The role of Gag in HIV-1 DNA synthesis and sensitivity to reverse transcriptase inhibitor drugs

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I, Claire Jane Kerridge confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
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Abstract

We hypothesise that HIV-1 DNA synthesis occurs inside intact viral capsid (CA) cores. We propose that dNTPs are transported into the CA via an electrostatic channel, formed by six positively charged arginines in the centre of CA hexamers. Here, we consider whether reverse transcriptase inhibitor (RTI) sensitivity is altered when the nature of the channel is changed, either by Gag mutation or exchange with Gag from a non-pandemic HIV isolate with a different structure.

There are two classes of RTIs: nucleoside/nucleotide based (NRTI) and non-nucleoside inhibitors (NNRTI). We hypothesised that negatively charged NRTIs would recruit to CA hexamers, to be transported into cores. However, NNRTIs are uncharged, yet potently inhibit DNA synthesis, suggesting that NNRTIs enter cores by diffusion or inhibit after uncoating.

We tested HIV-1 vector sensitivity to RTIs, either bearing lab adapted M-group, transmitted founder, O-group or mutant Gag sequences. Viral inhibition was measured by comparing IC50 and IC90 values in a range of cell lines. Our data shows that some differences in Gag demonstrate a cell type-dependent effect on viral sensitivity to RTIs.

We also tested the stage of RTI inhibition, measuring early and late–reverse transcription (RT) products of HIV-1 (M) and HIV-1 (O) virus in the presence of inhibitors. Our data show that both HIV-1 (M) and (O) vectors are inhibited after 2nd DNA strand transfer. We determined that a small number of vDNA strands are required to infect a U87 cell, which increases in the presence of RTIs or on R18G mutation.

We conclude that differences in Gag have some small cell type-dependent effects on RTI sensitivity. We hypothesise this may be due to differences in the timing of CA uncoating between cell types, supported by our finding that all RTIs tested inhibit RT predominantly after 2nd strand transfer.
**Impact Statement**

Human immunodeficiency virus type 1 (HIV-1) remains a pandemic. In 2016, the World Health Organisation (WHO) estimated that, of the 36.7 million people worldwide living with HIV, approximately 1 million died of HIV/ acquired immunodeficiency syndrome (AIDS)-related illnesses. Despite access to anti-retroviral therapy (ART), drug resistance and poor adherence to drug regimens remain problematic.

Recent data suggests that the use of anti-retrovirals for pre-exposure prophylaxis (PrEP) can significantly reduce HIV-1 incidence among specific at-risk populations. RTIs are used for this purpose. Yet, how RTIs work in early HIV-1 transmission is less well understood.

We recently proposed a model of early HIV-1 infection where CA has two possible fates: early uncoating or late uncoating. The finding that dNTPs were able to enter through a central electrostatic channel located in the centre of CA hexamers, supported a model of late uncoating and led us to question whether RTIs enter the core in a similar way, as dNTPs, to function. We asked whether differences in Gag conferred differences in viral sensitivity to RTIs. Briefly, we found that differences in Gag affects the sensitivity of HIV-1 to RTIs. Furthermore, only a small number of vDNA products are required for infection in the U87 cell line, yet this increased in the presence of RTIs.

Our research has impacted the academic field of HIV-1 research as follows. The discovery of a role for Gag in sensitivity to RTIs is a unique finding and further supports our hypothesis of a role for CA in RT, within the cell cytoplasm. We specifically found cell type-dependent differences in NNRTI sensitivity in the non-pandemic HIV-1 (O)\textsubscript{MVP} vector compared to the pandemic HIV-1 (M)\textsubscript{R9} vector, with O-group requiring two-fold as much NNRTI to inhibit 50% and 90% of infection (IC50/IC90). Previously, poor NNRTI efficacy in O-group infection was attributed to drug resistant mutations within RT. However, our conclusion that Gag may have a role on RT raises further questions within the field, such as differences between pandemic and non-pandemic Gag sequences and differences between transmitted founder and chronic clonal sequences.

Furthermore, our study of the timing of RTI function has supported published literature, demonstrating the increased inhibitory potential of RTIs on longer vDNA products, after the point of 2\textsuperscript{nd} strand transfer. This finding has impacted our better understanding of the timing of RT. More importantly, it demonstrates the need for only a small number of vDNA products for an infection. This supports our model describing two fates for CA, by suggesting that not all HIV-1 CA result in infection.
Further academic based impact of our research has been in the formation of international collaborations. Thus, promoting a multidisciplinary approach within our research. Due to the early stage of our research, the impact outside of academia is limited. However, we hope that further research in the area of RT and viral sensitivity to RTIs might provide the evidence base for the model-informed design of more effective RTIs.
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Key Abbreviations

3TC  Lamivudine
ABC  Abacavir
AIDS Acquired immune deficiency syndrome
ART Antiretroviral therapy
ATP Adenosine triphosphate
ATV Atazanavir
AZT Zidovudine
BHIVA British HIV association
BIV Bovine immunodeficiency virus
CA Capsid
CAMP Cyclic adenosine monophosphate
cART Combined antiretroviral therapy
CCR5 CC chemokine receptor 5
CD4 Cluster of differentiation 4
CD8 Cluster of differentiation 8
cGAMP Cyclic GMP-AMP
cGAS Cyclic GMP-AMP synthase
CPSF6 Cleavage and polyadenylation specificity factor 6
CRFs Circulating recombinant forms
CsA Cyclosporine A
CTL Cytotoxic lymphocyte
CXCL10 C-X-C motif chemokine 10
CXCR4 C-X-C chemokine receptor type 4 (also called CD184)
CypA Cyclophilin A
CypA-DsRed Tetrameric cyclophilin A-DsRed
d4T Stavudine
DAPY Diarylpyrimidine
DMEM Dubbecco’s modified eagle medium
DMSO Dimethyl sulphoxide
dNTP Deoxynucleotide
DRV Darunavir
dsDNA Double-stranded DNA
DTG Dolutegravir
EFV Efavirenz
EM Electron microscopy
Env Envelope
ERT Endogenous reverse transcription
ESCRT Endosomal sorting complex required for transport
ETV Elvitegravir
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS/FCS</td>
<td>Foetal bovine/calf serum</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>FOC</td>
<td>Fate of capsid</td>
</tr>
<tr>
<td>FSH</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>FTC</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>FWD</td>
<td>Forward</td>
</tr>
<tr>
<td>Gag</td>
<td>Group specific antigen</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFP+</td>
<td>Green fluorescent protein positive</td>
</tr>
<tr>
<td>gLUC</td>
<td>Gaussia luciferase</td>
</tr>
<tr>
<td>gp41</td>
<td>Glycoprotein 41kDa</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120kDa</td>
</tr>
<tr>
<td>GT</td>
<td>Genital tract</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HEPT</td>
<td>1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HIV-2</td>
<td>human immunodeficiency virus type 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human lymphotropic virus</td>
</tr>
<tr>
<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IC90</td>
<td>Concentration of drug required to inhibit 90% virion growth</td>
</tr>
<tr>
<td>IC99</td>
<td>Concentration of drug required to inhibit 99% virion growth</td>
</tr>
<tr>
<td>IDU</td>
<td>Intravenous drug use</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon alpha/beta receptor</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>INI</td>
<td>Integrase inhibitor</td>
</tr>
<tr>
<td>INSTI</td>
<td>Integrase strand transfer inhibitor</td>
</tr>
<tr>
<td>IU</td>
<td>Infectious unit</td>
</tr>
<tr>
<td>IU/mL</td>
<td>Infectious units per mL</td>
</tr>
<tr>
<td>IU/U RT</td>
<td>Infectious units per units of reverse transcription</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>Melittic acid</td>
<td>Hexacarboxybenzoene</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte derived macrophage</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MLV</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MRCA</td>
<td>Most recent common ancestor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MVV</td>
<td>Ovine Maedi-Visna virus</td>
</tr>
<tr>
<td>MxA</td>
<td>Myxovirus resistance protein A</td>
</tr>
<tr>
<td>MxB</td>
<td>Myxovirus resistance protein B</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NUP153</td>
<td>Nucleoporin 153kDa</td>
</tr>
<tr>
<td>NUP358</td>
<td>Nucleoporin 358kDa</td>
</tr>
<tr>
<td>NVP</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OWM</td>
<td>Old world monkey</td>
</tr>
<tr>
<td>P24</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>P24+</td>
<td>Capsid protein positive</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononucleocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pbs</td>
<td>Primer binding site</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PF74</td>
<td>2-Methyl-N-[(1S)-2-(methylphenylamino)-2-oxo-1-(phenylmethyl)ethyl]-1H-indole-3-acetamide, PF 3450074</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PIC</td>
<td>Preintegration complex</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypurine tract</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAL</td>
<td>Raltegravir</td>
</tr>
<tr>
<td>RELIK</td>
<td>Rabbit endogenous lentivirus type K</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of virion proteins</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>REV</td>
<td>Reverse</td>
</tr>
<tr>
<td>RLU</td>
<td>Reflective light units</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase/transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RTC</td>
<td>Reverse transcription complex</td>
</tr>
<tr>
<td>RTI</td>
<td>Reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>RTPCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>Sterile alpha motif and HD domain containing protein 1</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SEV</td>
<td>Sendai virus</td>
</tr>
<tr>
<td>SGA</td>
<td>Single genome amplification</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SIVsm</td>
<td>Simian immunodeficiency virus from Sooty Mangabeys</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TAR</td>
<td>Transactivation response region</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>T/F</td>
<td>Transmitted founder virus</td>
</tr>
<tr>
<td>TIBO</td>
<td>Tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin 2 (1H)-one and -thione</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMC125</td>
<td>Etravirine</td>
</tr>
<tr>
<td>TMC278</td>
<td>Rilpivirine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tenofovir</td>
</tr>
<tr>
<td>TNPO3</td>
<td>Transportin 3</td>
</tr>
<tr>
<td>TREX1</td>
<td>Three prime repair exonuclease 1</td>
</tr>
<tr>
<td>TRIM5</td>
<td>Tripartite motif-containing protein 5</td>
</tr>
<tr>
<td>U RT</td>
<td>Units of reverse transcription</td>
</tr>
<tr>
<td>U RT/ml</td>
<td>Units of reverse transcription per ml</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein r</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein u</td>
</tr>
<tr>
<td>Vpx</td>
<td>Viral protein x</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus G envelope protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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</table>
Chapter 1: Introduction

1.1 Human Immunodeficiency Virus

1.1.1 The HIV-1 lentivirus

Human immunodeficiency virus (HIV) was first described in 1981 and isolated in 1983 (1, 2). Prior to the isolation of HIV-1, an increasing number of men who have sex with men (MSM) were presenting with multiple opportunistic infections and malignancy, of unknown cause (3).

Retroviruses, such as HIV were previously classified into 3 subfamilies according to virion morphology: oncoviruses, such as HTLV, spumaviruses, such as foamy viruses and lentiviruses including HIV, feline (FIV), bovine (BIV), ovine (MVV) and simian (SIV) (Figure 1) (4). However, the use of sequencing techniques across various regions of the retroviral genome has now led to the phylogenetic inference of evolutionary relationships.

Figure 1: Evolutionary relationships of Pol sequences among lentiviruses

Sequences from multiple mammalian lentiviruses including: exogenous viruses (black), HIV-1, HIV-2 and SIVmac are highlighted in red. Endogenous viruses are shown in blue. Phylogenetic tree estimation produced from maximum likelihood methodology. Scale =0.10 amino acid replacement/ site. Figure from (Paul M. Sharp and Beatrice H. Hahn, 2011) (5).
The retroviridae family encode the same three structural genes: envelope (Env), polymerase (Pol) and group specific antigen (Gag), along with regulatory genes trans activator of transcription (Tat) and regulator of expression of virion proteins (Rev) and virus specific accessory genes. HIV-1 encodes Nef, Vif, Vpu and Vpr (Figure 2) while HIV-2, known to differ from HIV-1 in Env glycoproteins (6), encodes Vpx. A unique feature of all retroviruses is the copying of the diploid RNA genome into dsDNA within the host though the process of reverse transcription. Despite the early discovery of RNA dependent DNA polymerase in 1956 (7), the process of reverse transcription was not defined until 1970 (8, 9).

The 9.7 Kb HIV-1 genome undergoes cleavage of the three main genes into structural proteins. Gag is cleaved into structural proteins p17 matrix (MA), p24 capsid (CA) spacer peptides 1 and 2 (SP1 and SP2), p7 nucleocapsid (NC) and p6. Pol encodes multiple enzymes: p10 protease (pro) which cleaves Gag-Pol poly protein along with p7 and p6, the late domain of HIV-1, p51/p66 reverse transcriptase and p31 integrase (IN) and p15 RNAse. The Envelope encoded gene is cleaved by a host protease, furin, into 2 glycoproteins (gp) 120 and 41 (Figure 2).

![Figure 2: The Genome of HIV-1 M-group HXB2](image)

HIV-1 encodes 9 genes across 3 reading frames (Y-axis). 3 main genes: gag, pol, env and accessory genes: vpr, vpu, vif, tat, rev, nef. X-axis indicates nucleotide number. Figure adapted from Los Alamos HIV-1 gene map (10).

### 1.1.2 Origins of HIV-1 and HIV-2

The pandemic spread of HIV-1, after its isolation in 1983 was poorly understood. On isolation of HIV-2 in 1986, it was found that HIV-1 and HIV-2 were only distantly related. HIV-2, based on homology between retroviral sequence more closely resembles simian immunodeficiency virus (SIV), (11, 12).

Interestingly, SIVs derived from multiple primate hosts such as african green monkeys and sooty mangabeys are non-pathogenic. However, chimpanzee SIV is found to cause AIDS-like disease (13), interesting considering the close phylogenetic link between HIV-1 and chimpanzee SIV (Figure 3) (14). However, the phylogenetic link between HIV-2 and sooty mangaby suggests HIV-1 and HIV-2 are derived from multiple cross species.
transmission events (15, 16). This is a unique transmission feature of HIV, as most lentiviral species demonstrate horizontal transmission. Although a historical example, RELIK, now endogenous, transmits vertically (Figure 1) (5, 17).

HIV-1 is comprised of four lineages, termed groups. Each HIV-1 group has arisen from a separate cross-species transmission event: M (major group), O (outlier group), N (non-O or M) and P (Figure 3). Whilst HIV-1 M-group viruses have rapidly disseminated becoming pandemic, infecting ~60 million individuals worldwide, HIV-1 O-group is less prevalent, non-pandemic. HIV-1 O-group infection is restricted to Cameroon and Gabon and accounts for <1% of the global burden (~100,000 cases) (18, 19). Perhaps due to their lack of pandemicity, HIV-1 O, N and P-group viruses were not isolated until after HIV-1 M-group. The HIV-1 O group strain MVP 5180 was the first to be isolated in 1994 (20), followed by the identification, in 1998, of N-group HIV-1, which to this date has only been isolated in ~20 individuals, originating from Cameroon (21, 22). Finally, the HIV-1 P-group, only known to infect 2 individuals from Cameroon, was identified in 2008 (23, 24). Interestingly, despite their date of isolation, M and O-group epidemics have been dated as originating in the 20th century, while N and P groups are likely to be as a result of a more recent transmission event (25).

Phylogenetic analysis of HIV-1 suggests that M, N groups are more closely related to SIVcpz while the non-pandemic P-group clusters with SIVgor. The origin of O-group remained unclear, until recently when D’arc reported a new sequence of a SIVgor, isolated from central Cameroon, which was found to be closely related to HIV-1 O-group (Figure 3). Their data suggests that HIV-1 O-group therefore originated from a cross-species transmission event from gorillas (26).

Despite classification of the simian precursors to HIV-1, the exact point of zoonotic transmission remains unknown. However, repeated exposure to infected blood in Cameroon through, for example, the practice of bush meat hunting is a likely candidate for initial transmission. This is especially likely considering the high proportion of infected wild apes with genetically divergent SIV found in this region (27, 28).
1.1.3 The HIV-1/AIDS Pandemic

While sexual transmission still remains responsible for the global burden of disease, intravenous drug use (IDU) and mother-to-child transmission are alternate routes of transmission (29). Successful HIV-1 M-group pandemicity has been attributed to the rapid rate of transmission due to viral evolution through error prone copying of reverse transcriptase. However, considering that the HIV-1 O-group is as old as M-group virus, it is unclear why O-group has remained non-pandemic. Recently, structural attributes of HIV-1 M-group capsid have been proposed as the reasoning behind the pandemic success of this specific group of lentivirus, further discussed in section 1.4 (30). Despite rapid HIV-1 M-group global transmission, population bottlenecks have given rise to further sub-types and recombinant sub-types, which promote further diversity (Figure 3) (29, 31).

Figure 3: The evolution of HIV-1 and HIV-2

SIV sequences (black), HIV sequences (red). Figure from Stephane Hue (unpublished)
1.1.4 The clinical course of HIV-1/ AIDS

The clinical course of HIV-1 infection can be monitored by overt symptoms such as: wasting and opportunistic infections, the monitoring of blood levels of CD4+ T-cells and viral RNA quantification by PCR. High intra-patient variation in the timing of clinical progression often means that the basic clinical stages of disease do not remain the same in the absence of antiretroviral therapy (ART). Clinical stages of HIV infection, termed Fiebig stages, can be defined based on the results to diagnostic tests (Figure 4) (32).

The eclipse phase, defined as an unsymptomatic period of time between infection and positive detection of vRNA, lasts between 1-2 weeks post infection. During this time, HIV-1 rapidly replicates and spreads from the initial site of infection. Fiebig stages I-III are the earliest time points of vRNA detection by PCR during acute infection, which encompasses stages I-V. Fiebig stages III-IV are characterised by an increase in viremia, which can reach $>10^7$ vRNA copies / mL blood. Increased viremia up to 30-40 days post transmission is often associated with flu-like symptoms brought under control by the adaptive immune response mounted against viral proteins. However, prior to immune control, targeted CD4+ T-cells are infected and highly activated. By Fiebig stage V, on the mounting of an adaptive immune response, HIV-1 specific antibodies (Abs) can be detected by ELISA or western blot. The acute phase of HIV infection is marked by a decline in detectable virus, which rapidly reduces until reaching clinical latency at Fiebig stage VI. Despite immune control of infection, over the period of chronic infection CD4+ T-cells become exhausted, before the viral set point is reached. Continual loss of CD4+ T-cells results in the complete loss of immune control over HIV and opportunistic infections, and eventually acquired immune deficiency syndrome (AIDS) (Figure 4) (32, 33). Despite advances in HIV treatment, of the 36.7 million people living with HIV worldwide, in 2016 ~1 Million people died of HIV/AIDS related illness (WHO Global Health Observatory (GHO) data) (34).
**Figure 4: Fiebig stages of HIV-1 infection**

Classification of clinical stage of HIV-1 infection according to positive results to diagnostic test results. The eclipse phase is defined as the time between infection and positive detection of viral RNA. Acute infection, including Fiebig stages 1 to 5 mark the increase and later decrease of vRNA detected in plasma samples and increase in positive HIV-1 diagnostic assays. Chronic infection is defined by: the plateau of viremia, establishment of HIV-1 reservoirs and continued decrease of CD4+ T-cells. Figure adapted from (McMichael, A. J., et al., 2010) (32).

1.1.5 **Clinical treatment of HIV-1 in the UK**

Previously, NHS guidelines in the UK stipulated a CD4+ T-cell count of <350 cells/ mm$^3$ was required before the initiation of HIV-1 treatment. However, new guidelines in 2018 recommend the immediate initiation of ART from clinical diagnosis, irrespective of patients clinical stage (35). Whilst treatment regimens may vary the guidelines remain the same for HIV treatment throughout the UK. The primary aim of ART is “the prevention of the mortality and morbidity associated with chronic HIV infection at low cost of drug toxicity” as set by the British HIV Association (BHIVA) 2016 update (36). The combined/ highly active ART regimen (cART/ HAART) aims to suppress HIV to (<50 copies/ mL), through the initial combination of two nucleotide reverse transcriptase inhibitors (NRTIs) such as: tenofovir (TDF, or referred to throughout as TNF) and emtricitabine (FTC), with the addition of a 3rd agent such as protease inhibitors (PI): atazanavir (ATV) and darunavir (DRV), integrase strand transfer inhibitors (INSTIs): elvitegravir (ETV), dolutegravir (DTG), raltegravir (RAL) or the non nucleoside reverse transcriptase inhibitor (NNRTI) Rilpivirine (RPV, or as referred to throughout as TMC278). Virological failure during ART is classified as >200 copies/mL and requires rapid genotypic resistance testing and alteration of ART as appropriate (36).
In addition to ART since September 2017 pre-exposure prophylaxis (PrEP) Truvada, comprising 2 NRTIs emtricitabine and tenofovir disoproxil fumarate has been available in the UK. The introduction of PrEP has already demonstrated high efficiency as a preventative method of HIV treatment (37).

1.1.6 Reverse transcriptase inhibitors (RTIs)

1.1.6.1 Nucleotide/Nucleoside reverse transcriptase inhibitors (NRTIs)

First described for their efficacy against HTLV III, 2'-3'-deoxynucleotide analogues were found to be potent chain terminators (38, 39). From this group of DNA chain terminators, zidovudine (AZT), first synthesised in 1964, was approved for the treatment of HIV-1 in 1986 (38, 40, 41). Following the discovery of AZT seven nucleoside reverse transcriptase inhibitors and one nucleotide (tenofovir) reverse transcriptase inhibitor were approved for ART (Figure 5A-C). Although AZT is now rarely used, due to widespread resistance mutations located throughout pol, alongside significant toxicity and side effects in patients (42), alternative NRTIs remain the backbone of ART.

NRTIs are analogues of endogenous 2'-deoxy-nucleosides and nucleotides. After transportation into the host cell, by passive diffusion or carrier-mediated transport, NRTIs require the activation by phosphotransferase, a host kinase. On phosphorylation, NRTIs are activated to deoxynucleoside triphosphates (dNTPs) (43, 44) (Figure 5A-C). Active NRTIs compete with naturally occurring dNTPs binding between the palm and finger subdomains of DNA polymerase, contacting key residues of the p66 subunit: L74, Y115, M184 and Q151 (Figure 6B) (45, 46). However on binding, NRTIs function as obligate chain terminators of DNA synthesis, due to the absence of a 3’ hydroxyl group or the replacement of hydroxyl with a 3-azide (−N₃) group (Figure 6A) (38, 47, 48).

Viral resistance to NRTIs remains a complexity in the delivery of effective ART, especially from the transmission of drug resistant virus. NRTI resistance can occur by 2 different means: exclusion, the enhancement of incorporation discrimination against NRTIs, in favour of natural dNTPs, for example mutation at position M184 (46), or ATP-dependent excision, during which NRTIs are removed from the 5’ end of the template strand (49).
Figure 5: Examples of nucleotide reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) structures

NRTIs: (A) Tenofovir triphosphate (B) Zidovudine triphosphate (C) Stavudine triphosphate and NNRTIs: (D) Nevirapine and (E) Rilpivirine. All structures obtained from the PubChem database (50).

1.1.6.2 Non nucleoside reverse transcriptase inhibitors (NNRTIs)

The first NNRTIs HEPT (51, 52) and TIBO (53), described in 1989, were found to have targeted action against reverse transcriptase of HIV-1 but were inefficient against HIV-2. Following a screening programme in 1990, the first generation NNRTI nevirapine (NVP) was selected due to its high anti-viral activity in cell culture (54) (Figure 5D).

NNRTIs function through the direct binding of a hydrophobic pocket within reverse transcriptase. Located approximately 10 Å in distance away from the active substrate binding site (55), the NNRTI binding pocket comprises residues: L100, K101, K103, V106, T107, V108, V179, Y181, Y188, V189, G190, F227, W229, L234, Y318 from the p66 domain and E138 from the p51 domain (56-58) (Figure 6C-D). Structural studies have provided further functional evidence of NNRTI mechanism. On binding, NNRTIs cause repositioning of a beta-sheet within the p66 subunit, which allosterically alters the reverse transcriptase active site conformation, rendering it inactive. Interestingly, due to the close proximity of the NNRTI binding site to the reverse transcriptase active site, there is a functional connection between both sites. This therefore improves the effectiveness of inhibition on the combination of both NRTI and NNRTIs (59, 60).
However, as with NRTI treatment, NNRTI resistance soon became a problem for their use. Molecular modelling of NNRTIs in 1996 led to the discovery of the diarylpyrimidine (DAPY) group, of which etravirine (TMC125) was found to be highly effective in individuals with NNRTI resistance mutations (61, 62). Despite the development of second and third generation NNRTIs, such as efavirenz and etravirine, multi drug resistance remained a significant problem for their efficacy. The majority of NNRTI resistance mutations are located within or around the binding site such as: K103N and Y181C with L100I, K101E, V106A, V179D, Y188L, G190A, and P236L, all of which are still commonly observed (58). NNRTI resistance mutations can be grouped into 3 broad resistance mechanisms. Firstly, the loss or change of key interactions, such as mutations at sites 181, 188, and 227. Secondly, steric hindrance, such as mutation at sites 100 and 190 which change the NNRTI pocket conformation, and finally pocket entrance mutations such as K103N and K101E which prevent NNRTI entry into the binding site (59, 63-65). Amidst mounting NNRTI resistance, the year 2005 saw the development of the broad spectrum NNRTI Rilpivirine (TMC278) (66) (Figure 5E). TMC278 was found to be highly effective, even against multidrug resistant strains of HIV-1 (67). However, while NRTIs make up the backbone of HAART, protease inhibitors, NNRTIs or INSTIs are the third option drugs used in HAART.
Figure 6: Nucleos(t)ide reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) sites of action:

(A) During DNA synthesis incorporated NRTIs (red) induce chain termination on binding via their 5'-phosphate (-OH) group, the lack of 3'-OH group means no further dNTPs can join. Figure adapted from (Esposito, F., et al., 2012) (68). (B) Structural components of HIV-1 reverse transcriptase (purple) with bound chain terminated DNA (orange) from NRTI zidovudine (AZT) treatment (Das, K. and E. Arnold, 2013) (PDB 3V6D) (69). (C) Reverse transcriptase (purple) with bound NNRTI rilpivirine (TMC278) (Lansdon, E. B., et al., 2010) (PDB 3MEE) (57). (D) NNRTI binding pocket of reverse transcriptase, labelled residues show sites of known NNRTI resistance mutations, figure from (Sarafianos, S. G., et al., 2009) (58).
1.2 HIV-1 Transmitted/Founder viruses

1.2.1 HIV-1 transmission bottlenecks

Transmitted/founder (T/F) viruses are the first clones to establish infection within a recipient, on transmission from an infected person. Despite rapid clonal expansion and diversity within transmitter HIV-1 viral sequences soon after transmission, testing in early recipients shows clonal homology irrespective of the mode of transmission (70-72). In fact, from the majority of transmission events, 76-80% of sexual transmission, recipient infection is established from a single HIV variant, determined using a range of sequencing studies from peripheral blood (73-75).

Considering high within transmitter clonal diversity, it is surprising that infection within a recipient is established from so few clones. Three models have previously been put forward to explain low diversity among transmitted sequences: low inoculum level transmitted to the host, selective amplification within a new host, and finally, selective transmission of clones bearing an advantage in the mode of transmission, for example overcoming the mucosal barrier (76). However, with clonal restriction during transmission, it is probable that T/F viruses must overcome more than one bottleneck either within the transmitter, during transmission, to evade innate sensing mechanisms and establish infection within the first target cells.

1.2.1.1 Bottlenecks within a transmitter

Viral compartmentalisation within a transmitter affects the rate of amplification and subsequent clonal diversity within the genital tract (GT) during transmission (77). HIV-1 clones within a transmitter are also selected during transmission in the transmission fluid, due to the presence of neutralizing antibodies. Interestingly, the Env of HIV-1 sub-types A, C and D are less glycosylated, which is found to be beneficial in the evasion of antibody detection and therefore transmission. However, the transmission benefit of reduced glycosylation is dependent on the route of transmission (78, 79).

1.2.1.2 Bottlenecks within a recipient

The ability of HIV to enter a target cell such as CD4+ T-cells, is reliant on viral Env gp120 binding to host CD4 and the engagement of co-receptors, CCR5 or CXCR4. Co-receptor preference is selected for on transmission, interestingly, all T/F clones are R5 tropic. Although some T/F clones have dual tropism, the preference for R5 usage suggests this route is essential for entry into target cells during transmission (80, 81). Despite R5 tropism, T/F clones poorly infect macrophages, linked to low levels of CD4 expression (78, 82). During transmission, clones with high replication rate are also likely to be
selected. This is of benefit during the reversion of cytotoxic T-cell escape mutants within the recipient (83).

1.2.2 Acquisition of patient derived T/F sequences

1.2.2.1 Phylogenetic comparison between a chronic transmitter and newly infected recipient

An early method to study clonal selection during transmission was to examine peripheral blood samples from a maternal transmitter and their 2-4 month old child. Variation between samples was compared by the amplification of Env variable loops, by nested PCR, and sequencing. Although a simplistic approach to comparing transmitter to recipient variation, such studies were the first to demonstrate transmission from a single genotype. It was found that transmitted sequences were present within the transmitter but as a minor genotype (71, 76).

Later this methodology was used to assign T/F Env consensus sequences from sera of recently seroconverted patients. Such studies determined large intrapatient sequence variation, up to 11% distance between two sequences, while recipient sequences were homologous for several months after seroconversion (70, 72). Whilst use of this methodology led to the construction of neighbour joining trees to better understand transmission bottlenecks it did not identify true transmitted founder virus sequences (74).

1.2.2.2 Single genome amplification (SGA) direct sequencing

Keele et al were the first to use a novel technique to identify true T/F HIV Env sequences from acutely infected individuals. vRNA isolated from plasma was subjected to single genome amplification (SGA), using multiple primer sets, followed by direct sequence analysis of uncloned amplicons of Env variable regions. Sequences were analysed, using neighbour joining phylogenetic trees, which allowed for the tracing of a common ancestral sequence. This method, in multiple transmitters, confirmed 76% of infections were established from a single virus (75). The development of SGA promoted the study of sequence evolution, on the application of Monte Carlo simulations, which capture randomness of early HIV-1 evolution (84).

In 2009 SGA was used to study transmission pairs from the Lusaka cohort, Derdeyn et al (74), and an additional cohort from Kigali, Rwanda, through the analysis of complete Env gp160 sequences, further analysing the large bottleneck of transmission between heterosexual pairs. Through the use of SGA for example, it was found that 90% of transmission occurred from a single transmitter quasispecies, which represented less than 5% of the transmitters total quasispecies variation (85). Despite the transmission of a single or small number of clones, soon after transmission the sequencing of Env and
Gag genes shows the rapid diversification of a heterogeneous population of sequences. However, this diversity decreases over 1-2 months post transmission, following the outgrowth of a single dominant T/F sequence (86).

However, these studies are limited to the comparison of just a small fraction of the genome of interest and the error of unequal template amplification of sequences. Salazar-Gonzalez et al sought to overcome these complexities of SGA-bulk amplification by developing a new SGA methodology for full Env genes on un-cloned DNA amplicons (87). Resulting Env sequences were subjected to phylogenetic analysis. Having determined the use of T7 polymerase prevents Taq induced errors, highlighter analysis was then applied to the MRCA to identify early founder Env sequences (87).

1.2.2.3 Molecular cloning of full-length T/F virus
The study of early transmission bottlenecks and the effect of T/F genotype diversity on viral phenotype have focused on Env. However, Salazar-Gonzalez et al (2009) sequenced whole T/F genomes to gain further insight into the bias of studying just Env (88). Peripheral blood samples were obtained from 12 acutely infected individuals, from which full-length T/F sequences were obtained from un-cloned DNA amplicons (87). This method had previously been used to characterise near full-length infectious molecular clones (89). Evolutionary data of within patient sequence diversity showed the replacement of T/F sequences from 12 months post infection, suggesting rapid adaption post transmission (88). However, this could also be due to poor replication fidelity.

1.2.3 Phenotypic characterisation of T/F viruses
1.2.3.1 T/F coreceptor usage
T/F sequences obtained through comparison of matched transmitter-recipient sequences or SGA show a strong selective force for CCR5 co-receptor usage, only a few isolated T/F Envs demonstrate R5/X4 dual tropism (80, 90, 91). Distinguished by differences in the V3 loop region of Env, it is thought X4 tropism in T/F clones confers a reduction in transmission efficiency (75, 92). Despite R5 tropism being linked with viral infection in macrophages, all R5 T/F clones preferentially infect and replicate within R5 expressing CD4+ T-cells, more specifically T/F clones infect resting or activated memory subsets of CD4+ T-cells (88, 93, 94). Interestingly, as well as R5 tropism amongst T/F viruses, variants also have reduced sensitivity to CD4, measured by the decreased sensitivity to soluble CD4 (89). Envelope sequences isolated from chronic clones have increased sensitivity to soluble CD4 (75). Furthermore, for HIV-1 sub-type B infection, increased levels of either CD4 or CCR5 expression can compensate for a lower level of the other during infection. However, there are multiple sub-type differences in receptor and co-
receptor usage, with sub-type C clones requiring high levels of both CD4 and co-receptor on a host cell to infect (94).

1.2.3.2 Env incorporation and glycosylation
Aside from co-receptor preference, the study of T/F Env sequences has provided further insight into T/F virus specific differences that increase viral fitness. There is evidence for increased expression and packaging of Env in sub-type B and C T/F viruses and this was correlated with increased viral titre (95). However, selection of T/F clones with higher Env content was disputed by Iyer et al, who found variable Env packaging in T/F isolated from plasma of matched transmitters and recipients (96). Irrespective of Env content, T/F viruses demonstrate both increased infectivity and replication, compared to chronic clones (CC). This is thought to correspond to the evolutionary selection of a positively charged histidine residue at Env position 12 (96, 97).

Further studies have highlighted differences in Env glycosylation patterns. Env of some T/F clones have been characterised by a shortening in variable loops. This limits the number of glycosylation sites and therefore increases the percentage of utilised sites in T/F compared to chronic clones. This decrease in N-linked glycosylation in T/F clones is thought to promote the immunogenicity of Env, promoting early immune escape and facilitating CD4-CCR5 interactions, through co-receptor glycosylation signals (98, 99).

T/F Env glycosylation patterns also promote α4β7 binding to the Env gp120 subdomain (100). α4β7 is a gut homing integrin expressed on activated CD4+ T-cells found to bind with high efficiency to T/F gp120, however Env derived from chronic clones are unable to bind this integrin. T/F binding of α4β7 has been found to improve infectivity with some T/F clones demonstrating a two-fold increase in infectivity compared to CC, in the absence of α4β7 specific antibodies (95).

1.2.3.3 IFN resistance
Increased infectivity of T/F clones, compared to CC, is not entirely due to the recruitment of the α4β7 integrin. Increased T/F infection and viral titres can be partly explained by increased resistance to interferon-α (IFNα), as measured in CD4+ T-cell treated with Type 1 interferon (IFN-I) (95). Interestingly IFN-α treatment of CC reduces Env incorporation into viruses, due to an assembly defect. This suggests that T/F have adapted to evade this effect (101). Despite good evidence for the resistance of T/F to IFNα, the extent of this effect has recently been questioned by 2 groups, who report either no difference in T/F IFN sensitivity to CC or suggest T/F viruses have increased sensitivity to IFN (102, 103). Despite these studies, from the comparison of transmitter and recipient isolates it is clear that recipient isolates exhibit IC50 values 11-fold higher to CC on IFNα treatment or
71-fold on IFNβ treatment (96). T/F viruses also demonstrate reduced sensitivity to IFN-II. T/F resistance to IFNγ, has previously been shown to have potent antiviral activity in THP-1 and CD4+ T-cells, which maps to gp120 (104-106).

1.2.3.4 Acute HIV fitness and cytotoxic T lymphocyte (CTL) escape mutants

T/F viruses can be characterised by their homogenous genotype. However, by 6 weeks post infection, CTL immune adaptive mutations within variable regions of the HIV genome such as Env and regions of higher conservation such as Gag, are visible on sequencing (107). Acute viral CTL escape, from CD8+ T-cells, plays a large role in the evolution of HIV. However, CTL responses are restricted by an array of different human leukocyte antigen (HLA) molecules (108). One such example, of a HLA dependent CTL escape signature, is the association of HLA-B57/5801 with the Gag TSTLQEQIAW (TW10) epitope. For example, the dominant substitution of Thr by Asn, at position 242 within Gag (T242N).

The T242N signature has been observed in both B and C sub-types, after transmission and, therefore, is thought to result from positive selection during CTL escape within a recipient (109). Despite the clear fitness benefit of CTL escape mutants, some mutations located within Gag are associated with fitness costs (110). This is evidenced both by the fast reversion of T242N to the consensus sequence, and the lack of transmission of T/F bearing the T242N mutant. However, other CTL escape mutants also have a significant reduction in replicative capacity compared to NL4.3 (109, 111). Contradicting, the T242 mutant has no fitness defect on single-round infection and is known to stabilise the CA cone, by stabilising the electrostatic charge along Helix 6 in which it is located (111).

The cost to viral fitness is further signified by the reversion of CTL escape mutants within the first 6 months of acute infection (112, 113). In contrast, mutations arising during later infection are predominantly slow forward mutations, which mutate away from the consensus sequence, often not associated with fitness defects (114).

However, fitness defects can be overcome by compensatory mutations such as G248A and H219Q, both of which are found to compensate for the reduction in spreading efficiency in virus containing the Gag T242 mutation (111, 115). Despite bearing a fitness defect, CTL escape mutants have been shown to transmit successfully to a new recipient, such as mutations within the CypA binding loop of HIV-1 CA (115).
1.3 The HIV-1 Lifecycle

1.3.1 HIV-1 entry into a target cell

CD4+ T-cells are the primary targets of HIV-1. To infect both T-cells and macrophages, initial contact between HIV-1 envelope (Env) surface glycoprotein gp120 and the host CD4 binding site is required (116-118) (Figure 7B-C). This bridging connection during attachment results in the stimulation of the Env variable region to undergo rearrangement exposing a new binding site for coreceptors (119-121) (Figure 7D). Co-receptor binding, of CXCR5 or CXCR4, then promotes the insertion of the hydrophobic transmembrane gp41 fusion peptide into the host cell membrane (122).

On fusion with the host cell, the gp41 region of Env rearranges, bringing both the amino terminal helical region (HR-N) and carboxy terminal helical region (HR-C) together (121, 123, 124). This 6 helix structure is the final structure which brings both viral and cellular membranes together and drives the formation of a fusion pore (122, 124, 125) (Figure 7E).
Figure 7: HIV-1 attachment and entry into a host cell

(A) Mature HIV-1, on contact with a host cell, forms weak contacts. (B) Attachment is mediated by gp120 or acquired host cell surface proteins. (C) After initial attachment, host CD4 binds gp120. (D) CD4 binding alters conformation of Env variable loops exposing the co-receptor binding site promoting X4 or R5 binding. gp41 structural rearrangement promotes formation of the 6 helix bundle. (E) Collection of host cell membranes promotes viral fusion. Figure adapted from (Shattock and Moore, 2003) (118).
1.3.2 Early cytoplasmic trafficking of capsid

Formation of the fusion pore completes the joining of viral and host membranes, allowing for the release of the viral CA into the host cytoplasm. However, the events prior to the completion of vDNA replication and nuclear entry remain poorly defined. The difficulty in studying the traversing of HIV-1 capsid, within the cytoplasm, due to the lack of appropriate techniques, which has led to a lack of consensus in the field over the timing and location of CA uncoating. It is evident from microscopy studies that CA cones uncoat at different times in the cytoplasm, near the nuclear pore, or even in the nuclear compartment. However, the difficulty remains in determining which core leads to infection.

We recently proposed a model describing two possible fates for capsids (126). We propose that to infect a host the CA core must remain intact until the near completion of vDNA synthesis, by reverse transcription. Either on completion of reverse transcription, or on docking at the nuclear pore, uncoating is initiated (Figure 8.1). However, virions which have reduced infectivity, initiate uncoating on entry into the host cytoplasm (Figure 8.2) (126). Irrespective of the timing of uncoating, CA disassembly is required for nuclear entry and integration. Although, this hypothesis has been contested by Peng et al who reported nuclear import of CA structures in MDMs, therefore, suggesting a role for CA after nuclear entry (127).

1.3.2.1 Cytoplasmic capsid uncoating

One fate of HIV-1 CA cones on entry into the host is early CA disassembly, which may occur on completion of strong stop vDNA (Figure 9B) (128). On disassembly vRNA, vDNA or perhaps duplexes, are released along with viral proteins, packaged at the time of viral budding (129). Interactions between viral products and the complex network of the host cytoskeleton promotes the formation of a stable reverse transcription complex (RTC) (129). This notion is supported by the prevention of uncoating and viral trafficking on disruption of microtubules by siRNA against dynein or kinesin 1 (130).

Initially described for MLV, the formation of a HIV-1 RTC (Figure 9C) is partly supported by the strong association of RT, IN, MA and Vpr with vDNA post reverse transcription, while association to p24 CA protein is very weak. This was previously examined by immunoprecipitation of nuclear extracts (131). Electron microscopy studies have also visualised the early disassembly of cores after viral fusion through triple labelling of p24 (132). Although such studies are limited by the lack of virion tracking to determine if virions which uncoat early, productively infect a cell (132, 133).

The absence of CA in the RTC is also supported by the extraction of RTCs on separation by equilibrium density fractionation (134, 135). It was determined that while fractions containing intact purified RTC could effectively reverse transcribe in the presence of dNTPs, measured by the endogenous reverse transcription (ERT) assay (134), fractions
containing CA and matrix (MA) could not complete the synthesis of strong stop DNA (135). However, this method of reverse transcription quantification is flawed due to the measurement of much less vDNA synthesis past strong stop.

Other studies suggest RTC formation is initiated from a short nucleotide fragment (approximately 101 nucleotides in length), which contain the tRNA^Lys primer binding site, such as strong stop (136). The hypothesis that strong stop synthesis initiates uncoating and RTC formation, is supported by the discovery that inhibition of reverse transcription, by addition of the NNRTI nevirapine, both blocks early RNaseH activity and inhibits CA dissociation (128). These data have also been replicated using an in situ uncoating assay and suggest the process of 1st DNA strand transfer is the trigger for CA uncoating (137). Uncoating assays, however, cannot measure which virions are infectious.

Aside from biochemical assays, labelling of RTC or preintegration complexes (PICs) by GFP-Gag, GFP-Vpr or IN-GFP have allowed for the visualisation of complexes at the time of reverse transcription completion (138, 139). However, direct tagging and visualisation of CA was not possible until the use of a novel CypA-CA probe oligomeric cyclophilin A-DsRed chimera (CypA-DsRed). CypA-DsRed can be incorporated into CA particles without affecting stability (139). Visualisation of CypA-DsRed tagged virions found that the majority of CA was lost rapidly, taking around two minutes to complete uncoating after a fusion event. However, a small number of particles retained CA for longer, uncoating on progression of reverse transcription (139). Both this study and the later use of triple tagged p24, failed to identify which particles were infectious, a caveat to all such experiments (132, 139).

Supporting a model of two fates of capsid, thus both early and late uncoating, is data obtained from the EURT assay, which measures entry/ uncoating based on core-packaged RNA availability and translation. This unique assay determined the presence of two CA core types, one in which the genome is readily exposed and a second core, which decreases in stability during reverse transcription (140). However, while the process of reverse transcription seems the likely trigger for capsid uncoating, the exact stage of reverse transcription at which this process occurs remains controversial. This is further complicated by differences in data obtained in cell lines or primary cells.
Figure 8: A model for post entry CA behaviour

Some HIV-1 capsid cores will remain intact on entry into the host cytoplasm. Recruitment of the cofactor CypA may promote stability to further protect ongoing encapsidated reverse transcription. (1) On nearing the nuclear pore complex CA starts to disassemble to release newly transcribed vDNA. (2) However, some HIV-1 CA uncoat early. (3) While some vDNA is degraded by host exonucleases (4) unprotected vDNA triggers innate DNA sensors, such as cGAS (5) or polyglutamine-binding protein 1. (6) In MDM IFI16 may also sense vDNA. (7) Similarly, virions entering through endosomal routes can be sensed by toll like receptors (TLR), (8) which drive the activation of IL-1. Irrespective of the sensing pathway all pathways result in the activation of transcription factors IRF3 or NF-κB, which drive the production of type 1 IFN. Figure from (Sumner, R. P., et al., 2017) (126).
Figure 9: The HIV-1 lifecycle model with cytoplasmic uncoating

(A) On binding CD4 and coreceptors CXCL4 or CCR5 HIV-1 fuses with the host membrane. (B) On fusion the viral capsid is released into the host cytoplasm where it uncoats releasing viral RNA and proteins and (C) initiates reverse transcription. (D) On completion of reverse transcription, the preintegration complex is formed before translocation to the nucleus. (E) In the nucleus vDNA is integrated into the host genome and (E) transcribed and (F-G) translated into new vRNA and viral proteins, which translocate to the cell surface. (H) After assembly of immature virions, the new virus buds and is released where the virus is (I) matured on Gag cleavage by protease enzyme. Figure from (Barre-Sinoussi, F., et al., 2013) (2).

1.3.2.2 A model for late capsid uncoating

Whilst a number of HIV capsid cores undergo at least partial uncoating, in the host cytoplasm, we propose virions which uncoat early do not lead to productive infection in primary cells. We propose that this is due to exposed vDNA being detected by the host innate immune response (Figure 8.2) (126). Innate immune triggering within monocyte derived macrophages (MDMs) has previously been measured on infection with cofactor binding mutations, P90A, which prevents CypA binding, and N74D, preventing CPSF6 binding (141). Gao et al reported activation of the cGAS-STING pathway on MDM infection, with both SIV and MLV, suggesting these viral capsids, unlike wild type HIV-1, have a reduction in core stability (142).

Models of early CA uncoating cite that CA subunit association with the PIC negatively impacts integration and infection through the recruitment of host restriction factors such as TRIM (143, 144).

Effective integration has also been shown to require capsid binding of the karyopherin TNPO3, which is recruited by the same pocket as the host cofactor CPSF6 (145, 146).
However, direct TNPO3-CA binding has been widely contested, with Maertens et al compellingly showing TNPO3 binding to the host cofactor CPSF6 (147).

Further supporting the retention of capsid, until docking at the nuclear pore complex, is the measurable loss of CA prior to translocation in WT HIV-1. Virions lacking a central DNA flap (described in section 1.3.5) retain CA for much longer, with CA detected in the perinuclear region up to 73 hours post infection (133). These data suggest, that CA retention is both required for docking at the nuclear pore and the completion of reverse transcription prior to integration. Whilst it is evident that encapsidation of vDNA synthesis in primary cells is optimal for productive infection it is yet to be determined if cell lines, in which innate sensing pathways are inactive, also require the encapsidation of RT for optimal infection.

1.3.3 Cellular detection of reverse transcription products

Our recent model, describing two of the possible fates of capsid, suggest that while some capsids remain intact within the cytoplasm others undergo an early uncoating event which leaves both vRNA and newly synthesised vDNA exposed to the cell autonomous innate immune response (Figure 8.2) (126). In primary cells, which express high levels of host exonucleases, such as TREX1, exposed vDNA may be effectively degraded (Figure 8.3) (148). However, in the absence of TREX1, viral DNA is sensed by the DNA sensor cyclic GMP–AMP synthase (cGAS) (Figure 8.4) (148). cGAS on binding cytoplasmic DNA promotes the synthesis of GMP-AMP synthase (cGAMP) (149, 150). STING, an ER resident signalling protein, which senses dinucleotides, engages with newly synthesised cGAMP and is translocated through the Golgi, from the ER, to the perinuclear region (Figure 8.5) (149). Once near the nucleus, STING activates IKK and TBK, which enhances p65 and p50 activation and phosphorylates IRF3 (Figure 8.9). This signalling results in the activation of IRF3 and NF-κB promoting the production of IFN-I. IFN-I production then leads to a positive feed back regulatory loop of IFN production, between other uninfected cells in the surrounding location (151, 152).

vDNA is not the only pathogen associated molecular pattern (PAMP) recognised by cGAS. RNA:DNA hybrids have also been determined to promote IFN-I production along with viral ssRNA, which can be sensed by RIG-I and TLRs (153) (154). Exposure of viral nucleic acid therefore stimulates both the NFκB and IRF3 pathways and also drives the production of pro inflammatory cytokines (Figure 8.7-8.8) (155, 156).
1.3.4 HIV-1 cofactor requirements in early infection

1.3.4.1 CPSF6

Cleavage and polyadenylation specificity factor-6 (CPSF6) is a pre-mRNA processing protein mostly located within the nucleus of a host cell, however, CPSF6 is able to move between the nucleus and cytoplasm (157, 158). Manipulation of the CTD region of the protein alters the dynamics of CPSF6 movement causing cytoplasmic build up. The cytoplasmic retention of CPSF6 subsequently blocks HIV-1 WT infection, suggesting that CPSF6 is required for effective transportation of HIV between the nucleus and cytoplasm of a host cell (159).

CPSF6 directly binds a diverse range of lentiviral CA cores. The CPSF6 binding site, located within the HIV-1 CA NTD, is comprised of a pocket formed by helix 4, helix 3 and helix 5, via CPSF6 residues V314, L315 and F321 (Figure 10A) (160). Yet, the absence of CPSF6 binding does not directly impact CA stability, uncoating and infection. Rather, the inability to bind CPSF6 affects HIV after reverse transcription through the reduction in nuclear transport, by reduced nuclear pore associated nucleoporin recruitment to CA (160). This is also observed in the A77V CA mutant, which has reduced binding efficiency of CPSF6 which renders HIV infection independent from TNPO3, NUP358, and NUP153 (161). This suggests that CPSF6 guides HIV-1 to use a specific nuclear entry pathway, yet, how CPSF6 achieves this remains unclear.

We previously measured the production of IFN-I in MDMs on infection with the full length N74D mutant, which is unable to bind CPSF6. IFN-I production correlated with the block to both single-round infection and viral replication. These data clearly demonstrate the requirement for CPSF6 recruitment and that downstream interactions with NPC components is essential for HIV-1 to avoid triggering innate immune DNA sensors in primary cells (141). More recently, we have determined the requirement for CPSF6 binding in primary CD4+ T-cells. In CD4+ T-cells, the block to both single-round infection and replication of the N74D mutant is attributable to a block to early reverse transcription, from strong stop DNA (data unpublished). The role for CPSF6 recruitment on DNA synthesis and CA stability is also supported by the observation that expression of CTD truncated CPSF6 in cell lines is inhibitory for HIV-1 infection (162).

Interestingly, N74D, as well as the A87V CA mutant also has a loss of TNPO3, RanBP2 and NUP153 dependence. Considering these are all host nuclear pore associated proteins, involved in transportation via the nuclear pore complex (NPC), these data suggest a further role for the recruitment of CPSF6 for nuclear entry and integration (159, 163, 164).
While some models propose the binding of CPSF6 on entry of the CA to a host cytoplasm for cloaking, CPSF6 has also been found to play a more significant role during viral integration. The knockdown of CPSF6 in U2OS cells was found to decrease viral infectivity and proviral formation through a decrease in viral integration into transcriptionally active genes or regions of chromatin enrichment. Previously integration was attributed to the cofactor LEDGF/p75, which on binding integrase (IN) forms a complex to activate IN (165-168).

**Figure 10: CPSF6 and CypA recruitment to HIV-1 (M) capsid**

(A) Crystal structure of HIV-1 (M) CA (blue) bound CPSF6 (multi coloured sticks) with resolved β-hairpin (yellow) by (Price et al., 2012) (160) (PDB 4UOA). (B) Crystal structure of HIV-1 CA monomer (blue) bound CypA (green) through contact with key residues H87 (magenta) and P90 (red) by (Liu et al., 2016) (169) (PDB 5FJB).

### 1.3.4.2 Cyclophilin A (CypA)

Cyclophilin A (CypA) is a member of the wider family of human cyclophilin proteins which are conserved in sequence, of approximately 150 nucleotides in length, and defined by their prolyl-isomerase activity. Ranging from 18-40 kDa in size, the abundant cellular proteins have been located in a range of subcellular compartments, including the cytoplasm, ER and nucleus (170). First detected in 1984, from their binding to the immunosuppressive drug cyclosporine A (CsA) (171), family members CypA, B, C, 40, 60, CARS-CyP and NK-TRCyP were later determined to bind HIV-1 Gag (172).

CypA binds directly to the cyclophilin binding loop of the HIV-1 capsid NTD, by forming an eight stranded β-barrel structure, comprising a hydrophobic pocket (173, 174). This pocket can bind the Cyp binding loop of capsid via residues A88, G89 and P90 (175). It is the CypA pocket that the drug CsA also binds, effectively inhibiting CypA-HIV-1 CA binding at a 10 μM dose (176). However, it is known that CsA at 10 μM is toxic, therefore a better dose for experiments is 5 μM.
Crystallography studies of CypA binding have determined the preferential binding of multimerized capsid over monomeric subunits (174). A recent cryo-EM study measured the preferential binding of CypA to two CA hexamers, through forming a stabilising bridge over the CA cone (Figure 10B) (169). The use of atomic force microscopy has provided further mechanistic insight into the relationship between CypA recruitment and CA cone stabilisation. For example, it was determined that the increased input of CypA to promote a ratio of three CypA bound to six CA had a direct effect on CA stiffness by promoting hexamer-hexamer interactions. However, increasing the CypA:CA ratio to 6:6 had a negative impact on core stability, decreasing overall structural stiffness (177). Suggesting that CypA recruitment to CA must be at an optimal ratio.

This stabilising role of CypA binding to the CA cone is also supported by data from our lab. We demonstrated that the P90A CA mutant, which is unable to recruit CypA, had a significant block to single-round and spreading infection in MDMs. This infection defect was due to the triggering of an innate response, quantified by the production of IFN-I (141). From these data, we defined this stabilising role of CypA recruitment to CA as viral cloaking. While P90 seems to be a key residue in infection efficiency, mutants at CA positions 87, 88 and 89 have also been described as having a block to infection due to CA core instability (174, 178). Furthermore, single mutations in CA also prevent the packaging of CypA into HIV-1 virions (179). Building upon these data, demonstrating a structural and protective role in the early HIV-1 lifecycle (Figure 11), we have recently determined that CypA is recruited during infection of CD4+ T-cells for completion of reverse transcription. Our data demonstrate an early block to HIV-1 reverse transcription, by inhibiting strong stop synthesis, in the P90A mutant (data unpublished).

Aside from the recruitment of CypA to HIV-1 CA to promote core stability, the binding of CypA has also been found to impact viral integration site preferences. Schaller et al determined that CypA binding CA mutants P90A and G89V were also unable to bind nuclear pore associated protein NUP358, which bind to CA via the Cyp binding loop (163, 166). This lack of both CypA and NUP binding led to the integration of vDNA within an alternative region of the host genome, of high gene density (Figure 11). These effects could also be replicated on the treatment of WT virus with CsA (163).
On entry into the cytoplasm CA recruits host cofactors CPSF6 and CypA. After reaching the nuclear pore CA binds NUP358 and NUP153 uncoats and releases newly transcribed vDNA through the nuclear pore where it integrates in a region of optimal gene density in the host genome. Alterations to this pathway through disrupting CPSF6 binding by CA mutation or altering CPSF6 nuclear trafficking truncation of CPSF6 or altering CypA binding on addition of competitive drug CsA or CA mutation bypasses the pathway of integration, evidenced by reduced requirement of NUP358 and NUP153, resulting in less efficient viral integration into low or high gene density regions. Figure adapted from (Schaller, T., et al., 2011) (163).

1.3.5 Reverse transcription

1.3.5.1 The process of HIV reverse transcription

Despite contention over the location of vDNA synthesis the process of reverse transcription is well defined. A characteristic of all retroviridae is the process of copying single stranded viral RNA into double stranded DNA prior to integration into a host genome. The process of HIV-1 reverse transcription requires the function of two domains in RT p66 and p51 regions (Figure 6B) (69). RNaseH, which functions to cleave RNA part of the RNA:DNA duplex, while DNA polymerase, comprised of four subdomains (fingers, palm, thumb and connection) copies both RNA and DNA templates (56, 58). The separate functions of both RNaseH and polymerase domains of reverse transcriptase cooperate during the process of reverse transcription.

Reverse transcription is initiated from the 18 nucleotide long primer-binding site (pbs), at the 5’ end of the viral genome. This pbs functions as a template for the hybridisation of 18 complementary nucleotides from the 3’end of human tRNALys3, which functions as the
primer of reverse transcription (180). On tRNALys3 hybridisation, reverse transcriptase associated DNA polymerase initiates transcription of the 1st (-)DNA synthesis, along the RNA genome template. tRNA elongates until the end of the 5’ssRNA, producing the first strong stop (-)DNA. It has been estimated that the production of strong stop (-)DNA takes around two hours to complete (Figure 12 step 1) (181).

Synthesis of (-)DNA generates a RNA:DNA hybrid product, which functions as a substrate for the reverse transcriptase associate RNaseH. RNaseH is therefore able to degrade the RNA strand of the hybrid, freeing the 5’ R region of the (-)DNA. The 5’R binds to the 3’ end R region, of the viral ssRNA template, both 3’ and 5’ R ends are identical. This process is termed 1st strand transfer (Figure 12 step 2) (182).

After successful 1st strand transfer, (-)DNA synthesis continues from the 3’ to 5’ end of the RNA template (183). As DNA synthesis continues, RNaseH continues to cleave the RNA:DNA hybrids at multiple points. RNaseH cleavage sites do not appear to be sequence specific. However, two purine rich sequences, (polypurine tracts) cPPT and 3’PPT, are both resistant to RNaseH activity (Figure 12 step 3) (58).

The preserved 3’PPT and cPPT sequences now function as a primer for (+) strand DNA synthesis. Elongation of +strand DNA continues to the 5’ end of (-)DNA, also recruiting the first 18 nucleotides tRNA PBS sequence as a template (Figure 12 step 4) (184). However, the presence of a methyl A in the 3’ end tRNA sequence, the 19th nucleotide terminates elongation generating (+) strand strong stop DNA. Activity of RNaseH again cleaves the RNA portion of RNA: DNA hybrids promoting the annealing of the (+) strand pbs to its complementary sequence on the 3’ (-) strand DNA. While RNaseH of other retroviruses removes the entire tRNA at this stage, HIV-1 only cleaves 1 nucleotide from the tRNA/DNA junction. This leaves a ribo-A (ribonucleotide) at the –DNA 3’ which is required for second strand transfer (Figure 12 step 5) (185).

After 2nd strand transfer, removal of tRNA exposes (+)ssDNA, the sequence of which matches the PBS. This allows the 5’ end of (-)DNA to be copied over. Bidirectional DNA synthesis completes the dsDNA, however, due to earlier (-)strand elongation, vDNA is longer than the RNA from which it is derived. The resulting ssDNA central flap functions, on integration, as a template for the copying of new viral genomes by the host DNA dependent RNA polymerase (58). The central flap may also be removed later through cleavage, possibly by the flap endonuclease1 (FEN-1) (Figure 12 step 6) (68).

The final stage of HIV reverse transcription is the removal of PPT RNA primers to expose the viral integration sequence, promoting its insertion into an optimal region of the host chromosome (Figure 12 step 7).
Figure 12: The order of events during HIV reverse transcription

1. Host tRNA (yellow) hybridises to the 5' PBS on the (+) strand viral RNA (black line). (-) strand synthesis (purple arrow) initiates towards the 5' end using host tRNA as a primer.
2. RNase H (red arrow) hydrolysies the RNA:DNA hybrid exposing ssDNA, the (-) strand strong stop DNA.
3. (-) strand DNA is transferred by hybridisation to the 3' R region of the ssRNA viral genome and continues to extend.
4. RNase H cleaves RNA from RNA:DNA hybrid at multiple sections as DNA extends from 3' end, leaving intact resistant cPPT 3'PPT sections.
5. (+) strand DNA synthesis is initiated from both cPPT and 3'PPT sections which function as primers. RNaseH next hydrolysies these PPT segments and at the tRNA:DNA hybrid junction, thereby releasing the PBS of the (+) strand DNA.
6. The (+) strand DNA PBS is transferred and anneals to the (-) strand DNA PBS. On PBS joining (+) strand DNA synthesis continues.
7. On completion of dsDNA PBS primers are removed by cleavage. Expose of integration sequence promotes insertion into the host. Figure adapted from (Esposito, F., et al., 2012) (68).
1.3.5.2 The timing of reverse transcriptase inhibitor function

AZT triphosphate (AZTTP), the active form of the dTTP (thymidine triphosphate) analogue, was the first FDA approved reverse transcriptase inhibitor. Despite approval of AZT in 1986, the exact timing of inhibition during reverse transcription was unknown.

Early studies of HTLV-III replication in 1987, used a synthesised Klenow fragment of E.Coli RNA polymerase and reverse transcriptase from avian myeloblastosis, to mimic reverse transcription. The addition of ddNTPs, at low concentration, was found to terminate HTLV-III early reverse transcription measured by Sanger sequencing of fragments (186). In the same year, another publication reported the inhibition of single-round and spreading infection of the avian leukosis virus, a retrovirus, on addition of AZT. Southern blot analysis of vDNA found a 90% decrease in vDNA production, after strong stop completion, on treatment with a high dose of AZT, at 200 μM (187).

On purification of HIV-1 reverse transcriptase, reverse transcriptase inhibitor molecules were seen to be incorporated, by denaturing RNA gel electrophoresis, from 20 minutes after addition terminating DNA synthesis during the extension phase (188). Later, the NRTI 3TC was also found to terminate early reverse transcription during chain extension (189). St Clair et al., also determined that one molecule of inhibitor was inserted every 1.5-2.4 available initiation sites (188).

Whilst chain terminators were found to inhibit during early reverse transcription, due to insufficient techniques, the exact timing of NRTI function remained elusive. However, the extraction of NRTI truncated low molecular weight DNA and quantification by qPCR amplification found that three NRTIs: 3TC, AZT and ddI, truncated vDNA after the synthesis of (-)strong stop (190).

The use of PCR provided greater insight into the timing of both NRTI and NNRTI function. NRTIs such as AZT, were found to inhibit intermediate length vDNA products. While NNRTIs, such as NVP, had no effect on (-) strand DNA but greatly inhibited products greater than 4 kb in length (181). The timing of AZT inhibition has also been confirmed on measurement of the rate of polymerization during reverse transcription, however, this study found AZT also inhibited the production of (-)-strong stop vDNA (191).

More recently, the timing of RTI function has been measured in the natural endogenous reverse transcription (NERT) assay. The NERT assay involves the isolation of cell free viral particles and without the use of detergent to permeabilise the CA cone, the measurement of RT on the addition of dNTPs and membrane pore forming substance, melittin, in vitro (192, 193). Using the NERT assay, the NNRTI NVP was found to inhibit during jumping stages of reverse transcription, such as strand transfer (194, 195). NRTIs also inhibited during elongation stages of reverse transcription (194).
The development of strand specific DNA amplification and real time PCR quantification, using duel labelled TaqMan probes, allowed for the specific timings of each stage of reverse transcription to be measured. First quantified in the 293T cell line and primary CD4+ T-cells, it was determined that minus strand synthesis took 104-105 minutes, minus strand transfer 11 min, plus strand transfer 28 min, PPT initiation 12 min and cPPT initiation 31 min (196).

Using these timings, the addition of NRTIs AZT and d4T, at an IC90 dose, inhibited (-) strand DNA while another NRTI ddi and the NNRTI NVP had a greater inhibitory effect on (+)strand DNA (196). It is thought that NRTIs may preferentially inhibit during multiple stop sites which occur during the elongation stages of reverse transcription (197). While, even on the increase of NNRTI dosage to >IC90, NNRTIs do not inhibit (-) strand synthesis (181) (198). Variation in NNRTI structures has been linked to differing effects of inhibitor efficacy. Furthermore, the study of NNRTI efficacy has been complicated by differing IC50 values in polymerase of RNAseH based assays (199, 200).

1.3.6 Capsid or PIC docking at the nuclear pore

γ-retroviruses, such as MLV infect a host cell during cell division, at the stage of nuclear breakdown during mitosis. However, lentiviruses such as HIV are characterised by their ability to infect non-dividing cells. To achieve this, HIV has evolved the ability to utilise the host cell nuclear pore (201).

1.3.6.1 Nuclear pore complex structure

The nuclear pore complex (NPC) is a 50 MDa cylindrical shaped structure embedded within the nuclear envelope. First discovered in 1950, the NPC is comprised of multiple copies of around 30 different nucleoporins (NUP) (202). Each class of NUP, which are based around a central channel, form different substructures which link to the peripheral basket (Figure 13) (203, 204). The central channel/ pore is comprised of FG-NUPs, which contain up to 50 Phe-Gly repeat regions. FGs in NUPs are joined together by a hydrophilic spacer region and function during karyopherin mediated transport, functioning as docking sites for transport receptors (205).

The NPC functions to facilitate trafficking between the cytoplasm and nuclear compartments. However, transportation is limited by the size of the central channel, which is 90-100 Å, or 39-40 nm in diameter (206-208). Therefore, the NPC can only passively transport molecules that are 40 kDa or less (208). This raises a problem for the transportation of the HIV-1 PIC, which is approximately 56 nm in diameter (209) and the HIV-1 CA cone, which is 50-60 nm in diameter and 210 nm in length (133, 210). From these measurements, it is highly unlikely that either structure can traverse the NPC intact. Therefore, structures must disassemble at the pore, prior to vDNA entry (206-208).
The two-way directional movement of the nuclear channel is determined by a RanGTP gradient. However, specific nuclear localisation signals are required for the initiation of import and export perhaps initiated on docking of the PIC at the NPC (211).

1.3.6.2 The role of nucleoporins and transportin in the NPC

Previous genome wide RNAi screening identified three host proteins located at the NPC, nucleoporins: NUP153 and NUP358, also referred to as RANBP2, and transportin 3 (TNPO3), also referred to as TRN-SR2, although TNPO3 was first identified prior to this screen. All three were found to have capsid dependent functions, prior to viral integration (212-214).

NUP153 is a FG nucleoporin, so called due to the extensive repeats of phenylalanine-glycine (FG) domains, is anchored to the nuclear rim of the NPC by its NTD region (Figure 13). The CTD of NUP153 is highly flexible and 200 nm in length. This flexibility allows the FG regions of the NUP to reach through the nuclear pore to the cytoplasmic side, forming the major structural component of the cytoplasmic NPC filaments along with NUP358 (215-217). Interestingly, NUP153 has been determined to contribute to HIV-1 infection. On depletion by siRNA, in Jurkat T-cells, wildtype HIV-1 infection was blocked by more than 10 fold (218). Furthermore, the CPSF6 binding capsid mutant N74D, which is unable to bind TNPO3 (159), was also only inhibited by 10 fold, in NUP153 depleted cells (218). This was previously explained by the utilisation of an alternate NUP independent integration pathway for the N74D mutant (Figure 11) (163). NUP153 depletion, therefore, results in a reduction of 2-LTR circles and vDNA integration into suboptimal regions of the host genome (167, 219).

Aside from the protein’s role in integration site targeting, binding competition assays have measured the binding of NUP153 to HIV-1 CA, via the CPSF6 binding pocket (Figure 10A). NUP153 competitively binds CA reducing CA bound CPSF6. It has been hypothesised that this functions to orientate the viral CA at the nuclear pore. Therefore, NUP153 binding can also be prevented on treatment with the CPSF6 binding pocket targeted drug PF74 (160).

The nucleoporin NUP358 is the largest known FG nucleoporin (220). Comprising the cytoplasmic filaments of the NPC cytoplasmic ring NUP358 is anchored through interactions with other cytoplasmic facing NUPs such as NUP88 and NUP214 (221). Due to the cytoplasmic location and presence of 4 RanGTP binding domains and 2 homologous zinc finger domains, NUP358 functions as a Ran GTPase activator (222-225). NUP358 therefore functions during import, assisting the removal of importins bound to RanGTP (226) and during export in the removal of cargo from exportin complexes (217, 227).
Further to the role for NUP358 in the bidirectional transport through the nuclear pore, like NUP153, NUP358 plays role in the docking of the HIV-1 CA core to the NPC. In fact CA is the only known target of NUP358 (228). NUP358, binds to CA via the NTD CypA homologous domain but with weaker affinity than CypA (229). Aside from docking at the NPC, NUP358 binding to CA is associated with the triggering of uncoating on cis-trans isomerisation of the G89-P90 bond (229). Supporting this, it is known that the P90A, CypA binding mutant, is also unable to bind NUP358 (163). Depletion of NUP358 also reduces infection through a reduction in nuclear entry, which is evidenced by normal levels of reverse transcription but reduced 2-LTR circles and infection. Furthermore, depletion of NUP358 also leads to the misintegration of vDNA, into regions of lower gene density, and alters the morphology of the NPC (163, 166, 217).

The karyopherin transportin 3 (TNPO3) is a member of the β-importin super family. Able to bind RanGTP via the NTD, TNPO3 is involved in the import of SR rich splicing factors into the nucleus (212, 213, 230). TNPO3 also binds HIV-1 integrase (IN) with high affinity, although this is lost in RanGTP mutants. The biological relevance of TNPO3-IN binding is uncertain however, this suggests that IN might be the main cargo of TNPO3 through the nuclear pore (231, 232).

The HIV-1 host cofactor CPSF6 is also a cargo of TNPO3. TNPO3 has been found to bind to CPSF6 via arginine-serine dipeptide repeat domains within the cofactor. While, the depletion of TNPO3 is thought to have no significant effect on the localization of CPSF6, one study found CPSF6 moved to the cytoplasm on depletion of TNPO3 (146, 233). However, the depletion of TNPO3 has a negative effect on nuclear entry, as measured by the depleted production of 2-LTR circles (146, 159, 233, 234).

One study suggested that TNPO3 also binds to HIV-1 CA, the strength of which is increased in the presence of RanGTP and decreased in the CPSF6 binding mutant N74D. These data suggested that TNPO3 may function as a nuclear localization signal driving CA uncoating (145, 234, 235). However, this study does not fit with more compelling work demonstrating the role of TNPO3 to import the cofactor CPSF6 (147).
A cross section of the nuclear pore complex comprising the cytoplasmic ring and ring associated filaments such as NUP358 (red). The central transport channel traverses the nuclear membrane, comprised of structural scaffold NUPs (yellow). The nuclear ring and basket (blue) comprised of NUPs such as NUP153. Figure adapted from (Raices and D'Angelo, 2012) (202).

1.3.7 Integration

1.3.7.1 The process of HIV integration

Integration is an essential stage in the retrovirus lifecycle which is initiated by the virus encoded integrase (IN) protein. IN is delivered into the host cell nucleus either packaged in an intact capsid core or as part of the RTC, by association with newly transcribed vDNA (236). Integrase functions in close association with the host factor LEDGF/p75. LEDGF/p75 functions early in integration to tether integrase to the selected chromatin site in the host genome and protect it from proteolytic degradation (237, 238). However, not all vDNA, which enters the nucleus, is successfully integrated. The 2 ends of vDNA can be ligated together, to form 2-LTR circles, by non-homologous end joining. 1-LTR circles can also be formed by recombination.
Studying the X-ray crystallography structure of HIV-1 integrase and integration intermediates, has provided further insight into its mechanism (239). The process of integration is carried out by the intasome, a complex of nucleoproteins comprised of a tetramer of IN which is assembled on the ends of viral DNA (240).

The process of integration can be divided into stages (241). The first stage of integration is 3’ end processing, which is the removal of two nucleotides from each 3’ end of the newly transcribed linear vDNA (Figure 14A-B). 3’ end processing results in a 5’ over hang at each end of the vDNA (Figure 14B). The shortened 3’ ends now target phosphodiester bonds within the host DNA at 2 separate sites five nucleotides away from each other. On binding, this results in covalent bonding between viral 3’ and the targeted host DNA (Figure 14C). On bonding the integration intermediate structure is formed (Figure 14D). The completion of integration requires the repair of the single strand gaps in both viral and target DNA, by cellular enzymes followed by the ligation of the viral DNA 5’ end over hang (Figure 14E) (236).

![Figure 14: HIV-1 vDNA integration into the host genome](image)

(A) On entry into the nucleus vDNA must integrate into the host genome. (B) vDNA is processed at both 3’ ends, (C) resulting in a 5’ overhang. (D) The integration intermediate is formed (E) prior to the final stage of integration the repair of vDNA and host DNA gaps and ligation of the 5’ overhang. Figure from (Craigie and Bushman, 2012) (236).
1.3.7.2 Factors determining the HIV-1 pathway of integration

HIV-1 host cofactors CPSF6 and CypA are thought to have a role in HIV-1 capsid stability resulting in optimal infectivity. As previously discussed in section 1.3.6.2, both cofactors, CPSF6 and CypA, binding sites are shared with NUP153 and NUP358 located at the nuclear pore. It is perhaps, therefore, unsurprising that both cofactors determine the integration site of newly transcribed vDNA.

Integration site sequencing found that HIV-1 capsids able to recruit both CypA and CPSF6 led to interactions with NUP. This series of interactions within the nuclear pore complex, the binding order of which is unknown, promotes integration of vDNA, on entry into the nucleus, into an active region of the host genome. This is evidenced by the integration of vDNA into regions of low or very high gene activity. Termed ‘alternate pathways of integration’, this occurs on the absence of CypA or CPSF6 binding to CA on treatment with targeted drugs, such as CsA or PF74 or on CA mutation. During alternate pathways of integration, NUP153, NUP358 and TNPO3 binding is no longer a requirement for infection, evidenced by insensitivity to their depletion (159, 163, 166).

1.3.8 Transcription, virus assembly and budding

1.3.8.1 Transcription of HIV-1 proviral DNA to RNA

After vDNA integration, into the host genome, HIV proviral DNA is transcribed (Figure 9E). Transcription, driven by the host polymerase II, is initiated from the 5’ LTR on binding of the cellular factors NFκB, SP1 and TATA (242). The newly produced viral transcripts are then spliced and translated.

Tat is an absolute requirement for HIV-1, evidenced by ΔTat virus being uninfected. Furthermore, Tat greatly increases the efficiency of the elongation phase of transcription. Tat functions through the binding to the transactivation response region (TAR) of RNA, at the 5’ end, mediated through a 5’ 3 nt pyrimidine bulge in TAR (243-245). A 6 nt loop in TAR also promotes the recruitment of a transcription elongation factor, pTEFb (246).

pTEFb, on Tat dependent binding, functions to phosphorylate the RNA polymerase II CTD to enhance the elongation phase of transcription (247). pTEFb also assists in the recruitment of the TATA box binding protein to the 5’ LTR promoter, thus assisting in the formation of Pol II transcription complexes (Figure 15) (248, 249).
Figure 15: Tat driven HIV-1 transcription

Tat binding to the 5’ LTR TAR region drives the formation of transcriptional complexes comprising cellular factors NFκB, SP1 and TATA along with the phosphorylated RNA polymerase II which enhances transcriptional elongation. Figure from (Karn, J., 1999) (245).

1.3.8.2 RNA export, translation and viral assembly

Viral RNA subsequently must traffic out of the nucleus (Figure 9F) to function as both genomic RNA, for viral packaging, and messenger (mRNA) for translation. HIV-1 mRNA can either be singly or multiply spliced. Therefore, to overcome the block to single or unspliced RNA being exported from the nucleus, the HIV accessory protein Rev is required (242). Rev, on binding to the Rev response element (RRE), located in the env mRNA coding region, interacts with the chromosomal maintenance 1 (CRM1) RanGTP nuclear export complex (250-253).

Full length unplicated mRNA functions as a template for the Gag precursor (pr55) polypeptide and Gag-Pol translation. The main HIV structural proteins capsid (CA), matrix (MA) and nucleocapsid (NC) are then synthesised through host translation machinery (Figure 9G) (254).

After Gag cytoplasmic trafficking to the assembly site, the binding of Gag to genomic RNA, guided by packaging signals, causes Gag multimerisation. This drives the assembly of immature virions, which contain 2 copies of the capped and polyadenylated full-length RNA genome (Figure 9G) (255-257). Following assembly, virion budding is initiated through both the late domains within Gag and host cell class E vacuolar sorting proteins (VPS), such as the ESCRT family. Both Gag and ESCRT proteins drive the final stages of viral budding and release of immature virions from the host cell plasma membrane (Figure 9H) (258).
1.3.9 HIV-1 maturation and Gag cleavage

The final stage of the HIV-1 viral lifecycle is the maturation of immature virions. Both Gag and Gag-pol precursors undergo proteolytic cleavage by viral protease, in a cascade, to produce processed MA, CA, NC, p6, PR, RT, and IN proteins (259). The first cleavage site is located between spacer peptide 1 (SP1) and NC, followed by MA-CA, SP2-P6 and SP2 removal from NC. The final stage of maturation is always the removal of SP1 from CA (Figure 16) (260). On completion, mature HIV virions are now infectious and comprise a stable double RNA genome surrounded by the capsid cone structure. MA, associated with the viral membrane, forms an encompassing protective shell (Figure 9I) (261).

![Diagram of Gag cleavage](image)

**Figure 16: HIV-1 sequence of Gag cleavage, by protease, and virus maturation**

Gag processing by protease cleavage is an essential part of the viral lifecycle. (1) The maturation cascade starts with the cleavage between spacer peptide 1 (SP1) and nucleocapsid (NC), (2) before cleavage between both matrix (MA) and capsid (CA) and SP2 and p6. (3) SP2 is removed from NC and the final stage of the maturation cleavage cascade is the removal of SP1 from CA. Figure adapted from (Wang, M., et al., 2017) (262).
1.3.10 Restriction of HIV-1 during early stages of the viral lifecycle

Aside from targeted antivirals, which inhibit multiple stages of the HIV-1 lifecycle, such as: fusion, entry, reverse transcription, integration and protease driven maturation (Figure 16), the host cell also employs proteins with antiviral activity functioning as restriction or resistance factors. Here we briefly discuss antiviral factors targeting capsid, reverse transcription, nuclear entry and integration activity (Figure 17) (126).

![Figure 17: The HIV life cycle and cellular restriction factors](image)

On release into the host cytoplasm HIV-1 must overcome innate restriction factors. SERINC, prevent virus fusion but are antagonized by HIV-1 Nef. IFITMs, impair viral entry but are overcome by Env evolution. TRIM5 targets CA for degradation but is overcome on CA evolution. APOBEC3 suppresses reverse transcription, antagonized by Vif mediated degradation. SAMHD1 restricts infection efficiency by depleting nucleotides to inhibit RT, antagonized by Vpx. MxB restricts nuclear entry. Schlafen 11, restricts translation and tetherin inhibits viral release, antagonized by Vpu. Figure from (Sumner, R. P., et al., 2017) (126).

1.3.10.1 TRIM5α

On entry of the HIV-1 capsid into the cytoplasm, after fusion, TRIM5α is able to bind (Figure 17). First identified as an antiviral in old world monkeys (OWM), TRIM5α effectively inhibits multiple retroviruses. TRIM5α binding to HIV-1 CA is highly specific due
to the presence of unique C-terminal PRYSPRY domains, which are modified, in simian species, to target a CypA domain (263-265). The greatest insight into the role of TRIM5α has recently been determined from a structural study, demonstrating the formation of a hexagonal lattice of TRIM5α (266, 267). This complex network covers the entire CA cone, providing multiple PRYSPRY contacts. This has been found to promote early cytoplasmic CA disassembly, thus blocking the early stages of reverse transcription (268).

1.3.10.2 SAMHD1
Sterile alpha motif and histidine–aspartate domain containing protein 1 (SAMHD1), targets HIV-1 during DNA synthesis by reverse transcription (Figure 17) (269, 270). On activation, SAMHD1 functions to deplete the intra cellular dNTP pool, required to fuel the process of reverse transcription. The ability of SAMHD1 to deplete cellular dNTPs is regulated by the dNTPase activity of the catalytic core HD domain on activation by dephosphorylation (271). SAMHD1 is expressed in multiple cell lines and primary cell types and is sufficient to block infection in MDMs. However, even on activation, SAMHD1 is not sufficient to block HIV replication in dividing cell lines, perhaps due to the high level of dNTPs (272). Interestingly, while SIV has an accessory protein, Vpx, which targets and block the function of SAMHD1, SIVcpz does not have a Vpx. Therefore, on the evolutionary transmission of HIV-1 from SIVcpz (Figure 1) the ability to evade SAMHD1 restriction was lost (273).

1.3.10.3 APOBEC3
Apolipoprotein-B mRNA-editing, enzyme-catalytic, polypeptide-like 3 proteins (APOBEC3) are a family of ssDNA deaminases. Comprising seven enzymes, the APOBEC3 family function during HIV-1 reverse transcription (193, 274). APOBEC3G disrupts vDNA synthesis at multiple stages, either by preventing tRNA binding to the vRNA primer, thus disrupting (-) DNA strand transfer or inhibiting during the elongation stages of reverse transcription (Figure 17) (193, 275, 276). APOBEC3G also induces mutations during these crucial stages in reverse transcription rendering the resulting provirus uninfectious (277). To function, APOPEC3G must be packaged into virions prior to viral maturation. This is achieved through the interaction of APOBEC with vRNA bound to NC (278). HIV-1 is able to antagonise APOBEC by its accessory protein Vif. Vif functions to prevent the packaging of the restriction factor into a virion and also induces the degradation of APOBEC3. Vif is able to initiate APOBEC3 degradation by acting as the substrate for the Cullin5 ubiquitin ligase complex (279).
1.3.10.4 Myxovirus resistance (Mx) protein MxB

MxB is an interferon stimulated gene (ISG), which has limited antiviral activity against retroviruses, but is effective against HIV-1 (280). Despite the function of MxB remaining incompletely defined, studies suggest MxB functions after vDNA synthesis to inhibit vDNA nuclear entry (Figure 17) (281). However, MxB mutants also demonstrate reduced nuclear import, measured by a reduction in 2-LTR circles. These data suggest a secondary role for MxB, perhaps in the inhibition, or at least in reducing efficiency, of viral integration (282, 283).

1.4 The HIV-1 capsid cone

1.4.1 Structure of the HIV-1 capsid cone

Rearrangement of the Gag polyproteins during viral maturation, on cleavage by viral protease, results in the condensing of CA and NC around vDNA. This process forms the conical CA ‘core’ structure, which functions to protect and organise vDNA (284, 285). However, during the process of reverse transcription, vRNA also dictates the shaping of the cone, acting to concentrate NC-CA and neutralising charges across the structure (286). Visualisation of HIV-1 CA by cryo-EM has been a useful tool to the study of HIV-1 CA stability and uncoating, but has proven complex due to the different formations CA can take in solution. CA has been formed as spheres, tubes and cones dependent on conditions used (285, 287, 288).

The HIV-1 CA cone (Figure 18A) is comprised of hexameric (Figure 18B) and pentameric (Figure 18D) structural subunits. Generation of two all atomic CA fullerene cone models determined that the cone can be comprised of 1,356 monomeric subunits (model I) or 1,176 monomeric subunits (model II), which alters the shaping of the complete cone structure, dependent on conditions (289). Normally comprising 216 hexameric subunits, a complete cone must also contain exactly 12 CA pentamers (289).

The 12 pentameric CA ‘defects’ are specifically distributed at each end of the fullerene cone and provide structural curvature, which is required at each end to close the structure (Figure 18A). Seven pentamers are found at the wider end of the CA cone, and 5 at the thinner end. Due to the fundamental structural role of these 12 pentamers, CA cone models with differing distributions promote the formation of spheres or tubes rather than cones (285, 290).

Further to the study of the CA cone shape, the use of Cryo-EM has provided a unique insight into pentamer-hexamer CA-CTD dimer interface. It has been shown that these connections between pentamer and hexamer subunits promote capsid movement, which along with curvature of the pentamer subunits, allow for the closure of the CA cone structure (291).
CA hexamers (Figure 18B) are formed by connection of six CA monomers. Each monomer is formed from 2 independent domains the NTD and CTD, connected by a linker. This connector sequence (MYSPT) promotes some flexibility in the structure (Figure 18C) (174, 292). However, a series of experiments, by mass spectroscopy, have determined the linkage between NTD and CTD is intermolecular, therefore not between domains of the same CA as previously thought (293, 294). Cryo-electron tomography has also been used to measure the twist/tilt motion between the CTD-NTD flexible linker. Such techniques have further confirmed the flexibility in hexameric subunits (291).

Both crystallographic and EM studies have found the dimeric linkage between CA CTDs functions to connect adjoining hexamers and is therefore essential for promoting stability in the canonical structure (160, 295). Furthermore, CTD-CTD dimerization also promotes flexibility between hexamers during cone formation allowing for the incorporation of each NTD within the structure (296).

The CA NTD forms the outward facing hexameric structure of the intact cone, therefore the NTD comprises domains required for cofactor binding such as the CypA binding loop (Figure 10B) (290, 297, 298). NTD-NTD interactions are mediated through interaction between 3 helices, which can be disrupted through mutation, on substitution of surface facing residues in helices 1 and 2 (299).

### 1.4.2 Retroviral capsid structure

Despite a lack of Gag and CA sequence conservation between retroviruses, the structure of the CA NTD remains highly evolutionarily conserved. Spanning from the HIV-1 CA residue 153–172 the major homology region (MHR) between mammalian and avian retrovirus species is conserved, except for a few minor polymorphisms (300). Furthermore the structural components of retroviral particles are also conserved, comprising a CA structure, protecting a diploid RNA genome which is bound to NC (301).

Whilst all retroviruses encode CA the shape of the shell is variable. The HIV-1 CA assembles into a conical shape (Figure 18A), yet, Rous sarcoma virus (RSV) do not demonstrate uniform size or shapes (302), while MLV appear more spherical by cryo-EM (303). Like RSV, the delta-retrovirus HTLVs cores are also not uniform in shape (304).
Figure 18: Structural components of the HIV-1 capsid cone

(A) The complete CA cone (Zhao, G., et al., 2013) (PDB 3K3Q) (289) is comprised of 216 (B) hexameric subunits made from 6 (C) monomers. Each monomer has a NTD (blue) linked (black) to the CTD (red) providing a degree of flexibility in the structure (Jacques, D. A., et al., 2016) (PDB 5HGN) (30). (D) Further curvature, required to close the cone, is provided by the distribution of 12 pentameric subunits within the cone (Mattei, S., et al., 2016) (PDB5MCY) (291).

1.4.3 The HIV-1 capsid electrostatic channel

1.4.3.1 HIV-1 CA β-hairpin

Analysis of available HIV-1 NTD crystal structures have highlighted differences in CA helices 4, 5 and 7 (Figure 18C), which explain conformational differences between CA cones (299). Of particular interest is the β-hairpin region (Figure 19A), which forms after Gag maturation, and is present in all retrovirus CA structures. The CA β-hairpin is formed from the joining of Gag residues Pro 133 – Asn 137 (CA residues 1-5) and Gln 141-Gln 145 (CA residues 9-13). Located against helix 6, the residue Pro 133 forms a salt bridge
with Asp 183 (CA residue 51) (Figure 19B) (305). The β-hairpin region of CA is known to be essential for infectivity, however the exact reason for this remains elusive. This is because CA mutants, in which all or part of the β-hairpin is missing, are still able to assemble into tubular structures, suggesting that this region is not a requirement for overall CA stability on maturation (287, 305).

The study of β-hairpin has been complicated by the disordered nature of CA residues 1 to 11, in most x-ray structures. However, a study of MLV provided vital insight by demonstrating the assembly of six β-hairpins, one from each monomer, at the centre of a MLV CA hexamer (306).

Following this earlier study, Jacques et al recently proposed structural flexibility for the HIV-1 CA β-hairpin. On overlaying all published structures of the HIV-1 CA NTD, with resolved β-hairpin (Figure 19A), it was evident that this region crystallised in alternative conformations. The positioning of the β-hairpin differed by as much as 15 Å in distance between structures (30). The differing β-hairpin conformations, between structures, was due to the movement of the residue proline 1, required for the salt bridge formation to aspartic acid 51 (30, 305). This structural variability could be attributed to differences in buffer pH during crystallisation, with a low pH resulting in the residue D51 forming a secondary salt bridge with H12 (Figure 19B). While at high pH, and in stabilised hexamer crystals, the presence of a water molecule destabilises the H12 residue and forms a network of hydrogen bonds between residues 12, 48, 50 and 51 (Figure 19B) (30).

Interestingly, the pivoting movement of these residues results in an 'iris-like' motion of the six central β-hairpins. The movement of the six central β-hairpins could open or close access to a central pore (Figure 19C). The HIV-1 central pore, sometimes referred to as a chamber, or channel, was found to be 25 Å deep and 3,240 Å³ in volume and contains a ring of six basic arginines, contributed from each of the 6 monomers in the CA hexamer subunit at residue 18 (307).
**Figure 19: A role for the HIV-1 CA NTD beta-hairpin region**

(A) β-hairpin of the CA NTD can be crystallised in 3 conformations. (B) B-hairpin conformations are associated with the state of the hydrogen bond network involving residues P1, H12, T48 and D51, which correlate with the formation of the (C) CA pore at the 6-fold axis of symmetry. Green closed, yellow intermediate and pink open. Figure from (Jacques, D. A., et al., 2016) (30).

1.4.3.2 The arginine 18 (R18) ring

The R18 residues, located in CA helix 1, are known to be important for the formation and structure of the CA cone. Mutating arginine 18 to a leucine (L) results in a change in CA morphology, to spheres (298, 299). However, despite the conservation of the CA β-hairpin among lentiviruses, arginine at residue 18 is not conserved in MLV (uncharged serine), HSV or HTLV (positive charged lysine) (306). Aside from the structural role R18 has in HIV-1, these residues were recently found to have a major impact on viral infection in cell lines. The mutant R18G was found to form normal CA cone structures, originally observed
by EM. Yet, the mutant has a significant block to reverse transcription and therefore infection in cell lines (308, 309).

The 6 arginine 18 residues in the HIV-1 CA hexamer structure cluster at the central pore. The highly positive charge of arginine acts as an electropositive focus, the exposure and conformation of which is controlled by the conformation of the β-hairpin domain and surrounding residues. Jacques et al found the maximum electrostatic pore diameter was 8 Å, suggesting that dNTPs are able to pass through an open HIV-1 CA pore. On measuring binding by fluorescence anisotropy, Jacques et al measured high affinity binding (6-40 nM) of all four dNTPs. However, dNTP binding to both monomeric CA and the R18G mutant was completely absent (Figure 20A) (30). Interestingly a hexameric molecule, hexacarboxybenzoene, was found to crystallise within the open hexameric CA pore (Figure 20B). On measurement of reverse transcription, using the ERT assay, hexacarboxybenzoene was determined to effectively block reverse transcription. These data suggest that the CA electrostatic pore is a possible target site for new antiretroviral design (30).

![Figure 20: The role of arginine 18 in the HIV-1 hexameric CA pore](image)

(A) Arginine 18 residues (red) which line the electrostatic channel of the HIV-1 CA (blue) pore are able to bind dNTPs, (PDB 5HGM) and (B) a hexameric compound mellitic acid (PDB 5HGP). Structures from (Jacques, D. A., et al., 2016) (30).

### 1.4.3.3 Structural insight into the location of reverse transcription

The discovery that the central hexameric electrostatic channel is essential for viral reverse transcription and infectivity raises the question of the location of reverse transcription and the timing of CA uncoating. We hypothesise that the movement of β-hairpin, to either expose or protect the R18 ring controls access of nucleotides into an intact CA cone, to fuel encapsidated reverse transcription. However, the dynamic nature of the β-hairpin also functions to protect viral RNA and DNA from the hostile host cytoplasm. This hypothesis is supported by the recent discovery that non-pandemic HIV-1 O-group and HIV-2 viral CA hexamer pores are crystallised in an open conformation. Whilst HIV-1
O-group virus reverse transcribes at an increased rate, compared to pandemic HIV-1 M-group virus, O-group has a reduction in infectivity in MDM due to triggering IFN-I production (manuscript in preparation).

However, the regulation of dNTP entry to HIV-1 (M) viruses, possibly, to fuel encapsidated reverse transcription led us to question how reverse transcriptase inhibitors (RTI) are able to enter the core to function. Taking a molecular approach, we aimed to determine if differences throughout Gag, between M and O group, correlated to differences in viral sensitivity to both class of RTI, NRTI and NNRTIs.
Chapter 2: Materials and Methods

2.1 Plasmids and plasmid preparation

2.1.1 Plasmid list

<table>
<thead>
<tr>
<th>Plasmid Category</th>
<th>Plasmid Name</th>
<th>Genes or Proteins expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Envelope</td>
<td>pMDG</td>
<td>Vesicular stomatitis virus G envelope protein (VSV-G)</td>
</tr>
<tr>
<td>Genome/ Transfer</td>
<td>pCSGW</td>
<td>Green fluorescent protein (GFP)</td>
</tr>
<tr>
<td>Genome/ Transfer</td>
<td>pCSFLW</td>
<td>Firefly Luciferase</td>
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<tr>
<td>Genome/ Transfer</td>
<td>pEXN</td>
<td></td>
</tr>
<tr>
<td>Genome/ Transfer</td>
<td>Pc-SIREN</td>
<td></td>
</tr>
<tr>
<td>Packaging</td>
<td>P8.91 EX</td>
<td>Group specific antigen (Gag-) Pol, trans-activator of transcription (Tat), regulator of expression of virion proteins (Rev)</td>
</tr>
<tr>
<td>Packaging</td>
<td>pCRV</td>
<td>Gag- Pol of HIV-1</td>
</tr>
<tr>
<td>Packaging and Genome</td>
<td>pLAI (GFP)</td>
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</tr>
<tr>
<td>Packaging and Genome</td>
<td>pLAI (LUC)</td>
<td>Luciferase (LUC)</td>
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<tr>
<td>Full length</td>
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<tr>
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<tr>
<td>Full length</td>
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<tr>
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</tr>
<tr>
<td>Transformation control</td>
<td>pUC 19</td>
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</table>

Table 1: Plasmid List

Plasmid category, name and genes expressed. pMDG supplied by genescript, pUC 19 supplied from Invitrogen, CSFLW kindly donated by Gupta, R (UCL) and full-length primary isolates kindly donated by Hahn, B (University of Pennsylvania).

2.1.2 Preparation of competent bacteria

HB101 (RecA-) competent bacteria were used to inoculate 10 ml of lysogeny broth (LB) (autoclave sterilised and composed of: 10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl) at 37°C, shaking at 200 rpm (Kuhner ISF-1-W), for, approximately, 16 hours. 5 ml of culture was used to inoculate 200 ml LB, incubated, as above, until an optical density (OD) of OD 595 = 0.5-0.6. OD was measured using the WPA UV1101 spectrophotometer (Biotec Jencons). The 200 ml culture was divided into 50 ml Falcon tubes, incubated on ice for ten minutes and pelleted at 3,000 rpm (Sorvall RT, equivalent to 4,000g). Pellets were pooled, by resuspension, in 20 ml TFB1 buffer (0.45 μM filter sterilised, composed of: 30 mM KAc, 100 mM RbCl, 10 mM CaCl2, 50 mM MnCl2, 15% glycerol and water) and incubated on ice for five minutes and pelleted at 3,000 rpm for ten minutes. The pellet was re-suspended in 2 ml TFB2 buffer (0.45 μM filter sterilised and composed of: 0.1 mM PIPES, 75 mM CaCl, 10 mM RbCl and 15% glycerol) and incubated on ice for ten minutes. Cells were then aliquotted (50 μl) and snap-frozen on dry ice. HB101 competency was
determined on transformation of 10 pg pUC19. Good efficiency was determined by a count of > 100 colonies.

2.1.3 Transformation and selection

HB101 cells were thawed on ice, added to 1 ng of plasmid DNA (Table 1) and incubated on ice for a minimum of 30 minutes. Cells were heat-shocked at 42°C for 50 seconds and returned to ice for two minutes. Cells were plated onto ampicillin selective agar (100 μg/mL) and incubated at 37°C for approximately 16 hours. Agar was made by dissolving 17.5 g of LB agar (Merck Millipore) in 500 ml dH₂O and sterilised by autoclave.

A minimum of three individual colonies were selected, picked and used to inoculate 5 ml of LB with ampicillin. Cultures were incubated, shaking, at 37°C, or 30°C for full-length HIV plasmids, for approximately 16 hours.

2.1.4 Mini-prep of plasmids

5 ml of bacterial cultures were pelleted by centrifugation at 3,000 rpm for 30 minutes. Plasmid DNA was extracted using the QIAprep spin mini-prep kit, according to manufacturer's instructions, and eluted into 50 μl buffer (EB) (Qiagen). Plasmid DNA concentration was measured using a Nanodrop NDW1000 (Thermo Fischer).

2.1.5 Midi-prep of plasmids

5 ml of LB starter cultures were incubated, shaking at 37°C for eight hours. 200 μl was used to inoculate 200 ml LB plus selection. Cultures were incubated, shaking, at 37°C or 30°C for full-length HIV plasmids, for approximately 16 hours.

Cultures were pelleted by centrifugation at 3,000 rpm and 4°C for 20 minutes. Plasmid DNA was extracted using the QIAfilter Plasmid Midi Kit (Qiagen), according to manufacturer's instructions. However, all centrifugation stages were carried out at 3,000 rpm. Plasmid DNA was eluted into 150 μl H₂O/TE and DNA concentration measured by Nanodrop.
2.2 Site-directed mutagenesis (SDM)

2.2.1 SDM primer list

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gag mutation site</th>
<th>Forward Primer</th>
<th>Forward (FWD) Primer Sequence</th>
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<td>LH 129</td>
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<td>120R</td>
<td>LH 270</td>
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<td>H12Y</td>
<td>LH 314</td>
<td>GGCAAATGGTATATCAGGCAATATCCACC</td>
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<td>N74D</td>
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<td>H87A</td>
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<td>R18G</td>
<td>LH 280</td>
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<td>Q50Y</td>
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<td>120R</td>
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<td>GT 504</td>
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<td>H87A</td>
<td>CK 24</td>
<td>GGCCTTGGTGCAATAGGGCCCTTGCTGCCACTGATGCCACTCTATCC</td>
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</table>
Table 2: Gag point mutations

List of point mutations throughout Gag introduced by SDM. All point mutations were made in 8.91 vector, either expressing HIV-1 (M) Gag or HIV-1 (O) Gag, as indicated. Forward and reverse primer sequences used during SDM are indicated.

<p>| | | | | |</p>
<table>
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<td>LH 325</td>
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</table>

2.2.2 PCR for SDM

Single site point mutations in HIV-1 Gag were made using the following SDM protocol: 30 ng of template and SDM PCR Mix (35 μl water, 5 μl 10x PFU reaction buffer, 1 μl 25mM deoxynucleoside (dNTP) mix, 2 μl forward/reverse primer (10mM) and 2 μl PFU turbo polymerase) was added to 0.2 μl plastic tubes.

The PCR reaction was carried out using the following cycling conditions: denaturing 92°C (1 min) | (12x cycles) denaturing 92°C (1 min), annealing 55°C (1 min), extension 68°C (2 min per kb) | final extension 68°C (30 min)| HOLD|

2.2.3 Gel electrophoresis

6 μl of SDM product was removed and added to 6x loading dye. Samples were loaded onto a 1% agarose gel, made by dissolving 1 g of agarose powder (Sigma) in 100 ml 1x Tris-Acetate-EDTA (TAE), with 0.2 μg/ ml ethidium bromide (Sigma). Gels were run on medium voltage and with the GeneRuler 1 kb ladder (Thermo). Post-run, DNA bands were visualised by UV trans-illumination (BioDoc).

2.2.4 DNA digestion and PCR clean up of SDM product

The remaining 44 μl of product was digested at 37°C for two hours, with 1 μl Dpn1 (NEB) and 5 μl 10x cut smart (NEB restriction buffer 4). Dpn1 digested PCR product was PCR cleaned using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer’s instructions, with the following alterations: three volumes of buffer QG and one volume of isopropanol were added to digested PCR product. DNA was eluted into 30 μl ddH₂O.
2.2.5 Transformation and selection of SDM products

5 μl of PCR clean product was used to transform HB101 (described in section 2.1.3), with an initial incubation on ice for one hour, to increase efficiency. Resulting colonies were selected, cultured and prepared (described in sections 2.1.3 and 2.1.4). Plasmids were sent for sequencing (Eurofins) to determine successful mutant clones compared to wild type (WT) (DNA Dynamo).

2.2.6 Cloning of SDM Products

2.2.6.1 DNA Digest

DNA of parental plasmid (3 μg) or SDM product was cut for three hours at 37°C with promega or NEB supplied restriction enzymes (1 μl each) against unique sites with high buffer-type (5 μl) specificity.

2.2.6.2 Gel electrophoresis and gel extraction of DNA

Whole digest products (total 50 μl) were mixed with 10x loading dye and run on a 1% agarose gel (discussed in section 2.2.3) with the GeneRuler 1 kb ladder. DNA bands were visualised by UV transillumination (BioDoc) and excised using a scalpel.

The resulting gel bands were weighed and gel extraction performed using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer instructions. Vector or insert DNA concentrations were measured using a Nanodrop NDW1000 (Thermo Fischer).

2.2.6.3 Vector ligation and retransformation

Vector insert ratios of 1:2 or 1:3 were calculated based on 30-50 ng vector, using an online calculator (Insilico ligation calculator). Vector and insert were mixed 10x adenosine triphosphate (ATP) active ligase buffer (2 μl) and T4 DNA ligase (0.4 U) 1 μl. The mix was incubated at 22°C for one hour or 4°C for approximately 16 hours. 10 μl of ligated product was used to transform HB101 competent cells (as described in section 2.2.5).

2.3 Mammalian cell line culture

2.3.1 Mammalian cell line culture conditions

Mammalian cell lines were cultured in either Dulbecco’s modified eagle medium (DMEM) + L-glutamine or Roswell Park Memorial Institute medium 1640 (RPMI) (both Gibco Invitrogen), supplemented with 100 U/MI penicillin/ streptomycin. Cells were incubated at 5% CO₂, apart from HEK293T cells, incubated at 10% CO₂. Cell-washing steps were
carried out using 1x phosphate buffered saline (PBS Ca-, Mg-) (Lonza), and removal of adherent cells by addition of 1 ml 0.25% Trypsin +EDTA (Sigma) per 10 cm dish.

2.3.2 Cell counting

10 μl of cell suspension was mixed with 10 μl of trypan blue cell viability stain (Gibco, Thermo). 10 μl was added to a hemocytometer by capillary action. Live cells, in a minimum of two large grid-squares, were counted and the resulting value multiplied by $10^4$.

2.3.3 Cell storage

Cells were pelleted by centrifugation at 2,000 rpm for three minutes. Pellets were re-suspended in 0.2 μM filter sterilised foetal calf serum (FCS), 90% + 10% dimethyl sulfoxide (DMSO) (Sigma). Cells were aliquotted into cryovials, at a minimum cell density of $1\times10^6$/ ml, and frozen at -80°C for 24 hours in a Nalgene Mr Frosty freezing container (Sigma), prior to transfer to liquid nitrogen.

2.3.4 THP-1 differentiation

THP-1 cells were pelleted by centrifugation at 1200 rpm for five minutes and re-suspended at $4\times10^5$ cells/Ml. 50 ng/Ml phorbol 12-myristate 13-acetate (PMA) (Sigma) was added to the cell suspension. Cells were plated and incubated for 24 hours to differentiate, determined visually by cell adherence (310).
<table>
<thead>
<tr>
<th>Adherent or</th>
<th>Name</th>
<th>Cell line type</th>
<th>Culture Medium</th>
<th>Cell Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent</td>
<td>HEK293T</td>
<td>Embryonic human kidney</td>
<td>DMEM + 10% FCS</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Human cervical epithelial carcinoma</td>
<td>DMEM + 10% FCS</td>
<td>(1:4-1:10)</td>
</tr>
<tr>
<td></td>
<td>HeLa ISRE</td>
<td>HeLa cells stably expressing firefly luciferase reporter gene under IFN-α/ISRE control</td>
<td>DMEM + 10% FCS</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td></td>
<td>TE671</td>
<td>Embryonic human cerebellar medulloblastoma (rhabdomyosarcoma)</td>
<td>DMEM + 10% FCS</td>
<td>(1:4-1:10)</td>
</tr>
<tr>
<td></td>
<td>U87</td>
<td>Human astrocytoma (glioblastoma)</td>
<td>DMEM + 10% FCS</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td></td>
<td>CRFK</td>
<td>Crandall feline kidney cells</td>
<td>DMEM + 10% FCS</td>
<td>(1:4-1:10)</td>
</tr>
<tr>
<td></td>
<td>TZM-bi</td>
<td>Stable LUC indicator cell line. Parental line: JC53-bi (clone 13) HeLa cell line</td>
<td>DMEM + 10% FCS</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td></td>
<td>GHOST (3) CXCR4<em>CCR5</em></td>
<td>HOS derived reporter cells line stably transduced with MV7neo-T4 and hGFP construct</td>
<td>DMEM + 10% FCS Supplemented with: 500 μg/ml G418 and 100 μg/ml hygromycin</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td>Suspension</td>
<td>THP-1</td>
<td>Human leukemic monocyte</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
<tr>
<td></td>
<td>THP-1 – Lucia NF-κB</td>
<td>Stably integrated NF-κB LUC reporter cell line. Human THP-1 Monocytes</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
<tr>
<td></td>
<td>THP-1 IFIT1 gLUC</td>
<td>THP-1 cell line gaussia luciferase (gLUC) knock in under IFIT-1 promoter</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
<tr>
<td></td>
<td>Jurkat (JLTR5)</td>
<td>Human T-cell lymphoblast</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
<tr>
<td></td>
<td>SupT1</td>
<td>Human T-cell lymphoblastic lymphoma</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
<tr>
<td></td>
<td>A3.01</td>
<td>Human T-cell lymphoblastic leukaemia (CEM derivative)</td>
<td>RPMI + 10% FCS</td>
<td>Every 3 days</td>
</tr>
</tbody>
</table>

Table 3: Cell culture reagents list and culture conditions
2.4 Protein depletion or over expression in mammalian cell lines

2.4.1 Oligonucleoside design and cloning

The amplified DNA sequences were cloned into expression vector or shRNA construct (as explained in section 2.2.6).

2.4.2 Transfection and transduction of vectors

pSIREN and pEXN expression vectors were transected into HEK293T cells, in 10 cm plates of 80% confluency, using the following three vector system: 1 μg packaging plasmid, 1 μg envelope plasmid and 1.5 μg transfer plasmid. Vectors were incubated at room temperature for 20 minutes with 200 μl optimum (Gibco) and 10 μl Fugene 6 (Promega) before being added, drop wise, to HEK293T cells. The media was replaced 24 hours post-transfection and vector harvested, filtered (0.45 μm), aliquotted and stored at -80°C at 48 and 72 hours post-transfection.

Adherent or suspension cells were seeded at 8x10^5 cells in a six-well plate. Approximately 24 hours later, the media was replaced with 1 ml media and 1 ml vector and 8μg/mL polybrene (Sigma) polybrene. 48 hours post-transduction, cells were either expanded into 10 cm plate, or re-seeded in six-well plates for kill-curve selection (as discussed in section 2.4.3).

2.4.3 Kill-curve selection

Antibiotic selection was titrated onto seeded cells and toxicity determined visually either by cell death in adherent cells or by trypan blue staining for suspension cell lines. Selection media was replaced every two days, for seven days, or until cell death was noted in the transduction control cell line. The following antibiotic ranges were used: G418 0.1–2.0 mg/ml and puromycin 0.25–10 μg/ml.
<table>
<thead>
<tr>
<th>Target name</th>
<th>Cell line used</th>
<th>Knock-down or over-expression</th>
<th>Expression vector or construct</th>
<th>Oligonucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPSF6, C-terminus 50 amino acid deletion (NLS mutant)</td>
<td>HeLa</td>
<td>Over-expression</td>
<td>EXN</td>
<td>FWD-ATCGGAATTCTAGGGCGGAC&lt;br&gt;GGTGTGGACACATAGACA&lt;br&gt;TTTAC&lt;br&gt;REV-ATGCAGCCGCGCTCATCTCGTGATCTACTATGGTCCC</td>
</tr>
<tr>
<td>TNPO3</td>
<td>HeLa</td>
<td>Knock-down</td>
<td>EXN</td>
<td>FWD-ATGCGGAATTCATGCAGGAGA&lt;br&gt;GCAAGGCGGACATTGAGCG&lt;br&gt;TC&lt;br&gt;REV-ATGCGTCGACCTATCGAACA&lt;br&gt;CAACCTGTGGAAGTCTCGC&lt;br&gt;AAGG</td>
</tr>
<tr>
<td>NUP358</td>
<td>HeLa</td>
<td>Knock-down</td>
<td>HIV-1 SIREN</td>
<td>FWD-GTCACTCGAGATGAGGCAGC&lt;br&gt;AGCAGGGCTGACGTTAGGAGC&lt;br&gt;REV-GCATGTCGACCTATCCAGC&lt;br&gt;ATCAGATTGCAATATC</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>THP-1 And THP-1 IFIT1</td>
<td>Knock-down</td>
<td>HIV-1 SIREN</td>
<td>FWD-GATCCCAGGGCATCATCTTT&lt;br&gt;GGAATCCAAAATCGAGTTT&lt;br&gt;GGATTC&lt;br&gt;REV-CAAGATGATGGGTATT&lt;br&gt;AATTCAAAAAGCCATCATCT&lt;br&gt;TGGAATCCAAAATCGAGTTT&lt;br&gt;GGATCCCAAGATGTGGGCC&lt;br&gt;CGG</td>
</tr>
<tr>
<td>TREX1 V5 tagged</td>
<td>U87</td>
<td>Over-expression</td>
<td>EXN</td>
<td>FWD-ATGCCTCGAGGCACCATGG&lt;br&gt;GCTCGAGGCGCCCTGCCC&lt;br&gt;REV-TGCAGCCGCGGCTTACGTA&lt;br&gt;GAATCGAGACCGAG</td>
</tr>
</tbody>
</table>

Table 4: Oligonucleotide sequences and expression vectors

2.5 Full-length virus or viral vector production

2.5.1 Transfection of viral vector

Viral vectors were transfected in 80% confluent HEK293T cells. For small vector preparations, 10 cm dishes were used. Vectors were incubated at room temperature for 20 minutes with 200 μl optimum (Gibco) and 10 μl Fugene 6 (Promega), before transfection by drop wise addition to cells.

For large vector preparations, to be sucrose-purified by ultracentrifugation, 4x T150 flasks were transfected and vectors incubated with 500 μl optimum and 30 μl Fugene 6.
2.5.2 Transfection of full-length virus

Full-length HIV-1 viral plasmids were transfected in T150 flasks of 80% confluent HEK293T cells. 14 μg of plasmid was incubated for 20 minutes at room temperature with 500 μl optimum and 30 μl Fugene 6, per flask.

<table>
<thead>
<tr>
<th>Plasmid system</th>
<th>Plasmid category</th>
<th>Plasmid quantity in 1x 10 cm dish (μg)</th>
<th>Plasmid quantity in 1x T150 flask (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three plasmid transfaction system</td>
<td>Packaging (p8.91)</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Envelope (pMDG)</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Transfer (CSGW or CSFLW)</td>
<td>1.5</td>
<td>3.75</td>
</tr>
<tr>
<td>Two plasmid transfaction system</td>
<td>Envelope (pMDG)</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>pLAI GFP or pLAI LUC</td>
<td>2.5</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Table 5: Transfection of HIV-1 plasmids to produce single-round viral vectors

2.5.3 Virus or viral vector collection

24 hours after transfection, total media of 10 cm dishes or T150 flasks were replaced. Viral or vector supernatants were harvested at 48 and 72 hours post-transfection, 0.45 μm filtered, aliquotted and stored at -80°C.

2.5.4 DNase treatment of virus or viral vector

Viral or vector preparations from transfection of T150 flasks, for innate immune sensing assays or q-pcr, were DNase treated prior to purification by ultracentrifugation.

DNase powder (DN25, Sigma) was suspended in 0.2 μm DNase buffer (10 mM Tris-HCl (pH7.5), 50 mM NaCl, 10 mM MgCl2 and 1 mM DTT, made up 1:1 with sterile 100% glycerol). The virus or viral vector was treated with 15 U/ml of DNase (Affymetrix) and 10 mM MgCl2 (Sigma) for two hours at 37°C.

2.5.5 Purification of virus or viral vector by ultracentrifugation

Viral or vector supernatants were layered, in sterilised ultra centrifuge tubes, onto 5 ml of filter-sterilised 20% sucrose diluted in 1x PBS. Tubes were balanced by weight, then ultracentrifuged (Surespin 630 rotor and buckets) at 23,000 rpm for two hours at 4°C. Pellets were re-suspended and pooled in 600 μl RPMI 10% FCS, aliquotted and stored at -80°C.
2.6 Mammalian cell line infections

2.6.1 Infection of adherent and suspension cells with GFP expressing vector or full-length virus for quantification by flow cytometry

For infection of adherent cells, 4x10⁴ cells per well were seeded in a 24-well plate, or 2x10⁴ per well in a 48-well plate. Approximately 24 hours after seeding, cell media was replaced with fresh media containing the required dilution of vector and 8 μg/ Ml polybrene (Sigma) or virus. Cells were incubated for 48 hours at 37°C, harvested and fixed for analysis by flow cytometry (fluorescence-activated cell sorting, FACS).

For infection of suspension cells, T-cell lines were seeded at 1x10⁶ per well in a 24-well plate, or 5x10⁵ in a 48-well plate. THP-1 cells were seeded at 4x10⁵ per well in a 24-well plate, or 2x10⁵ in a 48-well plate. On the same day as plating, cells were infected with the required dilution of vector and 8 μg/ml polybrene (Sigma) or virus. The cells were incubated for 48 hours.

2.6.2 Infection of adherent or suspension cells with CSFLW expressing vector for LUC assay

For infection of adherent cell lines with LUC expressing vector, cells were seeded at 2x10⁴ cells per well in a 96-well plate and infected, before cell adherence, with required dilutions of vector and 8 μg/ml polybrene (Sigma). Cells were incubated for 48 hours before cell lysis and quantification of LUC with the Steady-Glo (Promega) reagent and GloMax® 96 Microplate Luminometer (Promega) (discussed in section 2.8.5). Suspension cell lines were seeded at 5x10⁴ cells per well in a 96-well plate, infected and analysed as above.

2.6.3 Infection of TZMbl or GHOST reporter cell lines with full-length virus for LUC assay or FACS quantification.

The TZMbl LUC reporter cell line was seeded at 2x10⁴ cells per well in a 96-well plate and, before cell adherence, with the required viral dilutions. Cells were incubated and harvested as detailed in section 2.7.2.

The GHOST GFP-reporter cell line was seeded at 4x10⁴ cells per well in a 24-well plate, or 8x10⁴ cells per well in a 12-well plate. Approximately 24 hours after seeding, cell media was replaced with fresh media containing the required dilutions of virus and incubated for 48 hours.
2.6.4 Infection of T-cell lines with full-length virus

1x 10^6 cells/ml of JLTR5 T-cells were infected with 0.5 units of reverse transcription per ml (U RT/mL) of virus for six hours in a 50 ml falcon tube. Cells were washed in PBS by centrifugation at 2,000 rpm for three minutes two times and plated, for 48 hours, at 1x10^6/ml or 0.5x10^6/ml in the required plate format.

2.6.5 Infection for TaqMan qPCR

Adherent cell lines were plated at 2x10^5 cells per well in a 12-well plate for approximately 12 hours prior to infection with single-round vector at a multiplicity of infection (MOI) of approximately 0.3. Suspension T-cells were plated at 2.5-5x10^5 cells per well in a 12-well plate and infected with 0.5 U RT/MI of full-length virus.

2.6.6 Infection for measuring IFN stimulated gene expression by qPCR

2x10^5 cells were plated per well in a 12-well plate. After 24 hours media was replaced with fresh media containing the required dilution of vector, either dosed on U RT/MI or to achieve approximately 30% of infection, and 8 μg/ml polybrene.
2.7 Drug assays

2.7.1 Drug list

<table>
<thead>
<tr>
<th>Drug Name and Abbreviation</th>
<th>Drug class</th>
<th>Empirical Formula</th>
<th>Top dose of drug</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abacavir ABC</td>
<td>NRTI</td>
<td>C14H18N6O</td>
<td>300 μM</td>
<td>AIDS reagents</td>
</tr>
<tr>
<td>Didanosine ddi</td>
<td></td>
<td>C10H10N4O2 · H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emtricitabine FTC</td>
<td></td>
<td>C8H10FN3O3S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamivudine 3TC</td>
<td></td>
<td>C8H11N3O3S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stavudine d4T</td>
<td></td>
<td>C10H12N2O4</td>
<td>100 μM</td>
<td></td>
</tr>
<tr>
<td>Tenofovir TNF</td>
<td></td>
<td>C9H14N5O4P · H2O</td>
<td>300 μM</td>
<td></td>
</tr>
<tr>
<td>Zidovudine AZT</td>
<td></td>
<td>C10H13N5O4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etravirine ETR (TMC125)</td>
<td>NNRTI</td>
<td>C20H15BrN6O</td>
<td>100 nM</td>
<td></td>
</tr>
<tr>
<td>Nevirapine NVP</td>
<td></td>
<td>C15H14N4O</td>
<td>6 μM</td>
<td></td>
</tr>
<tr>
<td>Rilpivirine TMC278</td>
<td></td>
<td>C22H18N6</td>
<td>4 μM</td>
<td></td>
</tr>
<tr>
<td>PF 3450074 PF74</td>
<td>Small molecule HIV inhibitor</td>
<td>C27H27N3O2</td>
<td>20 mM</td>
<td>James, L (LMB, Cambridge)</td>
</tr>
<tr>
<td>Cyclosporine A CsA</td>
<td>antibiotic</td>
<td>C62H111N11O12</td>
<td>5 μM</td>
<td>Sandoz</td>
</tr>
<tr>
<td>sarsosine-3(4- methylbenzoate)-CsA SmBz-CsA</td>
<td>CsA analogue</td>
<td>10 μM</td>
<td>Selwood, D Warne, J (medicinal chemistry, UCL)</td>
<td></td>
</tr>
<tr>
<td>Benzenehexacarboxylic acid Mellitic acid Carboxylic acid C6(CO2H)6 (linear formula)</td>
<td>50 μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JW 3-33 Prodrug of mellitic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6: List of drugs and inhibitors

Table abbreviations: NRTI = nucleotide/nucleoside reverse transcriptase inhibitors, NNRTI = non-nucleoside reverse transcriptase inhibitors, PF = 2-Methyl-N-[(1S)-2- (methylphenylamino)-2-oxo-1-(phenylmethyl)ethyl]-1H-indole-3-acetamide.

2.7.2 PF74 assay

HeLa cells were seeded in a 48-well plate at 2.5x10⁴ cells per well. 18 - 24 hours after seeding, cells were infected with single-round vector at MOI ~0.3. PF74 drug, or anhydrous DMSO (Sigma) control, was titrated onto infected cells (20 mM, 10 mM, 5 mM). Cells were incubated at 37°C, 5% CO₂ for 48 hours.

2.7.3 Cyclosporine A (CsA) assay

WT or siRNA knock down nucleoporin 358 kDa (NUP358) and transportin 3 (TNPO3) HeLa cells were seeded in a 24-well plate at 4x10⁵ cells per well. 18 to 24 hours after seeding, cells were infected with single-round vector, MOI approximately 0.3 IU. CsA (5 μM) or SmBz-CsA (10 μM) drugs, or DMSO control, were added. Cells were incubated at 37°C, 5% CO₂ for 48 hours.
2.7.4  JW3-33 assay

TE671 cells were seeded in a 48-well plate at 2.5x10^4 cells per well. 18 - 24 hours after seeding, cells were infected with single-round pLAI vector, 30% approximate infection (MOI 0.3). Mellitic acid, JW3-33 or anhydrous DMSO control, titrated (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 μM). Cells were incubated at 37°C, 5% CO₂ for 48 hours. Alternatively, adherent cells were pretreated with titrated JW3-33 or DMSO control for two hours. Cells were washed in 1x PBS prior to infection.

2.7.5  Measuring 50% inhibitory drug concentration (IC50) by LUC or GFP

Inhibitors, or anhydrous DMSO control, were titrated (1:3), in appropriate media, in a 96-well plate from top dose (as specified in table 6). Adherent cells were re suspended at 4x10^5/ ml or suspension cells at 1x10^6 / ml and viral vector or virus added to suspension, dose based on approximately 1x10^6 reflective light units (RLU) or 30% of no drug control. Cell and vector/ virus mix was added to titrated inhibitor at 2x10^4 cells/ well. Cells were incubated at 37°C, 5% or 10% CO₂ for 48 hours. Cells infected with vector encoding eGFP were prepared and analysed by flow cytometry. Firefly luciferase encoding vector, or TZMbI cells infected with full-length virus were analysed as follows. Cells were mixed with equal volume of Steady-Glo reagent (Promega) to media and incubated at room temperature for five minutes. Lysed cells were transferred into 96-well plates compatible with the GloMax® 96 Microplate Luminometer (Promega) and luminescence read using the appropriate Promega protocol. RLU-data obtained was normalised, transformed and the IC50 value calculated using non-linear regression (curve fit) for log inhibitor versus response (variable slope using four parameters), all performed using Prism 6 software. This protocol has previously described in (311).

2.8  Flow cytometry (FACS)

2.8.1  Cell staining for flow cytometry

The proportion of infected, P24, surface or intracellular marker and live versus dead cells were all quantified by flow cytometry (FACS), using either the BD Accuri C6 or BD FACS Calibur (BD Biosciences). Live cells were gated according to size on forward (FSC) and internal complexity or granularity on side scatter (SSC). For each sample 5,000 to 10,000 live cells were counted.
### Table 7: FACS antibody reagents list

<table>
<thead>
<tr>
<th>FACs antibody</th>
<th>Conjugate</th>
<th>Dose</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>P24</td>
<td>FITC</td>
<td>1 μl / 2x10^5 cells in 50 μl</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Live/ Dead fixable cell stain kit</td>
<td>APC</td>
<td>1.5 μl / 1,000 μl (50 μl / ≤1x10^6 cells)</td>
<td>ThermoFisher</td>
</tr>
</tbody>
</table>

#### 2.8.1.1 Gag^{P24} staining

Harvested cells were fixed in 4% paraformaldehyde (PFA) diluted in 1x PBS and pelleted, and then washed, three times, in 0.2 μm filter sterilised FACS wash buffer (PBS + 1% FCS) at 2,000 rpm for three minutes (Epindorf 5424). Cells were permibilised for 30 minutes at room temperature (10x BD Perm Buffer diluted in sterile water to 1x) and P24 antibody added, and then incubated in the dark at room temperature for 30 minutes. The cells were washed three times in faces wash buffer (FWB), re-suspended in FWB and analysed by FACS.

#### 2.8.1.2 Live dead cell staining

Harvested cells (2x10^5–1x10^6) were transferred into a round bottom 96-well plate and washed twice in 1x PBS, by centrifugation at 2,000 rpm for three minutes. Live dead stain (ThermoFisher) was prepared and added to the cells. The cells were incubated at 4°C for ten minutes in the dark. Stained cells were washed twice, as above, and fixed in 4% PFA.

#### 2.8.2 Quantification of GFP or P24 positive (GFP^+, P24^+) cells by flow cytometry

The percentage of live GFP^+ or P24^+ cells was determined 48 hours post-infection, unless otherwise stated, by flow cytometry. The positive cells were gated using an uninfected control from total live cells. Data obtained using the BD FACS Calibur was analysed using FlowJo software.

#### 2.8.3 Calculation of viral infectious units

From quantitation of GFP^+ or P24^+ cells, by FACS, infectious units per mL (IU/mL) of a vector or virus was quantified using the following formula:

\[
\text{(number of cells per well X GFP}^+ \text{ or p24}^+ \text{ cells)} / \text{(volume of virus per well (μl) / 1,000)}
\]
2.9 Nucleic acid extraction

2.9.1 RNA extraction

RNA was extracted from pelleted adherent or suspension mammalian cells using an RNeasy mini-kit (Qiagen), according to manufacturer’s instructions, and stored at -80°C.

2.9.2 cDNA synthesis

cDNA was synthesised according to the following protocol, using a Thermocycler GeneAmp PCR System 9700:

- First round: 500 ng-1 μg of RNA was incubated with 1 μl Oligo dT (50 μM) and 1 μl dNTP mix (10 mM) at 65°C for five minutes. Reactions were immediately transferred and incubated on ice for one minute.
- Second Round: Total product from round one (13 μl) was added to the second round mix of: 5x first strand buffer (4 μl), DTT (0.1 M) (1 μl), RNAse out (1 μl) and superscript III reverse transcriptase (RT) (1 μl). The mixture was incubated as follows: 50°C (60 min) | 70°C (15 min).

2.9.3 DNA extraction

DNA was extracted from pelleted adherent or suspension mammalian cells. Cells were washed twice in 1x PBS and re-suspended in 200 μl 1x PBS. 20 μl of proteinase K was added per sample prior to extraction using the QIAmp DNA blood mini-kit, according to manufacturer's instructions. DNA was eluted in 50 μl of supplied elution buffer and stored at -20°C. The DNA quantity and quality obtained was cell-type dependent ranging from 20 to 150 ng / μl.

2.10 Quantitative PCR (qPCR) / real time PCR (RT-PCR)

All qPCR protocols were run and analysed using the 7500 Fast RTPCR System and accompanying software (Thermo).

2.10.1 SYBR Green qRT-PCR

SYBR Green dye detects PCR products through direct binding to double-stranded DNA (dsDNA). Throughout the PCR, DNA polymerase amplifies the specific targeted sequence, while SYBR Green dye binds to newly synthesised dsDNA. Therefore, the increase in SYBR dye fluorescent intensity is dependent on the quantity of target product synthesised per reaction. Melt curve analysis post-PCR was used to determine binding specificity of primers and GAPHD was used as an expression control.
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10</td>
<td>TGGCATTCAAGGAGTACCTC</td>
<td>TTGTAGCAATGATCTCAACACG</td>
</tr>
<tr>
<td>ISG56</td>
<td>CCTCCTTGGGGTTGCTCTACA</td>
<td>GGCTGATATCTGGGTGCCTA</td>
</tr>
<tr>
<td>MxA</td>
<td>ATCCTGGGATTTTGGGGCTT</td>
<td>CCGCTTTGCTGCTGGTGTCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCCAGAAGACTGTGGATGG</td>
<td>TTCTAGACGGCAGGTCAAGGT</td>
</tr>
</tbody>
</table>

Table 8: Primers for SYBR Green qRT-PCR

2.10.1.1 q-RT-PCR to measure interferon stimulated gene (ISG) expression

cDNA was diluted 1:5 in molecular-grade water and added to the following mix: 5 μl SYBR Green Master Mix, 1 μl forward/reverse primers (10 μM) and 1 μl water. Samples were run as follows: polymerase activation 95°C (10 min) | (40x cycles) denaturing 95°C (15 sec), annealing and extension 60°C (60 sec) | melt curve 95°C (15 sec), 60°C (60 sec), 60°C-95°C (0.3°C/sec), 95°C (30 sec), 60°C (15 sec) |

2.10.1.2 Analysis of SYBR Green qPCR raw data

Data was analysed using Excel software as follows. Duplicates of cT values were averaged and the ΔcT value calculated by subtracting GAPHD1 control average cT from ISG average cT values. The ΔΔcT was calculated by subtracting the mock ΔcT value from each sample. Lastly, the RQ value was calculated from $2^{-\Delta\Delta cT}$ of each sample.

2.10.1.3 qRT-PCR to measure vector or virus reverse transcription (RT) by SG-PERT

Protocol previously described in references (312, 313). 5 μl diluted viral or vector supernatants were lysed at room temperature for ten minutes in 5 μl 2x lysis buffer (100 mM 1M Tris HCL pH7.4, 50mM 1M KCL, 0.25% Triton X-100, 40% glycerol made up to 10 ml with dH$_2$O) with 0.4 U/μl ribolock RNAse (Fermentas). Lysis was inactivated with 90 μl molecular-grade water. 10 μl of lysed virus, or prepared standards (10$^8$ pU/μl - 10$^1$ pU/μl) were added per well with master mix (Table 9). Samples were run using the following cycling parameters: reverse transcriptase/ transcription (RT) 42°C (20 min), Taq activation 95°C (15 min) | (40 cycles) denaturing 95°C (10 sec), annealing 60°C (30 sec), extension 72°C (15 sec).
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity per reaction (μl)</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitect SYBR Green Master Mix (2x)</td>
<td>12.5</td>
<td>Qiagen, Quantitect SYBR Green PCR kit</td>
</tr>
<tr>
<td>MS2 FWD primer (100 μm)</td>
<td>0.125</td>
<td>TCCTGCTCAACTTCCTGTCGAG</td>
</tr>
<tr>
<td>MS2 REV primer (100 μm)</td>
<td>0.125</td>
<td>CACAGGTCAAACCTCCTAGGAATG</td>
</tr>
<tr>
<td>MS2 RNA 0.7 pM/ μl</td>
<td>0.125</td>
<td>Roche</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 9: SG-PERT reagents

### 2.10.1.4 Analysis of SYBR Green SG-PERT raw data

After fluorescence acquisition, duplicate quantity values calculated post-run, using the standard curve, were averaged and pU RT calculated using the following formula: \(((\text{pU RT per well} \times 10) / 5) \times \text{dilution factor of vector or virus}\)

### 2.10.2 TaqMan qRT PCR

Fluorescently labelled probes used for TaqMan qPCR improve amplification product specificity as follows. The specific oligonucleotide probe consists of a reporter fluorescent dye at the 5’ end (FAM) and a quencher located at the 3’ end (TAMRA), the function of the quencher, in an intact probe, is to reduce emitted fluorescence. On annealing the probe to the target, downstream of a primer site, the primer is cleaved (separating the reporter and quencher) by 5’ nuclease activity of Taq polymerase. Primer extension continues until template strand completion. As this continues, reporter cleavage increases fluorescence intensity, proportional to the quantity of amplicon produced per reaction.
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Target DNA</th>
<th>Primer and probe sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 Strong Stop transcripts</td>
<td>LTR R repeat LTR U5</td>
<td>FWD- GCCTCAATAAAGCTCGTTGAAGCTCGTTGA AGAGTCACACACAGACGGGCACACACTATAMRA</td>
</tr>
<tr>
<td>HIV-1 1st Strand synthesis transcripts</td>
<td>eGFP gene</td>
<td>FWD- CAACAGCCACAACGTCTATATCATATCAT REV- ATGTTGTGGCGGATCTTGAAG PRO- FAM- CCGACAAGCAAGAAGACGGCATCAA-TAMRA</td>
</tr>
<tr>
<td>HIV-1 2nd strand transfer transcripts</td>
<td>LTR +ve strand PBS -ve strand</td>
<td>FWD- TAGTCAGTGAGAATCTCTTAGC REV- CTTCTAGCCTCCGCTAGTCAA PRO- FAM-TCGACGCAGGACTCGGCTTGCTTAMRA</td>
</tr>
<tr>
<td>HIV-1 2-LTR circles (314)</td>
<td>LTR-LTR junction</td>
<td>FWD- AACTAGAGATCCCCCTCACCCCTTTT REV- CTTTCTTCCGTTGGGAGTAATT PRO- FAM-CTAGAGATTTTCCACACTGAC-TAMRA</td>
</tr>
</tbody>
</table>

Table 10: Primers and probes for TaqMan qPCR

2.10.2.1 TaqMan qPCR mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x TaqMan Gene Expression Master Mix (ThermoFisher)</td>
<td>10</td>
</tr>
<tr>
<td>FAM/TAMRA Probe (7.5 μM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer FWD (7.5 μM)</td>
<td>1</td>
</tr>
<tr>
<td>Primer REV (7.5 μM)</td>
<td>1</td>
</tr>
<tr>
<td>Molecular-grade nuclease-free water</td>
<td>2.5</td>
</tr>
<tr>
<td>DNA</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 11: TaqMan q-PCR master mix

2.10.2.2 Cycling parameters of TaqMan qPCR to measure products of RT or integration

Strong stop, 1st strand synthesis and 2nd strand transfer PCR were run as follows: Holding 50°C (2 min), polymerase activation 95°C (10 min) | (40x cycles) denaturing 95°C (15 sec), annealing and extension 60°C (60 sec)|

2-LTR circle transcript PCR were run as follows: Holding 50°C (2 min), polymerase activation 95°C (10 min) | (50x cycles) denaturing 95°C (15 sec), annealing and extension 60°C (60 sec)|
2.10.2.3 Analysis of strong stop, 1st strand synthesis, 2nd strand synthesis or 2-LTR qPCR raw data

Standards for TaqMan qPCR were made as follows: strong stop pCSGW (2 camplicon copies per plasmid copy), 1st strand synthesis CSGW (1 amplicon copy per plasmid copy), 2nd strand transfer pCSGW (1 amplicon copy per plasmid copy) and 2-LTR circles p2LTR-LA. All standards were used at dilutions $10^{-7}$ to $10^{-9}$.

Duplicate quantity values, calculated post-run using the above standard curves, were averaged and the following calculations made:

- $RT$ per 200ng DNA = $(RT$ per ng DNA $\times 200)$
- $RT$ per $10^6$ cells = $(RT$ per cell $\times 10^6)$
- $RT$ per infection = $(RT$ per $10^6$ / number of infected cells per $10^6$)

2.11 Western Blotting

2.11.1 Sample preparation

Cells were washed in 1x PBS and lysed with 100 μl lysis buffer (10x, Cell Signalling), transferred to Eppendorf tubes and incubated on ice for ten minutes. 6x sample buffer was added to each lysed sample and boiled at 100°C for five minutes.

2.11.2 SDS gel preparation

Gels were cast, according to the recipe detailed in the following table, and set prior to use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Resolving gel (6%) 5 ml</th>
<th>Stacking Gel (5%) 1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td>SDS 20%</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>1mM Tris (pH8.8)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>0.5 mM Tris (pH6.8)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>TMED</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2.6</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 12: SDS Gels

2.11.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cast gels were placed in gel cassettes and placed in a tank of fresh 1x running buffer (diluted from 10x running buffer: Tris 30g, Glycine 144g, SDS 10g, water was added to 1 L) and a page ruler ladder or prepared sample loaded. Samples were run at low voltage (18v) through the resolving gel and later increased to 130v.
2.11.4 **Wet transfer**

Gels were equilibrated in 1x transfer buffer (diluted from 10x transfer buffer: Tris 15g, Glycine 72g, water to 500mL). Blots were assembled as follows: one sponge, filter paper, membrane, filter paper, sponge. The blotting cassette was placed in the tank with the transfer buffer and run in the fridge on low voltage overnight.

2.11.5 **Membrane blotting, primary and secondary antibody blotting and exposure**

After transfer, membranes were blotted in 5 ml 5% (w/v) milk powder in PBS for a minimum of 30 minutes. Primary antibodies were diluted in 5 ml 5% milk powder incubated, rolling, for approximately one hour at room temperature. Prior to adding the secondary antibody, membranes were washed for five minutes, three times, in PBS with Tween. Secondary antibodies were diluted (Table 24) in 5 ml % milk powder + 0.1% Tween and incubated, rolling, for two hours at room temperature. After secondary antibody blotting, membranes were washed three times in PBS + Tween and one time in PBS. Enhanced chemiluminescence, HRP substrate, was added to each blot prior to film-based development.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Raised in</th>
<th>Size (kD)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUP358</td>
<td>1:2,000</td>
<td>Rabbit</td>
<td>358</td>
<td>C288 Catavas, E</td>
</tr>
<tr>
<td>TNPO3</td>
<td>1:100</td>
<td>Mouse</td>
<td>104</td>
<td>Ab54353, Abcam</td>
</tr>
<tr>
<td>TREX1</td>
<td>1:250</td>
<td>Mouse</td>
<td>38</td>
<td>sc-271870, Santa Cruz</td>
</tr>
<tr>
<td>VCP</td>
<td>1:1,000</td>
<td>Rabbit</td>
<td>90</td>
<td>sc-271870, Santa Cruz</td>
</tr>
<tr>
<td>B-Actin</td>
<td>2:20,000</td>
<td>Mouse</td>
<td>40</td>
<td>ab6276, Abcam</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Raised in</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1:5,000</td>
<td>Sheep</td>
<td>NXA931, GE Healthcare</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1:5,000</td>
<td>Donkey</td>
<td>NA934V, GE Healthcare</td>
</tr>
</tbody>
</table>

Table 13: Western blot antibody list
Chapter 3: Determining the role of HIV Gag in reverse transcriptase inhibitor sensitivity

3.1 Chapter 3 Introduction

Many models of the early stages of HIV-1 infection propose that after HIV-1 virion fusion to a host cell, on CD4+ and coreceptor CCR5 or CXCR4 binding, the released HIV-1 capsid cone (referred to here as the capsid (CA) cone or core) disassembles, or uncoats, in the cytoplasm (135). Although the timing and cytoplasmic location of this uncoating step remains controversial, it has been proposed as being crucial for either the initiation or continuation of RT (128). The model of early cytoplasmic uncoating has been supported by data acquired from biochemical approaches, such as in vitro core stability assay (315, 316), the fate of CA assay (268) and in situ uncoating assay (135). The data from all of these assays demonstrates a loss of CA cone stability in the cytoplasm and shedding of CA, which is required for formation of the reverse transcription complex (RTC) (317). However, the exact location of CA uncoating still remains controversial, with in vitro uncoating assays demonstrating the requirement for CA dissociation only for the completion of RT. This suggests that at least the first stages of RT occur in core (137, 315, 316). Supporting the hypothesis of late CA uncoating, is the requirement of nuclear pore-associated proteins, such as nucleoporin 153kDa (NUP153), NUP358, to bind CA. Interactions between CA and nuclear pore-associated proteins have been shown to promote optimal nuclear entry and integration of vDNA (145, 146, 218). Furthermore, CA is the only known target of NUP358 (218). Such observations led us to propose a model whereby CA remains intact through the cytoplasm and uncoats on docking to the nuclear pore complex (NPC) (127, 133, 137).

Irrespective of the timing of CA uncoating, it remains unlikely that an intact CA cone can enter through a nuclear pore, considering the NPC diameter is 39-40 nm (206-208) and the CA cone between 50-60 nm and 120 nm in length, as determined by transmission electron microscopy (TEM) (133, 210). However, it is hypothesised that the NPC is flexible, thereby allowing transportation of an intact core.

Despite the location of CA uncoating, it is evident that CA requires the binding of two host cofactors, cleavage and polyadenylation specificity factor 6 (CPSF6) and cyclophilin A (CypA). We had previously determined in macrophages that cofactor-binding mutants, N74D and P90A, have a block to replication, due to innate immune triggering. This led us to suggest a role for cofactor recruitment in cloaking the virus from innate sensors within the cytoplasm (141). The stabilising, or cloaking role for CypA was further supported by the detection of IFNβ production in WT HIV-1 infection on treatment with CypA-targeting drug, CsA. However, it is evident that even on binding of specific host cofactors, CypA and CPSF6, some WT HIV-1 particles undergo early cytoplasmic uncoating, but do not
trigger an innate immune response. This is due to the degradation of vDNA by host exonucleases, such as TREX1. The role for TREX1 was determined by depletion in MDM and subsequent replication defect of HIV-1 (M) WT virus, due to the increased production of IFNβ (141, 148).

Primary cells, with intact RNA or DNA-sensing pathways, trigger in response to early CA uncoating, either by mutation or by inhibiting cofactor binding on addition of drugs such as PF74 or CsA. Yet, it is possible that cell lines with sensing pathway defects license early uncoating, thereby, allowing unprotected RT possibly within the RTC (126, 141).

The requirement of CA to bind nuclear pore associated proteins for optimal integration suggests that CA cores uncoat near to the NPC. However, it was previously difficult to determine which CA cores uncoated early or late. The recent development of live cell and single virion imaging has provided a way to measure the dynamics of uncoating. This was achieved through direct labelling of CA, with a tetrameric cyclophilin A-DsRed (CypA-DsRed) marker (139). Use of this labelling technique has determined that most cores docked at the nuclear pore have been protected from cytoplasmic degradation and, therefore, contain CypA-DsRed. However, only CA cores that lose the CypA-DsRed signal enter the nucleus. This suggests that, while CA-CypA binding is required in the cytoplasm, it is obsolete after docking with the nuclear pore, thus supporting a model of late CA uncoating in infectious cores (318).

However, it is evident that CA sub-units are able to enter the nucleus. Microscopy studies have located CA inside the nuclear compartment, by A3F-YFP labelling of HIV particles. The presence of CA in the nucleus has led some groups to propose a post-transcriptional role for CA (210). Despite evidence for the presence of some CA sub-units within the nucleus, there is poor evidence of the role of CA transport via the nuclear pore affecting integration. It is, however, well-evidenced that CA binding of CypA, CPSF6 and nuclear pore associated nucleoporins determines the pathway of viral integration.

Until recently, a mechanism of dNTP transport to fuel encapsidated RT had not been required. This was because the model of HIV-1 infection proposed the early uncoating of CA (Figure 9B) (2). The location of unprotected RT, within the cytoplasm, as part of the RTC provided accessibility of cellular dNTPs to fuel ongoing vDNA synthesis (135). Recently, research from Jacques et al demonstrated a role for HIV-1 M-group hexameric CA during vDNA synthesis (30). On comparing all available HIV-1 M-group CA crystal structures, with resolved N-terminal β-hairpin domain, Jacques et al determined differing β-hairpin conformations, dependent on the pH of crystal formation. Reconstruction of the β-hairpin in a hexameric CA structure resulted in a CA pore, 8Å in diameter at the six-fold
axis, in the centre of each CA structure. When β-hairpin domains were crystalised in an open conformation, a 25Å deep chamber was revealed (30). Furthermore, in an open conformation a ring of six basic arginine (R) side chains, from residue 18 (R18), are exposed within the chamber (30). However, this study did not implicate a role for cofactor binding in controlling the movement of the HIV-1 CA β-hairpin. This dynamic movement is only measureable at high pH during crystal formation.

Considering the positive charge of the R18 ring, Jacques et al., asked whether dNTPs could bind these residues, promoting the transport of dNTPs into an intact core, thus fuelling encapsidated RT. Through measuring affinity of binding by fluorescence anisotropy, Jacques et al determined that all dNTPs are recruited to and bind arginine residues with high 6-30 nM affinity. However, dNTP binding was lost on mutation of the R18 residue to a neutral glycine (R18G). Interestingly, it has previously been determined that R18G mutants produce mature particles, evidenced by the formation of CA cones observed by EM. However, Jacques et al determined R18G mutants were defective for RT (30, 308). However, the block to RT, and infection, in R18G mutants could be rescued. This was achieved by the production of chimeric CA cores containing differing ratios of R18 and R18G monomers in each CA hexamer. Increasing the ratio of WT to mutant monomer correlated with the increase to infection and RT (30). This experiment provided the first evidence for a role of HIV-1 CA in binding cellular dNTPs and promoting viral DNA synthesis.

This novel insight into pre-integration events in the HIV lifecycle has provided evidence in support of our model of encapsidated RT. However, it is yet to be determined how reverse transcriptase inhibitors (RTIs), a major class of drug used in antiretroviral therapy (ART), are able to enter the CA core to access and inhibit ongoing encapsidated RT.

We hypothesised that NRTIs would be able to bind the R18 residue with high affinity, as determined for dNTPs, based on their structural similarity and negative charge. However, we asked whether NNRTIs, which are uncharged, would traverse the CA pore, by passive diffusion, or require CA uncoating to gain access to reverse transcriptase. Taking a molecular genetics approach, we asked whether differences in Gag would result in differences viral sensitivity to RTIs. To determine the role for Gag we made single site mutations known to impact CA pore conformation, cofactor recruitment to CA or CA core stability. The CA structure of some of these mutant viruses has also been determined by crystallography (unpublished, by the Jacques, D. A).

Following on from the published worked by Jacques et al, we made mutations in HIV-1 M-group, which either fix the CA pore in an open (Q50Y+120R) or closed (H12Y) conformation determined by crystal structures (Figure 21). We determined that the open
pore mutant, Q50Y+120R, produced a similar quantity of vDNA as WT virus (data unpublished), as measured by the quantification of strong stop and 2nd strand transfer RT products by q-PCR (Figure 22). However, the closed pore mutant, H12Y, demonstrated a slower rate of RT, as previously determined by Jacques et al.

These observations led us to ask if differences in R18 accessibility and pore conformation, on CA mutation, would correlate to differences in the sensitivity of virus to RTIs. Here, we measured RTI IC50 and IC90 inhibitory concentrations and compared differences between HIV-1 vectors and Gag mutants. IC values are defined as the concentration of inhibitor required to inhibit 50% or 90% of infection. We chose to use single-round vectors bearing either M-group Gag, HIV-1 (M)R9 or O-group Gag HIV-1 (O)MVP, and mutants thereof, so that all vectors had the same reverse transcriptase. Single-round vectors were packaged with LUC. On comparing RTI IC50 and IC90 values, we observed some small, but consistent cell line dependent differences in vector sensitivity to inhibition.

IC50 data is summarised in table 14. Throughout these experiments, significance of IC50/IC90 values is set at greater than two-fold increase or decrease in value compared to WT. This limit accounts for both within and between experiment variability in the drug susceptibility assay. Despite measuring some two-fold effects on differences in Gag, either by mutations Q50Y+120R, N74D or in HIV-1 (O)MVP, we conclude that these are non-significant differences which have arisen from background noise of the assay. This conclusion that differences in Gag do not influence the concentration of RTIs required to inhibit infection is now supported by work from Mallery et al who propose a new role for the HIV-1 CA pore (319).
Figure 21: HIV-1 M-group hexameric CA mutant structures show fixed open and closed electrostatic channel positions

(A) Solving of HIV-1 (M) hexameric CA crystal at high pH (5HGL) shows the CA pore in a closed conformation due to β-hairpin (green) positioning. This is determined by the formation of a water molecule between key residues 12, 48 and 50. (B) Solving of the HIV-1 (M)_{Q50Y+120R} open CA mutant crystal at high pH demonstrates the exposure of the CA pore due to β-hairpin (pink) positioning. This is determined by the altered structural conformation on mutating CA residue 50. (C) Solving of the HIV-1 (M)_{H12Y} closed CA pore mutant crystal at low pH demonstrates the closed CA pore conformation (C). Figure from David Jacques, UNSW (manuscript in preparation).
Figure 22: Quantification of HIV-1 (M) and HIV-1 (M)_{Q50} or HIV-1 (M)_{Q50Y+120R} mutant early and late RT products in the THP-1 cell line

(A) Suspension THP-1 cells were infected with DNAse treated HIV-1 (M)_R9, HIV-1 (M)_{Q50} or HIV-1 (M)_{Q50Y+120R} GFP vectors equalised by SG-PERT (B) qPCR measurements of reverse transcripts of strong stop (early RT). (C) 2nd strand transfer (late RT) were made from one to nine hours post-infection. Data is from four independent experiments. Error bars +/- standard error of the mean (SEM). Paired t-test was performed to compare M-group versus O-group viruses at each time point. Figure from Lorena Zuliani-Alvarez, UCL (manuscript in preparation).
Table 14: Summary of IC50 and IC90 values for NRTI and NNRTIs in different cell lines on infection with HIV-1 (M) Gag mutants of HIV-1 (O) Gag vectors, compared to WT

Symbol + indicates a greater than two-fold increase in IC50/IC90 value. This indicates a reduction in sensitivity, to a RTI, of a vector compared to WT. Symbol – indicates a greater than two-fold decrease in IC50/IC90 value. This indicates an increased sensitivity, to a RTI, of a vector compared to WT. 0 indicates no significant difference in IC50/IC90 values in Gag mutants compared to WT. NRTI is abbreviated to N and NNRTI abbreviated to NN, grey squares indicates the absence of data.

3.2 HIV-1 M-group open (Q50Y+120R) and closed (H12Y) CA pore mutants do not confer a significant difference in sensitivity to RTI drugs compared to WT HIV-1

3.2.1 HIV-1 M-group open and closed pore mutant viruses have an infection defect in the HEK293T cell line

Using our HIV-1 (M) LUC vector we first sought to determine whether CA mutants Q50Y+120R or H12Y were as infectious as WT. From titration of both CA pore mutant viruses, compared to their WT controls HIV-1 (M) LUC, the control for mutant Q50Y+120R which is on an p8.91 background, and HIV-1 (M)CRV LUC, the control for H12Y which is on a pCRV background, we noted both mutants had a reduction in infectivity, with the open pore mutant HIV-1 (M)Q50Y+120R LUC demonstrating the largest infection defect (Figure 23). We found this result interesting as we had determined that the open pore mutant has a slight reduction in the rate of early RT, as measured in the THP-1 cell line, on measurement of both strong stop and 2nd strand transfer RT products by q-PCR (data unpublished) (Figure 22B, by Lorena Zuliani- Alvarez)
Figure 23: Titration of HIV-1 (M) LUC WT or CA pore mutant viruses in the HEK293T cell line shows mutant infection defects

Infection, shown as average RLUs, measured at 48 hours post-infection on reading RLUs using the steady glo protocol for HIV-1 (M)R9 WT (black circle), open pore mutant Q50Y+120R (open circle), HIV-1 (M)CRV WT (black square) and closed pore mutant H12Y (open square). Data representative of at least two independent experiments performed in biological duplicate. Error bars +/- SD.

Taking into account the infection defect for both Q50Y+120R and H12Y CA pore mutants, by normalising viral input to 1x10⁶ RLU, we asked whether differences in CA pore conformation altered vector sensitivity to RTIs, by measuring IC50 and IC90 values for NRTI and NNRTI. We hypothesised, based on changes to vector titre in the CA pore mutants, that mutation may affect the structural integrity of the CA cone and, therefore, might affect the accessibility of NRTI and NNRTI drugs.

Furthermore, we hypothesised that the open pore CA mutant may allow for increased access of NRTIs, through binding to a more accessible ring of R18 residues. Conversely, NNRTI sensitivity might be increased, through increased rates of passive diffusion through the larger diameter of an open CA pore. Conversely an open pore could reduce RTI concentration within the viral core. Therefore, an increased dose of inhibitor might be required to inhibit HIV-1 (M)Q50Y+120R infection. We also hypothesised that the opposite effect would be observed in the closed pore CA mutant due to the complete closure of the pore and, therefore, R18 residues would be inaccessible, due to the differing position of β-hairpin (30).

3.2.2 IC50 and IC90 measurements for open and closed pore mutants compared to WT in the HEK293T cell line

To distinguish between these models, we used a previously published single-replication cycle drug susceptibility assay (311). We used this drug susceptibility assay to measure IC50 and IC90 values (the dose of RTI required to inhibit 50% or 90% of infection) for our
panel of HIV-1 (M) LUC mutant and WT vectors in 293T cells. Considering the infection defect in the pore mutant vectors, vector input was normalised so that each biological replicate in the no drug control averaged $1 \times 10^6$ RLU. This was set to allow for a large inhibitory range on RTI addition. Thus, inhibitor comparisons were performed at similar MOI.

We first titrated a range of NRTIs, abacavir (ABC), stavudine (d4T), tenofovir (TNF) and zidovudine (AZT), and NNRTIs, nevirapine (NVP) and rilpivirine (TMC278). We then determined the percentage of inhibition for each drug curve by normalising the data to the no drug control (Figure 24). From these curves we evaluated the quality of data obtained from Hill slope and $R^2$ values (Table 15). The Hill slope factor is a measurement of curve steepness, with a standard steepness of a dose response curve being -1, where the higher the value the steeper the curve. The $R^2$ value is a measure of the fit of data points on the curve, therefore provides a value for the proportion of variance within the data set. A value of 1 is a conserved perfect fit, without scatter (320).

Next, we compared IC50 and IC90 values for NRTI and NNRTIs (Tables 14-16). We determined no significant difference in IC50 or IC90 values between HIV-1 (M) LUC and HIV-1 (M)$_{Q50Y+120R}$ LUC or HIV-1 (M)$_{CRV}$ LUC and HIV-1 (M)$_{H12Y}$ LUC vectors, in the HEK293T cell line, for either NRTI or NNRTIs. Significance for this assay was set at two-fold, due to experimental variability. However, we did measure a trend towards increased NRTI IC50 and IC90 values and increased NNRTI IC50 values for the closed pore mutant HIV-1 (M)$_{H12Y}$ LUC (Tables 15-17).

Despite small and consistent differences, we conclude that this data collectively demonstrates no significant impact in RTI sensitivity by these specific CA pore mutations in the HEK293T cell line.
Figure 24: HIV-1 (M) LUC WT and CA pore mutant vectors Q50Y+120R and H12Y are equally inhibited by multiple RTIs in 293T cells

293T cells infected with HIV-1 (M)R9 WT (black square), Q50Y+120R (blue circle), HIV-1 (M)CRV WT (maroon square) and H12Y (orange square) in the presence of titrated (A) NRTI drugs, ABC, d4T, TNF and AZT, or (B) NNRTI drugs, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of three independent experiments performed in biological replicate error bars +/- SD.
<table>
<thead>
<tr>
<th></th>
<th>ABC</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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Table 15: Calculation of IC50 values for HIV-1 (M) CA pore mutant LUC vectors, compared to HIV-1 (M) WT and HIV-1 (M) CRV WT show no significant differences for NRTIs in the 293T cell line. IC50 values average of three independent experiments performed in biological replicate calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
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Table 16: Calculation of IC50 values for HIV-1 (M) CA pore mutant LUC vectors, compared to HIV-1 (M) WT and HIV-1 (M) CRV WT, show no significant differences for NNRTIs in the 293T cell line.

IC50 values calculated from an average of three independent experiments performed in biological replicate calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
Table 17: Calculation of IC90 values for HIV-1 (M) CA pore mutant LUC vectors compared to HIV-1 (M) WT and HIV-1 (M) CRV WT, show no significant differences for NRTI or NNRTIs in the 293T cell line

Average IC90 values calculated from at least two independent experiments, performed in biological replicate.

3.2.3 Measuring the sensitivity of open and closed pore mutant vectors to NRTI and NNRTIs, compared to WT, in T-cell lines

Next, we asked whether the use of a more relevant cell line would provide a better model for IC50/IC90 measurements, using a single-round drug inhibition assay. Considering that HIV-1 predominantly infects CD4+ T-cells, we selected three T-cell lines: A3.01, SUP-T1 and Jurkat. We first determined vector titre in the T-cell lines, to ensure that the reduced titre for HIV-1 (M)Q50Y+120R LUC/GFP and HIV-1 (M)H12Y LUC/GFP was cell-type
independent. On calculation of infectious units (IU) of HIV-1 (M) GFP WT and CA pore mutant vectors, we measured an infection defect in both mutants in all three T-cell lines with the largest infection defect to mutants, compared with WT, measured in the Jurkat T-cell line (Q50Y+120R 32x and H12Y 4x) (Figure 25A). This data was also confirmed on infection of Jurkat cells with HIV-1 (M) LUC WT and CA pore mutant vectors. The open pore mutant HIV-1 (M)Q50Y+120R LUC demonstrated the biggest infection defect in Jurkat T-cells (Figure 25B), as previously shown and discussed in the HEK293T cell line (Figure 19).

We determined RTI IC50 and IC90 values using the single-replication cycle drug susceptibility assay for our panel of HIV-1 (M) LUC vectors in T-cell lines (311). Considering the infection defect in the pore mutant vectors, vector input was normalised so that each biological replicate in the no drug control averaged 1x10^6 RLU.

As part of the optimisation of the protocol for suspension cells, we initially calculated IC50 and IC90 values in the A3.01 (Figure 26A) and SupT1 (Figure 26B) T-cell lines on titrating NRTI, ABC. After determining the quality and fit of data from calculating Hill slope and R^2 values (Table 18), we compared both IC50 and IC90 values between HIV-1 (M) LUC and HIV-1 (M)Q50Y+120R LUC or HIV-1 (M)CRV LUC and HIV-1 (M)H12Y vectors. We found no significant difference in IC50 (Table 18) or IC90 (Table 19) between WT and CA pore mutants in the SupT1 and A3.01 cell lines for NRTI, ABC.

Having optimised our drug susceptibility assay in suspension cells, we then measured IC50 and IC90 values for NRTI, ABC, and NNRTI, NVP. We selected the Jurkat T-cell line, which demonstrated the biggest infection defect in both the open and closed pore mutant vectors (Figure 25B). We selected the NRTI, ABC, (Figure 27A) and NNRTI, NVP, (Figure 27B). On comparing IC50 (Table 20) and IC90 (Table 21) values, we did not measure any differences in values for the closed pore mutant HIV-1 (M)H12Y LUC for either RTI. However, we measured a two-fold decrease in NRTI, ABC, IC50 values for the open pore mutant HIV-1 (M)Q50Y+120R LUC (16 μM) compared to the WT (29 μM) (Table 20) and NNRTI, NVP, HIV-1 (M)Q50Y+120R LUC (0.05 μM), compared to the WT (0.13 μM). However, this difference was not evident in IC90 values (Table 21).
Figure 25: Titre of HIV-1 (M) GFP and HIV-1 (M) LUC WT and CA pore mutant vectors in T-cell lines

(A) Vector titre measured 48 hours post-infection by FACS on titration of HIV-1 (M)R9 WT GFP, Q50Y120R, HIV-1 (M)CRV WT or H12T in A3.01, SupT1 and Jurkat T-cell lines. IU were normalised to units of RT (IU/RT). (B) HIV-1 (M) LUC vectors were titrated in Jurkat T-cells for 48 hours prior to quantification of RLU by steady glo protocol. Data representative of at least two independent experiments. All error bars +/- SD.
Figure 26: HIV-1 (M) LUC WT and CA pore mutant vectors Q50Y+120R and H12Y are equally inhibited by NRTI, ABC, in A3.01 and SupT1 T-cell lines

(A) A3.01 or (B) SupT-1 suspension T-cell lines were infected with HIV-1 (M)R9 WT (black square), Q50Y+120R (blue circle), HIV-1 (M)CRV WT (maroon square) or HIV-1(M)CRV H12Y (orange square) Gag, in the presence of titrated NRTI drug, ABC, for 48 hours. The percentage of viral inhibition was calculated. Data from of two independent experiments performed in biological replicate. Error bars +/- SD.

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Table 18: Calculation of IC50 values for HIV-1 (M) WT or CA pore mutant LUC vectors show no significant differences for NRTI drug, ABC, in T-cell lines A3.01 or SUP-T1

IC50 values average of two independent experiments performed in biological replicate in T-cell lines A3.01 and SUP-T1, calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of two independent experiments.
Table 19: Calculation of IC90 values for HIV-1 (M) WT and CA pore mutant LUC

IC90 values calculated from an average of two independent experiments performed in biological replicate, as calculated in the statistical application GraphPad Prism.

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Figure 27: The HIV-1 (M)Q50Y+120R LUC open CA pore mutant has a 2x lower IC50 and IC90 value for NRTI, ABC, and NNRTI, NVP, in the Jurkat T-cell line

Jurkat (JLTR5) cells were infected with HIV-1 (M) \textsuperscript{WT} (black square), CA pore mutant Q50Y+120R (blue circle), HIV-1 (M) \textsuperscript{CRV WT} (maroon square) and HIV-1 CRV H12Y (orange square) in the presence of titrated (A) NRTI drug, ABC, and (B) NNRTI drug, NVP, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of three independent experiments performed in biological replicate. Error bars +/- SEM.
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Table 20: Calculation of IC50 values for HIV-1 (M) WT and CA pore mutant LUC vectors in Jurkat T-cells, shows a reduction in Q50Y+120R IC50 for both ABC and NVP

IC50 values average of three independent experiments performed in biological replicate in Jurkat JLTR5 T-cell line, as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.

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Table 21: Calculation of IC90 values for HIV-1 (M) WT and CA pore LUC mutants for NRTI or NNRTIs in the JLTR-5 T-cell line

IC90 values calculated in the statistical application GraphPad Prism from an average of three independent experiments performed in biological replicate.

3.3 Further characterisation of HIV-1 M-group CA pore mutants

Considering the infection defect of HIV-1 (M)Q50Y+120R and HIV-1 (M)H12Y CA mutant vectors in HEK293T and T-cell lines, we asked whether CA pore mutation had an effect on CA core stability (30, 141). We hypothesised that mutating the CA pore to a fixed open position would lead to exposure of vDNA, either through vDNA leaking out of the open, unregulated, pore or through early CA uncoating, thus leading to triggering of an innate immune response. However, we proposed that mutating the CA pore to a fixed closed position would not trigger an innate immune response, based on the early defect for RT
and assuming this mutation has no effect on CA stability. It has recently been suggested, Jacques, D.A that the H12Y mutant has a further role in CA stability. It is thought that the closed pore prevents the release of a packaged inositol hexaphosphate IP6. There is recent evidence for the role of bound IP6 stabilising HIV-1 CA through binding to and blocking the hexameric CA pore (manuscript in review).

3.3.1 HIV-1 CA pore mutants do not trigger an innate immune response in differentiated or suspension THP-1 cells

To further characterise the nature of the CA mutants, we sought to test whether they trigger an innate immune response, when used to infect THP-1 cells. To do this, we infected cells at an MOI of 0.3 and measured ISG expression. We infected PMA differentiated monocytic THP-1 cells, expecting these cells to most likely be innate immune competent (321). As previously determined in 293T and T-cell lines, the open pore mutant Q50Y+120R demonstrated the largest titre decrease in the THP-1 cell line (Figure 28A). We quantified RT capacity of each of our HIV-1 (M) GFP vectors by SG-PERT to standardise vector dose, and infected cells at low RT dose (0.1U) or high RT dose (0.5 U), equivalent to a MOI of 0.3 in HIV-1 (M)WT vector. 24 hours post-infection, we harvested cells and measured ISG expression (CXCL10, ISG56 and myxovirus resistance protein A (MxA)), normalised to GAPDH expression, by qRT-PCR, using SEV as a positive control for innate immune activation (Figure 24B). On quantification of CXCL10, ISG56 and MxA expression, we did not measure any ISG upregulation on infection with HIV-1 (M)WT GFP, mutants HIV-1 (M)Q50Y+120R GFP or HIV-1 (M)H12Y GFP or HIV-1 (O) GFP (Figure 28B). Like HIV-1 Q50Y+R120, the CA pore of HIV-1 (O) is fixed open, according to X-ray crystallography determination of CA hexamer structure.

In further support of our q-RTPCR data is recent data from our lab (experiments performed by Lorena Zuliani-Alvarez). Over a range of vector input (0.5 U RT – 0.05 U RT) in the differentiated THP-1 reporter cell line (Figure 29A), which express gLUC under the control of the endogenous IFIT1 promoter and are stably depleted for the sterile alpha motif and HD domain containing protein 1 (SAMHD1) restriction factor, we showed that neither WT HIV-1 GFP, open pore mutant or O-group GFP trigger the IFIT1 pathway (Lorena Zuliani-Alvarez, paper in preparation) (Figure 29B). We concluded from this data that the infection defect observed on mutating the CA pore is not due to an innate immune response in THP-1. Importantly, these experiments do not determine the stability of mutant cores.
Figure 28: HIV-1 WT and open and closed CA pore mutant vectors do not trigger an innate immune response in PMA differentiated THP-1 stably depleted for restriction factor SAMHD1 cells

(A) Titration of HIV-1(M) R9 or CRV WT and CA pore mutant LUC vectors Q50Y+120R or H12Y in PMA differentiated THP-1 shSAMHD1 cells shows an infection defect in CA pore mutants. (B) Quantification of ISG CXCL10, ISG56 and MxA expression in PMA differentiated THP-1 cells, infected with low (0.1 U RT) or high dose (0.5 U RT) of HIV-1 (M) WT or CA mutant GFP vectors or Sendai virus (SEV). Infection data from two independent experiments performed with biological replicate. Error bars +/- SEM. ISG quantification from one experiment.
Figure 29 HIV-1 WT (M)R9 WT, HIV-1 (O)MVP WT or open mutant HIV-1 (M)Q50Y+120R GFP vectors do not trigger an innate immune response in the differentiated SAMHD1 KD THP-1 IFIT1 cell line.

(A) Infection of IFIT1 cell lines of HIV-1 (M)R9 WT (black bars), HIV-1 (O)MVP WT (dark grey bars) or HIV-1 (M)Q50Y+120R (light grey bars) GFP vectors from high (0.5 U RT) to low (0.05 U RT) dose. (B) IFIT1 activation determined on quantification of RLU expression. Data from Lorena Zuliani-Alvarez, UCL (Manuscript in preparation). Data from three independent experiments. Error bars mean +/- SEM.
3.4 Addition of cofactor binding mutants to HIV-1 M-group CA has small but non-significant effects on viral sensitivity to RTIs

3.4.1 Recruitment of the cofactor CPSF6 to HIV-1 CA and its relationship to NNRTI sensitivity

We have previously shown that the CPSF6-binding CA mutant, N74D, has a significant block to infection and replication in MDM due to the triggering of type 1 IFN (141). Furthermore, recent data from our lab has demonstrated that the N74D mutant has a reduction to infection and replication in isolated primary CD4+ T-cells, due to an early block to RT at the level of strong stop (unpublished). However, a further role for CPSF6 recruitment to CA has been determined. CPSF6 is known to play a vital role in nuclear transport of vDNA, promoting HIV-1 CA to utilise the NPC and, therefore, has a direct role in viral integration (159, 163, 164). This data may also suggest that the loss of CPSF6 binding, perhaps at the NPC, might be a trigger for CA uncoating. After considering this data, we asked whether the N74D CA mutant would have altered sensitivity to RTIs.

Previously, mixed viral particles of WT and RT mutations, D110E or Y181I, were found to be more susceptible to inhibition by NNRTIs, efavirenz (EFV) and NVP, in JC53 BL13+ cells, a clone of the TZM-bl cell line. This result was attributed to the reduced number of RT molecules in mixed cores and, therefore, an overall reduction in RT activity (322). However, there is also evidence for a role HIV-1 CA plays in viral inhibition by NNRTIs. Ambrose et al previously determined that the CPSF6-binding CA mutant, N74D, has decreased ability to bind CypA. This was measured by the reduced recovery to infection on addition of CypA targeting drug CsA, compared to HIV-1 (M) WT infection. Furthermore, the N74D mutant was found to have fewer RT molecules associated to the RTC, possibly due to a reduction in CA cone stability (323). N74D was significantly more inhibited by NNRTI, NVP, due to the reduced activity of RT. However, treatment with NRTI, emtricitabine (FTC), demonstrated no difference in viral inhibition (323).

3.4.2 The CPSF-6 binding CA mutant N74D demonstrates increased NNRTI sensitivity, compared to WT virus, in HEK293T cells

We hypothesised, considering the reported impact of mutating the CPSF6-binding pocket (Figure 30A) on RT (323), that the HIV-1 (M)N74D LUC vector would have a lower IC50 for NNRTIs compared to the HIV-1 (M) WT vector. On titrating HIV-1 (M) LUC and HIV-1 (M)N74D GFP vectors in the HEK293T cell line and measuring IU/U RT, we did not measure a significant infection defect in the N74D mutant, as we have previously determined in the HeLa cell line (141) (Figure 31A). Also confirmed on titration of HIV-1 (M) LUC vectors on HEK293T cells (Figure 31B).
Figure 30: Mapping cofactor binding CA mutant sites to the HIV-1 (M) hexameric CA structure

(A) Hexameric CA 5HGM, structure from (Jacques, D. A., et al., 2016) (30) (blue), R18 (red) and locations of cofactor-binding mutants, CPSF6-binding mutant, N74D (yellow) and CypA-binding mutant, P90A (pink), and (B) CypA-binding loop polymorphic sites, H87 (orange) and A88 (yellow).

Next, we sought to determine differences in HIV-1 (M) WT and HIV-1 (M)N74D LUC sensitivity to NRTI and NNRTIs in 293T cells, through measurement of vector IC50 and IC90 values, using the method previously described. We titrated NRTI, TNF, and NNRTI, NVP, and determined the percentage of inhibition for each drug curve (Figure 32) and considered the quality of data obtained (Table 22).

We then compared IC50 (Table 22) and IC90 (Table 23) values for NRTI and NNRTI and determined no significant difference in IC50 or IC90 values for NRTI, TNF, between WT and N74D vectors. This suggests that the CPSF6-binding mutant, N74D, has no effect on viral sensitivity to NRTIs. However, on comparing IC50 values, we measured a two-fold decrease in IC50 for NNRTI, NVP, (0.04 μM) on infection with HIV-1 (M)N74D LUC compared to HIV-1 (M) WT LUC (0.08 μM). This suggests that the CA mutant has an increased sensitivity to NNRTIs. However, comparing IC90 values revealed that this was not replicated at a higher drug dose (Table 23). This led us to ask whether the difference in sensitivity measured was noise from the LUC assay.
Figure 31: The CPSF6-binding mutant, N74D, demonstrates no infection defect in HEK293T cells

(A) Vector IU/U RT measured at 48 hours post-infection by FACS in the HEK293T cell line. (B) Comparison of infection measured by average RLU on titration of HIV-1 (LUC) R9 WT or N74D mutant vectors. Data from at least two replicates. Error bars +/- SD.

Figure 32: The HIV-1 (M) CPSF6-binding mutant, N74D, demonstrates increased sensitivity to NNRTI, NVP, in 293T cells

293T cells were infected with HIV-1 (M)R9 WT (black circle) or CPSF-6 binding mutant HIV-1 (M)N74D (green triangle) LUC vectors in the presence of titrated (A) NRTI drug, TNF, or (B) NNRTI drug, NVP, for 48 hours. The percentage of inhibition was calculated from LUC readout. Data from of two independent experiments for TNF, and three independent experiments for NVP, performed in biological replicate. Error bars +/- SEM.
### Table 22: Calculation of IC50 values for HIV-1 (M) WT and CPSF6-binding mutant, HIV-1 (M)N74D, LUC vectors shows a decrease in NNRTI IC50 for N74D in the 293T cell line

IC50 values for NRTI, TNF (average of two independent experiments), and NNRTI, NVP (average of three independent experiments performed, in biological replicate), in 293T cells, as calculated in the statistical application GraphPad Prism. Hill slope and $R^2$ value representative of two or three independent experiments, as above.

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<tr>
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### Table 23: Calculation of IC90 values for HIV-1 (M) WT and CPSF6-binding mutant, HIV-1 (M)N74D, LUC vectors in the 293T cell line

IC90 values for NRTI, TNF (average of two independent experiments), and NNRTI, NVP (average of three independent experiments), in 293T cells, as calculated in the statistical application GraphPad Prism. Hill slope and $R^2$ value representative of two or three independent experiments, as above.

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</thead>
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<td>N74D</td>
<td></td>
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<td>N74D</td>
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3.4.3 Comparison of N74D RTI sensitivity to HIV-1 (M) WT vector in U87 cells

We have recently determined a requirement for cofactor recruitment, CPSF6 and CypA, for HIV-1 (M) infection in the U87 cell line. Supporting this observation, on titrating both cofactor binding mutants HIV-1 (M)\textsubscript{N74D} GFP and the CypA-binding mutant HIV-1 (M)\textsubscript{P90A} GFP vectors and calculating IU/U RT in comparison to WT vector, we measured an infection defect in both mutants with P90A (3.6x) demonstrating the largest infection defect compared to N74D (2x), as previously shown (141) (Figure 33A). These infection defects in cofactor binding mutants were replicated with our panel of LUC vectors (Figure 33B). Our infection data, therefore, suggests that HIV-1 infection in the U87 cell line is dependent on both CypA and CPSF6 recruitment.

From these observations, we hypothesised, based on our IC\textsubscript{50} data in the HEK293T cell line, that we would measure greater HIV-1 (M)\textsubscript{N74D} LUC mutant vector sensitivity to NNRTIs in the U87 cell line. Previously, Ambrose et al found that HIV-1 N74D had an increased sensitivity to NNRTIs in macrophages (323). We, therefore, sought to measure inhibitory concentration values for both mutant and WT vector in the U87 cell line.

Using the NRTIs, TNF and emtricitabine (FTC), (Figure 34A) and NNRTIs, NVP and TMC278, (Figure 34B), we determined the percentage of inhibition for each drug curve. We compared IC\textsubscript{50} (Table 24) and IC\textsubscript{90} (Table 25) values for NRTIs and NNRTIs. We found no significant difference in values for any NRTI or NNRTI tested. This was surprising, considering the two-fold decrease in NNRTI IC\textsubscript{50} measured in the HEK293T cell line for HIV-1 (M)\textsubscript{N74D} LUC and led us to question the significance of this small, but consistent effect. However, from this data in the U87 cell line, we conclude that there are no measurable differences in RTI sensitivity in the absence of CPSF6 binding to CA.
Figure 33 Cofactor binding mutants HIV-1 (M) N74D and P90A have a small infection defect in the U87 cell line

(A) IU for HIV-1 (M) R9 WT or CPSF6-binding mutant, N74D, and CypA-binding mutant P90A GFP vectors were measured 48 hours post-infection by FACS in the U87 cell line and normalised to U RT (IU/U RT). (B) Comparison of infection measured by average RLU on titration of HIV-1 (M) R9 WT (black circle) or N74D (green triangle) and P90A (red triangle) mutant LUC vectors. Data representative of at least two replicates. Error bars +/- SD.
Figure 34: HIV-1 (M)R9 WT and HIV-1 (M) CPSF6-binding mutant, N74D, LUC vectors are equally inhibited by both NRTI and NNRTI drugs in U87 cells

U87 cells were infected with HIV-1 (M)R9 WT (black circle) or CPSF-6 binding mutant N74D (green triangle) LUC vectors in the presence of titrated (A) NRTI drugs, TNF, FTC, or (B) NNRTI drugs, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicates. Error bars +/- SEM.
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<th>Hill slope</th>
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Table 24: Calculation of IC50 values for HIV-1 (M) WT and CPSF-6 binding mutant N74D LUC vectors in U87 cells shows no significant difference in inhibition by NRTI or NNRTI drugs

IC50 values for NRTI drugs, TNF and FTC, and NNRTI drugs, NVP and TMC278, average of three independent experiments performed with biological replicate in U87 cells, as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
IC90 values for NRTI drugs: TNF and FTC and NNRTI drugs: NVP and TMC278, average of three independent experiments performed with biological replicate in U87 cells as calculated in the statistical application GraphPad Prism.

### 3.4.4 Recruitment of CypA to HIV-1 CA

We have previously determined the importance of CypA recruitment to the CA core in MDM to cloak the virus and prevent innate sensing of vDNA (141). The CA mutant, P90A, unable to bind CypA also has an infection and replication defect in primary CD4+ T-cells, due to inefficient RT as early as strong stop (unpublished). Interestingly, we observe that mutant P90A has a larger infection defect compared to N74D, in primary cells (141), T-cell lines and in the U87 cell line. However, while both CA mutants P90A and N74D in the HeLa cell line do not affect RT, they do result in the integration of vDNA into a different region of the host genome, due to disruption of the nuclear entry pathway (163, 324).

Considering the role of CypA in core stability, regulation of RT, integration site selection and productive infection, we wanted to determine if adding the CypA-binding mutant, P90A, or CypA-binding loop mutants, A88M, A88V, H87A or H87P, demonstrated...
differences in HIV-1 (M) vector sensitivity to NRTIs or NNRTIs in the U87 cell line. We hypothesised that the CypA-binding mutant HIV-1 (M)P90A LUC, would demonstrate decreased sensitivity to RTIs due to cytoplasmic uncoating and the loss of a concentrated RTI pool within the CA cone.

### 3.4.5 Comparison of the CypA-binding CA mutant, P90A, to WT vector sensitivity to RTIs in the HEK293T and U87 cell lines

On normalising HIV-1 (M) WT and P90A LUC vector input to 10^6 RLU in the no drug control, we measured viral inhibition in the HEK293T cell line on titration of NRTI, TNF, (Figure 35A) and NNRTI, NVP, (Figure 35B). We also measured inhibition in the U87 cell line (Figure 36) on titration of NRTIs, TNF and FTC, (Figure 36A) and NNRTIs, NVP and TMC278, (Figure 36B). On determining data quality from Hill slope and R^2 calculations in both 293T (Table 26) and U87 (Table 28) cell lines, we measured IC50 and IC90 values for HIV-1 (M) WT LUC compared to the mutant HIV-1 (M)P90A LUC vector. On comparing IC50 (Table 26) and IC90 (Table 27) values in the HEK293T and U87 cell line, between WT and the P90A mutant (Tables 27-28), we measured no significant differences between WT and mutant vector. This data suggests that the CA mutant, P90A, has no measurable effect on viral sensitivity to RTIs in both cell lines tested.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 35: HIV-1 (LUC) WT and CypA-binding mutant, P90A, are equally inhibited by both NRTI and NNRTI drugs in HEK293T cells**

HEK293T cells were infected with HIV-1 (M) R9 WT (black circle) or CypA-binding mutant, P90A (red triangle) LUC vector in the presence of titrated (A) NRTI drug, TNF, or (B) NNRTI drug, NVP, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of two independent experiments performed in biological replicate. Error bars +/- SEM.
Figure 36: HIV-1 (LUC) WT and CypA-binding mutant, P90A, are equally inhibited by both NRTI and NNRTI drugs in U87 cells

U87 cells were infected with HIV-1 (M) R9 WT (black circle) or CypA-binding mutant, P90A (red triangle) LUC vector in the presence of titrated (A) NRTI drugs, TNF, and FTC or (B) NNRTI drugs, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of three independent experiments performed in biological replicate. Error bars +/- SEM.
Table 26: Calculation of IC50 values for HIV-1 (M) WT and CypA-binding mutant, P90A, LUC vectors in 293T cells shows no significant difference in inhibition by NRTI or NNRTI drugs

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<td></td>
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<td>P90A</td>
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<tr>
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Table 27: Calculation of IC90 values for HIV-1 (M) WT and CypA-binding mutant, P90A, LUC vectors in HEK293T cells

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IC50 values for NRTI drug, TNF, and NNRTI drug, NVP. Average of two independent experiments performed with biological replicate in 293T cells, as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of two independent experiments.

IC90 values for NRTI drug, TNF, and NNRTI drug, NVP. Average of two independent experiments performed with biological replicate in 293T cells, as calculated in the statistical application GraphPad Prism.
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**Table 28: Calculation of IC50 values for HIV-1 (M)R9 WT and CypA-binding mutant, P90A LUC vectors in U87 cells shows no significant difference in inhibition by NRTI or NNRTI drugs.**

IC50 values for NRTI drugs, TNF and FTC, and NNRTI drugs, NVP and TMC278. Average of three independent experiments in U87 cells, as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
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Table 29: Calculation of IC90 values for HIV-1 (M) WT and CypA-binding mutant, P90A, in U87 cells

IC90 values for NRTI drugs, TNF and FTC, and NNRTI drugs, NVP and TMC278. Average of three independent experiments in U87 cells, as calculated in the statistical application GraphPad Prism.

3.4.6 CypA-binding loop mutants, A88M and A88V, demonstrate no significant difference in sensitivity to RTIs in the U87 cell line

Aside from the CypA-binding CA mutant, P90A, other HIV-1 CA CypA-binding loop mutants have been described in HIV-1 (M) at CA positions, A88 and H87 (325). Interestingly, while HIV-1(M) viruses are 61.2% conserved with a histidine (H) at position 87, 31.8% have a glutamine (Q). 7.1% are alternative residues, such as proline (P), valine (V) or alanine (A) (326). At position 88, 95.3% of HIV-1 (M) viruses are conserved with an alanine (A) (326). However, 4.7% have alternative residues at this position, such as valine (V) or methionine (M). Interestingly, HIV-1 (O) viruses may have a V or M residue at position 88 (327).

On mutating HIV-1 (M) A88M, the CypA-binding loop has an altered structural conformation, due to the change of residue orientation from methionine (328). HIV-1 (M) A88V mutant has increased sensitivity to TRIM-Cyp (RhTC) (327) and reduced binding
and sensitivity to myxovirus resistance protein B (MxB) (280). However, A88V demonstrates less restriction of CypA-binding (4.8 fold) compared to the P90A CA mutant (36 fold). Measured by surface plasmon resonance (SPR), this data suggests that the A88V mutant is able to bind some CypA (329). After considering this data, we sought to determine whether mutating CA residues at positions 87 or 88 would convey any differences in RTI sensitivity.

First, we tested whether HIV-1 (O) A88M and A88V LUC mutants demonstrated an infection defect in the U87 cell line, as previously determined for the HIV-1 (M) P90A LUC mutant. On titration of our panel of HIV-1 (M) WT and mutant LUC vectors, we observed an infection defect for both A88M (three-fold) and A88V (four-fold) mutants, at the top dose. However, P90A demonstrated the greatest infection defect (57.8x) (Figure 37). Having determined an effect on viral titre on mutating residue 88 in CypA loop binding, we asked whether we could measure an effect in viral sensitivity to RTIs.

**Figure 37: CypA-binding loop mutants, HIV-1 (M) A88M and A88V, demonstrate an infection defect on titration in U87 cells**

HIV-1 (M)R9 WT (black circle) or HIV-1 (M) cypA loop binding mutants, P90A (red triangle), A88M (red square) and A88V (blue triangle), were titrated in U87 cell line for 48 hours. Infection was determined on LUC readout (average RLU). Data representative of at least two independent experiments performed with biological duplicate. Error bars +/- SD.
Figure 38: HIV-1 (M) WT and CypA-binding loop mutants, A88M and A88V, demonstrate no difference in sensitivity to RTIs

U87 cells were infected with HIV-1 (M)R9 WT (black circle), A88M mutant (red square) or A88V mutant LUC vectors (blue triangle) in the presence of titrated (A) NRTI drug, TNF, and (B) NNRTI drug, NVP, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicate. Error bars +/- SEM.

We titrated NRTI, TNF (Figure 38A), and NNRTI, NVP (Figure 38B), and determined the percentage of inhibition for each drug curve. We then compared both IC50 (Table 30) and IC90 (Table 31) values for NRTI and NNRTIs and measured no significant difference in values for either TNF or NVP in the U87 cell line. This suggests, as previously determined for CypA-binding loop residue, P90, that mutations in this region of CA do not confer differences in sensitivity to RTIs.
Table 30: Calculation of IC50 values for HIV-1 (M) WT and CypA-binding mutants, A88M and A88V, LUC vectors in the U87 cell line shows no difference in sensitivity to RTI drugs

IC50 values for NNRTI drug, TNF, or NNRTI drug, NVP. Average of three independent experiments performed in biological replicate in U87 cells, as calculated in the statistical application GraphPad Prism. Hill slope and $R^2$ value representative of three independent experiments.

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<tbody>
<tr>
<td></td>
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<td>WT A88M A88V</td>
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<tr>
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<tr>
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<td>1.00 0.72 0.78</td>
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<tr>
<td>$R^2$</td>
<td>0.99 0.99 1.00</td>
<td>0.99 1.00 1.00</td>
</tr>
</tbody>
</table>

Table 31: Calculation of IC90 values for HIV-1 (M) WT and CypA-binding mutants, A88M and A88V, LUC vectors in the U87 cell

IC90 values for NNRTI drug, TNF, or NNRTI drug, NVP. Average of three independent experiments performed in biological replicate in U87 cells, as calculated in the statistical application GraphPad Prism.

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<tr>
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<td>WT A88M A88V</td>
<td>WT A88M A88V</td>
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<tr>
<td>Average IC90 (μM)</td>
<td>24.76 21.32 19.83</td>
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<td>SEM</td>
<td>2.90 0.86 0.83</td>
<td>0.01 0.01 0.01</td>
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<td>Fold Difference</td>
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<tbody>
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<td></td>
<td>WT A88M A88V</td>
<td>WT A88M A88V</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
<td>0.28 0.22 0.23</td>
<td>0.01 0.01 0.01</td>
</tr>
<tr>
<td>SEM</td>
<td>0.01 0.01 0.01</td>
<td>0.01 0.01 0.01</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00 0.80 0.85</td>
<td>1.00 0.80 0.85</td>
</tr>
</tbody>
</table>
3.4.7 **CypA-binding loop CA mutants, H87A and H87P, demonstrate no significant difference in sensitivity to RTIs in the U87 cell line**

HIV-1 O-group is known to be polymorphic at CypA-binding loop residue 87. The polymorphism, H87A, in rhesus monkeys is able to overcome restriction by the host factor TRIM5α (330, 331). H87A does not demonstrate a large infection defect despite the known role for this position of CA in CypA binding. In fact, H87A mutant only has a 4.8-fold reduction in CypA binding, as measured by SPR (329). Another mutant at position 87, H87P, has previously been described in the context of escape from CA-binding inhibitor, PF74 (178). This mutant has poor fitness, both in cell lines and primary macrophages. However, the HIV-1 (M) H87P mutant also has a significant block to infection and resistance against the CPSF6-binding pocket targeting drug, PF74. Interestingly, HIV-1 CA mutant with five substitutions, referred to as 5Mut encoding Q67H, K70R, H87P, T107N, and L111I, is also insensitive to PF74 (332). This PF74-resistance has been described as being due to CA destabilisation by mutation (178, 332). However, evidence for this is limited.

We first tested whether HIV-1 (M)H87A and H87P LUC mutants had an infection defect in the U87 cell line. On titrating both WT and mutant vectors, we measured no infection defect in both HIV-1 (M)H87A and H87P LUC mutants (Figure 39). We quantified IC50 (Table 32) and IC90 (Table 33) values, from inhibition curves, for HIV-1 (M)H87A and H87P LUC (Figure 40). On comparing IC values against HIV-1 (M) WT vector, we measured no significant difference in vector sensitivity to NRTIs or NNRTIs. Collectively, our data from CPSF6 and CypA cofactor binding mutants, suggests that the prevention of cofactor recruitment does not significantly affect viral sensitivity to RTIs, in the HEK293T or U87 cell lines.

![Figure 39: HIV-1 (M) CypA-binding loop mutants, H87A and H87P, demonstrate a small infection defect in the U87 cell line](image-url)

HIV-1 (M)R9 WT (black circle) or CypA-binding loop mutants, H87A (red square) and H87P (orange triangle), were titrated in U87 cells. Data representative of at least two independent experiments performed in biological replicate. Error bars +/- SD.
Figure 40: HIV-1 (M) WT and CypA-binding loop mutants H87A and H87P demonstrate no differences in sensitivity to RTI drugs in the U87 cell line

U87 cells were infected with HIV-1 (M)R9 WT (black circle) or H87A (red square) and H87P (orange triangle) mutants in the presence of titrated (A) NRTI drug, TNF, and (B) NNRTI drug, NVP, for 48 hours. Percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicate. Error bars +/- SEM.

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<tr>
<td></td>
<td>WT</td>
<td>H87A</td>
<td>H87P</td>
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<tr>
<td>Average IC50 (µM)</td>
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<td>H87P</td>
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<td>Fold Difference</td>
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<td>1.53</td>
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<td>Hill slope</td>
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</tr>
<tr>
<td>R²</td>
<td>0.99</td>
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</tr>
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</table>

Table 32: Calculation of IC50 values for HIV-1 (M) WT and and CypA-binding loop mutants, H87A and H87P, in the U87 cell line shows no difference in sensitivity to RTI drugs in the U87 cell line

IC50 values for NNRTI drug, TNF, or NNRTI drug, NVP. Average of three independent experiments performed in biological replicate in U87 cells, as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
Table 33: Calculation of IC90 values for HIV-1 (M) WT and CypA-binding loop mutants, H87A and H87P, in the U87 cell line show no difference in sensitivity to RTI drugs

<table>
<thead>
<tr>
<th></th>
<th>TNF WT</th>
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<th>H87P</th>
</tr>
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<tbody>
<tr>
<td>Average IC90 (μM)</td>
<td>29.03</td>
<td>19.37</td>
<td>19.99</td>
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<tr>
<td>SEM</td>
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<table>
<thead>
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<th>H87P</th>
</tr>
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<tbody>
<tr>
<td>Average IC90 (μM)</td>
<td>0.23</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>SEM</td>
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<td>0.01</td>
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<tr>
<td>Fold Difference</td>
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<td>1.07</td>
<td>1.21</td>
</tr>
</tbody>
</table>

IC90 values for NNRTI drug, TNF, or NNRTI drug, NVP. Average of three independent experiments performed in biological replicate in U87 cells, as calculated in the statistical application GraphPad Prism.

3.5 Addition of hyper-stabilising mutations in HIV-1 CA does not alter viral sensitivity to RTIs in the U87 cell line

Previously, CA escape mutants, A92E and G94D (Figure 41), have been found to overcome CypA inhibition, in the presence of CsA, in some cell lines, but not others. This appears to be dependent on cellular levels of CypA. Mutant A92E becomes insensitive to CsA in cell lines with low CypA. However, in cells with high levels of CypA, HIV-1 CA mutant, A92E or G94D, becomes dependent on CsA. Therefore, high levels of cellular CypA inhibit A92E and G94D, but their infection can be restored by the addition of CsA (333, 334).

Despite having similar levels of RT to WT virus, mutants A92E G94D have an infection defect in HeLa cells due to high CypA levels and poor nuclear entry. Therefore, a hypothesis is that they do not effectively uncoat at the nuclear pore (335). In support of this observation, A92E and G94D mutants have also been shown to assemble CA cores and tubes with increased core stability. This is true for both mutants and, as such, they have been described previously as hyper-stable mutants (290).

Considering that CA mutants, A92E and G94D, do not require stabilisation by CypA, we asked whether mutants A92E and G94D have altered RTI sensitivity. We hypothesised that the CA cones of both A92E and G94D vectors might remain intact within the cytoplasm for longer and, therefore, allow for the concentration of RTIs within the CA core. Therefore, we expected both mutant vectors to have an increased sensitivity to RTIs, measurable by lower IC50 and IC90 values.
To test this hypothesis, first, we tested whether CA mutant’s HIV-1 (M)\textsubscript{A92E} and \textsubscript{G94D} had an infection defect in the U87 cell line compared to HIV-1 (M) WT vector. On titration, we measured HIV-1 (M)\textsubscript{A92E} LUC had a five-fold increase in infection in the U87 cell line, while HIV-1 (M)\textsubscript{G94D} LUC demonstrated the same infectivity as the HIV-1 (M) WT LUC vector (Figure 42).

Next, we asked whether mutating CA residue, A92 or G94, would affect viral sensitivity to the NRTI, TNF, (Figure 43A) and NNRTI, NVP, (Figure 43B). However, on comparing IC\textsubscript{50} (Table 34) and IC\textsubscript{90} (Table 35) values for NRTI and NNRTIs, we measured no significant difference in values for either CA mutant in the U87 cell line. This demonstrated that altering HIV-1 CA stability by mutation does not alter viral sensitivity to RTIs in the U87 cell line.

**Figure 41:** Mapping CA ‘hyper-stable’ mutants A92E and G94D to the HIV-1 (M) hexameric CA structure

HIV-1 M-group CA hexameric sub-unit, 5HGM (Jacques, D. A., et al., 2016) (blue), with marked positioning of hyper-stable mutation locations, A92 (yellow), G94 (orange) and R18 (red).
U87 cells were infected with HIV-1 (M) R9 WT (black circle), CA mutants A92E (orange diamond) or G94D (red diamond) LUC vectors for 48 hours. Infection was determined by RLU readout. Data representative of at least two independent experiments performed in biological duplicate. Error bars +/- SEM.

U87 cells were infected with HIV-1 (M) R9 WT (black circle), CA mutants A92E (orange diamond) or G94D (red diamond) in the presence of titrated (A) NRTI drug, TNF, and (B) NNRTI drug, NVP, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicate. Error bars +/- SEM.
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>A92E</td>
<td>G92D</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
<td>12.28</td>
<td>13.33</td>
<td>12.52</td>
</tr>
<tr>
<td>SEM</td>
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<tr>
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<td>$R^2$</td>
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Table 34: Calculation of IC50 values for HIV-1 (M)R9 WT and CA mutants, A92E and G94D, in the U87 cell line demonstrate no difference in viral sensitivity to NRTI or NNRTIs.

IC50 values for NRTI drug, TNF, and NNRTI drug, NVP. Average of three independent experiments in U87 cells, as calculated in the statistical application GraphPad Prism. Hill slope and $R^2$ value representative of three independent experiments.

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<tr>
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<td>G94D</td>
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<td>Average IC90 (μM)</td>
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<td>G94D</td>
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<td>Average IC90 (μM)</td>
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</table>

Table 35: Calculation of IC90 values for HIV-1 (LUC) WT and CA mutants, A92E and G94D, in the U87 cell line.

IC90 values for NRTI drug, TNF, and NNRTI drug, NVP. Average of three independent experiments in U87 cells, as calculated in the statistical application GraphPad Prism.
3.6 HIV-1 bearing M-group and O-group Gags have 2-fold difference in sensitivity to NRTIs

While HIV-1 M-group has spread to become a global pandemic, HIV-1 N-, P- and O-group viruses have not demonstrated similarly high rates of human-to-human transmission. First isolated in the Cameroon and Gabon HIV-1 O-group is so named because it is a genetic outlier in contrast to HIV-1(M) (336). HIV-1 O-group was first described as having large envelope (Env) sequence diversity (336). However, the CA of HIV-1 O-group isolate MVP5180 (MVP) and M-group viruses also demonstrate differences, in cofactor sensitivity. One such example is a reduced requirement for CypA binding for productive infection. While O-group virus packages CypA in the same way as HIV-1 M-group viruses, O-group infectivity is unaffected when treated with CypA targeting drug, CsA. This suggests that CypA is not an absolute requirement for O-group replication, despite conservation of CA position, P90, and the CypA-binding site in O-group viruses (Figure 44) (141, 337, 338). While HIV-1 M-group and O-group viruses both bind CypA, HIV-2 does not bind CypA (179, 339).

Of particular interest to our research question was the observation that over half of HIV-1 O-group sequences demonstrate high resistance to NNRTIs. This was previously linked to a mutation at residue 181 of RT (340). For example, Descamps et al found the O-group isolate, MVP, had a 16.5-fold increase in IC50 for NNRTI NVP compared to M-group isolates in monocytes, extracted from peripheral blood (340). However, no link has previously been made between differences in HIV-1 Gag and decreased HIV-1 O-group sensitivity to NNRTIs.

On solving the MVP CA hexamer crystal structure, our collaborators recently found the CA pore of MVP to be in an open conformation (Figure 45). In fact, the HIV-1 (O) CA hexamer pore was open despite low pH crystallography, which would usually result in a closed pore in HIV-1 (M) (Figure 45A). This showed that protonation of position H12 within the CA beta hairpin, usually associated with a closed structure, failed to close the O-group pore. Furthermore, differences in residues at position 50 (tyrosine) and 48 (valine), demonstrate, by not being able to coordinate with a water molecule, that MVP favours the open conformation (Figure 45B). We have recently shown the ability of HIV-1 M-group CA positively charged residue R18, within the electrostatic channel, to bind negatively charged dNTPs (30). Interestingly, R18 is highly conserved in O-group viruses (Figure 38), suggesting that O-group viruses may retain the ability to fuel encapsidated RT through this mechanism.

Interestingly, the open conformation of the O-group CA pore of HIV-1 (O)_MVP and HIV-1 (O)_RBF206 is associated with an increased rate of RT compared to HIV-1 (M) GFP vector. This was determined by Lorena Zuliani- Alvarez, on measuring strong stop and 2nd strand
transfer, by q-PCR, in suspension THP-1 cells (Figure 46). However, in macrophages O-group infection and replication is blocked due to triggering of IFN. This inability to evade innate sensing in macrophages might contribute to O-group viruses not becoming pandemic (Jane Rasaiyaah, manuscript in preparation).

Considering the data characterising the HIV-1 O-group Gag, we sought to understand whether differences in the structures of the CA pores of HIV-1 M-group and O-group viruses result in differences in sensitivity to RTIs. We hypothesised that vector bearing Gag from O-group virus MVP, in which the CA pore appears fixed open, would require an increased dose of RTIs to inhibit infection.

**Figure 44: HIV-1 M-group and O-group CA alignment shows high sequence conservation**

Figure 45: Comparison of HIV-1 M-group and O-group CA hexameric structures

(A) Solving of HIV-1 (M) hexameric CA structure at high pH (5HGL) shows the CA pore in a closed conformation due to β-hairpin (green) positioning. This is determined by the coordination of a water molecule between key residues 12, 48, 50 and 51. (B) Solving of the HIV-1 (O)MVP CA hexamer at high pH demonstrates the open CA pore due to β-hairpin (pink) positioning. This is determined by the altered structural conformation of O-group polymorphisms at CA residues 48 and 50. Figure from David Jacques, UNSW (manuscript in preparation).
Figure 46: Quantification of HIV-1 (M) and HIV-1 (O) early and late RT products in the THP-1 cell line

(A) Suspension THP-1 cells were infected with DNAse treated vectors bearing Gag from, HIV-1 (M)R9, HIV-1 (O)MVP or HIV-1 (O)RBF206 GFP vectors equalised by SG-PERT. (B) qPCR measurements of reverse transcripts of strong stop (early RT) and (C) 2nd strand transfer (late RT) were made from one to nine hours post-infection. Early RT products and late RT products. Data from four independent experiments. Error bars +/- SEM. Paired t-test was performed to compare M-group versus O-group viruses at each time point. P values refer to R9 versus MVP5180 (black stars), R9 versus RBF206 (grey stars) * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001. Figure from Lorena Zuliani-Alvarez, UCL (manuscript in preparation).

3.6.1 Determining the effect of TREX1 over expression in the U87 cell line on HIV-1 (M) and HIV-1 (O) infection

HIV-1 O-group has previously been determined to have an infection defect in MDM due to innate immune triggering (Jane Rasaiyaah, unpublished). We asked whether HIV-1 (O) WT LUC vector, which encodes O-group Gag fused to M-group chimeric junction in Pol, also demonstrated an infection defect in the U87 cell line.

To address this question, we considered whether over expression of the host exonuclease, TREX1, would affect viral titre in the U87 cell line. We hypothesised that HIV-1 (O) CA cones might undergo increased CA uncoating in the host cytoplasm. While this is known to result in triggering an innate immune response in MDM, uncoating may be licensed in the U87 cell line. Therefore, we hypothesised that the over expression of TREX1 would degrade DNA of early uncoating HIV-1 (O) virions, resulting in decreased infection.
To test these hypotheses, we stably over-expressed V5-tagged TREX1 in the U87 cell line (Figure 47B). We titrated HIV-1 (M)R9 and HIV-1 (O)MVP GFP vectors on TREX1 cells or control cells and measured IU normalised to URT (IU/U RT), quantified by SG-PERT (Figure 47A). We measured a 2.3-fold decrease in HIV-1 (O) infection in the TREX1-V5 cells compared to the control, while the HIV-1 (M) vector demonstrated no significant difference in vector titre on TREX1 over-expression (Figure 47A).

One possibility is that HIV-1 (O) CA instability results in the release of vDNA to the host cytoplasm. In U87 cells this does not cause triggering, therefore, early uncoating virions are still infectious. However, on the over-expression of TREX1, vDNA from uncoated virions is degraded, thus resulting in the decrease in the viral titre measured.

![Figure 47: HIV-1 (O)MVP GFP vector titre is reduced in U87 cell line over expressing the exonuclease TREX1](image)

(A) U87 cells over-expressing TREX1-V5 (white bars) or control plasmid (black bars) were infected with HIV-1 (M)R9 or HIV-1 (O) GFP vector. 48 hours post-infection IU were calculated by flow cytometry and normalised to mU of RT (IU/mU RT), quantified by SG-PERT method. (B) Immunoblot of TREX1 to determine over-expression in U87 cells. Data from two independent experiments. Error bars +/- SD.

### 3.6.2 The HIV-1 O-group vector has increased sensitivity to NNRTIs, compared to HIV-1 M-group vector, in the U87 cell line

Having demonstrated differences in HIV-1 (M) and HIV-1 (O) titre on over expression of TREX1, suggesting possible differences in CA uncoating, we next asked whether differences in HIV-1 (M) LUC and HIV-1 (O) LUC Gag would affect viral sensitivity to RTIs in WT cell lines. We hypothesised that if HIV-1 (O) LUC CA cones were uncoating early in the cytoplasm, this may have an effect on RTI sensitivity, because the drugs may more easily access the reverse transcriptase complex. However, an open pore and increased rate of early stages of reverse transcription may also increase the inhibitory dose of RTI required. We therefore, tested whether O-group vectors require an increased dose of RTIs to inhibit.
Wishing to further examine cell-type differences between HIV-1 (M) LUC and HIV-1 (O) LUC vector, focusing on sensitivity to inhibitors, we selected the U87 cell line. Tibet et al previously used the U87 cell line expressing CD4 and co-receptors, X4 and R5, to determine NRTI and NNRTI IC50 values of HIV-1 O-group primary isolates, compared to HIV-1 M-group. They determined that HIV-1 O-group primary isolates had differential sensitivity to NNRTI, NVP, compared to M-group isolates. Interestingly, HIV-1 O-group isolates had the same IC50 value as HIV-1 M-group isolates for NRTIs. Furthermore, Tibet et al found that while some isolates were only partially insensitive to inhibition by NNRTIs, other HIV-1 (O) isolates had a large decrease in sensitivity, evidenced by an increase in IC50 value. They concluded that such differences in sensitivity could be attributed to polymorphisms or point mutations located within the V3 loop of glycoprotein 120kDa (gp120) (envelope) (341). Considering these data, we sought differences in RTI sensitivity using our HIV-1 (M) LUC and HIV-1 (O) LUC vectors to attribute differential NNRTI sensitivity in O-group virus to differences in Gag.

To determine if HIV-1 (O) LUC demonstrated an infection defect compared to HIV-1 (M) LUC vector, we titrated both vectors in the U87 cell line. We confirmed the HIV-1 (O) LUC vector had a titre defect in this cell line (Figure 48).

We next asked whether differences in HIV-1 (M) LUC and HIV-1 (O) LUC Gag would affect viral sensitivity to RTIs in the U87 cell line (Figure 49). We compared HIV-1 (O) vector compared to HIV-1 (M) vector IC50 (Table 36) and IC90 (Table 37) values of NRTIs: TNF and FTC, and NNRTIs, NVP and TMC278, and measured no significant difference in O-group sensitivity to either NRTI, compared to M-group. Interestingly we found that the HIV-1 (O) LUC vector had a 2-fold reduction in sensitivity to NNRTIs, determined through measuring both IC50 and IC90 values, compared to the HIV-1 (M) vector in the U87 cell line. Our data is consistent with the data obtained by Tibet et al, although we observe a smaller effect size, and suggests that differences in O-group Gag confer small differences in RTI sensitivity.
Figure 48: Infection of HIV-1 (M) and HIV-1 (O) LUC vectors in the U87 cell line

HIV-1 (M)\textsubscript{R9} and HIV-1 (O)\textsubscript{MVP} LUC vectors were titrated in the U87 cell line and average RLU measured by steady glo protocol 48 hours post-infection. Data representative of at least two independent experiments. Error bars +/- SD.

Figure 49: HIV-1 (O) LUC is less inhibited by NNRTIs in U87 cells compared to HIV-1 (M) LUC vector

U87 cell line was infected with HIV-1 (M)\textsubscript{R9} (black circle) or HIV-1 (O)\textsubscript{MVP} (blue open square) vector in the presence of titrated (A) NRTIs, TNF or FTC, or (B) NNRTI, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of three independent experiments performed in biological replicate. Error bars +/- SEM.
<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th></th>
<th>FTC</th>
<th></th>
<th>NVP</th>
<th></th>
<th>TMC278</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
<td>5.89</td>
<td>5.88</td>
<td>0.23</td>
<td>0.42</td>
<td>0.03</td>
<td>0.06</td>
<td>0.44</td>
<td>1.18</td>
</tr>
<tr>
<td>SEM</td>
<td>0.17</td>
<td>0.29</td>
<td>0.05</td>
<td>0.06</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.85</td>
<td>1.00</td>
<td>2.08</td>
<td>1.00</td>
<td>2.67</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.15</td>
<td>-1.24</td>
<td>-1.36</td>
<td>-1.37</td>
<td>-1.07</td>
<td>-1.59</td>
<td>-1.03</td>
<td>-1.66</td>
</tr>
<tr>
<td>R²</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 36: Calculation of IC50 values for HIV-1 (M) and HIV-1 (O) LUC vectors in the U87 cell line shows an increased NNRTI IC50 for the HIV-1 (O) vector.

IC50 values for NRTI drugs, TNF and FTC, or NNRTI drugs, NVP and TMC278, average of three independent in U87 cells as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments, as above.
Table 37: Calculation of IC90 values for HIV-1 (M) and HIV-1 (O) in the U87 cell line shows an increased NNRTI IC90 for HIV-1 (O) LUC vectors

IC90 values for NRTI drugs, TNF and FTC, or NNRTI drugs, NVP and TMC278, average of three independent in U87 cells, as calculated in the statistical application GraphPad Prism.

The decrease in HIV-1 (O) vector sensitivity to NNRTIs in the U87 cell line led us to question whether RTI sensitivity could be attributed to the open conformation of the CA pore. Therefore, we sought to measure, in the U87 cell line, if the HIV-1 (M)Q50Y+120R open CA mutant vector also had reduced sensitivity to inhibition by NNRTIs. We hypothesised that if decreased vector sensitivity to NNRTIs was related to the open CA pore, then HIV-1 (M)Q50Y+120R would also demonstrate at least a two-fold reduction in IC50 and IC90 for NNRTI, NVP. Conversely, we hypothesised that the closed pore of the mutant HIV-1 (M)H12Y might have an increased sensitivity to NNRTIs.

However, on measuring IC50 (Table 38) and IC90 (Table 39) values for both HIV-1 (M)Q50Y+120R and HIV-1 (M)H12Y LUC vectors, compared to HIV-1 (M)R9WT or HIV-1(M)CRV WT control vectors, in the U87 cell line (Figure 50), we measured no significant differences in vector sensitivity to NRTI or NNRTI.

<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th>FTC</th>
<th>NVP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>O</td>
<td>M</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
<td>15.34</td>
<td>14.62</td>
<td>0.66</td>
</tr>
<tr>
<td>SEM</td>
<td>0.47</td>
<td>0.41</td>
<td>0.03</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>0.95</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>NVP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>O</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
<td>1.33</td>
<td>2.59</td>
</tr>
<tr>
<td>SEM</td>
<td>0.09</td>
<td>0.27</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>1.95</td>
</tr>
</tbody>
</table>
The open pore mutant HIV-1 (M) Q50Y+120R does not demonstrate a reduction in sensitivity to NNRTIs, compared to HIV-1 (M) WT, while HIV-1 (O) vector does. This suggests that CA pore conformation may not be the reason for differing sensitivity. As previously discussed, O-group viruses bear differences throughout Gag, compared to M-group, for example, throughout the CypA-binding loop. It is, therefore, possible that other residues, throughout Gag influence the cell type dependent reduction in O-group NNRTI sensitivity. For example, influencing the location of uncoating within the cell.

Figure 50: HIV-1 (M) LUC WT and CA pore mutant vectors Q50Y+120R and H12Y are equally inhibited by NRTI and NNRTI in the U87 cell line

U87 cells were infected with HIV-1 (M)R9 WT (black square), Q50Y+120R (blue circle), HIV-1 (M)CRV WT (maroon square) and HIV-1 CRV H12Y (orange square) Gag, in the presence of titrated (A) NRTI, TNF, or (B) NNRTI, NVP, for 48 hours. The percentage of viral inhibition was calculated. Data from of two independent experiments performed in biological replicate error bars +/- SD.
Table 38: Calculation of IC50 values for HIV-1 (M) WT or CA pore mutant LUC vectors show no significant differences in sensitivity to RTIs in the U87 cell line

<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Q50Y+120R</td>
<td>CRV</td>
<td>H12Y</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
<td>2.56</td>
<td>2.63</td>
<td>3.76</td>
<td>2.42</td>
</tr>
<tr>
<td>SEM</td>
<td>0.11</td>
<td>0.38</td>
<td>0.53</td>
<td>0.78</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>1.02</td>
<td>1.00</td>
<td>0.64</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.10</td>
<td>-1.11</td>
<td>-1.55</td>
<td>-1.36</td>
</tr>
<tr>
<td>R²</td>
<td>1.00</td>
<td>0.99</td>
<td>1.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NVP</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Q50Y+120R</td>
<td>CRV</td>
<td>H12Y</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
<td>0.53</td>
<td>0.54</td>
<td>0.60</td>
<td>0.59</td>
</tr>
<tr>
<td>SEM</td>
<td>0.46</td>
<td>0.46</td>
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<td>0.40</td>
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<tr>
<td>Fold Difference</td>
<td>1.00</td>
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<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.00</td>
<td>-1.25</td>
<td>-1.10</td>
<td>-1.55</td>
</tr>
<tr>
<td>R²</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 39: Calculation of IC90 values for HIV-1 (M) WT or CA pore mutant LUC vectors show no significant differences in sensitivity to RTIs in the U87 cell line

<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Q50Y+120R</td>
<td>CRV</td>
<td>H12Y</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
<td>40.05</td>
<td>30.56</td>
<td>18.65</td>
<td>19.20</td>
</tr>
<tr>
<td>SEM</td>
<td>12.56</td>
<td>9.37</td>
<td>0.36</td>
<td>3.01</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>0.76</td>
<td>1.00</td>
<td>1.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NVP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Q50Y+120R</td>
<td>CRV</td>
<td>H12Y</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
<td>0.75</td>
<td>1.03</td>
<td>1.45</td>
<td>0.90</td>
</tr>
<tr>
<td>SEM</td>
<td>0.03</td>
<td>0.08</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>1.37</td>
<td>1.00</td>
<td>0.62</td>
</tr>
</tbody>
</table>

3.6.3 Measuring sensitivity of HIV-1 O-group to NRTI and NNRTIs compared to M-group vector in the HEK293T cell line

On titrating both HIV-1 (M) WT LUC and HIV-1 (O) WT LUC vectors we noted a large infection defect for the O-group virus in the HEK293T cell line (Figure 51). We then compared IC50 (Table 40) and IC90 (Table 41) values for NNRTIs, ABC, TNF and AZT, (Figure 52A) and NNRTIs, NVP and TMC278, (Figure 52B). We found that both HIV-1 (M)
and (O) LUC vectors have the same sensitivity to NRTIs, as determined by the similarity in IC50 and IC90 values. However, the HIV-1 (O) LUC vector had a 2-fold increase in IC50 and IC90 value for NNRTIs. This suggested that HIV-1 (O) vector has a reduction in sensitivity to NNRTIs in the HEK293T cell line. This is consistent with previous literature. However, our data uniquely suggests a role for Gag in RTI sensitivity, not just reverse transcriptase (342).

![Figure 51 Comparison of HIV-1 (M)R9 and HIV-1 (O)MVP vector infection in the HEK293T cell line](image)

HEK293T cells were infected with HIV-1 (M)R9 WT (black circle) or HIV-1 (O)MVP (blue square) for 48 hours. Infection was determined by RLU readout, data representative of at least two independent experiments performed in biological duplicate. Error bars +/- SEM.
Figure 52: The HIV-1 (O) LUC vector is less sensitive to NNRTIs, compared to HIV-1 (M) LUC vector in the HEK293T cell line

HEK293T cells were infected with HIV-1 (M)R9 WT (black circle) or HIV-1 (O)MVP (blue open square) LUC vectors, in the presence of titrated (A) NRTI drugs, ABC, d4T, TNF and AZT, or (B) NNRTI drugs, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicate. Error bars +/- SEM.
|          | ABC       |          |  |          |  |
|----------|-----------|----------|  |          |  |
|          | M   | O   |  |          |  |
| Average IC50 (μM) | 2.27 | 3.33 |  |          |  |
| SEM      | 0.06 | 0.25 |  |          |  |
| Fold Difference | 1.00 | 1.47 |  |          |  |
| Hill slope | -1.48 | -1.47 |  |          |  |
| R²       | 0.99 | 0.99 |  |          |  |
|          |          |          |  |          |  |
|          | TNF      |          |  |          |  |
|          | M   | O   |  |          |  |
| Average IC50 (μM) | 0.73 | 0.83 |  |          |  |
| SEM      | 0.00 | 0.12 |  |          |  |
| Fold Difference | 1.00 | 1.14 |  |          |  |
| Hill slope | -1.22 | -0.96 |  |          |  |
| R²       | 0.99 | 1.00 |  |          |  |
|          |          |          |  |          |  |
|          | AZT      |          |  |          |  |
|          | M   | O   |  |          |  |
| Average IC50 (μM) | 0.17 | 0.11 |  |          |  |
| SEM      | 0.04 | 0.01 |  |          |  |
| Fold Difference | 1.00 | 0.65 |  |          |  |
| Hill slope | -0.62 | -0.61 |  |          |  |
| R²       | 0.99 | 0.97 |  |          |  |
|          |          |          |  |          |  |
|          | NVP      |          |  |          |  |
|          | M   | O   |  |          |  |
| Average IC50 (μM) | 0.07 | 0.12 |  |          |  |
| SEM      | 0.01 | 0.01 |  |          |  |
| Fold Difference | 1.00 | 1.85 |  |          |  |
| Hill slope | -1.34 | -1.33 |  |          |  |
| R²       | 1.00 | 0.99 |  |          |  |
|          |          |          |  |          |  |
|          | TMC278   |          |  |          |  |
|          | M   | O   |  |          |  |
| Average IC50 (μM) | 1.96 | 1.83 |  |          |  |
| SEM      | 0.57 | 0.54 |  |          |  |
| Fold Difference | 1.00 | 0.93 |  |          |  |
| Hill slope | -1.14 | -1.22 |  |          |  |
| R²       | 1.00 | 1.00 |  |          |  |

Table 40: Calculation of IC50 values for HIV-1 (M) and HIV-1 (O) LUC vectors in 293T cells

IC50 values for NRTI drugs: ABC, TNF and AZT and NNRTI drugs: NVP and TMC278 from an average of three independent experiments performed in biological duplicates as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments.
### Table 41: Calculation of IC90 values for HIV-1 (M) and HIV-1 (O) vectors in 293T

<table>
<thead>
<tr>
<th></th>
<th>ABC</th>
<th>TNF</th>
<th>AZT</th>
<th>NVP</th>
<th>TMC278</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
<td>M</td>
</tr>
<tr>
<td>Average IC90 (µM)</td>
<td>5.50</td>
<td>6.88</td>
<td>1.77</td>
<td>1.95</td>
<td>0.57</td>
</tr>
<tr>
<td>SEM</td>
<td>0.18</td>
<td>0.50</td>
<td>0.04</td>
<td>0.02</td>
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<tr>
<td>Fold Difference</td>
<td>1.00</td>
<td>1.25</td>
<td>1.00</td>
<td>1.10</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

IC90 values for NRTI drugs: ABC, TNF and AZT and NNRTI drugs: NVP and TMC278 from an average of three independent experiments performed in biological duplicates, as calculated in the statistical application GraphPad Prism.

#### 3.6.4 Measuring sensitivity of HIV-1 O-group to NRTI and NNRTIs compared to M-group vector in the Jurkat (JLTR-5) T-cell line

We previously determined that the HIV-1 (M)Q50Y+120R LUC CA open pore mutant has increased sensitivity to both NRTI and NNRTIs in the Jurkat T-cell line compared to HIV-1 (M) LUC vector. This finding is contrary to our demonstration of decreased NNRTI sensitivity of the HIV-1 (O) LUC vector, compared to HIV-1 (M). This suggests that CA pore conformation may not be the only contributing factor to RTI sensitivity in these vectors. Given these data, we tested whether the HIV-1 (O)MVP LUC vector has different sensitivity, compared to HIV-1 (M)R9 LUC vector, to RTIs in the Jurkat cell line.
We hypothesised that we would find a difference in HIV-1 (O) viral sensitivity to NNRTIs. This was based on previous literature, which measured increased IC50 values for NNRTIs on O-group infection, compared to HIV-1 (M)\textsuperscript{Lai} infection, in peripheral blood mononucleocytes (PBMCs) (340, 343).

To determine if the HIV-1 (O)\textsubscript{MVP} vector demonstrated an infection defect compared to HIV-1 (M) vector in the Jurkat T-cell line, we first measured IU/U RT on comparison to M-group to O-group GFP vector, we determined that O-group had an 8.5-fold decrease in IU/U RT (Figure 53A) compared to HIV-1 (M). Titration of HIV-1 (M) and (O) LUC vectors also confirmed there was an infection defect in the O-group Gag bearing vector (Figure 53B).

We next asked whether differences in HIV-1 (M) LUC and HIV-1 (O) LUC Gag would affect viral sensitivity to RTIs in the Jurkat T-cell line (Figure 54). On comparing HIV-1 (O) vector to HIV-1 (M) vector IC50 (Table 42) and IC90 (Table 43) values of NRTI, TNF, and NNRTI, TMC278, we measured no significant difference in HIV-1 (O) LUC vector sensitivity to NRTI or NNRTI in Jurkat T-cells. We found this result surprising considering that we had previously measured an effect for the open pore mutant, in the Jurkat cell line, and HIV-1 (O) LUC, in the 293T cell line. However, we found all cell-type differences measured, in both HIV-1 (M) and HIV-1 (O) vectors, are small, but highly repeatable. This suggests that vector sensitivity to RTIs is influenced by cell type differences.

### Figure 53: The HIV-1 (O) vector has an infection defect in the Jurkat T-cell line

(A) JLTR5 Jurkat T-cells were infected with HIV-1 (M)\textsubscript{R9} and HIV-1 (O)\textsubscript{MVP} GFP vectors and titre (IU/U RT) measured 48 hours post-infection by FACS. (B) JLTR5 Jurkat T-cells were infected with HIV-1 (M)\textsubscript{R9} and HIV-1 (O)\textsubscript{MVP} LUC vectors and average RLU measured by steady glo protocol 48 hours post-infection. Data representative of at least two independent experiments. Error bars +/- SD.
Figure 54: HIV-1 (M) and HIV-1 (O) LUC vectors are equally inhibited by NRTI and NNRTIs in JLTR5 Jurkat T-cells

JLTR-5 Jurkat T-cells were infected with HIV-1 (M) (black circle) or HIV-1 (O) (blue open square) vectors in the presence of titrated (A) NRTI, TNF, or (B) NNRTI drugs, NVP and TMC278, for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from three independent experiments performed in biological replicate. Error bars +/- SEM.
<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th></th>
<th>NVP</th>
<th></th>
<th>TMC278</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
<td>M</td>
<td>O</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
<td>25.07</td>
<td>20.01</td>
<td>0.12</td>
<td>0.14</td>
<td>1.96</td>
<td>1.83</td>
</tr>
<tr>
<td>SEM</td>
<td>2.25</td>
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<td>0.01</td>
<td>0.02</td>
<td>0.57</td>
<td>0.54</td>
</tr>
<tr>
<td>Fold Difference</td>
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<td>0.80</td>
<td>1.00</td>
<td>1.20</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-0.82</td>
<td>-1.02</td>
<td>-1.50</td>
<td>-1.33</td>
<td>-1.14</td>
<td>-1.22</td>
</tr>
<tr>
<td>R²</td>
<td>1.00</td>
<td>0.99</td>
<td>0.98</td>
<td>0.99</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 42: Calculation of IC50 values for HIV-1 (M) and HIV-1 (O) LUC vectors in the JLTR-5, Jurkat T-cell cell line

IC50 values for NRTI drug TNF and NNRTI drugs: NVP and TMC278 from an average of three independent experiments in JLTR-5 cells as calculated in the statistical application GraphPad Prism. Hill slope and $R^2$ value representative of three independent experiments.
<table>
<thead>
<tr>
<th></th>
<th>NVP</th>
<th>TMC278</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average IC90 (μM)</strong></td>
<td>0.26</td>
<td>4.61</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Fold Difference</strong></td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 43: Calculation of IC90 values for HIV-1 (M) and HIV-1 (O) LUC vectors in the JLTR-5 cell line

IC90 values NNRTI drugs: NVP and TMC278 from an average of three independent experiments in JLTR-5 cells as calculated in the statistical application GraphPad Prism.

3.6.5 Mutation of the CypA-binding site in HIV-1 (O) vector does not alter viral sensitivity to RTI drugs in the U87 cell line

We previously demonstrated that CypA binding is an absolute requirement for HIV-1 M-group infection in MDMs, evidenced in our lab by the block to infection and replication of the CypA-binding mutant, P90A, in MDM (141). Furthermore, both treatment of the HIV-1 (M)Rg vector with CsA or CA mutation P90A reduce viral titre in the U87 cell line. More recently, a block to HIV-1 (M)P90A RT has been measured in CD4+ T-cells (Maria Theresa Rodriguez, unpublished).

However, despite O-group virus incorporating CypA it is not an absolute requirement for infection, evidenced by insensitivity to CypA recruitment inhibition by CsA in Jurkat T-cells (141, 179, 338). As previously discussed, HIV-1 O-group is polymorphic at specific residues within the CypA-binding loop that interacts with CypA, specifically CA residue 88.

This led us to ask if mutating HIV-1 (M) at CA position 88 within the CypA-binding loop, to residues found in O-group isolates, would alter CypA binding and sensitivity of virus to RTIs. Despite measuring no significant differences in RTI sensitivity in the A88M and A88V mutant vectors, we questioned whether mutating HIV-1 (O) position 88 from a methionine (M) to an alanine (A) would affect viral sensitivity, in comparing HIV-1 (O). On titration of HIV-1 (O) and HIV-1 (O)M88A LUC, in the U87 cell line, we measured a decrease, of approximately 10-fold, in infection for the HIV-1 (M)M88A LUC vector (Figure 55).

We next asked whether the HIV-1 (O)M88A LUC mutant would demonstrate altered viral sensitivity to RTIs in the U87 cell line compared to HIV-1 (O) LUC. However, on measuring IC50 (Table 44) and IC90 (Table 45) values for HIV-1 (O)M88A and HIV-1(O) in the U87
cell line (Figure 56), we measured no significant differences in vector sensitivity to NRTI or NNRTI.

These data along with our previous results for HIV-1 (M) A88M and A88V mutants, suggests that, despite incurring fitness defects, mutating CA residue 88 in both M-group and O-group Gag does not confer differences in sensitivity to RTIs.

Figure 55: Measuring HIV-1 (O)MVP WT and HIV-1 (O)M88A CypA loop mutant infection in the U87 cell line

Average RLU measured by steady glo protocol 48 hours post-infection. Data representative of at least two independent experiments. Error bars +/- SD.

Figure 56: HIV-1 (O)MVP and CypA loop mutant HIV-1 (O)M88A LUC vectors demonstrate no differences in sensitivity to RTIs

U87 cells were infected with HIV-1 (O)MVP (blue square) or HIV-1 (O)M88A mutant (red triangle) in the presence of titrated (A) NRTI drug TNF and (B) NNRTI drug NVP for 48 hours. The percentage of viral inhibition was calculated from LUC readout. Data from of three independent experiments performed in biological duplicate. Error bars +/- SEM.
<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th></th>
<th>NVP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>M88A</td>
<td>WT</td>
<td>M88A</td>
</tr>
<tr>
<td>Average IC50 (μM)</td>
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<td>6.61</td>
<td>0.14</td>
<td>0.17</td>
</tr>
<tr>
<td>SEM</td>
<td>0.88</td>
<td>1.07</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Fold Difference</td>
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<td>0.93</td>
<td>1.00</td>
<td>1.25</td>
</tr>
<tr>
<td>Hill slope</td>
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<td>-1.42</td>
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<td>-2.17</td>
</tr>
<tr>
<td>R²</td>
<td>1.00</td>
<td>1.00</td>
<td>0.99</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Table 44:Calculation of IC50 values for HIV-1 (O)<sub>MVP</sub>WT and HIV-1 (O)<sub>M88A</sub>CypA-binding loop mutant in the U87 cell line

IC50 values for NRTI TNF or NNRTI drug NVP average of three independent experiments in U87 cells as calculated in the statistical application GraphPad Prism. Hill slope and R² value representative of three independent experiments as above.

<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th></th>
<th>NVP</th>
<th></th>
</tr>
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<tr>
<td></td>
<td>WT</td>
<td>M88A</td>
<td>WT</td>
<td>M88A</td>
</tr>
<tr>
<td>Average IC90 (μM)</td>
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<td>15.33</td>
<td>0.28</td>
<td>0.33</td>
</tr>
<tr>
<td>SEM</td>
<td>1.18</td>
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<td>0.03</td>
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<td>Fold Difference</td>
<td>1.00</td>
<td>0.91</td>
<td>1.00</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Table 45: Calculation of IC90 values for HIV-1 (O)<sub>MVP</sub>WT and HIV-1 (O)<sub>M88A</sub>CypA loop mutant in the U87 cell line

IC90 values for NRTI TNF or NNRTI drug NVP average of three independent experiments in U87 cells, as calculated in the statistical application GraphPad Prism.
3.7 Comparison of full-length HIV-1 M-group, HIV-1 O-group and HIV-2 CA sensitivity to RTIs

3.7.1 Comparison of HIV-1 (M), HIV-1 (O) and HIV-2 CA sequences

Having determined small cell-type dependent differences in HIV-1 (O) LUC sensitivity to NNRTIs, compared to HIV-1 (M) LUC, we next considered another non-pandemic HIV. We chose the HIV-2 lineage which is predominantly confined to West Africa (344) and is derived from simian immunodeficiency virus (SIV) from Sooty Mangabeys (SIVsm) by zoonosis into humans (15). HIV-2 has high genetic relatedness between strains (>80% at nucleotide level) (345). However, on comparing Gag-Pol, between HIV-1 and HIV-2, HIV-2 isolates have just 58-59.4% relatedness, considered low due to the high conservation of this region (345). On sequence alignment of HIV-1 (M) R9, HIV-1 (O) MVP and HIV-2 ROD10 CA sequences, there is low conservation throughout the CA sequence, especially within the β-hairpin region, CA residues 1-13 (Figure 57). However, a comparison of a higher number of sequences is required to determine the significance of this observation. Currently, the only crystal structure available for HIV-2 CA, in which both the CypA-binding loop and β-hairpin are resolved, is just of the NTD, solved by Price et al (327) (Figure 58A). Using this structure Jacques, D.A modelled the HIV-2 hexameric structure (Figure 58B). The HIV-2 hexameric CA model suggests the HIV-2 CA pore is open, exposing R18 residues (Figure 58B). The suggestion of an open HIV-2 CA pore is also based on the β-hairpin positioning in the NTD crystal structure, despite the key residues at HIV-2 ROD10 CA positions 12 (isoleucine, I) and 48 (proline, P) differing from those in the CA pore of HIV-1 (O)MVP (H12, V48, Y50).

Of relevance to this research, are previous measurements made by Shih et al who quantified IC50 values for NNRTI, NVP, using the RNAse H assay. The ribonuclease H (RNase H) assay allows the quantification of reverse transcriptase enzymatic activity. On titration of NNRTIs, RT activity is decreased, dose dependently. Therefore, an IC50 value can be calculated to describe the amount of inhibitor required to reduce RT activity by 50%.

Through use of the RNAseH assay, it was determined that HIV-2 had a significantly higher IC50 value for NNRTI, NVP, (>200 µM) compared to HIV-1 (M) (0.25 µM). The poor NNRTI inhibitory effect on HIV-2 could be mimicked in the HIV-1 (M)Y181V and HIV-1(M)Y188L reverse transcriptase mutants. The mutants also had an increased IC50 value for NVP, 56 µM and >200 µM, respectively (346). Interestingly, the NNRTI resistance of the mutant, Y181V, was later determined to be due to structural differences within the NNRTI pocket, conferring differences in NNRTI binding energy (347). Therefore, differences in the NNRTI binding site dictate sensitivity to NNRTI between HIV-1 and HIV-2.
We previously measured small, but consistent two-fold increase in NNRTI IC50 and IC90 values in the HIV-1 (O) vector, compared to HIV-1 (M) in the U87 cell line. After considering these data, we asked whether we could measure differences between HIV-1 (M)_{R9BAL} or HIV-1 (O)_{MVP} HIV-2_{ROD10} full-length CA chimeras, and attribute differences in RTI sensitivity to CA. We hypothesised, based on our previous data sets that differences in both HIV-1 (O)_{MVP} and HIV-2_{ROD10} CA chimeric R9BAL viruses would decrease viral sensitivity to NNRTIs.

**Figure 57: HIV-1 (M)_{R9}, HIV-1 (O)_{MVP} and HIV-2_{ROD10} CA alignment shows low sequence conservation**

HIV-1 (M)_{R9BAL}, HIV-1 (O)_{MVP} and HIV-2_{ROD10} sequence alignment performed in DNA Dynamo with the Clustal W multiple sequence alignment tool. Amino acid site scoring: conserved (*), conservative (:), semi-conservative (.), non-conservative ( ) polymorphisms.
Figure 58: Modeling the HIV-2 CA hexamer

(A) Crystal structure of HIV-1 CA NTD (2WLV) by (Price et al., 2009) (327), CA NTD (blue), β-hairpin (green) and CypA-binding loop (yellow). (B) Predicted hexameric structure of HIV-2 (blue) modeled from bound monomeric crystal suggests arginine residues (red) are exposed as observed for HIV-1 O-group viruses.

3.7.2 Comparison of full-length HIV-1 (M)R9BAL bearing HIV-1 (O)MVP and HIV-2ROD10 CA on viral inhibition by RTI drugs

We next determined whether full-length chimeric viruses, bearing either HIV-1 (O)MVP or HIV-2ROD10 CA sequences, inserted into WT HIV-1 (M)R9BAL by overlap PCR, had differences in sensitivity to RTIs compared to WT HIV-1 (M)R9BAL.

We first considered whether our panel of HIV-1 (M)R9BAL CA chimeric viruses bore any differences in viral titre. Using the GHOST cell line, which stably expresses X4 and R5 coreceptors, we measured viral infectious units (IU/μU RT) and found all CA chimeric viruses had the same titre as WT HIV-1 (M)R9BAL (Figure 59).

Next, we determined whether the HIV-1 (O)MVP and HIV-2ROD10 viruses, compared to HIV-1 (M)R9BAL WT virus, have altered sensitivity to RTIs in the TZM-bl, LUC reporter cell line (Figure 60). However, on calculation of IC50 (Table 46) values for NRTI, TNF, and NNRTI, NVP, we measured no significant differences in sensitivity to RTIs in either chimeric virus compared to R9BAL WT. We were unable to measure IC90 values due to the poor quality of the data set, exemplified by Hill slope and R² values (Table 46).
Figure 59: Full-length R9 BAL CA chimeras do not have an infection defect in the GHOST cell line

Viral titre of full-length R9 BAL or CA chimera O-group MVP and HIV-2 measured 48 hours post-infection by FACS in the GHOST cell line IU/U RT. Data representative of two independent experiments. Error bars +/- SD.

Figure 60: Measuring IC50 of full-length HIV-1(M)R9 BAL WT and CA chimeras HIV-1 (O)MVP and HIV-2 in TZM-BL cells

(A-B) TZMBL cells were infected with full-length HIV-1 (M)R9 Bal WT (black circle) R9 BAL CA chimeras: HIV-1 (O)MVP (Blue square) (C-D) or HIV-2ROD10 (green triangle) in the presence of titrated NRTI drug, TNF, and NNRTI drug, NVP, for 48 hours. The percentage of viral inhibition was calculated from LUC readout for NRTI. Data from two independent experiments performed in biological duplicate. Error bars +/- SEM.
Table 46: Calculation of IC50 values for full-length HIV-1 (M)R9 BAL and CA chimeras HIV-1 (O)R9 and HIV-2ROD10 in TZM-BL cells

IC90 values for NRTI TNF or NNRTI drug NVP average of two independent experiments performed in biological replicate in U87 cells as calculated in the statistical application GraphPad Prism. Hill slope and R² represent two independent experiments.

3.8 The HIV-1 CA pore as a new drug target

The discovery of the HIV-1 CA pore, by Jacques et al, has provided a new target for the development of antiretrovirals. Furthermore, recent drug development has focused on CA as a target using a variety of approaches, for example, the development of small molecule inhibitors, such as PF74, which at low doses prevents CPSF6 recruitment and at high doses inhibits RT (348). Other such examples of small molecule inhibitors are: C1, which is incorporated into HIV-1 destabilising the CA cone (262) and ebselen, which inhibits CA CTD dimerization (349).

Jaques et al recently crystalised the HIV-1 (M) CA hexamer bound to a hexameric compound, hexacarboxybenzene, also referred to here as mellitic acid. Mellitic acid binding was found to be coordinated through R18 residue interactions (30). Furthermore, mellitic acid partially blocked endogenous reverse transcription (ERT), providing evidence that the CA pore is required for efficient encapsidated RT of HIV-1 (M) and is, therefore, an attractive site for anti-HIV drug design (30).

Mellitic acid (Figure 61A) is unable to traverse cell membranes, so in collaboration with Justin Warne and David Selwood, department of medicinal chemistry UCL, we asked
whether addition of ester groups to mellitic acid, referred to as JW3-33, (Figure 62B) would effectively prodrug the molecule promoting entry into infected cells. Pro drugs, pharmacologically inactive derivatives of the active drug, are required to be highly stable prior to degradation within a host tissue. Esters can be used as an effective pro drug strategy, for drugs targeting tissues with abundant endogenous esterases, the hydrolase enzymes required to cleave the prodrug into its active form (350, 351). Therefore, we questioned whether JW3-33 would be hydrolysed in cell lines, for this, we chose the TE671 adherent cell line derived from cerebellar medulloblastoma.

Figure 61: Addition of ester groups to hexacarboxybenzoene

Six ester groups (blue circle) were added to each free oxygen of (A) mellitic acid to prodrug to (B) JW3-33. Structures from Justin Warne, UCL.
3.8.1 Determining mellitic acid pro-drug JW3-33 efficacy against HIV-1 M-group infection

We first asked whether the hexameric compound mellitic acid had any effect on cell viability or HIV-1 infection. We hypothesised, due to the inability of mellitic acid to traverse cell membranes, that addition of mellitic acid to HIV infected cells would have no effect on cell viability or infection.

First we infected the TE671 cell line with HIV-1 (M)\textsubscript{Lai Δ-env} GFP vector, at an approximate MOI of 0.3 (IU/ml, calculated from viral titration), and titrated mellitic acid from high to low dose (50-0.39 μM). We determined the effect of the compound on infection by flow cytometry measuring GFP\textsuperscript* infected cells. As expected, we measured no effect of the compound on HIV-1 (M)\textsubscript{Lai Δ-env} GFP infection (Figure 62A, black bars). We also measured the effect of mellitic acid on cell viability and on quantifying the percentage of live versus dead cells, by live/dead cell staining prior to flow cytometry. From viability measurements, we determined only a very small effect on cell viability at the top dose 50 μM), possibly accounted for by the volume of DMSO added with the molecule, 5 μl/mL (Figure 62B). However this is less that the level, 15%, previously found toxic in HeLa cells (352).

We next asked whether the prodrug JW3-33 had any effect on cell viability and HIV-1 infection and RT, compared to mellitic acid. We hypothesised that if JW3-33 was able to enter cells it would effectively reduce infection through a block to early RT, as previously measured for mellitic acid in the ERT assay by Jacques et al (30). The ERT assay can be used to quantify RT within isolated CA cores in solution (353).

We infected the TE671 cell line with HIV-1 (M)\textsubscript{Lai Δ-env} GFP vector, at an approximate MOI of 0.3, and titrated JW3-33 from high to low dose (50-0.39 μM). We determined the effect of the prodrug on HIV-1 (M)\textsubscript{Lai Δ-env} GFP infection by flow cytometry and found that JW3-33 blocked infection at high drug doses 50–6.25 μM, but had no effect on infection below 3.13 μM (Figure 62A, white bars). The infection data suggested that JW3-33 was able to successfully traverse the cell membrane. However, on determining the effect of JW3-33 on cell viability, we measured low cell viability between drug doses 50 – 3.13 μM, suggesting the block to infection was accompanied by, and possibly due to, high cell toxicity (Figure 62C).

From measuring RT products of strong stop (early RT) and 2\textsuperscript{nd} strand transfer (late RT) for HIV-1 (M)\textsubscript{Lai Δ-env} GFP vector in the presence of 25-0.78 μM JW3.33 we determined a dose dependent block to early RT between 25 – 6.25 μM (Figure 62D). The block to RT had an accumulated effect and, on measuring products of late RT, we noted a complete block to 2\textsuperscript{nd} strand transfer in JW3-33 doses 25 μM and 12.5 μM (Figure 62D). However, these measurements do not directly determine if the block to HIV-1 RT is due to the functioning of JW3-33 in blocking the CA pore or the high level of toxicity in the TE671 cell line.
We then measured whether JW3-33 toxicity was reduced by reducing the exposure time. To measure this, we pre-treated TE671 cells for two hours with JW3-33 at a range of doses 25 – 0.2 μM prior to infection. After two hours, we washed the cells twice, with PBS, and infected the cells with HIV-1 (M)\textsubscript{Lai Δ-env} GFP vector. We hypothesised that reduction of JW3-33 drug exposure would reduce the negative effect on cell viability and, therefore, allow us to determine the drugs inhibition of infection.

We determined the effect of JW3-33 pre-treatment on HIV-1 (M)\textsubscript{Lai Δ-Env} GFP infection by flow cytometry and measured a reduction to infection at high doses 25 - 1.56 μM (Figure 63A). However, on analysis of cell viability, we measured high cell toxicity in TE671 on the pre-treatment of cells with JW3-33 at doses 25 - 1.56 μM (Figure 63B). These data suggest that due to the high toxicity of JW3-33 it is an unsuitable drug for further development. Despite JW3-33 not proving effective as a CA pore targeting antiviral, we were able to determine the efficient entry of JW3-33 into a host cell, as demonstrated by the block to infection and drug effect on cell viability, within two hours post-treatment.
Figure 62: Inhibitor JW3-33 shows high cell toxicity at inhibitory concentrations

Mellitic acid or JW3-33 were titrated in TE671 adherent cells infected with HIV-1 (GFP) LAI delta-Env virus. 48 hours post-infection, cells were harvested and stained with LIVE/DEAD™ prior to fixation in PFA. Samples we analysed by flow cytometry (1X10^4 total live cells). Un-treated cells were harvested and hydrogen peroxide (0.3%) treated for dead gating control.

(A) FACS data was used to quantify percentage of infection and cell viability on (B) mellitic acid or (C) JW3-33 treatment. (D) Cells for quantification of RT product by q-PCR were harvested at six hours (strong stop) and ten hours post-infection. Data from one experiment, q-RTPCR performed in biological replicate. Error bars +/-SD.
Figure 63: Inhibitor JW3-33 is toxic at inhibitory concentrations despite drug washout two hours post-addition

JW3-33 were titrated in TE671 adherent cells for two hours, washed twice with PBS and infected with HIV-1 (GFP) LAI delta-Env virus. 48hours post-infection, cells were harvested and stained with LIVE/DEAD™ prior to fixation in PFA. Samples were analysed by flow cytometry (1X10⁴ total live cells). FACS data was used to quantify (A) percentage of infection and (B) cell viability, compared to hydrogen peroxide control. Data from one experiment.

3.9 Chapter 3 Discussion

The model of encapsidated RT (126) raises the question of how RTIs are able to enter an intact CA core to inhibit RT. Taking a molecular genetics approach, we sought to determine the affect on HIV sensitivity to RTIs on mutating Gag to fix the electrostatic channel in an open or closed conformation, perhaps altering the accessibility of R18 residues (30). We also made Gag mutants to alter cofactor recruitment. We achieved this through mutation and switching of different Gag sequences. Whilst we hypothesised that charged nucleoside analogues, NRTIs, bind the R18 residues of CA, as dNTPs do, we suggested that uncharged NNRTIs would need to passively diffuse through an open CA pore, or wait until the CA initiated uncoating.

Through measurement of IC50 and IC90 values for a range of NRTIs and NNRTIs in HIV-1 LUC vector-bearing different Gag, we found small, but consistent, cell type dependent differences in vector RTI sensitivity. We measured an increased sensitivity of the open CA pore mutant HIV-1 (M)Q50Y+120R to RTIs and a decrease in NNRTI sensitivity of HIV-1 (O)MVP vector to NNRTIs.

3.9.1 M-group, O-group and HIV-2 CA pore conformation and sensitivity to RTIs

We hypothesised that open pore vectors: HIV-1 (M)Q50Y+120R, HIV-1 (O)MVP or HIV-2 would require an increase concentration of NRTIs to compete with dNTPs and vice versa in HIV-1 (M)H12Y vector with closed CA pore. However, the alternative hypothesis may be true
that an open pore increases viral sensitivity to inhibitors, due to the increase in accessibility.

While we measured no difference in sensitivity of full-length HIV-2 and HIV-1 (O) viruses compared to HIV-1 (M)\textsuperscript{RB5AL}, we measured small cell specific differences between the HIV-1 (M)\textsuperscript{Q50Y+120R} mutant and HIV-1 (O) LUC vectors.

Despite the small two-fold differences being consistent within a cell line, we found high variation, both in IC50 and IC90 values and fold differences, between differing cell lines. This led us to question whether measurements of differing RTI sensitivity between vectors were artefacts of our LUC based single-round drug susceptibility assay, especially considering our use of vectors packaged with short genome. We questioned, therefore, if differences between RTI sensitivities were too small to characterise using a single-cycle assay. It is possible that we may observe greater differences over multiple rounds of infection.

To address cell type dependent IC50 and IC90 values differences, we considered the effect of differing intrinsic dNTP levels. For example, a decreased dNTP level in Jurkat cells could lead to a reduction in RT and, therefore, reduce NRTI competition and the dose of NNRTI required for inhibition of reverse transcriptase. In collaboration with Adolfo Sarardi, MRC Laboratory for Molecular Cell Biology, LMCB, we quantified dNTPs, by high-performance liquid chromatography (HPLC), in the JLTR-5 Jurkat cell line. Interestingly, we found JLTR-5 T-cells had a similar dNTP level compared to stimulated CD4+ T-cells. This suggested that Jurkats do not have low dNTP levels and, therefore, can be considered a good model. However, a direct comparison between Jurkat and HEK293T or U87 cell lines is now required to answer if differing dNTP levels are a contributing factor in our drug susceptibility assay.

A further experiment, to address the question of intrinsic dNTP levels impacting IC50 and IC90 measurements, is to quantify the strength of binding of NRTIs compared to dNTPs. While we hypothesise that NRTIs, would bind R18 in HIV-1 (M) WT hexameric CA as efficiently as dNTPs (30), it would be of interest to measure whether mutations in HIV-1 (M) CA allosterically alter binding to R18 and dNTP-RTI competition.

Furthermore, comparing differences between HIV-1 (M)\textsuperscript{Q50Y+120R} and HIV-1 (M) and HIV-1 (O) to HIV-1 (M) in the same cell line, our data suggested that differences in O-group NNRTI sensitivity were not related to an open pore conformation. This led us to suggest that differences in RTI sensitivity could be attributed to another region of Gag, aside from the major residues contributing to the CA hexamer pore, perhaps residue position 48. With a large number of differing residues throughout Gag we chose to focus on known CA mutants, to determine if cofactor recruitment played any part in vector sensitivity to RTIs. This originated from the hypothesis that cofactor recruitment within the cytoplasm might drive the opening of the CA pore and influx of dNTPs.
Furthermore, HIV-1 (O) virus is known to have reduced sensitivity to the inhibition of CypA binding, previously described as a requirement for protected encapsidated RT (141, 163). Despite measuring a 2x decrease in sensitivity to NNRTIs HIV-1 (O) vector, in the CypA dependent cell line U87 these data were not replicated in the full-length HIV-1 (O) CA chimeric full-length virus. Possibly due to differences between cell types, all full-length experiments were performed in the TZM-bl reporter cell line, not HEK293T or U87 as for single round vectors. Yet the CypA-binding loop mutants, P90A, H87A/P and mutants in both M-group and O-group vector at position 88 demonstrated no effect on viral sensitivity to NRTI or NNRTIs. This result was the same as data previously acquired in TZM-bl cells (196). Considering the compiled data, demonstrated cell type differences in IC50 and IC90 values, it suggested that the CypA recruitment conferred a difference in viral sensitivity to RTIs.

However, we were interested in the small effect measured for the CPSF6-binding mutant, N74D, which in 293T cells demonstrated a small increase in NNRTI sensitivity, but no difference in the U87 cell line. While it was previously suggested that N74D sensitivity is due to a reduction in active RT particles per virion (196), we do not measure a reduction in infection or RT in the HIV-1(M)N74D mutant in cell lines. However, since IC50 and IC90 values only differed in the 293T cell line, we questioned if this was an artefact of using single round vector.

Despite our data being well replicated between experiments, the small differences between IC50 and IC90 values, only just greater or less than two-fold, led us to question if the two-fold effect is an artefact. We questioned whether we had set our threshold too low and had not completely eradicated background noise from the LUC based assay. From these data we concluded that the two-fold threshold is too low to determine significance and therefore suggest that differences in HIV-1 Gag do not confer differences in viral sensitivity to RTIs. From this conclusion, we questioned whether measuring the quantity of drug required to inhibit RT, in an ERT based assay, would provide a better measure of differences in inhibitor sensitivity between CA mutants.

Our data led us to ask whether opening and closing of the pore, due to β-hairpin mobility, could promote early uncoating events within cell lines. We hypothesised that the block to infection measured in both the open and closed HIV-1 (M) mutants could be due to instability of the CA core. However, the open mutant did not induce an innate immune response in the THP-1 cell line this finding, we suggest there might be measurable differences in IFN-I production in MDM, as we have recently measured in HIV-1 (O) virus (manuscript in preparation). Without a direct measurement of CA instability in cell lines, we cannot currently measure the effect that CA uncoating has on viral sensitivity to RTIs.
3.9.2 The effect of CA stability on coreceptor usage and viral sensitivity to RTIs

We have shown that, while preventing CypA recruitment by mutation bears no effect on viral sensitivity to inhibitors, the prevention of CPSF6 recruitment has an effect in specific cell lines. While previously both CypA and CPSF6-binding mutants have been found to trigger innate immune responses, (141), we suggested that mutants that increase CA cone stability, A92E and G94D, may assist encapsidated RT. However, hyper-stabilising the CA bore no effect on sensitivity to RTIs. A possible explanation for these data, is that despite mutation at residues 92 and 94 the hexameric CA β-hairpin remains dynamic. Therefore, mutants still control the influx of dNTPs and RTIs into the core as WT vector. Whilst our current assay cannot answer these questions we propose the use of the ERT assay to measure RTI sensitivity to intact cores of HIV-1 (M) A92E and G94D mutants, compared to both HIV-1 (M) WT and HIV-1(O) WT to measure differing CA stability.

A further question, that use of the single-round drug inhibition assay raises, is the effect of differing vector RT and appropriate dosing of vectors to make direct comparisons between IC50 and IC90 values. We normalised all vector doses according to $10^6$ RLU, as previously described (311). However, considering differences in HIV-1 (M) mutants RT, and variation between viral preps, we question if normalising vector input based on either a quantification of RT, by SG-PERT, or normalising input to vector genome copies, as measured by q-RTPCR, would provide more robust comparisons between vectors with differences in infection.

In conclusion, we suggest that while we have measured small cell type differences in viral sensitivity to RTIs, which are Gag dependent, our IC50 and IC90 data alone does not sufficiently provide evidence of the specific region of Gag to determine viral sensitivity to RTIs. We propose that measuring affinity of RTI binding to hexameric CA, through biocore measurements, would provide better insight into differences in CA mutant sensitivity to NRTIs.

3.9.3 The HIV CA pore as a new drug target

The determination that hexameric compound, mellitic acid, could effectively block the HIV-1 electrostatic channel, reducing RT, as quantified by the ERT assay, suggested that the newly defined pore is an effective site for new drug design (30). Despite the addition of esters to mellitic acid (Figure 58) not providing a viable anti-viral compound, our preliminary data paves the way for future drug design and adaptation of alternative compounds.
Chapter 4: Determining the timing of block to HIV-1 DNA synthesis by RTIs

4.1 Chapter 4 Introduction

The effectiveness of reverse transcriptase inhibitors (RTIs) on HIV-1 M-group infection both in vitro and in vivo has been well characterised (43, 354, 355). However, one important question remaining has been to determine the exact stage of reverse transcription (RT) at which both classes of inhibitors function. This is an important question to consider in the development of new generations of RTI. Discussed in section 1.3.5, the process of HIV-1 vDNA replication by reverse transcription can be split into distinct sections of DNA elongation, and the transfer of first (negative strand) and second (positive strand) DNA (68). The first detectable synthesised product through our TaqMan primer and probe set is minus strand strong stop DNA, used as a measure of early reverse transcription (356). The synthesis of minus strand strong stop, a short 238 nt vDNA product, is initiated from the binding of 3’-human tRNALys3 to the 5’-PBS and continues to the end of the 5’-ssRNA template (307). We are also able to measure the DNA product from 1st strand DNA synthesis and 2nd strand DNA transfer (183). We also quantify the production of 2-LTR circles, formed by non-homologous end joining of complete newly synthesised vDNA, used as a measure of nuclear entry efficacy (Figure 64) (357).

Prior to RT product quantification by q-PCR, Arts et al. first sought to determine the timing of NRTI function during HIV-1 reverse transcription. Using an endogenous cell free reverse transcription assay (ERT) to measure the effect of NRTI treatment on vDNA product length, the NRTI zidovudine (AZT) was found to have no effect on strong stop DNA synthesis from full length HXB2. However, AZT treatment reduced template switching of longer products, between 284-609 nt in length, by approximately 33% (190). Template switching is a process common to all reverse transcriptases, where the enzyme jumps between templates during elongation, leading to genetic variation and recombination events.

Later, Quan et al. measured vDNA products by qPCR and found that the NRTI AZT and the NNRTI nevirapine (NVP), both dosed at 5 μM, had no effect on (-)ssDNA. However both AZT and NVP potently inhibited the production of intermediate and full-length vDNA products (181). Interestingly, the NNRTI NVP more potently inhibited reverse transcription (181), possibly due to the high dose of inhibitor used.

However, measurement of reverse transcription using the natural endogenous reverse transcription assay (NERT) found that a high dose of the NNRTI NVP (10-100 μM) inhibited reverse transcription from an early stage. NVP also reduced DNA strand transfer by 77% to 98% but had limited effect during the elongation stages of RT (194). Supporting these data, Thomas et al. measured an 85% or 95% inhibition of –ve strand DNA
synthesis on treatment with high dose, IC95-97, NNRTI or NRTI, using a novel strand specific amplification (SSA) assay (196). While it is evident that both classes of RTI inhibit early stages of reverse transcription, neither completely block DNA synthesis at –ve strand elongation and therefore must also function on +ve strand DNA synthesis. However, it is clear that NNRTI treatment more effectively inhibits +ve strand DNA synthesis (197). Collectively, the published data, through use of multiple techniques, suggests that both classes of RTI are able to function from early stages of reverse transcription. However, NNRTIs appear to have increased efficacy on longer vDNA strands, produced in the later elongation stages of reverse transcription. We suggest that the efficiency of RTIs later in reverse transcription is due to the increased probability of RTI incorporation into a longer strand of vDNA.

We have determined that differences in Gag sequence, either induced by site directed mutagenesis or polymorphisms in different HIV-1 sub-types i.e. HIV-1 (M) and HIV-1 (O) Gag, have no significant effect on the efficacy of RTIs in cell lines. While we demonstrated that cytoplasmic uncoating of the HIV-1 CA core in cell lines has no effect on single-round vector, due to the absence of innate sensing mechanisms, we asked if differential stability of CA cores could affect the exact timing of RTI function. Developing upon previous studies, we quantified differences in timing of reverse transcription inhibition in HIV-1 (M) or (O) vectors with short genome (CSGW), long genome (LAI) or full length constructs (R9BAL) on treatment with NRTI and NNRTIs.

We hypothesised that vectors packaged with longer genomes, such as LAIΔEnv (referred to here as HIV-1 (M)\textsubscript{LAIΔEnv}), or full length replication competent virus R9BAL, would be more greatly inhibited by NRTIs. We predicted that there would be a correlation between genome length and the timing of completion of DNA extension, therefore allowing increased opportunity for NRTI incorporation and thus inhibition. Considering that mutations in HIV-1 CA, to fix the CA pore in an open or closed conformation, bore no significant effect on viral sensitivity to RTIs, we hypothesised that both NRTI and NNRTIs would function as efficiently in both HIV-1 (M)\textsubscript{R9} closed pore, and HIV-1 (O)\textsubscript{MVP} open pore Gag bearing vectors.

Using RTI doses to inhibit either 50% (IC50) or 90% (IC90) of infection we quantified reverse transcription products from strong stop DNA, 1\textsuperscript{st} strand DNA synthesis and 2\textsuperscript{nd} strand DNA transfer, as well as 2-LTR circles using TaqMan q-RTPCR. Here we report that there are no significant differences in the timing of NRTI or NNRTIs between HIV-1 (M) and HIV-1 (O) vectors. Furthermore, we found that the production of just one complete dsDNA is required to infect each cell. However, on the pre-treatment of cells with high dose (~IC90) NRTI or NNRTI, an increased number of complete dsDNA is required per infectious event. We measured a plateau of both early and late reverse transcription products from 6 hours post infection in U87 cells infected with both HIV-1 (M)\textsubscript{R9} and HIV-
1 (O)_{MVP} GFP vectors, suggesting that reverse transcription is completed within this time in the U87 cell line.

Figure 64: Reverse transcription

(A) The process of reverse transcription is used by HIV-1 to convert viral ssRNA to dsDNA. ssRNA-bound tRNA primer joins at the 5’ end primer binding site (PBS). (B) Minus strand DNA synthesis is initiated (dark blue), RNaseH activity degrades RNA template (dashed lines). (C) Minus strand transfer occurs between R sequences. (D) From this jump, minus strand DNA synthesis continues along with RNA degradation from RNaseH. (E) RNaseH resistant polypurine tract (ppt) near U3 functions as a primer for the synthesis of plus strand DNA. (F) Plus strand DNA synthesis continues, copying the first 18 nucleotides of tRNA. RNaseH then cleaves the tRNA primer, leaving a small section of 3’ tRNA attached to (-)DNA. Removal of tRNA initiates plus strand DNA transfer. (G) Extension of both plus and minus strand completes the double stranded linear vDNA. Figure from (Hu and Hughes, 2012) (185).
4.2 Optimisation of early and late RT product measurement in RTI treated cell lines

We first aimed to optimise a cell-based protocol to measure reverse transcription products from strong stop (Figure 64B) and 2nd strand transfer (Figure 64F) in HIV-1 (M)R9 GFP vector on treatment with NRTI or NNRTIs.

4.2.1 Optimising NRTI and NNRTI drug dose and time of measurement for early and late RT products by q-RTPCR in TE671 and U87 cells

We first asked whether there were measurable differences between NRTI and NNRTIs on the timing of vDNA synthesis inhibition in TE671 cells infected with HIV-1 (M)R9 GFP vector (MOI 0.3). At the time of infection TE671 cells were treated with titrations of the NRTI tenofovir (TNF) (2.5-20 μM) or NNRTI nevirapine (NVP) (100-400 nM) aiming for approximate IC25, IC50 and IC90 values. 6 hours post infection, cells were harvested and products from strong stop (Figure 65B, E), 1st strand synthesis (Figure 65C, F) and 2nd strand transfer (Figure 65D, G) quantified by TaqMan qPCR. Efficacy of RTI treatment on infection was measured by flow cytometry 48 hours post infection (Figure 59A).

At 6 hours post infection we observed that the NRTI TNF demonstrated poor inhibition of strong stop production (Figure 65B). However, treatment with a high dose of the NNRTI NVP (400 nM) correlated with a small reduction in strong stop (Figure 65E), suggesting that the block to RT was after the completion of strong stop vDNA. From quantification of products from 1st strand synthesis we measured a dose dependent inhibition on treatment with the NNRTI NVP (Figure 65F). However, the NRTI TNF had no measurable effect on 1st strand transfer (Figure 65C). On quantification of 2nd strand transfer we measured a dose dependent block on addition of both NVP (Figure 65G) and TNF (Figure 65D), although the NNRTI NVP demonstrated increased efficacy. Considering the moderate effect of TNF, we questioned if 6 hours was too early to measure the complete block to reverse transcription by NRTIs. We therefore repeated the same experiment collecting samples at 12 hours post infection.

At 12 hours post infection we quantified a reduction in reverse transcription on addition of both TNF (Figure 66B) and NVP (Figure 66D) at the stage of strong stop. The block to reverse transcription increased to 2nd strand transfer as expected, considering the increased probability of inhibition of longer vDNA strands (Figure 66C, E). The NNRTI NVP, despite showing a reduced potency on infection, demonstrated a greater block to late reverse transcription, compared to TNF (Figure 66A). These data suggest that NRTI efficacy is improved on the increased time of RTI treatment.
Figure 65: Reverse transcriptase inhibitors function from 1st strand synthesis

TE671 cells were infected with DNase treated sucrose purified HIV-1 (M)\textsuperscript{GFP} in the presence of titrated NRTI tenofovir (TNF) (2.5 μM, 12 μM or 20 μM) or NNRTI nevirapine (NVP) (100 nM, 200 nM or 400 nM). (A) Infection was measured 48 hours post infection by flow cytometry. (B,E) Cells were harvested at 6 hours for quantification of early RT products, (C,F) mid RT products or (D,G) late RT products, by q-PCR, data represented as RT products per 200ng DNA. Infection was measured 48 hours post infection by flow cytometry (A). Data from 2 independent experiments, error bars +/- SD.
Figure 66: RTIs have increased efficacy at late reverse transcription on measurement at 12 hours post treatment

TE671 cells were infected with DNase treated and sucrose purified HIV-1 (M)\textsuperscript{res} GFP vector in the presence of titrated NRTI tenofovir (TNF) (12 μM, 20 μM, 25 μM) or NNRTI nevirapine (NVP) (100, 200, 400 nM). (A) Infection was measured 48 hours post infection by flow cytometry. (B,D) Cells were harvested 12 hours post infection and early RT products or (C,E) late RT products quantified by q-PCR, data represented as RT products per 200ng DNA. Data from 2 independent experiments, error bars +/- SD.

4.2.2 Determining the effect of pre-treating cells on early and late RT product measurement in the TE671 cell line

NRTIs are positively charged nucleotide analogues, and on entry into a cell they require phosphorylation into their active form (43). We questioned if this process was a rate-limiting step in NRTI efficacy on reverse transcription. We hypothesised that the pre-treatment of TE671 cells, with the NRTI Tenofovir, would increase the efficiency of RTIs.
To probe this hypothesis, we pre-treated TE671 cells with titrated TNF, 2.5, 12 and 20 μM. 2 hours post treatment we infected cells with the HIV-1 (M)r9 GFP vector and harvested cells. Cells were harvested at 6 hours and 12 hours post infection, for quantification of strong stop, 1st strand synthesis and 2nd strand transfer RT products by q-RTPCR. In the no drug control we observed an increase in reverse transcription products between 6 and 12 hours post infection, thus demonstrating the need for time points past 6 hours post infection (Figure 67).

On titration of the NRTI Tenofovir we measured a dose dependent reduction in RT products at both 6 and 12 hours post infection. The dose dependent block to RT accumulated from strong stop (Figure 67B) to 2nd strand transfer (Figure 67D). However, the main block to reverse transcription appeared to occur past 2nd strand transfer, as the block to 2nd strand transfer did not correlate with the overall decrease in infection measured at 48 hours post infection (Figure 67A). The reduction in 2nd strand transfer product also suggested that not all viral transcripts which complete strong stop go on to complete reverse transcription.

These data confirmed that pre-treatment of cells, prior to infection, increases the potency of NRTIs. Our data also highlights the importance of a time course to effectively measure multiple stages of reverse transcription.

4.2.3 Optimising the quantification of early and late RT products in RTI treated U87 cells infected with HIV-1 (M)LAI ΔEnv vector

Recent research in our lab has suggested that vectors packaged with a short GFP-encoding HIV-1 genome, CSGW, HIV-1 (M)r9 GFP, have increased CypA dependence, measured by difference in infection in the presence or absence of CsA (unpublished). This led us to question the effect of using the short CSGW genome to measure the timing of reverse transcription and RTI efficacy. We hypothesised that the increased length of the HIV-1 (M)LAIΔEnv genome would result in the increased length of time to complete reverse transcription, therefore increased inhibition of NRTI and NNRTIs.

To probe this hypothesis, we infected the CypA dependent U87 cell line with the single-round HIV-1 (M)LAIΔEnv GFP vector and treated cells with low dose (~IC50) of clinically relevant RTIs: NRTI tenofovir (TNF) at 2.6 μM and NNRTI rilpivirine (TMC278) at 10.5 nM (Figure 68A). We harvested cells at 4, 6 and 10 hours post infection and measured the effect of RTIs on strong stop and 2nd strand transfer.

From our quantification of strong stop vDNA, we measured a similar reduction in strong stop synthesis on both TNF and TMC278 addition from 4 hours post infection (Figure 68B). However, on quantification of 2nd strand transfer we observed an increased effect of both NRTI TNF and NNRTI TMC278 (Figure 68C), in accordance with the infection data (Figure 68A). However, as expected both classes of RTI inhibited longer DNA with greater efficiency. Despite RTIs inhibiting longer vDNA in both TE671 and U87 cell lines,
small differences in the rate of reverse transcription and timing of RTI function suggest cell type differences. Such differences could possibly be due to differences in dNTP levels or innate sensing pathways which might affect CA behaviour.

Figure 67: The efficiency of reverse transcription inhibition by tenofovir increases on pre-treatment prior to infection

TE671 cells were pre-treated with a titrated dose of NRTI tenofovir (2.5 μM, 12 μM and 20 μM) for 2 hours, prior to infection with DNase treated sucrose purified HIV-1 (M)RS GFP vector. (A) Infection was measured 48 hours post infection by flow cytometry. (B) Cells were harvested at 6 or 12 hours post infection and early RT products, (C) mid RT products or (D) late RT products quantified by q-PCR, data represented as RT products per 200ng DNA. Data from 1 experiment performed in biological replicate, error bars +/- SD.
Figure 68: Optimising the measurement of RT products in the U87 cell line infected with HIV-1 (M)$_{\text{LAI}}$ΔEnv GFP vector

U87 cells were infected with DNase treated sucrose purified HIV-1 (M)$_{\text{LAI}}$ΔEnv GFP vector (black square), or boiled control (black circle) in the presence of 2.6 μM NRTI tenofovir (TNF) (grey triangle) or 10.5 nM NNRTI rilpivirine (TMC278) (grey diamond). (A) Infection was measured 48 hours post infection by flow cytometry. (B) Cells were harvested at 4, 6 and 10 hours post infection and early RT products or (C) late RT products, quantified by q-PCR, data represented as RT products per 200ng DNA. Data from 2 independent experiments, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.
4.3 NRTI and NNRTI drugs inhibit both early and late stages of HIV-1 reverse transcription

4.3.1 High dose of the NRTI tenofovir and NNRTI rilpivirine inhibits HIV-1(M)\textsubscript{LAI\textsubscript{ΔEnv}} GFP vector 2\textsuperscript{nd} strand synthesis

Following our optimisation of early and late RT product quantification in the presence of RTIs, we aimed to determine if there were differences in the stage of RT at which NRTIs or NNRTIs inhibited. From our data collected from optimisation experiments we hypothesised that early reverse transcription, strong stop, of HIV-1 (M)\textsubscript{LAI\textsubscript{ΔEnv}} GFP vector would not be reduced on treatment of NRTI or NNRTIs.

To test this we pre-treated U87 cells for 2 hours with both a low (IC50) and high dose (IC90) of the NRTI tenofovir (TNF) 2.6 μM or 10.5 μM or NNRTI rilpivirine (TMC278) 4 nM or 10.5 nM. Cells were infected with HIV-1 (M)\textsubscript{LAI\textsubscript{ΔEnv}} GFP vector and harvested at time points from 0.5 to 6 hours post infection for quantification of strong stop, 1\textsuperscript{st} strand synthesis and 2\textsuperscript{nd} strand transfer RT products.

On measuring the rate of reverse transcription in the no drug control for strong stop, we found that RT products were limited between 0.5-1 hours post infection. However, a substantial increase in strong stop was measured between 1 and 2 hours. After 2 hours, the rate of early RT continued until a plateau at 5 hours post infection. (Figure 69B). On quantification of strong stop in the presence of RTIs, we measured a small inhibitory effect of both NRTI and NNRTI from 2 hours post infection, which increased between 5-6 hours post infection (Figure 69B, H). However, the block to strong stop, measured at 6 hours, on addition of both NRTI (IC50 1.2x and IC90 1.3x) and NNRTIs (IC50 1.3x and IC90 2.5x) was less than the block to infection, measured at 48 hours post infection.

We asked if TNF and TMC278 had a different amount of inhibition at 1\textsuperscript{st} strand synthesis and if this block equated to the infection block. On quantification of products from 1\textsuperscript{st} strand synthesis we measured a small inhibition to reverse transcription from 1 hour (Figure 69C). From 4-6 hours, we measured an increased effect of both NRTI (IC50 1.2x and IC90 1.7x) and NNRTI (IC50 1.6x and IC90 2.6x) (Figure 69C). However, the block to reverse transcription at 6 hours post infection was less than the block to infection measured at 48 hours post infection (Figure 69A). Suggesting that the block to RT occurs after the completion of 1 strand synthesis.

To determine if the block to RT on RTI treatment occurred during a later stage, we also compared the effect of NRTI and NNRTI treatment on reverse transcription products from 2\textsuperscript{nd} strand transfer. In the no drug control we observed a plateau phase from 0.5-2 hours post infection, followed by an increase in RT products from 2-3 hours reaching a plateau 5-6 hours post infection (Figure 69D). In the presence of TNF or TMC278 we measured a block to 2\textsuperscript{nd} strand transfer from 3 hours, which increased up to 6 hours post infection.
However, at 6 hours the block to 2nd strand transfer by both NRTI (IC50 1.9x and IC90 6.3x) and NNRTI (IC50 1.3x and IC90 3.0x) (Figure 69D) were less than the block to infection (Figure 69A) suggesting that the block to reverse transcription occurs after the completion of 2nd strand transfer. On replication of this experiment, we found the late block of reverse transcription was consistent (Extended Figure 1, in Appendix). Furthermore, the quantification of 2nd strand transfer products in the presence of RTI’s, compared to the block to infection, suggests that many of the 2nd strand transfer products are non-productive and therefore do not result in infection.

We also asked if there were measurable differences in 2-LTR circle production in the presence of either TNF or TMC278 at both high and low dose. On quantification of 2-LTR circles, from U87 cells harvested at 6 hours post infection, we measured a 9x reduction in 2-LTR circles on treatment with high dose TNF and an 8x reduction on treatment with high dose TMC278, compared to the no drug control (Figure 69K). This was surprisingly less than the block to infection, measured at 48 hours post infection, on addition of TNF 16x and TMC278 61.7x.

Interestingly on normalisation of strong stop (Figure 69H), 1st strand synthesis (Figure 69I) and 2nd strand transfer (Figure 69J) reverse transcription products per infected cell we determined that at 6 hours post infection just 1 dsDNA product was required to infect each cell. However, in the presence of RTI’s at high dose the number of dsDNA products required to infect a cell increased as follows: strong stop TNF 13x, TMC278 28x; 1st strand synthesis TNF 10x, TMC278 28x; and 2nd strand transfer TNF 2.6x, TMC278 8.3x. This was as expected, considering the presence of RTI’s prevents the synthesis of full length products.
Figure 69: High dose NRTI tenofovir effectively inhibits 2nd strand transfer of HIV-1 (M)LAIΔEnv GFP vector while high dose of NNRTI rilpivirine inhibits from strong stop

U87 cells were pre-treated for 2 hours with NRTI tenofovir (TNF) at an IC50 dose (2.6 μM) (purple triangle) or IC90 dose (10.5 μM) (red triangle) or NNRTI rilpivirine (TMC278) at an IC50 dose (4 nM) (dark blue diamond) or IC90 dose (10.5 nM) (light blue diamond) prior to infection with DNase treated sucrose purified HIV-1 (M)LAIΔEnv GFP vector. (A) Infection was measured 48 hours post infection by flow cytometry. (B) Cells were harvested at time points from 0.5 to 6 hours post infection and early RT products, (C) mid RT products or (D) late RT products quantified by q-PCR. Data represented as (B-D) RT products per 200ng DNA, (E-G) RT products per 10^6 cells and (H-J) RT products normalised to number of infected cells. Data from 1 experiment performed in biological replicate, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.
4.3.2 High dose of the NRTI tenofovir and NNRTI rilpivirine inhibits HIV-1 (M)$_{R9}$ reverse transcription from strong stop

We next asked at which stage of reverse transcription in the short CSGW genome NRTI and NNRTIs function. We hypothesised that vector containing a short genome would complete reverse transcription at an increased rate, which would be measured by the amount of reverse transcription product plateauing earlier. We therefore also suggested that vectors packaged with the CSGW genome would have reduced sensitivity to RTI. This would be an important observation, considering the majority of previous measurements characterising RTI sensitivity are performed using single round vectors with a short genome. Furthermore, we predicted that RTIs would have decreased efficiency, as they predominantly target longer vDNA products.

To test this hypothesis we pre-treated U87 cells with a high dose (~IC90) of the NRTI Tenofovir (TNF) 30 μM or NNRTI Rilpivirine (TMC278) 50 nM for 2 hours prior to infection with the HIV-1 (M)$_{R9}$ GFP vector. Strong stop and 2nd strand transfer reverse transcription products were quantified from 0.5-8 hours post infection by q-RTPCR. We first noticed that the amount of RT product in the no drug control, from both strong stop (Figure 70B and D) and 2nd strand transfer (Figure 70C and E) measurements plateaued from 4-8 hours post infection, an hour earlier than the plateau measured in HIV-1 (M)$_{LAI\Delta Env}$ GFP vector (Figure 69).

On treatment of HIV-1 (M)$_{R9}$ GFP with NRTI and NNRTI we measured a block to RT from 2-3 hours post infection (Figure 70B and D), similar to the timing of RT block by RTI in HIV-1 (M)$_{LAI\Delta Env}$ GFP vector (Figure 69). Interestingly, the block to HIV-1 (M)$_{R9}$ GFP strong stop at 8 hours post infection for TNF (4.3x) and TMC278 (9x) (Figure 70B) was less than the block to infection TNF (77x) and TMC278 (309x), measured at 48 hours post infection (Figure 70A).

On quantification of 2nd strand transfer products, we measured a plateau from 5-6 hours post infection in the no drug control, suggesting the completion of this stage of reverse transcription occurs within 6 hours in these experiments (Figure 70C). The block to 2nd strand transfer products, at 8 hours post infection in the presence of TNF (20x) or TMC278 (28x) (Figure 70C) was less than the block to infection measured (Figure 70A). These data suggest that the block to reverse transcription of both NRTI and NNRTIs is occurring at a later stage of reverse transcription, post 2nd strand transfer.

However, we are unable to measure after the completion of 2nd strand transfer using our TaqMan q-PCR method, as there is no unique template, due to the 1st DNA strand having the same sequence. We therefore measured 2-LTR circles as a late RT product.

Considering the difference in block to 2nd strand transfer product and infection on RTI treatment we questioned if there was a measurable block to the production of 2-LTR circles. On the quantification of 2-LTR circles, at 8 hours post infection, we measured a 12x block on treatment with TNF and a 21x block in TMC278 (Figure 72A) suggesting that the block to reverse transcription by both NRTI and NNRTIs does occur at later stages of
reverse transcription, after 2\textsuperscript{nd} strand transfer. On repeat of this experiment we found this late block to HIV-1 (M)\textsubscript{R9} GFP reverse transcription by both NRTI and NNRTIs to be consistent (Extended Figures 2 and 4).

**Figure 70:** High dose of NRTI tenofovir and NNRTI rilpivirine inhibit HIV-1 (M)\textsubscript{R9} GFP vector reverse transcription from strong stop

U87 cells were pre-treated for 2 hours with 30 μM NRTI tenofovir (TNF) (red triangle) or 50 nM NNRTI rilpivirine (TMC278) (blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (M)\textsubscript{R9} GFP vector. (A) Infection was measured 48 hours post infection by flow cytometry. (B) Cells were harvested at time points from 0.5-8 hours post infection. Early RT products or (C) late RT products were quantified by q-PCR. Data represented as (B-C) RT products per 200ng DNA and (D-E) RT products per 10\textsuperscript{6} cells. Data from 1 experiment performed with biological replicate, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.
4.3.3 High dose of the NRTI tenofovir and NNRTI rilpivirine inhibits HIV-1 (O)MVP reverse transcription from strong stop

We next asked if differences in HIV-1 Gag could confer differences in the timing of RTI function. Our collaborators recently solved the hexameric CA of an O-group virus, called MVP, and found the central hexameric pore was in a fixed open conformation. The HIV-1 (O)MVP CA pore also remained open under crystallography conditions in which the HIV-1 (M)R9 hexameric pore is in a closed conformation (manuscript in preparation). This led us to hypothesise that an open pore conformation may lead to an earlier block to reverse transcription, due to the increased accessibility of the RT complex, of both NRTIs and NNRTIs to encapsidated reverse transcription. However, considering we previously measured a 2x increase in the amount of NNRTI required to inhibit HIV-1 (O)MVP LUC infection compared to HIV-1 (M)R9 LUC in the HEK293T and U87 cell lines we questioned if this correlated with the increased accessibility of NRTIs and earlier block to reverse transcription.

To probe this hypothesis, we pre-treated U87 cells with a high dose of the NRTI tenofovir (TNF) 30 μM or NNRTI rilpivirine (TMC278) 50 nM for 2 hours prior to infection with HIV-1 (O)MVP GFP vector. From 0.5-8 hours post infection, cells were harvested for quantification of strong stop and 2nd strand transfer. Despite a lower infection of HIV-1 (O)MVP in the no drug control (Figure 71A) compared to HIV-1 (M)R9 (Figure 70A), we observed a good level of inhibition on both NRTI (25x) and NNRTI (12x) addition, measured at 48 hours post infection by flow cytometry. First comparing quantification of strong stop products we measured that the strong stop plateaued from 4-8 hours post infection (Figure 71B-D), as previously determined in HIV-1 (M)R9 GFP vector (Figure 70), suggesting that this stage of RT is completed within a similar time frame between the two viruses, irrespective of differences in the CA pore conformation. In the presence of TNF or TMC278 strong stop was inhibited from 2 hours post infection. At 8 hours post infection the block to HIV-1 (O)MVP strong stop, compared to the no drug control, for TNF was 4.3x and for TMC278 was 8.9x (Figure 71B). However, this was less than the block to infection by TNF (25x) and TMC278 (112x), measured at 48 hours post infection (Figure 71A).

Quantifying products of 2nd strand transfer in the no drug control we measured a plateau to late reverse transcription from 6-8 hours post infection (Figure 71C and E), 1 hour later than the plateau measured for HIV-1 (M)R9 2nd strand transfer (Figure 71). In the presence of TNF and TMC278 we measured a block to 2nd strand transfer from 2 hours post infection, however this greatly increased by 2 hours post infection. Interestingly, despite the NRTI TNF demonstrating a smaller inhibition to infection, compared to the NNRTI TMC278 (Figure 71A), TNF demonstrated a greater block to 2nd strand transfer at 8 hours post infection (63.8x) compared to TMC278 (29x) (Figure 71C). However, on quantification of 2-LTR circles, at 8 hours post infection, the NNRTI TMC278 demonstrated a greater block to 2-LTR circles (87x) than the NRTI TNF (37x), compared
to the no drug control (Figure 72B). The block to 2-LTR-circle formation in the presence of RTIs was similar to the block to infection, suggesting that the main inhibitory effect of both NRTI and NNRTIs occurs after 2nd strand transfer. These findings were consistent in repeat experiments (Extended Figures 3-4, Appendix) and, furthermore, support data from Lorena Zuliani-Alvarez, UCL, demonstrating HIV-1 (O)MVP initially reverse transcribes at an increased rate, compared to HIV-1 (M)R9, despite both resulting in equal reverse transcription products.

Figure 71: High dose of NRTI tenofovir and NNRTI rilpivirine inhibit HIV-1 (O)MVP GFP reverse transcription from strong stop

U87 cells were pre-treated for 2 hours with 30 μM NRTI tenofovir (TNF) (red triangle) or 50 nM NNRTI rilpivirine (TMC278) (blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (O)MVP GFP vector. (A) Infection was measured 48 hours post infection by flow cytometry. (B) Cells were harvested at time points from 0.5-8 hours post infection and early RT products or (C) late RT products quantified by q-PCR. Data represented as (B-C) RT products per 200ng DNA and (D-E) RT products per 10^6 cells. Data from 1 experiment performed with biological replicate, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.
U87 cells were pre-treated for 2 hours with 30 μM NRTI tenofovir (TNF) (red triangle) or 50 nM NNRTI rilpivirine (TMC278) (blue diamond) prior to infection with DNAse treated sucrose purified (A) HIV-1 (M)R9 GFP vector or (B) HIV-1 (O)MVP GFP vector. Cells were harvested 8 hours post infection for quantification of 2-LTR circles. Data represented as RT products per 200ng DNA. Data from 1 experiment performed with biological replicate, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.

4.3.4 Inhibition of full-length HIV-1 R9 BAL reverse transcription by RTIs in JLTR-5 T-cells

Having observed differences in timing of strong stop and 2nd strand transfer completion between HIV-1 (M)LAIΔEnv and HIV-1 (M)R9 GFP vectors and the timing of NRTI and NNRTI effect, we asked if there were differences between the two classes of RTIs in a replication competent virus. We hypothesised that replication competent virus R9BAL would complete both strong stop and 2nd strand transfer in a similar time to HIV-1 (M)LAIΔEnv because LAI Δ Env virus is just 500 nt shorter in length than full length virus. Furthermore, we hypothesised that both NRTI and NNRTIs would predominantly inhibit reverse transcription during a late phase, past the point of 2nd strand transfer.

To test these hypotheses we pre-treated JLTR5 T-cells for 2 hours with a high dose (IC90) of the NRTI tenofovir (TNF) (40 μM) and NNRTI rilpivirine (TMC278) (10 nM). T-cells were infected for 6 hours with 0.5 units of RT R9BAL virus, quantified by SG-PERT protocol. Cells were washed with PBS and plated at 0.5x10^6/ml then harvested at 20, 48 and 168 hours post infection and reverse transcription products from strong stop and 2nd strand transfer and 2-LTR circles quantified by q-RTPCR.

Upon the quantification of RT products from strong stop we measured a small block to reverse transcription from 20 hours post infection, TNF 1x and TMC278 1.2x (Figure 73C). The RTI block to strong stop did not increase between 20 and 48 hours post infection. Furthermore, the block to strong stop products by RTIs did not correlate with the block to infection, TNF 5-fold, TMC278 7-fold, measured at 48 hours post infection (Figure 73A), suggesting that the block to reverse transcription occurs, as expected, after strong stop. As expected, over a spreading infection of 7 days the block to strong stop by TNF increased (6x) while the block by TMC278 decreased (>1x) (Figure 73C). These data
were consistent with the reduction in efficacy of TMC278 on spreading infection, as determined by quantification of infection at 7 days post infection (Figure 73B). These data either suggest that TMC278 has reduced stability in culture over an extended time frame or demonstrate the increased efficacy of strong stop inhibition by NRTI treatment.

From the quantification of 2\textsuperscript{nd} strand transfer, we measured a similar block at 48 hours on addition of TNF (3x) and TMC278 (2-3x) (Figure 73D), as measured for strong stop (Figure 73C). However, by 7 days post infection we measured a 2x block to 2\textsuperscript{nd} strand transfer on rilpivirine treatment and 6x block on tenofovir treatment (Figure 73D), similar to the block measured for strong stop at this time point. These data suggest that HIV-1 (M)\textsubscript{R9BAL} virus reverse transcription is inhibited from strong stop. However, considering the large block to infection, measured at both 48 hours and 168 hours post infection, our data suggests that the main block to reverse transcription by both NRTI and NNRTIs occurs after the completion of 2\textsuperscript{nd} strand transfer. However, on quantification of 2-LTR circles at 20, 48 and 168 hours post infection we found the block to 2-LTR circles on treatment with both tenofovir (1.2x) and rilpivirine (2x) was similar to the block at early and late RT (Figure 73E). These data suggest that full length HIV-1 R9 BAL virus is moderately inhibited from strong stop, which carries through to 2\textsuperscript{nd} strand transfer and formation of 2-LTR circles. However, our data also suggests that not all of the products of R9BAL reverse transcription we measure result in productive infection in the JLTR-5 T-cell line.
Figure 73: NRTI tenofovir and NNRTI rilpivirine inhibit replication competent R9 BAL infection from strong stop

JLTR-5 T-cells were pre-treated for 2 hours with 40 μM NRTI tenofovir (TNF) (red triangle) or 10 nM NNRTI rilpivirine (TMC278) (blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (M)_{R9BAL} virus. (A) Infection was measured 48 hours and (B) 168 hours post infection by flow cytometry. (C) Cells were harvested at time points 20, 48 and 168 hours post infection and early RT products, (D) late RT products and (G) 2-LTR circles quantified by q-PCR. Data represented as (C-D) RT per 200ng DNA and (E-F) RT normalised to number of infected cells. Data from 1 experiment performed with biological triplicate for 20-48 hours, error bars +/- SD, numbers represent fold difference compared to the no drug DMSO control.
4.3.5 Determining the effect of vector production in the presence of reverse transcriptase inhibitors

Reverse transcriptase interacts with nucleic acids of differing structures, such as dsRNA, dsDNA and RNA-DNA duplexes (358). During the process of viral packaging, dsRNA and viral proteins are packaged into the HIV CA core. Post translation vRNA functions as a scaffold, tethering Gag proteins through interactions with nucleocapsid (NC) domains (359). Considering cellular dNTPs are packaged in core to initiate strong stop DNA (360), we questioned if reverse transcriptase inhibitors could be packaged into HIV-1 particles. To address this question we added high dose (20 μM), medium dose (6.7 μM) or low dose (2.2 μM) NRTI zidovudine (AZT) or NNRTI nevirapine (NVP) at the time of HIV-1 (M)R9 or HIV-1 (O)MVP LUC vector transfection. After 48 hours vectors were collected from the supernatant and diluted on HEK293T cells (1:10). Viral titre was subsequently measured by quantification of average RLUs. Final NRTI and NNRTI concentrations, present in the infections, were 0.2 μM, 0.067 μM and 0.022 μM. Comparing this to our IC50 values calculated for HIV-1 (M) LUC and HIV-1 (O) LUC in the HEK293T cell line, M-group AZT 0.17 μM and NVP 0.07, O-group AZT 0.11 μM and NVP 0.12, we concluded that our data may be influenced by the volume of inhibitor present in the viral supernatant.

On quantification of vector titre from HIV-1 (M)R9 made in the presence of RTIs we observed the addition of NVP greatly inhibited infection, in a concentration dependent manner (70x at 20 μM, 22x at 6.7 μM, and 4x at 2.2 μM), compared to the addition of AZT (14x at 20 μM, 5x at 6.7 μM, and 3x at 2.2 μM) (Figure 74A). HIV-1 (O)MVP LUC infection, was also inhibited on transfection in the presence of AZT (35x at 20 μM, 10x at 6.7 μM, and 4x at 2.2 μM) compared to NVP (26x at 20 μM, 7x at 6.7 μM, and 3x at 2.2 μM), demonstrating an increase in infection inhibition compared to HIV-1 (M) (Figure 74B). These data complement our discovery that the HIV-1 (O) vector has decreased sensitivity to NNRTIs (Chapter 3). Furthermore, these data suggest that RTIs can be packaged into HIV-1 particles. Considering our model of encapsidated reverse transcription it is an attractive hypothesis that RTIs are packaged into the CA cone. However, we have previously found RTIs remain stable in culture for 7 days post treatment (Figure 74). Therefore, the effect on viral titre is, in part, due to residual RTI present in the viral supernatant. This effect could be controlled for by vector purification by ultracentrifugation, after production in the presence of RTIs.
4.4 Characterising reverse transcription in the HIV-1 M group and O group capsid pore mutant R18G

Jacques et al. previously measured binding of negatively charged dNTPs to wild type hexameric capsid compared to capsid mutants R18G. R18 is a positively charged residue located in the CA NTD, helix 1. They determined that mutating R18 to a neutral G residue inhibited dNTP binding (30, 308), which correlated with a block to viral reverse transcription and infection. However, the block could be rescued, on the production of chimeric capsid with differing ratios of WT to mutant (30). While this data highlights the importance of R18 for effective reverse transcription we asked if dNTPs could passively diffuse through an open CA pore of the R18G mutant. We asked if reverse transcription of the R18G mutant would be initiated at a late timepoint to R18 WT vector.

4.4.1 HIV-1 M-group and O-group R18G capsid mutants have a large infection defect in the U87 cell line

We first measured the titre of purified HIV-1 (M)R9 and HIV-1 (O)MVP GFP WT vectors compared to HIV-1 (M)R9R18G and HIV-1 (O)MVP R18G mutants in the U87 cell line. 48 hours post infection we quantified infectious units normalised to units of reverse transcription, quantified by SG-PERT (IU/μU RT) (Figure 75). This is a control to normalise data for differences between viral preps. We measured a 6700x decrease in titre in the HIV-1 (M)R9 R18G compared to HIV-1 (M)R9 WT, while the HIV-1 (O)MVP R18G mutant had a 1400x
titre defect compared to the HIV-1 (O)_{MVP} WT vector. Demonstrating that both viruses had a severe block to infection and were effectively dead.

![Figure 75: HIV-1 (M)_{R9} and HIV-1 (O)_{MVP} CA pore mutants R18G have an infection defect in the U87 cell line](image)

U87 cells were infected with DNAse treated sucrose purified HIV-1 (M)_{R9} or HIV-1 (O)_{MVP} WT vectors or R18G mutants thereof. Infection was measured 48 hours post infection, by flow cytometry, and normalised to μU RT, quantified by SG-PERT. Data from 1 independent experiment performed in biological replicate, error bars +/- SD, numbers represent fold difference between R9 WT or MVP WT vector.

### 4.4.2 High dose of R18G mutants are unable to complete reverse transcription

We next asked if R18G mutants could initiate and complete any amount of reverse transcription if left for longer in cells. We hypothesised that if dNTPs were able to passively diffuse though the CA pore of the R18G mutant then reverse transcription would be initiated but at a slower rate than R18 WT vector.

To probe this hypothesis we infected U87 cells with a high dose of both HIV-1 (M) and (O) WT both at 5.1x10^9 pU RT/ml, MOI 0.5 IU/ml; HIV-1 (M)_{R18G} at 1.3x10^{11} pU RT/ml, MOI 0.04 IU/ml, or HIV-1 (O)_{R18G} at 7x10^{10} pU RT/ml, MOI 0.04 IU/ml, as measured by SG-PERT (mutant dosing was equivalent to using 1 concentrated vector prep per experiment). Cells were harvested at 0.5-96 hours post infection and reverse transcription products from strong stop and 2\textsuperscript{nd} strand transfer quantified by q-RTPCR.

On measurement of infection at 48 hours post infection both R18G mutant vectors infected less than 1% of cells (Figure 76A). Despite high vector input HIV-1 (M)_{R18G} had a 70x reduction in infection compared to WT, and HIV-1 (O)_{R18G} had a 357x reduction in infection compared to WT (Figure 76A). This implied that RT was not completed in either mutant by 48 hours post infection.
From the quantification of products from strong stop we measured increased products for both M-group and O-group WT vectors between 0.5-8 hours. Between 8-24 hours the amount of strong stop reached a plateau, before decreasing between 24-96 hours post infection (Figure 76B). These data show that the reverse transcription of strong stop DNA is complete by 8 hours post infection. We propose that the loss of strong stop product over 24-96 hours could be due to viral DNA degradation by host exonucleases such as TREX1. Interestingly, we measured a 8.9x increase in strong stop for HIV-1 (O) compared to HIV-1 (M) vector despite normalising vector input to RT. This suggests that O-group vector in the U87 cells produces more RT product over time compared to M-group. It is an attractive hypothesis that this is due to the increased accessibility of dNTPs due to the open CA pore of the O-group vector.

From the quantification of HIV-1 (M)\textsubscript{R18G} strong stop we found that the mutant was able to complete strong stop. The amount of strong stop products measured plateaued from 6-24 hours post infection, before decreasing between 24-96 hours post infection (Figure 76B). While we measured strong stop products for HIV-1 (M) WT and HIV-1 (M)\textsubscript{R18G}, the latter demonstrated a small 2x block. However, the HIV-1 (O)\textsubscript{R18G} mutant had a large 266x block to strong stop, compared to HIV-1 (O)\textsubscript{MVP} WT vector at 24 hours post infection. Furthermore, the O-group R18G mutant plateaued from 2 hours post infection, suggesting that the defect to reverse transcription occurred very early in this mutant (Figure 76B). As previously described for WT vectors, reverse transcription products for R18G mutants rapidly decreased between 24-96 hours post infection, possibly due to degradation of strong stop vDNA.

Considering both R18G mutants made strong stop vDNA we also asked if they were able to complete 2\textsuperscript{nd} strand transfer. First comparing HIV-1 (M) and (O) WT vectors we measured that the amount of RT product from 2\textsuperscript{nd} strand transfer increased from 0.5-6 hours post infection, while late RT appeared to reach a plateau from 6-24 hours post infection. Between 24-96 hours post infection the amount of 2\textsuperscript{nd} strand transfer products measured for both WT vectors rapidly decreased, suggesting that by 24 hours reverse transcription is complete in our single-round vector and any residual vDNA is possibly degraded by host exonucleases, as previously discussed.

Aside from binding dNTPs, the hexameric CA R18 residues have recently been found to bind a highly charged polyanion, IP6 (319). IP6 is incorporated into virions and binds to CA hexamers at a ratio of 1:1. The binding of one IP6 molecule per hexamer was found to greatly increase the stability of an intact CA core. Furthermore, addition of IP6 to an ERT assay greatly increased the number of reverse transcripts (319). Therefore, in the absence of IP6 binding to CA pores, on R18G mutation, these data suggest that the CA core is not stabilised and, therefore, falls apart. This, in part, explains
why both HIV-1 (M) and HIV-1 (O) R18G mutant vectors are unable to complete reverse transcription and therefore, are dead for infection.

Interestingly, we found that HIV-1 (O) vector had 7.6x more late RT products compared to HIV-1 (M) at 24 hours post infection, despite normalising vector input to units of reverse transcription (Figure 76C). On quantifying 2nd strand transfer products for R18G mutants we found that both mutants had a large defect in late RT, suggesting that the block to HIV-1 (M)R18G, which completed strong stop, was mostly during late reverse transcription. We found that HIV-1 (M)R18G had a 100x reduction in late RT compared to WT and HIV-1 (O)R18G had a 1924x reduction in late RT, as measured at 24 hours post infection compared to WT vector (Figure 76C).
Figure 76: R18G mutants are unable to complete reverse transcription

U87 cells were infected with DNAse treated and sucrose purified HIV-1 (M)\textsubscript{R9 WT} (5.1x10^9 pU RT/ml), HIV-1 (O)\textsubscript{MVP WT} (5.1x10^9 pU RT/ml) MOI 0.5, HIV-1 (M)\textsubscript{R18G} (1.3x10^{11} pU RT/ml) or HIV-1 (O)\textsubscript{R18G} (7x10^{10} pU RT/ml) MOI 0.004 GFP vectors. (A) Infection was quantified 48 hours post infection by flow cytometry. (B) Cells were harvested at time points from 0.5-96 hours post infection and early RT products or (C) late RT products quantified by q-PCR. Data shown as (B-C) RT products normalised to 200ng. Data from 1 experiment, q-PCR performed in biological duplicate, error bars +/- SD, numbers represent fold difference between R9 WT or MVP WT vectors.
4.5 Chapter 4 Discussion
We aimed to measure the timing of reverse transcription inhibition after treatment with NRTI and NNRTIs. We hypothesised that longer vDNA strands would be targeted by both NRTI and NNRTIs, and therefore we suggested that 2nd strand transfer would be more greatly affected compared to strong stop vDNA products. We also hypothesised that vectors packaged with a near full length genome, or replication competent virus, would require longer to complete reverse transcription. These would therefore demonstrate increased sensitivity to RTIs, due to increased opportunity during elongation for RTIs to function. However, we found that RTIs functioned earlier in vectors packaged with short CSGW genome, perhaps suggesting that vector particles packaged with shorter genomes are less stable, or have a reduction in DNA-RNA interactions in the CA core.

We also determined that while some strong stop was inhibited in the presence of RTIs the majority of the block to RT occurred late, after the measurement of 2nd strand transfer. Interestingly, differences between Gag of M-group and O-group vectors did not alter the efficacy of NRTIs or NNRTIs, or the stage of RT during which they functioned.

4.5.1 Comparison of NRTI and NNRTI efficacy on strong stop vDNA
Previous comparisons of HXB2 reverse transcription on NRTI treatment in the Jurkat T-cell line, found NRTIs had no effect on strong stop (190, 361). However, our data from single-round vectors, LAI, CSGW and replicative competent R9, suggest both inhibitor classes reduce strong stop. This was measurable 2-3 hours post infection, or 20 hours in the replicative competent virus R9BAL.

RTI inhibition of reverse transcription from strong stop was surprising because it has previously been reported that reverse transcription of strong stop vDNA is completed (360), or nearly completed (362), within the viral core prior to entry into a host cell. This is supported by the detection of strong stop products in defective cores which do not synthesise DNA past the stage of 1st strand transfer (353). However, our data suggests that reverse transcription, of strong stop vDNA, is initiated from dNTPs packaged in the core and this is completed within the host cytoplasm. We hypothesise that RTIs are unable to access the start of strong stop reverse transcription and may gain access to the viral genome at the same time as dNTPs in the cytoplasm. We suggest that the use of the ERT assay, providing the ability to manipulate dNTP levels in the assay, and therefore control rates of reverse transcription would provide further insight into the timing of RTIs.

Previously both classes of RTI have been found to function on intermediate to full length DNA products, with no measurable effect on (-)ssDNA inhibition (181). While we measure a small block to 1st strand synthesis of HIV-1 (M)LAIΔEnv, both in the presence of TNF and TMC278, we found that the main block to reverse transcription occurred after 2nd strand
A further experimental idea to demonstrate the efficacy of RTIs on short vDNA includes characterisation of the timing of early RT and RTI efficacy in primary cell types, such as human CD4+ cells and macrophages isolated from peripheral blood.

### 4.5.2 Comparison of NRTI and NNRTI efficacy on 2\textsuperscript{nd} strand transfer

Previous studies have measured the increased efficacy of RTIs on longer vDNA. While NRTIs target both early and late RT through incorporation into different lengths of vDNA, NNRTIs have previously been found only to inhibit after (+)ssDNA synthesis. However, there is disagreement in the field, with some groups suggesting NNRTIs also function on (-)ssDNA, after the point of 1\textsuperscript{st} strand transfer (181, 196).

Our observation that both NRTI and NNRTIs at high dose partially inhibit –ve strand DNA, led us to question if RTIs had a cumulative effect by 2\textsuperscript{nd} strand transfer. We found that both long and short genomes, irrespective of capsid pore conformation, were inhibited at the stage of 2\textsuperscript{nd} strand transfer by high dose NRTI and NNRTIs. However, we determined that vectors packaged with a short genome were more sensitive to both NRTI and NNRTI treatment. This was contrasted with RTI treatment of the full length R9BAL replication competent virus, which demonstrated no further block to RT past the stage of strong stop.

However, in agreement with the measured decrease to infection, we found that the NNRTI Rilpivirine demonstrated the greatest block to 2\textsuperscript{nd} strand transfer in both M and O-group CSGW vectors. The increased potency of NNRTI has previously been observed, however such studies have been flawed by not comparing the effect to infection and using very high doses of NNRTIs (181). Here we chose to use low dose of RTIs and compare the effect of RT to infection. Interestingly, in all of our experiments the block to late reverse transcription by both NRTI and NNRTIs was less than the total block to infection measured at 48 hours post infection. This led us to propose that the RTI block is occurring during the final elongation stage of reverse transcription, after the completion of 2\textsuperscript{nd} strand transfer.

However, our data goes against a previous finding that late reverse transcription of LAIΔEnv vector in the presence of 1 μM NVP is almost completely blocked, as is the production of 2-LTR circles (363). This was determined by the quantification of integrated products using Alu qPCR (364). However, the dose of NVP used during this experiment is especially high, it completely blocked all infection. Also these experiments were performed in monocyte derived macrophages (MDMs), known to have very low levels of dNTPs (365), so this data is not comparable to our work in the U87 cell line.

One caveat of our experimental design is the inability to measure RT products post 2\textsuperscript{nd} strand transfer, as there are no unique sites for the design of TaqMan primers and probes, due to the presence of 2 identical vDNA strands. Our hypothesis of RTI function on ds vDNA is supported by our infection data, which confirms that our dose of both NRTI and
NNRTI are effectively reducing infection by 90-99% and the previous description of the NNRTI nevirapine (NVP) preferentially inhibiting plus-strand DNA (196). Recently a high throughput sequencing strategy, for defining the 3’ termini of DNA products, has been used to measure APOBEC3G restriction of DNA synthesis (366). We propose that use of this assay to measure RTI effect on DNA synthesis would provide greater insight into the exact timing of RTI function. This method would enable us to measure specific sites at which vDNA is terminated and also provide the means to measure reverse transcription past 2nd strand transfer. Due to the high throughput nature of the assay, it would allow easier comparison between open, Q50Y+120R, and closed, H12Y, pore mutants, including further characterisation of O-group and HIV-2 capsids. Further to this, defining the structure of full length DNA products, through use of long-read sequencing, rather than just 3’ termini sequencing may be more informative, because DNA products may be defective elsewhere in the genome.

4.5.3 Comparison of NRTI and NNRTI efficacy through quantification of 2-LTR circle formation

Considering the caveat in our quantification of reverse transcription past the completion of 2nd strand transfer, by qPCR, we quantified 2-LTR circle formation as a measurement of the completion of RT, on treatment with both NRTI and NNRTIs. Considering 2-LTR circle formation occurs after nuclear entry of vDNA, after the completion of reverse transcription, we decided to use this measurement to quantify the block of RTIs to late RT.

In the presence of high dose (IC90) RTIs we measured a block to 2-LTR circle formation from 6-8 hours post infection for both HIV-1 (M)R9, HIV-1 (O)MVP and HIV-1 (M)LAIΔEnv. However, in all experiments the block to infection was greater than the measured block to 2-LTR circle formation. Although our current methodology is unable to address this, we propose that differences measured between the block to RT, 2-LTR circle formation and infection may in part be accounted for by the presence of reverse transcription products which do not go on to become infectious.

Furthermore, it has been shown that structural changes to MLV reverse transcriptase result in increased template switching, promoting the production of defective genomes (367). It is therefore likely that our q-PCR is detecting such RT products, which would not result in productive infection. It is also probable that RTIs might result in increased template switching, due to the slowing down of reverse transcription, thus, allowing increased time for template switching to occur.
4.5.4 Low numbers of vDNA are required per infection in the U87 cell line

Through measuring the effect of RTI treatment on different stages of reverse transcription we were able to make some novel measurements to determine the amount of strong stop or 2\textsuperscript{nd} strand transfer vDNA product required per cell for infection in the U87 cell line. We measured that HIV-1 (M)\textsubscript{LAI\textendash}Env required just 1 strong stop and 2\textsuperscript{nd} strand transfer vDNA product to infect U87 cells, while in the presence of both TNF and TMC278 this increases. The low numbers of both strong stop and 2\textsuperscript{nd} strand transfer DNA product required for infection in the U87 cell line, suggests that viral RT product is not degraded over time. Rather, once strong stop is made this continues on to 2\textsuperscript{nd} strand transfer and integration.

However, previous measurements in PBMCs suggest that higher numbers of vDNA, between 100 and 1000, are required for infection (368). This leads us to suggest that there are cell type differences in the numbers of vDNA required for infection. This is also supported by data from our lab demonstrating that between approximately 20-100 total vDNA copies are required per infectious event in αcd3 αcd28-stimulated CD4\textsuperscript{+} T-cells infected with CCR5-tropic HIV-1 NL4.3 BaL, donor dependent (Maria T Rodriguez-Plata, unpublished). However, there are multiple factors influencing the number of vDNA products required to infect a cell. For example, differences in the number or defective genomes produced or integration into the wrong site of the genome.
Chapter 5: Characterisation of HIV-1 T/F virus Gag

5.1 Chapter 5 Introduction

5.1.1 HIV transmitted founder viruses

HIV infection is established from transmission of one or a small number of transmitted/founder (T/F) clones (87). From the T/F sequence, clonal expansion promotes genome wide diversification on acquisition of mutations. Early mutations are caused by both errors in reverse transcription (RT) and driven by evasion of cytotoxic lymphocyte (CTL) detection. Selection pressures, both within the transmitter and the recipient, are reviewed in section 1.2.1.

CTL escape mutations are detectable by sequencing within the first six months of infection (75, 369). However, major CTL escape mutations, usually located within Gag such as those in residues 240 – 249 (TSTLQEIQGW), TW10, are costly to the virus and are often associated with a decrease in viral fitness (369).

Clinical markers can be used to distinguish stages of HIV infection from the initial acute stage through clinical progression to chronic infection, Fiebig stages (32). Due to patients often presenting at later time-points in clinics, post the eclipse phase, T/F sequences are no longer present within a patient's clonal diversity. Therefore, many early studies relied on sequencing within patient diversity over time. Such early sequencing data relied on sequencing both the recipient, between one to six months post infection, and the matched transmitter. Interestingly, sequences within the recipient between one and six months post-transmission were found to map to minor variants within the transmitter's clonal diversity (70, 76).

However, this method did not allow for the sequence of true T/F virus to be determined. This problem was resolved, more recently, through the development of single genome amplification (SGA). SGA can be used to derive individual cDNA molecules from defined regions of the HIV genome (370). cDNA, spanning a specific region of the genome, are then PCR amplified and sequenced in bulk. Therefore, SGA methodology avoids polymerase-induced recombination artefacts by eliminating the need to re-sample multiple clones from one initial template (371). Multiple individual SGAs are performed to acquire multiple clones, which are required for optimal sampling depth (372). SGA was initially applied to obtain T/F ENV sequences of 102 HIV-1, sub-type B samples. From these initial sequences it was determined that samples derived from later stages of acute infection bore a much higher percentage of Env diversity (75).

An alternative method to acquire true T/F sequences from specific regions of the genome is achieved through the construction of a phylogenetic tree, from within patient clonal
diversity. From this patient clonal tree, the determination of a common ancestral sequence can be traced, referred to as the transmitted sequence. Both of these approaches result in a true T/F sequence from patient samples (75, 88). More recently, the phylogenetic determination of a common ancestral sequence, has resulted in the acquisition of whole genome T/F sequences (75, 88).

Reviewed in section 1.2.3, phenotypic characterisation of T/F sequences has reported T/F to be predominantly CCR5-tropic, with only a few samples demonstrating duel R5 X4 tropism. T/F clones also demonstrate greater resistance to type-1 IFN, compared to chronic clones (75, 93, 95, 373, 374). Interestingly, despite the majority of T/F clones being CCR5-tropic, they are found to poorly infect and replicate within macrophages. Yet, the selection of R5 tropism is thought to assist T/F clones through early selection bottlenecks within mucosal membranes. This has been demonstrated through the correlation between R5 usage and resistance of T/F to IFITM mediated early restriction (89, 375, 376).

The transmission bottlenecks associated with mucosal transmission are not present through the route of injection drug use (IDU). Therefore, how T/F varied through this route of transmission was previously considered. Findings indicated that recipients, through IDU, were more likely to become infected from multiple-variant transmission (377). Furthermore, a wider range of T/F sequences could establish infection via this route than through hererosexual transmission. In fact, SGA amplicon sequencing, from plasma samples, determined multiple-variant transmission in IDUs was as high as 60%, compared to heterosexual transmission of 19% (378).

5.1.2 HIV cofactor usage

We have previously determined the importance of host cofactor, CPSF6 and CypA, recruitment to the HIV-1 (M) CA to evade innate sensing in macrophages, a process we termed ‘cloaking’ (141). More recently, we have measured the requirement for cofactor recruitment in CD4+ T-cells for efficient RT. Both cofactor binding mutants, N74D and P90A, had a significant block from early RT, strong stop, compared to HIV-1 (M) WT virus (data unpublished).

Interestingly, the abundant host protein, CypA, has previously been determined to have a role in early HIV-1 infection prior to integration. Shah et al demonstrated a role for CypA in stability of the CA cone. This was determined by the reduction of CA uncoating, quantified by the amount of CA present on gradient centrifugation observed on CypA binding (235). Liu et al also proposed a role of CypA through the formation of bridging interactions between two hexameric sub-units, determined through cryoEM (177).
However, it has also been proposed that CypA recruitment to CA has also been proposed to promote optimal vDNA integration. The CypA binding mutant, P90A, is unable to interact with NUP358 and TNPO3, located at the nuclear pore. Therefore, the HIV-1 (M)P90A utilises an alternate pathway of integration (163, 235).

5.1.3 Summary
The majority of published work on HIV-1 transmitted founder viruses has focused on HIV-1 T/F entry and innate immune evasion, both of which are associated with differences in T/F Env sequences. Therefore, most studies focussing on T/F Env have not considered the role of Gag in the early stages of T/F infection.

Using three T/F Gag-Pro sequences, (Trjo sub-type B, ZM246 sub-type C, and ZM247 sub-type C) (88), in a single-round HIV-1 (M) GFP vector, we sought to compare the requirement of known HIV-1 cofactors for T/F infectivity. Using a single-round drug inhibition assay, previously described in Chapter 3 (311), we measured differences in T/F sensitivity to RTIs compared to laboratory strain HIV-1 (M)RS LUC vector.
We found that all three T/F vectors demonstrated a requirement for CPSF6, CypA, NUP358 and TNPO3. Furthermore, all T/F were restricted by over expression of TRIM-Cyp. However, we measured differing sensitivities to both depletion of cofactors and expression of the restriction factor. Finally, all T/F viruses tested had similar sensitivity to RTIs, quantified by measurement of IC50 and IC90 values.

5.2 Chapter 5 Results

5.2.1 Conservation of HIV-1 CA sequence between T/F viruses
HIV-1 envelope sequences, from acute HIV-1 infected plasma samples, were found to rapidly diversify during seroconversion from a single virus or a small number of closely related sequences (87). However, sequence diversity is not limited to Env, as determined by acquisition of full-length HIV-1 sequences by SGA. In fact, significant synonymous changes arise throughout the whole HIV-1 genome within the first six months of infection, despite sequence diversity decreasing after each CTL escape bottleneck during the acute phase of infection (75, 379).
To compare some site polymorphisms in our three selected T/F Gag sequences: Trjo, ZM246 and ZM247, we performed a Clustal W multiple sequence alignment, compared to laboratory strain HIV-1 (M)RS sequence (Figure 77). However, this approach does not determine substitution rates of sequences at specific positions. For this, we would require position-specific scoring matrix (PSSM). Focusing on CA conservation (Figure 78) we found only three non-conserved positions ( ), eight semi-conserved positions (.) and 22
conserved site polymorphisms (\(\cdot\)). All key residues involved in the dynamics of the β-hairpin region are highly conserved (residue positions 12, 48, 50 and 51). However, we noted that Trjo was polymorphic at CA residue 61, located within the NTD-NTD interface. The L6I CA mutant has previously been associated with a reduction in viral fitness (380). Other CTL escape mutants and site polymorphisms at CA positions of interest, with regard to viral fitness, are compiled in Table 46.

Mapping the sites of T/F polymorphisms and CTL escape mutants onto the solved hexameric CA crystal structure, 5HGM (30), further exhibited the protein-wide differences between T/F viruses (Figure 79).
Figure 7: Gag protein alignment of T/F viruses to lab-adapted strain, HIV-1 (M) R9. Alignment performed by Clustal W multiple sequence alignment tool in DNA Dynamo with amino acid site scoring: conserved (*), conservative mutation (:), semi-conservative mutation (.), non-conservative mutation ().
Figure 78: CA protein alignment of T/F viruses to lab-adapted strains, R9 and LAI, locates multiple site polymorphisms


<table>
<thead>
<tr>
<th>CA position and mutant</th>
<th>T/F CA with mutant</th>
<th>Location in CA hexamer</th>
<th>Mutant phenotype</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>I15L</td>
<td>ZM247</td>
<td>Non-interface</td>
<td>Conservative mutant no effect on fitness</td>
<td>(381)</td>
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<tr>
<td>H87Q</td>
<td>Trjo, ZM247</td>
<td>CypA-binding loop</td>
<td>Reduced spreading infection</td>
<td>(308)</td>
</tr>
<tr>
<td>E98D</td>
<td>ZM247</td>
<td>Non-interface loop</td>
<td>Reduced spreading infection</td>
<td>(308)</td>
</tr>
<tr>
<td>T110N</td>
<td>ZM246, ZM247</td>
<td>Non-interface</td>
<td>Sub-type C escape mutation found in HLA-B<em>57 and B</em>5801</td>
<td>(382)</td>
</tr>
<tr>
<td>A116T/ A116G</td>
<td>ZM247/Trjo</td>
<td>TW10 Gag epitope</td>
<td>CTL escape mutations; both require compensation</td>
<td>(383)</td>
</tr>
<tr>
<td>G120H/ G120S</td>
<td>Trjo/ ZM247</td>
<td>Helix 6</td>
<td>CTL escape in the B57-TW10 epitope</td>
<td>(115)</td>
</tr>
<tr>
<td>V148T</td>
<td>Trjo</td>
<td>Non-interface loop</td>
<td>Reduced spreading and single-round viral titre</td>
<td>(308)</td>
</tr>
<tr>
<td>R154K</td>
<td>ZM246</td>
<td>Non-interface</td>
<td>Reduced spreading and single-round viral titre</td>
<td>(380)</td>
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<tr>
<td>F169Y</td>
<td>Trjo</td>
<td>NTD-CTD</td>
<td>Reduced viral titre</td>
<td>(308)</td>
</tr>
<tr>
<td>S178T</td>
<td>ZM247</td>
<td>CTD Non-interface</td>
<td>CTL escape. No effect on titre unless 242T present</td>
<td>(384, 385)</td>
</tr>
<tr>
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<td>Non-interface</td>
<td>Reduced viral fitness</td>
<td>(308)</td>
</tr>
<tr>
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<td>Non-interface</td>
<td>CTL escape</td>
<td>(308)</td>
</tr>
<tr>
<td>V230I</td>
<td>Trjo</td>
<td>Non-interface</td>
<td>Reduced viral fitness</td>
<td>(308)</td>
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</tbody>
</table>

Table 47: Known HIV-1 CA CTL escape mutants in T/F viral clones
Figure 79: Mapping of T/F virus site polymorphisms to HIV-1 M-group hexameric CA protein structure, 5HGM, shows protein-wide differences

Protein structure 5HGM (blue) PDB DOI: 10.2210/pdb5hgm/pdb, (Jacques, D. A., et al., 2016) (30) with mapped CA residue differences (yellow) for T/F viruses (A) Trjo, (B) ZM246 and (C) ZM247, and the conserved central R18 residue (red).

5.3 Measuring the impact of HIV-1 T/F Gag on vector titre in multiple cell lines

5.3.1 HIV-1 T/F vector titre is variable and cell-type dependent

Previously, the replicative capacity of near full-length T/F infectious molecular clones (IMCs), isolated from viral RNA or integrated proviral DNA, was tested in T-cells and monocyte derived macrophages (MDMs) (89, 375). In CD4+ T-cells, T/F clones demonstrated similar replicative capacity, measured over 10 days, compared to control viruses YU2, BaL and ADA. However, T/F virus infection in MDMs was greatly reduced, despite all clones being R5 tropic (89, 375). The reduction in spreading efficiency of T/F in MDMs, compared to YU2, has previously been linked to a block in RT efficiency (386). However, the block to reverse transcription (RT) and the resulting infection defect, in
MDMs, can be rescued, to near that of YU2, on addition of SIV\textsubscript{Mac} particles containing Vpx (386).

Differences in T/F viral titre have previously been linked to differences in tropism and Env sequence (89). Considering our proposed model of encapsidated vDNA synthesis (126) based on a role for HIV-1 CA in RT (30), we questioned if differences in T/F Gag conferred differences in infectivity. On comparing whether T/F CA aligned to lab strain R9, we located multiple polymorphisms in all three T/F sequences as compared to R9 (Table 47). Considering the presence of known CTL escape mutants in our panel of T/F, we hypothesised that T/F clones would demonstrate differences in titres in a range of cell lines. We speculated that differences in titre between T/F clones this may also be related to altered cofactor dependence.

To probe this hypothesis, we titrated HIV-1 (M)\textsubscript{R9 WT} GFP vectors bearing Gag-Pro derived from T/F clones, HIV-1 (M)\textsubscript{Trjo, ZM246 or ZM247 or, lab strain R9 in the adherent cell lines: HeLa and U87 (Figure 80A), T-cell lines, Jurkat, A3.01 and SupT1 (Figure 80B), and monocytic suspension THP-1 cells (Figure 80C). 48 hours post infection we quantified the percentage of GFP\textsuperscript{+ve} cells by flow cytometry and calculated infectious units (IU). Viral dose was normalised to units of reverse transcription (IU/U RT), quantified by the SG-PERT method to correct viral dosage, to allow for differences in efficiency of vector preparation. Importantly, all chimeric packaging plasmids containing T/F Gag-Pro, encode the same RT derived from HIV-1 R9.

In the adherent HeLa cell line we observed a significant three-fold decrease in HIV-1 (M)\textsubscript{ZM246} titre, compared to R9 (Figure 80A). However, in the U87 cell line, we measured a significant decrease in titre for both T/F vectors ZM246 (6.5x) and ZM247 (30.5x) (Figure 80A).

Full-length T/F are, predominantly, CD4\textsuperscript{+} T-cell tropic, despite CCR5 coreceptor usage. Considering this, we questioned whether differences in T/F Gag also accounted for differences in titre in a range of T-cell lines compared to HIV-1 (M)\textsubscript{R9} GFP vector. On measuring IU/U RT in Jurkat, A3.01 and SUPT1 T-cells, on titration of sucrose purified GFP vectors, the T/F HIV-1 (M)\textsubscript{ZM246} had a significant, but variable, reduction in titre in all three T-cell lines: 2-fold in Jurkats, 3-fold in A3.01 and 4-fold in SupT1 (Figure 80B).

Having determined an infection defect in the T/F HIV-1 (M)\textsubscript{ZM246} GFP vector in both adherent cell lines and T-cell lines, we asked whether the same defect could be measured in the monocytic THP-1 cell line. On measuring IU/U RT in the THP-1 cell line, we measured a 2.6-fold decrease in HIV-1(M)\textsubscript{ZM246} GFP vector titre (Figure 80C).
Collectively this data shows cell type dependent differences in titre of HIV-1 (M) GFP vectors bearing T/F Gag-Pro. However, the T/F ZM246 Gag bearing vector always demonstrated a significant decrease in titre compared to R9 in all cell lines we measured. This suggests that T/F Gag sequences do correlate with differences in viral fitness, as measured by variable titre in multiple cell lines.

Figure 80: HIV-1 (M) bearing T/F Gag GFP vector titre is variable dependent on cell line

(A) Viral titre measured 48 hours post-infection by FACS, calculated IU/mL and normalised to U/RT from titration of either HIV-1 (M) GFP vector-bearing lab strain HIV-1(M)R9 or T/F Gag. IU/ U RT were compared in adherent cell lines, HeLa and U87, (B) T-cell lines JLT-R5, A3.01 and SUP-T1 and (C) monocytic suspension cell line THP-1. Data representative of 3 independent experiments of three separate vector preparations, U87 representative of two independent experiments, RT quantification by SG-PERT. Error bars +/- SEM. Statistics unpaired t-test with Welch’s correction, * P ≤ 0.05, *** P ≤ 0.001 and **** ≤ 0.0001. Numbers show fold difference compared to HIV-1 (M) R9 vector.
5.3.2 CTL escape mutants and T/F Gag

Early immune pressure from cytotoxic T lymphocytes and selection bottlenecks drive the accumulation of CTL escape mutations across the whole HIV genome (387). While the signature TW10 of Gag residues 240 – 249 (TSTLQEQIGW) is associated with viral control in patients with HLA-B57 or HLA-B5801 (109, 388), specifically of interest to this study is the accumulation of three main CTL mutants within the 240-249 Gag epitope: T242N, V247I and G248A (109). While CTL escape mutations often arise after transmission, stable CTL escape mutations are found to be transmitted, often reverting back soon after transmission (109).

T242N, V247I and G248A CTL escape mutations are found to result in the complete loss of T-cell viral recognition (111, 369). More specifically, acquisition of the single T242N mutation, occurring predominantly in HIV-1 sub-type C, is associated with the significant decrease in viral fitness. This is determined from measurement in a single viral passage experiment in CD4+ T-cells (109, 369).

Gag alignment of T/F viruses with the reference strain R9 (Figure 77) illustrated that both ZM246 and ZM247 have the 242N mutant. While all T/F sequences were found to encode 247I, only ZM246 also bears the third mutant, 248A. The presence of all three major CTL Gag escape mutants has previously been associated with a reduction in fitness and, therefore, likely explains the consistent reduction in titre of T/F vector ZM246 in all cell lines tested (Figure 70) (109). However, we cannot conclusively determine this without making the mutants and testing them individually to ensure the effect isn’t associated with another region of Gag. Further to the three main escape mutants, T/F ZM246 and ZM247 also have additional mutations at two sites within the TW10 sequence, 246A and 247T. These additional mutations may further contribute to the decreased viral titre of these T/F clones (Figure 80). Therefore, differing infection between T/F Gag sequences might be explained by the acquisition of multiple CTL escape mutations, including 242N, 247I and 248A throughout Gag.

5.3.3 HIV-1 (M) vector-bearing lab strain R9 or T/F Gag-Pro single-round GFP vectors do not trigger an innate immune response

T/F clones demonstrate resistance, or reduced sensitivity, to type-1 interferon (IFN-I), compared to chronic clones (CC) (101). T/F have increased replicative capacity, compared to CC, in the presence of IFN-α (95). However, IFN sensitivity is sub-type specific, with sub-type B demonstrating the greatest resistance. These results were determined from 13 sub-type B T/F sequences and 14 sub-type C T/F sequences (95). While many clones demonstrate IFN-α and IFN-β resistance this is not true of all T/F clones. Fenton-May et al also characterised IFN sensitive full-length primary isolates
which all had reduced replication in the presence of titrated IFN-α or IFN-β (374). Recently, T/F clones sensitive to type 2 IFN, IFN-γ, have been characterised in MT4 and CD4+ T-cells. IFN-γ sensitivity was mapped to T192M substitution in Env (104).

IFN-I is produced as part of the acute innate response to viral infection and is, therefore, a good measure of early innate immune triggering (373). Considering clonal and sub-type differences in HIV-1 IFN resistance and the cell type dependent differences in T/F infection described here, we questioned whether HIV-1 (M) GFP vectors bearing T/F Gag-Pro would induce an innate immune response.

To address this question, we utilised the HeLa ISRE cell line. These cells express a firefly LUC reporter under control of the IFN-α ISRE response element, therefore indicate triggering of the JAK-STAT signal transduction pathway. On binding the IFNα receptor (IFNαR), on a cells surface, IFN-α initiates TYK2 and JAK1 phosphorylation. This event triggers a JAK-Stat signal transduction cascade downstream. Furthermore, IFN-α induces the formation of ISG factor 3 (ISGF3), comprised of STAT1, STAT2 and IRF9. ISGF3 binds IFN stimulated response elements (ISRE) which induces ISG transcription (389).

To measure triggering of the JAK-STAT pathway, we infected TE671, SupT1 and suspension THP-1 cell lines with HIV-1 (M)R9 or T/F Trjo, ZM246 or ZM247 vectors at a MOI of 0.3 IU/ml. After 24 hours, supernatants were removed from cell lines and added to HeLa ISRE cells. After 48 hours luciferase was read out from the HeLa ISRE LUC reporter cells. On quantification of average RLUs, we did not observe any significant increase in LUC production for HIV infection of HIV-1 (M)R9 WT or T/F Trjo, ZM246 or ZM247 vectors in any of the cell lines tested (Figure 81). These data suggest that neither R9 nor T/F Gag-Pro vectors induce the production of IFN-1 in any of the cell lines tested.
5.4 HIV-1 T/F vectors utilise cofactors for efficient infection in cell lines

5.4.1 HIV-1 (M) T/F GFP vectors require CPSF6 binding for optimum infection in HeLa cells

We have previously determined that blockage of CPSF6 binding, on addition of the N74D mutant, resulted in a 5-fold infection defect in MDMs on single-round infection. The production of IFN-I had a further accumulative effect on spreading infection. This block to infection could be rescued by adding the IFN-α/β receptor antibody (141). Deletion of the CPSF6 nuclear localisation signal (NLS) (158), or C-terminal truncation of CPSF6 (162), and over-expression in cell lines, also results in a defect in single-round HIV-1 (M) WT infection (141), through suppression of RT (162). However, IFN is not typically produced in cell lines when CPSF6 interactions are disrupted. This is likely due to the disruption of DNA sensing pathways in tumour derived cell lines, as studied by Xia, T et al., 2016 (390).

Aside from cloaking HIV-1 CA from cytoplasmic innate immune sensors, CPSF6 is also shown to determine the pathway of vDNA integration. The evidence for this was that the N74D mutant integrated into a higher region of gene activity. This effect may be explained by the cellular role of CPSF6 in transporting cargo via the nuclear pore. HIV-1 has hijacked this function in order to infect non-dividing cells (391).

Considering the early selective pressures on T/F clones, we questioned whether T/F clones demonstrate the same degree of CPSF6 requirement for infection of cell lines. The HIV-1 CPSF6-binding pocket, comprising CA residues 53, 56–57, 66–67, 70, 73–74, 105,
107, 109 and 130 (Figure 83A) (160), were well conserved between our T/F Gag sequences. Therefore, we hypothesised that all T/F would have the same requirement for CPSF6 recruitment for optimal infectivity.

To test this hypothesis, we infected HeLa cells stably expressing NLS-truncated CPSF6 (Δ-CPSF6). This mutant protein is able to bind to CA but prevents trafficking to the nucleus (159). We used HIV-1 (M)R9 and HIV-1 (M)T/F vectors and measured infectivity (IU/U RT) by flow cytometry normalised to RT, quantified by SG-PERT. As expected, expression of Δ-CPSF6 resulted in a significant reduction in the titre of both R9 and T/F vectors (Figure 82A). We observed that the T/F vector ZM246 demonstrated the largest sensitivity to CPSF6 (125x) (Figure 82A).

It is known that the HIV-1 cofactor CPSF6 binds to a conserved pocket in HIV-1 CA (Figure 83A) (160). The CPSF6 binding pocket, located between CA NTD helices 3, 4, 5 and 7, is also the binding site of the small molecule inhibitor, PF74. PF74 competitively binds to CA, thus preventing CPSF6 recruitment (Figure 83B) (160, 392, 393). The effect of PF74 on HIV-1 infection is found to be dose-dependent. At low doses, 2 μM or less, PF74 reduces HIV-1 infection but does not induce a block to RT. While higher doses of PF74, 5-10 μM, block viral RT, likely through driving uncoating of the CA core (178, 295). In fact, PF74 demonstrates an interesting triphasic dose response curve consisting of two inhibitory phases and a plateau phase (394).

Having found that HIV-1 (M)T/F GFP vectors require CPSF6-binding (Figure 82A) we questioned whether T/F vectors were equally sensitive to inhibition by PF74 as the HIV-1 (M)R9 vector. Considering the T/F ZM246 previously demonstrated the largest decrease in infection in the Δ-CPSF6 HeLa cell line, we hypothesised that adding PF74 would have the biggest inhibitory effect on HIV-1 (M)ZM246 infection.

To test this hypothesis, we selected two T/F GFP vectors, the least and most affected by CPSF6-truncation, HIV-1 (M)Trjo and ZM246, and titrated PF74 (0 μM, 5 μM, 10 μM and 20 μM) onto the HeLa cell line at the same time as infection. Both WT and T/F vectors were sensitive to PF74 titration, but interestingly, we observed that the T/F Trjo demonstrated the greatest sensitivity to high dose, 20 μM, not ZM246 as hypothesised (Figure 82B). This suggests that PF74 more efficiently out-competes CPSF6 in the T/F Trjo.
Figure 82: T/F vectors demonstrate the same requirement as HIV-1 (GFP) WT vector for CPSF6 binding

(A) IU/U RT of either HIV-1 (GFP)_R9 vector or vector-bearing T/F Gag were calculated in HeLa cells expressing CPSF6 NLS mutant (white bars) or expression vector control DNA (EXN) (black bars). (B) Effect on HIV-1 (M)_R9 and T/F: Trjo and ZM246, GFP vector infection on titration of HIV-1 CA targeting drug PF74 compared to DMSO control. Data from (A) representative of three independent experiments, error bars +/- SD; statistics multiple t-tests with Holm Sidak correction, * P ≤ 0.05. Data from (B) from one independent experiment. Within experiment error bars +/- SEM.
5.4.2 HIV-1 (M) T/F GFP vectors require NUP358-binding for optimum infection in HeLa cells

The NPC is comprised of over 30 different nucleoporins bearing differing function. For example, NUP358 functions in cell migration and cAMP signalling. While, NUP153 functions in cell migration, TGF-β signalling and DNA repair (395).

Of interest to the nuclear entry of HIV-1 is NUP358. NUP358 is a large FG-NUP containing multiple repeats of phenylalanine-glycine domains (FG) (396). It also functions as negative regulator of cyclic adenosine monophosphate (cAMP) (397). Previous studies have reported the post-reverse transcription block to HIV-1 infection on NUP358 depletion, is due to the reduction of HIV nuclear import, through reduced docking efficiency of the HIV-1 CA at the NPC (159, 163, 228). Despite all T/F viruses tested requiring CPSF6 for optimal infection, we questioned whether differences in T/F viral titre in cell lines would correlate to a reduction in NUP358 utilisation. We hypothesised that T/F Gag demonstrating reduced CPSF6 requirement would also demonstrate reduced NUP358 requirement. We hypothesised this, being aware of the NUP/TNPO3 independent integration pathways described in the N74D CA mutant (159).

To probe this hypothesis, we produced a HeLa cell line stably depleted for NUP358 (Figure 84B) and titrated our panel of HIV-1 (M) GFP vectors. By quantifying viral titre by flow cytometry, we were able to determine that all vectors had a modest infection defect on depletion of NUP358. However, we measured the largest infection defect in the T/F
ZM246 (11x). These data suggested that the ZM246 Gag had an increased requirement of NUP358 (Figure 84A). Indicating that, while the binding of NUP358 to CA is optimal for nuclear entry, it is not an absolute requirement (Figure 84B). We concluded this because Trjo and ZM247 demonstrated much smaller infection defects on depletion of NUP358 (4x) suggesting they were still able to effectively enter the nucleus using a NUP358 independent mechanism.

NUP358 contains a cyclophilin domain, which is recruited by HIV-1 CA with a similar binding affinity as CypA (163). We have previously determined that integration targeting of HIV-1 is CypA-dependent and retargeted after NUP358 depletion. The NUP358 dependent block to nuclear entry and thus infection can be partly rescued by the addition of the CypA targeting drug, CsA (163). We have concluded that the prevention of CypA binding to CA, through addition of CsA, forces the CA to use a NUP/TNPO3-independent pathway of integration (163).

Having previously shown the importance for CypA targeting through the CypA-NUP358 dependent integration pathway in HIV-1 (M)R9 virus, we asked whether this pathway of integration was conserved in our T/F viruses. Considering the small measurable differences in T/F sensitivity to NUP358 depletion, we hypothesised that infection by T/F clones would be rescued, to differing extents, on addition of CsA.

To test this hypothesis, we titrated single-round GFP vectors on HeLa cells depleted for NUP358, in the presence or absence of 5 μM CsA. The effect of CsA addition was measured by quantification of infection, by flow cytometry, IU/U RT. We found that adding CsA rescued both HIV-1 (M)R9 WT and T/F vector infection to levels near that of the control cell line (Figure 84C). Collectively these data demonstrate a conserved use and requirement for the CypA-NUP358 mediated entry pathway for optimal infection in T/F viruses.
Figure 84: T/F vectors demonstrate the same requirement as HIV-1 (M)R9 WT vector for NUP358-binding

(A) IU/U RT of HIV-1 (GFP) R9 vector or vector-bearing T/F Gag were calculated in HeLa cells knocked down for NUP358 (white bars) or shRNA control (shSIREN) (black bars). (B) Immunoblot for NUP358 in control HeLa cells and on stable depletion of NUP358 by shRNA. (C) Titre defect in shNUP358 cells is partially rescued on CsA treatment (5 μM) compared to DMSO control. Data from A representative of three independent experiments, error bars +/- SD. Data from C from two independent experiments, error bars +/- SD. All statistics performed using multiple t-tests with Holm Sidak correction, * P ≤ 0.05.
5.4.3 HIV-1 (M) T/F GFP vectors require TNPO3 expression in HeLa cells for optimum infection

TNPO3 karyopherin-β protein functions to import serine/arginine splicing factors in the host nucleus (398). After the identification of TNPO3 as an important host factor for HIV-1, the depletion of this protein was found to result in a large infection defect to HIV-1 (212, 213, 399). TNPO3 requires binding of CPSF6 and RanGTP to support HIV-1 replication. This was determined by mutation of TNPO3, to prevent CPSF6/ RANGTP binding, which resulted in a significant block to infection (147). These data support the importance of TNPO3 in nuclear transport and HIV-1 infection efficiency. However, TNPO3 also functions after nuclear transport. The depletion of TNPO3 is found to alter HIV-1 integration site selection, demonstrating multiple roles of TNPO3 during this stage of the HIV-1 life cycle (166).

Considering the importance of TNPO3 in HIV-1 (M)WT infection, we questioned whether our panel of HIV-1 (M) T/F GFP vectors had equal requirement for TNPO3 binding to CA. Considering the T/F, ZM246, was found to have increased sensitivity to NUP358, we hypothesized that this vector would also demonstrate a greater infection defect in the absence of TNPO3-binding.

To probe this hypothesis, we titrated our panel of HIV-1 (M) T/F GFP vectors in HeLa cells stably depleted for TNPO3 (Figure 85B). Infection was measured by flow cytometry, IU/UR T, quantified by SG-PERT (Figure 85A). We observed a significant defect in infection of both HIV-1 (M)WT and HIV-1 (M) T/F vectors. Interestingly all three T/F vectors had increased inhibition on TNPO3 depletion, compared to the WT cell line. We found that the T/F ZM247 had the largest infection inhibition 64x (Figure 85A), compared to the other T/F vectors. These data confirm that recruitment of TNPO3 is important for both R9 and T/F infection, but to differing extents.
Figure 85: T/F vectors demonstrate the same requirement as HIV-1 (GFP)$_{R9}$ vector for TNP03 binding

(A) IU/U RT of HIV-1 (GFP)$_{R9}$ vector or vector-bearing T/F Gag were calculated in HeLa cells knocked down for TNP03 (white bars) or shRNA control (shSIREN) (black bars). (B) Immunoblot of TNPO3 expression in control HeLa cells and on stable shRNA depletion. Data from (A) is representative of three independent experiments. Error bars +/- SD. Statistics multiple t-tests with Holm Sidak correction, * P ≤ 0.05.

5.4.4 HIV-1 (M) T/F GFP vectors are restricted by over-expression of TRIM-CypA

TRIM5 potently restricts HIV in old world monkeys, however, amongst new world monkeys only the owl monkey demonstrates HIV-1 restriction due to the expression of TRIMCyp. The expression of this was initially measured in owl monkey kidney cells (400, 401). Research in our lab previously found that TRIMCyp and FV1Cyp over-expression in the CRFK cell line efficiently restricts both HIV-1 and FIV infection. This restriction to infection is rescued on treatment with CsA, which binds to CypA, thereby preventing its recruitment to CA (402). We tested whether HIV-1 (M) T/F GFP vectors were equally restricted by the over-expression of TRIM-CypA in the CRFK cell line.

We titrated both HIV-1 (M)$_{R9}$ and HIV-1 (M) T/F GFP vectors on the CRFK cell line expressing the TRIM-CypA restriction factor. On quantification of infection, by flow cytometry, IU/U RT, we measured significant restriction by TRIM-CypA expression in all T/F vectors (Figure 86). However, the T/F Trjo was found to be most restricted with a 622-fold reduction of titre compared to the control CRFK cell line. Furthermore, T/F vectors, ZM246 and ZM247, demonstrated an approximately a 300-fold decrease in titre compared to 370-fold decrease in titre of the HIV-1 (M)$_{R9}$ vector (Figure 86). These data confirm that T/F are restricted by expression of TRIM-CypA. However, differences in T/F Gag sequences appear to confer differences in vector sensitivity to restriction.
Figure 86: HIV-1 (GFP) vectors bearing R9 or T/F Gag are equally restricted by over-expression of TRIM-CypA

Infectious units, normalised to units of reverse transcription, of either HIV-1 (M)\textsubscript{R9} GFP or HIV-1 (M)\textsubscript{T/F} GFP vectors in CRFK cells stably over-expressing TRIM-CypA compared to the control cell line. Data is representative of three independent experiments. Error bars +/- standard deviation. Statistics multiple t-tests, with Holm Sidak correction, * P ≤ 0.05.

5.5 Determining the role of HIV-1 T/F virus Gag in reverse transcriptase inhibitor (RTI) sensitivity

The defining of the hexameric CA pore, by Jacques et al, which is able to recruit dNTPs through R18 binding, provided a novel insight into pre-integration events of HIV. Furthermore, their data provided evidence towards a model of encapsidated RT. However, the late uncoating of the CA core raises questions regarding how RTIs are able to enter an intact core. We hypothesised that NRTIs would be able to bind the R18 residue with high affinity, as determined for dNTPs, based on their structural similarity and negative charge. However, we hypothesised that uncharged NNRTIs would enter the encapsidated core by diffusion. We addressed this in Chapter 3 and found that differences in Gag did confer small cell type dependent differences in viral sensitivity to RTIs. Interestingly we found the HIV-1 (O) vector had a two-fold reduction in NNRTI sensitivity compared to HIV-1 (M) vector. While RTI-resistance mutations, throughout reverse transcriptase, have previously been characterised, this is the first evidence for a role for Gag in viral sensitivity to RTIs (43, 69). This led us to question whether differences in T/F Gag would correlate to differences in sensitivity to RTIs, in comparison to lab strain R9.

5.5.1 T/F vectors are equally inhibited by RTIs compared to HIV-1 (M)\textsubscript{R9} WT vector in HEK293T cells

First, we asked whether HIV-1 (M)\textsubscript{T/F} LUC vectors had the same sensitivity to RTIs compared to HIV-1 (M)\textsubscript{R9} WT LUC vector in the HEK293T cell line.
We first sought to determine if T/F Gag-bearing LUC vectors had the same titre as R9 in the HEK293T cell line. From titration of HIV-1 (M)R9, HIV-1 (M)Trjo, HIV-1 (M)ZM246 and HIV-1 (M)ZM247 LUC vectors in the HEK293T cell line we measured small infection defects for the T/F vectors, ZM246 and ZM247, compared to T/F Trjo and R9 vectors (Figure 87A).

We next questioned whether we could measure differences in T/F sensitivity to RTIs. On normalising LUC vector input, to $10^6$ RLU in the no drug control, we titrated NRTIs abavacir (ABC), tenofovir (TF) and zidovudine (AZT) (Figure 87B), and the NNRTI nevirapine (NVP), (Figure 87C) and determined the percentage of inhibition for each drug curve, by normalising the data to the no drug control. From these curves we considered the quality of data obtained from calculation of Hill slope and R squared values (Table 48). On comparison of both IC50 (Table 48) and IC90 (Table 49) values for NRTI and NNRTIs, we measured no significant difference between HIV-1 (M)R9 LUC or HIV-1 (M)T/F vectors. This suggests that polymorphisms or CTL escape mutations in T/F Gag do not confer differences in sensitivity to RTIs in the HEK293T cell line.
Figure 87: HIV-1 (M)₉₀ and HIV-1 (M)₇₅₉ LUC vectors are equally inhibited by multiple RTI drugs in HEK293T cells

(A) Infection of HEK293T cells, shown as average RLUs, measured at 48 hours post infection on reading RLUs using the steady glo protocol for HIV-1 (M)₉₀ (black circle) or T/F Gag: Trjo (blue square), ZM246 (maroon triangle) and ZM247 (orange diamond). HEK293T cells infected in the presence of titrated (B) NRTIs: abacavir, tenofovir and zidovudine, or (C) NNRTI: nevirapine for 48 hours. Data from two independent experiments, with biological replicates, error bars +/- SEM.
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<thead>
<tr>
<th></th>
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<td>ZM246</td>
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<td>Average IC50 (μM)</td>
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<td>2.78</td>
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<td>SEM</td>
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<td>ZM246</td>
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<td>Average IC50 (μM)</td>
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<td>2.03</td>
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<td>Average IC50 (μM)</td>
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**Table 48: HIV-1 (M)R9 and T/F LUC vector IC50 values in the HEK293T cell line**

IC50 values averaged from two independent experiments, as calculated in the statistical application GraphPad Prism. Hill slope and R square value representative of two independent experiments.
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<th>NVP</th>
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<td>R9</td>
<td>Trjo</td>
<td>ZM246</td>
<td>ZM247</td>
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<td>Average IC90 (μM)</td>
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<td>5.80</td>
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Table 49: HIV-1 (M)R9 and T/F LUC vector IC90 values in the HEK293T cell line

IC90 values averaged from two independent experiments, as calculated in the statistical application GraphPad Prism.
5.5.2 T/F viruses are equally inhibited by RTIs in the U87 cell line

We have recently described the dependency of HIV-1 (M)\textsuperscript{R9} infection on CypA recruitment in the U87 cell line. This data suggests that the NUP358-CypA dependent pathway of integration is intact in this cell line (data not shown). Considering the importance of cofactor recruitment for CA stability and infectivity in MDMs (141), we questioned whether differences in T/F Gag conferred differences in sensitivity to RTIs in the U87 cell line.

First, we determined whether T/F LUC vectors had reduced titre in the U87 cell line, as previously measured in GFP vectors (Figure 88A). Considering that both Trjo and ZM247 bear a mutation in the CypA binding loop, H87Q, we hypothesised that both of these T/F vectors would have reduced infection in the U87 cell line. We titrated HIV-1 (M)\textsuperscript{R9}, HIV-1 (M)\textsuperscript{Trjo}, HIV-1 (M)\textsuperscript{ZM246} and HIV-1 (M)\textsuperscript{ZM247} LUC vectors in the U87 cell line. However, on comparing infection, T/F Trjo or ZM247 did not have a significant infection defect compared to R9. However, the titre of ZM246 was reduced (Figure 88A).

We then questioned whether differences in T/F Gag conferred difference in T/F sensitivity to RTIs. On normalising LUC vector input, to $10^6$ RLU in the no drug control, we titrated NRTI, TNF (Figure 88B) and NNRTI, NVP (Figure 88C). We calculated the percentage of inhibition for each drug curve by normalising the data to the no drug control. From these curves, we considered the quality of data obtained from calculation of Hill slope and R squared values (Table 50). Next, we compared both IC50 (Table 49) and IC90 (Table 51) values for TNF and NVP and measured no significant difference between HIV-1 (M)\textsuperscript{R9} LUC or HIV-1 (M)\textsuperscript{T/F} vectors. This confirmed that differences in T/F Gag do not affect viral sensitivity to RTIs in the U87 cell line.
Figure 88: HIV-1 (LUC)R9 and T/F viruses are equally inhibited by multiple RTI drugs in U87 cells

(A) Infection of U87 cells, shown as average RLUs, measured at 48 hours post infection on reading RLUs using the steady glo protocol for HIV-1 (M)R9 (black circle) or T/F Gag: Trjo (blue square), ZM246 (maroon triangle) and ZM247 (orange diamond). U87 cells infected in the presence of titrated (B) NRTI, TNF, or (C) NNRTI, NVP, for 48 hours. Data from three independent experiments, with biological replicates. Error bars +/- SEM.
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<td>R9</td>
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<tr>
<td>Average IC50 (μM)</td>
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<td>R9</td>
<td>Trjo</td>
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<td>ZM247</td>
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Table 50: HIV-1 (M)R9 and T/F LUC vector IC50 values in the U87 cell line

IC50 values averaged from three independent experiments, as calculated in the statistical application GraphPad Prism. Hill slope and R square value representative of three independent experiments.

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<td>SEM</td>
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Table 51: HIV-1 (M)R9 and T/F LUC vector IC90 values in the U87 cell line

IC90 values averaged from three independent experiments, as calculated in the statistical application GraphPad Prism.
5.6 Chapter 5 Discussion

Thus far, the majority of T/F virus characterisation has focused on differences in Env sequence, co-receptor usage, evasion of innate detection and detection of genome-wide CTL escape mutants (75, 89, 376, 387). Here we sought to determine whether differences in T/F Gag sequence, compared to lab strain R9, would alter the cofactor requirement of CA, the CA pathway of integration and resulting infection. Using single-round GFP vectors bearing R9 or T/F (Trjo, ZM246 or ZM247) Gag-Pol sequences we measured differences between T/F viral sensitivity to depletion of NUP358, TNPO3 and truncation of CPSF6. We also report differences in T/F restriction by TRIM-CypA expression. However, all T/F vectors are equally sensitive to inhibition by NRTIs and NNRTIs, in comparison to HIV-1 (M)R9 LUC vector. Collectively these data suggest that differences in HIV-1 T/F virus Gag sequence and co-receptor requirement, for optimal infection, do not significantly alter the efficacy of RTIs in cell lines.

5.6.1 HIV-1 T/F titre is variable and cell type-dependent, despite their use of host cofactors

We asked whether T/F residue differences mapped in Gag (Figure 77), would affect viral titre compared to lab strain vector HIV-1 (M)R9. We were especially interested in the comparison between T/F ZM246 and R9, because ZM246 has three CTL escape mutants that are associated with a decrease in titre in T-cells, 242N, 247I and 248A (111, 369, 388). Consistent with previous data, we characterised ZM246 as having a significant infection defect in all cell lines measured. Interestingly the T/F Trjo appeared to have similar fitness in all cell lines as HIV-1 (M)R9. This was surprising due to the presence of known CTL escape mutants 116G, 120H, 225G and 169Y mutation situated between the CA NTD and CTD. The presence of 242N and 247I escape mutations in ZM246 conferred poor viral fitness in multiple cell lines, despite the presence of some known compensatory mutations in all T/F Gag sequences (Table 52).

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Table 52: CTL escape compensatory mutation sites located within Gag

Transmitted/ Founder virus sequences obtained from (75, 87-90). Alignment and sequence comparison performed in DNADynamo.

We have previously determined that U87 cells have CypA dependency (data unpublished). We therefore suggest that the presence of CypA-binding mutant, 87Q, in ZM247 might explain the reduction in titre in the U87 cell line (308). However, the T/F Trjo also bears the CypA loop-binding mutant 87Q yet does not demonstrate a significant
infection defect, suggesting, as expected, that this is not the sole residue conferring reduced T/F fitness in cell lines. Cofactor binding mutants, P90A and N74D, have previously been determined to trigger an innate immune response in MDMs (141). None of the three T/F vectors we tested resulted in IFN-I production in T671, SUP-T1 or THP-1 cell lines, determined using HeLa ISRE reporter cell line. However, we propose a better experiment, to measure whether T/F infection defects are due to innate triggering, by adding the IFNα/β receptor antibody (IFNα/βR Ab). A rescue to T/F infection IFNα/βR Ab addition would more clearly demonstrate sensitivity to IFN-I.

In accordance with previous studies, all HIV-1 (M) vectors had reduced titre in HeLa expressing truncated CPSF6 (158, 162). However, the T/F ZM246, which demonstrated the biggest titre defect in cell lines, was most affected by CPSF6 truncation. This suggests that ZM246 has increased CPSF6-TNPO3 cofactor dependency and is reliant on the optimal pathway of integration (163).

Sub-type C T/F vectors, ZM247 and ZM246, demonstrate high dependency on TNPO3, the binding of which is vital for nuclear transport (147, 235). Depletion of TNPO3 in HeLaT4 cells has previously been found to reduce HIV-1 (M)NL4.3 2nd strand transfer, late RT, by three to five-fold (231). Furthermore, TNPO3 depletion prevents effective HIV-1 integration, measured by the lack of detectable 1-LTR and 2-LTR circular products (231). Despite not measuring a complete block to T/F infection on TNPO3, as measured by Shah et al (235), it would be of interest to quantify 2nd strand transfer and production of 2-LTR circles on T/F infection in HeLa cells depleted for TNPO3 to determine if there are measurable differences and a partial block to RT. TNPO3 and CPSF6 binding sites, T54, A77 and A105, have previously been characterised (403). Due to the deletion of residues 120-122 in ZM246 and 120-121 in ZM247 both T/F bear different residues at these sites. It would be of interest to measure T/F CA core stability and uncoating in HeLa depleted for TNPO3 and WT HeLa. While addition of TNPO3 in cell lines has previously been found to result in the increased uncoating of HIV-1 CA cores, we question whether T/F cores may be unstable (235, 324).

Therefore, an attractive hypothesis is that these differences may result in both ZM246 and ZM247 having reduced binding of CPSF6 and NUP358. Despite this hypothesis, the A77V CPSF6 binding mutant retains high titre in cells depleted of NUP358, TNPO3 and NUP153, through use of an alternate pathway of integration (159). To determine the effect on nuclear entry of T/F vectors, we should have quantified the production of 2-LTR circles, previously measured to be reduced in Δ-CPSF6 cell lines (163) and, more recently in our lab, in the N74D binding mutant (unpublished).
ZM246 is also sensitive to the depletion of NUP358, which is binds CA via interaction with the CypA-binding loop domain (Figure 89) (172, 229). CypA is highly abundant in cells, however, HIV-1 CA binds to the Cyp domain of NUP358 with greater affinity (16 μM) than CypA (7 μM), as measured by ITC (229). While TNPO3 promotes CA uncoating, NUP358 increases CA stability preventing uncoating by up to 50% (324).

![Figure 89: NUP358 and CypA binding stabilises HIV-1 CA](image)

(A) Crystal structure of CA NTD (blue), with resolved β-hairpin (yellow), bound NUP358 (light blue) demonstrating key interactions at P90 (red) and H87 (magenta) of the CypA-binding loop. (B) Structure 4LQW from Bichel et al (2013) (229). HIV-1 CA monomer (blue) interaction with CypA (green) via key residues, H87 (magenta) and P90 (red), stabilises HIV-1 CA through bridging of two HIV-1 CA monomers. Structure 5FJB from Liu et al (2016) (169).

To probe the conservation of NUP358 independent integration pathway in T/F vectors, we performed the same experiment described by Schaller et al and added CypA-targeting drug, CsA, to the NUP358-depleted HeLa cells at time of infection (163). We determined that all vectors were rescued to near that of the control infection. This suggests that, in the absence of an intact CypA-NUP358 dependent integration pathway, T/F are able to utilise an alternate pathway.

Despite ZM246 demonstrating increased requirement for cofactor binding, on measuring the restriction of T/F vectors on TRIM-CypA over-expression, Trjo had the more significant titre decrease. The restriction of HIV-1 infection by TRIMCyp expression has previously been recovered by the addition of CypA binding drug, CsA (404). However, HIV-1 with CypA-binding mutant, G89V, is less restricted by TRIM-CypA expression, even in the absence of CsA (404). While none of the T/F vectors we tested had a valine at CA residue 89, the CypA-binding mutant, 87Q, in Trjo had no effect in rescuing restriction. It would be interesting to further examine this by mutating key CypA binding loop resides in our T/F panel and to repeat the restriction experiment in the presence of CsA.
5.6.2 Differences in T/F Gag sequence do not confer differences in reverse transcriptase sensitivity measured by determination of inhibitor IC50 and IC90.

Currently two classes of RTIs are clinically available. The NRTIs and NNRTI. Our collaborator’s recent discovery of the central HIV-1 CA pore, which is able to transport dNTPs through binding of CA arginine 18 residues, provides a new mechanism for the fuelling of encapsidated reverse transcription (30). This discovery led us, in Chapter 3, to question whether differences in HIV-1 (M) and HIV-1 (O) Gag confer differences in viral sensitivity to RTIs.

We sought to determine whether natural polymorphisms or escape mutants throughout Gag in T/F Trjo, ZM246 and ZM247 would affect sensitivity to RTIs. From measurement of IC50 and IC90 values of HIV-1 (M)R9 and T/F vectors, we concluded that differences in T/F Gag do not affect viral sensitivity to RTIs in the HEK293T or U87 cell lines. However, there were measurable and consistent differences in IC50 values on comparison of vectors between cell lines. This suggests that differences in cell lines, such as nucleotide levels, may alter the rate of RT and therefore the dose of RTI required (Table 53).

<table>
<thead>
<tr>
<th>Virus/RTI</th>
<th>IC50 fold difference (U87:293T)</th>
<th></th>
<th>IC90 fold difference (U87:293T)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Trjo</td>
<td>1.7</td>
<td>1.5</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>ZM246</td>
<td>1.4</td>
<td>1.6</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>ZM247</td>
<td>1.4</td>
<td>1.8</td>
<td>2.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 53: Comparison (fold difference) of IC50 and IC90 values (µM) for NRTI, Tenofovir, and NNRTI, Nevirapine, obtained for HIV-1(M)R9 and T/F LUC vectors in HEK293T and U87 cell lines

However, we found it difficult to compare our data to published literature as most commonly EC50 values have previously been measured for full-length lab strains, such as NL4.3. Alternatively, full-length molecular clones with known resistance mutations in reverse transcriptase have been used. Interestingly, IC90 values have not been widely considered in the literature. However the measurement of EC50 values of the NNRTI Rilpivirine in MT4 cells has previously found differing values among full length clinical clones as laid out in table 54 (405). This, in conjunction with our data, suggests that differences in T/F Gag do not account for differential RTI sensitivity.
Table 54: EC50 data measured from infection of MT4 cells with primary isolates of differing sub-type and co-receptor tropism

Data from Azijn et al (405).

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Sub-type</th>
<th>Co-receptor usage</th>
<th>EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR-CSF</td>
<td>B</td>
<td>CCR5</td>
<td>0.51</td>
</tr>
<tr>
<td>93BR021</td>
<td>B</td>
<td>CCR5</td>
<td>0.23</td>
</tr>
<tr>
<td>WEJO</td>
<td>B</td>
<td>CXCR4</td>
<td>0.08</td>
</tr>
<tr>
<td>92BR025</td>
<td>C</td>
<td>CCR5</td>
<td>0.33</td>
</tr>
<tr>
<td>93IN101</td>
<td>C</td>
<td>CCR5</td>
<td>0.53</td>
</tr>
<tr>
<td>93MW959</td>
<td>C</td>
<td>CCR5</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Chapter 6: A model for the timing of reverse transcriptase inhibitor function during encapsidated reverse transcription

6.1 HIV-1 CA cores, with optimal stability, remain intact within the cytoplasm until completion, or near completion of RT

There is strong evidence, both in cell lines and primary cells, for the stabilising function of the host HIV-1 cofactor, CypA. CypA binding to HIV-1 CA core has been shown to promote innate immune evasion and promote optimal integration (163, 169, 406). However, the P90A CA mutation in MDMs results in significant titre defects, due to innate immune triggering (141). While the P90A CA mutant titre defect in CD4+ T-cells can be attributed to a reduction of RT (data unpublished). However, recent data from our lab has found that, on treatment with the CypA-targeting drug, CsA, the HIV-1 (M)R9 GFP vector has an infection defect in the U87 cell line (data unpublished). This suggests that infection of this cell line is cofactor-dependent. Interestingly, the vectors packaged with a short CSGW genome had increased sensitivity to CsA addition compared to the near full-length vector LaiΔEnv. However, HIV-1 (M) mutants, HIV-1 (O), which are less reliant on CypA or HIV-2 CAs (which not bind CypA), are still infectious in most cell lines (324), as is the CPSF6-binding CA mutant, N74D (338, 407).

We hypothesise that, on entry into a host cytoplasm, the HIV-1 CA core remains intact (30, 141, 145). One possible model suggests that, on binding the cofactor, CypA, the CA pore opens allowing an influx of dNTPs, which are transported via binding the R18 ring. The influx of dNTPs drives the completion of strong stop vDNA synthesis and fuels RT (Figure 90). From our measurements of strong stop inhibition, on treatment with high-dose NRTIs and NNRTIs, we hypothesise that RTIs may gain access to the CA core at the time of dNTP influx. While NRTIs bind the R18 ring, as dNTPs, we suggest that NNRTIs may passively diffuse into the core when the CA electrostatic channel is open. The early entry of RTIs is supported by our data, which demonstrates a reduction in strong stop RT products in the presence of high dose NRTIs and NNRTIs. However, we found that mutation of the CypA-binding loop, at various positions, bore no effect on the efficacy of RTIs in cell lines (Chapter 3). We propose that point mutations in CA do not significantly alter the CA enough to confer differences in RT and, therefore, RTI function in cell lines (Figure 90).

On completion of RT (either near to, or docked at, the NPC), the sequential binding of cofactors, CPSF6, NUP153 and NUP358, to CA, and TNPO3 to CPSF6 promote CA uncoating. Uncoating allows for the release of newly transcribed vDNA into the nucleus via the NPC, as reviewed by Hilditch and Towers (408). We have demonstrated, however, that the requirement of cofactor recruitment is variable amongst T/F vector Gag sequences, Trjo, ZM246 and ZM247, as measured in the HeLa cell line. This goes some
way to explaining their differing infectivity in cell lines (Chapter 5). However, T/F differences in cofactor usage had no effect on IC50 or IC90 values measured for NRTIs and NNRTIs compared to lab-strain HIV-1 (M)R9.

Having determined that cofactor binding mutants and differences in T/F Gag bore no difference in RTI IC50/IC90 values, we wanted to determine the exact timing of RTI function. Our measurement of late RT suggests RTIs inhibit longer viral transcripts, as determined by the decreased measurement of 2nd strand transfer products in the presence of either NRTI or NNRTI. However, this result was not surprising considering the mathematical likelihood of longer vDNA sequences being inhibited. Yet, despite the differing rates of RT between HIV-1 (M) and HIV-1 (O) CA in THP-1 cells, we did not observe any differences in the timing of RTI function between these vectors (Chapter 4). This suggests that the accessibility of R18, on the opening of the CA channel, does not affect the timing of RTI function. However, we did measure a cell type-specific small decrease in O-group Gag sensitivity to NNRTIs. Interestingly, O-group viruses are less dependent on the binding of CypA to infect in cell lines, while HIV-2 viruses have been found not to require CypA binding (407). Yet, mutating position residue 88 in the CA CypA-binding loop to the residue present in HIV-1 (M) CA bore no effect in HIV-1 (O) vector sensitivity to RTIs. Recent data from our lab suggests that the reduction of CypA dependency results in an innate immune response in MDMs, but not cell lines. This indicates that such changes to CA are licensed in cell lines. However, opposing this, Mamede et al characterised multiple HIV-2 clones which were reliant on CypA and NPC-associated nucleoporins, NUP358 and NUP153, (409). This may indicate that our findings are a characteristic of HIV-2ROD10, which is cell culture-adapted.

Furthermore, it is evident from our infection data that the majority of RT block occurs after the completion of 2nd strand transfer, during the late stage of RT that we are unable to measure using our current method. It is possible that before or after the completion of 2nd strand transfer the CA core has started, or completed, the process of uncoating. We, therefore, hypothesise that, during late RT, both NRTIs and NNRTIs readily access cytoplasmic vDNA (Figure 90).

6.2 The licensing of HIV-1 CA uncoating in some cell lines

We propose a second fate of CA, where CA cores with reduced stability uncoat on entry into the cytoplasm. We suggest uncoating may be initiated from completion of strong stop vDNA, cofactor binding or removal from CA, or the influx of dNTPs into the core (128). Furthermore, the process of CA uncoating may be a rapid complete or a gradual partial process. CA with cofactor-binding mutants, P90A or N74D, or HIV-1 O-group CA are sensed in MDM’s leading to the production of IFN-I, as reviewed by Sumner et al (126). It is, therefore, an attractive hypothesis that vDNA is exposed due to the instability and
uncoating of the HIV-1 CA, either on mutation or differences between HIV-1 (M) and (O) CA. Host exonucleases, such as TREX1, degrade exposed vDNA, however, in cells with high infection, or low TREX expression, undegraded vDNA is sensed, as reviewed by Sumner et al (126). As we found TREX1 levels in cell lines, such as U87 cells, are naturally low.

Many cell lines, including U87, do not have an intact DNA sensing pathway however. Therefore, viruses that trigger an innate immune response, in MDM’s are licensed to infect or replicate in cell lines. This leads us to question whether cell lines are appropriate models to measure differences in RTI sensitivity of differing CA mutants or Gag sequences.

We hypothesise that a model of infection where early CA uncoating is equally licensed in cell lines as the late uncoating of WT M-group infection. We suggest that, in cell lines, the timing of uncoating has no effect on the concentration of inhibitor required compared to encapsidated reverse transcribing vector. Furthermore, we suggest that, in cell lines, both NRTIs and NNRTIs inhibit at the same stage of RT (Chapter 4).

If our hypothesis is correct, differences in T/F viral sensitivity to RTIs cannot be efficiently measured in cell lines, in which CA stability and infection is independent of cofactor recruitment. Consequently, in cell lines where early uncoating is licensed, we propose that dNTPs and RTIs do not utilise the R18 residues of the CA channel. Therefore, the sequence of CA has no effect on RTI efficacy or timing of function during RT.
Figure 90: Model of cell line licensing of cytoplasmic HIV CA uncoating

(1) On entry into the cytoplasm the HIV-1 CA core recruits the abundant host cofactor, CypA. The stable CA core remains intact as it traverses the cytoplasm, protecting ongoing vDNA synthesis. The CA core uncoats near to, or docked at, the NPC. (2) Unstable CA cores uncoating within the cytoplasm, perhaps on completion of early RT. vDNA synthesis continues within the cytoplasm as part of a RTC. vDNA synthesis completes close to the NPC. Both NRTIs and NNRTIs can equally gain access to ongoing vDNA synthesis in both models, either by binding R18 or through passive diffusion.
Chapter 7: Future Work

Our research into the effect of Gag on viral sensitivity to RTIs could be furthered in several ways. From measuring cell type differences in Gag sensitivity to RTIs we initially suggested that differing levels of dNTPs, between cell types, might affect the competition of R18 binding. To quantify differences in competitive binding, between dNTPs and NRTIs, we suggest measuring differences in dNTP levels between the cell lines used in this study. This could be performed by HPLC-MS/MS using the previously described method by Chen et al., (410).

To further measure if NRTIs are outcompeted by dNTPs, for binding to the R18 ring of hexameric CA, we suggest using fluorescence anisotropy. This method would allow us to measure binding affinity compared to dNTPs as recently described, for the NRTI AZT, by our collaborator James, L (319). Malery et al., determined that AZT effectively bound to hexameric CA while the NNRTI NVP did not, as expected due to NNRTIs being uncharged. However, it would be of interest to also measure binding of clinically relevant NRTIs, to determine if there is increased affinity in 2nd and 3rd generation inhibitors. Furthering the measurement of NRTI binding to R18, we also propose the use of Biacore to quantify binding of our panel of NRTIs to hexameric CA.

From measuring cell type differences in RTI sensitivity in the open CA pore mutant, HIV-1 (M)Q50Y+120R, CPSF6 binding mutant HIV-1(M)N74D and non-pandemic Gag bearing vector HIV-1 (O)MVP we questioned if mutations in CA might alter the timing and location of core uncoating. In chapter 6, we hypothesised that many cell lines, in which sensing pathways are defective, allow for the early uncoating of viral CA. However, we also suggest that differences in Gag may also cause CA to uncoat late, perhaps docked to the NPC or within the nucleus. Determining the location of uncoating of infectious particles is difficult and has been an obstacle in the field of research. We therefore suggest that measuring CA stability of a range of HIV-1 (M) and HIV-1 (O) mutants might provide information into which mutants or CA are more likely to uncoat early within a cell. This may in turn provide further information to explain the differences in RTI sensitivity measured, especially between pandemic HIV-1 (M) and non-pandemic HIV-1 (O) Gag bearing vectors.

There are currently several methods for measuring CA stability, such as microscopy studies, which involve tagging CA and the fate of capsid assay (FOC), such approaches measure the behaviour of multiple CA cores. However, we favour the use of a new method, the single molecule CA uncoating assay, described by Marquez et al (411). This HIV uncoating assay allows for the tracking of multiple individual virions, measuring individual uncoating patterns (Figure 91). Use of this method, led Marquez et al., to describe two separate CA uncoating patterns, capsid opening and lattice disassembly (411).
Our measurements in chapter 4 of the timing of RTI function in U87 cells, suggested that both NRTIs and NNRTIs predominantly inhibit late RT, after the completion of 2\textsuperscript{nd} strand DNA transfer. However, we are unable to measure past this stage of RT with our current method of TaqMan q-PCR. We therefore suggest the use of a deep sequencing strategy, developed by Pollpeter et al., which enables the characterisation of reverse transcription products and their precise 3\textsuperscript{\textprime}-termini (366). We suggest that use of this assay would allow us to determine the exact stage of RT at which both NRTIs and NNRTIs inhibit, through the comparison of DNA strand lengths on RTI treatment at IC50 and IC90 doses. A further experiment to measure the stage of RTI function, is the use of the ERT assay, previously described (30). Use of the ERT assay would allow for the measurement of RT in a single CA cone, either WT or CA mutant, in the presence of RTI. This assay also allows for the manipulation of dNTP levels, possibly allowing us to reproduce those measured by HPLC-MS/MS.
Chapter 8: References

10. Databases H. Landmarks of the HIV-1 genome, HXB2 [Available from: https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html]


Relationship of potency and resilience to drug resistant mutations for GW420867X revealed by crystal structures of inhibi-


selective inhibition of human immunodeficiency virus type 1 by a novel 6-substituted


for specific anti-HIV-1 agents: 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine. J Med


of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science (New York,


of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure

of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance.


56. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. Crystal structure at 3.5 A

resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science (New York,


Structures of HIV-1 Reverse Transcriptase with Etravirine (TMC125) and Rilpivirine

(TMC278): Implications for Drug Design. Journal of Medicinal Chemistry. 2010;53(10):4295-

9.


59. Rittinger K, Divita G, Goody RS. Human immunodeficiency virus reverse transcriptase

substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside


60. Spence RA, Kati WM, Anderson KS, Johnson KA. Mechanism of inhibition of HIV-1


novel next-generation nonnucleoside reverse transcriptase inhibitor active against

nonnucleoside reverse transcriptase inhibitor-resistant human immunodeficiency virus type 1.


conformational and positional adaptability in structure-based design of TMC125-R185335

eviral) and related non-nucleoside reverse transcriptase inhibitors that are highly potent


64. Ren J, Nichols CE, Chamberlain PP, Weaver KL, Short SA, Chan JH, et al. Relationship of


65. Hsiou Y, Ding J, Das K, Clark AD, Jr., Boyer PL, Lewi P, et al. The Lys103Asn


a novel anti-HIV drug: multidisciplinary coordination in the discovery of 4-[[4-[(1E)-2-

cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile (R278474,


Bieniasz PD, Grdina TA, Bogerd HP, Cullen BR. Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. The EMBO journal. 1998;17(23):7056-65.


Demirov DG, Orenstein JM, Freed EO. The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. Journal of virology. 2002;76(1):105-17.


319. Mallory DL, Marquez CL, McEwan WA. IP6 is an HIV pocket factor that prevents capsid collapse and promotes DNA synthesis. 2018;7.


406. De Iaco A, Luban J. Cyclophilin A promotes HIV-1 reverse transcription but its effect on transduction correlates best with its effect on nuclear entry of viral cDNA. Retrovirology. 2014;11:11-.
Extended Figure 1: High dose NRTI TNF effectively inhibits 2nd strand transfer of HIV-1 (M)LAI ΔEnv GFP vector while high dose of NNRTI TMC278 inhibits from strong stop

U87 cells were pretreated for two hours with NRTI TNF at an IC50 dose (2.6 μM) (purple triangle) or IC90 dose (10.5 μM) (red triangle) or NNRTI TMC278 at an IC50 dose (4 nM) (dark blue diamond) or IC90 dose (10.5 nM) (light blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (M)LAI ΔEnv GFP vector. (A) Infection was measured 48 hours post-infection by flow cytometry. (B) Cells were harvested at time points from 0.5 to six hours post-infection and early RT products, (C) mid RT products or (D) late RT products quantified by q-PCR. Data represented as (B-D) RT per 200ng DNA, (E-G) RT per 10⁶ cells and (H-J) RT normalised to number of infected cells. Data from one experiment performed in biological replicate. Error bars +/- SD.
Extended Figure 2: NRTI TNF and NNRTI TMC278 inhibit HIV-1 (M)R9 infection from strong stop

U87 cells were pretreated for two hours with NRTI TNF at an IC90 dose (30 μM) (red triangle) or NNRTI TMC278 at an IC90 dose (50 nM) (blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (M)R9 GFP vector. (A) Infection was quantified 48 hours by flow cytometry. (B) Cells were harvested at time points from 0.5 to eight hours post-infection and early RT products or (C) late RT products quantified by q-PCR. Data represented as (B-C) RT per 200ng, (D-E) DNA RT per 10⁶ cells and (F-G) RT normalised to number of infected cells. Data from one experiment performed with biological replicate. Error bars +/- SD.
Extended Figure 3: NRTI TNF and NNRTI TMC278 inhibit HIV-1 (O)MVP GFP infection from strong stop

U87 cells were pretreated for two hours with NRTI drug TNF at an IC90 dose (30 μM) (red triangle) or NNRTI drug TMC278 at an IC90 dose (50 nM) (blue diamond) prior to infection with DNAse treated sucrose purified HIV-1 (O)MVP GFP vector. (A) Infection was quantified 48 hours post-infection by flow cytometry. (B) Cells were harvested at time points from 0.5 to eight hours post-infection and early RT products or (C) late RT products quantified by q-PCR. (B-C) Data represented as RT per 200ng DNA, (D-E) RT per 10^6 cells and (F-G) RT normalised to number of infected cells. Data from one experiment performed with biological replicate. Error bars +/- SD.
U87 cells were pretreated for two hours with NRTI TNF at an IC90 dose (30 μM) (red triangle) or NNRTI TMC278 at an IC90 dose (50 nM) (blue diamond) prior to infection with DNase treated sucrose purified (A) HIV-1 (M)R9 or (B) HIV-1 (O)MVP GFP vector. Cells were harvested at time points eight or 48 hours post-infection and 2-LTR circles quantified by qPCR. Data from one experiment performed with biological replicate. Error bars +/- SD.