NOVEL MECHANISMS OF RESISTANCE TO HIV-1 REVERSE TRANSCRIPTASE (RT) INHIBITORS: A MOLECULAR AND CLINICAL CHARACTERIZATION OF MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF RT

by

Jessica Holly Brehm

BS, College of Charleston, 2003

Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Jessica Holly Brehm

It was defended on

June 29, 2010

and approved by

John W. Mellors, M.D.
Professor, Dissertation Director
Department of Medicine
School of Medicine, University of Pittsburgh

Phalguni Gupta, Ph.D.
Professor, Committee Member
Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Jeremy Martinson, Ph.D.
Assistant Professor, Committee Member
Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Nicolas Sluis-Cremer, Ph.D.
Assistant Professor, Committee Member
Department of Medicine
School of Medicine, University of Pittsburgh

Joanne Yeh, Ph.D.
Professor, Committee Member
Department of Structural Biology
School of Medicine, University of Pittsburgh

John W. Mellors, M.D.

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ABSTRACT

Current antiretroviral therapy (ART) has reduced morbidity and mortality from HIV-1 infection, but the long-term efficacy of ART is limited by selection of HIV-1 drug-resistant variants. Most HIV-1 drug resistance mutations that have been studied are located in the polymerase domain of HIV-1 reverse transcriptase (RT) and this region of RT is sequenced in genotyping tests used clinically to guide ART. Recently, attention has focused on the connection and RNase H domains of RT as locations of drug resistance mutations, but the prevalence, molecular mechanisms, and impact of such mutations on response to ART are uncertain. We therefore performed a series of studies to address this uncertainty, including *in vitro* selection of HIV-1 resistant to 3'-azidothymidine (AZT), drug susceptibility studies, biochemical assays and genotype analysis of clinical samples to identify and characterize resistance mutations in the RT connection and RNase H domains. From this work, we provide several lines of evidence that connection and RNase H domain mutations emerge with ART and impact nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) susceptibility. First, the connection domain mutation

A371V and the RNase H domain mutation Q509L are selected *in vitro* with AZT and confer > 50-fold AZT resistance and low-level cross resistance to lamivudine, abacavir and tenofovir when in the context of thymidine analog mutations (TAMs) in the polymerase domain of RT. Second, we show that mutation Q509L in the RNase H domain promotes dissociation of RT from RNA/DNA template/primer bound in an RNase H competent mode, thereby decreasing secondary RNase H cleavage and destruction of the template/primer. As a consequence, template/primer binds in a polymerase competent mode allowing AZT-monophosphate excision, DNA polymerization and AZT resistance. Third, the connection domain mutation A360V emerges in patients after prolonged exposure to AZT monotherapy and increases resistance to AZT in the context of 3 or more TAMs. Fourth, connection and RNase H domain mutations are not more frequent at virologic failure in HIV-1 subtype B infected patients treated with 2 NRTI plus efavirenz when failure is defined as a small increase in plasma HIV-1 RNA. However, the connection domain mutation N348I emerges frequently at virologic failure in HIV-1 subtype C infected patients in South Africa who were treated with efavirenz/lamivudine/stavudine or nevirapine/lamivudine/stavudine when virologic failure is defined as confirmed plasma HIV-1 RNA > 1,000 copies/mL. This work provides strong evidence that RT connection and RNase H domain mutations emerge in HIV-1 infected patients treated with ART and these mutations are missed with currently available genotype tests. Mutations missed by routine genotyping tests pose a potential public health threat if left undetected and transmitted to others.

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ACKNOWLEDGEMENTS

Foremost, I am grateful to have had Dr. John Mellors as my Ph.D. advisor. Over the past five and half years, he has been a teacher; an inspiration and a true mentor who has guided me to become the scientist I am today. Dr. Mellors gave me numerous opportunities to present my research at some of the most prestigious national and international conferences which provided a challenging environment to improve my verbal and writing skills. Furthermore, Dr. Mellors worked with me to live in South Africa for a year. This broadened my worldwide perspective on the HIV-1 epidemic and this was an amazing experience that I am grateful Dr. Mellors allowed me to pursue.

I also owe a special thanks to Dr. Nicolas Sluis-Cremer for taking the time to teach me about the biochemical mechanisms of HIV-1 reverse transcriptase. Dr. Sluis-Cremer and his Ph.D. students Jessica Radzio and Brian Herman were always available to have stimulating discussions in biochemistry.

At the University of Witwatersrand in Johannesburg, South Africa I want to thank Dr. Wendy Stevens and Dr. Carole Wallis for their kindness and guidance during the nine months I worked at the University. I am also grateful to Raquel Viana, Michelle Bronze, Dr. Carole Wallis and the Wallis family for helping me adapt to life in South Africa; they have become good friends.

I thank my committee members Dr. Jeremy Martinson, Dr. Phalguni Gupta and Joanne Yeh for always challenging me and providing the assistance I needed to complete a multidisciplinary Ph.D. project.

I send my gratitude to the laboratory members past and present of the Dr. John Mellors lab. Especially, Dr. Urvi Parikh who before receiving her Ph.D. mentored me as a first year doctoral student; Dianna Koontz who was always there for me when I needed an extra pair of hands to complete a project; and Dr. Shauna Clark and Jeff Meteer who were my fellow student companions and were always around to discuss research ideas and share our numerous stories about being graduate students.

Finally, I thank my classmates Jing Jin, Greg Davenport and Mike Davies who were all able to graduate within six years. I especially want to thank my friends Greg, Adam and Tait who were there for me when times became tough. I want to thank Leandro Ferra for being a part of this journey as he listened and provided encouraging advice. Most of all I want to thank my parents Wendy Grim and Tom Brehm and my brother Webster Brehm for supporting my continued determination to pursue a higher education.

1 INTRODUCTION

In 1981, severe immune deficiency was reported in four previously healthy, homosexual men who had developed *Pneunmosistis carini pneumonia* and mucosal candidiasis [1]. This led to the recognition that the epidemic of Acquired Immunodeficiency Syndrome (AIDS) had spread globally and that the cause was Human Immunodeficiency Virus type 1 [2, 3].

1.1 ORIGIN OF HIV

Human immunodeficiency viruses have been divided into two categories, type 1 (HIV-1) and type 2 (HIV-2). HIV-1 is the more prevalent, virulent virus found in persons across the globe whereas HIV-2 is less virulent and is largely confined to persons living in West Africa [4]. HIV-1 originated in chimpanzees (*Pans troglodytes troglodytes)* as simian immunodeficiency virus (SIVcpz*Ptt*) and crossed into the human population through 7 or more instances of primate to human transmission [5-8]. HIV-2 arose from transmission of SIV in *Cercocebus torquatus atys* (sooty mangabey) to humans [6, 7, 9-11].

HIV-1 is classified genetically into three groups: major (M), outlier (O) and nonmajor/nonoutlier (N). More recent work has shown that HIV-1 group M, N and O originate from three independent primate populations in Western Africa. Group M was transmitted from

SIVcpz*Ptt* apes in southwestern Cameroon; group N is more genetically related to SIVcpz*Ptt* chimpanzees in south central Cameroon [11]. By contrast, the most recent evidence links group O HIV-1 to gorillas residing in Cameroon [8].

Group M is further classified genetically into 9 different subtypes: A, B, C, D, F, G, H, J and K [12]. Genetic recombination between two HIV-1 subtypes occurs in individuals dually infected with different HIV-1 subtypes. These recombinant viruses are classified as circulating recombinant forms (CRF) or unique recombinant forms (URF) [12]. In addition, HIV-2 has subtypes A-G [7, 9, 10, 13]. The remainder of this thesis will focus on HIV-1, the cause of the global AIDS pandemic.

1.2 HIV-1 TRANSMISSION AND SPREAD

HIV-1 is transmitted by direct contact with blood or fluids from an infected person. Common routes of transmission are direct sexual contact (homosexual or heterosexual); intrapartum, perinatal, or via breast milk from mother to child; and blood transfusions or injection drug use.

In 1981 the CDC first reported HIV/AIDS as being a disease found in homosexual men [14]. However, within one year AIDS was detected in injection drug users and patients with hemophilia, who became infected through infusion of contaminated clotting factors pooled from multiple donors. Between June 1, 1981 and September 15, 1982 there were 593 reported cases of AIDS, including 243 deaths (41%) [15]. The CDC published the first case definition of AIDS in Morbidity and Mortality Weekly Report on September 24, 1982: “a disease, at least moderately predictive of a defect in cell-mediated immunity, occurring in a person with no known cause for

diminished resistance to that disease [15].” The CDC’s definition was revised in 1985 after HIV was discovered as the underlying cause of AIDS and revised again in 1987 and 1993 [16-18].

In 1985 the United States Food and Drug Administration (FDA) approved the first commercial assay to test individuals for HIV-1 infection and it became clear that HIV-1 infection had spread worldwide. By 2008 33.4 million [31.1 million-35.8 million] people were living with HIV-1; ~2.7 million people were newly infected in 2008 and ~2.0 million had died from AIDS (Figure 1) [19]. Sub-Saharan Africa is the most severely affected region of the world with 22.4 million people living with HIV in 2008 (Figure 1) [19].

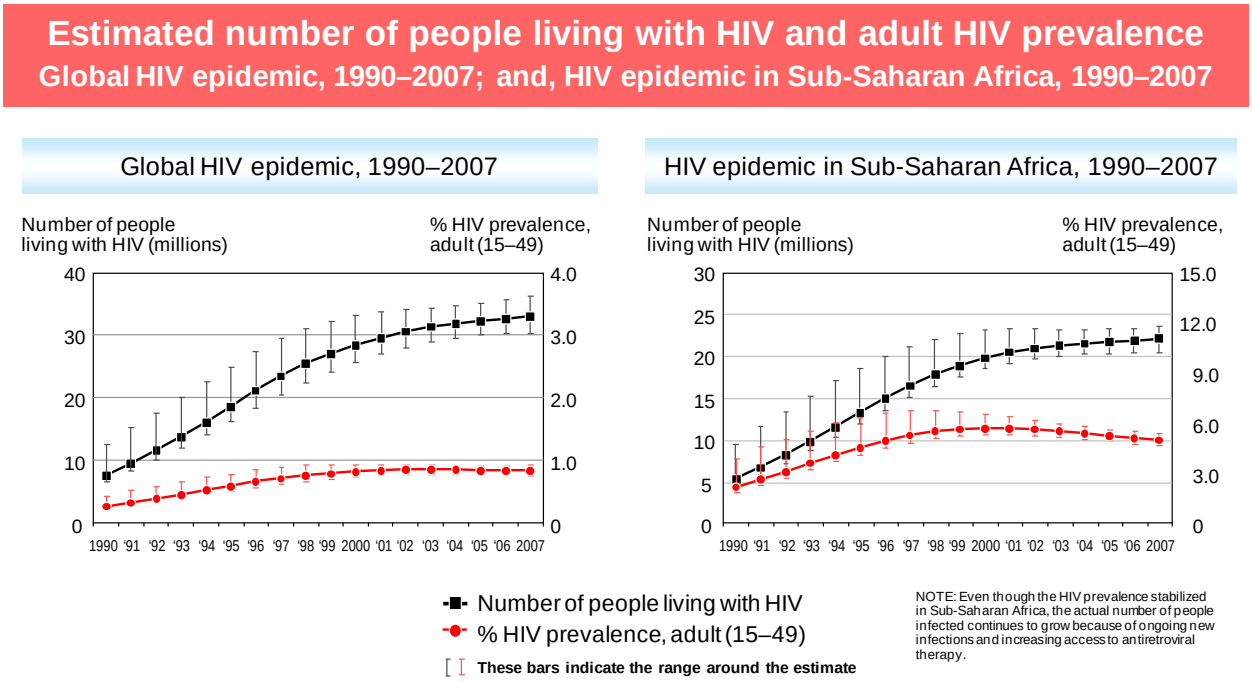


Figure 1. UNAIDS estimated number of people living with HIV and adult HIV prevalence. Global HIV epidemic and HIV epidemic in Sub-Saharan Africa, 1990-2007.
Reprint with permission from UNAIDS from 2008 Report of the Global AIDS Epidemic. 2008. UNAIDS.

1.3 PATHOGENESIS OF AIDS

The most common route for HIV-1 entry into cells is through binding of viral envelope proteins glycoprotein 120 and glycoprotein 41 to the cell surface receptor CD4 and co-receptors CCR5 or CXCR4, respectively. HIV-1 that enters using a CCR5 co-receptor are most common during the first 10 years of infection, but tropism often switches to CXCR4 (T-cell tropic) with immunodeficiency. After HIV-1 transmission, there are generally three clinical stages of disease: primary/acute infection, chronic asymptomatic infection and AIDS. Disease progression within an HIV-1 infected patient is routinely monitored by CD4 cell count [20] and/or levels of viral

RNA in plasma (viral load); HIV-1 RNA in plasma being the most accurate predictor of disease progression [21-23].

With primary HIV-1 infection, between 40%-90% of individuals experience flu-like symptoms that will persist for one to several weeks associated with rapid increases in plasma HIV-1 RNA that can be as high as >1 million copies of viral RNA/mL and a drop in CD4+ T lymphocyte (T-cell) count. The host's immune system responds to HIV-1 with a cytotoxic T lymphocyte response (CD8+ T-cells) specific for HIV infected cells, causing a reduction in viremia 10- to 100- fold, followed by partial restoration of CD4+ T-cells to greater than 600 cells/µl [24].

However, HIV-1 continues to replicate and there is a gradual decline in CD4+ T-cells. After about 8 to 10 years of asymptomatic infection, the CD4+ T-cell count drops below 200 cells/µl, symptoms appear, and the risk of opportunistic infections increases. The onset of AIDS is defined by the occurrence of opportunistic diseases such as pneumocystis jerovici pneumonia. Left untreated, once AIDS occurs, death follows on average within 18 months [24].

1.4 ANTIRETROVIRAL THERAPY (ART)

There are 25 FDA approved anti-HIV drugs in 6 different drug classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), entry/fusion inhibitors, CCR5 antagonists and integrase inhibitors. HIV cannot be cured by antiretroviral therapy (ART) because of persistent viral reservoirs; and as a

consequence, patients must continue treatment for life. Current goals are to increase patient adherence to ART to prevent emergence and spread of ART resistance.

1.4.1 History of antiretroviral therapy

Two years after the identification of HIV, Mitsuya and colleagues at the National Cancer Institute in collaboration with Burroughs-Welcome company discovered that 3'-azido-3'-deoxythymidne (AZT, zidovudine), a drug originally designed as an anti-cancer agent [25], was active against HIV *in vitro* [26]. The first clinical trials used AZT as a single compound to compare disease progression in patients treated with AZT monotherapy versus patients treated with placebo (BW 002 [27] and ACTG 016 [28]). In BW 002, patients treated with AZT monotherapy for 8 to 24 weeks had a lower frequency of opportunistic infections and reduced mortality compared to the placebo arm [27], and patients in ACTG 016 that were treated with AZT monotherapy for a median of 11 months were less likely to experience disease progression [28]. These trials provided initial proof that HIV-1 infection was treatable. In 1987, zidovudine became the first NRTI approved by the FDA for the treatment of HIV-1 infection.

However, in 1989 it was reported that mutations emerge in HIV-1 RT after prolonged AZT monotherapy that were associated with phenotypic resistance to AZT [29, 30]. Thereafter it was observed that in all studies where patients were treated with anti-HIV monotherapy, drug resistant HIV became the dominant viral population. Studies of two NRTI combinations showed delayed emergence of resistance to AZT but this was not sustained. Landmark studies of 2 NRTI plus a protease inhibitor (PI) or an NNRTI showed that HIV-1 replication could be suppressed indefinitely without the emergence of drug resistance [31, 32]. Today, the most commonly

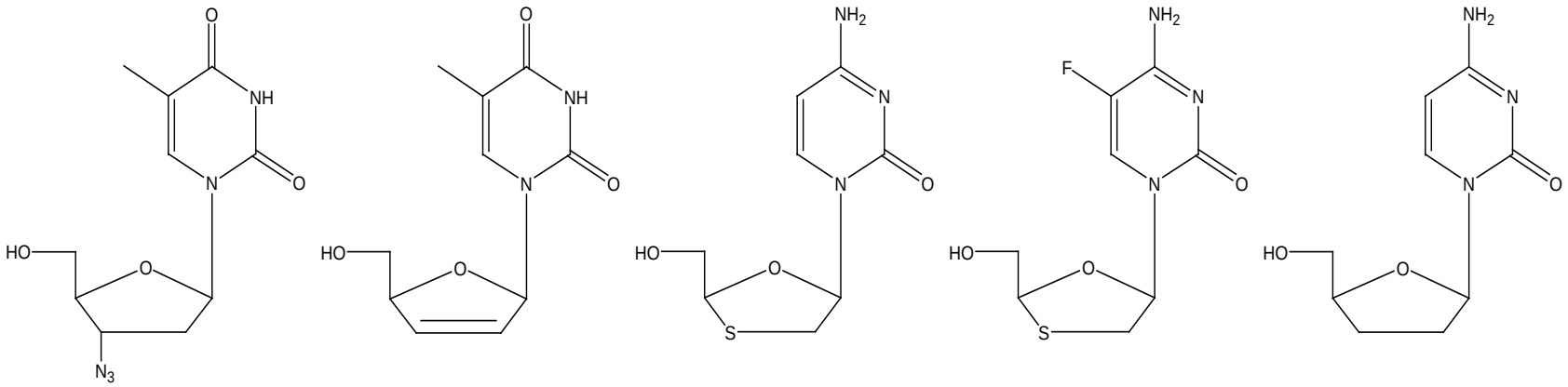
recommended regimen for HIV-1 infected patients remains 2 NRTI plus one NNRTI or a PI boosted with ritonavir [33].

1.4.2 FDA approved NRTI and NNRTI

NRTI are 2'-deoxy-nucleoside or -nucleotide analogs that lack the 3' hydroxyl (3'-OH) needed for DNA-elongation (Figure 2). NRTI are initially inactive and must be phosphorylated into nucleoside/tide triphosphates (NRTI-TP) by cellular kinases. NRTI-TP compete with natural intracellular dNTP for incorporation during HIV reverse transcription [34]. Since NRTI-TP lack the 3'-OH, once NRTI-TP are incorporated into DNA, reverse transcription is terminated and HIV is unable to replicate.

Eight NRTI were developed and approved by the FDA for treatment of HIV infection [34]. These include AZT, didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), tenofovir (TNV) disoproxil fumarate (TDF – prodrug of TNV) and emtricitabine (FTC) [Figure 2]. Zalcitabine was taken off of the market for HIV treatment in 2006 due to poor efficacy and toxicity [35, 36].

A second category of HIV therapeutics that target HIV RT is NNRTI. NNRTI are non-competitive inhibitors which bind to a pocket in reverse transcriptase (RT) formed by the drug itself. Once an NNRTI is bound, residues around the polymerase active site are repositioned and reverse transcription is blocked. FDA approved NNRTI include efavirenz (EFV), delavirdine (DLV), nevirapine (NVP) and etravirine (ETV), Figure 3 [37].



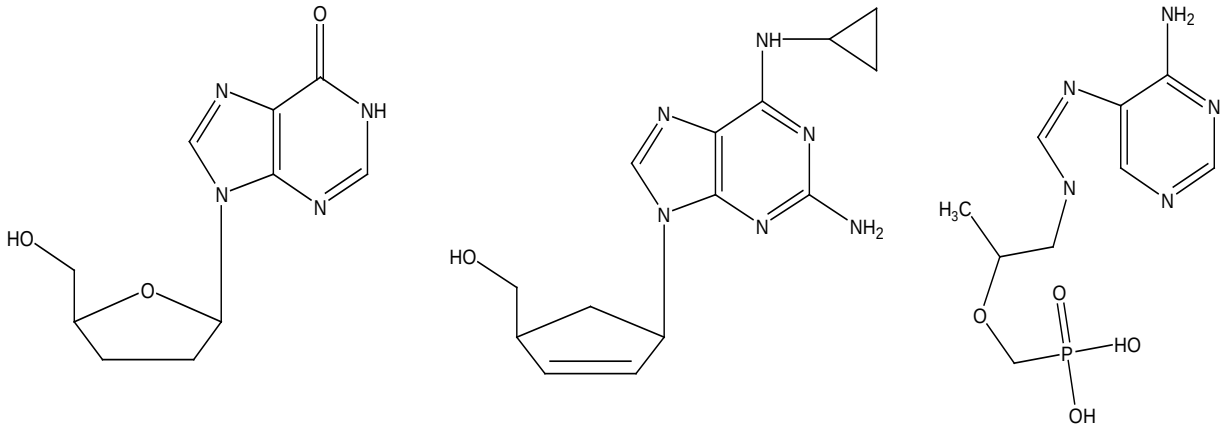
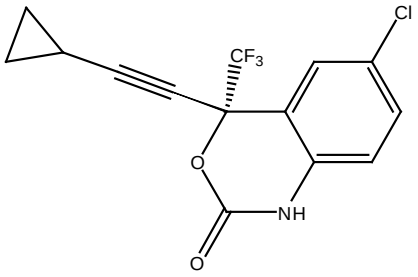
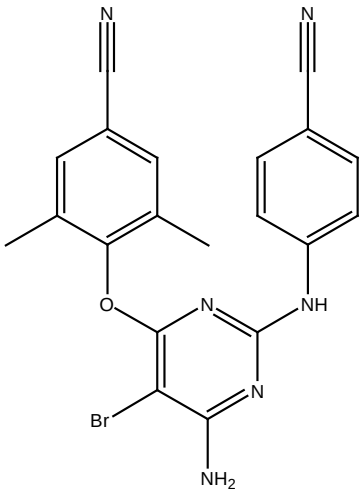


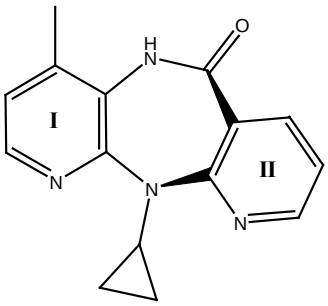
Figure 2. Structure of nucleoside/nucleotide reverse transcriptase inhibitors (NRTI).



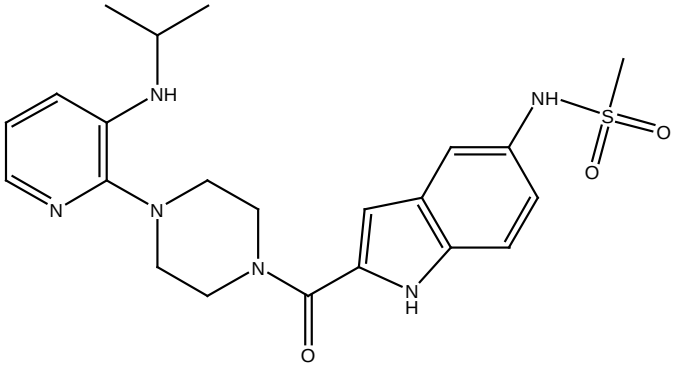
efavirenz
EFV



etravirine
ETV



nevirapine
NVP



delavirdine
DLV

Figure 3. Structure of non-nucleoside reverse transcriptase inhibitors (NNRTI).

1.4.3 FDA approved protease, entry, CCR5 antagonist and integrase inhibitors

Other approved antiretrovirals target various stages in the HIV life-cycle such as HIV entry into host cells (entry inhibitors), integration into cellular DNA (integrase inhibitors) and proteolytic cleavage of HIV Gag and Gag-Pol precursor proteins (protease inhibitors [PI]). PI approved by the FDA are atazanavir, darunavir, fosamprenavir (prodrug of amprenavir), indinavir, lopinavir, nelfinavir, saquinavir, ritonavir and tipranavir [38]. All PI except nelfinavir are recommended to be taken in combination with ritonavir; ritonavir reduces metabolism of a concomitantly administered PI improving bioavailability and half-life [38].

The recently approved entry inhibitor is enfuvirtide; CCR5 antagonist is maraviroc and integrase inhibitor is raltegravir [25].

1.4.4 Fixed dose combination regimens

Pill burden and dosing schedule are two limitations of combination ART. Fixed-dose combination regimens have been developed to increase adherence and reduce treatment failure. In 1997, the first fixed-dose combination pill, AZT+3TC (Combivir) was approved by the FDA. There are an additional five FDA approved fixed-dose combination regimens. These include lopinavir (LPV) boosted with ritonavir (LPV/r, Kaletra), ABC+3TC (Epzicom/Kivexa), TDF+FTC (Truvada), ABC+3TC+AZT (Trizivir) and TDF+FTC+EFV (Atripla) [39]. The latter combination, given as one pill once daily, has revolutionized ART.

1.4.5 ART in resource-rich countries

With the development of new treatments for HIV/AIDS, ART has been effective in decreasing AIDS mortality in the United States. Recommended first-line therapy consists of a combination of two NRTI plus one NNRTI or one PI. The recommended NRTI for first-line treatment are TDF and FTC [33]. Efavirenz is the recommended NNRTI or an FDA approved protease inhibitor boosted with ritonavir to be taken in combination with TDF/FTC. Specific regimens are not recommended for second-line treatment because treatment needs to be individualized so that it is active against resistant virus from first-line therapy [34, 40].

Current guidelines suggest that ART should start when CD4+ T-cell count drops below 350 cells/µl [40]. However, in resource-rich countries HIV-infected persons have the option to start ART even if their CD4+ T-cell count is above 350 cells/µl. There is continuing debate whether it is more beneficial to start ART at CD4+ T-cell counts above 500 cells/µl [41].

1.4.6 ART scale-up program in resource-limited countries (universal access)

Between 2000 and 2010, ART has become more affordable and accessible to HIV-infected populations in resource-limited countries. Current challenges include the number of low-cost anti-HIV drugs available to developing nations for second-line therapy and limited ability to monitor CD4+ T-cell count and plasma HIV-1 RNA.

The main goal of UNAIDS in partnership with the WHO is to provide treatment for a maximum number of HIV-infected persons in resource-limited countries and administer a standardized public health approach that can be used in all countries to administer and manage

ART [42]. WHO guidelines suggest that ART in resource-limited settings should be started once the patient's CD4+ T-cell count is below 350 cells/μl. If the country does not have resources to measure CD4+ T-cell count, treatment is to begin on the basis of clinical observation and WHO clinical staging [43, 44]. Drug selection in resource-limited countries is mainly driven by availability, affordability, methods of transport and temperature required for storage. The most widely used first-line regimen in developing nations is two NRTI (d4T/3TC or AZT/3TC) plus one NNRTI (NVP or EFV). When first-line ART failure occurs, the recommended second-line treatment is 2 NRTI plus a ritonavir-boosted protease inhibitor, although access to second-line therapy is limited.

Despite the many obstacles to providing ART in developing nations, ART scale-up has been highly successful. In 2003, UNAIDS and WHO launched the "3 by 5" initiative to provide three million people living with HIV/AIDS in low- and middle- income countries with ART by the end of 2005 [45]. With the "3 by 5" initiative acting as a catalyst, antiretroviral coverage rose from 7% in 2003 to 42% in 2008 [19]. Also in 2003, United States President George W. Bush launched the President's Emergency Plan for AIDS Relief (PEPAR) to combat the global HIV/AIDS pandemic [46]. During the initial phase of PEPFAR (2004-2008) the three main goals were to: increase supply of ART to patients in resource-poor countries, prevent new infections of HIV-1 worldwide and to care for 12 million people including 5 million orphans and vulnerable children infected with HIV-1. In 2006 the United Nations General Assembly agreed that countries should work toward universal access to ART, prevention, care and support and halt the spread of HIV by 2015 [47, 48]. More recently, United States President Barack Obama extended the PEPFAR program an additional 5 years [46].

1.5 HIV-1 REVERSE TRANSCRIPTASE

This section will focus on key concepts of HIV-1 reverse transcription and drug resistance to NRTI and NNRTI.

1.5.1 Overview and process of reverse transcription

RNA-dependent DNA-polymerase activity (reverse transcription) was first discovered in Rauscher mouse leukemia and Rous sarcoma virus particles by Baltimore and Temin in 1970 [49-51]. Reverse transcription is an essential component of the retroviral life-cycle, required to copy a viral genome before it is integrated into cellular DNA. HIV RT performs three enzymatic activities: RNA-dependent polymerization, DNA-dependent polymerization and RNase H cleavage, which are all necessary to copy single-stranded HIV RNA to double stranded DNA [24].

All retroviral RT enzymes require an RNA template and a short oligonucleotide to prime DNA synthesis. HIV is packaged with two strands of positive single-stranded RNA and cellular $tRNA^{Lys3}$. Before HIV enters a cell and/or after entry, packaged cellular $tRNA^{Lys3}$ binds to the primer binding site (PBS) located at the 5'-end of the HIV RNA genome to initiate minus-strand strong-stop DNA (-sssDNA) synthesis [24, 52, 53]. As RT copies viral RNA, a DNA/RNA hybrid is formed and RNase H activity of RT degrades the RNA template leaving single stranded DNA.

The 3' and 5' ends of an HIV genome are identical, sharing a repeated (R) region of bases. Newly formed -sssDNA will bind to the R region on the 3' end of positive strand viral RNA

(first strand transfer) and continue minus strand DNA synthesis while degrading viral RNA. RT does not degrade two purine rich sequences near the 3' end of the RNA genome known as the polypurine tract (PPT) and central PPT (cPPT). Instead, RT will bind to the PPT and cPPT to initiate second-strand DNA synthesis. Second-strand synthesis will continue until the first 18 nucleotides of $tRNA^{Lys3}$ have been copied. RT will then cleave the DNA/RNA hybrid formed by DNA/$tRNA^{Lys3}$ leaving one viral ribo-A at the 3'-end of minus strand DNA. The 5'-region of plus stranded DNA remaining after RNase H cleavage is complimentary to the PBS of on the 3'-end of minus strand DNA, facilitating a second strand transfer. After second strand transfer, both DNA strands will be elongated until a complete double stranded viral genome is formed with the same sequence on both ends, designated as the long terminal repeat (LTR).

1.5.2 Structure of RT polymerase domain

HIV RT is a heterodimeric enzyme composed of a 66 kDa (p66) catalytically active subunit and a 51kDa (p51) subunit without enzymatic activity (Figure 4) [54-57]. Both subunits are the product of HIV proteolytic cleavage of Gag-Pol polyproteins. The larger p66 subunit has been described as having 3 domains: polymerase domain (residues 1-318), connection domain (residues 319-426) and RNase H domain (residues 427-560) [54, 55]. However, some references depict the RNase H domain to begin at the proteolytic cleavage site between residues F440 and Y441 to form the p51 subunit [58]; therefore, the start of the RNase H domain will vary depending on the literature reviewed. The polymerase domain can be further described as mimicking the shape of a right hand with three subdomains: fingers (residues 1-85 and 118-155), palm (residues 86-117 and 156-236) and thumb (residues 237-318) [54, 55]. Both p66 and p51

subunits include the polymerase and connection domains; however, the smaller p51 subunit does not include the RNase H domain and is folded into a constrained conformation that serves as a scaffold for structural purposes [56, 57].

During DNA polymerization, a nucleic acid substrate fits within a DNA binding cleft formed by the polymerase and RNase H domains of p66 and connection and thumb domains of p51 to span an area of 17 to 18 nucleotides between the two active sites. Once the nucleic acid is bound, a conserved region of amino acids between the β12 and β13 hairpin in the p66 subunit helps position the 3'-OH group of the nucleic acid substrate near the polymerase active site (identified as the DNA primer grip [55]). Next, within the p66 palm subdomain three highly conserved residues D110, D185 and D186 are needed to form a divalent bond between two Mg^{2+} ions in the polymerase active site [55, 59]. Amino acids D185 and D186 are part of what is known as the YMDD motif in HIV. This is a highly conserved motif in retroviral RTs and the M is substituted with a V, L or A in other RNA-dependent polymerases [60].

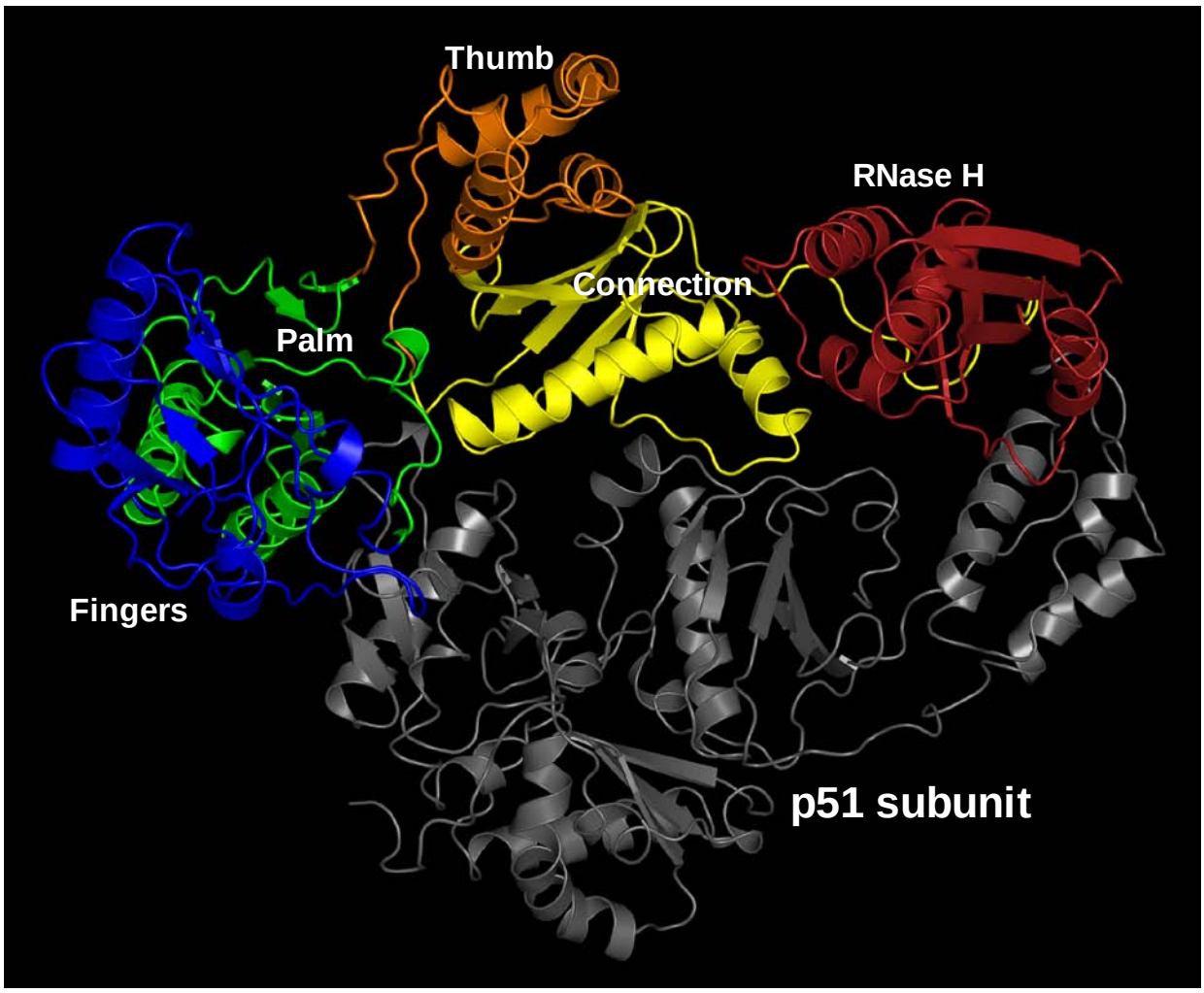


Figure 4. Structure of HIV-1 reverse transcriptase.
The fingers (blue), palm (green) and thumb (orange) subdomains are part of the polymerase domain. Connection and RNase H domains of the p66 subunit are colored yellow and red, respectively and the p51 subunit is in grey. Structure drawn using PyMOL based on coordinates from Ding *et.al.*, 1998, J Mol Biol 284: 1095; pdb access number 2HMI.

1.5.3 Detailed mechanism of DNA polymerization

During HIV RT polymerization (Figure 5), a double stranded template/primer (T/P) first binds to RT [61] in a configuration such that the 3'-OH of the primer strand is located in a binding pocket adjacent to the polymerase active site, known as the primer binding site (P site) [55]. The next incoming dNTP binds to the nucleotide binding site (N site) to form an RT-T/P-dNTP ternary complex [62]. Next, RT undergoes conformational changes such that the fingers subdomain of p66 folds over the polymerase active site to correctly align the 3'-OH of the primer strand and dNTP for catalysis.

In a two metal binding mechanism for phosphodiester bond formation, one Mg^{2+} ion binds to HIV-1 RT residue D185 and acts to lower the affinity of the 3'-OH for the hydrogen on the DNA primer strand to facilitate a 3' O^- attack on the α-phosphate of the incoming dNTP (Figure 5) [63, 64]. The second Mg^{2+} ion binds to RT residues D186 and D110 and helps with the release of the pyrophosphate group. After phosphodiester bond formation, the fingers open to release the pyrophosphate and allow translocation of the 3'-end of the nascent DNA to the P site (Figure 5) [65]. At this point, DNA polymerization will either continue by incorporating the next dNTP (processivity) or T/P will dissociate from the RT-T/P-dNTP ternary complex (dissociation) and must rebind to the same or different T/P to continue DNA polymerization. The reverse reaction to phosphodiester bond formation is phosphorolysis in which the terminal nucleotide is removed from the primer strand using ATP or pyrophosphate as a phosphate-donor [66]. However, this reaction is unfavorable but is enhanced by thymidine analog mutations that confer AZT or d4T resistance (described in more detail in the proceeding section).

Many pre-steady state and steady state biochemical studies have been used to determine the kinetics of dNTP binding and incorporation. Pre-steady state kinetics is the study of the first step in a reaction after enzyme and substrate are mixed together before products or intermediates have been formed. Pre-steady state occurs when an initial burst of product is formed. Once all enzyme-substrate complexes are generated, the rate of product formation plateaus over time representing steady-state kinetics.

During the first step of RT polymerization, RT binds to T/P with a dissociation constant in the nanomolar range [61, 66-68]. The second step occurs when an incoming dNTP binds to RT-T/P to form a ternary complex (binding [K_d] in the range of micromoles), followed by a conformational change and catalysis (k_{pol}). Pre-steady state and steady state enzyme kinetics are used to describe NRTI and NNRTI drug resistance mechanisms in the sections that follow.

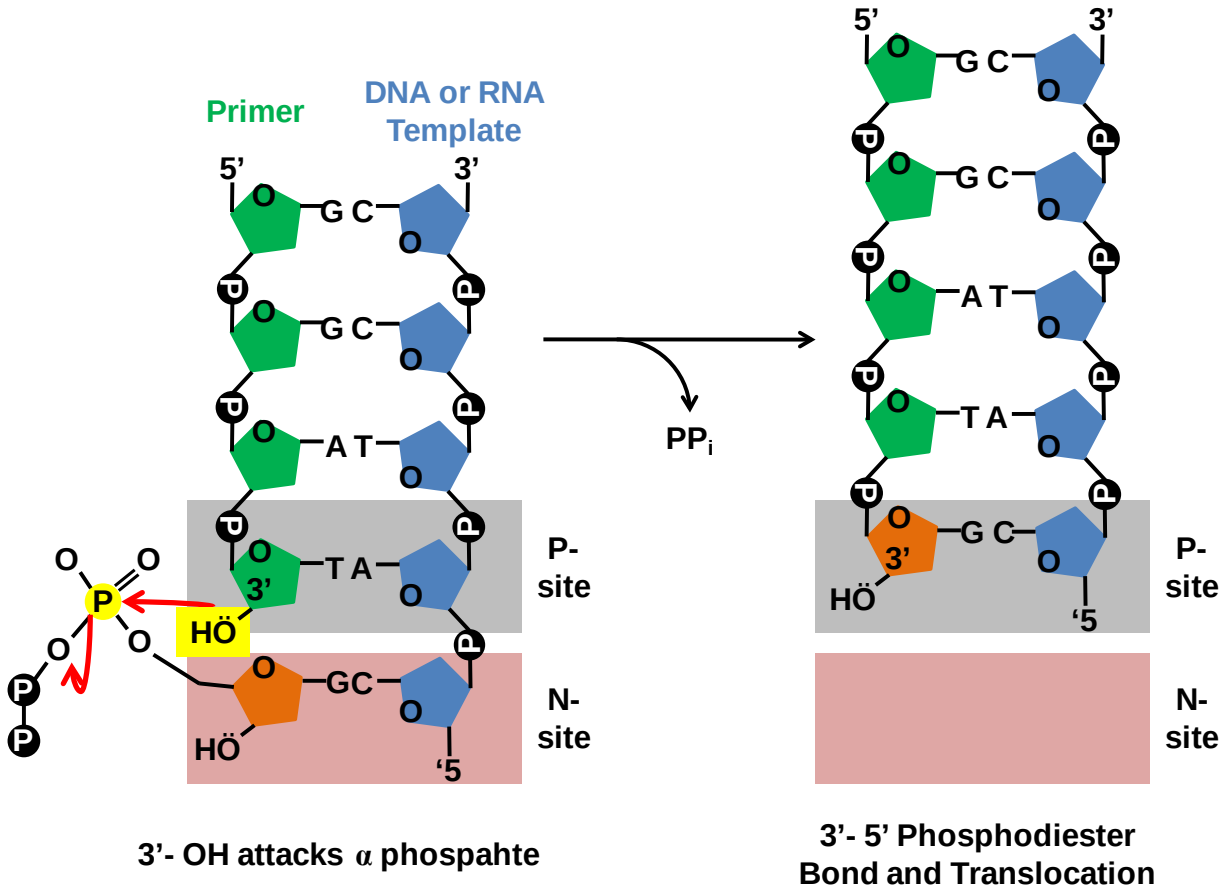


Figure 5. RT polymerization – phosphodiester bond formation.
A double stranded RNA or DNA template (blue)/ DNA primer (green) binds to RT so that the 3'-OH (yellow) of the primer strand is located in the primer binding site (P site - grey). The next incoming dNTP (orange) binds to the nucleotide binding site (N site - red) to form a ternary complex (RT-template/primer-dNTP). One Mg^{2+} ion binds to HIV-1 RT residue D185 and acts to lower the affinity of the 3'-OH (yellow) for the hydrogen on the DNA primer to facilitate a 3' O^- attack (red arrow) on the α-phosphate (yellow) of the incoming dNTP. The second Mg^{2+} ion binds to RT residues D186 and D110 and helps with the release of the pyrophosphate group (black P). After phosphodiester bond formation, the fingers open to release the pyrophosphate and allow translocation of the 3'-end (orange) of the nascent DNA to the P site.

1.5.4 RNase H activities

RNase H activity is just as important as polymerase activity in the process of HIV reverse transcription. If either polymerization or RNase H cleavage are inactivated by a mutation in the active site, HIV-1 cannot replicate [24]. The RNase H active site has several complex mechanisms for removing RNA template from the nascent DNA strand. These include 3'-directed cleavage (polymerase-dependent), 5'directed cleavage (polymerase-independent) and internal cleavage to remove residual RNA (Figure 6) [69].

Polymerase-dependent cleavage (3'-directed cleavage) occurs when RT is bound to an RNA/DNA hybrid with the 3'-OH group of the DNA primer resting in the polymerase active site. In this complex, the RNase H active site is 15-20 nucleotides away from the polymerase active site [67, 70, 71] and cleavage can occur during processive DNA polymerization at a rate of one cleavage per 100-200 bases incorporated [72]. Polymerase-dependent cleavage most commonly occurs during -sssDNA synthesis. Polymerase-independent cleavage (5'-directed cleavage) occurs when the RT polymerase domain binds to the 5' recessed end of an RNA template. In this complex, the RNA template strand will be cleaved 13-19 nucleotides from its 5' end. RNase H cleavage directed by the 5' end of the RNA template is essential for the removal of $tRNA^{Lys3}$ primer, polypurine tract (PPT) and cPPT tract during second-strand DNA synthesis [69]. The third type of RNase H cleavage reaction makes internal cuts in the RNA template with no restriction on binding by the DNA 3' or RNA 5'ends of a RNA/DNA duplex. Internal cleavage sites on an RNA template are sequence specific, guiding RT to bind to an RNA template to cleave during plus DNA strand synthesis [73].

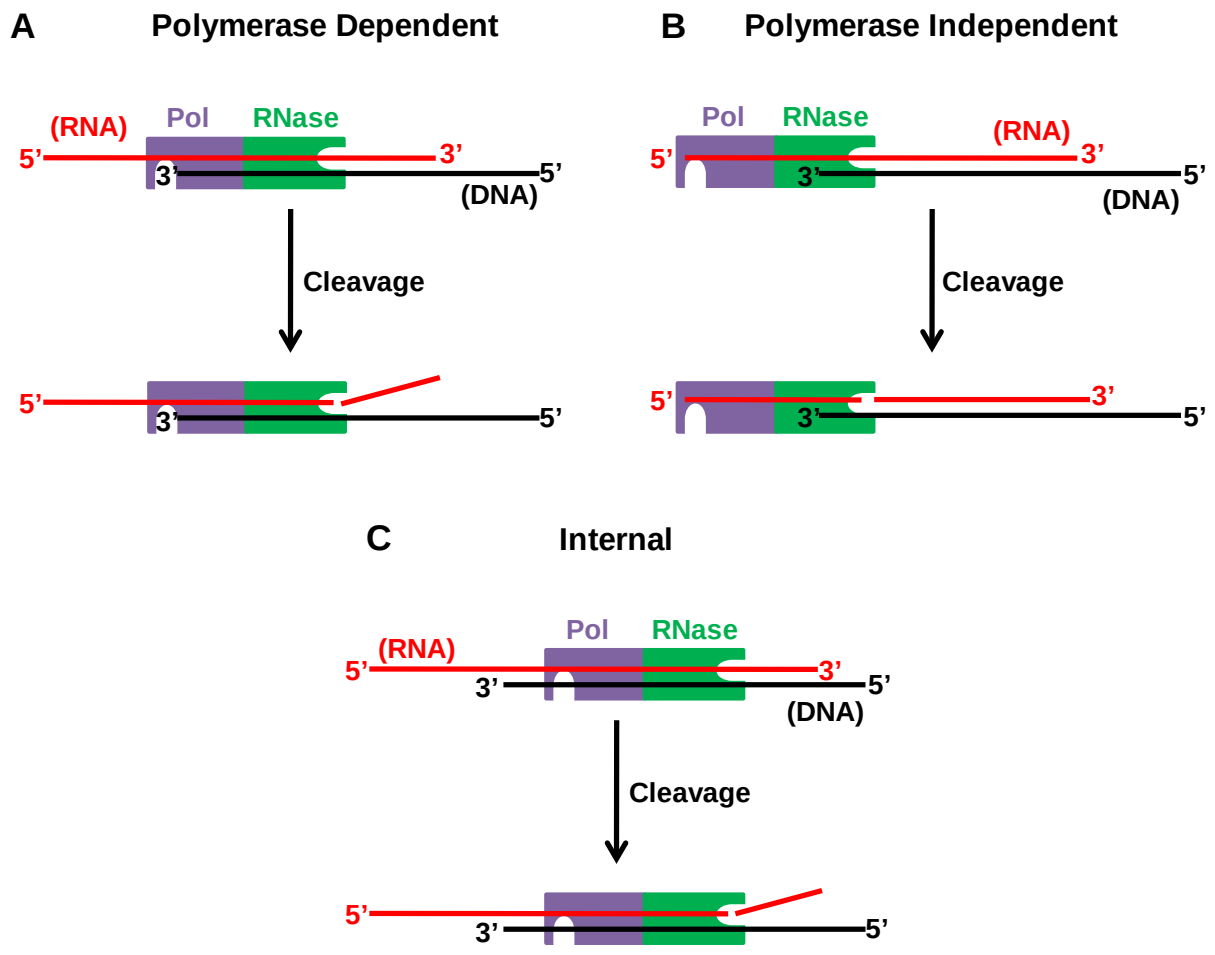


Figure 6. Mechanisms of RNase H cleavage.
(A) Polymerase Dependent (3' – DNA directed), RT binds to RNA/DNA template/primer with the polymerase active site positioned at the 3' end of the DNA primer strand and RT cleaves RNA ~18 nucleotides from the polymerase active site. (B) Polymerase Independent (5' – RNA directed), RT binds to RNA/DNA template/primer with the polymerase active site positioned at the 5' end of the RNA template and RT cleaves RNA ~18 nucleotides from the polymerase active site. (C) Internal, RT bound to RNA/DNA template/primer is sequence dependent and cleaves RNA ~18 nucleotides from the polymerase active site. RT-T/P complex formation is not dependent on 3'- or 5'- end of template or primer strand. Red line – RNA template; black line – DNA primer; purple rectangle – polymerase domain and green rectangle – RNase H domain.

1.5.5 Detailed mechanism of RNase H cleavage

To position the RNA template of an RNA/DNA hybrid in the RNase H active site, contacts are made between the DNA primer stand, RNA template strand and residues of the p51 and p66 RT subunits [74]. This region is referred to as the "RNase H primer grip." A 3.0 Å crystal structure of HIV-1 RT in complex with a HIV-1 RNA/DNA of the unique PPT region has been used to identify the highly conserved amino acids that make up the "RNase H primer grip" [74]. Residues that form contacts between the PPT and RT are K295 and E396 of the p51 subunit; G359, A360, H361 of the p66 connection domain and T473, N474, Q475, K476, Y501 and I505 of the p66 RNase H domain. Residues that make contact specifically with the RNA template are K390 of RT p51 and R448, N474, Q475, Q500 and H539 of RT p66 [74]. Site directed mutants of residues that form the RNase H primer grip result in decreased RNase H cleavage and decreased template switching [75].

The RNase H active site is formed by four acidic residues (D443, E478, D498 and D549) which are thought to coordinate with one or two Mg^{2+} or Mn^{2+} ions [58, 62]. Nowotny and Yang (2006) recently described a two metal cleavage mechanism which agrees with a model proposed by Steitz and Steitz in 1993 [63, 76]. In this model, one Mg^{2+} ion (A) is coordinated with RT residues D443 and D549 and water molecules (Figure 7). The second Mg^{2+} ion (B) coordinates with residues D443, E478 and D498 (Figure 7). After the RNA/DNA hybrid is correctly positioned in the RNase H active site, Mg^{2+} ion A activates the coordinated water molecule for nucleophilic attack. The two metal ions are then proposed to move closer to one another to form a pentacovalent transition state between the nucleophile and phosphorous atom and possibly neutralize the negative charge that is formed. In the final step, the intermediate is separated into

the 5’-phosphate and 3’-OH products and products dissociate [76]. Mg^{2+} ion B then relaxes and forms a normal octahedral coordination with water molecules as ligands.

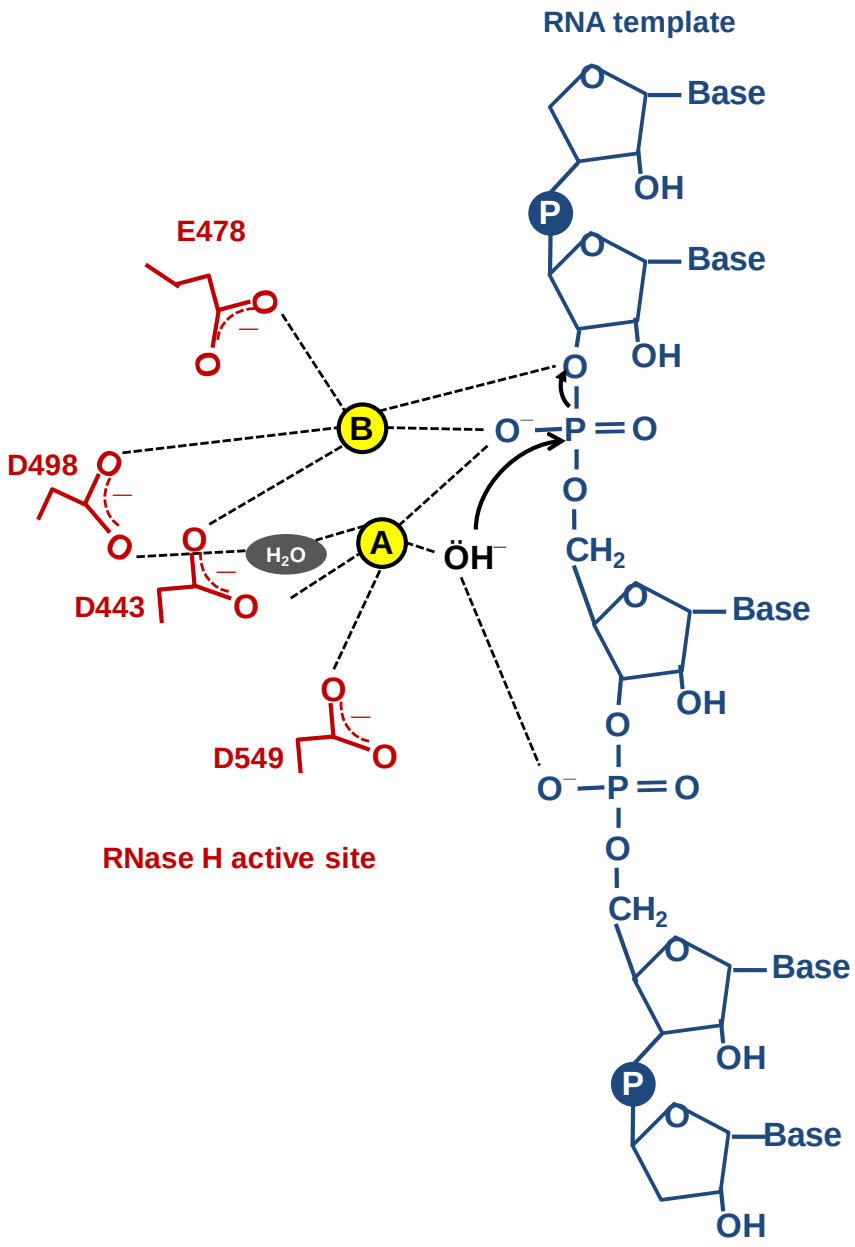


Figure 7. RNase H cleavage – two metal coordination.
Step 1 - Mg^{2+} ion (A) is coordinated with RT residues (red) D443 and D549 and water molecules (grey). Step 2 - The second Mg^{2+} ion (B) coordinates with residues (red) D443, E478 and D498. Step 3 - After the RNA/DNA hybrid (blue) is correctly positioned in the RNase H active site, Mg^{2+} ion A activates the coordinated water molecule for nucleophilic attack (black arrow). Step 4 - The Mg^{2+} ions move closer to one another to form a pentacovalent transition state between the nucleophile and phosphorous atom to neutralize the negative charge that is formed. Step 5 - The intermediate is separated into the 5'-phosphate and 3'-OH products and products dissociate. Mg^{2+} ion B then relaxes and forms normal octahedral coordination with water molecules as ligands. Figure adapted from Shultz and Champoux, 2008 [69].

1.5.6 Properties of RT that affect drug resistance

The engine behind genetic diversity in all retroviruses is the high replication rate and errors produced during viral replication [24, 77]. Mutations introduced into the HIV genome during reverse transcription can be neutral, negative or beneficial. Beneficial mutations will become the dominant species in a viral population under selective pressure such as that from RT inhibitors. Changes of the RT protein structure generated by mutations produce NRTI drug resistance through three main mechanisms: excision of a terminating NRTI to allow incorporation of the next dNTP; increased specificity for a natural dNTP over a NRTI-TP; and compensatory mutations that may restore RT function from resulting resistance mutations in the RT genome. Mutations that increase NNRTI resistance reposition residues around the RT polymerase active site to block reverse transcription.

Specific properties of HIV are important for the production of a diverse population of drug resistant virus [24]. First, HIV has a high rate of viral replication averaging about 10.3×10^9 virions per day with complete turnover of the viral population in 2.6 days [78]. Second, no proofreading ability has been identified for HIV RT (or other RNA polymerases) [79]. Consequently, the rate of nucleotide misincorporation during reverse transcription is between 10^{-4} to 10^{-5} per base per cycle [80-82]. It is estimated for an RNA genome 10^4 bp in length, a mutation will occur at each position of the genome numerous times a day [83]. In addition, recombination can occur between two RNA genomes packaged in each virion. The estimated rate of recombination is 2-3 cross-overs per genome per replication cycle [84, 85]. Recombination between two genomes within one viral particle may allow viral escape from replication errors incurred during reverse transcription and may increase the likelihood that new

synthesized HIV DNA has drug resistance mutations from both copies of RNA. This mechanism promotes the rapid emergence and spread of drug resistance throughout the viral population.

1.6 RT DRUG RESISTANCE MUTATIONS

HIV drug resistance is caused by a mutation or mutations in the HIV genome that enable mutant virus to replicate in the presence of higher drug concentrations than wildtype HIV. Drug resistance has developed to all FDA approved drugs. The genotype, phenotype and molecular mechanisms of resistance affecting each drug-class (i.e. reverse transcriptase, protease, integrase, entry inhibitors and CCR5 antagonists) have mainly been studied in HIV-1 subtype B.

Resistance to ART can be detected by phenotypic and genotypic assays. Phenotypic assays measure drug resistance directly by determining the ability of HIV to grow and replicate in the presence of varying concentrations of drug. Genotypic assays measure drug resistance indirectly by analyzing the genetic sequence obtained from HIV RNA to identify mutations associated with resistance. FDA approved genotyping tests for use with clinical samples are TRUGENE™ HIV-1 Genotyping Kit by Visible Genetics and ViroSeq™ HIV-1 Genotyping System by Celera. Both genotyping assays sequence the entire protease gene (residues 1-99) and residues within the polymerase domain of HIV RT: TRUGENE™ identifies mutations at RT residues 40-247 in the RT polymerase domain and ViroSeq™ identifies mutations at RT residues 1-319 in the polymerase domain and up to residue 335 in the connection domain. Other commercially available genotyping assays such as Virco®TYPE HIV-1 by Virco do not sequence beyond RT

amino acid 400 to identify possible resistance mutations in the RT connection and RNase H domains.

The following sections focus on well-characterized drug resistance mutations that develop in HIV-1 RT.

1.6.1 HIV-1 resistance to nucleoside/nucleotide reverse transcriptase inhibitors (NRTI)

1.6.1.1 Zidovudine (AZT) and stavudine (d4T)

Drug resistance was first identified in 1989 after use of AZT monotherapy to treat HIV-1 infected patients [30]. It was observed that patients receiving AZT monotherapy for greater than 6 months had a decrease in AZT susceptibility [29] and resistance occurred with emergence of RT mutation K70R followed by the M41L and T215 F or Y mutations. After continuing AZT treatment, mutations D67N and K219 E or Q also developed [30, 86-89].

Extensive resistance studies discovered that similar resistance mutations were selected by the NRTI d4T. Mutations selected by AZT and d4T were therefore termed “thymidine analog mutations” (TAMs) and include mutations M41L, D67N, K70R, L210W, T215F/Y and K219E/Q [30, 86, 87, 89]. TAMs occur in two separate pathways [90, 91], each pathway consisting of a combination of 2-5 TAMs: M41L-D67N-L210W-T215Y-K219E/N/Q (TAM 1) and M41L-D67N-K70R-T215F-K219E/N/Q (TAM 2). TAMs confer cross-resistance to all other NRTI; however, the level of resistance depends on the mutation and the number of TAMs in RT [30, 89, 92, 93]. Additional mutations (E44D, V118I, H208Y, R211K and L214F) are associated with TAMs and increase resistance to AZT and cross-resistance to NRTI when in combination with TAMs [94-96].

1.6.1.2 Abacavir (ABC) and didanosine (ddI)

Abacavir is a carboxylic guanosine nucleoside analog (Figure 2). During *in vitro* selection experiments, ABC selects virus with RT mutations K65R, L74V, Y115F and M184V [97]. These same mutations were identified in patient samples after 6-48 weeks of ABC monotherapy, the most common mutation being M184V and the most common combination being M184V-L74V [98, 99]. Low level ABC resistance occurs when M184V is present with two AZT resistance mutations. However, resistance to ABC increases substantially when M184V is present with three or more TAMs [99]. All ABC resistance mutations decrease susceptibility to ddC, ddI and 3TC but to varying degrees depending on mutation and presence of other ABC mutations [97].

Patients with decreased ddI susceptibility develop a similar set of resistance mutations as ABC. These include K65R and L74V [100, 101]. Mutation L74V is the most frequent and confers 4- to 10- fold increase in ddI resistance [100, 102, 103]. When L74V is present with AZT resistance mutation T215Y, AZT sensitivity is restored [101, 102, 104].

1.6.1.3 Lamivudine (3TC), emtricitabine (FTC) and zalcitabine (ddC)

Antiretrovirals 3TC and FTC are similar in chemical structure (Figure 2) and develop comparable resistance profiles with treatment failure. *In vitro* selection experiments using 3TC and FTC select for mutations M184V or I after 2 weeks [105, 106]. M184V/I are also the first mutations to arise in patients treated with 3TC- or FTC- containing regimens and confer >1000-fold resistance to both 3TC and FTC [105-107].

In vitro selection experiments with ddC select for K65R and M184V [108, 109]. Mutation K65R increases drug resistance to ddC and confers cross-resistance to 3TC, FTC, ddI, ABC and

TDF [108-110]. However, the most frequent mutation identified in patients treated with ddC monotherapy or combination therapy is T69D which increases ddC resistance 5-fold [111, 112].

1.6.1.4 Tenofovir disoproxil fumarate (TDF)

Tenofovir (TNV) is the NRTI after mucosal absorption of the oral prodrug tenofovir disoproxil fumarate (TDF). TNV is active against drug resistant strains including M184V, the multidrug resistant Q151M complex and virus with one or two TAMs, but TNV susceptibility decreases with three or greater TAMs specifically M41L and L210W [113]. Initially K65R was the only mutation seen to evolve in TDF treated patients and confers 3- to 9-fold TNV resistance [113, 114]. However with the use of triple NRTI therapy, K70E also develops in patients receiving regimens containing TDF [115-117].

1.6.2 HIV-1 resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI)

A second class of RT inhibitors developed to treat HIV infected patients are NNRTI. NNRTI bind to conserved residues within p66 forming a NNRTI "binding pocket" composed of two β-sheets (residues 100-110 and 180-190). All NNRTI are active against NRTI-resistant viruses and are therefore recommended as part of a triple-drug regimen composed of two NRTI plus one NNRTI.

The 4 FDA approved NNRTI used in combination ART are nevirapine (NVP), efavirenz (EFV), delavirdine (DLV) and etravirine (ETV). Common drug resistance mutations seen after NVP treatment are Y181C, Y188D/L/H and G190A [118, 119]. Greater than 100-fold resistance

to NVP is seen in viruses harboring the Y181C mutation and viruses with Y181C are cross-resistant to EFV and DLV [119, 120].

Efavirenz is an effective, well tolerated NNRTI and is the preferred drug in its class to be used with two NRTI [33]. Clinical data shows that 14 days of EFV monotherapy is sufficient to allow RT mutation K103N to emerge in patients. Other NNRTI mutations that occur in patients treated with EFV are L100I, K101E/Q, V106A, V108I, Y188H/L, G190S and P225H [121]. All mutations increase resistance to EFV and cause cross-resistance to NVP and DLV, except for P225H [110].

Delavirdine is rarely used because it has the largest pill burden and least antiviral activity [33]. The most frequent mutations in patients treated with DLV monotherapy are K103N and Y181C individually or as a double-mutant [122, 123] and in patients treated with AZT-DLV combination therapy, P236L usually occurs in the presence of a second NNRTI mutation [123]. Because cross-resistance occurs between NVP, DLV and EFV, it is not recommended to use a second NNRTI in salvage treatment after an NNRTI was used in first-line therapy [124].

Etravirine was recently FDA approved as an NNRTI in 2008. ETV is active against both HIV-1 and HIV-2 viruses and against DLV, EFV and NVP resistant virus except with the single mutants Y181I and F227C and the double-mutant L100I/K103N [125]. ETV resistance mutations are infrequently observed in NNRTI-experienced patients [126] and ETV resistance is usually only seen in patients with three or greater NNRTI mutations [125, 127]. Little information is known on etravirine's activity in treatment-naïve patients so it is not recommend for first-line therapy [33].

1.6.3 NRTI and NNRTI resistance mutations in HIV-1 subtype C

Drug resistance mutations in HIV-1 were initially characterized in subtype B virus which only includes < 10% of HIV/AIDS cases throughout the world [19]. Before initiation of "universal access" to ART across the globe, there was speculation that the same triple-regimen used to treat HIV-1 subtype B virus would not be successful to treat other HIV-1 subtype viruses, specifically subtype C [128]. Consequently, studies were completed to compare the RT and protease (PR) genome sequence from subtype B and C ART-naïve patients. Even though there were differences in polymorphisms found in subtypes B and C, no mutations that increase NRTI or NNRTI were found in subtype C sequence from patients that had never received ART [128, 129]. By contrast, PI resistance mutations were found in subtype C viruses from ART-naïve patients. PI resistance mutations commonly found in drug-naïve subtype C virus are K20R, M36I and H69K/Q [129, 130] but these mutations do not cause PI resistance without additional PR resistance mutations.

Subsequently, it was proposed that the same mutations that emerge in HIV-1 subtype B virus would also emerge in non-B subtype viruses and reduce antiretroviral activity. It was initially found that most mutations that cause antiretroviral resistance are similar across subtypes [131]. However, there are a few mutations that are more common in subtype C virus compared to subtype B. One NRTI mutation located at RT position 65 (K to R mutation) develops within 8 months of a d4T- or ddI- base regimen in subtype C patients, which is more frequent than in subtype B treated patients [128]. In addition, K65R emerged more rapidly in a subtype C RT backbone than subtype B in an *in vitro* selection experiment with tenofovir; however, definitive clinical data are not available to support more frequent emergence of K65R in subtype C infected patients [132, 133].

NNRTI mutations most often seen in subtype C HIV-1 include K103N and V106M with decreased susceptibility to NVP and EFV. Interestingly, V106M is more commonly seen in subtype C compared to V106A which is more frequent in subtype B [134, 135]. In HIV protease, the most common mutation in subtype C viruses and other non-B subtypes is L90M; whereas nelfinavir resistance arises with either a D30N or L90M change in subtype B. The D30N mutation is rarely seen in subtype C virus [136, 137].

In summary, with a few exceptions, the same resistance mutations that emerge in subtype B virus also emerge in subtype C virus.

1.7 NRTI DRUG RESISTANCE MECHANISMS

Two biochemical mechanisms of NRTI resistance have been described for mutations in the RT polymerase domain: discrimination and excision. Mutations that work through the discrimination mechanism change RT structure near the polymerase active site so that naturally occurring dNTPs are more likely to be incorporated instead of the competing NRTI-TP (Figure 8). Mutations that work through the excision mechanism increase removal of a dideoxynucleotide monophosphate (ddN-MP) or NRTI-MP from a terminated DNA primer (Figure 8). Different groups of polymerase domain mutations enable each mechanism and are described in detail below.

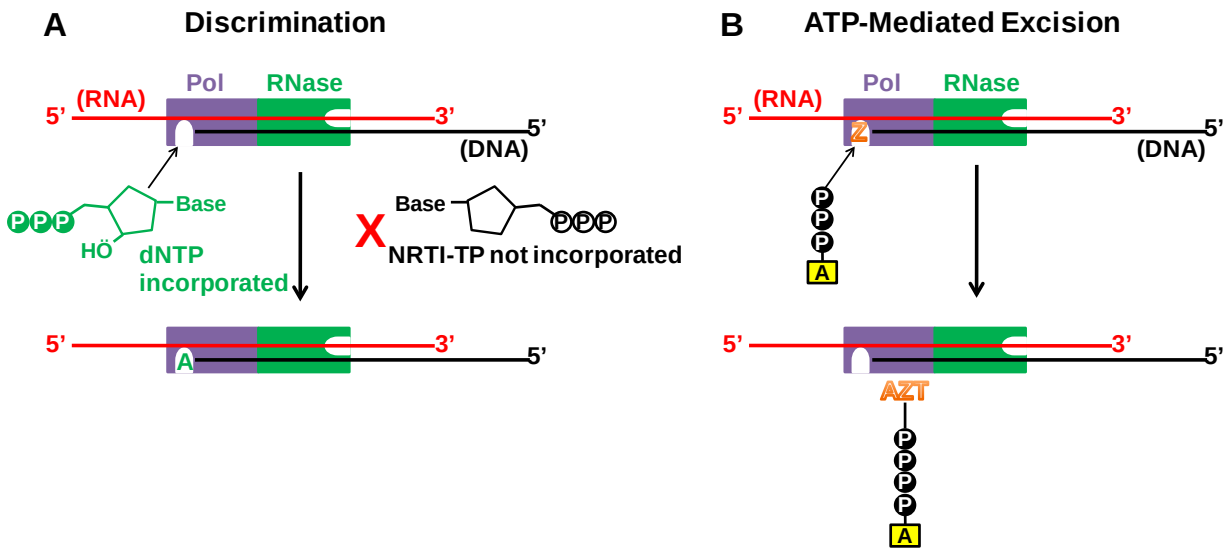


Figure 8. Schematic of biochemical mechanisms for NRTI resistance, discrimination and excision. (A) Discrimination – resistance mutations enhance binding of naturally occurring dNTPs (green) to the 3'-end of a DNA primer instead of terminating NRTI-TP (black). (B) Excision – TAMs help to increase binding of a phosphate donor (ATP or PPi) to the terminating NRTI-MP (AZT-MP) at the 3'-end of the primer stand to remove the terminating nucleotide and form a 5'-5'-dinucleoside tetraphosphate.

1.7.1 Excision

Drug resistance was first observed in AZT-treated patients soon after AZT was FDA approved. However, it took over a decade to successfully elucidate the biochemical mechanism that correlates with greater than 100-fold AZT resistance in cell culture. The mechanism for AZT resistance was unexpected and not clearly identified in early biochemical studies.

Discrimination was the first biochemical mechanism tested to explain AZT drug resistance. Unexpectedly, AZT-TP and dTTP had similar catalytic efficiencies (k_{pol}/K_m) for nucleotide incorporation [138-140]. Any small differences identified between AZT-TP and dTTP incorporation was not large enough to explain the greater than 100-fold resistance observed in cell culture.

Pyrophosphorolysis is the reverse reaction of polymerization. During this reaction cellular pyrophosphate (PP_i) attacks the monophosphate at the 3'-end of a primer to regenerate dNTP and $primer_{(n-1)}$/template (Figure 8B and Figure 9B). Pyrophosphorolysis is about 100 times slower than polymerization and incorporation of a dNTP onto the 3'-end of a primer is about 4 times more likely to occur within the cellular milieu than pyrophosphorolysis [138, 141]. Nevertheless, scientists hypothesized that AZT-MP is removed from the 3'-primer terminus by PP_i –mediated pyrophosphorolysis, and that thymidine analog mutations (TAMs) would increase the rate of AZT-MP removal (a.k.a. primer unblocking or excision). Initial experiments show that ddT-MP, ddA-MP, ddC-MP, AZT-MP, d4T-MP and 3TC-MP are removed from the 3'- primer terminus of a wildtype RT-T/P complex in the absence of dNTPs [142-145]. When dNTPs were included into PP_i-mediated primer unblocking experiments and wildtype RT was tested against TAMs containing RT, conflicting results were obtained. In experiments with low concentrations of RT,

TAMs increased the rate of pyrophosphorolysis [143, 145] and in experiments with high concentrations of RT, TAMs had no effect on rate of primer unblocking [139, 144].

Around the same time PP_i was studied as the substrate for pyrophosphorolysis, ATP was also considered as the phosphate-donor for excision [139, 145]. In ATP-mediated primer unblocking, ATP will attack the terminating AZT-MP on the 3'- end of the primer strand and remove AZT-MP to form 5'-5'-dinucleoside tetraphosphate and $primer_{(n-1)}$/template (Figure 8B and Figure 9B). Unlike PP_i excision, ATP-mediated primer unblocking is inefficient for wildtype enzyme[139]. The first ATP-mediated excision experiments showed that TAMs T215F and K219Q did not increase primer unblocking, but T215Y alone or combinations of D67N-K70R, M41L-T215Y, D67N-K70R-T215F-K219E/Q and D67N-K70R-T215Y-K219Q showed intermediate to high levels of primer unblocking and rescue of DNA synthesis [139, 145, 146]. The high level of primer unblocking observed in ATP-mediated excision experiments correlated well with high levels of AZT resistance identified in cell culture. Other nucleotides and NRTI that can be excised with ATP as the phosphate donor are d4T-MP efficiently and ddA-MP, ddC-MP, ddI-MP, 3TC-MP and CBV although with minimal efficiency [147].

The first structural model describing AZT-MP excision with ATP as the primary phosphate-donor was published by Boyer *et al* in 2001[65]. For excision to occur, the terminating NRTI-MP or ddN-MP must be positioned at the N-site of the polymerase active site [55, 62]. Mutations located around the polymerase active site (TAMs) increase RT affinity for ATP and facilitate NRTI-MP and ddN-MP excision from a terminated primer [65]. This model was later challenged with the notion that TAMs do not increase affinity of RT for ATP, but rather TAMs help to position ATP, RT and terminated primer to allow ATP-mediated phosphorolysis [148].

As described earlier, during polymerization an incoming dNTP binds to the N-site, followed by phosphate bond formation between the dNTP and 3'-end of the primer, then PPi release (Figure 9A). Before the next dNTP can bind to the N-site, the 3'-end of the primer strand must translocate into the P-site [65]. Recent studies suggest that the 3'- primer terminus is at equilibrium between both N- and P-sites until an incoming dNTP binds to the N-site for the next phosphate bond formation [149, 150]. Binding the next dNTP after a terminating NRTI-MP or ddN-MP will shift equilibrium so the incorporated NRTI-MP or ddN-MP translocates into the P-site (Figure 9C). Consequently, a diphosphate bond cannot form between the dNTP now located in the N-site and the NRTI-MP or ddN-MP in the P-site since NRTI-MP and ddN-MP lack a 3'-hydroxyl. A NRTI-MP or ddN-MP located in the P-site that cannot be excised forms a dead-end-complex (DEC) (Figure 9) [65, 149]. All NRTI-MP and ddN-MP are able to form a DEC except AZT-MP [142, 151]. It has been proposed that the 3'-azido group of AZT-MP is necessary to keep AZT-MP in the N-site, the position required for phosphate-mediated excision [65]. However, the 3'-azido group is not the only component of AZT that plays a role in AZT-MP excision. A more recent study has shown that RT containing TAMs D67N-K70R-T215F-K219Q confers resistance to 3'-azidopyrimidines (AZC, AZT and AZU) but not 3'-azidopurines (AZA and AZG) suggesting that structural differences such as the base component affect excision [152].

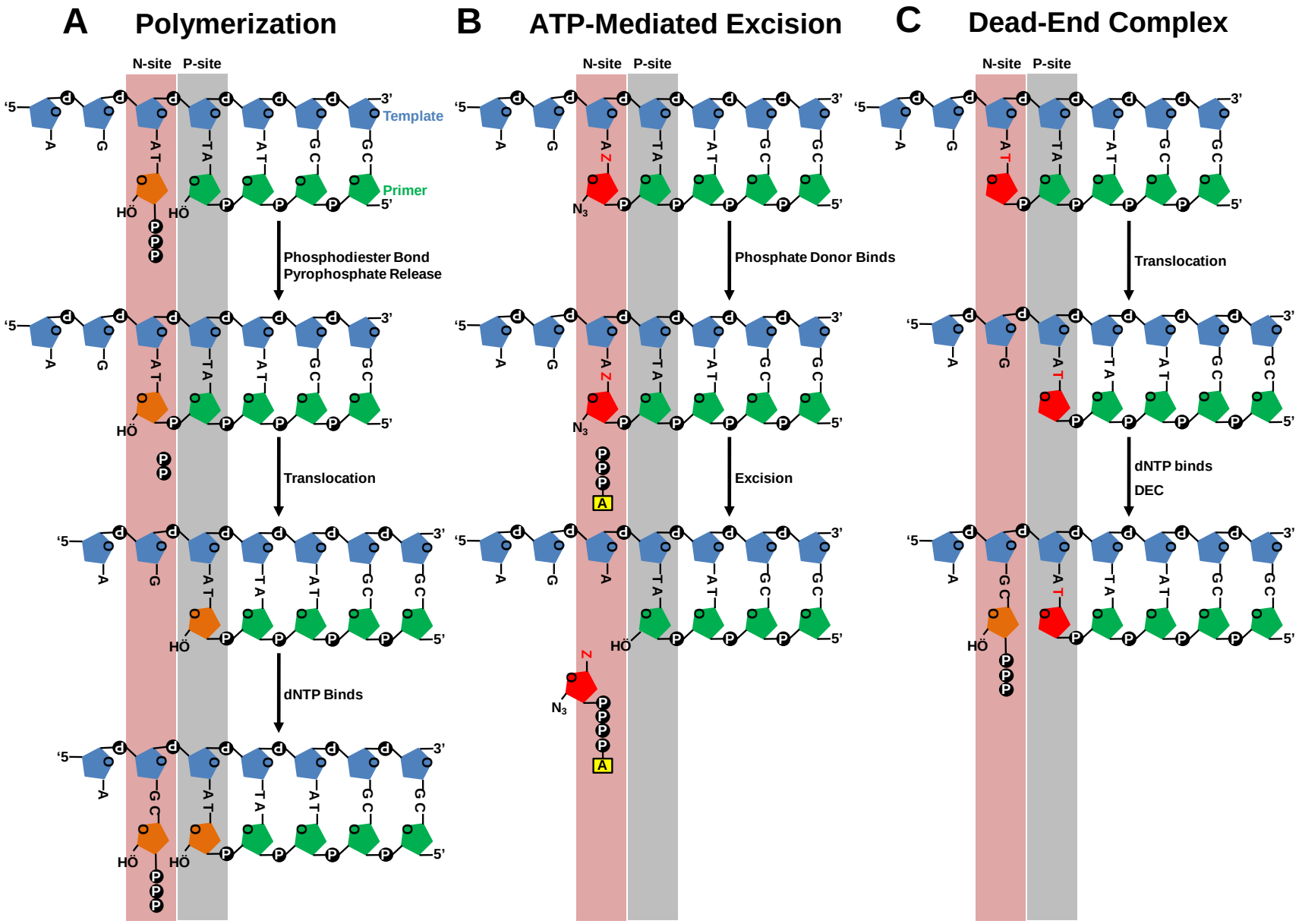

A
Polymerization
N-site
P-site
Template
Primer
Phosphodiester Bond
Pyrophosphate Release
Translocation
dNTP Binds
B
ATP-Mediated Excision
Phosphate Donor Binds
Excision
C
Dead-End Complex
Translocation
dNTP binds
DEC

Figure 9. Schematic of polymerization, ATP-mediated excision and dead-end-complex formation. (A) Polymerization: A phosphodiester bond is formed between the 3'-OH of the DNA primer strand and the α phosphate of a natural dNTP. Pyrophosphate is released and the newly bound dN-MP may translocate into the P-site allowing binding of the next dNTP to the N-site. (B) ATP-Mediated Excision: A phosphate donor (ATP) binds to the 3'-terminating NRTI-MP (AZT-MP) and removes AZT-MP forming a 5'-5'-dinucleoside tetraphosphate. (C) Dead-End-Complex (DEC): A terminating ddN-MP translocates to the P-site after phosphodiester bond formation and allows binding of the next dNTP at the N-site. The ddN-MP has no 3'-OH so a bond does not form between the new dNTP in the N-site and the ddN-MP in the P-site forming a DEC. Blue- RNA or DNA template; Green- primer; Orange – incoming dNTP; Red- terminating AZT-MP or ddN-MP; and Yellow A- ATP.

1.7.2 Discrimination

Discrimination occurs when mutations in the RT polymerase domain enhance incorporation of a naturally occurring dNTP into the primer strand instead of a competing NRTI-TP (Figure 8). Mutations identified in the polymerase domain known to increase the discrimination phenotype are K65R, L74V, M184V and the Q151M complex (A62V, V75I, F77L, F116Y and Q151M) [153]. This section will focus on key concepts of the discrimination mechanism using RT with M184V/I as an example.

M184V or I are the dominant mutations selected in cell culture and in patients treated with 3TC, FTC or ABC. M184V RT has weaker binding affinity for 3TC-TP than wildtype enzyme (K_m) and incorporates 3TC-TP at a slower rate (k_{pol}) than wildtype enzyme; 146-fold decrease in efficiency of incorporation (K_d/k_{pol}) on a DNA template and 120-fold decrease on an RNA template [154, 155]. In addition, biochemical studies show that the M to V or I mutation at 184 decreases DNA processivity and enzyme activity if found individually or in the presence of TAMs [156-158] and support studies that show M184V/I virus has reduced replication fitness in the absence of drug [157].

Biochemical data combined with structural studies have been used to propose two theories to explain increased discrimination by mutations M184V/I/T. First, crystal structures of M184I RT in the presence and absence of a DNA/DNA T/P illustrate that the β-branched methyl group of amino acid substitutions I, T and V at residue M184 is constrained by RT residues Y183 and Y115 resulting in steric hindrance between the β-methyl of M184V/I/T and the protruding oxatholine ring of 3TC [159]. In this case, binding of the natural dNTP would be favored over 3TC. Second, it has been proposed that T/P positioning within RT may be shifted by residue changes at amino acid 184 that alter the enzyme's ability to preferentially select for an incoming dNTP rather than a terminating NRTI-TP or ddN-TP [158-160]. Similar mechanisms have been described for HIV-1 RT containing K65R, L74V and the Q151M complex [62, 153, 161, 162].

1.8 DRUG RESISTANCE MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF RT

Clinical concern has grown that drug resistance mutations in the RT connection and RNase H domains are being missed with traditional genotyping methods. Kemp *et al.* was the first group to identify that connection domain mutation G333E emerges in patients treated with AZT-3TC dual-therapy [163]. It has been established that RT containing M184V decreases AZT-MP excision when in the context with TAMs [158] and therefore increases susceptibility for AZT [164]. However, RT containing G333D allows the enzyme to effectively discriminate between the normal substrate dCTP and 3TC-TP and enhances the ability of RT containing TAMs and M184V to bind AZT-MP terminated T/P, restoring ATP-mediated excision of AZT-MP [163, 165].

The first studies of conserved residues in the RNase H domain show that the mutations Q475E and H539N decrease RNase H activity and are defective in PPT cleavage [166, 167]. In addition, substitutions at RNase H primer grip residues G359, A360, H361, E396, T473, Q475, Y501 severely decrease viral replication and alter RNase H cleavage specificity [168-170]. In 2005 Nikolenko *et al.* tested the hypothesis that mutations in the RNase H domain that decrease RNase H cleavage will increase NRTI drug resistance via increasing NRTI excision. The authors showed that RNase H domain mutations D539N and H549N increase AZT resistance in cell-based drug susceptibility assays by 12-fold and 180-fold, respectively, and reduced d4T susceptibility by 2.4-fold and 10-fold, respectively [171]. Furthermore, when D549N was present with TAMs D67N-K70R-T215Y-K219Q, AZT and d4T resistance increased 1,230-fold and 12.5-fold, respectively. The authors proposed that mutations in the RNase H domain that

decrease RNase H activity, reduce RNA template degradation, thereby increasing the time for AZT-MP to be excised from the terminated primer and polymerization to resume on an intact template.

The *in vitro* experiments by Nikolenko *et al.* used two mutant enzymes with known RNase H deficient activities (D539N and H549N). However, it is not known if mutations in the RT connection or RNase H domains emerge *in vitro* or in patients treated with ART. The goal of this thesis is to identify mutations that evolve in the RT connection or RNase H domains *in vitro* and in patients treated with ART and elucidate the biochemical mechanism for the observed NRTI resistance.

2 HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Mutations in the HIV-1 connection and RNase H domains of reverse transcriptase (RT) that confer resistance to RT inhibitors emerge with antiretroviral selection and are missed using clinically available genotyping tests that only identify mutations in the RT polymerase domain. Mutations in the connection and RNase H domains cause resistance to NRTI by disrupting the normal equilibrium between DNA polymerization and RNase H cleavage.

Specific Aims

The above hypotheses will be tested through three approaches: virological, biochemical and clinical in the following specific aims:

1. Determine if mutations in the HIV-1 RT connection and RNase H domains are selected in cell culture with increasing AZT concentrations and elucidate the *in vitro* resistance profile of RT connection and RNase H domain mutations alone and in combination with polymerase domain mutations using cell-based drug susceptibility assays.
2. Investigate how mutations in the HIV-1 RT connection and RNase H domains in combination with TAMs affect RNase H cleavage activity and the rate of excision using pre-steady state and steady state biochemical assays.

3. Identify the prevalence of RT connection and RNase H domain mutations alone or in the presence of polymerase domain mutations in clinical samples from patients on failing antiretroviral therapy.

3 CHAPTER ONE. SELECTION OF MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE THAT INCREASE RESISTANCE TO 3'-AZIDO-3'-DIDEOXYTHYMIDINE

Jessica H. Brehm[1], Dianna Koontz[1], Vinay Pathak[2], Nicolas Sluis-Cremer[1], John W. Mellors[1]

[1]Division of Infectious Diseases, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, [2]National Cancer Institute, Fredrick, Maryland 21702

3.1 PREFACE

This chapter is adapted with permission from a published study (Brehm, J. H., D. Koontz, J. D. Meteer, V. Pathak, N. Sluis-Cremer, and J. W. Mellors. 2007. Selection of Mutations in the Connection and RNase H Domains of Human Immunodeficiency Virus Type 1 Reverse Transcriptase that Increase Resistance to 3'-azido-3'-dideoxythymidine. J Virol, 81(15): p. 7852-9). Copyright 2007 American Society for Microbiology.

Additionally, this study was presented in part as a poster at the 13th Conference on Retroviruses and Opportunistic Infections, Denver, CO, February 2006 (Abstract 601, Brehm, J., D. Koontz, V. Pathak, N. Sluis-Cremer, and J. Mellors. Does 3'-Azidothymidine Select Mutations in the RNase H Domain of HIV-1 Reverse Transcriptase?); in part as an oral abstract at the XV International HIV Drug Resistance Workshop, Sitges, Spain, June 2006 (abstract published in Brehm, J., D. Koontz, V. Pathak, N. Sluis-Cremer, and J. Mellors. 2006. AZT Selects Mutations in the Connection (A371V) and RNase H (Q509L) Domains of RT that Increase AZT Resistance in Combination with TAMs and Reduce Susceptibility to other NRTIs. Antiviral Therapy, 11: p. S141); and in part as an oral abstract at the 7th Annual Symposium on Antiviral Drug Resistance, Chantilly, VA, November 2006 (Brehm, J., D. Koontz, V. Pathak, N. Sluis-Cremer, and J. Mellors. Selection of Mutations in the Connection and RNase H Domains of HIV-1 RT that Increase Resistance to AZT).

The work presented in this chapter is in partial fulfillment of dissertation aim 1. Jessica Brehm performed all experimental work with the exception of AZT selection experiments completed by Dianna Koontz.

3.2 ABSTRACT

Recent work has suggested that mutations in the C-terminal domains of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) increase 3'-azido-3'-dideoxythymidine (AZT) resistance. Because it is not known if AZT selects mutations outside of the polymerase domain of RT, we carried out *in vitro* experiments in which HIV-1_{LAI} was passaged in MT-2 cells in increasing concentrations of AZT. The first resistance mutations to appear were 2 polymerase domain thymidine analog mutations (TAMs) - D67N and K70R - that together conferred 66-fold AZT resistance. These were followed by the acquisition of 2 novel mutations - A371V in the connection domain and Q509L in the RNase H domain - that in combination with D67N and K70R were associated with ~90-fold AZT resistance. Thereafter, the T215I mutation appeared but was later replaced by T215F, resulting in a large increase in AZT resistance (~16,000-fold). The roles of A371V and Q509L in AZT resistance were confirmed by site-directed mutagenesis: A371V and Q509L together increased AZT resistance ~50-fold in combination with TAMs. Mutagenesis studies also showed that HIV-1 containing D67N/K70R/T215F/A371V/Q509L conferred greater cross-resistance to lamivudine, abacavir and tenofovir than viruses without A371V/Q509L. Taken together, these results provide the first evidence that mutations in the connection and RNase H domains of RT are selected by AZT in combination with TAMs and confer significantly greater AZT resistance and cross-resistance to other nucleoside inhibitors.

3.3 GOAL OF STUDY

All nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) mutations included in the most widely used resistance tables, such as that from the International AIDS Society – USA (IAS-USA) expert panel [110], are located in the DNA polymerase domain of HIV-1 RT. This is the case, in part, because most commercial genotypic assays do not analyze the complete connection and RNase H domains of RT. In this regard, Nikolenko *et al.* reported that mutations introduced into the RNase H domain of HIV-1 reverse transcriptase (RT) that retard RNase H activity also cause resistance to 3'-azidothymidine (AZT). The authors proposed that mutations in the RNase H domain that decrease RNase H activity, reduce RNA template degradation, thereby increasing the time for AZT-MP to be excised from the terminated primer and polymerization to resume on an intact template [171].

It is not clear, however, whether mutations in the RNase H domain of RT are selected by AZT. We therefore carried out *in vitro* selections of AZT-resistant HIV-1, sequenced the entire coding region of RT to identify all drug-resistance related mutations, and characterized the effects of these mutations using site-directed recombinant viruses.

3.4 MATERIALS AND METHODS

3.4.1 NRTI

Zidovudine (AZT) and didanosine (ddI) were obtained from Sigma Chemical Corporation (St. Louis, MO). Lamivudine (3TC) and stavudine (d4T) were provided by Raymond Schinazi (Emory University, GA). Tenofovir (TNV) was provided by Gilead Sciences (Foster City, CA) and abacavir (ABC) by GlaxoSmithKline (Research Triangle Park, NC). NRTI were prepared as 10mM or 30mM stock solutions in dimethyl sulfoxide or sterile water and stored at -20˚C. The compounds were diluted immediately before use to desired concentrations in Dulbecco's modified Eagle medium, Phenol Red Free (DMEM-PRF, Gibco-BRL, Grand Island, NY).

3.4.2 Cells and viruses

MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were cultured in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES buffer, 50 IU/ml of penicillin and 50 mg/ml of streptomycin (referred to as R10). The P4/R5 reporter cell line (provided by Dr. Nathaniel Landau, Salk Institute, La Jolla, CA), which expresses the β-galactosidase gene under the control of the HIV long terminal repeat promoter that is transactivated by HIV-1 tat, was maintained in DMEM-PRF supplemented with 10% FBS, 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 0.5 μg/ml of puromycin (Clontech, Palo Alto, CA). Stock viruses were prepared in MT-2 cells as described

previously [172]. Briefly, 5 to 10 μg of plasmid DNA was electroporated into 1.3 x 10^7 MT-2 cells. Cell-free supernatants were collected 7 days after transfection at peak cytopathic effect (CPE) and stored at -80˚C. The infectivity of the virus stocks was determined by a threefold endpoint dilution in P4/R5 cells, and the 50% tissue culture infectivity dose ($TCID_{50}$) was calculated using the Reed and Muench equation [173]. To confirm the genotype of the stock viruses, viral RNA was extracted from cell-free supernatants and treated with 1 Unit/μl of DNase I for 2 hours. Codons 1-560 of RT were amplified using the following primers: RT forward 5'-AAGCTATAGGTACAGTATTAGTAGGACCTAC-3' and RT reverse 5'-TGCTCTCCAATTA CTGTGATATTTCTCA-3'. PCR products were purified (Wizard PCR purification system; Promega, Madison, WI) and sequenced using a Big Dye terminator kit (v.3.1) on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

3.4.3 Selection of AZT-resistant viruses

Resistant virus was selected in two independent experiments by the passage of wild-type HIV_{LAI} or HIV_{LAI} containing the M41L/L210W/T215Y mutations (AZT^R HIV_{LAI}) in MT-2 cells in increasing concentrations of AZT. To initiate each selection experiment, MT-2 cells ($1x10^6$) were pretreated for two hours with 0.5 μM and 25 μM AZT for wild-type HIV_{LAI} and AZT^R HIV_{LAI}, respectively, before virus was added. Viral replication was monitored by CPE. At +3/4 CPE (3 or 4 syncytia per field at 100× magnified field), cell-free supernatant was harvested and 0.1 ml of supernatant was added to fresh MT-2 cells to initiate a new passage. The concentration of AZT was doubled every three passages. The selection pressure was increased from an initial AZT concentration of 0.5 μM to a final concentration of 32 μM for wild-type HIV_{LAI}, and from

25 μM to 150 μM for the AZT^R HIV_{LAI}. The concentration of drug required to inhibit viral replication by 50% (IC_{50}) was calculated every five passages to identify changes in AZT susceptibility, and fold-resistance was determined by dividing the IC_{50} of the mutant virus by the IC_{50} of wild-type HIV-1_{LAI}. The genotype of the passaged virus was determined as described in Materials and Methods section 3.4.2.

3.4.4 Drug susceptibility assays

NRTI susceptibility was determined in P4/R5 cells as described previously [172]. Briefly, threefold dilutions of inhibitor were added to P4/R5 cells in triplicate and cells were infected with an amount of virus that produced 100 relative units of light (RLU) in no drug, virus control wells. After 48 hours, the cells were lysed (Gal-Screen; Tropix/Applied Biosystems, Foster City, CA) and the RLU was measured using a ThermoLabSystems luminometer (Waltham, MA). The IC_{50} and fold-resistance were calculated as described in Materials and Methods section 3.4.3. IC_{50} values from at least three independent experiments were $\log_{10}$ transformed and compared for statistically significant differences using the two-sample Student's *t* test.

3.4.5 Clonal analysis of HIV-1 RT for mutation linkage

The entire sequence of HIV-1 RT from passaged viruses was RT-PCR amplified using RT forward and RT reverse primers (defined in Materials and Methods section 3.4.2), and the PCR product was cloned into the TOPO TA® cloning vector (Invitrogen, Carlsbad, CA). After transformation into *Escherichia coli* TOP10 competent cells, clones containing the correct insert

were identified through blue-white screening. DNA from clones was purified and sequenced as described in Materials and Methods section 3.4.2.

3.4.6 Construction of mutant recombinant HIV-1

Mutant recombinant plasmid clones of virus were generated by oligonucleotide site-directed mutagenesis as described previously, using the p6HRT-MO plasmid. p6HRT-MO contains the entire RT and protease coding sequence as previously described [174] and four silent restriction sites (*XmaI, MluI, XbaI, and NgoMIV* from the 5' to 3' end of RT at codons 14, 358, 490 and 554, respectively). After site-directed mutagenesis (QIAamp kit, QIAGEN, Valencia, CA), the mutated RT was ligated into pxxHIV-1_{LAI} MO that contains the entire genome of HIV-1_{LAI} and the same silent restriction sites as p6HRT-MO. Infectious virus was generated by electroporating the mutated xxHIV-1_{LAI} MO plasmid into MT-2 cells as described in Materials and Methods section 3.4.2. All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

3.4.7 Assays of replication capacity and replication kinetics

The p24 (ng/ml) of each viral stock was determined by ELISA (Alliance HIV-1 p24 ELISA kit, PerkinElmer, Wellesley, MA) and single-cycle replication capacity was measured by adding 10 ng of viral p24 (MOI between 0.12 and 0.22) to 5×10^4 P4/R5 cells in a 96 well plate (6 wells per virus). After 48 hours, the cells were lysed and the RLU was measured as described in Materials and Methods section 3.4.4. Mean RLU from three independent experiments were compared for

statistically significant differences using the two-sample Student's *t* test. Multiple-cycle replication kinetics was determined in MT-2 cells. Virus (10 ng of p24, MOI between 0.12 and 0.22) was added to 5×10^6 MT-2 cells. After 2 hours, R10 was added to the infected MT-2 cells to give a final concentration of 1×10^6 cells/ml. An initial aliquot was taken after the 2 hour infection as background, and samples of 0.5 ml were collected every day for 7 days. The cultures were replenished with 0.5 ml of R10 after each aliquot was harvested. The p24 (ng/ml) concentration of each aliquot was measured, and values from three independent experiments were compared for statistically significant differences using the two-sample Student's *t* test.

3.4.8 Visualization of three dimensional structure of HIV-1 RT

The Molecular Operating Environment (MOE^{TM}) (Chemical Computing Group Inc., Montreal, Quebec, Canada) was used to visualize structural images of RT bound to an RNA/DNA template/primer (T/P) (pdb access number 1HYS; [74]).

3.5 RESULTS

3.5.1 Selection of AZT-resistant virus

Two independent AZT selection experiments were conducted. One starting with wild-type HIV-1_{LAI} and the second starting with AZT^R HIV-1_{LAI} (encoding M41L/L210W/T215Y). Both viruses were serially passaged in MT-2 cells in increasing concentrations of AZT. After every 5

passages, AZT susceptibility was measured in a single-cycle viral replication assay in P4/R5 cells (described in Materials and Methods sections 3.4.3 and 3.4.4). Viral RNA was extracted, converted to cDNA and the entire coding region of RT (residues 1-560) was PCR amplified and sequenced to monitor the appearance of mutations. In the selection experiment starting with HIV-1_{LAI}, AZT susceptibility was reduced 66-fold by passage 35 and two polymerase domain TAMs were identified: D67N and K70R (Table 1). By passage 40, the virus was ~ 90-fold AZT-resistant and had acquired two novel mutations in RT: A371V and Q509L in the connection and RNase H domains of RT, respectively. By passage 60, T215I appeared and was then replaced by T215F by passage 65, increasing AZT resistance to ~16,000-fold.

In the selection experiment starting with AZT^R HIV-1_{LAI}, AZT susceptibility was reduced >1,000-fold by passage 35 (Table 2). This decrease in AZT susceptibility was associated with the acquisition of two additional mutations in the DNA polymerase domain: D67N and L214F. Mutations in the connection or RNase H domains of RT were not detected.

Table 1. Selection of AZT-resistant virus starting with wild-type HIV-1_{LAI}

Passage	AZT Concentration (μM)	IC_{50} (μM)	Fold-Resistance[a]	Mutations
1	0.5	0.2	1.5	ND[b]
10	0.5	0.3	1.6	ND
20	0.5	0.7	5.1	ND
25	1.0	0.7	4.1	None
30	2.0	3.0	26	D67D/N, K70K/R
35	2.0	10	66	D67N, K70R
40	4.0	10	86	D67N, K70R, A371A/V, Q509Q/L
55	16	39	255	D67N, K70R, T215T/I, A371A/V, Q509Q/L
60	32	56	489	D67N, K70R, T215I, A371A/V, Q509L
65	32	810	>16200	D67N, K70R, T215I/F, A371A/V, Q509L

[a] Fold-resistance compared to wild-type HIV-1_{LAI} passaged in parallel without AZT.
[b] Not Done (ND).

Table 2. AZT selection starting with HIV-1_{LAI} encoding M41L, L210W, T215Y (AZT^R)

Passage	AZT Concentration (μM)	IC_{50} (μM)	Fold-Resistance[a]	Mutations
1	25	21	6.0	ND[b]
10	50	34	15	ND
25	100	126	39	M41L, D67D/N, L210W, L214F, T215Y
30	150	181	62	M41L, D67D/N, L210W, L214F, T215Y
35	150	>810	>1332	M41L, D67N, L210W, L214F, T215Y

[a] Fold-resistance compared to wild-type HIV-1_{LAI} passaged in parallel without AZT.
[b] Not Done (ND).

3.5.2 Linkage analysis of mutations

To evaluate whether D67N, K70R, T215F, A371V and Q509L were selected on the same viral genome in the first selection experiment, the RT coding region from passage 65 virus was amplified by RT-PCR, cloned into the TOPO TA® prokaryotic vector and transformed into *E. coli* TOP10 cells. Plasmid DNA was isolated from twelve bacterial colonies and the full-length RT coding region was sequenced (Table 3). All 12 clones contained D67N, K70R and Q509L. Six of the clones had all 5 mutations and three clones contained T215I with D67N, K70R, A371V and Q509L. The remaining 3 clones contained D67N, K70R, Q509L and either T215I or T215F, but not A371V. Additional mutations that were identified included R358K in only 4 clones and F416Y in 3 clones (Table 3).

Table 3. D67N, K70R, T215I/F, A371V and Q509L are linked on the same genome

HIV_{LAI}	Clone Number												% of Clones
	1	2	3	4	5	6	7	8	9	10	11	12	
G18	—	—	—	D	—	—	—	—	—	—	—	—	8
L26	—	—	—	—	—	—	—	—	—	—	—	S	8
T39	—	—	—	P	—	—	—	—	—	—	—	—	8
D67	N	N	N	N	N	N	N	N	N	N	N	N	100
K70	R	R	R	R	R	R	R	R	R	R	R	R	100
L73	—	—	—	—	—	—	—	S	—	—	—	—	8
I94	—	—	—	T	—	—	—	—	—	—	—	—	8
G99	—	—	—	—	—	—	—	E	—	—	—	—	8
K104	—	—	—	—	N	—	E	—	—	—	—	—	8(N), 8(E)
S117	—	—	—	—	L	—	—	—	—	—	—	—	8
V118	—	—	—	I	—	—	—	—	—	—	—	—	8
D123	—	G	—	—	—	—	—	—	—	—	—	—	8
G190	—	—	—	—	—	Q	—	—	—	—	—	—	8
S191	—	—	T	—	—	—	—	—	—	—	—	—	8
I202	—	—	—	—	—	T	—	—	—	—	T	—	16
T215	F	I	I	I	F	F	F	F	I	F	F	I	42(I), 58(F)
E122	—	—	—	—	—	—	—	—	G	—	—	—	8
Q242	—	—	—	—	—	—	—	—	—	—	R	—	8
I288	—	—	—	—	—	—	—	S	—	—	—	—	8
W337	—	—	—	—	R	—	—	—	—	—	—	—	8
R358	—	—	—	—	—	K	K	—	—	K	—	K	33
A371	V	V	V	—	V	V	—	V	V	V	V	—	75
K385	—	—	—	—	E	—	—	—	—	—	—	—	8
I393	—	—	L	—	—	—	—	—	—	—	—	—	8
F416	Y	—	—	—	—	—	—	—	Y	Y	—	—	25
I434	—	—	—	—	M	—	—	—	—	—	—	—	8
A445	—	—	—	—	—	V	—	—	—	—	—	—	8
L491	—	—	—	R	—	—	—	—	—	—	—	—	8
R461	—	—	—	—	K	—	—	—	—	—	—	—	8
N494	—	—	—	—	—	—	—	—	S	—	—	—	8
A502	T	—	—	—	—	—	—	—	—	—	—	—	8
Q509	L	L	L	L	L	L	L	L	L	L	L	L	100
L517	—	—	—	—	—	—	—	—	—	—	—	S	8
N519	—	—	—	—	—	—	—	—	S	—	—	—	8
A534	—	—	—	—	—	T	—	—	—	—	—	—	8
D549	—	—	N	—	—	—	—	—	—	—	—	—	8
A554	—	—	T	—	—	—	—	—	—	—	—	—	8
G555	Q	—	—	—	—	—	—	—	—	—	—	—	8
G555	—	—	K	—	—	—	—	—	—	—	—	—	8

3.5.3 Drug susceptibility of recombinant viruses

To confirm the role of the A371V and Q509L mutations in AZT resistance, recombinant mutant viruses were generated by site-directed mutagenesis. Five mutant viruses were constructed that represent the appearance of mutations in the AZT selection experiment at passages 35, 40, 60 and 65 (Table 1 and Table 4). An additional 10 mutant viruses were generated to delineate the roles of A371V and Q509L alone and together with different combinations of TAMs (Table 4). The A371V and Q509L mutations, alone or together, did not confer AZT resistance in the absence of TAMs. When the A371V mutation alone was added to viruses that contained different combinations of TAMs, AZT susceptibility was only marginally decreased (1.2- to 2-fold). By contrast, viruses that contained Q509L and different combinations of TAMs exhibited significantly greater resistance to AZT (3.0- to 11-fold). When both A371V and Q509L were combined with TAMs, the extent of AZT resistance was significantly greater (9- to 52-fold) compared with viruses that contained only one of the mutations or neither of them. Of note, there was only a small difference in AZT resistance between the D67N/K70R/A371V/Q509L mutant (39-fold) and the D67N/K70R/T215I/A371V/Q509L mutant (41-fold) [Table 4]. Thus, the selective advantage of T215I was not obvious from these drug-susceptibility analyses.

Table 4. AZT susceptibility of site-directed mutants

Mutation	IC_{50} (μM)[a]	Fold-Resistance[b]	p-value
Wild-type	0.2 ± 0.1	——	——
A371V	0.2 ± 0.04	0.7	0.2
Q509L	0.3 ± 0.2	1.3	0.6
A371V/Q509L	0.3 ± 0.06	1.7	0.4
67N/70R[c]	**1.1 ± 0.6**	**4.6**	**<0.001**
67N/70R/371V	1.4 ± 0.5	6.4	<0.001
67N/70R/509L	3.0 ± 1.0	14	<0.001
67N/70R/371V/509L	**9.1 ± 5.2**	**39**	**<0.001**
67N/70R/215I	0.3 ± 0.2	1.3	0.5
67N/70R/215I/371V	0.6 ± 0.3	2.6	0.07
67N/70R/215I/509L	3.0 ± 2.2	14	0.004
67N/70R/215I/371V/509L	**9.4 ± 6.7**	**41**	**<0.001**
67N/70R/215F	3.8 ± 2.1	18	0.002
67N/70R/215F/371V	4.9 ± 2.9	22	<0.001
67N/70R/215F/509L	**28 ± 17**	**128**	**<0.001**
67N/70R/215F/371V/509L	**203 ± 40**	**934**	**<0.001**

[a] Mean ± standard deviation from at least three experiments.
[b] Fold-resistance of mutants compared to wild-type.
[c] Mutation combinations in **bold** were selected *in vitro* by AZT.

3.5.4 Cross-resistance to other NRTIs

The effect of A371V and Q509L in combination with TAMs on cross-resistance to other NRTIs was also analyzed (Table 5). Statistically significant increases in cross-resistance to 3TC ($p = 0.047$ and 0.014 for D67N/K70R/A371V/Q509L and D67N/K70R/T215F/A371V/Q509L, respectively) and abacavir ($p = 0.020$ for D67N/K70R/A371V/Q509L) were noted in viruses that contained A371V and Q509L in combination with TAMs compared with those that contained only TAMs. Viruses that contained TAMs and A371V and Q509L also exhibited a trend toward decreased susceptibility to tenofovir ($p = 0.10$ and 0.058 for D67N/K70R/A371V/Q509L and D67N/K70R/T215F/A371V/Q509L, respectively), but not to d4T or ddI (Table 5).

Table 5. Cross-resistance of site-directed mutants to NRTIs

Mutation in HIV_{LAI} RT	IC_{50} (Fold-Resistance)[a]				
	lamivudine (3TC)	abacavir (ABC)	tenofovir (TNV)	stavudine (d4T)	didanosine (ddI)
Wild-type	0.5 ± 0.1	6.4 ± 0.5	3.3 ± 0.9	7.2 ± 0.6	4.2 ± 0.9
D67N/K70R	1.0 ± 0.3 (1.9)[c]	7.9 ± 0.4 (1.2)[c]	4.9 ± 1.5 (1.5)	10 ± 3.1 (1.4)	5.0 ± 0.9 (1.2)
D67N/K70R/A371V/Q509L	2.7 ± 1.4 (5.2)[c, d]	13 ± 2.5 (2.0)[c, d]	7.3 ± 1.3 (2.2)[c]	9.6 ± 2.9 (1.3)	4.9 ± 0.9 (1.2)
D67N/K70R/T215F	3.8 ± 0.59 (7.0)[b]	15 ± 1.5 (2.4)[b]	4.9 ± 0.7 (1.5)	17 ± 4.6 (2.4)[c]	6.8 ± 0.3 (1.6)[c]
D67N/K70R/T215F/A371V/Q509L	7.5 ± 1.8 (15)[b, e]	19 ± 4.5 (3.0)[c]	9.0 ± 3.5 (2.7)[c]	14 ± 4.4 (2.0)[c]	6.6 ± 0.4 (1.6)[c]

[a] Mean ± SD is from at least 3 independent experiments. Fold-resistance compared to wild-type in parentheses.
[b] IC_{50} is significantly different from wild-type, $p < 0.001$.
[c] IC_{50} is significantly different from wild-type, $p < 0.05$.
[d] IC_{50} is significantly different from D67N/K70R, $p < 0.05$.
[e] IC_{50} is significantly different from D67N/K70R/T215F, $p < 0.05$.

3.5.5 Replication capacity and replication kinetics of mutant viruses

Since the selective advantage of T215I was not evident from the drug-susceptibility analyses (Table 4), we next assessed replication capacity and kinetics of the 4 recombinant viruses with RT sequences identical to those in viruses from passages 35, 40 and 60. Replication capacity was assessed in a single-cycle assay in P4/R5 cells and replication kinetics was assessed using a multiple-cycle assay in MT-2 cells. Cells were infected with a standard inoculum (10 ng of p24, equivalent to MOI of 0.12-0.22) of each virus. Figure 10A shows that the replication capacity of the D67N/K70R/A371V/Q509L mutant was reduced to 48% of wild-type virus. This loss in replication capacity, however, was restored to wild-type levels by the addition of the T215I mutation. Similar results were observed in replication kinetic assays carried out in MT-2 cells over a 7 day period (Figure 10B). Specifically, the replication of the D67N/K70R and D67N/K70R/A371V/Q509L mutant viruses was reduced 46% and 37% on day 6, respectively, compared with wild-type virus. The impaired replication of the D67N/K70R/A371V/Q509L virus was restored to levels similar to wild-type by the T215I mutation.

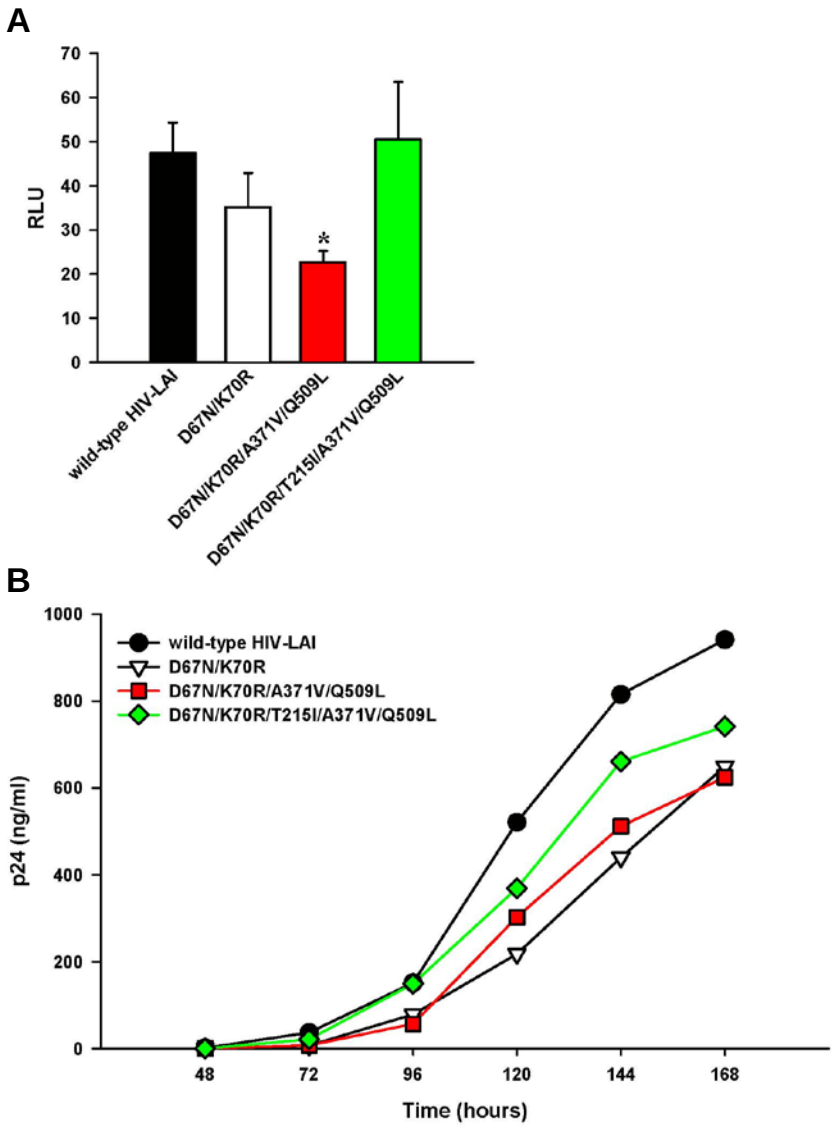


Figure 10. Single-cycle and multiple-cycle replication assays of recombinant HIV_{LAI} containing the A371V and Q509L mutations.
(A) Single-cycle replication was measured in P4/R5 cells infected with 10 ng p24 (MOI 0.12-0.22) in a 96 well plate. After 48 hours, cells were lysed and viral replication was measured using RLU. (B) Multiple–cycle replication was measured in MT-2 cells infected with 10 ng p24 (MOI 0.12-0.22). p24 was measured from cell-free supernatant harvested daily for 7 days. Recombinant viruses analyzed: wild-type xxLAI (black circle), D67N/K70R (white triangle), D67N/K70R/A371V/Q509L (red square) and D67N/K70R/T215I/A371V/Q509L (green diamond). Data represents the mean ± SD from 3 independent experiments. *p-values <0.05 were considered significant compared to wild-type HIV_{LAI}.

3.5.6 Location of residues A371 and Q509 in RT

Analysis of a crystal structure of HIV-1 RT in complex with an RNA/DNA polypurine tract T/P substrate reveals that both A371 and Q509 are located near the T/P DNA binding tract (Figure 11). A371 is 2.8 Å from K374, the side-chain of which interacts with the phosphate backbone of the RNA template strand through a hydrogen bond (Figure 11B). Q509 is close to the RNase H primer grip, in particular, residue I505. The RNase H primer grip of HIV-1 RT contacts the DNA primer strand and positions the template strand near the RNase H active site, influencing RNase H cleavage efficiency and specificity [74].

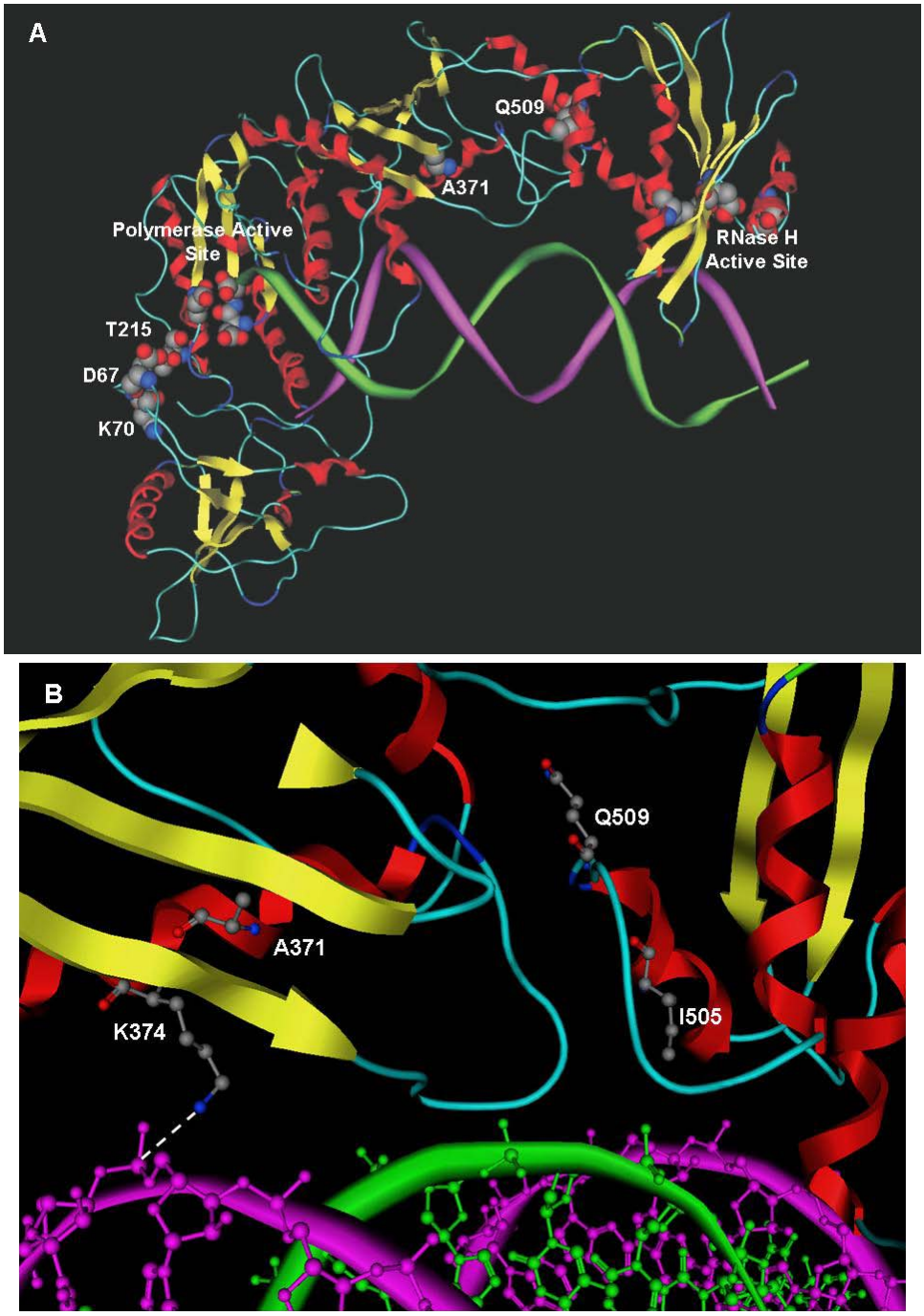


Figure 11. Structural representation of AZT selected mutations in the p66 subunit of RT.
(A) Location of residues A371 and Q509 in relation to TAMs D67, K70 and T215, the polymerase active site and the RNase H active site. (B) Both A371 and Q509 are located near the T/P DNA binding tract. A371 is 2.8 Å from K374, whose side chain interacts with the phosphate backbone of the RNA template strand (white line). Q509 is in proximity to I505, a residue that makes up the RNase H primer grip. p66 subunit of RT: cartoon; DNA Primer: green ribbon; RNA Template: purple ribbon. Structure drawn using MOE, based on coordinates from Sarafianos *et al*., 2001, EMBO J 20:1449. Pdb access number: 1HYS.

3.6 DISCUSSION

In this study we show that AZT selects novel mutations in RT, namely A371V in the connection domain and Q509L in the RNase H domain, that increase AZT resistance by ~50-fold with TAMs in the polymerase domain: D67N, K70R and T215I/F. This provides the first definitive virologic evidence that mutations in both the connection and RNase H domains are biologically relevant for HIV-1 resistance to AZT. In addition, we show that these mutations when combined with TAMs confer greater cross-resistance to 3TC and ABC, with a trend toward greater TNV resistance.

The only mutations that arose during the selection that started with AZT-resistant virus encoding the TAMs M41L, L210W and T215Y were D67N and L214F in the polymerase domain (Table 2), which increased AZT resistance by >1,332-fold at passage 35. No mutations were detected in the connection or RNase H domains. This indicates that very high-level AZT resistance is possible with mutations restricted to the polymerase domain, and suggests that A371V and Q509L mutations are only advantageous in certain TAM backgrounds.

Other evidence suggests that mutations outside of the polymerase domain of HIV-1 RT are involved in resistance to NRTI. For example, Nikolenko *et al* recently demonstrated that mutations that reduce RNase H activity, such as D549N and H539N, increase AZT resistance [171], but these mutations have not been identified in viruses from antiretroviral-experienced patients nor have they been selected by AZT *in vitro*. Initial analyses of clinical samples, however, have identified mutations in the connection and RNase H domains of RT that can increase AZT resistance [175-177]. For example, mutations G335C, N348I and A360I reduce AZT susceptibility 30-, 35- and 30-fold, respectively; when present with TAMs [175, 176]. In

addition, a polymorphism at RT amino acid 333 (G to E) has been observed in samples from patients on combination therapy with AZT and 3TC [163]. The G333E polymorphism counteracts the increase in AZT sensitivity of virus with the 3TC resistance mutation, M184V [163, 178].

Several retrospective statistical analyses of clinical genotype databases have also identified mutations in the connection and RNase H domains of RT that appear more frequently in samples from antiretroviral-experienced patients than antiretroviral-naïve patients [177, 179, 180]. However, the roles of these mutations in NRTI resistance have not been proven. The A371V and Q509L mutations have been identified in patient genotypes in the Stanford HIV Drug Resistance Database [180], and our preliminary analysis of this database reveals that patients treated with AZT show an increase in frequency of several mutations in the C-terminus of RT (amino acids 350-560). For example, A371V was detected in 5.5 % of 1509 subtype B samples from treatment-naïve individuals, and in 10.7% of 84 samples from subtype B infected patients treated with AZT monotherapy. Another mutation at codon 371 (A to T) is also seen at 2.3% frequency in AZT monotherapy samples. In addition, A371V is associated with mutations at T215 (F/I/S/Y) in 77% of the AZT monotherapy samples, and with 46%, 23%, 31%, 23% and 15% of the samples with M41L, D67N, K70R, L210W and K219Q, respectively. Only 14 full-length sequences (to codon 560) are available in the Stanford database, and none of these have mutations at codon 509. Additional full-length RT sequences from patients who have received AZT therapy are being generated to examine the RNase H domain including codon 509.

Two phenotypic mechanisms of NRTI resistance have been proposed. The first is NRTI discrimination and involves mutations in RT (such as K65R, K70E, L74V, Q151M and M184V) that enable RT to preferentially incorporate the natural dNTP substrate versus the NRTI-TP [158,

159, 181-183]. The second mechanism has been termed NRTI excision associated with TAMs. The available biochemical evidence suggests that TAMs increase the ability of HIV-1 RT to phosphorolytically excise AZT-monophosphate (AZT-MP) from the chain-terminated T/P [65, 184]. Because A371V and Q509L were selected in combination with TAMs and do not confer resistance to AZT alone, we hypothesize that these mutations enhance the RT-mediated excision reaction. Furthermore, the relatively small increases in cross-resistance to 3TC, ABC and TNV in viruses having the A371V and Q509L mutations suggest that these mutations may be largely specific for AZT.

Analysis of the crystal structure of RT bound to an RNA/DNA T/P showed that A371V and Q509L reside close to the DNA-binding tract in RT (Figure 11). This suggests that the mutations may affect either T/P interactions (in the case of A371V and Q509L) or RNase H activity (in the case of Q509L). With regard to the latter, several studies have clearly demonstrated that mutations in the RNase primer grip can significantly impact the rates and efficiency of RNase H cleavage [75, 169].

Because there was only a small difference between the IC_{50} values of viruses with D67N/K70R/A371V/Q509L and D67N/K70R/T215I/A371V/Q509L (Table 4), replication capacity and kinetics were performed to determine whether the T215I mutation affected viral replication capacity/kinetics. Single-cycle and multiple-cycle replication assays clearly showed that the T215I mutation restored replication capacity and kinetics of the D67N/K70R/T215I/ A371V/Q509L mutant to wild-type levels (Figure 10). This likely explains why the T215I mutant emerged without having a significant impact on AZT resistance. The T215I mutation was subsequently replaced by T215F at higher AZT selective concentrations. This replacement is likely explained by the T215F mutation conferring ~25-fold greater AZT resistance than T215I.

In summary, we have selected mutations *in vitro* in the 3' region of RT that increase AZT resistance. Biochemical analyses will be discussed in Chapter 2 to define the mechanisms involved, and studies of clinical isolates are discussed in Chapters 3-5 to define the occurrence and clinical significance of the mutations.

4 CHAPTER TWO. MECHANISM BY WHICH A GLUTAMINE TO LEUCINE SUBSTITUTION AT RESIDUE 509 IN THE RIBONUCLEASE H DOMAIN OF HIV-1 REVERSE TRANSCRIPTASE CONFERS ZIDOVUDINE RESISTANCE

Jessica H. Brehm[‡,§], John W. Mellors[‡], Nicolas Sluis-Cremer[‡]

‡ University of Pittsburgh, Department of Medicine, Division of Infectious Diseases, Pittsburgh, Pennsylvania 15261;§ University of Pittsburgh, Graduate School of Public Health, Department of Infectious Diseases and Microbiology, Pittsburgh, Pennsylvania 15261

4.1 PREFACE

This chapter has been reprinted and adapted with permission from (Brehm, J. H., J. W. Mellors, and N. Sluis-Cremer. 2008. Mechanism by which a Glutamine to Leucine Substitution at Residue 509 in the Ribonuclease H Domain of HIV-1 Reverse Transcriptase Confers Zidovudine Resistance, Biochemistry 47(52), 14020-14027). Copyright 2008 American Chemical Society.

Additionally, this study was presented in part as an oral abstract at the 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, February 2007 (Brehm, J., D. Koontz, S. Zelina, N. Sluis-Cremer, and J. Mellors. 2007. 3'-Azido-3'-Dideoxythimidine (AZT) Selects Mutations in the Connection (A371V) and RNase H (Q509L) Domains of Reverse Transcriptase that Increase AZT Resistance in Combination with Thymidine Analog Mutations without Affecting the Rate of AZT Excision on a DNA/DNA Template/Primer); in part as an oral abstract at the XVI International HIV Drug Resistance Workshop, Hilton Barbados, Barbados, June 2007 (abstract published in Brehm, J., D. Koontz , N. Sluis-Cremer and J. Mellors. 2007. HIV-1 Reverse Transcriptase Mutations A371V and Q509L Decrease DNA-Dependent RNase H Cleavage and Increase the Rate of AZT-MP Excision, Antiviral Therapy 12, S124); in part as an oral abstract at the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 2008 (Brehm, J., N. Sluis-Cremer and J. Mellors. 2008. Molecular Mechanisms for 3'-Azido-3'-Dideoxythymidine-resistance Conferred by Mutations in the Connection and RNase H Domains of HIV-1 Reverse Transcriptase); and in part as an oral abstract at the XVII International HIV Drug Resistance Workshop, Sitges, Spain, June 2008 (abstract published in Brehm, J., J. Mellors and N. Sluis-Cremer. 2008. Q509L in HIV-1 RT

Increases AZT Resistance by Promoting Polymerase-Competent versus RNase H-Competent Binding on RNA/DNA T/P with Short Duplex Lengths. Antiviral Therapy 13, A46).

The work presented in this chapter is in partial fulfillment of dissertation aim 2.

4.2 ABSTRACT

We recently reported that zidovudine (AZT) selected for the Q509L mutation in the ribonuclease H (RNase H) domain of HIV-1 reverse transcriptase (RT), which increases resistance to AZT in combination with the thymidine analog mutations D67N, K70R and T215F. In the current study, we have defined the mechanism by which Q509L confers AZT resistance by performing in-depth biochemical analyses of wildtype, D67N/K70R/T215F and D67N/K70R/T215F/Q509L HIV-1 RT. Our results show that Q509L increases AZT-monophosphate (AZT-MP) excision activity of RT on RNA/DNA template/primers (T/Ps) but not DNA/DNA T/Ps. This increase in excision activity on the RNA/DNA T/P is due to Q509L decreasing a secondary RNase H cleavage event that reduces the RNA/DNA duplex length to 10 nucleotides and significantly impairs the enzyme's ability to excise the chain-terminating nucleotide. Pre-steady-state kinetic analyses indicate that Q509L does not affect initial rates of the polymerase-directed RNase H activity but only polymerase-independent cleavages that occur after a T/P dissociation event. Further, competition binding assays suggest that Q509L decreases the affinity of the enzyme to bind T/P with duplex lengths less than 18 nucleotides in the polymerase-independent RNase H cleavage mode, while not affecting the enzyme's affinity to bind the same T/P in an AZT-MP excision competent mode. Taken together, this study provides the first mechanistic insights into how a mutation in the RNase H domain of RT increases AZT resistance and highlights how the polymerase and RNase H domains of RT function in concert to confer drug resistance.

4.3 GOAL OF STUDY

The molecular mechanisms by which thymidine analog mutations (TAMs) confer zidovudine (AZT) resistance have been well defined (see [185, 186] for recent reviews). By contrast, the biochemical mechanisms by which mutations outside of the DNA polymerase domain of reverse transcriptase (RT) augment AZT resistance have not been thoroughly evaluated. Recent studies by our group and others have investigated novel RT inhibitor resistance mutations in the connection domain of HIV-1 RT [165, 187-189]. To our knowledge, no studies have investigated the mechanism(s) by which mutations in the RNase H domain of RT confer drug resistance. In the current study, we sought to address this issue by determining the mechanism(s) by which Q509L in HIV-1 RT increases AZT resistance when combined with D67N, K70R and T215F.

4.4 MATERIALS AND METHODS

4.4.1 Reagents

Wildtype (WT) and mutant HIV-1 RTs were constructed, over-expressed in bacteria and purified to homogeneity, as reported previously [174, 190]. The protein concentration of the purified enzymes was determined spectrophotometrically at 280 nm using an extinction co-efficient (ε_{280}) of 260450 $M^{-1}cm^{-1}$, and by Bradford protein assays (Sigma-Aldrich, St. Louis, MO). The RNA- and DNA-dependent DNA polymerase activities of the purified WT and mutant enzymes were essentially identical (data not shown). 3'-azido-2', 3'-dideoxythymidine triphosphate (AZT-TP)

was purchased from TriLink Biotechnologies (San Diego, CA). [^{3}H]dTTP and dNTPs were purchased from GE Healthcare (Piscataway, NJ), and [γ-^{32}P]ATP was acquired from PerkinElmer Life Sciences (Waltham, MA). RNA and DNA oligonucleotides were synthesized by IDT (Coralville, IA).

4.4.2 Inhibition of WT and mutant RT by AZT-TP

Fixed time point assays were used to determine HIV-1 RT-associated RNA-dependent DNA polymerase activity, as reported previously [191]. Briefly, reactions were carried out in 50 mM Tris-HCl pH 7.5 (37°C), 50 mM KCl, 10 mM $MgCl_2$, 600 nM of poly(rA)-oligo$(dT)_{18}$ (the oligo$(dT)_{18}$ primer was biotinylated on the 5'-end), 25 μM [^{3}H]TTP, and variable concentrations of AZT-TP (0-500 nM). Reactions were initiated by the addition of 25 nM of RT, incubated for 20 min at 37°C and then quenched with 0.5 M EDTA. Streptavidin Scintillation Proximity Assay beads (GE Healthcare, Piscataway, NJ) were then added to each reaction, and the extent of radionucleotide incorporation was determined by scintillation spectrometry using a 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer, Waltham, MA).

4.4.3 AZT-monophosphate (AZT-MP) excision assays

A 26 nucleotide DNA primer (P; 5'-CCTGTTCGGGCGCCACTGCTAGAGAT-3') was 5'-radiolabeled with [γ-^{32}P] ATP and chain-terminated with AZT-MP to generate P_{AZT} as reported previously [152, 183]. P_{AZT} was then annealed to either a 35 nucleotide DNA (T_{DNA}; 5'-

AGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3') or RNA (T_{RNA}: 5'-AGAAUGGAAAAUCUCUAGCAGUGGCGCCCGAACAG-3') template. ATP-mediated AZT-MP excision assays were carried out by first incubating 20 nM T_{RNA}/P_{AZT} or T_{DNA}/P_{AZT} with varying concentrations of ATP, 10 mM $MgCl_2$, 1 μM dTTP and 10 μM ddCTP in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant RT. Aliquots were removed at defined times, quenched with sample loading buffer (98% deionized formamide, 1 mg/ml each of bromophenol blue and xylenecyanol), denatured at 95 °C for 8 min, and then product was resolved from substrate by denaturing polyacrylamide gel electrophoresis and analyzed, as reported previously [152, 183].

Excision assays using the P_{AZT} primer annealed to a series of 3'-recessed templates were also carried out as described previously [192]. Template/primer sequences for T_{RNA}^{18}/P_{AZT}, T_{RNA}^{16}/P_{AZT}, T_{RNA}^{12}/P_{AZT} and T_{RNA}^{10}/P_{AZT} are outlined in Figure 13C. Excision assays were carried out as described above.

4.4.4 Assay for RT RNase H activity

WT and mutant RT RNase H activity was evaluated using the same AZT-MP chain-terminated RNA/DNA T/P substrate described in Materials and Methods section 4.4.3, except the 5′-end of the RNA was ^{32}P-end-labelled. Assays were carried out using 20 nM T_{RNA}/P_{AZT}, 0.3 mM ATP and 10 mM $MgCl_2$ in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant HIV-1 RT. Aliquots were removed, quenched at varying times, and analyzed as described in Materials and Methods section 4.4.3.

4.4.5 Polyacrylamide gel electrophoresis analysis of RT polymerization products formed under continuous DNA polymerization conditions

Heteropolymeric RNA-dependent or DNA-dependent DNA polymerase T/Ps were prepared as reported previously [143, 193]. Briefly, a 230-nt RNA template (T_{RNA-2}) used for continuous round excision assays was constructed by *in vitro* transcription using T7 polymerase and MEGAscript® T7 kit (Ambion, Austin, TX). A 214-nt DNA template (T_{DNA-2}) used for continuous round excision assays was constructed by PCR amplification using a previously described protocol [143]. Concentration of both T_{RNA-2} and T_{DNA-2} were calculated spectrophotometrically. An 18-nt primer (P_{18}) was constructed to anneal to both T_{RNA-2} and T_{DNA-2} to be used for continuous polymerization assays: 5'-GTCCCTGTTCGGGCGCCA-3'.

DNA polymerization reactions were carried out by incubating 20 nM heteropolymeric T_{RNA-2}/P_{18} or T_{DNA-2}/P_{18} complex with 1 μM concentration of each dNTP, 2 μM of AZT-TP, 3 mM ATP and 10 mM $MgCl_2$ in buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant RT. After defined incubation periods, aliquots were removed from the reaction tube and quenched with equal volumes of gel loading dye. Products were separated by denaturing gel electrophoresis and quantified, as described in Materials and Methods section 4.4.3.

4.4.6 Pre-steady- state kinetic analyses of RNase H cleavage

Transient kinetic analyses were used to determine the initial rates of WT and mutant RT RNase H activity. All reactions described below were carried out using an RQF-3 Rapid Quench Instrument (Kintek Corporation, Clarence, PA). A 20 μL solution of 40 nM of T_{RNA}/P_{AZT} and 400 nM WT or mutant RT in 50 mM Tris-HCl (pH 7.5), 50 mM KCl and 2 mM EDTA was rapidly mixed with a 20 μL solution of 50 mM Tris-HCl (pH 7.5), 50 mM KCl containing 22 mM $MgCl_2$. The final concentrations of RT, T/P and $MgCl_2$ in the reaction were 200 nM, 20 nM and 10 mM, respectively. Reactions were quenched by mixing with 50 μl of 50 mM EDTA at times ranging from 17.5 ms to 30 s. Products were separated from substrates by denaturing gel electrophoresis and processed as described in Materials and Methods section 4.4.3.

4.4.7 Competition assays for T/P binding to WT or mutant RT

Competition binding assays were used to evaluate the affinity of WT or mutant HIV-1 RT for a T_{RNA}/P_{AZT} T/P that has a duplex length of 16 nucleotides (T_{RNA}^{16}/P_{AZT}; see Figure 13C for sequence). Either P_{AZT} or T_{RNA}^{16} was 5'end labeled with [γ-^{32}P]-ATP prior to T/P annealing to allow detection of excision or RNase H activity, respectively. 200 nM WT or mutant RT was first pre-incubated at 37 ˚C with 20 nM of T_{RNA}^{16}/P_{AZT} in 50mM Tris-HCl (pH 7.5), 50mM KCl and 0.5 mM EDTA for 15 min before the addition of 10.5 mM $MgCl_2$ and 3 mM ATP and varying concentrations (0-8 μM) of a nucleic acid trap (T_{RNA}^{16}/P that was not radioactively labeled or AZT-MP terminated). The residual RNase H or AZT-MP excision activities were

evaluated after 20 min or 120 min, respectively. Samples were processed as described in Materials and Methods section 4.4.3.

4.5 RESULTS

Two distinct mechanisms of HIV-1 resistance to NRTIs have been described [185, 186]. The mutations K65R, K70E, L74V, Q151M and M184V increase the selectivity of RT for incorporation of the natural dNTP substrate *versus* the NRTI-triphosphate (NRTI-TP) [154, 183, 194-196]. By comparison, TAMs, which include M41L, D67N, K70R, L210W, T215F/Y and K219Q/E, increase the ability of HIV-1 RT to excise a chain-terminating NRTI-monophosphate (NRTI-MP) from a DNA chain [139, 143]. In the experiments described below, we examined both the discrimination and excision phenotypes to elucidate the mechanism(s) by which Q509L confers zidovudine resistance. The enzymes included in this study were: wildtype (WT) RT, D67N/K70R/T215F (AZT^R) RT, AZT^R/A371V RT, AZT^R/Q509L RT and AZT^R/A371V/Q509L RT.

4.5.1 Incorporation of AZT-TP by WT and mutant HIV-1 RT

To determine whether the A371V and/or Q509L mutation affects the ability of RT to discriminate against the incoming nucleotide analog, we determined the concentration of AZT-TP required to inhibit the incorporation of dTTP into the homopolymeric poly(rA)-oligo$(dT)_{18}$

T/P by WT or mutant enzymes under steady-state assay conditions. The data show that each of the five recombinant enzymes was equally sensitive to inhibition by AZT-TP (Table 6), indicating that A371V or Q509L individually or in combination does not confer zidovudine resistance via a discrimination phenotype.

Table 6. Inhibition of WT, AZT^R, AZT^R/A371V, AZT^R/Q509L and AZT^R/A371V/Q509L HIV-1 RT by AZT-TP

Enzyme	IC_{50} (nM)[a]	Fold-Resistance[b]	p-value[c]
WT	291 ± 67	—	—
AZT^R	301 ± 133	1.0	0.91
AZT^R/A371V	220 ± 17	0.76	0.15
AZT^R/Q509L	273 ± 75	0.94	0.77
AZT^R/A371V/Q509L	257 ± 30	0.88	0.47

[a] Reported values are the mean and standard error of 3 independent experiments.
[b] Fold-resistance of mutant RT compared to WT RT.
[c] Mutant RT IC_{50} values were compared to WT IC_{50} for statistically significant differences using a two-sided Student's *t* test.

4.5.2 Excision of AZT-MP by WT and mutant HIV-1 RT from chain-terminated T/Ps

To determine whether A371V or Q509L independently or together directly altered the efficiency of AZT-MP excision, we investigated the ability of WT and mutant RTs to excise AZT-MP from chain-terminated DNA/DNA and RNA/DNA T/P at both high (3 mM) and low (0.3 mM) concentrations of ATP (Figure 12). These experiments allowed the determination of an apparent rate constant for AZT-MP excision ($k_{excision}$) and also the burst or total concentration of excision product generated during the reaction (Table 7).

Consistent with prior reports, AZT^R RT was more efficient than WT enzyme in excising AZT-MP from the 3'-end of the primer on both DNA/DNA and RNA/DNA T/P [152, 165, 187, 197]. In comparison with the WT enzyme, the rates of AZT-MP excision for AZT^R RT were increased 2.1- and 4.7-fold at 0.3 mM and 3.0 mM ATP, respectively, on DNA/DNA T/P; and 1.4- and 3.5-fold at 0.3 and 3 mM ATP, respectively, on the RNA/DNA T/P (Figure 12; Table 7). The efficiency of AZT-MP excision by AZT^R/A371V RT, AZT^R/Q509L and AZT^R/A371V/Q509L RT was identical to that of the AZT^R enzyme on DNA/DNA T/P, and this result was independent of the ATP concentration used in the assay (Figure 12A; Table 7). By contrast, differences in AZT-MP excision between the AZT^R and AZT^R/Q509L or AZT^R/A371V/Q509L enzymes were evident on the RNA/DNA T/P at low but not high concentrations of ATP (Figure 12B). At 0.3mM ATP, the AZT^R/Q509L and AZT^R/A371V/Q509L RT were more efficient at excising AZT-MP than the AZT^R enzyme, and this increase in excision efficiency was driven predominantly by an increase in the burst concentration and not by an increase in rate (Table 7). At 3 mM ATP, there was no difference in excision activity between the AZT^R/Q509L, AZT^R/A371V/Q509L and AZT^R enzymes. For RT

containing AZT^R and AZT^R/A371V mutations, there was no difference in burst or rate in AZT-MP excision regardless of ATP concentration or T/P used (Figure 12 and Table 7). Since A371V did not have an effect on dNTP discrimination or AZT-MP excision when in the context of TAMs, we decided to focus on the mechanism for AZT^R/Q509L RT for the remainder of this study.

Table 7. Kinetic rate constants for AZT-MP excision by WT and mutant HIV-1 RT at 0.3 mM and 3 mM ATP

Enzyme	0.3 mM ATP				3 mM ATP			
	DNA/DNA T/P		RNA/DNA T/P		DNA/DNA T/P		RNA/DNA T/P	
	Burst [a] (nM)	$k_{excision}$ [a] (min^{-1})	Burst (nM)	$k_{excision}$ (min^{-1})	Burst (nM)	$k_{excision}$ (min^{-1})	Burst (nM)	$k_{excision}$ (min^{-1})
Wild type	10.2 ± 2.1	0.029 ± 0.011	5.7 ± 0.7	0.025 ± 0.002	18.6 ± 2.2	0.051 ± 0.012	14.3 ± 1.6	0.031 ± 0.016
AZT^{R} [b]	17.9 ± 1.0	0.060 ± 0.011	12.8 ± 1.2	0.036 ± 0.006	19.1 ± 0.6	0.24 ± 0.09	19.1 ± 0.7	0.109 ± 0.020
AZT^{R}/A371V	18.0 ± 0.9	0.059 ± 0.006	13.6 ± 1.4	0.035 ± 0.009	19.3[c]	0.24[c]	18.2 ± 0.7	0.131 ± 0.025
AZT^{R}/Q509L	18.1 ± 0.9	0.059 ± 0.005	**16.1 ± 0.7**	**0.032 ± 0.006**	19.0 ± 0.2	0.31 ± 0.05	19.0 ± 0.3	0.135 ± 0.013
AZT^{R}/A371V/ Q509L	18.1 ± 0.6	0.054 ± 0.004	**15.6 ± 0.6**	**0.029 ± 0.004**	19.1 ± 0.6	0.18 ± 0.02	18.5 ± 0.9	0.100 ± 0.020

[a] Apparent excision rate constants ($k_{excision}$) were determined by fitting the excision isotherms in Figure 1 to the equation: $[product] = A[exp(-k_{excision}t)]$, where A represents the amplitude for product formation (i.e. burst). Data is the mean ± standard deviation from 3-4 independent experiments.

[b] AZT^{R} = RT containing AZT resistance mutations D67N/K70R/T215F.

[c] Assay only completed one time.

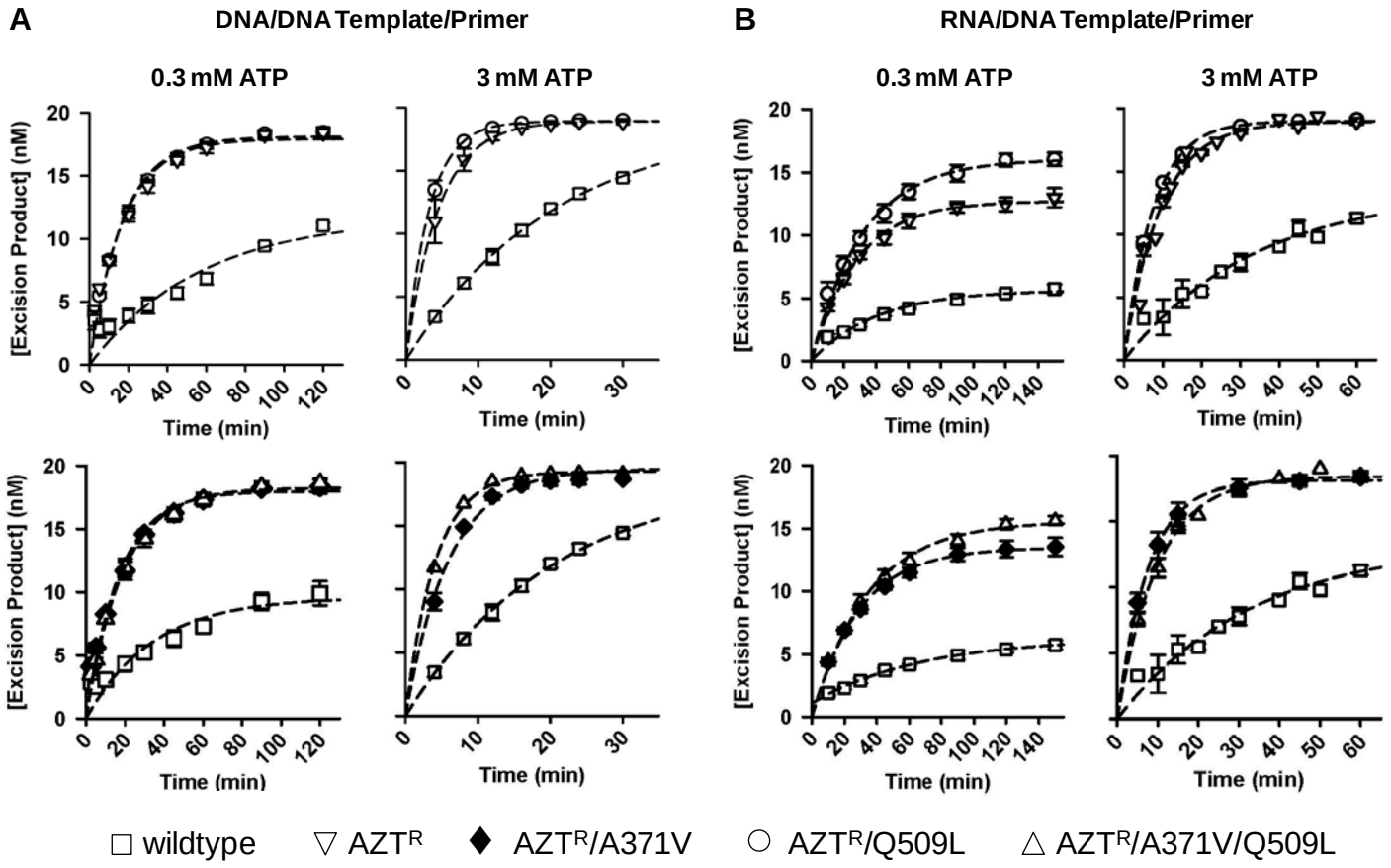


Figure 12. AZT-MP excision by WT and mutant HIV-1 RT at 0.3 mM and 3 mM ATP.
(A) ATP-mediated excision of AZT-MP from DNA/DNA T/P by WT and mutant HIV-1 RT at 0.3 mM and 3 mM ATP. WT, AZT^R, AZT^R/A371V, AZT^R/Q509L and AZT^R/A371V/Q509L RT are represented by the symbols □, ▽, ♦, o and △, respectively. Error bars represent standard errors from 2-5 repeated experiments. Some error bars are smaller than the size of the symbol. (B) ATP-mediated excision of AZT-MP from RNA/DNA T/P by WT and mutant HIV-1 RT at 0.3 mM and 3 mM ATP. The symbols for all five enzymes are the same as in A. Apparent excision rate constants ($k_{excision,}$ shown in Table 9) were determined by fitting the excision isotherms to the equation: $[product] = A[exp(-k_{excision}t)]$, where *A* represents the amplitude for product formation.

4.5.3 RNA template degradation by WT and mutant HIV-1 RT during the AZT-MP excision reaction

The data described above suggested to us that Q509L did not exert a direct effect on ATP-mediated excision because there was no change in the rates at which HIV-1 RT unblocked the AZT-MP chain-terminated primer on either DNA/DNA or RNA/DNA T/P. Instead, we only observed increased excision on RNA/DNA T/P at low ATP concentrations where the rates of ATP-mediated excision are slow (Table 7). Because Nikolenko *et al* hypothesized that mutations that decrease RNase H activity of RT may increase AZT resistance by limiting RNA template degradation [171], we next evaluated the RNase H activity that occurred during the ATP-mediated excision reaction by WT and mutant RTs, and also determined whether these cleavage events affected the efficiency of the excision reaction.

Figure 13A shows autoradiograms of the RNase H products generated during ATP-mediated excision assays by WT, AZT^R and AZT^R/Q509L RT. In comparison with the other two enzymes, AZT^R/Q509L RT accumulated more cleavage product with RNA/DNA duplex length of 15 or 16 nucleotides. There was also a significant decrease in the rate of appearance of a cleavage event that reduces the RNA/DNA duplex length to 10 nucleotides (Figure 13A, 13B); the apparent rate constants for this RNase cleavage event were calculated to be 0.034 min^{-1}, 0.036 min^{-1} and 0.016 $min^{-1}$for the WT, AZT^R and AZT^R/Q509L RTs, respectively.

To explore the relationship between the efficiency of AZT-MP excision and RNase H activity, we next evaluated the ability of WT and mutant HIV-1 RT to excise AZT-MP from a chain-terminated DNA primer that was annealed to different RNA templates that were recessed from the 3'-end, mimicking the T/P products generated by RNase H cleavage, as described

previously [192]. Consistent with our previous findings ([192]; Figure 13C), these analyses demonstrated that the efficiency of AZT-MP excision by WT and mutant RT was severely reduced when the RNA/DNA duplex length was decreased to 10 nucleotides - the duplex length arising from the secondary RNase H cleavage event described in Figure 13A. The finding that Q509L significantly decreases the formation of this 10 nucleotide duplex provides one mechanism by which this mutation enhances AZT-MP excision. It should also be noted that the efficiency of AZT-MP excision by AZT^R/Q509L RT on the 10 nucleotide duplex was significantly greater than that of either WT or AZT^R enzymes (Figure 13C), indicating a second mechanism whereby Q509L enhances AZT-MP excision.

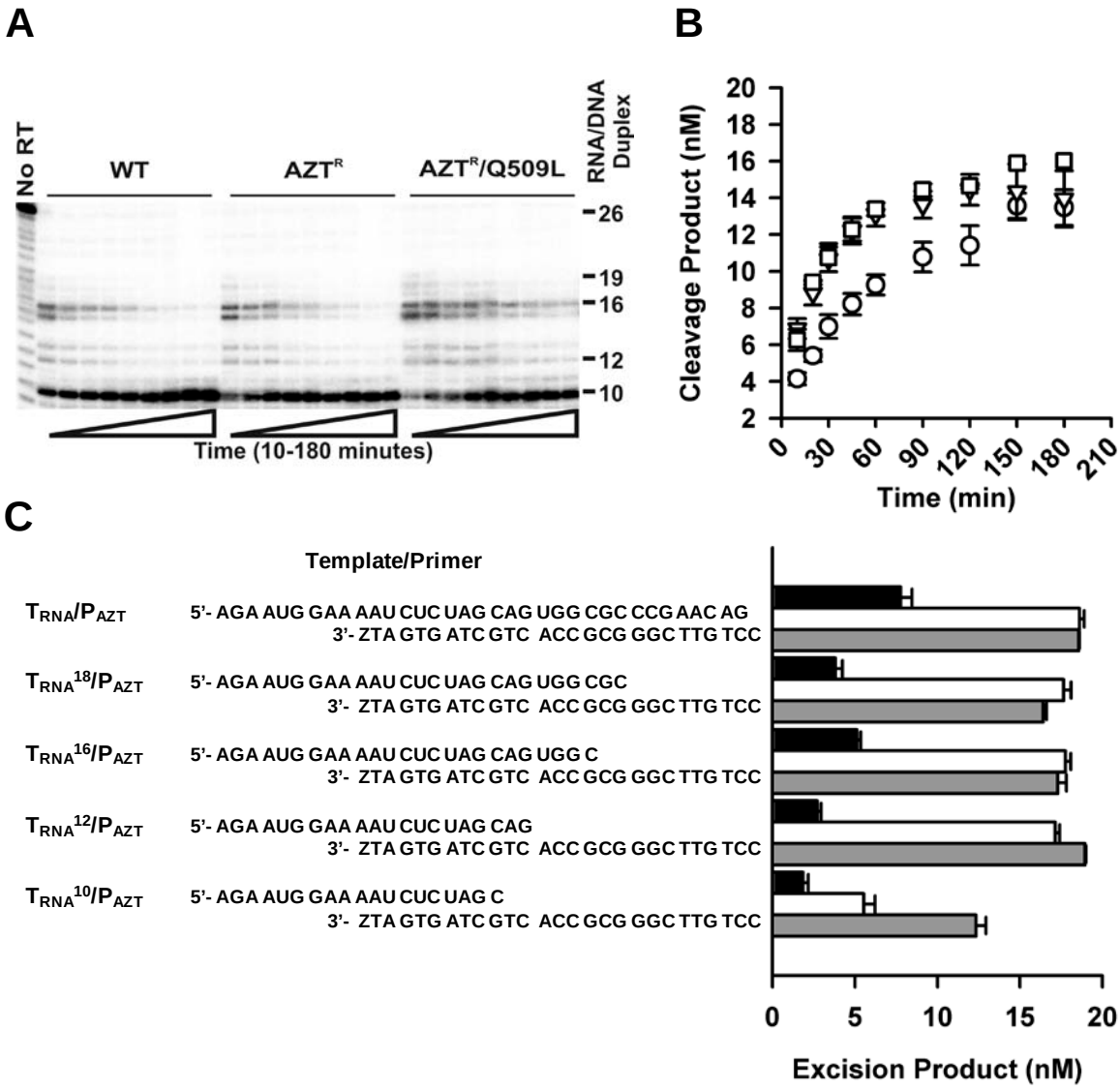


Figure 13. RNA template degradation by WT and mutant HIV-1 RT and AZT-MP excision on short RNA templates.

(A) Representative autoradiogram of RNase H activity of WT and mutant HIV-1 RT during ATP-mediated AZT-MP excision. The time points in the experiments were 10, 20, 30, 45, 60, 90, 120, 150 and 180 min, respectively. (B) Isotherm for the rate of appearance of the T_{RNA}^{10}/P_{AZT} RNase H cleavage product generated by WT and mutant HIV-1 during ATP-mediated AZT-MP excision. The intensity of the T_{RNA}^{10}/P_{AZT} RNase H cleavage product was determined by densiometric analyses using Bio-Rad GS525 Molecular Imager FX software. WT, AZT^R and AZT^R/Q509L RT are represented by the symbols □, ∇ and o, respectively. Data was fit to the equation *[cleavage product] = A[exp(-k_{RNaseH}t)]*, where *A* represents the amplitude for product formation and k_{RNaseH} is the apparent rate for RNase H cleavage. Error bars represent standard errors from 3 separate experiments. (C) Ability of WT and mutant RT to excise AZT-MP on RNA/DNA T/P with decreasing duplex lengths. The assay incubation time was 30 min. WT, AZT^R and AZT^R/Q509L RT are represented by black, white and grey bars, respectively. Error bars represent standard errors from 3 repeated experiments.

4.5.4 Cumulative effect of Q509L in assays that evaluate multiple AZT-TP incorporation and AZT-MP excision events

In the experiments described above, we evaluated the AZT-MP excision and RNase H activity of the WT and mutant enzymes on a defined (in terms of sequence and length) T/P. Because both the excision and RNase H activities of RT are likely affected by nucleic acid sequence and length, we next evaluated the ability of WT and mutant enzymes to synthesize DNA in the presence of AZT-TP and ATP using a long heteropolymeric RNA or DNA template, corresponding to the HIV-1 sequence used for (–) strong stop DNA synthesis, primed with a DNA oligonucleotide [143, 193]. The 173-nucleotide incorporation events needed to produce full-length DNA product in this assay system allow for multiple AZT-TP incorporation and AZT-MP excision events during the formation of full-length final product. In the presence of 3 mM ATP, the AZT^R/Q509L was noticeably more efficient than the AZT^R enzyme in synthesizing full-length product on the RNA/DNA T/P but not DNA/DNA T/P (Figure 14). These results reinforce the findings described in Figure 12, and further demonstrate that the Q509L mutation augments zidovudine resistance on an RNA/DNA T/P but not a DNA/DNA T/P.

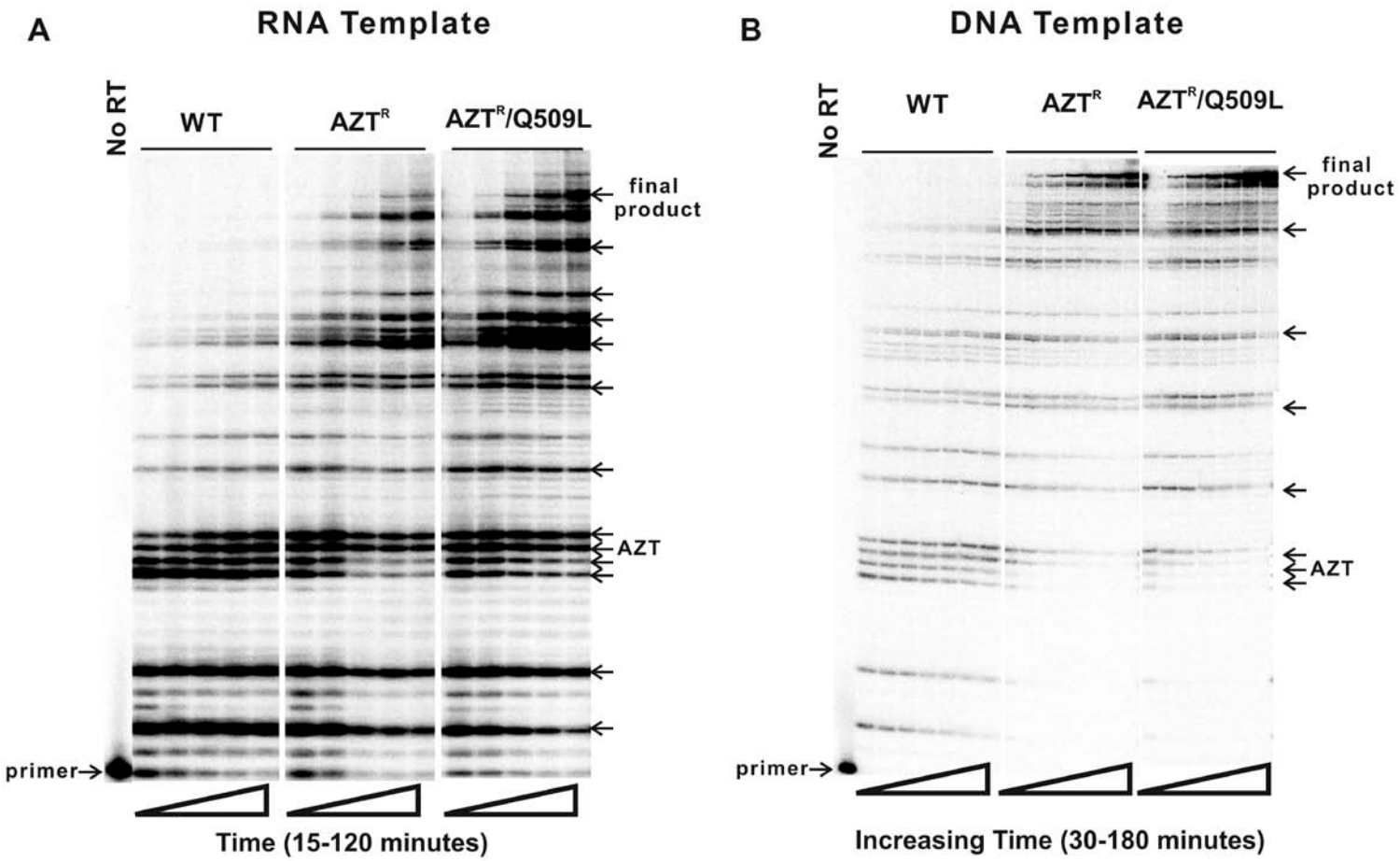


Figure 14. Multiple AZT-TP incorporation and AZT-MP excision events.
Autoradiogram of steady-state DNA synthesis by WT and mutant HIV-1 RT in the presence of AZT-TP and 3mM ATP using an (A) RNA template or (B) DNA template. Experiments were carried out as described in Materials and Methods. The primer, final product, and AZT-MP chain-termination sites are indicated.

4.5.5 Pre-steady-state kinetic analyses of RNase H cleavage by WT and mutant RT

Our analyses of RNase H activity (Figure 13) demonstrated that Q509L in RT decreases the formation of a secondary RNase H cleavage product that reduces the RNA template to 19 nucleotides (nt) and the RNA/DNA T/P duplex length to 10 nt. However, these assays were carried out using AZT-MP excision reaction conditions and RNase H cleavage was monitored only in the minute time range. Thus, these studies did not inform if Q509L also affected the primary RNase H cleavage event, which occurs in the millisecond time range, or the sequence of cleavage and T/P dissociation events that generate the 19 nt RNA product. Accordingly, we used pre-steady-state kinetics to evaluate the rates of initial and secondary RNase H cleavage. The data from these experiments show that the initial rates of RNase H cleavage were similar for the WT, AZT^R and AZT^R/Q509L enzymes (Figure 15A). However, as described above, the rate of appearance of the T_{RNA}^{10}/P_{AZT} was significantly decreased for AZT^R/Q509L RT compared with the WT and AZT^R enzymes (Figure 15B).

It should be noted that in these RNase H assays, the primary cleavage is polymerase-dependent (i.e. the 3'-end of the DNA primer resides in the polymerase active site), but all subsequent cleavages are polymerase-independent (the 3'-end of the primer cannot reside in the polymerase active site if the enzyme is poised for secondary RNase H cleavage). Because the RT-nucleic acid binding interactions must be different between these 2 modes of binding, we next determined whether RT dissociated from the T/P as it transitioned from one mode to the other by adding a nucleic acid trap to the RNase H reaction upon mixing. The results show that no cleavage products are formed that have RNA/DNA duplexes less than 15-nt for either the WT RT (Figure 15C) or for the AZT^R and AZT^R/Q509L enzymes (data not shown). These data

suggest that RT dissociates from $T_{RNA}^{15/16}/P_{AZT}$ and that it must rebind to this substrate in a polymerase-independent, RNase H competent mode to generate the T_{RNA}^{10}/P_{AZT} cleavage product.

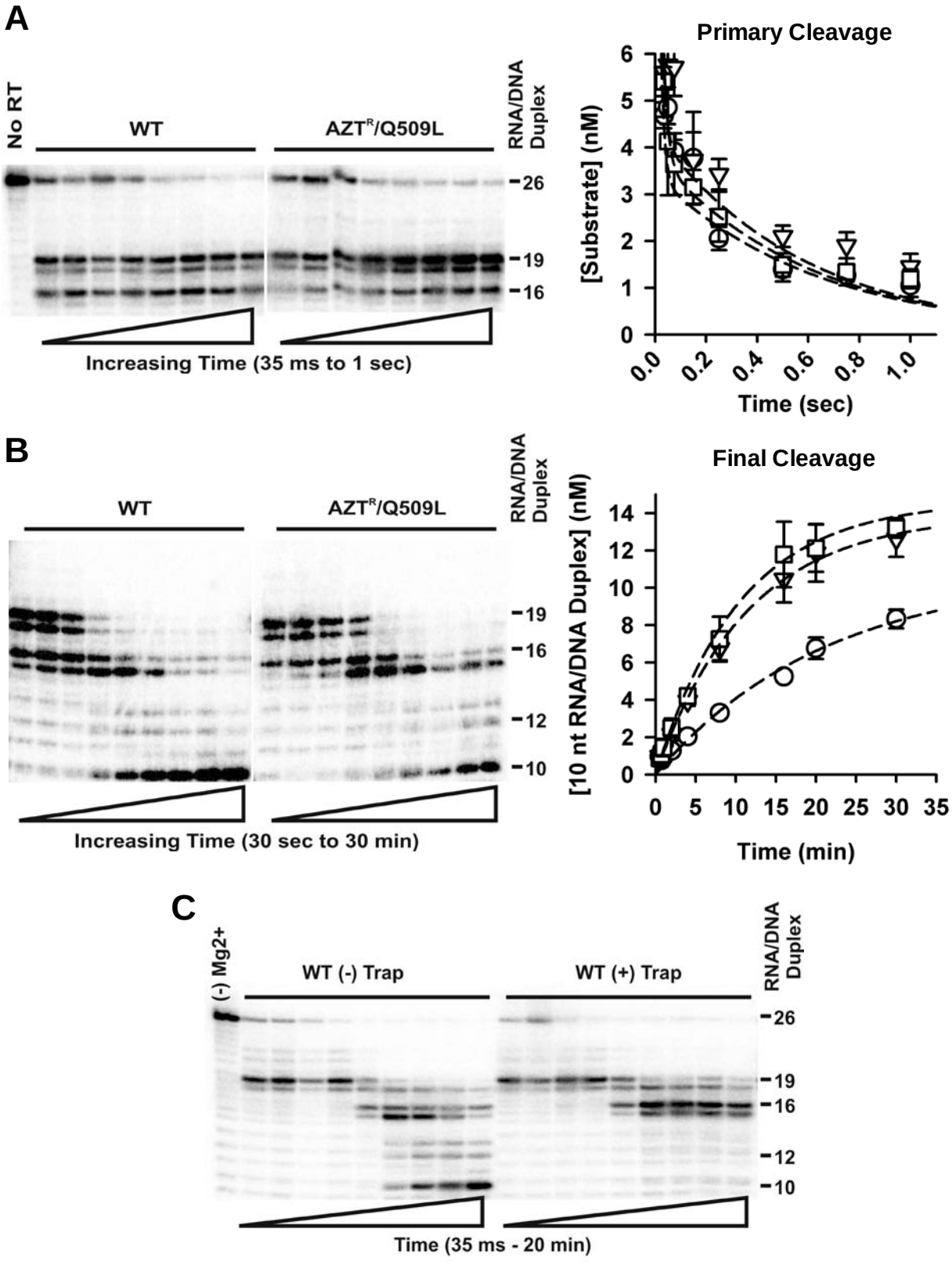

A
No RT
WT
AZT^R/Q509L
RNA/DNA Duplex
26
19
16
Increasing Time (35 ms to 1 sec)
Primary Cleavage
[Substrate] (nM)
Time (sec)
B
WT
AZT^R/Q509L
RNA/DNA Duplex
19
16
12
10
Increasing Time (30 sec to 30 min)
Final Cleavage
[10 nt RNA/DNA Duplex] (nM)
Time (min)
C
(-) Mg2+
WT (-) Trap
WT (+) Trap
RNA/DNA Duplex
26
19
16
12
10
Time (35 ms - 20 min)

Figure 15. Primary and secondary RNase H cleavage events by WT and mutant HIV-1 RT. (A) Representative autoradiogram and isotherm for the rates of primary polymerase-directed RNase H cleavage by WT (□), AZT^R (∇) and AZT^R/Q509L (o) HIV-1 RT. Experiments were carried out as described in Materials and Methods. Primary cleavage was determined by densiometric analyses of the full-length 35 nt RNA template band (or RNA/DNA duplex length of 26 nts) as a function of time (0.035, 0.050, 0.075, 0.15, 0.25, 0.5, 0.75 and 1 s, respectively). Data was fit to the equation $[cleavage\ product] = A[exp(-k_{RNaseH}t)]$, where A represents the amplitude for product formation and k_{RNaseH} is the rate for primary RNase H cleavage. (B) Representative autoradiogram and isotherm for the rate of appearance of the final secondary RNase H cleavage product by WT (□), AZT^R (∇) and AZT^R/Q509L (o) HIV-1 RT. Secondary cleavage was determined by densiometric analyses of the formation of T_{RNA}^{10}/P_{AZT} as a function of time (0.5, 0.75, 1, 2, 4, 8, 16, 20 and 30 min, respectively). Data were analyzed as described above. (C) Representative autoradiogram of RNase H cleavage by WT HIV-1 RT in the absence and presence of a nucleic acid trap. The time points were 0.035 s, 0.050 s, 0.15 s, 1 s, 30 s, 2 min, 4 min, 8 min and 20 min, respectively. Experiment was carried out as described in Materials and Methods.

4.5.6 RT-T/P dissociation from polymerase-dependent and polymerase-independent RNase H cleavage binding modes

Because RT dissociates and rebinds to T_{RNA}^{16}/P_{AZT} to generate T_{RNA}^{10}/P_{AZT}, we hypothesized that Q509L might decrease the efficiency of this cleavage by directly affecting the binding interactions involved. Accordingly, we next assessed the ability of WT or mutant RTs to bind T_{RNA}^{16}/P_{AZT} in an excision-competent mode or polymerase-independent, RNase H-competent mode by measuring AZT-MP excision or RNase H cleavage at defined times after the addition of trap to a pre-formed RT-T/P complex.

For the RNase H competition binding assays, WT or mutant RT was pre-incubated with T_{RNA}^{16}/P_{AZT} for 15 minutes before 10 mM $MgCl_2$ was added to initiate RNase H activity as well as varying concentrations of a nucleic acid trap. Reactions were terminated after 20 minutes and the amount of T_{RNA}^{10}/P_{AZT} formed for each trap concentration was compared to a no trap control. The concentrations of trap required to inhibit 50% of the enzyme's RNase H activity

were calculated to be 4.5 ± 0.4 μM, 4.2 ± 1.4 μM and 2.2 ± 0.8 μM trap for the WT, AZT^R and AZT^R/Q509L RTs, respectively (Figure 16). The lower IC_{50} value for the AZT^R/Q509L RT implies that this enzyme is more sensitive to inhibition by trap and therefore likely dissociates more readily from the T_{RNA}^{16}/ P_{AZT} substrate.

For the AZT-MP excision competition binding assays, WT or mutant RT was pre-incubated with T_{RNA}^{16}/ P_{AZT} for 15 minutes before the reaction was initiated with 3 mM ATP and 10 mM Mg^{2+} and varying concentrations of trap. Reactions were terminated after 120 minutes and the amount of AZT-MP excision product for each trap concentration was again compared to a no trap control. The concentrations of trap required to inhibit 50% of the enzyme's excision activity were calculated to be 47 ± 35 nM, 58 ± 37 nM and 65 ± 20 nM trap for the WT, AZT^R and AZT^R/Q509L RTs, respectively (Figure 16). Since all three enzymes exhibited similar IC_{50} values, we must conclude that Q509L does not affect the binding interaction between RT and T_{RNA}^{16}/ P_{AZT} when the enzyme is bound in a polymerase- or excision-competent mode.

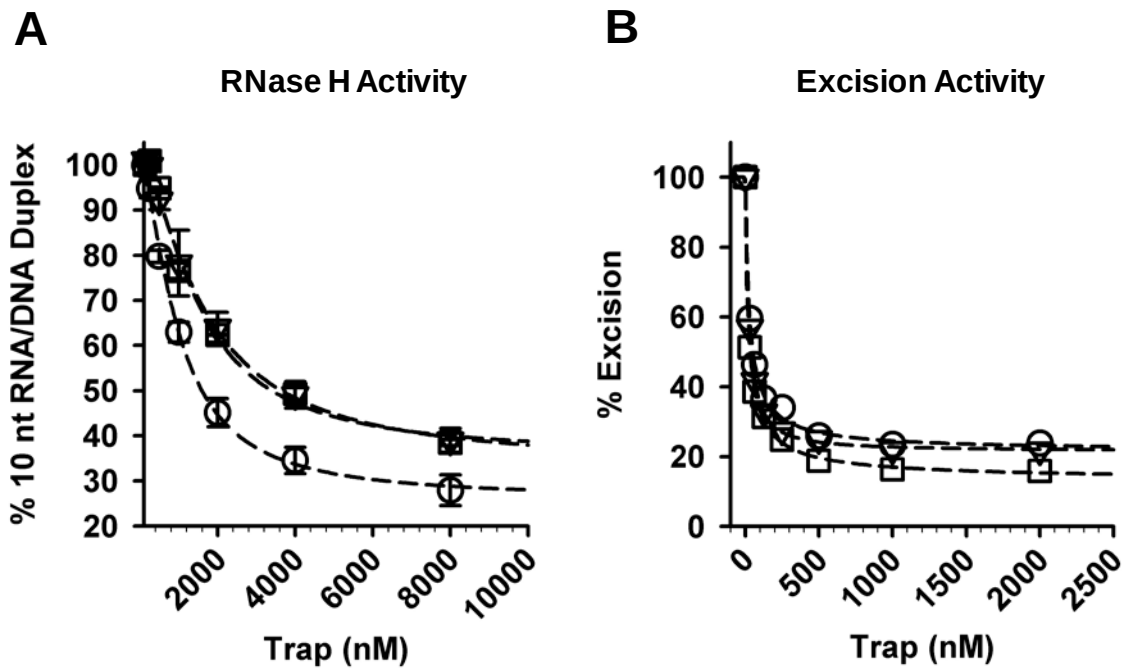


Figure 16. WT and mutant HIV-1 RT dissociation from polymerase-dependent and polymerase-independent RNase H cleavage binding modes.
(A) Sensitivity of WT (□), AZT^R (∇) and AZT^R/Q509L (o) RT to nucleic acid trap when bound to T_{RNA}^{16}/P_{AZT} in an RNase H competent mode. The concentration of trap required to inhibit 50% of the RNase H activity of WT, AZT^R and AZT^R/Q509L RT was calculated to be 4.5 ± 0.4 μM, 4.2 ± 1.4 μM and 2.2 ± 0.8 μM, respectively. (B) Sensitivity of WT (□), AZT^R (∇) and AZT^R/Q509L (o) RT to nucleic acid trap when bound to T_{RNA}^{16}/P_{AZT} in an excision competent mode. The concentration of trap required to inhibit 50% of the ATP-mediated AZT-MP excision activity of WT, AZT^R and AZT^R/Q509L RT was calculated to be 47 ± 35 nM, 58 ± 37 nM and 65 ± 20 nM, respectively. Data from both experiments are an average ± standard deviation of at least 2 independent experiments.

4.6 DISCUSSION

Recent studies of HIV sequence databases [187, 198, 199], and genotypic/phenotypic analyses of clinical isolates from patients failing NRTI therapies [163, 200] have identified several mutations in the connection domain of HIV-1 RT that are strongly associated with RTI resistance. Biochemical studies have demonstrated that these mutations impact NRTI sensitivity by several distinct mechanisms. For example, G333D in HIV-1 RT allows the enzyme to effectively discriminate between the normal substrate dCTP and lamivudine-triphosphate [165]. It also enhances the ability of RT containing TAMs and M184V to bind AZT-MP terminated T/P, thereby restoring ATP-mediated excision of AZT-MP [165]. We and others have also demonstrated that mutations, such as N348I, may increase AZT resistance by decreasing RNA template degradation [187, 189]. In addition, some of these mutations may also directly affect the excision activity of RT by an RNase H independent mechanism [188, 189].

In contrast to the identification of novel resistance mutations in the connection domain of RT, very few mutations in the RNase H domain have been identified that impact RTI resistance. This is likely due to the limited availability of sequence data for this domain from laboratory or clinical isolates. Recently, however, we carried out *in vitro* selections of HIV-1 with AZT and identified the A371V in the connection domain and Q509L mutation in the RNase H domain of RT were selected in combination with D67N, K70R, T215F [201]. Site-directed mutagenesis studies confirmed the role of these mutations in AZT resistance [201], and the goal of the current study was to define the biochemical mechanisms involved.

Our studies demonstrate that the Q509L mutation confers AZT resistance by increasing the AZT-MP excision activity of the enzyme via an RNase H-dependent mechanism. By using a

well-defined RNA/DNA T/P we show that AZT^R/Q509L increases the efficiency of AZT-MP excision by decreasing the frequency of a secondary RNase H cleavage event that reduces the RNA/DNA duplex to 10 nts. The resultant product (T_{RNA}^{10}/P_{AZT}) serves as an inefficient substrate for ATP-mediated excision of AZT-MP by RT. However, even on this T/P, the AZT^R/Q509L enzyme has a significantly better ability to unblock the chain-terminated primer than WT or AZT^R. It should be noted that under our assay conditions, increased excision was only observed at 0.3 mM ATP and not at 3 mM ATP. This is because at 3 mM ATP, the rate of formation of the T_{RNA}^{10}/P_{AZT} product by AZT^R RT is much slower (0.03 min^{-1}) than the rate of AZT-MP excision (0.10 min^{-1}); whereas at 0.3 mM ATP the rates of T_{RNA}^{10}/P_{AZT} product formation and AZT-MP excision (0.03 min^{-1}) are comparable, allowing competition between these two distinct activities. Because the Q509L mutation decreases the rate of T_{RNA}^{10}/P_{AZT} product formation (to 0.01 min^{-1}) it favors AZT-MP excision at 0.3 mM ATP. The fact that the increase in excision efficiency for the AZT^R/Q509L RT, observed in Figure 12B, is driven by an increase in burst concentration and not rate is entirely consistent with a model of competition between RNA template degradation and AZT-MP excision. It is also important to understand that the kinetics of AZT-MP excision are dependent on nucleic acid sequence and structure [202], and therefore Q509L will likely show a greater effect at positions where AZT-MP excision is slow. This hypothesis is supported by the results showing that AZT^R/Q509L RT exhibits a significant advantage over the AZT^R enzyme at 3 mM ATP in assays that evaluate multiple AZT-TP and AZT-MP excision events (Figure 14).

We also addressed the mechanism by which Q509L decreases the RNase H activity of RT. In this regard, Nikolenko *et al* hypothesized that any mutation in RT that directly decreases the RNase H activity of the enzyme will increase AZT resistance by preventing RNA template

degradation [171]. However, the replication fitness of viruses with decreased RNase H activities are likely to be compromised due to the important role of this activity in reverse transcription. In our study, we show by transient kinetic analyses that Q509L does not impact the rates of the initial polymerase-directed RNase H cleavage, but only polymerase-independent cleavages that occur after a T/P dissociation event (Figure 15). By contrast, Delviks-Frankenberry *et al* reported that NRTI-associated mutations in the connection domain of RT affect both the primary and secondary RNase H cleavages of RT [188]. However, these assays were carried out under steady-state assay conditions (i.e. [T/P] >> [RT]) in which the rate-limiting step of the reaction is T/P dissociation and, as such, they do not have the ability to resolve cleavage events that occur in the millisecond time-scale.

In Figure 17 we propose a model, based on our findings, to explain how Q509L decreases the RNase H activity of RT. The initial RNase H cleavages reduce the RNA/DNA duplex between 15 to18 nts in length (Figure 13). Because the distance between the DNA polymerase and RNase H active sites is 18 nts [203], RT has to bind the resultant T/P products in one of two distinct conformations to carry-out either excision or RNase H activity. Thus, after the primary RNase H cleavages and T/P dissociation event, an equilibrium forms in which RT binds the T/P in either of these configurations (Figure 17). Our data show that Q509L selectively decreases the affinity of RT binding to T_{RNA}^{16}/P_{AZT} in an RNase H competent mode, but not in an excision competent mode. The net effect of this is to decrease RNA template degradation and ultimately favor AZT-MP excision. Ehteshami *et al* have proposed a similar model for the N348I and A360V connection domain mutations [189].

RT with A371V did not have an effect on dNTP discrimination when in the context of AZT^R RT or AZT^R/Q509L RT and had a small effect on AZT-MP excision when in the context of

AZT^R/Q509L, thus the mechanism for AZT^R/A371V/Q509L RT may be similar to AZT^R/Q509L RT. Biochemical studies must be completed to confirm this mechanism.

In conclusion, our data provide evidence that Q509L in HIV-1 RT confers AZT resistance by affecting the balance between AZT-MP excision and RNase H activities of RT on RNA/DNA T/P. Q509L does not appear to directly decrease the RNase H activity of RT, rather it affects the enzyme's ability to bind T/P with short RNA/DNA duplexes in a polymerase-independent RNase H cleavage mode. Furthermore, this study, together with other biochemical studies on NRTI resistance mutations in the connection domain of RT, clearly demonstrates that the entire RT molecule functions in concert to confer drug resistance. Consequently, the inclusion of the C-terminal domains of RT in clinical genotype and phenotype assays could lead to more accurate determination of NRTI drug resistance.

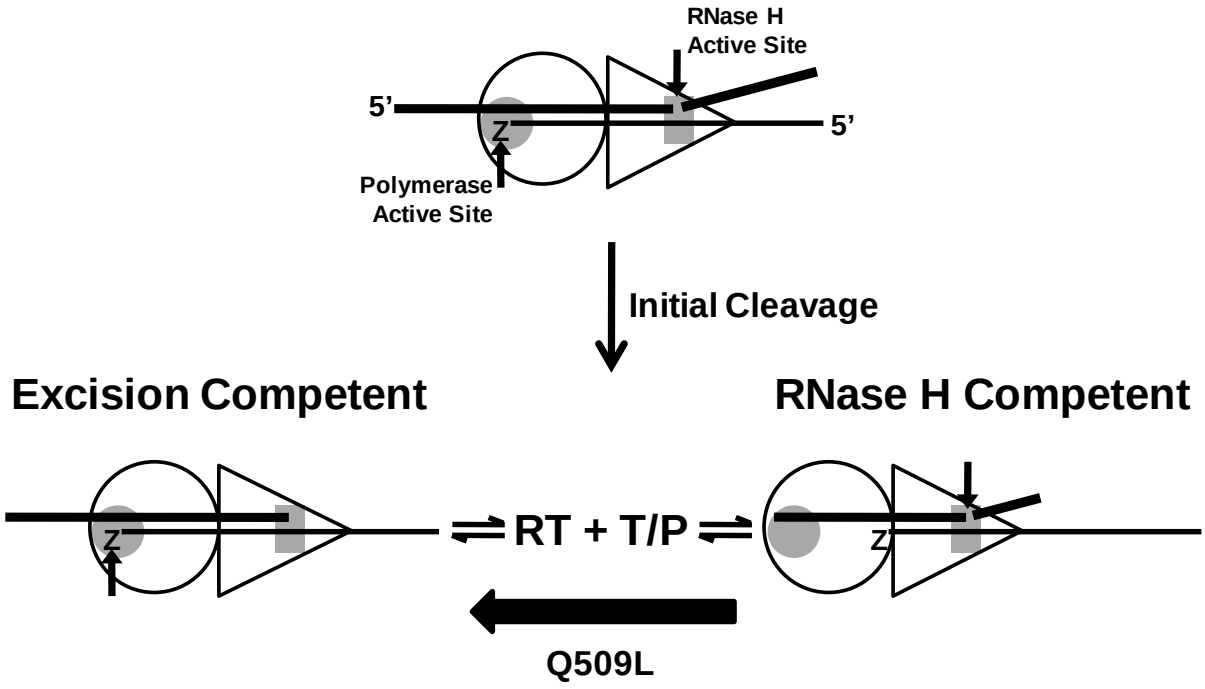


Figure 17. Proposed model for how Q509L in RT affects the balance between AZT-MP excision and RNase H activity on RNA/DNA T/P.
The initial RNase H cleavages reduce the RNA/DNA duplex to 15 to 18 nts in length. Because the distance between the DNA polymerase and RNase H active sites is 18 nts, RT has to bind the resultant T/P products in one of two distinct conformations to carry-out either excision or RNase H activity. Therefore, after the primary RNase H cleavages and T/P dissociation event, an equilibrium forms in which RT binds the T/P in both of these configurations. Q509L selectively decreases the affinity of RT to bind the T_{RNA}^{16}/P_{AZT} in an RNase H competent mode but not an excision competent mode.

5 CHAPTER THREE. EMERGENCE OF A360V IN THE CONNECTION DOMAIN OF HIV-1 REVERSE TRANSCRIPTASE AFTER PROLONGED ZIDOVUDINE MONOTHERAPY

5.1 PREFACE

The work presented in this chapter is in partial fulfillment of dissertation aim 3. All experiments were completed by Jessica Brehm with the exception of HIV-1 RNA assays performed by Kelley Gordon and drug susceptibility assays completed by Dianna Koontz.

5.2 ABSTRACT

We recently reported that HIV-1 reverse transcriptase (RT) connection and RNase H domain mutations A371V and Q509L are selected with thymidine analog mutations (TAMs) by zidovudine (AZT) *in vitro* and increase nucleoside reverse transcriptase inhibitor resistance when present with TAMs. Because it is not known whether A371V or Q509L emerge in HIV-1 infected patients treated with AZT, we compared full-length RT sequences from paired pre-therapy and AZT monotherapy samples in 23 patients from the AZT monotherapy arm of AIDS Clinical Trials Group (ACTG) study 175. In comparing pre-therapy to on therapy sequences, polymerase domain mutations that were significantly associated with AZT monotherapy were K70R (p=0.003) and T215I/Y (p=0.013). The connection domain mutation A360V was significantly associated with AZT monotherapy (p=0.041) and emerged at the same time or after TAMs. A371V was detected before therapy in one patient and during AZT monotherapy in 4 patients (p=0.25), and the Q509L mutation was not observed before or during AZT monotherapy in any patient. Recombinant infectious viral clones were constructed containing either the polymerase domain alone or full-length RT from pre-therapy and on therapy plasma samples from patients in whom the A360V mutation emerged. AZT susceptibility decreased 11-fold in one full-length clone with mutations M41L-L210W-T215Y and A360V compared to the clone with only polymerase domain mutations M41L-L210W-T215Y. No change in AZT resistance was seen in one full-length clone with K70R-A360V mutations. In conclusion, the A360V mutation in the connection domain was significantly associated with AZT monotherapy and conferred higher level AZT resistance than the polymerase domain alone in one patient with

multiple TAMs. The A360V mutation in the connection domain of RT should be included in the list of mutations that are selected by AZT monotherapy.

5.3 GOAL OF STUDY

The most common drug resistance mutations in RT are located in the polymerase domain and thus genotyping tests available for clinical use do not identify mutations in the RT connection or RNase H domains. However, recent studies have shown that mutations in the RT connection and RNase H domains can cause resistance to nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) alone (Table 8) or in combination with mutations in the polymerase domain [110, 163, 187, 200, 201, 204-210]. Mutations in the connection domain reported to be more frequent in treatment experienced patients are at residues G333, G335, N348, G359, A360, V365, K366, T369, A371, A376, K390, A400 and in the RNase H domain are at residues K451, L469, T470, L491, I506, Q524, K527, K530, Q547, A554 and K558 [198, 199, 207, 209]. By contrast, the mutations that are less frequent in treatment experienced than treatment naïve patients are at residue I326 in the connection domain and at residues T470 and K512 in the RNase H domain [198].

Currently recommended treatment for HIV-1 infection is two NRTI plus one NNRTI or one protease inhibitor. Consequently, it is difficult to determine which RTI combination drives the selection of mutations in the connection or RNase H domains. We therefore sought to determine if connection domain mutation A371V and/or RNase H domain mutation Q509L selected *in vitro* with zidovudine (AZT) or other novel C-terminal domain mutations emerged in patients treated with prolonged AZT monotherapy. We therefore amplified and sequenced full-length HIV-1 RT from serum samples obtained before and during treatment in ART-naïve patients enrolled in the AZT monotherapy arm of AIDS Clinical Trials Group study 175 (ACTG 175), a randomized,

double-blind, placebo-controlled trial designed to compare AZT- or didanosine- (ddI) monotherapy with combination therapy [211].

Table 8. Connection and RNase H domain mutations more frequent in treatment experienced patients than treatment naïve and resistance to NRTI or NNRTI

Connection Domain Mutation	RTI resistance with polymerase domain mutations[a]	RTI resistance without polymerase domain mutations[a]	Reference
G333E/D	AZT, 3TC	None	[163, 206]
G335C/D	AZT	None	[200, 206]
N348I	AZT, ddI, TNV, EFV, ETV, DLV, NVP	AZT, ddI, EFV, NVP, DLV	[187, 200, 204-206, 209, 210]
G359S[b]	Association with TAMs and NNRTI[c]	Not Done	[198, 212]
A360I/T/V[b]	AZT	None	[189, 198, 200, 206]
V365I	AZT	None	[200, 206]
K366R	Not Done	Not Done	[198]
T369I	AZT, DLV, EFV, NVP	AZT, DLV, EFV, NVP	[204]
A371V	AZT	None	[177, 198, 201, 206, 212]
A376S	AZT	NVP	[200, 206]
K390R[b]	Not Done	Not Done	[198]
A400T	AZT	None	[198, 208]
RNase H Domain Mutation	**RTI resistance with polymerase domain mutations**	**RTI resistance without polymerase domain mutations**	**Reference**
K451R	Not Done	Not Done	[209]
L469F/H/I/K/L/M/T	Not Done	Not Done	[199, 207]
T470E/K/P/S	Not Done	Not Done	[199]
L491P/S/T	Not Done	Not Done	[207]
I506L	Not Done	Not Done	[198]
Q524E/K	Not Done	Not Done	[207]
K527N/S	Not Done	Not Done	[198, 207]
K530R	Not Done	Not Done	[198]
Q547K	Not Done	Not Done	[198]
A554K/L/T	Not Done	Not Done	[199]
K558E/G/R	Association with TAMs[c]	Not Done	[199]

[a] AZT - zidovudine; 3TC – lamivudine; ddI – didanosine; TNV – tenofovir; EFV – efavirenz; ETV – etravirine; DLV – delavirdine; NVP – nevirapine.

[b] Residues in **bold** are part of the RNase H primer grip.

[c] Occurrence of connection or RNase H domain mutation was associated with TAMs or NNRTI resistance mutations in the polymerase domain. Resistance studies have not been done.

5.4 MATERIALS AND METHODS

5.4.1 Study design

ACTG 175 is a previously published randomized, double-blind, placebo-controlled trial designed to compare monotherapy with AZT or ddI to combination therapy consisting of AZT and ddI or AZT and zalcitabine (ClinicalTrials.gov number, NCT00000625) [211]. The study was approved at each site by an institutional review board and all patients provided written informed consent [211].

The primary study end-point was: i) CD4 count 50 percent below the average of two pre-treatment counts, confirmed within 3-21 days; ii) AIDS related end-points defined by the 1987 CDC criteria [17]; or iii) death [211]. Two hundred and sixty nine patients in the AZT monotherapy treatment arm had no previous antiretroviral exposure and reached the primary study end-point. Patients selected for this study either had stored serum samples from pre-therapy (week 0), longitudinal time points (weeks 8, 20 or 32) and reached the study end-point or had stored serum samples from pre-therapy (week 0), longitudinal time points (weeks 8, 20 or 32) and did not reach study end-point but serum was available after 20 weeks of AZT monotherapy.

5.4.2 HIV-1 RNA assays

HIV-1 RNA was determined with the Roche Amplicor Monitor HIV-1 Ultrasensitive Assay Kit 1.5 (Roche Diagnostics Corporation, Indianapolis, IN) using the ultrasensitive method. Statistical significance between HIV-1 RNA at pre-therapy and on therapy was calculated using the Mann-Whitney rank sum test.

5.4.3 Amplification and sequencing

Viral RNA was extracted from serum samples at pre-therapy, longitudinal and final available weeks from 23 patients (QIAamp® Viral RNA Mini kit, Qiagen, Valencia, CA). RNA was converted to cDNA using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, Carlsbad, CA) using a template specific primer and full-length RT (codons 1-560) was amplified by nested PCR. Primers (IDT, Coralville, IA) for RT-PCR were: 5'-AGGA AGATGGAAACCAAAAATGATAG-3' (OF) and 5'-CCTTGACTTTGGGGATTGTAGGGAA-3' (OR), and primers for PCR 2 were: OF and 5'-CACAGCTGGCTACTATTTCTTTGC- 3' (IR). PCR products were purified with ExoSAP-IT® (USB, Cleveland, OH) and bulk sequenced with six bidirectional sequencing primers using Big Dye terminator (v.3.1) on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing primers were (IDT, Coralville, IA): (A) 5'-GTAGGACCTACACCTGTCAACAT-3'; (B) 5'-TAAATCTGACTTGCC CA-3'; (C) 5'-GAAATAGGGCAGCATAGA-3'; (D) 5'-ATGGGTTCTTTCTCTAACTG-3'; (E) 5'-AGCCACCTGGATTCCTGA-3'; and (F) 5'-CATCGTTTTCTTTATCATCGGTCGACAC-

3'. Bidirectional sequences were assembled and analyzed using Sequencher 4.9 software (Gene Codes Corporation, Ann Arbor, MI).

Sequences from patients were examined at pre-therapy, longitudinal time points, study end-point or after 20 weeks of AZT treatment for known NRTI resistance mutations in the polymerase domain using the International AIDS Society – USA (IAS-USA) resistance table [110] and for novel mutations in the polymerase, connection and the RNase H domains of RT.

5.4.4 Statistical analysis

Sequences from 23 pre-therapy – AZT-treated pairs were compared for polymerase domain mutations listed in the IAS-USA 2009 mutation list and mutations that occurred more than in one patient in the polymerase, connection and RNase H domains, using two-sided exact McNemar's test. A p-value of <0.05 was considered statistically significant.

5.4.5 Construction of pxxLAI 3D

The full-length infectious HIV-1 clone (pHIV-1_{LAI}) designed by Peden *et al.* [213] and later modified to contain the restriction sites *XmaI* and *XbaI* in RT (pxxLAI) [214] was used to construct pxxLAI 3D. In this study we modified pxxLAI to form pxxLAI 3 domain (3D) and pxxLAI 3D Θ np to allow independent cloning of the three RT domains (Figure 18).

To construct pxxLAI 3D, site directed mutagenesis was used to introduce silent mutations into pxxLAI at RT codons 321-323, 358, 417-418, 554 and integrase codon 29-31 (QIAamp kit; QIAGEN, Valencia, CA) to create unique restriction sites *BstBI* (TTCGAA), *MluI* (ACGCGT),

HpaI (GTTAAC), *NgoMIV* (GCCGGC) and *SgrAI* (CACCGGTG), respectively (Figure 18). Primer sequences for site-directed mutagenesis were: BstBI-forward 5'- GGAGTGTATTATGA CCCTTCGAAAGACTTAA -3'; BstBI-reverse 5'- TTAAGTCTTTCGAAGGGTCATAATACA CTCC -3'; MluI-forward 5'- ATATGCAAGAACGCGTGGTGCCCACACTA -3'; MluI-reverse 5'- TAGTGTGGGCACCACGCGTTCTTGCATAT -3'; HpaI-forward, 5'-CTGAGTGGGAGTT TGTTAACACCCCTCCTTTAG-3'; HpaI-reverse, 5'-CTAAAGGAGGGGTGTTAACAAACT CCCACTCAG-3'; NgoMIV-forward 5'- GATAAATTAGTCAGTGCCGGCATCAGGAAAGT ACTAT -3'; NgoMIV-reverse 5'- ATAGTACTTTCCTGATGCCGGCACTGACTAATTTATC -5'; SgrAI-forward, 5'- AACCTGCCACCGGTGGTAGCAAAAGAAA-3'; and SgrAI-reverse, 5'-TTTCTTTTGCTACCACCGGTGGCAGGTT- 3' (restriction sites are underlined) [IDT, Coralville, IA].

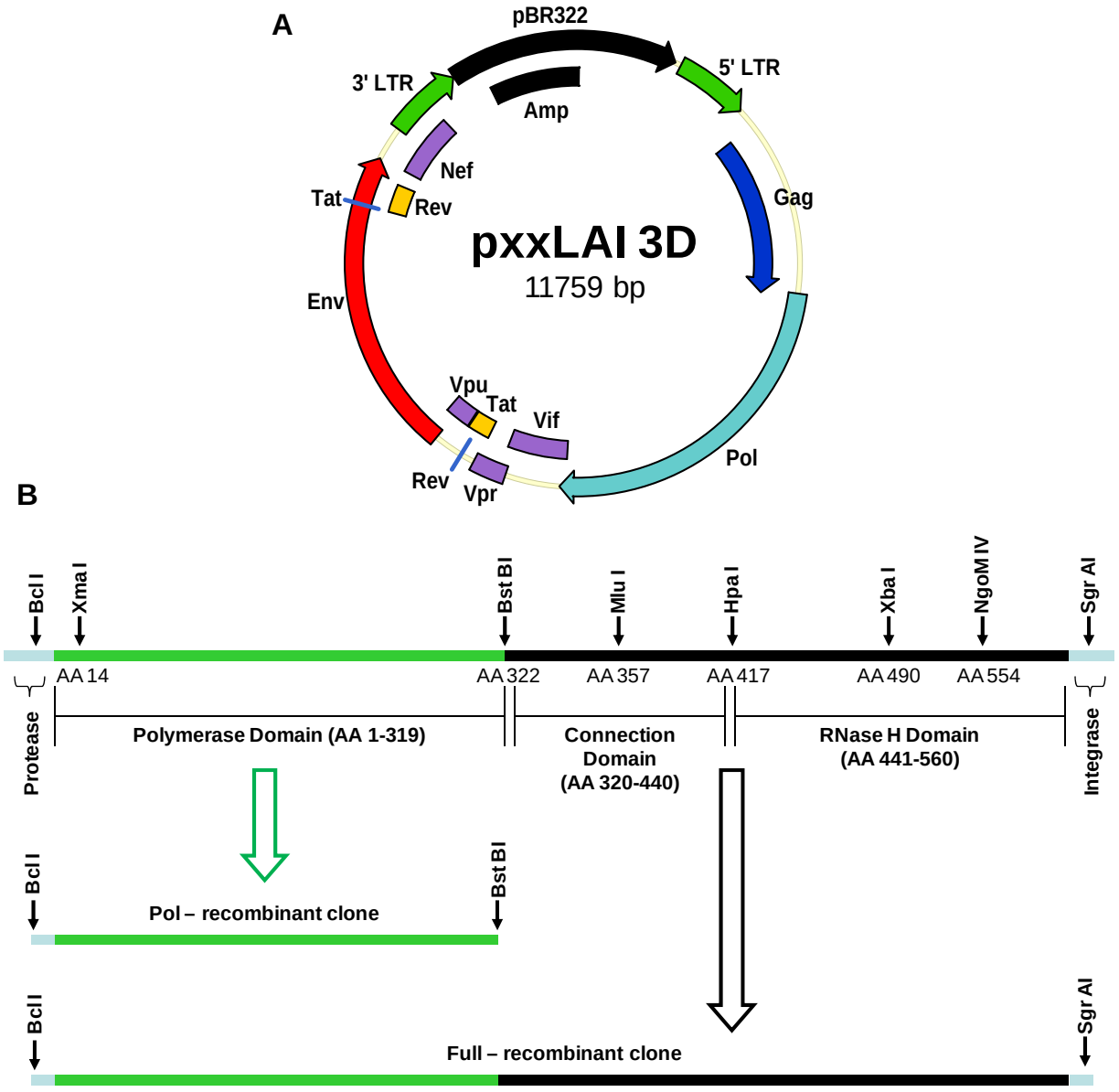


Figure 18. pxxLAI 3D plasmid map and RT restriction sites.
(A) Plasmid map for pxxLAI 3D. Plasmid contains the entire genome of HIV-1 LAI recombined with the ampicillin gene and ori from pBR322. (B) Location of unique restriction sites present in pxxLAI 3D: *BclI, XmaI, BstBI, MluI, HpaI, XbaI, NgoMIV* and *SgrAI*. Polymerase domain recombinant clones (pol) were created by digesting PCR product from clinical samples with restriction enzymes *BclI* and *BstBI* and ligation into wildtype pxxLAI 3D. Full-length clones (full) were created by digesting PCR product from clinical samples with restriction enzymes *BclI* and *SgrAI* and ligation into wildtype pxxLAI 3D.

5.4.6 Construction of pxxLAI 3D Θ np

The RT defective cloning vector was developed by deletion of RT polymerase domain codons 113 to 315, using unique restriction sites *NsiI* to *PflmI*, respectively. The *NsiI* to *PflmI* deletion was previously created in pxxLAI (pxxLAI Θ np) and described in detail by Shi *et al.*[214]. The deletion makes the pxxLAI Θ np vector replication incompetent and a full-length *XmaI - XbaI* RT fragment is needed to restore replication competency [214]. To form pxxLAI 3D Θ np, the polymerase domain of pxxLAI Θ np was PCR amplified with primers Bcl2-forward (5'-GTTTT ATCAAAGTAAGACAGTATGATCAGATA C-3') and BstBI2-reverse (5'-GTATTATGACCC TTCGAAAGACTTAATAGC-3') using Platinum® Taq High Fidelity (Invitrogen) to add the *BclI* (TGATCA) and *BstBI* (TTCGAA) restriction sites to the 5'- and 3'-ends, respectively. PCR products were concatamerized: (i) phosphates added to the 5'- and 3'- ends with T4 polynucleotide kinase (Promega Corporation, Madison, WI); (ii) dNTPs added with T4 polynucleotide kinase to make PCR products blunt ended (Promega Corporation, Madison, WI); (iii) and blunt ended PCR products ligated together with T4 DNA ligase (NEB, Ipswich, MA).

Concatamerized PCR product and pxxLAI 3D were digested with restriction enzymes *BclI* and *BstBI* (NEB, Ipswich, MA). The digested pxxLAI 3D backbone (10.67 kb) and pxxLAI Θ np insert (0.48 kb) were gel purified (Wizard® SV Gel and PCR Clean-Up System, Promega Corporation, Madison, WI), ligated together with T4 DNA ligase and transformed into C2925 cells (NEB, Ipswich, MA) to create pxxLAI 3D Θ np (Figure 19). Bacterial clones were sequenced to confirm the *NsiI - PflmI* deletion and correct DNA sequence.

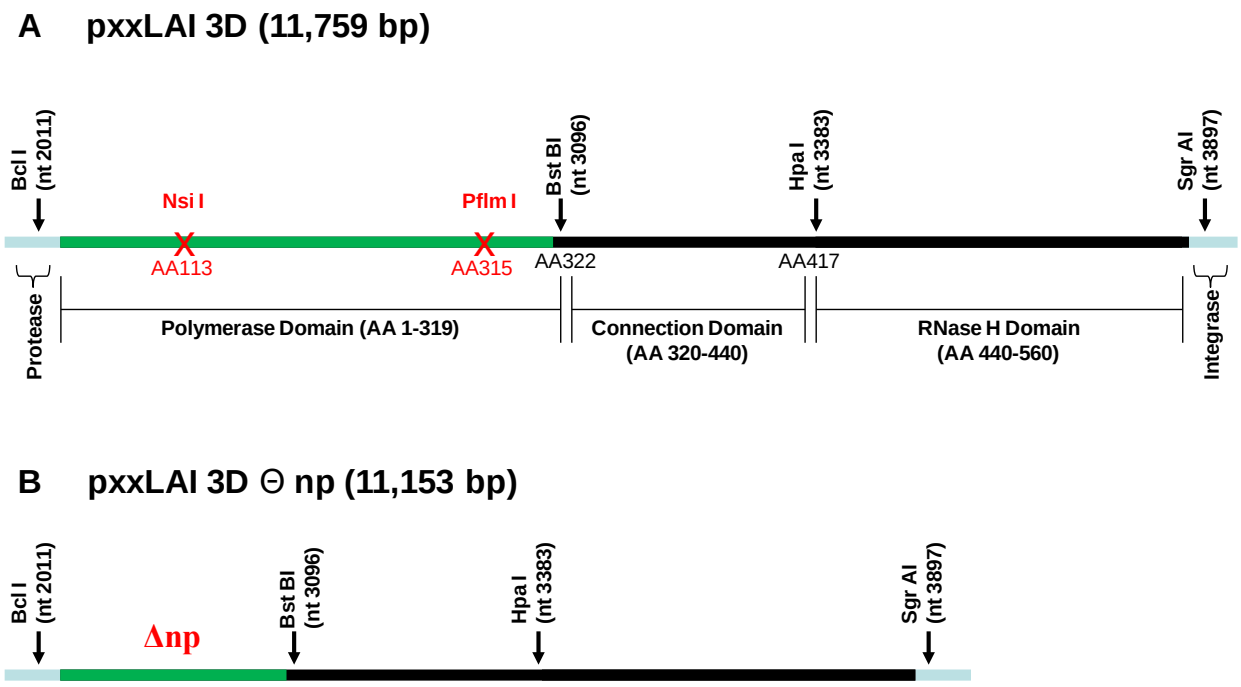


Figure 19. Generation of pxxLAI 3D Θ np.
(A) Full-length RT gene showing location of unique *NsiI* and *PflmI* restriction sites. (B) Region between Nsi I and Pflm I is deleted to form pxxLAI 3D Θ np .

5.4.7 Construction of RT polymerase domain recombinant virus

BclI and *BstBI* were added to the ends of nested PCR products as described above (see Material and Methods section 5.4.6) using BclI2-forward and BstBI2-reverse primers. PCR products were concatamerized (see Material and Methods section 5.4.6) and digested with *BclI* and *BstBI* to form a 1.09 kb insert. pxxLAI 3D Θ np was digested with *BclI* and *BstBI* restriction enzymes and the 10.67 kb backbone was gel purified (Wizard® SV Gel and PCR Clean-Up System, Promega Corporation, Madison, WI). pxxLAI 3D backbone and polymerase domain inserts were ligated with T4 DNA ligase and transformed into C2925 cells. The total volume of cells from transformation was used to grow a 100 mL LB (50 ng/µl ampicillin) culture to ensure collection of all recombinant clones and DNA was purified with PureYield™ Plasmid Midiprep System (Promega Corporation, Madison, WI). Polymerase domain recombinant clones were bulk sequenced to confirm similarity between the original viral population and the new polymerase domain recombinant clones (pol). Infectious virus was generated by electroporating the pol-clones (in bulk) into MT-2 cells as described previously (Chapter 1, Material and Methods section 3.4.7). All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

5.4.8 Construction of full-length RT recombinant virus

Recombinant clones were created as described above, except restriction site *SgrAI* (CACCGGTG) was added to the 3'-end of nested PCR products using primer SgrAI2-reverse (5'- CTACCACCGGTGGCAGGTTA -3'). PCR products were concatamerized and digested

with *BclI* and *SgrAI* to form a 1.89 kb insert. pxxLAI 3D Θ np was digested with *BclI* and *SgrAI* restriction enzymes and the 9.87 kb backbone was gel purified. pxxLAI 3D backbone and full-length RT inserts were ligated with T4 DNA ligase and transformed into C2925 cells. The total volume of cells from transformation was used to grow a 100 mL LB (50 ng/µl ampicillin) culture to ensure collection of all recombinant clones and DNA was purified. Full-length recombinant clones (full) were bulk sequenced to confirm similarity between the original viral population and the new recombinant clones. Infectious virus was generated by electroporating the full- clones (in bulk) into MT-2 cells as described above (Chapter 1, Material and Methods section 3.4.7). All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

5.4.9 Drug susceptibility

AZT susceptibility was determined in P4/R5 cells as described in Chapter 1, Material and Methods section 3.4.5. IC_{50} values from three to four experiments were compared for statistically significant differences using the two-sample Student's *t* test for data with a normal distribution or Mann Whitney Wilcoxon Rank Sum Test for non-parametric data.

5.5 RESULTS

5.5.1 Emergence of RT resistance mutations

Sixty-three out of 269 (23%) patients in the AZT monotherapy treatment arm had no prior antiretroviral exposure and reached a primary study end-point. Five of 63 of these patients who reached a study end-point had stored serum samples from pre-therapy (week 0) and longitudinal time points (weeks 8, 20 or 32). Eighteen of 206 patients who did not reach a study end-point had stored serum samples from pre-therapy (week 0) and longitudinal time points (weeks 8, 20 or 32). Pre-therapy serum HIV-1 RNA for patients who experienced study end-point (mean 33,261 c/mL; range 3,650 – 67,134 c/mL) was not significantly higher than for patients who did not reach a study end-point (mean 41,002 copies/mL; range 1,439 - 168,576 c/mL), p=0.875. At the final time point, HIV-1 RNA showed a higher trend for patients who reached a study end-point (mean 87,264 c/mL; range 35,227-216,219 c/mL) compared to those not reaching study endpoint (mean 37,424 c/mL; range 826-138,776 c/mL), p=0.06.

Table 9 shows polymerase domain mutations that were detected at a higher frequency (10% or greater) in samples after 20 weeks or more after AZT monotherapy compared with pre-therapy samples. RT polymerase domain mutations were detected in 70% (16/23) of patients. All five patients who reached a protocol end-point had mutations in the polymerase domain versus 11 of 18 (61%) who did not reach a protocol end-point. Only polymerase domain mutations K70R and T215I/Y were significantly more frequent after AZT monotherapy (4.3% in pre-therapy versus 52% in AZT-experienced, p=0.003 and 0.0% in pre-therapy versus 35% in AZT-experienced, p=0.013, respectively).

Table 9 also shows the connection and RNase H domain mutations that were more frequent after 20 weeks on AZT monotherapy. Connection domain mutation A360V was the only mutation in the C-terminus of RT that was significantly more frequent after AZT monotherapy (0.0% in pre-therapy versus 26% in AZT-experienced, p=0.041). The A360V mutation was present in 2 of 5 patients who reached a protocol end-point compared to 4 of 18 patients who did not. Of the 6 patients with A360V, the mutation was present as the dominant viral population (100% mutant) in only 2 patients and as part of a minor viral population (<50%) in 4 patients (Table 10). An A to T change at codon 360 was also observed but was not significantly more frequent after AZT monotherapy (N=3) than pre-therapy (N=2).

To determine if connection domain mutation A360V was selected by AZT before polymerase domain mutations, RNA from available serum samples at weeks 8, 20 or 32 was amplified and sequenced. The A360V mutation appeared at the same time or after RT polymerase domain mutations.

Table 9. Mutations ≥ 10% more frequent in patients treated with AZT monotherapy

Domain	Mutation	Protocol End-point (N=5)[a]		AZT for ≥ 20 weeks (N=18)[a]		Total (N=23)[a]		
		% Pre-Therapy (N)	% AZT Monotherapy (N)	% Pre-Therapy (N)	% AZT Monotherapy (N)	% Pre-Therapy (N)	% AZT Monotherapy (N)	p-value[b]
Polymerase (aa 1-319)	K20R	0.0 (0)	0.0 (0)	5.6 (1)	28 (5)	4.3 (1)	22 (5)	0.13
	M41L	0.0 (0)	40 (2)	0.0 (0)	5.6 (1)	0.0 (0)	13 (3)	0.25
	I47F	40 (2)	60 (3)	0.0 (0)	17 (3)	8.7 (2)	26 (6)	0.22
	D67N	0.0 (0)	40 (2)	0.0 (0)	17 (3)	0.0 (0)	22 (5)	0.074
	K70R[c]	**0.0 (0)**	**40 (2)**	**5.6 (1)**	**56 (10)**	**4.3 (1)**	**52 (12)**	**0.003**
	T215I/Y	**0.0 (0)**	**100 (5)**	**0.0 (0)**	**17 (3)**	**0.0 (0)**	**35 (8)**	**0.013**
	K219E/Q	0.0 (0)	20 (1)	0.0 (0)	11 (2)	0.0 (0)	13 (3)	0.25
Connection (aa 320-439)	A360T	20 (1)	20 (1)	5.6 (1)	11 (2)	8.7 (2)	13 (3)	1.0
	A360V	**0.0 (0)**	**40 (2)**	**0.0 (0)**	**22 (4)**	**0.0 (0)**	**26 (6)**	**0.041**
	A371V	0.0 (0)	20 (1)	5.6 (1)	17 (3)	4.3 (1)	17 (4)	0.25
RNase H (aa 440-560)	K558R	20 (1)	20 (1)	11 (2)	28 (5)	13 (3)	26 (6)	0.25

[a] 5 of 23 patients reached a protocol end-point and 18 of 23 patients did not but received AZT monotherapy for 20 weeks or greater.
[b] Two-sided McNemar's exact test between pre-therapy and AZT-experienced for N=23 pairs.
[c] p-value < 0.05 in **bold**.

5.5.2 Construction of RT polymerase domain and full-length RT recombinant clones

Since A360V emerged at the same time or after polymerase domain mutations, we hypothesized that A360V functions as an accessory mutation that increases AZT resistance in the context of polymerase domain mutations. Recombinant clones were created that included the RT polymerase domain alone or full-length RT (polymerase, connection and RNase H domains) from serum samples at the last available draw date (Figure 18). Table 10 shows mutation profiles of each recombinant clone. Patient 1 had polymerase domain mutations K70R (100% mutant), K219Q (mixture of 30% mutant and 70% wildtype) and connection domain mutation A360V (100% mutant) and patient 2 had polymerase domain mutations M41L (100% mutant), L210W (mixture of 40% mutant and 60% wildtype), T215Y (100% mutant) and connection domain mutation A360V (100% mutant). Patients 3, 4, 5 and 6 only had connection domain mutation A360V (mixture of 25%-35% mutant and 65%-75% wildtype) combined with polymerase domain mutation K70R (mixture of 25%-100% mutant and 0%-75% wildtype) or combined with a 100% mutant virus containing TAMs D67N, K70R, T215I, K219E (patient 3). Mutation mixtures identified in the original clinical sample were similar in recombinant clones.

Table 10. A360V with polymerase domain mutations increases AZT resistance

Patient-clone	Polymerase Mutations	Connection Mutations	IC_{50} (mM)	Fold-resistance[a] (Full vs Pol)	p-value[b]
Wildtype	—	—	0.28 ± 0.10	—	—
1-Pol[c]	100% K70R 30% K219Q	—	2.9 ± 2.1	10.3	0.551
1-Full[d]	100% K70R 30% K219Q	100% A360V	2.2 ± 1.8	7.75 (0.75)	
2-Pol	100% M41L 40% L210W 100% T215Y	—	4.6 ± 1.6	16.3	0.016
2-Full	100% M41L 40% L210W 100% T215Y	100% A360V	50 ± 30	180 (11)	
3-Pol	100% D67N 100% K70R 100% T215I 100% K219E	—	5.03 ± 1.9	18.0	0.165
3-Full	100% D67N 100% K70R 100% T215I 100% K219E	25% A360V	7.50 ± 3.3	26.8 (1.5)	
4-Pol	100% K70R	—	0.52 ± 0.30	1.86	0.306
4-Full	100% K70R	25% A360V	0.92 ± 0.49	3.29 (1.8)	
5-Pol	75% K70R	—	0.65 ± 0.28	2.32	0.806
5-Full	60% K70R	25% A360V	0.60 ± 0.06	2.14 (0.92)	
6-Pol	25% K70R	—	0.34 ± 0.05	1.21	0.074
6-Full	40% K70R	35% A360V	0.60 ± 0.18	2.14 (1.8)	

[a] Average fold-resistance compared to wildtype pxxLAI 3D (average fold-resistance of full-length recombinant virus compared to polymerase domain only recombinant virus).

[b] Two-sided Student's *t* test comparing IC_{50} between Full and Pol viruses.

[c] Pol – Recombinant virus containing the polymerase domain from patient sample and connection and RNase H domains from wildtype pxxLAI 3D.

[d] Full – Recombinant virus containing the polymerase, connection and RNase H domains from patient sample.

5.5.3 AZT susceptibility of recombinant clones

To determine if A360V decreases drug susceptibility in the context of polymerase domain mutations, recombinant clones were tested for AZT susceptibility (Table 10). Patient 2 had an 11-fold increase in AZT resistance between polymerase domain recombinant containing mutations M41L-L210W-T215Y and full-length recombinant containing mutations M41L-L210W-T215Y-A360V ($p=0.016$). However, AZT resistance did not increase in patient 1 with the addition of A360V to polymerase domain mutations K70R and a minor population of K219E (mixture of 30% mutant and 70% wildtype). There was no significant change in AZT susceptibility between polymerase domain recombinant and full-length recombinant clones in patients with TAMs plus A360V (as a mixture of 25%-35% mutant and 65%-75% wildtype); patients 3, 4, 5 and 6.

5.6 DISCUSSION

This study is the first to compare full-length RT sequences from paired samples obtained before and during AZT monotherapy. Mutations in the polymerase domain significantly associated with AZT monotherapy were K70R ($p=0.003$) and T215I/Y ($p=0.013$). The TAMs M41L, D67N, L210W and K219E/Q were also selected by AZT monotherapy but the associations with treatment were not significant ($p > 0.05$), probably because of the small sample size. The only mutation in the RT C-terminus considerably more frequent during AZT monotherapy was A360V in the connection domain ($p=0.045$). A360V was not seen at pre-therapy and emerged

simultaneously or after polymerase domain mutations in 6 out of 23 patients. Two patients developed a 100% A360V mutant population: in patient 1 A360V emerged in the presence of K70R (100% mutant) and K219Q (30% mutant); and in patient 2 A360V arose in the presence of M41L (100% mutant), L210W (40% mutant) and T215Y (100% mutant). In the remaining 4 patients with A360V the mutation occurred as a mixed mutant-wildtype population (25%-35% mutant), combined with D67N-K70R-T215I-K219E (all 100% mutant) in patient 3 or with K70R alone (25%-100% mutant) in patients 4, 5 and 6. The A360T mutation was also observed but it was present in 2 of 23 pre-therapy samples versus 3 of 23 on therapy samples and was not associated with polymerase domain mutations.

This is the only study to date that has compared samples obtained from the same patients before treatment and during AZT monotherapy for connection and RNase H domain mutations. Similar studies have analyzed the frequency of RT C-terminal mutations from AZT monotherapy treated patients but compare results to either untreated patients from separate clinical cohorts [198] or did not include a comparison to a treatment-naive group [215]. The former analysis showed that A360V was significantly more frequent in the group with AZT monotherapy (4/28) compared to treatment-naïve patients (0/87), and that A360V was only detected in subtype B patients [198]. In the latter analysis, N348I was detected in 3 of 50 patients exposed to AZT monotherapy but no other connection or RNase H domain mutations were evaluated [215].

In the current study we show that A360V conferred 11-fold increased AZT resistance when present with TAMs M41L, L210W, T215Y compared to virus with TAMs alone (Table 10). However, A360V did not decrease AZT susceptibility in the context of K70R (100% mutant) and K219Q (as a mixture of 30% mutant and 70% wildtype) indicating that A360V may only increase AZT resistance when in the presence of 3 or more TAMs. Testing of additional samples

is required to draw firm conclusions. AZT susceptibility studies by Nikolenko *et al.* show that A360V increases AZT resistance 22-fold in the presence of TAMs D67N, K70R, T215Y, K219Q and polymerase/connection domain mutations E297R/I341F.

HIV-1 RT crystal structures show that residue A360 is within the HIV-1 RT RNase H primer grip and contacts a DNA primer strand through hydrogen bonds [74]. Residues that make up the RNase H primer grip appear to be highly conserved within treatment-naive and treatment-experienced patients except at positions 359, 360, 390 and 395 [198, 209]. Previous studies show that mutations at residues within the RNase H primer grip region or residues contacting the RNA template near the RNase H active site will hinder RNase H cleavage [168-170, 216]. For example, when alanine is substituted at RNase H primer grip residues H361, K390, K395, T473 and I505 RNase H activity is decreased [216] and when substitutions are made at residues G359, A360, H361, E396, T473, Q475 and Y501 viral replication is severely decreased and RNase H cleavage specificity changes [168-170].

Biochemical studies suggest that A360V in the presence of TAMs will decrease RT binding to a 16 basepair RNA-DNA hybrid in an RNase H- competent mode required for RNase H cleavage. As a result, the amount of both primary and secondary RNA cleavage products formed is decreased. Subsequently, RT containing TAMs-A360V increases RT binding to a 16 basepair RNA-DNA hybrid in a polymerase-competent mode that favors AZT excision and DNA synthesis [189]. The net of these effects is preservation of RNA/DNA template/primer and increased binding of RT to template/primer in the excision competent mode. We provide strong evidence for a similar biochemical mechanism for the Q509L RNase H domain mutation in the context of TAMs (see Chapter 2; [217]).

Two other C-terminal domain mutations observed more frequently during AZT monotherapy compared to pre-therapy were A371V and K558R. However, both mutations were detected in some pre-therapy samples and were not significantly associated with AZT monotherapy ($p=0.25$). Connection domain mutation A371V has been previously shown to be associated with TAMs [201, 212] and to occur more frequently in treatment-experienced than in treatment naïve patients [177, 180, 198, 201, 212]. We have shown that when HIV-1 is passaged in cell culture with increasing concentrations of AZT, A371V is selected in the context of TAMs D67N-K70R-T215I/F and RNase H domain mutation Q509L. Alone, A371V does not increase AZT resistance but in the presence of D67N-K70R-T215F-Q509L susceptibility is decreased 9-fold compared to virus containing D67N-K70R-T215F-Q509L [201]. The biochemical mechanism of this A371V effect remains elusive. The second C-terminal domain mutation observed more frequently during AZT therapy compared to pre-therapy was K558R. Mutations K558 E, G and R have been reported to emerge in the context of TAMs in treatment experienced patients [199], but drug susceptibility studies have not been done.

The RNase H domain mutation Q509L that was selected with AZT *in vitro* was not observed in any of the 23 patients in the current study [201]. In addition, Q509L has been reported at 0.3% frequency in treatment experienced patients from the Stanford University HIV Drug Resistance Database [180]. The discrepancy between observation *in vitro* and *in vivo* may be due to differences in AZT concentrations between patient plasma (~2-4 μM; [218]) and that used for *in vitro* selection (up to 32 μM; see Chapter 1 [201]). Because drug concentrations and duration of treatment are different between *in vitro* experiments and patient treatment, mutations that arise *in vitro* are not always representative of mutations that emerge with ART in patients.

Other mutations in the C-terminus of RT that have been reported to be more frequent in treatment experienced patients compared to treatment naïve patients (Table 8) were not detected in this study. This may be due, in part, to exposure only to AZT in this study compared with others. As a consequence, only mutations that contribute to AZT resistance will emerge whereas mutations that increase drug resistance to one or multiple NRTI or NNRTI are more likely to emerge in combination therapy studies. Even though our study is the only paired pre-therapy versus failure comparison of full-length RT sequences from AZT monotherapy patients, the sample size (N=23 pairs) had limited power to detect mutations that emerge at low frequency.

In summary, this study of full-length RT sequences from paired treatment-naïve and AZT monotherapy patients revealed that the A360V mutation in the connection domain of RT is significantly associated with AZT monotherapy. A360V was detected in the same sample with TAMs or in subsequent samples after the appearance of TAMs. These findings suggest that the A360V mutation provides a selective advantage primarily in the presence of TAMs in the polymerase domain. Future mutagenesis studies are planned to confirm that A360V increases AZT resistance only in the context of TAMs.

6 CHAPTER FOUR. FAILURE OF INITIAL THERAPY WITH TWO NUCLEOSIDES AND EFAVIRENZ IS NOT ASSOCIATED WITH EMERGENCE OF MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF HIV-1 REVERSE TRANSCRIPTASE

Jessica H. Brehm[1], Christina M. Lalama[2], Michael D. Hughes[2], Richard Haubrich[3], Sharon A. Riddler[4], Nicolas Sluis-Cremer[4], John W. Mellors[4] for the AIDS Clinical Trials Group Study A5142 Protocol Team

[1]Graduate School of Public Health, Department of Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; [2]Harvard School of Public Health, Boston, MA 02115; [3]University of California San Diego, San Diego, CA 92103; [4]Department of Medicine, Division of Infectious Diseases, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

6.1 PREFACE

This study was presented in part as an oral abstract at the XVIII International HIV Drug Resistance Workshop, Fort Meyers, Florida, June 2009 (abstract published in Brehm, J., C. W. Sheen, M. Hughes, C. Lalama, R. Haubrich, S. Riddler, N. Sluis-Cremer and J. Mellors. 2009. Virologic Failure of Regimens Containing 2 NRTI + Efavirenz is Not Associated with the Selection of Mutations in the Connection or RNase H Domains of Reverse Transcriptase. *Antiviral Therapy* 14, A34) and in part as an oral abstract at the ACTG Network Meeting, Washington DC, August 2009 (Brehm, J., C. W. Sheen, M. Hughes, C. Lalama, R. Haubrich, S. Riddler, N. Sluis-Cremer and J. Mellors. 2009. Role of Mutations in the Polymerase, Connection or RNase H Domains of Reverse Transcriptase).

In addition, the manuscript for this study is in preparation. The work presented in this chapter is in partial fulfillment of dissertation aim 3.

6.2 ABSTRACT

It is uncertain how often mutations in the connection or RNase H domains of HIV-1 reverse transcriptase (RT) emerge with failure of first-line antiretroviral therapy. Full-length RT sequences in plasma obtained pre-therapy and at virologic failure were compared in 53 patients on first-line efavirenz-containing regimens from AIDS Clinical Trials Group study A5142. HIV-1 was mostly subtype B (48/53). Mutations in the polymerase but not in connection or RNase H domains of RT increased in frequency between pre-therapy and failure (K103N, p=0.001; M184I/V, p=0.016). Selection of mutations in C-terminal domains of RT is not common with failure of initial efavirenz-containing regimens.

6.3 GOAL OF STUDY

Genotype-based HIV-1 resistance tests available for clinical use only identify RT mutations in the polymerase domain and some portions of the connection domain. This restricted approach has raised concern that clinically important resistance mutations in RT are being missed. To address this concern, we have sequenced full-length RT in pre-therapy and virologic failure plasma samples from the 2 nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) plus efavirenz (EFV) arm of AIDS Clinical Trials Group (ACTG) study A5142 [219]. Sequences from pre-therapy and time of confirmed virologic failure were compared to assess if mutations in the reverse transcriptase (RT) polymerase, connection and RNase H domains emerged at virologic failure. In addition, pre-therapy sequences from patients who did not experience virologic failure were obtained and compared to pre-therapy sequences from patients with virologic failure to assess if polymorphisms in RT predispose to virologic failure of the 2 NRTI plus EFV regimen.

6.4 MATERIALS AND METHODS

6.4.1 Study design

ACTG A5142 was a phase III, multicenter, randomized, open-label trial among antiretroviral therapy- (ART)-naïve patients consisting of three arms: lopinavir/ritonavir (LPV/r) plus EFV, 2 NRTI plus LPV/r and 2 NRTI plus EFV (ClinicalTrials.gov number, NCT00050895) [219].

NRTI administered were lamivudine (3TC) plus zidovudine (AZT), stavudine or tenofovir disoproxil fumarate. All patients provided written informed consent and the study was approved at each site by an institutional review board or ethics committee [219].

Virologic failure was defined as i) confirmed HIV-1 RNA <1.0 $\log_{10}$ copies/mL reduction from pre-therapy and ≥200 copies/mL at/or after week 8 and prior to week 32, ii) lack of suppression to <200 copies/mL by week 32, iii) confirmed rebound >1000 copies/mL after confirmed suppression to <200 copies/mL before week 32, iv) confirmed rebound >1.0 $\log_{10}$ copies/mL from nadir and >1000 copies/mL without confirmed suppression to <200 copies/mL before week 32, or v) confirmed rebound ≥200 copies/mL after confirmed suppression to <200 copies/mL at/or after week 32. Sixty patients reached protocol-defined virologic failure among 250 patients randomized to 2 NRTI plus EFV in ACTG A5142; 53/60 had stored plasma samples from pre-therapy and failure time points with HIV-1 RNA >450 copies/mL.

One hundred forty-four patients among 190 patients in the 2 NRTI plus EFV arm of A5142 who did not experience protocol-defined virologic failure had an available full-length RT pre-therapy sequence.

6.4.2 Amplification and sequencing

Viral RNA was extracted from paired pre-therapy-failure plasma samples from 53 patients (QIAamp® Viral RNA Mini kit, Qiagen, Valencia, CA) and converted to cDNA using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, Carlsbad, CA) and template specific primers (see Chapter 3, Materials and Methods section 5.4.3). Full-length RT (codons 1-560) was amplified by nested PCR, products were purified with

ExoSAP-IT® (USB, Cleveland, OH) and bulk sequenced with six overlapping primers using Big Dye terminator (v.3.1) on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled and analyzed using Sequencher 4.9 software (Gene Codes Corporation, Ann Arbor, MI) [GenBank accession numbers: HM056533-HM056638].

Sequences from patients who experienced virologic failure were examined at failure for known NRTI and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations in the polymerase domain using the International AIDS Society-USA (IAS-USA) resistance table [110] and for novel mutations in the polymerase, connection and the RNase H domains. Sequences from pre-therapy plasma samples among patients who did not experience virologic failure were generated and obtained from Mina John and Simon Mallal [220]. These sequences were analyzed to identify associations between pre-therapy polymorphisms and virologic failure.

6.4.3 Statistical analysis

6.4.3.1 Emergence of mutations at virologic failure

Full-length RT sequences at time of failure and pre-therapy from 53 patients who experienced virologic failure were compared for IAS-USA polymerase domain mutations and mutations that occurred more than once in the connection and RNase H domains, using two-sided exact McNemar's test. The same comparison was performed within two subgroups: patients experiencing failure with at least one IAS-USA RT mutation (n=26) and those without any such mutations at failure (n=27).

6.4.3.2 Associations between pre-therapy mutations and virologic failure

Pre-therapy sequences from patients who did not experience virologic failure (non-failures, N=144) were compared to 53 pre-therapy sequences from patients who experienced failure for each mutation occurring more than once, using Fisher's exact test (two-sided). In addition, sequences from failure subgroups with (N=26) and without (N=27) known IAS-USA resistance mutations were compared to the 144 pre-therapy sequences from patients who did not experience virologic failure.

All sequences were compared to the appropriate reference subtype (B, C, D or circulating recombinant form (CRF) AB or AE). A p-value of <0.05 was considered statistically significant. P-values were not corrected for multiple comparisons.

6.5 RESULTS

6.5.1 Emergence of RT mutations at virologic failure

HIV-1 subtypes in the 53 patients experiencing virologic failure were predominantly B (48/53); 4/53 were C and 1/53 was CRF AE. RT inhibitor resistance mutations in the polymerase domain were identified in 26 of 53 (49%) failure samples. Table 11 shows polymerase domain mutations that were detected at a higher frequency (3% or greater) at failure than pre-therapy. Only K103N and M184V/I were significantly more frequent at failure than pre-therapy (p=0.001 and p=0.016, respectively). Table 11 also shows connection and RNase H domain mutations that were more frequent at virologic failure than pre-therapy, but none of the changes in frequency were

statistically significant (p-values ≥0.25). All of the pre-therapy mutations listed in Table 11 were also identified in the failure sample from the same patient except for one patient who did not have M377L in the failure sample.

Comparing pre-therapy and failure sequences within the two patient subgroups with (N=26) and without (N=27) IAS-USA resistance mutations at failure did not identify additional mutations in RT that were significantly more frequent at failure than at pre-therapy.

Table 11. RT mutations more frequent at virologic failure than at pre-therapy (N=53 pairs)[a]

Domain	Mutation	% Pre-therapy (N)[b]	% Failure (N)[b]	p-value[c]
Polymerase	K65R	0 (0)	7.6 (4)	0.13
	V90I	0 (0)	3.8 (2)	0.50
	K101E	0 (0)	5.7 (3)	0.25
	K103N	**1.9 (1)**	**23 (12)**	**0.001**
	V106I/M	1.9 (1)	9.4 (5)	0.13
	V179D	3.8 (2)	7.6 (4)	0.50
	M184I/V	**1.9 (1)**	**15 (8)**	**0.016**
	Y188H	0 (0)	3.8 (2)	0.50
	G190S	0 (0)	5.7 (3)	0.25
Connection	R358K	7.6 (4)	11 (6)	0.63
	A376S	1.9 (1)	5.7 (3)	0.50
	M377L	13 (7)	17 (9)	0.50
RNase H	V467I	17 (9)	23 (12)	0.25
	K530R	4.1 (2)	8.2 (4)	0.50

[a] Mutations ≥3% more frequent at virologic failure compared to pre-therapy.
[b] Total N=53 except 530R (N=52, missing first 16 amino acids for one sample).
[c] Exact McNemar's test (two-sided).

6.5.2 Associations between pre-therapy mutations and virologic failure

Of the 144 pre-therapy sequences from non-failures, 139 were subtype B, 3 were subtype C, 1 was subtype D and 1 was CRF AB. Pre-therapy mutations that were significantly associated with virologic failure (Table 12) included E6D (3% of non-failures versus 12% of failures, p=0.023), K103R (1% of non-failures versus 8% of failures, p=0.046) and Q174K (1% of non-failures versus 10% of failures, p=0.015) in the polymerase domain and Q334H in the connection domain (6% of non-failures versus 16% of failures, p=0.045).

Pre-therapy sequences from patient subgroups experiencing virologic failure with (N=26) and without (N=27) known IAS-USA mutations were also compared to pre-therapy sequences from the 144 patients who did not experience virologic failure (Table 12). In the subgroup with IAS-USA mutations at failure, mutations associated with failure were S68G (p=0.032), Q174H (p=0.026), Q174K (p=0.005), T200I (p=0.014), P243T (p=0.023), I244V (p=0.026) and E248D (p=0.031) in the polymerase domain; Q334E and Q334H (p=0.030) in the connection domain, and F440Y (p=0.047) and Q520L (p=0.023) in the RNase H domain. In the subgroup without IAS-USA mutations at failure, mutations associated with failure were I270C or F or S (p=0.013), E300V (p=0.024) in the polymerase domain and S322T (p=0.042) in the connection domain. The connection domain mutation D324E was only identified in pre-therapy sequences of non-failure samples (13%, p=0.046) in this subgroup.

Table 12. Associations of pre-therapy RT mutations with virologic failure

Domain	Mutation	All Patients			Subset with IAS Mutations at Failure			Subset without IAS Mutations at Failure		
		Controls[a] N=144[b]	Failures N=53[c]	p-value[d]	Controls N=144[b]	Failures N=26[c]	p-value[d]	Controls N=144[b]	Failures N=27[c]	p-value[d]
		% (N)	% (N)		% (N)	% (N)		% (N)	% (N)	
Polymerase	E6D	**3 (4)**	**12 (6)[e]**	**0.023**	3 (4)	12 (3)	0.73	3 (4)	12 (3)[e]	0.073
	S68G	3 (5)	11 (6)	0.072	**3 (5)**	**15 (4)**	**0.032**	3 (5)	7 (2)	0.31
	K103R	**1 (2)**	**8 (4)**	**0.046**	1 (2)	8 (2)	0.11	1 (2)	7 (2)	0.12
	Q174H	1 (2)	6 (3)	0.12	**1 (2)**	**12 (3)**	**0.026**	1 (2)	0 (0)	1.0
	Q174K	**1 (2)**	**10 (5)**	**0.015**	**1 (2)**	**15 (4)**	**0.005**	1 (2)	4 (1)	0.39
	T200I	5 (7)	14 (7)	0.052	**5 (7)**	**22 (5)**	**0.014**	5 (7)	8 (2)	0.63
	P243T	0 (0)	4 (2)	0.071	**0 (0)**	**8 (2)**	**0.023**	0 (0)	0 (0)	—
	I244V	1 (2)	6 (3)	0.12	**1 (2)**	**12 (3)**	**0.026**	1 (2)	0 (0)	1.0
	E248D	6 (8)	13 (7)	0.12	**6 (8)**	**19 (5)**	**0.031**	6 (8)	7 (2)	0.66
	I270C	1 (1)	6 (3)	0.060	0 (0)	0 (0)	—	**1 (1)**	**11 (3)**	**0.013**
	I270F	1 (1)	6 (3)	0.060	0 (0)	0 (0)	—	**1 (1)**	**11 (3)**	**0.013**
	I270S	1 (1)	6 (3)	0.060	0 (0)	0 (0)	—	**1 (1)**	**11 (3)**	**0.013**
	E300V	0 (0)	4 (2)	0.071	0 (0)	0 (0)	—	**0 (0)**	**7 (2)**	**0.024**
Connection	S322T	8 (12)	13 (7)	0.29	8 (12)	4 (1)	0.69	**8 (12)**	**22 (6)**	**0.042**
	D324E	13 (19)	8 (4)	0.33	13 (19)	15 (4)	0.76	**13 (19)**	**0 (0)**	**0.046**
	Q334E	6 (9)	12 (6)	0.22	**6 (9)**	**22 (5)**	**0.030**	6 (9)	4 (1)	1.0
	Q334H	**6 (9)**	**16 (8)**	**0.045**	**6 (9)**	**22 (5)**	**0.030**	6 (9)	12 (3)	0.40
RNase H	F440Y	2 (3)	6 (3)	0.35	**2 (3)**	**12 (3)**	**0.047**	2 (3)	0 (0)	1.0
	Q520L	0 (0)	4 (2)	0.071	**0 (0)**	**8 (2)**	**0.023**	0 (0)	0 (0)	—

[a] Controls did not reach protocol-defined virologic failure.
[b] For the control group, subtype C sequences (N=3) were excluded for analyses of codons 200 and 334 because they differed from subtype B consensus.
[c] For the failure group, subtype C sequences (N=4) were excluded from analysis at codons 200 and 334 because they differed from subtype B consensus. One CRF AE sequence was excluded at codon 174 for the same reason.
[d] Fisher's exact test (two-sided), p-value <0.05 in **bold**.
[e] Total N=52, missing first 16 amino acids for one sample.

6.6 DISCUSSION

This study is the first to compare full-length HIV-1 RT sequences in paired plasma samples obtained at pre-therapy and virologic failure time points. The only mutations that were significantly more frequent at virologic failure than pre-therapy were K103N (p=0.001) and M184V/I (p=0.016) in the polymerase domain, which confer resistance to EFV and 3TC, respectively. Other known polymerase domain mutations [110, 212] were not significantly associated with failure although, there were possible trends for K65R (p=0.13) and V106I/M (p=0.13). Mutations in the RT connection and RNase H domains were not significantly more frequent at virologic failure than at pre-therapy (p >0.25).

This study is the largest to date comparing pre-therapy and failure sequences from the same individuals (N=53). The only other similar study compared full-length RT sequences in one patient over three years [205]. In this patient, the N348I connection domain mutation was selected by AZT and/or didanosine therapy [205] and confers resistance to AZT, didanosine, nevirapine (NVP), EFV and delavirdine [187, 204, 205]. N348I was probably not detected in our study because EFV and not NVP was the NNRTI used for initial randomized therapy in ACTG A5142.

Other prior analyses of RT connection or RNase H domain mutations have compared sequences from unrelated ART-naïve patients and ART-experienced patients [163, 187, 198-200, 204-207, 209, 212]. These studies have identified a number of mutations in the C-terminus of RT that are more frequent in ART-experienced patients compared to ART-naïve including E312Q, G333D/E, G335C/D, N348I, A360I/V, A365I, T369I, A376S and K451R. Our study did not identify these mutations at virologic failure. This may be due, in part, to the strict definition of

virologic failure used in ACTG A5142 (see Materials and Methods section 6.4.1). As a consequence, the failure samples analyzed were early in the course of virologic breakthrough with limited time on failing therapy for drug-resistant variants to emerge. Nevertheless, our study does show that the first mutations to arise were in the polymerase domain and not in the connection or RNase H domains. Although our study is the largest paired pre-therapy versus failure comparison of RT sequences, the sample size (N=53 pairs) had limited power to detect mutations that emerge at low frequency.

Secondary analyses were performed to assess if pre-therapy RT polymorphisms predispose to virologic failure (Table 12). Polymerase, connection and RNase H domain mutations that were significantly associated with failure and occurred with known polymerase domain resistance mutations were S68G, Q174H, Q174K, T200I, P243T, I244V, E248D, Q334E, Q334H, F440Y and Q520L ($p < 0.05$). Mutations I270C, I270F, I270S, E300V and S322T were associated with virologic failure ($p < 0.05$) and occurred without known polymerase domain resistance mutations. P-values from these analyses were not corrected for multiple comparisons and thus must be interpreted with caution until confirmation in other datasets.

In summary, this study of full-length RT in paired sequences from pre-therapy and virologic failure did not identify mutations in the connection or RNase H domains associated with failure. These findings suggest that full-length RT sequencing is not essential for management of failure of first-line efavirenz-containing regimens, although analyses of larger datasets that include other regimens and more frequent non-B HIV-1 subtypes are needed before firm conclusions can be drawn.

7 CHAPTER FIVE. FREQUENT EMERGENCE OF N348I IN THE CONNECTION DOMAIN OF REVERSE TRANSCRIPTASE WITH VIROLOGIC FAILURE OF FIRST-LINE NNRTI-CONTAINING REGIMENS IN SOUTH AFRICA

7.1 PREFACE

This study was presented in part as a poster at the International HIV & Hepatitis Virus Drug Resistance Workshop & Curative Strategies, Dubrovnik, Croatia, June 2010 (abstract published in Brehm, J., C. L. Wallis, N. Sluis-Cremer, F. Conradie, P. Ive, C. Orrell, J. Zeinecker, I. Sanne, R. Wood, J. McIntyre, W. Stevens, J. Mellors and the CIPRA-SA Project 1 Study Team. 2010. Frequent Emergence of N348I in the Connection Domain of Reverse Transcriptase with Virologic Failure of First-Line NNRTI-Containing Regimens in South Africa. *Antiviral Therapy* 15, A126.). The work presented in this chapter is in partial fulfillment of dissertation aim 3.

7.2 ABSTRACT

It is not known how often mutations in the connection or RNase H domain of reverse transcriptase (RT) are selected with failure of first-line antiretroviral therapy (ART) in HIV-1 subtype C infected patients. We therefore compared full-length RT sequences in plasma samples obtained prior to ART and at confirmed virologic failure among patients enrolled in CIPRA-SA 'Safeguard the Household': a study of ART in resource poor settings evaluating care given by nurses versus physicians. Polymerase domain mutations significantly associated with failure of nevirapine/lamivudine/stavudine (NVP/3TC/d4T) were M184V ($p<0.0001$) and Y181C ($p=0.004$) and of efavirenz(EFV)/3TC/d4T were M184V ($p<0.0001$), K103N ($p<0.0001$) and V106M ($p=0.004$). K103N and Y181C mutations were detected more frequently at virologic failure in patients with prior exposure to single dose NVP (sdNVP). The connection domain mutation N348I was significantly associated with virologic failure of NVP-containing ART (0% pre-therapy versus 46% at failure, $p=0.002$) and was also observed with failure of EFV-containing ART (0% pre-therapy versus 12% failure, $p=0.062$). Analysis of longitudinal samples revealed that N348I generally emerged after M184V or NNRTI mutations in the polymerase domain. Prior sdNVP did not affect the frequency of N348I. Connection and RNase H domain mutations more common at pre-therapy in patients who experience failure with EFV/3TC/d4T and NVP/3TC/d4T were T359S ($p=0.015$), M377L ($p=0.002$), M377S ($p=0.008$) and E516G ($p=0.008$). This study identifies N348I in the connection domain of RT as an important resistance mutation in HIV-1 subtype C virus that is missed by restricting genotype analyses to the polymerase domain of RT. Other mutations identified in the connection and RNase H

domain may predispose to virologic failure, but analysis of additional datasets are required to confirm these associations.

7.3 GOAL OF STUDY

It is unclear how often mutations in the connection or RNase H domain of RT are selected with failure of first-line antiretroviral therapy (ART) in HIV-1 subtype C infected patients. We therefore compared full-length reverse transcriptase (RT) sequences in plasma samples obtained prior to ART and at virologic failure among patients enrolled in Comprehensive International Program of Research on AIDS in South Africa - 'Safeguard the Household' (CIPRA-SA); a study of ART in South Africa evaluating care given by nurses versus doctors [221]. We compared sequences obtained from patients at pre-therapy and confirmed virologic failure to identify novel mutations in the RT connection and RNase H domains. In a second analysis, sequences at pre-therapy from patients who did not fail first-line ART were compared with those who experienced virologic failure to determine if mutations before treatment can predict failure.

7.4 MATERIALS AND METHODS

7.4.1 Study design

CIPRA-SA "Safeguard the Household" is a randomized, controlled ART study evaluating care given by nurses versus doctors in resource poor settings (ClinicalTrials.gov number, NCT00255840) [221]. Phase one of CIPRA-SA was split into 2 study arms. In arm 1, HIV-1 infected individuals were administered first-line ART under the monitoring care of a HIV trained medical doctor and adherence counselors. In arm 2, HIV-1 infected individuals were administered first-line ART under the monitoring care of HIV-trained nurses and adherence counselors. First-line ART included 2 nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) [lamivudine (3TC) and stavudine (d4T)] plus one non-nucleoside reverse transcriptase inhibitor (NNRTI) chosen by a trained medical doctor or nurse. The NNRTI administered was preferably efavirenz (EFV). Nevirapine (NVP) or lopinavir/ritonavir (LPV/r) was given to women with child-bearing potential depending on whether the CD4 cell-count was less than or greater than 250 cells/mL, respectively. Pregnant women who were enrolled in the study after their first trimester were prescribed LPV/r or nelfinavir (NLF). All HIV-1 infected patients were ART-naïve at the start of the study or had been previously administered single dose NVP (sdNVP) to prevent mother-to-child transmission. Patients provided written informed consent and the study was approved at each site by an institutional review board [221].

Sixty-three patients experienced virologic failure using the definition of a confirmed plasma HIV-1 RNA >1000 copies/mL after 24 weeks of treatment. Paired plasma samples from pre-therapy and virologic failure from 63 patients were available for full-length RT sequencing.

Plasma obtained 12 weeks before failure or 12 weeks after failure were used when a sample from the virologic failure visit was not available. For patients in whom a N348I mutation was detected at failure, longitudinal plasma samples were obtained to assess the timing of emergence of N348I relative to known polymerase domain mutations.

Out of 441 patients who did not reach protocol-defined virologic failure, we randomly selected 202 patients who were administered EFV- or NVP- containing ART, who did not receive prior sdNVP and who had a pre-therapy plasma sample available for full-length RT sequencing.

7.4.2 Amplification and sequencing

Viral RNA was extracted from paired pre-therapy and failure plasma samples using an automated Roche MagNa Pure LC analyzer and MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Germany) or ViroSeq™ HIV-1 Sample Preparation Module (Celera Diagnostics, Alameda, CA) and converted to cDNA using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, Carlsbad, CA) and Reverse-1 primer (5'-CCTGACTTT GGGGATTGTAGGGAAT-3'. Full-length RT (codons 1-560) was amplified by nested PCR using primers: Forward-1 (5'-AGGAAAATGGAAACCAAAAATGATAG-3') and Reverse-1 for first-round PCR and Forward-1 and Reverse-2 (5'-CACAGCTAGCTACTATTTCTTTTGC-3') for second-round PCR. Products were purified with ExoSAP-IT® (USB, Cleveland, OH) and bulk sequenced with 6 bi-directional primers using Big Dye terminator (v.3.1) on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing primers were: (A) 5'–GTAGGACCTACACCTGTCAACAT-3', (B) 5'– TCAGGATGGAGTTCATA-3', (C)

5'–TATGAACTCCATCCTGA-3', (D) 5'–CTGCTCCATCTACATAGAA-3', (E) 5'–AGCCACCTGGATTCCTGA-3' and (F) 5'–TGCTCTCCAATTGCTGTG-3'. Bi-directional sequences were assembled and analyzed using SeqScape® Software Version 2.6 (Applied Biosystems, Foster City, CA). Base-pair mixtures with peak heights greater than 25% were counted as mutations. All primers were obtained from Whitehead Scientific (Pty) Ltd., IDT distributer in South Africa.

Sequences from patients who experienced virologic failure were examined at pre-therapy and virologic failure for known NRTI and NNRTI resistance mutations in the polymerase domain using the International AIDS Society USA (IAS-USA) resistance tables [110] and for novel mutations in the polymerase, connection and the RNase H domains. For patients in whom N348I was present at virologic failure, longitudinal sequences were analyzed and examined for known polymerase domain resistance mutations. Sequences from pre-therapy samples among patients who did not experience virologic failure were analyzed to identify associations between pre-therapy polymorphisms and virologic failure.

Sequences were evaluated for HIV-1 subtype using REGA HIV-1 Subtyping Tool Version 2.0 [222]. All sequences were HIV-1 subtype C. Phylogenetic analyses were performed on pre-therapy versus failure sequence pairs and longitudinal sequences to confirm appropriate clustering of sequences from an individual using ClustalW2 [223, 224].

7.4.3 Statistical analysis

7.4.3.1 Emergence of mutations at virologic failure

Sequences from 63 pre-therapy versus failure pairs from patients who experienced virologic failure with 3TC/d4T plus EFV or NVP were compared for IAS-USA polymerase domain mutations and mutations that occurred in more than one sample in the polymerase, connection and RNase H domains, using two-sided exact McNemar's test. A p-value of <0.05 was considered statistically significant.

7.4.3.2 Associations between pre-therapy mutations and virologic failure

Pre-therapy sequences from patients with no prior exposure to sdNVP and who did not experience virologic failure with EFV (N=158) or NVP (N=44) were compared with pre-therapy sequences from patients who experienced failure for mutations in the polymerase, connection and RNase H domains occurring in more than one sample, using Fisher's exact test (two-sided). A p-value of <0.01 was considered statistically significant. P-values were not corrected for multiple comparisons.

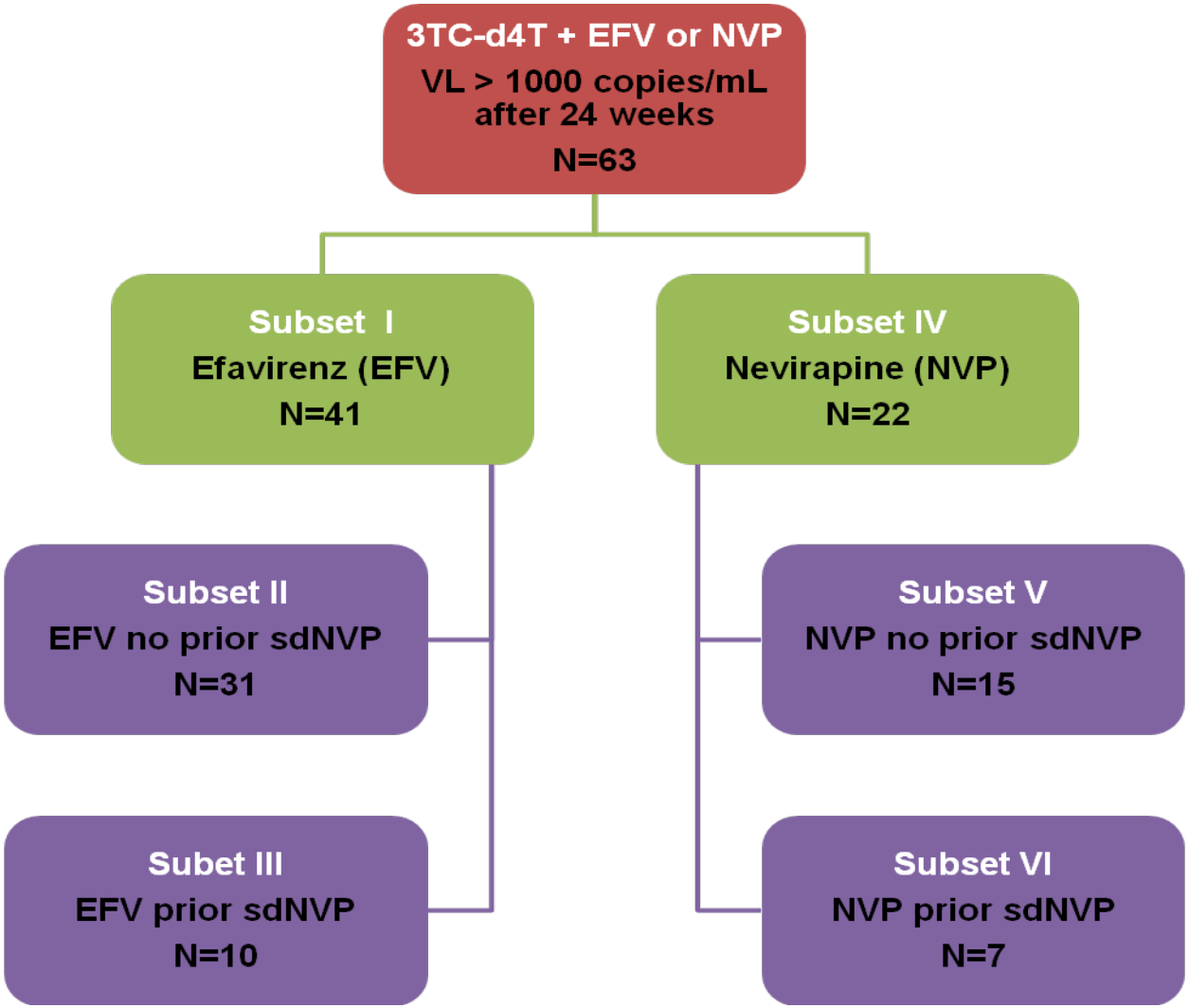


Figure 20. Treatment regimens for CIPRA-SA patients who experienced virologic failure.

7.5 RESULTS

7.5.1 Treatment regimens of patients studied

Figure 20 summarizes the 63 patients who experienced virologic failure with confirmed HIV-1 RNA >1,000 copies/mL and the subsets of patients used for data analysis. Subset I - total number of patients treated with EFV- containing ART, N=41. Subset II - patients treated with EFV/3TC/d4T with no prior-exposure to sdNVP, N=31. Subset III - patients treated with EFV/3TC/d4T with prior-exposure to sdNVP, N=10. Subset IV - total number of patients treated with NVP/3TC/d4T, N=22. Subset V - patients treated with NVP/3TC/d4T with no prior-exposure to sdNVP, N=15. And subset VI - patients treated with NVP/3TC/d4T with prior-exposure to sdNVP, N=7.

7.5.2 Emergence of polymerase domain mutations at virologic failure

Table 13 shows the frequency of polymerase domain resistance mutations (IAS-USA) identified in patients who experienced failure with EFV- or NVP- containing ART. Overall, 71-73% and 81-82% of patients who experienced virologic failure had NRTI or NNRTI mutations in the RT polymerase domain, respectively. NNRTI mutations K103N and V106M were more frequent with EFV-failure than pre-therapy (4.9% at pre-therapy versus 56% at failure, $p<0.001$ and 0% at pre-therapy versus 22% at failure, $p=0.004$, respectively), and Y181C was more frequent with NVP-failure (0% at pre-therapy versus 41% at failure, $p=0.004$) [Tables 14 and 15]. The only NRTI mutation significantly associated with failure of 3TC/d4T plus EFV or NVP treatment was

M184V/I (0% at pre-therapy versus 71% at failure, $p<0.0001$ and 0% at pre-therapy versus 45% at failure, $p<0.0001$, respectively).

The frequency of NNRTI mutations at virologic failure in subsets of patients who were pre-exposed to sdNVP (90% and 100% for EFV- and NVP- containing ART, respectively) was higher than in subsets of patients without prior exposure to sdNVP (77% and 73% for EFV- and NVP- containing ART, respectively) [Table 13]. Specifically, K103N emerged more frequently at failure in EFV/3TC/d4T treated patients who had prior exposure to sdNVP (70%) compared to those without prior sdNVP (52%). Similarly, Y181C appeared more frequently at failure in NVP/3TC/d4T treated patients with prior exposure to sdNVP (71%) compared to those without prior sdNVP (27%) [Table 14 and 15].

A number of IAS-USA polymerase domain mutations emerged with virologic failure but were not statistically significant. These included A62V in three EFV-treated patients; V75I in one EFV- and one NVP- treated patient; K65R in two EFV-treated patients; thymine analog mutations D67N, K70R and K219E in one EFV-treated patient; and NNRTI mutations V90I, A98G, K101E, V106A or I, V108I, E138A and V179D in several EFV- and NVP- treated patients (data not shown). NRTI mutations were always present with NNRTI mutations except for three patients whom M184V occurred alone with failure of EFV-containing ART.

At pre-therapy, NNRTI mutations K101E, K103N, V106M, E138A, V179D, Y181C and G190A were present in a few patients. Only K103N, V106M and E138A occurred in patients previously exposed to sdNVP.

Table 13. Frequency of IAS-USA polymerase domain mutations in CIPRA patients who experienced virologic failure

IAS-USA Polymerase Mutations	EFV			NVP		
	Prior sdNVP[a] N=10	No prior sdNVP N=31	Total N=41	Prior sdNVP N=7	No prior sdNVP N=15	Total N=22
NRTI	6 (60%)	23 (74%)	29 (71%)	7 (100%)	9 (60%)	16 (73%)
NNRTI	9 (90%)	24 (77%)	33 (81%)	7 (100%)	11 (73%)	18 (82%)
NRTI and/or NNRTI	9 (90%)	27 (87%)	36 (88%)	7 (100%)	11 (73%)	18 (82%)

[a] Women with prior exposure to sdNVP to prevent mother-to-child HIV-1 transmission.

Table 14. Mutations significantly more frequent at failure in patients treated with EFV

Domain	Mutation	EFV (N=41)			EFV no prior sdNVP (N=31)			EFV prior sdNVP (N=10)		
		% Pre-therapy (N)	% Failure (N)	p-value[a]	% Pre-therapy (N)	% Failure (N)	p-value[a]	% Pre-therapy (N)	% Failure (N)	p-value[a]
Polymerase	K103N[b]	4.9 (2)	56 (23)	<0.0001	3.2 (1)	52 (16)	<0.0001	10 (1)	70 (7)	0.031
	V106M[b]	0.0 (0)	22 (9)	0.004	0.0 (0)	23 (7)	0.016	0.0 (0)	20 (2)	0.500
	M184V[c]	0.0 (0)	71 (29)	<0.0001	0.0 (0)	74 (23)	<0.0001	0.0 (0)	60 (6)	0.031
Connection	**N348I[d]**	**0.0 (0)**	**12 (5)**	**0.062**	**0.0 (0)**	**13 (4)**	**0.125**	**0.0 (0)**	**10 (1)**	**–**

[a] Two-sided McNemar's exact test.
[b] Polymerase domain mutations resistant to EFV and cross-resistant to NVP and delavirdine (DLV).
[c] Polymerase domain mutation resistant to 3TC and cross-resistant to abacavir (ABC) and emtricitabine (FTC).
[d] N348I is a common connection domain mutation reported in literature. Resistant to AZT, didanosine (ddI), tenofovir (TNV), DLV, EFV, etravirine (ETV) and NVP [187, 204, 205, 210].

Table 15. Mutations significantly more frequent at failure in patients treated with NVP

Domain	Mutation	NVP (N=22)			NVP no prior sdNVP (N=15)			NVP prior sdNVP (N=7)		
		% Pre-therapy (N)	% Failure (N)	p-value[a]	% Pre-therapy (N)	% Failure (N)	p-value[a]	% Pre-therapy (N)	% Failure (N)	p-value[a]
Polymerase	Y181C[b]	0.0 (0)	41 (9)	0.004	0.0 (0)	27 (4)	0.125	0.0 (0)	71 (5)	0.062
	M184V[c]	0.0 (0)	73 (16)	<0.0001	0.0 (0)	60 (9)	0.004	0.0 (0)	100 (7)	0.016
Connection	**N348I[d]**	**0.0 (0)**	**45 (10)**	**0.002**	**0.0 (0)**	**40 (6)**	**0.031**	**0.0 (0)**	**57 (4)**	**0.125**

[a] Two-sided McNemar's exact test.
[b] Polymerase domain mutations resistant to NVP and cross-resistant to DLV, EFV and ETV.
[c] Polymerase domain mutation resistant to 3TC and cross-resistant to ABC and FTC.
[d] N348I is a common connection domain mutation reported in literature. Resistant to AZT, ddI, TNV, DLV, EFV, ETV and NVP [187, 204, 205, 210].

7.5.3 Emergence of connection domain mutation N348I at virologic failure

We next looked for connection and RNase H domain mutations that were more frequent at virologic failure than pre-therapy. The only connection domain mutation significantly more frequent at failure was N348I (Tables 14 and 15). N348I was observed with virologic failure of EFV-containing ART (0% pre-therapy versus 12% failure, p=0.062) and was significantly associated with failure of NVP-containing ART (0% pre-therapy versus 46% at failure, p=0.002). No additional connection domain or RNase H domain mutations were significantly associated with EFV/3TC/d4T or NVP/3TC/d4T failure.

7.5.4 N348I is usually selected after polymerase domain mutations

A previous study reported that N348I emerges early after failure of ART, at the same time as M184V/I and before the appearance of TAMs [187]. To delineate the longitudinal pattern for emergence of polymerase domain resistance mutations we sequenced plasma samples obtained at 4 weeks, 8 weeks, 12 weeks and at subsequent 12-week intervals after initiation of ART. In general, N348I emerged around 24 weeks or later after start of EFV- and NVP- containing ART (Figures 21 and 22). Sequence data could not be obtained for all longitudinal samples; therefore we were unable to determine if N348I emerged after or at the same time as M184V in 12 of 15 patients. Among the 3 patients with longitudinal sequence data available, N348I emerged between 12 and 48 weeks after the appearance of M184V. In addition, NNRTI resistance mutations in the polymerase domain emerged in 6 patients before N348I and five patients had NNRTI mutations before starting ART (2 in the EFV- and 3 in the NVP-treatment group).

Figure 21. N348I usually emerges after polymerase domain resistance mutations in patients who experienced failure with EFV/3TC/d4T (N=5).

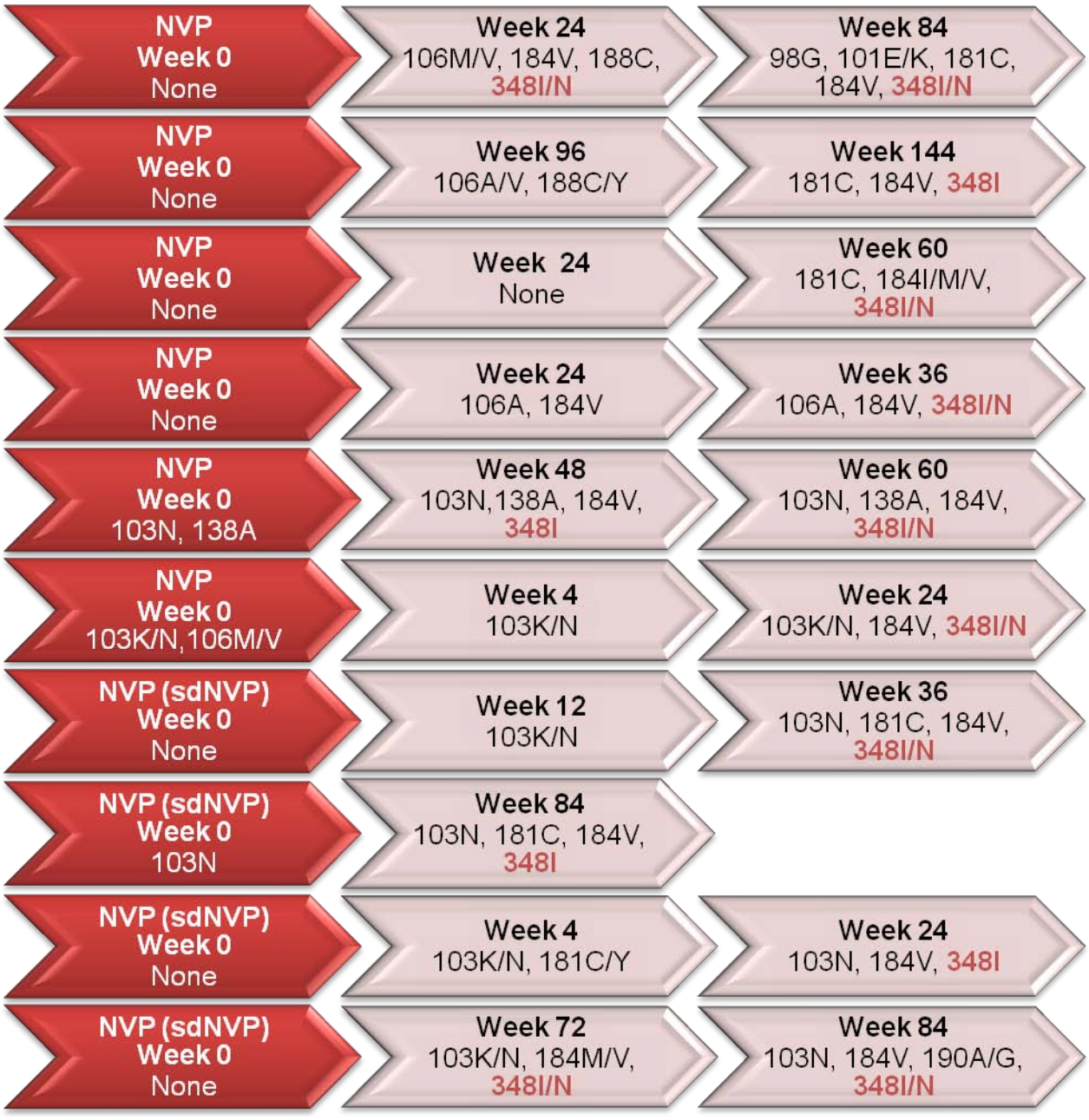


Figure 22. N348I usually emerges after polymerase domain resistance mutations in patients who experienced failure with NVP/3TC/d4T (N=10).

7.5.5 Associations between pre-therapy mutations and virologic failure

Of the 202 pre-therapy sequences from patients who did not experience virologic failure (non-failures), 44 were from patients treated with NVP-containing regimens and 158 were from patients treated with EFV-containing regimens (Tables 16 and 17). None of the non-failure patients received prior sdNVP. The following analyses were not corrected for multiple comparisons and therefore we used a p-value of ≤ 0.01 to define statistical significance.

In the patients who experienced failure with EFV-containing ART (N=41), IAS-USA reported polymerase domain resistance mutations associated with virologic failure were K103N (0% non-failure versus 5% failure, p=0.042) and G190A (0% non-failure versus 5%, p=0.042). One additional polymerase domain mutation was associated with virologic failure, K20R (p=0.006) [Table 16]. No polymerase domain mutations that occurred were significantly more frequent at pre-therapy in NVP/3TC/d4T treated patients who experienced failure compared to non-failure (Table 17).

We next evaluated whether pre-therapy mutations in the connection and RNase H domains were more frequent in failure versus non-failure samples. Connection domain mutations significantly associated with failure in EFV-treated patients (N=41) were: T359S (6% non-failure versus 20% failure, p=0.015), M377L (23% non-failure versus 49% failure, p=0.002) and M377S (0% non-failure versus 7% failure, p=0.008). These same connection domain mutations were significantly more frequent in the subset of EFV-treated patients with no prior exposure to sdNVP (N=31) [Table 16]. In addition, the E516G RNase H domain mutation was more frequent in pre-therapy sequences from patients experiencing failure with EFV-containing ART (N=41; 0% non-failure versus 7% failure, p=0.008) [Table 16]. No connection or RNase H domain

mutations were associated with failure in NVP-treated patients using a p-value of ≤0.01 as our cut-off for statistical significance (Table 17).

Table 16. Pre-therapy mutations significantly more frequent in patients with failure on EFV versus those not failing

Domain	Mutation	EFV Non-Failure (N=158) EFV (N=41)			EFV Non-Failure (N=158) EFV no prior sdNVP (N=31)		
		% Non-failure (N)	% Failure (N)	p-value[a]	% Non-failure (N)	% Failure (N)	p-value[a]
Polymerase	**K20R**	**6.0 (9)**	**20 (8)**	**0.010**	**6.0 (9)**	**23 (7)**	**0.006**
	E39A	1.0 (1)	5.0 (2)	0.108	0.0 (0)	0.0 (0)	—
	K103N	0.0 (0)	5.0 (2)	0.042	0.0 (0)	3.2 (1)	—
	G123K	0.0 (0)	5.0 (2)	0.042	0.0 (0)	6.0 (2)	0.026
	T165I	8.0 (12)	15 (6)	0.217	8.0 (12)	19 (6)	0.042
	G190A[b]	0.0 (0)	5.0 (2)	0.042	0.0 (0)	6.0 (2)	0.026
	K211Q	0.0 (0)	5.0 (2)	0.042	0.0 (0)	3.2 (1)	—
	K311R	**3.0 (5)**	**15 (6)**	**0.011**	3.0 (5)	10 (3)	0.126
Connection	**T359S[c,d]**	**6.0 (10)**	**20 (8)**	**0.015**	**6.0 (10)**	**23 (7)**	**0.010**
	M377L	**23 (36)**	**49 (20)**	**0.002**	**23 (36)**	**45 (14)**	**0.014**
	M377S	**0.0 (0)**	**7.0 (3)**	**0.008**	**0.0 (0)**	**10 (3)**	**0.004**
RNase H	I466V	34 (53)	49 (20)	0.101	34 (53)	55 (17)	0.040
	E492K	0.0 (0)	5.0 (2)	0.042	0.0 (0)	3.2 (1)	—
	E516G	**0.0 (0)**	**7.0 (3)**	**0.008**	**0.0 (0)**	**10 (3)**	**0.004**
	S534T	0.0 (0)	5.0 (2)	0.042	0.0 (0)	6.0 (2)	0.026
	S554N	15 (23)	29 (12)	0.037	15 (23)	26 (8)	0.181

[a] Two-sided Fisher's exact test. Not corrected for multiple comparisons.
[b] Polymerase domain mutation resistant to EFV and NVP and cross-resistant to ETV.
[c] Connection domain mutation reported in literature.
[d] Mutations in **bold** are have a p-value ≤ 0.01.

Table 17. Pre-therapy mutations significantly more frequent in patients with failure on NVP versus those not failing

Domain	Mutation	Non-Failure (N=44) NVP (N=22)			Non-Failure (N=44) NVP No prior sdNVP (N=15)		
		% Non-failure (N)	% Failure (N)	p-value[a]	% Non-failure (N)	% Failure (N)	p-value[a]
Polymerase	None	None			None		
Connection	M377L	27 (12)	50 (11)	0.100	27 (12)	60 (9)	0.031
	G436E	0.0 (0)	14 (3)	0.034	0.0 (0)	13 (2)	0.061
RNase H	E449D	11 (5)	32 (7)	0.086	11 (5)	40 (6)	0.023
	L469I	9.0 (4)	32 (7)	0.033	9.0 (4)	27 (4)	0.184
	T470S	9.0 (4)	27 (6)	0.072	9.0 (4)	33 (5)	0.038

[a] Two-sided Fisher's exact test. Not corrected for multiple comparisons.

7.6 DISCUSSION

This study is the first to show that connection domain mutation N348I emerges frequently in subtype C HIV-1 patients who experienced virologic failure with first-line ART consisting of NVP/3TC/d4T (p=0.002) or EFV/3TC/d4T (p=0.062) although less frequently with EFV-containing ART. NNRTI resistance mutations in the polymerase domain that were significantly more frequent at virologic failure than pre-therapy were K103N ($p < 0.0001$) and V106M (p=0.004) in EFV/3TC/d4T treated patients and Y181C (p=0.004) in NVP/3TC/d4T treated patients. The NRTI mutation most common at virologic failure in both treatment groups was M184V ($p < 0.0001$ compared to pre-therapy).

All patients who experienced failure with N348I also had the M184V mutation. Sequences from 3 patients show that M184V emerges before N348I by 12 to 48 weeks (Figures 21 and 22). In samples which we were unable to identify the timing of emergence of M184V versus N348I, NNRTI polymerase mutations always appeared before N348I. Mutations other than M184V that emerged in RT before N348I include NNRTI polymerase mutations V90I, K103N, V106A/M, E138A, Y181C, Y188C and G190A. This data is in contrast to findings by Yap *et al.* who reported that N348I occurs at the same time as M184V and NNRTI mutations [187]. In the latter study, the authors may not have had access to samples collected at short enough time intervals to distinguish whether N348I occurred before or after polymerase domain mutations.

This work is the first to compare full-length HIV-1 RT sequences in paired plasma samples from subtype C infected patients obtained at pre-therapy and virologic failure. Other studies have compared mutations at pre-therapy and failure; however, mainly using HIV-1 subtype B viruses. In one study, our group compared full-length RT sequences from virologic failure and pre-

therapy samples from 53 patients who experienced failure from the 2 NRTI plus EFV arm of AIDS Clinical Trials Group study (ACTG) A5142 (see Chapter 4). We found that mutations in the polymerase but not in connection or RNase H domains of RT increased in frequency between pre-therapy and failure (K103N, p=0.001 and M184I/V, p=0.016). We propose that connection or RNase H domain mutations were not identified in this study due to the strict definition of virologic failure used in ACTG A5142: lack of suppression of HIV-1 RNA viral load by 1 $\log_{10}$ at/after week 8 or rebound before week 32, or failure to suppress viral load to <200 copies/mL or confirmed rebound >200 copies/mL after week 32. By contrast, HIV-1 subtype C infected patients in the CIPRA-SA study were treated with failing first-line ART until plasma HIV-1 RNA was > 1,000 copies/mL. Patients receiving failing ART over longer periods of time are more likely to develop drug resistance and this may explain the different observations in our two studies. In addition, N348I was probably not detected in ACTG A5142 because EFV and not NVP was the NNRTI used for initial randomized therapy. However, we did observe that EFV can select for N348I in subtype C HIV-1 patients enrolled in CIPRA-SA. Additional studies must be done to clarify these differences.

In a second study, full-length RT sequencing was completed with longitudinal samples collected from one patient infected with HIV-1 subtype D virus and monitored over 3 years. Connection domain mutation N348I emerged with zidovudine and/or didanosine therapy [205]. Additional studies have been completed that do not compare sequences at longitudinal intervals; however, these reports show important data that suggest N348I is selected in patients treated with regimens containing AZT, AZT/ddI, d4T/ddI or AZT monotherapy [205, 215] and the emergence of N348I may be enhanced with the use of 3TC.

In vitro drug susceptibility assays identify N348I as an important drug resistance mutation that decreases susceptibility to both NRTI and NNRTI. N348I alone is able to reduce susceptibility to AZT, ddI, DLV, EFV and NVP [187, 204-206, 210]. When N348I is on the same genome as TAMs or NNRTI mutations the level of drug resistance increases by 2- to 11-fold compared to TAMs or NNRTI mutations alone [187, 204, 210]. RT crystal structures illustrate that residue 348 in the p66 subunit is in close proximity to the NNRTI binding pocket and the nucleic acid binding cleft; however, no RT structures have been solved to describe how N348I might decrease NRTI or NNRTI susceptibility.

Instead, *in vitro* biochemical data suggests two mechanisms by which N348I increases NRTI or NNRTI resistance. In one report, AZT resistance increases when N348I is in the background of TAMs [187, 205]. Specifically, N348I decreases template/primer binding to RT in an RNase H competent complex necessary for RNase H cleavage and increases RT binding in a polymerase-competent complex [189]. RNase H cleavage to form secondary cleavage products is thereby reduced and excision of AZT-MP from a terminated primer increases compared to enzyme with TAMs alone [187-189]. A similar mechanism has been described by our group to explain increased AZT resistance for enzymes with TAMs plus RNase H domain mutation Q509L (see Chapter 2; [217]). In support of a second mechanism, studies showing that mutations L74V, Y181C or M184V antagonize AZT resistance when in the context of TAMs, but when N348I is present with L74V/TAMs, Y181C/TAMs or M184V/TAMs, it counteracts the antagonism and restores AZT drug resistance to levels similar to enzyme with TAMs alone [215, 225]. In the current study, M184V and Y181C emerged first with EFV/3TC/d4T and NVP/3TC/d4T followed by N348I 12-48 weeks later. We hypothesize N348I emerges to counteract the antagonism between TAMs and M184V or Y181C and allow resistance to AZT or

d4T that are part of initial therapy. Mutagenesis and phenotypic studies are planned to test this hypothesis.

Secondary analyses were performed to determine if pre-therapy mutations in RT predict virologic failure. Of the mutations identified more frequently in pre-therapy sequences from patients who experienced failure with EFV/3TC/d4T, only the connection domain mutations T359S, M377L, M377S and RNase H domain mutation E516G were strongly associated (p-value <0.01). Residue 359 in the p66 subunit of RT is part of the RNase H primer grip [74], a region of RT necessary for binding and positioning RNA/DNA T/P for RNase H cleavage. Mutations in the RNase H primer grip severely decrease viral replication and RNase H cleavage specificity [168-170]. In subtype B virus, G (codon GGT) is the reference amino acid at residue 359 compared to the subtype C reference amino acid T (codon ACT). Nonetheless, we show that in subtype C virus, a T to S mutation (codons TCT, AGT, TCG or TCA) appears to predispose to virologic failure of EFV-containing ART.

The M (codon ATG) to L (codons CTG, TTG, CTA or TTA) or S (codon TCG) mutation at residue 377 is also of interest. In the current study M377L is significantly more common in pre-therapy sequences from patients that experienced failure with EFV/3TC/d4T compared to those not experiencing failure (p=0.002). In a separate analysis using samples from ACTG A5142, M377L was also seen more frequently at failure (17%) than pre-therapy (13%), but was not statistically significant (p=0.50) [see Chapter 4].

Finally, RNase H domain mutation E516G was strongly associated with failure of EFV-containing ART (p=0.008). No other studies have identified E516G as being associated with drug resistance. Additional clinical analyses and *in vitro* studies must be completed to investigate how these novel RT mutations may predispose to failure.

Other previously reported connection and RNase H domain mutations were not identified in the current study (see Table 10, Chapter 3). This may be due, in part, to small sample size. Even though our dataset of 63 pre-therapy versus failure pairs of subtype C infected patients failing first-line regimens is the largest to date, we still have limited power to detect mutations that influence NRTI and NNRTI drug resistance occurring infrequently.

In summary, the N348I connection domain mutation emerged frequently with virologic failure of NVP-containing first-line ART and also emerged with EFV-containing ART, although less frequently. The N348I mutation appeared to occur more frequently in subtype C (12%-45%) patients than subtype B (0% in ACTG A5142). This may be due, in part, to the duration of time a patient is treated with failing first-line ART or to genetic differences between subtypes; however, additional studies as outlined below must be completed to confirm these hypotheses.

8 FINAL SUMMARY AND FUTURE DIRECTIONS

At the time this study began, the only known drug resistance mutation in the C-terminus of RT in patients treated with antiretroviral therapy (ART) was G333E in the connection domain. The G333E mutation counteracts the antagonistic effect of M184V on thymidine analog mutations (TAMs) and thereby restores zidovudine (AZT) resistance [163]. It was not known if additional drug resistance mutations emerge in the connection or RNase H domains of HIV-1 RT with failure of ART.

An initial study by Nikolenko e*t al.* showed that introduction of mutations that reduce RNase H cleavage activity increase AZT resistance when present with TAMs. The proposed mechanism for drug resistance was that mutations in the RNase H domain increase AZT resistance by decreasing template/primer degradation, allowing more time for AZT-MP excision and continuation of DNA polymerization [171].

The current project aimed to: (1) characterize resistance and cross-resistance of connection and RNase H domain mutations selected with AZT in cell culture; (2) identify the biochemical mechanism of RNase H domain mutations through analysis of AZT-monophosphate excision and RNase H cleavage; (3) determine if connection or RNase H domain mutations emerge in HIV-1 patients treated with AZT monotherapy or currently recommended ART; and (4) establish the effect of HIV-1 subtype on emergence of RT mutations in patients treated with recommended ART in resource-poor versus resource-rich countries.

8.1 CONNECTION AND RNASE H DOMAIN MUTATIONS SELECTED IN CELL CULTURE

Our first goal was to identify if mutations in the reverse transcriptase (RT) connection or RNase H domains can be selected *in vitro.* We found that after 65 passages of HIV-1 in cell culture with increasing AZT concentrations up to 32 μM, the virus that emerged was >10,000-fold resistant and contained TAMs D67N-K70R-T215F on the same genome as connection domain mutation A371V and RNase H domain mutation Q509L (Chapter 1). Site-directed addition of Q509L and A371V-Q509L mutations to TAMs D67N-K70R-T215F increased AZT resistance 7- and 52-fold compared to TAMs alone, respectively. Mutant virus containing A371V-Q509L and TAMs also conferred 2-3 fold cross-resistance to lamivudine (3TC) and <2-fold for abacavir (ABC) and tenofovir (TNV) [Chapter 1]. This was the first study to show that mutations in the RT connection and RNase H domains are selected by AZT and decrease AZT susceptibility.

8.2 RT RNASE H DOMAIN MUTATION DECREASES RT BINDING TO TEMPLATE/PRIMER IN AN RNASE H COMPETENT MODE AND THEREBY INCREASES AZT-MONOPHOSPHATE EXCISION

It was well known that RT containing TAMs decrease drug susceptibility by influencing the positioning of RT, the terminated template/primer (T/P) and ATP (or PP_i) to increase ATP-mediated excision. At the time this work began, however, little was known about how mutations in the RNase H domains influence RT excision and RNase H cleavage.

In the current study we demonstrate that the RNase H domain mutation Q509L in the background of TAMs enhances the excision of AZT-MP from an AZT-MP terminated RNA/DNA T/P but not from a terminated DNA/DNA T/P. Pre-steady state kinetic analyses revealed that Q509L did not affect initial rates of the polymerase-directed RNase H activity but did reduce polymerase-independent cleavages that occur after a T/P dissociation event, thus refuting the hypothesis that overall RNase H activity is decreased. Competition binding assays provided evidence that Q509L decreases binding of RT to T/P duplexes less than 18 nucleotides in length in a polymerase-independent RNase H cleavage mode, while having no affect on the enzyme's ability to bind T/P in a polymerase-dependent AZT-MP excision competent mode (Chapter 2).

8.3 DO MUTATIONS IN THE CONNECTION AND RNASE H DOMAIN OF RT EMERGE IN HIV-1 INFECTED PATIENTS TREATED WITH ANTIRETROVIRAL THERAPY?

We showed that A371V and Q509L were selected by AZT in cell culture, but there were no data available on the *in vivo* emergence of these mutations with AZT therapy. We therefore initiated studies to identify the emergence of mutations in the connection and RNase H domains of RT using patient serum or plasma samples from 3 different clinical trials: a study of AZT monotherapy (ACTG 175), a study of current first-line ART in developed countries (ACTG A5412) and a study of current first-line ART in resource-limited settings (CIPRA-SA).

8.3.1 Connection domain mutation A360V associated with AZT monotherapy

Our first goal was to analyze full-length RT sequences from a study of patients treated with AZT monotherapy (ACTG 175). We show that connection domain mutation A360V emerges at the same time or after TAMs in patients exposed to AZT monotherapy for greater than 20 weeks and can increase resistance to AZT 11-fold in the background of TAMs in the polymerase domain compared to TAMs alone (Chapter 3).

Connection domain mutation A371V was more frequent after AZT monotherapy but the association was not significant (4.3% at pre-therapy versus 17% at failure, p=0.25). RNase H domain mutation Q509L was not selected in the 23 patients who received AZT monotherapy (Chapter 3). This is the case, in part, because of differences between drug concentration in patient plasma (~2-4 μM) and *in vitro* selection (up to 32 μM) and duration of treatment. Thus, mutations that arise *in vivo* are not always representative of mutations that emerge with ART in patients.

8.3.2 Connection and RNase H domain mutations are not selected in patients treated with currently recommended ART with early detection of virologic failure

Using samples from the ACTG study 175 we were able to show that A360V is significantly associated with AZT monotherapy and occurs with TAMs. However, it was still unknown if connection and RNase H domain mutations in RT would emerge in patients treated with currently recommend ART using strict criteria for virologic failure. Therefore, we sought to compare pre-therapy and failure sequences from predominantly subtype B HIV-1 infected

patients from ACTG study A5142 focusing on patients who received 2 NRTI + efavirenz (EFV) as initial therapy.

In this study, polymerase domain mutations significantly associated with failure were K103N and M184V/I. Unexpectedly, no connection or RNase H domain mutations were significantly more frequent at failure compared to pre-therapy (Chapter 4). In a second analysis, we found that a number of mutations in the polymerase, connection and RNase H domains may predispose failure. However, this finding must be interpreted with caution because of limited sample size and analyses were not corrected for multiple comparisons.

8.3.3 Connection domain mutation N348I is detected in patients from South Africa with virologic failure defined as HIV-1 RNA > 1,000 copies/mL

The last set of samples analyzed was from patients receiving ART regimens recommended for use in resource-limited settings (CIPRA-SA study). This group of patients differed from patients enrolled in ACTG A5142 in that they were infected with subtype C virus, were treated with lamivudine (3TC) - stavudine (d4T) plus EFV or nevirapine (NVP) and virologic failure was defined as greater than 1,000 copies/mL of HIV-1 RNA after week 24. We hypothesized that patients with higher level viremia at failure would more likely have virus with resistance mutations in the polymerase, connection and RNase H domains of RT.

Indeed, our data illustrates that N348I emerges in patients failing EFV- or NVP- containing ART (Chapter 5). Analyses of longitudinal samples from patients with N348I revealed that N348I generally emerges after polymerase domain mutations. This study provides the first conclusive evidence that connection domain mutation N348I emerges in patients treated with

3TC-d4T plus EFV or NVP regimens and that this mutation is missed by current HIV-1 genotyping tests available to clinicians. In secondary analyses, we found that a number of mutations in the polymerase, connection and RNase H domains may predispose to virologic failure including T359S, M377L, M377S and E516G. However, additional studies must be completed to confirm the association of these mutations with treatment failure.

8.4 PUBLIC HEALTH SIGNIFICANCE

In 2008, ~33.4 million people were living with HIV-1 and ~22.4 million of those infected reside in Sub-Saharan Africa. With no vaccine to prevent the spread of HIV, the only available treatment is lifelong ART to inhibit HIV-1 replication. Although ART has profoundly reduced morbidity and mortality from HIV-1 infection, the long-term efficacy is limited by the selection of drug-resistant variants of HIV-1.

8.4.1 Commercially available genotyping assays

HIV-1 drug resistance is identified using either genotypic assays, phenotypic assays or a combination of the two. Genotyping assays sequence regions within the HIV-1 genome to identify nucleic acid changes at residues that are known to confer drug resistance. Two FDA approved commercial genotyping kits available for use with patient samples are TRUGENE™ HIV-1 Genotyping Kit by Visible Genetics and ViroSeq™ HIV-1 Genotyping System by Celera. In general, the kits are designed to sequence the entire HIV-1 protease, the HIV-1 RT

polymerase domain and up to residue 335 in the HIV-1 RT connection domain. However, these kits do not sequence beyond RT residue 335, and our work indicates that these assays miss important drug resistance mutations in the RT connection and RNase H domains.

8.4.2 Should we extend clinical genotyping assays beyond the polymerase domain of RT?

We analyzed samples from pre-therapy and virologic failure from two independent clinical studies: ACTG A5142 in which subtype B infected patients were treated with currently recommended ART for resource-rich countries (2 NRTI plus EFV) and failure was defined with a strict definition of virologic failure and CIPRA-SA in which subtype C infected patients were treated with WHO recommended ART (3TC-d4T plus EFV or NVP) for resource-poor settings and virologic failure defined as >1,000 copies/mL. Connection and RNase H domain mutations did not emerge more frequently in subtype B patients who experience failure indicating that in patients treated with EFV-containing regimens and when virologic failure is detected early, genotyping tests do not need to be extended beyond the polymerase domain to identify resistance mutations in connection and RNase H domains of RT.

By contrast, connection domain mutation N348I did emerge in subtype C patients treated using WHO recommended ART and who experienced failure defined as HIV-1 RNA >1,000 copies/mL. It is not yet clear why N348I was selected in subtype C patients but not in subtype B; however, we propose 2 hypotheses. First, subtype C patients had a higher level viremia than subtype B patients at virologic failure, potentially allowing a longer duration of time for drug resistance mutations to emerge in subtype C than in subtype B patients. Second, in patients who were treated with d4T or AZT and developed 3TC and non-nucleoside reverse transcriptase

inhibitor (NNRTI) resistance, the N348I mutation may be required before selection of TAMs to counteract the antagonism conferred by viruses with both M184V/I or Y181C and TAMs.

The benefit of HIV genotyping is to identify HIV-1 drug resistance mutations that emerge in infected patients and use this information to select the most active drugs for subsequent antiretroviral regimens. Since current genotyping tests generally identify resistance mutations only in the RT polymerase domain, important drug resistance mutations in the RT connection and RNase H domains are being missed. Our study shows that connection domain mutation N348I is selected by 3TC/d4T plus EFV or NVP and may potentially be transmitted as a drug resistance mutation. We hypothesize that if new infections are with HIV-1 that contains N348I, these patients will have low-level resistance to NRTI and NNRTI and fewer active drugs will be available for first-line treatment. In this context, it would be beneficial to extend clinical genotyping tests to include the RT connection domain, especially in resource-limited countries where patients are likely treated with a failing first-line regimen for a longer duration of time than in developed countries.

8.5 FUTURE DIRECTIONS

8.5.1 Drug susceptibility studies

All drug susceptibility data available for mutations in the polymerase, connection and RNase H domains of RT have been observed in HIV-1 viruses with a subtype B genetic backbone. It is unknown if different genetic backbones of HIV-1 (*i.e.* subtype differences) will influence the phenotype of virus containing different drug resistance mutations. Drug susceptibility assays using mutant HIV-1 in subtype specific backbones will elucidate how mutations in the RT polymerase, connection or RNase H domains alter drug susceptibility. In this regard, we are interested in determining if the connection domain mutation N348I alone or in the context of M184V and/or NNRTI mutations has a different drug resistance profile in subtype C compared to subtype B HIV-1.

We plan to construct molecular clones using patient derived polymerase, connection and RNase H domains recombined with wildtype subtype C backbone and test drug susceptibility to all currently used NRTI and NNRTI.

8.5.2 Clinical studies

Additional clinical studies may be used to confirm the emergence of N348I with currently recommended ART. This study and others describe that N348I emerges before or at the same time as M184V/I and NNRTI mutations (Chapter 5) and almost always before TAMs [187]. In addition, N348I did not emerge in subtype B HIV-1 patients treated with 2 NRTI + EFV (ACTG

A5142) with early detection of virologic failure. We suggest that N348I may occur at a higher frequency in subtype C populations compared to subtype B for the following reason.

There is considerable genetic variability between HIV-1 group M. It may be that the different genetic backgrounds of subtypes will accelerate or delay the selection of specific drug resistance mutations. The N (aat) to I (att) codon change at residue 348 is the same for subtype B and C viruses; however, it is possible for neighboring codons to affect the emergence of mutations under selective pressure. ACTG A5175 is a phase IV, randomized, open-label trial developed to compare the emergence of drug resistance in resource poor countries between a once daily 2 NRTI + 1 NNRTI, to a once daily 2 NRTI + 1 protease inhibitor, to a twice daily 2 NRTI + 1 NNRTI. This study is unique in that about 50% of patients enrolled were infected with HIV-1 subtype C and 50% with subtype B. We plan to evaluate mutations that arise in the RT connection and RNase H domains of patients who reached virologic failure with 2 NRTI + 1 NNRTI to determine if there are differences in connection and RNase H domain mutations that emerge in patients with subtype B virus compared to subtype C.

8.5.3 Biochemical studies

We describe that N348I emerges frequently after M184V and NNRTI resistance mutations in patients treated with EFV- or NVP- containing ART. A recent study shows that N348I increases NNRTI resistance 2- to 4- fold as a single mutation and increases NNRTI resistance 3- to >400-fold when present with NNRTI resistance mutations L100I, K103N, Y181C or G190A [204]. Biochemical studies illustrate that RT containing N348I as a single mutation or in combination with NNRTI mutation Y181C decreases the amount of 10 nucleotide RNA/DNA duplex

cleavage product formed [225], suggesting that N348I alone and in combination with Y181C is able to decrease RT binding to T/P in an RNase H competent complex and therefore decrease RNase H cleavage and preserves terminated T/P for possible excision of the terminator. Additional biochemical assays are needed to determine the effect of N348I on RNase H cleavage activity and NNRTI susceptibility in subtype C RT.

In conclusion, this work has significant public health impact by providing an understanding of resistance and cross-resistance conferred by mutations in HIV-1 RT connection and RNase H domains that are likely to affect the efficacy of antiretroviral therapy worldwide. We have identified potential risk of transmitted NRTI and NNRTI drug resistance from the N348I mutation in the connection domain of RT in HIV-1 subtype C patients that is likely to be missed in both resource-rich countries where clinical available resistance tests do not include the connection domain and in resource-limited countries, where in addition, resistance testing is infrequently available. A better understanding of the biochemical mechanism of resistance from N348I, the effect of RT connection and RNase H domain mutations on drug susceptibility in specific HIV-1 subtypes and the importance of N348I for patients receiving currently recommended ART in resource-limited and resource-rich countries will enable us to assess the overall impact resistance mutations in connection and RNase H domains of RT on global efforts to control and end the AIDS epidemic.

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