the introduction of RAL, EVG, and DTG to effectively halt HIV-1 replication has been a major advancement for the HIV-1 treatment programme. Despite their effectiveness in HIV-1 therapy, INSTIs are threatened by the development of drug-resistant mutations. Such mutations may compromise the integrase-INSTI interaction rendering the drugs ineffective. Major pathways involved in RAL and EVG resistance include substitutions at positions 140, 148, and 155 (Hazuda, 2009, Wainberg et al., 2012, Blanco et al., 2011, Van Wesenbeeck et al., 2011, Quashie et al., 2013b) while DTG has shown substitutions mainly at position 263 (Oliveira et al., 2014, Ahmed et al., 2019).

Computational biology has contributed remarkably to understanding the mechanism of integration, drug binding, and resistance development (Malet et al., 2014). Tools such as molecular docking and molecular dynamics have increasingly become important to *in vitro* assays and studying the structure and function of biomolecules. Molecular docking is frequently used to analyse structure-based drug design. Two basic steps are involved (i) prediction of the ligand conformation and its orientation and position within the binding site, and (ii) assessment of the binding affinity.

Molecular dynamics simulations for studying the binding mechanism at the atomic level (Vlachakis et al., 2014), provide dynamical structural information and allows one to predict the time evolution of a system of interacting particles (atoms, molecules, granules, etc). They can be used to further optimize a homology modeled structure with docking results to analyse their stability during a specific duration of time. Existing lab experiments only provide partial information about the effects of mutations and to study protein dynamics *in vitro* is very expensive and time-consuming; the field of computational biology, therefore, complements the *in vitro* experiments.

In this study, RAMs were identified in patients failing an INSTI-based therapy, and molecular docking and dynamics simulations were used to investigate the effect of these RAMs, as well as other

polymorphisms on the structure of HIV-1C integrase enzyme. Understanding the effect of changes in integrase structure on its function is important for understanding treatment options, particularly for the Southern African region. It is also important to verify the effects of subtype-specific variations on integrase mutations.

Materials & Methods

Ethics and study approval

Study approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu Natal, South Africa (BE596/17), and permission to collect study samples was obtained from the KwaZulu Natal provincial Department of Health and the authorities of the King Edward VIII Hospital. Prior to sample and demographic data collection, signed informed consent was obtained from all study participants.

Laboratory Methods

Sample selection, collection and preparation for INSTI failures

Whole blood samples were obtained from seven patients who were referred to the King Edward VIII 3rd line regimen referral hospital with viral loads ≥1000 copies/mL while receiving INSTIs. Patients with viral loads <1000 copies/mL, or those not currently receiving INSTIs were excluded from the study. Samples were collected in EDTA tubes and processed on the same day by spinning at 1000 relative centrifugal force for 10 minutes at room temperature. Plasma was stored at −80°C for subsequent use.

Drug naïve sequences

HIV-1C integrase sequences obtained from stored plasma samples of nine patients failing a PI-based 2nd line treatment regimen were used as part of the INSTI-naïve control dataset. An additional 32 integrase HIV-1C sequences from treatment naïve patients were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to give a total of 41 INSTI-naïve sequences.

Viral RNA extraction and polymerase chain reaction

RNA was extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions for the spin protocol and stored at -80°C before use. The complete integrase region was amplified as follows: RNA was thawed to room temperature prior to usage and reverse transcriptase PCR (RT-PCR) was performed with SuperScript III RT/Platinum Taq enzyme (Invitrogen, Life Technologies, Carlsbad, CA). The primers used were INFORI (5'-GGA ATC ATT CAA GCA CAA CCA GA-3'; HXB2 location 4059→4081) and INREV-I (5'-TCT CCT GTA TGC AGA CCC CAA TAT-3'; HXB2 location 5244←5267). The reaction mix (25 µL) comprised 3 µL of DEPC treated water, 12.5 µL 2× reaction mix (a buffer with 0.4MM of each dNTP, 3.2 MM $MgSO_4$), 0.5 µL of each 10µM primer, 1 µL of SuperScript III RT/Platinum Taq mix enzyme and 7.5 µL of RNA. The PCR cycling conditions were as follows: one cycle of 55 °C for 30 min and one cycle of 94 °C for 2 min. This was followed by 35 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds and 68 °C for 2 min and a final extension at 68 °C for 5 min.

The presence and size of the PCR product were verified on a 1% agarose gel. Prior to sequencing, amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

Sanger sequencing

Cycle sequencing was conducted using the BigDye Terminator version 3.1, Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA). Generated nucleotide sequences were aligned using the BioEdit software v7.1.11 (Ibis Biosciences, An Abbott Company, CA. USA). These sequences were submitted to the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) for analysis and interpretation. From the sequencing results, three representative mutant samples were selected for further investigation, listed in Table 1.

Computational Methods

Structural modeling of the integrase protein

Homology modeling was performed on the three representative mutants (MuT-1, MuT-2 & MuT-3) and a wild-type sample. Briefly, protein sequences from patients were uploaded on the SWISS-MODEL web server (https://swissmodel.expasy.org), an integrated web-based modeling expert system. The criteria of the template were based on the best resolution structure and the highest sequence identity. HIV-1C integrase tetramers were generated using the cryoEM structure of HIV-1B intasome (PDB ID 5U1C) with 97.04%, 96.67% and 97.78 sequence identity for MuT-1, MuT-2, and MuT-3, respectively; 5U1C shares high sequence identity with HIV-1C integrase from South African cohorts (Brado et al.,

2018, Isaacs et al., 2020). Every model was accompanied by several quality verifications on the web server. Ramachandran analysis was used to analyse the stereochemistry of amino acid side chains around the peptide bond, with each amino acid side chain scored based on angular orientation around the psi (ψ) and phi (ϕ) torsion angles (Quashie et al., 2015a). Model quality was assessed based on qualitative model energy analysis (QMEAN) and global model quality estimation (GMQE) scoring functions. An integrase monomer was separated from the crystal structure to study molecular docking and dynamics simulations.

Docking

The wild-type and mutants HIV-1 integrase input files were prepared by a multi-step process through the UCSF Chimera software package version 1.13.1, a program for visualization and analysis of molecular structures (Pettersen et al., 2004). RAL, EVG, and DTG sequence structures were downloaded from PubChem and 3D structures prepared on the Avogadro software package (Hanwell et al., 2012)**.** Following ligand and receptor preparations, molecular docking was performed using the AutoDock-Vina Plugin available on Chimera v1.13.1 (Yang et al., 2012), with default docking parameters. An AutoDock-Vina grid box that specifies the ligand and receptor files was generated and AutoDock was initiated in Chimera v1.13.1 using the local option for executable location. A single docking experiment to study a single ligand with a single receptor at a time was performed.

Molecular Dynamics Simulation

To perform molecular dynamics simulations, AMBER 18 was accessed at the Centre for High Performance Computing (http://www.chpc.ac.za). After loading AMBER, antechamber automatically calculated charges and atom types using GAFF. Atom types and charges were manually validated, and AMBER topology files were generated using LEaP in AMBER 18. The LEaP module of AMBER 18 allows for the addition of hydrogen atoms, Na^+ as well as Cl counter ions for systems neutralization. The system was equilibrated at a constant temperature (300K) and pressure (1bar), mimicking an isobaric-isothermal ensemble. The total time for molecular dynamics simulations was 140 nanoseconds (ns).

Post Simulation Analysis

Simulation results were saved, and trajectories root mean square deviation (RMSD), root mean square fluctuation (RMSF) and solvent surface accessibility area (SASA) analyzed using the CPPTRAJ module employed in AMBER 18. The RMSD estimates the deviation in the C-α atoms of the protein residues and indicates the degree of protein stability (Dixit et al., 2006), while the RMSF provide insight into the residual flexibility of various regions of the integrase protein upon binding of INSTIs, and SASA

measures the exposure of the enzyme to solvent molecules (Ausaf Ali et al., 2014). Origin software (Origin Lab, Northampton, MA) was used for the plotting and generation of graphs.

Calculating Binding Free Energy

Free binding energy was calculated using the molecular mechanics / generalized Born and surface area continuum solvation method (MM/GBSA) (Ylilauri et al., 2013). The free binding energy (ΔG_{bind}) computed by this method for each molecular species (complex, ligand, and receptor) can be represented (Hayes et al., 2012) as :

$$\Delta G_{bind} = G_{complex} - G_{receptor} - G_{ligand} (1)$$

$$\Delta G_{bind} = E_{gas} + G_{sol} - TS \quad (2)$$

$$E_{gas} = E_{int} + E_{vdw} + E_{ele} (3)$$

$$G_{sol} = G_{GB} + G_{SA} (4)$$

$$G_{SA} = \gamma SASA \qquad (5)$$

Where E_{gas} denotes the gas-phase energy; E_{int} signifies internal energy; E_{ele} and E_{vdw} represent the electrostatic and the van der Waals contributions. T and S denote temperature and the total entropy of the solute, respectively. Entropy effects play an important role in drug binding (Sun et al., 2018) and their calculations are estimated using the normal mode analysis which has a large error margin and introduces significant uncertainty (Genheden and Ryde, 2015). In addition, these calculations are computationally expensive (Graham et al., 2013). In ligand-binding affinity, the entropic contribution is often neglected by end-point binding free energy calculation methods.

The E_{gas} was directly estimated from the FF14SB force field terms and the solvation-free energy, G_{sol}. The G_{GB} is derived from solving the GB equation, whereas G_{SA} contribution is estimated from SASA determined using a water probe radius of 1.4 Å.

Results

HIV-1C mutations & Structural Analysis

Figure 1 shows the frequency of mutations and polymorphisms detected in our study. Only one of the INSTI failures had major integrase mutations (G140A, Q148R) as well as a minor mutation (E157Q). The Stanford Drug Resistance Database identified several polymorphisms when compared to the subtype B reference strain namely: K14R, S17N, D25E, V31I, M5OI, I72V, F100Y, L101I, T112V, S119T, T124A, T125A, K136Q, V151I, V201I, T218I, L234I, R269K, D278A, and S283G. Of these, S119T and V151I were only seen in patients failing INSTI-based therapy. From Figure 1, representative sequences were selected for further investigation, Table 1.

The fragment encompassing the integrase 140s loop (i.e. G140-G149) is known to be important for catalytic function (Greenwald et al., 1999, Métifiot et al., 2010), and resistant mutants at this loop have been shown to reduce conformational flexibility (Perryman et al., 2010, Métifiot et al., 2010). We analysed changes that occurred in the integrase structure by comparing the WT and MuT-3's critical 140s loop. As illustrated in Figure 2, the G140A/Q148R mutant displayed a much higher dreiding energy. We further analysed the residue interaction network (RIN) in the loop, Figure 3, and our results show that MuT-3 is less stable than the WT.

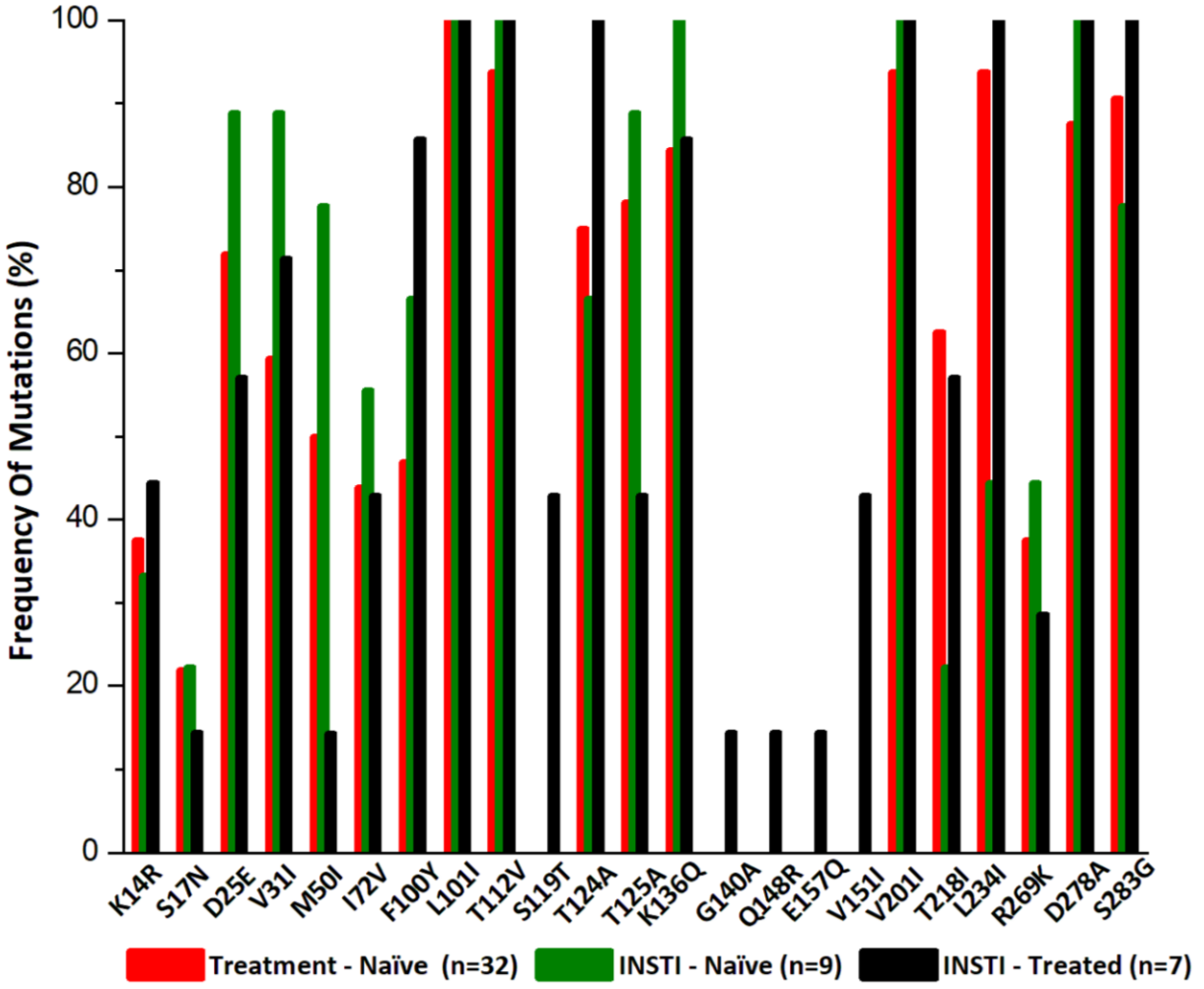


Figure 1 Resistance-associated mutations and polymorphisms in the integrase coding region of HIV-1C South African sequences. From the identified polymorphisms D25E, V31I, F100Y, L101I, T112V, T124A, T125A, K136Q, V201I, L234I, D278A and S283G were the most common amongst all study cohorts; S17N was the least common. The frequency of T125A and M50I was relatively low in INSTI-treated compared to INSTI-naive.

Table 1 Genotypic characteristics of MuT-1, MuT-2 and MuT-3

Sample ID	Third-line Treatment Regimen	Integrase Mutations Detected
MuT – 1	AZT + FTC + RAL	K14R, D25E, V31I, I72V, F100Y, L101I, T112V, S119T, T124A, K136Q, V151I, V201I, L234I, D278A, S283G
MuT – 2	3TC + AZT + RAL	K14R, D25E, M50I, I72V, F100Y, L101I, T112V, S119T, T124A, K136Q, V151I, V201I, T218I, L234I, R269K, D278A, S283G
MuT – 3	ETR + FTC + RAL	G140A, Q148R, E157Q, S17N, V31I, F100Y, L101I, T112V, T124A, T125A, K136Q, V201I, L234I, R269K, D278A, S283G

3TC , Lamivudine; AZT, Zidovudine; FTC , Emtricitabine; ETR, Etravirine; RAL , Raltegravir

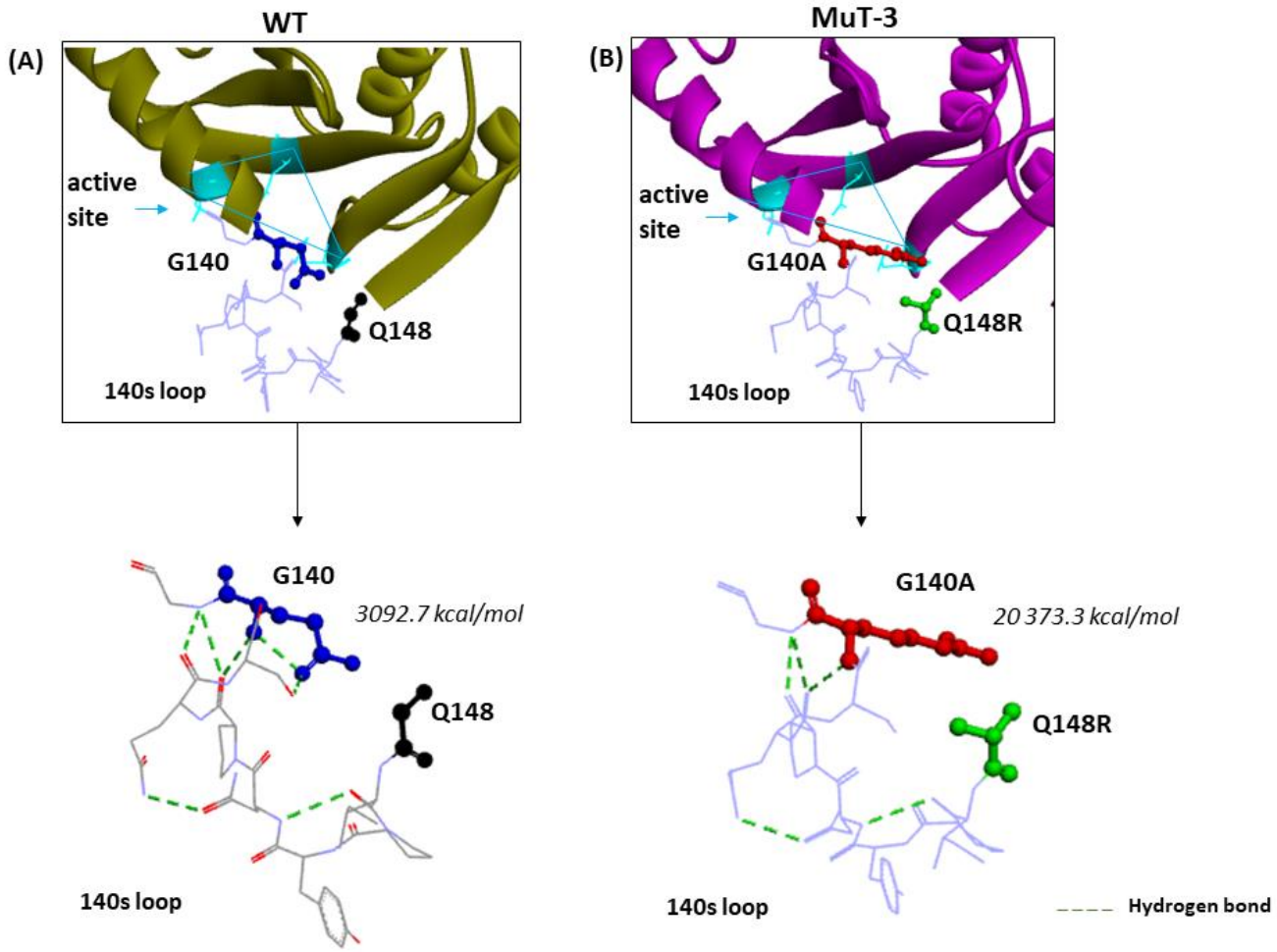


Figure 2 Comparing wild-type and MuT-3 integrase 140s loop region. (A) showing the wild-type 140s loop interaction with HIV-1 integrase catalytic residues (cyan); G140 is represented in blue ball and sticks and Q148 is represented in black ball and stick. (B) showing MuT-3 140s loop interaction with HIV-1 integrase catalytic residues (cyan); G140A represented in red ball and sticks and Q148R represented in green ball and stick. The wild-type 140s loop had a dreiding energy of 3092.7 kcal/mol and 7 hydrogen bond interactions, while the MuT-3 140s loop had a dreiding energy of 20373.3 kcal/mol and 5 hydrogen bond interactions.

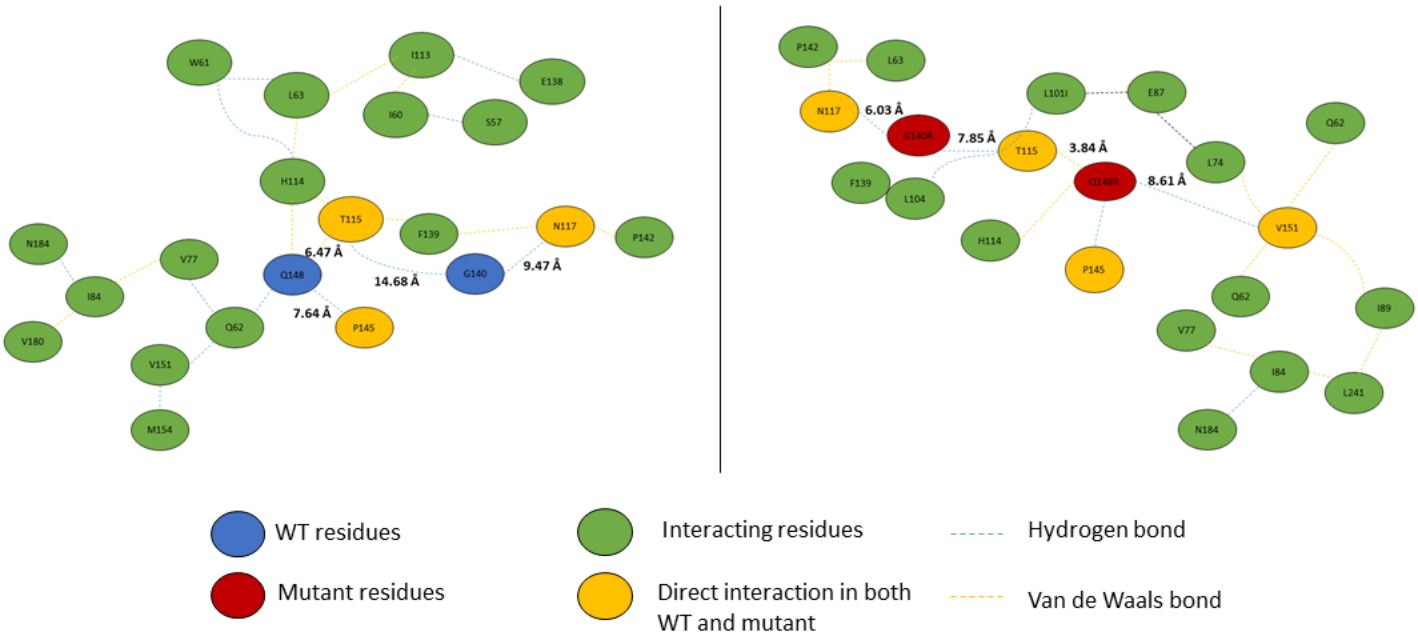


Figure 3 Residue interaction networks (RIN) showing the interaction between HIV-1 integrase 140's loop in the wild-type (G140+Q148) and mutated (G140A+Q148R) residues. RIN shows various interactions that take place in a protein in the form of a detailed network. Wild-type and MuT-3 proteins were submitted to the RING server (Martin et al., 2011) for RIN construction. Both wild-type and MuT-3 directly interact with residues T115, N117, P145 and V151.

Molecular Docking: Distance Measurement & Protein-Ligand Interaction

INSTIs tightly bind to a specific site close to the conserved DDE ($D_{64}D_{116}E_{152}$) motif. To understand structural changes that occur in the presence of mutations, we measured the distance between the side chains of the three conserved residues for WT and mutant proteins with docked INSTIs (Figure 4). The distance measurements and differences obtained are shown in Table 2.

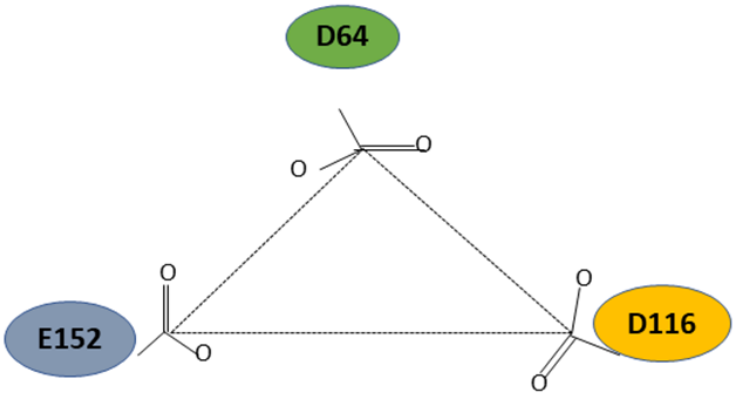


Figure 4 Scheme of the active site residues ($D_{64}D_{116}E_{152}$). The distance was measured between the three conserved catalytic residues for wild-type and mutant proteins with docked INSTIs.

Table 2 Distances (Å) calculated between wild-type and mutated integrases

Sample ID & INSTI	D64-D116	Differences Against The WT	D116-E152	Difference against WT	D152-D64	Differences Against The WT
WT-DTG	5.73		11.73		8.48	
WT-EVG	6.36		11.28		7.62	
WT-RAL	5.96		10.14		7.63	
MuT1-DTG	6.37	0.64	10.64	-1.09	8.31	-0.17
MuT1-EVG	6.11	-0.24	10.44	-0.84	7.86	0.24
MuT1-RAL	7.09	1.13	10.57	0.43	7.97	0.33
MuT2-DTG	5.94	0.21	11.97	0.24	9.01	0.53
MuT2-EVG	5.61	-0.75	10.43	-0.85	7.77	0.71
MuT2-RAL	6.32	0.36	10.12	-0.02	7.59	-0.04
MuT3-DTG	7.28	1.32	11.62	-0.11	7.42	-0.2
MuT3-EVG	4.57	-1.79	11.19	-0.09	8.16	0.53
MuT3-RAL	5.83	-0.13	10.88	0.74	7.77	0.14

WT, wild-type; MuT, mutant

To determine the WT and mutants protein stability with INSTIs, we analysed the protein-ligand interactions on the BIOVIA, Dassault Systèmes, Discovery Studio Visualizer Software, 2019. Key hydrogen bond interactions between RAL, EVG, and DTG complexes of WT, MuT-1, MuT-2, and MuT-3 integrases were analysed. Figure 5 shows that key residues such as S119 play a role in hydrogen bonding.

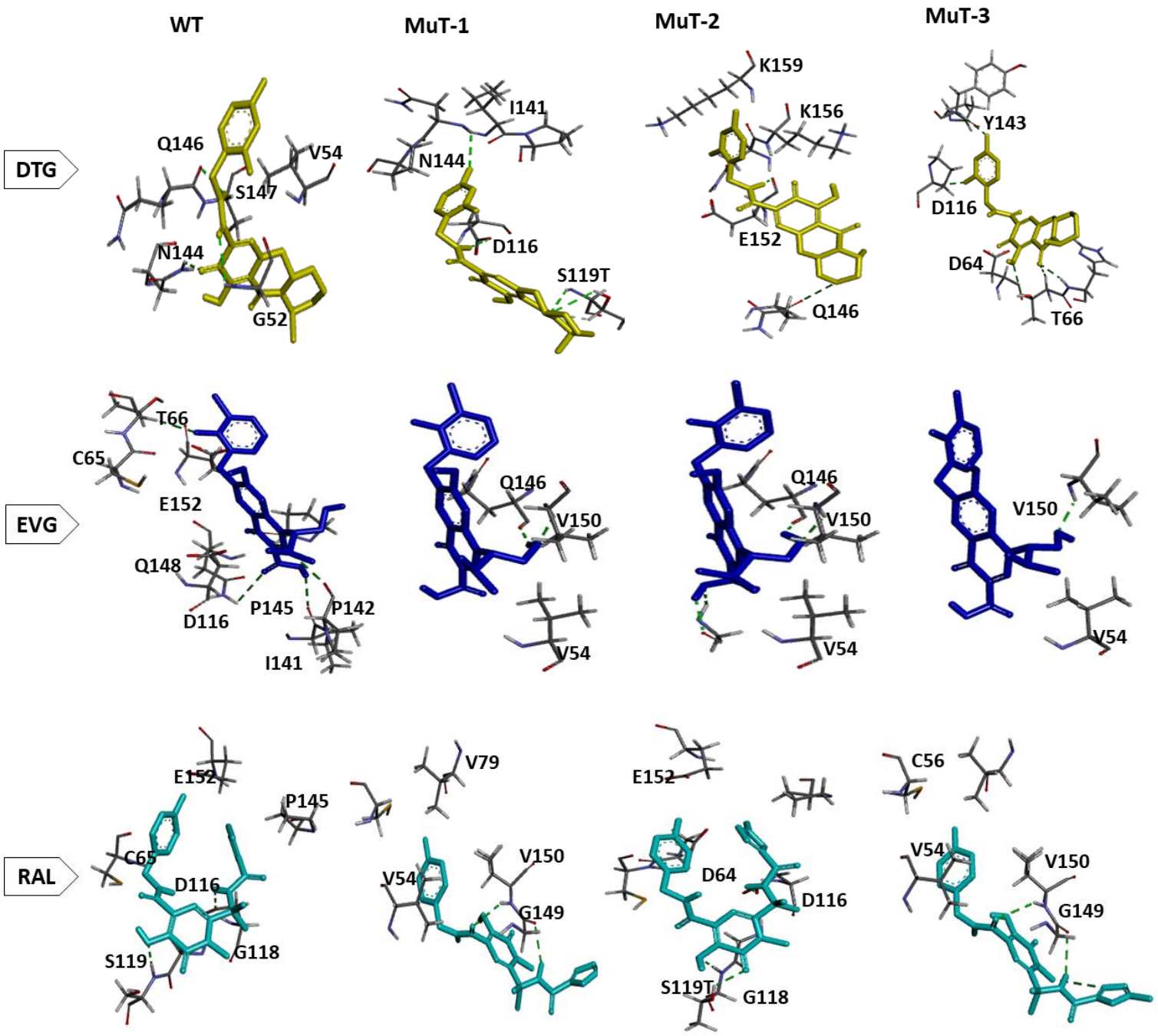


Figure 5 Protein-ligand interactions. Hydrogen bond interactions, shown in green dotted lines, within the protein-ligand of wild-type and mutant complexes with docked INSTIs. For DTG, the wild-type protein makes important hydrogen interactions with polar side chain residues (S147, Q146 and N144). The amino acid residues C65, D64 and D116 (key H bond amino acid residues) and two lysine residues (K156 & K159) are known to play an important role in stabilizing the protein (Eurtivong et al., 2019, de Carvalho et al., 2014). For EVG, key hydrogen bond amino acid residues are seen to interact with the wild-type, but the interaction is lost for mutants. Notably, RAL forms hydrogen bonds with residue 119.

Molecular Dynamics Simulations: Binding free energies and trajectory analysis of the simulated systems

We performed MM/GBSA calculations to get a quantitative estimation for the binding free energies and their components of HIV-1C integrase complexed with DTG, EVG, and RAL. The results were collected in Table 3 and are showing that the binding energies of the MuT-3 integrase were generally lower than the calculated free binding energies of the wild-type, but depended on the nature of the selected ligand for the other mutants.

The molecular dynamics simulations trajectory analysis of the integrase protein in contact with RAL, EVG, and DTG is shown in Figure 6. RMSD analysis indicated that the WT reached equilibrium after 40 ns together with MuT-1 and MuT- 2, for EVG and RAL (Figure 6A). Generally, mutants showed higher RMSD values compared to the WT. Analysis of the RMSF provided insight into the residual flexibility of various regions of the integrase protein upon binding of INSTIs (Figure 6B). The core of the protein (residues 52-210) was more structurally rigid, especially for WT and MuT-1, this is indicated with decreased flexibility. SASA analysis indicated that the WT surface area was more accessible than the mutants (Figure 6C).

Table 3 Binding free energies of INSTIs to various protein complexes using MM/GBSA

			Binding Affinity (kcal/mol)				
HIV-1 Sample ID	***IN- Inhibitor***	ΔE_{vdw}	ΔE_{ele}	ΔG_{gas}	ΔG_{sol}	ΔG_{SASA}	ΔG_{bind}
WT	DTG	-34.00	-18.06	-52.07	19.38	-4.08	-32.69
	EVG	-40.09	-11.17	-51.27	21.35	-5.27	-29.92
	RAL	-44.78	-31.44	-62.07	31.61	-5.65	-30.46
MuT -1	DTG	-26.90	-13.80	-40.71	21.35	-3.01	-19.35
	EVG	-38.37	-16.86	-55.24	19.68	-4.58	-35.56
	RAL	-39.56	-21.59	-61.16	30.75	-4.92	-30.40
MuT – 2	DTG	-43.74	-26.09	-69.83	40.02	-5.19	-29.80
	EVG	-41.25	-12.68	-53.94	21.59	-5.41	-32.34
	RAL	-39.94	-21.47	-61.42	31.77	-4.46	-29.64
MuT – 3	DTG	-31.44	-20.71	-52.16	27.01	-4.03	-25.14
	EVG	-17.84	-9.77	-27.62	15.12	-2.03	-12.49
	RAL	-20.54	-10.62	-31.17	19.71	-2.44	-11.45

DTG, Dolutegravir; EVG, Elvitegravir; RAL, Raltegravir; MuT, mutant; ΔE_{vdw}, van de Waals energy; ΔE_{ele}, electrostatic energy; ΔG_{gas}, gas-free energy; ΔG_{sol}, solvation free energy; ΔG_{SASA}, solvent accessible surface area; ΔG_{bind}, binding free energy.

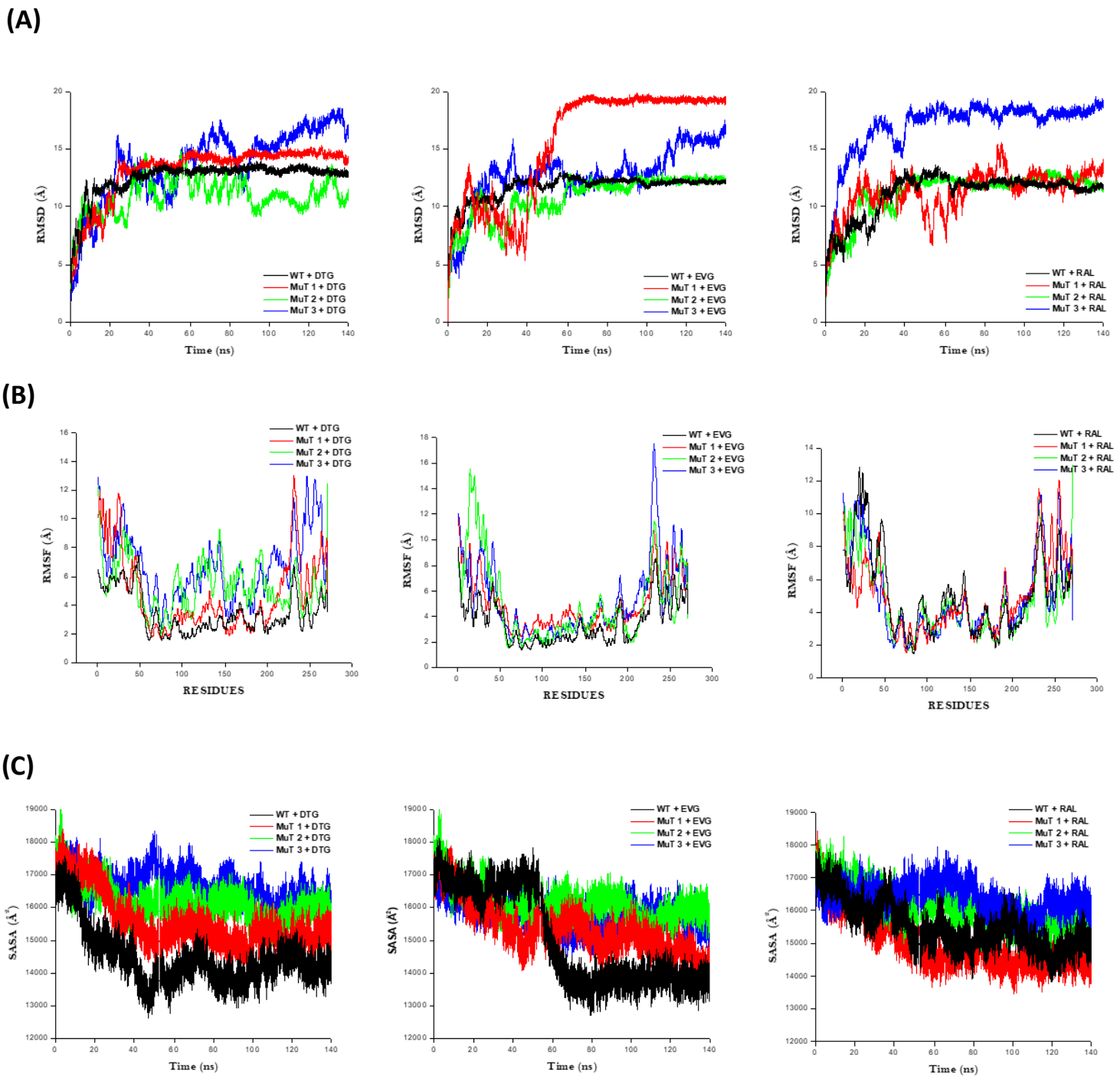


Figure 6 Trajectory analysis of the wild-type and mutant systems. (A) The monitored RMSD of the backbone atoms plotted for 140 ns. RMSD analysis indicated that the wild-type reached equilibrium after 40 ns together with MuT-1 and MuT- 2, for EVG and RAL. (B) Change in RMSF for the C-α residues plotted for 140 ns. The catalytic core was more structurally rigid, especially for wild-type and MuT-1. (C) Measurement of the enzyme's exposure to solvent molecules, SASA, showing that the wild-type surface was more accessible.

Discussion

With the increased use of INSTIs in HIV-1C as part of the third-line regimen in South Africa, and the WHO recommending the use of DTG in first-line treatment (Moorhouse et al., 2018), it is important to identify mutations that may affect the stability of integrase and binding of the drugs to the catalytic pocket of the protein. Therefore, in this study, we have analyzed HIV-1C integrase sequences from KwaZulu Natal, South Africa to identify polymorphisms and RAMs that affect drug binding as well as the stability of integrase. Low frequency of RAMs was seen in our study, with only one of the seven isolates (14.3%) presenting with clinically significant INSTI RAMs i.e. G140A, Q148R, and E157Q; Figure 1 and Table 1. This is in agreement with Brado et al. 2018 who reported a low frequency (2.85%) of major RAMs in South African HIV-1 integrase sequences (Brado et al., 2018). While the prevalence of RAMs is low, we identified S119T and V151I, which are not considered as INSTI resistance determinants, to be increased in integrase treatment failures each at a frequency of 42.9%. A structure-based study by Quashie et al, 2015 showed that subtype-specific effects on the integrase protein structure and function were caused by polymorphisms (Quashie et al., 2015a). Mutations such as S119T found close to the highly conserved integrase active site residues have been shown to prevent drug binding to the integrase enzyme and as a result affect drug susceptibility (Harper et al., 2001, Goethals et al., 2008, Malet et al., 2008, Smith et al., 2021b); with V151 near the active site residue, E152, mutating it to isoleucine will change the size of the active site which could potentially have an impact on INSTI binding. Our result suggests that S119T and V151I have an impact on the integrase protein, this is in contrast to the study by Isaacs et al that reported that polymorphisms alone have a negligible effect on drug binding (Isaacs et al., 2020). Our study is, however, in agreement with Brado et al who reported that polymorphisms affect the stability of the integrase protein complex (Brado et al., 2018).

Previous analysis on residue 119 showed that it plays a role in DNA recognition since it is appropriately situated to interact with cellular DNA during the insertion of viral DNA ends; substitutions such as S119T will alter integration target specificity (Harper et al., 2001, Harper et al., 2003). Residue S119 target site selection is a general property of integrase; in Visna and Rous sarcoma virus integrases, mutations at this residue change the target site preference (Harper et al., 2001, Harper et al., 2003). It has also been shown that mutating S119 contributes to a significant increase in DTG resistance (Hachiya et al., 2015). In Figure 5 we have shown that S119T directly interacts with both DTG (MuT-1) and RAL (MuT-2), suggesting that it might also be linked to protein-ligand interaction in INSTI-therapy. The V151I substitution has been previously reported to be associated with drug resistance and is believed to decrease drug susceptibility by disrupting drug binding to the region adjacent to the catalytic core (Hazuda and MM, 2007). In different HIV-1 subtypes, V151 has been suggested to act as

a secondary mutation associated with the primary RAL and EVG mutation N155H (Serrao et al., 2009, McColl and Chen, 2010, Ceccherini-Silberstein et al., 2010).

The integrase 140's loop is adjacent to the active site and also regulates binding (Métifiot et al., 2010), so mutations at one or more of these amino acid residues would change the flexibility of this loop and directly impact the active sites ability to interact with the drug (Abram et al., 2013). Residue Q148, in particular, forms part of the integrase inhibitors binding pocket (Sotriffer et al., 2000, Marchand et al., 2002), and mutating it to arginine which has a bulkier side chain may result in steric hindrance to INSTIs. As shown in Table 3, the wild-type protein showed a stronger binding affinity than MuT-3 which has the G140A+Q148R double mutant. A reduction of -17.43 kcal/mol (EVG) and -15 kcal/mol (RAL) could be associated with impaired drug binding and drug efficiency towards MuT-3. This was supported by differences indicated in the distance between the side chains of the active site residues (Figure 4 and Table 2).

The amino acid residue G140 act at the end of the loop act as a hinge together with G149 allowing the polypeptide backbone to rotate, this is crucial to maintaining the flexibility of the loop. To fully comprehend the effect of the double mutant on the flexible loop, we calculated dreiding energy between the wild-type and MuT-3 loop structures; Figure 2. Dreiding energy sums components assigned to bond lengths, bond angles, and dihedral angle torsion, with lower energy indicating a stable conformation (Mayo et al., 1990, Allen et al., 2010, Karimipour et al., 2020). The dreiding energy was found to be 3 092.7 kcal/mol in the wild-type and 20 373.3 kcal/mol in MuT-3's loop, which shows that the mutant loop is less stable compared to the wild-type one. The stability of the integrase protein structure largely relies on the network of hydrogen bonds which are fundamentally electrostatic interactions, important in protein stability and flexibility (Pace et al., 2014). Analyzing the RIN, we observed reduced hydrogen bond interactions in the MuT-3 140s loop network; Figure 3. Similarly, we observed less hydrogen bond interactions when comparing the wild-type and MuT-3 integrase 140s loop regions in Figure 2, further highlighting that MuT-3 is less stable than the wild-type.

Further molecular dynamics simulations were necessary to obtain the detailed interaction mode and relevant conformational changes occurring during the protein-ligand interaction process. Generally, mutants showed higher RMSD variation values compared to the wild-type (Figure 6A). It's worth mentioning that the core of the protein (52-210) was more structurally rigid; this is indicated by lower fluctuation degree in the catalytic core domain compared to the NTD and CTD (Figure 3B, RMSF). The higher SASA values in the presence of mutations could mean that the mutations may result in a conformational change that increases the SASA (Ausaf Ali et al., 2014). Like the RMSD and RMSF plots,

the SASA further revealed the negative impact mutations play on ligand-protein functionality by showing that mutations become less accessible to solvent (Figure 6C, SASA).

The integrase minor mutation E157Q found in the isolate with the double mutant may be of particular importance for patients receiving INSTI-based therapy since it has been previously reported to influence response to antiretroviral therapy when studied with R263K, the most commonly reported DTG resistance mutation (Anstett et al., 2016). This study reports on patient isolates where these mutations and polymorphism occurred together. To fully elucidate the effect of each mutation on drug binding, they should be measured individually using site-directed mutagenesis studies.

Conclusions

We were able to show variability in the integrase coding region in both INSTI-naive and INSTI-treated patients. While the low frequency of major INSTI RAMs augers well for their successful use in South Africa, the presence of polymorphisms associated with INSTI-based therapy failure needs to be considered when shifting patients to INSTI-inclusive therapy, and their effect on INSTI binding warrants for further investigation.

CHAPTER FOUR

EFFECT OF HIV-1 SUBTYPE C INTEGRASE INHIBITOR MUTATIONS ON THE VIRAL ENZYMATIC ACTIVITY

Link between chapter three and chapter four

After we identified key and some novel mutations in the previous chapter, they were shown to have a negative effect on the HIV-1C integrase protein structural stability and flexibility. In this chapter, we assessed their effect on the enzyme function by performing DNA binding and 3' processing assays on expressed and purified HIV-1C integrase proteins.

Abstract

HIV-1 integrase, essential for insertion of the viral genome into host DNA, has proven to be an important target for HIV-1 therapy. INSTIs are a class of antiretroviral drugs designed to inhibit the action of integrase. However, the long-term success of INSTIs is threatened by the development of RAMs. While several INSTI RAMs have been reported, it has been noted that the impact of mutations on integrase efficiency is highly dependent on the viral subtype. Therefore, we aimed to understand how INSTI RAMs and polymorphisms affect the enzymatic activity of HIV-1C integrase by performing DNA binding and 3' processing assays. Our results showed that the mutated HIV-1 integrase isolates exhibit lower DNA binding activity with a dissociation constant (K_d) ranging from 59.6 - 149.1nM compared to the wild-type (39.9nM), and all the isolates demonstrated a loss in 3'processing activity compared to the wild-type. This data suggest that in addition to known INSTI RAMs i.e. G140A, Q148R, and E157Q, two key substitutions that are close to the integrase catalytic site V151I and S119T, may play a role in the reduction of DNA binding activity and account for the loss in enzyme activity in HIV-1C.

Keywords: HIV-1C, integrase, drug resistance mutations, integrase strand transfer inhibitors, DNA binding, 3' processing

Introduction

Integrase is a 32 kDa, 288 amino acid protein that catalyses HIV DNA integration which is required to maintain the viral genome in the infected cell, and for efficient expression of viral proteins leading to the generation of new viral particles (Hazuda, 2009). The process of integration takes place in the nucleus of the host cell following reverse transcription of RNA into cDNA. Two major sequential reactions are catalyzed by the retroviral integrase, namely 3' processing and strand transfer (Delelis et al., 2008). Initially, integrase binds to a short sequence at each end of the viral DNA known as the LTR region. Integrase then catalyses endonucleotide cleavage also known as 3' processing which is the removal of a 3' dinucleotide from each end of the viral DNA in the pre-integration complex. The resulting cleaved DNA is then used as a substrate for the second step in integration, i.e. strand transfer, which results in covalent linkage of the viral DNA into the host cells.

Integrase inhibitors specifically target the strand transfer reaction and are therefore referred to as INSTIs. As mentioned in the previous chapters, there are five INSTIs that are currently approved for therapy namely RAL, EVG, DTG, BIC and CAB. They have been shown to effectively halt HIV-1 replication with few side effects. However, their long-term efficacy has been threatened by the development of RAMs. Subtype-specific mutations and polymorphisms have been reported to have differential effects on the integrase enzyme efficiency (Quashie et al., 2015b, Theys et al., 2019). It has also been shown that polymorphisms can cause subtype-specific effects which can significantly affect the function and activity of the protein even in the absence of INSTI major mutations (Quashie et al., 2015b, Acharya et al., 2020). Therefore, it is important to understand the enzyme activity of HIV-1C integrase as the development of resistance to INSTIs is likely to influence the use of these inhibitors in antiretroviral therapy.

Plate-based biochemical assays for the measurement of various integrase biochemical activities contributed to the development of viable integrase inhibitors (Hazuda et al., 1994, Hazuda et al., 2000). HIV-1 integrase-protein binding, strand transfer, and 3' processing activities can be produced *in vitro* in the presence of recombinant integrase using short double-stranded oligonucleotides mimicking the viral LTR U5 or U3 sequences (Leh et al., 2000b). The fluorescence microplate-based assay used here was modified for protein-DNA interactions (Zhang et al., 2003, Han et al., 2013). We investigated the ability of different HIV-1C integrase proteins, containing mutations and polymorphisms, to bind to the LTR substrate and their impact on 3'processing activity.

Materials & Methods

Ethics and study approval

As previously mentioned in chapter three, ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu Natal (BE596/17). The KwaZulu Natal Department of Health and the King Edward VIII Hospital authorities also gave permission to collect study samples. Signed informed consent was also obtained from all study participants prior to sample collection.

Sample selection, collection and preparation for INSTI failures

Samples were collected from patients with viral loads ≥1000 copies/mL while receiving INSTIs, as mentioned in chapter three. Whole blood samples were collected in EDTA tubes and processed by centrifugation and plasma was stored at −80 °C for future usage.

Viral RNA extraction and polymerase chain reaction

HIV-1 RNA extraction and polymerase chain reaction were performed as previously described in chapter three. Briefly, 140 µL plasma was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, USA) as per manufacturer's instructions. RT-PCR was performed using the SuperScript III RT/Platinum Taq enzyme (Invitrogen, Life Technologies, Carlsbad, CA) with primers: (5'-GGA ATC ATT CAA GCA CAA CCA GA-3'; HXB2 location 4059→4081) and INREV-I (5'-TCT CCT GTA TGC AGA CCC CAA TAT-3'; HXB2 location 5244←5267). RT-PCR was followed by gel electrophoresis, followed by amplicons purification using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions.

Sanger sequencing

The amplified products were sequenced as previously described in chapter three. Briefly, the BigDye Terminator version 3.1, Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) was used to generate nucleotide sequences. The sequences were aligned on the BioEdit software v7.1.11 (Ibis Biosciences, An Abbott Company, CA. USA) and submitted to the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) for analysis and interpretation. From the sequencing results, three representative mutant samples were selected for DNA binding and 3' processing activities i.e. MuT-1, MuT-2, and MuT-3, listed in chapter 3, Table 1.

Oligonucleotides

The oligonucleotides INT1 (5'TGTGGAAAATCTCTAGCAGT-3'), INT2 (5'-ACTGCTAGAGATTTTCCACA-3'), T35 (5'ACTATACCAGACAATAATTGTCTGGCCTGTACCGT-3'), SK70 (5'-ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT-3') correspond to the U5 LTR ends of the HIV-1 genome. They were purchased PAGE purified from Integrated DNA Technologies (IDT; Coralville, IA) with a covalently attached 6-carboxyfluorescein (6-FAM) moiety on either 5'- or 3' end. For DNA binding assays INT1/INT2 were used and for gel-based 3' processing assays T35/SK70 were used. To obtain double-stranded oligonucleotide substrates, T35/SK70 and INT1/INT2 were mixed, respectively, at a 1:1 molar ratio in annealing buffer (50μM in 10mM Tris pH 7.8, 0.1 mM EDTA), heated to 95 ^{o}C for 5 minutes and slowly cooled at room temperature, and stored at -20^{o}C until use.

Expression and purification of recombinant HIV-1 integrase

Wild type and the three mutated MBP-tagged integrase proteins (MuT-1, MuT-2, and MuT-3) were transformed in *Escherichia coli* BL21 (DE3) (New England Biolabs) for protein expression. Lauria-Bertani broth supplemented with ampicillin was used for bacterial growth at 37 °C. When bacterial cultures achieved an optical density of 0.5 at 600 nm, protein expression was induced by adding isopropyl β-D-thiogalacto-pyranoside (IPTG) to a final concentration of 0.3 mM. The cultures were then incubated for a further 2 hours at 37 °C, centrifuged (4000Xg for 10 minutes) to harvest cells, and resuspended in 10 mL column binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and stored at -20 °C. After expression, cells were lysed by multiple freeze-thaw followed by sonication and clarified by centrifugation (20000xg for 20 minutes).

Protein expression was measured with the Bradford Protein Assay kit (Bio-Rad Laboratories) and purified by affinity chromatography. Briefly, proteins were purified utilizing MBPTrap^{TM} HP Columns propylene columns (Life Sciences). MBP-tagged integrase proteins were equilibrated with five volumes of the binding buffer and applied to the columns using a syringe. Following that, five column volumes were eluted with elution buffer (10 mM maltose in binding buffer) and the eluates analysed on 10% SDS-polyacrylamide gels with Coomassie staining (Bio-Rad Laboratories). HIV-1 integrase protein was then verified using PAGE and western blot analysis.

The DNA binding assay

Microplate assays were performed according to the methods described by Han et al. in 2013 (Han et al., 2013) with minor modifications. Firstly, 6-FAM labelled double-stranded substrate INT1/INT2 was serially diluted in phosphate-buffered saline (PBS) at concentrations 0 to 40 nM, and 100 µL of each concentration was loaded onto Corning® 96 well, polystyrene, flat-bottom (chimney well) fluotrac™ 600 medium binding plates. Fluorescence signals were measured at excitation wavelength 490nm and emission wavelength 510 nm using the Turner Biosystems Modulus® microplate reader. To evaluate assay sensitivity, fluorescent signals against concentrations of substrates were plotted.

One-hundred µL volume protein concentrations (0 to 1000 nM) diluted in PBS were immobilized into the plate wells and incubated overnight at 4 ^{o}C; negative-control wells were coated with PBS and lacked DNA substrate. Excess unbound protein was removed by rapid inversion and each well was subsequently washed with 200 µL of PBS. Plates were then blocked with 5% bovine serum albumin (BSA) in PBS at room temperature for 2h. Following that, plates were washed once with 200 µL PBS and once with binding buffer (5mM DTT, 20 mM NaCl, 20 mM HEPES, and 7.5 mM $MnCl_2$). A 100 µL volume of LTR oligos in binding buffer was added into each well at a final concentration of 20nM and the plates incubated at room temperature in the dark for 1h, then washed once with 200 µL of PBS. After the final wash, 100 µL of PBS was added to each well and fluorescence signals were measured.

The following equation was used to calculate HIV-1 DNA Binding activity for each sample:

$BA_{sample} = (B_{total} - B_{background})$

Where $B_{background}$ is background signal from control reactions without integrase protein or LTR performed under the same reaction conditions and B_{total} is signals from reactions with immobilized HIV-1 integrase.

To determine the apparent dissociation constant (K_d) value, integrase (1000nM) was incubated with increasing concentrations of LTR oligos (0 to 200 nM) and K_d value calculated by directly fitting the titration curve using GraphPad Prism with nonlinear one-site binding regression.

3' processing assay

The 3' processing activity of purified HIV-1 integrase was determined by standard gel-based 3' processing assays as previously described (Han et al., 2012, Han et al., 2013). Briefly, 3' 6-FAM labelled T35/SK70 was annealed to generate a double-stranded LTR. Reactions were carried out in a final volume of 10 µL containing 0.5 µM LTR DNA with 1µM integrase in a buffer containing 50 mM HEPES

pH 6.8, 50 mM NaCl, 15 mM $MnCl_2$, and 50 µg/mL BSA; 3' processing reactions were initiated by incubation at 37 ^oC for 2h, and reactions stopped by adding an equal volume of gel loading mix (4.8g urea, 200 µL Tris pH 8, 0.025g bromophenol blue, 0.25g xylene cyanol and 0.1g SDS) and heated at 95^oC, 5 min. The reaction products were separated on 12.5% denaturing urea PAGE gel and scanned using the Bio-Rad Chemidoc Imaging system. The Image Lab software, Life Science Research Bio-Rad was used to analyse densitometry and relative 3'-processing activity was calculated.

Data Analysis

Data were expressed as mean ± standard deviation analyzed and using GraphPad Prism v5.03 software (GraphPad San Diego, CA) software. The GraphPad software – t test was also used to determine the p values for the HIV-1 DNA binding activity results. At least three independent experiments were performed, each in triplicates or duplicates. The Kd value was calculated by directly fitting the titration curve using GraphPad Prism with nonlinear one-site binding regression.

Results

HIV-1 integrase enzyme activity

Firstly, assays to determine the optimal concentration for HIV-1 integrase and LTR were performed. Figure 1 shows the calibration curve for 6 FAM attached INT/INT2-LTR that was free in PBS. Fluorescent signals at excitation 490nm and emission 510nm increased with increasing concentrations of INT1/INT2-LTR. For measuring wild-type and mutated DNA binding activity at various integrase concentrations the 20nM INT1/INT2-LTR concentration was used.

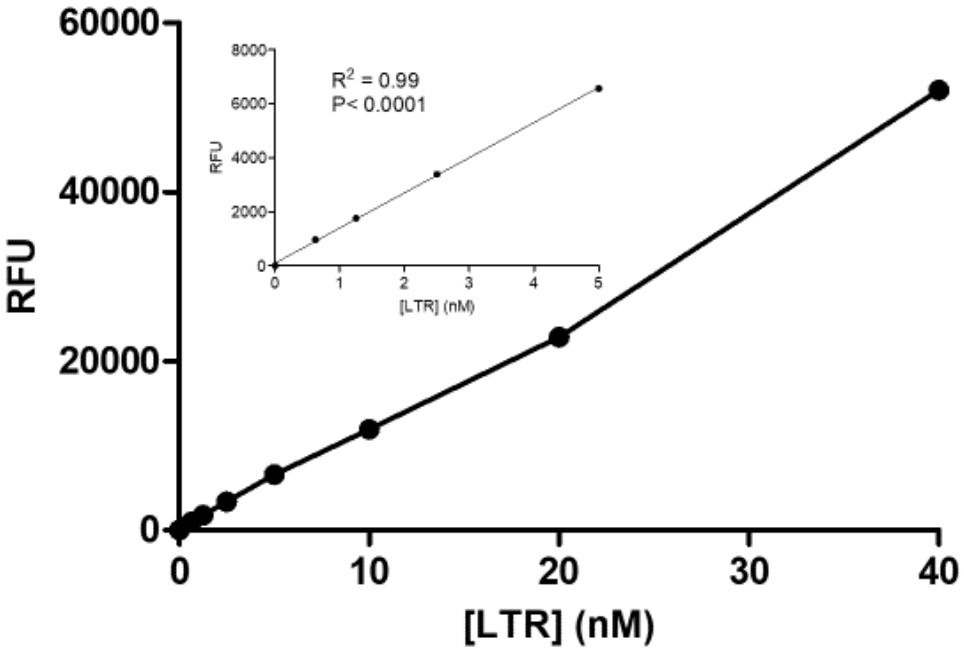


Figure 1 Fluorescence labelled INT1/INT2-LTR, free in PBS, calibration curve at concentration 0 to 40 nM. Data were fitted by linear regression at concentrations from 0 to 5 nM (see inset). Means ± standard error of the mean (error bars) was derived.

The enzyme HIV integrase has been shown to lose activity at high concentrations due to non-specific aggregation (Bushman et al., 1990, Jenkins et al., 1996, Jenkins et al., 1995, Dyda et al., 1994), therefore, the range of its activity before use in enzyme assays was determined. Firstly integrase was diluted in PBS at various concentrations and immobilised onto the surface of microplate wells. The LTR substrate concentration was kept constant at 20nM. As shown in Figure 2, the fluorescent signals increased with increasing concentrations of the immobilized HIV-1 integrase, with wild-type integrase showing increased signals than the mutants. The optimum integrase protein concentration of 1000nM was used to test for the DNA binding properties.

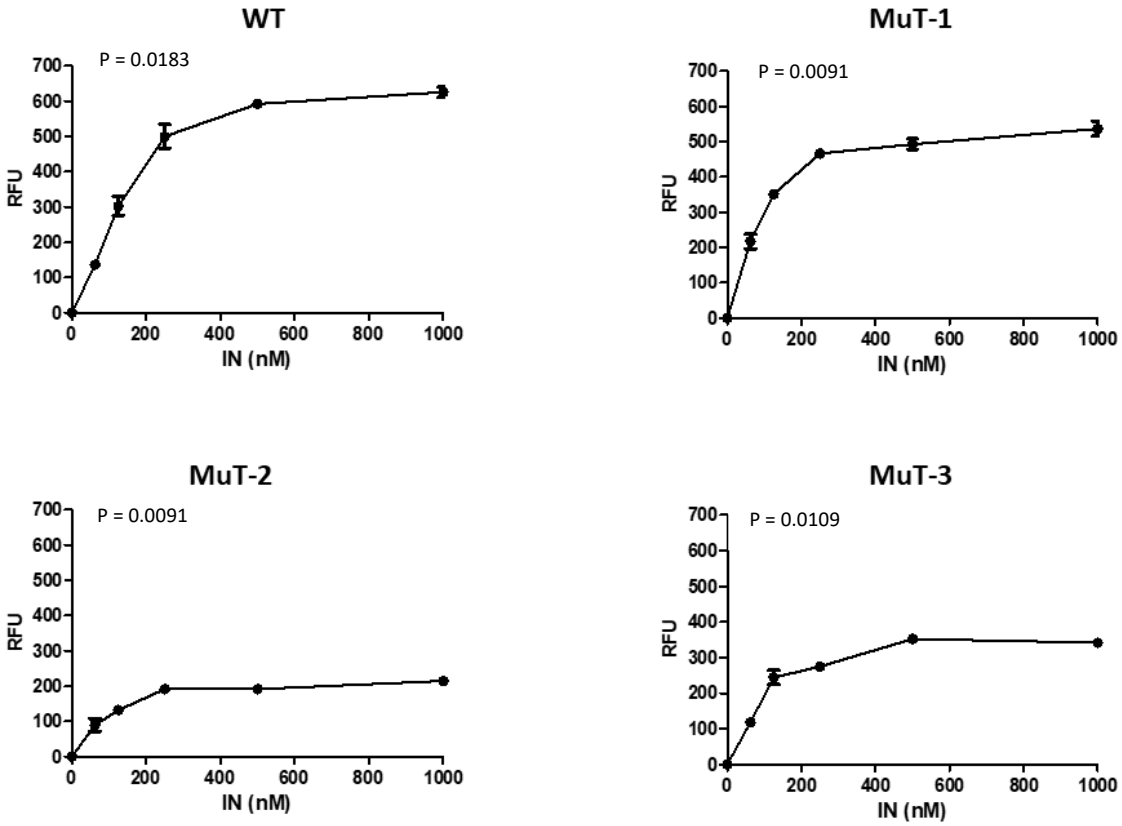
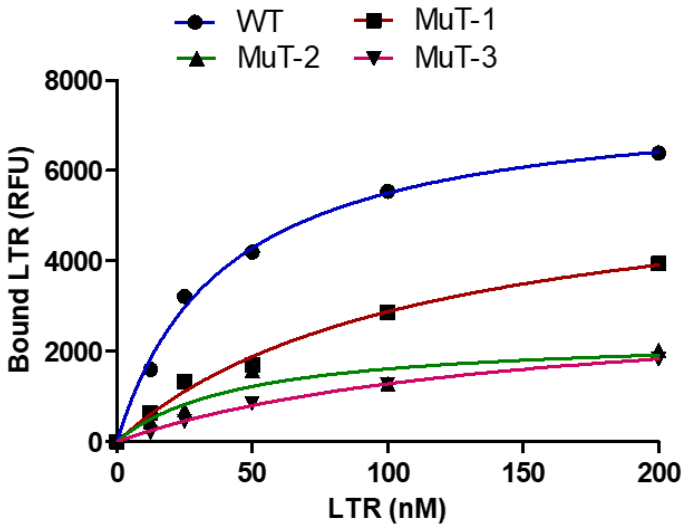


Figure 2 HIV-1 integrase DNA binding activity. DNA binding activity expressed in relative fluorescent units (RFU) in the presence of increasing wild-type and mutated integrase concentrations and 20nM INT1/INT2-LTR. Controls without integrase or LTR resulted in low fluorescence signals, showing that the high signal is due to integrase-DNA interaction. Means ± standard error of the mean (error bars) were derived. This graph was prepared with GraphPad Prism 5.03.

Integrases at 1000nM concentration were incubated with varying concentrations (0-200nM) of LTR substrates in binding buffer. Figure 3 shows the fluorescent signals from LTR bound to integrase. The results indicate that mutant integrases have lower DNA binding activity than wild-type integrase. The K_d values confirm this; with a Kd of 39.7 for the wild-type integrase, compared to 59.6, 112.1, and 149.1 for the mutants, respectively.



Integrase Protein	K_d Value (nM)
WT	39.7
MuT-1	59.6
MuT-2	112.1
MuT-3	149.1

Figure 3 Determination of the apparent dissociation constants for the binding of HIV-1 integrase (1000nM) to INT1/INT2-LTR concentrations 0-200nM. DNA binding reactions were performed in a binding buffer containing 7.5mM $MnCl_2$. The Kd values for wild-type and mutants were calculated by directly fitting the titration curve on GraphPad Prism 5.03 software using non-linear one-site binding regression.

DNA binding assays suggest a defect in the activity of mutated integrase, we evaluated the differences in LTR binding on the 3' processing activity using previously validated standard gel-based assays (Han et al., 2012). While each mutant showed a lower 3' processing activity than that of wild-type (Figure 4), there was no notable difference between MuT-2 and MuT-3's 3' processing activity.

3’ processing

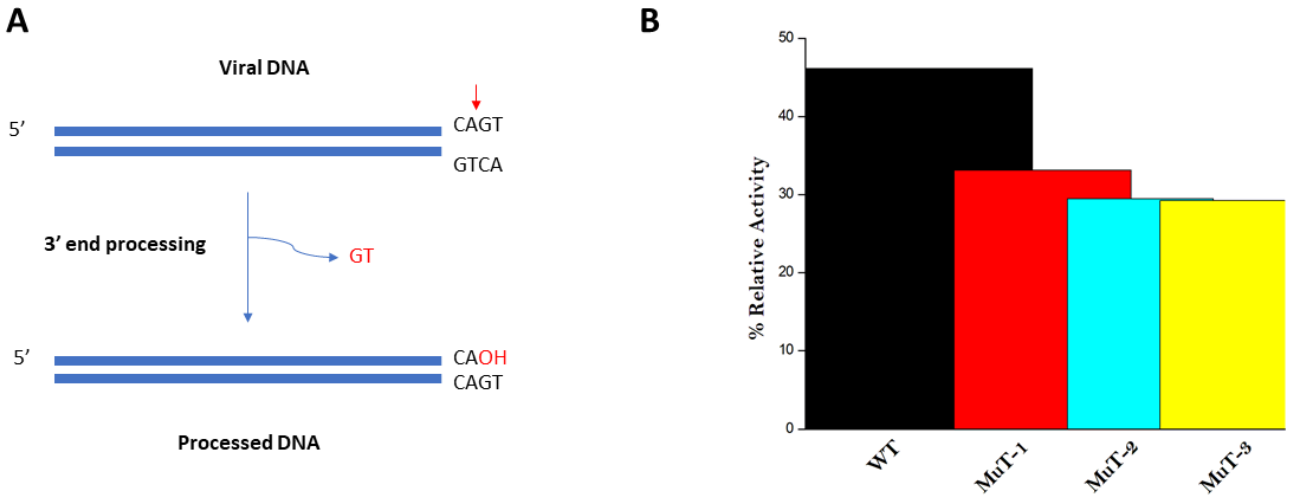


Figure 4 A) 3’ processing mechanism. **B)** 3’ processing activity of purified recombinant wild-type and mutated integrases. Calculated % relative activity after 2h incubation with 0.5 µM T35/SK70-LTR and 1µM protein

Figure 4A shows what happens when HIV- 1 integrase undergoes 3’ processing. The reaction starts with the integrase dimer binding to the active site; 3’ processing shown, is the cutting of the two nucleotides G and T from the 3’ end of viral DNA and gets replaced with a hydroxyl group. In Figure 4B the calculated % 3’ processing relative activity after 2h incubation with LTR and integrase proteins is shown. MuT-2 and MuT-3 activities had a similar 3’ processing activity, each mutant showed a lower 3’ processing activity than that of wild-type (Figure 4B).

Discussion

It has been demonstrated that subtype-specific differences are related to different transcriptional regulatory mechanisms between different subtypes, and that may affect replication kinetics (Montano et al., 1997, Gartner et al., 2020). Studies have also found that HIV-1C isolates demonstrate an additional nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) site compared to subtypes A, B, D, F, G, and AE (Jeeninga et al., 2000, Naghavi et al., 1999, Munkanta et al., 2005, Bachu et al., 2012). Similarly, genetic variation in HIV-1C integrase can influence the development of drug resistance in HIV-1 subtypes. To date, most published data on resistance to INSTIs have not focused on HIV-1C patient isolates from low-and middle-income countries (LMICs). For example, N155H is a clinically relevant HIV-1 integrase mutation that has been shown to exhibit increased resistance to RAL in subtype B molecular clones compared to subtype C (Sunpath et al., 2012, Bar-Magen et al., 2010a). With the second-generation INSTI, DTG, recently being recommended for use in the first-line combination antiretroviral therapy (cART) in LMICs (Moorhouse et al., 2018), it is important to identify and understand the possible role of INSTI RAMs and polymorphisms on the function of HIV-1C integrase enzyme, especially since it has been shown that INSTI mutations have differential effects on enzyme efficiency in different subtypes (Quashie et al., 2015b). Our previous study analysed the integrase region for the presence of INSTI treatment-associated mutations we used patient-derived isolates presenting with virologic failure after at least 6 months of RAL-based cART. The patient sequences contained known and novel mutations; only mutations S119T, G140A, Q148R, V151I, and E157Q were identified at treatment failure, of which G140A and Q148R are the most common and clinically significant INSTI resistance mutations (Brenner et al., 2011, Dicker et al., 2008, Anstett et al., 2017). In this study we aimed to determine the effect of INSTI treatment-associated mutations on integrase-LTR binding and 3'processing.

For subtype B, it is well established that INSTI RAMs reduce integrase activity (Anstett et al., 2017, Quashie et al., 2012a). Dicker et al, 2008 used a scintillation proximity assay to determine strand transfer kinetics (Dicker et al., 2008); while Métifiot et al, 2010, Quashie et al, 2012 and Quashie et al, 2013 examined 3' processing, strand transfer activity, and integrase-DNA binding of mutations using gel and microplate based-assays (Métifiot et al., 2010, Quashie et al., 2012a, Quashie et al., 2013a). These studies were all conducted on HIV-1B and have investigated mutations such as G118R, N155H, G140S, Q148R, and R263K (Métifiot et al., 2010, Quashie et al., 2012a, Quashie et al., 2013a). Overall, they showed decreased activity of the integrase variants compared to the wild-type, with the enzyme containing Q148R being the most decreased (Dicker et al., 2008, Quashie et al., 2012b). In this study, the viral isolates displayed loss in DNA binding (Figure 2) and 3' processing (Figure 4) compared to the wild-type integrase enzyme.

Métifiot et al, 2010 and Hassounah et al, 2014 has shown that the loss in DNA binding in the presence of mutations at amino acid residues 140-149, such as G140A/S and Q148H/R/K, referred to as the flexible loop is due to altered interactions with the retroviral DNA (Hassounah et al., 2014, Métifiot et al., 2010). In a cross-linking experiment, Q148 was shown as a possible contact for the 5'-adenine of the 5'-AC overhang (Esposito and Craigie, 1998). Mutating Q148 reduced the stability of the integrase-viral DNA complex as well as the strand transfer activity suggesting that it plays a role in the strand transfer reaction (Gerton et al., 1998, Dicker et al., 2008). The two glycines (G140 and G149) act as hinges at the end of the 140's loop region and allow a high degree of rotation on the polypeptide backbone due to their smaller side-chain size (Chiu and Davies, 2004, Greenwald et al., 1999); mutating residues 140 and 149 leads to minimized flexibility of the loop, therefore, restricted movement of the glycine residues (Greenwald et al., 1999).

The impact of mutations on the function of integrase could result from the longer side chain acquired in the Q148R mutation previously reported to alter viral DNA binding (Métifiot et al., 2010). Substitution to a more bulky residue, such as arginine, may result in steric hindrance (Hare et al., 2011) which would compromise the binding of INSTIs and cause a subtle change in the geometry of the active site (Hare et al., 2011). This in turn would affect the active site's ability to interact with the drug and substrate which can directly influence the enzyme's activity. The mutations G140A and Q148R are associated with resistance to RAL and EVG, from the manner they affect DNA binding *in vitro* it is possible to conclude that they can have an impact on the binding of INSTIs to the integrase enzyme, like it has been previously shown for various subtype B mutants (Quashie et al., 2013a, Dicker et al., 2008). However, mutations affecting DNA binding may not necessarily affect drug binding and drug efficacy. Therefore, studies using site-directed mutagenesis are needed to further evaluate the impact of mutations detected at RAL failure (S119T, G140A, Q148R, V151I, and E157Q) on integrase activity in the presence of each inhibitor to determine their susceptibility to INSTIs.

We further investigated the effect of these mutations on the dissociation constant (K_d value) since it indicates the binding affinity between integrase and LTR DNA, with a smaller Kd value indicating greater binding affinity of the ligand to its target. The presence of a metal cofactor Mn^{2+} or Mg^{2+} has been shown to have a large influence on the specificity of the integrase enzyme reaction (McNeely et al., 2011). In this study, we used Mn^{2+} since it is preferred for *in vitro* studies, as it has also been reported that integrase-DNA complexes are more stable in the presence of Mn^{2+} than in the presence of Mg^{2+} (Pemberton et al., 1998, Pemberton et al., 1996, Lesbats et al., 2008). The reported wild-type K_d value of 39.7 nM (Figure 3) is similar to previously reported K_d values of 35.95nM and 37.5nM by Han et al., 2013 and Deprez et al., 2014, respectively, for wild-type integrase proteins and is lower

than all the mutant proteins which are 59.6nM, 112.1nM and 149.1nM for MuT-1 to MuT-3, respectively (Deprez et al., 2004, Han et al., 2013). The decrease in all the mutant's K_d values is also reflected in their overall DNA binding (Figure 3) which further confirms that mutations selected at the catalytic site prevent the integrase enzyme's ability to bind LTR DNA (Thierry et al., 2017). The integrase catalytic core residue S119 is known to directly interact with the target DNA while residue V151, which is adjacent to the crucial active site residue E152, is known to be involved in DNA recognition and 3' processing (Calmels et al., 2004, Kessl et al., 2009, Serrao et al., 2014). The same residues when mutated are likely to reduce viral DNA binding affinity *in vitro* as shown for the isolates containing the S119T and V151 substitutions.

The 3' processing activity of the mutant isolates was markedly impaired compared to the wild-type. However, it is worth mentioning that the 3' processing activity of 33.12%, 29.44%, and 29.24% across the mutants alone, showed no appreciable differences compared to the mutant K_d values. Our findings indicate that the major effects caused by mutations on integrase activity were at the level of DNA binding. A study by Métifiot et al in 2010 demonstrated that the HIV-1B G140S+Q148H double mutant can restore the catalytic properties of integrase (Métifiot et al., 2010). Therefore, it is possible that in the double mutant isolate (MuT-3), each mutation has a distinct effect on the enzyme i.e., one of the mutations could have the ability to rescue the enzyme's 3' processing activity. This is a mechanism that has also been reported for HIV-1B H51Y+G118R double mutant where the enzyme's function was restored when the H51Y mutation acted as a secondary mutation compensating to G118R 3' processing activity (Quashie et al., 2013a, Métifiot et al., 2010). The E157Q minor mutation, also observed on MuT-3, has been previously reported at RAL failure (Charpentier and Descamps, 2018). Anstett et al in 2016 reported a loss in DNA binding activity in the presence of the E157Q mutation (Anstett et al., 2016). However, when combined with R263K, a major DTG mutation, E157Q was shown to have a beneficial effect on the integrase protein by restoring deficits caused by R263K on the enzyme activity (Anstett et al., 2016, Charpentier and Descamps, 2018). Future work is needed to quantify the contribution of G140A, Q148R, and E157Q mutations on HIV-1C integrase activity.

In conclusion, we present an analysis of DNA binding and 3'processing data to quantify the effects of RAMs and polymorphisms on HIV-1C. This study forms the basis for future research into the relevance of known mutations (G140A, Q148R, and E157Q) and most importantly polymorphisms (S119T and V151I) on the role they have in the development of drug resistance during INSTI-based cART since they have not been systematically investigated. The limitation of this study was that the sample size was small due to the low number of patients receiving INSTI-based therapy in South Africa and points to the need for continued surveillance of patients for the emergence of INSTI-RAMs after the initiation of INSTI-based cART.

CHAPTER FIVE

GENERAL DISCUSSION

GENERAL DISCUSSION

From their introduction, integrase inhibitors have been successful in terms of general patient outcomes, potency and *in vivo* pharmacokinetics (Mesplède et al., 2014); HIV-1 integrase has been regarded as the most promising therapeutic target (Trivedi et al., 2020). INSTIs potency and the improved resistance profiles of the second-generation drug classes such as DTG have made them effective for therapy in both treatment-experienced and treatment-naïve patients and are now included in the first-line cART in LMICs. Most recently, long waited advances in the field of cryo-electron microscopy provided high-resolution INSTI-bound structures of lentiviral intasomes from an NL43 strain and SIV_{rcm} (Cook et al., 2020, Passos et al., 2020). The structures helped clarify the INSTI binding mode within intasome active sites; this elucidation has led to a good understanding of the mechanism of resistance (Jóźwik et al., 2020). In this thesis, we have analysed HIV-1C integrase sequences from South African isolates, identifying RAMs and polymorphisms that affect INSTIs binding. We have also attempted to understand the effect of these mutations on the structure and functioning of HIV-1C integrase, as the prevalence of subtype C is high in LMICs and the impact of subtype-specific polymorphisms that may play a role in the development of drug resistance is not yet fully studied.

In South Africa, third-line cART in the public sector is accessed through a national committee that assesses eligibility and recommends a regimen on a case-by-case basis (Health and Africa, 2019, Moorhouse et al., 2019, Steegen et al., 2019). A 2019 report from the 3rd committee stated that only 1084 patients were approved for an INSTI-based 3rd line regimen nationally (Third-Line cART Committee May 2019 communication, (Steegen et al., 2019). As a result, there is a very low number of INSTI failures, and as a consequence data on integrase resistance in this region (KwaZulu Natal) is scarce. In this study, 7 HIV-1C isolates failing an INSTI-inclusive therapy referred for the 3rd line regimen referral hospital in Durban, South Africa were available for analysis. We also analyzed 41 HIV-1C INSTI-naïve control sequences (9 INSTI-naïve, treatment-experienced isolates amplified in our laboratory and 32 treatment naïve South African HIV-1C integrase sequences downloaded from GenBank). In 2018, Brado et al analysed HIV-1 integrase sequences from South Africa and reported the absence of major INSTI RAMs in naïve sequences, and in 2013 Bessong and Nwobegahay conducted a study in the North-East part of South Africa which showed a lack of major INSTI RAMs also in naïve HIV-1C integrase sequences (Brado et al., 2018, Bessong and Nwobegahay, 2013).

Resistance Associated Mutations

Sanger sequencing identified several mutations and polymorphisms within the integrase coding region, including some of the most clinically significant INSTI-RAMs i.e. G140A, Q148R and E157Q from the same patient at a frequency of 14.28%. Of the polymorphisms identified in our study, S119T and V151I were each only found in 3/7 (42.9%) patients failing an INSTI-containing cART regimen, shown in chapter 3 Figure 2. The G140A+Q148R double mutant has not been previously identified within HIV-1C infected patients (Chitongo et al., 2020). We, therefore, report for the first time in South African HIV-1C clinical samples, the presence of the G140A+Q148R double mutant. Previous biochemical analysis data on HIV-1B recombinant integrase showed that the double mutation at positions 140 and 148 is the most common combination to emerge *invitro* (Métifiot et al., 2010, Wensing et al., 2019) with G140A being associated with Q148R and G140S with Q148H/K. However, these mutations have also been identified in clinical studies (Malet et al., 2015, Johnson et al., 2013).

The major G140A and Q148R mutations are in the integrase flexible loop region of the α4 helix (amino acid residues 140-149) and are associated with resistance to RAL and EVG; any mutation in the 140's loop directly impacts the active site's ability to interact with drugs and DNA substrate (Métifiot et al., 2010). Table 2, Chapter 3 displaying integrase-INSTI binding affinities showed that the isolate containing the G140A+Q148R double mutant i.e. MuT-3 had the lowest binding affinity for both RAL and EVG. The lowered activity indicates that the mutations observed in the 140s loop have a negative functional impact on the protein that makes the enzyme less stable than the wild-type one. Moreover, Chapter 4 results (Figure 2 and 3) showed that integrase-LTR binding activity for MuT-3 was lowered compared to the wild-type, indicating that the integrase double mutant has the most effect on the binding step of integration. Previous studies on integrase have shown that the 140's loop flexibility is important for catalytic steps following DNA binding. In particular, Q148 was implicated in viral DNA binding, and mutations at this position reduce the stability of the integrase-viral DNA complex as well as integrase strand transfer and 3' processing activities (Lee et al., 2005, Métifiot et al., 2010, Gerton et al., 1998). The loss can be attributed to chemical cross-linking previously demonstrated between Q148 and the 5'AC overhang of the viral DNA (Esposito and Craigie, 1998). Docking studies have shown that Q148 forms part of the binding pocket for integrase inhibitors selective against the integrase strand transfer reaction (Marchand et al., 2002, Pais et al., 2002, Sotriffer et al., 2000). In addition, the acquired bulkier arginine side chains have been reported to inhibit integrase strand transfer and 3' processing activities (Métifiot et al., 2010).

While the integrase amino acid G140 has not been reported to directly interact with DNA, this residue is crucial in maintaining the flexibility of the 140's loop as it has been shown to act at the end of the

loop as a hinge together with G149 allowing the polypeptide backbone rotation. It is possible that with glycine residue at position 140 at the N-terminal end of the loop the acquired methyl side chain when substituted to alanine limits its interaction with residue G149, therefore, affecting the polypeptide backbone rotation. To evaluate changes caused by the double mutant in the 140s loop geometry, we calculated dreiding energy between the wild-type and mutant loop structures. Dreiding energy discussed by Mayo et al in 1990 sums components assigned to bond lengths, bond angles, and dihedral angle torsion, with lower energy indicating a stable conformation (Mayo et al., 1990, Allen et al., 2010, Karimipour et al., 2020). Chapter, Figure 3 shows a change from 3092.7 kcal/mol in the wild-type to 20 373.3 kcal/mol in the double mutants flexible loop. Such unstable conformation in the mutant loop structure could lead to insufficient integration such that integrase won't be able to efficiently perform its function. Analysis of the change in the trajectory of the double mutant isolate compared to the wild-type also suggested less stability of the double mutant system; chapter 3, Figure 2.

While the isolate containing the double mutant had the overall lowest binding affinity in relation to wild-type and the other isolates, with regards to the 3' processing activity, the decrease observed was not as considerable as observed with the DNA binding experiments. In 2010, Métifiot et al demonstrated that the G140S+Q148H double mutant is able to restore integrase 3' processing activity and in 2013, Quashie et al also showed that both H51Y+G118R and G118R+E138K double mutants fully restored integrase 3' processing activity (Quashie et al., 2013a, Métifiot et al., 2010). This could suggest that the double mutant (G140A+Q148R) in this isolate plays a role in the restoration of 3' processing, as it has been previously reported for HIV-1B double mutants.

The E157Q minor mutation is common with HIV-1 isolates resistant to INSTIs and has been previously reported after RAL exposure (Ghosn et al., 2009, Charpentier and Descamps, 2018). E157Q may be of particular importance for patients receiving INSTI-based therapy since it has been identified to decrease the integrase DNA binding activity when it was studied with R263K, the most commonly reported DTG resistance mutation (Anstett et al., 2016). Anstett *et al*, 2016 also suggested that E157Q may compensate for the effect conferred by R263K on the integrase protein (Anstett et al., 2016). Consistent with Anstett *et al*'s findings other reports have stated that E157Q is able to function as a compensatory mutation for N155H, a primary RAL and EVG mutation (Anstett et al., 2015, Hurt et al., 2014). Moreover, a RAL-containing cART failure has been previously reported in a patient harbouring E157Q alone (Danion et al., 2015). This study reports a patient isolate where these mutations (E157Q, G140A, and Q148R) known to cause resistance to INSTIs occurred together. Although RAL triple resistant mutants have been identified in patients and *in vitro* studies (Fransen et al., 2009a, Fransen et al., 2009b, Eron et al., 2013, Smith et al., 2021a), this is the first report on the occurrence of three mutations in one patient. Similar to the compensatory role for R263K and N155H, it is possible that

E157Q is compensating for the effects conferred by the mutations in the 140s loop. To fully elucidate the role of each mutation their activity should be measured individually and in combination using site-directed mutagenesis studies.

Mutations selected close to the integrase active site residues have been shown to prevent the binding of INSTIs resulting in the restoration of the integrase enzyme's ability to fulfil its viral DNA integration role (Malet et al., 2008, Goethals et al., 2008, Gerton et al., 1998). These mutations include positions G118 and S119 which are near the active site residue D116 and have been shown to affect DTG susceptibility (Smith et al., 2021b). Hachiya et al, 2015 also showed that mutation at residue S119 contributed to a significant increase in DTG resistance (Hachiya et al., 2015); V151 is also near the active site residue E152, mutating it with isoleucine reduces the size of the active site which could potentially affect INSTI binding. It has been suggested that residue 119, the second of four amino acids near the middle of the central domain of HIV-1 integrase, is one of the residues that contribute to the selection of target sites in non-viral DNA, thus making interactions with host DNA during catalysis (Harper et al., 2001). It was demonstrated in Visna and Rous sarcoma virus integrases that substitutions at this position (119) will change the target site preference suggesting that this residue's target site selection of host DNA is a general property of retroviral integrases (Harper et al., 2003). V151I, a mutation located close to the catalytic core has been suggested to act as a secondary mutation in different HIV-1 subtypes frequently associated with the N155H mutation (Serrao, 2009, McColl, 2010 (Ceccherini-Silberstein et al., 2010). While the Stanford Drug Resistance Database does not consider S119T and V151I substitutions as INSTI resistance determinants, in our subtype C isolates, they were both found at a frequency of 42.9% and only at virologic failure. Also, residue 119 forms hydrogen bond interactions with both RAL and DTG (Figure 6, chapter 3), this could mean that S119 may play an important role in drug binding and could be exploited in designing new INSTIs .

Implications for DTG treatment in LMICs

Despite DTG demonstrating a higher barrier to resistance than the first-generation INSTIs, treatment-experienced individuals experience DTG failure associated with the emergence of the R263K mutation (Wensing et al., 2019) showing that previous exposure to RAL and EVG can compromise the efficacy of DTG when used as a single antiretroviral agent; monitoring of drug resistance when patients switch to DTG salvage therapy should be recommended. On the other hand, HIV-1 subtype may influence the development of INSTI resistance mutations and the prevalence of R263K in subtype C has shown to be lower compared to subtype B (Mesplède et al., 2015, Inzaule et al., 2018). Also, limited cross-resistance has been observed between DTG and RAL/EVG, at positions Q148 and R263 compared to the significant cross-resistance observed between RAL and EVG at positions E138, G140, Y143, Q148,

N155, and R263 (Wainberg and Han, 2015, Orta-Resendiz et al., 2020, Oliveira et al., 2018). In treatment-naïve individuals, to date, first-line DTG resistance has been a rare occurrence in treatment naïve individuals unlike with first-generation drug classes RAL and EVG (Raffi et al., 2013, Mesplède et al., 2014, Blanco et al., 2011, Cevik et al., 2020) meaning the WHO decision to include DTG as a standard drug within the first-line cART for patients in LMICs could potentially be a critical benchmark moving forward in the arsenal against HIV-1.

Primarily, what separates DTG from RAL is the introduction of a different central pharmacophore scaffold i.e. a change from bicyclic to a tricyclic ring system responsible for metal chelation. As a result of the expanded scaffold, DTG makes additional contacts with integrase residues and retain potency against resistant viruses (Jóźwik et al., 2020). The ability of DTG to evade RAL and EVG primary substitutions by forming extensive hydrophobic stacking interactions with viral LTR makes it more difficult to develop resistance against DTG (Hare et al., 2011). In another study, DTG was reported to have a significantly longer binding half-life to HIV pre-integration complexes compared to both RAL and EVG (Dicker et al., 2008, Hightower et al., 2011). Overall the binding free energy reported in this study showed higher binding affinities between the wild-type HIV-1C integrase on all three drugs (i.e. DTG, EVG, RAL) compared to the mutant isolates; Table 2, chapter 3 Furthermore, when comparing each drug binding energies to the mutants, the work presented in chapter 3 showed that DTG had the highest binding affinity across the mutants suggesting that DTG will have more binding strength than RAL and EVG.

Our study utilized clinical isolates reflective of INSTI treatment-experienced patients harbouring HIV-1C and our analysis included recombinant constructs encoding patient-derived integrase. This study was limited by a small sample size. As few patients are receiving INSTI-based cART, there is currently a limited number of available INSTI failures in this region. This should be addressed in future studies especially because DTG is being introduced at a larger scale in LMICs.

Conclusions

This study serves as the first to report an occurrence of a double mutation in the 140s loop in HIV-1C with previous reports being for subtype B. Given the low number of patients on INSTIs in LMICs and consequently low number of INSTI failures, this study provides valuable data on the mutations that can arise at INSTI failure. While this study shows a low prevalence of RAMs in this cohort of INSTI failures, the identification of previously described RAMs (G140A, Q148R, and E157Q) and possible novel HIV-1C RAMs (S119T and V151I) suggest that proper drug resistance surveillance is needed

before switching to an INSTI-based cART for LMICs as the G140+Q148 combination has been related to DTG resistance in subtype B, implying that patients with pre-existing RAL resistance are likely to develop resistance to DTG. Our data suggest that DTG will be a better option than RAL for patients on salvage therapy to prevent any potential mutations that arise under RAL-treatment inclusive therapy. The current work highlights the fact that they are positions in integrase that have not previously been associated with drug resistance, however, we have shown that they were increased in treatment-experienced patients, and they impact drug binding. The clinical relevance of the double mutant and the possibility of this combination to restore integrase catalytic properties should be further investigated.

Future recommendations

In the current study, we experienced great difficulty to obtain a larger sample size. As DTG use increases in the South African public sector, future work should build upon this research findings to analyse drug resistance mutations to INSTIs on a larger scale and evaluate their impact on the functioning of the enzyme. Next generation sequencing can be conducted to detect low frequency integrase resistance mutations. Additionally, site-directed mutagenesis experiments on a subtype C backbone will be informative in future studies to identify INSTI-RAMs with significant impact on HIV-1 replication capacity.

CHAPTER SIX

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APPENDIX A - ETHICS CERTIFICATE

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28 January 2020

Ms N Mbhele
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Dear Ms Mbhele

PROTOCOL: HIV-1 integrase inhibitor mutations: Analysis of structural and biochemical effects.
Degree: PHD
BREC Ref No: BE596/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	27 February 2020
Expiration of Ethical Approval:	26 February 2021

I wish to advise you that your application for Recertification received on 20 January 2020 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 February 2020.

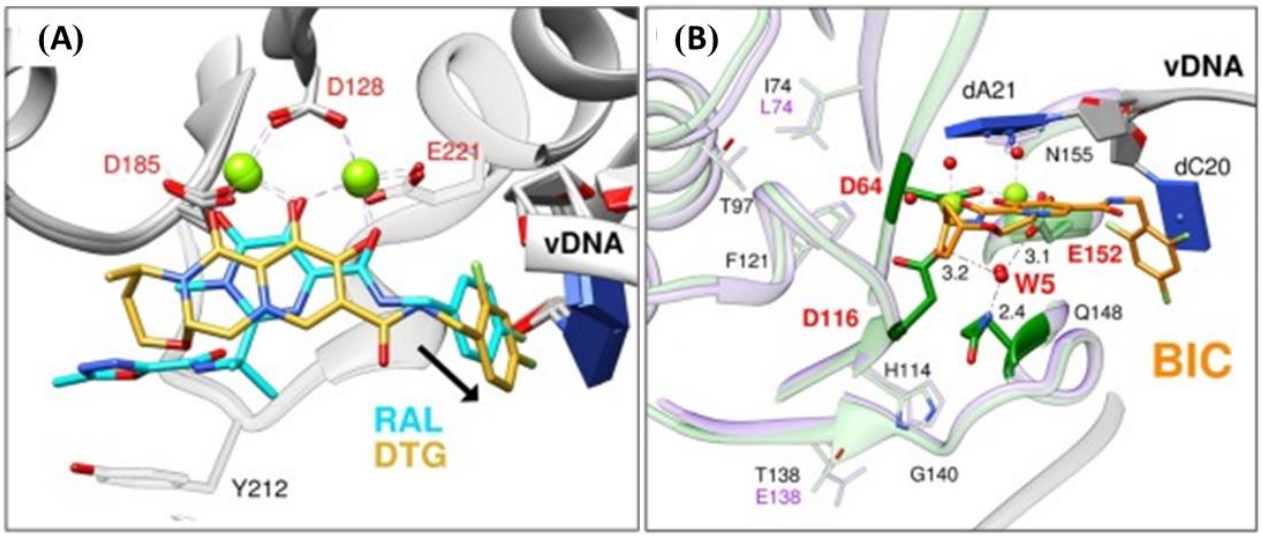
Yours sincerely

[redacted]
[redacted]rof V Rambiritch
[redacted]hair: Biomedical Research Ethics Committee

cc: dudhrajhp@ukzn.ac.za zolah.mbhele@gmail.com

APPENDIX B - SUPPLEMENTAL FIGURE

Chapter 2 supplemental figure



Supplemental Figure S1 Selected first and second-generation INSTIs binding characteristics. (A) DTG shown in gold sticks bound to PFV intasomes (PDB ID: 3S3M) superimposed with RAL, shown in cyan sticks bound to PFV intasome (PDB ID: 3OYA) depicting the deeper penetrance of DTG's halo benzyl substituent (black arrow) when compared to RAL. (B) Superimposed structure of BIC-bound to SIV_{rcm} (PDB ID: 6RWM; light green) and HIV (PDB ID 6PUW; green) intasomes.

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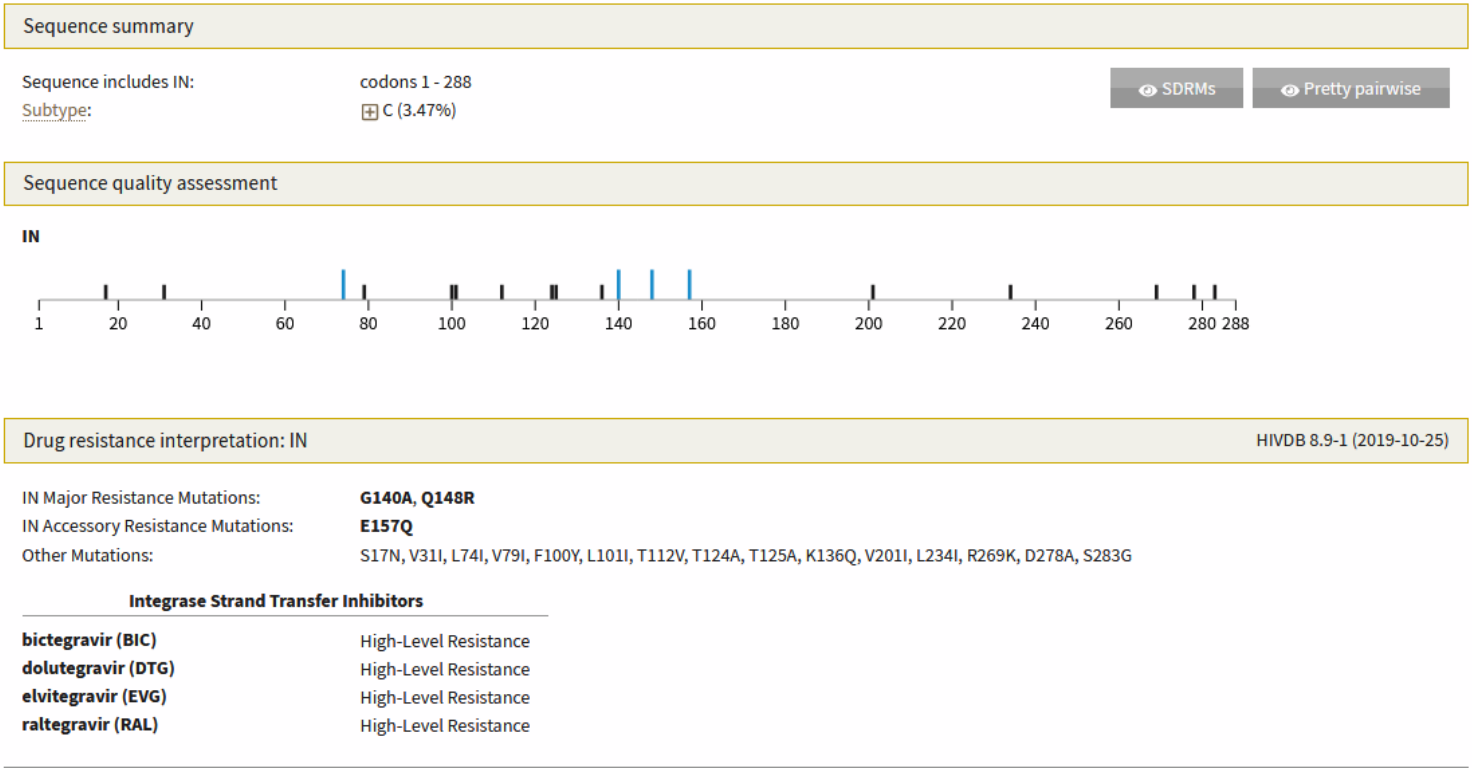
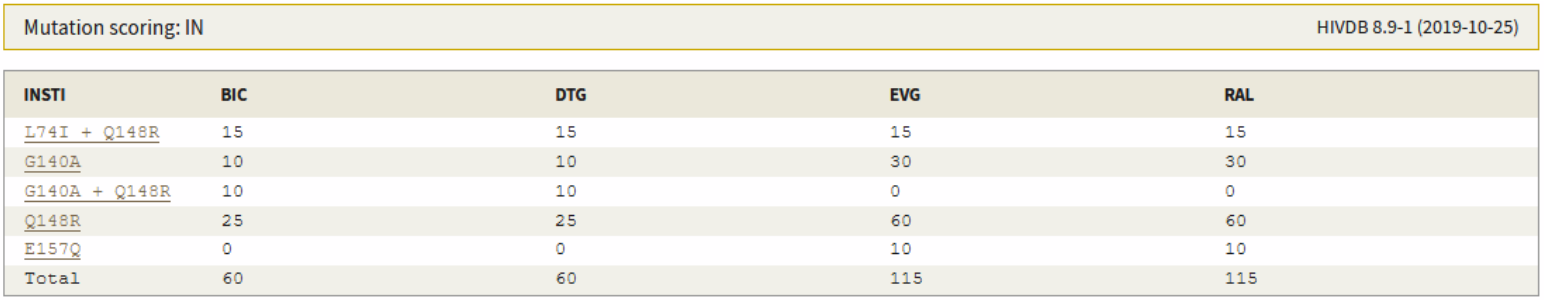
Chapter 3 supplemental figures

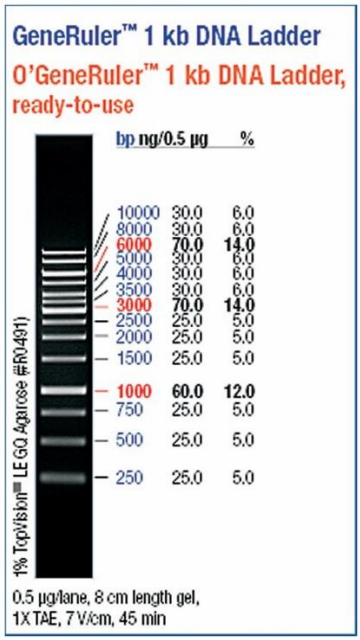


Supplemental Figure S1 Screenshot of output from the HIVdb algorithm showing mutations on MuT-3 and their level of resistance to INSTIs

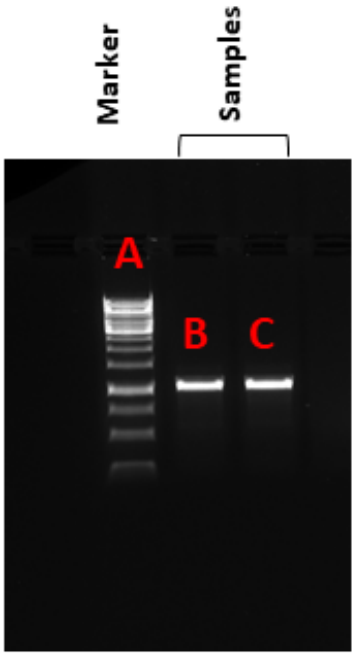


INSTI	BIC	DTG	EVG	RAL
L74I + Q148R	15	15	15	15
G140A	10	10	30	30
G140A + Q148R	10	10	0	0
Q148R	25	25	60	60
E157Q	0	0	10	10
Total	60	60	115	115

Supplemental Figure S2 Screenshot of output from the HIVdb algorithm showing mutations on MuT-3 and their scoring

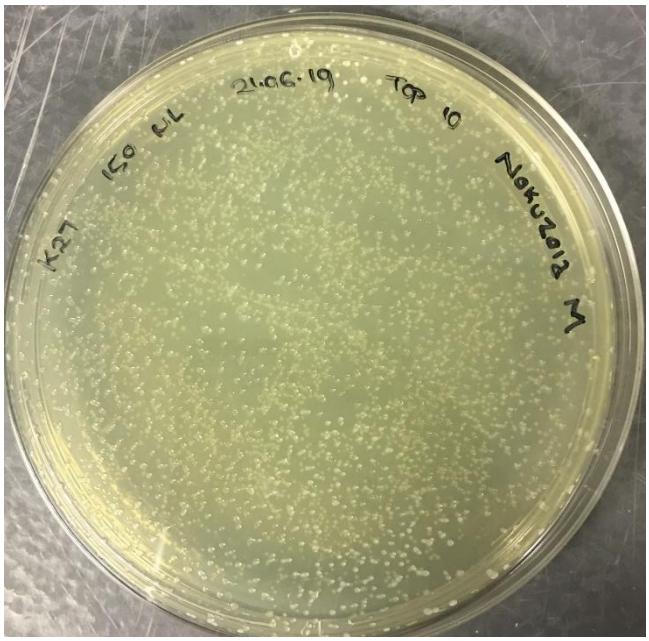


Supplemental Figure S3 One kb DNA ladder showing the different size products on a 1% Agarose gel.

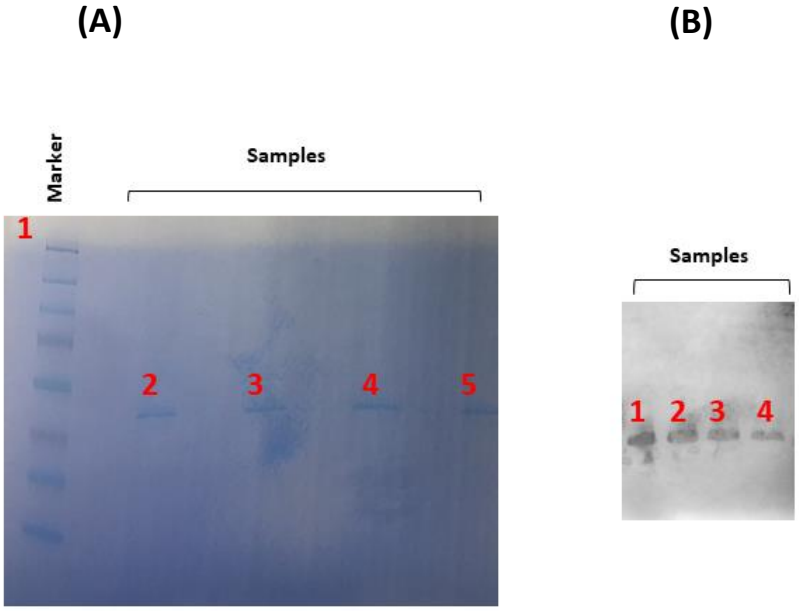


Supplemental Figure S4 PCR amplification of HIV-1 integrase

Chapter 4 Supplemental Figures



Supplemental Figure S1 Result of TOPO cloning technique



Supplemental Figure S2 Expression and purification of HIV-1C integrase. (A) 12% SDS-PAGE showing purified MBP tagged HIV-1C integrase: Lane 1 (protein marker), Lane 2-5 HIV-1C integrase mutant samples. (B) Western blot image of HIV-1C integrase mutant samples

APPENDIX C - SUPPLEMENTAL TABLE

Chapter 2 Supplemental Table

Supplemental Table S1 Structural differences between first and second-generation INSTIs.

First-generation INSTIs	**Second-generation INSTIs**
Have a bicyclic central pharmacophore scaffold ring system	Have a tricyclic central pharmacophore scaffold ring system
Does not make additional contact with integrase residues	Makes additional contact with integrase residues
Have a short linker connecting the pharmacophore and the halogenated benzyl moiety	Have a longer linker group connecting the central metal-chelating pharmacophore and the halogenated benzyl moiety
Have a limited ability to adjust compound binding in response to changes in the integrase active site	Have an unlimited ability to adjust compound binding in response to changes in the integrase active site
The nature and position of benzyl ring halogenation can limit inhibitor potency	The nature and position of benzyl ring halogenation can influence inhibitor potency

APPENDIX D – COVER PAGE OF PUBLISHED PAPER (CHAPTER TWO)

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HIV-1 integrase strand transfer inhibitors: a review of current drugs, recent advances and drug resistance

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ABSTRACT

Antiretroviral therapy has been imperative in controlling the human immunodeficiency virus (HIV) epidemic. Most low- and middle-income countries have used nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors extensively in the treatment of HIV. However, integrase strand transfer inhibitors (INSTIs) are becoming more common. Since their identification as a promising therapeutic drug, significant progress has been made that has led to the approval of five INSTIs by the US Food and Drug Administration (FDA), i.e. dolutegravir (DTG), raltegravir (RAL), elvitegravir (EVG), bictegravir (BIC) and cabotegravir (CAB). INSTIs have been shown to effectively halt HIV-1 replication and are commended for having a higher genetic barrier to resistance compared with NRTIs and NNRTIs. More interestingly, DTG has shown a higher genetic barrier to resistance compared with RAL and EVG, and CAB is being used as the first long-acting agent in HIV-1 treatment. Considering the increasing interest in INSTIs for HIV-1 treatment, we focus our review on the retroviral integrase, development of INSTIs and their mode of action. We also discuss each of the INSTI drugs, including potential drug resistance and known side effects.

1. Introduction

Human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS), was first reported in 1981 [1] and soon became a major epidemic and one of the greatest health challenges to humankind. Today, scientific and therapeutic progress against HIV has been remarkable. There has been a dramatic change in the life expectancy of people living with HIV as are recommended. The most common antiretroviral drugs used in low- and middle-income countries (LMICs) include reverse transcriptase inhibitors and protease inhibitors (PIs). Most first-line regimens use combinations of nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), with PIs being used in second-line and integrase strand transfer inhibitors (INSTIs) in third-line regimens. However, INSTIs are becoming more common in first-line ART, with the

APPENDIX D – COVER PAGE OF PUBLISHED PAPER (CHAPTER THREE)

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Structural effects of HIV-1 subtype C integrase mutations on the activity of integrase strand transfer inhibitors in South African patients

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Communicated by Ramaswamy H. Sarma

ABSTRACT

HIV-1 integrase enzyme is responsible for the integration of viral DNA into the host genomic DNA. Integrase strand transfer inhibitors (INSTIs) are highly potent antiretroviral agents that inhibit this process, and are internationally approved for the treatment of both naïve and treated HIV-1 patients. However, their long-term efficacy is threatened by development of drug resistance strains resulting in resistance mutations. This work aimed to examine the effect of INSTI resistance-associated mutations (RAMs) and polymorphisms on the structure of HIV-1 subtype C (HIV-1C) integrase. Genetic analysis was performed on seven HIV-1C infected individuals with virologic failure after at least 6 months of INSTI-based antiretroviral therapy, presenting at the King Edward VIII hospital in Durban, South Africa. These were compared with sequences from 41 INSTI-naïve isolates. Integrase structures of selected isolates were modeled on the SWISS model online server. Molecular docking and dynamics simulations were also conducted using AutoDock-Vina and AMBER 18 force fields, respectively. Only one INSTI-treated isolate (14.28%) harboured major mutations (G140A + Q148R) as well as the E157Q minor mutation. Interestingly, S119T and V151I were only found in patients failing raltegravir (an INSTI drug). Molecular modeling and docking showed that RAMs and polymorphisms associated with INSTI-based therapy affect protein stability and this is supported by their weakened hydrogen-bond interactions compared to the wild-type. To the best of our knowledge, this is the first study to identify a double mutant in the 140's loop region from South African HIV-1C isolates and study its effects on Raltegravir, Elvitegravir, and Dolutegravir binding.

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HIV-1C; integrase; drug resistance mutations; integrase strand transfer inhibitors; molecular docking; molecular dynamics simulations

Introduction

The HIV-1 Pol gene encodes for the enzyme integrase that catalyzes the chromosomal integration of newly synthesized double-stranded DNA into the host genomic DNA (Bessong & Nwobegahay, 2013). HIV-1 integrase is a 32 kDa protein comprising 288 amino acids (Sachithanandham et al., 2016), a class of nucleotidyltransferase enzymes with three conserved spatially arranged carboxylates, and three structural

RAL, EVG, and DTG to effectively halt HIV-1 replication has been a major advancement for the HIV-1 treatment programme. Despite their effectiveness in HIV-1 therapy, INSTIs are threatened by the development of drug-resistant mutations. Such mutations may compromise the integrase-INSTI interaction rendering the drugs ineffective. Major pathways involved in RAL and EVG resistance include substitutions at positions 140, 148, and 155 (Blanco et al., 2011; Hazuda,