# Exploring the Impact of Antiretroviral Drugs on the Cell-to-Cell Spread of HIV-1

**A Dissertation for the Degree of Doctor of Philosophy**

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## DECLARATION

I, Boghuma Kabisen Titanji, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

# ABSTRACT

Recent observations on the reduced susceptibility of HIV-1 cell-to-cell infection to inhibition by Reverse Transcriptase Inhibitors (RTIs) have raised questions on the bearing this mode of spread may have on the successful treatment of HIV-1, the maintenance of viral reservoirs and viral pathogenesis. This thesis presents a detailed assessment of the individual drug classes, which constitute first-line and second-line antiretroviral therapy, with regard to their ability to inhibit HIV-1 cell-to-cell infection in comparison to cell-free infection. Special emphases is given to the study of Protease Inhibitors (PIs), which have a mechanism of action different from RTIs, present a higher barrier to the selection of drug-resistant viruses, are highly potent and very important in both first-line and second-line treatment of HIV-1 infection. Also, PIs have not been studied before in the context of cell-to-cell spread of HIV-1. The results obtained show that different classes of antiretroviral drugs have different potencies against cell-to-cell spread of HIV-1. While PIs are equally effective at inhibiting cell-to-cell and cell-free spread of HIV-1, RTIs especially those of the Nucleoside Reverse Transcriptase Inhibitor (NRTI) class are ineffective inhibitors of cell-to-cell spread of the virus. This thesis also assesses the impact of combination antiretroviral therapy on these two modes of viral infection, using drug synergy analysis by the median effect principle. We show that combination antiretroviral therapy is effective against both cell-to-cell and cell-free HIV-1 infection. However in the context of antiretroviral drug resistance, cell-to-cell spread may contribute to a reduced efficiency of combination antiretroviral therapy in blocking the spread of infection. Overall, the study provides a better understanding of the impact of antiretroviral therapy on cell-to-cell spread of HIV-1 and within reason, bearing in mind the limitations of *in vitro* models, gives some insight on the possible clinical implications of these observations for current HIV-1 therapy.

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# List of Abbreviations

3TC - Lamivudine

ABC - Abacavir

AIDS - Acquired Immune Deficiency Syndrome

Alb - Albumin

ANOVA- Analysis of variance

APC – Antigen Presenting Cell

APOBEC - Apolipoprotein mRNA editing enzyme catalytic polypeptide

APV- Amprenavir

ARV- Antiretroviral

ATP – Adenosine Triphosphate

ATZ – Atazanavir

AZT- Azidothymidine

CA – capsid

CCR5 – Chemokine receptor type 5

CD - Cluster of designation

CDC – Centers for Disease Control

cDNA – complementary deoxyribonucleic acid

CFAR – Centre for AIDS research

Cpz - chimpanzee

CT – cytoplasmic tail

CTS – Central termination signal

CXCR4 – Chemokine (C-X-C motif) receptor 4

DC – Dendritic cell

DHHS – Department of Health and Human Services

DLV – Delaviridine

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulphoxide

DNA- Deoxy ribonucleic acid

dNTP- deoxyribonucleotidetriphosphate

DRV- Darunavir

EDTA – Ethylene diaminetetracetic acid

ELISA – Enzyme linked immunoabsorbent assay

Env - Envelope

ESCRT – Endosomal Sorting Complex Required for Transport

ETV - Etravirine

FACS – Fluorecence activated cell sorting

FCS – Fetal calf serum

FDA – Food and drug administration

Gag – Group-specific antigen

Gor – Gorilla

HAART – Highly active antiretroviral therapy

HB – Helix bundle

HEK – Human embryonic kidney

HIV – Human Immunodeficiency Virus

HRP – Horseradish peroxidase

HTLV – Human T Lymphocyte Virus

ICAM – Intercellular cell adhesion

Ig - Immbunoglobulin

IL - Interleukin

InSTI – Integrase strand transfer inhibitor

IS – Immunologic Synapse

LB – Luria Bertani

LEDGF- Lens epithelium derived growth factor

LFA-1- Lymphocyte function Antigen

LPV- Lopinavir

LTR- Long terminal repeat

MA – Matrix

MACS – Magnetic activated cell sorting

Mg – Magnesium

MHC – Major Histocompatibility Complex

Min -minutes

MLV – Murine Leukemia Virus

MOI – Multiplicity of Infection

MTOC – Microtubule organizing centre

MVC – Maraviroc

Nef – Negative factor

NIBSC – National Institute of Biological Standards and Control

NIH – National Institutes for Health

NNRTI – Non nucleoside reverse transcrptase inhibitor

NRTI- Nucleos(t)ide reverse transcriptase inhibitors

NUP – Nucleoporin

NVP- Nevirapine

P-TEFb – Positive transcription elongation factor b

PBMC – Peripheral blood mononuclear cells

PBS- Phosphate buffered saline

PCR- Polymerase Chain Reaction

PEP – Post exposure prophylaxes

PHA- Pytohemgglutinin

PI – Protease Inhibitor

PIC – Pre-integration complex

Pol - Polymerase

PPT – Polypurune tract

PreP – Pre-exposure prophylaxes

RAL- Raltegravir

Rpm – revolutions per minute

RPMI – Roswell Park Memorial Institute

RPV - Rilpivirine

RT-PCR – Real-time polymerase chain reaction

RTI - Reverse transcriptase

RTV – Ritonavir

SAMHD1 – Sam domain and HD domain containing protein 1

SAQ – Saquinavir

SDM – Site directed mutagenesis

SIV – Simian Immunodeficiency virus

Sp - Spacer

T-20 – Enfurvitide

TAE – Tris acetate EDTA

Tat - Transactivator

TBS- Tris buffered saline

TCR – T cell receptor

TFV - Tenofovir

TRIM5α – Tripartite motif 5 alpha

Vif- viral infectivity factor

Vpr – viral protein R

Vpu- viral protein U

VS – Virological synapse

WHO – World Health Organisation

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# 1 Introduction and Background

## 1.1 Introduction

The Human Immunodeficiency Virus (HIV) was first identified as the causative agent of the Acquired Immunodefiency Syndrome (AIDS) at the Pasteur Institute in Paris in 1983 (Barré-Sinoussi et al., 1983). Two related human retroviruses can cause AIDS in humans; HIV type-1 (HIV-1) (Barré-Sinoussi et al., 1983) has a worldwide distribution and is responsible for the global pandemic while HIV type-2 (HIV-2) (Clavel *et al*., 1986) which is primarily confined to West Africa, is responsible for a much smaller proportion of infections (Schim van der Loeff and Aaby, 1999). The discussions in this thesis will focus on HIV-1.

In the three decades that have followed its discovery, HIV/AIDS has caused over 36 million deaths and an estimated 65 million people have become infected with the virus worldwide (UNAIDS, 2013). In 2013 there were an estimated 35.3 million people living with HIV-1 infection globally (UNAIDS, 2013). These elements make HIV/AIDS one of the biggest epidemics of the last century and have spurred intense scientific research towards finding effective treatments for HIV/AIDS. There has been significant progress in the treatment of HIV/AIDS and the therapeutic options currently available have transformed what was once a death sentence for those infected, into a chronic and manageable condition. The current arsenal of antiretroviral agents allows infected individuals receiving treatment to live normal lives and have the same life expectancy as healthy uninfected individuals. With more people receiving life-saving antiretroviral therapy (ART) and widespread public health campaigns aimed at reducing transmission,

there has been a steady decline in the number of new infections from 3.4 million in 2011 to 2.3 million in 2013 (UNAIDS, 2013).

Despite the success of existing ART in controlling viral replication, treatment is not curative and remains a life-long commitment for infected individuals. HIV-1 also has the ability to persist in reservoirs within the body and to re-emerge if there are therapeutic lapses. This feature and the development of drug-resistant variants, continue to frustrate the efforts directed towards finding a definitive cure and an effective vaccine.

Furthermore, the roll out of antiretroviral therapy has not been without its challenges. In 2013, under the current WHO treatment guidelines, coverage of antiretroviral treatment remained low with only 34% of the 28.6 million people eligible for treatment having access to therapy (UNAIDS, 2013). Also, rising drug resistance to the available antiretroviral drugs poses a serious challenge for clinicians and patients (Gupta *et al*., 2012, WHO, July 2012) especially in resource poor settings where second and third-line treatment options are often limited or non-existent. Patients receiving ART do not necessarily always achieve full immune recovery and remain at a high risk of developing HIV-related malignancies as well as exhibiting increased levels of immune activation and persistent inflammation (Long *et al*., 2008, Hasse *et al*., 2011). A small subset of treated patients, termed CD4+ immunologic non-responders, have suppression of viral replication but their CD4+ T cell levels fail to rebound to normal on ART (Aiuti and Mezzaroma, 2006, Lewden *et al*., 2007, Gazzola *et al*., 2009, Valdez *et al*., 2002). In addition to the emergence of drug resistance and the ability of the virus to persist in reservoirs within the body, recent studies suggest that cell-to-cell spread of HIV-1 across a virological synapse (VS) may serve

as an additional mechanism of antiretroviral escape for the virus (Sigal *et al*., 2011).

Cell-to-cell spread is a very efficient mode of retroviral dissemination, which allows for directed virus transfer across a virological synapse, obviating the need for prolonged fluid-phase diffusion (Jolly and Sattentau, 2004, Jolly et al., 2004, Igakura et al., 2003, Sattentau, 2008, Sattentau, 2010, Martin et al., 2010). This mode of viral spread is more efficient than the classical mode of cell-free diffusion (Mazurov et al., 2010, Dimitrov et al., 1993, Johnson and Huber, 2002, Jolly et al., 2007, Sourisseau et al., 2007, Martin et al., 2010) and may be less sensitive to neutralisation by antibodies (Abela et al., 2012, Sourisseau et al., 2007, Sattentau, 2008, Sattentau, 2010, Piguet and Sattentau, 2004, Malbec et al., 2013). Cell-to-cell spread confers a replicative advantage for the virus. This is mediated by direct physical interaction between effector cells and target cells, and may be especially important in lymphoid tissues where densely packed CD4+ T lymphocytes increase the likelihood of frequent contacts. This could potentially contribute to the maintenance of the virus reservoir (Sewald *et al*., 2012, Sigal and Baltimore, 2012).

Indeed, BLT (bone-marrow, liver and thymus) humanised mice based studies using the technique of intravital imaging have recently validated the concept of the virological synapse *in vivo*, highlighting its putative relevance in the spread of retroviruses (Sewald *et al*., 2012, Murooka *et al*., 2012). Sigal *et al*. were the first group to suggest that cell-to-cell virus transfer may be a mechanism by which HIV-1 evades the effects of antiretroviral drugs and thus continues to replicate at low levels in treated patients (Sigal *et al*., 2011). In their study, they proposed that the large number of viral particles that are transmitted to an uninfected target cell during cell-to-cell transfer, increases the

probability that at least one virus particle will stochastically escape inhibition by drugs and proceed to infect the cell (Sigal *et al*., 2011). They tested this hypothesis by assessing the effects of Reverse Transcriptase Inhibitors (RTIs) on virus dissemination in an *in vitro* experimental model and found that cell-to-cell spread was less sensitive to inhibition by RTIs than cell-free transmission (Sigal *et al*., 2011). While interesting, these findings raise significant questions regarding the impact of other antiretroviral drug classes that constitute conventional cART, notably Protease Inhibitors (PIs) and Integrase Inhibitors (INIs), on HIV-1 cell-to-cell infection. Also the relevance of this observation in the context of cART has been brought to debate, given that triple therapy as currently prescribed is generally effective in patients treated for HIV-1 infection. In this thesis, *in vitro* co-culture systems are used to study the impact of antiretroviral drugs on HIV-1 cell-to-cell spread, with the aim of better defining the role and possible implications of cell-to-cell spread of HIV-1 in the context of antiretroviral therapy.

The data presented in this thesis provide a detailed assessment of the impact of the different components of ART on HIV-1 cell-to-cell spread in comparison to cell-free spread, with a focus on PIs and INIs, which have not been previously studied in the published literature. It also explores the effects of clinically relevant drug combinations on cell-to-cell spread of HIV-1 and provides an assessment of antiretroviral drug interactions in the context of cell-to-cell infection with wild type and drug-resistant variants of HIV-1. The HIV/AIDS pandemic is now thirty years old and there is a growing realisation that we may not be able to treat ourselves out of this pandemic. The untenable cost of lifelong therapy has shifted the focus towards a push for HIV-1 eradication. An understanding of the mechanisms of viral persistence and the establishment of viral reservoirs is crucial for developing novel

eradication strategies. In the present study, *in vitro* cell-to-cell assay systems are employed to address these specific questions in relation to antiretroviral therapy, cell-to-cell spread and antiretroviral drug resistance.

## 1.2 The origins, diversity and global distribution of HIV-1

Studies of primate lentiviruses using phylogenetic tools have enabled the origins of HIV-1 to be unravelled in recent years. Lentiviruses have been detected in more that 30 non-human primate species found in sub-saharan Africa (Sharp *et al*., 1995, Sharp and Hahn, 2011). These Simian Immunodeficiency Viruses (SIVs) appear to be for the most part non-pathogenic in their natural hosts (Sharp and Hahn, 2011). It is now generally accepted that the 4 distinct groups of HIV-1 that have so far been identified, came about as a result of at least four separate cross-species transmission events of SIVs from primates to humans (Sharp and Hahn, 2011). Pandemic HIV-1 group M is most closely related to SIVcpz, whose primary host is the chimpanzee (*Pan troglodytes*). Group M (Main), was the first to be identified and is responsible for almost the entire human pandemic of HIV-1. It is responsible for millions of infections worldwide and has been identified in virtually every location across the globe (Sharp and Hahn, 2011). Group N (New), has only be isolated from 13 individuals (Simon *et al*., 1998), all from Cameroon and like group M HIV-1 is most closely related to SIVcpz from chimpanzees. Group O (Outlier) is less prevalent, representing <1% of infections worldwide and is mainly restricted to small pockets in some central African countries like Cameroon and Gabon (De Leys *et al*., 1990, Gurtler *et al*., 1994, Peeters *et al*., 1997). Very recently the origin of HIV-1 group O has been traced to SIVgor, a SIV found in western low land gorillas in Cameroon (D'Arc, 2014). Group P HIV-1 is also closely related to SIVgor from gorillas. It is very rare and only two cases have so far

been reported in patients from Cameroon (Plantier *et al*., 2009, Vallari *et al*., 2011). A lot of what is known about HIV-1 with regard to transmission, pathogenesis, and treatment is based on studies of group M viruses. This group can be further sub-divided into 9 subtypes or clades (A-D, F-H, J, K) as well as over 40 circulating recombinant forms, which arise when infection of the same population by multiple sub-types occurs (Sharp and Hahn, 2011). The distribution of group M clades is illustrated in Figure 1.1. Subtype C is predominant in southern and eastern Africa, India, Nepal and China (Wainberg, 2004). A and A/G recombinants are mainly found in West and Central Africa (Wainberg, 2004). Clade B viruses are predominant in North America, Western Europe and Australia and most studies on drugs and resistance studies have been based on this subtype (Wainberg, 2004). The subtype classification is centred on the alignment of env sequences where 20-50% differences are seen between subtypes. More recent classifications also take into consideration protease and reverse transcriptase sequences, which show 10-12% variation at the nucleotide level or 5-6% variation at the amino acid level (Wainberg, 2004).

Some studies have suggested that the different sub-types of HIV-1 may possess unique biological properties and these could have implications for the rates of transmission, pathogenesis, disease progression and response to treatment. For example, infections with subtype D viruses have been associated with greater pathogenicity and more rapid disease progression in some east African studies (Kiwanuka *et al*., 2010). One study from Thailand suggested that subtype E viruses might be better transmitted through heterosexual contact than sub-type B viruses, explaining the predominance of sub-type E infections in this region (Kunanusont *et al*., 1995). Sub-type C viruses have spread very rapidly throughout southern Africa and the Indian sub-continent and

are now responsible for 50% of HIV-1 infections worldwide (Taylor and Hammer, 2008). This suggests that sub-type C viruses are inherently more transmissible in heterosexual populations than other subtypes. In a Kenyan study, the genital tract viral loads were found to be higher for women infected with subtype C viruses when compared to women infected with subtype A or D viruses, supporting the hypothesis of the better transmissibility of subtype C viruses (John-Stewart *et al*., 2005). Also in another study, Tanzanian mothers infected with sub-type A and C viruses where found to have an increased risk for mother-child transmission in comparison to mothers infected with subtype D virus (Renjifo *et al*., 2001, Renjifo *et al*., 2004). Another study found no differences in the mother-to-child transmissibility of the different sub-types of group M viruses, highlighting the controversy that still exists in the studies in this area (Eshleman *et al*., 2005).

Concerning antiretroviral therapy, several studies suggest that the response to antiretroviral therapy is similar among patients infected with different clades of group M virus (Alexander *et al*., 2002, Pillay *et al*., 2002, Bannister *et al*., 2006, Gatell, 2011, Scherrer *et al*., 2011). Some studies however have shown that differences exist in the frequency and pathways leading to the selection of drug-resistant variants in non-B clade viruses. Subtype D viruses appear to possess a natural resistance to non-nucleoside reverse transcriptase inhibitors, based on a single nucleotide substitution (Gao *et al*., 2004) and non-subtype B viruses appear to more frequently select for minor drug resistance mutations in protease (Pieniazek *et al*., 2000). The specific effects of subtype variability in relation to cell-to-cell spread of HIV-1 have not been investigated. Given the afore mentioned observations and the putative relevance of HIV-1 cell-to-cell spread for treatment and vaccine development, it would be interesting to find out whether all

HIV-1 subtypes exhibit comparable efficiencies in their ability to form virological synapses and spread efficiently from cell-to-cell.

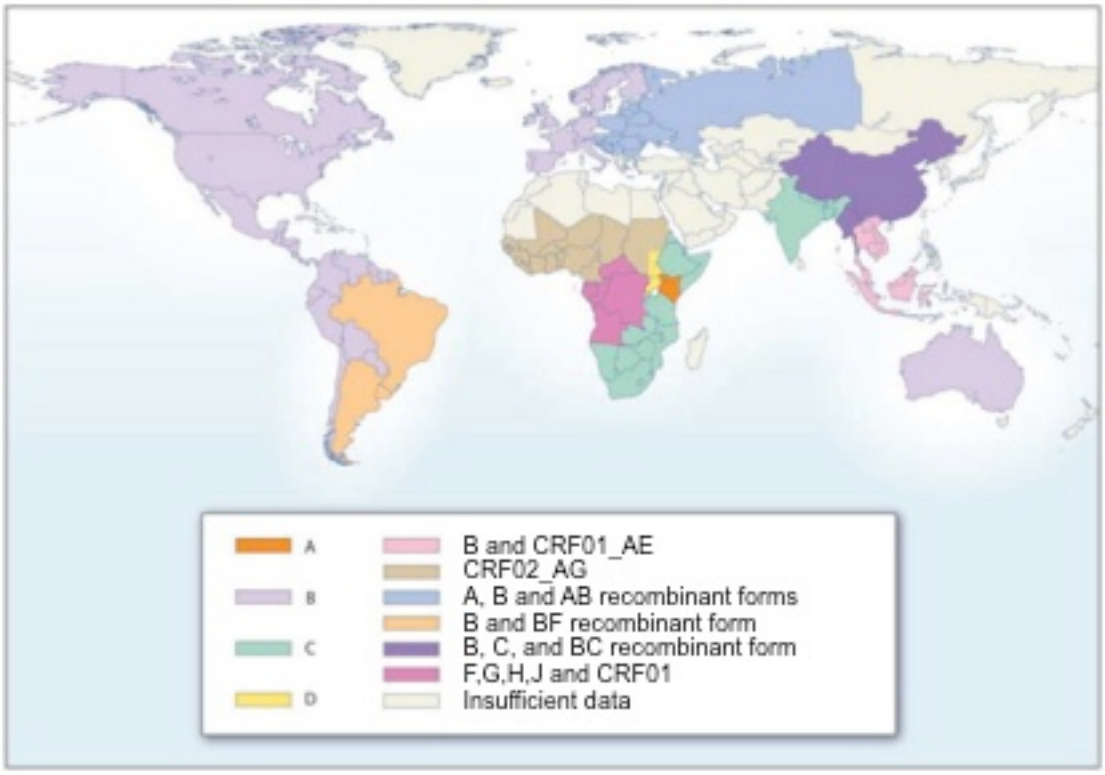


**Figure 1-1**: Current Global distribution of HIV-1 subtypes and recombinant forms (Taylor and Hammer, 2008)

# 1.3 HIV-1: Structure, Function and Replication

## 1.3.1 HIV-1 Structure and function

HIV-1 is a virus that belongs to the family of Retroviridae, the subfamily lentivirinae and the genus lentiviridae. The HIV-1 virion is spherical in shape and has an average diameter of 110nm. The viral genome consists of two copies of single stranded positive sense ribonucleic acid (RNA) in complex with viral enzymes, surrounded by a lipid-based envelope that is derived from the host cell membrane. The surface glycoprotein envelope (Env) of HIV-1 is initially synthesised as a precursor polypeptide gp160. This precursor polypeptide is subsequently cleaved by the cellular protease furin to generate the receptor glycoprotein or "spike" gp120 and the transmembrane and fusion-peptide containing component gp41 that remain non-

covalently associated (Freed, 2003, Sundquist and Krausslich, 2012). Gp120 projects from the surface of the viral envelope and mediates the attachment to target cell receptors (CD4) and co-receptors (CCR5 and CXCR4). The transmembrane glycoprotein gp41 facilitates fusion of the viral envelope with the target cell membrane and also contains essential trafficking determinants within its cytoplasmic tail (Checkley *et al.*, 2011). The viral lipid envelope surrounds an internal protein layer called the matrix (p17), which is derived from the Gag polyprotein (p55) following proteolytic processing and is anchored to the internal surface of the virus envelope. The HIV-1 genome is approximately 9.7 kilobases and encodes major structural and non-structural proteins common to all replication competent retroviruses. The viral genome and proteins are held in a cone-shaped protein core/capsid Gag (p24) that is also the result of Gag p55 processing.

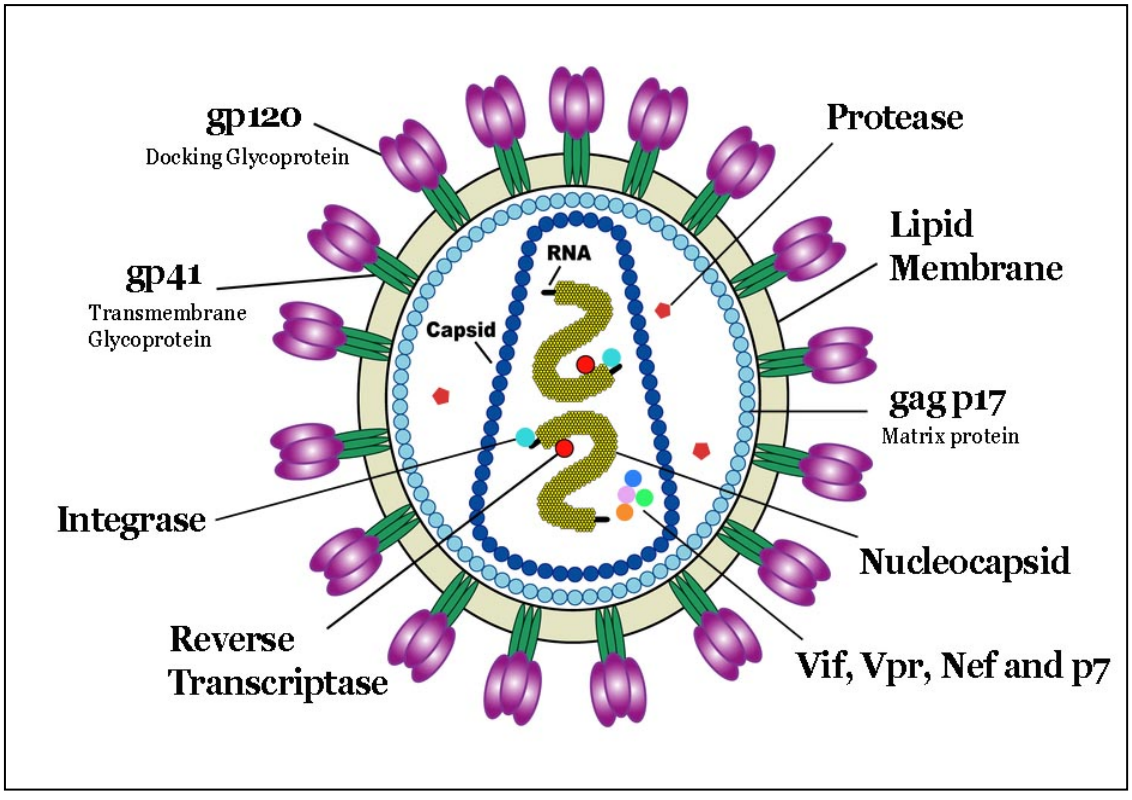


**Figure 1-2**: HIV-1 Structure (http://upload.wikimedia.org/wikipedia/commons/3/31/800px-HIV_Viron_es.png)

From the 5'- 3' ends, the HIV-1 genome is made up of 9 genes that code for 15 viral proteins. The three main virus genes are gag (group-specific antigen), pol (polymerase), and env (envelope). In addition to these, are 4 accessory genes; vif, vpr, vpu, and nef that generally modulate host responses to the virus and two regulatory genes tat and rev, which regulate steps in the replication cycle of the virus.

*Gag* - Encodes the polyprotein precursor Pr55Gag that is cleaved by the viral protease to mature structural proteins; matrix (p17), capsid (p24), nucleocapsid (NC or p7), p6 as well as the two spacer proteins p2 and p1 (Freed, 2003, Wiegers *et al*., 1998).

*Pol* - Codes for the polyprotein precursor Pr160GagPol that is processed by the viral protease into individual virus enzymes Protease (PR), Reverse Transcriptase (RT) and Integrase (IN)(Freed, 2003).

*Env* - Codes for a polyprotein precursor gp160 that is processed by host cell proteases during the trafficking of the virus envelope to the surface of the cell. Gp160 is cleaved into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41. These two surface glycoproteins remain non-covalently associated and are collectively referred to as Env (Freed, 2003, Sundquist and Krausslich, 2012).

*Vif* - Codes for the virion infectivity factor (Vif). Vif plays a role in suppressing the viral restriction factors APOBEC3G and APOBEC3F by binding and targeting these proteins for degradation via an ubiquitin pathway (Schrofelbauer *et al*., 2004, Sheehy AM, 2002).

*Vpr* - Codes for viral protein R, which is a moderate enhancer of virus infectivity and through its interaction with host cellular proteins has been implicated in post-entry nuclear import and cell cycle arrest. The

precise role of Vpr in the viral life cycle remains to be clearly defined (Kogan and Rappaport, 2011, Popov *et al*., 1998a, Popov *et al*., 1998b, Strebel, 2013).

*Rev*- Codes for the regulator of expression of viral proteins (Rev). Rev binds to a Rev responsive element (RRE) and plays an essential role in the nuclear export of unspliced viral mRNAs from the nucleus to the cytoplasm (Freed, 2003, Karn and Stoltzfus, 2012).

*Vpu* - Encodes for viral protein U (vpu). Vpu downregulates the expression of CD4 and MHC on the surface of infected host cells. It also counteracts the restriction factor tetherin by targeting it for degradation and by so doing induces virion release from the host cell surface (Dube *et al*., 2010, Neil *et al*., 2008a, Guo and Liang, 2012).

*Nef* - Codes for negative factor (Nef). Nef downregulates CD4 and MHC expression on the surface of infected host cells. It also modulates virus infectivity, blocks apoptosis and plays a role in determining pathogenicity of the virus (Foster and Garcia, 2007, Strebel, 2013).

*Tat* - Encodes for the transactivator of transcription (Tat) protein. Tat binds to the transacting response element (TAR) and acts as a potent activator of viral gene expression (Freed, 2003, Karn and Stoltzfus, 2012, Romani *et al*., 2010, Debaisieux *et al*., 2012, Van Lint *et al*., 2013).

Two untranslated repeat regions flank the HIV-1 RNA genome. At the 5' end of the repeat region internally is a unique 5' region (U5) and at the 3' end internally is a unique 3' region (U3) (Freed, 2003). At the end of reverse transcription of the viral RNA into proviral DNA, longer repeat regions are generated and the proviral DNA is flanked by 2 identical long terminal repeats (LTRs) containing the U3, R and U5 regions. The HIV-1 LTR is 630-640bp long and plays a vital role in initiating the

transcription of viral genes (Starcich *et al*., 1985, Karn and Stoltzfus, 2012). The U3 region contains the viral promoter and enhancer sequences, while the R regions contain the polyadenylation signal and the transactivation response element (TAR), to which the viral Tat protein binds (Starcich *et al*., 1985, Karn and Stoltzfus, 2012).

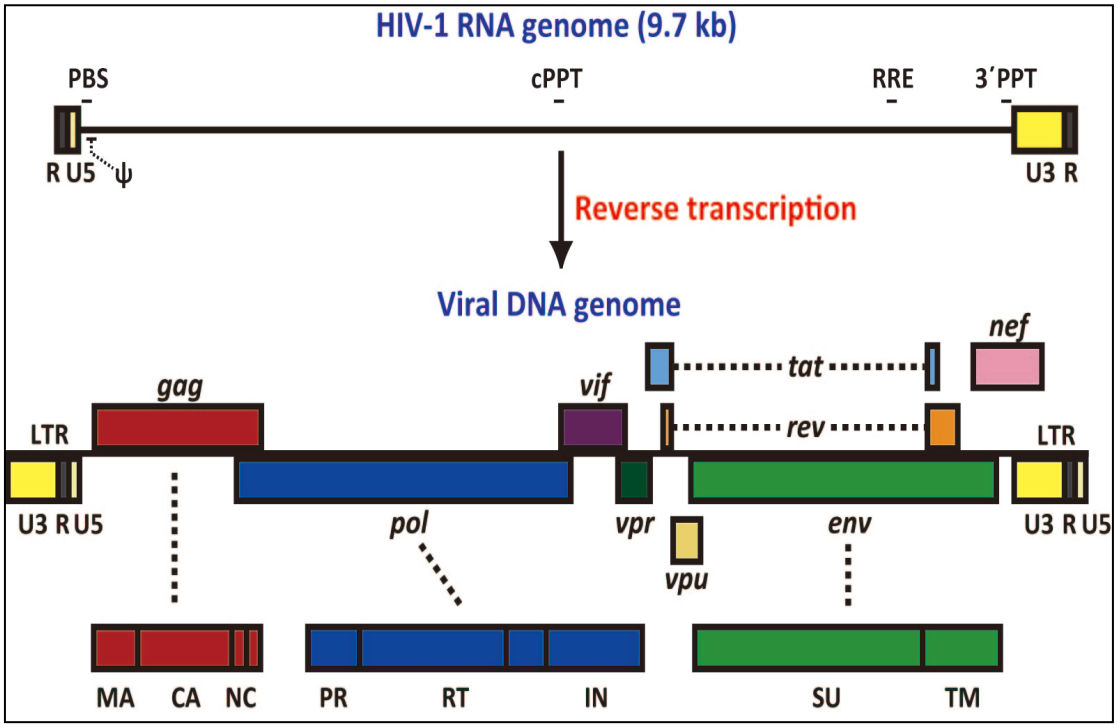


**Figure 1-3**: Organisation of the HIV-1 genome (Suzuki, 2011)

Targeting viral proteins and enzymes important for HIV-1 replication is the main strategy that has been adopted for the development of antiretroviral therapies currently used to treat HIV-1 infection. The steps in the replication cycle of HIV-1 are briefly described below.

## 1.3.2 HIV-1 Replication

### 1.3.2.1 Cell binding and entry

HIV-1 typically infects immune cells carrying the CD4 cell surface receptor and one or both of the co-receptors CCR5 and CXCR4. These cells include mainly helper T cells (CD4+ T cells) and macrophages, as well as some subsets of dendritic cells and langerhans cells. The viral

Env is a heavily glycosylated trimer of gp120 and gp41 heterodimers. The infection of a cell with HIV-1 begins when the virus surface glycoprotein gp120 binds to the CD4 receptor on the surface of the host cell. Gp120 has five relatively conserved domains (C1-C5) and five variable loops (V1-V5)(Wilen *et al*., 2012, Kwong *et al*., 1998). The variable loops are located predominantly on the surface of gp120 and are important for immune evasion and co-receptor binding. When gp120 binds to CD4 this triggers a reorganisation of the V1/V2 and subsequently V3 loops and also induces the formation of a bridging sheet facilitating co-receptor engagement (Wilen *et al*., 2012, Kwong *et al*., 1998). Engagement of the co-receptor triggers the exposure of the hydrophobic fusion peptide of gp41, which inserts into the host cell membrane and tethers the virus and host cell membranes (Chan *et al*., 1997, Weissenhorn *et al*., 1997, Wilen *et al*., 2012). The fusion peptide of each gp41 in the trimer folds at a hinge region, and this folding brings an amino terminal helical region and a carboxy terminal region from each gp41 subunit together to form a six-helix bundle (6HB) (Wilen *et al*., 2012, Chan *et al*., 1997, Weissenhorn *et al*., 1997). The formation of the 6HB drives the formation of the fusion pore and enables the fusion of the virus and host cell membrane. Membrane fusion is central to the infection process as it enables the introduction of the virus core into the cytoplasm of the host cell (Freed, 2003, Wilen *et al*., 2012).

#### 1.3.2.2 Reverse transcription

Following fusion, the core, which contains the genomic RNA, is released into the cytoplasm. The viral core uncoats and is converted to a reverse transcription complex (RTC) and then to a pre-integration complex (PIC). Reverse transcription of the viral RNA into double stranded DNA is performed by the viral reverse transcriptase protein (RT) in the RTC and is one of the defining steps in the life cycle of all retroviruses. The precise location and timing of uncoating remains a

matter of debate, and whether uncoating occurs before or during the process of RT remains unclear. Retroviruses generally use host cell tRNAs as primers to initiate the process of reverse transcription (Hu and Hughes, 2012). The 3' end of the $tRNA^{Lys3}$ in host cells is base paired to a complementary sequence at the 5' end of the HIV-1 RNA called the primer-binding site (pbs) and serves as the primer for initiating reverse transcription of the HIV-1 genome (Freed, 2003, Hu and Hughes, 2012). The pbs is 180 nucleotides from the 5' end of the viral genomic RNA. After binding of the $tRNA^{Lys3}$ primer to the pbs, RT synthesises a DNA/RNA hybrid and the RNA portion is degraded by RNaseH which is an inherent part of the RT holoenzyme, generating a fragment of DNA known as the minus strand strong stop. The ends of the viral RNA contain the R regions which serve as a bridge allowing the newly synthesised minus-strand DNA to be transferred or "jump" from the 5' to the 3' end of the genome in a process referred to as first-strand transfer. The 3' end of the strong stop DNA serves as a primer for the continuing synthesis of the minus strand. A purine–rich sequence (ppt) of RNA, resistant to RNaseH digestion at the 3' terminus (3'ppt) and the center of (central-ppt), remaining from minus strand synthesis, primes the synthesis of the plus strand (Hu and Hughes, 2012). The synthesis of the plus strand continues until the tRNA has been copied, allowing its removal by RNaseH and the second strand transfer to occur. Extension of the minus and plus strands completes the synthesis of the double-stranded linear viral DNA. The newly synthesised viral DNA remains associated with viral and cellular proteins in a large complex called the pre-integration complex (PIC) (Hu and Hughes, 2012). HIV-1 RT lacks the proofreading ability of cellular DNA polymerases and as such is highly error prone introducing an estimated 3.4×105 errors per base pair per cycle (Hu and Hughes, 2012). Also, the RT binds to its template with a very low affinity and is able to jump between the two strands of genomic RNA, potentially adding to RT-mediated sequence diversity

(Hu and Hughes, 2012). These characteristics of RT account for the sequence variability that is seen among HIV-1 isolates and the existence of a pool of viruses or quasi-species within an infected host.

### 1.3.2.3 Nuclear import and Integration

The PIC enters the nucleus through nuclear pore complexes (NPCs). The NPCs are specialised nuclear membrane channels, that are comprised of proteins called nucleoporins (Matreyek and Engelman, 2013). The precise mechanism by which the PIC is imported into the nucleus has been intensely studied but remains highly controversial. Based on recently reported findings, a popular model suggests that the PIC docks at the NPC by engaging the nucleoporin NUP358 through interactions with remaining CA proteins (Matreyek and Engelman, 2013, Schaller *et al*., 2011). Once docked, the PIC further engages with the cellular protein cleavage and polyadenylation specificity factor subunit 6 (CPSF6) and NUP153, an essential step in facilitating nuclear import of the PIC (Matreyek and Engelman, 2013). Transportin 3 (TNPO3) is a nuclear transport protein that also plays an important role in the nuclear import of the PIC and possibly nuclear trafficking and integration(Brass *et al*., 2008, Matreyek and Engelman, 2013, Diaz-Griffero, 2012). TPNO3 is required for proper nuclear localisation of CPSF6 and depletion of TPNO3 in human cells leads to a dramatic reduction in HIV-1 infection. Several viral factors associated with the PIC are also important for nuclear import, these include; MA, IN and Vpr, which all possess nuclear localisation signals (Popov *et al*., 1998b, Haffar *et al*., 2000, Bouyac-Bertoia *et al*., 2001, Matreyek and Engelman, 2013). These factors recruit nuclear transport proteins facilitating nuclear import of the PIC. The reverse transcribed genome also contains an important determinant of PIC nuclear import, a triple stranded DNA flap element generated through the action of the central polypurine tract (cPPT) and central termination signal

(CTS)(Matreyek and Engelman, 2013, Ao *et al*., 2004, Riviere *et al*., 2010). This DNA flap enhances the efficiency of both viral replication and nuclear import (Ao *et al*., 2004, Riviere *et al*., 2010). The process of integration mediated by the viral integrase is initiated in the PIC in the cytoplasm. Integrase binds to specific sequences in the LTR regions of the viral cDNA and is part of the PIC. Integrase cleaves the viral DNA at the 3' ends in a process known as 3' processing leading to the exposure of conserved CA dinucleotides at the 3' ends of the viral DNA (Craigie and Bushman, 2012).

Once the PIC is imported into the nucleus, the integration process proceeds by a strand transfer reaction in which the viral DNA is ligated to the host chromosomal DNA completing the process of integration. The viral DNA also undergoes several circularisation reactions leading to the generation of forms, which are unable to support replication and represent dead ends for the virus (Craigie and Bushman, 2012). 2 LTR circles are formed by ligation of the two ends of the viral DNA to each other (Craigie and Bushman, 2012). 1 LTR circles are also detected. HIV-1 exploits host cellular factors to enhance integration and integration site selection. The viral cDNA preferentially integrates into sites of active transcription within the host genome (Craigie and Bushman, 2012). LEDGF/p75 (Lens epithelium derived growth factor) is an important host derived co-factor required for integration of the viral DNA (Cherepanov *et al*., 2003). It tethers HIV-1 integrase and chromatin at the sites of active transcription, targeting integration to these locations (Cherepanov *et al*., 2003, Craigie and Bushman, 2012). The interaction between integrase and LEDGF/p75 is a promising target for the therapeutic inhibition of HIV-1 replication. Small molecules called LEDGINs, which are potent inhibitors of the LEDGF/p75-IN protein-protein interaction and allosteric inhibitors of the catalytic

function of integrase, are currently in the early stages of drug development (Christ and Debyser, 2013).

#### 1.3.2.4 Transcription and post-transcriptional regulation of HIV-1 gene expression

Once integrated into the host genome, the proviral DNA can remain silent or be transcribed by the cellular machinery if the infected CD4+ T cell is activated. Although the mechanisms by which HIV-1 establishes latency are not fully understood, the widely held view is that a small fraction of activated CD4+ T cells which are infected with the virus survive long enough to revert to a resting memory state in which transcription of viral genes is silent (Shan and Siliciano, 2013). It is also possible that HIV-1 is capable of directly infecting resting CD4+ T cells despite the very low efficiency of reverse transcription and integration in these cells (Agosto *et al*., 2007, Vatakis *et al*., 2009, Pace *et al*., 2012). These infected resting CD4+ T cells are believed to constitute an important component of the viral reservoir and can resume active production of infectious virions once they become re-activated. The integrated proviral DNA serves as a template for the synthesis of viral mRNAs that are translated into viral structural, regulatory and accessory proteins.

Transcription of the proviral DNA is initiated at the HIV-1 LTR and is greatly enhanced by the binding of Tat to the transactivation response element (TAR) (Romani *et al*., 2010, Karn and Stoltzfus, 2012). The HIV-1 promoter is located in the 5' LTR and bears important regulatory components needed for transcription. Transcription is initiated from the U3/R junction and is mediated by the cellular RNA polymerase II (Van Lint *et al*., 2013). Upstream of the promoter is a TATA box and binding sites for additional host transcriptional factors such as Sp1, NFkB and others (Van Lint *et al*., 2013). The viral genes, which encode for Tat, Rev and Nef are the first to be transcribed. Prior to the generation of Tat,

cellular transcription factors are solely responsible for activating the LTR. Once present, Tat strongly increases the activation of transcription and elongation via a positive feedback loop (Romani *et al*., 2010, Karn and Stoltzfus, 2012). When Tat binds to TAR, it recruits the cellular co-factor positive transcription elongation factor b (P-TEFb) complex to the promoter (Karn and Stoltzfus, 2012). The P-TEFb complex in turn phosphorylates the C-terminal domain of the RNA polymerase II and by so doing enhances its processivity and elongation function. Transcription produces a large number of viral RNAs; unspliced RNAs which are the mRNA for Gag and Gag-Pol precursor polyproteins (about 9kb), partially spliced mRNAs coding for Env, Vif, Vpu and Vpr proteins (about 4kb) and multiply spliced mRNAs which are translated into Rev, Tat and Nef proteins (about 2kb). Unspliced and partially spliced viral mRNAs are transported out of the nucleus with the help of the Rev protein. Rev binds to a Rev responsive element (RRE) to form a complex capable of interacting with the nuclear export machinery of the cell (Romani *et al*., 2010, Karn and Stoltzfus, 2012). This complex enables the transport of the unspliced and partially spliced mRNAs from the nucleus into the cytoplasm for translation, and because Rev possesses a nuclear localisation signal it is able to shuttle between the nucleus and the cytoplasm (Karn and Stoltzfus, 2012). Following the nuclear export of HIV-1 mRNAs, Gag and Gag-Pro-Pol polyproteins and most viral accessory proteins are translated in the cytosolic polysomes. The two viral membrane proteins Env and Vpu are encoded for by the same mRNA and are translated on the rough ER. Newly synthesised viral proteins are trafficked from their site of synthesis in the cytoplasm to the plasma membrane for assembly.

#### 1.3.2.5 Assembly, binding and release

All the necessary viral components needed for infectivity are packaged during assembly. These include two copies of the virus

genomic RNA, cellular $tRNA^{Lys3}$ required to prime cDNA synthesis during reverse transcription of the virus RNA, the viral envelope protein (Env) and the three viral enzymes RT, Protease and Integrase (Sundquist and Krausslich, 2012). Assembly of virions occurs at the plasma membrane and is coordinated by the HIV-1 Gag (and Gag-Pro-Pol) polyproteins. Gag domains play specific roles in the assembly process. Its amino terminal MA functions to bind the plasma membrane and possibly recruit the viral envelope protein, although the precise mechanism of Env incorporation and its interactions with Gag during virus assembly remain unresolved. Gag is targeted to the plasma membrane by a combination of viral and cellular factors, including the basic patch in MA, Gag myristoylation and also a plasma membrane specific lipid known as phosphatidylinositol (4,5) bisphosphate (PI (4,5) P2) that are collectively essential for targeting Gag to the plasma membrane. When MA binds to PI(4,5)P2 this causes the exposure of the amino terminal myristoyl group (myristoyl switch) enabling Gag to be stably anchored to the inner leaflet of the plasma membrane (Saad *et al*., 2006, Sundquist and Krausslich, 2012). The central domain of Gag known as CA is responsible for protein-protein interactions required for the assembly of immature virions and creates the core of the mature virus called the capsid. The nucleocapsid domain captures the viral RNA genome during assembly and is also believed to play a role in plasma membrane targeting. The carboxy terminal of Gag known as the p6 region possesses binding sites for the viral accessory protein Vpr and for cellular proteins involved in the ESCRT (endosomal cell sorting complex required for transport) pathway (Sundquist and Krausslich, 2012) that help mediate the process of budding. The spacer peptides in Gag Sp1 and Sp2 regulate conformational changes that are induced by the maturation process. Assembled virions acquire their lipid envelope and envelope proteins during the budding process. The virus envelope, an integral membrane protein, is inserted co-

translationally into the endoplasmic reticulum and goes through secretory pathways in the cell where it is glycosylated, assembled into trimeric complexes and processed into the transmembrane (gp41) and surface (gp120) subunits by the cellular protease furin (Sundquist and Krausslich, 2012). Gp120 and gp41 remain non-covalently linked and are delivered to the plasma membrane by the vesicular transport pathway. Although the viral Gag protein coordinates the packaging of co-factors and virus assembly, the virus hijacks the host cell ESCRT pathway for the final stages of budding and membrane pinching to release viral particles from the plasma membrane. Virus maturation occurs simultaneously during budding or immediately after budding. The viral protease is responsible for cleaving Gag and Gag-Pro-Pol polyproteins into fully functional sub-units MA, CA, NC, p6, PR, RT and IN. During the process of maturation the cleaved proteins are rearranged to produce the infectious virion, which is characterised by its cone-shaped core and electron dense morphology under the electron microscope. MA remains closely associated to the inner surface of the viral membrane and capsid surrounds the nucleocapsid. These mature virions are now fully capable of replicating in new target cells.

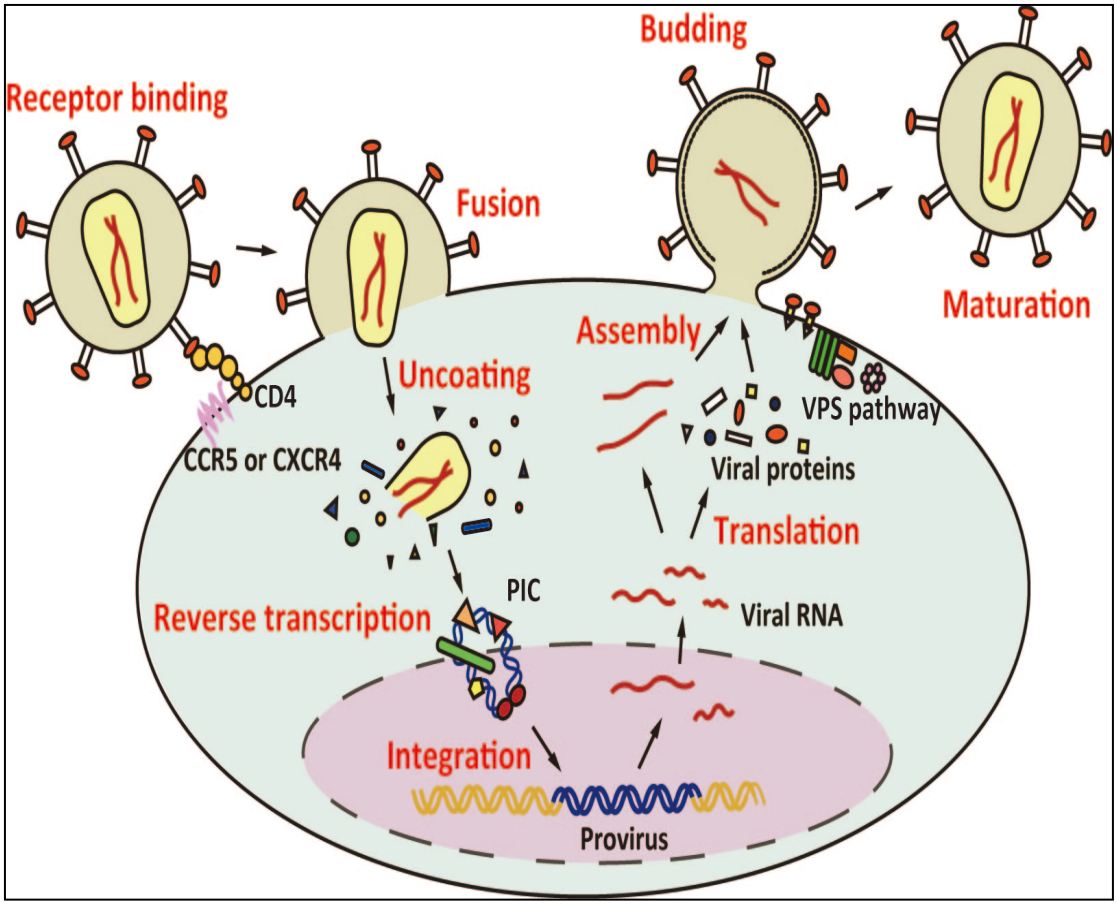


**Figure 1-4**: HIV-1 life-Cycle (Suzuki, 2011)

## 1.4 The course of HIV-1 infection

Once an effective person-to-person transmission event has occurred, HIV-1 infection follows a well-described course in infected individuals who are not treated.

Eclipse phase: The 7-21 days following a transmission event are known as the eclipse phase (Cohen *et al*., 2011, Keele et al., 2008, Lee et al., 2009). During this phase the virus freely replicates and spreads from the initial site of infection to other tissues. During this asymptomatic phase, HIV-1 RNA levels in the plasma are undetectable. Studies using plasma samples obtained from acutely infected donors collected before infection; at peak viraemia and during sero-conversion suggest that an early innate immune response occurs during this eclipse phase. This response is characterised by increase production of pro-inflammatory

cytokines and acute phase proteins (McMichael et al., 2010, Stacey *et al*., 2009). A recent study of SIV infection in macaques provides evidence that the viral reservoir is probably seeded during this eclipse phase of infection, before detectable viraemia (Whitney et al., 2014, Deng and Siliciano, 2014), a proposition which has important implications for virus eradication strategies (Deng and Siliciano, 2014, Whitney et al., 2014),

Acute phase: Occurring 2-4 weeks after infection, the phase of acute or primary infection is characterised by high levels of viral replication and HIV-1 RNA levels of up $10^7$ copies/ml of blood (Coffin and Swanstrom, 2013, Lee et al., 2009). Some individuals experience "flu-like" symptoms, fever and lymph node enlargement during this phase that is attributed to the inflammatory response in early HIV-1 infection also commonly referred to as a "cytokine storm". Around the time viraemia peaks, adaptive immune responses begin to appear. Antibodies against viral envelope proteins can be detected as well as cytotoxic CD8+ T cell responses targeting HIV-1 antigens. These HIV-1 antigens are coupled with MHC class I molecules and presented on the surface of infected CD4+ T cells which are then destroyed by cytotoxic CD8+ T cells. The initial antibody response is non-neutralising and doesn't lead to the selection of escape mutants (McMichael et al., 2010, Cohen et al., 2011). Antibodies capable of neutralising autologous virus only develop slowly about 12 weeks or more after the initial transmission event. 20% of patients are capable of generating broadly neutralising antibodies after several years of infection (McMichael et al., 2010). By the end of the acute phase of infection, the viral set point is established (Cohen et al., 2011). This set point represents a relatively steady plasma viral load turnover in a given patient. Infected individuals with a higher viral set point, progress more rapidly to AIDS and death. There is strong evidence to support that very

early treatment lowers this viral set point and reduces the size of the viral reservoir, slowing progression from clinical latency to full-blown disease (AIDS) (Mellors et al., 1997, Hogan et al., 2012, Fidler et al., 2013, Persaud et al., 2013, Strain et al., 2005, Whitney et al., 2014).

Latent phase: A phase of clinical latency follows the acute infection phase. During this phase patients remain asymptomatic and largely unaware of their infection. The duration of this clinical latency is variable from one individual to another but current estimates suggest that the average time from infection to the development of clinical symptoms is 8 -12 years (Bacchetti and Moss, 1989, Coffin and Swanstrom, 2013). The term "latency" to describe this phase of HIV-1 infection is misleading because there is strong evidence that virus replication and progressive decline of CD4+ T cells continues throughout this phase leading to progressive destruction of the immune system (Coffin and Swanstrom, 2013). In a small subset of individuals known as long-term non-progressors, CD4+ T cell decline is not observed and viral replication seems to be controlled by a range of genetic, virologic and immunologic mechanisms (Lambotte et al., 2005, Madec et al., 2005). In children infected at birth the latent phase may be shorter or entirely absent with progression to AIDS occurring very rapidly (De Rossi et al., 1996).

AIDS: After years of continuous CD4+ T cell decline, the level of these cells eventually falls below a tipping point <500 cells/μl of plasma (normal range: 600-1200cells/μl). Below this point, the first clinical signs of immune compromise begin to appear. The patient becomes more susceptible to opportunistic infections and malignancies. The control of infection is lost and the viral load rises (Coffin and Swanstrom, 2013). The viral set point established at the end of the acute phase of infection is an important determinant of the rate of progression to AIDS

(Cohen et al., 2011). The decline of CD4+ T cells typically continues until virtually all of these cells are lost. Severe immune compromise and the inability to fight common infections, as well an increased susceptibility to AIDS-related malignancies, eventually culminates in death of the patient.

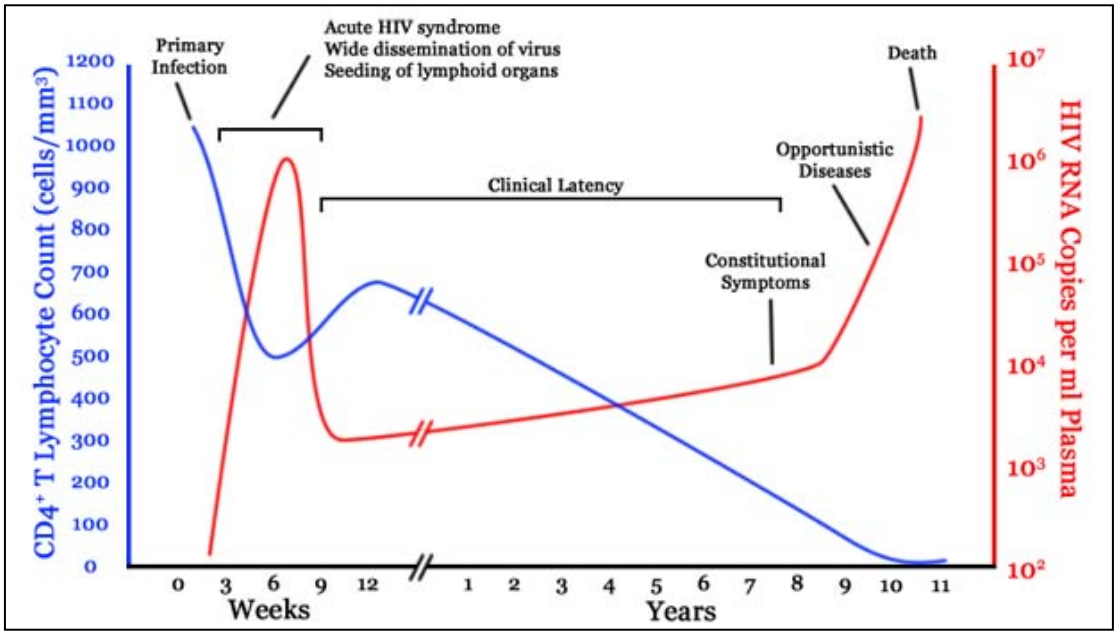


**Figure 1-5**: Time-course of typical HIV-1 Infection (Pantaleo et al., 1993)

## 1.5 Modes of HIV-1 transmission and spread between and within the host

### 1.5.1 HIV-1 Transmission

HIV-1 transmission from person-to-person occurs through the exchange of bodily fluids containing infectious virus particles via three main routes. These include: unprotected sexual intercourse, parenteral transmission (infected blood and tissue products and intravenous drug use) or mother-to-child transmission during pregnancy, delivery or breastfeeding. Transmission occurring across mucosal surfaces (vaginally or rectally) is the predominant mode of transmission and represents 80% of all adult infections while percutaneous and intravenous routes represent 20% of infections (UNAIDS, 2013). In order to gain a foothold within the host and establish infection, cell-free or cell-associated infectious virus particles must disseminate within the

host and infect target cells. Substantial evidence suggests that activated CD4+ T cells and Langerhans' cells are the initial target cells for HIV-1 during mucosal transmission (Cohen et al., 2011). Other dendritic cells play an important accessory role in facilitating spread of the virus from the site of initial entry, while macrophages can support productive infection and may serve as important long-lived reservoirs of the virus.

### 1.5.2 Modes of virus spread within the host

Three mechanisms of dissemination have been described for HIV-1 within the host (Piguet and Sattentau, 2004): In the "classical" mode of spread by diffusion of cell-free virions in bodily fluids, virions bind to CD4 receptors and coreceptors on permissive host cells and trigger the steps leading to viral entry and replication (Pierson and Doms, 2003, Piguet and Sattentau, 2004). In the second mode of spread, cells such as dendritic cells capture virus particles using cellular receptors notably DC-SIGN, without necessarily themselves becoming infected, and re-present the infectious virus particles to permissive target cells, in a mode of infection known as *in trans* (Geijtenbeek et al., 2000, Hu et al., 2004, Piguet and Sattentau, 2004, Cameron et al., 1992, Pope et al., 1994). The third mechanism of virus spread is through direct cell-to-cell transmission in which an HIV-1 infected cell is able to directly infect a target cell without the requirement for prolonged fluid-phase diffusion of cell-free virus in the extracellular milieu. It is important to highlight that in this mode of transmission, virus budding from an infected effector cell is polarised towards the target cell at the synaptic cleft and this does not involve fusion of donor and target cells. Rather the released virions have a short distance to travel before they can engage receptors on the target cell that is involved in the cell-to-cell contact (Jolly and Sattentau, 2004, Jolly et al., 2004, Sattentau, 2008, Piguet and Sattentau, 2004).

### 1.5.2.1 Cell-free spread of HIV-1

The classical paradigm of HIV-1 dissemination by diffusion of cell-free virions probably represents the best means of long distance dissemination for the virus. Following fluid phase diffusion, the virus particle attaches to the target cell bearing the required cellular receptors. The engagement of the CD4 receptor and co-receptors (CXCR4 and CCR5) by the virus drives the fusion of the virus envelope with the cellular membrane, leading to entry of the virion into the cytoplasm and its subsequent replication within the cell. HIV-1 can be found both in cell-free and cell-associated forms in semen (Van Voorhis et al., 1991, Miller et al., 1992, Quayle et al., 1997, Xu et al., 1997, Tachet et al., 1999, Ghosn et al., 2004, Anderson et al., 2010) and in breast milk (Koulinska et al., 2006, Ndirangu et al., 2012). A few studies have looked into the relative contributions of cell-free and cell-associated virus to transmission but so far no clear consensus has been reached as to which form of virus is predominantly responsible for person to person transmission (Sodora et al., 1998, Weiler et al., 2008, Salle et al., 2010). Cell-free spread allows for transmission of the virus from one host to another where the virus needs to exit the infected host and remain viable for a sufficient period of time to allow it to infect a new host. It also may enable long distance spread of the virus from the point of initial entry to distant tissues through diffusion in the bloodstream, although as discussed latterly, virus captured or infected migrating cells may also contribute to viral dissemination from the initial infection site. Despite these advantages, cell-free spread imposes a penalty to the virus by increasing its exposure to physical, kinetic and immunological barriers. HIV-1 has a fragile envelope and is prone to the decay of its infectivity over time, making it more challenging for the virus to remain infectious in the time required to cross mucosal membranes during sexual transmission. Also random diffusion in the blood stream increases the time it takes for the virus to encounter a target cell with the right

receptors (CD4, CCR5, CXCR4). These disadvantages highlight the benefits for a virus to have the ability to use alternative methods for dissemination in order to overcome these obstacles and effectively spread within the host.

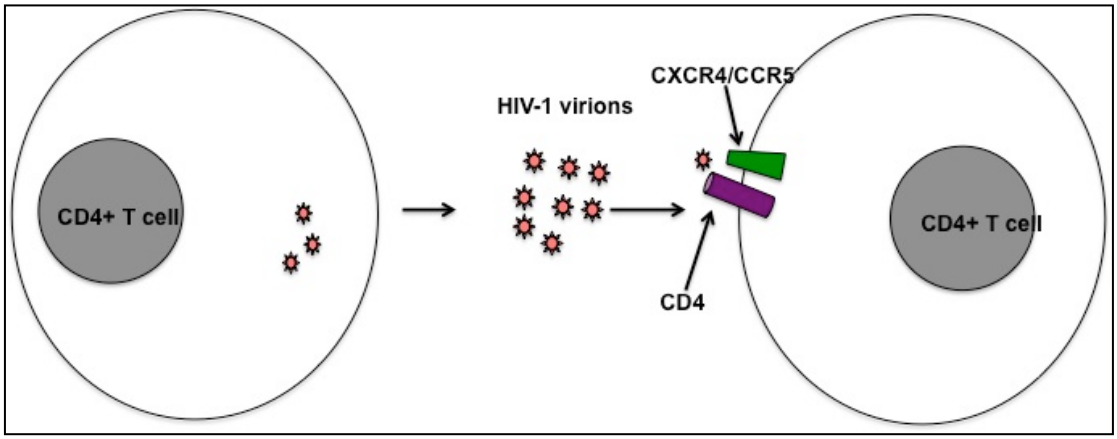


**Figure 1-6**: Cell-free spread of HIV-1 from T cell-to-T cell.

### 1.5.2.2 The virological synapse and T cell-to-T cell spread of HIV-1

Direct virus spread from cell-to-cell was first demonstrated for the herpes virus varicella zoster (Weller, 1953). Since then several other virus families including rhabdoviruses, poxviruses, paramyxoviruses and retroviruses have been shown to use this mechanism of propagation (Sattentau, 2010, Sattentau, 2008). HIV-1 is an enveloped retrovirus, which is fragile and extremely prone to infectivity decay when located outside its host cell. This singular fragility is largely due to the fact that its envelope glycoprotein spike is non-covalently assembled and highly prone to degeneration over time (McKeating et al., 1991, Layne et al., 1992). It is therefore primordial for its survival that the virus quickly finds and infects new host cells. About 10 years ago, seminal papers describing direct transfer of HIV-1 and HTLV-1 across a supramolecular structure termed a virological synapse, provided a mechanism for a highly efficient and rapid mode of retroviral dissemination through direct spread from T cell-to-T cell (Igakura et al., 2003, Jolly et al., 2004, Jolly and Sattentau, 2004), these findings were subsequently confirmed

by other groups (Sourisseau et al., 2007, Chen et al., 2007, Hubner et al., 2009).

A synapse has been defined as "a stable adhesive junction across which information is relayed by directed secretion" (Dustin and Colman, 2002). *In vivo*, HIV-1 predominantly infects and replicates in CD4+ T cells. T cells do not normally form stable contacts with other T cells and when these do occur, they are typically transient lasting less than 10 minutes (Sabatos et al., 2008). However during direct spread of HIV-1 from T cell-to-T cell, an HIV-1 infected T cell (effector cell) is capable of forming a stable intercellular junction with an uninfected cell (target) (Jolly and Sattentau, 2004). These stable junctions, which form when T cells are infected with HIV-1 are longer lasting, persisting for about 60 minutes before the cells come apart, (Jolly et al., 2004, Chen et al., 2007, Hubner et al., 2009, Martin and Sattentau, 2009) however some conjugates can last for several hours . At the point of contact, polarised virus assembly and budding occurs towards the engaged target cell (Jolly et al., 2004, Chen et al., 2007, Hubner et al., 2009, Rudnicka et al., 2009).

The virological synapse can be defined by the co-polarisation of HIV-1 Env and Gag on the infected cell and HIV-1 entry receptors (CD4 and co-receptor) on the target cell (Jolly et al., 2004). The initial event that leads to the formation of the virological synapse between an infected T cell and an uninfected T cell is the binding of the virus gp120 to the CD4 receptor. This interaction between the virus envelope proteins on the surface of the effector cell and the CD4 receptor and co-receptor (CXCR4 or CCR5) on the surface of the target cell is therefore an important factor that drives the co-polarisation of the viral Env and cellular receptors (Jolly et al., 2004). Drug inhibitors and antibodies, which disrupt the interaction between the CD4 receptor and Env,

impede formation of the virological synapse and this is substantiated by reduced clustering of Env, Gag, CD4 and co-receptors in the presence of these agents (Abela et al., 2012, Schiffner et al., 2013, Massanella et al., 2009, Sattentau, 2010, Durham et al., 2012, Su et al., 2012, Malbec et al., 2013). The stability of the synaptic junction is likely enhanced by the interaction between integrins such as lymphocyte function associated antigen-1 (LFA-1) and its cognate ligands intercellular adhesion molecules-1 and 3 (ICAM-1 and ICAM-3)(Jolly et al., 2007, Jolly et al., 2004) although this role of cellular adhesion molecules is not unanimously accepted (Puigdomenech et al., 2008, Rudnicka et al., 2009). However, when antibodies, inhibitory peptides or using T cells with mutated conformations of LFA-1, perturb LFA-1-ICAM binding the stability of the VS is reduced as well as cell-to-cell spread of HIV-1 (Jolly et al., 2007, Rudnicka et al., 2009). Moreover, both LFA-1 and ICAM are enriched at the VS (Jolly et al., 2004, Jolly et al., 2007) providing further evidence for their contribution to VS formation. The interaction between Env and CD4 triggers the recruitment of actin, more CD4, HIV-1 co-receptors (CXCR4 or CCR5) and adhesion molecules to the synaptic zone (Jolly et al., 2004, Jolly et al., 2007). The polarisation of HIV-1 proteins and cellular receptors at the synaptic junction creates a focal point for directed assembly and release of newly formed virus particles allowing the efficient infection of the engaged target cells. Thus the kinetic advantage offered by cell-to-cell spread can be explained by a combination of factors including 1)the localised recruitment of viral proteins and polarised budding, 2)the increased concentration of HIV-1 entry receptors at the contact zone and 3) the polarised release of virions that can rapidly engage the target cell that is in close physical contact.

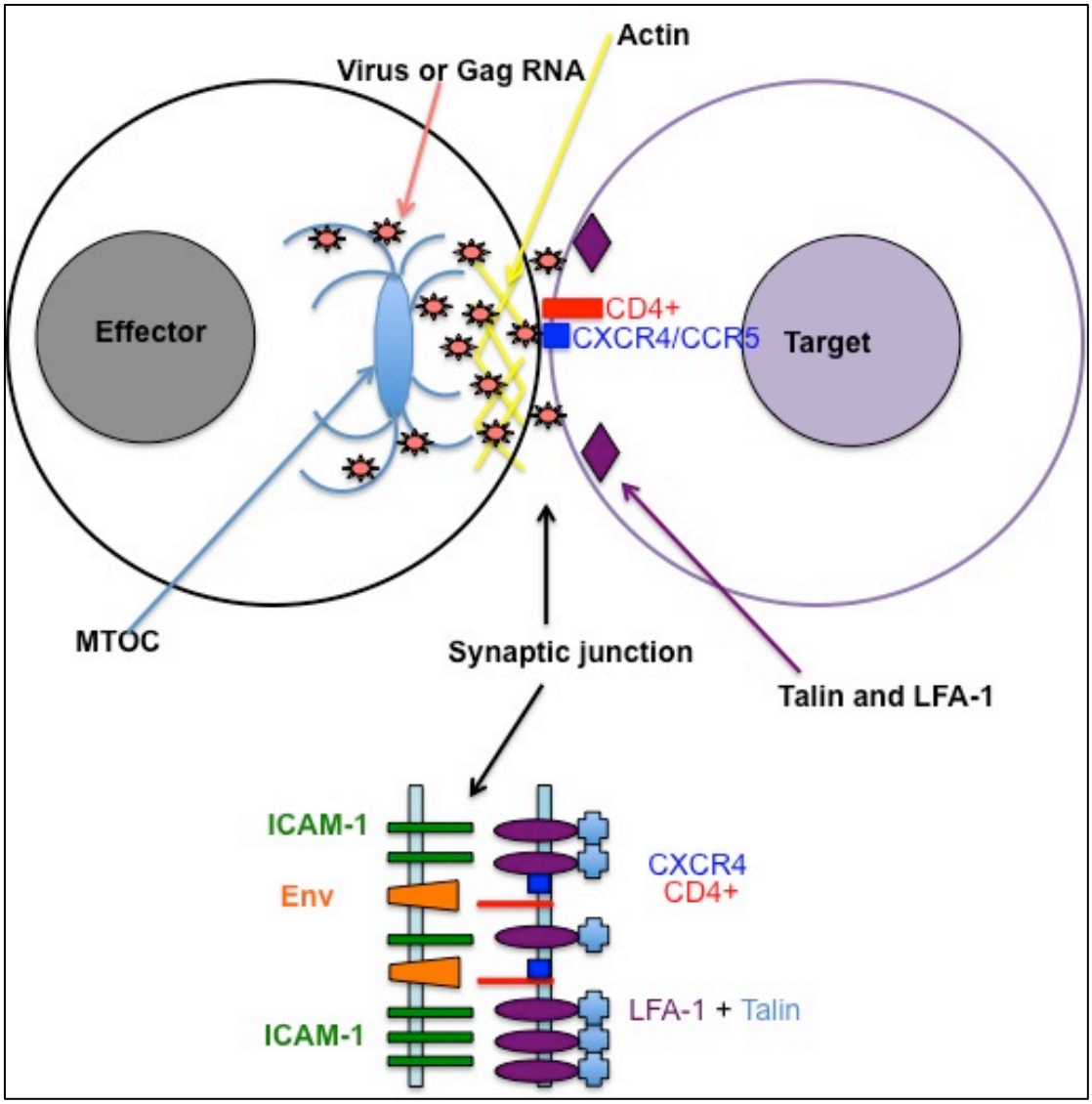


**Figure 1-7**: The virological (T cell) synapse. This schematic is based on the HIV-1 and HTLV-1 T cell virological synapses.

The microtubule organising centre (MTOC), mitochondria and other components of the cellular secretory apparatus are also polarised towards the virological synapse (Jolly and Sattentau, 2007, Jolly et al., 2011, Sol-Foulon et al., 2007) and it is likely that the recruitment of these organelles plays some role in VS formation and subsequent cell-to-cell spread. Besides cell-to-cell spread across VS, HIV-1 has also been observed to travel along long-tubular structures connecting infected T cells to uninfected cells (Rudnicka et al., 2009, Sowinski et al., 2008), these membrane nanotubes and filopodia however seem to be less frequently observed than virolological synapses. Simultaneous

transmission of HIV-1 from effector cells to multiple targets via polysynapses has also been observed *in vitro* (Rudnicka et al., 2009). Murine leukemia virus (MLV) a retrovirus that predominantly infects non-immune cells, notably fibroblasts has been shown to spread between cells by surfing on projections arising on the surface of infected cells termed filopodia (Sherer et al., 2007, Sherer et al., 2010). Similarly HIV-1 can move across filopodial bridges towards CD4/CXCR4-expressing cells (Sherer et al., 2007). It has now been unequivocally shown that HIV-1 spreads from cell-to-cell and recent studies applying intravital microscopy to humanised mouse models have allowed visualisation of the VS *in vivo* (Murooka et al., 2012, Sewald et al., 2012). Although the relative individual contribution of cell-to-cell spread at VS, via membrane nanotubes and through cell-free spread is difficult to quantify, cell-to-cell spread has been established as the predominant mode of HIV-1 dissemination in *in vitro* cell cultures (Sourisseau et al., 2007).

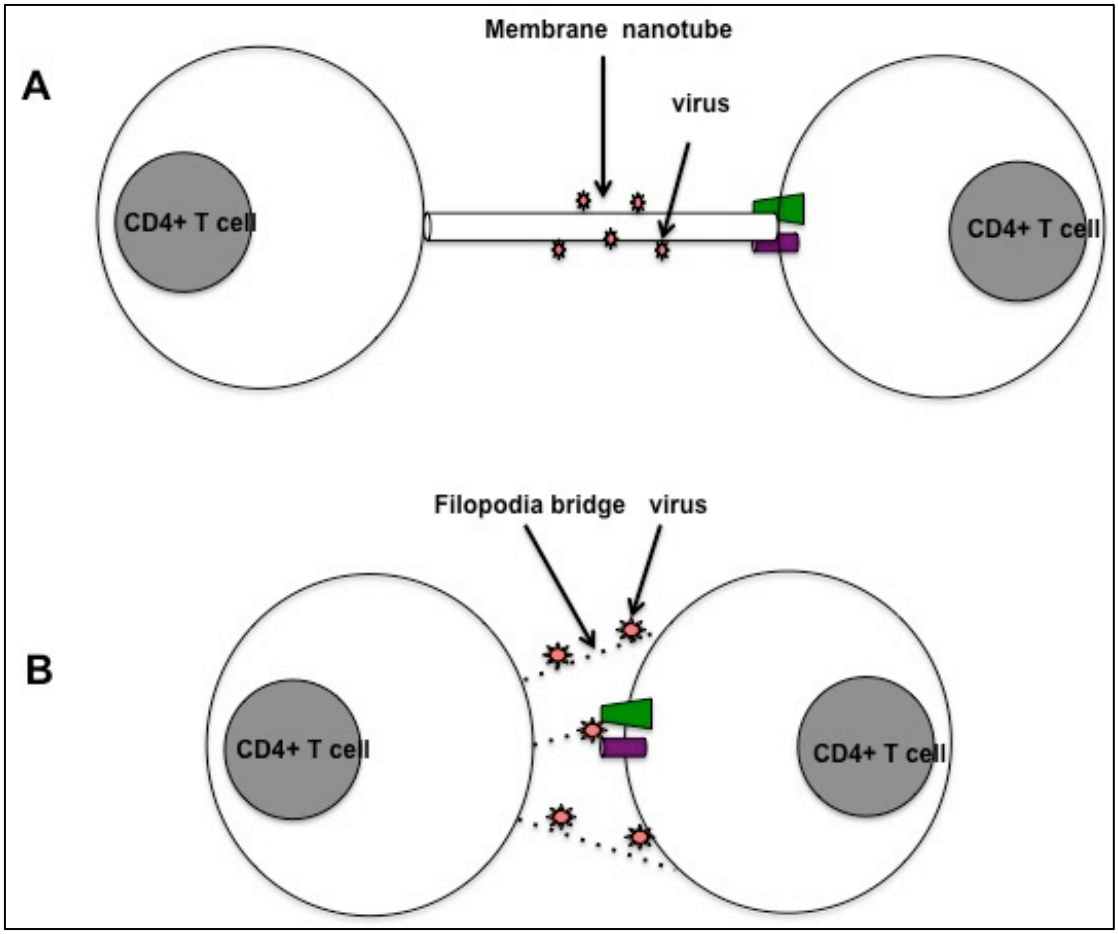


**Figure 1-8**: Cell-to-cell spread between T cell also occurs via membrane nanotubes **(A)** and filopodial bridges **(B)**.

Cell-to-cell spread provides many advantages to HIV-1 over the classical mode of cell-free spread (Sattentau, 2010, Sattentau, 2008). It has been shown to be up to three orders of magnitude quicker and more efficient than cell-free spread (Dimitrov et al., 1993, Johnson and Huber, 2002, Mazurov et al., 2010, Jolly et al., 2007, Sourisseau et al., 2007, Chen et al., 2007, Martin et al., 2010); it obviates the need for the rate limiting step of virus diffusion prior to attachment, and reduces the exposure of the virus to the neutralising effects of antibodies and complement, an observation that has been confirmed by several investigators (Sattentau, 2008, Sattentau, 2010, Sourisseau et al., 2007, Abela et al., 2012, Malbec et al., 2013, Martin et al., 2010). More recently it has been shown that cell-to-cell spread is less sensitive to inhibition by some reverse transcriptase inhibitors and as such may serve as a mechanism for antiretroviral escape for HIV-1 in the context

of ongoing antiretroviral therapy (Sigal et al., 2011). The impact of antiretroviral therapy on cell-to-cell spread of HIV-1 is of particular interest because of the possible implications for viral pathogenesis, the evolution of drug resistance and maintenance of viral reservoirs in the context of therapy.

### 1.5.2.3 Macrophages and cell-to-cell spread of HIV-1

Macrophages are a terminally differentiated, non-dividing subset of immune cells, which play an important role in the pathogenesis of HIV-1 infection (Waki and Freed, 2010). Unlike T cells, macrophages infected with HIV-1 survive for a long period post infection and the ability of these cells to traverse the blood-brain barrier enables the spread of HIV-1 infection into the central nervous system (Sharova et al., 2005, Gartner et al., 1986). Their inherent ability to resist virus-induced cytopathic effects also allows them to serve as long-term reservoirs of infection. Macrophages infected with HIV-1, like T cells and DCs are also capable of forming virological synapses to efficiently transfer HIV-1 to uninfected macrophages and T cells (Gousset et al., 2008, Groot et al., 2008). In comparison to the T cell and DC VS, cell-to-cell transfer across the VS in macrophages is less well described. Studies however suggest that the much of what has been elucidated on the VS in T cells and DCs will apply at least to a certain extent to macrophage induced VS. Cell-to-cell spread of HIV-1 from macrophages-to-T cells have also been shown to be less sensitive to inhibition by antiretroviral drugs in much the same way as T cell-to-T cell spread of HIV-1 (Duncan et.al, 2013).

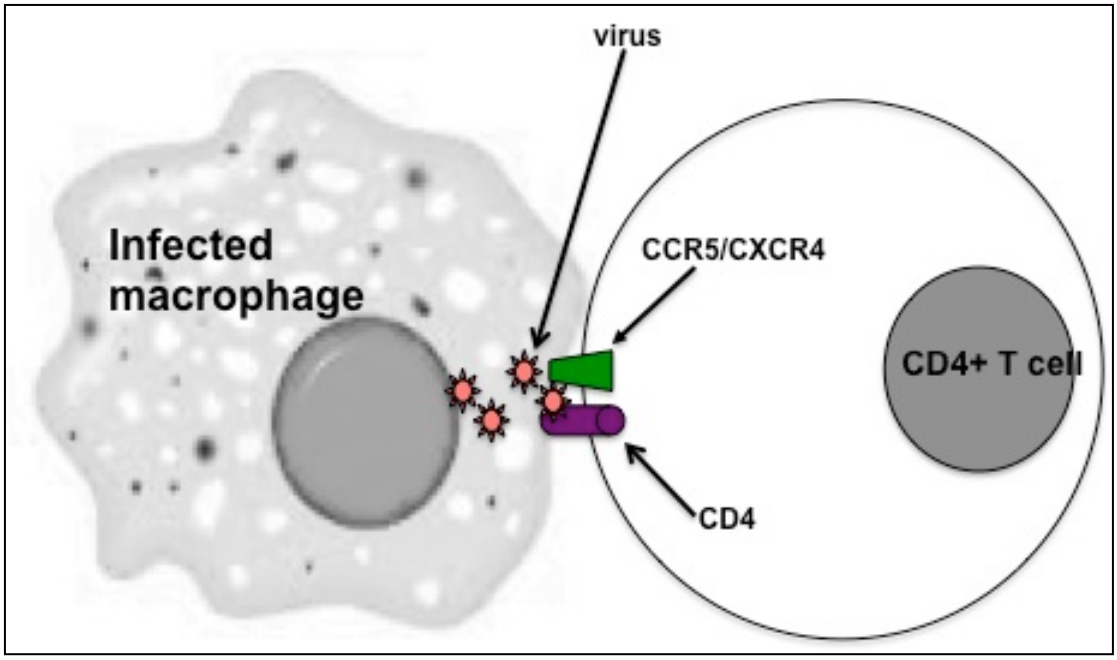


**Figure 1-9**: Macrophage to T cell spread of HIV-1

### 1.5.2.4Dendritic cells to T cell HIV-1 trans-infection

Dendritic cells are a diverse family of cells, which play an important role in coordinating innate and adaptive immune responses. These cells bind, internalise and degrade antigens picked up from the peripheral tissues and subsequently present these antigens in complex with HLA class II molecules to CD4+ T cells, triggering an adaptive immune response to the specific antigen (Banchereau et al., 2000). When a CD4+ T cell encounters a dendritic cell, cellular adhesion molecules like LFA-1 and ICAM arrest the movement of the T cell allowing it to probe the surface of the dendritic cell (Monks et al., 1998, Grakoui et al., 1999). If the correct Ag peptide/MHC-II complexes are presented to the T cell, it responds by concentrating T cell CD4 receptors and signalling molecules to the point of interaction, inducing the formation of an immunological synapse (Grakoui et al., 1999). HIV-1 is capable of exploiting the dendritic cell mediated immune function to allow for effective trans-infection of the virus through dendritic cells to T cells (McDonald, 2010). Dendritic cells are thought to be one of the first cell types that encounter HIV-1 following sexual transmission, due to their abundance in sub-mucosal tissue (McDonald, 2010). DCs were first

implicated in the pathogenesis of HIV-1 when it was observed that DCs exposed to HIV-1 could greatly amplify infection of T cells without themselves becoming infected (Cameron et al., 1992, Pope et al., 1994). The identification of DC-specific ICAM-3 grabbing non-integrin CD209 (DC-SIGN), a C-type lectin capable of binding the HIV-1 Env glycoprotein gp120, was pivotal in elucidating the mechanism of this trans-infection (Geijtenbeek et al., 2000, McDonald, 2010). DC-SIGN is an adhesion receptor with an important role in DC trafficking and in the formation of the immunological synapse (McDonald, 2010, Geijtenbeek et al., 2000). Interestingly, it was observed that when HIV-1 bound to DC-SIGN, it remained infectious and could be transferred to target cells without prior replication in the DC (Geijtenbeek et al., 2000). McDonald et.al confirmed this "Trojan horse" hypothesis by directly visualising trans-infection of GFP-tagged HIV-1 from DC to T cells (McDonald et al., 2003). They showed that DCs trans-infected T cells with HIV-1 by binding and concentrating the intact virus at the cellular interface and at the same time inducing the recruitment of HIV-1 receptors CD4, CCR5 and CXCR4 on the T cell, forming a structure similar to the immunological synapse, called the infectious synapse (McDonald et al., 2003, McDonald, 2010). The important distinction between the DC-T cell synapse (infectious synapse) and the T cell to T cell synapse (virological synapse) previously described is that formation of the latter requires interaction between the HIV-1 Env gp120 on the effector cell surface with CD4 on the target cell whereas the former relies on adhesion molecules employed during natural immune exchanges (McDonald, 2010).

There is no doubt that DCs play an important role in the establishment of HIV-1 infection and research efforts are currently aimed at developing therapeutic strategies which specifically target the interactions leading to trans-infection. Such agents will have the

potential to be used as topical treatments at the mucosa to block the initial DC mediated HIV-1 transmission events. The impact of currently existing antiretroviral drugs on specifically blocking trans-infection from DC to T cells has not been assessed. However a recent study, which aimed to determine whether the activity of antiretroviral drugs was limited by the mode of HIV-1 spread and the type of immune cell involved in transmission or was independent of these two variables, found that the high multiplicity characteristic of cell-to-cell transmission limited the efficacy of ARVs in HIV-1 cell-to-cell transmission from macrophages to T cells (Duncan et.al, 2013). It is reasonable to suggest that this may also apply to trans-infection from DC-T cells given that the reduced sensitivity to ARVs appears to be independent of the type of cell involved in cell-to-cell spread.

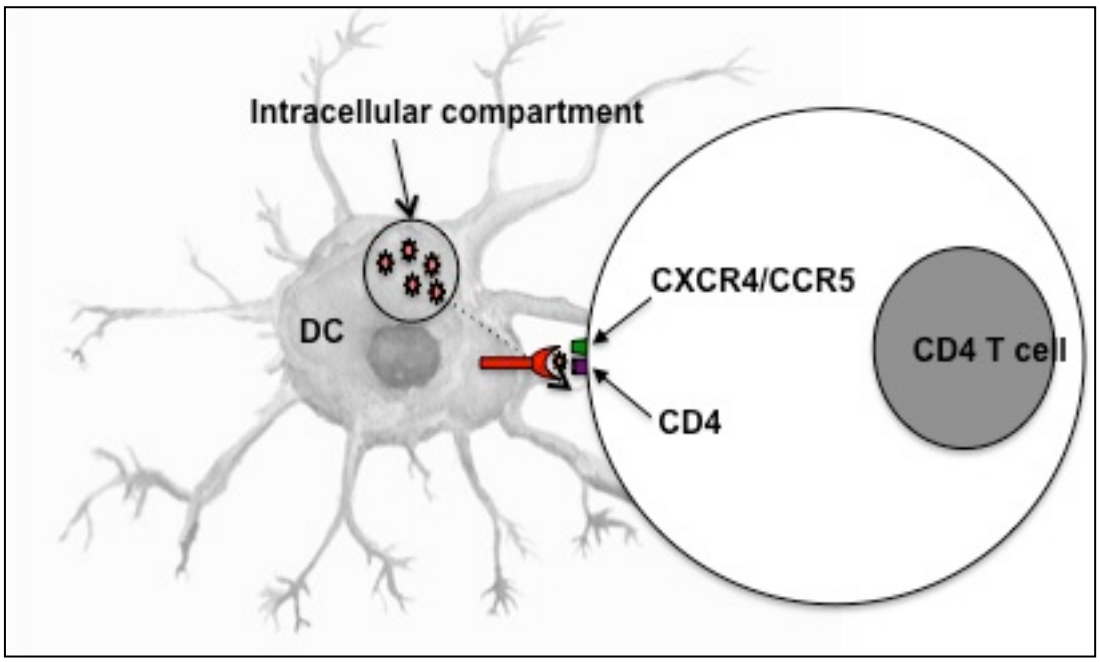


**Figure 1-10**: Dendritic cell to T cell spread of HIV-1

## 1.5.3 Inhibiting cell-to-cell spread of HIV-1

The ability of neutralising antibodies and drug inhibitors to block HIV-1 dissemination has typically been assessed by assays using cell-free virus infection systems. With increasing evidence that cell-to-cell infection of HIV-1 probably plays an important role in viral pathogenesis *in vivo*, several studies have assessed the role of neutralising antibodies,

interferon induced restriction factors and antiretroviral drugs on their ability to block infection mediated by this mechanism of virus transmission.

### 1.5.3.1 Cell-to-cell infection and neutralising antibodies

The effects of neutralising antibodies on cell-to-cell spread of HIV-1 have been extensively studied with results that sometimes appear paradoxical. These differences are likely due to the diverse experimental methods used (Schiffner et al., 2013). About 20% of HIV-1 infected individuals produce broadly neutralising antibodies (bNAbs) 2-4 years after infection (Kwong and Mascola, 2012). These naturally acquired bNAbs are insufficient to control or eliminate established infection. They have however been reproducibly shown to protect macaques against SIV and SHIV challenge (Moldt et al., 2012, Burton et al., 2011, Hessell et al., 2009, Parren et al., 2001) and to delay virologic rebound when infused in patients who have undergone a structured interruption of their antiretroviral treatment (Trkola et al., 2005). This makes them attractive targets for the development of prophylactic HIV-1 vaccines and immune based treatment strategies. Several factors are implicated in the inability of bNAbs to clear HIV-1 infection. These include: the high rates of escape mutations in Env allowing escape from the effects of antibodies (Wu et al., 2012, Bar et al., 2012), the existence of a latent virus reservoir established very early in infection (Eisele and Siliciano, 2012) and immune evasion mechanisms linked to the conformational flexibility of the viral Env and the masking effects of the glycan protein shield (Kwong and Mascola, 2012). Several studies have suggested that cell-to-cell HIV-1 infection serves as an additional explanation for the inability of bNAbs to clear HIV-1 infection in the host. This hypothesis was initially proposed by an early study which demonstrated that patient derived sera which effectively neutralised cell-free virus infection was ineffective in neutralising

infection in co-cultures of patient and donor peripheral blood mononuclear cells (PBMCs), over multiple rounds of infection (Gupta et al., 1989). With a better understanding of the mechanisms by which synaptic HIV-1 infection occurs, recent studies bearing in mind the differences in methodological approach, have convincingly shown that bNAbs are indeed able to inhibit infection by cell-to-cell spread albeit with a reduced efficiency in comparison to cell-free infection (Massanella et al., 2009, Durham et al., 2012, Abela et al., 2012, Su et al., 2012, Sagar et al., 2012, Malbec et al., 2013)(McCoy, 2014). This observation could have a significant bearing on the efficacy of prophylactic vaccines, underlining the need to assess the efficacy of vaccine candidates on their ability to effectively inhibit both cell-to-cell and cell-free modes of infection. There are several possible explanations for the relatively reduced sensitivity of synaptic infection to inhibition by bNAbs. These include the high multiplicity of infection and kinetic advantages characteristic of cell-to-cell infection, the steric barriers associated with cell-to-cell viral infection and the conformational changes in Env occurring during this mode of infection (Schiffner et al., 2013).

The increased multiplicity of infection of target cells, which characterises infection across the VS and the kinetic advantage of cell-to-cell infection, which is the result of clustering of receptors (CD4 and co-receptors) and viral Env proteins and Gag at the synapse (McDonald et al., 2003, Jolly et al., 2004, Schiffner et al., 2013), have been proposed to be at least partly responsible for the reduced *in vitro* efficiency of some bNAbs on cell-to-cell infection. The large number of virons which are transmitted during this mode of infection and the limited time of exposure to neutralising antibodies before virus attachment and infection of target cells, may limit the ability of bNAbs to fully inhibit HIV-1 cell-to-cell infections (Schiffner et al., 2013, Martin

and Sattentau, 2009, Massanella et al., 2009, Abela et al., 2012, Durham et al., 2012, Sagar et al., 2012, Malbec et al., 2013). This proposition however has been challenged by one study in which the effect of bNAbs were assessed for a DC-T cell mediated infection, and showed that even after equalising the infectivity of the virus for both cell-free and cell-to-cell modes of infection, some bNAbs directed against gp120 still displayed a reduced ability to neutralise cell-to-cell infection (Sagar et al., 2012).

In order to exert their neutralising effects NAbs must have access to the synapse or the compartment in which virions are held prior to synaptic release (in the case of DCs and macrophages). Steric hindrance is therefore a possible mechanism accounting for the reduced neutralisation efficiency observed with cell-to-cell HIV-1 infection. A study of the DC-T cell synapse using the 3D ion abrasion electron microscopy technique has provided a structural basis for steric hindrance (Felts et al., 2010). This study shows that T cells are enveloped by sheet-like membrane extensions from mature dendritic cells providing a shielded region for the formation of the VS. This compartment may be less accessible to some bNAbs, explaining their reduced efficiency (Felts et al., 2010). In contrast to this, a 3D reconstruction of the T cell-to-T cell VS reveals a more open structure, which remains accessible to NAbs that bind CD4 e.g. b12. This supports why such antibodies remain effective inhibitors of HIV-1 T cell-to-T cell infection .

Durham et.al showed that donor cells acutely transduced with HIV-1 appeared to transfer virions that expressed Env in conformations that were less susceptible to neutralisation by gp120 and gp41 antibodies. They also further demonstrated that truncating the cytoplasmic tail of gp41 significantly enhanced neutralisation of synaptic infection with

little or no effect on cell-free infection depending on the antibody assessed (Durham et al., 2012). Based on these results, they proposed that the cytoplasmic tail of gp41 regulates the exposure of key epitopes required for effective neutralisation during cell-to-cell spread and therefore plays an important role in the mechanism of immune evasion observed with this mode of infection (Durham et al., 2012). Seemingly contradicting these observations, synaptic infection mediated by chronically infected donor cells is equally sensitive to inhibition by gp120 and gp41 antibodies as cell-free infection. This dissimilarity may be attributed to observed differences in where viral fusion occurs depending on whether the effector cells are acutely transduced or chronically infected with HIV-1 (Schiffner et al., 2013). Some groups have suggested that infection with acutely transduced donor cells may involve endocytosis of immature virions following virus transfer and delayed CD4-dependent fusion from within endosomal compartments (Dale et al., 2011), while chronically infected cells produce mature viral particles which can immediately fuse following interaction with cell surface receptors on the target cell. However viral entry by endocytosis as an explanation for the reduced susceptibility to some NAbs is highly unlikely as this is now largely disproved. A recent study shows that viral fusion and entry occur predominantly at the plasma membrane during infection of T cell lines and CD4+ primary T cells (Herold et al., 2014).

#### 1.5.3.2 Cell-to-cell infection and interferon inducible restriction factors

Several interferon inducible host factors capable of inhibiting HIV-1 replication have been identified in recent years. These restriction factors constitute part of the innate immune response to virus challenge and present an interesting potential usefulness for immune modulation and gene therapy strategies, targeting HIV-1. Although the ability of restriction factors to inhibit cell-free infection has been

extensively studied, their role in inhibiting cell-to-cell spread of HIV-1 across the VS is less well defined. Studies are however emerging specifically addressing this question and so far the roles of some of these factors including: Tetherin, Trim5α (tripartite motif-containing protein 5α), APOBEC3G (Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) proteins, and SAMHD1 (sterile alpha motif and HD domain-containing protein-1) in inhibiting cell-to-cell infections have been considered.

***TRIM5α*** (Tripartite motif-containing protein 5α): Although human TRIM5α does not restrict HIV-1 infection, rhesus TRIM5α (rhTRIM5α) the Old World monkey orthologue of human TRIM5α, is a potent restriction factor of HIV-1 infection (Neil et al., 2008b, Stremlau et al., 2004, Malim and Bieniasz, 2012). There is interest in exploiting this property of rhTRIM5α in gene therapies by genetically modifying human cells to express rhTRIM5α as a strategy for treating HIV-1 infection (Malim and Bieniasz, 2012, Pertel et al., 2011). The mechanisms of HIV-1 restriction by rhTRIM5α are not fully understood but it is thought to affect several post entry steps in the viral replication cycle (Malim and Bieniasz, 2012). These include effects on the rate of capsid disassembly, disruption of the reverse transcription and integration steps (Malim and Bieniasz, 2012, Pertel et al., 2011). Richardson et.al studied the impact of rhTRIM5α on cell-to-cell spread of HIV-1 and found that cell associated infections were less susceptible to restriction than cell-free infection (Richardson et al., 2008). Given that the inhibitory effects of rhTRIM5α occur post-entry, it is possible that the high multiplicity in cell-to-cell infection increases the pool of incoming virus capsids capable of binding and saturating the intracellular rhTRIM5α and as such results in a reduced sensitivity of this mode of spread to rhTRIM5α mediated restriction (Richardson et al., 2008, Jolly, 2011). In fact this saturation of rhTRIM5α has been demonstrated for cell-free virus challenge in cell-

culture systems engineered to express rhTRIM5α (Stremlau et al., 2004, Anderson and Akkina, 2005).

***Tetherin***: The story is less clear-cut when the impact of tetherin on cell-to-cell spread of HIV-1 is considered. The studies that have addressed this question so far have generated conflicting results that seem at least in part, to be the result of the different experimental approaches used. Tetherin is a membrane protein which when expressed in the absence of the viral protein Vpu traps mature virions to the surface of the infected cells from which they are derived, thus preventing virus release. Kuhl et al. and Casartelli et al. suggested in their studies that tetherin was able to restrict HIV-1 cell-to-cell infection (Kuhl et al., 2010, Casartelli et al., 2010) while Jolly et al. showed a reduced susceptibility of cell-to-cell infections to the inhibitory effects of tetherin (Jolly et al., 2010). In the latter study Δvpu HIV-1 was also found to spread faster in T cell co-cultures in comparison to wild type HIV-1, under conditions in which tetherin inhibited cell-free virus spread (Klimkait et al., 1990, Yao et al., 1993, Schubert et al., 1995, Jolly et al., 2010). This finding bolsters the evidence supporting the putative importance of Vpu and cell-to-cell spread of HIV-1 *in vivo*. Vpu is highly conserved in transmitted founder viruses (Salazar-Gonzalez et al., 2009); and it has been demonstrated that tetherin can act as an innate immune sensor and thus may be important for Vpu conservation regardless of the mode of virus spread (Galao et al., 2012). The fact that cell-to-cell spread of HIV-1 remains efficient in the presence of tetherin may also be an indication that this mode of spread may be capable of bypassing the effects of an interferon induced virus restriction factor as has also been suggested for TRIM5$\alpha$ discussed above. All three studies on the effect of tetherin on cell-to-cell spread of HIV-1 though with different conclusions, demonstrated the presence of tetherin at the T cell virological synapse and showed that tetherin did not disrupt its

formation when expressed on the effector cells (Jolly et al., 2010, Casartelli et al., 2010, Kuhl et al., 2010). The apparent differences in results may be explained by; the different cell types used, the chronicity of infection of the effector cells and also the varying levels of tetherin expression in the cells, depending on the experimental approach. Future studies aiming to clarify the role of tetherin in cell-to-cell spread are needed and would have to consider potential confounding factors in the choice of experimental method.

***APOBECs***: APOBEC3G proteins are incorporated into newly assembled virions and restrict HIV-1 infection by their cytidine deaminase activity. They mediate post-synthetic editing of cytidine residues to uridine, causing a G to A hypermutation in the newly synthesised viral cDNA, thereby inhibiting reverse transcription and integration. They also appear to block the $tRNA^{Lys3}$ priming that is required for initiating reverse transcription. The viral infectivity protein (Vif) specifically counteracts the effect of APOBECs by binding and targeting these proteins for degradation and preventing their incorporation into newly formed virions. A recent study by Mohanram et al. has shown that using interferon-$\alpha$ to induce the expression of APOBEC3G, F, and A in immature dendritic cells limits the spread of HIV-1 to CD4+ T cells (Mohanram et al., 2013). Although this has not been specifically investigated for T cell-to-T cell infection, it is possible this cell-to-cell spread mechanism could also be sensitive to APOBEC restriction of viral replication like DC-to-T cell and cell-free infections.

***SAMHD1*** is a restriction factor that is expressed in DCs and other cells. It has a phosphohydrolase activity, which allows it to convert nucleotide triphosphates to a nucleoside and a triphosphate, and by so doing depletes the pool of intracellular dNTPs, thus preventing viral replication and infection in non-cycling cells (Laguette et al., 2011, Hrecka et al.,

2011, Lahouassa et al., 2012). The viral protein Vpx, an accessory protein encoded by HIV-2 and some SIVs, counteracts the effects of SAMHD1 by specifically binding to and targeting this protein for proteosomal degradation. Puigdomenech et al. have recently investigated the ability of SAMHD1 to inhibit cell-to-cell transmission from infected T cells to immature DCs and found that SAMHD1 significantly inhibits the productive cell-to-cell infection of target DCs (Puigdomenech et al., 2013). They also showed that through its ability to modulate the susceptibility of DCs to HIV-1 infection, SAMHD1 impacts on the ability of DCs to sense the virus and trigger an effective innate immune response. Although it is possible to hypothesise that during cell-to-cell infection dNTPs could be carried over from effector cells to target cells and as such counter the effects of SAMHD1 through a saturation mechanism, this study indicates that this is not the case at least for T cell to DC HIV-1 infection. It is therefore highly likely that SAMHD1 remains operative during HIV-1 intercellular spread and its activity in restricting virus replication is probably not saturable.

### 1.5.3.3 Cell-to-cell infection and antiretroviral therapy

Clinically available drug inhibitors have also been assessed for their ability to efficiently inhibit cell-to-cell spread of HIV-1. The fusion inhibitor Enfurvitide (T-20) and the attachment inhibitor Maraviroc (MVC) are equally efficient in blocking both cell-free and cell-to-cell infections (Abela et al., 2012, Agosto et al., 2014). Their efficiency is likely accounted for by their ability to block the functional interactions between Env and entry receptors during HIV-1 infection that is common to both cell-free and cell-to-cell spread. However, why these inhibitors that target attachment and entry should not show reduced efficacy during cell-to-cell spread, whereas NAbs that also target attachment and entry have been reported to do so remains unclear at present. Reverse transcriptase inhibitors have recently been shown to

have a reduced efficiency in inhibiting both T cell-to-T cell and macrophage-to-T cell mediated infection (Duncan et.al, 2013, Sigal et al., 2011, Sigal and Baltimore, 2012). The high multiplicity of infection that defines cell-to-cell infection has been proposed as the mechanism explaining this reduced sensitivity to RTIs. Sigal et al. proposed in a mathematical model confirmed using an *in vitro* system of T cell-to-T cell infection, that the high multiplicity in cell-to-cell infection stochastically increases the chance of a single virion escaping the effect of RTIs within the cell and going on to infect the cell (Sigal et al., 2011) .This has been confirmed for macrophage -to- T cell spread in the presence of RTIs (Duncan et.al, 2013). Permanyer et al. reported conflicting findings, stating that RTIs were equally effective against both cell-to-cell and cell-to-cell infection (Permanyer et al., 2012a). They explained their findings by proposing that the reporter gene assays used by Sigal et al. may have led to an overestimation of the level of target cell infection occurring during cell-to-cell spread in the presence of RTIs (Permanyer et al., 2012a). These discrepancies raise questions on the true effect of RTIs on cell-to-cell HIV-1 infections. Notably the effects of PIs, which are an important component of triple combination therapy and INIs, have not been considered for this mode of spread. These questions are specifically investigated in this thesis.

## 1.6 Antiretroviral therapy and drug resistance

In 1985-1986 3'-azido-2',3-dideoxythymidine, Zidouvidine (AZT), a nucleoside reverse transcriptase inhibitor, was the first molecule proven to effectively inhibit HIV-1 replication in cell culture (Furman et al., 1986). Over the course of the last 25 years several drugs have been developed for the treatment of HIV-1. In current clinical practice, physicians now have access to over 30 antiretroviral agents formulated either as single drugs or combinations to treat patients infected with HIV-1(Arts and Hazuda, 2012). These drugs target steps in the life-cycle

of the virus including; binding and entry of the virus into a target cell, reverse transcription of the viral RNA to form proviral DNA, integration of viral DNA into the host cell DNA and maturation of newly formed virions into fully formed infectious virus particles. Antiretroviral agents when administered in combination dramatically suppress the replication of the virus and reduce the HIV-1 viral load level in the plasma below the limits of detection by highly sensitive clinical assays. This leads to a significant recovery of the immune system as evidenced by an increase in CD4+ T cells with improved clinical outcomes and increased life expectancy for treated HIV-1 infected patients (Autran et al., 1997, Komanduri et al., 1998, Lederman et al., 1998). Since 2010, the HIV-1 treatment guidelines in the United States and the European Union recommend that antiretroviral therapy be initiated with three antiretroviral agents from at least two different drug classes (combined antiretroviral therapy = cART) when the peripheral CD4+ T cell counts fall to 350/mm3 (DHHS, 2014). Despite the many successes attributed to the introduction and widespread use of cART, these drugs are not capable of eliminating the HIV-1 infection and require strict adherence to a life-long treatment regimen for continued viral suppression (Arts and Hazuda, 2012). This presents major challenges as poor tolerance to drugs, drug interactions of antiretroviral agents with other medications and non-adherence can all lead to suboptimal levels of circulating drug, driving the evolution of drug resistance (Arts and Hazuda, 2012). In fact, drug resistance has been documented for all existing drug classes currently used in the treatment of HIV-1. This emphasises the need for developing new molecules that target HIV-1. A notable exception appears to be Dolutegravir (DTG), the most recent Integrase Inhibitor to be approved by the FDA. DTG is the only drug that has not selected for resistance mutations in the clinic (Mesplede and Wainberg, 2014). This is likely attributed to the long binding time of the drug to the integrase enzyme as well as the greatly reduced

replication capacity of the viruses that might become resistant to DTG (Bastarache et al., 2014, Mesplede and Wainberg, 2014).

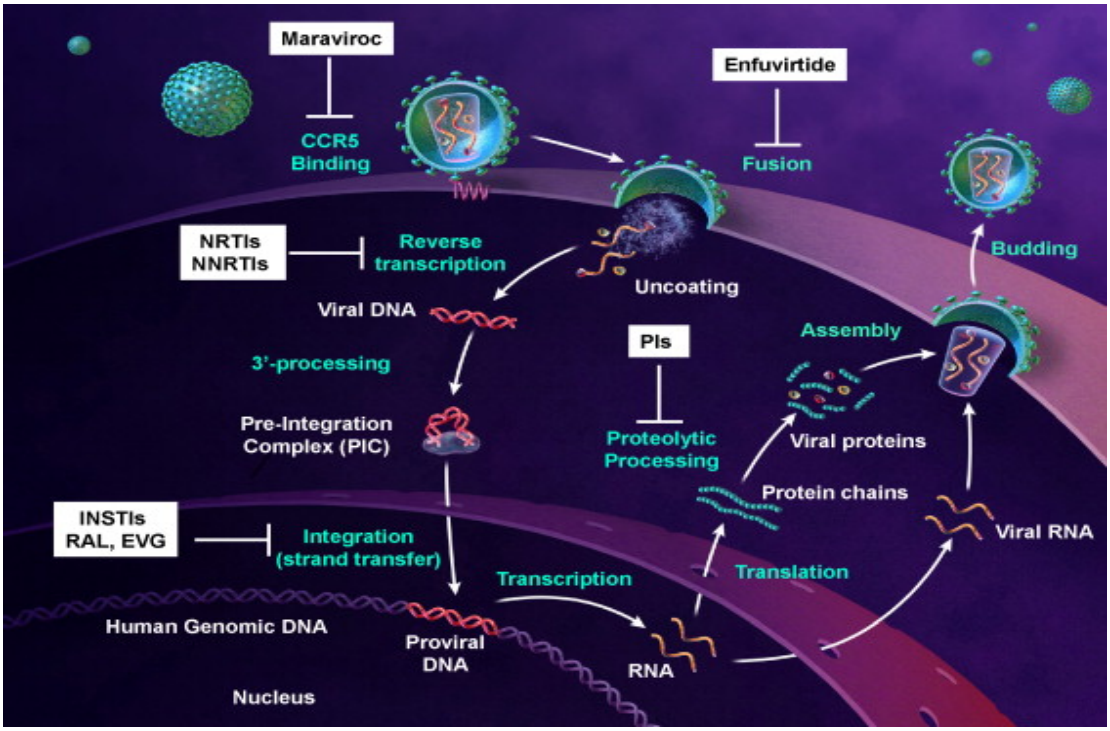


**Figure 1-11**: Antiretroviral agents targeting steps in the HIV-1 replication cycle (Suzuki, 2011)

## 1.6.1Reverse Transcriptase Inhibitors

### 1.6.1.1HIV-1 Reverse Transcriptase

HIV-1 RT is an asymmetric heterodimer composed of two related sub-units p66 and p51 (di Marzo Veronese et al., 1986, Lowe et al., 1988, Sarafianos et al., 2009). These sub-units come from the Gag-Pol polyprotein synthesised from unspliced virus mRNA and cleaved by the viral Protease (PR) following viral assembly and budding. RT possesses two enzymatic functions essential for copying the single stranded virus RNA into double stranded DNA that can be integrated into the host cell genome (Sarafianos et al., 2009, di Marzo Veronese et al., 1986, Lowe et al., 1988). The p66 sub-unit of RT contains the active sites for both enzymatic activities of the enzyme while the p51 sub-unit plays mainly a structural role. The crystal structure of unliganded RT has been solved and the three-dimensional structure of p66 is often compared to a right hand, with four domains (Kohlstaedt et al., 1992, Jacobo-Molina

et al., 1993): fingers (residues 1-85 and 118-155), palm (residues 86-117 and 156-236), thumb (237-318) and connection (319-426). The p51 sub-unit folds into the same four domains as the polymerase domain of p66, however the positioning of the sub-domains relative to each other is different in p66 and p51. The structural analyses of RT with various templates indicate that despite the sequence homology, p66 assumes a flexible structure while p51 is more compact playing a purely structural role devoid of any catalytic activity. This has led to the elucidation of the mechanism of action for the RT in which the "fingers" close around the primer-template and dNTP unit, allowing the precise alignment of the 3'-OH of the primer, the $\alpha$-phosphate and the polymerase active site, before the phosphodiester bond is formed (Huang et al., 1998, Kati et al., 1992, Sarafianos et al., 2009). The lengthening of the growing chain causes a relaxation of the "fingers", which open and allow the pyrophosphate to leave the active site (Sarafianos et al., 2009, Meyer et al., 2007, Kati et al., 1992). The nucleic acid substrate then translocates relative to RT to free the nucleotide-binding site so that the enzyme can bind to the incoming dNTPs (Sarafianos et al., 2009). The well recognised pivotal role of RT in the life-cycle of HIV-1 which has been discussed earlier, has led to the development of antiviral therapies specifically targeting RT. Nearly half of the drugs currently licensed for treating HIV-1 infection are reverse transcriptase inhibitors RTIs. Though some specific inhibitors of RNase H activity have been identified and tested *in vitro*, none have so far been approved for antiretroviral therapy (Sarafianos et al., 2009).

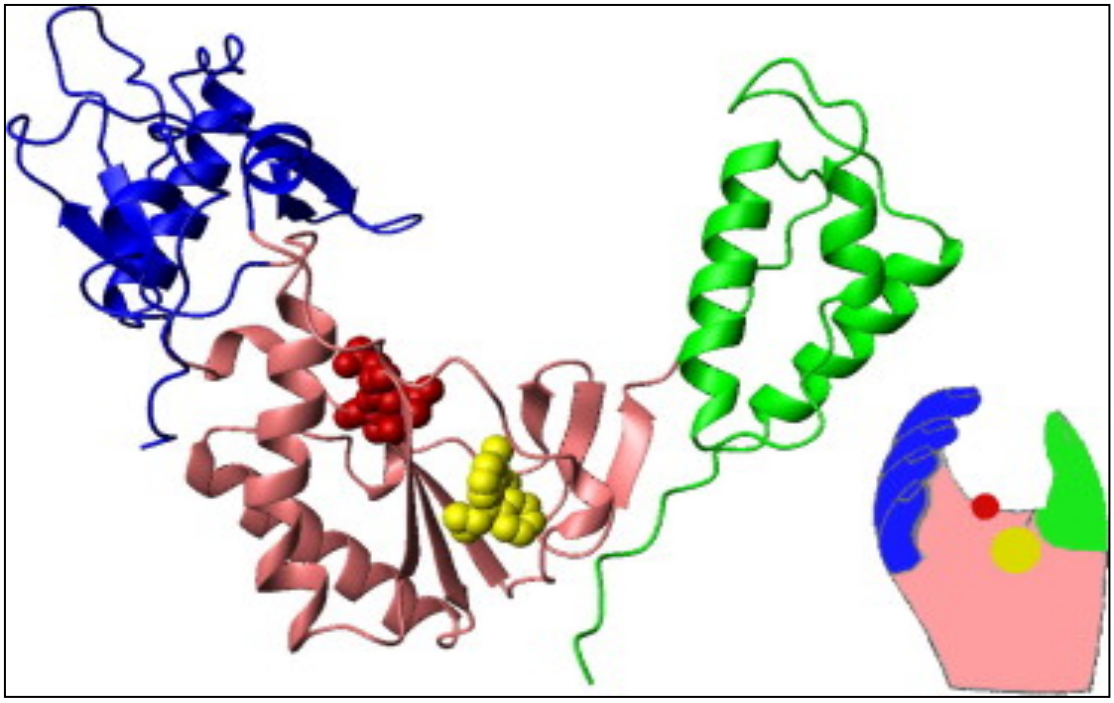

**Figure 1-12**: Ribbon representation of the active domain of HIV-1 RT illustrating the hand-like structure, showing fingers (blue), palm (pink) and thumb (green) the active site (red atoms) where DNA is elongated, is in the palm region. Also shown in yellow is an RT inhibitor drug in the pocket where it binds (Sarafianos et al., 2009).

### 1.6.1.2Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

NRTIs were the first established class of antiretroviral agents approved by the FDA and now constitute the backbone of cART. They are analogues of naturally occurring 2'-deoxy-nucleosides and nucleotides within the host cell. NRTIs are administered as pro-drugs and require phosphorylation by host cell kinases and phosphotransferases (Furman et al., 1986, Mitsuya et al., 1985, St Clair et al., 1987, Hart et al., 1992) to form the deoxynucleotide triphosphate analogues capable of inhibiting viral replication (Cihlar and Ray, 2010, Arts and Hazuda, 2012). Once converted to their triphosphate forms NRTIs compete with the natural dNTPs and become incorporated into the nascent proviral DNA chain. For some NRTIs the addition of the first phosphate is rate limiting and this has led to the development of NRTI pro-drugs, which already contain the first phosphate. An example of this is Tenofovir (TFV), which is administered as a pro-drug Tenofovir Disoproxil Fumarate (TDF) that only requires addition of the second and third phosphate

groups once in the cell. NRTIs lack a 3'-OH group on their 2'-deoxyribosyl moiety and this prevents the formation of a 3'-5'-phosphodiester bond between the NRTIs and incoming 5'-nucleoside triphosphate thus resulting in termination of the growing DNA chain (Mitsuya et al., 1985, Furman et al., 1986, St Clair et al., 1987, Hart et al., 1992). RT incorporates NRTI triphosphates with differing efficiencies, which may have some bearing on the relative potencies of the different existing NRTIs. Currently there are eight NRTIs approved by the FDA: Abacavir (ABC), Didanosine (ddI), Emtricitabine (3TC), Stavudine (d4T), Zidovudine (AZT) and Tenofovir disoproxil fumarate (TDF), the latter a nucleotide reverse transcriptase inhibitor (Cihlar and Ray, 2010, Arts and Hazuda, 2012).

Treatment with NRTIs as with all antiretroviral agents leads to the selection of drug-resistant HIV-1 variants, with a reduced susceptibility to these drugs. Drug resistance to NRTIs occurs by two well-elucidated mechanisms (Arts and Hazuda, 2012, Cihlar and Ray, 2010). The first mechanism of resistance affects the binding and rate of incorporation of the incoming nucleotide analogue and primarily implicates residues that are in direct contact with the incoming NRTI-triphosphate. Mutations in these residues enhance discrimination of the NRTI-triphosphate from naturally occurring triphosphates and as such prevent incorporation of NRTIs into the nascent chain. The classical example of such a mutation is M184V/I, a mutation that causes steric interference to the proper binding of 3TC and FTC in the HIV-1 RT binding site (Schinazi et al., 1993, Sarafianos et al., 1999). The second mechanism of drug resistance is through ATP-dependent removal of the NRTI-Triphosphate from the 3' end of the nascent chain after it has been incorporated, and the reversal of chain termination in a process known as pyrophosphorylysis. This mechanism of excision has been extensively studied and mutations linked to it are collectively known as

thymidine analogue mutations (TAMs). They promote pyrophosphorolysis and are implicated in resistance to AZT and d4T(Arion et al., 1998, Meyer et al., 1999, Boyer et al., 2001). Resistance mutations to NRTIs have been shown to reduce the fitness and replicative capacity of the virus to varying degrees when compared to wild-type virus (Arts and Hazuda, 2012, Cihlar and Ray, 2010).

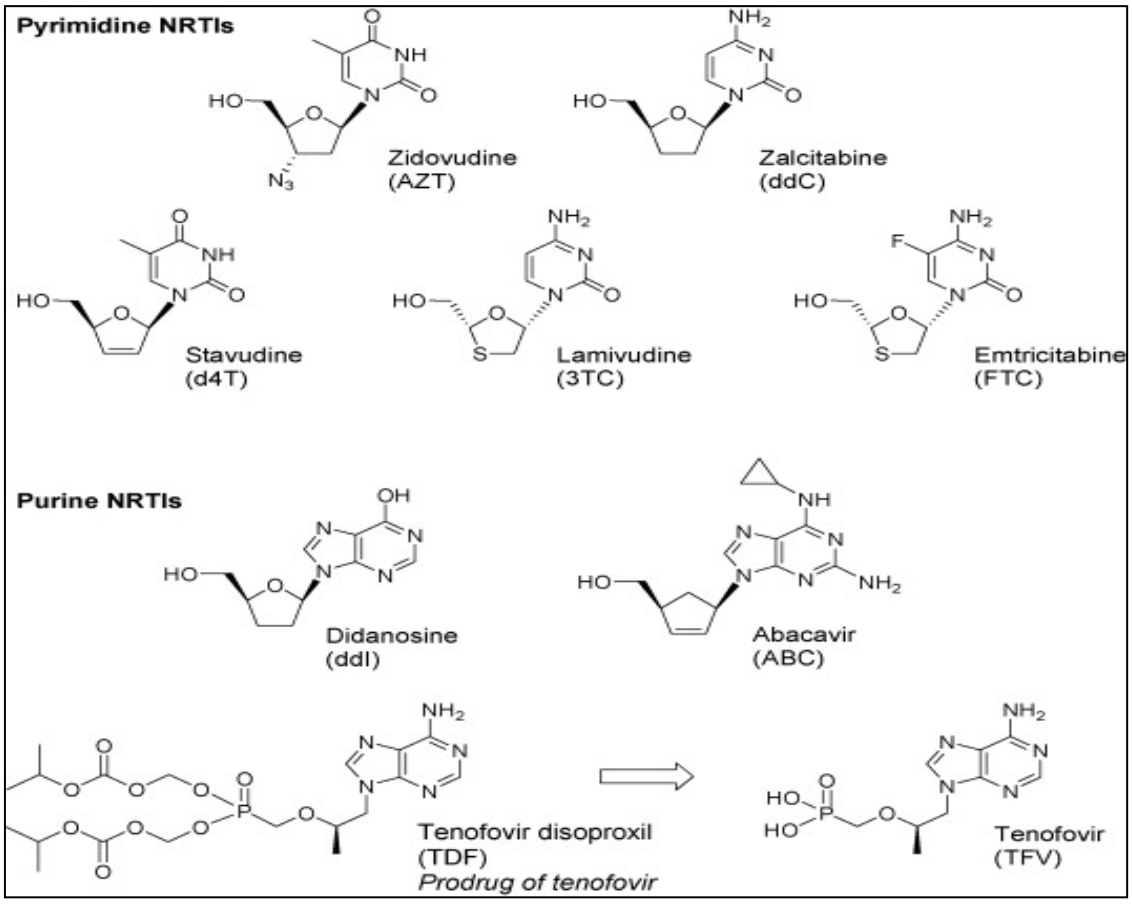


**Figure 1-13**: Chemical structure of approved NRTIs (Cihlar and Ray, 2010)

### 1.6.1.3Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs are a class of chemically diverse molecules with over 50 families (de Bethune, 2010, Jochmans, 2008). These non-competitive inhibitors of HIV-1 RT exert their inhibitory function by binding to the enzyme in a hydrophobic pocket located near the catalytic site of the enzyme at a distance of approximately 10Å. NNRTIs interact with this hydrophobic pocket, inducing a change in the spatial conformation of the binding site of RT that reduces its polymerase activity (Kohlstaedt et al., 1992,

Spence et al., 1995, Rodgers et al., 1995, Hsiou et al., 1996). The hydrophobic pocket is only created when the NNRTI binds to the enzyme and in the crystal structures of unliganded RT, the NNRTI binding pocket is not observed (Hsiou et al., 1996, Rodgers et al., 1995). Aromatic residues (Y181, Y188, F227, W229 and Y232), hydrophilic residues (K101, K103, S105, D132 and E224) and hydrophobic residues (Y181, Y188, F227, W229 and Y232) of the p66 subunit and two residues (I135 and E138) of the p51 subunit of RT line the hydrophobic pocket(de Bethune, 2010, Arts and Hazuda, 2012). It is worth noting that the shape of the pocket does not change significantly even though the NNRTI compounds are structurally very different (Spence et al., 1995, de Bethune, 2010). NNRTIs are highly specific to HIV-1 RT and do not inhibit the RT of other lentiviruses such as HIV-2 and SIVs (Kohlstaedt et al., 1992). There are currently four NNRTIs compounds approved for use in the treatment of HIV-1 and these include; Nevirapine (NVP), Efavirenz (EFV), Etravirine (ETV) and Delaviridine (DLV). Rilpivirine is in phase III clinical trials and there are several more in development.

Resistance to NNRTIs is generally the result of amino acid substitutions in the NNRTI binding pocket. These single nucleotide substitutions though capable of inducing high levels of resistance only cause a slight loss of replicative fitness for the virus (Dykes et al., 2001). The patterns of NNRTI resistance mutations are complex and alternative pathways to resistance have been described in individuals infected with non-subtype B viruses (Tantillo et al., 1994, Bacheler et al., 2000, Bacheler et al., 2001). K103N and Y181C are the most common NNRTI drug resistance mutations in subtype B viruses (Bacheler et al., 2000, de Bethune, 2010, Arts and Hazuda, 2012), however these mutations are also frequently selected in non-subtype B viruses (Akinsete et al., 2004) although the pathways to the development of resistance may be

slightly different (Lai et al., 2010). Most NNRTI drug resistance mutations engender varying degrees of cross resistance among the different NNRTIs and the selection of drug resistance to one member of the class generally limits the use of other members of the class in salvage second-line therapies following treatment failure (de Bethune, 2010). This is especially true for the first-generation NNRTIs (Nevirapine and Efavirenz), which are cornerstones of many first-line treatment regimens (Delaviridine is no longer used in clinical practice). The low barrier to development of drug resistance has confined these drugs to first-line treatments. This has driven the development of second generation NNRTIs with an improved resistance profile, with the aim of offering treatment experienced patients the chance to benefit from the convenient dosing and good tolerance profile of NNRTIs (de Bethune, 2010). Etravirine is the first of the new generation NNRTIs approved by the FDA and has been shown to be effective in the treatment of drug experienced adult patients with drug resistance to first generation NNRTIs and other antiretroviral agents (Madruga et al., 2007, Lazzarin et al., 2007). Rilpivirine is currently being assessed in phase III clinical trials for the same purpose.

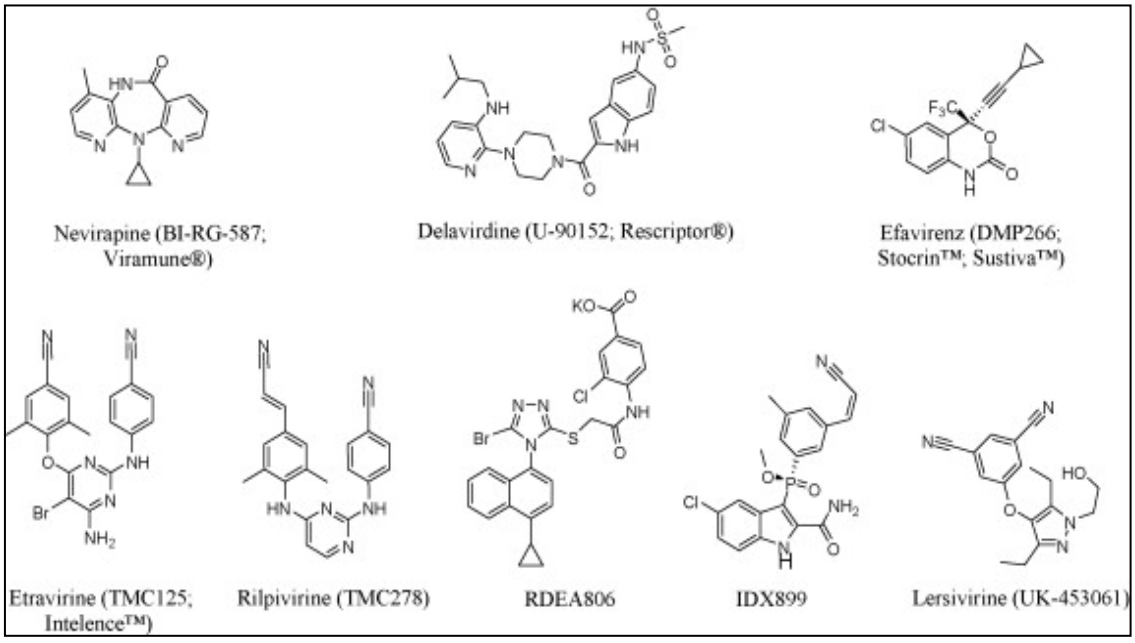


**Figure 1-14**: Chemical structure of first generation and next generation NNRTIs (de Bethune, 2010)

## 1.6.2 Protease Inhibitors (PIs)

### 1.6.2.1 HIV-1 Protease - Structure and Function

HIV-1 protease belongs to the family of aspartic proteases and exists as a homodimer consisting of two identical sub-units of 99 amino acids (Navia et al., 1989, Wlodawer et al., 1989). The active site is at the interface of the two sub-units and has three residues from each monomer Aspartic acid 25, Threonine 26 and Glycine 27. Residues 49-52 of each monomer form a flexible flap-like structure that extends over the substrate binding cleft (Wlodawer et al., 1989, Navia et al., 1989). The three important regions in the structure of the enzyme are therefore the active site cavity, the flexible flaps and the dimer interface. The flexibility of the flap region appears to be essential for the enzymatic activity of protease (Kräusslich et al., 1989, Weber and Agniswamy, 2009). The opening and closing of the flaps allow the movement of the substrate into and out of the active site. When inhibitors bind to the catalytic site with the flaps closed, the enzyme is essentially locked down preventing the processing of the substrates (Weber and Agniswamy, 2009).

HIV-1 protease cleaves precursor viral proteins, Gag and Gag-Pol, which accumulate at the plasma membrane during or shortly after the release of virus particles from infected cells (Park and Morrow, 1993, Miller, 2001). The viral protease therefore plays an essential role in the maturation of the virus, leading to the production of infectious virus particles. The HIV-1 protease recognises the asymmetric shape of the peptide substrates rather than specific amino acid sequences and all the cleavage sites on which the protease acts have a superimposable structure (Erickson-Viitanen et al., 1989, Wensing et al., 2010). Cleavage is an ordered and highly regulated process occurring at different rates for the various cleavage sites in Gag and following a set sequence (Wensing et al., 2010, Kräusslich et al., 1989, Pettit et al., 1994, Wiegers

et al., 1998, Erickson-Viitanen et al., 1989). The first cleavage event occurs at the C-terminal portion of p2 (MA-CA-p2/NC-p1-p6), this is followed by the cleavage of MA from CA-p2 (MA/CA-p2) and NC-p1 from p6 (NC-p1/p6), the two spacer proteins are the last to be cleaved p2 from CA (CA/p2) and p1 from NC (NC/p1). This processing of polyproteins by protease occurs during accumulation of the precursor polyproteins at the plasma membrane during or shortly after the assembled virions are released from the cell. Thus protease is not required for the production and release of virions but rather for the maturation of newly assembled virions into infectious particles.

### 1.6.2.2 Protease Inhibitor drugs

Solving the structure of the HIV-1 protease and its substrate led to the development of specific inhibitors of the viral protease (Craig et al., 1991, Kempf et al., 1995, Wensing et al., 2010, Arts and Hazuda, 2012). These molecules are designed to bind to the viral protease with a high affinity and by so doing prevent the enzyme from binding to and acting on its natural substrate. This leads to the production of immature non-infectious virions. In addition to its direct effect on HIV-1 protease, PIs have recently been shown to have multiple effects targeting other steps in the HIV-1 life cycle, with an inhibitory effect on viral entry, reverse transcription and post reverse transcription steps of viral replication (Rabi et al., 2013). In fact at therapeutic doses, approximately half of the inhibitory potential of PIs can be attributed to a block to viral entry, likely reflecting interactions between uncleaved Gag and the cytoplasmic tail (CT) of the envelope protein (Rabi et al., 2013).

The advent of PIs in the mid-nineties was a key moment in the development of antiretroviral therapy, which made possible the introduction of Highly Active Antiretroviral Therapy (HAART) combining

three active drugs from two different classes for the treatment of HIV-1 infection. Ten PIs are currently approved: Amprenavir (APV), Atazanavir (ATZ), Darunavir (DRV) Fosamprenavir, Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV) and Tipranavir (TPV)(Arts and Hazuda, 2012, Wensing et al., 2010). Due to extensive toxicity and high pill-burden associated with therapeutic doses of Ritonavir, it is no longer used as an antiretroviral in its own right. However its ability to reduce the metabolism of concomitantly administered PIs through inhibition of intestinal and hepatic cytochrome P450 3A4 enzyme, has led to its use as a pharmacokinetic enhancer for other PIs (Kempf et al., 1997). "Boosting" with Ritonavir enabled enhanced pharmacokinetics and more convenient twice-daily dosing schedules of other PIs (Kempf et al., 1997), except Nelfinavir which failed to show enhanced bioavailability when boosted, leaving this drug with a limited role as a relatively safe PI for use during pregnancy (Wensing et al., 2010). Boosting of Indinavir led to high peak plasma levels causing nephrotoxicity and as such has not been widely implemented (Voigt et al., 2002).

When they were initially developed, it was expected that resistance to PIs would be rare due to the small size of the viral protease (11kDa) and its vital role in the life cycle of the virus (Arts and Hazuda, 2012). However the protease gene has great plasticity and resistance has been described for all approved PIs, with polymorphisms observed in 49 of the 99 codons which constitute the protease gene and more than 20 substitutions shown to be associated with drug resistance (Arts and Hazuda, 2012, Wensing et al., 2010). Resistance to PIs develops in a step-wise manner and a mutation in the substrate-binding cleft is usually the first observed change (Molla et al., 1996). Resistance mutation in the binding site of the enzyme leads to its overall enlargement and reduces the ability of the inhibitor to bind, thus

leading to a decrease in the susceptibility of the drug (Wensing et al., 2010). This is generally accompanied by a decrease in the binding of the natural substrate and imposes a replicative and fitness cost to the mutated virus (Croteau et al., 1997, Mammano et al., 2000, Nijhuis et al., 1999). These mutations, which are first selected, and directly impact susceptibility to the PIs, are called primary or "major" resistance mutations. Secondary mutations or "minor" mutations do not by themselves confer resistance to PIs but emerge later to compensate for the loss in replicative fitness caused by the primary mutations (Nijhuis et al., 1999, Wensing et al., 2010). In addition to resistance mutations in the viral protease, several studies have also identified that mutations within the Gag protein, a substrate of protease, can also lead to PI drug resistance (Dam et al., 2009, Clavel et al., 2000, Gupta et al., 2010). These changes, which can occur within the eight major protease cleavage sites, improve the affinity of the substrate for the mutated protease and thus at least partially compensate for loss of viral fitness caused by primary protease resistance mutations (Doyon et al., 1996, Mammano et al., 1998, Miller, 2001, Nijhuis et al., 2001). All PIs share relatively similar chemical structures and as a result cross-resistance is commonly observed within this drug class.

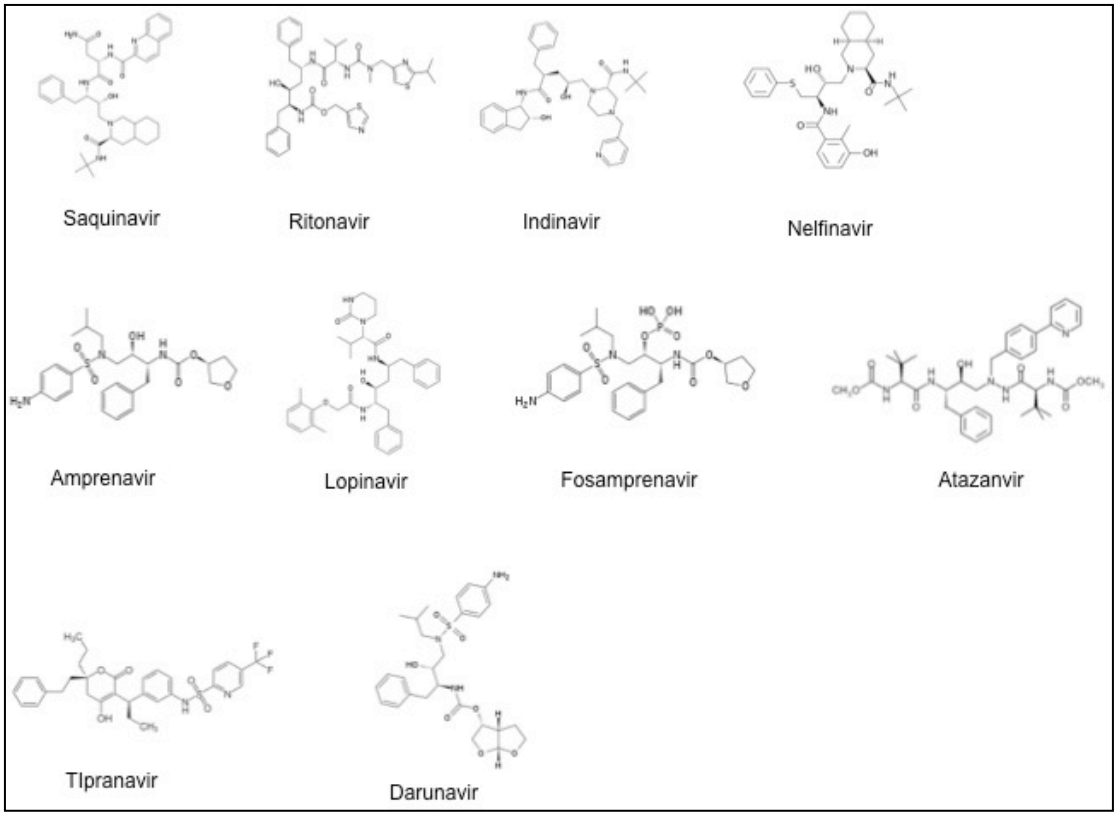


**Figure 1-15**: Structure of FDA approved Protease inhibitors (Wensing et al., 2010)

## 1.6.3 HIV-1 Entry Inhibitors

HIV-1 entry is a process that occurs through multiple steps mediated by interactions between the viral envelope proteins and cellular surface receptors and co-receptors as discussed earlier. Entry inhibitors have been developed to target specific steps including attachment of the virus particles to receptors on the target cell and fusion and entry into the cell. The drugs in this class can be divided into two main sub-groups: the fusion inhibitors and CCR5 small molecule antagonists.

### 1.6.3.1 Fusion Inhibitors

Fusion inhibitors disrupt gp41-mediated membrane fusion of the viral cellular membranes during entry (Tilton and Doms, 2010). Enfurvitide (T-20) was the first entry inhibitor approved for the treatment of HIV-1 in 2003 (Kilby et al., 1998, Lalezari et al., 2003). These drugs are synthetic peptides, which correspond to the HR1 and HR2 domains of gp41 (Tilton and Doms, 2010, Arts and Hazuda, 2012). These two domains

must interact with each other to promote fusion. The presence of a heterologous protein that is able to mimic these domains disrupts the interactions of these viral protein domains and as such prevents fusion from occurring. T-20 is a synthetic peptide with a sequence identical to part of the HR2 domain of gp41 and competes for binding to HR1 (Arts and Hazuda, 2012, Kilby et al., 1998). It has demonstrable potency against HIV-1 in clinical trials (Lalezari et al., 2003, Arts and Hazuda, 2012) and is currently used in salvage therapies for highly treatment-experienced patients (DHHS, 2014). However peptidic fusion inhibitors are not orally bioavailable and have to administered via parenteral routes. This has further limited their wider use in clinical practice.

Development of drug resistance to T-20 is mediated by mutations, which cluster within the HR1 domain of gp41 to which the drug binds (Wei et al., 2002, Tilton and Doms, 2010). While these mutations reduce the susceptibility of gp41 to T-20 they also reduce the efficiency of the six-helix bundle formation and the overall rate of fusion (Tilton and Doms, 2010).

### 1.6.3.2 CCR5 Antagonists

CCR5 antagonists are small molecules, which bind to the transmembrane helices of CCR5 (Dragic et al., 2000, Tsamis et al., 2003). They induce a conformational change of the receptor that causes it not to be recognised by the HIV-1 envelope (Tsamis et al., 2003, Tilton and Doms, 2010). Three CCR5 receptor antagonists Vicriviroc, Aplaviroc and Maraviroc, have so far shown inhibitory activity against HIV-1 in humans. Of these, Maraviroc (MVC) is currently the only one approved by the FDA since 2007 (Tilton and Doms, 2010, Arts and Hazuda, 2012). MVC binds a hydrophobic trans-membrane cavity of CCR5 causing a change in the conformation of the chemokine receptor that prevents interaction with the V3 loop of

gp120 (Dragic et al., 2000, Tilton and Doms, 2010, Arts and Hazuda, 2012).

The development of drug resistance to MVC follows a different pattern from other antiretroviral agents because MVC targets a host cell protein. Possible resistance mechanisms include; tropism switching i.e. the virus uses the CXCR4 co-receptor instead of CCR5 for entry, increased affinity for the co-receptor, using the inhibitor bound co-receptor and a faster rate of entry (Arts and Hazuda, 2012, Tilton and Doms, 2010). During the early stages of HIV-1 infection, most patients carry viruses that exclusively use CCR5 as co-receptor for infection (Lobritz et al., 2010). As infection progresses, variants, which use the CXCR4 co-receptor as well as dual tropic viruses begin to appear (Schuitemaker et al., 1992). In patients with a mixed population of circulating viruses i.e. CCR5-tropic and CXCR4-tropic viruses, administering CCR5 inhibitors can lead to outgrowth of CXCR4 viruses and treatment failure (Lobritz et al., 2010). Genotypic or phenotypic tropism testing is therefore a prerequisite for treatment with CCR5 inhibitors and only patients in whom no CXCR4 tropic viruses are detected are eligible for treatment with these drugs.

### 1.6.4 Integrase Inhibitors

Integrase inhibitors are the latest addition to the armamentarium of antiretroviral agents (Espeseth et al., 2000, Hazuda et al., 2004a, Hazuda et al., 2004b). The integrase enzyme catalyses two important reactions during the replication of HIV-1, notably the 3' processing of the double-stranded viral DNA ends and the transfer reaction which incorporates the viral DNA into the host chromosomal DNA forming a functional integrated viral DNA (Sherman and Fyfe, 1990, LaFemina et al., 1991, McColl and Chen, 2010). Integrase inhibitors are small molecules, which specifically target the strand transfer reaction

(McColl and Chen, 2010, Arts and Hazuda, 2012), and thus are more specifically referred to as Integrase strand transfer inhibitors (InSTIs). The FDA licensed Raltegravir (RAL) in 2007 and more recently Elvitegravir (2012) and Dolutegravir (2013). Elvitegravir like some PIs described above is co-formulated with the pharmacological enhancer Cobicistat, a CYP3A4 enzyme inhibitor, to improve the bioavailabilty of the drug. InSTIs bind to the specific complex between integrase and the viral DNA. These drugs interact with the essential Mg2+ ion cofactor in the active site of the enzyme and the DNA (McColl and Chen, 2010). InSTIs are comprised of 2 essential components, a phamacophore, which binds the $Mg^{2+}$ cofactor, and a hydrophobic group which interacts with the viral DNA and with the enzyme (McColl and Chen, 2010, Arts and Hazuda, 2012). This characteristic makes InSTIs the only antiretroviral drug class that interacts with two essential components explaining at least in part their broad efficacy against a wide variety of HIV-1 variants (Arts and Hazuda, 2012).

Mutations that cause drug resistance to InSTIs, are selected in the integrase-binding site, near the amino acid residues that are essential for the proper functioning of the Mg2+ cofactors (Arts and Hazuda, 2012, Hazuda et al., 2004a, Hare et al., 2010). Resistance mutations in integrase affect the replicative capacity of the virus as well as the functioning of the integrase enzyme (Marinello et al., 2008, Quercia et al., 2009). Three independent pathways have been identified for the development of drug resistance to Raltegravir, defined by primary signature mutations in residues Y143, N155 or Q148 of the integrase gene (Fransen et al., 2009, McColl and Chen, 2010, Arts and Hazuda, 2012). Cross-resistance is commonly observed with the drugs in this class, though low levels of clinical experience limits the understanding of the true impact of these resistance mutations in patients treated with InSTIs. In current clinical practice InSTIs are mostly used in second and

third-line therapies for highly treatment-experienced patients although rarely they can be used as part of first-line regimens for treatment naïve patients (DHHS, 2014).

## 1.7 Development and use of combination antiretroviral therapy

Before 1996, HIV-1 infection was a fatal disease with very few available therapeutic options. Treatment relied mainly on prophylaxes against opportunistic pathogens and treating AIDS-related conditions. This changed dramatically in the mid-nineties following a series of seminal studies that clearly described the viral dynamics and brought into sharp focus the fact that this was a viral infection requiring treatment. Ho et al., Perelson et al. and Wei et al. demonstrated the high turnover rate of HIV-1 and estimated that in an untreated individual there were $10^4$-$10^5$ or more particles per ml of plasma with a turnover rate of 10 billion virions per day (Ho et al., 1995, Wei et al., 1995, Perelson et al., 1996). Owing to its highly error-prone reverse transcription process, it was also estimated that a new mutation was introduced for every 1000-10000 nucleotides synthesised (Mansky and Temin, 1995, O'Neil et al., 2002, Abram et al., 2010). This high diversity leads to virus quasi-species within the host increasing the probability that HIV-1 variants with a reduced susceptibility to one or two antiretroviral drugs will exist even before treatment is initiated (Coffin, 1995, Frost and McLean, 1994). Using this knowledge on HIV-1 replication dynamics, mathematical modelling studies suggested that combinations of at least three drugs would be capable of providing durable inhibition of viral replication and would be better than combinations of two drugs (Frost and McLean, 1994, Coffin, 1995, Nowak et al., 1997, Stengel, 2008). These models were quickly verified in several randomised clinical trials (Staszewski et al., 1999, Walmsley et al., 2002, Robbins et al., 2003, Gallant et al., 2004, van Leth et al., 2004, Gulick et al., 2004) leading to

the birth of triple combination drug therapy initially popularised as Highly Active Antiretroviral Therapy (HAART)(Arts and Hazuda, 2012).

### 1.7.1 Antiretroviral drugs in current clinical practice

The antiretroviral drugs currently used in the treatment of HIV-1 infection cannot eradicate the virus from the body even when potent drugs are added to treatment regimens that already fully suppress viral replication below detection thresholds (Arts and Hazuda, 2012). This is a direct consequence of the existence of a virus reservoir consisting of a pool of latently infected CD4+ T cells, which is established in the very early stages of HIV-1 infection (Whitney et al., 2014). Antiretroviral therapy as currently prescribed therefore has the following aims:

- To reduce the morbidity and mortality associated with HIV-1 infection.
- To restore and preserve immune function.
- To suppress viral replication and reduce the plasma viral load.
- To prevent transmission from one person to another.

The World Health Organization (WHO) currently recommends initiating antiretroviral therapy with at least three drugs from two different drug classes in all patients with a confirmed diagnosis of HIV-1 infection, who have a CD4+ T cell count <500 cells/mm3, with priority given to initiating therapy among those with severe or advanced disease and CD4+ T cell counts of 350 cells/mm3 or less (WHO, July 2012). It also recommends that treatment be started regardless of the CD4+ T cell count in all infected patients with active Tuberculosis, Hepatitis B co-infection with chronic liver disease, all pregnant and breastfeeding women with HIV-1, children younger than 5 years infected with HIV-1 and all HIV-1 infected individuals in sero-discordant relationships (WHO, July 2012).

## 1.7.2 First-line antiretroviral therapy

The optimal antiretroviral treatment for a treatment-naïve patient consists of two NRTIs in combination with a third antiretroviral drug from one of three drug classes; an NNRTI, a PI boosted with ritonavir or an InSTIs. Below are listed preferred recommended first-line triple drug combinations (WHO, July 2012, DHHS, 2014, WHO, 2013).

NNRTI based treatment

EFV + 3TC(or FTC) +TDF

EFV + 3TC + AZT

NVP+3TC(or FTC) + TDF

NVP + 3TC (or FTC) + TDF

PI based treatment

LPV/r + 3TC(or FTC) + AZT

ATV/r + 3TC(or FTC) + AZT

ATV/r (or LPV/r) + 3TC (or FTC) + TDF

DRV/r + TDF + FTC

INSTI based treatment

DTG + ABC + 3TC (or FTC)

DTG + TDF + 3TC (or FTC)

ELV/cobi + TDF + 3TC (or FTC)

RAL + TDF + 3TC (or FTC)

It is worth mentioning that the treatment options available to patients in resource limited settings especially sub-Saharan Africa, the region most affected by the HIV-1 pandemic and home to an estimated 27 million HIV-1 infected individuals, are not the same as for patients in developed countries. Here NNRTI based treatments are the mainstay of first-line therapy with a limited pool of boosted PI-based regimens

mostly reserved for second-line treatment when first-line therapeutic options fail. This inequality in resources and treatment options is mainly due to existing patents on more recently approved drug classes and the overall cost of providing free anti-retroviral therapy to those who need treatment (WHO, 2013).

### 1.7.3 Monitoring antiretroviral therapy

Regular monitoring of patients who receive cART is essential to ensure successful treatment, promptly identify and correct adherence issues and to determine when to switch to second-line therapy in the event of treatment failure. The measurement of HIV-1 RNA (viral load) and the CD4+ T cell count are the two surrogate markers used to assess response to cART and disease progression in HIV-1 infected patients. Viral load is the most important indicator of an initial and sustained response to ART and is recommended as the preferred approach to diagnose and confirm treatment failure (DHHS, 2014, WHO, 2013). Viral load measurement not only provides an early and more accurate indication of treatment but also helps distinguish between treatment and non-adherence and can serve as an indicator for the transmission risk within a given population (Murnane et al., 2012, Das et al., 2010). With effective cART in a patient not harbouring any drug-resistant viruses, virological suppression is expected in 8-24 weeks following the initiation of cART (Thaker and Snow, 2003). The WHO recommends routine viral load monitoring every 6-12 months (WHO, 2013). In resource limited settings virological monitoring is challenging and not always available. CD4+ T cell counts, coupled with clinical monitoring are the main tools available for defining treatment failure and deciding when to switch to second-line therapy in this context. The CD4+ T cell count is the most important means of assessing immune function in HIV-1 infected patients. For most treated patients an adequate response to therapy is defined by a rise in the CD4+ T cell count of 50-150/$mm^3$

during the first year of treatment (WHO, 2013, DHHS, 2014). The WHO recommends CD4+ T cell count monitoring every 6-12 months (WHO, 2013).

### 1.7.4 Treatment failure, drug resistance testing and salvage therapy

Failure of antiretroviral therapy can be defined by clinical, immunological and virological criteria:

- Clinical failure is determined by a new or recurrent event indicating severe immunodeficiency after 6 months of effective cART (WHO, 2013, DHHS, 2014).
- Immunological failure: A fall in CD4+ T cell counts below baseline levels or a CD4 count persistently <100cells/$mm^3$ in a patient receiving effective cART (WHO, 2013, DHHS, 2014).
- Virological failure: A plasma viral load above 1000 copies/ml on 2 consecutive viral load measures after 3 months following initiation of cART with adherence support (WHO, 2013, DHHS, 2014).

Drug resistance mutations have been described for all antiretroviral drugs currently used as part of cART and remain the main cause of treatment failure in patients receiving treatment. Poor adherence to therapy, drug interactions between antiretroviral drugs and other medications as well as the side effects of treatment are also factors contributing to treatment failure. Drug resistance testing is recommended for patients presenting with virological failure after receiving adequate active triple combination therapy and at baseline for all HIV-1 infected patients entering care, to guide the choice of initial and second line therapies (DHHS, 2014).

Genotyping and phenotyping assays are currently available to test for drug resistance. Standard genotyping assays for drug resistance involve testing for mutations in HIV-1 genes (usually RT and PR), which can confer resistance to RTIs and PIs. These assays involve amplifying HIV-1 sequences from plasma samples with detectable viral load and identifying drug resistance mutations through sequencing. The main limitation with genotyping is that most commercially available assays do not assess determinants of drug resistance outside the virus RT and PR genes (such as Gag for PIs and Integrase for InSTIs) and could fail to identify the cause of drug resistance in some patients failing on regimens containing drugs from these classes (Hirsch et al., 2008).

Phenotyping assays measure the ability of clinically derived HIV-1 isolates to grow in the presence of drugs. They enable the assessment of virus replication at different drug concentrations and the results are used to calculate 50% and 90% inhibitory concentrations (IC50 and IC90) of a given drug for the isolate under investigation. Commercially available phenotyping assays amplify HIV-1 PR and RT as a unit from the plasma virus and generate a recombinant virus with other genes from a laboratory construct. Using reporter gene based systems the drug susceptibility of the construct carrying clinically derived RT and PR is assessed. With both standard genotyping and phenotyping assays only the predominant circulating viruses in the circulating pool of viruses are sampled, and as such minority drug-resistant species which can cause treatment failure or transmitted resistance are often missed (Hirsch et al., 2008). Ideally drug resistance testing should be performed while the patient is still taking the failing ARV regimen or within four weeks of discontinuing treatment (DHHS, 2014). Identifying treatment failure and making a decision to switch to second-line therapies relies mainly on clinical assessment in resource-limited settings, where the

cost and availability of technical capacities render wider use of drug resistance testing difficult (WHO, 2013).

Once treatment failure has been established, treatment guidelines currently recommend that the failing regimen be replaced with second-line therapies. Second-line treatments usually comprise of three drugs from at least two different drug classes which were not part of the initial treatment regimen and to which the patient doesn't have resistance mutations based on phenotyping or genotyping resistance assays (DHHS, 2014). In highly treatment-experienced patients with complicated drug resistance profiles the entry inhibitors Enfurvitide and Maraviroc as well as second generation NNRTIs that are currently not part of initial treatment options may be considered as part salvage therapy (DHHS, 2014).

### 1.7.5 Antiretroviral therapy for prevention of HIV-1 infection

Besides their use in treating patients infected with HIV-1, antiretroviral drugs have also been successfully used in strategies aimed at preventing infection. Currently there are four scenarios for which antiretroviral prophylaxes has proven successful and these have now been implemented to varying degrees globally in public health programs aimed at HIV-1 prevention.

#### 1.7.5.1 Prevention of mother to-child-transmission (PMTCT) of HIV-1

In the absence of any intervention the natural history of HIV-1 transmission during pregnancy carries a risk of 25-45%. In the mid-nineties following the FDA approval of increasing numbers of drugs for the treatment of HIV-1, the usefulness of antiretroviral therapy as a strategy to prevent mother-to-child transmission was demonstrated in several clinical trials (Connor et al., 1994, Shaffer et al., 1999, Wiktor et

al., 1999, Guay et al., 1999, Lallemant et al., 2004). These studies found that using AZT or NVP in monotherapy and dual therapy combinations antepartum, intrapartum and for the newborn, led to a reduction in the risk of mother-to-child transmission to 5-8% (Lallemant et al., 2004, Guay et al., 1999, Wiktor et al., 1999, Shaffer et al., 1999, Connor et al., 1994). These initial interventions where recommended by the WHO and other leading public health agencies and very quickly led to a significant reductions in the incidence of vertical transmission of HIV-1. Following the advent of HAART in the early 2000s, triple therapy combinations where assessed for PMTCT and found to be even more effective, cutting the transmission risk down to 1-2% (Marazzi et al., 2007, Marazzi et al., 2009, Marazzi et al., 2010).

The WHO now recommends cART for all pregnant and breastfeeding women with the option of discontinuing cART once the MTCT risk period has ceased for women not meeting the treatment eligibility criteria or lifelong cART for all pregnant women (WHO, 2013). ARVs as PMTCT reduce the circulating maternal viral load considerably and by so doing, reduce the likelihood that the baby will be exposed to infectious virus during pregnancy, at delivery and through breastfeeding. It is worth noting that due to limitations in resources, monotherapy and dual therapy interventions for PMTCT are still used in many middle and low-income countries despite the current recommendations. PMTCT has been associated with the development of drug resistance in the mother and transmitted drug resistance in babies born to infected mothers (Eshleman et al., 2001, Jourdain et al., 2004). Drug resistance associated to PMTCT may compromise future cART options and should be considered when choosing cART regimens for women who have previously received single or dual therapy ARVs for prophylaxes.

### 1.7.5.2Pre-exposure prophylaxes (PreP)

PreP is a fairly recent intervention for prevention aimed at individuals who are not infected with HIV-1 but have a substantial risk of becoming infected. The efficacy of oral PreP, a combination of two NRTIs Tenofovir and Emtricitabine in a single pill (Truvada®), has been demonstrated in several clinical trials assessing the impact of this intervention in different risk groups. In men who have sex with men (MSM), PreP reduced the risk of acquiring HIV-1 infection by as much as 92% (iPrex study)(Grant et al., 2010); among heterosexually active men and women the risk of transmission was reduced by 62% (TDF2 study) (Thigpen et al., 2012); in HIV-1 discordant couples PreP reduced transmission by up to 90% (Partners PreP study) (Baeten et al., 2012) and among injecting drug users a single dose of Tenofovir, one of the components of Truvada® used in the other three studies, reduced the risk of getting HIV-1 infection by 49% (Bangkok Tenofovir study)(Choopanya et al., 2013). The use of topical agents for PreP has also been assessed and the CAPRISA study showed 2% Tenofovir gel applied before and after sexual intercourse vaginally, reduced the risk of HIV-1 transmission by 54% for the women who used the intervention effectively (Abdool Karim et al., 2010).

Macaque models of vaginal infection with SIVs have given some insight into the early events around the mucosal transmission of HIV-1 (Heneine and Kashuba, 2012). These studies suggest that with mucosal transmission, there is an initial phase during which HIV-1 replicates at low levels at the point of entry (Heneine and Kashuba, 2012). PreP is designed to target this brief window period of vulnerability to block the virus infection from taking hold and establishing reservoirs within the host (Heneine and Kashuba, 2012). Despite the success seen in the PreP trials cited above, there remains considerable controversy in this area due to the failure of some trials to show any benefit in reducing

the risk of HIV-1 transmission with this intervention. The FEM-PreP (Van Damme et al., 2012) and VOICE trials were discontinued before completion because of futility. Also the variation in the degree of protection observed in the studies where PreP was found to be beneficial remains for the most part unexplained (Van Damme et al., 2012). Several factors could possibly account for the observed differences in results including; the different study populations enrolled in these trials, the degree of adherence to the prophylactic regimen and the route of transmission (rectal, vaginal or parenteral) for the population under study (Cohen and Baden, 2012).

With the recent observations on the reduced sensitivity of cell-to-cell viral spread to inhibition by antiretroviral drugs (Sigal et al., 2011, Duncan et.al, 2013), it is reasonable to contemplate whether this reduced sensitivity to drugs could confer an advantage to the virus allowing it to spread from the initial site of introduction and establish infection in the presence of PreP. This is especially important given that there is substantial evidence, albeit mainly derived from primate based transmission studies (Anderson et al., 2010), that cell-to-cell spread plays a role in the early steps of HIV-1 transmission at the mucosa. Cell-associated infection was demonstrated in SIV vaginal challenge of macaques and the concentration of infected cells needed for transmission in this model were shown to be within the physiological range, while cell-free infections required supra-physiological doses to establish infection (Salle et al., 2010, Weiler et al., 2008). The impact of antiretroviral drugs on cell-free and cell-to-cell modes of HIV-1 infection could have implications for the choice of ARVs used in PreP strategies.

The CDC and the WHO now recommend the use of oral PreP as a single daily dose of Truvada® for individuals who fit well-defined risk criteria for acquiring HIV-1 infection (Prevention, 2014, WHO, 2013). In

sub-Saharan Africa PreP is currently not widely used outside the context of clinical trials although mathematical modelling studies suggest that over a period of 10 years an effective PreP program could prevent 2.7-3.2 million new HIV-1 infections in this region (Heneine and Kashuba, 2012). Preliminary data on the risk of developing drug resistance following the implementation of PreP indicate that the benefits of PreP by far outweigh the risk associated with developing drug resistance in individuals who become infected despite receiving prophylaxis (van de Vijver and Boucher, 2010). Tenofovir and Emtricitabine, the components of Truvada®, are recommended as first-line choices for cART and as such monitoring for drug resistance in populations where PreP is being rolled out is important and recommended (Prevention, 2014).

#### 1.7.5.3Post-exposure prophylaxis (PEP)

PEP is short-term antiretroviral treatment to reduce the likelihood of HIV-1 infection after potential risky exposure to the virus which can be either occupational or through sexual intercourse. Data from simian infection models suggests that the post-exposure window during which infection can be cured ranges from 24 hours for IV injection (Tsai et al., 1998) to 48-72 hours for vaginal challenge (Otten et al., 2000). After the first few days elapse this curative window closes as HIV-1 drug insensitive reservoirs become established within the host (Sigal and Baltimore, 2012). PEP targets this window of opportunity to limit and possibly eliminate viral replication at the site of entry and prevent seeding of the virus in the lymphoid tissues and established infection. The WHO currently recommends using any of the first-line cART regimens for 28 days with the first dose being offered as soon as possible within 72 hours of the exposure (WHO, 2013).

## 1.8 Thesis Overview and Scope

Recent observations on the reduced susceptibility of HIV-1 cell-to-cell infection to inhibition by RTIs have raised questions on the bearing this mode of spread may have for the successful treatment of HIV-1, the maintenance of viral reservoirs and viral pathogenesis. RTIs are major components of first-line and second-line cART as previously discussed. This thesis presents a detailed assessment of the individual drug classes (PIs, RTIs and INIs), which constitute first-line and second-line antiretroviral therapies with regards to their ability to inhibit cell-to-cell HIV-1 infection in comparison to cell-free infection. Special emphasis is given to the study of PIs, which have a mechanism of action different from RTIs, present a higher barrier to the development of drug-resistant mutants, are highly potent and are very important for first-line and second-line treatment options. The results obtained from this study are presented in three sections as briefly summarised below:

In chapter 3, two assay systems, one with an indirect output measure (Tat-driven luciferase expression) and another with a direct output measure (HIV-1 *pol* DNA transcripts) of HIV-1 infection, are assessed for their use in studying the effect of inhibitors on HIV-1 cell-to-cell infection. The results presented in this section highlight specific limitations of using indirect output measures of infection when studying cell-to-cell spread of HIV-1, especially in the context of some types of inhibitors. It also justifies the use of the assay with a direct output measure of infection for the drug studies in my thesis. PI and RTI resistant mutants used in this thesis are also constructed, characterised and assessed for their ability to spread efficiently by a cell-to-cell mechanism in this section.

In chapter 4, a detailed study of the impact of PIs on cell-to-cell spread of wild type and drug-resistant HIV-1 is presented and compared to RTIs

already studied by other groups. This section highlights the variable effects of the different drug classes used in cART on cell-to-cell spread of HIV-1 and clearly demonstrates that PIs are equally effective at blocking all modes of virus infection, whereas RTIs show reduced efficacy, with NRTIs being a lot less effective than NNRTIs. The effect of INIs on cell-to-cell spread of HIV-1 between T cells is also studied and Raltegravir is shown to be effective against this mode of virus dissemination.

Chapter 5 addresses the question of drug combinations and cell-to-cell spread of HIV-1. Here cell-to-cell spread of HIV-1 is studied in the presence of clinically relevant PI and RTI-based combinations and compared to cell-free spread of the virus. The median effect principle is applied to assess the impact of the mode of virus transmission and drug resistance on drug interactions in the combinations tested.

Overall the study aims to provide a better understanding of the impact of antiretroviral therapy on cell-to-cell spread of HIV-1 and within reason, bearing in mind the limitations of *in vitro* models, gives some insight on the possible clinical implications of these observations for current HIV-1 treatment and prophylaxis.

# 2 Materials and Methods

This section contains a detailed listing of the reagents, organisms and description of the general methods used for this research project.

## 2.1 Materials

### *2.1.1* Enzymes

*DNA polymerase*

Taq DNA polymerase was purchased from Invitrogen® (Life Technologies Ltd, Paisley, UK). Hot Gold Star DNA polymerase used for real-time PCR was supplied in a RT-PCR Master mix plus low ROX from Eurogentec ® (Seraing, Belgium). Proofreading Pfx DNA polymerase was obtained from Invitrogen® (Life technologies Ltd, Paisley, UK).

*Restriction endonucleases*

Restriction enzymes and their 10x concentrated reaction buffers were obtained from New England Biolabs (NEB) (Ipswich, UK) Ltd and from Promega® UK Ltd (Southampton, UK).

*Alkaline Phosphatase*

Recombinant Alkaline phosphatase from bovine intestine was purchased from Roche® Diagnostics (Mannheim, Germany).

*T4 DNA Ligase*

T4 DNA ligase was obtained from Roche® Diagnostics (Mannheim, Germany).

### 2.1.2 Molecular weight markers (DNA and Protein)

*1Kb plus DNA Ladder* (Life Technologies Ltd, Paisley, UK)

DNA fragment sizes (base pairs): 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 369, 344, 298, 220,201, 154, 134, and 75.

*Protein molecular weight marker*

Pre-stained Amersham® SDS molecular weight standard mixture was obtained from GE healthcare Life Sciences (Uppsala, Sweden).

### 2.1.3 Deoxyribonucleotides (dNTPs)

dNTPs were supplied by Invitrogen® (Life Technologies Ltd Paisley, UK).

### 2.1.4 Oligonucleotide primers

Primers for PCR and DNA sequencing were designed using the computer software Sequencher® Gene Codes Corporation (Ann Arbor, Michigan, USA). All oligonucleotides were ordered from Eurogentec® (Seraing, Belgium). A list of oligonucleotide names and sequences is provided in the methods section.

### 2.1.5 Plasmids and molecular clones

*pNL4.3*

The HIV-1 clone was obtained from the NIH AIDS Research and Reference Reagent Program (ARRP). It is a plasmid that contains the full-length replication, and infection competent chimeric HIV-1 DNA derived from subtype B clinical isolates. Upon transfection, this clone directs the production of infectious virions in a wide variety of cells (Adachi et al., 1986).

*pCR®2.1TOPO*

This cloning vector was supplied in the TOPO® TA Cloning Kit from Invitrogen® (Life Technologies, Paisley, UK) and used for cloning to design drug-resistant HIV-1 molecular clones created by site directed mutagenesis of pNL4.3.

## 2.1.6 Bacteria strains

*Escherichia coli*

One Shot® TOP10 chemically competent Escherichia coli from Invitrogen® (Life Technologies Ltd, Paisley, UK) was used for plasmid propagation through out this thesis. The genotype of this strain is: F- mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galUgalKrpsL (StrR) endA1 nupG.

## 2.1.7 Cells

*Human embryonic kidney (HEK) 293T cells*

HEK293T cells were originally from ATCC® (American Type Culture Collection, Virginia, USA). The 293T cell line, originally referred to as 293tsA1609neo, is a highly transfectable derivative of human embryonic kidney 293 cells, and contains the SV40 T-antigen.

*HeLa TZM-bl cells*

HeLa (Henrietta Lacks) TZM-bl cells were obtained from the Center for AIDS reagents, National Institutes of Biological Standard and Control, UK (CFAR, NIBSC) and donated by J.Kappes, X.Wu and Tranzyme Inc. This cell-line is an engineered HeLa cell clone that expresses human CD4, CCR5 and CXCR4 and contains HIV-Tat regulated genes for firefly luciferase and β-galactosidase. HeLa TZM-bl is highly sensitive to infection with diverse isolates of HIV and SIV.

*Jurkat CE6.1*

This is a CD4+/CXCR4+ T cell line obtained through the AIDS Research and reference reagent program, Division of AIDS, NIAID, NIH (ARRP). The cell line was cloned from cells obtained from Dr. Kendall Smith and donated by Dr. Arthur Weiss.

*1G5*

This is a Jurkat derivative containing a stably integrated HIV-LTR luciferase construct. It was obtained from the AIDS Research and reference reagent program (ARRP), Division of AIDS, NIAID, NIH, donated by Dr. Estuardo Aguilar-Cordova and Dr. John Belmont.

*ACH-2*

This is a HIV-1 latent T cell clone with one integrated proviral copy. These cells are CD4-, CD5+, transferrin receptor +, Leu-1+ and HIV-1+. They are derived from A3.01 cells infected with LAV and cloned by limiting dilution. ACH-2 is a clone that survived infection and constantly produces low levels of RT and p24. The subclone A.3.01 is derived from CEM, a human T cell line originally isolated from a four year old caucasian female with acute lymphoblastic leukemia. This cell line is obtained from the NIH AIDS reagent program ARRP), division of AIDS, USA. Donated by Dr. Thomas Folks.

## 2.1.8 Antibodies

*Coating anti-HIV-1 p24 antibody (D7320)*

D7320 is a sheep polyclonal antibody, which was used for the p24 ELISA. Supplied by Aalto® Bioreagents (Dublin, Ireland).

*Biotinylated$\alpha$-p24 (BC1071-BIOT)*

BC1071-BIOT is a mouse monoclonal antibody, which was used for the p24 ELISA. Supplied by Aalto® Bioreagents (Dublin, Ireland).

*HIV-1 anti-Tat antibody (02-002)*

Mouse raised monoclonal antibody against recombinant Tat protein Tat protein of HIV-1 (BH10) origin with epitope mapping to amino acids 6-12. Supplied in a vial containing 100$\mu$g IgG1 in 1ml of PBS with < 0.1% sodium azide from Santa Cruz Biotechnology (Heidelberg, Germany).

*Normal Mouse IgG1 (purified)*

Mouse IgG1 isotype control antibody (supplied by Life Technologies Ltd, Paisley, UK). Supplied in a vial containing 100 $\mu$ g IgG1 in 1ml of PBS with <0.1% sodium azide.

*Anti-HIV-1 Gag p55 and p24 antibody*

This is a rabbit raised polyclonal IgG antibody that recognises p55Gag and p24CA it was obtained from the Center for AIDS research (CFAR) NIH, ARRP, and donated by Dr. G. Reid. This antibody was used for western blotting.

*Anti-HIV-1 p24 antibody-FITC*

This is an IgG goat polyclonal antibody to HIV-1 p24 which is conjugated to FITC (Fluorescein isothiocynate) obtained from Abcam® plc (Cambridge, United Kingdom).

## 2.1.9 General laboratory chemicals

General chemical reagents such as salts, alcohols, organic compounds and detergents were obtained from a range of suppliers including Fisher Scientific (Loughborough, UK), Sigma-Aldrich Company Ltd (Poole,UK), Life Technologies (Paisley, UK).

These include: agarose powder, acrylamide solution, sodium dodecyl sulphate (SDS), Tris, Ethylenediaminetetraacetic acid (EDTA), Dimethyl sulphoxide (DMSO), Tetramethylethylenediamine (TEMED), Ammonium Persulphate (APS), Acrylamide solution, NaOH (sodium hydroxide), NaCl (sodium chloride), HCl (hydrochloric acid), H2SO4 (sulphuric acid) , glacial acetic acid. Methanol, Ethanol, Ethidium Bromide, glycerol, X-gal. Tetramethylbenzidine (TMB), NaHCO3 (Sodium hydrogen carbonate).

## 2.1.10 Electrophoresis gels

*1% Agarose gel*

1g of agarose powder (Sigma-Aldrich) + 100ml of 1x TAE buffer. Microwaved for 2min or until the agarose dissolved and the solution became clear. Allowed to cool before pouring into cast.

*10% acrylamide gel (resolving gel) for SDS-PAGE*

The formula for two gels: 2.64ml 40% Acrylamide solution (Sigma-Aldrich Ltd, Poole,UK) + 2.96ml 1M Tris (Sigma-Aldrich) pH 8.8 + 79.2µl10% SDS + 2.3ml dH2O, 26.5µl Ammonium persulphate (APS) (Sigma-Aldrich Ltd, Poole, UK)+ 5.3µl TEMED (Sigma-Aldrich Ltd, Poole, UK ).

*Stacking gel for SDS-PAGE*

The formula for two gels: 1.66ml 40% Acrylamide solution + 1.24ml 1M Tris pH 6.8 + 99.2µl 10%SDS + 9.94ml dH2O + 49.6µl APS + TEMED.

## 2.1.11 Buffers and other solutions

*DMEM maintenance medium*

Dulbecco's Modified Eagle's Medium (DMEM) (with L-glutamine, 4500mg/l D-glucose and without sodium pyruvate) before use supplemented with 1% antibiotic solution and 10% fetal calf serum (FCS) and stored at 2°-8°C. Purchased from Life technologies Ltd, (Paisley, UK).

*RPMI-1640 maintenance medium*

Roswell Park Memorial Institute (RPMI)-1640 medium (with L-glutamine, without sodium pyruvate) before use supplemented with 1% antibiotic solution and 10% FCS and stored at 2°-8°C. Purchased from Life technologies Ltd (Paisley, UK).

*Luria Bertani (LB) medium*

To make 1L of LB broth 10mg of Bacto-tryptone, 5g-yeast extract and 5g NaCl were dissolved in one liter of water, and the pH was adjusted to 7.5 with 5M NaOH.

*Luria Bertani (LB) plates*

10g of agar powder (Sigma-Aldrich) was added to 1L of LB broth. The solution was autoclaved and allowed to cool to approximately 50°C before antibiotics were added and plates poured. Ampicillin was added to a final concentration of 100μg/ml.

*S.O.C Medium*

Composition: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose. Supplied by Invitrogen® Life Technologies Ltd (Paisley, UK).

*Trypsin solution*

Composed of 0.025% trypsin and 0.01% EDTA in Phosphate Buffered Saline (PBS) purchased from Invitrogen® Life Technologies Ltd (Paisley, UK).

*Antibiotic stock solutions*

Ampicillin 100mg/ml in sterile water and stored at -20°C.

Penicillin/Streptomycin solution from Invitrogen® Life Technologies Ltd (Paisley, UK) , 5000U/ml stored at -20°C.

*6x DNA loading buffer*

Bromophenol blue 0.25%(v/v), 0.25% xylene cyanol and 30% (v/v) glycerol in water, from Invitrogen® Life Technologies Ltd (Paisley, UK).

*10x PBS*

Obtained from Invitrogen®, Life technologies Ltd (Paisley, UK). Without magnesium and calcium, pH=7.4.

*10x Dulbecco's Phosphate buffered saline (DPBS)*

Obtained from Invitrogen, Life technologies Ltd (Paisley, UK). With magnesium and calcium, pH=7.4.

*10x Tris buffered saline (TBS)*

168.32g NaCl, 60.6g Tris (Sigma-Aldrich), 2L distilled H20. 30ml concentrated HCL was added to 2L TBS solution to adjust pH to 7.5.

*TBS/E/S (TBS with Empigen and serum)*

For 10ml of TBS/E/S: 1ml FCS (Life technologies Ltd, Paisley, UK), 1ml 10x TBS, 333µl Empigen® (Sigma-Aldrich Ltd. Poole, UK), 8.667µl distilled H20.

*TMT/SS*

Composition: 1xTBS, 2% Bovine serum albumin (Sigma-Aldrich), 20% FCS (Life Technologies Ltd, Paisley, UK).

*Streptavidin Horse Radish Peroxidase (HRP)*

Enzyme purified from Streptomyces avidii and supplied in PBS from Life Technologies Ltd (Paisley, UK).

*Coating Buffer (for p24 ELISA)*

100mM NaHCO3. For 20ml of coating buffer solution 168mg of NAHCO3 (Sigma-Aldrich Ltd, Poole, UK) in 20ml of dH2O.

*10x running buffer (Western blotting)*

30g Trizma base® (Sigma-Aldrich, Ltd, Poole, UK) + 144g glycine (Sigma-Aldrich Ltd, Poole, UK) + 1l dH20.

*1x running buffer (Western blotting)*

100ml of 10x running buffer + 10ml 10% SDS (Sigma-Aldrich Ltd, Poole, UK) + 1l H2O.

*Transfer buffer (Western blotting)*

100ml 10x running buffer + 700ml dH2O + 200ml methanol (Sigma-Aldrich Ltd, Poole, UK).

*50x TAE buffer stock solution (1l)*

100ml 0.5M EDTA solution (Sigma-Aldrich Ltd, Poole, UK) + 57.1ml glacial acetic acid (Sigma-Aldrich) + 242g Tris base (Sigma-Aldrich Ltd, Poole, UK) + 750ml H2O. Solutions were mixed thoroughly to dissolve the salts then the volume was adjusted to 1;. The stock solution was diluted as required with dH2O to obtain 10x and 1x TAE.

## 2.2 Methods

### 2.2.1 Transfecting plasmid DNA into chemically competent Escherichia coli (E.coli)

OneShot® TOP10 chemically competent E.coli (Invitrogen®) was used for all transformation procedures. 10pg-100ng of plasmid DNA was gently mixed with a vial of OneShot® cells thawed on ice according to manufacturer's instructions. The mixture was incubated on ice for 30min and then heat-shocked at 42°C for 30sec without shaking. The vials were placed on ice for 2min after heat shock and 250µl of pre-warmed S.O.C® medium added to each vial. The vials were placed for 1hour in a shaking incubator at 250rpm. 20-200µl of each transformation was spread unto a pre-warmed selective LB agar plate and incubated overnight at 37°C. The plates with colonies were stored at 4°C for a maximum of two weeks.

### 2.2.2 Glycerol stocks of plasmids

Bacterial glycerol stocks were made for long-term storage of all the plasmids used in the experiments. The addition of glycerol stabilises the frozen bacteria, preventing damage to the cells and keeping them alive. 0.5ml of overnight culture was added to 0.5ml 50% glycerol (Sigma-Aldrich Ltd, Poole, UK) in a 2ml cryovial and mixed thoroughly by gently pipetting. The cryovials were stored at -80°C.

### 2.2.3 Purification of plasmid DNA

DNA was extracted from a single transformed E.coli colony grown in 4ml of LB broth containing 100µg/ml of Ampicillin, overnight at 37°C in a shaking incubator at 250rpm. Mini-preps of the plasmid DNA were prepared using the QIAprep® spin miniprep kit (QIAGEN, Venlo Netherlands) according to manufacturer's instructions. Briefly, bacterial cells grown overnight were harvested by centrifugation at 3000rpm for

10min. All traces of supernatant were removed from the pelleted bacterial cells. The bacterial cells were re-suspended in 250µl P1 buffer and transferred to a microcentrifuge tube. 250µl of P2 buffer was added and mixed thoroughly by inverting the tube 4-6 times. 350µl buffer N3 was added and mixed thoroughly by inverting the tubes 4-6 times. The tubes were centrifuged for 10min at 13000rpm in a microcentrifuge. The supernatants were applied to the QIAprep® spin column by decanting and the columns centrifuged for 1min at 13000rpm and flow-through was discarded. To wash the QIAprep®, 0.5ml PB buffer was added to the column, centrifuged at 13000×g for 1minute and flow through discarded, this was followed by a wash with 0.75ml PE buffer. To elute DNA, 50µl elution buffer was added to the center of the column and columns centrifuged for 1minute. All DNA was stored at -20°C until use.

## 2.2.4 Extraction of DNA from cells

Total DNA from cells was purified using the DNeasy® blood and tissue kit (QIAGEN, Venlo, Netherlands) according to manufacturer's instructions. The cells from which DNA was to be extracted were re-suspended in 200µl PBS. 20µl proteinase K and 200µl buffer AL were added to cell suspension and mixed thoroughly by vortexing. The mixture was incubated at 56°C for 10min. After the incubation 200µl ethanol (100%) was added to the sample and mixed thoroughly by vortexing. The mixture was transferred to the DNeasy® mini spin column and placed in a 2ml collection tube and centrifuged at 8000rpm for 1min and the flow-through discarded. The column was washed first by adding 0.5ml buffer AW1 and centrifuging for 1min at 8000rpm, then adding 0.5ml buffer AW2 and centrifuging for 14000rpm for 3min to dry the DNeasy® membrane. The flow-through was discarded after each of the washes. The DNeasy® mini spin column was transferred to a clean 1.5ml microcentrifuge tube and 200µl buffer AE was added to

the DNeasy® membrane to elute the DNA and the columns centrifuged at 8000rpm for 1min. DNA was stored at -20°C.

## 2.2.5 Gel extraction of DNA

Gel extraction of DNA was performed using the QIAquick® gel extraction kit (QIAGEN, Venlo, Netherlands). Briefly, the PCR product was excised from the agarose gel and 3x the volume of QG buffer added to the gel (e.g. 300µl QG buffer to every 100µg of gel weight). The gel+QG buffer was incubated at 50°C until the gel had completely dissolved. The sample was then applied to a QIAquick® column and centrifuged at 10000g for 1minute to bind DNA to the column. 500µl buffer QG was added to the column and the column was centrifuged at 10000×g for 1minute to ensure the removal of all traces of agarose. 750µl buffer PE was added to wash the column and centrifuged at 10000×g for 1minute. Finally the DNA was eluted in 30µl DNAse/RNAse free elution buffer followed by one final 1min centrifugation at 10000×g. DNA was stored at -20°C.

## 2.2.6 DNA quantification

DNA was quantified in 1µl of purified DNA sample on a Nanodrop® ND-1000 UV-vis spectrophotometer (Nanodrop®, ThermoScientific, Wilmington, USA). Ethidium Bromide (Sigma-Aldrich Ltd, Poole, UK) staining and agarose gel electrophoresis were also used to quantify DNA by running a 5µl aliquot of purified DNA along with a DNA molecular marker (Invitrogen® Life technologies Ltd, Paisley, UK).

## 2.2.7 Agarose gel electrophoresis

Electrophoresis on a 1% agarose gel was used to separate and visualise DNA fragments. 5µl of Ethidium Bromide (Sigma-Aldrich Ltd, Poole, UK) was added to the pre-prepared gel before pouring it into a sealed gel

plate to a depth of 4-8mm. An appropriate sized comb was inserted into the gel and the gel was allowed to cool. Once cooled and solidified, the comb was removed and the gel submerged in 1× TAE buffer in the electrophoresis chamber. 1/5 sample volume of 6× loading dye (Invitrogen® Life Technologies Ltd, Paisley, UK) was added to each sample and loaded in the wells and DNA separated by electrophoresis at 120V for 1hour.

### 2.2.8 Restriction enzyme digestion of DNA

A typical digest reaction was set-up as detailed below using the recommended buffer.

DNA digest reaction:

2μg DNA

1U/μg of DNA restriction enzyme

5μl of 10× restriction enzyme buffer

Sterile deionised water up to 50μl

The reaction was incubated on a thermocycler for 1hour at the recommended temperature for the enzyme according to manufacturer's instructions.

### 2.2.9 Molecular cloning using TOPO pCR®2.1 and site-directed mutagenesis (SDM)

In order to create the full-length HIV-1NL4.3 drug-resistant mutants used in experiments in this thesis, the desired HIV-1NL4.3 fragment for SDM, from nucleotide position 740-2940, was amplified by PCR using a high fidelity polymerase enzyme, Platinum® Taq (Invitrogen®, Life Technologies Ltd, Paisley, UK) and target specific primers RT-forward and RT-reverse (**Table 2-2**: Sequencing and PCR primers). The DNA was then digested using the restriction enzymes SpeI and AgeI (New England Biolabs) according to manufacturer's instructions. The digested DNA fragments were visualised alongside a molecular ladder

marker using Ethidium Bromide (Sigma-Aldrich Ltd, Poole, UK) staining and agarose gel electrophoresis to check the fragment size. PCR products were purified using QIAquick® PCR purification kit (QIAGEN, Venlo, Netherlands) according to manufacturer's instructions.

PCR reaction mixture:
10x High Fidelity Buffer: 5µl
10mM dNTP mix: 1µl
50mM MgSO4: 2µl
Platinum® Taq: 0.2µl
10µM forward primer (RT forward): 2µl
10µM forward primer (RT reverse): 2µl
DNA template: 10ng
PCR-grade H2O: up to total reaction volume of 50µl

PCR program
94°C for 2min
94°C for 15s
55°C for 30s
68°C for 2min
Steps 2-4 repeated 30 times
72°C for 3min
4°C hold

## 2.2.10 Cloning of PCR products

To facilitate the cloning of products generated by the high fidelity polymerase into the TOPO® TA cloning vector pCR®2.1, 100ng of the PCR product with 15µl of 2mM dATP and 5 units of GoTaq® (Promega Ltd, Southampton UK) in a total reaction volume of 20µl was heated at 72°C for 30mins. 2µl of the freshly poly-A tailed PCR product was used for the TOPO® cloning reaction according to manufacturer's

instructions by mixing it with 1µl salt solution, 4µl of sterile water and 1µl of TOPO® vector in a total reaction volume of 6µl. The mixture was incubated for 5-20min and a2µl aliquot of this mixture was used to transform chemically competent OneShot® TOP10 cells (Invitrogen®, Life Technologies, Paisley UK), as previously described. The transformation reaction mixture was spread unto pre-warmed selective plates enriched with X-gal (Promega Ltd, Southampton, UK) and incubated overnight at 37°C. To analyse the transformants 6-10 white colonies where picked for DNA purification. The plasmid DNA wasdigested with the appropriate restriction enzymes and visualised by Ethidium Bromide staining and agarose gel electrophoresis to confirm the presence and orientation of the DNA insert in the cloning vector.

### 2.2.11 Site-directed mutagenesis (SDM)

Mutagenesis PCR was performed with Accuprime® pfx polymerase (Invitrogen® Life Technologies Ltd, Paisley, UK) according to the manufacturer's protocol.

PCR reaction mixture:

Accuprime® pfx supermix: 22.5µl

Forward and reverse mutagenesis primers (5µM): 0.5µl each

Plasmid DNA (20ng): 1µl

PCR-grade water: up to 25µl

SDM PCR-program:

95°C for 15min

95°C for 15s

60°C for 30s

68°C for 2min

Steps 2-4 30 times

68°C for 15min

4°C hold

The SDM PCR product was digested with DpnI restriction enzyme at 37°C for 1 hour. A 2µl aliquot of the DpnI digested PCR product was then used to transform TOP10 chemically competent cells. The transformations were plated on selective X-gal enriched agar plates as previously described. 6 white colonies were picked for DNA purification and subsequent sequencing to check for the presence of the desired mutation and any errors introduced by PCR.

**Table 2-1**: Mutagenesis Primers

| Primer name | Sequence (5'-3') | Function |
|---|---|---|
| **M184V-forward** | TCTATCAATACGTGGATGATTTGTATGTAGGATCTGACTTAG | M184V mutagenesis |
| **M184V-reverse** | AATCATCCACGTATTGATAGATGACTATGTCTGGATTTTG | M184V mutagenesis |
| **K103N-forward** | GCAGGGTTAAAACAGAACAAATCAGTAACAGTACTGG | K103N mutagenesis |
| **K103N-reverse** | ACATCCAGTACTGTTACTGATTTGTTCTGTTTTAAC | K103N mutagenesis |
| **V82A-forward** | GTAGGACCTACACCTGCCAACATAATTGGAAG | V82A mutagenesis |
| **V82A-reverse** | CAGATTTCTTCCAATTATGTTGGCAGGTGTAGG | V82A mutagenesis |
| **A431V-forward** | GAAAGATTGTACTGAGAGACAGGTTAATTTTTTAGG | A431V mutagenesis |
| **A431V-reverse** | GGCCAGATCTTCCCTAAAAATTAACCTGTCTCTCAGT | A431V mutagenesis |

## 2.2.12 DNA sequencing

DNA sequencing was carried out by COGENICS® (Beckman Coulter Genomics, Essex, UK). Typically, 100ng of plasmid DNA or gel purified PCR DNA was sent for DNA sequencing with the relevant primers at 5µM concentration. Sequencing was performed with BigDye® terminator chemistry and a 3730×1 analyser (Applied Biosystems®, Life Technologies Ltd, UK).

**Table 2-2**: Sequencing and PCR primers

| Primer/Probe name | Sequence (5'-3') | Function |
|---|---|---|
| **HXB2 forward** | GTGCTGGAATCAGGAAAGTACTA | HIV-1 *pol* qPCR (primer) |
| **HXB2 reverse** | ATCACTAGCCATTGCTCTCCAATT | HIV-1 *pol* qPCR (primer) |
| **HXB2 probe** | TGTGATATTTCTCATGTTCATCTTGGGCCTTATCT | HIV-1 *pol* qPCR (probe) |
| **Albumin forward** | GCTGTCATCTCTTGTGGGCTGT | HIV-1 *pol* qPCR (primer) |
| **Albumin reverse** | AAACTCATGGGAGCTGCTGGTT | HIV-1 *pol* qPCR (primer) |
| **Albumin probe** | CCTGTCATGCCCACACAAATCTCTCC | HIV-1 *pol* qPCR (probe) |
| **2-LTR forward** | AACTAGAGATCCCTCAGACCCTTTT | HIV-1 2-ltr qPCR (primer) |
| **2-LTR-reverse** | CTTGTCTTCGTTGGGAGTGAATT | HIV-1 2-ltrqPCR (primer) |
| **2-LTR-junction** | TTCCAGTACTGCTAGAGATTTTCCACACT | HIV-1 2-ltrqPCR (probe) |
| **Seq1-forward** | CATAGCAGGAACTACTAGTACC | Sequencing |
| **Seq2-reverse** | GCATTAGTAGAAATTTGTACAG | Sequencing |
| **Seq3-forward** | GTATGGTAAATGCAGTATACTTC | Sequencing |
| **Seq4-reverse** | AGAATCTCCCTGTTTTCTGCCA | Sequencing |
| **RT-forward** | GGAGGTTTTATCAAAGTAAGAC | PCR |
| **RT-reverse** | TCTTTTGATGGGTCATAATACACTCC | PCR |

## 2.2.13 Sub-cloning into HIV-1NL4.3 backbone

The DNA insert with the desired mutation (confirmed by sequencing) was extracted from the gel following electrophoresis of the double digest with AgeI and SpeI restriction enzymes of the TOPO® clone containing the insert. The DNA band corresponding to the HIV-1NL4.3 backbone was extracted from the agarose gel, following electrophoresis of the double digest of HIV-1NL4.3 with AgeI and SpeI. The DNA was stored at -20°C if not used immediately in ligation reactions. All ligation reactions were set-up using the Roche® Rapid DNA Dephos and ligation kit (Roche Diagnostics, Mannheim, Germany). The purified DNA of the vector backbone was first dephosphorylated prior to ligation to prevent vector re-ligation.

<u>Dephosphorylation reaction</u>

Vector DNA =x μl (up to 1μg)

10× Rapid Alkaline Phosphatase Buffer (Roche®) = 2μl

Rapid Alkaline Phosphatase (Roche®) = 1μl (1U)

Sterile water: added to make up a total volume of 20μl
The reaction was incubated at 37 °C for 30min and at 72°C for 5min to inactivate the alkaline phosphatase (Roche). The dephosphorylated vector DNA was used immediately in the ligation reaction or stored at -20°C.

Ligation reaction (molar ratio of vector DNA: insert DNA = 1:3)
Vector DNA = x μl (50ng)
Insert DNA = x μl (150ng)
5× DNA dilution buffer (Roche): 2μl
Sterile water: added to make up to a volume of 10μl and mixed thoroughly
2× T4 DNA ligase (Roche) added = 10μl
T4 DNA ligase (Roche) = 1μl (5U)

The ligation reaction was incubated at room temperature for 30min and 1/10 of the ligation reaction was used to transform competent E.coli as previously described.

## 2.2.14 Tissue culture techniques

All the cell and virus cultures described in this thesis were maintained in humidified incubators at 37°C with 10% CO2 for human embryonic kidney (HEK) 293T cells and 5% CO2 for all other cell lines.

### 2.2.14.1 Thawing cells

For long-term storage, frozen cell aliquots were stored in liquid nitrogen. The cells were removed from liquid nitrogen and rapidly thawed in a water bath at 37°C. Once thawed the cells were pelleted by spinning them at 1400rpm for 5min after which they were re-suspended in 15ml of culture media in a T25 tissue culture flask. The media were changed the next day and replaced with fresh pre-warmed media. HEK 293T

and HeLa TZM-bl cells were grown and maintained in DMEM (Invitrogen®, Life Technologies, Paisley UK). Jurkat cells and ACH-2 cells were grown and maintained in RPMI-1640 medium (Invitrogen® Life Technologies Ltd, Paisley, UK). The culture media were enriched with 10% FCS (Invitrogen® Life technologies Ltd, Paisley, UK) for cell lines and 20% FCS for primary T cells. 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen®, Life Technologies, Paisley, UK) were added to the media used for growing and maintaining cell lines.

### 2.2.14.2 Passaging cells

Adherent cells HEK 293T and HeLa TZM-bl cells were passaged when the cells were 90-100% confluent, as judged by inspecting cell cultures under a microscope. Under sterile conditions, 10ml of PBS was added to the T75-flask containing the cells and then aspirated in order to wash away the medium. 4ml of trypsin was added to the cells and left to incubate for 5min at 37°C. The flask was tapped several times to ensure the detachment of the cells and 6ml of culture medium added to dilute the trypsin (Invitrogen® Life Technologies Ltd, Paisley, UK). Depending on the desired density, an appropriate volume of the cell suspension was transferred into a new T75-flask and topped up with fresh medium. Suspension T cell lines (Jurkat and ACH-2 cells) were passaged when cultures reached a density of 1×106 cell/ml. This was usually every 4-5 days for cultures split to 1:10 (e.g. 3ml aliquot of cell suspension re-suspended in 27ml RPMI).

### 2.2.14.3 Freezing cells

Freezing media consisted of 60% culture medium, 20% FCS and 20% Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Poole, UK). Cells were pelleted by centrifuging at 1400rpm for 5min and re-suspended to a density of 1×107cells/ml. The suspension was aliquoted into sterile cryovials and placed into a Styrofoam container. The cells were initially

frozen overnight at -80°C and subsequently transferred to liquid nitrogen for long-term storage.

### 2.2.15 Isolating PBMCs from buffy coat

Buffy coat in blood bags was obtained from the national blood service (London, UK). The buffy coats from different donors were kept separate at all times. The blood was drained under standard sterile tissue culture conditions into a T75 flask. A Ficoll-Paque® (Sigma-Aldrich, Poole, UK) gradient was used to separate the white blood cells (WBCs) and serum from the red blood cells (RBCs). 10ml of Ficoll® was placed in a clean 50ml falcon tube and 25ml of blood transferred from the T75 flask was slowly layered on the Ficoll®. The falcon tubes containing blood layered on Ficoll® were centrifuged at 2000rpm for 20min at room temperature without brake. After the spin, a transfer pipette was used to carefully transfer the white layer containing PBMCs into a new falcon tube and topped up with 40ml of PBS. The PBMCs were centrifuged at 2000rpm for 10min to pellet the cells and the liquid was aspirated off. The cells were washed twice more by re-suspending them in 20ml PBS and spinning at 1500rpm for 5min at room temperature. After the washes, the cells were counted and re-suspended at a density of $1\times10^6$/ml in a T75 flask. PBMCs were maintained in RPMI-1640 medium with 20% FCS, 1μg/ml of phytohaemagglutinin (PHA), (Sigma-Aldrich, Poole, UK) and 10U/ml of interleukin-2 (IL-2), (NIBSC). PHA was removed after 2-3 days and cells were maintained thereafter in RPMI-1640 medium with 10U/ml of IL-2.

### 2.2.16 Isolating CD4+ T cells from activated PBMCs-Magnetic activated cell sorting (MACS®) depletion of non-CD4+ T cells

CD4+ T cells were sorted from activated PBMCs by negative selection, 3 days after isolation using the MACS® cell separation technology

(Miltenyi, Biotec®, Cologne, Germany) according to manufacturer's instructions of the CD4+ T cell isolation kit (Miltenyi, Biotec, Cologne, Germany). Briefly, PBMCs were washed in PBS and re-suspended in sterile and filtered MACS buffer (MACS buffer: PBS+0.5% FCS+2mM EDTA) - 40µl MACS® buffer/$10^7$ cells. 10µl/$10^7$ cells of biotin antibody cocktail was added to the cells, mixed thoroughly and incubated for 10min at 4°C. 30µl MACS® buffer and 20µl anti-biotin micro-beads/107 cells, mixed thoroughly and incubated for 15min at 4°C. Ten times the labelling volume of MACS® buffer was then added to the labelled cells suspension and centrifuged at 2000rpm for 5min (twice) after which the cells were re-suspended in 500µl of MACS buffer in preparation for magnetic separation (a maximum of 108 cells per column).Pre-chilled magnetic columns were equilibrated with 3ml of MACS buffer. The labelled cell suspension was then applied to the separation column and the flow-through collected in a cold 15ml falcon tubes. The flow-through was passed through the column a second time and 6ml of MACS buffer run through the column and collected into the same falcon tube. The cells were then pelleted and re-suspended at a density of $10^6$ cells/ml in RPMI-1640 + 20% FCS (Invitrogen® Life Technologies Ltd, Paisley, UK).

### 2.2.17 Transfection of HEK 293T-cells with full-length HIV-1 molecular clones

Full-length HIV-1 was produced by transfecting HEK 293T cells with molecular clone DNA. For transfection in a 6 well plate, $5\times10^5$ cells per well were plated 18-24h prior to transfection so that the monolayer cell density was optimally confluent (95% confluent) at the time of transfection. Complete culture medium supplemented with 10% FCS and 1% Penicillin/Streptomycin was freshly added to each well 30-60min before transfection. Fugene®HD (Promega® Ltd, Southampton, UK) transfection reagent was used for all transfection reactions. For

each well the complex was prepared by adding 9.9µl of Fugene®HD to 155µl of 0.020µg/l plasmid DNA solution in Opti-MEM® (Invitrogen®, Life Technologies Ltd, Paisley,UK). The complex was mixed by pipetting (15 times) then incubated at room temperature for 5-10min. 150µl of complex was added in a drop-wise manner to each well of cells and incubated at 37°C for 48h. After 48h the virus containing supernatant was collected, centrifuged to remove any cell debris carried over and aliquoted into cryovials for storage in liquid nitrogen. All transfections with full-length HIV-1 DNA and replication competent infectious virus were carried out in the containment level 3 facility following standard procedures as defined by the University College London (UCL) risk assessment guidelines.

### 2.2.18 Titration of virus stocks in HeLa TZM-bl cells

To assess the infectivity of the virus stocks, the tissue culture infectious dose was estimated using a HeLa TZM-bl based TCID50 assay adapted from the Duke University laboratory protocol for the titration of HIV-1 pseudotyped viruses and the TCID50 values were calculated using the method of Reed and Muench (Reed, 1938) and a TCID50 excel macro available from: http://www.hiv.lanl.gov/content/nab-reference-strains/html/TCID501.xls. A 5-fold dilution series of the virus stock was titrated on a white 96-well flat-bottomed tissue culture plate. 100µl of DMEM was placed in all wells on the plate. 25µl of neat thawed virus was added to the first 4 wells of a dilution series and a 5-fold dilution series was done. The 12th column wells were reserved as negative control wells. $1\times10^4$ HeLa TZM-bl in a volume of 100µl was added to each well. The plate was placed in an incubator at 37°C. After 48h, 100ul of medium was removed and 75µl SteadyGlo® luciferase added to lyse the cells for 5min. The plate was read using the SteadyGlo® protocol on a GloMax® 96 MicroplateLuminometer (Promega® Ltd,

Southampton, UK). The data was exported into Microsoft®Excel to calculate the TCID50.

### 2.2.19 HEK 293T drug susceptibility assay

An in-house assay designed by Dr. Chris Parry was used to determine the drug susceptibility of the drug-resistant mutant viruses compared to the wild-type virus (Gupta et al., 2010). HEK293T cells were transfected as described above, 16h after transfection the cells were seeded in the presence of a serial dilution of Protease Inhibitors. Virus supernatant was harvested 24h later and used to infect fresh target HeLa TZM-bl cells. For Reverse Transcriptase Inhibitors, the HeLa TZM-bl cells were infected in the presence of a serial dilution of the drug being tested. Replication was determined by measuring luciferase expression in infected target cells at 48h post-infection using SteadyGlo® Luciferase Assay system (Promega® Ltd, Southampton, UK) and expressed relative to that of a no-drug control. Fifty percent inhibitory concentrations (IC50s) were determined using Prism6® software (GraphPad, California, USA). The IC50 values were calculated as the mean of at least two independent experiments.

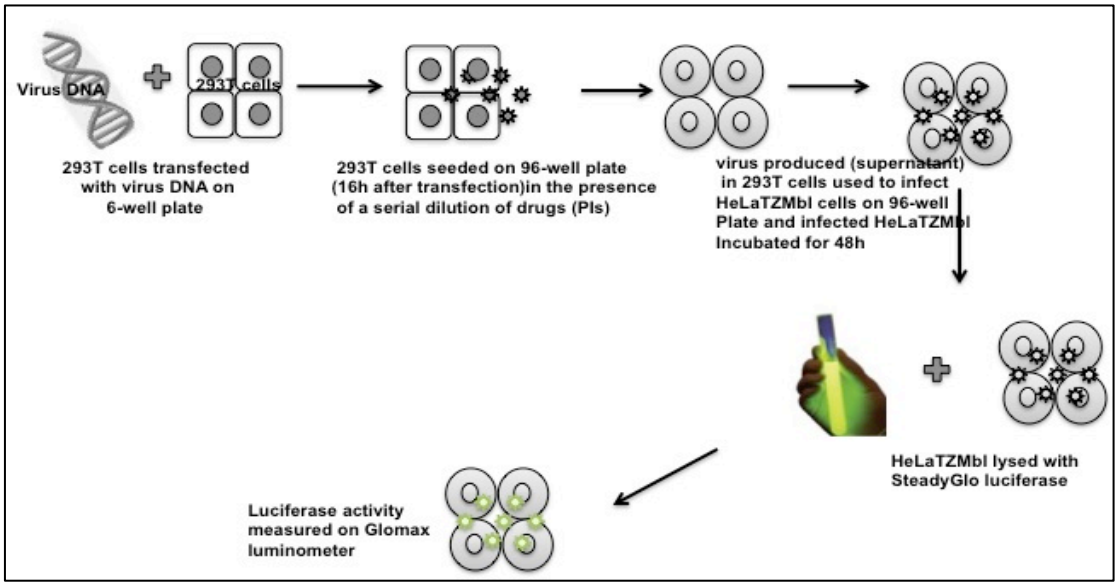


**Figure 2-1**: Drug susceptibility assay. For RTIs the HeLa TZM-bl cells were infected in the presence of a serial dilution of the drug.

## 2.2.20 Cell-to-cell and cell-free infection assays

### 2.2.20.1 Infection of donor cells

Jurkat T cells and Primary CD4+ T cells were used as donor cells in the experiments described. The cells were infected by adding 0.5-1 MOI of thawed virus stock to $3\times10^6$ cells re-suspended in a total volume of 500µl of culture medium in 15ml falcon tubes. The cells were spinoculated by centrifuging the tubes at 2000×g for 2h. After spinoculation the cells were re-suspended in 10ml of culture medium in a T25 flask and incubated at 37°C.

### 2.2.20.2 Gag p24 staining and FACS analysis of infected cells

Infected Jurkat cells were fixed with 3% paraformaldehyde (PFA) (Sigma-Aldrich Ltd, Poole, UK). The fixed cells were permeabilised by incubating for 30min with 1X BD™ Perm Buffer (Beckman Dickson Biosciences, Oxford, UK) and stained with anti-HIV-1 p24 monoclonal antibody conjugated to fluorescein isothiocyanate (HIV-1 p24 (24-4) FITC, monoclonal antibody). Ten thousand events were collected using a FACS Calibur® flow cytometer with Cellquest® software (Beckman Dickinson, Plymouth, UK). Data were analysed using FlowJo® software (with appropriate gating) to determine the percentage of Gag-positive cells.

### 2.2.20.3 RT-PCR based infection assays

To measure cell-to-cell transfer from an infected donor cell to an uninfected target cell, RT-PCR was used to detect *de novo pol*-transcripts as described in previous studies using this assay, (Jolly et al., 2007) with modifications to accommodate for the use of inhibitors (neutralising antibody, RTI and PIs). Donor cells were infected with either HIV-1NL4.3 (wild-type) or drug-resistant mutant virus. Three days after infection, the donor cells were stained for Gag and analysed by

flow cytometry. Only donor-cell cultures that were >80% infected were used for experiments. This minimised the background from spreading infection between donor cells after target cells were added to the culture. The virus input for infecting donor cells was standardised using the multiplicity of infection (MOI) for the different viruses tested (i.e. wild-type and drug-resistant mutants). 72h after infection by spinoculation, cultures infected with either wild type or drug-resistant viruses were similarly 85-90% infected. The donor cells were washed three times with 10ml of culture medium to remove all cell-free virus particles. After the washes the cells were counted, $2x10^5$ infected Jurkat cells (donors) per well on a 96 well plate were mixed with $8x10^5$ 1G5 cells/well in the presence of the maximum plasma concentration ($C_{max}$) of the inhibitor for time-course experiments or in the presence of a serial dilution of the inhibitor for IC50 determination. For time-course experiments, the co-cultures were incubated for 0h, 1h, 3h, 6h, 12h or 24h at 37°C before the cells for each time-point were pelleted and stored at -80°C until DNA extraction. For experiments to calculate the IC50 of the inhibitor, co-cultures in the presence of a serial dilution of the inhibitor under study were incubated for 24h before DNA extraction and RT-PCR. Total DNA extraction was performed using the DNeasy® Blood and Tissue kit according to manufacturer's instructions (QIAGEN®, Venlo, Netherlands) as previously described. Extracted DNA was stored at -20°C if RT-PCR was not performed on the same day.

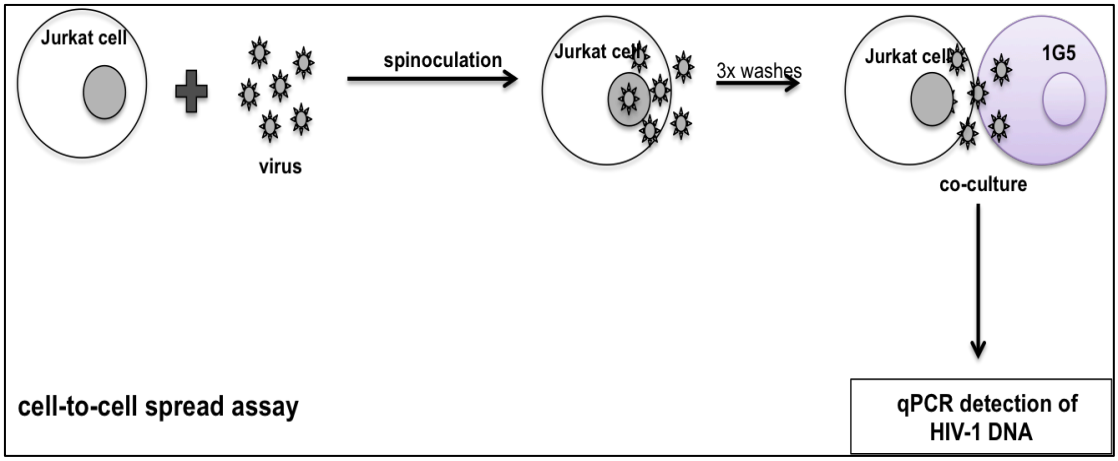


**Figure 2-2**: qPCR based cell-to-cell assay

For cell-free experiments, pre-washed $2x10^5$ donor cells/well were allowed to produce virus over 24h. 100µl of the virus supernatant was used to infect $1x10^6$ target cells/ well by spinoculation at 2000×g for 2h, in the presence of the maximum plasma concentration of the inhibitor under investigation for time-course experiments or in the presence of a serial dilution of the inhibitor for IC50 determination. Following infection by spinoculation, the target cells were incubated for 24h after which they were pelleted for total DNA extraction and subsequent RT-PCR.

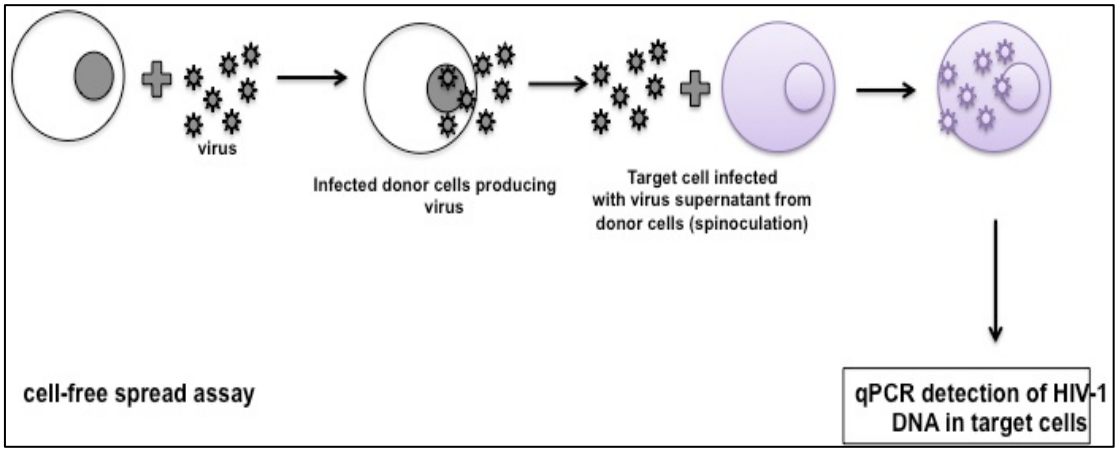


**Figure 2-3**: Cell-free qPCR based assay

## 2.2.21 RT-PCR

RT-PCR was used for the detection of HIV-1 *pol* transcripts using primers and probes specific for HIV-1 *pol* DNA and the housekeeping gene

Albumin (Jolly et al., 2007) and for the detection of 2LTR circles using primers and probes specific for HIV-1 2LTR circles (Apolonia et al., 2007). All RT-PCRs were carried out using a TaqMan® probe based assay (Jolly et al., 2007, Apolonia et al., 2007) with custom made probes and primers (Eurogenetec® Seraing, Belgium). Reactions were set-up in triplicate with 2× qPCRMastermix (Eurogentec®, Seraing, Belgium) and run on the Applied Biosystems® 7500 RT-PCR machine (Life Technologies Ltd, Paisley, UK).

#### 2.2.21.1 HIV-1 *pol* RT-PCR

HXB2 and Albumin master mixes were prepared separately. One master mix prepared with HXB2 *pol* primers and the other prepared with Albumin primers (see Table 2-1 for primer names and sequences)

Master mix for 110 wells:
Sterile deionised water: 528µl
Eurogentec® 2x master mix: 657µl
Probe (50µM): 2.75µl
Forward primer (100µM): 4.2µl
Reverse primer (100µM): 4.2µl

DNA samples were diluted before use 1/10 with sterile deionised water (so that the unknowns fell within the range of the standards). The standards were prepared in duplicate. DNA extracted from ACH-2 cells was used as the standard. In a 96-well PCR plate 2.5µl of either HxB2 or Albumin master mix (without DNA) was pipetted into each well. 2.5ul of each standard in duplicate from the standard dilution plate was transferred to the qPCR plate. A no template control was included in duplicate in the final two wells. 2.5µl of sample was transferred into the qPCR plate. Standards were run in duplicate and samples in triplicate. The plate was sealed and centrifuged for 1min. The qPCR plate was run

on the Applied Biosystems® 7500 real-time PCR system (Life Technologies Ltd, Paisley, UK).

#### 2.2.21.2 HIV-1 2LTR circles RT-PCR

Mastermix for 100 wells

- Eurogentec® 2x Mastermix: 625µl
- Forward Primer (100µM): 3.75µl
- Reverse Primer (100µM): 3.75µl
- Probe (50µM): 2.5µl
- Sterile water: 365µl

In a 96-well PCR plate 20µl of the master mix was pipetted into each well. 5µl DNA template was added to each well. Standards were run in duplicate and samples in triplicate. The plate was sealed and centrifuged for 1min, then run on the Applied Biosystems® 7500 real-time PCR system.

RT-PCR program:

The RT-PCRs were run on the Applied Biosystems® 7500 using the program detailed below:

50°C for 2min
95°C for 10min
90°C for 15s
60°C for 1min
Steps 3-4 repeated 40 times
50°C Hold

### 2.2.22 Transwell based infection assays

To assess the impact of the inhibitor Raltegravir on cell-to-cell and cell-free spread, $2x10^5$/well pre-washed infected donor cells were cultured directly together with $8x10^5$/well target cells on a 24-well plate in the presence or absence of the $C_{max}$ of Raltegravir. Co-cultured cells were

pelleted and stored at -80°C for DNA extraction and 2LTR qPCR and the supernatants stored for p24 ELISA. For cell-free experiments donor cells were separated from target cells by a 3μM transwells to allow for full diffusion of virus but not migration of the cells. $2x10^5$/well infected donor cells were suspended in 100μl of culture medium and placed in the top well and $8x10^5$/well uninfected target cells were suspended in 500μl and placed in the bottom well. Supernatant from untreated and drug treated targets were collected at 4, 7 and 10 days of culture. The target cells were pelleted and frozen down for subsequent DNA extraction and 2 LTR qPCR and the supernatants were stored at -80°C for measurement of p24 by ELISA.

### 2.2.23 p24 ELISA

p24 antigen detection was done using a twin site sandwich ELISA. 100-150μl of sample was inactivated by adding 5μl of 21% Empigen®BB detergent (Sigma-Aldrich Ltd, Poole, UK) to obtain a final concentration of 1%, and incubated for 30min at 56°C. Briefly, in this assay, the p24 antigen is captured from a detergent lysate of virions onto a polyclonal antibody adsorbed onto a solid phase. Bound p24 is detected with a biotinylated conjugated anti-p24 monoclonal antibody. In detail, a 96-well clear flat bottomed ELISA plate (Nunc®) was coated with 100μl/well of 10μg/ml capture antibody D7320, Aalto® overnight at room temperature. On the 2nd day the plates were washed 3x with wash buffer using a TECAN® plate washer (Tecan, Reading, UK). 200μl/well blocking buffer was added to the wells for 30min at room temperature. The wells were washed in 200μl of wash buffer 3x before transferring 100μl of diluted supernatant. A standard was prepared using a 2-fold serial dilution starting at 200ng/μl and 100μl transferred to the plate. Samples and standards were diluted in TBS/E/S. The plate was covered and incubated for 2h at 37°C. After this incubation the plate was washed 6x with 200μl/ well wash buffer. 100μl/

well of 1/1000 diluted stock biotynylated α-p24 antibody in TMT/SS was added to the wells and incubated at room temperature for 2h. The plate was washed 6x with 200µl wash buffer per well. 100µl/ml Streptavidin–Horse Radish Peroxidase (Serotec® Bio-Rad Laboratories, California, USA) in TMT/SS was added to the wells and the plate incubated for 1hour. The plate was washed 6x as described above and 100µl/well of TetraMethylBenzidine (TMB) (Sigma-Aldrich.Poole, UK) added to the plate. The reaction was stopped with 100µl/well of 0.5M Sulphuric acid ($H_2SO_4$). The plate was read on a multiscan FC absorbance plate reader (Thermo Scientific, Massachusetts, USA) at 450nm and the data were analysed using Prism® Software (GraphPad, California, USA).

### 2.2.24 Luciferase based infection assays

For cell-to-cell infection:

The experiment was set-up on a 96-well plate. $2x10^5$ donor cells/well were pre-incubated with a serial dilution of the inhibitor (PIs and NAb) under investigation (4h and 24h pre-incubation times were tested), in duplicate. No drug control wells were included on each plate. For experiments in which RTIs were used as inhibitors, donor and target cells were mixed in the presence of drug at the same time without prior pre-incubation of donor cells with the drug. $8x10^5$/well of 1G5/LTR-luciferase cells (target cells) were added to the donor cells and mixed thoroughly. When donor cells were pre-incubated with an inhibitor for 24h, the cells were centrifuged down, supernatant discarded and re-suspended in fresh inhibitor prior to the addition of target cells. This was to ensure that there was fresh non-degraded drug throughout the 48h of the assay. The co-culture (donor cells + target cells) was incubated for 24h to limit replication to a single cycle and minimise any cell-free infection. The infection of target cells was quantified by a SteadyGlo® (Promega, Southampton, UK) luciferase system. The cells were

centrifuged at 2000rpm for 3min and the supernatant removed. 75µl of SteadyGlo® luciferase (Promega, Southampton, UK) was added to each well to lyse the cells and the luciferase activity was measured using a Glomax® Luminometer (Promega, Southampton, UK). It is worth noting that for experiments with PIs and RTIs the highest concentration of drug tested was the maximum plasma concentration. In order to plot dose-response curves and calculate IC50s for the inhibitors tested, the average luciferase signal in the target cells in the absence of drug was considered to represent 100% infection. The luciferase signal in target cells for each drug concentration was expressed as a fraction of the "no-drug" positive controls and plotted against the drug concentrations on a logarithmic scale.

For cell-free infection:

$2x10^5$ cells/well of pre-washed infected donor cells were incubated in the presence of a serial dilution of the PI drug on a 96 well plate for 24h. After 24h, the cells were pelleted and 100µl of the virus supernatant was used to infect $8x10^5$/ml 1G5 cells (target cells) per well on a 96 well plate by spinoculating for 2h at 1200g. The infected target cells were incubated for 24h after which they were lysed with SteadyGlo® luciferase (Promega, Southampton, UK) and luciferase activity measured as described above.

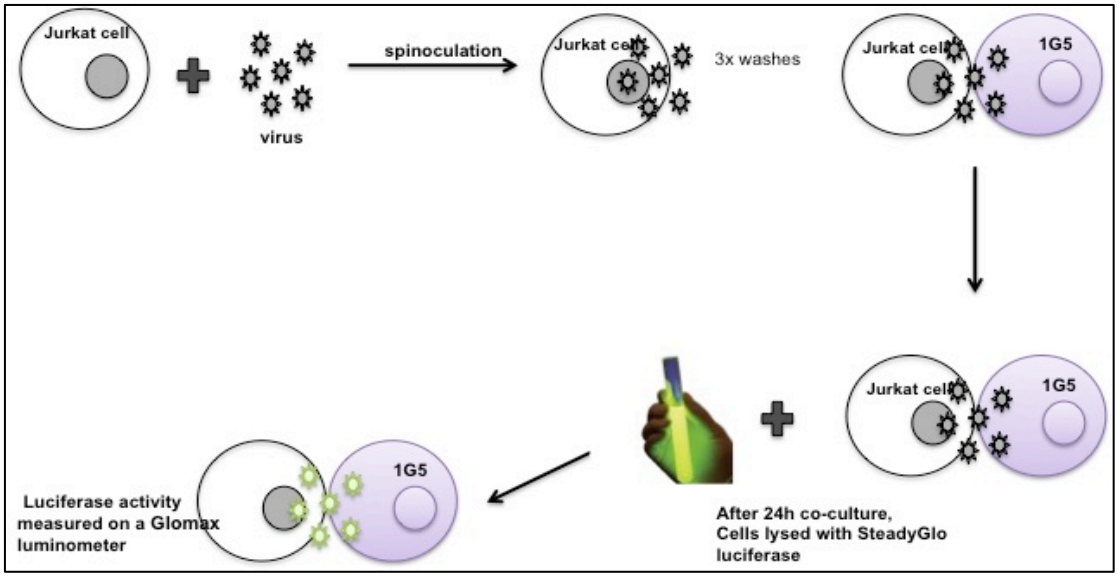


**Figure 2-4:** Luciferase based cell-to-cell assay

### 2.2.25 Drug combination studies

The RT-PCR based infection assays described above were used for the drug combination studies. Antiretroviral agents from the RTI and PI classes were tested in clinically relevant combinations. The drugs were combined in a ratio based on the IC50s of the individual drugs for cell-free infection. For example if the IC50 of drug A=50nM and the IC50 of drug B=100nM, to test these drugs in combination, A+B were combined in a ratio of 1:2 (Irene V. Bijnsdorp, 2011). Cell-to-cell and cell-free infection was assessed in the presence of a serial dilution of the combination and infection determined by qPCR as described above. The percentage inhibition at each concentration was determined and expressed as a fraction of the "no-drug" positive control. These values were used to determine the combination indices for the drug combination using the drug synergy analysis software Compusyn® (Paramus, New Jersey, USA).

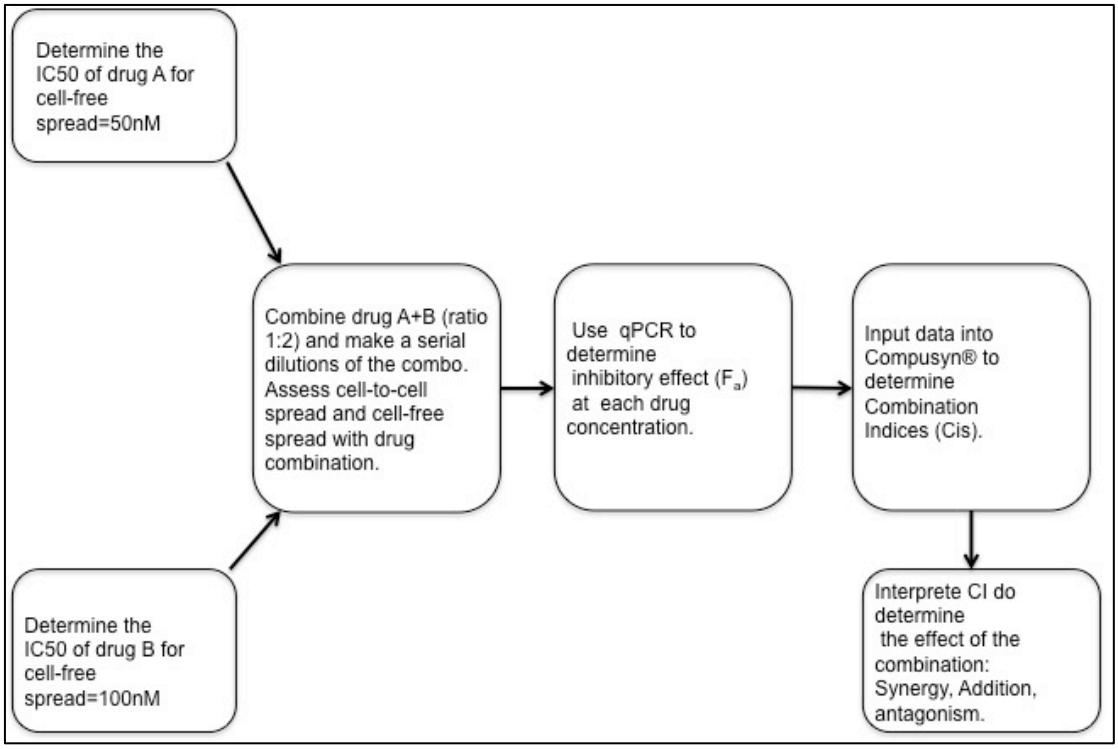


**Figure 2-5**: Scheme of drug combination experiments

## 2.2.26 SDS-PAGE and Western blotting

Cell-free virus supernatant collected from HIV-1 infected cell cultures treated with PIs was purified through a 25% sucrose gradient. 400µl of virus supernatant was carefully layered on 800µl of 25% sucrose solution in a 1.5ml micro centrifuge tube, followed by centrifugation at 10000×g for 90min at a temperature of 4°C. The sucrose was carefully poured off at the end of the centrifugation and the pelleted virus re-suspended in 40µl of PBS and stored at -80°C for SDS-PAGE. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the purified virus prior to detection of virus proteins by western blotting. 20µl of purified virus was loaded on the gel (see materials for composition of gel) along with 10µl of pre-stained protein marker (Invtrogen® Life Technologies Ltd, Paisley, UK) and proteins were separated by electrophoresis in 1x running buffer at 120V for 2h. Proteins were transferred unto a nitrocellulose membrane (Amersham®, Bioscience, Uppsala, Sweden) by electrophoresis at 4°C

overnight (18h) in transfer buffer. The membranes were blocked for 2h at in blocking buffer at room temperature and incubated for 1h on a rocker with the primary antibody (HIV-1 Gag antibody from CFAR, NIBSC), diluted in blocking buffer. Membranes were then washed 4 times for 15min in wash buffer and incubated with HRP coupled secondary antibody (goat anti-rabbit HRP, from DAKO), diluted in blocking buffer for 30min, on a rocker at room temperature. The membranes were washed as above and proteins were visualised by enhanced chemiluminescence (ECL) (Amersham®, Uppsala, Sweden).

### 2.2.27 Statistical methods

A two-tailed student t-test was performed to compare the mean IC50s for cell-free and cell-to-cell spread for PIs and RTIs. For comparisons of data with more than two groups a two-way ANOVA with Bonferroni post-test for multiple comparisons was used. All statistical analysis was done using GraphPad Prism® Software (California, USA.

# 3 Assessing two assay systems for studying the impact of drug inhibitors on cell-to-cell spread of HIV-1

## 3.1 Introduction

In studying the effects of antiretroviral drugs on cell-to-cell spread of HIV-1, *in vitro* co-culture systems using direct and indirect markers of infection have been used in the published literature, with conflicting results (Sigal et al., 2011, Permanyer et al., 2012b). Sigal et al. were the first group to report that HIV-1 cell-to-cell infection may be less susceptible to inhibition by RTIs than cell-free infection. In their experiments they used *in vitro* co-culture systems with surrogate markers of HIV-1 infection (p24 antigen staining and Tat-driven reporter gene expression) to test their hypothesis. Following the publication of these observations, Pemanyer et al. challenged the results by using *in vitro* co-culture systems with a direct marker of HIV-1 infection (qPCR detection of HIV-1 DNA transcripts) and obtained a different result finding that RTIs were effective inhibitors of cell-to-cell HIV-1 infection. They proposed that the use of surrogate markers for measuring target cell infection in co-culture assays, as used by Sigal et al., may be misleading due to the possibility of detecting these surrogate markers in the absence of true HIV-1 infection in target cells. These seemingly conflicting results led to some controversy with regards to the true impact of antiretroviral agents on HIV-1 cell-to-cell infection.

The primary objective of the studies presented in this thesis is to define the role of antiretroviral agents on HIV-1 cell-to-cell spread. It was therefore desirable to re-evaluate the co-culture assays available in our laboratory that use both surrogate and direct markers of HIV-1

infection, in order to establish the best method to use in answering my research questions. In this chapter, two co-culture assay systems, one using a surrogate marker of HIV-1 infection (Tat-driven reporter gene expression) and another using a direct marker of HIV-1 infection (qPCR-based detection of HIV-1 *pol* DNA copies), are used to study cell-to-cell and cell free spread of HIV-1 in the presence and in the absence of drug inhibitors. The results obtained suggest that assays with an indirect infection read-out such as reporter gene expression, are high throughput and useful for screening some inhibitors (attachment and entry inhibitors). However HIV-1 Tat protein derived from infected donor cells in co-culture may lead to reporter gene activation in the absence of true infection in target cells, even in the presence of inhibitors (PIs and RTIs). These findings provide the rationale for using a direct measure of HIV-1 infection (qPCR-based detection of HIV-1 *pol* DNA copies), to answer the research questions posed in this thesis. In addition to comparing the assay systems for testing the effect of drug inhibitors on HIV-1 cell-to-cell spread, the drug-resistant and wild type viruses used through out this thesis are also characterised in this chapter. The resistant phenotype of the viruses is confirmed in a drug susceptibility assay and their ability to spread efficiently via a cell-to-cell mechanism verified.

### 3.1.1 Specific Objectives

- To compare a reporter gene based assay and a qPCR-based assay for studying the effects of drug inhibitors on HIV-1 cell-to-cell spread.
- To construct PI and RTI drug-resistant viruses and validate their phenotypes in a drug susceptibility assay.
- To assess cell-to-cell spread of PI and RTI drug-resistant viruses compared to wild-type virus.

## 3.2 Results

### 3.2.1 Comparing direct and surrogate markers of infection for studying HIV-1 cell-to-cell spread

Due to conflicting reports on the impact of antiretroviral drugs on HIV-1 cell-to-cell spread, two assay systems with different output measures were compared for use in testing the effect of inhibitors on HIV-1 cell-to-cell spread. This was in order to determine the best method to use for the subsequent studies presented in this thesis and to clear the controversy stemming from previous studies by other groups. The qPCR-based assay directly quantifies infection of target cells by measuring HIV-1 *pol* DNA while the luciferase assay uses a surrogate marker of infection measuring Tat-driven reporter gene expression in target cells. Both assays have been used and validated in other studies of HIV-1 cell-to-cell infection (Jolly et al., 2007, Jolly et al., 2011, Martin et al., 2010). HIV-1 cell-to-cell spread was measured in the presence of drug inhibitors (PIs and RTIs) using these two assay systems.

A Jurkat CD4/CXCR4+ T cell line was used as donor cells in the two assays. Donor cells were infected with an MOI = 0.3-0.5 of CXCR4 tropic NL4.3 wild-type virus (HIV-$1_{WT}$), by spinoculating for 2h at 2000g. 72 hours after infection, an aliquot of the donor cell culture was fixed and stained for Gag and analysed by flow cytometry. Donor cell cultures were typically >80-90% infected by 72h. The use of a donor cell population in which >80% of cells are infected minimises background spreading infection between donor cells. A derivative of the Jurkat cell line (1G5) was used as target cells in both assays. 1G5 cells contain a stably integrated HIV-LTR-luciferase construct in which expression of the reporter gene is driven by HIV-Tat. The infected donor cells were washed three times with culture medium to remove as much cell-free virus as possible and co-cultured with the target cells in a ratio of 1:4 ($2\times10^5$ donor cells mixed with $8\times10^5$ target cells), in the presence of the

inhibitor. When PIs were tested, the donor cells were pre-incubated with the drug for 24h before co-culturing with target cells. With RTIs, the drugs were added at the time of mixing donor cells with target cells. The co-cultures were incubated for 24h for experiments with a single time-point and for variable durations: 1h, 3h, 6h, 12h and 24h for time-course experiments.

In the luciferase assay cell-to-cell infection was quantified by lysing the cells with SteadyGlo® luciferase and measuring luciferase expression on a GLOMAX® Luminometer. In the qPCR-based assay, pre-treated donor cells were co-cultured with target cells in the presence of the inhibitor as described above. Cell-to-cell spread of HIV-1 was measured by qPCR, to quantify *de novo* HIV-1 DNA *pol* copies arising from reverse transcription in the newly infected T cell population. The data were expressed as fold increase in HIV-1 *pol* DNA relative to the housekeeping gene Albumin. In this assay a synchronous population of HIV-1 infected donor cells, allows for reliable measurement of virus infection in target cells mediated by cell-to-cell dissemination with little or no contribution from the less efficient cell-free mode of infection (Jolly et al., 2007) (see methods for detailed description of assays).

The effect of inhibitors on cell-free infections was also tested for comparison. For cell-free assays, pre-washed infected donor cells were left to produce virus in the presence of PIs over 24h. The virus supernatant was collected and used to infect target cells by spinoculating for 2h. Infection of the target cell was measured by luciferase gene expression and by qPCR detection of HIV-1 *pol* DNA as described above.

As expected HIV-1 cell-free infection was potently blocked by LPV over a range of concentrations in both the luciferase assay system

(Figure3.1A) and the qPCR assay system (Figure 3.1B)). In contrast LPV appeared to be completely ineffective against cell-to-cell spread of HIV-1 over a wide range of concentrations when tested using the luciferase based assay (Figure 3.1C). RTIs also failed to inhibit cell-to-cell infection in this assay system (Figure 3.1D). To confirm these results the effect of LPV on HIV-1 cell-to-cell spread was tested in the qPCR-based assay system, which uses a direct output measure of infection, HIV-1 *pol* DNA copies as described earlier. Co-cultures where either left untreated or treated with $C_{max}$ LPV (12μM), and HIV-1 *pol* DNA copies detected by qPCR at different time-points (1h, 3h, 6h, and 12h). Surprisingly LPV potently blocked HIV-1 cell-to-cell infection in the qPCR-based assay system, indicating that these drugs were in fact effective against this mode of virus spread. This was evidenced by the absence of HIV-1 *pol* DNA transcripts in co-cultures treated with $C_{max}$ LPV (12μM) compared to a ready detection and a time dependent increase in HIV-1 *pol* DNA transcripts in untreated co-cultures (Figure 3.1E). These results seem paradoxical with LPV blocking HIV-1 cell-to-cell infection in one assay system and being completely ineffective in another assay system and reflect the conflicting results reported by other studies (Sigal et al., 2011, Permanyer et al., 2012b).

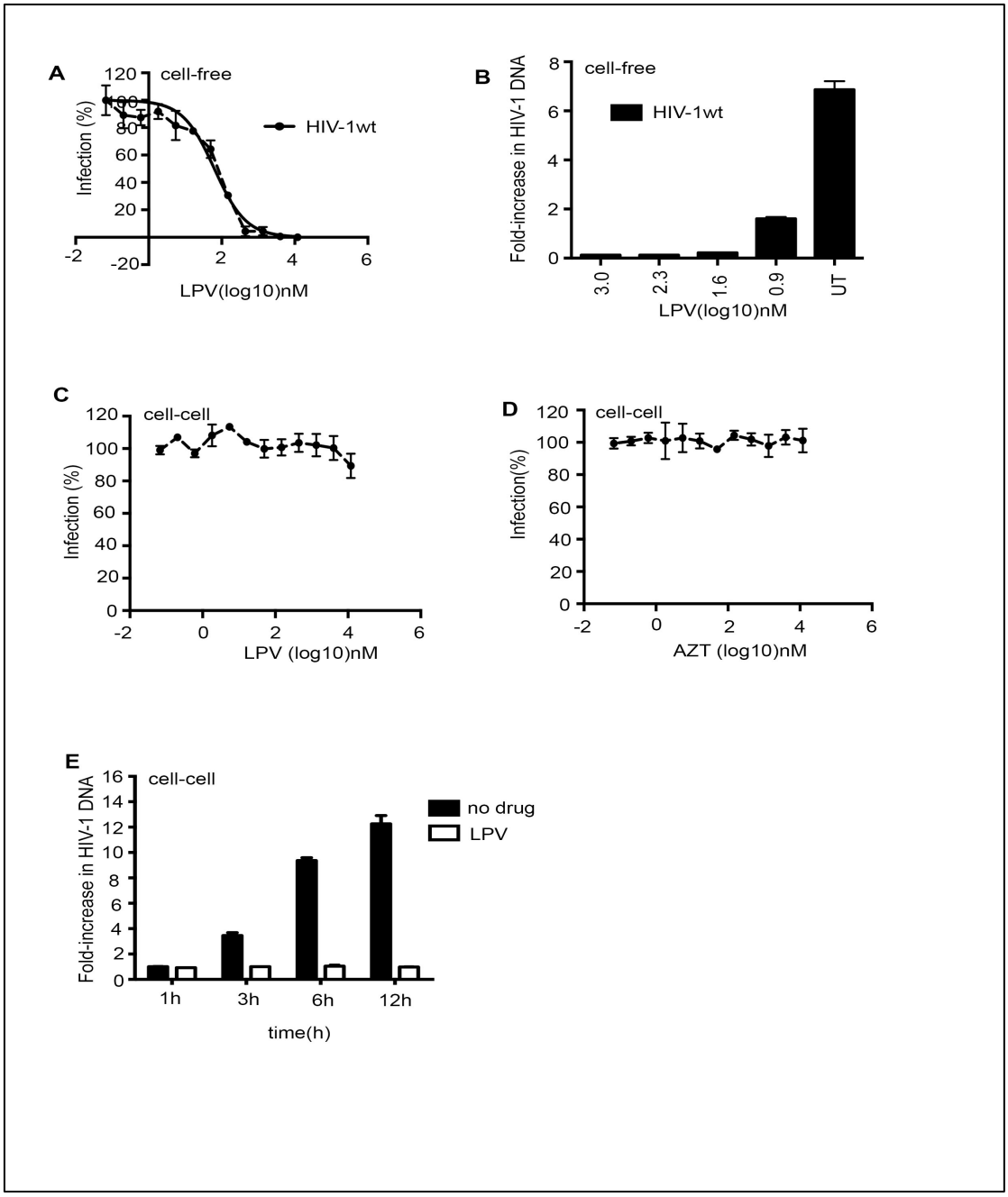


**Figure 3-1**: Comparing a reporter gene assay system and a qPCR-based assay system for studying the effect of drug inhibitors on HIV-1 cell-to-cell spread- LPV potently blocks cell-free spread of HIV-1 in **(A)** a Luciferase–based assay system and in **(B)** a qPCR-based assay system. Infected donor cells were allowed to produce virus in the presence of LPV, and this virus supernatant was used to infect target cells by spinoculation. Following 24h incubation, infection of target cells was quantified either by measuring luciferase activity **(A)** or qPCR detection of *pol* DNA **(B).** One representative experiment is shown and error bars represent the standard deviation (SD) of the mean of triplicates. **(C)** Protease inhibitors (LPV) **(D)** and Reverse Transcriptase Inhibitors appear ineffective against cell-to-cell spread of HIV-1 in a luciferase assay system. Infected donor cells treated with a serial dilution of PI or RTI were co-cultured with target cells and cell-to-cell infection measured by quantifying luciferase expression in target cells. A representative experiment of two independent repeats is shown. **(E)** LPV potently blocks cell-to-cell spread of HIV-1 in a qPCR based assay system. Donor cells pre-treated with LPV or untreated were co-cultured with target cells and HIV-1 *pol* DNA detected by qPCR at several time-points post mixing. A representative experiment of two independent repeats is shown. Error bars represent the SD of the mean.

To explore this further, I decided to test the effect of a Llama derived HIV-1 neutralising VHH J3 on cell-to-cell infection in both assay systems. J3 binds to the viral envelope and prevents its interaction with the CD4 receptor. By so doing J3 blocks the key interaction required for the formation of the virological synapse and hence should block HIV-1 cell-to-cell spread (McCoy et al., 2012, McCoy, 2014). Also this mechanism of inhibition differs from that of PIs and RTIs, which do not disrupt formation of the VS but rather affect later steps in the virus replication cycle notably entry and post entry steps for PIs (Craig et al., 1991, Wensing et al., 2010, Rabi et al., 2013) and reverse transcription for RTIs (Cihlar and Ray, 2010, de Bethune, 2010). For the experiments with J3, the donor cells were incubated with VHH for 1h before co-culturing with target cells. Surprisingly, in contrast to the PIs and the RTIs, VHH J3 potently blocked cell-to-cell infection across a range of concentrations in the luciferase assay system (Figure 2A) and qPCR assay system (Figure 2B and C). This suggests that in the luciferase-based assay Tat-driven reporter gene expression that is observed in PI and RTI-treated co-cultures may be occurring in the absence of true cell-to-cell infection of these cells.

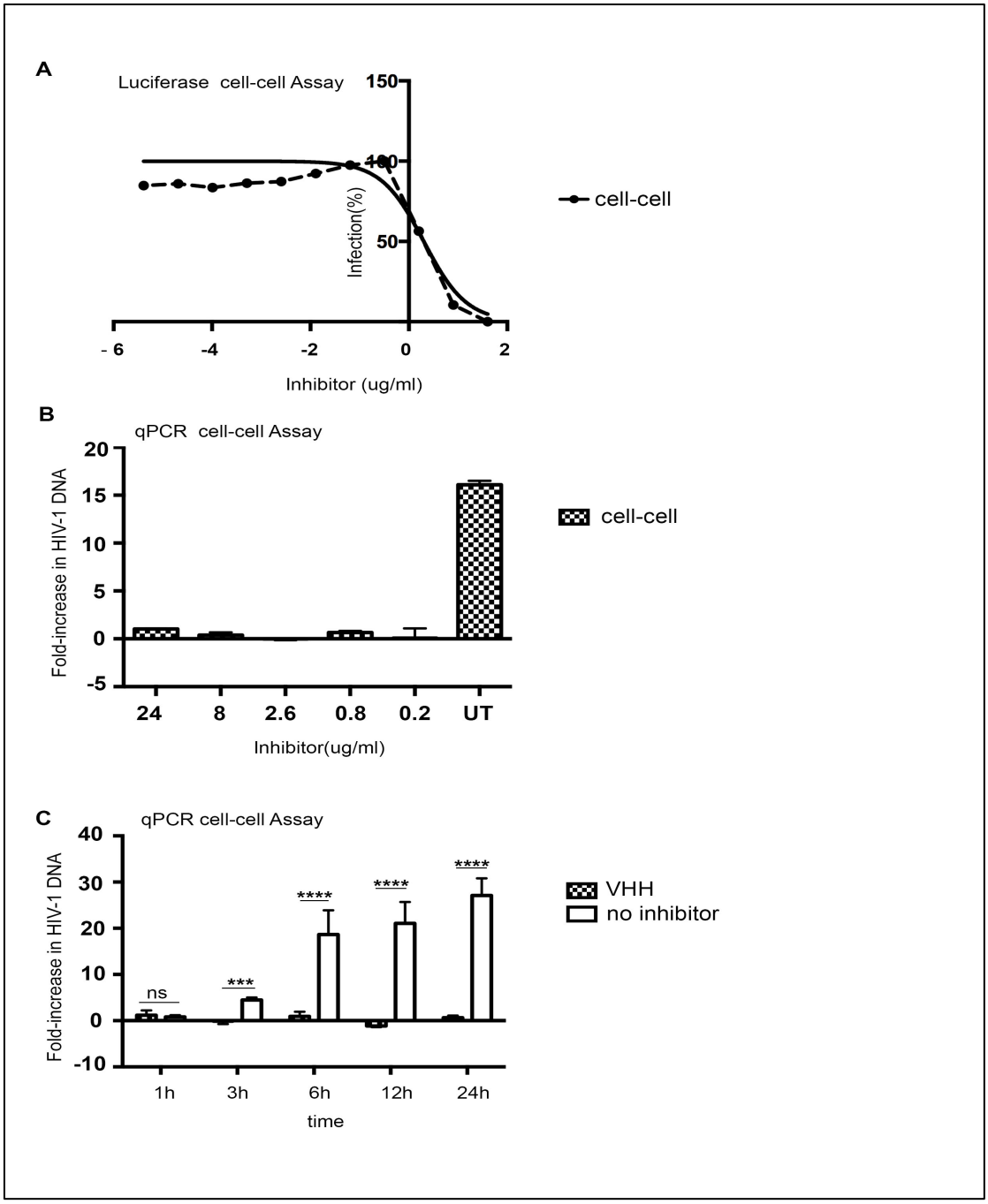


**Figure 3-2**: A Llama derived VHH J3 potently inhibits cell-to-cell spread of HIV-1 in a Luciferase based assay system and a qPCR-based assay system. Infected donor cells were co-cultured with target cells in the presence of the VHH J3. Following a 24h incubation, cell-to-cell infection was quantified by measuring luciferase expression and also by qPCR detection of HIV-1 *pol* DNA copies **(A)** J3 potently blocks cell-to-cell HIV-1 spread across a range of concentrations. A representative experiment is shown. Dotted line represents actual data points while bold line represents the non-linear regression curve fit, **(B)** and **(C)** J3 potently inhibits cell-to-cell spread of HIV-1 across a range of concentrations in the qPCR-based assay system. In (B) co-cultures were done in the presence of a titration of J3 VHH and in **(C)** co-cultures where treated with 8μg/ml of J3 and cell-to-cell infection measured by qPCR detection of HIV-1 *pol* transcripts at different time-points and compared to untreated co-cultures. A representative experiment is shown. Error bars represent the standard deviation of the mean of triplicates. Two-way ANOVA with Bonferonni post-test was applied for comparisons. ****p<0.0001, ***p<0.001, ns=not significant. UT= untreated.

Based on these results, I hypothesised that HIV-1 Tat protein secreted from infected donor cells into the co-culture could drive reporter gene expression in target cells. To test this hypothesis, donor cells were co-cultured with target cells in the presence of a $C_{max}$ of LPV and a serial dilution of an anti-HIV-1 Tat specific monoclonal antibody. This anti-Tat antibody specifically binds HIV-1 Tat protein, and should bind to free Tat protein secreted by the donor cells into the co-culture medium. I anticipated that if free Tat secreted by donor cells was causing reporter gene expression in the target cells in the absence of infection, then the presence of an anti-Tat antibody in the culture medium would reduce free Tat and hence the luciferase signal. After 24h incubation the cells were lysed and luciferase expression measured as previously described. There was a significant reduction (but not complete suppression) of luciferase expression in the co-cultures treated with the highest concentration (50μg/ml) of the anti-Tat antibody possible in our assay (Figure 3.3). Treating co-cultures with either LPV, the antibody diluent (PBS) or an irrelevant antibody isotype control, had no effect on the luciferase signal measured (Figure 3.3).

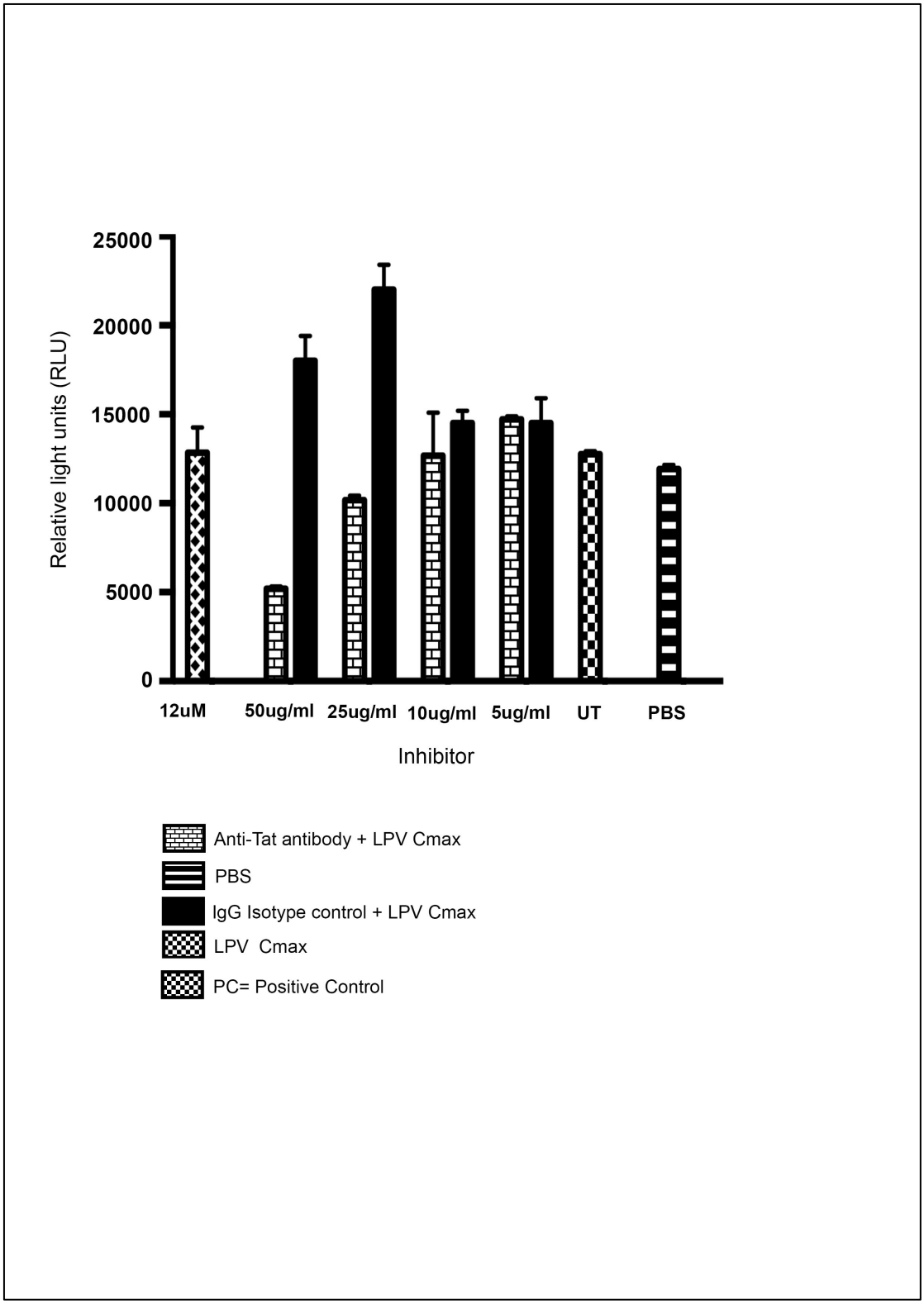


**Figure 3-3**:An Anti-tat antibody reduces the luciferase signal in co-cultures treated with $C_{max}$ LPV in the Luciferase assay system. Co-cultures of donor cells and target cells were treated with $C_{max}$ of LPV (12μM) and a titration of an HIV-1 anti-Tat antibody. In the presence of 50μg/ml of anti-Tat antibody, the luciferase signal detected in the co-cultures was significantly reduced. In co-cultures treated with $C_{max}$ LPV, PBS or a titration of IgG Isotype control + LPV $C_{max}$ or left untreated the levels of luciferase activity detected in the co-cultures was not affected. A representative experiment is shown. Error bars represent the SD of the mean of duplicates.

### 3.2.2 Drug susceptibility of wild type and drug-resistant viruses

PI and RTI drug-resistant viruses are used for the experiments presented in the subsequent chapters of my thesis. These viruses were generated by site-directed mutagenesis of wild type NL4.3 HIV-1 gag and pol (protease and reverse transcriptase) (Figure3.4). After mutagenesis, the mutated fragments were sequenced to confirm that the desired mutation had been introduced (Figure 3.4). Three drug-resistant viruses were designed; the PI drug-resistant virus HIVDM has the V82A mutation in protease and the A431V mutation in gag, the NRTI resistant virus HIV-$1_{M184V}$ that has the M184V mutation in reverse transcriptase and the NNRTI drug-resistant virus HIV-$1_{K103N}$ that has the K103N mutation in reverse transcriptase.

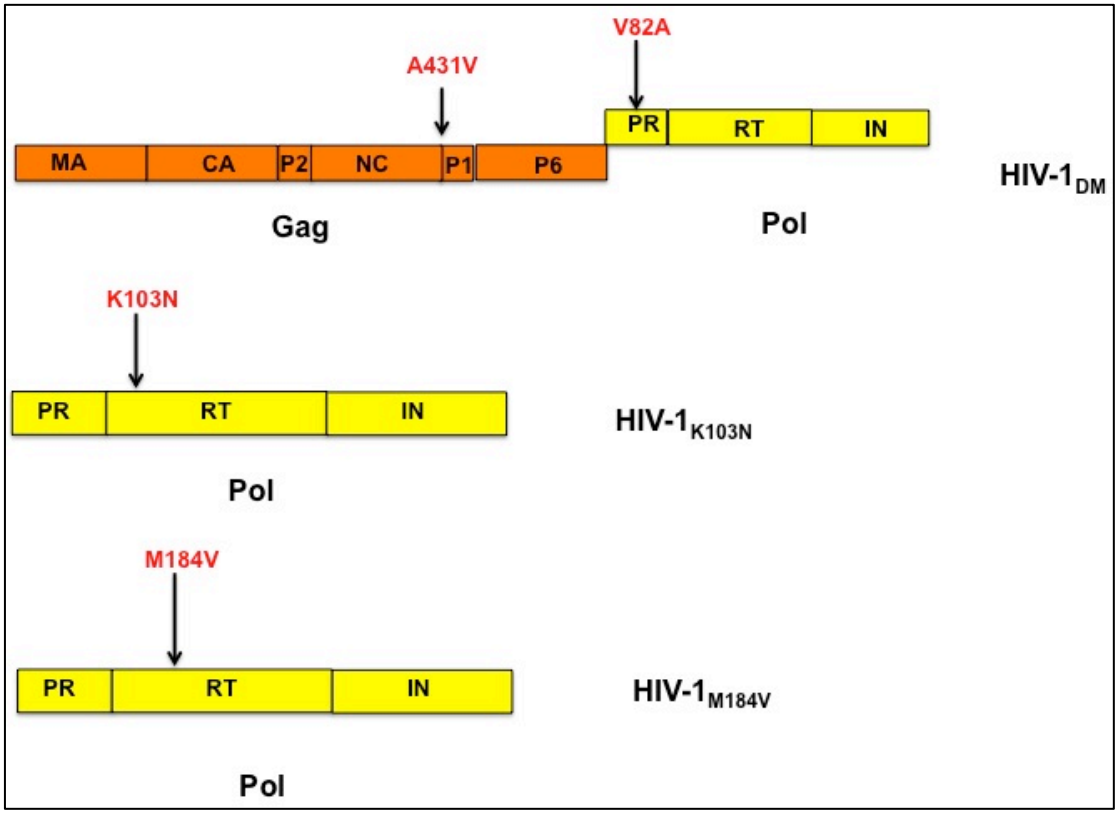


**Figure 3-4** Schematic of PI and RTI mutants showing point mutations introduced in Gag and Pol by site-directed mutagenesis.

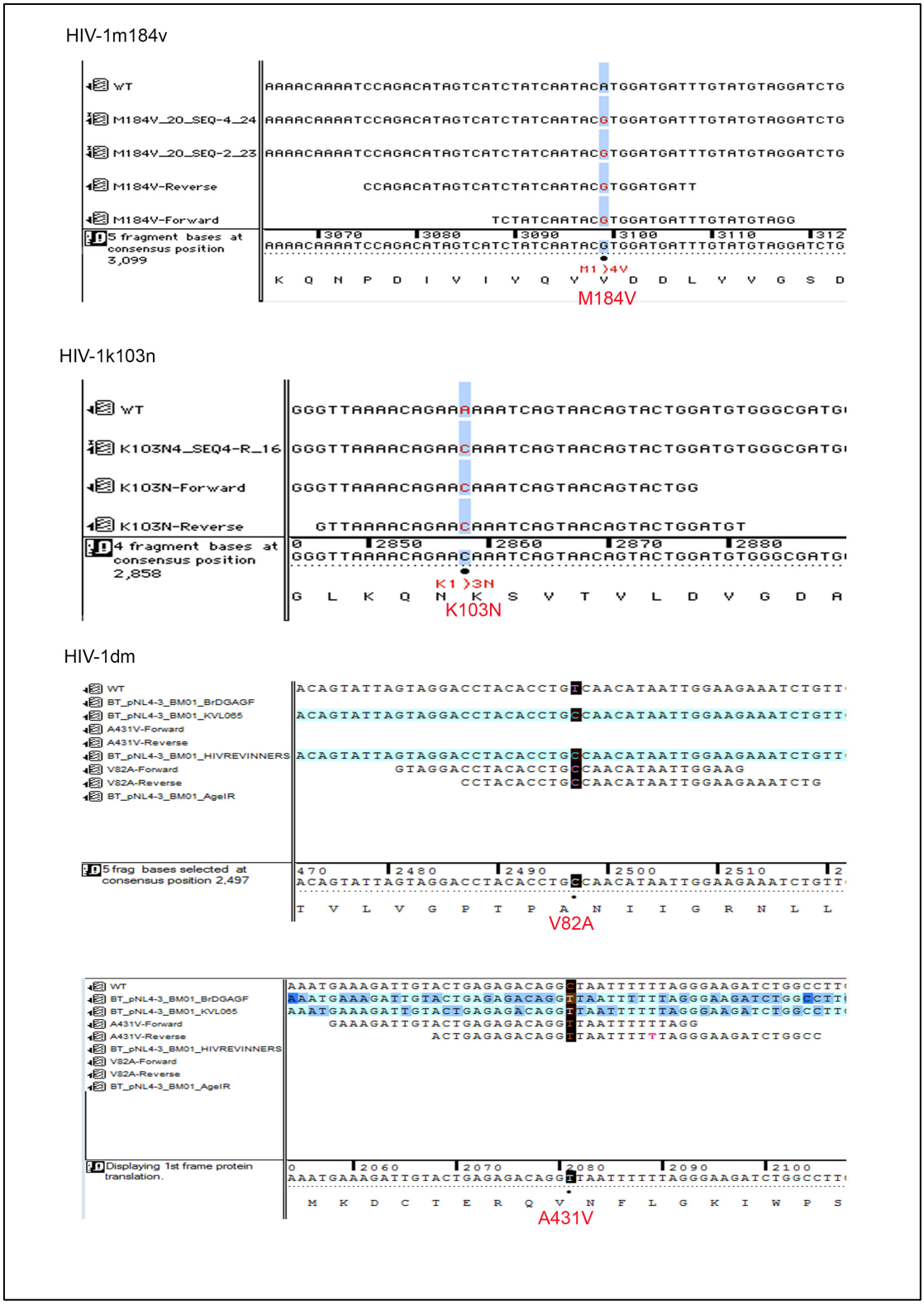


**Figure 3-5** Sequence alignments showing resistance mutations introduced by site-directed mutagenesis (SDM) compared to wild-type NL4.3 virus. After SDM, the sequences of the mutated DNA fragments were aligned and compared to wild-type sequences to check for the presence of the desired mutation. All sequences were aligned using Sequencher®. The alignments show the RT drug-resistant mutants HIV-$1_{M184V}$, and HIV-$1_{K103N}$ and the PI resistant mutant HIV-$1_{DM}$.

The resistant phenotypes of these viruses compared to wild-type virus were verified in a drug susceptibility assay (Gupta et al., 2010) prior to testing these viruses in cell-to-cell infection assays. Briefly HEK293T were transfected with virus DNA and allowed to produce virus in the presence of a serial dilution of drug (PIs). The virus supernatant was harvested after 48 hours and used to infect HeLa TZM-bl cells. For susceptibility to RTIs the virus produced by transfection of HEK293T cells was used to infect HeLa TZM-bl cells in the presence of a serial dilution of the RTI drug. The infected HeLa TZM-bl were incubated for 48h then lysed with SteadyGlo® luciferase and luciferase activity measured on a GLOMAX® luminometer (a detailed description of the drug susceptibility assay is provided in the methods section).

The PI drug-resistant virus HIV-$1_{DM}$ with a V82A mutation in protease and an A431V mutation in gag was 8.4 fold less susceptible to inhibition by LPV than HIV-$1_{WT}$ (Figure 3.6A and Table 3.1) but remained susceptible to DRV as expected (Figure 3.6B and Table 3.1). The NRTI drug-resistant mutant with an M184V mutation in reverse transcriptase (HIV-$1_{M184V}$) remained susceptible to AZT as expected (Figure 3.6C and Table 3.1) and was 120 fold more resistant to 3TC compared to HIV-$1_{WT}$ (Figure 3.6D and Table 3.1). The NNRTI drug-resistant mutant with a K103N mutation in reverse transcriptase (HIV-$1_{K103N}$) was 28-fold less susceptible to inhibition by NVP (Figure 3.6E and Table 3.1) and 650-fold less susceptible to inhibition by EFV as expected (Figure 3.6F and Table 3.1).

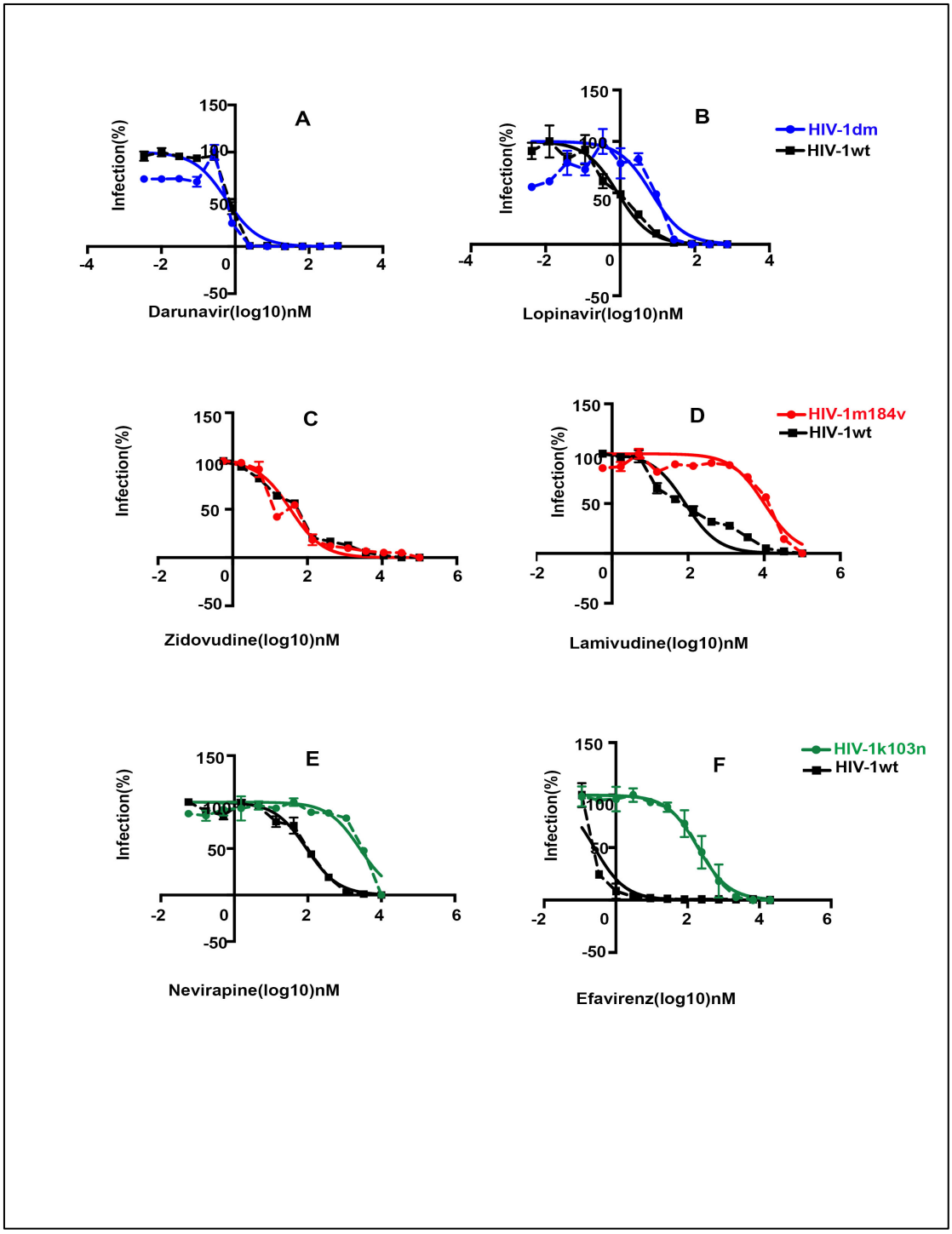


**Figure 3-6**: Drug susceptibility of drug-resistant viruses compared to wild-type virus: The drug susceptibility of the drug-resistant viruses was tested in a cell-free based HeLa TZM-bl drug susceptibility assay. The data were used to plot dose-response curves for the determination of IC50. The phenotypes of the viruses was confirmed in this assay and compared to wild-type virus **(A)** HIV-$1_{DM}$ was 8.4 fold more resistant to LPV than HIV-$1_{WT}$ but was **(B)** equally susceptible to DRV as HIV-$1_{WT}$. **(C)** HIV-$1_{M184V}$ was as expected susceptible to AZT but **(D)** 120 fold more resistant to 3TC than HIV-$1_{WT}$. **(E)** HIV-$1_{K103N}$ was 28 fold more resistant to NVP than HIV-$1_{WT}$, and was **(F)** 650 fold more resistant to EFV that HIV-$1_{WT}$. The dotted lines represent actual data points while the bold lines represent the non-linear regression curve fit of the data in Prism® GraphPad software. The error bars represent the standard deviation of the mean and a representative experiment of two independent repeats is shown.

**Table 3-1**: Summary Table of IC50s of drug-resistant viruses compared to wild-type virus

| Drugs | IC50 (nM) | | Fold-change in IC50 | (p-value) |
|---|---|---|---|---|
| **PIs** | HIV-$1_{WT}$ | HIV-$1_{DM}$ | | |
| **Lopinavir (LPV)** | 0.85 | 6.92 | 8.4 | < 0.001 |
| **Darunavir (DRV)** | 3.0 | 2.8 | 0.9 | 0.34 |
| **NRTIs** | HIV-$1_{WT}$ | HIV-$1_{M184V}$ | | |
| **Lamivudine (3TC)** | 84.6 | 10113 | 119.5 | < 0.0001 |
| **Zidovudine (AZT)** | 41.9 | 29.4 | 0.7 | 0.12 |
| **NNRTIs** | HIV-$1_{WT}$ | HIV-$1_{K103N}$ | | |
| **Nevirapine (NVP)** | 92.4 | 2605 | 28.2 | < 0.0001 |
| **EfavirenzEFV)** | 0.3 | 194.6 | 649 | < 0.0001 |

The IC50s were obtained from dose-response curves plotted using the drug susceptibility data. The IC50s of drug-resistant viruses were compared to the IC50 of wild-type virus using a paired student's t-test in Prism® GraphPad software.

### 3.2.3 Wild-type and drug-resistant HIV-1 viruses spread efficiently from cell-to-cell

After verifying and confirming the phenotype of the drug-resistant and wild type viruses, the qPCR assay described previously was used to assess the ability of these viruses to spread effectively by a cell-to-cell mechanism compared to a cell-free mechanism. Donor cells were infected with a standardised input of the virus being tested as previously described (MOI= 0.3-0.5). The infected pre-washed donor cells were then co-cultured with uninfected target cells and incubated for 24h. Infection was quantified following incubation by qPCR detection of HIV-1 *pol* DNA.

For HIV-$1_{WT}$, cell-to-cell spread was 6 fold more efficient than its cell-free spread both using the luciferase based assay system (Figure 3.7A) and the qPCR-based assay system (Figure 3.7B). The same differences were observed with the drug-resistant viruses tested; cell-to-cell spread of HIV-$1_{DM}$, HIV-$1_{K103N}$ and HIV-$1_{M184V}$ was 4-8 fold more efficient than their spread by a cell-free mechanism (Figure 3.7 C, D and E). Directly comparing cell-to-cell spread of the drug-resistant viruses to that of HIV-$1_{WT}$ showed that these viruses spread with a similar efficiency although subtle differences were noted for HIV-$1_{M184V}$ and HIV-$1_{K103N}$, which were statistically more efficient spreading by a cell-to-cell mechanism when compared to HIV-$1_{WT}$ (Figure 3.7F).

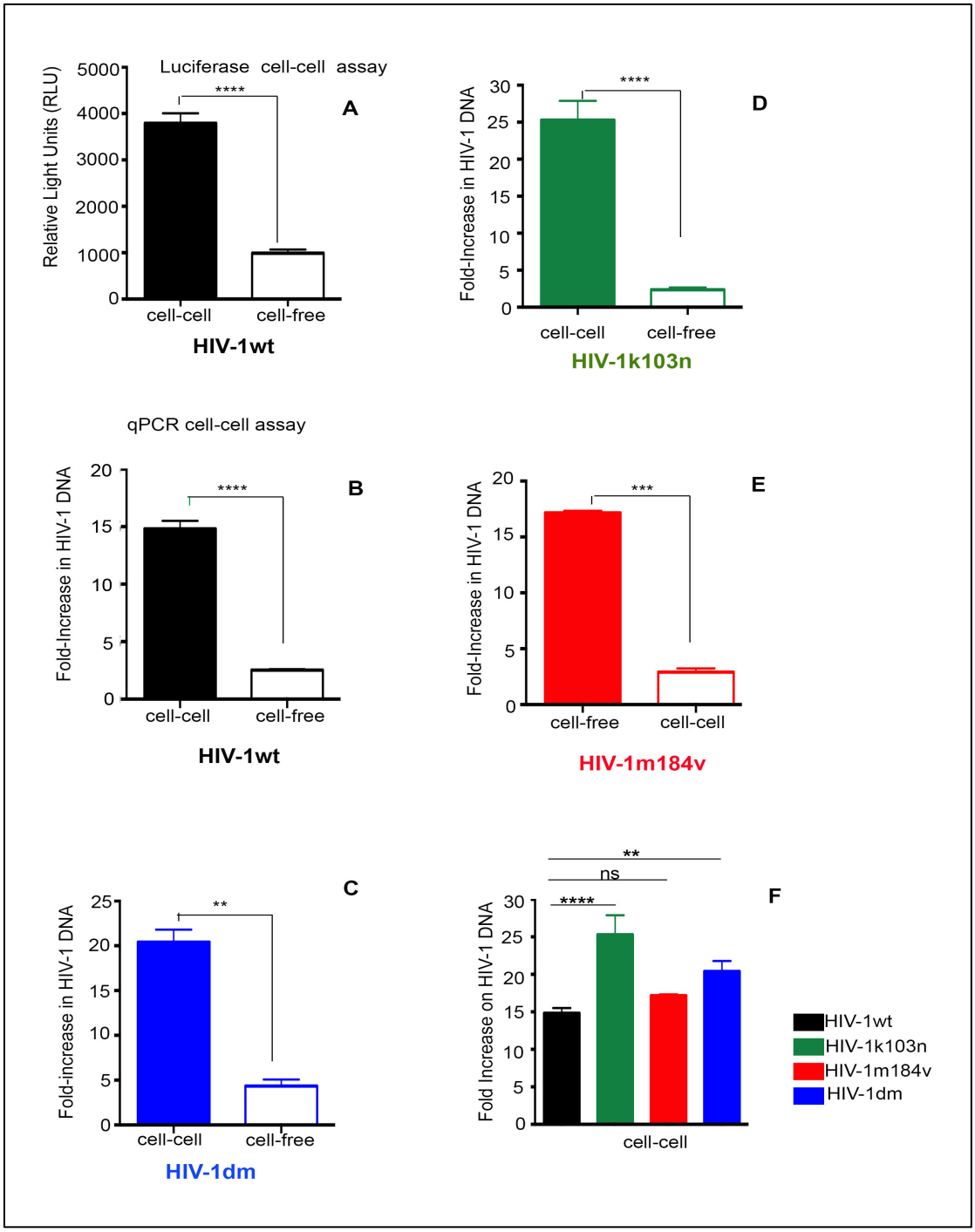


**Figure 3-7**: Comparing cell-to-cell spread vs. cell-free spread of wild type and drug-resistant HIV-1 viruses. Cell-to-cell spread of HIV-$1_{WT}$ was orders of magnitude more efficient than cell-free spread of this virus both in a luciferase assay system **(A)** and in the qPCR-based assay system **(B)**. Cell-to-cell spread of the PI and RTI resistant viruses HIV-$1_{DM}$ **(C)**, HIV-$1_{K103N}$ **(D)** and **(E)** HIV-$1_{M184V}$ was orders of magnitude more efficient than cell-free spread of these viruses. **(F)** cell-to-cell spread of the drug-resistant viruses was broadly similar in efficiency compared to cell-to-cell spread of wild-type virus with cell-to-cell spread of HIV-$1_{K103N}$ and HIV-$1_{M184V}$ being statistically more efficient when compared to cell-to-cell spread of HIV-$1_{WT}$. A representative experiment of two independent repeats is shown. Error bars represent the standard deviation of the mean. Statistical comparison were done using a paired student t-est. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, ns= not significant

## 3.3 Discussion

In this chapter, I compare two assay systems for studying the impact of drug inhibitors on HIV-1 cell-to-cell infection; a reporter gene assay using luciferase expression as a surrogate marker for HIV-1 infection and a qPCR based assay directly quantifying HIV-1 infection by detection of HIV-1 *pol* DNA. The aim of this comparison is to address existing controversy in the field brought about by conflicting results reported by two independent groups (Permanyer et al., 2012b, Sigal et al., 2011) and also, to determine the best and most accurate assay system to use in subsequent experiments presented in this thesis. Two groups have studied the effects of RTIs on cell-to-cell spread of HIV-1 using direct and indirect methods to measure target cell infection and come to different conclusions on the effects of RTIs on this mode of virus spread. In the present study, my results show that in a luciferase expression assay, Tat-driven reporter gene expression in target cells can occur in the absence of true infection. Direct measures of infection such as qPCR detection of HIV-1 DNA transcripts are less ambiguous and thus preferable when assessing the effects of inhibitors on this mode of virus spread.

### Tat-driven expression of reporter genes can occur in the absence of HIV-1 infection

The HIV-1 tat gene is an important regulatory gene in the HIV-1 genome. It encodes for the viral protein Tat. Tat is a potent transactivator protein, which greatly enhances the expression of virus genes through its interaction with the HIV-1 promoter regions contained within the HIV-1-LTR at the 5' end of the integrated provirus (detailed in the background). This property of Tat has been exploited to produce cell-lines containing stably integrated reporter genes under the control of the HIV-1-LTR promoter. When infected with HIV-1, Tat drives expression of the reporter gene contained in these cell-lines and their

expression provides a surrogate marker for productive HIV-1 infection. Assays based on this model are very useful in the field of HIV-1 research as they are usually high throughput, easy to perform and provide a quick way for measuring HIV-1 infection in target cells. The conflicting results obtained when using this system to assess the effects of drug inhibitors on cell-to-cell spread of HIV-1 suggests that there are factors, which may limit the usefulness of reporter gene assays when studying HIV-1 cell-to-cell spread in the context of some inhibitors.

While PIs and RTIs failed to inhibit reporter gene expression in the target cells of drug treated co-cultures (Figure 3.1 C and D), a Llama derived VHH HIV-1 neutralising antibody (J3) effectively suppressed luciferase expression in target cells in a dose dependent manner (Figure 3.2 A). J3 achieves its potent and broad neutralisation effect by directly interacting with the CD4 binding site on the HIV-1 envelope and preventing viral attachment to the target cell (McCoy et al., 2012, McCoy, 2014). This antibody is able to inhibit cell-to-cell spread both in the luciferase based assay and in the qPCR based assay (Figure 3.2 B and C) and also efficiently blocks cell-free infection (McCoy et al., 2012, McCoy, 2014). Several groups have shown that monoclonal antibodies that block CD4-Env interaction can block both cell-to-cell and cell-free HIV-1 infection (Chen et al., 2007, Jolly et al., 2004, Hubner et al., 2009, Jolly et al., 2007, Malbec et al., 2013, McCoy, 2014). The CD4-Env interaction is an important event in the formation of the virological synapse, which precedes actual cell-to-cell transfer of virions from the effector cell to a target cell. Blocking the attachment of viral Env to the CD4 receptor inhibits the formation of the VS and subsequent events leading to infection. The disruption of this interaction explains the potent inhibition of HIV-1 cell-to-cell infection by J3.

We hypothesised that Tat-driven reporter gene expression in target cells that occurred in the absence of infection, was the result of Tat secreted from infected donor cells into the co-culture, driving reporter gene expression in the target cells. To test this hypothesis, co-cultures were performed in the presence of the drug inhibitor ($C_{max}$ LPV) and a serial dilution of an anti-Tat antibody (Figure 3.3). The presence of the anti-Tat antibody significantly reduced the luciferase signal detected in co-cultures in a dose-dependent manner but did not abolish the signal. Several studies have previously shown that the extracellular form of HIV-1 Tat released from infected cells is able to enter nearby target cells and induce its effect of gene transactivation (Ensoli et al., 1990, Ensoli et al., 1993, Zauli et al., 1995, Ferrari et al., 2003, Zheng et al., 2005, Romani et al., 2010, Debaisieux et al., 2012). This extracellular secretion of Tat may play an important role in sustaining a paracrine loop required for optimal HIV-1 LTR transactivation (Zauli et al., 1995, Romani et al., 2010). It is reasonable to suggest that the presence of an anti-Tat mAb in the co-culture may interfere with the paracrine activation loop by binding free extracellular Tat and as such reduce luciferase expression in target cells.

Free extracellular Tat is probably not the only factor driving the expression of luciferase in the target cells in the absence of infection. We think this because in the presence of the highest concentration of anti-Tat antibody (50μg/ml) possible with the assay set-up, a luciferase signal is still detected in the target cells, treated with PI (LPV). This suggests that active secretion of Tat protein could also be occurring across the virological synapse (VS) from infected donor cells into the target cells. Virus transfer across the VS from donor cells to target cells is characteristically rapid and delivers a large dose of infectious virions into the target cells. If Tat secretion does occur across the VS, this may like virion transfer be very rapid and efficient and as such effectively

reduce the window available for complete neutralisation by an anti-Tat specific antibody as observed in this study. Before the first descriptions of the retrovirus induced virological synapse (Jolly and Sattentau, 2004, Igakura et al., 2003), an early study by Helland et al. suggested that direct cell-to-cell contact between an infected Tat producing effector cell (Jurkat-tat) and a target cell encoding a reporter gene under the control of the HIV-LTR promoter greatly enhanced transactivation of the reporter gene in the target cells (Helland et al., 1991). Was this early report a possible hint at Tat secretion across the VS? In the formation of the virological synapse, viral proteins such as Gag have been shown to co-localise to the point of contact between cells (Jolly and Sattentau, 2004, Jolly and Sattentau, 2005). It is conceivable that a viral protein like HIV-1 Tat will be able to hijack the synapse in a similar manner to mediate its function of inducing transactivation of gene expression in neighbouring target cells, although this has not been specifically investigated. This would explain why even though the anti-Tat mAb significantly reduced the luciferase signal probably by binding free extracellular Tat in the culture medium, it did not completely block transactivation of the reporter gene in the target cells.

The potent inhibition of Tat-driven luciferase gene expression in the target cells of co-cultures treated with the NAb J3 strengthens the case for possible Tat secretion across the VS as a source of HIV-1 LTR transactivation in target cells. J3 blocks the interaction between CD4 and Env and as such prevents formation of the VS. In the luciferase assay 2μg/ml of VHH NAb only suppresses luciferase expression by 50% (IC50) in target cells (Figure 3.2A). However this same dose of NAb completely suppresses the formation of *de novo* HIV-1 DNA in target cells measured by qPCR (Figure 3.2B). This supports the reasoning that the luciferase signal that is measured in the reporter gene based assay

system is very likely a combination of activation from extracellular free Tat from donor cells, Tat secreted across the synapse and possibly productive infection of target cells. It is difficult to say which of these modes of Tat driven expression predominates in a given co-culture system but this would likely depend on other variables such as the cell type used in the assay, the percentage of infected donor cells in culture, chronicity of donor cell infection etc. Another possible source of Tat induced reporter gene expression in the absence of target cell infection could be from the fusion of donor cells with target cells in co-culture. This is however unlikely as syncytium formation and cell-cell fusion are not commonly observed in T cell co-cultures of infected donor cells and target cells (Jolly et al., 2004) and it has been shown that the tetraspanin CD9 inhibits cell-cell fusion and syncytium formation at the VS (Weng et al., 2009).

In their work investigating the effects of RTIs on cell-cell spread of HIV-1, Permanyer et al. made similar observations on the occurrence of reporter gene expression in the absence of true infection of target cells in co-cultures (Permanyer et al., 2012b). They however did not propose a mechanism to explain these observations as we have done. My data show that high-level Tat-driven reporter gene expression can occur in the absence of true infection of target cells. Using Tat driven reporter gene expression as a measure of infection when studying cell-to-cell spread of HIV-1 would tend to overestimate true infection in target cells due to confounding sources of Tat driven reporter gene expression not related to target cell infection. This was probably the case in the study by Sigal et al. that found cell-to-cell spread of HIV-1 to be two orders of magnitude more resistant (>200 fold) to inhibition by RTIs than cell-free spread when using a Tat driven reporter gene expression assay system (Sigal et al., 2011). Their findings however are still noteworthy and relevant as they were also able to show this reduced susceptibility of

HIV-1 cell-to-cell infection to RTIs, using another surrogate marker of HIV-1 infection, which is independent of Tat (staining target cells for p24 antigen). This suggests that the reduced susceptibility though possibly over estimated when measured using a Tat driven reporter gene assay system is still genuinely present. Although Permanyer et al. contested this by using direct qPCR detection of HIV-1 DNA when assessing the impact of RTIs on HIV-1 cell-to-cell infection, in their study they adjusted the virus input so that cell-free and cell-to-cell spread resulted in a similar percentage of GFP+ infected cells in the untreated condition (Permanyer et al., 2012b). Under these conditions, the RTIs were found to be equally potent at inhibiting both cell-to-cell and cell-free infection. Normalising the virus input in this way removes the quantitative effects of high-multiplicity infection mediated by cell-to-cell spread and as such does not offer a true assessment of the effects of the inhibitor being studied on this mode of virus infection in comparison to cell-free infection.

Reporter gene assays remain useful for rapid screening of some inhibitors when studying the mechanisms of HIV-1 infection. However, their use warrants caution due to the existence of confounding factors, which may cause an overestimation of productive infection in target cells. In the subsequent chapters of my thesis, I use the qPCR based cell-to-cell assay for all other studies presented.

## Drug susceptibility and cell-to-cell spread of HIV-1 drug-resistant mutants

The PI and RTI drug-resistant HIV-1 viruses that are used in the experiments presented in this thesis were all tested in a drug susceptibility assay (Gupta et al., 2010) to confirm that they displayed the expected phenotype. The PI resistant mutant HIV-$1_{DM}$ has a V82A mutation in protease and an A431V mutation in gag (at the NC/p1

cleavage site). The V82A mutation is a common major PI resistance mutation, which is rapidly selected in patients who receive antiretroviral therapy containing Indinavir or Lopinavir (Luca, 2006). The presence of V82A reduces the ability of the viral protease to bind to its substrate and imposes a fitness cost on the virus (Zhang et al., 1997, Kantor et al., 2005). It decreases the susceptibility of the virus to inhibition by all PIs to variable degrees (low to high levels of resistance) except Darunavir (Clavel and Hance, 2004). In the evolution of PI resistance the selection of the V82A mutation is rapidly followed by the selection of the A431V mutation in gag. A431V is located at the NC/p1 cleavage site and its presence causes a conformational change that allows the binding and effective cleavage of the substrate (cleavage site) by the mutated protease containing the V82A (Dam et al., 2009). This gag mutation by itself is also capable of inducing a reduced susceptibility to PIs *in vitro* (Dam et al., 2009). In the drug susceptibility assay HIV-$1_{DM}$ was resistant to LPV but remained susceptible to DRV as expected (Figure 3.5 A and B).

The NRTI resistant virus HIV-$1_{M184V}$ has the M184V mutation in reverse transcriptase. M184V emerges very rapidly in all patients receiving non-suppressive therapy with Lamivudine (3TC) or Emtricitabine (FTC), reducing the virus susceptibility to these drugs by >100-fold (Eron et al., 1995, Marcelin, 2006). The changes in RT induced by M184V increase the fidelity of RT for the natural dNTP substrate but reduces the processivity of the enzyme thus leading to a reduction of viral fitness (Marcelin, 2006). In contrast the selection of this mutation increases the susceptibility of the virus to Stavudine, Tenofovir and Zidovudine. This phenotype was verified and confirmed in the drug susceptibility assay for Zidovudine and Lamivudine (Figure 3.3 C and D).

The NNRTI resistant mutant HIV-$1_{K103N}$ has the K103N mutation in reverse transcriptase. K103N is rapidly selected in 30-50% patients who receive non-suppressive antiretroviral therapy containing Nevirapine (NVP) or Efavirenz (EFV). This mutation is located in the hydrophobic NNRTI binding pocket of RT and reduces the affinity of the viral enzyme to these drugs (de Bethune, 2010). This mutation leads to high levels of resistance >50-fold to NVP and EFV (de Bethune, 2010) and this was confirmed in the drug susceptibility assay (Figure 3.3 E and F). The presence of this mutation does not negatively affect the replicative fitness of the virus.

The PI and RTI drug-resistant HIV-1 viruses were all able to spread efficiently from cell-to-cell similarly to wild type virus (Figure 3.7 C, D, E). The ability of HIV-1 to mutate its genome into resistant variants capable of circumventing the effect of drugs provides one of the biggest challenges to the success of HAART. Drug-resistant mutant viruses have a replicative advantage over wild-type variants in the presence of antiretroviral drugs. They achieve this through diverse mechanisms, discussed in detail in the background section of this thesis. Whether these drug-resistant variants are also better or less well adapted to spread from cell-to-cell when compared to wild-type variants is an interesting question that warrants further investigation. For example if these viruses are inherently fitter during cell-to-cell spread, this could be a contributing factor for the selection of resistant viruses in infected patients. This could be tested by directly comparing cell-to-cell and cell-free spread of these resistant viruses in the absence of inhibitors using a direct output measure like the qPCR assay described in this chapter. Given the apparent benefits of a cell-to-cell mode of viral dissemination over cell-free spread, it is reasonable to imagine that some drug-resistant viruses could evolve to be spread more efficiently by this means, providing them with an additional mechanism by which

to persist in the presence of therapy. Although our results hint at the existence of possible differences in the efficiency of cell-to-cell spread of some resistant viruses (Figure 3.7 F), we have only assessed three drug-resistant variants, which considerably limits our ability to make any meaningful conclusions. It is however interesting that a resistance mutation such as HIV-$1_{M184V}$ which has been shown to have a reduced replicative capacity in cell-free assays (Diallo et al., 2003) spreads efficiently in our in-vitro cell-to-cell assay system. A similar observation has been recently made with the drug resistant virus K263R selected by the integrase inhibitor Dolutegravir, which though unfit in the context of cell-free is not compromised in cell-to-cell transmission (Bastarache et al., 2014). Additional work with a larger panel of drug-resistant mutants is needed to explore the mechanisms underlying the apparent differences in the efficiency of spread of drug resistant virus by cell-free and cell-to-cell mechanisms. This however is not the focus of the work presented in this thesis.

# 4 Protease inhibitors effectively inhibit cell-to-cell spread of HIV-1 between T cells

## 4.1 Introduction

Despite advances in the clinical management of HIV-1 infection, finding an effective vaccine or a definitive cure for the disease continues to elude researchers. One of the main reasons for this is the ability of the virus to persist within the host in reservoirs and re-emerge when treatment is interrupted. It has been suggested that ongoing viral replication in patients receiving antiretroviral therapy may be a contributing factor to the maintenance of cellular reservoirs of the virus. This is however debated in the field, with evidence both in support of and against ongoing viral replication in the presence of antiretroviral agents. In favour of complete inhibition of viral replication with cART, patients who fully adhere to effective cART regimens do not show evidence of continuing viral evolution or treatment failure (Frenkel et al., 2003, Kieffer et al., 2004, Bailey et al., 2006, Kearney et al., 2014, Dinoso et al., 2009, McMahon et al., 2010, Gandhi et al., 2010). Nevertheless, some (but not all) treatment intensification studies with Raltegravir have demonstrated that there is an increase in episomal DNA and a reduction in the size of the viral reservoir when this agent is added to a triple therapy combination (Buzon et al., 2010, Yukl et al., 2010, Vallejo et al., 2012, Llibre et al., 2012). Also, several studies have demonstrated that low level viral replication may occur in specific anatomical compartments despite suppression of plasma HIV-1 RNA (Gunthard et al., 1998, Ruiz et al., 1999, Martinez et al., 1999, Martinez et al., 2001, Benito et al., 2004, Chun et al., 2008, Shiu et al., 2009). These

studies suggest that there may be a degree of ongoing residual viral replication with cART.

A study by Sigal et al. was the first to propose that cell-to-cell spread of HIV-1 may be a mechanism for such ongoing virus replication in the presence of antiretroviral therapy (Sigal et al., 2011). In this study the authors hypothesised that the high multiplicity of infection that typifies HIV-1 cell-to-cell dissemination, would increase the chance of at least one virus particle being able to stochastically escape inhibition by antiretroviral drugs and establish infection in target cells. They tested this hypothesis by assessing the impact of RTIs on cell-to-cell spread of HIV-1 in an *in vitro* assay system and showed that this mode of virus infection was less susceptible to inhibition by RTIs when compared to cell-free infection (Sigal et al., 2011). This report was challenged by another study in which the use of similar *in vitro* cell-to-cell assays showed RTIs to be equally effective against both cell-to-cell and cell-free modes of virus dissemination (Permanyer et al., 2012b).

The discrepancies between these two studies raise questions on the true impact that antiretroviral drugs have on HIV-1 cell-to-cell dissemination. Furthermore, because both studies restricted their analyses to RTIs, it remains unclear whether other classes of antiretroviral drugs vary in their ability to inhibit viral dissemination by a cell-to-cell mechanism. In this chapter the impact of Protease Inhibitors on cell-to-cell spread of HIV-1 in comparison to cell-free spread is studied using the qPCR-based *in vitro* assay evaluated in the previous chapter. This system is also used to reassess RTIs tested in afore mentioned studies for their relative efficacy against cell-to-cell vs. cell-free HIV-1 infection and comparing their effects to that of PIs. The reasons for focusing on PIs are manifold; PIs are important components of cART regimens by virtue of their potency in inhibiting viral replication

and the high barrier that they present against the selection of drug-resistant viruses (Wensing et al., 2010, Arts and Hazuda, 2012). This has led to PIs being the only class of antiretroviral drugs to be considered in clinical trials for use as monotherapy in the treatment of HIV-1 infection (Bierman et al., 2009, Perez-Valero and Arribas, 2011, Perez-Valero et al., 2011, Katlama et al., 2010, Arribas et al., 2010, Clumeck et al., 2011). Also, PIs are part of recommended first-line treatment options for HIV-1 infected patients, and constitute the mainstay of second-line regimens for patients who fail first-line therapies (WHO, 2013, DHHS, 2014). In resource challenged settings PIs are mainly reserved for use in second-line therapies when first-line RTI-based options fail. However in recent years, increasing prevalence of baseline drug resistance to RTIs in treatment naïve patients has led to wider use of PI-based therapies for initial treatments (Gupta et al., 2012, WHO, July 2012). This further highlights the importance of this drug class for the future of cART.

While the exact mechanisms by which PIs exert their potent inhibitory effects *in vivo* are not completely understood, recent studies suggest that in addition to preventing cleavage of viral polyproteins into functional sub-units leading to the production of immature non-infectious virions (Wensing et al., 2010), PIs also affect viral entry and post-entry steps in the replication cycle (Rabi et al., 2013). Cell-to-cell spread of HIV-1 is characterised by the polarisation of virus assembly and budding towards the point of contact between the donor cell and the target cell (Jolly et al., 2004, Jolly and Sattentau, 2004, Jolly et al., 2007, Jolly and Sattentau, 2007). It is therefore plausible that the assembly and maturation of newly formed virions at the virological synapse, coupled with more rapid virus transfer could limit the efficiency of PIs in blocking cell-to-cell dissemination of the virus. The impact of PIs on this mode of virus dissemination however has not been investigated prior to the present study.

In order to complete the panel of drugs tested, the impact of integrase inhibitors on T cell-to-T cell spread of HIV-1 was also tested. Integrase inhibitors are the latest addition to the arsenal of FDA approved antiretroviral agents and now constitute a part of first-line therapies as well as salvage therapy regimens for treatment-experienced patients who fail on first-line and second-line treatment options. For this reason it is interesting to assess their effect on T cell -to-T cell spread of HIV-1.

The results show that PIs are equally effective against both cell-to-cell and cell-free modes of HIV-1 dissemination. Also a PI-resistant mutant retains its resistant profile during cell-to-cell spread. By contrast, cell-to-cell spread is less susceptible to inhibition by RTIs. We also note existing intra-class variability in the ability of RTIs to effectively block this mode of virus dissemination, with NRTIs having much reduced potencies in comparison to NNRTIs against cell-to-cell infection. Lastly the results provide evidence that INIs are effective inhibitors of both cell-to-cell and cell-free spread of HIV-1 in T cells. These data suggest that if HIV-1 cell-to-cell dissemination does indeed contribute to ongoing viral replication and the maintenance of reservoirs in treated patients, this will likely be drug class dependent.

### 4.1.1 Specific objectives

- To assess the impact of Protease Inhibitors on cell-to-cell vs. cell-free spread of HIV-1.
- To assess the impact of Reverse Transcriptase Inhibitors on cell-to-cell vs. cell-free spread of HIV-1 and in comparison to PIs.
- To assess cell-to-cell spread of PI and RTI drug-resistant mutants in the presence of these respective drug classes.
- To assess the impact of Integrase Inhibitors on cell-to-cell spread of HIV-1.

## 4.2 Results

### 4.2.1 Protease inhibitors effectively inhibit cell-to-cell transfer of HIV-1

To investigate the effect of PIs on cell-to-cell spread, the qPCR cell-to-cell *in vitro* assay system that was assessed in the previous chapter was used. This assay has also been used and extensively validated in other studies of HIV-1 cell-to-cell spread (Jolly et al., 2007, Jolly et al., 2010, Casartelli et al., 2010). Briefly, infected donor cells (Jurkat T cells) untreated or pre-incubated with the PIs; Lopinavir (LPV) or Darunavir (DRV) for a maximum of 24h, were co-cultured with uninfected target T cells (Jurkat-1G5). The co-culture was incubated for 24h for experiments with a single time-point or for different durations (1h, 3h, 6h, 12h), for time-course experiments. Cell-to-cell spread of HIV-1 was measured by qPCR, to quantify *de novo* HIV-1 DNA *pol* copies arising from reverse transcription in the newly infected T cell population. The data were expressed as a fold increase in HIV-1 *pol* DNA relative to the housekeeping gene Albumin. In this assay a synchronous population of HIV-1 infected donor cells, allows for the reliable measurement of virus infection in target cells mediated by cell-to-cell dissemination with little or no contribution from the less efficient cell-free mode of infection (Jolly et al., 2007). Furthermore, subtracting the *pol* signal at t = 0h (baseline) from all subsequent time points removes the HIV-1 *pol* DNA signal arising from integrated proviral DNA within the donor cell population.

As expected, a time-dependent increase in the generation of HIV-1 *pol* DNA, indicative of cell-to-cell spread in the positive control (untreated co-cultures) was observed. In the co-cultures treated with the maximum plasma concentrations ($C_{max}$) achievable *in vivo* of LPV (Figure 4.1A) or DRV (Figure 4.1B) (14μM and 12μM respectively), cell-to-

cell spread was potently inhibited as evidenced by the absence of an increase in HIV-1*pol* DNA in these drug treated co-cultures.

It is expected that inhibiting the synthesis of new HIV-1 *pol* transcripts by blocking cell-to-cell infection will have an effect on the appearance of episomal DNA forms such as 2 LTR circles. This form of episomal DNA is frequently used as a marker for nuclear import, a step that precedes integration of proviral DNA into the host cell genome (Kalpana, 2008, Hazuda et al., 2000, Butler et al., 2001). After 24h incubation, 2 LTR circles were readily detected (635copies/100ng of DNA at 24h) in untreated co-cultures, however significantly fewer 2 LTR copies ($p<0.05$) were detected in co-cultures treated with PIs (<50 copies/100ng of DNA) (Figure 4.1C). Collectively, these results provide evidence that PIs are effective inhibitors of HIV-1 cell-to-cell infection between CD4+ T cells.

To confirm the activity of the PIs tested on Gag maturation and to verify that there was no overall defect in virus budding affecting cell-to-cell spread, a Western blot analysis of purified virus collected from PI–treated HIV-1 infected T cells was performed. Donor cells, which were treated with a $C_{max}$ of LPV or DRV displayed as expected a predominance of uncleaved p55Gag protein in virions, while in contrast untreated cell cultures or cell cultures treated with RTIs ($C_{max}$ of TFV=2μM or NVP=10μM) mainly generated virions with p24CA, indicating proper Protease mediated cleavage of Gag (Figure 4.1D).

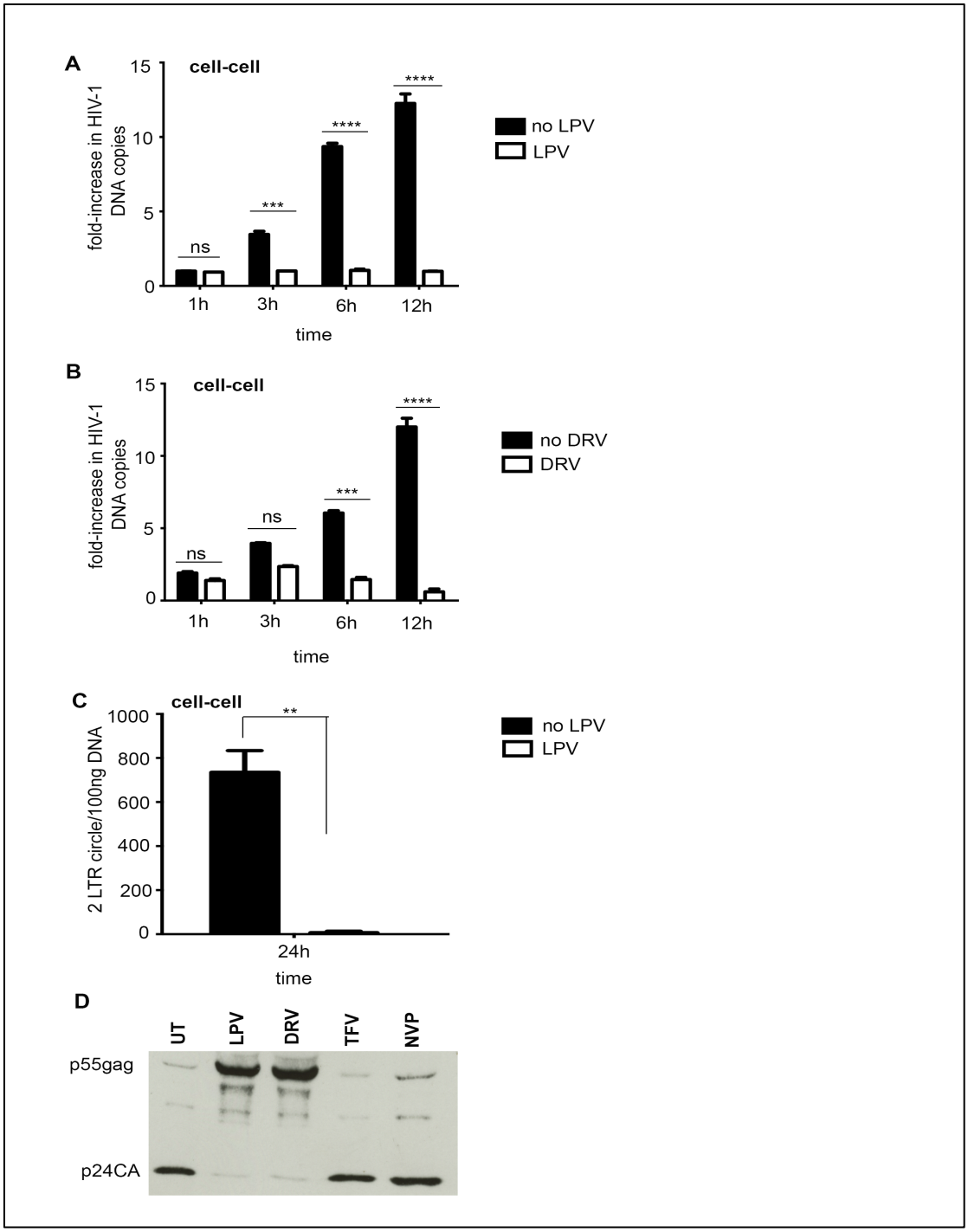


**Figure 4-1**: Protease Inhibitors effectively block cell-to-cell spread of HIV-1.**(A)** Quantification of cell-to-cell spread of HIV-1 in the presence of $C_{max}$ LPV (14μM) and **(B)** $C_{max}$ of DRV (12μM). HIV-1 infected Jurkat cells (donors) were pre-incubated with PI for 24h prior to co-culturing with target cells or co-cultured without pre-treatment with drugs. After co-culture, HIV-1 *pol* DNA was detected by qPCR. The data were normalised to the housekeeping gene Albumin and expressed as the fold increase in HIV DNA copy number over time relative to the baseline value at t=0h. Data show the mean of triplicates, error bars represent the standard deviation of the mean (SD). **** p<0.0001, ***p<0.001, ns: not significant, two-way ANOVA plus Bonferoni post-test applied for comparisons. **(C)** Reduced detection of 2 LTR circles following cell-to-cell spread of HIV-1 in the presence of LPV (14μM). After 24h co-culture of donor cells and target with or without LPV, 2 LTR circles were detected by qPCR. **p<0.05, unpaired student t-test. **(D)** Confirmation of PR Gag maturation defect in HIV-1. HIV-1+ donor cells incubated with PIs, RTIs or left untreated for 24 hours. Virus-containing supernatants were harvested, purified and equal volumes of virus were analysed by SDS-PAGE and Western blotting for HIV-1 Gag.

### 4.2.2 Protease inhibitors are equally effective at blocking both cell-free and cell-to-cell spread of HIV-1

In order to determine the efficacy of PIs over a range of concentrations at inhibiting both cell-to-cell and cell-free modes of HIV-1 dissemination infected donor cells mixed with target cells were co-cultured in the presence of serial dilutions of LPV or DRV. Cell-to-cell spread was measured by qPCR detection of HIV-1 *pol* DNA after a 24h incubation period as previously described, and these data were used to plot dose-response curves from which 50% inhibitory concentrations (IC50s) for the drugs tested were calculated. For cell-free infections, HIV-1 infected donor cells were incubated alone and allowed to produce virus in the presence of a serial dilution of PIs. Culture supernatants containing cell-free virus were subsequently harvested and used to infect target cells. Following 24h incubation, infection of target cells was measured by qPCR detection of HIV-1 *pol* DNA as previously described.

There were no significant differences in the IC50 of LPV (Figure 4.2A, Table 4.1) or DRV (Figure 4.2B, Table 4.1) for either cell-free infection (3.0nM and 2.5nM respectively) or cell-to-cell infection (2.9nM and 2.8nM respectively), demonstrating that PIs are equally effective against these two modes of virus dissemination. In the original study by Sigal et al., which showed that cell-to-cell spread of HIV-1 had a reduced susceptibility to inhibition by RTIs, the high MOI associated with cell-to-cell HIV-1 dissemination was proposed as the reason for this reduced susceptibility (Sigal et al., 2011). High multiplicity of infection has also been linked to the reduced susceptibility of cell-to-cell infection to inhibition by some neutralising antibodies (Martin and Sattentau, 2009, Abela et al., 2012, Durham et al., 2012, Massanella et al., 2009, Chen et al., 2007). Based on this, the effect of varying the

multiplicity of infection on the ability of PIs to remain effective at blocking HIV-1 cell-to-cell infection was assessed. This was done by modifying the donor cell to target cell ratio in the co-cultures to 5:1 compared to 1:5 that was used for the experiments previously described. Increasing the number of HIV-1 infected donor cells in the co-cultures led to a reduced ability of LPV to inhibit cell-to-cell infection when testing a drug concentration close to the IC50 (8nM). Increasing the concentration of LPV three-fold (24nM) restored the ability of the drug to effectively block cell-to-cell spread in the co-cultures with higher donor cell to target cell ratios (Figure 4.2C). It is worth noting that the concentration of LPV which effectively inhibited cell-to-cell spread in the co-cultures with increased number of donor cells and therefore effectively higher MOI, is still well below the minimum plasma concentration of the drug achievable *in vivo* in patients treated with LPV (Cmin=3μM). This suggests that PIs likely remain potent against cell-to-cell infections over a wide range of physiologically relevant drug concentrations.

The PI resistant mutant HIV-$1_{DM}$ was assessed for its ability to spread from cell-to-cell in comparison to HIV-$1_{WT}$ virus in the presence of LPV (Figure 4.2D) and DRV (Figure 4.2E). This drug-resistant virus carries the V82A mutation in protease and the A431V mutation in gag. In the drug susceptibility assay HIV-$1_{DM}$ is 14-fold more resistant to inhibition by LPV and is susceptible to DRV (see Chapter 3). This virus maintained its resistant phenotype to LPV (Figure 4.2D) and remained susceptible to DRV as expected (Figure 4.2E) when spreading by a cell-to-cell mechanism. These data indicate that the observations on the effects of PIs on cell-to-cell spread of HIV-1 are not a function of the viruses tested but really a reflection of the effect of the drugs assessed in our assays.

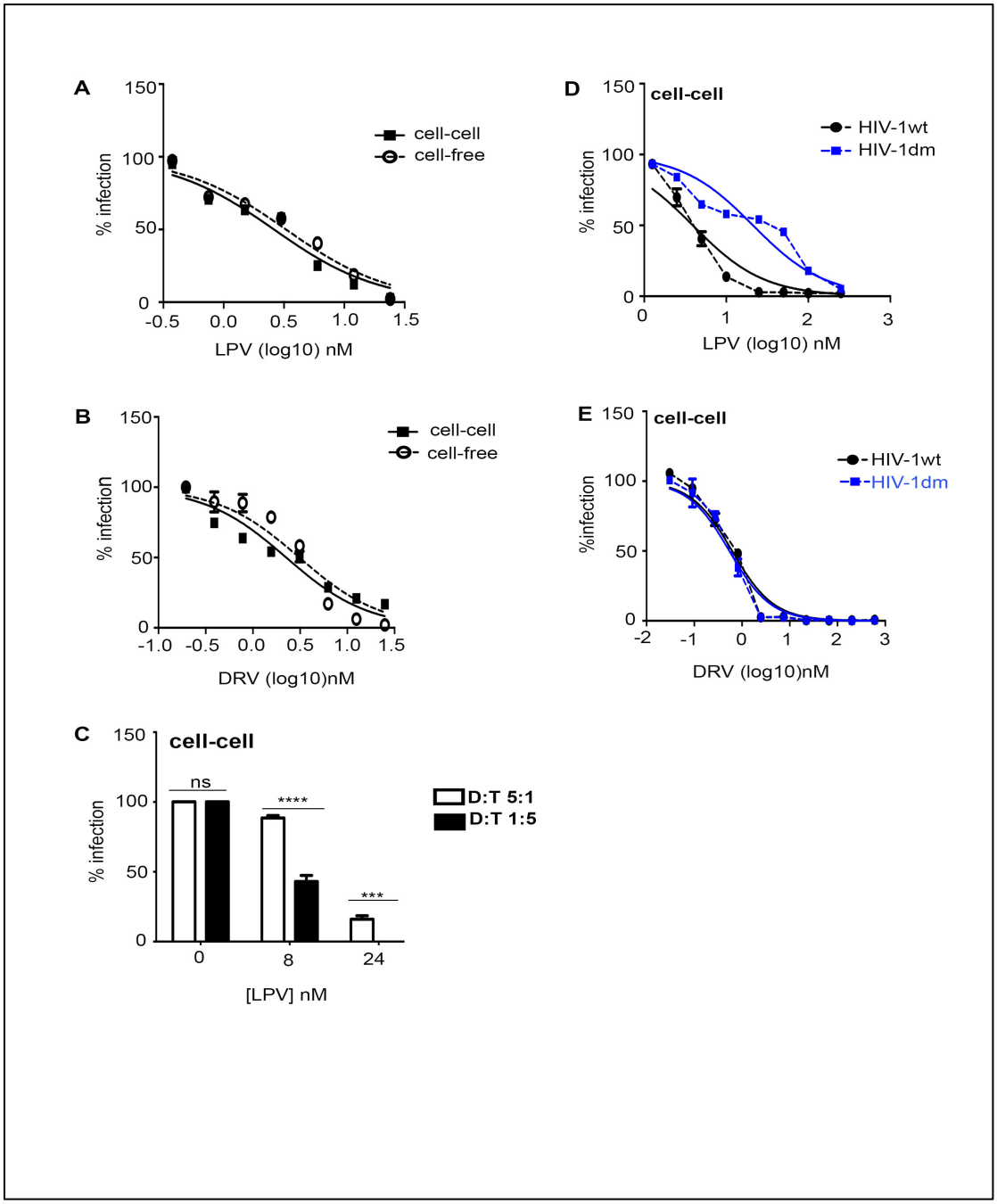


**Figure 4-2**: Protease Inhibitors are equally effective at inhibiting both cell-to-cell and cell-free spread of HIV-1. **(A)** LPV and **(B)** DRV equally block both cell-free and cell-to-cell spread of HIV-1. Infected donor cells were incubated with a serial dilution of the PIs for 24h prior to co-culturing with uninfected target cells. HIV-1 *pol* DNA was measured by qPCR following co-culture. For cell-free infections, virus supernatant collected from infected donor cells pre-incubated with PIs was used to infect target cells. 24h after infection, qPCR was performed to detect HIV-1 *pol* DNA and expressed as a fold increase relative to the Albumin housekeeping gene. The error bars represent the standard deviation of the mean of triplicates and a representative experiment is shown. **(C)** Increasing the donor: target cell ratio in co-culture reduces the efficacy of LPV in blocking cell-to-cell infection. **** $p<0.0001$, *** $p<0.001$, ns: not significant, two-way ANOVA with Bonferoni post-test applied for comparisons. **(D)** A PI drug-resistant mutant HIV-$1_{DM}$ maintains its resistant phenotype when spreading by a cell-to-cell mechanism, being less susceptible to inhibition by LPV than HIV-1wt and remains as expected (E) susceptible to inhibition by DRV as wild-type virus.

**Table 4-1**: Summary table showing IC50 of Protease Inhibitors; Lopinavir and Darunavir with cell-to-cell and cell-free spread of HIV-1.

| | **Protease Inhibitors** | | | |
|---|---|---|---|---|
| **Drugs** | Lopinavir | | Darunavir | |
| **Mode of Spread** | C-C | C-F | C-C | C-F |
| **Mean IC50 (nM)** | 2.9 | 3.0 | 2.8 | 2.5 |
| **SEM** | 0.2 | 0.2 | 0.4 | 0.1 |
| **p-value** | 0.7 | | 0.5 | |

### 4.2.3 PIs effectively inhibit cell-to-cell spread of HIV-1 mediated by infected primary T cells

The potency of PIs against cell-to-cell infection mediated by HIV-1 infected primary T cells was also tested. For this, CD4+ T cells were purified from PBMCs obtained from healthy donors. These CD4+ T cells were then stimulated with PHA and IL2 and infected with HIV-1. The infected donor cells (CD4+ T cells) were pre-incubated with $C_{max}$ of LPV (14μM) and then co-cultured with uninfected target cells (Jurkat T cells). The co-culture was incubated for 24h after which cell-to-cell infection was measured by qPCR as previously described. With primary CD4+ T cells as donor cells, it was not possible to obtain the same level of infection as with T cell lines (i.e. >90% Gag positivity by flow cytometry), only 60% HIV-1 Gag positivity was achieved in infected primary CD4+ T cell cultures. For this reason an additional control of infected primary CD4+ cells alone without addition of target cells was included in the experiment set-up, to control for spreading infection in this donor cell population. No increase in HIV-1 *pol* DNA overtime was observed in the donors cells cultured alone, indicating that there is minimal spreading infection in the primary CD4+ T cell population (Figure 4.3). The PI LPV effectively inhibited cell-to-cell spread from infected CD4+ T cells to Jurkat T cells as shown by the absence of a time dependent increase in the number of HIV-1 *pol* DNA copies detected in the drug treated co-cultures when compared to the untreated co-cultures (Figure 4.3).

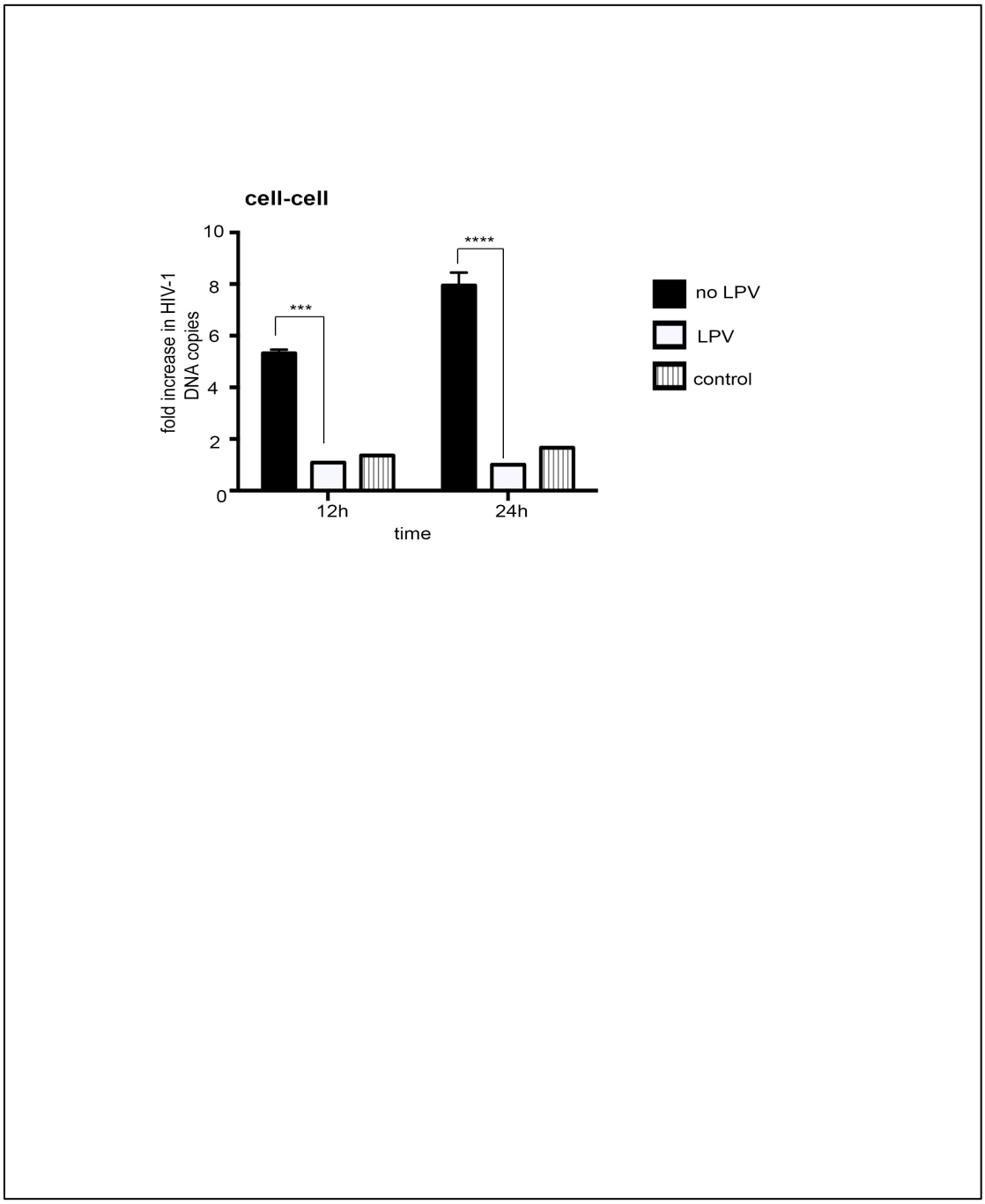


**Figure 4-3**: Protease Inhibitors block cell-to-cell transfer from HIV-1 infected primary T cells: HIV-1 infected primary CD4+ T cells (donor cells) were incubated with LPV (14μM) for 24h and mixed with uninfected Jurkat cells (target cells). Cell-to-cell infection was measured by qPCR detection of HIV-1 *pol* DNA. A representative of two independent experiments performed with primary cells isolated from two different donors is shown. The data are the mean of triplicates and error bars represent the standard deviation of the mean. **** $p<0.0001$, *** $p<0.001$, a two-way ANOVA with Bonferroni post-test was applied for comparisons.

### 4.2.4 Reverse Transcriptase Inhibitors are less effective inhibitors of HIV-1 cell-to-cell infection compared to cell-free infection

In light of the conflicting reports on the impact of RTIs on cell-to-cell spread of HIV-1, the potency of RTIs against cell-to-cell spread was re-assessed in this *in vitro* system and compared to the effects of PIs. To do this, co-cultures (infected donor cells mixed with uninfected target cells) and cell-free infection assays were performed in the presence of a serial dilution of RTIs; Zidovudine (AZT), Tenofovir (TFV), Lamivudine (3TC), Nevirapine (NVP), and Efavirenz (EFV). Infection was measured by qPCR detection of HIV-1 *pol* DNA as described earlier.

These data were used to plot dose-response curves and determine the IC50s for both modes of virus spread with these drugs. The results showed that most of the RTIs tested though effective against cell-free infections displayed reduced potencies against cell-to-cell infection. AZT (Figure 4.4 A) and TFV (Figure 4.4 B) were the least effective of these drugs with cell-to-cell infection being >20-fold less susceptible to inhibition by AZT (Figure 4.4A) or >10-fold less susceptible to inhibition by TFV (Figure 4.4B) when compared to cell-free spread. 3TC had a 6-fold reduced potency (Figure 4.4C) and NVP a 4-fold reduced potency against cell-to-cell spread in comparison to cell-free spread (Figure 4.4 D). The IC50s for cell-to-cell and cell-free infections are summarised on Table 4.2. Notably, EFV stood out in this drug class behaving in a similar fashion to PIs and displayed the same potency against both cell-to-cell and cell-free spread of HIV-1 (Figure 4.5E).

The effect of varying the multiplicity of infection on the potency of the RTIs that showed a reduced effectiveness against cell-to-cell infection was also investigated. Reducing the virus input 10-fold by decreasing the number of infected donor cells used in the co-cultures significantly

improved the ability of TFV and AZT to inhibit cell-to-cell infection (Figure 4.4F), suggesting that high MOI associated with cell-to-cell spread may contribute to the poor inhibitory potential of RTIs.

An NNRTI drug-resistant mutant HIV-$1_{K103N}$ (Figure 4.4G), which is resistant to inhibition by EFV and NVP and an NRTI drug-resistant mutant HIV-$1_{M184V}$ (Figure 4.4H), which is resistant to 3TC, maintained their resistant phenotypes when spreading by a cell-to-cell mechanism (Figure 4.4G and H). This indicates that the observations on the impact of the RTIs tested, on cell-to-cell spread of HIV-1, reflect the true effects of the drugs tested and are not a function of the viruses.

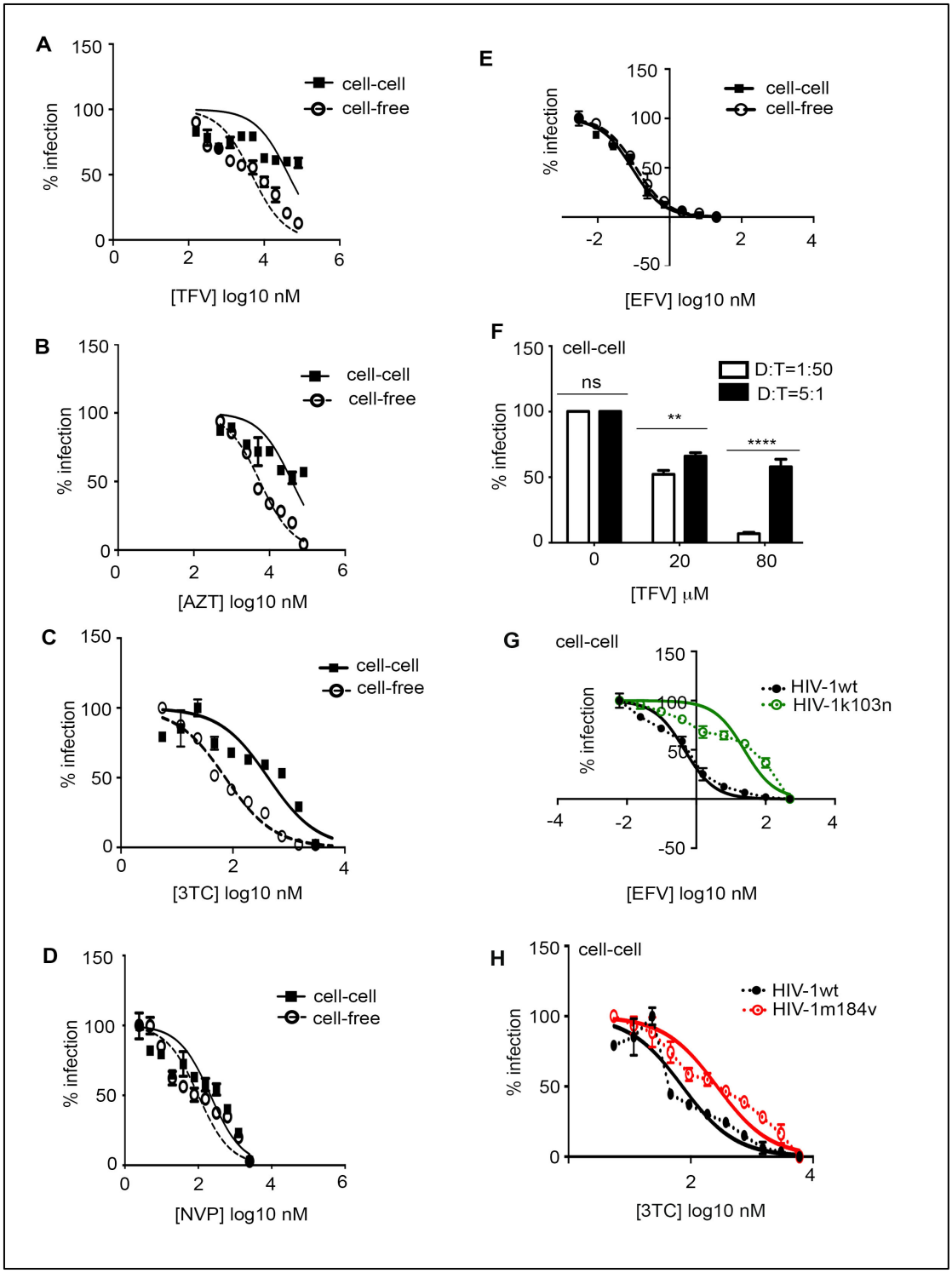


**Figure 4-4**: Reverse Transcriptase Inhibitors are less effective inhibitors of cell-to-cell spread of HIV-1 compared to cell-free spread: Infected donor cells were co-cultured with uninfected target cells in the presence of a serial dilution of RTIs. **(A)** TFV, **(B)** AZT, **(C)** 3TC and **(D)** NVP displayed reduced potency in their ability to inhibit cell-to-cell infection in comparison to cell-free infection. **(E)** EFV was equally effective at inhibiting both modes of virus spread. **(F)** Reducing the MOI by reducing the number of donor cells in the co-culture 10-fold restored the ability of an ineffective RTI, TFV to inhibit cell-to-cell spread of HIV-1. RTI mutants HIV-$1_{K103N}$ **(G)** and HIV-$1_{M184V}$ **(H)** maintain their resistance profile when spreading by a cell-to-cell mechanism. The data shown are a representative experiment from at least two independent repeats. Error bars represent the SD of the mean of triplicates. A two-way ANOVA with Bonferroni post-test was applied for group comparisons. **** $p < 0.0001$, *** $p < 0.001$, ns = not significant

**Table 4-2**: Summary Table showing IC50s of Reverse transcriptase inhibitors for cell-to-cell and cell-free infection

| | **Reverse Transcriptase Inhibitors** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Drugs** | AZT | | TFV | | 3TC | | NVP | | EFV | |
| **Mode of infection** | C-C | C-F | C-C | C-F | C-C | C-F | C-C | C-F | C-C | C-F |
| **IC50** | >80μM | 3.4μM | >80μM | 7.5μM | 428nM | 73nM | 360nM | 86nM | 0.23nM | 0.21nM |
| **SEM** | UD | 0.3 | UD | 0.7 | 34 | 6.3 | 89.5 | 9.2 | 0.04 | 0.017 |
| **p-value** | UD | | UD | | 0.001 | | 0.03 | | 0.62 | |

IC50s for NRTIs AZT, TFV, 3TC and NNRTIs NVP, EFV for cell-to-cell and cell-free infection are shown. UD= undetermined, SEM= standard error of the mean. C-F= cell-free, C-C = cell-cell.

### 4.2.5 Time of drug addition does not modify the effects of PIs and RTIs on HIV-1 cell-to-cell spread

The effect of the time of drug addition in the assays on the potency of the drugs tested was also investigated. To test this for PIs, HIV-1 infected donor cells were mixed with uninfected target cells in the presence of LPV or DRV without prior pre-incubation of donors with the drug (time of addition, t=0h). We noted that the PIs LPV (Figure 4.5A) and DRV (Figure 4.5B) remained effective against cell-to-cell infection regardless of the time of addition of the drugs i.e. either added at t=0h or with HIV-1 infected donor cells pre-incubated with drug for 24h prior to co-culturing with target cells as in the experiments previously described. A similar assessment was made for the RTIs with uninfected target cells pre-incubated with TFV (Figure 4.5C) or AZT (Figure 4.5D) for 24h prior to mixing with HIV-1 infected donor cells as opposed to adding the drug at t=0h. Infection was quantified by qPCR as previously described. Under these conditions we found that pre-incubating target cells with the RTIs as opposed to adding the drugs at the time of mixing the infected donor cells with the target cells, did not improve the ability of TFV (Figure 4.5C) and AZT (Figure 4.5D) to inhibit HIV-1 cell-to-cell spread. These data indicate that the effects of the tested antiretroviral agents on cell-to-cell spread of HIV-1 do not depend on the time of drug addition.

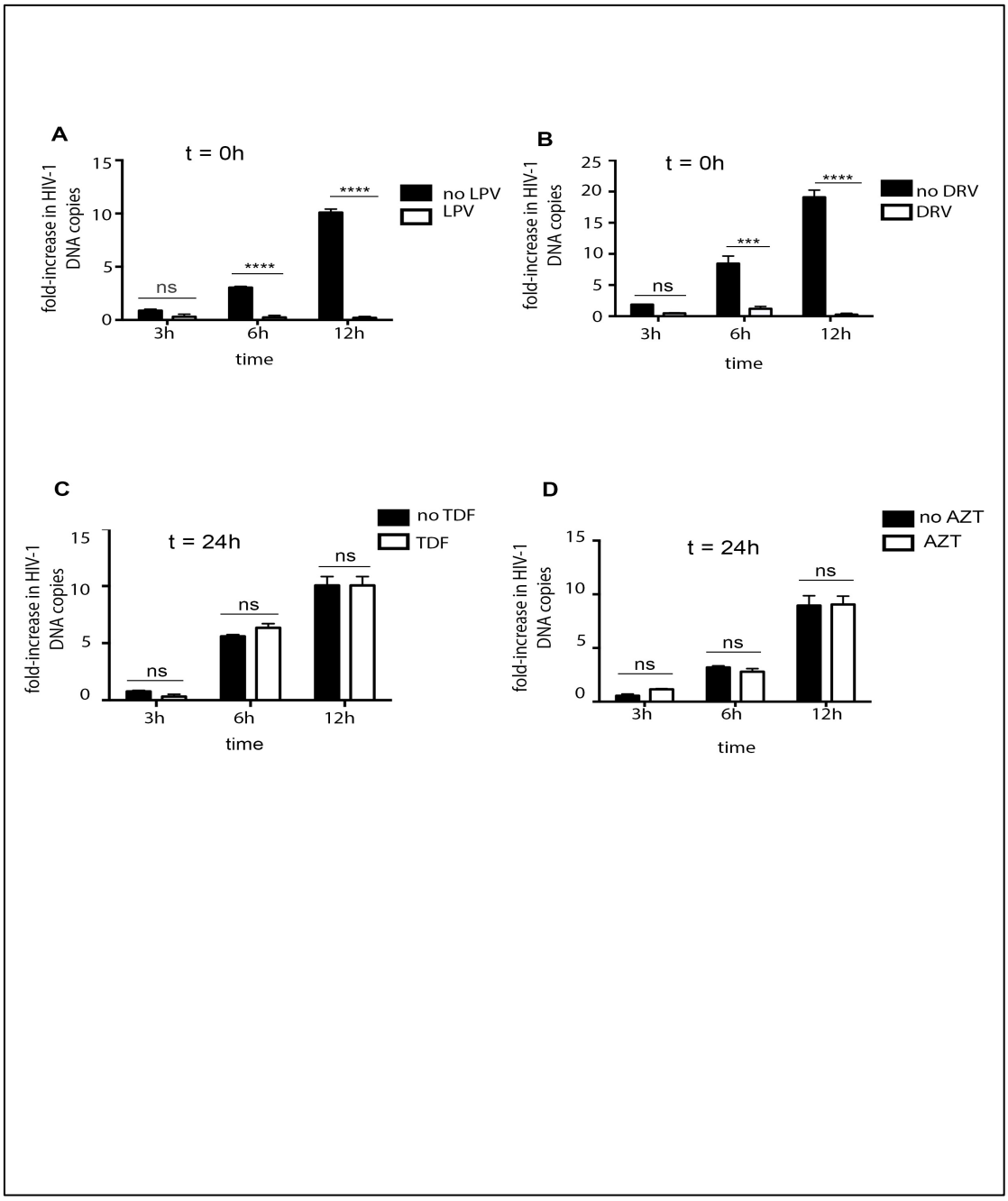


**Figure 4-5**: Time of drug addition does not modify the effects of PIs and RTIs on HIV-1 cell-to-cell spread. Infected donor cells were co-cultured with uninfected target cells in the presence of **(A)** LPV (14μM) and **(B)** DRV (12μM) without pre-incubation of the donor cells with the drug (time of drug addition t=0h). HIV-1 *pol* DNA was directed by qPCR for different time-points following co-culture. The data shown represent the mean of triplicates and the error bars are the standard deviation of the mean. For RTIs, uninfected target cells were pre-incubated with **(C)** TFV (2μM) or **(D)** AZT (10μM) for 24h prior to co-culture with infected donor cells. HIV-1 *pol* DNA was detected as previously described for different time-points following co-culture of donor and target cells and expressed as a fold increase relative to the Albumin housekeeping gene. Data show the mean of triplicates, error bars represent the SD of the mean. Comparisons were made using a two-way ANOVA with a Bonferroni post-test **** p< 0.0001, ***p<0.001, ns: not significant.

### 4.2.6 Integrase inhibitors effectively inhibit cell-to-cell spread of HIV-1 between T cells

Although the main focus of the work presented in this chapter is the impact of Protease Inhibitors on cell-to-cell spread of HIV-1, while completing this thesis Duncan et al. published a study assessing the impact of antiretroviral drugs on macrophage-to-T cell spread of HIV-1 (Duncan et.al, 2013). They studied the effects of RTIs and also Integrase Inhibitors (INIs) on this mode of virus dissemination. Given that the impact of INIs on T cell-to-T cell spread of HIV-1 has not been previously studied, to complete the panel of the drugs classes tested (RTIs and PIs); the impact of Raltegravir on cell-to-cell spread was tested. In the absence of a sufficiently accurate integrated DNA qPCR assay in our lab, a rapid assay using the quantification of p24 antigen in culture supernatants as a measure of infection was designed to explore the effects of the INI, Raltegravir (RAL) on T cell-to- T cell spread of HIV-1.

For cell-to-cell infections, $2x10^5$ infected donor cells (Jurkat T cells) were mixed with $8x10^5$ target cells in the presence of the $C_{max}$ of Raltegravir (6μM). Co-cultures without Raltegravir and cultures of donor cells alone were included in control wells. Culture supernatants were collected at 4, 7 and 10 days after co-culture, for the detection of p24 antigen by ELISA. For cell-free infections, cultures were performed on 24well plates with 3μM transwells to separate donor cell and target cell populations. The transwells allow full diffusion of virus but not migration of cells. The infected donor cells were suspended in the culture medium and placed in the top well, while the uninfected cells were placed in the bottom well. Target cells were treated with Raltegravir $C_{max}$ (6μM), or left untreated. Supernatants were collected from the target cells at 4, 7 and 10 days, for detection of p24 antigen by ELISA.

In the presence of INIs we expected to see an increase in non-integrated forms of viral DNA resulting from the block to integration. Detection of 2 LTR circles can be used as an indirect measure for assessing the effect of INIs (Hazuda et al., 2000, Butler et al., 2001). It is expected that 2 LTR circles would increase in the presence of INIs relative to untreated controls because blocking integration provides more substrate for the ligation to form 2 LTR circles (Hazuda et al., 2000). Cell-free and cell-to-cell infections in the presence of a serial dilution of Raltegravir were performed and generation of 2 LTR circles after 24h incubation detected by qPCR. More 2 LTR circles were generated in cell-to-cell co-cultures (Figure 4.6A) treated with Raltegravir, compared to cell-free infected cultures after 24h (Figure 4.6B) indicating that Raltegravir is indeed blocking integration in both modes virus infection. In addition, the increased absolute number of 2 LTRs detected in the cell-cell condition compared to cell-free likely represents the increase in viral transmission associated with cell-cell spread. In the p24 ELISA based assay, there was an increase in the amount of p24 antigen in untreated co-cultures over time, whereas in the Raltegravir treated co-cultures there was no increase in p24 antigen over time (Figure 4.6D). This was also true for cell-free infections (Figure 4.6E). This shows that Raltegravir effectively blocks both cell-free infection as well as cell-to-cell infection in co-cultures. This rapid assay was less adaptable for testing drug titrations needed to determine IC50s of Raltegravir for cell-to-cell and cell-free modes of infection compared to the qPCR-based assay used for testing RTIs and PIs. For this reason the IC50s of Raltegravir with the two modes of infection was not determined.

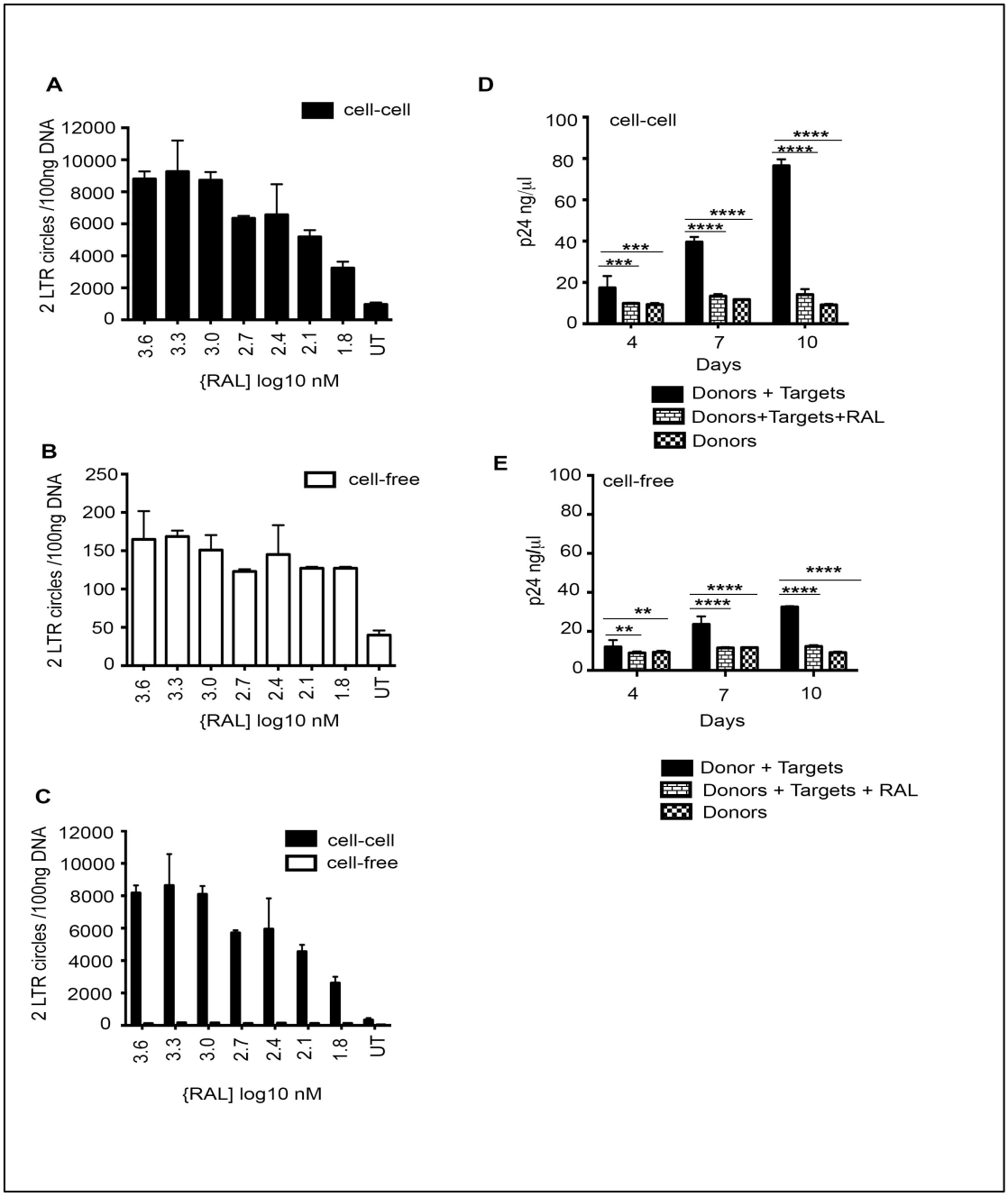


**Figure 4-6**: Integrase Inhibitors effectively inhibit cell-to-cell and cell-free spread of HIV-1 **(A)** More 2 LTR circles are generated in co-cultures of donor cells and target cells in the presence of a serial dilution of Raltegravir (RAL) in comparison to **(B)** target cells infected with cell-free virus in the presence of a serial dilution of RAL. 2 LTR circles were detected by qPCR. A representative experiment is shown, data represent the mean of triplicates and error bars are the SD of the mean. Graphs **(A)** and **(B)** are combined on one figure in **(C)**, directly comparing cell-cell and cell-free infection. Raltegravir effectively inhibits cell-to-cell **(D)** and cell-free spread **(E)** of HIV-1. For cell-to-cell infections, infected donor cells were co-cultured with uninfected donors cells in the presence of a $C_{max}$ of RAL, including untreated co-culture and donor cells only controls. p24 antigen in the culture supernatant was detected at 4, 7 and 10 days following co-culture. For cell-free infection donor and target cell population were separated by a 3µM transwell, which allows free diffusion of virus but prevents migration of cells. Untreated controls and donor cell only controls were included and p24 antigen detected in the target cell supernatant at 4, 7 and 10 days. One representative experiment is shown. The data represent the mean of duplicates and the error bars are the SD of the mean. A two-way ANOVA with Bonferroni post- test was applied for comparisons. **** p<0.0001, ***p<0.001 ,**p<0.01.

## 4.3 Discussion

In this chapter, the impact of PIs and to a lesser extent INIs on T cell-to-T cell spread of HIV-1 is investigated. The results presented demonstrate for the first time that PIs and INIs are potent inhibitors of HIV-1 cell-to-cell infection between T cells. In contrast we show that RTIs (AZT, TFV, 3TC and NVP) are less effective inhibitors of HIV-1 cell-to-cell spread in comparison to cell-free spread, with the exception of EFV, which like the PIs is equally potent against both modes of virus dissemination. Among the RTIs tested, AZT and TFV exhibit the greatest reduction in potency against cell-to-cell infection. In fact, for these drugs, doses exceeding the $C_{max}$ 20-fold fail to inhibit cell-to-cell infection below 50% levels in co-culture. PI and RTI drug-resistant viruses maintain their resistant phenotypes when spreading by a cell-to-cell mechanism, indicating that the observations are not a function of the viruses tested but rather provide a true reflection of the effects of PIs and RTIs on the modes of virus dissemination studied.

The difference in the potency of PIs and RTIs against cell-to-cell spread of HIV-1 is likely linked to the time window during which these drugs have to act, their biological functions and the multiplicity of infection that is a function of the mode of virus dissemination. PIs exert a wide range of biological effects, which likely account for their potency against HIV-1 dissemination. These drugs inhibit the cleavage of virus polyproteins into functional sub-units, an essential step required for the production of mature infectious virus (Wensing et al., 2010). Exposure to PIs therefore results in the generation of a pool of immature non-infectious virions and essentially reduces the multiplicity of infection regardless of whether these immature non-infectious virions spread from cell-to-cell across a virological synapse or by a cell-free diffusion. This may explain why PIs are equally effective against both cell-to-cell and cell-free modes of virus dissemination. This is supported by the

similar IC50 values for these drugs obtained with either mode of virus dissemination (Table 4.1).

One of the defining features of HIV-1 cell-to-cell spread is its high multiplicity of infection. This is the result of polarised virus budding at the VS, co-clustering of viral proteins and cellular receptors required for virus entry, and the close proximity between the infected donor cell and the target cell, all contributing to eliminate the need for prolonged virus diffusion (Jolly et al., 2004, Sourisseau et al., 2007, Hubner et al., 2009, Martin and Sattentau, 2009, Jolly et al., 2011, Duncan et.al, 2013). RTIs exert their inhibitory effects by blocking reverse transcription, a post-entry step in the replication cycle of the virus. When HIV-1 cell-to-cell infection is considered in the presence of RTIs, these drugs are faced with inhibiting a larger pool on incoming infectious virus particles. This may lead to RTIs becoming more easily saturated and tipping the scale in favour of the virus. As a result, infection of the target cell is more likely to occur, even in the presence of the RTI drug. This may explain why RTIs are less effective inhibitors of cell-to-cell spread compared to cell-free spread of the virus, which has a lower multiplicity of infection. In support of this proposition, when the multiplicity of infection in the assay was reduced by decreasing the number of infected donor cells in RTI treated co-cultures 10-fold, TFV regained potency to some degree against cell-to-cell spread of HIV-1 (Figure 4.4F). The reverse was also true, with the highly efficient PIs losing some of their potency when the multiplicity of infection was increased by raising the number of infected donor cells in the co-cultures treated with LPV (Figure 4.2C). The results confirm reports by other groups on the impact of RTIs on cell-to-cell spread of HIV-1 and role of MOI as a factor affecting drug potency (Sigal et al., 2011, Duncan et.al, 2013). Sigal et al. found greater fold differences in the reduced potency of RTIs against cell-to-cell infection compared to cell-free infection than

this study. However in their assays they used indirect measures of viral infection to quantify cell-to-cell and cell-free infection (p24 antigen detection and Tat-driven luciferase expression), likely leading to an overestimation of the effect. In this study infection has been measured by direct quantification of HIV-1 *pol* DNA, a better reflection of true infection than indirect methods. A detailed comparison of the methods for studying the effects of antiretroviral drugs on the modes of HIV-1 infection is provided in Chapter 3 of this thesis. In addition to their effects on blocking virus maturation, PIs also affect effective virus entry as well as post-entry steps in the virus replication cycle, notably reverse transcription and integration (Rabi et al., 2013). The ability of PIs to target multiple steps in the replication cycle of the virus probably also contributes to their superior potency against cell-to-cell infection in comparison to RTIs, which only target a single step in the virus replication cycle.

To confirm the potency of PIs in blocking cell-to-cell spread, nuclear entry in the presence of PIs was indirectly assessed by quantifying 2 LTR circles in co-cultures treated with PIs. Following 24h of co-culture in the presence of PIs, virtually no 2 LTR circles were detected compared to the untreated co-cultures in which 2 LTR circles were readily detected (Figure 4.1C). 2 LTR circles are non-functional forms of intracellular HIV-1 DNA, however they can serve as surrogate markers for the nuclear import of viral DNA as well as indicators for the completion of reverse transcription (Kalpana, 2008). In the presence of PIs, the earlier steps of HIV-1 replication leading to the formation of proviral DNA are blocked hence effectively reducing the substrate needed for the formation of 2 LTR circles. The potency of PIs in blocking cell-to-cell spread of HIV-1 was unchanged when primary CD4+ T cells were used as donor cells in the co-culture assay (Figure 4.3C) indicating that the observations are not affected by or dependent on the cell types mediating cell-to-cell

spread of HIV-1. This also shows that T cell lines provide a reliable model for studying the effect of inhibitors on cell-to-cell infection between T cells.

As mentioned earlier it was interesting to observe that unlike the other members of the RTI class tested, EFV, like the PIs, was equally potent against both cell-to-cell and cell-free spread of HIV-1 (Figure 4.4E). EFV is a member of the NNRTI sub-class and exerts its inhibitory effect by binding to a hydrophobic pocket near the active site of RT, thus preventing the efficient movement of the enzyme in carrying out its function of reverse transcription (de Bethune, 2010). This drug has the pharmacologic property of being an effective inhibitor of HIV-1 replication at very low drug concentrations its *in vitro* IC90 ranging between 1.7-25nM (www.hiv-druginteractions.org, 2011a). This is well below the minimum plasma concentrations of the drug achieved *in vivo* (Cmin= 5.6μM). This suggests that EFV might be inherently less easily saturated when compared to other members of the RTI drug class since a very small dose of the drug is capable of exerting a very potent inhibitory effect against virus dissemination. It is worth noting that potency in blocking HIV-1 infection at relatively lower drug concentrations is a feature EFV shares with the highly effective PIs discussed above and with INIs as well (www.hiv-druginteractions.org, 2011b, www.hiv-druginteractions.org, 2011c). Furthermore compared to the first generation NNRTIs Nevirapine and Delaviridine, the greater potency of EFV has been attributed to its much greater binding affinity for HIV-1 RT (Kd EFV= 0.63+/- 0.34, Kd NVP= 1550 +/- 441), this may also explain the effectiveness of this drug against HIV-1 cell-to-cell infection (Geitmann *et al*., 2006, Sluis-Cremer and Tachedjian, 2008).

Permanyer et.al reported contrasting findings to our observations and those of Sigal *et al*., reporting that RTIs were equally effective against

both modes of virus infection (Permanyer *et al.*, 2012b). However in their study they adjusted the virus input such that both cell-to-cell and cell-free spread gave rise to a similar percentage of infected cells (GFP+ cells) in the untreated state. Normalising the input of virus in this manner removes the higher multiplicity of infection mediated by cell-to-cell spread as a factor affecting the potency of the drugs tested. This as a consequence does not provide a true comparison of cell-to-cell and cell-free modes of virus dissemination. Mechanistically the steps of viral infection are the same for both modes of infection i.e. virus budding, attachment and entry. The main difference however is that cell-to-cell infection compared to cell-free infection occurs over a shorter distance with virus budding and attachment occurring in the synaptic space where virus proteins and cellular receptors are concentrated. This allows rapid and efficient transfer of more virions (a high MOI) from the effector cell to the target cell. In the present study, the virus input was not adjusted to achieve the same level of infection for both cell-to-cell and cell-free mode of virus infection. Therefore the quantitative properties of cell-to-cell spread were fully considered in comparing the effect of antiretroviral drugs on the two modes of virus dissemination.

The effect of Raltegravir on cell-to-cell and cell-free spread of HIV-1 was also investigated to complete the panel of drug tested. The results show that this drug is effective against both modes of infection and suppresses the production of p24 antigen in cultures infected by either a cell-to-cell or a cell-free mechanism. The experimental approach, using quantification of p24 antigen in culture supernatants to measure infection limits determination of IC50s. This assay is more difficult to adapt for the drug titrations needed for the calculation of IC50s, compared to the qPCR-based assay applied for testing PIs and RTIs. It is therefore impossible to comment with certainty on the relative potency

of INIs against to cell-to-cell infection when compared to cell-free infection based on my results.

INIs specifically target the viral integrase enzyme and prevent the integration of the proviral DNA into the host cell genome (Hazuda *et al*., 2000). The qPCR assay used to assess the effects of PIs and RTIs on cell-to-cell spread specifically detects HIV-1 *pol* DNA, because INIs act downstream of reverse transcription, HIV-1 *pol* DNA will still be detected in co-cultures treated with INIs regardless of a block to integration. This assay is consequently not appropriate for assessing the effect of INIs. Measuring 2 LTR circles is an indirect way of assessing the effect of INIs because an accumulation of episomal DNA is expected when nuclear entry and integration are blocked (Butler *et al*., 2001, Hazuda *et al*., 2000). This was verified by performing cell-to-cell and cell-free infections in the presence of a serial dilution of Raltegravir. As expected, more 2 LTR circles were detected in drug treated cultures regardless of the mode of infection, in comparison to untreated cultures (Figure 4.6A and Figure 4.6B). Significantly more 2 LTR circles were generated for cell-to-cell infections compared to cell-free infection in the presence of Raltegravir, probably reflecting the greater efficiency and higher multiplicity of the former mode of infection (Figure 4.6C). This indirect measure could however not be used to plot dose-response curves to calculate IC50s because of its limited accuracy.

Alternatively, the ideal direct parameter for assessing the effect of INIs is measuring integrated DNA by qPCR assays such as *Alu*-PCR. Such an assay could provide a yes or no answer with regards to the potency of INIs against cell-to-cell spread of HIV-1 (which we had already obtained through the p24 ELISA based assay). Nevertheless due to the heterogenous nature of HIV-1 integration sites within the host genome, the available assays for measuring DNA integration lack the precision

required for generating reliable dose-response curves for INIs to estimate IC50s. A possible approach to overcome this challenge could be using a luciferase reporter infectious molecular clone of HIV-1 for infection assays and separating pre-labelled donor cell and target cell populations by FACS following infection. With this set-up, target cell infection could be determined by measuring luciferase activity and these data used to plot dose-response curves. Unfortunately the biosafety level three laboratories where this study was carried out did not have a FACS sorting facility within it, limiting our ability to use this approach. The results obtained with the p24 ELISA based assay nonetheless agree with the findings of Duncan *et al*. They found that Raltegravir was effective against Macrophage-to-T cell HIV-1 infection as well as cell-free infection, with a similar potency for both modes of infection (Duncan et.al, 2013). The effects of drugs on the different modes of virus dissemination so far appear to be mainly dependent on the multiplicity of infection and independent of the cell-type used or type of virus tested. This allows us to speculate that INIs, which like PIs and EFV potently inhibit HIV-1 infection at very low concentrations of the drugs (www.hiv-druginteractions.org, 2011c), are likely to be less easily saturated even with high virus multiplicity. As such they should be expected to exhibit similar potencies against both cell-to-cell and cell-free spread of HIV-1, but this remains to be formally tested.

Cell-to-cell transmission takes advantage of interactions between immune cells allowing for effective delivery of virus from an infected cell to a target cell. Such interactions are likely to occur predominantly in lymphoid tissues where there is an abundance of target T cells and in anatomical sanctuary sites where close physical contact between cells is more likely (Sewald *et al*., 2012, Murooka *et al*., 2012). These sites have also been shown to have low penetration of antiretroviral drugs (Fletcher *et al*., 2014). Under these conditions it is feasible to speculate

that the high multiplicity of cell-to-cell spread combined with its reduced susceptibility to some antiretroviral drugs could indeed contribute to viral replication during ART (Fletcher *et al*., 2014, Sigal *et al*., 2011, Sigal and Baltimore, 2012). Although the studies assessing viral replication in treated patients have provided conflicting results till date, these studies have mainly relied on measuring plasma viraemia to assess ongoing infection (Frenkel *et al*., 2003, Kieffer *et al*., 2004, Bailey *et al*., 2006, Dinoso *et al*., 2009, McMahon *et al*., 2010, Buzon *et al*., 2010, Vallejo *et al*., 2012, Yilmaz *et al*., 2010). With the development of new techniques enabling intravital imaging of cell-to-cell contacts (Sewald *et al*., 2012, Murooka *et al*., 2012) and in light of the data presented in this thesis, it would be interesting to revisit the question in future clinical studies, this time sampling sanctuary sites in treated patients. This could help provide a clearer answer for the role of cell-to-cell spread of HIV-1 in the context of antiretroviral therapy *in vivo*.

Here were have assessed the effect of single drugs on cell-to-cell spread of HIV-1. However for the treatment of HIV infection antiretroviral drugs are administered in combination and are effective in stopping disease progression. It is therefore interesting to expand this study by exploring the impact of ART combinations on both cell-to-cell and cell-free spread of HIV-1, a question that is addressed in the next chapter of this thesis.

# 5 Impact of combination ART on cell-to-cell spread of wild-type and drug-resistant HIV-1

## 5.1 Introduction

The results presented in the previous chapter show that commonly used antiretroviral agents (PIs, INIs and RTIs) have variable effects on their ability to inhibit cell-to-cell spread of HIV-1 (Titanji *et al*., 2013). While PIs are equally effective at inhibiting both cell-to-cell and cell-free spread of the virus (Titanji *et al*., 2013), RTIs especially those of the NRTIs class, have a significantly reduced efficiency against HIV-1 cell-to-cell spread in comparison to cell-free spread (Sigal *et al*., 2011, Duncan et.al, 2013, Titanji *et al*., 2013). The data also suggest that the reduced efficiency of RTIs is likely a consequence of the high multiplicity of infection, characteristic of cell-to-cell infection, confirming the findings of other groups (Duncan et.al, 2013, Sigal *et al*., 2011).

The significance of the variable effects of single agents (monotherapy) on cell-to-cell spread of HIV-1 *in vitro* may appear paradoxical when considered in a clinical context. This is because antiretroviral drugs are prescribed in combination and these effectively suppress viral replication in treated patients *in vivo*, improving their survival and clinical outcomes (Perelson *et al*., 1997, Gulick *et al*., 1997, Walensky *et al*., 2006, Hammer *et al*., 1997, Arts and Hazuda, 2012). The success of cART is mainly attributed to the fact that combining drugs directed at two or more distinct molecular targets inhibits viral replication more effectively. Also it allows for beneficial interactions (addition and synergism) between the single agents in the combination, increasing

the overall potency of the drugs (Arts and Hazuda, 2012). This enhanced potency raises the barrier for the selection of drug-resistant forms of the virus capable of causing treatment failure (Perelson *et al*., 1997, Gulick *et al*., 1997, Arts and Hazuda, 2012).

The concept of drug synergy is complex but generally refers to the combination of multiple drugs producing a much greater effect than the simple arithmetic summation of the effects of the individual agents in the combination (Chou, 2006). Synergy has been clearly demonstrated *in vitro* between the two sub-classes of RTIs (NRTIs and NNRTIs) (Feng *et al*., 2009, Kulkarni *et al*., 2014, King *et al*., 2002), within the NRTI class (King *et al*., 2002, Feng *et al*., 2009), within the NNRTI class (Kollmann *et al*., 2001), and between PIs and RTIs (King *et al*., 2002, Beale and Robinson, 2000, Drusano *et al*., 1998, Deminie *et al*., 1996). Among the approved antiretroviral drug combinations, RTI-based combinations consisting of 2NRTIs + 1NNRTI are the oldest and most extensively studied. Clinical trials that have directly compared RTI-based combinations to PI-based combinations have reported that these combinations are equally effective at suppressing virus replication (Daar *et al*., 2011, DHHS, 2014, WHO, 2013). However, regimens that contain PIs although associated with a higher pill burden, present a higher barrier to the selection of drug resistance mutations (Lathouwers *et al*., 2011, Soriano *et al*., 2011).

RTI and PI based combinations therefore constitute the mainstay of first-line and second-line therapies for HIV-1 infection in current treatment guidelines (DHHS, 2014, WHO, 2013). These combinations have been assessed for their ability to inhibit HIV-1 cell-free infection. Since most RTIs as single agents have reduced potencies against HIV-1 cell-to-cell infection, it is interesting to study the effect of RTIs and PIs in combination against this mode of infection. In this chapter, the

question is specifically addressed using the *in vitro* qPCR-based cell-to-cell assay and the median effect principle of Chou and Talalay (Chou and Talalay, 1984, Chou, 2006, Chou, 2010) for drug interaction analyses. In addition, the effect of drug resistance to PIs and RTIs on combination antiretroviral therapy, in the context of cell-to-cell spread of HIV-1 is also explored.

During the early years of developing cART, drug interaction studies played an important role in identifying synergistic and additive drug combinations, most likely to provide the greatest combined inhibitory effect on viral replication. These studies were informative in guiding early ART clinical trials and also allowed the identification of antagonistic non-beneficial combinations to avoid, notably the combination of the two NRTIs Zidovudine (AZT) and Stavudine (d4T) (Ho and Hitchcock, 1989, Havlir *et al*., 2000, King *et al*., 2002). In recent years antiretroviral agents have found new uses in the prevention of HIV-1 transmission through PreP and recent antiretroviral drug interaction studies have been directed at assessing the beneficial interactions between potential new drug candidates for use in PreP (Gantlett *et al*., 2007, Schader *et al*., 2011, Chaowanachan *et al*., 2013). This further highlights the value of drug interaction studies in the process of drug development. There are several methods for analysing interactions (synergy, addition or antagonism) between drugs used in combination. One of the most widely used methods is the median effect principle of Chou-Talalay (Chou, 2010, Chou and Talalay, 1984). This method has been used and validated in several studies of antiretroviral drug combinations (Feng *et al*., 2009, Kulkarni *et al*., 2014, Beale and Robinson, 2000, Kollmann *et al*., 2001, King *et al*., 2002, Drusano *et al*., 1998, Deminie *et al*., 1996). We have adapted this method to assess antiretroviral drug combinations for synergy, addition,

or antagonism, in the context of cell-to-cell HIV-1 infection and antiretroviral drug resistance in this thesis.

The median effect principle is a mathematical modelling system derived from the Michaelis-Menten, Hill, Henderson-Hassellbach and Scatchard equations in biophysics and biochemistry with basic mass-action law considerations (Chou, 2010, Chou and Talalay, 1984). A full description of the mathematical derivations is beyond the scope of this thesis but I will briefly summarise its application in assessing the interactions between drugs in a combination. Applying the median effect principle to the study of drug combinations, Chou and Talalay introduced the combination index, which provides a numerical value to assess the combined effect of the drugs under study with regards to additive, synergistic or antagonistic interactions (Chou and Talalay, 1984, Chou, 2006). The computer software Compusyn® allows a simple determination of synergy using data derived from *in vitro* assays. The median effect principle is based on the assumption that two or more drugs alone or in combination will result in a sigmoidal shaped dose-response curve. Based on the slope of the curve (m), the 50% inhibitory concentration (Dm) can be estimated by transforming the dose-effect data to a logarithmic scale. A linear regression of the log-transformed data is then fitted to an equation in which the dose inhibitory effect of each drug (fa), the slopes of the curves (m) and the IC50s are incorporated, to calculate the combination index for each dose inhibitory effect (fa). The derived CI for two drugs is calculated using the following formula:

$$CI = [(D)1/(D1\text{-}fa)1] + [(D)2/(D1\text{-}fa)2] + [\alpha(D)1\,(D)2/\,(D1\text{-}fa)1\,(D1\text{-}fa)2]$$

Where (D)1 and (D)2 are the doses of the drugs in a fixed ratio while (D1-fa)1 and (D1-fa)2 are the doses of the individual drugs resulting in

the effect 1-fa.and$\alpha$=1 for mutually non-exclusive drugs (drugs with different mechanisms of action).

The combination indices obtained can then be interpreted to determine if the interaction between the drugs under study is synergistic, additive or antagonistic. Table 5-1 below summarises the interpretation of combination index values. To simplify the presentation of the results, the following cut-offs for CI values will be applied:
CI<0.9 =synergy, CI 0.9-1.2= addition, CI > 1.2= antagonism.

**Table 5-1**: Combination indices and their interpretation based on those described by Chou-Talalay (Chou and Talalay, 1984).

| Range of combination index | Description | Graded symbols |
|---|---|---|
| **<0.1** | Very strong synergism | +++++ |
| **0.1-0.3** | Strong synergism | ++++ |
| **0.3-0.7** | Synergism | +++ |
| **0.7-0.85** | Moderate synergism | ++ |
| **0.85-0.9** | Slight synergism | + |
| **0.9-1.2** | Additive effect | +/- |
| **1.2-1.45** | Moderate antagonism | -- |
| **1.45-3.3** | Antagonism | --- |
| **3.3-10** | Strong antagonism | ---- |
| **>10** | Very strong antagonism | ----- |
| | | |

In this chapter a panel of PI and RTI-based combinations commonly used in current clinical practice, have been tested for their ability to inhibit cell-to-cell spread of HIV-1. The results show that members of the RTI class, which are ineffective as single agents against this mode of spread, regain their potency upon combination with other members of this drug class or when combined with the highly effective PIs. Using the combination index as a measure of synergy, consistently stronger synergies are observed when RTI or PI-based combinations are tested on HIV-1 cell-free infection in comparison to cell-to-cell infection. Furthermore the results suggest that in the presence of a drug-resistant

mutation a virus spreading by a cell-to-cell mechanism can reduce the overall synergistic effects of a combination therapy.

### 5.1.1 Specific Objectives

- To assess the impact of RTI-based drug combinations on cell-to-cell spread of HIV-1.
- To assess the impact of PI-based drug combinations on cell-to-cell spread of HIV-1.
- To determine the impact of the mode of HIV-1 virus spread (cell-to-cell vs. cell-free) on the interaction between antiretroviral agents in combination.
- To determine the impact of antiretroviral drug resistance on combination antiretroviral therapy, in the context of cell-to-cell spread of HIV-1.

## 5.2 Results

### 5.2.1 RTI-based combination therapies effectively block cell-to-cell spread of HIV-1

The data presented in chapter 4 show that the RTIs; Tenofovir (TFV), Zidovudine (AZT), Lamivudine (3TC) and Nevirapine (NVP) have significantly reduced potencies against HIV-1 cell-to-cell infection when compared to cell-free infection, with TFV and AZT being the least effective drugs. These drugs are not used as monotherapy for the treatment of HIV-1 infected patients due to the high risk of selecting for drug-resistant variants (Larder *et al*., 1989, Rooke *et al*., 1989). When administered in combination, RTIs are effective in the treatment of HIV-1 (Gulick *et al*., 1997, Hammer *et al*., 1997, Perelson *et al*., 1997, Walensky *et al*., 2006). This appears paradoxical given observations on their inability to suppress cell-to-cell HIV-1 infection when administered as single agents. It was therefore interesting to assess the effect of RTIs in combination against HIV-1 cell-to-cell infection.

The median effect analysis based on the median effect principle of Chou and Talalay was applied to calculate the combination index (CI) and determine whether the interactions between the drugs in the combinations tested were; additive, synergistic or antagonistic and to compare this between HIV-1 cell-to-cell and cell-free infection. The qPCR-based cell-to-cell and cell-free infection assays validated and used in the two preceding chapters were applied for the drug combination studies. Three dual RTI combinations: AZT+TFV, TFV+EFV and AZT+NVP were tested. The drugs were combined in ratios based on their individual IC50s, determined in a cell-free infection assay as previously described in Chapters 3 and 4. Briefly, virus supernatant collected from HIV-1 infected donor cells was used to infect target cells in the presence of a serial dilution of the drug being considered. Following a 24h incubation, target cell infection was quantified by

qPCR detection of HIV-1 *pol* DNA as previously described and expressed as a fold increase relative to the Albumin housekeeping gene. These data were used to plot dose-response curves and calculate the IC50s in GraphPad Prism® software. Once the IC50s of the individual drugs had been determined, the drugs where then combined in a fixed dose ratio based on their individual IC50s. Cell-to-cell and cell-free infections were assessed in the presence of a serial dilution of this fixed dose combination by qPCR as previously described (see methods section). The inhibitory effect (fa) of each drug alone and as part of a combination was calculated and expressed as a fraction, representing inhibition of infection in the presence of the drug relative to the "no drug" control. These fa values were inputted in Compusyn® to determine CIs for each drug combination tested. Each fixed-dose combination experiment was repeated twice the mean CI values for 50%, 75%, 90% and 95% inhibition levels obtained from two independent experiments and the standard error of the mean (SEM) are presented. The steps in the drug combination studies are summarised in the schematic below:

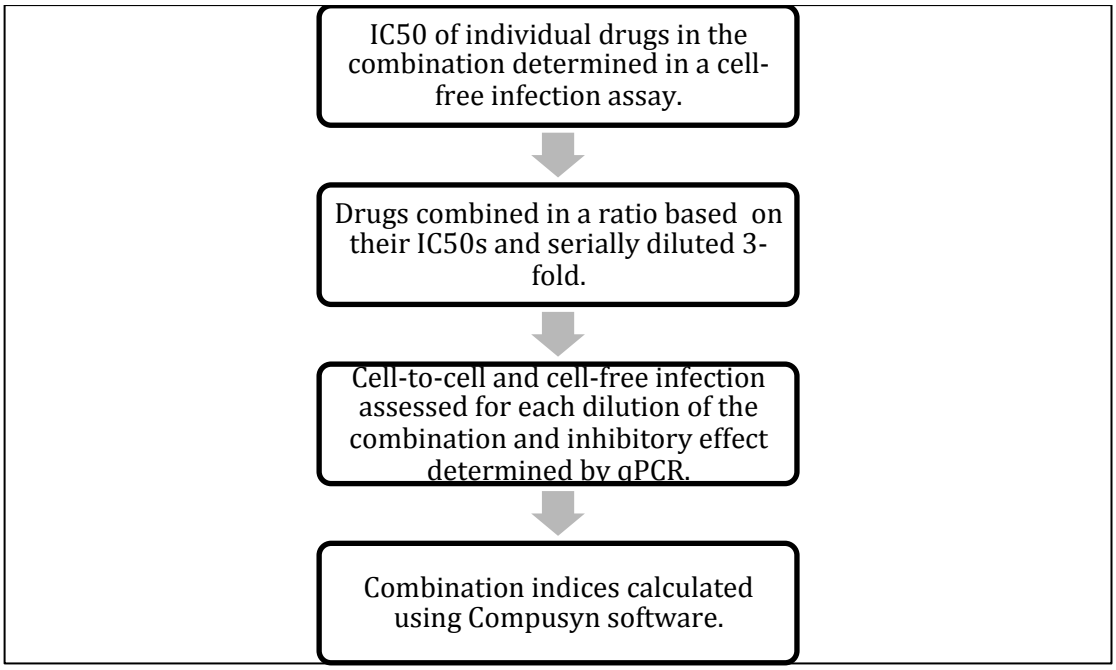


**Figure 5-1**: Steps in drug combination studies

Combining AZT and TFV, potently inhibited both cell-to-cell and cell-free HIV-1 infections (Figure 5.2A), however cell-to-cell spread still maintained an advantage over cell-free spread. This was striking because individually, AZT and TFV are >10-20 fold less effective against HIV-1 cell-to-cell infection compared to cell-free infection. The potency of this combination against both modes of HIV-1 infection was reflected in the combination index values, which were in the range of additive to synergistic effects against cell-to-cell infection and moderately synergistic effects against cell-free infection (Table 5.2).

AZT and TFV were then combined respectively with non-nucleoside reverse transcriptase inhibitors NVP and EFV. NVP as a single agent has a 4-fold reduced potency against cell-to-cell spread of HIV-1. Combining AZT+NVP potently inhibited both HIV-1 cell-to-cell and cell-free infections although cell-to-cell infection maintained a replicative advantage (Figure 5.2B). This was reflected by combination index values indicating an additive effect on cell-to-cell infection and a synergistic effect on cell-free infection (Table 5.3). EFV as a single agent was the only RTI that showed the same potency against both cell-free and cell-to-cell modes of viral infection. When combined with the less effective TFV, the combination effectively inhibited both cell-to-cell and cell-free infection (Figure 5.1C) with combined moderate to strongly synergistic effects against both modes of viral infection (Table 5.4).

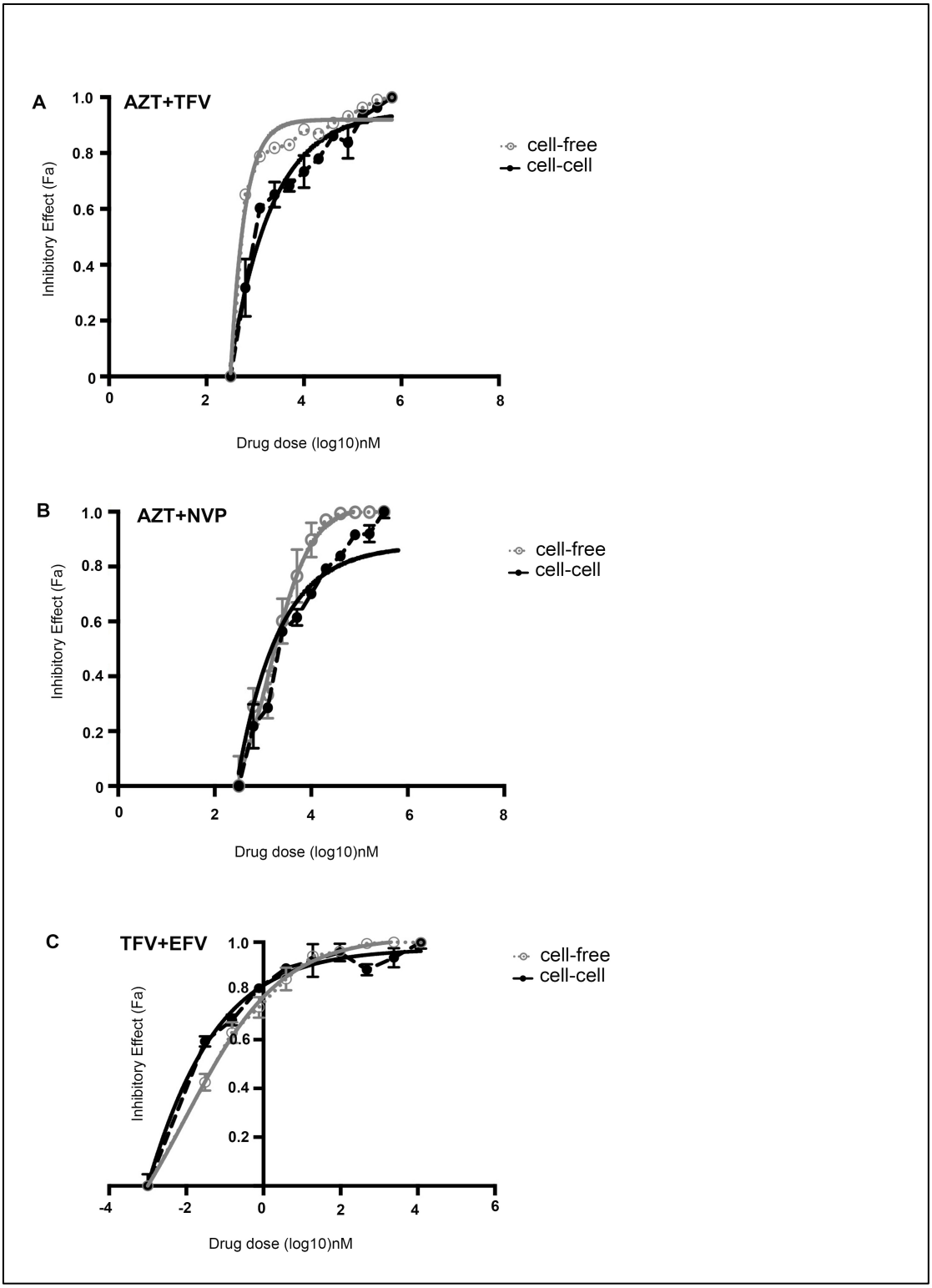


**Figure 5-2**: RTI-based combinations effectively inhibit both cell-to-cell and cell-free spread of HIV-1. Cell-to-cell and cell-free infections were assessed in the presence of a serial dilution of a dual RTI combination **(A)** AZT+TFV, (B) AZT+NVP and **(C)** TFV+EFV. The drugs were combined in a ratio based on their IC50s determined from a cell-free infection assay. Infection was measured by the number of HIV-1 *pol* DNA transcripts generated at each dilution of the combination and expressed as a fraction of the no drug control. The data were used to plot the dose-response curves, which are displayed on the graphs above; one representative from 2 independent experiments is shown. The error bars represent the standard deviation of the mean. The bold lines represent the non-linear regression curve-fit and dotted lines represent actual data points. The curves were fitted using GraphPad Prism curve fitting software.

**Table 5-2**: Combination indices for the combination of Zidovudine (AZT)+Tenofovir (TFV). The mean and standard error (SEM) (in parenthesis) obtained from two independent experiments are shown .

| Mode of Infection | Combination (ratio=1:1) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **Cell-Cell** | AZT+TFV | 0.9(0.1) | 0.85(0.1) | 0.82(0.05) | 0.74(0.12) | Additive/ synergistic |
| **Cell-Free** | AZT+TFV | 0.41(0.06) | 0.39(0.03) | 0.42(0.1) | 0.45(0.04) | Synergistic |

**Table 5-3**: Combination indices for the combination of Zidovudine (AZT) + Nevirapine (NVP). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Mode of Infection | Combination (ratio= 40:1) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **Cell-Cell** | AZT+NVP | 0.95(0.05) | 1.05(0.05) | 1.1(0.13) | 1.1(0.1) | Additive |
| **Cell-Free** | AZT+NVP | 0.97(0.06) | 0.89(0.04) | 0.79(0.06) | 0.76(0.1) | Additive/ Synergistic |

**Table 5-4**: Combination indices for the combination of Tenofovir (TFV) + Efavirenz (EFV). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Mode of Infection | Combination (ratio=1000:1) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **Cell-Cell** | TFV+EFV | 0.59(0.05) | 0.46(0.07) | 0.36(0.05) | 0.35(0.02) | Synergistic |
| **Cell-Free** | TFV+EFV | 0.1(0.02) | 0.13(0.01) | 0.22(0.05) | 0.39(0.1) | Synergistic |

### 5.2.2 PI-based combinations effectively block cell-to-cell spread of HIV-1

The results of chapter 4 showed that in contrast to RTIs, PIs are equally potent against HIV-1 cell-to-cell and cell-free infection. In the present study it was therefore interesting to explore the effects of combining PIs with less effective RTIs against cell-free and cell-to-cell modes of virus infection. Also, PIs are important components of second-line therapies, which are commonly recommended when first-line RTI-based combinations fail. The PI LPV was tested in combination with the NRTI TFV and in combination with the NNRTI NVP. Both combinations (LPV+TFV and LPV+NVP) potently inhibited HIV-1 cell-to-cell and cell-free infection (Figure 5.3). The combination of LPV+TFV was strongly synergistic for both modes of virus infection (Table 5.5) while the combination of LPV+NVP showed additive to synergistic effects for cell-to-cell infection and moderate synergy for cell-free infection (Table 5.6).

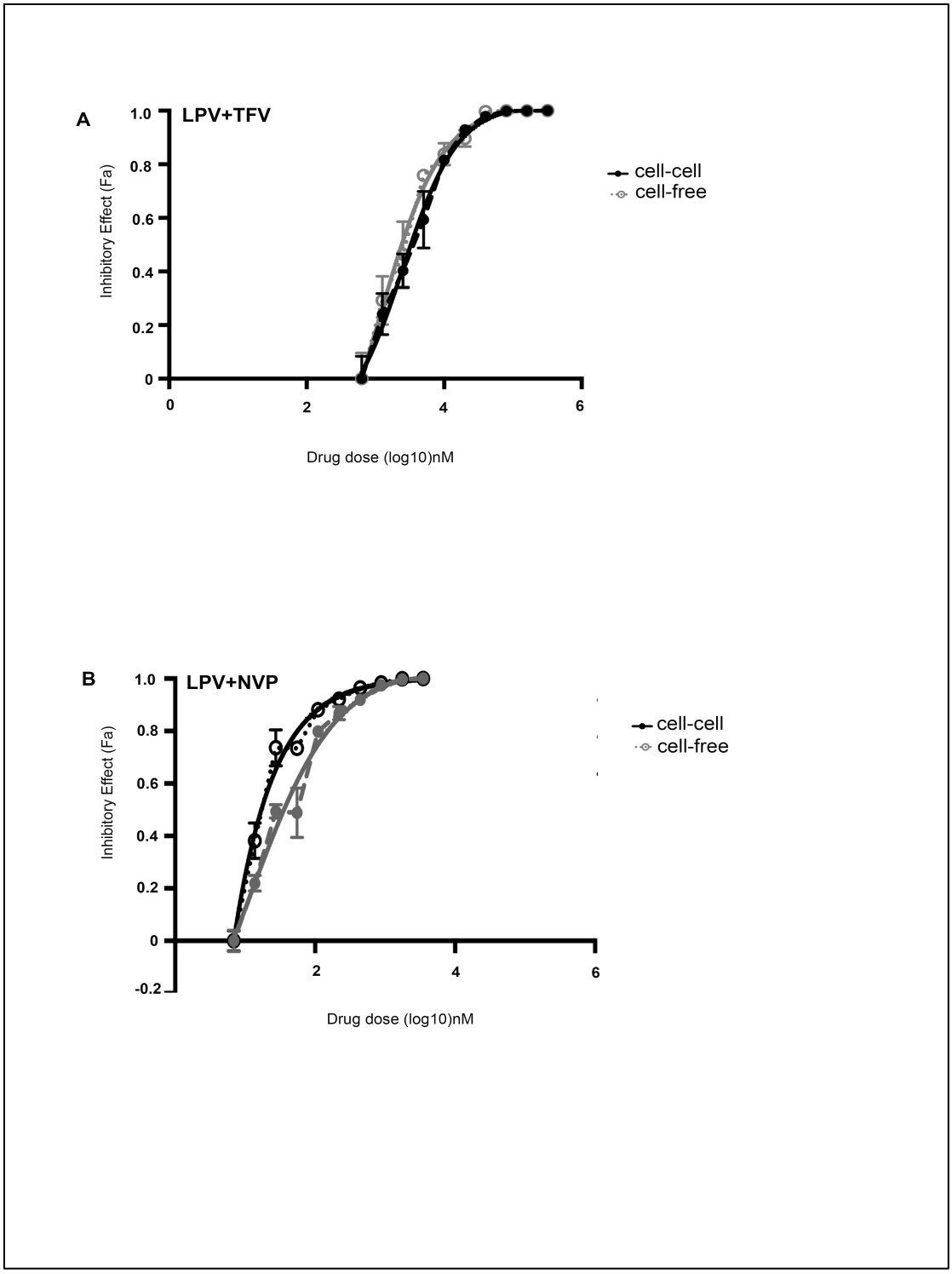


**Figure 5-3**: PI-based combinations effectively inhibit both cell-to-cell and cell-free spread of HIV-1. Cell-to-cell and cell-free infections were assessed in the presence of a serial dilution of a dual combination of a PI+RTI combination **(A)** LPV+TFV and **(B)** LPV+NVP. The drugs were combined in a ratio based on their IC50s determined from a cell-free infection assay. Infection was measured by the number of HIV-1 *pol* DNA transcripts generated at each dilution of the combination and expressed as a fraction of the no drug control. The data were used to plot the dose-response curves, which are displayed on the graphs above; a representative from 2 independent experiments is shown. The error bars represent the standard deviation of the mean. The bold lines represent the non-linear regression curve-fit and dotted lines represent actual data points. The curves were fitted using GraphPad Prism curve fitting software.

**Table 5-5**: Combination indices for the combination of Lopinavir (LPV) + Tenofovir (TFV). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Mode of Infection | Combination (ratio= 1:1000) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **Cell-Cell** | LPV+TFV | 0.03(0.06) | 0.1(0.01) | 0.12(0.04) | 0.36(0.2) | Synergistic |
| **Cell-Free** | LPV+TFV | 0.02(0.01) | 0.06(0.01) | 0.15(0.02) | 0.4(0.13) | Synergistic |

**Table 5-6**: Combination indices for the combination of Lopinavir (LPV) + Nevirapine (NVP). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Mode of Infection | Combination (ratio=1:25) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **Cell-Cell** | LPV+NVP | 1.1(0.07) | 0.92(0.2) | 0.74(0.24) | 0.71(0.26) | Additive/ Synergistic |
| **Cell-Free** | LPV+NVP | 0.86(0.03) | 0.72(0.03) | 0.6(0.04) | 0.46(0.04) | Additive/ Synergistic |

### 5.2.3Cell-to-cell spread of drug-resistant HIV-1 may compromise combination ART

The results presented so far show that PI and RTI-based combinations effectively inhibit HIV-1 cell-to-cell and cell-free infection, albeit with relatively weaker synergistic interactions against cell-to-cell infection compared to cell-free infection. The next question posed was whether drug-resistant viruses spreading by a cell-to-cell mechanism would affect the potency of combination therapies in fully suppressing HIV-1 replication. To answer this question, cell-to-cell spread of a PI drug-resistant virus HIV-$1_{DM}$ was tested in the presence of PI-based combinations and cell-to-cell spread of RTI mutants HIV-$1_{K103N}$ and HIV-$1_{M184V}$ tested in the presence of RTI-based combinations. This was compared to cell-to-cell spread of HIV-$1_{WT}$ in the presence of the same drug combinations. The combination indices for the different combinations tested with these viruses were determined using Compusyn® as previously described for HIV-$1_{DM}$ and HIV-$1_{K103N}$. The co-culture assays were set-up in the presence of a serial dilution of the fixed-dose combination of the drugs being tested as described earlier.

Cell-to-cell spread of HIV-$1_{DM}$ was assessed in the presence of LPV+TFV. The HIV-$1_{DM}$ mutant has an A431V mutation in Gag and a V82A mutation in Protease, making it 14-fold less susceptible to inhibition by LPV when compared to wild-type HIV-1 (HIV-$1_{WT}$). The results showed a replicative advantage of HIV-$1_{DM}$ over HIV-$1_{WT}$ in the presence of LPV+TFV (Figure 5.4A). This was reflected by an increase in CI values, shifting the combined effect of the drugs from strongly synergistic (observed with HIV-$1_{WT}$ (CI<0.3) to a moderately synergistic range (Table 5.7). Cell-to-cell spread of HIV-$1_{DM}$ was then tested in the presence of a triple combination of drugs, by adding EFV to the dual combination (LPV+TFV+EFV). In the presence of this triple combination, cell-to-cell spread of HIV-$1_{DM}$ was effectively suppressed to the same

extent as cell-to-cell spread of HIV-$1_{WT}$ (Figure 5.4C and D). Unfortunately, it was not possible to determine CIs for the triple combination because the dose-effect curves did not meet the criteria required for reliable CI estimates in Compusyn®. Due to nearly complete suppression of viral replication by the triple combination, the curves generated had R-squared values (goodness of fit, following non-linear regression) that were lower than the 0.9 which is required for accurate determination of combination indices.

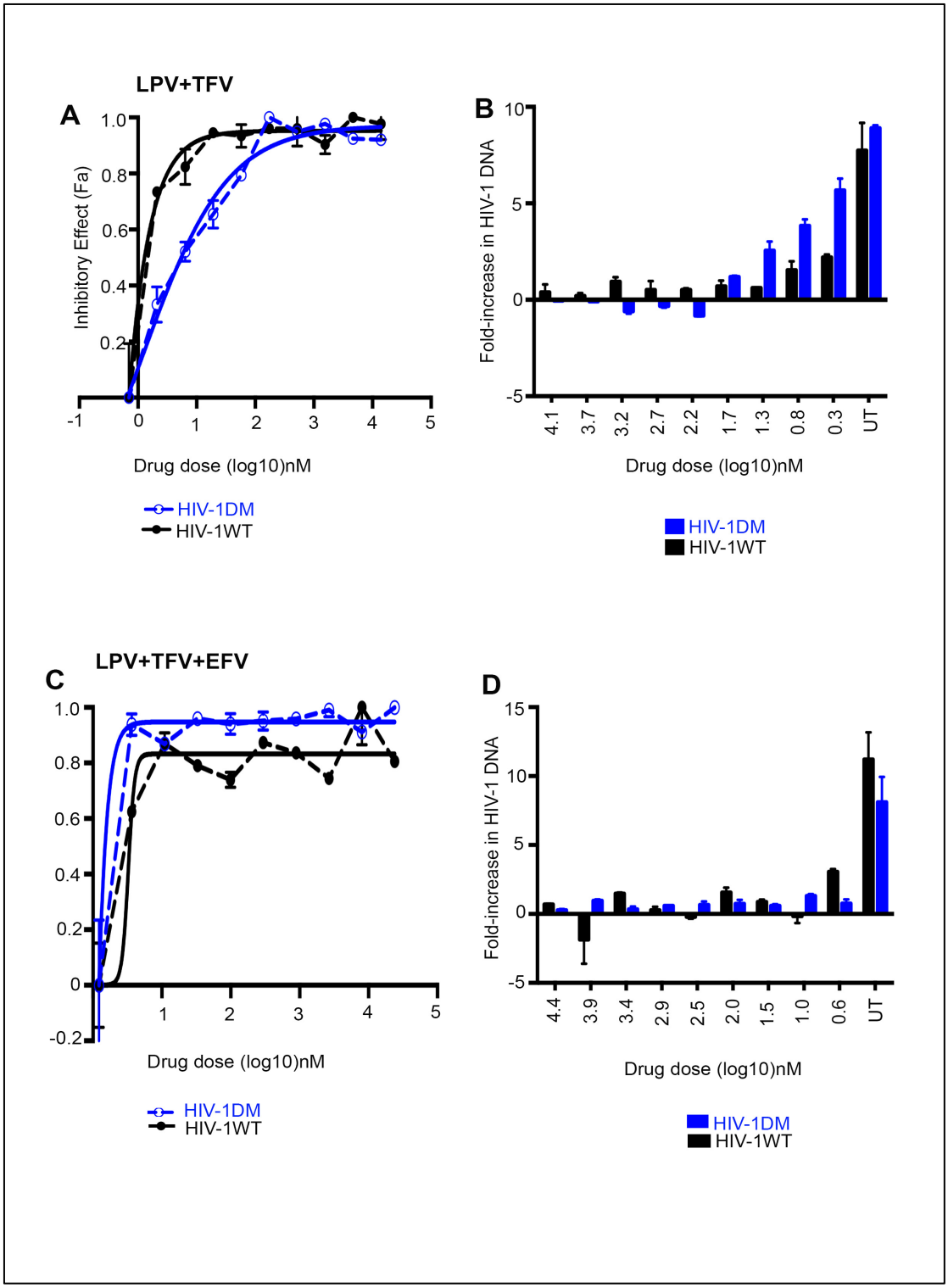


**Figure 5-4**: Cell-to-cell spread of a PI resistance mutant compared to wild-type virus in the presence of combination antiretroviral therapy **(A)** and **(B)** cell-to-cell spread of HIV-$1_{DM}$ was assessed in the presence of a serial dilution of a fixed dose combination of LPV+TFV and compared to cell-to-cell spread of HIV-$1_{WT}$ with the same combination. The error bars represent the standard deviation of the mean. HIV-$1_{DM}$ has a replicative advantage over HIV-$1_{WT}$ in the presence of this dual combination. In **(C)** and **(D)** a triple combination of LPV+ 3TC+ TFV potently inhibits both cell-to-cell spread of HIV-$1_{DM}$ and HIV-$1_{WT}$. The error bars represent the standard deviation and a representative of two independent experiments is shown. The bold lines represent the non-linear regression curve-fit and dotted lines represent actual data points. The curves were fitted using GraphPad Prism curve fitting software. UT=untreated control.

**Table 5-7:** Combination indices for the combination of Lopinavir (LPV) + Tenofovir (TFV) tested against cell-to-cell spread of a PI resistant mutant compared to wild-type virus. The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Virus | Combination (ratio=1:1000) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **HIV-$1_{WT}$** | LPV+TFV | 0.03(0.06) | 0.1(0.01) | 0.12(0.04) | 0.36(0.2) | Synergistic |
| **HIV-$1_{DM}$** | LPV+TFV | 0.14(0.01) | 0.21(0.01) | 0.29(0.04) | 0.49(0.1) | Synergistic |

The NNRTI drug-resistant mutant HIV-$1_{K103N}$ was also tested in a similar fashion. Cell-to-cell spread of this virus was assessed in the presence of TFV+EFV. The K103N mutation in RT renders the virus >650-fold more resistant to inhibition by EFV in comparison to HIV-$1_{WT}$. This combination, which efficiently suppressed cell-to-cell spread of HIV-$1_{WT}$, was less effective at suppressing cell-to-cell spread of HIV-$1_{K103N}$ (Figure 5.5A and B). The reduced efficiency of the combination on the drug-resistant virus was again reflected by an increase in the combination index, shifting the effect from a moderately synergistic range for HIV-$1_{WT}$ to a mildly synergistic to additive range for the HIV-$1_{K103N}$ (Table 5.8).

A third drug; 3TC was then added to TFV+EFV and cell-to-cell spread of HIV-$1_{K103N}$ and HIV-$1_{WT}$ tested in the presence of this triple combination (TFV+EFV+3TC). The triple combination effectively suppressed cell-to-cell spread of both the wild type and mutant virus (Figure 5.5 C and D), although HIV-$1_{K103N}$ appeared to maintain a small replicative advantage over the wild-type virus. The efficiency of the triple combination was reflected by a combined strongly synergistic interaction between the drugs in the combination against cell-to-cell spread of both HIV-$1_{WT}$ and HIV-$1_{K103N}$ (Table 5.9).

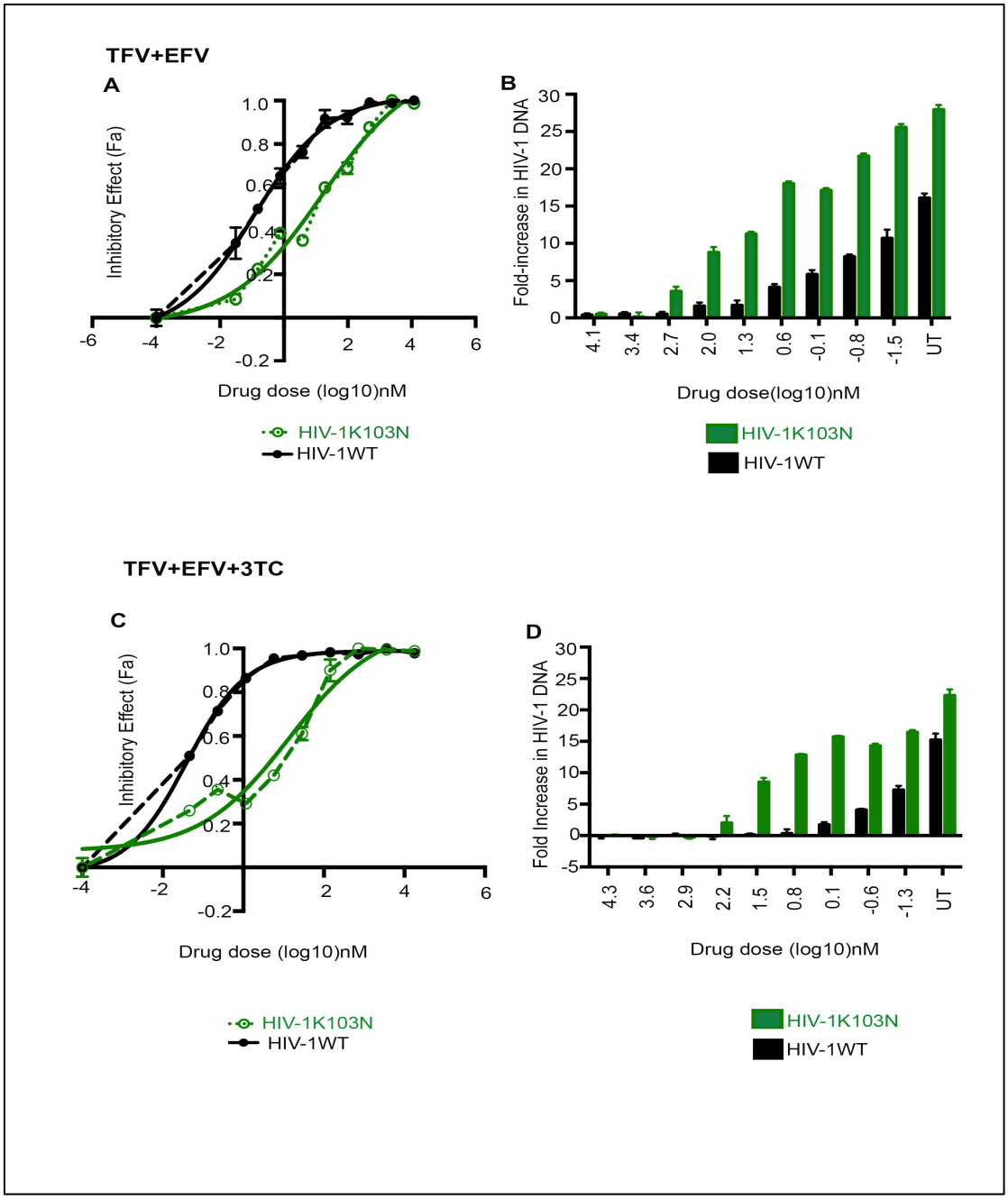


**Figure 5-5**: Cell-to-cell spread of a NNRTI resistance mutant compared to wild-type virus in the presence of combination antiretroviral therapy- **(A)** and **(B)** cell-to-cell spread of HIV-$1_{K103N}$ was assessed in the presence of a serial dilution of a fixed dose combination of EFV+TFV and compared to cell-to-cell spread of HIV-$1_{WT}$ with the same combination. The error bars represent the standard deviation of the mean. HIV-$1_{K103N}$ had a replicative advantage over HIV-$1_{WT}$ in the presence of this dual combination. In **(C)** and **(D)** a triple combination of EFV+ 3TC+ TFV potently inhibits both cell-to-cell spread of HIV-$1_{K103N}$ and HIV-$1_{WT}$., though HIV-$1_{K103N}$ retains a small replicative advantage over HIV-$1_{WT}$. The error bars represent the standard deviation of the mean and a representative from two independent experiments is shown. The bold lines represent the non-linear regression curve-fit and dotted lines represent actual data points. The curves were fitted using GraphPad Prism curve fitting software. UT=Untreated controls.

**Table 5-8**: Combination indices for the combination of Tenofovir (TFV) + Efavirenz (EFV). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Virus | Combination (ratio=1000:1) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **HIV-$1_{WT}$** | TFV+EFV | 0.69(0.05) | 0.46(0.07) | 0.36(0.05) | 0.35(0.02) | Synergistic |
| **HIV-$1_{K103N}$** | TFV+EFV | 1.1(0.02) | 0.93(0.01) | 0.82(0.05) | 0.79(0.1) | Synergistic/ Additive |

**Table 5-9**: Combination indices for the combination of Tenofovir (TFV) + Efavirenz (EFV) + Lamivudine (3TC). The mean and standard error of the mean (in parenthesis) obtained from two independent experiments are shown.

| Virus | Combination (1000:10:1) | Combination index (CI) | | | | Effect |
|---|---|---|---|---|---|---|
| | | 50 | 75 | 90 | 95 | |
| **HIV-$1_{WT}$** | TFV+3TC+EFV | 0.08(0.03) | 0.09(0.01) | 0.12(0.04) | 0.18(0.01) | Synergistic |
| **HIV-$1_{K103N}$** | TFV+3TC+EFV | 0.35(0.02) | 0.39(0.01) | 0.44(0.02) | 0.48(0.13) | Synergistic |

Finally, cell-to-cell spread of the NRTI drug-resistant virus HIV-$1_{M184V}$ was tested. The M184V mutation in RT mutation was particularly interesting for this study because while it confers a 120-fold resistance to inhibition by 3TC compared to wild-type virus in the drug susceptibility assay (see Chapter 3), this mutation is also well described for increasing the susceptibility of the virus to other NRTIs, notably TFV and AZT (Naeger *et al*., 2001, White *et al*., 2002, Diallo *et al*., 2003, Wolf *et al*., 2003). This feature was confirmed in the cell-free drug susceptibility assay (see Chapter 3). It was interesting to contemplate whether the increased susceptibility of HIV-$1_{M184V}$ to AZT would remain evident when infection was mediated by cell-to-cell mechanism of dissemination. This was even more interesting considering that cell-to-cell spread HIV-$1_{WT}$ is highly impervious to inhibition by AZT. To answer this question, cell-to-cell spread of the mutant virus was directly compared to that of the wild-type virus in the presence of AZT. While AZT was unable to fully suppress cell-to-cell spread of HIV-$1_{WT}$, the drug effectively inhibited cell-to-cell spread of HIV-$1_{M184V}$, (Figure 5.6A). Cell-to-cell spread of this resistant mutant was then tested with drug combinations. With the combination of 3TC+AZT, both HIV-$1_{WT}$ and HIV-$1_{M184V}$ cell-to-cell infections were effectively inhibited although HIV-$1_{M184V}$ showed a replication advantage albeit a modest one in the presence of this combination in comparison to HIV-$1_{WT}$ (Figure 5.6B). The triple RTI combination of 3TC+AZT+EFV potently blocked cell-to-cell spread of both viruses.

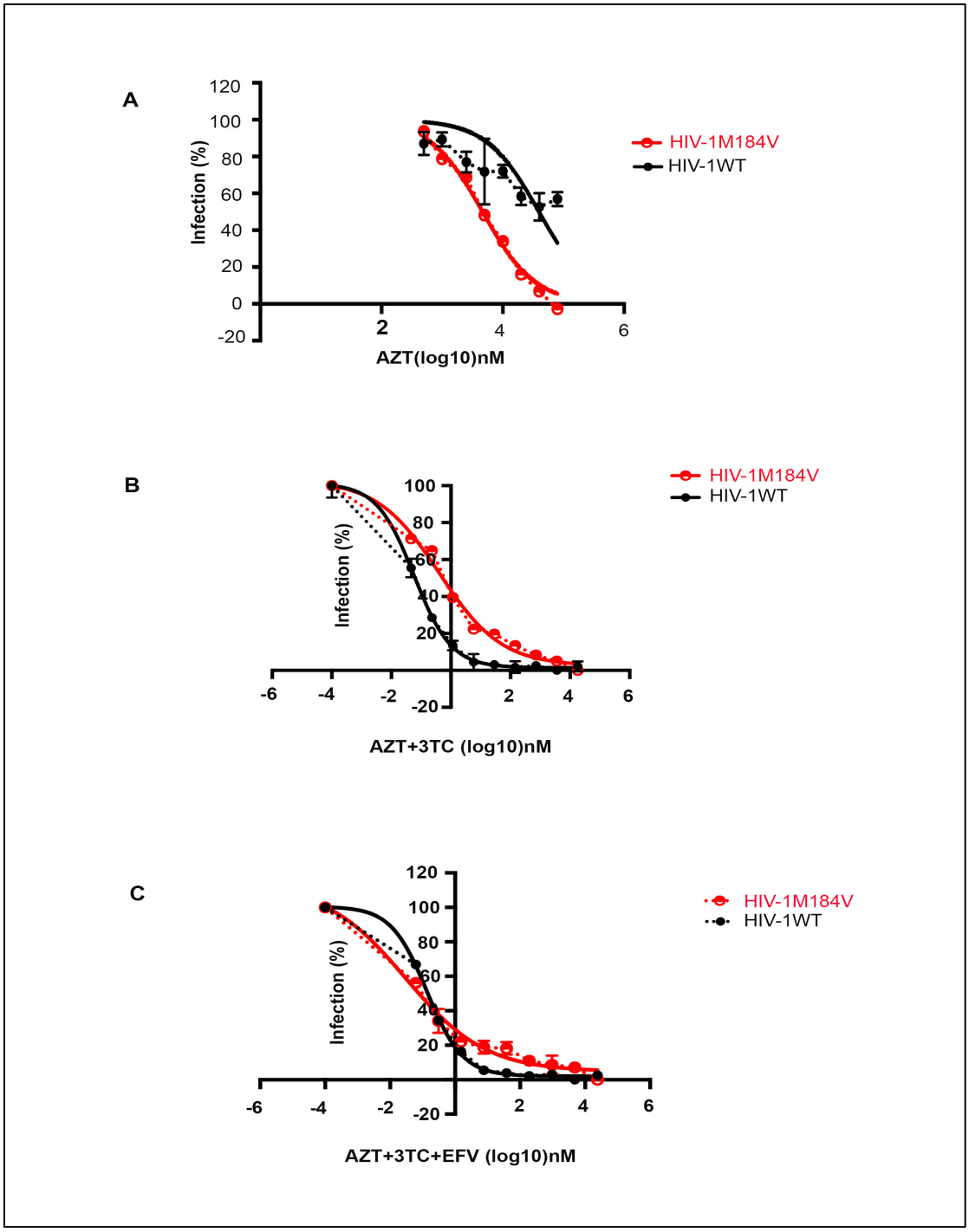


**Figure 5-6**: Cell-to-cell spread of NRTI mutant HIV-$1_{M184V}$ compared to cell-to-cell spread of HIV-$1_{WT}$ in the presence of RTI mono **(A)**, dual **(B)**, and triple therapy**(C)**. **(A)** AZT effectively inhibits cell-to-cell spread of HIV-$1_{M184V}$, though it is ineffective against cell-to-cell spread of HIV-$1_{WT}$. **(B)** In the presence of a serial dilution of a fixed dose combination of AZT+3TC cell-to-cell spread of HIV-$1_{WT}$ and HIV-$1_{M184V}$ are effectively blocked though HIV-$1_{M184V}$ has a replicative advantage over wild-type virus. **(C)** Triple combination of AZT+3TC+ EFV potently inhibits cell-to-cell spread of both HIV-$1_{WT}$ and HIV-$1_{M184V}$. The error bars represent the standard deviation of the mean and a representative from two independent experiments is shown. The bold lines represent the non-linear regression curve-fit and dotted lines represent actual data points. The curves were fitted using GraphPad Prism curve fitting software.

## 5.3 Discussion

The observations from this thesis and other studies on the variable potencies of commonly used antiretroviral drugs against HIV-1 cell-to-cell infection (Sigal *et al*., 2011, Duncan et.al, 2013, Titanji *et al*., 2013), have sparked an interesting debate on the true role of this mode of infection in viral pathogenesis and disease progression *in vivo*. We have shown that while PIs and INIs are equally effective at suppressing both cell-free and cell-to-cell modes of infection (Chapter 4), members of the widely used RTI class, which make up the backbone for most combination ART, have greatly reduced abilities to inhibit the latter mode of HIV-1 infection (Sigal *et al*., 2011, Duncan et.al, 2013, Titanji *et al*., 2013) This presents an interesting conundrum because, treating HIV-1 infected patients in the clinic with combinations of these seemingly "ineffective" drugs results in full suppression of virus replication and arrest of disease progression (Eron et al., 1995, Autran *et al*., 1997, Gulick *et al*., 1997, Lederman et al., 1998, Arts and Hazuda, 2012).

Some in the field have interpreted this as an indication that HIV-1 cell-to-cell infection may not be an important phenomenon *in vivo*, with cell-free infection which is susceptible to suppression by all currently approved RTIs, being likely predominant. In the present chapter this question is specifically addressed by testing RTI and PI-based combinations commonly prescribed in the clinic, on their ability to inhibit cell-to-cell spread of HIV-1 in comparison to cell-free spread. The role of drug resistance on the efficacy of cART in the context of cell-to-cell virus infection is also explored. The results show that cART potently inhibits both cell-to-cell and cell-free spread of HIV-1. This is most likely the result of the additive to synergistic interactions between antiretroviral drugs in combination being able to overcome the high multiplicity of infection that is a characteristic feature of cell-to-cell infection. Furthermore, consistently stronger combined synergistic or

additive effects are observed with the combinations tested when cell-free infection is compared to cell-to-cell infection. Finally, drug-resistant viruses are shown to have a replicative advantage over wild-type virus when spreading by a cell-to-cell mechanism, in the presence of "inadequate" combination therapies. While completing the experiments for my thesis, Agosto et al. published a similar study looking at the impact of NRTI-based combination therapies on cell-to-cell spread of HIV-1 (Agosto et al., 2014). Although they applied a different method to assess the inhibitory potential of drugs in combination on the different modes of HIV-1 infection, their results are similar to the findings presented here. The present study has however been expanded by testing more combinations including PIs and NNRTIs and also testing a panel of PI and RTI drug-resistant mutants, in order to provide a more complete picture.

TFV and AZT are the two members of the RTI drug class, which have consistently demonstrated significantly reduced potency against HIV-1 cell-to-cell infection *in vitro* when compared to cell-free infection (Agosto et al., 2014, Titanji et al., 2013, Duncan et.al, 2013, Sigal et al., 2011). The studies published so far have reported between 20 to 1000-fold decrease in susceptibility of this mode of virus infection to inhibition by TFV or AZT (Sigal et al., 2011, Duncan et.al, 2013, Titanji et al., 2013, Agosto et al., 2014). The effect of combining these two agents against HIV-1 cell-to-cell infection was investigated. Remarkably, when combined, both drugs were capable of potently inhibiting HIV-1 cell-to-cell infection almost as effectively as cell-free infection (Figure 5.2A). High MOI of infection is a key feature of cell-to-cell spread of HIV-1, which has been clearly demonstrated *in vitro* (Del Portillo et al., 2011, Russell et al., 2013, Zhong et al., 2013) and supported by some *in vivo* observations (Gratton et al., 2000, Jung et al., 2002). This attribute of cell-to-cell HIV-1 infection has been implicated as the primary driver of

ongoing cell-to-cell infection observed with some antiretroviral drugs *in vitro*, notably RTIs (Sigal et al., 2011, Duncan et.al, 2013, Titanji et al., 2013, Agosto et al., 2014). The synergistic interaction between TFV and AZT in combination, as determined by CI values (Table 5.2) explains the potency of this combination in blocking HIV-1 cell-to-cell spread. If we consider that cell-to-cell spread is 20 to 1000-fold less susceptible to TFV and AZT when these drugs are used as single agents (Duncan et.al, 2013, Titanji et al., 2013, Agosto et al., 2014, Sigal et al., 2011), an additive effect from combining both drugs will only double their effect and as such be likely insufficient to overcome cell-to-cell infection. Synergy however by definition produces an effect much greater than the simple arithmetic sum of the effect of the agents in combination and in this case, this combined effect is strong enough to overcome cell-to-cell spread of HIV-1. It is important to note nevertheless that cell-to-cell infection still maintains a slight advantage over cell-free infection, even in the presence of the combination, with stronger synergistic/additive effects observed between AZT and TFV for cell-free infection compared to cell-to-cell infection (Table 5.2).

AZT and TFV were also tested in combination with NNRTIs. As single agents, cell-to-cell spread of HIV-$1_{WT}$ was 4-fold less susceptible to inhibition by NVP, however EFV like the PIs and INIs was equally potent against both cell-to-cell and cell-free spread of the virus. A combination of AZT+NVP and TFV+EFV strongly inhibited both cell-to-cell and cell-free modes of infection (Figure 5.2B and C) with combined additive to synergistic effects (Table 5.3 and 5.4). Again, as observed with the AZT+TFV combination, the combined effect of the drugs was greater when the combinations were tested against cell-free infection in comparison to cell-to-cell infection. These observations show for the first time the effects of combining NRTIs + NNRTIs on HIV-1 cell-to-cell infection. They also confirm the findings by Agosto et al.

who showed that combining NRTIs leads to increased inhibitory potentials and enhanced potencies sufficient to overcome the high multiplicity of cell-to-cell infection (Agosto et al., 2014). They used the instantaneous inhibitory potential (IIP) as a parameter to assess the ability of drugs as single agents or in combination to inhibit virus dissemination. Shen et al. first defined this parameter as a measure of antiviral activity (Shen et al., 2008, Shen et al., 2009). The IIP directly measures the degree of inhibition by a given antiviral drug by taking into consideration the slope of its dose-response curve (Shen et al., 2008, Shen et al., 2009). Although it has been purported as a more accurate measure of antiviral activity, and in general correlates with clinical outcomes, its advantage over older methods is still debated (Henrich et al., 2010). Also there is limited experience with using this parameter as a method for assessing drug interactions in combination studies (Agosto et al., 2014). It is for this reason that the more extensively used median effect analysis and combination indices, validated in several combination studies of antiretroviral drugs was preferred (Chou and Talalay, 1984, Chou, 2006, Chou, 2010, Feng et al., 2009, Kulkarni et al., 2014, Kollmann et al., 2001, Beale and Robinson, 2000, Drusano et al., 1998, Deminie et al., 1996).

Synergy between drugs of the NRTI class and between NRTIs and NNRTIs has been extensively studied and well described in cell-free infection models *in vitro* (Feng et al., 2009, Kulkarni et al., 2014, King et al., 2002). This is verified *in vivo* by the efficiency of RTI-based combinations for the treatment of HIV-1 infected patients (Perelson et al., 1997, Gulick et al., 1997, Gulick et al., 2004, Staszewski et al., 1999, van Leth et al., 2004, Arts and Hazuda, 2012). NRTIs are synthetic analogues of naturally occurring dNTPs but they lack a 3'-Hydroxyl group. Their incorporation into the nascent viral DNA during the process of reverse transcription, leads to premature chain termination and as

such inhibits viral replication. Several mechanisms have been proposed to account for the synergy observed with combinations of RTIs. The combination of TFV+3TC, a popular backbone for many first-line cART regimens, increases the intracellular phosphorylation of both drugs and the available active metabolite of the drugs, enhancing the ability of the combination to out compete naturally occurring nucleotides (Borroto-Esoda et al., 2006). Although this enhanced phosphorylation has not been specifically investigated for other NRTI combination pairs, it is possible that this mechanism could be a contributing factor to the synergistic effects observed in this study with the combination of AZT+TFV. In the context of cell-to-cell spread, having more active metabolite to counter the incoming flux of infectious virions tips the scale in favour of the drug, allowing effective inhibition of cell-to-cell infection.

NNRTIs are non-competitive inhibitors, which bind to a hydrophobic pocket near the RT binding site and prevent movement of the protein domains of RT, which are required for effective reverse transcription, hence blocking viral replication (de Bethune, 2010). When NNRTIs are combined with NRTIs, the effects of the combination are additive to synergistic. In these combinations, the presence of NNRTIs has been shown to diminish the binding of ATP (Odriozola et al., 2003, Radzio and Sluis-Cremer, 2008), which is essential for pyrophosphorylysis, a process by which incorporated NRTIs are excised from the newly synthesised virus DNA by the HIV-1 RT, reversing chain termination and allowing reverse transcription to proceed (Arion et al., 1998, Meyer et al., 1998, Meyer et al., 1999, Ray et al., 2003). By so doing, NNRTIs enhance chain termination by the NRTI, bolstering its inhibitory potential (Odriozola et al., 2003, Radzio and Sluis-Cremer, 2008). Also RNAse H activity is enhanced in the presence of NNRTIs, effectively reducing the window of opportunity for NRTI excision by pyrophosphorylysis (Radzio and Sluis-

Cremer, 2008). Furthermore the formation of a stable complex mediated by NNRTI binding prolongs and promotes NRTI chain termination (Feng et al., 2009). All of these mechanisms collectively, likely account for the synergistic interactions between NRTIs and NNRTIs observed for both cell-to-cell and cell-free HIV-1 infection. It is worth noting that while the combination of the NRTI with EFV was strongly to moderately synergistic, combination of the NRTI with NVP only showed weakly synergistic to additive effects on both modes of virus infection. EFV has been shown in a large US and European cohort study of >20,000 patients to be a better NNRTI option than NVP for first-line treatment, in patients initiating cART for the first time (Collaboration, 2012). These findings have been confirmed in smaller cohort studies and clinical trials in sub-Saharan Africa and Asia (Nachega et al., 2008, Bock et al., 2013, Bonnet et al., 2013, Pillay et al., 2013). Regimens containing EFV were found to be associated with fewer AIDS–related opportunistic infections, improved survival and lower rates of treatment failure than NVP containing regimens (Collaboration, 2012). The reasons for this are not fully understood, however in light of our observations it is tempting to cautiously suggest that the stronger synergies seen when EFV is combined with NRTIs in comparison to NVP against both cell-to-cell and cell-free HIV-1 infection, may be a contributing factor to its superiority in a clinical context. This will of course be in addition to other factors such as the higher toxicities associated with NVP regimens, which make interruptions of such regimens and hence decreased efficiency more likely.

In chapter 4 we presented a detailed study of the impact of PIs on cell-to-cell spread of HIV-1. This antiretroviral drug class is highly effective against both cell-to-cell and cell-free modes of infection even with high virus MOIs. The FDA approval of highly potent PIs in the mid-nineties completely revolutionised antiretroviral therapy (Craig et al.,

1991, Kempf et al., 1995, Deeks et al., 1997, Wensing et al., 2010). Today, PIs in combination with RTIs are part of recommended initial treatments for patients starting cART and more importantly, these drugs are the mainstay of salvage therapies used for patients who fail treatment on first-line therapies (WHO, 2013, DHHS, 2014). Also, this is the only drug class, which to date has been used successfully as monotherapy for the treatment of HIV-1 in clinical trials (Arribas et al., 2009, Arribas et al., 2010, Clumeck et al., 2011, Valantin et al., 2012, Katlama et al., 2010). Despite the importance and great success of PIs, the precise steps in the virus life cycle affected by these agents under clinical conditions are yet to be clearly defined. *In vitro* studies have now identified that PIs target multiple steps in the virus replication cycle and this may explain their potency against HIV-1 infection and the high barrier that they present to the selection of drug-resistant viruses (Rabi et al., 2013). PIs inhibit the cleavage of virus precursor polyproteins into functional subunits required for maturation of the virus (Craig et al., 1991, Kempf et al., 1995, Deeks et al., 1997, Muller et al., 2009, Wensing et al., 2010). Virus maturation is important for early post entry steps of the virus replication cycle, including uncoating and reverse transcription (Lori et al., 1988, Kawamura et al., 1997, Muller et al., 2009). In the presence of PIs, recent findings show that interactions between uncleaved Gag and the cytoplasmic tail of Env present a barrier to effective viral entry. PIs therefore exert their inhibitory effects *in vitro* by inhibiting viral entry as well as post-entry steps of viral replication to varying degrees.

When the effect of combining PIs to RTIs was assessed, these combinations displayed additive to synergistic effects on both cell-free and cell-to-cell virus infection. Strong synergy was observed for combinations of PIs + NRTIs (LPV+TFV) (Table 5.5) and additive to mildly synergistic effects were observed with PI+NNRTI combinations

(LPV+NVP) (Table 5.6). These results confirm findings from previous studies, which have demonstrated synergy between PIs and NRTIs for cell-free infections (Drusano et al., 1998, King et al., 2002, De Meyer et al., 2005) and show for the first time this effect on HIV-1 cell-to-cell infection. PI and NNRTI are not usually combined in cART regimens and were only tested here for the sake of completeness. It is however interesting to observe that again weaker synergies are seen between NVP and PIs, as is the case when NVP is combined with NRTIs, discussed earlier. The multiple effects of PIs on several steps of viral replication probably explain the strong synergy observed when these drugs are combined with NRTIs.

It is tempting to speculate on a molecular mechanism for the reduced potency of NVP compared to EFV based combinations against cell-to-cell infection noted in this study. Especially since this is a feature not seen with *in vitro* cell-free infection systems. Efavirenz is a second generation NNRTI well recognised for its improved potency against HIV-1 RT compared to the first generation NNRTIs (Nevirapine and Delaviridine). Several studies on the interaction between NNRTIs and HIV-1 RT have shown that EFV has a greater affinity for HIV-1-RT compared to NVP and DLV (Kd NVP= 1550 +/- 441, Kd EFV= 0.63+/-0.34) (Geitmann et al., 2006, Sluis-Cremer and Tachedjian, 2008). The presence of hydrogen bonding between domains of the EFV molecule and HIV-1RT not seen with the HIV-1RT/NVP and HIV-1RT/DLV complexes contribute to the enhanced binding affinity of EFV (Nunrium et al., 2005). The effect of these differences in binding affinity on potency and ability to block RT may not be immediately obvious when these drugs are tested against cell-free HIV-1 infection. However when cell-to-cell infection is considered, the greater efficiency of EFV compared to NVP becomes more apparent due to the high MOI of this mode of infection. Combinations with EFV would likely be better at

binding and inhibiting HIV-1RT from a pool of incoming infectious virions compared to combinations containing NVP that binds RT with a lower affinity. The observations made with drug combinations are in line with the results presented in chapter 4, which show that while NVP is 4-fold less efficient against cell-to-cell infection compared to cell-free infection, EFV like PIs and INIs is equally potent against both cell-to-cell and cell-free HIV-1 infection. In addition to its high affinity for HIV-1 RT, EFV has the unique attribute within the NNRTI class of enhancing the processing of Gag and Gag-Pol polyproteins (Tachedjian et al., 2005, Figueiredo et al., 2006). This enhanced processing of viral polyproteins by EFV is associated with a decrease in the production of infectious virus particles (Tachedjian et al., 2005, Figueiredo et al., 2006). The binding of EFV to HIV-1 RT that is embedded in Gag-Pol is thought to promote the interaction between individual Gag-Pol polyproteins inducing the premature activation of the viral Protease and subsequent cleavage of the precursor polyproteins. This effectively reduces the amount of full-length viral proteins available for virus assembly and as a consequence the number of infectious virions produced (Tachedjian et al., 2005, Figueiredo et al., 2006). This additional function of EFV possibly contributes to reducing the MOI and this may provide an additional explanation for its superior effects over NVP in the context of HIV-1 cell-to-cell infection.

Having established the efficacy of RTI and PI-based combinations against cell-to-cell and cell-free infection, we proceeded to investigate the impact of cell-to-cell spread of HIV-1 drug-resistant viruses on combination therapy. The development of drug resistance is one of the most common causes of treatment failure in HIV-1 infected patients. As antiretroviral coverage becomes more widespread, clinicians have to contend with rising levels of drug resistance in treated populations and the threat of transmitted drug resistance

continues to grow. Cell-to-cell spread of a PI drug-resistant mutant HIV-$1_{DM}$, as well as two RTI resistance mutants HIV-$1_{K103N}$ andHIV-$1_{M184V}$ was tested in the presence of PI and RTI-based combinations. The PI mutant HIV-$1_{DM}$ displayed a replicative advantage over the wild-type virus HIV-$1_{WT}$ when spreading by a cell-to-cell mechanism in the presence of a combination of LPV+TFV. We had already shown that this combination was strongly synergistic and effective at inhibiting cell-to-cell spread of HIV-$1_{WT}$, however this synergy was reduced to moderate levels against HIV-$1_{DM}$, evidenced by an increase in CI values (Table 5.7). This was expected because the virus is resistant to LPV, which is potent against cell-to-cell spread of HIV-1, leaving only TFV that is a poor inhibitor of cell-to-cell infection to counter the effective spread of the virus. When a third drug EFV was added to the combination, the triple combination effectively inhibited both HIV-$1_{WT}$ and HIV-$1_{DM}$ cell-to-cell spread to the same degree. The HIV-$1_{DM}$ confers a 14-fold resistance to LPV in the drug susceptibility assay (see Chapter 3).

These results suggest that when combined with a drug such as TFV, which is ineffective in monotherapy against cell-to-cell spread, the drug-resistant virus is able to overcome the combined effects of the two drugs and still replicate efficiently when using a cell-to-cell mode of infection. EFV is very potent against cell-to-cell spread of HIV-1 unlike TFV, also the accumulation of multiple major and minor resistance mutations in Protease is usually required in a clinical context to compromise PI-based regimens (Molla et al., 1996). The PI mutant that has been tested in this thesis only carries two resistance mutations and therefore likely doesn't exhibit a resistance phenotype strong enough to compromise a triple combination regimen even with the virus spreading by the highly efficient cell-to-cell mechanism.

Similar observations were made for the RTI resistant mutants. HIV-$1_{K103N}$ had a replicative advantage over HIV-$1_{WT}$ in the presence of RTI-based dual and triple combinations TFV+EFV and TFV+EFV+3TC respectively. HIV-$1_{K103N}$ is 650-fold less susceptible to EFV when compared to wild type virus. The presence of this single point mutation is sufficient to compromise NNRTI-regimens containing EFV (Bacheler et al., 2001). Both TFV and 3TC have reduced potencies in their ability to inhibit cell-to-cell infections as has been previously demonstrated. A resistant virus such as HIV-$1_{K103N}$ with a strong resistance phenotype, gains an added advantage in its ability to compromise an antiretroviral combination by spreading through a cell-to-cell mechanism, especially when a priori some of the components of the combination have known reduced potencies against cell-to-cell infection.

Mutations conferring resistance to one drug can sometimes cause hyper susceptibility to a different compound or re-sensitise strains that were resistant to that drug. The HIV-1 M184V mutation in RT is an example of such a mutation. This mutation is usually selected in patients following treatment with 3TC (Diallo et al., 2003) and the presence of this mutation increases the susceptibility of the virus to other NRTIs including AZT and TFV (Boucher et al., 1993, Tisdale et al., 1993, Larder et al., 1995, Wainberg et al., 1999, Hertogs et al., 2000, Shulman et al., 2001). We hypothesised that this increased susceptibility to AZT and TFV, two drugs which when applied as monotherapy are highly inefficient against cell-to-cell infection, would restore the ability of these drugs to inhibit cell-to-cell spread of HIV-$1_{M184V}$. To test this hypothesis cell-to-cell spread of HIV-$1_{M184V}$ was tested in the presence of the AZT alone (Figure 5.6A) and in the presence of two combinations: 3TC+AZT and 3TC+AZT+EFV in comparison to cell-to-cell spread of HIV-$1_{WT}$. The results show that while the potency of AZT against cell-to-cell spread was restored for HIV-$1_{M184V}$, cell-to-cell

spread of HIV-$1_{WT}$ was not fully inhibited by this same drug. This confirms that the increased susceptibility to AZT mediated by the presence of the M184V mutation remains operational during highly efficient cell-to-cell mode of infection. The M184V mutation has been shown to reduce the selective excision of Zidovudine 5' monophosphate (AZTMP) by the RT of HIV-1 (Boyer et al., 2001). This mutation alters the polymerase active site in a way that specifically interferes with ATP mediated excision of the nucleoside analogue from the end of the primer strand (Boyer et al., 2001). In the presence of AZT and in the context of cell-to-cell spread this increased incorporation of AZTMP may allow more efficient inhibition of HIV-$1_{M184V}$ compared to HIV-$1_{WT}$ by tipping the scale in favour of the drug.

When AZT was tested in combination with 3TC, HIV-$1_{M184V}$ displayed a slight replicative advantage over HIV-$1_{WT}$ during cell-to-cell spread. Both viruses were however effectively suppressed by the triple combination of 3TC+AZT+EFV. HIV-$1_{M184V}$ is 120-fold less susceptible to inhibition by 3TC in the drug susceptibility assay; the combined effect of 3TC+AZT was not sufficient to allow inhibition of cell-to-cell spread of this virus to the same degree as HIV-$1_{WT}$ even considering its increased susceptibility to AZT. This may suggest that the ability of a given combination to inhibit viral replication in the context of drug resistance may be a balance between the potency of individual drugs in the combination against cell-to-cell infection, the strength of the synergistic interactions between drugs in the combination and the magnitude of the drug resistance phenotype of the virus in question. Cell-to-cell spread of both viruses was effectively blocked by the triple combination of 3TC+AZT+EFV. *In vivo* hyper susceptibility to NNRTIs, specifically EFV has been associated with the M184V mutation (Shulman et al., 2001)and may contribute to the enhanced potency

observed with the triple combination against both the wild type and drug-resistant virus.

Although the selection of drug resistance mutations to any drug in a cART regimen is an undesirable feature, usually leading to the drug in question being replaced, the M184V mutation is one of the rare mutations for which this rule may not always apply. In addition to increasing the sensitivity of the mutated virus to other NRTIs notably thymidine analogues, M184V leads to a reduction in the viral replication capacity (Diallo et al., 2003) and also raises the barrier for the selection of resistance mutations to other NRTIs (Naeger et al., 2001, Wolf et al., 2003). For this reason some clinicians favour maintaining patients on 3TC regimens even when this mutation is present. This strategy, although not part of the current WHO treatment guidelines, is supported by evidence from clinical studies showing that the ability of 3TC regimens to suppress viral replication is maintained in the presence of this mutation (Campbell et al., 2005, Castagna et al., 2006, Dunn et al., 2011). Such conservative strategies have the potential to be very useful in settings where scarce resources limit the pool of antiretroviral drugs to choose from when composing a salvage treatment regimen for patients with drug resistance. The findings that even when spreading in a cell-to-cell mechanism, HIV-$1_{M184V}$ remains susceptible to drug combinations containing 3TC offers an additional possible explanation as to why the presence of this mutation does not render a 3TC containing regimen useless for the treatment of HIV infected patients.

# 6 Conclusions and future directions

## 6.1 Conclusions

Three decades since the start of the HIV-1 pandemic, the pathogenesis of the disease is still not fully understood. Access to effective antiretroviral therapy has led to a rapid decline in disease-associated mortality and new infections. However, with the development of drug resistance coupled with the cost and toxicity of long-term antiretroviral therapy it seems evident that alternative strategies are needed in the fight against HIV-1 infection. In recent years the focus of HIV-1 research has shifted towards strategies aimed at eradicating the virus reservoir, alongside long-standing efforts to develop an effective vaccine to prevent new infections. These strategies are however far from being realised and antiretroviral drugs remain the best weapon to treat and potentially protect from HIV-1 infection. As the role of HIV-1 cell-to-cell spread across a virological synapse becomes more clearly defined, its putative importance in viral pathogenesis makes it an important element to consider in the context of antiviral therapy, ongoing viral replication in treated patients and the maintenance of the viral reservoir. In this thesis, I set out to explore and clearly define the impact of antiretroviral drugs on the unique mode of HIV-1 dissemination across a virological synapse. After identifying the best *in vitro* experimental approach to use to answer this question, I went on to assess the effects of PIs, INIs and RTIs alone and in clinically relevant combinations on HIV-1 cell-to-cell infection. My results provide insight into how antiretroviral drugs may affect HIV-1 cell-to-cell infection and allow us to speculate on what this could mean for their clinical use.

In my thesis I have assessed and validated an *in vitro* assay system for studying the impact of antiretroviral drugs on HIV-1 cell-to-cell infection. The findings presented help to resolve some of the methodological controversies that existed in the field prior to the start of this project. The

quantitative T cell line assay that directly measures the early steps of HIV-1 infection provides a suitable unambiguous approach for studying the impact of antiretroviral drugs on cell-to-cell spread of HIV-1. On the other hand a Tat-driven reporter gene expression assay though useful for screening is less accurate for studying the effects of some drug inhibitors on HIV-1 cell-to-cell infection. Applying the qPCR-based quantitative T cell line assay, I have explored for the first time the impact of PIs, and INIs on their ability to inhibit HIV-1 T cell-to-T cell infection as well as reassessed RTIs that were previously studied by other groups but with conflicting results (Sigal et al., 2011, Permanyer et al., 2012b). I have also for the first time assessed the impact of PI-based and RTI-based combination therapies on cell-to-cell spread of both wild type and drug-resistant HIV-1. The finding that PIs, INIs and RTIs have variable effects on their ability to inhibit HIV-1 cell-to-cell infection raises interesting questions on the implications for both prophylactic and therapeutic uses of these antiretroviral drugs.

Based on their work with RTIs Sigal et al. suggested that HIV-1 cell-to-cell infection could be a mechanism for antiviral escape in treated patients (Sigal et al., 2011). Their results are difficult to reconcile with the well-described effectiveness of antiretroviral therapy in suppressing viral replication in patients (Arts and Hazuda, 2012). This has generated considerable debate in the field on the true implications of cell-to-cell spread for treatment and driving ongoing replication to maintain viral reservoirs. My results show that different antiretroviral drug classes exhibit variable potencies against cell-to-cell spread of HIV-1. However when these drugs are used in combination they are effective against both cell-to-cell and cell-free HIV-1 infections. I also clearly demonstrate that drug-resistant variants of the virus gain an advantage when spreading by a cell-to-cell mechanism in the presence of cART. The potential for escape by a cell-to-cell

mechanism of spread is likely to be greater than with a cell-free mechanism of spread considering the higher multiplicity of infection and efficiency of the former. My results therefore suggest that cell-to-cell spread could play a role as a mechanism of escape during monotherapy or inadequate treatment regimens, thus driving the selection and replication of drug-resistant variants. Antiretroviral therapy is required life-long and the side effects associated with treatment have serious implications for effective adherence in patient populations. This is particularly relevant in resource-constrained settings where uninterrupted supply of cART is not always guaranteed. Under such conditions, cell-to-cell spread of the virus could play a significant role in fostering drug resistance and therapeutic failure.

The impact of cell-to-cell spread on ongoing replication and the maintenance of reservoirs with effective cART is less clear-cut. Using highly sensitive single copy assays, trace levels of viraemia can still be detected in effectively treated, fully adherent patients (Palmer et al., 2008). Whether this trace viraemia represents ongoing viral replication with cART or release of virus particles from the latent reservoir (Chun et al., 1997), is a subject of intense debate. *In vivo* HIV-1 cell-to-cell spread most likely occurs predominantly in lymphoid tissues where there is an abundance of CD4+ T cells and effective antiviral drug penetration may be sub-optimal (Fletcher et al., 2014). Also in other anatomic sanctuary sites with reduced drug penetration(Cu-Uvin et al., 2010, Trono et al., 2010, Deleage et al., 2011, Fletcher et al., 2014); niches with cells in close proximity and the absence of sheer flow are likely to favour cell-to-cell spread. It is plausible to suggest that some degree of ongoing replication mediated by cell-to-cell spread is possible in these sites where drug penetration may be suboptimal, even during seemingly "fully suppressive" cART. Some studies have failed to detect evidence of viral evolution in patients receiving fully suppressive cART

arguing against ongoing viral replication in treated patients (Kieffer et al., 2004, Bailey et al., 2006, Evering et al., 2012, Josefsson et al., 2013). It is however important to note that due to the difficulty in sampling sanctuary sites where cell-to-cell spread is most likely to occur, these studies have mostly relied on the detection of HIV-1 in blood samples and thus may not be a reflection of what happens in other compartments. A few studies have shown viral evolution during cART, however in these studies concomitant drug measurements were absent making it impossible to exclude poor adherence as the driving force behind the observed viral evolution (Frenkel et al., 2003, Tobin et al., 2005). Treatment intensification studies with Raltegravir have been shown to reduce immune activation, inflammation and induce a transient increase in 2 LTR circle copies detected (Buzon et al., 2010, Llibre et al., 2012, Hatano et al., 2013). 2 LTR circles are episomal forms of viral DNA, which do not replicate and are generated during new infections suggesting that their detection in some Raltegravir intensification studies may indicate viral replication during therapy. It is nevertheless difficult to reconcile these results with findings from other intensification studies that fail to find evidence of ongoing replication (Dinoso et al., 2009, Yukl et al., 2010, Gandhi et al., 2010). It is interesting to note that in the Raltegravir intensification studies showing increased detection of 2 LTR circles, this effect was mainly observed in patients receiving PIs (Buzon et al., 2010, Llibre et al., 2012, Hatano et al., 2013). This could possibly be a reflection of the unique pharmacokinetic and pharmacodynamic properties of PIs, as opposed to a sign of ongoing replication during cART (Rabi et al., 2013, Laskey and Siliciano, 2014). At the moment it is impossible to say with certainty whether or not ongoing viral replication occurs during effective cART as there is substantial evidence both in support of and against this phenomenon. With the advent of intravital imaging techniques and humanised mice models, allowing the visualisation of cell-to-cell spread *in vivo* (Sewald

et al., 2012, Murooka et al., 2012), it would be interesting to revisit this question using these new techniques and sampling multiple sanctuary sites, to hopefully put the controversy to rest.

Besides their use for treating HIV-1 infection, antiretroviral drugs have gained a lot of traction as prophylactic approaches (PreP and PEP), to prevent HIV-1 infection pre-exposure and post-exposure. Prophylactic antiretroviral therapy targets very early infection with the aim of clearing infections before the formation of drug insensitive latent reservoirs. PreP is now a strategy that is recommended for well-defined risk groups as a way of preventing the spread of HIV-1 infections (WHO, 2013, DHHS, 2014). The effectiveness of this strategy has been assessed in several clinical trials with encouraging but variable results. The CAPRISA 004 and VOICE trials tested the application of Tenofovir gel by women before sexual intercourse (Abdool Karim et al., 2010, Hankins and Dybul, 2013), while five studies tested oral Tenofovir in combination with Emtricitabine or as monotherapy for PreP (Baeten et al., 2012, Grant et al., 2010, Thigpen et al., 2012, Van Damme et al., 2012). The CAPRISA 004 trial showed a 39% reduction in HIV-1 transmission (Abdool Karim et al., 2010) but the VOICE trial was discontinued due to futility. Three of the five trials of oral PreP showed the effectiveness of this intervention ranging from 44-73% protection (Baeten et al., 2012, Grant et al., 2010, Thigpen et al., 2012) but two trials failed to show a demonstrable protective effect (Van Damme et al., 2012). It is worth noting that besides the CAPRISA 004 study, the other three trials that did show effectiveness of the intervention were of the oral combination of Tenofovir and Emtricitabine, two NRTIs. Although the explanations for why some trials failed to show a protective effect of the intervention are likely multifactorial including adherence, the type of sexual exposure, and choice of study populations. It is nonetheless attractive to speculate that biological factors such as the mechanism of

transmission and drug insensitivity could also contribute to infection breakthrough even with PreP, explaining some of the variability observed in these studies.

If cell-to-cell transmission is a significant mechanism of virus spread during early transmission events, this may cause a reduced sensitivity to some of the drugs, which have been assessed for PreP. This would increase the potential for infection to spread from the site of entry and establish distant foci of productive infection. There is considerable evidence from *in vitro* and primate based studies in support of cell-to-cell transmission during initial exposure to HIV-1 (Kingsley et al., 2009, Weiler et al., 2008, John et al., 2001, Salle et al., 2010, Rousseau et al., 2004). The studies on PreP to date have shown that this intervention is not 100% effective in preventing transmission of HIV-1. RTIs such as TFV, which is a key component of PreP, have up to 1000-fold reduced potencies against HIV-1 cell-to-cell infection (Sigal et al., 2011, Titanji et al., 2013, Duncan et.al, 2013, Agosto et al., 2014). We can therefore suggest that even a small decrease in susceptibility to antiretroviral PreP may lead to an increase in the number of people infected while receiving PreP. It would consequently be important to include interventions that effectively target cell-to-cell spread when assessing future microbicidal and oral PreP options. My data show that PIs and INIs are highly effective against cell-to-cell spread of HIV-1. These drug classes have not been assessed for their usefulness in PreP interventions and may be worth considering with the caveat that other factors such as drug penetration of mucosal tissue, pill burden and cost would need to be considered as well.

We can also speculate on what the variable effects of antiretroviral drugs on HIV-1 cell-to-cell infection means for post-exposure prophylaxis (PEP). The window during which HIV-1 infection can be

cleared following exposure has been defined using non-human primate infection models as 24h (for intravenous injection)(Tsai et al., 1998) and 48h (for vaginal challenge)(Otten et al., 2000). A recent study has shown that the latent virus reservoir is likely seeded much earlier in the course of infection than previously thought (within 72h) (Whitney et al., 2014). It is reasonable to suggest based on existing evidence that the reservoir requires an acute phase of infection and viral replication in order to seed sufficient numbers of cells (Chun et al., 1998). Hence although the viral reservoir may be present within 72h it is likely not to be fully formed. This is supported by the fact that early treatment of HIV-1 infection within a month of exposure reduces the viral set point during established infection (Steingrover et al., 2008, Ananworanich et al., 2012, Wyl et al., 2011, Hocqueloux et al., 2013, Saez-Cirion et al., 2013, Fidler et al., 2013). In the study by Whitney et al. macaques exposed to SIV were initiated on suppressive cART 3, 7, 10 and 14 days after infection and viral rebound was observed in all animals following treatment interruption (Whitney et al., 2014). It is valuable to work out whether the formation of early reservoirs established in the presence of cART involves drug insensitive cycles of infection possibly mediated by HIV-1 cell-to-cell spread. This question is made more compelling in light of rebound viraemia in the "Mississippi baby" who was treated with suppressive cART within thirty hours of life (NIH, 2014). The existence of such drug-insensitive cycles of infection remains to be proven. However if they do exist, this could provide the missing link between a curable infection and an established infection with a drug insensitive reservoir.

As the AIDS epidemic has evolved through the decades, the growing realisation that antiretroviral drugs offer only a temporary solution to controlling the infection has shifted the focus more towards the search for a cure. One of the more popular cure strategies involves the use of

histone deacetylase inhibitors to reactivate the virus in latent reservoirs with the hope that these activated cells would be eliminated by the immune system, the cytotoxic effects of the virus and concomitant administration of antiretroviral drugs to prevent actively infected cells from infecting new cells (Siliciano et al., 2007, Sagot-Lerolle et al., 2008, Lehrman et al., 2005, Archin et al., 2012). It would be important to bear in mind the reduced sensitivity of HIV-1 cell-to-cell infection to inhibition by some antiretroviral drugs in the optimisation and testing of such cure strategies.

## 6.2 Future Directions

For this thesis a laboratory-adapted molecular clone of HIV-1 (NL4.3) was used to study cell-to-cell spread of the virus between T cells. Laboratory-adapted strains of HIV-1 can infect and replicate better in cultured T cell lines compared to most primary clinical isolates. It would be informative to expand this study by assessing the impact of antiretroviral drugs on cell-to-cell spread of different HIV-1 strains and clinical isolates. Although to date several studies have demonstrated that cART is equally efficient against HIV-1 infection with different clades of group M virus (Alexander et al., 2002, Pillay et al., 2002, Bannister et al., 2006, Bouchaud et al., 2011, Gatell, 2011, Scherrer et al., 2011), differences have been identified in the frequency and pathways to the selection of drug resistance viruses (Gao et al., 2004, Pieniazek et al., 2000). For instance one report suggests that some subtype D viruses may possess a natural resistance to non-nucleoside reverse transcriptase inhibitors, based on a single nucleotide substitution (Gao et al., 2004) and non-subtype B viruses appear to more frequently select for minor drug resistance mutations in protease (Pieniazek et al., 2000). My results show that drug-resistant variants of HIV-1 gain a replicative advantage when spreading from cell-to-cell in the context of cART. It would therefore be interesting to consider the

efficiency of cART on cell-to-cell spread of different HIV-1 clades especially in the context of drug resistance. This could be further extended to include non-group M viruses, which though responsible for only a small proportion of HIV-1 infections, may possess natural polymorphisms that make them intrinsically resistant or less susceptible to some components of cART.

I have focused my study on T cell-to-T cell spread of HIV-1, however other cell types notably macrophages and dendritic cells also mediate HIV-1 cell-to-cell spread. Duncan et al. assessed the impact of RTIs and INIs on macrophage-to-T cell spread of HIV-1. They found that while RTIs were less effective against this mode of spread in an MOI dependent manner, INIs were effective against this mode of infection (Duncan et.al, 2013). Macrophages are long-lived cells, and their ability to traverse the blood brain barrier enables the spread of HIV-1 infection into sanctuary sites in the central nervous system (Sharova et al., 2005, Gartner et al., 1986). It would be attractive to expand the work I have done with drug combinations and drug-resistant viruses to macrophage-to-T cell spread of HIV-1 in order to complete the picture.

Dendritic cells do not become productively infected with HIV-1, however they mediate infection of T cells by capturing HIV-1 and re-presenting virus particles to T cells in a process known as trans-infection (Pope et al., 1994, Geijtenbeek et al., 2000, McDonald, 2010). This mode of spread between DC and T cells is thought to play a role in the initial phases of infection following the transmission event, allowing the virus to rapidly seed lymphoid tissues. To date only one study has assessed the impact of antiretroviral drugs on DC-to-T cell spread of HIV-1 (Muratori et al., 2009). Muratori et al. assessed the impact of PIs on cell-to-cell spread between DCs and T cell and found PIs to be effective inhibitors of this mechanism of spread. It will be informative to

explore the impact of other antiretroviral drug classes notably RTIs and INIs on DC-to-T cell trans-infection.

The results obtained with RTIs confirm the previous suggestion that high MOI can drive viral escape during cell-to-cell infection. It is difficult to evaluate whether the high MOI required for RTI escape can be achieved *in vivo*. This is however feasible in sanctuary sites such as lymphoid tissues where cell-to-cell infection predominates and antiviral diffusion can be reduced (Fletcher et al., 2014). With the advent of intravital imaging techniques that can visualise cell-to-cell spread *in vivo* (Murooka et al., 2012, Sewald et al., 2012), it would be to interesting to apply these tools to study the effect of antiretroviral drugs on synaptic spread of HIV-1 *in vivo*. This would provide a better understanding of what likely happens in HIV-1 infected patients who receive cART.

Protease inhibitors are one of the main foci of the work presented in this thesis. They stand out from the RTIs by being equally potent against both cell-to-cell and cell-free modes of HIV-1 infection (Titanji et al., 2013). The unique pharmacology of this antiretroviral drug class continues to be unraveled (Rabi et al., 2013, Laskey and Siliciano, 2014). Recent findings show that PIs inhibit multiple steps in the virus replication cycle with the block to entry being the most significant (Rabi et al., 2013). This block to entry has been attributed to the interactions between the uncleaved Gag and the cytoplasmic tail of Env, which inhibit entry until Gag is cleaved by Protease (Rabi et al., 2013). Some env mutations appear to confer resistance to PIs by allowing entry when Gag is not fully cleaved (Rabi et al., 2013). While we have explored cell-to-cell spread of wild type HIV-1 and a PI resistant virus carrying mutations in protease and gag, it would be interesting to assess env mutants conferring resistance to PIs in the

same manner. One of the enduring questions associated with PI-based treatments is failure in the absence of any discernable drug resistance mutations. Exploring the interplay between env mutations associated with PI failure and the mode of virus dissemination may provide some insight into why some patients fail on PI-based therapy without apparent protease or gag mutations.

A brief study of Raltegravir in this thesis has demonstrated its effectiveness against cell-to-cell spread of HIV-1 between T cells for the first time. It will be useful to extend this study to clarify its relative efficacy against cell-to-cell compared cell-free spread of HIV-1, which has not been assessed. Also I have not explored the impact of INI-based combinations on cell-to-cell spread of HIV-1 in my work. INIs are not yet widely used in first-line and second-line treatment regimens but INI-containing salvage regimens are emerging as highly potent options for treatment-experienced patients. Understanding their combined effects with other drugs against the different modes of virus dissemination will provide useful information on this drug class, which is a fairly recent addition to the arsenal of antiretroviral agents.

It now seems unequivocal that cell-to-cell spread of HIV-1 occurs *in vivo* and likely plays a role in viral pathogenesis. In view of our findings and bearing in mind the limitations of *in vitro* studies, the variable effects of antiretroviral drugs on cell-to-cell spread of HIV-1 would need to be considered for future prophylactic, therapeutic and eradication strategies in the fight against HIV-1 infection. The results presented in this thesis show that both PI and RTI-based combination therapies are potent against HIV-1 cell-free infection and highly efficient cell-to-cell infection. The variable potency of PIs and RTIs as single agents against cell-to-cell infection can therefore not be taken as an indication that this mode of virus dissemination is not relevant *in vivo*. Instead these

results suggest that both cell-to-cell and cell-free spread likely play a role in driving viral escape and treatment failure in the context of inadequate combination therapies. Predicting the outcomes of antiretroviral therapy in patients, though highly desirable, remains very difficult in current clinical practice. As new therapies are developed for the treatment of HIV, being able to assess the efficacy of novel combinations against all modes of virus dissemination will serve as a valuable tool for predicting their efficacy, prior to clinical testing. Employing a simple *in vitro* assay like the one used for this study provides a straightforward way of doing this.

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