

https://dspace.org/

In vitro selection and characterisation of human immunodeficiency virus type-1 subtype C integrase strand transfer inhibitor resistant mutants

Mphahlele, Morore Katlego

2016

http://hdl.handle.net/10539/19625

Downloaded from a DSpace repository. https://dspace.org/

In vitro selection and characterisation of human immunodeficiency virus type-1 subtype C integrase strand transfer inhibitor resistant mutants

Morore Katlego Mphahlele

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine

Johannesburg 2015

Declaration

I, Morore Katlego Mphahlele affirm that the work presented in this dissertation is my own. It is being submitted for the degree of Doctor of Philosophy in Medicine in the University of Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other University.



.....

3rd day of November, 2015

Dedication

I, "give thanks to the Lord, for he is good; his love endures forever." Psalm 118: 1

I thank my wonderful family for their unwavering support and love:

My wife, Mokgadi Mphahlele

My dearest mother, Mokgohloe Mphahlele for things too many to list: her selflessness and love.

My sons, Neo Mphahlele and Mphela Mphahlele

My daughter, Mpho Mphahlele

My uncles, brothers and sisters

My many nieces and nephews

"Oh, the depth of the riches of the wisdom and knowledge of God…
For from him and through him and to him are all things,
to him be glory forever!"
Romans 11: 33-36

Publications and Conference proceedings

Publications……

Morore Mphahlele, Raymond Hewer, Irene Ketseoglou, Nichole Cerutti, Telisha Traut, Salerwe Mosebi, Simon Travers and Maria Papathanasopoulos. *In vitro* selection and characterisation of human immunodeficiency virus type-1 subtype C integrase strand transfer inhibitor resistant mutants. *In preparation*

Conference Oral Presentations…….

MK Mphahlele, R Hewer, M Bronze, K Steegen, S Carmona, WS Stevens and MA Papathanasopoulos. Resistance-associated mutations in HIV-1 subtype C primary viruses after *in vitro* passage with the integrase inhibitor, raltegravir. 6TH SAAIDS Conference 18th - 21st June 2013, ICC Durban, South Africa

Conference Poster Presentations…….

Morore Mphahlele, Raymond Hewer, Irene Ketseoglou, Salerwe Mosebi, Simon Travers, and Maria Papathanasopoulos. The impact of in vitro selected drug resistance mutations on HIV-1 subtype C integrase strand transfer capacity. MAM-14/7th International Symposium on Macro- and Supramolecular Architectures and Materials. Emperors Palace, Johannesburg - South Africa, November 23 - 27, 2014

MK Mphahlele, R Hewer, M Bronze, S Mosebi, K Steegen, S Carmona, WS Stevens and MA Papathanasopoulos. Resistance-associated mutations in HIV-1 subtype C primary viruses after *in vitro* passage with the integrase inhibitors. International Workshop on HIV & Hepatitis Virus Drug Resistance and Curative Strategies 4-8 June 2013, The Fairmont Royal York Hotel, Toronto Canada

Abstract

The currently approved integrase strand transfer inhibitors (INSTIs), raltegravir (RAL) and elvitegravir (EVG) effectively halt HIV-1 replication but their use is limited by their low genetic resistance barrier and cross resistance. For instance, integrase amino acids N155 and Q148 represent genetic pathways selected by both drugs and are associated with considerable cross resistance to both RAL and EVG. Dolutegravir (DTG) is a second generation drug manufactured to exhibit a more robust resistance profile than RAL and EVG, and retains activity against RAL and EVG resistant isolates. Most research on drug resistance patterns have been carried out with emphasis on HIV-1 subtype B and inadequately assessed in HIV-1 subtype C. Thus, the aim of this study was to establish the drug resistance mutation profiles of HIV-1 subtype C primary virus isolates that evolve/emerge under selective pressure of the INSTIs RAL, EVG and DTG, and evaluate their impact on strand transfer. *In vitro* selection experiments were carried out using six primary virus isolates (three wild-type, FV, and three reverse transcriptase drug resistant, MR, viruses) grown in peripheral blood mononuclear cells in the presence of increasing concentrations of RAL, EVG and DTG, and monitored to beyond virus break-through. Viral RNA was extracted from various time points and the *pol* region was RT-PCR amplified and sequenced using conventional Sanger-based sequencing and next generation sequencing (Illumina MiSeq). HIV-1 subtype C FV6 wild-type and mutant recombinant integrase (generated by site-directed mutagenesis) were expressed, purified and used in strand transfer assays and surface plasmon resonance (SPR) experiments to establish the binding affinities of IN-DNA. Wild-type FV primary viruses were successfully grown in the presence of increasing concentrations of RAL, EVG and DTG, up to 266 nM, 66 nM and 32 nM, respectively. Drug resistant MR viruses were successfully grown in the presence of increasing concentrations of RAL, EVG and DTG, up to 266 nM, 16 nM and 8 nM, respectively. Sequence analysis on both platforms revealed the presence of the previously described drug resistance mutations T66IK, E92Q, F121Y, Q148R, N155H and R263K in some viruses, and additionally H114L was detected. RAL was observed to select for substitutions Q148R and N155H/H114L in isolates FV6 and MR69, respectively. EVG selected F121Y, T66I/R263K, T66K and T66I in FV3, FV6, MR69, MR81, and MR89, respectively. DTG selected substitutions E92Q and M50I in FV3 and MR81, respectively. *In silico* data exhibited changes in hydrophilicity, hydrophobicity and side chain changes as well as changes in polarity, and all substitutions displayed acceptable minimisation energies and

distances between the atoms. Seven IN mutants were expressed and purified, and thereafter tested for efficiency in strand transfer. All mutant $FV6^{T66I}$, $FV6^{E92Q}$, $FV6^{H114L}$, $FV6^{F121Y}$, $FV6^{Q148R}$, $FV6^{N155H}$ and $FV6^{R263K}$ IN enzymes demonstrated an overall loss in strand transfer capacity of 37.1%, 21.5%, 66.1%, 63.2%, 60.2%, 30.5% and 3.4%, respectively. This is the first report on loss of strand transfer activity associated with H114L. The loss in strand transfer capacity in all the mutants was not reflected by their overall binding affinities to donor DNA, as determined by surface plasmon resonance, likely attributed to the role of different residues associated with DNA and drug binding in the IN quaternary structure. In conclusion, this is the first report describing IN drug selection experiments using primary HIV-1 subtype C isolates, and a detailed genotypic and biochemical characterisation of the associated mutations.

Acknowledgements

My utmost gratitude to my supervisor Prof Maria Papathanasopoulos; you have been a consistent source of knowledge, support and guidance through the course of the study. You will forever remain an inspiration to me.

I thank my co-supervisors Dr Raymond Hewer and Dr Salerwe Mosebi for their assistance during the course of the study.

Much thanks to Dr Irene Ketseoglou, Prof Simon Travers and Dr Nichole Cerutti for their respective assistance with the next generation sequencing (Illumina Miseq), analysis of Illumina Miseq data and surface plasmon resonance studies. Thanks to Dr Michelle Bronze, Dr Azwidowi Lukhwareni and Esrom Letsoalo for their assistance with genotyping. Thanks to Dr Telisha Traut-Johnstone for her guidance through the Accelrys software and modelling.

I thank my lab friends, Qasim Fish, Shaakira Abrahams, Angela Harrison, Denise Le Noury, Michelle Grant and Dr Mark Killick for the splendid time in the laboratory.

I'm also thankful to Mintek, the University of the Witwatersrand and the National Research Foundation: Thuthuka Programme for funding their financial assistance.

"I have set the Lord always before me:
Because he is at my right hand,
I shall not be shaken." Psalm 16:8

Table of Contents

Publications and Conference proceedings III
Abstract IV
Acknowledgements VI
Table of Contents VII
List of Tables X
List of Abbreviations XI
CHAPTER 1: INTRODUCTION 1
1.1. Introduction to HIV and AIDS 1
1.1.1. History and global epidemiology of HIV 1
1.1.2. Nomenclature and phylogeny of HIV-1 2
1.1.3. Transmission of HIV-1 4
1.1.4. Geographical distribution of HIV 6
1.1.5. HIV-1: The virion 8
1.1.6. The HIV-1 life cycle 11
1.1.7. HIV-1 infection mechanisms 19
1.1.8. Disease progression 19
1.2. HIV-1 treatment 20
1.3. HIV-1 IN drugs 23
1.3.1. Early anti-HIV-1 IN agents 23
1.3.2. First generation clinically approved INIs 24
1.3.3. Stribild 25
1.3.4. Early second generation INIs 26
1.3.5. DTG and S/GSK-1265744 27
1.5. The mechanisms of HIV-1 drug resistance 29
1.6. The significance of this study 37
1.7. Hypothesis 39
1.8. Study objective 39
CHAPTER 2: MATERIAL AND METHODS 40
2.1. Materials and methods 40
2.2. Mammalian cells and primary HIV-1 subtype C preparation 40
2.2.1. Isolation of PBMCs 41
2.2.2. Virus expansion 41
2.2.3. Virus $TCID_{50}$ determination 42
2.3. *In vitro* selection of INSTIs in primary HIV-1 grown in PBMC's 42
2.4. HIV ARV drug resistance genotyping 43
2.4.1. Nucleic acid extraction 43

2.4.2. Nested PCR 44
2.4.3. DNA purification, cycle sequencing and DNA sequencing 45
2.4.4. Sequencing using the Illumina MiSeq 46
2.5. *In silico* analysis of the major *in vitro* selected mutations 47
2.6. Mutant and WT enzyme generation 48
2.6.1. Site-directed mutagenesis, plasmid preparation and sequencing 49
2.6.2. Recombinant protein expression 51
2.6.3. Nickel-chelating column chromatography 51
2.7. Polyacrylamide gel electrophoresis (PAGE) and Western-blot analysis 52
2.8. HIV-1 strand transfer assay 53
2.9. Binding kinetic analysis of W/T and mutant IN DNA and INSTI binding 54
CHAPTER 3: RESULTS 55
3.1. *In vitro* selection and virus break-through 55
3.2. Genotyping 57
3.2.1. Sanger-based sequencing 57
3.2.2. High through-put sequencing: Illumina MiSeq 60
3.3. *In silico* analysis of mutations using Accelrys 65
3.4. Multiple sequence alignment exhibiting relevant site-directed mutations 68
3.4. Western-blot analysis of purified WT and mutant integrase 69
3.5. HIV-1 subtype C mutant strand transfer capacity 69
3.6. HIV-1 subtype C $FV6^{WT}$ and $FV6^{MT}$ IN DNA binding capacity 70
CHAPTER 4: DISCUSSION 73
4.1. *In vitro* selection 74
4.2. Drug resistance-associated mutations selected *in vitro* 75
4.2.1. Resistance to RAL 76
4.2.2. Resistance to EVG 78
4.2.3. Resistance to DTG 81
4.3. Genetic drug resistance barrier and cross resistance 82
4.4. HIV-1 IN strand transfer efficacy 83
4.5. HIV-1 IN DNA binding kinetics 84
4.6. Overall impact of mutations on structure and function 86
4.7. Concluding remarks 88
4.8. Ongoing/future work 88
CHAPTER 5: REFERENCES 90
APPENDIX 146

List of Figures

Figure 1.1: Phylogenetic tree illustration of genome sequence relationships of HIV-1 and HIV-2 in humans with respect to primate SIV.. 5

Figure 1.2: Schematic diagram of the anatomy of HIV-1..... 8

Figure 1.3: The complete HIV-1 genome. 9

Figure 1.4: A schematic representation of the HIV-1 cycle..... 11

Figure 1. 5: A schematic representation of the HIV-1 integrase 3'-processing and strand transfer reaction..... 17

Figure 1. 6: The chemical structure of RAL. 24

Figure 1.7: The chemical structure of EVG. 25

Figure 1.8: The chemical structure of DTG. 27

Figure 3.1: Graphical representation of the HIV-1 subtype C MR (A) and FV (B) viruses during *in vitro* selection and virus break-through.. 55

Figure 3.2: Dose-escalation curves for the *in vitro* selection studies with RAL, EVG and DTG. 56

Figure 3.3: The *in vitro* selection process showing dose-escalation concentrations of RAL (A) and EVG (B) in PBMCs infected with primary virus isolates FV6 and MR69. 61

Figure 3.4: A three-dimensional docking model showing the positions at which the integrase inhibitor selected substitutions T66I, E92Q, H114L, F121Y, Q148R and N155H within the HIV-1 integrase catalytic core domain occur. 65

Figure 3.5: A three-dimensional docking model showing the amino acid substitutions of A) T66I, B) E92Q, C) F121Y, D) H114L, E) N155H, F) Q148R, and G) R263K. 66

Figure 3.6: Alignment of the predicted $FV6^{WT}$ integrase enzyme amino acid sequence with the FV6 mutants 68

Figure 3.7: Western-blot analysis of a purified wild-type and mutant HIV-1 subtype C integrase enzymes. 69

Figure 3.8: HIV-1 subtype C mutant ST capacity with respect to the WT IN subtype C enzyme 70

Figure 3.9: HIV-1 subtype C mutant donor (d) DNA binding capacity with respect to the FV6 WT IN enzyme.. 71

List of Tables

Table 1.1: HIV-1 proteins and their functions. 10
Table 1.2: Current US FDA-approved HIV/AIDS antiretroviral drugs....21
Table 1.3: Fold changes in susceptibity to major and alternative resistance pathways to current INSTIs.. 32
Table 1.4: Mutant 3'-processing, strand transfer activity and replication capacity with respect to wild-type integrase enzyme and virus....36
Table 2.1: A summary of NRTI/NNRTI resistant mutations from baseline MR virus isolates....40
Table 2.2: A summary of RT-PCR, PCR and sequencing primers used in the *pol* genotyping assays. 44
Table 2.3: Primers used in site-directed mutagenesis of the integrase gene. 49
Table 3.1: Baseline IN sequences of primary HIV-1 isolates selected. 58
Table 3.2: Major antiretroviral drug resistance mutations that emerged in the HIV-1 subtype C primary virus isolates during *in vitro* selection cultures with increasing concentrations of RAL, EVG and DTG. 59
Table 3.3: Resistance mutations as selected by RAL and identified using NexGen sequencing. 62
Table 3.4: Resistance mutations as selected by EVG and identified using NexGen sequencing. 63
Table 3.5: Resistance mutations as selected by DTG and identified using NexGen sequencing. 64
Table 3.6: A summary of minimisation energies and the RMSD's of the single point mutations in IN. 67
Table 3.7: The kinetics of donor DNA binding to the HIV-1 FV6 IN mutants with respect to the IN WT....72

List of Abbreviations

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APV	Amprenavir
ARV	Antiretroviral
ATC	Apricitabine
ATP	Adenosine triphosphate
ART	Antiretroviral therapy
ATV	Atazanavir
AVIP	AIDS Vaccine Integrated Project
AZT	Azidothymidine
BSA	Bovine serum albumin
CA	Capsid
CAN	Clean amplified NTA plate
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA
CHR	C-terminal heptad repeat
COBI	Cobicistat
CRFs	Circulating recombinant forms
CRA	co-receptor antagonists
CXCR4	C-X-C chemokine type 4 receptor
ddC	Zalcitabine
ddI	Didanosine
DKA	Diketo acid
DLV	Delavirdine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	double-stranded DNA
DRC	Democratic Republic of Congo
DRV	Darunavir
DTG	Dolutegravir
DTT	Dithiothreitol
d4T	Stavudine
E.coli	*Escherichia coli*
EFV	Efavirenz
Env	Envelope gene
Env	Envelope

ESCRT	Endosomal sorting complexes required for transport
ENF	Enfuvirtide
ETR	Etravirine
EVG	Elvitegravir
FBS	Fetal bovine serum
FDCs	Fixed dose combinations
FP	Fusion peptide
FPV	Fosamprenavir
FTC	Emtricitabine
FI	Fusion inhibitors
Gag	Group-specific antigen gene
gp160/120/41	envelope glycoprotein-160/120/41
HAART	Highly Active Antiretroviral Therapy
HIV-1/2	Human immunodeficiency virus type-1/2
HRP	Horseradish peroxidase
HTLV-III	Human T-lymphotropic Virus Type-III
IDV	Indinavir
IL-2	Interleukin-2
IN	Integrase
INI	Integrase Inhibitor
INSTIs	Integrase strand transfer inhibitors
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAV	Lymphadenopathy Associated Virus
LPV	Lopinavir
LTR	Long terminal repeat
Lys	Lysine
LB	Luria Bertani
MA	Matrix
MP	Microplate
MT	Mutant
mRNA	messenger RNA
MVC	Maraviroc
NC	Nucleocapsid
Nef	Negative regulatory factor
NFV	Nelfinavir
NHR	N-heptad repeat
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleotide reverse transcriptase inhibitors
NsRTIs	Nucleoside reverse transcriptase inhibitors

NTA	Nitriloacetic acid/ Nextera XT tagment amplicon
NVP	Nevirapine
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	Primer binding site/ Phosphate buffered saline
PCR	Polymerase Chain Reaction
PHA-P	Phytohemagglutinin
PI	Protease inhibitors
PICs	Pre-integration complexes
PMTCT	Prevention of mother-to-child transmission of HIV
Pol	Polymerase gene
PPT	Polypurine tract
PR	Protease
PVDF	Polyvinylidene difluoride
RAL	Raltegravir
RC	Replication capacity
Rev	Regulator of virion expression
RNA	Ribonucleic acid
RNAse H	Ribonuclease H
RPV	Rilpivirine
RT	Reverse transcriptase
RTCs	Reverse transcription complexes
RT-PCR	Reverse transcription polymerase chain reaction
RTV	Ritonavir
SA	South Africa/n
SANBS	South African National Blood Service
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGRs	Second generation recombinants
SIV	Simian immunodeficiency virus
SOPs	Standard operating procedures
SPR	Surface plasmon resonance
SQV	Saquinavir
SSC buffer	Saline-sodium citrate buffer
ST	Strand transfer
TAMs	Thymidine analogue mutations
Tat	Trans-Activator of Transcription
TAR	Transactivation response region
TB	Tuberculosis

TBS	Tris Buffered Saline
TBST	Tris Buffered Saline-Tween
$TCID_{50}$	Tissue Culture Infectious Dose-50
TDF	Tenofovir
TMB	Tetramethylbenzidine
TPV	Tipranavir
tRNA	Transfer RNA
T-20	Enfuvirtide
UNAIDS	The Joint United Nations Programme on HIV/AIDS
URFs	Unique recombinant forms
USA	United States of America
US FDA	United States Food and Drug Administration
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein unique
WHO	World Health Organization
3'-P	3'-end processing
3TC	Lamivudine
6-HB	six-helix bundle

Units of measurement

kDa	kilodaltons
M	molar
mg	Milligram
mM	Millimolar
mol	Mole
ng/ml	Nanogram per millilitre
nm	Nanometers
nM	Nanomolar
µl	Microlitre
µM	micromolar
rpm	Revolutions per minute
RU	response unit
°C	Degrees celsius
%	Percent

CHAPTER 1: INTRODUCTION

1.1. Introduction to HIV and AIDS

1.1.1. History and global epidemiology of HIV

Acquired immunodeficiency syndrome (AIDS) was first diagnosed in 1981, in a homosexual male patient that exhibited an immune deficiency state associated with opportunistic microbial infections (Gottlieb *et al*., 1981). It was in subsequent years that AIDS was confirmed to be caused by a retrovirus, then designated Lymphadenopathy associated virus (LAV) (Barre-Sinoussi *et al*., 1983) and later designated Human T-lymphotropic Virus Type (HTLV)-III (Popovic *et al*., 1984). In 1986 HTLV-III was appropriately named Human Immunodeficiency Virus Type-1 (HIV-1), since infection of humans with the pathogen resulted in immune depletion (Coffin *et al*., 1986). Although the initial diagnosis of HIV-1 was in the early 1980's; the virus has been sequenced from samples dating back to the 1970s, 1960s and 1950s (Froland *et al*., 1970; Jonassen *et al*., 1997; Zhu *et al*., 1998; Keele *et al*., 2006). For instance, clinical samples from Norwegian patients dated 1971 and 1976 have exhibited infection with HIV-1 group O (William *et al*; 1983; Froland *et al*., 1988; Jonassen *et al*., 1997). Other historical HIV-1 tissue samples include ZR59 obtained in 1959 and DR60 obtained in 1960 (Zhu *et al*., 1998; Keele *et al*., 2006). The ZR59 tissue sample is the oldest known HIV-1 sample, obtained from an African male patient in The Democratic Republic of Congo (DRC) (Zhu *et al*., 1998; Keele *et al*., 2006). Sequence analysis of the HIV-1 sample ZR59 has shown close phylogenetic relation with Simian Immunodeficiency Virus (SIV) strains isolated from primate *Pan Troglodytes troglodytes* (Keele *et al*., 2006). The HIV-1 sample DR60 is a lymph node biopsy which was preserved in paraffin wax since 1960 (Worobey *et al*., 2008). Sequence analysis of DR60 has shown a 12% nucleotide variation with respect to ZR59, a disparity caused by introduction of mutations. This mutational variation suggests that HIV-1 infections were present in humans for a considerable period before 1960 (Worobey *et al*., 2008). This notion is supported by statistical sequence analysis that estimated the existence of HIV-1 as early as 1915-1941; with the year 1931 being an optimum estimate for a common ancestor of HIV-1 group M (Korber *et al*., 2000).

Since its discovery in the early 1980's, HIV- infection has been reported in every country of the world. The Joint United Nations Programme on HIV/AIDS (UNAIDS) report on the global AIDS epidemic, has estimated that approximately 35.3 million (32.2 million – 37.2 million) people were living with HIV by the end of 2013. Of these 2.1 million (1.9 – 2.4 million) were new HIV infections which included 240 000 (210 000 – 280 000) newly HIV-infected children (UNAIDS World AIDS Day Report - Fact sheet, 2014). HIV/AIDS associated global mortality rates for year 2013 were estimated at 1.5 million (1.4 million -1.7 million) worldwide compared to 1.6 million (1.4 million – 1.9 million) from year 2012, of which approximately 1.4 million (1.2 million – 1.7 million) adults and approximately 210 000 (190 000 – 250 000) being children under the age of 15 years (UNAIDS, 2013; UNAIDS World AIDS Day Report - Fact sheet, 2014).

Sub-Saharan Africa remains the most burdened region by the global pandemic with an HIV/AIDS prevalence of approximately 24.7 million people by end of 2013, of which 58% are women. In the same year (2013) new HIV infections in sub-Saharan Africa were estimated to be 1.5 million (1.3 million – 1.6 million), and approximately 1.1 million (1 million – 1.3 million) died from HIV/AIDS related deaths (UNAIDS World AIDS Day Report - Fact sheet, 2014). South Africa remains the country with the highest HIV-1 prevalence in the world with an estimated 6.1 million living with HIV (UNAIDS, 2013; Avert, accessed 2015-01-26). Statistics South Africa reported a 93% increase in HIV/AIDS associated deaths between 1997 and 2006, and a mortality rate of 82% between 2006 and 2010 (Statistics South Africa, 2013).

1.1.2. Nomenclature and phylogeny of HIV-1

HIV can be classified into two types, HIV-1 and HIV-2; both are from the Retroviridae family, Orthoretrovirinae subfamily and the genus Lentivirus (The International Committee on Taxonomy of Viruses, Virology Division-IUMS http://ictvonline.org/virusTaxonomy.asp?taxnode_id=20124900 accessed 2014 January 6).

Both AIDS viruses originated from primate SIV, and were obtained by humans through multiple zoonotic transmissions in West and Central Africa (Hemelaar, 2012) (Figure 1.1). HIV-1 and HIV-2 represent two distinct lineages of primate reservoir (Hirsch *et al.*, 1989;

Huet *et al*., 1990; Gao *et al*., 1992; Sharp *et al*., 1995). For instance, HIV-2 originates from SIV-infected sooty mangabeys (*Cercobus atys*) and this was identified through genome sequence relations of HIV-2 with SIVsmm; a virus common in sooty mangabey (Hirsch *et al*., 1989; Huet *et al*., 1990; Gao *et al*., 1992; Sharp *et al*., 1995). HIV-2 has been divided into seven non-recombinant groups A, B, C, D, E, F and G; and one circulating recombinant form (CRF) HIV2_CRF01_AB (HIV sequence database: http://www.hiv.lanl.gov/content/sequence/HelpDocs/subtypes.html, accessed 7 January 2015).

The HIV-1 primate reservoir was identified to be the chimpanzee subspecies *P.t. troglodytes* and *P.t. schweinfurthii* common in central and eastern Africa. These primates were identified to be natural carriers of SIVcpz and SIVcpz-ant; viruses closely related to HIV-1 (Peeters *et al*., 1989; Peeters *et al*., 1992; Janssens *et al*., 1994; Van Haesevelde *et al*., 1996). The close genome sequence relationship between SIVcpz strains from primates in Cameroon with HIV-1 add to existing evidence of cross species infections from primates as a source of HIV-1 into humans (Simon *et al*., 1998; Gao *et al*., 1999; Corbet *et al*., 2000; Sharp *et al*., 2001).
The currently circulating HIV-1 has shown extensive diversity and phylogenetic analysis has divided the virus into four distinct groups, namely; HIV-1 groups M (major), N (non M non O), O (outlier) and group P. HIV-1 group P was isolated in 2009 from a Cameroonian woman residing in France, and genome sequence analysis proved the primate reservoir to be gorillas due to the close sequence relations with SIVgor (HIV sequence database: http://www.hiv.lanl.gov/content/sequence/HelpDocs/subtypes.html, accessed 7 January 2014; Plantier *et al*., 2009). HIV-1 group M represents the predominant group, and consist of 9 non-recombinant subtypes A (with sub-subtypes A1, A2), B, C, D, and F (with sub-subtypes F1, F2), G, H, J and K (HIV sequence database: http://www.hiv.lanl.gov/content/sequence/HelpDocs/subtypes.html, accessed 7 January 2014). Moreover, HIV-1 group M currently consists of 72 circulating recombinant forms (CRFs), unique recombinant forms (URFs) and second generation recombinants (SGRs) (Robertson *et al*., 2000; Hemelaar *et al*., 2006; McCutchan, 2006; Carr *et al*., 2010; Hemelaar, 2012; Lau *et al*., 2013; HIV sequence database: http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html, accessed 19 January 2015).
CRFs are known to emerge as a result of recombination between two HIV subtypes in epidemiologically unlinked individuals, and are responsible for major epidemics. For

instance, recombination between HIV-1 subtypes B and F has produced CRF_12BF; the designated number 12 is a representation of the order in which the CRF was reported. URFs are formed during recombination of three or more epidemiologically unlinked HIV-1 subtypes (Lau and Wong, 2013).

1.1.3. Transmission of HIV-1

Transmission routes of HIV-1 include sexual, parenteral and vertical transmission; and each route is associated with a distinctive risk of infection (Royce *et al*., 1997; Galvin and Cohen, 2004). The sexual route is the main method of transmission and includes male-to-female, female-to-male, male-to-male and fellatio; and is associated with a 1 in 200 to 1 in 2000, 1 in 700 to 1 in 3000, 1 in 10 to 1 in 1600 and 1 in 10 to 1 in 1600 risk of infection, respectively (Royce *et al*., 1997; Galvin and Cohen, 2004). Parenteral transmission includes transfusion of HIV-1 infected blood and needle stick injuries. Vertical transmission is from mother-to-child and has less than a 1 in 10 risk of infection during azidothymidine (AZT) treatment, and a 1 in 4 risk of infection when individuals are untreated (Royce *et al*., 1997; Galvin and Cohen, 2004).

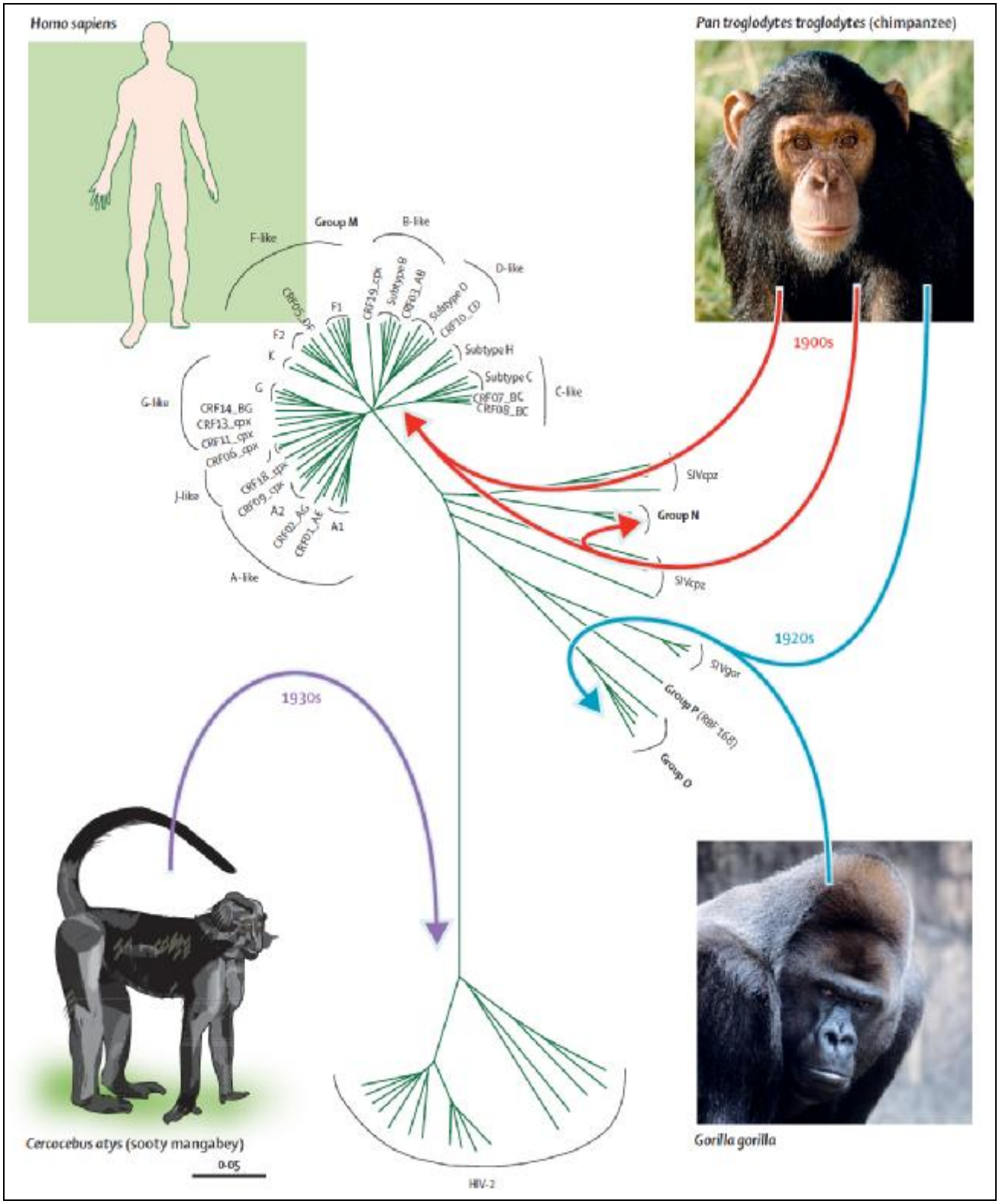


Figure 1.1: Phylogenetic tree illustration of genome sequence relationships of HIV-1 and HIV-2 in humans with respect to primate SIV. This figure was obtained from Tebit and Arts, (2011).

1.1.4. Geographical distribution of HIV

The HIV pandemic is immensely diverse in geographical distribution, with regional epidemics harbouring varying subsets in global diversity. For instance, HIV-2 has primarily been confined to Africa while HIV-1 exhibits a global prevalence. HIV-2 group A has been identified mainly in Angola and Mozambique, with isolated incidences in Brazil and India (Faria *et al*., 2012). HIV-2 group B has been in identified in isolated cases in West Africa (Marx *et al*., 2001; Santiago *et al*., 2005).

The HIV-1 pandemic is dominated by HIV-1 group M infections; however, HIV-1 group O and HIV-1 group N are associated with regional epidemics, generally limited to Cameroon and Senegal in Central Africa (Peeters *et al*., 1997; MacCutchan, 2006; Yamaguchi *et al*., 2006). As mentioned previously, HIV-1 group P has only been identified in France in a female immigrant from Cameroon (Plantier *et al*., 2009). HIV-1 group M was initially only limited to the Congo River basin which is the original site of initial zoonotic infections and HIV-1 group M diversification. However, HIV-1 group M diversification has since become complex and dynamic due to its evolution into numerous subtypes and CRFs (Tebit and Arts, 2011). The subtypes of HIV-1 form phylogenetic clusters with amino acid variations of 25%-40%, 20% and 10% in the *env* gene, *gag* gene and the *pol* gene, respectively (Gao *et al*., 1998; Brenner, 2007; Santoro and Perno, 2013). Some subtypes were found to be related genetically, and linked according to migration patterns between neighbouring countries (Gao *et al*., 1998).

Subtype distribution and epidemiology has indicated that HIV-1 subtype B is predominant in Western countries such as in Europe, North America and Australia; while HIV-1 subtype C is the predominant subtype worldwide. Nonetheless, due to influx of immigrants from Africa and Asia the Western countries have seen increased prevalence of HIV-1 non-B strains (Ndembi *et al*., 2004; Hemelaar *et al*., 2006; Tebit and Arts, 2011). For example, HIV-1 subtype A was initially isolated to sub-Saharan Africa but has since been identified in Russia (Tebit and Arts, 2011). Diversification of HIV-1 has over the last years led to rapid emergence of new subtypes and intermixing of strains and this has resulted in changes in geographical distribution and epidemiology (Robertson *et al*., 2000). For instance, HIV-1 subtypes A and F have evolved into sub-subtypes A1 and A2 and F1 and F2, respectively (Tebit and Arts, 2011). Furthermore, in countries such as Angola classification of HIV-1 sub-

subtypes A5 and A6 might be required (Bartolo *et al.*, 2009). From the Congo River basin westwards towards Nigeria, Côte d'Ivoire, Ghana, Senegal and Mali, the HIV-1 geographical distribution is reported to have changed from a previously predominant HIV-1 subtype A and D epidemic, to a regional epidemic where CRF02_AG is most prevalent (Tebit and Arts, 2011). Another country reported to have a high CRF prevalence and diversification is Burkina Faso, with CRF06_cpx and SGR's namely, CRF02_AG/CRF06_cpx having been identified (Rambaut *et al.*, 2001; Montavon *et al.*, 2002; Ouedraogo-Traore *et al.*, 2003; Ghys *et al.*, 2003; Tebit *et al.*, 2006).

Sub-Saharan Africa and the Congo River basin contain the most diverse HIV-1 strains, as virtually all subtypes, sub-subtypes and CRFs were reported in regions such as the DRC and Cameroon (Vidal *et al.*, 2000; Tebit *et al.*, 2002; Vidal *et al.*, 2003; Ndembi *et al.*, 2004). Initially, east African countries like Uganda, Kenya and Tanzania exhibited the highest HIV-1 prevalence, but in the late 1990s there was a shift in the epidemic with southern African countries namely, South Africa, Lesotho, Botswana and Zimbabwe exhibiting the highest regional prevalence (Tebit and Arts, 2011). The epidemic in Southern Africa is mainly due to HIV-1 subtype C similar to East Asia including China where HIV-1 subtype B and C CRFs (CRF07_BC and CRF08_BC) have been reported (Rambaut *et al.*, 2001; Ghys *et al.*, 2003; Tebit and Arts, 2011). South America was reported to exhibit HIV-1 diversity in countries like Brazil and Argentina, which have resulted in subtypes B, C and F recombinants (BC and BF recombinants). However, the HIV-1 subtype C epidemic remains the prevalent epidemic in South America, especially in Brazil where the incidence of the HIV-1 BC recombinant form was reported to be the highest (Soares *et al.*, 2005; Tebit and Arts, 2011). Although modest in their global prevalence, HIV-1 unique recombinant forms were reported to be abundant in South American countries like Brazil and Argentina; in addition to Nigeria and Cameroon in West and Central Africa, respectively. URFs were reported by McCutchan in 2006 to have 30% prevalence in East African countries like Kenya, Rwanda, Tanzania and Uganda (McCutchan, 2006).

1.1.5. HIV-1: The virion

HIV-1 is an enveloped virus with a diameter of approximately 100-120nm and consists of a cylindrically shaped core capsid (p24 structural protein) (refer to Figure 1.2) (Gallo *et al.*, 1984; Montagnier, 1999). The envelope of HIV-1 is a lipid bilayer obtained from the host cell plasma membrane during the virus budding process (Arthur *et al.*, 1992); and is studded with the viral surface glycoprotein projections; namely, the transmembrane glycoprotein (gp41) and the outer trimeric surface glycoprotein gp120 all derived from the precursor protein gp160 (Gelderblom, 1991; Turner and Summers, 1999). A matrix shell of the p17 protein lines the inner surface of the viral membrane as shown in Figure 1.2 (Turner and Summers, 1999).

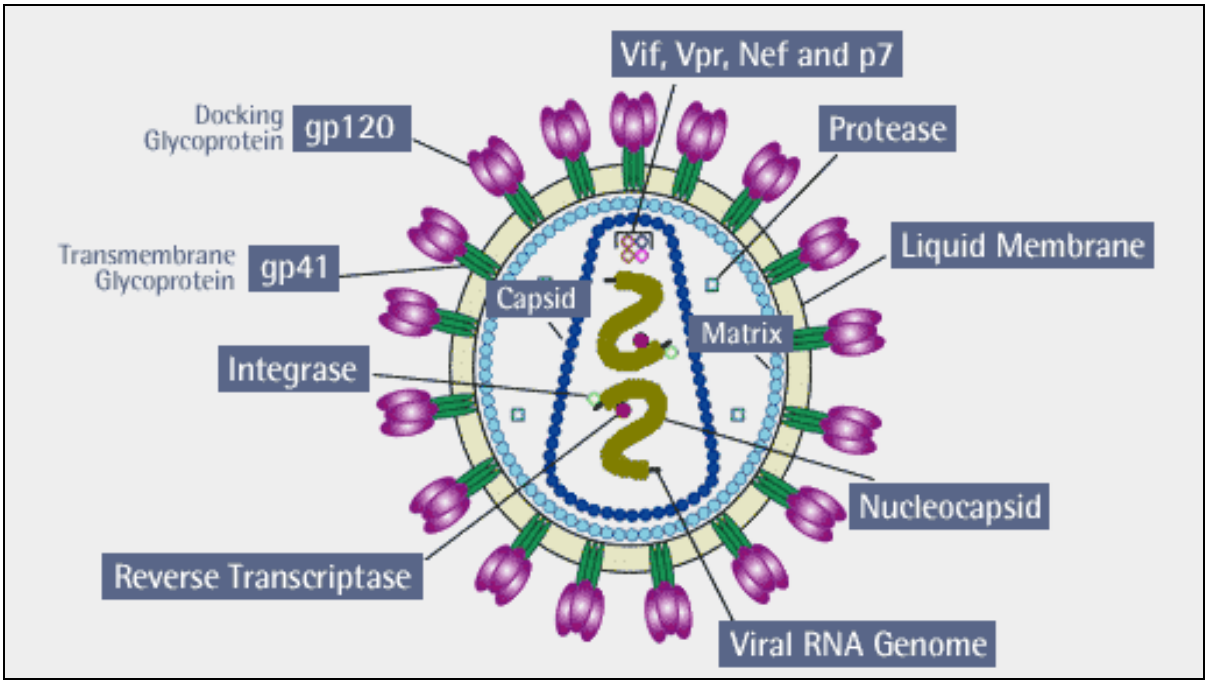


Figure 1.2: Schematic diagram of the anatomy of HIV-1. The envelope consists of protruding glycoprotein gp120, which stems from the fusion protein gp41. The virus matrix encloses the capsid which protects the two copies of genomic RNA, reverse transcriptase, integrase and protease. This figure was obtained from: http://www.infohow.org/science/biology-ecology/hiv-viron/ accessed 2015-06-22.

The HIV-1 genome comprises full length diploid linear ribonucleic acid (RNA) of positive polarity (Coffin, 1992; Lu *et al.*, 2011), which is stabilised by a ribonucleoprotein complex p7. The HIV-1 genome encodes enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN) in addition to structural, regulatory and accessory proteins (Gelderblom, 1991; Turner and Summers, 1999). In total, HIV-1 comprises of nine genes classified into three

functional groups as shown in Figure 1.3. The HIV-1 *gag*, *pol* and *env* genes are classified as structural genes; *tat* and *rev* genes are classified as regulatory genes; and *vpu*, *vpr*, *vif* and *nef* genes classified as accessory genes (Costin, 2007). The function of each of these genes is outlined in Table 1.1.

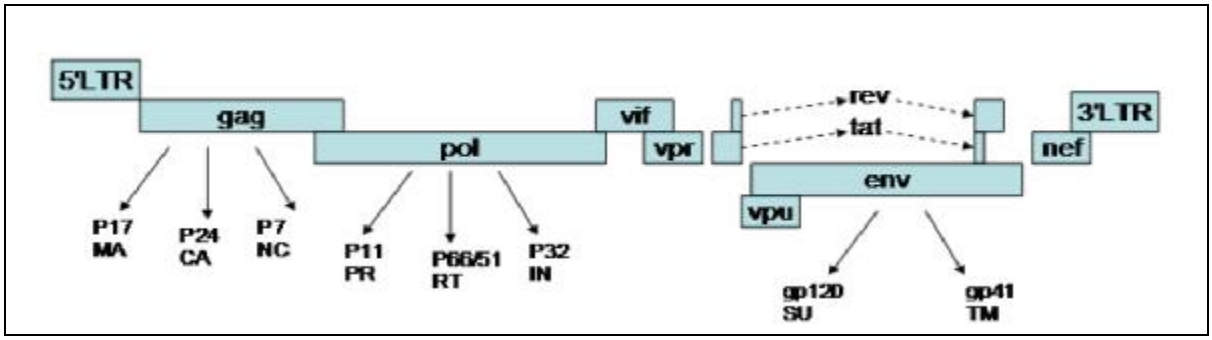


Figure 1.3: The complete HIV-1 genome. The open rectangles represent the coding sequences. The *Gag*, *Pol*, and *Env* genes encode polyproteins which serve as precursors that are cleaved to yield established virus proteins. The figure was obtained from Costin, 2007 and illustrates the three main structural genes of HIV-1: *gag*, *pol* and *env*. The HIV-1 diagram also depicts regulatory (*tat* and *rev*) and accessory (*nef*, *vif*, *vpr* and *vpu*) genes essential for the HIV-1 life cycle.

Table 1.1: HIV-1 proteins and their functions.

	Name	Size (kDa)	Function
Regulatory proteins	Tat/Trans-Activator of Transcription	14	Binds integrated HIV-1 DNA and initiates genome transcription.
	Rev/Regulator of virion expression	18	Regulates viral replication by a controlled translocation of spliced and unspliced HIV-1 RNA into the cytoplasm from the nucleus.
Accessory proteins	Nef/Negative Regulatory Factor	27-35	Down regulates CD4 expression and optimizes T-cell receptor stimuli to the favour of optimum virus proliferation.
	Vpr/Viral Protein R	14	Nuclear translocation of the HIV-1 pre-integration complex (PIC), through selective cation- ion channel activity. Enables HIV-1 replication in non-dividing cells.
	Vpu/Viral Protein Unique	16	Enables the virus budding process from the host cell plasma membrane, by interacting with Tetherin. Translocation of CD4 proteins toward ubiquitin-mediated degradation in lysosomal compartments.
	Vif/Viral infectivity factor	23	Inhibits antiviral activity of the host cell enzyme APOBEC, by translocation toward ubiquitin-mediated degradation.
Gag (Structural protein)	Gag	55	Precursor protein for MA, CA and NC
	Matrix (MA)	17	Binds and targets RNA to the plasma membrane, and incorporates the host envelope into virions during assembly. Attaches to the inner cell membrane via the myristoylated.
	Capsid (CA)	24	Forms the characteristic core capsid of the virion, which encloses the viral genome.
	Nucleocapsid (NC or p7; P6, p1 and p2)	6-7	p1 determines the rate of Gag proteolysis p2 cleavage regulates Gag proteolysis Stabilizes the viral RNA genome. p6 facilitates virus budding and incorporation of Vpr into virus particles.
Env (Structural protein)	gp160	160	Inactive precursor molecule for gp120 and gp41.
	gp120	120	Surface component for CD4 receptor binding and co-receptor (CCR5/CXCR4) binding.
	gp41	41	Transmembrane component for fusion of virus membrane to host cell membrane.
Gag Pol (Structural protein)	Protease/PR	11	Sequential proteolytic processing of the Gag and Gag-Pol polyproteins into mature chains.
	Reverse transcriptase/RT (RNAse H/polymerase)	66 (p66) 51 (p51)	Conversion of single-stranded viral RNA into double-stranded proviral DNA. Degrades RNA from RNA/DNA duplexes
	Integrase/IN	32	Responsible for irreversible integration of the viral DNA into the host chromosomal DNA, through 3'-processing and the strand transfer reaction.

The information for the table was compiled from Graves *et al*., 1988; Helseth *et al* ., 1991; Jaskolski *et al*., 1991; Sherman *et al*., 1991; Werner *et al*., 1991; Decroly *et al*., 1994; Bour *et al*., 1995; Franzusoff *et al*., 1995; Wan and Loh, 1995; Piller *et al*., 1996; Pettit *et al*., 1998; Bukrinsky and Adzhubei A, 1999; Doms *et al*., 2000; Montal, 2003; Pierson and Doms, 2003; Strebel, 2003; Levesque *et al*., 2004; Sluis-Cremer *et al*., 2004; Pugliese *et al*., 2004; Endo-Munoz., 2005; Fiorentini *et al*., 2006; Muthumani *et al*., 2006; Costin, 2007; Miller *et al*., 2007; Stanley *et al*., 2008; Mascarenhas and Musier-Forsyth , 2009; Blanco *et al*., 2011; Abraham and Fackler, 2012; Van Maarseveen et al., 2012; Kopietz *et al*., 2012; reviewed by Solbak *et al*., 2013 and Banerjee *et al*., 2014.

1.1.6. The HIV-1 life cycle

The life cycle of HIV-1 is comprised of a number of complex stages such as attachment, entry, uncoating, reverse transcription, integration, transcription, translation, assembly, budding and maturation, as summarised in Figure 1.4 below.

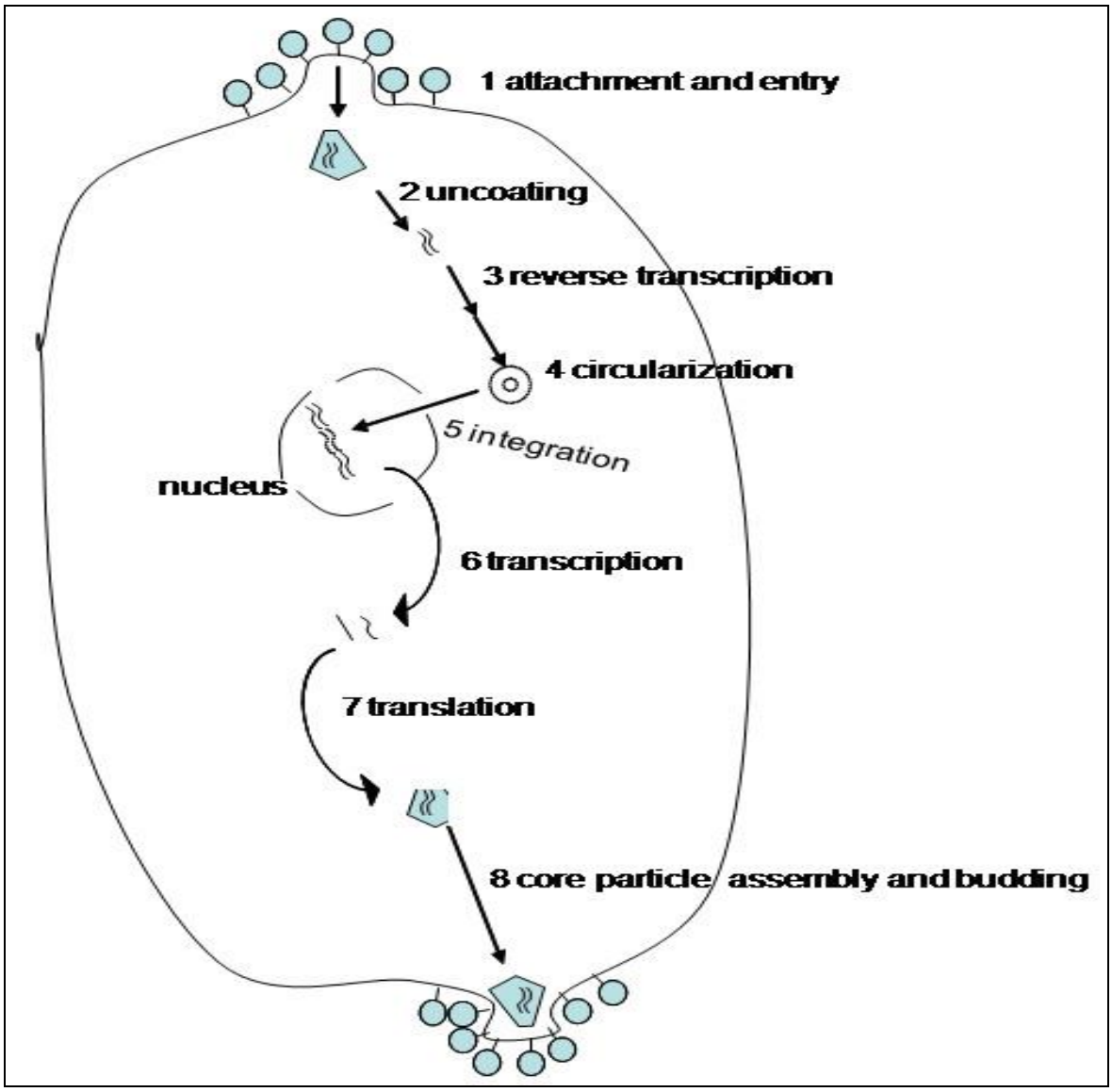


Figure 1.4: A schematic representation of the HIV-1 cycle. The figure was obtained from Costin, 2007 and shows attachment of virus to the receptors/and co-receptors which is followed by fusion with cell membrane. Virus nucleocapsid entry into cytoplasm precedes reverse transcription; integration occurs within the host-cell nucleus. Cellular transcription factors carry out transcription of viral RNAs which are later translated into regulatory and structural viral proteins. Daughter virions assemble and bud through cell membrane, following maturation through virus protease cleavage.

1.1.6.1. HIV-1 entry and tropism

HIV-1 entry is initiated by virus binding to target $CD4^+$ T cells, which involves CD4 binding by gp120, and results in conformational changes that expose the coreceptor binding site of the gp120 protein (Doms, 2000; Foti *et al.*, 2002). Following coreceptor binding, the gp120 is shed, triggering the gp41 component for fusion. Fusion introduces sequential conformational changes in gp41's native state to a pre-hairpin fusion intermediate, then a fusogenic state and finally a post-fusion state. During the fusogenic state the N-heptad repeat (NHR) and C-terminal heptad repeat (CHR) form a six-helix bundle (6-HB) core structure that allows fusion of the viral and host membranes (Lu *et al.*, 1995; Chan and Kim, 1997; Weissenhorn *et al.*, 1997; Cha *et al.*, 1998). The fusion process involves the gp41 conformational transition following gp120-CD4 binding and coreceptor binding, which leads to pore-formation. This step involves the interaction of the HR1 and HR2 peptide motifs which results in the formation of a six-helix bundle. However, before the formation of the hairpin structure that supports membrane fusion, the gp41 pre-hairpin intermediate structure is formed which exposes HR1 and HR2 portions (Wild *et al.*, 1993; Wild *et al.*, 1994; Chen *et al.*, 1995; Kliger *et al.*, 2001; Chen *et al.*, 2002; Lalezari *et al.*, 2003; Lazzarin *et al.*, 2003). This enables the virus core (p24) entry into the target cell cytoplasm and is, thus, essential to the HIV-1 life cycle.

Viral tropism is the ability of viruses to bind specific host cell receptors such as C-C chemokine receptor type 5 (CCR5) or the C-X-C chemokine type 4 (CXCR4) receptors (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Feng *et al.*, 1996; Vandekerckhove *et al.*, 2011). Viruses that bind the CCR5 chemokine coreceptor are designated R5 variants, and viruses that bind the CXCR4 chemokine receptors are designated X4 variants. R5 viruses are common in the early stages of HIV-1 infection, while X4 variants emerge later in HIV-1 infection and are associated with clinical disease progression and precipitous loss of $CD4^+$ T-cells (Koot *et al.*, 1993; Mass *et al.*, 2000; Sheperd *et al.*, 2008; Weisser *et al.*, 2008). Dual tropic viruses, namely, R5X4 use both the CCR5 and CXCR4 receptors, although preference for one receptor over the other is associated with R5X4 viruses (Glushakova *et al.*, 1999; Yi *et al.*, 1999; Li *et al.*, 2001; Coakley *et al.*, 2005).

The hypervariable V3 region of gp120 has been identified as the key determining factor of viral tropism (Cho *et al*., 1998; Smyth *et al*., 1998; Verrier *et al*., 1999; Li *et al*., 2001). The V3 loop was observed to have a high-affinity CD4 binding site; and the region complementary to the CD4 binding site is located in the conserved domains C3 and C4 of the gp120 protein (Freed, 2001). Factors such as amino acid sequence, number of basic residues and the overall net charge of the V3 loop are known to influence preference in tropism (De Jong *et al*., 1992; De Jong *et al*., 1992; Harrowe and Cheng-Meyer, 1995; Speck *et al*., 1997; Smyth *et al*., 1998; Xiao *et al*., 1998; Kato *et al*., 1999; Rizzuto and Sodroski, 2000; Wang *et al*., 2000). In addition to the V3 loop, other regions within the gp120 protein such as the V1 and V2 region are associated with co-receptor usage (Freed, 2001).

1.1.6.2. HIV-1 uncoating

Subsequent to the HIV-1 host cell fusion and entry, the virus core is then released into the host cell cytoplasm and successively the capsid (CA) is uncoated. Uncoating is a poorly understood process (Chen *et al*., 2013; Xu *et al*., 2013); however, a few theoretical models of p24 uncoating exist. The first theory involves dissociation of the viral capsid at the plasma membrane immediately after its insertion into the cytoplasm, activated by the immediate change in environment in which the viral core capsid finds itself (Dvorin and Malim, 2003; Bukrinsky, 2004; Lehmann-Che and Saïb, 2004; Suzuk and Craige, 2007). A second model proposes that uncoating and reverse transcription occur at the same time while the capsid is being gradually transported towards the nucleus (Warrilow *et al*., 2009; Arhel, 2010). A third model proposes that uncoating only occurs after the core capsid has reached the nuclear membrane, upon completion of reverse transcription as this provides optimum stoichiometry (Klarmann *et al*., 1993; Arhel, 2010). Uncoating allows the conversion of reverse transcription complexes (RTCs) into pre-integration complexes (PICs) which are integration competent (Ellison *et al*., 1990; Farnet *et al*., 1990).

1.1.6.3. HIV-1 reverse transcription

Reverse transcription is an essential step in the life cycle HIV-1, although such proteins as nucleocapsid protein (NC or p7), matrix protein, and IN, Tat, Nef and Vif are known to contribute in the regulation of the process. Reverse transcription is wholly dependent on the catalytic abilities of the viral RT enzyme. (Sova and Volsky, 1993; Aiken and Trono, 1995;

Goncalves *et al*., 1996; Harrich *et al*., 1997; Kiernan *et al*., 1998; Wu *et al*., 1999; Harrich and Hooker, 2002; Sluis-Cremer *et al*., 2004). HIV-1 RT is an asymmetric heterodimer that consists of subunits p66 (560-amino-acid) and a p51 (440-amino-acid) derived from the Gag-Pol precursor protein (Kohlstaedt *et al*., 1992). The primary function of RT is to convert HIV-1 RNA into double-stranded complementary DNA (Telesnitsky *et al*., 1997; Jonckheere *et al*., 2000; Sluis-Cremer *et al*., 2004). RT is a multi-functional enzyme with an endonucleolytic ribonuclease H (RNAse H, 15kDa) subunit which catalyses the degradation of RNA from DNA/RNA duplexes (Sluis-Cremer *et al*., 2004). The p66 subunit has an open hand conformation with a DNA polymerization site at the centre of the palm which facilitates the incoming transfer (t)-RNA binding during transcription (Arnold *et al*., 1992; Kohlstaedt *et al*., 1992; Kohlstaedt and Steitz., 1992; Jacobo-Molina *et al*., 1993; Freed, 2000). The p51 subunit consists of RNaseH (15kDa) and RNA binding sites (Warren *et al*., 2009).

The process of reverse transcription is initiated when tRNA binds to the primer binding site (PBS), of the full length and unspliced RNA template. The PBS is about 200 nucleotides downstream of the 5'-end. The host encoded tRNA primer consists of a free 3'-OH group to allow DNA elongation (Götte *et al*., 1999; Sluis-Cremer and Tachdjian, 2008). Once the primer is bound on the RNA template, RT recognises the binary tRNA/RNA complex, and elongates the primer through its RNA-dependent DNA polymerase activity extending from the exposed 3'-OH group until the 5'-end of the HIV-1 RNA (Barat *et al*., 1989; Isel *et al*., 1996; Gotte *et al*., 1999; Sluis-Cremer and Tachdjian, 2008). This process produces a minus-strand strong stop DNA, and subsequently the RNA template is hydrolysed through RNaseH activity from the RNA/DNA hybrid; leaving the RNA/$tRNA^{Lys3}$ intact to serve as primers for hybridization of nascent DNA during strand transfer (Schatz *et al*., 1990; Furfine and Reardon, 1991; Gopalakrishnan *et al*., 1992; Gotte *et al*., 1995; Gotte *et al*., 1999; Sluis-Cremer and Tachdjian, 2008). Elongation of the nascent DNA strand is catalysed by RT's RNA dependent primed DNA polymerase (RDDP) activity, and subsequently RNaseH is used to hydrolyse all of the remaining viral segment used as a primer except the polypurine tract (PPT), which is used as a primer for the initiation of plus strand DNA synthesis (Huber and Richardson, 1990; Wöhrl and Moelling, 1990; Charneau and Clavel, 1991; Klarmann *et al*., 1997). Plus strand DNA synthesis uses the PPT and $tRNA^{Lys3}$ as primers, which are later removed through RNaseH activity allowing a second strand transfer to occur by interaction of the complementary PBS sequences (Huber and Richardson, 1990; Li *et al*., 1994; Das *et al*.,

1995; Wakefield *et al*., 1995; Zhang *et al*., 1998). This results in provirus synthesis from the minus and plus strands which then serve as substrates for integration into the host cell genome catalysed by IN (Sluis-Cremer, 2008; Sarafianos *et al*., 2009).

1.1.6.4. HIV-1 proviral integration

The HIV-1 IN (288 amino acids) is encoded by the *pol* gene and released from the Gag-Pol polypeptide precursor through viral protease-mediated cleavage (Delelis *et al*., 2008). HIV-1 IN functions in a multimeric form stabilised by the metal cofactor Zn^{2+} (Lee *et al*., 1997); the enzyme consists of the N-terminal domain (residues 1-50), the central core domain (residues 51-212) and the C-terminal domain (residues 213-288). The N-terminal domain consist of a zinc binding motif, the central core domain consist of a catalytic active site while the C-terminal domain has been shown to exhibit DNA binding activity (Kulkosky *et al*., 1992; Zheng *et al*., 1996; Chen *et al*., 2000; Woodward *et al*., 2002; Chiu and Davies, 2004; Levin *et al*., 2009; Esposito and Craigie, 1999). The catalytic activity of HIV-1 IN has been shown to require the divalent metal cofactor Mg^{2+}/Mn^{2+} for efficient 3'-processing and DNA strand transfer (ST). However, Mg^{2+} was shown to induce a more optimum IN catalytic activity *in vitro* (Engelman and Craigie, 1995).

Reverse transcription produces a linear double-stranded proviral DNA which comprises of an identical Long Terminal Repeat (LTR) sequence at each end; the proviral DNA serves as a recombination intermediate. The viral IN binds the LTR ends and first carries out 3'-end processing (3'-EP; Figure 1.5). The 3'-EP involves cleavage of two terminal nucleotides (Cytosine and Adenine) from each 3'-end of the nascent viral cDNA (Craigie, 2001; Krishnan and Engelman, 2012). The cleaved proviral DNA is then transported into the nucleus as part of the preintegration complex (PIC) through sucrose velocity gradients between 160S and 640S after treatment with RNase (Bowerman *et al*., 1989; Farnet and Haseltine, 1990; Karageorgos *et al*., 1993; Miller *et al*., 1997). HIV-1 PICs are derived from the virion core structures and contain viral IN, RT, Vpr, the host cellular mobility group protein HMG 1(Y), LEDGF, and reduced amounts of viral MA (Karageorgos *et al*., 1993; Miller *et al*., 1997, Shun *et al*., 2007). The cleaved proviral DNA is then transported into the nucleus as part of the preintegration complex (PIC) where IN catalyses the ST reaction (Figure 1.5). Strand transfer involves ligation of exposed 3'-OH groups on the proviral DNA

onto the cleaved chromosomal DNA (reviewed by Pandey and Grandgenett, 2008). The host cell DNA is nucleophilically hydrolysed in a staggered manner during ST, and then the exposed 3'-oxygen atoms are used to cleave chromosomal DNA (Fujiwara and Mizuchi, 1988; Brown *et al*., 1989; Engelman *et al*., 1991; Krishnan and Engelman, 2012). The resulting DNA recombination intermediate is then ligated on the 5'-phosphates of target DNA to the unjointed 5'-ends of chromosomal DNA by host cell ligases to yield the integrated provirus (Fujiwara and Mizuchi, 1988; Brown *et al*., 1989; Engelman *et al*., 1991; Krishnan and Engelman, 2012).

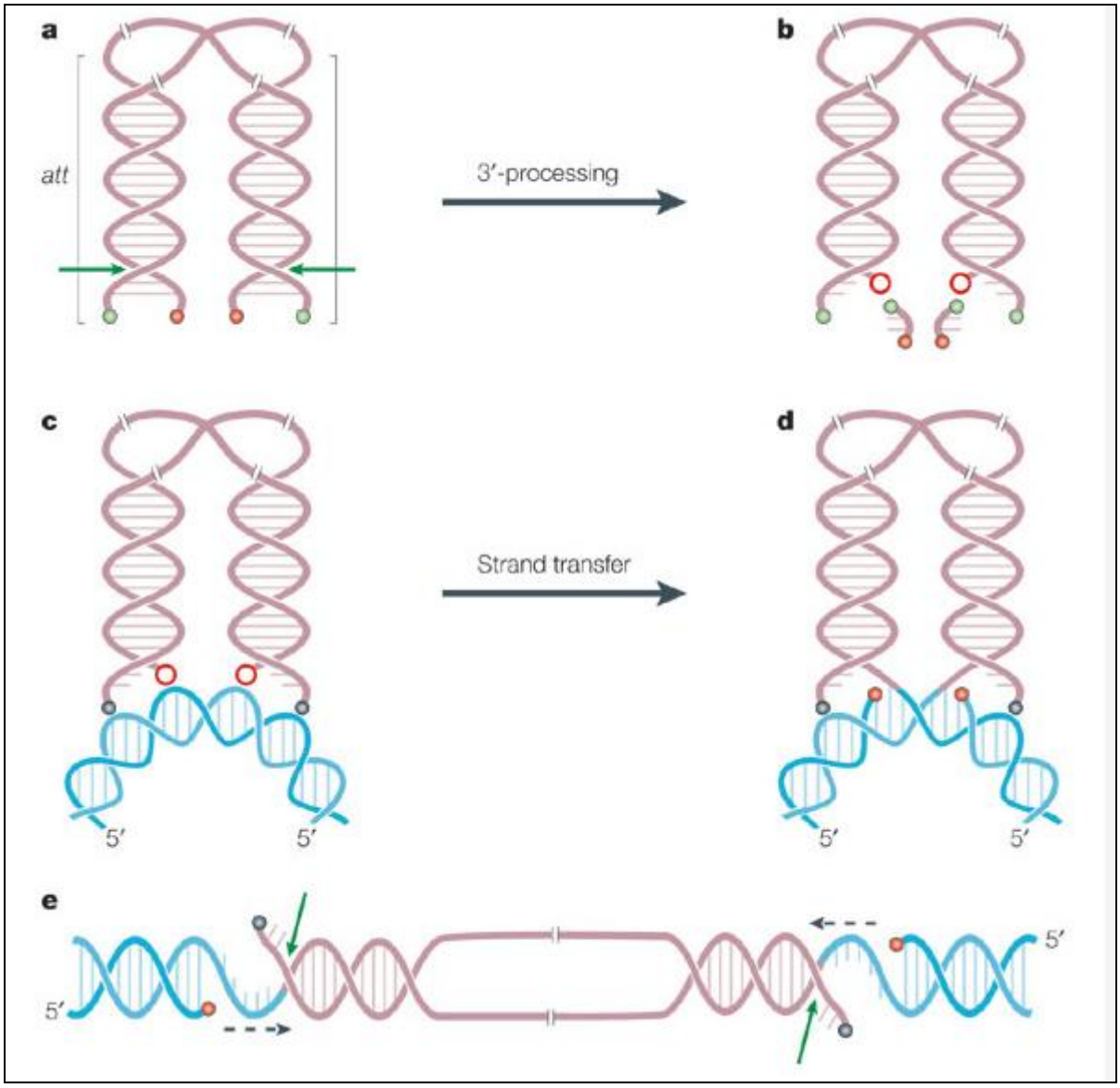


Figure 1. 5: A schematic representation of the HIV-1 integrase 3'-processing and strand transfer reaction. The figure was obtained from Pommier *et al*., 2005. (a) 3' processing is cleavage of the viral DNA immediately 3' from the conserved CA dinucleotide. (b) The viral DNA cleavage generates reactive 3'-OH groups on the viral DNA ends (red circles). The subsequent reaction is the strand transfer reaction which involves ligation of the cleaved 3'-ends into the host-cell chromosome (c-e: blue DNA structure). (d) Each of the 3'-OH viral DNA ends react with DNA phosphodiester bonds on the 5'-end of the host genome. This results in a five-base-pair stagger across the DNA major groove, a single stranded gap at each junction and two-base flap at the 5'-ends of the viral DNA (d and e). (e) Ligation is carried out by cellular repair enzymes which ligate the single stranded gap and release of the unpaired 5'-ends of the viral DNA (arrows).

1.1.6.5. HIV-1 transcription and translation

The integrated HIV-1 provirus then functions as a transcription template for viral RNA and is used to encode viral proteins (Freed and Martin, 2001; Karn and Stoltzfus, 2012). RNA transcription is initiated by RNA polymerase II (Pol II) binding to the HIV-1 LTR region

which harbours *cis*-acting elements required for RNA synthesis. The LTR consists of regions: U3 (for unique, 3'-end), R (for repeated) and U5 (for unique, 5'-end). RNA Pol II binds specifically to the U3/R intersection to initiate transcription from the provirus DNA template (Ross *et al*., 1991; Freed, 2001). Initiation of RNA synthesis also requires the Tat protein to bind the transactivation response region (TAR), resulting in the recruitment of the human P-TEFb complex to the TAR bulge. The Tat/human P-TEFb complex binding to DNA leads to the phosphorylation of the C-terminal domain of RNA Pol II, resulting in stimulation of RNA synthesis (Berkhout *et al*., 1989; Selby *et al*., 1989; Freed, 2001; Karn and Stoltzfus, 2012). The transcription process produces the generation of a large number of unspliced RNAs/mRNA, partially spliced mRNAs (5 kb) and small (1.7 kb to 2.0 kb)-multiply spliced mRNAs (Purcell and Martin, 1993; Freed, 2001). The unspliced mRNA function is eventually packaged as genomic RNA. The partially spliced RNA is translated into Gag, Gag-Pol precursors, Env, Vif, Vpu and Vpr proteins, while the small-multiply spliced mRNAs are translated into Rev, Tat, and Nef proteins (Freed, 2001).

1.1.6.6. HIV-1 assembly, budding and maturation

HIV-1 virus assembly packages components required for virus infectivity such as the two positive sense viral RNA strands, cellular $tRNA^{Lys}$ molecules used as primers for cDNA synthesis, the trimeric viral envelope (Env) gp120/gp41 protein, the Gag-Pol polyprotein, the PR enzyme , RT enzyme and the IN enzyme (Sundquist and Kräusslich, 2012). The process of virion assembly occurs at the host cell plasma membrane within specialised microdomains and is mediated by HIV-1 Gag (and Gag-Pol) polyprotein. The HIV-1 Gag polyprotein is known to mediate sequential protein-protein interactions at the plasma membrane which results in the formation of the virus core-structure and subsequent packaging of genomic RNA, and the relevant structural proteins used to create the virus core (Sundquist and Kräusslich, 2012). Virus assembly is followed by the budding process mediated by host cell endosomal sorting complexes required for transport (ESCRT). The virion acquires its envelope from the host cell membrane during budding and thereafter develops a lipid envelope interspersed by the viral gp120/gp41 trimeric spikes (Sundquist and Kräusslich, 2012). During the budding process the Gag polyprotein precursor is cleaved by PR into virus constituents MA, CA, NC and p6 proteins forming a mature and infectious virus (Sundquist and Kräusslich, 2012).

1.1.7. HIV-1 infection mechanisms

HIV-1 re-infection can occur subsequent to budding when a free and mature virus interacts with cells through $CD4^+$ T cell receptor binding (Anderson *et al*., 2010). Other methods of virus infection include cell-to-cell transmission when infected cells are in contact with uninfected or infected cells (re-infection) (Mothes *et al*., 2010). Cell-to-cell transmission may also occur through phagocytosis or during cell replication processes such as mitosis (Phillips, 1994; Noursadeghi *et al*., 2006).

1.1.8. Disease progression

HIV infection is known to progress in phases. The initial acute retroviral syndrome phase is associated with high levels of virus production (10^8 HIV-1 RNA copies/ml), rapidly reduced CD4 levels and flu-like symptoms in some patients (Piatak *et al*., 1993). Eventually the immune system reduces virus levels to a virus set point, and the patient enters an asymptomatic phase known as the clinical latency stage. Clinical latency is also associated with recovered CD4 levels, although CD4 cell count may not return to pre-infection levels. During clinical latency, the majority of patients progress to AIDS within 10 years, whereas approximately 5% patients remain asymptomatic for over 10 years, and 10-15% of patients progress to AIDS within two to five years (Kan, 2007). The symptomatic AIDS phase is associated with CD4 cell count below 200/350 cells/mm^3; however, even patients with CD4 cell count between 500 and 1600 cells/mm^3 who develop opportunistic infections are considered to have progressed to AIDS (Levy, 2007; AVERT, 2014 available on line: http://www.avert.org/stages-hiv-infection.htm). In the absence of antiretroviral therapy (ART; see below), symptomatic AIDS patients will die within two years of entering this phase.

The national consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults (http://www.sahivsoc.org/upload/documents/HIV%20guidelines%20_Jan%202015.pdf) outlines the standardised national eligibility criteria for initiating HIV/AIDS treatment. Adults and adolescents are eligible for ART regimens when their CD4 count is ≤500 cells/mm^3 regardless of infections with Hepatitis B virus (HBV), drug sensitive/drug resistant

tuberculosis (TB), Cryptococcus meningitis/TB meningitis and WHO clinical stage (WHO: The use of antiretroviral drugs for treating and preventing HIV: 2013). HIV-infected pregnant women/breast feeding mothers require fast-track ART within seven days of being eligible. Patients with CD4 less than 350 cells/mm^3 are placed on a wellness programme to monitor their CD4 count every six months and regular counselling. The national eligibility criterion for infants and children suggests all children below the age of five years are entitled for ART regardless of CD4 count. More importantly, children under the age of 1 year, particularly those with TB infection and CD4 counts less than 200 cells/mm^3 require fast-tracked ART (The South African Antiretroviral Treatment Guidelines; 2013: accessed 2015-01-27).

1.2. HIV-1 treatment

The discovery of HIV-1 as the causative agent of AIDS resulted in the research and development of anti-HIV-1 drugs (Barre-Sinoussi *et al*., 1983; Popovic *et al*, 1984); and over three decades later approximately 31 drugs have been US FDA-approved for HIV/AIDS treatment (reviewed in De Clerq, 2009; De Clerq, 2010; De Clerq, 2013). Suramin and HPA-23 were the first compounds described to have anti-HIV-1 activity (Mitsuya *et al*., 1984; Rozenbaum *et al*., 1985; De Clerq, 2013). However, AZT was the first antiretroviral drug licensed for HIV/AIDS treatment (Mitsuya *et al*., 1984; reviewed in De Clerq, 2010; De Clerq, 2013); and subsequently didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) were approved for clinical treatment of HIV/AIDS (Mitsuya and Broder, 1986; reviewed in De Clerq, 2010; Martin *et al*., 2010). Current HIV/AIDS drugs used in treatment are classified according to their mechanism of action (refer to Table 1.2 below), and the groups include nucleoside RT inhibitors (NsRTIs), nucleotide RT (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PI), HIV-1 co-receptor antagonists (CRA), fusion inhibitors (FI) and HIV-1 IN ST inhibitors (INSTIs).

Table 1.2: Current US FDA-approved HIV/AIDS antiretroviral drugs*.

Class	Name	Abbreviation	Mechanism of Action
NRTIs	Azidothymidine	AZT	Deoxyribonucleoside analogues that get phosphorylated into diphosphate forms (NRTI-TPs) without the 3'-OH group and inhibit transcription by acting as chain-terminators of DNA synthesis.
	Didanosine	ddI	
	Stavudine	d4T	
	Lamivudine	3TC	
	Abacavir	ABC	
	Emtricitabine	FTC	
NsRTIs	Tenofovir	TDF	Deoxyribonucleotide analogues that get phosphorylated into triphosphate forms (NRTI-TPs) without the 3'-OH group and inhibit transcription by acting as chain-terminators of DNA synthesis.
NNRTIs	Efavirenz	EFV	Non-competitive binding to RTs allosteric site which is ~15 Å from active site. This binding changes enzyme conformation to that which doesn't support RTs catalytic activity.
	Nevirapine	NVP	
	Delavirdine	DLV	
	Etravirine	ETR	
	Rilpivirine	RPV	
PI	Saquinavir	SQV	PIs are transition-state analogues comprising of a hydroxyethylene scaffold which mimics the normal peptide linkage (cleaved by the HIV protease), which cannot be cleaved. Thus once bound competitively to the active site proteolytic processing of precursor viral proteins into mature viral proteins is prevented.
	Ritonavir	RTV	
	Indinavir	IDV	
	Nelfinavir	NFV	
	Amprenavir	AMPL	
	Lopinavir	LPV	
	Atazanavir	ATV	
	Fosamprenavir	FPV	
	Tipranavir	TPV	
	Darunavir	DRV	
CRA	Maraviroc	MVC	Interacts with co-receptor CCR5 used by R5 HIV-1 strains. This interaction prevents HIV-1 gp120 binding to the co-receptor.
FI	Enfuvirtide	T-20	Enfuvirtide is homologous to a segment of the HR2 region of gp41 corresponding to amino acids 643-678. It binds to HIV-1 gp41 and interferes with its ability to approximate the two membranes. FI exploit the gp41 conformational transition that follows gp120-CD4 binding and co-receptor binding, and precedes pore formation.
INSTIs	Raltegravir	RAL	Inhibit IN ST reaction. Like most nucleotidyltransferase enzymes, IN requires two divalent cations bound at the active site for activity. Therefore INSTIs described inhibit HIV-1 IN by chelation of bound cations in a dose-dependent manner.
	Elvitegravir	EVG	
	Dolutegravir	DTG	

***Table 1.2:** was adapted from: Goody *et al.*, 1991; De Clerq, 1998; Marchand *et al.*, 2002; De Clerq *et al.*, 2004; Pauwels, 2004; Westby *et al.*, 2005; Perros, 2007; Sluis-Cremer and Tachedjian, 2008; De Clerq, 2009; Hazuda *et al.*, 2009; Quashie *et al.*, 2012; De Clerq, 2013.

1.2.1.1. Highly active anti-retroviral therapy (HAART)

Combination therapy/HAART regimens have been shown to successfully inhibit the disease progression of HIV/AIDS, although the treatment does not eradicate the virus in infected patients (Menendez-Arias, 2002; Schulenberg and Le Roux, 2008). However, HAART has for many years shown to sustain low plasma (<50 RNA copies/ml) viral load levels determined through ultra-sensitive viral load assays (Weller and Williams, 2001; Schulenberg and Le Roux, 2008). HAART treatment plans have been effective because of the combination of different classes of drugs, which has been observed to effectively delay the emergence of antiretroviral (ARV) drug resistant viral strains (Menendez-Arias, 2002; Schulenberg and Le Roux, 2008). HAART treatment plans usually consists of a cocktail of three/more ARV drugs; either one/two NRTIs/NsRTIs in combination with one NNRTI and/or a PI (Rainey, 2002; Wynn *et al*., 2004; Schulenberg and Le Roux, 2008). A fourth agent may be added when patients exhibit emergence of drug resistance due to treatment failure. Current first-line therapy cocktails approved for use in South Africa include: TDF+FTC/3TC+FDC /EFV/NVP, AZT+3TC+EFC/NVP, d4T+3TC+EFV/NVP, ABC+3TC+EFC/NVP and TDF+FTC/3TC+EFV with preferences dependent on side effects (psychiatric co-morbidity/tolerance, neuropsychiatric toxicity, renal disease and anaemia) associated with use of particular drugs. Second-line therapy cocktails usually consist of AZT+3TC+LPV/r and TDF+3TC (or FTC) LPV/r in patients that exhibit virologic failure to TDF and d4T based 1st line regimen. LPV/r can be switched to ATV/r when toxicity is observed (The South African Antiviral Treatment Guidelines, Version 14 March 2013). Third-line regimens are available through specialist referral and may consist of RAL, DRV and ETR. These become available once patients display virological failure during second-line therapy, and the regimen is adjusted according to HIV drug resistance genotypic interpretation (Schulenberg and Le Roux, 2008; The South African Antiretroviral Treatment Guidelines, Version 14 March 2013).

The World Health Organization (WHO) guidelines for scaling up ART with a focus on resource-limited settings was an indication of clinically integrated thinking aimed at curbing the severity of the HIV/AIDS epidemic in remote settings (WHO, 2002; Coetzee *et al*., 2004). It has since been reported that approximately 61% (57%-66%) of people eligible for ARV treatment in low- and middle income countries had obtained HIV/AIDS treatment; in total 12.9 million people living with HIV/AIDS were receiving ARVs in 2013 (UNAIDS Fact

Sheet, 2014). The expansion in ARV treatment produced clear health gains as a reduction in the number of new HIV-1 infections was observed in areas were ARV treatment was scaled-up. For instance, an estimated 1.5 million [1.4 million–1.7 million] people died from HIV/AIDS-related deaths in 2013, compared to the 2.4 million [2.2 million–2.6 million] in 2005 (UNAIDS Fact Sheet, 2014). The five-year ART rollout implemented in 2003 by the South African government had already resulted in an estimated increase of 11.3 years in life expectancy in the KwaZulu-Natal province by year 2011 (UNAIDS, 2013).

1.3. HIV-1 IN drugs

1.3.1. Early anti-HIV-1 IN agents

The history of the discovery of IN inhibitors (INI) comprises of a diverse group of different molecules such as peptides, nucleotides, DNA complexes, natural products and rationally designed compounds (Jing and Xu, 2001; Singh *et al.*, 2001; Sluis-Cremer *et al.*, 2002; Maurin *et al.*, 2003; Brigo *et al.*, 2005). Some of these molecules showed potential as INI and progressed into pre-clinical trials, but their development was halted by *in vivo* toxicity and non-specificity (de Soultrait *et al.*, 2003; Gupta and Nagappa, 2003; Maurin *et al.*, 2003). Of note were a few 4-aryl-2-,4-diketobutanic acid (DKA) that demonstrated notable anti-HIV-1 activity in cell culture from a large library of 250 000 compounds from Merck and Co; and *in vitro* selection and sequencing of the outgrown viruses showed resistant mutations within the IN gene, therefore confirming their mechanism of action as INIs (Hazuda *et al.*, 2000). L-731988 was one of these DKAs that demonstrated specificity through inhibition of the HIV-1 IN ST reaction (IC_{50} = 80 nM) in comparison to 3'-processing reaction (IC_{50} = 6 µM) (Hazuda *et al.*, 2000). The inhibition of the ST reaction was described to be by chelation of bound cations within the D64 D116 E152 motif by a prototype DKA namely, 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone (5-CITEP) within the IN active site (Goldgur *et al.*, 1999; Derwent, 1999; Marchand *et al.*, 2002). The backbone of 5-CITEP served as a template for the design and development of INI with enhanced efficacy, specificity, tolerability and bioavailability such as S1360. S1360 advanced into clinical trials but its success was halted by its short half-life *in vivo* due to rapid glucuronidation (Rosemond *et al.*, 2004).

1.3.2. First generation clinically approved INIs

RAL is a pyrimidine carboxamide developed using the backbone 5-CITEP variations namely, L-731988 and L-870812 (Summa *et al*., 2008). INIs L-731988 and L-870812 were optimized in Merck pharmaceuticals by substituting the β-diketo acid chemical moiety to various forms using ketones, diketones and naphthyrine carboxamides which resulted in enhanced chemical stability (Lataillade and Kozal, 2006) of one N-[(4-fluorophenyl) methyl]-1,6-dihydro-5-hydroxy-1-methyl-2-[1-methyl-1-[[5-methyl-,3,4-oxadiazol-2-yl)carbonyl]amino]ethyl]-6-oxo-4-pyrimidine carboxamide monopotassium salt (RAL) (Cocohoba *et al*., 2008; Figure 1.6). RAL was later approved by the US FDA in October 2007 and now forms part of third-line ART salvage treatment in South Africa in combination with ETV and DRV (http://www.avert.org/starting-monitoring-switching-hiv-treatment.htm).

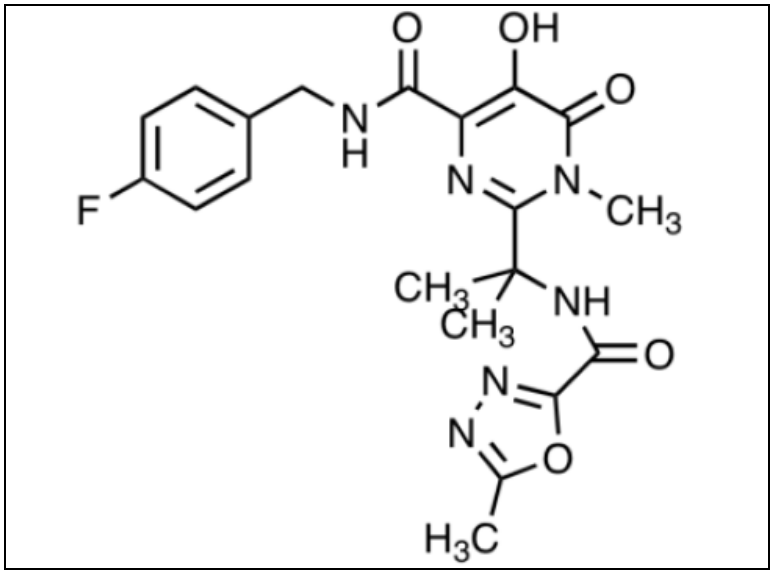


Figure 1. 6: The chemical structure of RAL. RAL is produced by Merck & Co and exhibits potent anti-HIV-1 IN activity (IC50 =2-7 nM), and anti-HIV activity (EC90 = 8.9 nM). The chemical structure and data were obtained from a review by Metifiot *et al*., 2010.

Further optimizations of DKAs by the Japan Tobacco Company led to four variations of 4-quinolone-3-glyoxylic acids which consisted of a single pair of coplanar ketone and carboxylic groups, designated according to their functional DKA motif (Sato *et al*., 2006; Wills *et al*., 2012). One of these variations of 4-quinolone-3-glyoxylic acids contained only a β-ketone functional group and a carboxylic acid functional group, and exhibited good HIV-1 IN ST inhibition (IC_{50} = 1.6 μM) and was used as the backbone for 6-[(3-chloro-2-fluorophenyl)methyl]-1-[(2S)-1-hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxoquinoline-3-carboxylic acid (EVG) synthesis (Figure 1.7) which demonstrated high potency (IC50 = 7.2

nM) in direct anti-HIV IN assays and good anti-HIV-1 (EC_{50} = 0.9 nM) inhibition (Sato *et al.*, 2006; Al-Mawsawi *et al.*, 2008). In 2012, the second first generation INI, EVG obtained US FDA approval as a component of the quad pill Stribild.

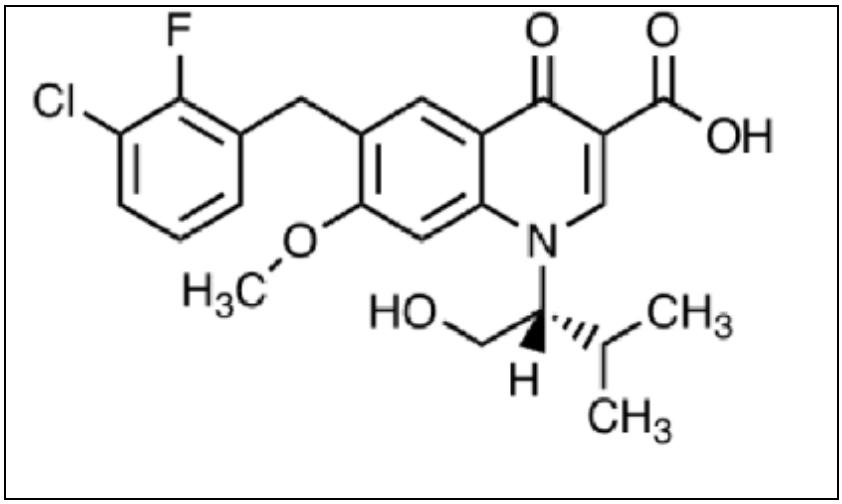


Figure 1. 7: The chemical structure of EVG which is produced by Gilead Sciences and Japan Tobacco Inc. The chemical structure was obtained from a review by Metifiot *et al.*, 2010.

1.3.3. Stribild

Fixed dose drug combinations (FDCs) have been introduced, and these include Combivir® (AZT+3TC), Trizivir® (AZT+3TC+ABC), Truvada® (FTC+TDF), a once daily combination pill Atripla® (TDF+FTC+EFV) approved in 2006, Complera®/Eviplera® (TDF+FTC+RPV) approved in 2011, and the only INI containing FDC, a "quad" pill designated Stribild (TDF+FTC+EVG+cobicistat (COBI)) approved in August 2012 (reviewed in De Clerq, 2013). Other quad pills currently in clinical trials contain (i) ATV+COBI+TDF+FTC and (ii) DRV+COBI +TDF+FTC (iii) EVG+COBI+TDF+FTC (reviewed in De Clerq, 2013).

Stribild contains 150 mg EVG, 200mg FTC, 300mg TDF and a pharmaco-enhancer without anti-HIV activity; COBI (150 mg) (https://www.stribild.com/). As mentioned in Table 1.2, EVG is an INSTI that binds the IN catalytic core domain and halts HIV-1 proviral DNA integration (DeJesus *et al.*, 2006; Hicks and Gulick, 2009; Deeks, 2013; Messiaen *et al.*, 2013); and virus proliferation of HIV-1 clinical isolates A, B, C, D, E, F, G, O and some NRTI, NNRTI and PI resistant virus isolates. Stribild was approved following observations of EVGs synergistic effects in combination with NRTIs (ABC, ddI, FTC, 3TC, d4T, TDF and AZT), NNRTIs (EFV, ETR and NVP), PIs (APV, ATV, DRV, IDV, LPV, NFV, RTV,

SQV), FI (ENF) and the CCR5 receptor antagonist (MRV). EVG exhibited additive effects in combination with RAL and antagonistic effects against other HIV-1 drugs are yet to be reported (Gilead Sciences Inc. US prescribing information for Stribild®, 2013; European Medicines Agency, 2013). The FTC in Stribild is a fluorine-containing cytidine analogue that inhibits HIV-1 RT following phosphorylation by cellular kinases into an active 5'-triphosphate that terminates host cell DNA synthesis/RNA transcription (Darque *et al.*, 1999; Rousseau *et al.*, 2001; Goodman *et al.*, 2011). TDF is also an acyclic nucleoside phosphonate that inhibits HIV-1 RT following diester hydrolysis and phosphorylation by cellular kinases into an active metabolite TDF-diphosphate. TDF-diphosphate inhibits HIV-1 RT through competition with the purine nucleoside, adenosine, leading to a halted viral DNA strand elongation (reviewed by De Clercq, 2013).

COBI is an inhibitor of enzymes involved in the metabolism of EVG and PIs (ATV and DRV). The metabolism of EVG involves the cytochrome P450 enzyme and UDP glucuronosyltransferase (UGT) 1A1/3 which catalyses glucuronidation (Ramanathan et al., 2008; Adams *et al.*, 2012; Olin *et al.*, 2012; Deeks, 2013). COBI was observed to extend the bioavailability and half-life of EVG by 21 hours (Adams *et al.*, 2012; Olin *et al.*, 2012). COBI also inhibits p-glycoprotein, BCRP, and OATP1B1/1B3 involved in substrate transportation (Lepist *et al.*, 2012; Custodio *et al.*, 2012). However, COBI similarly requires phosphorylation by cellular enzymes into an active intermediate by CYP3A4 and to a lesser extent CYP2D6 (Ernest *et al.*, 2005; Xu *et al.*, 2010; Deeks *et al.*, 2014).

1.3.4. Early second generation INIs

RAL and EVG are associated with a low to moderate genetic resistance barrier *in vitro* and in AIDS patients, as a result of the emergence of single mutations or a combination of primary and secondary mutations (Geretti *et al.*, 2012; Mesplède *et al.*, 2013; Quashie *et al.*, 2013; Grobler and Hazuda, 2014). This led to renewed research focus on the development of second generation INSTIs with a high genetic resistance barrier and activity against RAL and EVG resistant virus isolates. Initial developments of second generation INSTIs included the discovery of a tricyclic 10-hydroxy-7, 8-dihydropyrazinopyrrolopyrazine-1,9-dione compound which served as parent compounds for INI MK-2048 (Vacca *et al.*, 2007; Al-Mawsawi *et al.*, 2008). MK-2048 exhibited direct IN strand transfer inhibition and tissue

culture anti-HIV-1 activity (EC95 <50nM) (Vacca *et al*., 2007), but its progression was halted by poor pharmacokinetic properties observed during clinical development (Vacca *et al*., 2007; Al-Mawsawi *et al*., 2008; Pandey *et al*., 2010; Goethals *et al*., 2011; Van Wesenbeeck *et al*., 2011).

1.3.5. DTG and S/GSK-1265744

DTG (Figure 1.8) was developed along with S/GSK-1265744 which served as a back-up drug. Although S/GSK-1265744 exhibited good anti-HIV-1 efficacy, pharmacokinetic properties and exhibited good tolerability in patients, its development was halted by the US FDA approval of DTG (Highleyman, 2009). DTG was discovered by Shionogi Pharmaceuticals, Japan and further developed through a joint venture between Shiniogi-ViiV Healthcare-GlaxoSmithKline (Eron *et al*., 2010; Yoshinaga *et al*., 2010). DTG was developed with the primary objective of finding a drug that allowed once-daily dose (<100 mg) without the need for pharmacoenhancers, acceptable drug-drug interactions, and without cross-resistance (and a higher genetic resistance barrier) to RAL and EVG (Blanco *et al*., 2011; Jonhs *et al*., 2013).

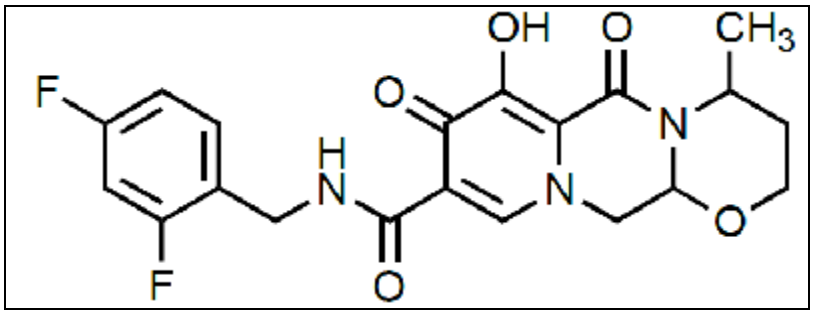


Figure 1. 8: The chemical structure of DTG. DTG has potent anti-HIV-1 IN activity of approximately 2.7 nM and anti-HIV-1 activity of 2 nM in tissue culture. The chemical structure and data were obtained from a review by Metifiot *et al*., 2010.

1.4. Antiretroviral drug resistance

Treatment of HIV/AIDS with multiple drugs/HAART suppresses virus replication more effectively than monotherapy; and effective suppression of HIV-1 proliferation results in the "arrest" of emerging new drug resistant variants (Clavel and Hance, 2004). The use of HAART has proven remarkably effective in controlling the progression of HIV/AIDS, and is

associated with reduced AIDS-related mortality rates and a prolonged life span of AIDS patients. These successes are, however, limited, by the emergence of antiretroviral drug resistant isolates (termed acquired resistance) during HAART treatment (Palella *et al*., 1998; Clavel and Hance, 2004). The emergence of drug resistant isolates has also been associated with transmission of such strains, which threaten the effectiveness of HAART in newly infected HIV/AIDS patients (termed transmitted drug resistance).

HIV infection is associated with the inevitable emergence of diverse quasispecies within infected individuals harbouring drug resistance mutations (Saag *et al*., 1988; Wainberg *et al*., 1996). Factors known to result in HIV-1 drug resistance include the low-fidelity of RT which is notoriously error prone. RT introduces on average one mutation per viral genome transcription, in combination with drug selection and immune pressure created during long-term patient treatment (Roberts and Bebenek, 1988; Preston *et al*., 1992; Wainberg *et al*., 1996). Most of the mutations obtained are base substitutions, but duplications, insertions and recombination have been reported (Coffin, 1995; Malim and Emerman, 2001; Bonhoeffer and Sniegowski, 2002; Clavel and Hance, 2004; Levy *et al*., 2004). Retroviral recombination occurs during coinfection of a cell by two/more viral isolates; and involves transfer of the nascent DNA provirus from one RNA template to that of another isolate, therefore resulting in mosaic DNA provirus (Levy *et al*., 2004). Studies have shown that RT alternates between RNA strands of distinct isolates on average three times per replication cycle and this capacity is believed to enhance the evolution of drug resistance mutations considerably (Jetzt *et al*., 2000; Zhaung *et al*., 2002; Bretscher *et al*., 2004). The development of HIV-1 drug resistant variants over time is associated in part with the eventual failure of treatment strategies (Richman *et al*., 1994; Wainberg *et al*., 1996). Mutations can confer some selective advantage to the virus, such as a decrease in its susceptibility to an antiretroviral agent (Clavel and Hance, 2004). However, certain strains such as HIV-2 exhibit natural resistance to most NNRTIs; likewise some HIV-1 non-B subtypes are naturally less susceptible to PIs/NNRTIs than HIV-1 subtype B, against which most of the ARV drugs were developed (Shafer *et al*., 1997; Descamps *et al*., 1997; Palmer *et al*., 1998; Descamps *et al*., 1998; Witvrouw *et al*., 1999).

The control of acquired and transmitted HIV drug resistance can be achieved through evidence-based treatment strategies which include the uninterrupted rational use of ARVs

with programmatic surveillance and monitoring. This will allow tracking of global drug resistance trends and rational choices in containment activities in areas with increasing drug resistance rates and high prevalence (WHO, 2014).

1.5. The mechanisms of HIV-1 drug resistance

1.5.1.1. RTI resistance

The mechanisms of resistance to nucleoside/tide analogues are associated with impaired analogue incorporation into the host DNA. In the case of M184, mutations are located within the catalytic site of RT, and are typically replaced with valine (M184V) which interferes with proper positioning of the 3TC triphosphate within the catalytic site; thus resulting in resistance (Sarafianos *et al.*, 1999). Another resistance mechanism involves the removal of the inserted nucleoside/tide analogue from a terminated DNA chain; and these are termed "thymidine analogue mutations (TAMs)." The development of TAMs is known to be catalysed by coenzyme adenosine triphosphate (ATP)-or pyrophosphate which phosphorylates the inserted chain terminator from the 3'-end of the DNA strand (Arion *et al.*, 1998; Meyer *et al.*, 1999). ATPs and pyrophosphates are abundant in human lymphocytes which do not participate in the DNA-polymerization reaction, but the structure of the RT expressing TAMs facilitates their attack of the phosphodiester bond adjacent to the incorporated analogue (Boyer *et al.*, 2001; Chamberlain *et al.*, 2002).

NNRTIs are designed to bind with high affinity to the hydrophobic pocket in close proximity to the RTs catalytic domain. This binding changes RTs conformation such that the enzyme flexibility is inhibited, thereby hindering the RT DNA polymerization capacity (Esnouf *et al.*, 1997). All NNRTI resistant mutations emerge within the hydrophobic pocket such that NNRTI affinity is reduced considerably (Boyer *et al.*, 1993; Richmann *et al.*, 1994; Esnouf *et al.*, 1997; Bacheler *et al.*, 2000; Ren *et al.*, 2001).

1.5.1.2. PI resistance

PIs are designed to exhibit high affinity for the HIV-1 PR active site, as they are structurally related to viral peptides normally recognized and cleaved by the PR enzyme. PIs lack a PR cleavage site; therefore, their active site binding inhibits PR catalytic activity through prolonged occupation of site (Roberts *et al.*, 1990; Erickson *et al.*, 1994). PI drug resistance

mutations (i.e. V82A) are associated with reduced amino acid residue size, which therefore only allows binding of the natural viral substrate (Prabu-Jeyabalan *et al*., 2002). Therefore, because PIs large size contributes to their maximum binding affinity; the reduced active site size due to mutations ensures non-binding of PIs. The natural substrates of PR are variable in size and have a lower affinity for the catalytic site, which promotes ordered sequential cleavage of the polyproteins required for proper assembly of virions (Clavel and Allan, 2004).

1.5.1.3. Entry inhibitor resistance

The gp41 protein is considered a highly conserved region of the envelope glycoprotein, with minor differences on the HR1 binding site among HIV-1 group M subtypes (Cilliers *et al*., 2004; Aghokeng *et al*., 2005; Holguin *et al*., 2007). The development of T-20 resistance has been associated with mutations within the highly conserved amino acid triad within the HR1 region at positions: 36-38 and 36-45 with varying levels of resistance (Poveda *et al*., 2002; Greenberg and Cammack, 2004; Mink *et al*., 2005). T-20 resistance mutations are associated with reduced binding effectiveness between the HR2/HR1 interactions, a prerequisite for the fusion process (Poveda *et al*., 2002).

HIV-1 resistance to CCR5 antagonists has been observed both *in vitro* and *in vivo*; and remains associated with emergence of multiple sequence mutations within the V3 region which results in dependency on the CCR5 N-terminus (NT) (Tsibris *et al*., 2008; Berro *et al*., 2009; Nolan *et al*., 2009; Ogert *et al*., 2010). Another, but rare, pathway involves mutations within the fusion peptide (FP) of the gp41 protein; but in all cases, resistant viruses gain cell entry through a free/inhibitor bound CCR5 receptor (Pugach *et al*., 2007; Anastassopoulou *et al*., 2009).

1.5.1.4. INSTI resistance

The majority of INSTI resistance mutations described emerge within the catalytic core domain of IN and are associated with decreased drug susceptibility, as shown in Table 1.3. Since the focus of this work is INSTI resistance, the mutations are described in detail. Mutations known to confer resistance to RAL have been described at positions 143, 148 and 155 within the putative INI binding pocket, and each represents mutually distinct and non-

overlapping pathways for RAL resistance (Goethals *et al*., 2008; Fransen *et al*. 2009; Malet *et al*., 2009; Whitmer and Danovich, 2009; Kobayashi *et al*., 2011; Fransen *et al*., 2012; Fransen *et al*. 2012). RAL and EVG were observed to exhibit cross-resistance as both drugs select for similar resistant mutations (N155H, Q148RHK and E92Q); with the exception of T66I only selected through EVG treatment (Goethals *et al*., 2008; Goodman *et al*., 2008; Malet *et al*., 2009). Other amino acid substitutions that have been selected during INSTI treatment both *in vivo* and *in vitro* include: V72I, L74I/M, E92Q, T97A, F212Y, E138K, G140S/A, V151I, E157Q, G163R, I203M and S230R (Low *et al*., 2009; Malet *et al*., 2009). To date, all RAL and EVG resistant isolates exhibit susceptibility to DTG; with Q148HRK being an exception. The Q148HRK substitutions have been shown to display limited resistance to DTG in combination with L101I, T124A, G118R, R263K and S153Y (Kobayashi *et al*., 2011; Mesplède *et al*., 2012; Quashie *et al*., 2012; Wares *et al*., 2014); and this suggests additional secondary mutations may induce considerable resistance to DTG (Mesplède *et al*., 2012). Combinations shown to exhibit notable resistance to DTG include E138K/Q148K and Q148R/N155H, while virus isolates with combinations as follows: Q148R/ G140CS and T66K/L74M and I151L have been reported to exhibit moderate reduction in DTG susceptibility (Kobayashi *et al*., 2011). Other DTG selected mutations include substitutions at position 153 which include S153F, S153Y; and S153 in combination with other mutations (L101/IS153F, T124A/S153Y and L101IT/124A/S153F) of unknown relevance (Kobayashi *et al*., 2011).

Along with known INSTI resistance pathways N155H, Q148HRK, and Y143RC; accessory mutations such as E92Q and G140S are associated with recovery of reduced viral fitness/replication capacity (RC; Malet *et al*., 2009; Hu and Kuritzkes, 2010; Canducci *et al*., 2010; Hatano *et al*., 2010). In isolated instances, INI resistant mutations have been observed to revert (or back-mutate); which demonstrates the dynamic nature and complexity of INI resistance development as the virus then regains the lost RC (Ferns *et al*., 2009; Canducci *et al*., 2010).

Table 1.3: Fold changes in susceptibity to major and alternative resistance pathways to current INSTIs. Data was compiled from Abram *et al*., (2013), Quashie *et al*., (2013) and Wainberg and Han (2015).

		Fold Resistance		
Resistance Pathways		**RAL**	**EVG**	**DTG**
Y143 pathway	Y143C	<10	<2	<2
	Y143H	2.1	1.2	0.9
	Y143R	<50	<2	<2
	T97A/Y143C	>100	<2	<2
	T97A/Y143R	>100	<2	<2
	L74M/T97A/Y143G	<50	-	<2
	L74M/T97A/E138A/Y143C	<20	-	<2
	T97A	1.2	2.4	0.5
N155 pathway	N155N	<50	50	<2
	E92Q/N155H	<100	>100	<10
	L74M/N155H	<50	<50	<2
Q148 pathway	Q148H	<20	<10	<2
	Q148K	<100	<100	<2
	Q148R	<50	<100	<2
	E138K/Q148H	<10	<20	<2
	E138K/Q148K	>100	>100	<10
	E138K/Q148R	>100	>100	<10
	G140S/Q148H	>100	>100	<20
	G140S/Q148K	<10	<100	<2
	G140S/Q148R	>100	>100	<10
	E138A/G140S/Y143H/Q148H	>100	-	<50
R263K pathway	R263K	<10	<10	<50
	H51Y/R263K	3-5	3	4-6
G118R pathway	G118R	<10	<2	<10
	H51Y/G118R	-	-	-
	L74M/G118R	<50	<10	-
	G118R/E138K	<10	<2	<10
	E138K	<10	<10	<10
T66 pathway	T66I	<2	<10	<2
	T66A	<2	<10	<2
	T66K	<10	<100	<50
	T66I/L74M	<10	<50	<2
	T66K/L74M	<50	<100	<10
	T66K/R263K	<2	<100	-
E92Q pathway	E92Q	<10	<50	<10
	E92G	1.5	9	1
	E92Q/S147G	<10	>100	-
	S147G	<2	<10	1
S153 mutations	S153F	<2	<10	<2
	S153Y	<2	<10	<10

- No information.

1.5.1.5. Effect of resistance mutations on RC

The inevitable emergence of ARV drug resistance is often associated with incompletely suppressive drug regimens or treatment interruptions by patients. It is generally acknowledged that the emergence of most ARV drug resistance mutations will decrease the RC of HIV-1. Interestingly, treatment interruption by patients infected with multi-drug resistant HIV-1 has been reported to result in the replacement or outgrowth of resistant viruses by wild-type (WT) virus. Furthermore, in the same patients a decreased CD4+ T-cell count was observed which suggests a greater replicative/pathogenic potential by WT viruses (Deeks *et al*., 2001; Clavel and Hance, 2004). Thus, in the absence of drugs, as ARV drug resistant viruses revert back to WT, the viral RC can be "restored".

The emergence of ARV drug resistance in HIV-1 occurs by the introduction of mutations within the *pol* gene (or other relevant gene targets), which have been shown to modify key viral proteins which results in various deleterious effects (Barbour *et al*., 2003; Clavel and Hance, 2004), and are often also associated with reduced RC/viral fitness and reduced pathogenicity (Deeks *et al*., 2001; Prado *et al*., 2005). For example, with the inhibitors targeting the RT, PR or IN enzymes, the loss in virus RC is often owed to structural changes in these enzymes expressed from the mutated *pol* gene which results in inefficiencies in binding capacity to natural substrates and loss in enzyme catalytic efficacy (Dauber *et al*., 2002; Malim *et al*., 2001). Nonetheless, the cost of replication impairment has been observed to vary among virus subtypes and clinical virus isolates from drug-naïve patients (infected with viruses carrying resistant natural polymorphisms) resistant to a particular ARV treatment (Wrin *et al*., 2001; Cingolani *et al*., 2002; De Luca *et al*., 2005). Such impaired viral/enzyme functions have been shown to be partially corrected by secondary mutations that compensate for the decreased viral/enzyme fitness (Kaplan *et al*., 1994; Nijhuis *et al*., 1999; Clavel and Hance, 2004; Lataillade *et al*., 2007).

Several NRTI, NNRTI and PI are associated with reduced viral fitness. For instance, NRTI resistance mutations M184IV, M41L, L210W, T125Y, K65R and L74V are known to exhibit reduced virus fitness (reviewed by De Luca, 2006). Interestingly, the presence of a single primary drug mutation such as M184V (confers resistance to 3TC) is associated with a lower incidence of TAMs and with increased susceptibility to other RT inhibitors such as AZT in viral variants that contain drug resistance mutations to AZT during simultaneous treatment

with 3TC. The M184V mutation also has an effect on RT fidelity and processivity, reducing spontaneous mutagenesis and impacting on viral replication fitness, respectively. NNRTI resistance mutation V106A, Y188C and G190S are also associated with lower viral fitness, but resistance mutations K103N and Y181C exhibit a trivial reduction in virus fitness although still comparable to WT virus (reviewed by De Luca, 2006).

Considerable loss in virus RC has been reported in clinical isolates/molecular clones containing PI resistance mutations D30N, M46I/L, G48V, I50V, I54V, V82A/T, I84V, N88DS and L90M. Substitution L90M is associated with resistance to SQV and was established to exhibit less RC when compared to NFV resistant D30N isolates (Martinez-Picado *et al*., 1999; Resch *et al*., 2002; Prado *et al*., 2002). FI (T20) resistance has also been associated with reduced virus fitness, as a result of resistance-associated substitutions introducing conformational changes in gp41 that lead to delayed and reduced efficiency in the fusion process (Reeves *et al*., 2005). Reduced virus/IN fitness has been reported as a result of selection of INSTI major resistance mutations (i.e. N155H and Q148H) within the *pol* gene following ARV treatment (reviewed by Quinones-Mateo 2008; Hu and Kuritzkes, 2010; see section 1.5.1.6 below).

Drug susceptibility/resistance scores remain the most important determinants of treatment responses during the clinical management of patients. South Africa has recently introduced the use of an INSTI as a component of the 3rd line salvage regimen. There is currently limited knowledge on the emergence of INSTI drug resistance in this treatment experienced HIV-1 subtype C infected population. Thus, a better understanding of the impact of antiretroviral drug-resistance mutations on HIV-1 RC and their contribution to levels of fold resistance are critical to derive maximum clinical benefit in our resource limited setting.

1.5.1.6. Effect of INSTI selected resistance mutations on RC and IN fitness

All IN resistance mutations are associated with reduced HIV-1 RC and reduced IN 3'-P/and ST as shown in Table 1.4. Abram *et al*., (2013) reported on the impact of primary EVG resistance-associated mutations on viral RC using single mutations T66AIK (69%/84%/9%), E92GQ (6%/64%), T97A (68%), Y143CHR (49%/78%/75%), Q148HKR (54%/36%/70%) and N155H (72%). Furthermore, double mutations T66I/E92Q, T66I/Q148R, T66I/N155H,

E92Q/Q148R, E92Q/N155H and Q148R/N155H exhibited 25%, 20%, 15%, 4%, 40% and 15% RCs, respectively (Abram *et al*., 2013). Preceding studies on corresponding mutations selected by RAL have described similar results (Buzon *et al*., 2008; Goethals *et al*., 2008; Shimura *et al*., 2008; Fransen *et al*., 2009; Nakahara *et al*., 2009; Quercia *et al*., 2009). In addition, RAL and EVG primary drug resistant substitutions are often associated with subsequent emergence of secondary mutations known to play a compensary role by enhancing RC/enzymatic activity (reviewed by Mesplède and Wainberg, 2014). However, selection of mutation R263K with DTG is not only associated with diminished viral RC and enzymatic 3'-P/ and ST activity; but the secondary mutations selected in combination with R263K are also associated with impaired (>80%) virus RC, and further diminution of HIV-1 IN activity (reviewed by Mesplède and Wainberg, 2014). This raises the possibility that low-level resistance against DTG might not lead to virological failure or adverse clinical consequences; as such viruses are truly at a severe replication disadvantage in comparison with wild-type HIV-1 (reviewed by Mesplède and Wainberg, 2014).

Table 1.4: Mutant 3'-processing, strand transfer activity and replication capacity with respect to wild-type integrase enzyme and virus. The data was obtained from a review by Métifiot *et al*., 2010.

	IN		Virus
Mutations	**3'-P**	**ST**	**RC**
H51Y	-	40-80%	-
H51Y/E92Q/S147G	-	10-40%	-
H51Y/E92Q/S147G/E157Q	-	40-80%	-
T66A	10-40%	40-80%	40-80%
T66I	80-100%	80-100%	40-80%
T66I/E92Q	-	0-10%	-
L74M	80-100%	80-100%	-
E92Q	80-100%	80-100%	40-80%
E92Q/S147G	-	10-40%	-
E92Q/N155H	-	-	40-80%
E138K/Q148HRK	-	-	40-80%
G140S	80-100%	10-40%	40-80%
G140A	40-80%	10-40%	-
G140S/Q148H	40-80%	80-100%	80-100%
G140S/Q148K	40-80%	10-40%	40-80%
G140S/Q148R	0-10%	10-40%	40-80%
G140A/Q148H	0-10%	10-40%	10-40%
G140A/Q148K	0-10%	0-10%	40-80%
G140A/Q148R	10-40%	10-40%	40-80%
G140S/Q148H/S230N	-	-	80-100%
Y143R	10-40%	40-80%	10-40%
Y143C	10-40%	10-40%	10-40%
Y143R/Q148H/S230N	-	-	40-80%
S147G	-	10-40%	-
Q148K	0-10%	10-40%	10-40%
Q148R	10-40%	10-40%	40-80%
Q148H	0-10%	10-40%	40-80%
Q148H/N155H	-	-	10-40%
Q148K/G163R	-	-	-
N155H	80-100%	80-100%	40-80%
E157Q	-	40-80%	-
S230R	80-100%	80-100%	-

-: No information; 3'-P: 3'-processing activity; ST: strand transfer activity; RC: virus replication capacity;

1.6. The significance of this study

HIV-1 subtype B has been the subject of most ARV drug resistant research with respect to INSTI (and all other ARVs), due to its predominance in those countries in which ARVs first become available (Wainberg and Brenner, 2012). As a result, data on non-C HIV-1 drug resistance to the new class (INSTI) of drugs is limited. HIV-1 naturally varies in genetic content by as much as 35% among subtypes, and variation is higher in some areas of the genome, particularly *Env*: 25-40%, and lower within the *Pol-Gag* gene: 8-10% (Brenner, 2007; Santoro and Perno, 2013). Inter-subtype variability in IN is relatively low: 8-12% at the amino acid level (Brenner, 2007; Santoro and Perno, 2013; Martinez-Cajas *et al*., 2009; Bar-Magen *et al*., 2009; Bar-Magen *et al*., 2010). Interestingly, subtype-specific amino acid differences are often found in close proximity to known ARV drug resistance sites.

Since its introduction in the government sector in April, 2004, HAART has dramatically increased the life expectancy of people infected by HIV-1 in South Africa. However, the success of HAART is limited by the emergence of antiretroviral drug resistance which results in virological failure (Ajose *et al*., 2012). Thus, the May 2013 addition of the INSTI, RAL to the third line regimen available in the South African public sector represented an important advance for the treatment of ARV treatment-experienced patients. Moreover, the latest FDA approval of EVG and DTG, suggests these INSTIs are likely to be approved by the Medicines Control Council of South Africa in the near future. RAL, EVG and DTG resistance data is limited for HIV-1 subtype C which is highly endemic in sub-Saharan Africa (UNAIDS, 2011), and particularly in South Africa. IN mutations T97A, E157Q, V165I and V72I have been reported in therapy naïve South African patients from the Charlotte Maxeke Johannesburg Academic Hospital (Fish *et al*., 2010). Additional IN mutations, specifically G163R and V151I previously associated with RAL use, were also reported in cohorts recruited for the AIDS Vaccine Integrated Project (AVIP) study at the Baragwanath Hospital in Johannesburg (Papathanasopoulos *et al*., 2010). Minor resistance mutations (L74M, Q95K), and polymorphisms (Q95P, E157K, I203M and R263S) associated with RAL resistance have been reported in six different subjects (Bessong and Nwobegahay, 2013); but to date no major/accessory INSTI associated resistance mutations have been reported in Southern Africa particularly in South Africa. Moreover, HIV-1 subtype B and C integrase enzymes have been shown to exhibit differential patterns of resistance to integrase inhibitors in *in vitro* biochemical assays (Bar-Magen *et al*., 2009).

Since preliminary evidence suggest the possibility that HIV-1 subtypes may differ in their sensitivity to INSTI it is important to provide genotypic and phenotypic data on these drug targets in the context of the evolving HIV genetic landscape (Marconi *et al*., 2008; Fish *et al*., 2010; Eshleman *et al*., 2009). Thus, this study aimed to select for ARV drug resistance in HIV-1 subtype C primary viruses grown in the presence of INSTIs, in order to understand how antiviral resistance may emerge to new drug agents as well as to direct future research and development of the next generation INSTIs with improved efficacy against known mutants (Eron *et al*., 2010).

In vitro selection of RAL, EVG and DTG resistance using primary HIV-1 subtype C isolates from South Africa could yield INSTI mutation profiles specific to the region. As the development of resistance to RAL, EVG and DTG is likely to influence the use of those inhibitors in HAART regimens; resistance profiling would be instructive to the requirements of subsequent generations of HIV-1 IN inhibitors. The analysis of large patient resistance data groups will result in an improved understanding of resistance pathways in HIV-1 subtype C, but *in vitro* selection can also indicate prospective resistance pathways as mutations produced are likely to be similar to those observed *in vivo*. Moreover, *in vitro* selection enables the rapid genotyping and phenotyping of mutant viruses, and validation with site-directed mutants allows the description of the change in drug susceptibility due to each specific mutation. In this way a database comprising of the degree of susceptibility, cross resistance and the identification of possible compensatory mutants and other resistance-associated mutations to a wide range HIV-1 subtype C IN inhibitors can be built.

1.7. Hypothesis

In vitro passage of WT and ARV resistant HIV-1 subtype C primary virus isolates in the presence of increasing concentrations of RAL, EVG and DTG could provide defined resistance pathways associated with HIV-1 subtype C prevalent in South Africa.

1.8. Study objective

The overall objective of this study was to establish the ARV drug resistance mutation profiles of HIV-1 subtype C primary virus isolates that evolve/emerge under selective pressure of the INSTIs RAL, EVG and DTG. This was to be accomplished through the fulfilment of the following aims:

- To perform *in vitro* selection of primary HIV-1 subtype C drug resistant mutant viruses grown in peripheral blood mononuclear cells (PBMCs) with increasing concentrations of the INSTIs RAL, EVG and DTG.
- To genotypically characterise the emergence of mutations associated with ARV drug resistance to RAL, EVG and DTG.
- To determine the impact of drug resistance-associated mutations on RC/enzyme fitness.
- To determine the effect of drug resistance-associated mutations on retroviral DNA binding.

CHAPTER 2: MATERIAL AND METHODS

2.1. Materials and methods

2.2. Mammalian cells and primary HIV-1 subtype C preparation

PBMC isolation, virus expansion and tissue culture infectious dose ($TDIC_{50}$) determination assays were carried out in accordance with standard operating procedures (SOPs) from the HIV Pathogenesis Research Laboratory, Department of Molecular Medicine and Haematology at the Faculty of Health Sciences, University of the Witwatersrand. Six primary HIV-1 subtype C virus isolates designated FV3, FV5, FV6, MR69, MR81 and MR89 were randomly selected for the INSTIs resistance study. The FV (FV3, FV5 and FV6) virus isolates were isolated from treatment naïve HIV/AIDS patients at the Charlotte Maxeke Johannesburg Academic Hospital in Johannesburg, South Africa (Connell *et al*., 2008). The MR (MR69, MR81 and MR89) virus isolates were isolated from HIV/AIDS treatment experienced patients, and were known to contain NNRTI/NRTI resistant mutations (as shown in Table 2.1 below).

Table 2.1: A summary of NRTI/NNRTI resistant mutations from baseline MR virus isolates.

HIV-1Virus Isolate	NRTI Resistant mutations	NNRTI Resistant mutations
MR69	None	K103N
MR81	M184V	K103N/K238N
MR89	M184V	K103N

K103N is associated with high-level resistance to RFV and NVP; M184V is associated with high-level resistance to FTC and 3TC; K238N is associated with resistance to EFV and NVP (resistance information was obtained online from: http://hivdb.stanford.edu/DR/).

2.2.1. Isolation of PBMCs

Buffy coats (3 pooled) were collected from the South African National Blood Service (SANBS), South Africa. For PBMC isolation, buffy coats were first pretreated with the RosetteSep® Human CD8 Depletion Cocktail (50 µl/ml) (StemCell Technologies, Canada) following manufacturer's instructions. The CD8 depleted buffy coats were then mixed (1:1) with pre-warmed Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, USA); and subsequently layered over Ficoll-Paque (4:3) (Amersham Biosciences, United Kingdom) prior to centrifugation at 360 x g for 30 minutes (no brakes and acceleration). The interphase PBMC layer was carefully aspirated and washed (x2) with 45ml PBS (22 °C) by centrifuging at 400 x g with maximum (9) brakes and acceleration, to remove platelets. PBMCs were resuspended to a concentration of $1.5x10^6$ /ml in 10% growth media (89% RPMI (Sigma Aldrich, USA), 10% fetal bovine serum (FBS) (Highveld Biologicals PTY LTD, US) and 1% 100x penicillin/streptomycin (Highveld Biologicals PTY LTD, USA). To stimulate the PBMCs, 1µg/ml phytohemagglutinin (PHA-P) (Sigma-Aldrich, USA) was added and cells were incubated over 72 hours in a 37 °C, 5% CO_2 incubator.

2.2.2. Virus expansion

Approximately 1000 $TCID_{50}$ primary HIV-1 subtype C virus (concentrations determined as described in section 2.2.3 below) was added to 3 ml PHA-stimulated PBMCs ($4.5 x10^6$ cells) in 10% growth media (supplemented with 5% Interleukin-2 at 200 U/ml (IL-2; Roche, Switzerland)) and incubated as before. After 96 hours, 1.5 ml of supernatant was replaced with fresh 10% growth media and then incubated for a further 72 hours as before. Virus growth was monitored using an HIV-1 p24 assay (bioMérieux, France) according to manufacturer's instructions. Briefly, a stored sample (100 µl) from the initial day of infection was tested against a sample from the final day of virus expansion. Approximately 25 µl disruption buffer was added onto 100 µl samples/controls, gently mixed and incubated for 1 hour at 37 °C. Subsequently, the wells were aspirated and washed (x4) with PBS (300 µl) prior to addition of 100 µl conjugate solution (containing horseradish peroxidase labeled anti-p24 antibody), followed by a further 1 hour incubation at 37 °C. The experimental wells were washed as before, followed by the addition of 100 µl tetramethylbenzidine (TMB) substrate and 30 minutes incubation at room temperature. A stop solution was added and the assay was quantified using a micro-plate reader at a wavelength of 450 nm with a 620 nm reference

wavelength. All supernatants containing high p24 values (>50 ng/ml) were clarified by centrifugation (400 x g), and transferred into RPMI supplemented with 20% FBS and aliquots were stored at -80 °C.

2.2.3. Virus $TCID_{50}$ determination

PHA-stimulated PBMCs ($1.5x10^6$ cells/ml) were prepared and incubated at 37 °C, 5% CO_2 with humidity for 72 hours. Subsequently, 150 µl 10% growth medium was transferred to empty wells designated 4^{-3} to 4^{-8} (rows C to E, columns 4 to 9 on a 96 well round bottom plate). One aliquot of each of the 6 expanded viruses was thawed and diluted 1:12 in 10% growth media (supplemented with 5% IL-2) and added at 200 µl/well into empty wells designated 4^{-2} (columns 3 in rows C to E). Subsequently, serial dilutions (50 µl) were carried out from virus wells designated 4^{-2} into wells designated 4^{-3} until wells designated 4^{-8} (column 9), and the excess 50 µl virus after wells designated 4^{-8} was discarded accordingly. The already prepared PBMCs (PHA-P stimulated) were then seeded 50 µl/well in virus containing wells (4^{-2} to 4^{-8}), prior to incubation of the assay at 37 °C, 5% CO_2 with humidity for 96 hours. Cells were then resuspended and approximately 125 µl of resuspended cells were replaced with an equal volume of fresh 10% growth media; before further 72 hours of incubation as before. Virus concentrations were determined using the HIV p24 (bioMérieux, France) following manufacturer's instructions as before; and the $TCID_{50}$ was calculated according to the Spearman-Karber equation (Finney, 1952).

2.3. *In vitro* selection of INSTIs in primary HIV-1 grown in PBMC's

In vitro selection assays were carried out using the dose-escalation method adapted from protocols described previously (Kobayashi *et al*., 2011; Margot *et al*., 2012). *In vitro* selection assays were carried out using PHA-P stimulated PBMCs ($2x10^6$ cells/ml) isolated on day 1. Following PHA-P stimulation, PBMCs were infected with each of the six primary HIV-1 subtype C viruses (250 $TCID_{50}$) and incubated for an hour to allow virus-cell binding. All *in vitro* selection assays were carried out in 10% growth media supplemented with 5% IL-2, and the increasing concentrations of RAL (NIH AIDS Reagent Program, USA), EVG (Selleck Chemicals, USA) and DTG (Selleck Chemicals, USA) prepared (10 mg/ml) in dimethyl sulfoxide (DMSO; Sigma Aldrich, USA). Virus proliferation was monitored once weekly using an HIV-1 p24 antigen assay (bioMérieux, France) as before. Cultures were

maintained twice weekly by replacing half (1.5 ml) the supernatant with fresh 10% RPMI media; followed by replacement of half the cultures by feeding cells with PHA-P stimulated donor PBMCs. All subcultures included INSTIs at appropriate concentrations. Cultures that exhibited low level p24 antigen were maintained at an unchanged drug concentration, while cultures with high p24 levels (>50 ng/ml) had two to four fold increases in drug concentration. All harvested supernatants were clarified by centrifugation (400 x g), made up to 20% FBS and stored at -80 °C for subsequent use in the genotyping assays.

2.4. HIV ARV drug resistance genotyping

The protocol used for genotyping and sequencing of the entire *pol* gene was adapted from van Baelen *et al*., (2009), and the primers used were obtained from Genbank using their designated accession numbers.

2.4.1. Nucleic acid extraction

Supernatant from all virus samples was thawed and used to extract total RNA using the EasyMag extraction kit (bioMérieux, France) and the NucliSens EasyMAG automated instrument (bioMérieux, France) following manufacturer's instructions. Briefly, the input virus supernatant was 500 µl, which was mixed with appropriate volumes of lysis buffer and incubated at room temperature for 10 minutes. The lysed sample was subsequently transferred into a plastic vessel with 50 µl of silica beads, and RNA separation was carried out using automatic magnetic separation into 25 µl elution buffer.

2.4.2. Nested PCR

Table 2.2 below summarizes all forward and reverse primers used in the nested polymerase chain reaction (PCR) and Sanger-based DNA sequencing reactions.

Table 2.2: A summary of RT-PCR, PCR and sequencing primers used in the *pol* genotyping assays.

Primer	Sequence	Alignment	Function
PR F1	5'-CCC TCA AAT CAC TCT TTG GCA ACG AC-3'	Forward primer	RT-PCR
VIF R3	5'-CTC CTG TAT GCA GAC CCC AAT ATG-3'	Reverse primer	RT-PCR
PR F3	5'-GCT CTA TTA GAT ACA GGA GCA GAT G-3'	Forward primer	PCR
VIF R5	5'-GGG ATG TGT ACT TCT GAA CTT-3'	Reverse primers	PCR
F7	5'-GTA CTG GAT GTG GGT GAT GC-3'	Forward primer	Sequencing
F8	5'-GTG GGA AAA TTG AAT TGG G-3'	Forward primer	Sequencing
PR_F2376	5'-TGG AAA CCA AAA ATG ATA GG-3'	Forward primer	Sequencing
F3771	5'-GCC ACC TGG ATT CCT GAG TG-3'	Forward primer	Sequencing
IN_F_4074	5'-CAA CCA GAT AAA AGT GAA TCA G-3'	Forward primer	Sequencing
IN_F_4540	5'-TAG CAG GAA GAT GGC CAG T-3'	Forward primer	Sequencing
RT_R_3304	5'-TGT ATG TCA TTG ACA GTC C-3'	Reverse primer	Sequencing
R1	5'-CTC CCA CTC AGG AAT CC-3'	Reverse primer	Sequencing
RT_R_4150	5'-CTT TGT GTG CTG GTA CCC ATG-3'	Reverse primer	Sequencing
IN_R_4348	5'-CTC CTT TTA GCT GAC ATT TAT CAC-3'	Reverse primer	Sequencing
VIF_R_5193	5'ATG TGT ACT TCT GAA CTT-3'	Reverse primer	Sequencing
Inseq2R	5'-CTG CCA TTT GTA CTG CTG TC-3'	Reverse primer	Sequencing

Primers used were obtained from van Baelen *et al*., 2009.

Extracted viral RNA was used in RT-PCR to produce complementary DNA (cDNA) encoding the entire RT, RNAseH and IN genes (referred to as RT-IN, 2898 bp in HXB2, Gen-bank accession number K03455) (van Baelen *et al*., 2009). RT-Outer PCR was modified using a one-step Super-Script III RT/Platinum Taq High Fidelity (Invitrogen, USA) with primers PR F1 (0.2 µM) (positions 2252-2277 in HXB2) and VIF R3 (0.2 µM) (5243-5266) with the following thermal cycling conditions: reverse transcription for 30 minutes at 56 °C, followed by outer amplification for 2 minutes at 94 °C; 30 cycles of 15 seconds at 92 °C, 30 seconds at 62 °C and 3 minutes and 30 seconds at 68 °C; and a final elongation for 10 minutes at 68 °C. The outer PCR was carried out with 2 µl cDNA product using the Expand High Fidelity Polymerase kit (Roche Diagnostics, Germany) following the manufacturer's instructions. Briefly, primers used in the assay were 0.304 µM VIF R5 (5193-5213) and 0.304 µM PR F3 (2316-2340) with the following thermal cycling conditions: 2 minutes at 94

°C; 35 cycles of 15 seconds at 94 °C, 30 seconds at 60 °C and 3 minutes at 68 °C; and a final elongation for 10 minutes at 68 °C (van Baelen *et al*., 2009).

2.4.3. DNA purification, cycle sequencing and DNA sequencing

Amplicons were purified using the QiaQuick PCR purification kit (Qiagen, USA) following the manufacturer's instructions. Briefly, 500 µl of Buffer BP was added to 100 µl PCR product and the mixture was transferred onto a QIAquick spin column supported by a 2 ml collection tube. The samples were centrifuged at 17 900 x g for 60 seconds, and subsequently the flow-through was discarded. Approximately 750 µl Buffer PE was added to the column and the sample was centrifuged as before. To remove residual ethanol from Buffer PE an additional centrifugation into an empty QIAquick tube was carried out for 60 seconds, and subsequently the column was transferred onto a sterile collection tube. Purified DNA was eluted with 25 µl elution buffer in a sterile 1.5 ml micro-centrifuge tube, prior to analysis using 2% agarose gel analysis stained with 5 µg/ml ethidium bromide (Bio-Rad, USA) and visualized on a Chemidoc (Bio-Rad, USA) system.

2.4.3.1. DNA sequencing and analysis

Cycle sequencing was carried out using the BigDye Terminator cycle sequencing kit (including the relevant sequencing primers listed in Table 2.2) according to manufacturer's instructions (Applied Biosystems, USA). The thermal cycling conditions for cycle sequencing were 1 minute at 96 °C; 25 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C and 4 minutes at 60 °C; and a final elongation for 10 minutes at 72 °C. The sequencing reactions were then cleaned as per manufacturer's instructions. The final sequencing reactions involved addition of 10 µl HIDI™ Formamide (Applied Biosystems, USA) and the amplicons were sequenced using the ABI3730*xl* automated sequencer overnight (Applied Biosystems, USA). All sequencing data was analysed using the Sequencher® version 4.8 (Gene Codes Corporation, USA) sequence analysis program. Nucleotide sequences were aligned in Clustal X 2.0 (Thompson *et al*., 1997), and converted to amino acids in GeneDoc 2.7 (Informer Technologies, USA). The nucleotide and amino acid sequence alignment was extensively analysed for the presence of any mutations associated with resistance to known INIs, as well as RTI and PI (http://hivdb.stanford.edu).

The INSTI mutations listed in the Stanford University: HIV Drug Resistance Database (http://hivdb.stanford.edu/DR/INIResiNote.html) that were relevant for each inhibitor used in this study were:

RAL - T66A/K, L68V/I, L74M, E92Q, Q95K, T97A, F121Y, E138K/A, G140S/A/C, Y143C/R/H, Q148H/K/R, N155H/S, E157Q, G163R/K.
EVG - H51Y, T66A/I/K, L68V/I, E92Q, Q95K, T97A, H114Y, F121Y, E138K/A, G140S/A/C, P145S, Q146P, S147G, Q148H/K/R, S153Y, N155H/S, E157Q, R263K.
DTG - E92Q, E138K/A, G140S/A, Q148H/R/K.

2.4.4. Sequencing using the Illumina MiSeq

In order to detect minority variants or quasispecies, an aliquot of the DNA amplicons obtained in section 2.4.2 were also sequenced on the MiSeq instrument (Illumina, USA). The DNA was quantified using the Qubit® dsDNA BR quantification kit and a Qubit® 2.0 fluorimeter (Life Technologies, USA). Libraries were prepared using the Nextera® XT DNA sample preparation kit in accordance with manufacturer's instructions (Illumina Nextera® XT DNA sample preparation guide, 2012). Briefly, Tagment DNA buffer was transferred into the Nextera XT tagment amplicon plate (NTA; Illumina, USA) at 10 µl/well; and 5 µl input DNA was subsequently added at 0.2 ng/ul (per well) and mixed gently. Amplicon tagment mix (Illumina, USA) was subsequently added at 5 µl/well and mixed as before. The NTA plate was sealed and centrifuged (280 x g at 20 °C for 1 minute), and subsequently incubated (55 °C for 5 minutes, hold at 10 °C) in a thermal cycler. Neutralize Tagment buffer (Illumina, USA) was subsequently added 5 µl/ sample and were mixed gently, and then centrifuged as before. The NTA plate was incubated at room temperature for 5 minutes, and subsequently 1µl of sample was assessed for tagmentation using the HS bioanalyzer chip (Illumina, USA). For PCR amplifications, a 15 µl/well Nextera PCR master mix (Illumina, USA) was added to each column on the experimental NTA plate, followed by 5 µl/well (to each row) of index primers (i7 and i5) and mixed gently. The experimental NTA plate was then sealed and centrifuged as before. The plate was subsequently placed in a thermocycler and PCR was carried out under the following conditions: 72 °C for 3 minutes, 95 °C for 30 seconds, 12 cycles of: (95 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds), 72 °C for 5 minutes, and a final hold at 10 °C.

A PCR clean-up was carried by centrifugation (280 x g for 1 minute at 20 °C), and subsequently the PCR product was transferred at 50 µl/well into a Clean amplified plate (CAA; Illumina, USA). AMPure XP beads were vortexed and added (30 µl/well), mixed and then incubated at room temperature for 5 minutes. The experimental CAA plate was then placed on a magnetic stand and beads were washed (x2) with 200 µl of 80% ethanol before air drying on the bench for 15 minutes. Resuspension buffer was added at 52.5 µl/well, mixed and incubated for 2 minutes at room temperature. The CAA plate was placed back onto a magnetic stand for another 2 minutes, and subsequently 50 µl of supernatant was transferred onto a sterile Clean amplified NTA plate (CAN). Thereafter, 20 µl of the supernatant was transferred into a MIDI 96-well Library normalisation plate (LNP; Illumina, USA), prior to addition of the Library normalisation additives (LNA1; Illumina, USA)/Library normalisation beads (LNB1; Illumina, USA) mix (45 µl/well). The LNP plate was then kept on a microplate shaker at 1800 rpm for 30 minutes before it was positioned onto a magnetic stand for 2 minutes/until the supernatant was clear. Subsequently the supernatant was gently removed and discarded; and the wells were washed (x2) using Library normalisation wash 1 (45 µl; with shaking at 1800 rpm for 5 minutes; and magnetic stand as before). A 30 µl 0.1 N NaOH solution was added to the beads to elute the sample and incubated at room temperature (with shaking 1800 rpm for 5 minutes). An internal control was prepared by thoroughly mixing (vortexed and centrifuged at 280 × g for 1 minute) 5 µl of 4 nM PhiX library with 5 µl of 0.2 M NaOH and incubated (denatures the PhiX library) at room temperature for 5 minutes. The PhiX library (10 µl) was added to a pre-chilled HT1 (990 µl). Subsequently, a Library normalisation storage buffer was added at 30 µl to the samples which were sealed and centrifuged to 112 x g for 1 minute before the all samples were pooled (including the control PhiX library) and diluted with 571 µl Hybridization buffer (Illumina, USA) and loaded onto a Miseq reagent cartridge kit version 2 (Illumina, USA), subjected to pair end 2×250 base-pair reads (maximum output of 7.5) and sequenced according to the Miseq System User Guide (part # 15027617).

2.5. *In silico* analysis of the major *in vitro* selected mutations

A three-dimensional docking model of the HIV-1 IN catalytic core domain was prepared based on PDB entry 1QS4 (Goldgur *et al.*, 1999) as a starting point (Accelrys Discovery Studio Version 3.5). This structure includes three identical chains of the HIV-1 IN catalytic

core, determined at a resolution of 2.1 Å. All water molecules as well as chains B and C were removed and an unresolved loop region (residues 141 – 144) was built onto the existing chain A. The second magnesium ion was placed in the active site based on the closely related two-metal active site of Tn5 transposase (PDB entry 1MM8). The protein structure of the monomeric catalytic core domain was prepared using the Protein Preparation tool in Accelrys Discovery Studio, and all hydrogen atoms were added. The protonation state and side-chain orientation of all residues were optimised for hydrogen bonding. The molecule was typed with the CHARMm forcefield and minimised using the smart minimiser protocol in DS4.0 with a maximum of 5000 steps to an energy change of 0.0 kJ. Quality indication of the protein model was verified using Ramachandran plots, verify modeller and verify score. Point mutations were manually introduced to reflect experimental results and literature reports.

2.6. Mutant and WT enzyme generation

Attempts were made to expand the viral isolates that harboured antiretroviral drug resistance mutations in PBMCs, but very low p24 antigen levels were routinely obtained following storage at -80 °C. This was indicative of some replicative fitness cost associated with these mutations. Thus, the decision was made to express, purify and characterize recombinant IN harbouring the relevant mutations.

2.6.1. Site-directed mutagenesis, plasmid preparation and sequencing

A pET-15b vector (Novagen, Germany) which encodes the full-length $FV6^{WT}$ integrase gene (HIV-1 subtype C 05ZAFV6) (Fish *et al.*, 2010) was available for use in this study. The $FV6^{WT}$ recombinant plasmid was used as a template to introduce relevant mutations identified in sections 2.4.3.1 above. Table 2.3 below summarizes all forward and reverse primers used in site-directed mutagenesis assays for induction of the HIV-1 IN mutants.

Table 2.3: Primers used in site-directed mutagenesis of the integrase gene.

Mutation	Sequence	Alignment	TM*
N155H	5'-GAGTAGTAGAATCTATGCATAAAGAATTAAAG-3'	Forward primer	60.81
	5'-CTTTAATTCTTTATGCATAGATTCTACTACTC-3'	Reverse primer	
T66I	5'-GCAATTAGATTGTATACATTTAGAAGGAAAAG-3'	Forward primer	60.81
	5'-CTTTTCCTTCTAAATGTATACAATCTAATTGC-3'	Reverse primers	
Q148R	5'-AATCCCCAAAGTCGGGGAGTAGTAGAATC-3'	Forward primer	67.45
	5'-GATTCTACTACTCCCCGACTTTGGGGATT-3'	Reverse primer	
F121Y	5'-GATAATGGCAGTAATTACACCAGTGCTGCAG-3'	Forward primer	67.29
	5'-CTGCAGCACTGGTGTAATTACTGCCATTATC-3'	Reverse primers	
R263K	5'-CATAAAAGTAGTACCAAGAAAGAAAGCAAAAATC-3'	Forward primer	62.25
	5'-GATTTTTGCTTTCTTTCTTGGTACTACTTTTATG-3'	Reverse primer	
E92Q	5'-CAGAAGTTATCCCAGCACAAACAGGACAAGAAAC-3'	Forward primer	68.28
	5'-GTTTCTTGTCCTGTTTGTGCTGGGATAACTTCTG-3'	Reverse primers	
H114L	5'-GGCCAGTCAAAGTAATACTTACAGATAATGGCAG-3'	Forward primer	67.34
	5'-CTGCCATTATCTGTAAGTATTACTTTGACTGGCC-3'	Reverse primers	

- Desired mutation; *TM- melting temperature

Mutagenesis was carried out using the QuikChange Lightning Site-Directed Mutagenesis kit according to manufacturer's instructions (Agilent Technologies, USA). Briefly, sample reactions were set up using 5 µl of 10× reaction buffer, 1 µl (100 ng) of double-stranded (ds) DNA template, 1.25 µl (125 ng) of each reverse and forward primer (shown in Table 2.3), 1 µl of dNTP mix, 1.5 µl of QuickSolution reagent, 39 µl of high purity water and then 1 µl of QuickChange® Lightning enzyme was added. The reactions were thermocycled using the following conditions: 2 minutes at 95 °C, 18 cycles of 20 seconds at 95 °C, 10 seconds at primer melting temperature 60 °C (dependent on GC content, Table 2.3), 30 seconds at 68 °C; and a final elongation for 5 minutes at 68 °C. Subsequently, 2 µl of *Dpn*I restriction enzyme (Agilent Technologies, USA) was added directly to each reaction, mixed, spun down and incubated at 37 °C for 5 minutes to digest the parental dsDNA.

Plasmids containing mutated cDNA sequences were then transformed into Stellar™ competent *Escherichia (E.) coli* cells (Clontech Laboratories, USA). Briefly, 50 µl of the competent bacterial cells were gently mixed with 5µl of site-directed mutagenesis DNA product (1-100 ng) and incubated on ice for 30 minutes in a sterile Eppendorf tube. Subsequently the cells were heat shocked at 42 °C for exactly 30 seconds, and thereafter incubated again on ice for 2 minutes. Approximately 250 µl of pre-warmed (37 °C) SOC media (without antibiotics) (Clontech Laboratories, USA) was added to the competent cells, followed by 1 hour of incubation at 37 °C with shaking (250 rpm). The transformed cells (20 µl) were then incubated overnight (12-16 hours) at 37 °C on Luria Bertani (LB) agar (Laboratorios Conda, Spain) plates supplemented with 100 µg/ml ampicillin (Melford Laboratories Ltd, UK). Subsequently, one colony was inoculated into LB Broth (Laboratorios Conda, Spain) supplemented with 100 µg/ml ampicillin and incubated for another 12-16 hours with shaking at 250 rpm.

Plasmid DNA purification was carried out using a kit (Machenery-Nagel GmbH & Co. KG, Germany) according to manufacturer's instructions. Briefly, 400 ml transformed bacterial cells were cultivated and harvested by centrifugation at 11000 x g for 30 seconds. The bacterial cells were lysed by resuspension of the pellet in 250 µl suspension buffer, followed by addition of 250 µl lysis buffer. The lysate was incubated for 5 minutes at room temperature before 300 µl of neutralization buffer was added. The lysate was clarified using centrifugation at 11000 x g for 10 minutes, and the supernatant was loaded onto a NucleoSpin® Plasmid column supported by a collection tube. The DNA was bound onto the column by centrifugation at 11000 x g for 1 minute and flow through was discarded, before a wash with 500 µl buffer AW followed by a wash with 600 µl buffer A4 (supplemented with 80% ethanol) at 11000 x g for 1 minute each. The dry silica membrane binding desired DNA was air dried at 11000 x g for 2 minutes, prior to elution (50µl elution buffer) by centrifugation at 11000 x g for 1 minute. Plasmid DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) at a wavelength of 260 nm, and a known concentration of each mutated plasmid was sequenced using the Universal T7 promoter and the T7 terminator primers at Inqaba Biotech, South Africa.

2.6.2. Recombinant protein expression

Competent *E.coli* BL21 (DE3) pLyS cells (Lucigen, USA) were transformed using the extracted recombinant plasmids as before (refer to section 2.5.1). The transformed cells (20 µl) were then incubated overnight (12-16 hours) at 37°C °C on LB agar (Laboratorios Conda, Spain) plates supplemented with antibiotic (100 ug/ml ampicillin and 100ug/ml chloramphenicol; Sigma Aldrich, USA). Overnight recombinant *E. coli* BL21 (DE3) cultures of wild-type and INSTI mutants were each diluted 100x into 400 ml LB broth (supplemented with 100 µg/ml ampicillin) and grown to log phase (OD reading: 0.4 - 0.6 at a 600 nm wavelength) by incubation at 37 °C for 2-3 hours with shaking (250 rpm). Subsequently, the IN overexpression was induced by addition of 400 µl of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich, USA), and the bacterial cultures were incubated as before for 3 hours. Bacterial cultures were then centrifuged at 1200 x g for 20 minutes at 4 °C; supernatant was discarded and pellets were stored at -20 °C overnight. Samples from before over-expression and samples obtained following over-expression were ran on the Mini-Protean® TGX Stain-Free™ Precast Gels (Bio-Rad, USA) and visualized on the ChemiDoc MP System (Bio-Rad, USA).

2.6.3. Nickel-chelating column chromatography

Purification of all the recombinant wild-type and mutant IN proteins was carried out using Nickel Chelating Column Chromatography. The nickel-bound resin (Macherey Nagel, Germany) was recharged with 2.5 ml of 0.1 M $NiSO_4$ (Sigma Aldrich, USA) and equilibrated with 10 column volumes of binding buffer (20 mM HEPES (pH 7.2; Sigma Aldrich, USA), 1 M NaCl (Sigma Aldrich, USA), 5 mM Imidazole (Sigma Aldrich, USA), 10% glycerol (Melford, UK), 0.05% Igepal (Sigma Aldrich, USA)) on the Acta Prime plus (GE Healthcare, UK). The frozen BL21 (pLys) pellets were resuspended in 50 ml lysis buffer (20 mM HEPES (pH 7.2), 1 M NaCl, 5 mM Imidazole, 10 mM $MgCl_2$ (Sigma Aldrich, USA), 0.25% CHAPS, 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma Aldrich, USA) and 10 ul DNase 1 (Sigma Aldrich, USA)), homogenised and incubated on ice for 30 minutes with gentle stirring. The Labsonic® M sonicator (Sartorius Stedim, Germany) was used (75% at 0.6 cycles) to disrupt cells (1 minute/sonication). The bacterial lysate was subjected to centrifugations at 15000 x g at 4 °C for 30 minutes, and supernatant was pooled into 50 ml tubes. The 50 ml supernatant was then loaded onto the super loop (washed with 50 ml binding buffer) and bound at a 1 ml/minute flow-rate with a 0.3 MPa maximum pressure.

Subsequently, the bound protein was washed with 5 column volumes of binding buffer, then washed with 10 column volumes of wash buffer 1 (20 mM HEPES (pH 7.2), 1 M NaCl, 60 mM Imidazole, 10% glycerol, 0.05% Igepal) followed by 10 column volumes of wash buffer 2 (20 mM HEPES (pH 7.2), 1 M NaCl, 150 mM Imidazole, 10% glycerol, 0.05% Igepal).

A gradient of 75 ml at 100% elution buffer (20 mM HEPES (pH 7.2), 1 M NaCl, 600 mM Imidazole, 10% glycerol, 0.05% Igepal) final was then set; with 3 ml fraction sizes. Collected fractions were concentrated using ultrafiltration membrane (10 000 Dalton, Dia: 44.5 mm, cellulose) (Millipore, USA) under nitrogen pressure. An equilibrated PD-10 column (GE Healthcare, UK) was used for buffer exchange in which the IN concentrate was transferred into 25 ml storage buffer (20 mM HEPES (pH 7.5), 1 M NaCl, 0.1 mM EDTA, 1 mM DTT (Melford, UK), 10% Glycerol). The collected flow-through was measured for protein on the NanoDrop at a wavelength of 280 nm and Beer-Lambert law was used to calculate the IN concentration.. Purified IN was aliquoted, snap-frozen in liquid nitrogen and stored in -70 °C.

2.7. Polyacrylamide gel electrophoresis (PAGE) and Western-blot analysis

The HIV-1 IN protein was verified using PAGE and Western-blot analysis. Briefly, 4-15% tris-glycine precast stain-free gels (Bio-Rad, USA) were used and the target IN enzyme was identified at 32 kDa using the ChemiDoc MP system (Bio-Rad, USA). The gels were then incubated in gel transfer buffer (20% methanol, 80% high purity water) for fifteen minutes. An iBlot gel transfer stack (Life Technologies, USA) was used to transfer protein onto a polyvinylidene difluoride (PVDF; Life Technologies, USA) membrane in accordance with manufacturer's instructions. An automated Western-blot assay was carried out using the Benchpro 4100 (Life Technologies, USA). Briefly, the PVDF membrane was incubated in blocking buffer (420 mM Tris pH 7.6, 137 mM NaCl, 5% BSA and 0.1% Tween 20), and subsequently washed in Tris Buffered Saline with Tween® 20 (TBST-1x) (420 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween 20) prior to incubation with anti-His.H8 (Abcam, UK) primary antibodies) (1:1000) for ninety minutes. The membrane was washed again as before, and subsequently incubated for an hour with secondary rabbit anti-mouse horseradish peroxidase linked IgG secondary antibody (1:2000; KPL, USA). HIV-1 IN detection was carried out on the ChemiDoc using the Supersignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, USA) following manufacturer's instructions.

2.8. HIV-1 strand transfer assay

The direct HIV-1 IN strand transfer assay was adapted from Mphahlele *et al.*, 2012, and carried out to determine the strand transfer capacity of HIV-1 subtype C mutants with respect to the WT HIV-1 subtype C enzyme. Donor (d) DNA (150 nM; biotin 5'-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA-3'/ 5'-ACTGCTAGAGATTTTCCACACTGACTAAAAG-3'; Inqaba Biotech Pretoria, South Africa) was prepared in Tris-buffer-A (10 mM Tris-HCl, 0.1 M NaCl; pH 7.2) and transferred into streptavidin coated microplate (MP) at 100 µl/well and subsequently incubated at 25 °C (with shaking 50 rpm) for an hour. Therefter, the wells were washed with working solution of PBS (250 µl). HIV-1 IN (2 µM) enzyme was prepared in reaction-buffer (20 mM HEPES, 30 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.05% Igepal; pH 7.5) (Chow, 1997) and transferred to 100 µl/well; the assay was then incubated for thirty minutes as before. Subsequently, the wells were washed twice with 250 µl reaction buffer, and approximately 90 µl reaction buffer was added to wells prior to a thirty minute incubation at 37 °C. About 2.5 µM target DNA (5'-TGACCAAGGGCTAATTCACT-3' Fluorescein and 5'-AGTGAATTAGCCCTTGGTCA-3' Fluorescein; Inqaba Biotech, Pretoria, South Africa) was prepared in reaction buffer and added to assay 10 µl/well; and the assay was incubated for an hour at 37 °C. The assay was then washed with 250 µl working solution of saline-sodium citrate (SSC) buffer (0.03 M sodium citrate, 0.3 M NaCl; pH 7.0), and subsequently monoclonal anti-FITC alkaline phosphatase (1:10 000) (Sigma Aldrich, USA) prepared in Tris-buffer-B (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20; pH 7.4) was added 200 µl/well. The assay was incubated for two hours at 25 °C and subsequently washed with Tris-buffer-B (without 0.05% Tween 20). A FirePhos™ Microwell Phosphate substrate system was prepared in accordance with manufacturer's instructions (KPL, USA) and added 100 µl/well. The assay was then incubated for thirty minutes at 37 °C, stopped and quantified at a wavelength of 505 nm on the xMARK™ (Bio-Rad, USA).

Although IN function in a multimeric form is stabilized by zinc, it was not included in the assay reagents described above due to the fact that the use of the metal co-factor manganese has been shown to be adequate (Lee and Han, 1996).

2.9. Binding kinetic analysis of W/T and mutant IN DNA and INSTI binding

The kinetics of the unlabelled pre-processed (3'-end artificially cleaved) dDNA binding to the HIV-1 subtype C IN mutants were tested with reference to the WT subtype C IN enzyme using surface plasmon resonance (SPR). All SPR experiments were carried out using the ProteOn^{TM} XPR36-Protein Interaction Array System (Bio-Rad, USA) at 25 °C. For direct IN-DNA binding kinetics, an HTG (a nitriloacetic acid (NTA)-linked) chip (Bio-Rad, USA) was preconditioned using 0.5% SDS, 50 mM NaOH, 100 mM HCl and 0.3 M EDTA (pH 8.5) and subsequently the HTG chip was charged with 0.5 M $NiSO_4$. The WT and WT- IN mutants were prepared (0.6 mg/ml) in Tris buffer (50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.005% Tween 20; pH 7.6) and immobilized on separate flow cells within the same sensor HTG chip (5 proteins immobilised per kinetic experiment) using a flow rate of 25 µl/min to a final response unit (RU) of between 250 and 4560 (final RU reached: IN-W/T 1300 RU, N155H 4560 RU, T66I 1944 RU, Q148R 630 RU, R263K 1100 RU, F121Y 250RU, H114L 1300 RU and E92Q 720 RU). The RU signal was calculated as the difference between immobilised protein and the corresponding $NiSO_4$ channel surface. Protein immobilization using HTG occurs through bio-molecular interactions that involve binding of the IN-histidine tag to the nickel (II) sulphate surface. IN-dDNA interactions were initiated by flowing (100 µl/min) dDNA at concentrations of 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM for T66I, N155H and R263K and 5 µM, 2.5 µM, 1.25 µM, 0.625 µM and 0.31255 µM for W/T, E92Q, H114L, F121Y and Q148R. Buffer injections and control surface (0.5 M $NiSO_4$ only surface) interactions were subtracted for all data in all binding experiments. All data was fitted to a Langmuir 1:1 binding algorithm using the ProteOn Manager Software (Version 3.1.0.6; Bio-Rad, USA).

2.10. Statistical analysis

The GraphPad software QuickCalcs (GraphPad Software Inc, USA) - t test calculator (available online at: http://graphpad.com/quickcalcs/ttest1.cfm?Format=SD) was used to determine the p-values for the HIV-1 subtype C mutant strand transfer capacity results.

CHAPTER 3: RESULTS

3.1. *In vitro* selection and virus break-through

All six selected primary HIV-1 subtype C isolates were successfully expanded in PHA-stimulated PBMCs, their $TCID_{50}$ established, and appropriate concentrations used in the subsequent *in vitro* selection assays. The *in vitro* selection assays were carried out using a dose-escalation method in the presence of RAL, EVG and DTG, and drug concentrations were gradually increased depending on viral proliferation, which was monitored using an HIV-1 p24 antigen assay. Subjecting HIV-1 to increasing INSTI drug concentrations often led to pressure on viral replication (decrease in p24 antigen detected), and drug concentrations were maintained until there was recovery of viral proliferation. For example, for a period of 16 days a reduction in p24 levels was observed with virus isolates MR69 (Figure 3.1A) and FV6 (Figure 3.1B) under EVG pressure; and subsequent recovery in p24 levels from day 56 onwards. A similar reduction in HIV-1 subtype C levels under RAL pressure was observed from day 32 (Figure 3.1A) and 48 (Figure 3.1B) with MR69 and FV6, respectively.

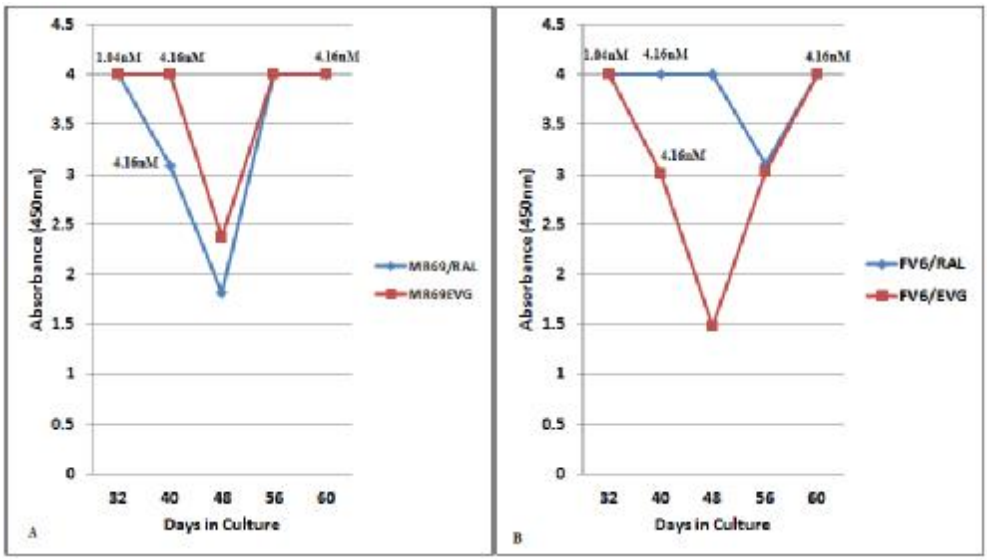


Figure 3.1: Graphical representation of the HIV-1 subtype C MR (A) and FV (B) viruses during *in vitro* selection and virus break-through. Virus break-through during selection was defined through a reduction followed by an increase of HIV-1 p24 antigen levels in the culture supernatant over a period of time.

Increases in INSTI drug concentrations were adjusted appropriately to permit the emergence of resistant variants. The FV and MR viruses replicated equally well in the presence of increasing concentration of RAL, but the presence of pre-existing NRTI and/or NNRTI mutations (in the 3 MR viruses) appeared to delay the INSTI dose-escalation for EVG and DTG (Figure 3.2).

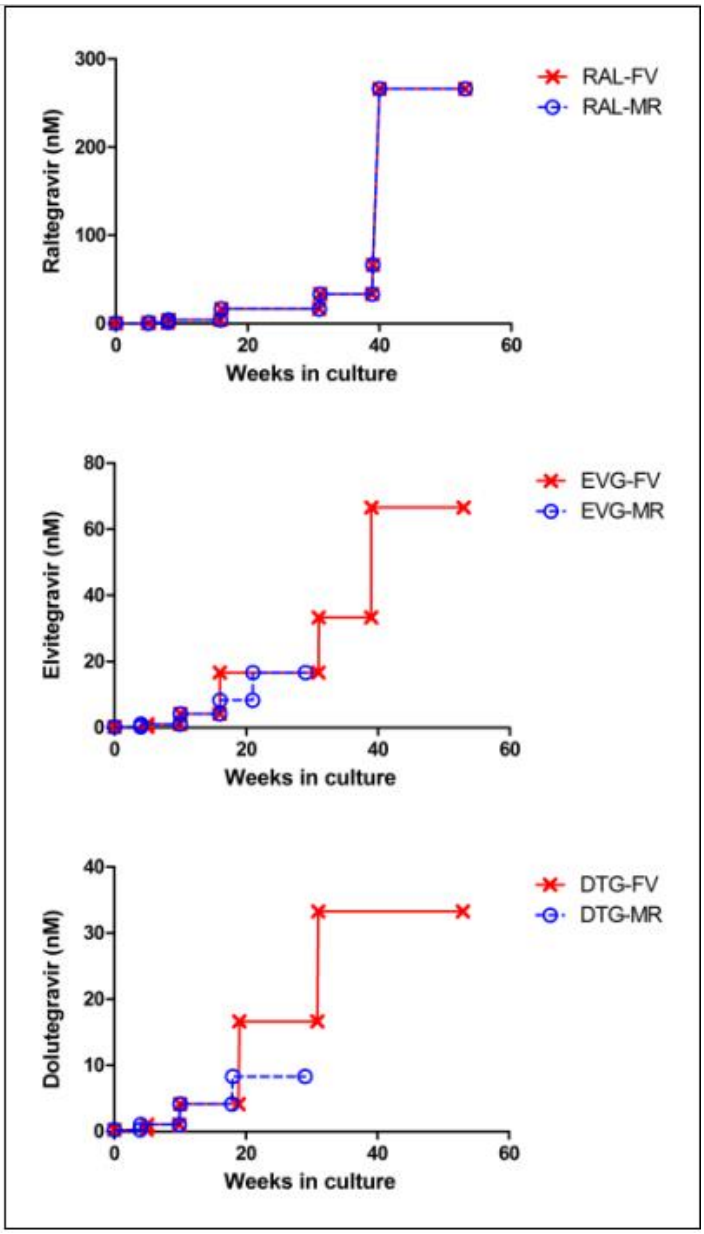


Figure 3.2: Dose-escalation curves for the *in vitro* selection studies with RAL, EVG and DTG in the HIV-1 subtype C FV (antiretroviral drug naïve) and MR (reverse transcriptase inhibitor resistant).

3.2. Genotyping

3.2.1. Sanger-based sequencing

The acquisition of INSTI mutations by primary HIV-1 subtype C isolates during the *in vitro* selection studies with RAL, EVG and DTG was confirmed by population-based sequencing with baseline HIV-1 isolates as reference (Table 3.1). A summary of the major primary INSTI resistance mutations, major accessory mutations and rare primary INSTI resistance mutations is presented in Table 3.2.

Under RAL pressure, the primary virus isolates FV6, MR69 and MR89 developed the major drug resistance mutations Q148R, N155H and N155H, respectively (Table 3.2). *In vitro* selection with EVG induced major INSTI resistance mutations T66I, T66I and T66K in virus isolates in FV6, MR69 and MR81, respectively. In addition, the rare primary INSTI-resistance mutation F121Y and the major INSTI accessory mutation R263K emerged with EVG pressure in FV3 and FV6, respectively (Table 3.2). Drug resistance-associated mutation E92Q emerged under DTG pressure as a primary mutation in virus isolate FV3 (Table 3.2), in combination with mutations V31LV/A38AP/T93K/D116N/S119C/K136Q. Additionally, the rare primary drug resistance-associated mutation M50I was selected in virus isolate MR81 (Table 3.2). Naturally occurring polymorphisms believed to be associated with DTG resistance at positions 101, 124 and 201, were observed from baseline primary HIV-1 subtype C in all virus isolates.

The pre-existing reverse transcriptase mutations in virus isolates MR69, MR81 and MR89 remained unchanged during culture.

Table 3.1: Baseline IN sequences of primary HIV-1 isolates selected.

HIV-1 subtype C isolates	Sequences
FV3	D25E, L101I, T112V, T124A, T125A, K136Q, Q177L, V201I, T218I, L234I, A265V, R269K, D278A, S283G
FV5	K7R, E11D, D25E, F100Q, L101I, K111T, T112V, T124A, T125A, K136Q, D167E, V201I, K215N, L234I, D278A, R284G, D286N
FV6	D25E, V31I, M50T, I72V, F100Y, L101I, T112V, T124A, T125A, K136Q, V201I, T218I, L234I, R269K, D278A, S283G, R284G, D286N
MR69	S17N, D25E,V31I, M50T, I72V, Y99X, F100Y, L101I, T122V, T124A, T125A, K136Q, D167E, V201I, T218I, Q221S, L234I, D278A, S283G
MR81	D25E,V31I, M50T, F100Y, L101I, T122V, T124A, T125A, K136Q, V201I, T218I, L234I, D278A, S283G
MR89	S17N, D25E,V31I, M50I, I84M, L101I, T122V, T124A, T125A, K136Q, V201I, T218I, L234I, A265V, R269K, M275V, D278A, S283G

Table 3.2: Major antiretroviral drug resistance mutations that emerged in the HIV-1 subtype C primary virus isolates during *in vitro* selection cultures with increasing concentrations of RAL, EVG and DTG. The major mutations are highlighted in bold red.

Integrase Inhibitor **Virus isolate**		**RAL**	**EVG**	**DTG**
FV3	Baseline	No DRM[¥]		
	Passage (P) sequenced	No DRM (P44)	**INSTI:** F121Y* (P44)	**INSTI: E92Q** (P44)
FV5	Baseline	No DRM		
	Passage sequenced	**INSTI:** No DRM (P44)	**INSTI:** No DRM (P44)	**INSTI:** No DRM (P39)
FV6	Baseline	No DRM		
	Passage sequenced	**INSTI: Q148R** (P44)	**INSTI: T66I**, R263K** (P44)	**INSTI:** ND***
MR69	Baseline	**NNRTI:** K103N		
	Passage sequenced	**NNRTI:** K103N **INSTI: N155H,** H114L (P39)	**NNRTI:** K103N **INSTI: T66I** (P44)	**NNRTI:** K103N **INSTI:** ND
MR81	Baseline	**NRTI:** M184V **NNRTI:** K103N, K238N		
	Passage sequenced	**NRTI:** M184V **NNRTI:** K103N, K238N **INSTI:** ND	**NRTI:** M184V **NNRTI:** K103N, K238N **INSTI: T66K** (P44)	**NRTI:** M184V **NNRTI:** K103N, K238N **INSTI:** M50I[#]
MR89	Baseline	**NRTI:** M184V **NNRTI:** K103N		
	Passage sequenced	**NRTI:** M184V **NNRTI:** K103N **INSTI: N155H/**M50I (P44)	**NRTI:** M184V **NNRTI:** K103N **INSTI:** ND	**NRTI:** M184V **NNRTI:** K103N **INSTI:** ND

[¥]DRM, drug resistance mutations; *F121Y is a rare primary INI-resistance mutation; **R263K is a major INSTI accessory mutation; [#] M50I is an accessory mutation; ***ND, Not Done, no amplification attributed to low p24 antigen levels in culture supernatant. K103N-is a major NNRTI resistance mutation; M184V-is a major NRTI resistance mutation; K238N-is an accessory mutation associated with NNRTI/NRTI resistance.

3.2.2. High through-put sequencing: Illumina MiSeq

High through-put sequencing results of the longitudinal culture samples allowed us to track the dynamic evolution of the quasispecies in response to the drug selection pressure. Quasispecies that were present at ≥1% of the variants were included in the analyses, and the prevalence of each mutation (in percentages) is shown in Figure 3.3 and Tables 3.3 to 3.5.

Viruses FV3 and FV5 adapted to grow in the presence of up to 266.32 nM of RAL without the emergence of INI drug resistance mutations (Table 3.3). The FV6 virus introduced a mutation at position 148 (Q148R) which was detected at 1% of the quasispecies population as early as P15 (4.16 nM RAL), and was the predominant population (97%) by P33 (33.28 nM). Similarly, the N155H/H114L and N155H mutations emerged in viruses MR69 and MR89 respectively. Q148R and N155H confer major resistance to RAL.

Under EVG pressure, all six viruses developed INI drug resistance mutations at varying proportions (Table 3.4). F121Y was detected in FV3, whereas T66I/K was detected in FV3, FV6, MR81 and MR89 as the predominant quasispecies by the end of the study. Additionally, the major accessory mutation R263K emerged (22% at P39) in FV6. Q148K was detected at 31% of the total viral population by passage 27. F121Y, T66I/K and Q148K confer high level resistance to EVG.

No major INI resistance mutations were detected in proportions greater that 20% in the viral cultures grown in the presence of DTG (Table 3.5). The V151L mutation was detected in FV3 (16% at P39), and is associated with low-level resistance to DTG. The G163K mutation was present in MR69 at proportions of 17%, but does not alter the susceptibility of HIV-1 to DTG. Low proportions of mutations were detected in MR81, with G140A (7%, which confers potential low level resistance to DTG), P145S (8%, virus is susceptible to DTG, but confers high level resistance to EVG) and Q148K (9%, low level resistance to DTG, but high level resistance to EVG and RAL).

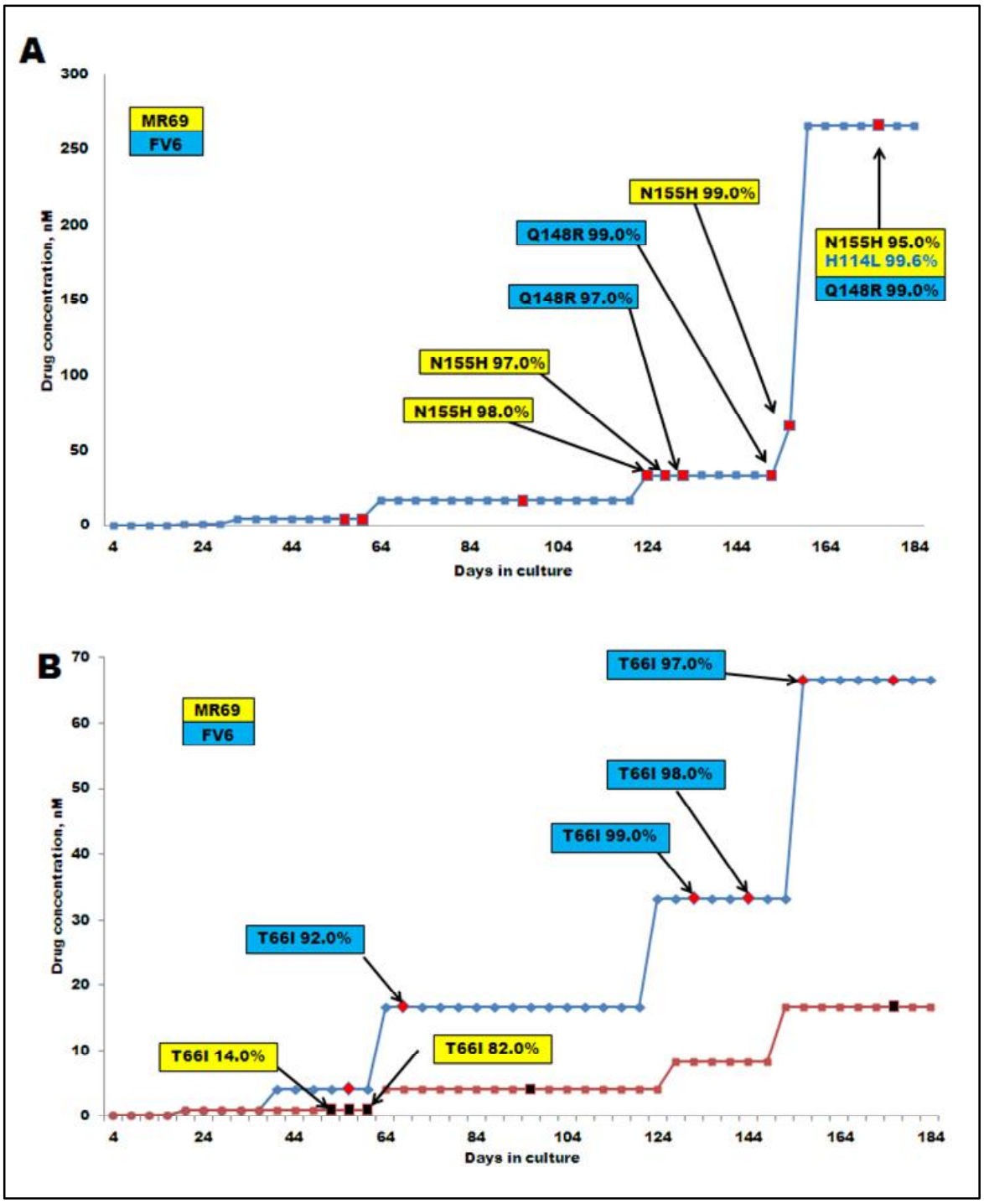


Figure 3.3: The *in vitro* selection process showing dose-escalation concentrations of RAL (A) and EVG (B) in PBMCs infected with primary virus isolates FV6 and MR69. The resulting INSTI mutations found in the depicted virus during the passages are shown in the matched colour boxes. Points highlighted in red represent the passages that were sequenced.

Table 3.3: Resistance mutations as selected by RAL and identified using NexGen sequencing.

	T66I	L74I	L74M	E92V	Q95K	T97A	E138K	Y143H	Y143K	Y143R	Y143S	Q146P	S147G	Q148H	Q148K	Q148R	V151A	V151L	S153F	S153Y	N155H	N155S	E157Q	G163R	S230R
FV3 RAL P24											1		1												
FV6 RAL P15	1		2													1									1
FV6 RAL P24			5											1		3	1								
FV6 RAL P33					2											97		1							
FV6 RAL P38																99									
FV6 RAL P44			1				4									99									
MR69 RAL P14															1		1		1					2	
MR69 RAL P24			1				4								1	96				1					1
MR69 RAL P31														1		1					98			1	
MR69 RAL P32					2											1					97			1	
MR69 RAL P39			1										3	1	1						99			2	1
MR69 RAL P44	1		1		5	1	1						1	1	1			1		1	95			1	
MR81 RAL P13		3			12		2		2	1	1	1	1	1	3	1		2				1	1	2	
MR81 RAL P17					2		1			1					2									1	
MR81 RAL P27			1													60					13				
MR81 RAL P39																1									
MR81 RAL P44			1			3							1	1	1	1		2	1						1
MR81 RAL P44			5			2			1				1	7	6			1	1	3					
MR89 RAL P16			1	1										1	1			1		1					
MR89 RAL P17					1			1																1	
MR89 RAL P41			2				1				1		1	3	3					1	97				

Table 3.4: Resistance mutations as selected by EVG and identified using NexGen sequencing.

	H51Y	T66A	T66I	L74I	L74M	E92G	E92Q	E92V	Q95K	T97A	G118R	F121Y	E138K	G140C	G140S	
FV3 EVG P14												25				
FV3 EVG P33												99				
FV3 EVG P44												100				
FV5 EVG P18		1												1	1	
FV5 EVG P27										2	1		3		3	
FV6 EVG P17	1		92													
FV6 EVG P33			99					1		2						
FV6 EVG P36			98						1	3						
FV6 EVG P39			97						1	2					1	
MR 69 EVG P14			14		1											
MR 69 EVG P24			99										6			
MR 69 EVG P24			98													
MR69 EVG P44			98		1	1	3		1							
MR 89 EVG P25			45													
MR 89 EVG P29			91	1					4				5			
MR89 EVG P29			93						3	1			5			
	Y143C	Y143K	Y143F	Y143S	P145S	Q148H	Q148K	Q148R	V151L	S153Y	N155S	N155T	E157Q	G163R	S230R	R263K
FV3 EVG P14								1		1						
FV3 EVG P33						1	1									
FV3 EVG P44						1	1									
FV5 EVG P18		1	2			3	3		2	1	2			2	1	
FV5 EVG P27							31							1		
FV6 EVG P17						1	1				1					
FV6 EVG P33													1			4
FV6 EVG P36						1	1			1	1			2	1	3
FV6 EVG P39				1		2	3							1		22
MR 69 EVG P14					12	1	1	3								
MR 69 EVG P24									2						1	
MR 69 EVG P24			3			1			1	1	1			2		0
MR69 EVG P44						1	1			1			2	1		
MR 89 EVG P25						1										
MR 89 EVG P29	1			1			1		1	4		1		3		2
MR89 EVG P29						1	1		1	2				5		3

Table 3.5: Resistance mutations as selected by DTG and identified using NexGen sequencing.

	T66A	L74I	L74M	F121Y	E138K	G140A	Y143R	P145S	Q146P	Q148H	Q148K	Q148R	V151L	S153Y	N155S	E157Q	G163K	G163R	R263K
FV3 DTG P17	1						1				1		1		1	1		1	
FV3 DTG P31			1							1									
FV3 DTG P33							1			1	1								
FV3 DTG P39			1					1		1	1		16						
FV5 DTG P26															1				1
FV5 DTG P39										1	1			1					
FV6 DTG P26					3												3		
FV26 DTG P26																			
MR69 DTG P23					2												17		
MR69 DTG P26		1									1							1	
MR81 DTG P26				1		7		8	1		9	1	1						

3.3. *In silico* analysis of mutations using Accelrys

The position of the four major primary mutations (T66I, E92Q, Q148R and N155H), the major accessory mutation R263K and miscellaneous INI-associated mutation (H114L) selected studies were mapped using Accelrys Discovery Studio 3.5. Figure 3.4 shows a three-dimensional docking model of the HIV-1 IN catalytic core domain prepared using PDB entry 1QS4 as a starting point, showing the catalytic triad residues (D64, D116 and E152) in green and the two magnesium ions as purple spheres. Primary amino acid mutations (T66I, E92Q, Q148R and N155H) in the linear sequence of the wild-type protein shown in orange.

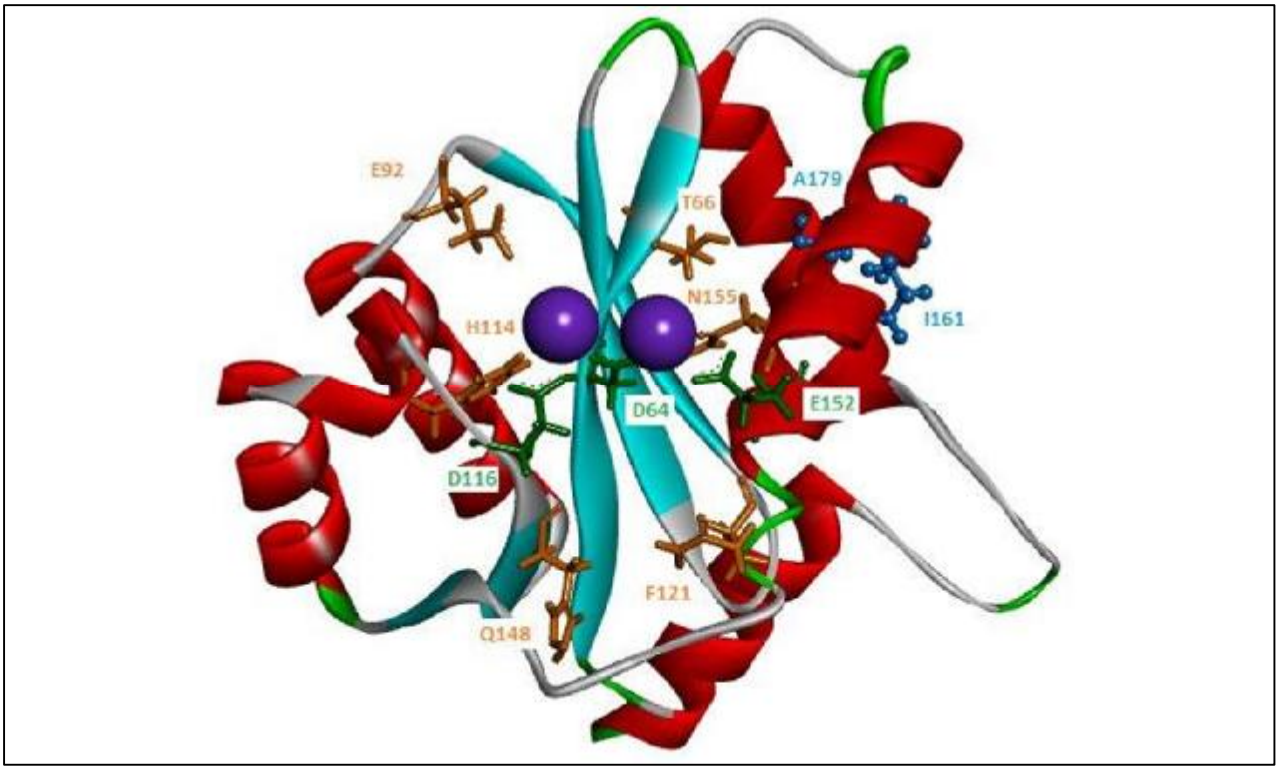


Figure 3.4: A three-dimensional docking model showing the positions at which the integrase inhibitor selected substitutions T66I, E92Q, H114L, F121Y, Q148R and N155H within the HIV-1 integrase catalytic core domain occur.

Figure 3.5 shows the changes in each amino acid substitution. For instance, T66I shows WT amino acid threonine (T66) with a hydrophobic methyl (CH_3) group which was substituted by isoleucine (I66) comprising of a bulkier hydrophobic hydroxyl (OH) group. The E92Q substitution replaces the negatively charged carboxylate group (COO^-) in glutamic acid (E92)

with a neutral and bulkier amine group in glutamine (Q92). The H114L substitution substitutes the planar hydrophilic aromatic group ($C_6H_{6)}$ from the histidine (H114) with a bulkier hydrophobic group from leucine (L114). The F121Y mutation substitutes the planar hydrophilic group in phenylalanine (F121) with a hydrophilic aromatic group from tyrosine (Y121). The Q148R mutation substitutes (glutamine with arginine) the hydrophobic carboxylic amine (an acid) with three bulky nitrogens (hydrophilic). The N155H single point mutation replaces the amine and the carboxylic group from asparagine (N155) with a planar aromatic group in histidine (H155) and remains hydrophilic. R263K (neutral) substitutes the (arginine with lysine) three amines with a less bulky amine, and obtained a negative charge and a minor change in orientation.

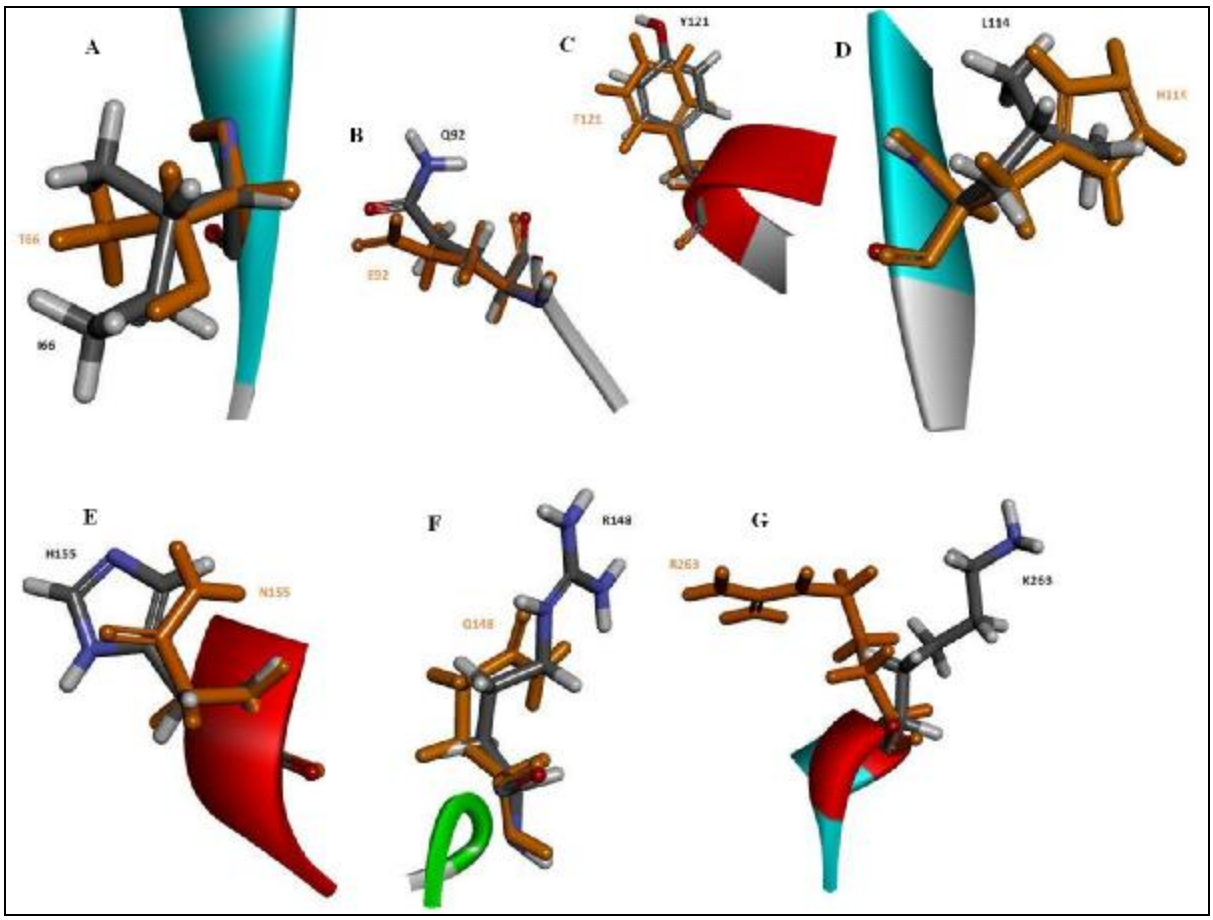


Figure 3.5: A three-dimensional docking model showing the amino acid substitutions of A) T66I, B) E92Q, C) F121Y, D) H114L, E) N155H, F) Q148R, and G) R263K. The orange colour represents the wild-type amino acid and the grey is the single point mutation.

The minimisation energy and the root mean square deviation (RMSD) from modelling of the IN single point substitutions using Accelrys Discovery Studio 3.5 are detailed in Table 3.6. The amino acid substitutions exhibited overall acceptable minimisation energies ranging from the highest -13.07 (R263K) to the lowest -178.16 (F121Y). The lower the minimisation energy, the more stable the amino acid and the higher the minimisation energy the less stable the single point mutation. The RMSD measures the differences between the WT amino acid and the mutant; and all obtained substitutions exhibited RMSD values below 0.3 Å. The higher the RMSD value the greater the difference.

Table 3.6: A summary of minimisation energies and the RMSD value of the single point mutations in IN, as determined by Accelrys Discovery Studio 3.5 software.

	Minimisation Energy	**RMSD (Å)**
T66I	-51.66	0.209
E92Q	-99.03	0.101
H114L	-84.98	0.016
F121Y	-178.16	0.041
Q148R	-21.92	0.139
N155H	-57.13	0.055
R263K	-13.07	0.195

3.4. Multiple sequence alignment exhibiting relevant site-directed mutations

Based on the Sanger and MiSeq sequencing results, seven mutations were targeted to evaluate their impact on HIV-1 subtype C WT IN activity. These included FV6^{T66I}, FV6^{E92Q}, FV6^{H114L}, FV6^{F121Y}, FV6^{Q148R}, FV6^{N155H} and FV6^{R263K}. A pET-15b vector with a cloned full-length FV6^{WT} IN gene was used as a backbone to successfully generate the seven mutants by site-directed mutagenesis. Nucleotide sequence analysis and an amino acid sequence alignment of the FV6^{WT} IN and mutants (FV6^{MT}) confirmed that all the desired mutations had been correctly introduced into the FV6^{WT} IN backbone (Figure 3.6).

```
                 *        20         *        40         *        60         *
05ZAFV6 : -FLDGIDKAQEEHEKYHSNWRAMASEFNLPPIVAKEIVASCDKCQLKGEATHGQVDCSPGIWQLDCTHLEGKVIL :  74
T66I    : M.............................................................I..... :  75
E92Q    : M............................................................... :  75
H114L   : M............................................................... :  75
F121Y   : M............................................................... :  75
Q148R   : M............................................................... :  75
N155H   : M............................................................... :  75
R263K   : M............................................................... :  75

            80         *       100         *       120         *       140         *
05ZAFV6 : VAVHVASGYIEAEVIPAETGQETAYYILKLAGRWPVKVIHTDNGSNFTSAAVKAACWWAGIQQEFGIPYNPQSQG : 149
T66I    : ..................................................................... : 150
E92Q    : ................Q.................................................... : 150
H114L   : ..................................L.................................... : 150
F121Y   : ..................................Y.................................... : 150
Q148R   : ..................................................................R. : 150
N155H   : ..................................................................... : 150
R263K   : ..................................................................... : 150

              160         *       180         *       200         *       220
05ZAFV6 : VVESMNKELKKIIGQVRDQAEHLKTAVQMAVFIHNFKRKGGIGGYSAGERIIDIIATDIQTKELQKQIIKIQNFR : 224
T66I    : ..................................................................... : 225
E92Q    : ..................................................................... : 225
H114L   : ..................................................................... : 225
F121Y   : ..................................................................... : 225
Q148R   : ..................................................................... : 225
N155H   : .....H............................................................... : 225
R263K   : ..................................................................... : 225

             *       240         *       260         *       280         *
05ZAFV6 : VYYRDSRDPIWKGPAKLLWKGEGAVVIQDNSDIKVVPRRKAKIIKDYGKQMAGADCVAGGQNED- : 288
T66I    : ..................................................................- : 289
E92Q    : ..................................................................- : 289
H114L   : ..................................................................- : 289
F121Y   : ..................................................................- : 289
Q148R   : ..................................................................- : 289
N155H   : ..................................................................- : 289
R263K   : ..................................K...............................- : 289
```

Figure 3.6: Alignment of the predicted FV6^{WT} integrase enzyme amino acid sequence with the FV6 mutants, indicating the introduced mutations in red, and the position highlighted in yellow. Dots indicate identity to the FV6^{WT} sequence.

3.4. Western-blot analysis of purified WT and mutant integrase

The $FV6^{WT}$ and $FV6^{MT}$ recombinant IN enzymes were successfully expressed in *E.coli* BL21 (pLys) cells by IPTG induction on a large scale, and purified using nickel chelating column chromatography. The purified IN stock concentrations were determined using the NanoDrop as to: $FV6^{WT}$ (600 µM), $FV6^{N155H}$ (114 µM), $FV6^{T66I}$ (109 µM), $FV6^{Q148R}$ (86 µM), $FV6^{F121Y}$ (101 µM), $FV6^{R263K}$ (78 µM), $FV6^{H114L}$ (117 µM) and $FV6^{E92Q}$ (156 µM). The recombinant IN proteins were confirmed using a Western-blot ELISA assay specific to the histadine tagged on al $FV6^{WT}$ and $FV6^{MT}$ IN enzymes (Figure 3.7).

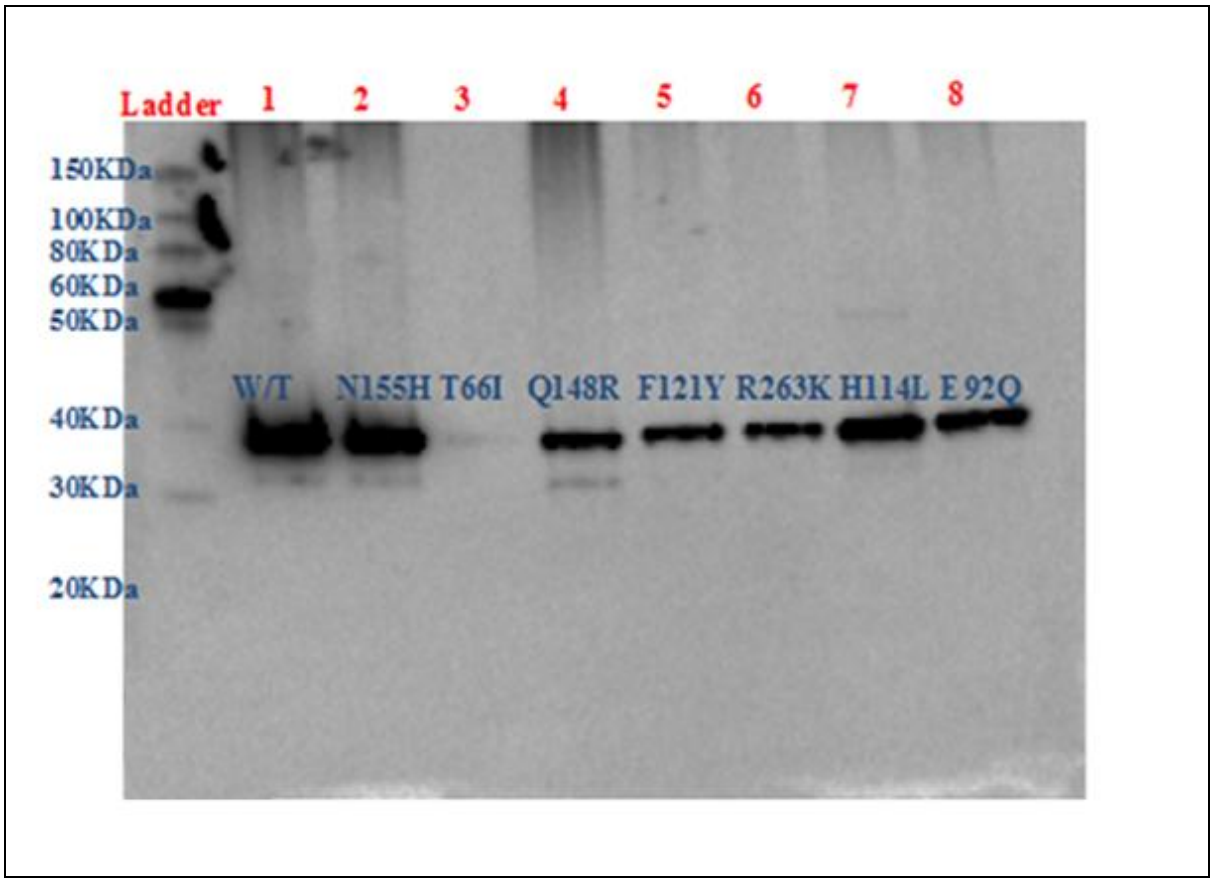
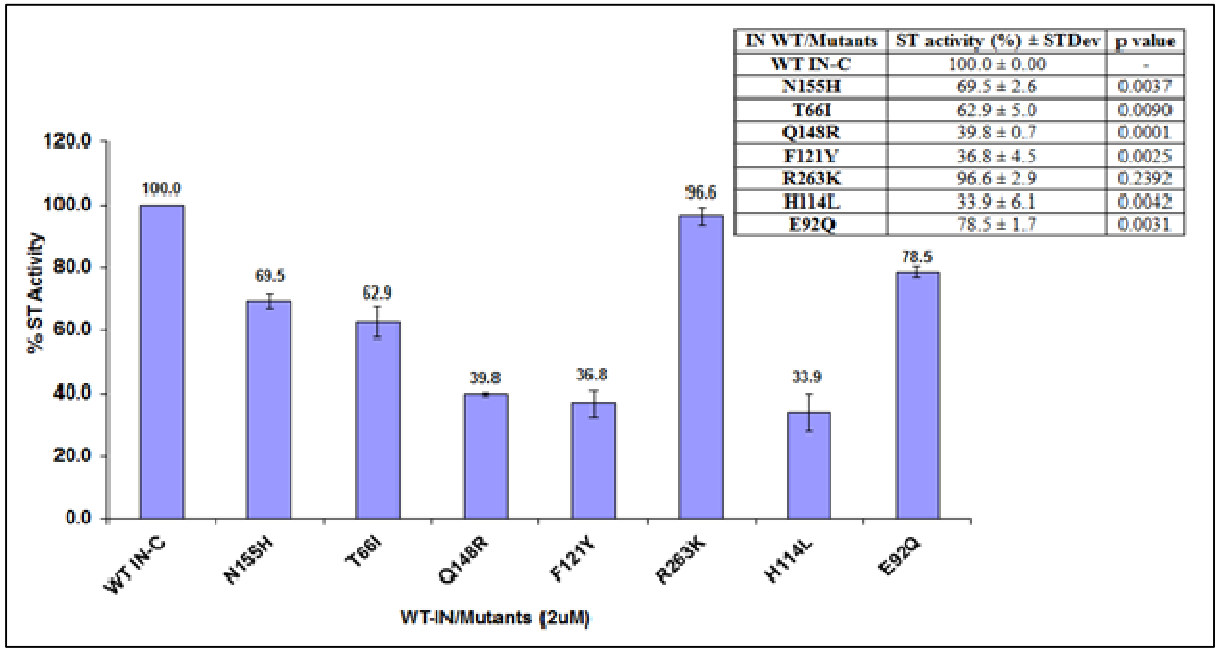


Figure 3.7: Western-blot analysis of a purified wild-type and mutant HIV-1 subtype C integrase enzymes (32 KDa) with Western-blot markers: SuperSignal® Molecular Weight Protein Ladder (Thermo Scientific, USA). The Western-blot does not include the standardization of protein concentration loaded onto the gel, but all HIV-1 IN concentrations were determined and input amounts were standardized for subsequent functional assays.

3.5. HIV-1 subtype C mutant strand transfer capacity

All purified $FV6^{WT}$ and $FV6^{MT}$ were tested for their ability to initiate HIV-1 IN ST reaction. Mutants containing single mutations at positions $FV6^{N155H}$, $FV6^{T66I}$, $FV6^{Q148R}$, $FV6^{F121Y}$, $FV6^{R263K}$, $FV6^{H114L}$ and $FV6^{E92Q}$ exhibited 30.5%, 37.1%, 60.2%, 63.2%, 3.4%, 66.1% and

21.5% reduced ST capacity as compared to wild-type, respectively (Figure 3.8). All mutants with an exception of FV6^{R263K} exhibited significant reduction (p-value < 0.05) in ST activity.



IN WT/Mutants	ST activity (%) ± STDev	p value
WT IN-C	100.0 ± 0.00	-
N155H	69.5 ± 2.6	0.0037
T66I	62.9 ± 5.0	0.0090
Q148R	39.8 ± 0.7	0.0001
F121Y	36.8 ± 4.5	0.0025
R263K	96.6 ± 2.9	0.2392
H114L	33.9 ± 6.1	0.0042
E92Q	78.5 ± 1.7	0.0031

Figure 3.8: HIV-1 subtype C mutant ST capacity with respect to the WT IN subtype C enzyme, using a plate-based ELISA assay. – WT IN-C –WT IN HIV-1 subtype C; HIV-1 IN ST (%) Activity – HIV-1 IN ST percent activity. P-values were calculated using GraphPad QuickCalcs: t test calculator: http://graphpad.com/quickcalcs/ttest1.cfm?Format=SD

3.6. HIV-1 subtype C FV6^{WT} and FV6^{MT} IN DNA binding capacity

All purified FV6^{WT} and FV6^{MT} were tested for their donor-DNA binding ability required for HIV-1 IN ST reactions. Figure 3.9 shows the SPR sensograms exhibiting an increase in RU due to the increasing retroviral DNA concentrations following interactions between FV6^{WT} and FV6^{MT}.

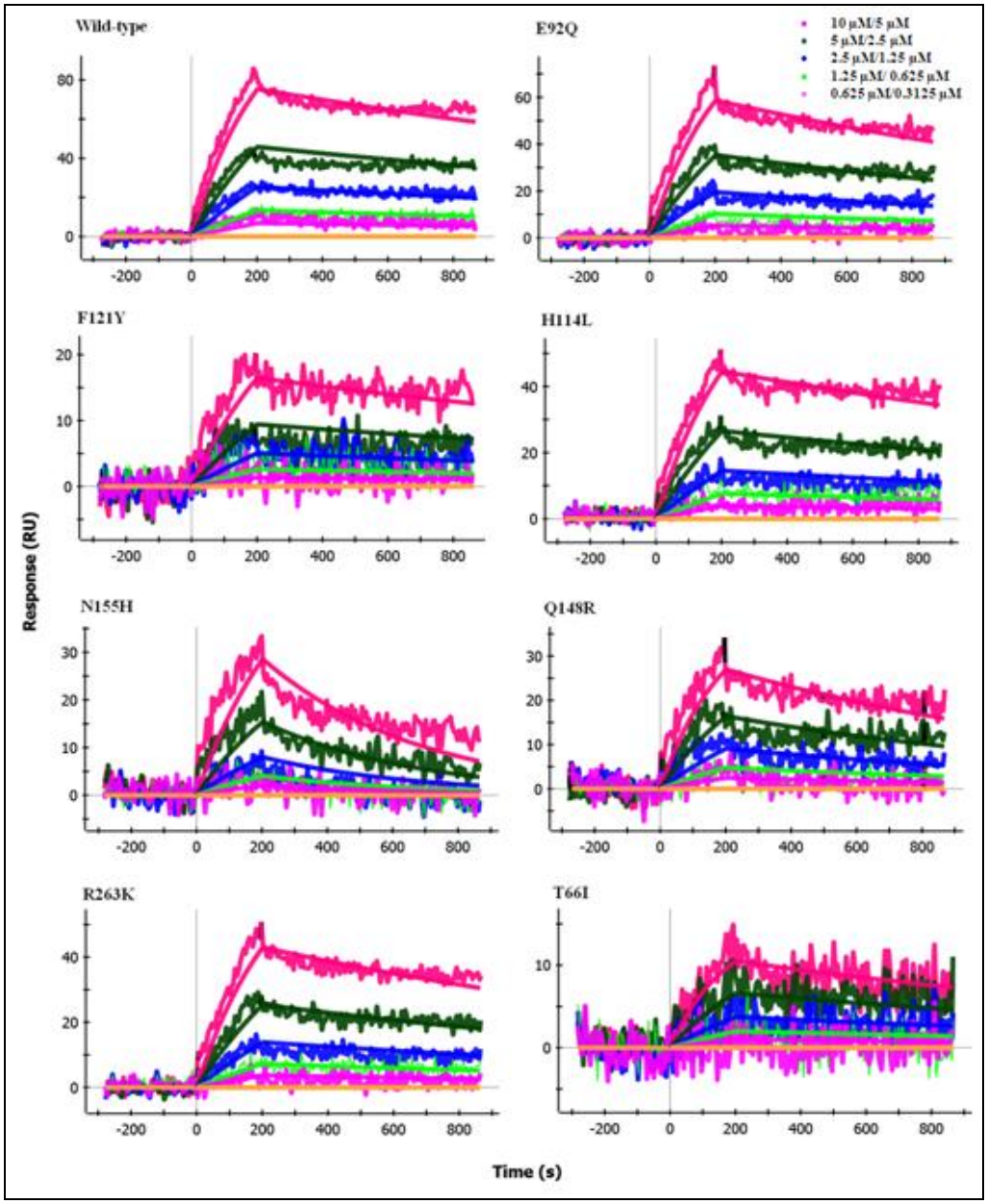


Figure 3.9: HIV-1 subtype C mutant donor (d) DNA binding capacity with respect to the FV6 WT IN enzyme. The analyte (dDNA) concentrations used are shown in the key, and the analyte concentrations for the relevant FV6 IN mutants are delineated in section 2.9 of the text.

Overall, with the exception of $FV6^{MT}$ T66I, the overall affinity (K_D) of each mutant was lower than the $FV6^{WT}$ (Table 3.7). $FV6^{MT}$ T66I and N155H have an increased association rate (k_a) relative to WT (an order of magnitude higher). $FV6^{MT}$ N155H also exhibited the fastest dissociation rate (k_d), an order of magnitude higher than the WT. The R_{max} (<200) and χ^2 (≤10% of R_{max}) values included in the Table are an indication of the goodness-of-fit to the Langmuir 1:1 binding model.

Table 3.7: The kinetics of donor DNA binding to the HIV-1 FV6 IN mutants with respect to the IN WT.

IN	k_a **(Ms^{-1})**	k_d **(s^{-1})**	K_D **(µM)**	R_{max}	χ^2
WT	8.82×10^{2}	3.78×10^{-4}	0.429	131.940	11.650
T66I	5.50×10^{3}	5.44×10^{-4}	0.099	16.340	2.800
E92Q	4.21×10^{2}	5.39×10^{-4}	1.280	102.400	9.480
H114L	3.90×10^{2}	3.92×10^{-4}	1.005	88.420	5.140
F121Y	5.80×10^{2}	4.11×10^{-4}	0.709	38.690	3.590
Q148R	8.99×10^{2}	7.97×10^{-4}	0.887	48.460	5.790
N155H	1.63×10^{3}	2.09×10^{-3}	1.282	123.090	8.260
R263K	3.74×10^{2}	5.17×10^{-4}	1.382	96.180	5.620

K_D: Donor DNA overall affinity for the IN enzyme
k_a: Rate at which donor DNA binds the IN enzyme
k_d: Rate at which donor DNA dissociated from the IN enzyme
R_{max}: maximum dDNA binding capacity on IN surface
χ^2: Chi^2-value is the goodness-of-fit of the sensograms

CHAPTER 4: DISCUSSION

The INSTI RAL has recently been approved for use in third line salvage therapy in South Africa. Although EVG and DTG are yet to be included as components in any of the current South African ARV treatment guidelines, they are likely to be considered as future components of salvage therapy. There is currently no published data on any South African patients failing a RAL-containing regimen. Thus, to better understand the generation of ARV drug resistance against INSTIs in treatment-naïve and treatment-experienced HIV-1 subtype C infected South African patients, we conducted *in vitro* selection studies using RAL, EVG or DTG against a panel of WT and ARV drug resistant primary virus isolates. ARV drug resistant primary viruses harbouring combinations of the NRTI and/or NNRTI mutations M184V, K103N and K238N (Table 2.1) were specifically included in this study to explore their impact on the emergence of INSTI mutations, and enzyme fitness. Several groups have already shown that the interactions between RT and IN drug resistant mutations are important for the development of resistance and viral fitness (Buzon *et al*, 2009; Gupta *et al*, 2009; Hu and Kuritzkes, 2014).

Baseline IN sequences of the six selected virus isolates confirmed the absence of any primary INI resistance mutations, and the presence of various combinations of subtype C signature polymorphic mutations (Table 3.1; positions 25, 31, 50, 72, 100, 101, 112, 124, 125, 136, 201, 218, 234, 265, 269, 278 and 283; Rhee *et al*., 2008; Oliveira *et al*., 2012) in the different isolates. Overall, the impact of these naturally occurring polymorphisms on drug susceptibility remains unclear. In this study, some mutations were present in all six isolates (100%), for example, codons 101, 124 and 201. Polymorphic mutations at codons 101 and 124 have been previously associated with DTG resistance (Garrido *et al*., 2011). Moreover, Marcelin *et al.*, (2010) reported a significantly higher rate of T124A and L101I+T124A in patients failing RAL than in INI-naïve patients. In contrast, V201I did not appear to alter INSTI susceptibility (Da Silva *et al*., 2010; Ceccherini-Silbersteinet *et al*., 2010). Interestingly, I72V, which is associated with secondary drug resistance to INSTIs, was seen

in virus isolates FV6 and MR69 (Oliveira *et al*., 2012). Thus, it is possible that the presence of these natural polymorphic mutations in HIV-1 subtype C may or may not alter susceptibility to INSTIs.

Natural polymorphisms at the sites of secondary mutations are common in non-B subtypes (Brenner *et al*., 2011; Fish *et al*., 2010; Garrido *et al*., 2010; Sierra *et al*., 2011). Of particular interest is the M50I polymorphism identified in MR89. M50I has been associated with drug resistance and IN enzyme impairment in combination with R263K in HIV-1 subtype B (Wares *et al*., 2012); and earlier reports identified M50I in 10-25% of INSTI-naïve patients infected with HIV-1 subtype B (Ceccherini-Silberstein *et al*., 2009). However, analysis of HIV-1 subtype C IN sequences in the Los Alamos database (www.hiv.lanl.gov; accessed 8 June, 2015) showed that variation at this position is common, and its effect on INSTI susceptibility warrants further investigation.

4.1. *In vitro* selection

To better understand the possible generation of resistance against INIs we conducted *in vitro* selection studies using RAL, EVG and DTG using a dose-escalation method in PBMCs infected with ARV drug naïve- and RTI drug resistant HIV-1 subtype C primary viruses. Selection studies using dose-escalation have been successfully used to adapt viruses in the presence of RTIs and INIs (Vingerhoets *et al*., 2005; Kobayashi *et al*., 2011). PBMCs were selected for use in the experiments since they most closely represent the natural infection setting. It should be noted that the choice in cell-line has also been observed to influence the development of resistance mutations during selection. For instance, selection with DTG in MT-2 cells has been associated with the development of substitutions T124A, S153F/Y and L101I in HIV-1 subtype B virus, and selection in cord blood monocytic cells (CBMCs) is associated with the emergence of the stochastic R263K mutation (Jones *et al*., 2007; Goethals *et al*., 2008; Shimura *et al*., 2008; Goethals *et al*., 2010; Quashie *et al*., 2012).

Drug concentrations were progressively increased over a period of 184 days. Drug concentrations were kept constant to allow the virus to adapt, and virus break-through was associated with a reduction of levels of HIV-1 p24 in the supernatant, followed by a recovery period. Overall, there were differences in viral replication between the WT and RTI resistance viruses in the presence of the different INSTIs, likely attributed to the genetic

barriers to the emergence of mutations. Both the FV and MR isolates retained sufficient viral replication under RAL pressure, with identical drug pressure profiles until selection was terminated at P53 under RAL (266.24 nM, up to 38-133 fold higher than the IC_{50} 2-7 nM) pressure (Figure 3.2). In contrast, the MR viruses grown under EVG and DTG pressure did not replicate at concentrations higher than 16.32 nM and 8.32 nM, respectively. Drug concentrations for the FV viruses could not be increased beyond 66.56nM for EVG and 32.64 nM for DTG. Thus, MR and FV viruses could replicate at EVG and DTG concentrations that were 5.8-23.8 -fold higher than the IC_{50} (2.8 nM), and approximately 3.1-12.1 fold higher than the IC_{50} (2.7 nM), respectively. These results highlight that RAL pressure has a lower impact on viral replication than EVG and DTG in primary HIV-1 subtype C viruses. This could be attributed to the presence of the HIV-1 subtype C signature polymorphic mutations described above. Moreover, there is likely a fitness cost associated with pre-existing RTI resistance mutations in the MR viruses exposed to increasing concentrations of EVG and DTG. The RTI resistant mutant, M184V, is associated with loss (4-33%) in replication efficacy with respect to the WT HIV-1 (Wakefield *et al*., 1992; Back *et al*., 1996; Paredes *et al*., 2009), and K103N was reported to demonstrate a virus fitness cost of 4.7-7.9-fold with respect to WT (Cong *et al*., 2007) due to processivity defect in the RT enzyme (Back *et al*., 1996). An alternative hypothesis is that the MR viral isolates were hypersusceptible to the INSTIs used in thus study, or their replication capacity/fitness could delay the onset of resistance to INSTIs.

4.2. Drug resistance-associated mutations selected *in vitro*

Overall, *in vitro* selection experiments with RAL, EVG and DTG resulted in the emergence of INSTI resistance mutations in some primary viruses, and Sanger-based and NexGen sequencing identified several mutations within the IN coding region, including the most common clinically significant INI-resistance mutations T66IK, E92Q, Q148RK, N155H, G118R, the rare F121Y as well as the nonpolymorphic mutation R263K that confers low-level resistance to these drugs. INSTI resistance mutations were identified at P40-P53 for 266.24 nM RAL, P39-P53/P21-P29 for 66.56 nM/16.32 nM EVG and P31-P53 for 66.56 nM DTG (Tables 3.2-3.5).

4.2.1. Resistance to RAL

Population-based Sanger sequencing identified the emergence of the major primary mutations Q148R, N155H and N155H in FV6 (P44), MR69 (P39) and MR89 (P44), respectively. Thus only two of the three (Q148H/K/R, N155H and the less common Y143H) signature pathways associated with RAL resistance were observed (Hazuda *et al.*, 2007; Johnson *et al.*, 2007). These results were verified using NexGen sequencing technology, where the major primary mutations were the predominant quasispecies. Additionally, the known secondary mutations associated with resistance to RAL, that is, L74M, T97A, E138K, G140S and G163R (Malet *et al.*, 2008) were detected as minority quasipecies (≤5%) at various passages. Only quasispecies detected at proportions greater that 1% were reported. Next generation sequencing has not been used previously in detecting mutations during *in vitro* selection experiments. A higher degree of genetic variation and quasispecies was expected than from the Sanger-based sequencing, however, very few quasispecies were detected, with the majority of the populations detected being either WT or harbouring a major primary mutation. This is likely attributed to the selection process during passage in the PBMCs which enabled particular quasispecies to grow and predominate. Although several of these mutations were present during early passages, for example, the major accessory mutation E138K comprised 4% of the MR69 quasispecies detected at P24; it did not proliferate/expand in subsequent passages, implying a fitness cost. Of interest, MR69 resistance to RAL appeared to emerge using the Q148R pathway by P24 (Q148R was present at 96% and E138K was present at 4%). The emergence of E138K in combination with Q148R it is associated with >100-fold reduced RAL and EVG susceptibility (Underwood *et al.*, 2012; Eron *et al.*, 2013; Castagna *et al.*, 2014). However, by P31 there were changes in resistance pathways to RAL with the emergence of N155H mutation. This is in contrast to previous reports where N155H mutant viruses emerged first and were subsequently replaced by Q148H (Canducci *et al.*, 2009; Delelis *et al.*, 2010).

Resistance to RAL is known to confer cross- resistance to EVG. Q148R and N155H are non-polymorphic mutations previously selected *in vitro* and in patients receiving RAL and EVG (Fransen *et al.*, 2009; Garrido *et al.*, 2012). Q148R alone is associated with reduced susceptibility (30 to 100-fold) to RAL and EVG (Goethals *et al.*, 2008; Canducci et al., 2011; Kobayashi *et al.*, 2011; Garrido *et al.*, 2012; Abram *et al.*, 2013), whilst N155H alone is

associated with reduced efficacy of RAL (~15-fold) and EVG (~30-fold) (Kobayashi *et al*., 2011; Margot *et al*., 2012). The Q148H and N155H substitutions are associated with conformational changes within the catalytic pocket leading to an increase in the binding energy of INSTIs (Hare *et al*., 2010). The conformational changes observed are possibly due to the removal of the hydrophobic carboxylic amine in Q148 with the three nitrogen's in R148 (Figure 3.5F); and the replacement of the carboxylic group and amine in N155 with the planar aromatic group in histidine (H155) shown in Figure 3.5E.

Efficacy of RAL (and EVG) is further reduced when N155H occurs in combination with E92Q or other accessory INI-resistance mutations (Abram *et al*., 2013). M50I was detected at baseline in virus isolate MR89, and later N155H emerged resulting in a double mutant (Table 3.1). Moreover, the M50I substitution has been associated with moderate resistance to DTG in combination with R263K, and without impact on enzyme fitness (Ceccherini-Silberstein *et al*., 2009; Quashie *et al*., 2012; Wares *et al*., 2014).

Primary viruses FV3 and FV5 were able to efficiently replicate in the presence of 266.24nM RAL without the emergence of associated major primary mutations. It is possible that the baseline naturally occurring polymorphisms in these viruses contributed to their "natural resistance", or alternatively other as yet unidentified changes may have contributed to this. Other mutations selected under RAL pressure were I161T and D41N in primary HIV-1 subtype C isolates FV3 and FV5 respectively (data not shown). This has also been reported in other patient samples without known signature resistance mutations toward RAL. The D41N substitution is associated with a drug burden index score between 5 and 9 (Casadella *et al*., 2014). Although information regarding I161T is limited it is located within the C4 α-helix of the CCD (shown in Figure 3.4), thus conformational changes induced may affect drug binding and IN inhibition.

As mentioned previously, several known secondary mutations associated with resistance to RAL (and EVG, DTG) were detected as minority quasipecies (≤5%) at various passages. During the selection experiments, they never emerged as predominant quasispecies, so their relevance in altering INSTI susceptibility could not be determined. For example, the E92V substitution which was detected in P16 in MR89 has only been selected *in vitro* using an experimental INI GS-9160, and was observed to increase resistance to RAL and EVG by 10

and 40-fold respectively (Jones *et al*, 2009). The Y143HS substitutions detected in FV3 (P24), and MR89 (P17, P41) are extremely rare nonetheless were reported in patients on RAL treatment and are associated with a 5-10-fold resistance to RAL and minimal effects on EVG and DTG (Canducci *et al*., 2010; Delelis *et al*., 2010; Metifiot *et al*., 2010; Kobayashi *et al*., 2011; Van Wesenbeeck *et al*., 2011; Garrido *et al*., 2012; Geretti *et al*., 2012; Abram *et al*., 2013; Van der Borght *et al*., 2013). S147G observed in MR69 (P39, P44) and MR89 (P41) has been identified in patients receiving EVG treatment and is associated with 5-10-fold resistance to EVG, and negligible resistance to RAL (Shimura *et al*., 2008; Kobayashi *et al*., 2011; Margot *et al*., 2012; Abram *et al*., 2013). Other mutations such as Q95K, Q146P, V151AL, S153FY and E157Q affect the fold resistance to RAL, EVG and DTG (Jones *et al*, 2009; Kobayashi *et al*., 2011; Vavro *et al* 2013; Shimura *et al*., 2007; Fun *et al*., 2010). The S230R substitution is associated with negligible resistance to INIs (Goethals *et al*., 2008; Goethals *et al*., 2010; Blanco *et al*., 2011; Geretti *et al*., 2012).

The *pol* from MR81 grown under RAL selection pressure could not be amplified despite adequate p24 antigen levels in the culture supernatant, hence the presence/absence of INSTI drug resistance mutations could not be verified. Different amplification primers could be designed for future work. Overall, three of the five primary HIV-1 subtype C isolates developed the common RAL resistance mutations, implying that HIV-1 subtype C infected South African patients receiving a RAL-containing regimen are likely to develop drug resistance using the Q148R and N155H pathways, as reported for subtype B. Understanding why the two WT primary viruses could effectively replicate in the presence of high concentrations of RAL warrants further investigation.

4.2.2. Resistance to EVG

Population-based Sanger and NexGen sequencing confirmed the emergence of the rare primary INI-resistance mutation F121Y in FV3, and the major primary mutations T66I, T66I and T66K in FV6, MR69 and MR81, respectively, following selection with EVG in primary HIV-1 subtype C virus isolates. No Sanger data was obtained for MR89, but NexGen data confirmed the presence of T66I. F121Y, T66I and T66I/R263K have been reported before in HIV-1 subtype B, selected under INSTI pressure in a MT-2 cell line (Margot *et al*., 2012) and under RAL (both *in vitro* and in patients) and EVG (Rowley, 2008; Shimura *et al*., 2008).

Additionally, the major accessory mutation R263K was detected in combination with T66I in FV6, and D232N and A179G (data not shown) was detected in FV3 and FV5, respectively. NexGen sequencing also identified the presence of Q148K in FV5 at 31% of the quasispecies at P27. This percentage falls within the cut-off limit of detection for Sanger sequencing, so may have not been recorded. Q148R constituted 60% of the quasispecies of MR81 at P27, but this coincided with the emergence of N155H (13%) and T66K (7%), following which T66K then became the predominant quasispecies. It is likely that these mutations are separate pathways to EVG resistance, with T66K being more favourable to optimal MR81 replication.

This is the first report of selection of the rare polymorphic mutation F121Y in primary HIV-1 subtype C viruses from South Africa. F121Y is associated with high-level resistance (>5-fold) to RAL, and is associated with over 10-fold resistance to EVG (Shimura *et al*., 2007; Kobayashi *et al*., 2008; Geretti *et al*., 2012). F121Y exhibits >100-fold resistance in combination with known secondary resistance mutations, but does not appear to affect DTG efficacy (Kobayashi *et al*., 2008; Margot *et al*., 2012). This mutation is associated with the replacement of the planar hydrophilic group with a hydrophilic aromatic group during F121Y substitution (Figure 3.5C). Interestingly, the D232N substitution is considered a secondary mutation associated with RAL resistance (Malet *et al*., 2009; Loizidou *et al*., 2009); therefore it could be speculated that the combination of F121Y/D232N observed in FV3 may affect drug resistance, enzyme fitness and *in vitro* retroviral DNA binding. EVG was also observed to induce drug resistance-associated mutation A179G in FV5 located within the C3 α-helix of the catalytic core domain essential for DNA binding (Figure 3.4). However, the biochemical impact of substitution A179G on IN function, enzyme fitness and DNA binding is yet to be established.

T66I has been selected both in culture and in patients on EVG treatment (Shimura *et al*., 2008; Goethals *et al*., 2008; Kobayashi *et al*., 2008; McColl and Chen, 2010; Molina *et al*., 2012; Margot *et al*., 2012), whilst the R263K was identified through *in vitro* selection with EVG and DTG and again isolated from patients on separate RAL and DTG treatment (Blanco *et al*., 2011; Margot *et al*., 2012; Quashie *et al*., 2012; Cahn *et al*., 2013; Mesplède *et al*., 2013). McColl and Chen, 2010 reported T66I in combination with accessory mutations R263K, F121 and S153Y in non-subtype C HIV. T66I is associated with reduced efficacy to EVG by approximately 15-fold (Kobayashi *et al*., 2011; Margot *et al*., 2012) but does not

affect the efficacy of RAL (Kobayashi *et al*., 2011; Margot *et al*., 2012; Van der Borght *et al*., 2013) or DTG (Abram *et al*., 2013). The R263K substitution is associated with 2-fold (RAL and EVG) and 3-5-fold (DTG) reduced efficacy (Margot *et al*., 2012; Garrido *et al*., 2012; Quashie *et al*., 2012; Cahn *et al*., 2013; Mesplède *et al*., 2013).

As with RAL pressure, selection with EVG induced a greater variety of mutations at low proportions (<20%) such as the major primary mutations E92V (FV6), Y143KRS (MR81), Q148HKR (all six isolates), N155HS (FV6, MR81); major accessory mutations T97A (FV5, FV6, MR81, MR89), G140S (FV5, FV6), V151L (MR81, MR89), S153SY (FV3, FV6, MR81, MR89), G163KR (FV5, FV6, MR81, MR89), S230R (FV6), R263K (FV6, MR89); rare primary INI-resistance mutations G118R (FV5), P145S (MR69), E157Q (FV6, MR81); and miscellaneous INI-associated mutations Q95K (FV6, MR81, MR89). Because none of these mutations ultimately emerged as predominant quasispecies, their contribution to EVG resistance is unknown.

The impact of some of these mutations were discussed above with an exception of T66A, E92GQ, G118R, G140CS, Q148HK and N155T. The T66A mutation was previously reported in patients receiving EVG treatment and was established to have approximately 10-fold EVG resistance (Hatano *et al*., 2010; Winters *et al*., 2012; Molina *et al*., 2012; Kobayashi *et al*., 2011; Margot *et al*., 2012; Abram *et al*., 2013; Van der Borght *et al*., 2013). The E92GQ mutations have been selected in patients and *in vitro* under EVG pressure and are associated with respective 10-fold resistance and 30-fold resistance to EVG (Van Wesenbeeck *et al*., 2011; Margot *et al*., 2012; Abram *et al*., 2013). Interestingly, the G118R substitution has only been selected using RAL (*in vivo*) and using DTG (*in vitro*), and is associated with increased resistance to RAL (10-fold) and EVG/DTG (3-fold) (Kobayashi *et al*., 2008; Malet *et al*., 2011; Geretti *et al*., 2012; Quashie *et al*., 2012; Quashie *et al* 2013). The G140CS substitutions are associated with over 100-fold increased resistance towards RAL and EVG and up to 10-fold resistance towards DTG, only in combination with INI resistant mutations at position Q148. These nonpolymorphic mutations (G140CS) have been selected in patients receiving RAL/EVG treatment (Hatano *et al*., 2010; Blanco *et al*., 2011; Winters et al., 2012; Fransen *et al*., 2012; Garrido *et al*., 2012; Hurt *et al*., 2014). The substitutions Q148HK are associated with increased resistance of 5-fold and 10-fold towards RAL and EVG respectively, and have been selected both *in vitro* and in patients receiving

RAL and EVG treatment. The N155ST substitution has been identified following selection with experimental agents and was observed to increase resistance to RAL and EVG (Jones *et al*, 2009; Kobayashi *et al*., 2011; Van der Borght *et al*., 2013).

Overall, all the primary HIV-1 subtype C isolates developed EVG resistance mutations, implying that HIV-1 subtype C infected South African patients receiving an EVG-containing regimen in future are likely to easily develop drug resistance using the T66IK pathway, as reported for subtype B (assuming they do not have any RAL-associated mutations from failing a RAL-containing regimen). T66IK emerged early, implying a low genetic barrier to development of drug resistance, whereas Q148K emerged more slowly. The emergence of F121Y is reported to be rare for subtype B, but may be a more common pathway for HIV-1 subtype C. Thus, future work should focus on investigating this further.

4.2.3. Resistance to DTG

Population-based Sanger sequencing revealed that *in vitro* selection with DTG induced a non-polymorphic mutation E92Q in FV3 (P44). NexGen sequencing data for FV3 P44 could not be obtained due to limited sample volumes. The E92Q has been isolated previously in patients receiving RAL (Blanco *et al*., 2011; Molina *et al*., 2012) and EVG (DeJesus *et al*., 2012; Winters *et al*., 2012) treatment; and following *in vitro* selection with RAL (Kobayashi *et al*., 2011). This serves as the first report of *in vitro* selection of E92Q using primary HIV-1 subtype C in a PBMC-culture. The E92Q has been described as the primary EVG-resistance mutation associated with virological failure in non-subtype C viruses (Molina *et al*., 2012; White *et al*., 2013). The E92Q substitution has been shown to decrease susceptibility to RAL (5-10-fold), EVG (~30-fold) and DTG (~1.5-fold) (Shimura *et al*., 2008; Jones *et al*., 2009; Kobayashi *et al*., 2011; Van Wesenbeeck *et al*., 2011; Abram *et al*., 2013). As shown in Figure 3.5B E92Q substitution is associated with a change in charge due to the replacement of the negatively charged carboxylate group (COO^-) with a neutral amine (bulky) group in Q92, therefore resulting in INSTI resistance.

Selection with DTG induced the same mutations at low proportions (<20%) as described above; namely, T66A, Y143R and N155S, the rare F121Y and a miscellaneous INI-associated mutation E157Q substitution. Similarly, because quasispecies harbouring these mutations never became the predominant quasispecies, the impact of these mutations remains

unclear. The T66A was previously selected *in vitro* using DTG, but is associated with negligible DTG resistance (Van Wesenbeeck *et al*., 2011; Abram *et al*., 2013). There are no reports of selection of Y143R using DTG and the substitution is not associated with reduced potency of DTG similar to N155S and F121Y (Kobayashi *et al*., 2011; Van Wesenbeeck *et al*., 2011; Margot *et al*., 2012; Abram *et al*., 2013; Van der Borght *et al*., 2013) selected in this study. The E157Q mutation has no known effects on DTG susceptibility, and its selection using DTG is yet to be reported. However, of the major accessory mutations only L74M, E138K, S153Y and R263K (Table 3.5) have been selected before *in vitro* and in patients. L74M and E138K single mutations are not associated with increased resistance towards DTG. Resistance was only observed when L74M and E138K were in combination with primary INI resistance mutations. Nonetheless, S153Y and R263K are known to induce 2-fold resistance towards DTG (Quashie *et al*., 2012; Underwood *et al*., 2012; Cahn *et al*., 2013; Eron *et al*., 2013; Vavro *et al*., 2013; Castagna *et al*., 2014). Although, the G140A and V151IL substitutions have only been observed in this study through selection with DTG; they are associated with a 10-fold and 3.6-fold resistance towards DTG (Brenner *et al*., 2011; Garrido *et al*., 2011; Kobayashi *et al*., 2011; Underwood *et al*., 2012).

Overall, our results support existing data and confirmed that the genetic barrier to the development of drug resistance to the second generation INSTI, DTG was higher than RAL or EVG (reviewed by Wainberg and Han Y-S, 2015). This implies that HIV-1 subtype C infected South African patients receiving a DTG-containing regimen in future are unlikely to experience virological failure easily.

4.3. Genetic drug resistance barrier and cross resistance

Genetic drug resistance barrier is defined as the accumulative number of drug resistance-associated mutations required by a virus to escape drug-selective pressure (Maiga *et al*., 2009; Van de Vijver *et al*., 2006) and is an essential aspect that contributes to the emergence of drug resistant isolates. A comparison in genetic drug resistance barriers between HIV-1 subtypes was evaluated following selection with RAL and EVG (Piralla *et al*., 2011). The occurrence of single point mutations during selection with RAL (Q148R and N155H) and EVG (T66I and F121Y) known to confer high-level resistance (Malet *et al*., 2008; Garrido *et al*., 2008) was a demonstration that HIV-1 subtype C shares a modest genetic resistance

barrier with non-subtype C isolates. As described above, our results confirm that WT and RTI drug resistant HIV-1 subtype C primary viruses have a modest genetic resistance barrier to RAL and EVG. Moreover, the two drugs share extensive cross resistance. For instance, the RAL signature mutations (N155H and Q148R) which emerged in FV6, MR69 and MR89 would render these viruses resistant to EVG. The only RAL-associated mutations yet to be selected by EVG are Y143C/R/H, which have been observed to remain susceptible to EVG (Métifiot *et a*l., 2011). Similarly, the selection of primary mutations (T66IK, F121Y and Q148K) by all six virus isolates using EVG suggests substantial cross resistance with RAL which was previously established (McColl *et al*., 2007; Van Baelen *et al*., 2008; Ceccherini-Silberstein *et al*., 2009). However, DTG demonstrated little to no cross resistance with RAL since it was shown to exhibit considerable antiviral activity towards isolates containing RAL and EVG resistance mutations (Sato *et al*., 2010; Kobayashi *et al*., 2011). The selection of E92Q could lead to increased resistance toward DTG. Therefore, it is essential that genetic variability within the HIV-1 subtype C is monitored, even though no considerable cross resistance was observed between DTG and the first generation INSTIs RAL and EVG.

4.4. HIV-1 IN strand transfer efficacy

It is well established that all major INI resistance mutations are associated with reduced subtype B IN activity and viral replication capacity (Hu and Kuritzkes, 2010). All the recombinant HIV-1 subtype C mutants used in this study exhibited varying degrees of loss of enzyme fitness with respect to the WT IN enzyme (Figure 3.8). Single mutant enzymes consisting of $FV6^{Q148R}$, $FV6^{F121Y}$ and $FV6^{H114L}$ displayed a substantial loss (60.2%, 63.2% and 66.1%, respectively) in ST capacity.

Results for the ST capacity of the $FV6^{Q148R}$ mutant correlate with findings from Abram *et al*., (2013), who showed an approximately 62% reductions in ST capacity for the HIV-1 subtype B LAI/IIB IN. This implies that the impact of the Q148R substitution on HIV-1 subtypes B and C ST capacity is similar. Interestingly, a substantial loss (>90%) in ST activity due to the Q148R substitution was reported for HIV-2 group B IN using *in vitro* gel-based enzymatic activity assays (Ni *et al*., 2011). Little data exists on the rare F121Y IN mutant ST capacity. Marinello *et al*., (2008) showed that among the HIV-1 subtype B HXB2 IN mutants tested (Q148K/R, T66I, F121Y, N155H and E92Q), F121Y demonstrated the highest reduction in

ST capacity comparable to the ~25% to ~50% ST activity reported using a dual ST/3'-P gel-based assay. This is comparable to our data (36.8% ST activity). Interestingly, only the single mutation H114Y, and not the $FV6^{H114L}$ (Table 3.2) has been reported by Goethals *et al*., (2008) with observations of negligible reduction in subtype B virus RC. This is the first report showing a substantial loss in IN ST efficiency due to the H114L substitution in IN as a result of RAL pressure.

The N155H exhibited a 30.5% reduction in ST activity which is within the reported range of 40% to ~75% reported previously in subtype B IN (Marinello *et al*., 2008; Metifiot *et al*., 2011; Abram *et al*., 2013). The R263K substitution alone is associated with impaired (by >50%) ST activity in subtype B IN across the range of protein concentrations tested (23-1440 nM; Mesplède *et al*., 2013) in contrast to the insignificant loss (3.4%) in ST efficiency of $FV6^{R263K}$ observed at IN concentrations of 200 nM using a similar plate-based IN assay.

Varying results have been reported in the literature for the ST capacity for T66I and E92Q IN mutants. ST capacities of mutant INs T66I and E92Q have been reported at approximately 20% and 50% in subtype B IN, respectively (Marinello *et al*., 2008). This is different from our findings, where $FV6^{T66I}$ and $FV6^{E92Q}$ exhibited 62.9% and 78.8% ST activity, respectively. Additional studies show more differential patterns. For example, in Malet *et al* (2008) the E92Q mutant demonstrated only a 12% ST activity in a gel-based IN subtype B ST reaction, whereas T66I has also been reported to exhibit 88%-103% ST activity in subtype B IN (Fikkert *et al*., 2003). Abram *et al*., (2013) reported approximately 40% and 70% for T66I and E92Q mutants, respectively.

Overall, the ST capacity of some HIV-1 subtype C mutants are different from subtype B, and warrant further investigation to evaluate their impact (and the impact of double mutants) on RC and virological failure in IN-treated patients in South Africa.

4.5. HIV-1 IN DNA binding kinetics

SPR was used for the first time in this study to investigate the affinity of the selected IN mutants on retroviral dDNA binding with respect to the $FV6^{WT}$ IN enzyme, a step which is essential for the HIV-1 IN ST reaction.

Previous work looking at the kinetics of strands transfer is limited, and is based on determining kinetics using a scintillation proximity assay (Dicker *et al.*, 2008), and IN-DNA binding on a microplate based assay (Quashie *et al.*, 2012). Dicker *et al.*, (2008) conducted biochemical analyses on the HIV-1 subtype B NL_{4-3} WT and eight MT (T66I, V75I, E92Q, Q148R, N155H, T66I/E92Q, T66I/N155H and V75I/M154I) enzymes. Overall, they showed lower affinity binding of the INSTI to the integrase complex, as a result of faster dissociation rates. Moreover, Q148R was the most defective enzyme. Although different methodologies were used, our results showed that only $FV6^{N155H}$ exhibited a faster dissociation rate compared to the $FV6^{WT}$ IN. The overall affinity of the donor DNA for IN (K_D) was 4.36 fold higher in the T66I mutant, attributed to the increased association rates compared to the $FV6^{WT}$ IN, whereas all other IN mutants exhibited 2.99, 2.34, 1.65, 2.07, 2.99 and 3.23 fold lower overall affinities for donor DNA of IN^{E92Q}, IN^{H114L}, IN^{F121Y} IN^{Q148R}, IN^{N155H} and IN^{R263K}, respectively.

The fold increases/decreases of overall DNA binding affinities of the IN variants does not parallel the ST data (as presented in Figure 3.8 and discussed in section 4.3 above). For example, despite the 3.23 fold lower overall affinity of DNA binding to the IN^{R263K}, the enzyme is still capable of performing the ST step with 96.6% efficiency. The structure of the full length HIV-1 IN remains unresolved, but Hare *et al.*, (2010ab) has reported a full length crystal structure of a related prototype foamy virus (PFV) IN (Hare *et al.*, 2010a) and cocrystal structures of the PFV intasome with RAL and EVG (Hare *et al.*, 2010b) . The PFV IN serves as a reliable surrogate for structural analyses due to active-site similarities (Abram *et al.*, 2013). Hare *et al.*, (2012) showed that the main chain amides of the PFV IN involved in DNA contact were Tyr212 and Gln186, which structurally correspond to HIV-1 Tyr143 and Asn117, respectively (Hare *et al.*, 2010b). In the PFV structure, the Tyr212 is located directly next to the drug binding pocket and the PFV side chain of Tyr212 (analogous to HIV-1 Tyr143) makes π-π stacking interactions with bound RAL; these interactions are very likely substantial contributors to inhibitor binding in the PFV IN. Furthermore, due to the similarity in the structure and function of the PFV and HIV-1 integrase enzymes, it is possible to draw conclusions on the manner in which mutations at position Tyr143 of HIV-1 IN can impact on drug efficacy (Hare *et al.*, 2012). However, mutations affecting drug efficacy may not necessarily affect DNA binding, just as direct INSTI and amino acid residue

interaction is not required for drug resistance. For example, PFV IN residues Ser217 and Asn224, corresponding to HIV-1 IN Gln148 and Asn155 respectively, do not directly contact bound INSTIs in the cocrystal structures, leaving the molecular basis of resistance for these major pathways open to further research (Hare *et al*., 2012). Moreover, substitutions at positions Ser217 (analogous to HIV-1 Q148) and Asn224 (analogous to HIV-1 N155) were observed to hinder metal coordination by only binding a single metal atom, due to subtle conformational changes within the active site (Hare *et al*., 2010).

4.6. Overall impact of mutations on structure and function

HIV-1 IN is comprised of an N-terminal zinc finger domain, a C-terminal domain vital for DNA binding and both flank the central catalytic core domain (CCD), key for catalytic activity (Engelman *et al*., 1991; Dyda *et al*., 1994). The T66I, E92Q, H114L Q148R and N155H substitutions border the catalytic pocket (Figure 3.4) in proximity to the catalytic DDE triad (D116, D64 and E152) (Hare *et al*., 2010a). The T66 amino acid borders the active site, whilst two of the DDE triad residues (D64 and E152) surround N155, the E92 residue is surrounded by DDE triad residues (D64 and D116), whilst all three catalytic DDE triad (D116, D64 and E152) residues are in close proximity to the Q148 residue. Since the Q148 residue lies within the catalytic β4-α2 loop, Q148RHK substitutions would change the flexibility of this loop, and could result in reduced efficiency of D64 and E152 metal ion binding (Dicker et al., 2007), impede retroviral DNA 5'-end binding (Johnson *et al*., 2006), and induce decreased hydrogen-bond interactions between Q148 and E152 (Hare *et al*., 2011). The T66 residue is located within the β2 sheet distal from the catalytic DDE triad in close proximity to the viral DNA 3'-end and the N155 residue. Substitutions at this T66 position are associated with reduced viral DNA binding due to altered interactions with the retroviral DNA 3'-end. The T66 substitution also alters metal ion coordination due to its effects on residue N155 primarily associated with metal cofactor binding (Goethals *et al*., 2008; Hare *et al*., 2010b; Hightower *et al*., 2011). The N155 residue is located within the α4-helix and orientates directly into the active site. The H155 substitution forms hydrogen bonds with the key metal ion binding residue D116, which is associated with altered metal binding and reduced ST efficiency (Hazuda *et al*., 2004). Moreover, substitutions of residue N155 alter the salt bridge with the phosphate of the terminal 3'-adenosine resulting in a widened

base of the catalytic pocket, and similarly alter metal ion coordination within the catalytic triad (at residue E152) (Hare *et al*., 2010b; Hightower *et al*., 2011).

The E92 residue is positioned within a β3-α1 loop in close proximity with the active site. The E92Q substitution is associated with a loss of the negative charge, which makes metal ion coordination and hydrogen bonding with water molecules less favourable (Goethals et al., 2008). The R263 residue is positioned within the C-terminal domain essential for DNA binding. The R263K substitution is associated with a loss of three hydrophilic amines, neutral in charge, replaced by one negatively charged amine and a change in amino acid orientation. Overall, these INSTI resistance mutations introduce subtle modifications in the local IN secondary structure, which results in altered metal ion coordination and changes in the interactions with retroviral DNA.

Amino acid substitutions will impact on IN stability, which results from changes in the degree of hydrophilicity, hydrophobicity, charge and size. For instance, T66I and H114L were shown to gain hydrophobic side chains, whereas F121Y (positioned on the opposite side of the active site on the α-helix) and Q148R were shown to gain hydrophilic side chains (Figure 3.5B, D and Figure 3.5C, F). The fragment encompassing amino acid residues 140-149 (the flexible loop (G140-G149)) is associated with low diffraction, and is therefore consistently not well resolved (Chiu and Davies, 2004; Jaskolski *et al*., 2009). The two glycines (G140 and G149) act at each end as hinges; due to their small side chain size they allow a high degree of rotation of the poly-peptide backbone. Greenwald *et al*., (1999) demonstrated conformational restriction by substitution of the two glycine molecules with alanine leading to complete resolution and minimized flexibility (Greenwald *et al*., 1999). Esposito and Craigie (1998) demonstrated a loss in 3'-P and ST activities due to substitutions Q148HKR, attributed to the loss in interaction efficiency with the tip of viral DNA LTR, as well as chemical cross linking between the IN residue at position 148 and the 5'-C on the overhang of the viral DNA lower strand. The bulkier side chains of the acquired amino acids were also reported to alter viral DNA binding, thereby inhibiting both IN 3'-P and ST reactions (Métifiot *et al*., 2010b). Overall, the mutated amino acids are associated with subsequent changes in IN function and overall structure, as defined by their side chains.

4.7. Concluding remarks

HIV-1 subtype C primary virus isolates were selected *in vitro* in primary PBMCs under INI pressure and found to select for the described major IN mutations, similar to what has been reported from patient clinical samples worldwide. Although most of the mutations identified herein have been described before, this study serves as the first description of resistance mutations and one H114L selected in HIV-1 subtype C within the South African context. Mutations selected with RAL (Q148R and N155H) and EVG (T66IK) served as a confirmation of cross resistance between both drugs in HIV-1 subtype C comparable to subtype B, as reported in western countries where the drugs first become available. Interestingly, DTG selected for negligible levels of substitutions at positions (associated with resistance to RAL and EVG) T66, Y143, Q148 and N155. However, these mutants remained inhibited as minor quasispecies, and likely did not expand due to drug pressure. This led to the supposition that, while cross resistance (to RAL and EVG) mutations were selected by DTG, for these mutations to gain prevalence within HIV-1 subtype C quasispecies and induce resistance towards DTG, requires accumulation of a combination of substitutions at resistance-associated (or potentially unknown) positions.

This study serves as the first to report on the impact of HIV-1 subtype C integrase mutations on ST activity within the South African context, and the first time that SPR has been utilized as a technique to describe the DNA binding affinities to IN. All resistance mutations selected were confirmed to induce loss in ST efficiency, and the biochemical (losses in ST efficiency) patterns proved significant (with the exception of R263K) and dissimilar to non-subtype C IN ST patterns reported previously. The loss in ST efficiency can be associated to $FV6^{MT}$ DNA binding and this can only be confirmed to be one of many contributing factors (i.e. metal ion coordination, effects of mutations on IN local secondary structure), especially with substitutions that were determined to be in close proximity to the active site.

4.8. Ongoing/future work

This study forms the basis for continued research into the relevance of naturally occurring polymorphisms as well as the known major (and rare) primary and accessory mutations in HIV-1 subtype C IN, in order to better inform patient responses and outcomes on INSTI-containing regimens.

Because we experienced great difficulty in expanding the mutated primary viruses in PBMCs for growth competition assays to compare relative viral fitness of WT and mutant viruses, future work should focus on cloning the IN mutants into a backbone to generate replication competent virus to be used in these assays. Phenotypic inhibition assays in the presence of each INSTI could then give an accurate indication of the fold differences in drug susceptibility. Site-directed mutagenesis can be used to further evaluate the impact of all the detected naturally occurring polymorphisms on enzyme activity. Additionally, SPR experiments can be repeated in the presence of each inhibitor to provide more insights into enzyme functions.

As RAL use in the South African public sector increases, it is expected that patients with virological failure will develop drug resistance mutations to RAL. Emerging data on the mutation patterns will be used to generate additional mutants and evaluate their impact on the biological functioning of the enzyme.

CHAPTER 5: REFERENCES

Abram ME, Hluhanich RM, Goodman DD, Andreatta KN, Margot NA, Ye L, Niedziela-Majka A, Barnes TL, Novikov N, Chen X, Svarovskaia ES, McColl DJ, White KL and Miller MD. Impact of primary elvitegravir resistance-associated mutations in HIV-1 integrase on drug susceptibility and viral replication fitness. *Antimicrobial Agents and Chemotherapy* 2013, 57: 2654 - 2663

Abraham L and Fackler OT. HIV-1 Nef: a multifaceted modulator of T cell receptor signalling. *Cell Communication and Signaling* 2012, 10: 39-50

Aghokeng AF1, Ewane L, Awazi B, Nanfack A, Delaporte E, Zekeng L and Peeters M. Enfuvirtide binding domain is highly conserved in non-B HIV type 1 strains from Cameroon, West Central Africa. *AIDS Research and Human Retroviruses* 2005, 21: 430-433

Aiken C and Trono D. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *Journal of Virology* 1995, 69: 5048–5056

Ajose O, Mookerjee S, Mills EJ, Boulle A and Ford N. Treatment outcomes of patients on second-line antiretroviral therapy in resource-limited settings: a systematic review and meta-analysis. *AIDS* 2012, 26: 929-938

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM and Berger EA. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996, 272: 1955-1958

Al-Mawsawi LQ, Al-Safi RI, Neamati N: Anti-infectives: clinical progress of HIV-1 integrase inhibitors. *Expert Opinion on Emerging Drugs* 2008, 13: 213-225

Anastassopoulou, CG, Ketas TJ, Klasse PJ and Moore JP. Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41. *Proceedings of the National Academy of Sciences USA* 2009, 106: 5318–5323

Anderson DJ, Politch JA, Nadolski AM, Blaskewicz CD, Pudney J and Mayer KH. Targeting Trojan horse leukocytes for HIV prevention. *AIDS* 2010, 24: 163–187

Arion D, Kaushik N, McCormick S, Borkow G and Parniak MA. Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* 1998, 37: 15908-15917

Arhel N. Revisiting HIV-1 uncoating. *Retrovirology* 2010, 7: doi: 10.1186/1742-4690-7-96

Armenia D, Vandenbroucke I, Fabeni L, Van Marck H, Cento V, D'Arrigo R, Van Wesenbeeck L, Scopelliti F, Micheli V, Bruzzone B, Lo Caputo S, Aerssens J, Rizzardini G, Tozzi V, Narciso P, Antinori A, Stuyver L, Perno CF and Ceccherini-Silberstein F. Study of genotypic and phenotypic HIV-1 dynamics of integrase mutations during raltegravir treatment: a refined analysis by ultradeep 454 pyrosequencing. *The Journal of Infectious Diseases* 2012, 205: 557 - 567

Arnold E, Jacobo-Molina A, Nanni RG, Williams RL, Lu X, Ding J, Clark AD Jr, Zhang A, Ferris AL, Clark P, Hizi A and Hughes SH. Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. *Nature* 1992, 357: 85 - 89

Arthur LO, Bess JW, Sownder RC, Benveniste RE, Mann DL, Chermann JC and Henderson LE. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccine. *Science* 1992: 18, 1935-1938

Avert, South Africa HIV & AIDS Statistics. http://www.avert.org/south-africa-hiv-aids-statistics.htm. Accessed 2014-01-15

Bacheler LT, Anton ED, Kudish P, Baker D, Bunville J, Krakowski K, Bolling L, Aujay M, Wang XV, Ellis D, Becker MF, Lasut AL, George HJ, Spalding DR, Hollis G and Abremski K. Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. *Antimicrobial Agents and Chemotherapy* 2000, 44: 2475-2484

Back NK, Nijhuis M, Keulen W, Boucher CA, Oude Essink BO, van Kuilenburg AB, van Gennip AH and Berkhout B. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *The EMBO Journal* 1996, 15: 4040-4049

Banerjee A, Benjamin R, Balakrishnan K, Ghosh P and Banerjee S. Human protein Staufen-2 promotes HIV-1 proliferation by positively regulating RNA export activity of viral protein Rev. *Retrovirology* 2014, 11: doi:10.1186/1742-4690-11-18

Barbour JD, Wrin T, Grant RM, Martin JN, Segal MR, Petropoulos CJ and Deeks SG. Evolution of phenotypic drug susceptibility and viral replication capacity during long-term virologic failure of protease inhibitor therapy in human immunodeficiency virus-infected adults. *Journal of Virology* 2002, 76: 11104-11112

Bar-Magen T, Donahue DA, McDonough EI, Kuhl BD, Faltenbacher VH, Xu H, Michaud H, Sloan RD and Wainberg MA. HIV-1 subtype B and C integrase enzymes exhibit differential patterns of resistance to integrase inhibitors in biochemical assays. *AIDS* 2010, 24: 2171–2179

Bar-Magen T, Sloan RD, Faltenbacher VH, Donahue DA, Kuhl BD, Oliveira M, Xu H and Wainberg MA. Comparative biochemical analysis of HIV-1 subtype B and C integrase enzymes. *Retrovirology* 2009, 6: 103-113

Barre-Sinousi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L. Isolation of a T-lymphotropic retrovirus from patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* 1983, 220: 868-871

Bártolo I, Rocha C, Bartolomeu J, Gama A, Marcelino R, Fonseca M, Mendes A, Epalanga M, Silva PC and Taveira N. Highly divergent subtypes and new recombinant forms prevail in the HIV/AIDS epidemic in Angola: New insights into the origins of the AIDS pandemic. *Infection, Genetics and Evolution* 2009, 9: 672-682

Barat C, Lullien V, Schatz O, Keith G, Nugeyre MT, Grüninger-Leitch F, Barré-Sinoussi F, LeGrice SF and Darlix JL. HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *The EMBO Journal* 1989, 8: 3279–3285

Berkhout B, Silverman RH and Jeang K-T. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell* 1989, 59: 273-282

Berro R, Sanders RW, Lu M, Klasse PJ and Moore JP. Two HIV-1 variants resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. *Plos Pathogens* 2009, 5: 1-17

Bessong PO and Nwobegahay J. Genetic Analysis of HIV-1 integrase sequences from treatment naive individuals in northeastern South Africa. *International Journal of Molecular Sciences* 2013, 14: 5013-5024

Blanco JL, Varghese V, Rhee SY, Gatell JM and Shafer RW. HIV-1 integrase inhibitor resistance and its clinical implications. *Journal of Infectious Disease* 2011, 203: 1204-1214

Bonhoeffer S and Sniegowski P. Virus evolution: The importance of being erroneous. *Nature* 2002, 420: 367-369

Bour S, Schubert U and Strebel K. The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. *Journal of Virology* 1995, 69: 1510–1520

Bowerman B, Bishop PO, Bishop JO and Varmus HE. A nucleoprotein complex mediates the integration of retroviral DNA. *Genes and Development* 1989, 3: 469-478

Boyer PL, Sarafianos SG, Arnold E and Hughes SH. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *Journal of Virology* 2001, 75: 4832-4842

Boyer PL, Currens M J, McMahon JB, Boyd MR and Hughes SH. Analysis of nonnucleoside drug-resistant variants of human immunodeficiency virus type 1 reverse transcriptase. *Journal of Virology* 1993, 67: 2412–2420

Brenner BG. Resistance and viral subtypes: how important are the differences and why do they occur*? Current Opinion in HIV & AIDS* 2007, 2: 94–102

Brenner BG, Lowe M, Moisi D, Hardy I, Gagnon S, Charest H, Baril JG, Wainberg MA and Roger M. Subtype diversity associated with the development of HIV-1 resistance to integrase inhibitors. *Journal of Medical Virology* 2011, 83: 751 - 759

Bretscher MT, Althaus CL, Müller V and Bonhoeffer S. Recombination in HIV and the evolution of drug resistance: for better or for worse? *BioEssays* 2004, 26: 180-188

Brown PO, Bowerman B, Varmus HE and Bishop JM. Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. *Proceedings of the National Academy of Sciences USA* 1989, 86: 2525-2529

Bukrinsky M and Adzhubei A. Viral protein R of HIV-1. *Reviews in Medical Virology* 1999, 9: 39–49

Bukrinsky M. A hard way to the nucleus. *Molecular Medicine* 2004, 10: 1-5

Cahn P, Pozniak AL, Mingrone H, Shuldyakov A, Brites C, Andrade-Villanueva JF, Richmond G, Buendia CB, Fourie J, Ramgopal M, Hagins D, Felizarta F, Madruga J, Reuter T, Newman T, Small CB, Lombaard J, Grinsztejn B, Dorey D, Underwood M, Griffith S and Min S. Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study. *Lancet* 2013, 382: 700-708

Canducci F, Barda B, Ceresola E, Spagnuolo V, Sampaolo M, Boeri E, Nozza S, Cossarin F, Galli A, Gianotti N, Castagna A, Lazzarin A and Clementi M. Evolution patterns of raltegravir-resistant mutations after integrase inhibitor interruption. *Clinical Microbiology and infectious Diseases* 2011, 17: 928-934

Canducci F, Marinozzi MC, Sampaolo M, Boeri E, Spagnuolo V, Gianotti N, Castagna A, Paolucci S, Baldanti F, Lazzarin A and Clementi M. Genotypic/phenotypic patterns of HIV-1 integrase resistance to raltegravir. *Journal of Antimicrobial Chemotherapy* 2010, 65: 425-433

Canducci F, Sampaolo M, Marinozzi MC, et al. Dynamic patterns of human immunodeficiency virus type 1 integrase gene evolution in patients failing raltegravir-based salvage therapies. *AIDS* 2009, 23: 455–460

Carr JK, Wolfe ND, Torimiro JN, Tamoufe U, Mpoudi-Ngole E, Eyzaguirre L, Birx DL, McCutchan FE and Burke DS. HIV-1 recombinants with multiple parental strains in low-prevalence, remote regions of Cameroon: evolutionary relics? *Retrovirology* 2010, 7: 39-47

Casadellà M, van Ham PM, Noguera-Julian M, Pou C, Struck D, van Kessel A, Clotet B, Boucher C, Paredes R and Wensing AM on behalf of the SPREAD programme. Primary resistance to integrase strand-transfer inhibitors in Europe. Poster 580 Conference on Retroviruses and Opportunistic Infections (CROI) 2014, Boston, USA

Castagna A, Maggiolo F, Penco G, Wright D, Mills A, Grossberg R, Molina JM, Chas J, Durant J, Moreno S, Doroana M, Ait-Khaled M, Huang J, Min S, Song I, Vavro C, Nichols G, and Yeo JM for the Viking-Study Group. Dolutegravir in antiretroviral-experienced patients with raltegravir- and/or elvitegravir-resistant HIV-1: 24-week results of the phase III VIKING-3 study. *Journal of Infectious Diseases* 2014, 210: 354 - 362

Ceccherini-Silberstein F, Malet I, D'Arrigo R, Antinori A, Marcelin AG and Perno CF. Characterisation Characterisation and structural analysis of HIV-1 integrase conservation. *AIDS Reviews* 2009, 11: 17-29

Ceccherini-Silberstein F, Van Baelen K, Armenia D, Trignetti M, Rondelez E, Fabeni L, Scopelliti F, Pollicita M, Van Wesenbeeck L, Van Eygen V, Dori L, Sarmati L, Aquaro S, Palamara G, Andreoni M, Stuyver LJ and Perno CF. Secondary integrase resistance mutations found in HIV-1 minority quasisspecies in integrase therapy-naïve patients have little or no effect on susceptibility to integrase inhibitors. *Antimicrobial Agents and Chemotherapy* 2010, 54: 3938-3948

Chan DC and Kim PS. HIV entry and its inhibition. *Cell* 1998, 93: 681-684

Chan DC, Fass D, Berger JM and Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 1997, 89: 263-273

Chamberlain PP, Ren J, Nichols CE, Douglas L, Lennerstrand J, Larder BA, Stuart DI and Stammers DK. Crystal structures of Zidovudine- or Lamivudine-resistant human immunodeficiency virus type 1 reverse transcriptases containing mutations at codons 41, 184, and 215. *Journal of Virology* 2002, 76: 10015-10019

Charneau P and Clavel F. A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract. *Journal of Virology* 1991, 65: 2415-2421

Charneau P, Alizon M and Clavel F. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. *Journal of Virology* 1992, 66: 2814-2820

Chen CH, Mathews TJ, McDanal CB, Bolognesi DP and Greenberg ML. A molecular clasp in the human immunodeficiency virus (HIV) type 1 TM protein determines the anti-HIV activity of gp41 derivatives: implication for viral fusion. *Journal of Virology* 1995, 69: 3771-3777

Chen RY, Kilby JM and Saag MS. Enfuvirtide. *Expert Opinion on Investigational Drugs* 2002, 11: 1837-1843

Chen N-Y, Zhou L, Gane PG, Price A, Zufferey M, Luban J, James L, Selwood D and Fassati A. HIV-1 uncoating in human CD4+ T cells: Kinetic and functional analyses. *Retrovirology* 2013, 10: P13-P13 (Poster Presentation)

Chen Z, Luckay A, Sodora DL, Telfer P, Reed P, Gettie A, Kanu JM, Sadek RF, Yee J, Ho DD, Zhang L and Marx PA. Human immunodeficiency virus type-2 (HIV-2) seroprevalence and characterisation characterisation of distinct HIV-2 genetic subtype from the natural range of SIV-infected sooty managabeys. *Journal of Virology* 1996, 71: 3953-3960

Chen Z, Yan Y, Munshi S, Li Y, Zugay-Murphy J, Xu B, Witmer M, Felock P, Wolfe A, Sardana V, Emini EA, Hazuda D and Kuo LC. X-ray structure of simian immunodeficiency virus integrase containing the core and c-terminal domain (residues 50-293) - an initial glance of the viral DNA binding platform. *Journal of Molecular Biology* 2000, 296: 521–533

Chesebro B, Nishio J, Perryman S, Cann A, O'Brien W, Chen IS and Wehrly K. Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *Journal of Virology* 1991, 65: 5782-5789

Chiu TK and Davies DR. Structure and function of HIV-1 integrase. *Current Topics in Medicinal Chemistry* 2004, 4: 965-977

Cho MW, Lee MK, Carney MC, Berson JF, Doms RW and Martin MA. Identification of determinants on a dual tropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. *Journal of Virology* 1991, 72: 2509-2515

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C and Sodroski J. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 1996, 85: 1135-1148

Chow SA. In vitro assays for activities of retroviral integrase. *Methods* 1997, 12: 306-317

Cilliers T1, Patience T, Pillay C, Papathanasopoulos M and Morris L. Sensitivity of HIV type 1 subtype C isolates to the entry inhibitor T-20. *AIDS Research and Human Retroviruses* 2004, 20: 477-482

Cingolani A, Antinori A, Rizzo MG, Murri R, Ammassari A, Baldini F, Di Giambenedetto S, Cauda R and De Luca A. Usefulness of monitoring HIV drug resistance and adherence in individuals failing highly active antiretroviral therapy: a randomized study (ARGENTA). *AIDS* 2002, 16: 369-379

Clavel F and Hance AJ. HIV drug resistance. *New England Journal of Medicine* 2004, 350: 1023-1035

Coakley E, Petropoulos CJ and Whitcomb JM. Assessing chemokine co-receptor usage in HIV. *Current Opinion in Infectious Diseases* 2005, 18: 9–15

Cocohoba J and Dong BJ. Raltegravir: the first HIV integrase inhibitor. *Clinical Therapeutics* 2008, 30: 1747–1765

Codoñer FM, Pou C, Thielen A, García F, Delgado R, Dalmau D, Santos JR, Buzón MJ, Martínez-Picado J, Alvarez-Tejado M, Clotet B, Ruiz L and Paredes R. Dynamic escape of pre-existing raltegravir-resistant HIV-1 from raltegravir selection pressure. *Antiviral Research* 2010, 88: 281-286

Coffin JM. Genetic variation in AIDS viruses. *Cell* 1986, 46: 1-4

Coffin JM. Genetic diversity and evolution of retroviruses. *Current Topics in Microbiology and Immunology* 1992, 176: 143-164

Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* 1995, 267: 483-489

Cong M, Heneine W and García-Lerma JG. The Fitness Cost of Mutations Associated with Human Immunodeficiency Virus Type 1 Drug Resistance Is Modulated by Mutational Interactions. *Journal of Virology* 2007, 81: 3037-3041

Connell BJ, Michler K, Capovilla A, Venter WDF, Stevens WS and Papathanasopoulos MA. Emergence of X4 usage among HIV-1 subtype C: evidence for an evolving epidemic in South Africa. *AIDS* 2008, 22: 896–899

Corbet S, Müller-Trutwin MC, Vermisse P, Delarue S, Ayouba A, Lewis J, Brunak S, Martin P, Brun-Vezinet F, Simon F, Bare-Sinoussi F and Mauclére P. *env* sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. *Journal of Virology* 2000, 74: 529-534

Costin JM. Cytopathic mechanism of HIV-1. *Virology Journal* 2007, 4: 1-22

Coetzee D, Hildebrand K, Boulle A, Maartens G, Louis F, Labatala V, Reuter H, Ntwana N and Goemaere E. Outcomes after two years of providing antiretroviral treatment in Khayelitsha, South Africa. *AIDS* 2004, 18: 887-895

Craigie R. HIV integrase, a Brief Overview from chemistry to therapeutics. *The Journal of Biological Chemistry* 2001, 276: 23213-23216

Das AT, Klaver B and Berkhout B. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA (3Lys). *Journal of Virology* 1995, 69: 3090-3097

Da Silva D, Van Wesenbeeck L, Breilh D, Reigadas S, Anies G, van Baelen K, Morlat P, Neau D, Dupon M, Wittkop L, Fleury H and Masquelier B. HIV-1 resistance patterns to integrase inhibitors in antiretroviral-experienced patients with virological failure on raltegravir-containing regimens. *Journal of Antimicrobial Chemotherapy* 2010, 65: 1262-1269

Dauber DS, Ziermann R, Parkin N, Maly DJ, Mahrus S, Harris JL, Ellman JA, Petropoulos C and Craik CS. Altered substrate specificity of drug-resistant human immunodeficiency virus type 1 protease. *Journal of Virology* 2002, 76: 1359-1368

De Clercq E. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in therapy of HIV-1 infection. *Antiviral Research* 1998, 38: 153-179

De Clercq E. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): past, present and future. *Chemistry and Biodiversity* 2004, 1: 44-64

De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *International Journal of Antimicrobial Agents* 2009, 33: 307-320

De Clercq E. Antiretroviral drugs. *Current Opinion in Pharmacology* 2010, 10: 507-515

De Clercq E. Tenofovir disoproxil fumarate (TDF): discovery and clinical development. In Antiviral Drugs: Biology, Chemistry, Clinic. Edited by Kazmierski WM. John Wiley Sons Inc., Hoboken, NJ; 2010

De Clercq E. Antivirals: Past, present and future. *Biochemical Pharmacology* 2013, 85: 727-744

Deeks E. Cobicistat: A review of its use as a pharmacokinetic enhancer of Atazanavir and duranavir in patients with HIV-1 infection. *Drugs* 2013, 74: 195-206

Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK and Grant RM. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *New England Journal of Medicine* 2001, 344: 472-480

DeJesus E, Berger D, Markowitz M, Cohen C, Hawkins T, Ruane P, Elion R, Farthing C, Zhong L, Cheng AK, McColl D, Kearney BP and the 183-0101 Study Team. Antiviral Activity, Pharmacokinetics, and Dose Response of the HIV-1 Integrase Inhibitor GS-9137 (JTK-303) in Treatment-Naive and Treatment-Experienced Patients. *Journal of Acquired Immune Deficiency Syndromes* 2006, 43 1-5

DeJesus E, Rockstroh JK, Henry K, Molina JM, Gathe J, Ramanathan S, Wei X, Yale K, Szwarcberg J, White K, Cheng AK and Kearney BP. Co-formulated elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate versus ritonavir-boosted atazanavir plus co-formulated emtricitabine and tenofovir disoproxil fumarate for initial treatment of HIV-1 infection: a randomised, double-blind, phase 3, non-inferiority trial. *Lancet* 2012, 379: 2429 – 2438

Decroly E, Vandenbranden M, Ruysschaert JM, Cogniaux J, Jacob GS, Howard SC, Marshall C, Kompelli A, Basak A, Jean F, Lazure C, Bedannet S, Chrétien M, Day R and Seidah NG. The convertases furin and PC1 can both cleave the human immunodeficiency virus (HIV)-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-I TM). *Journal of Biological Chemistry* 1994, 269: 12240-12247

Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK and Grant RM. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *New England Journal of Medicine* 2001, 344: 472-480

De Jong JJ, De Ronde A, Keulen W, Tersmette M and Goudsmit J. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *Journal of Virology* 1992, 66: 6777-6780

De Jong JJ, Goudsmit J, Keulen W, Klaver B, Krone W, Tersmette M and de Ronde A. Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. *Journal of Virology* 1992, 66: 757-765

Delelis O, Carayon K, Deprez E and Mouscadet J-F. Integrase and integration: biochemical activities of HIV-1 integrase. *Retrovirology* 2008; 5: 1-13

Delelis O, Thierry S, Subra F, Simon F, Malet I, Alloui C, Sayon S, Calvez V, Deprez E, Marcelin AG, Tchertanov L and Mouscadet JF. Impact of Y143 HIV-1 integrase mutations on resistance to raltegravir *in vitro* and *in vivo*. *Antimicrobial Agents and Chemotherapy* 2010, 54:491–501

De Luca A. The impact of resistance on viral fitness and its clinical implications. In.: Geretti AM, editor. Antiretroviral resistance in clinical practice. London: Mediscript; 2006. Chapter 12. Available online at: http://www.ncbi.nlm.nih.gov/books/NBK2244/

De Luca A, Bates M and Di Giambenedetto Sl. HIV-1 replication capacity (RC) in HAART-failing patients predicts virologic and immunologic responses when accounting for viral susceptibility to the salvage regimen: results from the Argenta trial. *10th Conference on Retroviruses and Opportunistic Infections* 2005, Boston. (Abstract. 692)

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Scahll TJ, Littman DR and Landau NR. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996, 381: 661-666

Descamps D, Colin G, Letourneur F, Apetrei C, Damond F, Loussert-Ajaka I, Simon F, Saragosti S and Brun-Vézinet F. Susceptibility of human immunodeficiency virus type 1 group O isolates to antiviral agents: in vitro phenotypic and genotypic analyses. *Journal of Virology* 1997, 71: 8893-8898

Descamps D, Apetrei C, Collin G, Damond F, Simon F and Brun-Vézinet F. Naturally occurring decreased susceptibility of HIV-1 subtype G to protease inhibitors. *AIDS* 1998, 12: 1109-1111

Derwent Abstract No. 1999-580735/49, "New indole derivatives are integrase inhibitors useful as antiviral and anti-HIV agents" *abstract of WO 99/50245 Shionogi & Co, Ltd* 1999

Dicker IB, Samanta HK, Li Z, Hong Y, Tian Y, Banville J, Remillard RR, Walker MA, Langley DR and Krystal M. Changes to the HIV long terminal repeat and to HIV integrase differentially impact HIV integrase assembly, activity, and the binding of strand transfer inhibitors. *Journal of Biological Chemistry* 2007, 282: 31186-31196

Dicker IB, Terry B, Lin Z, Li Z, Bollini S, Samanta HK, Gali V, Walker MA and Krystal MR. Biochemical analysis of HIV-1 integrase variants resistant to strand transfer inhibitors. *The Journal of Biological Chemistry* 2008, 283: 23599-23609

Doms RW. Beyond receptor expression: the influence of receptor conformation, density and affinity in HIV-1 infection. *Virology* 2000, 276: 229-237

Doms RW and Trono D. The plasma membrane as a combat zone in the HIV battlefield. *Genes and Development* 2000, 14: 2677-2688

Dvorin JD and Malim MH. Intracellular trafficking of HIV-1 cores: journey to the center of the cell. *Current Topics in Microbiology and Immunology* 2003, 281: 179-208

Dyda F, Hickman AB, Jenkins TM, Engelman A, Craigie R and Davies DR. Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. *Science* 1994, 266: 1981-1986

Ellison V, Abrams H, Roe T, Lifson J and Brown P. Human immunodeficiency virus in a cell-free system. *Journal of Virology* 1990, 64: 2711-2715

Endo-Munoz L, Warby T, Harrich D and McMillan NAJ. Phosphorylation of HIV Tat by PKR increases interaction with TAR RNA and enhances transcription. *Virology Journal* 2005, 2: 1-13

Engelman A, Mizuuchi K and Craigie R. HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell* 1991, 67: 1211-1221

Engelman A and Craigie R. Efficient magnesium-dependent human immunodeficiency virus type 1 integrase activity. *Journal of Virology* 1995, 69: 5908-5911

Erickson J and Kempf D. Structure-based design of symmetric inhibitors of HIV-1 protease. *Archives of Virology Supplement* 1994, 9: 19-29

Eron JJ, Young B, Cooper DA, Youle M, Dejesus E, Andrade-Villanueva J, Workman C, Zajdenverg R, Fätkenheuer G, Berger DS, Kunar PN, Rodgers AJ, Shaughnessy MA, Wlaker ML, Barnard RJO, Miller MD, DiNubile MJ, Nguyen B-Y, Leavitt R, Xi X and Sklar P for the SWITCHMARK 1 and 2 investigators. Switch to a raltegravir-based regimen versus continuation of a lopinavir-ritonavir-based regimen in stable HIV-infected patients with suppressed viraemia (SWITCHMRK 1 and 2): two multicentre, double-blind, randomised controlled trials. *The Lancet* 2010, 375: 396-407

Eron JJ, Clotet B, Durant J, Katlama C, Kumar P, Lazzarin A, Poizot-Martin I, Richmond G, Soriano V, Ait-Khaled M, Fujiwara T, Huang J, Min S, Vavro C and Yeo J. Safety and efficacy of dolutegravir in treatment-experienced subjects with raltegravir-resistant HIV type 1 infection: 24-week results of the VIKING study. *Journal of Infectious Diseases* 2013, 207: 740-748

Eron J, Livrozet JM, Morlat P, Lazzarin A, Katlama C, Hawkins T, Fujiwara T, Cuffe R, Vavro C, Santiago J, Ait-Khaled M, Min S and Yeo JM. Activity of the integrase inhibitor S/GSK1349572 in subjects with HIV exhibiting raltegravir resistance: week 24 results of the VIKING study (ING112961). *Journal of the International AIDS Society* 2010, 13 (Suppl 4): O51

Eshleman SH, Hudelson SE, Smith P, Hackett J, Holzmayer V, Swanson P, Devare SG and Marlowe N. Analysis of pol integrase sequences in diverse HIV type 1 strains using a prototype genotyping assay. *AIDS Research and Human Retroviruses* 2009, 25: 343-345

Esnouf RM, Ren J, Hopkins AL, Ross CK, Jones EY, Stammers DK and Stuart DI. Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proceedings of the National Academy of Sciences USA* 1997, 94: 3984-3989

Esposito D and Craigie R. Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein DNA interaction. The *EMBO Journal* 1998, 17: 5832-5843

Esposito D and Craigie R. HIV integrase structure and function. *Advances in Virus Research* 1999, 52: 19-333

Farnet CM and Haseltine WA. Integration of human immunodeficiency virus type 1 DNA in vitro. *Proceedings of the National Academy of Sciences USA 1990*, 87: 4164-4168

Faria NR, Hodges-Mameletzis I, Silva JC, Rodes B, Erasmus S, Paolucci S, Ruelle J, Pieniazek D, Taveira N, Trevin A, Goncalves MF, Jallow S, Xu L, Camacho RJ, Soriano V, Goubau P, de Sousa JD, Vandamme A-M, Suchard MA and Lemey P. Phylogeographical footprint of colonial history in the global dispersal of human immunodeficiency virus type 2 group A. *Journal of General Virology* 2012, 93: 889–899

FDA notifications. FDA approves raltegravir for HIV-1 treatment-naïve patients. AIDS Alert 2009, 24(9):106-107

Feng Y, Broder CC, Kennedy PE and Berger EA. HIV-1 entry cofactor: functional cDNA cloning of seven-transmembrane, G protein-coupled receptor. *Science* 1996, 272: 872-877

Ferns RB, Kirk S, Bennett J, Cook PM, Williams I, Edwards S and Pillay D. The dynamics of appearance and disappearance of HIV-1 integrase mutations during and after withdrawal of raltegravir therapy. *AIDS* 2009 23: 2159-2164

Development of Resistance against Diketo Derivatives of Human Immunodeficiency Virus Type 1 by Progressive Accumulation of Integrase Mutations. *Journal of Virology* 2003, 77: 11459–11470

Finney DJ. Statistical Method in Biological Assay. Hafner Publishing Co., New York, 1952. Illustrated. xix + 661 pp. 22

Fiorentini S1, Marini E, Caracciolo S and Caruso A. Functions of the HIV-1 matrix protein p17. *The New Microbiologica* 2006, 29: 1-10

Fish MQ, Hewer R, Wallis CL, Venter WDF, Stevens WS and Papathanasopoulos MA. Natural polymorphisms of integrase among HIV type 1-infected South African patients. *AIDS Research and Human Retroviruses* 2010, 26: 489-493

Foti M, Phelouzat MA, Holm A, Rasmusson BJ and Carpentier JL. P56Lck anchors CD4 to distinct microdomains on microvilli. *Proceedings of the National Academy of Sciences USA* 2002, 99: 2008-2013

Fransen S, Gupta S, Danovich R, Hazuda D, Miller M, Witmer M, Petropoulos CJ and Huang W. Loss of raltegravir susceptibility by human immunodeficiency virus type 1 is conferred via multiple nonoverlapping genetic pathways. *Journal of Virology* 2009, 83: 11440-11446

Fransen S, Gupta S, Frantzell A, Petropoulos CJ and Huang W. Substitutions at amino acid positions 143, 148 and 155 of HIV-1 integrase define distinct genetic barriers to raltegravir resistance In vivo. *Journal of Virology* 2012, 86: 7249-7255

Franzusoff A, Volpe AM, Josse D, Pichuantes S and Wolf JR. Biochemical and genetic definition of the cellular protease required for HIV-1 gp160 processing. *The Journal of Biological Chemistry* 1995, 270: 3154-3159

Freed EO. HIV replication. *Somatic Cell and Molecular Genetics* 2001, 26: 13-33

Freed EO and Martin MA. HIVs and their replication. In Knipe DM, Howley PM, Griffin D, Lamb RA, Roizman B, Martin MA, Strauss SE, eds. Fields Virology, 4th ed. Philadelphia Lippincott, Williams, and Wilkins 2001: 1971-2041

Froland SS, Jenum P, Lindboe CF, Wefring KW, Linnestad PJ and Böhmer PJ. HIV-1 infection in Norwegian family before 1970. *Lancet* 1988, 1: 1344-1345

Fujiwara T and Mizuuchi K. Retroviral DNA integration: structure of an integration intermediate. *Cell* 1988, 54: 497-504

Fun A, Van Baelen K, van Lelyveld SF, Schipper PJ, Stuyver LJ, Wensing AM and Nijhuis M. Mutation Q95K enhances N155H-mediated integrase inhibitor resistance and improves viral replication capacity. *Journal of Antimicrobial Chemotherapy* 2010, 65: 2300 - 2304

Furfine ES and Reardon JE. Reverse transcriptase. RNase H from the human immunodeficiency virus. Relationship of the DNA polymerase and RNA hydrolysis activities. *Journal of Biological Chemistry* 1991, 266: 406-412

Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J and Safai B. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 1984, 224: 500-503

Galvin SR and Cohen MS. The role of sexually transmitted diseases in HIV transmission. *Nature Reviews Microbiology* 2004, 2: 33-42

Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, ArthurLO, Peeters M, Shaw GM, Sharp PM and Hahn BH. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature* 1999, 397: 436-441

Gao F, Robertson DL, Carruthers CD, Morrison SG, Jian B, Chen Y, Barré-Sinoussi F, Girard M, Srinivasan A, Abimiku AG, Shaw GM, Sharp PM and Hahn BH. A comprehensive panel of near-full-length clones and reference for sequences for non-subtype B isolates of human immunodeficiency virus type 1. *Journal of Virology* 1998, 72: 5680-5698

Gao F, Yue L, White AT, Pappas PG, Barchue J, Hanson AP, Greene BM, Sharp PM, Shaw GM and Hahn BH. Human infection by genetically diverse SIVsm-related HIV-2 in West Africa. *Nature* 1992, 358: 495-499

Garrido C, de Mendoza C and Soriano V. [Resistance to integrase inhibitors]. *Enfermedades Infecciosas y Microbiología Clínica* 2008, 26: 40-66

Garrido C, Geretti AM, Zahonero N, Booth C, Strang A, Soriano V and De Mendoza C. Integrase variability and susceptibility to HIV integrase inhibitors: impact of subtypes, antiretroviral experience and duration of HIV infection. *The Journal of Antimicrobial Chemotherapy* 2010, 65: 320-326

Garrido C, Soriano V, Geretti AM, Zahonero N, Garcia S, Booth C, Gutierrez F, Viciana I and de Mendoza C. Resistance associated mutations to dolutegravir (S/GSK1349572) in HIV-infected patients--impact of HIV subtypes and prior raltegravir experience. *Antiviral Research* 2011, 90: 164-167

Garrido C, Villacian J, Zahonero N, Pattery T, Garcia F, Gutierrez F, Caballero E, Van Houtte M, Soriano V and de Mendoza C on behalf of the SINRES Group. Broad phenotypic cross-resistance to elvitegravir in HIV-infected patients failing on raltegravir-containing regimens. *Antimicrobial Agents and Chemotherapy* 2012, 56: 2873-2878

Gelderblom HR. Assembly and morphology of HIV: potential effect of structure on viral function. *AIDS* 1991, 5: 617-637

Geretti AM, Armenia D and Ceccherini-Silberstein F. Emerging patterns and implications of HIV-1 integrase inhibitor resistance. *Current Opinion in Infectious Diseases* 2012, 25: 677-686

Ghys PD, Saidel T, Vu HT, Savtchenko I, Erasilova I, Mashologu YS, Indongo R, Sikhosana N and Walker N. Growing in silence: selected regions and countries with expanding HIV/AIDS epidemics. *AIDS* 2003, 17 (sppl 4): S45- S50

Gilead Sciences Inc. US prescribing information for Stribild ®, 2013. Available on line at: http://www.gilead.com/~/media/Files/pdfs/medicines/hiv/stribild/stribild_pi.pdf. [Accessed 2015-04-23]

Glushakova S, Yi Y, Grivel JC, Singh A, Schols D, De Clercq E, Collman RG and Margolis L. Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue *ex vivo*. *The Journal of Clinical Investigation* 1999, 104: R5-R11

Goethals O, Clayton R, Van Ginderen M, Vereycken I, Wagemans E, Geluykens P, Dockx K, Strijbos R, Smits V, Vos A, Meersseman G, Jochmans D, Vermeire K, Schols D, Hallenberger S and Hertogs K. Resistance mutations in human immunodeficiency virus type 1 integrase selected with elvitegravir confer reduced susceptibility to a wide range of integrase inhibitors. *Journal of Virology* 2008, 82: 10366-10374

Goethals O, van Ginderen M, Vos A, Cummings MD, van Der Borght K, van Wesenbeeck L, Feyaerts M, Verheyen A, Smits V, van Loock M, Hertogs K, Schols D, Clayton RF: Resistance to raltegravir highlights integrase mutations at codon 148 in conferring cross-resistance to a second generation HIV-1 integrase inhibitor. *Antiviral Research* 2011, 91: 167-176

Goethals O, Vos A, Van Ginderen M, Geluykens P, Smits V, Schols D, Hertogs K and Clayton R. Primary mutations selected in vitro with raltegravir confer large fold changes in susceptibility to first-generation integrase inhibitors, but minor fold changes to inhibitors with second-generation resistance profiles. *Virology* 2010, 402: 338–346

Goldgur Y, Craigie R, Cohen GH, Fujiwara T, Yoshinaga T, Fujishita T, Sugimoto H, Endo T, Murai H, and Davies DR. Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: A platform for antiviral drug design. *Proceedings of the National Academy of Sciences USA* 1999, 96: 13040 - 13043

Goodman D, Hluhanich R, Waters J, Margot N, Fransen S, Gupta S, Huang W, Parkin NT, Borroro-Esoda K, Svarovskaia E, Miller M and McColl D. Integrase inhibitor resistance involves complex interactions among primary and secondary resistance mutations: a novel mutation L68V/I associates with E92Q and increases resistance. *Antiviral Therapy* 2008, 13: A15

Goncalves J, Korin Y, Zack J, and Gabuzda D. Role of Vif in human immunodeficiency virus type 1 reverse transcription. *Journal of Virology* 1996, 70: 8701–8709

Goody RS, Muller B and Restle T. Factors contributing to the inhibition of HIV reverse transcriptase by chain-terminating nucleotides *in vitro* and *in vivo*. *FEBS Letters* 1991, 7: 1-5

Gopalakrishnan V, Peliska JA and Benkovic SJ. Human immunodeficiency virus type 1 reverse transcriptase: spatial and temporal relationship between the polymerase and RNase H activities. *Proceedings of the National Academy of Sciences USA* 1992, 89: 10763-10767

Götte M, Li X and Wainberg MA. HIV-1 reverse transcription: A brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Archives of Biochemistry and Biophysics* 1999, 365: 199-210

Götte M, Fackler S, Hermann T, Perola E, Cellai L, Gross HJ, Le Grice SF and Heumann H. HIV-1 reverse transcriptase-associated RNase H cleaves RNA/RNA in arrested complexes: implications for the mechanism by which RNase H discriminates between RNA/RNA and RNA/DNA. *The EMBO Journal* 1995, 14: 833-841

Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA and Saxon A. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *New England Journal of Medicine* 1981, 305: 1425-1431

Graves MC, Lim JJ, Heimer EP and Kramer RA. An 11-kDa form of human immunodeficiency virus protease expressed in *Escherichia coli* is sufficient for enzymatic activity. *Proceedings of the National Academy of Sciences USA* 1988, 85: 2449–2453

Greenberg ML and Cammack N. Resistance to enfuvirtide, the first HIV fusion inhibitor. *Journal of Antimicrobial Chemotherapy* 2004, 54: 333-340

Greenwald J, Le V, Butler SL, Bushman FD and Choe S. The mobility of an HIV-1 integrase active site loop is correlated withcatalytic activity. *Biochemistry* 1999, 38: 8892-8898

Grobler JA and Hazuda DJ. Resistance to HIV integrase strand transfer inhibitors: in vitro findings and clinical consequences. *Current Opinion in Virology* 2014, 8: 98–103

Hare S, Gupta SS, Valkov E, Engelman A and Cherepanov P. Retroviral intasome assembly amd inhibition of DNA strand transfer. *Nature* 2010, 464: 232-236a

Hare S, Maertens GN and Cherepanov. 3'Processing and strand transfer catalysed by retroviral integrase in crystallo. *The EMBO Journal* 2012, 31: 3020-3028

Hare S, Smith SJ, Metifiot M, Jaxa-Chamiec A, Pommier Y, Hughes SH and Cherepanov P. Structural and functional analyses of the second-generation integrase strand transfer inhibitor dolutegravir (S/GSK1349572). Molecular Pharmacology. 2011, 80:565–572

Hare S, Vos AM, Clayton RF, Thuring JW, Cummings MD and Cherepanov P. Molecular mechanisms of retroviral integrase inhibition and the evolution of viral resistance. *Proceedings of the National Academy of Sciences USA* 2010, 107: 20057-20062b

Harrich D and Hooker B. Mechanistic aspects of HIV-1 reverse transcription initiation. *Reviews of Medical Virology* 2002, 12: 31-45

Harrich D, Ulich C, García-Martínez LF and Gaynor RB. Tat is required for efficient HIV-1 reverse transcription. *The EMBO Journal* 1997, 16: 1224-1235

Harrowe G and Cheng-Mayer C. Amino acid substitutions in the V3 loop are responsible for adaptation to growth in transformed T-cell lines of a primary human immunodeficiency virus type 1. *Virology* 1995, 210: 490-494

Hatano H, Lampiris H, Fransen S, Gupta S, Huang W, Hoh R, Martin JN, Lalezari J, Bangsberg D, Petropoulos C and Deeks SG. Evolution of integrase resistance during failure of integrase inhibitor-based antiretroviral therapy. *Journal of Acquired Immune deficiency Syndrome* 2010, 54: 389-393

Hazuda DJ, Anthony NJ, Gomez RP, Jolly SM, Wai JS, Zhuang L, Fisher TE, Embrey M, Guare JP Jr, Egbertson MS, Vacca JP, Huff JR, Felock PJ, Witmer MV, Stillmock KA, Danovich R, Grobler J, Miller MD, Espeseth AS, Jin L, Chen IW, Lin JH, Kassahun K, Ellis JD, Wong BK, Xu W, Pearson PG, Schleif WA, Cortese R, Emini E, Summa V, Holloway MK and Young SD. A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proceedings of the National Academy of Sciences* USA 2004, 101: 11233-11238.

Hazuda DJ, Felock P, Witmer M, Wolfe A, Stillmock K, Grobler JA, Espeseth A, Gabryelski L, Schleif W, Blau C, Miller MD: Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* 2000, 287: 646-650

Hazuda D, Iwamoto M and Wenning L. Emerging pharmacology: inhibitors of human immunodeficiency virus integration. *Annual Review of Pharmacology and Toxicology* 2009, 49: 377-394

Hazuda DJ, Miller MD, Nguyen BY and Zhao J. Resistance to HIV-integrase inhibitor raltegravir; Analysis of protocol 005, a phase 2 study in patients with triple-class resistant HIV-1 infection. *Antiviral Therapy* 2007, 12: S10

Hemelaar J. The origin and diversity of the HIV-1 pandemic. *Trends in Molecular Medicine* 2012, 18: 182-192

Hemelaar J, Gouws E, Ghys PD and Osmanov S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* 2006, 20: W13-W23

Hicks C and Guilick R. Raltegravir: the first HIV type 1 integrase inhibitor. *Clinical Infectious Diseases* 2009, 48: 931-939

Highleyman L: Early studies demonstrate potent activity and safety of experimental integrase inhibitor S/GSK1265744. http://www.hivandhepatitis.com/2009icr/icaac/docs/092209_a.html

Hightower KE, Wang R, Deanda F, Johns BA, Weaver K, Shen Y, Tomberlin GH, Carter HL, III, Broderick T, Sigethy S, Seki T, Kobayashi M, Underwood MR. Dolutegravir (S/GSK1349572) exhibits significantly slower dissociation than raltegravir and elvitegravir from wild-type and integrase inhibitor-resistant HIV-1 integrase-DNA complexes. *Antimicrobial Agents and Chemotherapy* 2011, 55: 4552–4559

Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH and Johnson PR. An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* 1989, 339: 389-392

Holguín A, Faudon JL, Labernardière JL and Soriano V. Susceptibility of HIV-1 non-B subtypes and recombinant variants to Enfuvirtide. *Journal of Virology* 2007, 38: 176-180

Hu Z and Kuritzkes DR. Effect of raltegravir resistance mutations in HIV-1 integrase on viral fitness. *Journal of Acquired Immune Deficiency Syndrome* 2010, 55: 148-155

Huber HE and Richardson CC. Processing of the primer for plus strand DNA synthesis by human immunodeficiency virus 1 reverse transcriptase. *The Journal of Biological Chemistry* 1990, 265: 10565-10573

Huet T, Cheynier R, Meyerhans A, Roelants G and Wain-Hobson S. Genetic organisation of a chimpanzee lentivirus related to HIV-1. *Nature* 1990, 345: 356-359

Hurt CB, Sebastian J, Hicks CB and Eron JJ. Resistance to HIV integrase strand transfer inhibitors among clinical specimens in the United States, 2009-2012. *Clinical Infectious Diseases* 2014, 58: 423 – 431

Illumina Nextera® XT DNA Sample Preparation guide, 2012

Isel C, Lanchy JM, Le Grice SF, Ehresmann C, Ehresmann B and Marquet R. Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA3Lys. *The EMBO Journal* 1996, 15: 917-924

Jacobo-Molina A, Ding J, Nanni RG, Clark AD, Lu X, Tantillo C, Williams RL, Kamer G Feris AL, Clark P, Hizi A, Hughes SH and Arnold E. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proceedings of the National Academy of Sciences USA* 1993, 90: 6320-6324

Janssens W, Fransen K, Peeters M, Heyndrickx L, Motte J, Bedjabaga L, Delaporte E, Piot P and van der Groen G. Phylogenetic tree analysis of a new chimpanzee lentivirus SIVcpz-gab2 from a wild-captured chimpanzee from Gabon. *AIDS Research and Human Retroviruses* 1994, 10: 1191-1192

Jaskolski M, Alexandratos JN, Bujacz G and Wlodawer A. Piecing together the structure of retroviral integrase, an important target in AIDS therapy. *FEBS Journal* 2009, 276: 2926-2946

Jaskolski M, Tomasselli AG, Sawyer TK, Staples DG, Heinrikson RL, Schneider J, Kent SBH and Wlodawer A. Structure at 2.5-.ANG. resolution of chemically synthesized Human Immunodeficiency Virus Type 1 protease complexed with a hydroxyethylene-based inhibitor. *Biochemistry* 1991, 30: 1600–1609

Jetzt AE, Yu H, Klarmann GJ, Ron Y, Preston BD and Dougherty JP. High rate of recombination throughout the human immunodeficiency virus type 1 genome. *Journal of Virology* 2000, 74: 1234-1240

Jing N, Xu X: Rational drug design of DNA oligonucleotides as HIV inhibitors. *Current Drug Targets - Infectious Disorders* 2001, 1: 79-90

Johnson VA, Brun-Vézinet F, Clotet B., Gunthard HF, Kuritzkes DR, Pillay D, Schapiro JM and Richman DD. Update of the drug resistance mutations in HIV-1: 2007. *Topics in HIV Medicine* 2007, 15: 119-125

Jonassen TO, Stene-Johansen K, Berg ES, Hungnes O, Lindboe CF, Frøland SS and Grinde B. Sequence analysis of HIV-1 group O from Norwegian patients infected in the 1960s. *Virology* 1997, 231: 43-47

Jonckheere H, Anné J and De Clercq E. The HIV-1 reverse transcription (RT) process as target for RT inhibitors. *Medicinal Research Reviews* 2000, 20: 129-54

Johns BA, Kawasuji T, Weatherhead JG, Taishi T, Temelkoff DP, Yoshida H, Akiyama T, Taoda Y, Murai H, Kiyama R, Fuji M, Tanimoto N, Jeffrey J, Foster SA, Yoshinaga T, Seki T, Kobayashi M, Sato A, Johnson MN, Garvey EP and Fujiwara T. Carbamoyl pyridone HIV-1 integrase inhibitors 3. A diastereomeric approach to chiral nonracemic tricyclic ring systems and the discovery of dolutegravir (S/GSK1349572) and (S/GSK1265744). *Journal of Medicinal Chemistry* 2013, 56: 5901 – 5916

Johnson AA, Santos W, Pais GC, Marchand C, Amin R, Burke TR Jr, Verdine G and Pommier Y. Integration requires a specific interaction of the donor DNA terminal 5'-cytosine with glutamine 148 of the HIV-1 integrase flexible loop. *Journal of Biological Chemistry* 2006, 281: 461-467

Jones G, Ledford RM, Yu F, Chen X, Miller MD, Tsiang M and McColl DJ. *In Vitro* Resistance Profile of HIV-1 Mutants Selected by the HIV-1 Integrase Inhibitor, GS-9137 (JTK-303). 14th Conference on Retroviruses and Opportunistic Infections (CROI), Los Angeles, California 2007

Jones GS, Yu F, Zeynalzadegan A, Hesselgesser J, Chen X, Chen J, Jin H, Kim CU, Wright M, Geleziunas R and Tsiang M. Preclinical evaluation of GS-9160, a novel inhibitor of human immunodeficiency virus type 1 integrase. *Antimicrobial Agents and Chemotherapy* 2009, 53:1194-1203

Kan V. Effectiveness and durability of non-nucleoside reverse transcriptase inhibitor based therapy in HIV clinical practice. *AIDS* 2007, 21: 880–882

Karageorgos L, Li P and Burrell, C. J. (1993). Characterisation of HIV replication complexes early after cell-to-cell infection. *AIDS Research and Human Retroviruses* 1993, 9: 817-823

Karn J and Stoltzfus CM. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harbor Perspectives in Medicine* 2012, 2: a006916-a006933

Kaplan AH, Michael SF, Wehbie RS, Knigge MF, Paul DA, Everitt L, Kempf DJ, Norbeck DW, Erickson JW and Swanstrom R. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. *Proceedings of the National Academy of Sciences USA* 1994, 91: 5597-5601

Kato K, Sato H and Takebe Y. Role of naturally occurring basic amino acid substitutions in the human immunodeficiency virus type 1 subtype E envelope V3 loop on viral coreceptor usage and cell tropism. *Journal of Virology* 1999, 73: 5520-5526

Keele BF, Van HF, Li Y, Bailes E, Takehisa J, Santiago ML, Bibollet-Ruche F, Chen Y, Wain LV, Liegeois F, Loul S, Ngole EM, Bienvenue Y, Delaporte E, Brookfield JF, Sharp PM, Shaw GM, Peeters M and Hahn BH. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* 2006, 313: 523-526

Kiernan RE, Ono A, Englund G and Freed EO. Role of matrix in an early post-entry step in the human immunodeficiency virus type 1 life cycle. *Journal of Virology*. 1998, 72: 4116–4126

Klarmann GJ, Schauber CA and Preston BD. Template-directed pausing of DNA synthesis by HIV-1 reverse transcriptase during polymerization of HIV-1 sequences in vitro. *Journal of Biological Chemistry* 1993, 268: 9793-9802

Klarmann GJ, Yu H, Chen X, Dougherty JP and Preston BD. Discontinuous plus-strand DNA synthesis in human immunodeficiency virus type 1-infected cells and in a partially reconstituted cell-free system. *Journal of Virology* 1997, 71: 9259-9269

Klibanov OM: Elvitegravir, an oral HIV integrase inhibitor, for the potential treatment of HIV infection. *Current Opinion in Investigational Drugs* 2009, 10: 190-200

Kliger Y, Gallo SA, Peisajovich SG, Munoz-Barroso I, Avkin S, Blumenthal R and Shai Y. Mode of action of an antiviral peptide from HIV-1. Inhibition at a post-lipid mixing stage. *Journal of Biological Chemistry* 2001, 276: 1391-1397

Kobayashi M, Nakahara K, Seki T, Miki S, Kawauchi S, Suyama A, Wakasa-Morimoto C, Kodama M, Endoh T, Oosugi E, Matsushita Y, Murai H, Fujishita T, Yoshinaga T, Garvey E, Foster S, Underwood M, Johns B, Sato A and Fujiwara T. Selection of diverse and clinically relevant integrase inhibitor-resistant human immunodeficiency virus type 1 mutants. *Antiviral Research* 2008, 80: 213-222

Kobayashi M, Yoshinaga T, Seki T, Wakasa-Morimoto C, Brown KW, Ferris R, Foster SC, Hazen RJ, Miki S, Suyama-Kagitani A, Kawauchi-Miki S, Taishi T, Kawasuji T, Johns BA, Underwood MR, Garvey EP, Sato A and Fujiwara T. *In Vitro* antiretroviral properties of S/GSK1349572, a next-generation HIV integrase inhibitor. *Antimicrobial Agents and Chemotherapy* 2011, 55: 813-821

Kohlstaedt LA and Steitz TA. Reverse transcriptase of human immunodeficiency virus can use either human tRNA (3Lys) or Escherichia coli tRNA (2Gln) as a primer in an in vitro primer-utilization assay. *Proceedings of the National Academy of Sciences USA 1992*, 89: 9652-9656

Kohlstaedt LA, Wang J, Friedman JM, Rice PA and Steitz TA. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 1992, 256: 1783-1790

Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, Miedema F, Schellekens PT and Tersmette M. Prognostic value of HIV-1 synctium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Annals of Internal Medicine* 1993, 118: 681-688

Kopietz F, Ayyappan A, Vasudevan J, Krämer M, Muckenfuss H, Sanzenbacher R, Cichutek K, Flory E and Münk C. Interaction of human immunodeficiency virus type 1 Vif with APOBEC3G is not dependent on serine/threonine phosphorylation status. *Journal of General Virology* 2012, 93: 2425-2430

Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, Hahn BH, Wolinsky S and Bhattacharya T. Timing the ancestor of the HIV-1 pandemic strains. *Science* 2000, 288: 1789-1796

Krishnan L and Engelman A. Retroviral integrase proteins and HIV-1 DNA integration. *Journal of Biological Chemistry* 2012, 287: 40858-40866

Kulkosky J, Jones KS, Katz RA, Mack JP and Skalka AM. Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. *Molecular and Cell Biology* 1992, 12: 2331-2338

Lalezari JP, Henry K, O'Hearn M, Montaner JS, Piliero PJ, Trottier B, Walmsley S, Cohen C, Kuritzkes DR, Eron JJ Jr, Chung J, DeMasi R, Donatacci L, Drobnes C, Delehanty J, Salgo M and TORO 1 Study Group. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. *New England Journal of Medicine* 2003, 348: 2175-2185

Lataillade M, Chiarella J and Kozal M. Natural polymorphism of the HIV-1 integrase gene and mutations associated with integrase inhibitor resistance. *Antiviral Therapy* 2007, 12: 563-570

Lataillade M and Kozal MJ. The hunt for HIV-1 integrase inhibitors. *AIDS Patient Care STDs* 2006, 20: 489–501

Lau KA and Wong JL. Current trends of HIV recombination worldwide. *Infectious Disease Reports* 2013, 5: 15-20

Lazzarin A, Clotet B, Cooper D, Reynes J, Arasteh K, Nelson M, Katlama C, Stellbrink HJ, Delfraissy JF, Lange J, Huson L, DeMasi R, Wat C, Delehanty J, Drobnes C, Salgo M and TORO 2 Study Group. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. *New England Journal of Medicine* 2003, 348: 2186-2195

Lee SP and Han MK. Zinc Stimulates Mg2+-Dependent 3'-Processing Activity of Human Immunodeficiency Virus Type 1 Integrase *in vitro*. *Biochemistry* 1996, 35: 3837-3844

Lee SP, Xiao J, Knutson JR, Lewis MS and Han MK. Zn^{2+} promotes the self-association of human immunodeficiency virus type-1 integrase *in vitro*. *Biochemistry* 1997, 36: 173-180

Levesque K, Finzi A, Binette J and Cohen EA. Role of CD4 receptor down-regulation during HIV-1 infection. *Current Opinion in HIV Research* 2004, 2: 51-59

Levin A, Armor-Omer A, Rosenbluh J, Melamed-Book N, Graessmann A, Waigmann E and Loyter A. Inhibition of HIV-1 integrase nuclear import and replication by a peptide bearing integrase putative nuclear localization signal. *Retrovirology* 2009, 6: 1-16

Levy DN, Aldrovandi GM, Kutsch O and Shaw GM. Dynamics of HIV-1 recombination in its natural target cells. *Proceedings of the National Academy of Sciences* 2004, 101: 4204-4209.

Levy J. HIV and the Pathogenesis of AIDS. (ASM Press: Washington DC, 2007)

Li X, Mak J, Arts EJ, Gu Z, Kleiman L, Wainberg MA and Parniak MA. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. *Journal of Virology* 1994, 68: 6198-6206

Li Y, Rey-Cuillie M-A and Hu S-L. N-Linked glycosylation in the V3 region of HIV type 1 surface antigen modulates coreceptor usage in viral infection. *AIDS Research and Human Retroviruses* 2001, 17: 1473-1479

Loizidou EZ, Kousiappa I, Zeinalipour-Yazdi CD, Van de Vijver DA and Kostrikis LG. Implications of HIV-1 M group polymorphisms on integrase inhibitor efficacy and resistance: genetic and structural *in silico* analyses. *Biochemistry* 2009, 48: 4-6

Low A, Prada N, Topper M, Vaida F, Castor D, Mohri H, Hazuda D, Muesing M and Markowitz M. Natural polymorphisms of human immunodeficiency virus type 1 integrase and inherent susceptibilities to a panel of integrase inhibitors. *Antimicrobial Agents and Chemotherapy* 2009, 53: 4275-4282

Lu M, Blacklow SG and Kim PS. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nature Structural Biology* 1995, 2: 1075-1082

Lu K, Heng X and Summers MF. Structural determinants and mechanism of HIV-1 genome packaging. *Journal of Molecular Biology* 2011, 410: 609–633

Maas JJ, Gange SJ, Schuitemaker H, Coutinho RA, Van Leeuwen R and Margolick JB. Strong association between failure of T cell homeostasis and the syncytium-inducing phenotype among HIV-1-infected men in Amsterdam cohort study. *AIDS* 2000, 14: 1155-1161

Maignan S, Guilloteau JP, Zhou-Liu Q, Clement-Mella C and Mikol V. Crystal structures of the catalytic domain of HIV-1 integrase free and complexed with its metal cofactor: high level of similarity of the active site with other viral integrases. *Journal of Molecular Biology* 1998, 282: 359-368

Maïga AI, Malet I, Soulie C, Derache N, Koita V, Amellal B, Tchertanov L, Delelis O, Morand-Joubert L, Mouscadet JF, Murphy R, Cisse M, Katlama C, Calvez V and Marcelin AV. Genetic barriers for integrase inhibitor drug resistance in HIV type-1 B and CRF02_AG subtypes. *Antiviral Therapy* 2009, 14: 123-129

Malet I, Delelis O, Soulie C, Wirden M, Tchertanov L, Mottaz P, Peytavin G, Katlama C, Mouscadet JF, Calvez V and Marcelin AG. Quasispecies variant dynamics during emergence of resistance to raltegravir in HIV-1-infected patients. *Journal of Antimicrobial Chemotherapy* 2009, 63: 795-804

Malet I, Delelis O, Valantin MA, Montes B, Soulie C, Wirden M, Tchertanov L, Peytavin G, Reynes J, Mouscadet JF, Katlama C, Calvez V and Marcelin AG. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor *in vitro. Antimicrobial Agents and Chemotherapy* 2008, 52: 1351-1358

Malet I, Wirden M, Fourati S, Armenia D, Masquelier B, Fabeni L, Sayon S, Katlama C, Perno CF, Calvez V, Marcelin AG and Ceccherini-Silberstein F. Prevalence of resistance mutations related to integrase inhibitor S/GSK1349572 in HIV-1 subtype B raltegravir-naive and -treated patients. *The Journal of Antimicrobial Chemotherapy* 2011, 66: 1481-1483.

Malim MH and Emerman M. HIV-1 sequence variation: drift, shift, and attenuation. *Cell* 2001, 104: 469-472

Marchand C, Zhang X, Pais GC, Cowansage K, Neamati N, Burke TR and Pommier Y. Structural determinants for HIV-1 integrase inhibition by be-diketo acids. *Journal of Biological Chemistry* 2002, 277: 12596-12603

Marconi VC, Sunpath H, Lu Z, Gordon M, Koranteng-Apeagyei K, Hampton J, Carpenter S, Giddy J, Ross D, Holst H, Losina E, Walker BD, Kuritzkes DR and South Africa Resistance Cohort Study Team. Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa. *Clinical Infectious Diseases* 2008, 46: 1589-1597

Margot NA, Hluhanich RM, Jones GS, Andreatta KN, Tsiang M, McColl DJ, White KL and Miller MD. *In vitro* resistance selections using elvitegravir, raltegravir, and two metabolites of elvitegravir M1 and M4. *Antiviral Research* 2012. 2: 288–296

Marinello J, Marchand C, Mott B, Bain A, Thomas C.J, and Pommier Y. Comparison of raltegravir and elvitegravir on HIV-1 integrase catalytic reactions and on a series of drug-resistant integrase mutants. *Biochemistry* 2008, 47: 9345–9354

Martin JC, Hitchcock MJM, De Clercq E and Prusoff WH. Early nucleoside reverse transcriptase inhibitors for the treatment of HIV: a brief history of stavudine (D4T) and its comparison with other dideoxynucleosides. *Antiviral Research* 2010, 85: 34-38

Martinez-Cajas JL, Pai NP, Klein MB and Wainberg MA. Differences in resistance mutations among HIV-1 non-subtype B infections: a systematic review of evidence (1996–2008). *Journal of the International AIDS Society* 2009, 12: 11-22

Martinez-Picado J, Savara AV, Sutton L and D'Aquila RT. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *Journal of Virology* 1999, 73: 3744–3752

Marx PA, Alcabes PG and Drucker E. Serial human passage of simian immunodeficiency virus by unsterile injections and the emergence of epidemic human immunodeficiency virus in Africa. *Philosophical Transactions of the Royal Society B: Biological Sciences* 2001, 356: 911-920

Mascarenhas AP and Musier-Forsyth K. The capsid protein of human immunodeficiency virus: interactions of HIV-1 capsid with host protein factors. *The FEBS Journal* 2009, 276: 6118–6127

Maurin C, Bailly F and Cotelle P. Structure-activity relationships of HIV-1 integrase inhibitors–enzyme-ligand interactions. *Current Medicinal Chemistry* 2003, 10: 1795 - 1810

McColl DJ, Fransen S and Gupta S. Resistance and cross resistance to first-generation integrase inhibitors: Insights from a phase II study of elvitegravir (GS-9137). In: 16th International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications 2007, Barbados, West Indies

McCutchan FE. Global epidemiology of HIV. *Journal of Medical Virology* 2006, 78: S7-S12

Menendez-Arias L. Targeting HIV: Antiretroviral therapy and development of drug resistance. *Trends in Pharmacological Science* 2002, 23: 381-388

Metifiot M, Marchand C, Maddali K and Pommier Y. Resistance to integrase inhibitors. *Viruses* 2010, 2: 1347 – 1366a

Metifiot M, Maddali K, Naumova A, Zhang X, Macharnd C and Pommier Y. Biochemical and pharmacological analyses of HIV-1 integrase flexible loop mutants resistant to raltegravir. *Biochemistry* 2010, 49: 3715-3722b

Métifiot M, Vandegraaff N, Maddali K, Naumova A, Zhang X, Rhodes D, Marchand C and Pommier Y. Elvitegravir overcomes resistance to raltegravir induced by integrase mutation Y143. *AIDS* 2011, 25:1175-1178

Messiaen P, Wensing AM, Fun A, Nijhuis M, Brusselaers N and Vandekerckhove L. Clinical use of HIV integrase inhibitors: a systematic review and meta-analysis. *PLoS One* 2013, 8: e52562

Mespléde T., Quashie PK and Wainberg MA. Resistance to HIV integrase inhibitors. *Current Opinion in HIV AIDS* 2012, 7: 407

Mesplède T, Quashie PK, Osman N, Han Y, Singhroy DN, Lie Y, Petropoulos CJ, Huang W and Wainberg MA. Viral fitness cost prevents HIV-1 from evading dolutegravir drug pressure. *Retrovirology* 2013, 10:22 doi: 10.1186/1742-4690-10-22

Meyer PR, Matsuura SE, Mian AM, So AG and Scott WA. A Mechanism of AZT Resistance: An increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Molecular Cell* 1999, 4: 35-43

Miller MD, Farnet CM and Bushman F D. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *Journal of Virology* 1997, 71: 5382-5390

Miller JH, Presnyak V and Smith HC. The dimerization domain of HIV-1 viral infectivity factor Vif is required to block virion incorporation of APOBEC3G. *Retrovirology* 2007, 4: 81-92

Mink M, Mosier SM, Janumpalli S, Davison D, Jin L, Melby T, Sista P, Erickson J, Lambert D, Stanfield-Oakley SA, Salgo M, Cammack N, Matthews T and Greenberg ML. Impact of human immunodeficiency virus type 1 gp41 amino acid substitutions selected during enfuvirtide treatment on gp41 binding and antiviral potency of enfuvirtide *in vitro*. *Journal of Virology* 2005, 79: 12447–12454

Mitsuya H, Popovic M, Yarchoan R, Matsushita S, Gallo RC and Broder S. Suramin protection of T-cells *in vitro* against infectivity and cytopathic effect of HTLV-III. *Science* 1984, 226: 172-174

Mitsuya H and Broder S. Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proceedings of the National Academy of Sciences USA* 1986, 83: 1911-1915

Molina JM, Lamarca A, Andrade-Villanueva J, Clotet B, Clumeck N, Liu YP, Zhong L, Margot N, Cheng AK and Chuck SL. Efficacy and safety of once daily elvitegravir versus twice daily raltegravir in treatment-experienced patients with HIV-1 receiving a ritonavir-boosted protease inhibitor: randomised, double-blind, phase 3, non-inferiority study. *The Lancet: Infectious Diseases* 2012, 12: 27 - 35

Montagnier L. Human Immunodeficiency Viruses (Retroviridae). *Encyclopedia of Virology* (2nd Ed.) 1999, 763-774

Montal M. Structure-function correlates of Vpu, a membrane protein of HIV-1. *Febs Letters* 2003, 552: 47-53

Montavon C, Toure-Kane C, Nkengasong JN, Vergne L, Hertogs K, Mboup S, Delaporte E and Peeters M. CRF06-cpx: A new circulating recombinant form of HIV-1 in West Africa involving subtypes A, G, K, and J. *Journal of Acquired Immune Deficiency Syndrome* 2002, 29: 522-530

Mortuza GB, Haire LF, Stevens A, Smerdon SJ, Stoye JP and Taylor I. High resolution structure of a retroviral capsid hexameric amino-terminal domain. *Nature* 2004, 431: 481-485

Mothes W, Sherer NM, Jin J and Zhong P. Virus cell-to-cell transmission. *Journal of Virology* 2010, 84: 8360-8368

Mphahlele M, Papathanasopoulos M, Cinellu MA, Coyanis M, Mosebi S, Traut T, Modise R, Coates J and Hewer R. Modification of HIV-1 reverse transcriptase and integrase activity by gold (III) complexes in direct biochemical assays. *Bioorganic & Medicinal Chemistry* 2012, 20: 401-407

Muthumani K, Choo AY, Zong WX, Madesh M, Hwang DS, Premkumar A, Thieu KP, Emmanuel J, Kumar S, Thompson CB and Weiner DB. The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. *Nature Cell Biology* 2006, 8: 170–179

Ndembi N, Takehisa J, Zekeng L, Kobayashi E, Ngansop C, Songok EM, Kageyama S, Takemura T, Ido E, Hayami M, Kaptue L and Ichimura H. Genetic diversity of HIV type 1 in rural eastern Cameroon. *Journal of Acquired Immune Deficiency Syndrome* 2004, 37: 1641-1650

Ni XJ, Delelis O, Charpentier C, Storto A, Collin G, Damond F, Descamps D and Mouscadet JF. G140S/Q148R and N155H mutations render HIV-2 Integrase resistant to Raltegravir whereas Y143C does not. *Retrovirology* 2011, 8: 86

Nijhuis M, Schuurman R, de Jong D, Erickson J, Gustchina E, Albert J, Schipper P, Gulnik S and Boucher CA. Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* 1999, 13: 2349-2359

Nolan K.M, Del Prete GQ, Jordan AP, Haggarty B, Romano J, Leslie GJ and Hoxie JA. Characterisation Characterisation of a human immunodeficiency virus type 1 V3 deletion mutation that confers resistance to CCR5 inhibitors and the ability to use aplaviroc-bound receptor. *Journal of Virology* 2009, 83: 3798–3809

Noursadeghi M, Katz DR and Miller RF. HIV-1 infection of mononuclear phagocytic cells: the case for bacterial innate immune deficiency in AIDS. *The Lancet Infectious Diseases* 2006, 6: 794-804

Ogert RA, Hou Y, Ba L, Wojcik L, Qiu P, Murgolo N, Duca J, Dunkle LM, Ralston R, and Howe JA. Clinical resistance to vicriviroc through adaptive V3 loopmutations in HIV-1 subtype D gp120 that alter interactions with the N-terminus and ECL2 of CCR5. *Virology* 2010, 400: 145–155

Oliveira MF, Ramalho DB, Abreu CM, Vubil A, Mabunda N, Ismael N, Francisco C, Jani IV and Tanuri A. Genetic diversity and naturally polymorphisms in HIV type 1 integrase isolates from Maputo, Mozambique: implications for integrase inhibitors. *AIDS Research and Human Retroviruses* 2012, 28:1788-1792

Ouédraogo-Traoré R, Montavon C, Sanou T, Vidal N, Sangaré L, Sanou I, Soudré R, Mboup S, Delaporte E and Peeters M. CRF06-cpx is the predominant HIV-1 variant in AIDS patients from Ouagadougou, the capital city of Burkina Faso. *AIDS* 2003, 17: 441-452

Pandey KK, Bera S, Vora AC and Grandgenett DP: Physical trapping of HIV-1 synaptic complex by different structural classes of integrase strand transfer inhibitors. *Biochemistry* 2010, 49: 8376-8387

Parczewski M, Bander D, Urbanska A and Boron-Kaczmarska A. HIV-1 integrase resistance among antiretroviral treatment naive and experienced patients from Northwestern Poland. *BMC Infectious Diseases* 2012, 12:368. doi:10.1186/1471-2334-12-368

Paredes R, Sagar M, Marconi VC, Hoh R, Martin JN, Parkin NT, Petropoulos CJ, Deeks SG and Kuritzkes DR. In vivo fitness cost of the M184V mutation in multidrug-resistant human immunodeficiency virus type 1 in the absence of lamivudine. *Journal of Virology* 2009, 83 (4):2038-2043

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ and Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine* 1998, 338: 853-860

Palmer S, Alaeus A, Albert J and Cox S. Drug susceptibility of subtypes A, B, C, D and E human immunodeficiency virus type 1 primary isolates. *AIDS Research and Human Retroviruses* 1998, 14: 157-162

Pandey KK and Grandgenett DP. HIV-1 integrase strand transfer inhibitors: Novel insights into their mechanism of action. *Retrovirology: Research and Treatment* 2008, 2: 11–16

Papathanasopoulos MA, Vardas E, Wallis C, Glashoff R, Buttơ, Poli G, Malnati M, Clerici M and Ensoli B. Characterisation Characterisation of HIV type 1 genetic diversity among South African participants enrolled in the AIDS Vaccine Integrated Project (AVIP) study. *AIDS Research and Human Retroviruses* 2010, 26: 705-709

Pauwels R. New non-nucleoside reverse transcriptase inhibitors (NNRTIs) in development for the treatment of HIV infections. *Current Opinion in Pharmacology* 2004, 4: 437–446

Peeters M, Gueye A, Mboup S, Bibollet-Ruche F, Ekaza E, Mulanga C, Ouedrago R, Gandji R, Mpele P, Dibanga G, Koumare B, Saidou M, Esu-Williams E, Lombart JP, Badombena W, Luo N, Vanden Haesevelde M and Delaporte E. Geographical distribution of HIV-1 group O viruses in Africa. *AIDS* 1997, 11: 493498

Peeters M, Honoré C, Huet T, Bedjabaga L, Ossari S, Bussi P, Cooper RW and Delaporte E. Isolation and partial characterisation characterisation of an HIV-related virus occurring naturally in chimpanzees in Gabon. *AIDS* 1989, 3: 625-630

Peeters M, Fransen K, Delaporte E, Van den Haesevelde M, Gershy-Damet GM, Kestens L, van der Groen G and Piot P. Isolation and characterisation characterisation of a new chimpanzee lentivirus (simian immunodeficiency virus isolate cpz-ant) from a wild-captured chimpanzee. *AIDS* 1992, 6: 447-451

Perros M. CCR5 antagonists for the treatment of HIV infection and AIDS. *Advances in Antiviral Drug Design* 2007, 5: 185-212

Pettit SC, Sheng N, Tritch R, Erickson-Vitanen S and Swanstrom R. The regulation of sequential processing of HIV-1 Gag by the viral protease. *Advances in Experimental Medicine and Biology* 1998; 436: 15–25

Phillips DM. The role of cell-to-cell transmission in HIV infection. *AIDS* 1994, 8:719-31

Piatak M, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM and Lifson JD. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 1993, 259: 1749–1754

Pierson TC and Doms RW. HIV-1 entry and its inhibition. *Current Topics in Microbiology and Immunology* 2003, 281: 1-27

Piller SC, Ewart GD, Premkumar A, Cox GB and Gage PW. Vpr protein of human immunodeficiency virus type I forms cation-selective cahnnels in planar lipid bilayers. *Proceedings of the National Academy Sciences USA* 1996, 93: 111-115

Piralla A, Paolucci S, Gulminetti R, Comolli G and Baldanti F. HIV integrase variability and genetic barrier in antiretroviral naïve and experienced patients. *Virology Journal* 2011, 8: 149 doi: 10.1186/1743-422X-8-149

Plantier J-C, Leoz M, Dickerson JE, De Oliveira F, Cordonnier F, Lemee V, Damond F, Robertson DL and Simon F. A new human immunodeficiency virus derived from gorillas. *Nature Medicine* 2009, 15: 871-872

Pommier Y, Johnson AA, and Marchand C. Integrase inhibitors to treat HIV/AIDS. *Nature Reviews Drug Discovery* 2005, 4: 236-248.

Popovic M, Sarngadharan MG, Read E and Gallo RC. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 1984, 224: 497-500

Prabu-Jeyabalan M, Nalivaika E and Schiffer CA. Substrate Shape Determines Specificity of Recognition for HIV-1 Protease: Analysis of crystal structures of six substrate complexes. *Structure* 2002, 10: 369–381

Prado JG, Wrin T, Beauchaine J, Ruiz L, Petropoulos CJ, Frost SD, Clotet B, D'Aquila RT and Martinez-Picado J. Amprenavir-resistant HIV-1 exhibits lopinavir cross-resistance and reduced replication capacity. *AIDS* 2002, 16: 1009–1017

Prado JG, Parkin NT, Clotet B, Ruiz L and Martinez-Picado J. HIV type 1 fitness evolution in antiretroviral-experienced patients with sustained CD4+ T cell counts but persistent virologic failure. *Clinical Infectious Diseases* 2005, 41: 729–737

Pugach P, Marozsan AJ, Ketas TJ, Landes EL, Moore JP and Kuhmann SE. HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. *Virology* 2007, 361: 212–228

Pugliese A, Vidotto V, Beltramo T, Petrini T and Torre D. A review of HIV-1 Tat protein biological effects. *Cell Biochemistry and Function*, 2005, 23: 223-227

Purcell DF and Martin MA. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication and infectivity. *Journal of Virology* 1993, 67: 6365-6378

Quashie PK, Sloan RD and Wainberg MA. Novel therapeutic strategies targeting HIV integrase. *BMC Medicine* 2012, 10: doi:10.1186/1741-7015-10-34

Quashie PK, Mespléde T, Han Y-S, Oliviera M, Singhroy DN, Fujiwara T, Underwood ME and Wainberg MA. Characterisation Characterisation of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir. *Journal of Virology* 2012, 86: 2696-2705

Quashie PK, Mesplède T, Han YS, Veres T, Osman N, Hassounah S, Sloan RD, Xu HT and Wainberg MA. Biochemical analysis of the role of G118R-linked dolutegravir drug resistance substitutions in HIV-1 integrase. *Antimicrobial Agents and Chemotherapy* 2013, 57: 6223–6235

Quashie PK, Mesplède T and Wainberg MA. Evolution of HIV integrase resistance mutations. *Current Opinion in Infectious Diseases* 2013, 26: 43-49

Quinones-Mateu ME, Moore-Dudley DM, Jegede O, Weber J and Arts EJ. Viral drug resistance and fitness. *Advances in Pharmacology* 2008, 56: 257–296

Rainey P. HIV drug interactions. The good, the bad and the other. *Therapeutic Drug Monitoring* 2002, 24: 26-31

Resch W, Ziermann R, Parkin N, Gamarnik A and Swanstrom R. Nelfinavir-resistant, amprenavir-hypersusceptible strains of human immunodeficiency virus type 1 carrying an N88S mutation in protease have reduced infectivity, reduced replication capacity, and reduced fitness and process the Gag polyprotein precursor aberrantly. *Journal of Virology* 2002, 76: 8659–8666

Rhee SY, Liu TF, Kiuchi M, Zioni R, Gifford RJ, Holmes SP and Shafer RW. Natural variation of HIV-1 group M integrase: implications for a new class of antiretroviral inhibitors. *Retrovirology* 2008, 5: 74. doi: 10.1186/1742-4690-5-74

Rambaut A, Robertson DL, Pybus OG, Peeters M and Holmes EC. Human immunodeficiency virus: Phylogeny and the origin of HIV-1. *Nature* 2001, 410: 1047-1048

Ren J, Nichols C, Bird L, Chamberlain P, Weaver K, Short S, Stuart DI and Stammers DK. Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors. *Journal of Molecular Biology* 2001, 312: 795-805

Richman DD, Havlir D, Corbeil J, Looney D, Ignacio C, Spector SA, Sullivan J, Cheeseman S, Barringer K, Pauletti D, Shih C-K, Myers M and Griffin J. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *Journal of Virology* 1994, 68: 1660-1666

Rizzuto C and Sodroski J. Fine definition of a conserved CCR5-binding region on the human immunodeficiency virus type 1 glycoprotein 120. *AIDS Research and Human Retroviruses* 2000, 16: 741-749

Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Kröhn A, Lambert RW, Merrett, JH, Mills JS, Parkes KEB, Redshaw, S, Ritchie AJ, Taylor DL, Thomas GJ and Machin PJ. Rational design of peptide-based HIV proteinase inhibitors. *Science* 1990, 248: 358-361

Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK. Gao F, Hahn BH, Khalish ML, Kuiken C, Learn GH, Leitner T, McCutchan F, Osmanov S, Peeters M, Pieniazek D, Salminen M, Sharp PM, Wolinsky S and Korber B. HIV-1 nomenclature proposal. *Science* 2000, 288: 55-56

Ross EK, Buckler-White AJ, Rabson AB, Englund G and Martin MA. Contribution of NF-KB and SP1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: Distinct patterns of viral growth are determined by T-cell types. *Journal of Virology 1991*, 65: 4350-4358

Rowley M. The discovery of raltegravir, an integrase inhibitor for the treatment of HIV infection. *Progress in Medicinal Chemistry* 2008, 46: 1 - 28

Royce RA, Sena A, Cates W and Cohen MS. Sexual transmission of HIV. *New England Journal of Medicine* 1997, 336: 1072-1078

Rozenbaum W, Dormont D, Spire B, Vilmer E, Gentilini M and Griscelli C. Antimoniotungstate (HPA 23) treatment of three patients with AIDS and one with prodrome. *Lancet* 1985, 1: 450-451

Saag MS, Hahn BH, Gibbons J, Li Y, Parks ES, Parks WP and Shaw GM. Extensive variation of human immunodeficiency virus type-1 *in vivo*. *Nature* 1988, 334: 440–444

Santiago ML, Range F, Keele BF, Li Y, Bailes E, Bibollet-Ruche F, Fruteau C, Noë R, Peeters M, Brookfield JFY, Shaw GM, Sharp PM and Hahn BH. Simian immunodeficiency virus infection in free-ranging Sooty Mangabeys (Cercocebus atys atys) from the Taï Forest, Côte d'Ivoire: Implications for the origin of epidemic human immunodeficiency virus type 2. *Journal of Virology* 2005, 79: 12515-12527

Santoro MM and Perno CF. HIV-1 genetic variability and clinical implications. *ISRN Microbiology* 2013. doi: 10.1155/2013/481314

Sarafianos SG, Das K, Clark AD Jr, Ding J, Boyer PL, Hughes SH and Arnold E. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proceedings of the National Academy of Sciences USA* 1999, 96: 10027-10032

Sarafianos SG, Marchand B, Das K, Himmel DM, Parniak MA, Hughes SH and Arnold E. Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *Journal of Molecular Biology* 2009, 385: 693-713

Sato A, Kobayashi M, Seki T, Morimoto CW, Yoshinaga T, Fujiwara T, Johns BA and Underwood M. S/GSK1349572: a next-generation integrase inhibitor (INI) with limited or no cross resistance to first generation INIs or other classes of antivirals. Abstracts of the 8th European HIV Drug Resistance Work-shop, Sorrenti 2010

Sato M, Motomura T, Aramaki H, Matsuda T, Yamashita M, Ito Y, Kawakami H, Matsuzaki Y, Watanabe W, Yamataka K, Ikeda S, Kodama E, Matsuoka M and Shinkai H: Novel HIV-1 integrase inhibitors derived from quinolone antibiotics. *Journal of Medicinal Chemistry* 2006, 49: 1506 – 1508

Schatz O, Mous J and Le Grice SFJ. HIV-1 RT-associated ribonuclease H displays both endonuclease and 3'----5' exonuclease activity. *The EMBO Journal* 1990, 9: 1171-1176

Schumlenburg E and Le Roux PJ. Antiretroviral therapy and anaesthesia. *Southern African Journal of Anaesthesia and Analgesia* 2008, 14: 31-38

Selby MJ, Bain ES, Luciw P and Peterlin BM. Structure, sequence and position of the stem-loop in TAR determine transcriptional elongation by Tat through the HIV-1 long terminal repeat. *Genes & Development* 1989, 3: 547-558

Serrao E, Odde S, Ramkumar K and Neamati N. Raltegravir, elvitegravir, and metoogravir: the birth of ‘‘me-too’’ HIV-1 integrase inhibitors. *Retrovirology* 2009, 6: 25 doi:10.1186/1742-4690-6-25

Shafer RW, Eisen JA, Merigan TC and Katzenstein DA. Sequence and drug susceptibility of subtype C reverse transcriptase from human immunodeficiency virus type 1 seroconverters in Zimbabwe. *Journal of Virology* 1997, 71: 5441-5448

Sharp PM, Bailes E, Chaudhuri RR, Rodenburg CM, Santiago MO and Hahn BH. The origins of acquired immune deficiency syndrome viruses: Where and when? *Transactions of Royal Society of London, B* 2001, 356: 867-876

Sharp PM, Robertson DL and Hahn BH. Cross-species transmission and recombination of AIDS viruses. *Philosophical Transactions of Royal Society of London, B* 1995, 349: 41-47

Shimura K, Kodama E, Sakagami Y, Matsuzaki Y, Watanabe W, Yamataka K, Watanabe Y, Ohata Y, Doi S, Sato M, Kano M, Ikeda S and Matsuoka M. Broad antiretroviral activity and resistance profile of the novel human immunodeficiency virus integrase inhibitor elvitegravir (JTK-303/GS-9137). *Journal of Virology* 2008, 82: 764-774

Sherman MP, De Noronha CM, Heusch MI, Greene S and Greene WC. Nucleocytoplasmic shuttling by human immunodeficiency virus type I Vpr. *Journal of Virology* 2001, 75: 1522-1532

Sherperd JC, Jacobson LP, Qiao W, Jamieson BD, Phair JP, Piazza P, Quinn TC and Margolick JB. Emergence and persistence of CXCR4-tropic HIV-1 in a population of men from the multicentre AIDS cohort study. *Journal of infectious disease* 2008, 198: 1104-1112

Shun M-C, Raghavendra NK, Vandegraaff N, et al. LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes and Development* 2007, 21: 1767-1778

Sierra S, Lübke N, Walter H, Schülter E, Reuter S, Fätkenheuer G, Bickel M, da Silva H, Kaiser R, Esser S and the SnoB-Study group. The SnoB study: frequency of baseline raltegravir resistance mutations prevalence in different non-B subtypes. *Medical Microbiology and Immunology* 2011, 200: 225 - 232

Simon F, Mauclére P, Roques P, Loussert-Ajaka I, Müller-Trutwin MC, Saragosti S, Georges-Courbot MC, Bare-Sinoussi F and Brun-Vézinet F. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Medicine* 1998, 4: 1032-1037

Singh SB, Jayasuriya H, Salituro GM, Zink DL, Shafiee A, Heimbuch B, Silverman KC, Lingham RB, Genilloud O, Teran A, Vilella D, Felock P and Hazuda D: The complestatins as HIV-1 integrase inhibitors. Efficient isolation, structure elucidation, and inhibitory activities of isocomplestatin, chloropeptin I, new complestatins, A and B, and acid hydrolysis products of chloropeptin I. *Journal of Natural Products* 2001, 64: 874 - 882

Sluis-Cremer N, Arion D, Abram ME and Parniak MA. Proteolytic processing of an HIV-1 pol polyprotein precursor: insights into the mechanism of reverse transcriptase p66/p51 heterodimer formation. *The International Journal of Biochemistry & Cell Biology* 2004, 36: 1836–1847

Sluis-Cremer N and Tachedjian G. Mechanisms of inhibition of HIV replication by nonnucleoside reverse transcriptase inhibitors. *Virus Research* 2008, 134: 147-156

Sluis-Cremer N and Tachedjian G. Modulation of the oligomeric structures of HIV-1 retroviral enzymes by synthetic peptides and small molecules. European *Journal of Biochemistry* 2002, 269: 5103 - 5111

Sluis-Cremer N, Temiz NA and Bahar I. Conformational changes in HIV-1 reverse transcriptase induced by nonnucleoside reverse transcriptase inhibitor binding. *Current HIV Research* 2004, 2: 323-332

Smyth RJ, Yi Y, Singh A and Collman RG. Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. *Journal of Virology* 1998, 72: 4478-4484

Soares EA, Martínez AM, Souza TM, Santos AF, Da Hora V, Silveira J, Bastos FI, Tanuri A and Soares MA. HIV-1 subtype C dissemination in southern Brazil. *AIDS* 2005, 19 (SUPPL 4): S81-S86

Solbak SM, Reksten TR, Hahn F, Wray V, Henklein P, Henklein P, Halskau O, Shubert U and Fossen T. HIV-1 p6-a structured to flexible multifunctional membrane-interacting protein. *Biochimica et Biophysica Acta* 2013, 1828: 816-823

Sova P and Volsky D. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with vif-negative human immunodeficiency virus type 1. *Journal of Virology* 1993, 67: 6322-6326

Speck RF, Wehrly K, Platt EJ, Atchison RE, Charo IF, Kabat D, Chesebro B and Goldsmith MA. Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop. *Journal of Virology* 1997, 71: 7136-7139

Stanley BJ, Ehrlich ES, Short L, Yu Y, Xiao Z, Yu X-F and Xiong Y. Structural insight into the human immunodeficiency virus Vif SOCS box and its role in human E3 ubiquitin ligase assembly. *Journal of Virology* 2008, 82: 8656–8663

Statistics South Africa (2013). Mortality and causes of death in South Africa, 2010: Findings from death notification. Available online at: http://www.avert.org/south-africa-hiv-aids-statistics.htm#sthash.gTa3v6iP.dpuf

Strebel K. Virus-host interactions: role of HIV proteins Vif, Tat and Rev. *AIDS* 2003, 17 (Suppl 4): S25-S34

Summa V, Petrocchi A, Bonelli F, Crescenzi B, Donghi M, Ferrara M, Fiore F, Gardelli C, Paz OG, Hazuda DJ, Jones P, Kinzel O, Laufer R, Monteagudo E, Muraglia E, Nizi E, Orvieto F, Pace P, Pescatore P, Scarpelli R, Stillmock K, Witmer MV and Rowley M. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. *Journal of Medicinal Chemistry* 2008, 51: 5843 – 5855

Sundquist WI and Krausslich H-G. HIV-1 assembly, budding and maturation. *Cold Spring Harbor Perspectives in Medicine* 2012. 2: a006924-a006948

Suzuki Y and Craigie R. The road to chromatin-nuclear entry of retroviruses. *Nature Reviews Microbiology* 2007, 5: 187-196

Taiwo B, Zheng L, Gallien S, Matining RM, Kuritzkes DR, Wilson CC, Berzins BI, Acosta EP, Bastow B, Kim PS, Eron JJ Jr and ACTG A5262 Team. Efficacy of a nucleoside-sparing regimen of darunavir/ritonavir plus raltegravir in treatment-naive HIV-1-infected patients (ACTG A5262). *AIDS* 2011, 25: 2113 - 2122

Tebit DM and Arts EJ. Tracking a century of global expansion and evolution of HIV to drive understanding and combat disease. *The Lancet* 2011, 11: 45-55

Tebit DM, Ganame J, Sathiandee K, Nagabila Y, Coulibaly B and Krausslich HG. Diversity of HIV in rural Burkina Faso. *Journal of Acquired Immune Deficiency Syndrome* 2006, 43: 144- 152

Tebit DM, Zekeng L, Kaptue L, Salminen M, Krausslich HG and Herchenroder O. Genotypic and phenotypic analysis of HIV type 1 primary isolates from western Cameroon. *AIDS Research and Human Retroviruses* 2002, 18: 39-48

Telesnitsky A and Goff SP. Reverse transcription and the generation of retroviral DNA. In: Coffin JM, Hughes SH, Varmus HE, eds Retroviruses. Cold spring Harbor: Cold Spring Harbor Laboratory Press 1997, 121-160

Temesgen Z and Siraj D. Raltegravir: first in class HIV integrase inhibitor. *Therapeutics and Clinical Risk Management*, 2008. 4, 493-400

The South African Antiretroviral Treatment Guidelines, Department of Health, Republic of South Africa, 2013. Version 14 March 2013. Available on line at: http://www.sahivsoc.org/upload/documents/2013%20ART%20Guidelines-Short%20Combined%20FINAL%20draft%20guidelines%2014%20March%202013.pdf

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 1997, 25: 4876-4882

Tsibris AM, Sagar M, Gulick RM, Su Z, Hughes M, Greaves W., Subramanian M, Flexner C, Giguel F, Leopold KE, Coakley E and Kuritzkes DR. *In vivo* emergence of vicriviroc resistance in a human immunodeficiency virus type 1 subtype C-infected subject. *Journal of Virology* 2008, 82: 8210–8214

Turner BG and Summers MF. Structural biology of HIV. *Journal of Molecular Biology* 1999, 285: 1-31

UNAIDS (World AIDS Day 2014 Report - Fact sheet). Available on line at: http://www.unaids.org/en/resources/campaigns/World-AIDS-Day-Report-2014/factsheet

UNAINDS Fact Sheet 2014, Global Statitics: http://www.unaids.org/sites/default/files/en/media/unaids/contentassets/documents/factsheet/2014/20140716_FactSheet_en.pdf

UNAIDS (The Joint United Nations Programme on HIV/AIDS) report on the global AIDS epidemic, 2013. Available on line at: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf

Underwood MR, Johns BA, Sato A, Martin JN, Deeks SG and Fujiwara T. The activity of the integrase inhibitor dolutegravir against HIV-1 variants isolated from raltegravir-treated adults. *Journal of Acquired Immune Deficiency Syndrome* 2012, 61: 297-301

Vacca J. Wai J, Fisher T, Embrey M, Hazuda D, Miller M, Felock P, Witmer M, Gabryelski L and Lyle T: Discovery of MK-2048 – subtle changes confer unique resistance properties to a series of tricyclic hydroxypyrrole integrase strand transfer inhibitors. Poster discussion: 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention (2007): Abstract no. WEPEA088

Van Baelen K, Rondelez E, Van Eygen V, Ariën K, Clynhens M, Van den Zegel P, Winters B and Stuyver L. A combined genotypic and phenotypic human immunodeficiency virus type 1 recombinant virus assay for the reverse transcriptase and integrase genes. *Journal of Virological Methods* 2009, 161: 231-239

Van Den Haesevelde MM, Peeters M, Jannes G, Janssens W, van der Groen G, Sharp PM and Saman E. Sequence analysis of a highly divergent HIV-1 related lentivirus isolated from a wild captured chimpanzee. *Virology* 1996, 221: 346-350

Vandekerckhove LPR, Wensing AMJ, Kaiser R, Brun-Vezinet F, Clotet B, De Luca A, Dressler S, Garcia F, Geretti AM, Klimkait T, Korn K, Masquelier B, Perno CF, Shapiro JM, Soriano V, Sonnerborg A, Vandamme A-M, Verhofstede C, Walter H, Zazzi M and Boucher CAB on behalf of the European Consensus Group on clinical management of tropism testing. European guidelines on the clinical management of HIV-1 tropism testing. *The Lancet* 2011, 11: 394-407

Van Baelen K, Van Eygen V, Rondelez E and Stuyver LJ. Clade-specific HIV-1 integrase polymorphisms do not reduce raltegravir and elvitegravir phenotypic susceptibility. *AIDS* 2008, 22: 1877–1880

Van der Borght K, Verheyen A, Feyaerts M, Van Wesenbeeck L, Verlinden Y, Van Craenenbroeck E and van Vlijmen H. Quantitative prediction of integrase inhibitor resistance from genotype through consensus linear regression modeling. *Virology Journal* 2013, 10: 8 doi: 10.1186/1743-422X-10-8

Van de Vijver DA1, Wensing AM, Angarano G, Asjö B, Balotta C, Boeri E, Camacho R, Chaix ML, Costagliola D, De Luca A, Derdelinckx I, Grossman Z, Hamouda O, Hatzakis A, Hemmer R, Hoepelman A, Horban A, Korn K, Kücherer C, Leitner T, Loveday C, MacRae E, Maljkovic I, de Mendoza C, Meyer L, Nielsen C, Op de Coul EL, Ormaasen V, Paraskos's D, Perrin L, Puchhammer-Stöckl E, Ruiz L, Salminen M, Schmit JC, Schneider F, Schuurman R, Soriano V, Stanczak G, Stanojevic M, Vandamme AM, Van Laethem K, Violin M, Wilbe K, Yerly S, Zazzi M and Boucher CA. The calculated genetic barrier for antiretroviral drug resistance substitutions is largely similar for different HIV-1 subtypes. *Journal of Acquired Immune Deficiency Syndrome* 2006, 41: 352 - 360

Van Hal SJ, Herring B, Deris Z, Wang B, Saksena NK and Dwyer DE. HIV-1 integrase polymorphisms are associated with prior antiretroviral drug exposure. *Retrovirology* 2009, 6: 1-3

Van Maarseveen NM, Andersson D, Lepšík M, Fun A, Schipper PJ, de Jong D, Boucher CAB and Nijhuis M. Modulation of HIV-1 Gag NC/p1 cleavage efficiency affects protease inhibitor resistance and viral replicative capacity. *Retrovirology* 2012, 9: 29-41

Van Wesenbeeck L, Rondelez E, Feyaerts M, Verheyen A, Van der Borght K, Smits V, Cleybergh C, De Wolf H, Van Baelen K and Stuyver LJ. Cross-resistance profile determination of two second-generation HIV-1 integrase inhibitors using a panel of recombinant viruses derived from raltegravir-treated clinical isolates. *Antimicrobial Agents and Chemotherapy* 2011, 55: 321 – 325

Vavro C, Hasan S, Madsen H, Horton J, DeAnda F, Martin-Carpenter L, Sato A, Cuffe R, Chen S, Underwood M and Nichols G. Prevalent polymorphisms in wild-type HIV-1 integrase are unlikely to engender drug resistance to dolutegravir (S/GSK1349572). *Antimicrobial Agents and Chemotherapy* 2013, 57: 1379 - 1384

Verrier F, Borman AM, Brand D and Girard M. Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. *AIDS Research and Human Retroviruses* 1999, 15: 731-743

Vidal N, Peeters M, Mulanga-Kabeya C, Nzilambi N, Robertson D, Ilunga W, Sema H, Tshimanga K, Bongo B and Delaporte E. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *Journal of Virology* 2000, 74: 10498-10507

Vidal N, Koyalta D, Richard V, Lechiche C, Ndinaromtan T, Djimasngar A, Delaporte E and Peeters M. High genetic diversity of HIV-1 strains in Chad, West Central Africa. *Journal of Acquired Immune Deficiency Syndrome* 2003, 33: 239-246

Vingerhoets J, Azijn H, Fransen E, De Baere I, Smeulders L, Jochmans D, Andries K, Pauwels R and de Béthune M-P. TMC125 displays a high genetic Barrier to the development of resistance: Evidence from *in vitro* selection experiments. *Journal of Virology* 2005, 79: 12773-12782

Wainberg MA. The impact of the M184V substitution on drug resistance and viral fitness. *Expert Review of Anti-infective Therapy* 2004 2:147–151

Wainberg MA and Bluma G. The Impact of HIV Genetic Polymorphisms and Subtype Differences on the occurrence of resistance to antiretroviral drugs. *Molecular Biology International* 2012: doi:10.1155/2012/256982

Wainberg MA, Drosopoulos WC, Salomon H, Hsu M, Borkow G, Parniak MA, Gu Z, Song Q, Manne J, Islam S, Castriota G and Prasad VR. Enhanced fidelity of 3TC-selected mutant HIV-1 reverse transcriptase. *Science* 1996, 271: 1282-1285

Wainberg M and Han Y-S. Will drug resistance against dolutegravir in initial therapy ever occur? *Frontiers in Pharmacology* 2015, 6:90. doi:10.3389/fphar.2015.00090

Wakefield JK, Jablonski SA and Morrow CD. In vitro enzymatic activity of human immunodeficiency virus type 1 reverse transcriptase mutants in the highly conserved YMDD amino acid motif correlates with the infectious potential of the proviral genome. *Journal of Virology* 1992, 66: 6806-6812

Wakefield JK, Wolf AG and Morrow CD. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA (3Lys). *Journal of Virology* 1995, 69: 6021-6029

Wan M and Loh BN. Expression and purification of active form of HIV-1 protease from E.coli. *Biochemistry and Molecular Biology International* 1995, 35: 899-912

Wang WK, Dudek T, Essex M and Lee TH. Hypervariable region 3 residues of HIV type 1 gp120 involved in CCR5 coreceptor utilization: therapeutic and prophylactic implications. *Proceedings of the National Academy of Sciences USA* 1999, 96: 4558-4562

Wares M, Mespléde T, Quashie PK, Osman N, Han Y and Wainberg MA. The M50I polymorphic substitution in association with the R263K mutation in HIV-1 subtype B integrase increases drug r3esistance but does not restore viral replicative fitness. *Retrovirology* 2014, 11: doi:10.1186/1742-4690-11-7

Warren K, Warrilow D, Meredith L and Harrich D. Reverse transcriptase and cellular factors: regulators of HIV-1 reverse transcription. *Viruses* 2009, 1: 873-894

Warrilow D, Tachedjian G and Harrich D. Maturation of the HIV reverse transcriptase complex: putting the jigsaw together. *Reviews in Medical Virology* 2009, 19: 324-337

Weiser B, Pjilpott S, Klimkait T, Burger H, Kitchen C, Burgisser P, Gorgievski M, Perrin L, Piffaretti JC and Ledergerber B. HIV-1 coreceptor usage and CXCR4-specific viral load predict clinical disease progression during combination antiretroviral therapy. *AIDS* 2008, 22: 469-479

Weissenhorn W, Dessen A, Harrison SC, Skehel JJ and Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 1997, 387: 426-430

Weller I and Williams I. ABC of AIDS. *British Medical Journal* 2001, 322: 1290–1293

Werner T, Ferroni S, Saermark T, Brack-Werner R, Banati RB, Mager R, Steinaa L, Kreutzberg GW and Erfle V. HIV-1 Nef protein exhibits structural and functional similarity to scorpion peptides interacting with k+ channels. *AIDS* 1991, 5: 1301-1308

Westby M and Van der Ryst E. CCR5 antagonists: host-targeted antivirals for the treatment of HIV infection. *Antiviral Chemistry & Chemotherapy* 2005, 16: 339-354

WHO: The use of antiretroviral drugs for treating and preventing HIV: June 2013. Availale on line at: http://apps.who.int/iris/bitstream/10665/85321/1/9789241505727_eng.pdf?ua=1

White K, Abram ME, Kulkarni R, Rhee M, Szwarcberg J and Miller MD. Emergent drug resistance from the HIV-1 phase 3 elvitegravir/cobicistat/emtricitabine.tenofovir disoproxil fumarate studies through week 96 [Abstract 596]. 20th Conference on Retroviruses and Opportunistic Infections, Atlanta, GA 2013

William G, Stretton TB and Leonard JC. AIDS in 1959? *Lancet* 1983, 1136

Wills T and Vega V. Elvitegravir: a once-daily inhibitor of HIV-1 integrase. Expert Opinion on Investigational Drugs 2012, 21: 395 – 401

Winters B, Llyod RJ, Miller M and Holodniy M. Evolution of IN inhibitor resistance mutations in patients failling elvitegravir-containing regimens. 19th Conference on Retroviruses and Opportunistic Infections (CROI), Seatle WA 2012

Witmer M and Danovich R. Selection and analysis of HIV-1 integrase strand transfer inhibitor resistant mutant viruses. *Methods* 2009, 47: 277-282

Witvrouw M, Pannecouque C, Van Laethem K, Desmyter J, De Clerq E and Van Damme AM. Activity of non-nucleoside reverse transcriptase inhibitors against HIV-2 and SIV. *AIDS* 1999, 13: 1477-1483

Wöhrl BM and Moelling K. Interaction of HIV-1 ribonuclease H with polypurine tract containing RNA-DNA hybrids. *Biochemistry* 1990, 29: 10141-10147

Woodward CL, Wang Y, Dixon WJ, Htun H and Chow SA. Subcellular localization of feline immunodeficiency virus integrase and mapping of its karyophilic determinant. *Journal of Virology* 2003; 77: 4516-4527

World Health Organization. Scaling up antiretroviral therapy in resource limited settings: Guidelines for a public health approach. Geneva: 2002

World Health Organization and International AIDS Society building. The global HIV drug resistance surveillance network. 2014. Available online at: http://www.who.int/drugresistance/hivaids/en/HIVdrugnetwork.pdf?ua=1

Worobey M, Gemmel M, Teuwen DE, Haselkorn T, Kunstman K, Bunce M, Muyembe J-J, Kabongo J-MM, Kalengayi RM, Van Marck E, Thomas M, Gilbert P and Wolinsky SM. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. *Nature* 2008, 455: 661-665

Wrin T, Gamarnik A, Whitehurst N, Beauchaine J, Whitcomb JM, Hellmann NS and Petropoulos CJ. Natural variation of replication capacity measurements in drug-naïve/susceptible HIV-1. *Antiviral Therapy* 2001, 6**:** 20

Wu X, Liu H, Xiao H, Conway JA, Hehl E, Kalpana GV, Prasad VR and Kappes JC. Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex. *Journal of Virology* 1999, 73: 2126-2135

Wynn G, Zapor M, Smith B, Wortmann G, Oesterheld JR, Armstrong SC and Cozza KL. Antiretrovirals Part 1: Overview, history and focus on protease inhibitors. *Psychosomatics* 2004, 45: 262-270

Xiao L, Owen SM, Goldman I, Lal AA, deJong JJ, Goudsmit J and Lal RB. CCR5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage. *Virology* 1998, 240: 83-92

Xu H, Franks T, Gibson G, Huber K, Rahm N, De Castillia CS, Luban J, Aiken C, Watkins S, Sluis-Cremer N and Ambrose Z. Evidence for biphasic uncoating during HIV-1 infection from a novel imaging assay. *Retrovirology* 2013, 10: doi: 10.1186/1742-4690-10-70

Yamaguchi J, Coffey R, Vallari A, Ngansop C, Mbanya D, Ndembi N, Kaptué L, Gürtler LG, Bodelle P, Schochetman G, Devare SG and Brennan CA. Identification of HIV type 1 group N infections in a husband and wife in Cameroon: viral genome sequences provide evidence for horizontal transmission. *AIDS Research and Human Retroviruses* 2006, 22: 83-92

Yi Y, Isaacs SN, Williams DA, Frank I, Schols D, De Clercq E, Kolson DL and Collman RG. Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism. *Journal of Virology* 1999, 73: 7117-7125

Yoshinaga T, Kanamori-Koyama M, Seki T, Ishida K, Akihisa E, Kobayashi M, Sato A and Fujiwara Shionogi T. Strong Inhibition of Wild-Type and Integrase Inhibitor (INI)-Resistant HIV Integrase (IN) Strand Transfer Reaction by the Novel INI S/GSK1349572. International HIV and Hepatitis Drug Resistance Workshop. Vol. Poster. Dubrovnik Croatia; 2010 (http://www.natap.org/2010/ResisWksp/ResisWksp_32.htm)

Zhang Z, Yu Q, Kang SM, Buescher J and Morrow CD. Preferential completion of human immunodeficiency virus type 1 proviruses initiated with tRNA3Lys rather than tRNA1, 2Lys. *Journal of Virology* 1998, 72: 5464-5471

Zheng R, Jenkins TM and Craigie R. Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization and enhances catalytic activity. *Proceedings of the National Academy of Sciences USA* 1996, 93: 13659-13664

Zhuang J, Jetzt AE, Sun G, Yu H, Klarmann G, Ron Y, Preston BD and Dougherty JP. Human immunodeficiency virus type 1 recombination: rate, fidelity and putative hot spots. *Journal of Virology* 2002, 76: 11273-11282

Zhu T, Korber BT, Nahmias AJ, Hooper E, Sharp PM and Ho DD. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* 1998, 391: 594-597

APPENDIX

R14/49 Mr Morore Mphalele

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130385

NAME: Mr Morore Mphalele
(Principal Investigator)

DEPARTMENT: Molecular Medicine & Haematology
Medical School

PROJECT TITLE: In Vitro Selection and Characterization of Human Immunodeficiency Virus Type-1 Subtype C Integrase Strand Transfer Inhibitor Resistant Mutants

DATE CONSIDERED: Ad hoc

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Prof M Papathanasopoulos

APPROVED BY:

Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 08/04/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES