ANTAGONISTIC EVOLUTIONARY PATHWAYS OF HIV-1 RESISTANCE TO NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS: A VIROLOGICAL, BIOCHEMICAL AND CLINICAL INVESTIGATION

by

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ABSTRACT

K65R, a lysine to arginine change at codon 65 of HIV-1 reverse transcriptase (RT), has been selected *in vitro* by many NRTIs (nucleoside analog RT inhibitors), but until recently, was infrequently detected in patients. Located in the fingers subdomain of RT, K65R causes NRTI resistance through a discrimination mechanism by increasing selectivity for natural deoxynucleotide triphosphate substrates (dNTP) incorporation over triphosphorylated NRTI incorporation. The thymidine analog mutations (TAMs) include the following amino acid changes in RT: M41L, D67N, K70R, L210W, T215F/Y and K219Q. Different combinations of TAMs facilitate AZT resistance by a primer unblocking mechanism, also known as an "excision" mechanism, in which the chain-terminating NRTI is removed from the nascent DNA strand to allow polymerization to resume. From *in vitro* and clinical observations, we proposed that K65R and TAMs represent two different pathways of NRTI resistance that exhibit bi-directional phenotypic antagonism and counterselection *in vivo*. We have generated several lines of evidence in support of this hypothesis: (1) HIV encoding K65R has reduced susceptibility to all NRTIs tested except those with a 3' azido in the pseudosugar component (AZT and AZA); (2) in a large clinical database, K65R is increasing in prevalence in patient isolates, whereas TAMs are decreasing in prevalence; (3) a strong negative relation exists between the frequency of K65R

and specific TAMs among HIV-1 isolates in a large clinical database; (4) K65R reverses viral resistance to AZT caused by TAMs and TAMs reverse resistance to abacavir and tenofovir caused by K65R; (5) K65R antagonizes the primer unblocking activity of TAMs and TAMs antagonize discrimination by K65R; and (6) in plasma samples in which both K65R and TAMs were detected by population sequencing, K65R was not found on the same genome with T215F/Y and 2 or more other TAMs, except when the Q151M multi-drug resistance complex was also present. HIV-1 drug resistance is a significant public health problem. This work contributes to the understanding of NRTI resistance and will help to optimize current and future therapy for HIV-1 infection.

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Acknowledgements

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1. INTRODUCTION

1.1. Historical Perspective

Human immunodeficiency virus (HIV), of the family retroviridae and genus lentivirus (39), is a relatively new human pathogen believed to have originated from the SIVcpz strain endemic in the *Pan troglodytes troglodytes* chimpanzee population in Western Africa (63). Acquired immunodeficiency syndrome (AIDS) was first officially identified in 1981 in a homosexual man (71). Advances in the isolation of the cytokine interleukin-2 and in the development of methods enabling the culture of T cells contributed greatly to the discovery of the AIDS-virus (60-62, 71, 142, 159). Amidst controversy, the new virus was named HIV (37, 38). The clinical definition of AIDS by the CDC was revised in 1993 as a decline in peripheral CD4 T cells to below 200 cells/ml, and modified by the World Health Organization to include a total lymphocyte count of less than 1000 cells/ml (4, 26). Additionally, a plasma RNA level greater than 100,000 copies/ml is a strong predictor of disease progression to AIDS (127, 128).

1.2. The Current Global Epidemic of HIV

In 1982, the Centers for Disease Control reported 593 cases of AIDS in the United States, with death rate oft 41% (243 cases) (27). Since then, the epidemic has exploded worldwide. As of December 2004, 39.4 million people are living with HIV globally, 4.9 million people were newly infected in 2004, and 3.1 million people died in 2004 (199). The mortality has been significantly reduced in the United States from antiretroviral therapy (Figure 1); however the death toll from

the epidemic continues to grow worldwide because of insufficient funds to provide therapy to infected persons in resource limited settings. Many efforts are ongoing to address these problems, including the World Health Organization's 3x5 Initiative to provide 3 million individuals with antiretroviral therapy by the end of the year 2005 (208) or the Global Fund to Fight AIDS, Tuberculosis and Malaria (1).

In the absence of a preventive vaccine, efforts to curb infection have been limited to antiretroviral therapy.

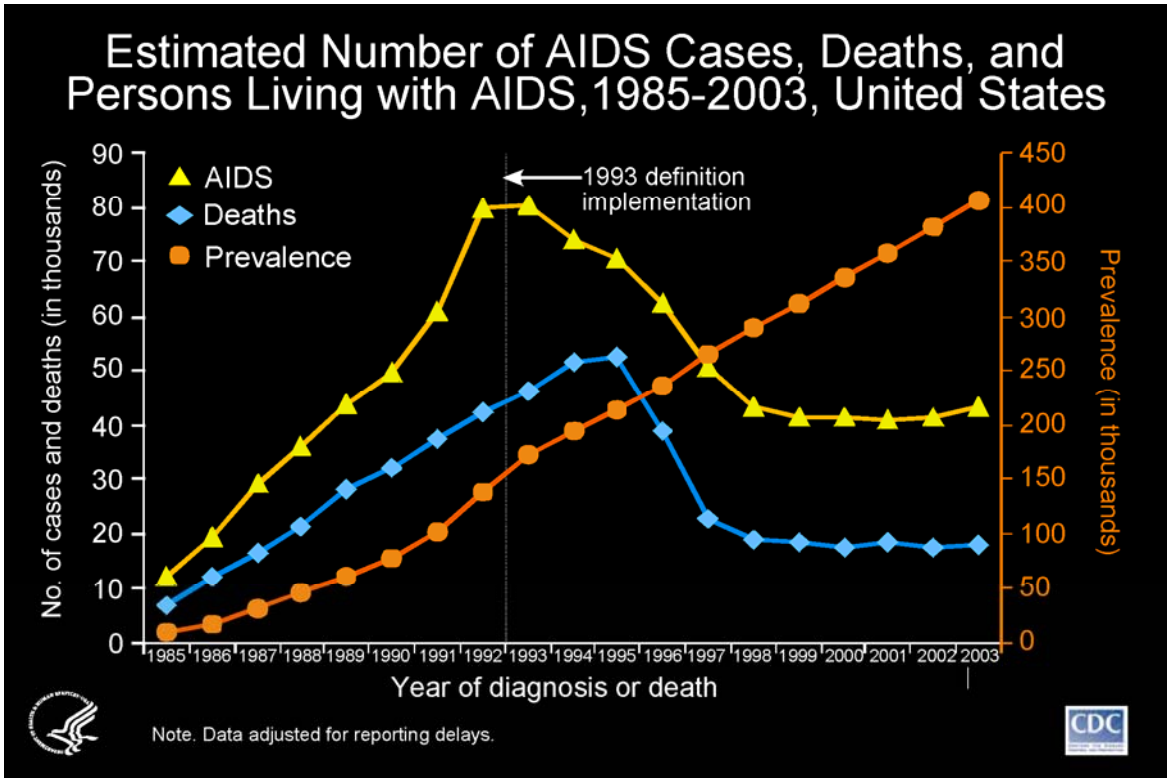


Figure 1. Center for Disease Control's Estimated Number of AIDS Cases, Deaths and Persons Living with AIDS, 1985-2003, United States.
Reproduced from publicly available epidemiology data from the Center for Disease Control, Decatur, GA, United States: www.cdc.gov

1.3. Antiretroviral Therapy

In 1985, 3'-azidothymidine (zidovudine, AZT) was discovered to be a potent *in vitro* inhibitor of HIV, known at the time as human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV) (140). A year later, AZT was tested in HIV-infected persons with advanced immunodeficiency (215) and soon became the first antiretroviral to gain approval by the United States Food and Drug Administration (FDA) (49, 99).

Subsequently, seven other nucleoside or nucleotide analog reverse transcriptase inhibitors (NRTI) have been FDA-approved (51). These include didanosine (ddI, approved Oct 1991) (216), zalcitabine (ddC, approved June 1992) (139), stavudine (d4T, approved June 1994) (10, 119), lamivudine (3TC, approved Nov 1996) (189), abacavir (ABC, approved Dec 1998) (40, 201), tenofovir (TDF, approved Oct 2001) (13, 197) and most recently, emtricitabine (FTC, approved July 2003) (174). All currently approved NRTIs are modifications of the natural deoxynucleoside substrate, lacking a 3' OH group, as highlighted in Figure 2. Nucleoside analogs must be metabolized by the cell to an active triphosphate form in order to compete with the cell's endogenous deoxynucleoside triphosphates (dNTPs) for substrate binding to RT. Once incorporated into a nascent DNA chain, the nucleoside analog acts as a chain terminator due to its lack of a 3' OH group. Metabolism of compounds can differ in efficiency, reliant on variables like cell type, cell cycle stage and infection status (191). The current guidelines for standard of care in the United States for HIV treatment strongly recommend a regimen of a two NRTI backbone prescribed in combination with either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or with a protease inhibitor (PI) (16).

Mutations in reverse transcriptase that cause resistance have been identified for each of these compounds both through *in vitro* viral selection studies and clinical studies (150). HIV has developed resistance to every FDA-approved antiretroviral, including those in other drug classes (150). Despite the improved efficacy of combination antiretroviral therapy, drug resistance remains a significant public health problem. Patients with drug resistant virus have poorer virologic outcome and increased risk of death in the absence of alternative treatment options (72, 147). Thus, understanding the mechanisms of resistance remains a crucial area of research. This study will focus on mechanisms of resistance specifically to NRTIs.

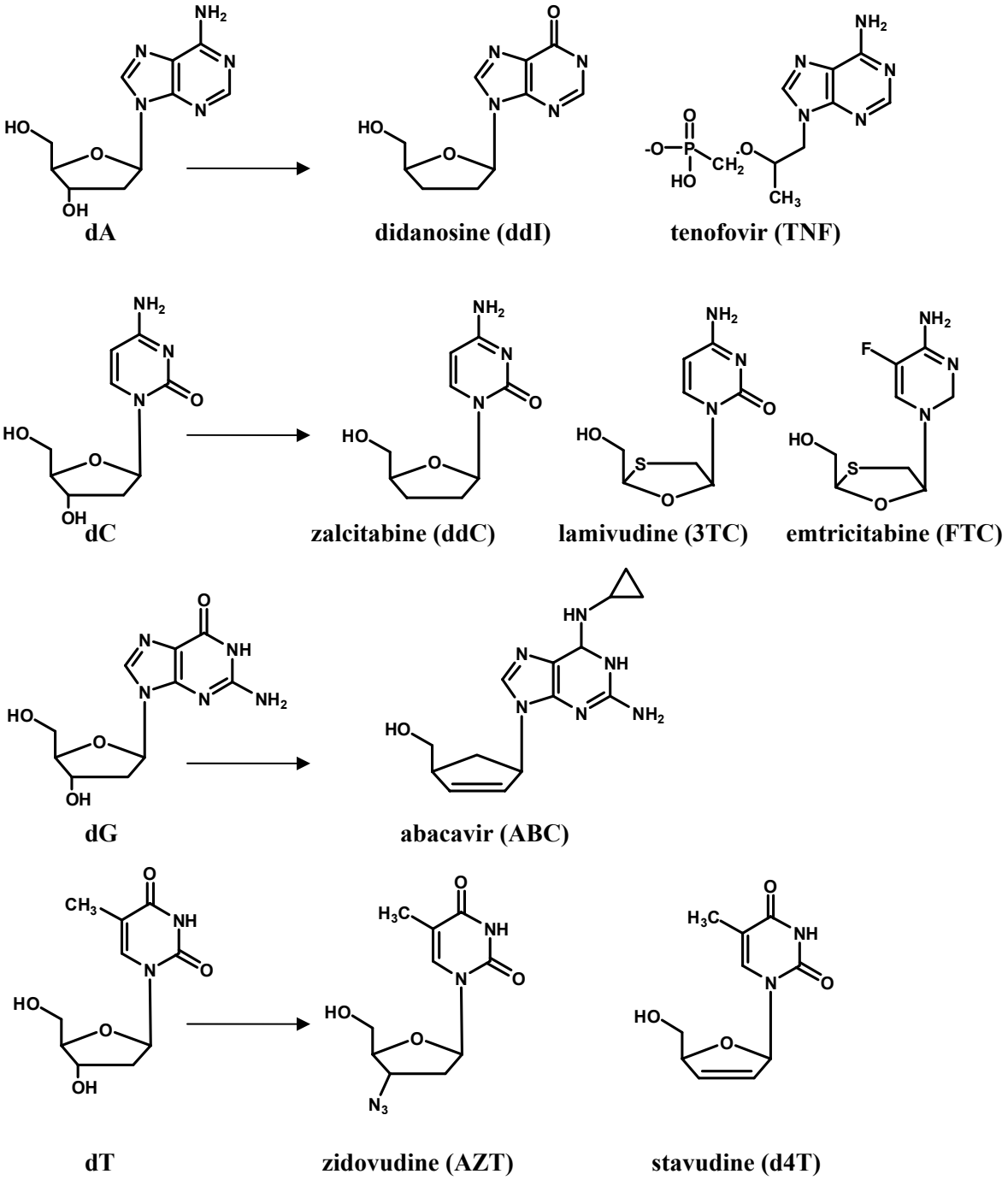


Figure 2. Currently FDA-Approved NRTIs for Use against HIV-1 infection
Natural nucleosides are shown on the left; corresponding NRTI structures are shown on the right. All structures were drawn using ChemDraw Ultra 7.0, CambridgeSoft Co., Cambridge, MA.

1.4. Biology of HIV-1 as it Relates to NRTI Resistance

This section will focus on key aspects of HIV replication that are essential for understanding NRTI resistance.

1.4.1. The Process of Reverse Transcription

1.4.1.1. Overview

The discovery of the reverse transcriptase enzyme (RT) by Baltimore and Temin (12, 195) challenged the central dogma of molecular biology that the flow of genetic information was from DNA to RNA, and proved to be essential for understanding retroviral replication. Characteristic of the family retroviridae, the genetic information of HIV is contained in a single stranded RNA genome present in two copies inside the virion (39). A nucleocapsid pre-integration complex containing the virion-associated capsid, reverse transcriptase, integrase and both viral RNA particles forms in the cytoplasm immediately after entry into the host cell (50). The success of the reverse transcription process is dependent on several enzymatic properties of RT: (1) RT catalyzes both DNA-dependent and RNA-dependent polymerization; (2) RT undergoes template-switching strand-transfer reactions or "jumps"; and (3) RT has a ribonuclease H (RNAse H) domain that degrades RNA from a DNA-RNA hybrid.

Through a complex series of steps, the viral reverse transcriptase enzyme (RT) generates a double-stranded DNA copy of the viral RNA genome (155). This well-defined mechanism is summarized by Coffin and others (39): First, (-)-strand priming and synthesis begins from the primer binding site (PBS) and completes at the 5' RNA terminus, creating a (-)-strand

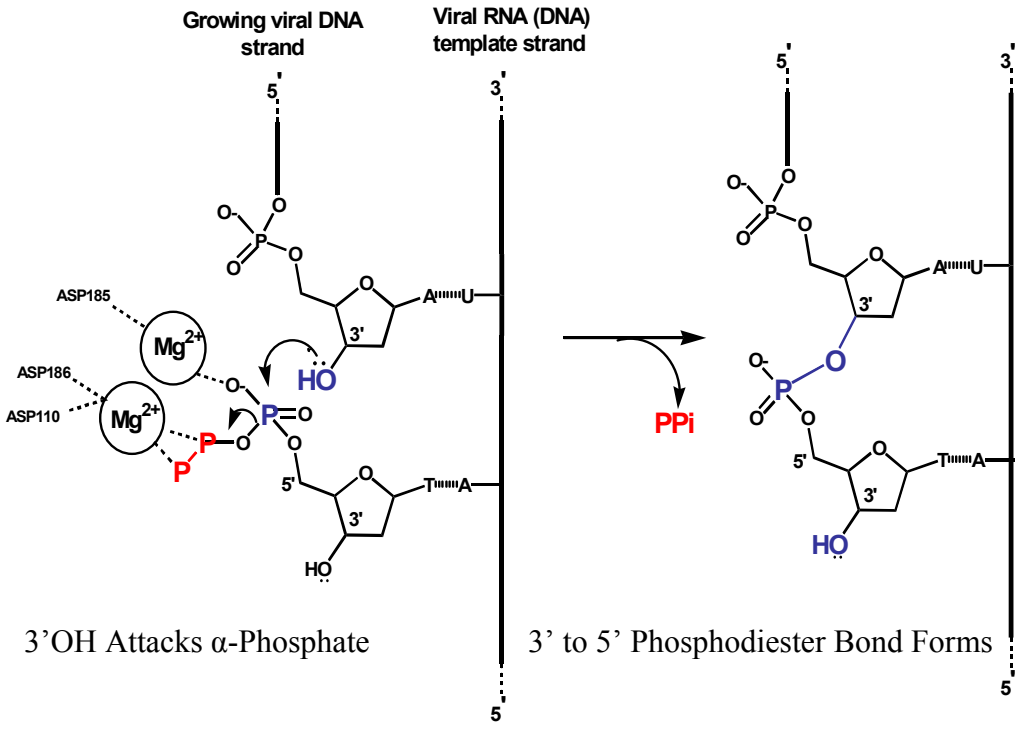
strong-stop DNA fragment (-sssDNA). The RNA fragment of the RNA/-sssDNA hybrid is degraded by RNAse H, after which the first strand transfer occurs. This "jump" of RT results in the usage of –sssDNA to prime DNA synthesis from the 3' to 5' end of RNA. These processes highlight the RNA-dependent polymerase activity of RT (39).

Then, a second RNAse H-mediated degradation of the original RNA strand occurs leaving a short polypurine tract (PPT) which serves as the primer for (+)-strand DNA generation. This process results in the formation of a (+)-strand strong stop DNA fragment (+sssDNA) followed by the second strand transfer reaction which relocates RT to the 5' end of the viral genome. Finally, (+)- and (-)-strand synthesis resumes until a complete, double stranded DNA copy of the viral genome is generated in preparation for integration into the host genome. These processes highlight the DNA-dependent polymerase activity of RT (39).

1.4.1.2. Detailed Mechanism

Enzymatic analyses of RT have elucidated the detailed mechanism of the incorporation of a single deoxynucleotide triphosphate (dNTP) into the nascent DNA chain (31, 85, 87, 213) . The reaction begins with the association of the template/primer (T/P) with RT to form an RT-T/P complex. In the presence of Mg^{2+}, the incoming dNTP binds RT in the substrate binding pocket, creating an RT-T/P-dNTP complex. The affinity of dNTP binding to RT is described by the kinetic constant K_d. After substrate binding, RT undergoes a conformational change which is the rate limiting step in this reaction. Then, polymerization occurs through nucleophilic attack of the α-phosphate of the incoming dNTP by the 3'-hydroxyl of the terminal nucleotide. The formation of a 3' to 5' phosphodiester bond and the release of inorganic pyrophosphate results in the incorporation of the dNTP into the nascent DNA chain

(Figure 3). The rate of this polymerization reaction (which includes the conformational change and chemistry step together) is described by the kinetic parameter k_{pol}. This process can be investigated by pre-steady state kinetic analyses (also known as single turnover analyses) (Figure 4). The reverse of polymerization, phosphorolysis (also known as primer unblocking or excision), can occur, resulting in the removal of the terminal nucleotide from the nascent DNA chain, but this process is inefficient in the absence of thymidine analog resistance mutations (TAMs).



3'OH Attacks α-Phosphate

Figure 3. Phosphodiester Bond Formation during DNA Polymerization.
The 3'OH from the terminal nucleotide attacks the α-Phosphate of the incoming dNTP resulting in the generation of a 3' to 5' phosphodiester bond, and the release of inorganic pyrophosphate. Schematic provided by Dr. Michael Parniak, University of Pittsburgh.

The values for K_d and k_{pol} are influenced by the three-dimensional structure of the T/P and the substrate (dNTP or NRTI-triphosphate [TP]), and by mutations present in RT. Using an instrument that enables the reaction of RT-T/P with substrate and Mg^{2+} at millisecond time points (generally a rapid quench machine), K_d and k_{pol} have been determined for the incorporation of several NRTIs by various RT mutants, including AZT-TP incorporation by RT with TAMs (96), and tenofovir-diphosphate (TNF-DP) incorporation by K65R with and without M184V (44). In the current project, one goal was to determine kinetic constants for binding and incorporation of tenofovir-diphosphate and AZT-TP by RT mutants with both K65R and TAMs. The impact of these mutations on NRTI resistance will be discussed in a later section of the Introduction.

Reverse transcription can occur in a processive manner, where a second dNTP binds immediately after the incorporation of one dNTP is complete. Following the same pathway described above, RT can consecutively incorporate several nucleotides before dissociating from the T/P (Figure 4). Wild-type RT is estimated to incorporate on average 50 bases (and up to 200-300 bases) before dissociating, and processivity can be affected both by mutations in RT and also the sequence of the template (87). Alternatively, after the incorporation of a dNTP, RT can dissociate from the T/P, and then re-associate either on the same T/P or a different T/P. This is referred to as distributive polymerization, and is less efficient than processive polymerization.

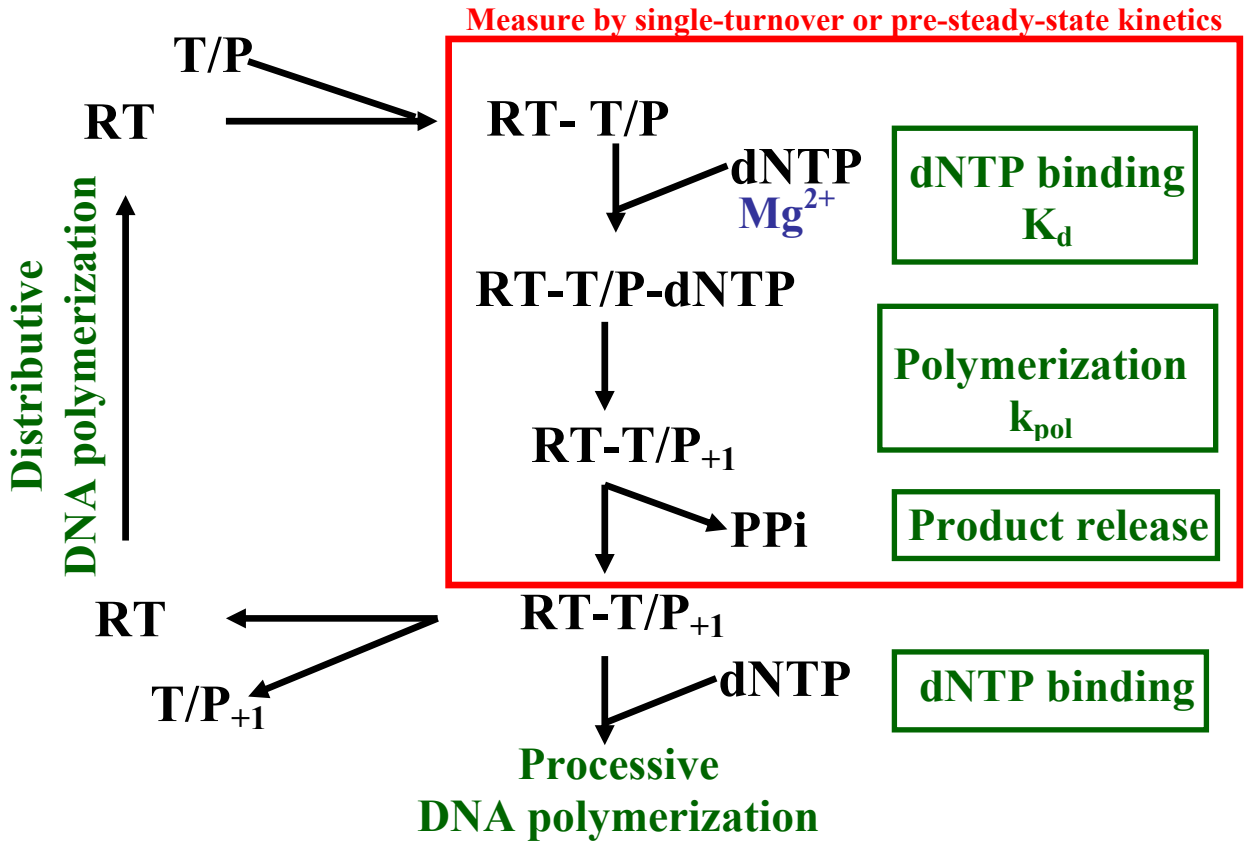


Figure 4. Enzymatic Reactions during DNA Polymerization
Abbreviations: reverse transcriptase (RT), template/primer (T/P), deoxynucleotide triphosphate (dNTP).

A model by Boyer et al. describes the movement of the 3'-terminal nucleotide in the nascent viral DNA chain. Immediately after the addition of a dNTP or NRTI-TP, the 3' primer terminus is located in the nucleotide binding site (N-site). A translocation event then occurs, resulting in the movement of the 3' primer terminus by 1 template base, from the N-site to the primer site (P-site). This event creates an opportunity for the incoming nucleotide to form hydrogen bonds with its complimentary base, before incorporation (Figure 5A).

For the reverse reaction (phosphorolysis) to occur, the terminal NRTI-MP must be located at the N-site. It is necessary for RT to catalyze phosphorolysis of the bound NRTI-MP before the next complementary dNTP or NRTI-TP binds, which results in the formation of a stable "dead end" complex, for which excision is no longer possible (Figure 5B).

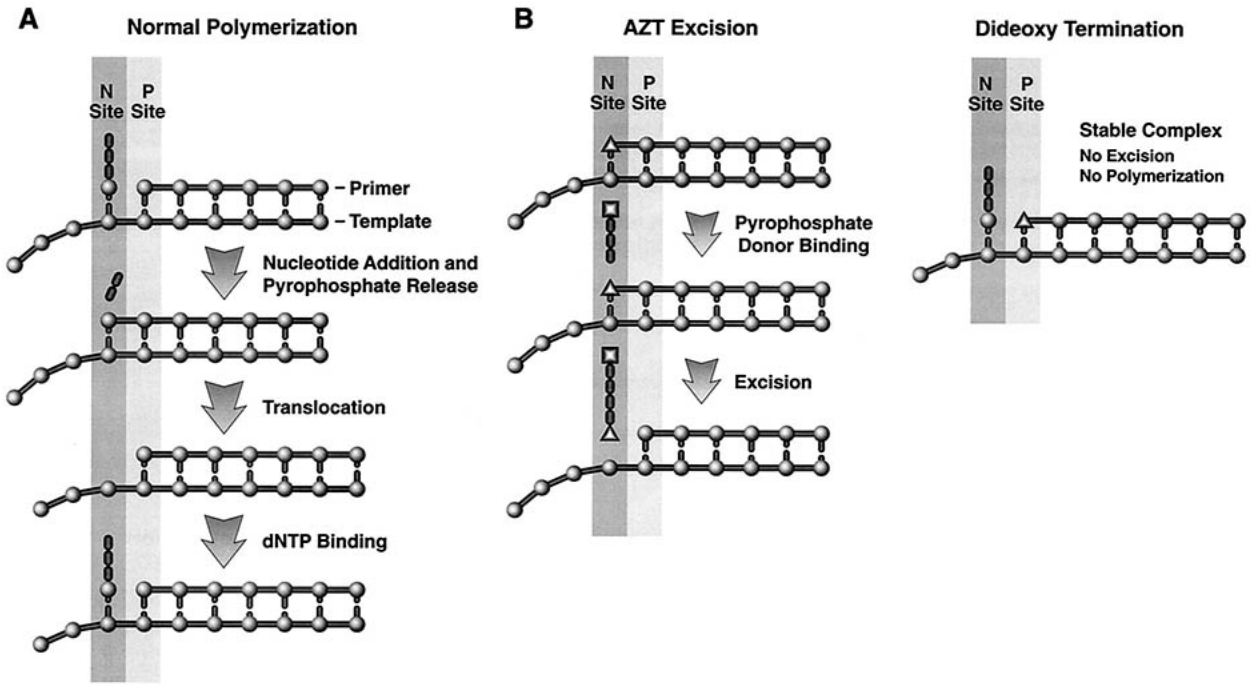


Figure 5. Schematic of Primer Translocation from P-site to N-site During Polymerization and Excision.
Reprinted with permission from the American Society for Microbiology from Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes. 2002. The M184V Mutation Reduces the Selective Excision of Zidovudine 5'-Monophosphate (AZTMP) by the Reverse Transcriptase of Human Immunodeficiency Virus Type 1. J. Virol. 76:3248-3256.

1.4.2. HIV-1 Reverse Transcriptase

1.4.2.1. Structure of Reverse Transcriptase

Reverse transcriptase is a heterodimer composed of two subunits: a 66 kDa subunit (p66) that includes a 15 kDa RNAse H domain, and a 51 kDa subunit (p51) which is generated by proteolytic cleavage of the p66 subunit. The overall tertiary structure of RT is described using an analogy of a right hand, with the functional subdomains of p66 named the fingers, palm, thumb and connection (98) (Figure 6).

During polymerization, the DNA duplex (primer and template) binds between the fingers and the thumb domains, and stretches from the polymerase active site to the RNAse H active site. Several residues in RT form a dNTP-binding pocket, and not surprisingly, many of these codons are also sites at which mutations associated with NRTI resistance occur. Arg72 and Lys65 are both involved in dNTP binding. Arg72 interacts with the α-phosphate of the incoming dNTP, and of particular interest for this thesis, Lys65 (K65) interacts with the β- and γ-phosphates of the incoming dNTP. Met184 may interact with the primer terminus to position it for binding of the dNTP. Although Leu74 does not directly affect dNTP binding, it may be involved in orienting the side chains of Arg72 or Gln151. Finally, a key residue in the binding pocket is Gln151, which interacts directly with the 3'OH in the deoxyribose ring. Together, these interactions position the dNTP for efficient incorporation. Upon binding of a dNTP, conformational changes in RT occur where the tips of the fingers press down towards the palm resulting in base pairing of the dNTP with the templating base (82, 86, 172).

The natural role of codons at which AZT resistance mutations occur is not clearly defined, although structural studies have provided some insights. Codon 70, like other residues in the fingers subdomain, may interact with the γ-phosphate of the incoming dNTP.

Residue 67 and 219 may form a 3'-dNTP binding pocket, whereas residues 215 and 41 are thought to accommodate the bulky azido in the binding pocket. Further, codon 215 may function to facilitate proper orientation of the pyrophosphate donor molecule in the excision reaction (described in further detail in Section 1.5.1) (20, 29, 86, 124, 172). The role of mutations at TAM positions will be discussed later in the Introduction.

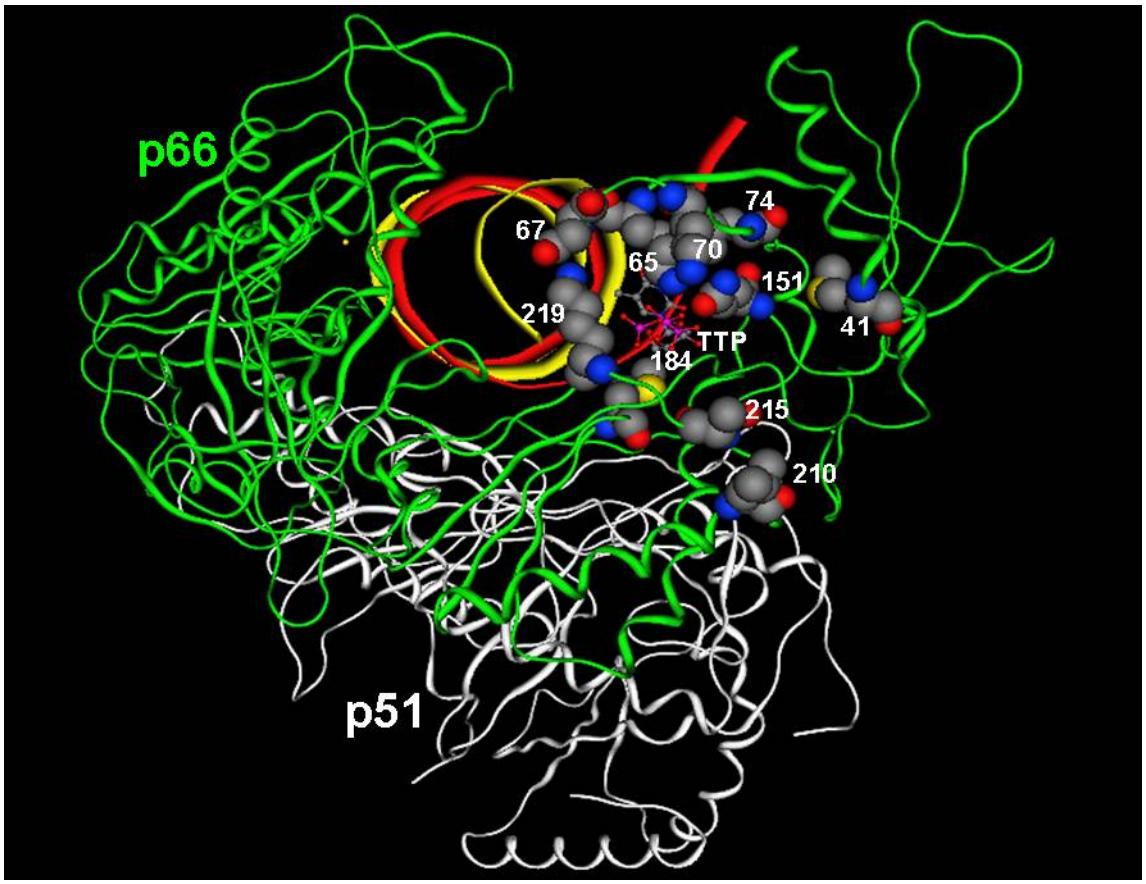


Figure 6. Structure of RT Highlighting Codons Where NRTI Mutations Frequently Occur
Structure drawn using MOE (Molecular Operating Environment Software), based on coordinates from Huang H, Chopra R, Verdine GL, and Harrison SC. Science. (1998) 282:1669-75. Pdb access number: 1RTD. The RT molecule is depicted as ribbons, with the p51 subunit of RT in white, and the p66 subunit in green. The bound primer/template is depicted as cartoon, with the template in red, and the primer in yellow. The incorporation of TTP is modeled as ball-and-stick, highlighting the triphosphates in pink. The codons at which NRTI mutations frequently occur are as labeled as indicated, depicted as space-filling, and include amino acids where TAMs occur (Met41, Asp67, Lys70, Leu210, Thr215, Lys219) and amino acids associated with decreased NRTI-TP incorporation (Lys65, Leu74, Met184, Gln151).

1.4.2.2. Properties of Reverse Transcriptase Important for Drug Resistance

HIV-1 RT can accommodate modest changes in specific structural regions without abrogating function, indicating flexibility (11). The mutations associated with resistance to NRTIs may increase selectivity of NRTI-TP over dNTP, allow excision of an incorporated NRTI, or serve as compensatory mutations to partially restore functional defects caused by other mutations. Although there is often a fitness cost of resistance mutations, mutant RT generally have advantage over wildtype RT in the presence of drug.

Several properties of RT contribute to the rapid development of resistance. One is the lack of a 3'-5' exonuclease proofreading mechanism, resulting in a misincorporation rate of 1/29,000 nucleotides per replication cycle (118). This high error rate contributes to the generation of a diverse population (quasi-species) in infected individuals and outgrowth of drug resistant variants when suboptimal drug pressure is applied. Second, recombination adds to the diversity of HIV, with the rate estimated to be 1-3 events per cycle of replication, and to be 3-10-fold higher than the mutation rate (30, 117, 220). Third, HIV infection is characterized by a rapid, continuous turnover of virus, estimated to be 4.4×10^{10} virions per day, with a complete replacement of population in 2.6 days (141, 157). Combined, these characteristics facilitate viral evolution and escape from immune and drug selection.

1.5. NRTI Resistance

At least four factors contribute to the development of NRTI resistance by HIV: (1) the viral mutation frequency, (2) the mutability of RT, i.e., the ability of RT to tolerate changes in protein composition, (3) the selective pressure of the NRTI (also related to the *in vivo* potency), and (4) the rate and magnitude of viral replication (165). *In vitro* and *in vivo* analyses have demonstrated resistance to all currently approved NRTIs, as well as all inhibitors in other drug classes (non-nucleoside reverse transcriptase inhibitors [NNRTIs], protease inhibitors [PIs]). Further, resistance has been demonstrated to most investigational compounds from all classes (150) with discovery of resistant mutants likely for other compounds as further analysis progresses.

Resistance to NRTIs generally correlates with amino acid changes near or distal to active sites of RT, enabling the enzyme to function efficiently despite the presence of enzyme inhibitors (115). Recent studies have elucidated two major mechanisms of NRTI resistance: discrimination and excision. As illustrated by Figure 7, resistance can occur by a discrimination mechanism, in which changes in RT structure reduce the incorporation of an NRTI versus the normal dNTP substrate. Mutations such as K65R, M184V, L74V and Q151M improve RT's discrimination activity. Alternatively, resistance can occur via an excision mechanism, where changes in RT (primarily TAMs) promote the ATP-catalyzed removal of an incorporated NRTI. This excision mechanism removes the chain-terminating NRTI, unblocking the primer and exposing the 3'-OH, enabling polymerization to resume (for recent review, see Clavel et. al, 2004) (34).

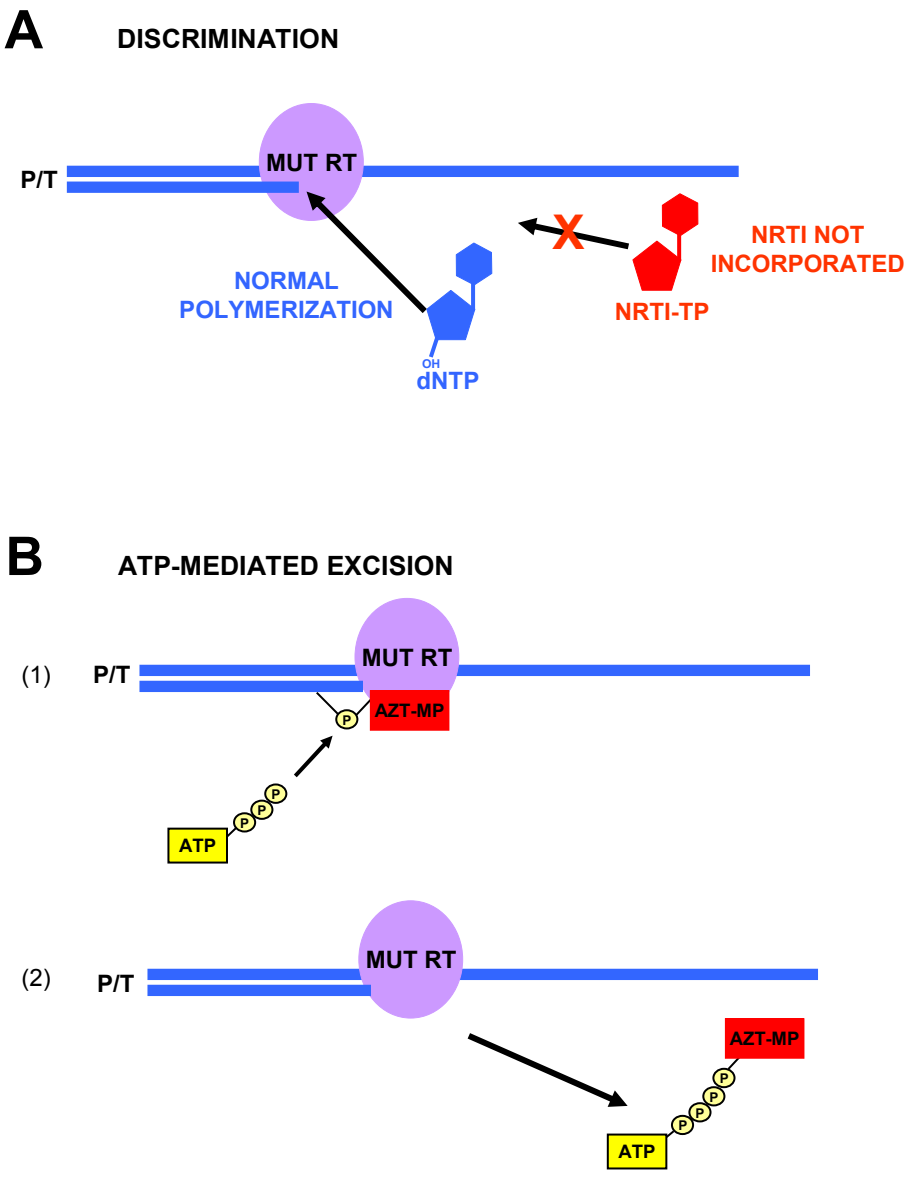


Figure 7. Schematic of the Two Pathways of Resistance to NRTIs: Discrimination and Excision.
(A) Mutant RT catalyzes the preferential incorporation of a dNTP over NRTI-TP resulting in prevention of chain-termination. (B) ATP catalyzes the removal of an incorporated NRTI by mutant RT, enabling polymerization to resume.

1.5.1. Thymidine Analog Mutations and Resistance to AZT

Resistance to AZT was noted soon after its administration as monotherapy to patients (123). In several key studies, Larder and colleagues identified mutations in the reverse transcriptase gene in viral isolates from AZT-treated patients and confirmed through site-directed mutagenesis and *in vitro* susceptibility testing that these codon changes (D67N, K70R, T215F or Y, and K219Q) were the cause of AZT resistance (108-110). Others confirmed the presence of these mutations in AZT treated patients: as many as 30% of patients had AZT resistance mutations after 1 year of AZT monotherapy, and 93% of patients developed resistance after 3 years of AZT monotherapy (103, 203).

Mutations that cause resistance to AZT are collectively known as "thymidine analog mutations" or TAMs, and include the following: M41L, D67N, K70R, L210W, T215F/Y, K219Q (80, 84, 94, 110, 125). They are known as TAMs because they can also be selected by therapy with the thymidine analog 2',3'-didehydro-3'-deoxythymidine (d4T) (36, 156). K70R is generally the first substitution to appear, followed by a substitution at codon 215 to F or Y. Mutations at 41, 210 and 219 generally occur after prolonged therapy. Additionally, all TAMs are not needed in order for the patient to have reduced response to AZT (18, 35). More recent studies have suggested that although K70R is the first to appear in virus from patients on AZT monotherapy, T215F/Y actually appears first in virus from patients on dual NRTI therapy that includes AZT (53). Other amino acid polymorphisms or substitutions have also been linked with TAMs, including E44D, H208Y, R211K and L214F. These mutations may enhance resistance to AZT or confer cross-resistance to NRTIs in combination with classical TAMs (68, 143, 168, 193).

Studies of linkage of TAMs have shown that TAMs can occur in any combination, but they most often occur in two different patterns: M41L/L210W/T215Y and D67N/K70R/T215F/K219Q (95, 120). The reason why these two different patterns occur has not been clearly elucidated (138) but the present study used both combinations of TAMs to further understand any potential mechanistic difference of these mutations in conjunction with the K65R mutation.

For a decade, the mechanism by which TAMs cause high level AZT resistance was not understood. In steady-state work using varying combinations of homodimeric and heterodimeric RT, as well as varying primer/template combinations, Lacey and others found only minor differences in AZT-TP incorporation between RTs from wildtype and mutant virus (102). In congruence with Lacey's work, Krebs and colleagues did not find any differences in incorporation of AZT-TP by DNA/DNA or DNA/RNA primer/templates using pre-steady state quenched-flow methodology (101). Also in similar pre-steady state work, Kerr and Anderson concluded that there was a 4-fold decrease in selectivity (k_{pol}/K_d) for AZT-TP incorporation by AZT-resistant RT compared to wildtype RT using an DNA/RNA primer/template, but no difference in selectivity by mutant versus wildtype RT using a DNA/DNA primer/template (96). In all of these reports, differences in AZT-TP incorporation could not account for high level AZT-resistance observed in cell-based assays and in patients.

Other explanations for AZT resistance by TAMs were pursued. It was speculated that TAMs may cause decreased processive DNA synthesis leading to a decrease in viral replication (23). In contrast, Arts and colleagues used *in vitro* tissue culture-based viral infection studies to propose that resistance to different NRTI combinations is actually due to a stimulation of reverse transcription by M41L and T215Y (but not K70R) in the presence of AZT, and this may

contribute to cross-resistance to other NRTIs including ddI and ddC (9). A structural hypothesis was also proposed that TAMs may induce long range conformational changes in RT that can affect resistance to AZT and other NRTIs (161). Thymidine kinase activity was found to be low in a several CD4+ T cell lines, but this also could not account for high level AZT resistance (45). As a minor advance, Canard et al. hypothesized that enhanced binding of AZT-resistant RT to an AZT-terminated primer-template may indicate that DNA repair activity is involved in the mechanism of AZT resistance by TAMs (25).

Finally, in a breakthrough study, Arion and colleagues reported that TAMs increase pyrophosphate (PPi)-mediated excision of AZT (6). At the same time, Meyer et al. reported that ATP-dependent primer unblocking (i.e. excision) is the mechanism for AZT resistance (130, 131). Using structural analysis, Boyer and colleagues proposed that some TAMs create or enhance an ATP binding site (20). The current consensus is that ATP is the relevant electron donor for the excision reaction; however, the possibility remains that PPi is involved as well (7, 20, 69).

1.5.2. The T215F/Y Mutation in HIV-1 Reverse Transcriptase

Of all the TAMs, most attention has been given to the T215F/Y substitution in HIV-1 RT. T215F/Y is most frequently selected in virus from patients on AZT therapy; and as mentioned previously, T215F/Y may be the first to be selected in virus from patients on combination NRTI therapy that includes AZT (53, 100). T215F/Y also appears to be the key mutation to predict clinical failure of AZT (Rey, Hughes et al. 1998). Additionally, T215Y alone is sufficient to confer a 2.3-fold increase in ATP-dependent removal of AZT-MP from a terminated primer-template; however, efficiency increases with the addition of other TAMs (132).

1.5.3. The K65R Mutation in HIV-1 Reverse Transcriptase

The K65R substitution results from a single nucleoside transition (from AAA to AGA). K65R was first discovered from *in vitro* passage of ddC (74) and has subsequently been selected *in vitro* by many NRTIs (17, 54, 66, 67, 78, 79, 163, 202, 219). Previous work from our lab by J. Hammond showed that every sugar/pseudosugar variation of D-enantiomers studied (including dideoxy [dd], d4, 2'F-d4 and oxathiolane) selected K65R *in vitro*.

Codon 65 is located in the fingers subdomain of RT, and interacts with the β and γ phosphates of the incoming dNTP, properly aligning the dNTP for incorporation (86). Early biochemical studies reported that K65R may increase polymerase processivity to compensate for decreased dNTP incorporation and have increased fidelity (the number of correct incorporations) compared to wild type RT (5, 179). In contrast to TAMs, K65R causes resistance to NRTIs by incorporating ddNTPs less efficiently than dNTPs, resulting in effective discrimination against ddNTPs (176, 186).

Although K65R was well studied *in vitro*, its *in vivo* relevance was uncertain. Studies examining viral fitness and kinetics of dNTP incorporation concluded that the cost of the mutation was too great for virus encoding K65R to survive *in vivo* (207). Indeed, one study reported a replication capacity of HIV-1 with K65R from patient-derived virus of only 10-32% that of wild-type. However the study criteria did not exclude the presence of other mutations in the virus; therefore, these additional mutations may have contributed to the diminished fitness seen in HIV-1 with K65R (205). Another study, using patient-derived virus with only K65R and no other NRTI mutations, reported replication capacity of 65% that of wild-type. The same study reported M184V replication capacity at 58% of wild-type (44). The diminished replication capacity of M184V does not preclude its appearance in patients; indeed, M184V is one of the

most frequently occurring mutations in patients (33). Hence, diminished replication capacity alone is an unlikely explanation for why K65R had been infrequently observed *in vivo*.

Recently, the advent of tenofovir therapy has probably contributed to the rise in prevalence of K65R (192). Nevertheless, the question remains why so many nucleoside analogs select K65R *in vitro* but not *in vivo*. The present study aimed to address this discrepancy by analyzing the conditions that favor selection of K65R. These conditions included the NRTIs used in combination therapy, and interaction of K65R with other NRTI mutations.

1.5.4. Other NRTI Mutations

L74V and M184V are two other well-described NRTI mutations. L74V was first identified as causing ddI resistance and reversing AZT resistance (190). L74V causes resistance via a discrimination mechanism by reducing dideoxynucleotide triphosphate incorporation (48, 180, 182). M184V was also discovered through *in vitro* passage of virus in ddI (75) and 3TC (173, 196). M184V causes resistance to all NRTIs that are L-enantiomers because of steric hindrance between the β-methyl group of valine and the L-pseudosugar, resulting in reduced incorporation (64, 78, 171). HIV-1 with L74V and/or M184V shows increased susceptibility to AZT. This may be because L74V alone, M184V alone, and the L74V/M84V double mutant all have diminished efficiency of ATP-catalyzed removal of AZT-MP from a blocked primer/template (19, 55, 70, 138).

1.5.5. Multi-drug Resistance (MDR) Mutations

Several combinations of mutations have been identified that cause resistance to multiple NRTIs. One such example is the Q151M multi-drug resistance (MDR) complex, comprised of Q151M

with A62V, V75I, F77L and F116Y. The selection of this 5-mutation complex was first noted in patients on combination AZT/ddI therapy or on long-term nucleoside therapy (93, 177). The Q151M MDR complex causes resistance via a discrimination mechanism by reducing electrostatic interactions between the incoming NRTI-TP and RT. This generalized mechanism results in a loss of activity for all NRTIs currently in therapeutic use (43). The replication rate of clones with all 5 MDR complex mutations was similar to wild-type (116), suggesting that reversion of this mutant upon removal of drug pressure may be unlikely.

A second example is the insertion mutations at codon 69 of RT. Alternating AZT and ddI therapy over 2.5 years resulted in multiple mutations, including TAMs and those at codon 69 with a final outcome of resistance to both drugs (52). In contrast to Q151M which can cause AZT-resistance without requiring excision, insertion mutations at codon 69 function in conjunction with TAMs to enhance excision (124, 129). Unusual insertions have been noted, ranging from 2 amino acids in length, to a clinical case where a 15-amino acid insertion between codons 69 and 70 was found that caused 4-fold to 371-fold resistance to AZT, 3TC, ABC, d4T, ddI and ddC without compromising replication capacity (22, 114).

1.6. Combination Antiretroviral Therapy and Interaction between NRTI Mutations

Since the introduction of NRTI therapy, HIV with multiple codon changes in RT has been isolated from patients. Many studies have attempted to understand the combined effect of drug resistance mutations. Early *in vitro* selection studies found that it was difficult to simultaneously select TAMs and M184V when HIV-1 was passaged in the presence of both AZT and 3TC (111). Subsequent studies showed that M184V antagonizes ATP-mediated primer unblocking activity, thus diminishing the ability of TAMs to excise AZT-MP (19). L74V in the context of TAMs similarly diminishes AZT-MP excision capability of RT (138). In separate earlier studies, D. Richman and B. Larder noted that combining AZT with ddC delays ddC resistance but not AZT resistance (106, 166). L74V and K65R are both associated with ddC resistance. The possibility exists that AZT prevented the emergence of L74V or K65R in these patients.

Recently, K65R has also been studied in the context of other NRTI mutations. The K65R/L74V combination is found to be replication deficient and unlikely to occur on the same genome (183, 211). The K65R/M184V combination mutant also has diminished replication capacity (44, 207) . In contrast to K65R/L74V, the M184V mutant does appear on the same genome as K65R, and may have a synergistic resistance effect (15). Of particular interest, K65R has been noted to reverse phenotypic AZT resistance in the context of TAMs (17).

The low prevalence of K65R in clinical samples remains unexplained. K65R exhibits similar characteristics to other mutations frequently seen *in vivo*. Like L74V, K65R is located in the fingers subdomain of RT, and confers resistance by decreased incorporation of NRTI-TP over dNTP. Like M184V, K65R also has diminished replication capacity (44, 207). However, it is not likely that diminished replication capacity alone can explain the low frequency of K65R *in*

vivo. Like the Q151M complex, K65R appears to cause resistance to multiple NRTIs. Q151M may be rare *in vivo* because the emergence of Q151M requires a two base change, possibly through an unfavorable intermediate of Q151L; in addition, 4 other codon changes in RT are required for effective multi-NRTI resistance (43, 65). The difficulty in evolving multiple mutations may explain the low prevalence of the Q151M complex in patients. In contrast, K65R is a point mutation of only one base change that is readily selected *in vitro* (78).

The most important determinant of what mutations are selected *in vivo* may be the baseline genetic composition of the virus present in the patient. Pre-existing mutations, like TAMs, either from transmitted variants or prior therapy, could prevent the selection of antagonistic pathways of resistance mediated by M184V, L74V or K65R. The idea that mutations may have interactions between them was conceived early on, as was the idea that mutations that cause resistance to some NRTIs, could reverse resistance to others (mainly AZT) (24, 107, 190). This thesis sought to further explore these mechanisms of antagonism between mutations with the goal of explaining the low frequency of K65R. Specifically, this study will focus on elucidating the mechanistic basis for multi-NRTI resistance by K65R, and exploring its interaction with TAMs. This work will contribute to the understanding of NRTI resistance which will help to optimize current and future therapy for HIV-1 infection.

2. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Specific combinations of nucleoside reverse transcriptase inhibitors (NRTIs) cause HIV-1 to evolve either in a pathway of resistance mediated by TAMs, or by K65R but not both. This is because K65R and TAMs are bi-directionally antagonistic: these mutations (1) confer different mechanisms of resistance (excision or discrimination); (2) restore susceptibility to several NRTIs when both are present on the same genome; and (3) are counter-selected in patients.

Specific Aims

The overall goal of this project is to determine how HIV-1 evolves specific mutations in reverse transcriptase in response to combinations of nucleoside reverse transcriptase inhibitors (NRTIs). The hypothesis will be tested through three lines of evidence: clinical, virological, and biochemical, by the following specific aims:

1. Determine the *in vitro* resistance profile of K65R alone and in combination with TAMs using cell-based drug susceptibility assays.
2. Analyze how the K65R mutation alone and in combination with TAMs affects the rate of incorporation and rate of excision of HIV-1 RT using steady-state and pre-steady state biochemical assays.
3. Determine the prevalence of K65R alone and in combination with TAMs in clinical samples.

3. CHAPTER ONE. IN VITRO ACTIVITY OF STRUCTURALLY DIVERSE NUCLEOSIDE ANALOGS AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENCODING THE K65R MUTATION IN REVERSE TRANSCRIPTASE

3.1. Preface

This study was presented in part as an oral abstract at the XII International Drug Resistance Workshop, Cabo San Lucas, Mexico, June 2003 (abstract published in Parikh, U. M., D. L. Koontz, J. L. Hammond, L. T. Bacheler, R. F. Schinazi, P. R. Meyer, and J. W. Mellors. 2003. K65R: A Multi-Nucleoside Resistance Mutation of Low but Increasing Frequency. Antivir Ther. 8 (Suppl.): S152). Additionally, this chapter is adapted from a published study (Parikh, U. M., D. L. Koontz, C. K. Chu, R. F. Schinazi, and J. W. Mellors. 2005. *In vitro* Activity of Structurally Diverse Nucleoside Analogs against Human Immunodeficiency Virus Type 1 with the K65R Mutation in Reverse Transcriptase. Antimicrob Agents Chemother. 49:1139-44) reprinted with permission from the American Society for Microbiology. Work described in this chapter is in partial fulfillment of specific aim 1.

3.2. Abstract

Human immunodeficiency virus type 1 (HIV-1) with a lysine-to-arginine substitution at codon 65 (HIV-1_{65R}) of reverse transcriptase (RT) can rapidly emerge in patients being treated with specific combinations of nucleoside analog RT inhibitors (NRTIs). A better understanding of the activity of approved and investigational NRTIs against HIV-1_{65R} is needed to select optimal therapy for patients infected with this mutant and to devise strategies to prevent its emergence. Therefore, we tested a broad panel of NRTIs that differed by enantiomer, pseudosugar, and base component against HIV-1_{65R} to determine how NRTI structure affects activity. Drug susceptibilities of recombinant wild-type (HIV-1_{65K}) or mutant HIV-1_{65R} were determined using a single-replication-cycle susceptibility assay with P4/R5 cells and/or a multiple-replication-cycle susceptibility assay with MT-2 cells. All D, L, and acyclic NRTIs were significantly less active against HIV-1_{65R} than against HIV-1_{65K} except for analogs containing a 3'-azido moiety. Pseudosugar structure and base component but not enantiomer influenced NRTI activity against HIV-1_{65R}. These findings support the inclusion of 3'-azido-3'-deoxythymidine (AZT) in drug combinations to treat patients having HIV-1_{65R} and to prevent its emergence.

3.3. Introduction

The lysine to arginine (AAA to AGA) mutation at codon 65 (K65R) in HIV-1 RT has been selected *in vitro* by several NRTI inhibitors including zalcitabine (219), adefovir (54) , tenofovir (202), (-)β-D-dioxolane-guanosine (D-DXG) (17), (+/-)-2'-deoxy-3'-oxa-4'-thio-5-fluorocytidine [(+/-)dOTFC] (163), (-)-2'-deoxy-3'-oxa-4'-thiocytidine [(-)DOTC], β-D-2',3'-didehydro-3'-deoxycytidine (D-d4C), β-D-2'-fluoro-2',3'-didehydro-2'3'-dideoxyadenine (D-2'F-d4A) (78), β-D-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (D-d4FC) (67, 79) and stavudine (66).

Paradoxically, the K65R substitution had been rarely observed in patients failing combination antiretroviral therapy. In recent clinical trials, however, HIV-1_{65R} has emerged in 24 to 92% of patients with treatment failure on regimens containing tenofovir and lamivudine combined with a third NRTI (abacavir or didanosine) or the non-nucleoside RT inhibitor efavirenz (88, 97, 104, 137). The reason for the high frequency of K65R in these trials is not known but may be related to the exclusion of 3'-azido-3'-deoxythymidine (AZT) from the treatment regimens studied (209, 210).

The mechanism by which K65R causes reduced susceptibility to dideoxy NRTIs has recently been proposed. The wild-type lysine at residue 65 lies in the fingers subdomain of HIV-1 RT and interacts with the γ-phosphate of the deoxynucleotide triphosphate (dNTP) substrate, properly aligning it for incorporation into the nascent DNA chain. The larger arginine residue of K65R likely alters the position of the dNTP, decreasing its incorporation relative to wild-type RT (186). A hydrogen bond between the 3' hydroxyl and one of the β-phosphate oxygen of the incoming dNTP may maintain proper positioning so that catalysis occurs, albeit at a lower rate than for wild-type RT. In the case of dideoxynucleoside triphosphates (ddNTP),

which lack the 3' hydroxyl, the hydrogen bond between the 3' hydroxyl and the oxygen of the β-phosphate is absent such that proper positioning of the ddNTP is not maintained and catalysis is decreased further (176). As a consequence, K65R RT is likely to incorporate ddNTP less efficiently than dNTP, resulting in effective discrimination against ddNTP and resistance to these analogs (176, 186). Other studies have shown that RT with the K65R mutation is less susceptible to 5'-triphosphorylated 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) than to thymidine and guanosine analog triphosphates, but the influence of NRTI structure on activity against K65R RT is not well defined (5, 73, 74). Therefore, we investigated the structural features of NRTIs that affect their activity against HIV-165R. This is the first detailed analysis of the activity of NRTIs against HIV-165R by the use of a large panel of approved and investigational NRTI analogs that differ by base, enantiomer, and pseudosugar structure.

3.4. Materials and Methods

3.4.1. Chemicals

The following analogs were provided by Raymond Schinazi, Ph.D.: β-D-2’,3’-didehydro-2’,3’-dideoxy-5-fluorocytidine (D-d4FC), β-L-2’,3’-didehydro-2’,3’-dideoxy-5-fluorocytidine (L-d4FC), β-D-2’-fluoro, 2’,3’-didehydro-2’,3’-dideoxy-5-fluorocytidine (D-2’F-d4FC), β-L-2’-fluoro, 2’,3’-didehydro-2’,3’-dideoxy-5-cytidine (L-2’F-d4C), β-D-2’-fluoro-2’,3’-didehydro-2’3’-dideoxyadenine (D-2’F-d4A), β-(+)-2’,3’-dideoxy-3’-thiacytidine [(+)-BCH-189 or (+)-3TC], (-)-β-2’,3’-dideoxy-3’-thiacytidine [(-)-3TC], β-D-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(+)-FTC], β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC], β-D-dioxolanefluorocytidine

(D-DOFC), β-L-dioxolane-5-fluorocytidine (L-DOFC), β-D-dioxolanecytidine (D-DOC), β-L-dioxolanecytidine (L-DOC), β-D-dioxolaneguanosine (DXG), β-D-2',3'-didehydro-2'3'-dideoxycytidine (D-d4C), β-D-3'-deoxy-2',3'-didehydrothymidine (d4T), and 3'-azido-2',3'-dideoxycytidine (AZC). 9-[2-(phosphonomethoxy)ethyladenine (PMEA, adefovir) and 9-[*(R)*-2-(phosphonomethoxy)propyl]adenine (PMPA, tenofovir) were provided by Gilead Sciences (Foster City, CA). 3'fluoro-3'-deoxythymidine (FLT) and 3'fluoro-3'-deoxyguanosine (FLG) were obtained from Medivir AB (Huddinge, Sweden). 3'-azido-2',3'-dideoxyadenine (AZA) and 3'-azido-2',3'-dideoxyguanosine (AZG) were obtained from Trilink (San Diego, CA). 3'-azido-3'-deoxythymidine (AZT) was obtained from Trilink and Sigma Chemical Corporation (St. Louis, MO). 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) were also obtained from Sigma Chemical Corporation (St. Louis, MO). 2',3'-dideoxythymidine (ddT) was obtained from Calbiochem (LaJolla, CA). β-D-2'-deoxy-3'-oxa-4'-thiocytidine [(-)-dOTC] and β-L-2'-deoxy-3'-oxa-4'-thiocytidine [(+)-dOTC] were obtained from Biochem Pharma (Laval, Quebec, Canada). *(*1*S,cis)*-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt) (2:1) (abacavir) was obtained from GlaxoWellcome (Research Triangle Park, NC). 2',3'-dideoxyguanosine (ddG) was obtained from ICN Biomedicals, Inc. (Irvine, CA).

The compounds were dissolved in dimethyl sulfoxide or sterile water as 10 mM or 30 mM stock solutions and stored at –20^{o}C. Compounds were diluted immediately before use to the desired concentration in RPMI 1640 culture medium (Whittaker MA Bioproducts, Walkersville, MD) or Dulbecco's Modified Eagle Medium, Phenol Red Free (DMEM-PRF: Gibco-BRL, Grand Island, NY). Pseudosugar structures were drawn using ChemDraw Ultra 7.0.

3.4.2. Cells

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 medium supplemented with 10% fetal bovine serum (FBS: HyClone, Logan, UT), 10 mM HEPES buffer, 50 IU/ml penicillin and 50μg/ml streptomycin. The P4/R5 reporter cell line (provided by Ned Landau, Salk Institute, LaJolla, CA) is a HeLa cell line stably transfected with a Tat-activated β-galactosidase gene under the control of an HIV-LTR promoter. P4/R5 cells were cultured in DMEM-PRF supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 50μg/ml streptomycin, and 0.5 μg/ml puromycin (Clontech, Palo Alto, CA). 293T cells (provided by John Julias and Steven Hughes, NCI, Frederick, MD) were maintained in DMEM supplemented with 5% fetal bovine serum, 5% fetal calf serum (HyClone, Logan, UT), 50 IU/ml penicillin and 50μg/ml streptomycin.

3.4.3. Generation of Mutant Recombinant HIV-1

Mutations in RT were introduced by site-directed mutagenesis using the Altered Sites II kit (Promega, Madison, WI). Silent 5'XmaI and 3'XbaI restriction sites in the xxRT clone (184) facilitated subcloning of the mutated RT fragment into the pxxHIV-1_{LAI} clone, which was then used to generate infectious virus.

3.4.4. Viruses

Stock viruses were prepared using the xxHIV-1_{LAI} clones encoding either 65K or 65R by electroporating (BIO-RAD Gene Pulser®, Hercules, CA) 5-10 μg of plasmid DNA into 1.3 x 10^7 MT-2 cells. At 7 days post-transfection, cell-free supernatant was harvested and stored at -80°C.

Alternatively, 5-10 μg of plasmid DNA was transfected into 293T cells using a calcium phosphate solution (2M $CaCl_2$, 1.37M NaCl, 0.05M KCl, 0.007M $Na_2HPO_4 \cdot 7H_2O$, 0.06M Dextrose, 1M Hepes). Transfected cells were washed with phosphate buffered saline at 8, 24 and 32-hours post-transfection. Cell-free supernatant was harvested 48-52 hours post-transfection, and aliquots were stored at -80°C. The genotype of stock viruses was confirmed by extracting RNA from virions (QIAamp kit, QIAGEN, Valencia, CA), treating the extract with DNAse I (Roche, Indianapolis, IN), amplifying the full length coding region (aa 1-550) of RT by RT-PCR, purifying the PCR product (Wizard PCR Purification System, Promega, Madison, WI), and sequencing the PCR product using the Big Dye terminator kit v.3.1 on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster city, CA). The 50% tissue culture infective dose ($TCID_{50}$) of the virus stock was determined in MT-2 cells or P4/R5 cells by three-fold endpoint dilution assays (six wells per dilution) and calculated using the Reed and Muench equation (160).

3.4.5. Single Replication Cycle Drug Susceptibility Assay

In a 96-well plate, 2 or 3-fold serial dilutions of an inhibitor were added to P4/R5 cells in triplicate. The cells were then infected at an MOI of 0.05 as determined by endpoint dilution in P4/R5 cells. Alternatively, cells were infected with the amount of virus that would yield a relative light unit (RLU) value of 100 in the no drug, virus-infected control wells. 48-hours post-infection, a cell lysis buffer and luminescent substrate (Gal-Screen, Tropix/Applied Biosystems, Foster City, CA) was added to each well and RLU were determined using a luminometer (ThermoLabSystems, Waltham, MA). Inhibition of virus replication was calculated as the

concentration of compound required to inhibit virus replication by 50% (IC_{50}). Fold-resistance was determined by dividing the IC_{50} of mutant HIV-1 by the IC_{50} of wildtype HIV-1.

3.4.6. Multiple Replication Cycle Drug Susceptibility Assay

In a 96-well plate, 3-fold serial dilutions of an inhibitor were added to MT-2 cells in triplicate. The cells were infected at an MOI of 0.01 as determined by endpoint dilution in MT-2 cells. Seven days post-infection, culture supernatants were harvested and treated with 0.5% Triton-X 100. The p24 antigen concentration in the supernatants was then determined using a commercial ELISA assay (DuPont, NEN Products, Wilmington, DE). IC_{50} and fold-resistance was calculated as described above.

3.4.7. Statistical Analysis

IC_{50} values from at least three independent experiments were $\log_{10}$ transformed and compared using a two sample Student's t-test. P-values less than 0.05 were considered to be statistically significant.

3.5. Results

3.5.1. Activity of FDA-Approved NRTIs against HIV-1_{65R}

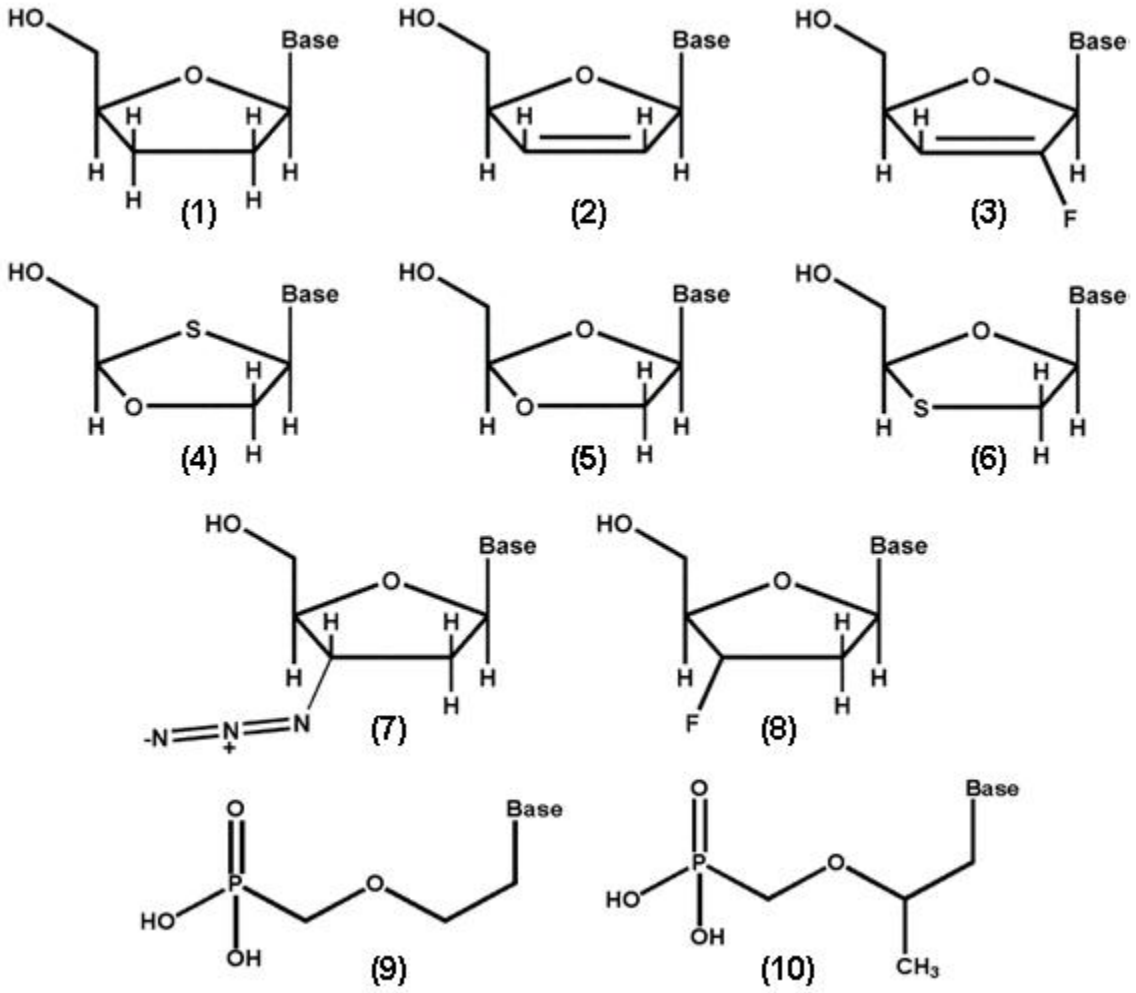
We first studied the activity of the 8 FDA-approved NRTI against HIV-1_{65R} including PMEA (adefovir), which is approved for treatment of hepatitis B virus infection but also has activity against HIV-1. In the single replication cycle susceptibility assay in P4/R5 cells, each of the approved NRTIs showed significantly reduced activity against HIV-1_{65R} except for AZT (Table 1). Fold-decreases in activity against HIV-1_{65R} ranged from 2.5-fold for tenofovir to 77-fold for 3TC. AZT was similarly active against HIV-1_{65R} and wildtype HIV-1_{65K} (1.1-fold decrease). Similar results were obtained with the multiple replication cycle susceptibility assay in MT-2 cells (Table 1). The approved NRTI studied differ by stereochemistry (3TC and FTC have the L enantiomeric configuration, all others have the D configuration), by base (AZT and d4T are thymidine analogs, 3TC and ddC are cytidine analogs, ddI and tenofovir are adenine analogs, and abacavir is a guanosine analog), and by pseudosugar structure. PMPA and PMEA both have acyclic pseudosugar structures (Figure 8).

Table 1. Activity of FDA-Approved NRTIs against HIV-1 Encoding K65R

	Single Cycle Replication Assay (IC_{50}, uM)[a,c]			Multiple Cycle Replication Assay (IC_{50}, uM)[b,c]		
Compound	**HIV-1_{WT}**	**HIV-1_{65R}**	**Significance**	**HIV-1_{WT}**	**HIV-1_{65R}**	**Significance**
AZT (Zidovudine)	0.20 ± 0.12	0.22 ± 0.16 (1.1)	p = 0.77	0.031 ± 0.024	0.028 ± 0.009 (0.9)	p = 0.58
d4T (Stavudine)	5.72 ± 3.34	21.8 ± 23.8 (3.8)	p < 0.001	1.87 ± 0.76	3.93 ± 1.34 (2.1)	p < 0.05
ddC (Zalcitabine)	1.35 ± 0.70	6.79 ± 3.98 (5.0)	p < 0.0001	0.13 ± 0.18	0.29 ± 0.23 (2.3)	p = 0.01
ddI (Didanosine)	2.32 ± 0.98	6.26 ± 2.92 (2.7)	p < 0.0001	0.49 ± 0.32	2.37 ± 1.80 (4.8)	p < 0.0001
ABC (Abacavir)	6.21 ± 1.66	26.3 ± 4.18 (4.2)	p < 0.0001	0.076 ± 0.010	0.60 ± 0.11 (7.9)	p < 0.0001
3TC (Lamivudine)	0.78 ± 0.48	60.5 ± 66.8 (77)	p < 0.0001	0.60 ± 0.46	15.3 ± 10.8 (25)	p < 0.0001
FTC (Emtricitabine)	0.17 ± 0.07	3.75 ± 1.75 (22)	p < 0.0001	0.044 ± 0.036	0.90 ± 0.42 (20)	p<0.001
PMPA (Tenofovir)	4.67 ± 2.31	11.4 ± 3.8 (2.5)	p < 0.0001	0.54 ± 0.61	3.82 ± 1.91 (7.1)	p < 0.005
PMEA (Adefovir)[d]	6.21 ± 0.91	23.3 ± 7.9 (3.7)	p < 0.001	not tested	not tested	not tested

a IC_{50} values determined by measuring inhibition of luminescence in P4/R5 cells.
b IC_{50} values determined by measuring inhibition of p24 production in MT-2 cells.
c Mean ± standard deviation from at least three independent experiments. Fold-resistance relative to wild-type shown in parentheses
d Approved for treatment of Hepatitis B virus, not HIV-1

Figure 8. Pseudosugar Structure of NRTIs Tested



(1) 2',3'-dideoxynucleosides (ddC, ddT, ddI, ddG)
(2) 2',3'-didehydro-2'3'-dideoxynucleosides (d4C, d4T)
(3) 2'-fluoro-2',3'-didehydro-2'3'-dideoxynucleosides (2'-F-d4FC, 2'-F-d4A)
(4) 2'-deoxy-3'-oxa-4'-thiocytidines (dOTC)
(5) dioxolane-nucleosides (DOC, DXG)
(6) 2',3'-dideoxy-3'-thiacytidines (3TC, FTC)
(7) 3'-azido-3'-deoxynucleosides (AZT, AZC, AZG, AZA)
(8) 3'-fluoro-3'deoxynucleosides (FLT, FLG)
(9) 9-[2-(phosphonomethoxy)ethyl nucleotide (PMEA)
(10) 9-[(R)-2-(phosphonomethoxy)propyl] nucleotide (PMPA)

3.5.2. Activity of Investigational NRTIs against HIV-1_{65R}

The reduced activity of the approved NRTIs against HIV-1_{65R} led us to further explore the structural features of NRTIs that influence activity against this mutant. A panel of 30 NRTIs that differed by stereochemistry, base, and pseudosugar structure were tested against HIV-1_{65R} and wildtype HIV-1_{65K}. All compounds were tested in the single cycle susceptibility assay; compounds that were not active in P4/R5 cells were tested in the multiple cycle assay in MT-2 cells.

Figure 8 shows the pseudosugar structures of the investigational NRTI studied. All lack a 3' OH group (structures 1-11); the 3'OH has been replaced by a hydrogen (structure 1), a double bond (2 and 3), an azido group (7) or a fluorine (8). In structures 4-6, the 3'carbon of the pseudosugar ring has been replaced by oxygen (4 and 5) or sulfur (6). DOTC (4) also has a sulfur atom replacing the normal oxygen of the pentose ring. The enantiomeric configuration of the analogs (D vs. L) and the base component linked to the pseudosugar also vary; bases include adenine, cytosine, guanine, thymine, inosine, 5-fluorocytosine (FC), and 2-amino-6-(cyclopropylamino)-purine (abacavir).

3.5.3. Activity of NRTI Differing by Base Component against HIV-1_{65R}

HIV-1_{65R} was tested against dideoxy compounds that differed only by base component: ddC, ddG, ddI and ddT. The activity of ddC, ddG, ddI, and ddT was decreased 5.0, 4.0, 2.7 and 2.2-fold, respectively, against HIV-1_{65R} compared to wildtype HIV-1_{65K} (Table 2), indicating a moderate influence of base component on NRTI activity. Several compounds differing only by cytosine fluorination were tested including both D-enantiomers (D-d4C vs. D-d4FC and D-DOC vs. D-DOFC) and L-enantiomers (L-DOC vs. L-DOFC and (-)-3TC vs. (-)-FTC). Fluorination

of cytosine did not significantly affect activity against HIV-1_{65R} ($p>0.05$) (Table 1, Table 2, Table 3).

3.5.4. Activity of NRTI Differing by Enantiomer against HIV-1_{65R}

All L-enantiomeric NRTI tested had lower activity against HIV-1_{65R} than wildtype, including (-)-3TC (77-fold), (-)-FTC (22-fold), L-DOFC (10-fold), (+)-DOTC (8.1-fold), L-DOC (8.1-fold) and L-d4FC (5.2-fold). The corresponding D-enantiomeric compounds (+)-3TC, (+)-FTC, D-DOFC, (-)-DOTC, D-DOC and D-d4FC all had similar reductions in activity as their L-enantiomer counterparts (Table 2-3), indicating that enantiomer did not affect activity against HIV-1_{65R}.

3.5.5. Activity of NRTI Differing by Pseudosugar Component against HIV-1_{65R}

The dideoxy compounds differ from natural nucleosides only by substitution of the 3'hydroxyl by a 3'hydrogen. Dideoxy compounds were 3.4-fold less active on average against HIV-1_{65R} compared with wildtype HIV-1_{65K}. Replacing the 3'hydrogen with a double bond in d4 compounds further reduced activity against HIV-1_{65R} by an average of 6.7-fold. Adding a 2'fluorine to D-d4FC did not significantly affect activity against HIV-1_{65R} (D-d4FC, 12-fold decrease; D-2'F-d4FC, 11.5-fold decrease). However, altering the base from a cytosine to an adenine (D-2'F-d4FC vs. D-2'F-d4A) significantly increased activity against HIV-1_{65R} ($p<0.0005$); D-2'F-d4FC was 11.5-fold less active against HIV-1_{65R}, whereas D-2'F-d4A was only 2.1-fold less active. This provides further evidence that base component can affect NRTI activity against HIV-1_{65R}.

Dioxolanes contain an oxygen in place of the 3'carbon in the pseudosugar ring. 3TC and FTC are oxathiolane analogs that contain a 3' sulfur in the pseudosugar ring structure. Compounds with a substitution of oxygen or sulfur for the 3'carbon showed the greatest loss of activity against HIV-1_{65R}. Both enantiomeric forms of 3TC showed >20-fold loss of activity (Table 3). (-)-FTC showed >20-fold loss of activity against HIV-1_{65R} and (+)-FTC had no activity against HIV-1_{65R} at the highest concentration tested (90 μM). The dioxolanes also showed decreased activity: D-DOC (11-fold), (-)-DOTC (10-fold), D-DOFC (6.1-fold), and D-DXG (6-fold).

FLT and FLG have a 3' fluorine in place of the 3'hydroxyl. FLG and FLT were 4.0-fold and 2.3-fold less active, respectively, against HIV-1_{65R} than wild-type HIV-1_{65K} (Table 3). The 3'fluorine in FLT and FLG have a similar position and negative charge as the natural 3' hydroxyl group, which may explain the relative preservation of their activity against HIV-1_{65R}.

Table 2. *In vitro* Activity of Analogs with Dideoxy- and D4-Pseudosugars against HIV-1 Encoding K65R in RT

Compound[a]	Mean IC_{50} (μM) ± SD[b]			P-value
	$HIV\text{-}1_{WT}$	$HIV\text{-}1_{65R}$	Fold Increase[c]	
ddC	1.35 ± 0.70	6.79 ± 3.98	**5.0**	<0.0001
ddG	3.11 ± 0.64	12.5 ± 1.5	**4.0**	<0.0001
ddI	2.32 ± 0.98	6.26 ± 2.92	**2.7**	<0.0001
ddT[d]	10.2 ± 4.8	22.3 ± 9.3	**2.2**	0.01
d4C	2.63 ± 1.20	17.9 ± 6.4	**6.8**	<0.0001
d4T	5.72 ± 3.34	21.8 ± 23.8	**3.8**	<0.001
d4FC	1.26 ± 0.51	14.6 ± 7.1	**12**	<0.0001
2'-F-d4A	2.19 ± 0.91	4.35 ± 1.4	**2.1**	<0.01
2'-F-d4FC[d]	7.35 ± 5.6	84.5 ± 9.6	**11.5**	0.001
L-d4FC	0.23 ± 0.14	1.20 ± 0.17	**5.2**	0.005

a Compounds are D-enantiomers except for L-d4FC

b Mean ± standard deviation from at least three independent experiments. All assays done in P4/R5 cells using a single replication cycle assay unless otherwise noted.

c Fold-increase in IC_{50} relative to wild-type.

d Assay done with MT-2 cells (see Materials and Methods).

Table 3. *In vitro* Activity of D- and L-enantiomers of Analogs with 3'C or 3'OH Substitutions against HIV-1 Encoding K65R

Compound	Mean IC_{50} (μM) ± SD[a]			P-value
	HIV-1_{WT}	HIV-1_{65R}	Fold Increase[b]	
D-enantiomers				
(+)-3TC	1.20 ± 0.56	32.9 ± 10	**27**	<0.0001
(+)-FTC	11.6 ± 3.4	>90	**7.7**	<0.0001
D-DOC	0.34 ± 0.07	3.57 ± 1.4	**11**	<0.0001
D-DOFC	0.89 ± 0.56	5.41 ± 1.6	**6.1**	<0.0001
(-)-DOTC[c]	6.34 ± 1.74	65.5 ± 37	**10**	<0.01
DXG	3.60 ± 0.31	21.8 ± 8.2	**6.0**	<0.005
FLG	1.02 ± 0.41	4.05 ± 0.89	**4.0**	<0.005
FLT	0.18 ± 0.05	0.41 ± 0.03	**2.3**	<0.005
L-enantiomers				
(-)3TC	0.78 ± 0.48	60.5 ± 66.8	**77**	<0.0001
(-)-FTC	0.17 ± 0.07	3.75 ± 1.75	**22**	<0.0001
L-DOC	0.082 ± 0.04	0.66 ± 0.18	**8.1**	<0.001
L-DOFC	0.032 ± 0.01	0.34 ± 0.21	**10**	<0.0005
(+)-DOTC[c]	11.2 ± 4.4	>90	**8.1**	<0.001

a Mean ± standard deviation from at least three independent experiments.
b Fold-resistance relative to wild-type.
c Assay done with MT-2 cells (see Materials and Methods).

3.5.6. Activity of 3' Azido Analogs against HIV-1_{65R}

3'-azido-containing analogs were the only compounds that did not show consistent loss of activity against HIV-1_{65R} (Table 4). AZT and AZA showed no loss of activity against HIV-1_{65R} (1.1- and 0.9-fold, respectively). AZG and AZC were 2.0- and 2.5-fold less active, respectively, against HIV-1_{65R} than HIV-1_{65K} but this reduction in activity was not significant ($p = 0.09$ for AZG) or showed borderline significance ($p = 0.05$ for AZC).

Table 4. *In vitro* Activity of 3'- Azido Analogs against HIV-1 Encoding K65R

Compound	Mean IC_{50} (μM) ± SD[a]			P-value
	HIV-1_{WT}	HIV-1_{65R}	Fold Change[b]	
AZA	3.92 ± 1.1	3.72 ± 0.30	**0.9**	0.89
AZG[c]	7.90 ± 4.63	15.8 ± 9.8	**2.0**	0.09
AZC[c]	15.0 ± 10.7	37.8 ± 28.5	**2.5**	0.05
AZT	0.20 ± 0.12	0.22 ± 0.16	**1.1**	0.77

a Mean ± standard deviation from at least three independent experiments.
b Fold-change relative to wild-type.
c Assay done in MT-2 cells

3.6. Discussion

Prior studies have shown that the K65R mutation in HIV-1 RT decreases susceptibility to various NRTIs including ddC, PMEA, PMPA, D-DXG, (+/-)dOTC, D-d4FC and d4T (17, 54, 66, 67, 163, 202, 219). Our study is the first to systemically analyze the structural components of NRTIs that influence activity against HIV-1_{65R} using the same susceptibility assays. We found that all D- and L- and acyclic NRTIs tested had significantly reduced activity against HIV-1_{65R} except those containing a 3'azido moiety (AZT and AZA). In addition, the structural features of NRTIs that influence activity were identified.

Pseudosugar structure had the greatest impact on activity against HIV-1_{65R}. Comparing cytidine analogs that differed only by pseudosugar structure, the greatest loss of activity was seen with (+)-3TC (77-fold), whereas the least was seen with ddC (5.0-fold). The rank order of greatest to least loss of activity against HIV-1_{65R} for D or (+) cytidine pseudosugars is: 3TC ≅ FTC>dOTC>DOC>d4C>ddC. The fold-resistance for 3TC was significantly different from DOC, d4C and ddC ($p \leq 0.05$) with a trend towards significance from dOTC ($p=0.06$). The fold-resistance for dOTC and DOC were also significantly different from ddC ($p<0.05$). A similar pattern was seen for different pseudosugars with other base components, although the differences in fold-resistance were not significant. For example, DXG showed greater loss of activity than ddG (6.0-fold vs. 4.0-fold), as did d4T (3.8-fold) compared with ddT (2.2-fold). A conclusion cannot be made for DXT (data not shown) because this compound was poorly active in both P4/R5 cells and MT-2 cells.

The role of HIV-1_{65R} in resistance to d4T has not been well defined. In our study, HIV-1_{65R} did exhibit significantly reduced susceptibility to d4T in P4/R5 cells (3.8-fold;

$p<0.001$) and in MT-2 cells (2.1-fold; $p<0.05$). The recent report by Garcia-Lerma indicates that K65R can be selected by d4T *in vitro* (66). In addition, K65R has been reported to be selected in two clinical trials of d4T-containing regimens that excluded tenofovir. In the Gilead Study 903, 2 patients (0.7%) on d4T/3TC/Efavirenz failed with virus encoding K65R (58), and in a Danish study, 5 out of 8 patients who failed d4T/ddI/ABC therapy had virus with K65R (167). Collectively, these data support the view that K65R reduces susceptibility to d4T and that this reduction in susceptibility can be clinically relevant.

As noted above, (+)- or (-)-3TC and (+)- or (-)-FTC showed the greatest loss of activity against HIV-1_{65R}. In addition, greater than 10-fold loss of activity was also observed for most of the dioxolane compounds and the dioxathiolane, dOTC. This suggests that the K65R mutation in RT preferentially interferes with incorporation of analogs containing pseudosugars with 3' oxygen or sulfur components. The 3' oxygen or sulfur, which is larger and more negatively charged than the normal 3' carbon, may distort the positioning of the analog triphosphates to a greater extent than for other pseudosugars. Further, the 3' hydroxyl of the incoming dNTP makes a stabilizing intramolecular hydrogen bond with one oxygen atom of the β-phosphate (176). The lack of the 3' hydroxyl group and the negatively charged oxygen or sulfur at the 3' position is likely to further distort the configuration of the analog triphosphate. As a consequence, natural dNTPs are likely to be much more efficiently incorporated by HIV_{65R} RT than 3' oxygen or sulfur substituted analogs, leading to high level resistance to these analogs.

Our results show that base component has a moderate effect on NRTI activity against HIV-1_{65R}. Comparing compounds that were structurally identical except for base component shows that the rank order of greatest to least loss of activity is C>G>A>T. For example, the rank order of activity loss for the dideoxy compounds is ddC>ddG>ddI>ddT. However, only the

differences in activity of ddC vs. ddI or ddT are statistically significant (p=0.02). Although all the possible variations of base component for other pseudosugar structures are not active against HIV-1, a similar pattern is apparent for the active analogs tested. Specifically, the loss of activity was greater for D-DOC than D-DXG (p=0.02), FLG than FLT (p=0.04) and d4C than d4T (p=0.13). Biochemical studies have showed a similar trend with HIV_{65R} RT, showing greater discrimination against ddCTP compared to ddTTP (176, 186). The influence of base component on activity against HIV-1_{65R} is unexplained but may be mediated through differences in binding affinity or position of the analog in the substrate binding site.

Stereochemical differences (L- versus D-enantiomer) did not influence NRTI activity against HIV-1_{65R}. Six sets of compounds that differed only by stereochemistry were tested: (+)- and (-)-3TC, (+)- and (-)-FTC, L- and D-DOFC, L- and D-dOTC, L- and D-DOC, and L- and D-d4FC. The loss of activity was not significantly different for any enantiomeric pair (p>0.5). One hypothesis to explain this is that altering the enantiomer from D to L does not further affect the position of the 5'-triphosphates of the analog, which are linked to the pseudosugar by flexible single bonds.

AZT and AZA showed no loss of activity against HIV-1_{65R} whereas AZG and AZC showed a trend toward significant loss of activity (2.0 and 2.5-fold decrease, respectively). Thus, base component has a role in the activity of the 3'azido-containing compounds with C and G analogs showing greater loss of activity than A or T analogs as was observed with other pseudosugar structures. The relative preservation of activity of 3' azido analogs may be explained by interaction of the 3' azido with the β and γ phosphates of the analog triphosphate, restoring the correct configuration of the analog for incorporation. RT with K65R has reduced excision capability compared to wild type, allowing AZT to remain incorporated (133, 153).

The decreased excision capacity of K65R may help explain the preservation of activity of compounds with 3'-azido groups against HIV-1_{65R}. The use of AZT in combination with other NRTIs is likely to prevent the emergence of HIV-1_{65R} because this substitution does not confer a selective advantage for the virus in the presence of AZT. Recent clinical data support this hypothesis (46, 76, 209, 210).

In summary, the NRTI structures that retain activity against HIV-1_{65R} are those having a thymine or adenine base and a 3' azido or 3' fluorine component. NRTI with the 3' carbon replaced by a sulfur or oxygen have the least activity in either the D or L conformation. The data presented provide evidence that K65R is a multi-NRTI resistance mutation, and support the use of AZT in drug combinations to treat patients with HIV-1_{65R} and to prevent its emergence.

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4. CHAPTER TWO. BI-DIRECTIONAL PHENOTYPIC ANTAGONISM BETWEEN THE K65R MUTATION AND THYMIDINE ANALOG MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE

4.1. Preface

This study was also presented in part as an oral abstract at the XII International Drug Resistance Workshop, Cabo San Lucas, Mexico, June 2003. Part of this study was also presented as an oral abstract at the 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, February 2004 (Abstract 54: Parikh, U. M., D. L. Koontz, N. Sluis-Cremer, J. L. Hammond, L. Bacheler, R. F. Schinazi, and J. W. Mellors. K65R: A Multi-nucleoside Resistance Mutation of Increasing Prevalence Exhibits Bi-directional Phenotypic Antagonism with TAMs). The work presented in this chapter is in partial fulfillment of specific aim 1.

4.2. Abstract

The K65R mutation in HIV-1 RT is selected in vitro by many D-nucleoside analog RT inhibitors but had been paradoxically rare in patients treated with combination therapies containing AZT. In recent clinical trials, however, K65R emerged in 24-92% of patients experiencing virologic failure on antiretroviral regimens consisting of 3 nucleoside RT inhibitors not including AZT. The reason for this difference in the frequency of K65R is uncertain. To gain further insight, we examined trends in the prevalence of K65R and other NRTI mutations, examined the association of K65R with thymidine analog mutations (TAMs), and determined the viral susceptibility profile of K65R alone and in combination with TAMs. Of the >60,000 clinical samples submitted for resistance testing that contained one or more NRTI resistance mutations, the frequency of K65R increased from 0.4% in 1998 to 3.6% in 2003. In examining the frequency of other NRTI mutations that occurred with K65R, a strong negative relation was noted with M41L, D67N, L210W, and T215Y/F ($p < 0.0001$). This suggested that K65R is antagonistic to TAMs. The addition of K65R to two different HIV-1 clones encoding TAMs (M41L/L210W/T215Y or D67N/K70R/T215F/K219Q) reduced AZT resistance from >30-fold to <3-fold. In addition, TAMs altered the phenotypic effect of K65R, reducing resistance to ddC, ddI, d4T, abacavir, and tenofovir. Thus, K65R and TAMs exhibit bidirectional phenotypic antagonism. Combining AZT with NRTIs that select K65R is likely to prevent K65R from emerging.

4.3. Introduction

The lysine (K) to arginine (R) substitution at residue 65 (K65R) in human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) results from a single G to A transition (AAA to AGA). This mutation was first identified *in vitro* by serial passage of HIV-1 in peripheral blood mononuclear cell cultures containing the nucleoside analog RT inhibitor, 2',3'-dideoxycytidine (ddC) (219), and has subsequently been selected *in vitro* by many other nucleoside reverse transcriptase inhibitors (NRTIs) (17, 54, 66, 78, 79, 192, 202).

The lysine at codon 65 in RT interacts with the γ phosphate of the incoming dNTP, properly positioning it for incorporation into the nascent DNA chain (86). The arginine is believed to alter this interaction, favoring incorporation of dNTP over NRTI-triphosphate, resulting in resistance from increased selectivity (73, 176, 186). To clarify the structural determinants of NRTIs that influence resistance by K65R, we recently analyzed a diverse panel of NRTIs that varied in base component, pseudosugar structure and enantiomer. The only NRTIs that retain activity against HIV-1 with K65R are those having a both a thymine or adenine base and a 3'-azido component in the pseudosugar structure (151). Of the 8 NRTIs currently approved by the Food and Drug Administration (FDA) for use against HIV-1, only zidovudine (AZT) fits these criteria.

The frequency of K65R in NRTI-treated patients has depended greatly on the treatment regimen used. In trials of abacavir monotherapy (CNA2001 and CNA2002), K65R was selected in 13 of 127 (10%) patients. The frequency of K65R was lower (3 of 86 [3.5%]), in patients failing therapy with both abacavir and AZT. Of note, 2 of these 3 patients were on a regimen where AZT was added after 4 weeks of abacavir monotherapy. In addition, K65R was never

detected in patients treated with abacavir who had baseline virus with TAMs (CNA2007) (105). These observations suggested an interaction between TAMs and K65R.

Recently, K65R has been observed frequently in patients on failing treatment regimens that excluded AZT (83, 113, 137, 175, 200). For example, 4/7 (57%) treatment-naïve patients in one study and 2/6 (33%) in another study had early virologic failure with K65R after initiating dual nucleoside therapy with tenofovir and didanosine (113, 158) (Table 5). The frequency of K65R was even higher (44-92%) in studies of triple nucleosides in which AZT was excluded (56, 88, 97, 104, 167) (Table 5). The reason for these striking differences in the frequency of K65R has not been well defined.

Resistance to AZT results from combinations of mutations collectively referred to as "thymidine analog mutations" or TAMs, which occur most often in two patterns: M41L/L210W/T215Y and D67N/K70R/T215F/K219Q (120). These mutations improve the ATP- or pyrophosphate-catalyzed primer unblocking activity of RT, resulting in the removal of the chain-terminating NRTI and allowing continued DNA polymerization (6, 20, 69, 130). This mechanism is different than that for K65R and M184V, both which selectively decrease NRTI incorporation (64, 73, 176, 186). Although the individual mechanisms of resistance for K65R and TAMs have been elucidated, the effect of these mutations in combination is not known. Recent observations from clinical trials (Table 5) suggest that K65R and TAMs are counter-selected, i.e. the presence of one mutation (or mutation set) prevents the emergence of the other. We therefore aimed to explore potential antagonism between K65R and TAMs.

We examined changes in the prevalence of K65R and individual TAMs in a large clinical database, analyzed associations of K65R and individual TAMs from the same database, and determined the resistance profile of K65R in combination with TAMs against the 8 FDA-

approved NRTIs. Because all triple nucleoside failures with K65R also had M184V, we additionally analyzed resistance to NRTIs conferred by K65R and M184V in combination.

Table 5. Number and Percent of Failures with K65R in Clinical Trials of 2-4 NRTI Regimens That Included and Excluded Zidovudine (AZT)

Regimen[a]	Failures with K65R	Study/Reference
No AZT		
3TC + TDF + *EFV*	7/29 (24%)	Miller et al. 2003 (137)
DDI + TDF + *EFV*	2/6 (33%)	Podzamczer et al. 2005 (158)
3TC + ABC + TDF	4/9 (44%)	Khanlou et al. 2005 (97)
3TC + DDI + TDF	10/20 (50%)	Jemsek et al. 2004 (89)
DDI + TDF + (*EFV or NVP*)	4/7 (57%)	Leon et al. 2005 (113)
DDI + D4T + ABC[b]	5/8 (63%)	Roge et al. 2003 (167)
3TC + ABC + TDF[b]	23/36 (64%)	Gallant et al. 2003 (57)
3TC + ABC + TDF[b]	11/12 (92%)	Landman et al. 2004 (104)
With AZT		
TZV qd + TDF	1/8 (13%)	Elion et al. 2004 (47)
TZV bid	0/82 (0%)	Gulick et al. 2004 (77)

a Regimen components abbreviated as follows: NRTIs are lamivudine (3TC), abacavir (ABC), zidovudine (AZT), didanosine (DDI), stavudine (D4T) tenofovir (TDF) and trizivir (abacavir + lamivudine + zidovudine, TZV). Non-nucleoside reverse transcriptase inhibitors (NNRTIs; highlighted in *italics*) are efavirenz (EFV), and nevaripine (NVP). Regimen dosing abbreviated as follows: once daily (qd) and twice daily (bid)

b All failures with K65R in 3 NRTI regimens that excluded AZT also had the M184V mutation.

4.4. Materials and Methods

4.4.1. Clinical Samples and Database Queries

To obtain frequency data, the Virco database (VircoLab, Inc, Durham, NC) was searched for presence of NRTI mutations as defined by the International AIDS Society—United States of America (IAS-USA) published report (90). The data analyzed for this study are from a subset of 66,224 genotypes in the Virco database, consisting of samples and sequences submitted for resistance analysis as routine clinical testing, between 1998 and 2003. Samples originating from clinical trials of investigational or FDA-approved drug regimens were excluded from the analysis. Treatment histories were not available for the patients from whom the samples were obtained. Genotyping of virus from patient plasma was done by VircoLab, as previously described (81). A mutation was considered to be present if it was detected in the sequencing reaction either as a pure mutant or as a mixture with another variant at the same position.

To examine the associations of K65R with other NRTI mutations, the Virco genotype database was searched by first selecting the subset of genotypes that had one or more of the IAS-USA list of mutations associated with resistance to NRTIs (90), then dividing the subset into those with K65R and those without K65R. Lastly, the prevalence of each NRTI mutation was calculated for each subgroup. Chi-square analysis was performed using GraphPad software (GraphPad Software, Inc., San Diego, Calif.).

All genotype data for this study were obtained without patient identifiers.

4.4.2. Chemicals

(-)-β-2',3'-dideoxy-3'-thiacytidine [(-)-3TC], β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC], and β-D-3'-deoxy-2',3'-didehydrothymidine (d4T) were kindly provided by Raymond Schinazi, Ph.D., Emory University, GA. 9-[*(R)*-2-(phosphonomethoxy)propyl]adenine (PMPA, tenofovir) was provided by Gilead Sciences (Foster City, CA). 3'-azido-3'-deoxythymidine (AZT) was obtained from TriLink Biotechnologies (San Diego, CA) and Sigma Chemical Corporation (St. Louis, MO). 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) were also obtained from Sigma Chemical Corporation (St. Louis, MO). *(1S,cis)*-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt) (2:1) (abacavir) was obtained from GlaxoWellcome (Research Triangle Park, NC). The compounds were dissolved in dimethyl sulfoxide or sterile water as 10 mM or 30 mM stock solutions and stored at –20^{o}C. Compounds were diluted immediately before use to the desired concentration in Dulbecco's Modified Eagle Medium, Phenol Red Free (DMEM-PRF: Gibco-BRL, Grand Island, NY).

4.4.3. Generation of Mutant Recombinant HIV-1

Mutations in RT were introduced by site-directed mutagenesis using the Altered Sites II kit (Promega, Madison, WI) as described earlier (Section 3.4.3). Mutants encoding the following changes in RT were made: K65R, M184V, K65R/M184V, M41L/L210W/T215Y, M41L/L210W/T215Y + K65R, D67N/K70R/T215F/K219Q, and D67N/K70R/T215F/K219Q + K65R. Stock viruses were prepared using the xxHIV-1_{LAI} clones by electroporating (BIO-RAD Gene Pulser®, Hercules, CA) 5-10 μg of plasmid DNA into 1.3 x 10^7 MT-2 cells. At 7 days post-transfection, cell-free supernatant was harvested and stored at -80°C. The genotype of

stock viruses was confirmed by extracting RNA from virions (QIAamp kit, QIAGEN, Valencia, CA), treating the extract with DNAse I (Roche, Indianapolis, IN), amplifying the full length coding region (aa 1-550) of RT by RT-PCR, purifying the PCR product (Wizard PCR Purification System, Promega, Madison, WI), and sequencing the PCR product using the Big Dye terminator kit v.3.1 on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster city, CA).

4.4.4. Single Replication Cycle Drug Susceptibility Assay

Assays were done as previously described (Section 3.4.5). Briefly, 3-fold serial dilutions of an inhibitor were added in triplicate to P4/R5 cells (provided by Ned Landau, Ph.D., Salk Institute, LaJolla, CA), which is a HeLa cell line stably transfected with a Tat-activated β-galactosidase gene under the control of an HIV-LTR promoter. Cells were infected with the amount of site-directed mutant (generated using QuikChange, Invitrogen, Carlsbad, CA) that would yield a relative light unit (RLU) value of 100 in the no drug, virus-infected control wells. A cell lysis buffer and luminescent substrate (Gal-Screen, Tropix/Applied Biosystems, Foster City, CA) were added to each well 48-hours post-infection, and RLU were determined using a luminometer (ThermoLabSystems, Waltham, MA). Fold-resistance was determined by dividing the concentration of compound required to inhibit virus replication by 50% (IC_{50}) of wildtype HIV-1, by the IC_{50} of mutant HIV-1. IC_{50} values from at least three independent experiments were $\log_{10}$ transformed and compared using a two sample Student's t-test. P-values less than 0.05 were considered to be statistically significant.

4.5. Results

4.5.1. Change in Prevalence of K65R and TAMs

We analyzed a large clinical database to examine the changes over time in the frequency of NRTI resistance mutations. The subset of genotypes analyzed was from samples submitted to VircoLab for routine clinical testing between 1998 to the beginning of 2003 that were not part of a clinical trial. Of 62,222 such samples, the only mutation that showed an increase in frequency over time was K65R, increasing from 0.4% in 1998 to 3.6% by the beginning of 2003 (Figure 9, Table 6). By contrast, other NRTI mutations, including all TAMs (M41L, L210W, T215F/Y, K219E/N/Q) and M184I/V, decreased in frequency during the same time period. Of the TAMs, T215Y had the greatest decrease, a 19% change from 1998 to 2003, followed by M41L (17% decrease) and L210W (13% decrease) (Figure 9, Table 6). The prevalence of the 69-insert mutation, Q151M and V118I remained low and changed 1.1% or less during this time period (Table 6).

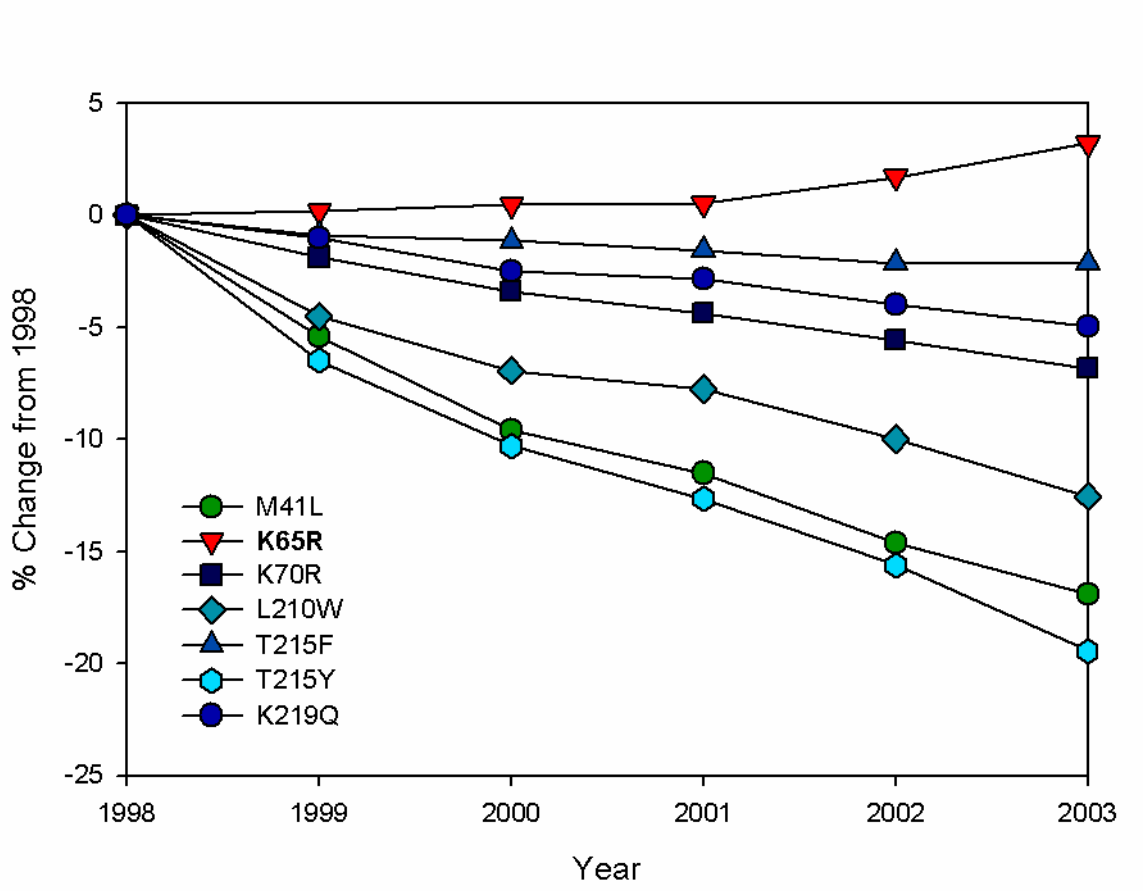


Figure 9. Changes in Prevalence of K65R and TAMs, 1998-2003

Table 6. Change in Prevalence of NRTI Mutations from Virco Database, 1998-2003

Mutation in HIV-1 RT[a]	YEAR 1998 (n=1721)	1999 (n=9789)	2000 (n=19,305)	2001 (n=17,298)	2002 (n = 12,519)	2003[b] (n = 1590)	TOTAL (n = 62,222)	Change[c]
Increased in Prevalence								
K65R	13 (0.4%)	77 (0.6%)	170 (0.9%)	162 (0.9%)	266 (2.1%)	60 (3.6%)	748 (1.2%)	**+3.0%**
Decreased in Prevalence								
T215Y	653 (37.9%)	3079 (31.5%)	5339 (27.7%)	4375 (25.3%)	2795 (22.3%)	294 (18.5%)	16,535 (27%)	**-19%**
M41L	667 (38.8%)	3265 (33.4%)	5630 (29.2%)	4713 (27.2%)	3020 (24.1%)	347 (21.8%)	17,642 (28%)	**-17%**
L210W	426 (24.8%)	1981 (20.2%)	3439 (17.8%)	2939 (17.0%)	1847 (14.8%)	194 (12.2%)	10,826 (17%)	**-13%**
K70R	362 (21.0%)	1876 (19.2%)	3405 (17.6%)	2883 (16.7%)	1937 (15.5%)	226 (14.2%)	10,689 (17%)	**-6.8%**
V118I	299 (17%)	1459 (15%)	2560 (13%)	2344 (14%)	1569 (13%)	172 (11%)	8403 (14%)	**-6.6%**
M184I/V	790 (46%)	4487 (46%)	8401 (44%)	7728 (45%)	5115 (41%)	629 (40%)	27150 (44%)	**-6.3%**
E44D	180 (10%)	805 (8%)	1316 (7%)	1146 (7%)	707 (6%)	70 (4%)	4224 (7%)	**-6.1%**
T69D/N	254 (15%)	1340 (14%)	2332 (12%)	1849 (11%)	1182 (9%)	144 (9%)	7100 (11%)	**-5.7%**
K219Q	22 (12.8%)	1158 (11.8%)	1993 (10.3%)	1726 (10.0%)	1105 (8.8%)	125 (7.9%)	6328 (10%)	**-5.0%**
K219E/N	161 (9%)	896 (9%)	1641 (9%)	1354 (8%)	918 (7%)	103 (6%)	5073 (8%)	**-2.9%**
T215F	159 (9.2%)	816 (8.3%)	1563 (8.1%)	1328 (7.7%)	889 (7.1%)	113 (7.1%)	4868 (7.8%)	**-2.1%**
Minimal Change (≤ 1.1%)								
Y115F	10 (0.6%)	54 (0.6%)	171 (0.9%)	207 (1.2%)	182 (1.5%)	27 (1.7%)	651 (1.0%)	**+1.1%**
T69-ins	13 (0.8%)	83 (0.8%)	149 (0.8%)	102 (0.6%)	84 (0.7%)	16 (1.0%)	447 (0.7%)	**+0.3%**
Q151M	39 (2.3%)	173 (1.8%)	318 (1.6%)	304 (1.8%)	186 (1.5%)	18 (1.1%)	1038 (1.7%)	**-1.1%**

a K65R highlighted in red, TAMs highlighted in blue, all other NRTI-associated mutations in black.

b Results are only from the beginning of 2003. Updated data, listed as follows, is only available for K65R: 2003, 417/11,692 (3.6%); January 2004, 26/737 (3.5%)

c Change calculated as (% Frequency of Mutation in 2003 - % Frequency of Mutation in 1998).

4.5.2. Negative Association of K65R and TAMs

Because of the divergent trends in the frequency of K65R and TAMs, we queried the Virco database to determine whether a negative association exists between K65R and TAMs. As a control, we also queried the prevalence of K65R with Q151M, in which a positive association has been reported (3), and M184V, which is also frequently seen with K65R (Table 5). Chi-square analysis was used to determine statistical significance.

Of the 65,535 isolates from 1998 to 2003 that had at least one NRTI-associated mutation, 32,356 isolates, or 49.4% of isolates had M41L (Table 7). Of the 689 isolates that had K65R, only 71, or 10.3% also had M41L. The frequency of having both K65R and M41L in the same isolate was significantly lower than expected ($p<0.001$), indicating that there is a negative association of these two mutations that is not simply due to chance. Significant negative associations were noted for other TAMs, including D67N, L210W, T215Y, and T215F. Only K70R was not negatively associated with K65R; the subset of isolates with K70R was similar in the group without K65R and the group with K65R (27.2% vs. 26.7%, $p = 0.081$).

By contrast, K65R was positively associated with Q151M, as reported previously (175, 204, 209, 210). Only 2.4% of samples without K65R had Q151M, compared to 55% of samples with K65R. Q151M causes resistance through selective decrease in NRTI incorporation (43).

K65R was negatively associated with M184V ($p < 0.001$). However, of the 689 total isolates with K65R, over half (360,689 or 52.2%) also had M184V (Table 7).

Table 7. Frequency of Association of K65R with Other NRTI Mutations

Mutation	Number of Isolates[a] (% of Total)[b]		Statistical Significance[c]	Association with K65R
	Without K65R (n = 65,535)	With K65R (n = 689)		
M41L	32,356 (49.4%)	71 (10.3%)	**p < 0.0001**	**negative**
D67N	24,286 (37.1%)	60 (8.7%)	**p < 0.0001**	**negative**
K70R	17,820 (27.2%)	184 (26.7%)	p = 0.081	no association
L210W	18,854 (28.8%)	28 (4.1%)	**p < 0.0001**	**negative**
T215Y	30,751 (46.9%)	51 (7.4%)	**p < 0.0001**	**negative**
T215F	8034 (12.3%)	5 (0.7%)	**p < 0.0001**	**negative**
K219Q or E	15,643 (23.9%)	128 (18.6%)	**p < 0.005**	**negative**
M184V or I	44,869 (68.5%)	360 (52.2%)	**p < 0.0001**	**negative**
Q151M	1568 (2.4%)	379 (55.0%)	**p < 0.0001**	**positive**

a Sample subset includes only those isolates with one or more NRTI-associated mutations identified during 1998 to 2003. "Wild-type" isolates, or isolates with no NRTI-associated mutations were excluded from this sample population.

b % of Total calculated by dividing number with mutation (column 2) by 689 (number with K65R), or number with mutation (column 3) by 65,535 (number without 65R).

c Chi-Square analysis performed to determine statistical significance between number of isolates with an NRTI mutation with and without K65R. A p-value less than 0.05 was considered to be statistically significant, and is indicated in **bold**.

4.5.3. Site-Directed Mutants

To gain a better understanding of the negative association of K65R and TAMs through analysis of viral phenotypes, we generated site-directed mutants with K65R alone (HIV-1_{65R}) and mutants with two different combinations of TAMs: M41L/L210W/T215Y (HIV-1_{TAM41}) and D67N/K70R/T215F/K219Q (HIV-1_{TAM67}). Additionally, we generated site-directed mutants with K65R in combination with both sets of TAMs: M41L/L210W/T215Y + K65R (HIV-$1_{TAM41/65R}$) and D67N/K70R/T215F/K219Q + K65R (HIV-$1_{TAM67/65R}$). Figure 10 illustrates the recombinant mutants used in this study.

4.5.4. K65R Antagonizes Resistance of TAMs to AZT

As previously reported in Chapter 2, HIV-1 with K65R alone did not have decreased susceptibility to AZT (1.1-fold). As expected (110), both pathways of TAMs conferred high level resistance to AZT, with a >50-fold change in IC_{50} for both HIV-1_{TAM41} and HIV-1_{TAM67}. The addition of K65R to TAMs significantly decreased this resistance to 2.1-fold for HIV-$1_{TAM41/65R}$ and to 1.5-fold for HIV-$1_{TAM67/65R}$ (Table 8). This reduction in resistance to AZT clearly demonstrates phenotypic antagonism of TAMs by K65R.

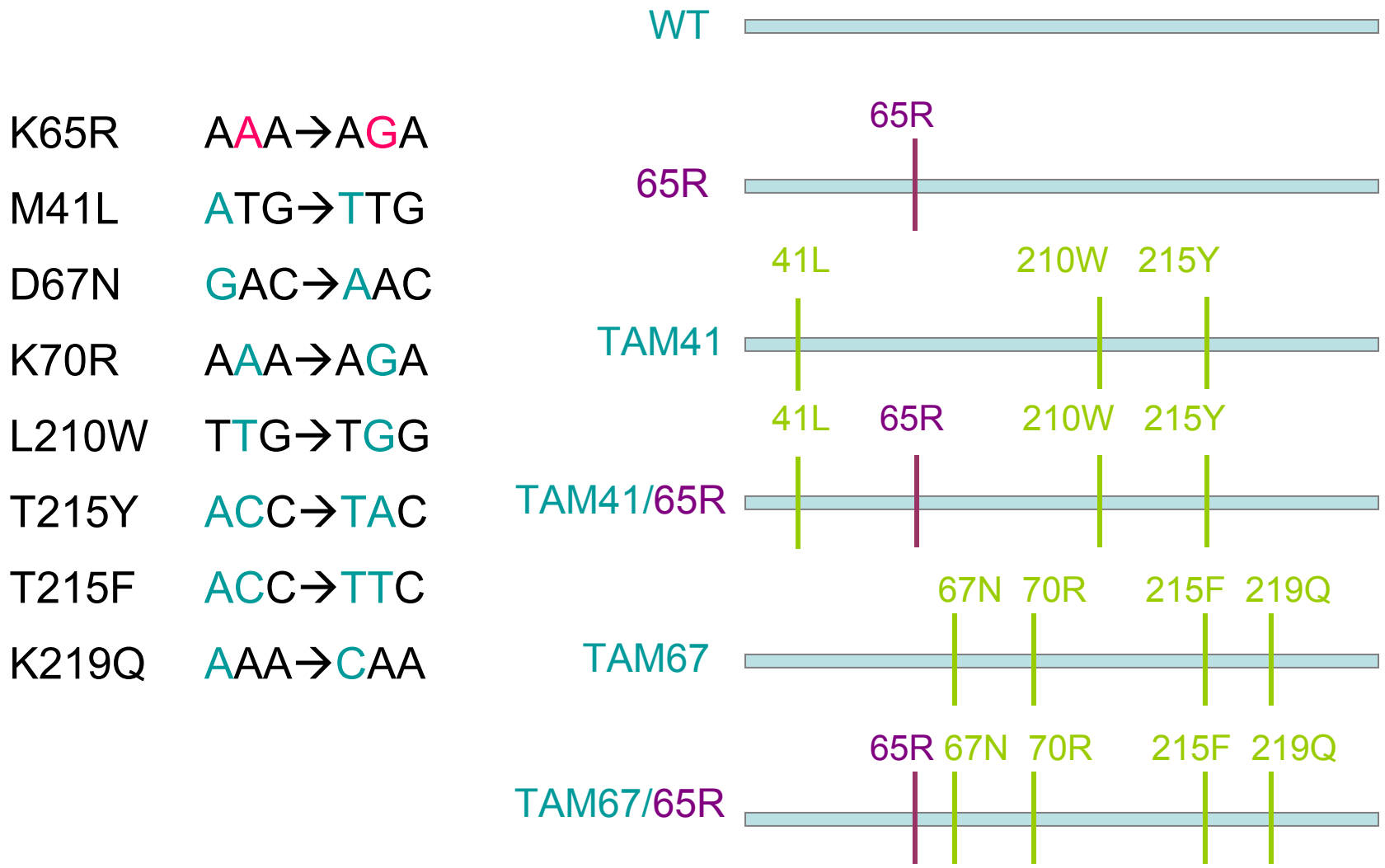


Figure 10. Site-Directed Recombinant Mutants of xxLAI
Mutations in HIV-1 RT were generated by site-directed mutagenesis, and cloned into pxxLAI for virus generation (See Materials and Methods).

Table 8. *In vitro* Activity of AZT against HIV-1 Encoding K65R and/or TAMs in RT

HIV-1_{LAI} Mutations in RT	Mean IC_{50} (μM) ± SD[a]	
	Zidovudine (AZT)	**Fold Change[b]**
wildtype	0.19 ± 0.12	-
K65R	0.22 ± 0.16	**1.1**
TAM41	12.0 ± 12.0	**62**
TAM41/65R	0.42 ± 0.28	**2.2[c]**
TAM67	10.4 ± 9.0	**54**
TAM67/65R	0.29 ± 0.17	**1.5[d]**

a Mean ± standard deviation from at least three independent experiments.

b Fold-change relative to wild-type.

c $P < 0.001$ for IC_{50} TAM41 compared to IC_{50} TAM41/65R.

d $P < 0.001$ for IC_{50} TAM67 compared to IC_{50} TAM67/65R.

4.5.5. TAMs Antagonize Resistance of K65R to ABC and TNF

To analyze the effect of TAMs on the phenotype of K65R, we evaluated the *in vitro* susceptibility of HIV-$1_{wildtype}$, HIV-1_{65R}, HIV-1_{TAM41}, HIV-1_{TAM67}, HIV-$1_{TAM41/65R}$ and HIV-$1_{TAM67/65R}$ to several FDA-approved NRTIs, including ddC, ddI, d4T, ABC, and TDF. HIV-1_{TAM41} had wildtype susceptibility to ddC, ddI, d4T, ABC and TDF (fold change 0.8 to 1.2, $p > 0.05$). HIV-1_{TAM67} exhibited significant low level decrease in susceptibility only to d4T and ABC (2.3 and 2.0-fold respectively). As expected (Table 1 from Chapter 1), HIV-1_{65R} had reduced susceptibility to all five NRTIs, ranging from 2.7-fold to 5.0-fold (Table 9).

The most striking antagonism of K65R by TAMs was observed for abacavir and tenofovir. The addition of the TAM67 combination significantly reduced resistance of HIV-1_{65R} to abacavir from 4.2-fold to 2.6-fold ($p<0.005$), whereas the addition of the TAM41 combination significantly reduced resistance of HIV-1_{65R} to abacavir from 4.2-fold to 2.4-fold ($p<0.005$). Surprisingly, only the TAM67 combination significantly diminished resistance of K65R to tenofovir (2.4-fold to 1.0-fold, $p<0.005$); the TAM41 combination did not (2.3-fold for HIV-$1_{TAM41/65R}$ compared to 2.4-fold for HIV-1_{65R} alone) (Table 9).

For the other three NRTIs tested, trends of antagonism of TAMs to K65R were observed. Resistance of HIV-1_{65R} to ddC was decreased both by the TAM41 combination (4.8-fold to 2.2-fold, $p = 0.06$) and the TAM67 combination (4.8-fold to 2.1-fold, $p = 0.05$). Resistance of K65R to ddI (2.7-fold) was reduced slightly more by the TAM67 combination (1.8-fold) than the TAM41 combination (2.1-fold) but these differences were not significant ($p > 0.05$). Similarly, resistance of HIV-1_{65R} to d4T (3.8-fold) was diminished by both sets of TAMs (HIV-$1_{TAM41/65R}$, 1.5-fold; HIV-$1_{TAM67/65R}$, 2.1-fold) but these differences were also not significant ($p > 0.05$) (Table 9).

Table 9. *In vitro* Activity of d4T, ddC, ddI, ABC and TDF against HIV-1 Encoding K65R and/or TAMs in RT

$HIV\text{-}1_{LAI}$ Mutations in RT	Mean IC_{50} ± St. Dev. (Fold-Change)[a]				
	stavudine (d4T)	zalcitabine (ddC)	didanosine (ddI)	abacavir (ABC)	tenofovir (TDF)
WT	5.68 ± 3.28	1.35 ± 0.70	2.29 ± 0.97	6.33 ± 1.68	4.38 ± 2.02
TAM41	6.74 ± 5.91 (1.2)	1.28 ± 0.70 (1.0)	1.91 ± 0.90 (0.8)	7.22 ± 4.96 (1.1)	3.98 ± 1.24 (0.9)
TAM67	13.2 ± 7.4 (2.3)[b]	1.19 ± 0.53 (0.9)	2.75 ± 1.60 (1.2)	12.8 ± 4.1 (2.0)[b]	3.55 ± 1.27 (0.8)
K65R	21.7 ± 23.8 (3.8)	6.79 ± 3.98 (5.0)	6.26 ± 2.92 (2.7)	26.3 ± 4.2 (4.2)	12.1 ± 4.3 (2.8)
TAM41/65R[c]	**8.44 ± 5.15 (1.5)**	**2.90 ± 1.21 (2.2)[d]**	4.78 ± 2.91 (2.1)	**16.3 ± 4.2 (2.6)[e]**	10.7 ± 6.9 (2.4)
TAM67/65R[c]	**11.8 ± 4.8 (2.1)**	**2.81 ± 1.15 (2.1)[d]**	**4.23 ± 2.45 (1.8)**	**15.1 ± 6.3 (2.4)[e]**	**4.52 ± 0.77 (1.0)[e]**

a IC_{50} values determined by measuring inhibition of luminescence in P4/R5 cells. Mean ± standard deviation from at least 3 and up to 78 independent experiments. Fold-change relative to wild-type is shown in parentheses.
b IC_{50} is significantly different from wild-type, $p < 0.01$.
c Antagonism or trend in antagonism indicated in bold.
d $P = 0.06$ for $HIV\text{-}1_{TAM41/65R}$ compared to $HIV\text{-}1_{65R}$. $P = 0.05$ for $HIV\text{-}1_{TAM67/65R}$ compared to $HIV\text{-}1_{65R}$.
e IC_{50} is significantly different from $HIV\text{-}1_{65R}$, $p < 0.005$.

4.5.6. TAMs Do Not Antagonize Resistance of K65R to 3TC or FTC

We also tested 3TC and FTC to determine whether TAMs antagonized resistance of K65R to these compounds. HIV-1_{65R} was highly resistant to both 3TC (58-fold) and FTC (21-fold). The addition of TAM41 to K65R did not alter susceptibility to 3TC (from 58-fold to 65-fold, p = 0.90) but did decreased susceptibility to FTC (from 21-fold to 57-fold, p = 0.04).

HIV-1 with TAM67 alone demonstrated significant resistance (p < 0.001) to both 3TC (4.0-fold) and FTC (4.5-fold). The addition of TAM67 to K65R did not increased resistance of K65R to 3TC (from 58-fold to 79-fold, p = 0.58) or to FTC (from 21-fold to 44-fold, p = 0.16) (Table 10).

Table 10. *In vitro* Activity of 3TC and FTC against HIV-1 Encoding K65R and/or TAMs in RT

HIV-1_{LAI} Mutations in RT	Mean IC_{50} (µM) ± SD[a]			
	Lamivudine (3TC)	Fold-Change	Emtricitabine (FTC)	Fold-Change
WT	0.70 ± 0.44		0.19 ± 0.08	
TAM41	1.08 ± 0.64	1.4	0.36 ± 0.27	1.9
TAM67	2.81 ± 1.10	4.0[b]	0.88 ± 0.54	4.5[b]
K65R	40.7 ± 35.0	58[b]	4.15 ± 1.51	21[b]
TAM41/65R	45.2 ± 42.6	65[b]	11.0 ± 9.4	57[b,c]
TAM67/65R	55.0 ± 41.5	79[b]	8.5 ± 7.4	44[b]

a IC_{50} values determined by measuring inhibition of luminescence in P4/R5 cells. Mean ± standard deviation reported is from at least 3 independent experiments.
b IC_{50} is significantly different from wild-type, p < 0.0001.
c IC_{50} is significantly different from K65R alone, p = 0.04.

4.5.7. K65R Enhances Resistance of M184V to ABC, ddI and TDF

Finally, to assess whether the observed antagonism is a phenomenon specific to K65R and TAMs, recombinant mutants of HIV-1 with both M184V and K65R (HIV-$1_{65R/184V}$) were generated to examine the combined effect of mutations with similar resistance mechanisms (i.e. discrimination). We hypothesized that due to the association of K65R and M184V seen in patients (Table 5) and because 52.2% of samples with K65R also had M184V (Table 7), resistance to NRTIs would have a synergistic or enhanced effect from both mutations. Indeed, this was the case. K65R significantly increased resistance of HIV-1_{184V} to zalcitabine (from 1.8-fold to 4.9-fold), didanosine (from 1.5-fold to 3.4-fold) and abacavir (from 2.7-fold to 11-fold) ($P < 0.001$). Of note, HIV-1_{65R} reversed hypersusceptibility of HIV-1_{184V} to tenofovir (0.5-fold), restoring HIV-$1_{65R/184V}$ to wild-type susceptibility (1.1-fold) ($P < 0.001$).

There was no discernable effect for the double mutant K65R/M184V for several NRTIs tested, including 3TC, FTC, AZT and d4T. Resistance to 3TC and FTC was maximal at the highest drug concentration tested (90 μM) for HIV-1 with M184V alone or with K65R. Lastly, HIV-1_{184V} and HIV-$1_{65R/184V}$ both had wildtype susceptibility to AZT and to d4T.

Table 11. *In vitro* Activity of FDA-Approved NRTIs against HIV-1 Encoding M184V Alone or in Combination with K65R in RT

	Mean IC_{50} (μM) ± SD[a] (Fold-Change)[b]		
NRTI	**M184V**	**K65R/M184V**	**Significance[a]**
Zidovudine (AZT)	0.18 ± 0.16 (0.9)	0.12 ± 0.03 (0.6)	P = 0.65
Stavudine (d4T)	5.61 ± 0.85 (1.0)	7.39 ± 1.39 (1.3)	P = 0.08
Zalcitabine (ddC)	2.42 ± 0.45 (1.8)	6.62 ± 0.61 (4.9)	**P < 0.001**
Didanosine (ddI)	3.54 ± 0.64 (1.5)	7.86 ± 1.33 (3.4)	**P < 0.001**
Abacavir (ABC)	17.4 ± 3.7 (2.7)	69.6 ± 22.1 (11)	**P < 0.001**
Lamivudine (3TC)	>90 (>120)	>90 (>120)	Cannot determine
Emtricitabine (FTC)	>90 (>470)	>90 (>470)	Cannot determine
Tenofovir (TNF)	2.02 ± 0.12 (0.5)	4.77 ± 1.28 (1.1)	**P < 0.001**

a IC_{50} values determined by measuring inhibition of luminescence in P4/R5 cells. Mean ± standard deviation reported is from at least 3 independent experiments.

b Fold-Change relative to wild-type shown in parentheses. Wild-type IC_{50} values are as shown in Table 8 (AZT), Table 9 (ABC, ddI and TDF), and Table 10 (3TC).

c Significance determined between Fold-R of K65R/M184V compared to Fold-R of M184V alone. P-values less than 0.05 (indicated in **bold**) are considered to be statistically significantly.

4.6. Discussion

This is the first study to demonstrate bi-directional phenotypic antagonism between K65R and TAMs. Two lines of evidence support the existence and relevance of this antagonism: (1) clinical and epidemiological observations of increased K65R prevalence and negative association with TAMs; and (2) a systematic analysis of mutants containing K65R with two different clinically relevant combinations of TAMs against all eight FDA-approved NRTIs.

From our clinical studies conducted using a large database of over 60,000 samples, we observed that the prevalence of K65R increased while the prevalence of all individual TAMs decreased in the same time period. We had first noticed this increase in 2003 (152) and our updated findings confirm that the trend has continued. Since then, other studies have also observed positive trends in the frequency of K65R (21, 91). For instance, Valer and colleagues found that the prevalence of K65R increased from 0.6% in 1999 to 11.5% in 2004 in their database of 1846 HIV-infected patients in Spain (200). In a retrospective analysis, Winston and colleagues noticed a significant increase in K65R, from 1.7% in 2000 to 4% in 2002, from 997 regimen-failing patients from Chelsea and Westminster Hospital, U.K. (210). The reason for this increase is not known. It is possible that the use of tenofovir without AZT as first line therapy may have contributed to the increase in K65R, as well as the decrease in TAMs. In our study, however, therapy information for the patients was not available to confirm this hypothesis.

Using a large sample size (66,224 isolates) we statistically confirmed that K65R is negatively associated with specific TAMs, including M41L, D67N, L210W, and T215F/Y. TAMs function to facilitate the excision reaction by increasing RT's interaction with ATP through mutations at 215 and 219 (29). K65R may alter RT's interaction with ATP to decrease

excision. Thus, RT may not be able to structurally accommodate both changes at K65R and TAMs without compromising the improved excision conferred by TAMs.

In the database, we found that K70R was not among the TAMs negatively correlated with K65R. Crystal structure predicts that like K65R, K70R may play a role in interaction with the γ-phosphate of the incoming dNTP (86). In addition, K70R in the absence of other AZT-resistance conferring mutations may have minor discrimination activity against AZT and ddI (181). Thus, in the absence of other TAMs, K70R alone may not have an antagonistic effect on K65R.

We aimed to explain these two clinical observations (divergent prevalence trends of K65R and TAMs, and negative association of K65R with TAMs) by analyzing the phenotypic effect of both mutations (K65R and TAMs) on susceptibility to the 8 FDA-approved NRTIs. We found that K65R significantly diminishes AZT resistance (from >50-fold to <2.5-fold) in the context of two clinically relevant combinations of TAMs (M41L/L210W/T215Y and D67N/K70R/T215F/K219Q) (Table 8). Reversal of resistance to AZT by K65R was first reported by Bazmi and colleagues in a different, clinically less common TAM background (17). Our study extends the finding that K65R antagonizes TAMs effect on AZT. M184V, which also has an antagonistic effect on TAMs, has been shown to diminish the primer unblocking activity of TAMs (19, 70). Biochemical studies have shown that K65R exerts its effect on TAMs in a similar manner (133, 153).

TAMs significantly diminished resistance to abacavir and tenofovir conferred by K65R. A trend of antagonism by TAMs to K65R is also noted with didanosine, stavudine and zalcitabine. TAMs may diminish K65R's function of discriminating NRTIs by partially restoring catalytic rate of NRTI-TP incorporation. This may be because K65R discriminates by preventing proper positioning of the γ phosphate of the incoming dNTP or NRTI-triphosphate

(NRTI-TP). Having additional mutations in the fingers region may restore essential molecular interactions needed for incorporation. Biochemical studies exploring this antagonism will be discussed in the next chapter.

Finally, susceptibility of virus with K65R and M184V was determined to assess whether the antagonism was specific to K65R and TAMs. We hypothesized that the K65R and M184V mutations, which increase RT discrimination against incorporation of NRTIs, would result in greater resistance together than either alone. This proved to be the case. The K65R/M184V double mutant had significantly increased resistance to zalcitabine, didanosine and abacavir compared to M184V alone. Additionally, M184V is known to have hypersusceptibility to tenofovir (212) and K65R reversed this hypersusceptibility. Pre-steady state enzymatic analysis may explain this synergy between K65R and M184V. Deval and colleagues showed that discrimination by K65R RT is due to decreased catalytic rate of incorporation of NRTI-TP compared to WT RT with little effect on binding efficiency. Conversely, discrimination by M184V RT is due to decreased binding affinity of NRTI-TP compared to WT RT with little effect on catalytic rate of incorporation. The double mutant K65R/M184V however, has both decreased binding affinity and decreased catalytic rate of incorporation compared to wild-type RT (44). This may lead to greater discrimination of most NRTI-TP by the double mutant. Similar biochemical analyses are needed to explain antagonism between K65R and TAM and are in progress (see Chapter 3).

Although viruses with K65R in combination with M184V have diminished replicative capacity and replicative fitness, there is enough clinical advantage for the double mutant to be selected in patients (44, 205). Indeed, over half of all isolates from the Virco database with K65R also have M184V and preliminary work from our lab indicates that K65R and M184V do

occur on the same genome in patients (15). K65R and M184V may emerge in failure of triple NRTI regimens that lack AZT because they increase NRTI resistance.

To conclude, both clinical and virological evidence strongly suggest that K65R and TAMs are counter-selected in patients due to antagonistic mechanisms of resistance. K65R negates resistance to AZT caused by TAMs, while TAMs negate resistance to tenofovir and abacavir caused by K65R. Thus, there is no benefit for the virus to evolve both pathways of mutations. Our findings may explain why K65R is increasingly selected in patients failing regimens that do not include AZT, while K65R is rarely found in patients failing regimens that do include AZT.

Acknowledgements. We would like to thank Lee Bacheler from VircoLabs for providing data for the clinical analyses.

5. CHAPTER THREE. BIOCHEMICAL MECHANISMS OF ANTAGONISM BETWEEN K65R AND THYMIDINE ANALOG MUTATIONS IN HIV-1 REVERSE TRANSCRIPTASE

5.1. Preface

This study was presented in part as an oral abstract at the 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, February 2004 (Abstract 54: Parikh, U. M., D. L. Koontz, N. Sluis-Cremer, J. L. Hammond, L. Bacheler, R. F. Schinazi, and J. W. Mellors. K65R: A Multi-nucleoside Resistance Mutation of Increasing Prevalence Exhibits Bi-directional Phenotypic Antagonism with TAM) and in part as an oral abstract at the XIV International Drug Resistance Workshop, Quebec City, Canada, June 2005 (abstract published in Parikh, U. M., N. Sluis-Cremer and J. Mellors. 2005. Kinetic Mechanism by Which Thymidine Analog Mutations Antagonize K65R in HIV-1 Reverse Transcriptase. Antivir Ther. 10: S95). The work presented in this chapter is in partial fulfillment of dissertation aim 2.

5.2. Abstract

The K65R mutation in HIV-1 reverse transcriptase (RT) decreases *in vitro* susceptibility to all nucleoside reverse transcriptase inhibitors (NRTIs) except 3'-azidothymidine (AZT) by selectively decreasing analog incorporation (discrimination mechanism). Different combinations of thymidine analog mutations (TAMs) in HIV-1 RT at residues 41, 67, 70, 210, 215 and 219 cause resistance to AZT through ATP-catalyzed primer unblocking of the terminated primer (excision mechanism). Previously, we demonstrated bi-directional phenotypic antagonism between K65R and TAMs in HIV-1 susceptibility assays: recombinant virus clones with both K65R and TAMs show markedly decreased resistance to AZT compared to clones with TAMs alone; and decreased resistance to tenofovir and abacavir compared to clones with K65R alone. In this study, we sought to elucidate the biochemical mechanisms involved in this antagonism. We used purified, recombinant RT with K65R alone or in combination with TAMs (M41L/L210W/215Y or D67N/K70R/T215F/K219Q) to measure ATP-catalyzed removal of AZT-MP from AZT-MP-terminated primers; and to determine pre-steady state kinetic parameters for the single nucleotide incorporation of TTP, AZT-TP, dATP and tenofovir-diphosphate (TFV-DP). K65R reversed the ATP-catalyzed primer unblocking activity of TAMs. K65R increased selectivity against AZT-TP by 2.4-fold, but neither set of TAMs affected this discrimination. D67N/K70R/T215F/K219Q partially restored (4-fold) the rate of TFV-DP incorporation (k_{pol}) by K65R RT (16-fold lower than wildtype). M41L/L210W/T215F antagonized K65R to a lesser degree than TAM67 by decreasing selectivity for dATP versus TNF-DP. These biochemical data further support the model that K65R and TAMs have mutually antagonistic resistance mechanisms that can be exploited to optimize NRTI therapy.

5.3. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) are key components of current antiretroviral therapy. The 8 NRTIs approved by the Food and Drug Administration (FDA) for use in the treatment of HIV-1 infection are zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), abacavir (ABC), tenofovir (TFV) and emtricitabine (FTC) (16). AZT was the first of these to be used therapeutically (215). When administered alone, AZT rapidly selects for drug-resistant HIV-1 (109). AZT resistance is caused by different combinations of mutations in reverse transcriptase (RT), collectively referred to as thymidine analog mutations (TAMs) that most often occur in two different patterns: M41L/L210W/T215Y (TAM41) and D67N/K70R/T215F/K219Q (TAM67) (120). Early studies showed that these mutations did not decrease incorporation of AZT-5'-triphosphate (AZT-TP) sufficiently to explain the high level of AZT resistance (>100-fold) observed in cell-based phenotypic assays (94, 96, 109). More recent studies have shown that primer unblocking is the major mechanism of AZT resistance for TAMs, with ATP or pyrophosphate being the phosphate donor for the phosphorolytic removal of the AZT-MP from terminated primers (6, 7, 131, 133, 146).

The K65R mutation has been found to reduce HIV-1 susceptibility to all FDA-approved NRTIs except AZT. The frequency of K65R in plasma samples from HIV-infected patients is increasing, whereas TAMs are decreasing (Chapter 2) (151, 153). Several studies have examined the mechanism whereby K65R in confers resistance to NRTIs. The lysine (K) at residue 65 of RT is located in the flexible β3-β4 loop in the fingers subdomain of HIV-1 RT and interacts with the γ-phosphate of the incoming dNTP substrate, properly aligning it for incorporation into the elongating DNA chain (86). The longer side chain of arginine (R)

compared to lysine (K) is believed to alter the positioning of the incoming dNTP or dideoxynucleoside-5'-triphosphate (ddNTP), favoring incorporation of the dNTP over the ddNTP.

In prior studies of HIV-1 isolates, we showed that K65R and TAMs exhibit bi-directional phenotypic antagonism. Site-directed mutants of HIV-1 with both K65R and TAMs (TAM41 or TAM67) were 10-20-fold less resistant to AZT compared to those with TAMs alone, and showed decreased resistance to d4T, ddC, ABC (both TAM41 and TAM67), and tenofovir and ddI (TAM67 only) compared to K65R alone (Chapter 2) (153). To elucidate the biochemical basis for this, we conducted steady-state and pre-steady state analysis of HIV-1 RT with K65R alone and in combination with the TAM41 and TAM67 mutational patterns.

5.4. Materials and Methods

5.4.1. Reagents

dNTPs and 2', 3'-dideoxyguanosine 5'-triphosphate (ddGTP) were obtained from Amersham Biosciences (Piscataway, NJ). 3'-azido-2', 3'-dideoxythymidine triphosphate (AZT-TP) was purchased from Sierra Bioresearch (Tucson, AZ). Tenofovir diphosphate (TFV-DP) was kindly provided by Michael Miller (Gilead, Foster City, CA). Adenosine triphosphate (ATP) was purchased from Roche Diagnostics (Indianapolis, IN).

5.4.2. DNA Substrates

The sequences of DNA template and primer combinations used are summarized in Table 12. The 45mer and 57mer DNA templates were synthesized by Integrated DNA Technologies (Coralville, PA). The 214mer DNA template was generated by PCR-amplifying a 214-base region from the beginning of HIV-1_{LAI} to 15 bases past the primer binding site, using the forward primer 5'-GGTCTCTCTGGTTAGACCAG-3' and the 5'-phosphorylated reverse primer 5'-CCCTTTCGCTTTCAAGTCCC-3' (IDT, Coralville, PA). PCR product was purified (Promega Wizard PCR Purification kit, Madison, WI) and incubated with 0.1 U/µl λ-exonuclease (Invitrogen, Carlsbad, CA) at 37°C for 30 min to generate single-stranded DNA. Resulting oligonucleotide was purified by phenol chloroform extraction and ethanol precipitation. All primers (DNA19AZT, DNA18, DNA20A, and DNA19T) were synthesized by Integrated DNA Technologies (Coralville, PA).

Primers were 5'end-labeled using T4 polynucleotide kinase (Fisher Scientific, Pittsburgh, PA) and γ^{32}-ATP (Amersham Biosciences, Piscataway, NJ) and purified using a NAP-5 column (Amersham Biosciences, Piscataway, NJ). Purified, labeled primer was annealed to a DNA template (95°C for 7 min, slow cooling to room temperature) at a 1:2 molar ratio of primer:template in the combinations described in Table 12. To generate AZT-chain terminated primers, the primer/template (P/T) (DNA19T/45mer, Table 12) was first incubated at 37°C with polynucleotide kinase buffer (Fisher Scientific, Pittsburgh, PA), AZT-TP and wild-type RT. Labeled, AZT-terminated primer-template was then speed-vacuumed to dryness, and gel-purified using a 7 M urea-16% polyacrylamide sequencing gel (BioRad, Hercules, CA). Primer was eluted from gel piece using a Maxim-Gilbert Diffusion Buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS), NAP-5 column purified, and re-annealed to template as described above.

Table 12. DNA Oligonucleotides

Primer/Template	Sequence (DNA)
pr19AZT/45mer[a]	5′-TAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACA-3′ 3′-ZCACCGCGGGCTTGTCCCTG-5′-[^{32}P]
pr18/214mer[b]	5′-GGTCTCTCTGGTTAGACCAGATTTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCT CAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCT CAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTGAAAGCGAAAGGG-3′ 3′-ACCGCGGGCTTGTCCCTG-5′-[^{32}P]
pr20A/57mer[c]	5′-CTCAGACCCTTTTAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACA-3′ 3′-GAGATCGTCACCGCGGGCT-5′-[^{32}P]
pr19T/45mer[d]	5′-TAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACA-3′ 3′-CACCGCGGGCTTGTCCCTG-5′-[^{32}P]

a DNA template corresponds to HIV-1_{LAI} sequence from base 155 to base 200. Primer corresponds to region near primer binding site. Primer is AZT-terminated (represented by "Z").

b DNA template corresponds to first 214 bases of HIV-1_{LAI}. Primer corresponds to primer binding site.

c DNA template corresponds to HIV-1_{LAI} sequence from base 143 to base 200. Primer corresponds to region near the primer binding site.

d Template and primer similar to (a) except primer is not AZT-terminated.

5.4.3. Reverse Transcriptase

Mutants containing K65R and TAMs (combinations of M41L/L210W/T215Y hereafter referred to as TAM41, or D67N/K70R/T215F/K219Q, hereafter referred to as TAM67) were generated in the expression vector p6HRT (provided by Stuart LeGrice, PhD, National Cancer Institute, Frederick, MD). p6HRT contains the entire RT and protease sequence, which results in post-transcriptional processing to heterodimeric RT. p6HRT was co-transformed with pDM1.I (pDM1.I overproduces the lac repressor to allow high levels of protein expression) (28, 112) into JM109 cells, then cultured in Power Prime broth (Athena Enzyme Systems, Baltimore, MD) at 37°C until OD_{600} reached approximately 0.5. After inducing the culture with isopropyl-beta-D-thiogalactopyranoside (IPTG) overnight at 30°C, bacteria were pelleted (11,000 rpm for 10 min, 4°C), re-suspended in lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.8; 1 mM β-mercaptoethanol), then lysed using a French Press at 16,000 psi (Thermo Electron Corporation, West Palm Beach, FL). Lysed cells were centrifuged again (11,000 rpm for 10 min, 4°C) to remove cell debris. Protein from supernatant was purified using a BD Talon column (BD Biosciences, Franklin Lake, NJ) and eluted from beads using elution buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 6.0, 0.6 M NaCl, 200 mM imidazole, and 1 mM β-mercaptoethanol). Protein was dialyzed in dialysis buffer (50 mM Tris/HCl, pH 8.2, 25 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM tris(2-carboxyethyl)phosphine hydrochloride [TCEP]) using a 50K Millipore filtration tube (Millipore, Billerica, MA). The protein concentration was determined spectrophotometrically at OD_{280nm} using an extinction co-efficient (ε280) of 260,450 $M^{-1}cm^{-1}$. SDS-PAGE confirmed equivalent amounts of p51 and p66 subunits (Figure 11A).

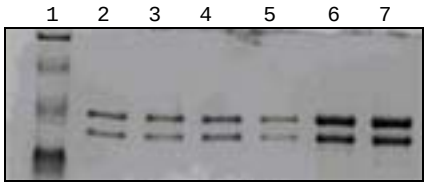
Pre-steady state burst experiments were used to determine active site concentrations for all enzymes. Specifically, 130 nM RT and 260 nM [^{32}P]-labeled DNA:DNA P/T (pr19T/45mer,

Table 12) in buffer (50 mM Tris pH 7.5, 50 mM KCl, 1 mM EDTA pH 8.0) were rapidly combined with 25 μM TTP in buffer (50 mM Tris pH 7.5, 50 mM KCl, 21 mM $MgCl_2$) in a rapid quench machine (KinTek Corporation, Clarence, PA). The reaction was quenched with 0.35 M EDTA after timepoints ranging from 50 ms to 5 s. Sample loading buffer (0.05% bromphenol blue, 0.05% xylene cyanole, deionized formamide) was added to quenched reactions. Samples were denatured by heating at 95°C for 7 min, and run on a denaturing polyacrylamide sequencing gel (19:1 acrylamide:bisacrylamide, urea, TBE). The gel was exposed to an FX Imaging Screen (Bio-Rad Laboratories, Inc, Hercules, CA) and results were read on a Bio-Rad GS525 Molecular Imager. Personal FX software (Quantity One, version 4.3.0) was used to quantify the bands. The ratio of product formation over time was analyzed by non-linear burst regression using Sigma Plot software (Jandel Scientific) with the equation as follows [1]:

$$[20\text{ nt product}] = A[1-\exp(-k_{obs}t)] + (k_{ss}t) \qquad [1]$$

where A represents the burst amplitude, k_{obs} is the burst rate constant for single turnover incorporation of TTP, k_{ss} is the steady-state rate constant for TTP incorporation, and t is time. The % active enzyme was calculated by multiplying maximum burst amplitude by concentration of enzyme used in the assay; with corrections for dilution factors (Figure 11B).

A



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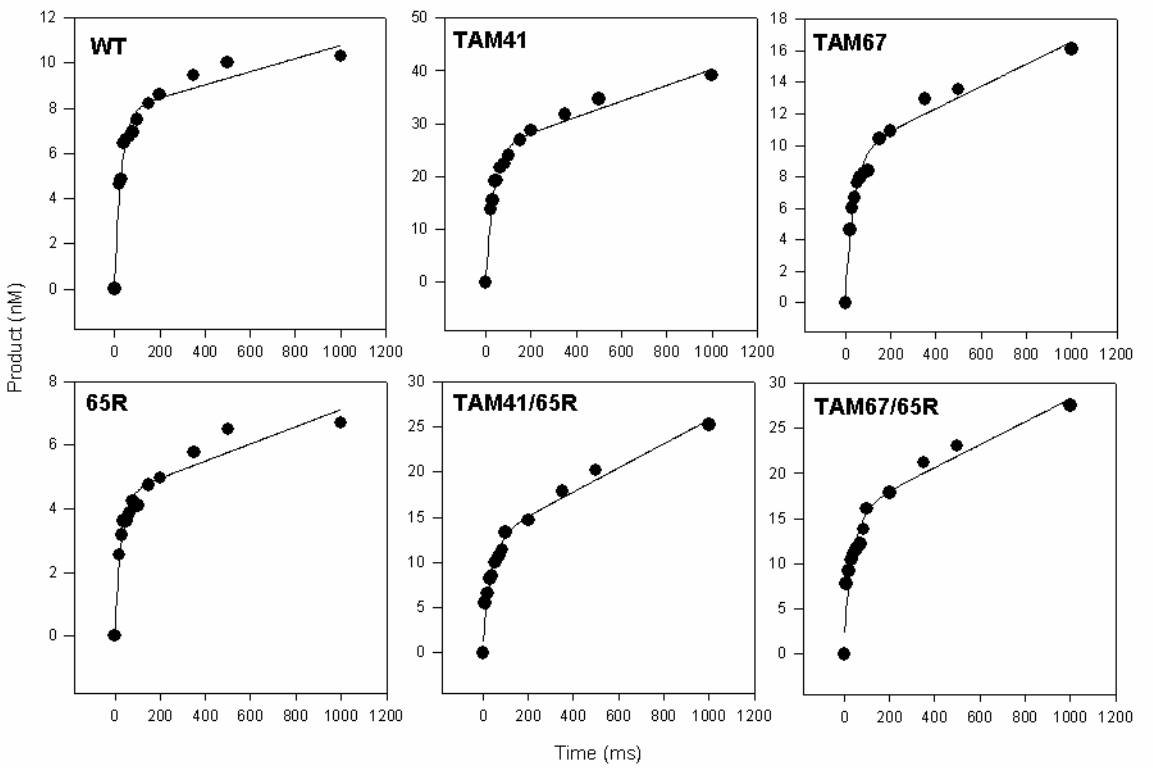


Figure 11. SDS-PAGE and Active Site Determination of Purified Recombinant RT
(A) SDS-PAGE of newly generated proteins confirms approximately equivalent quantities of each subunit of RT. Lane 1 is the size marker, lanes 2-6 are RT as follows: wild-type, K65R, TAM41, TAM67, TAM41/65R and TAM67/65R. (B) Pre-steady state burst and single turnover experiments were performed to determine % active sites of newly generated RT. Reaction was done by mixing 130 nM P/T pre-incubated with 37-70 nM RT, and 100 mM TTP under pre-steady state conditions (See Materials and Methods). % Active Site determined from amplitude of curve is as follows: WT, 73.5%; 65R, 40.8%; TAM41, 49.7%; TAM67, 30.6%; TAM41/65R, 32.6% and TAM67/65R, 41.5%.

5.4.4. Single Nucleotide Excision and Rescue Assay

20 nM AZT-terminated DNA:DNA P/T (pr19AZT/45mer, Table 12) was pre-incubated with 100 nM active RT in buffer (50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$). 3 mM ATP, 20 μM TTP and 20 μM ddGTP were pre-incubated with 0.01 U/ml inorganic pyrophosphatase (Sigma-Aldridge Corporation, St. Louis, MO), then added to P/T/RT mix to begin the reaction. Aliquots were collected at time points ranging from 30 s to 2 hours, and the reaction was quenched in sample loading dye containing EDTA (98% deionized formamide, 10 mM EDTA and 1 mg/ml each of bromphenol blue and xylene cyanole). Samples were resolved using 7 M urea-16% acrylamide denaturing gel electrophoresis as described above.

Formation of a product one nucleotide longer than the primer (corresponding to "excision" or removal of AZT-MP catalyzed by ATP, and "rescue" or addition of TTP and ddGTP onto the primer) was analyzed using phosphoimaging. The apparent pseudo-first order rate for rescue was determined through SigmaPlot using the following equation:

$$[\text{20 nt product}] = A[1-\exp(-k_{ATP}t)] \qquad [2]$$

where A represents the amplitude for product formation, k_{ATP} is the apparent rate of rescue when 3 mM ATP is used to catalyze the excision reaction, and t is time. The k_{ATP} was determined for wild-type RT (RT^{WT}), RT with K65R (RT^{65R}), RT with TAMs (RT^{TAM41} and RT^{TAM67}), and RT with both TAMs and K65R ($RT^{TAM41/65R}$ and $RT^{TAM67/65R}$).

5.4.5. Competitive Assay for Substrate Incorporation

An assay under steady-state conditions was performed to determine whether RT_{65R} preferentially incorporates TTP over AZT-TP when both molecules are available as substrates for DNA chain elongation. 20 nM heteropolymeric DNA:DNA P/T (pr19T/45mer, Table 12) was pre-incubated with 100 nM active RT_{WT} or RT_{65R}. P/T/RT was combined with 5 μM ddGTP, 5 μM TTP, and varying concentrations (ranging from 0.5 μM to 50 μM) AZT-TP in buffer (10 mM Tris pH 8.0, 10 mM KCl, 2 mM $MgCl_2$). Incorporation of AZT-TP resulted in a 20 nt product due to chain termination after only one polymerization step. Incorporation of TTP resulted in a 21 nt product due to subsequent incorporation of ddGTP and chain termination after two polymerization steps. Control experiments (Figure 13) confirmed that incubation of P/T/RT with substrate for 5 min at 37°C was sufficient to ensure that the reaction occurred to completion. The reaction was quenched with sample loading dye containing EDTA, and resolved on a denaturing polyacrylamide sequencing gel as described above. The ratio of amount of 20 nt product formation was compared with amount of 21 nt product formation to estimate the preference of TTP vs. AZT-TP usage for each enzyme.

5.4.6. Pre-Steady State Single Nucleotide Incorporation Assay

Varying concentrations of dNTP or NRTI-TP in buffer (50 mM Tris pH 7.5, 50 mM KCl, 21 mM $MgCl_2$) were rapidly mixed with heteropolymeric DNA:DNA [^{32}P]-labeled P/T (pr20A/57mer for experiments with dATP and TNF-DP, and pr19T/45mer for experiments with TTP and AZT-TP, Table 12) at time points ranging from 50 ms to 20 s in a KinTek rapid quench machine (KinTek Corporation, Clarence, PA). Samples were quenched with 0.35 M EDTA, and diluted in sample loading dye (0.05% bromphenol blue/0.05% xylene cyanole in deionized

formamide). Elongation of primer by one nucleotide was resolved on a polyacrylamide sequencing gel and analyzed using a BioRad Molecular Imager as described above. Kinetic data were analyzed by nonlinear regression using Sigma Plot software with the following equations. The apparent burst rate constant (k_{app}) for each particular concentration of dNTP was determined by fitting the time courses for the formation of the 20 nt product with equation [3]:

$$[21 \text{ nt product}] = A[1-\exp(-k_{app}t)] \qquad [3]$$

where A represents the burst amplitude, and t is time. The turnover number (k_{pol}) and apparent dissociation constant for dNTP or NRTI-TP (K_d) were then obtained by plotting the apparent catalytic rates, k_{app}, against dNTP or NRTI-TP concentrations and fitting the data to the following hyperbolic equation [4]:

$$k_{app} = (k_{pol}[dNTP])/([dNTP] + K_d) \qquad [4]$$

5.4.7. Steady-State Multiple Polymerization Assay

RT polymerization products were electrophoretically analyzed under continuous DNA polymerization conditions. 50 nM (active site) RT was pre-incubated with 50 nM heteropolymeric DNA:DNA P/T (pr18/214mer, Table 12). Substrate composition varied in concentration of NRTI-TP and dNTP and experiments were conducted in the presence and absence of ATP (AZT-TP incorporation studies only). All substrate mixes also contained 0.01 U/ml inorganic pyrophosphatase and buffer with divalent metal ions to start the reaction (10 mM Tris-HCl pH 8.0, 10 mM KCl, 2 mM $MgCl_2$). Substrate was combined with P/T/RT and

incubated at 37°C. Aliquots were collected at time points varying from 5 min to 90 min, quenched in sample loading dye containing EDTA, and resolved on a denaturing polyacrylamide sequencing gel (as described above).

5.4.8. Statistical Analysis

Student's t test was used (SigmaStat, Systat Software, Inc., Point Richmond, CA) to assess significant differences between K_d and k_{pol} values.

5.5. Results

5.5.1. K65R Diminishes Primer Unblocking Activity of TAMs

In prior virologic studies, we observed bi-directional phenotypic antagonism between TAMs and K65R. Specifically, recombinant HIV-1 mutants with TAMs (TAM41 or TAM67) exhibited >50-fold resistance to AZT, but with the addition of K65R, this resistance decreased to <2.5-fold (Chapter 2, Table 8). To explain this antagonism, we hypothesized that K65R diminishes the primer unblocking activity of RT with TAMs. ATP-mediated "excision and rescue" analyses were performed to determine the apparent pseudo-first order rate constant for recovery (k_{ATP}) for phosphorolytic removal of AZT-MP by the following recombinant RTs: (1) wildtype (RT^{WT}); (2) 2 different TAM combinations—M41L/L210W/T215Y (RT^{TAM41}) and D67N/K70R/T215F/K219Q (RT^{TAM67}); (3) K65R alone (RT^{65R}), and; (4) K65R in combination with TAM41 ($RT^{TAM41/65R}$) or TAM67 ($RT^{TAM67/65R}$). "Excision" was defined as the ATP-catalyzed removal of AZT-MP by RT, and "rescue" or "recovery" was defined as the elongation

of the primer by 1 base due to the addition of TTP, followed by incorporation of the chain-terminating ddGTP.

RT with TAMs showed 1.9 to 4.4-fold increases in the k_{ATP} for recovery compared with RT^{WT} (Table 13, Figure 12), which is consistent with previous reports (8, 20, 130, 146, 187). We found that the rate of recovery was higher for RT^{TAM67} (4.4-fold) than RT^{TAM41} (1.9-fold), which has not been previously reported. By contrast, the rate of recovery for RT^{65R} was half that of wild-type (0.035 min^{-1} vs. 0.065 min^{-1}). This diminished rate is due only to decreased excision by K65R because the differences in incorporation efficiency between the enzymes (measured in seconds) are negligible in the time scale (minutes) used in this assay. Additionally, control experiments demonstrate that both wildtype and K65R RT can fully incorporate TTP and ddGTP by 5 minutes (Figure 13). When the K65R mutation was introduced into either combination of TAMs, the apparent rate of recovery was diminished to 0.5-fold and 0.6-fold that of wildtype RT (Table 13, Figure 12), indicating that K65R reversed the excision activity of RT with TAMs.

Table 13. k_{ATP} of Recovery of AZT-MP-Terminated Primer

HIV-1 RT	k_{ATP} (min^{-1})[a,b]	Fold-change relative to wild-type	Maximum Product Recovered[c], nM (%)
wildtype	0.065 ± 0.006	1	16.1 (81%)
65R	0.035 ± 0.003	0.5	6.9 (35%)
41L/210W/215Y	0.124 ± 0.013	1.9	18.3 (91%)
41L/210W/215Y + 65R	0.032 ± 0.013	0.5	11.9 (59%)
67N/70R/215Y/219Q	0.288 ± 0.024	4.4	17.9 (90%)
67N/70R/215Y/219Q + 65R	0.036 ± 0.006	0.6	14.1 (70%)

a Data is mean ± standard deviation of three independent experiments

b k_{ATP} represents apparent pseudo-first order rate of recovery. Excision was catalyzed by addition of 3 mM ATP.

c Maximum product recovery possible is 20 nM (see Materials and Methods). The value shown is the average of 3 experiments, with variance less than 10%.

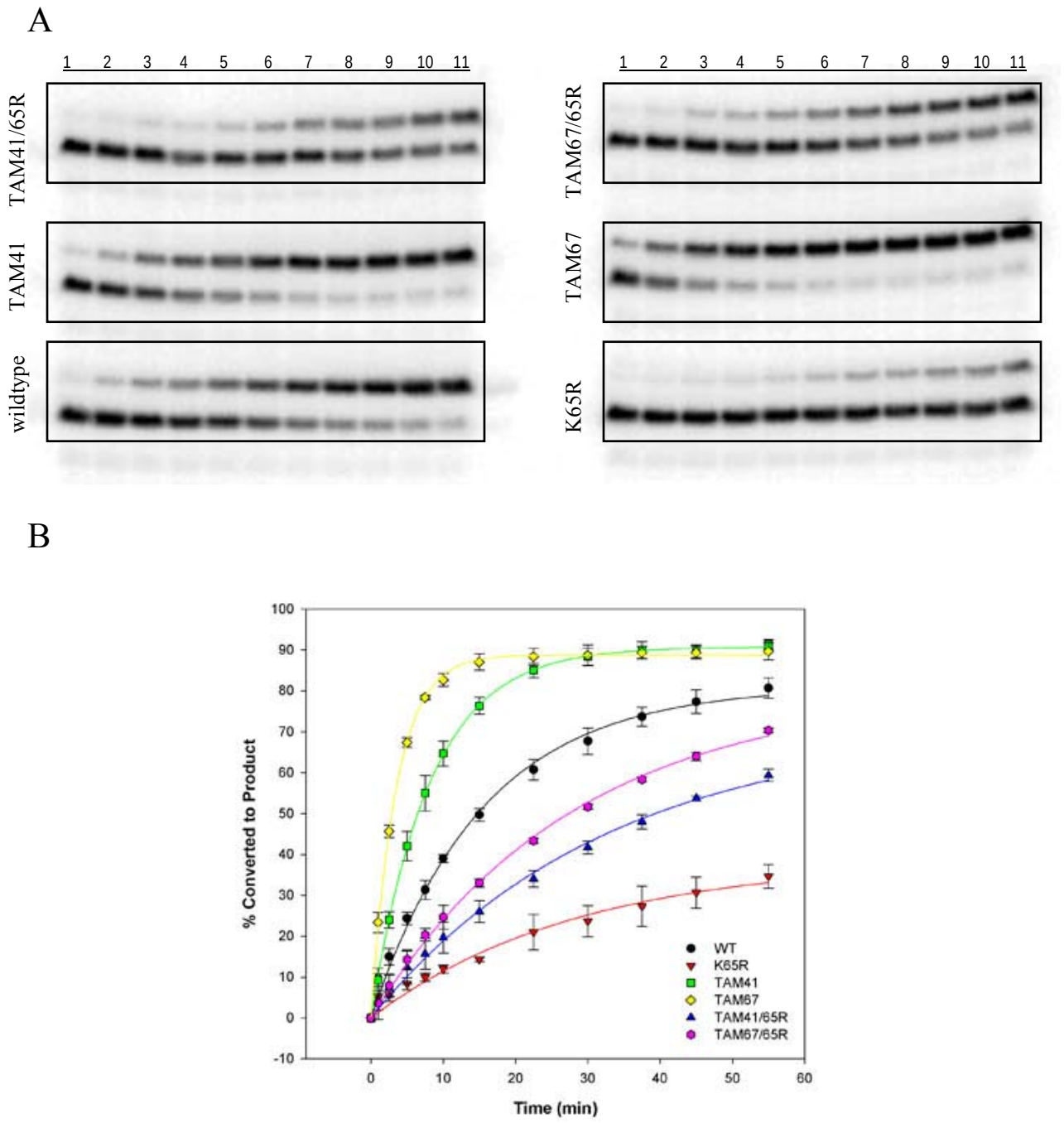


Figure 12. Effect of K65R and TAMs on AZT Excision and Recovery
Single excision and rescue experiments were performed as described in Materials and Methods. (A) Representative gel. Reaction products were quenched with sample loading buffer and run on a polyacrylamide sequencing gel. Timepoints 1-11 correspond to 1, 2, 5, 5, 7.5, 10, 15, 22.5, 30, 37.5, 45, and 55 min respectively. WT RT or with mutations used to catalyze the reaction are as indicated. (B) A ratio of the top band (final product, primer +1) to the bottom band (initial product) was determined for each time point for each enzyme using the Personal FX Quantity One software. The results are plotted as mean ± standard deviation % Product Formed as a function of time.

5.5.2. RT^{65R} Selectively Incorporates TTP over AZT-TP

Although the excision activity for AZT-MP was diminished by 50% for RT^{65R} compared to RT^{WT}, HIV-1_{65R} and HIV-1_{WT} had similar susceptibility to AZT in virologic assays (Chapter 2, Table 8). If the excision activity of K65R is diminished, then HIV-1_{65R} would be expected to show increased susceptibility to AZT compared to wildtype HIV-1, unless incorporation of AZT-TP were also reduced. To explore this possibility, we compared the ability of RT^{WT} and RT^{65R} to incorporate TTP in the presence of varying concentrations of AZT-TP. The potential outcomes of the polymerization reactions were either: (A) incorporation of AZT-TP resulting in primer termination, preventing further elongation, and yielding a +1 product (+1); or, (B) the incorporation of TTP followed by incorporation of ddGTP terminating the primer and yielding a +2 product. Substrate ratios were varied from 1:10 (AZT-TP:TTP) to 10:1 (AZT-TP:TTP) for the polymerization reactions. In theory, if no discrimination of AZT-TP over TTP occurred, the probability of incorporating AZT-TP would be equivalent to the proportion of AZT-TP used in the reaction.

Our results demonstrate that wild-type RT incorporated AZT-TP and TTP with approximately equal probability. When AZT-TP was used at a ratio of 1:10 (AZT-TP:TTP), only 9% of the (+1) product was formed. Similarly, when AZT-TP was used at a ratio of 10:1 (AZT-TP:TTP), 86% of (+1) product was formed. Overall, for wildtype RT, the amount of (+1) product formed was very similar to what was expected for all ratios of substrate tested (1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, AZT-TP:TTP). In contrast, K65R RT catalyzed less (+1) product formation for each ratio of substrate tested. The diminished incorporation of AZT-TP ranged from 11% less than expected for 1:10 (AZT-TP:TTP) to 19% less than expected for 10:1

(AZT-TP:TTP) These data indicate that K65R preferentially incorporates TTP over AZT-TP (Figure 13).

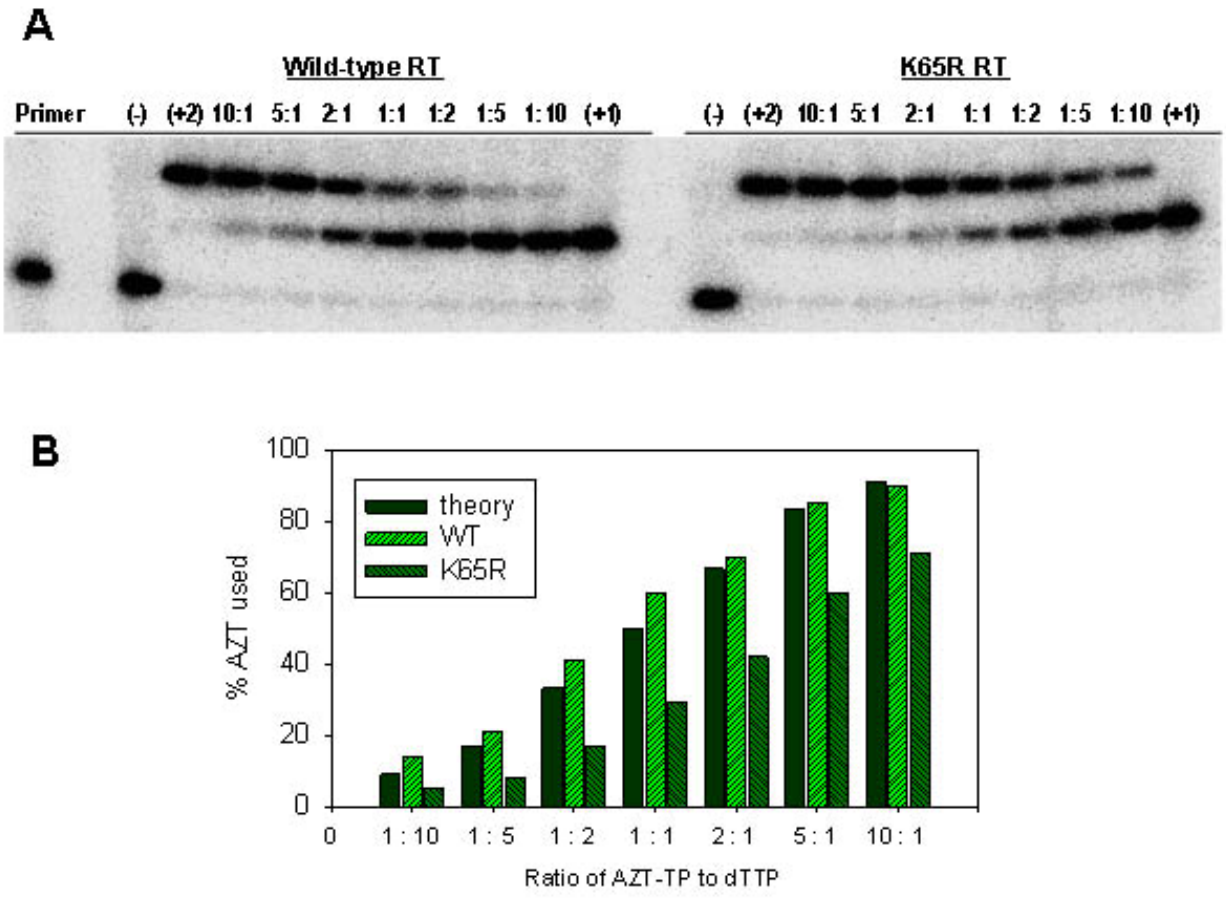


Figure 13. Preferential Incorporation of TTP over AZT-TP by K65R RT
(A) "Primer" indicates primer only, (-) indicates ddGTP only (no AZT-TP or TTP), (+2) indicates TTP and ddGTP only (no AZT-TP), (+1) indicates AZT-TP only (no TTP or ddGTP). Ratios reported as AZT-TP:TTP. (B) Theory (dark green solid bars) is calculated as % AZT that should be incorporated at each ratio if the probability of incorporating AZT was equal to the probability of incorporating TTP. The wildtype RT bars (light green left diagonals) indicates % AZT incorporated. The % AZT incorporated was determined using the Personal FX software by taking the ratio of AZT used to total product formed on the gel. A similar calculation was done for K65R RT (medium green right diagonals).

5.5.3. Pre-Steady State Incorporation of TTP and AZT-TP by RT^{WT} and RT^{65R}

In previous virologic studies (Chapter 1), we found that HIV-1_{65R} causes decreased susceptibility to all NRTIs tested except those with a 3'-azido component in the pseudosugar (specifically AZT and AZA, the adenine-analog equivalent of AZT—See Chapter 1). This decreased susceptibility to NRTIs may result from a general discrimination mechanism. The results described above showing that K65R preferentially incorporates TTP over AZT-TP suggest that all NRTIs, including AZT, are subject to discrimination by K65R. In agreement with this, White and others recently reported that the incorporation of AZT-TP by K65R RT is decreased compared to wildtype RT as determined using steady-state enzymatic analysis (206). To gain a detailed understanding of the mechanism by which K65R discriminates AZT-TP, we conducted a pre-steady state analysis of TTP and AZT-TP incorporation by RT^{WT} and RT^{65R}. Through this analysis, we compared for RT^{WT} and RT^{65R} the maximum rate of incorporation (k_{pol}), the substrate binding affinity or dissociation constant (K_d), and the catalytic efficiency of incorporation (k_{pol}/K_d). (For a detailed explanation of these kinetic constants, please see Figure 4).

The catalytic efficiency for the incorporation of the natural substrate TTP was 2.3-fold less for RT^{65R} compared to RT^{WT}. This diminished catalytic efficiency for RT^{65R} was entirely due to a reduction in the catalytic rate (29.6 s^{-1} for WT^{RT} compared to 11.9 s^{-1} for RT^{65R}); the binding constants (K_d) for TTP were similar for both enzymes (43.2 μM for RT^{WT} and 40.6 μM for RT^{65R}). These results are consistent with those from Deval and colleagues, who found that the K65R mutation generally affects polymerization, not binding of natural dNTPs (44). The diminished catalytic efficiency of RT^{65R} was even more pronounced for AZT-TP, being 8.3-fold less for RT^{65R} than RT^{WT} (15.1 s^{-1} for RT^{WT} vs. 1.81 s^{-1} for RT^{65R}). This resulted in a selectivity

(or preferential incorporation of TTP over AZT-TP), of 5.8 for RT^{65R} calculated as the ratio of k_{pol}/K_d for TTP versus k_{pol}/K_d for AZT-TP) (Table 14, Figure 14). Taken together, RT^{65R} was 2.4-fold resistant to AZT-TP, calculated as the ratio of selectivity of RT^{65R} versus selectivity for RT^{WT}.

5.5.4. Pre-Steady State Incorporation of TTP and AZT-TP by RT^{65R} with TAMs

Since RT^{65R} was found to discriminate against AZT-TP incorporation, we aimed to determine whether TAMs would antagonize this discrimination. Pre-steady state analyses of TTP and AZT-TP incorporation were performed using RT with TAM41 and TAM67 alone, or in combination with K65R. Consistent with previous findings, wildtype RT and RT with TAMs had similar catalytic efficiency (k_{pol}/K_d) for TTP (0.44 to 0.68 $s^{-1}\mu M^{-1}$) and AZT-TP incorporation (0.15 to 0.28 $s^{-1}\mu M^{-1}$) (96, 101, 187). RT with K65R alone or RT with K65R and TAMs had similar reductions in catalytic efficiencies for both TTP (0.28 to 0.29 $s^{-1}\mu M^{-1}$) and AZT-TP (0.046 to 0.051 $s^{-1}\mu M^{-1}$) incorporation compared to wildtype RT or RT with TAMs alone (Table 14, Figure 14), although the mechanism of decreased efficiency differed by TAM pathway. Compared to RT^{65R} alone, $RT^{TAM67/65R}$ had decreased binding affinity (K_d) but increased catalytic rate (k_{pol}) for both TTP and AZT-TP. By contrast, $RT^{TAM41/65R}$ had similar binding affinity and catalytic rate as RT^{65R} (Figure 16, Table 14). Despite these differences in K_d and k_{pol}, the net fold-resistance (fold-R) to AZT-TP was similar for $RT^{TAM67/65R}$ (2.4-fold), $RT^{TAM41/65R}$ (2.6-fold) and RT^{65R} (2.4-fold) (Table 14). These results indicate that there is little effect of TAMs on K65R for discrimination against AZT-TP, although the mechanism of this preserved selectivity differed by TAM combinations (Figure 15).

Table 14. Pre-steady state kinetic constants of TTP and AZT-TP incorporation by HIV-1 RT

HIV-1 RT	TTP			AZT-TP			Selectivity[b]	Fold-R[c]
	k_{pol} (s^{-1})[a]	K_d (µM)[a]	k_{pol}/K_d (s^{-1}•µM^{-1})	k_{pol} (s^{-1})[a]	K_d (µM)[a]	k_{pol}/K_d (s^{-1}•µM^{-1})		
WT	29.6 ± 3.0	43.2 ± 8.1	0.68	15.1 ± 3.1	53.8 ± 2.3	0.28	2.4	-
TAM41	43.8 ± 25	73.8 ± 15	0.59	13.2 ± 0.49	52.9 ± 6.9	0.25	2.4	1.0
TAM67	22.3 ± 7.5	50.9 ± 11	0.44	9.06 ± 0.94	60.3 ± 30	0.15	2.9	1.2
65R	11.9 ± 2.1	40.6 ± 12	0.29	1.81 ± 0.33	35.5 ± 22	0.051	5.8	**2.4**
TAM41/65R	15.9 ± 5.2	55.1 ± 13	0.29	1.97 ± 0.24	43.0 ± 24	0.046	6.3	**2.6**
TAM67/65R	25.2 ± 1.4[d]	90.0 ± 2.4[d]	0.28	5.38 ± 1.8[d]	111 ± 20[d]	0.048	5.8	**2.4**

a Data reported is mean ± standard deviation of three independent experiments.
b The selectivity is calculated as the ratio of [kpol/Kd (TTP)]/[kpol/Kd (AZT-TP)].
c Fold-Resistance is calculated as the ratio of [selectivity (mutant RT)/selectivity (wild-type RT)].
d Value significantly higher than 65R, $p < 0.05$, as determined by Student's T test.

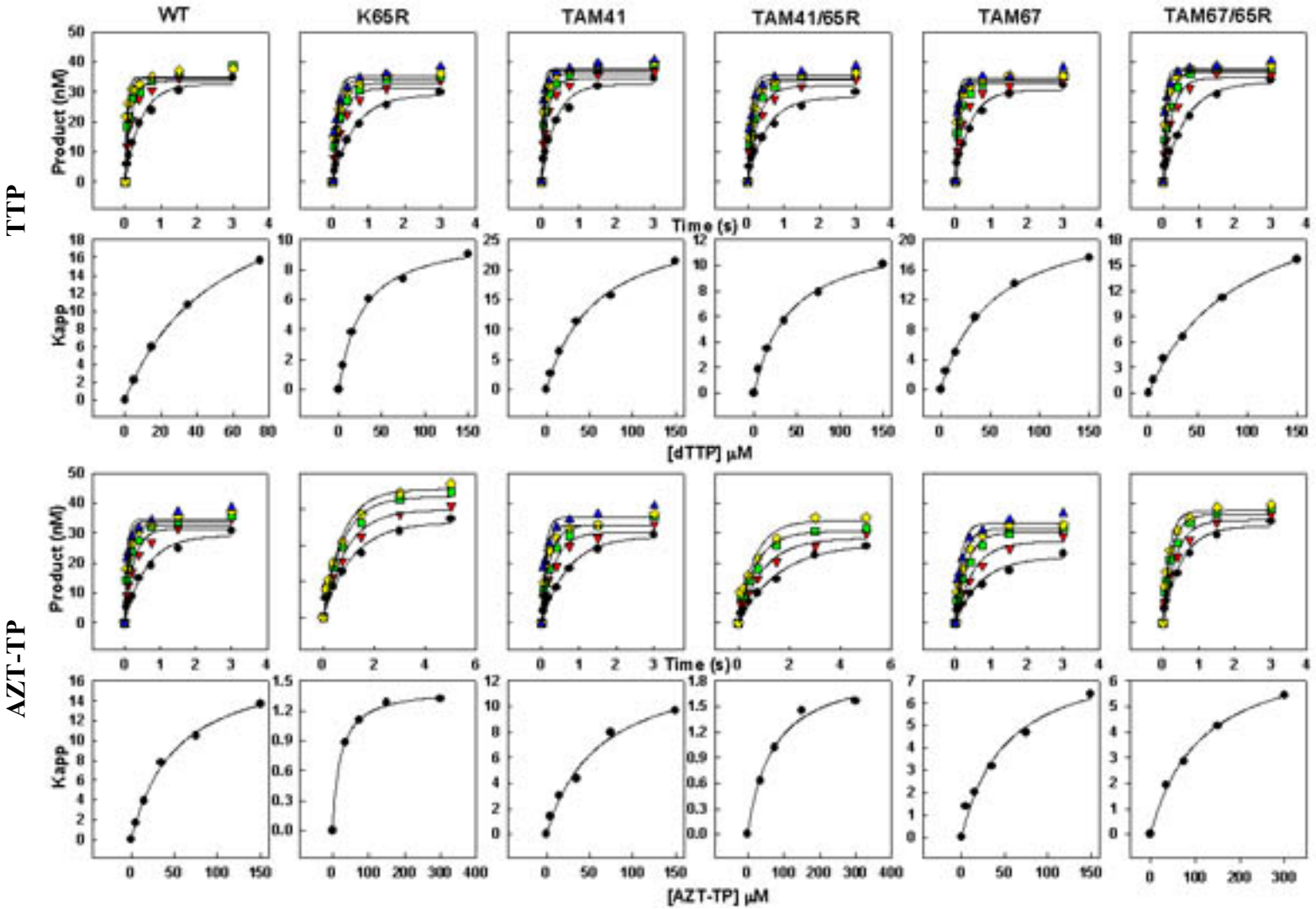


Figure 14. Representative Data for Pre-steady State Analysis of TTP or AZT-TP Incorporation
Representative data is shown from three independent experiments for each enzyme and substrate.

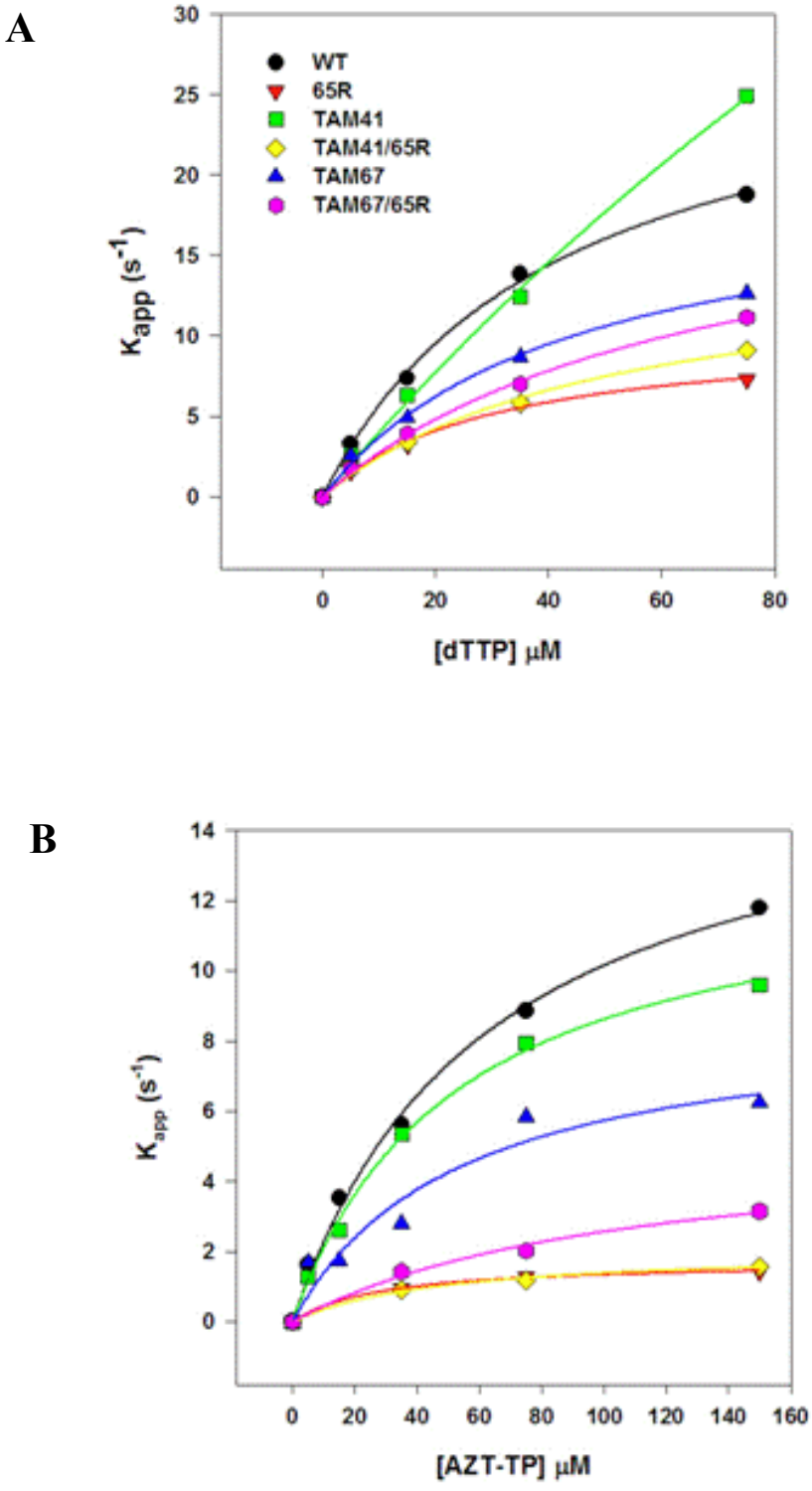


Figure 15. Product Formed as a Function of Time: TTP or AZT-TP Incorporation. Data is plotted as mean K_{app} from each substrate concentration. 3 independent experiments were conducted. (A) Incorporation of TTP and (B) incorporation of AZT-TP catalyzed by RT (WT, K65R, TAM41, TAM41/65R, TAM67 and TAM67/65R).

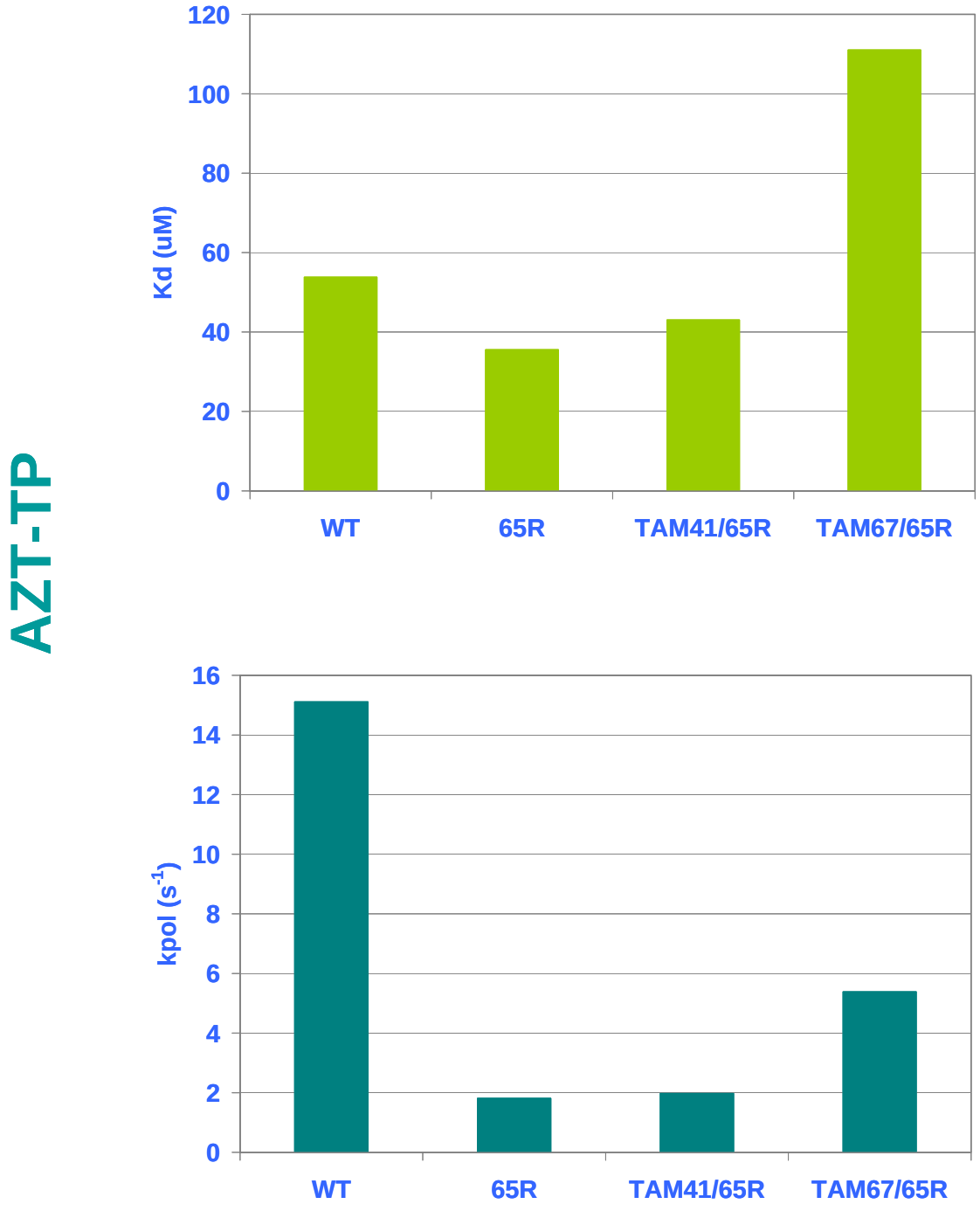


Figure 16. Comparison of K_d and k_{pol} for AZT-TP Incorporation by HIV-1 RT with K65R alone or with TAMs

Data shown is the mean K_d and k_{pol} from three independent experiments. Top graph, comparison of K_d; bottom graph, comparison of k_{pol} for RT (WT, K65R, TAM41/65R and TAM67/65R).

5.5.5. Full-Length Polymerization in the Presence of AZT-TP and/or ATP

To analyze the combined effect of discrimination and excision over multiple nucleotide incorporations, we examined steady-state DNA synthesis using a long heteropolymeric template that corresponds to the first 214 nucleotides of the primer binding site in the HIV-1 genome. Polymerization was catalyzed by wildtype RT, RT with K65R alone, or RT with K65R in combination with TAMs (TAM41 or TAM67). RT reactions contained all four dNTPs, AZT-TP, and a physiologically relevant concentration of ATP (3 mM) (Figure 17).

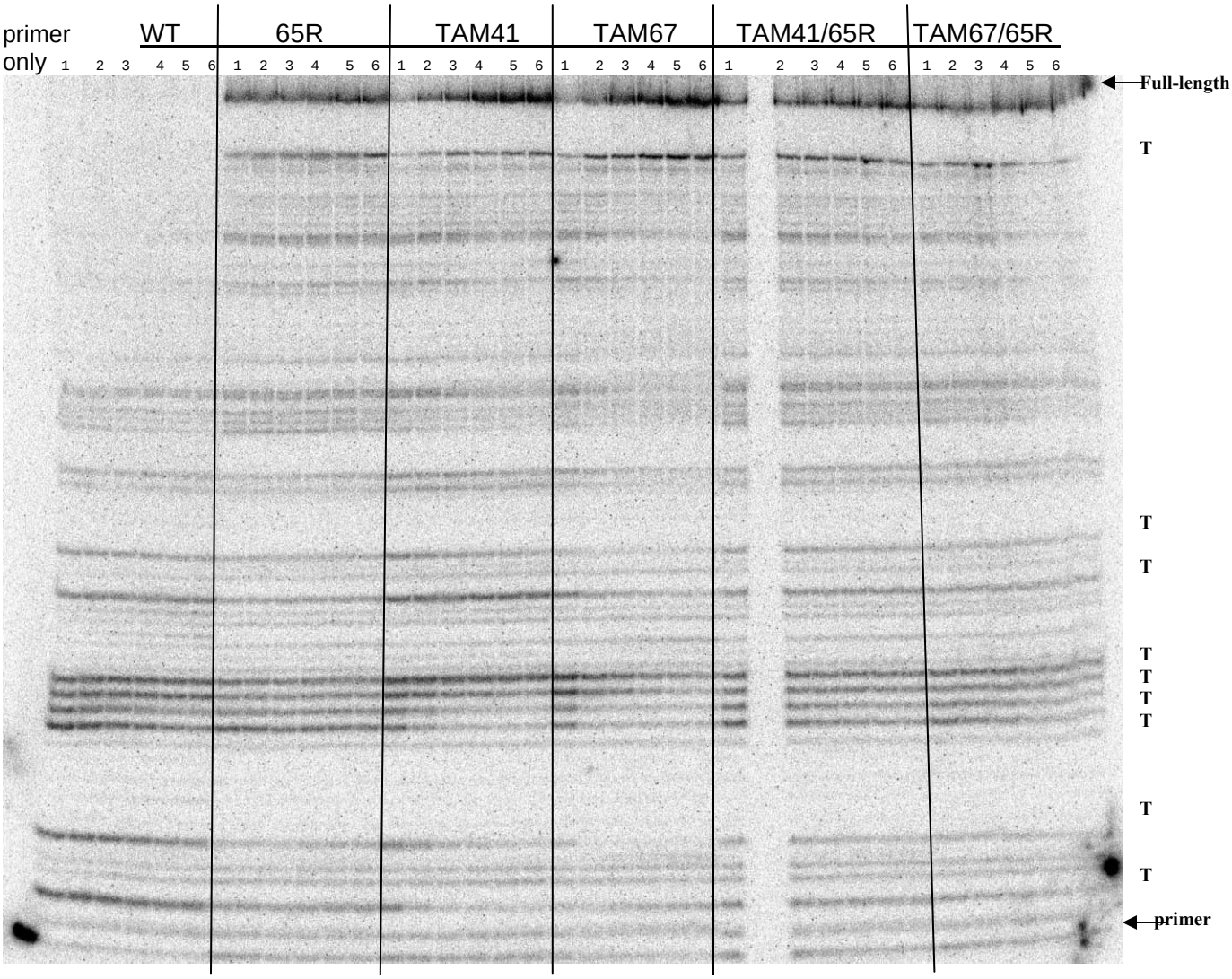
As expected, wildtype RT did not catalyze full-length product formation due to inefficient excision of AZT-MP. By contrast, RT with either combination of TAMs (TAM41 or TAM67) did catalyze full length product formation equally efficiently, indicating that AZT-TP in the substrate mix was not a significant barrier to polymerization. RT^{65R} alone or in combination with TAMs also catalyzed full-length product formation, albeit at a lower level than TAMs alone (Figure 17).

To demonstrate that incomplete product formation was not due to poor activity of the wildtype enzyme, experiments in the absence of AZT-TP and ATP were performed. As expected, wildtype RT catalyzed equivalent full-length product formation as other RTs (Figure 18).

Finally, to demonstrate that full-length product synthesis by RT^{65R}, $RT^{TAM41/65R}$, and $RT^{TAM67/65R}$ (Figure 17) was due to discrimination and not excision, we performed experiments in which ATP was excluded (to prevent excision) but AZT-TP was included. Under these conditions, only RT with K65R alone or in combination with TAMs was able to catalyze full-length product formation (Figure 19). These data confirm that TAMs enable RT to avoid chain-termination by AZT-TP through an excision mechanism, whereas K65R prevents chain-

termination by AZT-TP through discrimination. The combined effects of K65R, decreased excision and increased discrimination, explains the wildtype susceptibility of HIV-1_{65R} to AZT observed in viral assays.

A



B

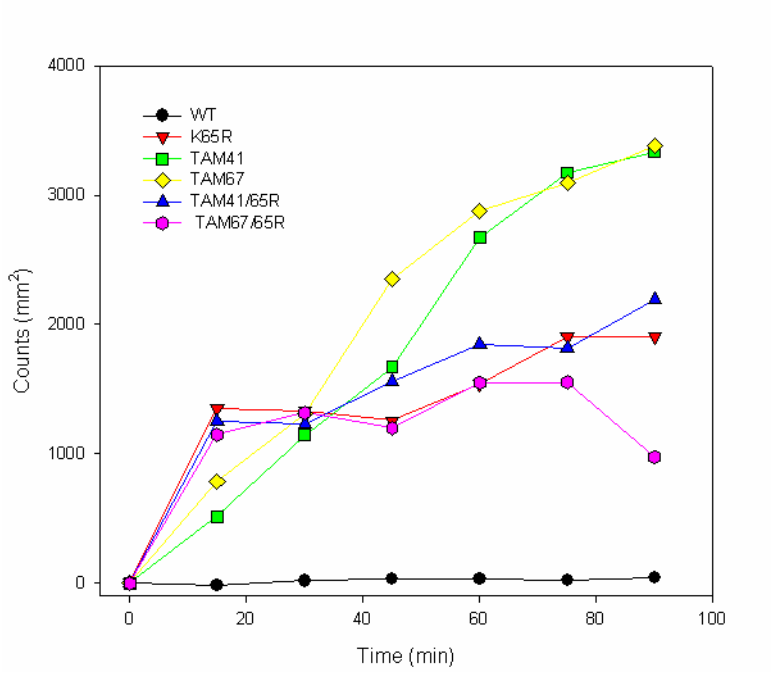


Figure 17. Full-Length Polymerization by RT with K65R and TAMs in the Presence of AZT-TP and 3 mM ATP

(A) Polyacrylamide gel time points 1, 2, 3 ,4, 5 and 6 refer to 15, 30, 45, 60, 75 and 90 minutes respectively. (B) Densitometry (Personal FX software Quantity One) was used to compare the topmost band of the gel (full length product) for each time point for each enzyme. The data is plotted as counts mm2 (area of band) by time.

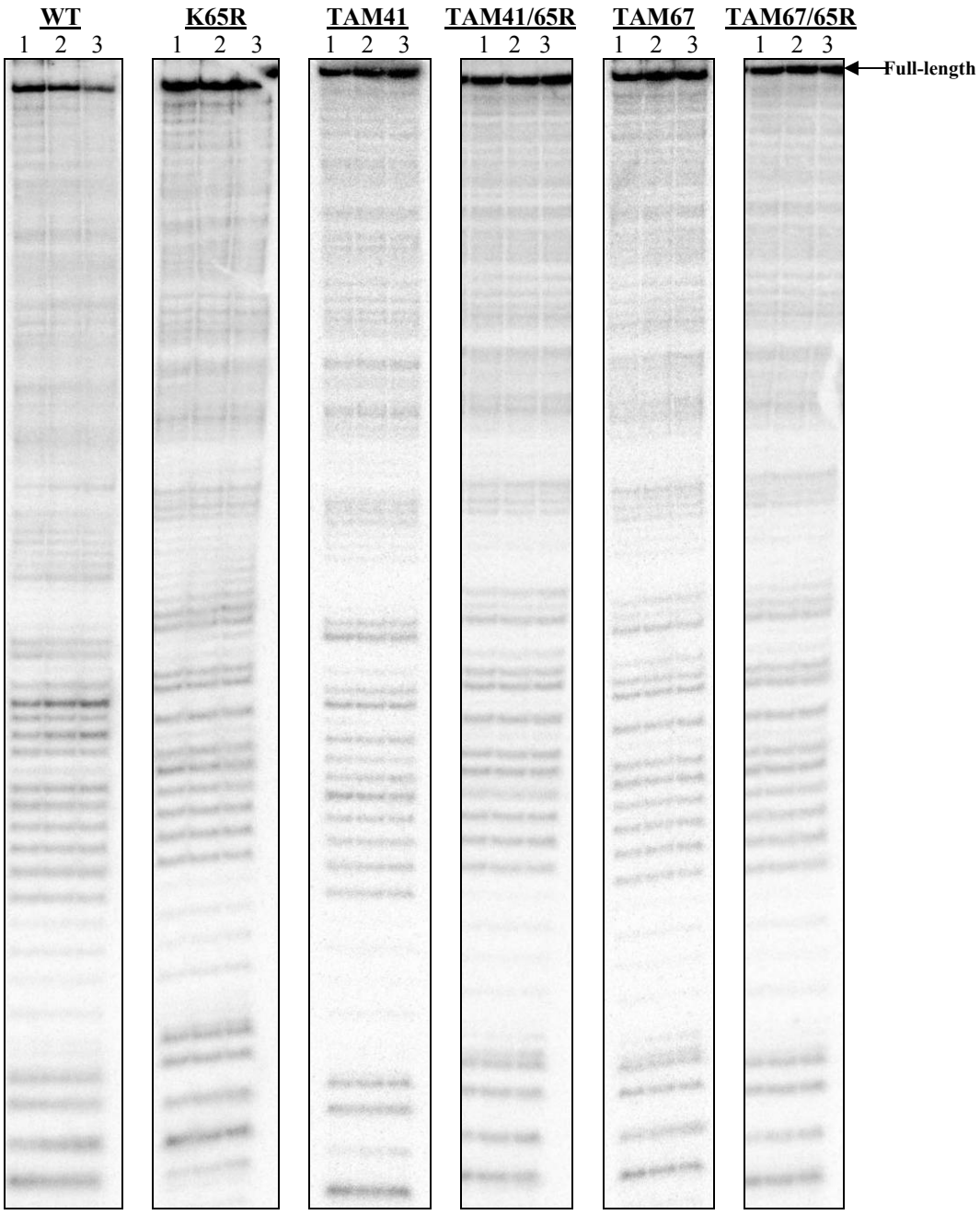


Figure 18. Full-Length Polymerization by RT with K65R and TAMs in the Absence of AZT-TP and ATP

Assay components included 10 mM P/T (See Materials and Methods), Reaction Buffer (50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$), 0.01 U/ml pyrophosphatase, 200 nM active RT (WT, 65R, TAM41, TAM67, TAM41/65R and TAM67/65R), and 5 μM of each dNTP. Time points correlate to (1) 30 min, (2) 60 min, and (3) 90 min.

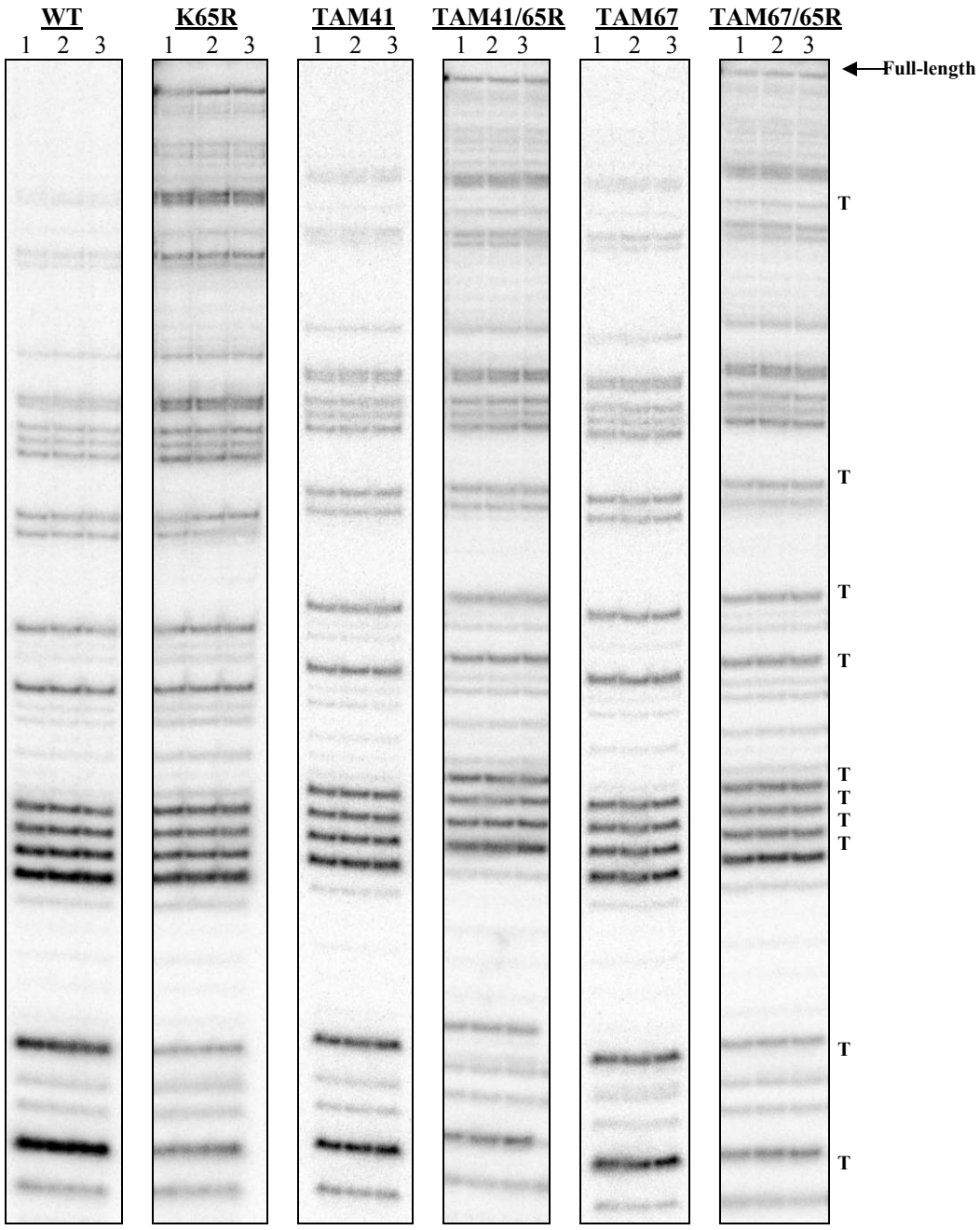


Figure 19. Full-Length Polymerization by RT with K65R and TAMs in the Presence of AZT-TP, But No ATP

Assay components included 10 mM P/T (See Materials and Methods, chapter 3), Reaction Buffer (50 mM Tris, 50 mM KCl, 10 mM MgCl2), 0.01 U/ml pyrophosphatase, 200 nM active RT (WT, 65R, TAM41, TAM67, TAM41/65R and TAM67/65R), 5 µM of each dNTP, and 1 µM AZT-TP. Time points correlate to (1) 30 min, (2) 60 min, and (3) 90 min.

5.5.6. Pre-Steady State Incorporation of dATP and TNV-DP by RT with K65R and TAMs

K65R is a primary mutation selected by tenofovir *in vitro* and in patients (122, 169, 202). We recently demonstrated that the TAM67 combination decreases K65R's resistance to tenofovir from K65R in viral susceptibility assays, from 2.8-fold for HIV-1 with K65R to wildtype susceptibility for HIV-1 with TAM67/65R (Table 9, Chapter 3) (153). To elucidate the biochemical mechanism for this antagonism, we conducted pre-steady state analyses to determine kinetic constants for dATP and TNF-DP incorporation by wildtype RT and RT with K65R alone or in combination with TAMs.

Wildtype RT and RT with TAMs alone had similar binding affinity, catalytic rate and catalytic efficiency for both dATP and TNV-DP. TAMs alone did not confer resistance to tenofovir through discrimination: fold-change was 1.1 (TAM67) and 0.9 (TAM41) compared to wildtype. This is consistent both with virological data (Chapter 3) (153) and TAMs being poor discriminators of NRTIs (96, 101). As previously reported, K65R greatly reduced incorporation of TNV-DP mainly due to a loss of catalytic rate with minimal effect on binding affinity (Table 15, Figure 20, Figure 22) (44).

In our study, we were interested in whether TAMs would alter the discriminatory effect of K65R on TNV-DP incorporation. Antagonism of K65R by TAMs was observed for both TAM combinations but the mechanism differed between pathways. The TAM41 pathway exerted its effect on K65R by decreasing selectivity for TNF-DP compared to dATP. That is, the catalytic efficiency of $RT^{TAM41/65R}$ for TNF-DP incorporation was comparable to RT^{65R} ($0.040\ s^{-1}\mu M^{-1}$ vs. $0.061\ s^{-1}\mu M^{-1}$) but the catalytic efficiency for dATP incorporation was lower for $RT^{TAM41/65R}$ ($1.7\ s^{-1}\mu M^{-1}$) than for RT^{65R} ($4.0\ s^{-1}\mu M^{-1}$) (Figure 22, Table 15).

By contrast, TAM67 antagonized K65R by restoring incorporation of TNV-DP, primarily by increasing the catalytic rate of TNV-DP incorporation. The catalytic rate (k_{pol}) of TNV-DP incorporation by RT^{65R} alone (0.45 s^{-1}) was increased 4-fold for $RT^{TAM67/65R}$ to 2.1 s^{-1}. Binding affinity (K_d), however, was decreased in the combined mutant (7.31 μM for RT^{65R} compared to 13.5 μM for $RT^{TAM67/65R}$) (Table 15, Figure 22).

These data demonstrate how TAMs antagonize the effect of K65R on tenofovir resistance. K65R conferred 12-fold resistance to tenofovir, and this resistance was decreased to 5.0-fold by TAM67, and to 8.0-fold by TAM41. Figure 21 illustrates the change in apparent rates of incorporation of TNV-DP as a function of substrate concentration. The graph shows that the maximum rate of TNF-DP incorporation is similar for RT^{WT}, RT^{TAM41} and RT^{TAM67}, is reduced for $RT^{TAM67/65R}$ compared to RT^{WT}, and is minimal for RT^{65R} and $RT^{TAM41/65R}$. These maximum rates are consistent with viral susceptibility data, in which antagonism of K65R primarily occurs with the TAM67 pattern and not with the TAM41 pattern.

Table 15. Pre-steady state kinetic constants of dATP and TNF-DP incorporation by HIV-1 RT

HIV-1 RT	dATP			TNF-DP			Selectivity[b]	Fold-R[c]
	k_{pol} (s^{-1})[a]	K_d (µM)[a]	k_{pol}/K_d (s^{-1}•µM^{-1})	k_{pol} (s^{-1})[a]	K_d (µM)[a]	k_{pol}/K_d (s^{-1}•µM^{-1})		
WT	24.4 ± 3.9	3.22 ± 1.7	7.6	6.16 ± 1.0	4.29 ± 1.0	1.4	5.3	-
TAM41	18.6 ± 0.86	3.18 ± 0.86	5.9	6.05 ± 0.83	4.92 ± 3.7	1.2	4.8	0.9
TAM67	19.2 ± 4.3	2.83 ± 1.0	6.8	5.93 ± 2.1	5.18 ± 1.2	1.1	5.9	1.1
65R	12.6 ± 1.9	3.19 ± 0.68	4.0	0.45 ± 0.062	7.31 ± 2.7	0.061	65	**12**
TAM41/65R	17.0 ± 7.2	9.85 ± 3.7[d]	1.7	0.79 ± 0.36	19.5 ± 9.8	0.040	43	**8.0**
TAM67/65R	29.0 ± 6.0[d]	7.25 ± 2.2[d]	4.0	2.06 ± 0.25[d]	13.5 ± 1.9[d]	0.15	26	**5.0**

a Data reported is mean ± standard deviation of three independent experiments.
b The selectivity is calculated as the ratio of [k_{pol}/K_d (dATP)]/[k_{pol}/K_d (TNF-DP)].
c Fold-Resistance is calculated as the ratio of [selectivity (mutant RT)/selectivity (wild-type RT)].
d Value significantly different than 65R, $p < 0.05$, as determined by Student's T test.

dATP

TNF-DP

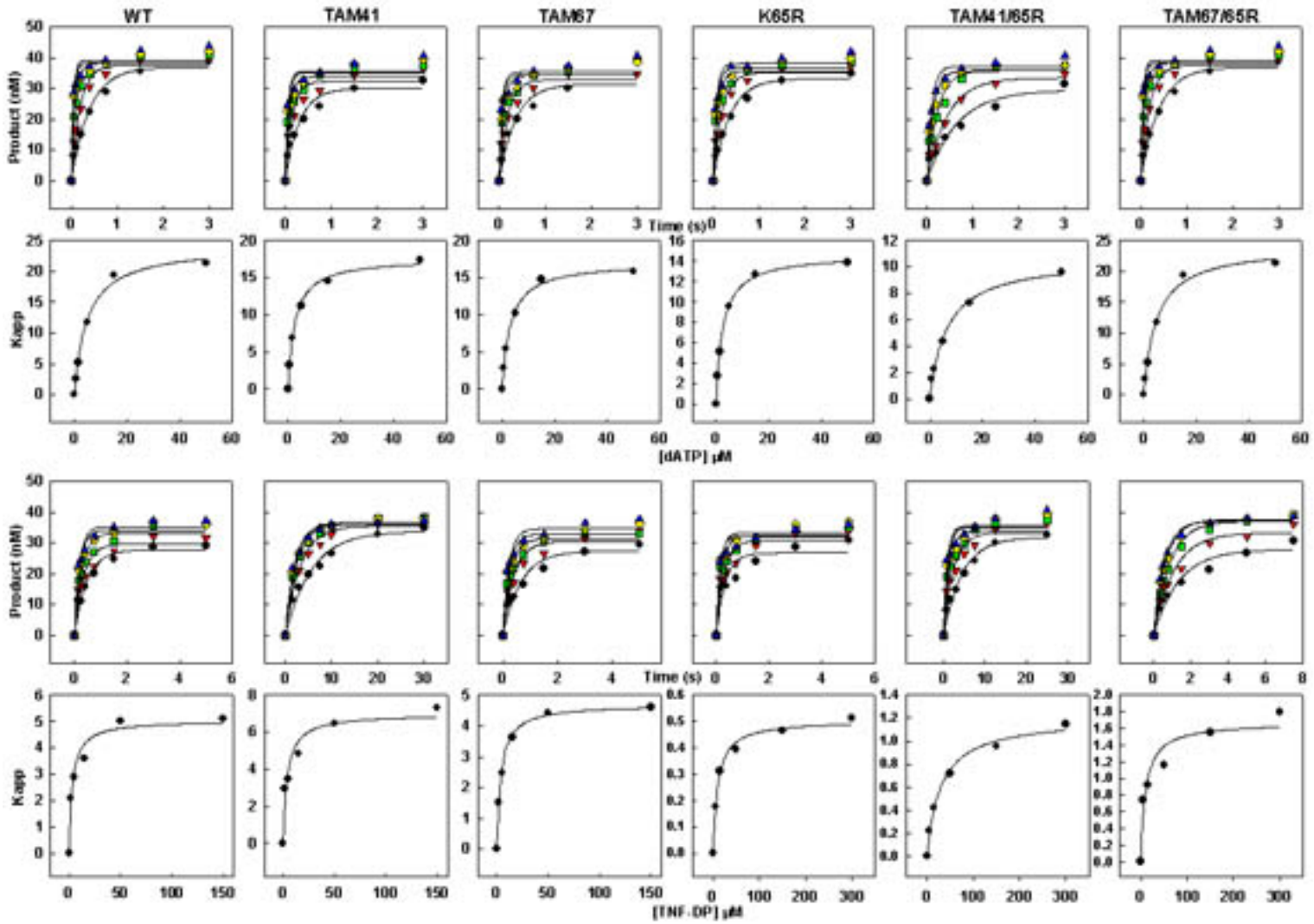


Figure 20. Representative Data for Pre-steady State Analysis of dATP or TNF-DP Incorporation
Representative data is shown from three independent experiments for each enzyme and substrate.

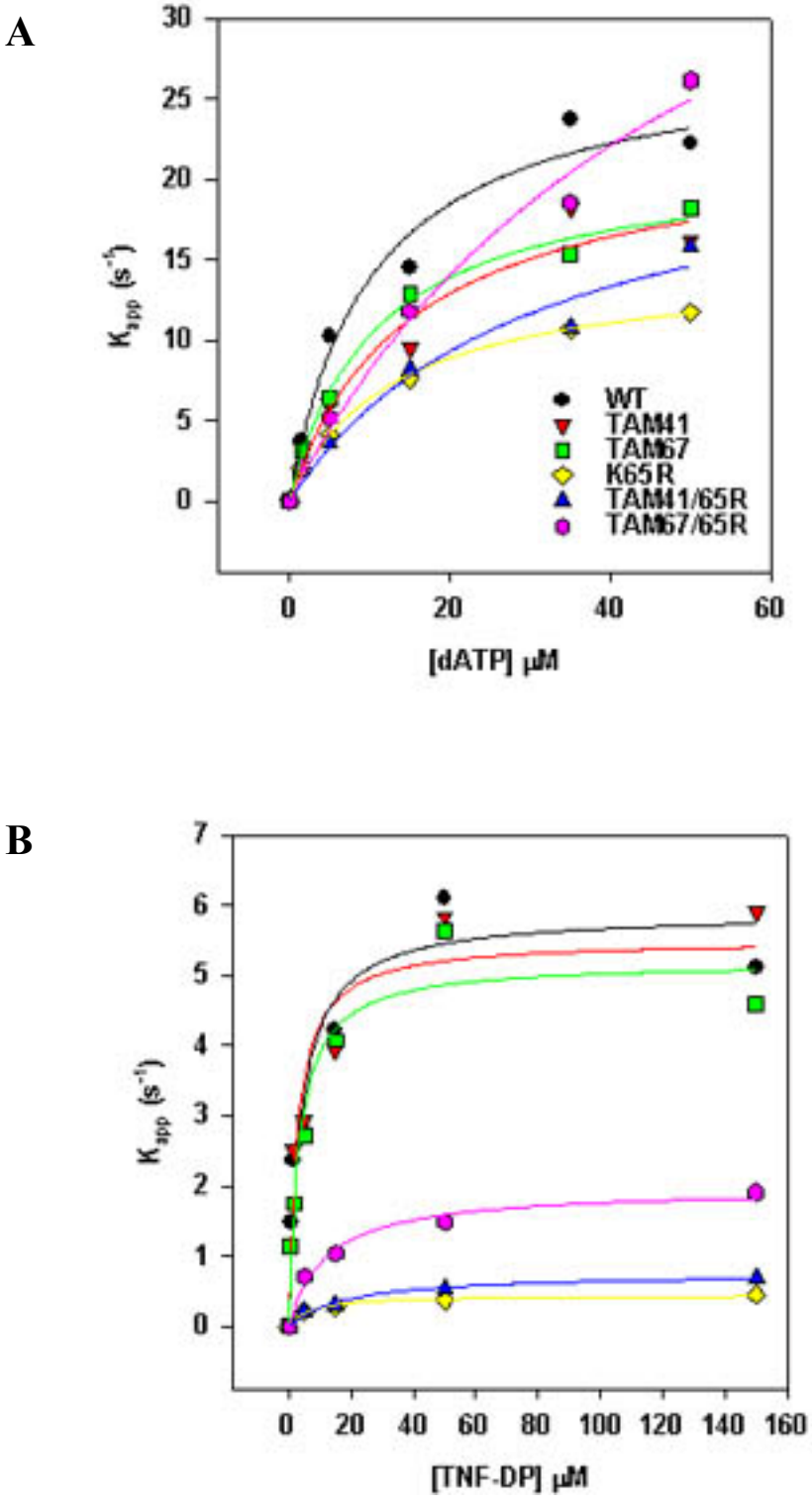


Figure 21. Comparative k_{app} as a Function of dATP or TNF-DP Concentration
Data shown is the mean Kd and kpol from three independent experiments.

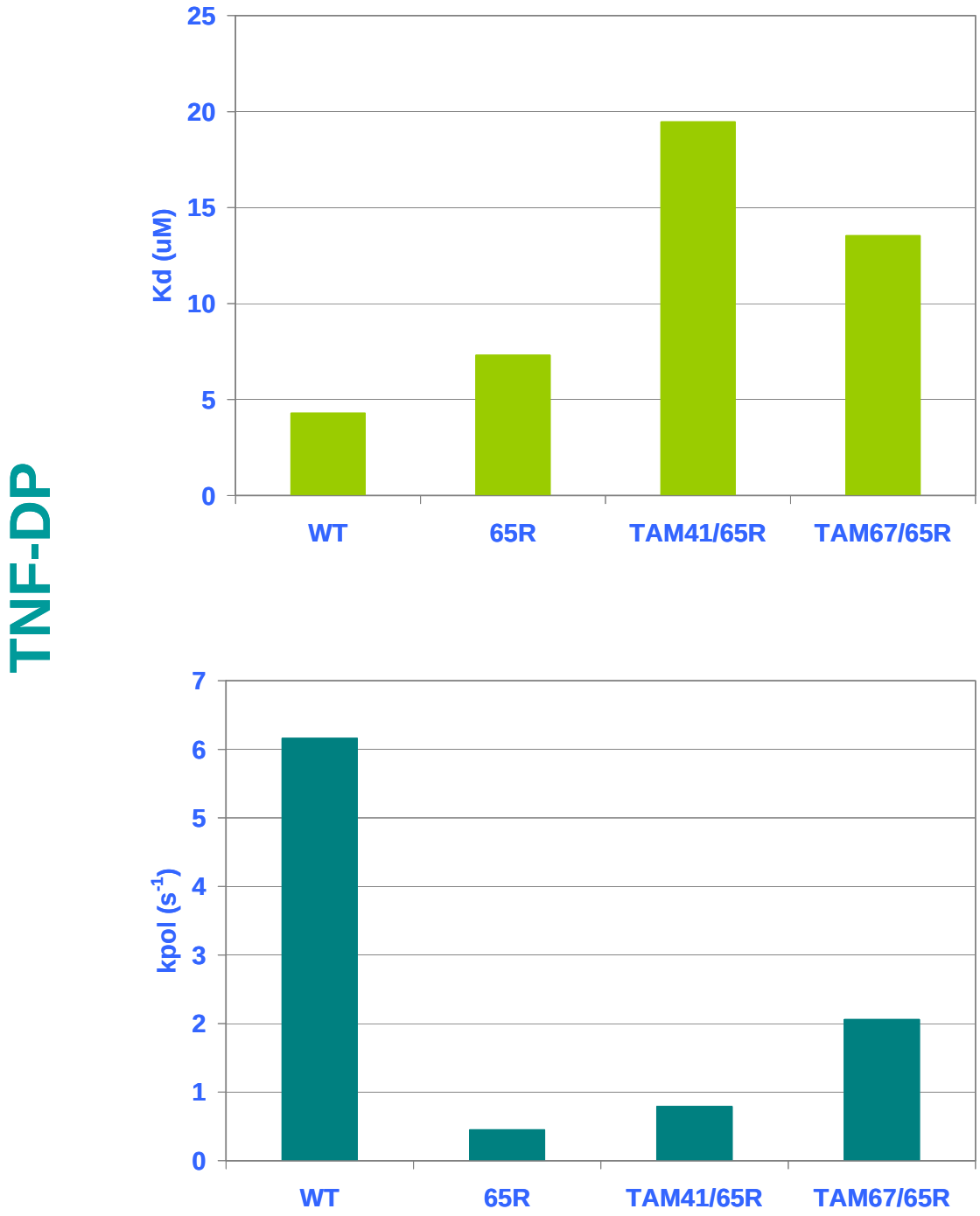


Figure 22. Comparison of K_d and k_{pol} for TNF-DP Incorporation by HIV-1 RT with K65R alone or with TAMs

Data shown is the mean K_d and k_{pol} from three independent experiments. Top graph, comparison of K_d; bottom graph, comparison of k_{pol} for RT (WT, K65R, TAM41/65R and TAM67/65R).

5.5.7. Full-Length Polymerization in the Presence of TNF-DP

Finally, to assess the antagonistic effect of TAMs on K65R over multiple incorporations, we conducted steady-state analyses using a long template primed by a DNA oligonucleotide (pr18/214mer, Table 12). Consistent with pre-steady state data, we found that full-length product generation catalyzed by RT^{WT}, RT^{TAM41} and RT^{TAM67} were similar. As expected, full-length product generated by RT^{65R} was greater than that for RT^{WT} because of discrimination by RT^{65R}. Product formation by $RT^{TAM41/65R}$ was similar to RT^{65R}, again indicating that there is little antagonism of K65R by TAM41. By contrast, product formation by $RT^{TAM67/65R}$ was diminished compared to RT^{65R}, demonstrating that TAM67 antagonizes the discrimination of K65R against TNV-DP incorporation (Figure 23).

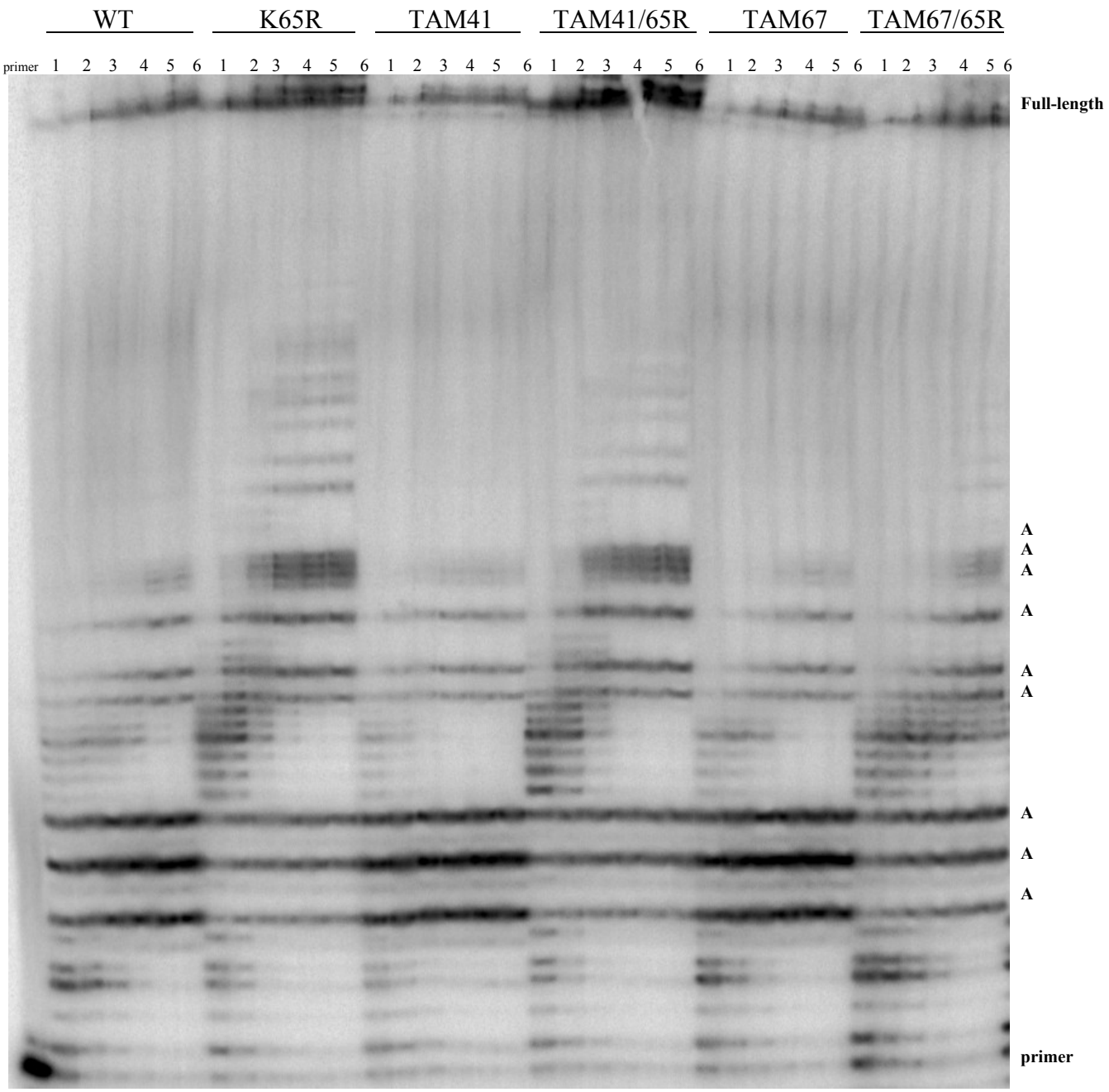


Figure 23. Full-Length Polymerization by RT with K65R and TAMs in the Presence of TNF-DP

50 nM active site RT and 50 nM P/T (See Materials and Methods) was combined with 5 μM TNF-DP and 1 μM each dNTP. Time points 1-6 as indicated above are 2.5, 5, 10, 20, 30 and 60 min.

5.6. Discussion

In previous work, we demonstrated bi-directional phenotypic antagonism between K65R and TAMs in HIV-1 susceptibility assays. The current study provides biochemical explanations for this antagonism.

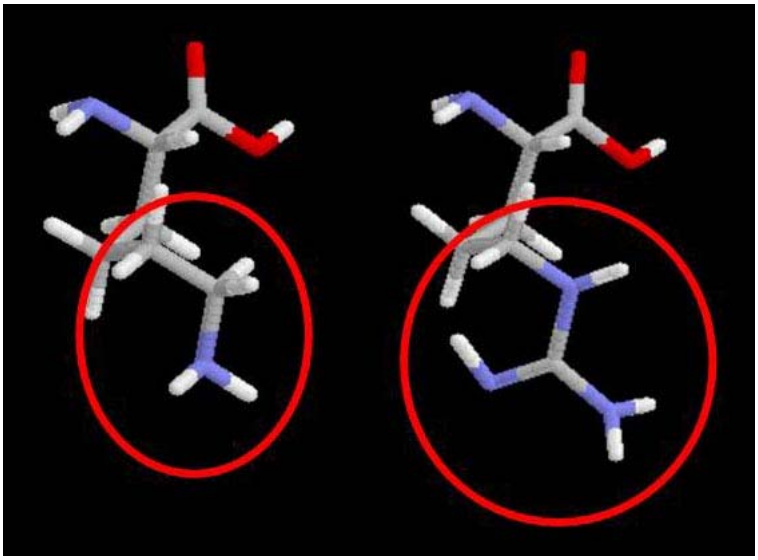
We first analyzed the mechanism of reversal of AZT resistance by K65R. TAMs cause resistance to AZT by increasing ATP-mediated primer unblocking activity of RT (for recent review see Goldschmidt et al, 2004) (69). Using a physiological concentration of ATP (3 mM), our study confirmed that the removal of AZT-MP from a terminated primer/template catalyzed by RT^{TAM41} and RT^{TAM67} was 1.9-fold and 4.4-fold more efficient than wild-type, respectively. By contrast, the rate of excision and rescue by RT^{65R} was decreased to only 50% that of RT^{WT}. The addition of either TAM pattern to RT with K65R did not improve the primer unblocking activity. Similar results were obtained by Meyer and colleagues using the clinically uncommon TAM combination D67N/K70R/T215Y/K219Q with K65R (Meyer, Matsuura et al. 2003).

Antagonism of TAMs by other NRTI mutations has been reported previously. The M184V mutation was first observed to maintain the clinical efficacy of AZT (111), and subsequent studies demonstrated that M184V reverses AZT resistance by reducing the primer unblocking activity of TAMs (70). Boyer and colleagues suggested that the combination of M184V and TAMs may produce subtle conformational changes in the DNA polymerase active site that perturb the alignment of the γ-phosphate of ATP with the phosphodiester bond between the penultimate nucleotide and the NRTI at the 3'-end of the primer (19). L74V also antagonizes TAMs by counteracting the increased rate of primer unblocking activity by RT with TAMs (138). Finally, foscarnet resistance mutations including W88G, E89K, A114S, S117T, F160Y,

M164I and Q161L (alone or with H208Y) also suppress resistance to AZT in the background of TAMs (126). The A114S mutation may decrease pyrophosphorolysis, or increase the rate of RT-T/P dissociation (8). E89K and W88G are proposed to reverse AZT resistance by affecting the positioning of the primer terminus in either the "N" or the "P" site (8, 133). The "N" and "P" site describe the location of the primer terminus before and after translocation respectively, and excision can occur only if the primer terminus is at the "N" site (20).

A similar mechanism may be proposed for antagonism of RT excision activity by K65R. A structural representation of the interaction of Lys65 with ATP suggests that the lysine at codon 65 may interact with the α and β phosphates of ATP to properly align it for nucleophilic attack for removal of AZT-MP. The larger side chain of arginine (Figure 24A) may alter the position of ATP such it is not properly aligned for excision (Figure 24B). The presence of TAMs, which are proposed to increase ATP binding, are not sufficient to overcome this misalignment and restore excision efficiency.

A



B

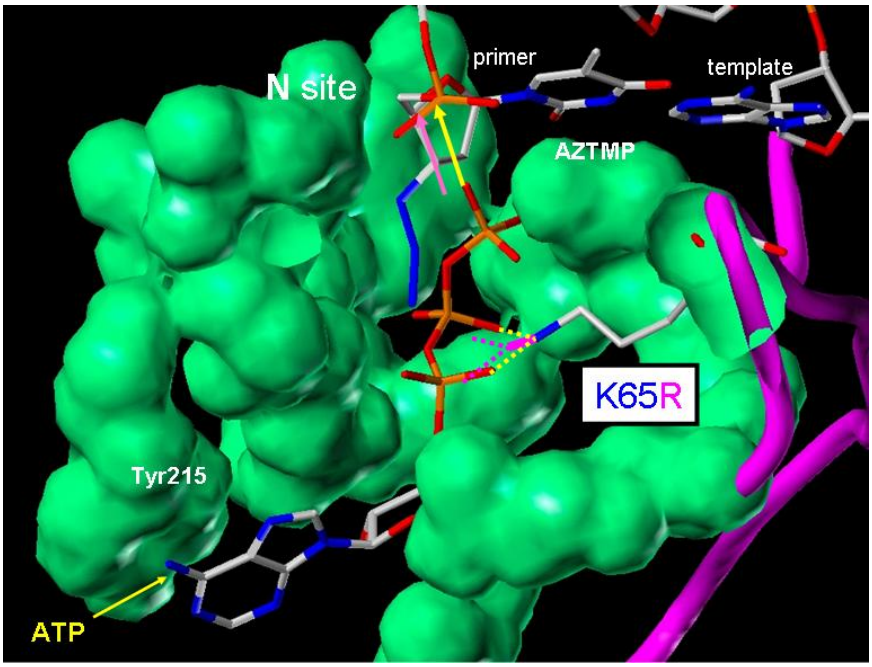


Figure 24. The Larger Side Chain of R65 May Alter ATP Interaction with AZT-MP to Decrease Excision

(A) Model drawn using ChemDraw and RasMol. (B) Adapted from model courtesy of Stephan Sarafianos, Rutgers University, based on Pdb access number 1RTD (86, 170) .

Interestingly, our data show that excision reactions carried out by HIV-1 RT containing K65R also produce much less final product than those reactions carried out by wildtype or TAM RT (i.e. the burst amplitude is decreased; Figure 12). Table 13 indicates the % final product formed by each enzyme. Although RT^{WT}, RT^{TAM41} and RT^{TAM67} extend 81-91% available primer by 55 minutes, $RT^{TAM41/65R}$ and $RT^{TAM67/65R}$ produce only 59-70% final product, and RT^{65R} produces only 35% final product. The slowest-step (or rate-limiting) step of both the DNA polymerization and excision reactions is RT-T/P dissociation (92). The excision rate of RT containing K65R may be decreased similar to the rate of dissociation of the T/P complex from the enzyme. Specifically, if the rate of excision is too slow, RT^{65R} may dissociate from the P/T before the excision reaction can occur. Experimental data from further biochemical and structural studies are needed to confirm these hypotheses.

The second goal of this study was to determine how TAMs antagonize resistance to tenofovir by K65R. Of note, in viral susceptibility assays, only the TAM67 pathway reversed K65R resistance to tenofovir, whereas the TAM41 pathway had no effect (Chapter 2). Using pre-steady state kinetic analysis, we confirmed the work of Deval and colleagues that K65R discriminates against TNF-DP by decreasing the rate of incorporation without affecting the NRTI binding interaction (44). TAM67 antagonizes K65R by partially restoring incorporation of TNF-DP. TAM41 antagonizes K65R to a lesser degree than TAM67 by decreasing selectivity for dATP vs. TNF-DP. Figure 21 clearly shows that the maximum rate of TNF-DP incorporation is similar for K65R and TAM41/65R, but partially restored for TAM67/65R. These data probably explain why phenotypic antagonism to K65R is seen with the TAM67 pathway but not the TAM41 pathway. The full-length polymerization results support these

findings. Full-length product generation by RT^{65R} and $RT^{TAM41/65R}$ occurred with equivalent efficiency, while full-length product generation by $RT^{TAM67/65R}$ was reduced. This also indicates decreased discrimination of TNV-DP by the TAM67 pattern.

Using a structural representation based on coordinates from Tuske and others (198), we hypothesize that the three mutations K65R, D67N and K70R which are in proximity in the fingers subdomain of RT, may alter the orientation of the β3-β4 hairpin loop to partially restore incorporation (k_{pol}) of TNV-DP, thus antagonizing the selectivity of K65R. M41L and L210W are more distant from the polymerase active site and as a result would not be expected to affect catalysis. Minor decreases in TNV-DP binding for both pathways of TAMs may be due to the structural changes in RT induced by the T215F/Y mutation.

Finally, our results explain the wildtype susceptibility to AZT by K65R observed in HIV-1 susceptibility assays (Table 8, Chapter 2). Prior steady-state studies by White and others have reported that K65R evades NRTIs by a combination of both decreased incorporation and decreased excision (206). Using pre-steady state analysis, we confirmed that K65R discriminates AZT due to a minor decrease in binding, but primarily by a decrease in catalytic rate of AZT-TP incorporation. This is consistent with the mechanism of discrimination K65R employs for other NRTI-TP including TNF-DP (44). Excision and rescue analysis demonstrated that excision activity of RT^{65R} is only half that of RT^{WT}. The combined effect of multiple polymerizations and excisions was noted in the full-length polymerization assays. RT^{65R} had reduced full-length product generation compared to RT with TAMs due to decreased excision; however, RT^{65R} had greater full-length product generation compared to RT^{WT} due to increased discrimination.

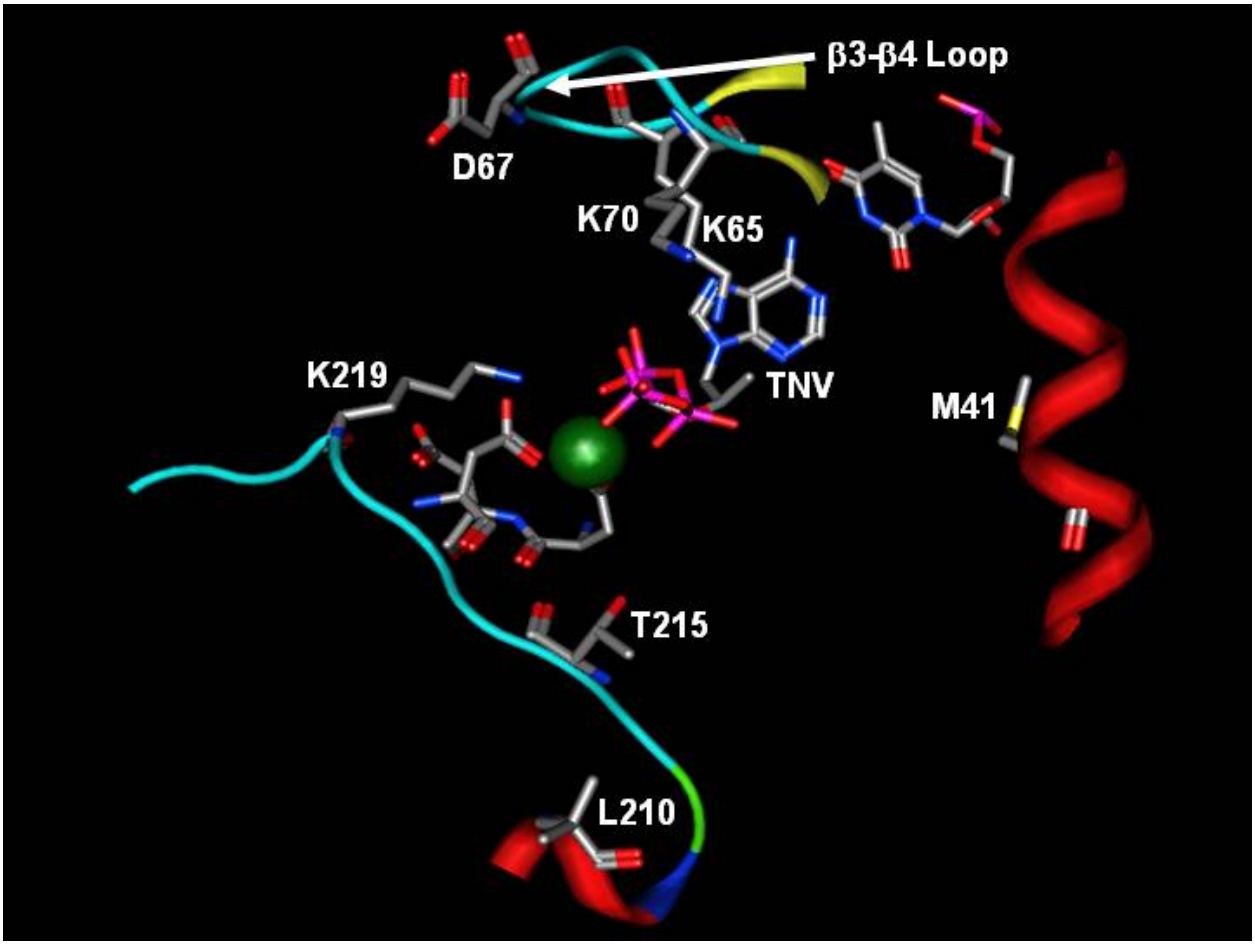


Figure 25. Structural Representation of Reverse Transcriptase
Three mutations in close proximity in the fingers subdomain of RT (D67N, K70R and K65R) may alter the orientation of the β3-β4 hairpin loop and restore incorporation (kpol) of TNF-DP, thus antagonizing discrimination by K65R. Structure drawn using MOE, based on coordinates from Tuske et al. Nat Struct Mol Biol, 2004: Pdb access number: 1T05.

In conclusion, TAMs confer resistance to AZT via a primer unblocking mechanism, and we demonstrate that this effect of TAMs is reversed by K65R. Conversely, K65R confers resistance to all known NRTIs via a discrimination mechanism, and TAMs reverse this effect by restoring catalytic rate, as demonstrated by pre-steady state analysis of TNV-DP incorporation. Taken together, these results indicate that there is no advantage for HIV-1 to have both K65R and TAMs on the same genome. In support of this, several clinical studies have reported that K65R and TAMs are rarely selected in the same patient (175, 209, 210). These kinetic data further support the model that K65R and TAMs have mutually antagonistic resistance mechanisms that can be exploited to optimize NRTI therapy.

6. CHAPTER FOUR. ANTAGONISM BETWEEN THE HIV-1 REVERSE TRANSCRIPTASE MUTATIONS K65R AND T215F/Y AT THE GENOMIC LEVEL

6.1. Preface

This study was presented in part as an oral abstract at the 12th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, February 2005 (Parikh, U, D. Barnas, C. Bixby, H. Faruki and J. Mellors. Abstract 98. K65R and T215Y Are Not Present on the Same Viral Genome in Plasma Samples with Both Mutations Detected by Population Sequencing). The work presented in this chapter is in partial fulfillment of dissertation Aim 3.

6.2. Abstract

The lysine (K) to arginine (R) change at codon 65 in HIV-1 RT (K65R) is a multi-nucleoside reverse transcriptase inhibitor (NRTI) resistance mutation, reducing susceptibility to all FDA-approved NRTI except zidovudine (AZT). Resistance to AZT is mediated by combinations of thymidine analog mutations (TAMs), which include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E/N. Of the TAMs, those at codon 215 are critical for ATP-mediated excision of AZT and other NRTI. Prior virologic and biochemical studies have shown marked phenotypic antagonism between K65R and TAMs in site-directed laboratory mutants. We hypothesized, based on this antagonism, that K65R and T215Y/F with 2 or more other TAMs would not be selected on the same viral genome. We therefore searched the 2003 and 2004 LabCorp database (n = 59,262) for the frequency of K65R in combination with 3 or more TAMs as determined by population sequencing. K65R and multiple TAMs were rarely detected (<0.1%) in the same plasma sample by population sequencing. Those samples with both K65R and 3 or more TAMs (n = 21) were analyzed by single genome sequencing. K65R was never found on the same genome with T215F/Y and 2 or more other TAMs, except in the presence of the Q151M complex. These findings demonstrate at the genomic level the antagonism and mutual exclusivity of the K65R and the T215Y/F pathways of NRTI resistance.

6.3. Introduction

HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs) occurs through two major mechanisms: selective decrease in NRTI incorporation (i.e. NRTI discrimination) primarily mediated by the K65R, L74V, or M184V mutations in reverse transcriptase (RT), or selective increase in NRTI excision mediated by "thymidine analog mutations" (TAMs), which include combinations of M41L, D67N, K70R, L210W, T215F/Y, and K219Q (20, 34, 120, 188, 209). TAMs function to increase ATP-mediated removal of the incorporated analog through phosphorolysis, resulting in primer unblocking and continued polymerization (20, 130). Of the TAMs, T215F/Y is critical for high-level AZT resistance (95) and is believed to be the most important TAM for the excision phenotype (53, 132, 214).

The K65R mutation in HIV-1 RT causes decreased susceptibility to all FDA-approved NRTIs except AZT. We have shown that K65R reverses the phenotypic effects of TAMs by decreasing ATP-catalyzed phosphorolysis (Chapter 3) (17, 69, 153). The antagonism between K65R and TAMs is bi-directional: TAMs also reverse K65R-mediated resistance to abacavir and tenofovir. Additionally, kinetic analyses of RT show that TAMs reduce the selectivity of K65R for natural dATP incorporation over tenofovir diphosphate (TNV-DP) incorporation.

Recent clinical observations support the hypothesis that K65R and TAM-mediated resistance are antagonistic and that there is counter-selection of these mutations. In a genotypic analysis of over 60,000 samples submitted for clinical testing, K65R was negatively associated with specific TAMs, including M41L, D67N, L210W and T215F/Y (Chapter 2). Others have also reported a negative association of K65R and TAMs in genotype databases, particularly with T215F/Y (153, 175, 200). This led to the suggestion that the use of thymidine analogs may

prevent the emergence of K65R (204, 210). Recent data from clinical trials support this suggestion. In studies of triple NRTI regimen that excluded AZT, 24-92% patients failing therapy had virus with K65R (58, 88, 97, 104, 137, 167). By contrast, only 1 of 90 failures in trials of 3-4 NRTIs that included AZT had virus with K65R (46, 76). The one failure with K65R was in a patient who received once daily AZT. Other clinical and *in vitro* studies have reported that the selection of K65R and TAMs is generally mutually exclusive (2, 105, 121, 192, 204).

These observations suggest that the existence of K65R and TAMs on the same genome is unfavorable for HIV-1. Yet, in a small number of patients, K65R and TAMs have been detected in plasma samples by standard genotype analysis (also referred to as population or bulk genotype). Whether these mutations exist on the same or different HIV-1 genomes can not be determined by population sequencing. We therefore used a new single genome sequencing (SGS) method (148) to determine whether or not K65R and TAMs including T215F/Y are linked on the same genome. Compared to standard genotype analysis which only provides a composite of mutations present in the population of patient virus, SGS enables a more detailed analysis of the composition of HIV-1 populations by sequencing DNA from many single viral genomes in a plasma sample, and allows analysis of linkage of mutations (148).

In our study, we searched the LabCorp database to determine the frequency of patient isolates that had both 65R and TAMs as determined by standard genotyping, and used SGS to determine whether K65R and TAMs, in particular T215F or Y, occurred on the same genome. These studies provide valuable insight on the emergence and evolution of NRTI resistance to aid in the appropriate design of combination therapy for the treatment of HIV-1 infection.

6.4. Materials and Methods

6.4.1. Plasma Samples

Samples analyzed consisted of 59,262 anonymized specimens submitted to Laboratory Corporation of America (LabCorp) for HIV-1 genotyping over a 2 year period from January 2003 through December 2004. Samples were predominantly from community-based clinical specimens, although they did include a small number of clinical trial specimens (658 samples). None of the plasma samples analyzed by single genome sequencing were from clinical trials. Patient treatment history was not available for analysis. HIV-1 RNA testing and standard genotyping was performed by Laboratory Corporation of America. Samples were considered for single genome sequencing if standard genotyping identified K65R and 3 or more TAMs (including or excluding T215F/Y), or K65R with T215F/Y alone or with any number of other TAMs. Samples were selected randomly and based on availability from the subset of patients who were infected with virus that met these criteria. Testing of samples was approved by the Institutional Review Board of the University of Pittsburgh.

6.4.2. Database Queries

The LabCorp genotype database was initially searched for the number of specimens with (1) K65R; or (2) one or more TAMs. Group 2 (one or more TAMs) was further divided into subsets as follows: (2a) samples with 1 or 2 TAMs; (2b) samples with 3 or more TAMs; (2c) samples with T215F/Y only or with 1 other TAM; or (2d) samples with T215F/Y with 2 or more other TAMs. For these searches, TAMs were defined as any of the following mutations unless otherwise specified: M41L, D67N, K70R, L210W, T215F, T215Y, or K219Q. In addition to the simple searches described above, combination queries were conducted to determine the

number of samples that fit 2 or more search criteria combined with the Boolean operator "AND". Two such searches were conducted.

The first combination search queried the association of K65R with TAMs. Query criteria were: (3) samples with K65R AND (3a) no TAMs; (3b) 1 or 2 TAMs; or (3c) 3 or more TAMs. In addition, the same query was conducted to identify samples with (4) K65R AND T215F/Y AND (4a) 0-1 other TAM; or (4b) 2 or more other TAMs. The second combination search queried the presence of TAMs without K65R. Query criteria were: (5) samples WITHOUT K65R that have (5a) 1 or 2 TAMs; or (5b) 3 or more TAMs. In addition, queries were conducted to assess the frequency of T215F/Y specifically with the following criteria: (6) samples WITH T215F/Y BUT NOT K65R AND (6a) 0-1 other TAM; or (6b) with 2 or more other TAMs.

6.4.3. Standard Genotype

HIV-1 RNA was pelleted by ultracentrifugation from 0.5-1.0 ml plasma. RNA was extracted from the pellet using the QIAamp viral RNA kit (Qiagen, Chatsworth, CA). The viral RNA equivalent of 50-100 µl of plasma was reverse transcribed and PCR amplified. A 1.7 kb fragment was then sequenced, including the entire protease gene and the 5' coding region of the RT region through codon 399. PCR products were sequenced in both directions using fluorescent dye-labeled sequencing terminators (ABI Prism BigDye Terminators, Applied Biosystems, Foster City, CA) followed by capillary electrophoresis on an ABI 3700 (Applied Biosystems). Sequence assembly, alignment and analysis was performed using Sequencher Software (Genecodes, Ann Arbor, MI) with comparison to HIV-1 reference strain HXB2. Manual examination of the electropherogram was used to confirm the sequence calls made by the software. Resistance associated mutations (International Aids Society-USA drug resistance

mutations) (217) were captured in the GenoSure database (LabCorp, Research Triangle Park, NC) database.

6.4.4. Single Genome Sequencing

To obtain sequences from individual cDNAs, the single genome sequencing (SGS) method was used as described in Palmer *et al*. (148). Briefly, HIV-1 RNA was extracted from plasma samples by centrifugation, treatment of the virion pellet with proteinase K, guanidinium isothiocyanate and glycogen, precipitation with 100% isopropanol, and washing with 70% ethanol. The purified RNA was resuspended in Tris-HCl, and then combined with deoxynucleotide triphosphates, and random hexamers (Promega, Madison, WI). After denaturation, cDNA was synthesized by reverse transcribing viral RNA using the following components: $MgCl_2$, RNase Out (Invitrogen, Carlsbad, CA), dithiothreitol, 1X RT Buffer (Invitrogen) and Superscript II RT (Invitrogen). cDNA was serially diluted, and PCR amplified. By Poisson distribution, if 30% of the PCR reactions are positive, it is expected that the amplification product arose from a single cDNA molecule in 4 out of 5 instances. The positive PCR reactions were detected using an E-gel system (Invitrogen), and sequenced using ABI Prism BigDye Terminator version 3.1 dideoxyterminator cycle sequencing (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencher software (Gene Codes Corp., Ann Arbor, MI) and those that contained mixtures at any position were excluded from the dataset. Resistance mutations from analyzed sequences were identified using the Stanford HIV Drug Resistance Database algorithm (178).

6.4.5. Statistical Analysis

Chi-square analysis was performed using GraphPad software (GraphPad Software, Inc., San Diego, CA).

6.5. Results

6.5.1. Frequency of K65R and TAMs by Standard Genotyping

To determine whether K65R and TAMs are negatively associated, we first searched a large clinical database (n = 59,262) to examine the frequency of plasma samples having both K65R and TAMs as detected by standard genotyping. In our query, the following mutations were included as TAMs: M41L, D67N, K70R, L210W, T215F, T215Y, or K219Q. The majority of plasma samples in the database (98.9%) were clinical samples randomly submitted for resistance testing in 2003-2004; 1.1% of samples were from clinical trials. Although these clinical trial samples were included in the database queries, they were not used for single genome sequencing analysis. Of the 59,262 samples, 3.2% had K65R and 31.9% of samples had one or more TAMs (Table 16), which is consistent with analyses of other databases (153, 175, 204, 210). More specifically, 14.8% of samples had 1 or 2 TAMs, and 17.1% had 3 or more TAMs. Further, 7.8% had T215F/Y alone or with 1 other TAM, and 14.2% had T215F/Y with 2 or more other TAMs (Table 16).

Using probability theory, the expected number of samples with K65R and 1 or 2 TAMs was similar to the actual number in this database (280 expected vs. 305 actual). This difference was not statistically significant, suggesting the lack of significant mutational interaction between

K65R and 1 or 2 TAMs. However, K65R was present in samples with 3 or more TAMs significantly less frequently than expected (322 expected vs. 52 actual, $p<0.001$). Additionally, significantly fewer samples than expected (73 vs. 416; $p<0.001$) had both K65R and T215F/Y with any number of other TAMs (Table 16). This strong negative association between K65R and TAMs that include T215Y/F is consistent with previous virologic and biochemical studies that demonstrate bi-directional phenotypic antagonism between these mutations (Chapter 2, Chapter 3). This led us to hypothesize that even though the K65R and T215F/Y mutations can be rarely detected in plasma samples by standard genotyping, they will not be encoded on the same genome. We therefore performed single genome sequencing on plasma samples from the three subsets that had significantly lower frequencies of K65R and TAMs than expected: samples with K65R, T215F/Y and 2 or more TAMs, samples with K65R, T215F/Y with 0-1 other TAM, and samples with K65R and 3 or more TAMs excluding T215F/Y (Table 16).

Table 16. Frequency and Association of K65R, T215F/Y and other TAMs in the LabCorp Database

Database Subset	Frequency		*P*[b]
	Actual Number (%)	Expected Number (%)[a]	
Total Number of Samples	59,262	NA[c]	NA
Samples with 65R	1,881 (3.2%)	NA	NA
Samples with any number of TAMs	18,889 (31.9%)	NA	NA
Samples with 1 or 2 TAMs	8,752 (14.8%)	NA	NA
Samples with 215F/Y only or with 1 TAM	4,597 (7.8%)	NA	NA
Samples with 3 or more TAMs	10,137 (17.1%)	NA	NA
Samples with 215F/Y and 2 or more TAMs	8,411 (14.2%)	NA	NA
Samples with 65R			
AND 1 or 2 TAMs	305 (0.51%)	280 (0.47%)	0.072
AND 3 or more TAMs	52 (0.088%)	322 (0.54%)	<0.0001
Samples with 65R AND 215F/Y			
AND 0-1 TAM	49 (0.083%)	147 (0.24%)	<0.0001
AND 2 or more TAMs	24 (0.040%)	269 (0.45%)	<0.0001

a The expected number was calculated using probability theory, in which the actual frequency of two subsets were multiplied to obtain the expected frequency. The expected frequency was multiplied by sample size to get the expected number.

b Statistical significance was determined using Chi-Square analysis.

c NA = not applicable

6.5.2. Single Genome Sequences from Plasma Samples with K65R, T215F/Y and 2 or more TAMs

From the LabCorp database, 24 samples had K65R, T215F/Y and 2 or more other TAMs as determined by population sequencing (Table 16), and ten of these samples were available for single genome sequencing (SGS). Antiviral treatment history was not available for the patient samples. We sequenced 19-22 genomes from each plasma sample, except for two samples which had low HIV-1 RNA (5-11 genomes sequenced) and one which was difficult to amplify (2 genomes sequenced).

Sequences obtained from single genomes were individually analyzed using Sequencher software, and mutations that differ from consensus clade B sequence were identified using the Stanford database algorithm (178). Table 17 shows the HIV-1 RNA (copies/ml) in the plasma samples, the NRTI mutations identified by standard genotyping, and the proportion of single genomes with the K65R mutation and/or TAMs. Other mutations known to be associated with NRTI resistance, as reviewed by the IAS-USA (90) are also shown for single genomes.

The main goal of this analysis was to determine whether K65R and T215F/Y occurred on the same genome. From this data, no genomes (0 of 161 genomes) had K65R, T215F/Y and 2 or more TAMs. We observed two circumstances in which K65R and T215F were on the same genome: (1) in the presence of Q151M and at least 2 other mutations comprising the 151-multi-drug resistance complex (22 of 161 genomes), or when T215F/Y was present with only one other TAM (5 of 161 genomes). All five of these genomes had K65R with M41L/T215Y on the same genome, and were from the same patient plasma sample (Table 17).

Table 17. Genotypes from Samples with K65R and T215F/Y with 2 or More Other TAMs

Patient Plasma Sample	Viral Load (c/ml)	Standard Sequencing	Single Genome Sequencing			
		NRTI Mutations	Genomes/ Total Genomes	NRTI Mutations[a]		
				65	TAMs	Other NRTI Mutations[b]
A1	74,115	41L, 65R, 70R, 210W, 215Y	2/20	-	-	-
			1/20	65R	-	**151M**
			9/20	-	*67G*, 210W, 215Y	-
			7/20	-	41L, *67G*, 210W, 215Y	-
			1/20	-	41L, *67G*, 70R, 215Y	-
A2	20,123	41L, 65R, 210W, 215Y	4/21	-	-	-
			1/21	65R	-	-
			1/21	-	*215S*	-
			4/21	-	215Y	-
			1/21	-	215Y, *219R*	-
			5/21	-	210W, 215Y	-
			2/21	-	210W, 215Y	118I
			1/21	-	41L, 44D, 215Y, *219R*	-
			1/21	-	41L, 67N, 210W, 215Y	74V, 118I
			1/21	-	41L, 44D, 67N, 210W, 215Y	74V, 118I, 184V
A3	271,335	41L, 62V, 65R, 67N, 75I, 116Y, 151M, 184V, 210W, 215Y	2/22	65R	41L, 67N, 210W, *215D*	**75I, 116Y, 151M**
			1/22	65R	41L, 67N, 210W, *215D*	**62V, 75I, 116Y, *151W***
			5/22	65R	41L, 67N, 210W, *215D*	**62V, 75I, 116Y, 151M**
			1/22	-	210W, 215Y	184V
			1/22	-	41L, 215Y	184V
			3/22	-	41L, 67N, 215Y	-
			1/22	-	41L, 215Y	-
			1/22	-	41L, 67N, 210W, *215D*	**116Y, 151M**
			2/22	-	41L, 67N, 210W, *215D*	**75I, 116Y, 151M**
			4/22	-	41L, 67N, 210W, 215Y	**62V, 75I, 116Y, 151M**
			1/22	-	41L, 67N, 210W, 215Y	**62V, 75I, 116Y, 151M**, 184V
A4	768	41L, 65R, 67N, 69D/N, 70R, 184V, 210W, 215F, 219Q	1/5	-	70R	-
			1/5	-	*67E*, 70R	-
			1/5	-	67N, 70R, 219Q	-
			1/5	-	*67K*, 70R, 215Y	*69A*
			1/5	-	41L, 67N, 70R, *215S*, 219Q	*69A*, 184V
A5	3,260	41L, 65R, 184V, 210W, 215Y	1/20	-	41L	184V
			15/20	-	41L, 215Y	184V
			1/20	-	41L, *44K*, 215Y	184V
			3/20	-	41L, 210W, 215Y	184V
A6	18,893	41L, 65R, 67N, 75I, 115F, 116Y, 151M, 210W, 215Y, 219E	1/21	65R	41L, 67N, 210W, 219E	**75I,** 115F, **116Y, 151M**
			1/21	65R	41L, 67N, 210W, 215Y, 219Q	**75I, 116Y, 151M**
			5/21	65R	41L, 67N, 210W, 215Y, 219E	**75I, 116Y, 151M**
			4/21	65R	41L, 67N, 210W, 215Y, 219E	**75I,** 115F, **116Y, 151M**
			1/21	65R	41L, 67N, 210W, 215Y, 219E	*69A*, **75I, 116Y, 151M**
			9/21	65R	41L, 67N, 210W, 215Y, 219E	*69A*, **75I,** 115F, **116Y, 151M**
A7	1,434	41L, 65R, 210W, 215Y	5/20	65R	-	-
			1/20	-	*70E*	-
			1/20	-	210W, 215Y	-
			13/20	-	41L, 210W, 215Y	-
A8	1,043	41L, 65R, 67N, 70R, 215Y, 219Q	1/11	65R	41L	-
			5/11	65R	41L, 215Y	-
			2/11	-	41L, 215Y	-
			1/11	-	41L, 215Y	184V
			1/11	-	41L, 67N, 70R, 215Y, 219Q	184V
			1/11	-	*41I*, 67N, 70R, 215Y. 219Q	184I
A9	3,747	41L, 65R, 67N, 70R, 215Y, 219E	1/19	65R	-	***75L***
			6/19	-	41L, 67N, 70R, 215Y, 219E	-
			9/19	-	41L, 67N, 70R, 215Y, 219E	184V
			1/19	-	41L, 67N, 70R, 215Y, *219R*	184V
			1/19	-	41L, 67N, 70R, *215I*, 219E	184V
			1/19	-	41L, *44K*, 67N, 70R, 215Y, 219E	184V
A10	129,948	41L, 65R, 67N, 69D, 70R, 75I, 116Y, 118I, 151M, 215F, 219Q	1/2	65R	41L, 67N, 70R, 215F, 219Q	69D, **75I, 116Y,** 118I, **151M**
			1/2	65R	41L, 67N, 70R, 215F, 219Q	69D, **75I, 116Y,** 118I, ***151I***

a Non-standard amino acid changes at codons known to be associated with NRTI resistance are noted in *italics* while

b Mutations associated with the multi-NRTI resistance 151M complex are noted in **bold**.

Unusual codons or polymorphisms were detected in each of the ten plasma samples, including mutations that may be TAM revertants. At TAM positions, these changes include the following: M41I, E44K, D67E/G/K, K70E, T215D/I/S and K219R.

6.5.3. Single Genome Sequences from Plasma Samples with K65R and T215F/Y with 0-1 Other TAMs

To further explore whether K65R and T215F/Y can occur on the same genome in samples with fewer than 2 TAMs, we performed single genome sequencing of 8 plasma samples that had K65R and T215F/Y alone or with one other TAM. In these plasma samples, K65R was never on the same genome as T215F/Y and 1 other TAM (0 of 98 genomes). In one plasma sample, K65R occurred on the same genome as T215F alone (Sample B2, 6/25 genomes), and in one plasma sample, K65R occurred on the same genome as T215Y alone (Sample B7, 8/12 genomes) (Table 18, Table 20). These samples also included unusual amino acid changes at TAM positions in 4 of 8 samples, including K70T, T215S/V, and K219R/T.

Table 18. Genotypes from Samples with K65R, T215F/Y and less than 2 Other TAMs

Patient Plasma Sample	Viral Load (c/ml)	Standard Sequencing	Single Genome Sequencing			
		NRTI Mutations	Genomes/ Total Genomes	NRTI Mutations[a]		
				65	TAMs	Other NRTI Mutations[b]
B1	287,903	41L, 65R, 74V, 215F	2/14	-	-	-
			5/14	65R	-	-
			3/14	-	215Y	-
			4/14	-	41L, 215Y	-
B2	7,232	65R, 74V, 215F	4/25	65R	*215V*	-
			12/25	65R	*215S*	-
			6/25	65R	215F	-
			3/25	-	215F	74V
B3	24,664	65R, 215F	14/18	65R	-	-
			2/18	-	215F	-
			1/18	-	41L, 215F	-
			1/18	-	*219R*	-
B4	62,931	65R, 184V, 215F	5/5	-	215F	184V
B5	6,471	65R, 184V, 215Y	6/7	65R	-	184V
			1/7	-	*215S*	184V
B6	421,600	65R, 74V, 215Y	8/12	65R	-	-
			1/12	-	-	74V
			1/12	-	215Y	74V
			2/12	-	215Y	-
B7	26,374	65R, 115F, 215Y	3/12	65R	215Y	*74I*, 115F
			5/12	65R	215Y	115F
			1/12	65R	*70T*, 215Y	115F
			3/12	65R	215Y, *219T*	115F
B8	1,229,600	65R, 74I, 184V, 215Y	1/5	-	215F	*74I*, 184V
			3/5	-	215Y	184V
			1/5	-	215Y	*74I*, 184V

a Non-standard amino acid changes at codons known to be associated with NRTI resistance are noted in *italics*.

6.5.4. Single Genome Sequences from Plasma Samples with K65R and 3 or More TAMs without T215F/Y

Finally, we performed SGS analysis of three plasma samples with K65R and 3 or more TAMs other than T215Y/F detected by standard genotyping. Of the 62 genomes analyzed, K65R occurred with 4 TAMs (M41L/D67N/K70R/219E) in 3 of 30 genomes from one sample (C1). Although these genomes did not have Q151M, all three genomes had V75I, which is associated with the Q151M-complex. K65R occurred with 3 TAMs in 1 of 9 genomes from sample C2 (67N/70R/219Q) and K65R occurred with 2 TAMs (K70R/K219Q) in 4 of 17 genomes from sample C3 (Table 19). Although these samples did not have T215F/Y, 40 of 62 genomes had T215I (Table 19).

We also used this subset to explore the association of K65R with other TAMs. K65R was infrequently detected on the same genome as other TAMs, being on the same genome with D67N in 4 of 44 genomes, with K70R in 8 of 58 genomes, and with K219Q/E in 8 of 62 genomes (Table 19). The frequency of M41L was low (only 5 single genomes) in this sample set.

Table 19. Genotypes from Samples with K65R and 3 or More TAMs without T215F/Y

Patient Plasma Sample	Viral Load (c/ml)	Standard Sequencing	Single Genome Sequencing			
		NRTI Mutations	Genomes/ Total Genomes	NRTI Mutations[a]		
				65	TAMs	Other NRTI Mutations[b]
C1	481,972	41L, 62V, 65R, 67N, 70R, 75I, 219E	3/34	65R	41L, 67N, 70R, 219E	**75I**
			1/34	-	41L, 67N, 70R, 219E	**75I**
			1/34	-	41L, 67N, 70R, *215I*, 219E	**62V, 75I**
			1/34	-	67N, 70R, 219E	**75I**
			3/34	-	67N, 70R, 219E	**62V, 75I**
			25/34	-	67N, 70R, *215I*, 219E	**62V, 75I**
C2	2,815	65R, 67N, 70R, 184V, 219Q	1/9	65R	67N, 70R, 219Q	184V
			1/9	-	67N, 70R, 219Q	184V
			7/9	-	67N, 70R, 219Q	74V, 184V
C3	23,523	65R, 67N, 69D/N/S, 70R, 184V, 219Q	3/19	65R	70R, 219Q	69S
			1/19	65R	70R, 219Q	69S, 184V
			1/19	-	67N, 70R, 219Q	69S
			2/19	-	*67H*, 70R, *215I*, 219Q	69D
			1/19	-	70R, *215I*, 219Q	69N
			10/19	-	67N, 70R, *215I*, 219Q	69D
			1/19	-	67N, 70R, *215I*, 219Q	69N

a Non-standard amino acid changes at codons known to be associated with NRTI resistance are noted in *italics*.

b Mutations associated with the multi-NRTI resistance 151M complex are noted in **bold**.

Table 20. Summary of Standard Genotype versus Single Genome Sequence

Standard Genotype Category[a]	N	Single Genome Sequences		
		3 or more TAMs with 65R	1 or 2 TAMs with 65R	No TAMs, 65R Only
65R, 215F/Y and ≥ 2 TAMs	95	**0% (0/41)**	14% (6/42)[b]	54% (7/13)
65R, 215F/Y and ≥ 2 TAMs **and Q151M**	59	62% (31/50)[c]	0% (0/12)	33% (1/3)
65R, 215F/Y with 0- 1 other TAMs	98	-	41 % (18/44)[d]	91% (49/54)
65R + ≥ 3 TAMs, without 215F/Y	62	7.3 % (4/55)	57 % (4/7)	-

a All sample sets exclude plasma samples with 151M or 151M-complex associated mutations unless otherwise noted.
b All 6 genomes were from the same plasma sample. Five genomes had 65R + 41L/215Y and one genome had 65R + 41L.
c Of 28 single genomes, 8 did not have 215F/Y.
d All 18 genomes had 65R and 215F/Y with no other TAMs.

6.6. Discussion

The high rates of recombination between HIV quasispecies within an infected individual (134, 220) should result in linkage between K65R and T215F/Y with other TAMs even in the absence of negative selection. Based on the actual number of specimens with K65R (1,881 specimens) and with 215F/Y with 2 or more TAMs (8,411 specimens) from our database of 59,262 specimens, we expected to have 269 specimens with both K65R and 215F/Y with 2 or more TAMs, as calculated by Chi Square analysis (Table 16). However, the number of plasma samples that had both K65R and T215F/Y with 2 or more TAMs as determined by standard sequencing was significantly lower than expected (24 specimens, $p < 0.001$). Additionally, from the 321 total genomes tested from 21 patient plasma samples, K65R was never found on the same genome as T215F/Y with 2 or more TAMs (except when Q151M was present). Strong functional antagonism between these mutations is the most likely explanation for this mutual exclusivity between K65R and T215F/Y.

Evidence for antagonism between K65R and TAMs has previously been demonstrated virologically (Chapter 2) and biochemically (Chapter 3). Recombinant HIV-1 with K65R and two different combinations of TAMs (one combination included T215F and a second combination included T215Y) resulted in reversal of AZT resistance, and reversal or significant decrease in tenofovir and abacavir resistance conferred by K65R (153). Further, kinetic analysis showed that K65R diminishes the increased excision activity of TAMs, and TAMs antagonize the selectivity for dNTP over NRTI-TP incorporation of K65R (154). The current study strongly corroborates these *in vitro* findings by showing that linkage between K65R and TAMs on the same genome rarely occurs.

We found only two circumstances in which linkage between K65R and T215F/Y occurred: when Q151M and several of its associated mutations of the multi-NRTI resistance complex were also present, and when T215F/Y was present with 0-1 other TAMs. To illustrate, of the 43 single genomes that had both K65R and T215F/Y, 20 genomes also had Q151M and at least 2 other mutations from the Q151M multi-NRTI resistance complex (A62V, V75I, V77L, and/or F116Y). Q151M confers low-level multi-NRTI resistance, while the other mutations in the complex contribute to the phenotype and help retain replication capacity (116, 149, 185). The primary mutation in this complex, Q151M, decreases the rate of incorporation of NRTIs, but has been found to have minimal, if any, excision activity (43). Its co-existence on the same genome with K65R and multiple TAMs suggests that the Q151M complex is a viral escape mechanism required for NRTI-resistance in the context of the bi-directionally antagonistic K65R and TAM pathways (Figure 26). The evolution and drug selection leading to this complex genotype are not defined.

Of the 23 single genomes with 65R and T215F/Y in the absence of the Q151M multi-NRTI complex, all had T215F/Y alone or with only 1 other TAM. 6 genomes from 1 patient had K65R/T215F, 12 genomes from 1 patient had K65R/T215Y, and 5 genomes from 1 patient had K65R/M41L/T215Y. The reason for the requirement of at least 3 TAMs for antagonism between K65R and T215F/Y is not defined. One explanation may be that the antagonism of K65R is not significant with less than 3 TAMs or without T215F/Y. This may also explain why other TAMs were present in low frequency on the same genome as K65R (Table 19). Alternatively, few TAMs suggest that there was not continued selection for high-level excision of AZT and other NRTIs (e.g. AZT therapy was stopped). A study by Meyer and others showed that ATP-dependent removal of AZT-MP is least efficient with T215Y alone, and increases in

excision efficiency correlates with the presence of additional TAMs (132). *In vitro* phenotyping studies from our lab (Mellors et al, unpublished) corroborated these findings.

Because therapy information is not available for the patients from whom the plasma samples were obtained, it is not possible to know in which order mutations appeared. The presence of mutations such as T215D/I suggest that that the TAM pathway is reverting, possibly from the cessation of AZT therapy. The presence of Q151M and varying numbers of secondary mutations suggest that this pathway of resistance is developing. Retrospective samples were not available for this study, but it would be valuable to know how mutational patterns evolved in patients with K65R, TAMs and Q151M, or in patients with unusual changes at TAM codons.

Several unusual codon changes at TAM positions were detected in single genomes, many of which have been previously reported, including M41I, E44K, D67E/G/H/K, K70E/T, T215D/I/S/V and K219R/T. These mutations are rare, with only 3-59 instances reported in a database of HIV-1 sequences from over 7000 persons (162). Some mutants at TAM positions appear to cause resistance to other NRTIs, including D67G to (+) and (-) enantiomers of dOTC (2'-deoxy-3'-oxa-4'-thio-5-fluorocytidine) (164) and K70E to PMEA or tenofovir (32, 41, 136). K219R has also been reported in patients; however, its contribution to drug resistance is not clear (168, 194). Several changes at 215 were also seen, including T215D/I mutants, which are likely to be revertants from T215F/Y (59, 218).

In conclusion, we propose a model describing the pathways of NRTI resistance (Figure 26). HIV-1 evolves resistance to ddI, ddC, d4T, 3TC, abacavir, tenofovir, and FTC via a pathway of discrimination, primarily mediated by K65R, L74V or M184V. Of these three mutations, K65R is of greatest concern, because it can reduce HIV-1 susceptibility to almost all investigational and FDA-approved NRTIs tested to date except those with an azido group

(Chapter 1) (151). Alternatively, HIV-1 evolves resistance to AZT or d4T and cross-resistance to other NRTIs via a pathway of excision mediated by TAMs, with T215F/Y as a key mutation. The K65R and TAM pathways are bi-directionally antagonistic, and counter-selected in patients. Simultaneous therapy of AZT and other NRTIs may prevent the emergence of K65R. Sequential therapy of discontinued AZT followed by non-thymidine analog NRTIs may lead to genotypic profiles that may serve as an intermediate for Q151M emergence.

The implications of this study are that there is a high genetic barrier to combinations containing AZT + NRTIs that select K65R. Thus, 3-4 NRTI regimens containing AZT should be studied. Some of these trials are ongoing, including the DART study, with tenofovir + combivir (145) and the TIMS study with tenofovir and trizivir. It should be remembered that when designing therapy, factors other than genetic barrier are important for regimen efficacy and must be considered. These include drug exposure, safety/tolerability and antiretroviral potency.

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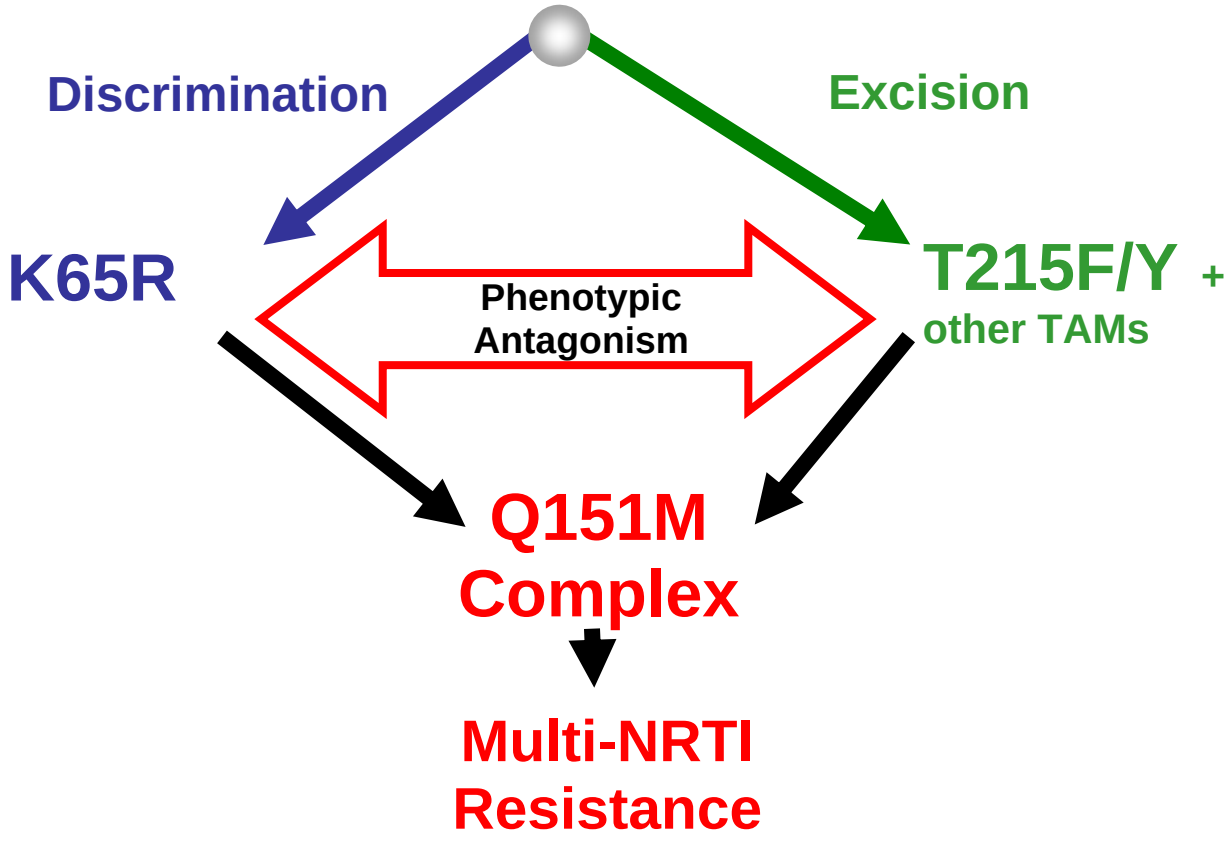
Figure 26. Model for Two Pathways of NRTI Resistance Involving K65R and TAMs



7. SUMMARY AND FINAL DISCUSSION

At the time this study began, the K65R substitution in HIV-1 RT was well recognized as a drug resistance mutation selected *in vitro*, but with little known clinical relevance because of its rarity *in vivo*. K65R confers resistance by a mechanism of discrimination, or decreased incorporation of dideoxynucleoside-triphosphates (ddNTP), in favor of the natural nucleoside triphosphates (dNTP) (86). It remained unexplained, however, why a potentially important multi-NRTI resistance-causing mutation that required only a relatively simple one base transition to evolve had such low frequency in patients (<1%).

Shortly before beginning this study, the mechanism by which TAMs cause AZT resistance had been elucidated to be one of primer unblocking catalyzed by a pyrophosphorolytic donor such as inorganic pyrophosphate (PPi) or adenosine triphosphate (ATP) (6, 131). This mechanism of resistance by TAMs was different than the mechanism of resistance by K65R. In addition, data from our laboratory indicated that K65R reversed resistance to AZT caused by TAMs (17), but the mechanism responsible for this reversal was not known. The hypothesis emerged that K65R and TAMs are distinct pathways of resistance evolution for HIV-1 that are bi-directionally antagonistic.

The current project aimed to: (1) characterize the phenotype of antagonism by K65R and TAMs through viral susceptibility studies; (2) explain the biochemical basis for the mechanism of antagonism through steady state and pre-steady state kinetic analysis; and (3) determine whether the bi-directional model held true *in vivo* by analyzing whether K65R and TAMs occurred on the same viral genome *in vivo.*

7.1. Preliminary Studies: K65R is a Multi-NRTI Resistance Mutation of Clinical Relevance

Prior work both from our lab and by others had shown that K65R is readily selected by HIV-1 passaged in the presence of D-nucleoside analog RT inhibitors (17, 54, 66, 67, 163, 202, 219). To gain a better understanding of resistance by K65R, we investigated the relationship between NRTI structure and activity against HIV-1_{65R}.

7.1.1. K65R Causes Resistance and Cross-Resistance to Multiple NRTIs

Our first goal was to systematically characterize NRTI resistance conferred by K65R. This involved testing a broad panel of NRTIs that differed by pseudosugar structure, base component, and stereochemistry against recombinant HIV-1 with and without K65R. We found that K65R causes decreased susceptibility to all NRTIs except AZT and AZA (the adenine analog equivalent of AZT). Pseudosugar structure had the greatest influence on activity, followed by base component. Stereochemistry did not affect activity against the K65R mutant (Chapter 1). These data showed that K65R is a multi-nucleoside resistance mutation.

Interestingly, the only structures that retained activity against HIV-1 with K65R were those with a 3'azido group in the pseudosugar component, specifically AZT and AZA. However, biochemical analysis demonstrated that RT with K65R preferentially incorporates TTP over AZT-TP. Compared to wildtype RT, K65R RT had a decreased catalytic rate of incorporation of AZT-TP (Chapter 3). This finding was not consistent with viral susceptibility studies showing that HIV-1 with K65R was equally sensitive to AZT as wildtype virus. This seeming paradox required explanation.

In ATP-catalyzed primer unblocking experiments, K65R was found to have a 50% decrease in ability to excise AZT-MP from a terminated primer/template compared to wildtype RT (Chapter 3). As previously suggested by White *et al*. (206), the wild-type susceptibility of HIV-1_{65R} to AZT seen in viral phenotypic assays is likely due to the combined effect of increased discrimination but decreased excision.

7.1.2. K65R is Clinically Relevant and Increasingly Observed *in Vivo*

Although K65R has potential as a multi-NRTI resistance mutation, several arguments have been made against its clinical relevance. First, K65R does not confer high-level resistance to most NRTIs and thus resistance may be overcome by high concentrations of NRTI-5'-triphosphate (NRTI-TP). Second, K65R has been shown to have decreased replication capacity compared to wild-type HIV-1. Recent studies using recombinant mutants or patient-derived virus have reported decreases in replication efficiency ranging from 10% to 58% that of wild-type (44, 205, 207). Because of decreased fitness and retention of partial drug susceptibility of viral mutants with K65R, it has been suggested that patients with K65R may continue to benefit from tenofovir or other NRTI therapy (135, 205).

Despite these arguments, several counterpoints suggest that K65R is important. For instance, the level of resistance to tenofovir or abacavir from K65R is modest compared to the high levels of resistance to 3TC or FTC from M184V, or with AZT by TAMs; however, any decrease in drug potency may be clinically significant. Second, patients on long-term therapy may experience greater toxicity if increased doses are needed to maintain efficacy. This is particularly relevant for K65R because it causes broad NRTI resistance. Third, the selection of K65R may pave the way for the selection of NRTI mutations associated with greater multi-drug

resistance, such as the Q151M complex. Several studies, including the present study, have shown that Q151M is frequently associated with K65R (Chapter 2) (3, 42, 200).

To gain further evidence for the clinical significance of K65R, we used a large clinical database (Virco) of 62,222 patient isolates to examine changes in the frequency of K65R *in vivo.* The prevalence of K65R was low in 1998 (0.4%) but steadily increased each year, to 3.6% by the end of 2003. Since the presentation of this work, others have observed similar increases in the frequency of K65R in patients (21, 91, 200, 210). The prevalence of K65R was similar (3.2%) in another large clinical database (LabCorp) we analyzed that contained 59,262 patient-derived isolates collected between January 2003 and December 2004 (Chapter 4). The increase in the frequency of K65R may be due to the use of tenofovir therapy, which was FDA-approved in 2001. K65R decreases incorporation of all NRTIs by a general discrimination mechanism, which may partially explain why tenofovir selects K65R *in vivo.* In addition, tenofovir has an incomplete ring in its pseudosugar component, resulting in an overall smaller molecular structure and greater flexibility compared to other NRTIs. This structure may facilitate transition of the primer to the P-site, resulting in the formation of a dead-end-complex in the presence of the next incoming dNTP (198). The poor excision of tenofovir-monophosphate may explain why tenofovir does not select for TAMs. Increased use of tenofovir without including AZT in the NRTI combination may have contributed to the rise in K65R prevalence in patients.

In keeping with this hypothesis, we found that the prevalence of individual TAMs in the Virco database decreased from 1998 to 2003. Further, we found that K65R was negatively associated with specific TAMs, including M41L, D67N, L210W, T215F/Y and K219Q/E. Others

have corroborated these findings (175, 200, 204). These data provided the first clinical observation of antagonism between K65R and TAMs (Chapter 2).

7.2. Mechanism of Antagonism between K65R and TAMs

The present study provides virologic, biochemical, and clinical evidence that supports the model of bi-directional antagonism between K65R and TAMs. Consistent with our hypothesis, we show that K65R and TAMs: (1) confer different mechanisms of resistance (discrimination vs. excision); (2) restore susceptibility to some NRTIs when both are present on the same genome; and (3) are not selected in the same viral genome *in vivo*. The following briefly summarizes our findings:

7.2.1. Clinical Observation of Antagonism between K65R and TAMs

In addition to the decrease in prevalence of TAMs and negative association of TAMs with K65R mentioned above, recent clinical trials supported the negative interaction of K65R and TAMs. A review of the literature found that K65R was selected in 24 to 92% of patients failing therapy on trials of 2 to 3 NRTIs that did not include AZT. In contrast, only 1 out of 90 patients failed with K65R in trials that did include AZT. Of note, the one failure was on a regimen in which AZT was only administered once daily (Table 5). These observations suggested that the selective environment in the pre-tenofovir era (most regimens included AZT) may explain the infrequent selection of K65R *in vivo.* In addition, the pre-existence of TAMs in patient virus may also prevent the selection of

K65R, particularly if the selective pressure for TAMs was maintained by AZT or other NRTIs to which TAMs confer cross-resistance.

7.2.2. Virologic (Phenotypic) Evidence of Antagonism between K65R and TAMs

We generated recombinant viral mutants genetically engineered to contain K65R with two different clinically relevant pathways of TAMs. These studies showed that K65R in the context of different TAMs reversed AZT resistance, which extends prior findings (17). Additionally, TAMs reversed resistance by K65R to abacavir and tenofovir. This was the first report of the bi-directional nature of antagonism between K65R and TAMs (Chapter 2).

7.2.3. Biochemical Evidence of Antagonism between K65R and TAMs

We generated purified recombinant reverse transcriptase enzyme with the same RT genes tested in viral susceptibility assays to determine the biochemical basis of the phenotypic antagonism. We found that K65R reverses the excision activity of TAMs to levels 50% below that of wild-type RT. Conversely, we found that the TAM67 pathway partially restores the diminished catalytic rate of NRTI-TP incorporation (AZT-TP and TNF-DP) of RT with K65R. A novel finding was that antagonism of K65R by TAMs depended on the combination of TAMs. TAM67 antagonized K65R by restoring incorporation of TNF-DP, while TAM41 antagonized K65R to a lesser degree than TAM67 by decreasing selectivity for dATP versus TNF-DP (Chapter 3).

7.2.4. Clinical Evidence of Counter-Selection of K65R and TAMs

To further investigate the mutual antagonism between K65R and TAMs, we examined single genomes from clinical isolates found to have both K65R and TAMs as determined by population sequencing to assess if the antagonism was sufficient to prevent both mutational pathways from occurring on the same genome. An epidemiological survey showed that samples with both K65R and TAMs detected by standard genotype analysis were rare. Only 24 samples from 59,262 patient isolates had K65R and T215F/Y with 2 or more other TAMs. Of these, we examined 19-22 single genomes each from 7 patient samples and up to 11 genomes each from 3 patient isolates with low plasma HIV-1 RNA or inefficient amplification (161 total genomes). We found that none of the single genome sequences had K65R and T215F/Y with 2 or more other TAMs except in the presence of the Q151M multi-NRTI resistance complex (Chapter 4).

Together, these data support our hypothesis that K65R and TAMs are mutually exclusive pathways of resistance that are counter-selected in patients. This work made an important contribution to the understanding of HIV-1 drug resistance. Through a comprehensive virologic, biochemical and genetic analysis, we explained the mechanism of antagonism between two clinically relevant pathways of drug resistance.

7.3. Future Directions

7.3.1. Structural Studies

A better structural understanding of RT is needed. Specific to this work, K65R was found to antagonize excision activity of TAMs. From structural models of RT, we may speculate that Arg65 alters the interaction with the α and β phosphates of ATP, preventing the proper alignment needed for nucleophilic attack of the phosphodiester bond between the penultimate nucleotide and the terminal AZT-MP. Analysis of a crystal structure of a mutant RT containing K65R and TAMs bound to an AZT-terminated primer/template and ATP would help elucidate the interactions occurring with each of these molecules. In addition, TAMs were found to antagonize discrimination by K65R. The presence of multiple mutations in the fingers subdomain such as K65R, D67N and K70R, may modify RT structure such that discrimination by K65R is antagonized. Due to these close proximity of mutations, a greater effect may be seen with the TAM67 pattern rather than the TAM41 pattern. Analysis of a crystal structure of RT with K65R and TAMs bound to a primer/template and incoming dNTP or NRTI-TP would provide insight into how TAMs antagonize K65R discrimination function.

7.3.2. Multi-NRTI Resistance Escape Pathways

Single genome sequencing analysis may aid in furthering the understanding of virus evolution. In one patient, we found that 18/19 single genomes analyzed contained K65R, 4-5 TAMs, Q151M and at least two other mutations associated with the Q151M complex (including V75L and F116Y). Two genomes also had T69A. Although Q151M may be an effective route to

escape inhibition by all NRTIs, there is a high genetic barrier to evolving a virus with 9 or more NRTI resistance mutations. This unusual observation leads to several considerations:

a. The possibility of selecting such a highly mutated species seems rare, and it would be very useful to determine what combinations of NRTIs and what sequence of NRTIs were prescribed to this patient. Detailed analysis of the ordered appearance of mutations including briefly appearing intermediates would advance the understanding of resistance evolution.
b. Our single genome sequencing analysis identified several mutations associated with Q151M development, or with reversion of TAMs. In particular, determining viral susceptibility to NRTIs by mutants containing combinations of TAM reversion mutations, K65R, and/or Q151M pathway intermediates may provide insight into the influence of such mutants on resistance to therapy in patients.

Overall, continuation of three major pursuits in the field of HIV drug resistance is suggested: (1) We showed antagonism between two major pathways of NRTI resistance (K65R and TAMs) but we also found that HIV-1 does have an escape route to evade this antagonism through the Q151M complex. Although the frequency of Q151M is currently low (~1%), lessons from the K65R story should encourage continued study of mutants having Q151M. (2) A better structural understanding of how mutations in RT affect its function is needed to provide insight for the design of better inhibitors of RT. Finally, (3) NRTIs are an essential component of combination therapy; however, the development of other viral targets is essential. Current progress has been

made in the development of entry inhibitors, RNAse H inhibitors and fusion inhibitors among others (for a recent review see Barbaro *et al*.) (14), but further study is necessary.

In conclusion, a better understanding of HIV evolution, the mechanisms by which mutations in HIV cause resistance, and interactions among mutations will enable us to prolong the health and life of those infected. This work has had significant public health impact, by establishing K65R as a multi-drug resistance mutation that should be prevented in patients, by providing biochemical, virologic and genetic explanations for how AZT prevents emergence of K65R, and by influencing the design of ongoing clinical trials of new NRTI combinations, which include the DART study (145), TIMS study (144), and ACTG 5231.

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