DISTINCT MECHANISMS OF HIV-1 HYPERSUSCEPTIBILITY TO NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS: A DISCUSSION OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR INDUCED MUTATIONS V118I, H208Y, AND T215Y

by

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DISTINCT MECHANISMS OF HIV-1 HYPERSUSCEPTIBILITY TO NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS: A DISCUSSION OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR INDUCED MUTATIONS V118I, H208Y, AND T215Y

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Hypersusceptibility (HS) to non-nucleoside reverse transcriptase inhibitors (NNRTI) improves HIV infected patients' virological responses to NNRTI-containing regimens. NNRTI HS is associated with nucleoside RT inhibitor (NRTI) mutations, especially those conferring resistance to 3′azidothymidine (AZT). Recent logistic regression analyses of a large genotype-phenotype dataset showed the NRTI mutations most strongly associated with NNRTI HS are T215Y, H208Y and V118I. We hypothesized that NRTI mutations V118I, H208Y, and T215Y in combination cause NNRTI HS and that this phenotype is due to multiple mechanisms including a decrease in enzyme activity and/or deficient viral replication due to decreased virion packaged RT. Therefore we sought to: (1) determine the phenotypic effects of these mutations alone and in combination on the susceptibility of infectious molecular clones and recombinant reverse transcriptase proteins to efavirenz, delavirdine, and nevirapine; and (2) elucidate differences in viral replication, protein production and packaging for virus containing mutations V118I, H208Y, and/or T215Y. We established different patterns of NNRTI HS and replication capacity depending on the combination of mutations present. HIV-1 viruses containing H208Y + T215Y were HS to all NNRTI; whereas the V118I + T215Y virus was only HS to delavirdine and nevirapine. H208Y + T215Y viruses exhibited reduced replication capacity compared to wildtype HIV-1. In comparison, the V118I + T215Y virus replicated as efficiently as wildtype

virus. Upon further investigation we found the amount of HIV-1 RT incorporated into the H208Y + T215Y viruses was significantly reduced compared with wildtype virus due to decreased viral packaging of GagPol precursors. Interestingly, the RT content in the V118I + T215Y virus was similar to wildtype virus. Furthermore, purified recombinant RT containing the H208Y+ T215Y mutations were not NNRTI HS. By contrast, the V118I/T215Y mutant RT showed five-fold increased susceptibility to NNRTI. Our work highlights the complexity of the HS phenotype and provides an in-depth understanding of how NRTI mutations V118I, H208Y, and T215Y contribute to increased NNRTI susceptibility. This work has significant public health impact because it can help facilitate the design of inhibitors of HIV that exploit the mechanisms of hypersusceptibility during rational drug design and can increase the overall knowledge of interactions between drug classes for improved patient therapy.

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1.0 INTRODUCTION

1.1 HIV AND AIDS PANDEMIC

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). Progression to AIDS is most notably characterized by a depletion of the host immune system, especially CD4 T cells, leading to severe clinical manifestations. According to the Centers for Disease Control (CDC) AIDS is diagnosed if an HIV infected person has 1) a CD4 T cell count less than 200 cells/mm^3 of blood and/or 2) an AIDS defining condition such as candidiasis, *Pneumocystis carinii* pneumonia, or Kaposi's sarcoma.

There are two types of HIV described as HIV-1 and HIV-2. HIV-2 is primarily found in West Africa and although infection also leads to the development of AIDS, HIV-2 is less pathogenic and the progression to AIDS caused by HIV-2 infection is usually slower than that caused by HIV-1 infection (Bock and Markovitz 2001). This work focuses solely on HIV-1. HIV is primarily transmitted through sexual contact or percutaneous infection, such as through contaminated needles for injected drug use. In addition, HIV can be vertically transmitted from mother to child during child birth or breast feeding (Hirschel and Francioli 1998; Palella, Delaney et al. 1998) .

Ninety-six percent of the 6800 new HIV-1 infections per day in 2007 were in people living in low and middle income countries (Figure 1). Moreover, 68% of the total number of people living with HIV worldwide reside in Sub-Saharan Africa with more than 60% of the adults (age 15 and older) infected with HIV being women (UNAIDS 2007 AIDS epidemic update). In South and Southeast Asia, the number of people living with HIV increased from 3.5 million in 2001 to 4 million in 2007. Although the rising number of people in Asia becoming HIV infected is alarming, evidence from some African nations suggests behavior modifications and prevention practices are effective methods for reducing viral transmission and incidence of new infections. In 2006, Kenya reported that sex with non-regular partners (among women) decreased and condom use with non-regular partners (among men and women) increased, coinciding with a 9% decrease in the prevalence of HIV from 2001-2005 (UNAIDS 2007 AIDS epidemic update). Decreases in the prevalence of HIV infection have also been reported in Botswana, Sierra Leone, and Cameroon during this same period.

Although low income countries generally bear the largest HIV burden, certain populations in high-income countries are disproportionately infected. The Centers for Disease Control (CDC) reported that in 2004 in the United States, HIV infection was the leading cause of death among black women aged 25-34 years. Additionally, black Americans comprised 13% of the US population in 2006 but accounted for 49% of the AIDS cases (www.CDC.gov). Though HIV is far more prevalent in some regions/populations of the world, the HIV/AIDS pandemic is a health, social, and economic burden on the entire global community.

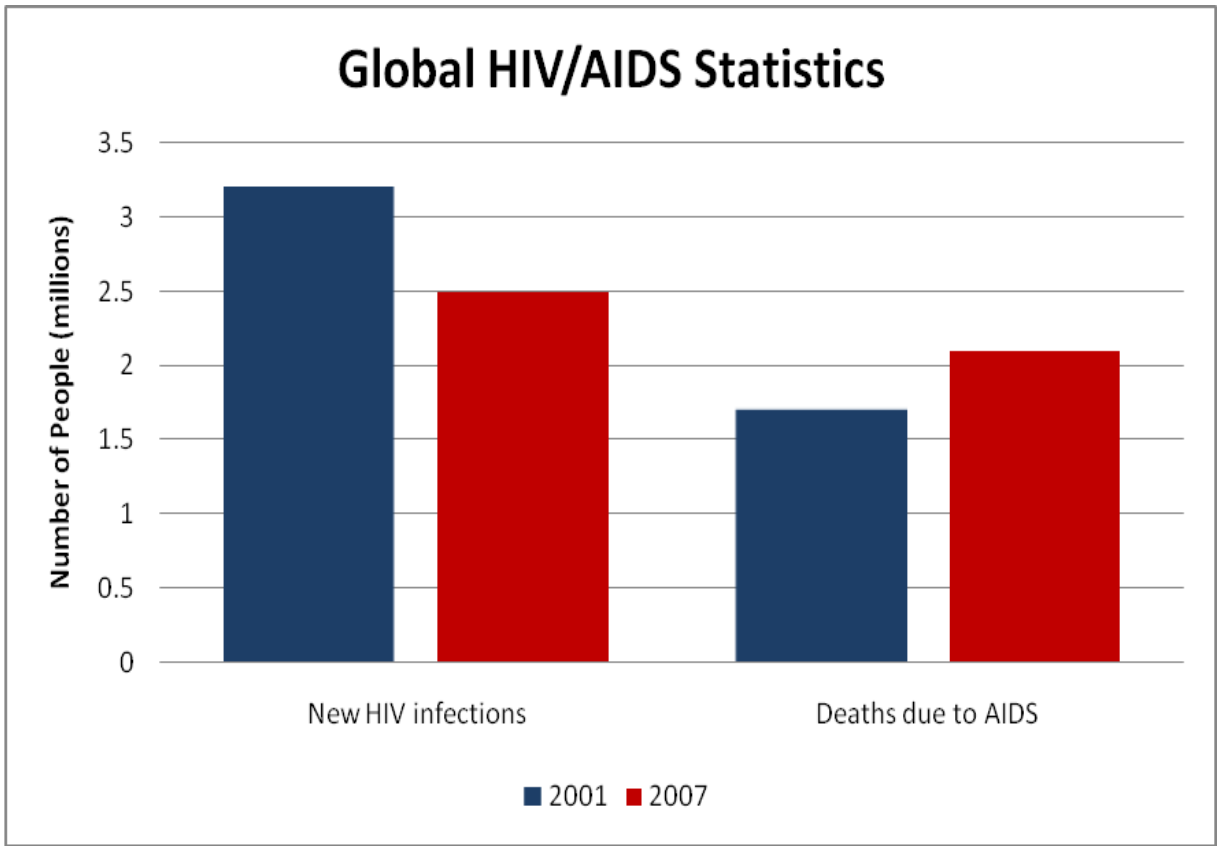


Figure 1. Global HIV/AIDS Statistics

According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) there were 2.5 million new HIV infections globally in 2007, down from 3.2 million in 2001. The number of deaths attributed to AIDS went from 1.7 million in 2001 to 2.1 million in 2007. Shauna, the reduction in infections is probably not from treatment. UNAIDS revised their estimates based on further surveillance data.

1.2 HIV OVERVIEW

1.2.1 Key Elements of HIV

HIV belongs to the family retroviridae, genus lentivirus, characterized by retroviruses with a long incubation period and slow progression to disease. HIV-1 can be divided into three groups M, N, and O (Gurtler, Hauser et al. 1994; Simon, Mauclere et al. 1998; Gao, Bailes et al. 1999). Group M can be further divided into nine subtypes or clades (A, B, C, D, F, G, H, J, K) based on phylogenetic comparison of structural genes (Gao, Bailes et al. 1999).

HIV-1 virions contain two copies of a single-stranded positive RNA genome encoding nine viral genes (*gag, pol, env, vif, tat, rev, nef, vpr,* and *vpu*, Figure 2) along with the enzymes reverse transcriptase (RT), protease (PR), and integrase (IN), and proteins nucleocapsid and p6. The viral RNA is surrounded by capsid proteins (Figure 3). Surrounding the capsid are matrix proteins that are further enclosed in a highly glycosylated envelope composed of a lipid bilayer from the host cell and viral proteins gp120 and gp41.

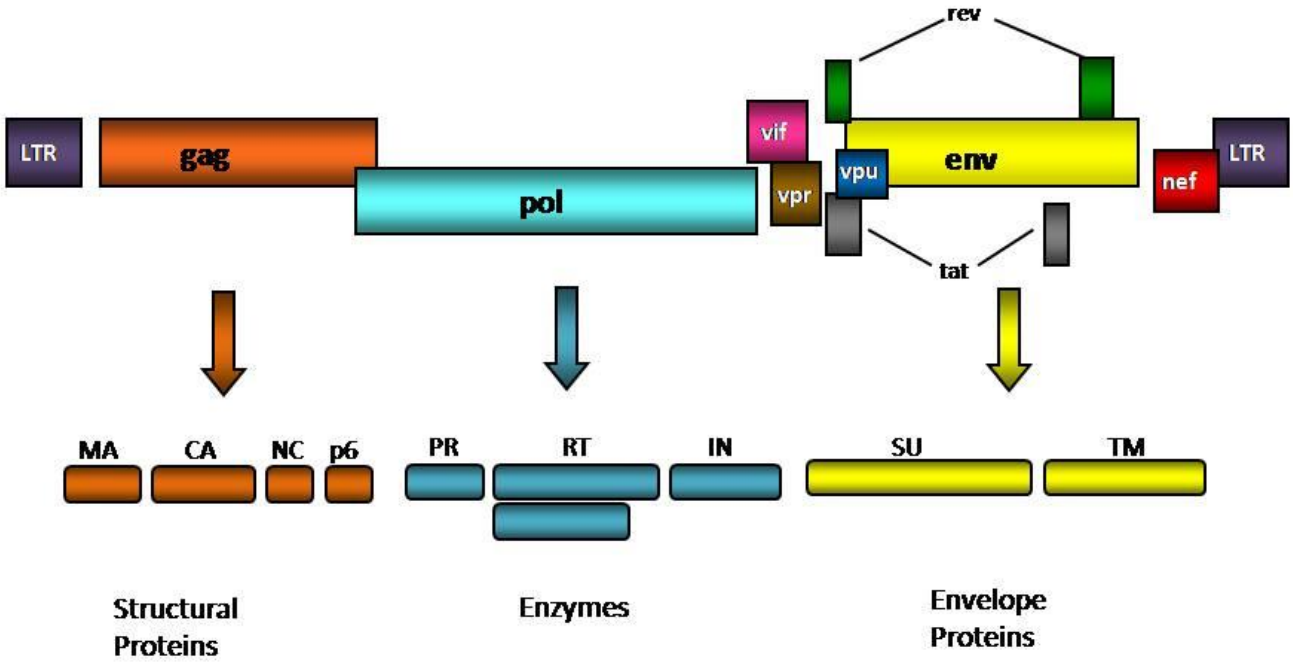


Figure 2. HIV-1 Genome Organization.

HIV-1 encodes genes for 9 viral proteins. The *gag* gene, shown in orange, encodes the structural proteins capsid (CA), matrix (MA), nucleocapsid (NC), and p6. *pol* (shown in blue) encodes the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). The viral *env* gene encodes the surface (SU or gp120) and transmembrane (TM or gp41) coat proteins. The remaining accessory genes encode proteins that regulate various aspects of the HIV life cycle.

1.2.2 Life cycle[1]

1.2.2.1 Early Events

The life cycle of HIV-1 begins with recognition and binding of the viral surface protein gp120 to the host cell receptor CD4 found mainly on T-cells and macrophages (Figure 3). During the complex interaction of gp120 with CD4, a conformational change occurs in the viral

[1] This section provides an overview of the HIV-1 life cycle modified from the Retroviruses textbook (Coffin *et al* 1997) except where noted.

transmembrane protein gp41, including exposure of the fusion peptide at the amino terminus of gp41, allowing fusion of viral and cellular membranes (Kilby, Hopkins et al. 1998). Recently, the three dimensional structures of the native trimeric Env structure (formed by gp41 and gp120) in both CD4 liganded and unliganded states were derived through electron tomography and molecular modeling (Liu, Bartesaghi et al. 2008). It is now clear that CD4 binding results in reorganization of the Env trimer causing displacement of each of the gp120 monomers as well as rearrangement of gp41 leading to intimate contact between the virus and cellular target (Liu, Bartesaghi et al. 2008). Although CD4 is required for viral binding to T-cells and macrophages it is not sufficient for infection. Either chemokine receptor CCR5 or CXCR4 can act as a co-receptor for viral entry (Deng, Liu et al. 1996; Feng, Broder et al. 1996).

Reverse Transcription

Although it is generally accepted that once the viral core has entered the host cell the viral genomic RNA is released from the surrounding capsid proteins (Auewarakul, Wacharapornin et al. 2005; Warrilow, Meredith et al. 2008), there is evidence suggesting uncoating occurs after reverse transcription (Arhel, Souquere-Besse et al. 2007). In any event, it is at this stage that the single-stranded viral RNA is converted to double-stranded DNA (Figure 3) via the viral enzyme reverse transcriptase (RT). Using genomic RNA as a template and transfer RNA (tRNA) as a primer, a short minus strand DNA is the first product of reverse transcription. The RNA portion of the RNA/DNA heteroduplex is then degraded by the RNase H activity of reverse transcriptase. A strand transfer occurs allowing the 3' end of the genomic RNA to be used as a template for further synthesis of the minus strand DNA. A small portion of the genomic RNA containing a polypurine tract resists degradation by RNase H and is used

as a primer for plus strand DNA synthesis. Plus strand synthesis continues until the tRNA primer is removed allowing complementation of the plus strand DNA to sequences near the 5' end of the minus strand DNA. After a second strand transfer occurs, both plus and minus strand DNA synthesis resume until a full length double-stranded DNA is formed.

Integration

Upon completion of a full length double-stranded DNA intermediate by RT another viral enzyme, integrase (IN), is responsible for the integration of the double stranded viral DNA (vDNA) into host chromosomal DNA. IN is a 32 kD protein made up of 288 amino acids (Katz and Skalka 1994). The 3' termini of the double-stranded DNA are cleaved by integrase providing the site for attachment of the vDNA to host DNA. A pre-integration complex consisting of both cellular and viral proteins along with the vDNA enters the nucleus of the host cell and integrates into host DNA (Shimotohno and Temin 1980; Fujiwara and Mizuuchi 1988; Neamati, Marchand et al. 2000; Freed 2001).

1.2.2.2 Late Events

Transcription and Translation

Integrated proviral DNA requires host RNA polymerase II for transcription as well as the viral transcription transactivator Tat. Post-transcriptional modification including 5' capping and 3' polyadenylation occurs for all synthesized viral RNAs. Large transcripts are either packaged in virions as genomic RNA or used as mRNA for translation of polyprotein precursors for Gag and GagPol, while smaller transcripts encode other structural and accessory proteins (Figure 3).

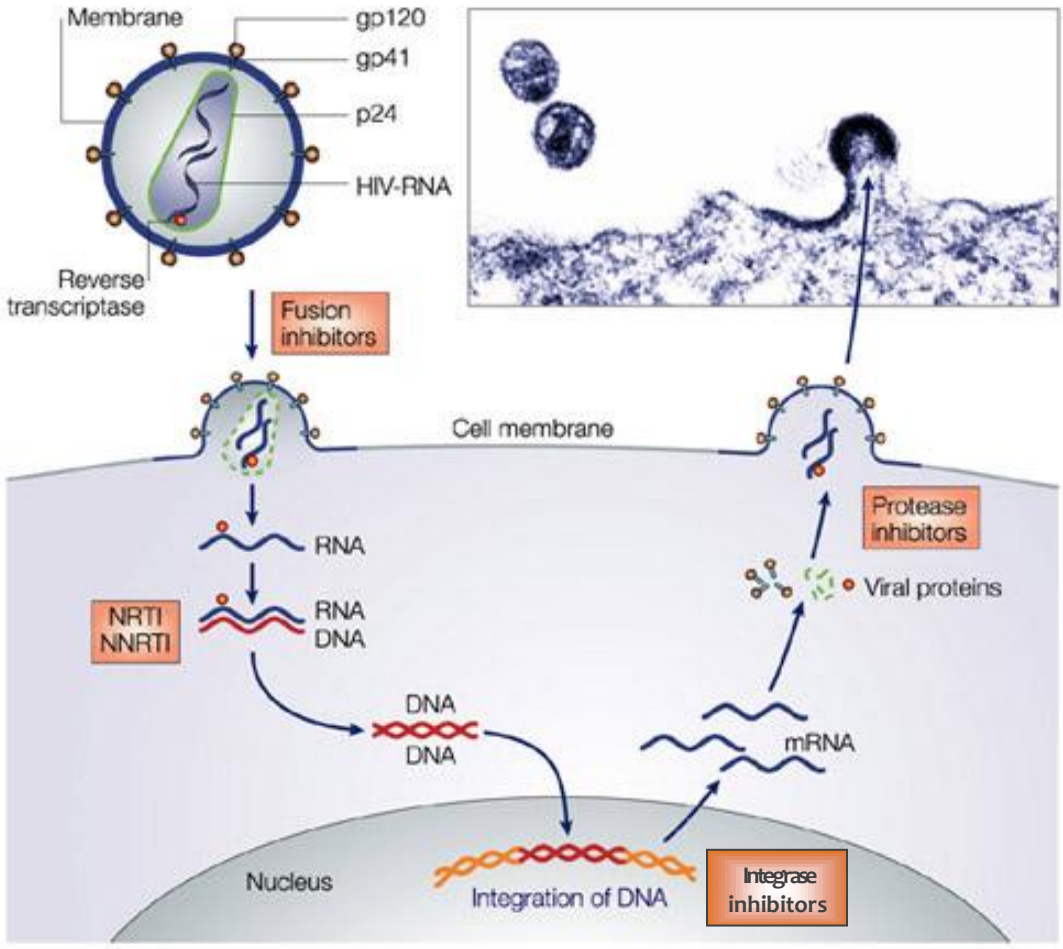


Figure 3. HIV-1 Life Cycle

This figure depicts key aspects in the HIV life cycle and highlights them as targets of current HIV therapy. Fusion/binding inhibitors inhibit the entry of virions into a new target cell. Reverse transcription is targeted using nucleoside analogues or non-nucleoside reverse-transcriptase inhibitors (NRTI and NNRTI, respectively). Integrase inhibitors prevent strand transfer of the viral DNA into the host chromosome. Protease inhibitors block proteolytic processing of the viral Gag and GagPol polyproteins, resulting in the production of non-infectious particles. Electron micrograph showing the budding of virions is courtesy of P. Bieniasz, Aaron Diamond AIDS Research Center, USA. (Simon and Ho 2003)

Assembly and Maturation

Following viral protein synthesis, genomic RNAs and polyprotein precursors are assembled at the plasma membrane. Targeting to the membrane occurs due to myristylation of the N terminal of the Gag polyprotein (Zhou, Parent et al. 1994). Budding of the assembled viral components takes place at the plasma membrane due to signals in the p6 portion of Gag referred to as late or L domains (Garnier, Bowzard et al. 1998)

Cleavage of the Gag and GagPol polyproteins by the viral enzyme PR occurs quickly after the release of immature particles and marks virion maturation. Active PR is required for viral infectivity (Kohl, Emini et al. 1988). The mature enzyme is a 10 kD homodimer and is formed from two GagPol polyprotein precursors (hereafter referred to as GagPol). HIV PR is an aspartyl protease, characterized by its active site triad including Asp-Thr/Ser-Gly corresponding to amino acids 25-27. The two catalytic Asp 25 residues are adjacent in the protein cleft and both subunits of the enzyme contain parallel β sheets called flaps (Tozser, Yin et al. 1997). In their native conformation the flaps overlap and form an intersubunit hydrogen bond that would prevent substrate binding (Tozser, Yin et al. 1997). However, these flaps are flexible and act during catalysis by "pinning" the substrate in the active site (Tozser, Yin et al. 1997; Louis, Weber et al. 2000).

1.3 A CLOSER LOOK AT REVERSE TRANSCRIPTASE

Reverse transcriptase (RT) is a product of the *pol* gene whose 5' end overlaps with the *gag* gene (Figure 2). Pol is translated as a GagPol fusion protein due to a -1 ribosomal frame shift and requires proteolytic processing to form mature heterodimeric RT (Jacks, Power et al. 1988; Pettit, Simsic et al. 1991; Pettit, Clemente et al. 2005). This ribosomal frame shift occurs infrequently allowing a 20:1 ratio of Gag:GagPol (Jacks, Power et al. 1988). The ratio of GagPol produced relative to Gag is important for viral assembly. In murine leukemia virus (MLV), a mutation that resulted in the production of 100% GagPol (no Gag) prevented proteolytic processing of GagPol and the assembly of virions (Felsenstein and Goff 1988). GagPol is incorporated into assembling particles through interactions within the capsid (CA) domains of Gag and GagPol (Srinivasakumar, Hammarskjold et al. 1995; Huang and Martin 1997; Hill, Tachedjian et al. 2005), although it has been shown that Pol can be packaged outside of the GagPol context (Cen, Niu et al. 2004). Defects at any of these steps could compromise viral particle assembly, incorporation, and/or processing leading to decreases in viral replication and the amount of virion associated RT.

1.3.1 Enzyme Structure

RT is responsible for the conversion of the single-stranded RNA genome into a double-stranded DNA intermediate and is essential for HIV replication . RT is a heterodimeric protein consisting of 66 kD (p66) and 51 kD (p51) subunits. Two models have been proposed to

explain the formation of the mature heterodimer (Sluis-Cremer, Arion et al. 2004). The first is the concerted model in which each of the subunits arises from proteolytic cleavage of two separate GagPol polyproteins that then assemble to form the heterodimer. The second, more favored model is referred to as sequential, whereby two 66 kD subunits derived from proteolytic cleavage of two GagPol polyproteins, form a homodimer followed by additional processing of one of the 66 kD subunits into a smaller 51 subunit (Sluis-Cremer, Arion et al. 2004). The polymerase domain of the p66 subunit has been likened to a right hand containing fingers, palm, and thumb subdomains (Kohlstaedt, Wang et al. 1992). In addition, the p66 subunit contains a connection and RNase H domain. There is a large cleft in the polymerase domain similar to the Klenow fragment of *E.coli* DNA polymerase I (Kohlstaedt, Wang et al. 1992). Unlike p66, p51 has no cleft and the aspartic acid residues involved in catalysis are buried (Kohlstaedt, Wang et al. 1992) leaving the smaller subunit to serve a predominantly structural function.

1.3.2 Enzymatic Function

1.3.2.1 Polymerase

There are two polymerase functions of RT: (1) RNA Dependent DNA polymerase activity (RDDP) and (2) DNA Dependent DNA polymerase activity (DDDP). RDDP is the polymerization of the initial (-) DNA strand using RNA as the template while DDDP uses a DNA template for synthesis. DNA synthesis follows a series of steps that have been described mechanistically by several groups (Cheng, Dutschman et al. 1987; Huber, McCoy et al. 1989; Kati, Johnson et al. 1992; Hsieh, Zinnen et al. 1993). Free RT binds the template/ primer (T/P) forming a binary complex followed by binding of the incoming dNTP; to form the ternary

complex RT-T/P-dNTP. Formation of the ternary complex allows nucleophilic attack, facilitated by Mg^{2+}, of the 3' hydroxyl group of the primer on the α-phosphate of the newly bound dNTP (Parniak and Sluis-Cremer 2000). Pyrophosphate is released with the formation of the phosphodiester bond. At this point reverse transcription can continue in a processive manner in which the binary complex+1dNTP immediately continues incorporating dNTPs or in a distributive manner in which RT and the T/P+1dNTP dissociate (Parniak and Sluis-Cremer 2000). In distributive polymerization after dissociation of the binary complex RT either binds a new T/P or re-binds T/P+1. The processive form of polymerization is a more efficient enzyme activity. In addition, RT lacks formal proofreading ability and is consequently error prone leading to a high rate of genomic mutation (Roberts, Bebenek et al. 1988; Bebenek, Abbotts et al. 1993).

1.3.2.2 RNase H

In addition to its polymerase functions RT also exhibits endonuclease activity conferred by the RNase H domain in the C-terminus of the p66 subunit (Hansen, Schulze et al. 1988). RNase H degrades the RNA strand of RNA: DNA duplexes removing the polypurine tract and tRNA primers to continue synthesis of double-stranded DNA. The RNase H primer grip, which is adjacent to the RNase H active site, plays a role in substrate binding at both the polymerase and RNase H active sites. Therefore this region of RNase H can influence both polymerization and RNA degradation (Schultz and Champoux 2008).

1.4 THERAPEUTICS DIRECTED AGAINST RT

Due to the essential role of reverse transcriptase in the HIV-1 life cycle, it has long been a major target of therapeutic intervention. To date there are twenty-five FDA approved drugs for the treatment of HIV infection (not including combination drugs) (www.fda.gov/oashi/aids/virals.html). Fifty percent of those drugs target the reverse transcriptase enzyme highlighting its importance in the viral life cycle and its relative ease as a target. Currently, there are two major types of approved inhibitors targeting RT: nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). NRTIs block viral DNA synthesis by acting as DNA chain terminators (Mitsuya, Weinhold et al. 1985; Yarchoan, Mitsuya et al. 1989) whereas NNRTI bind to a hydrophobic pocket near the RT active site distorting the conformation of the enzyme required for efficient catalysis (Spence, Kati et al. 1995).

1.4.1 Nucleoside Reverse Transcriptase Inhibitors (NRTI)

Until 3' azido-3'-deoxythymidine (commonly referred to as zidovudine or AZT) was approved for treatment of HIV/AIDS on March 19, 1987 being infected with HIV-1 had a bleak prognosis with no recourse. It wasn't until October 9, 1991 that the next nucleoside analog, didanosine (ddI), would be approved for therapeutic use in HIV infected patients. To date there are eight FDA approved NRTIs (not including varying formulations) for clinical use. Initially inhibitors were used sequentially, however early on it was discovered that NRTIs used in combination were more effective than monotherapy (Meng, Fischl et al. 1990; 1996; Hammer,

Katzenstein et al. 1996). At the third Conference on Retroviruses and Opportunistic Infections (CROI) data was reported on the benefits of using the protease inhibitors indinavir, ritonavir, and saquinavir in combination with NRTI as potent anti-HIV therapy (Grossman 2006). At the World AIDS Conference later that year there were reports of a "new AIDS cocktail" that would include RT inhibitors as well as protease inhibitors and the expression highly active antiretroviral therapy (HAART) was coined (www.hivmedicine2007.org).

1.4.1.1 Mechanism of Action

NRTIs are competitive substrate inhibitors that structurally mimic naturally occurring nucleosides but lack the 3' hydroxyl necessary for phosphodiester bond formation with the incoming deoxynucleotide triphosphate resulting in termination of DNA chain elongation (Figure 4). Nucleosides must be phosphorylated once inside the cell to their active triphosphate form by cellular kinases. Once triphosphorylated, NRTIs can also inhibit cellular DNA polymerases resulting in cytotoxicity.

1.4.1.2 Resistance

NRTI therapy is initially successful in reducing plasma viral load but long-term use results in the development of drug resistance by viral RT rendering the inhibitors less effective. Resistance to NRTI is caused by two major known mechanisms. The first allows reverse transcriptase to discriminate between the natural substrate and the analog with a single point mutation (Figure 5A). In the second mechanism, referred to as excision, a specific group of

mutations enable the enzyme to remove the chain terminating analog and continue DNA synthesis (Figure 5B).

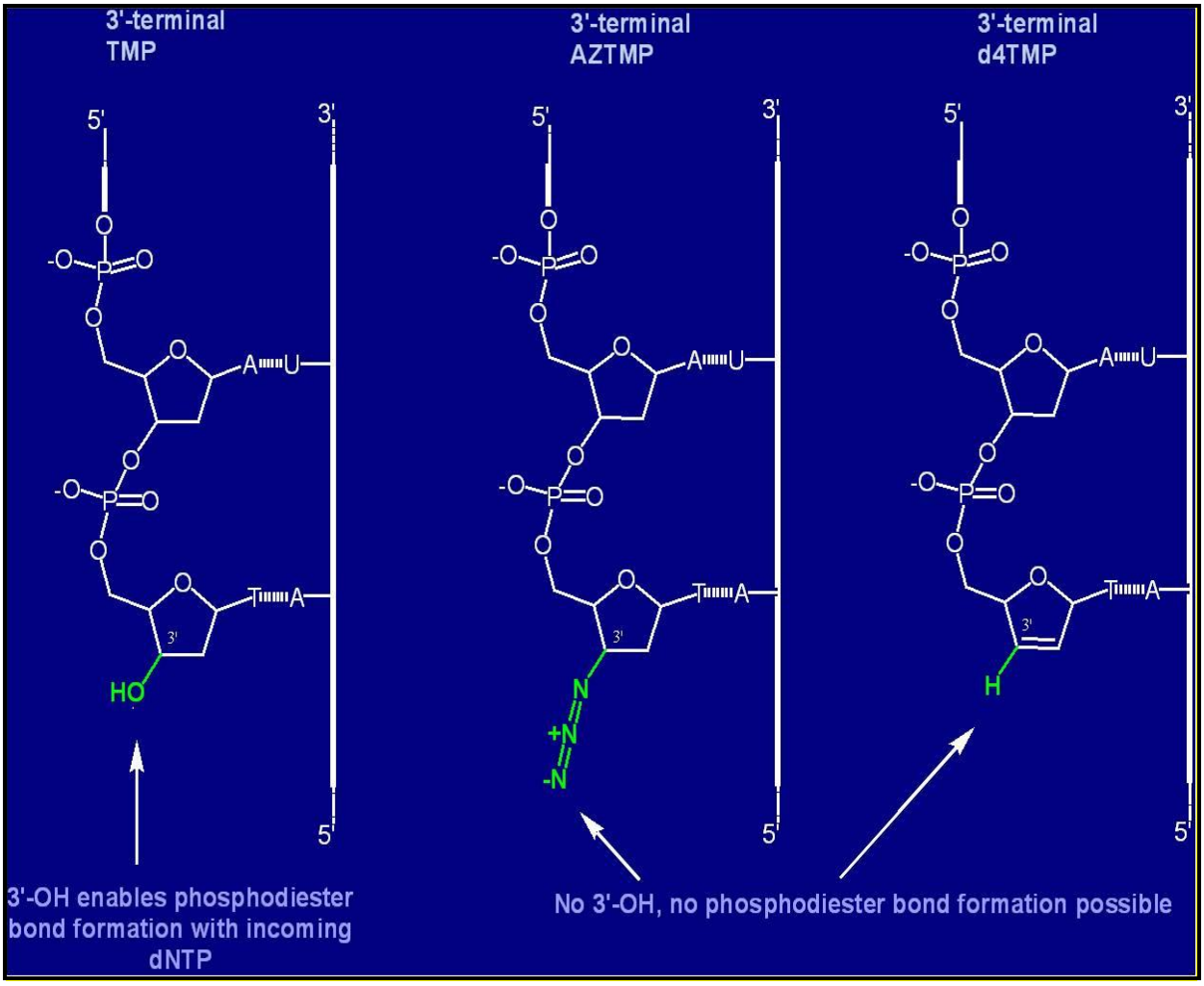


Figure 4. NRTI Mechanism of Action.

The left panel illustrates the incorporation of a normal thymine with a 3' hydroxyl (shown in green) that allows bond formation with the incoming dNTP for DNA chain elongation. The right two panels (AZT in the middle, d4T on the right) illustrate NRTI that lack this 3'hydroxyl thereby preventing further dNTP incorporation.

An example of a discrimination inducing mutation is the methionine to valine change at position 184 (M184V). M184V confers substantial resistance to 3TC as shown in a study by Schinazi et al. By week seven of a selection experiment in which HIV-1 was grown in increasing concentrations of 3TC, viruses containing the M184V mutation were more than 1000 fold resistant to 3TC (Schinazi, Lloyd et al. 1993). The substantial resistance caused by M184V was later attributed to dramatic decreases for incorporation efficiency of 3TC-triphosphate (3TC-TP) versus the natural substrate dCTP caused by reduction in binding affinity of 3TC-TP to the enzyme/template-primer complex (Feng and Anderson 1999).

The mechanism of resistance to AZT posed a conundrum to investigators for many years. In 1989 multiple mutations including D67N, K70R, T215F/Y, and Q219K conferring high level to resistance to AZT were identified (Larder and Kemp 1989). Later mutations M41L and L210W were added to the list of mutations conferring high level resistance to AZT now referred to as thymidine analog mutations or "TAMS" (Kellam, Boucher et al. 1992; Harrigan, Kinghorn et al. 1996; Hooker, Tachedjian et al. 1996). However it soon became clear that the mechanism of resistance to AZT was not due to the inability of the TAM containing enzyme to incorporate AZT-TP (Lacey, Reardon et al. 1992; Kerr and Anderson 1997) but rather an ATP- dependent reaction catalyzed by HIV-1 RT could remove the chain terminating inhibitor (Figure 5B), allowing primer extension to occur (Meyer, Matsuura et al. 1998; Meyer, Matsuura et al. 1999).

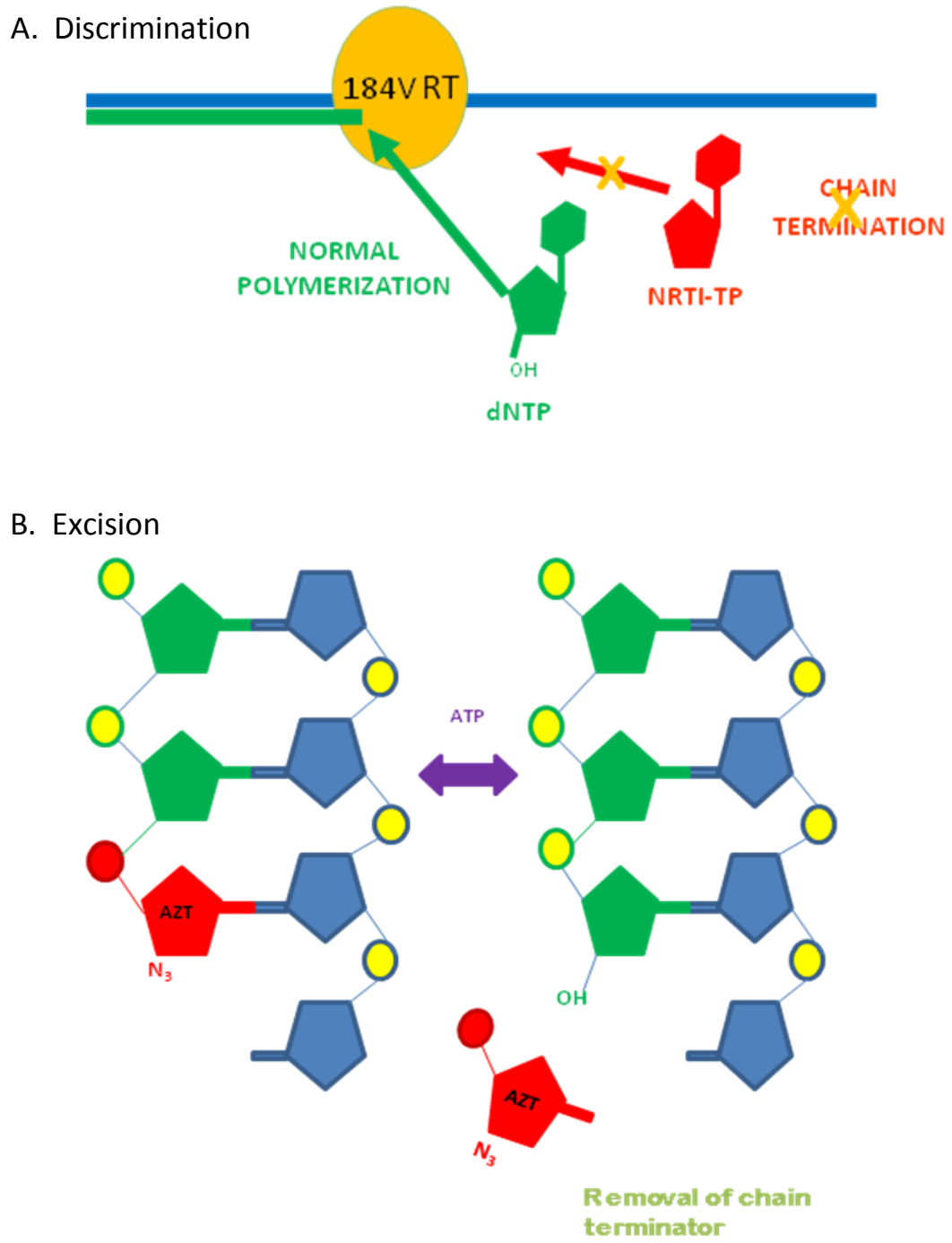


Figure 5. Mechanisms of NRTI resistance.

In discrimination (A) RT preferentially incorporates the natural dNTP substrate over the NRTI. In the excision mechanism (B) the chain terminator is catalytically removed by RT in the presence of a phosphate donor such as ATP.

1.4.2 Non-nucleoside Reverse transcriptase inhibitors (NNRTI)

There are currently four non-nucleoside RT inhibitors (NNRTI) approved for therapeutic use by the U.S. FDA including etravirine, efavirenz, delavirdine, and nevirapine. NNRTI are non-competitive RT inhibitors that are structurally distinct (Figure 6), small hydrophobic compounds (Sluis-Cremer and Tachedjian 2008) which bind to a site within the palm subdomain of the p66 subunit of RT (Kohlstaedt, Wang et al. 1992; Ren and Stammers 2008). This "pocket" is located approximately 10Å away from the polymerase active site (Smerdon, Jager et al. 1994; Ren, Esnouf et al. 1995).

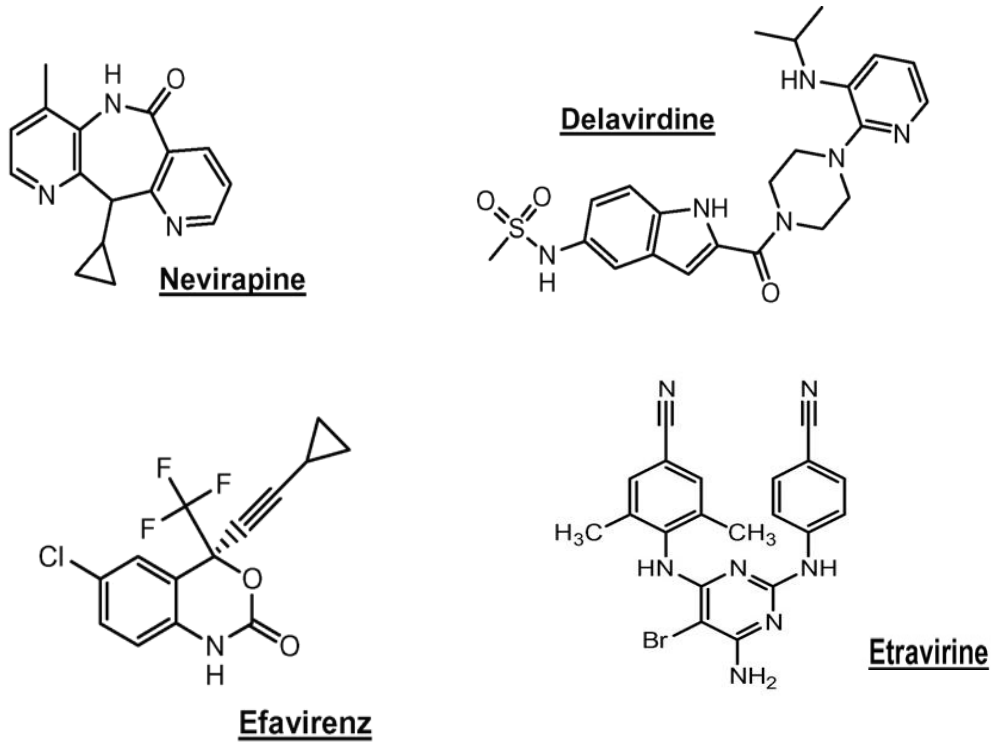


Figure 6. Structure of FDA approved NNRTI

At the initiation of this study three NNRTI were FDA approved for clinical use including efavirenz, delavirdine, and nevirapine. A fourth NNRTI, etravirine, was FDA approved for clinical use January 2008. The drugs are structurally distinct and bind to a hydrophobic pocket in the p66 subunit of RT. Scheme obtained from Sluis-Cremer and Tachedjian *Virus Research 2008 Vol. 134 (1-2):147-56*, used with permission from Elsevier Limited.

1.4.2.1 Mechanism of Action

Binding of NNRTI to RT forms a hydrophobic binding pocket in which the side chains of the crucial tyrosine 181 and tyrosine 188 residues move from a down to an up position (Figure 6) (Esnouf, Ren et al. 1995; Rodgers, Gamblin et al. 1995; Spence, Kati et al. 1995; Ren and Stammers 2008). Several mechanisms have been proposed to explain the inhibition of HIV-1 replication by NNRTIs. Early on, using crystallography, Kohlstaedt et al. proposed two mechanisms responsible for the inhibition of RT by nevirapine. The first included the inhibitor acting as a stick or a pole being jammed in the gears of a machine preventing movement of the thumb subdomain thereby preventing catalytic function (Kohlstaedt, Wang et al. 1992). However, examination of different crystal structures bound with various NNRTI showed significant variations in relative domain position leaving no clear evidence that NNRTI binding induces a single positioning of the p66 thumb subdomain (Esnouf, Ren et al. 1998; Ren and Stammers 2008). The second mechanism proposed by Kohlstaedt et al. was that nevirapine could indirectly affect the conformation of critical active site aspartic residues (Kohlstaedt, Wang et al. 1992). Comparison of NNRTI-bound and unbound RT showed that inhibitor specificity was achieved by emulating the protein-protein interactions that stabilize the structure of the p51 subunit, resulting in a distortion of the polymerase active site by movement of the key aspartic acid residues (Esnouf, Ren et al. 1995). A third mechanism of NNRTI action has been proposed in which the position of the polymerase primer grip is significantly displaced in RT bound to NNRTI versus unbound enzyme (Das, Ding et al. 1996). Displacement of the primer grip would affect the positions of the primer terminus and the polymerase active site, possibly explaining biochemical data that showed NNRTI binding to HIV-1 RT reduced efficiency of the chemical step of DNA polymerization, but does not prevent binding of either dNTPs or DNA (Das, Ding

et al. 1996). More recently studies have shown that NNRTIs also inhibit reverse transcription by altering RNase H function (Shaw-Reid, Feuston et al. 2005; Hang, Li et al. 2007; Radzio and Sluis-Cremer 2008; Sluis-Cremer and Tachedjian 2008). Current studies also show that NNRTI can influence other areas of the viral life cycle including GagPol processing (Tachedjian, Moore et al. 2005; Figueiredo, Moore et al. 2006) and RT dimerization (Tachedjian, Orlova et al. 2001; Mulky, Sarafianos et al. 2005; Figueiredo, Zelina et al. 2008).

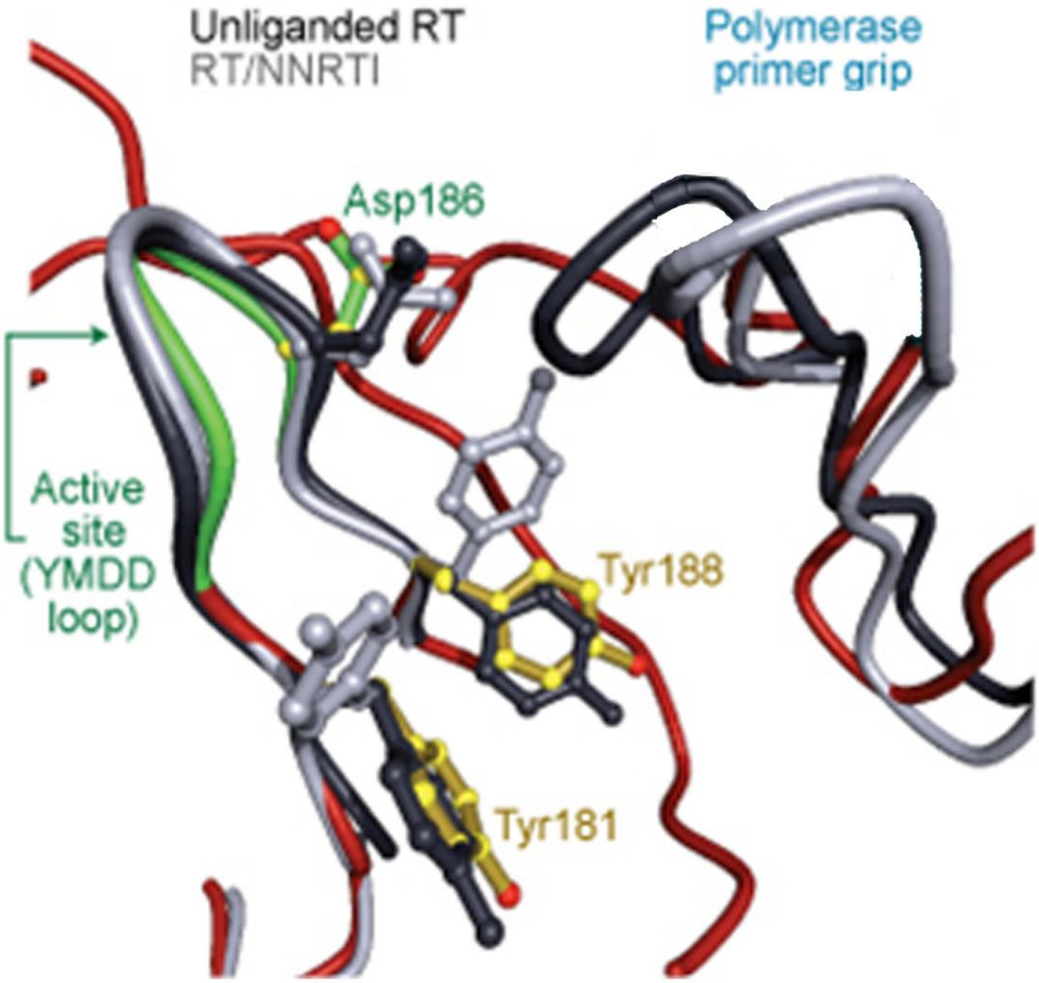


Figure 7. Conformational changes in RT due to NNRTI binding.

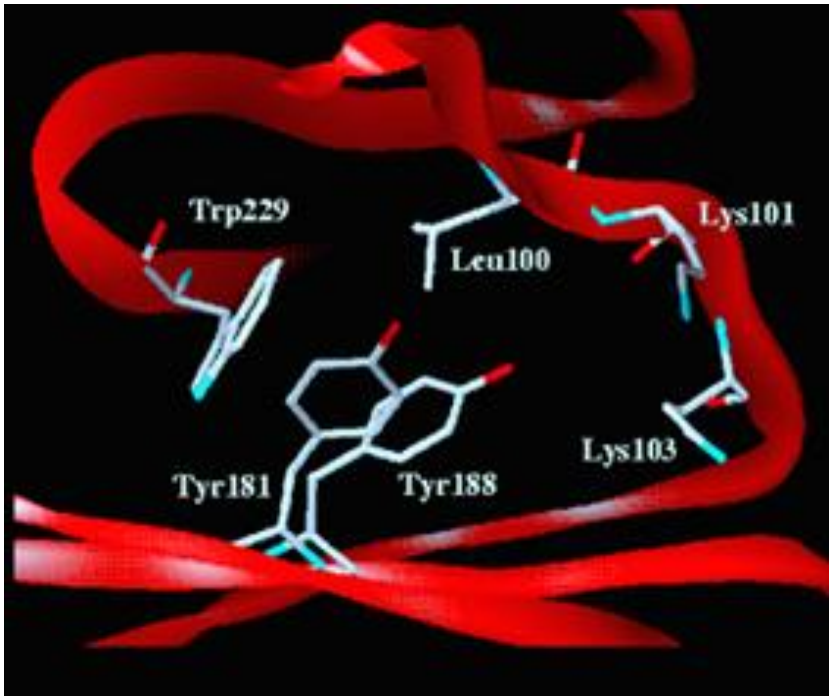
NNRTI bind to a hydrophobic pocket in the p66 subunit of RT. Unliganded RT is pictured in black and RT with an NNRTI bound is shown in grey. For comparison, the conformational changes due to binding of the RNase H inhibitor dihydroxyl benzoyl naphthyl hydrazone (DHBNH) are shown in color. When binding occurs Tyr188 and Tyr 181 move from a down to an up position (shown in grey). NNRTI binding also causes a change in the position of the primer grip region of RT when compared to the unliganded structure. This figure was obtained from *Himmel et al ACS Chemical Biology 2006 Vol.1 (11): 702-12* and used with permission from American Chemical Society (Himmel, Sarafianos et al. 2006).

1.4.2.2 Resistance

First generation inhibitors (including nevirapine and delavirdine) generally bind to the same pocket region of RT and interact with two crucial tyrosine residues. There are significant ring stacking interactions between the first generation NNRTI and tyrosines 181 and 188. Replacement of the tyrosines with cysteine residues results in dramatic loss of the ring stacking interactions leading to first generation NNRTI resistance and cross- resistance (Ren and Stammers 2008). No doubt this is why Y181C and Y188C were two of the first NNRTI resistance mutations identified (Mellors, Dutschman et al. 1992; De Clercq 1994; Ren and Stammers 2008). However, inhibitors such as efavirenz have less extensive interaction with the tyrosine residues. Therefore, mutations at these regions show smaller losses in efavirenz potency. Inhibitor flexibility has also been proposed as a reason some second generation NNRTIs, such as etravirine, are less susceptible to the effects of mutations at these key residues (Hsiou, Das et al. 1998; Ren and Stammers 2008).

K103N is a commonly reported mutation associated with NNRTI resistance. Loss of NNRTI efficacy when lysine is substituted by asparagines at 103 is not as easily explained as the aforementioned substitutions because most NNRTI do not directly contact the side chains of lysine 103 (Ren and Stammers 2008). The exception is delavirdine which makes hydrogen bonds to the main chain of lysine 103 (Esnouf, Ren et al. 1997). It has been proposed that the substitution of K103N leads to the formation of a hydrogen bond with the side chain of Tyr188 (Figure 8) stabilizing the residue in its apo-conformation and creating a barrier to NNRTI binding (Maga, Amacker et al. 1997).

A.



B.

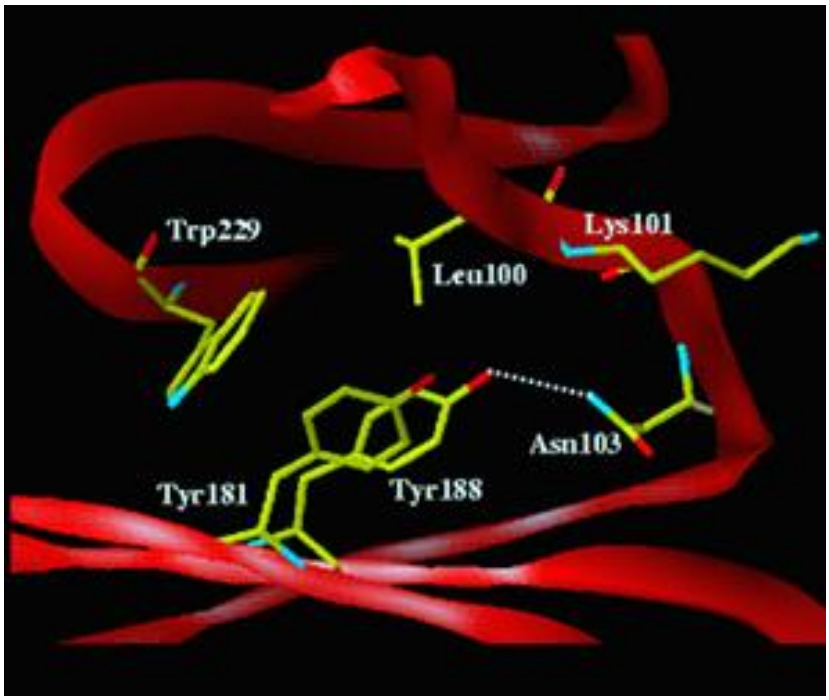


Figure 8. NNRTI resistance caused by K103N mutation.

Residue 103 is near the entrance to the NNRTI pocket. The two figures show the region around the entrance to the NNRTI binding pocket for wild-type RT (A) and the K103N mutant (B). An additional hydrogen bond between K103N and Y188 (dotted line in B) keeps the entrance to the pocket closed, making it more difficult for NNRTIs to enter the pocket. Obtained from *Sarafianos et al. http://www.retrovirus.info/rt/NNRTI_fig4.html*

1.5 HYPERSUSCEPTIBILITY

1.5.1 A Common Example

HIV-1 resistance to each type of inhibitor readily occurs when used independently; therefore the compounds are typically used in combination leading to more effective control of viral replication than either class alone. Although the inhibitors have different mechanisms of action and act at different sites in RT, there are examples of mutations induced by one class of compounds that increase viral susceptibility to the other class of inhibitors. A firmly established example is the Y181C mutation which arises in response to NNRTI exposure. Y181C in the NNRTI-binding pocket has been shown to increase viral susceptibility to the NRTI zidovudine (AZT) in the presence of AZT resistance mutations (Larder 1992; Selmi, Deval et al. 2003). Introduction of the Y181C mutation in a background of TAMS suppresses the excision phenotype (Larder 1992) by reducing ATP binding to the enzyme thereby re-sensitizing the enzyme to AZT (Selmi, Deval et al. 2003).

1.5.2 NNRTI Hypersusceptibility

Recently, a phenotype was observed due to regular drug resistance testing of HIV-1 infected patients in which some viral strains demonstrated significantly increased drug susceptibility to NNRTIs compared with wild type virus (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002; Katzenstein, Bosch et al. 2003). The increased sensitivity to NNRTI, termed NNRTI hypersusceptibility (HS), was positively correlated with previous NRTI experience. In fact, a longer duration of NRTI therapy and reduced NRTI susceptibility

was associated with efavirenz HS (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002). In a study examining over 17,000 patient-derived plasma samples, NNRTI HS was observed more frequently among viruses from NRTI experienced/NNRTI naïve patients than among patients whom experienced both drug classes or were NRTI naïve (Whitcomb, Huang et al. 2002).

1.5.2.1 Prevalence and Statistical Cut-offs

The prevalence of NNRTI HS has been reported in 11-50 % of viral isolates containing no major NNRTI mutations from NRTI-experienced/NNRTI naïve patients (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002; Shulman, Bosch et al. 2004).

In the previously mentioned studies, hypersusceptibility was defined as a fold change in viral sensitivity to a compound < 0.4 when compared to wildtype sensitivity to the same compound. Originally this cut-off was determined based on assay variability, however in a 2003 study a more clinically relevant threshold for efavirenz hypersusceptibility was sought (Bosch, Downey et al. 2003). Multiple potential cut-offs were examined in the ACTG 364 trial database. The variable cut-offs were related to the virological outcome of treatment over 144 weeks. It was determined that the 0.4 fold change cut-off for NNRTI HS showed the maximum likelihood estimate and provided the smallest hazard ratio (Bosch, Downey et al. 2003).

1.5.2.2 Clinical Relevance

In a prospective cohort analysis, more than 100 NNRTI naïve HIV-1^+ patients that were failing a stable antiretroviral regimen were placed on an NNRTI containing regimen (Haubrich, Kemper et al. 2002). After six months of treatment the mean change in $\log_{10}$ HIV RNA was

greater in patients with hypersusceptible virus than in patients without hypersusceptible virus at baseline and persisted for twelve months. $CD4^+$ T cell increases were also greater in those patients with NNRTI HS virus at baseline compared to patients without NNRTI HS virus (Figure 9). In a randomized trial of multi-drug regimens containing efavirenz in NRTI-experienced patients, baseline EFV HS increased the likelihood of HIV-1 RNA suppression to below 200 copies /mL at 24 and 48 weeks after the initiation of the regimen (Hammer, Vaida et al. 2002).

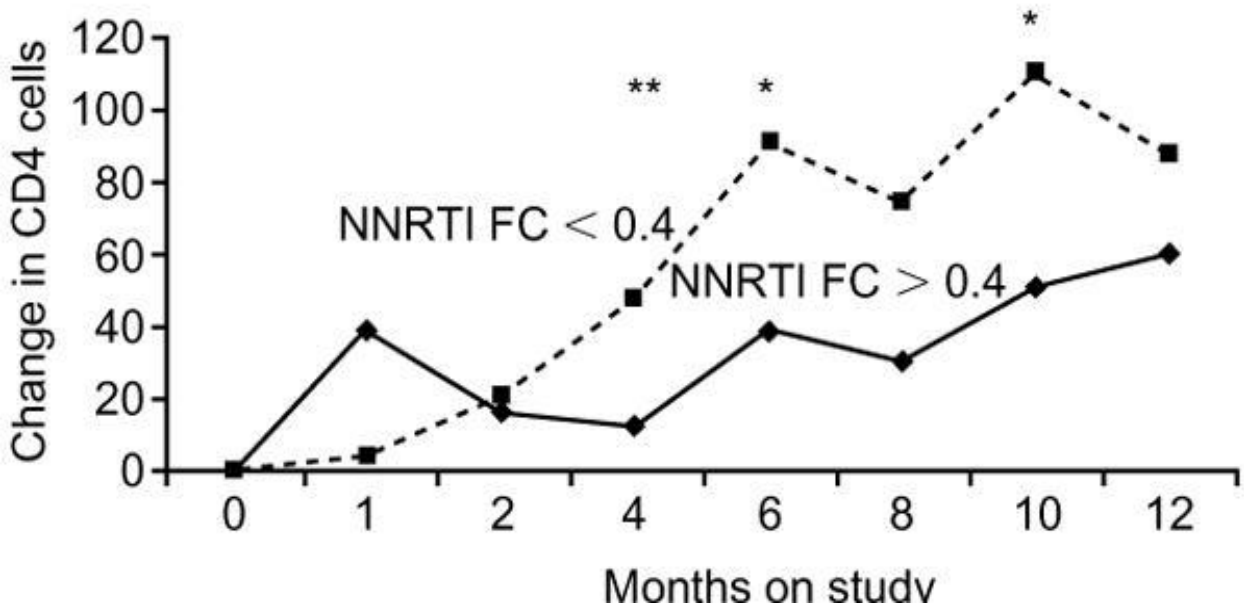


Figure 9. Increase in CD4 T cell count in patients with HS virus.

In an analysis of more than 100 NNRTI naïve HIV^+ patients failing an antiretroviral regimen, those patients with hypersusceptible (HS) virus at baseline (dotted line) had superior increases in CD4 T cell counts compared to patients who did not have NNRTI HS virus (solid line) at baseline. *P = 0.1, **P = 0.06. {Haubrich, 2002 #29}

1.5.2.3 Genetic Correlates of NNRTI HS

Recently, a study of paired baseline genotypes and phenotypes were obtained from 444 subjects entering one of five ACTG studies (290, 359, 364, 370, or 398) that statistically correlated specific NRTI mutations with NNRTI HS (Shulman, Bosch et al. 2004). All subjects were NRTI experienced and NNRTI naïve at the beginning of the study. Using Fishers exact tests, recursive partitioning (Classification and Regression Trees, CART), and logistic regression, RT mutations at positions 118, 208, and 215 were predictive of and most associated with efavirenz HS (Figure 10).

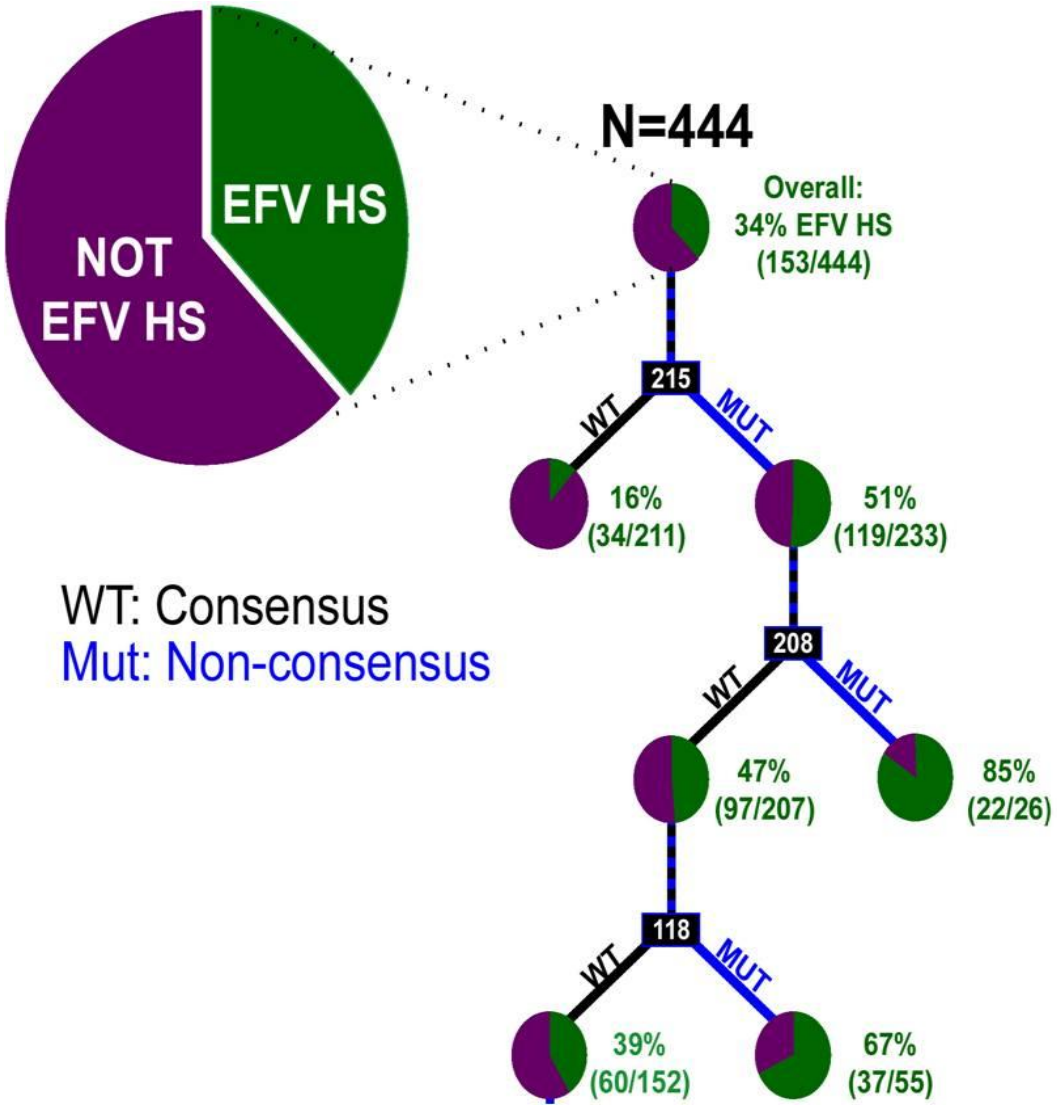


Figure 10. Genetic Correlates of Efavirenz Hypersusceptibility.

Statistical analyses including recursive partitioning and Classification and Regression Tree (CART) were used to identify reverse transcriptase mutations associated with efavirenz hypersusceptibility. Combinations of mutations at RT codons 215, 208, and 118 were most associated with efavirenz hypersusceptibility: of 26 isolates with mutations at 208 and 215 85% were HS to EFV and of 55 isolates with mutations at 118 and 215 67% were HS to EFV. Figure obtained from *Shulman et al AIDS 18(13):1781-178 2004*, reprinted with permission from Lippincott Williams & Wilkins.

1.6 THE ROLE OF MUTATIONS 118I, 208Y, AND 215Y IN NRTI RESISTANCE

Mutation T215Y is firmly established as a drug resistance mutation evidenced by its inclusion in the current list of HIV-1 drug resistance mutations by the International AIDS Society (IAS) and the Stanford University HIV Drug Resistance Database (Stanford DB). V118I is not included in the IAS panel of resistance mutations but is listed by the Stanford DB as causing "low-level resistance to 3TC and possibly other NRTI" when in the presence of other resistance mutations. Although mutations at residue 208 are not included on the most current IAS list or the Stanford DB of HIV-1 drug resistance mutations there is growing evidence supporting a role for both V118I and H208Y in drug resistance (Johnson et al Topics in HIV Medicine 2008).

1.6.1 V118I

In 2000, a new mutational pattern that leads to moderate resistance to the NRTI lamivudine (3TC) in the absence of M184V was reported (Hertogs, Bloor et al. 2000). When present in a background of TAMS viral isolates with V118I alone or in combination with E44D showed 4-50 fold resistance to 3TC (Hertogs, Bloor et al. 2000). Analysis of two HIV-1 treatment and resistance databases revealed a prevalence of approximately 12% for the valine to isoleucine mutation at codon 118 in RT in NRTI treated patients (Romano, Venturi et al. 2002). Genotypic analysis of 344 patient plasma samples found V118I in 78 out of 261 isolates from patients previously treated with antiretrovirals (Delaugerre, Mouroux et al. 2001). The valine to isoleucine change was always found in an AZT resistance background (the mutation was never

found in drug naïve patients) and although the change was associated with 3TC resistance, its presence was independent of the M184V mutation (Hertogs, Bloor et al. 2000; Delaugerre, Mouroux et al. 2001; Montes and Segondy 2002; Romano, Venturi et al. 2002). In a study of 1083 patient samples, V118I was found at high frequencies in the presence of ZDV resistance (Hertogs, Bloor et al. 2000). V118I occurred at the highest frequency (approximately 40%) when AZT resistance was greater than 10 fold and 3TC resistance was between 4-50 fold (Figure 11). The V118I mutation was also implicated in resistance to d4T and ddI (Larder, Hertogs et al. 2000). Biochemical studies determined that V118I-containing RT enzymes showed dramatic reductions in rates of incorporation of AZT-MP and 3TC-MP indicating the V118I mutation conferred increased discrimination of the inhibitor compared to the natural substrate. This same study also showed decreased unblocking of an AZT-terminated primer when V118I was present. However a mutation at codon 116 was also present in these enzymes leaving the sole contribution of V118I undefined (Girouard, Diallo et al. 2003). V118 is proximal to the ATP binding site (Figure 12) allowing mutations at this residue to affect the binding and positioning of ATP (Sarafianos, Das et al. 2004).

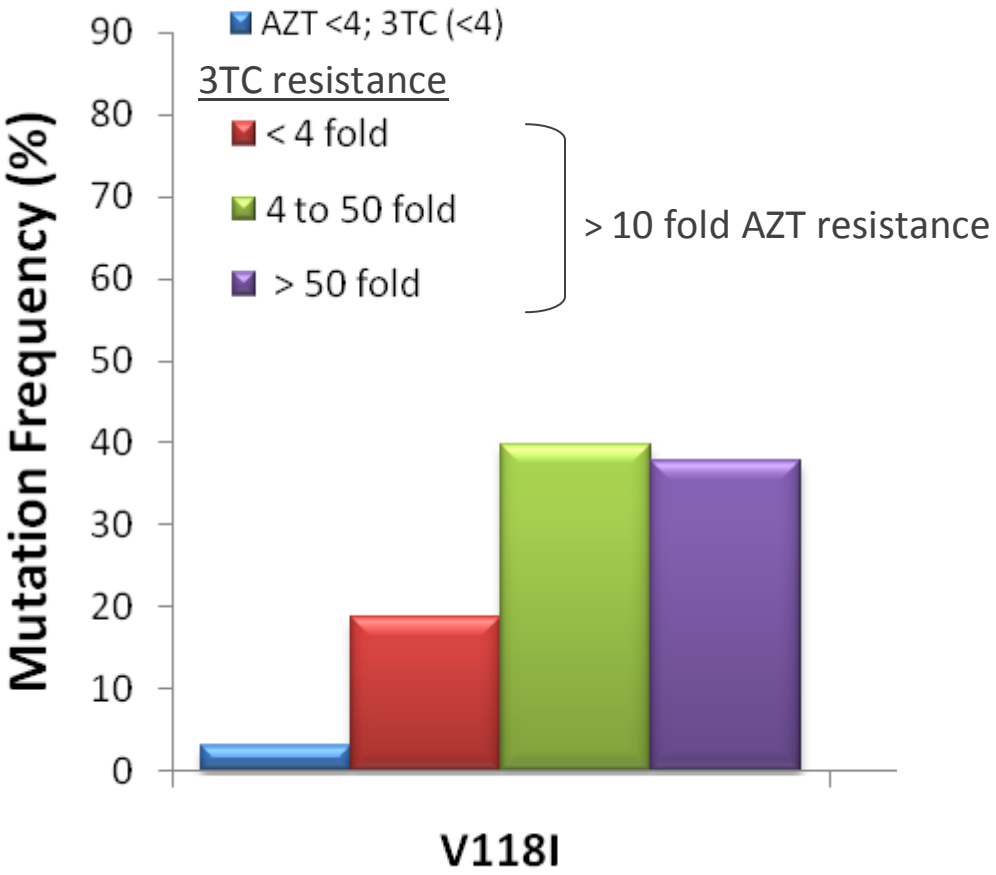


Figure 11. Frequency of V118I in clinical samples.

The frequency and distribution of NRTI associated mutation V118I according to 3TC and AZT susceptibility. Data from 1083 plasma samples obtained during routine clinical testing in Europe and US. Bars represent frequency of V118I in samples with: less than 4 fold resistance to AZT and 3TC (blue bar); > 10 fold AZT resistance and < 4 fold 3TC resistance (red bar); > 10 fold AZT resistance and moderate (4-50 fold) 3TC resistance (green bar); > 10 fold AZT resistance and high (> 50 fold) 3TC resistance (purple bar). V118I is present at low frequencies in the absence of AZT resistance. V118I occurred at the highest frequency when AZT resistance was greater than 10 fold and 3TC resistance was between 4-50 fold. (Hertogs, Bloor et al. 2000)

1.6.2 H208Y

In 1995 while doing an *in vitro* selection of HIV-1 replication in the presence of the pyrophosphate analog foscarnet, a histidine to tyrosine change at residue 208 of RT (along with other mutations) resulted in approximately 8.5 fold resistance to foscarnet (Mellors, Bazmi et al. 1995). Further investigation of the individual contributions of the mutations showed that H208Y alone contributed 2.4 fold resistance to foscarnet. In an investigation of AZT resistance in 223 clinical samples a mutation at 208 enhanced AZT resistance in the presence of TAMS and M184V (Sturmer, Staszewski et al. 2003). The H208Y mutation (present with 211K and 214F) increased AZT resistance 21 fold and was only detected in highly AZT resistant samples. This group found that although H208Y was present in 12.56% of the study population, the presence of M41L and T215Y was a prerequisite for the appearance of H208Y (Sturmer, Staszewski et al. 2003).

Residue 208 is located at the C terminus of an alpha helix in the palm domain (Figure 12), and has been implicated in the stabilization of this secondary structure (Meyer, Matsuura et al. 2003; Sturmer, Staszewski et al. 2003; Svicher, Sing et al. 2006). In a comparison of the "open" binary and "closed" ternary configurations of crystallized RT, H208 occupied the same position in both structures and did not play a role in positioning of the incoming dNTP (Meyer, Matsuura et al. 2003). However, 208 is proximal to the ATP binding site (Figures 12 and 13) and alteration of the geometry of this site may allow efficient excision even in the presence of M184V (Meyer, Matsuura et al. 2003; Sarafianos, Das et al. 2004).

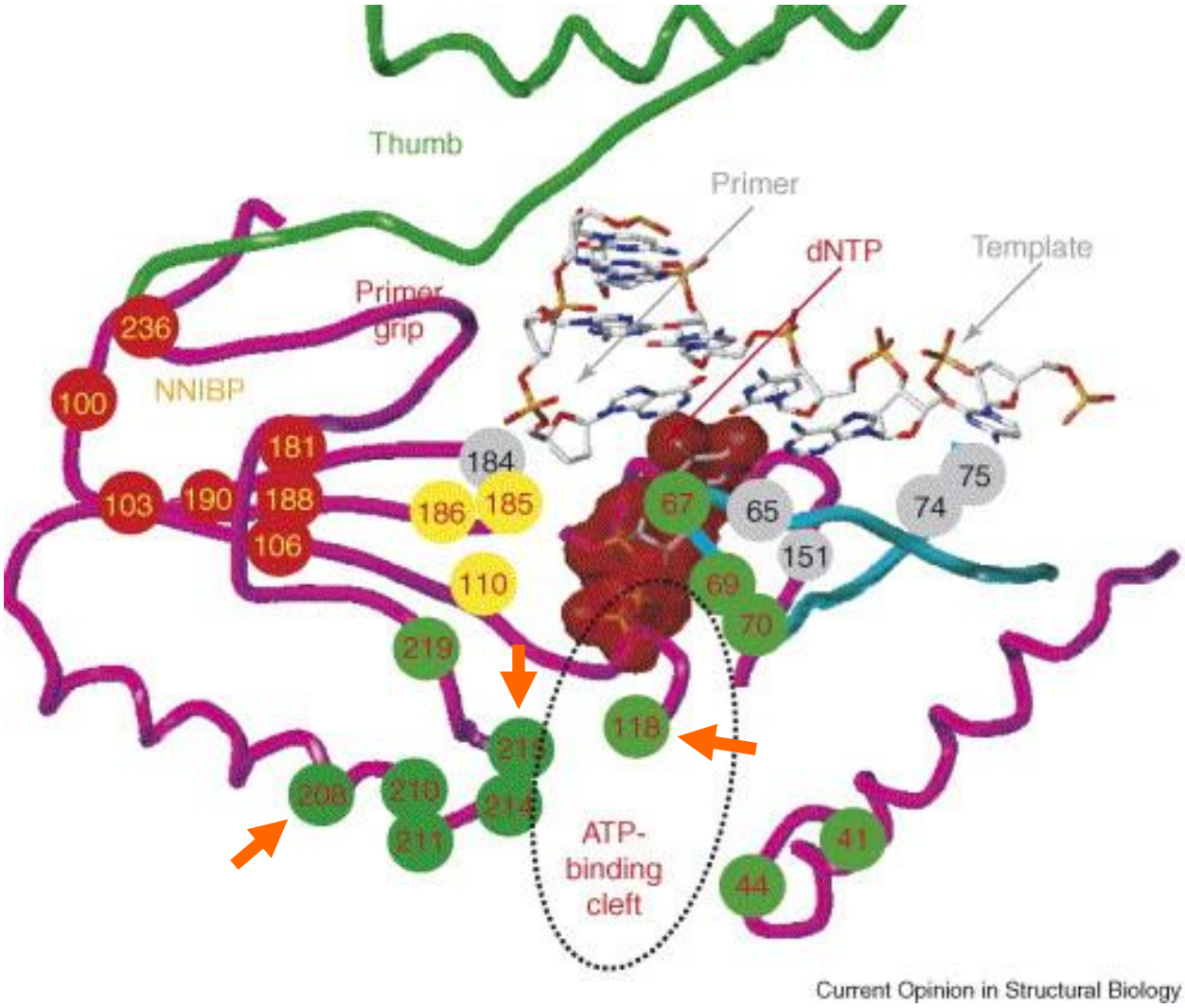


Figure 12. Site of Drug Resistance Mutations in HIV-1 RT.

The structure of HIV-1 RT in the region near the polymerase active site is shown. The subdomains are color coded (palm, magenta; thumb, green; fingers, cyan). A template–primer is shown; the van der Waals volume of the incoming dNTP is colored maroon. The NNRTI binding pocket (NNIBP) is labeled; the ATP binding cleft that would participate in the excision reaction is shown. The catalytic aspartates are shown in yellow circles; residues that enhance the excision reaction are shown in green circles; other residues involved in NRTI resistance are shown in gray circles; residues that cause NNRTI resistance are shown in red circles. Mutations most associated with efavirenz HS are highlighted with orange arrows. Figure obtained from *Sarafianos et al. Current Opinion in Structural Biology 14:716-730, 2004*, reprinted with permission from Elsevier Ltd.

A study to define an association of H208Y with NRTI exposure and NRTI resistance showed that a tyrosine residue at position 208 was prevalent in only 0.2% of treatment naïve and approximately 4% of treatment experienced patients and 11.3% in patients having experience with four or more NRTI (Svicher, Sing et al. 2006; Nebbia, Sabin et al. 2007). H208Y was strongly associated with prolonged NRTI-selective pressure (Nebbia, Sabin et al. 2007).

1.6.3 T215Y

The role of T215Y in AZT resistance was established with the first reports of the genetic basis of AZT resistance in patients (Larder and Kemp 1989). Larder et al. examined 12 HIV-1 isolates from patients, and 7 of the 12 isolates showed phenotypic resistance to AZT. All of the AZT sensitive isolates had wildtype threonine at position 215 while the resistant isolates had either phenylalanine or tyrosine at residue 215. In that study 70% of the resistant isolates had a tyrosine at 215 instead of phenylalanine. Through modeling experiments it was shown that a crucial component of AZT resistance was the direct contact of T215Y with the adenine ring of ATP (Figure 12), which enhances binding of ATP by properly positioning ATP and allows more efficient use of the substrate for the excision reaction (Boyer, Sarafianos et al. 2001). This positioning allows the γ-phosphate of ATP to be near the phosphate that joins the last two nucleotides of the primer (Sarafianos, Das et al. 2004).

2.0 HYPOTHESIS AND SPECIFIC AIMS

2.1 STUDY RATIONALE

Waning therapeutic options available to HIV-1 infected patients due to NRTI drug resistance leads to loss of viral suppression and ultimately increased morbidity and mortality in the infected population. A benefit of specific NRTI resistance mutations such as V118I, H208Y, and T215Y is that they improve NNRTI susceptibility, which is associated with better virologic outcome (Shulman, Zolopa et al. 2001; Hammer, Vaida et al. 2002; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002). One of the current standards of care for HIV-infected patients consists of combination therapy using NRTI with NNRTI. Therefore, defining mechanisms of NNRTI hypersusceptibility due to NRTI-induced resistance mutations is critical in improving combination therapies, particulary for patients with limited treatment options. In addition, elucidating mechanisms of NNRTI HS caused by mutations V118I, H208Y, and T215Y is intrinsic to the rational design and development of RT inhibitors capable of exploiting these mechanisms to enhance therapeutic regimens.

Patient isolates demonstrating phenotypic HS often contain a multitude of resistance-associated mutations (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002) making it difficult to discern the contribution of specific NRTI resistance mutations to NNRTI HS. Although statistical correlations linking NRTI mutations V118I,

H208Y, and T215Y with NNRTI HS have been made (Shulman, Bosch et al. 2004), causality has not been determined and the mechanistic basis of NNRTI HS remains undefined.

NRTIs and NNRTIs have different antiretroviral mechanisms and are active in different regions of RT, leaving the correlation between NRTI resistance mutations and NNRTI HS unclear (Figure 13). Therefore mutations in RT that cause NRTI resistance may contribute to HS through impaired viral replication, decreased amounts of virion-associated RT, and/or diminished enzyme susceptibility resulting in an overall reduction in the concentration of NNRTI needed to inhibit production of infectious virus. This study aimed to define the impact of specific mutations involved in NNRTI HS and to elucidate mechanisms leading to phenotypic HS.

How Do NRTI Mutations V118I, H208Y, and T215Y Affect NNRTI Susceptibility?

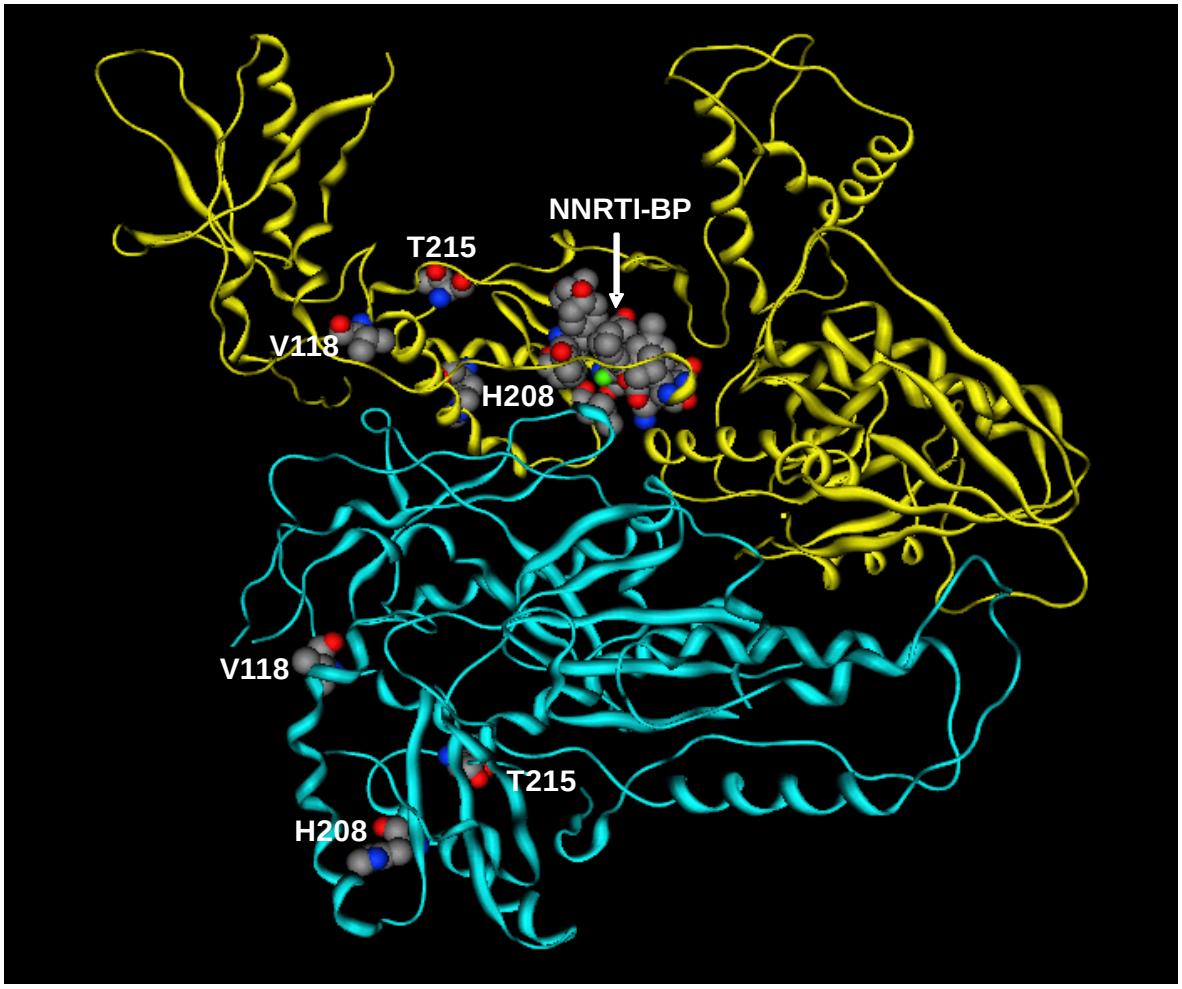


Figure 13. Location in RT of Codons V118, H208, and T215.

A ribbon diagram of HIV-1 RT with the p66 subunit highlighted in yellow and the p51 subunit highlighted in blue. The positions of amino acids V118, H208, and T215 in both subunits are space filled along with the NNRTI binding pocket (NNRTI-BP).

2.2 HYPOTHESIS

We hypothesize that *NRTI mutations 118I, 208Y, and 215Y in combination cause NNRTI HS and that this phenotype is due to multiple mechanisms including deficient viral replication due to decreased virion packaged RT and decreased enzyme NNRTI susceptibility*.

2.3 SPECIFIC AIMS

The overall objective of this project was to assess the contribution of HIV-1 reverse transcriptase mutations V118I, H208Y, and T215Y alone and in combination to NNRTI hypersusceptibility and to elucidate mechanisms responsible for the HS phenotype. The hypothesis was tested by the following specific aims:

1) Establish the contribution of mutations 118I, 208Y, and 215Y to the NNRTI hypersusceptibility phenotype using:
    a. Cell based viral drug susceptibility assays
    b. Cell free purified enzyme NNRTI susceptibility assays
2) Elucidate differences in viral replication, protein production and packaging for virus containing mutations 118I, 208Y, and/or 215Y.

3.0 CHAPTER ONE. NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR INDUCED MUTATIONS 118I, 208Y, AND 215Y CAUSE HIV-1 HYPERSUSCEPTIBILITY TO NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

PREFACE

This chapter is adapted from a published study (Clark SA[1], Shulman NS[2], Bosch RJ[3], and Mellors JW[1]. 2006. Reverse Transcriptase Mutations 118I, 208Y, and 215Y Cause HIV-1 Hypersusceptibility to Nonnucleoside Reverse Transcriptase Inhibitors. AIDS 20:981-984) reprinted with permission from Lippincott Williams & Wilkins Publishing. Work described in this chapter is in fulfillment of specific aim one.

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3.1 ABSTRACT

HIV-1 hypersusceptibility (HS) to non-nucleoside reverse transcriptase inhibitors (NNRTI) improves the response to NNRTI-containing regimens. The genetic basis for NNRTI HS was partially defined in our prior analyses of a paired genotype-phenotype dataset of viral isolates from treatment-experienced patients, in which reverse transcriptase (RT) mutations V118I, H208Y, and T215Y were strongly associated with NNRTI HS. We evaluated the role of these mutations in NNRTI HS by site-directed mutagenesis and phenotypic analysis of HIV-1 recombinants. Drug susceptibility was determined in viral single and multiple cycle replication assays as well as in cell free enzyme susceptibility assays. HS was defined by a statistically significant (P <0.01; Student's t-test) mean fold change (FC) in IC_{50} of < 0.4. The single mutations V118I, H208Y, and T215Y did not increase viral NNRTI susceptibility. The H208Y/T215Y and V118I/H208Y/T215Y mutants showed marked viral HS to efavirenz. Additionally, viruses containing mutation combinations H208Y/T215Y, V118I/T215Y, and V118I/H208Y/T215Y were also hypersusceptible to delavirdine and nevirapine. When assessed for enzyme NNRTI susceptibility, RT containing mutations at codons 208 and 215 were not hypersusceptible to NNRTI. In contrast, the V118I/T215Y RT showed significantly increased susceptibility to efavirenz and nevirapine (Fold-change = 0.2, P = 0.001 and Fold-change = 0.2, P = 0.002, respectively). These findings indicate that combinations of NRTI mutations V118I, H208Y, and T215Y produce NNRTI HS and depending on the mutation profile virus may be HS to some NNRTI and not others. In addition, a lack of HS for mutant enzymes containing H208Y

and T215Y combined with the significant increases in enzyme susceptibility for V118I/T215Y mutants provide the first evidence of distinct mechanisms of NNRTI HS.

3.2 INTRODUCTION

Phenotypic testing of HIV-1 infected patient samples identified viral variants that were more susceptible to non-nucleoside reverse transcriptase inhibitors (NNRTI) than wildtype virus (Shulman, Zolopa et al. 2001). This phenotype has been termed NNRTI hypersusceptibility (HS). HS is generally defined by a fold change in the test virus IC_{50} of < 0.4 compared to wildtype control virus run in parallel. NNRTI HS is more common in viral isolates from patients who are NRTI-experienced and NNRTI-naïve than in patients who are treatment naïve or experienced to both NRTI and NNRTI (Whitcomb, Huang et al. 2002). Overall, NNRTI HS is common; 17% of approximately 18,000 patient plasma samples tested during a two year period showed HS to at least one of the three FDA-approved NNRTI (Whitcomb, Huang et al. 2002). Several studies have shown that patients with virus that is NNRTI HS have better virologic and CD4 responses to NNRTI-containing regimens than patients without NNRTI HS virus (Shulman, Zolopa et al. 2001; Hammer, Vaida et al. 2002; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002; Katzenstein, Bosch et al. 2003). For example, in a randomized trial of multi-drug regimens containing efavirenz (EFV) in NRTI -experienced patients, baseline HS to EFV significantly increased the likelihood of plasma HIV RNA suppression to below 200 copies/mL at 48 weeks after initiation of the regimen (Hammer, Vaida et al. 2002).

Previously, we reported genetic correlates of NNRTI HS among 444 NRTI-experienced patients having paired genotypes and phenotypes (Shulman, Bosch et al. 2004). Several statistical methods were used to identify mutations associated with NNRTI HS including univariate analyses, stepwise binary regression, and recursive partitioning (classification and

regression trees, CART). Mutations at RT codons 118, 208, and 215 were most predictive of EFV HS (Shulman, Bosch et al. 2004). In the CART analysis, EFV HS was present in 51% of the isolates with mutations at codon 215, 69% with mutations at codons 118 and 215, and 85% with mutations at codons 215 and 208. The role of these mutations in NNRTI HS was inferred but not directly demonstrated. Therefore, the goal of this study was to assess the specific contribution of mutations at codons 118, 208, and 215, alone and in combination, to NNRTI HS by phenotypic testing of site-directed mutant virus and enzyme. Defining the contribution of specific mutations to NNRTI HS is an essential step in elucidating the virological mechanisms involved.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

The non-nucleoside reverse transcriptase inhibitors (4S)-6-chloro-4-cyclopropylethynyl-4-trifluoro methyl-1,4-dihydro-beno[d][1,3]oxazin-2-one (**Efavirenz**), and 11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b: 2'3'-e][1,4][diazepin-6-one] (**Nevirapine**) were provided by the NIH AIDS Reference and Reagent Program, Division of AIDS NIAID. The non-nucleoside reverse transcriptase inhibitor 1-[3-[(1-methylethyl) amino]2-pyridinyl]-4-[[5-[(methylsulfonyl)amino]-1H-indol-2-yl] carbonyl]-piperazine (**Delavirdine**) was purchased from Biomol International (Plymouth Meeting, PA). The nucleoside reverse transcriptase inhibitor (R)-9-(2-phosphonylmethoxypropyl) adenine (**Tenofovir**) was provided by Gilead Sciences (Foster City, CA). 3'-azido-3'deoxythymidine (**AZT**) was obtained from

Sigma Chemical Corporation (St. Louis, MO). 2',3'-deoxy-2'-3'-didehydrothymidine (**d4T**) was provided by Raymond Schinazi, PhD (Emory University). The following inhibitors were obtained from the NIH AIDS Research and Reference Reagent Program: protease inhibitors 1,3-thiazol-5-ylmethyl[3-hydroxy-5-[3-methyl-2[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl] carbamoyl]amino-butanoyl] amino-1,6-diphenyl-hexan-2-yl] amino formate (**Ritonavir**), and (2S-N-[(2S,4S,5S)-5{2-(2,6-dimethylphenoxy) acetyl]amino}-4-hydroxy-1,6-diphenyl-hexan-2-yl]-3methyl-2-(2-oxo-1,3-diazinan-1-yl) butanamide (**Lopinavir)**, fusion inhibitor acetyl-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-amide (**Enfuvirtide**), and integrase inhibitor 4-[3-(azidomethyl) phenyl]-2-hydroxy-4-oxo-2-butenoic acid (**118-d-24**).

The compounds were dissolved in the appropriate buffers and stored at -20℃. Compounds were diluted immediately before use to the desired concentrations in RPMI 1640 culture medium (Whittaker MA Bioproducts, Walkersville, MD), Dulbecco's Modified Eagle Medium, phenol red free (DMEM-PRF: Gibco-BRL, Grand Island, NY), or dimethyl sulfoxide (DMSO).

3.3.2 Cells

The P4/R5 reporter cell line (provided by Ned Landau, Salk Institute, LaJolla, CA) is a CCR5 tropic HeLa cell line stably transfected to express a Tat-activated β-galactosidase gene under the control of an HIV-1 LTR promoter. P4/R5 cells were cultured in DMEM-PRF supplemented with 10% fetal bovine serum (FBS: HyClone, Logan, UT), 50 IU/mL penicillin, 50 µg/mL streptomycin, and 0.5 µg/mL puromycin (Clonetech, Palo Alto, CA). MT-2 cells (AIDS Research and Reference Reagent Program National Institute of Allergy and Infectious

Diseasee, National Institutes of Health) were cultured in RPMI 1640 supplemented with 10% FBS, 10 mM Hepes buffer, 50 IU/mL penicillin, and 50 µg/mL streptomycin.

3.3.3 Generation of Recombinant HIV-1

Mutations were introduced into the RT gene of the xxHIV-1_{LAI} infectious clone using the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Silent 5' XmaI and 3' XbaI restriction sites allowed subcloning of the mutated RT fragments into the xxHIV-1_{LAI} clone (Shi and Mellors 1997). The presence of the desired mutations was verified by DNA sequencing.

3.3.4 Generation of Virus

Virus was produced by transfection of wildtype or HIV-1_{LAI} plasmids containing mutations V118I alone; H208Y alone; T215Y alone; V118I/T215Y; H208Y/T215Y; and V118I/H208Y/T215Y (Figure 14) by electroporating (BIO-RAD Gene Pulser ®, Hercules, CA) 5-10 µg DNA into MT-2 cells. Culture supernatants were harvested seven days post-transfection and stored at -80°C. The genotype of harvested virus was confirmed by extracting RNA from virions (QIAamp kit, QIAGEN, Valencia, CA), treating the extract with DNase I (Roche, Indianapolis, IN), amplifying the RT coding region using RT-PCR, purifying the PCR product (Wizard PCR Purification System, Promega, Madison, WI), and sequencing the PCR product. The 50% tissue culture infectious dose ($TCID_{50}$) of each virus stock was determined in P4/R5 or MT-2 cells by three fold endpoint dilution assay and calculated using the Reed and Muench equation.

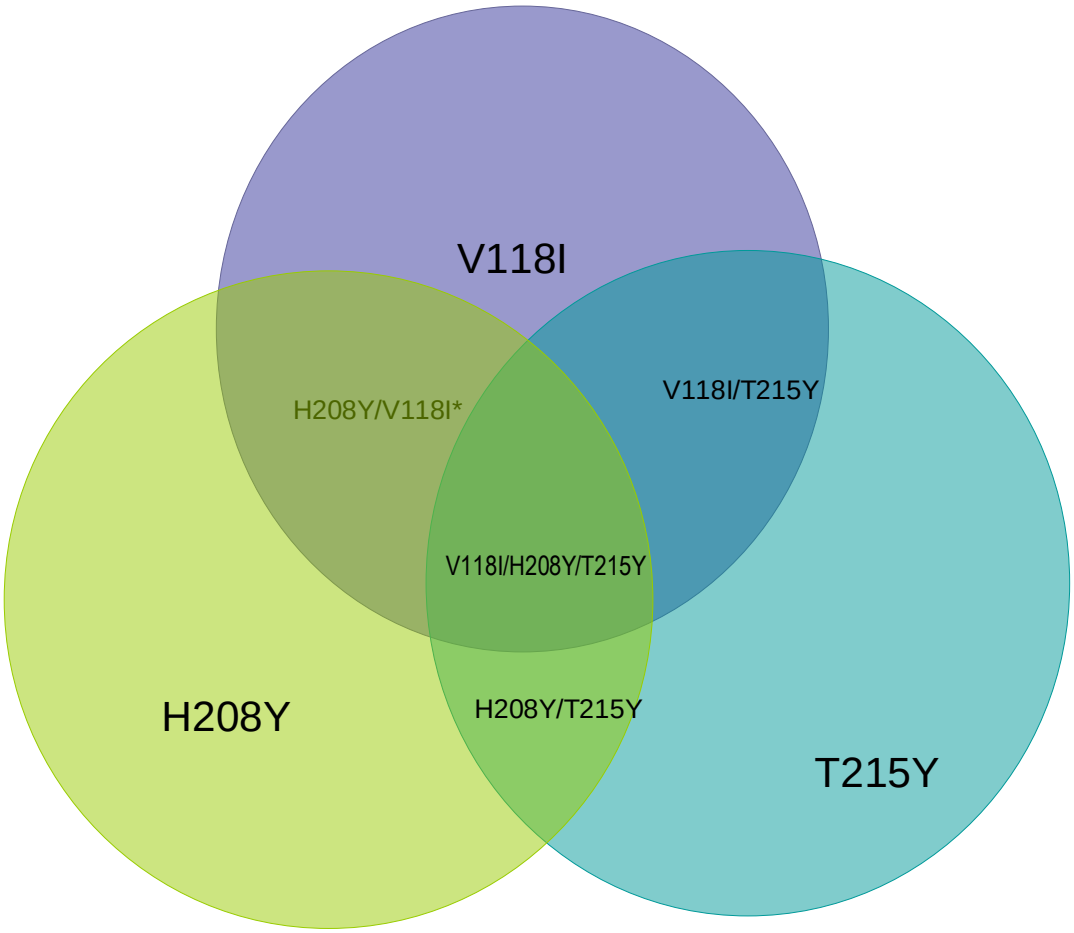

Figure 14. NRTI mutant combinations studied

Site directed mutants containing these mutations were used to study NNRTI hypersusceptibility. *The H208Y/V118I mutant combination was never found in patient isolates in the five ACTG studies used to determine statistical correlates of HS and was therefore not included in this study.

3.3.5 Single Cycle Drug Susceptibility Assay

Serial 2-fold drug dilutions were added in triplicate to 5 x 10^3 P4/R5 cells/well in a 96 well plate. Wells were inoculated with sufficient virus to produce 100 relative light units (RLU) in no-drug control wells. Forty-eight hours after infection, a cell lysis buffer and luminescent substrate (Gal-Screen; Tropix/Applied Biosystems) were added to each well, and the RLU values were determined using a luminometer (ThermoLabSystems, Waltham, MA). The 50% inhibitory concentration (IC_{50}) was calculated as the concentration of drug needed to inhibit 50% of viral replication. The fold-change (FC) in virus susceptibility was calculated by dividing the IC_{50} of mutant virus by the IC_{50} values of wildtype HIV_{LAI}.

3.3.6 Multiple Cycle Drug Susceptibility Assay

Serial dilutions of an inhibitor were added in triplicate to MT-2 cells in a 96-well plate. The cells were infected at an MOI of 0.01 determined by endpoint dilution in MT-2 cells. Cells were monitored daily for syncytium formation. Seven days post-infection, culture supernatants were harvested and treated with 0.5% Triton-X 100. The p24 antigen concentration in the supernatants was determined using a commercial ELISA assay (DuPont, NEN Products, Wilmington DE). IC_{50} and fold-change were calculated as described above.

3.3.7 Protein Purification and Expression

Mutant HIV-1_{LAIxx} (Shi and Mellors 1997) RT made using the Quickchange site directed mutagenesis kit (Stratagene LaJolla, California USA) was inserted into the protein expression plasmid p6HRT-PR-xx (p6HRT) which contains XmaI and XbaI restriction sites, a six consecutive histidine residue tag at the N-terminus, and the complete protease (PR) sequence (Le Grice and Gruninger-Leitch 1990). Expression of an HIV-1 protease sequence allows processing of RT into the p51 and p66 subunits during protein generation. p6HRT and pDMI.1 (expresses a *lac* repressor) were co-transformed into *E. coli* JM109 cells. Cultures were grown in PowerPrime broth to an OD_{600nm} 0.4 at 37°C. Protein production was induced with the addition of 1 M isopropyl β D-1-thiogalactopyranoside (IPTG) with overnight incubation at 30°C. Proteins were purified using TALON® Polyhistidine-Tag Purification Resins and 2 mL disposable gravity columns (ClonTech Mountainview, CA). RT concentrations were determined by spectrophotometry at 280 nm using an extinction coefficient (ε280) of 260450 $M^{-1}cm^{-1}$ and by Bradford assay (Bradford 1976). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with a VersaDoc imaging system (BioRad Laboratories, Inc., Hercules, CA) to ensure equal amounts of p66 and p51 subunits. Following the addition of glycerol, proteins were stored at -20°C or -80°C until further use.

3.3.8 Generating Primer/Template

SPA primer/ template

Polyadenylic acid (Poly rA) obtained from Amersham Biosciences (USA) and a biotinylated thymidine primer were dissolved in Tris-EDTA (TE) buffer. Poly rA –biotin dT were annealed in TE buffer by heating for 15 minutes at 100°C followed by slow cooling to room temperature.

3.3.1 Enzyme NNRTI Susceptibility Assays (SPA)

The NNRTI concentration effective at inhibiting 50% (IC_{50}) of RT activity for wildtype and mutant enzymes was measured by incubating 25 nM wildtype or mutant RT, 600 nM rA-dT template-primer, 25 mM Tris, 10 mM $MgCl_2$ and 25 µM [^{3}H]-TTP at 37°C for 20 minutes in the presence and absence of varying drug concentrations. The reactions were stopped with the addition of streptavidin coated scintillation beads (GE Healthcare, Piscataway, NJ USA) diluted in EDTA. Reaction mixtures and beads were agitated for thirty minutes at room temperature and plates were read on a MicroBeta TriLux Scinitllation Counter (Perkin Elmer, Shelton, CT).

3.3.2 Statistical Analyses

Student's *t*-test was used to assess the statistical significance of differences in IC_{50} values between mutant and wild type virus or protein. P-values less than or equal to 0.01 were considered to be statistically significant. Hypersusceptibility was defined by two criteria: IC_{50} values significantly less than wildtype ($P < 0.01$) and fold change values of < 0.4.

3.4 RESULTS

3.4.1 Role of mutations 118I, 208Y, and 215Y in HIV-1 Efavirenz hypersusceptibility

To assess the role of mutations at RT codons 118, 208, and 215 in NNRTI HS, mutant recombinant viruses encoding each of these mutations alone and in combination (combinations of mutations are shown in Figure 14) were produced and assayed initially for susceptibility to EFV, the most widely used NNRTI. Table 1 shows the mean EFV IC_{50} values for each of the mutants, the fold-change (FC) in IC_{50} compared to wildtype, and the statistical significance of the difference in mean IC_{50} values. The single mutants V118I, H208Y, and T215Y did not show significantly increased susceptibility to EFV, having FC values of 0.58, 0.55, and 0.70, respectively. The H208Y/T215Y double mutant showed HS to EFV with a mean IC_{50} value of 0.39 ± 0.2 (FC of 0.27; P = 0.0007). By contrast, the V118I/T215Y mutant was not HS to EFV with a mean IC_{50} value of 1.04 ± 0.61 nM and FC of 0.71 (P = 0.73). The triple mutant containing V118I/H208Y/T215Y showed the greatest HS to EFV of the isolates tested, with a mean IC_{50} value of 0.29 ± 0.18 nM and FC of 0.20 (P= 0.00001). Testing the same site-directed mutants in a pNL43 background gave similar results (Table 7, Appendix).

Table 1. Viral susceptibility to Efavirenz

Mean IC_{50} (nM) ± Standard Deviation (fold-change)			
HIV_{LAI}	Efavirenz		P-value
WT	1.46 ± 0.29		
118I	0.85 ± 0.25	(0.58)	0.01
208Y	0.81 ± 0.24	(0.55)	0.001
215Y	1.02 ± 0.27	(0.70)	0.12
118I/215Y	1.04 ± 0.61	(0.71)	0.73
208Y/215Y	0.39 ± 0.20	(0.27)*	0.0007
118I/208Y/215Y	0.29 ± 0.18	(0.20)*	0.00001

Mean IC_{50} ± standard deviation from at least three independent experiments. Fold-change comparing mutant and WT IC_{50}. Single cycle assay in P4/R5 cells was used to determine drug susceptibility. Specifically, 2 fold drug dilutions were added in triplicate to P4/R5 cells. Viral replication in the presence and absence of drug was detected 48 h post-infection; relative light unit values were determined using a luminometer. The Students *t*-test was used to assess statistical significance. P-values less than or equal to 0.01 were considered statistically significant. *Indicates hypersusceptibility (Fold change < 0.4 and $P < 0.01$).

3.4.2 Role of mutations 118I, 208Y, and 215Y in HIV-1 Delavirdine Susceptibility

We next determined the susceptibility of the mutants to delavirdine (DLV) another FDA-approved NNRTI (Table 2). None of the single mutants were hypersusceptible to DLV. The H208Y/215Y double mutant was HS to both DLV with FC values of 0.19 ($P \leq 0.001$), as was the triple V118I/H208Y/T215Y mutant with a FC value of 0.14 ($P < 0.001$). Although the V118I/T215Y double mutant was not HS to EFV (mean FC 0.71, $P = 0.73$), it was HS to DLV with a mean FC of 0.25 ($P = 0.002$).

Table 2. Viral Susceptibility to Delavirdine

Mean IC_{50} ± Standard Deviation (fold-change)			
HIV_{LAI}	Delavirdine		P-value
WT	63.3 ± 19		
118I	36.5 ± 23	(0.58)	0.02
208Y	52.3 ± 19	(0.83)	0.3
215Y	39.5 ± 6.0	(0.62)	0.02
118I/215Y	15.5 ± 3.9	(0.25)*	0.002
208Y/215Y	12.3 ± 4.3	(0.19)*	0.0004
118I/208Y/215Y	8.90 ± 0.6	(0.14)*	0.0002

Mean IC_{50} ± standard deviation from at least three independent experiments. Fold change compared to WT. Single cycle assay in P4/R5 cells were used to determine drug susceptibility. Specifically, 2 fold drug dilutions were added in triplicate to P4/R5 cells. Viral replication in the presence and absence of drug was detected 48 h post-infection; relative light unit values were determined using a luminometer. The Student's *t*-test was used to assess statistical significance. P-values less than or equal to 0.01 were considered statistically significant. *Indicates hypersusceptibility (Fold change < 0.4 and $P < 0.01$).

3.4.3 Role of mutations 118I, 208Y, and 215Y in HIV-1 Nevirapine Susceptibility

Finally, the susceptibility of the mutants to the FDA approved NNRTI nevirapine (NVP) was determined (Table 3). None of the single mutants were hypersusceptible to NVP. Although both V118I and H208Y had approximately 0.55 fold changes in NVP susceptibility, these susceptibility increases were not statistically significant. The H208Y/215Y double mutant was HS to NVP with a FC value of 0.25 ($P \leq 0.001$), as was the triple V118I/H208Y/T215Y mutant with a FC value of 0.16 ($P < 0.001$). Interestingly, the V118I/T215Y double mutant was also hypersusceptible to NVP with a FC of 0.34 ($P = 0.003$).

Table 3. Viral Susceptibility to Nevirapine

Mean IC_{50} ± Standard Deviation (fold-change)			
HIV_{LAI}	Nevirapine		P-value
WT	85.5 ± 27		
118I	48.1 ± 17	(0.56)	0.016
208Y	48.5 ± 13	(0.57)	0.016
215Y	60.1 ± 29	(0.70)	0.170
118I/215Y	29.2 ± 12	(0.34)*	0.003
208Y/215Y	21.6 ± 8.5	(0.25)*	0.001
118I/208Y/215Y	13.3 ± 8.8	(0.16)*	0.0005

Mean IC_{50} ± standard deviation from at least three independent experiments. Fold change compared to WT. Single cycle assay in P4/R5 cells were used to determine drug susceptibility. Specifically, 2 fold drug dilutions were added in triplicate to P4/R5 cells. Viral replication in the presence and absence of drug was detected 48 h post-infection; relative light unit values were determined using a luminometer. The Student's *t*-test was used to assess statistical significance. P-values less than or equal to 0.01 were considered statistically significant. *Indicates hypersusceptibility (Fold change < 0.4 and $P < 0.01$)

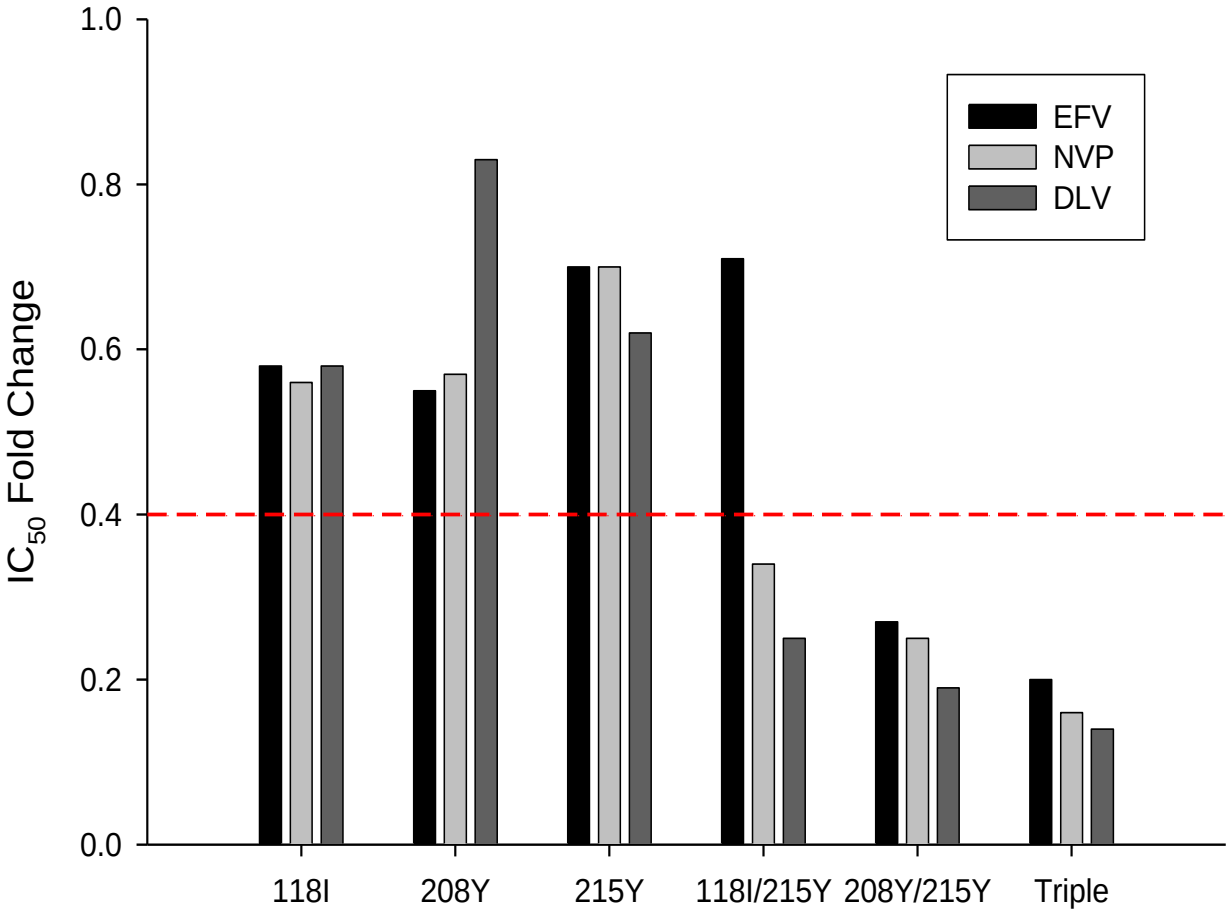

Figure 15. Summary of HIV-1 Susceptibility to Non-nucleoside RT Inhibitors

The dotted red line indicates a 0.4 fold change in drug susceptibility. All bars below the dotted line specify hypersusceptibility to the respective drug.

3.4.4 Susceptibility of HIV-1 Mutants to NRTI

Although mutations V118I, H208Y, and T215Y have been implicated in decreased susceptibility of HIV-1 to NRTI in the context of additional mutations (such as thymidine analog mutations or TAMs), the individual contribution of these mutations to NRTI resistance is not clear and no studies to date have assessed the contribution of mutant combinations V118I/T215Y, H208Y/T215Y, and V118I/H208Y/T215Y to NRTI resistance. Therefore susceptibility to stavudine, tenofovir, and zidovudine was tested in single cycle replication assays. Table 4 shows that although none of the single mutants were significantly resistant to any of the NRTI tested, the H208Y single mutant did reduce susceptibility to stavudine by almost two fold and the T215Y mutant decreased susceptibility to zidovudine by three fold. All three mutant combinations showed decreased susceptibility to stavudine though only the difference in susceptibility between the triple mutant and wildtype virus was statistically significant (fold change 2.3; $P = 0.007$). All three combinations also showed decreases in susceptibility to zidovudine and tenofovir, with the V118I/T215Y combination reducing zidovudine susceptibility by almost three fold; however the differences from wildtype were not statistically significant (Table 4).

3.4.5 Susceptibility of HIV-1 Mutants to non-RT Inhibitors

To further demonstrate that hypersusceptibility due to mutations V118I, H208Y, and T215Y is NNRTI specific, we tested the susceptibility of these mutants to inhibitors that exert their effects on other areas of the viral life cycle. Four inhibitors were tested; ritonavir, lopinavir, 118-d-24, and T-20. Ritonavir and lopinavir are FDA approved protease inhibitors,

while T-20 is an FDA approved fusion/entry inhibitor. 118-d-24 is an azido-containing diketo-acid derivative that disrupts the strand transfer reaction of HIV integrase (Svarovskaia, Barr et al. 2004). Table 5 shows that all three mutants exhibited wildtype susceptibility to the integrase and entry inhibitors. H208Y/T215Y had wildtype susceptibility to both protease inhibitors and V118I/T215Y had increased susceptibility to ritonavir, though it did not meet the HS cut-offs. However, the triple mutant was hypersusceptible to the protease inhibitor ritonavir (FC = 0.1, $P < 0.0001$) but not lopinavir (FC = 1.3, $P = 0.2$) (Table 5).

Table 4. Viral NRTI Susceptibility

Mean IC_{50} (μM) ± Standard Deviation (FC)						
HIV_{LAI}	**Stavudine**	P-value	**Tenofovir**	P-value	**Zidovudine**	P-value
WT	4.98 ± 1.90		3.04 ± 1		0.44 ± .27	
118I	5.41 ± 1.90 (1.1)	0.69	2.07 ± 0.39 (0.7)	0.05	0.27 ± 0.15 (0.6)	0.44
208Y	9.37 ± 3.97 (1.9)	0.15	2.42 ± 0.97 (0.8)	0.11	0.31 ± 0.19 (0.7)	0.57
215Y	4.35 ± 0.36 (0.9)	0.17	5.48 ± 1.80 (1.8)	0.36	1.31 ± 0.45 (3.0)	0.02
208/215	7.99 ± 1.70 (1.6)	0.11	3.22 ± 0.40 (1.1)	0.79	0.74 ± 0.33 (1.7)	0.29
118/215	8.23 ± 1.20 (1.7)	0.07	3.77 ± 0.60 (1.2)	0.36	1.28 ± 0.71 (2.9)	0.13
118/208/215	14.3 ± 3.10 (2.3)[#]	0.007	5.49 ± 0.20 (1.8)	0.19	0.71 ± 0.41 (1.6)	0.39

Means ± standard deviation from at least three independent experiments. Fold change (FC) compared to WT. Single cycle assay in P4/R5 cells were used to determine drug susceptibility. Specifically, 2 fold drug dilutions were added in triplicate to P4/R5 cells. Viral replication in the presence and absence of drug was detected 48 h post-infection; relative light unit values were determined using a luminometer. Inhibiton concentrations calculated as stated in Materials and Methods. The Student's *t*-test was used to assess statistical significance. [#] P-values less than or equal to 0.01 were considered statistically significant.

Table 5. Non-RT Inhibitor Susceptibility

Mean IC_{50} ± Standard Deviation (FC)								
HIV_{LAI}	Ritonavir	P-value	Lopinavir (nM)	P-value	118-d-24 (µM)	P-value	T-20 (µg/mL)	P-value
WT	73.9 ± 8.3		7.00 ± 3.7		20.7 ± 5.8		0.57 ± 0.3	
118I/215Y	38.5 ± 9.5 (0.5)	0.02	8.29 ± 3.2 (1.2)	0.2	21.8 ± 8.8 (1.1)	0.9	0.57 ± 0.2 (1.0)	0.4
208Y/215Y	62.6 ± 11 (0.9)	0.2	7.36 ± 4.2 (1.1)	0.2	24.2 ± 12 (1.2)	0.8	0.35 ± 0.1 (0.6)	0.7
118I/208Y/215Y	7.63 ± 4.5 (0.1)*	< 0.0001	8.84 ± 4.5 (1.3)	0.2	22.3 ± 1.3 (1.1)	0.7	0.48 ± 0.2 (0.8)	0.3

Values reported as means ± standard deviation (SD) from at least three independent experiments with fold-change (FC) in parenthesis. Integrase inhibitor (118-d-24) and entry inhibitor (T-20) susceptibility were determined in P4/R5 cells as described above. Protease susceptibility was determined using a multiple cycle assay in MT-2 cells (see Materials and Methods for details). The Student's *t*-test was used to assess statistical significance between wildtype (WT) and mutant IC_{50} values. P-values less than or equal to 0.01 were considered statistically significant. * denotes hypersusceptibility.

3.4.1 Enzyme NNRTI Susceptibility

We determined that HIV-1 viruses harboring RT mutations V118I/T215Y, H208Y/T215Y, and V118I/H208Y/T215Y were hypersusceptible to NNRTI. Resistance mutations in reverse transcriptase that decrease viral susceptibility often alter enzyme structure and/or function consequently eliciting the same drug susceptibility phenotype in purified enzymes that is seen in virus. Therefore the susceptibility of purified recombinant RT enzymes to efavirenz, nevirapine, and delavirdine was evaluated. Purified recombinant wildtype and mutant RT enzymes were incubated with $MgCl_2$, rA/dT, and [^{3}H]-TTP in the presence and absence of drug, and polymerase activity was measured by incorporation of ^{3}H into the DNA chain. Previous studies show that enzymes with the E138K mutation exhibited low level resistance (2-5 fold) to all NNRTIs in therapeutic use (hivdb.standford.edu; (Sato, Hammond et al. 2006) and were therefore used as a control for the detection of small differences in drug susceptibility. Table 6 shows the susceptibility of each mutant RT combination to efavirenz, nevirapine, and delavirdine. Enzyme V118I/T215Y was hypersusceptible to efavirenz and nevirapine with fold changes of 0.2 (P = 0.001), and 0.2 (P =0.002) respectively. Susceptibility to delavirdine was increased compared to wildtype (fold-change 0.2; P =0.04) however the P-value did not meet the specified cut-off of < 0.01. Interestingly, neither H208Y/T215Y nor V118I/H208Y/T215Y was hypersusceptible to delavirdine, nevirapine, or efavirenz. As expected E138K showed low-level decreases in NNRTI susceptibility. The lack of NNRTI hypersusceptibility of purified recombinant mutant proteins containing H208Y + T215Y while V118I/T215Y enzymes showed increased susceptibility to all NNRTI tested provides evidence of multiple mechanisms of NNRTI HS depending on the mutation profile.

Table 6. NNRTI Susceptibility of Purified Enzymes

	Mean $IC_{50} \pm$ Standard Deviation (Fold-Change)					
RT	**Efavirenz (nM)**	P-value	**Delavirdine (μM)**	P-value	**Nevirapine (μM)**	P-value
WT	34.3 ± 11		3.48 ± 1.1		5.04 ± 0.2	
118I/215Y	7.24 ± 4.9 (0.2)**	0.001	0.78 ± 0.2 (0.2)*	0.04	0.74 ± 0.15 (0.2)**	0.002
208Y/215Y	27.8 ± 6.0 (0.8)	0.2	2.35 ± 0.9 (0.7)	0.13	3.52 ± 1.3 (0.7)	0.253
118I/208Y/215Y	17.6 ± 3.4 (0.5)	0.1	2.82 ± 0.8 (0.8)	0.55	4.66 ± 0.08 (0.9)	0.136
138K	43.8 ± 4.4 (1.3)	0.36	6.83 ± 1.8 (2.0)	0.14	26.9 ± 15 (5.3)	0.17

Values represent mean ± standard deviation from at least three independent experiments. Scintillation proximity assay used to determine RT susceptibility to NNRTI. Briefly, RT added in duplicate to 96 well plates followed by varying concentrations of inhibitor and master mix (rA/dT, ^{3}HTTP, $MgCl_{2,}$ KCl). Reactions were incubated for 30 minutes at 37ºC followed by incubation with streptavidin scintillation beads for 1.5 hrs. Plates were read on a liquid scintillation counter. Student's *t*-test was used to determine statistical significance. $^{**}P < 0.01$ (hypersusceptible), $^{*}P < 0.05$. Interestingly, only the V118I/T215Y RT was hypersusceptible to NNRTI.

3.5 DISCUSSION

Our prior analyses of a large genotype-phenotype database identified V118I, H208Y, and T215Y to be strongly associated with NNRTI HS. It was not clear from these analyses, however, whether the mutations were genetic markers or causal of the NNRTI HS phenotype. Through our current analysis of site-directed mutants we provide the first report of mutations at codons 118, 208 and 215 causing NNRTI HS. We found that the V118I and H208Y single mutant viruses showed significantly increased EFV susceptibility, but the T215Y mutant did not. No single mutants were HS to NVP or DLV. Both $HIV_{H208Y/T215Y}$ and HIV_{Triple} mutant viruses were HS to all three NNRTI, whereas $HIV_{V118I/T215Y}$ was HS to DLV and NVP but not EFV.

Of note, mutations V118I and H208Y are not included as drug resistance mutations listed by the International AIDS Society panel, and are sometimes considered polymorphisms. However, a study of 344 plasma samples from HIV infected people analyzed by genotypic resistance testing showed that V118I was never found in the treatment naïve population and was present in 30% of those patients previously treated with antiretroviral therapy (Delaugerre, Mouroux et al. 2001). In addition, a study of the prevalence of H208Y that examined 6352 genotypic resistance tests linked to a clinical database showed that H208Y was present in 0.3% of treatment naïve samples and 4.1% in treatment experienced samples (Nebbia, Sabin et al. 2007). Emergence of both V118I and H208Y were strongly associated with other NRTI mutations, especially TAMS (Delaugerre, Mouroux et al. 2001; Nebbia, Sabin et al. 2007). In our analysis of the contribution of these mutations to NRTI susceptibility we found that the

H208Y and T215Y single mutants reduced susceptibility to stavudine (d4T) and zidovudine (AZT) by 2 and 3 fold respectively. The single V118I mutant had no affect on the susceptibility of d4T, TNF, or AZT. When assessing the contribution of the mutations in combination to NRTI susceptibility, all three mutants showed reduced susceptibility to the three inhibitors tested. This data provides further evidence that mutations V118I and H208Y reduce NRTI susceptibility when combined with other mutations and these mutations should possibly be included in resistance mutation lists/panels.

The origin of drug resistance due to mutations selected in the presence of drug can often be pinned on amino acid changes that alter protein structure and function. For example, the lysine to arginine at residue 103 in RT decreases NNRTI susceptibility by creating an additional hydrogen bond with the tyrosine at residue 188 that keeps the entrance to the pocket closed, making it more difficult for NNRTIs to enter the pocket causing a barrier to enzyme drug binding. In order to determine if mutations V118I, H208Y, and T215Y have similar phenotypic effects on the enzyme as were seen in the virus we made site directed mutants in a protein expression vector to obtain purified recombinant proteins. When RT containing V118I/T215Y, H208Y/T215Y, and V118I/H208Y/T215Y were assessed for NNRTI susceptibility we found that both enzymes containing H208Y and T215Y were not NNRTI HS. In contrast, the V118I/T215Y mutant was HS to efavirenz and nevirapine, and had increased susceptibility to delavirdine (Table 6).

Interestingly, the V118I/H208Y/T215Y mutant was hypersusceptible to the protease inhibitor ritonavir but not lopinavir (Table 5). Studies of protease hypersusceptibility show that many viral isolates with increased susceptibility to ritonavir have low replication capacity (Martinez-Picado, Wrin et al. 2005). Interestingly, although ritonavir hypersusceptibility was

associated with low replication capacity, lopinavir susceptibility had the lowest correlation with replication capacity (Martinez-Picado, Wrin et al. 2005) possibly explaining the divergent results for the two protease inhibitors. It is possible that the V118I/H208Y/T215Y mutant is severely replication deficient, in this instance ritonavir but not lopinavir susceptibility would be affected. Notably, only 2% (9 of 444) of the viral isolates in our prior study had all three mutations, while combinations H208Y/T215Y and V118I/T215Y were present in 6% and 12% of the isolates tested, respectively (Shulman et al 2004). The low frequency of the triple mutant in that patient population may be due to impaired viral fitness. The replication capacity for the triple mutant may be defective and requires further investigation (see Chapter 2).

The mechanistic role of each of the mutations studied in NNRTI HS is uncertain. The V118I mutation has been reported to decrease ATP-catalyzed excision of chain terminators and to increase the selectivity of RT for normal substrate (Girouard, Diallo et al. 2003). The H208Y mutation confers low-level resistance to the pyrophosphate analog foscarnet (Mellors, Bazmi et al. 1995) and is commonly associated with mutations at residue 215 (Shulman, Bosch et al. 2004). The T215Y mutation has been proposed to interact favorably with the adenine ring of ATP, improving the ability of RT to catalyze ATP-mediated excision of chain terminators (Boyer, Sarafianos et al. 2001). How these mutations interact to cause HS is unknown and will require further investigation. Our findings have facilitated this investigation by defining key mutations and combinations of mutations involved. The variable effects of the mutations on HS at both the viral and enzyme levels suggest that more than one mechanism is likely to be involved in HS. Investigation of the basis for NNRTI HS caused by the V118I, H208Y, and T215Y mutations are described further in chapter two.

4.0 CHAPTER 2. DECREASED VIRION ASSOCIATED REVERSE TRANSCRIPTASE LEVELS CAUSED BY MUTATIONS H208Y + T215Y INCREASE HIV-1 SUSCEPTIBILITY TO NNRTI

PREFACE

The replication capacity studies in this chapter are adapted from a published study (Clark SA[1], Shulman NS[2], Bosch RJ[3], and Mellors JW[1]. 2006. Reverse Transcriptase Mutations 118I, 208Y, and 215Y Cause HIV-1 Hypersusceptibility to Non-nucleoside Reverse Transcriptase Inhibitors. AIDS 20:981-984) reprinted with permission from Lippincott Williams & Wilkins Publishing.

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The remaining sections are being prepared for journal submission. The work described in this chapter is in fulfillment of specific aim two.

4.1 ABSTRACT

Previous analysis of a large clinical genotype-phenotype dataset identified three NRTI mutations (118I, 208Y, and 215Y) statistically correlated with NNRTI hypersusceptibility (HS). In chapter one we showed that site-directed mutant HIV-1 containing combinations of the three NRTI mutations V118I, H208Y, and T215Y elicited varying degrees of NNRTI hypersusceptibility. Specifically, single mutations moderately increased NNRTI susceptibility, with double and triple mutants exhibiting more dramatic increases in susceptibility. However, the mechanism by which these mutations in RT led to NNRTI HS was not defined. The goal of this study was to investigate the impact that these mutations have on the ability of the virus to replicate and package proteins in the absence of drug, providing mechanistic insights in to the cause of NNRTI HS. Single cycle replication assays revealed reduced replication capacity in H208Y/T215Y and V118I/H208Y/T215Y mutant viruses (40% and 35% of wildtype, respectively) while the V118I/T215Y mutant virus replicated as efficiently as the wildtype virus. Western blot analysis showed significant reductions in virion associated HIV-1 RT in H208Y/T215Y and V118I/H208Y/T215Y mutant viruses compared to wildtype virus (47% and 30% of wildtype, respectively). The RT content in the V118I/T215Y virus was similar to the wildtype virus. Decreases in quantifiable viral RT and virion associated polymerase activity were found for the H208Y/T215Y and V118I/H208Y/T215Y mutants while V118I/T215Y had viral RT activity similar to wild type. Therefore we conclude that NNRTI HS of viruses containing both NRTI mutations H208Y and T215Y is due to decreased levels of virion RT, however the level of virion associated RT for the V118I/T215Y mutant was the same as wildtype, suggesting an alternate mechanism of HIV-1 hypersusceptibility to NNRTI for V118I/T215Y mutants.

4.2 INTRODUCTION

HIV-1 reverse transcriptase (RT) lacks formal proof reading activity leading to high error rates, with estimated mutation rates of 5 X 10^{-4} to 3 X 10^{-5} per nucleotide base per cycle of replication (Preston, Poiesz et al. 1988; Robertson, Sharp et al. 1995). Together with a high mutation rate, rapid replication of HIV *in vivo* produces a large viral population carrying pre-therapy mutations (Coffin 1995). Combining the high error rate and large viral population with drug pressure often leads to the preferential growth of viruses containing drug resistance mutations. Mutations selected by drug pressure frequently decrease viral enzyme function, diminishing overall viral fitness.

The methionine to valine change at position 184 (M184V) of RT is an example of a drug resistance mutation that diminishes overall viral fitness. M184V arises in the presence of the NRTI 3TC pressure conferring up to 1000 fold drug resistance in cell culture and *in vivo* compared to wildtype 3TC susceptibility (Boucher, Cammack et al. 1993; Gao, Gu et al. 1993; Schinazi, Lloyd et al. 1993; Ait-Khaled, Rakik et al. 2003). M184 is part of the highly conserved YMDD motif in the catalytic core of RT and mutations in this region drastically alter enzyme function and viral replication (Back, Nijhuis et al. 1996; Feng and Anderson 1999; Dykes and Demeter 2007).

Replication of HIV-1 is dependent on the ability of RT to properly convert the viral RNA genome in to dsDNA for incorporation into host DNA. RT is a product of the *pol* gene which is translated as a GagPol fusion protein due to a -1 ribosomal frame shift and requires proteolytic processing to form mature heterodimeric RT (Jacks, Power et al. 1988; Pettit, Simsic et al. 1991; Pettit, Clemente et al. 2005). This ribosomal frameshift occurs infrequently to maintain a 20:1 ratio of Gag:GagPol (Jacks, Power et al. 1988). GagPol is incorporated into assembling particles

through interactions within the capsid (CA) domains of Gag and GagPol (Srinivasakumar, Hammarskjold et al. 1995; Huang and Martin 1997; Hill, Tachedjian et al. 2005). Defects at any of these steps could compromise viral particle assembly, incorporation, and/or processing leading to decreases in viral replication and the amount of virion associated RT.

We previously found that nucleoside reverse transcriptase inhibitor (NRTI) mutations V118I, H208Y, and T215Y introduced as single mutations in viral RT did not significantly alter viral susceptibility to the FDA approved non-nucleoside reverse transcriptase inhibitors (NNRTI) nevirapine, delavirdine, and efavirenz (see Chapter 1, (Clark, Shulman et al. 2006)). However, introduction of various combinations of these mutations (including V118I/T215Y; H208Y/T215Y; and V118I/ H208Y/T215Y) resulted in dramatic increases in viral NNRTI susceptibility. Here, we investigated virological factors including replication capacity, protein packaging, and RT activity that may contribute to the increased NNRTI susceptibility caused by combining these NRTI-induced mutations.

4.3 MATERIALS AND METHODS

4.3.1 Cells

The P4/R5 reporter cell line (provided by Ned Landau, Salk Institute, LaJolla, CA) is a HeLa cell line stably transfected to express a Tat-activated β-galactosidase gene under the control of an HIV-1 LTR promoter. P4/R5 cells were cultured in Dulbecco's Modified Eagle Medium- phenol red free (DMEM-PRF) supplemented with 10% FBS (HyClone, Logan, UT),

50 IU/mL penicillin, 0.5 µg/mL puromycin (Clonetech, Palo Alto, CA) and 50 µg/mL streptomycin (from here on referred to as D10). MT-2 cells (AIDS Research and Reference Reagent Program National Institute of Allergy and Infectious Disease, National Institutes of Health) were cultured in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum, 10 mM Hepes buffer, 50 IU/mL penicillin, and 50 µg/mL streptomycin (from here on referred to as R10).

4.3.2 Generation of Recombinant HIV-1

Virus was made as previously described in Chapter 1. Briefly, wildtype or HIV-1 plasmids containing mutations V118I/T215Y, H208Y/T215Y, and V118I/H208Y/T215Y were transfected by electroporating (BIO-RAD Gene Pulser ®, Hercules, CA) 5-10 µg of proviral DNA into MT-2 cells (AIDS Research and Reference Reagent Program, NIAID, NIH). Culture supernatants were harvested seven days post-transfection and stored at -80°C. The genotype of harvested virus was confirmed by extracting RNA from virions (QIAamp kit, QIAGEN, Valencia, CA), treating the extract with DNase I (Roche, Indianapolis, IN), amplifying the RT coding region using RT-PCR, purifying the PCR product (Wizard PCR Purification System, Promega, Madison, WI), and sequencing. Culture supernatants were assessed for p24 antigen content using a commercially available enzyme-linked immunosorbent assay (Alliance HIV-1 p24 ELISA kit; Perkin Elmer, Wellesley, MA).

4.3.3 Single Cycle Replication Capacity Assay

Five-thousand P4/R5 cells/well were added to a 96 well plate. Cells were infected with a standard inoculum containing 10 ng p24 antigen and incubated at 37°C, 5% CO_2 for 48 hours. Following infection, a cell lysis buffer and luminescent substrate (Gal-Screen; Tropix/Applied Biosystems) were added to each well and virus production assessed by measuring relative light units (RLU) on a luminometer (ThermoLabSystems, Waltham, MA). Values shown are the mean ± standard deviation of three independent experiments. An asterisk indicates $P < 0.01$ compared to WT (Student's *t*-test).

4.3.4 Quantification of virion associated Pol proteins

Protein composition of mutant and wildtype virus was determined by ultracentrifugation (Sorvall Thermo-Scientific Waltham, MA USA) of transfection supernatants (175,000 X g) containing 5 µg p24 antigen through a 20% sucrose cushion. Viral pellets were lysed with RIPA lysis buffer (20 mM Tris-Cl [pH 8.0] containing 120 mM NaCl, 2 mM EDTA, 0.5% [v/v] DOC, 0.5% [v/v] NP-40 as well as 2 µg/mL PMSF, 10 µg/mL apoprotein and 10 µg/mL pepstatin A) and reassessed for p24 antigen content. Protein loading buffer was then added to each sample and proteins separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE proteins were electrophoretically transferred to a PVDF membrane and blocked for two hours while shaking at room temperature with Tris-buffered saline containing Tween (T-TBS) and 5% milk. T-TBS buffer (with 2% milk) containing either mouse anti-HIV-1 RT or anti-p24 monoclonal antibodies (NIH AIDS Research and Reference

Reagent Program) were used to probe PVDF membranes for proteins of interest for two hours at room temperature while shaking. After incubation the PVDF membranes were repeatedly washed in T-TBS and a secondary HRP-conjugated goat anti-mouse antibody (NIH AIDS Research and Reference Reagent Program) was added to the membrane and incubated at room temperature for 30 minutes while shaking. Membranes were briefly incubated in the SuperPico ECL Substrate System for detection of peroxidase-labeled antibody (PIERCE, Rockford, IL, USA) and bands visualized using Versa Doc Imaging System followed by densitometric quantitation using Quantity One v4.3.0 software (BioRad, Hercules, CA). Values shown are from five independent experiments where * denotes $P < 0.01$ using Student's *t*-test.

4.3.5 Virion associated RT activity

Mutant and wildtype virus associated reverse transcriptase polymerase activity was assessed by ultracentrifugation of transfection supernatants (175,000 x g) containing 5 μg p24 through a 20% sucrose cushion. Viral pellets were lysed with non-denaturing lysis buffer (0.5% Triton X-100, 0.8 M NaCl, 20% glycerol, 50 mM Tris pH 7.8, protease inhibitor tablet, and distilled water). Lysates were added to reaction mixtures containing 60 mM Tris pH 7.8, 12 mM $MgCl_2$, 6 mM dithiothreitol, poly (rA)-oligo (dT) , [^{3}H]-TTP (Moravek Biochemicals, Brea, CA), and dH_20. Reactions were incubated for 30, 60, 120, 180, and 240 minutes at 37ºC, followed by the addition of scintillate coated streptavidin beads (Amersham Biosciences, Piscataway, NJ) in EDTA. Plates were read on a MicroBeta TriLux scintillation counter (Perkin Elmer, Shelton, CT). Values presented are the mean $\pm$ standard deviation of three independent experiments. Statistical significance was assessed using Student's *t*-test to compare mutant and wildtype polymerase activity. * denotes $P \leq 0.01$.

4.3.6 HIV protein levels in the cell

HIV_{LAI} infected MT-2 cells were pelleted, washed extensively with fresh R10 media, lysed with RIPA lysis buffer and subjected to SDS-PAGE followed by Western blot. Membranes were probed with either HIV-Ig or mouse anti-RT (8CA) monoclonal antibody (AIDS Reference and Reagent Program, National Institutes of Health) followed by appropriate HRP-conjugated secondary antibody. The mean values of three independent experiments are shown. Student's *t*- test was used to define statistical significance ($P < 0.01$) denoted by *.

4.3.7 Virion associated precursor proteins

Protein composition of mutant and wildtype virus grown in the presence of 10 µM ritonavir in R10 was determined by ultracentrifugation (175,000 x g) through a 20% sucrose cushion. Viral pellets were lysed with RIPA lysis buffer and equivalent amounts of total protein determined by Bradford assay were subjected to SDS-PAGE followed by Western blot. Membranes were probed using mouse anti-HIV-1 RT and anti-p24 monoclonal antibodies for protein identification followed by secondary HRP-conjugated goat anti-mouse antibody. Bands were visualized using Versa Doc Imaging System and quantified by densitometry.

4.4 RESULTS

4.4.1 NNRTI HS mutations H208Y + T215Y decrease viral replication capacity

We previously showed that combinations of mutations at codons 118, 208, and 215 in reverse transcriptase cause hypersusceptibility to efavirenz, delavirdine, and nevirapine (Chapter 1; Clark 06). The mechanisms by which mutations at these specific sites cause hypersusceptibility are unknown. Recently, increased drug susceptibility was linked to defects in viral replication (Huang, Gamarnik et al. 2003; Martinez-Picado, Wrin et al. 2005; McColl, Chappey et al. 2008). In order to determine if defective viral replication may play a role in NNRTI hypersusceptibility we performed replication capacity assays. Generally, viruses containing single mutations demonstrated no significant reduction in single cycle replication assays compared to wildtype (Figure 16), although HIV-1_{215Y} replicated at 80% of wildtype, this difference was not statistically significant. The V118I/T215Y mutant HIV-1 replicated as well as wildtype while the H208Y/T215Y and V118I/H208Y/T215Y mutants replicated at 42% and 35% of wildtype, respectively (Figure 16). A methionine to valine at position 184 in RT is known to diminish replication capacity (Sharma and Crumpacker 1999; Wei, Liang et al. 2002) and was used as a control for the detection of decreased replication. Similar to previous findings, replication of HIV-1 containing M184V was significantly reduced to less than 60% of wildtype virus.

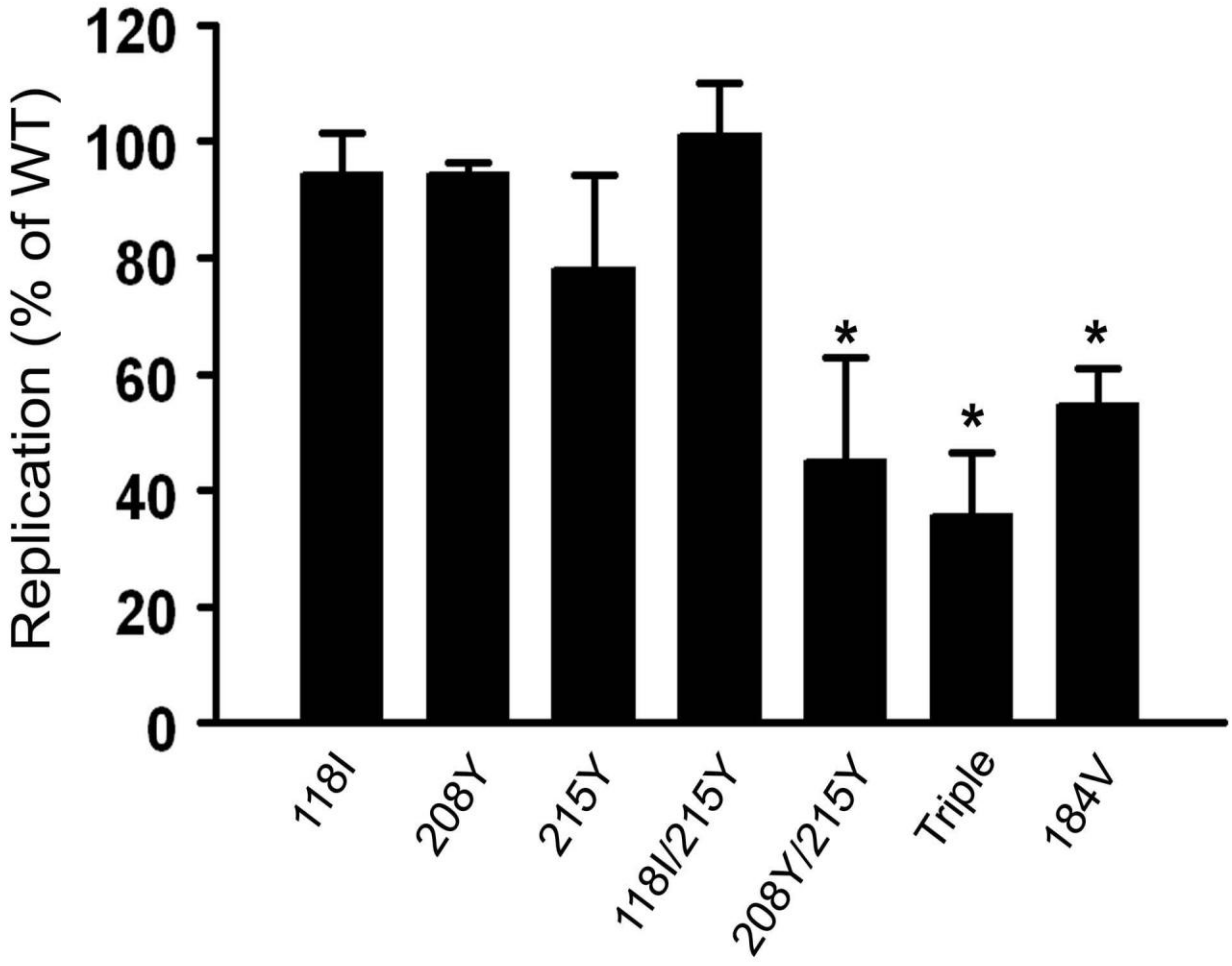

Figure 16. Single cycle replication capacity.

Briefly, P4/R5 cells were infected with a standard inoculum (10 ng p24) and incubated at 37ºC for 48 hours. Viral replication was measured as described in Materials and Methods. Triple = V118I/H208Y/T215Y. Bars represent the mean ± standard deviation of three independent experiments. An asterisk indicates $P < 0.01$ compared to wildtype (Student's *t*-test).

4.4.2 NNRTI HS mutations decrease RT packaged in virions

A recent study investigating HIV-1 particles containing different levels of RT activity suggested a relationship between the level of RT packaged in virions and NNRTI susceptibility (Ambrose, Julias et al. 2006). The decrease in replication capacity of the H208Y/T215Y and V118I/H208Y/T215Y mutants and the unaltered viral replication capacity of V118I/T215Y HIV-1 led us to explore the cause of this divergent pattern among the mutants. Therefore, we investigated whether decreased levels of RT in the virion was a possible cause of the diminished replication and increased NNRTI susceptibility for the H208Y/T215Y and V118I/H208Y/T215Y mutants. To assess RT incorporation, viral lysates were immunoblotted and probed with anti-RT monoclonal or anti-p24 antibodies (Figure 17A). RT and p24 bands were quantified by densitometry and the mean levels of viral RT corrected for lysate p24 content from five independent experiments are shown (Figure 17B). We found that the V118I/T215Y mutant had levels of RT in the virion similar to wildtype virus which correlates well with the virus maintaining wildtype levels of replication. However, the H208Y/T215Y and V118I/H208Y/T215Y mutants had significantly decreased levels of RT in the virion compared to wildtype virus (42% and 35% respectively), corresponding with decreases in replication capacity (Figures 16). In previous reports, HIV-1 containing the G190S mutation showed decreased replication capacity, diminished virion associated RT, and increased delavirdine susceptibility (Huang, Gamarnik et al. 2003), thus virus containing the G190S mutation served as a control for decreased RT packaged in the virion. In agreement with previously reported data, G190S mutants showed severe reductions in the amount of detectable virion associated RT (Figures 17A and 17B).

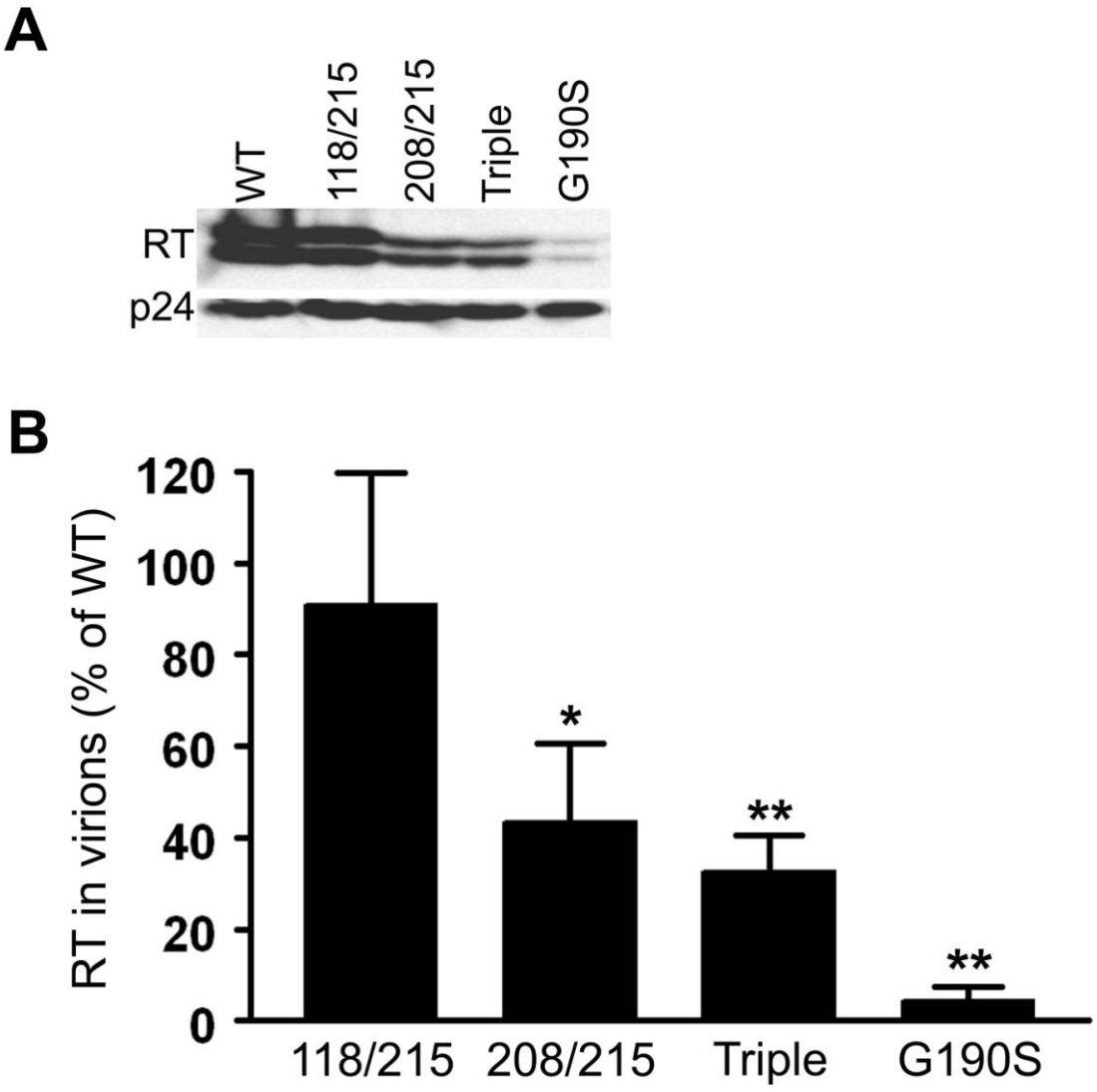

Figure 17. Reverse Transcriptase levels in mutant and wildtype virus

RT levels in viral lysates were determined as stated in Materials and Methods. (A) Representative western blot of five individual experiments. (B) Mean ± Standard Deviation (error bars) percent of wildtype RT in virions detected among the five individual experiments. * and ** denote $P < 0.01$ and $P < 0.001$ respectively, using Student's *t*-test.

4.4.3 Virion associated RT activity

The intravirion reverse transcriptase activities for the hypersusceptible mutants as well as wildtype virus were also examined (Figure 18). Due to the diminished level of RT detected in virus containing the H208Y/T215Y and V118I/H208Y/T215Y mutations it was expected that these viruses (containing less RT) would polymerize less efficiently compared to wildtype and V118I/T215Y HIV-1. Accordingly, virus containing the V118I/T215Y reverse transcriptase mutations elongated the homopolymeric poly rA-oligo dT template-primer as well as wildtype HIV-1 (Figure 18). Both the H208Y/T215Y and V118I/H208Y/T215Y virion associated enzymes polymerized 50% ($P < 0.01$) and 62% ($P < 0.01$) of wildtype, respectively (Figure 18). The G190S reverse transcriptase had significantly impaired polymerase activity, yielding approximately 30% of wildtype activity (Figure 18), consistent with previous findings.

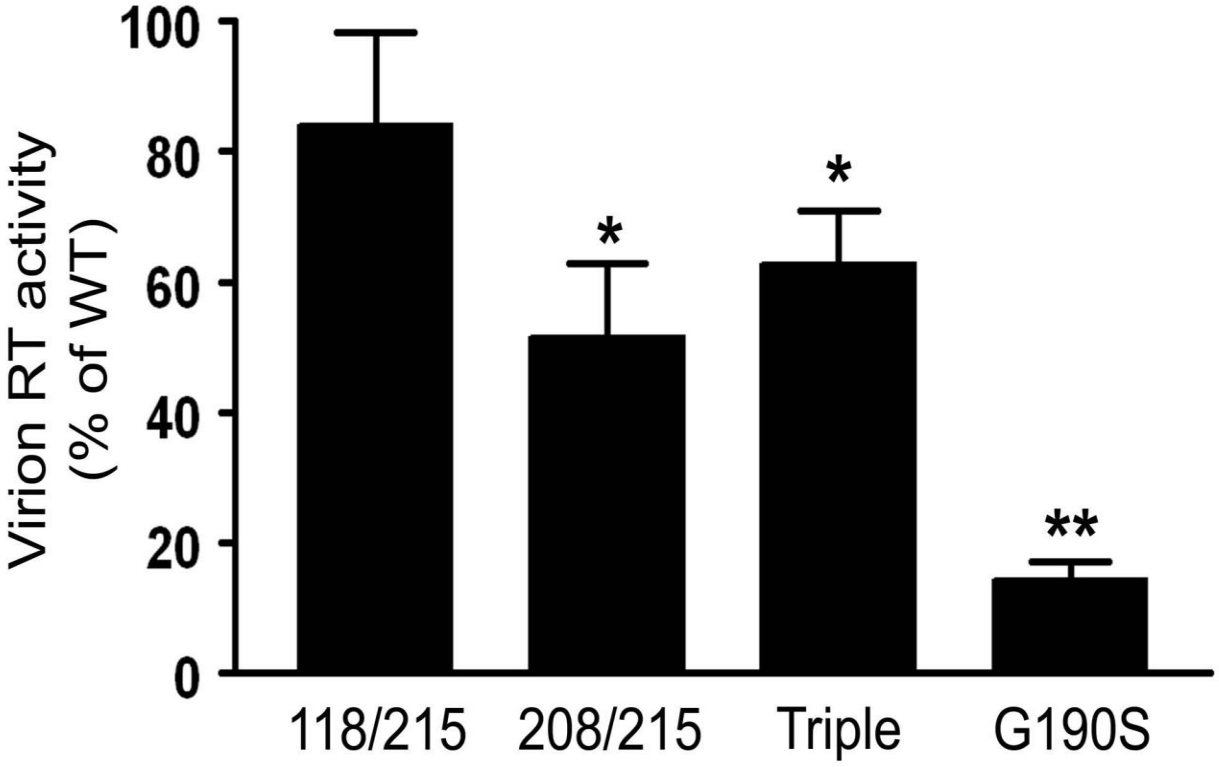

Figure 18. Crude virion associated RT activity.

Crude virion associated RT activity of mutant and wildtype virus was assessed by incubating non-denatured viral lyaste with Tris, $MgCl_2$, DTT, poly (rA)-oligo (dT), ^{3}HTTP, and dH_20 for 1 hour at 37ºC, followed by the addition of scintillate coated streptavidin beads. Plates were read on a scintillation counter. * and ** denotes $P < 0.01$ and $P < 0.001$ respectively, using Student's *t*-test. Error bars represent the standard deviation of three independent experiments.

4.4.4 Effect of hypersusceptibility mutations on HIV protein levels in the cell

A possible explanation for decreases in the amount of detectable RT for HS mutants is that the GagPol precursor is not efficiently produced. An alternative explanation is that GagPol may be degraded in the cell or mislocalized within the cell, and thus is not incorporated into virions. To investigate the former possibility for the observed decreases in virion RT levels, an analysis of cell associated HIV precursor and fully processed proteins was performed. Cell lysates infected with mutant or wildtype virus were probed with HIV-Ig or anti-RT monoclonal antibodies (mAb). Figure 15A shows there was no difference between wildtype HIV-1 or the mutants when comparing all major cell associated HIV proteins detected with HIV-Ig. Although there was a detectable band at 160 kD this position corresponds to the envelope precursor gp160, not the Pol precursor $GagPol^{160}$. When the membrane was probed specifically for RT with the anti-RT monoclonal mAb, there was no difference in the amount of p66/p51 in cell lysates normalized for p24 content when comparing infection with wildtype and V118I/T215Y HIV-1(Figure 15B and 15C). The H208Y/T215Y mutant and the triple mutant had less RT (71% and 73% respectively; Figure 15B and 15C) in cell lysates compared to wildtype.

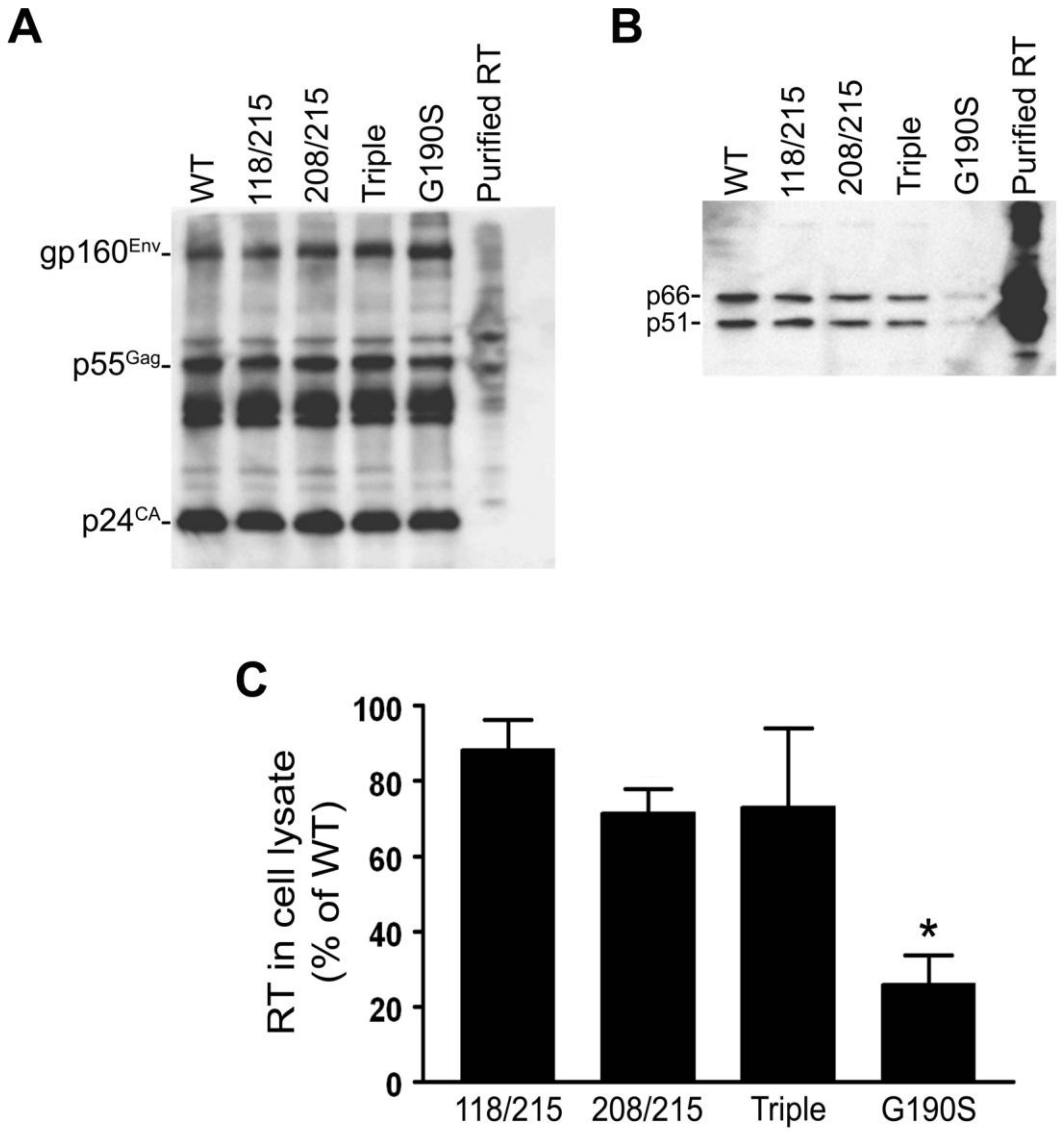

Figure 19. Cell associated HIV proteins.

HIV protein levels in the cell. Seven days post-transfection cells were washed, pelleted, and lysed. Whole cell lysate was run on an SDS-PAGE followed by western blotting. The blots were probed with either (A) HIV-Ig or (B) a monoclonal anti-RT antibody. Bands from blot B were visualized using VersaDoc Imaging system and three independent blots were quantified (C) using densitometry. Bars represent the mean of three independent experiments and error bars the standard deviation. * denotes $P \leq 0.01$.

4.4.5 Effect of mutations on viral packaging of unprocessed precursors

Another possible explanation for the diminished amount of detectable virion RT is that GagPol is made efficiently in the cell but is not incorporated into virions. To explore this hypothesis, wildtype and mutant viruses were cultured in the presence of the protease inhibitor ritonavir which inhibits processing of viral proteins by viral protease allowing visualization of unprocessed viral precursors that would normally be processed too quickly to observe. Interestingly, the levels of $GagPol^{160}$ incorporated into V118I/T215Y mutant virions were similar to wildtype (Figure 16A and 16B). The H208Y/T215Y and V118I/H208Y/T215Y mutants had 55% and 61%, respectively, of the amount of $GagPol^{160}$ in the virion compared with wildtype virus. The G190S mutant had the least amount of detectable $GagPol^{160}$ in the virion (20%) when compared to wildtype HIV-1. Decreased levels of unprocessed precursor protein per particle indicate that $GagPol^{160}$ is not as efficiently incorporated into the H208Y/T215Y, V118I/H208Y/T215Y, and G190S mutant virions when compared to wildtype.

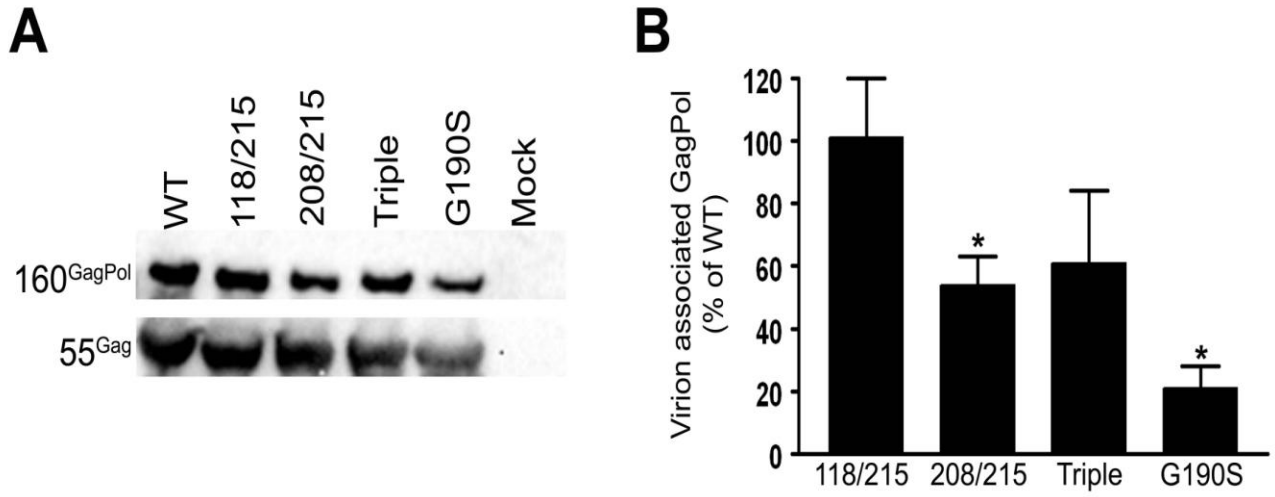

Figure 20. Accumulation of Polyprotein Precursors in Virions

Protein composition of mutant and wildtype virus grown in the presence of 10 µM RTV was determined by ultracentrifugation (175,000 X g) through a 20% sucrose cushion followed by lysis of viral pellets and subjecting equivalent amounts of total protein by Bradford assay to SDS-PAGE followed by Western blot. A) Mouse anti-HIV-1 RT or anti-p24 monoclonal antibodies were used to probe the membrane for proteins of interest followed by secondary HRP-conjugated goat anti-mouse antibody. B) Bands were visualized using Versa Doc Imaging System and quantified by densitometry. GagPol was normalized by Gag content and graphed as percent of wildtype. Shown is the mean ± standard deviation of three independent experiments. Student's *t*-test was used to determine statistical significance. * denotes $P < 0.01$.

4.5 DISCUSSION

We previously showed that viruses containing reverse transcriptase (RT) mutations V118I/T215Y, H208Y/T215Y, and V118I/H208Y/T215Y exhibit varying degrees of hypersusceptibility to the NNRTIs efavirenz, delavirdine, and nevirapine in cell based assays (Chapter 1; (Clark, Shulman et al. 2006). However, possible causes of the hypersusceptible phenotype were not examined. In this study, replication kinetics, virion and cell associated RT and GagPol protein levels, and virion associated RT activity were compared for wildtype and NNRTI HS mutants to determine causes of hypersusceptibility.

4.5.1 Replication Kinetics

Efficient replication of HIV-1 is dependent upon properly functioning viral enzymes (protease, integrase, and reverse transcriptase). Virus inhibited at any of the three enzymatic steps can reduce or abrogate production of infectious virions. Hypersusceptibility to protease inhibitors has been associated with low viral replication capacity (Ziermann, Limoli et al. 2000; Resch, Ziermann et al. 2002; Leigh Brown, Frost et al. 2004; Martinez-Picado, Wrin et al. 2005). Therefore, examining the ability of the NNRTI HS mutants to replicate was done to determine if defective replication is a factor in NNRTI HS. The single cycle assay directly measures the ability of virus to efficiently perform the early stages of the viral life cycle. In this system, an increase in relative light units is expected when the production of tat-activated β-galactosidase increases due to HIV-1 infection of the cells.

In single cycle replication assays HIV-1 containing V118I/T215Y replicated at levels equal to wildtype virus. Strikingly, the H208Y/T215Y mutant showed a dramatic reduction in single cycle replication (42% of wildtype HIV-1) which was further decreased in the presence of all three mutations (118I/208Y/215Y, 35% of wildtype; Figure 12). Our studies of viral replication capacity showed that NNRTI hypersusceptibility was not always associated with impaired replication capacity. In particular, the V118I/215Y mutant was hypersusceptible to nevirapine and delavirdine (Chapter 1, Tables 2 and 3) but replicated as well as wildtype. By contrast, the V118I/H208Y/T215Y mutant showed the greatest reduction in replication capacity and was the most hypersusceptible to NNRTI. Interestingly, H208Y/T215Y and V118I/H208Y/T215Y mutants showed significant decreases in replication capacity and were found in 5.8% and 2.0%, respectively, of the 444 patient isolates from various ACTG clinical studies tested for hypersusceptibility, while the V118I/T215Y mutant combination was detected in 12.4% of the isolates tested (Shulman, Bosch et al. 2004). Deficiencies in replication may explain why mutants carrying the H208Y+T215Y mutations are less prevalent in NRTI experienced patients than the V118I/T215Y mutant. However, the therapeutic regimen of the patient population is also a key factor in the evolution of drug resistance mutations. Perhaps more patients were receiving drugs that were more likely to select V118I instead of H208Y. Despite similar decreases in replication capacities of the H208Y/T215Y and V118I/H208Y/T215Y mutants the H208Y/T215Y genotype was found in almost three times more patient isolates than isolates containing all three mutations. One possible explanation is that the combination of all three mutations may render the virus replication defective below a certain threshold for efficient growth. In addition, it is possible that other mutations exist in the patient isolates that may compensate for the loss of replication in the H208Y/T215Y mutants that

are not selected in mutants containing all three mutations, which could explain the higher prevalence of H208Y/T215Y versus V118I/H208Y/T215Y.

4.5.2 The effect of NRTI mutations on virion-associated RT

Although drug resistance mutations offer an advantage to viruses under the selective pressure of therapy, they are often at least partially detrimental to the viral life cycle. A range of effects on the virus have been defined for a variety of mutations or mutation profiles. Mutations in highly conserved regions of the viral genome can completely abrogate viral infectivity, virion particle formation, or enzymatic functions. For example, substituting the glycine at residue 190 in reverse transcriptase with cysteine, serine, glutamic acid, glutamine, threonine, or valine is associated with significant decreases in the viruses' ability to replicate (Huang, Gamarnik et al. 2003). Diminished replication has been associated with reduced amounts of reverse transcriptase packaged in virions (Garcia Lerma, Yamamoto et al. 1998; Bleiber, Munoz et al. 2001; Huang, Gamarnik et al. 2003; Marozsan, Fraundorf et al. 2004; Ambrose, Julias et al. 2006; Olivares, Mulky et al. 2007). Therefore we determined if NNRTI HS mutants packaged decreased amounts of RT. Again, differences among the mutants were evident based on the mutation profile. Virus containing mutations H208Y and T215Y had a significant decrease in the amount of detectable RT packaged in virions and this decrease correlated with diminished viral replication capacity. The V118I/T215Y mutant had levels of virion associated RT similar to wildtype virus, also correlating with the data obtained in replication capacity studies. Our data show that hypersusceptibility for virus containing mutations H208Y and T215Y is due to decreased virion associated reverse transcriptase and that hypersusceptibility of V118I/T215Y is due to a different mechanism.

We evaluated different causes of the reductions in RT proteins packaged into NNRTI HS virions including decreased cellular RT and decreased incorporation of GagPol into virions.

The mature protein may be unstable with increased susceptibility to proteolytic degradation. In this instance, one would expect that premature degradation would also occur in the HIV infected cell, decreasing the amount of p66/p51 detected in cell lysates. Our data show that the level of detectable RT in infected cells is similar to wildtype for V118I/T215Y, decreased for mutants containing H208Y + T215Y, and drastically decreased for G190S. The decrease in cell associated proteins for mutants containing H208Y + T215Y is not as great as the decreased RT in virions suggesting that other factors may be involved. One possibility is that the precursor proteins may not be efficiently incorporated into virions.

Lack of GagPol incorporation could be due to defects in trafficking to the cytoplasm and/or inefficient assembly at the membrane. In cells infected with wildtype virus, GagPol polyproteins are trafficked to the cell membrane via interactions within the matrix (MA) domains of Gag and GagPol (Freed, Orenstein et al. 1994; Chazal, Gay et al. 1995). The major homology region (MHR) in CA is required for efficient incorporation and oligomerization of GagPol into assembling viral particles (Trono, Feinberg et al. 1989; Hong and Boulanger 1993; Mammano, Ohagen et al. 1994). Reportedly, mutations in the CA-MHR can inhibit GagPol packaging without affecting the formation of Gag particles indicating that different sequences are involved in Gag/Gag interactions compared to Gag/GagPol interactions (Tachedjian, Moore et al. 2005). Our data show less GagPol detected in virions containing mutations H208Y + T215Y compared to wildtype, possibly indicating a defect in the interactions necessary for proper membrane trafficking and/or packaging. Therefore, we conclude that diminished levels of detectable virion associated RT are due to reduced packaging of the polyprotein precursor (Figure 21). Interestingly, both HIV-1 and murine leukemia virus (MLV) Pol proteins have been shown to be

packaged when not expressed in the context of GagPol, indicating that sequences in Pol may be important for packaging (Buchschacher, Yu et al. 1999; Cen, Niu et al. 2004) and that GagPol/Gag interactions and trafficking may be affected by amino acid changes in the Pol region. Further investigation is needed to determine if trafficking and assembly play a role in NNRTI HS.

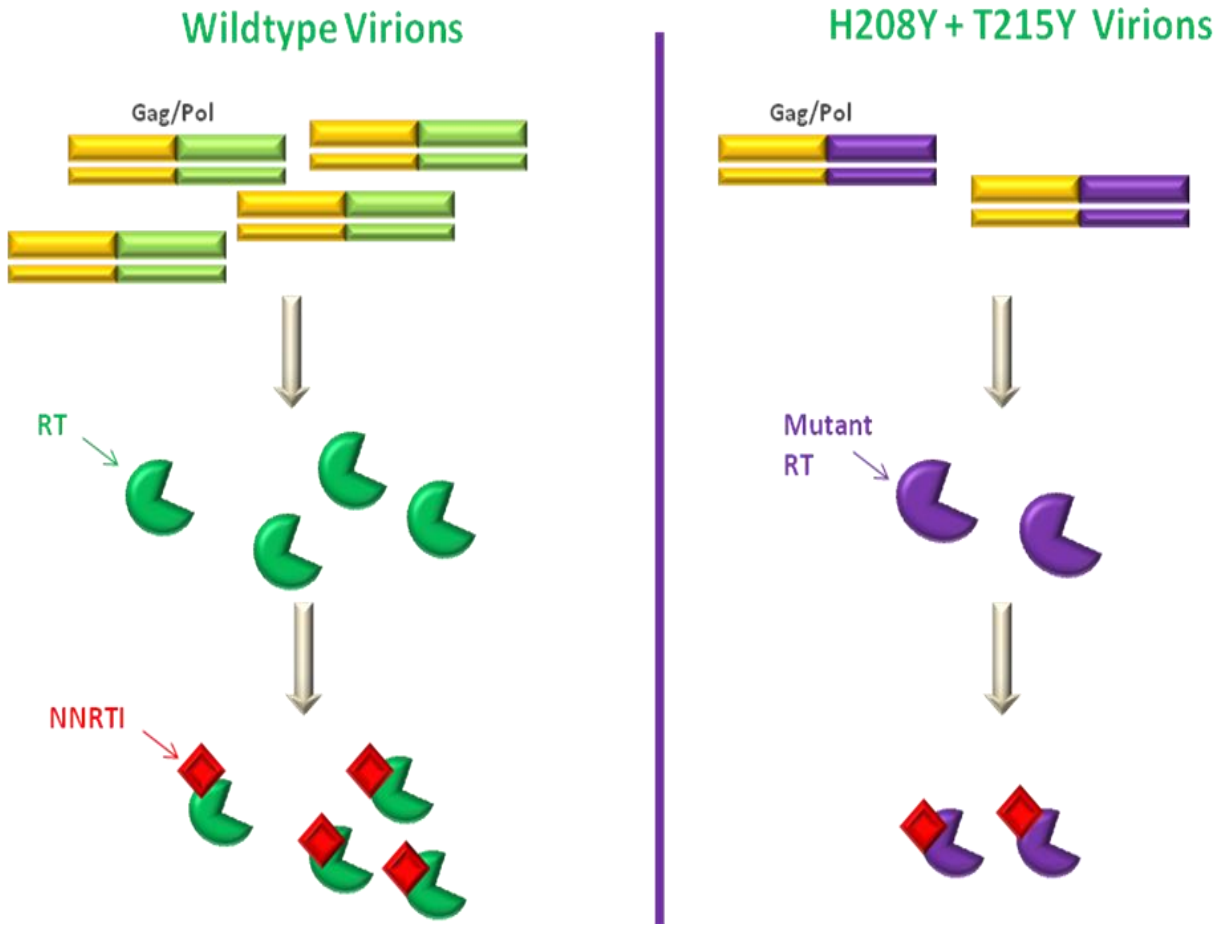

Figure 21. Cartoon summary of NNRTI HS due to mutations H208Y and T215Y

(Left panel) In wildtype virions, GagPol (yellow and green bars) is incorporated and proteolytically processed into mature RT (dark green partial circles). NNRTIs (red diamonds) bind to RT and inhibit the enzyme's ability to polymerize. (Right panel) In HS mutant virus containing RT mutations H208Y and T215Y, less GagPol (yellow and purple bars) is incorporated into virions leading to a decrease in the amount of mature RT (purple partial circles) formed. This decrease in RT reduces the amount of NNRTI needed for inhibition of reverse transcription.

4.5.3 Virion Associated RT Activity

The observation that NNRTI HS mutant viruses package less RT in virions than wildtype virus leads to questions concerning the functionality of the virion associated enzyme. Specifically, do the HS mutants with decreased virion associated RT exhibit less RT activity than wildtype or does the virus/enzyme select mutations that compensate for the decrease in protein by increasing activity? The G190S mutation (and viruses with other G190X mutations) was shown to significantly reduce virion associated RT activity (Huang, Gamarnik et al. 2003). We therefore determined if RT activity of HS mutants was altered compared to wildtype virion associated RT activity. Significant losses in RT activity were seen for the G190S mutant virus, and viruses containing H208Y + T215Y mutations polymerized less efficiently than wildtype in samples normalized for p24 content. However, both H208Y/T215Y and V118I/H208Y/T215Y virion associated enzymes made more detectable polymerase product in crude virion associated assays (Chapter 2, Figure 18) than expected considering the large decreases in virion associated protein. This finding suggests that the enzymes present in the H208Y + T215Y HS mutant viruses are functional and capable of synthesizing DNA but the low level of enzyme present is largely responsible for the replication impairment and HS phenotype.

5.0 OVERALL DISCUSSION

HIV-1 resonates around the world as one of the most significant public health issues facing the human race. The advent of therapy options to treat HIV-1 infection has considerably reduced the associated morbidity and mortality. Currently, two major classes of inhibitors (nucleoside and non-nucleoside RT inhibitors) target the reverse transcriptase enzyme due to its importance in the viral life cycle. The inhibitors drastically reduce viral load and slow disease progression, however emergence of mutations conferring resistance to the available inhibitors can increase viral load comparable to pre-therapy levels and ultimately limit therapeutic options. Interestingly, there are usually costs (or side effects) to viral fitness due to these mutations. A benefit of resistance mutations has been reported in patients that are NNRTI naïve and NRTI experienced (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002). Patient isolates containing NRTI resistance mutations have been shown to exhibit increased susceptibility to NNRTI. Often these patient isolates contain a multitude of resistance-associated mutations (Shulman, Zolopa et al. 2001; Haubrich, Kemper et al. 2002; Whitcomb, Huang et al. 2002) making it difficult to discern the contribution of specific NRTI resistance mutations to NNRTI HS. This study provided evidence that combinations of NRTI resistance mutations V118I, H208Y, and T215Y cause HIV-1 hypersusceptibility to NNRTI. Not only did our study offer evidence that mutations induced through NRTI experience significantly increase susceptibility to NNRTI, we also presented two mechanisms that can

potentially be exploited during rational drug design aimed at suppressing HIV-1 replication in patients.

NNRTI HS Mechanism 1 - H208Y + T215Y

Neither single mutation H208Y or T215Y elicited viral HS to efavirenz, nevirapine, or delavirdine. There was a statistically significant increase in efavirenz (P = 0.001) and nevirapine (P = 0.016) susceptibility conferred by the H208Y single mutant, but the fold-change in susceptibility did not meet the clinically relevant 0.4 cut-off (Tables 1-3). However, combining the two mutations on the same genome conferred viral hypersusceptibility to all NNRTI. Interestingly the addition of V118I to H208Y/T215Y considerably increased viral susceptibility to NNRTI.

We have provided evidence of the involvement of H208Y in HIV-1 NNRTI HS. We found that H208Y alone did not significantly change NRTI susceptibility and when combined with T215Y susceptibility was decreased although not significantl from wildtype HIV-1 susceptibility. However, when all three mutations V118I, H208Y, and T215Y were combined there was a significant decrease in stavudine susceptibility. This data suggests that H208Y on its own is not sufficient to cause resistance but like other known resistance mutations, such as T215Y and other TAMS; it requires the presence of other mutations to significantly alter NRTI susceptibility. Currently, H208Y is not included as a resistance mutation in the Stanford HIV Drug Resistance Database or the International AIDS Society (IAS) panel of resistance mutations. However, in addition to the *in vitro* susceptibility data from our study, there is growing clinical evidence that H208Y should be considered an NRTI resistance mutation. Recent studies have correlated the presence of H208Y with prolonged NRTI exposure (Svicher,

Sing et al. 2006; Nebbia, Sabin et al. 2007). In an analysis of 6352 genotypic resistance tests in a clinical database H208Y was prevalent in only 0.3% of a treatment naïve patient population but was present at greater than 11% in patients experienced with four or more NRTI (Nebbia, Sabin et al. 2007) . In fact, the prevalence of H208Y was higher in genotypes with M184V and TAMs than in genotypes without these mutations (P = 0.0001), possibly suggesting a role for H208Y in resistance to zidovudine and stavudine in the presence of M184V.

To our surprise, when testing the susceptibility of H208Y + T215Y viruses to non-RT inhibitors we found that V118I/H208Y/T215Y (but not H208Y/T215Y) was hypersusceptible to the protease inhibitor ritonavir, though this virus showed wildtype susceptibility to lopinavir (another protease inhibitor). Ritonavir hypersusceptibility is related to low replication capacity (Leigh Brown, Frost et al. 2004; Martinez-Picado, Wrin et al. 2005). In a large group of clinical samples lacking major protease inhibitor resistance mutations, decreased replication capacity was significantly correlated with increased susceptibility to ritonavir, however replication ability was not correlated with lopinavir susceptibility (Martinez-Picado, Wrin et al. 2005). Our study showed that virus with mutations H208Y + T215Y had considerable decreases in replication capacity (Chapter 2), with the triple mutant showing the most significant reduction in replication compared to wildtype; suggesting that the triple mutant was HS to ritonavir (and not lopinavir) due to severely compromised replication. One way to investigate this theory would be to determine PR inhibitor susceptibility of additional viruses with mutations in RT that cause severe decreases in replication capacity (*i.e.* G190S). One would expect that the significantly replication deficient viruses would also be ritonavir HS with no change in lopinavir susceptibility. Our findings of reduced viral replication for NNRTI HS mutants are important because reductions in viral replication may improve HIV-1 disease outcome.

Inefficient viral replication is associated with reduced plasma viremia, delayed emergence of resistance mutations, and improved immunological response (Coffin 1995; Petrella and Wainberg 2002). In fact it was hypothesized that a two-fold reduction in the number of productively infected cells could reflect similar increases in mean clinical latency (Coffin 1995).

Although decreased replication capacity may contribute to NNRTI hypersusceptibility, it is not the main contributing factor to this phenotype as there are other NRTI mutations that decrease replication capacity but do not cause NNRTI hypersusceptibility. For example in the 2004 statistical analysis of genetic correlates of EFV HS, M184V was not associated with EFV HS despite its deleterious affect on replication capacity (Shulman, Bosch et al. 2004; Clark, Shulman et al. 2006). Importantly in our study, loss of replication ability of NNRTI HS viruses with mutations H208Y + T215Y (with and without V118I) was attributed to significant reductions in virion associated RT (Figure 17), leading to the NNRTI HS phenotype. This finding is in agreement with previous findings in the field in which the NNRTI HS was caused by decreased virion associated RT (Huang, Gamarnik et al. 2003; Ambrose, Julias et al. 2006). However, this is the first description to my knowledge of NRTI selected mutations causing decreased levels of virion associated RT.

We also showed significant decreases in the amount of virion associated GagPol in mutants with H208Y + T215Y mutations (54% and 61% of wildtype for H208Y/T215Y and V118I/H208Y/T215Y, respectively). Reductions in viral GagPol and RT were caused by the inability of H208Y + T215Y viruses to properly package the GagPol polyprotein. Reverse transcriptase is a product of the *pol* gene whose 5' end overlaps with the *gag* gene. Pol is translated as a GagPol fusion protein due to a -1 frame shift and requires proteolytic processing

to form mature heterodimeric RT (Jacks, Power et al. 1988; Pettit, Clemente et al. 2005). Therefore, if GagPol is not properly packaged in the virion, the amount of RT in the virion would subsequently be decreased. However, the cause of decreased GagPol in virions remains undefined. Although the levels of virion associated GagPol were decreased in HIV-1 containing H208Y + T215Y mutations leading to NNRTI HS, viral susceptibility to IN and PR was largely unaltered (except the triple mutant ws ritonavir HS). One explanation for the unaltered protease and integrase susceptibility despite decreased amounts of protein may be that these inhibors are less sensitive to changes in the amount of protein present in the virion.

In our study we investigated the levels of cellular GagPol by measuring the amount of RT detectable in cell lysates, in retrospect a more prudent method would have been to directly measure the amount of GagPol in cell lysates. The levels of detectable cellular RT were similar for all mutants (except G190S which showed significant decreases), providing no further information on the cause of the decrease in virion associated GagPol.

Interestingly, enzymes containing H208Y+T215Y showed wildtype susceptibility to all NNRTI tested supporting our findings that the mechanism of HS conferred by this mutant combination is reduced virion RT and is not related to enzyme function.

NNRTI HS Mechanism 2 - V118I/T215Y

HIV-1 with a valine to isoleucine substitution at codon 118 in RT showed increased drug susceptibility to efavirenz (fold-change 0.58, $P = 0.01$), delavirdine (fold-change 0.58, $P = 0.02$), and nevirapine (fold-change 0.56, $P = 0.016$). However, as with the H208Y single mutant, the increases did not meet the clinically relevant 0.4 fold-change cut-off. Interestingly, when mutation V118I was combined with T215Y the virus was not hypersusceptible to efavirenz

(fold-change 0.71, P = 0.73) but was hypersusceptible to delavirdine (fold-change 0.25, P = 0.002) and nevirapine (fold-change 0.34, P = 0.003). The reasons for the drug dependent increases in NNRTI susceptibility remain unclear, however similar discordant patterns of NNRTI susceptibility have been observed for other resistance mutations. In fact, the NNRTI resistance mutation G190S confers high level resistance to efavirenz and nevirapine, intermediate resistance to etravirine (recently FDA approved NNRTI), and increased susceptibility to delavirdine (www.hivdb.stanford.edu). Another example of a drug resistance mutation causing discordant NNRTI susceptibility is the P236L mutation. P236L is found at the entrance of the NNRTIBP, and causes high level delavirdine resistance but does not alter the susceptibility of HIV-1 to efavirenz, nevirapine, or etravirine (Fan, Evans et al. 1995; Sarafianos, Das et al. 2004). In both examples the differences may be due to the structural variability of the inhibitors and the residues in RT with which they contact upon binding. Crystallographic studies of RT bound to various NNRTI show the diverse conformations of the drugs when complexed with RT. For instance most first-generation NNRTIs, such as nevirapine, bind in what is considered a "butterfly-like" mode, whereas delavirdine binds in an extended mode, and occupies additional pocket volume near P236 (Ding, Das et al. 1995; Ren, Esnouf et al. 1995; Esnouf, Ren et al. 1997; Sarafianos, Das et al. 2004). This additional volume may explain why a mutation at residue 236 causes high level resistance to delaviridine, but does not change susceptibility of HIV-1 to the other NNRTI. In addition, diarylpyrimidine (DAPY) drugs like etravirine and the compound rilpivirine have the ability to bind RT in more than one conformation, potentially explaining their effectiveness even in the presence of traditional NNRTI resistance mutations (Das, Clark et al. 2004; Das, Bauman et al. 2008).

The increased susceptibility caused by V118I/T215Y was not due to any of the viral parameters we investigated. Indeed, viral replication and the amount of RT packaged were

similar to wildtype for this mutation profile (Figures 12-13). In addition, virion associated RT activity was similar to wildtype for the mutant V118I/T215Y (Figure 14). Therefore another mechanism of NNRTI HS was investigated.

Drug resistance to HIV detected in cell culture systems is caused by mutations in RT that often affect enzyme susceptibility and function as well. Biochemical data typically correlates with phenotypic data obtained in cell culture. In fact, biochemical data has elucidated two major mechanisms of HIV resistance to NRTI as being that of increased discrimination between analogs and normal substrate or increased excision of therapeutic analogs. The role that biochemical investigations have played in elucidating mechanisms of resistance prompted us to investigate whether NNRTI HS mutations increased NNRTI susceptibility at the enzyme level in cell free polymerization assays. Consequently, the susceptibility of purified recombinant RT enzymes to efavirenz, nevirapine, and delavirdine was evaluated. Interestingly, purified recombinant $RT^{118I/215Y}$ showed increased susceptibility to all three NNRTI tested (Table 6). The finding that $RT^{118I/215Y}$ was HS to efavirenz was unexpected because HIV-1 with these same mutations was not efavirenz HS. Possible explanations for these divergent findings could lie within the assays used. Reportedly, HIV-1 with a methionine to valine change at RT codon 184 replicated as well as wildtype in some cell culture systems but showed moderate to severely defective polymerase activity in cell free biochemical systems (Wakefield, Jablonski et al. 1992; Back, Nijhuis et al. 1996; Feng and Anderson 1999; Feng and Anderson 1999). There are both cellular and viral factors that are present in cell culture susceptibility assays that are not present in cell free susceptibility assays that could be responsible for differences observed between the two methodologies. For example, both viral accessory proteins Vif and Tat have been implicated in optimal reverse transcription efficiency in HIV infected cells, although the mechanisms by which these proteins increase efficiency is not known (Apolloni, Hooker et al. 2003; Warrilow

and Harrich 2007). In addition, Nef deficient virions showed a reduction in reverse transcription in cells (Schwartz, Marechal et al. 1995). These and other viral factors could increase the efficiency of reverse transcription in cell culture systems and this increased efficiency would be lost in virion-free biochemical assays. Cellular factors such as a survival motor neuron (SMN)-interacting protein 1 (Gemin2) which is thought to associate with IN as part of a reverse transcriptase complex (RTC), have also been implicated in the efficient conversion of the HIV RNA genome into its DNA intermediate (Hamamoto, Nishitsuji et al. 2006).

In this report we did not define a cause of the increased NNRTI susceptibility of $RT^{V118I/T215Y}$. The major mechanism of action of NNRTI is distortion of the enzyme upon binding which interrupts enzyme function. Resistance to NNRTI is often attributed to decreased inhibitor-enzyme binding (Ren, Nichols et al. 2001; Ren, Nichols et al. 2004; Sarafianos, Das et al. 2004; Ren, Nichols et al. 2006; Ren and Stammers 2008), therefore a possible explanation for increases in enzyme susceptibility could be increased RT-NNRTI binding. However, the NNRTI HS causing mutations that we have investigated here are approximately 10 Ångstrom away from the NNRTIBP in close proximity to the polymerase active site, specifically V118I and T215Y are close to the ATP binding cleft and may therefore also affect polymerase function. A decrease in polymerase function would also result in increased NNRTI sensitivity. Imagine that a runner (wildtype RT) is asked to run a race carrying a sack that will be filled with bricks (NNRTI) until the athlete can no longer run due to the weight of the sack. Now imagine that the runner is injured (mutant RT) and is asked to run with increasing amounts of bricks until she is unable to perform. In this situation one would expect that the already injured runner would be hampered by less bricks than the runner in good physical condition. One would also expect that the injured athlete would perform worse than the non-injured athlete even in the absence of bricks. Future studies of enzyme function could shed light on NNRTI HS mediated by

V118I/T215Y. In addition, studies of other NRTI resistance mutations show altered polymerase function for the mutant enzymes compared to wildtype as a mechanism of resistance. For example K65R reportedly decreases dNTP affinity and increases processivity of the enzyme resulting in viral replication that is similar to wildtype (Arion, Borkow et al. 1996; Deval, Navarro et al. 2004). Perhaps the V118I/T215Y mutant enzyme also has defects that hinder one enzyme function necessitating the use of lower concentrations of NNRTI for effective inhibition, but increase another enzyme function resulting in no overall change in viral replication. The correlation of RT function and viral replication capacity however is complex, especially when considering the impact of these parameters on hypersusceptibility across drug class, and requires further investigation.

Overall, our findings support two distinct mechanisms of NNRTI HS. HIV-1 with mutations H208Y +T215Y have decreased virion associated RT leading to a decrease in the amount of NNRTI necessary to inhibit viral growth. The diminished levels of virion RT, caused by decreased GagPol polyprotein precursor in the virion, decreases the amount of NNRTI necessary to inhibit reverse transcription. A second, less characterized mechanism includes RT enzymes with mutations V118I/T215Y having increased susceptibility to NNRTI. Factors involved in NNRTI HS for the V118I/T215Y mutant may include defective polymerase activity, changes in RNase H activity, and/or altered enzyme drug-binding.

5.1 FUTURE DIRECTIONS

NNRTI HS in primary human cells

Examining HIV-1 containing mutations V118I, H208Y, or T215Y in human peripheral blood mononuclear cells (PBMC), which contain 33-1000 fold-less dNTP than cell lines (see Table 7), would provide more information on the ability of these mutants to replicate in the presence and absence of NNRTI. Reports of dNTP dependent polymerase activity (Back, Nijhuis et al. 1996; Giacca, Borella et al. 1996; Back and Berkhout 1997; Bouchonnet, Dam et al. 2005; Jamburuthugoda, Chugh et al. 2006; Jamburuthugoda, Santos-Velazquez et al. 2008) confirm that studies of this nature are also warranted to further substantiate the hypothesis that low dNTP concentrations result in a less replication competent V118I/T215Y virus.

In addition, replication and drug susceptibility experiments in the P4R5 and MT-2 cell lines used in this study could be done in the presence of a compound that inhibits ribonucleotide reductase, such as hydroxyurea (HU). Ribonucleotide reductases are responsible for the reduction of ribonucleotides to deoxyribonucleotides. Using HU to deplete cellular dNTP pools in the P4 cell line (similar to P4R5 cell line) led to the conclusion that the impact of resistance mutations on viral replication is more profound in cell populations characterized by small dNTP pools (Bouchonnet, Dam et al. 2005). Pre-treating cell lines with HU in a dose dependent manner would gradually reduce the cellular dNTP pool allowing comparison of decreasing levels of dNTP pools on viral replication between HS and wildtype HIV-1 within the same cell culture system used in our previous work (Chapters 1 and 2).

Enzyme kinetics

The underlying cause of increased enzyme NNRTI susceptibility in the V118I/T215Y RT and the unaltered susceptibility of the H208Y/T215Y mutant enzymes should be further

investigated. Preliminary studies of the ability of NNRTI HS RT to efficiently catalyze multiple rounds of polymerization showed decreases in polymerase activity. Differences between wildtype and HS mutant enzymes detected during multiple rounds of steady state polymerase activity should be investigated in the presence and absence of drug. Also, additional studies using a transient kinetic approach would allow closer examination of each step in the polymerization pathway (Kati, Johnson et al. 1992). Single turnover experiments would allow investigation of chemical catalysis during a single enzyme turnover. For instance, Feng and Anderson used transient kinetics to show that M184V reduced the efficiency of incorporation of dCTP and 3TC-TP during RNA dependent polymerization (Feng and Anderson 1999). Investigation of kinetic parameters such as maximum rate of nucleotide incorporation (k_{pol}), nucleotide binding affinity (K_d), and incorporation efficiency (k_{pol}/K_d) would provide further mechanistic insight into NRTI resistance mutation induced NNRTI HS.

Effect of NNRTI HS mutations on enzyme-drug binding

Although residues 118, 208, and 215 are not part of the NNRTIBP, it is possible that mutations at these residues could have long range effects on the binding of NNRTI. Preliminary studies of the binding affinity of radio-labeled delavirdine to recombinant HIV-1 RT using equilibrium dialysis showed no significant difference in the ability of wildtype and H208Y + T215Y containing enzymes to bind delavirdine. However, further analysis is necessary to definitively rule on altered RT-drug binding as a mechanism of NNRTI HS especially for the V118I/T215Y mutant RT. Additionally, the NNIBP is flexible and its shape depends on the size and structure of the bound NNRTI (Ren, Nichols et al. 2001; Ren, Nichols et al. 2004;

Sarafianos, Das et al. 2004), therefore efavirenz and nevirapine should be included in the analysis since all NNRTI are structurally distinct and have diverse interactions with RT.

Investigation of the origin of decreased virion GagPol

The origin of decreases in virion GagPol could provide insight into basic HIV virology as well as offer further insight into mechanisms of NNRTI HS. A variety of possible explanations for the decrease should be investigated such as mislocalization of the GagPol polyproteins in the cell, defective trafficking of the oligomerized polyproteins to the plasma membrane, and decreased assembly of the precursors into viral particles. Using immunofluorescence microscopy to study subcellular localization of RT showed that although HIV-1 RT can be efficiently incorporated into virus like particles (VLP) outside of the GagPol context, mislocalization of RT was common and accounted for decreases in RT incorporation into VLP (Liao, Huang et al. 2007). To determine the cellular localization, trafficking, and assembly of GagPol polyproteins, immunofluorescence experiments could be performed. In these experiments a comparison of the localization of wildtype and NNRTI HS GagPol would determine if mislocalization is responsible for decreases in the amount of precursor packaged into virions. These experiments could also determine possible trafficking defects of NNRTI HS HIV-1 by taking images of the immunofluorescently labeled proteins at different time points to visualize the movement and oligomerization of polyprotein precursors.

Etravirine

In the time that this manuscript was being prepared the HIV-1 non-nucleoside reverse transcriptase inhibitor etravirine (ETR) was approved for clinical use by the FDA. ETR is a

diarylpyrimidine (DAPY) compound that exhibits flexibility and isomerism allowing the drug to bind to RT in multiple conformations (Das, Clark et al. 2004). Interestingly, K103N (which confers high level resistance to NVP, DLV, and EFV) by itself has no effect on HIV-1 susceptibility to ETR, likely due to its ability to change conformation. It would be interesting to determine if the NNRTI HS mutations would affect HIV-1 susceptibility to ETR.

Investigation of V118I/H208Y HIV-1

In the analysis of 444 patient isolates for EFV HS, the V118I/H208Y viral variant was never found, we therefore excluded this mutant from our study. However, including this mutant in future investigations may provide helpful insight into the mechanisms of NNRTI HS and could further elucidate the role of each of these mutations in the HS phenotype. Perhaps the combination of these mutations is severely detrimental to viral replication and/or enzyme function, indicating that T215Y may compensate for such defects.

Selection of NNRTI resistance mutations in the presence of pre-existing NNRTI HS mutations

The low genetic barrier to acquiring high level resistance to DLV, NVP, and EFV can limit therapeutic options. Allowing HIV-1 containing NNRTI HS mutations to replicate in the presence of increasing concentrations of NNRTI could help determine if these mutations slow the selection of virus with NNRTI resistance mutations such as K103N that cause resistance to three of the four FDA approved inhibitors in this class.

APPENDIX – ADDITIONAL FIGURES

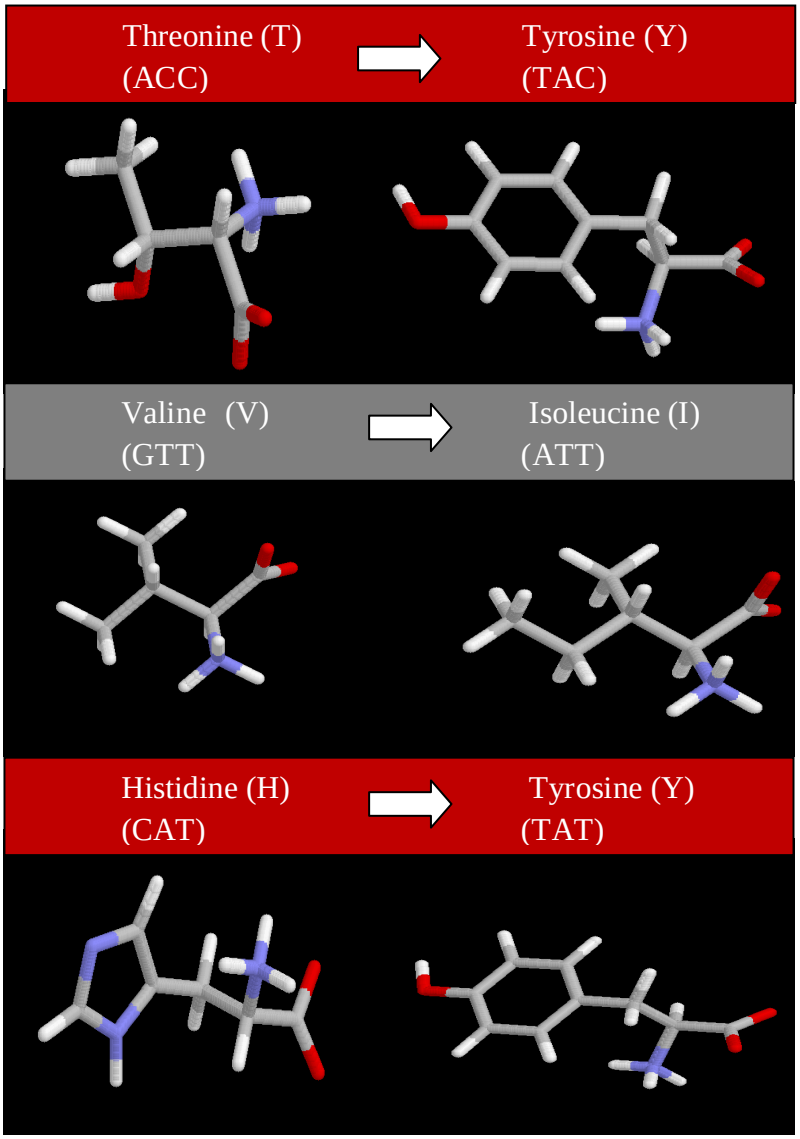

Figure 22. Amino Acid Changes in RT Associated with NNRTI HS.

Molecular depiction of the NRTI resistance mutations T215Y (top), V118I (middle), and H208Y (bottom) are shown using RasMol.

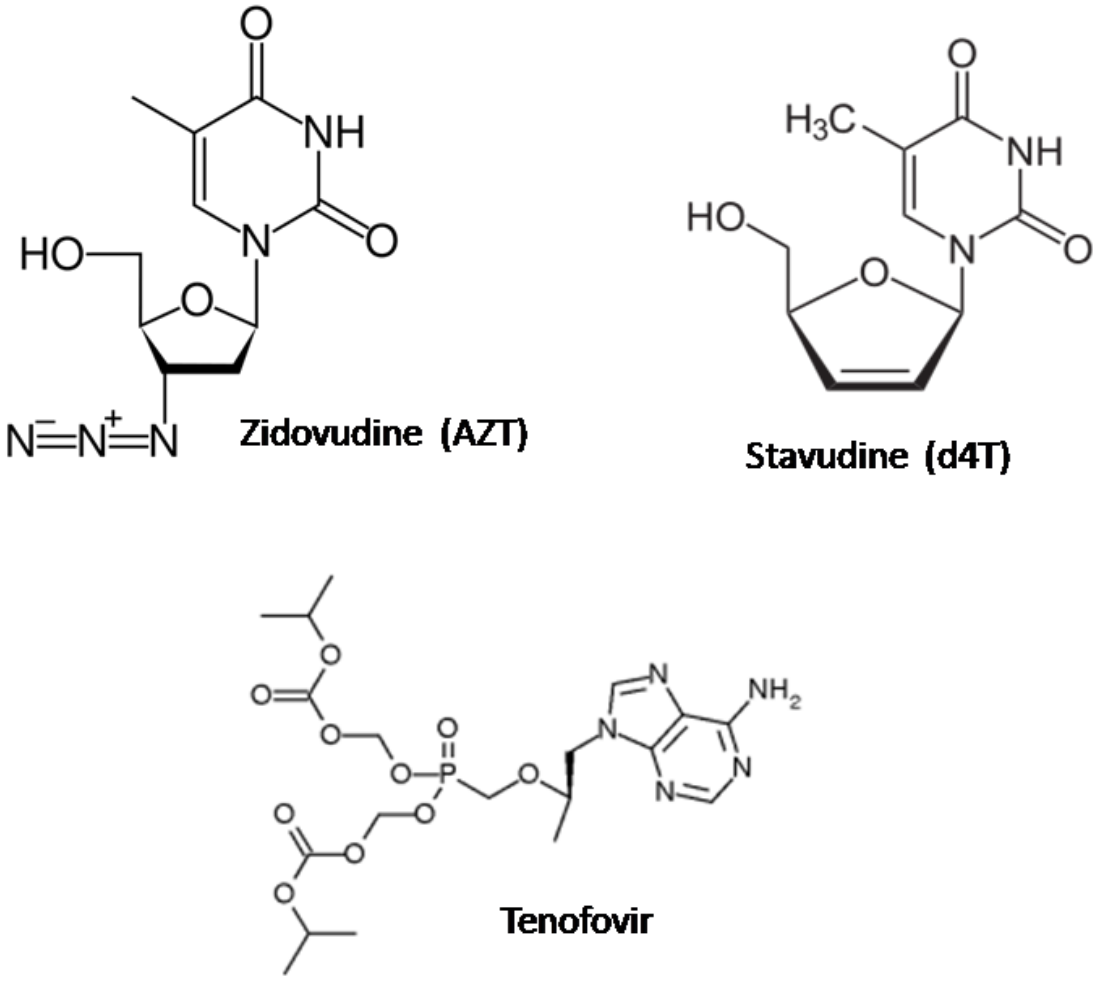

Figure 23. Structure of NRTIs used in this study.

Thymidine analogues zidovudine and stavudine along with nucleotide analogue tenofovir were used in viral drug susceptibility assays (Chapter 1).

Table 7. HIV-1_{pNL43} NNRTI Susceptibility

Fold Change in Mean IC_{50} (nM) Compared to Wildtype			
HIV_{pNL43}	Efavirenz	Nevirapine	Delavirdine
118I	ND	ND	ND
208Y	0.12	0.21	0.1
215Y	0.46	0.55	0.31
208Y/215Y	0.23	0.29	0.13
118I/215Y	ND	ND	ND
118I/208Y/215Y	0.12	0.21	0.06

Values represent the fold change in NNRTI susceptibility when comparing wildtype and mutant virus from three independent experiments using Phenosense HIV Assay (Monogram Biosciences, INC. San Francisco, CA). ND = not determined. Fold change < 0.4 indicates HS.

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