



Assessment of the *in vitro* phenotypic drug susceptibility of HIV-1 subtype C drug-resistant variants to Doravirine (DOR)

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“The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day.”

Albert Einstein

(14 March 1879 – 18 April 1955)

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1. **N. Reddy**, K. Steegen and A. E. Basson, *In vitro* Phenotypic Doravirine Susceptibility of Prevalent HIV-1 Subtype C NNRTI-Resistance Mutations, September 2023, 30th International Drug Resistance Conference, Cape Town, South Africa.

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Abstract

The high prevalence of antiretroviral drug resistance warrants approval of novel antiretroviral drugs that are effective against drug resistant-variants. Doravirine (DOR) is a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) with an improved safety profile, requires only a single daily dose, and has shown activity against prevalent NNRTI drug resistance mutations. To understand the impact of NNRTI drug resistance mutations on DOR in South Africa, in which HIV-1 subtype C is prevalent, this study assessed the *in vitro* phenotypic drug susceptibility of DOR against prevalent HIV-1 subtype C drug-resistant variants. Replication-defective HIV-like pseudoviruses (PSV) containing the most prevalent NNRTI drug resistance mutation combinations (n=20), as well as the single mutations (n=15) they contain, were generated and screened in a single-cycle *in vitro* phenotypic assay for susceptibility to DOR. An initial screen of DOR against wild-type PSVs showed no significant differences in Islatravir (ISL) potency among subtypes B and C. Furthermore, the combination of DOR with ISL showed a significant synergistic inhibition of a wild-type HIV-1 subtype C PSV. High-level DOR resistance was observed with the V106M and Y188L single mutants, while the remaining single mutants remained susceptible or showed a low level of resistance to DOR. Combinations of mutations that included V106M, Y188L, and P225H showed a high level of resistance to DOR. Given the lower prevalence of the latter mutations in treatment-naïve and -experienced patients in South Africa, DOR would likely be an effective treatment option. However, genotypic drug resistance testing may be advised prior to initiation of DOR-containing regimens for NNRTI-experienced individuals.

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List of Abbreviations

3TC	Lamivudine
ABC	Abacavir
ADR	Acquired Drug Resistance
AIDS	Acquired Immunodeficiency Deficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ART	Anti-Retroviral Therapy
ATP	Adenosine triphosphate
ATV	Atazanavir
AZT	Zidovudine
BAF	Barrier-to-Auto-integration Factor
BICD2	Dynein Adapter Protein Bicaudal D2
bp	base pairs
CAB-LA	Long-Acting injectable Cabotegravir
cART	combination Antiretroviral Therapy
CC ₂₀	20% Cytotoxic Concentration (cell viability reduced to 80%)
CC ₅₀	50% Cytotoxic Concentration (cell viability reduced to 50%)
CCR5	Co-receptor C-C Chemokine receptor 5
CDC	Center for Disease Control
CDC	Centre for Disease Control and Prevention
cDNA	complementary DNA
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CPSF6	Cleavage and Polyadenylation Specificity Factor 6
CRF	Circulating Recombinant Forms
CXCR4	CXC Chemokine Receptor 4
d4T	Stavudine
ddI	Didanosine
ddNTPs	dideoxynucleotides
DLV	Delavirdine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

dNTP	deoxynucleoside Triphosphate
DOR	Doravirine
DPBS	Dulbecco's Phosphate Buffered Saline
DRM	Drug Resistance Mutation
DRV	Darunavir
DTG	Dolutegravir
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
eEF1A	Elongation Factor zeta 1
EFV	Efavirenz
EI	Entry Inhibitor
ETR	Etravirine
FC	Fold Change
FTC	Emtricitabine
Gemin2	Survival motor neuron-interacting protein 2
gp	glycoproteins
HAART	Highly Active Antiretroviral Treatment
HDAC1	Histone Deacetylase 1
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HMGA1	High-Mobility Group protein A1
HTLV-III	Human T-cell Lymphotropic Virus type III
IC ₅₀	50% Inhibitory Concentration
INI1	Integrase Interactor 1
INSTI	Integrase Inhibitor
ISL	Islatravir
kbp	kiolbase pairs
KLD	Kinase-Ligase- <i>Dpnl</i>
LAV	Lymphadenopathy Associated Virus
LB	Luria-Bertani
LEDGF/p75	Lens Epithelium-Derived Growth Factor

LAV	Lymphadenopathy Associated Virus
LPV	Lopinavir
LTR	Long Terminal Repeat
mRNA	messenger RNA
NAM	Nucleotide Associated Mutations
NFAT	Nuclear Factor of Activated T cells
NFkB	kappa-light-chain-enhancer of activated B cells
NHD	National Health Department
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NPV	Nevirapine
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase translocation Inhibitor
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PDR	Pre-treatment Drug Resistance
PEP	Post-exposure Prophylaxis
PI	Protease Inhibitor
PIC	Pre-Integration Complex
PrEP	Pre-exposure Prophylaxis
PSV	pseudovirus
p-TEFb	positive Transcription Elongation Factor
r	Ritonavir
RLU	Relative Light Units
RNA	Ribonucleic Acid
RPV	Rilpivirine
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
RTC	Reverse Transcription Complex
SAHPRA	South African Health Products Regulatory Authority
SAP18	Sin3A-Associated Protein
SDM	Site-Directed Mutagenesis
SIV	Simian Immunodeficiency Virus
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
TAF	Tenofovir Alafenamide

TAMs	Thymidine Analog Mutations
TAR	Trans-Activating Response
tat	trans-activator of transcription
TBE	Tris-Borate- EDTA
TCO	Technical Cut-Off
TDF	Tenofovir
TDR	Transmitted Drug Resistance
TLD	TDF + 3TC + DTG
T _m	melting temperature
URF	Unique Recombinant Forms
UTR	Untranslated Region
VL	Viral Load
VSV-G	Vesicular Stomatitis Virus G protein
WHO	World Health Organisation
WT	Wild Type
ZDV	Zidovudine

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1. Background

1.1 A brief overview of the HIV epidemic

Human Immunodeficiency Virus (HIV) is a retrovirus that targets the host's immune system. The virus particularly targets CD4⁺ T cells which are central to the adaptive immune response (Vijayan *et al.*, 2017). This results in a compromised immune system making the host susceptible to opportunistic infections. Untreated HIV infection develops into Acquired Immunodeficiency Deficiency Syndrome (AIDS).

The first cases of AIDS were 5 patients diagnosed with *Pneumocystis pneumonia* in June of 1981 (Centres for Disease Control (CDC), 1981) eight male patients who were homosexual and diagnosed with Kaposi's sarcoma (Hymes *et al.*, 1981). Throughout the year the cases of opportunistic infections in homosexual men were rising causing concern (CDC, 1981b). The term AIDS was first used in September of 1982 (CDC, 1982a) and the retrovirus responsible was termed Human T-cell Lymphotropic Virus type III (HTLV-III) and Lymphadenopathy Associated Virus (LAV) in 1983 by Barré-Sinoussi *et al.* (1983) and Gallo *et al.* (1984). The virus spreading worldwide would later be known as HIV type 1 (HIV-1). Early cases observed in haemophiliacs (CDC, 1982b), infants (CDC, 1982c), heterosexuals (CDC, 1982a) and homosexual men (CDC, 1982d), indicated that the transmission of HIV was through blood and intimate contact (CDC, 1984). In 1986, the virus causing AIDS was officially termed HIV by the International Committee on the Taxonomy of Viruses. The first case of HIV in South Africa was published in 1983, with two cases of AIDS reported in homosexual men (Ras *et al.*, 1983). Although initially, the epidemic mirrored observations overseas where the disease was prevalent in white homosexual men, in 1989 the epidemic shifted to predominantly affect black heterosexual men and women (Sher, 1989).

The origin of HIV was not understood for many years after its discovery. The finding that would help scientists understand HIV's origin was the identification of a virus from two AIDS patients in West Africa that was similar to HIV-type 1 (HIV-1) in its biology and morphology, but its envelope glycoproteins were distinct and it was termed LAV-II which is now known as HIV type 2 (HIV-2) (Clavel *et al.*, 1986). They also noted that the virus was genetically more similar to the simian virus (SIV) than HIV-1 (Chakrabarti *et al.*, 1987; Guyader *et al.*, 1987). This allowed scientists to understand that HIV was a zoonotic virus that had been infecting several primate species in Africa and had transferred to humans (Hahn *et al.*, 2000). The origins of HIV-1 were traced back to chimpanzees (Huet *et al.*, 1990), while HIV-2 is genetically related to a virus infecting sooty mangabeys (Hirsch *et al.*, 1989).

HIV-1 is the most prevalent type globally, accounting for approximately 95% of infections (<https://www.avert.org/professionals/hiv-science/types-strains>). HIV-2 is relatively uncommon and prevalent in West Africa (Visseaux *et al.* 2016). HIV-1 is divided into four groups, namely M (Major), N (non-M, non-O), O (Outlier), and P (Burke, 1997; Plantier *et al.*, 2009; Robertson *et al.*, 2000; Vallari *et al.*, 2011). These HIV-1 groups resulted from four independent inter-species transmission events from the simian immunodeficiency virus infecting African apes (D'arc *et al.*, 2015). Group M makes up the majority of HIV infections and it is divided into subtypes (i.e. A, B, C, D, F, G, H, J and K) as well as circulating recombinant forms (CRF) and unique recombinant forms (URF) (Perrin *et al.*, 2003). The subtypes arose through the combination of the selective pressure by the immune system, reverse transcriptase's high error rate, time, the founder effect and geographical isolation, thus resulting in genetically diverse variants that cluster together. The genetic variation between the subtypes is 25-35%, while the genetic variation within a subtype ranges from 15-20% (Santoro and Perno, 2013). The two most prevalent HIV-1 subtypes are geographically distributed, where subtype B predominantly circulates in North and South America, Western Europe, and Australia, whereas subtype C is primarily found in Eastern Africa, Southern Africa and India (Bbosa *et al.*, 2019).

1.2 HIV Statistics

1.2.1 Global Statistics

The HIV epidemic has had a significant impact on the health of the global population since 1981 with 85.6 million (65.0 – 113.0 million) people infected with HIV and claiming the lives of 40.4 million (32.9 – 51.3 million) people (UNAIDS, 2023a). The HIV epidemic has declined since its peak in 2005 but the disease still affects a large percentage of the global population (UNAIDS, 2005). As of 2022, there were 39 million (33.1 – 45.7 million) people living with HIV, 630 000 (480 000 – 880 000) people who died from HIV-related causes and 1.3 million (1.0 – 1.7 million) people were infected with HIV (WHO, 2023). The prevalence of HIV worldwide was 0.7% in 2022.

1.2.2 Southern and South African Statistics

Africa carries a high portion of the global HIV burden, accounting for more than two-thirds of the global population living with HIV with a prevalence of 3.2% in 2022 (WHO, 2023). There were 25.6 million people living with HIV on the African continent in 2022 and 660 000 new HIV infections were reported for that year (WHO, 2023). Eastern and Southern Africa had 20.8 million people living with HIV, 260 000 AIDS-related deaths and 500 000 new HIV infections in 2022 (UNAIDS, 2023b). The epidemic has a high prevalence of 17.8% (11.9% - 23.2%) in South Africa as of 2023 (<https://cfs.hivci.org/>). The number of people living with HIV in South Africa is 7.6 million (5.4 – 9.9 million) in 2023. The number of new HIV infections was 160 000 (120 000 – 230 000) and 45 000 (31 000 – 90 000) people died due to HIV in South Africa, as of 2023. South Africa has a proportionally high population living with HIV compared to the rest of the countries in the world. However, 75% (53%-98%) of people in need of treatment were receiving anti-retroviral therapy (ART) in 2023. HIV-1 subtype C has accounted for 46.6% of infections in the past decade and continues to be the dominant subtype globally (Bbosa *et al.*, 2019). HIV subtype C is the predominant subtype in South Africa responsible for 98.44% of HIV infections (Elangovan *et al.*, 2021; Giovanetti *et al.*, 2020; Williams *et al.*, 2023).

1.3 HIV Characteristics

1.3.1 HIV Genome and Structure

HIV's genome consists of identical long terminal repeats (LTR) on the 5' and 3' ends and three overlapping reading frames which encode nine genes and fifteen viral proteins (Seitz, 2016) (Figure 1.1 A). These viral proteins together comprise a functional HIV virus (Figure 1.1 B). HIV-1 is enveloped with a lipid bilayer which is obtained from the host cell after the virus buds from the host cell. The envelope proteins (gp160) are glycoproteins (gp) that are embedded in the lipid bilayer, forming spikes. The gp120 (SU) binds to CD4 receptor on the target cell (a CD4 T lymphocyte that play a key role in regulating the immune response), while gp41 (TM) mediates the fusion of the virus's and host cell's membranes through interaction with the CD4 T cell receptor (Chan *et al.*, 1997). Additionally, gp120 is heavily glycosylated which allows it to evade recognition by the immune system (Rathore *et al.*, 2017). These envelope proteins are encoded by the *env* gene. The viral components are encapsulated by matrix proteins (MA, p17). The virus contains two identical single-stranded ribonucleic acids (RNA) molecules of 9750 nucleotides in length (Ratner *et al.*, 1985). The RNA is bound to nucleocapsid proteins (NC, p7) and late assembly proteins (p6), which is then encapsulated by an icosahedral capsid composed of the viral capsid protein (CA, p24) (Rossi *et al.*, 2021). The structural proteins (MA, NC, CA) are encoded by the *gag* gene. HIV has three enzymes that are critical during its replication. Reverse transcriptase (RT) is responsible for reverse transcribing HIV's RNA genome into deoxyribonucleic acid (DNA) which is inserted into the host cell's genome by integrase (IN) (Tasara *et al.*, 2001). Protease (PR) is involved in the cleavage of the polypeptide chain produced (Gag-Pol) as a result of the replication of the integrated DNA, to form a functional virion (Gulnik *et al.*, 2000). These enzymes are encoded by the *pol* gene.

The accessory proteins (nef, vif, vpr, vpu) function as adaptor molecules connecting cellular substrates to cellular pathways to improve HIV's replication by weakening the host's cells' defence system (nef, vif), guiding RNA to the host cell's nucleus (vpr) or aiding in viral escape (vpu) (Strebel, 2013). The regulatory proteins (tat, rev) are

responsible for regulating the transcription of the viral genome during replication (Faust *et al.*, 2017).

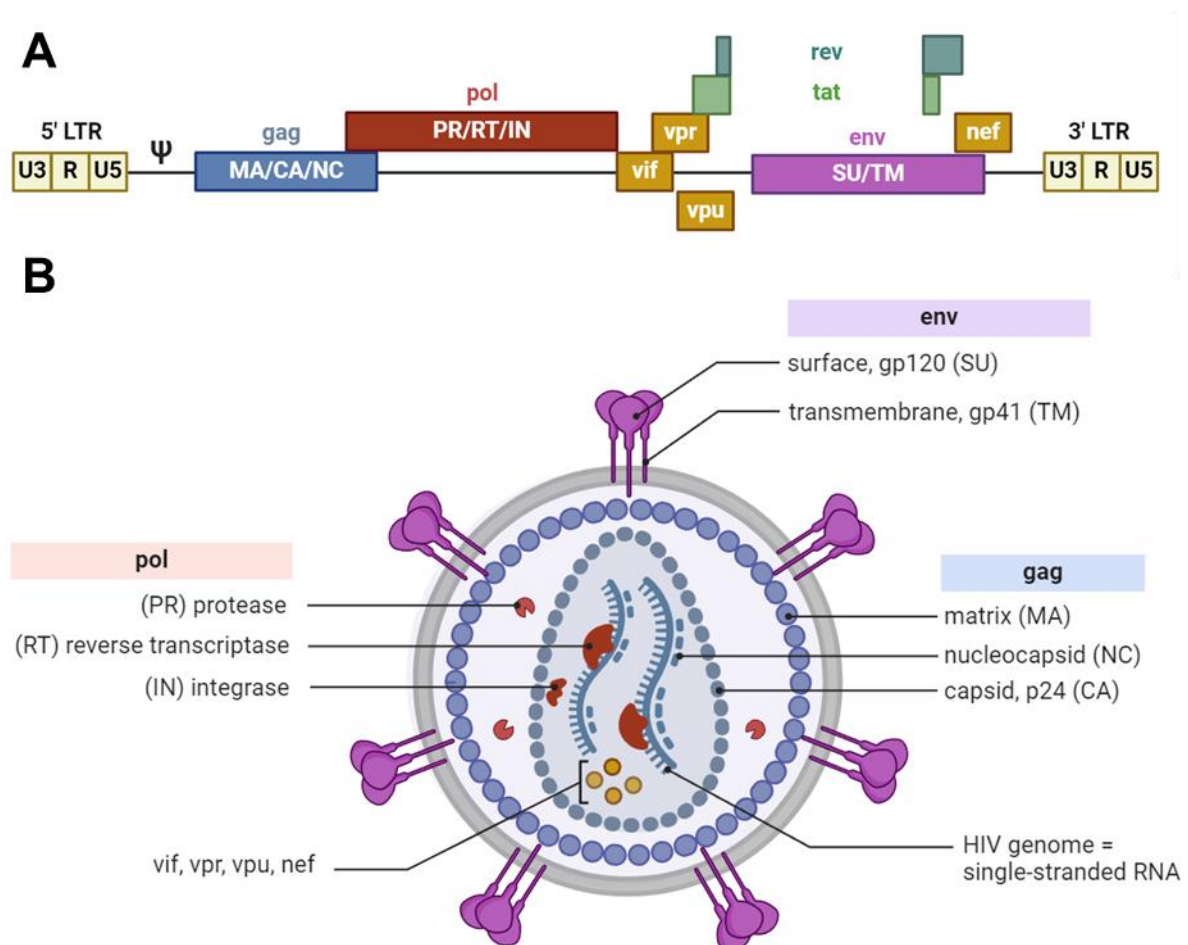


Figure 1.1: Annotated structure and the genome of HIV-1

The viral genome consists of *gag*, *pol* and *env* with 5' and 3' LTRs on either end (A). The genome has three open reading frames, which result in the transcription of the structural proteins (encoded by *env* and *gag*), viral enzymes (encoded by *pol*), accessory proteins (encoded by *vif*, *vpr*, *vpu*, *nef*) and regulatory proteins (encoded by *tat* and *rev*). The mature virus contains the envelope proteins (gp120, SU and gp41, TM) embedded in a lipid bilayer which is coated by the matrix (MA) protein, the HIV genome with nucleocapsid (NC) bound encapsulated by capsid (CA), viral enzymes protease, reverse transcriptase, integrase and accessory proteins (*nef*, *vif*, *vpu*, *vpr*) (B). This figure has been modified using Biorender (<https://app.biorender.com>).

1.3.2 HIV Replication Cycle

The proteins previously discussed that comprise the structure of HIV all play a role in viral replication. HIV-1 binds to the host cell's CD4 receptor and chemokine co-

receptor (CCR5 and/or CXCR4) via its gp120 glycoproteins (Figure 1.2 – step 1) (Berger *et al.*, 1999; Clapham and McKnight, 2002). The process of viral replication begins when gp120 specifically binds CCR5 or CXCR4 depending on the tropism of the HIV (Markosyan *et al.*, 2003). The viral membrane then fuses with the cell membrane and the viral RNA enters the cell (Figure 1.2 – step 2). The binding of gp120 to CCR5 causes gp41 to fold into a hairpin conformation and results in a six-helical bundle which induces membrane fusion by bringing the two lipid membranes together (Chen, 2019). This results in the release of the viral genomes coated with the viral capsid into the host cell (Figure 1.2 – step 3).

Reverse transcription is initiated during the import of the capsid to the nucleus (Figure 1.2 – step 4). Reverse transcriptase requires a primer, for this, it recruits tRNA^{Lys3} from the host cell for this purpose, which binds to the primer binding site on the HIV RNA and the first five nucleotides are added to the primer (Dobard *et al.*, 2007; Isel *et al.*, 1996; Luban and Goff, 1995). Reverse transcription involves the conversion of the HIV RNA genome into double-stranded DNA. This process starts with the formation of the stable reverse transcription complex (RTC) (Das *et al.*, 2019; Larsen *et al.*, 2018). The RTC comprises of viral RNA, host cell tRNA^{Lys3}, synthesised DNA, nucleic acids, viral factor (HIV matrix protein, HIV capsid, HIV nucleocapsid protein, integrase, vpr) and host factors (Fassati and Goff, 2001; Iordanskiy *et al.*, 2006; Nermut and Fassati, 2003). Reverse transcription will occur in this complex, beginning during the uncoating of capsid and completing in the nucleus just before uncoating is completed, in time for its release, since reverse transcription is a slow process taking 8-12 hours while nuclear import is faster taking 0-2 hours (Arhel, 2010; Selyutina *et al.*, 2020). The plus-strand of the DNA is synthesised in a discontinuous manner with multiple sites initiating transcription and occurring rapidly (Klarmann *et al.*, 1997), while the minus strand is synthesised at a slower rate (Thomas *et al.*, 2007). The rate of DNA synthesis is also dependent on the state of the target cell, and this is observed in quiescent cells which will replicate HIV at a slower rate (Zack *et al.*, 1992). The uncoating of the capsid leaves the HIV transcribed genome vulnerable to nuclease degradation, thus the newly synthesised DNA is wrapped around the host and viral proteins to form the pre-integration complex (PIC) (Khiytani and Dimmock, 2002). The host cellular factors, which are bound to integrase and are involved in this process include integrase

interactor 1 (INI1), survival motor neuron-interacting protein 2 (Gemin2), histone deacetylase 1 (HDAC1) and sin3A-associated protein (SAP18) (Hamamoto *et al.*, 2006; Kalpana *et al.*, 1994; Rain *et al.*, 2009; Sorin *et al.*, 2009). INI1 and Gemin2 associate with the RTC/PIC and promotes integrase activity as well as reverse transcription. SAP18 and HDAC1 form a complex involved in the initiation of reverse transcription.

To deliver its genome to the host cell, the virus undergoes partial uncoating and transport to the nucleus (Figure 1.2 – step 5). This process involves the host cell's cytoskeleton which is a network of microtubules and microfilaments. The nuclear transport and uncoating were initially believed to involve the cytoplasmic proteins dynein and viral proteins HIV capsid, nef and vif. However, studies have since shown that several other cytoplasmic proteins are involved (Delaney *et al.*, 2017; Lukic *et al.*, 2014; McDonald *et al.*, 2002). Microtubule-associated proteins were found to tether HIV capsid (p24) to the microtubules promoting their stability and hence trafficking of the capsid to the nucleus and transportin-1 similarly was discovered to promote nuclear import of capsid, but also mediates the uncoating of capsid (Fernandez *et al.*, 2019, 2015). Dynein adapter protein bicaudal D2 (BICD2) and Elongation Factor zeta 1 (eEF1A) respectively, actively promote and regulate the movement of HIV's genetic information to the nucleus (Dharan *et al.*, 2017; Malikov *et al.*, 2015). Reverse transcription and uncoating are completed in the nucleus (Burdick *et al.*, 2020; Dharan *et al.*, 2020) (Figure 1.2 – step 6).

Integration of the reverse-transcribed HIV genome into the host cell's genome is an essential step in viral replications (Figure 1.2 – step 7). The PIC is responsible for integration and the host proteins in this complex include high-mobility group protein A1 (HMGA1) and barrier-to-auto-integration factor (BAF), while the viral proteins involved are XRCC6, TFRC and HSP70 (Lin and Engelman, 2003; Schweitzer *et al.*, 2013). Topoisomerase II α and β are also present in the PIC and are essential for replication (Kondapi *et al.*, 2006). Lens epithelium-derived growth factor (LEDGF/p75) binds integrase and specifically binds the AT-rich regions in chromosomal DNA to ensure that the viral genome is actively transcribed (Ciuffi *et al.*, 2005; Llano *et al.*, 2006). Cleavage and polyadenylation specificity factor 6 (CPSF6) have also been

implicated in the locational preference for integration in euchromatin (Bejarano *et al.*, 2019; Burdick *et al.*, 2020).

Integrase is responsible for the integration of the DNA into the host genome and IN1 stimulates the DNA-joining activity of the enzyme (Turelli *et al.*, 2001). Integrase first cleaves the 3' ends of the viral DNA leaving a hydroxyl group on the adenine nucleotide. This cleavage occurs in the cytoplasm before nuclear import. In the nucleus, integrase binds to the host DNA and finally joins the two strands by simultaneously cleaving five base pairs of the host DNA and covalently ligating the viral genome at its 3' end hydroxyl group (Ciuffi, 2016). The host's DNA repair machinery then removes the protruding DNA 5' ends and repairs the five unpaired bases of the host's genome (Ciuffi, 2016).

The virus either undergoes active transcription (Figure 1.2 – step 8) or latency at this point. Active transcription will result in the production of new viral particles, while the viral genes will not be transcribed and translated during latency and leave the viral genome dormant. HIV latency is the reason that ART is not curative (Chun and Fauci, 1999) since ART suppresses active viral replication but doesn't affect latent virus. When these latent cells become transcriptionally active, they can produce viral particles to initiate new infections (Jubault *et al.*, 1998). Viral transcription utilises the host's machinery to generate mRNA encoding HIV. The 5'-LTR serves as the initiation site of transcription and it contains the binding site for multiple host transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and nuclear factor of activated T cells (NFAT) (Böhnlein *et al.*, 1988; Kinoshita *et al.*, 1998; Siekevitz *et al.*, 1987). These T cell mitogens are responsible for T cell activation and they were also found to regulate HIV-1 gene expression by binding to the 5' LRT (Siekevitz *et al.*, 1987).

It is common knowledge that HIV's high genetic variation, which allows the virus to evade the immune system and treatment, is attributed to the reverse transcriptase enzyme's lack of proofreading ability. However, replication errors may also arise from host DNA polymerase which, replicates the host's genome with HIV's genome integrated and host RNA polymerase II which transcribes messenger (mRNA) from

the integrated HIV genome (Figure 1.2 – step 8). The fidelity of DNA polymerase is believed to be higher than RNA polymerase II and reverse transcriptase since it possesses a proofreading ability (Kunkel *et al.*, 1989). This makes the number of errors contributes significantly less than the other two enzymes, especially when considering HIV high replication rate (Hu and Hughes, 2012). However, the error rate contributed by each RNA polymerase II and reverse transcriptase has shown conflicting results (Abram *et al.*, 2014). Mutations in the HIV genome have been observed to occur during reverse transcription, and are caused by a cellular factor known as apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G). APOBEC3G is a cytidine deaminase that converts cytosine to uracil in the minus strand of DNA while being produced (Bishop *et al.*, 2004). These mutations are therefore present as adenine mutations in the RNA of the new virus produced, resulting in genetic variation and possibly resistance depending on their position.

Transcription is induced by the binding of viral tat to the trans-activating response (TAR) site in the 5' LTR (Schulze-Gahmen and Hurley, 2018). This binding is mediated by the positive transcription elongation factor (p-TEFb) when it concurrently binds tat and facilitates binding to the TAR site, as well as phosphorylating RNA Polymerase II. This allows for evasion of the pause that the polymerase usually takes during transcription of the viral genes. The mRNA is spliced by the host machinery and the process is facilitated by the HIV regulatory elements (Sertznig *et al.*, 2018) and the mRNA is transported out of the nucleus into the cytoplasm using the host cell's mRNA transport system for translation (Figure 1.2 – step 9). In the cytoplasm, viral proteins tat and rev protect the viral mRNA from degradation by host cellular proteins (Chukwurah *et al.*, 2017; Lee *et al.*, 2018). Translation is accomplished using the host's eukaryotic translational machinery. The ribosomal is recruited by HIV's 5' capped untranslated region (UTR) mRNA and the TAR element (Chukwurah *et al.*, 2017). HIV factors target the host's translational factors involved with the steps of translation (initiation, elongation and termination) to disrupt the host's translational machinery. The HIV proteins are translated in a cap-dependent (majority) or independent mechanism. This is when the frameshifting occurs in the ribosome, to generate all of the proteins HIV requires (Korniy *et al.*, 2019).

The HIV proteins are assembled near the cell membrane (Figure 1.2 – step 10). The envelope protein which is expressed as gp160 is cleaved by furin to produce gp120 and gp41 (Ward and Wilson, 2015). They then self-assemble into a trimeric spike (Donlon and Frankel, 2012). This involves the packaging of the two strands of viral RNA, host tRNA^{Lys3}, HIV envelop protein and the Gag-Pol polyprotein (Korniy *et al.*, 2019). The Gag-Pol polyprotein mediates assembly and once complete the virus buds from the plasma membrane acquiring its lipid membrane from the host cell (Figure 1.2 – step 11). The envelope proteins which are embedded in this membrane now spike outwards and protease becomes active, cleaving the Gag-Pol polyprotein into HIV's structural proteins which triggers condensation of the HIV genome and capsid assembly thus resulting in a mature HIV particle (Figure 1.2 – step 12).

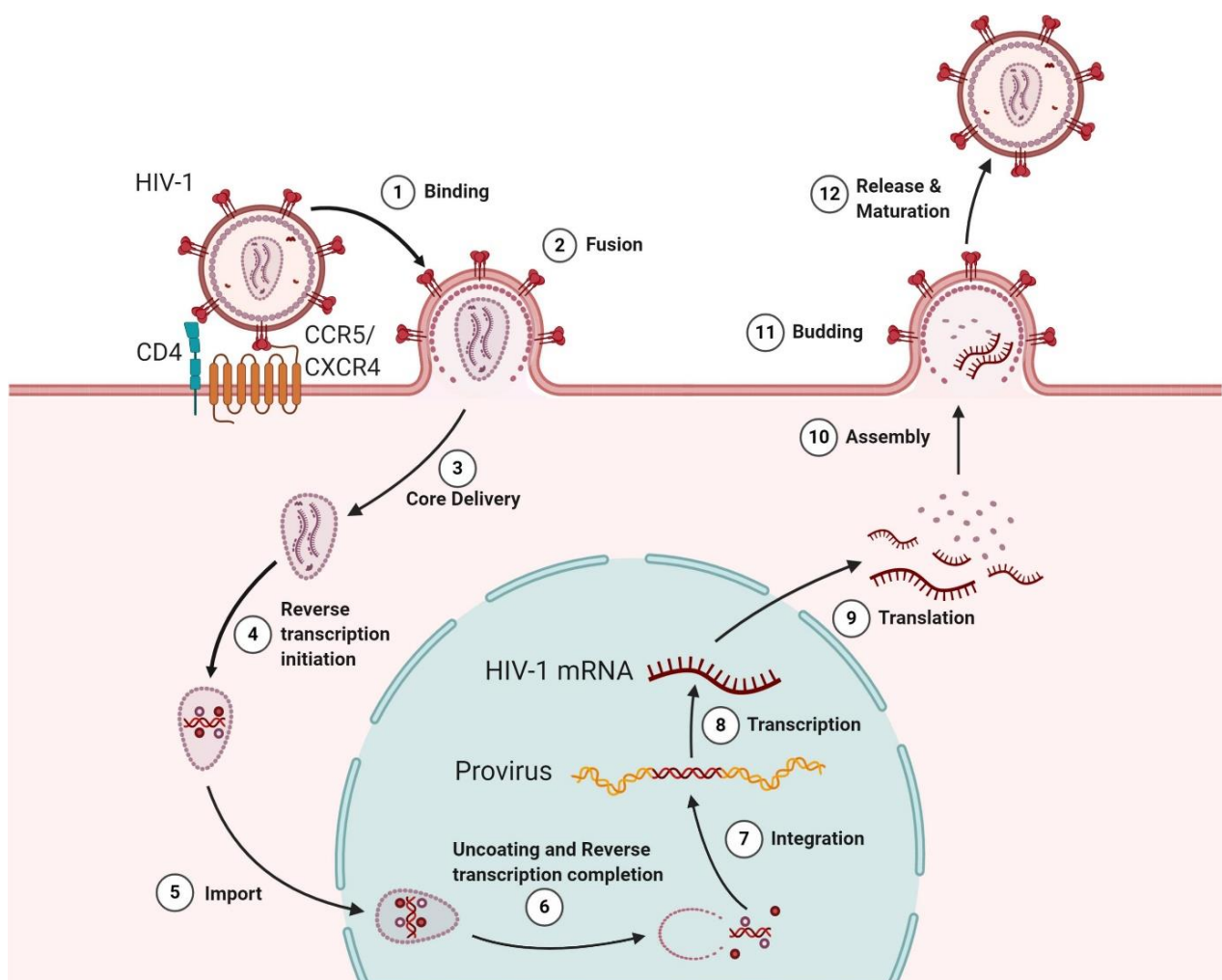


Figure 1.2: The HIV replication cycle in a CD4 cell

To replicate HIV undergoes a 12-steps process in a host cell, resulting in a mature virus particle. HIV-1 gp120 binds to the CD4 receptor and chemokine co-receptor (CCR5 and/or CXCR4) (1)

subsequently fusing with its membrane (2), which results in the release of the viral capsid containing the viral genetic information (3). Reverse transcription is initiated (4) and the capsid begins uncoating as it is imported to the nucleus by the host's cytoskeleton (5). In the nucleus, reverse transcription and uncoating are completed simultaneously (6). The resultant viral DNA is then integrated by integrase into the host's genome (7). The HIV-1 DNA is then transcribed using the host's machinery and HIV's regulatory proteins (8). The viral proteins are translated as polypeptides (Gag-Pol polyprotein and gp160) (9). The viral proteins are assembled into a viral particle near the host cell membrane (10). The viral particle then buds out of the cell membrane acquiring its lipid membrane (11) and the Gag-Pol protein is cleaved into the structural proteins resulting in a mature viral particle (12) (Ramdas *et al.*, 2020).

1.4 HIV prevention and treatment strategies

There is currently no cure for HIV and this is due to HIV latency and the integration of the viral DNA into the host cell's genome (Cummins and Badley, 2015). Vaccines have been investigated as a prevention strategy, but none have been successful yet. This is due to HIV's high mutation rate and thus ability to escape the immune response (Lu *et al.*, 2013). Therefore, preventative and treatment strategies have been used to manage the HIV epidemic.

1.4.1 HIV Prevention

HIV is transmitted through blood (drug users and perinatal transmission), sexual fluids (sexual transmission) and breast milk (Nisida *et al.*, 1996). Therefore, the prevention strategies involve preventing contact with these fluids. There are three types of prevention strategies: behavioural, biomedical and structural interventions (Rotheram-Borus *et al.*, 2009).

Behavioural interventions include educating the public on the disease and encouraging biomedical interventions (Coates *et al.*, 2008). Some examples of these strategies include: encouraging people to get tested for HIV, drug users to use sterilised needles, people to limit their number of sexual partners and practice safe sex. A successful campaign was launched in Lesotho called 'Know Your Status'

(WHO, 2008). The campaign was launched in 2004 when only 50 000 people knew their HIV status, but by 2007 over 240 000 people knew their status.

Biological interventions include male circumcision, barrier methods (condoms, diaphragm), antimicrobial products used for pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) (Rotheram-Borus *et al.*, 2009). Male circumcision reduces the chance of HIV infection by 50-60% according to the Centre for Disease Control and Prevention (CDC) during heterosexual contact (CDC, 2022), while the use of male condoms reduces the contraction of HIV by 70%-95% (Anderson, 2003; Foss *et al.*, 2004). The most effective intervention thus far has been the early implementation of ART, thus resulting in the 90-90-90 UNAIDS goals, wherein 90% of all people living with HIV should know their HIV status, 90% should be on ART and 90% of on ART should be virally suppressed (Mitchell *et al.*, 2022; Sohail *et al.*, 2020).

PrEP and PEP both involve the use of anti-retrovirals, which inhibits the replication ability of HIV. Individually takes the antiretroviral drug (ARV) either before (PrEP) or after HIV exposure (PEP) so that it is present at the site of infection and hence prevents HIV from establishing a permanent infection in the cell. PrEP should be taken by people who have a substantial risk for HIV infection such as sex workers and injecting drug users (Bekker *et al.*, 2020). The World Health Organisation (WHO) recommended a Tenofovir Disoproxil Fumarate (TDF) based PrEP since 2015 (WHO, 2016). They have also recommended the Dapivirine ring for women (WHO, 2021) and the long-acting injectable Cabotegravir (CAB-LA) as PrEP (WHO, 2022). In South Africa, the combination of TDF and Emtricitabine (FTC) as a single oral tablet is recommended for PrEP in adults by the National Health Department (NHD) (NDH, 2021).

PEP should be taken by people who are HIV-negative and have been exposed to HIV in the period of 72 hours (Sultan *et al.*, 2014). It is a regimen consisting of three ARVs and must be taken for 28 days. The current recommendation for PEP in South Africa is TDF/Lamivudine(3TC)/Dolutegravir(DTG) as a fixed-dose combination (NDH, 2020).

Structural interventions involve changing the social, economic, organisational and environmental factors that influence an individual's HIV prevention or risk behaviour (Sumartojo, 2000). These include providing clean syringe needles for injecting drug users, establishing a 100% condom use policy for sex workers and providing sufficient funding for HIV testing, prevention and treatment (Blankenship *et al.*, 2006).

1.4.2 HIV Treatment

The development of highly active antiretroviral treatment (HAART) in 1995 was a major turning point in the HIV/AIDS global epidemic (James, 1995). HAART comprises of a combination of antiretroviral drugs inhibiting viral replication via different mechanisms. HAART evolved into ART or combination antiretroviral therapy (cART). ART has decreased mortality and increased the lifespan of people living with HIV, thus combating the global epidemic (Roser and Ritchie, 2018). However, ART must be taken throughout the patient's lifetime. This is because ART suppresses the virus to low levels, but isn't curative as the virus will rebound within two weeks after ART is ceased (Davey *et al.*, 1999). This requirement for patients to adhere to their treatment is challenging due to numerous factors including; ART has many unpleasant side effects, the regimen of drugs must be taken daily and drug-drug interactions complicate the treatment (Legesse and Reta, 2019). If these hurdles are not overcome and the patient doesn't adhere to treatment, antiretroviral resistance could develop (Danel *et al.*, 2009). This would drastically limit the treatment options and is a major challenge in HIV treatment.

1.4.2.1 ARV drug classes

ART regimens are generally initially composed of a least three ARVs which are from two different classes. There are six classes of FDA-approved ARVs: Entry inhibitors (EI), Integrase strand transfer inhibitors (INSTIs), protease inhibitors (PI), reverse transcriptase inhibitors which include nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and capsid inhibitors (Arts and Hazuda, 2012). FDA-approved ARVs are available online

(<https://hivinfo.nih.gov/understanding-hiv/fact-sheets/fda-approved-hiv-medicines> (NIH, 2023)).

1.4.2.1.1 **Entry or Attachment Inhibitors**

HIV binds to the CCR5 co-receptor to attach to the CD4 cell, leading to a conformational change in gp41 which results in the virus fusing with the cell membrane. Two drugs have been developed to inhibit these two steps. MVC which inhibits the binding of HIV to CCR5 and Enfuvirtide which inhibits gp41 preventing fusion.

1.4.2.1.1.1 **Attachment inhibitors**

Maraviroc (MVC) is the only FDA-approved attachment inhibitor to date (Dorr *et al.*, 2005; Tsamis *et al.*, 2003) and binds to the hydrophobic pocket within the transmembrane helices of CCR5, thus stabilising the co-receptor in a conformation which is not recognised by the HIV envelope protein, gp120 (Dragic *et al.*, 2000). MVC will only inhibit viruses that are CCR5 tropic, not CXCR4. This means the patient must first undergo tropism testing (genotypic/phenotypic testing) which is expensive (Tremblay *et al.*, 2013). Other attachment inhibitors under investigation include Fostemsavir, Ibalizumab and Enfuvirtide (Orkin *et al.*, 2022) No attachment inhibitors for CXCR4-tropic variants are currently available.

1.4.2.1.1.2 **Fusion inhibitors**

These ARVs mimic one of the homologous domains of gp41 that bundle together to promote fusion, thus binding to the domains and disrupting the bundling and thus inhibiting fusion of the HIV lipid membrane and the host cell membrane (Wilén *et al.*, 2012). Enfuvirtide (T-20) has been the only EI developed with this mechanism and it has demonstrated potent activity *in vivo* (Kilby *et al.*, 1998; Lalezari *et al.*, 2003).

1.4.2.1.2 **Reverse Transcriptase Inhibitors**

These ARVs inhibit reverse transcription and have been the backbone of ART (Battini and Bollini, 2019; Rai *et al.*, 2018). The ARVs bind to reverse transcriptase at the active binding site (NRTI) or the allosteric binding site (NNRTI) (Namasivayam *et al.*, 2019; Peddi *et al.*, 2018). These two target sites have been declared the optimal target sites for the development of ARVs.

1.4.2.1.2.1 Nucleoside Reverse Transcriptase Inhibitors

NRTIs are the most commonly used ARVs since they are recommended in combination with all other ARV drug classes. These ARVs inhibit reverse transcription through chain termination. The ARV mimics deoxynucleoside triphosphates (dNTPs) thus they are incorporated in the transcribed HIV DNA (Cheng *et al.*, 1987; Yates and Seley-Radtke, 2019). However, they lack a 3' hydroxyl group at the sugar moiety which prevents the formation of a phosphodiester bond with the next dNTP in the synthesised HIV DNA strand. This results in the termination of the complementary DNA strand during extension, specifically either the DNA synthesised from RNA (minus strand) or from DNA (plus-strand).

This was the first class of drugs approved for the treatment of HIV with the FDA approving the use of Zidovudine (ZDV) in 1987 to treat HIV in monotherapy (Young, 1988). Since, there have been several approved NRTIs including Abacavir (ABC), FTC, 3TC, TDF, Tenofovir Alafenamide (TAF), Didanosine (ddI) and Stavudine (d4T).

TDF which was approved in 1991 and TAF which was approved in 2016 are the most commonly used NRTIs (Li *et al.*, 2022). TAF is a new formulation of TDF (DeJesus *et al.*, 2018), displaying superiority over TDF, as it has shown to increase total cholesterol and cause less adverse effects (renal and bone) (Wassner *et al.*, 2020). TDF is often recommended in ART guidelines as the first of two NRTIs in the ART regimen (WHO, 2016). (Markowitz and Sarafianos, 2018; Michailidis *et al.*, 2009). TAF has displayed higher viral suppression rates and risk of bone and renal events, but TDF is recommended generally and particularly in South Africa due to its lower cost and TAF's association with weight gain (Hill *et al.*, 2018; Willem D. F. Venter *et al.*, 2019).

3TC which was approved in 1995 and FTC which was approved in 2003 have similar molecular structures (Modrzejewski and Herman, 2004). These two drugs have been commonly recommended in ART guidelines as the second NRTI and are used interchangeably (WHO, 2016). Their efficacy is the same, thus the decision of which to use in ART is generally determined by the drugs that are co-formulated with them (Modrzejewski and Herman, 2004).

The latest addition to this class is a nucleoside reverse transcriptase translocation inhibitor (NRTTI), Islatravir (ISL). The drug has not been approved but has shown to be efficacious and promising due to the fact that the ARV has two inhibitory mechanisms including translocation inhibition and delayed chain termination (Markowitz and Sarafianos, 2018; Michailidis *et al.*, 2009).

1.4.2.1.2.2 Non-Nucleoside Reverse Transcriptase Inhibitors

NNRTIs bind to reverse transcriptase forming at a hydrophobic pocket proximal to the active site and thus inhibit reverse transcription allosterically (Peddi *et al.*, 2018). More specifically, they bind to the allosteric pocket in reverse transcriptase's p66 subunit causing a conformational change in the active site (Esnouf *et al.*, 1995). This change involves the rearranging of a three-stranded β -sheet in the active site of the p66 subunit, locking it into an inactive conformation. The drugs are classified according to their time of development with the initial NNRTIs released being deemed the first generation ARVs. Nevirapine (NVP) was the first approved NNRTI (1996) which is known as a first generation NNRTI (Li *et al.*, 2022). The remaining first-generation NNRTIs include Delavirdine (DLV) approved in 1997 and Efavirenz (EFV) approved in 1998 (Usach *et al.*, 2013). The efficacy of the first-generation NNRTIs has severely decreased over the years due to their heavy usage (Shirvani *et al.*, 2019), which resulted in a high prevalence of NNRTI drug resistance. DLV is no longer prescribed due to its low genetic barrier, high pill burden and severe adverse events (Scott and Perry, 2000; Wang *et al.*, 2019). Similarly, NVP and EFV have poor pharmacokinetic properties and confer unwanted side effects. NVP causes severe rashes and EFV is associated with central nervous system side effects (Best and Goicoechea, 2008; Podzamczar and Fumero, 2001).

The second generation of NNRTIs was thus developed with a higher genetic barrier and included Etravirine (ETR) approved in 2008 and Rilpivirine (RPV) approved in 2011 (Li *et al.*, 2022). These NNRTIs are effective against the mutations selected for by the first-generation NNRTIs (i.e. K103N, Y181C).

A number of third-generation NNRTIs are therefore currently under investigation to circumvent the problems with side effects and efficacy. Doravirine (DOR) is an approved third-generation NNRTI that displays high potency against viruses harbouring the mutations K103N and Y181C (Lai *et al.*, 2009).

1.4.2.1.3 Integrase Strand Transfer Inhibitors (INSTIs)

These ARVs have been favoured for the treatment of HIV in recent years (WHO, 2016). INSTIs prevent the transfer of the viral DNA into the host's genome by binding to integrase and the viral DNA, as well as interacting with the critical magnesium metal ion co-factors in the active site (Grobler *et al.*, 2002). This means that all of the ARVs have a hydrophobic group which binds integrase with viral DNA and a metal-binding pharmacophore which binds the magnesium ions. The currently approved INSTIs are Bictegravir (BIC), CAB, DTG, Raltegravir (RAL) and Elvitegravir (EVG) (Scarsi *et al.*, 2020a). RAL and EVG were the first generation INSTIs, while the second generation of INSTIs include DTG, BIC and CAB which have a higher genetic barrier to resistance as well as retain effectivity against mutations selected for by RAL and EVG (Malet *et al.*, 2012; Scarsi *et al.*, 2020b). DTG is recommended as the basis of first-line ART worldwide (WHO, 2016), while CAB-LA is currently recommended as PrEP (WHO, 2022). DTG has been favoured due to its high genetic barrier to the development of resistance, thus it was recommended for first-line ART rather than an NNRTI (Llibre *et al.*, 2015). However, a concern is the fact that INSTIs have been observed to cause significant weight gain compared to NNRTIs and PIs (Sax *et al.*, 2020). The combination of DTG with TAF is of particular concern since the combination results in the highest weight gain, particularly affecting African women (Venter *et al.*, 2019).

1.4.2.1.4 **Protease Inhibitors**

These ARVs bind to protease in the catalytic site mimicking a peptide and thus preventing protease from cleaving viral proteins (such as the gag-pol polypeptide) into functional proteins during HIV maturation (Dakshinamoorthy *et al.*, 2023). The approved PIs are Ritonavir which was approved in 1996, Indinavir, Nelfinavir, Saquinavir, Amprenavir, Lopinavir, Atazanavir, Fosamprenavir, Tipranavir and Darunavir (DRV). PIs are metabolised by cytochrome P450 CYP3A4 isoenzyme (Becker, 2003), which significantly reduces the half-life of the drug in the body. Therefore, all PIs must be boosted with Ritonavir (r) which inhibits cytochrome P450 CYP3A4 isoenzyme (Zeldin and Petruschke, 2004).

1.4.2.1.5 **Capsid Inhibitors**

This is the most recent class of HIV inhibitors developed and approved. HIV capsid proteins play important roles during replication. They are responsible for maturation after replication during budding. These key roles have made capsid a desirable target for the development of ARVs (Thenin-Houssier and Valente, 2016). Lenacapavir (LEN) is the only approved ARV in this class and as a long-acting drug offered to patients with multi-drug resistant HIV (Paik, 2022). LEN has displayed 100% efficacy in HIV prevention in cisgender women receiving subcutaneous LEN every 26 weeks (Bekker *et al.*, 2024).

1.4.2.2 **ART Initiation and Monitoring**

The WHO guidelines recommend initiating ART for all people, including adults, pregnant and breastfeeding women, infants, adolescents and children, with HIV immediately after diagnosis, regardless of their CD4 T cell count and their clinical stage of HIV infection (WHO, 2021). This improves the patient's health and decreases HIV transmission as rapid initiation of ART has been shown to rapidly improve the time to viral suppression and prevent viral transmission (Michienzi *et al.*, 2021; Pathela *et al.*, 2021). The patient's viral load (VL) is then monitored over the course of treatment. This is recommended after 6 months of starting ART, then at 12 months, and finally

yearly (NDH, 2023; WHO, 2021). CD4 count and clinical monitoring are used to assess treatment failure only when VL testing is not available (WHO, 2021). A VL < 50 copies/mL is considered undetectable and patients are maintained on their current ART regimen. Patients with a VL between 50 and 1000 copies/mL are given adherence counselling. Patients with two confirmed VLs $\geq$ 1000 copies/mL are experiencing virological treatment failure are switched onto a different ART regimen. Genotypic resistance testing is performed to determine the regimen, if the patient was failing on a DTG-based regimen and no DTG resistance mutations are detected the patient will remain on DTG-based ART.

1.4.2.3 ART in South Africa

The South African National ART Clinical Guidelines recommend that newly HIV-infected patients are started on first-line therapy which is INSTIs-based (TLD) (NDH, 2023, 2019). Before 2019, first-line therapy was NNRTI based with the guidelines recommending a regimen of TDF + 3TC/FTC + EFV/NVP and after patients experienced virological failure they were switched onto a second-line ART regimen of ZDV + 3TC + LPV/r (NDH, 2015). In 2019, TDF + 3TC + DTG (i.e., “TLD”) was introduced as the preferred first-line regimen for initiation of ART (NDH, 2019). The patients already on an NNRTI-based first-line regimen could either remain on their current regimen or transition onto TLD. ART is monitored using viral load testing with the first test performed after 3 months of ART initiation and if the VL is undetectable the second test is performed after 10 months and if the patient remains suppressed yearly tests are performed (NDH, 2023). Treatment failure is defined as two or more consecutive test results with VL $\geq$ 1000 copies/mL, after which the patient is given adherence counselling and switched onto TLD if they were previously on NNRT-based ART. If the patient was taking TLD and on a DTG-based regimen for at least two years, genotypic testing is performed.

1.5 HIV Drug Resistance

ART has increased the lifespan of people living with HIV and improved their quality of life (Samji *et al.*, 2013). However the viruses' high replication and error rate, allows

HIV to be highly adaptable and escape drug inhibition (Preston *et al.*, 1988; Wei *et al.*, 1995). The virus will rapidly replicate and mutations will arise due to reverse transcriptase's lack of a proofreading activity. The ARVs create selection pressure, favouring replication of the virus with resistance to that specific ARV, thus creating a dominant population of these mutant viruses (Domingo and Holland, 1997). These mutations affect the ability of the ARVs to bind and inhibit viral replication using their particular class mechanism, therefore some mutations often result in cross-resistance to the other ARVs in the same drug class (Delaugerre *et al.*, 2001). It is for this reason that three drugs from two different drug classes are used in ART.

However, drug resistance may still develop, during viral suppression in plasma and this is due to the varying concentration of the drug over time in the body tissues (Feder *et al.*, 2021). This means that the ARVs are not present in all the body compartments at the same concentrations resulting in the optimal environment in some tissues for the development of resistance. The ability of patients to adhere to their treatment regimen is also challenging (Legesse and Reta, 2019). The different half-lives of the three drugs coupled with suboptimal adherence results in suboptimal concentrations of the ARVs in the body and offer the virus an opportunity to develop resistance and propagate. It is important to note that when the selective pressure of ARVs is removed, the replicating viral population typically reverts back to wild-type (no resistance mutations) usually due to the reduction in fitness caused by the mutation (Gandhi *et al.*, 2003). Mutation-containing viruses are either transmitted to patients during infection or acquired by patients when they experience suboptimal treatment adherence. Low abundance variants are the mutant/s present as the minority in an individual's body where the wild-type HIV population have become the majority (Geretti *et al.*, 2022). Therefore, the drug resistance strains are still present but cannot be detected through standard Sanger-based genotypic drug resistance tests. However, these mutations do not affect virological outcomes and thus there is no need to screen for these mutations during typical drug resistance testing (Wang *et al.*, 2023).

1.5.1 Transmitted Drug Resistance

Transmitted drug resistance (TDR) is detected in a newly infected ART-naïve patient. TDR is not found in high frequencies especially compared to other parasites such as malaria (Read and Huijben, 2009). These estimates vary across studies since the measurement of TDR often relies on patients or clinics reporting previous ART treatment. The worldwide prevalence of TDR in 2021 was 9.3% with NNRTIs (5.7%) displaying the highest prevalence of TDR across the ARV classes (Guo *et al.*, 2021). South Africa has shown a rapid increase in TDR since the implementation of ART (Gupta *et al.*, 2012). The TDR prevalence was below 5% in 2009 and rose to 11.9% in 2016. More recent studies have estimated this percentage is between 5% (Lippman *et al.*, 2020) and 21.8% (Moyo *et al.*, 2020). This supports the change from an NNRTI-based first-line regimen to an INSTIs-based regimen due to the ARVs' high genetic barrier (Chimukangara *et al.*, 2019).

1.5.2 Acquired Drug Resistance

Acquired drug resistance (ADR) mostly results from sub-optimal adherence since studies have demonstrated a strong link between adherence and drug resistance (Ekstrand *et al.*, 2011). This is supported by drug resistance being twice as common in patients with sub-optimal adherence (adherence of 80% to 95%) (Lima *et al.*, 2008) and by the fact that treatment interruptions result in the increased presence of resistance mutations (Danel *et al.*, 2009). The prevalence of ADR is significantly higher than TDR and there is a higher prevalence observed within the NRTI and NNRTI drug class. In 2023, the prevalence of DRMs was 34% for NRTIs, 27% for NNRTIs and 9% for PIs in patients on ART, while for people failing therapy, the DRM prevalence was 50% for NRTIs, 49% for NNRTIs, 11% for PIs and 1% for INSTIs (Mirzaei *et al.*, 2024). This was significantly higher than TDR which was 3% for NRTIs and 5% for NNRTIs. The prevalence of ADR is significantly higher in South Africa with Chimukangara *et al.* (2021) reporting it at 92.2% in patients failing NNRTI-based ART, with 89% displaying dual-class resistance to NRTIs and NNRTIs. ADR is more prevalent in low-income countries and this could be due to unplanned treatment interruptions such as drug shortages and less frequent viral load monitoring (Marcellin *et al.*, 2008; NDH, 2023). Although the majority of the resistance observed is due to sub-optimal adherence, mutations still develop under optimal adherence and the percentage of acquired

resistance has been observed to increase with the number of years of on ART (Phillips *et al.*, 2005; Stadel and Richman, 2013).

1.5.3 Pre-treatment Drug Resistance

Pre-treatment drug resistance (PDR) is resistance detected in people initiating or re-initiating first-line ART, therefore it can include both acquired and transmitted drug resistance. This type of drug resistance is less complex to measure than TDR which can only be detected in recently infected individuals. The prevalence of PDR has been steadily increasing over the years worldwide (Gupta *et al.*, 2018). There is a high prevalence of resistance to NNRTIs with Southern Africa having the highest increase in the prevalence of NNRTI resistance at 11% in 2016. This increase in PDR prevalence particularly in South Africa would have implications on the efficacy of new ARVs (Chimukangara *et al.*, 2019). The rise in PDR in South Africa along with the benefits of including DTG in first-line therapy resulted in the alteration of the first-line ART regimen from NNRTI-based to INSTI-based (NDH, 2019). A study by Moyo *et al.* (2020) found that the PDR prevalence among laboratory-confirmed ART-negative patients was 22.8% and that 18.9% of all drug-resistant patients were resistant to NNRTIs.

1.5.4 Drug Resistance Testing

HIV drug resistance testing is critical in monitoring the treatment of highly ART-experienced patients failing cART. In South Africa, a genotypic drug resistance test is recommended after PI-based second-line ART (NDH, 2023, 2019). The results from the drug resistance testing inform clinicians which ARVs would be effective against their drug-resistant HIV strain actively replicating at the time of testing. There are two types of drug resistance tests genotypic and phenotypic testing. Genotypic drug resistance testing is commonly used to assess drug resistance due to the fact that they can easily be conducted in clinical laboratories and produce rapid results (Weinstein *et al.*, 2001). Phenotypic drug resistance testing is commonly used during research on the susceptibility of DRMs *in vitro*.

1.5.4.1 Genotypic Drug Resistance Testing

Genotypic drug resistance testing identifies the mutations associated with treatment failure (Ji and Sandstrom, 2022). This is performed using a blood sample of a person living with HIV failing ART. The plasma is isolated by centrifugation and the viral RNA is then extracted. The RNA is reverse transcribed and amplified using reverse transcriptase polymerase chain reaction (RT-PCR). The *pol* gene is amplified and sequenced using Sanger sequencing. This gene is selected because it encodes for the enzymes reverse transcriptase, protease and integrase which are the binding sites of the four main classes of ARVs used in ART (i.e., NRTIs, NNRTIs, PIs and INSTIs). The results are then compared to the wild-type HIV, to identify mutations. There are many different online tools for this interpretation (Liu and Shafer, 2006). The most widely used interpretation tool is the Stanford University HIV Drug Resistance Database which uses a predictive algorithm to determine whether the mutations present in the virus will cause resistance to specific approved ARVs. The algorithm is based on published studies conducted with the ARVs including clinical observations and *in vitro* experiments.

This type of testing allows for the detection of the known resistance mutations that are present. There are several disadvantages with genotypic testing. Sequencing is population-based and cannot identify individual HIV mutant variants (D'Amato *et al.*, 1998). Drug resistance mutations present below $\pm 20\%$ prevalence in the samples may be missed (Halvas *et al.*, 2006). The predictive algorithms don't integrate the results of previous ART history, VL and CD4 T cell count. This means that a clinician is required to interpret the results and the algorithm cannot advise on the appropriate ART regimen (Liu and Shafer, 2006). The complicated relationship between virological failure and drug resistance is not well understood therefore the results from the genotypic testing may be discordant with clinical outcomes (Shafer, 2002).

There are more sensitive genotypic testing techniques that have been developed to detect minor populations of drug-resistant HIV such as; single genome sequencing (Palmer *et al.*, 2005), allele-specific Polymerase Chain Reaction (PCR) (Metzner *et al.*, 2005), hybridisation assays (Shi *et al.*, 2004) and massively-parallel sequencing

(Margulies *et al.*, 2005). Each of these methods has advantages and disadvantages, however in most regions Sanger sequencing remains the standard of care for drug resistance testing. Oligonucleotide ligation assays use an immunosorbent-linked assay to detect the mutations present in a sample, with a single probe detecting a single mutation limiting the number of mutations that can be detected (Tobe *et al.*, 1996). Next-generation sequencing involves massive parallel sequencing which can identify minor mutant populations, but it is expensive and time-consuming (Ávila-Ríos *et al.*, 2020).

1.5.4.2 Phenotypic Testing

Phenotypic susceptibility assays measure the ability of a virus to replicate *in vitro* in the presence of an ARV. Similarly, to the preparation for genotypic testing, HIV RNA is isolated and RT-PCR is performed. The amplified portion of the HIV genome (usually *gag* or *pol*) is then inserted into a recombinant HIV expression vector and used to produce viral particles for testing in an *in vitro* phenotypic assay. The concentration of the drug which inhibits the viral particle's activity is compared to a wild-type reference to determine the susceptibility of the patient's viral strain to the ARV under evaluation. A commercial assay is offered by Monogram Bioscience (South San Francisco, United States), the PhenoSense Assay.

The first version of the phenotypic assay was a replicative competent assay and relied on the key principles described above (Kellam and Larder, 1994). In this assay, the peripheral blood mononuclear cells from healthy donors were used as a control and the viral activity was measured using the production of HIV-1 p24 antigen. The assay displayed variable results, depending on the cell type used in the assay and it was time-consuming. A single-cycle replication assay which used recombinant HIV clones which contain the amplified HIV genes from the patient, Human Embryonic Kidney (HEK) 239T cells which produce good quality virus-like particles and a luciferase reporter to measure viral activity was an improvement over the original assay (Petropoulos *et al.*, 2000). This assay is the 'golden-standard' for phenotypic testing since it is reliable and accurate (Hertogs *et al.*, 1998; McMahon *et al.*, 2009), yet it is still time-consuming and expensive.

This assay relies on cut-off values to determine the susceptibility/resistance of a viral particle, which is used to determine the efficacy of a particular ARV for treatment. The clinical cut-offs were originally designed based on the reproducibility of the wild-type in the phenotypic assay, and were denoted as the ‘technical cut-off’. This was not a good reflection of the clinical outcomes since it merely indicated the mutations with large differences in susceptibility rather than significant values that would reflect an outcome. An improvement was the method of classifying resistance/susceptibility using a ‘biological cut-off’ which is defined as the average viral activity reducing ARV plus two standard deviations measured in 1000 ARV-naïve patients (Harrigan *et al.*, 2001). The ideal standard for determining susceptibility/resistance would be the use of a ‘clinical cut-off’ which is determined by performing phenotypic testing and linking the result with the clinical outcomes observed.

The phenotypic test has an advantage over genotypic testing wherein the susceptibility of a patient’s HIV population to specific ARVs is measured (Blaise *et al.*, 2002). This allows the clinician to make an informed choice on the change of ART, since the results specify which ARVs will not be efficacious in the patient. The test can also provide information on previously unidentified resistance-causing mutations. There are several disadvantages including that the assay cannot identify minority mutant populations, the assay is expensive, complicated and time-consuming and the cut-off values used to determine susceptibility require sufficient data to be established (Blaise *et al.*, 2002).

1.5.5 Resistance in the ARV classes

1.5.5.1 Entry or Attachment Inhibitors

Resistance to this drug class develops in the envelop proteins (gp41 and gp120) since they are crucial for binding and are thus targeted by the inhibitors. Mutations to the attachment inhibitors have been observed in the third variable loop of gp120 such as K305R, S306P, T307I, F316I, K315R, T318R and G319E (Lobritz *et al.*, 2010). Mutations have also been identified outside this region such as N425K in gp120 which causes resistance to MVC (Ratcliff *et al.*, 2013). Additionally, tropism switching of the

patient's HIV from CCR5 to CXCR4 allows the virus to escape inhibition by MVC (Westby *et al.*, 2006).

Mutations to the fusion inhibitors arise in gp41's amino-terminal heptad repeat region (Rimsky *et al.*, 1998). Enfuvirtide which is the only fusion inhibitor available, has the following resistance-associated mutations: G36D, I37T, V38AM, N42T, N42D and N43K (Wei *et al.*, 2002). These mutations also result in a viral fitness reduction since they affect the envelope protein which plays an important role in viral replication (Reeves *et al.*, 2005).

1.5.5.2 Nucleoside Reverse Transcriptase Inhibitors

There are two mechanisms by which HIV establishes resistance to an NRTI, namely mutations that either increase reverse transcriptase's ability to distinguish between NRTIs and dNTPs blocking the inhibitors from being incorporated into the growing DNA strand (Huang *et al.*, 1998; Sarafianos *et al.*, 1999), or promote the removal of incorporated NRTIs through ATP-dependent pyrophosphorolysis (Meyer *et al.*, 1998). The NNRTI mutations are classified as nucleotide-associated mutations (NAMs) and thymidine analogue mutations (TAMs).

The most prevalent NAMs are M184V and K65R. M184V is selected by and causes high-level resistance (reducing susceptibility > 100 fold) to 3TC (Boucher *et al.*, 1993) and FTC (Tisdale *et al.*, 1993). The mutation is also selected for by ABC (3-fold reduction) but doesn't reduce susceptibility as much as 3TC/FTC (Miller *et al.*, 2000). This means while the presence of M184V alone results in resistance to 3TC/FTC, while an additional mutation is required before ABC is not effective (Katlama *et al.*, 2000). The M184V mutation is very common presenting in 1% of new HIV infections globally (Rhee *et al.*, 2020). K65R is found more frequently in HIV-1 subtype C (Invernizzi *et al.*, 2009). This is a result of subtype C possessing five consecutive adenine nucleotides before the K65 codon, at which reverse transcriptase displays more-accentuated pausing than subtype B resulting in mutagenesis (Coutsinos *et al.*, 2009). This mutation in combination with M184V is the second most common NRTI mutation in patients failing an NNRTI-based regimen containing TDF (Gregson *et al.*,

2016). The mutation is selected for by d4T and ABC and will not appear in a regimen containing ZDV (García-Lerma *et al.*, 2003; Margot *et al.*, 2002; Parikh *et al.*, 2006; Wainberg *et al.*, 1999).

TAMs are non-polymorphic mutations selected by ZDV and d4T (Meyer *et al.*, 2002; Naeger *et al.*, 2002). The majority of the NRTI mutations cause a significant reduction in the effectiveness of ZDV (Vandamme *et al.*, 1999). This is understandable as it was the first ARV used to treat HIV in monotherapy. The most common ZDV mutations are at positions M41L, D67N, K70R, L210W, T215YF and K219QE (Harrigan *et al.*, 1996; Hooker *et al.*, 1996; Kellam *et al.*, 1992; Larder and Kemp, 1989). The presence of multiple (≥ 3) TAMs results in high-level drug resistance (Goodall *et al.*, 2017). The TAMs cause resistance to NRTIs via two distinct mutation pathways (Marcelin *et al.*, 2004). TAM-1 includes the mutations M41L, L210W, and T215Y, while TAM-2 includes the mutations D67N, K70R, T215F, and K219Q/E (Cozzi-Lepri *et al.*, 2005).

1.5.5.3 Non-Nucleoside Reverse Transcriptase Inhibitors

The mutations conferring resistance to this drug class are positioned in the NNRTI-binding pocket and include mutations L100I, K101P, K103N, E138A, V179D, Y181C and Y188L (Tantillo *et al.*, 1994). There are a number of resistance causing amino acid substitutions at a single amino acid position (i.e. K103N/S/H/T/R/Q/E). These single mutations alone cause high-level resistance to the other NNRTIs (Frentz *et al.*, 2014). As discussed previously this ARV class is the most prevalent class in TDR (19%) and ADR (74%) (Moyo *et al.*, 2020; Zou *et al.*, 2020). However, these mutations do not result in a significant loss of fitness capacity which is often observed in mutants to non-NNRTI classes (Deeks, 2001; Dykes *et al.*, 2001; Imamichi *et al.*, 2001).

K103N is the most commonly transmitted drug resistance mutation (DRM) worldwide (Rhee *et al.*, 2021 and in South Africa (Hauser *et al.*, 2022). This mutation accounts for over 50% of the total resistance caused by NNRTI mutations (Rhee *et al.*, 2003; Shafer, 2006). The mutation is selected for under treatment with NVP and EFV, to which the mutation causes high-level resistance (Bachelier *et al.*, 2000; Reuman *et al.*, 2010). The mutation is commonly found in combination with Y181C and L100I during

treatment with EFV (Bacheler *et al.*, 2000). K103N does not affect the fitness of the virus, thus explaining the high prevalence of the mutation in the population (Deeks, 2001). Y181C is the second most prevalent NNRTI mutation and it causes high-level resistance to EFV, RPV and ETR (Melikian *et al.*, 2014; Rimsky *et al.*, 2012; Tambuyzer *et al.*, 2010).

G190A is selected by NVP and EFV exposure (Melikian *et al.*, 2014; Rimsky *et al.*, 2012) and the mutation is prevalent in subtype C dominant populations such as South Africa (Basson *et al.*, 2020; Derache *et al.*, 2016; Hauser *et al.*, 2022; Kityo *et al.*, 2017; Steegen *et al.*, 2016). V106M is selected for by NVP and EFV (Melikian *et al.*, 2014). V106M is predominantly found in HIV-1 subtype C, while V106A is observed in subtype B (Grossman *et al.*, 2004).

L100I is selected for by EFV, ETR and RPV (Bacheler *et al.*, 2000; Croxtall, 2012; Rimsky *et al.*, 2012). It is usually found in combination with K103N and together they reduce the susceptibility of all NNRTIs including DOR (Melikian *et al.*, 2014; Rhee *et al.*, 2023). K101P is selected for by all the approved NNRTIs (EFV, ETR, NVP, RPV) except DOR (Bacheler *et al.*, 2000; Martin *et al.*, 2020; Rimsky *et al.*, 2012; Tambuyzer *et al.*, 2010). E138A is selected for mainly by people on treatment with RPV (Rimsky *et al.*, 2012), but is also selected for to a lesser extent by people receiving treatment with ETR (Lai *et al.*, 2014), but the mutations are commonly observed in people who have previously taken RPV than ETR. V179D is an accessory mutation and it causes reduced susceptibility to ETR but only when in combination with other mutations (Vingerhoets *et al.*, 2010). When V179D is combined with K103R they have a synergistic reduction on the susceptibility of EFV and NVP (Parkin *et al.*, 2006a). Y188L is selected for by EFV and NVP (Bacheler *et al.*, 2000; Reuman *et al.*, 2010).

1.5.5.3.1 **Integrase Inhibitors**

These mutations are located in the active site of integrase near the amino acids groups that coordinate the magnesium ions which are essential for the integration of the viral DNA into the host's genome (Hazuda *et al.*, 2004). The relevant INSTI mutations are N155H, Y143R, G140S, Q148H/K/R, G118R and R263K (Hare *et al.*, 2011; Kobayashi

et al., 2011; Margot *et al.*, 2012). The Q148 substitution is observed in combination with G140S (Q148H), E138K (Q148K) and N155H (Q148R). These mutations result in a significant decrease in viral fitness (Marinello *et al.*, 2008) and cross-resistance has been observed within the class (Fransen *et al.*, 2009). This explains the primary set of mutations observed (N155H, Q148H/K/R, Y143R) followed by accompanying secondary mutations such as E138K (Fransen *et al.*, 2009). Mutations in the 3' polypurine tract (PPT) cause INSTI resistance, but they do not prevent integration. They cause a change during reverse transcription resulting in the accumulation of 1-LTR circles rather than the usual linear DNA, which leads to viral replication (Richetta *et al.*, 2022). There is significant cross-resistance between RAL and EVG particularly with N155H (Garrido *et al.*, 2012), Q148H/K/R, but DTG displays limited cross-resistance with only G118R reducing the susceptibility of all three ARVs (Wainberg and Han, 2015). DTG-selected mutations include R263K, G118R, N155H and Q148H/R (Rhee *et al.*, 2019).

The prevalence of INSTI resistance has been rising since its addition to global treatment guidelines in 2019 (Muccini *et al.*, 2023). In South Africa, there has been a steady rise in resistance to DTG (Hans *et al.*, 2023; Steegen *et al.*, 2019).

1.5.5.3.2 **Protease Inhibitors**

PIs bind to protease's catalytic site therefore the mutations conferring resistance are found in this region of the enzyme. The mechanism of PIs relies on mimicking a polypeptide resulting in them being structurally similar, thus conferring cross-resistance across the ARV class (Hertogs *et al.*, 2000). The accumulation of multiple mutations is required to alter the effectiveness of PIs (Condra *et al.*, 1996; Kempf *et al.*, 2001; Molla *et al.*, 1996). This is usually acquired in a stepwise process due to the deleterious impact these mutations have on fitness (Menéndez-Arias and Delgado, 2022). Common PI mutations include D30N, G48V, I50V, V82A and I84V (Condra *et al.*, 1996; Hertogs *et al.*, 1998; Markowitz *et al.*, 1998; Tisdale *et al.*, 1995). Mutations have also been reported to occur at the cleavage site of gag polypeptide, to overcome the viral fitness reduction (Doyon *et al.*, 1996).

1.5.5.4 Capsid Inhibitors

The novelty of this drug as well as its complex nature wherein it inhibits during multiple stages of the replication cycle result in minimal data on the mechanism of drug resistance to capsid inhibitors. The mutations L56I, M66I, Q76H, K70N, Q67H+N74S and Q67H+T107N displayed *in vitro* resistance to LEN (Link *et al.*, 2020), while in patients who displayed resistance to LEN the following mutations were detected: M66I, K70H and Q67H+K70HR (Segal-Maurer *et al.*, 2022).

1.5.6 Drug Resistance in HIV-1 Subtypes

ARVs have the same potency across all the HIV-1 subtypes resulting in equivalent clinical outcomes across subtypes A, B, C and D (Geretti *et al.*, 2009). However, natural polymorphisms may have a higher prevalence within specific subtypes, and thus result in different drug resistance profiles which can affect drug susceptibility. Supporting this hypothesis NNRTIs are not effective against HIV-1 subtype O as well as HIV-2 (Usach *et al.*, 2013). They both display high-level resistance to NVP and EFV (Descamps *et al.*, 1997; Hizi *et al.*, 1993; Shih *et al.*, 1991; Yang *et al.*, 1996). Additionally, the genetic variation results in different frequencies of mutations present in subtype populations, as demonstrated by the subtypes displaying distinct mutation pathways (Lai *et al.*, 2010). Resistance mutations in different HIV-1 subtypes therefore can result in different susceptibility profiles (Lessells *et al.*, 2012).

Compounding this with the fact that genotypic drug resistance test results are interpreted with web-based algorithms (such as the Stanford Drug Resistance Database algorithm), in which phenotypic data on non-B subtypes for newer drugs is limited, therefore examining the effect of mutations in subtype C (either through clinical investigation or *in vitro* phenotypic testing) could improve ARV drug resistance predictive outcomes for subtype C. HIV-1 group M showed greater variability in NNRTI susceptibility between HIV isolates than with NTRIs or PIs (Brown *et al.*, 2000). Further supporting their investigation is the observation that NNRTI mutations have different resistance pathways among HIV-1 subtypes (Gao *et al.*, 2004).

1.6 ART Maintenance Regimens

A three-drug combination regimen is the standard in ART due to the decrease in mortality that was observed when a combination of ARVs was prescribed, compared to single and dual drug therapies (James, 1995). The three-drug regimen is also favoured due to the quick development of drug resistance when fewer drugs are prescribed (James, 1995). However, there has been a recent drive towards two-drug maintenance regimens with high genetic barriers to resistance, to improve the patient's quality of life, due to the concerns of toxicity, pill burden, and drug-drug interactions of the three-drug regimens (Moreno *et al.* 2019).

The TANGO study showed that a combination of DTG and 3TC was non-inferior to three-drug and four-drug TAF-based regimens (van Wyk *et al.*, 2020). Several clinical trials have shown that M184V does not reduce its efficacy (Ndashimye and Arts, 2021). In the GEMINI trial, the same drug combination showed non-inferiority to DTG with FTC and TDF (Cahn *et al.* 2019). The DTG/3TC regimen has been approved as first-line therapy in the United States (FDA, 2019). The LATTE-1 and 2 studies have shown promising results with long-acting CAB and RPV, with LATTE-2 investigating administering CAB-LA and RPV as an injectable (Margolis *et al.*, 2017, 2015). The participants in both studies first achieved viral suppression with oral CAB with ABC+3TC and then they were sustained on CAB+RPV. The oral dose and injectable ARVs maintained viral suppression. These compelling results have promoted Phase III maintenance trials comparing regimens containing INSTI+ 2 NRTIs vs long-acting CAB+RPV in the ATLAS (NCT0295152) and FLAIR (NCT02938520) trials, as well as comparing a 4-week vs 8-week dosage timing in the ATLAS-2M (NCT03299049) trial.

A Phase IIb, randomized, double-blind clinical trial (MK-8591-011; NCT03272347) investigated a two-drug regimen composed of two novel ARVs, namely DOR and ISL. At 96 weeks into the trial, the two-drug regimen showed non-inferiority in viral suppression (81.1%) compared to the triple-drug regimen (80.6%; DOR + 3TC + TDF). DOR + ISL had a good safety profile and was well tolerated in patients, and

furthermore, patients reported fewer adverse events, with only two patients withdrawing because of adverse events (Molina *et al.* 2021).

The numerous studies conducted on these two-drug regimens support their implementation, since they have the ability to reduce side effects (Gibas *et al.*, 2022). This would improve the quality of people living with HIV, which is important as ART has increased their lifespan. Two drug regimens could also be more cost-effective depending on the combination of ARVs, as demonstrated by the DTG+3TC combination (Girouard *et al.*, 2016), which would be ideal in South Africa.

1.6.1 Islatravir

ISL is the first nucleoside reverse transcriptase translocation inhibitor (NRTtI). ISL has a unique chemical structure which ensures favourable characteristics such as high antiviral potency, long intracellular half-life, desirable pharmacological properties, and a robust resistance profile (Markowitz and Grobler 2020). It inhibits reverse transcription by chain termination similar to the other NRTIs. However, after being incorporated at the 3' end of the nucleic acid primer it additionally prevents translocation therefore preventing the binding of other nucleotides during transcription (Michailidis *et al.*, 2009). The chain termination is not a result of the lack of a 3' hydroxyl group like the other NRTIs, but rather a result of a strong interaction between ISL and the dNTP binding site delaying chain termination (Markowitz and Sarafianos, 2018).

This ARV is not yet approved for the treatment of HIV infection and it is currently still under clinical investigation. A Phase II clinical trial called GS-US-563-6041 (NCT05052996) examined the efficacy of switching patients to a ISL+LEN (oral dose) regimen after viral suppression compared to remaining on BIC+FTC+TAF (Gilead Sciences, 2024). The participants who switched maintained a high rate of viral suppression, non-inferior to BIC+FTC+TAF (Colson *et al.*, 2024). There are numerous trials ongoing examining the efficacy of a DOR+ISL regimen: IMAGINE-DR (NCT04564547), GS-US-563-6041 (NCT05052996), ILLUMINATE SWITCH A (NCT04223778), ILLUMINATE SWITCH B (NCT04223791), ILLUMINATE HTE (NCT4233216), ILLUMINATES NAÏVE (NCT04233879), MK-8591A-051

(NCT05631093), MK-8591A-052 (NCT05630755), MK-8591A-053 (NCT05705349) and MK-8591A-054 (NCT057663501). However, it is important to note that the IMAGINE-DR (NCT04564547) was briefly halted in November 2021 due to observations that ISL reduced CD4 T cell counts (MERCK, 2021a). The study was continued in December 2021 with a lower dose (MERCK, 2021b) that showed no detrimental impact on CD4 T cell counts (MERCK, 2023).

ISL has a high genetic barrier to resistance and the only mutations observed to arise with treatment have been M184IV and A114S, of which the latter is a unique mutation not observed with the NRTI class (Diamond *et al.*, 2022). When used in combination with DOR, it displayed a higher genetic barrier to resistance than DOR + 3TC and DTG + 3TC. DOR & ISL inhibited viral breakthrough at lower concentrations. Additionally, the number of mutations that were present during viral breakthrough with the combination was lower than the individual ARVs (Lai *et al.*, 2022). The mutations that were selected for when DOR and ISL were used in combination were V106A, V108I, M184I, P236L and Y318F.

1.6.2 Doravirine

DOR is a third-generation NNRTI, and it inhibits reverse transcription allosterically by binding to a hydrophobic pocket near the active site. The structure of this compound is displayed in Figure 1.3. The ARV has displayed high *in vitro* potency in HIV-1 strains with the three most prevalent NNRTI mutations, namely, K103N, G190A, and Y181C (Feng *et al.*, 2016; Fulco and Herity, 2021; Wong *et al.*, 2019). DOR additionally has a favourable pharmacokinetic profile supporting a daily dose with a single oral tablet (Anderson *et al.*, 2015). This has also resulted in DOR possessing a better safety profile and lower potential for drug-drug interactions than other NNRTIs (Boyle *et al.*, 2019). This means DOR results in fewer adverse events and side effects compared to the other NNRTIs. DOR is approved in Europe and the United States for the treatment of naïve or suppressed HIV patients (FDA, 2019) and it is currently part of a three-drug regimen (DOR+3TC+TDF) (EACS, 2019). DOR has been approved in South

Africa as a fixed dose combination with 3TC and TDF (Delstrigo) in 2021 and as a single tablet (Pifeltro) in 2022 (SAHPRA, 2024). Unfortunately, it is not currently available in the national treatment program (NDH, 2023).

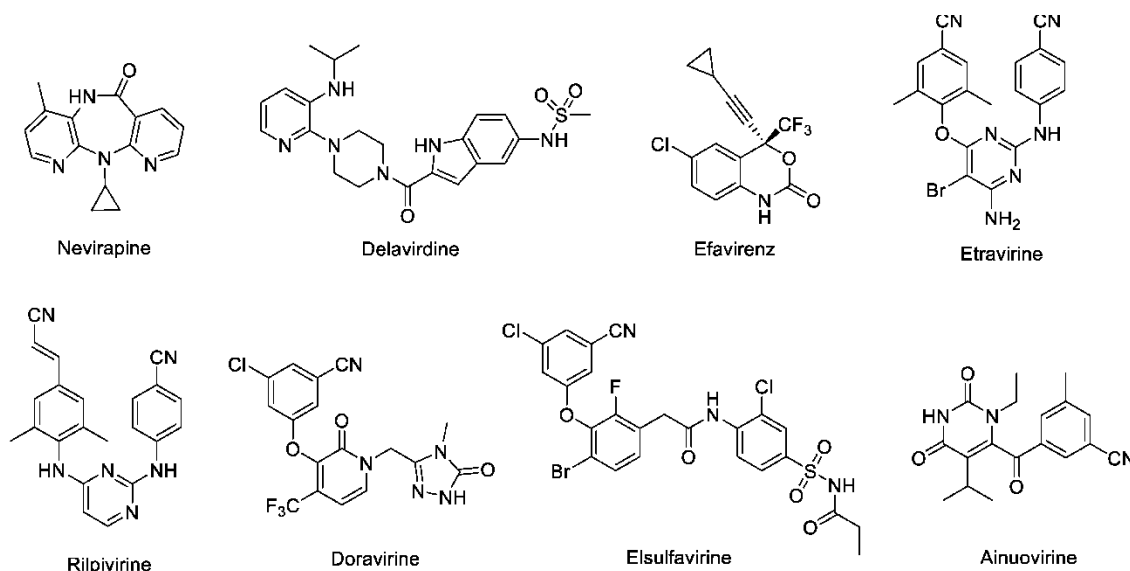


Figure 1.3: Structure of DOR compared to other NNRTI compounds

The chemical structure of currently FDA-approved NNRTIs Delavirdine (DLV), Doravirine (DOR), Efavirenz (EFV), Etravirine (ETR), Nevirapine (NVP), Rilpivirine (RPV), as well as Elvitegravir (ESV) which is approved for the treatment of HIV in Russia and Atravirine (ANV) which is approved for HIV treatment in China (Kang *et al.*, 2022).

DOR was approved as a result of two Phase III clinical trials conducted in ART-naïve patients, namely the DRIVE-FORWARD (NCT02275780) and AHEAD (NCT02403674) trials. The DRIVE-FORWARD trial compared a single daily dose of either DOR to DRV/r in combination with two NRTIs and they observed non-inferiority in the efficacy of these two regimens for up to 48 weeks, with DOR displaying a superior lipid profile (Molina *et al.*, 2020). The DRIVE-AHEAD study compared the regimen of DOR+3TC+TDF to a regimen of EFV+FTC+TDF and found non-inferiority in their efficacies for up to 96 weeks (Orkin *et al.*, 2021). The DOR-containing regimen displayed a favourable safety profile, with fewer neuropsychiatric side effects and fewer patients discontinuing treatment due to adverse events. The development of drug resistance was also observed to cease after 48 weeks with the DOR regimen but not the EFV regimen. A DRIVE-SWITCH (NCT02397096) trial investigated

DOR+3TC+TDF as an option for patients who are virally suppressed on a boosted PI/Elvitegravir or NNRTI-based regimen (Kumar *et al.*, 2021). They observed that the majority (90%) of the patients who were switched onto the DOR-based regimen remained virally suppressed for over 2 years. The DOR regimen similarly displayed a favourable safety profile, an improved lipid profile, minimal weight gains and the absence of DOR resistance.

The DOR-associated resistance mutations include V106AIM (Feng *et al.*, 2014; Lai *et al.*, 2014; Orkin *et al.*, 2019), Y188L (Feng *et al.*, 2014; Lai *et al.*, 2014; Orkin *et al.*, 2019) and F227L (Brenner *et al.*, 2021; Smith *et al.*, 2016). This could be a result of their close proximity/binding to DOR (Figure 1.4). Other NNRTI mutations have also been observed to reduce DOR susceptibility and they include V108I, G190ES, H221Y, M230IL, L234I and P236L (Brenner *et al.*, 2021; Feng *et al.*, 2014; Fulco and Herity, 2021). The positions of this amino acid are displayed in Figure 1.4, wherein it is clear DOR is effective in the presence of K103N since it directly binds to the position of the substitution. DOR has displayed a significantly improved susceptibility profile compared to the other NNRTIs (EFV/NVP/ETR/RPV), making its drug resistance profile unique (Asante-Appiah *et al.*, 2021). The majority of these studies have been conducted in HIV-1 subtype B and considering that polymorphisms amongst the subtype may effect on susceptibility, the effectiveness of DOR in the presence of these mutations in subtype C should be examined, to assess the efficacy of DOR in subtype C. This is particularly important in South Africa where HIV-1 subtype C strains have a high prevalence of NNRTI drug resistance.

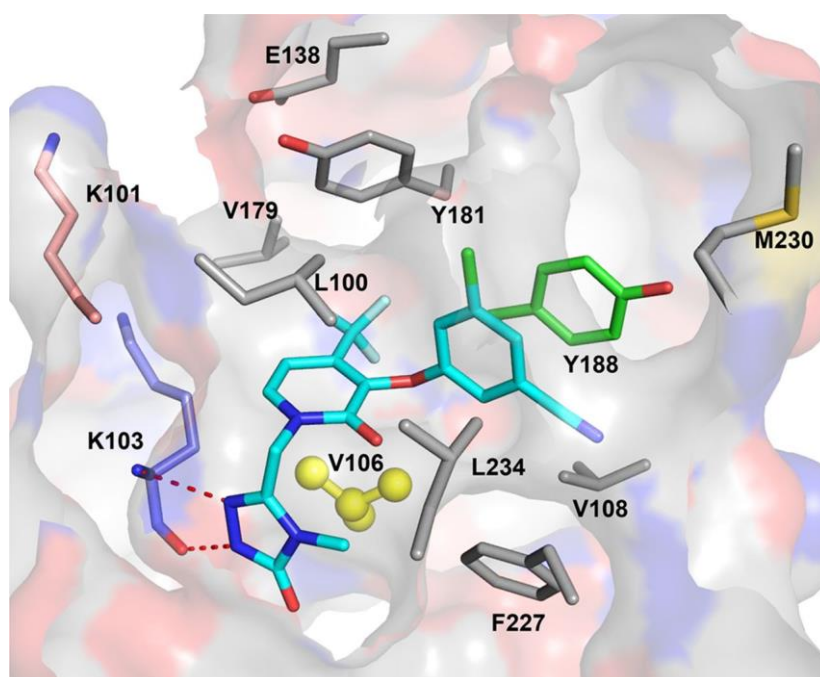


Figure 1.4: DOR bound to reverse transcriptase and the amino acids interactions.

This figure shows the crystal structure of reverse transcriptase with DOR bound and the positions of DOR-selected mutations (V108I, V106AM, Y188L, F227CLV, L234I). The red dotted line indicates hydrogen bonds (Feng *et al.*, 2014).

1.7 Problem Statement and Aim

1.7.1 Problem Statement

HIV remains a public health problem today with 39 million people living with HIV globally at the end of 2022 with 1.3 million new infections (UNAIDS, 2023a). The disease particularly affects Southern Africa where 7.6 million people are living with HIV in 2022 (<https://cfs.hivci.org/>). ARV therapy has significantly reduced the number of AIDS-related deaths and new infections (UNAIDS, 2023a), however, the rising prevalence of pre-treatment drug resistance warrants the development and implementation of novel ARV drugs (Gupta *et al.*, 2018). DOR is a novel NNRTI effective against the three most prevalent NNRTI mutations (K103N, G190A and Y181C), and supports the use of a single daily dose (Boyle *et al.*, 2019; Feng *et al.*, 2016; Wong *et al.*, 2019). The implementation of DOR could improve adherence and delay the development of drug resistance, however, the drug has not yet been approved in South Africa (Cohen *et al.*, 2013). Its implementation would be beneficial due to South Africa's high prevalence of drug resistance particularly to NNRTIs (Moyo *et al.*, 2020). However, the majority of the studies conducted with DOR on drug resistance were conducted in countries where HIV-1 subtype B is dominant therefore it is unclear whether its benefits will hold true in a subtype C context (Asante-Appiah *et al.*, 2021). Therefore, it is important to examine the effectiveness of DOR on common drug-resistant profiles associated with HIV-1 subtype C in South Africa, to understand the relevance of this drug in a South African context.

1.7.2 Aim

The aim of this study is to assess the *in vitro* phenotypic drug susceptibility of Doravirine (DOR) in the context of HIV-1 subtype C drug-resistant variants.

1.7.3 Objectives

1. To generate wild-type HIV-1 subtype B and subtype C pseudoviruses (PSVs) from lab adapted strains.
2. To compare the potency of DOR in the wild-type HIV-1 subtype B and C PSVs.
3. To assess potential synergy between DOR and Islatravir (ISL) against wild-type HIV-1 subtype C reference isolate.
4. To perform site-directed mutagenesis (SDM) to generate mutant HIV-1 subtype C PSVs.
5. To perform *in vitro* phenotypic DOR susceptibility testing on mutant HIV-1 subtype C PSVs.

2. Materials and Methods

2.1 Materials

2.1.1. Antiretroviral drugs

Doravirine (MK-1439), which was used in the *in vitro* phenotypic testing of the NNRTI mutants, was purchased from MedChemExpress (New Jersey, USA, <https://www.medchemexpress.com/Doravirine.html>, CAS No. 1338225-97-0).

Islatravir (MK-8591, EFdA), which was used in the *in vitro* phenotypic testing of the synergy between the ISL and DOR combination, was also obtained from MedChemExpress (New Jersey, USA, <https://tinyurl.com/3a8be8x4>, CAS No. 865363-93-5).

Efavirenz (EFV) is a NNRTI, which is approved for the treatment of HIV infected patients in South Africa (South African Department of Health, 2023). Prior to 2019, EFV with 3TC (or Emtricitabine) and Tenofovir was the recommendation for first-line therapy (South African Department of Health, 2015). Currently, an EFV based regimen is recommended when Dolutegravir is poorly tolerated (Southern African HIV Clinicians Society, 2023). EFV was obtained from HIV Reagent Program (Virginia, USA, <https://tinyurl.com/37jbh8ff>, CAS No. 154598-52-4). This drug was used as the control NNRTI drug in the *in vitro* phenotypic drug susceptibility assays.

3TC is an NRTI currently recommended as part of first-line therapy, which comprises of Tenofovir, Dolutegravir and 3TC or Emtricitabine (South African Department of Health, 2023). 3TC was similarly obtained from HIV Reagent Program (Virginia, USA, <https://tinyurl.com/2s49bb68>, CAS No. 134678-17-4). It was used as a control NRTI drug in the synergy assay with EFV.

All the drugs (DOR, ISL, EFV, 3TC) that were used in this study were obtained as a powder. The stock solution for each drug was made by dissolving 5 mg of the powder in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; CAS No. 67-68-5) to a final

concentration of 10mM. A working stock solution of 20 μ M was made for each drug by diluting in complete DMEM described in 2.2.1.2.1 (see page 47). For toxicity and phenotypic testing, the drugs were similarly diluted in complete DMEM to the appropriate concentration as determined by the activity assay. Drug stock solutions for use in the phenotypic drug resistance assay was stored in 1.5 mL aliquots, at -80°C. The stocks were thawed at room temperature and the phenotypic testing stock was never thawed more than twice.

2.1.2. Plasmids

This study employed three plasmids to produce replication-defective virus-like particles (PSV) for use in the *in vitro* phenotypic drug susceptibility and synergy assays. The HIV-1 packaging plasmids (p8.9NSX+, p8.9MJ4) were obtained from Dr. Deenan Pillay (University College London, London, United Kingdom), and the pCSFLW firefly luciferase transfer plasmid was obtained from Dr. Nigel Temperton (University College London, London, United Kingdom). Plasmid pHEF.VsVg (ARP-4693) expressing vesicular stomatitis virus G protein gene (VSV-G) for use to produce VSV-G enveloped PSVs was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH (contributed by Dr. Lung-Ji Chang). Please refer to the Appendix A (Figures A.1-3) for the plasmid maps.

These three plasmids were utilised because they each provide an essential component in the generation of PSVs upon the co-transfection of HEK293T cells as depicted in Figure 1. p8.9NSX+ and p8.9MJ4 encode the PSVs' internal structural components via HIV-1 *gag* (matrix, capsid, nucleocapsid) and enzymes via HIV-1 *pol* (*protease, reverse transcriptase, integrase*). pCSFLW encodes the RNA transcript for firefly luciferase and pHEF.VsVg encodes for VSV-G. This results in a PSV (depicted in Figure 2.1) with the structure and enzymes of HIV-1 containing the firefly luciferase RNA transcript and enveloped by VSV-G. The PSVs are non-replicating, therefore they only infect the cell in a single event.

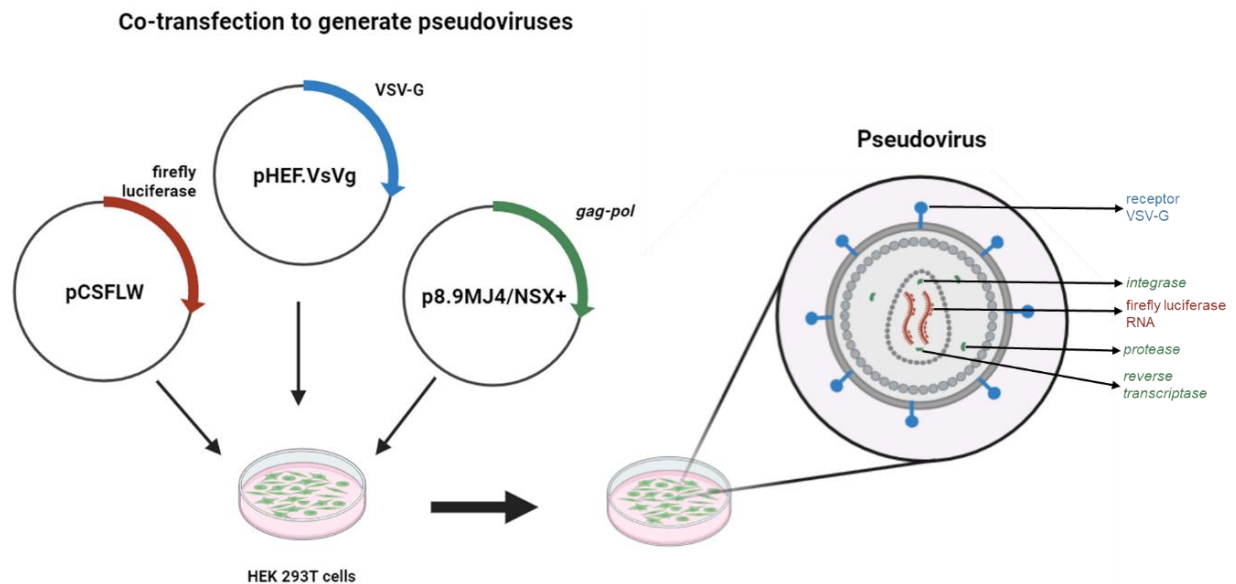


Figure 2.1: The role of each plasmid in HIV-1 PSV production.

HEK293T cells were transfected with pCSFLW, pHEF.VsVg and p8.9MJ4/NSX+ to produce PSVs. These three plasmids provide the essential components to produce an HIV-1 PSV of the desired subtype and containing the mutations that were investigated. pCSFLW contains the **RNA template for firefly luciferase** which will be integrated and expressed in the target cells, allowing for viral quantification. pHEF.VsVg encodes for the **vesicular stomatitis virus G protein**, which allows entry into the HEK293T cells. p8.9MJ4 encodes for subtype C and p8.9NSX+ encodes for subtype B *gag* (Matrix, Capsid, Nucleo-capsid) and *pol* (*Protease*, *Reverse Transcriptase*, *Integrase*).

The VSV-G membrane facilitates entry into the target cells by receptor mediated endocytosis specifically using the clathrin-mediated endocytic pathway (Sun *et al.*, 2010). The internalised PSV releases its firefly luciferase transcript into the target cell's cytosol. The replication cycle is initiated wherein firefly luciferase transcript is reverse transcribed and integrated into the target cell's genome. Antiretroviral drugs inhibit the reverse transcription step (by NNRTIs and NRTIs) and integration step (by INTSIs). The expression of firefly luciferase is initiated by the Spleen Focus Forming Virus promoter. Firefly luciferase then converts D-luciferin (substrate which is added) to oxyluciferin and light is emitted as a by-product of this reaction (de Wet *et al.*, 1987). The amount of light produced is directly proportional to the amount of firefly luciferase present in the target cells, therefore the level of pseudoviral infection can be deduced by this direct linear relationship. The addition of ARVs would inhibit the replication cycle and thus no firefly luciferase will be produced. Therefore, the effect of mutations

in HIV-1 *reverse transcriptase* (and *integrase*) on ARV drug susceptibility can be assessed using the *in vitro* phenotypic assay.

Plasmids p8.9NSX+ (Parry *et al.*, 2009) and p8.9MJ4 (Gupta *et al.*, 2010) are derivatives of the non-replicating packaging construct pCMV Δ R9 (Naldini *et al.*, 1996), which encodes for HIV-1 *gag-pol*, *tat* and *rev*. The plasmid is defective in HIV-1 *env* and the Vpu accessory protein. Plasmid p8.9NSX+ contains HIV-1 subtype B *gag-pol*, while the *protease* and most of *reverse transcriptase* were replaced with that of the MJ4 HIV-1 subtype C isolate (GenBank accession number AF321523.1) (Ndung'u *et al.*, 2001) in p8.9MJ4. Modifications were made to both these plasmids by Dr Basson by introducing a PvuI endonuclease restriction site at nucleotide positions 10 – 15 (amino acid positions 4 - 6) in *reverse transcriptase* (HXB2 nucleotides 2560 – 2565) and a HpaI endonuclease restriction site at nucleotide positions 1,100 – 1,106 (amino acid positions 366 – 369) (HXB2 nucleotides 3682 – 3588). The PvuI endonuclease restriction site at the beginning of *protease* in p8.9NSX+ was silenced. Plasmids p8.9NSX+ and p8.9MJ4 were used for the generation of site-directed mutants, while the modified version of the two plasmids were used for the cloning of lab-adapted viral strains.

2.1.3. Lab-adapted HIV-1 strains:

Four wild-type HIV-1 subtype B lab-adapted strains (i.e., DS9, LTNP5, SM1 and SM2) and four wild-type HIV-1 subtype C lab-adapted strains (i.e., CM9, DU151, DU178 and DU422) were used in this study (Table 2.1). These were obtained from the National Institute for Communicable Diseases (Nation Health Laboratory Services, Johannesburg, South Africa) as culture supernatants stored at -80°C.

Table 2.1: HIV-1 subtype B and C lab-adapted strains

HIV-1 Subtype	Strain	GeneBank accession number	Publication
B	DS9	MH234640	Obasa <i>et al.</i> , 2019
	LTNP5	AY835779	Mikhail <i>et al.</i> , 2005
	SM1	KY060873	Ledwaba <i>et al.</i> , 2019
	SM2	KF469956	Maldarelli <i>et al.</i> , 2013
C	CM9	AF411967	Papathanasopoulos <i>et al.</i> , 2002
	DU151	AF544009	Williamson <i>et al.</i> , 2003
	DU179	AY043174	van Harmelen <i>et al.</i> , 2001
	DU422	AF544005	Williamson <i>et al.</i> , 2003

2.1.4. General reagents:

2.1.1.1. PEI MAX

Polyethylenimine “Max” (PEI MAX) (Polysciences; CAS No. 49553-93-7) was used as the transfection reagent when generating PSV. PEI is a cationic polymer and thus binds strongly to negatively charged DNA. This imparts a positive charge on the condensed DNA particle. These particles bind to the anionic cell wall and are taken up by endocytosis. The PEI max was prepared to a final concentration of 1 mg/mL. The solution was compiled by dissolving 0.01 g of PEI max powder in 10 mL of deionised water. The pH of the solution was adjusted to 7. The solution was filter sterilised with a 0.2 µM syringe filter and stored at 5°C in 1 mL aliquots.

2.1.1.2. Alamar Blue solution

“Alamar Blue” or Resazurin sodium salt (Sigma-Aldrich; CAS No. 62758-13-8) was used for cell viability testing during the toxicity screens. The solution was made by dissolving 15 mg of Resazurin sodium salt in 100 mL of 1X Dulbecco’s Phosphate

Buffered Saline (DPBS) (PAN Bio; Cat No. P04-36500). The solution was filter sterilised with a 0.2 µM syringe filter and stored as 10 mL aliquots at -20°C. Resazurin in its oxidised form is a blue non-fluorescent compound. The compound is cell permeable so enters the cell's cytosol and upon contact with dehydrogenase (a mitochondrial enzyme) is reduced by accepting electrons from NADPH, FADH, FMNH, NADH, and cytochromes, thus changing the compound into Resorubin. This causes the colour to change from blue (from Resazurin) to a fluorescent pink (to Resorubin) (Page *et al.*, 1993). Alamar blue is frequently used to assess therapeutic drugs in cytotoxicity assays (Anywar *et al.*, 2023).

2.1.1.3. Bright-Glo™ Luciferase Assay System

Bright-Glo™ Luciferase (Promega; Cat No. E2650) was used in the phenotypic assays to assess viral activity. The solution was prepared by combining 500 µL of Bright-Glo™ Luciferase Assay Buffer (Promega; Cat No. E264B) with Bright-Glo™ Luciferase Assay Substrate (Promega; Cat No. E263B). The solution was stored at -80°C in 50 µL aliquots. The enzyme firefly luciferase catalyses the conversion of D-luciferin to oxyluciferin in a multistep process resulting in the release of a photon of light which was detected by the luminometer. Firefly luciferase requires Mg^{2+} , adenosine triphosphate (ATP) and O_2 for these reactions (de Wet *et al.*, 1987). The Bright-Glo™ Luciferase assay system is commonly used in pseudoviral quantification and viral inhibition assays (Fernandez *et al.*, 2022).

2.2 Methods

2.2.1 Fundamental Methodologies

2.2.1.1 Bacterial Cell Culture

2.2.1.1.1 Preparation of antibiotics

The plasmids used in this study contained the β -lactamase gene which confers resistance to ampicillin. Ampicillin and carbenicillin contain a beta-lactam ring (Demain and Elander, 1999). Transcription of the β -lactamase gene results in production of the enzyme β -lactamase which hydrolyses this ring resulting in its destruction and compromising the antibiotic's structure. Carbenicillin is more stable than ampicillin in Luria-Bertani (LB) growth media, therefore it was used in this study. Carbenicillin was made to a stock concentration of 100 mg/mL by dissolving 1 mg of carbenicillin disodium salt (Merck & Co., New Jersey, US, CAS No. 4800-94-6) in 10 mL of deionised water. The solution was sterilised with a 0.2 μ M filter and stored at -20°C in 1 mL aliquots.

2.2.1.1.2 Preparation of LB agar and LB broth

The agar plates were prepared by adding 2.5 g of Tryptone, 1.25 g of NaCl, 1.25 g of Yeast extract and 3.75 g of agar to 250 mL of deionised water. The agar was autoclaved at 121°C for 20 minutes and after cooling, 250 μ L of 100 mg/mL Carbenicillin (final concentration of 100 μ g/mL) was added. The agar was poured into sterile 10 cm petri dishes and left to cool. The plates were sealed with Parafilm and stored inverted in a 5°C fridge.

The LB broth was prepared by adding 2.5 g of Tryptone, 1.25 g of NaCl and 1.25 g of Yeast extract to 250 mL deionised water. The LB broth was autoclaved at 121°C for 20 minutes, allowed to cool and 250 μ L of 100 mg/mL carbenicillin (final concentration of 100 μ g/mL) was added. The LB broth was stored at 5°C.

2.2.1.1.3 Preparation of NZY+ broth

The NZY+ broth was prepared by adding the following to 1 L of deionised water: 10 g of NZ amine (casein hydrolysate), 5 g of Yeast Extract, 5 g of NaCl and 2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of the NYZ broth was adjusted to 7.5 using NaOH. The NYZ+ broth was autoclaved at 121°C for 20 minutes and stored at -20°C in 2 mL aliquots.

2.2.1.1.4 Preparation of transformation buffer

The transformation buffer was prepared by combining 100 mM CaCl_2 , 10 mM PIPES•HCl and 15% Glycerol with 100 mL of deionised water. The pH of the buffer was adjusted to 7 using NaOH and it was autoclaved at 121°C for 20 minutes. The transformation buffer was stored at 5°C.

2.2.1.1.5 Preparation of chemically competent cells

Dh5 α bacterial cells were used for general bacterial transformation by heat-shock. To prepare the chemically competent Dh5 α bacterial cells, an aliquot of 50 μL was removed from storage at -80°C, thawed on ice and subsequently added to 4 mL of LB broth (without antibiotic). The cells were incubated at 37°C while shaking at 200 rpm overnight. The following day, 1 mL of the overnight culture was added to 40 mL of LB broth (without antibiotic). The cell culture was grown until it reached an O.D.₆₀₀ of 0.4, after which the cells were centrifuged at 1500 rpm for 10 minutes and the supernatant was discarded. The cells were resuspended in 1 mL of cold transformation buffer, after which they were incubated on ice for 20 minutes. Aliquots of 50 and 100 μL were prepared and stored at -80°C.

2.2.1.1.6 Bacterial transformation through heat shock

Chemically competent Dh5 α bacterial cells were removed from storage at -80°C and thawed on ice, after which 2 μL of the reaction (e.g., ligation reaction) was added to 25 μL of Dh5 α bacterial cells. The cells were incubated on ice for 30 minutes after which they were heat shocked in a water bath at 42°C for 35 seconds. The cells were immediately placed on ice for 5 minutes and 500 μL of NZY+ broth was added. The bacterial cells were incubated at 37°C for 1 hour while shaking at 200 rpm. The culture was centrifuged at 4000 rpm for 4 minutes to pellet the bacterial cells and 450 μL of the supernatant was removed. The bacterial pellet was then re-suspended by aspirating in the remaining NZY+ broth. The cell suspension was then spread on an carbenicillin containing agar plate and incubated overnight at 37°C.

2.2.1.2 Mammalian Cell Culture

2.2.1.2.1 Preparation of complete culture medium

Dulbecco's Modified Eagles Medium (DMEM) (PAN Biotech; Cat No. P04-03590, with 4.5 g/L Glucose, 584 mg/L L-Glutamine, 110 mg/L Sodium Pyruvate and 3.7 g/L NaHCO₃ was supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich; Cat No. F0679), 22.12 mM HEPES (ThermoFisher Scientific; Cat No. 15630130) and 13.27 µg of Gentamycin (Sigma-Aldrich; Cat No. G1264-1G). This complete culture media was used to maintain HEK293T cell cultures and for *in vitro* experiments.

2.2.1.2.2 Maintenance of continuous mammalian culture cell line

The HEK293T cell line (Lin *et al.*, 2014) is an immortalised adherent clonal cell line derived from a human embryonic kidney cell, which expresses the T-antigen of SV40 (DuBridge *et al.*, 1987). It is commonly used for transfection in PSV production, since it results in high viral titers (Voss *et al.*, 2019).

Cells that were stored in 10% DMSO under liquid nitrogen were thawed in a water bath at 30°C. To reduce the DMSO concentration because it is toxic to the cells, 20 mL of complete culture media was added and the cells were centrifuged at 1600 rpm at 25°C for 10 minutes. The supernatant was discarded and the cells were resuspended in 1 mL of FBS with 9 mL of complete culture media. The cells were added to a 75 cm² NEST Cell Culture Flask with a sealed cap (NEST; Cat No. 708001). The cells were maintained at 37°C with 5% CO₂ in a humidified incubator. The confluency and morphology of cells were inspected under a microscope and they were passaged when they were 70-80% confluent, which occurred every 2-3 days. This entailed, removal of the 10 mL of spent culture media, rinsing the cells with 5 mL of 1X DPBS. The DPBS contained no calcium or magnesium ions as this would inhibit trypsin activity. The cells were trypsinized with 1 mL of TrypLE™ Express Enzyme X1 (ThermoFisher Scientific; Cat No. 12604021). After the cells detached, they were resuspended in 4 mL of complete culture media. The culture flask was reseeded with 1 mL of cells in 10 mL of complete culture media. The morphology of the cells was

monitored and if any changes in morphology or adherence was observed a new batch of cells was thawed.

To store the cells, they were passaged as described above. The cells were resuspended in freeze media (i.e., FBS with 10% DMSO) at $1 - 5 \times 10^6$ cells/ml. The cells were then stored in cryogenic storage vials as 1 mL aliquots in liquid nitrogen.

2.2.1.2.3 Mammalian cell counting

Trypan blue is not membrane-permeable, therefore live cells that have an intact membrane are not permeated by the stain while dead cells that do not have an intact membrane are labelled with the blue stain. To count the number of living cells, 10 μ l of the suspended cells were added to 90 μ l of Trypan Blue Stain 0.4% (ThermoFisher Scientific; Cat No.15250061). Then, 10 μ l of the cells with the stain were loaded onto the hemocytometer. The cells were automatically counted using the Countess™ II Automated Cell Counter (ThermoFisher Scientific; Cat No. AMQAX1000).

2.2.1.3 Molecular Biology

2.2.1.3.1 Agarose gel electrophoresis

The DNA fragments under investigation ranged in size from 100 – 12,000 bp, therefore a 1.2% TAE agarose gel was sufficient for proper resolution of the DNA fragment. A mixture of 100 mL of 1X TAE buffer (0.4 M Tris-acetate (pH 8.3); 0.01 M ethylenediaminetetraacetic acid (EDTA)) with 1 g agarose was brought to boil until the agarose was completely dissolved. After this solution had cooled, 4 μ l of ethidium bromide solution (10 mg/mL) (Bio-rad; Cat. No. 1610433) was added and the gel was left to set in a casting tray containing a comb. The appropriate amount of a 6X Purple Gel Loading Dye (New England Biolabs; Cat No. B7024S) was added to the DNA samples. The GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific; Cat No. SM0314) was loaded alongside the sample for size determination. Electrophoresis was performed at 100 V (400 mAmp) for 60 minutes. The agarose gel was visualised

under UV light on the Gel Doc XR Imaging System (BioRad; Cat No. BGDXR). The DNA fragment sizes were estimated with reference to the molecular weight marker.

2.2.1.3.2 Plasmid DNA extraction

Following bacterial transformation in section 2.2.1.1.6 (page 46), several bacterial colonies were picked from the agar plate and each were inoculated into 4 mL of LB broth with carbenicillin. The cell culture was incubated at 37°C while shaking at 200 rpm overnight. The bacteria culture was centrifuged at 4500 rpm for 10 minutes at 4°C to pellet the bacteria. The supernatant was discarded

Plasmid extraction was performed using the QIAprep Spin Miniprep Kit (Qiagen; Cat No. 27104) according to the manufacturer's instructions. Briefly, 250 µl of resuspension buffer P1 was added to the bacterial pellet. The cells were vortexed to aid in their resuspension and they were subsequently transferred to a 2 mL tube. To lyse the cells, 250 µl of buffer P2 was added. The reaction was neutralised by adding 350 µl of buffer N3 and the reaction was centrifuged at 13 000 rpm for 10 minutes. This resulted in a white pellet of protein and cell debris at the bottom of the tube while the nucleic acids remained in the clear supernatant. The supernatant was removed carefully and added to a spin column attached to a collection tube. The spin column was centrifuged at 13 000 rpm for one minute. The nucleic acids remained bound to the silica membrane.

The filtrate was discarded and 500 µl of wash buffer PB was added to remove endonucleases. The column was centrifuged at 13 000 rpm for 1 minute, the filtrate was discarded and 750 µl of an ethanol containing wash buffer PE was added to remove organic salts. The column was centrifuged at 13 000 rpm for 1 minute and the filtrate was discarded. The empty column was centrifuged at 13 000 rpm for 1 minute again to remove any remaining ethanol containing wash buffer, which would prevent the elution of the nucleic acids in solution. The collection tube was removed and the column was placed in an empty 2 mL recovery tube. The DNA was eluted by incubated the silica membrane with 55 µl of elution buffer (10 mM Tris•Cl pH 8.5) for 2 minutes. The column with the tube was then centrifuged at 13 000 xg for 1 minute and the spin column was discarded.

2.2.1.3.3 DNA quantification

The DNA was quantified using a NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific; Cat No. ND2000CLAPTOP). Generally, 1.5 µl of the sample was used for quantification. The concentration and the absorbance ratios of A_{280}/A_{260} and A_{260}/A_{230} were examined to assess the organic compound and protein contamination, respectively, thus informing on the purity of the extracted DNA. A ratio of $A_{280}/A_{260} \geq 1.8$ and $A_{260}/A_{230} = 2.0$ -2.2 would indicate a pure DNA sample.

2.2.1.3.4 Sanger sequencing

Sanger Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Cat No. 4337455). This method of DNA sequencing relies on the binding of a single primer and amplification by DNA polymerase and chain terminating fluorescently labelled dideoxynucleotides (ddNTPs). Multiple primers are usually used for ample coverage of the desired region wherein a minimum of two sequence reads are required for accurate sequencing. The following was combined to a final volume of 10 µl in the wells of a MicroAmp™ Optical 96-Well Reaction Plate with Barcode (Applied Biosystems; Cat No. 4306737): 2 µl of 5x Sequencing Buffer, 1 µl of BigDye™ Terminator v3.1 Ready Reaction Mix, 1.5 µl of 3.5 µM sequencing primer, 300 ng of template DNA and PCR grade water. Four sequencing primers (Table 2.2) were used to sequence the portion of *reverse transcriptase* that is relevant to the NNRTI DRMs investigated in this study.

Table 2.2: Sanger sequencing primers covering reverse transcriptase

Primer Name	Sequence (5' → 3')	Primer direction	Position relative to HXB2
PFOR-IN	CACCTGTCAACA	forward	2491 → 2502
PREV-IN	AATTTCCCTGTT	reverse	3599 → 3610
PolSexAF	TTAAAACCAGGTATGGAT	forward	2583 → 2600
Pol101R	GTA CTGATATCTAATCCCTGG	reverse	2967 → 2987

The plate was centrifuged at 3000 rpm for 2 minutes to collect droplets on the sides of the wells and then thermal cycling was carried out with the PCR System 9700 GeneAmp Thermocycler (Applied Biosystems; Cat No. 4303481H) under the following conditions: 96°C for 1 minutes, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. This resulted in the amplification of the DNA in different fragment sizes with a fluorescent ddNTP on the end. The colour of the fluorescent molecule is dependent on the nucleotide at the end of the DNA fragment. The reactions were stored at 4°C until the clean-up was performed.

To clean up the sequencing reactions, 50 µl of an ethanol and sodium acetate solution was added to the wells. This solution was prepared by mixing sodium acetate (3M, pH 5.25) and 100% ethanol in the ratio of 1:25, respectively. The plate was centrifuged at 2000 xg for 30 minutes at 4°C to precipitate the nucleic acids into a pellet. The plate was inverted and centrifuged at 100 xg for 20 seconds to remove the salt and ethanol solution. Afterwards, 150 µl of cold 70% ethanol was added to each well of the plate and centrifuged at 2000 xg for 5 minutes to wash away residual salts. The plate was inverted once again and centrifuged at 50 xg for 30 seconds to remove the ethanol. The plate was then incubated at 95°C for 3 minutes to evaporate any remaining ethanol. The DNA pellets were then resuspended in 10 µl of Hi-Di™ Formamide (Applied Biosystems; Cat No. 4311320) in preparation for sequencing. The pellets were incubated at 65°C for 5 minutes and vortexed well to dissolve the DNA. The plate was centrifuged at 1000 xg for 2 minutes to collect droplets on the sides of the wells and the plate was loaded in the 3730xl DNA Analyzer (Applied Biosystems; Cat No. A41046).

The DNA Analyzer has 96 capillaries which contain a polymer (POP-7 (ThermoFisher Scientific; Cat No 4363929)). The DNA fragments from each reaction were separated by size (short to long) through capillary electrophoresis. Simultaneously, a laser excited the fluorescently labelled ddNTP as the DNA fragments pass through the polymer. A detector then records a peak in fluorescence intensity known as a chromatogram. The colour of the fluorescent dye and the order of the peaks allow the DNA sequence to be established.

The sequence trace files (.abi) were processed with Geneious version 2022.2.2 (Biomatters, Auckland, New Zealand, <https://www.geneious.com>). This entailed trimming the ends of the trace files that displayed poor sequence quality and aligning the four sequence trace files to an appropriate reference sequence (i.e., p8.9MJ4 or p8.9NSX+ *reverse transcriptase*). The sequences were reverse complimented when necessary. The consensus sequence was uploaded to the Stanford HIV Drug Resistance Database (Stanford University, Palo Alto, California USA, <https://hivdb.stanford.edu/>, (Liu and Shafer, 2006)), to check the quality of the sequences and confirm the presence or absence of mutations in the sequence.

2.2.2 Objective specific methodologies

2.2.2.1 Objective 1: Assessment of *in vitro* DOR susceptibility of wild-type HIV-1

Purpose:

The purpose of this objective was to

- a. assess potential differences in DOR potencies against wild-type subtype B and subtype C HIV-1 variants
- b. assess potential synergy between DOR and ISL combinations against the wild-type HIV-1 subtype C reference isolate

Overview:

The RNA from the lab-adapted strains was extracted and reverse transcribed to cDNA. PCR was used to amplify a portion of *reverse transcriptase* and simultaneously introduce restriction sites on the 5'-ends (*PvuI*) and 3'-ends (*HpaI*) of the amplicons for cloning. The amplicons were cloned into the HIV-1 expression vectors through subcloning. The clones were sequenced to confirm the identity of the inserts. PSVs were subsequently generated through co-transfection of HEK293T cells, and screened *in vitro* for susceptibility to DOR over non-toxic concentrations. To investigate potential synergy between DOR and a novel, investigational NRTI (i.e.,

ISL), various ratios of the two drugs were combined and assessed in our *in vitro* phenotypic assay against the subtype C expression vector (i.e., p8.9MJ4).

2.2.2.1.1 Objective 1A: Comparing *in vitro* DOR susceptibility of subtype B and C strains

2.2.2.1.1.1 Preparation of strain specific PSVs

2.2.2.1.1.1.1 Nested PCR

RNA extraction

The viral RNA of the viruses was extracted using the QIAamp Viral RNA Mini Kit (Qiagen; Cat No. 52904) according to the manufacturer's instructions. Briefly, the viral culture supernatants were removed from storage at -80°C and thawed on ice. An aliquot of 140 µl culture supernatant was transferred to a 1.5 mL tube containing 560 µl of lysis buffer AVL and 5.6 µl of carrier RNA. This buffer lysed the viral envelope and capsid to release the viral RNA. The purpose of the carrier RNA was to bind to small RNA fragments (< 200 bp) or low amounts of RNA and allow for improved binding of the RNA to the silica membrane of the column. The presence of chaotropic salts in the buffer ensured the binding of the RNA to the silica membrane, whilst guanidine isothiocyanate inhibited RNAases. The lysis reaction was incubated at ~24°C for 10 minutes and centrifuged briefly to collect droplets on the side of the tubes. Then, 560 µl of 100% ethanol was added to the reaction to precipitate the RNA. The solution was mixed well by pulse-vortexing for 15 seconds. A QIAamp Mini column was prepared and 630 µl of the solution was added to the membrane. The column was centrifuged at 6000 rpm for 1 minute, after which the filtrate was discarded and the 2 mL collection tube was replaced with a new tube. The remaining solution was added to the column and centrifugation was repeated. After replacing the collection tube, 500 µl of wash buffer AW1 was added to remove endonucleases. Similarly, the column was centrifuged at 6000 rpm for 1 minute, the filtrate was discarded and the collection tube was replaced. Then, 500 µl of wash buffer AW2 was added to remove organic salts and the column was centrifuged at 13 000 rpm for 3 minutes. The filtrate was

discarded and a new collection tube added. The empty column was centrifuged at 13 000 rpm for 1 minute to remove any remaining ethanol which would prevent the elution of the nucleic acids in solution. The column was removed from the collection tube and placed in a labelled 1.5 mL tube.

The viral RNA was eluted from the silica membrane with the addition of 60 µl of buffer AVE to the column and centrifugation at 13 000 rpm for 1 minute. Buffer AVE created a low salt-condition that caused the hydrophilic RNA to return to solution. The viral RNA was stored at -80°C.

cDNA synthesis

The resultant viral RNA was reverse transcribed into cDNA with the SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific; Cat No. 18080051). Reverse transcription is catalysed by reverse transcriptase, which is an RNA-dependent DNA polymerase. The following was combined on ice to a final volume of 10 µl; 1 µl of 2 µM primer 3016R (Table 2.3), 1 µl of 10 µM dNTP mix, and nuclease-free water. The reaction components were mixed and incubated at 65°C for 5 minutes to denature the nucleic acids. The reaction was stopped by placing the tubes immediately on ice for 1 minute. The following was combined in a separate tube: 2 µl of 10X *reverse transcriptase* buffer, 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl 4 U RNaseOUT and 1 µl 200 U SuperScrip III RT. To reduce the di-sulfide bonds, 0.1 M DTT was added resulting in the reduction of RNA in its secondary structure and allowing *reverse transcriptase* to bind. This mixture was added to the 10 µl reaction containing the RNA. The combined reaction was incubated at 50°C for 50 minutes to facilitate the reverse transcription of the RNA. The *reverse transcriptase* was then inactivated at 85°C for 5 minutes. The reaction was chilled on ice for 1 minute and then 1 µl of RNase H was added to remove the template RNA, so the DNA is pure and the RNA cannot effect downstream applications. The reaction was carried out at 37°C for 20 minutes. The cDNA was stored at -20°C.

PCR

The *reverse transcriptase* gene fragments, which were 1096 bp in size, were amplified with nested PCR. Nested PCR entails the use of two amplification reactions, each with a unique set of primers. The PCR product from the first round of amplification serves

as the template for the second round of amplification. This process relies on the primers from the second round binding internally to the PCR product from the first round. This results in the amplification of a specific PCR product and also allows for the introduction of the two restriction sites required for cloning. The procedure is depicted in Figure 2.2 (see page 56), wherein the first round of amplification was guided by the outer primers 2528F and 3660R. The second round of amplification was carried out with the inner primers 2560F and 3628R, which introduced the *PvuI* and *HpaI* restriction sites on the 5'- and 3'-ends, respectively. Please refer to Table 2.3 below for the primer sequences.

Table 2.3: Primers used in nested PCR to amplify a portion of reverse transcriptase

PCR round	Primer Direction	Primer Name	*Sequence (5' → 3')	Position relative to HBX2	T _m (°C)
1 st	forward	2528F	GCTTGGATGCACACTAAATT TTCC	2528 → 2551	55.2
	reverse	3660R	CCATGGCTATTTTTTGCAC GC	3660 → 3681	55.5
2 nd	forward	2560F	CGATCG AAACTGTACCAGTA AAATTAAAGC	2560 → 2589	49.8
	reverse	3628R	GTTAACTG TTTTACATCATTA GTGTGGG	3628 → 3655	50.7

* the restriction sites are indicated in bold

The first round of amplification was performed using the Roche Expand™ High Fidelity PCR System (Roche; Cat No. 11732641001) and the first primer sets to amplify the *reverse transcriptase* fragment. The second round of PCR was performed using PCR reagents from the TOPO™ XL-2 PCR Cloning kit (ThermoFisher Scientific; CAT No. 45-20810) and the second set of primers to amplify the first round PRC product. This was done to prepare the PCR products for cloning into a TOPO vector for the TOPO-TA cloning.

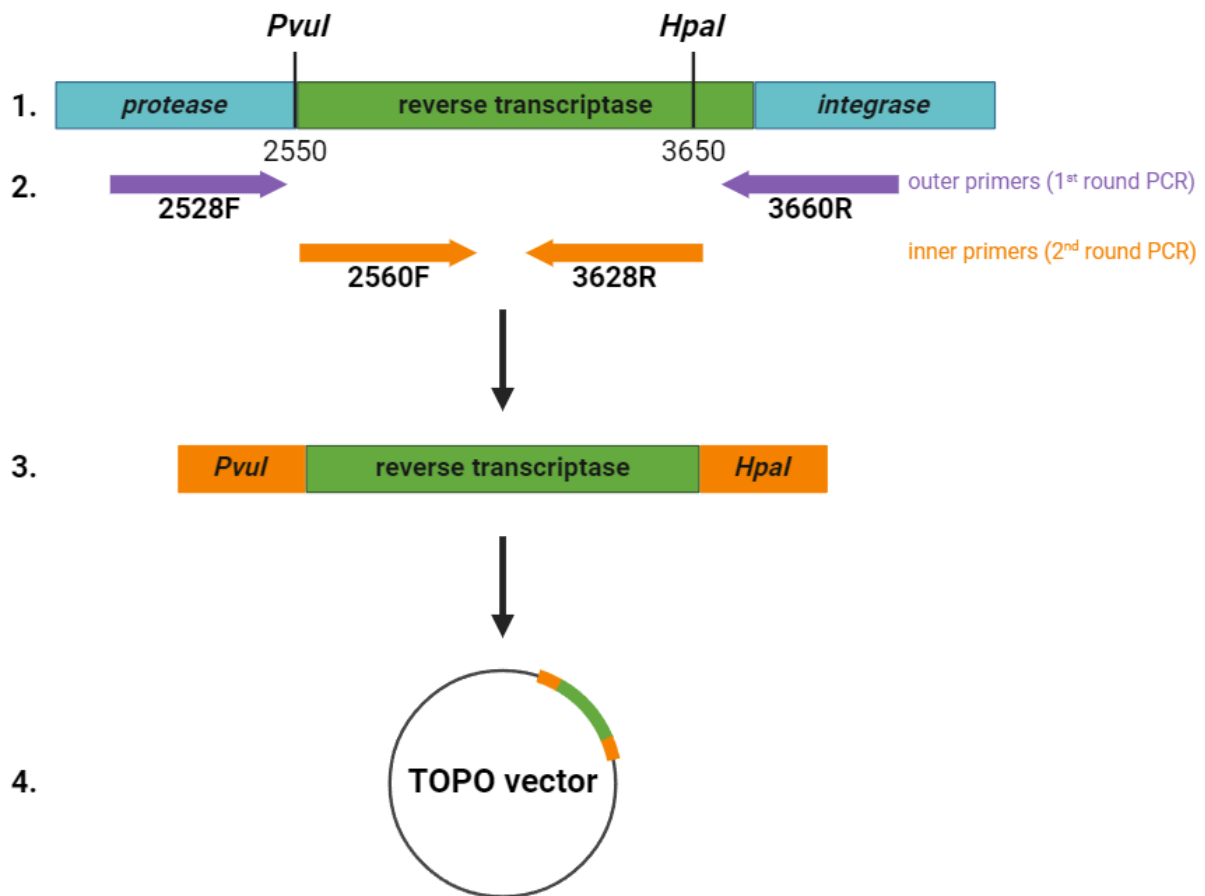


Figure 2.2: Overview of the cloning of amplicons from lab-adapted strains into TOPO vectors.

A portion of the *reverse transcriptase* gene of the HIV-1 subtype B and C was amplified from the lab-adapted strains. After reverse transcription of the viral RNA, the cDNA (1) from for each subtype C (n = 4) and subtype B (n = 4) lab-adapted strain was used as the template for nested PCR (2) using two sets of primers. The outer primers 2528F and 3660R were used in the first round of amplification and the inner primers 2560F and 3628R, which contain the *PvuI* and *HpaI* restriction sites, were used in the second round of amplification. This resulted in a 1096 bp *reverse transcriptase* amplicon with *PvuI* (5') and *HpaI* (3') restriction sites on either end (3). The amplicons were then cloned into the TOPO vector using TA cloning (4).

1st round PCR

The reactions for first round PCR were prepared by combining the reagents listed in Table 2.4 and thermal cycling was carried out as conditioned in Table 2.5 with the PCR System 9700 GeneAmp Thermocycler (Applied Biosystems; Cat No. 4303481H).

Table 2.4: First-round PCR reaction components

Reagent	Concentration	Volume (μl)	Final concentration
dNTPs	10 mM	1.0	200 μM
sense primer 2528F	3 μM	1.0	0.3 μM
antisense primer 3660R	3 μM	1.0	0.3 μM
cDNA		2.5	0.1 - 250 ng
Enzyme mix	3.5 U/μl	0.8	0.05 U/μl
PCR buffer	10X	5.0	1X
dH ₂ O		19.3	
Final volume		50	

Table 2.5: First-round PCR reaction conditions

Number of cycles	PCR Step	Temperature	Time
1	initial denaturation	94°C	2 minutes
	denaturation	94°C	15 seconds
10	annealing	55.5°C	30 seconds
	extension	72°C	1 minute, 10 seconds
20	denaturation	94°C	15 seconds
	annealing	55.5°C	30 seconds
	extension	72°C	1 minute, 20 seconds*
1	final extension	72°C	7 minutes

* + 5 minutes for each successive cycle

2nd round PCR

The reactions for the second round PCR were compiled by combining the reagents in Table 2.6. Thermal cycling was performed as conditioned in Table 2.7 with the PCR System 9700 GeneAmp Thermocycler (Applied Biosystems; Cat No. 4303481H).

Table 2.6: Second-round PCR reaction components

Reagent	Concentration	Volume (μl)	Final concentration
Platinum™ SuperFi™ Green PCR Master Mix	2X	25	1x
sense primer 2560F	10 μM	2.5	0.3 μM
antisense primer 3628R	10 μM	2.5	0.3 μM
The PCR product from first round PCR		5.0	
SuperFi™ GC Enhancer	5X	10	1X
dH ₂ O		5.0	
Final volume		25	

Table 2.7: Second-round PCR reaction conditions

Number of cycles	PCR Step	Temperature	Time
1	initial denaturation	98°C	30 seconds
35	denaturation	98°C	10 seconds
	annealing	55.5°C	10 seconds
	extension	72°C	1 minute
1	final extension	72°C	4 minutes

Clean-up of PCR products

The PCR products were cleaned up using the PureLink™ PCR Purification Kit (Invitrogen; Cat No. K310001) for downstream use in cloning so that the buffers and enzymes do not inhibit the ligation reaction. This kit relies on the same principle as the RNA extraction kit, wherein nucleic acids bind selectively to a silica membrane and the other PCR reagents are washed away using buffers, after which the DNA is eluted off the membrane. To the 45 μl PCR reaction, 180 μl of Binding Buffer (B2) was added. The solution was added to spin column placed in a 2 mL collection tube. The column was centrifuged at 10 000 rpm for 1 minute. The filtrate was discarded and 650 μL of Wash Buffer was added to the spin column and the spin column was centrifuged at 10 000 rpm for 1 minute. The filtrate was discarded and the empty column was centrifuged again at 13 000 rpm for 2 minutes to evaporate the remaining ethanol. The spin column was then placed in a collection tube and 50 μl of Elution Buffer (E1) was

added. The column was incubated for 1 minute and then centrifuged at 13 000 rpm for 1 minute. The purified PCR products were quantified using a NanoDrop™ 1000 Spectrophotometer (ThermoFisher Scientific; Cat No. ND2000CLAPTOP) and stored at -20°C.

Sequencing and phylogenetic analysis

Sanger sequencing was performed as described previously (see 2.2.1.3.4 Sanger sequencing, page 50) with the second round PCR products. The consensus sequences were aligned to the HIV-1 subtype reference sequences A to J obtained from the Los Alamos HIV Database (<https://www.hiv.lanl.gov/content/index>). The sequences were submitted to Stanford's HIV drug resistance database to confirm their subtype. A phylogenetic tree was drawn with the consensus sequences to ensure there was no cross-contamination using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al.*, 2021) with the following parameters: tree build method – Neighbour-Joining, test of phylogeny – Bootstrap method and No. of bootstrap replication – 1000. A phylogenetic tree was drawn with the reference sequences for subtype B and C obtained from the Los Alamos HIV database.

2.2.2.1.1.2 Cloning through TOPO-TA

The TOPO™ XL-2 PCR Cloning kit (ThermoFisher Scientific; CAT No. 45-20810) was used to clone the *reverse transcriptase* fragments of the lab adapted strains into a TOPO vector (see plasmid map in the Appendix A, Figure A.4). TOPO-TA cloning allows for the insertion of a PCR fragment into a backbone without the use of restriction enzymes and DNA T4 ligase (Figure 2.3, page 60). This method was selected because *PvuI* and *HpaI* are endonucleases. This means they cleave the phosphodiester bond of the DNA, thus they prefer to cleave DNA internally (Pingoud and Jeltsch, 2001). This often results in their failure to cleave sites on the end of the DNA fragment, therefore makes it desirable to restrict the fragment in a vector rather than restricting the ends of the fragment (Kaufman and Evans, 1990).

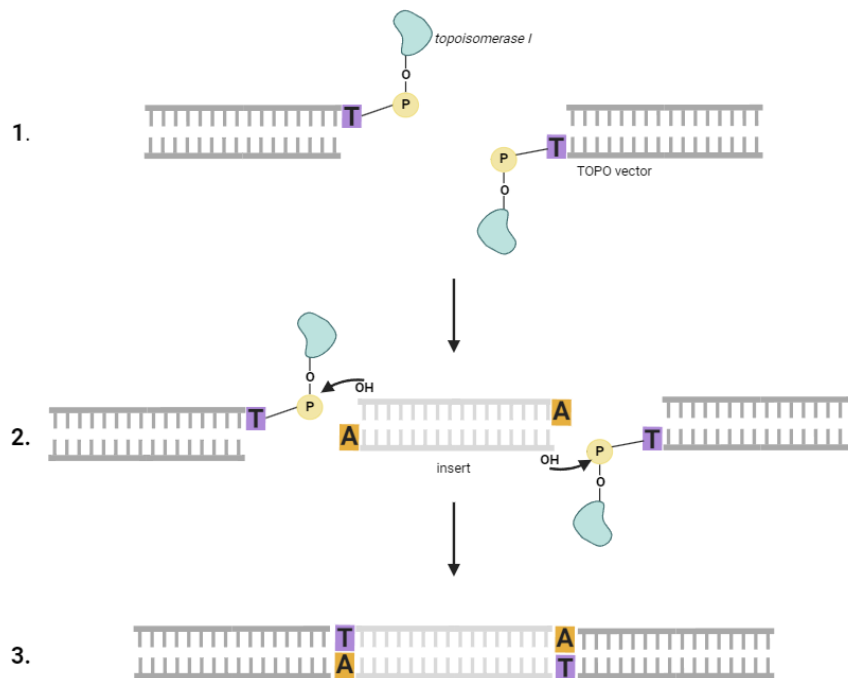


Figure 2.3: The principle of TOPO-TA cloning

The principle of TOPO-TA cloning is dependent on T-A overhangs and *topoisomerase I*, rather than restriction digest and ligation. *Topoisomerase I* which was bound to each 3' end of the TOPO vector backbone, cleaves at 5'-CCCTT-3' resulting in a 'T' overhang. The energy from the cleavage was stored as a covalent bond between the phosphate of the 'T' and *topoisomerase I* (1). The insert which contained 'A' overhangs resulting from PCR, binds to the 'T' overhang. During this process the hydroxyl group of the insert attacked the covalent bond (2). This released *topoisomerase I* as the strands joined, resulting in the *reverse transcriptase* fragment in the TOPO vector (3).

TOPO-TA cloning relies on the vaccinia virus DNA *topoisomerase I*. Two *topoisomerase* enzymes are covalently bound to the 3' ends of the double-stranded DNA of the TOPO vector. The enzyme cleaves the phosphodiester bond after 5'-CCCTT-3' on a single strand (Shuman, 1991). This results in a 5' thymine overhang in the vector and the energy from this broken phosphodiester bond is stored as a covalent bond between the 3' phosphate of the cleaved strand and *topoisomerase's* tyrosyl residue (Tyr-274) (Shuman, 1994). The insert contains the adenine overhang due to *Taq's* lack of 3' to 5' exonuclease proofreading activity. During binding of the thymine to adenine, the 5' hydroxyl group of the insert attacks the covalent bond between the phosphate and Tyr-274, joining the ends and the enzyme is released. This results in the *reverse transcriptase* fragment being joined into the TOPO vector.

The TOPO vector contains a disrupted *lacZ α -ccdB* gene with the PCR insertion site in the middle, which allows for blue-white screening of the clones. The *lacZ α -ccdB* gene encodes for β -galactosidase which cleaves X-gal into a blue product. Therefore, clones without the PCR product inserted will result in blue bacterial colonies. This allows for the selection of the colonies where the *reverse transcriptase* fragment was successfully inserted into the TOPO vector. When this occurs the *lacZ α -ccdB* gene remains disrupted and no β -galactosidase is produced, so X-gal is not cleaved into blue product. Therefore, clones with inserts will appear as the white bacterial colonies on the agar plate.

The TOPO™ Cloning reaction was compiled by mixing: 1 μ l of salt solution, 1 μ l PCR-XL-2-TOPO™ Vector (10 ng/ μ l), and 4 μ l of PCR product. The reaction was incubated at 25°C for 5 minutes and then immediately placed on ice. A vial of One Shot™ OmniMAX™ 2T1R Chemically Competent *E. coli* (ThermoFisher Scientific; Cat No. C854003) containing 50 μ l was thawed. To the *E. coli* cells, 2 μ l of TOPO cloning reaction was added. The cells were incubated on ice for 30 minutes and heat-shocked for 30 seconds in a 42°C water bath. The cells were incubated on ice for 2 minutes and 250 μ l of SOC Outgrowth Medium (New England Biolabs; Cat. No. B9035) was added. The tube was shaken at 225 rpm for 1 hour at 37°C. The bacterial cells were pelleted by centrifuged at 4000 rpm for 2 minutes. The supernatant (200 μ l) was removed and the bacterial cells were resuspended in the remaining 50 μ l. To prepare the agar plates for blue-white colony screening, the LB agar plates containing 100 μ g/mL carbenicillin were incubated at 37°C for 30 minutes with 120 μ l of X-Gal (20 mg/mL) and 40 μ l of Isopropyl β -d-1-thiogalactopyranoside (IPTG, 100 mM) spread evenly on the agar. All the bacterial cells were spread on the warm LB agar plates, which were inverted and incubated overnight at 37°C.

2.2.2.1.1.1.3 Screening through colony PCR

The white bacterial colonies were further screened with colony PCR to confirm that they contained the PCR insert. Each white colony was picked and resuspended in 50 μ l of LB broth (with carbenicillin) in a sterile 96-well plate. The cultures were incubated at 37°C for 2 hours with shaking at 200 rpm. The PCR reaction was prepared by

combining the reagent in Table 2.8 and thermal cycling was carried out under the conditions detailed in Table 2.9. The forward and reverse inner primers from the nested PCR were selected because they both bind to the *reverse transcriptase* fragment insert. Therefore, if the insert was present in the TOPO vector, both primers would bind and produce a PCR product of 1096 bp. In the absence of the insert, no PCR product would be produced.

Table 2.8: Colony PCR screening reaction components

Reagent	Concentration	Volume (µl)	Final concentration
One Taq Quick-Load 2X Master Mix (New England Biolabs; Cat No. SM0486S)	2X	5.0	1x
sense primer 2560F (Table 2.3, page 55)	10 µM	0.2	0.2 µM
antisense primer 3628R (Table 2.3, page 55)	10 µM	0.2	0.2 µM
LB broth containing the bacterial cell outgrowth		1.0	
dH ₂ O		3.6	
Final volume		10	

Table 2.9: Colony PCR screening reaction conditions

Number of cycles	PCR Step	Temperature	Time
1	initial denaturation	94°C	30 seconds
30	denaturation	98°C	30 seconds
	annealing	55.2°C	60 seconds
	extension	68°C	1 minute 20 seconds
1	final extension	68°C	1 minutes

The PCR products were visualised with a 1.2% agarose gel as described previously in 2.2.1.3.1 Agarose gel electrophoresis (page 48). The clones were considered successful if their amplicons were of the expected size of 1096 bp. For successful

clones, the 50 µl bacterial culture was added to 4 mL of LB broth (with carbenicillin) and shaken at 200 rpm overnight at 37°C. DNA extraction was performed as described in 2.2.1.3.2 Plasmid DNA extraction (page 49) and Sanger sequencing was performed as outlined before in 2.2.1.3.4 Sanger sequencing (page 50) to confirm the TOPO vector contains the *reverse transcriptase* gene fragment of the correct lab-adapted strain.

2.2.2.1.1.1.4 Subcloning of *reverse transcriptase* into p8.9MJ4 and p8.9NSX+

The amplicons were excised from the TOPO vectors and cloned into either the modified p8.9MJ4 (for subtype C lab-adapted strains, n = 4) or the modified p8.9NSX+ (for subtype B lab-adapted strains, n = 4) to create strains-specific HIV-1 expression vectors. A double restriction digest with the restriction enzymes *PvuI* and *HpaI* were performed on the PCR products and the modified p8.9MJ4 and p8.9NSX+ plasmids. Restriction with *PvuI* results in a sticky end, whereas restriction with *HpaI* results in a blunt end. The use of two different restriction enzymes and the different ends they produce allowed for directional cloning of the PCR fragments into the vector plasmids. This ensures that the PCR products being inserted into their expression vector in the correct orientation.

The restriction digests were prepared by combining the following to a final volume of 70 µl: template DNA (4000 ng of modified p8.9MJ4 or p8.9NSX+ , or 1000 ng of TOPO clone), 0.5 µl of 10 U/µl *HpaI* (New England Biolabs; Cat No. R0105S), and 0.5 µl of 10 U/µl *PvuI*-HF (New England Biolabs (NEB); Cat No. R3150S), 7 µl of rCutSmart™ Buffer (New England Biolabs; Cat No. B6004S), 1 µl of Shrimp Alkaline Phosphatase (New England Biolabs; Cat No. M0371S) for the modified p8.9MJ4 and p8.9NSX+ or 1 µl of T4 Polynucleotide Kinase for the TOPO clones (New England Biolabs; Cat No. M0201S) and deionised water. Shrimp Alkaline Phosphatase dephosphorylates the restricted modified p8.9MJ4 and p8.9NSX+ backbones. This prevents re-circularisation of the backbone during ligation. T4 Polynucleotide Kinase phosphorylates the restricted inserts from the TOPO clones. This ensures a 5' phosphate group on the ends of the insert for ligation. The reactions were incubated at 37°C for 60 minutes. The restricted expression vectors and PCR products were

electrophoresed on a 0.7% and 1.2% TAE agarose gel, respectively, as previously described (page 48) to confirm that the products are of the expected sizes: 1 130 bp for the PCR products and 11 048 bp for the expression vectors.

The digested *reverse transcriptase* fragments and the plasmid backbones were ligated together with the Quick Ligation Kit (New England Biolabs; Cat No. M2200). The ligation reaction was carried out by T4 DNA ligase which catalyses a phosphodiester bond formation between a 5' phosphate group on the insert and the 3' hydroxyl group of the vector plasmid. The ligation reaction was performed with a ratio of 1:3 (backbone: insert). The reaction was prepared by adding the following to a final volume of 10 µl: 55.2 ng of restricted and dephosphorylated vector plasmid (p8.9MJ4 or p8.9NSX+), 184.3 ng of restricted and phosphorylated *reverse transcriptase* fragments, and deionised water to 10 µl. To the mixture, 10 µl of Quick Ligase Reaction Buffer (2X) and 1 µl of Quick Ligase was added. After mixing, the reaction was incubated at 25°C for 5 minutes. T4 DNA Ligase's optimal temperature is 37°C. However, sticky ends are short and thus cannot stably bind DNA at high temperatures therefore a lower temperature (10-16°C) is favourable for ligation (Dugaiczky *et al.*, 1975). Ligation with blunt ends can be performed at a slightly higher temperature (15-20°C), but not at 37°C (Dugaiczky *et al.*, 1975). The insert and backbone used in this study has blunt and sticky ends, therefore a temperature between the optimal T4 ligase temperature (37°C) and the optimal temperature for the ends was selected. The ligation was placed on ice and 5 µl of the reaction was used for bacterial transformation which was performed as described in 2.2.1.1.6 Bacterial transformation through heat shock (page 46). The ligation reactions were stored at -20°C.

The bacterial colonies were screened with colony PCR as previously described in 2.2.2.1.1.1.3 Screening through colony PCR (page 61) to determine whether they contained the correct inserts. The nested PCR for this colony screen contained the outer forward primer (2528F in Table 2.3 on page 55) and inner reverse primer (3628R in Table 2.3 on page 55) of the nested PCR protocol. The forward outer primer would bind to the modified p8.9MJ4/NSX+ backbone while the reverse primer would bind to the *reverse transcriptase* fragment insert. Therefore, if both the backbone and insert are present both primers would bind and produce a PCR product of 1128 bp. However, if the insert is missing, no PCR product is produced. The presence of a PCR amplicon

would also confirm directionality of the insert since the reverse primers would bind in the incorrect direction, no product would be amplified. The PCR was carried out under the same thermal cycling conditions described in 2.2.2.1.1.1.3 Screening through colony PCR (page 61), except an annealing temperature of 55.5°C was used.

The PCR products were visualised with a 1.2% agarose gel as described in section 2.2.1.3.1 (page 48). The clones were considered successful if they were the expected size of 1128 bp. The 50 µl bacterial culture was added to 4 mL of LB broth (with carbenicillin) shaken at 200 rpm overnight at 37°C. DNA extraction was performed as described in 2.2.1.3.2 Plasmid DNA extraction (page 49) and Sanger sequencing was performed as outlined in 2.2.1.3.4 Sanger sequencing (page 50) to confirm the expression vector contains the *reverse transcriptase* gene of the lab-adapted strains. A phylogenetic tree was constructed with the sequences of the PCR products, TOPO clones and the clones used for PSV production and phenotypic testing using MEGA11, as described above in section 2.2.2.1.1.1.1 Nested PCR on page 53.

2.2.2.1.1.2 Generation and titration of PSVs

To produce replication-defective PSVs, the packaging plasmids (p8.9MJ4 or p8.9NSX+ containing *reverse transcriptase* from the lab adapted strains) were each co-transfected with a transfer plasmid (pCSFLW) and envelope plasmid (pHEF.VsVg). Prior to transfection, 8 million HEK239T cells were seeded in 10 mL of complete cell culture medium in a 10 cm transfection dish. The cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere for 24 hours. The three plasmids were combined in the following amounts: 1 µg of the modified p8.9MJ4 or p8.9NSX+ clones, 0.25 µg of pHEF.VsVg and 1.5 µg of pCSFLW. The plasmids were co-transfected in the ratio of 1:0.25:1.5 respectively. To the plasmid mixture, 50 µl of DMEM (without supplements) and 20 µl of Polyethylenimine “Max” (1 mg/ml) was added and mixed by pipetting. This mixture was incubated for 20 minutes at room temperature. The HEK293T cells were checked to be attached to the petri dish and the confluency was confirmed to be ~60%. The plasmid mixture was added dropwise to the media of the transfection dish containing the HEK239T cells. The media in the transfection dish was gently swirled around to distribute the encapsulated DNA evenly. The transfection dish was incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours.

The PSVs were harvested after 48 hours by collecting ~9 mL of media from the transfection dish and centrifuging it at 2000 rpm for 10 minutes to pellet cellular debris. The PSV-containing supernatants were stored at -80°C in 1 mL aliquots. After the first harvest, 10 mL of complete cell culture medium was added back to the transfection dish containing the HEK293T cells. The transfection dish was incubated at 37°C with 5% CO₂ in a humidified atmosphere for 24 hours and a second harvest was performed as describe above.

Viral titers were determined to standardize of the amount of virus used in the phenotypic susceptibility assay. The assay was set up in a Nunc™ Edge™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate (ThermoFisher Scientific; Cat No. 167425 (Figure 2.4, page 66). The assay was set up by adding 100 µl of PSVs to row H in duplicate. To the remaining wells, 50 µl of complete cell culture medium was added. A two-fold serial was performed from row H to A, which resulted in the highest concentration of PSV in row H and the lowest concentration of PSV in row A. Finally, 2000 HEK293T cells in 50 µl of complete culture medium was added to all the wells and the plate was incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours.

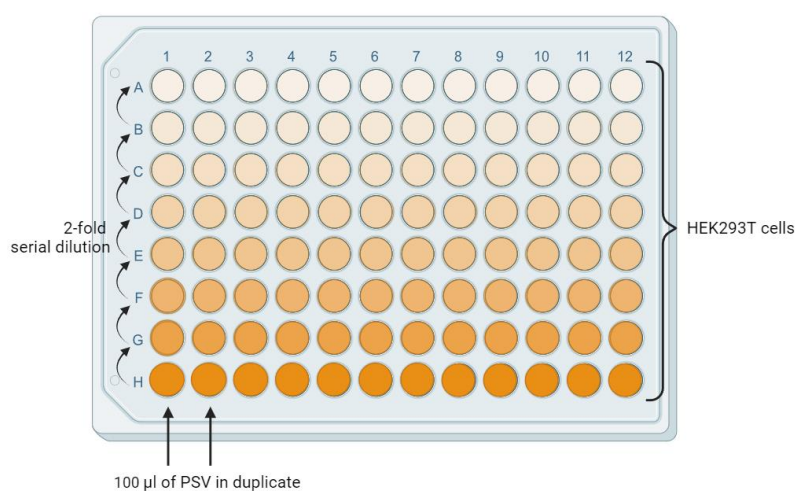


Figure 2.4: Plate layout for viral titration.

PSV was quantified using a viral titer assay. The produced PSVs were diluted and combined with cells to examine the number that were infected.

The plates were developed using the Bright-Glo™ Luciferase Assay System (Promega; Cat No. E2610) to assess of the level of firefly luciferase expression in

pseudoviral-containing cultures. To each well of 100 μ l of culture, 100 μ l of Bright-Glo Luciferase substrate was added. The cells with substrate were incubated for 2 minutes at room temperature and then mixed thoroughly by aspirating before being transferred to the corresponding wells of a white 96-well plate. A white plate was used to maximise the light output signal on the luminometer, since white reflects light. The luminescence of each well was quantified with the GloMax[®] 96 Microplate Luminometer (Promeg; Cat No. E6501) (emission at 560 nm) and the relative light unit (RLU) values were collected. Viral inputs for the phenotypic assays were standardized to a PSV dilution that corresponds to an RLU value of 1×10^6 of the media (no drug) control.

2.2.2.1.1.3 Assessing the toxicity of antiretroviral drugs to HEK293T cells

The toxicity of antiretroviral drugs (DOR, ISL, EFV, 3TC, DMSO solvent) to the HEK293T cells was examined before the phenotypic assay was executed, to ensure that viral inhibition observed in the phenotypic assay was not from antiretroviral drug cytotoxicity.

The plate set-up for the toxicity assay is displayed in Figure 2.5 (page 68). The assay was prepared on a Nunc[™] Edge[™] 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplates and 75 μ l of drug at 100 μ M was added to row H in triplicate. In the other wells in the column 50 μ l of culture medium was added. A three-fold serial dilution was carried out by transferring 25 μ l of each drug (DOR, ISL, EFV, 3TC, DMSO, culture medium). Fifty microliters of complete culture medium was added to the third column of each drug, which did not received any cells and served as a control for background fluorescence. A no-drug control, with complete culture media instead of drug, was also included to represent 100% cell viability. Lastly, 2000 HEK293T cells in 50 μ l of complete cell culture medium was added to all the remaining wells. The plate was incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours.

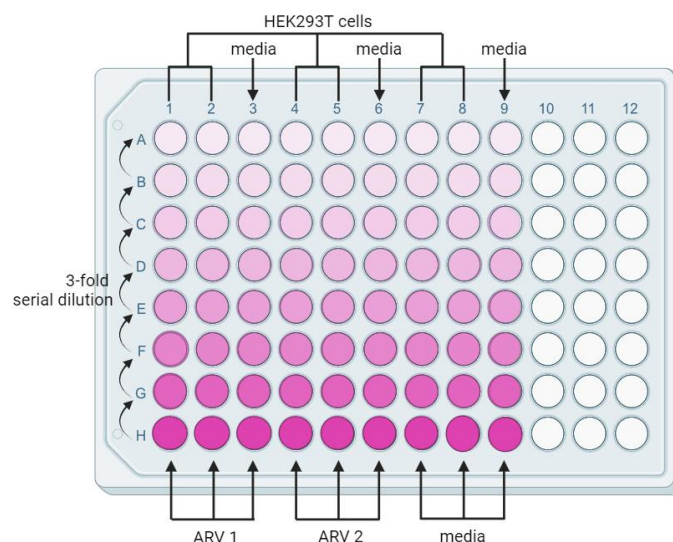


Figure 2.5: Plate set-up to measure the cytotoxicity of ARVs.

A cell viability assay was used to determine the cytotoxicity of the ARVs and DMSO solvent to the HEK293T cells. The drugs were diluted serially and HEK293T cells were added. A no-drug control and a control for background fluorescence was included.

To develop the plate, 20 μ l Alamar Blue solution (made up as described in 2.1.1.2 Alamar Blue solution (pg. 5)) was added to all wells and the plate was incubated at 37°C with 5% CO₂ in a humidified atmosphere for $\pm$ 2 hours.

The GloMax® 96 Microplate Luminometer was used to read the plates. The plate was shaken for 10 seconds first at 300 cycle/minute with linear shaking to shear the cells releasing the fluorescent molecules. The wells were excited at 520 nm. For each drug, values of the third column wells (containing no cells) were then subtracted from the values of the corresponding first and second column wells (wells containing cells). This was done to eliminate background fluorescence. Cell viability was calculated by divided by the average RLU values of the drug-containing wells by the average RLU value of the no-drug control (which represents 100% cell viability). Sigmoidal dose-response curves of cell viability (%) against drug concentration ($\log_{10}$ μ M) were generated for each drug with Microsoft Excel. The concentrations of antiretroviral drug that resulted in a cell viability of 80% (CC₂₀) and 50% (CC₅₀) were predicted from the cell viability values with the FORECAST formula in Microsoft Excel. All antiretroviral drugs were initially screened in the *in vitro* phenotypic drug susceptibility assay at initial concentrations $\leq$ CC₂₀, to prevent cytotoxicity being mistaken as viral inhibition. The

concentration ranges used in the *in vitro* phenotypic assays were optimised by repeated testing at various concentration ranges to obtain full viral suppression at the highest drug concentration and full viral activity at the lowest drug concentration.

2.2.2.1.1.4 *In vitro* phenotypic drug susceptibility testing of wild-type HIV-1 subtype B and C PSVs

In vitro phenotypic drug susceptibility testing was performed in Nunc™ Edge™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate, for which the setup is depicted in Figure 2.6 (page 69). In column 1, 75 µl of the ARV drugs were added in duplicate. To the remaining wells, 50 µl of complete cell culture medium was added and a three-fold serial dilution of the antiretroviral drugs was performed by transferring 25 µl of the drugs from column 1 to 11. The wells in column 12 did not received any ARV drug and served as the media (no-drug) control. A mixture of 2000 HEK293T cells in 50 µl of complete cell culture medium and PSVs (standardised to 1×10^6 RLU) was added to all the wells. The plate was incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours.

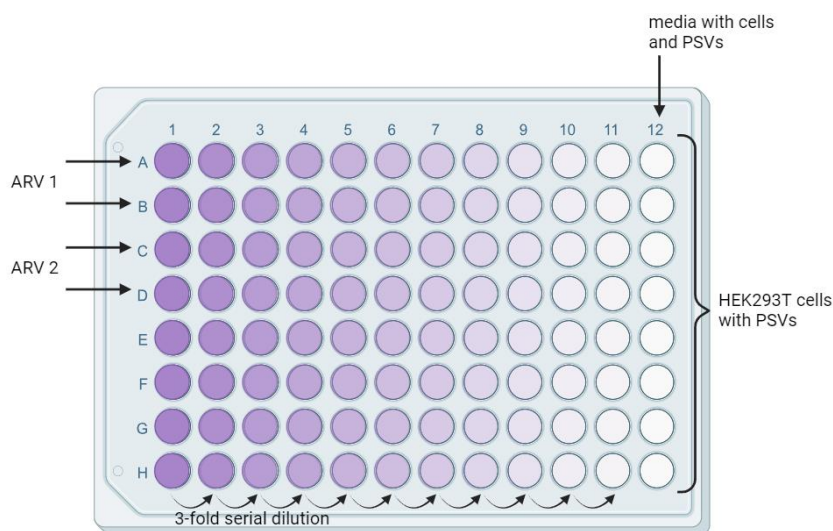


Figure 2.6: *In vitro* phenotypic assay set-up used to test ARV drug susceptibility.

To determine the susceptibility of the lab-adapted strains to a particular ARV, 3-fold dilutions of the ARV were produced and the lab-adapted PSVs were applied to the drug along with HEK293T cells.

The plates were developed with BrightGlo Luciferase as described previously in 2.2.2.1.1.2 Generation and titration of PSVs (page 65). The viral activity (%) of each

ARV drug concentration was calculated by dividing their RLU's by the average RLU of the media (no-drug) control. A dose-response curve was plotted of the viral activity (%) against the concentration of the antiretroviral drugs. The concentration of drug that reduced viral activity by 50% (IC₅₀) was calculated using the 'FORECAST' formula in Microsoft Excel. The IC₅₀ values were compared to determine differences in potency of the antiretroviral drugs on HIV-1 subtype B and C lab adapted strains. PSVs generated from the p8.9NSX+ and p8.9MJ4 HIV expression vectors were also included in the assays for reference.

2.2.2.1.2 Objective 1B: Investigating *in vitro* dual drug synergies with wild-type HIV-1

2.2.2.1.2.1 *In vitro* dual drug synergy assay

To determine whether using DOR and ISL in combination has a greater inhibitory effect on HIV-1 than using them individually, the synergy between the two ARV drugs was assessed. The same *in vitro* phenotypic drug susceptibility assay was used as described above in 2.2.2.1.1.4 *In vitro* phenotypic drug susceptibility testing of wild-type HIV-1 subtype B and C PSVs (page 69), but with a modified setup (Figure 2.7, page 71). Since DOR and ISL is a combination of an NNRTI and an NRTI, the combination of EFV (an NNRTI) and 3TC (an NRTI) was used as a reference to compare synergy. EFV and 3TC were part of the triple therapy recommended for first-line treatment prior to 2019 (South Africa National Department of Health, 2019).

Five serial dilutions of DOR and ISL over non-toxic concentrations were prepared separately. The concentrations of DOR were 0.5 µM, 0.03 µM, 0.002 µM, 0.0001 µM, 0.00001 µM. The concentrations of ISL were 0.1 µM, 0.007 µM, 0.0004 µM, 0.00003 µM, 0.000002 µM. These concentrations were combined as depicted in Figure 2.7 (indicated by the black box). The final volume of the drug combinations in each well was 50 µl, which was then combined with 50 µl of 2000 HEK239T cells in 50 µl of cell culture medium and HIV-1 wild-type subtype C PSV (standardised to 1x10⁶ RLU). Well 1A contained no drug. This grid of drug dilutions was prepared in duplicate on the same 96-well plate, after which cells and PSV were similarly applied. The plate was

incubated at 37°C with 5% CO₂ in a humidified atmosphere for 48 hours. The same procedure was repeated with 3TC and EFV. The five serial dilutions that were prepared for EFV were 0.1 µM, 0.007 µM, 0.0004 µM, 0.00003 µM, 0.000002 µM. The five serial dilutions that were prepared for 3TC were 10 µM, 7 µM, 0.4 µM, 0.03 µM, 0.002 µM.

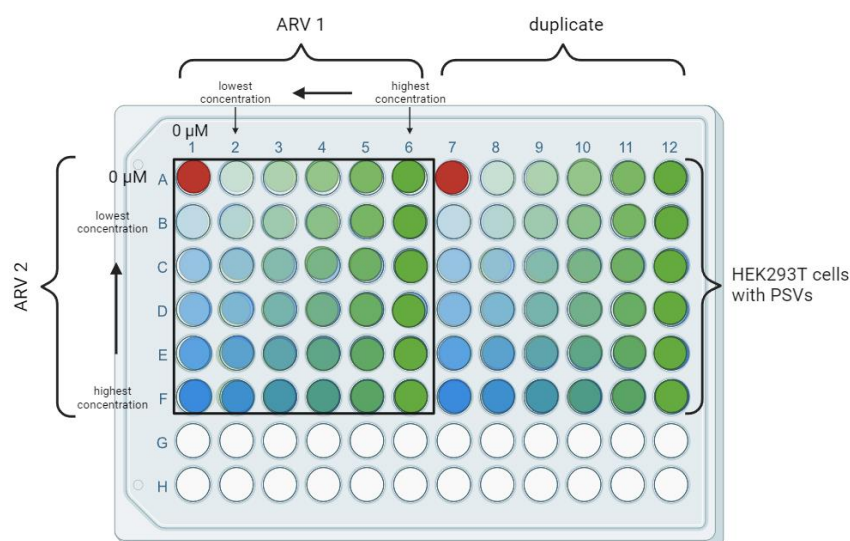


Figure 2.7: *In vitro* phenotypic assay to determine the synergistic effect of two ARV drugs.

To assess the synergy between DOR and ISL, a grid of various combined drug dilutions were produced and HEK239T cells with HIV-1 subtype C wild-type PSV was applied. A duplicate was included on the same plate. Well 1A served as a no drug control, containing only cells, culture media and PSV.

The plates were developed with BrightGlo Luciferase as described previously in 2.2.2.1.1.2 Generation and titration of PSVs (pg. 65). The data was analysed with SynergyFinder version 30.04.2023-R-3.8.2 (Ianevski *et al.*, 2017). A synergy score was generated and used to classify the interaction between the two drugs. This score indicated the excess response observed over the predicted response due to drug interaction. The programme plotted a 3-D graph of the drug concentrations on the x and y axis' with the synergy score on the y-axis. The program used four different synergy models (ZIP, Lowewe, Bliss and HSA) generate a synergy scores. Each model calculated synergy using different conditions. The ZIP model made the assumption that the drugs do not have an effect on each other. The Loewe model predicted an expected response as if both drugs were the same drug. The Bliss model made the assumption that the drugs act independently. HSA calculated the synergy

as excess response seen over the highest effect of the two individual drugs. The average synergy score of each plot was calculated and used to determine synergy. The p-value was calculated to indicate significant synergy. An example of two established MK-1775 and Niraparib synergistic drugs MK-1775 and Niraparib were used as a reference to visualise true synergy and compare the synergy scores to known significant synergy scores (O'Neil *et al.*, 2016).

2.2.2.2 Objective 2: Assessment of *in vitro* DOR susceptibility of NNRTI resistant HIV-1

Purpose:

The purpose of this objective was to:

- a. generate HIV-1 subtype C PSVs containing prevalent single and multi-NNRTI DRMs
- b. assess the *in vitro* phenotypic drug susceptibility of these mutant PSVs against DOR

Overview:

The top twenty prevalent NNRTI DRM combinations in South Africa were identified from an HIV-1 genotypic drug resistance testing database obtained from the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). These combinations of mutations, as well as the single mutations present in the combinations, were generated in the p8.9MJ4 HIV-1 subtype C expression vector via SDM. *In vitro* phenotypic DOR susceptibility testing was performed on these PSVs to understand the effect that common NNRTI resistant mutations in South Africa would have on DOR susceptibility.

2.2.2.2.1 Objective 2A: Identification of prevalent NNRTI resistance mutations in South Africa

The selection of NNRTI DRMs to generate and screen for DOR resistance was based on prevalence. The HIV-1 *reverse transcriptase* sequences (n = 7749) from January

2016 to December 2020 were acquired from the CMJAH database, as part of routine genotypic drug resistance testing for NNRTI-experienced patients failing mostly second-line cART. The twenty most prevalent combination NNRTI DRM profiles (n = 20) were prioritised in this study. The combination DRM profiles were defined as a mutation profile which contained more than one major NNRTI resistance-causing mutation, according to Wensing *et al.* (2022). These profiles were generated in p8.9MJ4 and phenotypic susceptibility testing was performed. To understand the level of drug resistance each of the individual mutations in the profiles contributed to the overall reduction in susceptibility observed in the combination DRM profiles, the individual mutations present in the DRM profiles were also generated in p8.9MJ4 and phenotypic susceptibility testing was performed.

2.2.2.2.2 Objective 2B: Generation of drug resistant HIV-1 plasmids through SDM

2.2.2.2.2.1 SDM primer design and mutagenesis

The selected DRMs were introduced into the reference HIV-1 subtype C expression vector (i.e., p8.9MJ4) through SDM. Two approaches were followed for the design of the mutagenesis primers. The majority of mutants were generated through the use of non-overlapping mutagenesis primers. However, when this approach failed, an alternative approach was followed that used overlapping mutagenesis primers.

2.2.2.2.2.1.1 Mutagenesis using non-overlapping primers

The non-overlapping primers were designed with NEBaseChanger version 1.3.3 (New England Biolabs, Ipswich, Massachusetts USA, <https://nebasechanger.neb.com/>). The desired amino acid change was made by introducing the least amount of nucleic acid base changes to the wild-type codon to generate the desired amino acid change in the Reverse Transcriptase. The altered base(s) was on (or close to) the 5' end(s) of either or both the forward and reverse primer(s). Only a single mutation was introduced into the expression vector per round of SDM. For combinations of DRMs, multiple rounds of SDM was performed to introduce the desired mutations consecutively. The

melting temperatures of the mutagenesis primers were initially calculated with the NEB T_m calculator version 1.16.4 (New England Biolabs, Ipswich, Massachusetts USA, <https://tmcalculator.neb.com/#!/main>). The melting temperature difference between the forward and reverse primers were confirmed to be within 2-4°C using the OligoAnalyzer™ Tool (Integrated DNA Technologies, Coralville, Iowa USA, <https://eu.idtdna.com/calc/analyzer>).

The workflow of the mutagenesis procedure is depicted in Figure 2.8. This entails the PCR amplification of the HIV-1 expression vector DNA using a primer set containing the desired mutation. The ends of the amplification product are phosphorylated, ligated to circularize, and the original plasmid template is digested. This results in an HIV-1 expression vector that contains the desired mutation(s).

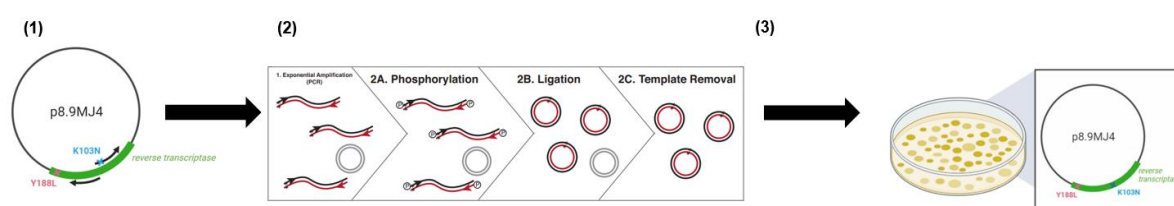


Figure 2.8: Introducing a DRM into p8.9MJ4 using SDM.

The figure depicts the generation of the combination drug resistance profile K103N, Y188L. (1) Primers are designed to introduce the K103N DRM into a plasmid that already contains the Y188L DRM. The resulting plasmid would therefore have both K103N and Y188L. (2) PCR is used to amplify the plasmid. The primers containing the desired nucleotide change(s) ensures that the amplified product contains the desired change(s). (2A-C) The ends of the amplification product are then phosphorylated and ligated to circularise the plasmid DNA. The original template plasmid (indicated in grey) is then digested by *Dnpi*. (3) Bacterial cells are transformed with the reaction product, grown overnight on an antibiotic-selective agar plate to yield bacterial colonies that contain the expression vector with the desired mutation profile. This picture in (2) was altered from the SDM NEB product manual (<https://tinyurl.com/SDMproductmanual>).

The SDM reaction was performed by combining the reagents in Table 2.10. Thermal cycling of the reaction was performed under the conditions described in Table 2.11 and the SDM reaction was stored at 4°C.

Table 2.10: SDM reaction components

Reagent	Concentration	Volume (μl)	Final concentration
Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs; Cat. No. M0492S)	2X	12.5	1x
sense primer	10 μM	1.3	0.5 μM
antisense primer	10 μM	1.3	0.5 μM
Template DNA (i.e., p8.9MJ4)	25 ng/μl	1.0	1 ng/μl
dH ₂ O		9.0	
Final volume		25	

Table 2.11: SDM reaction conditions

Number of cycles	PCR Step	Temperature	Time
1	initial denaturation	98°C	30 seconds
25	denaturation	98°C	10 seconds
	annealing	50-72°C	30 seconds
	extension	72°C	10 minute (30 seconds/kb)
1	final extension	72°C	2 minutes

The SDM reactions (5 μl) were visualised on a 1.2% TAE agarose gel, as described previously in section 2.2.1.3.1 (page 48), to determine if there was successful amplification before proceeding. A single distinct bright ~12 000 bp band indicated the successful amplification of the expression vector. In the absence of a band, or to increase the amount of product, the SDM PCR was repeated at a different annealing temperature.

The Kinase-Ligase-DpnI (KLD) reaction was prepared by combining the following to a final volume of 10 μl: 1 μl of SDM reaction, 1 μl of *DpnI* (ThermoFisher Scientific; Cat No. ER1701, 10 U/μl), 1 μl of T4 Polynucleotide Kinase ThermoFisher Scientific; Cat No. EK0031, 10 U/μl), 1 μl of T4 DNA Ligase (ThermoFisher Scientific; Cat No.

EL0012, 5 U/μl), 1 μl of 10X T4 DNA Ligase buffer and 5 μl of nuclease-free water. This reaction was incubated at 24°C for 1 hour. Bacterial transformation was then performed with 5 μl of the KLD reaction as described previously in section 2.2.1.1.6 Bacterial transformation through heat shock (page 46). For each SDM reaction, ±4 colonies were selected and grown up overnight in 4 mL of LB broth (with carbenicillin). The DNA from these colonies was extracted with the QIAprep Spin Miniprep Kit as described in previously section 2.2.1.3.2 Plasmid DNA extraction (pg. 49). Sanger sequencing was then performed as described previously in 2.2.1.3.4 Sanger sequencing (page 50) and the consensus sequences were submitted to the Stanford HIV Drug Resistance Database to confirm the presence of the expected DRMs. The p8.9MJ4 mutants which contained the correct DRMs were used for the generation of PSVs and *in vitro* phenotypic drug susceptibility testing.

2.2.2.2.1.2 Mutagenesis using overlapping primers

The overlapping primers were designed since the success rate of the non-overlapping primers was low. This method of SDM was based on a method established by Yang *et al.* (2022). This method uses four sets of primer to amplify the plasmid. The first set of overlapping primers bind to the ampicillin resistance gene while the second set of overlapping primers bind to the desired region and contain the mutation.

The overlapping primers with the mutation were designed with QuickChange Primer Design (Agilent, California USA, <https://tinyurl.com/5x9j6kt8>). The mutations were designed as described above. The melting temperatures of the primer sets were checked with the OligoAnalyzer™ Tool. The workflow of the SDM reaction are displayed in Figure 2.9. This entails two PCR reaction per mutant, with each containing one primer from each set and thus result in the plasmid in two fragments. The plasmid in then assembled after bacterial transformation by recombination in *E. coli*, resulting in an HIV-1 expression vector that the contains desired mutation.



Figure 2.9: Alternative approach for introducing a DRM into p8.9MJ4 using SDM.

The figure depicts the generation of the mutation V106M. (1) Primers are designed to introduce the V106M using two complementary overlapping primers. PCR is used to amplify the plasmid in two fragment. This was accomplished by using an Amp forward primer and mutation containing reverse primer in one reaction and an Amp reverse primer and mutation containing forward primer in a separate PCR reaction. (2) This results in two plasmid fragments with one contained the desired mutation. The original template plasmid (indicated in grey) is digested by *DpnI*. (3) Bacterial cells are transformed with the two PCR product fragments. The plasmid assembled using *E. coli*'s *in vivo* recombination system. The bacteria are grown overnight on an antibiotic-selective agar plate to yield bacterial colonies that contain the expression vector with the desired mutation.

The reagents in Table 2.12 were combined for each SDM reaction with the appropriate primer. Thermal cycling was performed under the conditions described in Table 2.13 and reaction were stored at 4°C.

Table 2.12: SDM reaction components

Reagent	Concentration	Volume (μl)	Final concentration
Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs; Cat. No. M0492S)	2X	12.5	1x
sense primer AmpF1* or Mutant forward	10 μM	1.3	0.5 μM
antisense primer Mutant reverse or AmpR1*	10 μM	1.3	0.5 μM
Template DNA (i.e., p8.9MJ4)	25 ng/μl	1.0	1 ng/μl
dH ₂ O		9.0	
Final volume		25	

* the Amp primers were designed by Yang *et al.* (2022)

Table 2.13: SDM reaction conditions

Number of cycles	PCR Step	Temperature	Time
1	initial denaturation	98°C	30 seconds
25	denaturation	98°C	10 seconds
	annealing	50-72°C	30 seconds
	extension	72°C	10 minute (30 seconds/kb)
1	final extension	72°C	2 minutes

The SDM reactions (5 µl) similarly visualised on a 1.2% TAE agarose gel, as described previously in 2.2.1.3.1 Agarose gel electrophoresis (page 48), to determine if there was successful amplification before proceeding. A two distinct bright bands of ~5000 bp and ~6000 bp indicated the successful amplification. As described above the annealing temperature was altered to increase the amount of product.

To digest the original template plasmid, a digest with *DpnI* was performed. The following were combined to a final volume of 10 µl: 1 µl of SDM reaction, 1 µl of *DpnI* (ThermoFisher Scientific; Cat No. ER1701, 10 U/µl), 1 µl of 10X Buffer Tango and 5 µl of nuclease-free water. This reaction was incubated at 37°C for 2 hours. Bacterial transformation was then performed with 1.5 µl of each of the two KLD reactions for the mutants, as described previously in section 2.2.1.1.6 Bacterial transformation through heat shock (page 46). The bacterial colonies were grown up as described above and their DNA was extracted as described in previously section 2.2.1.3.2 Plasmid DNA extraction (pg. 49). Sanger sequencing was then performed as described previously in 2.2.1.3.4 Sanger sequencing (page 50) and the consensus sequences were submitted to the Stanford HIV Drug Resistance Database to confirm the presence of the expected DRMs. The plasmids which contained the correct DRMs were used for the generation of PSVs and *in vitro* phenotypic drug susceptibility testing.

2.2.2.2.3 Objective 2C: Screening mutant PSVs for DOR susceptibility

2.2.2.2.3.1 *In vitro* phenotypic DOR susceptibility testing of mutant HIV-1 subtype C PSVs

To determine the DOR susceptibility of the drug mutation profiles, *in vitro* phenotypic testing was performed with the generated PSVs. The same process was used to test these mutants as was used to test the wild-type PSVs (as described in section 2.2.2.1.1.4 *In vitro* phenotypic drug susceptibility testing of wild-type HIV-1 subtype B and C PSVs (page 69). The mutants were tested in duplicate in a minimum of two independent phenotypic assays. EFV was used as a control drug since, it was the NNRTI included in the first-line therapy before 2019 (South Africa National Department of Health, 2019). The wild-type PSV's EFV susceptibility was tested in every independent assay. This allowed for the comparison of the standard deviation between the IC₅₀ of the assays.

The level of phenotypic DOR susceptibility was expressed as a “fold-change” (FC) in IC₅₀, which was the IC₅₀ of the mutant PSV under investigation, relative to the IC₅₀ of the wild-type reference PSV (i.e., original p8.9MJ4). The lower technical cut-off (TCO) for the assay was determined as the 99th percentile of the average IC₅₀ of the wild-type reference PSV, determined over multiple repeat assays. For our purposes, DOR susceptibility/resistance was classified as follows: $FC \leq TCO$ = susceptible, $TCO > FC < 2 \times TCO$ = potential low-level resistance, $2 \times TCO > FC < 3 \times TCO$ = low-level resistance, $3 \times TCO > FC < 4 \times TCO$ = intermediate resistance and $FC > 4 \times TCO$ = high-level resistance. Please note that these cut-off values were not linked to clinical outcomes and could not be used to predict a clinical outcome based on the presence of the mutations assessed in this study.

The DOR FC values and resistance classifications for each of the mutants in our assay were compared to the DOR drug resistance scores and FC values (where available) on Stanford's HIV Drug Resistance Database. The Stanford Drug Resistance Database makes predictions based on submitted sequences and the corresponding clinical outcomes or phenotypic responses. In the case where a major discrepancy was observed for a specific mutant between our data and the predicted outcome provided by the Stanford's HIV Drug Resistance Database, that mutation was introduced into the HIV-1 subtype B expression vector (i.e., p8.9NSX+) through SDM,

PSVs were generated and screened in our assay for a head-to-head comparison with the HIV-1 subtype C mutant PSV.

2.2.3 Statistical Analysis

In regards to *in vitro* phenotypic testing, the average FC and IC₅₀ values and their respective standard deviations were calculated for the replicates. The intra-subtype variations were assessed using the FC values and a parametric unpaired Student's t-test with Welch's correction which does not assume equal standard deviation was used to calculate statistical significance. The inter-subtype variations were assessed using the IC₅₀ values and a Brown-Frosythe and Welch one-way ANOVA was performed to calculate statistical significance.

3. Results

3.1 *In vitro* phenotypic DOR susceptibility of wild-type subtype B and C lab adapted strains

3.1.1 Preparation of the wild-type lab adapted strain-derived PSVs

3.1.1.1 Amplification of *reverse transcriptase* from wild-type lab adapted strains

The RNA was extracted from four HIV-1 subtype B (i.e., DS9, SM1, SM2, LTNP5) and from four HIV-1 subtype C (i.e., DU151, DU179, DU422, CM9) lab adapted strains. After reverse transcription, a 1110 base pairs (bp) fragment of their *reverse transcriptase* genes were amplified using nested PCR. The products were visualised on a 1.2% agarose. All lab adapted strains were successfully amplified and the sizes of the bands of both the 1st (1154 bp) and 2nd round (1096 bp) products were as expected (Figure 3.1 on page 82). For the second round reaction, an additional band of unspecific PCR product could be observed around 2 kilobase pairs (kb) in all reactions. The amount of product significantly increased after 2nd round PCR. There were no bands visible after cDNA synthesis, since there was less than 10 ng present.

The 2nd round PCR products were sequenced using Sanger sequencing. The chromatograms of two amplicons (i.e., DS9 and CM9) are shown in Figure 3.2 A (Figure 3.2 on pages 83-86). The sequencing primers PolSexAF, PREV-IN and Pol101R showed distinct peaks representing each nucleotide in the sequence. The PFOR-IN sequencing primer did not successfully amplify and hence was not included in the alignment. An alignment and phylogenetic analysis with the subtype reference sequences from the Los Alamos HIV Database confirmed the subtypes of the lab-adapted strains (Figure 3.2 B and C). The sequences of the DS9 and CM9 amplicons align to the *reverse transcriptase* of their respective references sequences without any polymorphisms. In the phylogenetic tree, the sequences from DS9, LTNP5, SM1 and SM2 cluster with the HIV-1 subtype B reference sequences while the sequences from

CM9, DU151, DU179 and DU422 cluster with the HIV-1 subtype C reference sequences.

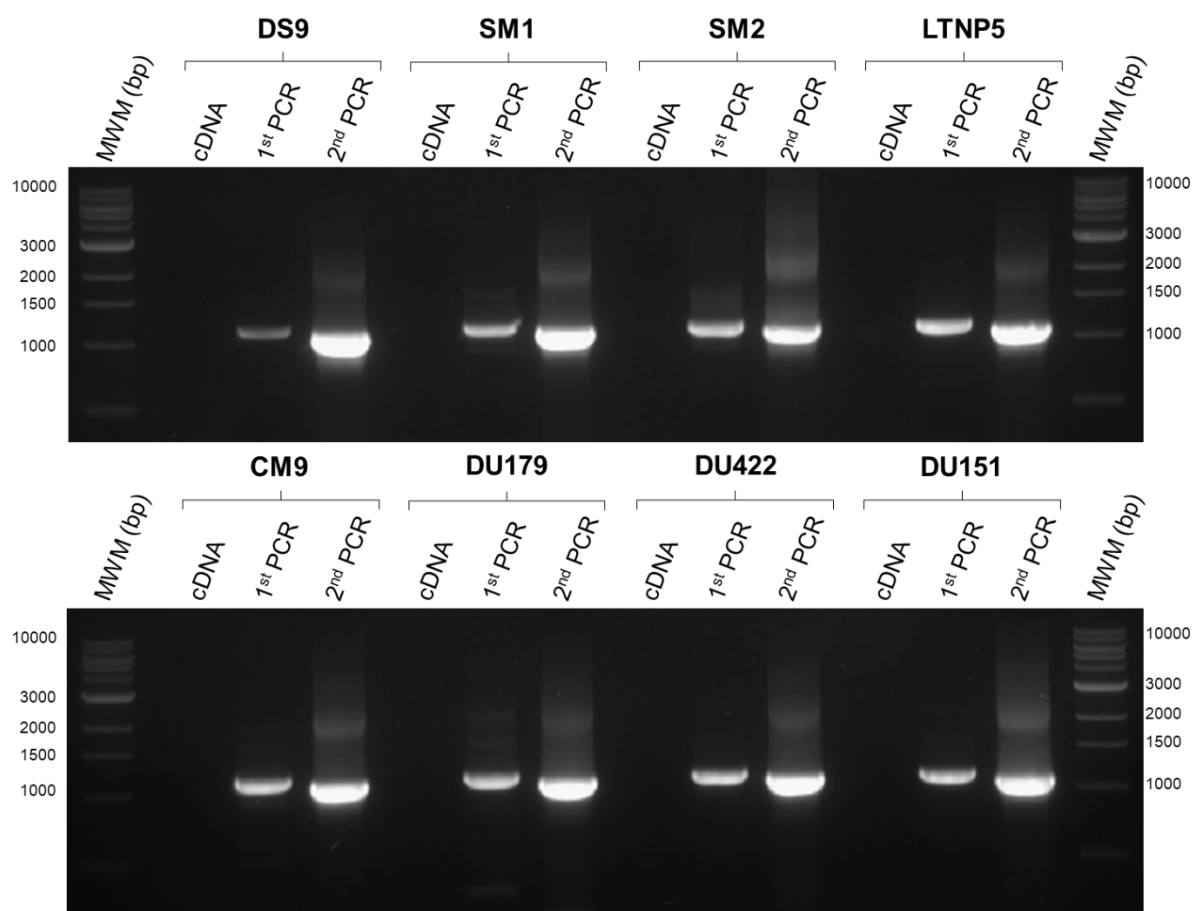
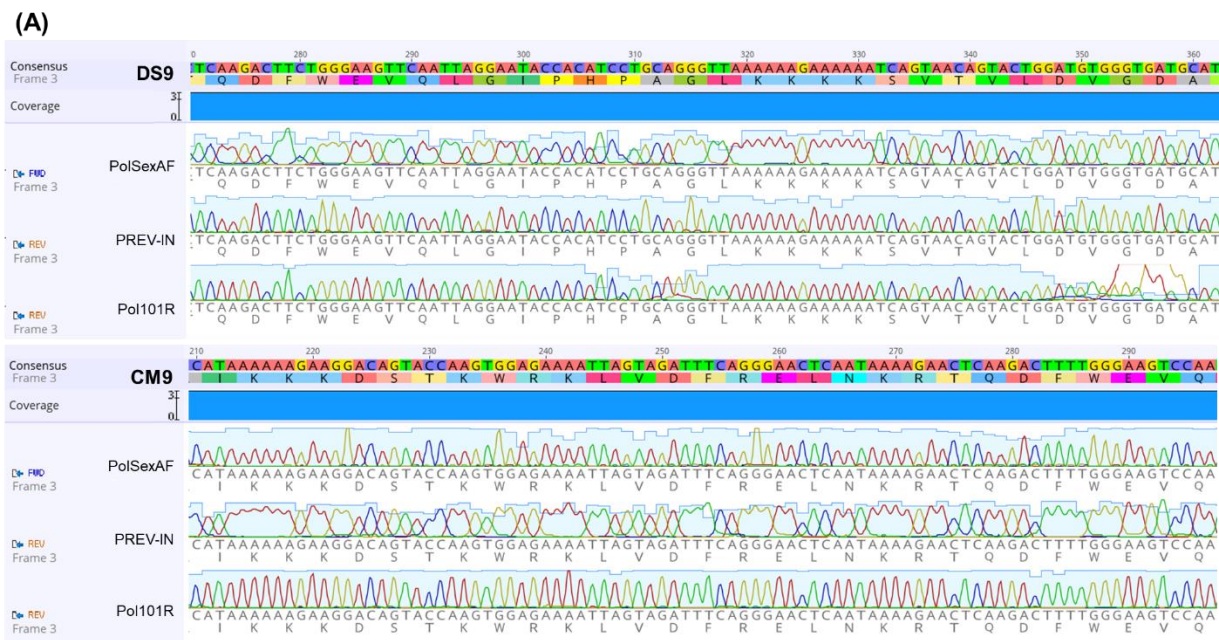


Figure 3.1: Amplification of HIV-1 lab adapted strains reverse transcriptase gene fragments. RNA was extracted from viral culture supernatants and reverse transcribed. A portion of the *reverse transcriptase* gene was amplified with nested PCR. Bands with the expect size (1.1 kb) was observed for 2nd round PCRs of all strains. All lab adapted strains were successfully amplified. MWM – molecular weight marker.

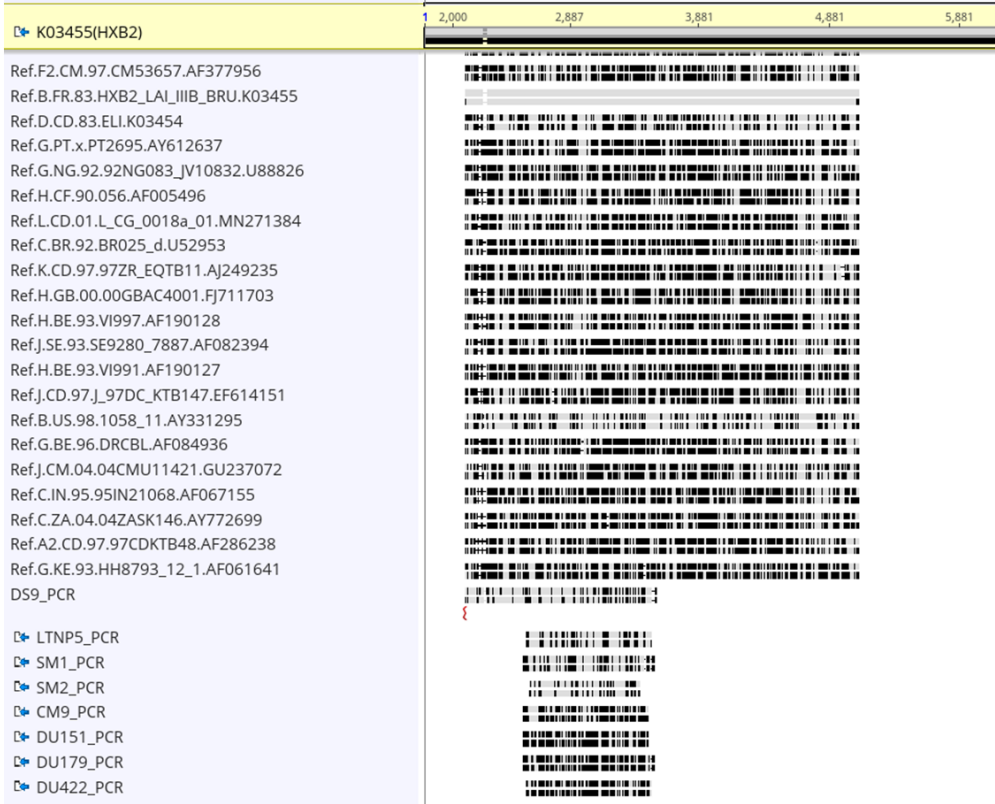
This confirms the expected HIV-1 subtypes of the lab-adapted strains. To confirm identity of the amplicons and the absence of cross-contamination during the PCR, the lab adapted strains were aligned with their published sequences (i.e., DS9 and CM9 are shown as an example in Figure 3.2 D) and with one another. A phylogenetic analysis was then performed (Figure 3.2 E). The alignment of DS9 and CM9 to their corresponding published sequences showed the correct lab adapted strains have been amplified. The phylogenetic tree showed two distinct clusters for HIV-1 subtype B and C. One cluster contained the sequences of the subtype B lab adapted strains (i.e., DS9, LTNP5, SM1 and SM2) while the other cluster contained the sequences of

subtype C lab adapted strains (i.e., CM9, DU151, DU179 and DU422). This indicates that there was no cross contamination in the nested PCR. The lab adapted strains clustered with their reference sequences indicating they are closely related to their reference sequences and thus there was no cross-contamination within the subtypes.

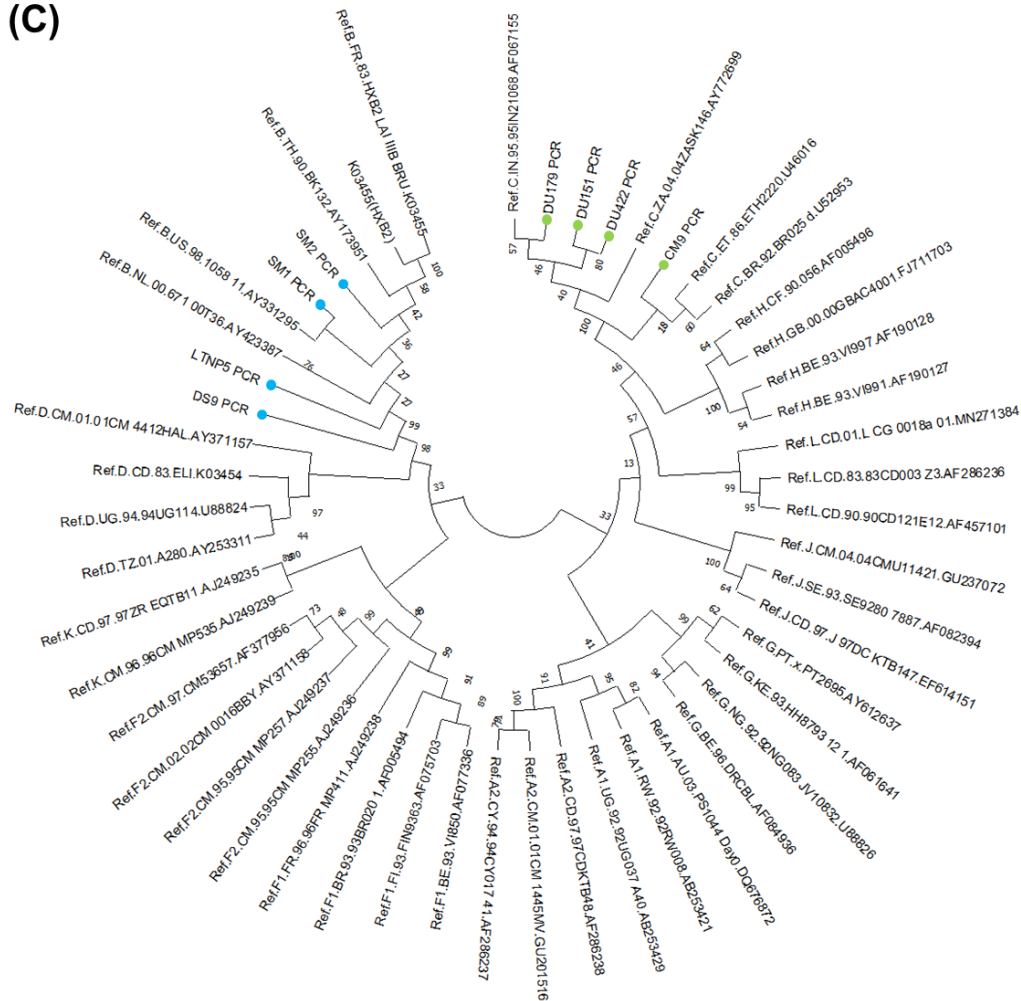


Consensus

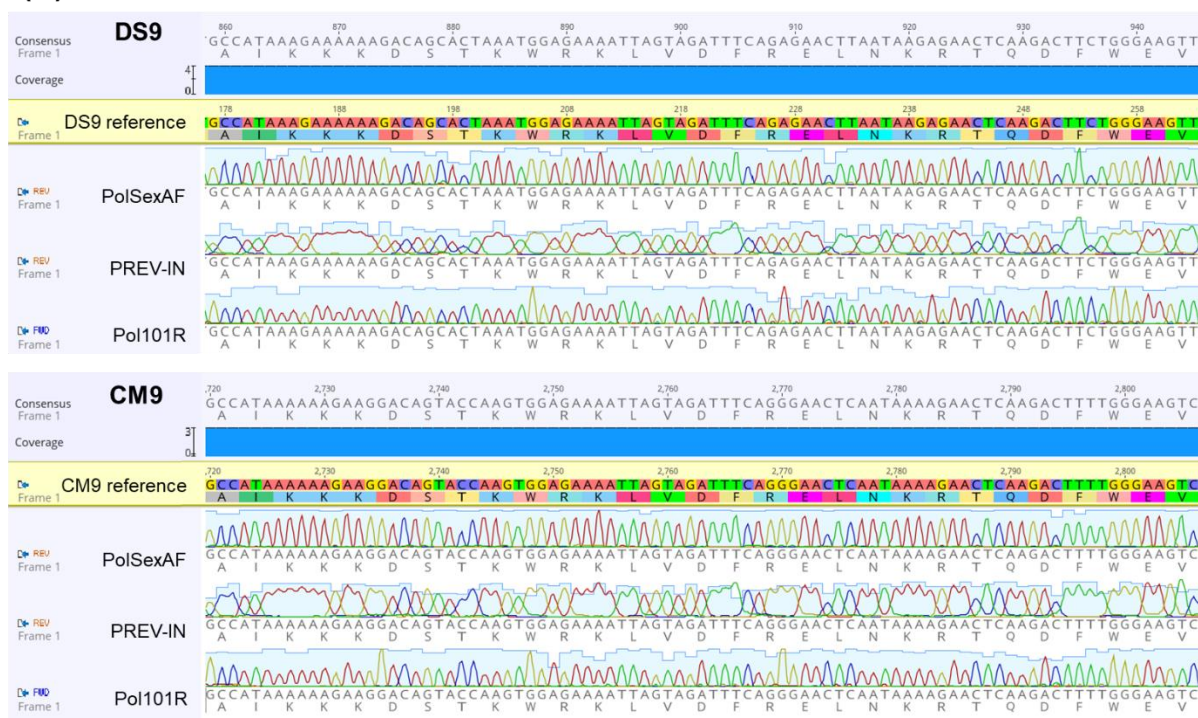
Coverage



(C)



(D)



(E)

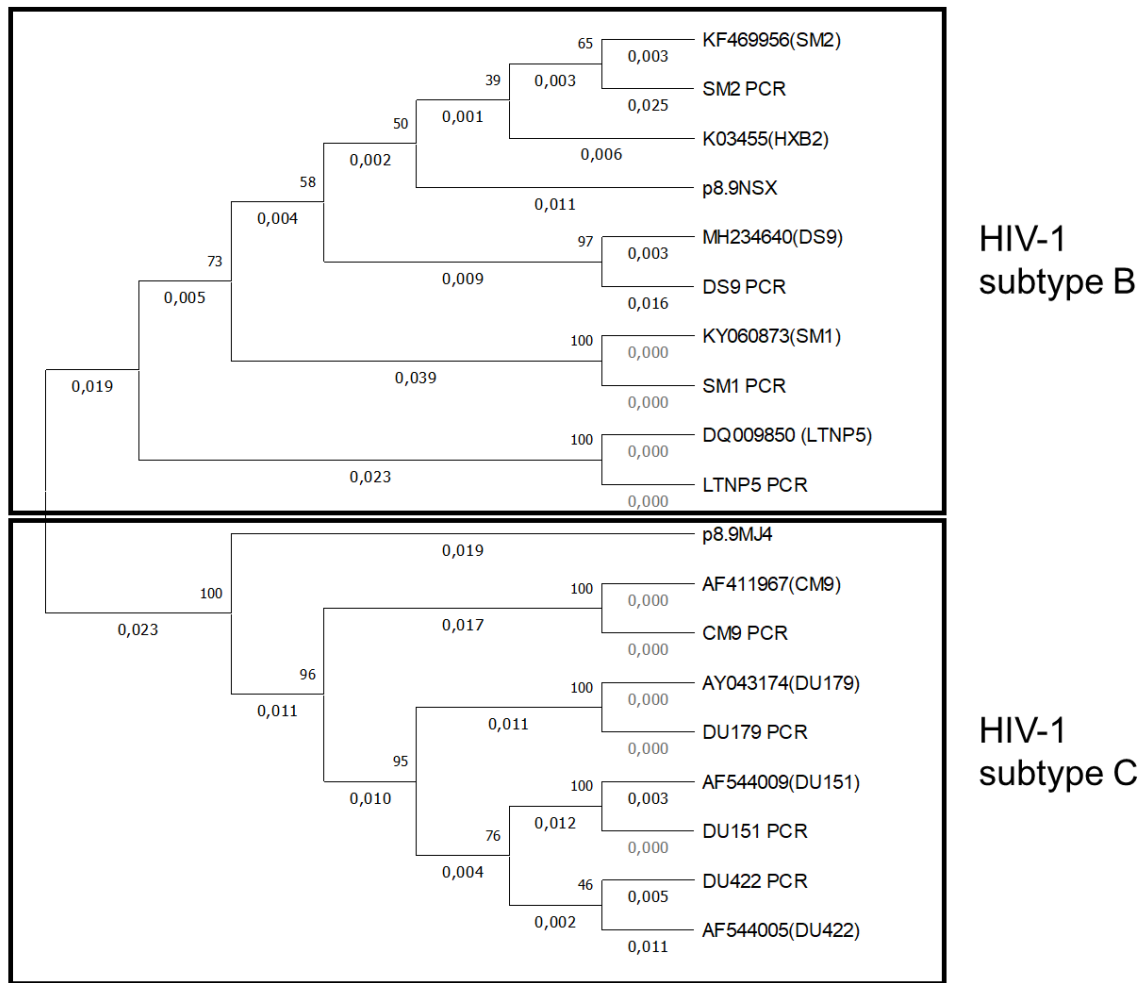
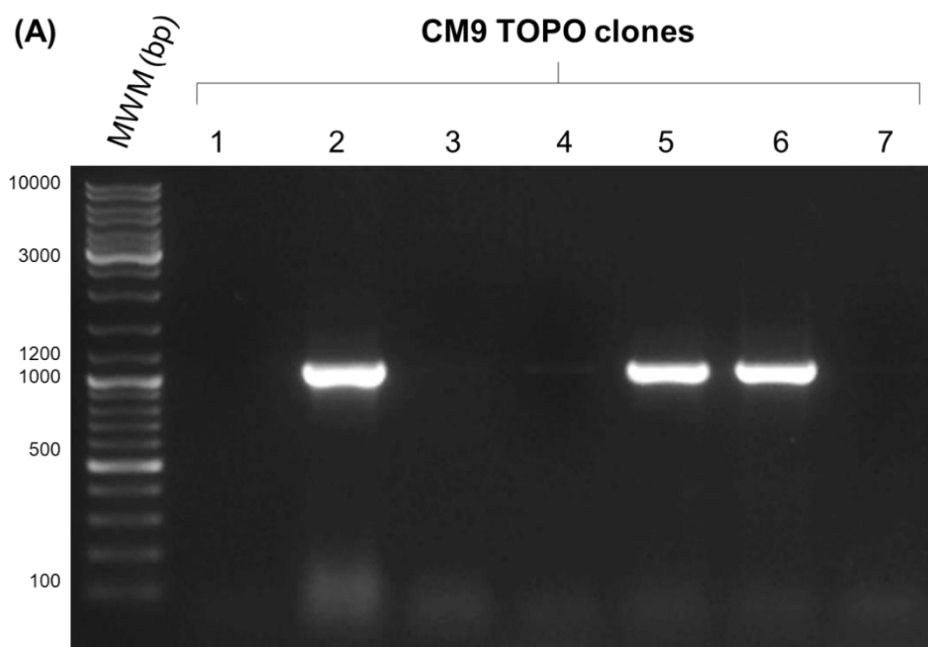


Figure 3.2: Alignments and phylogenetic trees of the amplified lab adapted strains.

(A) The sequence reads (i.e., chromatograms) of the sequencing primers were aligned to obtain the consensus sequence for each lab strain. The single peaks of chromatograms indicate the quality of the CM9 and DS9 sequences are accurate. (B) The consensus of the sequence reads was aligned with subtype reference sequences from the Los Alamos HIV Database. (C) A neighbour-joining phylogenetic tree was constructed with subtype A to J reference sequences and the subtype C (●) and subtype B (●) lab adapted strain amplicon sequences. To confirm the absence of cross-contamination during PCR, sequences were (D) aligned with the published sequences (i.e., DS9, CM9) and (E) a neighbour-joining phylogenetic tree was constructed. The lab adapted strains' sequences clustered with the expected subtype reference sequences and were closely related to their reference sequence. This shows that the correct lab strains were amplified without cross-contamination during the PCRs.

3.1.1.2 PCR screening of TOPO-TA clones

The second round PCR amplicons, containing *PvuI* and *HpaI* restrictions site on their 5' and 3' end, respectively, were cloned into a TOPO vector for sub-cloning into the modified p8.9MJ4 or p8.9NSX plasmids. Bacteria were transformed with the ligation reaction and the bacterial colonies were screened using PCR. The inner forward primer (i.e. 2560F) and inner reverse primer (i.e., 3628R) and of the second round PCR protocol were used to screen the TOPO-TA colonies for the presence of the insert. Figure 3.3 shows the screening process for CM9, as an example. Of the seven TOPO-TA bacterial colonies that were screened for CM9, three colonies (i.e., 2, 5 and 6) produced an amplicon of the expected size (i.e., 1096 bp) (Figure 3.3 A). Sanger sequencing was performed on plasmid from colonies 2, 5 and 6 for confirmation of the identity of the insert. Figure 3.3 B shows that the inserts of colonies 2 and 5 were indeed from CM9, since the *reverse transcriptase* sequence of the TOPO clones matches CM9's reference sequence. However, colony 6 had differences from the CM9 reference sequence and was excluded from downstream cloning.



(B)

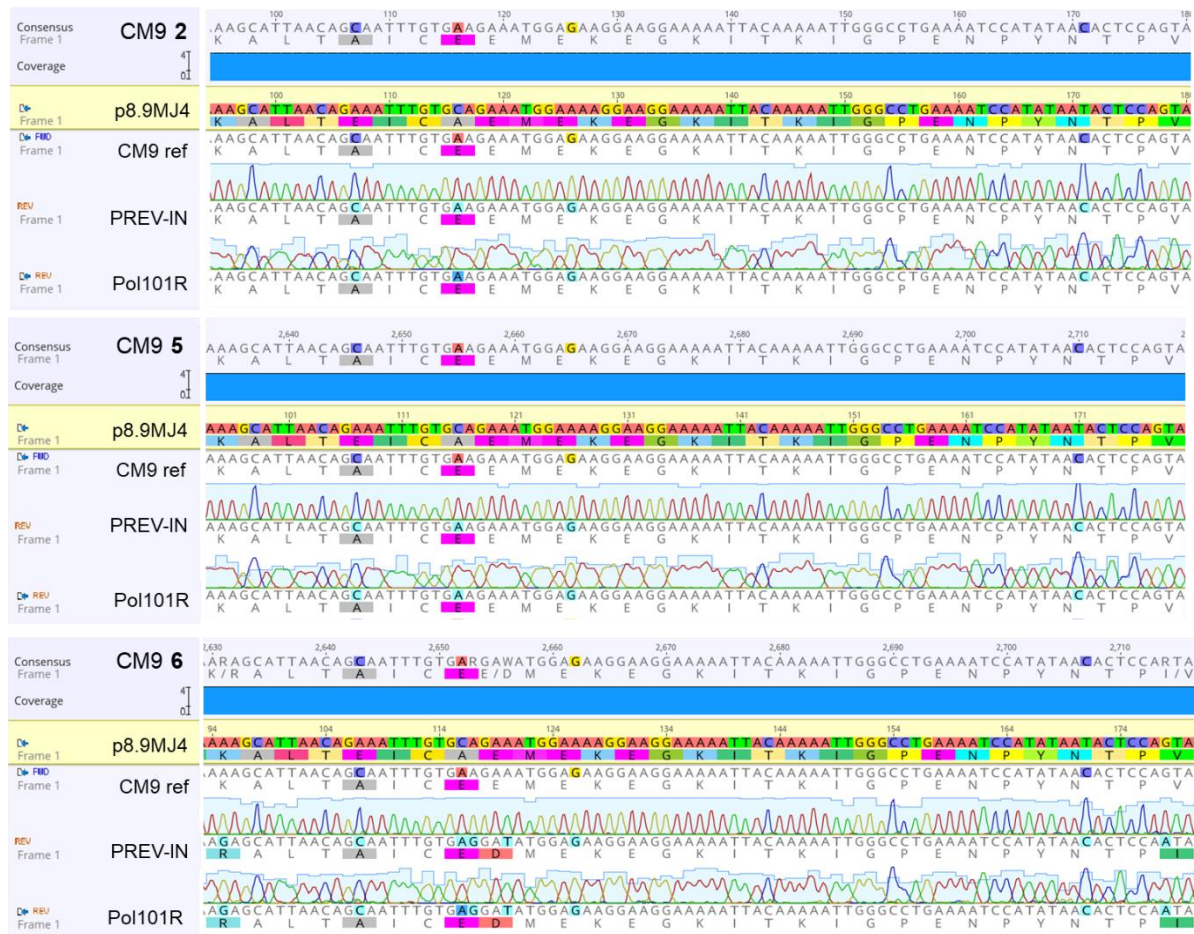


Figure 3.3: An example of colony PCR and sequencing of TOPO-TA clones.

(A) The PCR of bacterial colonies resulting from the transformation with the TOPO-TA ligation reaction of CM9 is shown here. Amplicons were analysed on a 1% agarose gel and a PCR product of 1096 bp was indicative of the presence of the insert. It can be seen on this figure that the bacteria in three of seven CM9 bacterial colonies (i.e., 2, 4 and 5) contained the insert in their TOPO-TA plasmid DNA. (B) Colonies with the correct sized amplicons were cultured, their plasmid DNA extracted, and the inserts sequenced. An alignment with the CM9 reference (ref) sequence confirm that the inserts from colonies 2 and 5 were indeed from CM9. MWM – Molecular Weight Marker.

3.1.1.3 Cloning of the TOPO-TA inserts into the modified p8.9MJ4/NSX

The inserts of the four subtype B and four subtype C lab adapted strains were excised from their respective TOPO-TA plasmids through *PvuII/HpaI* double digests and ligated into their corresponding HIV-1 expression vectors. After bacterial transformation, colony PCR screening was performed with the outer forward primer (i.e. 2528F) and inner reverse primer (i.e., 3628R) of the nested PCR protocol to confirm the presence

of an insert with the correct size. Figure 3.4 shows the screening process for CM9, as an example, to determine the success of the cloning reaction. Seven (i.e., 2, 3, 4, 6, 8, 9 and 11) of the twelve CM9 bacterial colonies that were screened produced an amplicon of the expected size (i.e., 1128 bp) (Figure 3.4 A). Colonies 2, 3 and 4 were sequenced to confirm of the identity of the insert. Figure 3.4 B shows that the inserts of colony 3 were indeed from CM9 since the sequence of the primers match CM9's reference sequence.

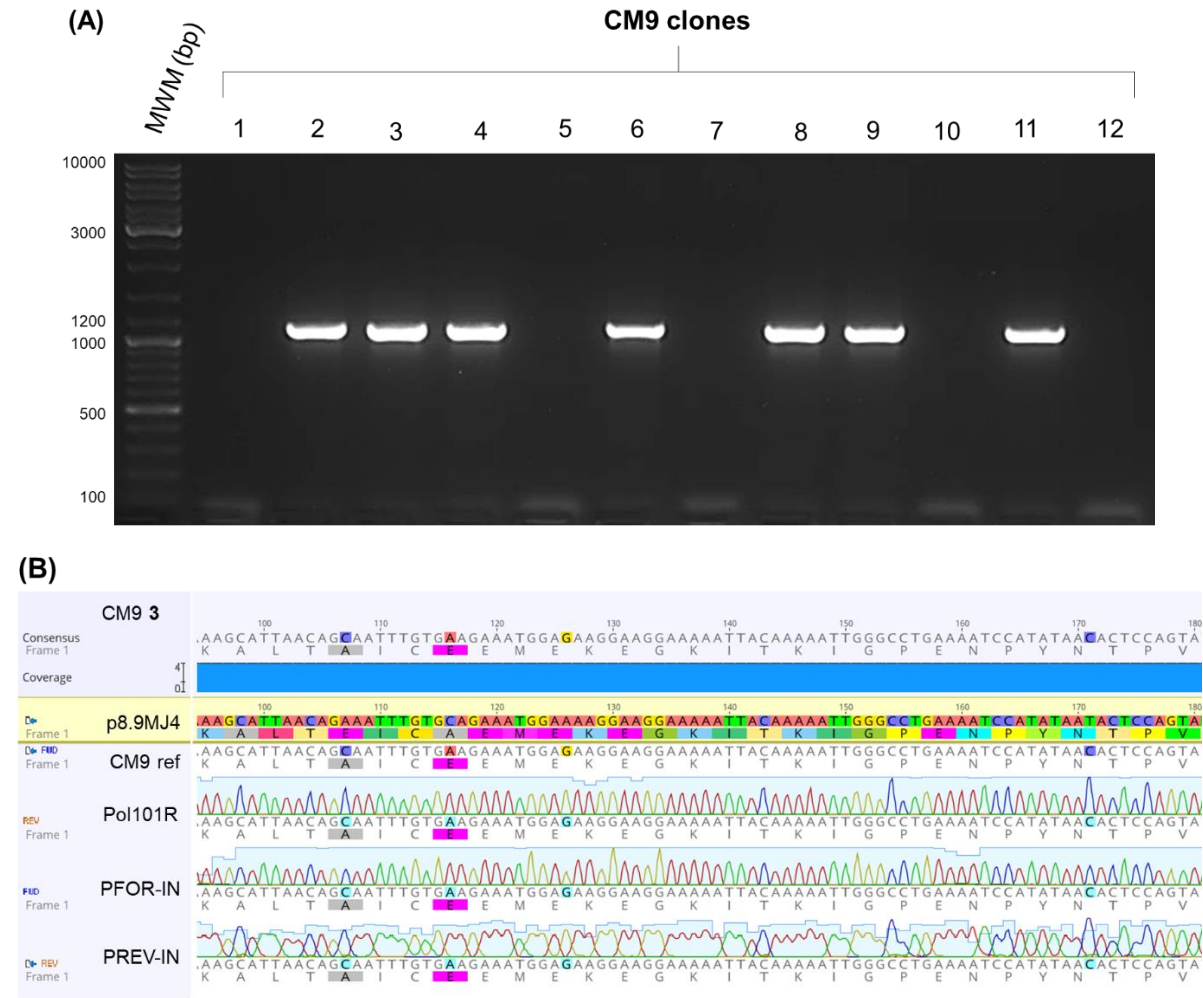


Figure 3.4: An example of colony PCR and sequencing of clones used for phenotypic testing.

(A) The PCR of bacterial colonies resulting from the transformation with the ligation reaction of the insert from TOPO and modified p8.9MJ4 for CM9. Amplicons were analysed on a 1% agarose gel and a PCR product of 1128 bp indicates the presence of the insert. It is depicted in this figure that seven of twelve CM9 bacterial colonies (i.e., 2, 3, 4, 6, 8, 9 and 11) contained an insert of the correct size in their plasmids. (B) Colonies with the correct sized amplicons were cultured, their plasmid DNA extracted, and the inserts sequenced. The alignment of colony 3 to the CM9

reference (ref) sequence is shown and confirmed the insert was from CM9. MWM – Molecular Weight Marker.

The clones that were confirmed to be from the correct lab strains were aligned and a neighbour-joining phylogenetic tree was constructed (Figure 3.5). The two distinct clusters were observed that corresponded to the respective subtypes: HIV-1 subtype B (i.e., p8.9NSX+, DS9, LTNP5, SM1 and SM2) and HIV-1 subtype C (i.e., p8.9MJ4, CM9, DU151, DU179 and DU422). All the lab adapted strains' products from every step of cloning (i.e., amplification, cloning into TOPO and subcloning into modified p8.9NSX/MJ4) indicated there was no cross contamination during the process of cloning (Figure 3.5). The DU422 TOPO clone sequence, however, clustered with the sequences from CM9 and DU179. Although this could possibly be indicative of cross-contamination with CM9 or DU179 during the cloning and subcloning, closer inspection of the sequences confirmed that they were indeed from DU422. The sequences that were used to generate the tree were very short (± 1 kb), and could have contributed to the inaccurate clustering. After confirmation of the identity of the inserts in the modified p8.9MJ4/NSX plasmids, PSVs were generated for *in vitro* DOR phenotypic susceptibility testing.

3.1.1.4 Generation and titration of HIV-1 wild-type subtype B and C PSVs

PSVs were produced by co-transfecting HEK293T cells with modified p8.9MJ4 or p8.9NSX containing *reverse transcriptase* fragments (from the lab adapted strains), pCSFLW and pHEF.VsVg. The harvested PSVs were quantified using a viral titration assay. The resultant RLU values were used to determine the dilution at which the PSVs were to be used in the *in vitro* phenotypic drug susceptibility assays, (i.e., an RLU value of 1×10^6 would be obtained in the absence of ARV drug). A linear relationship was observed between the PSV dilutions of both subtypes and their respective RLU values, indicating that with an increasing dilution factor the lower the concentration of PSV particles present (Figure 3.6). The high RLU values for the subtype B PSVs indicated that they had high concentrations of viable PSV particles (Figure 3.6 A). Similarly, the high RLU values for the subtype C suggest high concentrations of PSVs were obtained (Figure 3.6 B). Both the subtype B and C PSVs

had a plateau in RLU values at the two lowest dilution factors. This plateau is more visible in subtype C and is at a lower RLU value than subtype B. The plateau is indicative of potential cytopathic effects induced by very high viral titres or maximal infection of the number of cells in the wells. Therefore, in subtype B lab adapted strains, more PSVs were produced than subtype C. The dilution factors of the PSVs to produce RLU values in the absence of ARV drugs were as follows (Table 3.1): DS9 – 414.24 times, LTNP5 – 406.27, SM1 – 375.2, SM2 – 421.91 times, CM9 – 427.45 times, DU151 – 375.40 times, DU179 – 431.38 times and DU422 – 382.43 times.

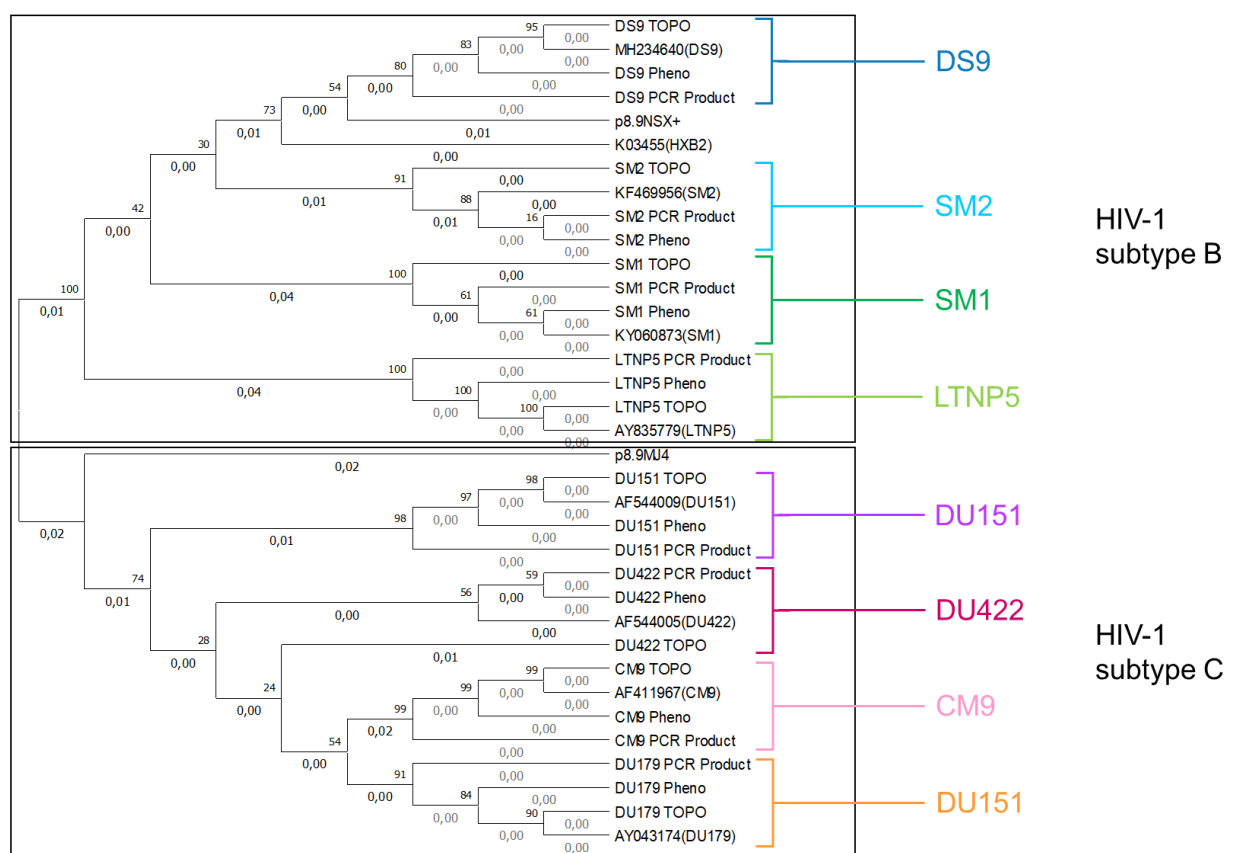


Figure 3.5: Phylogenetic tree of the lab adapted strains' amplicon and inserts.

The inserts from the TOPO-TA clones were excised from the TOPO-TA vectors with *PvuI* and *HpaI*, and ligated into p8.9MJ4 (for subtype C) or p8.9NSX+ (for subtype B). The inserts of the resulting clones were sequenced (labelled Pheno) and aligned with the sequences of the second-round PCR amplicons (labelled PCR product), the TOPO-TA inserts (labelled TOPO) and the reference sequences. A neighbour-joining phylogenetic tree was constructed, which is shown in this figure.

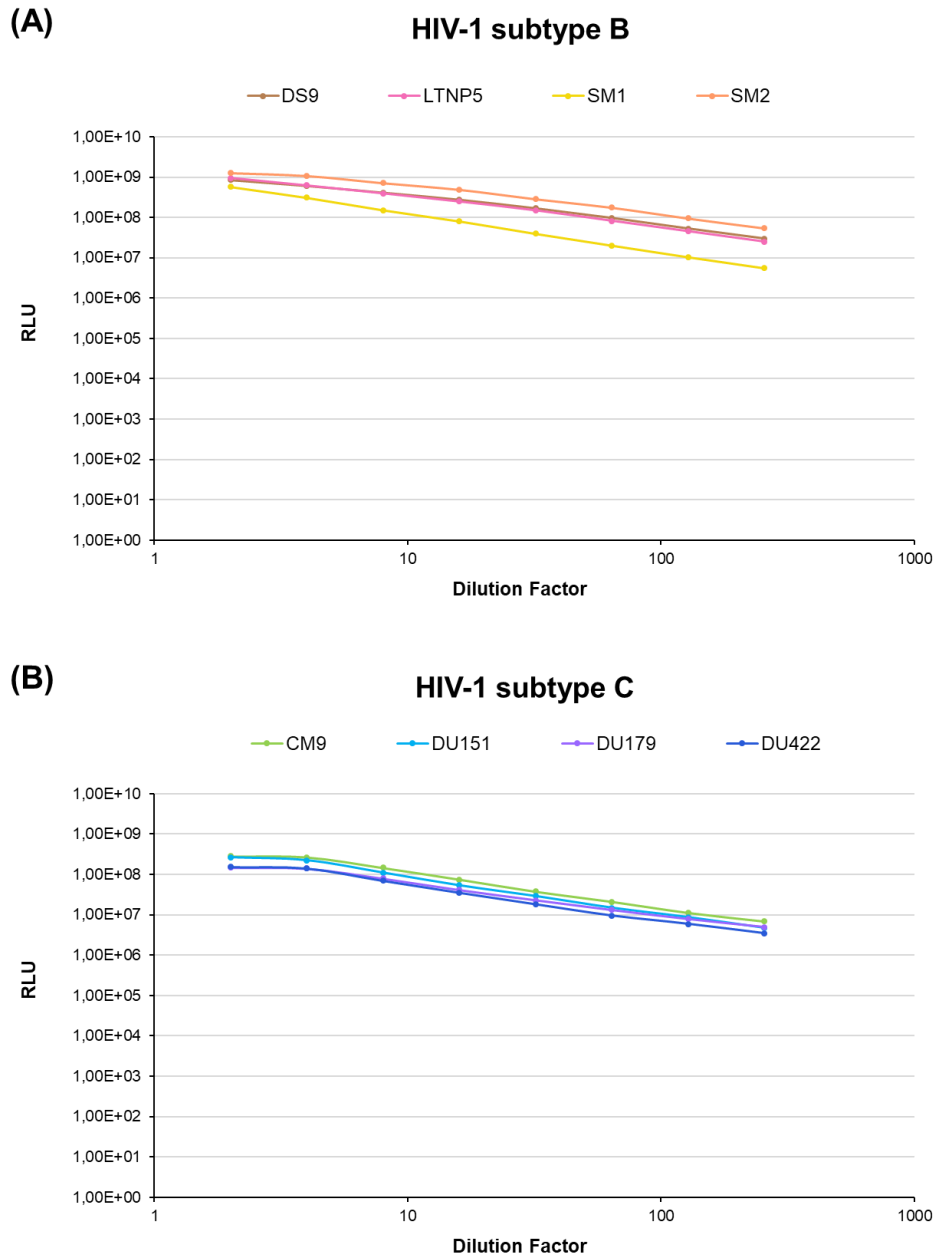


Figure 3.6: Pseudoviral titration curve for lab adapted strain-derived PSVs.

The PSV produced was quantified using a viral titration assay and the average RLU values of two replicates are shown above for (A) subtype B and (B) subtype C PSVs. The amount of PSV added to the phenotypic assay was standardised to 1×10^6 RLU.

Table 3.1: Calculated dilutions of PSVs used for *in vitro* phenotypic testing

HIV-1 Subtype	PSV	Dilution Factor	Amount added to the <i>in vitro</i> phenotypic assay (µl)*
B	DS9	414.24	6.76
	LTNP5	406.27	6.89
	SM1	375.26	7.46
	SM2	421.91	6.64
C	CM9	427.45	6.55
	DU151	375.40	7.46
	DU179	431.38	6.49
	DU422	382.43	7.32

*the dilution factor was halved to compensate for the 2-fold dilution of the PSVs after the addition to the drug in the *in vitro* phenotypic assay. PSV - pseudovirus

3.1.2 Phenotypic DOR susceptibility of wild-type HIV-1 subtype B and C lab adapted strains

3.1.2.1 Toxicity of ARV drugs to HEK293T cells

The toxicity of DOR, EFV (the control ARV drug) and DMSO solvent to the HEK293T cells was examined before *in vitro* phenotypic testing. This was done to ensure that viral inhibition seen in the phenotypic assay was due to inhibitory activity of the ARV drugs, and not from toxicity of the drugs to the HEK293T cells. EFV appeared to be the most toxic ARV drug, with a steep decline in cell viability at >10 µM (Figure 3.7). DOR appears to be the least toxic to the HEK293T cells, since cell viability decreases only at >33.3 µM (Figure 3.7). The DMSO solvent contributes to cell toxicity but only at concentrations >0.33% (>33.3 µM drug equivalent). The CC₅₀ values and CC₂₀ values for DOR and EFV were calculated to be as follows: DOR (CC₅₀ >100 µM, CC₂₀ >100 µM), EFV (CC₅₀ = 42.51 µM ± 14.30 µM, CC₂₀ = 21.66 µM ± 8.53 µM) and DMSO (CC₅₀ >1.00%, CC₂₀ = 0.89% (Table 3.2). The CC₂₀ concentrations were initially used in phenotypic assays to determine the optimal concentration ranges of DOR and EFV to use in the assay.

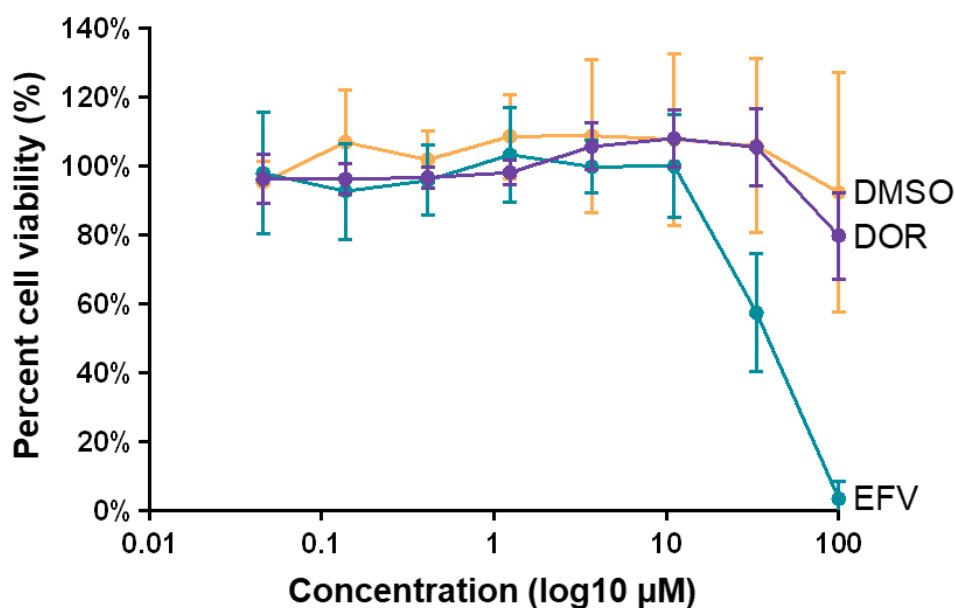


Figure 3.7: Toxicity of ARV drugs to HEK293T cells.

Cell viability in the presence DOR, EFV and the DMSO solvent was tested to ensure that viral inhibition seen in the phenotypic assay was due to antiviral activity and not from cytotoxicity. The error bar indicates the standard deviation observed between the four repeats of toxicity screening.

Table 3.2: CC₅₀ and CC₂₀ values from toxicity assays

Drug	Average CC ₅₀ (n = 3)	CC ₅₀ Standard Deviation	Average CC ₂₀ (n = 3)	CC ₂₀ Standard Deviation
DOR	>100.00 μM	-	>100.00 μM	-
EFV	42.51 μM	14.30 μM	21.66 μM	8.53 μM
DMSO	>1.00%	-	0.89%	0.44%

CC₂₀ – 20% cytotoxic concentration (cell viability reduced to 80%), CC₅₀ – 50% cytotoxic concentration (cell viability reduced to 50%)

3.1.2.2 The optimal ARV drug concentration for phenotypic testing

Initial phenotypic assays were performed to determine the optimal concentrations to test for each antiviral drug. The range of concentrations were adjusted to cover the entirety of the sigmoidal curve including, 100% viral activity, the IC₅₀ value, and 100% viral inhibition. The CC₂₀ of each drug were initially used as the highest testing concentration, but the concentrations were adjusted over several assays until they provided a sigmoidal dose-response curve (Figure 3.8.). 3TC was the least potent of

the ARVs with the highest IC₅₀ (Table 3.3). ISL was the most potent drug with the lowest IC₅₀, followed by EFV and DOR. The optimal final starting concentrations for phenotypic testing were as follows: DOR = 0.5 µM, ISL = 0.1 µM, 3TC = 100 µM, EFV = 0.1 µM (Table 3.3). These concentrations were used in downstream testing.

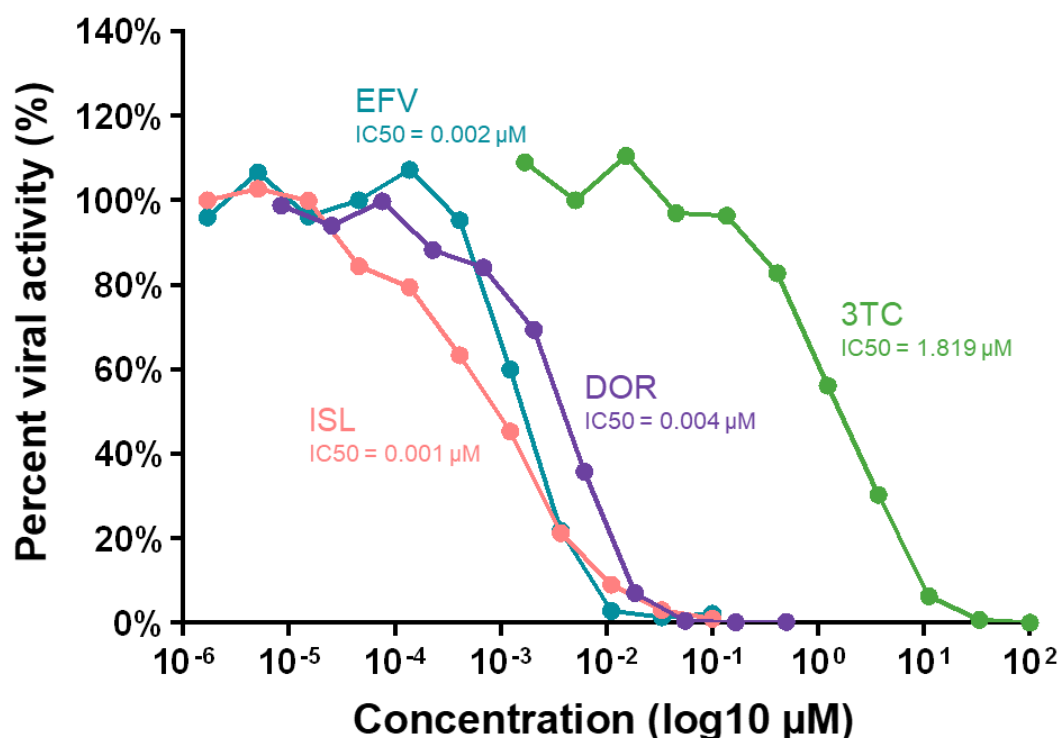


Figure 3.8: Optimal DOR, ISL, 3TC and EFV concentrations.

Phenotypic assays were performed with MJ4 WT PSVs to determine the optimal concentrations to perform phenotypic testing. The optimal concentrations used for phenotypic testing were: DOR = 8.5×10^{-6} µM to 0.5 µM, ISL = 1.7×10^{-6} µM to 0.1 µM, 3TC = 1.7×10^{-3} µM to 100 µM, EFV = 1.7×10^{-6} µM to 0.1 µM.

Table 3.3: Average IC₅₀ values from the activity assays

Drug	Average IC ₅₀ (µM)	Final Starting Concentration for testing (µM)
DOR	0.004	0.50
EFV	0.002	0.10
ISL	0.001	0.10
3TC	1.819	100

IC₅₀ – 50% inhibitory concentration

3.1.2.3 Phenotypic testing of the lab adapted strains susceptibility to DOR

Phenotypic assays were performed to determine the potency of DOR on PSVs from subtype B and C lab adapted strains. DOR susceptibility of subtype B PSVs were compared to the susceptibility of the NSX PSV (Figure 3.9, Table 3.4). The FC values were DS9 = 0.81 ± 0.18 , LTNP5 = 1.49 ± 0.13 , SM1 = 1.54 ± 0.28 , SM2 = 1.46 ± 0.28 and NSX = 1 ± 0.32 (Figure 3.9). LTNP5, SM1 and SM2 had higher FC values than NSX, but only LTNP5 showed a statistically significant difference ($p = 0.025$). The FC of DS9 was lower than the other subtype B PSVs, but this was not statistically significant. The susceptibility of the subtype C PSVs were compared to the MJ4 PSV. The FC values were CM9 - 0.85 ± 0.05 , DU151 - 0.68 ± 0.08 , DU179 - 0.91 ± 0.11 , DU422 - 0.87 ± 0.17 and MJ4 - 1 ± 0.18 (Figure 3.9, Table 3.4). All PSV were more susceptible to DOR than MJ4, however, only DU179 had a significantly lower FC to MJ4.

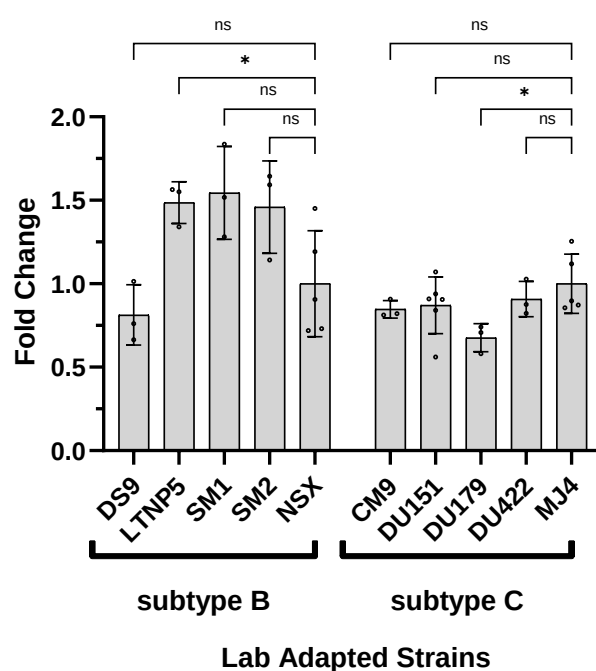


Figure 3.9: Phenotypic susceptibility of subtype B and C lab adapted strains to DOR

In vitro phenotypic assays were performed to assess the susceptibility of the lab adapted strains to assess the potency of DOR. Susceptibility was expressed as FC which was defined as the IC_{50} of the lab adapted strain divided by the subtype reference (MJ4/NSX). The IC_{50} and FC values for this graph are in Table 3.4.

Table 3.4: IC₅₀ and FC values of lab adapted strains

Lab adapted strain	Average IC ₅₀ (n = 3, standard deviation) (μM)		Average Fold Change (n = 3, standard deviation)		p-value*
DS9	0.0026	(0.0006)	0.81	(0.18)	0.330
LTNP5	0.0048	(0.0004)	1.49	(0.13)	0.025
SM1	0.0050	(0.0009)	1.54	(0.28)	0.053
SM2	0.0047	(0.0009)	1.46	(0.28)	0.085
NSX	0.0033	(0.0010)	1.00	(0.32)	-
CM9	0.0046	(0.0003)	0.85	(0.05)	0.132
DU151	0.0047	(0.0009)	0.68	(0.08)	0.256
DU179	0.0037	(0.0005)	0.91	(0.11)	0.014
DU422	0.0049	(0.0006)	0.87	(0.17)	0.397
MJ4	0.0055	(0.0022)	1.00	(0.18)	-

* an unpaired Student's t-test test compared the FC of each lab adapted strain to the subtype wild-type reference and the significant p-values are bolded. IC₅₀ – 50% inhibitory concentration

The subtype B PSVs showed higher FC values to DOR than the subtype C PSVs (Figure 3.9, Table 3.4). This could indicate that DOR has a slightly higher potency in HIV-1 subtype C. However, since potency was expressed relative to the IC₅₀ of the reference (i.e., either NSX or MJ4), there is a possibility that the reference IC₅₀ value could have biased the potencies. When comparing the IC₅₀ value of NSX (IC₅₀ = 0.0033 ± 0.0010 μM) to that of MJ4 (IC₅₀ = 0.0055 ± 0.0022 μM), it is clear that the lower IC₅₀ value of NSX (compared to all other PSVs) have biased the FC of the subtype B PSVs to lower DOR potencies. A one-way ANOVA performed on the IC₅₀ values between the PSV indicated no significant difference in potency between the subtypes, except for DS9 and MJ4 (p = 0.032) (Table 3.5). DOR, therefore, showed similar potencies in subtypes B and C lab-adapted PSVs.

Table 3.5: P-values comparing potency of subtype B and C lab adapted strains

		subtype B				
		DS9	LTNP5	SM1	SM2	NSX
subtype C	CM9	0.103	0.994	0.992	>0.999	0.335
	DU151	0.076	>0.999	>0.999	>0.999	0.382
	DU179	0.483	0.248	0.542	0.711	0.995
	DU422	0.079	>0.999	>0.999	>0.999	0.257
	MJ4	0.032	0.937	0.999	0.974	0.123

* a one-way ANOVA compared the mean IC₅₀ of each lab adapted strain to each other and p-values <0.05 were significant (■)

3.2 Phenotypic testing of the synergy between DOR and ISL

3.2.1. Toxicity of DOR & ISL to HEK293T cells

The toxicity of DOR and ISL in combination had to be assessed first before synergistic phenotypic testing could be performed, so that cell toxicity was not mistaken for observed viral inhibition. A combination of DOR and ISL at six concentrations were applied to HEK293T cells. Since the combination of EFV with 3TC or EFV with TDF were used as a comparator in the synergy assay, their combined toxicities were also examined.

The various combinations of DOR & ISL resulted in a cell viability of 80%-120% (Figure 3.10 A). The three highest concentrations of DOR only (i.e., 0.002, 0.033, 0.500 µM) reduced cell viability, while the ISL only concentrations didn't reduce cell viability much. All the combinations of EFV & 3TC have a similar cell viability ranging from 80%-100%, except for 3TC at 0.4 µM and 6.7 µM which had cell viabilities of 101% and 103%, respectively (Figure 3.10 B). DOR & 3TC similarly had a consistent cell viability of 100%-120% across all combinations, apart from 6.7 µM 3TC with 0.500 µM

and 0.033 μM DOR which have viabilities of 123% and 128%, respectively. The combination of 6.7 μM 3TC with various concentrations of DOR increases cell viability.

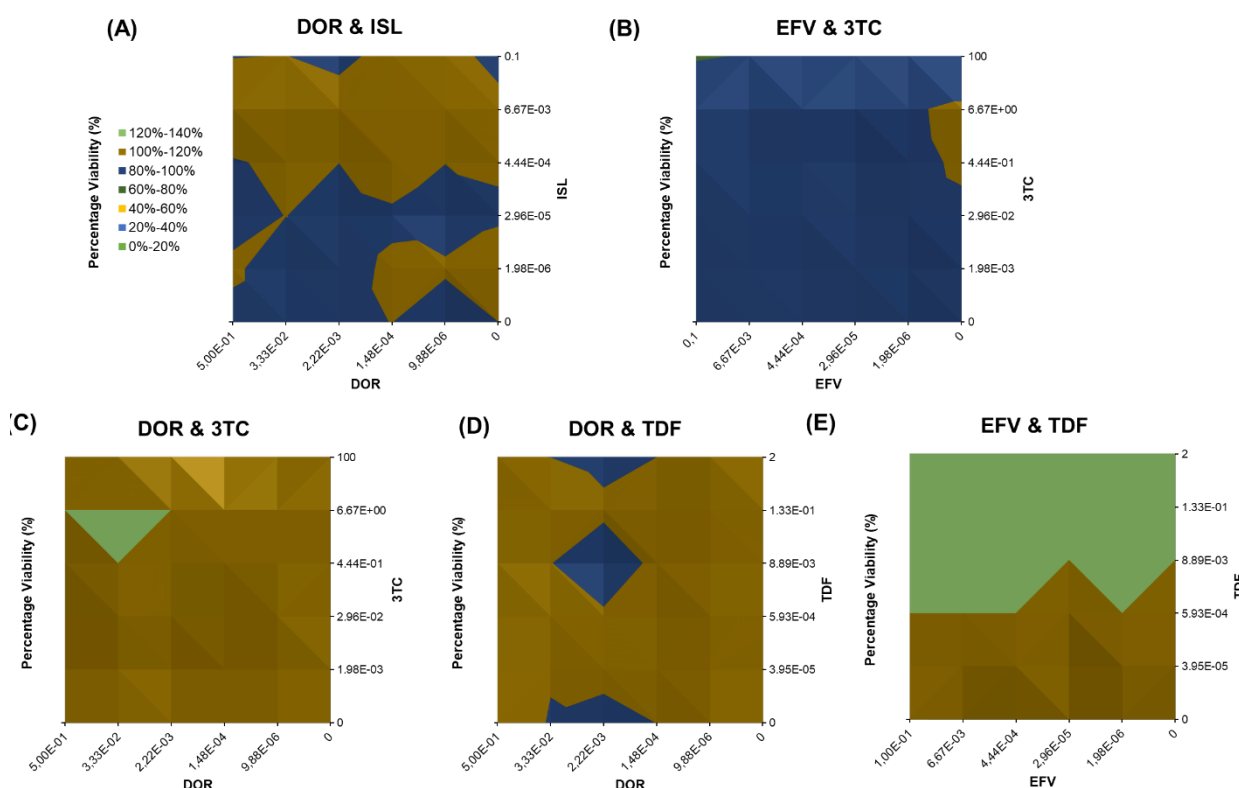


Figure 3.10: DOR & ISL are not toxic to HEK293T cells in combination.

A six by six grid of drug combinations that were tested for toxicity to HEK293T cells. The response of the cells to DOR & ISL (A), EFV & 3TC (B), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E) were measured. These graphs show the average of the three independent toxicity assay preformed in duplicate. The areas shown in green (■) indicate cell viability >100%, (■) indicate cell viability 100%-120%, while the areas shown in brown (■) indicate cell viability <100%. None of the five combinations were toxic (<80% cell viability) to HEK293T cells over all concentrations.

DOR & TDF displays a similar viability of 100%-120% at all combinations, however the cell viability does decrease to 80%-100% at the following combinations; 2 μM and 0.009 μM TDF with 0.002 μM DOR and 0.002 μM DOR alone. The combination of 0.2 μM DOR with various concentrations of TDF and alone increases cell viability to 120%-140%. EFV & TDF shows a clear divide in viability over the various combinations. The three lowest concentrations (0 μM , 0.00004 μM , 0.0006 μM) of TDF display a viability of 100%-120% across all EFV combinations, while the three highest concentrations (2 μM , 0.1 μM , 0.009 μM) of TDF display a viability of 120-140%. High concentrations of

TDF increase cell viability alone and when combined with EFV. This is likely due to TDF being less cytotoxic at higher concentrations in HEK293T cells. This is supported by the observation that TDF is less cytotoxic in human cell types than other NTRIs (Cihlar *et al.*, 2002). EFV & 3TC displays higher toxicity over the combination of concentrations than the other ARV combinations (Figure 3.10). The cell viabilities of all combinations were generally above 80% and therefore suitable to use for synergistic phenotypic testing.

3.2.2. Testing the synergistic effect of DOR and ISL on wild-type HIV-1

The combination of DOR & ISL were tested to determine if any combination showed synergism towards inhibiting wildtype HIV-1 subtype C. DOR and EFV were also tested in combination with two other NRTIs (3TC, TDF), for comparison. Before submitting the activity data to SynergyFinder, it was first inspected by plotting curves in Microsoft Excel.

All drugs, individually, showed the expected sigmoidal curves at their highest concentrations (Figure 3.11, left side and back of each graph). Near complete viral inhibition was observed at the highest drug concentration combinations for DOR & ISL (A), DOR & TDF (D) and EFV & TDF (E). Inhibition of >80% was observed for EFV & 3TC (B) and DOR & 3TC (C) at these combined concentrations. Three-dimensional sigmoidal curves were observed for all combination, indicating increased viral inhibition at higher combined concentrations and decreased viral inhibition at lower combined concentrations. The graphs of DOR & ISL (A) has a smaller volume than the graphs of EFV & 3TC (B), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E), indicating that the combination of DOR & ISL is more potent than the other combinations. The viral activity “peaks” (100%-120%) observed for ISL & DOR (A), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E) are likely due to variation in the assay. No unexpected “dips” in viral activity, indicative of synergistic inhibition, was observed for any of the combinations.

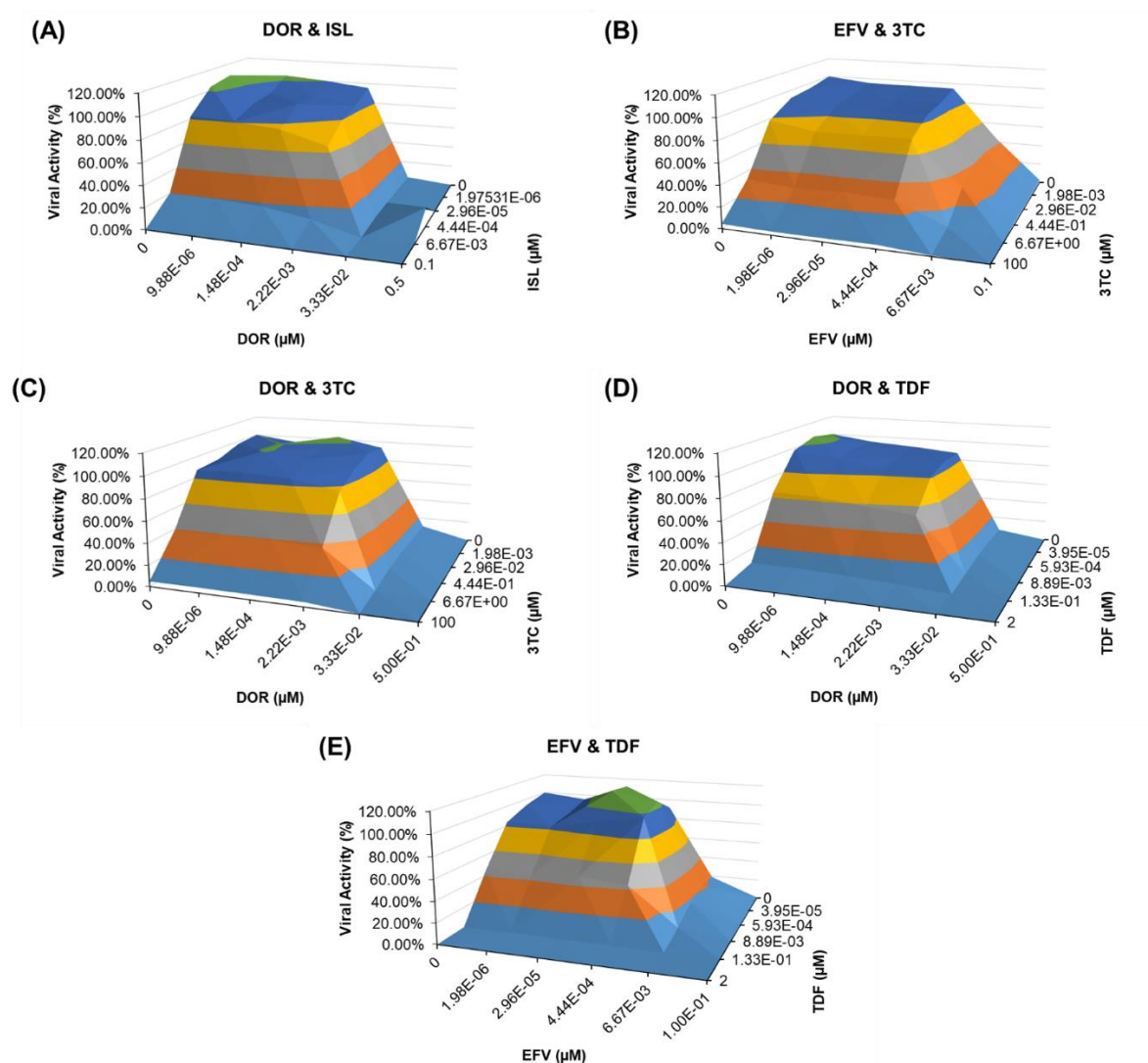


Figure 3.11: Viral activity response to DOR & ISL and EFV & 3TC.

The optimal concentrations previously established were used to test the phenotypic susceptibility of HIV-1 subtype C wild-type PSV to the combinations of DOR & ISL (A), EFV & 3TC (B), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E). Three repeats were performed in duplicate with the average viral activity displayed in the figure. The colours correspond to the viral activity percentage as follows: green – 100%-120%, dark blue – 80%-100%, yellow – 60%-80%, grey – 40%-60%, orange – 20%-40% and light blue 0%-20%.

In SynergyFinder, four mathematical models (ZIP, Loewe, Bliss, HSA) were used to calculate a synergy score for the ARVs. Figure 3.12 depicts the average synergy scores for DOR & ISL (A), EFV & 3TC (B), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E) over the six concentration combinations (Figure 3.12). The synergy scores for the three repeats are displayed in Table 3.6. A positive synergy score is indicative of synergy while a negative score is indicative of antagonism.

Table 3.6: Synergy scores for the five ARV combinations

ARV combination	Experiment Number	ZIP model*	p-value**	HSA model*	p-value**	Loewe model*	p-value**	Bliss model*	p-value**
DOR & ISL	1	5.50	0.015	6.20	0.001	5.04	0.008	5.88	0.004
	2	4.07	0.008	4.18	0.003	3.84	0.003	5.75	0.002
	3	2.43	0.002	0.79	0.379	-0.79	0.235	5.25	0.001
	Average***	2.12	0.067	3.63	0.018	2.54	0.049	4.82	0.011
EFV & 3TC	1	0.57	0.069	3.84	0.0001	2.86	0.0002	1.74	0.009
	2	0.13	0.815	4.58	0.0003	4.62	0.001	-0.16	0.780
	3	3.26	0.0002	0.50	0.512	0.93	0.113	3.84	0.0002
	Average***	1.45	0.0001	5.32	0.00002	4.65	0.000002	1.88	0.000001
DOR & 3TC	1	1.32	0.088	2.10	0.126	2.15	0.009	0.53	0.715
	2	-1.01	0.106	-4.76	0.017	-5.44	0.002	-4.53	0.008
	3	-0.14	0.675	-0.53	0.443	-0.82	0.180	-0.86	0.117
	Average***	-0.27	0.394	-0.02	0.977	-0.30	0.508	-1.51	0.095
DOR & TDF	1	5.18	0.022	6.20	0.001	5.72	0.002	5.88	0.004
	2	-0.95	0.013	-1.11	0.159	-1.71	0.016	-2.42	0.007
	3	-0.14	0.709	-0.11	0.908	-0.55	0.564	-0.60	0.362
	Average***	0.27	0.328	1.93	0.003	1.32	0.017	1.09	0.012
TDF & EFV	1	-0.09	0.943	-6.62	0.075	-8.31	0.047	-3.37	0.313
	2	-0.64	0.099	-1.00	0.198	-1.43	0.012	-1.89	0.024
	3	-0.51	0.433	1.72	0.169	1.15	0.287	-0.93	0.264
	Average***	-0.46	0.430	-1.30	0.330	-1.56	0.270	-2.00	0.113

* this score represents the average synergy across the entire matrix in Figure 3.12

** the significant (p-value < 0.05) synergy scores (■)

*** the average viral activity of the three repeats was submitted to SynergyFinder to obtain this score

DOR – Doravirine, ISL – Islatravir, EFV – Efavirenz, 3TC – Lamivudine

Comparing the overall synergy of the five combinations, DOR & ISL (A), EFV & 3TC (B) and DOR & TDF (D) display significant synergism over at least three models. DOR & 3TC (C) and EFV & TDF (E) display antagonism, although this was not significant (Figure 3.12). In general, the observed synergy or antagonism for each combination was observed within the intermediate concentration combinations. DOR & ISL displays synergy across the highest range of concentrations (Figure 3.12), additionally it had the highest statistically significant synergy scores across the repeat tests (Table 3.6). DOR & TDF showed the least synergism (Figure 3.12, Table 3.6). EFV & TDF

displayed the highest amount of antagonism across the 25 combination of concentrations, and DOR & 3TC exhibited the lowest amount of antagonism (Figure 3.12, Table 3.6).

The combination of DOR & ISL (Figure 3.12 A) showed synergism across the three lowest concentrations of DOR (2.22×10^{-3} μM , 1.48×10^{-4} μM and 9.88×10^{-6} μM) in combination with the three lowest concentrations of ISL (4.44×10^{-4} μM , 2.96×10^{-5} μM and 1.98×10^{-6} μM). The combination of 2.22×10^{-3} μM DOR with various combination of ISL, particularly 2.96×10^{-5} μM showed the highest level of synergy. However, the concentration 5.00×10^{-1} μM of DOR in combination with various concentrations of ISL results in antagonism, particularly with 4.44×10^{-4} μM ISL (Figure 3.12 A). DOR & ISL had the highest number of significant synergy scores across the repeats and showed the most agreement between the four models (Table 3.6). Therefore, among the ARV combinations, DOR & ISL displayed the highest degree of synergy (Figure 3.12 A, Table 3.6).

EFV & 3TC in combination displayed synergy across all the combinations of concentrations (Figure 3.12 B). The combination of 6.67×10^{-3} μM EFV with 4.44×10^{-1} μM and 6.67×10^0 μM 3TC showed a higher level of synergism than the other combinations, with the latter displaying the highest synergy. EFV & 3TC had the second highest number of significant synergy scores across the repeats (Table 3.6). The combination displayed the strongest significant average synergy scores, however there was less agreement between the models across the repeats than DOR & ISL.

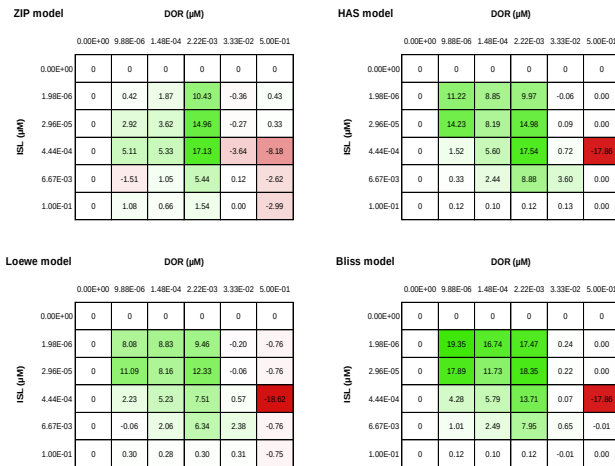
DOR & 3TC displayed a mixture of synergism and antagonism across the concentration combinations with majority showing some level of antagonism (Figure 3.12 C). The combination of 3.33×10^{-2} μM DOR with 6.67×10^0 μM 3TC and 2.22×10^{-3} μM DOR with 4.44×10^{-1} μM 3TC both show the highest level of synergy. The combination of 9.88×10^{-6} μM DOR with 2.96×10^{-2} μM 3TC show the highest level of antagonism. However, all of these notable concentration combinations synergy scores vary amongst the four mathematical models. DOR & 3TC showed a mixed response in its significant synergy score values (Table 3.6). Repeat 1 showed two positive significant synergy scores and repeat 2 showed two negative significant synergy

scores (Table 3.6). However, none of the average synergy scores which are depicted in Figure 3.12 C were significant.

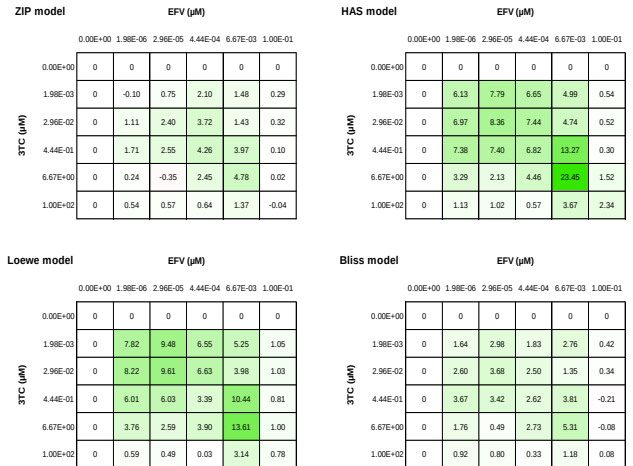
DOR & TDF also shows a mixture of synergy and antagonism across the concentration combinations, although synergy is dominant (Figure 3.12 D). Notable combinations include $2.22 \times 10^{-3} \mu\text{M}$ DOR with $8.89 \times 10^{-3} \mu\text{M}$ TDF and $1.48 \times 10^{-4} \mu\text{M}$ DOR with $5.93 \times 10^{-4} \mu\text{M}$ TDF, but they are only present in two of the four models. There was a mixed response observed in DOR & TDF's significant synergy scores, but the average scores were significantly positive indicating synergy although they were not as high as DOR & ISL and EFV & 3TC (Table 3.6). Additionally, these scores were not in as much agreement across the four models as the other two synergistic combinations.

EFV & TDF displayed a stronger mixed response in the synergy and antagonism across the concentration combinations Figure 3.12 E). However, antagonism was predominantly displayed across the grid. The combination $6.67 \times 10^{-3} \mu\text{M}$ EFV with $8.89 \times 10^{-3} \mu\text{M}$ TDF displayed a high level of synergy, but this was not consistent across the models. The combinations of $4.44 \times 10^{-4} \mu\text{M}$ EFV with $3.95 \times 10^{-5} \mu\text{M}$ TDF, $4.44 \times 10^{-4} \mu\text{M}$ EFV with $5.93 \times 10^{-4} \mu\text{M}$ TDF and $2.96 \times 10^{-5} \mu\text{M}$ EFV with $3.95 \times 10^{-5} \mu\text{M}$ TDF showed the highest level of antagonism. The average synergy scores were negative indicating antagonism, but they were not significant (Table 3.6). EFV & TDF displayed a higher level of antagonism than DOR & 3TC.

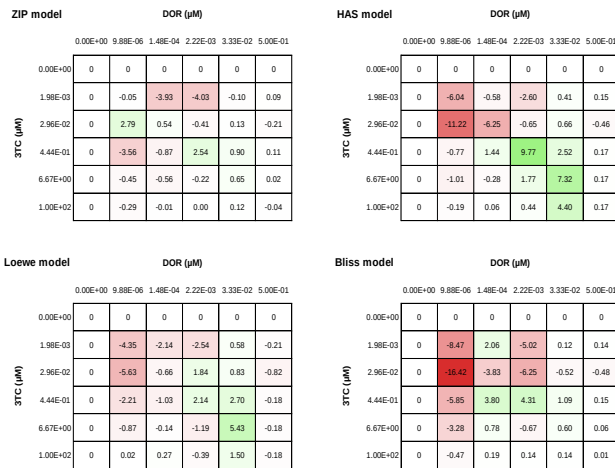
(A) DOR and ISL



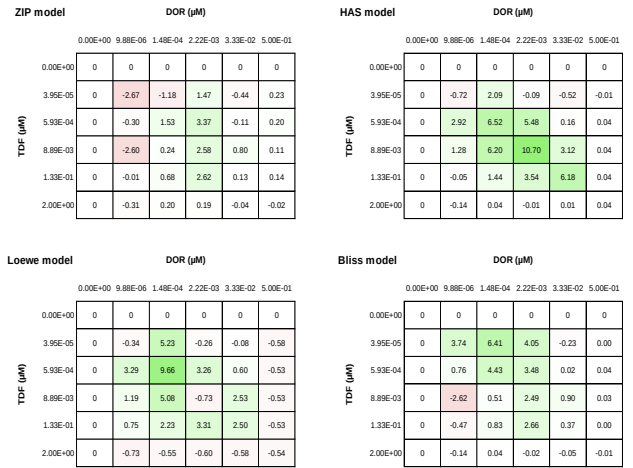
(B) EFV and 3TC



(C) DOR and 3TC



(D) DOR and TDF



(E) EFV and TDF

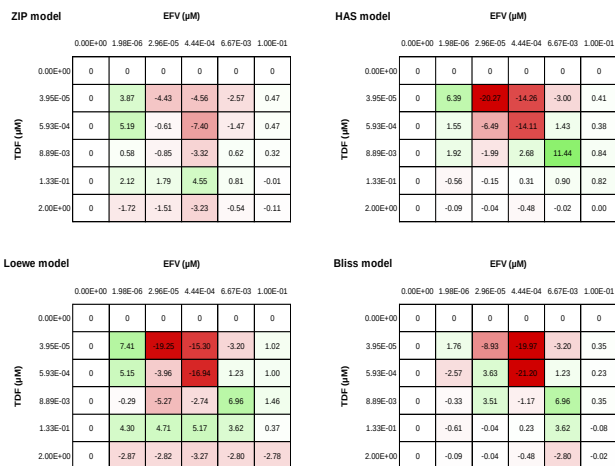


Figure 3.12: 3D synergy plot for DOR & ISL (A), EFV & 3TC (B), DOR & 3TC (C), DOR & TDF (D) and EFV & TDF (E)

Synergy was assessed with an *in vitro* phenotypic synergy assay and SynergyFinder was used to generate the 3D synergy plots using the average RLU values from three repeats. These plots

represent the average over three repeats. Four major synergy models were used to calculate the synergy score; ZIP, Loewe, Bliss, and HAS. Each model quantifies synergy under different conditions. The synergy score represents the excess response over the predicted response which was observed due to drug interactions. A positive synergy score indicates synergy (■) and a negative synergy score indicates antagonism (■) with the maximum being 20 and -20 respectively. DOR with ISL (B) displayed the highest level of synergy compared the other ARV combinations.

3.3 *In vitro* phenotypic DOR susceptibility of mutant HIV-1

3.3.1 Identification of the most prevalent NNRTI mutations in South Africa

The CMJAH database included 7 749 patient samples of people failing ARV treatment from 2016 to 2020, in line with the resistance testing guidelines, the most common ARV regimen observed in the database was PI-based for 70.89% of patients (Table 3.7). The remaining patients were on an unknown (14.87%), NNRTI-based (12.67%), INSTI-based (0.84%) or other (0.74%) regimen (Table 3.7). The NNRTI commonly used in the NNRTI-based regimens was EFV. Among the 7 749 patients included in the database, NNRTI drug resistance was observed in 5 928 (76.5%).

Table 3.7: ARV regimens of the patients.

ARV based regimen	Frequency in CMJAH database (% (n))	
PI	70.89%	(5493)
Unknown*	14.87%	(1152)
NNRTI	12.67%	(982)
INSTI	0.84%	(65)
Other**	0.74%	(57)

*no ARV drugs were recorded on the drug resistance test request form

**some ARV drugs in the regimen were not recorded

PI – protease inhibitors, NNRTI – non-nucleoside reverse transcriptase inhibitors, INSTI – integrase strand transfer inhibitors

A total of 20 most prevalent NNRTI drug resistance combination profiles as well as 15 single mutations were identified from the CMJAH database (Table 3.8). These NNRTI mutations were introduced in p8.9MJ4 for *in vitro* phenotypic DOR susceptibility testing. The most prevalent single NNRTI mutation was notably K103N (14.59%) and K103N+P225H (6.46%) was the most prevalent combination mutation (Table 3.8).

Y181C is known to cause high-level resistance to EFV (Melikian *et al.*, 2014), RPV (Rimsky *et al.*, 2012) and ETR (Tambuyzer *et al.*, 2010), additionally the mutation causes high-level resistance to EFV, ETR, NVP and RPV when it is combined with other NNRTIs mutations (Basson *et al.*, 2015). This along with the high prevalence of Y181C in South African patients failing first-line NNRTI-based ART, thus making this an important mutation to include in the study (Basson *et al.*, 2020, 2015; Steegen *et al.*, 2017). The K101P and F227L mutations were only observed in combination with other NNRTI mutations and were not observed in isolation. The K103N and V106M mutations were present in the majority of the combinations.

Table 3.8: The prevalence of single and combination NNRTI mutations in patients with NNRTI DRMs.

Single NNRTI DRMs (n = 15) (in isolation)	Prevalence in CMJAH database (% (n))	
K103N	14.59%	(865)
E138A	2.82%	(167)
Y188L	1.54%	(91)
A98G	1.43%	(85)
G190A	1.21%	(72)
V179D	1.01%	(60)
Y181C	0.91%	(54)
P225H	0.74%	(44)
K103S	0.39%	(23)
V106M	0.37%	(22)
K101E	0.34%	(20)
V108I	0.12%	(7)
L100I	0.02%	(1)
K101P*	0.00%	(0)
F227L*	0.00%	(0)
NNRTI DRM combination profiles (n = 20)	Prevalence in CMJAH database (% (n))	
K103N,P225H	6.46%	(383)
A98G,K103N,P225H	2.61%	(155)
L100I,K103N	2.41%	(143)
K103N,V106M	2.02%	(120)
V106M,V179D	1.59%	(94)
K103N,E138A	1.30%	(77)
K103N,V108I	1.11%	(66)
A98G,K103N	1.03%	(61)
V106M,G190A,F227L	1.01%	(60)
V106M,V179D,F227L	1.01%	(60)
K103S,V106M	0.93%	(55)
K103N,Y188L	0.89%	(53)
V106M,G190A	0.83%	(49)
V179D,Y188L	0.64%	(38)
L100I,K103N,P225H	0.61%	(36)
V106M,Y188L	0.61%	(36)
K103N,V106M,F227L	0.57%	(34)
K101P,K103N	0.57%	(34)
K101E,V106M,G190A	0.56%	(33)
K103N,V108I,P225H	0.51%	(30)

*these mutations were only present in combination with other NNRTI mutations

NNRTI – non-nucleoside reverse transcriptase inhibitors, DRM – drug resistance mutation

3.3.2 Generation of the NNRTI mutants *via* SDM

The combination and single mutants were generated in p8.9MJ4 using SDM. The primers used in SDM reactions as well as the optimised annealing temperature for a successful PCR reaction are listed in Table 3.9 on page 110. All of the SDMs were performed using non-overlapping primers.

Table 3.9: SDM primers used to generate the single and combination NNRTI profiles.

DRM Profile	Primer Name*	Mutation	Sequence (5' → 3')**	optimised T _m (°C)
A98G, K103N, P225H	A98G, K103N forward	A98G	AGGGTTAAAAAAGAATAAATCAGTAACAGTATTAG	63
A98G, K103N	A98G, K103N reverse		CCTGGGTGCGGTATTCCTAATTGAAC	63
L100I, K103N	L100I, K103N forward	L100I	AAAAAAGAATAAATCAGTAACAGTATTAGATGTG	56.9
L100I, K103N, P225H	L100I, K103N reverse		ATCCCTGCTGGGTGCGGTATTCCTAA	55.1
K101P, K103N	K101P, K103N forward	K101P	AAAGAATAAATCAGTAACAGTATTAGATGTGGGG	61.3
	K101P, K103N reverse		GGTAACCCTGCTGGGTGCGGTATTCC	
K103N, V106M, F227L	K103N, V106M forward	K103N	TAAATCAATGACAGTATTAGATGTGGGGGATG	63
K103N, V106M	K103N, V106M reverse	V106M	TTCTTTTTTAACCCTGCTGGGTGCGG	62
K101E, V106M, G190A	V106M forward	V106M	GACAGTATTAGATGTGGGGGATGCA	64.4
	K101E, V106M reverse		ATTGATTTTTTCTTCTCTAACCCTGC	
K103S, V106M	K103S, V106M reverse		ATTGATTTGCTCTTTTTTAACCCTGC	
K103N, V108I	K103N, V108I forward	V108I	ATTAGATGTGGGGGATGCATATTTTTTC	63.2
K103N, V108I, P225H	K103N, V108I reverse		ATTGTTACTGATTTATTCTTTTTTAACCCTG	63.2
K103N, E138A	E138A forward	E138A	AATAGTCATCTATCAATATATGGATGATTTGTATG	59.6
	E138A reverse		GCTGGATTTTGTGTCCTAAAGGGCTC	
K103N, Y188L	Y188L forward	Y188L	AGTAGGATCTGATTTAGAAATAGGGC	54
V106M, Y188L	Y188L reverse		AACAAATCATCCATATATTGATAGATGACTATTTC	55
V179D, Y188L	V179D, Y188L reverse		AACAAATCATCCATATATTGATAGATGTCTATTTC	57
V106M, G190A	G190A forward	G190A	ATCTGATTTAGAAATAGGGCAACATAG	54
V106M, G190A, F227L	G190A reverse		GCTACATACAAATCATCCATATATTGATAGATG	54
V106M, V179D, F227L	F227L forward	F227L	ACTTTGGATGGGGTATGAACTCCATC	62.7
	F227L reverse		AATGGGGGTTCTTTCTGATGTTTCTTGTC	

* Some sets of primers were occasionally used to introduce more than one nucleotide change

** The nucleic bases that were changed to introduce mutations (■)

DRM – drug resistance mutation, T_m – melting temperature

The SDM reactions were visualised on a 1.2% TAE agarose gel to determine whether there was successful amplification before proceeding. An example is shown in Figure 3.13, wherein two combination mutations (A98G+K103N and A98G+K103N+P225H) were successfully generated. For both, a prominent band at 10 kb could be observed indicating the entire plasmid was amplified successfully (Figure 3.13). For most PCR reactions, a single amplicon was observed but for some reactions (such as A98G+K103N in Figure 3.13) additional amplicons were observed. Smaller amplicons would not interfere with the KLD reaction but would still be present after the reaction. After transformation bacteria with the fragment would not grow on the antibiotic containing plate, since the fragment likely does not contain the antibiotic resistance gene since neither of the primers bind to this gene and the length of the fragment is too short. Additionally, there was significantly more product of the correct size produced, thus it will be likely more bacterial cells are transformed with this product.

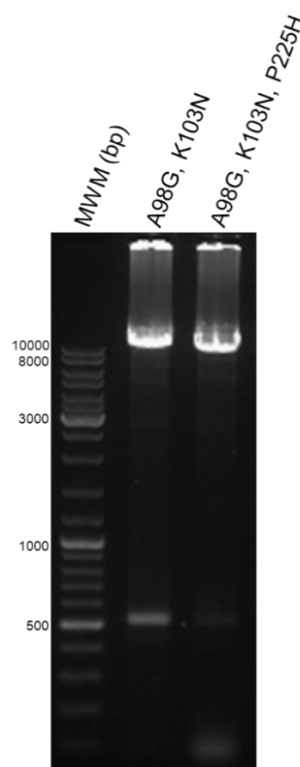
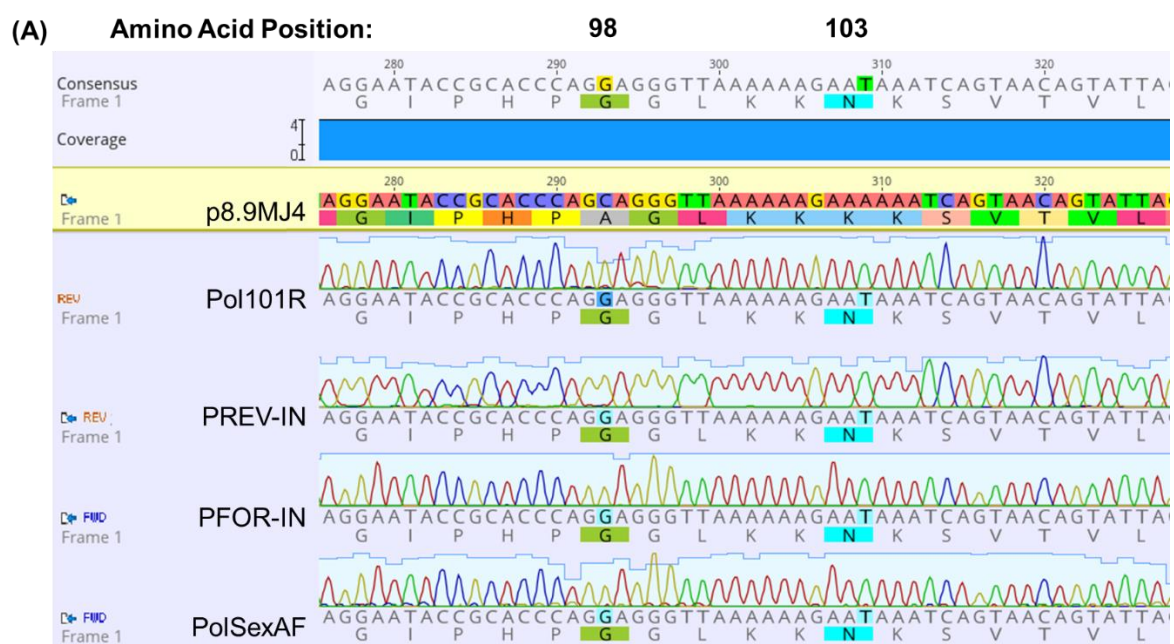


Figure 3.13: Successful amplification of the A98G+K103N and A98G+K103N+P225H SDMs. The SDM products were visualised on a 1% TAE agarose gel using electrophoresis. The presence of a single ± 10 kb band indicated successful amplification. The two combination mutation profiles depicted here were successfully generated using SDM. A98G+K103N also shows an unspecific product of 520 bp.

The SDM products were sequenced and submitted to the Stanford Drug Resistance Database to confirm the presence of the mutations. The four sequence reads were aligned to each other to form a consensus sequence and then the consensus was aligned to p8.9MJ4 as a reference. An example of an alignment (Figure 3.14 A) and a report from the Stanford Drug Resistance Database (Figure 3.14 B) for A98G+K103N is shown below. The chromatograms show distinct peaks at the mutation sites indicating the correct nucleotide change were made and the desired amino acid mutations were generated (Figure 3.14 A). Stanford's report confirms that A98G and K103N was generated in a subtype C plasmid and that the quality of the sequence was good (Figure 3.14 B). Additionally, Stanford's report showed the ARV drugs the mutation would be susceptible to and generated a drug resistance score indicating the level of resistance to the ARV drugs predicted by the algorithm.



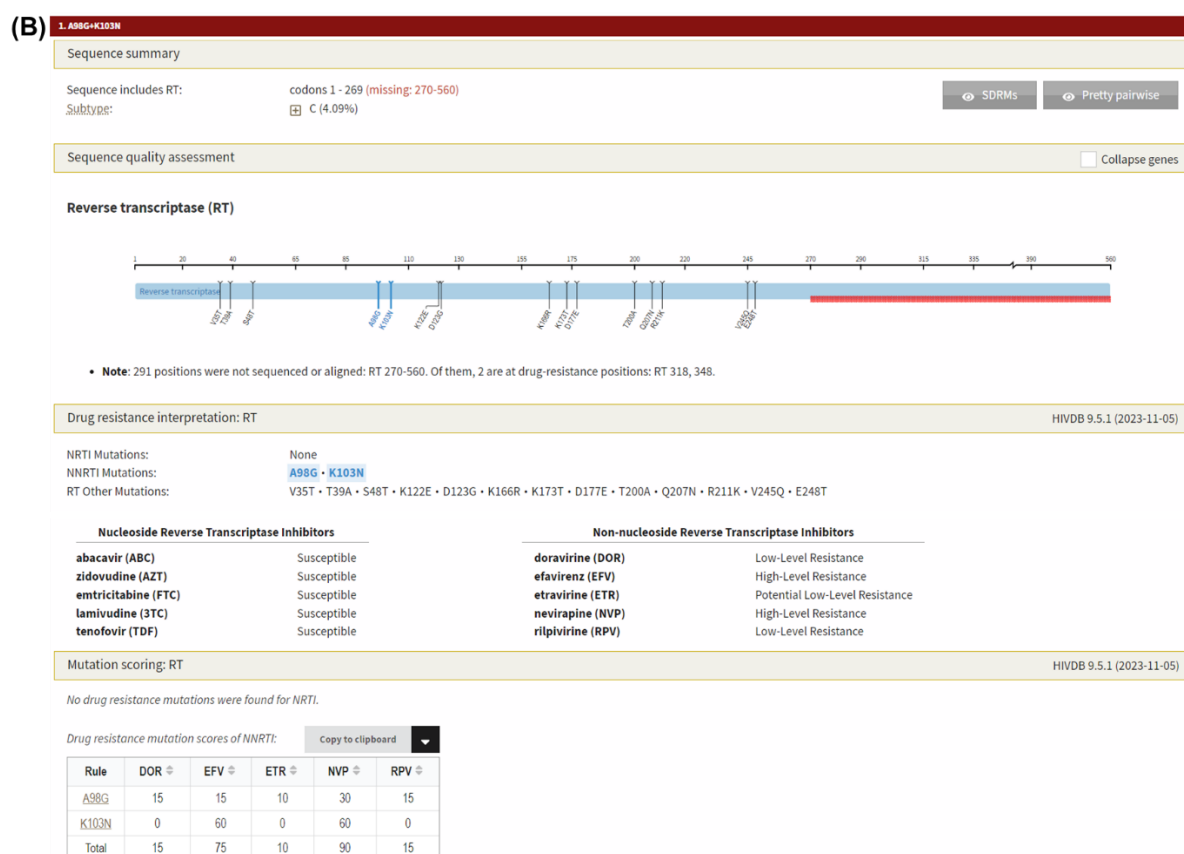


Figure 3.14: Successful generation of DRM profile A98G+K103N.

(A) The sequence reads (i.e., chromatograms) were aligned to the wild-type reference sequence (i.e., p8.9MJ4). The mutations are shown in colour as they are different from the reference sequence. (B) This sequence was submitted to the Stanford Drug Resistance Database (<https://hivdb.stanford.edu/>, (Liu and Shafer, 2006)) and an example of the relevant portions of the report is shown. The mutations in the sequence are described in ‘Drug resistance interpretation: RT’. The DRM score was noted from ‘Mutation scoring: RT’. The nucleotide changes that encode for A98G and K103N were therefore successfully generated in p8.9MJ4 reverse transcriptase.

PSVs were produced using the plasmids containing the mutations, a transfer plasmid and a packing plasmid and was quantified using a viral titration assay. An example of the viral titration curve of A98G+K103N is shown in (Figure 3.15). The high RLU values for mutation profile indicated that there are high concentrations of viable PSV particles (Figure 3.7 A). The dilution factors of the PSVs to produce RLU values in the absence of ARV drugs were as follows (Table 3.10): Harvest 1 – 271.76 times and Harvest 2 – 269.96 times. Harvest 1 showed almost similar PSV yield to harvest 2.

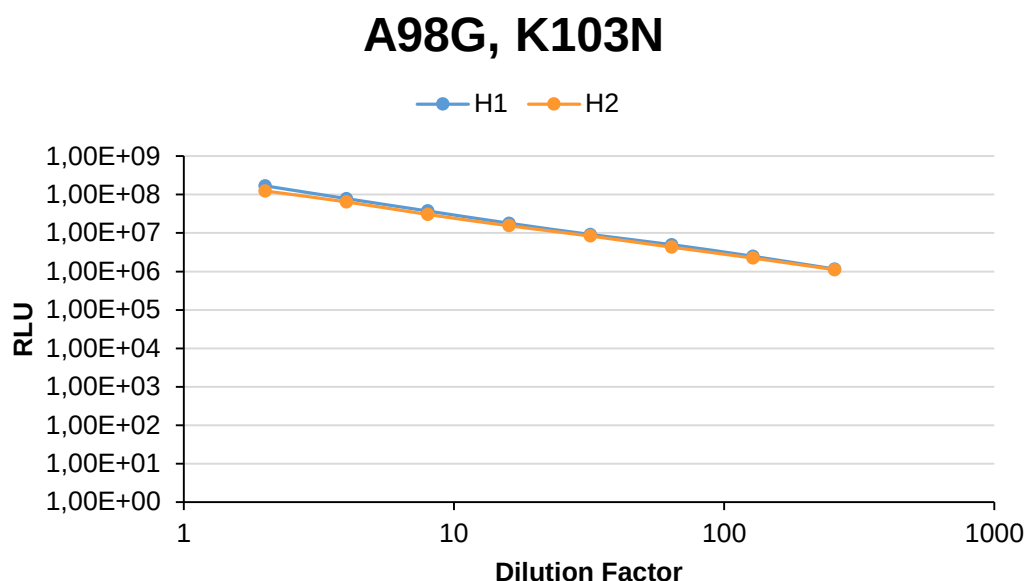


Figure 3.15: Pseudoviral titration curve for A98G+K103N.

The amount of PSVs produced were quantified using a viral titration assay and the resultant RLU values are shown above. The amount of PSVs added to the phenotypic assay was standardised to 1×10^6 RLU. H1 – 1st harvest of PSVs. H2 – 2nd harvest of PSVs

Table 3.10: Dilution of PSV used for *in vitro* phenotypic testing

PSV	Dilution Factor	Amount added to the <i>in vitro</i> phenotypic assay (μl)*
Harvest 1 (H1)	271.76	10.30
Harvest 2 (H2)	269.96	10.37

*the dilution factor was halved to compensate for the 2-fold dilution of the PSVs after the addition to the drug in the *in vitro* phenotypic assay. PSV - pseudovirus

3.3.3 *In vitro* phenotypic susceptibility testing of NNRTI resistant mutants to DOR

3.3.3.1 Phenotypic DOR susceptibility testing of the single NNRTI mutants

PSV stocks which each contain one of the 15 single NNRTI mutants were generated in an HIV-1 subtype C backbone (p8.9MJ4) and *in vitro* phenotypic susceptibility testing was performed against DOR. The results from the phenotypic assay were expressed as FC. The cut-offs used to determine resistance/susceptibility were calculated as described in the materials and methods and these were as follows:

susceptible ≤ 1.31 FC, potential low-level resistance = 1.31 to 2.62 FC, low-level resistance = 2.62 to 3.93 FC, intermediate resistance = 3.93 to 5.24 FC and high-level resistance > 5.24 FC.

The mutants K101P (0.85 ± 0.20 FC), K103N (0.96 ± 0.02 FC), V179D (0.64 ± 0.03 FC) and Y181C (0.35 ± 0.07 FC) were susceptible to DOR. (Figure 3.16, Table 3.12). A statistically significant decrease in FC was observed for V179D and Y181C, suggesting they display increased (or hyper-) susceptibility to DOR. L100I (1.36 ± 0.5 FC), K101E (1.79 ± 0.51 FC), K103S (1.23 ± 0.29 FC), E138A (1.56 ± 0.16 FC) and F227L (1.34 ± 0.24 FC) displayed susceptibility to potential low-level resistance to DOR (Figure 3.16, Table 3.12). F227L was the only mutant that showed a statically significant difference decrease from MJ4. Mutants A98G (2.99 ± 0.02 FC), V108I (2.84 ± 0.21 FC) and G190A (2.52 ± 0.55 FC) showed low-level DOR resistance (Figure 3.16). The phenotypic response of V108I and G190A ranged from potential low-level resistance to low-level resistance. A98G, V108I and G190A showed statistically significant differences between their FC and MJ4.

The mutation P225H (3.86 ± 1.60 FC) displayed intermediate resistance to DOR, with its FC values range from low to high-level resistance (Figure 3.16, Table 3.12). A statistically significant difference was not observed between P225H and MJ4. V106M (17.33 ± 5.31 FC) and Y188L (89.16 ± 0 FC) displayed high-level resistance to DOR (Figure 3.16, Table 3.12). The level of resistance conferred by V106M and Y188L to DOR is significantly higher than all the other single mutations and was statistically significant compared to MJ4.

The phenotypic results were compared to the DRM score from the Stanford HIV Drug Resistance Database. The classification of susceptibility/resistance according to Stanford based on the DRM score generated by their algorithm are described in Table 3.11. Although the *in vitro* phenotypic results are mostly in agreement with predictions by the Stanford HIV DR Database, there are several notable contradictions. V106M (DRM score = 30) displayed high-level DOR resistance, although intermediate resistance was predicted, and Y181C (DRM score = 10) displayed susceptibility to DOR despite predicted low-level resistance (Figure 3.16). P225H (DRM score = 20)

displayed intermediate resistance while low-level resistance was predicted. F227L displayed low-level resistance, although high-level resistance was predicted (Figure 3.16). Since, V106M and F227L had large discrepancies between Stanford's predictions and our phenotypic data, they were generated in the subtype B and C lab-adapted PSVs and tested for DOR susceptibility in the *in vitro* assay to validate the discrepancies.

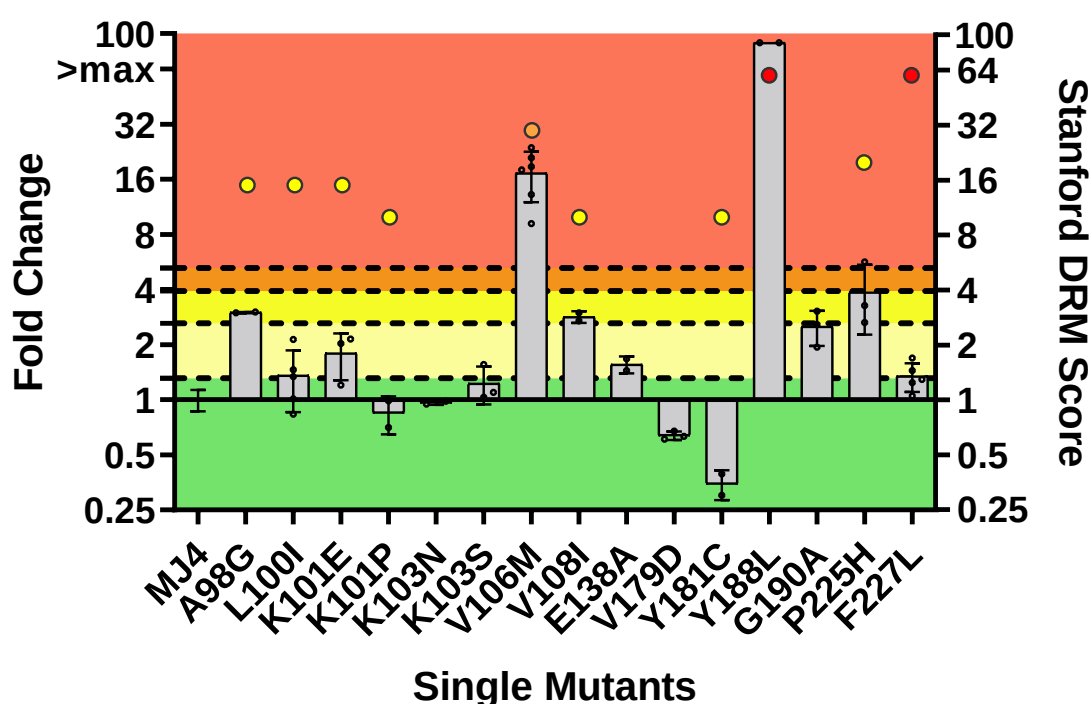


Figure 3.16: Phenotypic susceptibility of single NNRTI mutations to DOR.

In vitro phenotypic assays were performed to assess the susceptibility of each mutant. The lower TCO for DOR susceptibility in the assay was 1.31. DOR susceptibility/resistance was classified as follows: $FC \leq TCO$ = susceptible (■), $TCO > FC < 2 \times TCO$ = potential low-level resistance (■), $2 \times TCO > FC < 3 \times TCO$ = low-level resistance (■), $3 \times TCO > FC < 4 \times TCO$ = intermediate resistance (■) and $FC > 4 \times TCO$ = high-level resistance (■). The coloured dots above each bar graph represents the Stanford's HIV Drug Resistance score. It indicates the level of resistance predicted by their algorithm for a particular mutation (low-level resistance (■), intermediate resistance (■), high-level resistance (■)). The bars without dots (e.g. K103N, V179D) were predicted to be susceptible to DOR, as their score would be 0 the dots are not depicted. V106M and Y188L displayed high-level resistance to DOR. F227L showed low-level DOR resistance despite a predicted high-level resistance.

Table 3.11: Classification of mutations according to Stanford's DRM score

Resistance/susceptibility classification	Stanford DRM score
Susceptible	0 - 9
Potential low-level resistance	10 - 14
Low-level resistance	15 - 29
Intermediate resistance	30 - 59
High-level resistance	≥60

DRM – drug resistance mutation

Table 3.12: IC₅₀ values and FC for the single mutants

Mutation	Average IC ₅₀ (standard deviation) (μM)		Average Fold Change (standard deviation)		p-value**
A98G	0.017	(0.000)	2.99	(0.02)	<0.0001
L100I	0.008	(0.003)	1.36	(0.50)	0.188
K101E	0.010	(0.003)	1.79	(0.51)	0.114
K101P	0.005	(0.001)	0.85	(0.20)	0.465
K103N	0.005	(0.000)	0.96	(0.02)	0.274
K103S	0.007	(0.002)	1.23	(0.29)	0.300
V106M	0.097	(0.030)	17.33	(5.31)	0.001
V108I	0.016	(0.001)	2.84	(0.21)	0.046
E138A	0.009	(0.001)	1.56	(0.16)	0.119
V179D	0.004	(0.000)	0.64	(0.03)	<0.0001
Y181C	0.002	(0.000)	0.35	(0.07)	0.014
Y188L	0.500*	(0.000)	89.16	(0.00)	<0.0001
G190A	0.014	(0.003)	2.52	(0.55)	0.041
P225H	0.022	(0.009)	3.86	(1.60)	0.090
F227L	0.008	(0.001)	1.34	(0.24)	0.031
MJ4	0.006	(0.002)	1.00	(0.33)	-

*IC₅₀ values exceed the maximum concentration tested which was 0.5 μM

**an unpaired Student's t-test test compared the FC of each mutant to MJ4 and the significant p-values are bolded

*** each mutant was tested in at least two independent assays in duplicate

IC₅₀ – 50% inhibitory concentration

3.3.3.2 Generating V106M and F227L in HIV-1 subtype B and C lab-adapted PSVs

V106M and F227L displayed large discrepancies between the phenotypic results and Stanford's predictions, therefore these mutations were generated in four subtype B

and four subtype C lab adapted strains along with NSX and MJ4. Stanford's database is mostly comprised of subtype B data, since the majority of sequencing is performed in countries where subtype B is prevalent. This could result in their predictions being more accurate for subtype B. Therefore, the V106M and F227L mutations were introduced into the subtype B and C lab adapted PSVs to determine whether this difference was due to a difference in HIV-1 subtype. SDM reactions were successfully carried out using the primers and melting temperatures listed in Table 3.13.

Table 3.13: SDM primers used to generate single mutants in p8.9NSX+

Mutation	Primer direction	Sequence (5' → 3')***	optimised T _m (°C)
V106M*	forward	CCACATCCAGTACTGT C ATTGATTTTTC	58.2
	reverse	TTTTTAA TTAAAAAAGAAAAAATCA A T G ACAGTACT GGATGTGG	
V106M** in NSX	forward	G ACAGTACTGGATGTGGGCG	59
	reverse	A T TGATTTTTTCTGTTTAAACCCTGCAGG	
F227L**	forward	A CTTTGGATGGGTTATGAACTCCATC	58
	reverse	AATGGAGGTTCTTTCTGATGTTTTTTGTC	

*SDM with overlapping primers was performed

**SDM with non-overlapping primers was performed

***The nucleic bases that were changed to introduce mutations are highlighted (■)

T_m – melting temperature to table

3.3.3.2.1 Phenotypic DOR susceptibility testing of mutants in lab adapted strains

The cut-offs used to determine susceptibility of the subtype C lab adapted strains were the same as the cut-off used for the single mutants. The cut-offs for the subtype B lab adapted strains were calculated using the same method but with a subtype B reference (NSX) and were as follows: susceptible ≤ 1.55, potential low-level resistance = 1.55 to 3.09, low-level resistance = 3.09 to 4.64, intermediate resistance = 4.64 to 6.18 and high-level resistance > 6.18.

The four subtype B and four subtype C lab adapted PSVs all display high-level resistance to DOR (Figure 3.17 on page 120). The subtype B mutants FC values were:

DS9 = 13.40 ± 1.66 FC, LTNP5 = 30.69 ± 2.75 FC, SM1 = 20.55 ± 4.31 FC, SM2 = 26.93 ± 5.63 FC and NSX = 12.69 ± 1.86 FC. All subtype B lab strains had statistically significant higher FC values than NSX supporting the high-level DOR resistance observed (Table 3.14 on page 121). The FC values of the subtype C mutants were CM9 = 23.59 ± 2.36 FC, DU151 = 7.73 ± 0.64 FC, DU179 = 37.67 ± 9.02 FC, DU422 = 29.46 ± 4.03 FC and MJ4 = 17.33 ± 5.31 FC (Table 3.14). DU179 had the highest FC values thus was the most resistant. The FC values of all subtype C strains were statistically significant compared to MJ4 (Figure 3.17, Table 3.14). MJ4 with V106M compared to MJ4 WT (wild type) had the highest statistical significance, confirming the high-level resistance V106M confers.

The IC_{50} values of the V106M mutant lab adapted PSVs were compared across subtypes (Table 3.15 on page 121). NSX WT was statistically significant when compared to CM9, DU151, MJ4 and MJ4 WT. MJ4 WT displayed statistically significant lower IC_{50} values than DS9 and LTNP5. MJ4 WT was more susceptible than NSX WT and this difference was statistically significant. V106M in CM9 resulted in significantly more resistance to DOR than DS9 and NSX. There was a statistically significant difference between LTNP5 and DU151. This indicates that the resistance of V106M is significantly lower in DU151 compared to LTNP5.

Although there are significant differences in the inter- and intra-subtype comparisons of DOR FC and IC_{50} values, the phenotype of all subtypes of the lab adapted (and control) PSVs showed a high level of DOR resistance, despite Stanford's predicted intermediate resistance to DOR.

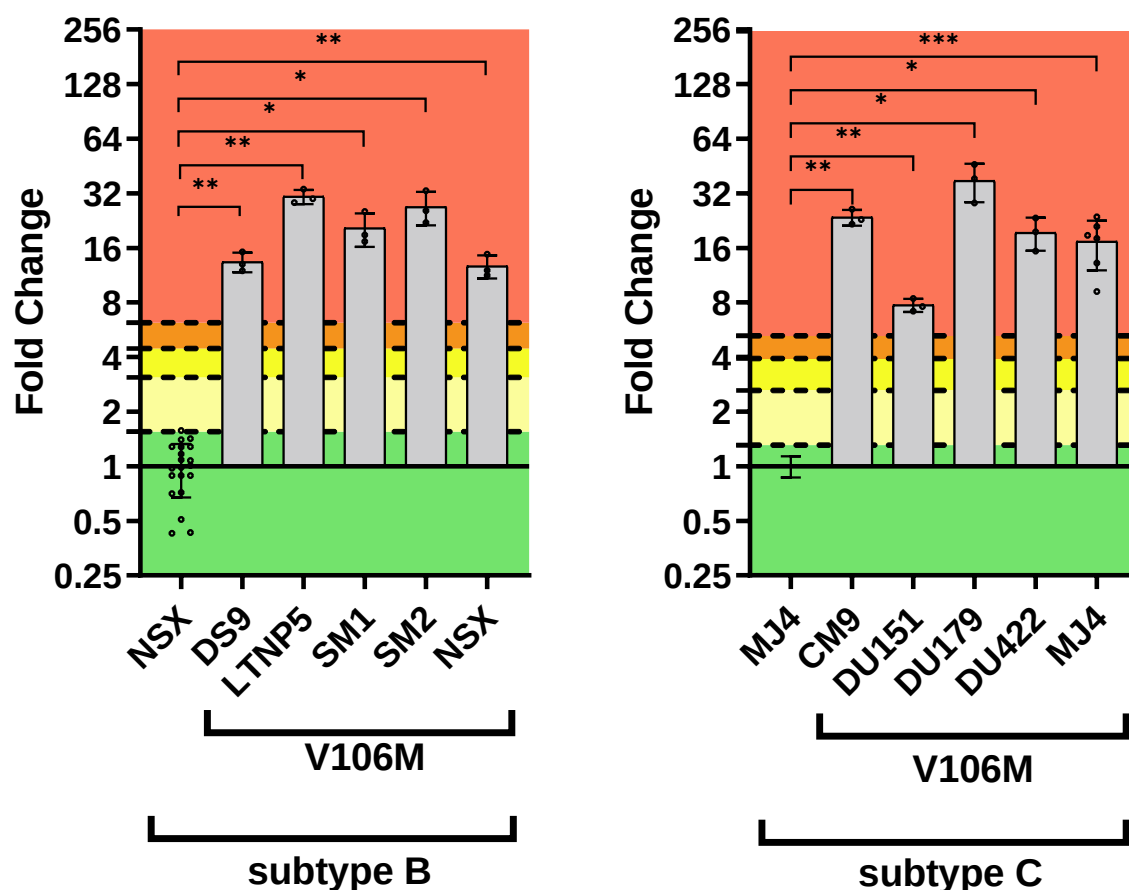


Figure 3.17: Phenotypic susceptibility of V106M in lab adapted strains to DOR.

In vitro phenotypic assays were performed to assess the susceptibility of V106M and was expressed as FC. The lower TCO for DOR susceptibility in the assay was 1.31 for subtype C and 1.55 for subtype B. DOR susceptibility/resistance was classified as follows: $FC \leq TCO$ = susceptible (■), $TCO > FC < 2 \times TCO$ = potential low-level resistance (■), $2 \times TCO > FC < 3 \times TCO$ = low-level resistance (■), $3 \times TCO > FC < 4 \times TCO$ = intermediate resistance (■) and $FC > 4 \times TCO$ = high-level resistance (■). Intermediate resistance was predicted by the Stanford HIV Drug Resistance Database. The subtype B and C lab adapted strains displayed high-level resistance to DOR.

Table 3.14: IC₅₀ values and FC for V106M in lab adapted strains

Mutation	Lab Adapted Strain	Average IC ₅₀ (standard deviation) (μ M)		Average Fold Change (standard deviation)		p-value*
V106M	DS9	0.044	(0.005)	13.40	(1.66)	0.006
	LTNP5	0.101	(0.009)	30.69	(2.75)	0.003
	SM1	0.068	(0.014)	20.55	(4.31)	0.016
	SM2	0.089	(0.019)	26.93	(5.63)	0.015
	NSX	0.042	(0.006)	12.69	(1.86)	0.008
	NSX WT	0.003	(0.003)	1.00	(0.33)	-
V106M	CM9	0.132	(0.013)	23.59	(2.36)	0.004
	DU151	0.043	(0.004)	7.73	(0.64)	0.003
	DU179	0.211	(0.051)	37.67	(9.02)	0.020
	DU422	0.109	(0.023)	19.46	(4.03)	0.016
	MJ4	0.097	(0.035)	17.33	(5.31)	0.0007
	MJ4 WT	0.006	(0.002)	1.00	(0.33)	-

* an unpaired Student's t-test test compared the FC of each lab adapted strain to the subtype wild-type reference and the significant p-values are bolded

** each lab strain was tested in at least two independent assays in duplicate

IC₅₀ – 50% inhibitory concentration, WT – wild type

Table 3.15: Comparing V106M in subtype B and C lab adapted strains

		subtype B						
		V106M					WT	
		DS9	LTNP5	SM1	SM2	NSX	NSX	
subtype C	V106M	CM9	0.022	0.278	0.052	0.286	0.019	0.019
		DU151	>0.9999	0.026	0.450	0.254	>0.9999	0.01
		DU179	0.152	0.311	0.188	0.249	0.147	0.104
		DU422	0.186	1.000	0.450	0.957	0.171	0.081
		MJ4	0.094	>0.9999	0.683	>0.9999	0.079	0.009
	WT	MJ4	0.037	0.02	0.092	0.088	0.051	<0.0001

* a one-way ANOVA compared the mean IC₅₀ of each lab adapted strain to each other and p-values <0.05 were significant (■)

F227L was similarly generated in the lab adapted PSVs and the phenotypic results are depicted in Figure 3.18 (page 123). This mutation displayed potential and low-level resistance to DOR in the subtype B lab adapted PSVs. DS9 (1.3 ± 0.11 FC) and NSX (0.97 ± 0.17 FC) displayed susceptibility to DOR (Table 3.16 on page 124). LTNP5 (3.75 ± 1.01 FC) and SM2 (3.90 ± 0.35 FC) displayed low-level DOR resistance, however LTNP5 had a higher standard deviation resulting in it ranging from potential low-level to intermediate resistance. SM1 (2.45 ± 0.17 FC) displayed potential low-level resistance. LTNP5, SM1 and SM2 displayed statistically significant higher FC values than NSX WT (Table 3.16).

F227L in the subtype C lab adapted strains displayed susceptibility and hyper-susceptibility to DOR (Figure 3.18). CM9 (1.35 ± 0.11 FC), DU151 (0.91 ± 0.11 FC) DU179 (0.26 ± 0.07 FC), DU422 (0.69 ± 0.14 FC) and MJ4 (1.34 ± 0.24 FC) displayed DOR susceptibility (Table 3.16). CM9 and MJ4 range in phenotype from susceptible to low-level resistance. They displayed statistically significant higher FC values than MJ4 WT. DU179 showed a statistically significant increase in susceptibility to DOR than MJ4 WT. Therefore, DU179 displays potential hyper-susceptibility to DOR.

F227L in subtype B and C lab adapted PSVs showed different susceptibility profiles, where majority of subtype B displayed low-level DOR resistance and majority of subtype C displayed susceptibility/hyper-susceptibility to DOR. Comparing the IC_{50} values of all the subtype B and C lab adapted strains, NSX WT was statistically significant compared to CM9, DU178, DU422 and MJ4 (Table 3.17 on page 124). MJ4 WT was more susceptible than NSX WT and this difference was statistically significant. SM1 and SM2 displayed significantly higher resistance than all the other lab adapted PSVs. This difference was statistically significant between SM2 and all subtype C lab adapted PSVs, but only between SM1 and DU151, DU179 or DU422. DU179 was the most susceptible lab strain containing F227L displaying a significantly higher susceptibility than all other lab strains. However, these differences were only statistically significant for DS9, SM1 and SM2. All the subtype C lab adapted strains with F227L display more susceptibility to DOR than subtype B.

However, this difference was only significant in CM9 which displayed statistically significant higher IC₅₀ values than DS9 and NSX and MJ4 which was significantly higher than NSX with F227L. F227L was predicted by Stanford to confer high-level resistance to DOR. The phenotypic results of subtype B and C lab adapted PSVs both contradict Stanford's predictions, since they display hyper-susceptibility to low-level resistance to DOR.

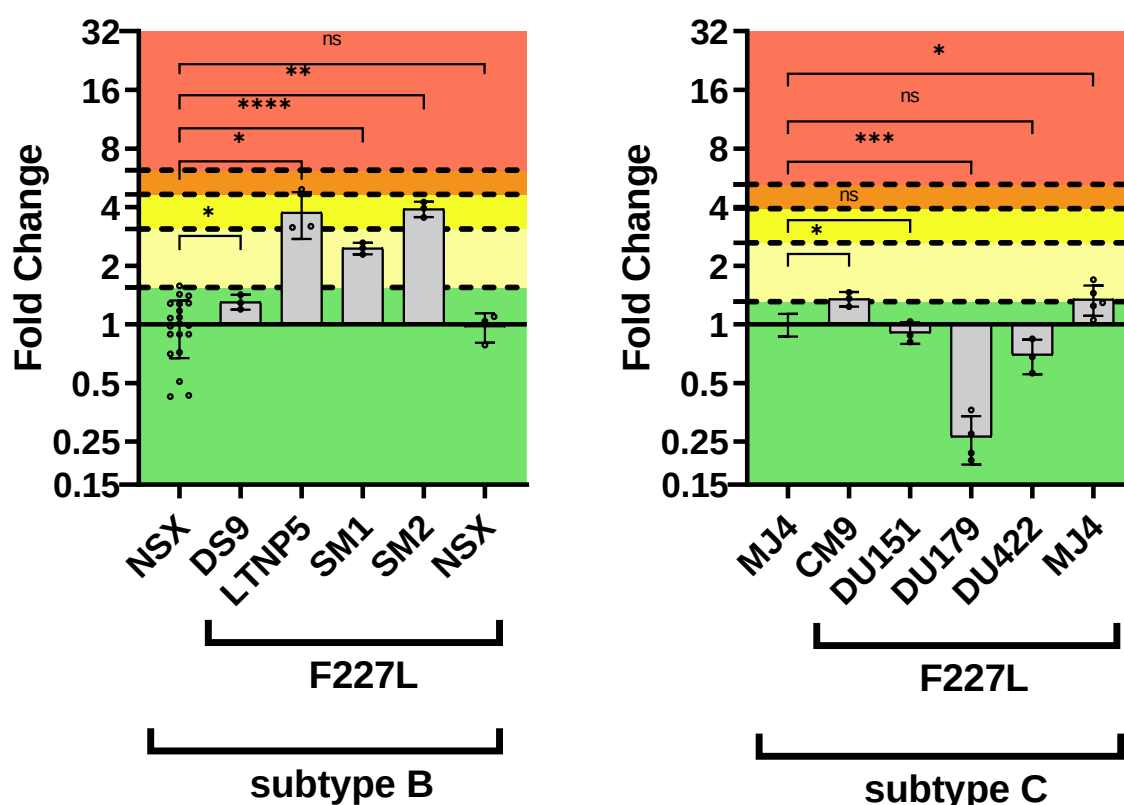


Figure 3.18: Phenotypic susceptibility of F227L in lab adapted strains to DOR.

In vitro phenotypic assays were performed to assess the susceptibility of F227L and was expressed as FC. The lower TCO for DOR susceptibility in the assay was 1.31 for subtype C and 1.55 for subtype B. DOR susceptibility/resistance was classified as follows: $FC \leq TCO$ = susceptible (■), $TCO > FC < 2 \times TCO$ = potential low-level resistance (■), $2 \times TCO > FC < 3 \times TCO$ = low-level resistance (■), $3 \times TCO > FC < 4 \times TCO$ = intermediate resistance (■) and $FC > 4 \times TCO$ = high-level resistance (■). Intermediate resistance was predicted by the Stanford HIV Drug Resistance Database. The subtype B lab adapted strains displayed potential and low-level resistance to DOR. The subtype C lab adapted strains displayed susceptibility to DOR.

Table 3.16: IC₅₀ values and FC for F227L in lab adapted strains

Mutation	Lab Adapted Strain	Average IC ₅₀ (standard deviation) (μM)		Average Fold Change (standard deviation)		p-value*
F227L	DS9	0.004	(0.0003)	1.30	(0.11)	0.013
	LTNP5	0.012	(0.003)	3.75	(1.01)	0.040
	SM1	0.008	(0.001)	2.45	(0.17)	<0.0001
	SM2	0.013	(0.001)	3.90	(0.35)	0.002
	NSX	0.003	(0.001)	0.97	(0.17)	0.824
	NSX WT	0.003	(0.003)	1.00	(0.33)	-
F227L	CM9	0.008	(0.001)	1.35	(0.11)	0.023
	DU151	0.005	(0.001)	0.91	(0.11)	0.290
	DU179	0.001	(0.000)	0.26	(0.07)	<0.0001
	DU422	0.004	(0.001)	0.69	(0.14)	0.053
	MJ4	0.008	(0.001)	1.34	(0.24)	0.0307
	MJ4 WT	0.006	(0.002)	1.00	(0.33)	-

* an unpaired Student's t-test test compared the FC of each lab adapted strain to the subtype wild-type reference and the significant p-values are bolded

** each lab strain was tested in at least two independent assays in duplicate

IC₅₀ – 50% inhibitory concentration, WT – wild type

Table 3.17: Comparing F227L in subtype B and C lab adapted strains

		subtype B						
		F227L					WT	
		DS9	LTNP5	SM1	SM2	NSX	NSX	
subtype C	F227L	CM9	0.035	0.566	0.978	0.048	0.012	0.008
		DU151	0.750	0.310	0.043	0.016	0.183	0.156
		DU179	0.005	0.156	0.001	0.011	0.128	0.003
		DU422	0.996	0.235	0.027	0.008	0.949	0.962
		MJ4	0.056	0.573	0.998	0.030	0.015	0.016
	WT	MJ4	0.079	0.352	0.057	0.040	0.061	<0.0001

* a one-way ANOVA compared the mean IC₅₀ of each lab adapted strain to each other and p-values <0.05 were significant (■)

3.3.3.3 Phenotypic DOR susceptibility testing of the combination NNRTI mutants

PSVs containing the prevalent NNRTI combination mutants (n = 20) were generated in an HIV-1 subtype C backbone (p8.9MJ4) and *in vitro* phenotypic susceptibility testing was performed against DOR. The established cut-off values for susceptibility/resistance (as for the single mutants) were used. Seventeen of the twenty most prevalent NNRTI combination mutants displayed high-level resistance to DOR (Figure 3.19 on page 127). Nine of the twelve combination mutants that contained K103N displayed high-level resistance to DOR. A98G+K103N (8.1 ± 2.3 FC), A98G+K103N+P225H (80.5 ± 14.8 FC), L100I+K103N (6.3 ± 1.5 FC), L100I+K103N+P225H (29.3 ± 10.2 FC), K103N+V106M (81.9 ± 12.5 FC), K103N+V106M+F227L (89.2 ± 0 FC), K103N+V108I+P225H (17.5 ± 0.3 FC), K103N+Y188L (89.2 ± 0 FC) and K103N+P225H (9.7 ± 2.2 FC) display high-level DOR resistance, with K103N+V106M+F227L and K103N+Y188L having IC₅₀ values greater than the maximum testing concentration of 0.5 μ M. K103N+V106M and A98G+K103N+P225H had the highest IC₅₀ values close to the highest testing concentration (Table 3.18 on page 128). Amongst the high-level resistant mutants, the remaining mutants decreased in resistance as follows; L100I+K103N+P225H, K103N+V108I+P225H, K103N+P225H, A98G+K103N and L100I+K103N. The FC of L100I+K103N ranged from intermediate resistance to high-level resistance. All the mutants displaying high-level resistance possessed statistically significant higher FC values than MJ4 WT. K103N+V108I (2.8 ± 0.3 FC) displayed low-level resistance to DOR. K101P+K103N (0.8 ± 0.1 FC) and K103N+E138A (1.2 ± 0.2 FC) were susceptible to DOR. K103N+V108I, K103N+E138A and K101P+K103N were not statistically significant when compared to MJ4 WT.

All of the combination mutation profiles containing V106M showed high-level resistance to DOR. K101E+V106M+G190A (89.2 ± 0 FC), K103S+V106M (89.2 ± 0 FC), V106M+V179D+F227L (89.2 ± 0 FC), V106M+Y188L (89.2 ± 0 FC), V106M+G190A+F227L (89.2 ± 0 FC) and V179D+Y188L (89.2 ± 0 FC) displayed high-level DOR resistance with IC₅₀ values exceeding the maximum testing concentration. V106M+G190A (85.3 ± 6.7 FC) and V106M+V179D (6.7 ± 0.3 FC) displayed high-level resistance with V106M+V179D having a lower IC₅₀ values. V179D+Y188L (89.2 ± 0

FC) also display high-level resistance to DOR. All the V106M mutants as well as V179D+Y188L had statistically significant higher FC change values than MJ4 WT, with the mutants displaying maximum IC₅₀ values showing the strongest significance. The V106M mutants were significantly less susceptible than the K103N mutants.

The phenotypic results were compared to Stanford's DRM score which were previously described in Table 3.11 on page 117. Generally, the high FC values obtained for the mutation combinations are in agreement with the higher DRM scores provided by Stanford. However, some discrepancies were observed. A98G+K103N (DRM score = 75) displayed high-level resistance while low-level DOR resistance was predicted. K101P+K103N (DRM score = 10) showed DOR susceptibility, while low-level resistance was predicted. K103N+E138A (DRM score = 0) displayed potential low-level DOR resistance, although susceptibility was predicted. A98G+K103N+P225H (DRM score = 45), L100I+K103N (DRM score = 30), L100I+K103N+P225H (DRM score = 50), K103N+V106M (DRM score = 30), K103N+V108I+P225H (DRM score = 40), K103N+P225H (DRM score = 30), K101E+V106M+G190A (DRM score = 50), K103S+V106M (DRM score = 30), V106M+V179D (DRM score = 30) and V106M+G190A (DRM score = 30) all were predicted to have intermediate DOR resistance, but displayed high-level resistance. K103N+ V106M+F227L (DRM score = 90), K103N+Y188L (DRM score = 60), V106M+V179D+F227L (DRM score = 90), V106M+Y188L (DRM score = 90), V106M+G190A+F227L (DRM score = 90) and V179D+Y188L (DRM score = 60) display high-level resistance as predicated.

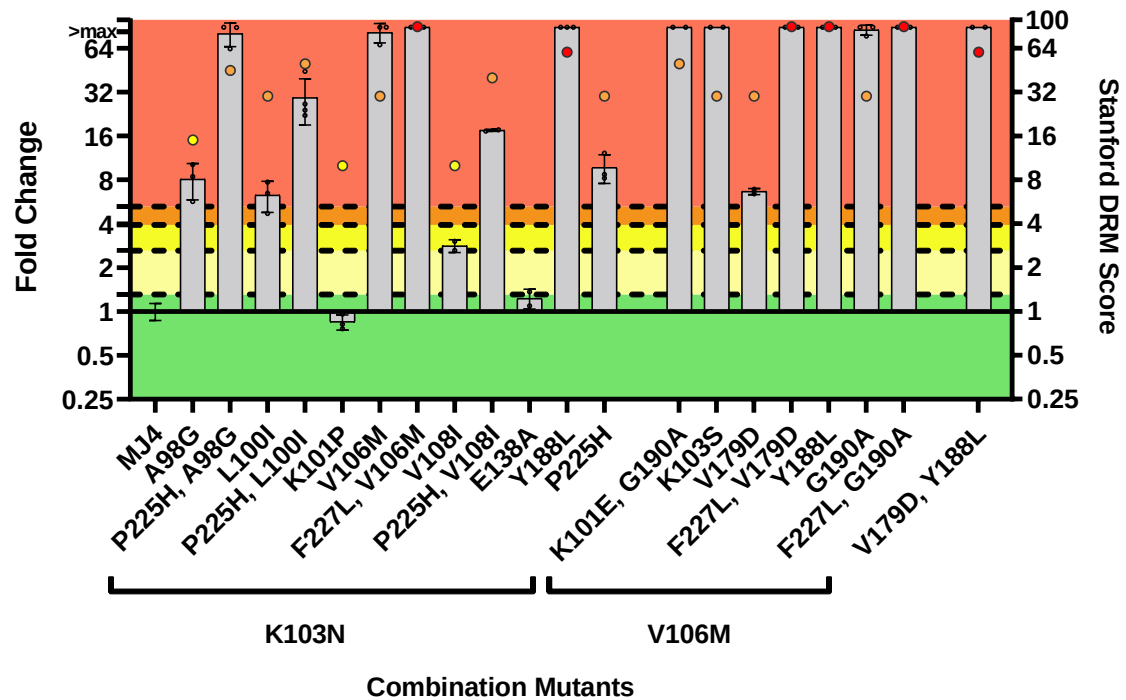


Figure 3.19: Phenotypic susceptibility of combination NNRTI mutations to DOR.

In vitro phenotypic assays were performed to assess the susceptibility of each mutant and susceptibility was expressed as FC. The lower TCO for DOR susceptibility in the assay was 1.31. DOR susceptibility/resistance was classified as follows: $FC \leq TCO$ = susceptible (■), $TCO > FC < 2 \times TCO$ = potential low-level resistance (■), $2 \times TCO > FC < 3 \times TCO$ = low-level resistance (■), $3 \times TCO > FC < 4 \times TCO$ = intermediate resistance (■) and $FC > 4 \times TCO$ = high-level resistance (■). The coloured dots above each bar graph represents the Stanford's HIV Drug Resistance score for the DRM profile. It indicates the level of resistance predicted by their algorithm for a particular mutation (low-level resistance (■), intermediate resistance (■), high-level resistance (■)). Majority of the mutants (17 of 20) displayed high-level resistance to DOR.

Table 3.18: IC₅₀ values and FC for combination mutants

Mutation	Average IC ₅₀ * (standard deviation) (μM)		Average Fold Change (standard deviation)		p-value**
A98G,K103N	0.045	(0.013)	8.1	(2.3)	0.032
A98G,K103N,P225H	0.451	(0.083)	80.5	(14.8)	0.011
L100I,K103N	0.035	(0.008)	6.3	(1.5)	0.026
L100I,K103N,P225H	0.164	(0.057)	29.3	(10.2)	0.012
K101P,K103N	0.005*	(0.001)	0.8	(0.1)	0.104
K103N,V106M	0.459	(0.070)	81.9	(12.5)	0.008
K103N,V106M,F227L	0.500*	(0.000)	89.2	(0.0)	<0.0001
K103N,V108I	0.016	(0.002)	2.8	(0.3)	0.067
K103N,V108I,P225H	0.098	(0.001)	17.5	(0.3)	0.006
K103N,E138A	0.007	(0.001)	1.2	(0.2)	0.334
K103N,Y188L	0.500*	(0.000)	89.2	(0.0)	<0.0001
K103N,P225H	0.054	(0.012)	9.7	(2.2)	0.020
K101E,V106M,G190A	0.500*	(0.000)	89.2	(0.0)	<0.0001
K103S,V106M	0.500*	(0.000)	89.2	(0.0)	<0.0001
V106M,V179D	0.037	(0.002)	6.7	(0.3)	0.025
V106M,V179D,F227L	0.500*	(0.000)	89.2	(0.0)	<0.0001
V106M,Y188L	0.500*	(0.000)	89.2	(0.0)	<0.0001
V106M,G190A	0.478	(0.038)	85.3	(6.7)	0.002
V106M,G190A,F227L	0.500*	(0.000)	89.2	(0.0)	<0.0001
V179D,Y188L	0.500*	(0.000)	89.2	(0.0)	<0.0001
A98G,K103N	0.006	(0.002)	89.2	(0.0)	0.032
MJ4	0.045	(0.013)	1.00	(0.3)	0.011

* IC₅₀ values exceed the maximum concentration tested which was 0.5 μM

** an unpaired Student's t-test test compared the FC of each mutation profile to wild type subtype C (MJ4) and the significant p-values are bolded

*** each combination mutation was tested in at least two independent assays in duplicate

IC₅₀ – 50% inhibitory concentration

4. Discussion

The increasing prevalence of drug resistance warrants the development and implementation of ARV drugs that are effective against viruses with prevalent DRMs (Chimukangara *et al.*, 2019). DOR is a novel NNRTI effective against the three most prevalent NNRTI mutations (K103N, G190A and Y181C) (Feng *et al.*, 2016; Fulco and Herity, 2021; Wong *et al.*, 2019). Although studies have demonstrated DOR's effectiveness in DRMs associated with HIV-1 subtype B, its effect on subtype C remains unclear (Asante-Appiah *et al.*, 2021). Therefore, the aim of this study is to assess the *in vitro* phenotypic drug susceptibility of DOR in the context of HIV-1 subtype C drug-resistant variants. An initial screen on wild-type variants showed no significant differences in DOR's potency across subtypes B and C PSVs, although strain-specific differences may be observed. The combination of DOR with a novel NRTI (i.e., ISL) showed low but significant synergy in inhibiting a wild-type subtype C PSV. Most of the prevalent single NNRTI drug resistance mutants (e.g., K103N, Y181C) were susceptible to DOR, while the single V106M and Y188L mutants displayed high-level resistance to DOR. The majority of K103N and V106M combination mutants showed intermediate to highly-level resistance to DOR.

The CMJAH genotypic drug resistance database with sequences from 2016 – 2020 was employed in this study to identify the most prevalent DRMs. During this period the South African National ART Clinical Guidelines recommended an NNRTI-based with a regimen of TDF + 3TC/FTC + EFV/NVP (NDH, 2015) for first-line ARV treatment. Patients experiencing virological failure (defined as VL $\geq$ 1000 copies/ml) at two consecutive clinic visits two months apart despite good adherence were switched to PI-based second-line regimen of ZDV + 3TC + LPV/r. In 2019, TDF + 3TC + DTG (i.e., "TLD") was introduced as preferred first-line regimen for initiation of ART (NDH, 2019). Patients receiving an NNRTI-based first-line regimen had the option to remain on the regimen or to transition to TLD.

Both the 2015 and 2019 guidelines recommended genotypic drug resistance testing only after failure on a PI or INSTI-based regimen, defined as VL $\leq$ 1000 copies/ml on at least three occasions over the course of two years or with signs of immunological

or clinical failure (NDH, 2015; NDH, 2019). The CMJAH genotypic drug resistance database contained data from 7749 patients of which 70.89% were failing PI-based therapy, which aligns with these recommendations. A much smaller proportion of patients (i.e., 12.67%) were genotyped while failing an NNRTI-based regimen. However, this number is not high enough to justify the high prevalence of NNRTI drug resistance (76.5%) in the database and implies that patients failing an NNRTI-based regimen retain NNRTI mutations during PI-based treatment. This persistence of NNRTI mutations after the discontinuation of NNRTI therapy is commonly observed. Jenny-Avital and Stein (2004) compared the persistence of NNRTI mutations to PI mutations and observed a high persistence and prevalence of NNRTI mutations compared to PI mutation after therapy was ceased for over 2 years. They also observed NNRTI resistance mutations in patients 9 years after ART was discontinued. In an epidemiological study conducted by Machnowska *et al.* (2019), it was concluded that the high prevalence of NNRTI-transmitted drug resistance is likely a result of the longevity of the NNRTI resistance mutations, which largely results from their limited effect on viral fitness.

Since most sequences in the CMJAH genotypic drug resistance database were obtained from patients who have not recently been exposed to NNRTIs, it was unclear whether the prevalent NNRTI DRMs observed were representative of the prevalent NNRTI DRMs that are typically observed during NNRTI-based first-line failure. In, a national survey conducted in South Africa, Steegen *et al.* (2017) reported that among 788 patients failing NNRTI-based therapy the K103N/S (48.9%) was the most common mutation observed, but V106A/I/M (34.9%), Y181C/V (26.1%) and G190A/S (21.4%) were also notable. Majority of the patients were infected with HIV-1 subtype C and being treated with EFV. The other NNRTI mutations observed in the study all had a prevalence below 15% (V90I, A98G, L100I, K101E/H/P, V108I, E138A/G/K/Q, V179D/F/L/T, Y188C/H/L, H221Y, P225H, F227L, M230L and N348I). Similarly, K103N was the most prevalent mutation in this study, but V106M, Y181C and G190A were not commonly observed. This could be attributed to V106M, Y181C and G190A conferring lower fitness capacities than the wild-type virus (Armstrong *et al.*, 2011).

However, other studies reported that the NNRTI mutations commonly observed after first-line failure in a South African cohort are K103N, V106M, Y181C and G190A (Basson *et al.*, 2020, 2015; Derache *et al.*, 2016). Therefore, all of these mutations were included in this study as prevalent single NNRTI mutations and were tested for DOR susceptibility. Hauser *et al.* (2022) examined virological failure in 3915 patients from 19 studies of which 13 were conducted in South Africa. The patients were being treated with NNRTI-based therapy with 76.4% of them on EFV and 23.1% on NVP. The group found that K103N/R/S was the most frequent mutation. V106A/I/M, Y181C/I/V and G190A/E/R/S were prevalent with Y181C/I/V more prevalent after treatment with NVP rather than EFV. All of the mutations detected in patients failing NNRTI-based therapy were examined in this study apart from: K101E, E138G/K/Q, V179F/L/T, Y181I/V, Y188C/H, G190E/R/S, H221Y, M230L and N348I, which all have a prevalence below 22%. However, the most prevalent mutations in South African patients failing NNRTI therapy across several studies include K103N, V106M, Y181C and G190A (Chimukangara *et al.*, 2019; Etta *et al.*, 2017). Although the reported prevalence of the mutations may differ from what was observed in our database, we consider the mutations investigated in this study to be representative of the mutations observed during NNRTI-based first-line failure.

In this study, DOR has demonstrated higher potency in subtype C lab adapted strains than subtype B lab strains, although after statistical analysis of the IC₅₀ values no significant differences in DOR potencies were observed between subtype B and C. A similar conclusion was drawn by Lai *et al.* (2014), who observed no difference in the potencies of DOR, EFV, ETR or RPV amongst 10 different HIV subtypes in a single-cycle assay. Others have also demonstrated similar potencies or clinical outcomes for patients infected with different subtypes and treated with NNRTIs (Geretti *et al.*, 2009). DOR would therefore be suitable for treatment of wild-type HIV-1, irrespective of subtype.

Dual-drug synergy assessments in this study revealed synergy between DOR & ISL, EFV & 3TC and DOR & TDF, while DOR & 3TC and EFV & TDF displayed antagonism. Previous studies have also reported on the synergy between NRTIs and NNRTIs (approved or unapproved) combinations: ZDV & NVP (Richman *et al.*, 1991), ZDV &

Tivirapine (i.e., R-86183) (Pauwels *et al.*, 1994), ZDV & Emivirine (i.e., MKC-442) (Brennan *et al.*, 1995), Didanosine & Delavirdine (Chong and Pagano, 1997) Zidovudine & Delavirdine (Chong and Pagano, 1997), ZDV or 3TC & EFV (King *et al.*, 2002), TDF or FTC & EFV and (Feng *et al.*, 2009). Similarly, we observed synergy between EFV & 3TC and DOR & ISL and DOR & TDF. There have been several theories regarding the mechanism of synergy observed between these two drug classes. Radzio and Sluis-Cremer (2008) have demonstrated that EFV increases HIV reverse transcriptase's ribonuclease H activity, which reduces the rate at which incorporated ZDV is removed from the complementary viral DNA. Also, according to Basavapathruni *et al.* (2004) synergy is considered to be as a result of NNRTIs inhibiting the rate of excision of NRTIs by decreasing the rate of the chemical binding of ATP. Another mechanism was revealed by Feng *et al.* (2009), wherein they observed the independent formation of stable NNRTI complexes, which lengthened and enhanced NRTIs' inhibition mechanism of chain termination.

In this study, the combination of DOR (at 2.22×10^{-3} μ M) and ISL (at 2.96×10^{-5} μ M) showed the highest level of synergy across all the tested ARV combinations. An *in vitro* selection study performed by Lai *et al.* (2022) against wild-type HIV-1 showed that the DOR & ISL (DOR – 10.8 nM, ISL – 2.37 nM) combination inhibited viral breakthrough at lower drug concentrations than the DOR & 3TC (DOR – 25.2 nM, ISL – 2604 nM) and DTG & 3TC (DOR > 28.8 nM, ISL > 2976 nM) combinations. Furthermore, viral breakthrough was inhibited at 8-fold lower concentrations for the DOR & ISL combination than when DOR and ISL were tested individually. They observed fewer mutations after breakthrough in the presence of DOR & ISL in combination than with ISL or DOR alone, concluding that the drug combination has a high genetic barrier to the development of resistance. This synergistic effect suggests that DOR and ISL could provide efficacy at lower concentrations when used in combination. This would translate to reduced side effects, pill burden, drug-drug interactions and thus improve adherence in the clinical setting (Moreno *et al.*, 2019). There were two Phase III clinical trials investigating viral suppression in patients who switch to a DOR/ISL regimen after achieving viral suppression with BIC+FTC+TAF compared to patients remaining on a three drug ART regimen (Asante-Appiah, 2024). They found that the DOR/ISL regimen maintained viral suppression for up to 96 weeks,

even in the presence of NNRTI resistance mutations. Further, supporting the implementation of these two drugs together.

Antagonism was observed in this study with the DOR & 3TC and EFV & TDF dual-drug combinations. Antagonism is not commonly observed with ARVs, but was previously reported with EFV & NVP (King *et al.*, 2002). That study noted that the measurement of synergy is largely dependent on the concentration of the compounds and the ratio of the combination of the compounds and this was observed in previous studies as well (Hoggard *et al.*, 1996). Lai *et al.* (2014) screened DOR in combination with 18 different ARVs, including 5 NNRTIs and 9 NRTIs. No antagonism was observed with any of the ARVs, including with 3TC and TDF. This supports our findings for DOR & TDF, but contradicts our findings for DOR & 3TC. This could be due to differences in the assays (i.e., the concentrations and ratios of the compounds tested), or it could be related to how they defined synergy since quantitatively summarizing a synergistic interaction is challenging (Ma and Motsinger-Reif, 2019). A published algorithm that employed four different models to define synergy (i.e., SynergyScore (Zheng *et al.*, 2022)) was used in this study and may have been more rigorous than the one employed by Lai *et al.* (2014). In summary, the NRTI's used in conjunction with DOR may impact on its efficacy due to differences in their interactions, as highlighted in this study. Trials that evaluate the clinical outcomes of patients on combinations of DOR with various NRTIs are therefore needed. The impact of NRTI and NNRTI DRMs on these combinations may also provide valuable information for the treatment of ART-experienced patients and should be explored in future studies.

Phenotypic susceptibility of HIV-1 subtype C mutant PSVs to DOR was assessed in this study through an *in vitro* single cycle non-replicative assay. The highly prevalent K103N and Y181C single NNRTI mutations were found to be susceptible to DOR. K103N is the most prevalent NNRTI resistance mutation worldwide (Rhee *et al.*, 2021), as well as in South Africa (Hauser *et al.*, 2022). This is the result of selection of K103N by NVP and EFV, which were amongst the first ARVs prescribed for the treatment of HIV infections (Bachelier *et al.*, 2000; Reuman *et al.*, 2010). DOR has previously been established to be effective against the K103N mutation (Feng *et al.*, 2016; Lai *et al.*, 2014) since the central ring of DOR interacts with the backbone, and not the side

chain, of the lysine (K) at amino acid position 103 in reverse transcriptase. The substitution of the lysine with an asparagine (N) at this position therefore has no effect on the interaction with DOR (Feng *et al.*, 2014). We also observed susceptibility of K103N in HIV-1 subtype C to DOR.

Y181C is selected for by EFV (Melikian *et al.*, 2014), RPV (Rimsky *et al.*, 2012) and ETR (Tambuyzer *et al.*, 2010), and thus displays high-level drug resistance to all three of these NNRTIs. Studies have shown that the Y181C single mutation is susceptible to DOR (Asante-Appiah *et al.*, 2021; Feng *et al.*, 2016; Lai *et al.*, 2014). We observed potential hyper-susceptibility of the Y181C single mutant to DOR. This could be explained by the fact that DOR does not interact with the tyrosine (T) at amino acid at position 181 when bound to reverse transcriptase (Feng *et al.*, 2014). We also observed potential hyper-susceptibility to DOR for the V179D single mutant. Although this has not been reported by other studies, a study performed by Asante-Appiah *et al.* (2021) using clinical isolates showed that V179D mutants had the lowest FC for DOR among the 24 single NNRTI mutations tested. Hyper-susceptibility to NNRTIs have also been reported in the clinical setting (Haubrich *et al.*, 2002).

Several single mutants (i.e., L100I, K101EP, K103S, E138A, F227L) were susceptible to DOR in this study. L100I is selected for by the NNRTIs EFV, ETR and RPV (Bachelier *et al.*, 2000; Rimsky *et al.*, 2012; Vingerhoets *et al.*, 2005). K101E is commonly observed in combination with other NNRTI-resistance mutations and in combination reduces the susceptibility of EFV, ETR and RPV (Melikian *et al.*, 2014; Saladini *et al.*, 2020). K101P is selected for by the NNRTIs EFV, ETR, NVP and RPV and individually reduces their susceptibility (Melikian *et al.*, 2014; Parkin *et al.*, 2006b). K103S is selected for by EFV and NVP and is most commonly found in people who already have K103N (Harrigan *et al.*, 2005). This is likely due to the fact that two base pair changes are required to transition from a lysine (K, codon: AAA) to a serine (S, codon: AGT/C), while two nucleotide changes are required to transition to an asparagine (N, codon: GAT/C). F227L is selected for by EFV and NVP and is found in combination with V106A (Balzarini *et al.*, 1998; Brenner *et al.*, 2021; Reuman *et al.*, 2010). These mutations (L100I, K101EP, K103S, E138A, F227L) have also previously displayed susceptibility to DOR (Asante-Appiah *et al.*, 2021; Lai *et al.*, 2014).

The single A98G, V108I, G190A and P225H mutants in HIV-1 subtype C displayed low to intermediate levels of resistance to DOR in our study. A98G and V108I displayed low-level DOR resistance. A98G and V108I are accessory NNRTI mutations (Reuman *et al.*, 2010). A98G has previously been associated with susceptibility to DOR (Asante-Appiah *et al.*, 2021), while the susceptibility of V108I to DOR has not been tested previously. The G190A mutation is selected for and displays high-level resistance to NVP and EFV (Melikian *et al.*, 2014; Rimsky *et al.*, 2012). The mutation has previously been shown to be susceptibility to DOR (Asante-Appiah *et al.*, 2021; Lai *et al.*, 2014). Our results suggest that the single G190A mutations may be of concern with implementing DOR treatment in South Africa where G190A is prevalent (Steegen *et al.*, 2017). P225H display intermediate resistance to DOR and similarly the mutation has been observed to cause low-level resistance to DOR in clinical isolates (Asante-Appiah *et al.*, 2021). However, this mutation has a lower prevalence in South Africa in HIV-1 subtype C strains (Steegen *et al.*, 2017).

Two single mutations (i.e., V106M, Y188L) displayed a significant decrease in DOR susceptibility in our study. Mutations at position V106 are selected for by NVP and EFV (Melikian *et al.*, 2014). An alanine (A) is frequently selected for at this position in subtype B, while a methionine (M) is selected for in subtype C (Grossman *et al.*, 2004). This “preference” is largely driven by the nucleotide background in the two subtypes (Brenner *et al.*, 2003). For subtype C, only a single nucleotide change is required to transition from a valine (V, codon: GTG) to a methionine (M, codon: ATG), while two nucleotide changes are required to transition to an alanine (A, codon: ATT/C/A). Although the single V106M mutants displayed high-level resistance to DOR in our study, the Stanford’s Drug Resistance Database (Liu and Shafer, 2006) predicted only an intermediate level of resistance to DOR for this mutation. However, irrespective of the level of DOR resistance, both our study and the predicted response indicate that this mutation causes a significant decrease in DOR susceptibility. This finding is also in agreement with other studies that have shown that V106M is a major DOR-associated DRM that is selected for by DOR (Feng *et al.*, 2014; Lai *et al.*, 2014; Orkin *et al.*, 2019). Mechanistically, this can be explained by the observation that the valine at position 106 is important for the interaction with the central ring of DOR. Thus, weakening of this interaction by amino acid substitutions will weaken DOR’s binding

affinity (Feng *et al.*, 2014). Since this mutation is the second most prevalent mutation amongst NNRTI-experienced patients in South Africa (Steege *et al.*, 2017), it raises concerns for the efficacy of DOR in the treatment-experienced patients.

Y188L is selected for by NVP and EFV (Bachelier *et al.*, 2000; Reuman *et al.*, 2010) and is also a major DOR-associated DRM (Feng *et al.*, 2014; Lai *et al.*, 2014; Orkin *et al.*, 2019). According to the Stanford HIV Drug Resistance Database (Rhee *et al.*, 2003; Shafer, 2006), it causes high-level resistance to all NNRTIs except to ETR. Feng *et al.* (2014) showed that the interaction between the tyrosine (Y) at position 188 and DOR is crucial for its binding to reverse transcriptase. Mutations at this position would therefore significantly decrease DOR binding, leading to DOR resistance. Although this mutation is of major concern for the efficacy of DOR in NNRTI-experienced patients, it has a low prevalence among treatment-experienced patients in South Africa (Chimukangara *et al.*, 2019; Etta *et al.*, 2017).

Two single mutations (i.e., V106M, F227L) that showed *in vitro* phenotypic responses that were discrepant to the Stanford HIV Drug Resistance Database (Liu and Shafer, 2006) predictions were further investigated in lab-adapted PSVs in this study to understand whether the discrepancy could be as a result of subtype bias. No significant difference was observed between the phenotypic response of the single V106M mutation in subtypes B and C. Both subtypes displayed high-level resistance to DOR in our assay, which is in agreement with the literature. As discussed above the, V106M is commonly present in subtype C (not subtype B), and it is uncertain if the under-estimated resistance prediction by the Stanford HIV Drug Resistance Database could be as a result of limited data with regards to the response of V106M mutants to DOR in non-B subtypes. The single F227L mutation displayed susceptibility to DOR in our study, but high-level resistance was predicted by the Stanford HIV Drug Resistance Database (Liu and Shafer, 2006). The mutation is selected for by EFV, NVP and DOR and is commonly found in conjunction with V106A (Balzarini *et al.*, 1998; Brenner *et al.*, 2021; Reuman *et al.*, 2010; Smith *et al.*, 2016). In our subtype analysis, F227L displayed low-level resistance to DOR in three of the subtype B PSVs and susceptibility/hyper-susceptibility to DOR in all four subtype C PSVs. These differences between subtypes B and C PSVs were statistically significant and

appeared to be strain specific. Although this analysis could not explain the discrepancy with the predicted response, it does point out that the Stanford HIV Drug Resistance Database (Liu and Shafer, 2006) may over-estimate drug resistance associated with F227L to DOR amongst HIV-1 subtype C and some subtype B strains. Validation of our results with clinically derived viral variants may shed more light on the issue. Since the single F227L did not reduce DOR susceptibility in our study and has a low prevalence in South Africa (Etta *et al.*, 2017; Steegen *et al.*, 2017), F227L as single mutation would not impact on DOR treatment in NNRTI-experienced patients.

The assessment of DOR phenotypic drug susceptibility of HIV-1 subtype C mutant PSVs with combinations of NNRTI DRMs showed high-level resistance for the majority of mutants, particularly for combinations that contain V106M. This was less apparent for combinations that contained K103N. It has been well documented that the susceptibility to ARVs is reduced with an increasing number of resistance associated mutations (Mansky, 2002; Taiwo *et al.*, 2011). Asante-Appiah *et al.* (2021) have demonstrated that, in clinical isolates, EFV/NVP susceptibility decreased in the presence of one or more mutations, ETR/RPV susceptibility decreased in the presence of three or more mutations, while DOR susceptibility decreased in the presence of 5 or more mutations. Although we have observed high-level DOR resistance with only two combinations of NNRTI mutations, Asante-Appiah *et al.* (2021) did not specify which combinations of NNRTI mutations were tested in their study. They have noted, though, that a single NNRTI mutation (e.g., Y188L) has also caused resistance to DOR. Therefore, both the type and number of NNRTI DRMs will impact on DOR susceptibility. Another study published by Saladini *et al.* (2020) reported a reduced susceptibility to DOR in the presence of three or more NNRTI mutations, which included some of the mutations combinations we reported in this study (e.g., K101E, K103N, G190A, F227L, etc.).

A study by Rhee *et al.* (2023) analysed all the published *in vitro* phenotypic data on DOR and found that among NNRTI drug resistance combinations containing two or three mutations that the presence of V106A, F227C, Y188L and M230L significantly reduced DOR susceptibility, while the susceptibility of the three mutant combinations was reduced with V106A, F227C and M230L. Two of the studies included in this

analysis support our findings, as they both observed high-level DOR resistance in NNRTI combination mutation profiles containing V106A/M (Brenner *et al.*, 2021; Smith *et al.*, 2016). Smith *et al.* (2016) observed DOR susceptibility in L100I+K103N (FC = 3) and resistance in K103N+P225H (FC = 38), K103N+Y181C (FC = 18), while all of the combination mutations containing V106M showed high-level DOR resistance with FC>64. Brenner *et al.* (2021) did not examine combination mutations with K103N, but they similarly observed a FC>64 for combination mutants containing V106M.

In this study, the single mutations and the combination mutations were generated in the same backbone vector and PSVs were produced using the same methods, therefore the contribution of the single mutations to the combination mutations can be drawn by comparing their FC values. Six single mutations (i.e., A98G, V106M, V108I, Y188L and G190A) caused a significant decrease in DOR IC₅₀ compared to the wild-type. The V106M and Y188L single mutations caused the highest decrease in DOR susceptibility, which is also reflected in their combination with other mutations. The V106M+V179D combination's decrease in DOR susceptibility was not as extreme, but could be justified by the potential hyper-susceptible phenotype of V179D. Interestingly, V179D did not impact on DOR resistance in the V106M+V179D+F227L and V106M+V179D+Y188L combinations. A98G and V108I were not observed in combination with V106M, but in combination with K103N. Although both single mutations showed a low level of resistance to DOR, the addition of K103N to A98G reduced DOR susceptibility much more than the addition of K103N to V108I. The further addition of P225H to the combinations lead to a drastic reduction in DOR susceptibility. In fact, the K103N+P225H combination also showed a high level of resistance to DOR. All combinations that contained either the G190A or F227L mutations also contained the V106M mutation, which causes a high level of resistance to DOR. Thus, the contribution of these two mutations in combinations with other NNRTI mutations cannot be gauged from this dataset. The addition of K103N to either K101P or E138A, all of which were mostly susceptible to DOR when tested in isolation, did not lead to a decrease in DOR susceptibility.

K103N+P225H, K103N+Y188L displayed high-level DOR resistance (Lai *et al.*, 2014; Smith *et al.*, 2016). L100I+K103N had conflicting results with Smith *et al.* (2016) finding

the combination susceptible whereas Lai *et al.* (2014) and this study found it resistant. These results support our findings wherein all these mutations caused high-level resistance. L100I+K103N was resistant in our findings. Also supporting our findings, K101P+K103N was shown to be susceptible to DOR (Saladini *et al.*, 2020). Y181C was not tested in combination with other NNRTI mutations since these combinations were not prevalent (K103N+Y181C is 0.44%) in our setting. K103N and Y181C were tested in combination in the following studies due to the prevalence of the individual mutations.

Basson *et al.* (2015) found that the combination of K103N and Y181C was not susceptible to ETR (FC = 9.5), RPV (FC = 5.7), EFV (FC = 29.8) and NVP (FC = 90). A reduction in susceptibility to these four NNRTIs was similarly reduced when Y181C was combined with A98G, K101E/H, V179F, G190A/S and H221Y. Lai *et al.* (2014) observed a similar reduction in susceptibility to EFV, RPV and ETR by K103N+Y181C with all of them having an FC>10. The combination of K103N and Y181C also reduced susceptibility to DOR with FC values of 4.3 and 4.9 in Lai *et al.* (2014) and Saladini *et al.* (2020) respectively, while viruses with individual mutations remained susceptible. This reduction in susceptibility is not as high for EFV, ETR, NVP and RPV.

A study by Saladini *et al.* (2020) supported this study finding that resistance to DOR significantly increased (p-value = 0.005) for combination mutations. They noted that multiple mutations selected for by ETR and RPV confer cross-resistance to DOR. Their results correlate with our results, showing that DOR does not overcome the NNRTI resistance in combination mutants previously observed. Stanford also underestimated the level of resistance of the combination mutants to DOR, where high-level resistance was only predicted for six of the combination mutants. There is minimal literature on the effect of DOR in the presence of multiple NNRTI mutations, likely due to the fact that NNRTI resistance is usually conferred with a single mutation. However, this is important particularly in South Africa where there is a high prevalence of NNRTI mutations thus there is an increased risk of multiple NNRTI mutations being present. Therefore, more studies need to be conducted on the impact of combination NNRTI mutations in subtype C on DOR. This suggests that use of DOR as third-line ART in South Africa may not be feasible, since patients failing PI-based second line

therapy will likely retain the NNRTI mutations from first-line therapy. Therefore, DOR should only be recommended to patients who were previously on treatment with an NNRTI after genotypic testing.

The Stanford Drug Resistance database (Rhee *et al.*, 2003; Shafer, 2006) acknowledges the following NNRTI DRMs as transmissible: L60I, K101E, K101P, K103NS, V106AM, V179F, Y181CIV, Y188CHL, G190AES, P225H and M60L. A study performed by Rhee *et al.* (2023) analysed 168 studies on ART-naïve patients from 2017 to 2021, and found that majority of ART-naïve patients had a single NNRTI mutation (82.6%), with the most prevalent mutations being V106I, K103N, V108I, G190A and Y181C. Several studies have reported on transmitted NNRTI DRMs in South Africa, and mutations at positions 100, 101, 103, 106, 181 and 188 were commonly observed in ART-naïve patients (Chimukangara *et al.*, 2019; Gupta *et al.*, 2018; Hunt *et al.*, 2019; Lippman *et al.*, 2020). Since V106M and Y188L showed significant reductions in DOR susceptibility in our study, the presence of these two mutations would be of greatest concern regarding the efficacy of DOR on transmitted NNRTI mutants. The prevalence of these mutations in ART-naïve patients was 0.80% for V106M and 1.19% for Y188L with the combination of these two mutants having a prevalence of 0.03% the study by Rhee *et al.* (2023). This suggests that only a small portion of newly HIV-infected patients will be resistant to DOR this supports the implementation of DOR in first-line ART. This also makes DOR a good candidate for PrEP, since it will remain effective against the prevalent transmitted NNRTI mutations.

DTG has a high genetic barrier to the development of INSTI resistance (Llibre *et al.*, 2015) and has been included in the South African National Treatment guidelines since 2019 (NDH, 2019). However, a steady increase in DTG resistance has been observed since its implementation in first-line ART (Hans *et al.*, 2023; Steegen *et al.*, 2019). Additionally, there has been a concern regarding weight gain on DTG-based regimens in South African women (Venter *et al.*, 2019). The unique pharmacokinetics of DTG may also lead to drug-related sensitivities in certain individuals (Cattaneo *et al.*, 2017; de Boer and Brinkman, 2017). Therefore, despite DTG's potency, it may not be an accommodating treatment option for some, and DOR may then be a possible alternative. In fact, DOR is recommended in Botswana as an alternative to DTG during

first-line therapy for people who present with intolerance to DTG, and is recommended in second-line therapy in conjunction with Darunavir (DRV) (Republic of Botswana Ministry of Health, 2023). An additional benefit of DOR is that, unlike most other ARVs (including DTG), it does not interact with antacids (Kandel and Walmsley, 2015). This means patients can take the antacids without increasing the dosage of DOR, thus decreasing the side-effects they would experience. Therefore, DOR may be offered as a solution to patients experiencing undesired side effects or drug-interactions on DTG, with genotypic drug resistance testing recommended if the patient has previously been exposed to an NNRTI.

Clinical cut-off's for *in vitro* phenotypic drug resistance tests are currently not available for DOR. A lower technical cut-off was established for DOR susceptibility in our *in vitro* assay, and the susceptibility/resistance classifications were based on incremental increases of this cut-off value. As such, our classifications were not linked to clinical outcomes and may have under- or over-estimated resistance to DOR. The discrepancies that we've observed between our data and the predictions made by the Stanford HIV Drug Resistance Database (Liu and Shafer, 2006) may be as a result of this. Another method of classifying resistance/susceptibility is by using a biological cut-off which is defined as the average FC + 2 standard deviations measured in 1000 ARV-naïve patients as this would provide an improved threshold for defining abnormal changes in drug susceptibility (Harrigan *et al.*, 2001). A study by Asante-Appiah *et al.* (2021) used a cut-off of 3-fold to classify susceptibility in clinical isolates to DOR, in a similar assay (i.e., Monogram Biosciences) to the one used in our study. Although their cut-off was higher than the one we established for our assay (i.e., 1.31 FC), we defined low-level resistance only at a FC > 2.6, which is close to their cut-off value. This supports the use of 2.6 FC as lower cut-off in our analysis. and hence classification of DOR resistance/susceptibility. However, a study performed by Parkin *et al.* (2004) investigated the difference between biological and clinical cut-offs for seven ARVs in a similar assay (i.e., Monogram Biosciences). They noticed that the biological cut-offs were lower than the clinical cut-offs and the biological cut-offs established in other phenotypic assays. This study demonstrates that clinical cut-offs are essential to inform on clinical outcomes for a drug and that more studies have to be conducted to determine biological and clinical cut-offs for DOR. This would involve phenotypic

testing of clinically-derived variants obtained from patients with known clinical outcomes on DOR-based treatment. The result in this study should be confirmed by testing the NNRTI mutations in clinical isolates to get a better understanding of the clinical outcome profiles associated with these mutations.

In summary, V106M and Y188L, either individual or in combination with other NNRTI DRMs, showed the largest decreases in DOR phenotypic susceptibility in this study. Although the highly prevalent K103N mutation was susceptible to DOR by itself, its combination with P225H (and other mutations), led to a large decrease DOR susceptibility. The K103N, V106M and P225H mutations are preferentially selected for by EFV (Reuman *et al.*, 2010) while Y181C, Y188L is preferentially selected for by NVP. Currently, NVP has been phased out of first-line ART recommendations and South Africa is transitioning away from the EFV-based first-line regimen to the DTG-based first-line regimen. It can therefore be expected that a decline in the above-mentioned mutations, in both acquired and transmitted drug resistance, will be observed. However, due to the persistence of NNRTI DRMs after cessation of NNRTI exposure, as we have observed in the sequences of PI-based failures in this study, DOR would most likely not be suitable for approximately an estimate of 30% of NNRTI- and PI-experienced patients in South Africa based on the prevalence of the DOR resistant mutations after first-line NNRTI failure. Genotypic drug resistance testing would likely be required to identify NNRTI DRMs before including DOR in the regimens. Since genotypic drug resistance testing is recommended before switching to a third-line regimen, the efficacy of DOR could be assessed after second-line failure. This would not, however, affect the presence of archived NNRTI drug resistance that may impede DOR treatment. Therefore, DOR would currently be most suitable for the treatment of ART-naïve patients, and preferentially in combination with its synergistic partner ISL when this novel NRTI is approved.

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A.Appendix: Supplementary Figures

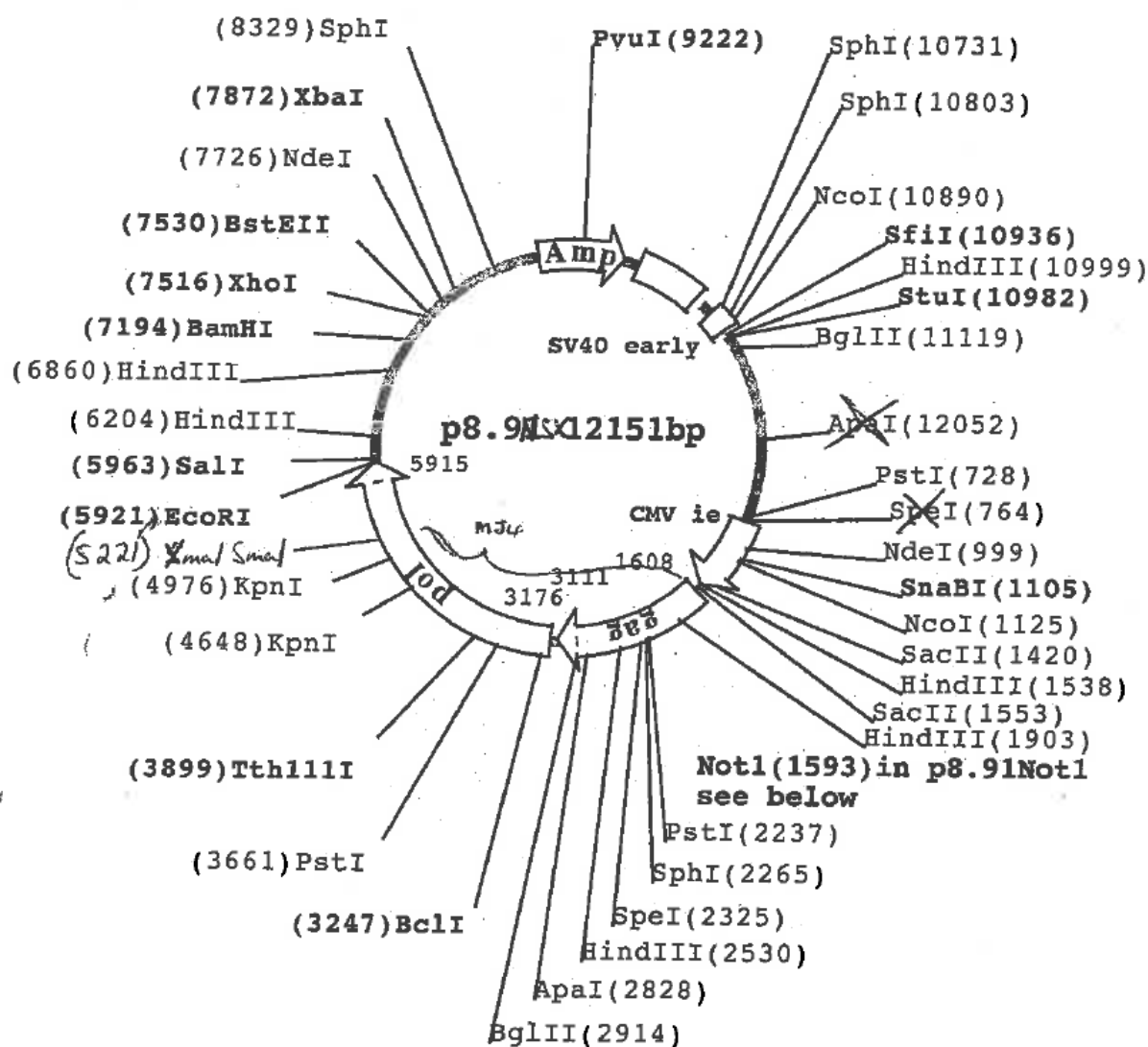


Figure A.1: Plasmid map of p8.9NSX+/p8.9MJ4 (packing plasmid)

This plasmid map is taken from (Naldini *et al.*, 1996). p8.9NSX+ contains HIV-1 subtype B *gag-pol*. p8.9MJ4 contains MJ4 (GenBank accession number AF321523.1) HIV-1 subtype C *gag*, *protease* and majority of the *reverse transcriptase* genes Ndung'u *et al.* (2001).

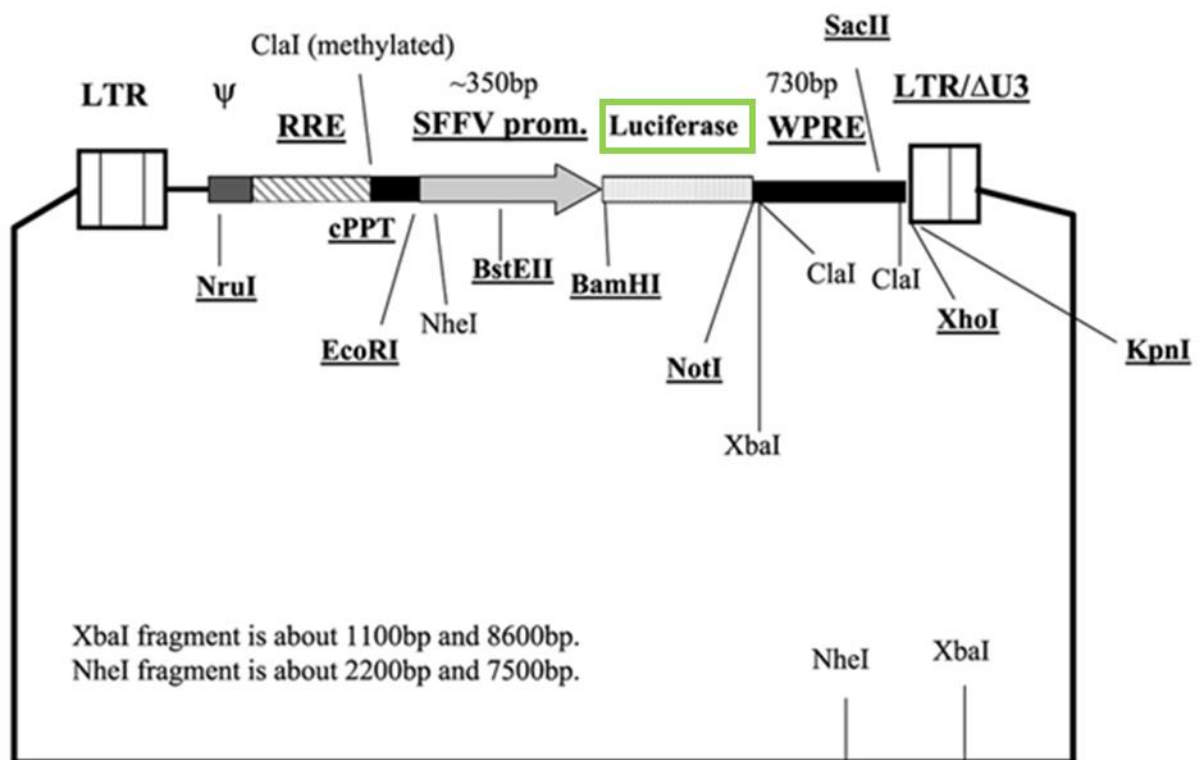


Figure A.2: Plasmid map of pCSFLW (transfer plasmid) containing the luciferase gene

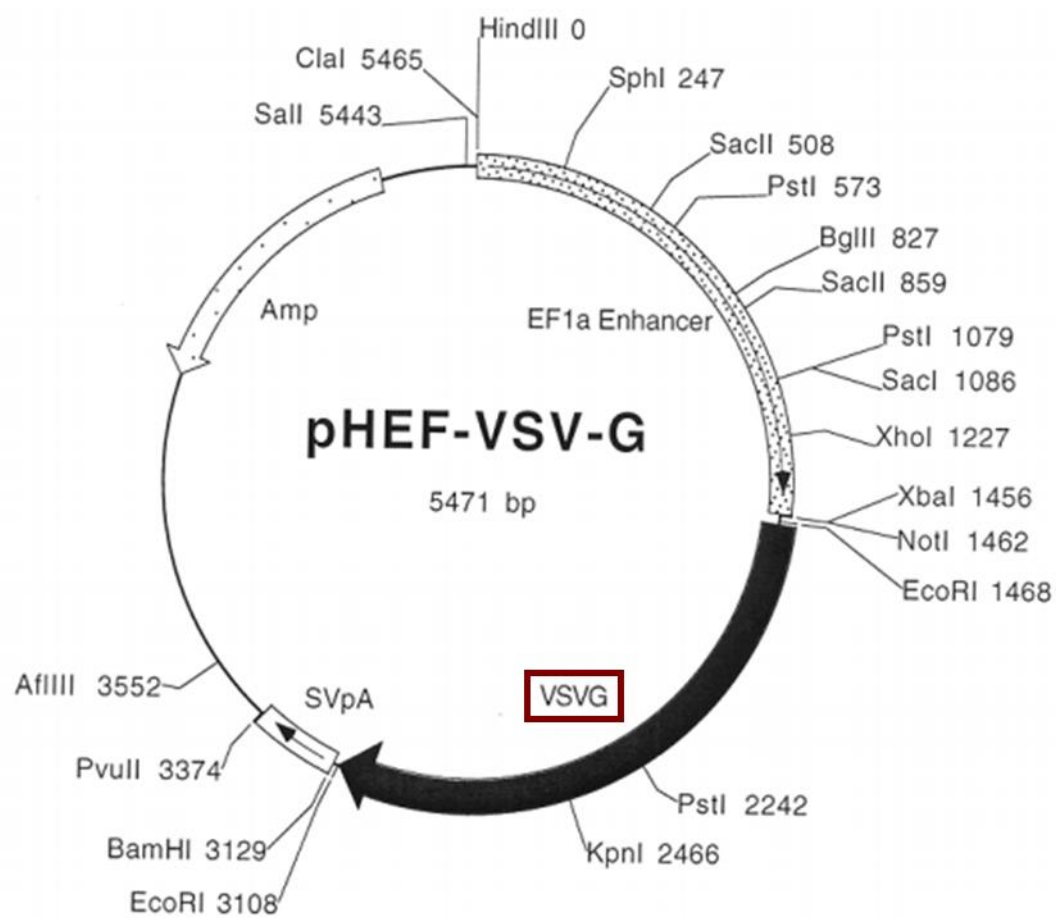
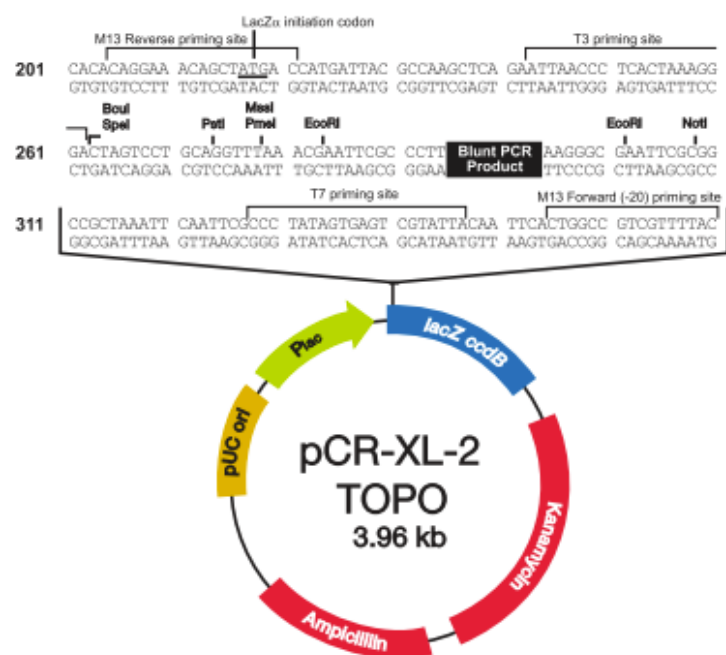


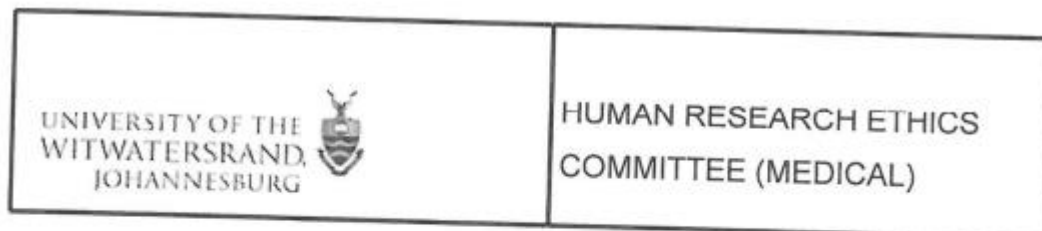
Figure A.3: Plasmid map of pHEF.VsVg (envelop plasmid)



Element	Position
<i>lac</i> promoter region	bases 2–216
Transcription start site	base 179
M13 Reverse priming site	bases 205–221
LacZα- <i>ccdB</i> gene fusion	bases 217–810
T3 priming site	bases 243–262
TOPO™ Cloning site	bases 294–295
T7 priming site	bases 328–347
M13 Forward (–20) priming site	bases 355–370
Kanamycin resistance gene	bases 1159–1953
Ampicillin resistance gene	bases 2203–3063
pUC origin	bases 3161–3834

Figure A.4: Plasmid map of the TOPO vector

B.Appendix: Ethics Waiver



Office of the Deputy Vice-Chancellor (Research & Innovation)

TO: Dr AE Basson
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CC: Supervisor: N/A <N/A>
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FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 03/05/2022

REF: R14/49

PROTOCOL NO: W-CBP-220503-01 (This is your ethics application study reference number.
Please quote this reference number in all correspondence relating to this
study)

PROJECT TITLE: *Screening of synthetic compounds and natural products for
activity against HIV-1*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.



MSWorks2000/Iain0007/ClearScanWaiver.wps

 UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
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Office of the Deputy Vice-Chancellor (Research & Innovation)

03/05/2022

Ref: W-CBP-220503-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Dr AE Basson
Student No. (if appropriate):
Staff No. (if appropriate): A0033617

Supervisor: N/A

School: Pathology
Department: Molecular Medicine and Haematology
HIV Pathogenesis Research Unit
Medical School
University

Project title: *Screening of synthetic compounds and natural products for activity against HIV-1*

Reason: In vitro laboratory study
No human participants will be involved in the study



Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

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C.Appendix: Plagiarism Report



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Assessment of the in vitro phenotypic drug susceptibility of HIV-1 subtype C drug-resistant variants to Doravirine (DOR)

Nikita Reddy
1840175

A dissertation submitted to the Faculty of Health Sciences,
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in fulfillment of the requirements for
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