The Role of the HIV-1 Reverse Transcriptase Mutation H208Y to Drug Resistance and Viral Fitness

A thesis submitted to the University College London for the degree of

Doctor of Philosophy (PhD)

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Declaration of Authorship

I declare that the work in the thesis was carried out in accordance with the regulations of the University College London. The work is original, except where indicated by special references in the text, and no part of the thesis has been submitted for any other academic award.

Signed: Sarah Malik

Abstract

Accessory mutations are thought to arise alongside major or primary drug resistance mutations in order to augment resistance, restore viral fitness, or both. The H208Y mutation in the HIV-1 reverse transcriptase (RT) gene was hypothesised to be an accessory mutation. This thesis characterises the H208Y mutation in terms of linkage of H208Y to other major and accessory resistance mutations, and examines two phenotypic aspects, drug susceptibility and viral fitness. The HIV Resistance Database held at the Royal Free Hospital was searched for genotypes containing the H208Y mutation. The prevalence of H208Y in antiretroviral treatment naïve and treatment experienced patients was 5/3783 (0.1%) and 12/1304 (0.9%), respectively, indicating a high degree of conservation of position 208 in wild type virus and an increase in prevalence under selective drug pressure. Four patients were chosen to conduct further analysis of virus with H208Y, comprising a treatment naïve patient with subtype B virus, and three treatment experienced patients harbouring subtype A, subtype B and subtype C virus respectively. The RT gene from these patients was cloned and sequenced. H208Y was found to be associated with the thymidine analogue mutations (TAMs), particularly mutations at positions D67, T215 and K219. H208Y was always associated with accessory mutations at positions V35, K122 and T200. Recombinant viruses containing patient derived RT genes with and without H208Y were constructed to examine the impact of H208Y on drug susceptibility and viral fitness. A multiple cycle drug susceptibility assay showed that H208Y conferred a reduced susceptibility to the nucleotide RT inhibitor tenofovir in the context of subtype B wild type RT and subtype B RT containing

TAMs. Growth competition assays were used to examine the fitness effects of H208Y using allele-specific PCR to differentiate between competing strains with and without H208Y. In the context of subtype B wild type RT, H208Y conferred a reduced viral fitness both in the presence and absence of drug. The effect may be proposed to contribute to the overall high degree of conservation of position 208. In contrast, H208Y did not appear to impact on viral fitness in the context of subtype B and subtype A RT containing TAMs.

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Abbreviations

ABC	Abacavir
AIDS	Acquired Immune Deficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
ATV	Atazanavir
ATP	Adenosine triphosphate
AZT	3'-Azido-3'-deoxythymidine
bp	Base pair
CA	Capsid
CCR5	chemokine (C-C motif) receptor 5
CD4	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CPE	Cytopathic effect
CRFs	Circulating recombinant forms
CTS	Central Termination Sequence
CypA	Cyclophillin A
D4T	2'-3'-didehydro-2'-3'-dideoxythymidine (Stavudine)
DRV	Darunavir
DDC	Zalcitabine
DDI	Didanosine
DC	Dendritic cells
DLV	Delviradine
dNTP	Deoxyribonucleotide triphosphate
EFV	Efavirenz
EM	Electron Microscopy
ESCRT	Endosomal sorting complex required for transport machinery
FEN1	Enzyme Flap Endonuclease 1
FPV	Fosamprenavir
FTC	Emtricitabine
Gag/*gag*	Group specific Antigen (Protein/*gene*)
gp	Glycoprotein
HA	Hemagglutinin
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HR	Heptad Repeat
HTA	heteroduplex tracking assay
HTLV-III	Human T-cell Lymphotropic virus type 3
IDAV	Immunodeficiency Associated Virus
IC50/90	Drug concentration to inhibit virus replication by 50/90%
IDU	Injecting Drug Users
Ig	Immunoglobulin g
IL	Interleukin
IDV	Indinavir
kDa	Kilodalton
3TC	Lamivudine
LAV	Lymphadenopathy-Associated Virus

LEDGF/p75	Lens Epithelium-Derived Growth Factor/p75
LPV	Lopinavir
LTNP	Long Term Non-Progressor
LTR	Long Terminal Repeat
MA	Matrix
MSM	Men who have Sex with Men
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nef	Negative Factor
NES	Nuclear Export Signal
NC	Nuclear Capsid
NLV	Nelfinavir
NLS	Nuclear localisation signal
NNRTI	Non Nucleotide Reverse Transcriptase Inhibitor
NRTI	Nucleotide Reverse Transcriptase Inhibitor
ETV	Etravirine
NVP	Nevirapine
PBMCs	Peripheral Blood Mononuclear Cells
PCP	Pneumocystis carinii pneumonia
PIC	Pre-Integration Complex
PIs	Protease Inhibitors
PPT	Polypurine Tract
P-TEF-b	Positive Transcription Elongation Factor b
Rev	Regulatory factor for HIV expression
RTV	Ritonovir
RMVA	recombinant marker virus assay
RNase H	Ribonuclease H
RRE	Rev responsive element
RT	Reverse Transcriptase
RTC	Reverse Transcription complex
RTIs	Reverse Transcriptase Inhibitors
RVA	recombinant virus assay
SQV	Saquinavir
SU	Surface Subunit
TAMs	Thymidine Analogue Mutations
TAR	Transactivation Response element
Tat	Transactivator of HIV gene expression
TCID50/ml	50% Tissue Culture Infectivity Dose per millilitre
TDF	Tenofovir
TPV	Tipranavir
TRIM	Tripartite Motif
UTR	Untranslated Region
URF	Unique recombinant form
Vif	Viral infectivity factor
VL	Viral Load
Vpr	Viral protein R
Vpu	Viral protein u

Chapter one

1 Introduction

1.1 **HIV discovery**

In 1981 the CDC received reports of a disease affecting young homosexual men in San Francisco, New York and Los Angeles (CDC Weekly, 1982c;CDC Weekly, 1982a;CDC Weekly, 1982b). These patients presented with *Pneumocystis carnii* pneumonia infection (PCP) and Kaposi's sarcoma with or without other opportunistic infections. These patients showed evidence of cellular immune dysfunction with the hallmark of the disease being the depletion of $CD4^+$ T cells. Therefore the disease was named Acquired Immune Deficiency Syndrome (AIDS).

The virus was isolated in the Pasteur Institute by Luc Montagnier's group in 1983 and named lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983). Later at the NIH, Robert Gallo's group also isolated the virus and named it human T-lymphotropic viruses type III (HTLV-III) (Popovic et al., 1984). At the time, the virus was also known by other names such as immunodeficiency-associated virus (IDAV) and AIDS-associated retrovirus (ARV). In May 1986, the International Committee on the Taxonomy of Viruses published an article suggesting a common name for the etiological agent of AIDS be named and Human Immunodeficiency Virus (HIV) was designated (Coffin et al., 1986).

1.2 **Strains and subtypes**

HIV can be divided into two types, HIV-1 and HIV-2. The majority of HIV infections are caused by HIV-1 which can be divided into four main phylogenetic groups thought to represent three distinct transmission events from chimpanzees and one distinct transmission event from gorillas (Hahn et al., 2000). Group M (main) is

responsible for the majority of HIV-1 infections worldwide. Group O (outlier) and group N (non-M/non-O) are limited to West-Central Africa, particularly Cameroon (Buonaguro et al., 2007) Recently, a French group isolated a virus from a Cameroonian woman that was related to SIV in gorillas. Since this virus was distinct from HIV-1 groups M, N and O, a new group P was designated (Plantier et al., 2009).

Within group M there are 9 clades or subtypes, A-D, F-H, J and K which have a 15% and 25% amino acid variability for the *gag* and *env* genes respectively (Kandathil et al., 2005;Geretti, 2006). Subtypes can be further divided into additional phylogenetic clusters called sub subtypes. For example, subtype F is divided into F1 and F2 and subtype A is divided into A and A2 (TakebE et al., 2004b). A major source of HIV-1 diversity is the ability of two HIV-1 virions from different subtypes infecting the same cell, leading to the generation of hybrid viruses from each of the subtypes. Recombinant forms are classified epidemiologically as circulating recombinant forms (CRF) and unique recombinant forms (URF) (Kandathil et al., 2005).

CRFs must be identified by full genome sequencing in at least three unlinked individuals. The first CRF was characterised by sequencing viruses from Thailand and central Africa and was originally designated subtype E until it was shown to contain regions of subtype A and as yet an unidentified pure subtype E (TakebE et al., 2004a;Thomson et al., 2002b). At least 20 CRFs have been reported since, with the majority of originating from Africa. The relevance of CRFs is becoming

increasingly significant as they account for 18% of HIV-1 infections (Buonaguro et al., 2007).

Unique recombinant forms contain unique mosaic structures that are observed in a few epidemiologically linked individuals without any evidence of spread. The majority of URFs are located in areas where multiple subtypes are circulating particularly in central Africa (Thomson et al., 2002a;Thomson and Najera, 2005). Recombinant strains are generated within individuals that either acquire dual HIV-1 infection or experience superinfection (McCutchan, 2006).

HIV-2 is most closely related to SIV from sooty mangabeys and is thought to have entered the human population on at least eight occasions, generating 8 distinct groups. Despite there being eight distinct groups only two, group A and group B are endemic with the remaining groups consisting of single person infections which are mainly confined to Western Africa (de Silva et al., 2008;Hirsch et al., 1989).

1.3 **Epidemiology of HIV-1**

Estimations of the first introduction into humans of the common ancestor of group M strains have been elucidated to be around 1931 (1915-1941) (Hahn et al., 2000;Korber et al., 2000). Since then, the radiation of different subtypes has occurred throughout the world. As shown in figure 1-1, five strains dominate the current global epidemic, subtype A, B and C and the CRFs CRF01_AE and CRF02_AG. Subtype A is observed in central and eastern Africa and eastern Europe. Subtype B is the dominating strain circulating in western and eastern Europe, the Americas and Australia. Subtype C accounts for nearly 50% of HIV-1 infections and resides in southern Africa and India. In terms of the CRFs, CRF02_AG dominates west and west central Africa whilst CRF01_AE is detected in Southeast Asia (Buonaguro et al., 2007;McCutchan, 2006). Subtype D is seen in eastern Africa and subtypes F, G, H, J and K dominate in central Africa (Geretti, 2006).

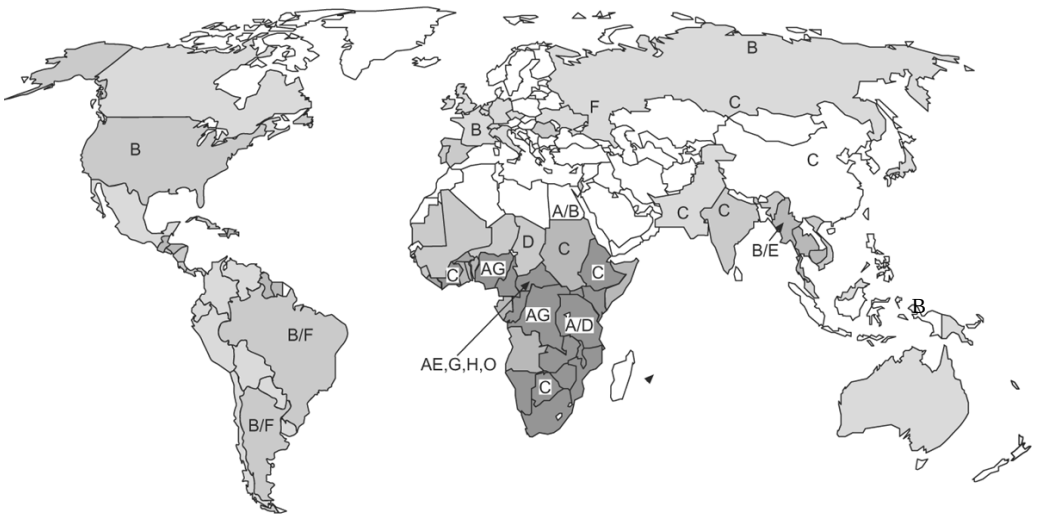


Figure 1.1 Geographical distribution of HIV-1 subtypes around the world. Letters represent the dominant subtype in the selected countries.

The prevalence of HIV-1 is now widespread with an estimated 33.4 million people infected with the virus. The majority (22.4 million) of which are living in sub-Saharan Africa. There were an estimated 2.7 million newly infected in 2009 (UNIAIDS, 2009).

The current pandemic can be depicted in two patterns. The first pattern involves sexual transmission, which includes both homosexual and heterosexual transmission. The second pattern involves parenteral transmission, which includes transmission via infected blood and injecting drug users (IDUs). In the majority of countries in sub-Saharan Africa, the epidemic is attributed to heterosexual transmission. Similarly, the main mode of transmission in the Caribbean is via heterosexual means. In Asia and Eastern Europe, the most common transmission route is via heterosexual transmission and IDUs. In Latin America, heterosexual and homosexual transmission account for the majority of infections. In North America, homosexual, followed by heterosexual transmission is still the predominant mode of transmission (UNIAIDS, 2009).

In the UK there are two types of epidemics occurring within the population, that occurring in the homosexual population and that occurring in the heterosexual population. Within the heterosexual population, the majority of infections are of non-B subtypes, suggesting that these viruses originate through immigration, predominantly from sub-Saharan Africa (Hughes et al., 2009). Within the homosexual population, the majority of infections are of B subtype. However, a recent study showed that there was an increase of non-B subtype infections within

the homosexual population, with the most commonly detected subtypes being subtype C (102/437) and subtype A (53/437). This suggests that there maybe an increase in non-B subtypes within the homosexual community as a result of mixing between individuals who harbour non-B subtypes from abroad with those individuals who are born in the UK (Fox et al., 2010).

1.4 Human Immunodeficiency Virus

HIV is a lentivirus and is part of the bigger family called the retroviridae family. Mature HIV-1 virions have a spherical shape and are 100-120nm in diameter (Barre-Sinoussi et al., 1983). As with other lentiviruses, HIV is enveloped by a lipid bilayer and is studded with around 72 spikes of the Env glycoproteins. The envelope surrounds the nucleocapsid core which contains two strands of the viral RNA genome, three essential enzymes; integrase, reverse transcriptase (RT) and protease; accessory proteins and some cellular factors (figure 1.2a). The RNA genome is 9.2kb in length and encodes the structural proteins common to other retroviruses. The ends of the HIV-1 genome are flanked by long terminal repeats (LTR). From the 5' end there are the gag-pol genes, which encode two polyprotein precursors gag and pol. The Env gene encodes gp120 and gp41. Accessory genes include Nef, Vif, Vpr, Tat, Vpu and Rev (figure 1.2b).

Gag encodes a polyprotein that is cleaved into matrix (MA or p17), capsid (CA or p24), and nucleocapsid (NC or p7). In addition, p6 and the two spacer domains p2 and p1 are also present in the gag polyprotein. Gag is responsible for the assembly, budding and release of HIV-1 virions. Matrix associates with the plasma membrane and targets gag and pol proteins to the site of assembly. The main role of capsid is to homo oligomerise with around 1500 capsid molecules and assemble to form a conical capsid that encapsidates the viral RNA, nucleocapsid, RT and integrase. Nucleocapsid plays essential roles in assembly, reverse transcription and maturation. During the assembly process, nucleocapsid is responsible for binding to RNA and ensuring its packaging into the virion. Another important step during particle

assembly involves the incorporation of tRNA molecules into virions. Nucleocapsid promotes the annealing of tRNA to the RNA so that reverse transcription can by initiated. A critical step in viral maturation involves the formation of a stable dimer complex between the two molecules of RNA. The nucleocapsid aids this process by assisting the RNA to find the most stable structure. The p6 protein is situated at the end of the gag polyprotein and has a role in viral budding. The p6 protein interacts with the host cellular factors Tsg101 and ALIX. Tsg101 is a component of the ESCRT-I complex which is involved in the budding of proteins. ALIX is known to interact with Tsg101 and also binds to p6 however binding of p6 and ALIX alone is not sufficient for viral budding (Turner and Summers, 1999).

The pol gene also encodes a polyprotein which is cleaved into the three essential enzymes, protease, RT and integrase. Protease is a homodimeric aspartyl enzyme consisting of 99 amino acids. The main function of protease is to recognise specific sites in the gag and pol polyproteins and cleave them into their constitutive proteins. The RT is a heterodimer composed of two subunits, p51 which has mainly a structural role and p66, which carries out the catalytic activities of the enzyme. The two functions of RT include the synthesis of a double stranded DNA copy from a single stranded RNA template and RNase H activity. Integrase catalyses the incorporation of the double stranded viral DNA into the host chromosomes so that a provirus is established. Once the viral genome is integrated, transcription of viral genes occurs and new viruses are produced.

The Env gene encodes gp120 (surface subunits) and gp41 (transmembrane subunits). Together these proteins instigate the entry of HIV by binding to the host cell

receptors CD4. The gp120 and gp41 proteins are linked by non-covalent bonds and three of the gp120-gp41 heterodimers make up the envelope glycoprotein knob. Both the subunits are heavily glycosylated and these sites are conserved between viral isolates signifying their importance in infectivity.

Tat and Rev are regulatory proteins that bind viral RNA. Tat is a transcriptional activator that enhances processivity of RNA polymerase II so that longer viral transcripts are produced. Tat has also been implicated in interfering with cellular processes such as proliferation and apoptosis. Rev is a protein involved in the nuclear transport of RNA. It promotes the nuclear export of unspliced and singly spliced viral transcripts (Seelamgari et al., 2004).

Nef is a multi functional protein that plays a role in the down regulation of CD4, enhancing virion infectivity and signal transduction in T cells. CD4 is downregulated on the surface of infected cells to prevent immune recognition. Virions produced in the absence of Nef exhibit a severe defect in viral entry and viral synthesis is impaired (Jere et al., 2010;Glushakova et al., 2001;Piguet et al., 2000).

The accessory proteins Vpr, Vpu and Vif are expendable for virus replication in vitro (Trono, 1995). Vpr is an accessory protein that is involved in infectivity, apoptosis, cell cycle control, viral transcription and nuclear import of the pre integration complex (Romani and Engelbrecht, 2009). The main function of Vif is to interact with the host proteins APOBEC 3G and APOBEC 3F. The APOBEC family induce the deamination of cytidine to uridine resulting in hypermutation in the HIV-1 genome. The interaction of Vif to APOBEC induces its degradation by the

proteosome and no hypermutation can occur (Malim, 2009;Henriet et al., 2009). Vpu has two primary functions, degradation of CD4 in the endoplasmic reticulum and the enhancement of virion release (Nomaguchi et al., 2008).

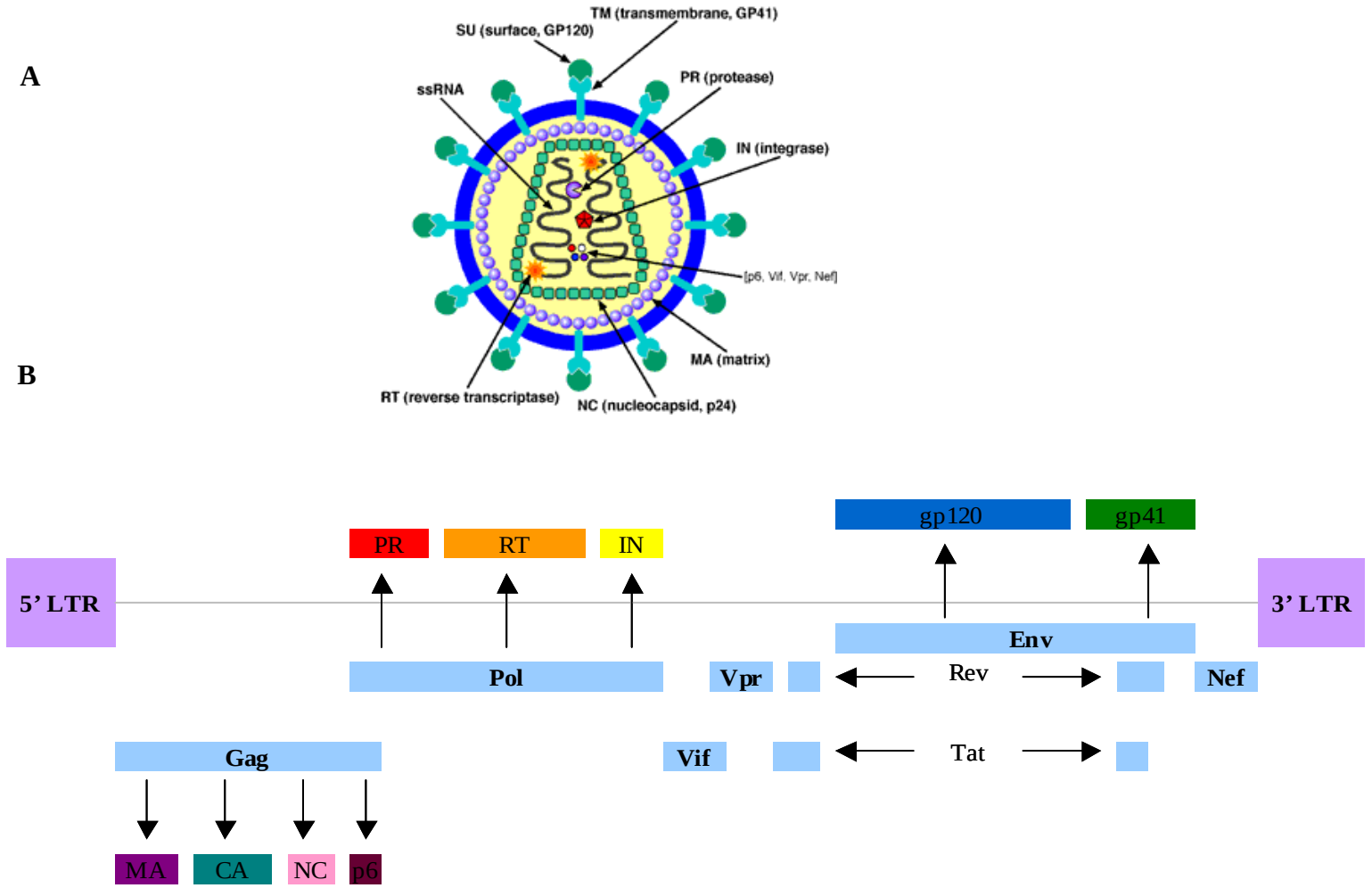


Figure 1.2 Structure and organisation of the HIV-1 genome. A mature HIV-1 virion is shown in panel A. In panel B, the structural organisation of the HIV-1 genome is illustrated. The genome is 9.2kb in length and codes for structural and non structural genes (figure taken from Freed EO 2004).

1.5 HIV life cycle

The steps of the HIV-1 life cycle are shown in figure 1.3. The first step involves entry into the host cell, followed by release of the viral genome into cytoplasm where reverse transcription occurs. The transcribed RNA genome is transported into the nucleus where it is integrated into the host genome forming the provirus. The viral proteins are translated and assembled at the plasma membrane before budding from the cell.

1.5.1 Attachment and entry of HIV-1

Virus entry begins with binding of the viral envelope to the primary receptor CD4 on host cells. The CD4 receptor interacts with class II MHC molecules to participate in the signal transduction of T cell activating signals (Doyle and Strominger, 1987). CD4 has four immunoglobulin domains D1 to D5 with gp120 binding to D1. The HIV-1 viral membrane contains 14 $\pm$7 spikes of gp160 complexes (Zhu et al., 2006). Each gp160 consists of three gp120 (surface glycoprotein) proteins that are linked to the gp41 (transmembrane glycoprotein) protein in a tripod shape. The gp120 subunit contains hypervariable regions V1 to V5, defined as areas where less than 30% of the amino acids are conserved and conserved regions C1 to C6, where the amino acid is relatively constant. The V3 region plays a major role in determining the cellular tropism and which co-receptor the virus will bind to.

The chemokine co-receptors CCR5 and CXCR4 are a class of G protein-coupled receptors that have a seven transmembrane architecture. During the early stages of infection, CCR5 infecting viruses predominate whereas the progression to AIDS is

associated with CXCR4 viruses (Deng et al., 1997). Other chemokine receptors have been reported for HIV-1 including CCR2 (Frade et al., 1997) and CCR3 (Choe et al., 1996). Mutations within the CCR5 co-receptor, namely a 32 base pair deletion at position 32 (CCR5Δ32), result in a truncated CCR5. Individuals who are homozygous for the deletion are resistant to HIV-1 infection and individuals who are heterozygous for the deletion have a slower progression to AIDS (Dean et al., 1996).

Once gp160 binds to the CD4 receptor, a conformational change is triggered in gp120 revealing the binding site of the chemokine co-receptors and depending on the tropism of the virus, either CCR5 or CXCR4 becomes engaged (Gomez and Hope, 2005). Binding of the co-receptor instigates a series of conformational changes that ensue with the formation of a six helical bundle that completes the fusion of the viral envelope and the plasma membrane (Melikyan et al., 2000).

1.5.2 Uncoating

Once fusion has occurred, the HIV-1 core disassembles and leads to the generation of the reverse transcription complexes (RTCs) and the preintegration complexes (PICs). Recent studies have shown that uncoating is not a spontaneous process and that the release of capsid proteins during uncoating, is triggered by cellular factors. This includes the cellular factor cyclophilin A (CypA) which has been shown to bind to capsid and aid the uncoating process (Auewarakul et al., 2005;Warrilow et al., 2008;Aiken, 2006). A recent study showed that peptidyl-prolyl isomerase (Pin 1) also assists in the uncoating process of the capsid core. Capsid is phosphorylated after release from an infected cell and interacts directly with Pin1 after entry into the host cell so that uncoating can take place. It has been shown that the depletion of

Pin1 resulted in dysfunctional uncoating of HIV (Misumi et al., 2010). Viral proteins such as integrase have also been shown to play a role in the uncoating process by promoting capsid core stability (Briones et al., 2010).

1.5.3 Reverse transcription

The next stage in the life cycle is reverse transcription which results in synthesis of a double stranded viral DNA molecule from a single stranded RNA template. Initiation of reverse transcription occurs following fusion of the viral and host membranes prior to nucleocapsid uncoating. The steps of reverse transcription are shown in figure 1.4. Reverse transcription begins with the binding of a tRNA molecule acquired from the host, to the primer binding site (PBS). Synthesis of the minus strand DNA is initiated and proceeds towards the 3' end of the RNA genome, producing U5 and R sequences in a DNA-RNA hybrid. The RNase H activity of RT degrades the U5 and R sequence of the RNA. The 3' end of the RNA genome also contains an R sequence and during the first strand transfer event, the newly synthesized U5 and R DNA sequence are transferred to the 3' end of the plus strand RNA genome. The newly synthesised R sequence is complementary to the R sequence at the 3' end and the U5 and tRNA overhang at the 3' end. Synthesis of the minus stand continues towards the 5' end of the RNA genome with the RNase H activity of RT degrading the RNA template once as the DNA is synthesised. There are two stretches of purine rich RNA sequences that are not degraded; the central polypurine tract (cPPT) and the 3' polypurine tract (3' PPT). The cPPT and PPT are used as priming sites for synthesis to occur towards the 5' end of the plus strand DNA, again RNase H activity degrades the PPT RNA and tRNA concomitantly. The second transfer event involves the transfer of the plus strand DNA to the 3' end of the

minus strand DNA where complementary base pairing occurs between the PBS sequences. Plus strand synthesis continues until it reaches the central termination signal (CTS). At this point, the RT is ejected and a central DNA flap is created. Minus and plus strand synthesis resumes, with each strand using the other as a template until double stranded DNA which contains the LTR is produced. The central flap is removed after integration by the host enzyme flap endonuclease 1 (FEN1). The DNA is now ready to be transported to the nucleus via the preintegration complex (PIC) (Basu et al., 2008;Freed, 2001a;Jonckheere et al., 2000).

1.5.4 Integration

The PIC contains viral DNA, RT, integrase, matrix and Vpr as well as cellular factors such as LEDGF/p75 (Ciuffi and Bushman, 2006). The matrix protein contains two nuclear localisation signals (NLS) enabling it to direct the PIC to the nucleus. Although Vpr does not have a NLS, it also has a role in directing the PIC to the nucleus by binding to members of the importin alpha family. The importin family are involved in protein transport and recognises the NLS. Once the protein has bound to importin alpha, it then binds to importin beta and together, this complex docks at the nuclear pore and allows translocation through into the nucleus (Nitahara-Kasahara et al., 2007;Gorlich et al., 1996). The cellular factor LEDGF/p75 has been shown to bind to integrase and it contains a NLS, which drives the translocation of the PIC across the membrane (Cherepanov et al., 2003).

Viral DNA can integrate into any sequence in the host but integration has been shown preferentially to integrate into actively transcribed regions in order to promote

efficient viral gene expression (Marshall et al., 2007). Integration can be divided into two stages, 3' processing and strand transfer. The first stage involves the recognition of specific DNA sequences at the end of each LTR. Integrase catalyses the removal of two nucleotides from the 5' end exposing a 3'-OH group (Engelman et al., 1991). In the strand transfer reaction, target sites on the host are cleaved via a nucleophilic attack exposing a 5 base pair at each end. The viral DNA is integrated into host genome at the 5' end. The two nucleotides at the 5' end of the viral DNA removed and the gaps are filled by cellular repair enzymes. The integrated viral DNA is now called a provirus and serves as the template for the synthesis of structural, regulatory and accessory proteins required for viral production (Delelis et al., 2008;Jegede et al., 2008).

1.5.5 Gene expression

The provirus is transcribed to produce the full RNA genome as well as shorter RNA products that are produced through splicing. The two regulatory proteins Tat and Rev are required for viral gene expression. The expression of the viral genome is regulated by enhancer and promoter elements found within the 5' LTR. The TATA box is a key sequence where transcription is initiated.

A key interaction of Tat is with the positive transcriptional elongation factor b (P-TEDb) which aids the initiation of transcription by binding to a RNA stem loop structure known as the tat response element (TAR), located at the 5' end of viral transcripts. The interaction between TAR and the P-TEDb complex result in the phosphorylation of the C terminal domain of RNA polymerase II which enhances the processivity of polymerase II complex so that full length transcripts are produced

(Zhu et al., 1997). In the absence of Tat, shorter transcripts are produced and it has been suggested that this is due to the poor processivity of RNA polymerase II (Pagans et al., 2005;Strebel, 2003b).

When levels of Rev reach a specific level, the production of single spliced and unspliced transcripts is initiated. The transcription of the longer transcripts occurs when the Rev protein binds to a stem loop structure known as the Rev response element (RRE) located in the env gene. Rev facilitates the export of RRE containing RNA out of the nucleus before further splicing can occur. It has a nuclear export signal (NES) and hijacks the host export pathway to export the transcripts out of the nucleus (Groom et al., 2009;Strebel, 2003a). Host proteins including CRM-1 and B23 are also involved in this process (Szebeni et al., 1997;Fukuda et al., 1997).

Once the transcripts enter the cytoplasm, they are ready to be translated by host ribosomes. The gag and gag-pol polyproteins are translated by ribosomes in the cytoplasm whereas the env polyprotein is translated on ribosomes in the endoplasmic reticulum. The gag and gag-pol proteins are modified with the addition of the 14-carbon fatty acid myristate at the N terminus end of matrix. This modification is important for promoting membrane association of gag and particle formation (Gottlinger, 2001;Resh, 2005;Zhou et al., 1994).

1.5.6 Assembly, release and budding

The assembly of gag particles requires nucleocapsid, capsid and matrix and occurs at the membrane in areas that are rich in lipids. Matrix targets assembly of viral proteins towards regions that are cholesterol and sphingolipid rich so that the virus

can acquire a plasma membrane that is rich in cholesterol and lipids. This is important, as cells that are depleted of these lipids are more likely to have an impaired infectivity and viral particle production (Ono et al., 2007).

In order for the virus to bud from the cell membrane, HIV-1 exploits the endosomal sorting complex required for transport (ESCRT) machinery. The ESCRT machinery is comprised of 3 subunits, ESCRT-I, ESCRT-II and ESCRT-III. Short sequences in gag, known as the late domain (L domain) are involved in binding to Tsg101 and ALIX of ESCRT-I and ESCRT-III respectively. Through a series of interactions with subunits of the ESCRT machinery, the virion envelope is detached from the cell membrane (Roxrud et al., 2010;Bieniasz, 2009;Strack et al., 2003;VerPlank et al., 2001).

Before transport to the cell membrane, host enzymes add sugar side chains to env producing monomers of gp160, which aggregate to form oligomers. The oligomers are transported to the Golgi apparatus where further processing of the sugar side chains occurs, and eventually gp160 is cleaved into gp120 and gp41 by the host enzyme furin. Once the gp120-gp41 complex arrives at the cell surface, it is internalised. The incorporation of the env proteins into a budding particle is not well understood but matrix is thought to play an important role (Freed, 1998).

1.5.7 Maturation

The final stage of the cell cycle is virion maturation which involves the cleavage of the gag and gag-pol polyproteins by protease. The cleavage of gag takes place at five different sites, all of which are important for infectivity. The order of cleavage

occurs in the following sequence, NC-SP1, MA-CA, SP2-p6, NC-SP2 and CA-SP1. This cleavage results in a dramatic change in virion morphology, with immature virions appearing doughnut shaped by electron microscopy and mature virions containing conical cores (Pornillos et al., 2009;Freed, 2001b).

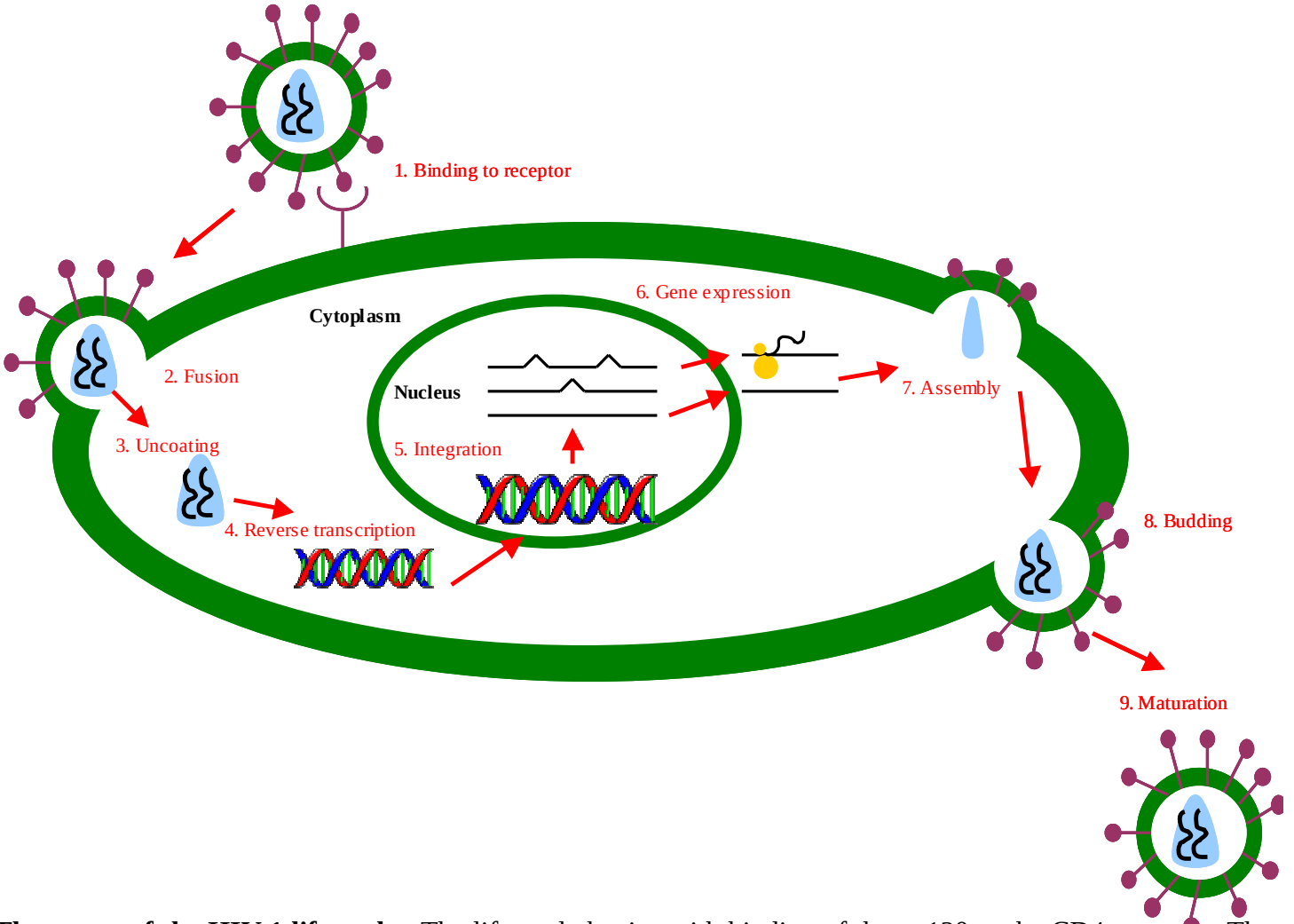


Figure 1.3 The stages of the HIV-1 life cycle. The life cycle begins with binding of the gp120 to the CD4 receptor. Through a series of conformational changes, gp41 is exposed and the V3 region of gp41 binds to the chemokine co receptor CCR5 or CXCR to initiate fusion of the viral membrane and the host cell membrane. The viral genome is uncoated and reverse transcription transcribes the RNA to DNA followed by production of a second complementary DNA strand. The double stranded viral DNA is transported into the nucleus via the pre integration complex. The viral DNA is integrated into the host chromosome and the provirus is established. The provirus serves as a template for the production of new viral proteins and RNA genomes. The newly transcribed transcripts are transported out of the nucleus and they assemble at the cell membrane, where the viral particle is released. The final stage is maturation of the viral particle, characterised by the processing of viral polypetides into discrete proteins.

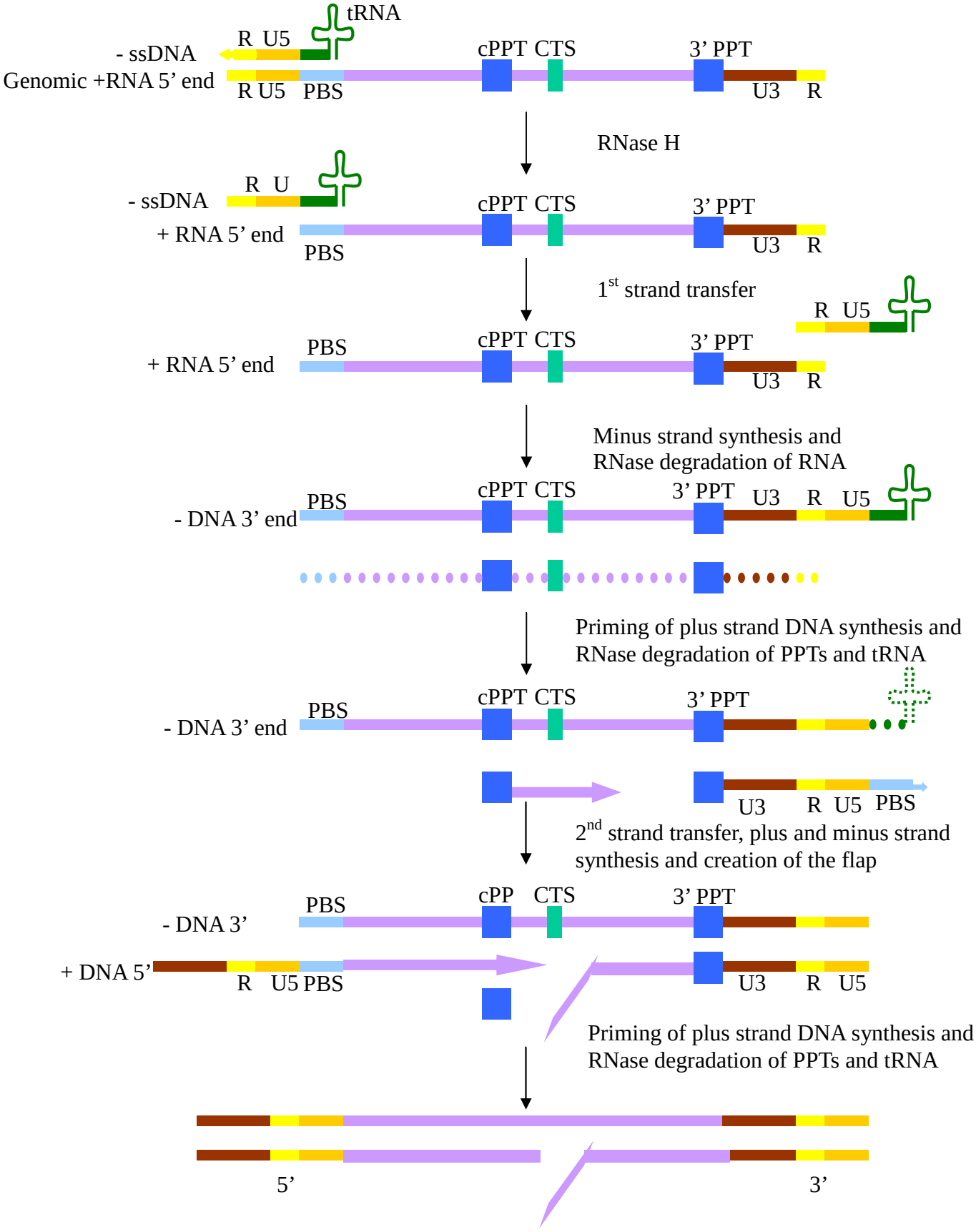


Figure 1.4 Steps in reverse transcription. Reverse transcription is initiated by tRNA at the primer binding site (PBS) and proceeds towards the 5' end. After two strand transfer events, two copies of the viral DNA genome are produced.

1.6 Transmission routes

HIV can be transmitted horizontally through sexual contact or via infected blood or blood products, and vertically from mother to child during pregnancy, at delivery or through breastfeeding. The most common route of transmission is via sexual contact (UNAIDS 2007,). The transmission of HIV from other bodily fluids such as salvia is very rare (Campo et al., 2006).

1.7 Pathogenesis of HIV-1

HIV-1 infection is acquired across the mucosal membrane during sexual and perinatal transmission. The transmission of HIV from an infected to an uninfected individual is correlated with the concentration of HIV-1 in the bodily fluid that is the source of the infection. In a retrospective study carried out in Uganda, HIV transmission was studied among couples. Individuals with a plasma HIV-1 RNA level of <3,500 copies per millilitre did not transmit the virus onto their partner. Individuals with a plasma HIV-1 RNA level between 10,000 and 49,999 copies per millilitre showed a 40% rate of transmission to their partner (Quinn et al., 2000). A Swiss meta-analysis of heterosexual discordant couples showed that the risk of HIV transmission from the positive partner to the negative partner was reduced by 92% if the positive partner was treated with antiretroviral therapy and had a suppressed HIV-1 RNA level in plasma (Attia et al., 2009). A similar reduction in risk of transmission as a result of HIV suppression with antiretroviral therapy was recently reported among discordant heterosexual couples in Africa (Donnell et al., 2010).

Since dendritic cells are located within the mucosal and the lymphoid tissues, it had been suggested that they are among the first cells that encounter HIV-1 and are responsible for transmitting the virus onto $CD4^+$ T cells (Embretson et al., 1993;Wu and KewalRamani, 2006). There are three stages of HIV-1 infection, the acute phase, the chronic phase and AIDS as shown in figure 1.5. During the acute phase of the infection, HIV-1 replicates within the host producing a viraemia over a period of a few weeks since initial exposure. The viraemia is partially controlled by the host immune system. The acute phase of the infection may occur asymptomatically or in the presence of symptoms together forming the Acute Seroconversion Syndrome. This typically includes flu-like symptoms, rash, fever, sore throat, fatigue and lymphadenopathy for a couple weeks. During the acute phase, antibodies to HIV-1 may not be detected although the level of infectious virus within the patient is very high. The development of a negative to positive antibody response is known as seroconversion and this usually occurs within a few weeks following infection. The acute phase is characterised by a significant depletion of $CD4^+$ T cells, followed by a partial recovery once the initial burst of virus replication subsides. The viral load level decreases to a nearly stable level known as the viral set point. The set point has been shown to predict the rate of disease progression and varies between individual to individual (Mellors et al., 1996). The range of the viral set point is usually between 10^3 and 10^5 RNA copies per millilitre.

The next stage is the chronic phase of infection, which comprises a generally long asymptomatic phase before the development of AIDS. Despite this phase being clinically silent or nearly silent, virus replication continues at high level accompanied by chronically high levels of immune activation. This leads to progressive immune

dysfunction and depletion of $CD4^+$ T cells, leading to the onset of the clinical manifestations of AIDS. The time between infection and development of AIDS is approximately seven to ten years, but progression can be more rapid in approximately 10% of patients (rapid progressors) and conversely delayed for over 10 years in about 5-10% of patients (slow progressors and long-term non progressors).

The immune deterioration that occurs during the chronic phase eventually results in the appearance of opportunistic conditions. This stage is known as AIDS and is characterised by diseases such as *Pneumocystis carnii* pneumonia and Kaposi's sarcoma.

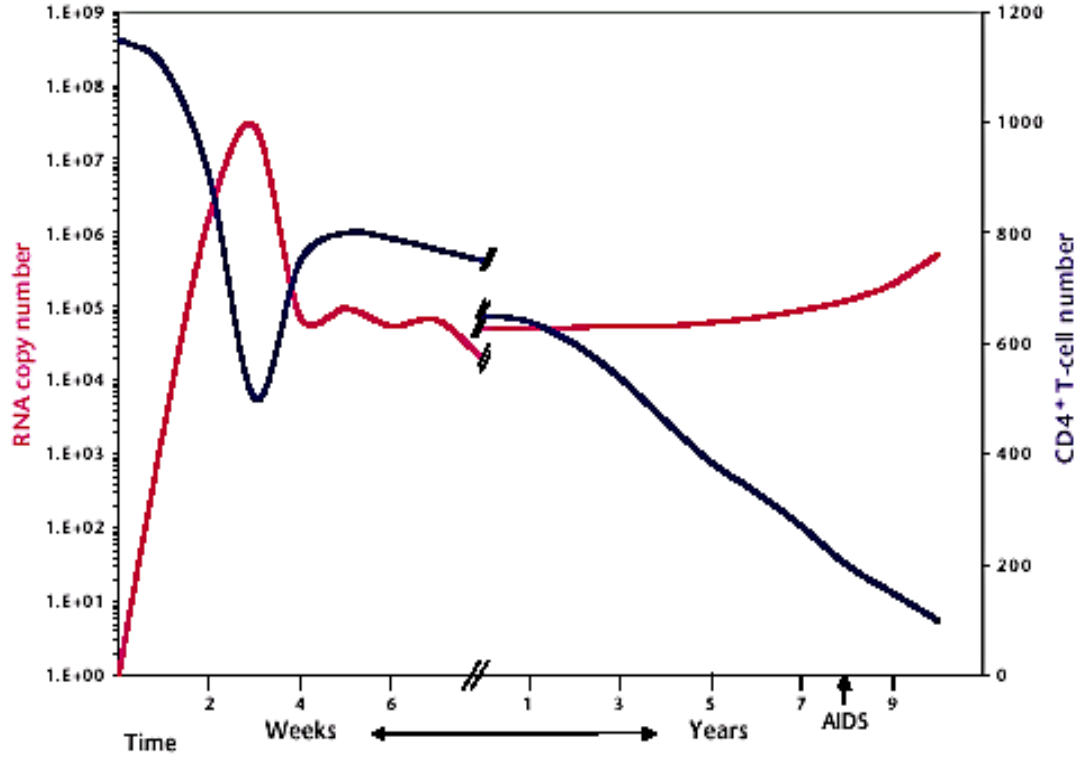


Figure 1.5 Typical course of HIV-1 infection. The acute phase of infection is characterised by the depletion of CD4 T cells and a high viraemia. The chronic phase is characterised by a generally long asymptomatic phase during which virus replication continues at high level while immune deterioration progresses. The final stage is AIDS which is characterised by the appearance of opportunistic conditions, ultimately resulting in death. Figure taken from (Feinberg and Moore, 2002).

1.8 HIV-1 reverse transcriptase

1.8.1 Discovery of HIV-1 RT

Before the 1970's, the central dogma of molecular biology was that genetic information proceeded from DNA to RNA to protein. The discovery of RT in 1970 by Temin and Baltimore contradicted this central dogma since it was shown that RT could allow genetic information to proceed from RNA to DNA. Temin worked on Rous sarcoma virus to prove that the RNA genome could be converted into a provirus and in doing so, the virus could lie dormant in cells (Temin and Mizutani, 1970). Baltimore worked on virus replication and looked for the presence of RNA and DNA in virions of Rauscher murine leukaemia virus. Together, these experiments showed that DNA could be synthesised from a DNA template (Baltimore, 1970). Their discoveries of RT lead to their receiving the Nobel Prize for Physiology and Medicine in 1975.

1.8.2 Functions of RT

RT is a key enzyme in the life cycle of HIV-1 as shown in step 3 of the virus life cycle in figure 1.3. RT has three functions; an RNA dependent DNA polymerase activity to synthesize a single stranded DNA copy from the RNA template, an DNA dependent DNA polymerase activity to complete the synthesis of the newly transcribed cDNA and an RNase H activity is required to degrade the RNA template from the RNA-DNA hybrid (Sluis-Cremer et al., 2000b).

1.8.3 Structural features of RT – fingers/palm/thumb domains

The HIV-1 RT is a heterodimer composed of two subunits, p66 (66kDa) and p51 (51kDa) as shown in figure 1.6. The p51 subunit carries out essentially a structural

role by serving as a scaffold to stabilize p66. It is initially processed as a polyprotein that has both a pol and RNase H domain. Subsequent cleavage of the polyprotein cleaves the RNase H domain producing subunits with and without a RNase H domain corresponding to p66 and p51 respectively. The p66 subunit is responsible for the catalytic activities of the enzyme and its structure is said to resemble a human hand in that it contains five subdomains named thumb, fingers, palm, connection and RNase domain. The p51 subunit shares the same subdomains as p66 except the RNase sub domain (Shafer, 2002).

The palm and connection subdomains consist of five beta sheets with two alpha helices on one side, the thumb subdomain consists of a four helical bundle. The fingers subdomain consists of a beta sheet and three alpha helices. The beta strands of the palm subdomain form hydrogen bonds with the beta strands at the base of the thumb subdomain that results in hydrogen bonding to the connection beta sheet (Kohlstaedt et al., 1992).

Despite the fact that subunits p51 and p66 share the same amino acid sequence, there are some significant differences on how the subunits are folded. The p51 subunit has no cleft and the residues that are involved in the catalytic functions of the enzyme are buried. In the p51 subunit, the finger subdomain is positioned towards the palm subdomain and the palm subdomain is situated further away from the fingers and palm subdomains then in p66. A vital difference between these subunits is observed in the connection domain. The p66 connection subdomain contacts the RNase H, the bottom of the thumb and the connection subdomain of p51. The p51 subunit on the other hand contacts the other subdomains and like p66, also contacts the connection

subdomain of p66 (Kohlstaedt et al., 1992). It is interesting to consider why the RNase H subdomain is cleaved from one of the subunits. It has been suggested that there is an evolutionary pressure to increase the amount of information of viral genomes. Therefore, the ability of the HIV-1 to encode two protein subunits from the same gene through proteolytic cleavage gives the virus the ability to produce proteins that can adopt more than one structure and function (Castro et al., 2006).

1.8.4 Comparison to other RT's

The genomic organisation of HIV-2 is similar to that of HIV-1 with the two viruses sharing between 60-70% sequence homology in the RT gene (Le Grice et al., 1988;Shaharabany and Hizi, 1992). Similar to other retroviral RTs, the HIV-2 RT has a RNA dependent DNA polymerase activity as well as an RNase H activity. The HIV-2 RT is composed of a heterodimer consisting of p68, which is equivalent to p66 in HIV-1 RT and p55, which is equivalent to p51 in HIV-1 RT (Muller et al., 1991). The connection domain in HIV-2 is much longer than that of HIV-1 RT and this has been attributed to the specificity of the protease (Fan et al., 1995). A significant difference between HIV-1 RT and HIV-2 RT is in the RNase H activity. HIV-2 RT has a ten times slower RNase H activity than HIV-1 RT although the pattern of cleavage is the same in both enzymes (Sevilya et al., 2001).

Moloney murine leukaemia virus RT (MoMLV RT) shares functional and sequence similarities to the HIV-1 RT. An important site on RT is the polymerase active site. Similar to HIV-1 RT, which has three aspartate in its catalytic active site at positions Asp110, Asp185 and Asp186, MoMLV RT also shares these aspartate residues in its active site at positions Asp150, Asp224 and Asp225 (Kohlstaedt et al., 1992;Boyer et

al., 2001a). Furthermore, the dNTP binding site in HIV-1 RT requires residues K65 and R72 whereas in MoMLV RT, the corresponding residues are K103 and R110.

As mentioned previously, the HIV-1 RT is a heterodimer composed of 560 amino acids and contains a fingers, palm, thumb, connection and RNase H subdomains. Structurally, the MoMLV RT is a monomer of 671 amino acids and consists of the same subdomains as HIV-RT, namely fingers, palm, thumb, connection and RNase H (Roth et al., 1985;Tanese and Goff, 1988). Within the fingers and palm subdomains, there is a 25% sequence identity between HIV-1 RT and MoMLV RT and the connection domain has the least sequence identity with only 6% homology (Cote and Roth, 2008).

The fidelity of RT can be defined as the accuracy at which the template strand is copied. Enzyme processivity can be defined as the number of nucleotides incorporated by RT before the RT dissociates from the template. The fidelity and processivity of HIV-1 RT, HIV-2 RT and MoMLV RT have been compared and it was found that the MoMLV RT had a higher fidelity, than both HIV-1 RT and HIV-2 RT, which both had a similar fidelity (Bakhanashvili and Hizi, 1992b;Bakhanashvili and Hizi, 1992a;Bakhanashvili and Hizi, 1993). The processivity of HIV-2 RT was shown to be less than that of HIV-1 RT on a single stranded M13 DNA template (Boyer et al., 2006).

The *Escherichia coli* Pol I Klenow fragment was also found to resemble a hand and this likeness was extended to include the HIV-1 RT. However there does not appear to be much sequence similarity but it can be said that these polymerases are related to

each other to some extent in terms of structure. This conservation is particularly seen in the subdomain that contains the catalytic site (Ollis et al., 1985).

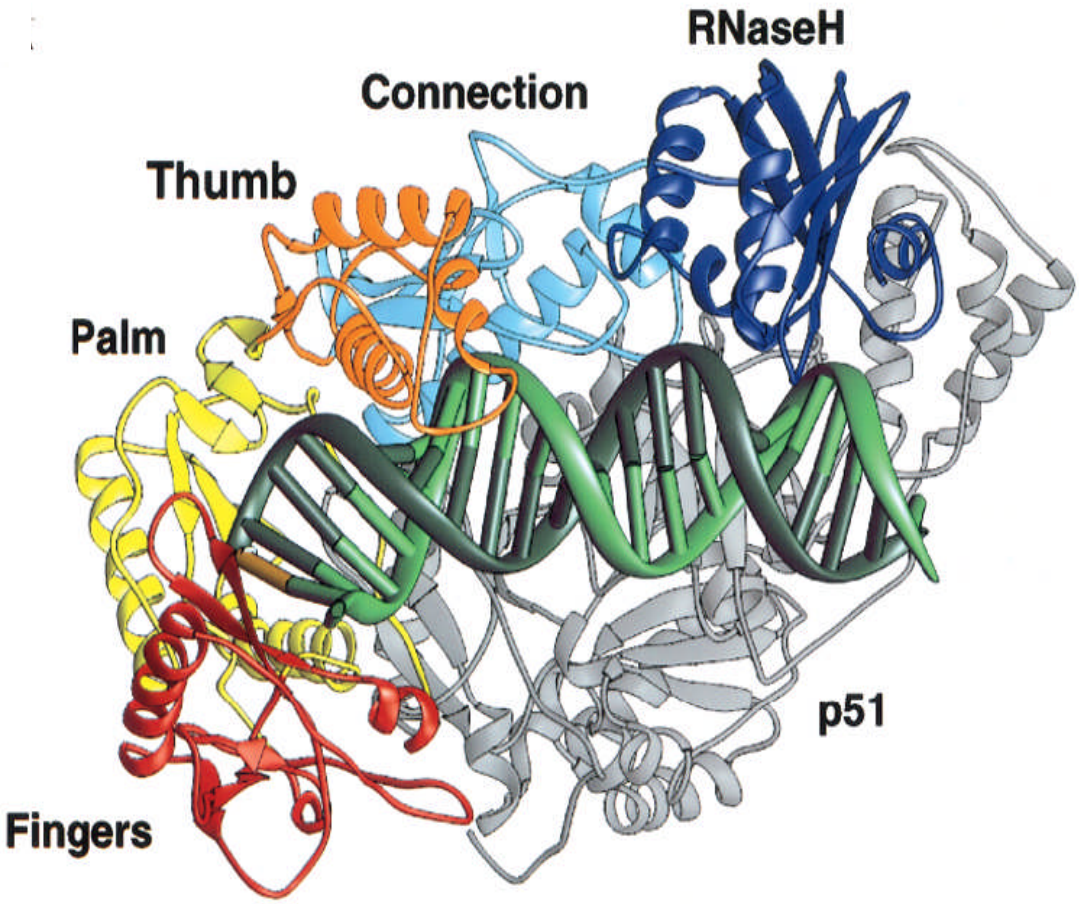


Figure 1.6 The structure of HIV-1 RT. The two domains p66 (in colour) and p51 (in grey) are shown. Within the p66 domain, the subdomains are shown as fingers (red), palm (yellow), thumb (orange), connection (cyan) and RNase H (blue). The DNA template (light green) and primer (dark green) strands are also shown. Figure taken from (Kohlstaedt et al., 1992).

1.9 Drug resistance

1.9.1 Current drug targets

The principal goals of antiretroviral therapy are to reduce morbidity, prolong survival, improve quality of life, restore and preserve immunological function and prevent transmission. Suppression of HIV-1 RNA in plasma (the "viral load") is used as a surrogate marker for these desired outcomes. Antiretroviral therapy, typically combinations of three antiretroviral agents, can suppress the viral load to below the detection limits of routine viral load assays (typically 50 copies per millilitre) and this is considered to be the measure of successful therapy (Hammer et al., 2008).

Following the introduction of highly active antiretroviral therapy (HAART), it was thought that a 'hit early hit hard' approach could reduce morbidity and mortality, prevent immune deterioration, and even eradicate the disease (Ho, 1995). The rationale behind early treatment was that it would prevent damage to the immune system and by controlling virus replication and lead to HIV eradication within estimated three years of treatment (Ho, 1995). However, this approach was mitigated by the following considerations. Firstly, HIV-1 infection cannot be eradicated due to its persistence in latently infected T cells; the viral load rebounds rapidly after stopping suppressive antiretroviral therapy, and treatment must therefore be regarded as life-long. This raises concerns about the patient's ability to maintain adherence long-term, the risk of treatment failure with accumulating resistance and exhaustion of treatment options, and long-term toxicity of antiretroviral drugs. However antiretroviral therapy has evolved over time and is currently safer and

simpler than in the past. A large number of options are available that allow a tailored treatment, improving both adherence and tolerability. Emerging clinical data have clearly shown the benefit of starting therapy earlier than it was recommended until recently, in terms of improved morbidity and mortality. The beneficial effect of treatment is seen not only in terms of reduced incidence of opportunistic conditions, but also incidence of numerous co-morbidities that are triggered by HIV-associated immune activation and inflammation (Baker et al., 2010). Thus, treatment guidelines have been recently revised to recommend earlier initiation of therapy, before the CD4 cell counts declines below 500 cells per microliter in developed countries (Thompson et al., 2010) and 350 cells per microliter in resource-limited settings (WHO 2009, 2010). The benefit of treating patients with a CD4 cell count above 500 cells per microlitre is unclear (Volberding and Deeks, 2010;Gazzard et al., 2008). In all guidelines however, emphasis is placed on the fact that treatment should be considered in patients with CD4 cell counts above the recommended threshold for therapy initiation in the presence of specific circumstances. These include for example hepatitis B or hepatitis C infection, high cardiovascular disease risk, renal disease, age >50 years, high viral load, or a rapid CD4 cell decline.

Currently antiretroviral therapy targets viral proteins at six main stages in the HIV-1 life cycle. This includes RT, protease and integrase, which inhibit reverse transcription, proteolytic cleavage during maturation, and integration of proviral DNA into the host genome respectively. The viral protein gp41 and the host cell proteins CCR5, which are involved in virus entry, are also targeted.

Novel therapeutic approaches to inhibit HIV-1 replication include targeting lipid rafts, uncoating, RNase H activity, matrix, capsid, nucleocapsid, p6, accessory proteins such as Vif, Vpu and Nef and also the host protein LEDGF/p75 which is involved in the nuclear import of the PIC. The plasma membrane has regions of microdomains that rich in cholesterol and lipids. Studies have shown that HIV-1 uses lipid rafts as platforms for viral particle assembly and release (Ono et al., 2007;Waheed and Freed, 2009). Using compounds such as amphotericin B methyl ester (AME) that bind to the virion lipid bilayer, viral particle production and infectivity have been shown to be inhibited (Waheed et al., 2008;Waheed et al., 2006). The process of uncoating has also been investigated. The TRIM5α protein targets partially uncoated incoming capsid resulting in either premature disassembly or by recruiting the virion for proteasomal degradation. Therefore TRIM5α like molecules that could restrict HIV-1 could be developed (Adamson and Freed, 2010;Wilkin et al., 2010).

The introduction of antiretrovirals and in particular HAART has vastly improved the outcome of HIV infected patients. However, there is a significant risk of emergence of resistance to these antiretrovirals and there are many factors that contribute to the surfacing of resistance. Firstly, the RT is highly error prone and lacks a proofreading ability, introducing an average of one to two mutations for each viral genome transcribed (Bebenek et al., 1989;Ji and Loeb, 1992). Furthermore HIV-1 has a very high replication rate, reaching up to 10^9-10^{12} virus particles produced daily in untreated patients (Perelson et al., 1996). Therefore HIV infected patients harbour numerous virus variants known as the quasispecies that can vary in their sensitivity to antiretrovirals. Within the quasispecies, certain strains dominate and this

represents the dynamic equilibrium between escape for selective pressure (e.g. immune responses, drug therapy) and preserved ability to replicate and infect.

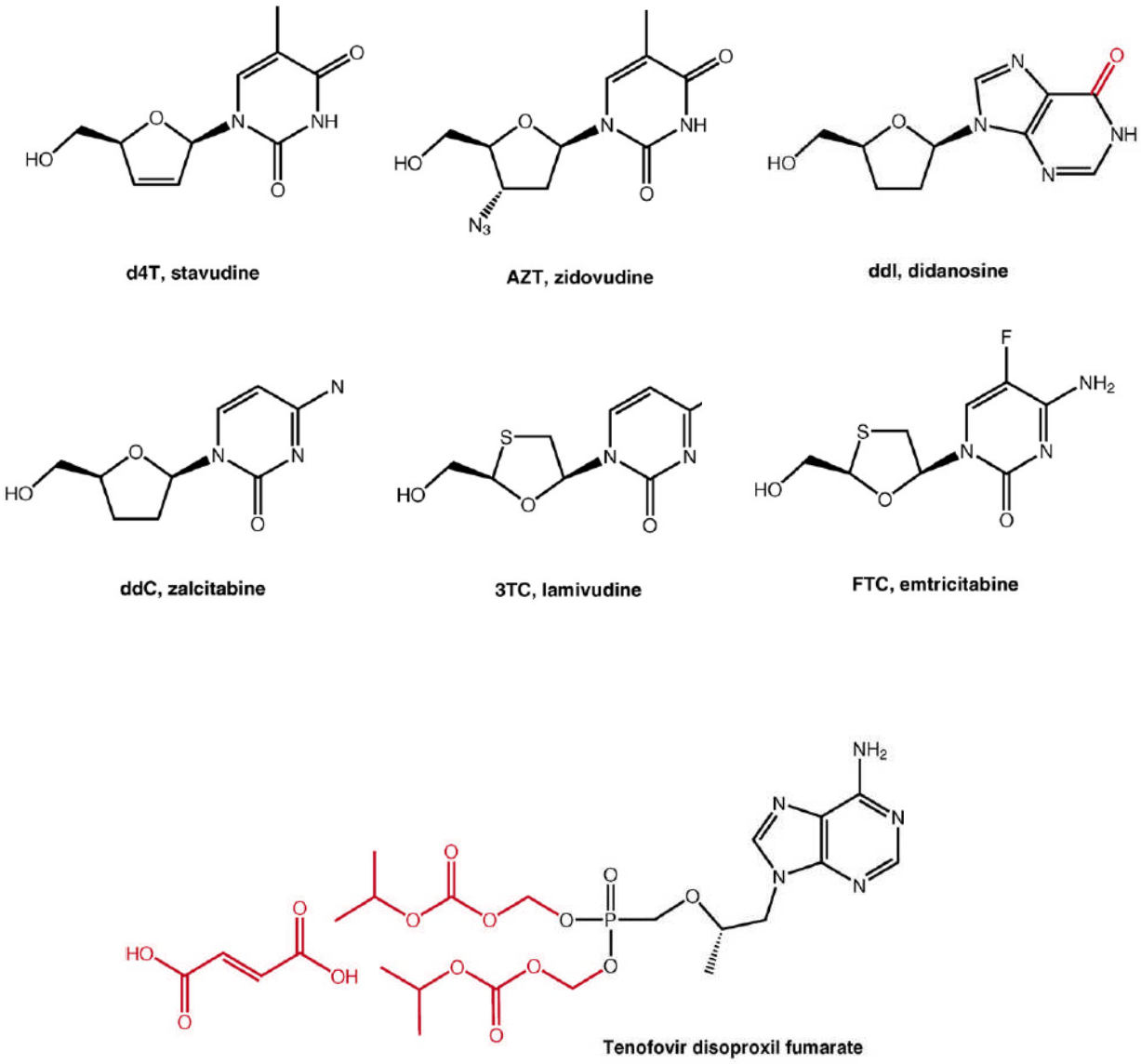


Figure 1.7 Structure of nucleoside and nucleotide RT inhibitors (NRTIs). Nucleosides include zidovudine (AZT), stavudine (D4T), zalcitabine (DDC), didanosine (DDI), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC) and one nucleotide tenofovir (TDF).

1.10 **RT Inhibitors**

Inhibitors of RT can be divided into two groups, nucleoside and nucleotide RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs).

1.10.1 NRTIs

The first NRTI to be developed was zidovudine (AZT). It was originally developed in 1964 to treat cancer but was found to be ineffective in killing tumour cells. A collaboration with the National Cancer Institute and the Burroughs Wellcome Company led to finding that AZT could inhibit HIV-1 replication (Mitsuya et al., 1985). NRTIs are competitive inhibitors that compete with the natural dNTP substrate for incorporation into the growing DNA chain. NRTIs therefore share a similar structure to dNTP's in that they both have a nitrogenous base and are attached to a ribose sugar. The discriminating feature is that the 3' hydroxyl group of dNTPs is absent which prevents further bonding and thus results in DNA chain termination (El et al., 2007;Zdanowicz, 2006). There are currently 8 NRTIs, comprising seven nucleosides, zidovudine (AZT), stavudine (D4T), zalcitabine (DDC), didanosine (DDI), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC) and one nucleotide tenofovir (TDF) as shown in figure 1.7. Once NRTIs enter cells they need to be phosphorylated by cellular kinases to become active. The nucleoside analogues are administered in an unphosphorylated form and require triphosphorylation. TDF is administered as a monophosphate and requires biphosphorylation. The first phosphorylation event is the rate-limiting step for all nucleosides except for AZT where the second phosphorylation event is the rate-limiting step (Balzarini et al., 1989;Furman et al., 1986).

1.10.2 Mechanisms of resistance to RT inhibitors

There are at least two mechanisms of resistance to the NRTIs. The first mechanism is mediated by mutations that interfere with binding and/or incorporation of the NRTI figure 1.8. Examples include M184V, Q151M, L74V, K70E, and K65R. The second mechanism is known as NRTI excision figure 1.9. Examples include the thymidine analogue mutations (TAMs).

1.10.2.1 Binding and incorporation

The M184V mutation is selected by treatment with 3TC and FTC. This mutation is situated close to the active site of the enzyme in the palm subdomain, which is highly conserved among retroviral RTs. The change of amino acid from methionine to valine causes steric hindrance between the oxathiolane ring of the 3TC and the side chain of valine. Mutant RT containing M184V decreases the catalytic efficiency of incorporation of the 3TC triphosphate however whether this is attributed to the K_D (dissociation constant) or k_{pol} (polymerisation rate) is not known, but both are thought to be important (Huang et al., 1998;Sarafianos et al., 1999a;Krebs et al., 1997;Deval et al., 2004b).

The Q151M mutation is located within the palm subdomain of RT and interacts with the nitrogen base of the dNTP resulting in the altered recognition and a reduced incorporation of the NRTI. It is selected by multiple NRTIs, typically by combinations such as AZT and DDI, and confers resistance to all available NRTIs, albeit less so to 3TC and TDF (Sluis-Cremer et al., 2000a).

The L74V mutation is located within the fingers sub domain and is selected by DDI or ABC. The DDI resistance effects of L74V are related to the incorporation rates of the inhibitor whereas ABC resistance is related to differences in the discrimination of the inhibitor and natural substrate (Deval et al., 2004a;Winters et al., 1997)

The K70 residue is located in the β3-β4 hairpin loop of RT and is associated primarily with TDF resistance. Nucleotide discrimination appears to be the major mechanism of resistance conferred by K70E, in particular a decreased incorporation rate of the inhibitor (Sluis-Cremer et al., 2007).

The K65 residue interacts with the γ-phosphate of the incoming dNTP. The K65R mutation is selected by TDF, ABC and DDI and is located in the fingers subdomain. The K65R mutation mediates nucleotide discrimination by decreasing the k_{pol} for the incorporation of the inhibitor (Huang et al., 1998;Selmi et al., 2001).

Several of these mutations, typically M184V, but also K65R and L74V reduce viral replication capacity or fitness (Vivet-Boudou et al., 2006).

1.10.2.2 NRTI excision

The second mechanism of resistance is NRTI excision or primer unblocking. This mechanism involves the selected removal of the incorporated NRTI from the end of the growing DNA chain and is demonstrated by a group of mutations known as the thymidine analogue mutations (TAMs), which are selected by the thymidine analogues AZT and D4T. The evolution of TAMs can be divided into two pathways; the TAM-1 pathway consists of M41L, L210W, T215Y and sometimes D67N. The

TAM-2 pathway consists of D67N, K70R, T215F and K219Q/E. The first pathway is associated with greater levels of AZT resistance and significant NRTI cross-resistance than the second pathway (Marcelin et al., 2005;Miller, 2004;Hu et al., 2006). The TAMs emerge in sequential order and their accumulation over time is associated with increasing levels of resistance to AZT and D4T, as well as ABC, DDI and TDF. The first TAM to appear is usually K70R followed by T215Y/F which arises with M41L and L210W and D67N, K219Q/E can appear after either K70R or T215Y/F (Garcia-Lerma, 2005).

The NRTI excision mechanism mediated by the TAMs occurs through a pyrophosphorolysis reaction using cellular pyrophosphate or ATP as an acceptor. The T215Y or T215F mutations are selected because the aromatic ring of the side chain stacks with the adenine ring of ATP enhancing the ability of RT to bind to ATP (Boyer et al., 2001b;Dharmasena et al., 2007).

The efficiency of the excision reaction is influenced by many factors including whether the template strand is DNA or RNA, the specific NRTI blocking synthesis and the presence of other resistance mutations. NRTI excision is less efficient when the template strand is RNA rather than DNA (Nikolenko et al., 2005). The thymidine analogues AZT and D4T are very efficiently removed whereas the cytidine analogues 3TC and DDC are not very efficiently removed (Boyer et al., 2001b;Meyer et al., 2000;Meyer et al., 1999). Other NRTI resistance mutations such as K65R, L74V, M184V and the NNRTI resistance mutation Y181C are able to enhance susceptibility to AZT and frequently also D4T in the presence of TAMs.

These mutations have been shown inhibit excision of AZT (Gotte et al., 2000;Selmi et al., 2003;White et al., 2006;Miranda et al., 2005).

A growing body of evidence has emerged implicating a role in drug resistance for mutations in the connection and RNase H domains. These mutations have been demonstrated to augment AZT resistance by altering the balance between NRTI excision and RNase H activity. The mutations specifically reduce the RNase H activity of RT, allowing more time for the RT to excise AZT from the terminated primer template. Connection domain mutations such as E312Q, G335C/D, N348I, A360I/V, A371V, V365I and A376S have been shown to increase resistance to AZT (Nikolenko et al., 2007;viks-Frankenberry et al., 2007;viks-Frankenberry et al., 2008;Yap et al., 2007). Mutations in the RNase H domain have also been shown to increase AZT resistance including H539N, D549N and Q509L. These mutations are also associated with TAMs (Brehm et al., 2007;Brehm et al., 2008;Nikolenko et al., 2005).

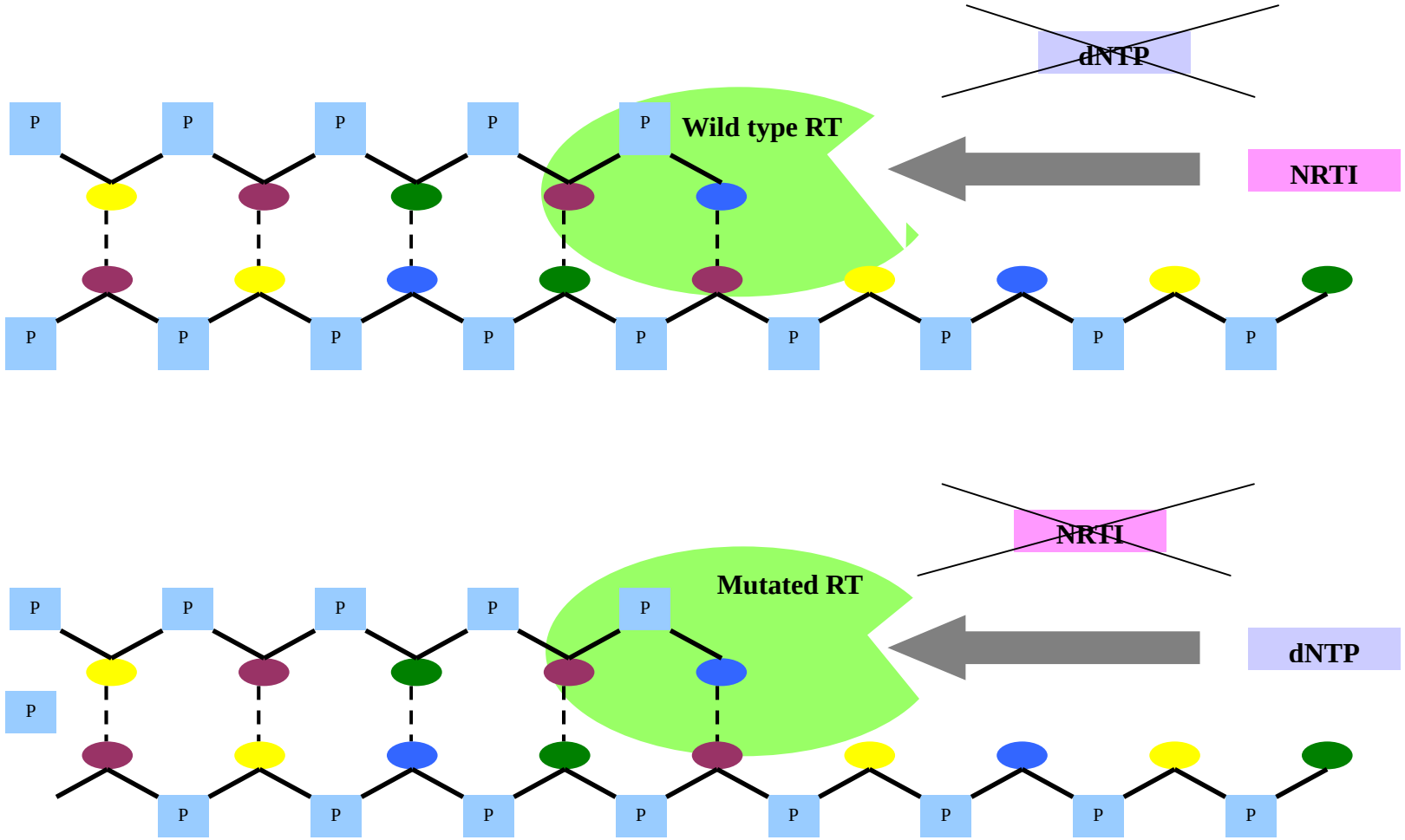


Figure 1.8 Mechanisms of NRTI resistance. Binding and incorporation. A drug sensitive virus has its replication blocked when the NRTI binds. A virus containing the mutations M184V, L74V, K70E or K65R interfere the binding and incorporation of the NRTI.

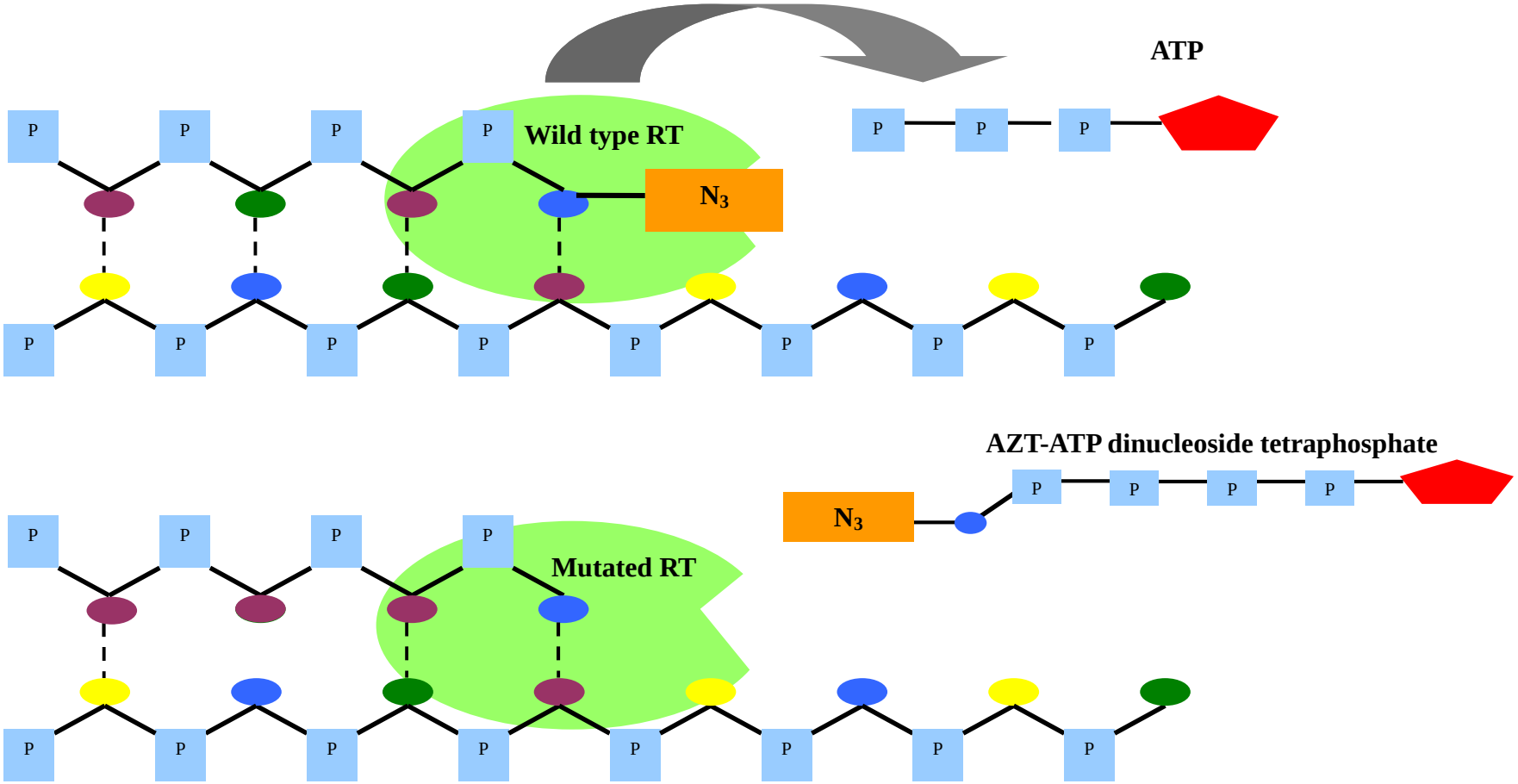


Figure 1.9 Mechanisms of NRTI resistance. NRTI excision mediated by the TAMs. A drug sensitive has its replication blocked despite the presence of ATP. The virus containing TAMs excises the bound NRTI using ATP as a pyrophosphate donor, allowing chain synthesis to continue.

1.10.3 NNRTIs

Currently there are four approved NNRTIs for treating HIV-1 infection. Nevirapine (NVP), efavirenz (EFV) and delavirdine (DLV) are first generation NNRTIs with largely overlapping resistance pathways. Second generation NNRTIs can retain activity against certain virus strains resistant to first generation NNRTIs (e.g., K103N) and include etravirine (ETV), which is already licensed, and rilpivirine which is in advanced stages of development (figure 1.10). NNRTIs are non-competitive inhibitors of RT. They bind to a hydrophobic pocket in the p66 subunit that is close but distinct from the enzyme active site and NRTI binding site. The NNRTI binding pocket can only form once the NNRTI is bound. Once the NNRTI is bound, conformational changes occur in the RT that impact the catalytic ability of the enzyme (Sluis-Cremer et al., 2004). All NNRTIs bind at the same site in RT and this binding site is located in the palm subdomain of p66 approximately 10Å from the catalytic active site. The hydrophobic pocket is lined with aromatic, hydrophobic and hydrophilic residues. Binding of the NNRTI induces a conformational change that rotates the side chains of residues Y181 and Y188 (Hsiou et al., 1996). NNRTIs are highly specific inhibitors of HIV-1 and have no activity against HIV-2 (Tucker et al., 1996).

Single amino acid substitutions are usually sufficient to confer high-level resistance to the NNRTIs. These mutations are often clustered around the NNRTI binding pocket, in particular, mutations at positions K103, Y181, Y188 and G190 cause significant effects on resistance. These mutations effect the stacking interactions between their aromatic side chains and the pyridine group of the NNRTI (Ren et al., 2001). The K103N mutation is located at the rim of the NNRTI binding pocket and

its side chain protrudes out. A hydrogen bond between the Y188 hydroxyl group and the amide group of K103N is formed which creates an energy barrier reducing drug potency (Ren et al., 2000). Mutations at positions L100, K101, V106, V108 and E138 have also been shown to contribute to NNRTI resistance. Second generation NNRTIs such as ETV have been developed to be effective against viruses containing K103N, but are vulnerable to the effect of mutations at positions E138 (Tambuyzer et al., 2010) and Y181 (Andries et al., 2004).

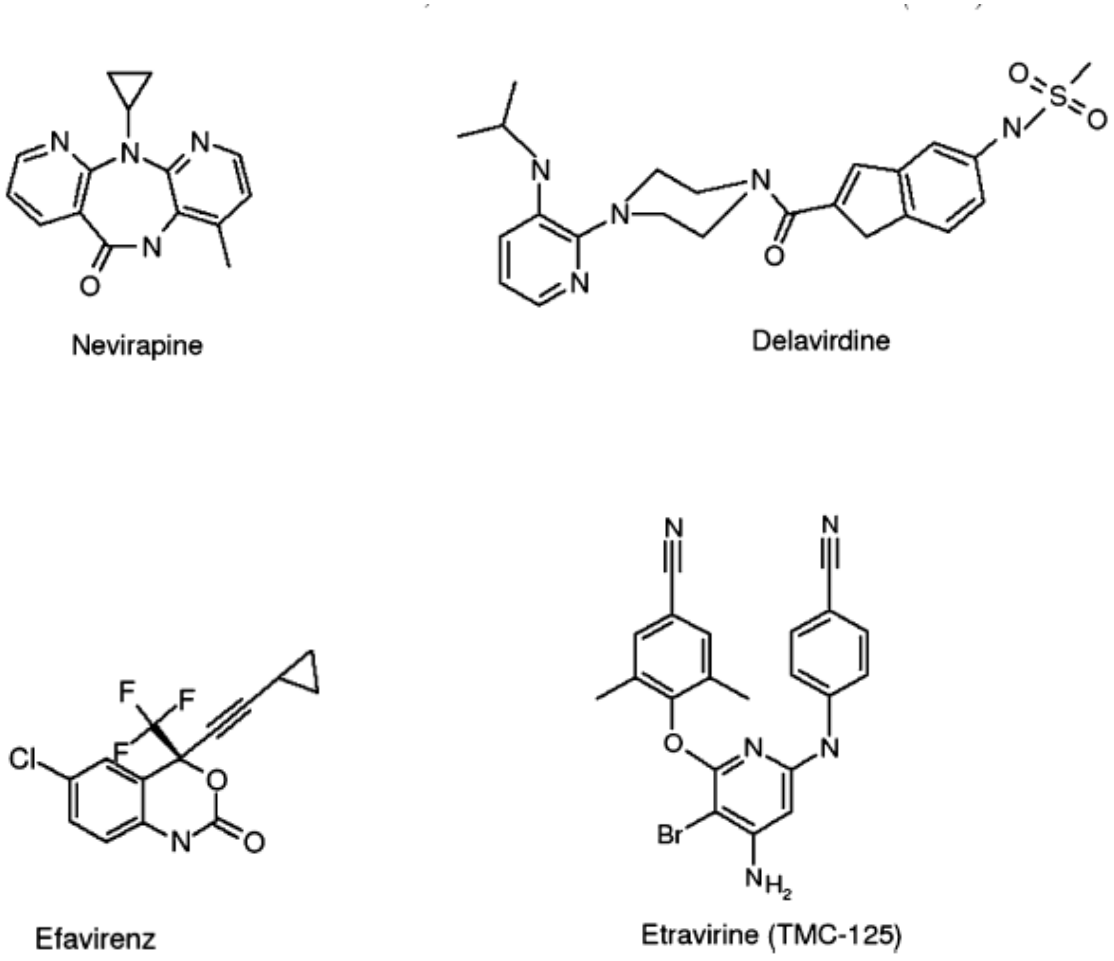


Figure 1.10 Structure of the non-nucleoside RT inhibitors (NNRTIs). Nevirapine (NVP), efavirenz (EFV), delavirdine (DLV) and etravirine (ETV).

1.11 **Protease Inhibitors**

HIV-1 protease is involved in the maturation of the HIV-1 particle. It cleaves the Pr55gag and Pr160gag-pol polyproteins to release structural and functional proteins, including matrix, capsid, nucleocapsid, RT, protease and integrase. Protease recognises the asymmetric shape of the peptide substrate rather than specific amino acid sequences and consequently the amino acid sequence of all cleavage sites differ (Prabu-Jeyabalan et al., 2002). Protease belongs to the family of aspartic proteases and has an aspartic acid at position 25 in the active site. It is composed of two identical subunits of 99 amino acids per monomer. Three main domains of protease are often described, the active site cavity, the dimerisation domain and the flaps (Navia et al., 1989).

Protease inhibitors (PIs) are competitive inhibitors and have been designed to bind to the viral protease with high affinity. Once bound, they result in the production of immature HIV particles that lack infectious abilities (Craig et al., 1991;Vacca, 1994). Currently there are nine PIs in clinically use, saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), lopinavir (LPV) (co-formulated with ritonavir), fosamprenavir (FPV) (as a prodrug of amprenavir), atazanavir (ATV), tipranavir (TPV) and darunavir (DRV) (Mastrolorenzo et al., 2007). PIs are metabolised via the cytochrome P450 enzyme system (CYP450) in the liver and gastrointestinal tract. These pathways both act in different ways to restrict drug exposure. Processing in the gastrointestinal tract reduces the proportion of the PI in the blood stream while processing in the liver clears the PI already present in the blood stream. Hence the half-life of the PI is reduced. To improve the bioavailability of PIs, a cytochrome

P450 enzyme inhibitor known as ritonavir is used to 'boost' the plasma levels and therefore the action of the PIs (Barry et al., 1997). PI can be divided into three groups, first generation inhibitors (IDV, SQV, NFV and RTV), second generation inhibitors (FPV, LPV, and ATV) and third generation inhibitors (TPV and DRV). The first generation inhibitors were poorly adaptable to mutations in protease and consequently the presence of a one or a few mutations caused resistance (Velazquez-Campoy et al., 2002). In contrast the newer PIs have a higher affinity for the enzyme active site and can tolerate the presence of a few resistance mutations without loss of activity (Ohtaka et al., 2004).

The development of PI resistance is a stepwise process whereby mutations in the substrate binding cleft are usually first to appear and this includes mutations at positions D30, V32, M46, I47, V48, I50, I54, T74, L76, I84, V82 and N88. These mutations cause an increase in the size of the catalytic site and as a result, there is decreased binding of the PI. These mutations are known as primary or major mutations. Secondary or minor mutations emerge later and although in isolation they do not have a significant effect on resistance, in combination with primary or major resistance mutations, these mutations contribute to an increased resistance and fitness (Nijhuis et al., 1999;Mammano et al., 2000).

1.12 **Integrase inhibitors**

Integrase is required for the integration of the viral genome into the host chromosome so that viral proteins can be produced. The process of integration can be divided in to two stages, 3' processing and strand transfer (Pommier et al., 2005). Raltegravir is the first integrase inhibitor to be licensed for use in the treatment of HIV infected patients. It is an inhibitor of the strand transfer step of integration. It binds tightly to integrase and chelates the metal ions in the catalytic triad resulting in sequestration of the crucial metal ion cofactors required for integrase to function (Grobler et al., 2002). When integrase is inhibited, host enzymes circularise the viral DNA and 2-long terminal repeats accumulate within the nucleus (Svarovskaia et al., 2004).

Resistance to integrase inhibitors has been described as occurring in residues surrounding the catalytic core and can be divided into three main pathways. The first pathway consists of the N155H mutation, the second pathway consists of the Q148K/R/H mutation and the third pathway consists of the Y143C/R mutation. These mutations are primary resistance mutations and are associated with secondary mutations that restore viral fitness (Delelis et al., 2009;Marinello et al., 2008;Kobayashi et al., 2008).

1.13 **Fusion inhibitors**

HIV-1 entry into the target cells is initiated through binding of the viral gp120 envelope glycoprotein to the CD4 host cell surface receptor. Following binding, gp120 undergoes a conformational change enabling its interaction with a chemokine co-receptor. The gp41 subunit is responsible for fusion of the viral and cell membranes. It contains two heptad repeats, HP1 and HP2 and three monomers of gp41 lie parallel to one another to make a six helical bundle. The formation of the six helical bundle brings the two membranes closer together so that the fusion peptide region capable of piercing the cell membrane is brought forward to form a pore (Chan et al., 1997).

Enfuvirtide is the only fusion inhibitor that is currently in clinical use. Enfuvirtide binds to the HR1 region gp41 and blocks the formation of the six helical bundle and consequently fusion is blocked. Resistance to enfuvirtide is associated with changes in a conserved triad of amino acids at positions 36-45 in HR1 of gp41 (Wei et al., 2002;Mink et al., 2005).

1.14 **Entry inhibitors**

CCR5 and CXCR4 are the major chemokine co-receptors that HIV-1 uses for entry and their distribution on CD4 positive cells determines viral tropism. Entry inhibitors targeting the chemokine co-receptor represent the first example of an antiretroviral drug that targets a host cell protein rather than a viral protein. Maraviroc was the first and to date the sole co-receptor antagonist approved for use in the treatment of HIV (Macarthur and Novak, 2008). It is a non-competitive inhibitor of the CCR5 co receptor. Maraviroc binds within a cavity in the membrane spanning regions of the CCR5 helices, resulting in conformational changes within the CCR5 receptor that prevents interaction with the V3 crown of gp120 (Watson et al., 2005;Dragic et al., 2000).

Given the high variability in the envelope gene of HIV, it is not surprising that the development of resistance to CCR5 is complex. Two mechanisms of resistance have been suggested. The first mechanism involves the more efficient use of inhibitor free CCR5. The second mechanism involves emergence of changes in the V3 loop that allow the virus to bind the CCR5 co-receptor despite the presence of the antagonist. These resistant variants retain the use of the natural CCR5 receptor as well as binding to the altered inhibitor bound CCR5 (Westby et al., 2007;Pugach et al., 2007). The use of CCR5 antagonists has raised concerns about whether there will be an evolution towards CXCR4-using viruses. Emergence of CXCR4-using viruses has been observed in patients experiencing virological failure during CCR5 antagonist therapy as a result of the selection of pre-existing variants. This however has not been associated with a negative impact on CD4 cell counts. In addition, the selected

CXCR4-using variants are rapidly outgrown by R5 strains once the drug selective pressure is discontinued, indicating a lack of intrinsic fitness advantage of CXCR4-using strains under conditions of relatively preserved immunity and cell target availability (Westby and van der, 2010).

1.15 **Phenotypic assays**

The development of drug resistance is a contributory factor that leads to treatment failure (Alcorn and Faruki, 2000). To address this problem, resistance testing has been used to help design new regimens for patients who harbour drug resistant viruses (Hirsch et al., 2000). There are two types of resistance assays, genotypic and phenotypic assays.

Genotypic assays involve amplification and sequencing of genes such as protease, RT or integrase. Regions are reverse transcribed *in vitro* and amplified by either a single or nested PCR reaction. Automated DNA sequencing is used to obtain the sequence, which is compared to a wild type reference sequence such as HXB2. Mutations are designated as those differing from the wild type reference sequence. Drug resistance mutations are classified according to interpretation systems such as the Stanford HIV Drug Resistance Interpretation Algorithm, French National Agency for AIDS Research, International AIDS Society-USA and Rega Institute (Grant and Zolopa, 2009).

For reliable amplification from plasma, most resistance assays require a viral load between 500 to 1000 copies per millilitre, although amplification at viral loads less than 500 copies per millilitre is possible. However, whether this would be an accurate representation of the viral population within the patient is debatable (Alcorn and Faruki, 2000). Nonetheless use of resistance testing at viral load of just a few hundred copies has been shown to provide clinically useful information (Mackie et al., 2010). It should also be noted that the population sequencing only detects viral populations that represent more than 20% of the population (Schmidt et al., 2002a).

In the absence of drug pressure, wild type virus tends to rapidly outgrow drug resistant variants, which may thus become a minor species in the viral population. Drug resistant variants may also be archived within proviral DNA in latently infected cells. Once treatment is resumed, reselection of the resistant variants can occur rapidly. Drug resistance should therefore be regarded as long lived. Although a resistant mutant may be undetectable by routine resistance testing, it may be detectable by more sensitive research methods. At least in the case of NNRTI resistant mutants, these low frequency variants have been shown to impact significantly on therapeutic responses. This concept has been well illustrated in studies of single dose nevirapine to prevent mother to child transmission, patients experiencing virological failure and patients with transmitted drug resistance (Lecossier et al., 2005;Flys et al., 2005). Research tools that can achieve increased sensitivity of detection include allele specific PCR, ultra deep sequencing and single genome sequencing (SGS) (Johnson et al., 2008;Metzner et al., 2009;Paredes et al., 2009;Johnson and Geretti, 2010;Geretti et al., 2009;Palmer et al., 2005). While with some of these assays it is possibly to obtain a sensitivity well below 1%, cut-offs are usually applied to aid interpretation and circumvent the issue of detection of the natural background of resistant variants in the quasispecies and technical errors intrinsic to the methodologies.

Phenotypic assays are another method of analysing the effect that resistance mutations have on drug susceptibility. In their most common format, they involve inserting a particular gene of interest, e.g. RT or protease into a defective vector to produce a replicating recombinant virus. The virus and a wild type control are then passaged into cells in varying concentrations of antiretrovirals. The drug

concentration required to inhibit virus replication by 50% (IC50) or 90% (IC90) relative to a control virus is calculated. The fold difference (fold change) between the wild type and mutant virus are used as a measure of drug susceptibility (Sebastian and Faruki, 2004).

Previously, phenotypic assays were performed on peripheral blood mononuclear cells (PBMCs) in the presence of increasing concentrations of drug. The patient virus and PBMCs were isolated and cultivated for 2-8 weeks. The readout for this assay was typically p24 antigen production in the supernatant. Although this method has the advantage of using cells and virus from the patient, there are major disadvantages. These include the fact that the assay is time consuming, that given that the virus is cultured for more than two weeks drug resistance mutations can be selected during passage, and that reproducibility can be a problem due to natural biological variations (Schmidt et al., 2002b;Japour et al., 1993). Therefore, a simpler phenotypic method was developed based on the recombinant virus technique. The assay involves amplifying the gene of interest and co transfecting with a defective molecular clone to produce a recombinant virus. The readout for this assay was initially a tetrazolium colorimetric assay that measured cell death (Kellam and Larder, 1994).

The recombinant virus assay was subsequently modified into two different types of assays, a single cycle assay and a multiple cycle assay. The single cycle assay allows a single cycle of replication to occur whereas the multiple cycle allows multiple cycles of replication. The single cycle has the advantage that it is a fairly rapid assay compared to the multiple cycle assay, and results of replicate experiments generally

show smaller variation. A commercial assay utilising the single cycle approach for measuring drug susceptibilities is provided by Monogram Biosciences in the United States. The multiple cycle assay has the advantage of the multiple cycle method is the fact that the virus has multiple rounds of replication and therefore mimics more closely the conditions that the virus experiences *in vivo*. However, the assay is more complex and time consuming than the single cycle assay and retains the potential that the virus may mutate during passage. Virco in Belgium provides a commercial assay utilising the multiple cycle approach for measuring drug susceptibilities (Youree and D'Aquila, 2002;Demeter and Haubrich, 2001).

To compare drug susceptibilities, the fold change is used. To aid interpretation, cut offs are frequently used in routine diagnostics. The technical cut off is generated by measuring the variation seen with repeat testing of the same samples. The biological cut off represents the phenotypic variability observed within treatment naïve patients. The clinical cut off represents the value that discriminates between treatment responders and non responders among treatment experience patients. It should be noted that phenotypic assays do not take into account drug-drug interactions which may influence resistance (Hsu et al., 1998) and do not take into account the effect of drug combinations as they assay each drug independently.

Despite the limitations of both genotypic and phenotypic assays, both methods have been used to correlate virological failure with resistance (Deeks et al., 1999;Lorenzi et al., 1999). It is accepted that resistance testing improves the virological outcome of patients who have the benefit of its use and therefore, resistance testing is an integral component of the management of HIV infected patients.

1.16 **Virus Fitness**

Virus fitness can be defined as the ability of a virus to replicate and produce progeny (Domingo et al., 1997). There are two stages to describe the evolution of viral fitness whilst on therapy. The first stage is characterised by viruses that not only have reduced drug susceptibility but an impaired replicative capacity relative to the wild type. During the second stage, additional mutations arise that alone do not confer drug resistance but in combination with the primary mutations enhance the replicative capacity of the virus. These mutations are known as accessory mutations (Nijhuis et al., 1999). It can be said that the natural evolution of HIV under drug pressure is towards increasing levels of resistance, cross resistance and fitness.

There are a number of ways to measure virus fitness and these methods can be divided into either in vivo or ex vivo methods. In vivo methods include calculating fitness by comparing the amount of wild type and mutant viruses detectable in raw sequence electropherogram data (Devereux et al., 2001). This method has also been used to estimate the viral dynamics of CMV (Emery et al., 1999).

There are also many ex vivo methods that are employed to measure fitness, including monoinfection assays, enzymatic assays and growth competition assays. Monoinfection assays involve quantifying virus replication. This includes measuring specific viral proteins such as p24 by ELISA or RT activity. Enzymatic assays that measure enzyme activity such as protease processing or maturation or RT RNase H activity offer good insights into the effect drug resistance mutations can have on enzyme functioning (Martinez-Picado and Martinez, 2008). Another method to measure virus replication is to use a reporter gene like luciferase for instance in a

single cycle assay (Dykes and Demeter, 2007). Advantages of using a single cycle system to measure fitness include the fact that they are rapid and reproducible (Deeks et al., 2001b;Petropoulos et al., 2000b). One drawback to single cycle assays is that they do not measure significant differences in fitness between viruses that may differ by only a single nucleotide.

Growth competition assays are regarded as the gold standard for assessing virus fitness *in vitro*. They measure the relative fitness between two virus strains. The two strains are mixed together at specific ratios and passaged into cells. The outgrowth of one strain is a reflection of greater fitness. These assays are accurate but time consuming. They have the advantage that small changes in fitness can be detected and that they provide as close as possible a reproduction of conditions *in vivo*. A major challenge when setting up growth competition assays is developing the appropriate technique to differentiate between mutant and wild type strains. Previous studies have used sequencing, real time PCR or a recombinant marker assay (Quinones-Mateu and Arts, 2002).

Generally drug resistance mutations are associated with a lower fitness compared to wild type virus in the absence of drug. An interesting question is whether the drug resistant mutations can affect likelihood of transmission from person to person. In support of this hypothesis, studies have demonstrated that the 3TC mutation M184V, which has a significant impact on viral fitness, has a high prevalence among treated patients but is rarely seen in drug naive patients (Catucci et al., 1999). However the older studies relied on population sequencing as a way of detecting resistance. Recent studies using ultrasensitive methodologies have found a relatively high

frequency of M184V among treatment naive patients (Geretti et al., 2009;Johnson et al., 2008;Johnson and Geretti, 2010;Metzner et al., 2009;Paredes et al., 2007).

Given the fact that an unfit virus would replicate less efficiently, it is also reasonable to speculate that if an unfit virus was transmitted, a lower viral load set point could be established. A recent study found the drug-naive patients with the M184V mutation (detected by routine resistance testing) had indeed a lower viral load than patients without the mutation (Harrison et al., 2010). Since the viral load set point has been directly associated with disease progression, it is not unreasonable to speculate that viral fitness could also be associated with disease progression. One study examined the impact of fitness on $CD4^+$ T cell counts and viral load in acutely infected HIV-1 infected patients (Barbour et al., 2004). Using the single cycle fitness assay provided by ViroLogic, the fitness of the wild type viruses varied widely with only 11/191 (6%) attributed to the presence of drug resistance mutations. No correlation was seen between fitness and the viral load level recorded at baseline. Furthermore, a threshold value of 42% (compared to the reference control) appeared to be the best predictor of the baseline $CD4^+$ T cell count. Patients showing fitness over 42% having an average $CD4^+$ T cell count of 512 cells per microlitre compared to patients showing a fitness of less than 42% having an average $CD4^+$ T cell count of 663 cells per microlitre. It should be noted that the assay variability was high, with 95% confidence intervals of 12% to 93%. The same group studied viral fitness in acutely infected HIV-1 patients using the multiple cycle system by Monogram. They found no correlation between fitness and viral load or $CD4^+$ T cell counts at baseline (Barbour et al., 2006). It should be noted that these studies do not take into

consideration other determinants of fitness such as the contribution of Env and immunological escape.

In a study looking at viral fitness in long-term non progressors, a reduced fitness was seen in 2/4 (50%) of patients. This study used virus and PBMCs isolated from the patients and measured fitness by monitoring p24 antigen levels. These patients showed evidence of HIV specific cellular immune responses and high tires of neutralising antibodies, which may account for the attenuated severity of the disease (Cao et al., 1995). A similar study using long-term non progressors also showed that their viruses had a reduced fitness (Blaak et al., 1998).

The influence of drug resistance mutant with a reduced fitness may influence clinical outcome. However, larger studies that take into account confounding variables such as the immune response and host genetic factors need to be done to provide more information regarding the impact of virus fitness on clinical outcomes.

1.17 **Accessory mutations**

When therapy fails to suppress viral replication, resistance mutations emerge that generally reduce virus fitness relative to wild type virus. If replication continues to occur, new mutations emerge alongside major resistance mutations, which may either further augment resistance or restore virus fitness. The mutations have mainly been identified through statistical analyses of large databases, comparing the frequency of different mutations in untreated and treated persons and identifying clinical correlates. As a result the individual in vitro effects of the mutations are often unknown and the mutations are not generally included in the routine interpretation of genotypic resistance.

Currently, 61 novel mutations at 44 positions have been associated with NRTI resistance (Perno et al., 2006) (tables 1.1 and 1.2). These mutations can be classed into two groups. The first group includes mutations that that are present in treatment experienced patients and very rarely present in drug naïve patients, and are usually observed with major NRTI resistance mutations. Two conclusions can be inferred from these observations, firstly that they require drug pressure to emerge and secondly, that they emerge after prolonged exposure to NRTIs. Therefore these mutations could contribute to increasing resistance and/or fitness (Gonzales et al., 2003;Rhee et al., 2005). One example includes the K20R mutation, which was found in 1/47 (2%) treatment naïve patients and in 29/47 (62%) patients that were treatment experienced. K20R has been shown to confer a reduced susceptibility to 3TC (Saracino et al., 2006;Svicher et al., 2006). Another example includes the E40F and K43E mutations, which cause resistance and have effects on viral fitness.

Introduction of these mutations on a M41L + T215Y RT backbone resulted in a 135 fold increase in resistance to AZT compared to HXB2. Growth competition experiments showed that the virus containing M41L, T215Y, E40F and K43E was more fit than the M41L and T215Y virus (Huigen et al., 2008).

The second group includes mutations that are common polymorphisms in drug naïve patients but their prevalence increases further in treatment experienced patients. Unlike the mutations in the first group, these mutations may not contribute significantly to fitness but may have an effect on drug resistance (Ceccherini-Silberstein et al., 2005;Gonzales et al., 2003;Rhee et al., 2005). This group includes the G196E mutation, which was found in 88/551 (16%) of treatment naïve patients and 120/417 (29%) of treatment experienced patients and found to contribute to AZT and 3TC resistance (Stoeckli et al., 2002;Svicher et al., 2006). Another example is the K122E mutation, which was found in 121/551 (22%) of treatment naïve patients and 175/417 (42%) of treatment experienced patients and found to confer an increased resistance to AZT, D4T and TDF (Svicher et al., 2006).

These two groups of mutations have been shown to contribute to NRTI resistance in a positive way in that their presence increases resistance. However, there are mutations that have been associated with a negative impact on NRTI resistance. Characteristics of these mutations include that they are commonly observed in drug naïve patients and their prevalence decreases in treatment experienced patients, in particular those who have experienced virological failure. Furthermore, these mutations are rarely observed with NRTI resistance mutations and if they are present together, these mutations confer an increased susceptibility to the NRTIs. Examples

include V35I and R83K. The V35I mutation was found in 127/551 (23%) of treatment naïve patients and 58/417 (14%) treatment experienced patients. Similarly, the R83K mutation was found in 182/551 (33%) of treatment naïve patients and 83/417 (20%) of treatment experienced patients. R83K has also been associated with AZT, DDI and D4T resistance (Svicher et al., 2006;Ceccherini-Silberstein et al., 2005).

Novel mutations associated with the NNRTIs have also been identified, with 33 novel mutations at 22 positions associated with NNRTI exposure (table 1.3). The majority of these mutations are found within the hydrophobic binding pocket. The I132M has been shown to contribute to an increased susceptibility to AZT, 3TC and TDF but a decreased susceptibility to NVP, EFV and DLV (Nissley et al., 2007;Ambrose et al., 2009).

Table. 1.1 Accessory mutations associated with NRTIs exposure.

NRTI Associated Accessory Mutations[a]																			
K 20 R	V 35 M	T 39 A	E 40 F	K 43 EN Q	N 57 H	V 60 I	S 68 GR	D 113 EG	**Y 115 HN**	K 122 E	Q 145 LM	P 157 S	S 162 A	S 163 N	G 196 E	T 200 AE IK	I 202 V	E 203 K	Q 207 ED	H 208 Y	R 211 KG S

Table. 1.2 Accessory mutations associated with NRTIs exposure.

NRTI Associated Accessory Mutations[a]																		
F 214 L	D 218 E	**K 219 R**	K 223 Q	L 228 HM R	G 333 DE	G 335 C	N 348 I	R 356 K	G 359 S	A 360 I	T 369 I	A 371 V	T 377 L	E 399 D	L 469 T	Q 509 L	H 539 N	D 549 N	K 558 R

[a]Positions highlighted in bold indicate residues that can also show major mutations associated with NRTI resistance. NRTI accessory mutations were classified according to (Svicher et al., 2006;Perno et al., 2006;Cane et al., 2007).

Table. 1.3 Accessory mutations associated with NNRTIs exposure.

NNRTI Associated Accessory Mutations[a]																						
1 3 L	E 6 K	V 35 L	E 40 K	S 48 T	**A** **62** **V**	**L** **74** **V**	V 90 I	**K** **101** **Q**	**V** **106** **I**	I 134 A M	I 135 KL MR TV	**V** **179** **FI**	**Y** **181** **HS** **W**	G 196 R	H 221 Y	K 223 EQ	**F** **227** **C**	L 228 HR	L 283 I	N 348 I	T 369 I	T 386 A	E 399 D

[a]Positions highlighted in bold indicate residues that can also show major mutations associated with NNRTI resistance. NNRTI accessory mutations were classified according to (Svicher et al., 2006;Perno et al., 2006;Cane et al., 2007).

1.18 **H208Y**

It has been suggested that the H208Y mutation is an accessory mutation that arises in order to increase resistance and or viral fitness. H208Y is located in the fingers subdomain of RT.

The H208Y mutation was identified following passage of recombinant HIV-1 in the presence of foscarnet. Foscarnet is a pyrophosphate analog that demonstrates a broad activity against both RNA and DNA viruses including CMV, HSV-1 and HIV-1. It binds to the pyrophosphate binding site on RT and blocks HIV-1 replication (Crumpacker, 1992). After 13 passages the foscarnet resistant virus demonstrated an 8.5 fold foscarnet resistance and also an increased susceptibility to AZT. Sequencing the RT gene showed the emergence of G161L and H208Y, which were suggested to be responsible for foscarnet resistance (Tramontano et al., 1998).

Further work carried out on HIV patients who had been treated with various antiretrovirals including NRTIs, NNRTIs and PIs demonstrated a strong association of H208Y with the TAMs M41L and T215Y. M184V was not a prerequisite for the presence of H208Y. Site directed experiments showed that the triple mutant H208Y/R211K/L214F in the background of TAMs resulted in a 21-fold AZT resistance (Sturmer et al., 2003). These findings were supported by the study carried out by Svicher et al. 2006. This study also showed that H208Y in the presence of M41L/L210W/T215Y conferred resistance to all NRTIs including AZT and D4T.

Despite these findings indicating a role of H208Y conferring resistance to AZT, a contradictory study showed that H208Y when present in isolation decreased

susceptibility to AZT and NNRTIs. This study also showed using a single cycle replicative capacity assay, that the H208Y mutation had a similar replicative capacity to the wild type strain (Clark et al., 2006). Currently however, the H208Y mutation is not recognised as a major NRTI mutation.

The H208Y mutation has been suggested to play a role in NRTI resistance for many reasons. Firstly, the incidence of H208Y in treatment naïve patients is very low at 0.3% (n=2347) compared to treatment experienced patients where it is 4.1% (n=4005) (Nebbia et al., 2007). The low incidence in naïve patients compared to treatment experienced patients suggests that the H208Y requires drug selective pressure to arise. Furthermore, H208Y has been shown to appear alongside other known major resistance mutations such as M184V and TAMs suggesting that it may further increase the level of resistance associated with NRTI exposure, or possibly increase the replicative capacity of a heavily mutated NRTI resistant virus. This is consistent with the finding that the H208Y mutation is rarely observed in the absence of major NRTI mutations M184V and TAMs (Cane et al., 2007;Sturmer et al., 2003;Nebbia et al., 2007;Stoeckli et al., 2002).

In this thesis the H208Y mutation has been characterised to test the hypothesis that H208Y is an accessory mutation. In order to test this hypothesis, three main aspects were investigated. Firstly, the association of H208Y to major resistance mutations and other accessory mutations was examined. Also, the contribution of H208Y to conferring drug resistance and viral fitness was explored.

Chapter two

2 Materials and Methods

2.1 Materials

2.1.1 Bacteria

HB101: F–, *thi*-1, *hsd*S20 (r_B^-, m_B^-), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^r), *xyl*-5, *mtl*-1.

TOP10F': F´ {*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

XL1 blue supercompetent cells: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)].

2.1.2 Mammalian cell lines

MT4: human T cells isolated from patient with T-cell leukaemia (Harada et al., 1985).

2.1.3 Bacterial Media

LB: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride and deionised water to a final volume of 1 litre. Ampicillin and IPTG were added at a concentration of 50 ug/ml and 100 mM respectively. LB agar was made by adding 1.5% agar.

Terrific broth: 12 g tryptone, 24 g yeast extract, 4 ml glycerol, 100 mL of 0.17M KH2PO4 and 0.72M K2HPO4 and deionised water to a final volume of 1 litre.

NZY broth: 10 g of NZ amine (casein hydrolysate), 5 g of yeast extract, 5 g of sodium chloride, 12.5 ml of 1M magnesium chloride, 12.5 ml of 1M magnesium sulphate, 10 ml of 2M glucose and deionised water to a final volume of 1 litre. The pH was adjusted to 7.5 using sodium hydroxide.

S.O.C: 20 g tryptone, 5 g yeast extract, 0.5 g sodium chloride, 2.5 ml 1M potassium chloride, 20mls 1M glucose and deionised water to a final volume of 1 litre. The pH was adjusted to 7 with sodium hydroxide.

2.1.4 Cell culture

Cells were grown in RPMI (GIBCO, Invitrogen, Paisley, UK) supplemented with 100 U/ml of penicillin, 100 ug/ml of streptomycin and 10% fetal bovine serum (FBS) (BioSera, Ringmer, UK).

2.1.5 Antiretrovirals

NRTIs: zidovudine, abacavir, lamivudine, tenofovir and didanosine.

NNRTIs: nevirapine, efavirenz and etravirine.

All antiretrovirals were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Table. 2.1 Primer sequences

Primer name	Sequence 5'-3'	Position in HXB2
Res 1	GAAGAAATGATGACAGCATGTCAGGG	1822-1844
Res 2	TAATTTATCTACTTGTTCATTTCCTCCAAT	4173-4199
Pin 2	GGCTGTACTGTCCATTTATCAGG	3254-3276
Pin 18	CAGTATTAGTAGGACCTACACC	2472-2493
Pout 3	AAGGGCTGTTGGAAATGTGG	2019-2038
PK pout 3	CATTGCTCTCCAATTACTGTGATATTTCTCATG	4263-4295
PK pout 5	GGTACAGTATTAGTAGGACCTACACCTGTCAACAT	2469-2503
PK pin 3	TCTATTCCACAAAAATAGTACTTTCCTGATTCC	4212-4246
PK pin 5	AATTTTCCCATTAGTCCTATTGAAACTGTACCAG	2544-2577
Res 3	ATGGYTCTTGATAAATTTGATATGTCC	3559-3582
Res 4	AGACAGGCTAATTTTTTAGGGA	2077-2095
Seq 1	CAAGGCCATTGACAGAAG	2616-2634
Seq 2	GGATCACCAGCAATATTCCA	3012-3031
Seq 3	CAAGGCCAATGGACATATCA	3549-3568
Seq 4	CTTCTATGTAGATGGGGC	3866-3883
Seq 5	TGGGCCATCCATTCCTGGCTT	2586-2606
Seq 6	CATCCCTGTGGAAGCACATT	2998-3007
Seq 7	TCTGCTATTAAGTCTTTTGAT	3512-3532
Seq 8	GGAAAGTACTATTTTTAGATGGAATAGA	4219-4246
Pyro forward	ATGGAAAGGATCACCAGCAA	3005-3024
Pyro reverse	TGTTGAGGTGGGGACTTACC	3175-3194
Pyro seq 1	CAAAAATAGAGGAG	3148-3161
Pyro seq 2	GAGGAGCTGAGACAA	3156-3170
Pyro seq 3	AAAATAGAGGAGCTGAGACA	3150-3169
TaqMan forward	GACATAGTTATCTATCAATACAT	3078-3100
TaqMan reverse	GATAAATGGACAGTACAGCC	3258-3277
ASPCR mt st A	GCATAGAGCAAAAATAGAAGAGTTAAGAGGTT	3140-3171
ASPCR wt st A	CATAGAGCAAAAATAGAAGAGTTAAGAGGTC	3141-3171
ASPCR mt st B	CATAGAGCAAAAATAGAGGAACTRAGACGAT	3141-3171
ASPCR mt st B	CATAGAGCAAAAATAGAGGAACTRAGACGAC	3141-3171
SDM st B forward	GAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAG GTGGGG	3145-3187
SDM st B reverse	CCCCACCTCAACAGATGTTGTCTCAGCTCCTCTATTTTT GTTC	3145-3187
SDM st A forward	GAGCAAAAATAGAAGAGTTAAGAGCTCATCTATTGAGC TGGGG	3145-3187
SDM st A reverse	CCCCAGCTCAATAGATGAGCTCTTAACTCTTCTATTTTT GCTC	3145-3187
SDM stA V184M forward	CCAGAAATAATTATCTATCAATATATGGATGATTTGTATGT AGGATC	3075-3121
SDM stA V184M reverse	GATCCTACATACAAATCATCCACATATTGATAGATAATTA TTTCTGG	3075-3121

Table. 2.2 Probe sequences

Probe Name	Sequence	Position in HXB2
ASPCR	ATGGGTTATGAACTCCATCCTGA	3237-3259
TaqMan mt	CTAAGACAATATCTGTT	3162-3178
TaqMan wt	CTAAGACAACATCTGTT	3162-3178

2.2 Methods

2.2.1 Identification of patients

All HIV-1 infected patients that attend the Royal Free Hospital for drug resistance testing have their *pol* gene sequences and genotypic profile entered into a database. This database also includes treatment regimens, plasma HIV-1 RNA load and HIV-1 subtype. In March 2010, this database contained approximately 8500 *pol* sequences. Upon examination of the database 17 patients harbouring H208Y were identified. From these 17 patients, 12 were treatment experienced and 5 were treatment naïve. Ethics approval for use of HIV infected patient samples in phenotypic studies was obtained prior to beginning the study.

2.2.2 Molecular Biology

2.2.2.1 Extraction of viral RNA

All nucleic acid extraction methods are based on the Boom extraction method (Boom et al., 1990). Both automated and manual extraction methods were used. The automated method was carried out by the EasyMag automated extractor (Nuclisens, Biomerieux, Boxtel Netherlands). Nucleic acid was extracted from 500µl of patient plasma according to the manufacturer's instructions. RNA was eluted into 55µl elution buffer and stored at -80ºC until required.

Manual RNA extractions were done using the Qiagen QIAamp Viral RNA kit (Qiagen, Crawley, UK). Briefly, 200 µl of HIV infected culture supernatant was lysed under denaturing conditions using guanidine thiocyanate and bound to the silica membrane in the spin column thorough centrifugation. Contaminants and proteins in the sample were removed by washing with specific buffers. Viral RNA

was eluted into 60 µl of RNase-free water containing 0.04% sodium azide. RNA was stored at -80ºC until required.

2.2.2.2 Random priming

10 µl of eluted RNA was denatured at 65°C for 30 seconds followed by 5 minutes at 42°C. 10 µl of reverse transcription mix was added to the RNA. The mix consisted of 5XRT buffer containing 1.5 mM $MgCl_2$, (Promega, Southampton, UK), 250 uM of each dNTP, 500 ng random primers (Promega, Southampton, UK), 200 units of MMLV reverse transcriptase (Promega, Southampton, UK) and 20 units of RNase inhibitor (Promega, Southampton, UK). Cycling conditions were 42°C for 60 minutes followed by 99°C for 5 minutes.

2.2.2.3 One step RT-PCR

The Qiagen one step RT-PCR kit (Qiagen, Crawley, UK) was used to amplify codons 1-345 of the HIV-1 RT gene. The RT-PCR mix contained primers res 1 and res 2 (table 2.1) at a final concentration of 600 uM each, 400 µM of each dNTP, 5X RT-PCR buffer containing 1.25 mM $MgCl_2$, 2 µl of RT-PCR enzyme, 10 µl of RNA and made up to a final reaction volume of 50 µl with water. Reaction conditions were one cycle of 50ºC for 35 minutes, 95ºC for 15 minutes, 95ºC for 30 seconds, 65ºC for 45 seconds, 72ºC for 3 minutes, 95ºC for 30 seconds, 60ºC for 45 seconds. 72ºC for 3 minutes, followed by 45 cycles of 95ºC for 30 seconds, 58ºC for 45 seconds, 72ºC for 3 minutes, finally 72ºC for 10 minutes and a hold at 4ºC.

2.2.2.4 Amplification of codons 1-335 of the reverse transcriptase gene

20 µl of cDNA was used to amplify codons 1-335 of the reverse transcriptase (RT) gene. The PCR mix consisted of 10Xbuffer containing 2.5 mM $MgCl_2$, 250 uM of each dNTP, 5U AmpliTaq Gold Polymerase (Applied Biosystems, UK) and 200 uM of primers pin 18 and pout 3 (table 2.1). PCR cycling conditions were 35 cycles of 95°C for 15 minutes, 95°C for 30 seconds, 54°C for 45 seconds, 72°C for 2 minutes followed by 72°C for 10 minutes and a hold at 4°C.

2.2.2.5 Amplification of codons 1-560 of the reverse transcriptase gene

Codons 1-560 of the reverse transcriptase gene were amplified using 20 µl of cDNA in a 100 µl PCR mix which consisted of 10Xbuffer containing 1.5 mM $MgCl_2$, 375 uM of each dNTP, 150 uM of primers and 2.5units of *PfuUltra* high fidelity DNA Polymerase (Stratagene, Cheshire, UK). PCR cycling conditions were 35 cycles of 95°C for 3 minutes, 90°C for 1 minute, 55°C for 30 seconds, 72°C for 3 minutes followed by 72°C for 10 minutes and a hold at 4°C. Primers PK pout 3/5 were used in the 1st round PCR and PK pin 3/5 were used in the nested PCR (table 2.1).

Nested PCR was done using 10 µl of the first round PCR product. Cycling and reagent conditions were the same as the first round PCR conditions.

2.2.2.6 Gel electrophoresis

Agarose gel electrophoresis was used to confirm the correct size of the PCR product. 1.5 g of agarose was dissolved in TAE buffer in a microwave and once cooled, 10

mg/ml ethidium bromide was added. PCR products were mixed with 5Xloading dye and loaded onto the gel with the DNA mass ladder hyperladders I-IV (Bioline, London, UK). Gels were run for approximately 1-2 hours at 100 volts, depending on the size of the band expected. All gels were visualised using a UV transilluminator (Biorad, Hertfordshire, UK)

2.2.2.7 Purification of PCR product

PCR products were visualised using ethidium bromide staining and agarose gel electrophoresis. The desired product was either excised using the QIAQuick Gel Extraction Kit (Qiagen, Crawley, UK) or purified using the QIAQuick PCR Purification kit (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions. Briefly, the PCR product was added to the buffer containing the chaotropic agent guanidine thiocyanate and bound to the silica membrane in the QIAquick spin column through centrifugation for 1 minute at 13,000rpm. Impurities and contaminants were removed through washing with an ethanol containing buffer and DNA was eluted into a low salt and pH containing buffer.

2.2.2.8 A-tailing

To enable efficient TA cloning of products generated with high fidelity polymerases, purified PCR products were added to a mix of 250 uM dATP, 10Xbuffer containing 2.5 mM $MgCl_2$, and 2.5 units of Amplitaq Gold Polymerase. The mix was heated to 95^{o}C for 10 minutes followed by 20 minutes at 72^{o}C.

2.2.2.9 PCR cloning

The TOPO TA cloning kit (Invitrogen, Paisley, UK) was used to clone the HIV reverse transcriptase gene. TOPO TA cloning uses *Topoisomerase I* to ligate the PCR product with an A nucleotide overhang into the vector which contains the T nucleotide overhang. All PCR products were cloned into pCR 2.1-TOPO. The ligation reaction consisted of 4 ul PCR product, 1 ul salt solution and 1 ul TOPO vector. Ligation reactions were incubated at room temperature for 5 minutes and then stored on ice ready for transformation. 2 ul of the ligation reaction was transformed into a vial of 50ul TOP10F' *E. coli* cells and incubated on ice for 5-30 minutes. Cells were then heat shocked at 42ºC for 45 seconds and cooled on ice for 2 minutes. 250 ul of SOC medium (Invitrogen, Paisley, UK) was added to the cells and incubated in a 37ºC orbital shaker for 1 hour. Cells were plated onto LB agar plates containing 50 ug/ml ampicillin and 100mM IPTG and incubated overnight at 37ºC.

2.2.2.10 Site directed mutagenesis

Site directed mutagenesis was carried out using Quickchange Muliti/Site Directed Mutagenesis Kit (Stratagene, Cheshire, UK) to insert desired mutations. All primer combinations were designed specifically to incorporate the desired mutation and are shown in table 2.1. 50 ng plasmid and 125 ng of the primers containing the required mutation were used in the following PCR, 12-18 cycles of 95°C for 30 seconds, 90°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes and a hold at 4°C. In order to degrade the parental DNA plasmid, the PCR product was incubated at 37ºC for at least one hour with the restriction enzyme *Dpn1*. The digested PCR product was then transformed into XL1-blue supercompetent cells and plated onto LB agar plates

containing 50 ug/ml ampicillin, 80 ug/ml Xgal and 20 mM IPTG. Plates were incubated overnight at 37ºC. The desired mutations were confirmed by sequencing the full RT gene using Sanger sequencing.

2.2.2.11 Minipreps

Plasmid DNA was extracted from bacterial cells using the methodology described by (Birnboim and Doly, 1979).

Plasmid DNA was extracted from bacterial cells using the QIAPrep Spin Miniprep Kit (Qiagen, Crawley, UK). in accordance with the manufacturer's instructions. Briefly, DNA was extracted from 2 mls of LB broth containing 50 ug/ml ampicillin overnight cultures, inoculated using a single transformed white colony. Bacterial cells were resuspended and lysed under alkaline conditions. The lysate was then neutralised with acetic acid and bound to the silica membrane of the QIAprep spin column through centrifugation for 1 minute at 13,000rpm. Remaining impurities were washed away using an ethanol based buffer. The plasmid DNA was then eluted under low salt conditions into RNase-free water.

Maxipreps of the vectors was done using the JETstar plasmid purification kit (Genomed-DNA, Löhne, Germany) according to the manufacturer's instructions. Briefly, DNA was extracted from 100 mls of LB broth containing 50 ug/ml ampicillin overnight cultures. Bacterial cells were resuspended and neutralised with acetic acid. The plasmid was bound to the anion exchange resin and all impurities

were washed away using an alcohol based buffer. The plasmid DNA was eluted and concentrated by alcohol precipitation.

2.2.2.12 Restriction digest

5 ul of miniprep were digested with 1ul *EcoRI* (Invitrogen, Paisley, UK), 2ul buffer containing 1 mM $MgCl_2$, and 12ul RNase free water. Digests were incubated for 1 hour at 37ºC and visualised using ethidium bromide staining and agarose gel electrophoresis. Positive clones were identified as those harbouring the correctly sized inserts.

2.2.2.13 Sequencing

All DNA was sequenced using Sanger sequencing. Plasmids identified as containing the PCR insert were then sequenced using the BioDye Sequencing mix v1.1. Primers seq 1 to seq 8 (table 2.1) were used to get an overlapping readable sequence. The sequencing PCR conditions were as follows, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes and a hold at 4°C. Sequences were analysed using a 3100-Avant Genetic Analyzer (Applied Biosystems, UK).

2.2.3 Cell Culture

2.2.3.1 Passaging cells

MT4 cells are adherent cells derived from lymphocytes. They were passaged twice a week in RPMI 1640 + 10%FCS + 100 U/ml penicillin and 100 μ□g/ml streptomycin in 5% CO_2 at 37ºC.

2.2.3.2 Construction of recombinant viruses

The recombinant virus assay was used to create a fully replicative competent virus. The molecular clone pHIVΔRTBstEII contains the complete HIV-1 genome except for a 1.4kb deletion in the reverse transcriptase gene. It also contains the T7 RNA polymerase promoter, a *BstEII* restriction site, ampicillin and β-galactosidase markers. This clone in not infectious but once it is transfected into MT4 cells with full-length reverse transcriptase PCR product, a replicative virus is produced through homologous recombination.

pHIVΔRTBstEII was linearised by incubation with *BstEII* at 60ºC for at least 2 hours. The linearised plasmid was purified using the QIAQuick Purification kit (Qiagen, Crawley, UK) as described previously. 10 μg of the molecular clone was used per transfection. MT4 cells were split 1:2 (volume:volume) the day before the transfection. For each transfection, $2x10^6$ MT-4 cells and 12 μl of the transfection reagent DMRIE-C ((1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol) (Invitrogen, Paisley, UK) were used. MT4 cells were resuspended at a concentration of $7.5x10^5$ /ml. Serum free OptiMEM medium was added to 6 well plates followed by DMRIE-C and 2 μg DNA. Following an

incubation of 15-30 minutes at room temperature, MT4 cells were added and the plates were incubated at 37ºC with 5% CO_2 for 5 hours. Cells were observed for CPE and each day and fresh medium was added. Once CPE was observed, the cell free supernatant was harvested and stored in aliquots at –80°C.

2.2.3.3 MTT titration assay

Virus titres were obtained in MT4 cells and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) was used to determine cell viability. $2x10^6$ MT-4 cells were used per titration at a concentration of $7.5X10^5$/ml. Viruses were serially diluted (0.5 $\log_{10}$) in RPMI (no phenol red). MT4 cells were resuspended in supplemented RPMI (no phenol red) and 50 µl was added to the wells. Each dilution was performed in quadruplicate.

Following a 5 day incubation, 20 µl of MTT (Sigma-Aldrich, Dorset, UK-Aldrich) (5 mg/ml in PBS) was added and the plates were incubated for 2 hours. 170 µl of acidified isopropanol (86% Isopropanol, 11% dH_2O, 2.7% NP-40 and 0.3% concentrated HCl) was added and the absorbance was measured at 595nm and the $TCID_{50}$ (Tissue culture infectivity dose) determined by the Spearman-Karber method.

2.2.3.4 Cytopathic effect titration assay

100 µl of supplemented RPMI was added to a 96 well plate. 23 µl of neat virus was added to the plate and nine serial fivefold dilutions were made. 50 µl of MT4 cells were added to the wells at a concentration of $6X10^5$ per ml. Plates were left to incubate for five days. After five days, all wells were scored for the presence of

CPE. TCID50 was calculated using the Reed and Muench method. The equation used was as follows; M = inv log{x_1 + [(x_2 - x_1)((y_1 - 50) / y_1 - y_2)]}, where y_1 = percent of well scored positive closest to, but higher than 50% at a certain virus dilution, y_2 = percent of well scored positive closest to, but lower than 50% at a certain virus dilution, x_1 = the log (dilution of the virus where was observed), x_2 = the log (dilution of the virus where was observed).

2.2.3.5 Drug susceptibility assays

All drug susceptibility assays required $2X10^6$ MT4 cells per assay. Cells were washed first with PBS and then with supplemented RPMI (no phenol red). The cell pellet was resuspended using the virus supernatant and volumes were equalised with medium so that the same volume of liquid was added to each of the assays. Cells and virus were incubated for two and a half hours at 5% CO_2 at 37ºC. 50 µl of supplemented RPMI (no phenol red) was added to each of the wells and 50 µl of concentrated drug (4X) was added to one column. 10 serial dilutions of the drug were done to give a 1X concentration of drug (following the addition of 50 µl of MT4 cells). Infected cells were resuspended in supplemented RPMI (no phenol red) at a concentration of $7.5x10^5$ /ml and 50 µl of these cells were added to all the wells. Uninfected MT4 cells and infected MT4 cells containing no drug controls were also set up. Plates were left to incubate at 5% CO_2 at 37ºC for 4 days. On day four MTT was used to analyse results as described previously. All assays were done in quadruplicate and the average of the four was used to calculate the IC50.

2.2.3.6 Growth competition assay

Growth competition assays were set up according to (Harrigan et al., 1998). Briefly, $7.5x10^5$ MT4 cells were used per growth competition assay. For each assay 750 TCID50 units total of 2 viruses were added to MT4 cells. Cells and viruses were left to incubate for 2 hours at 5% CO_2 at 37ºC and cells were resuspended every 30 minutes. After 2 hours, cells were spun down and washed twice with PBS. Infected cells were then resuspended in 5 ml of supplemented RPMI and incubated for 6 days. On day 6, aliquots of virus supernatant were collected and stored at –80°C. 50 µl of virus supernatant was used to re-infect fresh MT4 cells and resuspended in 5ml of supplemented RPMI. This was continued for four passages. For competitions done in the presence of antiretrovirals, 1ml of virus supernatant was used to start a new passage. The antiretroviral drug was added to 5 ml of supplemented RPMI. Controls of monoinfections and uninfected MT4 cells were also set up. RNA was extracted from 200 µl of virus supernatant and used in a quantitative real time PCR to assess proportions of mutant and wild type viruses.

2.2.4 Pyrosequencing

2.2.4.1 Pyrosequencing – preparation of PCR product

For pyrosequencing a 200bp fragment encompassing codons 176-242 of the reverse transcriptase gene was amplified using primers Pyro forward and reverse (table 2.1). The PCR mixture contained 50 ng DNA, 200 uM of primers, 250 uM of each dNTP, 10X enzyme buffer and 2.5 units of *PfuUltra* high fidelity DNA Polymerase (Stratagene, Cheshire, UK) in a total volume 50 µl. The reverse primer was biotinylated at the 5’ end to allow capture onto the streptavidin beads. PCR cycling conditions consisted of 40 cycles of 95°C for 5 minutes, 95°C for 30 seconds, 55°C

for 30 seconds, 72°C for 30 seconds followed by 72°C for 5 minutes and a hold at 4°C. The correct sized fragment was confirmed by gel electrophoresis.

2.2.4.2 Pyrosequencing – sample preparation

A mixture of 0.3 µM sequencing primer in 40 µl of annealing buffer was made for each of the samples and aliquoted into the pyrosequencing plate. 20 µl of biotinylated PCR product was added to 3 µl of streptavidin sepharose beads, 37 µl of binding buffer and 20 µl of high purity water. The PCR product-bead mix was added to a single well in a 96 well plate. To enable the biotinylated product to be captured onto the streptavidin beads, the plate was mixed at 1000rpm for 10 minutes. To secure the single stranded biotinylated PCR DNA onto the filter probe, a vacuum pump was used. The beads were then washed with 70% ethanol, denatured and given a final wash with the washing buffer to remove the non biotinylated strand. The DNA was then released into the plate containing the sequencing and annealing buffer mixture. To allow binding of the DNA to the sequencing primer, the mixture was heated at 80ºC for 2 minutes and allowed to cool. Samples were run on the PSQ96 MA (biotage) and analysed using the allele quotient mode in order to determine the proportion of mutant and wild type nucleotide at position 208.

2.2.5 Quantitative real time PCR – Taqman PCR

Primer3 software was used to design two taqman PCR probes that would specifically bind to mutant and wild type DNA (table 2.2). Primers TaqMan forward and reverse (table 2.1) amplified a 200bp fragment encompassing the region of interest. 200 ng of DNA was added to a 50 µl PCR mix containing 150 nM primers, 100 nM taqman probe, and 12.5 µl of 2 X superscript platinum qRT-PCR mix (Invitrogen, Paisley,

UK). Cycling conditions were as follows, 50°C for 15 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

2.2.6 Allele specific PCR (ASPCR)

Mutant and wild type forward primers were specifically designed with incorporated G nucleotide two base pairs away from the SNP. Primers were designed to be subtype specific. To amplify subtype B sequences, primers ASPCR st B mt and wt were used and to amplify subtype A sequences, primers ASPCR st A mt and wt were used (table 2.1).

300 ng of total RNA was used in the real time PCR with a PCR mix containing 150 nM primers, 100 nM of probe ASPCR, and 12.5 µl of 2X superscript platinum qRT-PCR mix. Cycling conditions for the real time PCR were 95°C for 11 minutes, 45 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 60°C for 32 seconds.

Chapter three

3 Clonal analysis of H208Y

3.1 **Introduction**

The HIV-1 reverse transcriptase (RT) is a heterodimer composed of two subunits, p66 (66kDa) and p51 (51kDa). The p51 subunit carries out essentially a structural role whereas the p66 subunit is responsible for the catalytic activities of the enzyme. The p66 subunit contains five subdomains named thumb, fingers, palm, connection and RNase domain. RT is one of the major drug targets of antiretroviral therapy and the majority of RT associated resistance mutations are located within the fingers and palm domain (Sarafianos et al., 1999b;Sluis-Cremer et al., 2000b). However, mutations within the connection and RNase H domain have also been shown to contribute to NRTI (nucleos(t)ide RT inhibitors) and NNRTI (non-nucleoside RT inhibitors) resistance (Brehm et al., 2007;Brehm et al., 2008;Ehteshami et al., 2008;Nikolenko et al., 2007;Yap et al., 2007).

When antiretroviral therapy fails to suppress HIV replication, mutations emerge that confer drug resistance but generally reduce virus fitness relative to wild type virus. If replication continues to occur, new mutations emerge alongside major resistance mutations, which may either augment resistance or restore virus fitness. These mutations are called accessory mutations and are associated with major resistance mutations. Although some accessory mutations may occur spontaneously in drug naïve persons, their frequency increases significantly in treated persons (Cane et al., 2007;Perno et al., 2006;Svicher et al., 2006). These mutations have mainly been identified through statistical analyses of large resistance databases, comparing the frequency of different mutations in untreated and treated persons and identifying clinical correlates. As a result, the individual and cumulative *in vitro* effects of the

mutations are often unknown and the mutations are not generally included in the routine interpretation of genotypic resistance.

To date, accessory mutations associated with NRTI treatment and major NRTI resistance mutations have been identified at 40 positions in RT and this includes the H208Y mutation (Cane et al., 2007;Gonzales et al., 2003;Perno et al., 2006;Sturmer et al., 2003;Svicher et al., 2006). The thymidine analogue mutations (TAMs) are a group of major NRTI resistance mutations that confer resistance to stavudine (D4T) and zidovudine (AZT). TAMs can be divided into 2 pathways, the TAM-1 pathway includes M41L, L210W and T215Y and the TAM-2 pathway includes D67N, K70R, T215F and K219Q/E. The TAM-1 pathway is known to confer a higher level of resistance and cross-resistance than the TAM-2 pathway (Miller, 2004). The accessory mutation H208Y has been shown to cluster preferentially with the TAM-1 pathway (Cane et al., 2007;Gonzales et al., 2003;Sturmer et al., 2003;Svicher et al., 2006).

Previous studies examining the phenotypic effect of the H208Y mutation have reported conflicting results. Some studies have shown that in the presence of TAMs, H208Y confers a decreased susceptibility to AZT (Sturmer et al., 2003;Svicher et al., 2006). Despite these findings indicating a role for H208Y in conferring resistance to AZT, another study showed that H208Y in isolation could confer an increased susceptibility to AZT (Clark et al., 2006).

The aim of the study described in this chapter was to investigate which major NRTI resistance mutations and recognised accessory mutations co-exist with H208Y on the same viral genome.

3.2 **Methods**

3.2.1 Identification of patients

In the Virology Diagnostic Department at the Royal Free Hospital, the HIV-1 *pol* sequences (protease codons 1-99 and RT codons 1-335) of patients attending for routine care are routinely examined for the presence of drug resistance mutations by automated population sequencing. The sequences are obtained from plasma samples using either the Viroseq^{TM} HIV-1 genotyping system (Celera Diagnostics, USA) according to the manufacturer's instructions or an in-house methodology, and analysed using a 3100-Avant Genetic Analyzer (Applied Biosystems, UK). All sequences are stored in the local HIV Genotyping Drug Resistance Database. This database also contains information about HIV-1 subtype, antiretroviral treatment history, HIV plasma RNA load and CD4 cell counts at the time of testing. In March 2010 there were 8500 *pol* sequences stored in the database. The database was searched to identify patients who harboured the H208Y mutation in their genotypic resistance test.

3.2.2 RNA extraction

Nucleic acid from plasma samples were extracted using the EasyMag automated extractor (Nuclisens, Biomerieux, Boxtel Netherlands). RNA was eluted into 55µl elution buffer and stored in aliquots at -80ºC until required.

3.2.3 Amplification of viral RNA

Random priming was used to convert RNA to cDNA. Codons 1-560 and 1-335 of RT were amplified by nested PCR using 20µl of cDNA.

3.2.4 Cloning of the RT gene

The PCR product from each patient was purified and cloned into the commercial vector pCR 2.1-TOPO. Positive clones were identified as those harbouring inserts of the correct size after a restriction digest.

3.2.5 Sequencing the RT gene

All DNA was sequenced using Sanger sequencing. Plasmids identified as containing the PCR insert were then sequenced using the BioDye Sequencing mix v1.1 (Applied Biosystems, UK). Four forward and reverse primers were used to obtain overlapping sequences. Sequences were analysed using a 3100-Avant Genetic Analyzer (Applied Biosystems, UK).

3.2.6 Nomenclature

Sequences were analysed for the presence of mutations relative to HXB2 wild type. Mutations were classed into the following groups: major RT (NRTI and NNRTI) resistance mutations (http://hivdb.stanford.edu/), accessory NRTI mutations in the polymerase domain and connection domain (Cane et al., 2007;Perno et al., 2006;Svicher et al., 2006;Torti et al., 2004) and other mutations with unknown effects on drug susceptibility or viral fitness. Accessory mutations in the RNase H domain were not investigated.

3.3 Results

3.3.1 Database Analysis

The HIV Genotyping Database was searched for patients with H208Y present in their genotypic resistance test. Samples with missing treatment status were excluded from the analysis. A total of 5087 patients were identified, with 1304 being treatment experienced and 3783 being treatment naive. Upon examination of the database, 17/5087 (0.3%) patients harbouring the H208Y mutation were identified. A total of 12/17 (71%) patients had an extensive treatment history and had experienced NRTIs, NNRTIs and PIs (protease inhibitors). However, 5/17 (29%) patients were antiretroviral treatment naïve. The prevalence of H208Y was 5/3783 (0.1%) in treatment naïve patients and 12/1304 (0.9%) in treatment experienced patients. The majority of treatment experienced patients harbouring H208Y (8/12, 67%) were infected with subtype B virus followed by subtype C (2/12, 17%), subtype A (1/12, 8%) and subtype G (1/12, 8%) virus (table 3.1). Similarly, the majority of treatment naïve patients (2/5, 40%) had a subtype B virus and one each with subtype C, CRF02_AG and CRF06_cpx.

From these 17 patients, 5 were selected for further analysis as shown in table 3.2. Since the majority of patients harbouring H208Y had a subtype B virus, both a treatment naive patient and a treatment experienced patient with subtype B were chosen for further analysis. Two treatment experienced patients harbouring the highly prevalent subtype A and subtype C virus were also chosen to assess whether there could be any subtype related differences associated with H208Y.

Table. 3.1 Prevalence of H208Y according to treatment status and HIV-1 subtype[a]

Subtype	Treatment Experienced Patients		Treatment Naïve Patients	
	Number of patients analysed	Patients with H208Y, n (%)	Number of patients analysed	Patients with H208Y, n (%)
A	104	1 (1.0)	258	0
B	514	8 (1.6)	1621	2 (0.1)
C	415	2 (0.5)	1134	1 (0.1)
G	34	1 (2.9)	105	0
CRF01_AE	25	0	309	1 (0.3)
CRF06_cpx	8	0	33	1 (3.0)
Other subtypes	204	0	323	0
All	1304	12 (0.9)	3783	5 (0.1)

[a]Patients with H208Y were identified from the HIV-1 drug resistance database held at the Royal Free Hospital.

Table. 3.2 RT population sequencing results in patients with H208Y[a]

Patient	Subtype	Major NRTI Resistance Mutations	Major NNRTI Resistance Mutations	NRTI Accessory Mutations	Other RT Mutations
1	B	None	None	G196E, H208Y	D123E, D177E, I178M, I195T, R211K, A272P, K277R, A288T, I293V, E297K, Q334L
2	B	M41L, D67N, V75M, V118I, L210W, T215Y, K219DN	A98G, G190Q	K43E, V60I, K122E, I135V, T200A, H208Y,	V35L, E40F, W88C, I94L, I178L, R211K
3	A	D67N, T69N, K70KR, M184V, T215F, K219E	None	K20KR, V60I, K122E, I135T, V179I, H208HY, T200A,	E6K, V35T, D123S, K173L, Q174K, D177E, Q207A, R211S, V245Q, A272S, T286A, E291D, I293V, E312D, Q334E
4	C	M41L, E44D, D67N, L74V, V118IV, M184V, L210W, T215Y, K219R	A98G, K101H, V108I, Y181C, G190A	E203K, H208Y, T200A, D218E, K223Q	V35L, T39E, V111I, K122P, D123N, A158S, S162N, K173T, Q174K, D177E, L205F, Q207E, R211K
5	B	M41L, E44D, T69N, L74I, V118IV, M184V, L210W, T215Y, K219R	A98G, K103N, V108I, F227L	K43Q, K122E, I135L, V179I, G196EG, T200A, E203K, H208Y, K223DE, L228H,	E6K, V111I, E194EQ, , R211K, Q242H, V245Q, I293V, P294Q, V317A

[a]Mutations that were present in bulk sequences spanning codons 1-335 of the RT gene from five patients that harboured the H208Y mutation, including one treatment naïve (no 1) and 4 treatment experienced (no 2-5) patients. Major NRTI and NNRTI resistance mutations were classified according to the Stanford HIV Drug Resistance Interpretation Algorithm. NRTI accessory mutations associated with TAMs were classified according to (Cane et al., 2007;Perno et al., 2006;Svicher et al., 2006). Other RT mutations were classified as differing from the HXB2 wild type reference strain. NRTIs = Nucleos(t)ide RT inhibitors; NNRTIs= non-nucleoside RT inhibitors.

Patient 1 was treatment naïve and did not have any major RT resistance mutations. Among the treatment experienced individuals, patients 2, 4 and 5 showed TAM-1 mutations (M41L, L210W, T215Y) accompanied by one or more TAM-2 mutations (D67N, K219D/N/R), with or without the lamivudine (3TC) associated resistance mutation M184V. Patient 3 showed TAM-2 mutations with M184V. In addition, patients 2, 4 and 5 showed V118I and/or E44D which are mutations typically seen in NRTI-experienced patients with TAMs, while patients 4 and 5 showed L74V/I, which confers resistance to didanosine (DDI) and abacavir (ABC). Patient 2 had the V75M mutation, which is associated with resistance to DDI and D4T. Patients 2, 4 and 5 also showed major NNRTI mutations conferring resistance to nevirapine (NVP), efavirenz (EFV) and etravirine (ETV).

NRTI accessory mutations other than H208Y were detected in all patients and comprised G196E in patient 1, K43E, V60I, K122E, I135V and T200A in patient 2, K20K/R, V60I, K122E, I135T, V179I and T200A in patient 3, T200A, E203K, D218E and K223Q in patient 4, and K43E, K122E, I135L, V179I, G196E, T200A, E203K, K223DE and L228H in patient 5.

3.3.2 Cloning and sequencing of the RT gene encompassing codons 1-335

Population sequencing that occurs routinely in clinical settings can only identify virus variants that represent over 20% of the viral population (Alcorn and Faruki, 2000), and since it produces a consensus sequence from all dominant variants it cannot be used to examine linkage of mutations. To examine which mutations were linked to H208Y at the genomic level, codons 1-335 of the RT gene from patients 2, 4 and 5 were cloned and 35 clones from each patient were sequenced from one PCR reaction.

Table 3.3 shows the major RT resistance mutations and NRTI accessory mutations found within the clones. For the major resistance mutations, in all three patients, all clones showed a combination of TAM-1 and TAM-2 mutations. These comprised M41L and L210W together with mutations at codon 215 (T215Y, T215N or T215C) and codon 219 (K219G, K219E, K219N or K219R) with or without D67N. The major resistance mutations M184V with mutations at codon 74 (L74I or L74V) were found in all clones from patients 4 and 5; E44D was present in all clones from patient 4 and in 34/35 (97%) clones from patient 5; all patients had V118I present in all clones except for patient 4 who had V118I present in 10/35 (29%) clones.

NRTI accessory mutations other than H208Y were also found in the clones. Patients 2 and 5 had mutations at codon 43 (K43E or K43Q), codon 135 (I135L or I135V) and K122E present in all clones. Patients 2 and 5 had all clones with T200A and patient 4 had 33/35 (94%) of clones with T200A. Patients 4 and 5 had mutations at codon 203 (E203D or E203K) and codon 223 (K223E or K223Q) present in their

clones. Patient 4 had 19/35 (54%) clones with E203K and 3/35 (9%) with E203D and patient 5 had 34/35 (97%) clones with E203K. Patient 4 had 19/35 (54%) clones with K223Q and patient 5 had 21/35 (60%) clones with K223Q and 6/35 (17%) clones with K223E. Other NRTI accessory mutations present in the clones included V60I for patient 2, D218E for patient 4, and G196E/D, and L228H in clones from patient 5.

NNRTI accessory mutations other than H208Y were also found in the clones. Patient 4 had all clones with V35L and patient 2 had all clones with V35I, and patient 5 had all clones with E6K and V179I.

Table. 3.3 Mutations detected in RT clones spanning amino acids 1-335[a]

	Major NRTI Resistance Mutations																		NNRTI Accessory Mutations					NRTI Accessory Mutations													
Patient	M 41 L	E 44 D	D 67 N	L 74 I	L 74 V	T 69 N	T 69 D	V 75 M	V 118 I	M 184 V	L 210 W	T 215 Y	T 215 N	T 215 C	K 219 G	K 219 E	K 219 N	K 219 R	E 6 K	V 35 L	V 35 I	V 179 I	K 43 E*	K 43 Q	V 60 I	K 122 E*	I 135 V	I 135 L	G 196 E	G 196 D	T 200 A	E 203 D	E 203 K*	H 208 Y*	D 218 E*	K 223 E	K 223 Q*	L 228 H*
2																																						
4									10						1			34													33	3	19				19	
5		34				34	1					33	1	1		1		28					1	34					28	1			34			6	21	20

[a]Major RT resistance mutations and accessory mutations that were detected in clones generated from three patients that harboured the H208Y mutation. The clones spanned RT amino acids 1-335. For each patient, 35 clones were sequenced. White boxes indicate absence of the mutation in all 35 clones; shaded boxes indicate presence of the mutation in either all 35 clones (no number), or in a subset of clones (number given in the box). * indicates accessory mutations associated with exposure to both NRTIs and NNRTIs.

3.3.3 Cloning and sequencing of full-length RT gene

Recent studies have shown that mutations within the C terminus of RT also contribute to NRTI resistance (Brehm et al., 2007;Brehm et al., 2008;Ehteshami et al., 2008;Ehteshami and Gotte, 2008;Nikolenko et al., 2007;Yap et al., 2007;Hachiya et al., 2008). Therefore, in order to examine associations of H208Y and mutations in the C terminus of RT, the full RT gene was cloned and sequenced in four patients. In order to improve sampling of the viral quasispecies, more than one PCR reaction was used to amplify the RT gene in each patient, excluding patient 3. The PCR products were then pooled prior to cloning. Clinical details, the number of PCR reactions used for the amplification reactions and the number of clones sequenced from each patient are shown in table 3.4.

Table. 3.4 Number of PCR reactions used for amplification and number of clones sequences in each patient

Patient	Subtype	Viral load (log_{10} copies/ml)	Treatment history	Number of PCR reactions	Number of clones sequenced
1	B	5.5	None	2	10
2	B	4.4	DDI, TDF, LPV/R SQV	2	11
3	A	6	3TC, LPV/r, SQV	1	6
4	C	5.2	3TC, TDF, FPV, SQV/R, T-20	3	20

3.3.3.1 Full-length RT clones from patient 1 (n=10)

Patient 1 was treatment naïve and infected with subtype B, and harboured no major RT resistance mutations by population sequencing. This was confirmed in the clonal analysis of full-length RT. Figure 3.1 shows the NRTI accessory mutations present within the 10 clones from this patient. Consistent with the results of population sequencing, all 10 clones showed the NRTI accessory mutations G196E and H208Y in the polymerase domain. In the connection domain all 10 clones showed the G359S mutation, which has been previously associated with TAMs (Cane et al., 2007). However this patient did not have TAMs. Within the RNase H domain, all 10 clones had the Q509K mutation. In combination with D67N, K70R and T215F, the Q509L mutation has been shown to increase AZT resistance by 7.4 fold (Brehm et al., 2007).

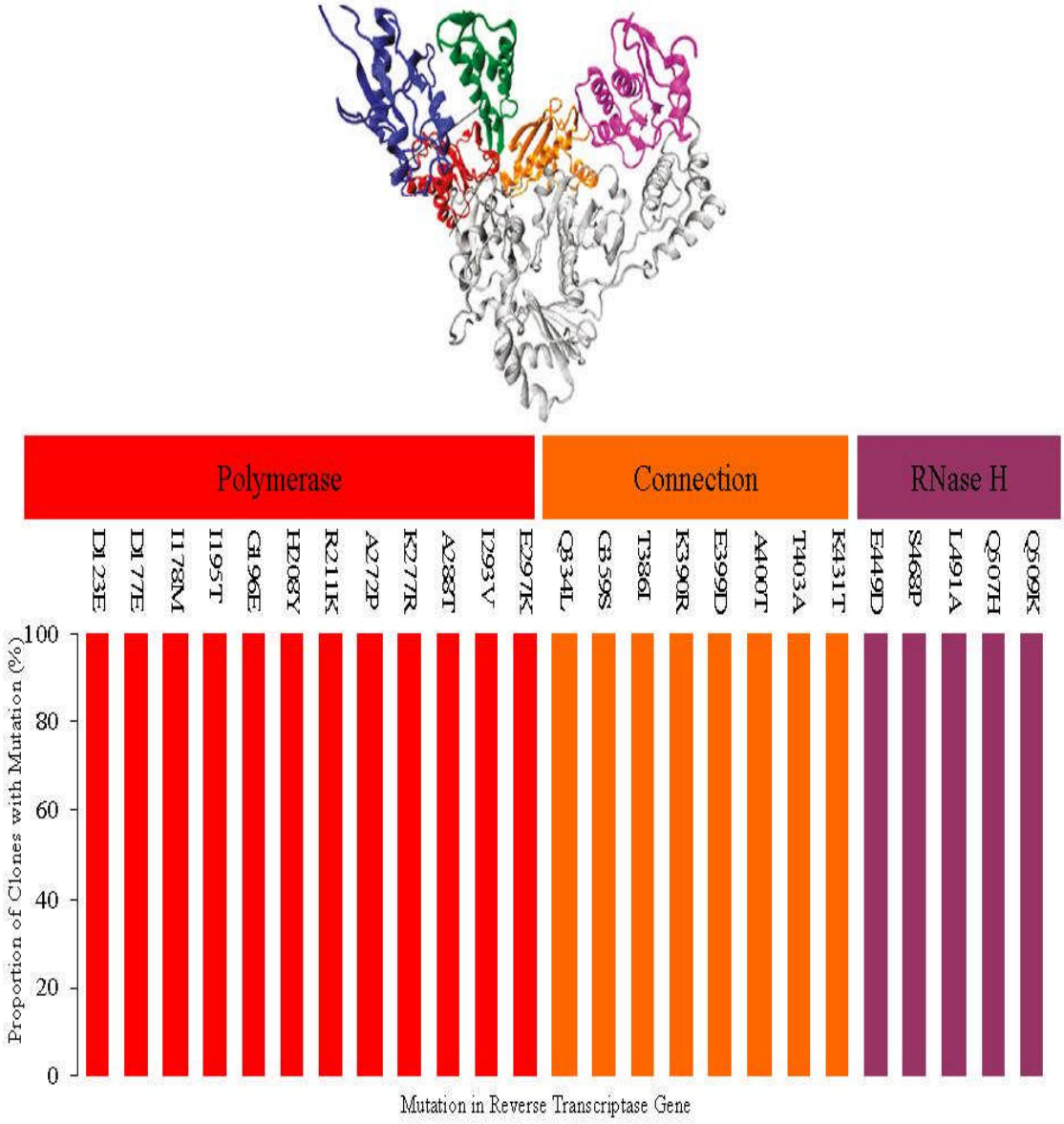


Figure 3.1 Clonal analysis from patient 1. The p66 subunit of RT is shown with the 5 subdomains; fingers (blue), palm (red), thumb (green), connection (orange) and RNase H (purple). The full-length RT gene from patient 1 was cloned and sequenced. The proportions (%) of clones (n=10) with mutations are shown. Sequences were analysed for the presence of mutations relative to HXB2 wild type. Mutations were classified according to the Stanford Genotypic Resistance Interpretation Algorithm Version 6.0.8. Bars in red denote mutations in the polymerase domain, bars in orange represent mutations in the connection domain and bars in purple represent mutations in the RNase H domain.

3.3.3.2 Full-length RT clones from patient 2 (n=11)

Patient 2 was treatment experienced and infected with subtype B and both population sequencing and clonal analysis of RT codons 1-335 showed several major RT resistance mutations and NRTI accessory mutations. Figure 3.2 shows the mutations present within the 11 full-length RT clones from this patient. They comprised as major NRTI resistance mutations the TAMs M41L, D67N, L210W, T215Y and K219N as well as V75M and V118I in all clones. The major NNRTI resistance mutations A98G and G190Q were also present in all clones.

Within the polymerase domain, patient 2 had the NRTI accessory mutations K43E, V60I, K122E, I135V and T200A in all clones with H208Y. Within the connection T377I was present in 1/11 (9%) clones. No known resistance or accessory mutations were found within the RNase H domain.

There was however variability in the clones relative to the HXB2 reference strain with several other mutations detected alongside H208Y that have unknown effects on drug susceptibility and viral fitness. In the polymerase domain these other mutations comprised of L310I, which was present in 2/11 (18%) clones. Within the connection domain, K350R was present in 8/11 (73%) clones, K390R was present in 7/11 (64%) clones and T386I was present in 4/11 (36%) clones. Within the RNase H domain, all clones had the mutations D460N, S468P, Q480E, H483N, L491P and K512Q.

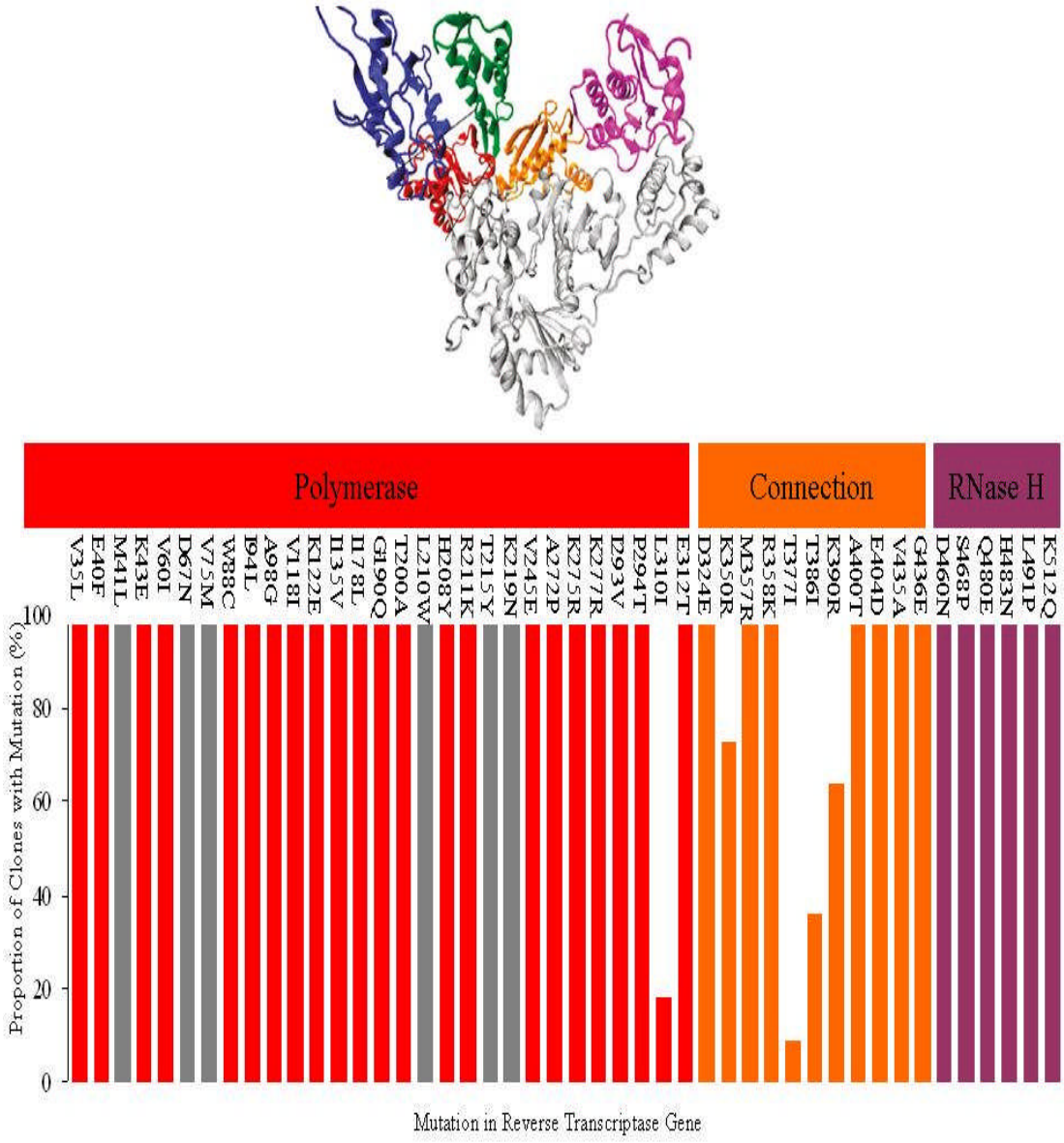


Figure 3.2 Clonal analysis from patient 2. The p66 subunit of RT is shown with the 5 subdomains; fingers (blue), palm (red), thumb (green), connection (orange) and RNase H (purple). The full-length RT gene from patient 2 was cloned and sequenced. The proportions (%) of clones (n=11) with mutations are shown. Sequences were analysed for the presence of mutations relative to HXB2 wild type. Mutations were classified according to the Stanford Genotypic Resistance Interpretation Algorithm Version 6.0.8. Bars in grey denote major NRTI resistance mutations in the polymerase domain, bars in red denote mutations in the polymerase domain, bars in orange represent mutations in the connection domain and bars in purple represent mutations in the RNase H domain.

3.3.3.3 Full-length RT clones from patient 3 (n=6)

Patient 3 was treatment experienced and infected with subtype A and both population sequencing and clonal analysis of RT codons 1-335 showed several major RT resistance mutations and NRTI accessory mutations. Figure 3.3 shows the mutations present within the 6 full-length RT clones from this patient. They comprised as major NRTI resistance mutations the TAMs D67N, T215F and K219E, as well as T69N and M184V present in all clones. K70KR was seen a mixture in the population sequencing but was not found in any of the 6 clones. No major NNRTI resistance mutations were found within the clones from patient 3.

Within the polymerase domain, patient 3 had the NRTI accessory mutations K20R, V60I, K122E, V179I and T200A in all clones with H208Y. H208Y was present in 1/6 (17%) of clones whereas in the population sequencing, patient 3 showed a mixture of H208HY. Within the connection domain, three NRTI accessory mutations associated were found, G359A, A371V and T377L which were present in all clones with H208Y.

There was however variability in the clones relative to the HXB2 reference strain with several other mutations detected alongside H208Y that have unknown effects on drug susceptibility and viral fitness. In the polymerase domain these other mutations comprised of I2L, which was present in 1/6 (17%) clones. Within the connection domain T369A was present in 1/6 (17%) clones. Within the RNase H domain, K454R and V466I were present in 2/6 (33%) of clones and T477A was present in 4/6 (67%) of clones.

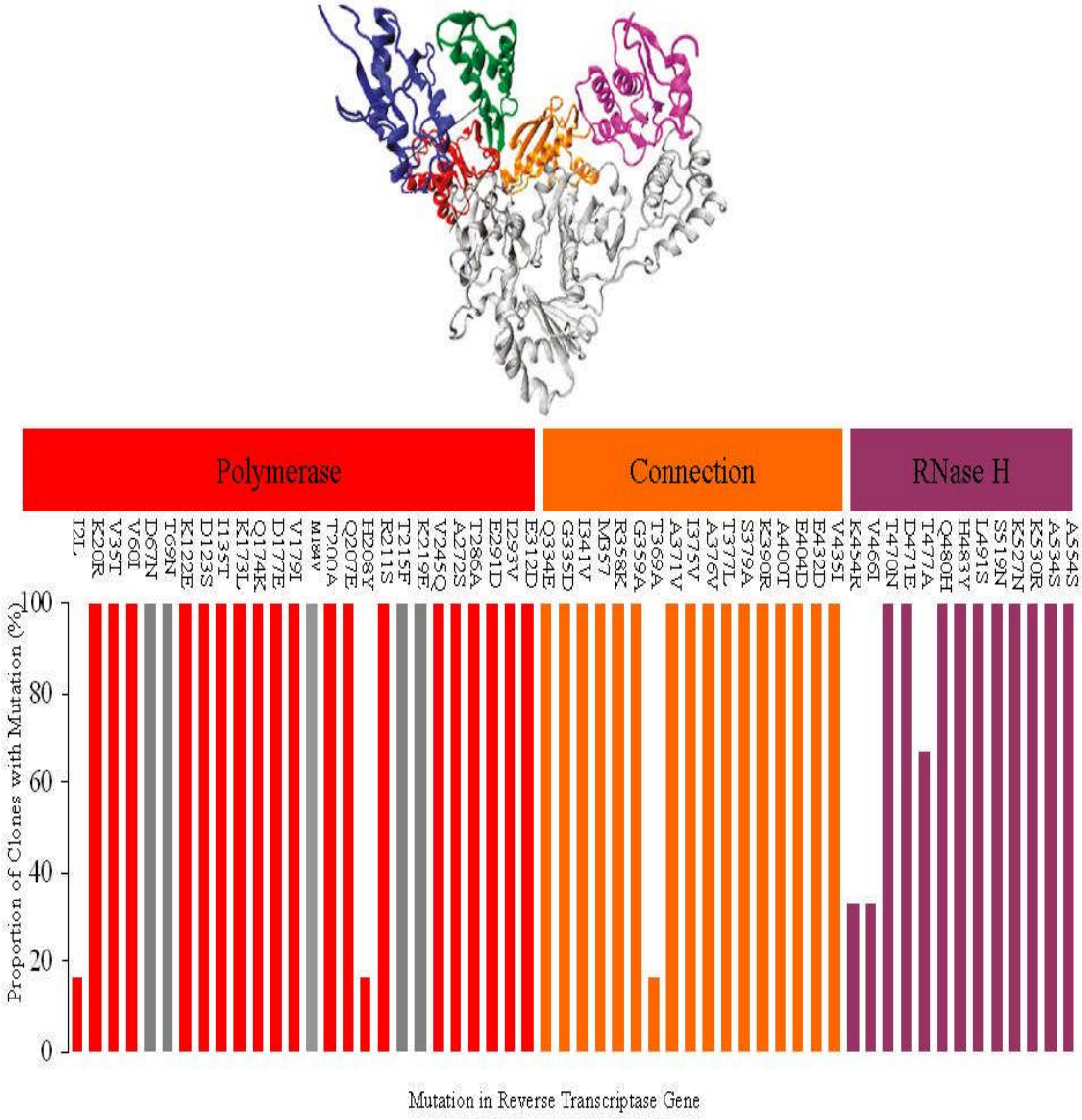


Figure 3.3 Clonal analysis from patient 3. The p66 subunit of RT is shown with the 5 subdomains; fingers (blue), palm (red), thumb (green), connection (orange) and RNase H (purple). The full-length RT gene from patient 3 was cloned and sequenced. The proportions (%) of clones (n=6) with mutations are shown. Sequences were analysed for the presence of mutations relative to HXB2 wild type. Mutations were classified according to the Stanford Genotypic Resistance Interpretation Algorithm Version 6.0.8. Bars in grey denote major NRTI resistance mutations in the polymerase domain, bars in red denote mutations in the polymerase domain, bars in orange represent mutations in the connection domain and bars in purple represent mutations in the RNase H domain.

3.3.3.4 Full-length RT clones from patient 4 (n=20)

Patient 4 was treatment experienced and infected with subtype C and both population sequencing and clonal analysis of RT codons 1-335 showed several major RT resistance mutations and NRTI accessory mutations. Figure 3.4 shows the mutations present within the 20 full-length RT clones from this patient. They comprised as major NRTI resistance mutations the TAMs M41L, D67N, L210W, T215Y and K219R as well as E44D, L74V, V118I and M184V present in all clones with H208Y. The major NNRTI resistance mutations A98G, K101H, Y181C and G190A were also present in all clones and V108I was present in 10/20 (50%) of clones with H208Y.

Within the polymerase domain, patient 4 had the NRTI accessory mutations T200A, E203K and K223Q present in clones with H208Y. E203K was found in 14/20 (70%) of clones, T200A were present in all clones and K223Q was present in 12/20 (60%) of clones. The G335D, R356K and T377L mutations were also found within the connection domain and present in all clones. The G335D mutation has been associated with contributing to AZT resistance in the context of TAMs (Zelina et al., 2008).

There was however variability in the clones relative to the HXB2 reference strain with several other mutations detected alongside H208Y that have unknown effects on drug susceptibility and viral fitness. In the polymerase domain these other mutations comprised of D123N and L205F, which were present in 8/20 (40%) of clones. Mutations S162N, R206K, H221Y, A272P and K277R were present in 2/20 (10%) of clones. Within the connection domain T386I mutation was present in 4/20 (20%) of clones, K390R was present in 6/20 (30%) of clones and G436E was present in 2/20

(10%) of clones. Within the RNase H domain mutations Q500L and K558I were present in 2/20 (10%) of clones and K527N was present in 8/20 (40%) of clones.

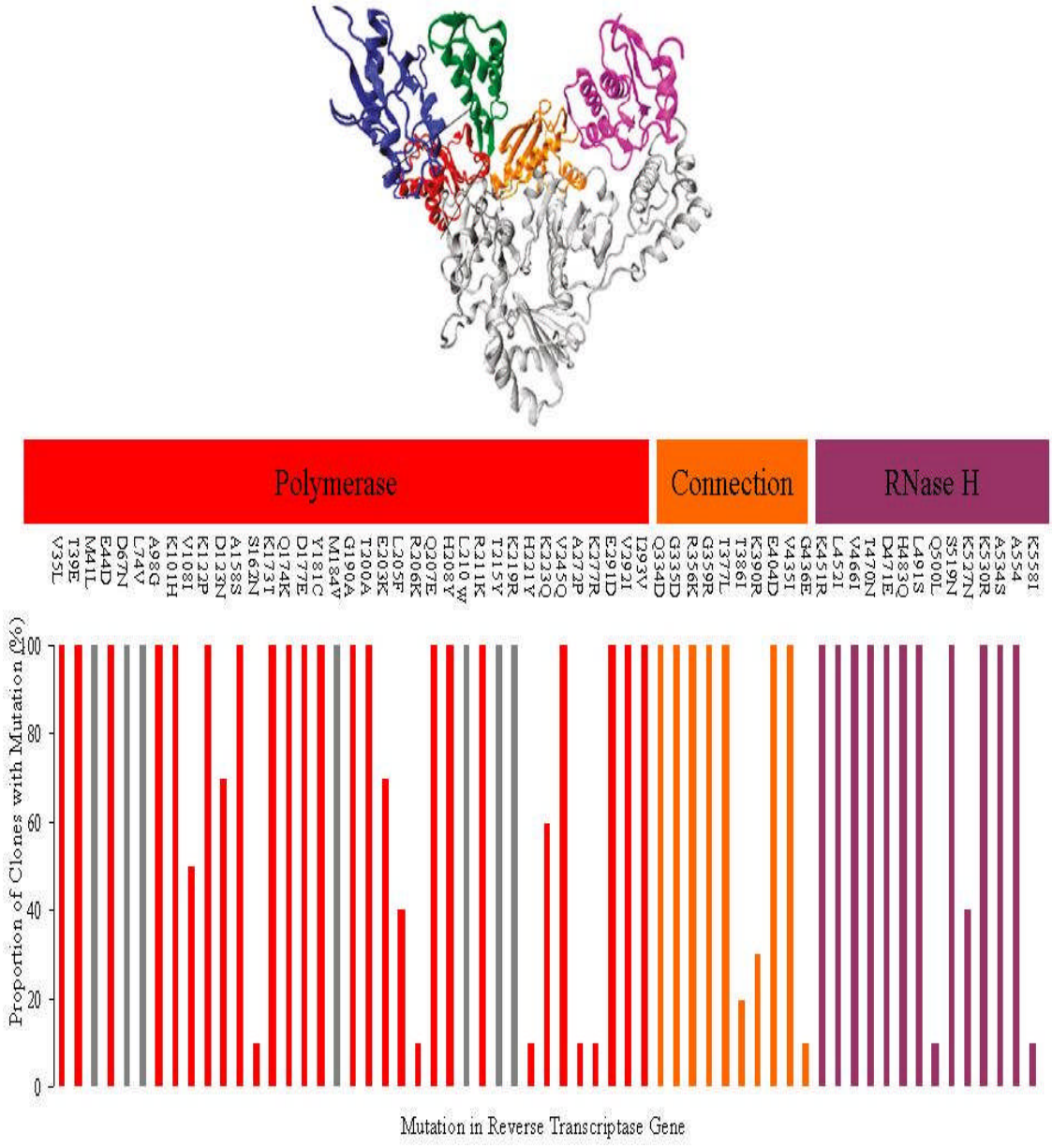


Figure 3.4 Clonal analysis from patient 4. The p66 subunit of RT is shown with the 5 subdomains; fingers (blue), palm (red), thumb (green), connection (orange) and RNase H (purple). The full-length RT gene from patient 4 was cloned and sequenced. The proportions (%) of clones (n=20) with mutations are shown. Sequences were analysed for the presence of mutations relative to HXB2 wild type. Mutations were classified according to the Stanford Genotypic Resistance Interpretation Algorithm Version 6.0.8. Bars in grey denote major NRTI resistance mutations in the polymerase domain, bars in red denote mutations in the polymerase domain, bars in orange represent mutations in the connection domain and bars in purple represent mutations in the RNase H domain.

3.4 **Discussion**

There have been two separate studies looking at the prevalence of H208Y in treatment naive and treatment experienced patients. The first study reported a prevalence of 7/2347 (0.3%) in treatment naïve patients and 165/4005 (4.1%) in treatment experienced patients (Nebbia et al., 2007). The other study reported a prevalence of 0.1% in treatment naïve patients and 12.8% in patients who had >4 TAMs (Cane et al., 2007). The HIV Drug Resistance Database maintained by Stanford University USA (http://hivdb.stanford.edu/) reports a prevalence of 24/18812 (0.1%) in treatment naïve patients and 2119/23212 (9%) in treatment experienced patients. It should be noted that although the prevalence of H208Y reported by the Stanford Database in treatment experienced patients is higher than that reported by published studies, the numbers used in the Stanford database are much larger than those used by (Nebbia et al., 2007) or (Cane et al., 2007). Furthermore, the treatment history and perhaps the HIV-1 subtype are also likely to influence prevalence estimates as the Stanford database contains many sequences from treatment experienced patients and subtype B viruses. It should also be taken into account that the studies by Gaia et al. and Cane et al. both used sequences from the UK HIV Drug Resistance Database and therefore they reflect similar patient populations in terms of treatment history and distribution of HIV-1 subtypes. Upon examination of the HIV Resistance Database held at the Royal Free Hospital, 17 patients harboured H208Y. The prevalence of H208Y in treatment experienced patients was 12/1304 (0.9%) and higher than that found in treatment naïve patients 5/3783 (0.1%) and was in agreement with the previously mentioned reports from the

UK. These observations support the hypothesis that the H208Y mutation is an accessory mutation that emerges as a result of drug pressure.

Regarding subtype differences, Nebbia et al. 2007 reported that in the 165 treatment experienced patients who harboured H208Y, H208Y was most commonly observed in subtype B patients with a prevalence of 118/165 (71.6%), followed by 26/165 (15.6%) in subtype C patients. The Stanford Database showed that in patients who had been exposed to RT inhibitors, H208Y was most common in subtype F with a prevalence of 60/461 (13%). This was followed by 1959/17806 (11%) in subtype B, 20/751 (2.7%) in subtype G, 48/1831 (2.6%) in subtype C and 13/560 (2.3%) in subtype A. In treatment naïve patients, the prevalence reported by the Stanford Database for subtypes B, C, D and CRF02_AG was similarly low at 0.1-0.2%. Similar to the study by Nebbia et al., in the treatment experienced population described in this study, 8/12 (67%) of patients with H208Y had a subtype B virus, followed by 2/12 (17%) of patients with a subtype C virus. The remaining two patients had a subtype A and subtype G virus. Although these findings are not consistent with those from the Standard Database, it should be noted that the number of samples used to calculate prevalence in the Stanford Database and in this study varies considerably between subtypes and therefore, the prevalence reported may be biased. For instance the total number of patients with subtype F and subtype G viruses in the HIV Database held at the Royal Free Hospital was 16 and 139 respectively, compared to 461 subtype F and 751 subtype G samples from the Stanford Database. Analysis of the prevalence of H208Y in people with different subtypes should keep in mind one important caveat. The increased prevalence of the mutation in people with multiple TAMs suggests selection through prolonged drug

pressure with the thymidine analogues AZT and D4T. This is more likely to have occurred in resource-rich regions where antiretroviral therapy has been available for many years and where subtype B predominates.

The H208Y mutation results from a single nucleotide change from CAT to TAT. To examine the sequence variation at codon 208 between subtypes, consensus sequences for subtypes A, B, C, D, F, G, CRF01_AE and CRF02_AG were obtained from the Los Alamos Database (http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html The nucleotide sequence at codon position 208 was examined and for all subtypes, the nucleotide sequence was always CAT at codon 208. This argues against a sequence related preferential selection of H208Y and indicates a high degree of conservation at this position.

Consistent with previous observations (Cane et al., 2007;Gonzales et al., 2003;Sturmer et al., 2003;Svicher et al., 2006), population RT sequencing of the treatment experienced patients with the H208Y mutation demonstrated that it occurred with multiple major RT resistance mutations, particularly multiple TAMs. The results from the clonal analysis of RT codons 1-335 confirmed the linkage with TAMs. Importantly, the clones from the treatment experienced patients were found to be homogenously resistant. The clonal analysis was extended to full-length RT to assess the presence of mutations in the C terminus of the gene which may contribute to NRTI resistance (Brehm et al., 2008;Ehteshami and Gotte, 2008;Nikolenko et al., 2007;Yap et al., 2007;Hachiya et al., 2008). In order to minimise the chances of over sampling the same clone, multiple PCR reactions were used to generate clones for each patient. Again the full-length RT clones were homogenous within each patient

with regard to the proportion of clones that contained major NRTI resistance mutations. This may be expected in heavily treated persons in whom selective pressure leads to a highly mutated quasispecies becoming dominant. Population sequencing in routine use in clinical practice can only identify virus variants that represent over 20% of the population (Alcorn and Faruki, 2000). The clones selected for analysis also represent the dominant quasispecies within each patient.

The subtype A virus from the treatment experienced patient showed a mix of H208HY in the population sequence, indicating a mixture was present in the dominant quasispecies and indeed the clonal analysis identified 1/6 clones that contained the H208Y mutation with the remaining 5/6 clones with H208. It should be noted that only six clones were sequenced for this patient. The remaining patients had all clones with the H208Y mutation indicating it is present in the dominant quasispecies of these patients.

The plasma HIV-1 RNA load of the sample is an important factor that will influence sampling of the viral quasispecies. This is because if the viral load is low, it is unlikely that the subsequent PCR and cloned sequence will have been derived from different RNA templates and therefore a true representation of the genetic diversity within the patient will not be obtained (Liu et al., 1996).

All the patient samples had a viral load over 400,000 copies per millilitre and the results from the sequencing analysis showed that there was variability in the patient clones. Excluding the treatment naïve patient, who had identical clones, the clones from the three treatment experienced patients showed more variability. The clones

from the treatment experienced subtype B and A patients had 11% of clones that were unique. The clones from the treatment experienced subtype C patient had 26% of clones that were unique. This demonstrates that although there was not large variability between clones, a representation of the quasispecies was sampled for each patient, as the clones were not all identical to one another. It should also be noted that only a small number of clones were sequenced for each patient.

In order to obtain a wider representation of low frequency variants within the quasispecies, a large number of clones would need to be sequenced or alternative sequencing methods (e.g., ultra-deep pyrosequencing) employed, which was beyond the scope of these studies. Clonal analysis of the dominant quasispecies however allows an analysis of the linkage of mutations at the genomic level. Given that H208Y is a mutation that usually emerges following the appearance of major NRTI resistance mutations, it is most likely to be present within the dominant quasispecies of a patient once major resistance mutations have evolved.

Another important consideration is that PCR amplification may introduce errors though the incorporation of incorrect nucleotides. Errors introduced during the early round of PCR amplification will result in the subsequent clones containing the PCR induced sequencing errors (Varghese et al., 2010). This problem can be overcome to an extent by using a proof-reading polymerase such as *Pfu* high-fidelity polymerase which has 3'-5' exonuclease activity, as done in this study. An alternative way to address this issue is to perform limiting dilution of the cDNA so that a single template is used in the PCR and sequencing reactions. By using this technique, even if an error is introduced during the early stages of PCR, the error will not be present

at greater than 25% of the bases at that position in the mixture and therefore will not result in an erroneous base call when the DNA is sequenced (Learn, Jr. et al., 1996).

The TAMs are a group of major NRTI resistance mutations that confer resistance to D4T and AZT (Miller, 2004). The mechanism of NRTI resistance mediated by the TAMs is known as NRTI excision or pyrophosphorolysis. In the presence of ATP or pyrophosphate, the TAMs can mediate the excision of the incorporated NRTI through a nucleophilic attack on the phosphodiester bond between the NRTI and the DNA. This results in the removal of the NRTI and allows chain synthesis to continue (Acosta-Hoyos and Scott, 2010;Clavel and Hance, 2004a).

The H208Y mutation has previously been associated with TAMs and this association is augmented with an increasing number of TAMs. The TAM-1 pathway (M41L, L210W and T215Y) has been predominantly associated with the H208Y mutation (Cane et al., 2007;Nebbia et al., 2007;Saracino et al., 2006;Svicher et al., 2006). The findings of this study show that there is a strong genetic linkage with TAMs within viruses that are highly drug resistant. Among the treatment experienced patients, the TAMs were the predominant group of mutations that were associated with the H208Y mutation and were present in all clones for all patients. Two of these patients had clones containing the TAM-1 pathway mutations and one patient had clones containing the TAM-2 pathway mutations. The TAMs that were always detected with H208Y in the clones from treatment experienced patients included D67N, T215Y or F and various mutations at codon 219. Based upon these observations, it can be speculated that H208Y may have a role in augmenting NRTI resistance by aiding the pyrophosphorolysis effect of T215Y or T215F. A key mutation in

pyrophosphorolysis is T215Y/F. The aromatic ring of the ATP interacts with the aromatic ring of the tyrosine amino acid through π-π interactions. This interaction changes the binding of ATP and affects its orientation enabling more efficient binding (Singh et al., 2010;Chamberlain et al., 2002;Meyer et al., 2002;Arion et al., 1998;Sarafianos et al., 2009).

The H208Y mutation did not show a consistent linkage with M184V. The M184V mutation causes high level resistance to 3TC and emtricitabine (FTC) by decreasing the incorporation of the two NRTIs and allowing DNA synthesis to continue. M184V has been reported to be present in approximately 44% of patients that already harbour the H208Y mutation (Nebbia et al., 2007). This is consistent with the findings reported in this study, showing that half of the patients with H208Y had the M184V mutation, but also implying that M184V is not a prerequisite for the H208Y mutation. M184V carries a significant fitness cost for the virus, for which compensatory mechanisms have not been yet clearly identified (Diallo et al., 2003), possibly because of its positioning near the catalytic site of the enzyme (Perno et al., 2006). M184V has also been shown to antagonise NRTI pyrophosphorolysis mediated by mutations at position T215, partially restoring susceptibility to AZT, D4T and TDF (Gotte et al., 2000;Menendez-Arias, 2008). It would be tempting to speculate that H208Y either counterbalances the fitness reduction caused by M184V or interferes with its antagonists effects on NRTI susceptibility, but the fact that H208Y occurs independently of M184V at both the population and clonal level does not support the hypothesis.

There were a number of other NRTI and NNRTI resistance associated mutations that were consistently present in the clones with H208Y, including the NRTI resistance mutations E44D, T69N/D, L74V, V75M and V118I. The V118I mutation was present in clones from two treatment experienced patients. Several of these mutations (E44D, T69N/D and V118I) also tend to occur in NRTI-experienced patients in combination with TAMs (Miranda et al., 2005;Girouard et al., 2003;Zaccarelli et al., 2007). NNRTI resistance mutations present were A98G, K101H, V108I, Y181C and G190A/Q. Among the three treatment experienced patients, A98G and G190A/Q were present with H208Y in all clones from two patients. The presence of NRTI and NNRTI resistance mutations reaffirms that patients with H208Y usually have had extensive treatment exposure.

Some NNRTI resistance mutations have been shown to increase susceptibility to the NRTIs and this includes I132M and Y181C. The Y181C mutation confers high-level resistance to NVP and delavirdine (DLV) and low-level resistance to EFV (Johnson et al., 2009;Balzarini et al., 1994). It has also been shown to confer an increased susceptibility to AZT when present with M41L and T215Y (Baldanti et al., 2003;Byrnes et al., 1994;Larder, 1992). The mechanism of this increased susceptibility is a reduced ability of the RT to excise AZT-monophosphate from chain-terminated primers (Selmi et al., 2003).

The I132M mutation also confers high-level resistance to NVP and DLV and low-level resistance to EFV (Nissley et al., 2007). It has also been shown to confer an increased susceptibility to 3TC and TDF and have a lower fitness compared to the wild type virus. The mechanism of increased susceptibility to 3TC and TDF is

different to that mediated by Y181C and is based on the ability of the mutant RT to discriminate between the natural nucleotide and the nucleoside analogue (Ambrose et al., 2009).

Among the treatment experienced patients, Y181C and H208Y were present in all clones from the subtype C patient. No previous studies have looked at the association of H208Y with Y181C and looking at the patient clones within this study, no strong association of these mutations appear to occur since Y181C is not a prerequisite for H208Y. Furthermore, since the hypothesis remains that H208Y is associated with the TAM group of mutations which mediate NRTI excision, it is perhaps not surprising that there is no association of these mutations together since Y181C causes re-sensitisation to AZT.

Many studies have showed the importance of mutations within the connection and RNase H domains in resistance, which confer resistance to NRTIs and NNRTIs. Mutations in the connection domain that have confer NRTI and NNRTI resistance includes N348I which confers resistance to AZT, DDI and NVP (Yap et al., 2007), A371V which confers resistance to AZT, 3TC and ABC (Brehm et al., 2007), G335D which confers resistance to AZT and 3TC (Zelina et al., 2008) and Q509L which confers resistance to AZT, 3TC and ABC (Brehm et al., 2007;Hachiya et al., 2008;Nikolenko et al., 2007;Roquebert and Marcelin, 2008a).

In this study, two connection domain mutations were found in clones with H208Y, G335D and A371V. The G335D mutation has been demonstrated to confer AZT and 3TC resistance in two ways. Firstly in combination with M184V, G335D confers an

increased level of discrimination of the NRTI compared to the natural dNTP. Secondly, in combination with TAMs and M184V, the G335D mutation can help to increase the excision of the NRTI from the DNA chain and thus allow chain synthesis to resume (Roquebert and Marcelin, 2008b;Zelina et al., 2008). The two patients with this mutation also showed M184V and TAMs, which is in agreement with these findings. The A371V mutation in combination with TAMs has been demonstrated to increase AZT resistance (Hachiya et al., 2008;Santos et al., 2008). The patient with A371V had TAMs but also the M184V mutation, which has not been associated with A371V (Brehm et al., 2007).

Three mutations in the RNase H domain have been shown to confer resistance to AZT, H539N, D549N and Q509L (Brehm et al., 2008;Roquebert and Marcelin, 2008a). None of these mutations was found within the clones from treatment experienced patients, although all clones from the treatment naïve patient had Q509K. To develop into Q509L, Q509K would require multiple nucleotide changes (from AAA or AAG to CTC, CTT, CTA, CTG, TTA or TTG), and its detection has therefore unclear significance in terms of potential effects on drug susceptibility.

Other NRTI accessory mutations previously associated with TAMs were also found within all patient clones. These were E40F and K43E, which were found within all clones with H208Y from patient 2. Recent data have further indicated that E40F confers resistance to AZT up to 187 fold if present in the background of TAMs (Huigen et al., 2008). The K43E mutation, when present in isolation does not show significant effects on drug susceptibility or viral fitness. In combination with E40F in a background of TAMs, however K43E increases resistance to AZT by up to 135

fold. The virus replicative capacity as a measure of viral fitness is also increased (Huigen et al., 2008).

There were three NRTI associated accessory mutations within the polymerase domain that were consistently present in the clones with H208Y. These were found at codon positions V35, K122 and T200 (Cane et al., 2007;Perno et al., 2006). All three treatment experienced patients from whom RT clones were generated had mutations present at RT position V35 comprising V35L and V35T. The V35M mutation has been identified as an NRTI accessory mutation, which is associated with ABC and TDF resistance. Interestingly, the V35I mutation is not associated with NRTI resistance and is a common polymorphism in treatment naïve patients (Perno et al., 2006;Svicher et al., 2006). Patient 3 had the V35T mutation present in all its clones with H208Y whereas patients 2 and 4 had the V35L mutation present with H208Y in all their clones, which has not been associated with resistance. All three treatment experienced patients from whom RT clones were generated had mutations present at RT position K122 comprising K122E and K122P. The K122E mutation has been identified as an NRTI accessory mutation, which is associated with AZT, D4T and TDF resistance (Perno et al., 2006;Svicher et al., 2006). Patients 2 and 3 had the K122E mutation present in all its clones with H208Y whereas patient 4 the K122P mutation present with H208Y in all their clones, which has not been associated with resistance. All three treatment experienced patients had clones with H208Y and T200A.

There were a number of NRTI associated accessory mutations within the connection domain that were consistently present in the clones with H208Y. These were

G359A/R/S and T377L (Cane et al., 2007;Torti et al., 2004). Mutations at position G359 were present in all clones with H208Y from the treatment naïve patient and the subtype A and C treatment experienced patients. Mutations at position T377 were present in all clones with H208Y from the treatment experienced subtype A and C patients and in 1/11 (9%) of clones from the subtype B treatment experienced patient.

The presence of H208Y and mutations within the connection and RNase H domain that have been associated with contributing to NRTI resistance does not indicate that H208Y is linked to other NRTI resistance mutations. However, the presence of these additional mutations in some of the patients is consistent with the general observation that these viruses have been subjected to prolonged drug pressure with NRTIs and have evolved multiple mechanisms for coping with this pressure.

In summary, the findings showed that H208Y was associated with TAMs, in particular mutations at positions D67, T215 and K219. The clones from the treatment experienced patients had H208Y always present with accessory mutations at positions V35, K122 and T200 and other mutations with unknown effects on drug susceptibility and fitness at positions V245, A272, I293, T377, K390, E404, V435, H483 and L491. Since there is a strong association with H208Y and the TAMs, it can be speculated that H208Y works in two ways. Firstly, H208Y could further augment NRTI resistance to NRTIs conferred by the TAMs and secondly, H208Y could increase the fitness of the TAM virus.

Chapter four

4 Phenotypic impact of H208Y on drug susceptibility

4.1 Introduction

In the previous chapter, the RT gene from patients harbouring H208Y was cloned and sequenced. The results from the clonal analysis showed that the H208Y mutation was always associated with mutations at codon positions D67, T215 and K219. These mutations are members of the thymidine analogue mutation (TAM) group of mutations, which are known to contribute significantly to NRTI resistance (Johnson et al., 2009). The results were consistent with previous observations that the H208Y mutation is most often observed in patients who have an extensive treatment history and harbour TAMs (Cane et al., 2007;Sturmer et al., 2003;Svicher et al., 2006). It was therefore hypothesized that H208Y is an accessory mutation that arises in a background of major resistance mutations in order to increase drug resistance or improve viral fitness. In order to test this hypothesis, recombinant viruses containing patient derived RT genes with and without H208Y were constructed. These recombinant viruses were used in drug susceptibility assays to determine whether H208Y had an impact on resistance to the RT inhibitors NRTIs and NNRTIs. Based upon the observed association with TAMs, it was hypothesised that the H208Y mutation would confer an increased resistance to the NRTIs, and particularly to the thymidine analogues AZT and D4T.

There are two main methods to measure drug susceptibilities *in vitro*, a single and multiple cycle system. Both methods involve cloning the gene of interest into a defective molecular clone to produce a recombinant virus. This virus is then incubated in the presence of increasing concentrations of drug, and the drug concentration required to reduce replication of the recombinant virus by 50% relative

to a control virus (IC50) is calculated. The main difference between these assays is that the single cycle system only allows one cycle of virus replication to occur whereas the multiple cycle assay allows continuous rounds of replication (Quinones-Mateu and Arts, 2002). In this study, the multiple cycle method was used in order to determine the drug susceptibilities of the viruses constructed. The reasons for choosing this method were to gain a close as possible reproduction of conditions *in vivo* and obtain viruses that could be used in growth competition experiments.

4.2 Methods

4.2.1 Patient group

Plasma samples from four patients with H208Y were used for further analysis. This included a subtype B treatment naïve patient, a subtype B treatment experienced patient, a subtype A treatment experienced patient and a subtype C treatment experienced patient. The treatment naïve subtype B patient harboured no major resistance mutations. The treatment experienced subtype B patient harboured the TAMs M41L, D67N, L210W, T215Y and K219N together with V75M, A98G and G190Q. The treatment experienced subtype A patient harboured the TAMs D67N, K70R, T215F and K219E together with T69N, A98G and G190Q. The treatment experienced subtype C patient harboured the TAMs M41L, D67N, L210W, T215Y and K219R together with E44D, L74V, A98G, K101H, V108I, Y181C and G190A/Q. The treatment experienced subtype A and C patients also harboured M184V which was back mutated to eliminate its effect in the drug susceptibility assay.

4.2.2 Site directed mutagenesis

The most commonly occurring clone from each patient was selected for further analysis. These clones all had H208Y and were reverted to the wild type codon 208 amino acid (tyrosine to histidine) by site directed mutagenesis. All site directed mutants underwent Sanger sequencing to confirm the correct amino acid was present.

4.2.3 Production of recombinant viruses

Patient derived PCR product containing the RT gene with and without H208Y and the molecular clone pHIVΔRTBstEII were transfected into MT4 cells. Cells were

monitored for CPE and once peak CPE was observed, the virus supernatant was aliquoted. RNA was extracted from the virus supernatant and Sanger sequencing was used to confirm the correct mutations were present.

4.2.4 Virus titrations

Once the recombinant viruses were constructed two methods were used to determine their viral titre. These were the CPE titration method and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) titration method (table 4.1). The CPE titration method was a qualitative assay that involved scoring wells based on whether any CPE was present. The MTT titration method was a quantitative assay that used MTT as a cell viability marker to measure the number of live cells present. The Reed and Muench equation was used to calculate the tissue culture infectious dose (TCID50) of each virus. All virus titrations were done in MT4 cells. For MTT titrations, 2-3 independent replicate experiments were performed and CPE titrations were done once.

4.2.5 Optimisation of incubation time in drug susceptibility assay

Drug susceptibility assays were performed in quadruplicate and the average of the four was used to calculate the IC50. Results are presented as mean IC50 of 3-5 replicate experiments for each virus/drug combination. HXB2 was used as a wild type control in all assays.

4.2.6 Alignment of sequences

The RT amino acid sequences from patients were aligned using ClustralW2. Subtype A and C sequences were obtained from the Los Alamos Database

(http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html) and used to create consensus sequences. To create the consensus subtype A and C sequence, 153 subtype A and 761 subtype C sequences were used. The consensus sequences were compared to corresponding patient sequence. For the subtype B sequences, HXB2 was used as the consensus sequence.

4.3 Results

4.3.1 Virus titrations

Table 4.1 shows the virus titres determined using the two different methods. The titres obtained with both methods were comparable as they lied within a 2-fold difference of each other. The MTT titres were used for the drug susceptibility assays.

4.3.2 Optimisation of incubation period in drug susceptibility assay

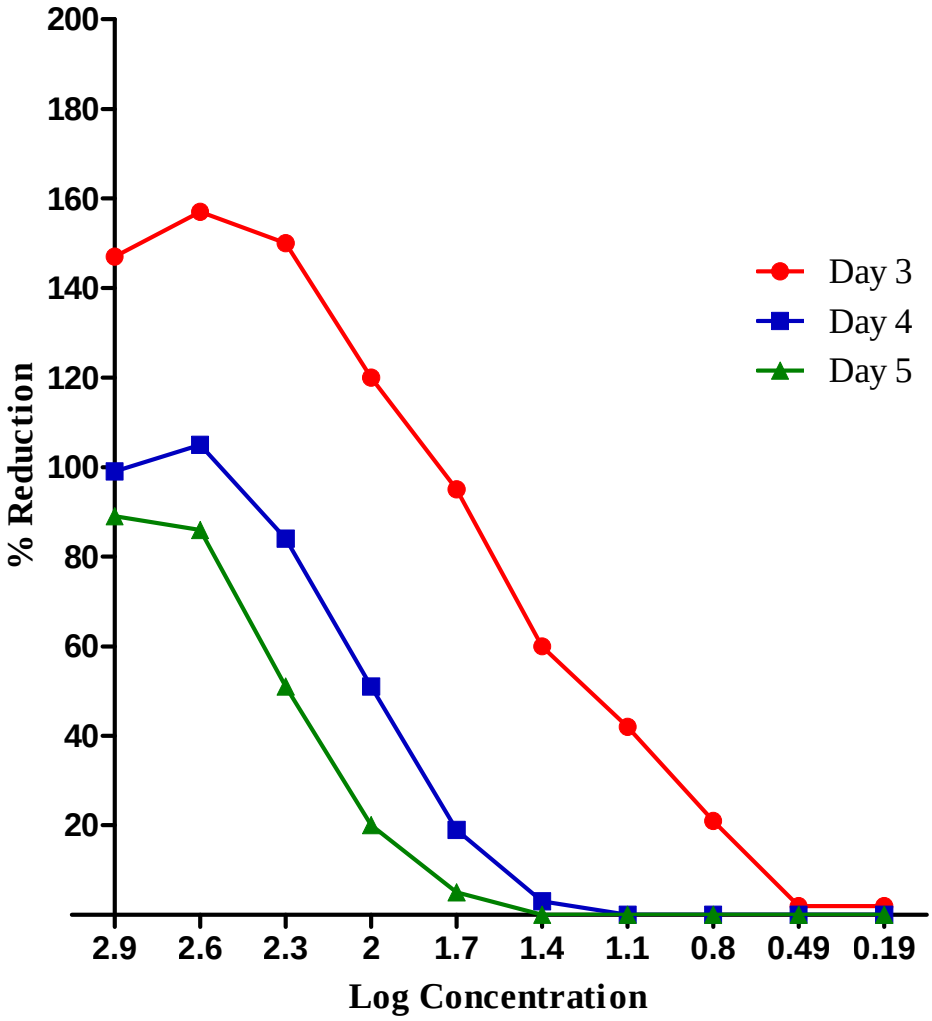
As shown in figure 4.1 the IC50 values obtained over the three days were variable between the different days. There was over a 5-fold difference in the IC50 value obtained on day 3 and day 4, but there was less variability between values obtained on day 4 and day 5 with only a 2 fold difference. Table 4.2 shows how the IC50 values for AZT obtained in this study compared to those available from published studies. Table 4.2 also shows published IC50 values for other NRTIs and results obtained with both HXB2 and TAM-containing viruses (Hertogs et al., 1998;Naeger et al., 2001;Ross et al., 2001;Garcia-Lerma et al., 2003;Garcia-Perez et al., 2007;Pernas and Lopez-Galindez, 2008;Taylor et al., 1999). It should be noted that the IC50 values were obtained using different phenotypic assays.

The published AZT IC50 range for HXB2 using the MTT/MT4 assay ranges between 0.007µM and 0.1µM. In this study, the IC50 obtained on day 4 was within this range and therefore day 4 post-infection was selected for the analysis of all drug susceptibility assays.

Table. 4.1 Recombinant virus titres[a]

Virus[b]	MTT Titration $TCID_{50}$/ml	CPE Titration $TCID_{50}$/ml
HXB2 Wild type	$2.0X10^4$	$2.0X10^4$
Subtype B naïve H208	$6.3X10^4$	$6.0X10^4$
Subtype B naïve H208Y	$4.7X10^4$	$5.0X10^4$
Subtype B experienced H208	$3.6X10^3$	$7.0X10^3$
Subtype B experienced H208Y	$2.7X10^3$	$5.8X10^3$
Subtype A experienced H208	$2.4X10^4$	$2.0X10^4$
Subtype A experienced H208Y	$1.8X10^3$	$1.5X10^3$
Subtype C experienced H208	$2.5X10^4$	$5.0X10^4$
Subtype C experienced H208Y	$6.3X10^4$	$5.8X10^4$

[a]Average of 2-3 independent replicate experiments. The TCID50 was calculated using the Reed and Muench equation. The equation was as follows; $M = \text{inv log}\{x_1 + [(x_2 - x_1)((y_1 - 50) / y_1 - y_2)]\}$, where y_1 = percent of well scored positive closest to, but higher than 50% at a certain virus dilution, y_2 = percent of well scored positive closest to, but lower than 50% at a certain virus dilution, x_1 = the log (dilution of the virus where was observed), x_2 = the log (dilution of the virus where was observed). [b]Viruses with H208Y were reverted to H208 by side directed mutagenesis.



Day	IC50 (uM)
3	0.017
4	0.098
5	0.197

Figure 4.1 Drug susceptibility curves and IC50µM values for AZT obtained against HXB2 on day 3, 4 and 5 post-infection.

Table. 4.2 Published NRTIs IC50 values with HXB2 and TAM containing viruses

Virus	Assay	Drug	IC50 (µM)	Reference
HXB2[a]	Multiple cycle MTT/MT4	ABC DDI 3TC TDF AZT D4T	2.17 4.840 1.977 23.288 0.099 1.953	This study
HXB2	Multiple cycle MTT/MT4	ABC DDI 3TC TDF AZT D4T	1.36 0.75 - 10 0.91 - 7 0.48 – 4.4 0.007 – 0.1 1 – 8	Garcia-Lerma et al. 2003; Hertogs et al. 1998; Taylor et al. 1999; Naeger et al. 2001; Pernas et al. 2008
HXB2 + D67N, K70R, T215F, K219Q	MTT/MT4	AZT	0.6	Taylor et al. 1999
HXB2 + D67N, K70R, T215Y	XTT/MT2	AZT TDF 3TC	5.1 ± 1.40 8.2 ± 1.2 7.4 ± 0.9	Naeger et al. 2001
HXB2 + ≥ 3 TAMs	Virco (multiple cycle)	D4T	6.7	Ross et al. 2001
pNL4-3 + ≥ 3 TAMs	Phenosense (single cycle)	D4T	2	
pNL4-3 + M41L, K70R, T215F, K219R		AZT D4T 3TC TDF	0.408 0.12 0.432 4.42	
pNL4-3 + M41L, E44D, D67N,V118I, L210W, T215Y, K219R	MT2 luciferase (single cycle)	DDI ABC AZT D4T 3TC TDF DDI ABC	0.510 0.298 0.624 0.179 0.696 4.96 0.867 0.269	Garcia-Perez et al. 2007

[a]The IC50 values obtained with the MTT/MT4 assay performed in this study using HXB2 wild type and a panel of NRTIs are shaded.

4.3.3 Impact of H208Y on drug susceptibility

We studied the plasma viruses from four patients. These comprised a treatment naïve patient who unusually showed the presence of H208Y in the absence of major resistance mutations in RT, and three treatment experienced patients who showed H208Y in the presence of TAMs and other major resistance mutations. The RT gene with H208Y and a SDM without H208Y derived from the former were cloned into the subtype B molecular clone pHIVΔRTBstEII, and tested in quadruplicate in 3-5 independent replicate experiments in the presence of RT inhibitors.

4.3.3.1 H208Y in the context of subtype B wild type virus from a treatment naïve patient

Figure 4.2 shows the mean IC50 (μM) values obtained with a panel of NRTIs. Compared to the H208 SDM, the presence of H208Y in the absence of major resistance mutations increased susceptibility to 3TC, AZT, ABC, DDI and D4T, but decreased susceptibility to TDF.

Hypersusceptibility is usually defined as a virus that has a fold change of at least 0.4 compared to the wild type control virus (Clark et al. 2006). The presence of H208Y conferred hypersusceptibility to AZT by 0.24 fold, to ABC by 0.29 fold and to EFV by 0.28 fold.

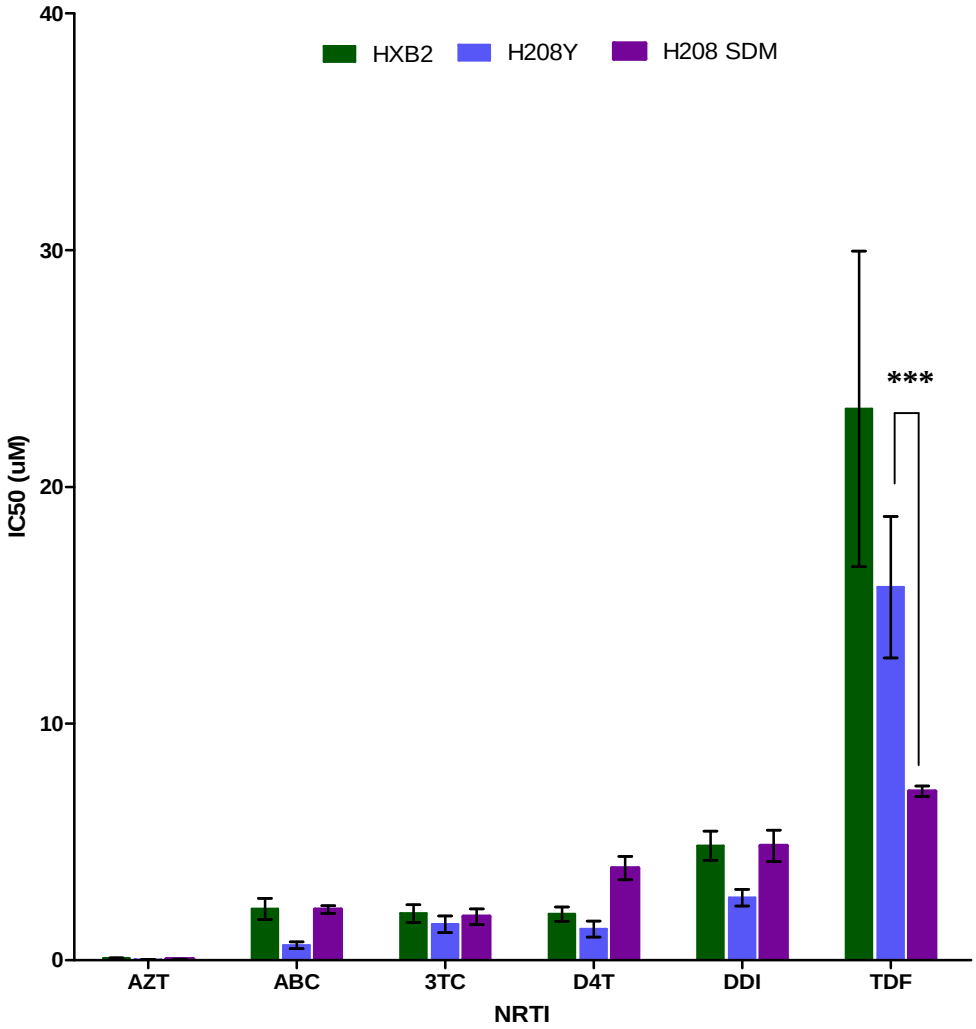
For 3TC, the IC50 values were 1.52μM and 1.84μM with and without H208Y, with a fold change relative to the HXB2 wild type control of 0.77 and 0.93 (Δ +0.16) respectively. For AZT, the IC50 values were 0.024μM and 0.044μM with and without H208Y, with a fold change of 0.24 and 0.44 (Δ +0.20) respectively. For

ABC, the IC50 values were 0.63µM and 2.14µM with and without H208Y, with a fold change of 0.29 and 0.99 (Δ +0.70) respectively. For DDI, the IC50 values were 2.65µM and 4.83µM with and without H208Y, with a fold change of 0.55 and 1 (Δ +0.45) respectively. For D4T, the IC50 values were 1.32µM and 3.89µM with and without H208Y, with a fold change of 0.67 and 2 (Δ +1.33) respectively. For TDF, the IC50 values were 15.75µM and 7.14µM with and without H208Y, with a fold change of 0.68 and 0.31 (Δ -0.37) respectively.

Figure 4.3 shows the drug susceptibility profile of the same virus with a panel of NNRTIs that consisted of NVP, EFV and ETV. H208Y increased susceptibility to NVP and ETV but reduced susceptibility to EFV.

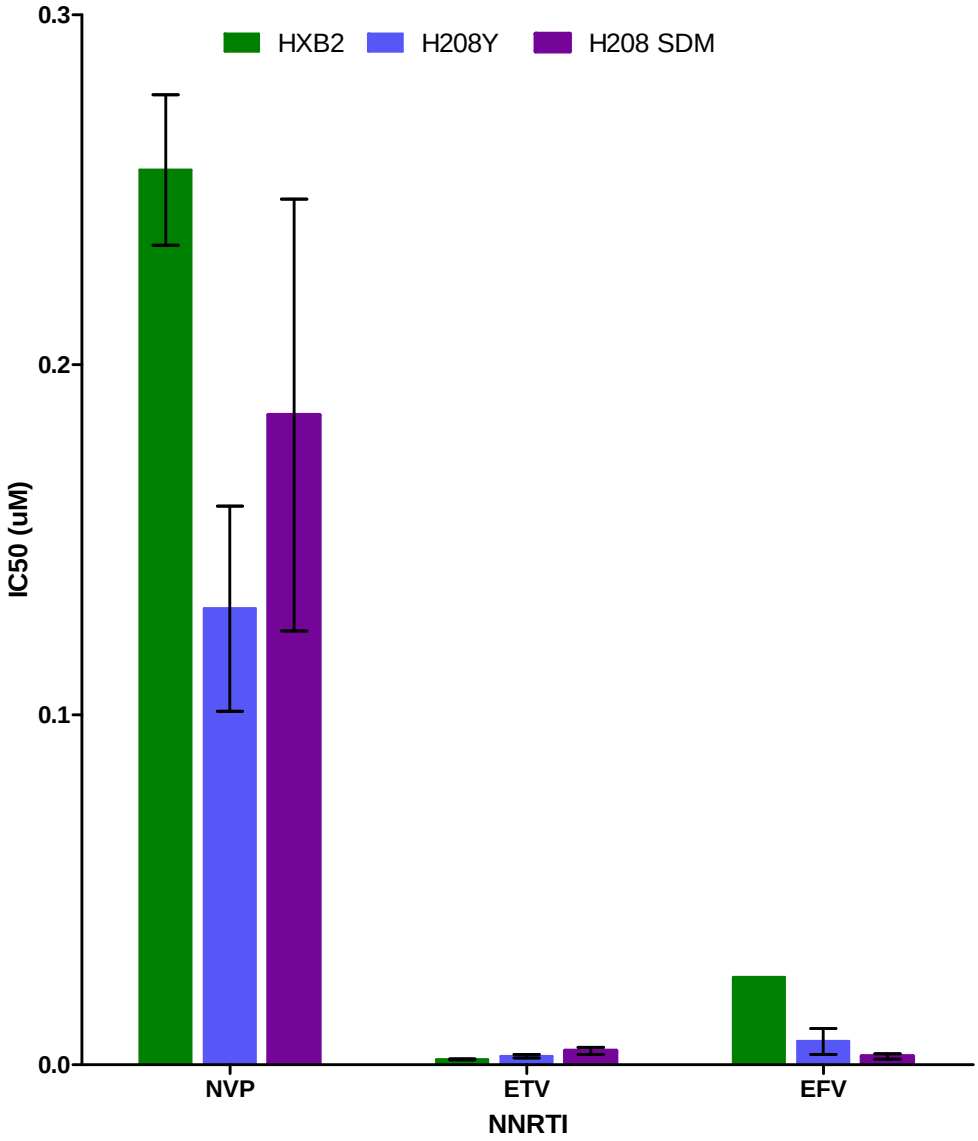
For ETV, the IC50 values were 0.003µM and 0.004µM with and without H208Y, with a fold change of 1.56 and 2.5 (Δ +0.94) respectively. For NVP, the IC50 values were 0.130µM and 0.186µM with and without H208Y, with a fold change of 0.51 and 0.73 (Δ +0.22) respectively. For EFV, the IC50 values were 0.007µM and 0.002µM with and without H208Y, with a fold change of 0.28 and 0.1 (Δ -0.18) respectively.

From the replicate experiments, the IC50 values obtained for viruses without and without H208Y in the subtype B treatment naïve patient between replicates experiments were all within a 2.5-3 fold difference of each other. There were two exceptions where a 4 fold difference was seen with AZT and NVP for the viruses with and without H208Y respectively.



Drug	HXB2 IC50(μM)	H208Y		H208 SDM		Average Δ (standard deviation)
		Mean IC50 μM (range IC50)	Mean fold change (range)	Mean IC50 μM (range IC50)	Mean fold change (range)	
AZT	0.099	0.02 (0.01-0.04)	0.24 (0.1-0.36)	0.04 (0.03-0.06)	0.44 (0.30-0.61)	+0.20 (0.24)
ABC	2.173	0.63 (0.35-0.83)	0.29 (0.16-0.38)	2.14 (1.84-2.35)	0.99 (0.85-1.08)	+0.70 (0.18)
3TC	1.977	1.52 (0.83-2.48)	0.77 (0.42-1.25)	1.84 (1.45-2.82)	0.93 (0.73-1.43)	+0.16 (0.48)
D4T	1.953	1.32 (0.75-2.18)	0.67 (0.38-1.1)	3.89 (2.57-4.67)	2 (1.32-2.39)	+1.33 (0.57)
DDI	4.840	2.65 (2.29-3)	0.55 (0.47-0.62)	4.83 (4-6.14)	1 (0.83-1.27)	+0.45 (0.21)
TDF	23.288	15.75 (10.16-20.4)	0.68 (0.44-0.88)	7.14 (6.84-7.58)	0.31 (0.29-0.33)	-0.37 (0.38)

Figure 4.2 NRTI Drug susceptibility profile from the subtype B treatment naive patient. Virus was obtained from a treatment naïve patient with the RT mutation H208Y in the absence of major resistance mutations, compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. Mean values with range were calculated from 3 to 4 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged. *** indicates significant difference $p<0.001$ as determined by two tailed ANOVA.



Drug	HXB2 IC50(µM)	H208Y		H208 SDM		Average Δ (standard deviation)
		Mean IC50 µM (range IC50)	Mean fold change (range)	Mean IC50 µM (range IC50)	Mean fold change (range)	
NVP	0.256	0.130 (0.072-0.164)	0.510 (0.28-0.64)	0.186 (0.102-0.306)	0.730 (0.4-1.2)	+0.22 (0.20)
ETV	0.0016	0.003 (0.002-0.003)	1.560 (1.25-1.88)	0.004 (0.002-0.005)	2.500 (1.25-3.13)	+0.94 (0.44)
EFV	0.025	0.007 (0.005-0.014)	0.280 (0.20-0.56)	0.002 (0.001-0.004)	0.100 (0.04-0.16)	-0.18 (0.22)

Figure 4.3 NNRTI Drug susceptibility profile from the subtype B treatment naive patient. Virus was obtained from a treatment naïve patient with the RT mutation H208Y in the absence of major resistance mutations, compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. Mean values with range were calculated from 3 to 4 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged.

4.3.3.2 H208Y in the context of subtype B virus from a treatment experienced patient

Plasma virus from the subtype B treatment experienced patient harboured the pathway 1 TAMs M41L, L210W and T215Y together with D67N, K219N and V75M in all clones. The NNRTI resistance mutations A98G and G190Q were also present in all clones.

Figure 4.4 shows the mean IC50 (μM) values obtained with a panel of NRTIs. Compared to the H208 SDM, the presence of H208Y on a background of TAM-1 pathway mutations increased susceptibility to ABC and D4T, but reduced susceptibility to 3TC, DDI and TDF.

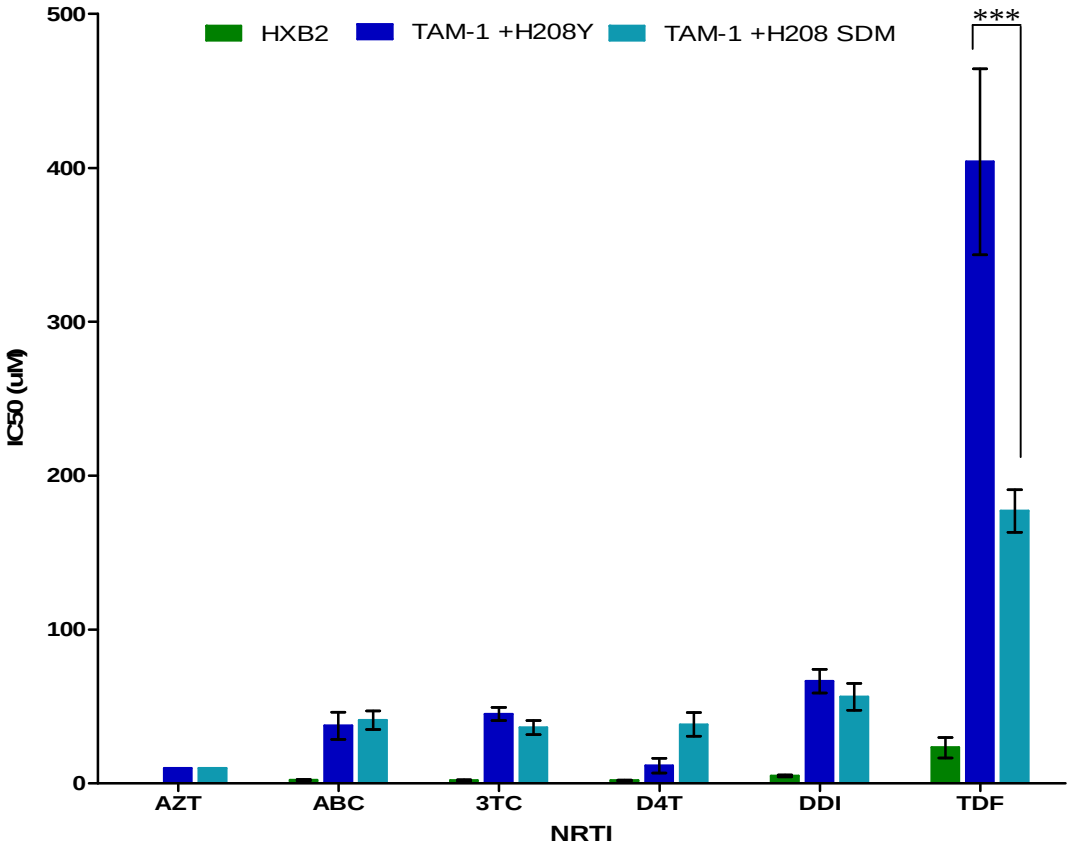
For ABC, the IC50 was 37.50μM and 41.10μM with and without H208Y, with a fold change of 17.26 and 18.91 (Δ +1.65) respectively. For D4T, the IC50 was 11.53μM and 38.28μM with and without H208Y with a fold change of 5.9 and 19.6 (average Δ +13.70) respectively. For 3TC the IC50 was 45.13μM and 36.23μM with and without H208Y respectively with a fold change of 22.83 and 18.33 (Δ –4.50) respectively. For DDI, the IC50 was 66.5μM and 56.23μM with and without H208Y with a fold change of 13.74 and 11.62 (Δ –2.12) respectively. For TDF, the IC50 was 404μM and 177μM with and without H208Y respectively with a fold change of 17.3 and 7.6 (Δ –9.70) respectively.

Figure 4.5 shows the drug susceptibility profile of the same virus with a panel of NNRTIs that consisted of NVP, EFV and ETV. H208Y also conferred an increased

susceptibility to ETV. The IC50 was 0.004μM and 0.0097μM with and without H208Y with a fold change of 2.5 and 6 (Δ + 3.8) respectively.

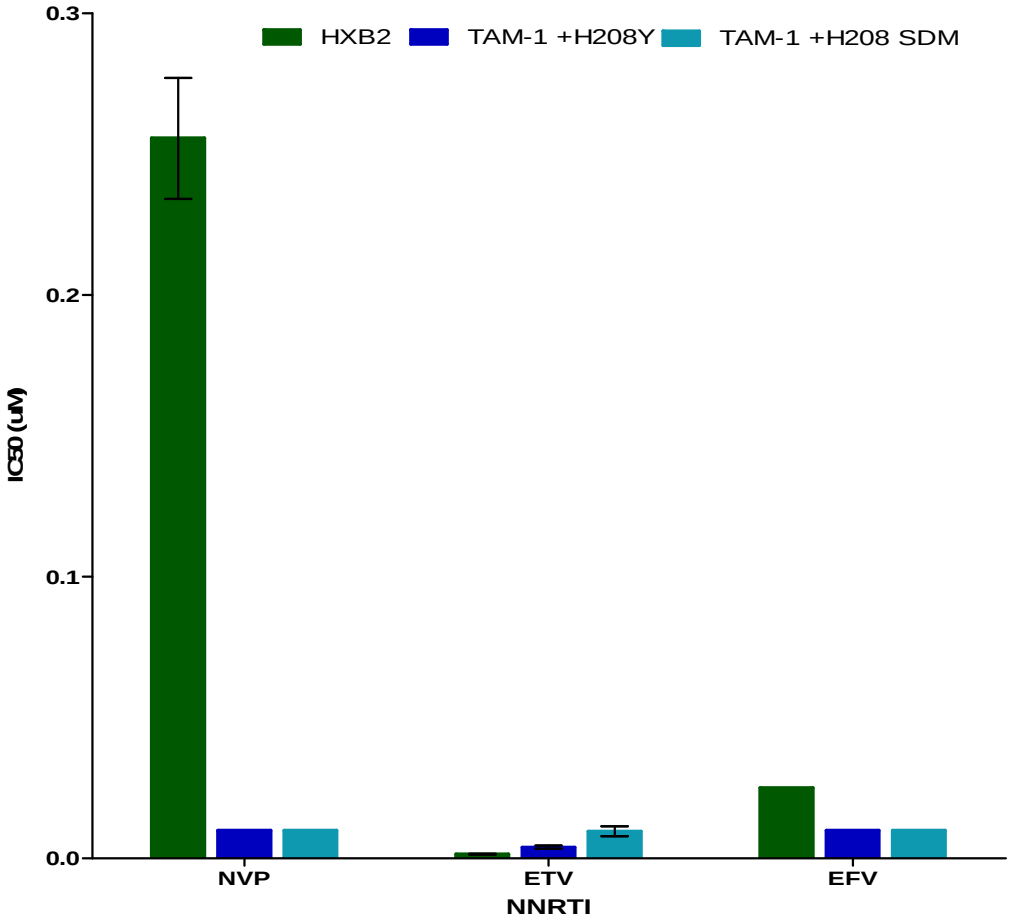
From the replicates experiments, the IC50 values obtained for viruses with and without H208Y in the subtype B treatment experienced patient were all within a 2.5 fold difference of each other.

Despite repeat attempts no IC50 values were obtained for AZT, EFV and NVP. For AZT this was due to the fact the viruses were highly drug-resistant due to the presence of multiple TAMs, and therefore the drug levels required to inhibit the viruses exceeded the limit of the assay and were toxic to cells.



Drug	HXB2 IC50(μM)	TAM-1 H208Y		TAM-1 H208 SDM		Average Δ (standard deviation)
		Mean IC50 μM (range IC50)	Mean fold change (range)	Mean IC50 μM (range IC50)	Mean fold change (range)	
AZT	0.099	>10	NA	>10	NA	NA
ABC	2.173	37.50 (21.2-51.5)	17.26 (9.8-23.7)	41.10 (34-53)	18.91 (15.6-24.4)	+1.65 (8.42)
3TC	1.977	45.13 (36.8-51)	22.83 (18.6-25.8)	36.23 (28.2-43.9)	18.33 (14.3-22.2)	-4.50 (6.11)
D4T	1.953	11.53 (6.8-16.3)	5.9 (3.5-8.4)	38.28 (23-53)	19.60 (11.8-27)	+13.70 (5.13)
DDI	4.840	66.50 (52.5-79.4)	13.74 (10.8-16.4)	56.23 (39.2-67)	11.62 (8.1-13.8)	-2.12 (3.99)
TDF	23.288	404 (294-500)	17.3 (12.6-21.5)	177 (150-196)	7.6 (6.4-8.4)	-9.70 (4.47)

Figure 4.4 NRTI Drug susceptibility profile from the subtype B treatment experienced patient. Virus was obtained from a treatment experienced with the RT mutation H208Y in the presence of M41L, D67N, V75M, A98G, V118I, G190Q, L210W, T215Y and K219N compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. Mean values with range were calculated from 3 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged. *** indicates significant difference $p<0.001$ as determined by two tailed ANOVA.



Drug	HXB2 IC50(µM)	TAM-1 H208Y		TAM-1 H208 SDM		Average Δ (standard deviation)
		Mean IC50 µM (range IC50)	Mean fold change (range)	Mean IC50 µM (range IC50)	Mean fold change (range)	
NVP	0.256	<0.01	NA	<0.01	NA	NA
ETV	0.0016	0.004 (0.003-0.005)	2.5 (1.9-3.1)	0.01 (0.007-0.013)	6.3 (4.4-8.1)	+3.8 (2.53)
EFV	0.025	<0.01	NA	<0.01	NA	NA

Figure 4.5 NNRTI Drug susceptibility profile from the subtype B treatment experienced patient. Virus was obtained from a treatment experienced patient with the RT mutation H208Y in the presence of M41L, D67N, V75M, A98G, V118I, G190Q, L210W, T215Y and K219N, compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. Mean values with range were calculated from 3 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged.

4.3.3.3 H208Y in the context of subtype A virus from a treatment experienced patient

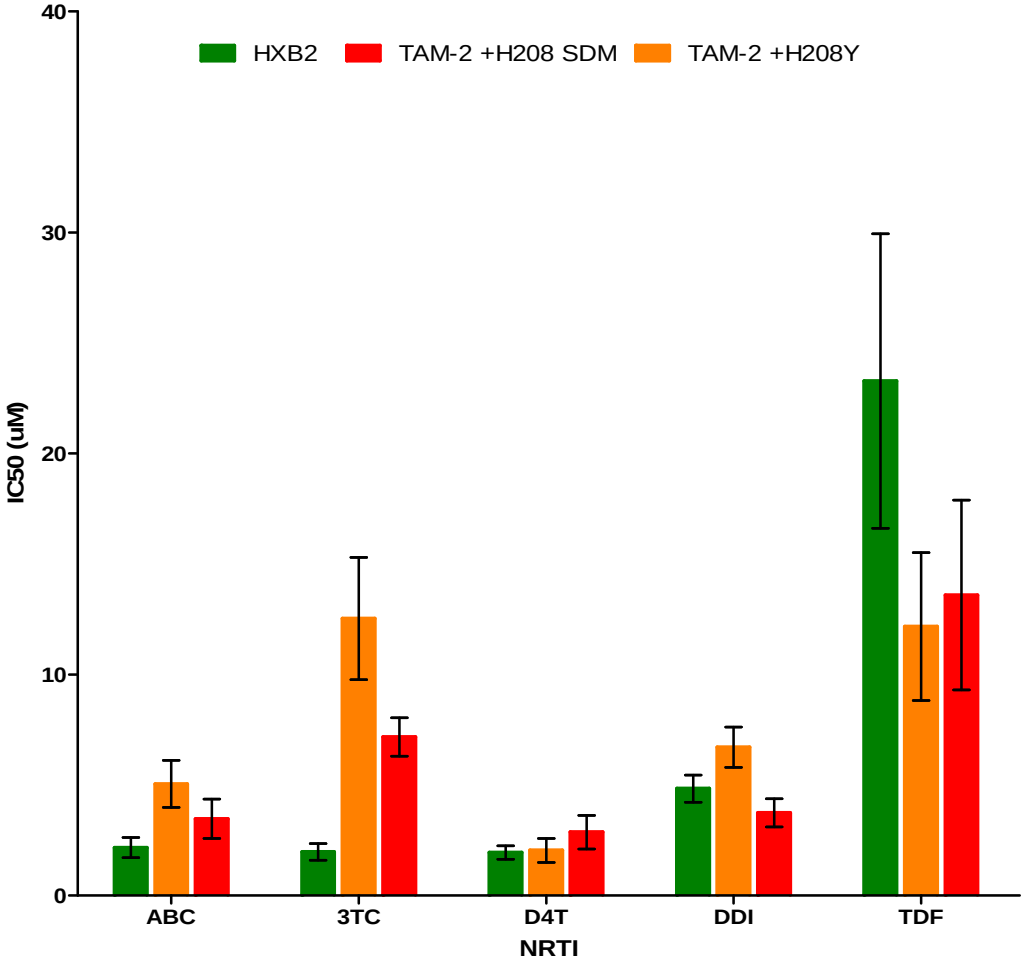
The subtype A patient harboured the pathway 2 TAMs D67N, K70R, T215F and K219E. This patient also had the M184V and T69N mutations present in all clones. The M184V mutation was back mutated to the wild type amino acid methionine by side directed mutagenesis in order to eliminate its strong effects on drug susceptibility, including high-level resistance to 3TC and FTC, and increased susceptibility to ZDV, D4T and TDF. The NNRTI resistance mutations A98G and G190Q were present in all clones.

Figure 4.6 shows the mean IC50 (μM) values obtained with a panel of NRTIs. The presence of H208Y in the context of TAM-2 mutations increased susceptibility to D4T, but reduced susceptibility to ABC, DDI and 3TC. No effects were seen for TDF.

For 3TC, the IC50 was 12.54μM and 7.18μM with and without H208Y respectively, with a fold change of 6.34 and 3.63 (Δ -2.71) respectively. For ABC the IC50 was 5.06μM and 3.48μM with and without H208Y respectively with a fold change of 2.3 and 1.6 (Δ -0.66) respectively. For DDI the IC50 was 6.71μM and 3.74μM with and without H208Y respectively with a fold change of 1.39 and 0.77 (Δ -0.62) respectively. For D4T the IC50 was 2.05μM and 2.87μM with and without H208Y with a fold change of 1.05 and 1.45 (Δ +0.42) respectively.

From the replicates experiments, the IC50 values obtained for viruses with and without H208Y in the subtype A treatment experienced patient were all within a 3 fold difference of each other. The two exceptions were with ABC and 3TC for the

virus with H208Y, where a 3.4 fold difference was seen for both drugs. For both these drugs, the number of replicates was increased to 4 and 5 for ABC and 3TC respectively to obtain a more reproducible IC50 value. The subtype A viruses with and without H208Y were not tested for their susceptibility to AZT and the NNRTIs as the previous data indicated that due to the presence of TAMs in these viruses, no reliable results would be obtained for AZT, EFV and NVP.



Drug	HXB2 IC50(μM)	TAM-2 H208Y		TAM-2 H208 SDM		Average Δ (standard deviation)
		Mean IC50 μM (range IC50)	Mean fold change (range)	Mean IC50 μM (range IC50)	Mean fold change (range)	
AZT	0.099	ND	ND	ND	ND	ND
ABC	2.173	5.06 (1.9-6.6)	2.33 (0.9-3)	3.48 (2-5.9)	1.67 (0.92-2.7)	-0.66 (0.94)
3TC	1.977	12.54 (5.7-19.3)	6.34 (2.9-9.8)	7.18 (5.8-8.8)	3.63 (2.9-4.4)	-2.71 (2.68)
D4T	1.953	2.05 (1.1-3.0)	1.05 (0.6-1.6)	2.87 (1.7-4.3)	1.45 (0.9-2.2)	+0.42 (0.70)
DDI	4.840	6.71 (6-8.54)	1.39 (1.2-1.8)	3.74 (2.8-5)	0.77 (1.6-1)	-0.62 (0.45)
TDF	23.288	12.17 (8.4-18.9)	0.5 (0.4-0.8)	13.60 (6.9-21.6)	0.58 (0.3-0.9)	+0.08 (0.53)

Figure 4.6 NRTI Drug susceptibility profile from the subtype A treatment experienced patient. Virus was obtained from a treatment experienced patient with the RT mutation H208Y in the presence of D67N, T69N, T215F and K219E, compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. The patient's virus also had the major RT mutation M184V which was back mutated to the wild type amino acid methionine by site directed mutagenesis. Mean values with range were calculated from 3 to 5 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged.

4.3.3.4 H208Y in the context of subtype C wild type virus from a treatment experienced patient

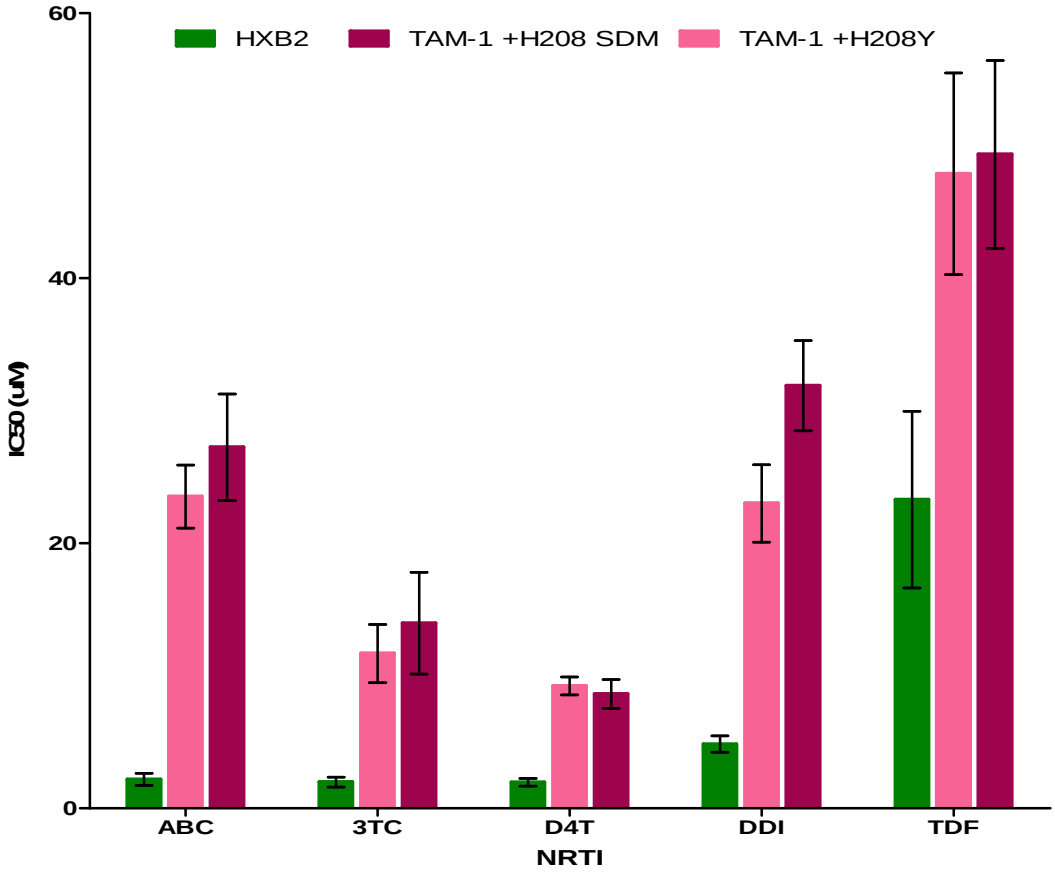
The subtype C patient harboured the pathway 1 TAMs M41L, L210W and T215Y with D67N and K219R. This patient also had the E44D, L74V and M184V mutations present in all clones. The M184V mutation was back mutated to the wild type amino acid methionine by side directed mutagenesis in order to eliminate its strong effects on drug susceptibility. The NNRTI resistance mutations present were A98G, K101H, V108I, Y181C and G190A/Q. All NNRTI mutations were present in 100% of clones except for V108I which was present in 50% (10/20) of clones.

Figure 4.7 shows the mean IC50 (μM) values obtained with a panel of NRTIs. The presence of H208Y in the context of TAM-1 mutations increased susceptibility to ABC, DDI, and 3TC and reduced susceptibility to D4T. No effects were seen for TDF.

For DDI the IC50 was 23.01μM and 31.89μM with and without H208Y with a fold change of 4.75 and 6.59 (Δ+1.84). For ABC the IC50 was 23.53μM and 27.26μM with and without H208Y with a fold change of 10.83 and 12.29 (Δ+1.46). For 3TC the IC50 was 11.68μM and 13.97μM with and without H208Y respectively with a fold change of 5.9 and 7.06 (Δ+1.16).

For D4T the IC50 was 9.23μM and 8.63μM with and without H208Y respectively with a fold change of 4.73 and 4.42 (Δ-0.31). No effect was observed against TDF.

From the replicates experiments, the IC50 values obtained for viruses with and without H208Y in the subtype C treatment experienced patient were all within a 2.5 fold difference of each other. The subtype C viruses with and without H208Y were not tested for their susceptibility to AZT and the NNRTIs as the previous data indicated that due to the presence of TAMs in these viruses no reliable results would be obtained for AZT, EFV and NVP.



Drug	HXB2 IC50(µM)	TAM-1 H208Y		TAM-1 H208 SDM		Average Δ (standard deviation)
		Mean IC50 µM (range IC50)	Mean fold change (range)	Mean IC50 µM (range IC50)	Mean fold change (range)	
AZT	0.099	ND	ND	ND	ND	ND
ABC	2.173	23.53 (18.8-26.4)	10.83 (8.6-12.1)	27.26 (16.5-36)	12.29 (7.6-16.6)	+1.46 (3.06)
3TC	1.977	11.68 (7.4-14.5)	5.9 (3.7-7.3)	13.97 (8.9-21.5)	7.06 (4.5-10.9)	+1.16 (5.29)
D4T	1.953	9.23 (8.1-10.5)	4.73 (4.2-5.4)	8.63 (7.3-10.8)	4.42 (3.8-5.5)	-0.31 (1.26)
DDI	4.840	23.01 (18.8-28.6)	4.75 (3.9-5.91)	31.89 (25.4-36.8)	6.59 (5.2-7.6)	+1.84 (1.14)
TDF	23.288	47.88 (32.7-56.5)	2 (1.4-2.4)	49.36 (41.5-63.5)	2.12 (1.8-2.7)	+0.12 (0.48)

Figure 4.7 NRTI Drug susceptibility profile from the subtype C treatment experienced patient. Virus was obtained from a treatment experienced patient with the RT mutation H208Y in the presence of M41L, E44D, D67N, L74V, A98G, K101H, Y181C, G190A, L210W, T215Y and K219R, compared to a reference wild type HXB2 and to a site directed mutant lacking H208Y. This patient's virus also had the major RT mutation M184V, which was back mutated to the wild type amino acid methionine by site directed mutagenesis. Mean values with range were calculated from 3 replicate experiments each performed in quadruplicate. Delta (Δ) indicates the difference in fold change measured for the virus with H208Y and without H208Y. The delta was calculated for each replicate experiment and then averaged.

Table. 4.3 Overall impact of the RT mutation H208Y on susceptibility to RT inhibitors

Subtype	Major RT Mutations	RT Inhibitors								
		AZT	ABC	3TC	D4T	DDI	TDF	NVP	ETV	EFV
B	None	↑	↑	↑	↑	↑	↓***	↑	↑	↓
B	M41L, D67N, V75M, A98G, V118I, G190Q, L210W, T215Y, K219N	ND	↑	↓	↑	↓	↓***	ND	↑	ND
A	D67N, T69N, K70R, T215F, K219E	ND	↓	↓	↑	↓	↑	ND	ND	ND
C	M41L, E44D, D67N, L74V, A98G, K101H, Y181C, G190A, L210W, T215Y, K219R	ND	↑	↑	↓	↑	↑	ND	ND	ND

Delta (Δ) values were calculated as the difference in fold change (relative to the reference wild type HXB2) between virus with and without H208Y. For interpretation purposes, a positive delta value was regarded as increased susceptibility (↑); a negative delta value was regarded as decreased susceptibility (↓); ND= not done; *** indicates a significant difference $p<0.001$ as determined by two tailed ANOVA.

Table 4.3 summarises the overall impact of the H208Y mutation on drug susceptibility in the four patients using the delta values obtained when comparing the virus with and without H208Y (relative to the reference wild type HXB2). An increased susceptibility was defined as a positive delta value and a decreased susceptibility was defined as a negative delta value. For most of the drugs no statistically significant effect was observed except from the reduced susceptibility to TDF seen in the subtype B patients. Statistical analysis was performed using two-tailed ANOVA.

In the absence of major resistance mutations in subtype B RT, H208Y increased susceptibility to AZT, ABC, 3TC, D4T, DDI, NVP and ETV and reduced susceptibility to TDF and EFV. The magnitude of the effect was such that it can be concluded that H208Y conferred hypersusceptibility (at east 0.4 fold) to AZT, ABC and NVP. In the presence of the TAMs M41L, D67N, L210W, T215Y, K219N and V75M, A98G, V118I and G190Q in subtype B RT, H208Y increased susceptibility to ABC, D4T and ETV and reduced susceptibility to 3TC, DDI and TDF. In subtype C, with the same TAMs plus E44D, L74V, A98G, K101H, V108I, Y181C, G190A and K219R RT, H208Y increased susceptibility to ABC, 3TC, TDF and DDI and reduced susceptibility to D4T. In the presence of the TAMs D67N, T215F, and K219E together with T69N, A98G and G190Q in subtype A RT, H208Y increased susceptibility to D4T and TDF and reduced susceptibility to ABC, 3TC and DDI.

4.3.4 Amino acid sequence alignment of RT sequences from the 4 patients

The presence of H208Y in RT from different HIV-1 subtypes showed variable results regarding increased and decreased susceptibility to NRTIs and NNRTIs. However, with TDF, a statistically significant effect was observed with the subtype B RT. In this context, the presence of H208Y conferred a decreased susceptibility to TDF. In order to gain insights into the different phenotypic effects seen for TDF with the different viruses, the clonal RT amino acid sequences from the subtype A and subtype C patients were compared with the subtype B sequences (figure 4.8).

The subtype B treatment naïve patient harboured no major RT resistance mutations. Major resistance mutations shared between the three treatment experienced patients were D67N, T215Y/F and K219E/N/R. Both the subtype B and C treatment experienced patients had the A98G, G190A/Q, M41L and V118I mutations present. Mutations not shared by the treatment experienced patients included V75M for the subtype B patient, T69N for the subtype A patient and E44D, L74V, K101H, K103N, V108I and Y181C for the subtype C patient.

Excluding major drug resistance mutations from this alignment, 12 amino acid positions were identified that were the same in the two subtype B sequences but different in the subtype A and C sequences. These positions are highlighted in red in figure 4.8. Most of the mutations were found within the RNase H domain (6/12, 50%), followed by the polymerase domain (4/12, 33%) and the connection domain (2/12, 17%). The majority of these changes were not mutations known to be

associated with NRTI resistance. Of interest, two positions were identified that appear to play a role in NRTI resistance involving codons 207 and 335. At position 207, both subtype B patients harboured Q (glutamine) whereas subtype A and subtype C viruses harboured A (alanine) and E (glutamic acid) respectively. At position 335, both subtype B patients harboured a G (glycine) whereas subtype A and subtype C viruses harboured a D (aspartic acid).

To examine how the differences related to subtype, subtype A and subtype C consensus sequences were downloaded from the Los Alamos Database (http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html) in order to perform a comparison of these 12 positions. This analysis showed that the majority of changes were subtype-related differences with only 1/12 (8%) for subtype A and 4/12 (33%) for subtype C having a mutation present (table 4.4). The positions at 335 and 207 were wild type for the respective subtype A and C patient sequences. No accessory mutations were shared between the four patients.

```
A              PISPIETVPVKLKPGMDGPRVKQWPLTEEKIKALTEICTEMEKEGKISKIGPENPYNTPI 60
C              PISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALLEICEELEKDGKISKIGPENPYNTPV 60
Exp_B          PISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALIEICTFLEEEGKISKIGPENPYNTPI 60
Naive          PISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYNTPV 60

A              FAIKKKNSNKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPL 120
C              FAIKKKNSTKWRKVVDFRELNKRTQDFWEVQLGIPHPGGLHKKKSVTVLDIGDAYFSIPL 120
Exp_B          FAIKKKNSTKWRKLMDFRELNKRTQDFCEVQLGLPHPGGLKKKKSVTVLDVGDAYFSIPL 120
Naive          FAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPL 120

A              DESFRKYTAFTIPSTNNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRLKNPEIII 180
C              DPNFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPSIFQSSMTKILEPFRTKNPEIVI 180
Exp_B          DEDFRKYTAFTIPSVNNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPDLVI 180
Naive          DKEFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPEMVI 180

A              YQYMDDLYVGSDLEIGQHRAKIEELRAYLLSWGFFTPDEKHQKEPPFLWMGYELHPDKWT 240
C              CQYMDDLYVASDLEIGQHRAKIKELREYLWKWGFYTPERKHQQEPPFLWMGYELHPDKWT 240
Exp_B          YQYMDDLYVQSDLEIGQHRAKIEELRQYLWKWGFYTPDNKHQKEPPFLWMGYELHPDKWT 240
Naive          YQYMDDLYVGSDLETEQHRTKIEELRQYLLKWGFTTPDKKHQKEPPFLWMGYELHPDKWT 240

A              VQPIQLPEKDSWTVNDIQKLVGKLNWASQIYSGIKVKQLCKLLRGAKALTDVVPLTEEAE 300
C              VQPIQLPEKDSWTVNDIQKLVGKLNWASQIYPGIKVKQLCKLLRGTKALTDIVPLTEEAE 300
Exp_B          VQPIELPEKDSWTVNDIQKLVGKLNWASQIYPGIRVRQLCKLLRGTKALTEVVTLTREAE 300
Naive          VQPIVLPEKDSWTVNDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKTLTEVVPLTKEAE 300

A              LELAENREILKDPVHGVYYDPSKDLIAEIQKQGEDQWTYQVYQEPFKNLKTGKYARRKAA 360
C              LELAENREILKEPVHGVYYDPSKDLIAEIQKQGDDQWTYQIYQEPFKNLKTGKYAKRRTA 360
Exp_B          LELAENREILKTPVHGVYYDPSKELIAEIQKQGQGPWTYQIYQEPFKNLRTGKYARRKGA 360
Naive          LELAENREILKEPVHGVYYDPSKDLIAEIQKQGLGQWTYQIYQEPFKNLKTGKYARMRSA 360
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A               HTNDVKQLTEVVQKVVLEAIVIWGKTPKFRLPIQKETWETWWTDYWQATWIPEWEFVNTP 420
C               HTNDVKQLTEAVQKIALESIVIWGKTPKFKLPIQKETWEAWWTDYWQATWIPEWEFVNTP 420
Exp_B           HTNDVKQLTEAVQKIATESIVIWGKTPKFRLPIQKETWETWWTDYWQATWIPEWEFVNTP 420
Naive           HTNDVKQLTEAVQKIATESIVIWGKIPKFRLPIQKETWDTWWAEYWQATWIPEWEFVNTP 420

A               PLVKLWYQLEKDPIIGAETFYVDGAANRETKLGKAGYVTDRGRQKVVSLNETTNQKAELH 480
C               PLVKLWYQLEKEPIIGAETFYVDGAANRETRIGKAGYVTDRGRQKIVSLNETTNQKTELQ 480
Exp_B           PLVKLWYQLEKEPIAEAETFYVDGAANRETKLGKAGYVTNRGRQKVVPLTDTTNQKTELE 480
Naive           PLVKLWYQLETEPIVGAETFYVDGAANRDTKLGKAGYVTDRGRQKVVPLTDTTNQKTELQ 480

A               AIYLALQDSGSEVNIVTDSQYALGIIQAQPDKSESELVNQIIEQLINKERVYLSWVPAHK 540
C               AIQLALQDSGSEVNIVTDSQYALGIIQAQPDKSESELVNQIIEQLIKKERVYLSWVPAHK 540
Exp_B           AINLALQDSGPEVNIVTDSQYALGIIQAQPDQSESELVSQIIEQLIKKEKVYLAWVPAHK 540
Naive           AIHLALQDSGAEVNIVTDSQYALGIIHAKPDKSESELVSQIIEQLIKKEKVYLAWVPAHK 540

A               GIGGNEQVDKLVSSGIRKVL 560
C               GIGGNEQVDKLVSNGIRKVL 560
Exp_B           GIGGNEQVDKLVSAGIRKVL 560
Naive           GIGGNEQVDKLVSAGIRKVL 560
```

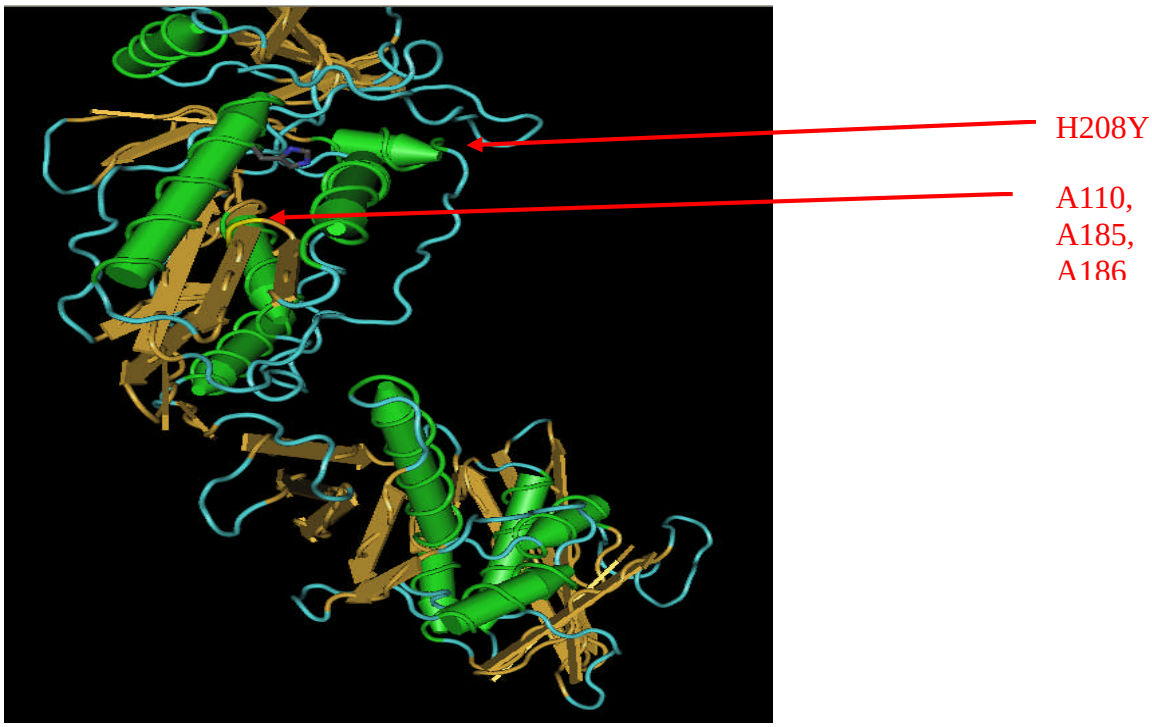
Figure 4.8 Amino acid sequence alignment of the RT derived from the plasma virus of four patients. Subtype B treatment naïve (Naïve), subtype B treatment experienced (Exp_B), subtype A treatment experienced (A) and subtype C treatment experienced (C). Amino acids in red represent positions that are conserved in the subtype B sequences but differ in the subtype A and subtype C sequences. Amino acids in green represent major NRTI and NNRTI resistance mutations.

Table. 4.4 Subtype related sequence variation[a]

Subtype B Amino Acid	Subtype A Amino Acid	Subtype C Amino Acid
K173	S173L	A173T
Q174	K174	Q174K
Q207*	A207	E207
K277R	K277	R277K
E291	D291	D291
G335*	D335	D335
T468P	S468	S468
D471	E471	E471
S519	N519	N519
K530	K530	R530
A534	S534	S534
A554	S554	S554N

[a]12 amino acid positions were identified that were conserved in the two subtype B sequences but differed in the subtype A and C sequences. The consensus sequences for subtype A and C were obtained by aligning 153 and 761 subtype A and subtype C sequences respectively from the Los Alamos Database. The consensus sequences were compared to the patient sample of the corresponding subtype and mutations were identified as those differing from the consensus sequence. * indicates positions that have been proposed as playing a role in NRTI resistance.

Figure 4.9 Structural representation of the p66 subunit of the HIV-1 reverse transcriptase gene. The arrows shows the position of H208Y and the three catalytic aspartic residues A110, A185 and A186.



4.3.5 Structure of RT with the H208Y mutation

Figure 4.9 shows a graphical representation of the HIV-1 RT p66 subunits backbone secondary and tertiary structure. The H208 residue is shown in red and in yellow are the catalytically important residues A110, A185 and A186. It can be seen that residue 208 is located at a distal alpha-helix at the base of the palm subdomain, with its side-chain protruding out into the surrounding solvent.

4.4 Discussion

In this study, we determined the impact of the RT mutation H208Y on phenotypic susceptibility to RT inhibitors using a multiple cycle recombinant virus assay. In the context of subtype B RT without major resistance mutations and a subtype B RT with the TAMs M41L, D67N, L210W, T215Y and K219N, the H208Y mutation conferred a reduced susceptibility to TDF. This effect was not observed with subtype A RT harbouring the TAMs D67N, K70R, T215F and K219E and with subtype C virus harbouring the TAMs M41L, D67N, L210W, T215Y and K219R. The resistance effects of H208Y were not seen with other NRTIs, rather the mutation was shown to confer an increased susceptibility to some NRTIs and NNRTIs, although these effects were not statistically significant when comparing virus with and without H208Y.

Phenotypic assays most commonly assess virus susceptibility to antiretroviral drugs using recombinant viruses. The inhibitory concentration required to inhibit 50% of the virus (IC50) is calculated and compared to a wild type laboratory strain as reference. Currently there are two types of phenotypic assays which both utilise recombinant virus. They involve cloning the gene of interest into either a replicative competent vector (multiple cycle assay) or into a replication defective vector that allows only one round of replication to occur (single cycle assay). The read out of these two assays differs. The multiple cycle assay uses the MTT lysis method whereas the single cycle method uses reporter genes such as luciferase or ß-galactosidase.

There are advantages and drawbacks with both methodologies. Advantages of the single cycle assay include the fact that it is a fairly rapid assay compared to the multiple cycle assay, and results of replicate experiments generally show smaller variation. A commercial assay utilising the single cycle approach for measuring drug susceptibilities is provided by Monogram Biosciences in the United States. The intra-assay reproducibility of this test was evaluated by using duplicate aliquots of 9 clinical samples in a single experiment. The authors found that there was <2.5 fold variability in the results given by 99% of tests (n=107) (Demeter and Haubrich, 2001). The major drawback to this method is that only a single round of replication occurs and therefore it can be argued that the assay does not fully reproduce the *in vivo* effects of resistance mutations.

Another commercial phenotypic assay is provided by Virco in Belgium. The assay uses a multiple cycle approach to determine drug susceptibilities. The Virco assay has been shown to be highly reproducible across independent experiments. The fold change range compared to wild type virus was between 1.2 and 2.5 with 16 samples tested in 10 independent experiments (Demeter and Haubrich, 2001). An advantage of the multiple cycle method is the fact that the virus undergoes multiple rounds of replication and therefore the assay conditions mimic more closely the conditions that the virus experiences in vivo. This advantage is also a potential drawback in that it is possible for the virus to mutate during passaging, particularly in the presence of drugs, and therefore the input virus may not necessarily be the same virus at the end of the assay. This is particularly true for drug susceptibility assays that are done over a period of 2 to 3 weeks. The drug susceptibility assays presented in this study were analysed following a short incubation of 4 days. The likelihood that the virus will

mutate significantly enough to cause a phenotypic effect in 4 days can be considered low. An i*n vitro* study showed that the selection of AZT resistant mutations in wild type HXB2 appeared after a median of 54 days in culture in the presence of AZT. The TAMs first to emerge were K70R and D67N (Garcia-Lerma et al., 2004).

When analysing independent replicate experiments in this study, variability was usually within a 2.5 to 3 fold change range. There were some incidences however where the range in fold change was greater than 3 and in this case the number of replicate experiments was increased to give confidence in the results. Thus, when testing the subtype A virus with H208Y for susceptibility to ABC and 3TC, the replicate experiments showed initially a difference in the fold change range of 3.4 and therefore the number of replicates was increased to 5 and 4 respectively. Differences in the fold change range were also seen with the subtype B virus with H208Y when tested for susceptibility to AZT, and the subtype B virus with H208Y when tested for susceptibility to EFV.

The majority of IC50 values obtained in this study were consistent with those available in the literature. The variability observed between replicates is not overly surprising given the fact that these were clinical samples tested in a multiple cycle system. It is important to consider that although the same assay was used, differences will occur resulting from the intrinsic variability of tissue culture conditions. One important factor in reducing the impact of such variability is to use fold changes in IC50 relative to the wild type virus tested under the same experimental condition, rather than using absolute IC50 values. This is currently the gold standard method of analysing phenotypic data.

Previous studies examining the phenotypic effect of the H208Y mutation have yielded conflicting results. These studies have reported both increased and reduced susceptibility to AZT in the presence of H208Y. One study used the multiple cycle assay and created site directed mutants to show that the triple mutant H208Y/R211K/L214F in the context of the TAMs M41L, D67N, K70R, L210W, T215Y and K219E resulted in a 21-fold reduced susceptibility to AZT (Sturmer et al., 2003). These findings were supported by the study carried out by (Svicher et al., 2006). This study sent samples to Virco for phenotypic testing. In the Virco multiple cycle assay, in the presence of M41L, L210W and T215Y in the context of subtype B RT, H208Y reduced susceptibility to all NRTIs including TDF. Despite these findings indicating a role of H208Y in conferring resistance to AZT, another study showed that in the absence of TAMs, H208Y caused increased susceptibility to AZT, D4T, NVP and EFV, but reduced susceptibility to TDF. This study created site directed mutants on subtype B RT backbone and used a single cycle assay to determine drug susceptibility (Clark et al., 2006). The phenotypic data presented here confirm that H208Y confers reduced susceptibility to TDF in both wild type subtype B virus and subtype B virus containing the TAMs M41L, D67N, L210W, T215Y and K219N. A novel finding was that the effect was not seen with subtype A and subtype C virus showing the TAMs D67N, K70R, T215F and K219E and M41L, D67N, L210W, T215Y and K219R respectively.

There are at least two mechanisms of resistance to the NRTIs. The first mechanism is known as NRTI excision or primer unblocking. The excision is mediated by TAMs, particularly T215Y. Excision occurs through a pyrophosphorolysis reaction

using cellular pyrophosphate or more commonly ATP as an acceptor. This results in the release of the incorporated NRTI and a free hydroxyl group enabling DNA synthesis to resume (Goldschmidt and Marquet, 2004;Sluis-Cremer et al., 2000b).

The second mechanism is mediated by mutations that interfere with binding and/or incorporation of the NRTI. Examples of these mutations are K65R and K70E, which confer TDF resistance, and M184V which confers resistance to 3TC and FTC. These mutations allow the enzyme to discriminate between the natural dNTP substrate and the NRTI and consequently decrease incorporation of the NRTI, allowing chain synthesis to continue (Sluis-Cremer et al., 2007). These mutations are also known to have an antagonistic relationship with the TAMs. The side chain of K65R interacts with the side chain of R72 (arginine) leading to the formation of a bridge which restricts the movement of the RT. Consequently there is reduced NRTI incorporation and NRTI excision (Sluis-Cremer et al., 2007;Das et al., 2009).

Neither of the subtype B clones harboured K70E or K65R, eliminating their role in conferring TDF resistance. It can be speculated that the mechanism whereby H208Y confers resistance to TDF is not the same as the traditional TDF resistance mutations. Since the H208Y mutation was associated with TAMs, and consistently with T215Y/F, the mechanism could be related to NRTI excision or primer unblocking. This argument is further strengthened by the fact that H208Y was always associated with mutations at position D67, T215 and K219. The T215Y/F mutation has been suggested to increase the efficiency of ATP binding during pyrophosphorolysis (Arion et al., 1998;Boyer et al., 2001b;Chamberlain et al., 2002;Meyer et al., 2002;Sluis-Cremer et al., 2007;Boyer et al., 2002). These mutations also destabilise

the RT allowing efficient release of the NRTI (Boyer et al., 2002;Meyer et al., 2003). Therefore it is plausible that the mechanism of resistance of H208Y is related in NRTI excision and perhaps aids the process.

There are several possible reasons why the phenotypic effect of H208Y on susceptibility to TDF was observed only with the subtype B viruses. This includes subtype-related or drug-induced sequence differences and possibly structural differences in the enzyme. The TAMs present in the viruses from the three treatment experienced patients were similar but not identical. The subtype B virus had the TAMs M41L, D67N, L210W, T215Y and K219N alongside V75M, V118I and the NNRTI resistance mutations A98G and G190Q. The subtype A virus had the TAMs D67N, K70R, T215F and K219E alongside T69N and the NNRTI resistance mutations A98G and G190Q, while the subtype C virus had the TAMs M41L, D67N, L210W, T215Y and K219R alongside E44D, L74V, and the NNRTI resistance mutations A98G, K101H, V108I, Y181C and G190A/Q. Thus the mutation profile was similar albeit not identical for the subtype B and subtype C viruses, but rather different for the subtype A virus. It is known that the pathway 1 TAMs M41L, L210W and T215Y have stronger effects on NRTIs susceptibility than the pathway 2 mutations K70R, T215F and K219E/Q. Pathway 1 TAMs confer cross resistance to DDI and TDF whereas pathway 2 TAMs do not (Hu et al., 2006;Marcelin et al., 2005;Miller, 2004).

We also investigated whether other mutations were present that may explain the differences observed between subtypes. Aligning the RT amino acid sequences from the four patients showed that there were 12 positions which were the same in the two

subtype B patients, but differed in the subtype A and subtype C patients. The majority of these changes were polymorphisms with unknown effect on drug susceptibility. However, differences were observed at codons 207 and 335. These two positions are known to play a role in NRTI resistance. At codon 207, the subtype B viruses harboured glutamine (Q), whereas the subtype A and subtype C viruses harboured glutamic acid (E) and alanine (A) respectively. The Q207D mutation has been shown to increase resistance by 2.7 fold to AZT when present in an AZT-resistant RT but had no effect on AZT susceptibility when present in a wild type RT (Lu et al., 2005). Therefore, it can be speculated that the mutation may have played a role in conferring resistance, however it should be noted that none of the patients had the Q207D mutation present and the phenotypic effect of Q207A/E have not been investigated. Similarly, at position 335, the subtype B viruses harboured glycine (G) whereas both the subtype A and C viruses had aspartic acid (D). The G335D mutation has been shown to confer resistance to 3TC and AZT when present alongside M184V and TAM-1 mutations respectively however, the phenotypic impact of G335D alone has not been investigated (Zelina et al., 2008). The subtype A and C viruses did not have the M184V present and they were not tested against AZT however, the subtype A virus did show a decreased susceptibility to 3TC with a average delta of Δ -2.71 but this effect was not statistically significant. As a result it is difficult to make any assumptions regarding the impact of mutations at residues Q207 and G335 when present with H208Y on phenotypic susceptibility.

It is also interesting to look at the wild type consensus sequences of subtype A and C and compare the 12 differences found between subtype B and subtypes A and C. For subtype A, only 1/12 positions were mutated in the patient virus relative to the

subtype A consensus wild type sequence, this was S173L. For subtype C, 4/12 positions were mutated, these were A173T, Q174K, R277K and S554N. The phenotypic effect of these mutations have not been investigated thus, available data on these mutations do not explain the lack of a phenotypic effect of H208Y for TDF with the non-B subtype viruses.

It is also important to remember that the same subtype B molecular clone was used to generate all recombinant viruses and therefore it may be speculated that different results may be obtained with a subtype-specific vector. HIV-1 subtypes differ from one another by 10-12% on a nucleotide level and 5-6% in the protease and RT genes on an amino acid level (Kantor et al., 2005). Therefore it is plausible that using subtype specific molecular clones could have given a different result. However one study used a subtype C RT in both a subtype B and C recombinant vector and showed that there was not a significant difference in the drug susceptibility measurements (Choe et al., 2007).

Looking at the structure of RT, residue 208 is located at a distal alpha-helix at the base of the palm subdomain, with its side-chain protruding out into the surrounding solvent. As this residue has minimal contact with other residues in the local tertiary structure, it can be speculated that a mutation from histidine to tyrosine will not have a significant direct effect on important residues in the surrounding region. However, the mutation from a hydrophilic histidine to a hydrophobic tyrosine could be speculated to have a slight impact on the tertiary structure of the local region, which includes the catalytic residues of the active site (A110, A185 and A186). The hydrophobic tyrosine could try to reorient itself to make contacts with surrounding

hydrophilic residues such as 167. This could have an effect on the tertiary structure of the active site, resulting in the drug not binding as well.

Previous studies have shown that hypersusceptibility is a common phenomenon in treatment experienced patients with major drug-resistance mutations (Whitcomb et al., 2002). Mutations in RT that are known to confer resistance to certain drugs while increasing susceptibility to others include M184V, which confers resistance to 3TC (and FTC) while increasing susceptibility to AZT, d4T and TDF; K65R, which confers resistance to TDF, DDI, D4T, ABC, and 3TC (and FTC) while increasing susceptibility to AZT; and L74V, which confers resistance to ABC and DDI while increasing susceptibility to AZT. These mutations confer increased susceptibility to AZT whilst conferring increased resistance to the remaining NRTIs. These mutations interfere with AZT susceptibility by inhibiting excision of incorporate AZT-triphosphate from the DNA chain, which is mediated by TAMs. The subtype A and subtype C patients did harbour the M184V mutation and in order to prevent interference from the strong phenotypic effects of M184V, the valine in the viral RT was back-mutated to the wild type amino acid methionine. An association between major NRTI resistance mutations and NNRTI hypersusceptibility has also been reported, and one of the main NRTI resistance mutations that have been implicated in this respect is T215Y (Shulman et al., 2004).

The H208Y mutation has been previously implicated in contributing to EFV and NVP susceptibility. One study using site directed constructed mutants on a wild type background showed that H208Y contributed to an increased susceptibility to the two NNRTIs. When the T215Y mutation was added alongside H208Y, this increased

susceptibility effect was further augmented (Clark et al., 2006). In this study, H208Y in the context of wild type subtype B virus showed increased susceptibility to NVP. However, the effect was not evident with EFV. In fact, the difference in fold change for EFV in the presence and absence of H208Y was Δ+0.17, which indicates a small decrease in susceptibility. This discrepancy maybe explained by the use of a multiple cycle assay and patient-derived clinical samples, whereas the study by (Clark et al., 2006) used a single cycle assay with site directed mutants on a wild type background. There are no previously published susceptibility data regarding the effect of H208Y on susceptibility to ETV. In this study, we demonstrated that at least in the context of subtype B virus the presence of H208Y conferred an increased susceptibility to ETV.

The mechanistic basis of NNRTI hypersusceptibility is unknown, structurally, the NNRTI binding pocket is situated close to the active site, which could provide one possible explanation (Kohlstaedt et al., 1992). Another possible explanation is that NRTI mutations could enhance the binding of NNRTIs (Shulman et al., 2004).

In summary, the H208Y mutation showed a decreased susceptibility to TDF in both the treatment naïve and experienced subtype B patients. However this effect was not recapitulated with the treatment experienced subtype A and C patients.

Chapter five

5 Development of a growth competition assay to measure viral fitness

5.1 Introduction

The results from the drug susceptibility assays indicated that in the context of subtype B RT with and without major resistance mutations, H208Y conferred reduced susceptibility to TDF. To complete the analysis of the phenotypic effects of H208Y, the next step was to address whether the H208Y mutation conferred a fitness advantage. Fitness is defined as the ability of an organism to adapt to a given environment. For HIV, fitness can be interpreted as the ability of the virus to produce progeny (i.e., infect and replicate) in a given environment (Domingo et al., 1997).

There are several methods that measure viral fitness and these include single cycle assays and growth competition assays. Single cycle assays measure viral growth kinetics such as p24 antigen or reporter genes such as luciferase and ß-galactosidase (Deeks et al., 2001a). Growth competition assays involve incubating two viruses in differing proportions over a period of time and measuring the growth of each virus (Quinones-Mateu and Arts, 2002).

(Quinones-Mateu and Arts, 2002) compared the different methods of measuring fitness for protease and reverse transcriptase mutations. The authors showed that there was variability in the fitness result with all the methods, possibly reflecting different methodologies or the use of clinical isolates versus laboratory strains.

One drawback to single cycle assays is that they do not detect significant differences in fitness between viruses that may differ by only a single nucleotide. However,

single cycle assays offer the advantage of good reproducibility with minimal variability, and a relative simple format (Deeks et al., 2001a;Petropoulos et al., 2000a).

Growth competition assays have the advantage that small changes in fitness can be detected and provide as close as possible a reproduction of conditions *in vivo*. Different methods can be used to distinguish the two competing viral strains. Current methods include real-time PCR and pyrosequencing (de Ronde A. et al., 2001;Lahser et al., 2003).

Real-time PCR using TaqMan probes is based on conventional PCR but uses fluorescent reporter dyes to monitor the progression of the PCR reaction as it is occurring in real time (de Ronde A. et al., 2001;Heid et al., 1996). It uses sequence specific probes that are labelled with a fluorescent reporter at the 5' end and a quencher at the 3' end. Once the primers and probe have bound to its target sequence, the 5'-3' exonuclease activity of *Taq* polymerase hydrolyses the bond between the reporter and quencher. The dissociated reporter is then free to emit its energy (figure 5.1). The fluorescence emitted by the reporter dye is directly proportional to the amount of PCR product produced. A fixed threshold is set which takes into account the background fluorescence in the reaction. The threshold cycle (C_T) is a parameter defined as the cycle number at which the fluorescence exceeds the fixed threshold. The more concentrated the PCR product, the lower the C_T value is (Nolan et al., 2006;Vanguilder et al., 2008).

Pyrosequencing is a method of DNA sequencing that is based on the detection of pyrophosphate as nucleotides are incorporated (figure 5.2). A single stranded biotinylated DNA strand is bound onto sepharose beads where the sequencing primer is hybridised. A mix of ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphosulphate (APS) and luciferin are added to the DNA strand. Each of the four nucleotides are added in turn to the DNA strand. As DNA polymerase catalyses the incorporation of the complementary dNTP into the DNA strand, pyrophosphate is released as a proportion to the amount of incorporated nucleotide. In the presence of APS, ATP sulfurylase converts the released pyrophosphate to ATP. ATP is then used to mediate the conversion of luciferin to oxyluciferin which generates light in proportion to the amount of ATP. The light is detected by a charge coupled device (CCD) camera, and is displayed as peaks in the resultant pyrogram. The height of the peak is proportional to the number of nucleotides incorporated into the DNA strand. Apyrase degrades the unincorporated nucleotides and ATP before the addition of the next dNTP (Royo et al., 2007;Ahmadian et al., 2006;Diggle and Clarke, 2004;Ronaghi et al., 1998).

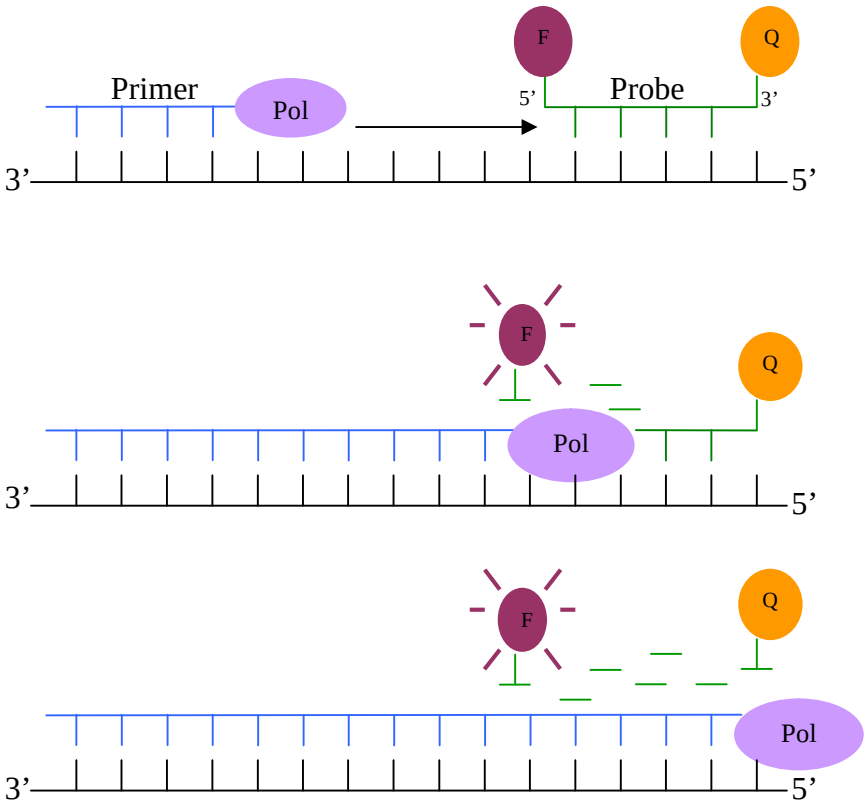


Figure 5.1 Principle of TaqMan real-time PCR. Once the primer and probe are bound onto the template, *Taq* polymerase catalyses the incorporation of dNTP's into the DNA strand. When *Taq* polymerase reaches the probe, the bond between the reporter and quencher is broken. This allows the reporter to emit its florescence and this is detected by a CCD camera. *Taq* polymerase is indicated in the lilac circle (Pol), the fluorescent reporter in the purple circle (F) and the quencher in the orange circle (Q).

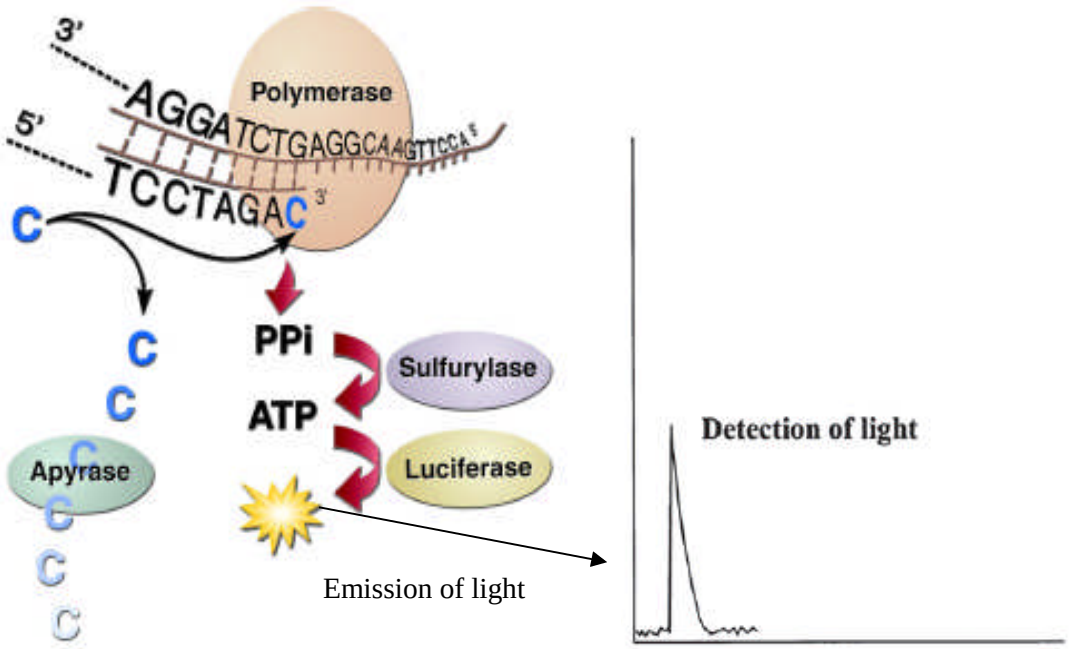


Figure 5.2 Principle of pyrosequencing. The sequencing primer is annealed to the single stranded DNA template. A mix of enzymes, APS, luciferin and dNTPs are added and light is released in proportion to the amount of nucleotide that is incorporated. The light emitted is represented as peaks in the pyrogram. Figure taken from (Diggle and Clarke, 2004).

The technique of allele-specific PCR (ASPCR) was first introduced in the mid 1980's and was previously known by many synonymous names such as PCR amplification of specific alleles (PASA), allele-specific amplification (ASA), and amplification refractory mutation system (ARMS) (Sarkar et al., 1990). This technique has been used to identify polymorphisms in genetic diseases such as sickle cell anaemia (Wu et al., 1989) and haemophilia (Bottema et al., 1990).

With regard to HIV research, ASPCR was used to address the issue that some HIV genotying methods could not detect viral populations that represented less than 20% of the total population. (Hance et al., 2001) used ASPCR to study the kinetics of viral populations within patients who harboured drug resistant virus. These patients underwent structured treatment interruptions and the rate at which the resistant quasi species was replaced by wild type was monitored using ASPCR.

ASPCR is based on the principle of having a single nucleotide mismatch at the 3' end of the primer which destabilises the 3' end and increases specificity. The mismatched primer binds more strongly to the wild type sequence than the mutant sequence and as a result, the Ct value of the mutant sequence is higher than the Ct value of the wild type sequence. ASPCR is a rapid, sensitive and quantitative assay and as a result has often been used to characterise minority species in drug resistant HIV populations (Metzner et al., 2009;Liu et al., 2010;Johnson et al., 2008;Bottema et al., 1990;Paredes et al., 2007).

In the studies presented in this thesis, viral fitness was determined using the growth competition assay. This chapter describes the optimisation of the method for

differentiating H208Y and the H208 SDM in growth competition assays. Three methods were investigated: allele specific PCR, TaqMan real time PCR and pyrosequencing.

5.2 Method

5.2.1 DNA template

Plasmids were produced using the RT gene with H208Y and the H208 SDM derived from the plasma virus of the treatment naïve and treatment experienced patients infected with subtype B. Plasmids from the treatment experienced patient infected with subtype A and the HXB2 wild type were also used. The plasmids were mixed in combinations containing 100% H208Y/0% H208SDM; 90% H208Y/10% H208 SDM, and 0% H208Y/100% H208 SDM before testing.

5.2.2 Allele-specific PCR

Subtype specific primers targeting H208Y and H208 were designed and tested with the H208Y and H208 SDM plasmids in three independent experiments. Each experiment was performed in duplicate and the mean was calculated. Water and no template were used as negative controls in each experiment. The no template control contained water instead of a DNA template.

5.2.3 TaqMan real-time PCR

Specific mutant and wild type probes were designed and tested against H208Y and H208 SDM DNA. In each run, triplicate wells were used for each sample and the mean ct was calculated. Water and no template controls were run in each assay. The no template control contained water instead of a DNA template. This was repeated on three separate occasions.

5.2.4 Pyrosequencing

Forward and reverse biotinylated primers were designed for the PCR. Water was run as a negative control in all runs. Pyrosequencing reactions were analysed using the SQA and QA analysis programs. The SQA run provides the sequence of the given sample once the dispensation order has been determined. From these sequences, the peak heights can be used to determine the proportions of mutant and wild type codons at position 208. The QA program automatically determines the percentage of wild type and mutant codons within the samples when a specific dispensation order is given.

5.3 Results

5.3.1 Allele-specific PCR

Figure 5.3 shows the sequences of the subtype specific primers used in the ASPCR assay. Two primers were designed for each subtype, one to amplify H208 SDM DNA and one to amplify H208Y DNA. In all cases, the mismatched nucleotide was positioned two nucleotides positions from the SNP site and is shown in red in figure 5.3.

Figure 5.4 shows the amplification plots on both the H208 SDM DNA and H208Y DNA using mutant and wild type specific primers. Figure 5.4a shows the mutant primers amplified at a Ct value of 8 and the wild type primers amplified at a Ct of 17 (Δ=9) with the H208Y DNA template. In contrast figure 5.4b shows the mutant primers amplified at a Ct of 18 and the wild type primers amplified at a Ct of 10 (Δ=8) with the H208 SDM DNA template.

The same experiments were repeated for the plasmids containing the RT from HXB2 wild type and the two treatment experienced patients infected with subtype A and subtype B. To check reproducibility, the assay was repeated on three separate occasions. The results are shown in table 5.1, indicating that for the subtype B plasmids, the delta Ct was between 7.5-9 and between 11-12 for the subtype A plasmids.

The coefficient of variance was calculated for the Ct values obtained using both the mutant and wild type primers. All CV values were below 10% for mutant and wild type primers. For the plasmid from the subtype A infected patient using the wild

type primers, a CV of 0% was obtained as there was no variation in the Ct value obtained in the replicate experiments.

In order to quantify proportions of H208Y and H208 DNA present, standard curves were used. For each patient, two standard curves were constructed, one to quantify the H208 SDM DNA and one to quantify the H208Y DNA. This is shown in Figure 5.4 (c-d). Standard curves with a slope range of -3.3 to -3.5 and an R^2 value of 0.99 were accepted and any curves outside of this range were repeated. In the standard curve, the range of plasmid copies was from 10^5 to 10^{-1} copies per millilitre.

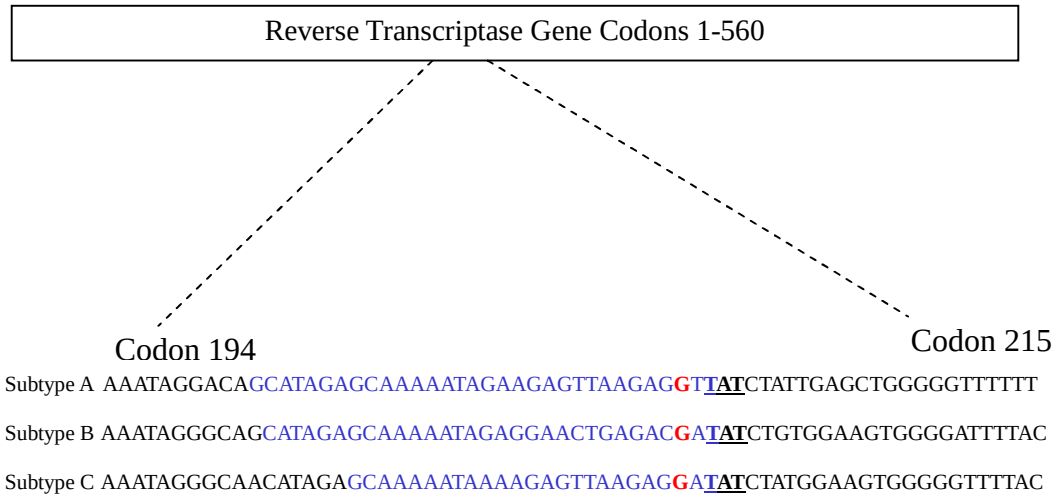


Figure 5.3 Schematic of reverse transcriptase gene. Nucleotides encompassing codons 194-215 are shown. The H208 position is shown in bold and underlined. The nucleotide change from histidine to tyrosine is CAT to TAT. The subtype specific mutant and wild type primers are shown in blue and the designed mismatch at the 3' of the primer is shown in red.

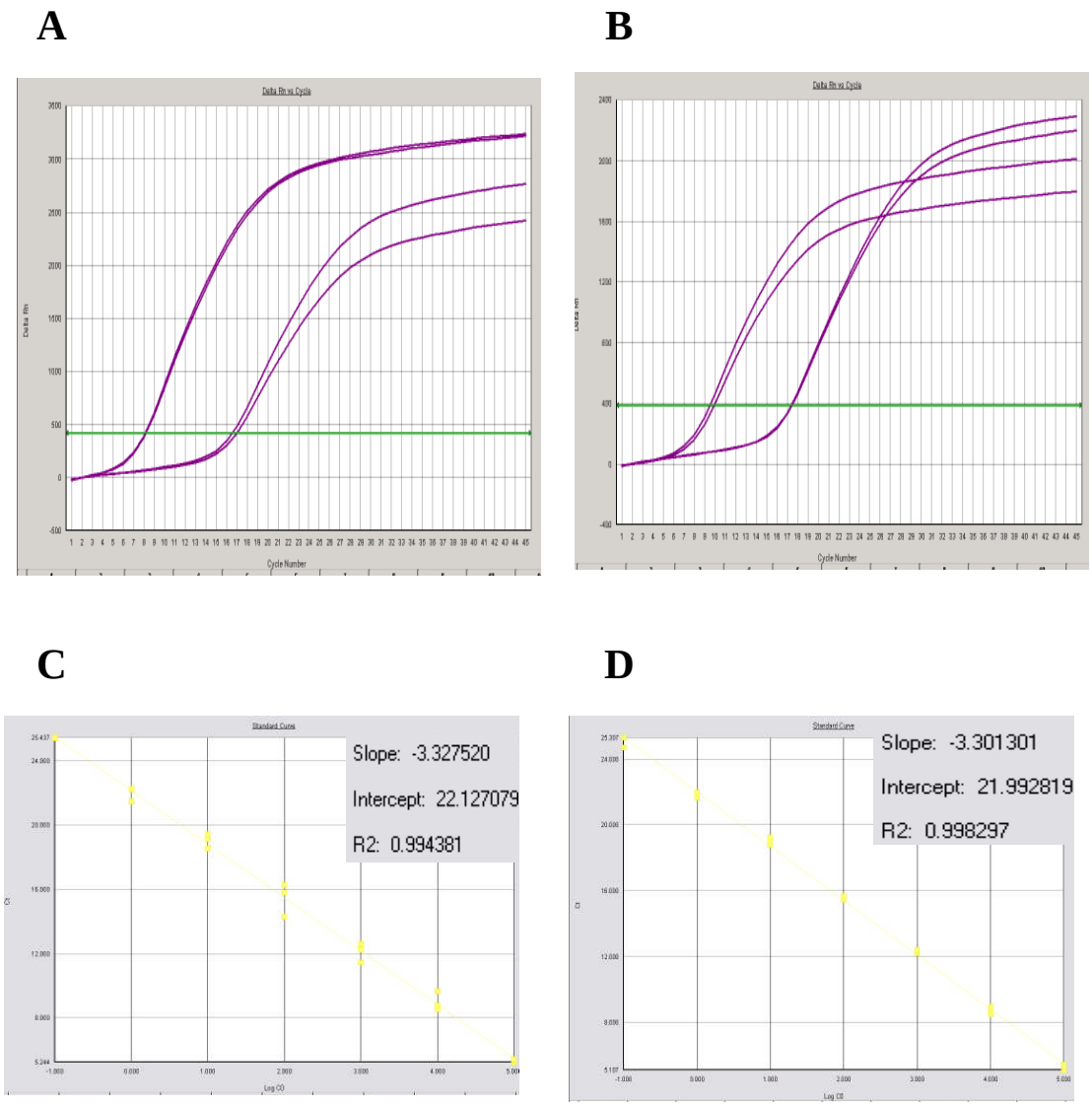


Figure 5.4 Amplification curves from the ASPCR. Plasmids containing the RT gene with H208Y and the H208 SDM derived from the plasma virus of the treatment naïve patient were used as the DNA template. a) amplification using the mutant and wild type primers with the H208Y DNA template. b) amplification using the wild type and mutant primers with the H208 SDM DNA template. c) standard curve obtained using mutant primers with the H208Y DNA template. The range of the plasmid copies was from 10^5 to 10^{-1}. d) standard curve obtained using wild type primers with the H208 SDM DNA template. The range of plasmid copies was from 10^5 to 10^{-1}.

Table. 5.1 Ct values obtained using different plasmid DNA templates with subtype specific mutant and wild type primers

Template	Mutant Primers			Wild Type Primers			Δ Ct (range)	Replicates
	Ct Mean (range)	Ct Standard Deviation	Coefficient of Variance (CV)	Ct Mean (range)	Ct Standard Deviation	Coefficient of Variance (CV)		
Subtype B Naive	19 (17-20)	1.73	9.1%	10 (10-12)	0.58	6%	9 (7-8)	3
Subtype B Naïve +H208Y	8 (7-10)	0.58	6.9%	17 (17-18)	0.58	3.3%	9 (10-8)	3
Subtype B Experienced	16 (15-17)	1.00	6.3%	8 (8-8)	0	0%	8 (7-9)	3
Subtype B Experienced +H208Y	7.5 (7-8)	0.50	6.7%	15 (15-15)	0	0%	7.5 (8-7)	3
Subtype A Experienced	23 (23-24)	0.58	2.5%	12 (11-13)	1.00	8.3%	11 (12-11)	3
Subtype A Experienced +H208Y	12 (11-13)	1.00	8.3%	24 (23-24)	0.58	2.4%	12 (12-11)	3
HXB2 Wild type	19.5 (19-20)	0.50	2.6%	10.5 (10-11)	0.50	4.8%	9 (9-9)	3

[a]Delta Ct (**Δ**) refers to the difference in Ct between the mutant and wild type primers. The coefficient of variance was calculated by dividing the Ct standard deviation by the mean Ct and multiplied by 100.

5.3.2 Real-time PCR – TaqMan PCR

For the TaqMan real-time PCR, specific probes to amplify H208Y or H208 SDM DNA were designed as shown in figure 5.5.

Probes were first optimised by titrating primer and probe concentrations. Primer concentrations varied from 50nM to 300nM and probe concentrations were varied from 25nM and 300nM. Figure 5.6 shows the primer probe optimisation amplification plots for the two probes.

Figure 5.6 shows that some DNA is amplified using the H208 SDM probe for all the primer probe combinations however no amplification was detected with the H208Y probe.

To improve the sensitivity of the PCR, magnesium sulphate was added to the reaction but still no amplification occurred. The annealing temperature of the mutant was also increased but again no amplification occurred.

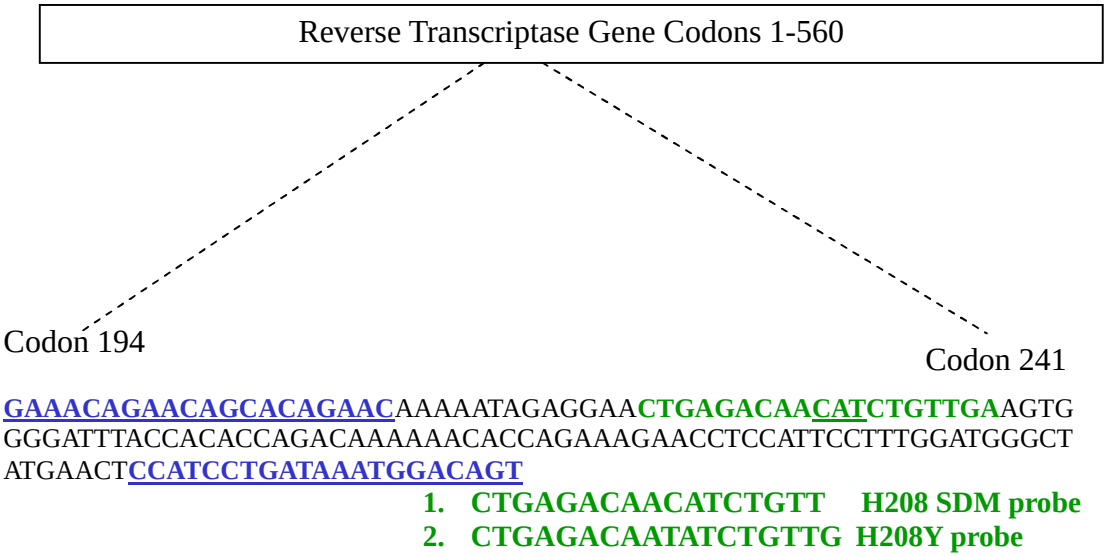
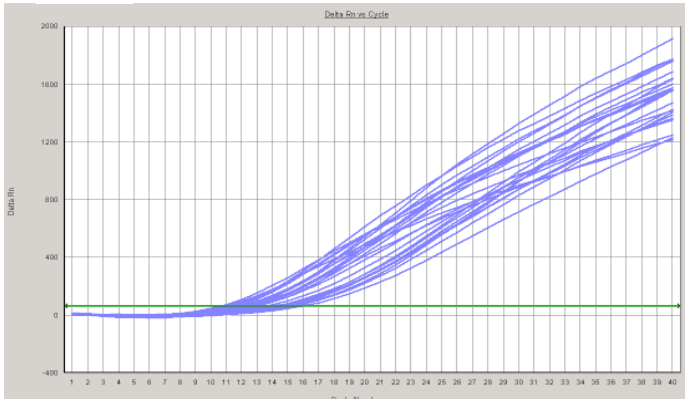


Figure 5.5 Schematic of reverse transcriptase gene encompassing codons 194-241. PCR primers are in blue and the H208Y codon is shown in green and underlined. Probe sequences are shown in green.

A



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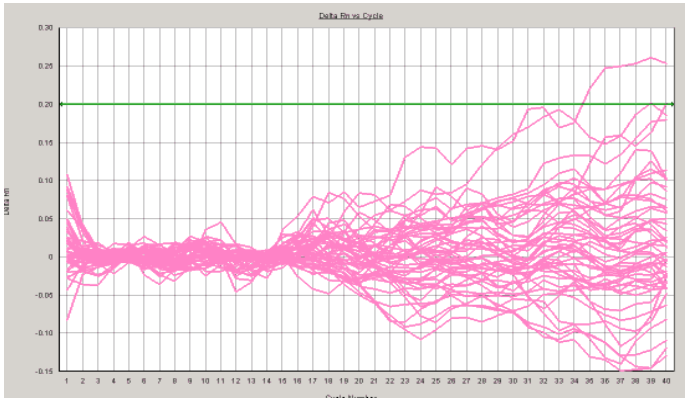


C

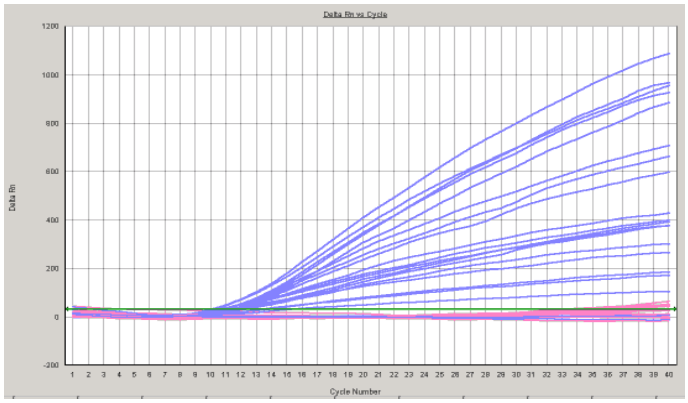


Figure 5.6 Real-time PCR amplification plots using TaqMan probes. a) Amplification plots for the H208 SDM probe (purple) with H208 SDM DNA template. b) Amplification plots for the H208Y probe (pink) with H208Y DNA template. c) Amplification plot of the H208Y and H208 SDM templates together.

5.3.3 Pyrosequencing

Primers were designed so that a 189bp fragment encompassing the H208Y mutation was amplified. Plasmid mixtures containing the RT gene with H208Y and H208 SDM from the treatment naïve patient were made and consisted of the following combinations 100% H208Y/0% H208SDM; 90% H208Y/10% H208 SDM, and 0% H208Y/100% H208 SDM. In order to check that the primers amplified a product, a PCR was done. Figure 5.7 shows the positioning of the pyrosequencing PCR and sequencing primers and the gel electrophoresis picture of the PCR using these primers. The gel electrophoresis picture shows that a clean band is obtained in all the samples at the 200bp position except for the negative control which contained water instead of a DNA template.

Different sequencing primers were designed that aligned at different positions in order to increase the chance of obtaining a clean sequence. The sequencing primers were designed so that they were between 0 and 4 nucleotides from the SNP. These sequencing primers were used in the pyrosequencing assay against the 12 mixture samples at a concentration of 0.3µM and 0.4µM. The pyrosequencer was programmed for both the SQA and QA analysis.

Table 5.2 shows the analysis from the SQA pyrosequencing runs. Run 1 shows that in all the samples, there were mixed wild type (C) and mutant (T) nucleotide detected except for the 100% H208Y sample which failed. In the majority of the samples, the wild type (C) nucleotide was detected and within these samples, the range of wild type (C) nucleotide was between 20-89%. The amount of mutant (T) nucleotide

detected was the highest in the 90% and 80% samples, with 79% and 80% respectively being detected. In the 0% H208Y sample, 16% detected was mutant and 84% detected was wild type.

In run 2 4/11 samples detected 100% wild type (C) nucleotide and this included the 0% H208Y sample. Samples 100%, 90%, 80% and 70% H208Y all detected the mutant nucleotide (T) at higher percentages than the wild type nucleotide (C) and the 100% H208Y sample did have the highest percentage of the mutant nucleotide from all the samples with 85%. The remaining samples detected higher levels of wild type nucleotide than the mutant nucleotide.

The 3rd run had no incidences where 100% of either mutant or wild type nucleotides were detected. In the majority of samples, the wild type nucleotide (C) was detected at a higher frequency than the mutant nucleotide (T) except for the 100% H208Y sample where the mutant (T) was detected at 87.5%.

Since the SQA did not give accurate proportions of mutant and wild type amino acids in the mixture samples, the allele quantification (QA) program was used. Table 5.3 shows the results of the QA from 3 runs. In run 1, the majority of the samples detected 100% wild type (6/11). Samples 100%, 90% and 80% H208Y all detected 79% of the mutant nucleotide and the samples 20% and 0% detected wild type nucleotide at 82% and 84% respectively.

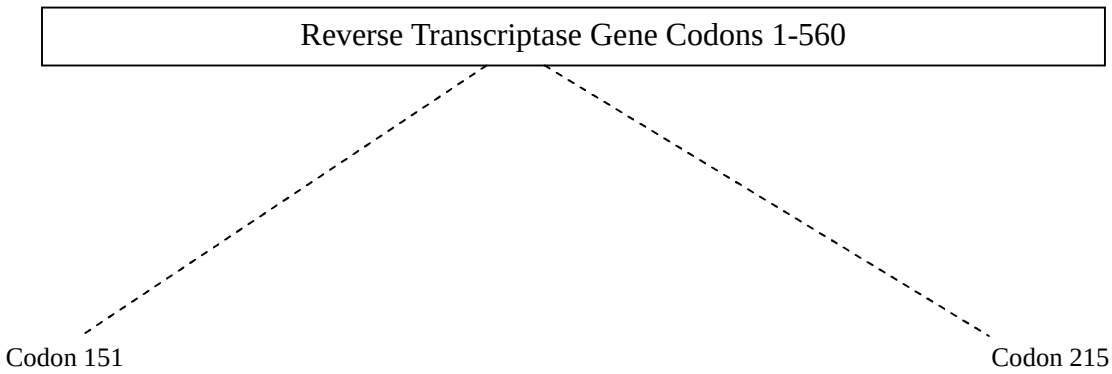
In 5/11 samples from run 2, the wild type nucleotide was detected at a range of 97-100%. The remaining samples detected higher detection levels of the mutant

nucleotide than wild type amino acid. The highest proportion of mutant (T) nucleotide was detected in the 100% mutant samples, which detected 96% mutant and 4% wild type.

Similar to the previous 2 runs, in run 3 the wild type and mutant nucleotides were not accurately detected in the samples. In the majority of cases the wild type nucleotide was detected at a higher percentage. In samples, 100%, 90%, 80% and 70%, the mutant nucleotide was detected at a higher percentage although the wild type nucleotide was also detected in these samples.

In order to improve the accuracy of this assay, different sequencing primers at different concentrations were used as well as using fresh PCR samples for each run. However, the pyrosequencing was not able to differentiate mutant and wild type nucleotides at an accurate level.

A



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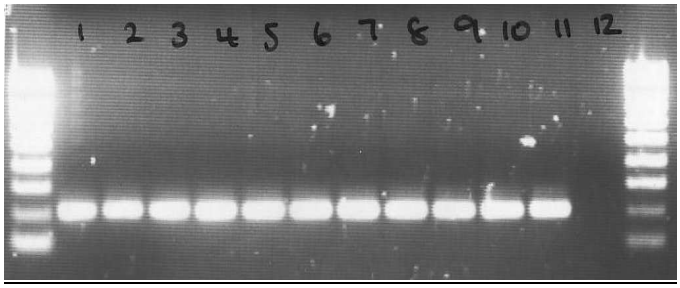

1. 100% mutant
2. 90% mutant
3. 80% mutant
4. 70% mutant
5. 60% mutant
6. 50% mutant
7. 40% mutant
8. 30% mutant
9. 20% mutant
10. 10% mutant
11. 0% mutant
12. Negative control

Figure 5.7 a) Schematic of reverse transcriptase gene. Nucleotides encompassing codons 151-215 are shown with the PCR primers shown in blue, the H208Y codon shown in red and the sequencing primers in green. Three sequencing primers were used and these are listing 1 to 3 in the figure. b) Gel electrophoresis picture of the PCR used in the Pyrosequencing reaction. Lanes 1 to 11 are mixtures of mutant and wild type and the water as the negative control.

Table. 5.2 Pyrosequencing SQA analysis

% Mutant (T)	Run 1		Run 2		Run 3	
	% C	% T	% C	% T	% C	% T
100%	FAILED	FAILED	15%	85%	12.5%	87.5%
90%	21%	79%	24%	76%	87%	13%
80%	20%	80%	21%	79%	80%	20%
70%	89%	11%	24%	76%	89%	11%
60%	85%	15%	100%	0%	94%	6%
50%	86%	14%	100%	0%	95%	5%
40%	84%	16%	89%	11%	91%	9%
30%	83%	17%	89%	11%	88%	12%
20%	88%	12%	87%	13%	90%	10%
10%	88%	12%	100%	0%	92%	8%
0%	84%	16%	100%	0%	93%	7%

Table. 5.3 Pyrosequencing QA analysis

% Mutant (T)	Run 1		Run 2		Run 3	
	% C	% T	% C	% T	% C	% T
100%	21%	79%	4%	96%	15%	85%
90%	21%	79%	18%	82%	24%	76%
80%	21%	79%	17.5%	82.5%	21.5%	78.5%
70%	100%	0%	17%	83%	24%	76%
60%	100%	0%	15.5%	84.5%	100%	0%
50%	100%	0%	22%	78%	100%	0%
40%	100%	0%	97%	3%	89%	11%
30%	100%	0%	100%	0%	89.5%	10.5%
20%	83%	17%	100%	0%	87%	13%
10%	100%	0%	100%	0%	100%	0%
0%	84%	16%	100%	0%	100%	0%

5.4 **Discussion**

In order to distinguish between the two competing viral strains in the growth competition assay, three methods were tested; AS-PCR, pyrosequencing and TaqMan real-time PCR. In this study the allele-specific PCR assay was the most accurate technique that was able to differentiate the two viral strains compared to real-time PCR and pyrosequencing assays.

Growth competition assays involve incubating cells with two viruses and passaging these viruses over a period of time. The different viruses are monitored and the outgrowth of one virus is considered to be a measure of fitness (Holland et al., 1991). One advantage of this assay is that because viruses are monitored over a period of days, small changes in fitness can be measured. Also, the input ratio of the viruses can be altered, giving further insights into the relative fitness of these viruses.

There are two major drawbacks to using growth competition assays to measure fitness. The first drawback is that since the viruses are incubated for a period of days, and given the plasticity of HIV, the virus may mutate and the output virus may be altered from the input virus. Another disadvantage is that it is notoriously difficult to develop a method that is able to distinguish the virus variants, particularly when they differ at a single nucleotide position. Methods that have been used to differentiate the different viruses within growth competition assays include heteroduplex tracking assay (HTA) (Quinones-Mateu et al., 2000b), sequencing (Harrigan et al., 1998) and real-time PCR (de Ronde A. et al., 2001).

Not only was the ASPCR the most accurate method of differentiating between the mutant and wild type templates in this study but it was also highly reproducible. The coefficient of variance was calculated to look at the variation between replicates for both the mutant and wild type primers using the different DNA templates. The inter assay coefficient of variance was below 10% for all samples and a CV of <10% has been suggested to be satisfactory (Murray W et al., 1993).

The delta Ct was determined for all plasmids and the highest delta was seen with the subtype A plasmids with a delta Ct of 11-12 for mutant and wild type primers. The deltas for the subtype B patients were between 7.5 and 9. Having a larger delta Ct value indicates that the primers are binding with good specificity and would be able to reliably differentiate between mutant and wild type DNA. Therefore, for this assay the primers appeared to be differentiating between mutant and wild type DNA with good specificity. The basis of specificity is the designed mismatched nucleotide positioned two nucleotides from the SNP. Previous studies have demonstrated that the mismatched nucleotide in the primer is very disruptive in that it prevents chain elongation and causes a delay of several Ct cycles (Hance et al., 2001;Bottema and Sommer, 1993;Bergroth et al., 2005).

There are two possible reasons why the pyrosequencing was unable to accurately differentiate proportions of mutant and wild type template. These related to the design of the assay or the practical side of the experiment. In terms of assay design, the sequencing primer could have misprimed to another region in the PCR product. There are two potential sites consisting of a stretch of A nucleotides upstream of H208 that the sequencing primers may have annealed. In doing so, the mutant T

nucleotide would not have been measured and this may explain why in many cases there was no accurate presence of the T nucleotide reported by the pyrosequencing. The other possible reason is that the template itself may self-prime resulting in the formation of secondary structures within the DNA and therefore hindering the action of the DNA polymerase and/or the sequencing primer. This pyrosequencing assay was designed using the Pyrosequencing Assay Design Software Version 1.0 which does take into account formation of secondary structures and mispriming of primers however that does not completely rule out the possibility of secondary structures forming.

Regarding the practical side, the pyrosequencing procedure requires use of a vacuum prep tool which is awkward to handle. The vacuum prep tool is used to first capture the sepharose beads containing the biotinylated PCR product and then wash and neutralise the PCR product. The PCR product is then released into a tray where the sequencing primer can anneal. One study redesigned the vacuum prep tool as they found the original tool caused residual liquid to remain causing product dilution and also unequal aspiration (Gharizadeh et al., 2006).

In the TaqMan real-time PCR assay, the mutant probe did not detect any DNA. The wild type probe in both cases did produce amplification plots. Possible reasons for the no amplification in the mutant probe include operational error, template impurity and secondary structure. Operational error includes factors such as failure to mix the reaction components or excluding one of the reaction components. However, this assay was repeated a number of times and since in all cases the wild type probe hybridised and produced amplification suggests that there was not a problem with

operational error. The template stock of the template was also changed to rule out the possibility of using the incorrect template. However, this did not resolve the problem. Similar to the pyrosequencing, it is possible that secondary structures formed which prevented the probe from binding. Since these methods were being developed simultaneously and the ASPCR gave promising results, no further time was invested in improving the real-time PCR and pyrosequencing assays.

Chapter six

6 The impact of H208Y on viral fitness

6.1 Introduction

Results from the previous chapters indicated that in the context of a subtype B that was either wild type or harboured pathway 1 TAMs, H208Y reproducibly conferred a reduced susceptibility to tenofovir (TDF). To investigate whether the H208Y mutation conferred a fitness advantage, growth competition experiments were performed using an optimised allele specific PCR (AS-PCR) assay to differentiate between mutant and wild type strains. In this chapter the results of the growth competition assay are presented.

Many studies have shown that the majority of heterosexual infections result from the transmission of a single virus (Keele, 2010;Keele and Derdeyn, 2009;Kearney et al., 2009;Haaland et al., 2009). However due to the combined effect of a high viral replication rate and an error-prone reverse transcriptase that lacks proof-reading activity, a quasispecies is quickly formed whereby virus variants that are related but genetically distinct are produced (Domingo et al., 1985). The dominant quasispecies is constantly subjected to various selective forces such as drug pressure, and as a result, the dominant quasispecies changes accordingly to adjust to the new environment. The predominating quasispecies at a given time represents the population that is best equipped to infect and replicate, i.e., it is most fit under the given selective pressures (Coffin, 1995). In this scenario variants of higher fitness out compete variants of lower fitness to become the dominant quasispecies. Hence, within a HIV infected patient, there are virus variants residing with differing degree of fitness.

Major or primary drug resistance mutations are generally associated with a reduced fitness, often referred to as the “fitness cost” of drug resistance. With ongoing virus replication under drug pressure, additional mutations emerge that may either increase the level of resistance or compensate for decreased fitness. These mutations are known as compensatory, accessory or secondary mutations. These mutations have been well characterised in the case of the protease gene, but data are more limited in the case of the RT gene (Nijhuis et al., 1999;Mammano et al., 2000;Borman et al., 1996;Molla et al., 1996;Condra et al., 1995). To date, there has been only one study that has investigated the effects of accessory mutations in the RT gene. This study identified the E40F and K43E mutations and showed that they conferred resistance to zidovudine (AZT) if present in the background of TAMs however, in isolation, no resistance was conferred by these mutations. The virus with E40F, K43E and TAMs was also shown to be more fit than the virus with TAMs (Huigen et al., 2008).

Mathematical models have often been used to compare the fitness of different viral strains. However there have been a number of different equations used to calculate relative fitness and this has hindered the ability of comparing results obtained from different studies. One mathematical model uses the selection coefficient (s) to measure fitness and takes into account data collected from two time points. The relative fitness of the variant, defined as 1+s, is the relative contribution of that variant to the next generation. If mutant and wild type strains are compared and the mutant strain is more fit than the wild type strain, 1+s will be greater than 1. If the mutant strain is less fit than the wild type strain, 1+s will be less than 1 (Dykes and Demeter, 2007;Maree et al., 2000). The first study that used the selection coefficient in relation to HIV drug resistant variants was Goudsmit et al. 1996. This study

looked at the dynamics of variants from a patient who was infected with a strain containing the AZT resistance mutation T215Y. They found that in the absence of drug, the T215Y variant was replaced by T215S as it was estimated to be 0.4 to 2.3% more fitter than T215Y (Goudsmit et al., 1996).

The aim of the study described in this chapter was to use growth competition assays to investigate whether the H208Y mutation contributes to viral fitness in the context of both wild type virus and highly resistant viruses harbouring TAMs alongside other major RT resistance mutations.

6.2 Methods

6.2.1 Growth competition assay

Competitions were set up between H208Y and H208 SDMs derived from subtype B wild type virus, subtype B virus with pathway 1 TAMs, and subtype A virus with pathway 2 TAMs. The subtype C virus with TAMs was not tested as the AS-PCR was unable to differentiate between H208Y and H208 viruses accurately with this subtype and optimisation could not be completed in time. Competitions were set up at three H208Y:H208 input ratios: 50:50, 25:75 and 75:25. The two competing viruses were added to MT4 cells. After 2 hours, cells were washed with PBS and resuspended in medium. On day 6, aliquots were stored at -80ºC and 50µl of virus supernatant was used to reinfect fresh MT4 cells to start a new passage. This was continued for 4 passages over 24 days. As controls in all assays, single virus infections as well as uninfected cells were also set up. Competitions were repeated at least twice on separate occasions for each combination. The mean proportions from the replicates experiments are shown. For competitions done in the presence of drug, the IC50 values obtained for these viruses with tenofovir (TDF) were used. TDF was added to the medium and replenished at each new passage.

6.2.2 RNA extraction

Viral RNA was extracted using the Qiagen QIAamp Viral RNA kit (Qiagen) from 200µl of HIV infected culture supernatant. RNA was stored at -80ºC until required.

6.2.3 Allele-specific PCR

RNA was nanodroped using the NanoDrop^{TM} 8000 and 300ng RNA was used in the AS-PCR assay. Subtype specific H208Y and H208 primers were designed for each subtype and both primers were tested against mutant and wild type DNA. In each run, duplicates wells were used for each sample and the mean was calculated. Water and no template controls were run in each assay.

6.2.4 Sequencing

From each competition, virus recovered from passages 1 and 4 was sequenced using Sanger sequencing with BioDye Sequencing mix v1.1 (Applied Biosystems, UK). Four forward and reverse primers were used to obtain overlapping sequences. Sequences were analysed using a 3100-Avant Genetic Analyzer (Applied Biosystems, UK).

6.2.5 Fitness Calculations

Fitness calculations were performed as described by (Quinones-Mateu et al., 2000a) and (Hu and Kuritzkes, 2010). The mean from at least two independent experiments was used to calculate the fitness difference and selection coefficient. The proportion of virus recovered relative to the initial inoculum was determined as the amount of virus produced at day 12 or 18 divided by the proportion present in the initial inoculum. The relative fitness was determined as the average virus produced from the three different ratios. The fitness difference was determined as the ratio of relative fitness values $W_D = W_M/W_L$, where W_M represents the more fit virus and W_L represents the less fit virus in the growth competition assay. The selection coefficient was determined using the following formula: $s = (1/t \ \ln[q(t)p(0)/p(t)(q(0)])*100$,

where t is the time in days, $p(t)$ is the proportion on day 12 or day 18 of the more fit virus, $q(t)$ is the proportion on day 12 or day 18 of the less fit virus, $p(0)$ corresponds to the initial proportion in the inoculum of the more fit virus and $q(0)$ corresponds to the initial proportion in the inoculum of the less fit virus.

6.3 Results

6.3.1 Growth competitions with wild type subtype B virus from a treatment naïve patient

Figure 6.1 shows the growth competitions from the subtype B treatment naïve patient. Plasma virus from this patient harboured no major resistance mutations. Input ratios for the H208Y vs H208 competitions were 50:50, 25:75 and 75:25 in the absence of drug and 50:50 in the presence of drug. All competitions were set up on three separate occasions and the mean proportions of the H208Y and H208 viruses recovered were calculated.

In the 50:50 H208Y vs H208 competitions, the H208 virus outgrew the H208Y virus. By day 12, the proportion of H208Y virus dropped from the initial 50% input to less than 10%. In the 25:75 competitions, the H208 virus out competed the H208Y virus. In the 75:25 competitions, the H208 virus outgrew the H208Y virus from the outset.

The phenotypic data showed that compared to the H208 SDM, the presence of H208Y in the absence of major resistance mutations decreased susceptibility to TDF. The IC50 values were 15.75μM and 7.14μM with and without H208Y, with a fold change of 0.68 and 0.31 (Δ -0.37) respectively. In competition experiments in the presence of TDF, the IC50 for TDF was used. By day 6 the H208 SDM virus started to outgrow the H208Y virus and by day 12, the proportion of the H208Y virus fell from 40% to 18% and remained at low proportions until day 24.

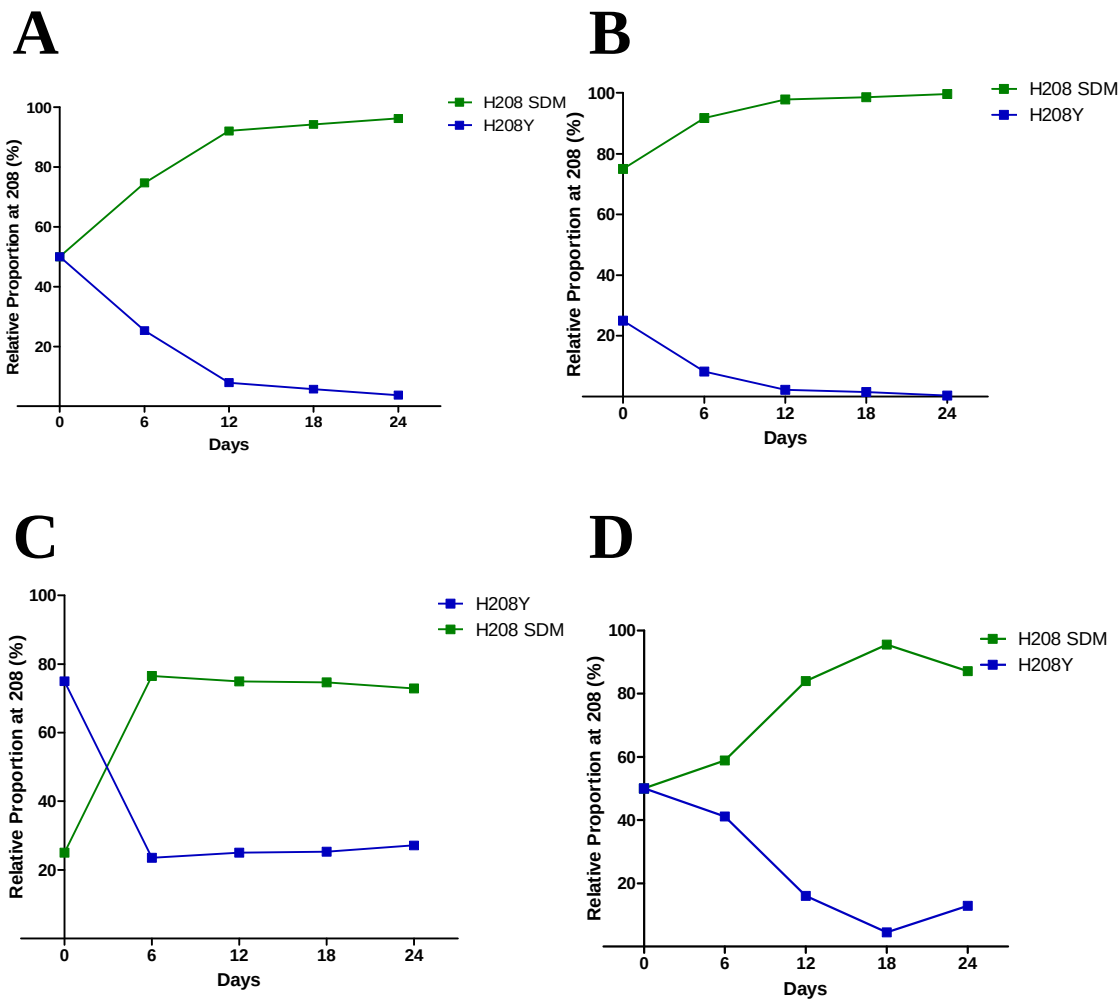

Figure 6.1 Growth competitions using wild type virus with H208Y and the SDM virus with H208 from the subtype B treatment naïve patient, in the presence and absence of TDF. The virus harboured no resistance mutations. The mean from three independent experiments is shown. The input ratios for H208Y vs H208 were a) 50:50; b) 25:75; c) 75:25 without TDF; and d) 50:50 in the presence of TDF. The green line indicates proportion of the H208 SDM virus and the blue line indicates the proportion of the H208Y virus.

6.3.2 Growth competitions with drug resistant subtype B virus from a treatment experienced patient

Plasma virus from the subtype B treatment experienced patient harboured the pathway 1 TAMs M41L, L210W and T215Y together with the TAM-2 D67N and K219N and V75M. The NNRTI resistance mutations A98G and G190Q were also present.

Figure 6.2 shows the results of the growth competitions between the virus (referred to as TAM-1) with and without H208Y. Input ratios for TAM-1 H208Y vs. TAM-1 H208 in the competitions were 50:50, 25:75 and 75:25 in the absence of drug, and 50:50 in the presence of drug. All competitions were set up on three separate occasions and the mean proportions of the TAM-1 virus with H208Y and the SDM with H208 were calculated.

From the 50:50 competitions, while the TAM-1 H208Y appeared to prevail at day 6, by day 12 it had been outcompeted by H208. After day 12 the proportions of the two viruses remained constant. In the 25:75 and 75:25 competitions, the proportions remained relatively constant throughout the experiments.

The phenotypic data showed that compared to the TAM-1 H208 SDM, the presence of H208Y on a background of TAM-1 mutations decreased susceptibility to TDF. The IC50 was 404µM and 177µM with and without H208Y respectively with a fold change of 17.3 and 7.6 (Δ –9.7) respectively. In the 50:50 competitions in the presence of TDF, the IC50 obtained for TDF was used. There were no major changes in the proportions of the two viruses until day 12. After day 12, the TAM-1

H208 SDM virus started to outgrow the TAM-1 H208Y virus and by day 24, the proportion of the TAM-1 H208 SDM and TAM-1 H208Y viruses were 65% and 35% respectively.

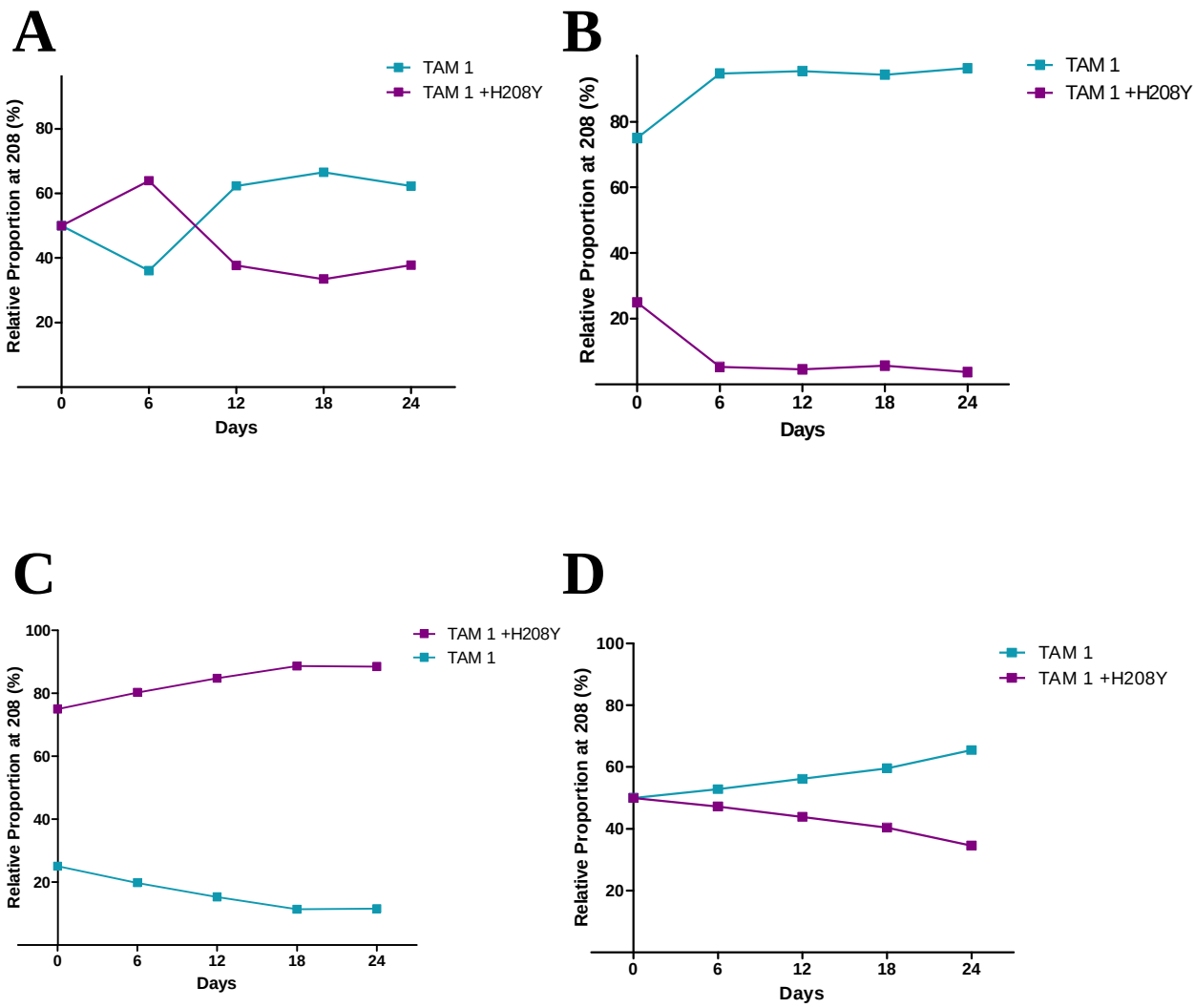

Figure 6.2 Growth competitions using drug resistant virus with TAM-1 H208Y and the SDM virus with TAM-1 H208 from the subtype B treatment experienced patient, in the presence and absence of TDF. The virus harboured the pathway 1 TAMs M41L, L210W and T215Y together with the TAM-2 D67N and K219N with V75M. The NNRTI resistance mutations A98G and G190Q were also present. The mean from three independent experiments is shown. The input ratios for TAM-1 H208Y vs TAM-1 H208 SDM were a) 50:50; b) 25:75; c) 75:25 without TDF; and d) 50:50 in the presence of TDF. The blue line indicates proportion of the TAM-1 H208 SDM virus and the purple line indicates the proportion of the TAM-1 H208Y virus.

6.3.3 Growth competitions with drug resistant subtype A virus from a treatment experienced patient

Plasma virus from the subtype A treatment experienced patient harboured the pathway 2 TAMs D67N, K70R, T215F and K219E together with M184V and T69N. The M184V mutation was back mutated to the wild type amino acid methionine by side directed mutagenesis in order to eliminate the effect this mutation would have on viral fitness. The NNRTI resistance mutations A98G and G190Q were also present.

Figure 6.3 shows the results of the growth competitions between the TAM-2 virus with and without H208Y. Input ratios for TAM-2 H208Y vs. TAM-2 H208 in the competitions were 50:50, 25:75 and 75:25 in the absence of drug, and 50:50 in the presence of drug. All competitions were set up on two separate occasions and the mean proportions of the TAM-2 virus with H208Y and the SDM with H208 were calculated.

In all replicate experiments from the 50:50 competitions, the proportions of the viruses remained relatively constant as the input ratios up to day 12. By day 24, the proportion of the TAM-2 H208 SDM and TAM-2 H208Y viruses were 68% and 32% respectively. In both the 25:75 and 25:75 competitions, the proportions remained relatively unchanged from the input ratios throughout the experiments.

The phenotypic data showed that H208Y did not modify significantly phenotypic susceptibility to TDF relative to virus without H208Y. The IC50 was 12.17µM and 13.60µM with and without H208Y respectively with a fold change of 0.50 and 0.58

(Δ +0.08) respectively. In the 50:50 competitions in the presence of TDF, the IC50 obtained for TDF was used. By day 6, the TAM-2 H208 SDM outgrew the TAM-2 H208Y virus and by day 24 represented 83% of the virus population.

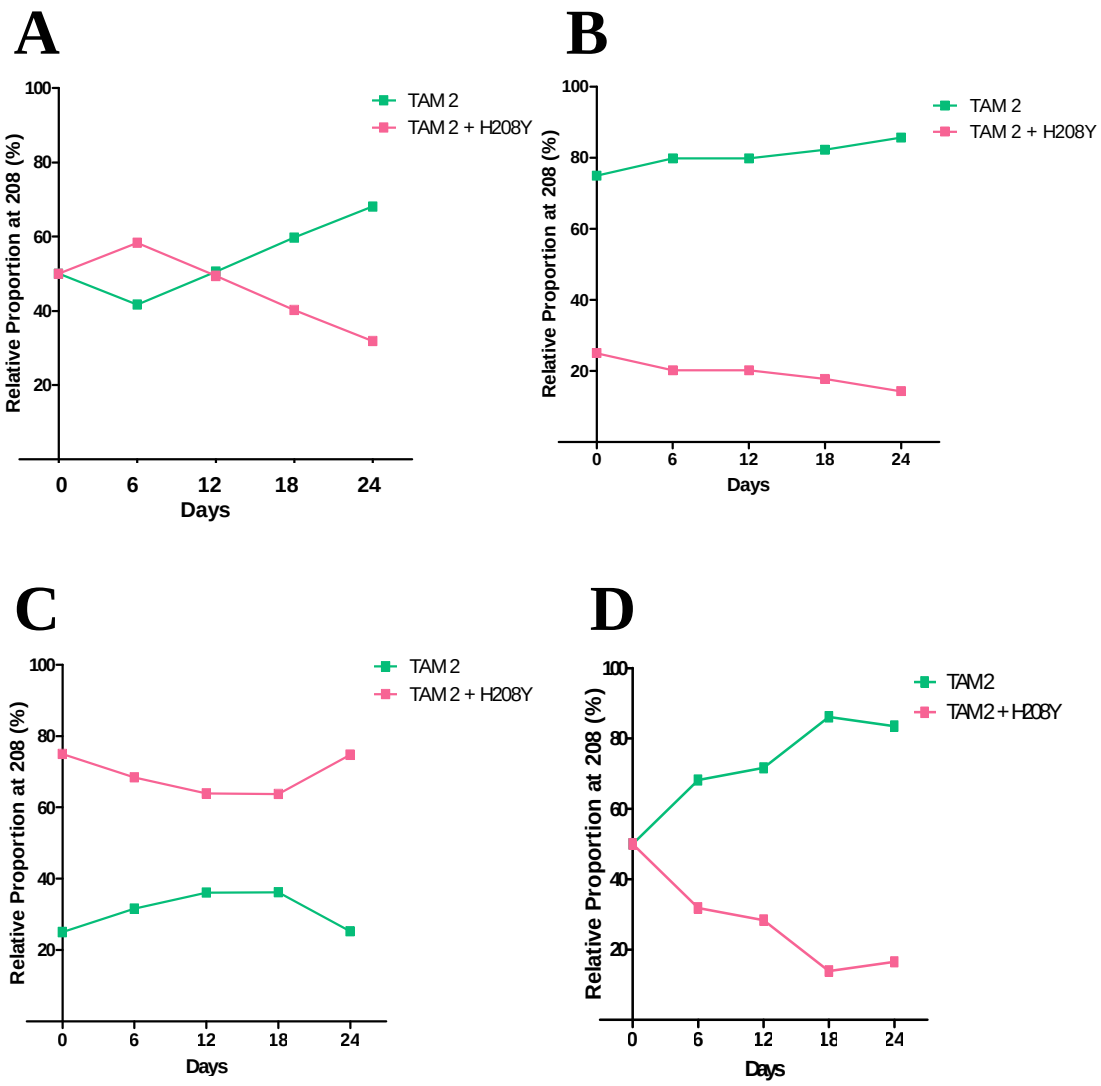

Figure 6.3 Growth competitions using drug resistant virus with TAM-2 H208Y and the SDM virus with TAM-2 H208 from the subtype A treatment experienced patient, in the presence and absence of TDF. The virus harboured the pathway 2 TAMs D67N, K70R, T215F and K219E together with M184V and T69N. The mean from two independent experiments is shown. The input ratios for TAM-2 H208Y vs TAM-2 H208 SDM were a) 50:50; b) 25:75; c) 75:25 without TDF; and d) 50:50 in the presence of TDF. The teal line indicates proportion of the TAM-2 H208 SDM virus and the pink line indicates the proportion of the TAM-2 H208Y virus.

Table. 6.1 Relative fitness of viruses with and without H208Y

Virus	TDF	Proportion of Virus Relative to Initial Inoculum[a]			Relative Fitness[b]	Fitness Difference (fold)[c]	Selection Coefficient[d] (%)
		50:50	25:75	75:25			
H208Y	-	0.14	0.08	0.33	0.18	11.44	21.5
H208 SDM	-	1.86	1.31	3	2.06		
H208Y	+	0.32	ND	ND	0.32	5.25	13.8
H208 SDM	+	1.68	ND	ND	1.68		
TAM-1 H208Y	-	0.74	0.2	1.13	0.69	1.51	4.4
TAM-1 H208 SDM	-	1.26	1.27	0.6	1.04		
TAM-1 H208Y	+	0.88	ND	ND	0.88	1.27	2.0
TAM-1 H208 SDM	+	1.12	ND	ND	1.12		
TAM-2 H208Y	-	1	0.8	0.85	0.88	1.32	2.3[e]
TAM-2 H208 SDM	-	1	1.07	1.44	1.17		
TAM-2 H208Y	+	0.56	ND	ND	0.56	2.57	7.9
TAM-2 H208 SDM	+	1.44	ND	ND	1.44		

[a]The proportion of virus relative to the initial inoculum was determined as the amount of virus produced at day 12 divided by the proportion present in the initial inoculum. Values represent the mean from at least two separate experiments. [b]The relative fitness was determined as the average virus produced from the three different ratios. [c]The fitness difference was determined as the ratio of relative fitness values $W_D = W_M/W_{L,}$ where W_M represents the more fit virus and W_L represents the less fit virus in the growth competition assay. [d]The selection coefficient was determined using the following formula, $s = (1/t \ln[q(t)p(0)/p(t)(q(0)])*100$, where t is the time in days, $p(t)$ is the proportion on day 12 of the more fit virus, $q(t)$ is the proportion on day 12 of the less fit virus, $p(0)$ corresponds to the initial proportion in the inoculum of the more fit virus and $q(0)$ corresponds to the initial proportion in the inoculum of the less fit virus. The mean from at least two separate experiments was used to determine the fitness difference. [e]The selection coefficient for this competition used values obtained from day 18 rather than day 12 because there was no difference in proportion obtained on day 12.

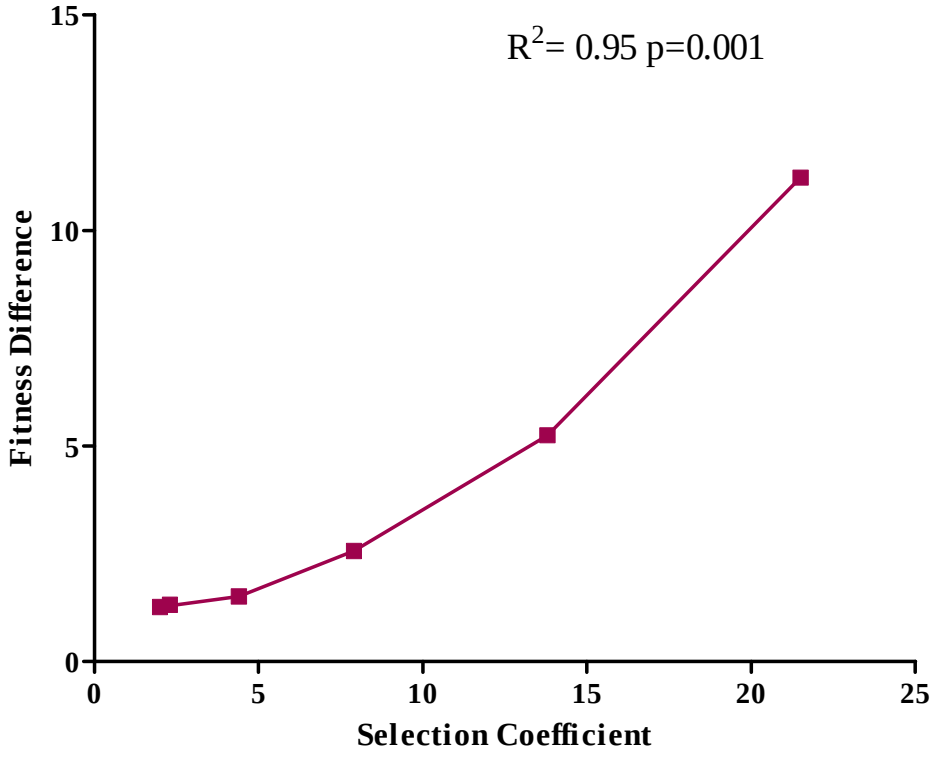

Figure 6.4 Line graph plotting the selection coefficient (s) against the fitness difference. There was a direct correlation between the selection coefficient and the fitness difference, r^2 = 0.95, P = 0.001 as determined using Pearson's product moment correlation coefficient.

6.3.4 Relative fitness of viruses containing H208Y

Fitness calculations as described by (Quinones-Mateu et al., 2000a;Hu and Kuritzkes, 2010) were used to obtain a quantitative estimate of viral fitness on day 12. Day 12 was chosen because in the majority of cases, the fitness effect did not alter significantly after this time. The one exception was for the competitions with TAM-2 subtype A virus done in the presence of TDF. On day 12, the proportions were unchanged; therefore the next time point (day 18) was used in the fitness calculations.

As shown in table 6.1, the relative fitness of all viruses with H208Y was lower than that of viruses without H208Y. The relative fitness differences for the TAM-containing viruses were lower than those observed for wild type subtype B virus. From the competitions of the subtype B wild type virus, the relative fitness difference of the H208 SDM virus was 11.44 fold greater than that of the H208Y virus in the absence of TDF. In the presence of TDF the relative fitness difference if the H208 SDM virus was 5.25 fold greater than that of the H208Y virus. From the competitions of the TAM-1 subtype B virus, the relative fitness difference of the H208 virus was 1.51 fold greater than that of the H208Y virus in the absence of TDF. In the presence of TDF, the relative fitness of the H208 virus was 1.27 fold greater than that of the H208Y virus. From the competitions of the TAM-2 subtype A virus, the relative fitness difference of the H208 virus was 1.32 fold greater than that of the H208Y virus in the absence of TDF. In the presence of TDF, the relative fitness of the H208 virus was 2.57 fold greater than that of the H208Y virus.

The selection coefficient was also calculated for the competitions as shown in table 6.1. For both the subtype B wild type and subtype B TAM-1 containing viruses, the selection coefficient in the presence of TDF was lower than the selection coefficient in the absence of TDF. However the selection coefficient for the subtype A TAM-2 containing virus had a higher selection coefficient in the presence of TDF compared to the absence of TDF.

The competitions with the wild type subtype B virus both in the presence and absence of TDF had the highest *s* value of 21.5 and 13.8 respectively. This indicates that H208Y is less fit compared to H208 in the presence and absence of TDF and that this fitness cost is most apparent within the competitions of the subtype B wild type virus. This was also observed with the TAM-1 subtype B virus albeit to a lesser effect that the wild type subtype B virus. The selection coefficient was 4.4 and 2.0 in the absence and presence of TDF respectively.

The selection coefficient of the subtype A TAM-2 containing viruses were 2.3 and 7.9 in the absence and presence of TDF respectively. This demonstrates that similar to the subtype B wild type and TAM-1 containing viruses, H208Y is less fit in a subtype A TAM-2 virus but also that the effect is more apparent in the presence of TDF compared to the absence of TDF.

To see if there was a correlation between the two different calculation methods used to calculate the relative fitness, the selection coefficient was plotted against the relative fitness (figure 6.4). There was a direct correlation between the selection coefficient and the relative fitness difference with an $r^2 = 0.95$ and $P = 0.001$,

indicating that both calculation methods showed that the viruses with H208Y were less fit than the viruses without H208Y.

Table. 6.2 IC50 and IC90 values obtained for tenofovir against patient viruses with and without H208Y[a]

Virus	IC50 (μM)	IC90 (μM)
H208Y	15.75	29
H208 SDM	7.14	48
TAM-1 H208Y	404	2942
TAM-1 H208 SDM	177	4673
TAM-2 H208Y	12.17	63
TAM-2 H208 SDM	13.60	193

[a]Drug susceptibility assays were performed in quadruplicate and the average of the four was used to calculate the IC50. Mean values were calculated from three replicate experiments for each virus/drug combination. HXB2 was used as a wild type control in all assays.

6.3.5 Drug susceptibility results for TDF

Table 6.2 shows the IC50 and IC90 values obtained for TDF for the viruses with and without H208Y from the different patients. To compare the IC90 values of the viruses obtained against TDF, the IC90 was calculated using the same dose response curve that the IC50 was obtained. In some cases an IC90 value was not available from the dose response curve because the percentage reduction for the particular virus-drug combination did not reach 100%, therefore although the IC50 could be calculated, the IC90 could not be calculated. In such cases, adding a trendline on the dose response curve and using the equation from the graph, a projected IC90 was calculated. The IC90 from at least two replicate experiments was used to obtain an average IC90 value.

For the viruses from the subtype B treatment naïve patient, the IC50 was 15.75µM and 7.14µM with and without H208Y, with a fold change of 0.68 and 0.31 (Δ -0.37) respectively. The IC90 was 29µM and 48µM with and without H208Y respectively. For the viruses from the subtype B treatment experienced patient, the IC50 was 404µM and 177µM with and without H208Y respectively with a fold change of 17.3 and 7.6 (Δ –9.7) respectively. The IC90 was 2952µM and 4673µM with and without H208Y respectively. For the viruses from the subtype A treatment experienced patient, the IC50 was 12.17µM and 13.60µM with and without H208Y respectively with a fold change of 0.50 and 0.58 (Δ +0.08) respectively. The IC90 was 63µM and 193µM with and without H208Y respectively.

6.4 **Discussion**

In the previous chapters, it was shown that the H208Y mutation conferred a reduced susceptibility to TDF in the context of a subtype B wild type and TAM-1 RT sequence. A growth competition assay was developed to investigate whether the H208Y also had effects on viral fitness. A subtype specific AS-PCR assay was designed to differentiate between the H208Y mutant and H208 wild type viruses in the growth competition assays. In the context of a subtype B wild type RT sequence, the H208 SDM virus was more fit than the H208Y virus regardless of the input ratios. This was true for competitions done both in the absence and presence of TDF. In the context of a TAM-1 subtype B and TAM-2 subtype A RT sequence, there was no appreciable difference in fitness as at all input ratios, the viruses maintained stable proportions throughout the competition. This was true for the competitions done in the both in the absence and presence of TDF. TDF is a nucleotide analogue that unlike the nucleoside analogues contains a phosphate group and so requires two rather than three phosphorylation steps (Menendez-Arias, 2008).

The finding that in the context of wild type RT the H208 SDM virus was more fit than the H208Y virus is consistent with the high degree of conservation and therefore infrequent detection of H208Y among treatment naïve patients. The prevalence of H208Y in the treatment naïve population has been reported to be between 0.1 and 0.3% (Cane et al., 2007;Nebbia et al., 2007). In this study, prevalence in the Royal Free treatment naive population was 0.1%. The low prevalence of H208Y in treatment naïve patients and its moderate increases among heavily treatment

experienced patients support the hypothesis that H208Y is an accessory mutation that arises under drug pressure.

Interestingly, in the context of TAM-1 subtype B virus and TAM-2 subtype A virus H208Y showed no significant effect on relative fitness. Regardless of the initial input ratio, the viruses maintained stable proportions throughout the experiments, with the virus that was initially present in the highest proportion remaining the prevalent virus by the end of the competition. In the 50:50 competitions, there was a slight growth advantage TAM-1 and TAM-2 H208 SDM viruses over H208Y viruses. These results suggest that that whereas in the context of wild type RT H208Y has a detrimental effect on viral fitness, in the presence of TAM-1 or TAM-2 pathway mutations, there is no appreciable effect of the H208Y mutation on viral fitness.

The fitness contribution of the H208Y has not been extensively explored with only one study looking at its effect. This study created site directed mutants and used a single cycle assay to measure fitness in the absence of drug. Results indicated that in isolation, the H208Y SDM showed a similar fitness to HIV_{LAI} H208 wild type virus. In contrast, the H208Y + T215Y double SDM was less fit than both the HIV_{LAI} wild type H208 and single SDM with either H208Y or T215Y (Clark et al., 2006). The findings presented in this thesis showed that H208Y did not influence the fitness of TAM containing viruses. While these results are not consistent with the previous published literature, an important difference that should be appreciated is in the experimental design, as different assays were used to measure fitness. It should also be noted that whereas the study from Clark et al. concluded that the T215Y single SDM had only a slightly lower fitness than the wild type virus, other studies have

shown a greater impact of T215Y on viral fitness (Harrigan et al., 1998;Cong et al., 2007). Although one would not expect significant differences in the fitness effect between different methodologies used, contradictory results have been obtained when comparing fitness of drug resistance mutations to wild type strains. These differences have been attributed to the methodology used, the use of primary HIV-1 isolates or laboratory strains (Quinones-Mateu and Arts, 2002).

Several studies have looked at the fitness of TAMs and have found that the TAM-1 pathway mutations, consisting of M41L, L210W and T215Y, are more fit than the TAM-2 pathway of mutations, consisting of D67N, K70R and K219Q both in the presence and absence of zidovudine (AZT) (Hu et al., 2006;Paintsil et al., 2006;Armstrong et al., 2009a). The TAM-1 mutations also contribute to a greater cross-resistance than the TAM-2 mutations (Miller, 2004). In growth competition assays, one study used SDMs to show that the TAMs K70R, T215Y, M41L and M41L + T215Y were all less fit compared to the HXB2 wild type in the absence of drug. However, once AZT was added to the competitions, the TAM containing viruses were more fit than the HXB2 wild type virus (Harrigan et al., 1998). This is an expected result, as one would anticipate that in the absence of drug pressure, resistance mutants are less fit than the wild type virus; once drug is added, the resistant mutants acquire a selective advantage.

The hypothesis of this study was that H208Y was an accessory mutation that arose in highly mutated viral genomes that have acquired multiple mutations, in particular the TAMs. As a result, the H208Y could increase NRTI resistance or improve the fitness of this heavily mutated unfit virus particularly in the presence of drug. The expected

result would be that since the H208Y mutation conferred a reduced susceptibility to TDF, once TDF was added to the competitions, the viruses with H208Y would be fitter than the viruses without H208Y. The competitions done in the presence of TDF in this study did not show this expected result and instead, there was not much difference between the competitions in the presence or absence of TDF. These findings may reflect the complex dynamics that characterise the relationship between viral fitness and escape from drug pressure *in vivo*. They may also be explained by methodological considerations, including the type of cells used in the assay, which may be rather permissive for HIV replication and therefore prevent the appreciation of fine changes in viral fitness. In addition, differences in drug concentration may influence the findings, as well as virus evolution during passaging.

Single cycle assays have the advantage that they can be completed in a shorter time frame but the viruses are limited to a single cycle of replication. Multiple cycle assays can detect smaller changes in fitness because there are multiple rounds of replication and the differences between two variants can be amplified over many rounds of replication. The use of whole virus rather than recombinant virus also changes the nature of the fitness assay. Whole virus assays involve culturing virus isolated directly from the patient and therefore have the major advantage that all viral components can contribute to the measured outcomes. However, these assays are notoriously difficult to standardise, at least in part due to the common use of peripheral blood mononuclear cells that must be activated to support HIV replication. Recombinant virus assays involve amplifying a gene of interest and cloning the gene into a laboratory derived recombinant vector. The advantage of this assay is that isolation of the infectious HIV-1 is not required and a greater degree of

standardization can be achieved. The vector can be modified to incorporate reporter genes such as luciferase (Dykes and Demeter, 2007;Quinones-Mateu and Arts, 2002). A study that compared the fitness of whole virus with that of recombinant virus containing the corresponding envelope gene demonstrated comparable results (Rangel et al., 2003). It should be noted however that the envelope is a key determinant of viral fitness thus it is conceivable that its effects in isolation will be close to those of the whole virus. In this study, the growth competition assay was used to measure fitness because they have the advantage that small changes in fitness can be detected and the fact that they provide as close as possible reproduction of conditions *in vivo*.

The use of cell lines and primary human cells has also been shown to affect the *in vitro* fitness of drug resistant mutants. For instance, the M184V mutation, which confers resistance to lamivudine, was reported to have a reduced fitness compared to the wild type in primary human cells but the observation was not repeated in a lymphoid cell line. Similar findings have been found with K65R and the TAMs (Back et al., 1996;Perez-Bercoff et al., 2007).

It is also important to note that the use of patient-derived RT containing multiple mutations rather than a site directed mutant containing few selected mutations on HXB2 RT. Thus, while this may make it difficult to appreciate the effect of any individual mutation, it is also more representative of the in vivo scenario where H208Y is known to emerge in the context of highly mutated virus that has been subjected to extensive selective pressure.

One other possible reason for the lack of a fitness effect of H208Y in the presence of TDF may be related to the long half-life of the drug. (Delaney et al., 2006). The phenotypic assay involved incubating infected cells in the presence of drug for 4 days. However, the drug in the growth competition assays was replenished every 6 days. Therefore it is plausible that the IC50 level of drug was not maintained throughout the competition and this may have affected the relative fitness reported. Maintaining the correct drug levels in the growth competition assays is a critical parameter, which could alter the outcome of the assay if not maintained accurately. Having too a high level of drug would mean that replication would be suppressed whereas having a low concentration of drug would mean that virus replication would not be suppressed to the correct levels.

The major resistance mutation associated with TDF is K65R. It is possible that the continuous passage of these viruses in the growth competition assays in the presence of TDF could have selected K65R and this could have affected the fitness result. It has been reported that it would take at least 75 weeks to select K65R when passaging wild type virus in the presence of TDF (Brenner et al., 2006). To rule out the possibility of mutations arising in the RT during passage that may influence the fitness result, the RT gene from passages 1 and 4 were sequenced from each competition (data not shown). No non-synonymous mutations were found in the sequences. However since only the RT was sequenced, this does not rule out the possibility that a mutation else where in the virus may have developed and had an effect on the fitness. However, this possibility seems unlikely given that TDF exerts pressure on the RT gene.

The gold standard for growth competition assays involves passaging viruses over a period of 12-24 days (Cong et al., 2007;Hu et al., 2006;Hu and Kuritzkes, 2010;Huigen et al., 2008;Armstrong et al., 2009b). It is possible that by increasing the number of passages and decreasing or increasing the amount of supernatant used to passage the virus could have influenced the fitness result. However, due to time constraints, these options could not be tested.

The fact that both wild type and drug resistant RT containing H208Y showed a reduced susceptibility to TDF in the absence of appreciable fitness advantages in the presence of TDF was an unexpected result. An important consideration is the slope of the dose response curve achieved in the phenotypic assay. Conventional phenotypic assays use the IC50 value as a measure of resistance and do not report the slope of the dose response curve. It has been shown that viruses with the same IC50 values but differing steepness of the dose response curve may achieve different inhibition at clinically relevant concentrations (Shen et al., 2008). Furthermore, the IC90 may also be relevant. Mutant viruses that have a higher IC50 than the wild type virus may have a lower IC90 than the wild type virus due to the slope of the dose response curve. As a result, if the correct drug levels were not obtained, one virus may have had an advantage over the other in the growth competition assay.

In all cases, the IC90 was higher for the viruses with H208 compared to the viruses with H208Y. Therefore it is possible that if a too high drug concentration were used, viruses with H208Y would be at a growth disadvantage.

Despite these possible explanations to address why a fitness effect was not observed in the growth competition assays in the presence of drug, it is possible that the

H208Y mutation does not contribute to viral fitness and is purely a resistance mutation. There are examples whereby mutations confer resistance but do not have a significant effect on fitness. The L74V mutation confers high level resistance to didanosine (DDI) and intermediate level resistance to abacavir (ABC) (McColl et al., 2008;Trivedi et al., 2008). In a multiple cycle system, p24 antigen measurements were used to measure fitness of L74V compared to wild type. The L74V mutation has been shown to have the same fitness as wild type (Deval et al., 2004a). Another example includes the NNRTI resistance mutation K103N which confers high level resistance to efavirenz (EFV), nevirapine (NVP) and delaviridine (DLV) (Clavel and Hance, 2004b;Dykes and Demeter, 2007). Despite having a significant effect on resistance, K103N does not have a significant effect on fitness. Growth competition assays have shown that K103N had a lower fitness compared to the wild type virus in the absence of drug (Collins et al., 2004). In the presence of 3nM EFV or 30nM NVP the K103N containing virus had a similar fitness to the wild type virus, however with higher concentrations of EFV and NVP, the K103N virus was more fit than the wild type virus (Gatanaga et al., 2006).

In summary, the growth competition assays showed that in the context of a subtype B wild type RT sequence, the H208Y mutation was detrimental to viral fitness, both in the absence and presence of drug. However in the context of a subtype A TAM-2 and subtype B TAM-1 containing RT sequence, H208Y had a similar fitness to the viruses without H208Y. In the context of drug resistant RT, an appreciable effect on fitness was not observed, which may be either due to the fact that the viruses were too highly mutated and “adapted” to be impacted significantly by just one small change, or methodological issues that require further work to be elucidated.

Chapter seven

7 General discussion

7.1 Discussion

The emergence of resistance can be divided into two stages. The first stage involves the emergence of mutations that directly affect the functioning of the drug and cause resistance. These mutations are called primary or major resistance mutations and are generally associated with a decreased fitness, commonly referred to as the "fitness cost" of drug resistance. The second stage involves the emergence of secondary or accessory mutations that may contribute to increased resistance or may compensate for the reduced fitness. The latter are also known as compensatory mutations.

Accessory mutations are initially identified through statistical analyses of large databases that compare the frequency of mutations in treatment naïve and treatment experienced patients. Accessory mutations are found more frequently in treatment experienced patients that treatment naïve patients as they require drug pressure to arise (Cane et al., 2007;Perno et al., 2006;Svicher et al., 2006). The characterisation of accessory mutations in reverse transcriptase (RT) has not been extensively studied. The work presented in this thesis characterises the H208Y mutation as an accessory mutation in RT.

The HIV Resistance Database held at the Royal Free Hospital contains the *pol* gene population sequences of all patients undergoing drug resistance testing locally. The patient population is highly diverse, with approximately 46% of sequences showing a HIV-1 subtype other than B, including all prevalent non B subtypes, common recombinant forms and some complex sequences of unclear subtype assignment. The database was searched for RT sequences with the H208Y mutation. The prevalence

of the H208Y mutation was found to be 5/3783 (0.1%) in treatment naïve patients and 12/1304 (0.9%) in treatment experienced patients. Four patients were chosen for further analysis; a treatment naïve patient infected with a subtype B virus and three treatment experienced patients infected with subtype A, subtype B and subtype C virus respectively. Cloning the gene RT from these patients showed that the H208Y was most often observed with the thymidine analogue mutations (TAMs), in particular, the TAM-1 pathway of mutations, which consists of M41L, L210W and T215Y. H208Y was always associated with TAM mutations at positions D67, T215 and K219. TAMs accumulate in step-wise fashion in patients receiving therapy with the thymidine analogues zidovudine (AZT) and stavudine (D4T) and take several months of incompletely suppressive therapy to emerge. In addition to conferring resistance to AZT and D4T, TAMs reduce susceptibility to all other available nucleoside and nucleotide RT inhibitors (NRTI), including abacavir (ABC), didanosine (DDI) and tenofovir (TDF), and to a lesser extent lamivudine (3TC) and emtricitabine (FTC). The effects on resistance and cross-resistance grow progressively with increasing numbers of TAMs. Taken together these initially findings confirmed the high degree of conservation of the RT residue 208 in wild type HIV-1 across a wide range of subtypes, and the emergence of H208Y under selective drug pressure in the context of a highly mutated and NRTI resistant RT (Cane et al., 2007;Nebbia et al., 2007;Svicher et al., 2006). The clones from the treatment experienced patients showed that H208Y was consistently associated with accessory mutations at positions V35, K122 and T200 and other mutations with unknown effects on drug susceptibility and fitness at positions V245, A272, I293, T377, K390, E404, V435, H483 and L491.

In order to examine the contribution of H208Y to drug susceptibility and viral fitness, the RT gene from plasma virus recovered from the four patients with H208Y was used to create recombinant viruses to be tested in drug susceptibility and growth competition assays. For this purpose, strains with H208Y were subjected to site directed mutagenesis to produce sequences with H208. While it may have been possible to simply compare site directed mutants (SDMs) with and without H208Y, the advantage of this approach was that it allowed the impact of the mutation to be studied in the context of the sequence within which it emerged. The drawback of this approach however was that comparisons among viruses derived from different patients were difficult to interpret given the significant differences observed in terms of major and accessory mutations co-existing with H208Y. One attempt at circumventing this problem was the reversion of strains with M184V to strains with M184, to remove the strong phenotypic effects of M184V on drug susceptibility (partial resensitisation to AZT, D4T and TDF) and viral fitness (high fitness cost).

The drug susceptibility assay showed that the H208Y mutation conferred a reduced susceptibility to TDF in the context of both wild type subtype B RT and subtype B RT containing TAM-1 mutations. This effect was statistically significant ($p<0.001$) as determined by two tailed ANOVA. The IC50 values from replicate experiments were all within a 2 fold range of one another showing that this effect was reproducible.

The resistance effects of H208Y were not observed with other NRTIs and the mutation was shown to confer an increased susceptibility to some NRTIs and

NNRTIs, although these effects were not statistically significant when comparing viruses with and without H208Y. Previous data on the phenotypic impact of H208Y have been contradictory and have indicated both increased and decreased susceptibility to AZT (Clark et al., 2006;Sturmer et al., 2003). These studies created site directed mutants on a subtype B RT backbone and used a single cycle and multiple cycle assay respectively. In this work, a phenotypic effect could not be demonstrated for AZT, and this is due to the fact that the treatment experienced patients harboured highly AZT-resistant viruses with multiple TAMs and any phenotypic effect of H208Y was clearly too small to be appreciated in this context. However, the virus from the treatment naïve patient showed an increased susceptibility to AZT and D4T but this effect was not statistically significant.

The resistance effects for TDF seen with H208Y were not recapitulated with the subtype A or subtype C viruses. Possible explanations include subtype related or drug induced sequence differences and structural differences in the enzyme. The TAMs present in the treatment experienced patients are similar although not identical. The subtype B virus had the TAMs M41L, D67N, L210W, T215Y and K219N alongside V75M, V118I and the NNRTI resistance mutations A98G and G190Q. The subtype A virus had the TAMs D67N, K70R, T215F and K219E alongside T69N and the NNRTI resistance mutations A98G and G190Q, while the subtype C virus had the TAMs M41L, D67N, L210W, T215Y and K219R alongside E44D, L74V, and the NNRTI resistance mutations A98G, K101H, V108I, Y181C and G190A/Q.

Alignment of the RT sequences from the four patients identified 12 positions that were the same in the subtype B sequences but differed in the subtype A and subtype C sequences. From these 12 positions, 2 positions were associated with NRTI resistance and these were at positions Q207 and G335. These mutations contribute to AZT and 3TC resistance when present with TAMs and TAMs with M184V respectively (Lu et al., 2005;Zelina et al., 2008). No phenotypic effect for AZT was measured for the subtype A and subtype C viruses. But for 3TC, the subtype A viruses conferred a reduced susceptibility to 3TC and the subtype C patient conferred an increased susceptibility to 3TC but these results were not statistically significant. Therefore, the impact of these mutations when present with H208Y is unclear.

It should also be noted that a subtype B molecular clone backbone was used for all viruses and perhaps using subtype specific molecular clones may have yielded different results.

In order to assess the contribution of H208Y to viral fitness, an allele-specific PCR (AS-PCR) was developed. The assay was used to differentiate between competing strains with and without H208Y and quantify proportions of mutant and wild type viruses in growth competition assays. The growth competitions with virus from the subtype B treatment naïve patient showed that the presence H208Y was detrimental and made the virus less fit relative to a SDM without the H208Y. This observation may contribute to explain the high degree of conservation of H208Y among viruses from treatment naïve patients. The mutation appears to be equally rare among different HIV-1 subtypes, suggesting the fitness effect observed with subtype B is likely to be reproduced across different clades. However, no samples were available

from treatment naïve patients with non B subtypes and H208Y to test this hypothesis. Further work will require testing non-B RT with H208Y introduced by site directed mutagenesis.

In the presence of TAM-1 or TAM-2 mutations, viruses with H208Y maintained stable proportions throughout the competitions, suggesting there was no measurable impact of H208Y on viral fitness. Viral fitness data on H208Y are limited, with only one study that has explored its impact. This study created site directed mutants with T215Y and H208Y and showed that it was less fit than the wild type and single mutants with T215Y or H208Y alone (Clark et al., 2006). In this work, an effect was not appreciated. Possible reasons include the fact that patient derived RT containing multiple TAMs was used rather than using a wild type RT with one or two mutations, which would be easier to look however, how useful this would be since they are unlikely to occur just in pairs. Methodological issues such as the ensuring the correct concentration of drug was maintained and the half life of the drug may also have influenced the result.

In summary, the findings presented in this thesis show the H208Y is highly conserved among treatment naïve patients infected with diverse HIV-1 subtypes. The conservation may be explained by its negative impact on viral fitness seen with wild type subtype B virus. The findings also showed that H208Y was linked on the same viral genome with the TAM-1 pathway of mutations, and occurred consistently with mutations at positions V35, K122, T200, V245, A272, I293, T377, K390, E404, V435, H483 and L491. In both wild type and TAM-1 containing subtype B virus,

H208Y reproducibly reduced susceptibility to TDF. In the context of highly mutated, drug-resistant RT, H208Y showed no appreciable effect on viral fitness.

The finding that in the presence of the TAMs, H208Y did not increase fitness does not support the hypothesis that the H208Y mutation is a compensatory mutation. This is because compensatory mutations are defined as occurring alongside major resistance mutations and augmenting viral fitness. This leads to the question of whether H208Y is in fact an accessory mutation that acts by augmenting resistance.

Future work will focus on determining the mechanism of TDF resistance conferred by H208Y. For instance, RT fidelity and processivity as well as NRTI excision could be examined. Methodological modifications to the growth in the presence of TDF could also be done. This includes increasing the concentration of TDF used or using AZT, increasing the number of passages and the volume of supernatant used to continue the passage. Furthermore, using primary cell lines in the growth competition assays could also be done.

Chapter 8

8 References

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