STUDIES ON THE BASIS OF HIV-1 VARIATION AND ITS PURPOSEFUL INCREASE

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DEDICATION

To my late grandmother Joanne Zylka, whose battle with cancer inspired my curious nature and passion for discovery.

To my mother, for her patient devotion and contagious display of happiness.

ABSTRACT

Unconventional measures are needed in an effort to outpace the HIV/AIDS pandemic. Even with access to the six classes of FDA-approved antiretrovirals, adverse drug effects and evolution of drug resistance still pose obstacles, and represent issues that will inevitably arise in the developing world. Along with the absence of a proven vaccine strategy, these shortcomings necessitate the continual search for new therapeutic targets. One such novel approach is to target HIV-1's low copying fidelity with a deliberate increase in viral mutational load using exogenous small molecule mutagens. However, inasmuch as this concept of viral *lethal mutagenesis* has gained notoriety, it is also essential to understand fundamental enzymatic components (i.e., reverse transcriptase) that contribute to variation. The goal of this dissertation was to advance basic models to better understand the causes and consequences of HIV-1 variation.

The components that influence variation within an HIV-1 population structure are critical to predict the emergence and direction of viral evolution (e.g., drug resistance). For these studies, the relationship between viral fitness and mutation rate was investigated. A panel of 10 reverse transcriptase mutants – most having drug resistance phenotypes – were analyzed for their effects based on these two biological properties. Mutation rate differences were measured using single-cycle vector assays, while fitness differences were identified using *ex vivo* head-to-head competition assays. As anticipated, viral mutants possessing either higher or lower mutation rate had a corresponding loss in fitness. These observations provided the first description of an interrelationship between HIV-1 fitness and mutation rate and support the conclusion that mutator and antimutator phenotypes correlate with reduced viral fitness.

A second focus of this dissertation was directed at studying novel mechanisms by which viral mutagens diminish HIV-1 infectivity. I have detailed the antiviral mechanisms of the ribonucleoside analog 5-azacytidine. It was demonstrated that the primary antiviral activity of 5-azacytidine can be attributed to its effect on the early phase of HIV-1 replication mediated by reverse transcriptase. Furthermore, the antiviral activity was associated with an increase in the frequency of viral mutants. Sequencing analysis showed enrichment in guanosine-to-cytidine (G-to-C) transversion mutations. These results indicated that 5-azacytidine was incorporated into viral DNA following its 2'-OH reduction to 5-aza-2'-deoxycytidine. Incorporation into the viral DNA led to an increase in mutant frequency, which is consistent with lethal mutagenesis.

Lastly, studies were directed at understanding concomitant exposure of two unrelated mutagenic agents. Because the APOBEC3 proteins' restrictive nature associated with cytosine deamination, I wanted to investigate its interplay with the small molecule cytosine analog, 5-azacytosine. Reduced viral infectivity and increased viral mutagenesis were observed with both the viral mutagen 5-azacytosine (i.e., G-to-C mutations) and the host restriction factor APOBEC3G (i.e., guanosine-to-adenosine (G-to-A) mutations); however, when combined, they had complex interactions. Nucleotide sequence analysis revealed that concomitant HIV-1 exposure to both 5-azacytosine and APOBEC3G resulted in an increase in G-to-A viral mutagenesis at the expense of G-to-C mutagenesis. Also, APOBEC3G catalytic activity was required for the diminution in G-to-C mutagenesis. These findings provided the first demonstration for potentiation of the mutagenic effect of a cytosine analog by APOBEC3G expression, resulting in concomitant HIV-1 lethal mutagenesis.

In summary, the studies conducted in this dissertation 1) provide the first direct experimental evidence of an interrelationship between HIV-1 fitness and mutation rate, 2) demonstrate that the primary antiviral mechanism of 5-azacytidine can be attributed to its ability to increase the HIV-1 mutation frequency through viral DNA incorporation during reverse transcription, and 3) provide the first demonstration for potentiation of the mutagenic effect of a cytosine analog (i.e., 5-azacytosine) by APOBEC3G expression, resulting in concomitant HIV-1 lethal mutagenesis.

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2. **Dapp MJ**, Heineman, RH, Mansky LM. 2012. Interrelationship Between HIV-1 Fidelity and Mutation Rate. ***Journal of Molecular Biology*** [Article in press].

3. **Dapp MJ**, Holtz CM, Mansky LM. 2012. Concomitant Lethal Mutagenesis of Human Immunodeficiency Virus Type 1. ***Journal of Molecular Biology*** 419:158-70.

4. **Dapp, M.J**., Clouser, C.L., Patterson, S.E. and L.M. Mansky. 2009. 5-Azacytidine can Induce Lethal Mutagenesis in Human Immunodeficiency Virus Type 1. ***Journal of Virology*** 83:11950-58.

CHAPTER I

GENERAL INTRODUCTION

Portions reprinted with permission from: Dapp, M.J., Patterson, S.E., and Mansky, L.M. 2012. Back to the future: revisiting HIV-1 lethal mutagenesis. Trends in Microbiology [Article in press].

The HIV-1 Replication Cycle

Human immunodeficiency virus type 1 (HIV-1) was discovered as the causative agent of acquired immunodeficiency syndrome (AIDS) almost 30 years ago (Barre-Sinoussi *et al.* 1983; Gallo *et al.* 1983). Despite modern medicine and technology, HIV/AIDS continues to remain one of most deadly scourges in human history resulting in nearly 30 million deaths since its early 1980s outbreak (UNAIDS 2012). One reason for its success as a human pathogen is its tropism for a particular subset of immune cells. Direct and indirect killing of CD4+ T cells and macrophages cause dysregulation of the immune system, which enables a foothold for secondary infections and associated malignancies (Alimonti *et al.* 2003).

HIV-1 requires the CD4 cell surface marker as an entry receptor to initiate virus binding. Subsequent to attachment, a T cell (CXCR4) or macrophage (CCR5) co-receptor is required for viral envelope rearrangements to trigger virus-cell membrane fusion. This fusion event allows the membrane-bound virus particle to deposit its core into the cytoplasm of the infected cell. HIV-1 particles contain two copies of the RNA viral genome and all of the enzymatic proteins that enable the genome's conversion to double-stranded DNA for incorporation into cellular chromosome. Inherent to all retroviruses, HIV-1 employs the remarkable reverse transcriptase (RT) heterodimer (Fig. 1-1); an enzyme that not only redefined the central dogma of molecular biology, but paved the way for an entire biotechnology industry and a new era of cancer biology research (Baltimore 1970; Temin *et al.* 1970). The multifunctional enzyme is a RNA- and DNA-dependent DNA polymerase with ribonuclease activity of DNA-RNA hybrids (RNase H) and inherent strand melting abilities. RT is active during early infection events to convert (i.e.,

reverse transcribe) the positive-sense, single-stranded RNA genome into a double-stranded complementary DNA copy through an elegant process involving region- and strand-specific nucleotide removal and multiple template and enzyme "jumps" (Fig. 1-2). The final product is processed by viral integrase (IN), which first processes the 3'-ends of double-stranded viral DNA and then facilitates its integration into chromosomal DNA. The ~10kb integrated genome is defined as a provirus which is another unique feature among retroviruses. A provirus can be copied by the host cellular DNA replication machinery and, for the most part, gene expression machinery. This trait allows retroviruses to remain permanently established within a host, sometimes latent over long periods of time, as a continual reservoir for new virus production.

HIV-1 is a complex retrovirus with many accessory genes, some of which help to control virus production in a controlled manner. Briefly, the virus is able to temporally regulate expression of tandem-spliced accessory genes, single-spliced enzymatic and structural proteins, and unspliced full-length viral genome. This complexity is compounded by the intricate HIV-1 genomic structure which: 1) encodes multiple overlapping genes on all three open reading frames including multiple splice acceptor sites; 2) contains necessary secondary structure for efficient transcription, nuclear export, and genome packaging; and 3) maintains critical *cis* elements for translational frameshifting and proficient genome copying during reverse transcription processes (Fig. 1-3).

Yet, this relatively sophisticated framework of gene expression and processing provides the necessary means for infectious virus particle assembly and release. The process is generally described as a membrane assembly of full-length genomic RNA, within Gag structural proteins and Gag-associated polyproteins, which bud off as ~120 nm par-

ticles that contain processed and carbohydrate-decorated envelop proteins. Subsequent to particle egress, the retroviral protease (PR) homodimerizes to self-cleave from the Gag polyprotein thereby processing other enzymatic (i.e., RT and IN) and structural (i.e., nucleocapsid, capsid, matrix) proteins, by which the latter transform the immature particle into one that is fully infectious.

HIV-1 Diversity and the Mutation Rate

An important observation that has become fully appreciated in the absence of an effective vaccine, evident by dedicated HIV-patient sequence databases, is the tremendous diversity among HIV-1 isolates. Based on sequence alignments, there are two human primate lentivirus species, most likely derived from cross-species zoonotic reservoirs, denoted as HIV-1 and HIV type 2 (HIV-2). These retroviral species also show a distinct regional prevalence, host virulence patterns, and drug resistance evolution. Within HIV-1 there are four distinct groups (i.e. M: major, N: new, O: outlier, P: pending), each thought to occur through four independent zoonotic events from chimpanzees, and possibly, one species jump from gorilla. Still, HIV-1 sequences within group M (composed of roughly 90% of all HIV-1 sequences) can be designated into at least 9 different subtypes (i.e. A-D, F-H, J-K), among the subtype recombinant forms that exist.

What drives this diversity within HIV-1? Geneticists argue that inter-subtype and intra-subtype recombination can, and does quite frequently, spawn a high degree (roughly 10 crossovers per genome) of genetic diversity (Neher *et al.* 2010; Batorsky *et al.* 2011). However, biochemists and molecular biologists focus on RT as the main driver of HIV-1 mutation rate (Hahn *et al.* 1986; Preston *et al.* 1988; Roberts *et al.* 1988; Goodenow *et al.*

1989; Preston 1997). Initial RT fidelity studies performed using purified enzyme found an inordinately high mutation rate, even by RNA virus standards (Preston *et al.* 1988). However, these measurements were improved upon with the development of an *in vivo* forward mutation rate assay. The assay was designed to include all viral components necessary for a single round of replication, and the mutation rate was calculated as 3.4 x 10^{-5} mutations per basepair per generation (about 20-fold lower that previous *in vitro* studies and on par with the retroviral mutation rate average) (Mansky *et al.* 1995). Nonetheless, discoveries made in HIV-1 biology continue to unravel unforeseen mechanisms, pathways, and components that all may contribute to HIV-1's genetic diversity. For instance, the human apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3 (APOBEC3) proteins can generate site-specific cytosine-to-uracil (C-to-U) mutations on nascent minus-strand DNA during viral genome copying; these lesions appear as guanosine-to-adenosine (G-to-A) mutations within the plus strand of the proviral DNA (discussed below). Furthermore, HIV-1's viral protein R (Vpr) has a role in recruiting cellular uracil-DNA glycosylase (UNG) into viral particles, which is thought to enhance replication fidelity (Mansky *et al.* 2000; Chen *et al.* 2004). There is also evidence, albeit in cell culture based studies, that nucleoside analogs, antimetabolites, and even RT variants themselves, can influence the mutation rate of HIV-1 (Mansky *et al.* 2000; Mansky *et al.* 2002; Mansky 2003; Mansky *et al.* 2003; Chen *et al.* 2005). In fact, over the past decade, the Mansky laboratory has characterized the fidelity of many HIV-1 RT drug resistant variants using *in vivo* mutation rate assays (Mansky *et al.* 2000; Mansky *et al.* 2002; Mansky *et al.* 2003; Chen *et al.* 2005). Reported findings have found that mutation rates can vary by up to 4- fold that of wildtype virus in just a single replication cycle.

Reverse Transcriptase Drug Resistance: Mechanisms

Antiretroviral therapy has become the mainstay for HIV-infected individuals ever since the mid-90s renaissance with the implementation, by David Ho and colleagues, of combined drug approach known as highly-active antiretroviral therapy (HAART) (Markowitz *et al.* 1995; Perelson *et al.* 1996). Most patients with access to HAART are able to live to the same life expectancy as uninfected individuals (Antiretroviral_Therapy_Cohort_Collaboration 2008). However, even with access to the six classes of FDA-approved antiretrovirals, adverse drug effects and evolution of drug resistance provide the impetus to continually seek new therapeutic targets along with improved next-generation compounds.

Specifically focusing on RT as a drug target, there are two classes of clinically-approved antiretrovirals: the nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). The former are prodrugs that must be metabolized by cellular kinases to active triphosphate analogs; these compounds are chain terminators designed to be incorporated by RT during reverse transcription. Nucleotide polymerization is abruptly terminated upon NRTI incorporation because of the absence of a 3'-OH functional group. Meanwhile, the NNRTI drug class functions at a region adjacent to the enzyme active site. These compounds allosterically inhibit RT function by disrupting the natural flexibility required for polymerization. Interestingly, the range in HIV diversity is such that HIV-2 is naturally resistance to NNRTIs; the lack of susceptibility exists because of polymorphic sites within the HIV-2 RT drug binding pocket (Witvrouw *et al.* 1999).

Drug resistance studies continue to provide new insights into a drug's mechanism

of action as well as to help researchers gauge the barriers to resistance of a given drug. Currently, and strictly speaking for NRTIs, there are two well-founded drug resistance mechanisms [reviewed (Sarafianos *et al.* 2009; Menendez-Arias 2010)]. The first mechanism is increased discrimination of natural nucleotide triphosphates relative to the chain-terminating nucleotide analogs. Biochemical studies have revealed that RT is able to reconfigure residues surrounding the polymerase active site to selectively exclude unnatural nucleotides (Sarafianos *et al.* 1999; Selmi *et al.* 2001). Antiretrovirals such as lamivudine (3TC), emtricitabine (FTC), tenofovir (TDF), and abacavir (ABC) select for virus with this discrimination resistance phenotype, i.e., possessing one or more of the mutations: K65R, K70E, L74V, Y115F, M184V, or Q151M multi-drug resistance complex. The second NRTI resistance pathway is an excision mechanism that removes the chain-terminating nucleotide before an irreversible dead-end RT-primer complex is formed (Arion *et al.* 1998; Meyer *et al.* 1999). This mechanism is exclusive to thymidine analogs, such as zidovudine (AZT) and stavudine (d4T). Resistance stems from accumulation of thymidine-associated mutations (TAMs: M41L, D67N, K70R, L210W, T215Y/F, and K219E/Q), which surround the dNTP binding site. These mutations reconfigure this pocket to better accommodate a pyrophosphate donor molecule (most often ATP because of its intracellular concentrations). TAMs allow the ATP molecule to attack the primer strand's phosphodiester bond, thus reversing the chemistry of the most recent nucleotide, to efficiently excise the chain-terminating thymidine analog.

Reverse Transcriptase Drug Resistance: Viral Fitness

Fitness models, stemming from evolutionary biology, have been readily applied to

the field of virology (Morse 1994; Domingo E 2008). In essence, viral fitness is defined as the ability of a virus to replicate itself in a given environment (Domingo *et al.* 1997). A useful parameter in the field is relative fitness, which measures the abundance of a particular viral strain against a competing strain over a given amount of time, or number of generations (Holland *et al.* 1991). Traditional experimental approaches replicated the contending strains in separate, parallel cultures and then compared the number of infectious virus. However, more sophisticated molecular detection techniques have now allowed simultaneous co-culturing of the viral strains to enable direct head-to-head competition (Martinez-Picado *et al.* 2008).

By definition, wildtype virus is the most fit strain within a natural environment because it exists as the most predominant virus, albeit surrounded by a cloud of related variants (Fig. 1-4A). Once the environmental conditions are altered, through introduction of an inhibitor or new host species, the non-random process of natural selection is observed as clearance of unfit virus to give rise to variants (i.e., mutants) with superior replicative abilities. One realization of this process is that mutations are not generated in response to environmental changes; rather, variants must preexist within the population prior to the action of selective forces.

Viral Quasispecies and the Error Threshold

RNA viruses, such as HIV-1, have extended our understanding of viral population dynamics by paving the way for more advanced models and therapeutic approaches. These organisms are appreciated because of their robust population sizes, short generations times, and extreme mutation rates on the order of 1 per genome replication event

(Coffin 1995; Sanjuan *et al.* 2010). Each of these factors contributes to the tremendous variation within RNA viral populations; however, experimental evidence for this did not exist until seminal work by Esteban Domingo, Charles Weissmann and colleagues characterizing the population heterogeneity of the RNA phage Q-beta (Domingo *et al.* 1978). Coincidently around this time, Manfred Eigen was developing models to help understand the evolution of primordial macromolecules (Eigen 1971; Eigen M 1977). His original *hypercycle* model helped to describe the coupling of self-replicating units through a continual autocatalytic cycle (Eigen M 1977). Ultimately this framework was used to develop the quasispecies model in viruses (Novak 1992). This model is better suited than traditional population genetics to explain the evolutionary processes in highly mutable viruses. For instance, because of the high mutation rate and near infinite population size, selection occurs on the mutant ensemble rather than individual sequences (Fig. 1-4A). Mutation and selection are coupled so that the quasispecies evolves as a single cooperative unit (Eigen 1993; Bull *et al.* 2005). Although the quasispecies model is well-validated mathematically and *in silico*, its application to virus populations remains controversial (Holmes *et al.* 2002). Yet, many in the RNA virus field continue to use the model as a useful framework to describe these dynamic populations.

Another concept introduced by Eigen's quasispecies model is the existence of an error threshold (Fig. 1-4B) (Eigen 1993; Bull *et al.* 2007). Because of the aforementioned RNA virus characteristics, together with their constrained genome size and organization, there are limits to the population's mutational load (Elena and Sanjuan 2005). Quasispecies theory predicts that these highly mutagenic populations will exist in evolutionary neutral regions of a fitness landscape, located within "flat" and highly connected

sequence space (Sardanyes *et al.* 2008). These populations have greater mutational robustness, i.e., greater propensity to absorb mutations while maintaining a viable phenotype. At any rate, evidence with experimental chemical mutagens suggests that, even with a flat fitness peak, RNA virus populations exist very near the threshold for viral extinction. Error catastrophe, or more appropriately called extinction catastrophe, is an irreversible meltdown in the genetic material of a population through accumulated deleterious mutations (Fig. 1-4B) (Anderson JP 2004; Bull *et al.* 2005; Bull *et al.* 2007). This approach has been validated in numerous RNA viruses through use of purposeful mutagenesis, called lethal mutagenesis (Loeb *et al.* 1999; Smith RA 2005; Bull *et al.* 2007; Graci *et al.* 2008; Graci *et al.* 2008; Dapp *et al.* 2009).

Viral Lethal Mutagenesis

Effects of chemical mutagens in living systems has been the subject of investigation that dates back to the 1940s (Auerbach *et al.* 1947). Over this era, the DNA damaging potential of mutagens has become appreciated both as harmful carcinogenic agents as well as development into anti-tumor chemotherapeutics. The realization that small molecules could possess such properties helped to further establish the impetus for improved regulatory processes of compounds vying FDA approval. Pre-clinical tests include the Ames test that measures the mutagenic potential of a compound in bacteria (Ames *et al.* 1973), *in utero* studies in both rodent and non-rodent animal models to measure a chemical's teratogenic potential (Ito *et al.* 2011), and dose-escalation studies in animal models to assess chronic-compound exposure leading to carcinogenesis, among others (U.S._FDA 2012).

Conventional anti-cancer drug development was based solely on non-specific poisoning of rapidly-dividing cells which inherently causes numerous unpleasant side effects (Davis *et al.* 1964). Newer generation cancer chemotherapeutics are based on rational drug design in which selectively target aberrant cellular proteins or rogue cancer cell-surface markers, making these compounds more patient-tolerable (Sawyers 2004; Stratton 2011).

As therapeutics become more patient-specific and disease-specific, the era of broad-spectrum chemical mutagen therapy has been reinvigorated by the study of lethal mutagenesis with viruses. Provided a high enough mutational burden, an irreversible genetic meltdown of the entire population causes its elimination (Bull *et al.* 2007). Fortunately, the immense population size, genome structure, and adaptive requirements of most virus populations have positioned them very near the extinction threshold. Evidence for this first emerged in picornavirus studies where a mere 2-fold increase in viral mutation frequency was required to reduce viral titers by 99% (Holland *et al.* 1990). Lethal mutagenesis has since been observed in many other RNA virus systems, including: HIV-1 (Harris *et al.* 2005; Dapp *et al.* 2009), poliovirus (Crotty *et al.* 2000; Crotty *et al.* 2001), foot and mouth disease virus (FMDV) (Sierra *et al.* 2000), and lymphochoriomeningitis virus (LCMV) (Ruiz-Jarabo *et al.* 2003). Evidence in cell culture systems indicate that the clinically approved ribavirin regimen, used to treat HCV since 1998, elicits antiviral activity via lethal mutagenesis though this mechanism has not been fully established *in vivo* (Contreras *et al.* 2002; Cuevas *et al.* 2009). These early studies provided evidence for lethal mutagenesis, but less attention was focused on potential mechanisms. The early HIV-1 studies with 5-hydroxydeoxycytidine suggested the slow accumulation model,

whereby lethal mutagenesis occurred by a slow accumulation of mutations over multiple rounds of replication, ultimately causing virus extinction (Loeb *et al.* 1999). By analogy, many of the other lethal mutagenesis studies – even if not directly stating such a model – inferred this type of model for mutation accumulation leading to high mutational loads and eventual lethal mutagenesis.

Cell culture-based studies have shown promise for several viral systems, but the demonstration of lethal mutagenesis *in vivo* has been elusive. For example, phase II clinical trial results for KP-1461 a prodrug of KP-1212 (5,6-dihydro-5-aza-2'-deoxycytidine), a first-in-class viral mutagen for HIV-1, demonstrated there was perturbation of the mutation spectra, but no reduction in viral loads (Mullins *et al.* 2011). In particular, deep-sequence analysis of virus in these subjects revealed differences in the virus mutation spectra, but drug-exposed subjects showed no detectable differences in viral loads or CD4+ T cell counts, which are key prognosis markers for the level of HIV-1 infection. One question that arises from these studies is whether KP-1461 could be effective as a monotherapy (Mullins *et al.* 2011; Hicks *et al.* 2012), especially since the combined triple-therapy drug cocktail approach has been the standard for HIV-1 chemotherapy since the mid-1990s. Also, the clinically approved ribavirin regimen for treatment HCV is administered in combination with interferon (Brok *et al.* 2010). While monotherapy drug exposure typically leads to the rapid development of drug resistance, it is of note that there are no publications that document drug resistance to KP-1461.

In another cell culture study, a combination of two clinically-approved cytosine analogs, gemcitabine (2'-deoxy-2',2'-difluorocytidine) and decitabine (5-aza-2'-deoxycytidine, a cytosine analog similar to KP-1461), described a new experimental ap-

proach to extinction of HIV-1 infectivity (Clouser *et al.* 2010) and had *in vivo* antiretroviral activity (Clouser *et al.* 2011; Clouser *et al.* 2012). Future studies will need to address if the *in vivo* antiretroviral activity correlates with virus extinction. Viral mutagens may be more effective in causing virus extinction when used with standard non-mutagen antivirals. In tissue culture studies performed with FMDV (an RNA virus), viral populations were rapidly driven to extinction only when sequential treatment of inhibitor first, followed by viral mutagen (Perales *et al.* 2009). This was far superior to the reversed sequential treatment or the simultaneous combination of inhibitor and mutagen. Taken together, these data suggest that a clinical trial modeled after this sequential approach with KP-1461 would likely yield improved efficacy data.

Lethal mutagenesis can be induced not only by drugs, but also by the human APOBEC3 (A3) family of proteins (Harris *et al.* 2003; Lecossier *et al.* 2003; Mangeat *et al.* 2003; Mariani *et al.* 2003; Zhang *et al.* 2003; Bishop *et al.* 2004; Turelli *et al.* 2004; Sasada *et al.* 2005; Chen *et al.* 2006; Zennou *et al.* 2006). The mechanism by which A3 proteins, the prototype being A3G, hypermutate retroviral genomes has been well-established [reviewed in (Harris *et al.* 2004), (Malim *et al.* 2008), and (Chiu *et al.* 2008)]. Briefly, A3G is packaged into budding virions, after which the virion matures and binds to a target cell. In the target cell, A3G deaminates cytosines (C) present in the negative-sense viral DNA during reverse transcription process. The deamination of C to uracil (U) causes a pre-mutatgenic lesion. This site templates for adenine (A) during plus strand DNA synthesis rather than guanine (G). A3G's deamination of C in the minus strand DNA during reverse transcription generates G-to-A mutation signatures in the resulting plus-strand DNA of the provirus (Harris *et al.* 2003). However, its ability to mutate the

viral genome depends on how well it can overcome HIV-1's viral infectivity factor (Vif) countermeasure. In a host specific manner, Vif targets A3G proteins for proteosomal degradation. By saturating A3G levels or less-stringent Vif alleles, A3G proteins can gain access to the nascent virions and mutate the viral genome as described above. A deaminase-independent mechanism has been proposed for HIV-1, however, this model remains controversial (Shindo *et al.* 2003; Bishop *et al.* 2006; Schumacher *et al.* 2008; Browne *et al.* 2009).

Cell culture work convincingly shows that these mutagen-inducing proteins can dispose of viral genomes in short order, i.e., over 90% loss in viral infection within a single replication cycle. Because of A3G's processivity once it engages the viral genome, evidence suggests the mutagenesis is a discrete all-or-nothing process (Chelico *et al.* 2009; Armitage *et al.* 2012). However, some studies suggest that A3G can contribute to sublethal mutagenesis (Sadler *et al.* 2010), which include shaping the emergence of HIV-1 drug resistance (Mulder *et al.* 2008; Jern *et al.* 2009; Kim *et al.* 2010). Currently, the extent to which A3 proteins have contributed HIV-1 evolution remains controversial (Ebrahimi *et al.* 2011); although, it is clear that HIV-1-infected patient samples reveal that A3-signature mutations are actively found (Pace *et al.* 2006; Vazquez-Perez *et al.* 2009).

A3G and A3F remain at the center of much recent focus as powerful HIV-1 mutators [reviewed in (Albin *et al.* 2010) and (Rosenberg *et al.* 2007)]. Both A3G and A3F, along with A3B, possess the capacity to restrict other retroviral genera as well, including: the murine leukemia virus (MLV, gammaretrovirus) (Harris *et al.* 2003; Mangeat *et al.* 2003; Bishop *et al.* 2004), human T-lymphotropic virus 1 (HTLV-1, deltaretrovirus)

(Sasada *et al.* 2005), foamy viruses (spumaviruses) (Delebecque *et al.* 2006), as well as the equine infectious anemia virus (EIAV, lentivirus) (Mangeat *et al.* 2003). In addition to retroviruses, hepatitis B virus (HBV, a hepadnavirus), and adeno-associated virus (AAV, a parvovirus), are also susceptible to members of the A3 family (Turelli *et al.* 2004; Chen *et al.* 2006).

Dissertation Objectives

The objectives of this dissertation were two-fold. The first was to understand the *causes* of HIV-1 variation, while the second to determine the *consequences* of its purposeful increase. These objectives extend upon the initial observational experiments with hypothesis-driven methodology to reveal mechanistic conclusions.

Previous work in the Mansky laboratory has established methodologies for measuring retroviral mutation rates. This work has been extended to look at the fidelity of HIV-1 drug resistant variants within RT, which may have important clinical, and drug development, implications. Although the fidelity for NRTI drug resistant variants has been published on, the analysis has not been exhaustive. Therefore, Chapter I and Appendix I, provide *in vivo* mutant frequency measures for at total of 14 drug resistant RT variants (i.e., A62V, K65R, L74V, K103N, V108I, Y115A/F, F116T, V148I, Q163N, M184I/V, K65R/M184V, L74V/Y115F/M184V), 6 of which had never been analyzed using an *in vivo* assay.

Additional experiments were performed on these HIV-1 drug-resistant variants to better understand the relationship between viral mutation rate and fitness. Previous reports in the literature reasoned that organisms may evolve an optimal mutation rate

(Kimura 1967; Leigh 1970; Eshel 1973; Leigh 1973; Earl *et al.* 2004; Lynch 2010). Therefore Chapter I describes experiments performed to measure viral fitness of these RT mutants using sophisticated head-to-head competition assays. Increases or decreases in viral fidelity were hypothesized to directly correlate with a fitness loss. This work paves the way for more mechanistic approaches to quantify rates of reverse transcription as well as population-based sequencing that will flush out the fitness costs associated with increased and decreased fidelity, respectively.

Another approach to better understand causes of HIV-1 variation was through use of chemical and protein mutagens. What began as a blinded, small molecule screen of anti-cancer drugs against HIV-1, led to interesting virological and biochemical results for the ribonucleoside analog, 5-azacytidine (5-AZC, Fig. 1-5C). In fact, previous work had shown the antiretroviral and mutagenic potential of 5-AZC (Bouchard *et al.* 1990; Pathak *et al.* 1992); however, the work described herein (Chapter II) provides more thorough pharmacologic and mechanistic data for 5-AZC's mutagenic and antiretroviral abilities.

Additional assays were used to test 5-AZC's antiretroviral activity among distinct HIV-1 targets. Chapter II describes the bifunctional inhibitory action of 5-AZC and provides a model for its lethal mutagenesis-inducing behavior. Subsequent studies with 5-AZC sought to understand its role in concomitant mutagen exposure against HIV-1 (Chapter III). These pioneering studies utilized APOBEC3G to be combined with 5-AZC, each with distinct mechanisms and mutational patters, G-to-A and G-to-C, respectively. Results from this work showed an interesting phenomenon in which 5-AZC stimulated the hypermutagenic potential of APOBEC3G.

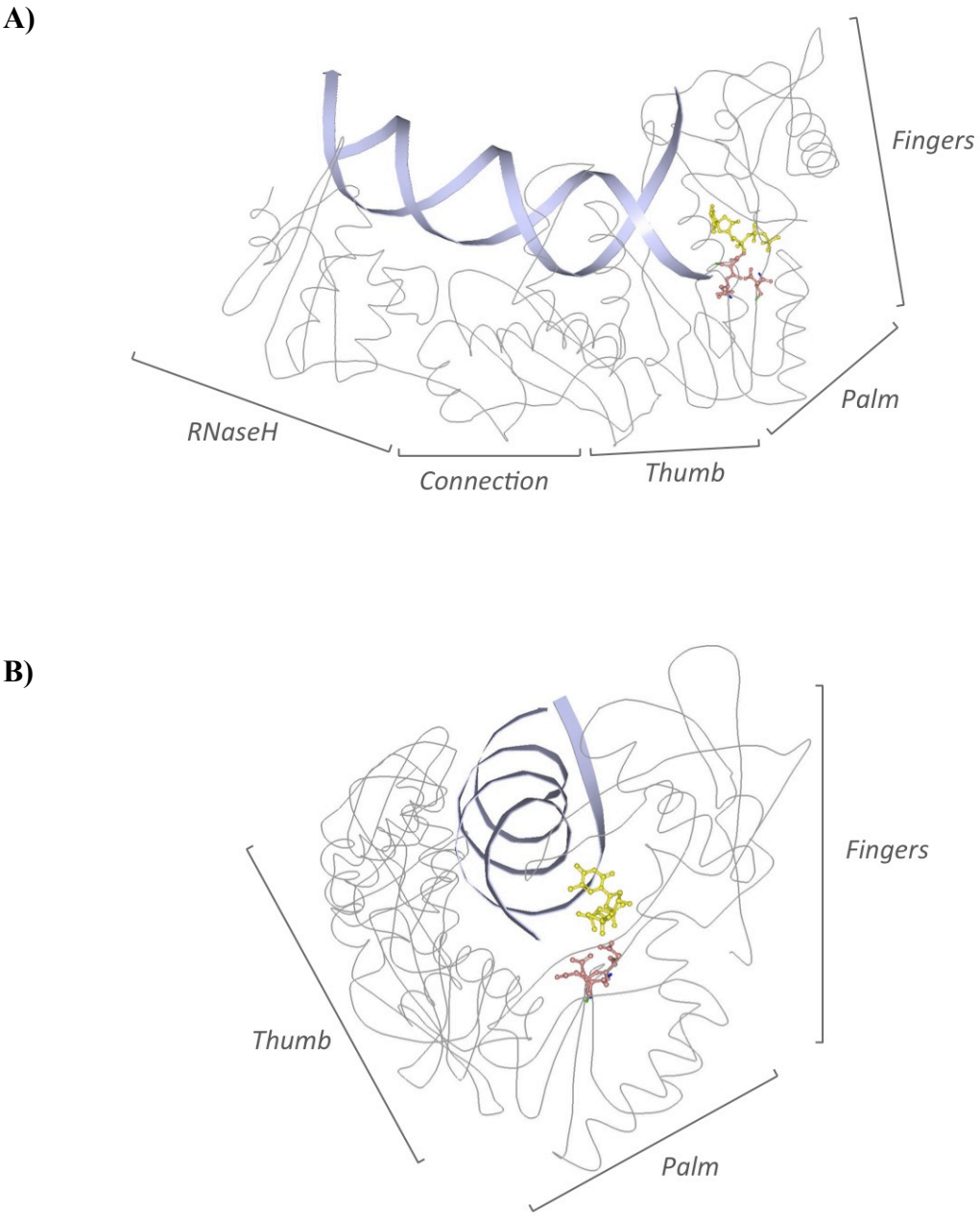


Figure 1-1. Ribbon diagram of the HIV-1 RT structure. The p66 catalytically active subunit possesses both the DNA polymerase activity and ribonuclease H activity. Represented by side-view (A) and head-on view (B) each of the subdomains is labeled: fingers, palm, thumb, connection, and RNase H. The DNA double helix primer:template is colored in blue; a dideoxynucleotide sits at the polymerase active site; the catalytic carboxylates D110, D185, D186 are colored red. [PDB ID: 1RTD; (Huang *et al.* 1998)].

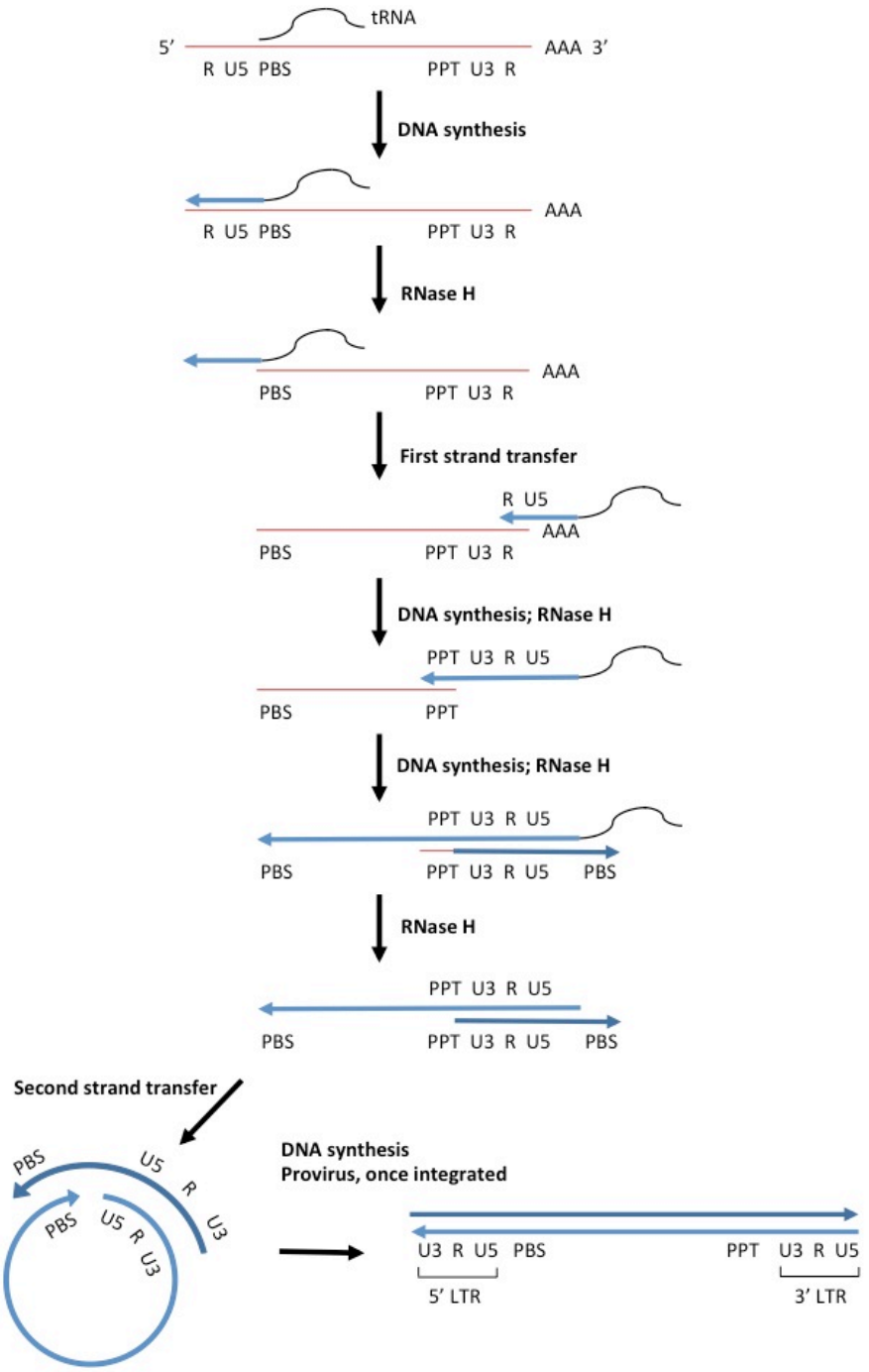


Figure 1-2. Retroviral reverse transcription of genomic viral RNA to double-stranded complementary DNA. Details are provides above in text. (*Red line*) RNA; (*blue lines*) DNA. tRNA = transfer RNA; R = repeat region; U5 = unique 5' region; U3 = unique 3' region; PPT = polypurine tract; AAA = poly adenosine (A) tail; LTR = long terminal repeat.

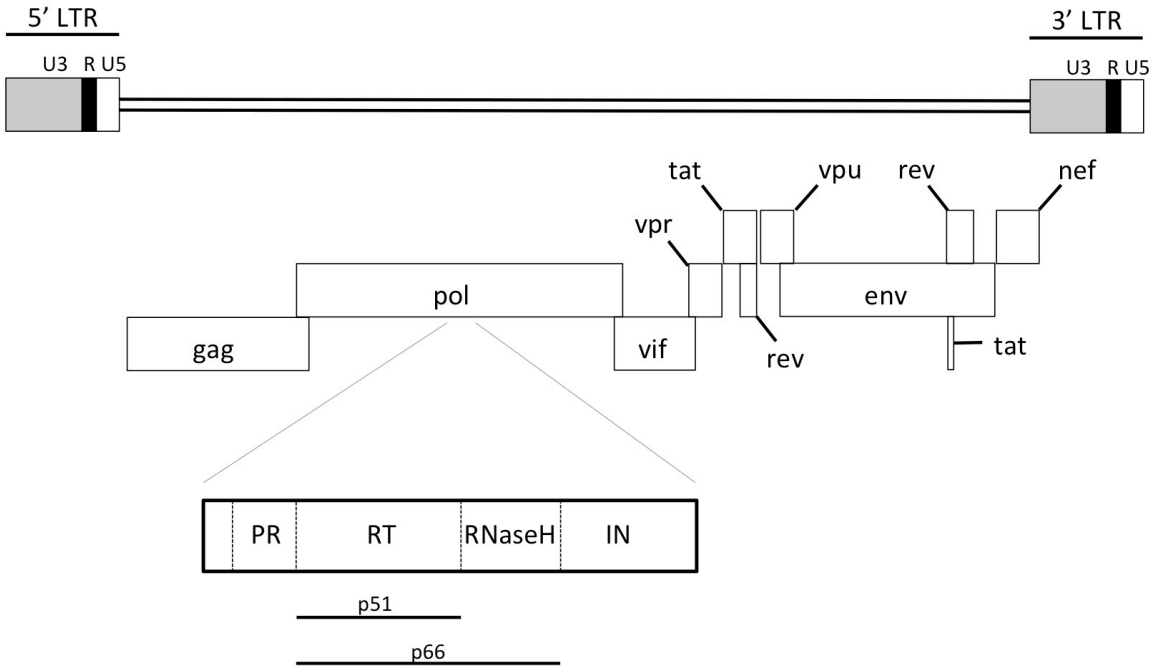
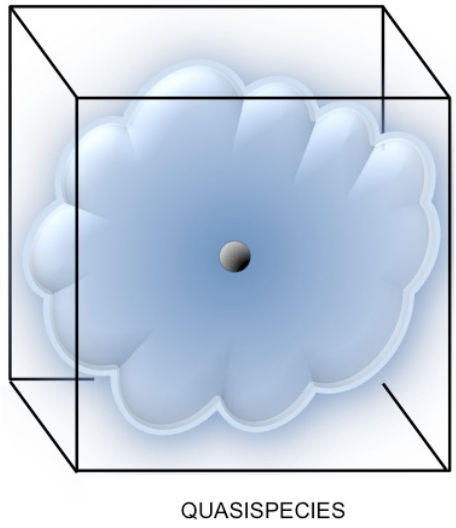


Figure 1-3. HIV-1 genome organization. Layout of a typical HIV-1 proviral DNA sequence. Long terminal repeats (LTR) flank the structural (*i.e.*, gag), enzymatic (*i.e.*, pol), envelope (*i.e.*, env), and accessory (*i.e.*, tat, rev, vpr, vpu, vif, and nef) genes. Genome length is 9719 and each row of genes represents a different reading frame. The tat and rev genes require post-transcriptional splicing. The pol gene encodes protease (PR), reverse transcriptase (RT, including the RNase H domain), and integrase (IN). p51 and p66 denote the heterodimeric subunits of RT. R = repeat region; U5 = unique 5' region; U3 = unique 3' region

A)


QUASISPECIES

B)

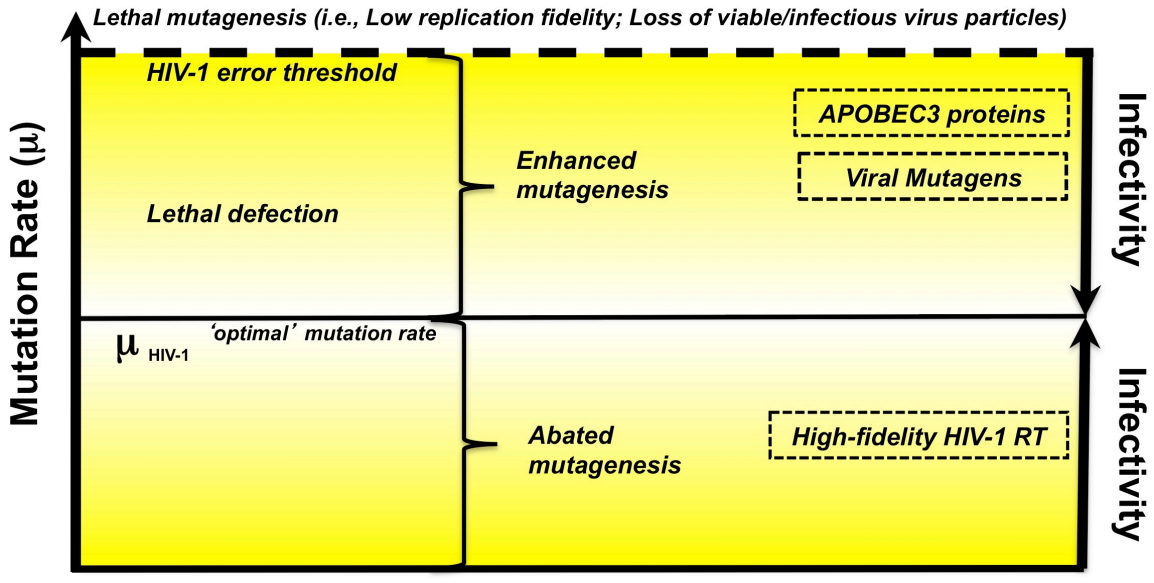

Lethal mutagenesis (i.e., Low replication fidelity; Loss of viable/infectious virus particles)
HIV-1 error threshold
Lethal defection
Enhanced mutagenesis
APOBEC3 proteins
Viral Mutagens
Infectivity
Mutation Rate (μ)
μ HIV-1
'optimal' mutation rate
Abated mutagenesis
High-fidelity HIV-1 RT
Infectivity

Figure 1-4. Quasispecies and extinction threshold models. A) Three-dimensional nucleotide sequence space quasispecies model depicting a hypothetical virus population as a "cloud" or "swarm" of related genetic variants. Each axis represents sequence space, while the center dot represents the most fit master sequence. **B)** Diagram to describe the concept of HIV-1 lethal mutagenesis (extinction catastrophe). The left and right y-axes describe the inverse relationship between mutation rate (μ) and viral infectivity, respectively. Viable virus populations exist below the virus extinction threshold. Small molecules (e.g., nucleotide analogs) and cellular proteins (e.g., APOBEC3G) can act as viral mutagens and increase the viral mutation rate. An increase in mutation rate could result in either sublethal mutagenesis (including lethal defection, an intermediary step in the path towards virus extinction) or directly past the error threshold (dashed line) to induce extinction catastrophe, i.e., lethal mutagenesis. As the HIV-1 mutation rate is decreased (e.g., due to HIV-1 reverse transcriptase variants with higher fidelity), viral infectivity also diminishes, most likely due to replicative fitness costs.

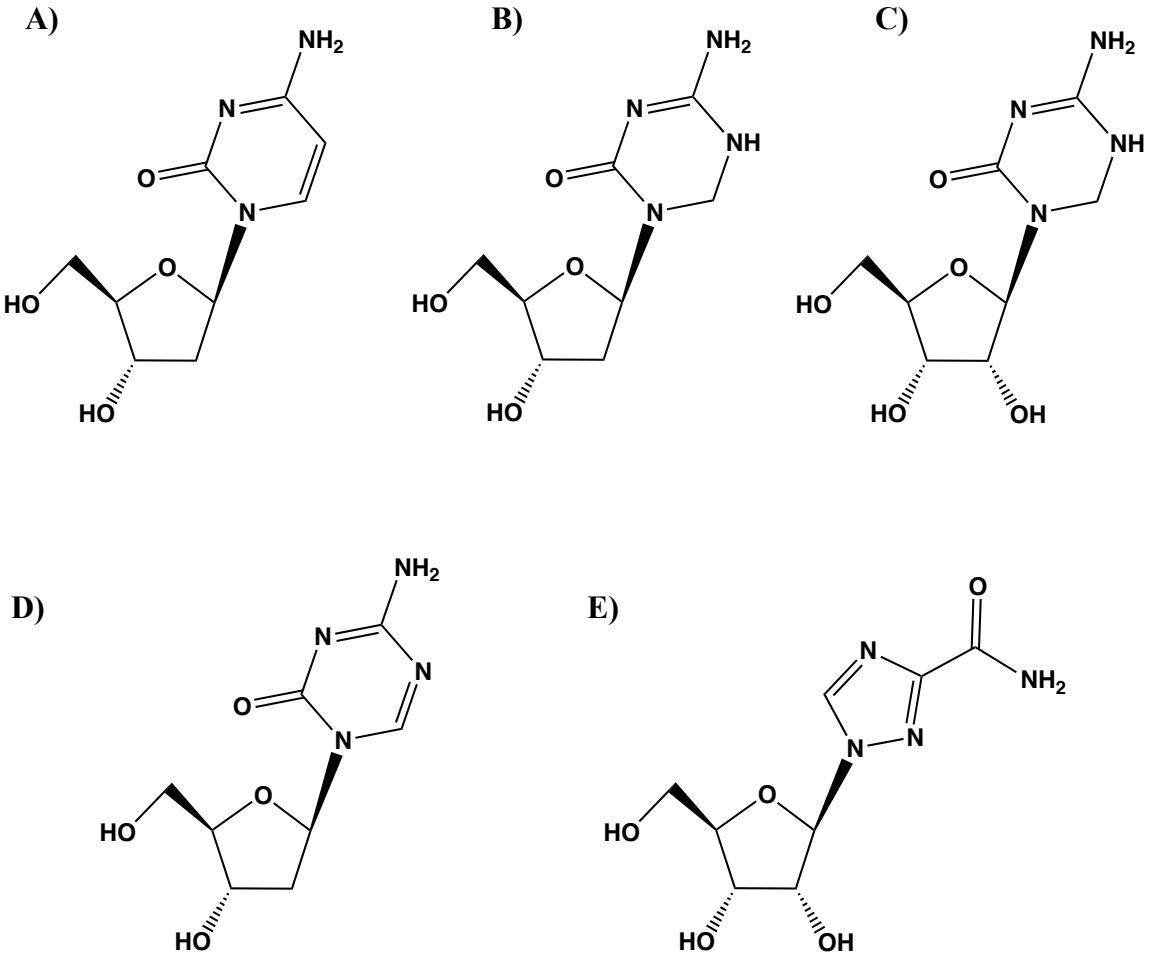


Figure 1-5. Chemical structures of natural deoxycytidine nucleoside and related viral mutagens. **A)** 2'-deoxycytidine [dC]; **B)** 5-aza-2'-deoxycytidine [Decitabine]; **C)** 5-azacytidine [Vidaza]; **D)** 2′-deoxy-5,6-dihydro-5-azacytidine [KP-1212]; **E)** 1-(β-D-Ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide [Ribavirin]. Structures drawn using ChemBio Draw Ultra 12.0, PerkinElmer.

CHAPTER II

INTERRELATIONSHIP BETWEEN HIV-1 FITNESS AND MUTATION RATE

Introduction

Mutation rates among viruses and other microbes range between 1.1 x 10^{-3} – 5.0 x 10^{-10} mutations per site per generation (Drake *et al.* 1998; Drake *et al.* 1999; Lynch 2006; Sanjuan *et al.* 2010). Much of this range can be explained by an inverse relationship between mutation rate and genome size (Drake 1969; Drake *et al.* 1998; Sanjuan *et al.* 2010). DNA-based microbes, for example, possess a near-constant mutation rate (0.003-0.004 mutations/genome/generation) over a genome size of 10,000 base pairs (Drake 1991; Drake *et al.* 1998). However, RNA viruses mutate at a considerably higher rate (10-100 fold) than DNA viruses of comparable size. (Drake *et al.* 1999; Sanjuan *et al.* 2010). The observed high mutation rates of RNA viruses makes them convenient model systems to investigate selection pressures on the viral mutation rate.

Human immunodeficiency virus type 1 (HIV-1) has a high mutation rate, determined to be 1.2 – 3.4 x 10^{-5} mutations per target base per round of replication (Mansky *et al.* 1995; Abram *et al.* 2010). Many of these mutations are deleterious or lethal, and therefore have limited long-term contribution to genetic diversity. In general, the high mutation rate of RNA viruses has been hypothesized to push the virus to the brink of either error catastrophe (Tsimring *et al.* 1996; Gerrish *et al.* 2007; Rouzine *et al.* 2008) or extinction (Bull *et al.* 2007). In fact, drugs and host proteins that increase the HIV-1 mutation rate possess antiviral activity (Loeb *et al.* 1999; Harris *et al.* 2003; Anderson JP 2004; Yu *et al.* 2004; Harris *et al.* 2005; Smith RA 2005; Dapp *et al.* 2009; Dapp *et al.* 2012).

The high mutation rate of HIV-1 is often thought to be important for adaptation (Nowak *et al.* 1991; Mansky 1998; Lee *et al.* 2008), including the ability infect new host

cells and the emergence antiviral drug resistance (Coffin 1995; Perelson *et al.* 1996; Mansky 2002). Mutator phenotypes have been found in bacteria (Gross *et al.* 1981; LeClerc *et al.* 1996; Matic *et al.* 1997; Horst *et al.* 1999) and lytic RNA viruses (Suarez *et al.* 1992), as well as HIV-1 (Mansky *et al.* 2000; Gutierrez-Rivas *et al.* 2001; Mansky *et al.* 2002; Chen *et al.* 2005). They commonly have an advantage in new or fluctuating environments (Sniegowski *et al.* 1997; Giraud *et al.* 2001; Pal *et al.* 2007; Cooper *et al.* 2010). Mutations that increase mutation rate can become associated with adaptive changes and therefore 'hitchhike' to a greater frequency within a population (Leigh 1970; Sniegowski *et al.* 1997; Dawson 1998; Johnson 1999; Tenaillon *et al.* 1999). Experimental data on selection for a high mutation rate in viruses is limited. Studies in vesicular stomatitis virus (VSV) and HIV-1 found that anti-mutator strains did not impede the ability of these viruses to adapt to novel environments or to develop drug resistance (Lee *et al.* 1997; Keulen *et al.* 1999). On the other hand, a poliovirus variant with a viral polymerase possessing 3 times higher fidelity than wt was far less virulent in mice, suggesting a reduced ability for virus adaptation (Pfeiffer *et al.* 2005; Vignuzzi *et al.* 2008). HIV-1 adaptation may be important for avoidance from the host immune response that allows viral replication in long-term non-progressors (Sheppard *et al.* 1993; Williamson 2003). To date, there is no strong evidence that retroviruses or RNA viruses require more rapid phenotypic adaptation than DNA viruses (which generally possess lower mutation rates) (Elena *et al.* 2005).

An alternative explanation is that the high mutation rate of HIV-1 is due to some level of genetic constraint. RNA-dependent RNA polymerases of RNA viruses, as well as HIV-1 reverse transcriptase (RT), lack intrinsic 3'-to-5' proofreading activity. Nonethe-

less, there is a wide range of mutation rates across RNA and retroviruses (Sanjuan *et al.* 2010). The mutation rates of these viruses can be elevated by drugs that either increase mutation rate or terminate polymerase extension with 2',3'-dideoxynucleoside analogues. Drug resistant variants have been found to have lower mutation rates in RNA viruses (Pfeiffer *et al.* 2005) as well as in HIV-1 (Mansky *et al.* 2000; Mansky *et al.* 2003; Chen *et al.* 2005).

If HIV-1 has the ability to evolve to a lower mutation rate, increasing replication fidelity is likely limited by polymerase kinetics or processivity (Bessman *et al.* 1974; Goodman *et al.* 1974; Hopfield 1974). Evidence with VSV anti-mutator strains suggest that direct fitness costs are imposed as measured by viral replication (Furio *et al.* 2005). A meta-analysis approach combining eleven previous studies found that the HIV-1 mutation rate correlated with the biochemical parameter k_{cat}^{-1}, i.e., the rate at which a polymerase extends past a terminal base mispair (Furio *et al.* 2007). This and other studies (Bessman *et al.* 1974; Goodman *et al.* 1974; Hopfield 1974; Back *et al.* 1996; Pandey *et al.* 1996; Dawson 1998) suggest that polymerase fidelity is intrinsically linked to replication rate – that is, more energy and/or time for base recognition, nucleotide synthesis, and/or polymerase translocation is required for the polymerase to incorporate the correct base. According to this model, the higher mutation rate of RNA viruses and retroviruses may be due to a relatively greater cost to the virus in increasing replication fidelity.

HIV-1 provides a powerful system for the study of constraints on the viral mutation rate. In this study, a library of ten HIV-1 mutants with varying fidelity (mainly amino acid substitutions in HIV-1 RT that confer drug resistance to nucleoside RT inhibitors) was used to investigate the relationship of mutation rate to viral fitness. Most of the RT

variants are not only clinically relevant, but define a range in viral fidelity that maintains viral infectivity. By measuring mutation rate and fitness of HIV-1 under near identical conditions, a cost of replication fidelity was identified. Viruses with both higher and lower mutation rates - relative to wt,- led to reductions in viral fitness, suggesting that wt HIV-1 may be close to an optimum. These studies provide the first demonstration of the relationship between HIV-1 mutation rate and viral fitness and aid in gaining greater insight into their interaction.

Results

In the present study, a panel of ten viruses encoding RT variants was generated to test the hypothesis that both lower and higher mutation rates reduce HIV-1 fitness below that of wt (Fig. 2-1 A and Fig. 2-1 B). Of these ten mutants, eight confer resistance to one or more clinically approved nucleoside reverse transcriptase inhibitors (NRTIs), while the remaining two (i.e., Y115A and V148I) have the largest magnitude impact on HIV-1 fidelity (Table 2-1). Table 2-1 provides a comprehensive summary of previous fitness, processivity, and fidelity measures for each mutant. Although there is some general agreement regarding the findings with any particular RT variant, the phenotypic measurements of the RT mutants vary greatly between studies, which are likely due to discrepancies in reagents, assays, and laboratories. Additionally, the table includes viruses with RT variants having previously uncharacterized fitness and/or mutation rates. In order to investigate the potential interrelationship between viral fitness and mutation rate, direct parallel comparisons of fitness and mutation rate were conducted here in this study under standardized experimental conditions.

Drug-resistance conferring substitutions in HIV-1 RT influence the fidelity of replication

A single-cycle vector assay was used to measure differences in HIV-1 mutant frequency relative to the NL4-3 wildtype (wt) reference strain. This assay uses flow cytometry analysis to score target cells for expression of a pair of marker genes (Fig. 2-1A). Cells expressing only one of the two marker genes are interpreted as being infected yet harboring a mutated virus. This assay increases the ability of identifying mutations that arise during HIV-1 replication. Since the marker genes have not been exhaustively studied to determine precisely the target bases that result in a scorable phenotypic difference, the changes in mutation rate that can be determined are analyzed relative to that of the wt HIV-1 reference vector. This dual reporter system has been previously established to quantify increased mutational load in the presence of known viral mutagens (i.e. 5-azacytidine, decitabine, and APOBEC3G), and validated by sequencing analysis (Dapp *et al.* 2009; Clouser *et al.* 2010; Dapp *et al.* 2012).

In parallel analyses, each HIV-1 vector harboring a RT variant was used to transduce the CEM T-cell line. The results from the single-cycle assay predictably led to the grouping of the RT variant viruses into 3 main categories: 1) mutant frequency higher than wt; 2) mutant frequency lower than wt; 3) mutant frequency comparable to wt virus. Three of the RT variant viruses (i.e., A62V, Y115F/A) had a higher mutant frequency than wt, six had a lower mutant frequency (i.e., K65R, V148I, M184I/V, K65R/M184V, and L74V/Y115F/M184V), and one RT variant (L74V) was found to have comparable mutant frequency to wt (Fig. 2-2 and Table 2-2). These results are generally consistent with previous work, though there are some inconsistencies noted (Table 2-1).

Drug-resistant conferring substitution mutations in HIV-1 RT reduce viral fitness

In order to assess the fitness of the panel of RT variant viruses under investigation, each virus was subjected to a head-to-head competition assay against wt (Fig. 2-1B). This assay was performed in a close derivative of CEM (i.e., CEM-GFP), so we could measure fitness and fidelity under similar cellular conditions. Viral stocks were titered on CEM-GFP cells to determine infectious unit equivalents of mutant and wt virus. Each assay was initiated with a 1:1 ratio of wt and mutant virus and passaged for 10 days before quantification of each clone by qPCR.

A significant decrease in viral fitness relative to wt virus was observed for 5 variants (i.e., K65R, Y115A, V148I, M184I, and K65R/M184V) (Fig. 2-3). Three of the other variants analyzed (i.e., A62V, Y115F, and M184V) appeared to have somewhat lower fitness (i.e., 18%-43% less fit than wt, Table 2-1), but these and the remaining 2 mutant viruses were not significantly different than wt. In control experiments, wt virus competed against a wt reference strain showed no discernable fitness differences (Fig. 2-3 and Table 2-3).

Interconnection between HIV-1 fitness and mutation rate

An optimality model for the HIV-1 mutation rate in a constant environment would suggest that RT variant viruses with higher or lower mutation rates would be associated with reduced fitness. To investigate this, mutant viruses were grouped by their fidelity relative to wt and the relationship between fitness and fidelity was analyzed by Pearson's correlation and linear regression. Analysis of the 6 RT variant viruses with lower mutation rate than wt, along with L74V (which has no effect on mutation rate) and the wt reference strain, demonstrated a significant correlation between lower mutation rates and lower fitness (two-tailed Pearson correlation coefficient $r = 0.903$, P value = 0.0022, Fig.

2-4A). These same analyses were performed without wt to confirm that the correlation was not driven merely by a high fitness of wt RT and low fitness of mutants; the pattern was unchanged (two-tailed Pearson correlation coefficient r = .9244, P value = 0.0028). Exclusion of the K65R/M184V and L74V/Y115F/M184V mutants, to avoid retesting of the same mutations in a different genetic background, also did not affect results (two-tailed Pearson correlation coefficient r = 0.980, P value = 0.0032). These analyses support the conclusion that there is a fitness cost to high fidelity.

The effect of viral variants with higher mutation rates (including L74V) were next tested for their effect on fitness. Inclusion of wt into analysis resulted in a significant correlation (two-tailed Pearson correlation coefficient r = -0.945, P value = 0.0152, Fig. 2-4B). This correlation was no longer significant after removal of wt from the analysis (two-tailed Pearson correlation coefficient r = -0.948, P value = 0.0518). The high mutation rate and low fitness of the Y115A RT mutant virus strongly influences the correlation, which helps to explain the decreased significance of the Spearman's correlation, a nonparametric test that is more resistant to outliers (two-tailed Spearman correlation coefficient r = -1.00, P value = 0.0834). Taken together, while the total number of mutants in this study with a high mutation rate is relatively small, the results from our analysis suggest that the wt virus has a mutation rate near the optimum, at least under these culture conditions, and in the absence of strong adaptive pressures.

Discussion

In this study, the relationship between HIV-1 fitness and mutation rate was investigated in parallel analyses. Advantages to performing this analysis with HIV-1 include

its high mutation rate (which has clinical implications) and the wealth of data published on HIV-1 RT – including *in vitro* biochemical measurements (i.e., fidelity and processivity) as well as many measurements of viral fitness (Table 2-1). While these previous measurements have clearly enhanced the field, there are significant advantages to using a single data set from parallel analyses to explore the interaction between fitness and mutation rate. For instance, the fitness assay methodology used here in this study (i.e., direct *ex vivo* competition measurements) is more accurate than *in vitro* studies of catalytic rates; the rate of polymerization during viral replication may not always be a limiting factor to viral reproductive rate (Furio *et al.* 2007). Most importantly, the parallel analyses allow for more direct comparisons between fitness and mutation rate, which is important for deciphering the relationships between these two important biological properties.

There is evidence supporting at least three evolutionary factors that govern mutation rate (Fisher 1930; Kimura 1967; Leigh 1970; Orr 2000; Sniegowski *et al.* 2000). A low mutation rate may reduce the rate of adaptation (though this will be the limiting factor for adaptation only under certain conditions). A high mutation rate can cause an increase in mutational load in the population, or decrease individual fitness by causing lethal mutations in progeny. Finally, there may be physiological limits on polymerase extension as fidelity is improved. The first of these forces varies greatly as adaptive conditions change; but if the latter two dominate, a relatively stable and optimal fidelity may exist.

As demonstrated in this study, a decrease in mutation rate (i.e., increased replication fidelity) comes at a cost in regards to viral fitness (Fig. 2-4A). This observation is consistent with a recent meta-analysis of HIV-1, in which an inverse correlation between

the enzymatic incorporation rate K_{cat}^{-1} and viral fidelity was shown, suggesting that the fitness cost of increased fidelity is tied to energetic constraints of nucleotide synthesis (Furio *et al.* 2007). Such a fidelity-cost model has been postulated by enzymologists over the past few decades to explain enzyme kinetic observations of antimutator polymerases (Bessman *et al.* 1974; Goodman *et al.* 1974; Hopfield 1974; Back *et al.* 1996; Pandey *et al.* 1996; Dawson 1998). If the cost to increased fidelity is greater in RNA viruses and retroviruses, this might explain the lower fidelity of these viruses overall, even in the absence of a strict constraint. A detailed analysis of RT mutant behavior during single-cycle reverse transcription events would likely be informative; our results cannot distinguish between, for example, longer generation time and a decreased rate of successful infection.

Our findings also suggest that higher mutation rates were associated with lower fitness (Fig. 2-4B). Some studies have found mutator strains to have higher fitness under conditions where adaptation was important (Gibson *et al.* 1970; Chao *et al.* 1983; Giraud *et al.* 2001; Loh *et al.* 2010). However, when adaptation is less important, mutators are generally at a disadvantage (Funchain *et al.* 2000; Giraud *et al.* 2001; Notley-McRobb *et al.* 2002; Denamur *et al.* 2006). In addition, numerous studies have shown that viral mutagens (i.e., 5-azacytosine and ribavirin) and the APOBEC3 host proteins can reduce infectivity of HIV-1 and other RNA viruses (Harris *et al.* 2003; Dapp *et al.* 2009; Clouser *et al.* 2010; Dapp *et al.* 2012). This current study with HIV-1 supports the general notion that even relatively small increases to the mutation rates of RNA viruses and retroviruses decrease fitness, reinforcing the promise of lethal mutagenesis as an antiviral intervention strategy (Crotty *et al.* 2001; Anderson JP 2004; Graci *et al.* 2008).

Much of our mutation rate data is in good agreement with previously published

studies using various methodologies (Table 2-1 and Fig. 2-2). For example, the measured mutation rates for the K65R, L74V, Y115A, V148I, M184I, and M184V RT variants generally coincided with previous findings. Detailed analysis of the HIV-1 mutation rate using the *lac*Za gene as a mutation target led to similar but not identical determinations (Mansky *et al.* 1995; Abram *et al.* 2010). In the current study, a more qualitative assay was utilized with a mutation target that has not been as extensively characterized. One limitation of this is not being able to calculate mutation rates with high precision. Nonetheless, this assay provides an appropriate surrogate measure of relative differences in mutation rate, as has been previously validated (Dapp *et al.* 2009; Clouser *et al.* 2010; Dapp *et al.* 2012).

Of the four remaining RT variants analyzed in this study, A62V and K65R/M184V have, to our knowledge, never been analyzed for their influence on the HIV-1 mutation rate, while minor discrepancies were noted with Y115F and L74V/Y115F/M184V (Fig. 2-2 *versus* Table 2-1). The Y115F RT mutant had previously been shown to slightly decrease viral fidelity, but these trends were not found to be significant (Martin-Hernandez *et al.* 1997; Chen *et al.* 2005); in this study this RT variant was shown to have a 20% (1.2-fold) higher mutant frequency (Fig. 2-2 and Table 2-1). Previous analysis of the triple mutant L74V/Y115F/M184V showed an almost 2-fold increase in frameshift mutations (Chen *et al.* 2005), while current analysis revealed a 30% (1.3-fold) decrease in mutant frequency (Fig. 2-2 and Table 2-1). This discrepancy is likely due to the inherent differences between mutation assays. For instance, the previously published *in vivo* single-cycle frameshift assay measures the effect of an RT variant to cause frameshifts at a poly-thymidine tract (Chen *et al.* 2005). The fidelity assay used

in this study, however, provides a more global analysis of both substitution mutations as well as frameshift mutations.

While many of our analyses of HIV-1 fitness among the RT variant viruses are consistent with previous observations, there were discrepancies (Fig. 2-3 and Table 2-1). These differences can be attributed to either: 1) the type of fitness assay (i.e., parallel growth *versus* single-cycle *versus* dual competition *versus in vivo* reversion); 2) the end-point assay measure and its representation (i.e., p24 capsid levels *versus* viral copy number and percent difference *versus* fold difference); and 3) the type of reagents used (i.e., molecular clones *versus* cloned patient samples and cell lines *versus* primary cells). Although there is no consensus fitness assay in the field, several labs have popularized the co-culturing dual competition assay as a sensitive and internally controlled assay (Ball *et al.* 2003; Abraha *et al.* 2005; Troyer *et al.* 2005; Anastassopoulou *et al.* 2007; Liu *et al.* 2007).

Of the ten HIV-1 RT variant viruses that were analyzed in the dual competition assay, seven had been previously assayed for their effects on viral fitness (Table 2-1). The fitness difference measures of these 7 mutants (i.e., A62V, K65R, L74V, Y115A, M184I, M184V, and K65R/M184V) were generally similar to previously reported ranges (Fig. 2-3 and Table 2-1). Two minor discrepancies were L74V and M184V. The fitness analysis in this study indicated that L74V had no difference from wt (Fig. 2-3), while 3 of 5 previous studies found a subtle, 11% to 2-fold, defect to viral fitness (Table 2-1). The current analysis revealed that the M184V mutant had an insignificant 1.4-fold decrease in viral fitness, while 7 of 8 previous studies found this variant's fitness loss to range from 4% to 14-fold relative to wt (Table 2-1). These inconsistencies may be explained by any

number of the aforementioned differences among fitness assays. It is also possible that certain cell types, especially with low dNTP pool concentrations, may exacerbate subtle replication defects (Diamond *et al.* 2004; Weiss *et al.* 2004; Gao *et al.* 2008; Jamburuthugoda *et al.* 2008). It may be that the CEM cell line, chosen to limit variability when comparing RT variant measures between the two assays, minimizes fitness differences present in other cell types.

Taken together, the data presented in this study support a relatively simple optimality model for the HIV-1 mutation rate, in which extreme high and low mutation rates are selected against. While this mutational optimum may depend on the niche (e.g., transmission event, virus spread in the lymph node), and may be easily disrupted in the presence of novel adaptive conditions, it is interesting that in this study the mutational optimum was near that of the reference wt strain. In summary, this is the first study to directly compare fidelity and fitness measures in HIV-1, and the first description of an interrelationship between HIV-1 fitness and mutation rate.

Material and Methods

Plasmid constructs and cell lines. The HIV-1 vector pHIG, used in the mutant frequency assays, has been previously described (Dapp *et al.* 2009). Briefly, a ~2.0-kbp dual reporter cassette composed of the murine heat stable antigen CD24 (HSA), an internal ribosome entry site (IRES), and enhanced green fluorescent protein (eGFP) was placed in-frame and 3' to the NL4-3 nef start codon. The original vector pNL4-3.HSA.R+.E- was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Germantown, MD) and contributed by N. Landau (He

et al. 1995). The HIV-1 NL4-3 molecular clones used in the fitness assays were a kind gift from E. Arts and described here (Anastassopoulou *et al.* 2007). To generate the 10 drug-resistance mutants in HIV-1 pol, the 2100-5983 region was subcloned into pCR®2.1-TOPO® (Invitrogen). Site-directed mutagenesis was performed to introduce point mutations using site-directed mutagenesis (QuickChange II Site-Directed Mutagenesis, Stratagene Santa Clara, CA). Correct clones were confirmed by sequencing analysis and cloned back into the pHIG vector, using SbfI (2844) and AgeI (3486) restriction enzymes (New England Biolabs, NEB, Ipswich, MA); or back into the NL4-3 molecular clone using MscI sites (2683 and 4545). Inserts were, again, sequenced confirmed for orientation and quality.

The G glycoprotein of vesicular stomatitis virus (VSV-G) envelope expression plasmid HCMV-G was used to pseudotype virions and was a kind gift from J. Burns (University of California, San Diego). The phycoerythrin (PE)-conjugated antibody to HSA (anti-CD24) was purchased form BD Pharmingen (San Diego, CA). The human embryonic kidney (HEK 293T) cell line was purchased from ATCC (Manassas, VA) and maintained in Cellgro DMEM (Manassas, VA) plus 10% HyCLone FetalClone III (FC3; Thermo-Scientific). The CEM-GFP cell line was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Germantown, MD), contributed by J. Corbeil (Gervaix *et al.* 1997). The CEM cell line was a kind gift from M. Malim. Both CEM and CEM-GFP lines were maintained in RPMI (Gibco, Life Technologies Invitrogen, Grand Island, NY) plus 10% FC3.

Virus production and virus titer assays. Both the pHIG vector virus and the NL4-3 molecular clones were produced by transfection of HEK 293T cells. The calcium

phosphate method was used to transfect 2 x 10^6 cells with 20 ug plasmid viral DNA and 2.5 ug plasmid HCMV-G envelop. Viral supernatants were collected 48 h post-transfection and passed through 0.2-um filter. To determine infectious units (IU) of virus per mL supernatant the tissue culture infectious dose (50% $TCID_{50}$) end-point dilution assays was used. For each wt and mutant virus stock, 100 uL of 10-fold serial diluted supernatant was added to 50,000 CEM-GFP indicator cells in 250 uL total volume of a 96-well plate. Each dilution series consisted of n=6. Cells were maintained every other day for 10 days. At day 10, the number of GFP-positive wells was determined by visual inspection. To calculate the $TCID_{50}$, the number of GFP-positive wells was multiplied by 1/6 and summed with 0.5; 10 to the negative power of this sum is the $TCID_{50}$, and the $TCID_{50}$ divided by 0.1 mL (100 uL added at each dilution) is the number of IU in each mL of supernatant. An MOI of 0.0001 can then be computed by calculating the amount of supernatant with 50 IU (MOI = IU / # of cells; 0.0001 x 500,000 cells = 50 IU).

Mutant frequency analysis by flow cytometry. Vector virus, both wt and the panel of 10 RT variants, was used to transduce 50,000 CEM cells by 2 h spinoculation at 1200 x g. Each experimental replicate consisted of 4 to 6 biological replicates. Viral stocks were titered on the CEM line to maintain 15-30% transduction efficiency. This range of infection ensures an MOI of less than 1 to reduce the probability of co-infection but also was not low enough to be subject to background anti-HSA staining (see below). Cells were prepared and stained as previously described (Dapp *et al.* 2009). Briefly, 48 h post-transduction, cells were resuspended in 75 uL PBS/2% FC3 with 1:250 anti-HSA-PE and incubated for 20-min at 4 °C. Cells were then washed with 1 mL PBS/2% FC3 and finally resuspended in 200 uL PBS/2% FC3 for flow cytometry analysis. Cells were analyzed

with a FACScan (BD Biosciences). Gates were chosen based on live cell morphology of forward scatter channel (FSC) and side scatter channel (SSC) of 10,000 cells. Excitation with the 488 nm Argon laser provided detection of GFP emission at 507 nm in fluorescence channel 1 (FL-1), and detection of PE-anti-HSA at 578 nm in fluorescence channel 2 (FL-2). Compensation was set based on single-color controls to eliminate spillover and re-verified based on the geometric mean of the single-color positive to negative-detected populations. All flow cytometry data was analyzed by FlowJo software v 9.2 (Ashland, OR). Mutant frequency calculations were determined from the percentage of target cells expressing a single reporter gene relative to the percentage of total infected cells (i.e., %[HSA+/GFP-] plus %[HSA-/GFP+] divided by the % of total infected cells). Mutant frequencies were set relative to wt for each experimental replicate.

Dual competition assay. Each of the 10 RT variants was replicated in the presence of the isogenic wt NL4-3 clone. In each head-to-head competition assay, the wt and RT mutant virus clone can be independently quantified by qPCR based on probes designed to a specific region in vif; either the wt subtype B consensus denoted as vifA or the same region consisting of 11 synonymous point mutations denoted as vifB. Briefly, 5 x 10^5 CEM-GFP cells were infected with a 1:1 ratio of wt to RT mutant virus at an MOI of 0.0001. Virus growth was maintained for 10 days, while cells were split and fresh culture media replenished every 2 days. Each pair-wise competition was repeated 3 independent times consisting of 4 biological replicates.

TaqMan Duplex qPCR assay. Competition experiments were analyzed using a duplex qPCR assay with modifications from here (Anastassopoulou *et al.* 2007). At day 10 in the competition assay, cells were collected and resuspended in 200 uL PBS. Total

genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit (Roche Applied Science) and eluted into 50 uL total volume. First, 5 uL of extracted DNA were subjected to a brief, external PCR amplification reaction in 50 uL, using VifOut+ (5'-GCA AAG CTC CTC TGG AAA GGT GAA GGG-3') and VifOout- (5'-CTT CCA CTC CTG CCC AAG TAT CCC-3') primers. Reactions were performed with Platinum PCR Supermix (Invitrogen) under the following cycling conditions: 1 cycle at 94 °C for 2 min; 10 cycles at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s; and 1 cycle at 68 °C for 5 min. The reaction was then purified with GenElute PCR Clean-Up Kit (Sigma) and eluted into 35 uL.

The TaqMan assay utilized probes to differentiate between the two forms of the NL4-3 backbone (vifA and vifB). The vifA probe sequence was 5'-AGG ATC TCT ACA GTA CTT GGC ACT AGC A-3' and vifB probe sequence was 5'-AGG AAG CTT GCA ATA TCT AGC GTT AGC A-3'. The probes were labeled with Cy5 and Black Hole Quencher 2 (vifA) and FAM and Black Hole Quencher 1 (vifB) at 5'- and 3'-ends, respectively. The reactions were run on a BioRad CFX96 Touch Real-Time PCR Detection System (BioRad). For the duplex qPCR reaction, the forward primer was VifIn+ (5'-GAA AGA GAC TGG CAT TTG GGT CAG GG-3') and reverse primer was VifIn- (5'-GTC TTC TGG GGC TTG TTC CAT CTG TCC-3'). Primers were optimized and added at a final concentration of 375 nM each, while vifA probe added at 250 nM and vifB probe added at 125 nM. To this, 2 uL of PCR product from above, was added to the primer/probe in 10X TaqMan® Gene Expression Master Mix (Applied Biosystems) to a 20 uL total reaction. Reaction conditions were: 95 °C for 10 min, the 95 °C for 15 s and 53 °C for 1 min for 40 cycles. All reactions were performed in duplicate, including a 9-

log range in plasmid DNA template (5 x 10^9 to 50 copies), as well as a negative control (no-template). The PCR efficiency, E, of each standard curve (vifA or vifB) was calculated based on the curves slope, $E = (10^{-1/s} - 1) \times 100\%$. To maximize sample data confidence, E of VifA and VifB standard curves were within 5% and R^2 of each standard curve was ≥99%. Because the competing strains were added at an equal ratio, differences in relative amounts of each clone after appropriate viral growth were used to calculate fitness differences. Co-cultured viral clones were quantified using duplex qPCR, which relies on mutant- and wt-specific probes to specifically differentiate the two strains. This assay was both specific and sensitive over a 6-log range in viral copy numbers, well within the range of viral detection (Fig. 2-5).

Calculation of viral fitness. Fitness differences (W_D) were calculated for each wt versus RT mutant co-culturing competition assay. For each of the 4 biological replicates, the frequency of the mutant strain was calculated as follows [mutant copy # / (mutant copy # + wt copy #)]. This frequency is then arcsine transformed [arcsine (√mutant frequency)] to convert the proportional data to a normal distribution. Next, the mean of the arcsine transformed data is calculated and untransformed by squaring the sine of each mean. Data from the 3 experimental replicates were compiled to show the fitness differences.

Statistical analyses. All statistical analysis and graphical representation was done using GraphPad Prism version 5.0 (La Jolla, CA). A one-way ANOVA was used to determine differences between wt HIV-1 and the 10 RT mutants for both mutant frequency and fitness. Relative mutant frequency differences were analyzed by first log transforming the data. A Dunnett's multiple comparison post-hoc test was used to compare differ-

ences between wt and each mutant RT measure. A two-tailed Pearson's coefficient of correlation was used on the mutant frequency and fitness measures to determine the chance that there was zero correlation between the parameters. An analysis by the non-parametric two-tailed Spearmen's rank correlation, robust to the effect of outliers, had similar results and is generally not reported. Linear regression was used to identify R-squared values.

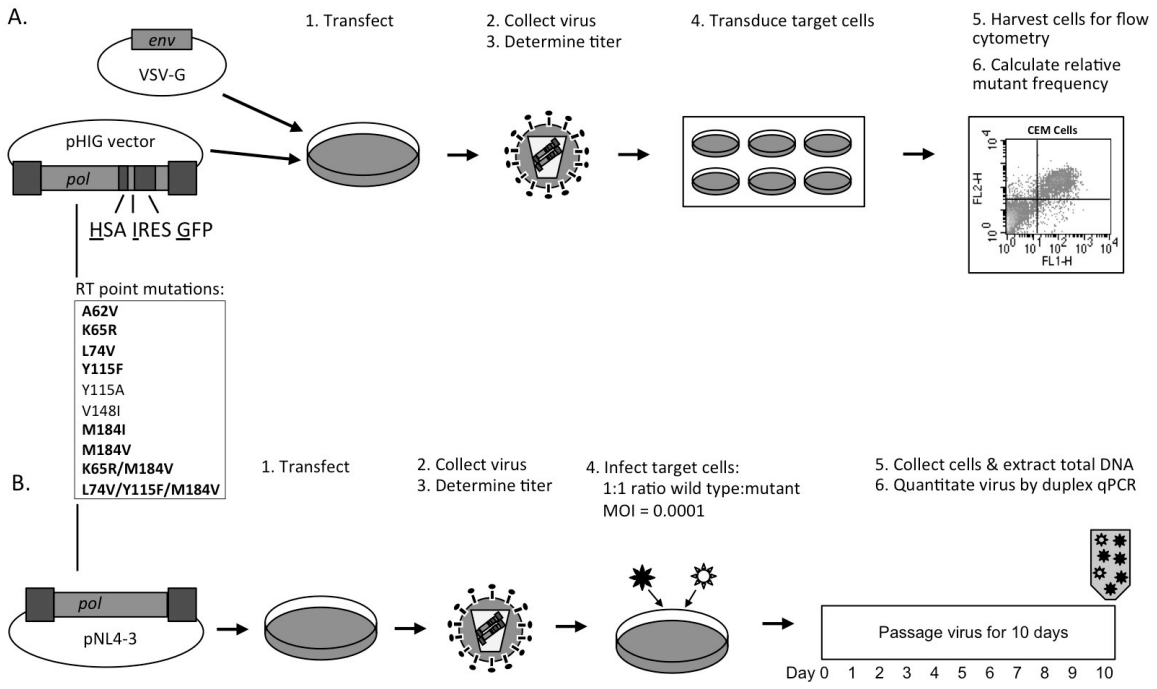


Figure 2-1. Experimental approach. Measurement of A) viral fidelity and B) viral fitness, among panel of 10 HIV-1 RT mutants. **A)** An HIV-1 vector (pHIG) (Dapp *et al.* 2009; Clouser *et al.* 2010; Dapp *et al.* 2012) sensitive to changes in fidelity was manipulated to consist of NL4-3 wt or one of ten RT mutants. Each of these vectors was co-transfected with a VSV-G envelope expression plasmid into a producer cell line. Cell culture supernatants were collected, filtered to remove cellular debris, and titered for downstream application. Viral equivalents were used to transduce CEM target cells to calculate relative mutant frequencies by flow cytometry analysis. **B)** The same 10 RT mutants were engineered into the NL4-3 molecular clone. Together with wt, these clones were generated and titered. Each of the mutant viruses was competed against wt by infecting CEM-GFP target cells at an equal MOI of 0.0001. Virus was passaged for ten days and then relative amounts of viral nucleic acid were quantified by duplex qPCR. The RT point mutations listed in bold text are nucleoside RT inhibitor mutations. Abbreviations: HSA, murine heat stable antigen; IRES, internal ribosome entry site; GFP, green fluorescence protein; VSV-G, vesicular stomatitis virus-glycoprotein; FL1-H, fluorescence channel 1, height of intensity; FL2-H, fluorescence channel 2, height of intensity; *pol*, HIV-1 gene consisting of protease, RT, and integrase; MOI = multiplicity of infection; qPCR, quantitative PCR.

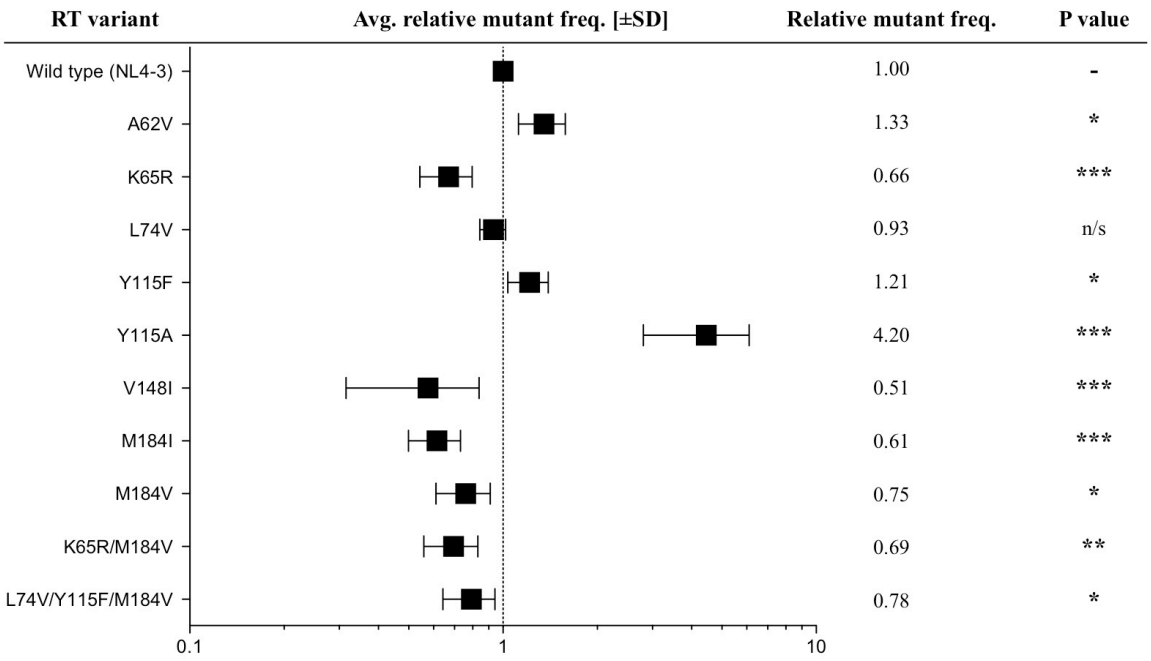


Figure 2-2. HIV-1 RT mutants influence virus mutant frequencies. Each of the 10 RT mutants was measured for differences in fidelity relative to the wt reference strain. Mutants are displayed in ascending order of their RT position. Mutant frequencies were calculated relative to wt and are depicted on log-scale. The actual measurements are listed in the adjacent column. Values represent at least 4 biological replicates that were experimentally repeated 4 to 8 times. SD is standard deviation. * P value < 0.05; ** P value < 0.01; *** P value < 0.001. n/s is not significant.

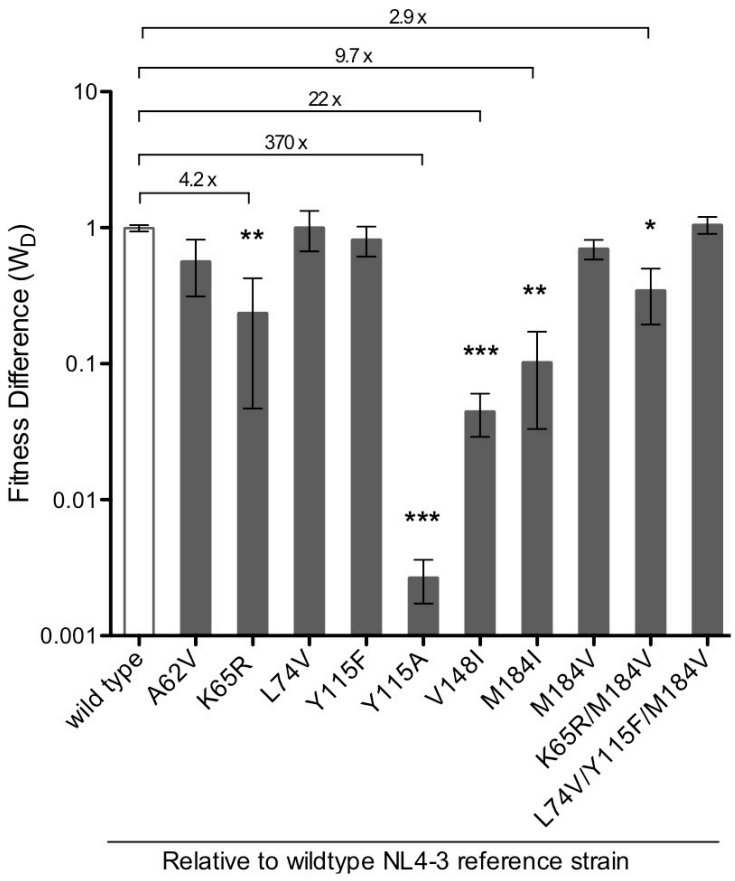
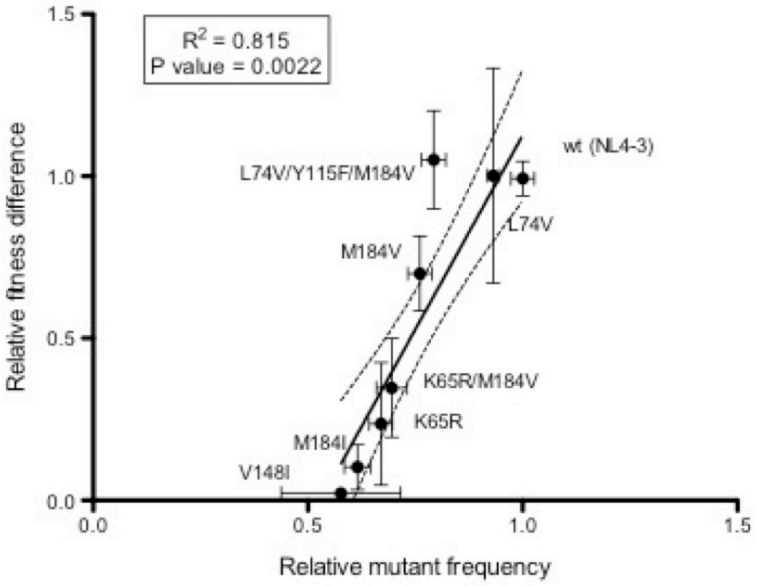


Figure 2-3. Impact of HIV-1 RT mutants on viral fitness. Each of the 10 RT mutants, and a wt control, was passaged with a wt reference strain in a head-to-head competition assay. Mutants are assembled by RT position, and respective fitness differences (W_D) are displayed on a log-scale. Asterisk symbols denote level of significance with numerical values along horizontal line showing fold difference from wt. Error bars represent SEM, standard error of the mean. * P value < 0.05; ** P value < 0.01; *** P value < 0.001.

A)



B)

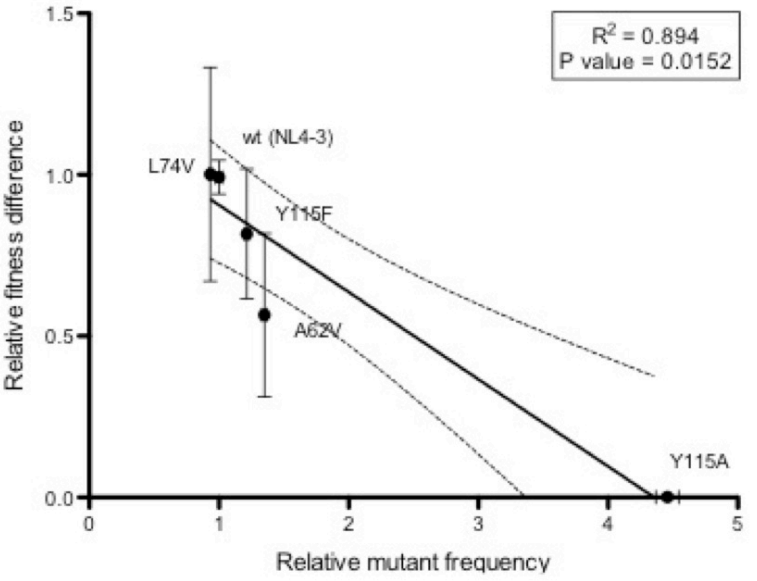


Figure 2-4. Correlative influence of HIV-1 RT variants on both virus mutant frequency and fitness. Assembly of RT mutants that have **A)** higher, or **B)** lower, fidelity relative to wt and the corresponding fitness difference measurements. L74V and wt are included in both assemblies. Lines represent the linear regression calculated from best-fit values. R^2 is the squared value of the Pearson's correlation coefficient. Error bars represent SEM, standard error of the mean. Dashed lines indicate 95% confidence interval of linear regression.

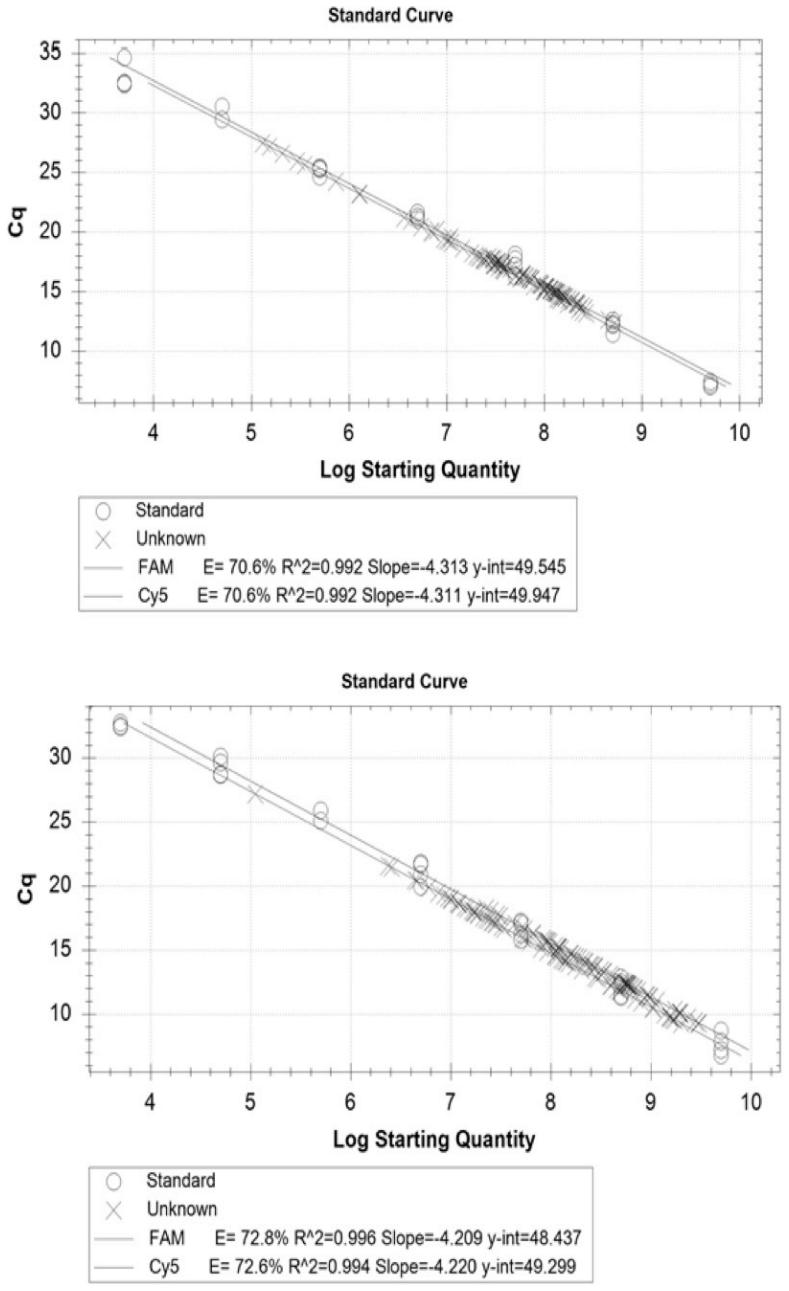


Figure 2-5. Sensitivity and specificity of qPCR dual competition primer probe sets. Quantitative PCR plots of plasmid standards (open circles) and experimental unknowns (X; irrelevant). Standard curves are fit to 10-fold serially diluted standard plasmid copy number ($5x10^9$ – $5x10^3$ copies). Cyanine 5 (Cy5) and 6-carboxyfluorescein (FAM) used to label Vif A and Vif B probes, respectively. Cq = threshold cycle, Log Starting Quantity = log plasmid standard copy number. E = PCR efficiency, see Materials and Methods.

Table 2-1. Summary of fitness and fidelity measures for HIV-1 RT variants.

RT variant	Location	Resistance Phenotype	Effect on Fitness	Assay	Effect on Fidelity	Assay	Citation
A62V	Finger subdomain	Q151M complex	No Change	Parallel growth			(Maeda et al. 1998)
K65R	Finger subdomain	ABC, ddI, ddC, 3TC FTC, TDF	↓ (29-fold)	Dual competition			(Maeda et al. 1998)
			↓ (40%)	Single-cycle RC			(Deval, White et al. 2004)
			No Change	Parallel growth			(Deval, Navarro et al. 2004)
			↓ (47%)	Single-cycle RC			(White, Margot et al. 2002)
							(Sharma et al. 2009)
					↑ (8.1-fold)	*In vitro* FMA	(Shah, Curr et al. 2000)
			↓ (80%)	Single-cycle RC: patient samples			(Weber et al., 2005)
			↓ (15%)	Dual competition			(Svarovskaia et al. 2008)
					↑ (3.3-fold)	*In vivo* single-cycle FMA	(Mansky et al. 2003)
L74V	Finger subdomain	ABC, ddI	↓ (11%)	Parallel growth			(Sharma et al. 1997)
			↓	Parallel growth			(Sharma et al. 1997)
			↓ (2-fold)	Dual competition			(Cong, Heneine et al. 2007)
			No Change	Parallel growth			(Deval, Navarro et al. 2004)
							(Sharma et al. 2009)
					↑ (1.7-fold)	*In vitro* FMA	(Shah, Curr et al. 2000)
					↑ (2-6-fold)	Gel-based assay	(Rubinek et al. 1997)
					↑ (3.5-fold)	*In vitro* FMA	(Jonckheere et al. 2000)
					No Change	*In vivo* single-cycle FMA	(Mansky et al. 2003)
			No Change	Parallel growth			(Maeda et al. 1998)
Y115F	dNTP binding site	ABC					(Martin-Hernandez 1996)
					No Change	*In vitro* FMA	(Martin-Hernandez 1997)
					No Change	*In vivo* single-cycle FSA	(Chen et al. 2005)
Y115A	dNTP binding site	N/A					(Martin-Hernandez 1996)
							(Martin-Hernandez 1997)
			↓ (100%)	Parallel growth			(Olivares et al. 1999)
					↓ (4.0-fold)	*In vitro* FMA	(Jonckheere, et al. 2000)
					↓ (2.3-fold)	*In vivo* single-cycle FMA	(Mansky, et al. 2003)
V148I	Finger subdomain	N/A			↑ (8.1-fold)	*In vitro* FMA	(Diamond et al. 2003)
					↑ (8.7-fold)	*In vitro* FMA	(Weiss, Chen et al. 2004)
					↑ (3.33-fold)	*In vivo* single-cycle FMA	(Weiss, Chen et al. 2004)
M184I	dNTP binding site	ABC, 3TC, FTC	↓	Parallel growth			(Back, Nijhuis et al. 1996)
			↓ (12-22%)	*In vivo* reversion			(Devereux et al. 2001)
			↓ (10-fold)	Dual competition			(Li, Li et al. 2009)
			↓ (5-fold)	Single-cycle RC			(Hu and Kuritzkes 2011)
			No Change	Single-cycle RC			(Julias, Boyer et al. 2004)
			↓ (30%)	Single-cycle RC			(Van Cor-Hosmer 2010)
							(Boyer and Hughes 1995)
					↑ (4-fold)	*In vitro* FMA	(Rezende et al. 1998)
			↓ (0-99.5%)	Single-cycle RC			(Jamburuthugoda 2008)

(Table 2-1 cont.)

M184V	dNTP binding site	ABC, 3TC, FTC	↓	Parallel growth			(Back, Nijhuis et al. 1996)
			↓ (12-22%)	*In vivo* reversion			(Devereux et al. 2001)
			↓ (14-fold)	Dual competition			(Cong, Heneine et al. 2007)
			↓ (4-fold)	Single-cycle RC			(Hu and Kuritzkes 2011)
			↓ (35%)	Single-cycle RC			(Deval, White et al. 2004)
							(Sharma et al. 2009)
			No Change	Single-cycle RC			(Julias, Boyer et al. 2004)
			↓ (4-8%)	*In vivo* reversion			(Paredes, Sagar et al. 2009)
					↑ (1.2 fold)	*In vitro* FMA	(Jonckheere et al. 2000)
							(Boyer and Hughes 1995)
					↑ (1.6-fold)	*In vitro* FMA	(Rezende et al. 1998)
					↑ (1.6-fold)	*In vitro* FMA	(Drosopoulos Prasad 1998)
			↓ (3-6 fold)	Parallel growth			(Aquaro et al. 2005)
					↑ (1.3-fold)	*In vivo* single-cycle FMA	(Mansky Bernard 2000)
K65R/M184V		ABC, 3TC, FTC	↓ (65%)	Single-cycle RC			(Deval, White et al. 2004)
			↓ (76%)	Single-cycle RC			(White, Margot et al. 2002)
L74V/Y115F/M184V		ABC			↓ (1.91-fold)	*In vivo* single-cycle FSA	(Chen et al. 2005)

Table 2-1 legend.

Q151M multidrug resistance complex consists of A62V/V75I/F77L/F116Y/Q151M. ABC = Abacavir; ddI = Didanosine; ddC = Zalcitabine; FTC = Emtricitabine; 3TC = Lamivudine; TDF = Tenofovir. N/A means not applicable. Parallel growth describes fitness assay that replicated wt and mutant HIV-1 clones in separate cultures. Dual competition describes fitness assay that replicated wt and mutant HIV-1 clones together in co-culturing conditions. Single-cycle RC, replicative capacity, describes assay that compares transduction efficiency of wt and mutant HIV-1 vectors after one round of replication. *In vivo* reversion describes a fitness measure that takes into account the reversion time of a particular mutant back to wt after therapy is withdrawn. *In vitro* FMA describes a cell-free forward mutation assay. Gel-based assay describes a cell-free assay that compares the ability of wt and mutant RTs to insert and extend mispairs on a specified template. *In vivo* single-cycle FMA is a cell-based forward mutation assay. *In vivo* single-cycle FSA is a cell-based frame shift detection assay.

Table 2-2. Supplementary table of mutant frequency data. Data collected and used to generate Fig 2-2.

	Average relative mutant frequency					
RT variant	Number of replicates	Mean	Std. Deviation	Std. Error	Lower 95% CI	Upper 95% CI	P-value
wild type	8	1.000	N/A	N/A	N/A	N/A	N/A
A62V	8	1.334	0.0711	0.0214	1.195	1.489	*
K65R	8	0.658	0.0863	0.0273	0.571	0.759	***
L74V	8	0.928	0.0416	0.0132	0.866	0.994	n/s
Y115F	8	1.205	0.0592	0.0171	1.105	1.313	*
Y115A	4	4.203	0.1775	0.0888	2.193	8.056	***
V148I	4	0.510	0.2782	0.1391	0.184	0.707	***
M184I	8	0.605	0.0862	0.0305	0.513	0.714	***
M184V	8	0.749	0.0790	0.0279	0.644	0.872	*
K65R/M184V	5	0.685	0.0805	0.0360	0.544	0.863	**
L74V/Y115F/M184V	8	0.780	0.0941	0.0284	0.682	0.893	*

N/A is not applicable; n/s is not significant. * P value < 0.05; ** P value < 0.01; *** P value < 0.001

Table 2-3. Supplementary table of mutant frequency data. Data collected and used to generate Fig 2-3.

	Fitness difference (W_D) relative to reference strain										
	Replicate number						Mean	STD	Dunnett's Multiple Comparison Test		
	1	2	3	4	5	6			Mean diff.	95% CI of diff.	P-value
wild type	0.888	0.918	1.220	1.070	0.956	0.904	0.993	0.129	N/A	N/A	N/A
A62V	0.202	0.442	1.052				0.565	0.438	0.427	-0.077 to 0.504	n/s
K65R	0.036	0.614	0.058				0.236	0.327	0.757	0.088 to 0.669	**
L74V	0.874	0.502	1.628				1.001	0.574	0.009	-0.295 to 0.286	n/s
Y115F	0.448	1.142	0.860				0.817	0.349	0.176	-0.203 to 0.369	n/s
Y115A	0.0045	0.0013	0.0023				0.003	0.002	0.990	0.204 to 0.786	***
V148I	0.051	0.068	0.015				0.045	0.027	0.948	0.183 to 0.765	***
M184I	0.242	0.033	0.033				0.103	0.121	0.890	0.154 to 0.736	**
M184V	0.692	0.504	0.904				0.700	0.200	0.293	-0.144 to 0.437	n/s
K65R/M184V	0.085	0.614	0.342				0.347	0.265	0.646	0.032 to 0.614	*
L74V/Y115F/M184V	1.132	0.758	1.262				1.051	0.262	0.058	-0.320 to 0.262	n/s

N/A is not applicable; n/s is not significant. * P value < 0.05; ** P value < 0.01; *** P value < 0.001

CHAPTER III

5-AZACYTIDINE CAN INDUCE LETHAL MUTAGENESIS IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Introduction

Significant progress has been made in the clinical management of HIV-1 infection using antiretroviral drugs (De Clercq 2009). Nonetheless, the emergence of drug resistance limits the efficacy of current HIV-1 drugs and drives the need for novel therapeutics (Marsden *et al.* 2009). Although current therapy relies on combination drug therapy, low levels of viral replication coupled with the high HIV-1 mutation rate makes drug resistance difficult to prevent (Mansky 1998; Mansky *et al.* 2000). The development of novel anti-HIV-1 drugs that have a high barrier to the emergence of drug resistance would offer new treatment options as the current drugs become ineffective from the emergence and transmission of drug resistance.

The HIV-1 mutation rate represents a novel drug target that may offer a higher genetic barrier to the emergence of drug resistance relative to current anti-HIV-1 drugs. The high mutation rate of HIV-1, as well as other RNA viruses, enables virus survival in the face of a rapidly changing host environment (e.g., host immune response to viral infection) (Domingo *et al.* 1985). However, because most mutations are detrimental, the high mutation rate leads to a large proportion of non-infectious virions (Coffin 1995). Thus, HIV-1 may have evolved a mutation rate that carefully balances the need for viral adaptation with the need to replicate with enough fidelity to remain viable. Based upon this high mutation rate, a novel therapeutic strategy is to tip this balance in favor of a higher mutation rate such that the virus is unable to replicate with enough fidelity to remain infectious. This strategy, termed lethal mutagenesis, is based on the concept that only a modest increase in the viral mutation rate is needed to render the virus non-viable (Loeb *et al.* 1999; Holmes 2003; Domingo *et al.* 2005).

The validity of lethal mutagenesis as an antiviral strategy is supported by experimental evidence showing an inverse correlation between mutation rate and infectivity of several RNA viruses (e.g., polio virus, foot and mouth disease virus, and Hantaan virus) as well as retroviruses (e.g., spleen necrosis virus and HIV-1) (Pathak *et al.* 1992; Harris *et al.* 2005; Chung *et al.* 2007; Graci *et al.* 2007; Agudo *et al.* 2008). Nucleoside analogs have been shown to effectively increase viral mutation rates (Holland *et al.* 1990; Loeb *et al.* 1999; Sierra *et al.* 2000; Crotty *et al.* 2001; Harris *et al.* 2005). However, there has yet to be a nucleoside analog of acceptable efficacy and safety to be clinically relevant for the purpose of lethal mutagenesis. One of the concerns regarding the use of mutagenic nucleoside analogs is the potential for toxicity and carcinogenicity since these compounds can be directly incorporated into the host genome.

Ribonucleoside analogs with mutagenic potential have also been explored for their antiviral activity against riboviruses (Suzuki *et al.* 2005; Graci *et al.* 2008; Cuevas *et al.* 2009). The use of ribonucleoside analogs for blocking retroviral replication have been used, but the mechanism of action is not clear. There are three models that could explain the mechanism by which ribonucleoside analogs inhibit retrovirus replication. First, ribonucleoside analogs could be incorporated into HIV-1 RNA during transcription of the genomic length RNA (Li *et al.* 1970; Loeb *et al.* 1999; Graci *et al.* 2004). Alternatively, the ribonucleoside analog could be incorporated into viral DNA by reverse transcriptase (RT) following its reduction to the 2'-deoxynucleotide form. Finally, ribonucleoside analogs may exert antiviral activity through incorporation into both DNA and RNA. To date, a detailed understanding of how ribonucleosides manifest an antiretroviral effect is not well established, including those ribonucleosides with mutagenic potential.

5-azacytidine (5-AZC) is a ribonucleoside analog that is used clinically to treat myelodysplastic syndromes (Issa *et al.* 2005; Yoo *et al.* 2006). 5-AZC has previously been shown to inhibit HIV-1 infectivity (Bouchard *et al.* 1990). Based on its structure and its effect on the poliovirus mutation rate, 5-AZC was proposed to exert its antiviral activity by increasing the HIV-1 mutation rate although no data has been published to support lethal mutagenesis as its mechanism of action. As a ribonucleoside, 5-AZC was proposed to increase the HIV-1 mutation rate through direct incorporation into viral RNA. However, 5-AZC could also be incorporated into viral DNA after reduction by cellular ribonucleotide reductase. In this study, we examined the mechanism by which 5-AZC inhibits HIV-1. Our data reveal that 5-AZC exerts its antiviral activity through incorporation into both viral RNA and DNA. Interestingly, the most potent antiviral activity was attributed to incorporation of 5-AZC into viral DNA following its reduction to the 2'-deoxy form. Further investigation into the mechanism of 5-AZC revealed that incorporation of 5-AZC into viral DNA during reverse transcription leads to lethal mutagenesis which is characterized by a significant increase in transition mutations within the provirus.

Results

5-AZC inhibits HIV-1 replication and increases viral mutant frequency. To elucidate the stage of viral replication that 5-AZC inhibits, we used a single round vector assay that enables differentiation of early and late stages of viral replication (Fig. 3-1). Specifically, cells cotransfected with envelope-deficient HIV-1 plasmid and a plasmid encoding the HIV-1 envelope support the late stages of viral replication by enabling viral

transcription, translation, and particle production. In contrast, virus-infected target cells support the early stages of viral replication by enabling viral entry, reverse transcription, and integration. The stage at which 5-AZC exerts its antiviral activity can be determined by specifically treating either the virus-producing cells, to examine late stages of replication, or the target cells to examine the early stages of viral replication. From previous studies, it was expected that ribonucleosides exert their antiviral activity in the late phase of the HIV replication cycle (Loeb *et al.* 2000; Smith RA 2005). In support of this, 5-AZC led to a concentration-dependent decrease in HIV-1 infectivity with a half maximal inhibitory concentration (IC_{50}) of 112 μM (Fig. 3-2A). Importantly, there was no concentration-dependent effect of the deoxynucleoside 5-aza-dC during the late phase of viral replication suggesting that the antiviral activity is dependent on 5-AZC's influence on viral RNA. Additionally, this decrease in infectivity was not due to changes in virus production (Fig. 3-7). Unexpectedly, 5-AZC led to a more potent inhibition of HIV-1 infectivity in the early phase of the HIV-1 replication cycle with an IC_{50} of 57 μM (Fig. 3-2C).

Since 5-AZC could cause mutations through its incorporation into viral RNA during transcription, we next examined whether 5-AZC increased HIV-1 mutant frequency. To do this, the single cycle assay described in Figure 3-1 was used to determine HIV-1 mutant frequency when either virus-producing cells or target cells were treated with 5-AZC. The single cycle assay enables the determination of mutant frequency through the use of two reporter genes, HSA and GFP. HSA serves as a reporter for infectivity, while the other reporter gene, GFP, is used as a mutation target. Specifically, cells that express one target gene, but not the other, represent cells that are infected, but harbor a mutation that abrogates expression of one of the target genes. Since 5-AZC is a ribonucleoside

analog, it was expected that an increase in mutant frequency would be observed in the late phase of the HIV-1 replication cycle. As expected, 5-AZC increased the HIV-1 mutant frequency by 2-fold when virus-producing cells were treated at the respective [IC_{50}] (Fig. 3-2B). This indicates that 5-AZC increases mutant frequency during the late phase of HIV-1 replication. Figure 3-2D shows that 5-AZC also increased the mutant frequency by 3-fold when target cells were treated also with the respective [IC_{50}]. These data suggest that 5-AZC may inhibit HIV-1 infectivity by increasing the mutant frequency at two distinct stages of the HIV-1 replication cycle.

Although the loss of infectivity coincided with an increase in mutant frequency, it is possible that the loss of infectivity could be attributed to compound toxicity. To investigate this, the toxicity of 5-AZC was analyzed for two different endpoints including relative cellular ATP levels as well as mitochondrial reductase activity (data not shown) when either virus producing cells or permissive target cells were treated with 5-AZC. Figure 3-3 shows that loss of viral infectivity was not correlated with cellular toxicity. Similar results were observed when mitochondrial reductase activity was used as an endpoint for toxicity (data not shown).

Analysis of HIV-1 mutation spectra during viral replication in the presence of 5-AZC. Based on the data shown in Figure 3-2, we hypothesized that 5-AZC increased mutation frequency by two different mechanisms. First, 5-AZCTP could induce mutations through its direct incorporation into viral RNA. Second, 5-AZC could be incorporated into viral DNA after its 2'-OH reduction by ribonucleotide reductase. Since incorporation during either RNA or DNA synthesis would result in mutations in the provirus, we examined the mutation spectra of the proviral GFP gene after treating either virus produc-

ing cells or target cells with 5-AZC. To do this, cells infected by mutant HIV were isolated by cell sorting. In this case, cells expressing HSA but not GFP (HSA+/GFP-) were sorted and the mutant GFP gene was sequenced.

Figure 3-4 shows the location and mutation type of over 100 individual GFP gene sequences recovered from target cells. The mutation spectra revealed a dramatic enrichment in G-to-C transversion mutations from virus exposed to 5-AZC in the early phase of replication compared to untreated virus producing cells (Figs 3-4A and 3-4B). Besides a dramatic change in the mutation spectra, 5-AZC increased the average number of mutations per nucleotide sequenced by 2.3-fold compared to virus obtained from untreated cells (Table 3-1). This indicates that 5-AZC not only changes mutation type but also increases the mutation frequency. The finding that treatment of permissive target cells dramatically altered the HIV-1 mutation spectra suggests that 5-AZC was likely incorporated into viral DNA during reverse transcription after its reduction by cellular ribonucleotide reductase since a similar mutation spectra has been reported in cells exposed to 5-aza-dC (Pathak *et al.* 1992; Jackson-Grusby *et al.* 1997).

Similar G-to-C transversion mutations were observed when virus-producing cells were treated with 5-AZC (Fig 3-4C). However, in addition to an increase in G-to-C mutations, there was also an increase in C-to-G mutations consistent with previous observations that 5-AZC could alter mutation spectra of spleen necrosis virus (Pathak *et al.* 1992). Additionally, 5-AZC treatment increased the average number of mutations per nucleotide sequenced by 1.5-fold when compared to untreated virus-producing cells (Table 3-1).

Analysis of the average number of mutations per GFP gene sequenced reveals a general trend towards increased mutational load when comparing 5-AZC early and late

phase treatments with that of the no drug treatment (Fig 3-4D). The GFP proviral sequences analyzed had mutational loads ranging from 0 to over 10 mutations per GFP target gene. The high mutation load is unlikely to produce infectious virus and suggests that 5-AZC's ability to increase the HIV-1 mutation rate leads to a significant decrease in viral replication capacity.

Susceptibility of replication-competent HIV-1 to 5-AZC. Although the results so far suggest that 5-AZC inhibits HIV-1 infectivity by increasing the mutation rate, we wanted to confirm that these results would extend to replication-competent HIV-1. Additionally, we hypothesized that 5-AZC would inhibit replication-competent virus at lower concentrations compared to that used in the single cycle assay. This hypothesis is based on two factors. First, unlike the experiments described thus far, both phases of the replication cycle will be exposed to 5AZC during treatment of cells infected with replication-competent HIV-1. Second, if the antiviral activity of 5-AZC is due to an increase in mutation frequency, then multiple rounds of replication would allow for the accumulation of mutations resulting in a greater proportion of lethally mutagenized viruses. The results in Figure 3-5 show that significantly lower concentrations were required to inhibit replication-competent HIV-1 compared to the concentrations required to inhibit HIV-1 in the single cycle assay.

Discussion

The emergence and transmission of drug resistance as well as the halt of the Merck vaccine trial emphasizes the continual need for the development of novel HIV-1 drugs as well as a return to fundamental aspects of vaccine development (Girard *et al.*

2008; Kozal 2009). As an antiviral strategy, lethal mutagenesis offers a novel drug target (i.e., the viral mutation rate) and is likely to have a high barrier to drug resistance. However, little progress has been made in identifying compounds with enough therapeutic potential to be used clinically to promote HIV-1 lethal mutagenesis. The ribonucleoside analog, ribavirin, is the only clinically approved ribonucleoside identified so far that may act as a lethal mutagen to inhibit viral replication - specifically, hepatitis C virus (Cuevas *et al.* 2009). While ribavirin is not effective against HIV-1, other ribonucleoside analogs such as 5-AZC have been shown to have anti-HIV activity in cell culture (Bouchard *et al.* 1990). However, little is known about its mechanism of action.

In this study, we examined the antiviral activity, mechanism, and toxicity of 5-AZC. Since 5-AZC is a ribonucleoside analog, it was hypothesized that its antiviral activity would primarily be attributed to its incorporation into viral RNA and subsequent increase in HIV-1 mutation frequency. In support of this, several previous studies have shown that 5-AZC can be incorporated into RNA (Cihak *et al.* 1965; Doskocil *et al.* 1967; Paces *et al.* 1968). One study demonstrated that 5-AZC was weak competitive inhibitor having a 20-fold lower affinity than CTP for RNA polymerase II (Lee *et al.* 1977). However, our results show that the most potent antiviral activity of 5-AZC is actually attributed to its effect on the early phase of HIV-1 replication, which includes reverse transcription. In fact, while 5-AZC increased HIV-1 mutation frequency in both the late and early phases of HIV-1 replication, it had a greater effect on the early phase of replication. These data suggest that 5-AZC exerts its antiviral activity at both phases of replication through an increase in mutation frequency. Although 5-AZC led to a modest increase in mutant frequency, similar increases in mutation rates have been shown sufficient to le-

thally mutagenize other RNA viruses (Holland *et al.* 1990; Julias *et al.* 1998; Loeb *et al.* 1999; Sierra *et al.* 2000; Harris *et al.* 2005). In fact, the theory of lethal mutagenesis suggests that small increases in viral mutation rates should lead to a disproportionately larger decrease in viral infectivity (Eigen 2002; Domingo 2003).

Both phosphorylation and 2'-OH reduction are prerequisites for 5-AZC's clinical use as a DNA hypomethylating agent (reviewed therein (Issa *et al.* 2005; Yoo *et al.* 2006)). Similarly, our results suggest that 5-AZC is likely to be phosphorylated and reduced prior to its incorporation into viral DNA by RT. Phosphorylation of 5-AZC is likely performed by uridine-cytidine kinase as this enzyme is responsible for the phosphorylation of CMP, UMP, dCMP, as well as many pyrimidine analogs used for cancer and antiviral therapy (Hsu *et al.* 2005). Additionally, 5-AZC was shown to be a suitable substrate for uridine-cytidine kinase (Liacouras *et al.* 1975).

After phosphorylation, it is inferred that 5-aza-CDP is reduced by cellular ribonucleotide reductase before incorporation into viral DNA. It has been shown that 10-20% of 5-AZC is incorporated into cellular DNA, suggesting a metabolic pathway through ribonucleotide reductase (Li *et al.* 1970). To further support a role for ribonucleotide reductase in 5-AZC metabolism, the ribonucleotide reductase inhibitor hydroxyurea, was found to block the epigenetic hypomethylation activity of 5-AZC *in vivo* (DeSimone *et al.* 1982). Furthermore, there is indirect mutational data to support the conversion 5-AZC and its incorporation of into DNA. Specifically, our data show a significant increase in G-to-C transversion mutations in proviral DNA after target cells were treated with 5-AZC (Fig. 3-4 B and Table 3-1) and a similar increase in G-to-C mutations was reported for cellular DNA exposed to 5-aza-dC (Jackson-Grusby *et al.* 1997). To our knowledge,

there have been no biochemical studies which have looked at the interaction between 5-AZCTP (or 5-aza-dCTP) and purified RT; however, it is well established that the DNA polymerase activity of RT is specific for dNTPs by preferentially excluding ribonucleoside-triphosphates (rNTPs) from entering the polymerase active site (Gao *et al.* 1997). Moreover, previous reports have shown that DNA polymerase a have similar affinities and rate of incorporation for 5-aza-dCTP and dCTP (Bouchard *et al.* 1983).

Based on the data presented here, we propose a model that accounts for 5-AZC's antiviral effect on the early phase of the HIV-1 replication cycle (Fig. 3-6). In this model, 5-AZC is first reduced to 5-aza-dCDP by ribonucleotide reductase. Next, 5-aza-dCTP is incorporated into viral DNA during reverse transcription. Once incorporated into DNA, the 5-aza-cytosine triazine ring (*i.e.*, the base), can undergo a ring-opening step which would enable it to base pair with cytosine (Jackson-Grusby *et al.* 1997). Thus, as shown in Figure 3-6, cytosine would be incorporated into the plus strand DNA opposite of 5-aza-dC. Finally, our model shows that integration is a key step in repairing the 5-AZC-induced mutations. The model proposes that 5-aza-dC is excised by host DNA repair machinery during integration. 5-aza-dC would be replaced with guanosine since it can base pair with the cytosine located in the plus strand DNA opposite from the abasic site. When transcribed, the minus strand DNA then results in viral progeny carrying G-to-C mutations. In contrast, 5-aza-dC could be incorporated into the positive strand viral DNA across from guanosines present in the minus strand. However, during integration, the DNA repair machinery would likely excise the 5-aza-dC and replace it with a cytosine which would not lead to a mutation.

The use of ribonucleoside analogs as lethal mutagens offer the benefit of being able to predict the type of mutations that give rise to virus lethality (Loeb *et al.* 2000;

Smith RA 2005). The increase in G-to-C mutations caused by 5-AZC could be predicted based on the base pairing properties of 5-AZC and 5-aza-dC. Thus, it is possible that ribonucleoside analogs could be designed to specifically target certain nucleotides for mutation. Ribonucleoside analogs may be superior to current HIV-1 drugs in their ability to delay the emergence of drug resistance. For high-level resistance to emerge against ribonucleoside analogs that function like 5-AZC, it is likely that mutations would have to be acquired in both RT and RNA Pol II. Although RT is likely to accumulate mutations, there is little pressure on RT to select for mutations that would exclude mutagenic nucleoside analogs since these drugs do not appear to prevent RT-mediated polymerization. This is in contrast to nucleoside analogs such as azidothymidine (AZT) which prevent replication by chain termination, and therefore efficiently select for any mutations in RT that restore viral DNA synthesis.

A limitation to the development of ribonucleosides as potential antiretroviral agents are the relatively high concentrations needed to observe an antiviral effect (Fig. 3-2 A and 3-2 C). However, the high concentrations of 5-AZC shown here may be attributed in part by the cell lines used in this study. A previous meta-analysis study documented that cell lines have up to a 5-fold higher concentration of rNTPs than primary cells and that cellular rNTPs are 10-100 fold more abundant that dNTPs (Traut 1994). This suggests that during late phase replication, 5-AZCTP must compete with an intracellular concentration of cytidine triphosphate (CTP) of 109 – 455 μM. Similarly, dividing cells were found to have intracellular dCTP concentrations ranging from 27 – 50 μM (Traut 1994). Because the current study investigates treatment with the ribonucleoside 5-AZC, which has the potential to undergo a num- ber of nucleos(t)ide metabolic pathways, it

is difficult to predict the intracellular concentration of the rNTP and dNTP forms of 5-AZC. Nonetheless, these particular cell lines were chosen because of technical reasons during the virological assays as well as requirements for the shear number of cells needed for the sequencing experiments.

Differences in dNTP pools in the cell types used here may also account for the discrepancy in IC_{50} values when comparing the single-cycle assay (Fig. 3-2) to the multiple round assay (Fig. 3-5) although differences in transport pathways and metabolism could also play a role. Future studies will be needed to precisely measure intracellular 5-AZCTP and 5-aza-dCTP levels when exposing cells to ribonucleosides. It may also be of interest to measure endogenous dNTP pool levels when treating cells with nucleoside analogs because this has been shown to cause alterations in natural dNTP pool levels (Vela *et al.* 2008) and thus may contribute to an increase in retroviral mutation rate (Julias *et al.* 1997; Julias *et al.* 1998; Mansky *et al.* 2000; Jewell *et al.* 2003; Mansky 2003; Chen *et al.* 2005). However, the presence of the specific G-to-C transversion in the presence of 5-AZC seems to argue against this notion (Fig. 3-4 B and Table 3-1).

Since our data support a model in which 5-AZC is converted to the corresponding deoxyribonucleoside triphosphate, it is possible that it could be incorporated into the host genome. This raises concerns over the possible genotoxicity of potential mutagenic ribonucleosides. However, toxicity was not significant at the concentrations required to inhibit viral replication. Additionally, a nucleoside analog currently in development, KP1212/KP1461, induces mutations in viral DNA, but does not appear to do so in the host cell genome (Harris *et al.* 2005; Murakami *et al.* 2005). It is likely that the host DNA repair machinery is sufficiently effective to eliminate any of these analogs that are

incorporated into genomic DNA. However, the novelty of these drugs warrants further investigation into potential long-term effects.

Previous studies have demonstrated the anti-HIV activity of 5-AZC (Bouchard *et al.* 1990). Based on the structure of 5-AZC, it was speculated that its antiviral activity was due to its ability to be incorporated into viral RNA during transcription. Here, our data show that while 5-AZC does demonstrate antiviral activity by this mechanism, its more potent anti-HIV activity can be attributed to its reduction to 5-aza-dCTP followed by incorporation into viral DNA during reverse transcription. Thus, 5-AZC inhibits HIV infectivity through its incorporation into both viral RNA and DNA. This incorporation significantly increases the HIV-1 mutation frequency to a point consistent with lethal mutagenesis. Compounds with a similar mechanism of action could represent an important new class of anti-HIV compounds to explore for clinical viability.

Materials and Methods

Plasmids, cell lines and reagents – The HIV-1 based vector, HIG, was constructed by cloning the IRES-eGFP sequence from pIRES2-eGFP (Clontech, Mountain View, CA) in the Xho I site of pNL4-3.HSA.R+.E- (the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH, Germantown, MD) (Connor *et al.* 1995; He *et al.* 1995). The resulting vector, pHIG, has a vector cassette with the murine heat stable antigen (HSA) gene, IRES and GFP. The Env expression vector pIIINL4env was a kind gift from Eric Freed (NCI Drug Resistance Program, NCI, Frederick, MD). U373-MAGI$_{CXCR4}$ and CEM-GFP cell lines were also obtained from the AIDS Reagent Program (via M. Emerman and J. Corbeil, respectively) (Gervaix *et al.* 1997; Vodicka *et al.*

1997). The PE-conjugated antibody to mouse HSA was obtained from BD Pharmingen (San Diego, CA). The CellTiter-Glo and CellTiter 96 cell proliferation kits were obtained from Promega (Madison, WI). 5-azacytidine was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in DMSO at 1M stock concentrations and stored at -20 °C. The high Pure PCR Template Preparation Kit was from Roche Applied Science (Indianapolis, IN) and the pCR2.1 TOPO cloning vector was from Invitrogen (Carlsbad, CA).

Transfections, infections, drug treatments, and mutant frequency - To produce the HIG vector as a virus, a calcium phosphate transfection protocol was used. Briefly, 1.5 x 10^6 HEK 293T cells were transfected with 10 μg pNL4-3.HIG.E- and 1 μg pIIINL4env. Cells were washed the next day with PBS and resuspended in culture media (DMEM + 10% fetal calf serum). Cell culture supernatants were harvested 48 h post-transfection and passed through a 0.2 μm filter. For drug treatment during virus particle production, 5-AZC or 5-aza-dC was added for 12 h prior to the harvesting of cell culture supernatants. The amount of drug that could potentially be carried over while in the producer cell supernatant was calculated to be negligible after ensuing dilutions. U373-MAGI$_{CXCR4}$ cells were used as permissive target cells for infections and were maintained in DMEM supplemented with 10% fetal calf serum and in the presence of 0.2 mg/mL neomycin, 0.1 mg/mL hygromycin B, and 1.0 μg/mL puromycin. Cells were infected at an MOI of 0.3 in the absence of selectable media. For treatment of target cells, 5-AZC was added 2 hours prior to infection and maintained for 24 hours post-infection. Mutant frequency was determined by the percentage of target cells expressing one of the reporter genes relative to that of the entire infected population (i.e. [HSA+/GFP-] + [HSA-/GFP+] relative to total percent of infected cells). Mutant frequencies were normalized to virus repli-

cation in the absence of drug treatment.

Virus particle capsid protein (p24) assay- ELISA plates (96 well) were incubated overnight with 1:1000 rabbit p24 antiserum (AIDS Reagent Program Cat. #4250). Plates were then washed with PBST (PBS/0.5% Tween 20) and blocked for 1 h with 3% milk in PBST. Samples and standards were prepared by adding 1:1 volume of PBS/0.1% Empigen and incubating at 56 °C for 30 minutes. Samples were then added to the plate and incubated at 37 °C for 1 h, wells washed with PBST, and then incubated with mouse anti-p24 in PBST/1% milk at 22 °C for 1 h. Incubation was then done with a secondary anti-mouse HRP in PBST/1% milk for 30 minutes, and wells washed with PBST and PBS before the addition of the TMB substrate. Reactions were stopped by the addition of 1M sulfuric acid and the absorbance was determined at 450 nm.

Cellular toxicity analysis – Both 293T and U373-MAGI$_{CXCR4}$ were diluted out on 96-well plates to determine the linear range for cell number in each assay. 5,000 293T cells and 6,000 U373-MAGI $_{CXCR4}$ were plated and the CellTiter-Glo and Cell Titer96 assays were performed as described by manufacturer's protocol. Briefly, the CellTiter-Glo assay generates a luminescent signal, which is proportional to ATP levels while the CellTiter-96 assay measures the amount of a colorimetric product produced after a substrate is reduced by mitochondrial reductase.

Flow cytometry analysis - Infected target cells were prepared for flow cytometry by harvesting cells with trypsin-EDTA. Cells were then centrifuged at 200 x g for 5 minutes, and cell pellets were then resuspended at a final concentration of 5 x 10^6 cells/mL in PBS/2% fetal calf serum. Cells (2.5 x 10^5) were incubated for 20 minutes on ice with 1:250 anti-HSA PE (BD Pharmingen). Cells were then washed with

PBS/2% fetal calf serum, centrifuged at 200 x g for 5 min, and then resuspended in PBS containing 1% FC3 and 1% paraformaldehyde. Cells were analyzed using a FACScan (BD Biosciences) with CellQuest software. Cells were gated for morphology and a minimum of 10,000 cells counted. Excitation was done at 488 nm; FL1 detected emission at 507 nm and FL2 detected emission at 578 nm. Compensation was set at FL2 - 99% FL1.

Cell sorting and proviral DNA sequence analysis - Target cells were collected and single cells sorted using a FACSAria (BD Biosciences) 48 hrs post-infection. Approximately ten thousand cells from the PE-HSA+/GFP- quadrant were collected. Total genomic DNA was purified from these cells (Roche High Pure PCR Template Preparation Kit) and used as a template for nested PCR to amplify the GFP gene. Outer primer pair: 5'-CTGAAGGATGCCCAGAAGG-3' and 5'-TGCTTCTAGCCAGGCACAAGC-3'; inner primer pair: 5'-TTACATGTGTTTAGTCGAGG-3' and 5'-GCTACTTGTGATTGCTCCATA-3'. The resulting PCR products were purified, ligated to pCR 2.1 (Invitrogen), and transformed into the DH5α strain of E. coli. Plasmid DNA was purified from cells (Invitrogen Quick Plasmid Miniprep Kit) and used for DNA sequencing analysis. Sequence alignment was performed using SeqMan program of the Lasergene 7 software package (DNASTAR, Madison, WI).

Analysis of 5-AZC with replication competent HIV-1 - CEM-GFP cells (1.5×10^6) were infected with the HIV-1 NL4-3 molecular clone at an MOI of 0.05. 5-AZC or DMSO alone was added to each culture at a 1:1000 dilution. Cultures were monitored every two days by flow cytometry to determine the percentage of infected cells.

Statistical analysis - All statistical analyses, graphical representation and curve fitting were done using GraphPad Prism version 5.0 (La Jolla, CA).

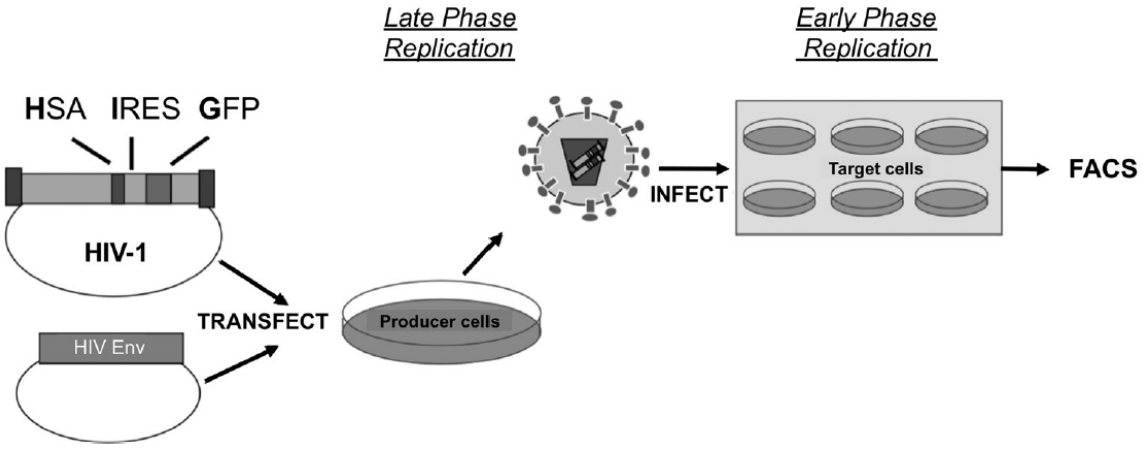


Figure 3-1. Human immunodeficiency virus type 1 (HIV-1) vector for monitoring viral infectivity and mutant frequency in the presence of antiretroviral drugs. An envelope and Nef-deficient HIV-1 vector was constructed with a gene cassette containing the mouse heat stable antigen (HSA), an internal ribosomal entry site (IRES) element and the GFP gene. This vector was cotransfected into 293T cells along with a HIV envelope expression plasmid for producing vector virus. Forty-eight hours posttransfection, cell culture supernatants were collected, filtered, and used to infect permissive U373-$MAGI_{CXCR4}$ target cells at a multiplicity of infection of 0.3. Forty-eight hours postinfection, cells were harvested, stained with a PE-conjugated HSA antibody and analyzed by flow cytometry. Virus-producing cells or permissive target cells were pretreated with drug or DMSO for 12 hrs after either transfection or 2 hrs prior to infection and treatment continued until cell culture supernatant or cells were collected, respectively.

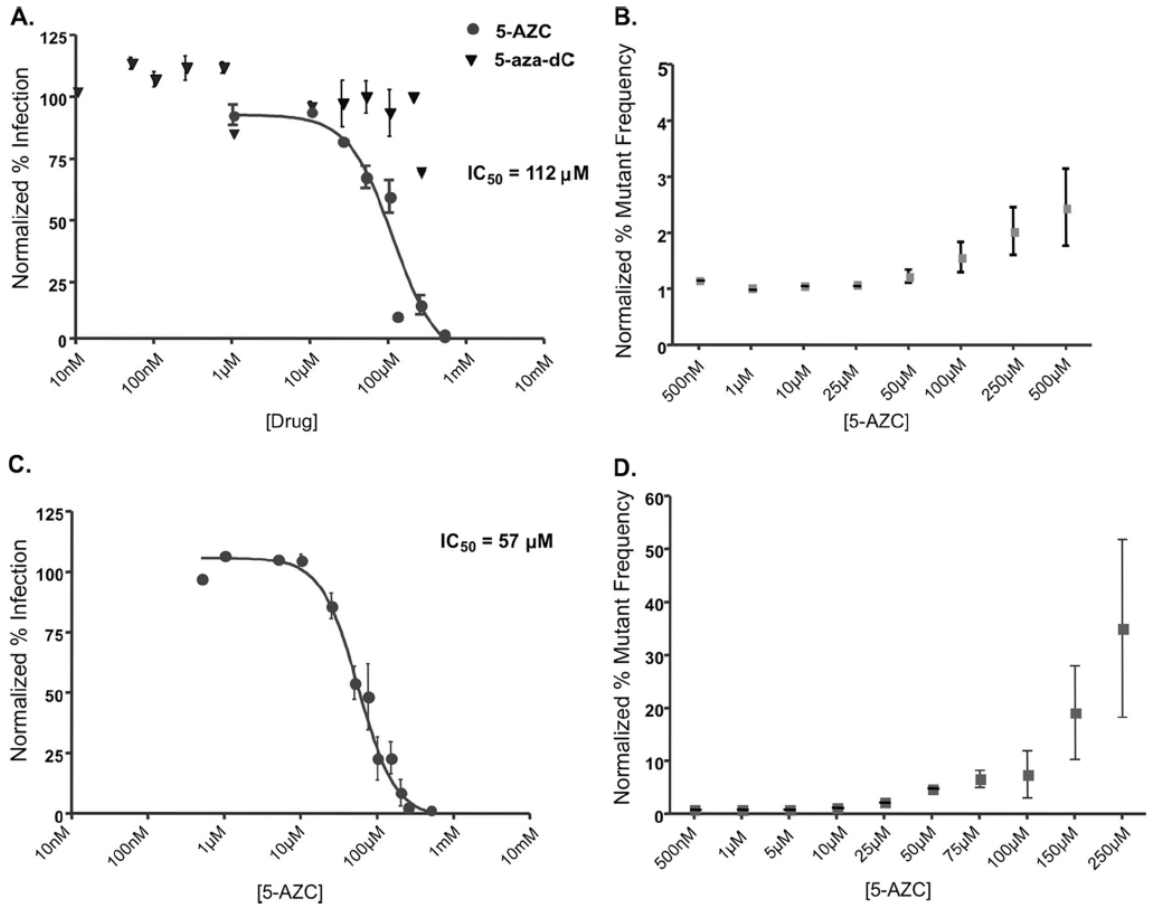


Figure 3-2. Concentration-dependent effects of 5-azacytidine (5-AZC) on HIV-1 infectivity and mutant frequency. 293T or U373-MAGI$_{CXCR4}$ cells were treated with the indicated concentrations of compound prior to transfection (late phase replication) or infection (early phase replication), respectively. Infected cells (MOI of 0.3) were analyzed by flow cytometry. Only the ribonucleoside 5-AZC had a concentration-dependent effect on viral infectivity during treatment at late phase replication **(A)** and this coincided with an increase in viral mutant frequency **(B)**. A more potent antiviral effect was observed with 5-AZC treatment during early phase replication **(C)**. This antiviral activity coincided with a much more dramatic increase in viral mutant frequency **(D)**. Data shown is mean ± SD of 5 independent experiments.

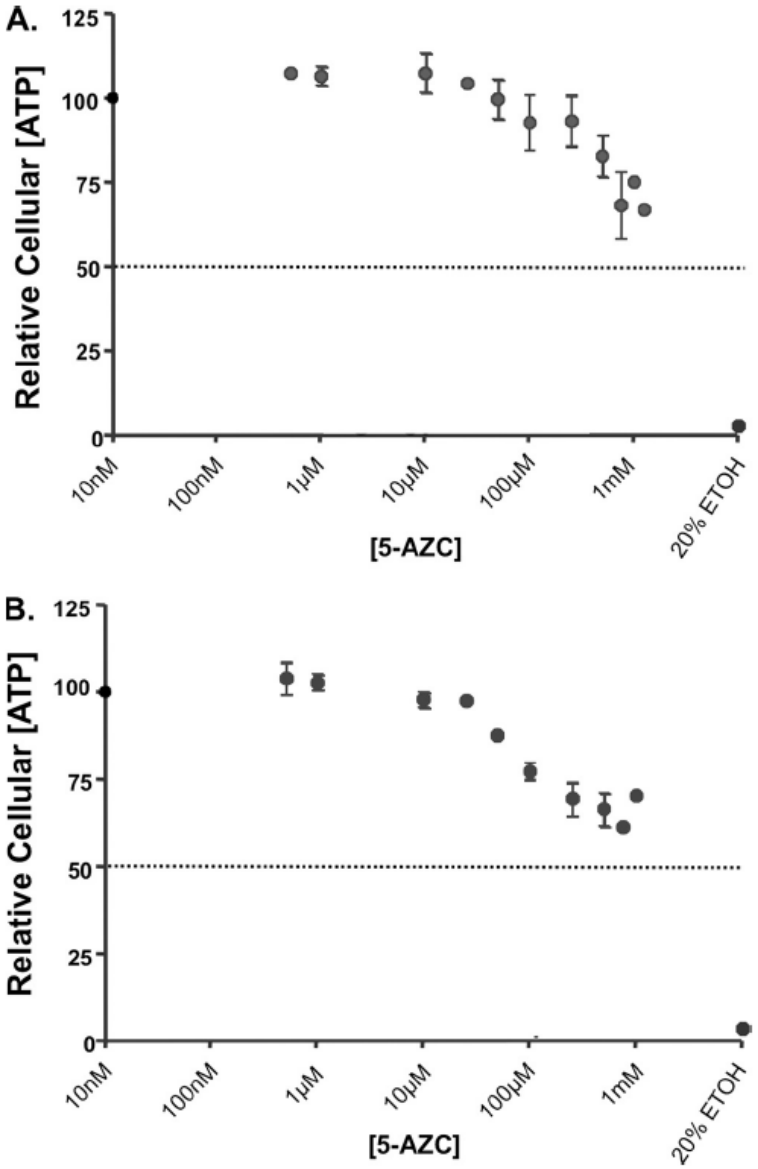
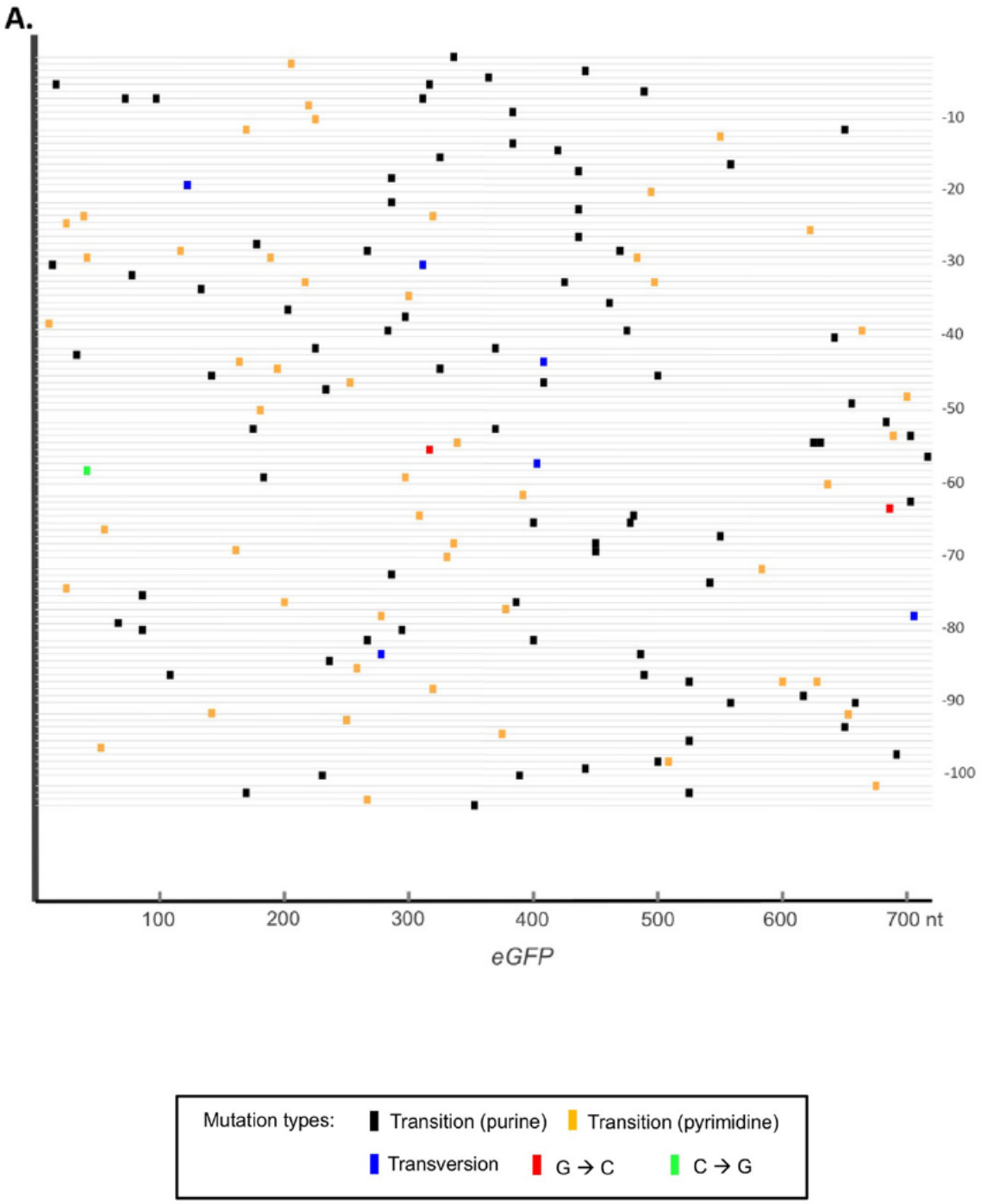
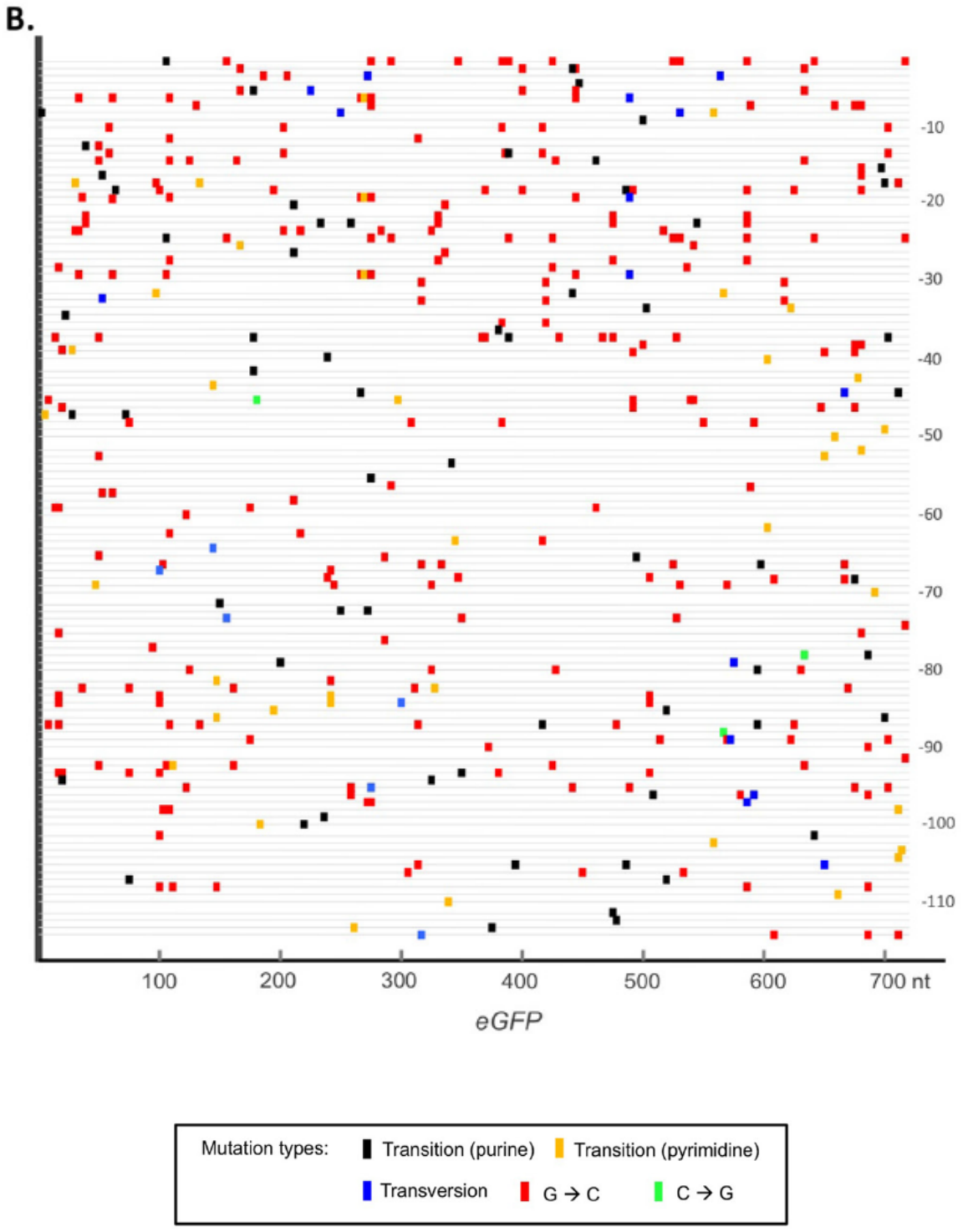


Figure 3-3. Cytotoxicity of 5-AZC. 293T **(A)** or U373-MAGI$_{CXCR4}$ **(B)** cells were treated at the indicated concentrations of 5-AZC and were analyzed for cell viability by measuring cellular ATP levels. Data represent the mean ± SD of 4 independent experiments.

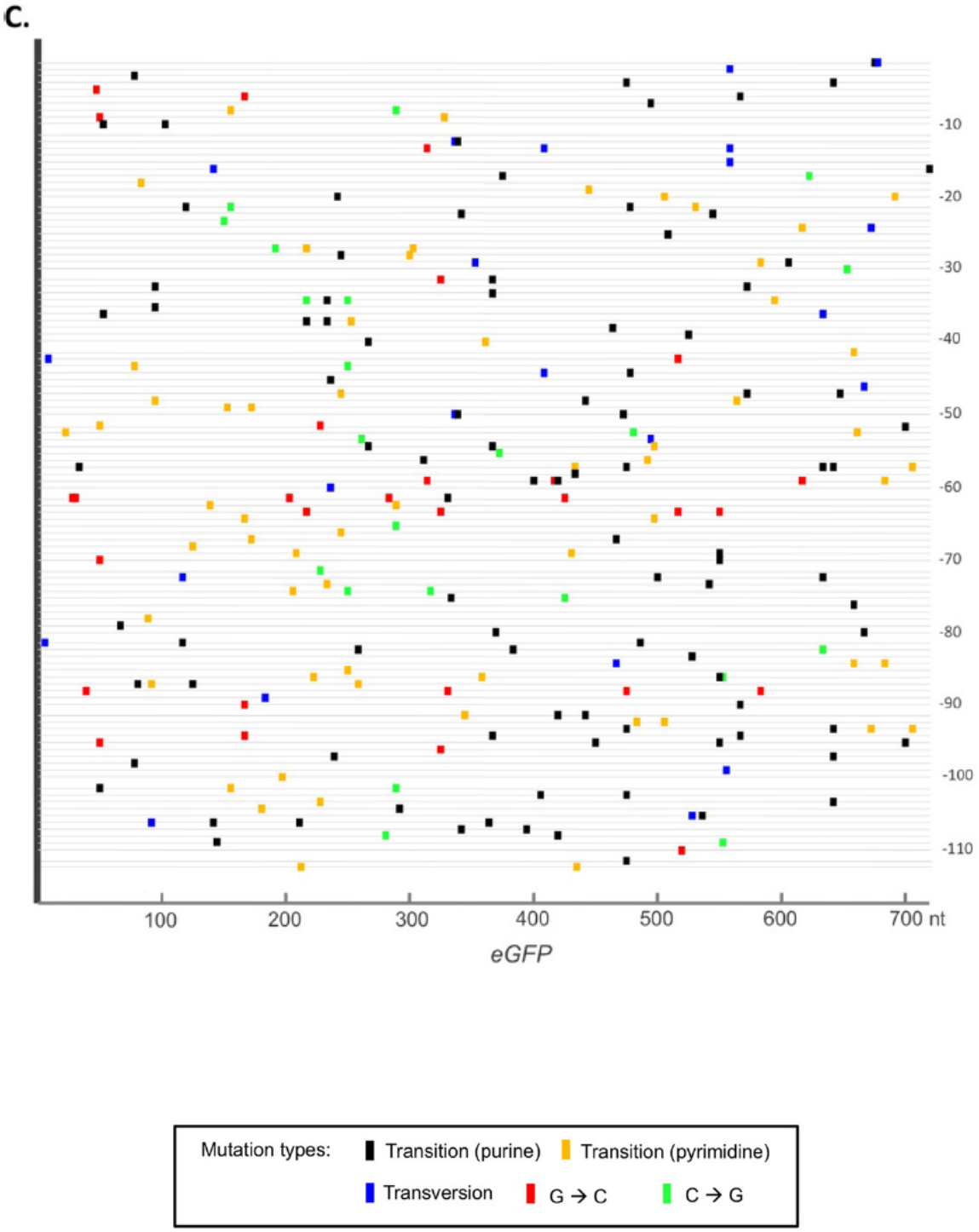
No drug control



5-AZC treatment of permissive target cells



5-AZC treatment of virus-producing cells



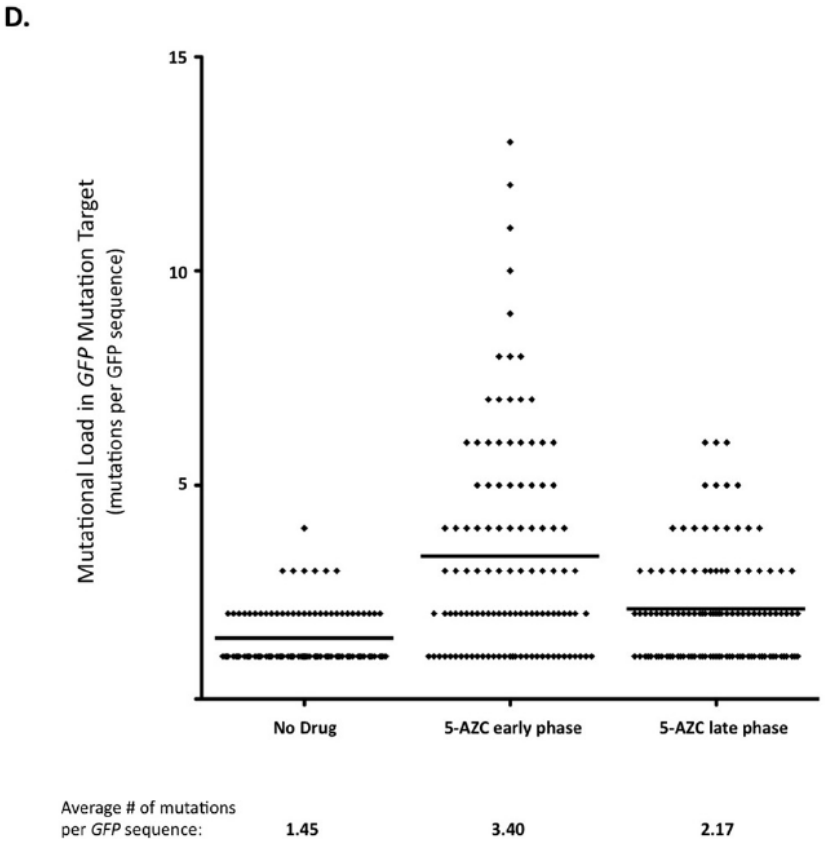


Figure 3-4. Mutation spectra of GFP gene sequences from vector proviral DNAs. The HSA^+, GFP^- cell population from infected U373-MAGI cells was sorted from cells treated with 5-AZC (Fig. 3-1). The sequence results from at least 100 mutants are shown from **(A)** no drug, **(B)** 5-AZC treatment of permissive target cells and **(C)** 5-AZC treatment of virus-producing cells. Each GFP gene sequence analyzed is represented by a thin gray horizontal line. The location of a mutation in the GFP gene sequence is indicated by a colored box perpendicular to the line relative to the 5'-end of the 720 bp ORF. Transition mutations are represented by either black (purine) or yellow (pyrimidine) rectangular boxes. G-to-C and C-to-G transversion mutations are indicated by red and green rectangular boxes, respectively. Transversion mutations (other than G-to-C and C-to-G) are indicated by blue rectangular boxes. **(D)** Mutational load in GFP gene from proviral DNAs. The summary of the mutational load (all mutation types) in the GFP gene from either no drug, 5-AZC early phase and 5-AZC late phase is shown, with each diamond symbol representing a proviral GFP sequence. The calculation of the average number of mutations per GFP target gene sequence indicated.

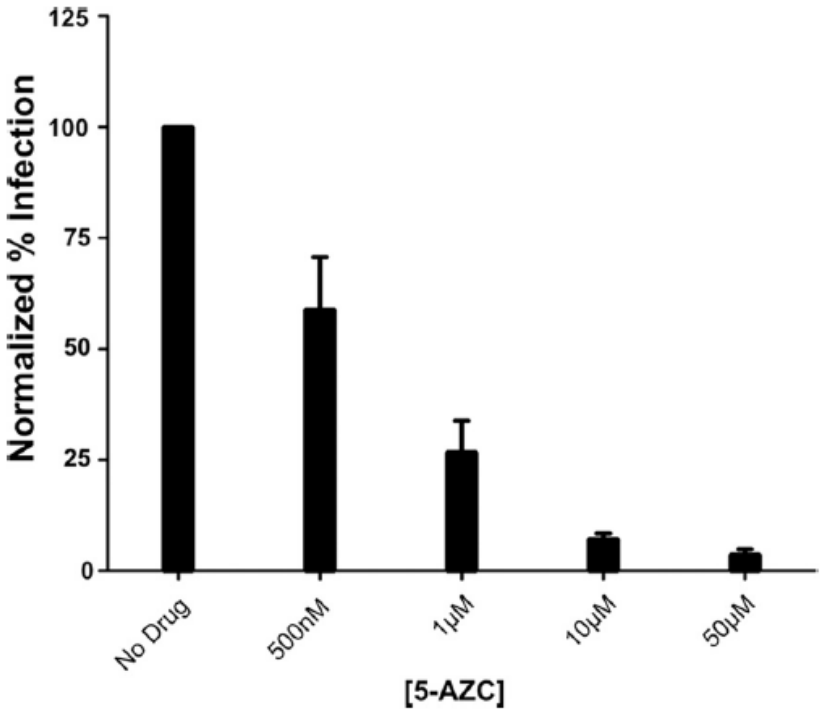


Figure 3-5. 5-AZC inhibits replication-competent HIV-1. The HIV-1 NL4-3 molecular clone was transfected into 293T cells to produce an infectious virus stock that was used to infect CEM-GFP cells. Cells were treated with 5-AZC at the indicated concentrations. Cells were split every 2 days and fresh media and 5-AZC was added. Flow cytometry was used to determine the percentage of infected cells every 2 days. Data represent the mean ± SE of parallel experiments done in triplicate from 8 days post treatment and are representative of 3 independent experiments.

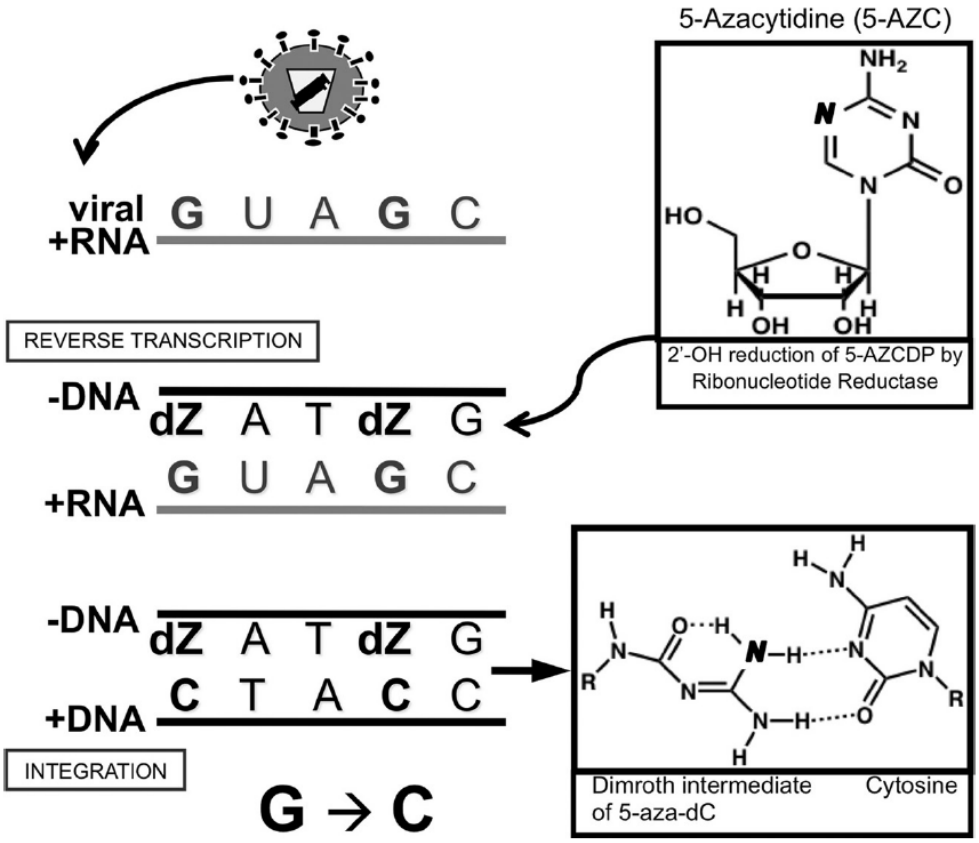


Figure 3-6. Model of 5-AZC mutagenesis during minus-strand DNA synthesis during HIV-1 reverse transcription. Ribonucleotide reductase converts 5-AZCDP to 5-aza-dCDP. After the incorporation of 5-aza-dC (dZ) triphosphate into minus-strand viral DNA, a spontaneous cytosine ring opening to a Dimroth intermediate occurs, which allows the base to pair with deoxycytidine. During integration, 5-aza-dC is excised by DNA repair machinery and replaced with a guanosine since it base pairs with the cytosine present opposite of the abasic site in the plus strand DNA. When transcribed, the guanosine in the minus strand codes for a cytosine thereby leading to an overall G-to-C mutation.

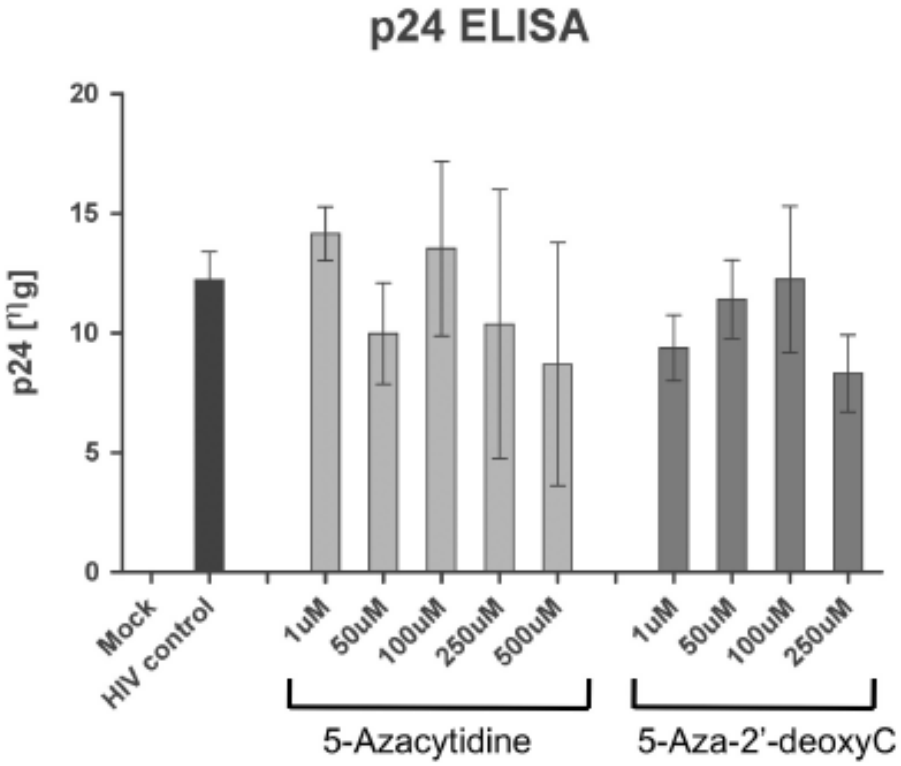


Figure 3-7. **Effects of 5-AZC on virus particle production.** 293T cells were transfected with the HIV-1 vector and HIV Env expression plasmid. Forty-eight hrs post-transfection, cell culture supernatants were collected and used for the detection of the HIV capsid protein using a p24 ELISA . Data represent the mean ± SD of 3 independent experiments.

TABLE 3-1. Mutation spectra in the enhanced GFP reporter gene from background and 5-AZC treatments

Mutation from:	Mutation to (%):											
	No drug[a]				5-AZC early phase[b]				5-AZC late phase[c]			
	A	C	G	T	A	C	G	T	A	C	G	T
A to		1	38	0		0	13	0		2	30	4
C to	1		1	11	0		1	7	1		10	13
G to	18	1		1	3	66		5	10	13		1
T to	1	23	0		0	5	0		1	13	1	
Indel	4				0				2			

[a] Total no. of sequences, 104; total no. of mutations, 152; no. of nucleotides sequenced, 74,880; mutation frequency, 0.2030%.
[b] Total no. of sequences, 114; total no. of mutations, 386; no. of nucleotides sequenced, 82,080; mutation frequency, 0.4703%; Δ in mutation frequency, 2.3167 ($P < 0.001$).
[c] Total no. of sequences, 112; total no. of mutations, 243; no. of nucleotides sequenced, 80,640; mutation frequency, 0.3013%; Δ in mutation frequency, 1.4845 ($P < 0.001$).

CHAPERTER IV

CONCOMITANT LETHAL MUTAGENESIS OF

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Reprinted with permission from: Dapp MJ, Holtz CM, Mansky LM. 2012. Concomitant Lethal Mutagenesis of Human Immunodeficiency Virus Type 1. J Mol Biol. 2012 Jun 8;419(3-4):158-70. Epub 2012 Mar 15.

Introduction

The quasispecies model describes how rapidly evolving viruses exist within a mutation-selection balance (Eigen 1993; Bull *et al.* 2005). The model asserts that high viral mutation rates drives the formation and maintenance of the quasispecies and allows for these viruses to readily adapt to a changing environment. However, the quasispecies also presents a significant obstacle in the successful treatment of rapidly evolving viruses such as HIV. It has been predicted that a purposeful increase in viral mutation rate would cause an irreversible meltdown of the genetic information (Crotty *et al.* 2001; Anderson JP 2004; Smith RA 2005; Graci *et al.* 2008). This process, termed extinction catastrophe, has been exploited as a novel therapeutic approach, yet findings from short-term cell culture based studies fail to predict the shortcomings observed clinically. For example, RNA viruses have been lethally mutagenized *in vitro* by various nucleoside analogs, including: ribavirin, 5-flurouracil, and 5-AZC (Sierra *et al.* 2000; Dapp *et al.* 2009). Specifically, ribavirin was shown to lethally mutagenize poliovirus and hepatitis C virus (Crotty *et al.* 2001; Contreras *et al.* 2002), while 5-flurouracil was shown to be an active viral mutagen against foot-and-mouth-disease virus (Sierra *et al.* 2000). The compound, 5-AZC, was also demonstrated to lethally mutagenize HIV in cell culture through induction of G-to-C mutations (Dapp *et al.* 2009). A related compound, KP1212 was shown to lethally mutate HIV in cell culture; however, the compound did not decrease viral loads or increase viral mutation loads in patient samples (Harris *et al.* 2005; Mullins *et al.* 2011). Similarly, abundant mutations were identified in patient-derived hepatitis C virus suggesting purposeful mutagenesis by the ribavirin-interferon regimen (Cuevas *et al.* 2009); however, a second study showed only a transient increase in mutation rate in pa-

tients on ribavirin monotherapy indicating that lethal mutagenesis may not be the sole antiviral mechanism (Lutchman *et al.* 2007).

Lethal mutagenesis can be induced not only by drugs, but also by the APOBEC3 (A3) family of proteins (Harris *et al.* 2003; Lecossier *et al.* 2003; Mangeat *et al.* 2003; Mariani *et al.* 2003; Zhang *et al.* 2003; Bishop *et al.* 2004; Turelli *et al.* 2004; Sasada *et al.* 2005; Chen *et al.* 2006; Zennou *et al.* 2006). These proteins have emerged as innate restriction factors that induce targeted hypermutagenesis of viral genomes. While their importance is suggested by the A3 loci's rapid evolutionary expansion, most APOBEC3 proteins seem to be active against retroviruses and retroelements. However, A3G and A3F exert potent anti-HIV activity through lethal mutagenesis (reviewed in (Albin *et al.* 2010) and (Rosenberg *et al.* 2007)). Both A3G and A3F, along with A3B, possess the capacity to restrict other retroviral genera, including: the murine leukemia virus (MLV, gammaretrovirus) (Harris *et al.* 2003; Mangeat *et al.* 2003; Bishop *et al.* 2004), human T-lymphotropic virus 1 (HTLV-1, deltaretrovirus) (Sasada *et al.* 2005), foamy viruses (spumaviruses) (Delebecque *et al.* 2006), as well as the equine infectious anemia virus (EIAV, lentivirus) (Mangeat *et al.* 2003). In addition to retroviruses, hepatitis B virus (HBV, a hepadnavirus), and adeno-associated virus (AAV, a parvovirus), are also susceptible to members of the A3 family (Turelli *et al.* 2004; Chen *et al.* 2006).

The mechanism by which A3G hypermutates retroviral genomes has been well established (reviewed in (Harris *et al.* 2004), (Malim *et al.* 2008), and (Chiu *et al.* 2008)). Briefly, A3G is packaged into budding virions, after which the virion matures and binds to a target cell. In the target cell, A3G deaminates cytosines (C) present in the single-stranded negative-sense viral DNA during reverse transcription process. The deamina-

tion of C leads to uracil (U) and this pre-mutatgenic lesion templates for adenine (A) during plus strand DNA synthesis rather than guanine (G). A3G's deamination of C during reverse transcription generates G-to-A mutation signatures in the resulting provirus(Harris *et al.* 2003). However, A3G's ability to mutate the viral genome depends on its ability to overcome viral countermeasures such as the HIV-1 Vif protein. In a host specific manner, Vif targets A3 proteins for proteosomal degradation. However, through saturating A3G levels or less-stringent Vif alleles, A3G proteins can gain access to the nascent virions and mutate the viral genome as described above. The ability of A3G to escape Vif is evident in patient samples where A3G signature mutations are found (Pace *et al.* 2006; Vazquez-Perez *et al.* 2009). A deaminase-independent mechanism has been proposed for HIV, but this model remains controversial (Shindo *et al.* 2003; Bishop *et al.* 2006; Schumacher *et al.* 2008; Browne *et al.* 2009).

Many of the compounds that lethally mutagenize HIV-1 are C analogs including KP1212, 5-OH-dC, and 5AZC(Johnson 1999; Harris *et al.* 2005; Dapp *et al.* 2009). Competitive replacement by C mutagens could interfere with A3G-mediated deamination. For instance, the kinetics of 5-AZC- and A3G-generated mutations indicate that 5-AZC incorporation into viral DNA proceeds A3G's ability to catalyze cytosine deamination. Replacement of C with 5-AZC may remove potential sites that would otherwise be mutated by A3G. Therefore, compounds targeting C residues may not be the most efficient at inducing lethal mutagenesis in the presence of APOBEC3 proteins. To examine this type of interaction, we investigated mutagen-specific alterations to both mutation spectra as well as the mutational load. Interestingly, our results show that exposure of HIV-1 to both 5-AZC and A3G simultaneously increased the frequency of G-to-A mutations at the

expense of G-to-C mutagenesis. Furthermore, the diminution of G-to-C mutations were dependent on A3G catalytic activity. These observations provide the first demonstration of potentiation for the antiviral effect of a mutagenic cytosine analog by concomitant A3G expression.

Results

Concomitant antiviral effects of 5-AZC and A3G. A single cycle vector assay was used to assess the concomitant antiviral effect of 5-AZC and A3G (Fig. 4-1). This HIV-1 vector system utilizes a dual reporter cassette to monitor both transduction efficiency as well as mutant frequency. Infectious vector virus used in this assay was produced by transfection of HIV-1 vector and HIV-1 env into previously characterized HEK 293 cell lines that stably express A3G at physiological levels (Sadler *et al.* 2010). Three of these cell lines were used in this study including: 1) a 293-A3G null vector control which does not express A3G, 2) a cell lines expressing low A3G levels (clone #4 in ref. 39) referred to here as A3G4 low and 3) a cell line expressing high A3G levels (clone #10 in ref. 39) referred to herein as A3G10 high (Sadler *et al.* 2010). Production of virus from these cells results in packaging of A3G into the virions. These virions are used to transduce permissive target cells that have been treated with increasing concentrations of 5AZC as indicated in the legend of Figure 4-2A. This system allows for both mutagens to be present during reverse transcription of the genome. Any decrease in antiviral activity can be assessed by flow cytometry as a decrease in double positive HSA/GFP-expressing cells.

Similar to previous reports (Harris *et al.* 2003; Mangeat *et al.* 2003; Zhang *et al.* 2003), A3G decreased HIV-1 replication (Fig. 4-2A) and this decrease was greater as the

expression of A3G increased (compare A3G4 low and A3G10 high to vector) (Sadler *et al.* 2010). Specifically, vector virus produced from cells expressing low A3G levels (A3G4 low) reduced viral infectivity by one-third, while virus produced from cells expressing high A3G levels (A3G 10 high) reduced viral infectivity by two-thirds. When permissive target cells were treated with various concentrations of 5-AZC (i.e., 10 μM, 50 μM or 200 μM), A3G potentiated 5-AZC's antiviral effect (Fig. 4-2A). For example, virus treated with 50 μM 5-AZC reduced infectivity by 2-fold compared to vector alone; however, this inhibitory effect was potentiated by 3.4- and 5.8-fold with concomitant exposure to A3G4 low and A3G10 high, respectively (Fig. 4-2A). The same trend was observed with concomitant treatment of 200 μM 5-AZC and increasing amounts of A3G, indicating A3G potentiates the inhibitory activity of 5-AZC (Fig. 4-2A).

The process of lethal mutagenesis suggests that a decreased infectivity should correlate with an increase in mutation frequency. Therefore, we investigated if the loss of infectivity seen in Figure 4-2A correlated with an increase in mutant frequency. To do this, the single cycle assay was used, but the data was analyzed for expression of both marker genes. For example, targets cells expressing a single marker gene (*i.e.*, [HSA+/GFP-] or [HSA-/GFP+]) indicate a loss-in-phenotype mutation arose in the vector. As indicated in Figure 4-2B, A3G expression increased viral mutant frequency. Similarly, mutant frequency was significantly increased when target cells were treated with either 50 μM or 200 μM 5-AZC. Again, the 5AZC-mediated increase in mutant frequency was potentiated by A3G expression. For example, vector virus treated with 50 μM 5-AZC increased the mutant frequency by 3.2-fold, while concomitant A3G exposure potentiated this effect by 4.3- and 4.6-fold, A3G4 low and A3G10 high, respectively (Fig. 4-2B).

Similar trends were found with concomitant A3G exposure in the presence of 200 μM 5-AZC (Fig. 4-2B).

Concomitant Exposure to 5-AZC and A3G alters HIV-1 Mutation Spectra. The increase in mutant frequency suggests that the viral genome was heavily mutated by A3G and 5-AZC. Since both mutagens target cytosines and induce distinct mutations, we examined provirus that had been exposed to A3G, 5AZC (EC_{50} concentration), or the combination of the two. Since proviruses are expected to be heavily mutated, primers were designed to minimize PCR bias caused by heavily mutated sequences (see Methods and Materials). Table 4-1 shows the cumulative mutation spectra with each mutation type denoted as the percentage of total number of mutations. These data are compiled from the sequencing data shown in Figure 4-4. As expected, A3G levels correlated to an increase in the percentage of G-to-A mutations. For example, 65% of mutations were G-to-A when sequencing provirus exposed to low A3G levels (293-low A3G) whereas 70% of mutations were G-to-A when sequencing provirus exposed to high A3G levels (293-high A3G). The lack of a more dramatic difference in the % G-to-A mutations may be due to the HIV-1 Vif protein's ability to partially restrict A3G activity in these cells or because of the unexpectedly high number of G-to-A mutations at non A3G signature sites (5'-GG-3') found in the 293-low A3G expressing vector (Fig. 4-3C). In contrast, 5AZC induced a robust increase (61%) in the number of G-to-C transversions compared to untreated cells (Table 4-1) as previously reported (Dapp *et al.* 2009).

Since both A3G and 5AZC induce mutations by targeting C residues, we next examined viral mutation spectra of combined expression of A3G with 5AZC treatment. As shown in Table 4-1, viral genomes exposed to the 5-AZC EC_{50} with increasing levels of

A3G, significantly decreases the percentage of G-to-C transversion mutations. For example, 61% of mutations were G-to-C in provirus that was exposed to 5-AZC alone whereas 41% of mutations were G to C when provirus was exposed to both 5-AZC and a low level of A3G. Finally, only 20% of mutations were G-to-C when provirus was exposed to both 5-AZC and a high level of A3G. The increase in the number of G-to-A and G-to-C mutation types, suggests that A3G and 5-AZC significantly increased the viral mutation frequency, which is consistent with lethal mutagenesis.

A statistical analysis was performed to determine if either 5-AZC-induced G-to-C mutations (denoted by blue marks in Fig. 4-3) or A3G-induced G-to-A mutations (denoted by red marks in Fig. 4-3) were influenced by preference sites (hotspots). As expected by 5-AZC's random substitution for C bases, the hotspot prediction algorithm CLUSTERM found no G-to-C hotspots within GFP. Alternatively, as described in previous A3G studies, the clustering of G-to-A mutations at GG sites was predicted by the algorithm [(Rogozin *et al.* 2003; Rogozin *et al.* 2003) and personal communications with I.B. Rogozin]. Of the 51 di-G nucleotide positions in *eGFP*'s plus strand, 26 such locations were found to be A3G preference sites.

Disparate Mutational Load with Concomitant 5-AZC and A3G Exposure. While mutational spectra analysis offers a population view of the 5-AZC and A3G exposed virus, analysis of mutational load can discern differences among individual viral clones. The mutational load was determined for each mutation type of interest (i.e. G-to-C and G-to-A) by finding the mean number of mutations per viral clone (Table 4-2). As expected, the G-to-A mutational load increased from 0.03% to 0.35% and 0.37%, when comparing viral clones unexposed to A3G to those exposed to A3G low and A3G high

levels, respectively (Table 4-2). The G-to-C mutational load was also increased to 0.31% relative to no drug controls (Table 4-2). Unexpectedly, the G-to-A mutational load was 18% higher ($p = 0.026$) when A3G and 5-AZC exposure was combined, compared to this amount of A3G alone (Table 4-2). Moreover, the concomitant exposure of A3G and 5-AZC also diminished the G-to-C mutational load by 36% ($p = 0.013$) compared to drug alone. These results suggest that 5-AZC and A3G may interact to influence the G-to-A and G-to-C mutational load.

A3G catalytic activity is required for reductions in 5-AZC-mediated G-to-C mutations. Since A3G has been shown to possess catalytic-independent antiviral activity, we next examined the effect of a catalytically inactive A3G on the transduction efficiency. Results show that the A3G_E259Q mutant has no effect on transduction efficiency or mutant frequency, compared to vector alone (Fig. 4-4 A and 4-4 B). Similarly, there was no difference in G-to-C mutation spectra under concomitant exposure to 5-AZC and A3G_E259Q compared to 5-AZC alone, 57% *versus* 59%, respectively (Table 4-3). Immunoblot analysis of A3G virion incorporation indicated that the A3G_E259Q was as efficiently packaged into HIV-1 particles as wt A3G (Fig. 4-4C). These data indicate that A3G catalytic activity is necessary for the decrease in transduction efficiency as well as the potentiation of mutant frequency during concomitant 5-AZC exposure.

Discussion

Lethal mutagenesis as a therapeutic strategy has gained momentum, especially after ribavirin and KP-1212 gained clinical use (Contreras *et al.* 2002; Mullins *et al.* 2011). Most of the exploratory HIV viral mutagens target C analogs including the tria-

zole base, 5-AZC. Since the APOBEC3 protein subfamily also targets C bases for deamination, these mutagens may exert redundant mechanisms when combined. This would suggest that compounds targeting C bases could be less effective mutagens in the presence of A3G. Alternatively, incorporation of 5-AZC could have secondary effects by altering A3G substrate specificity or target site architecture. In this study, we examined concomitant exposure of HIV-1 to 5-AZC and A3G. The ability of A3G to induce G-to-A hypermutagenesis provides an example of an evolutionary conserved mechanism that eliminates HIV infectivity by lethal mutagenesis. Editing of retroviral genomes by A3G in the face of concomitant mutagens has not been previously explored.

5-AZC is a first-in-class hypomethylating agent as substitution of N-5 can no longer be methylated (Kaminskas *et al.* 2005). The ribonucleoside 5-AZC undergoes anabolic metabolism to the corresponding triphosphate, but a fraction is converted to the nucleotide triphosphate, 5-aza-2'-deoxycytidine (5-AZdC), and is substituted for dCTP in DNA (Paces *et al.* 1968; Li *et al.* 1970). Because some azapyrimidines are chemically unstable, ring-opened intermediates may have the potential for non-Watson-Crick base pairing, leading to base mispairing (Rogstad *et al.* 2009). In fact, 5-AZC has been implicated in several studies as a mutagen, specifically introducing a rare GC-to-CG transversion type (Cupples *et al.* 1989; Pathak *et al.* 1992; Jackson-Grusby *et al.* 1997; Dapp *et al.* 2009). Experimentation with an HIV-1 vector system found that 5-AZC increased the preponderance of only G-to-C mutations, suggesting that the mutagenic product of azacytosine is able to template for C:C mispairings when incorporated during minus-strand DNA synthesis (Dapp *et al.* 2009).

Intriguingly, the majority of retroviral minus-strand DNA is also transiently sin-

gle-stranded during replication, and ssDNA is the preferential substrate of the APOBEC3 subfamily (Harris *et al.* 2003). During HIV-1 reverse transcription, A3G gains access to ssDNA, honing to cytosine residues for deamination to pre-mutagenic lesions (Yu *et al.* 2004). These kinetics, of A3G and 5-AZC-based mutagenesis, suggest that access to minus-strand ssDNA by A3G is subsequent to RT incorporation of 5-AZdC during minus-strand DNA synthesis.

The interaction between A3G and the non-canonical 5-azacytosine base could result in two possible obvious outcomes: 1) A3G is unable to engage 5-azacytosine or its ring-opened decomposition products. This would be observed as a decrease in G-to-A mutations with no difference in the number of G-to-C mutations; 5-azacytosine would effectively antagonize A3G deaminase activity. 2) A3G is able to catalyze deamination of 5-azacytosine to 5-azauracil and this uracil analog, results in increased G-to-A mutations at the expense of G-to-C mutation types. However, other mechanisms cannot be excluded at this time, such as: the ability of 5-azacytosine (or its ring-opened products) to alter local DNA secondary structure and subsequent ssDNA availability, or the influence of 5-azacytosine on A3G processivity or substrate specificity due to close-proximity base interactions, as suggested by Rausch et al. (Rausch *et al.* 2009).

In order to understand mutation type-specific differences of concomitant mutagen exposure, proviral DNA was sequenced. The G-to-A mutational load (i.e., # of G-to-A mutations/clone) was increased up to 18% in virus exposed to both 5-AZC and A3G, as compared to A3G exposure alone. This effect was at the expense of a 36% decrease to the 5-AZC-induced mutational load (i.e., # of G-to-C mutations/sequence). These results invoke a model whereby 5-AZC incorporation into retroviral DNA causes complex inter-

actions with A3G, to inversely shift the G-to-A and G-to-C mutational loads. Furthermore, the E259Q catalytically inactive A3G with 5-AZC showed no difference in G-to-C transversions compared to drug alone, suggesting a requirement for fully functional A3G.

One potential concern regarding the sequencing data is biased amplification due to the oligonucleotides selected. Particular attention to primer design helped ensure universal and unbiased PCR. For example, the location of each oligonucleotide was carefully adjusted such that no di-GG nucleotide motif was positioned in the forward primer (and no di-CC nucleotide motif in the reverse primer) to eliminate the potential for misannealing due to the creation of A3G signatures. Moreover, no more than two G nucleotides we positioned at the forward primer regions (no more than two C nucleotides in the reverse primer region) while these potentially mutable bases were substituted with the degenerate S (50% G and 50% C), to exclude 5-AZC biased amplification. These primers amplified heavily mutated sequences in each treatment group (23 mutations/ 720 bp), indicating that the sequenced were not biased against any mutation type.

Presently, concomitant use of viral mutagens and chain terminators shows efficacy against RNA viruses (Pariente *et al.* 2001; Pariente *et al.* 2003; Perales *et al.* 2009; Perales *et al.* 2009; Clouser *et al.* 2010; Perales *et al.* 2011). For example, Perales et al. demonstrated that in foot-and-mouth disease virus, sequential treatment with a traditional antiviral inhibitor followed by the viral mutagen ribavirin was much more effective in extinguishing picornaviridae replication than these compounds used together, or either one alone (Perales *et al.* 2009). Future progress of mutagen utilization hinges on understanding the mutational constraints within RNA virus population structure. For example, sub-restrictive editing of retroviral genomes by APOBEC3 proteins has the potential to

negatively influence therapeutic intervention, by rapid generation of drug-resistant mutants (Mulder *et al.* 2008; Kim *et al.* 2010). Furthermore, quasispecies theory predicts that populations will evolve more robust genomes, termed survival of the flattest, in the face of increased mutational load (Codoner *et al.* 2006; Sanjuan *et al.* 2007). Alternative adaptive strategies include selection of anti-mutator viral polymerases (Pfeiffer *et al.* 2003; Levi *et al.* 2010; Arribas *et al.* 2011) or enhanced discrimination between correct and mutagenic nucleotides (Sierra *et al.* 2007). Evolution of drug resistance, or increased robustness, of mutagens can threaten such therapeutic approaches; yet, discovery of novel viral mutagens, as well as optimal applications, may lead to alternative therapeutic strategies.

Understanding molecular details of potential mutagens as well as sequence space limitations of specific viral pathogens may provide a rationale for tailored therapeutic intervention rather than lengthy trial-and-error pursuits. Generally, HIV-1 viral mutagens are stealth nucleosides directly utilized by RT during replication to induce site-specific mutations. These compounds are referred to as universal bases because they can mispair with more than one of the canonical Watson-Crick base pairs. For instance, the viral mutagen KP1212, can base pair with either G or A due to a tautormeric shift in the pyrimidine base. While, 5-AZC, a close derivative to KP1212, induces a unique G-to-C transversion mutational pattern during HIV-1 replication because of its ability to mispair with C bases (Jackson-Grusby *et al.* 1997; Dapp *et al.* 2009; Rogstad *et al.* 2009). Even by understanding specific molecular details, viral mutagens still lack a reliable framework to help predict successful treatment outcomes. Many parameters involved in understanding virus behavior are not fully understood, including: sequence space, mutational robustness,

effective population size, as well as the natural fitness landscape. Similarly, since nucleotide base composition among viral *genera* is known to be quite distinct, purposeful alterations to the mutational bias of a particular virus may pose a greater defect to fitness. For example, since HIV-1 has an unusually A-rich genome (Berkhout *et al.* 1994), it is not clear if mutagens that cause more N-to-A mutations (versus A-to-N) are more detrimental to viral fitness.

In summary, we describe concomitant HIV-1 mutagenesis using two unrelated classes of viral mutagens. Our findings indicate a combined yet intricate interaction between the 5-AZC and A3G. The combined antiviral effect indicated that A3G potentiated the antiviral effect of 5-AZC. Sequencing analysis revealed that the combined mutagenic effect resulted in an increase in the frequency of G-to-A mutations at the expense of G-to-C mutations, suggesting a complex interaction between A3G and 5-AZC upon incorporation into viral DNA. Future studies to further define the molecular interaction between A3G and 5-AZC will provide greater details into the molecular mechanism.

Materials and Methods

Plasmids constructs and cell lines. The HIV-1 vector pHIG has been previously described (Dapp *et al.* 2009). Briefly, a ~2.0 kbp dual reporter cassette comprised of the murine heat stable antigen (HSA), an IRES element, and eGFP was placed in frame and 3' to the NL4-3 *nef* start codon. The G protein of vesicular stomatitis virus (VSV-G) envelope expression plasmid HCM-VG was used to pseudotype virions and was a kind gift from J. Burns (UCSD). The A3G-expressing cell lines 293-A3G clone 4 and 293-A3G clone10 were previously characterized in Sadler et al. (Sadler *et al.* 2010). The APO-

BEC-expression plasmids pcDNA3.1^{+}-A3G-3xHA and pcDNA3.1^{+}-A3G_E259Q_-3xHA were a kind gift from R. Harris (University of Minnesota). U373-MAGI$_{CXCR4}$ target cells lines were obtained from the AIDS Reagent Program (from M. Emerman).

Single-cycle transduction and mutant frequency assays. pHIG vector virus was produced by transient transfection of 293 cell lines. Producer cell lines were transfected with 10μg pHIG and 1μg VSV-G env plasmids using Genjet ver. II reagent (SignaGen Rockville, MD) as per manufacturer's recommendations. Twenty-four hours after transfection, viral supernatants were collected and passed through a 0.2-μm filter. Viral stocks were normalized by transfection efficiency, measured by %GFP-expressing producer cells. Respective viral stocks were either used to transduce target cells or stored at -80°C. The single-cycle transduction efficiency for the control A3G null virus was set to 30%. U373-MAGI$_{CXCR4}$ target cells were plated in flat bottom 12-well dish at concentration of 62.5 x10^{5} cells per mL 24-hr prior to transduction. Experiments with the viral mutagen 5-AZC (Sigma-Aldrich St. Louis, MO) included a 2-hr pre-treatment of 1:1000 dilution prior to addition of normalized viral supernatant up to 1mL total volume. At 24-hr post-transduction, culture media was changed to remove residual drug and virus.

Flow cytometry analysis. Target cells were prepared for flow cytometry to quantify transduction efficiency (via both GFP and HSA reporter gene expression) and mutant phenotype (via lack of GFP or HSA reporter gene expression). At 72-hr post transduction cells were collected and stained with 1:250 anti-HSA-PE (BD Pharmingen). Following 20-min incubation at 4°C, cells were washed 1x in PBS-2% FC3, and resuspended at an adequate concentration for downstream flow cytometry analysis. Mutant frequency analysis was determined from the % target cells expressing a single reporter gene relative

to entire transduced population (i.e., %[HSA+/GFP-] plus %[HSA-/GFP+] divided by % of total transduced cells). Mutant frequencies were set relative to virus null for A3G in the absence of drug. Cells were analyzed with a FACScan (BD Biosciences) and CellQuest software. Cells were gated by morphology (FFS vs. SSC) by counting 10,000 cells for fluorescence analysis. Excitation was done at 488 nm; fluorescence channel 1 (FL1) detected GFP emission at 507nm, and FL2 detected PE emission at 578nm. Compensation was set based on single color controls to eliminate spill-over and re-verified based on the geometric mean of single-color positive to negative-detected populations.

Proviral DNA sequence analyses. Approximately 25,000 target cells from each treatment group (+/- A3G and +/- 5-AZC) were collected for total genomic DNA isolation (Roche High Pure PCR Template Preparation Kit) and subjected to nested PCR of the vector GFP gene. Primer sets were carefully designed as to remove any bias in amplicon pool considering mutagenic potential of the agents used. Specifically, forward primers did not contain any 5'-GG-3' motifs while reverse primers were absent of 5'-CC-3' motifs to remove the selection by A3G. Additionally, forward primers were located to regions containing only 2 Gs (same for reverse, however regions contained only 2 Cs) but these sites were designed to contain a G or C, denoted by S. This approach limited the 5-AZC-induced G-to-C bias that may be excluded from the amplicon pool. Within the pHIG vector these primer sets would amplify a 944pb region of GFP: outer primer pair, 5'-CTCAATSCCACASCCATA-3' and 5'-GTSTTSTTTGGGAGTGAATTAG-3'; inner primer pair, 5'-CTCTCCTCAASCSTATTCAAC-3' and 5'-GGTATGGSTGATTATGATSTAGAGT-3'. Both PCR reactions were run using Platinum PCR Supermix (Invitrogen), while the initial PCR reaction was purified with GenE-

lute PCR Clean-up Kit (Sigma). Amplicons were verified for correct size and purity by DNA gel imaging before 24-hr ligation at 4 °C into the pGEMT vector (Promega). Plasmids were transformed into *E. coli*, and insert containing-vectors were purified using (DirectPrep 96 Miniprep Kit , Qiagen) then sent for sequence analysis (Functional Bio-sciences; Madison, WI). Sequence alignments were performed using SeqMan of Lasergene 7 (DNAStar; Madison, WI). Only sequence traces covering the 720bp ORF of GFP were considered for analysis. This region was chosen because it has been subjected to extensive sequencing analysis by independent studies related to A3G and 5-AZC target site preferences and mutation spectra (Harris *et al.* 2003; Liddament *et al.* 2004; Hache *et al.* 2005; Dapp *et al.* 2009).

Immunoblot analysis of transiently transfected A3G-containing particles. HEK 293T cells were transfected with 10 μg pHIG, 1 μg VSV-G env, and either 5 μg pcDNA3.1^{+}-A3G-3xHA or pcDNA3.1^{+}-A3G_E259Q_-3xHA or 5μg empty vector, using Genjet ver. II reagent as per manufacturer's recommendations. Twenty-four hours after transfection, viral supernatants were collected and passed through a 0.2-μm filter. 1.5mL of each virus supernatant was spun on tabletop centrifuge 16,000xg for 2-hr at 4°C. Supernatant was removed and 2.5 μL of 10X RIPA buffer and 2.5 μL of 10X protease inhibitor cocktail was added for incubation on ice for 15 min. Samples were then denatured with addition of 6X loading dye and 10 min boil before fractionation on a 4-20% denaturing gel. Proteins were transferred to nitrocellulose membrane using BioRad Semi-dry transfer system. To detect A3G and A3G_E259Q -containing particles, the blot was probed with the 1° monoclonal HA.11 (Covance; Emeryville, CA) and 2° goat anti-mouse HRP conjugate (Invitrogen). To detect capsid p24, the blot was probed with 1°

rabbit anti-p24 (Advanced Biotechnologies; Columbia, MD) and 2° goat anti-rabbit IgG (H+L)-HRP conjugate (BioRad). The blot was imaged with a BioRad ChemiDoc Imager with Quantity One software (version 4.5.2, BioRad).

Statistical analysis. All statistical analyses and graphical representation were done using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Bars graphs of transduction efficiency and mutant frequency data are shown with mean +/- standard deviation. Statistical analysis of mutational load differences was performed using a chi-squared contingency table, comparing the number of mutated sites to the number of non-mutated sites amongst each group (G-to-A and G-to-C). A3G hotspot prediction was performed using CLUSTERM (http://www.itba.mi.cnr.it/webmutation and ftp://ftp.bionet.nsc.ru/pub/biology/dbms/ CLUSTERM.ZIP) This algorithm predicts a hotspot threshold for the number of mutations at a particular site. The threshold number is established by analyzing the frequency distribution of mutations amongst a predetermined genomic region (Rogozin *et al.* 2003).

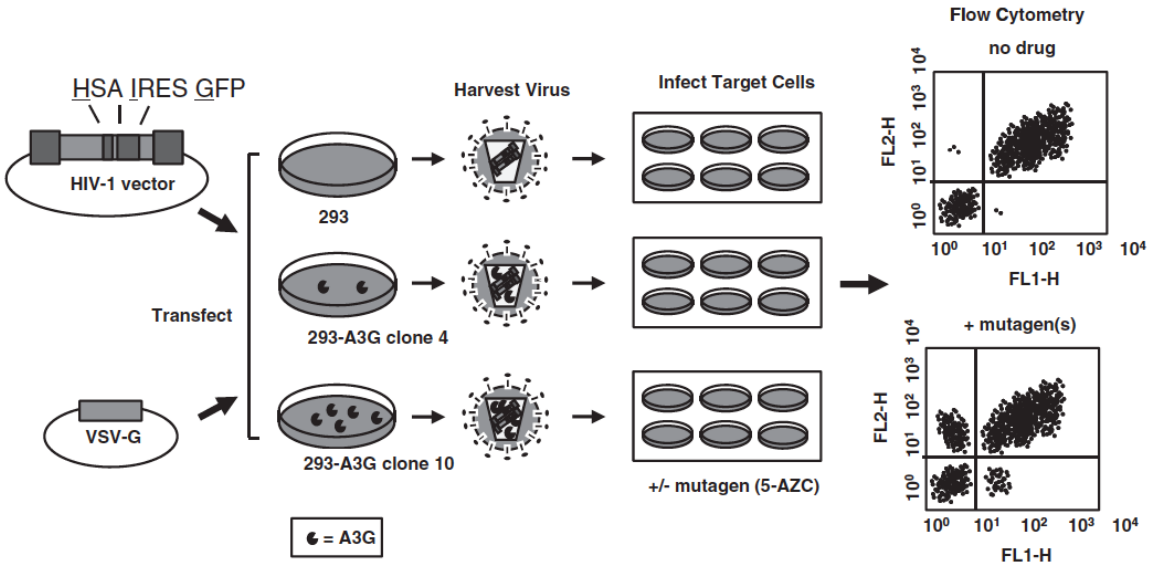


Figure 4-1. HIV-1 vector assay for analysis of viral mutagens. The HIV-1 vector (Dapp *et al.*) was co-transfected with a VSVG envelope plasmid into previously characterized 293 cell lines, expressing a physiologic range in APOBEC3G protein levels (Sadler *et al.*). Cell culture supernatants were collected, filtered, and virus was titred on permissive U373-MAGI$_{CXCR4}$ target cells. After respective viral titre normalization, target cells were pre-treated for 2-hr with 5-AZC and transduced at an MOI of 0.30. 72-hr post-transduction, cells were collected for flow cytometry and sequence analysis. MOI, multiplicity of infection. VSVG, vesicular stomatitis virus glycoprotein. HSA, murine heat stable antigen (CD24). IRES, internal ribosome entry site. GFP, green fluorescent protein. A3G, APOBEC3G. FL1-H and FL2-H, fluorescence channel, 1 and 2, height of intensity.

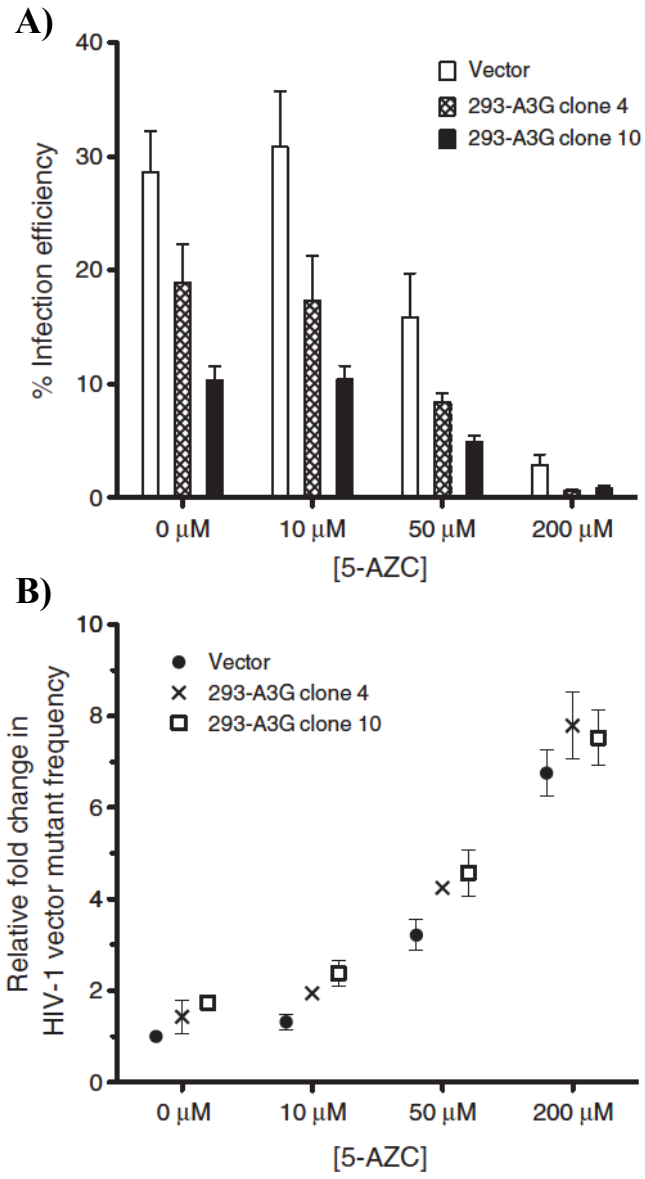
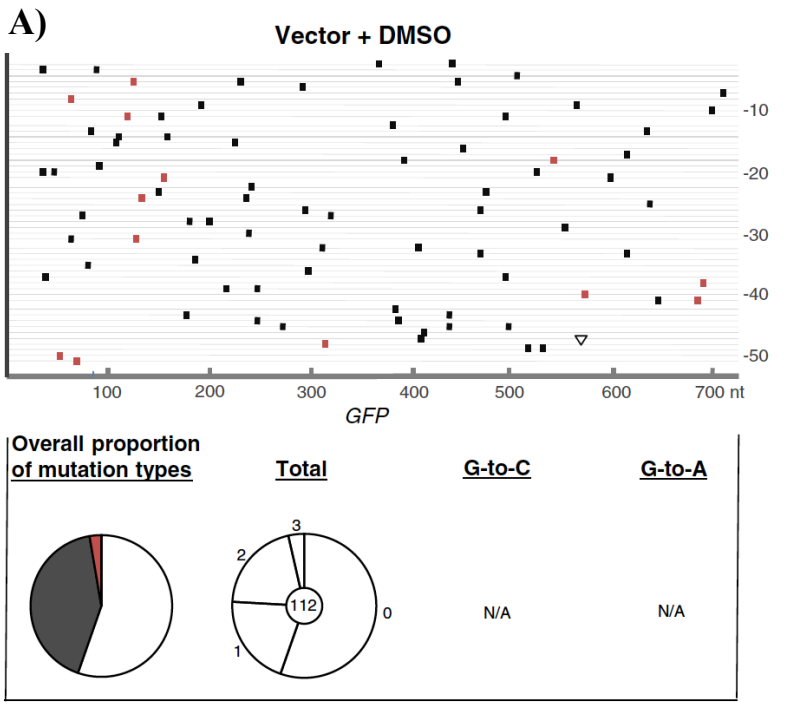
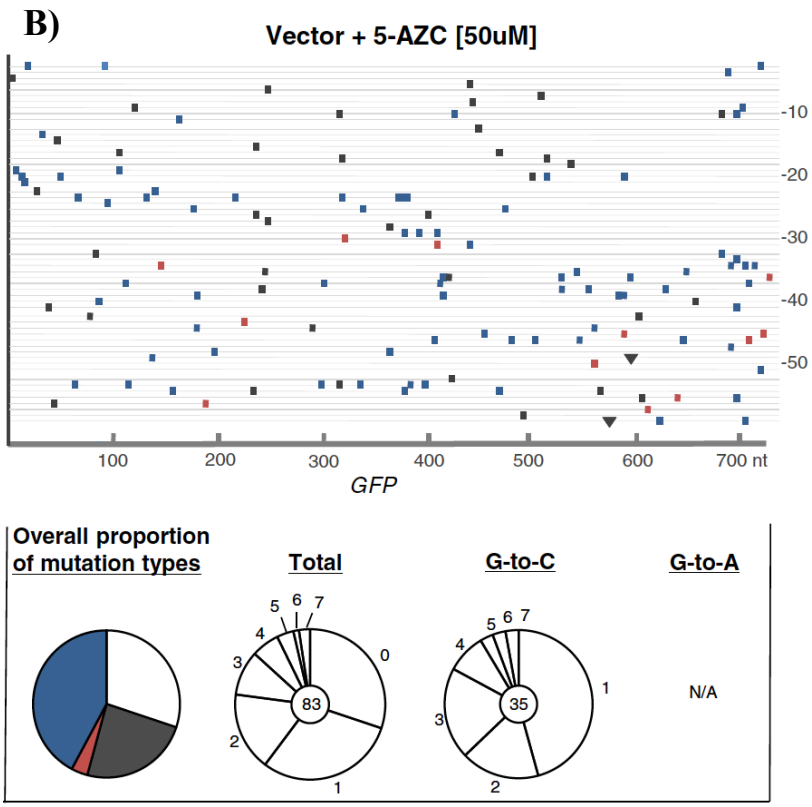
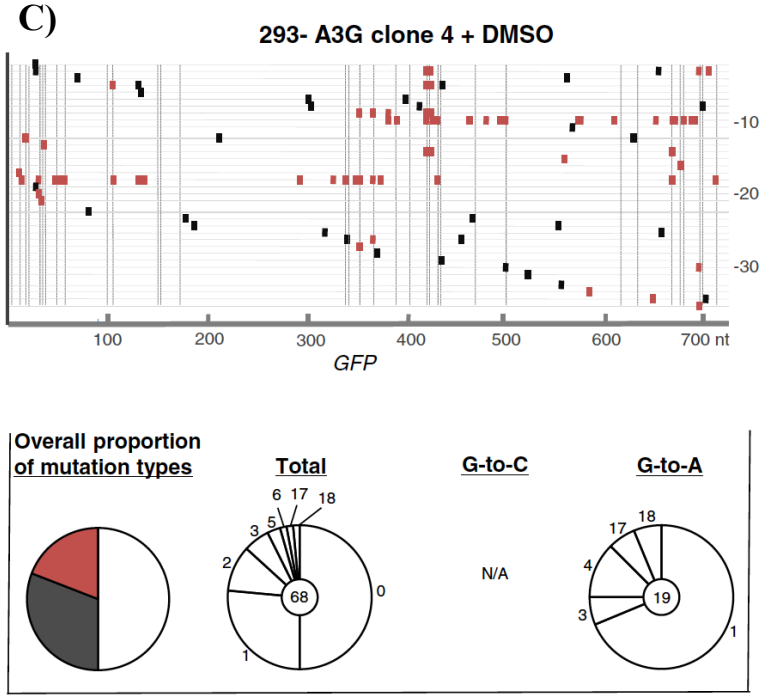


Figure 4-2. Concentration-dependent inhibition of 5-AZC of HIV-1 replication in the presence of A3G. A) Viral stocks, produced from A3G-expressing cell lines (Sadler *et al.* 2010) were normalized. U373-MAGI$_{CXCR4}$ cells were pretreated for 2-hr with the indicated concentrations of 5-AZC prior to addition of viral supernatant. Transduction efficiency determined by positive expression of both marker genes. **B)** Fold-change shows mutant frequency relative to vector + vehicle control. Data represent mean fold change ± SD from at least 3 independent replicates. Mutant frequency calculated by phenotypic expression of marker genes within pHIG vector, see Materials and Methods.

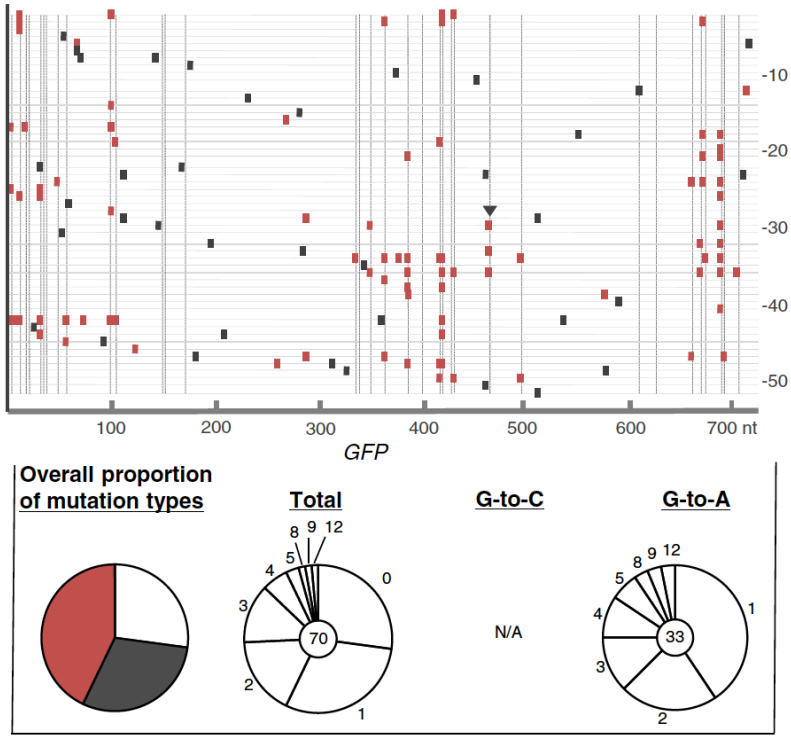

A)
Vector + DMSO
-10
-20
-30
-40
-50
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
3
2
112
0
1
N/A
N/A


B)
Vector + 5-AZC [50uM]
-10
-20
-30
-40
-50
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
5
6
7
4
3
83
0
2
1
5
6
7
4
35
1
3
2
N/A

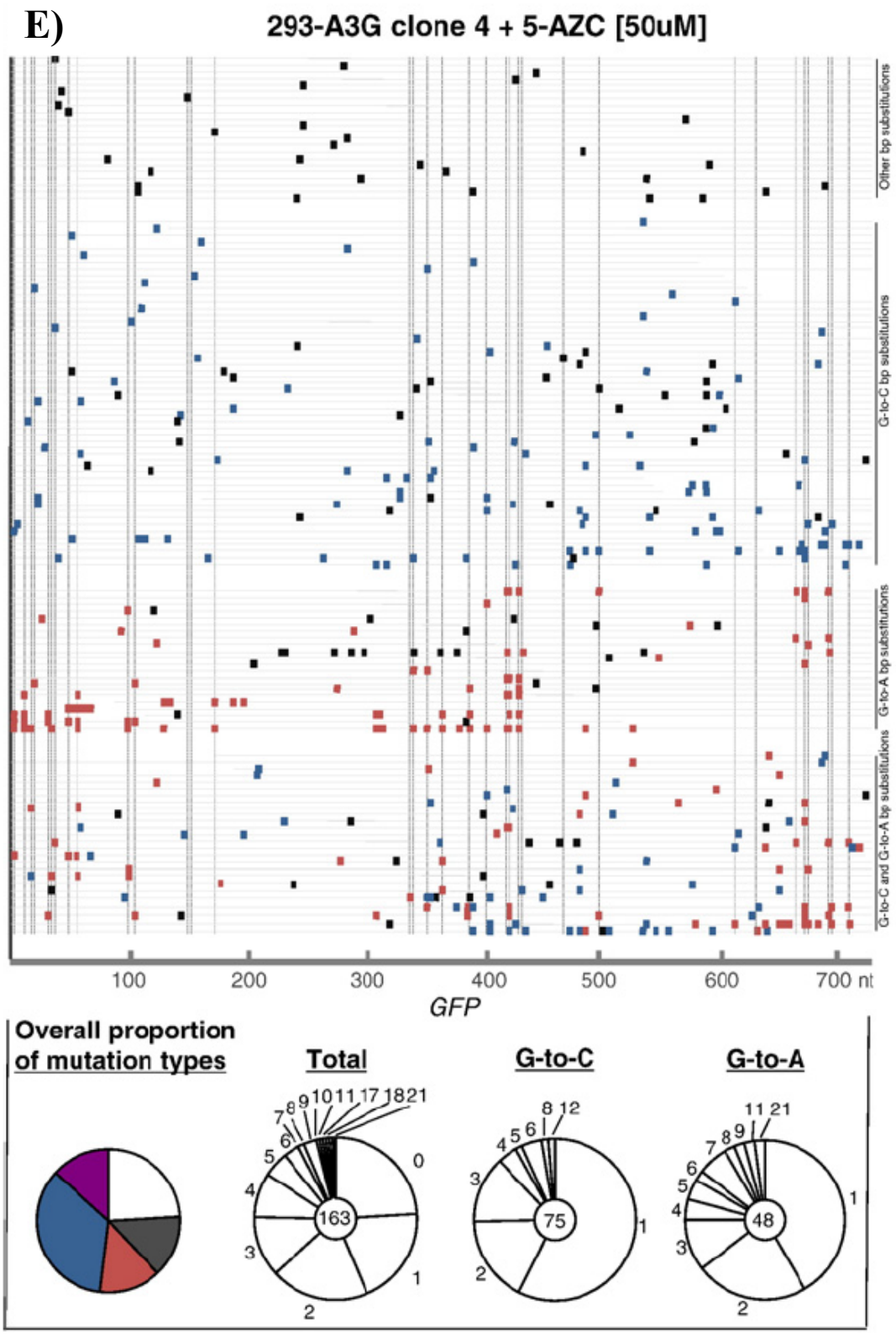
C)


293- A3G clone 4 + DMSO
-10
-20
-30
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
6 17 18
3 5
2
68
0
1
N/A
17 18
4
19
3
1

D)


293-A3G clone 10 + DMSO
-10
-20
-30
-40
-50
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
8 9 12
5
4
0
3
70
2
1
N/A
9 12
8
5
4
1
33
3
2

E)


293-A3G clone 4 + 5-AZC [50uM]
Other bp substitutions
G-to-C bp substitutions
G-to-A bp substitutions
G-to-C and G-to-A bp substitutions
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
163
75
48

F)

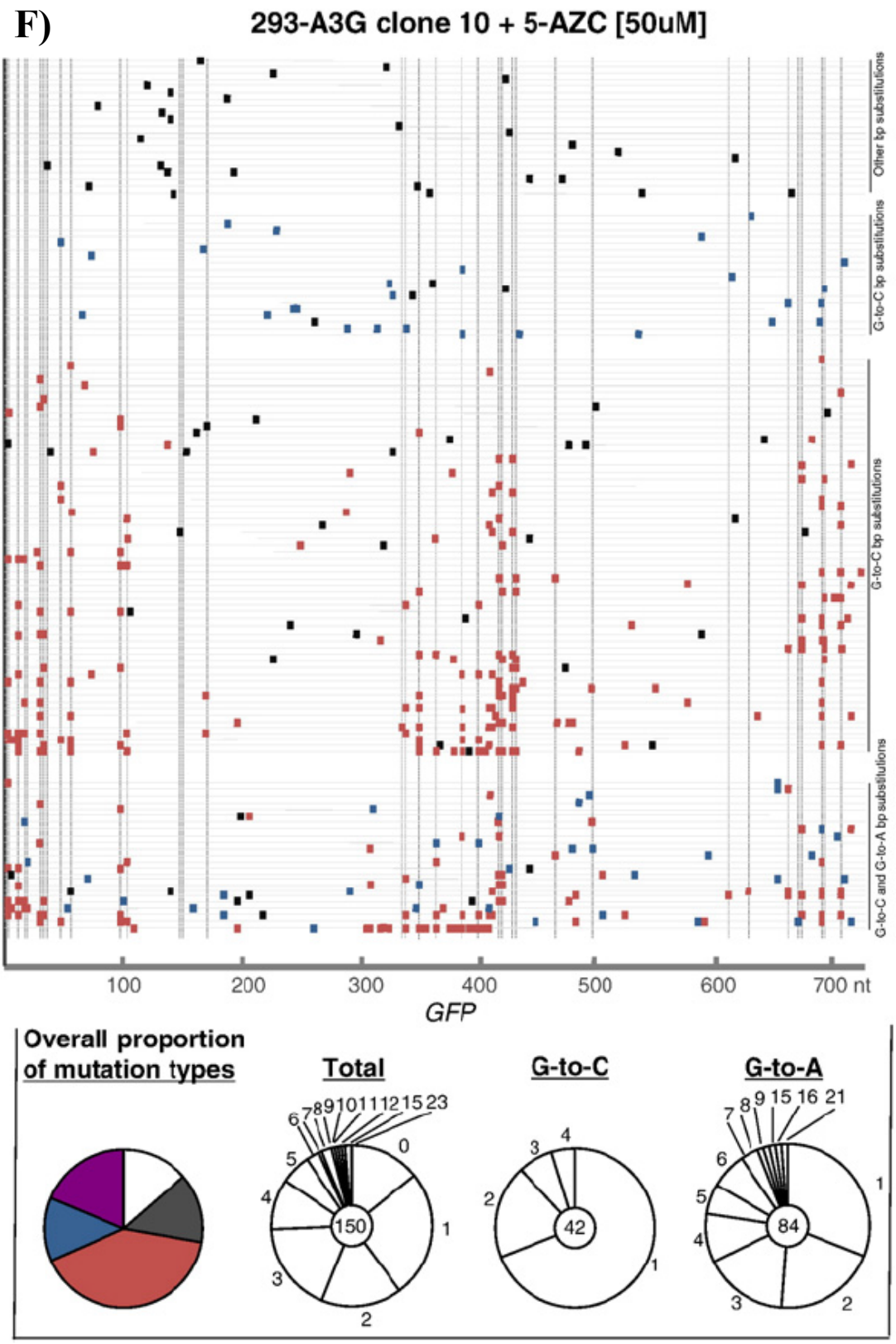

293-A3G clone 10 + 5-AZC [50uM]
Other bp substitutions
G-to-C bp substitutions
G-to-C bp substitutions
G-to-C and G-to-A bp substitutions
100
200
300
400
500
600
700 nt
GFP
Overall proportion of mutation types
Total
G-to-C
G-to-A
150
42
84

Figure 4-3. Mutation spectra of GFP sequences obtained from proviral clones. Proviral HIV-1 DNA was purified, amplified, and cloned for individual sequence analysis. **A)** Vector + vehicle control, **B)** vector + 5-AZC, **C)** A3G4 (low) + vehicle, **D)** A3G10 (high) + vehicle, **E)** A3G4 (low) + 5-AZC, **F)** A3G10 (high) + 5-AZC. Each horizontal line denotes an individual non-redundant proviral GFP gene sequence. The location of a mutation is indicated by a colored box perpendicular to the line, relative to the 720-bp eGFP open reading frame. Predominate mutation types are denoted in the legend, represented by pink (G-to-A) or blue (G-to-C) boxes. Black boxes (other) denote all other transition and transversion mutation types. Insertions (filled triangle) and deletions (open triangle) are also represented, although found infrequently. Dashed vertical lines in panels **C-F** signify predicted A3G hotspots using CLUSTERM analysis (see Materials and Methods).

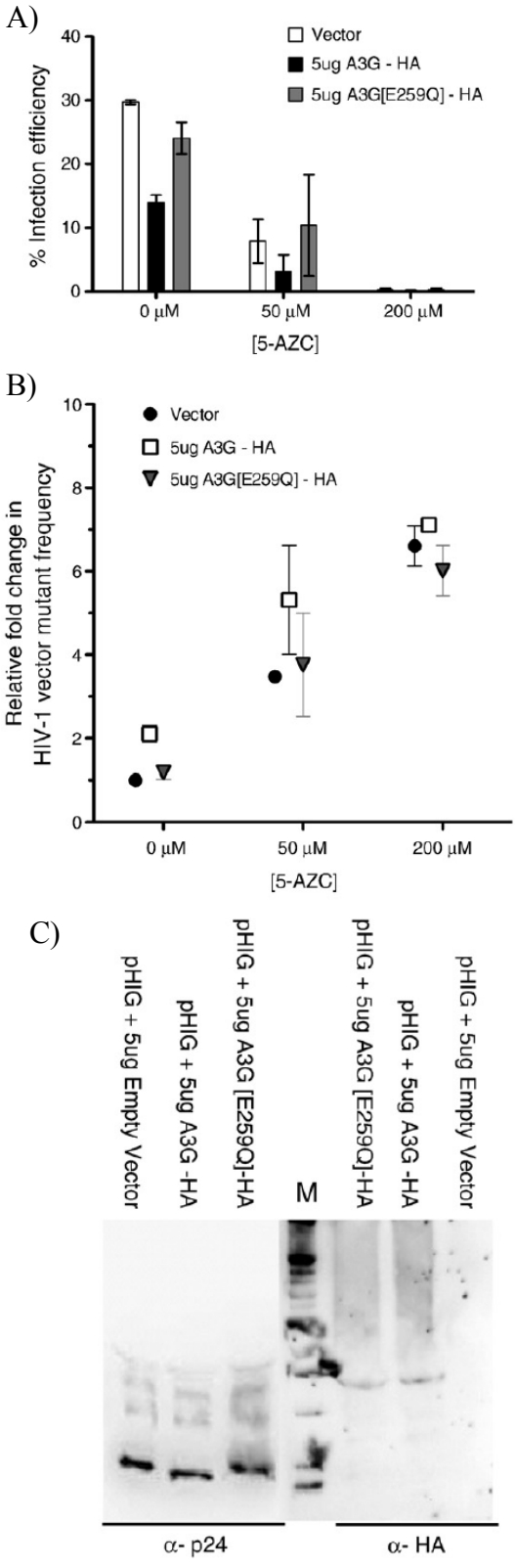

A)
Vector
5ug A3G - HA
5ug A3G[E259Q] - HA
% Infection efficiency
40
30
20
10
0
0 μM
50 μM
200 μM
[5-AZC]
B)
Vector
5ug A3G - HA
5ug A3G[E259Q] - HA
Relative fold change in HIV-1 vector mutant frequency
10
8
6
4
2
0
0 μM
50 μM
200 μM
[5-AZC]
C)
pHIG + 5ug Empty Vector
pHIG + 5ug A3G -HA
pHIG + 5ug A3G [E259Q]-HA
M
pHIG + 5ug A3G [E259Q]-HA
pHIG + 5ug A3G -HA
pHIG + 5ug Empty Vector
α- p24
α- HA

Figure 4-4. Requirement of APOBEC3G catalytic activity for reducing the frequency of 5-AZC-mediated G-to-C mutations. 293T cells were transfected with the HIV-1 vector (pHIG), VSV-G envelope expression plasmid, and a A3Gexpression vector, the A3G(E259Q) catalytically inactive mutant expression vector, or vector only. Twenty-four hours post-transfection, cell culture supernatants were harvested and used to infect 5-AZC-treated target cells to assay for either viral infectivity **(A)** or viral mutant frequency **(B)** determined or used for immunoblot analysis of HIV-1 Gag or A3G **(C)**.

TABLE 4-1. Mutation spectra in the GFP gene of vector proviral sequences

Mutation from	Mutation to % (±SD)																							
	Vector+vehicle control[a]				Vector+ 5-AZC [50 μM][b]				A3G clone 4 +vehicle[c]				A3G clone 10 +vehicle[d]				A3G clone 4+5-AZC [50 μM][e]				A3G clone 10+5-AZC [50 μM][f]			
	A	T	C	G	A	T	C	G	A	T	C	G	A	T	C	G	A	T	C	G	A	T	C	G
A to	—	0	2(2)	39(5)	—	0	0	13(9)	—	0	0	16(5)	—	0	1(2)	14(1)	—	0	1(1)	11(5)	—	0	0	6(3)
T to	2(3)	—	24(8)	0	0	—	8(4)	2(3)	0	—	10(5)	0	0	—	9(4)	1(1)	0	—	5(1)	0	0	—	3(2)	0
C to	0	13(5)	—	0	0	6(3)	—	1(1)	0	9(8)	—	0	0	2(2)	—	1(2)	1(1)	7(4)	—	1(1)	0	5(1)	—	0
G to	19(6)	0	0	—	4(4)	5(2)	61(12)	—	65(12)	0	0	—	70(5)	1(2)	0	—	28(9)	4(2)	41(16)	—	64(6)	1(1)	20(10)	—

[a] Total number of sequences, 112; total number of mutations, 81; number of nucleotides sequenced, 80,640; mutation frequency $\times 10^{-3}$, 1.035 (±0.136)
[b] Total number of sequences, 83; total number of mutations, 132; number of nucleotides sequenced, 59,760; mutation frequency $\times 10^{-3}$, 1.953 (±0.459)
[c] Total number of sequences, 68; total number of mutations, 95; number of nucleotides sequenced, 48,960; mutation frequency $\times 10^{-3}$, 1.602 (±0.368)
[d] Total number of sequences, 70; total number of mutations, 127; number of nucleotides sequenced, 50,400; mutation frequency $\times 10^{-3}$, 2.203 (±0.335)
[e] Total number of sequences, 158; total number of mutations, 363; number of nucleotides sequenced, 113,760; mutation frequency $\times 10^{-3}$, 3.255 (±0.383)
[f] Total number of sequences, 155; total number of mutations, 468; number of nucleotides sequenced, 111,600; mutation frequency $\times 10^{-3}$, 4.272 (±0.305)

Table 4-2. Mutational load in the GFP gene of vector proviruses

	Vector+ 5-AZC [50 μM]		A3G clone 4+DMSO		A3G clone 4+ 5-AZC [50 μM][a]		A3G clone 10+DMSO		A3G clone 10+ 5-AZC [50 μM][a]	
Number of G-to-A mutations	12	0.03%	48	0.35%	148	0.43%	87	0.37%	287	0.48%
Total number of G-to-A mutable positions[b]	41,760		13,680		34,560		23,760		59,760	
% change in G-to-A mutational load						+12% ($p > 0.2$)				+18% ($p = 0.026$)
Number of G-to-C mutations	78	0.31%	—	0.00%	157	0.27%	—	0.00%	63	0.20%
Total number of G-to-C mutable positions[b]	25,200		—		58,320		—		30,960	
% change in G-to-C mutational load						−13% ($p > 0.3$)				−36% ($p = 0.013$)

[a] p values determined by $\chi 2$ analysis.
[b] Number of clones multiplied by the 720 nucleotide positions in the GFP target sequence.

Table 4-3. Effect of catalytically inactive A3G expression in virus-producing cells on mutation spectra in the GFP gene of vector proviruses

Mutation from	Mutation to % (±SD)											
	Vector + 5-AZC (50 μM)[a]				A3G E259Q + DMSO[b]				A3G E259Q + 5-AZC (50 μM)[c]			
	A	T	C	G	A	T	C	G	A	T	C	G
A to	—	0	0	19 (3)	—	2 (2)	2 (1)	19 (0)	—	0	0	14 (3)
T to	0	—	3 (2)	5 (2)	0	—	29 (5)	2 (2)	0	—	7 (1)	0
C to	0	3 (2)	—	0	0	17 (10)	—	0	0	10 (3)	—	1 (1)
G to	10 (4)	5 (3)	59 (5)	—	26 (3)	2 (2)	0	—	10 (3)	2 (2)	57 (10)	—

[a] Total number of sequences, 36; total number of mutations, 59; number of nucleotides sequenced, 25,920; mutation frequency $\times 10^{-3}$, 2.276 (±0.346).

[b] Total number of sequences, 53; total number of mutations, 42; number of nucleotides sequenced, 38,160; mutation frequency $\times 10^{-3}$, 1.101 (±0.371).

[c] Total number of sequences, 90; total number of mutations, 103; number of nucleotides sequenced, 64,800; mutation frequency $\times 10^{-3}$, 1.590 (±0.312).

CHAPTER V

DISSERTATION SUMMARY AND FINAL DISCUSSION

Dissertation Summary

The work presented in this dissertation provides a new understanding of clinically relevant reverse transcriptase (RT) variants and their fidelity-fitness relationship, as well as insight into the mechanisms of purposeful mutagenesis in HIV-1. Drug-resistance conferring RT mutants were investigated for their contribution to viral fidelity. Of the 14 mutants, 6 were never analyzed by *in vivo* mutation rate assay. Differences in fidelity were observed, which helped establish an optimality model for HIV-1 mutation rate. The model was supported by viral fitness measures, as increased fidelity mutants positively correlated with fitness and decreased fidelity mutants negatively correlated with fitness. More mechanistic studies are needed to show the fitness costs associated with these fidelity mutants.

Aside from RT mutants that contribute to HIV-1 variation, the cytidine analog 5-azacytidine (5-AZC) was also investigated for its ability to exploit purposeful mutagenesis. Remarkably, this anti-cancer compound showed two distinct antiretroviral pathways. Both the early phase of HIV-1 replication (i.e., during reverse transcription) as well as the late phase of viral production (i.e., viral RNA transcription) were inhibited by 5-AZC in a concentration-dependent manner. Furthermore, the antiviral activity coincided with an increased GC-to-CG transversion mutational load. Interestingly, 5-AZC, a ribonucleoside, was most potent when delivered prior to reverse transcription, although RT strongly favors incorporation of deoxynucleotide. Our current model suggests that 5-AZC is intracellularly converted to 5-aza-2'-deoxycytidine triphosphate, and becomes a substrate for RT. A second interesting observation was 5-AZC's mutational pattern subsequent to RT incorporation. Only G-to-C transversion mutations were observed; not the reciprocal

C-to-G type. Evidence had already existed to explain how the triazole ring could undergo spontaneous ring-opening to allow a azacytosine:cytosine mispair; however, the unlikely biased G-to-C mutational pattern is explained by strand-specific templating during reverse transcription. Only when the azacytosine base is incorporated during negative strand synthesis is it able to template with a cytosine during positive strand synthesis to induce the G-to-C mutations; incorporation during positive strand synthesis is most likely excised and repaired with no opportunity to template for mispairs.

It has been argued by John Coffin that the only effective anti-HIV drug is one that the virus can evolve resistance to (Hultquist *et al.* 2009). This, and the novelty of HIV-1 evolving resistance to a mutagen, was the impetus to passage HIV-1 in the presence of 5-AZC. Preliminary results are discussed in Appendix II. Population-based sequencing of the HIV-1 pol gene shows two fixed mutations in the RT coding region. These mutations are threonine to isoleucine at position 215 (T215I) and aspartic acid to glutamic acid at position 237 (D237E). Interestingly, structural analysis revealed that one of the mutations was within proximity of the RT polymerase active site. T215I is a transitory mutation to 215 tyrosine (Y) or phenylalanine (F), which is part of the thymidine analog mutation (TAM) complex for thymidine analog resistance (AZT-zidovudine and d4T-stavudine) (Rhee *et al.* 2003; Shafer 2006). RT position 215 resides at the pocket surrounding incoming deoxynucleotide triphosphates (dNTPs). D237E has never been found to be associated with resistance to any clinical or experimental nucleoside/non-nucleoside analog (Rhee *et al.* 2003; Shafer 2006). It is located distal to the non-nucleoside RT inhibitor (NNRTI) binding pocket which is 10 Å from the polymerase active site. However, introduction of these mutations back into HIV-1 molecular clones

showed no effect on viral susceptibility. Thus, these selection experiments are ongoing.

This theme of lethal mutagenesis was further explored in HIV-1 by examining the outcome of concomitant mutagen exposure. A subsequent chapter explored how 5-AZC and APOBEC3G would interact to inhibit HIV-1 replication. As expected, the combined exposure of both mutagens produced an additive inhibitory effect, suggesting that the mutagens had distinct, non-overlapping mechanisms. Yet, sequencing analysis revealed that concomitant mutagen treatment caused a significant increase in the percentage of G-to-A, APOBEC3G-induced mutations at the expense of 5-AZC's G-to-C mutational pattern. More mechanistic studies are needed. However, these suggest that 5-AZC incorporation into viral DNA somehow stimulates APOBEC3G's ability to deaminate cytosines, or possibly that 5-AZC is be preferentially deaminated.

Mechanistic Studies of Fitness Costs associated with HIV-1 Mutation Rate

The hypotheses in Chapter II set out to test a relationship between HIV-1 fitness and mutation rate. Similar to previous findings (Furio *et al.* 2005; Furio *et al.* 2007), our study revealed that viral mutation rates correlate with viral fitness (Fig. 2-4); however, these studies warrant further investigation into mechanism. To examine the positive correlation between increased fidelity and viral fitness, DNA synthesis rate comparisons should be done. This study would analyze *ex vivo* rates of reverse transcription by measuring viral population-based completion of early and late RT products. The approach would emulate the methodology of a novel study by V. Pathak (Thomas *et al.* 2007). This work used strand-specific probes to monitor RT products by qPCR and was able to measure cell culture-based rates of nucleotide synthesis as well as strand transfer events.

Interestingly, the *ex vivo* DNA synthesis rate was calculated at 68-70 nucleotides/minute for wildtype virus, similar to other published accounts (Hulme *et al.* 2011). The experimental approach is to synchronously transduce target T cells in parallel with wildtype and a series of RT mutant vectors. Next, viral DNA would be collected at half hour to hour intervals so that population-based RT product accumulation curves could be modeled to calculate relative DNA synthesis rates. The hypothesis being tested is that increased fidelity correlates with fitness costs because RT mutations that influence fidelity also impair completion of reverse transcription.

Additional experiments should be done to determine the exact mechanism for the relationship between decreased fidelity and viral fitness. The foremost hypothesis is that increased mutational load causes the population to succumb to extinction catastrophe (Fig 1-3 B). Current evidence using a phenotypic mutant frequency vector assay suggests this is the case, but next-generation deep sequencing would be more convincing. This approach could be used to quantify the mutation frequency of various RT mutants versus wildtype over a single replication event. Furthermore, the most direct way to show existence of an inverse correlation between decreased fidelity and fitness is to perform full-length viral sequencing. This would demonstrate, based on a population average, that viruses with error-prone RT cause mutational load increases that are defective for long-term replication.

Molecular Interaction between 5-Azacytosine and APOBEC3G

APOBEC3 proteins are zinc-coordinating cytosine deaminases and some family members possess strong antiretroviral activity through mutation-mediated restriction

(Harris *et al.* 2004). The discovery that A3G is a powerful antiviral factor against HIV-1 has stirred interest in harnessing its potential for lethal mutagenesis. To date, small molecule Vif-A3G interface disruptors have been reported, which work by relieving Vif's proteosomal targeting of A3G (Nathans *et al.* 2008; Cen *et al.* 2010).

There is also interest in APOBEC3's target site specificity. The R. Harris laboratory has published a study that details the discovery and structure-activity relationship of small molecule cytosine deaminase inhibitors (Li *et al.* 2012). Other reports have detailed A3G's target site preference by analyzing neighboring bases with non-canonical structures (Rausch *et al.* 2009). These results showed that most unnatural base analogs interfere with A3G's deaminase ability. Interestingly, the data discussed in Chapter IV provides evidence that suggests that azacytosine stimulates A3G's deaminase activity. Three possibilities remain plausible to explain the increased G-to-A mutational load: 1) azacytosine incorporation into viral DNA alters secondary structural elements, 2) azacytosine incorporation influences local cytosine target site preference sites, and/or 3) azacytosine is preferentially deaminated by A3G to induce G-to-A mutations and the expense of G-to-C mutations. But the question remains of whether A3G can directly deaminate 5-azacytosine to 5-azauracil.

Efforts were taken to directly test this latter possibility as it had never been described in the literature. This approach was initiated by custom order of a 39-mer oligonucleotide. Because of the sensitive nature of the 5-azacytosine base, special phosphoramidite chemistry was required for its synthesize (Eritja *et al.* 1997; Garcia *et al.* 2001; Rogstad *et al.* 2009). Unfortunately the out-sourced 5-azacytosine-containing oligonucleotide continued to possess stability issues when University of Minnesota collaborators

(N. Tretyakova) attempted to run control experiments. Continued efforts are underway to have the custom oligonucleotide resynthesized.

The standard cytosine deaminase activity assay measures the relative cleavage of an abasic oligonucleotide (Iwatani *et al.* 2006). The abasic site is generated when uracil DNA glycosylase (UDG) removes its substrate, uracil, from a position acted on by the cytosine deaminase. However, this experimental approach will not be able to detect A3G's deaminase activity upon 5-azacytosine because UDG's substrate specificity is such that it may not be able to recognize and excise a 5-azauracil base (Krokan *et al.* 1981; Bensen *et al.* 1986; Duraffour *et al.* 2007; Jiang *et al.* 2010). These findings indicate that the deaminase activity must be measured before the assay's uracil excision step. For instance, it was proposed that mass spectroscopy techniques could be used to detect the -1 mass shift from NH_2 to O of the deaminase reaction (Zhao *et al.* 2004; Liu *et al.* 2006).

Drug Resistance Selection of Viral Mutagens

To date, there is no published account of an HIV-1 clone resistant to a small molecule chemical mutagen. This remains even as the HIV-1 viral mutagen KP-1212 has shown substantial pre-clinical data to be granted investigational new drug status, for human use. Viral mutation rate is an innovative drug target, though several resistance mechanisms can be envisioned which include: 1) Increased discrimination/excision, 2) increased polymerase fidelity, and/or 3) increased mutational robustness of virus population. So far, experimental evidence of mutagen resistance exists for three other RNA viruses: poliovirus, FMDV, and Q-beta. The Kirkegaard laboratory passaged poliovirus in

the presence of ribavirin to select out resistant isolates (Pfeiffer *et al.* 2003). A variant in the polymerase gene was discovered and the mutation actually increased overall enzyme fidelity. This approach was also used by E. Domingo's laboratory in an effort to determine mutagen resistance in FMDV (Arias *et al.* 2005; Sierra *et al.* 2007). These studies showed that FMDV used both discriminatory, as well as increased fidelity, mechanisms to confer resistance to ribavirin. Lastly, a recent RNA phage Q-beta study showed that 5-AZC-resistant virus is selected through polymorphic mutations in the replicase gene, suggesting that the resistance mechanism is directed at the population rather than a single clone (Arribas *et al.* 2011).

Extension to the antiviral studies of 5-AZC (Chapter II), were established to determine HIV-1 resistance-conferring mechanisms. These studies involve continual passaging of virus in the presence of increasing amounts of drug until virus with reduced susceptibility emerges. A passage is defined as placing cell-free viral supernatant onto fresh target cells – this approach is continually repeated and at roughly every two weeks, the drugs concentration is doubled. Importantly, virus and cells stocks are frozen down at regular intervals to remain as an archive, to restart the experiment in case virus becomes extinguished and as a depository for sequencing analysis. In the event that resistant virus is detected virologically, population-based and clonal sequencing are used to find fixed mutations. These mutations are then cloned back into the parental virus and the experiment is effectively restarted to measure susceptibility differences between wildtype and putative mutant clones. Preliminary data and details of these experiments investigating HIV-1 resistance to 5-AZC is discussed in Appendix II.

BIBLIOGRAPHY

Abraha, A., R. M. Troyer, M. E. Quinones-Mateu and E. J. Arts (2005). "Methods to determine HIV-1 ex vivo fitness." *Methods in molecular biology*. **304**: 355-368.

Abram, M. E., A. L. Ferris, W. Shao, W. G. Alvord and S. H. Hughes (2010). "Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication." *Journal of Virology*. **84**(19): 9864-9878.

Agudo, R., A. Arias, N. Pariente, C. Perales, C. Escarmis, A. Jorge, A. Marina and E. Domingo (2008). "Molecular characterization of a dual inhibitory and mutagenic activity of 5-fluorouridine triphosphate on viral RNA synthesis. Implications for lethal mutagenesis." *J Mol Biol*. **382**(3): 652-666.

Albin, J. S. and R. S. Harris (2010). "Interactions of host APOBEC3 restriction factors with HIV-1 in vivo: implications for therapeutics." *Expert reviews in molecular medicine*. **12**: e4.

Alimonti, J. B., T. B. Ball and K. R. Fowke (2003). "Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS." *The Journal of general virology*. **84**(Pt 7): 1649-1661.

Ames, B. N., F. D. Lee and W. E. Durston (1973). "An improved bacterial test system for the detection and classification of mutagens and carcinogens." *Proc Natl Acad Sci U S A*. **70**(3): 782-786.

Anastassopoulou, C. G., A. J. Marozsan, A. Matet, A. D. Snyder, E. J. Arts, S. E. Kuhmann and J. P. Moore (2007). "Escape of HIV-1 from a small molecule CCR5 inhibitor is not associated with a fitness loss." *PLoS pathogens*. **3**(6): e79.

Anderson JP, D. R., Loeb LA (2004). "Viral error catastrophe by mutagenic nucleosides." *Annu Rev Microbiol*. **58**: 183-205.

Antiretroviral_Therapy_Cohort_Collaboration (2008). "Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies." *Lancet*. **372**(9635): 293-299.

Aquaro, S., V. Svicher, F. Ceccherini-Silberstein, A. Cenci, F. Marcuccilli, S. Giannella, L. Marcon, R. Calio, J. Balzarini and C. F. Perno (2005). "Limited development and progression of resistance of HIV-1 to the nucleoside analogue reverse transcriptase inhibitor lamivudine in human primary macrophages." *The Journal of antimicrobial chemotherapy*. **55**(6): 872-878.

Arias, A., R. Agudo, C. Ferrer-Orta, R. Pérez-Luque, A. Airaksinen, E. Brocchi, E. Domingo, N. Verdaguer and C. Escarmís (2005). "Mutant Viral Polymerase in the Transition of Virus to Error Catastrophe Identifies a Critical Site for RNA Binding." *J Mol Biol*. **353**(5): 1021-1032.

Arion, D., N. Kaushik, S. McCormick, G. Borkow and M. A. Parniak (1998). "Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase." *Biochemistry*. **37**: 15908-15917.

Armitage, A. E., K. Deforche, C. H. Chang, E. Wee, B. Kramer, J. J. Welch, J. Gerstoft, L. Fugger, A. McMichael, A. Rambaut and A. K. Iversen (2012). "APOBEC3G-induced hypermutation of human immunodeficiency virus type-1 is typically a discrete "all or nothing" phenomenon." *PLoS genetics*. **8**(3): e1002550.

Arribas, M., L. Cabanillas and E. Lazaro (2011). "Identification of mutations conferring 5-azacytidine resistance in bacteriophage Qbeta." *Virology*.

Auerbach, C. and J. M. Robson (1947). "The production of mutations by chemical substances." *Proc R Soc Edinb Biol*. **62**: 271-283.

Back, N. K., M. Nijhuis, W. Keulen, C. A. Boucher, B. O. Oude Essink, A. B. van Kuilenburg, A. H. van Gennip and B. Berkhout (1996). "Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme." *EMBO J.* **15**: 4040-4049.

Ball, S. C., A. Abraha, K. R. Collins, A. J. Marozsan, H. Baird, M. E. Quinones-Mateu, A. Penn-Nicholson, M. Murray, N. Richard, M. Lobritz, P. A. Zimmerman, T. Kawamura, A. Blauvelt and E. J. Arts (2003). "Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C." *J Virol*. **77**(2): 1021-1038.

Baltimore, D. (1970). "RNA-dependent DNA polymerase in virions of RNA tumor viruses." *Nature (London)*. **226**: 1209-1211.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum and L. Montagnier (1983). "Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)." *Science*. **220**(4599): 868-871.

Batorsky, R., M. F. Kearney, S. E. Palmer, F. Maldarelli, I. M. Rouzine and J. M. Coffin (2011). "Estimate of effective recombination rate and average selection coefficient for HIV in chronic infection." *Proceedings of the National Academy of Sciences of the United States of America*. **108**(14): 5661-5666.

Bensen, R. J. and H. R. Warner (1986). "The Partial Purification and Characterization of Nuclear and Mitochondrial Uracil-DNA Glycosylase Activities from *Zea mays* Seedlings." *Plant Physiol.* **83**: 6.

Berkhout, B. and F. J. van Hemert (1994). "The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins." *Nucleic acids research*. **22**(9): 1705-1711.

Bessman, M. J., N. Muzyczka, M. F. Goodman and R. L. Schnaar (1974). "Studies on the biochemical basis of spontaneous mutation. II. The incorporation of a base and its analogue into DNA by wild-type, mutator and antimutator DNA polymerases." *Journal of molecular biology*. **88**(2): 409-421.

Bishop, K. N., R. K. Holmes and M. H. Malim (2006). "Antiviral potency of APOBEC proteins does not correlate with cytidine deamination." *Journal of virology*. **80**(17): 8450-8458.

Bishop, K. N., R. K. Holmes, A. M. Sheehy, N. O. Davidson, S. J. Cho and M. H. Malim (2004). "Cytidine deamination of retroviral DNA by diverse APOBEC proteins." *Current biology : CB*. **14**(15): 1392-1396.

Bouchard, J. and R. L. Momparler (1983). "Incorporation of 5-Aza-2'-deoxycytidine-5'-triphosphate into DNA. Interactions with mammalian DNA polymerase alpha and DNA methylase." *Mol Pharmacol*. **24**(1): 109-114.

Bouchard, J., M. C. Walker, J. M. Leclerc, N. Lapointe, R. Beaulieu and L. Thibodeau (1990). "5-azacytidine and 5-azadeoxycytidine inhibit human immunodeficiency virus type 1 replication in vitro." *Antimicrob Agents*

Chemother. **34**(2): 206-209.

Boyer, P. L. and S. H. Hughes (1995). "Analysis of mutations at position 184 in reverse transcriptase of human immunodeficiency virus type 1." *Antimicrobial agents and chemotherapy*. **39**(7): 1624-1628.

Brok, J., L. L. Gluud and C. Gluud (2010). "Ribavirin plus interferon versus interferon for chronic hepatitis C." *Cochrane database of systematic reviews*(1): CD005445.

Browne, E. P., C. Allers and N. R. Landau (2009). "Restriction of HIV-1 by APOBEC3G is cytidine deaminase-dependent." *Virology*. **387**(2): 313-321.

Bull, J. J., L. A. Meyers and M. Lachmann (2005). "Quasispecies made simple." *PLoS computational biology*. **1**(6): e61.

Bull, J. J., R. Sanjuan and C. O. Wilke (2007). "Theory of lethal mutagenesis for viruses." *Journal of Virology*. **81**(6): 2930-2939.

Bull, J. J., R. Sanjuán and C. O. Wilke (2007). "Theory of lethal mutagenesis for viruses." *J. Virol*. **81**(6): 2930-2939.

Cen, S., Z. G. Peng, X. Y. Li, Z. R. Li, J. Ma, Y. M. Wang, B. Fan, X. F. You, Y. P. Wang, F. Liu, R. G. Shao, L. X. Zhao, L. Yu and J. D. Jiang (2010). "Small molecular compounds inhibit HIV-1 replication through specifically stabilizing APOBEC3G." *The Journal of biological chemistry*. **285**(22): 16546-16552.

Chao, L. and E. C. Cox (1983). "Competition Between High and Low Mutating Strains of Escherichia coli." *Evolution*. **37**(1): 125-134.

Chelico, L., P. Pham and M. F. Goodman (2009). "Stochastic properties of processive cytidine DNA deaminases AID and APOBEC3G." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. **364**(1517): 583-593.

Chen, H., C. E. Lilley, Q. Yu, D. V. Lee, J. Chou, I. Narvaiza, N. R. Landau and M. D. Weitzman (2006). "APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons." *Current biology : CB*. **16**(5): 480-485.

Chen, R., E. Le Rouzic, J. A. Kearney, L. M. Mansky and S. Benichou (2004). "Vpr-mediated incorporation of UNG2 into HIV-1 particles is required to modulate the virus mutation rate and for replication in macrophages." *The Journal of biological chemistry*. **279**(27): 28419-28425.

Chen, R., M. Yokoyama, H. Sato, C. Reilly and L. M. Mansky (2005). "Human immunodeficiency virus mutagenesis during antiviral therapy: impact of drug-resistant reverse transcriptase and nucleoside and nonnucleoside reverse transcriptase inhibitors on human immunodeficiency virus type 1 mutation frequencies." *J Virol*. **79**(18): 12045-12057.

Chiu, Y. L. and W. C. Greene (2008). "The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements." *Annual review of immunology*. **26**: 317-353.

Chung, D. H., Y. Sun, W. B. Parker, J. B. Arterburn, A. Bartolucci and C. B. Jonsson (2007). "Ribavirin reveals a lethal threshold of allowable mutation frequency for Hantaan virus." *J Virol*. **81**(21): 11722-11729.

Cihak, A., J. Vesely and F. Sorm (1965). "Incorporation of 5-azacytidine into liver ribonucleic acids of leukemic mice sensitive and resistant to 5-azacytidine." *Biochim Biophys Acta*. **108**(3): 516-518.

Clouser, C. L., C. M. Holtz, M. Mullett, D. L. Crankshaw, J. E. Briggs, J. Chauhan, I. M. VanHoutan, S. E. Patterson and L. M. Mansky (2011). "Analysis of the

ex vivo and in vivo antiretroviral activity of gemcitabine." *PLoS One*. **6**(1): e15840.

Clouser, C. L., C. M. Holtz, M. Mullett, D. L. Crankshaw, J. E. Briggs, M. G. O'Sullivan, S. E. Patterson and L. M. Mansky (2012). "Activity of a novel combined antiretroviral therapy of gemcitabine and decitabine in a mouse model for HIV-1." *Antimicrob Agents Chemother*. **56**(4): 1942-1948.

Clouser, C. L., S. E. Patterson and L. M. Mansky (2010). "Exploiting drug repositioning for discovery of a novel HIV combination therapy." *Journal of virology*. **84**(18): 9301-9309.

Codoner, F. M., J. A. Daros, R. V. Sole and S. F. Elena (2006). "The fittest versus the flattest: experimental confirmation of the quasispecies effect with subviral pathogens." *PLoS pathogens*. **2**(12): e136.

Coffin, J. (1995). "HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy." *Science*. **267**: 483-489.

Coffin, J. M. (1995). "HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy." *Science*. **267**(5197): 483-489.

Cong, M. E., W. Heneine and J. G. Garcia-Lerma (2007). "The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions." *Journal of Virology*. **81**(6): 3037-3041.

Connor, R. I., B. K. Chen, S. Choe and N. R. Landau (1995). "Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes." *Virology*. **206**: 935-944.

Contreras, A. M., Y. Hiasa, W. He, A. Terella, E. V. Schmidt and R. T. Chung (2002). "Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system." *J Virol*. **76**(17): 8505-8517.

Cooper, T. F. and R. E. Lenski (2010). "Experimental evolution with E. coli in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations." *BMC evolutionary biology*. **10**: 11.

Crotty, S., C. E. Cameron and R. Andino (2001). "RNA virus error catastrophe: direct molecular test by using ribavirin." *Proc Natl Acad Sci U S A*. **98**(12): 6895-6900.

Crotty, S., D. Maag, J. J. Arnold, W. Zhong, J. Y. Lau, Z. Hong, R. Andino and C. E. Cameron (2000). "The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen." *Nat Med*. **6**(12): 1375-1379.

Cuevas, J. M., F. Gonzalez-Candelas, A. Moya and R. Sanjuan (2009). "The effect of ribavirin on the mutation rate and spectrum of Hepatitis C virus in vivo." *J Virol*. **in press**.

Cuevas, J. M., F. Gonzalez-Candelas, A. Moya and R. Sanjuan (2009). "Effect of ribavirin on the mutation rate and spectrum of hepatitis C virus in vivo." *Journal of Virology*. **83**(11): 5760-5764.

Cupples, C. G. and J. H. Miller (1989). "A set of lacZ mutations in Escherichia coli that allow rapid detection of each of the six base substitutions." *Proc Natl Acad Sci U S A*. **86**: 5345-5349.

Dapp, M. J., C. L. Clouser, S. Patterson and L. M. Mansky (2009). "5-Azacytidine can induce lethal mutagenesis in human immunodeficiency virus type 1." *Journal of virology*. **83**(22): 11950-11958.

Dapp, M. J., C. M. Holtz and L. M. Mansky (2012). "Concomitant Lethal Mutagenesis

of Human Immunodeficiency Virus Type 1." *Journal of molecular biology*.

Davis, W. and L. F. Larionov (1964). "Progress in Chemotherapy of Cancer." *Bulletin of the World Health Organization*. **30**: 327-341.

Dawson, K. J. (1998). "Evolutionarily stable mutation rates." *Journal of theoretical biology*. **194**(1): 143-157.

De Clercq, E. (2009). "Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV." *Int J Antimicrob Agents*. **33**(4): 307-320.

Delebecque, F., R. Suspene, S. Calattini, N. Casartelli, A. Saib, A. Froment, S. Wain-Hobson, A. Gessain, J. P. Vartanian and O. Schwartz (2006). "Restriction of foamy viruses by APOBEC cytidine deaminases." *Journal of virology*. **80**(2): 605-614.

Denamur, E. and I. Matic (2006). "Evolution of mutation rates in bacteria." *Mol Microbiol*. **60**(4): 820-827.

DeSimone, J., P. Heller, L. Hall and D. Zwiers (1982). "5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons." *Proc Natl Acad Sci U S A*. **79**(14): 4428-4431.

Deval, J., J. M. Navarro, B. Selmi, J. Courcambeck, J. Boretto, P. Halfon, S. Garrido-Urbani, J. Sire and B. Canard (2004). "A loss of viral replicative capacity correlates with altered DNA polymerization kinetics by the human immunodeficiency virus reverse transcriptase bearing the K65R and L74V dideoxynucleoside resistance substitutions." *The Journal of biological chemistry*. **279**(24): 25489-25496.

Deval, J., K. L. White, M. D. Miller, N. T. Parkin, J. Courcambeck, P. Halfon, B. Selmi, J. Boretto and B. Canard (2004). "Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations." *The Journal of biological chemistry*. **279**(1): 509-516.

Devereux, H. L., V. C. Emery, M. A. Johnson and C. Loveday (2001). "Replicative fitness in vivo of HIV-1 variants with multiple drug resistance-associated mutations." *Journal of medical virology*. **65**(2): 218-224.

Diamond, T. L., M. Roshal, V. K. Jamburuthugoda, H. M. Reynolds, A. R. Merriam, K. Y. Lee, M. Balakrishnan, R. A. Bambara, V. Planelles, S. Dewhurst and B. Kim (2004). "Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase." *J Biol Chem*. **279**(49): 51545-51553.

Diamond, T. L., G. Souroullas, K. K. Weiss, K. Y. Lee, R. A. Bambara, S. Dewhurst and B. Kim (2003). "Mechanistic understanding of an altered fidelity simian immunodeficiency virus reverse transcriptase mutation, V148I, identified in a pig-tailed macaque." *The Journal of biological chemistry*. **278**(32): 29913-29924.

Domingo, E. (2003). "Quasispecies and the development of new antiviral strategies." *Prog Drug Res*. **60**: 133-158.

Domingo, E., C. Escarmís, E. Lázaro and S. C. Manrubia (2005). "Quasispecies dynamics and RNA virus extinction." *Virus Res*. **107**(2): 129-139.

Domingo, E. and J. J. Holland (1997). "RNA virus mutations and fitness for survival." *Annu Rev Microbiol*. **51**: 151-178.

Domingo, E., E. Martinez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortin, C. Lopez-Galindez, P. Perez-Brena, N. Villanueva, R. Najera, S. VandePol, D. Steinhauer, N. DePolo and J. Holland (1985). "The quasispecies (extremely

heterogeneous) nature of viral RNA genome populations: Biological relevance-a review." *Gene*(40): 1-8.

Domingo E, P. C., and Holland JJ (2008). *Origin and Evolution of Viruses*, Elsevier Ltd.

Domingo, E., D. Sabo, T. Taniguchi and C. Weissmann (1978). "Nucleotide sequence heterogeneity of an RNA phage population." *Cell*. **13**(4): 735-744.

Doskocil, J., V. Paces and F. Sorm (1967). "Inhibition of protein synthesis by 5-azacytidine in Escherichia coli." *Biochim Biophys Acta*. **145**(3): 771-779.

Drake, J. W. (1969). "Comparative rates of spontaneous mutation." *Nature*. **221**: 1132.

Drake, J. W. (1991). "A constant rate of spontaneous mutation in DNA-based microbes." *Proc. Natl. Acad. Sci. USA*. **88**: 7160-7164.

Drake, J. W., B. Charlesworth, D. Charlesworth and J. F. Crow (1998). "Rates of spontaneous mutation." *Genetics*. **148**(4): 1667-1686.

Drake, J. W. and J. J. Holland (1999). "Mutation rates among RNA viruses." *Proc. Natl. Acad. Sci.* **96**: 13910-13913.

Drosopoulos, W. C. and V. R. Prasad (1998). "Increased misincorporation fidelity observed for nucleoside analog resistance mutations M184V and E89G in human immunodeficiency virus type 1 reverse transcriptase does not correlate with the overall error rate measured in vitro." *Journal of Virology*. **72**(5): 4224-4230.

Duraffour, S., A. A. Ishchenko, M. Saparbaev, J. M. Crance and D. Garin (2007). "Substrate specificity of homogeneous monkeypox virus uracil-DNA glycosylase." *Biochemistry*. **46**(42): 11874-11881.

Earl, D. J. and M. W. Deem (2004). "Evolvability is a selectable trait." *Proceedings of the National Academy of Sciences of the United States of America*. **101**(32): 11531-11536.

Ebrahimi, D., F. Anwar and M. P. Davenport (2011). "APOBEC3 has not left an evolutionary footprint on the HIV-1 genome." *Journal of virology*. **85**(17): 9139-9146.

Eigen, M. (1971). "Selforganization of matter and the evolution of biological macromolecules." *Die Naturwissenschaften*. **58**(10): 465-523.

Eigen, M. (1993). "Viral quasispecies." *Scientific American*. **269**(1): 42-49.

Eigen, M. (2002). "Error catastrophe and antiviral strategy." *Proc Natl Acad Sci U S A*. **99**(21): 13374-13376.

Eigen M, S. P. (1977). "The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle." *Naturwissenschaften*. **64**: 541-565.

Elena, S. F. and R. Sanjuan (2005). "Adaptive value of high mutation rates of RNA viruses: separating causes from consequences." *J Virol*. **79**(18): 11555-11558.

Eritja, R., V. E. Marquez and R. G. Garcia (1997). "Synthesis and Properties of Oligonucleotide Containing 5-Aza-2'-deoxycytidine." *Nucleosides Nucleotides & Nucleic Acids*. **16**(7): 4.

Eshel, I. (1973). "Clone-selection and Optimal Rates or Mutation." *Journal of applied probability*. **10**(4): 11.

Fisher, R. A. (1930). "The genetical theory of natural selection." *Oxford, England: Clarendon Press.*

Funchain, P., A. Yeung, J. L. Stewart, R. Lin, M. M. Slupska and J. H. Miller (2000). "The consequences of growth of a mutator strain of Escherichia coli as measured

by loss of function among multiple gene targets and loss of fitness." *Genetics*. **154**(3): 959-970.

Furio, V., A. Moya and R. Sanjuan (2005). "The cost of replication fidelity in an RNA virus." *Proceedings of the National Academy of Sciences of the United States of America*. **102**(29): 10233-10237.

Furio, V., A. Moya and R. Sanjuan (2007). "The cost of replication fidelity in human immunodeficiency virus type 1." *Proceedings. Biological sciences / The Royal Society*. **274**(1607): 225-230.

Gallo, R. C., P. S. Sarin, E. P. Gelmann, M. Robert-Guroff, E. Richardson, V. S. Kalyanaraman, D. Mann, G. D. Sidhu, R. E. Stahl, S. Zolla-Pazner, J. Leibowitch and M. Popovic (1983). "Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS)." *Science*. **220**(4599): 865-867.

Gao, G., M. Orlova, M. M. Georgiadis, W. A. Hendrickson and S. P. Goff (1997). "Conferring RNA polymerase activity to a DNA polymerase: a single residue in reverse transcriptase controls substrate selection." *Proc Natl Acad Sci U S A*. **94**(2): 407-411.

Gao, L., M. N. Hanson, M. Balakrishnan, P. L. Boyer, B. P. Roques, S. H. Hughes, B. Kim and R. A. Bambara (2008). "Apparent defects in processive DNA synthesis, strand transfer, and primer elongation of Met-184 mutants of HIV-1 reverse transcriptase derive solely from a dNTP utilization defect." *The Journal of biological chemistry*. **283**(14): 9196-9205.

Garcia, R. G., A. S. Brank, J. K. Christman, V. E. Marquez and R. Eritja (2001). "Synthesis of Oligonucleotide Inhibitors of DNA (Cytosine-C5) Methyltransferase Containing 5-Azacytosine Residues at Specific Sites." *Antisense & Nucleic Adid Drug Development*. **11**: 10.

Gerrish, P. J., A. Colato, A. S. Perelson and P. D. Sniegowski (2007). "Complete genetic linkage can subvert natural selection." *Proceedings of the National Academy of Sciences of the United States of America*. **104**(15): 6266-6271.

Gervaix, A., D. West, L. M. Leoni, D. D. Richmond, F. Wong-Staal and J. Corbeil (1997). "A new reporter cell line to monitor HIV infection and drug susceptibility in vitro." *Proc Natl Acad Sci U S A*. **94**(9): 4653-4658.

Gibson, T. C., M. L. Scheppe and E. C. Cox (1970). "Fitness of an Escherichia coli mutator gene." *Science*. **169**(3946): 686-688.

Girard, M. P. and G. P. Bansal (2008). "HIV/AIDS vaccines: a need for new concepts?" *Int Rev Immunol*. **27**(6): 447-471.

Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman, M. Fons and F. Taddei (2001). "Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut." *Science*. **291**(5513): 2606-2608.

Goodenow, M., T. Huet, W. Saurin, S. Kwok, J. Sninsky and S. Wain-Hobson (1989). "HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions." *J. AIDS*. **2**: 344-352.

Goodman, M. F., W. C. Gore, N. Muzyczka and M. J. Bessman (1974). "Studies on the biochemical basis of spontaneous mutation. III. Rate model for DNA polymerase-effected nucleotide misincorporation." *Journal of molecular biology*. **88**(2): 243-235.

Graci, J. D. and C. E. Cameron (2004). "Challenges for the development of

ribonucleoside analogues as inducers of error catastrophe." *Antivir Chem Chemother*. **15**(1): 1-13.

Graci, J. D. and C. E. Cameron (2008). "Therapeutically targeting RNA viruses via lethal mutagenesis." *Future virology*. **3**(6): 553-566.

Graci, J. D., D. A. Harki, V. S. Korneeva, J. P. Edathil, K. Too, D. Franco, E. D. Smidansky, A. V. Paul, B. R. Peterson, D. M. Brown, D. Loakes and C. E. Cameron (2007). "Lethal Mutagenesis of Poliovirus Mediated by a Mutagenic Pyrimidine Analogue." *J Virol*.

Graci, J. D., K. Too, E. D. Smidansky, J. P. Edathil, E. W. Barr, D. A. Harki, J. E. Galarraga, J. M. Bollinger, Jr., B. R. Peterson, D. Loakes, D. M. Brown and C. E. Cameron (2008). "Lethal mutagenesis of picornaviruses with N-6-modified purine nucleoside analogues." *Antimicrob Agents Chemother*. **52**(3): 971-979.

Gross, M. D. and E. C. Siegel (1981). "Incidence of mutator strains in Escherichia coli and coliforms in nature." *Mutation research*. **91**(2): 107-110.

Gutierrez-Rivas, M. and L. Menendez-Arias (2001). "A mutation in the primer grip region of HIV-1 reverse transcriptase that confers reduced fidelity of DNA synthesis." *Nucleic Acids Research*. **29**(24): 4963-4972.

Hache, G., M. T. Liddament and R. S. Harris (2005). "The retroviral hypermutation specificity of APOBEC3F and APOBEC3G is governed by the C-terminal DNA cytosine deaminase domain." *J Biol Chem*. **280**(12): 10920-10924.

Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks and W. P. Parks (1986). "Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS." *Science*. **232**: 1548-1553.

Harris, K. S., W. Brabant, S. Styrchak, A. Gall and R. Daifuku (2005). "KP-1212/1461, a nucleoside designed for the treatment of HIV by viral mutagenesis." *Antiviral Res*. **67**(1): 1-9.

Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger and M. H. Malim (2003). "DNA deamination mediates innate immunity to retroviral infection." *Cell*. **113**(6): 803-809.

Harris, R. S. and M. T. Liddament (2004). "Retroviral restriction by APOBEC proteins." *Nature reviews. Immunology*. **4**(11): 868-877.

He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan and N. R. Landau (1995). "Human immunodefiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity." *J. Virol.* **69**: 6705-6711.

Hicks, C., P. Clay, R. R. Redfield, J. P. Lalezari, R. Liporace, S. Schneider, M. Sension, M. McRae and J. P. Laurent (2012). "Safety, Tolerability and Efficacy of KP-1461 as Monotherapy for 124 Days in Antiretroviral-Experienced, HIV-1-Infected Subjects." *AIDS research and human retroviruses*.

Holland, J. J., J. C. de la Torre, D. K. Clarke and E. Duarte (1991). "Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses." *Journal of Virology*. **65**(6): 2960-2967.

Holland, J. J., E. Domingo, J. C. De La Torre and D. A. Steinhauer (1990). "Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis." *J. Virol.* **64**: 3960-3962.

Holmes, E. C. (2003). "Error thresholds and the constraints to RNA virus evolution." *Trends Microbiol*. **11**(12): 543-546.

Holmes, E. C. and A. Moya (2002). "Is the quasispecies concept relevant to RNA viruses?" *Journal of Virology*. **76**(1): 460-465.

Hopfield, J. J. (1974). "Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity." *Proceedings of the National Academy of Sciences of the United States of America*. **71**(10): 4135-4139.

Horst, J. P., T. H. Wu and M. G. Marinus (1999). "Escherichia coli mutator genes." *Trends in microbiology*. **7**(1): 29-36.

Hsu, C. H., J. Y. Liou, G. E. Dutschman and Y. C. Cheng (2005). "Phosphorylation of Cytidine, Deoxycytidine, and Their Analog Monophosphates by Human UMP/CMP Kinase Is Differentially Regulated by ATP and Magnesium." *Mol Pharmacol*. **67**(3): 806-814.

Hu, Z. and D. R. Kuritzkes (2011). "Interaction of reverse transcriptase (RT) mutations conferring resistance to lamivudine and etravirine: effects on fitness and RT activity of human immunodeficiency virus type 1." *Journal of Virology*. **85**(21): 11309-11314.

Huang, H., R. Chopra, G. L. Verdine and S. C. Harrison (1998). "Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance." *Science*. **282**: 1669-1675.

Hulme, A. E., O. Perez and T. J. Hope (2011). "Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription." *Proceedings of the National Academy of Sciences of the United States of America*. **108**(24): 9975-9980.

Hultquist, J. F. and R. S. Harris (2009). "Leveraging APOBEC3 proteins to alter the HIV mutation rate and combat AIDS." *Future virology*. **4**(6): 605.

Issa, J. P. and H. Kantarjian (2005). "Azacitidine." *Nat Rev Drug Discov*. **Suppl**: S6-7.

Ito, T., H. Ando and H. Handa (2011). "Teratogenic effects of thalidomide: molecular mechanisms." *Cellular and molecular life sciences : CMLS*. **68**(9): 1569-1579.

Iwatani, Y., H. Takeuchi, K. Strebel and J. G. Levin (2006). "Biochemical activities of highly purified, catalytically active human APOBEC3G: correlation with antiviral effect." *Journal of Virology*. **80**(12): 5992-6002.

Jackson-Grusby, L., P. W. Laird, S. N. Magge, B. J. Moeller and R. Jaenisch (1997). "Mutagenicity of 5-aza-2'-deoxycytidine is mediated by the mammalian DNA methyltransferase." *Proc Natl Acad Sci U S A*. **94**: 4681-4685.

Jamburuthugoda, V. K., J. M. Santos-Velazquez, M. Skasko, D. J. Operario, V. Purohit, P. Chugh, E. A. Szymanski, J. E. Wedekind, R. A. Bambara and B. Kim (2008). "Reduced dNTP binding affinity of 3TC-resistant M184I HIV-1 reverse transcriptase variants responsible for viral infection failure in macrophage." *The Journal of biological chemistry*. **283**(14): 9206-9216.

Jern, P., R. A. Russell, V. K. Pathak and J. M. Coffin (2009). "Likely role of APOBEC3G-mediated G-to-A mutations in HIV-1 evolution and drug resistance." *PLoS pathogens*. **5**(4): e1000367.

Jewell, N. A., R. Chen, R. Raices and L. M. Mansky (2003). "Nucleoside reverse transcriptase inhibitors and HIV mutagenesis." *J Antimicrob Chemother*. **52**(4): 547-550.

Jiang, Y. L., X. Gao, G. Zhou, A. Patel and A. Javer (2010). "Selective recognition of uracil and its derivatives using a DNA repair enzyme structural mimic." *The Journal of organic chemistry*. **75**(2): 324-333.

Johnson, T. (1999). "Beneficial mutations, hitchhiking and the evolution of mutation rates in sexual populations." *Genetics*. **151**(4): 1621-1631.

Jonckheere, H., E. De Clercq and J. Anne (2000). "Fidelity analysis of HIV-1 reverse transcriptase mutants with an altered amino-acid sequence at residues Leu74, Glu89, Tyr115, Tyr183 and Met184." *European journal of biochemistry / FEBS*. **267**(9): 2658-2665.

Julias, J. G., P. L. Boyer, M. J. McWilliams, W. G. Alvord and S. H. Hughes (2004). "Mutations at position 184 of human immunodeficiency virus type-1 reverse transcriptase affect virus titer and viral DNA synthesis." *Virology*. **322**(1): 13-21.

Julias, J. G., T. Kim, G. Arnold and V. K. Pathak (1997). "The antiretrovirus drug 3'-azido-3'-deoxythymidine increases the retrovirus mutation rate." *J Virol*. **71**(6): 4254-4263.

Julias, J. G. and V. K. Pathak (1998). "Deoxyribonucleoside triphosphate pool imbalances in vivo are associated with an increased retroviral mutation rate." *J. Virol.* **72**: 7941-7949.

Kaminskas, E., A. T. Farrell, Y. C. Wang, R. Sridhara and R. Pazdur (2005). "FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension." *The oncologist*. **10**(3): 176-182.

Keulen, W., A. van Wijk, R. Schuurman, B. Berkhout and C. A. Boucher (1999). "Increased polymerase fidelity of lamivudine-resistant HIV-1 variants does not limit their evolutionary potential." *AIDS*. **13**: 1343-1349.

Kim, E. Y., T. Bhattacharya, K. Kunstman, P. Swantek, F. A. Koning, M. H. Malim and S. M. Wolinsky (2010). "Human APOBEC3G-mediated editing can promote HIV-1 sequence diversification and accelerate adaptation to selective pressure." *Journal of virology*. **84**(19): 10402-10405.

Kimura, M. (1967). "On the evolutionary adjustments of spontaneous mutation rates." *Genetical Research*. **9**(01): 11.

Kozal, M. J. (2009). "Drug-resistant human immunodefiency virus." *Clin Microbiol Infect*. **15 Suppl 1**: 69-73.

Krokan, H. and C. U. Wittwer (1981). "Uracil DNa-glycosylase from HeLa cells: general properties, substrate specificity and effect of uracil analogs." *Nucleic Acids Research*. **9**(11): 2599-2613.

LeClerc, J. E., B. Li, W. L. Payne and T. A. Cebula (1996). "High mutation frequencies among Escherichia coli and Salmonella pathogens." *Science*. **274**(5290): 1208-1211.

Lecossier, D., F. Bouchonnet, F. Clavel and A. J. Hance (2003). "Hypermutation of HIV-1 DNA in the absence of the Vif protein." *Science*. **300**(5622): 1112.

Lee, C. H., D. L. Gilbertson, I. S. Novella, R. Huerta, E. Domingo and J. J. Holland (1997). "Negative effects of chemical mutagenesis on the adaptive behavior of vesicular stomatitis virus." *J Virol*. **71**(5): 3636-3640.

Lee, H. Y., A. S. Perelson, S. C. Park and T. Leitner (2008). "Dynamic correlation between intrahost HIV-1 quasispecies evolution and disease progression." *PLoS computational biology*. **4**(12): e1000240.

Lee, T. T. and R. L. Momparler (1977). "Kinetic studies with 5-azacytidine-5'-triphosphate and DNA-dependent RNA polymerase." *Biochem Pharmacol*. **26**(5): 403-406.

Leigh, E. G. (1970). "Natural selection and mutability." *Amer. Naturalist*. **104**: 301-305.

Leigh, E. G. (1970). "Natural Selection and Mutability." *The American Naturalist*. **104**(937): 5.

Leigh, E. G. (1973). "The evolution of mutation rates." *Genetics Suppl.* **73:**: 1-18.

Levi, L. I., N. F. Gnadig, S. Beaucourt, M. J. McPherson, B. Baron, J. J. Arnold and M. Vignuzzi (2010). "Fidelity variants of RNA dependent RNA polymerases uncover an indirect, mutagenic activity of amiloride compounds." *PLoS pathogens*. **6**(10): e1001163.

Li, J., L. Li, H. P. Li, D. M. Zhuang, S. Y. Liu, Y. J. Liu, Z. Y. Bao, Z. Wang and J. Y. Li (2009). "Competitive capacity of HIV-1 strains carrying M184I or Y181I drug-resistant mutations." *Chinese medical journal*. **122**(9): 1081-1086.

Li, L. H., E. J. Olin, H. H. Buskirk and L. M. Reineke (1970). "Cytotoxicity and mode of action of 5-azacytidine on L1210 leukemia." *Cancer research*. **30**(11): 2760-2769.

Li, M., S. M. Shandilya, M. A. Carpenter, A. Rathore, W. L. Brown, A. L. Perkins, D. A. Harki, J. Solberg, D. J. Hook, K. K. Pandey, M. A. Parniak, J. R. Johnson, N. J. Krogan, M. Somasundaran, A. Ali, C. A. Schiffer and R. S. Harris (2012). "First-in-class small molecule inhibitors of the single-strand DNA cytosine deaminase APOBEC3G." *ACS chemical biology*. **7**(3): 506-517.

Liacouras, A. S. and E. P. Anderson (1975). "Uridine-cytidine kinase. Purification from a murine neoplasm and characterization of the enzyme." *Arch Biochem Biophys*. **168**(1): 66-73.

Liddament, M. T., W. L. Brown, A. J. Schumacher and R. S. Harris (2004). "APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo." *Current biology : CB*. **14**(15): 1385-1391.

Liu, Y., J. McNevin, H. Zhao, D. M. Tebit, R. M. Troyer, M. McSweyn, A. K. Ghosh, D. Shriner, E. J. Arts, M. J. McElrath and J. I. Mullins (2007). "Evolution of human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitopes: fitness-balanced escape." *Journal of Virology*. **81**(22): 12179-12188.

Liu, Z., G. Marcucci, J. C. Byrd, M. Grever, J. Xiao and K. K. Chan (2006). "Characterization of decomposition products and preclinical and low dose clinical pharmacokinetics of decitabine (5-aza-2'-deoxycytidine) by a new liquid chromatography/tandem mass spectrometry quantification method." *Rapid communications in mass spectrometry : RCM*. **20**(7): 1117-1126.

Loeb, L. A., J. M. Essigmann, F. Kazazi, J. Zhang, K. D. Rose and J. I. Mullins (1999). "Lethal mutagenesis of HIV with mutagenic nucleoside analogs." *Proc. Natl. Acad. Sci.* **96**: 1492-1497.

Loeb, L. A. and J. I. Mullins (2000). "Lethal mutagenesis of HIV by mutagenic ribonucleoside analogs." *AIDS Res Hum Retroviruses*. **16**(1): 1-3.

Loh, E., J. J. Salk and L. A. Loeb (2010). "Optimization of DNA polymerase mutation rates during bacterial evolution." *Proceedings of the National Academy of Sciences of the United States of America*. **107**(3): 1154-1159.

Lutchman, G., S. Danehower, B. C. Song, T. J. Liang, J. H. Hoofnagle, M. Thomson

and M. G. Ghany (2007). "Mutation rate of the hepatitis C virus NS5B in patients undergoing treatment with ribavirin monotherapy." *Gastroenterology*. **132**(5): 1757-1766.

Lynch, M. (2006). "The origins of eukaryotic gene structure." *Molecular biology and evolution*. **23**(2): 450-468.

Lynch, M. (2010). "Evolution of the mutation rate." *Trends in genetics : TIG*. **26**(8): 345-352.

Maeda, Y., D. J. Venzon and H. Mitsuya (1998). "Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance." *The Journal of infectious diseases*. **177**(5): 1207-1213.

Malim, M. H. and M. Emerman (2008). "HIV-1 accessory proteins--ensuring viral survival in a hostile environment." *Cell host & microbe*. **3**(6): 388-398.

Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin and D. Trono (2003). "Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts." *Nature*. **424**(6944): 99-103.

Mansky, L. M. (1998). "Retrovirus mutation rates and their role in genetic variation." *J. Gen. Virol.* **79**: 1337-1345.

Mansky, L. M. (2002). "HIV mutagenesis and the evolution of antiretroviral drug resistance." *Drug Resist Updat.* **5**: 219-223.

Mansky, L. M. (2003). "Mutagenic outcome of combined antiviral drug treatment during human immunodeficiency virus type 1 replication." *Virology*. **307**: 116-121.

Mansky, L. M. and L. C. Bernard (2000). "3'-azido-3'-deoxythymidine (AZT) and AZT-resistant reverse transcriptase can increase the in vivo mutation rate of human immunodeficiency type 1." *J. Virol.* **74**: 9532-9539.

Mansky, L. M. and K. S. Cunningham (2000). "Virus mutators and antimutators: roles in evolution, pathogenesis and emergence." *Trends in Genetics*. **16**: 512-517.

Mansky, L. M., E. Le Rouzic, S. Benichou and L. C. Gajary (2003). "Influence of reverse transcriptase variants, drugs, and Vpr on human immunodeficiency virus type 1 mutant frequencies." *J Virol*. **77**: 2071-2080.

Mansky, L. M., D. K. Pearl and L. C. Gajary (2002). "Combination of drugs and drug-resistant reverse transcriptase results in a multiplicative increase of human immunodeficiency virus type 1 mutant frequencies." *J Virol*. **76**: 9253-9259.

Mansky, L. M., S. Preveral, L. Selig, R. Benarous and S. Benichou (2000). "The interaction of Vpr with uracil DNA glycosylase modulates the human immunodeficiency virus type 1 *in vivo* mutation rate." *J. Virol.* **74**: 7039-7047.

Mansky, L. M. and H. M. Temin (1995). "Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than predicted from the fidelity of purified reverse transcriptase." *J. Virol.* **69**: 5087-5094.

Mariani, R., D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, C. Munk, H. Nymark-McMahon and N. R. Landau (2003). "Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif." *Cell*. **114**(1): 21-31.

Markowitz, M., M. Saag, W. G. Powderly, A. M. Hurley, A. Hsu, J. M. Valdes, D. Henry, F. Sattler, A. La Marca, J. M. Leonard and et al. (1995). "A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection." *The New England journal of medicine*. **333**(23): 1534-1539.

Marsden, M. D. and J. A. Zack (2009). "Eradication of HIV: current challenges and new directions." *J Antimicrob Chemother*. **63**(1): 7-10.

Martin-Hernandez, A. M., E. Domingo and L. Menendez-Arias (1996). "Human immunodeficiency virus type 1 reverse transcriptase: role of Tyr115 in deoxynucleotide binding and misinsertion fidelity of DNA synthesis." *The EMBO journal*. **15**(16): 4434-4442.

Martin-Hernandez, A. M., M. Gutierrez-Rivas, E. Domingo and L. Menendez-Arias (1997). "Mispair extension fidelity of human immunodeficiency virus type 1 reverse transcriptases with amino acid substitutions affecting Tyr115." *Nucleic Acids Research*. **25**(7): 1383-1389.

Martinez-Picado, J. and M. A. Martinez (2008). "HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo." *Virus research*. **134**(1-2): 104-123.

Matic, I., M. Radman, F. Taddei, B. Picard, C. Doit, E. Bingen, E. Denamur and J. Elion (1997). "Highly variable mutation rates in commensal and pathogenic Escherichia coli." *Science*. **277**(5333): 1833-1834.

Menendez-Arias, L. (2010). "Molecular basis of human immunodeficiency virus drug resistance: an update." *Antiviral research*. **85**(1): 210-231.

Meyer, P. R., S. E. Matsuura, A. M. Mian, A. G. So and W. A. Scott (1999). "A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase." *Molecular Cell*. **4**: 35-43.

Morse, S. (1994). *Evolutionary Biology of Vireses*, Raven Press Ltd.

Mulder, L. C., A. Harari and V. Simon (2008). "Cytidine deamination induced HIV-1 drug resistance." *Proceedings of the National Academy of Sciences of the United States of America*. **105**(14): 5501-5506.

Mullins, J. I., L. Heath, J. P. Hughes, J. Kicha, S. Styrchak, K. G. Wong, U. Rao, A. Hansen, K. S. Harris, J. P. Laurent, D. Li, J. H. Simpson, J. M. Essigmann, L. A. Loeb and J. Parkins (2011). "Mutation of HIV-1 genomes in a clinical population treated with the mutagenic nucleoside KP1461." *PloS one*. **6**(1): e15135.

Murakami, E., A. Basavapathruni, W. D. Bradley and K. S. Anderson (2005). "Mechanism of action of a novel viral mutagenic covert nucleotide: molecular interactions with HIV-1 reverse transcriptase and host cell DNA polymerases." *Antiviral Res*. **67**(1): 10-17.

Nathans, R., H. Cao, N. Sharova, A. Ali, M. Sharkey, R. Stranska, M. Stevenson and T. M. Rana (2008). "Small-molecule inhibition of HIV-1 Vif." *Nature biotechnology*. **26**(10): 1187-1192.

Neher, R. A. and T. Leitner (2010). "Recombination rate and selection strength in HIV intra-patient evolution." *PLoS computational biology*. **6**(1): e1000660.

Notley-McRobb, L., S. Seeto and T. Ferenci (2002). "Enrichment and elimination of mutY mutators in Escherichia coli populations." *Genetics*. **162**(3): 1055-1062.

Novak, M. A. (1992). "What is a quasispecies?" *Trends Ecol Evol*. **7**: 118-121.

Nowak, M. A., R. M. Anderson, A. R. McLean, T. F. Wolfs, J. Goudsmit and R. M. May (1991). "Antigenic diversity thresholds and the development of AIDS." *Science*. **254**(5034): 963-969.

Olivares, I., V. Sanchez-Merino, M. A. Martinez, E. Domingo, C. Lopez-Galindez

and L. Menendez-Arias (1999). "Second-site reversion of a human immunodeficiency virus type 1 reverse transcriptase mutant that restores enzyme function and replication capacity." *Journal of Virology*. **73**(8): 6293-6298.

Orr, H. A. (2000). "The rate of adaptation in asexuals." *Genetics*. **155**(2): 961-968.

Pace, C., J. Keller, D. Nolan, I. James, S. Gaudieri, C. Moore and S. Mallal (2006). "Population level analysis of human immunodeficiency virus type 1 hypermutation and its relationship with APOBEC3G and vif genetic variation." *Journal of virology*. **80**(18): 9259-9269.

Paces, V., J. Doskocil and F. Sorm (1968). "Incorporation of 5-azacytidine into nucleic acids of Escherichia coli." *Biochimica et biophysica acta*. **161**(2): 352-360.

Pal, C., M. D. Macia, A. Oliver, I. Schachar and A. Buckling (2007). "Coevolution with viruses drives the evolution of bacterial mutation rates." *Nature*. **450**(7172): 1079-1081.

Pandey, V. N., N. Kaushik, N. Rege, S. G. Sarafianos, P. N. Yadav and M. J. Modak (1996). "Role of methionine 184 of human immunodeficiency virus type-1 reverse transcriptase in the polymerase function and fidelity of DNA synthesis." *Biochemistry*. **35**(7): 2168-2179.

Paredes, R., M. Sagar, V. C. Marconi, R. Hoh, J. N. Martin, N. T. Parkin, C. J. Petropoulos, S. G. Deeks and D. R. Kuritzkes (2009). "In vivo fitness cost of the M184V mutation in multidrug-resistant human immunodeficiency virus type 1 in the absence of lamivudine." *Journal of Virology*. **83**(4): 2038-2043.

Pariente, N., A. Airaksinen and E. Domingo (2003). "Mutagenesis versus Inhibition in the Efficiency of Extinction of Foot-and-Mouth Disease Virus." *Journal of Virology*. **77**(12): 7131-7138.

Pariente, N., S. Sierra, P. R. Lowenstein and E. Domingo (2001). "Efficient virus extinction by combinations of a mutagen and antiviral inhibitors." *J Virol*. **75**(20): 9723-9730.

Pathak, V. K. and H. M. Temin (1992). "5-azacytidine and RNA secondary structure increase the retrovirus mutation rate." *J. Virol.* **66**: 3093-3100.

Perales, C., R. Agudo and E. Domingo (2009). "Counteracting quasispecies adaptability: extinction of a ribavirin-resistant virus mutant by an alternative mutagenic treatment." *PloS one*. **4**(5): e5554.

Perales, C., R. Agudo, S. C. Manrubia and E. Domingo (2011). "Influence of mutagenesis and viral load on the sustained low-level replication of an RNA virus." *Journal of molecular biology*. **407**(1): 60-78.

Perales, C., R. Agudo, H. Tejero, S. C. Manrubia and E. Domingo (2009). "Potential benefits of sequential inhibitor-mutagen treatments of RNA virus infections." *PLoS pathogens*. **5**(11): e1000658.

Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard and D. D. Ho (1996). "HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time." *Science*. **271**(5255): 1582-1586.

Pfeiffer, J. K. and K. Kirkegaard (2003). "A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity." *Proceedings of the National Academy of Sciences of the United States of America*. **100**(12): 7289-7294.

Pfeiffer, J. K. and K. Kirkegaard (2005). "Increased fidelity reduces poliovirus fitness

and virulence under selective pressure in mice." *PLoS pathogens*. **1**(2): e11.

Preston, B. D. (1997). "Reverse transcriptase fidelity and HIV-1 variation." *Science*. **275**: 228-231.

Preston, B. D., B. J. Poiesz and L. A. Loeb (1988). "Fidelity of HIV-1 reverse transcriptase." *Science*. **242**: 1168-1171.

Rausch, J. W., L. Chelico, M. F. Goodman and S. F. Le Grice (2009). "Dissecting APOBEC3G substrate specificity by nucleoside analog interference." *The Journal of biological chemistry*. **284**(11): 7047-7058.

Rezende, L. F., W. C. Drosopoulos and V. R. Prasad (1998). "The influence of 3TC resistance mutation M184I on the fidelity and error specificity of human immunodeficiency virus type 1 reverse transcriptase." *Nucleic Acids Research*. **26**(12): 3066-3072.

Rhee, S. Y., M. J. Gonzales, R. Kantor, B. J. Betts, J. Ravela and R. W. Shafer (2003). "Human immunodeficiency virus reverse transcriptase and protease sequence database." *Nucleic Acids Research*. **31**(1): 298-303.

Roberts, J. D., K. Bebenek and T. A. Kunkel (1988). "The accuracy of reverse transcriptase from HIV-1." *Science*. **242**: 1171-1173.

Rogozin, I. B., V. N. Babenko, L. Milanesi and Y. I. Pavlov (2003). "Computational analysis of mutation spectra." *Briefings in bioinformatics*. **4**(3): 210-227.

Rogozin, I. B. and Y. I. Pavlov (2003). "Theoretical analysis of mutation hotspots and their DNA sequence context specificity." *Mutation research*. **544**(1): 65-85.

Rogstad, D. K., J. L. Herring, J. A. Theruvathu, A. Burdzy, C. C. Perry, J. W. Neidigh and L. C. Sowers (2009). "Chemical decomposition of 5-aza-2'-deoxycytidine (Decitabine): kinetic analyses and identification of products by NMR, HPLC, and mass spectrometry." *Chemical research in toxicology*. **22**(6): 1194-1204.

Rosenberg, B. R. and F. N. Papavasiliou (2007). "Beyond SHM and CSR: AID and related cytidine deaminases in the host response to viral infection." *Advances in immunology*. **94**: 215-244.

Rouzine, I. M., E. Brunet and C. O. Wilke (2008). "The traveling-wave approach to asexual evolution: Muller's ratchet and speed of adaptation." *Theoretical population biology*. **73**(1): 24-46.

Rubinek, T., M. Bakhanashvili, R. Taube, O. Avidan and A. Hizi (1997). "The fidelity of 3' misinsertion and mispair extension during DNA synthesis exhibited by two drug-resistant mutants of the reverse transcriptase of human immunodeficiency virus type 1 with Leu74-->Val and Glu89-->Gly." *European journal of biochemistry / FEBS*. **247**(1): 238-247.

Ruiz-Jarabo, C. M., C. Ly, E. Domingo and J. C. Torre (2003). "Lethal mutagenesis of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV)." *Virology*. **308**(1): 37-47.

Sadler, H. A., M. D. Stenglein, R. S. Harris and L. M. Mansky (2010). "APOBEC3G contributes to HIV-1 variation through sublethal mutagenesis." *Journal of virology*. **84**(14): 7396-7404.

Sanjuan, R., J. M. Cuevas, V. Furio, E. C. Holmes and A. Moya (2007). "Selection for robustness in mutagenized RNA viruses." *PLoS genetics*. **3**(6): e93.

Sanjuan, R., M. R. Nebot, N. Chirico, L. M. Mansky and R. Belshaw (2010). "Viral

mutation rates." *Journal of virology*. **84**(19): 9733-9748.

Sarafianos, S. G., K. Das, A. D. Clark, Jr., J. Ding, P. L. Boyer, S. H. Hughes and E. Arnold (1999). "Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids." *Proceedings of the National Academy of Sciences of the United States of America*. **96**(18): 10027-10032.

Sarafianos, S. G., B. Marchand, K. Das, D. M. Himmel, M. A. Parniak, S. H. Hughes and E. Arnold (2009). "Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition." *Journal of molecular biology*. **385**(3): 693-713.

Sardanyes, J., S. F. Elena and R. V. Sole (2008). "Simple quasispecies models for the survival-of-the-flattest effect: The role of space." *Journal of theoretical biology*. **250**(3): 560-568.

Sasada, A., A. Takaori-Kondo, K. Shirakawa, M. Kobayashi, A. Abudu, M. Hishizawa, K. Imada, Y. Tanaka and T. Uchiyama (2005). "APOBEC3G targets human T-cell leukemia virus type 1." *Retrovirology*. **2**: 32.

Sawyers, C. (2004). "Targeted cancer therapy." *Nature*. **432**(7015): 294-297.

Schumacher, A. J., G. Hache, D. A. Macduff, W. L. Brown and R. S. Harris (2008). "The DNA deaminase activity of human APOBEC3G is required for Ty1, MusD, and human immunodeficiency virus type 1 restriction." *Journal of virology*. **82**(6): 2652-2660.

Selmi, B., J. Boretto, S. R. Sarfati, C. Guerreiro and B. Canard (2001). "Mechanism-based suppression of dideoxynucleotide resistance by K65R human immunodeficiency virus reverse transcriptase using an alpha-boranophosphate nucleoside analogue." *The Journal of biological chemistry*. **276**(51): 48466-48472.

Shafer, R. W. (2006). "Rationale and uses of a public HIV drug-resistance database." *The Journal of infectious diseases*. **194 Suppl 1**: S51-58.

Shah, F. S., K. A. Curr, M. E. Hamburgh, M. Parniak, H. Mitsuya, J. G. Arnez and V. R. Prasad (2000). "Differential Influence of Nucleoside Analog-resistance Mutations K65R and L74V on the Overall Mutation Rate and Error Specificity of Human Immunodeficiency Virus Type 1 Reverse Transcriptase." *J. Biol. Chem.* **275**: 27037-27044.

Sharma, P. L. and C. S. Crumpacker (1997). "Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation." *Journal of Virology*. **71**(11): 8846-8851.

Sharma, P. L., J. H. Nettles, A. Feldman, K. Rapp and R. F. Schinazi (2009). "Comparative analysis of in vitro processivity of HIV-1 reverse transcriptases containing mutations 65R, 74V, 184V and 65R+74V." *Antiviral research*. **83**(3): 317-323.

Sheppard, H. W., W. Lang, M. S. Ascher, E. Vittinghoff and W. Winkelstein (1993). "The characterization of non-progressors: long-term HIV-1 infection with stable CD4+ T-cell levels." *AIDS*. **7**(9): 1159-1166.

Shindo, K., A. Takaori-Kondo, M. Kobayashi, A. Abudu, K. Fukunaga and T. Uchiyama (2003). "The enzymatic activity of CEM15/Apobec-3G is essential for the regulation of the infectivity of HIV-1 virion but not a sole determinant of its antiviral activity." *The Journal of biological chemistry*. **278**(45): 44412-44416.

Sierra, M., A. Airaksinen, C. Gonzalez-Lopez, R. Agudo, A. Arias and E. Domingo (2007). "Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe." *Journal of virology*. **81**(4): 2012-2024.

Sierra, S., M. Davila, P. R. Lowenstein and E. Domingo (2000). "Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity." *J Virol*. **74**(18): 8316-8323.

Smith RA, L. L., Preston BD (2005). "Lethal mutagenesis of HIV." *Virus Res*. **107**(2): 215-228.

Sniegowski, P. D., P. J. Gerrish, T. Johnson and A. Shaver (2000). "The evolution of mutation rates: separating causes from consequences." *BioEssays : news and reviews in molecular, cellular and developmental biology*. **22**(12): 1057-1066.

Sniegowski, P. D., P. J. Gerrish and R. E. Lenski (1997). "Evolution of high mutation rates in experimental populations of E. coli." *Nature*. **387**(6634): 703-705.

Stratton, M. R. (2011). "Exploring the genomes of cancer cells: progress and promise." *Science*. **331**(6024): 1553-1558.

Suarez, P., J. Valcarcel and J. Ortin (1992). "Heterogeneity of the mutation rates of influenza A viruses: isolation of mutator mutants." *Journal of Virology*. **66**(4): 2491-2494.

Suzuki, T., T. Okada, C. Otsuka, K. Moriyama, D. Loakes and K. Negishi (2005). "Mutagenic properties of ribonucleotide analogues in reverse transcription with HIV and AMV reverse transcriptases." *Nucleic Acids Symp Ser (Oxf)*(49): 97-98.

Svarovskaia, E. S., J. Y. Feng, N. A. Margot, F. Myrick, D. Goodman, J. K. Ly, K. L. White, N. Kutty, R. Wang, K. Borroto-Esoda and M. D. Miller (2008). "The A62V and S68G mutations in HIV-1 reverse transcriptase partially restore the replication defect associated with the K65R mutation." *Journal of acquired immune deficiency syndromes*. **48**(4): 428-436.

Temin, H. M. and S. Mitzutani (1970). "RNA-dependent DNA polymerase in virions of Rous sarcoma virus." *Nature (London)*. **226**: 1211-1213.

Tenaillon, O., B. Toupance, H. Le Nagard, F. Taddei and B. Godelle (1999). "Mutators, population size, adaptive landscape and the adaptation of asexual populations of bacteria." *Genetics*. **152**(2): 485-493.

Thomas, D. C., Y. A. Voronin, G. N. Nikolenko, J. Chen, W. S. Hu and V. K. Pathak (2007). "Determination of the ex vivo rates of human immunodeficiency virus type 1 reverse transcription by using novel strand-specific amplification analysis." *Journal of Virology*. **81**(9): 4798-4807.

Traut, T. W. (1994). "Physiological concentrations of purines and pyrimidines." *Mol Cell Biochem*. **140**(1): 1-22.

Troyer, R. M., K. R. Collins, A. Abraha, E. Fraundorf, D. M. Moore, R. W. Krizan, Z. Toossi, R. L. Colebunders, M. A. Jensen, J. I. Mullins, G. Vanham and E. J. Arts (2005). "Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression." *Journal of virology*. **79**(14): 9006-9018.

Tsimring, L. S., H. Levine and D. A. Kessler (1996). "RNA virus evolution via a fitness-space model." *Physical review letters*. **76**(23): 4440-4443.

Turelli, P., B. Mangeat, S. Jost, S. Vianin and D. Trono (2004). "Inhibition of hepatitis

B virus replication by APOBEC3G." *Science*. **303**(5665): 1829.

U.S._FDA. (2012, 05/17/2012). "Guidance, Compliance & Regulatory Information: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm065007.htm." Retrieved 07/02/2012.

UNAIDS. (2012). "Global Report Fact Sheet." Retrieved 7-1-12, 2012.

Van Cor-Hosmer, S. K., W. Daddacha and B. Kim (2010). "Mechanistic interplay among the M184I HIV-1 reverse transcriptase mutant, the central polypurine tract, cellular dNTP concentrations and drug sensitivity." *Virology*. **406**(2): 253-260.

Vazquez-Perez, J. A., C. E. Ormsby, R. Hernandez-Juan, K. J. Torres and G. Reyes-Teran (2009). "APOBEC3G mRNA expression in exposed seronegative and early stage HIV infected individuals decreases with removal of exposure and with disease progression." *Retrovirology*. **6**: 23.

Vela, J. E., M. D. Miller, G. R. Rhodes and A. S. Ray (2008). "Effect of nucleoside and nucleotide reverse transcriptase inhibitors of HIV on endogenous nucleotide pools." *Antivir Ther*. **13**(6): 789-797.

Vignuzzi, M., E. Wendt and R. Andino (2008). "Engineering attenuated virus vaccines by controlling replication fidelity." *Nature Medicine*. **14**(2): 154-161.

Vodicka, M. A., W. C. Goh, L. I. Wu, M. E. Rogel, S. R. Bartz, V. L. Schweickart, C. J. Raport and M. Emerman (1997). "Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses." *Virology*. **233**(193-198).

Weber, J., B. Chakraborty, J. Weberova, M. D. Miller and M. E. Quinones-Mateu (2005). "Diminished replicative fitness of primary human immunodeficiency virus type 1 isolates harboring the K65R mutation." *Journal of Clinical Microbiology*. **43**(3): 1395-1400.

Weiss, K. K., R. Chen, M. Skasko, H. M. Reynolds, K. Lee, R. A. Bambara, L. M. Mansky and B. Kim (2004). "A role for dNTP binding of human immunodeficiency virus type 1 reverse transcriptase in viral mutagenesis." *Biochemistry*. **43**(15): 4490-4500.

White, K. L., N. A. Margot, T. Wrin, C. J. Petropoulos, M. D. Miller and L. K. Naeger (2002). "Molecular mechanisms of resistance to human immunodeficiency virus type 1 with reverse transcriptase mutations K65R and K65R+M184V and their effects on enzyme function and viral replication capacity." *Antimicrobial agents and chemotherapy*. **46**(11): 3437-3446.

Williamson, S. (2003). "Adaptation in the env gene of HIV-1 and evolutionary theories of disease progression." *Molecular biology and evolution*. **20**(8): 1318-1325.

Witvrouw, M., C. Pannecouque, K. Van Laethem, J. Desmyter, E. De Clercq and A. M. Vandamme (1999). "Activity of non-nucleoside reverse transcriptase inhibitors against HIV-2 and SIV." *AIDS*. **13**(12): 1477-1483.

Yoo, C. B. and P. A. Jones (2006). "Epigenetic therapy of cancer: past, present and future." *Nat Rev Drug Discov*. **5**(1): 37-50.

Yu, Q., R. Konig, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J. M. Coffin and N. R. Landau (2004). "Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome." *Nature structural & molecular biology*. **11**(5): 435-442.

Zennou, V. and P. D. Bieniasz (2006). "Comparative analysis of the antiretroviral activity of APOBEC3G and APOBEC3F from primates." *Virology*. **349**(1): 31-40.

Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam and L. Gao (2003). "The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA." *Nature*. **424**(6944): 94-98.

Zhao, M., M. A. Rudek, P. He, C. Hartke, S. Gore, M. A. Carducci and S. D. Baker (2004). "Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography." *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. **813**(1-2): 81-88.

APPENDIX I

FIDELITY OF HIV-1 REVERSE TRANSCRIPTASE VARIANTS

Summary of Preliminary Data

The dataset presented in Chapter II focused on a panel of 10 NRTI mutants (i.e., A62V, K65R, L74V, Y115F/A, V148I, M184I/V, K65R/M184V, L74V/Y115F/M184V), predominately in and around the RT dNTP binding site. This panel of mutants were considered to share enough commonalities to warrant their findings be published as a stand-alone unit. However, the fidelity of an additional four RT mutants (i.e., V75I, F116T, K103N, Q/S163N) was determined and remains unpublished, pending additional studies. Fidelity measurements for RT variants are shown in Figure I-1 and Table I-1. The assay used to measure mutant frequency is the same of that described in Chapter II (Fig. 2-1 and Materials and Methods).

The V75I and F116T mutants are among five mutations that constitute the Q151M multidrug resistance (MDR) complex observed in HIV; the others being A62V, F77L, and notably Q151M. Interestingly, V75I was found to have increased fidelity (Fig. I-1 and Table I-1). Previous fidelity measurements have only investigated the valine (V)-to-threonine (T) substitution at this position; results showed that the 75T mutant had no affect on RT's frameshift activity (Chen *et al.* 2005). Fidelity of F116T was found to be no different from wildtype (Fig. I-1 and Table I-1), while previous fidelity studies of this mutant have not been published. The Mansky laboratory is currently pursuing fidelity and fitness of these, and other, combinations of the Q151M MDR complex.

The K103N variant is the sole non-nucleoside RT inhibitor (NNRTI) conferring mutation investigated by the *in vivo* fidelity assay. Previous work, inline with current measurements, found K103N to have no effect on viral fidelity (Chen *et al.* 2005). This observation seems to fit well with predictions from RT's structure-function relationship

because the mutation is outside of the polymerase active site or incoming nucleotide contacts. Although, in other viruses mutations outside of these regions can serve as fidelity checkpoints, at least in other polymerases, such as poliovirus's 3Dpol (Pfeiffer *et al.* 2003).

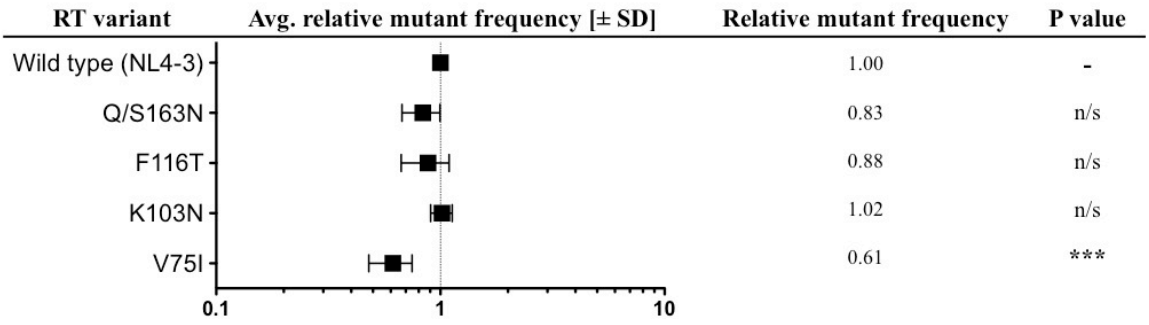


Figure I-1. Fidelity of HIV-1 RT mutants. Each of the 4 RT mutants was measured for fidelity differences relative to the wt reference strain. Mutant frequencies were calculated relative to wt and depicted on log-scale. Relative mean mutant frequency differences are listed in the adjacent column. Values represent at least 4 biological replicates experimentally repeated 4 to 8 times. SD is standard deviation, represented by error bars. * P value < 0.05; ** P value < 0.01; *** P value < 0.001.

Table I-1. Supplementary table of mutant frequency data. Data collected and used to generate Fig. I-1.

	Average relative mutant frequency						
RT variant	Number of replicates	Mean	Std. Deviation	Std. Error	Lower 95% CI	Upper 95% CI	P-value
wild type	8	N/A	N/A	N/A	N/A	N/A	N/A
V75I	5	0.600	0.1029	0.0460	0.447	0.805	***
K103N	8	1.011	0.0486	0.0146	0.938	1.090	ns
F116T	6	0.859	0.1064	0.0434	0.664	1.111	ns
S/Q163N	8	0.821	0.0821	0.0247	0.723	0.932	ns

N/A is not applicable; ns is not significant.
One-way ANOVA with Dunnett's Post test: * P value < 0.05; ** P value < 0.01; *** P value < 0.001

APPENDIX II

SELECTION OF HIV-1 DRUG RESISTANCE TO 5-AZACYTIDINE

Objectives

In the field of infectious disease medicine for which pharmacologic intervention is available, drug resistance is a perpetual obstacle. This frustrating spectacle is similar to the *molecular arms race* between host and pathogen as each entity coevolves more superior mechanisms to withstand the ever-changing environment. In essence, this phenomenon is evolution by natural selection. Inasmuch, as scientists seek to develop a "magic bullet" therapeutic for any given pathogen, attempts are futile. Research must be continually directed toward future generation pharmacologics – for increased potency, reduced off-target effects, and greater barrier to resistance – as well as novel molecular targets.

Viral resistance to small molecules can emerge by an almost infinite number of crafty mechanisms, including: mutation of drug binding site (Sarafianos *et al.* 1999; Selmi *et al.* 2001), removal of drug from active site (Arion *et al.* 1998; Meyer *et al.* 1999), compensation through *cis* (Pfeiffer et al. 2003; Arias et al. 2005) and *trans* (Nikolenko *et al.* 2010) enzymatic activity, and perhaps even population dynamics effects (Arribas *et al.* 2011). Drug resistance selection experiments are also useful to determine mechanism of action by pinpointing the genes undergoing positive selection. Furthermore, a temporal catalogue of the resistance mutations can help to delineate primary and secondary mutations. The former being involved in the mechanistic underpinning of the loss in drug susceptibility, while the latter most often serve as compensatory mutations that help to restore the initial fitness cost.

Of interest to the 5-AZC antiretroviral work in Chapter III, are continued studies to 1) help to further elucidate its mechanism of action and 2) be the first to show how HIV-1 evolves resistance to a small molecule lethal mutagen. This section describes the

preliminary dataset of attempts to select for 5-AZC-resistant HIV-1.

Preliminary Findings

A CEM-GFP T-cell line was infected with the NL4-3 HIV-1 strain, and virus was passaged at regular intervals with increasing concentrations of 5-AZC. Preliminary viral kinetics growth curves were used to gauge wt HIV-1 susceptibility to 5-AZC [Fig. II-1 and (Dapp *et al.* 2009)]. The IC_{50} for replication-competent HIV-1 was determined to be approximately 1 μM 5-AZC. The selection experiment was thus initiated at a 1000-fold lower concentration that the IC_{50}, therefore, at 1 ηM 5-AZC. The concentration of 5-AZC was doubled approximately every 9 days. Every four weeks, cells were collected for sequencing analysis of proviral DNA. Initially, individual sequences were cloned into sequencing vectors, while the latter approach consisted of population-based sequencing of the PCR amplicon to identify mutations present in the consensus sequence.

After two months of passaging virus in the presence of 5-AZC, virus was able to replicate at IC_{50} concentrations. Sequencing analysis was then used to reveal acquisition of mutations within the viral population. Analysis was performed for the entire RT 1680 base pair (bp) open reading frame (ORF). Of three independent virus populations analyzed, the majority (over 50%) of the individual proviral clones revealed the novel RT mutations threonine to isoleucine at position 215 (T215I) and aspartic acid to glutamic acid at position 237 (D237E) (Fig. II-2 A). Interestingly, some of the clones showed acquisition of T215I and D237E but never D237E alone, suggesting that T215I may have emerged first (sequencing of archived virus was unavailable to support this claim).

Over the next 8 weeks, 5-AZC was maintained at 10X the IC_{50} (or 10 μM 5-AZC).

Subsequent sequencing analysis over the next 4 and 8 weeks also showed that the viral population consensus had T215I and D237E fixed mutations (Fig. II-2 B and Fig. II-2 C, respectively). These mutations were fixed in the majority of the six independent passaging experiments. Interestingly, structural analysis revealed that one of the mutations was within proximity of the RT polymerase active site (Fig. II-3). T215I is a transitory mutation to 215 tyrosine (Y) or phenylalanine (F), which is part of the TAM complex for thymidine analog resistance (AZT-zidovudine and d4T-stavudine) (Rhee *et al.* 2003; Shafer 2006). RT position 215 resides at the pocket surrounding incoming dNTPs. D237E has never been found to be associated with resistance to any clinical or experimental nucleoside/non-nucleoside analog (Rhee *et al.* 2003; Shafer 2006). It is located distal to the NNRTI binding pocket more than 10 Å from the polymerase active site.

To show that either, or both, of these putative 5-AZC mutations confer resistance, they were cloned into a parental HIV-1 wt vector and molecular clone for phenotypic virological analyses. This set of experiments would rule out the dependence of undefined mutations throughout the virus population and show that T215I and/or D237E are necessary and sufficient to confer 5-AZC resistance. Quite interestingly, single-cycle vector assays found no difference in the susceptibility of either, or both, mutations to 5-AZC (Fig. II-4 A-D). This was also the case when the mutations were cloned into replication-competent HIV-1 and subjected to passaging at high concentrations of 5-AZC (Fig. II-5 B & D). Of note, these RT mutations do not seem to impose replicative defects when measured by parallel growth kinetics (Fig. II-5 A and Fig. II-5 C).

Summary and Conclusions

Long-term HIV-1 passaging experiments revealed emergence of two putative 5-AZC resistance mutations. Sequencing analysis showed that the viral population consensus sequence (through amplicon-based sequencing) possessed fixed T215I and D237E mutations in the HIV-1 *pol* gene. Although virus was able to be continually passaged at 10-25X the IC_{50} 5-AZC, these mutations alone do not confer resistance to the azapyrimidine compound. Experiments are ongoing, but thus far, the data suggest that either: 1) T215I and D237E, in concert with mutations outside of the RT ORF, confer resistance to 5-AZC; or 2) T215I and D237E have serendipitously become fixed in multiple independent selection experiments, through cross-contamination, while unidentified mutations outside of the RT ORF confer 5-AZC resistance; or 3) a quasispecies population effect of complex viral mutations is able to confer reduced susceptibility to 5-AZC. Interestingly, the latter of the three has been recently described as the bacteriaphage Q-beta's mechanism for 5-AZC resistance (Arribas *et al.* 2011); however, this phenomenon has yet to be validated in another viral system.

Materials and Methods

Plasmid constructs and cell lines and reagents. The CEM-GFP indicator T cell line, derived from CEM-SS parental line, was used to passage virus and visually monitor viral infection. A HEK 293T cell lines was used to produce replication-competent virus upon transfection. Cell lines were maintained in RPMI and DMEM with 10% fetal calf serum, respectively. The wt HIV-1 NL4-3 molecular clone was used to initiate the drug-selection experiments. The 5-AZC stock compound was purchased from Sigma-Aldrich and stored at -20 °C. Working stocks of 5-AZC were made by dissolving DMSO and

storage at -20C.

Passaging of virus with 5-AZC. Passaging experiments consisted of 250,000 CEM-GFP cells infected at an MOI of 0.01 across six biological replicates. The IC_{50} for replication-competent HIV-1 was determined to be approximately 1 μM 5-AZC. The selection experiments were initiated at a 1000-fold lower concentration that the IC_{50}, therefore, at 1 ηM 5-AZC. The concentration of 5-AZC was doubled approximately every 9 days, while cells were also split for archival stocks stored at -80 C. Approximately, every four weeks, cells were collected for sequencing analysis of proviral DNA.

Isolation of proviral DNA and sequencing analysis. Total genomic DNA was isolated from infected CEM-GFP cells using the Roche High Pure PCR Template Preparation Kit (Roche Applied Science). DNA was amplified using HIV-1 RT specific primers with degenerate bases to accommodate 5-AZC G-to-C mutations. 2550+_opti TCC CAT TAS TCC TAT TSA SAC; and 4176-_opti AAT TSS TTT GTG TGS TGG TA (S = 50%/50% G/C mix). The thermocycler conditions were as follows: 94C for 2:00 min; 94C for 30s, 47.5C for 30s, 72C for 2:00 min for 5 cycles; 94C for 30s, 50C for 30s, 72C for 2:00 min for 25 cycles, then a final 7:00 min extension at 72C. The 1626bp PCR products were visualized by separation on a 1% TAE DNA gel. Amplicons were then extracted (Wizard® SV Gel and PCR Clean-Up System) and either cloned into a sequencing plasmid (Promega pGEM®-T Vector Systems) or directly sequenced. All sequencing was performed off-site using standard Sanger sequencing methods (Functional Biosciences; Madison, WI). Sequence alignments were performed using DNAStar SeqMan software (Lasergene; Madison, WI). Sequencing primers for pGEM-T clones were: T7 promoter 5'-TAA TAC GAC TCA CTA TAG GG-3' and SP6 promoter 5'-

GCT ATT TAG GTG ACA CTA TAG-3'. The sequencing primer for PCR-based sequencing was the 2550+_opti forward oligonucleotide.

Phenotypic test of putative 5-AZC resistance mutations. The T215I and D237E RT mutations were generated individually and in tandem by site directed mutagenesis (Stratagene). They were subcloned into the pNL4-3 HIV-1 molecular clone and the pHIG *env*- HIV-1 vector. Both infectious virus and vector virus were produced off of HEK 293T producer cells using PEI transfection methods. Vector virus was pseudotyped with the VSV envelope glycoprotein. Single-cycle vector assays of wt and the three mutants transduced U373-MAGI cells that were pretreated for 2 hrs with increasing 5-AZC. Titered NL4-3 virus was used to infect CEM-GFP cells at MOI of 0.01. After 24 hr, 5-AZC was added to triplicate wells at increasing concentrations. Every two days, cells were split as needed, and fresh media and compound were added. Experiment was terminated after peak of virus replication, typically 10-14 days. Scoring for %GFP+ populations (surrogate for infectious virus) was performed on BD LSRII flow cytometer.

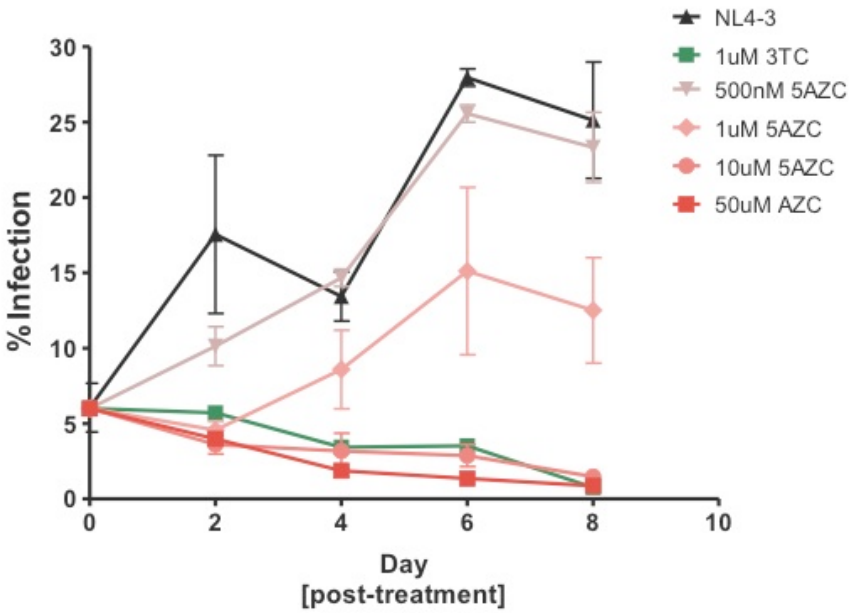
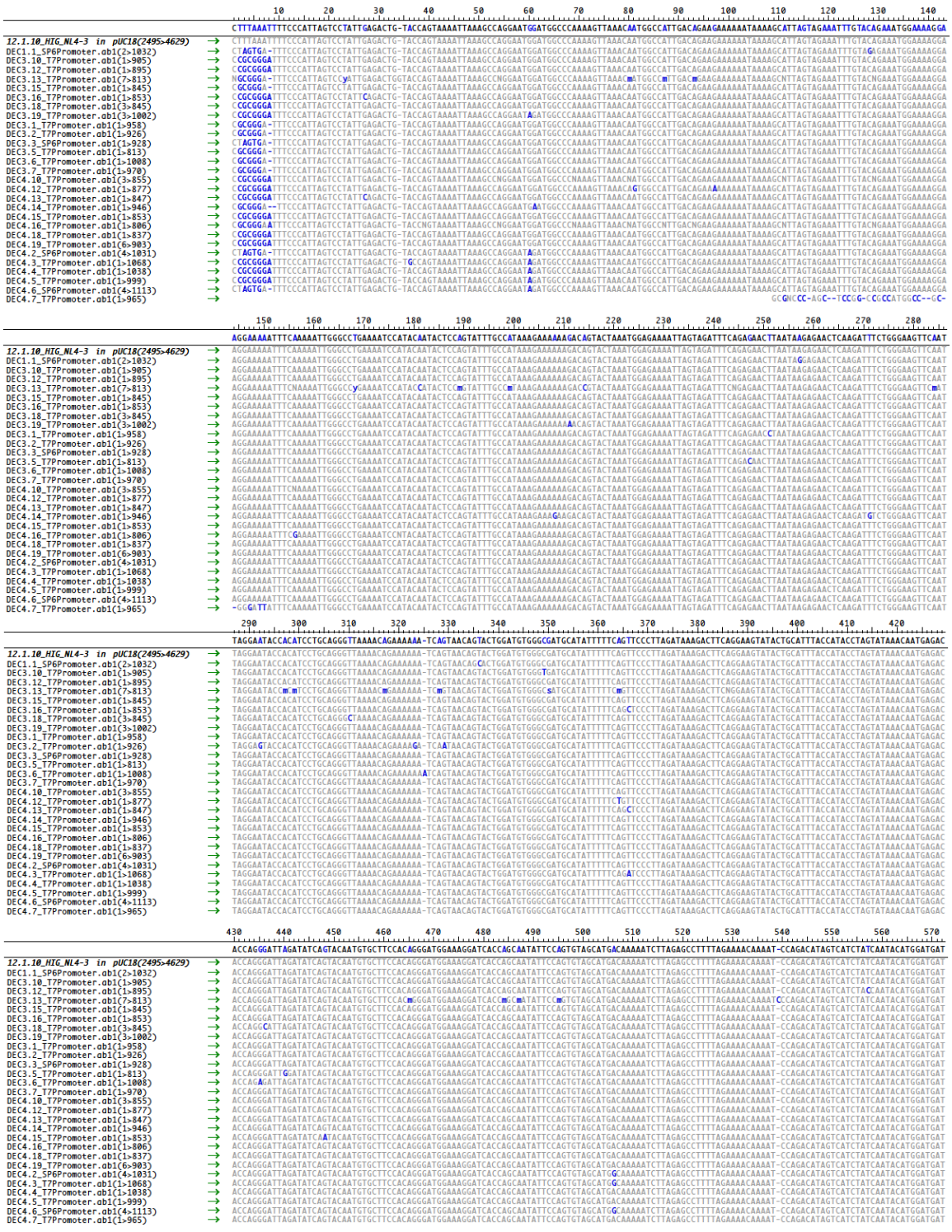
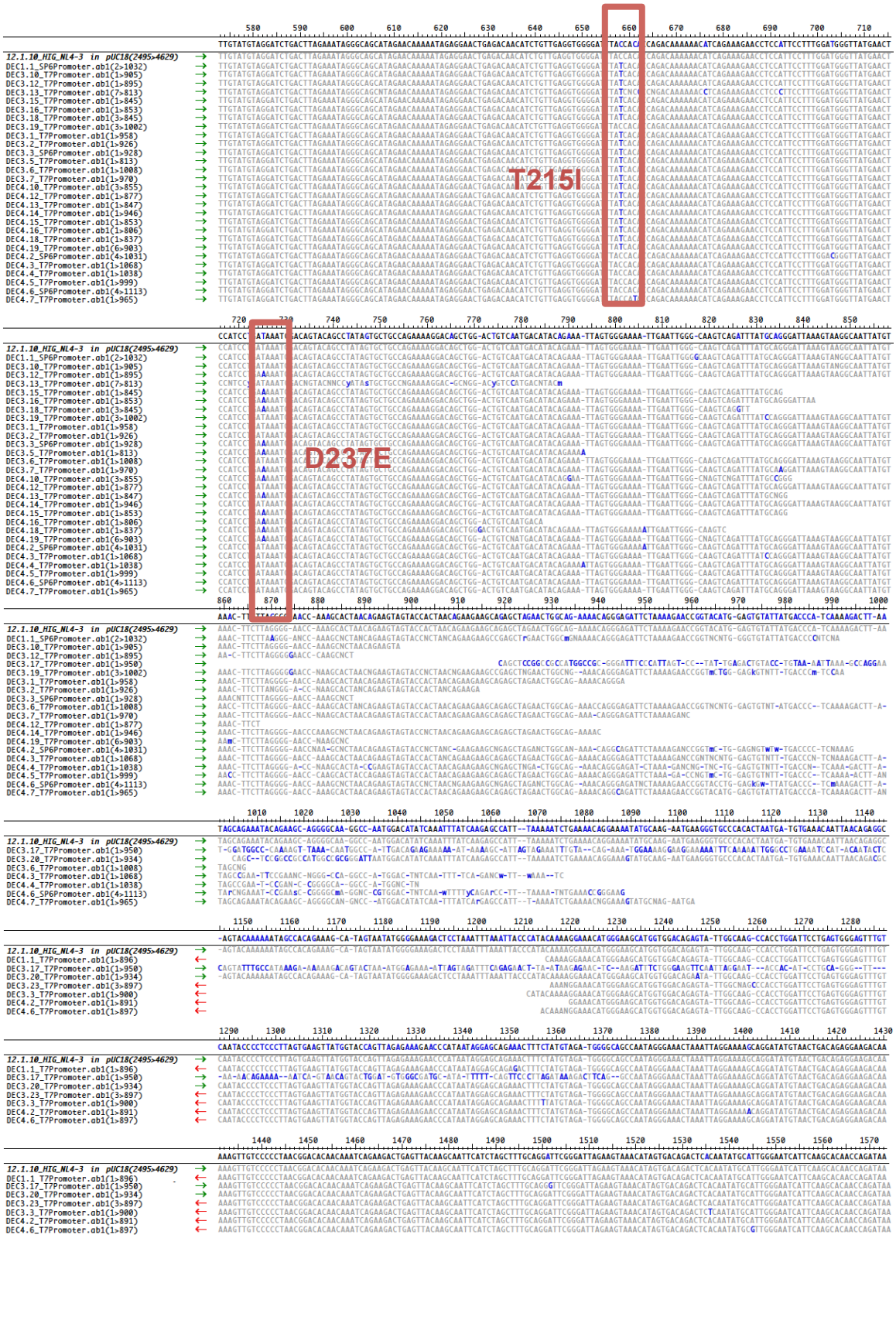
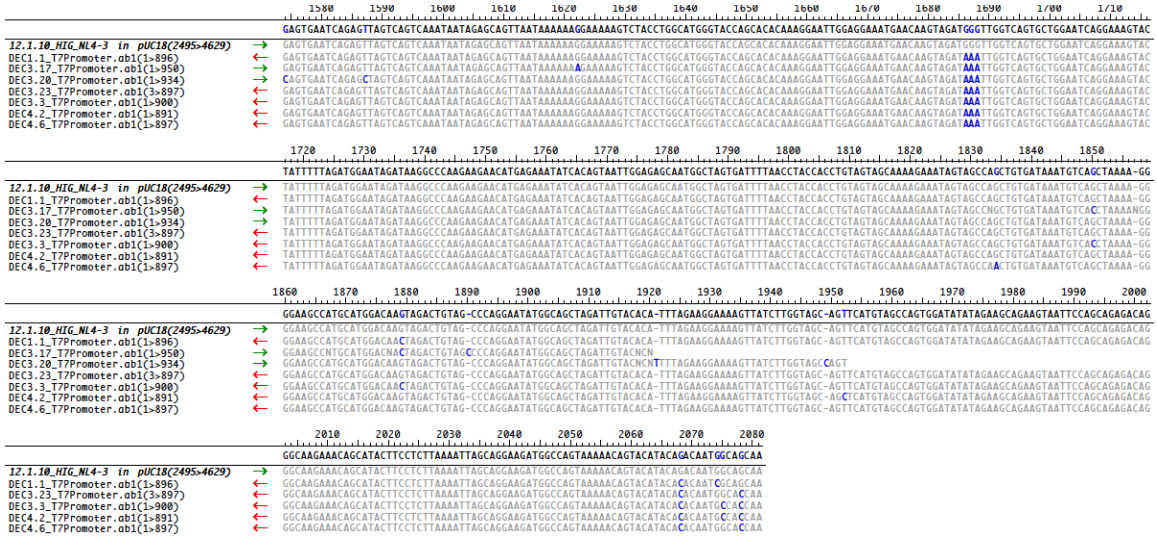


Figure II-1. Viral kinetics of HIV-1 at increasing concentrations of 5-AZC. Wildtype HIV-1 molecular clone (NL4-3) was passaged for 8 days at increasing concentrations of 5-AZC (increasing intensity of pink lines). A positive (3TC [2',3'-dideoxy-3'-thiacytidine], dideoxynucleoside NRTI; green line) and negative (no drug, black line) are displayed. Each point represents the mean of three biological replicates. Error bars are ±SEM.

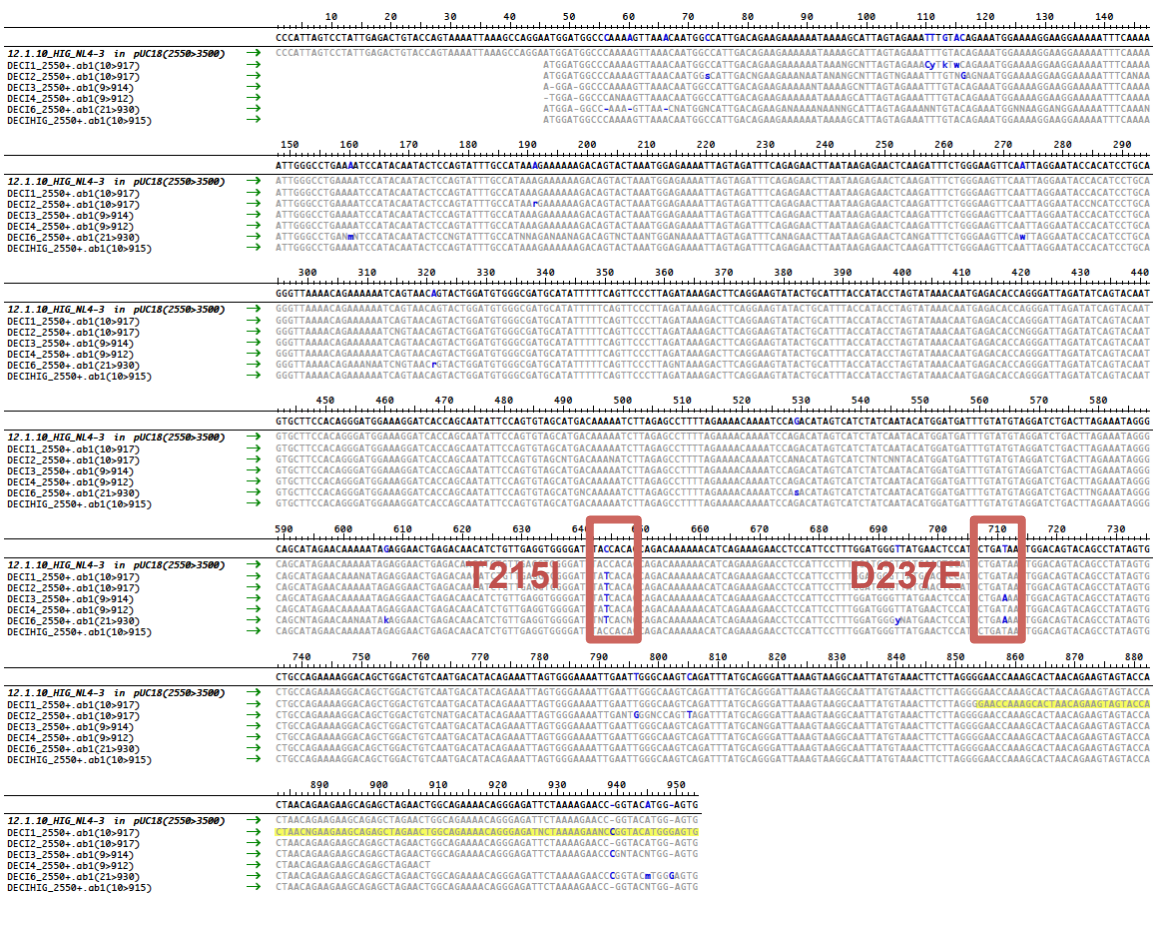
A)




T215I
D237E



B)



C)

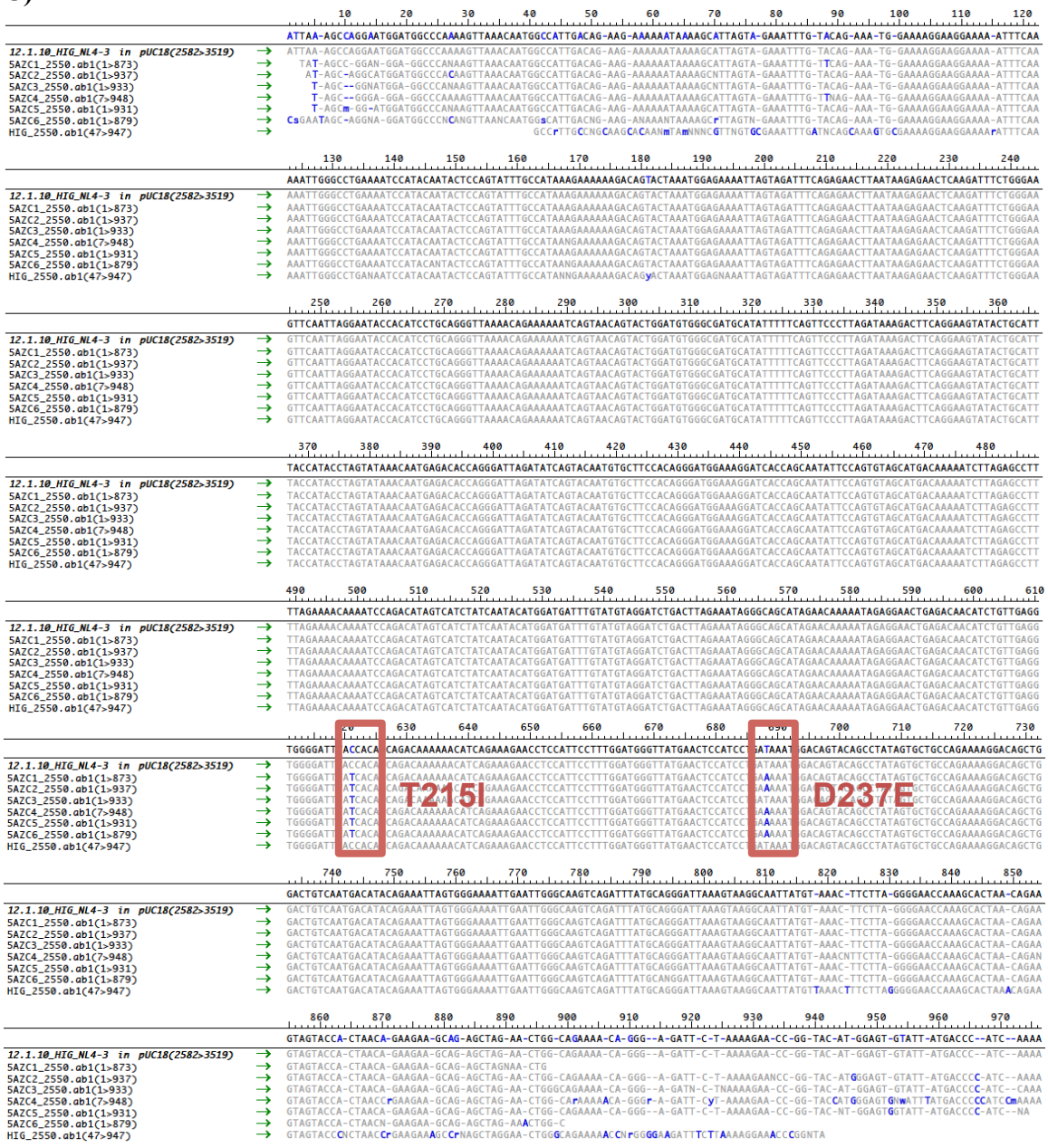


Figure II-2. Sequencing analyses of HIV-1 exposed to 5-AZC. (A) Alignment of individual proviral DNA sequences from three independent passaging experiments (1, 13, and 14 respectively) after approximately two months of passaging virus with 5-AZC. The alignment spans through the RT ORF position 2550 to 4230. Noted are the fixed mutations T215I and D237E in over half of the individual proviral clones sequenced.

Alignment of proviral consensus sequences from PCR amplicons at three **(B)** and four **(C)** months of resistance selection. These alignments represent five **(B)** and six **(C)** cultures independently passaged with 5-AZC. All alignments were performed using DNAStar SeqMan software (Lasergene Madison, WI). The topmost horizontal read is the consensus sequence and directly beneath this is the wildtype reference sequence (*12.1.12_HIG_NL4-3*). The bottom most sequence (*HIG*_2550) is a control denoting PCR amplification and sequencing of wildtype plasmid viral DNA. All other sequences depict either **(A)** individual or **(B) & (C)** consensus sequences isolated and PCR-amplified from proviral DNA.

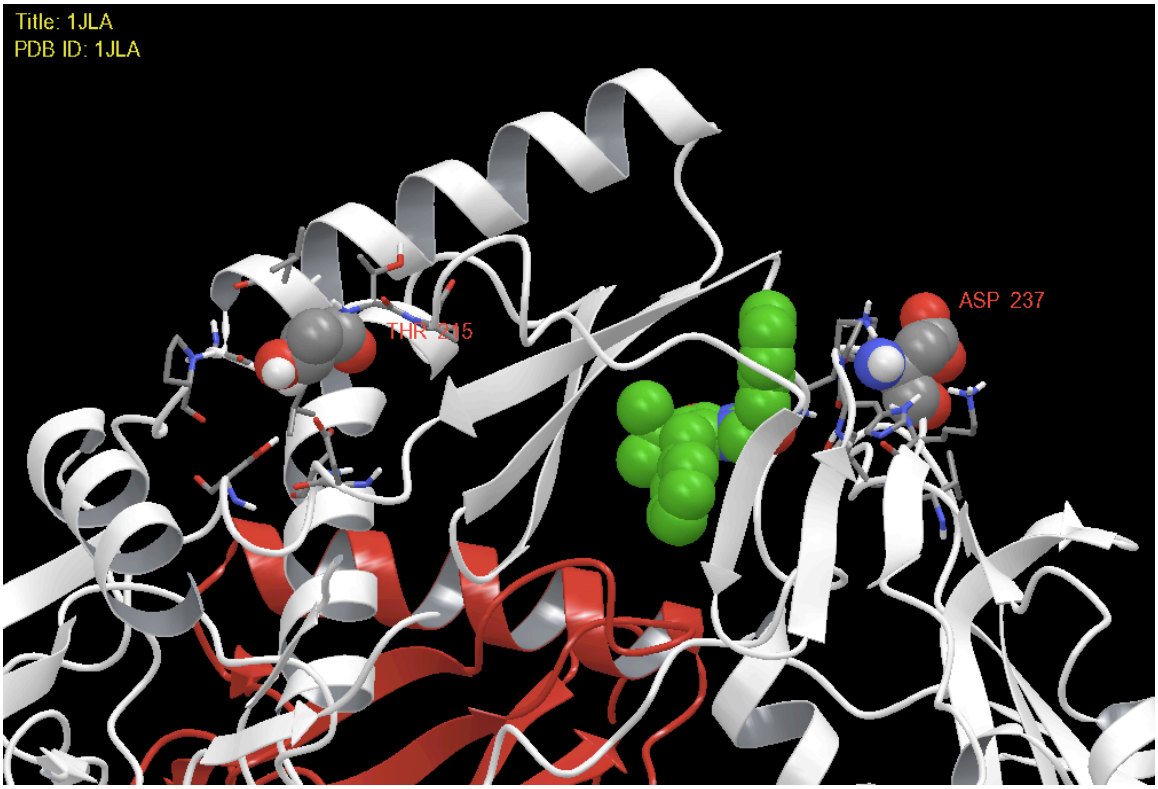


Figure II-3. Ribbon diagram of putative 5-AZC resistance mutations: T215 and D237. Image depicts RT ribbon diagram with threonine (T) 215 and aspartic acid (D) 237 as space filled amino acids. Green molecule represents location of incoming dNTP into enzyme active site. (Image courtesy Y. Sham, Center for Drug Design)

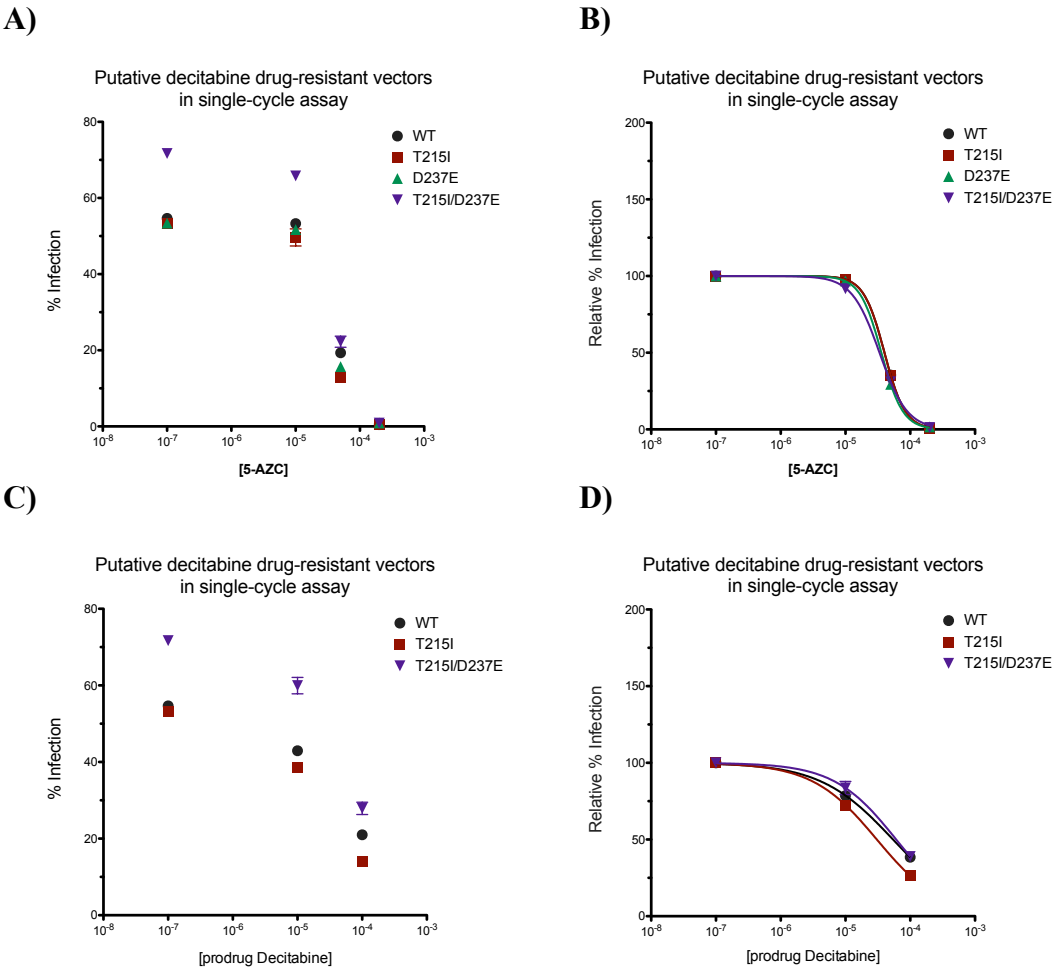


Figure II-4. Single-cycle HIV-1 concentration response curves and mutant frequency analysis of putative 5-AZC resistant mutants. The 215I and D237E RT mutants were cloned individually and in tandem into HIV-1 vectors. Vector virus was created and used to infect target cells. Transduction efficiency of wt and mutant clones showed no difference in 5-AZC **(A & B)** or related azapyrimidine decitabine **(C & D)** susceptibility; depicted as raw transduction efficiency **(A & C)** and relative to wt no-drug control **(B & D)**. Neither was there any difference in the susceptibility to either azapyrimidines' mutagenic effects, as measured by single-cycle HIG assay (see Chapter 3 Material and Methods; Fig. 3-1).

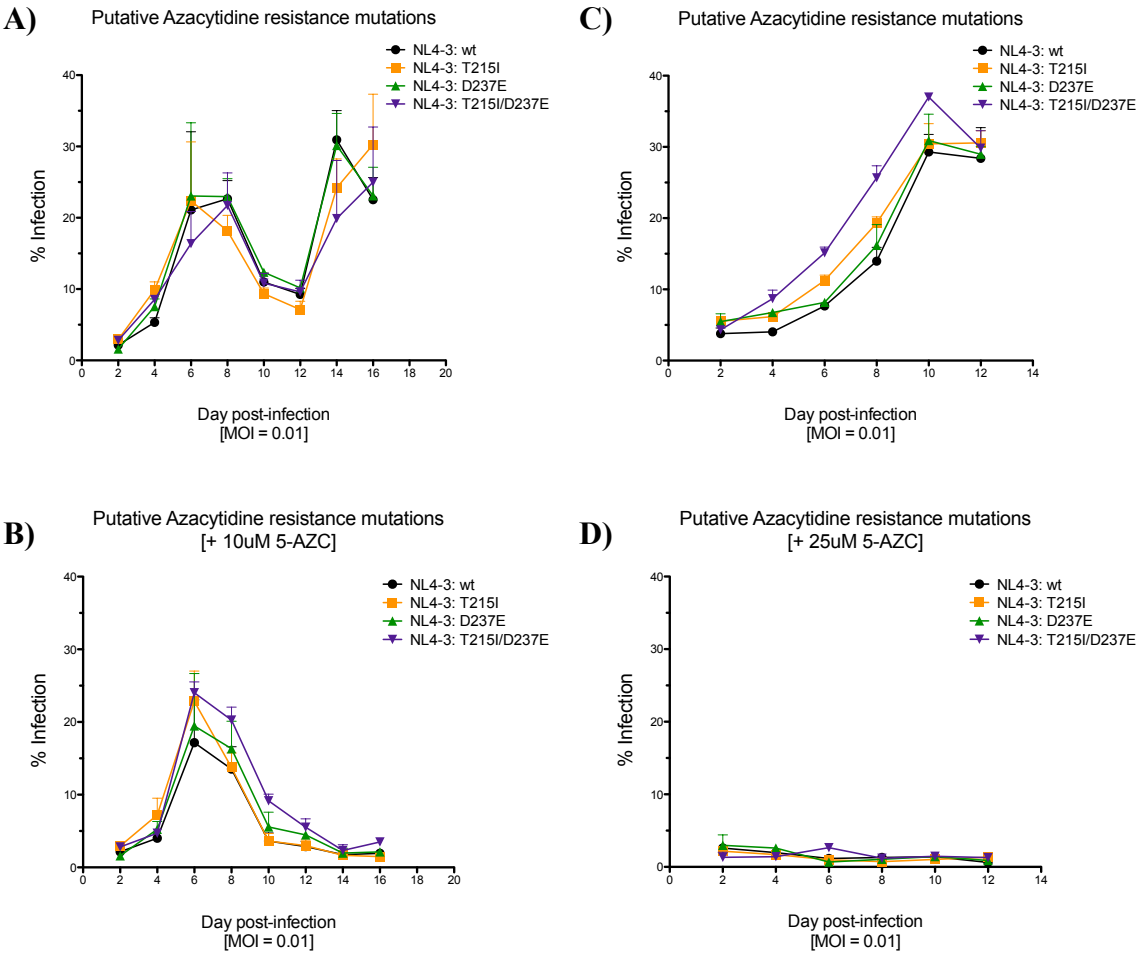


Figure II-5. Replication-competent HIV-1 concentration response curves of putative 5-AZC resistance mutants. Wt and the three mutant HIV-1 molecular clones were used to infect indicator T-cell line, CEM-GFP as equivalent MOI = 0.01. Viral infectivity was monitored for 12-15 days in the presence of 10 μM 5-AZC **(B)** or 25 μM 5-AZC **(D)**. Matched no drug controls show that there are no obvious replication defects of the mutant virus, **(A)** and **(C)**, respectively. Each point represents the mean of three biological replicates. Error bars are ±SEM.

BIBLIOGRAPHY

Arias, A., R. Agudo, C. Ferrer-Orta, R. Pérez-Luque, A. Airaksinen, E. Brocchi, E. Domingo, N. Verdaguer and C. Escarmís (2005). "Mutant Viral Polymerase in the Transition of Virus to Error Catastrophe Identifies a Critical Site for RNA Binding." *J Mol Biol*. **353**(5): 1021-1032.

Arion, D., N. Kaushik, S. McCormick, G. Borkow and M. A. Parniak (1998). "Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase." *Biochemistry*. **37**: 15908-15917.

Arribas, M., L. Cabanillas and E. Lazaro (2011). "Identification of mutations conferring 5-azacytidine resistance in bacteriophage Qbeta." *Virology*.

Chen, R., M. Yokoyama, H. Sato, C. Reilly and L. M. Mansky (2005). "Human immunodeficiency virus mutagenesis during antiviral therapy: impact of drug-resistant reverse transcriptase and nucleoside and nonnucleoside reverse transcriptase inhibitors on human immunodeficiency virus type 1 mutation frequencies." *J Virol*. **79**(18): 12045-12057.

Dapp, M. J., C. L. Clouser, S. Patterson and L. M. Mansky (2009). "5-Azacytidine can induce lethal mutagenesis in human immunodeficiency virus type 1." *Journal of virology*. **83**(22): 11950-11958.

Meyer, P. R., S. E. Matsuura, A. M. Mian, A. G. So and W. A. Scott (1999). "A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase." *Molecular Cell*. **4**: 35-43.

Nikolenko, G. N., K. A. Delviks-Frankenberry and V. K. Pathak (2010). "A novel molecular mechanism of dual resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors." *Journal of Virology*. **84**(10): 5238-5249.

Pfeiffer, J. K. and K. Kirkegaard (2003). "A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity." *Proceedings of the National Academy of Sciences of the United States of America*. **100**(12): 7289-7294.

Rhee, S. Y., M. J. Gonzales, R. Kantor, B. J. Betts, J. Ravela and R. W. Shafer (2003). "Human immunodeficiency virus reverse transcriptase and protease sequence database." *Nucleic Acids Research*. **31**(1): 298-303.

Sarafianos, S. G., K. Das, A. D. Clark, Jr., J. Ding, P. L. Boyer, S. H. Hughes and E. Arnold (1999). "Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids." *Proceedings of the National Academy of Sciences of the United States of America*. **96**(18): 10027-10032.

Selmi, B., J. Boretto, S. R. Sarfati, C. Guerreiro and B. Canard (2001). "Mechanism-based suppression of dideoxynucleotide resistance by K65R human immunodeficiency virus reverse transcriptase using an alpha-boranophosphate nucleoside analogue." *The Journal of biological chemistry*. **276**(51): 48466-48472.

Shafer, R. W. (2006). "Rationale and uses of a public HIV drug-resistance database." *The Journal of infectious diseases*. **194 Suppl 1**: S51-58.

APPENDIX III

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