STUDIES ON THE ANTIRETROVIRAL MECHANISM OF ACTION OF CLOFARABINE

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Dedication

To my family and friends and people of all backgrounds who identify with or support bisexual, transgender, queer, questioning, intersex, asexual, lesbian, gay and straight allied (BTQQIALGA) communities. I would not have been able to navigate my way through life or the academy without your strength, courage, vision and guidance.

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Abstract

Since the beginning of the AIDS epidemic over thirty years ago, human immunodeficiency virus type 1 (HIV-1) has infected seventy-five million people and has claimed the lives of over thirty-six million people worldwide, making HIV/AIDS one of the most devastating global infectious disease epidemics in history. To date, no preventative vaccine or curative treatment exists for HIV-1 infection. The availability of drugs to treat HIV-1 infection has led to drug resistance, which limits the utility of antiviral therapy. This has provided the basis for the continual need for identifying new targets for antiviral drugs.

This dissertation investigated the antiretroviral activity and mechanism of action for clofarabine, a purine nucleoside antimetabolite. Clofarabine was demonstrated to exert antiretroviral activity against both HIV-1 and human immunodeficiency virus type 2 (HIV-2). Studies directed at elucidating the antiretroviral mechanism of action support a model in which clofarabine acts as an inhibitor of ribonucleotide reductase, leading to imbalances in cellular dNTP pools, which reduces viral infectivity through an increase in the HIV-1 mutation rate.

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Chapter 1

General Introduction

The HIV Replication Cycle

Since 1981, the human immunodeficiency virus (HIV) has been reported to infect seventy-five million people and has claimed the lives of over thirty-six million people worldwide (UNAIDS, 2013), making HIV/AIDS one of the most devastating global infectious disease epidemics the world has ever seen. HIV is a member of the lentivirus genus in the *Retroviridae* family of viruses. The ability of HIV to transform its single stranded, positive sense RNA genome into double stranded DNA revolutionized the central dogma of biology and is the genetic hallmark of all retroviruses. Many retrovirologists see reverse transcriptase (RT), the enzyme responsible for catalyzing this reaction, as the defining protein of this viral family.

Six major steps comprise the life cycle of HIV: 1. Binding and Fusion 2. Uncoating and Reverse Transcription 3. Integration 4. Viral RNA and Protein Expression 5. Budding 6. Maturation (Figure 1-1). When an HIV viral particle infects a cell, the viral gp120 envelope protein interacts with the CD4 receptor of a susceptible cell. Following this interaction, gp120 undergoes a conformational change, exposing the viral gp41 fusion peptide. Depending upon the target cell type, the fusion peptide interacts with either a CCR5 coreceptor (macrophages) or a CXCR4 co-receptor (CD4-positive T-cells). Following another conformational change, the bilayers of the target cell and the viral particle fuse together. Fusion results in entry of the viral core into the cellular cytoplasm, which is followed by

uncoating and the continuation of reverse transcription, which begins in the viral core.

The exact timing of the uncoating of the viral capsid vis-à-vis reverse transcription is a matter of debate, as reviewed in (Arhel, 2010). Viral particles contain both the RT enzyme and two copies of the HIV RNA genome within their cores (Nikolaitchik *et al.*, 2013). The capsid lattice of the HIV-1 core has gaps of up to ten nanometers (Li *et al.*, 2000), large enough for dNTP's from the target cell and for small molecule inhibitors of RT to passively diffuse into it. The activation state and dNTP concentration of the target cell can influence the timing and successful completion of reverse transcription (Doitsh *et al.*, 2010).

Reverse transcription is a complicated, but well-studied process, as reviewed in (Sarafianos *et al.*, 2009). Briefly, reverse transcription initiates when a tRNA-lys_3 primer binds to the primer binding site (PBS) in the HIV genomic RNA. The heterodimeric RT begins synthesis of single stranded viral DNA, while simultaneously degrading the genomic RNA template. This single stranded strong stop DNA hybridizes with a complementary RNA sequence on the opposite end of the viral RNA genome, undertaking the "first jump." Elongation of the minus strand follows, which is in turn followed by positive strand DNA synthesis. Positive strand synthesis requires priming from the central polypurine tract (PPT) and uses the minus strand vDNA as a template. Strand displacement synthesis extends the positive stranded vDNA. RNA primer removal follows,

which facilitates the second jump and resulting circularization of the double stranded viral DNA.

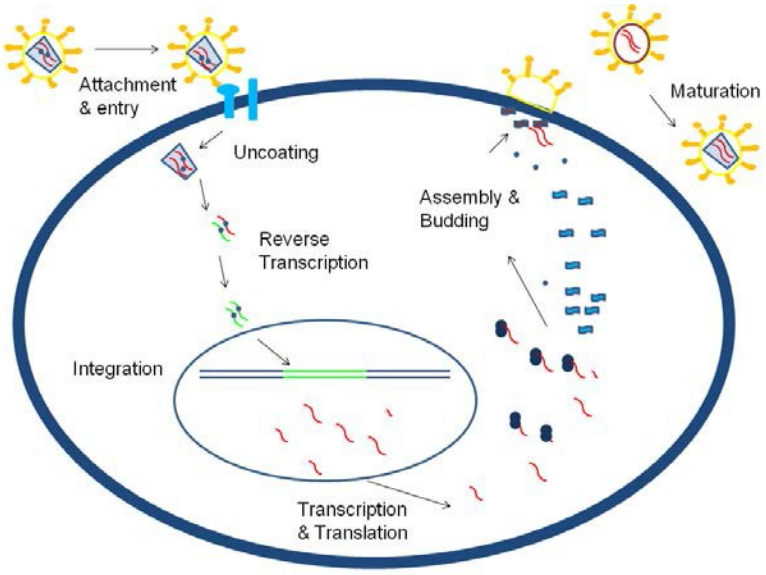

Figure 1-1 The HIV Life Cycle. The HIV life cycle is defined by six major steps: 1. Attachment and entry to the receptor/coreceptor 2. Uncoating of the viral capsid 3. Reverse transcription 4. Transcription and translation of viral mRNAs and proteins 5. Assembly and budding and 6. Maturation of the viral particle to produce a conical shaped core.

Upon completion of reverse transcription, the newly formed double-stranded viral DNA linearizes, is actively imported into the nucleus and integrates into the host chromosomal DNA (Katz *et al.*, 2003). Following integration, the cellular machinery makes RNA copies of the virus, which viral accessory proteins help export. Subsequently, cellular proteins translate some of these viral RNA's into viral proteins. Other, full length RNAs dimerize and are packaged into budding HIV-1 particles as viral genomes, along with many viral and some host proteins. Upon budding, HIV protease processes the viral Gag protein, and the resulting viral capsid monomers multimerize to form the mature viral core, in a

shape approximating a fullerene cone. The resulting mature HIV particles may then infect susceptible target cells.

Antiretroviral Drugs: HIV Treatment and Prevention

Disruption of any of the steps in the HIV life cycle represents an opportunity to stop viral replication. No preventative vaccine or curative treatment exists for HIV. Antiretroviral drugs have provided the most powerful tool to stop the loss of life associated with the HIV/AIDS epidemic. Currently, the United States Food and Drug Administration (FDA) has approved six classes of medications to treat HIV-1, including entry inhibitors, fusion inhibitors, nucleoside reverse transcription inhibitors (NRTIs), non-nucleoside reverse transcription inhibitors (NNRTIs), integrase inhibitors and protease inhibitors (Arts & Hazuda, 2012). In the late 1990's, Dr. David Ho, who first suggested the concept of highly effective antiretroviral therapy (HAART), proposed long-term suppression of HIV in patients required treatment with at least three drugs from two classes of anti-HIV medications (Perelson *et al.*, 1996). Soon after, clinical trials featuring different combinations of three HIV drugs confirmed the success of the HAART approach in patients (Gulick *et al.*, 1997; Montaner *et al.*, 1998).

Today, HAART remains the gold standard in HIV treatment. HAART has saved millions of lives and has allowed many people to achieve undetectable viral plasma loads (UNAIDS, 2013). The benefits of HAART, however, extend beyond individual life-saving treatment. The utilization of effective HAART can also prevent new HIV infections on a public health level. In San Francisco, the

reduction in community viral loads due to widespread HAART led to lower transmission of HIV throughout the population (Das *et al.*, 2010). Subsequent studies demonstrated HAART could reduce HIV transmission in HIV-1 serodiscordant couples by up to ninety-six percent (Anglemyer *et al.*, 2013; Cohen *et al.*). In 2011, Science magazine named “HIV treatment as prevention” the scientific breakthrough of the year (Cohen).

The ability of anti-HIV drugs to prevent not only the transmission but also the acquisition of HIV has also been demonstrated. In 2012 the FDA approved the NRTI’s emtricitabine and tenofovir, marketed together in one pill as Truvada, for use in pre-exposure prophylaxis (PrEP), after clinical trials showed daily Truvada reduced the rates of HIV-1 acquisition for populations at high risk for HIV-1 infection (Baeten & Celum, 2013; Celum, 2011; Murnane *et al.*). These findings followed in the intellectual footsteps of the earlier success of the use of HIV-1 drugs for prevention of mother to child transmission of HIV (PMTCT) (CDC, 1994a; b; Mofenson *et al.*, 2009) and post-exposure prophylaxis for HIV-1 (PEP) (Cardo *et al.*, 1997). Many anti-HIV microbicides under development to prevent HIV also contain anti-retroviral drugs.

HIV Drug Resistance Creates a Constant Need for Novel HIV Therapeutics

Though anti-HIV-1 drugs ushered in a life-saving revolution to the HIV/AIDS epidemic, drug resistance immediately presented a threat to the sustained success of the fight against the virus (Larder *et al.*, 1989a; Larder & Kemp, 1989; Larder *et al.*, 1989b). HIV has evolved drug resistance to every

drug in each class of FDA approved anti-HIV therapeutics. HIV drug resistance is such a well-established issue, FDA guidance states sponsors developing anti-HIV-1 drugs should include characterization of HIV-1 drug resistance mutations and cross-resistance profiles in their submissions to the agency (HHS, 2013).

Drug resistance reduces the efficacy of HAART and results in rebounds in HIV-1 viral loads. In individual patients, viral load increases are correlated with the reductions in CD4+ T-cell populations that can lead to AIDS. Viral load increases in patients experiencing drug resistance related treatment failure can also result in the transmission of drug resistant virus. The transmission of drug resistant virus decreases the treatment options for newly HIV infected people. The Centers for Disease Control (CDC) recently reported an increase in the percentage of transmitted drug resistant HIV-1 in the United States (Taylor *et al.*, 2013). Although it is has not yet been demonstrated as a serious problem, increased resistance to anti-HIV-1 drugs could also result from the use of these drugs to prevent HIV-1 infection. In order to extend the lives of treatment experienced patients and of patients newly infected with drug resistant HIV, scientists must constantly develop more anti-HIV drugs with different drug resistance profiles. The high cost of drug resistance and the essential role drugs play in combating the epidemic (Krentz *et al.*, 2013) have led to extensive studies describing how drug resistance develops and spreads.

The Evolution of HIV Drug Resistance and its Impact on Viral Fitness

Mutations resulting in amino acid changes in HIV viral proteins provide the molecular genetic basis for the acquisition of drug resistance (Menendez-Arias, 2013; Wainberg *et al.*, 1993). HIV drug resistance evolves so easily in large part because of the error prone nature of the HIV-1 reverse transcriptase enzyme (Boyer *et al.*, 1992). RT-based errors provide the primary force responsible for HIV's high mutation rate (Mansky & Temin, 1995). When combined with the staggering replicative capacity of HIV-1 in untreated patients (Perelson *et al.*, 1996), theoretical calculations show the HIV-1 mutation rate results in the mutation of every base in the roughly 9.7 kilobase HIV-1 genome every day. Experimental evidence has confirmed the high mutation rate of HIV-1(Mansky & Temin, 1995). The high number of HIV viral mutants results in the development of a genetically diverse HIV-1 quasispecies, rather than a genetically identical wild type HIV-1 population in the host. HIV-1 drug treatment results in the outgrowth of viral mutants that confer a selective advantage toward replicating in the presence of the drug, allowing a rebound in viral load in HIV infected patients.

Reverse transcriptase, therefore, fundamentally drives the evolution of HIV, allowing the virus to respond to selective pressures. RT synthesizes the double stranded DNA copy of the HIV genome that serves as the template for the production of all viral proteins and subsequently produced RNA genomes. Errors made in the viral DNA can lead to drug resistance mutations in any part of the HIV genome – including in the coding region of RT itself. Mutations in RT not

only lead to drug resistance to RT inhibitors, they can also alter the course of HIV evolution and viral fitness. Drug resistance mutations in RT impact the mutation rate (Mansky *et al.*, 2003), catalytic rate (Deval *et al.*, 2005), structure (Arnold *et al.*, 1996; Huang *et al.*, 1998; Tu *et al.*, 2010; Zhan *et al.*, 2009), dNTP and dNTP analog binding ability (Gao *et al.*, 2000) and dNTP analog excision rate (Matamoros *et al.*, 2004; Xu *et al.*, 2009) of the virus.

Changes in these RT related parameters in turn can result in alterations to the replicative capacity and reduce the overall fitness of the virus, in laboratory experiments and in patients (Bouchonnet *et al.*, 2005; Dapp *et al.*, 2013a; Weber *et al.*, 2007). Dynamic mathematical models have been generated as a means to estimate the impact of drug resistance mutations on HIV fitness and evolution (von Kleist *et al.*, 2012). Anti-HIV drug therapies that intentionally drive HIV evolution towards less fit viral genotypes have been proposed, but have yet to be developed. Investigations of the impact of NRTI drug resistance mutations on the course of HIV evolution suggest that resistance, in particular the M184V/M184I and K65R/L74V mutations, can reduce the fitness of the virus, especially under limiting dNTP conditions (Back & Berkhout, 1997; Bouchonnet *et al.*, 2005; Deval *et al.*, 2004). Ongoing drug development efforts, including the investigation of ribonucleotide reductase inhibitors (RNRIs) (Rawson *et al.*, 2013) and lethal mutagens (Clouser *et al.*, 2010; Dapp *et al.*, 2009), also may provide the additional tools and information necessary to pave the way for this objective.

NRTIs: Mechanism of Action and Drug Resistance and the Impact on Viral Fitness

Of all HIV proteins, researchers first successfully targeted RT as an antiviral drug target. The thymidine analog zidovudine (AZT) was the first anti-HIV-1 drug to show activity in clinical trials (Fischl *et al.*, 1987). AZT, like all other nucleoside reverse transcriptase inhibitors (NRTIs), acts as a viral DNA chain terminator to inhibit HIV replication. The structure of NRTIs contributes to their antiviral activity - NRTIs lack a 3'-hydroxyl group on their sugar moiety. When RT incorporates a phosphorylated NRTI into HIV DNA, the lack of the 3'-hydroxyl prevents the formation of the 5′,3'-phosphodiester bond that is necessary for extension of the DNA chain. Currently, seven unique NRTIs are FDA approved to treat HIV-1 infection - abacavir (ABC), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), zidovudine (AZT), didanosine (ddI) and tenofovir disoproxil fumarate (TDF). The drug resistance mutations and pathways for all of these NRTIs have been extensively characterized, as discussed in (Arts & Hazuda, 2012; Lawyer *et al.*, 2011; Menendez-Arias, 2013; Sarafianos *et al.*, 2009).

At least two mechanistic pathways – excision and exclusion – can lead to HIV viral RT mutants resistant to NRTIs. In the excision mechanism, the thymidine analog mutations (TAM's) mutations in RT, including T215F/T215Y, K219E/K219Q, M41L, D67N, K70R, and L210W increase the enzyme's ability to remove chain-terminating activated NRTIs from the growing viral DNA chain via ATP mediated phosphorlysis (Meyer *et al.*, 1999). Interestingly, HIV-1, but not

HIV-2, can use the excision mechanism to facilitate drug resistance (Boyer *et al.*, 2012; Boyer *et al.*, 2006). Accordingly, the TAM mutations have been reported to have no effect on zidovudine sensitivity for HIV-2 and are rarely seen in HIV-2 positive individuals on NRTI containing treatment regiments (Charpentier *et al.*, 2014; Smith *et al.*, 2009).

In the exclusion mechanism, RT mutations, including M184I, M184V, K65R, K70E, V75I, and L74V grant the enzyme a heightened ability to prevent triphosphorylated NRTIs from incorporating into the viral DNA (Miranda *et al.*, 2005; Tisdale *et al.*, 1993). Some of these mutations can also decrease the ability of RT to incorporate endogenous nucleotides, especially under low dNTP concentrations, thereby contributing to fitness reductions (Bouchonnet *et al.*, 2005; Deval *et al.*, 2004). Both HIV-1 and HIV-2 can utilize the exclusion mechanism of RT resistance, and these resistance mutations have been described in individuals infected with either virus (Boyer *et al.*, 2012; Charpentier *et al.*, 2014; Menendez-Arias & Alvarez, 2014; Parkin & Schapiro, 2004).

When dNTP pool levels reach critically low levels, neither HIV-1 nor HIV-2 RT can continue to synthesize viral DNA (Boyer *et al.*, 2012). Given that RT competitively incorporates endogenous dNTPs and NRTIs into vDNA, the ratio of endogenous dNTPs to NRTIs can impact the development of drug resistance (Amie *et al.*, 2013a). Accordingly, drugs or cellular factors that affect the endogenous dNTP pool levels or modulate the phosphorylation levels of NRTIs can also influence how sensitive drug resistant RT mutants become to NRTIs

(Back *et al.*, 1996; Balzarini, 2000). Ribonucleotide reductase inhibitors have been used in combination with NRTIs to enhance the chain terminating activity of these drugs, as well as to resensitize NRTI drug resistant viruses to NRTI treatment (Frank *et al.*, 2004a; Heredia *et al.*, 2000; Palmer *et al.*, 1999). The combination of RNRIs and viral mutagens also can increase the HIV-1 mutation frequency and synergistically reduce HIV-1 infectivity (Clouser *et al.*, 2010). These results, along with the anti-viral effect of RNRIs (Gao *et al.*, 1993; Heredia *et al.*, 2000) and the antiviral cellular host restriction factor triphosphohydrolase SAMHD1 (Amie *et al.*, 2013a; Baldauf *et al.*, 2012; Berger *et al.*, 2011), indicate dNTP pool levels themselves may represent an anti-viral drug target.

The Cellular Maintenance of dNTP Pools: Ribonucleotide Reductase and the Innate Immune Response

All cellular organisms as well as DNA viruses and viruses that go through a DNA intermediate in their life cycles require dNTPs to replicate and repair their DNA-containing genomes. Cells tightly regulate the production, timing and ratios of dNTP pools, which helps maintain nuclear and mitochondrial genome integrity and prevent viral infection and cancer (Kim *et al.*, 2012; Rampazzo *et al.*, 2010). The ancient and highly conserved enzyme ribonucleotide reductase (RNR) serves as the master creator and regulator of dNTP pools in cells (Reichard, 1985). As the sole human enzyme responsible for reducing NDPs to dNDPs, RNR governs the rate limiting step in dNTP synthesis (Reichard *et al.*, 1961; Szekeres *et al.*, 1997). Comprised of a large, constitutively expressed regulatory

subunit (RRM1) and a small, cell cycle dependent (RRM2) or DNA damage induced (p53R2) catalytic subunit (Tanaka *et al.*, 2000), RNR has served as both an anti-cancer and an antiviral drug target (Szekeres *et al.*, 1997).

For successful replication, viruses that use DNA as the building blocks of their genomes must gain access to cellular concentrations of dNTPs in excess of the Kd of the polymerases that synthesize viral DNA. More efficient viral replication occurs when cellular dNTP concentrations exceed the Km of the polymerases (Diamond *et al.*, 2004). RNR produces high concentrations of dNTPs in the cytoplasm during S phase of the cell cycle (Leeds *et al.*, 1985). Resting cells, however, have much lower concentrations of cytoplasmic dNTPs (Hakansson *et al.*, 2006). Many DNA-containing viruses that replicate in the cytoplasm can only efficiently infect cells undergoing cell division, because the dNTP concentrations in many resting cell types are too low to support the complete synthesis of their genomes (Baldauf *et al.*, 2012; Capella *et al.*, 2012; Pieroni *et al.*, 1999). Interestingly, some herpes viruses encode viral ribonucleotide reductases in order to help overcome this block, further demonstrating the crucial role dNTP levels can play in viral replication (Swain & Galloway, 1986).

Intrinsic cellular immune defenses have evolved to lower cellular dNTP pool concentrations to defend against viral infection. The expression of the triphosphohydrolase SAMHD1 in myeloid cell lineages renders these cell types highly impermissive to HIV-1 infection (Laguette *et al.*, 2011). HIV-2 encodes the

Vpx protein to target SAMHD1 for degradation (Ahn *et al.*, 2012), allowing the virus to replicate in macrophages (Amie *et al.*, 2013a) and, unlike HIV-1, also in dendritic cells (Lahaye *et al.*, 2013). Without Vpx, however, HIV-2 cannot efficiently infect macrophages (Kappes *et al.*, 1991). The HIV-2 RT enzyme has lower activity than HIV-1 RT (Boyer *et al.*, 2012), which may partially help explain why HIV-1, which does not encode Vpx, can (albeit inefficiently) infect macrophages (Diamond *et al.*, 2004), while HIV-2 heavily relies on Vpx for this ability. SAMHD1 has also been shown to prevent other DNA viruses, including HSV-1 and Vaccinia virus, from infecting cell types with low dNTP pools (Hollenbaugh *et al.*, 2013; Kim *et al.*, 2013). The increasingly understood impact on fluctuations in dNTP pools on the permissiveness of cells to DNA-dependent virus infection suggests RNR may be a timely drug target to revisit for the treatment and prevention of DNA viruses and retroviruses, including HIV-1 and HIV-2 (Allouch *et al.*, 2013).

Antiviral Ribonucleotide Reductase Inhibitors and Their Mechanisms of Action

The Food and Drug Administration has approved many small molecule inhibitors of ribonucleotide reductase (RNRIs). Collectively, RNRIs include drugs that target the large or small subunits of RNR and utilize a diverse variety of mechanisms to inhibit the enzyme, as reviewed in (Nordlund & Reichard, 2006). RNR inhibition decreases one or more dNTP pools in cells, which can be reversible or irreversible. Hydroxyurea (HU), a non-nucleoside RNR, was the first

in class to be FDA approved. Subsequently, many more nucleoside and non-nucleoside analog RNRIs have become available on the U.S. market.

The RNRIs hydroxyurea (Lori *et al.*, 1994), gemcitabine (Clouser *et al.*, 2010), resveratrol (Heredia *et al.*, 2000) and deferoxamine (Rawson *et al.*, 2013) have anti-HIV activity in cell culture. In the 1990s and early 2000s, many physicians prescribed HU off-label to treat HIV-1 infection. HU, while having no anti-HIV-1 efficacy as monotherapy in patients (Frank *et al.*, 2004a; Giacca *et al.*, 1996), decreases HIV-1 viral loads, when used in combination with NRTIs, especially ddI (De Antoni *et al.*, 1997; Frank *et al.*, 2004a). Subsequent toxicity concerns, however, have largely led to the discontinued use of HU as a component of HAART.

The mechanistic studies of HU mediated inhibition of HIV show HU's ability to decrease the concentration of dNTP pools correlates with its antiviral effect (Bakshi *et al.*, 2007). Primer extension assays illustrate that in stimulated PBMCs, HU can deplete dATP pools for over 48 hours, while simultaneously increasing other dNTP pools (Gao *et al.*, 1995). Another antiviral non-nucleoside RNRI, resveratrol, in contrast, has been reported to deplete all dNTP pools 24 hours after treatment of HL-60 cells (Horvath *et al.*, 2005). Depletions of dNTP pools that alter the naturally occurring cellular ratios of the four endogenous dNTPs to one other can lead to the inhibition of viral replication in at least two distinct ways: 1. By increasing the viral mutation rate beyond the error threshold

of the virus (lethal mutagenesis) and 2. By reducing viral DNA synthesis. Each of these mechanisms and their complexities are addressed.

Lethal Mutagenesis: Cellular dNTP Pool Modulations Increase HIV Mutation

Eurkaryotic cells maintain dNTP pools at levels consistent with the DNA replication needs of the cell. Though the concentrations, ratios, and overall levels of dNTP pools can vary from cell type to cell type, the endogenous concentrations of the four dNTP pools are not equimolar in mammalian cells (Mathews & Ji, 1992; Vartanian *et al.*, 1997). In most mammalian cells the concentrations of the dNTP pools vary, in the order of [TTP] > [dATP] > [dCTP] > [dGTP] (Mathews & Ji, 1992; Zhang & Mathews, 1995). In primary cells, the non-equimolar balance of eurkaryotic dNTP pools has been found to promote increased fidelity over equimolar dNTP pools in vitro (Martomo & Mathews, 2002). In contrast, groups have suggested these unequal pool levels are non-ideal for HIV-1 replication. Notably, the addition of thymidine to PMBCs has an antiviral effect that can be partially reversed by the addition of deoxycytidine (Meyerhans *et al.*, 1994). Though Meyerhans et. al. did not characterize the molecular mechanisms responsible for the antiviral effect of thymidine, they note the incomplete recovery of viral infectivity upon addition of deoxycytidine after thymidine treatment could have been due to an increase in viral mutation (Meyerhans *et al.*, 1994).

The idea that increases in viral mutational burden can have antiviral effects is related to the concept of lethal mutagenesis. In the context of viral

infection, lethal mutagenesis is defined as the point where an increase in mutation leads to the increasing production of non-infectious progeny virions, eventually resulting in extinction of the viral population. The specific threshold at which the viral population experiences genetic collapse and the virus can no longer successfully replicate has been referred to as extinction catastrophe (Dapp *et al.*, 2013b). The amount of mutation required for different viruses to reach their extinction catastrophe thresholds varies. The theoretical measure of robustness evaluates the extent of mutation a virus can withstand before reaching its extinction catastrophe threshold (Elena *et al.*, 2006).

As an antiviral strategy, lethal mutagenesis is theorized to work best for viruses that have low robustness (Elena *et al.*, 2006). Lethal mutagenesis can have genetic or environmental causes. The genetic destruction of a viral population might occur due to a mutation in key viral replication or repair enzymes that lead to a strong mutator phenotype. Or, mutagenic compounds might be added to the viral replication environment, leading to enhanced viral mutation through mutagen incorporation, changes in dNTP pools leading to overall increases in base misincorporation, increases in DNA extension without proofreading after base misincorporation, increases in one or more “next nucleotide” nucleotide sequence dependent errors, chemical modification of DNA by alkylating or other similar agents (Drablos *et al.*, 2004), or a combination of these mechanisms.

Viral population size, basal mutation rate, genetic robustness, replication rate, and the accessibility of mutagenic factors to reach the entire viral population can all impact the timing, evolution and dynamics of lethal mutagenesis. It has been suggested that the high mutation rate and low robustness of RNA viruses and retroviruses make them easily amenable to extinction via lethal mutagenesis (Dapp *et al.*, 2013a; Loeb *et al.*, 1999). Indeed, the lethal mutagenesis of RNA viruses (Ortega-Prieto *et al.*) including HIV (Clouser *et al.*, 2010; Dapp *et al.*, 2009) have been demonstrated in cell culture via the incorporation of mutagenic drugs. What remains uncertain, however, is whether an imbalance in dNTP pools alone can be mutagenic enough to cause the lethal mutagenesis of HIV.

At least one study has demonstrated a correlation between increasing HU concentration and corresponding increases in both dNTP pool imbalances and higher retroviral mutation rates in murine leukemia virus (MLV) and spleen necrosis virus (SNV) (Julias & Pathak, 1998). This same study also found dose-dependent increases in thymidine increased retroviral mutation rates even more than HU, despite resulting in a very different profile of dNTP pool imbalances (Julias & Pathak, 1998). Despite finding an association between reduced viral titer and an increase in mutation rate, the authors speculated heightened mutation rates were not responsible for the majority of the antiviral effect of either HU or thymidine. Instead, they state the near five-fold increase in viral mutation rate they observed in one cycle of replication could theoretically only account for 10% of the greater than 60% or 90% reductions in titer reduction observed in

their study from thymidine or HU treatment, respectively. Julias *et. al.* suggest the majority of the HU's antiviral effect likely came from a reduction in DNA synthesis due to overall lowered dNTP pools and that thymidine's antiviral effect could be due to cytotoxicity (Julias & Pathak, 1998). Neither of these hypotheses were directly tested in this study, though notably others had previously demonstrated HU treatment is associated with depletions in vDNA synthesis and that HU's potency is higher in cell lines with lower dNTP pools (Lori *et al.*, 1994).

Other authors have noted HU treatment in cell culture results in a decrease in viral titer and a corresponding increase in HIV-1 RT template switching and recombination (Nikolenko *et al.*, 2004). This increase in template switching was hypothesized to be due to a dNTP depletion related reduction in DNA synthesis (Nikolenko *et al.*, 2004). Notably, in this study, no tests were done to measure whether HU increased or decreased viral mutant frequency. In the absence of HU treatment, however, this same study reported that many NRTI drug resistance mutations also led to an increase in template switching, and that this was correlated with reduced dNTP binding, increased pyrophosphorlysis, greater RT fidelity, lower viral titer, and lower mutant frequencies (Nikolenko *et al.*, 2004). It is not clear whether some or all of these trends would hold true under HU treatment.

Other studies suggest that if Nikolenko *et. al.* had investigated the impact of HU, they may have found that unlike some NRTI drug resistance mutations that increase RT fidelity, HU can increase mutant frequency. HU treatment has

been shown to be associated with a 6.2 fold increase in the mutant frequency of wild type HIV-1 RT, even in one round of replication in a cell line undergoing continuous division (Mansky, 2003). When multidrug resistant HIV-1 RT was treated with HU, the mutant frequency increased 21.8 times vis-à-vis wild type HIV-1 RT with no drug treatment (Mansky, 2003). In contrast to Julias *et. al.*, the authors of this study report HU's increase in viral mutation frequency likely accounted for the decrease in viral titer observed under HU treatment (Mansky, 2003). Similar results in these studies were found with thymidine treatment (Mansky, 2003). Using a different assay, studies with the RNRIs gemcitabine and resveratrol have also independently shown these drugs increase HIV-1 mutant frequency in a dose-dependent fashion (Rawson *et al.*, 2013). These authors also suggest that non-specific increases in viral mutation rate play a large role in the anti-HIV-1 activity of these drugs. Mutant spectra result showing an increase in mutations on a genetic level further support the finding of increased mutagenicity using phenotypic assays.

When HIV-1 proviruses have been sequenced from RNRI-treated cells, mutant spectra analysis has revealed most of the detected mutations are G-to-A transitions (Rawson *et al.*, 2013). For many years, wild type HIV-1 proviral clones have also been isolated that contain high rates of G-to-A transition mutations, including some sequences with extensive G-to-A sequence hypermutation (Li *et al.*, 1991b; Mansky & Temin, 1995). Prior to the discovery of APOBEC3 family members, the high TTP, lower dCTP pyrimidine dNTP pool bias in HIV-1 target

cells was hypothesized to be responsible for these G-to-A hypermutants (Martomo & Mathews, 2002; Vartanian *et al.*, 1997).

In the early 2000s, however, APOBEC3 proteins were found to restrict HIV-1 replication at least in part by causing G-to-A hypermutation (Sheehy *et al.*, 2002; Zheng *et al.*, 2004). Today, G-to-A hypermutation is nearly universally considered the hallmark of APOBEC3 mutation, especially when the majority of the individual mutations are in the sequence context of GG and GA dinucleotides (Chaipan *et al.*, 2013). To cause hypermutation, APOBEC3 proteins must be packaged into budding virions (Mangeat *et al.*, 2003; Mariani *et al.*, 2003). During reverse transcription, the APOBEC3 proteins APOBEC3G, APOBEC3F, APOBEC3DE, and APOBEC3H can processively deaminate cytidine to uracil in minus strand single stranded viral DNA (Chelico *et al.*, 2006; Hultquist *et al.*, 2011). This leads to G-to-A transition mutations in the positive strand of HIV DNA. High levels of uracil in the resulting single-stranded DNA transcripts can lead to the degradation of viral DNA (Mangeat *et al.*, 2003). Any HIV-1 viral DNA that does integrate, moreover, often contains G-to-A hypermutated DNA (Mangeat *et al.*, 2003; Russell *et al.*, 2009).

Interestingly, different target cell types infected with Vif-containing HIV-1 viral like particles produced from the same cell line can show different rates of G-to-A hypermutation (Holtz & Mansky, 2013). These findings suggest the target cell environment may also influence the extent of APOBEC3 associated hypermutation. *In silico* modeling studies have questioned whether APOBEC3

alone or APOBEC3 in combination with endogenous dNTP pool imbalances explains the G-to-A hypermutation observed in HIV-1 infected cells (Deforche *et al.*, 2007). Taken together with the older literature demonstrating the TTP to dCTP pool level ratio (Martomo & Mathews, 2002; Vartanian *et al.*, 1997) and "next nucleotide" effects (Zhang & Mathews, 1995) can impact the rate and sequence context of G-to-A hypermutation, these studies suggest modulations in dNTP pools associated with SAMHD1 or RNRI treatment may also influence the G-to-A hypermutation of APOBEC3 proteins.

Regardless of the contributions APOBEC3 proteins vs. target cell dNTP pools make to G-to-A hypermutation, components of the intrinsic host immune response to HIV infection and perhaps also the homeostatic state of dNTP pools in immune cells selectively increase viral mutation. Experimental evidence from tissue culture indicates increases in G-to-A hypermutation correlate with reductions in viral infectivity. To date, high G-to-A proviral hypermutation has not been shown to cure HIV in any patient, though higher patient APOBEC3G levels have been associated with lower HIV proviral burden (De Pasquale *et al.*, 2013). On an infected cell basis, processive G-to-A hypermutation may theoretically lethally mutate an individual HIV provirus that subsequently integrates, leading to the production of only non-infectious viral particles from that cell. Especially in the presence of Vif, APOBEC3 proteins may also sub-lethally mutate HIV-1 proviruses (Sadler *et al.*, 2010). Sublethal APOBEC3 associated mutations may contribute to the evolution of HIV drug resistance mutations (Mulder *et al.*, 2008).

The advent of drug resistance mutations, however, does not mean HIV drug resistance will immediately emerge. Other factors, including dNTP pool levels in target cells, also affect whether the phenotype of drug resistance will emerge in an HIV-1 infected individual on drug treatment.

As previously mentioned, many studies have shown that HU and other RNRIs can re-sensitize NRTI resistant HIV RT mutants to NRTI treatment (Balzarini, 2000; Heredia *et al.*, 2014; Heredia *et al.*, 2000; Heredia *et al.*, 2013; Lori *et al.*, 1997). The results from at least one small clinical study, however, have also shown HU treatment may result in the enhanced generation of drug resistance mutations (De Antoni *et al.*, 1997). De Antoni and colleagues reported that when comparing HU + ddI vs. ddI monotherapy treated patients, 50% of patients in the HU + ddI treatment arm developed resistance to ddI, while only 25% of those in the ddI monotherapy arm developed these resistance mutations over a 24 week time period (De Antoni *et al.*, 1997). Patients in the HU + ddI treatment arm, however, also experienced a one log greater reduction in their viral loads (De Antoni *et al.*, 1997). The authors indicate the comparative reduction in viral load was likely due to HU's reduction in dATP pools allowing for a relative greater potency of ddI, resulting in the continued inhibitory effects of ddI even in the presence of resistance mutations (De Antoni *et al.*, 1997).

The potential lesson from these and similar studies (Frank *et al.*, 2004b) is that even if the imbalanced and depleted cellular dNTP pools resulting from RNRI treatment do lead to an increases in HIV-1 drug resistance mutations, it is

likely because these RNRIs also result in an overall increase in HIV-1 mutation frequency. This increased mutation frequency correlates with decreases in viral loads in HIV-1 positive patients, when RNRIs are given in combination with NRTIs, when compared to NRTI treatment alone. These data suggest part of the antiviral mechanism of action of RNRIs is likely related to lethal mutagenesis.

Importantly, these results also suggest even if HIV-1 drug resistance genotypes do increase under RNRI treatment, drug resistance mutations may not actually lead to the phenotype of NRTI drug resistance in HIV-1 positive patients taking both NRTIs and RNRIs. This effect can occur through multiple mechanisms, including that RNRI's can increase the relative ratio of NRTIs vis-à-vis dNTP's by reducing one or more endogenous dNTP pools and/or by increasing the phosphorylation of the NRTIs (Gao *et al.*, 1995). These modulations in dNTP pools and changes in phosphorylation patterns allow HIV-1 RT to incorporate NRTIs, even in the presence of drug resistance mutations that lead to reduced NRTI binding and/or increased excision of these chain terminating drugs.

RNRIs Reduce DNA Synthesis Through Depletions in dNTP Pools

In addition to enhanced mutation, reductions in viral DNA synthesis also likely contribute to the anti-HIV activity of RNRIs, much like the antiviral mechanisms of some host immune factors. A reduction in one or more dNTP pools caused by antiviral immune responses or by RNRI administration can cause RT to pause until the limiting dNTP becomes available. If the required

dNTP does not materialize, RT may either attempt to switch genomic templates, (Nikolenko *et al.*, 2004), or may fall off the template entirely, leading to abortive HIV infection.

Hydroxyurea treatment administered to PBMCs during reverse transcription results in a near complete inhibition of HIV-1 infectivity (Gao *et al.*, 1994; Meyerhans *et al.*, 1994) that subsequent adenosine administration largely overcomes (Meyerhans *et al.*, 1994). Similarly, HU treatment of macrophages results in a reduction of viral DNA synthesis that dNTP precursor treatment can mostly reverse (Kootstra *et al.*, 2000). These results indicate HU inhibits viral DNA synthesis by depleting dNTP pools during HIV-1 reverse transcription. The effects of cellular dNTP pool level modulations associated with cell cycle phase and/or the enzymatic activities of antiviral proteins further support this model.

Notably, the naturally lower dNTP pools in non-dividing cells can result in lower rates of viral DNA synthesis. The low dNTP pools found in resting CD4 positive T-cells lead to abortive HIV-1 infection that maps to the reverse transcription step of the HIV-1 life cycle (Zack *et al.*, 1990; Zack *et al.*, 1992). Stimulation of infected resting CD4 positive T-cells results in the rescue of HIV-1 infection (Zack *et al.*, 1992), likely because of the stimulation-associated increase in dNTP pools (Meyerhans *et al.*, 1994). The extremely low dNTP concentrations in macrophages also result in comparatively less efficient HIV viral DNA synthesis (Diamond *et al.*, 2004).

In addition, some host immune factors exploit lowering the rate of viral DNA synthesis as part of their antiviral mechanisms. To achieve its full antiviral effect, APOBEC3G not only hypermutates viral DNA, it also slows down the rate of reverse transcription by binding to the viral RNA template (Adolph *et al.*, 2013). The slower rate of reverse transcription allows APOBEC3G more time to deaminate cytosines to uracils in minus strand HIV DNA, demonstrating a mutually beneficial link between a decrease in DNA synthesis rate and mutagenesis as antiviral mechanisms (Adolph *et al.*, 2013).

In the absence of Vpx, SAMHD1 associated decreases in dNTP pools upon HIV infection leads to a slower rate or a complete collapse of viral DNA synthesis (Kim *et al.*, 2012). Given that SAMHD1 does not dephosphorylate NRTIs, a combination of SAMHD1 triphosphohyrdrolase activity leading to a depletion in endogenous dNTPs and NRTI treatment in macrophages may lead to an irreversible inhibition of HIV-1 viral DNA synthesis (Amie *et al.*, 2013a).

Summary

Collectively, this chapter demonstrates the cellular maintenance of dNTP concentrations above the level required for RT catalysis is important for successful HIV viral DNA replication and infection (Amie *et al.*, 2013b). Reductions in dNTP pools below the Km of HIV RT, whether naturally occurring or induced by RNRI's, result in the slowing of viral DNA synthesis rates. Slower vDNA synthesis can result in the increased incorporation of: i. incorrect dNTP's ii. ribonucleoside triphoshphates, iii. deoxynucleoside analogs, or iv. nucleoside

analogs, raising HIV's mutation rate. Slower RT DNA catalysis rates also lead to more opportunity for APOBEC3G-associated hypermutation. Additionally, slower viral DNA replication decrease the overall number of viral genomes produced in an infected patient, per unit of time, thereby reducing viral fitness.

Evidence from the literature suggests the intentional lowering of dNTP pools may contribute to the lethal mutation of many proviruses in HIV-infected patients or in cell culture. Whether or not the mutagenic effects of dNTP pool disruptions acting alone could ever result in the successful lethal mutagenesis of HIV, however, remains an open question. To date, the only examples of the lethal mutagenesis of HIV have been attributed to mutagenic nucleoside analogs acting alone or mutagenic analogs in combination with drugs that lower dNTP pools.

Separation of the relative contributions increased mutation and reductions in viral DNA levels make to the molecular mechanism of action of RNRI's poses a great technological and intellectual challenge. Increasing the difficulty of this challenge is the ability for the presence of many DNA lesions to lead to DNA destruction. Groups have reported viral DNA transcripts carrying many APOBEC3G-induced mutagenic lesions can be degraded before integration, showing APOBEC3 related increases in mutation can lead directly to reductions in viral DNA levels (Russell *et al.*, 2009). It may be that the increased mutagenesis of viruses commonly results in associated depletions of viral DNA

that “muddy the waters” of the theoretical mutation-only mechanism of action attributed to lethal mutagenesis.

Whether RNRIs act mostly through increased viral mutation rates or by a more general inhibition of viral DNA synthesis, their clinical utility as antiviral agents merits reconsideration. The human immune system itself uses both lethal mutagenesis and dNTP pool limitations to counteract viral infection. The HIV restriction of APOBEC3G, APOBEC3F, APOBEC3DE and APOBEC3H via G-to-A hypermutation demonstrates the human immune system exploits lethal mutagenesis as part of its natural defense mechanisms. The antiviral mechanism of action of SAMHD1 also shows the human body has naturally evolved to regulate dNTP pool levels as part of the antiviral response. RNRI’s have the unique ability to simultaneously inhibit wild type HIV replication and to re-sensitize NRTI resistant HIV mutants to NRTI treatment. This dual-pronged approach toward inhibiting HIV should be seen as increasingly important in a world where antiretroviral agents are used not only to treat HIV infection, but also to prevent the spread of the virus.

Chapter 2

Characterization of antiretroviral drug activity using the time of drug addition assay

Abstract

Human immunodeficiency virus type 2 (HIV-2) infects approximately 2 million people worldwide. Compared to human immunodeficiency virus 1 (HIV-1), HIV-2 has significantly fewer treatment options and evolves drug resistance more quickly. The ribonucleotide reductase inhibitor (RNRI) hydroxyurea has been used clinically to treat HIV-1 infection. RNRIs deplete cellular dNTP pools, resulting in an inhibition of reverse transcription. To date, no systematic analysis has been conducted to test the efficacy of RNRIs against HIV-2, though studies have indicated HIV-2 may be more sensitive to reductions in dNTP pools. Our findings demonstrate the RNRIs gemcitabine, resveratrol and clofarabine as well as the mutagen 5-azacytidine have potent antiviral activity against both HIV-2 and HIV-1 in parallel vector virus assays. Statistical analysis revealed that the EC_{50} of 5-azacytidine, clofarabine and resveratrol are lower for HIV-2 compared to HIV-1. Time of drug addition assays confirm that RNRIs act during the reverse transcription phase of HIV-1 and HIV-2 replication. These results indicate that RNRIs and viral mutagens may be viable drug classes to add to the treatment repertoire for HIV-2 patients, especially those for whom other treatment options are limited.

Introduction

HIV-2 infected individuals are primarily of west African descent and many of the HIV-2 cases worldwide are attributed to immigrant populations of west Africans living abroad.(Campbell-Yesufu & Gandhi, 2011; Costarelli *et al.*, 2008;

Rey *et al.*, 1989; van der Ende *et al.*, 1990). The low prevalence of HIV-2 compared to HIV-1 is attributed to its low infectivity. HIV-2 is considered a naturally attenuated infection and HIV-2 infected individuals are more likely to have lower viral RNA levels and are less likely to progress to AIDS compared to HIV-1 infected individuals (Bouree *et al.*, 1995; Campbell-Yesufu & Gandhi, 2011; Costarelli *et al.*, 2008; Soares *et al.*, 2011). However, when HIV-2 does progress to AIDS, infected individuals have fewer treatment options than those infected with HIV-1, because not all anti-HIV-1 drugs inhibit replication of HIV-2. Furthermore, HIV-2 has been reported to have lower genetic barriers to the evolution of multidrug resistance than HIV-1, further narrowing the already-limited HIV-2 drug treatment options (Gottlieb *et al.*, 2009; MacNeil *et al.*, 2007; Menendez-Arias & Alvarez, 2014; Rodes *et al.*, 2000; Smith *et al.*, 2009; Witvrouw *et al.*, 2004).

The RNRI hydroxyurea (HU) was used alone and in combination with NRTIs to treat HIV-1 before more specific drugs were available (Biron *et al.*, 1995; Frank *et al.*, 2004a; Lori *et al.*, 1997). However, the toxicity of hydroxyurea made it a less than ideal drug for use in HIV-1 infected individuals. Several studies have since demonstrated the anti-HIV-1 activity of other, potentially less toxic, RNRIs in cell culture and in mouse models (Clouser *et al.*, 2012a; Clouser *et al.*, 2011; Clouser *et al.*, 2012b; Rawson *et al.*, 2013). HU and other RNRIs work by inhibiting RNR, a cellular enzyme that reduces NDPs to dNDPs in the cytoplasm of cells (Xie *et al.*, 2014). Through the reduction of NDPs, RNR

provides cells with the dNTPs which are used to replicate the host DNA, but which can also be used to enable replication of DNA viruses and retroviruses. Both HIV-1 and HIV-2 require cellular dNTPs to complete reverse transcription.

Multiple independent lines of evidence led us to hypothesize HIV-2 RT might be more sensitive than HIV-1 RT to reductions in dNTP pools. HIV-2 RT is less processive than HIV-1 RT (Boyer *et al.*, 2012; MacNeil *et al.*, 2007; Post *et al.*, 2003), and this effect is enhanced under reduced dNTP pools (Boyer *et al.*, 2012). Thus, the depletion of dNTP pools by RNRIs could hinder the ability of HIV-2 to complete reverse transcription to a greater extent than HIV-1. Additionally, unlike HIV-1, HIV-2 encodes the Vpx protein (Clavel *et al.*, 1986), which degrades the cellular triphosphorhydrolase SAMHD1 (Ahn *et al.*, 2012). SAMHD1 degradation results in an increase in dNTP pool concentrations, which allows HIV-2 to replicate in cells with low dNTP concentrations such as macrophages (Baldauf *et al.*, 2012; Kim *et al.*, 2012; Lahouassa *et al.*, 2012; St Gelais *et al.*, 2012). In contrast, HIV-1 which does not encode Vpx, can replicate in macrophages (Diamond *et al.*, 2004), without counteracting SAMHD1(Nguyen *et al.*, 2014). In summary, efficient replication of HIV-2 is dependent on the HIV-2 virally encoded protein Vpx, while HIV-1 replication is not. Thus, the role of Vpx may be to maintain levels of dNTPs that are sufficient for HIV-2 replication. These studies suggest that replication of HIV-2 may be more dependent on the availability of dNTPs compared to HIV-1. Thus, small molecules that lower the dNTP pools, including RNRIs, may be more effective against HIV-2 than HIV-1.

RNRIs used in combination with NRTIs may be able to slow the development of HIV-2 drug resistance since, unlike HIV-1, HIV-2 RT cannot excise NRTIs and instead relies only on the exclusion of dNTP analogs to develop NRTI drug resistance (Boyer *et al.*, 2012; Boyer *et al.*, 2006; Ntemgwa *et al.*, 2009). The combination of an RNRI that depletes the corresponding dNTP pool of an NRTI analog could enhance NRTI incorporation during HIV-2 chemotherapy. Since HIV-2 RT can only exclude NRTIs, but not excise NRTIs that have already been incorporated, the combination of NRTIs and RNRIs during the treatment of HIV-2 infected individuals may delay the emergence of HIV-2 drug resistance.

Despite a more limited drug repertoire for the treatment of HIV-2 infection due to natural occurring resistance polymorphisms (Gottlieb *et al.*, 2009; MacNeil *et al.*, 2007; Menendez-Arias & Alvarez, 2014; Rodes *et al.*, 2000; Smith *et al.*, 2009; Witvrouw *et al.*, 2004), and the potential promise of RNRIs to selectively inhibit HIV-2 replication, no studies have examined the efficacy of RNRIs against HIV-2. Accordingly, in this study, we examined the ability of RNRIs gemcitabine, resveratrol, and clofarabine to inhibit replication of HIV-2. The ability of these RNRIs to inhibit HIV-1 and HIV-2 replication were compared, and time of addition assays were performed to examine at what point in the viral life cycle the RNRIs were acting.

Materials and Methods

In all of these assays, HIV-1 and HIV-2 plasmids containing an identical dual fluorescent reporter cassette, pNL43-MIG and pROD10-MIG, respectively, were used to produce vector virus stocks. The construction of pNL43-MIG and the methods used to produce and titer viral stocks have been described previously (Rawson *et al.*, 2013). The molecular clone pROD10-MIG was created by inserting the *mCherry* gene from pHIV-1 MIG (Rawson *et al.*, 2013) in place of the mouse *heat stable antigen* from pROD10-HIG (Rawson et al. *submitted*), as follows. First, a fragment of pHIV-2 HIG spanning *env*, *hsa*, and IRES was amplified and ligated into pGEM-T (Promega Corp; Madison, WI). Next, the resulting construct and pHIV-1 MIG were digested with BamHI and AvrII (New England Biolabs; Ipswich, MA). The appropriate fragments were purified and ligated together using T4 DNA ligase (New England Biolabs), thus swapping *hsa* for *mCherry* within the pGEM-T subclone. Last, the pGEM-T subclone and pHIV-2 HIG were digested with PmlI, which cleaves within *env* and IRES. The vector was treated with Antarctic Phosphatase (New England Biolabs), and the appropriate fragments were ligated together using T4 DNA ligase. Similar to pNL43-MIG, pROD10-MIG expresses mCherry and EGFP and all viral proteins except Env and Nef.

For drug treatment experiments, VSV-G pseudotyped HIV-1 and HIV-2 vector virus stocks produced in HEK 293T cells obtained from ATCC (Manassas, VA) were used to infect 10,000 Magi-U373-CXCR4$_{CEM}$ cells/well in a 96-well

plate format that had been pre-treated for 2 hours with increasing concentrations of drugs or with vehicle, DMSO. The small molecules tested in these experiments were zidovudine, tenofovir, nevirapine, raltegravir, 5-azacytidine, clofarabine, resveratrol and gemcitabine. Zidovudine, tenofovir, nevirapine and raltegravir and the Magi-U373-CXCR4$_{CEM}$ cell line were all obtained from the NIH AIDS Reagent Program. Resveratrol was obtained from Sigma Aldrich (St Louis, MO), 5-azacytidine and gemcitabine were obtained from Carbosynth (Compton, UK). Clofarabine was obtained either from the National Cancer Institute or from Alfa Aesar (Ward Hill, MA). Flow cytometry detecting mCherry and EGFP was used to quantify infectivity generated from the dose responses using these drugs, and EC_{50} values were calculated in GraphPad Prism6 (San Diego, California, USA). GraphPad was also used to determine if HIV-1 and HIV-2 EC_{50} values were statistically different from one another, as indicated in Table 1.

Results and Discussion

Table 2-1 reports the EC_{50} values calculated for each member of an eight drug panel from at least four different independent experiments. Dose response curves and EC_{50} values were obtained for each independent experiment using nonlinear regression models that generated unambiguous EC_{50} values for both HIV-1 and HIV-2 and that gave acceptable fits in a combined replicates test. These replicate EC_{50} values were subjected to an unpaired two-tailed t-test to generate a compiled EC_{50} value for each drug and to compare whether the EC_{50} values differed for HIV-1 and HIV-2 (Mamotte *et al.*, 1999). Our results show that

all compounds tested with the exception of nevirapine possessed potent anti-HIV-1 and HIV-2 activity. Consistent with previous publications, nevirapine inhibited HIV-1, but not HIV-2 replication (Balzarini, 2004). Resveratrol, clofarabine, and 5-azacytidine showed statistically different EC_{50} values, as indicated by a p value of 0.05 or less. In contrast, zidovudine, tenofovir and raltegravir did not show any significant differences in their comparative HIV-1 and HIV-2 EC_{50} values, in agreement with previously published reports (Smith *et al.*, 2008; Smith *et al.*, 2011; Witvrouw *et al.*, 2004). Notably, this report demonstrates for the first time both the anti-HIV-1 and HIV-2 activity of clofarabine.

Our lab has previously reported the toxicity of 5-azacytidine (Dapp *et al.*, 2009), resveratrol, gemcitabine (Clouser *et al.*, 2012a; Clouser *et al.*, 2010; Rawson *et al.*, 2013), and clofarabine (Beach et al, unpublished data) using the same cell line as reported here. Based on our previous reported toxicity data, it is unlikely that the antiviral activity shown here can be attributed to cytotoxic effects. In support of this, microscopic inspection of the cells as well as forward and side scatter by flow cytometric analysis did not reveal any evidence of increased cell death or abnormalities with any of the drug treatments reported here. Although we have not previously reported the cytoxicity of nevirapine, our finding that nevirapine inhibits HIV-1 but not HIV-2 suggests that the antiviral activity is specific and not due to cytotoxicity.

The results showing a similar antiviral efficacy for zidovudine, tenofovir, and raltegravir, but not for nevirapine, correlate with previously reported findings comparing anti-HIV-1 and anti-HIV-2 activity (Andreatta *et al.*, 2013; Roquebert *et al.*, 2008; Witvrouw *et al.*, 2004). It was found that HIV-2 is more sensitive to the RNRIs gemcitabine, clofarabine, and resveratrol than HIV-1 by approximately 2.04 fold, 3.49 fold, or 4.21 fold, respectively. These findings support the hypothesis that HIV-2 would be more sensitive to RNRIs when compared to HIV-1. Notably, only resveratrol and clofarabine showed a statistically significant decrease in the EC_{50} value in these assays, but all RNRIs tested showed a trend of inhibiting HIV-2 more than HIV-1. Interestingly, we also found HIV-2 was approximately 1.4 fold more sensitive than HIV-1 to inhibition with 5-azacytidine. The antiviral mechanism of action of 5-azacytidine has been reported to be the lethal mutagenesis of HIV-1 (Dapp *et al.*, 2009). These findings suggest further study of RNRIs and lethal mutagens against HIV-2 may be warranted.

After characterizing the drug dose responses in our panel, we performed vector virus time-of-addition (TOA) assays to identify the phase of the HIV-1 and HIV-2 life cycle that was inhibited by these compounds. Infections and the analysis of infectivity were performed using the same methods as described for the dose response assays described above, except that rather than pre-treating with drugs for 2 hours before infection, drugs were added immediately at the time of infection, or at varying time points thereafter. Cell culture media was changed 24 hours after each drug treatment for every time point. All drugs were given at

concentrations that extinguished viral infectivity in the dose-response assays. The infectivity observed at the 0 hour time point for each drug was subtracted and infectivity was subsequently normalized to the no drug control at the 24 h time point. We performed each TOA three independent times. In TOA assays, the last time point a drug consistently stops HIV replication correlates with the step of the HIV life cycle the drug inhibits (Daelemans *et al.*, 2011). Based on the timing of the HIV retroviral life cycle, we conducted our vector virus TOA assays to measure the ability of drugs to inhibit HIV replication through the integration phase of the viral life cycle.

Based on previous literature, we *a priori* defined any drugs that stopped inhibiting replication at or before 1 hour as entry or fusion inhibitors; any drugs that stopped inhibiting replication from 3 to 4 hours were defined as reverse transcriptase inhibitors; and any drugs that inhibited replication after 6 hours were defined as integrase inhibitors (Daelemans *et al.*, 2011). It was hypothesized that depending on when RNRIs depleted dNTP pools, RNRI-mediated inhibition of infectivity could map after fusion but before traditionally reported RT times. This hypothesis was based on the fact that RNRIs inhibit HIV RT indirectly, by inhibiting dNTP pools, rather than by directly causing chain termination or the steric hindrance of RT polymerization, like NRTIs and NNRTIs, respectively. Similar to previous publications, the time of drug inhibition was qualitatively defined in the assays in this study as the time point after which infectivity was consistently detected above baseline (Daelemans *et al.*, 2011).

The results indicate RNRIs inhibit HIV-1 and HIV-2 during the reverse transcription phase of the viral life cycle (Figures 2-1 and 2-2, respectively). Interestingly, gemcitabine and resveratrol lose their antiviral efficacy at earlier time points than clofarabine, but within a similar time frame as defined by the bounds of the loss of inhibition of tenofovir, zidovudine, and, for HIV-1, nevirapine. In this assay, we observed FDA approved RT inhibitors exert their inhibitory effects between 2 and 4 hours. This suggests that in this assay inhibition from 2 to 4 hours should be interpreted as selective for the RT phase of the HIV life cycle. The breadth of this window could reflect different timing of cellular uptake and phosphorylation of nucleoside analogs in the Magi-U373-$CXCR4_{CEM}$ line. For the RNR inhibitors, it is also possible that these variations reflect differences in the timing, longevity, and efficiency by which they inhibit RNR. Additional studies would be needed to investigate these mechanistic possibilities.

The timing of the inhibition of RT was distinguishable from the inhibition of integrase in the assay. The integrase inhibitor raltegravir inhibited HIV -1 through 6 hours and HIV-2 through 8 hours, the penultimate time points tested. In the drug panel, only raltegravir inhibited HIV-1 or -2 replication at 6-8 hours - no other drug inhibited HIV-1 replication past 4 hours or HIV-2 replication past 6 hours.

Overall, these studies indicate the antiviral activity of the RNRIs gemcitabine, resveratrol, and clofarabine as well as the mutagen 5-azacytidine is

selective for the reverse transcription phase of the HIV life cycle. The data also show that the drugs were comparable in their inhibition of HIV-1 and HIV-2 in all cases except for resveratrol, clofarabine, and 5-azacytidine, which showed greater anti-viral activity against HIV-2, and nevirapine, which showed greater anti-viral activity against HIV-1. These results do not reveal a mechanism responsible for the increased potency of RNRIs against HIV-2. Recent reports, however, demonstrate that HIV-2, but not HIV-1 must degrade the dNTP pool regulator SAMHD1 for successful infection of macrophages and other myeloid cells, which are known to have extremely low dNTP pools (Diamond *et al.*, 2004; Kim *et al.*, 2012). The increased dNTP pools that arise through Vpx-induced SAMHD1 degradation have been hypothesized to be responsible for the rapid emergence of NRTI drug resistance in HIV-2 infected individuals (Amie *et al.*, 2013a). It is tempting to speculate that RNRI treatment of HIV-2 infection could decrease dNTP pool levels below the Km of HIV-2 RT in macrophages, resting CD4 T cells, and dendritic cells, disrupting the progression of HIV-related pathogenesis and slowing the pace of the evolution of NRTI drug resistance in patients. Future studies will be needed to investigate the ability of RNRIs to inhibit HIV-2 replication in these cell types. RNRI's may hold unique potential for use as antiretroviral agents for HIV-2 infection - a forgotten virus with few effective treatment options.

Table 2-1 EC_{50} values of drugs under study for HIV-1 and HIV-2 infection.

Drug	HIV-1 EC_{50} (μM) [95% confidence interval][d]	HIV2 EC_{50} (μM) [95% confidence interval][d]	Significant difference in EC_{50} values ($p < 0.05$)[e]	EC_{50} ratio HIV-1/HIV-2
gemcitabine[a]	**1.3×10^{-3}** [2.28×10^{-4} - 2.81×10^{-3}]	**6.36×10^{-4}** [3.95×10^{-4} -1.67×10^{-3}]	N	2.04
clofarabine[b]	**1.12×10^{-1}** [3.06×10^{-2} -1.94×10^{-1}]	**3.21×10^{-2}** [9.52×10^{-4} -63.2×10^{-2}]	Y	3.78
resveratrol[c]	**25.6** [20.1-31.0]	**6.08** [4.49-7.68]	Y	4.21
5-azacytidine[c]	**20.2** [18.7-21.8]	**14.5** [10.3-18.8]	Y	1.39
raltegravir[b]	**1.72×10^{-3}** [8.47×10^{-4} - 4.30×10^{-3}]	**1.85×10^{-3}** [4.43×10^{-3} -4.15×10^{-3}]	N	0.93
tenofovir[b]	**1.9×10^{-1}** [49.8×10^{-2} -3.31×10^{-1}]	**1.30×10^{-1}** [1.40×10^{-2} -2.73×10^{-1}]	N	1.46
zidovudine[a]	**3.67×10^{-2}** [2.72×10^{-2} -4.62×10^{-2}]	**4.46×10^{-2}** [2.73×10^{-2} -6.19×10^{-2}]	N	0.823
nevirapine[a]	**6.2** [5.31-7.12]	No activity	-	-

[a]log(inhibitor) vs. normalized response was used to determine EC_{50}

[b]log(inhibitor) vs. response (three parameters) was used to determine EC_{50}

[c]log response (inhibitor) vs. response (variable slope) was used to determine EC_{50}.

[d]Curve fitting was performed using log inhibitor vs normalized response. If the goodness of fit was not appropriate as determined by the replicates test and residuals, the curves were fit using either the log inhibitor vs response or log inhibitor vs response (variable slope) as indicated.

[e]EC_{50} values for each replicate were used to perform an unpaired two-tailed t-test to determine differences between HIV-1 and HIV-2. Experiments were performed in parallel with HIV-1 and HIV-2 for each drug at least 4 times.

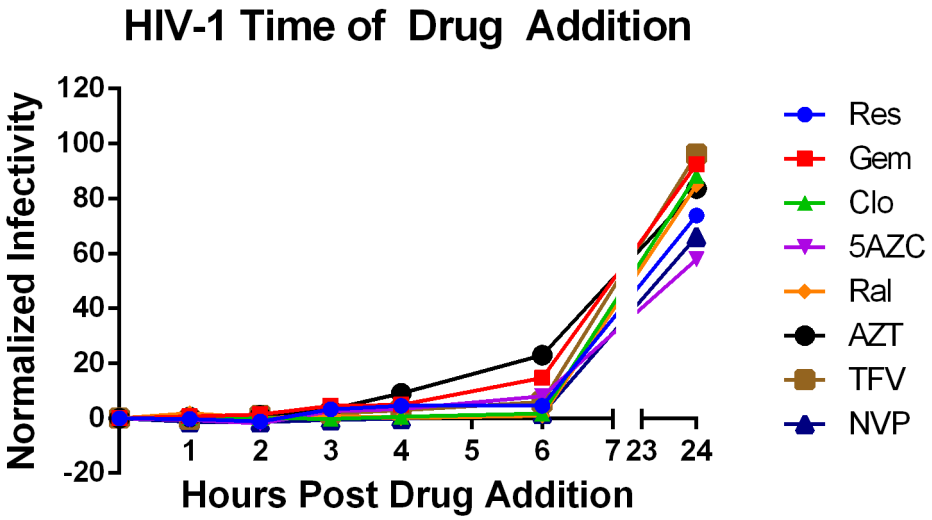

Figure 2-1 HIV-1 Time of Drug Addition Assay. Magi cells seeded into 96 well plates were infected with VSV-G pseudotyped HIV-1 vector virus. At 0, 1, 2, 3, 4, 6, and 24 hours post infection, infected cells were treated with DMSO or with inhibitory concentrations of clofarabine (Clo), zidovudine (AZT), tenofovir (TFV), resveratrol (Res), raltegravir (Ral), nevirapine (NVP), 5-azacytidine (5AZC), gemcitabine (Gem). Infections were performed three times, background infectivity at the 0 hour time point was subtracted, and infectivity for drug treatments was normalized to DMSO infectivity at the 24 hour time point. Values represent the averages of three independent experiments, with each treatment at each time point treated in duplicate. Infectivity was normalized to no drug, which was set at 100 at the 24 h time point. The measured no drug infectivity average value at 24 h was 46%.

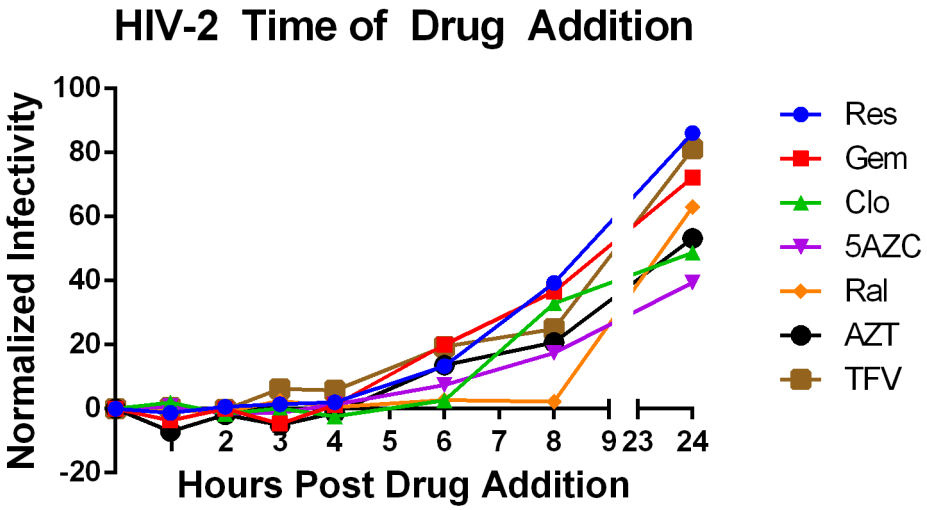

Figure 2-2. **HIV-2 Time of Drug Addition Assay.** Magi cells seeded into 96 well plates were infected with VSV-G pseudotyped HIV-2 vector virus. At 0, 1, 2, 3, 4, 6, 8 and 24 hours post infection, infected cells were treated with DMSO or with inhibitory concentrations of clofarabine (Clo), zidovudine (AZT), tenofovir (TFV), resveratrol (Res), raltegravir (Ral), 5-azacytidine (5AZC), or gemcitabine (Gem). Infections were performed three times, background infectivity at the 0 hour time point was subtracted, and infectivity for drug treatments was normalized to DMSO infectivity at the 24 hour time point. Values represent the averages of three independent experiments, with each treatment at each time point treated in duplicate. Infectivity was normalized to no drug, which was set at 100 at the 24 h time point. The measured no drug infectivity average value at 24 h was 55%.

Chapter 3

Investigation of the antiretroviral mechanism of action of clofarabine

Abstract

The deoxyadenosine analog and ribonucleotide reductase inhibitor (RNRI) 2-Chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-9*H*-purin-6-amine, or clofarabine, is FDA approved to treat refractory pediatric acute lymphoblastic leukemia. In this study, we show for the first time that clofarabine also has anti-HIV-1 activity. Our studies show the selectivity index of clofarabine to be 3349 in Magi-U373-CXCR4$_{CEM}$ cells and 35.9 in CEM-GFP cells. Further characterization of clofarabine's anti-HIV-1 activity in the Magi-U373-CXCR4$_{CEM}$ cell line revealed an increase in HIV-1 mutational loads when compared to no drug controls. At clofarabine's EC_{50} concentration, we observed a 2.03 fold increase in HIV-1 mutant frequency. Sequencing of the proviral DNA revealed significant clofarabine-associated differences in the HIV-1 mutation spectra. In particular, clofarabine-treatment was associated with significantly higher G-to-A transition mutations, in the context of G-to-A hypermutated sequences. Analysis of dNTP pools revealed that at non-toxic, antiviral concentrations, clofarabine significantly depleted dATP and dGTP nucleotide pools. Our data demonstrates that clofarabine exerts potent anti-HIV-1 activity, increases HIV-1 mutation, and depletes purine dNTP pools in the Magi-U373-CXCR4$_{CEM}$ cell line.

Introduction

For over three decades, the HIV/AIDS epidemic has been one of the world's most pressing global public health challenges. Currently there are six FDA-approved classes of anti-HIV-1 medications that allow people living with HIV who receive timely treatment to expect a near normal lifespan (Johnson *et al.*, 2013), (Nakagawa *et al.*, 2012). In the past three years, the success of treatment as prevention (Anglemyer *et al.*, 2013) and pre-exposure prophylaxis (Celum, 2011) have also shown these same medications are effective at blocking the transmission and acquisition of HIV-1. These findings have led to the recommendation that all HIV-1 infected people be placed onto early treatment, both to benefit their own health and to protect the health of the community (Cohen *et al.*, 2013).

Since the first anti-HIV-1 medications entered clinical development, drug resistance associated with mutations in the HIV-1 genome has been known to reduce the effectiveness of HIV-1 treatment (St Clair *et al.*, 1991). Given the important role antiretroviral drugs play in both HIV-1 treatment and prevention, drug resistance presents an increasing threat not only against the health of HIV infected individuals, but also in the community level fight against HIV. The CDC reports cases of newly transmitted HIV-1 drug resistance are on the rise in the USA (Taylor *et al.*, 2013). As increasing numbers of people living with HIV access treatment, community viral loads will decrease. Reduction in community HIV-1 viral load has been shown to reduce population level transmission rates of

HIV-1 (Das *et al.*, 2010). As more people living with HIV attain undetectable viral loads, drug resistance associated treatment failures may increasingly drive HIV-1 transmission. To keep up with the future of the HIV/AIDS epidemic, tactical pharmacological innovations in treatment and prevention are warranted. In particular, the development of drugs that could re-sensitize drug resistant mutants to existing HIV medications could play a crucial role under the new public health paradigm of HIV-1 treatment as both treatment and prevention.

The history of antiretroviral drug development reveals that in the context of the current HIV-1 epidemic, ribonucleotide reductase (RNR) may be strategic to revisit as an anti-HIV-1 drug target. The RNRIs hydroxyurea (Lori *et al.*, 1994), resveratrol (Heredia *et al.*, 2000), (Clouser *et al.*, 2012a), (Rawson *et al.*, 2013), and gemcitabine (Clouser *et al.*, 2010) have independent anti-HIV-1 activity in cell culture. Recently, knockdown of the small subunit of RNR, RRM2, has also been shown to be antiviral, demonstrating that RNR is a valid, anti-HIV-1 target (Allouch *et al.*, 2013). Notably, the ribonucleotide reductase inhibitors (RNRI)s hydroxyurea (HU) and resveratrol (RV) can re-sensitize NRTI-resistant HIV-1 mutants to NRTI treatment (Balzarini, 2000), (Heredia *et al.*, 2000), (Heredia *et al.*, 2013).

The ability of RNRIs to inhibit HIV-1 replication and to resensitize drug resistant HIV-1 variants has been associated with their ability to deplete dNTP pools (Balzarini, 2000). The relationship between low dNTP pool levels and inhibition of HIV infection has long been known (Gao *et al.*, 1993), (Johns & Gao,

1998). The naturally low dNTP pools in macrophages helps limit HIV-1 replication in these cells (Diamond *et al.*, 2004), (Allouch *et al.*, 2013). The antiviral mechanism of SAMHD1 has been attributed to its ability to deplete dNTP pools (Amie *et al.*, 2013a), (Kim *et al.*, 2012).

In this study, the RNRI clofarabine was investigated to determine if the drug possessed anti-HIV-1 activity. Clofarabine triphosphate (Clo-TP) is a well known allosteric inhibitor of the regulatory subunit of ribonucleotide reductase, RRM1 (Aye *et al.*, 2012; Aye & Stubbe, 2011; Chang & Cheng, 1980). Clofarabine, therefore, is a prodrug that needs activation first to its monophosphate form (Clo-MP), then to its diphosphate and triphosphate forms by monophosphate and diphosphate kinases, respectively, before it can inhibit RNR (Lotfi *et al.*, 1999; Nagai *et al.*, 2011; Xie & Plunkett, 1995). Clofarabine is a deoxyadenosine analog that is FDA approved to treat refractory or relapsed pediatric acute lymphoblastic leukemia (Faderl *et al.*, 2005). We chose to study clofarabine because of its high oral availability (Jacoby *et al.*, 2014), its comparatively long half life (Bonate *et al.*, 2004), its high stability, and its low prevalence of associated toxic metabolites (Bonate *et al.*, 2011), when compared to other RNRIs. Our results show that at non-toxic antiviral concentrations, clofarabine depletes dATP pools by ten fold and dGTP pools by 3.7 fold and increases the mutant frequency of HIV-1 in Magi-U373-CXCR4$_{CEM}$ cells by two fold. Recently, our lab has also reported the anti-HIV-1 activity of the RNRIs resveratrol and gemcitabine is associated with an increase in the mutant

frequency of HIV-1 (Rawson *et al.*, 2013). This study demonstrates for the first time the anti-HIV-1 activity of clofarabine. It is also the first study that reports simultaneously the effect of an RNRI on dNTP pools, HIV-1 mutation frequency, and HIV-1 proviral mutation spectra in the same cell culture model. Ongoing studies will be performed to characterize the molecular mechanism of clofarabine against HIV-1 and will investigate its ability to resensitize HIV-1 drug resistant mutants to approved anti-HIV-1 medications. RNRIs with improved anti-viral efficacy and reduced toxicity may bring new promise to the fight against the acquisition and transmission of HIV-1 drug resistance.

Results

Clofarabine Has Potent Anti-HIV-1 Activity

To determine if clofarabine had anti-HIV activity, we performed both single cycle vector virus and spreading HIV-1 live virus in the presence or absence of clofarabine. To perform vector virus experiments, we used the recently reported dual-fluorescent reporter plasmid, pNL43-MIG (Rawson *et al.*, 2013). The data shows that clofarabine has an EC_{50} value of 854 nM, with a 95% confidence interval of 723 nM to 1.01 μM (Figure 3-1). This EC_{50} places clofarabine among the RNRI's with the most potent anti-HIV activity in tissue culture. Gemcitabine, resveratrol, and hydroxyurea have previously reported EC_{50} values in the low nanomolar, low micromolar and low millimolar concentrations, respectively (Clouser *et al.*, 2010; Lori *et al.*, 1994; Rawson *et al.*, 2013).

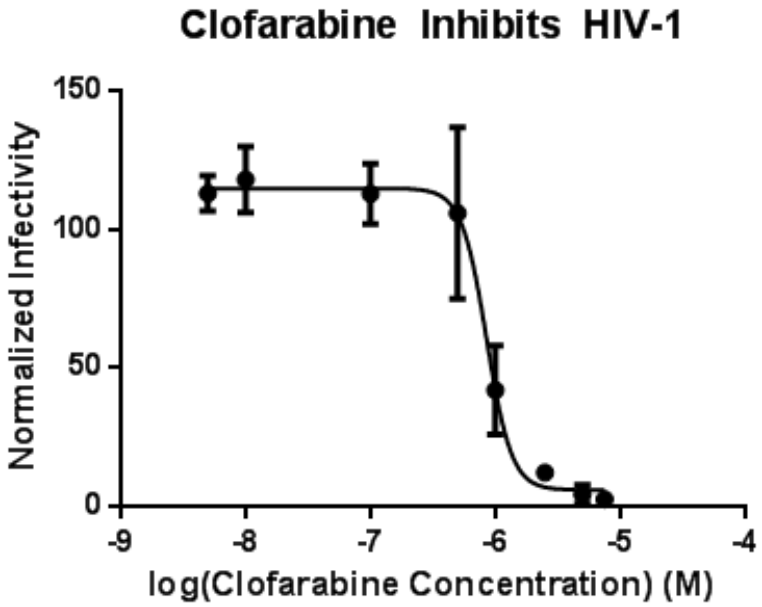

Figure 3-1 Clofarabine Inhibits HIV-1 in a Vector Virus Model. Magi-U373-CXCR4$_{CEM}$ cells pre-treated with increasing concentrations of clofarabine or with vehicle (DMSO) were infected with pNL43-MIG. Flow cytometry was used to monitor viral infectivity. Infections were normalized to the DMSO control, and the normalized values were entered into GraphPad Prism 6 and log transformed. The EC_{50} values were calculated using the log(inhibitor) vs. normalized response - variable slope nonlinear regression test. Replicates analysis confirmed the appropriateness of the statistical test. The experiment was performed three independent times. Error bars represent standard deviation (SD). The EC_{50} of clofarabine was 854 nM, with 95% CI values of 723 nM to 1010 nM.

Clofarabine's low EC_{50} value in the vector virus model inspired us to determine its efficacy in a spreading HIV-1 infection. Briefly, we infected CEM-GFP cells with the infectious HIV-1 molecular clone, NL4-3, at an MOI of 0.01. Dose responses obtained from these experiments revealed the antiviral activity of clofarabine in a spreading HIV-1 infection to appear to be more potent, with an EC_{50} of 15.8 nM (Appendix I). Collectively, these results demonstrate clofarabine has a powerful antiviral effect in cell culture that appears in line not only with the

potency of other RNRI's, but also with FDA approved anti-HIV-1 medications (Parkin & Schapiro, 2004).

Anti-HIV-1 Effects of Clofarabine are not Explained by Cytotoxicity

Clofarabine, like gemcitabine and hydroxyurea, is an anti-cancer drug that is used clinically for its cytotoxic effect (Jeha *et al.*, 2004). One obvious potential explanation that could spuriously account for clofarabine's anti-viral activity in our cell culture models is cytotoxicity. To examine if the observed antiviral activity could be due to cytoxicity, we examined the effects of clofarabine on cell toxicity using an ATP-based cell viability assay. The data shows that the concentrations of clofarabine that exert antiviral activity were not toxic to the cells. The TC_{50} of clofarabine in MAGI-U373-CXCR4CEM cells was 2.86 mM, (Figure 3-2). The TC_{50} of clofarabine in the CEM-GFP cell line was 567 nM (Figure 3-2).

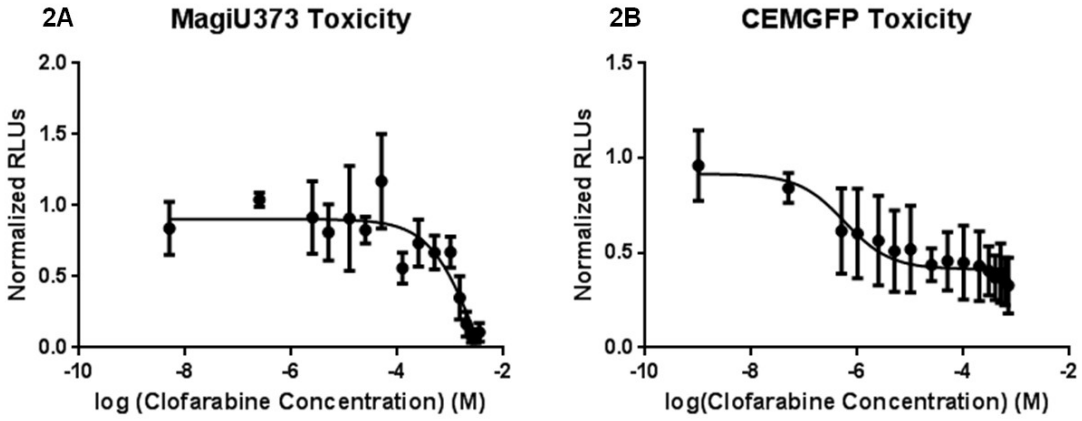

Figure 3-2 Clofarabine Has Limited Toxicity in the Magi-U373-CXCR4$_{CEM}$ and CemGFP Cell Lines. Toxicity analysis of clofarabine was performed using the Promega CellTiter-Glo Luminescent Cell Viability Assay Kit according to the manufacturer's instructions. All samples were normalized to the no drug control. The TC_{50} value of clofarabine in the Magi cell line was 2.86 mM, with a 95% CI of 640.2 µM to 12.8 mM. The TC_{50} value of clofarabine in the CEMGFP line was 567 nM, with a 95% CI of 172 nM to 1.87 µM. For Magi-U373-CXCR4$_{CEM}$ (Figure 2A) and for CemGFP (Figure 2B) cells, experiments were performed three independent times. The error bars represent the standard deviation.

A comparison of the EC_{50} and TC_{50} values of clofarabine indicates that the anti-HIV-1 efficacy of clofarabine cannot be explained by cytotoxicity at the doses used in our model systems. The selectivity index of clofarabine in the Magi-U373-$CXCR4_{CEM}$ cell line is 3.35×10^3 and 35.9 in the CEM-GFP line.

Clofarabine Depletes dATP and dGTP Pools in Magi-U373-$CXCR4_{CEM}$ Cells

Since clorafabine is an RNRI, we examined whether its anti-HIV activity corresponded to alterations in the dNTP pools. (Lotfi *et al.*, 1999; Parker *et al.*, 1991; Xie & Plunkett, 1996).To examine the effects of clofarabine on dNTP pools, a dual ion-pair LCMS-MS method was used. Three million Magi-U373-$CXCR4_{CEM}$ cells were treated with 0.5 or 5.0 μM clofarabine for 3, 6, or 24 hours before harvesting the cells and extracting dNTP's. The results show that at 3 and 6 hours post treatment, both the low and high clofarabine concentrations significantly depleted dATP and dGTP, but not dCTP or TTP pools (Figure 3-3).

Time of drug addition experiments in cells and in patients have shown in one round of replication, reverse transcription ends approximately 4.5 to 6 hours post initial infection with HIV-1 (Daelemans *et al.*, 2011; Murray *et al.*, 2011). Therefore, our dNTP pool studies show that clofarabine can deplete dNTP pools during the same time frame of the reverse transcription phase of the HIV-1 life cycle in the Magi-U373-$CXCR4_{CEM}$ cell line.

The dNTP pool analysis also showed the inhibition by clofarabine of dATP and dGTP pools is relatively long lived. At 24 hours, clofarabine continued to effectively inhibit dATP and dGTP pools, implicating Clo-TP was still present

within these cells. This finding agrees with patient studies where over 50% of Clo-TP was found to be present in leukemic cells 24 hours after initial clofarabine infusion (Gandhi *et al.*, 2003). Interestingly, clofarabine treatment after 24 hours also led to a significant increase in TTP pools. The dCTP pools, which previously had exhibited a non-significant downward trend, were unaffected. These findings indicate that at antiviral concentrations, clofarabine treatment was not toxic in Magi-U373-CXCR4$_{CEM}$ cells, an increase in dNTP pools would be inconsistent with drug induced cell death or cytostaticity. These findings, combined with previous reports from clinical trials, also provide promising evidence that Clo-TP may persist long enough to be compatible with once daily dosing schedules for treatment of HIV-1, though more extensive testing in patients would be needed.

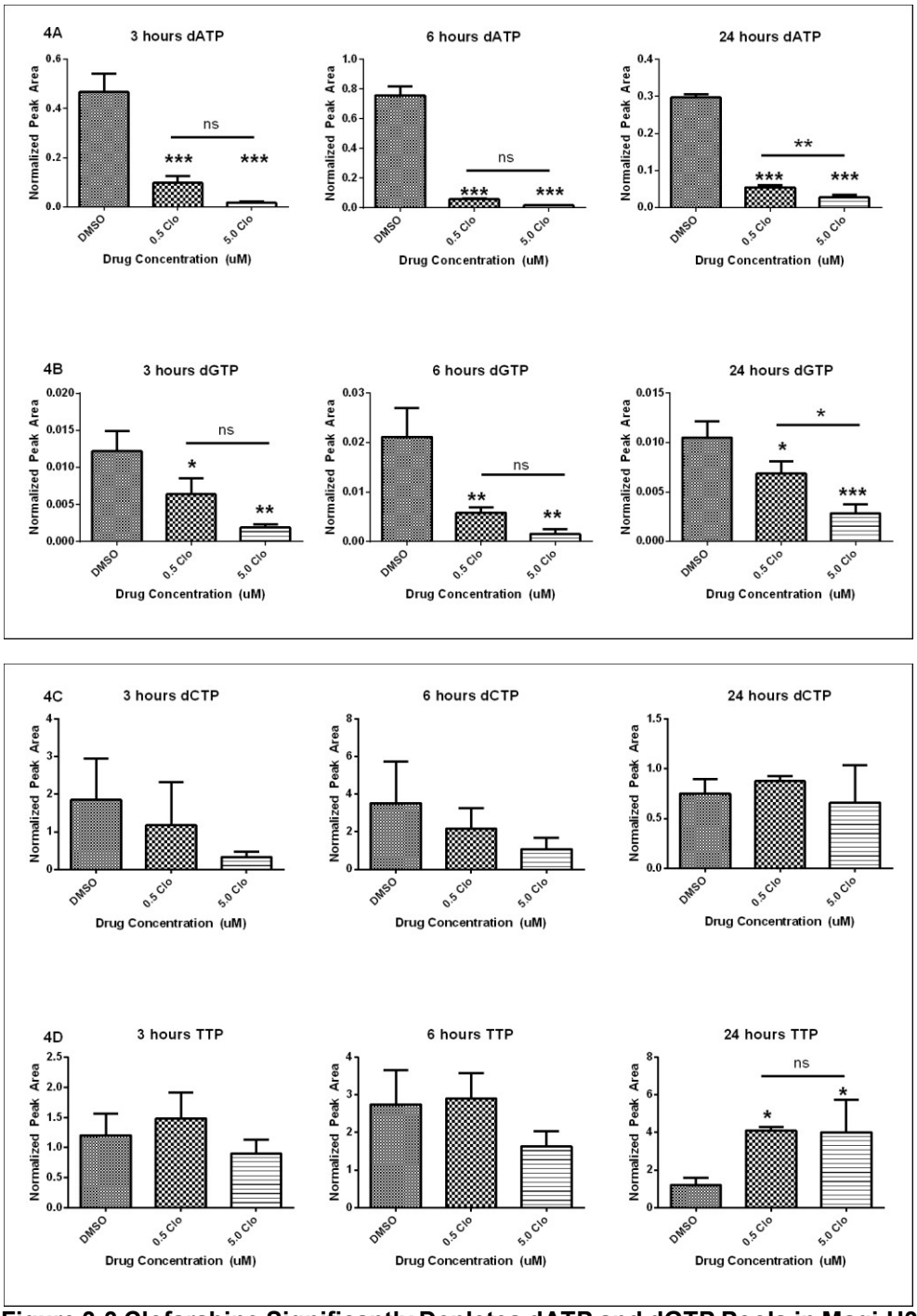

Figure 3-3 Clofarabine Significantly Depletes dATP and dGTP Pools in Magi-U373-CXCR4$_{CEM}$ cells. Magi-U373-CXCR4$_{CEM}$ cells were treated with DMSO, 0.5 μM or 5 μM clofarabine for 3, 6, or 24 hours before being harvested for dual ion pair LCMS-MS dNTP pool analysis. The mean and the standard deviation were calculated and plotted after normalization to the internal standard. A one-way ANOVA with Tukey's post-test was used to assess differences among all treatment groups. Clofarabine significantly depleted deoxypurine but not deoxypyrimidine nucleotide pools. * P < 0.05, **P <.01, ***P < 0.001.

Anti-HIV-1 Activity of Clofarabine Correlates with an Increase in HIV-1 Mutant Frequency

Many groups have reported even small imbalances in dNTP pools to be mutagenic, both for viruses and for cells (Ahluwalia & Schaaper, 2013; Julias & Pathak, 1998; Kumar *et al.*, 2011; Rampazzo *et al.*, 2010). Imbalances in dNTP pool have been found to affect the fidelity of HIV-1 reverse transcriptase (Bebenek *et al.*, 1992). RNRIs both alone (Rawson *et al.*, 2013) and in combination with mutagens (Clouser *et al.*, 2010) have been reported to enhance HIV-1 mutant frequency. Since the HIV-1 mutation frequency is sensitive to changes in cellular dNTP pools and clofarabine imbalances deoxypurine pools, it the ability of clofarabine treatment to increase mutant frequency was next examined.

A dual fluorescent reporter vector virus system was used to assess whether clofarabine increased HIV-1 mutant frequency. The vector virus system enables both the infectivity and mutant frequency values to be assessed from the same samples. The results demonstrated a correlation between loss of infectivity and an increase in mutant frequency (Figure 3-4). At concentrations nearly extinguishing HIV-1 infection, in one round of replication, clofarabine increased HIV-1 mutant frequency by over 7 fold. At its EC_{50} concentration, the mutant frequency value observed in the presence of clofarabine was 2.03 times higher than the no drug control. These values place the HIV-1 mutation frequency values in one round of replication in the presence of clofarabine above the

reported values using the same assay for resveratrol and gemcitabine, and even above the values observed for KP-1212, a compound that had entered clinical trials as a lethal mutagen of HIV-1 (Rawson *et al.*, 2013).

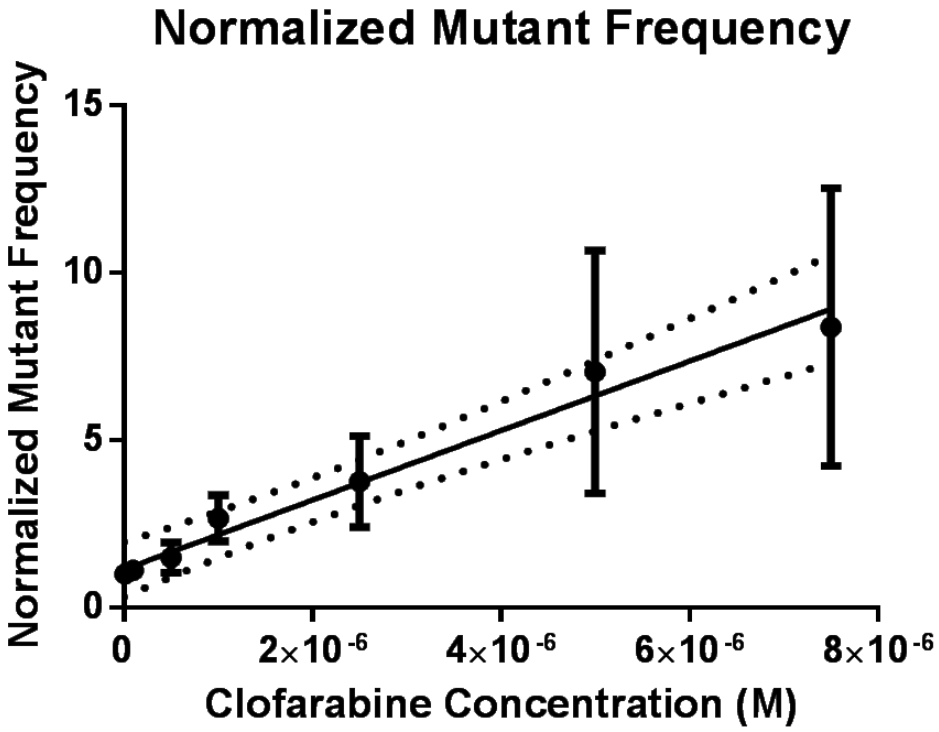

Figure 3-4 Clofarabine Increases the Mutant Frequency of HIV-1. To determine mutant frequency, cells were pre-treated with increasing concentrations of clofarabine or with DMSO, then infected with pNL43-MIG. Dual color flow cytometry was performed and mutant frequency was calculated as described in the Materials and Methods. At the EC_{50} concentration, clofarabine's mutant frequency was 2.03 times higher than the DMSO vehicle control, with lower and upper 95% confidence interval values of 1.32 fold and 2.74 fold, respectively. The data are from three independent experiments, and error bars represent the standard deviation.

The ability of clofarabine to enhance HIV-1 mutant frequency raises a concern that at non-cytotoxic levels, clofarabine may also act as a mutagen to cells. In its FDA regulatory approval package, clofarabine is reported not to show any activity in the bacterial Ames test. Regulatory agencies use Ames test results as a surrogate to indicate that a drug does not increase toxic frameshift or point

mutations (Li *et al.*, 1991a) in the human genome. Results from a mammalian mutation detecting HGPRT assay referenced in (Clouser *et al.*, 2010) show that neither non-toxic, antiviral concentrations of gemcitabine alone, nor of the synergistically mutagenic combination of decitabine and gemcitabine, increased the mutation frequency of mammalian cellular DNA. While this manuscript does not report the ability of clofarabine to modulate the mutation of cellular DNA, clofarabine's lack of toxicity at antiviral concentrations, combined with its lack of signal in the Ames test indicates that it is unlikely to be mutagenic to the human genome.

Clofarabine is Associated with Increased Mutation in HIV-1 Proviral DNA

The increase in mutant frequency combined with the decrease in dATP and dGTPs suggest that clofarabine may affect the mutation spectra of HIV. To determine if clofarabine affected the mutant spectra of HIV-1, proviral DNA was sequenced from infected cells treated with clofarabine or DMSO. Sequencing of the *egfp* gene revealed that clofarabine treatment increased G-to-A transition mutations, in the context of G-to-A hypermutated sequences (Figure 6). When all mutations were included, clofarabine treatment was associated with statistically higher G-to-A, T-to-C, and C-to-T transition mutations. No significant differences were found for A-to-G or for transversion mutations.

When G-to-A mutations in the context of G-to-A hypermutated sequences were removed from the mutational analysis, no significant differences between the mutant spectra of clofarabine and DMSO treatment, with the exception of an

overall statistically higher number of combined transversions detected in clofarabine when compared to DMSO treated proviral sequences. No single transversion mutation, however, was found to be significantly higher in clofarabine treated cells (Appendix IV).

Unlike in past studies (Clouser *et al.*, 2010; Holtz & Mansky, 2013; Rawson *et al.*, 2013), sorting of single positive fluorescent infected cells to enrich for mutations was not done in this study. Infected cells positive for only one marker gene could be less likely to contain extensively hypermutated sequences. Viruses with higher numbers of G-to-A mutations may have both marker genes knocked out, resulting in cells that actually contain hypermutated viruses, but which appear uninfected. Though a rare event, G-to-A hypermutation has a large impact on mutant spectra because of how many mutations accumulate in one sequence.

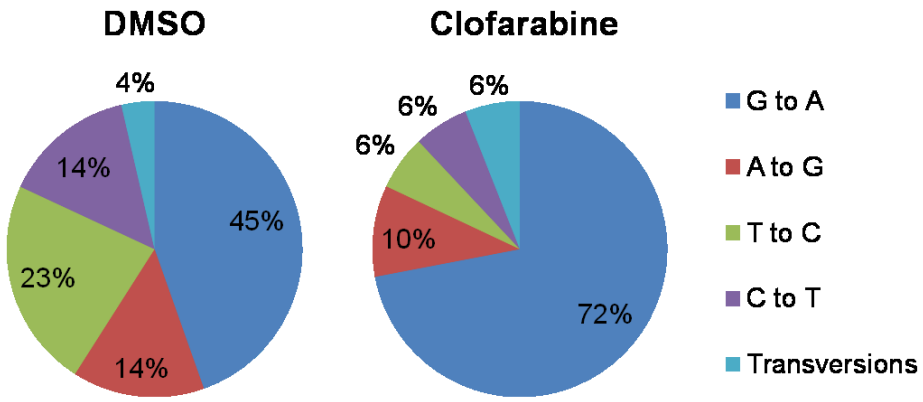

Figure 3-5 Mutant Spectra of Clofarabine vs. DMSO. Cells were treated with 5 μM clofarabine or with DMSO and infected with DNaseI treated pNL4-3-MIG. Genomic DNA was extracted and DpnI treated. The *efgp* sequence was PCR amplified and ligated into a bacterial cloning vector, Sanger sequenced and analyzed for mutations. For DMSO, 83 mutations were detected from 132 sequences, and for clofarabine,107 mutations were detected from 69 sequences. Sequences with duplicated mutations were only counted once. All G-to-A transition mutations in hypermutated sequences are included in this analysis.

Table 3-1 Mutant Spectra Statistical Analysis.

Mutation	P-Value	Significance by Fisher's Exact Test
G-to-A	0.0002	*** significant
A-to-G	0.5019	ns
T-to-C	0.0014	** significant
C-to-T	0.0471	* significant
All Transversions	0.4694	ns

[a]Mutations recovered from 69 clofarabine treated sequences and 132 DMSO treated sequences were analyzed using Fisher's exact test. The mutations analyzed included G-to-A mutations from hypermutated sequences.

[b]All mutation types analyzed except A-to-G and total compiled transversion mutations were significantly different between the two treatments. * P <0.05, **P < 0.01, ***P < 0.001.

Discussion

Clofarabine, a ribonucleotide reductase inhibitor (RNRI), is FDA approved to treat refractory pediatric acute lymphoblastic leukemia. Previously, other FDA-approved RNRIs have been found to exert anti-HIV-1 activity. These RNRIs have been shown to change the mutant spectra and increase the mutation frequency of HIV-1(Clouser *et al.*, 2010; Rawson *et al.*, 2013). The mechanism responsible for these changes, however, has not yet been thoroughly established. In this study, we report that clofarabine has anti-HIV-1 activity associated with increases in HIV-1 mutant frequency and an increase in G-to-A transition mutations in the context of G-to-A hypermutation.

Hypermutated G-to-A retroviral sequences have been reported since the early days of the HIV-1 epidemic, both in patients and in cell culture (Li *et al.*, 1991b; Mansky, 1996). In the early 2000's, members of the APOBEC3 protein family were reported to cause this G-to-A hypermutation (Sheehy *et al.*, 2002; Zheng *et*

al., 2004). Today, APOBEC3G, APOBEC3F, APOBEC3DE and APOBEC3H have all been found to be packaged into HIV-1 viral particles, leading to the G-to-A hypermutation that restricts HIV-1(Hultquist *et al.*, 2011). Given the prevailing view that APOBEC3 proteins must be packaged into retroviral particles to cause G-to-A hypermutation, it is not clear why in a single cycle vector virus assay, different target cell types would have different rates of G-to-A hypermutation (Holtz & Mansky, 2013), or why clofarabine treatment would be associated with more G-to-A hypermutation than DMSO treatment in target cells. Target cell activity has been reported for APOBEC3C, (Bourara *et al.*, 2007), which is known to be expressed in Magi-U373-CXCR4$_{CEM}$ cells. However, this activity was not found to cause extensive hypermutation (Bourara *et al.*, 2007), nor did APOBEC3C expression correlate with the different levels of hypermutation found in different target cell types (Holtz & Mansky, 2013).

Interestingly, at least one paper has proposed a relationship between APOBEC3G activity and deoxynucleotide pools (Plesa *et al.*, 2007). These authors proposed that in resting CD4 cells, APOBEC3G and APOBEC3F could be partially responsible for the slower RT kinetics observed in these cells, which the addition of deoxynucleosides helps overcome. Recent reports of the SAMHD1 restriction factor have shown that fluctuations in dNTP pools can affect target cell susceptibility to HIV-1 infection. SAMHD1 has been reported to restrict HIV at the step of reverse transcription (Laguette *et al.*, 2011) by depleting dNTP pools below the concentrations necessary to sustain reverse transcription

(Goldstone *et al.*, 2011). Vpx degrades SAMHD1 and can counteract the restriction factor's ability to block HIV-1 infection in monocyte derived macrophages (MDM's) (Goldstone *et al.*, 2011; Hrecka *et al.*, 2011; Laguette *et al.*, 2011; Lahouassa *et al.*, 2012). It has been shown that when increasing amounts of exogenous dNTP's are added to SAMHD1 containing MDM's infected with Vpx containing HIV-1 VLPs, the ability of SAMHD1 to restrict HIV-1 is depleted in a dose responsive fashion (Lahouassa *et al.*, 2012). HU treatment can block the recovery of HIV-1 infectivity associated with Vpx, demonstrating that RNR inhibition prevents the cell from synthesizing the dNTP's that would allow for recovery of reverse transcription and viral infectivity (Lahouassa *et al.*, 2012). These results, as well as studies showing variations in the concentration of cellular dNTPs affect the kinetics of reverse transcription (Diamond *et al.*, 2004), strongly suggest that dNTPs in the cytoplasm of the infected cell can gain access to the retroviral reverse transcription complex.

The mutant spectra results from our studies may suggest some insight into the relationship between dNTP pool levels and APOBEC3 restriction factor activity. Our results show that clofarabine depletes dNTP pools during the time frame reverse transcription would occur in the Magi-U373-CXCR4$_{CEM}$ cells. Depletions in dNTP pools due to RNRI treatment have been shown to slow down the kinetics of HIV-1 reverse transcription (Lori *et al.*, 1994). The portions of HIV-1 viral DNA that remain single stranded the longest have been shown to have more G-to-A hypermutation (Chelico *et al.*, 2006). APOBEC3G has also been

reported to slow down the kinetics of HIV-1 reverse transcription utilizing a deamination-independent mechanism, which would result in the enhancement of its deamination-dependent antiviral restriction (Adolph *et al.*, 2013; Iwatani *et al.*, 2007). Taken together, these studies implicate a relationship between dNTP pool levels, RT kinetics and APOBEC3 restriction, whereby depleted dNTP pools would lead to slower HIV RT kinetics and therefore be associated with more extensive APOBEC3 induced G-to-A hypermutation in target cells. This model is also consistent with Plesa *et al's* observation that APOBEC3G and APOBEC3F have opposite effects on RT kinetics in resting CD cells as deoxynucleoside addition. This model assumes target cell dNTP's readily diffuse into the retroviral core during reverse transcription.

Ongoing work is needed to characterize clofarabine's ability to deplete viral DNA products and affect RT kinetics. It is possible that in addition to dNTP pool depletion, any decrease in viral DNA we observe could be due to Clo-TP's ability to be incorporated and act as a mutagen or as a non-canonical DNA chain terminator. Our mutant spectra results did not reveal a specific mutational signature for clofarabine, suggesting that Clo-TP, if incorporated, does not cause a specific mutation type. No other studies have ever suggested that Clo-TP would have mutagenic properties upon incorporation into DNA, and the Ames test results reported in the FDA approval package for clofarabine indicates that clofarabine is not mutagenic in a prokaryotic cellular system. In contrast, Clo-TP has been shown to compete with dATP and to inhibit DNA polymerase alpha

after its incorporation into DNA (Parker *et al.*, 1991), suggesting that at least one human DNA polymerase recognizes Clo-TP as a dATP molecule. The finding that heavily mutated EGFP sequences can be recovered from clofarabine treated cells, however, argues against viral DNA chain termination being the sole mechanism responsible for the anti-HIV-1 activity of clofarabine.

Overall, these studies demonstrate that clofarabine possesses potent anti-HIV-1 activity, and that this antiviral activity is associated with an increase in viral mutational burden. Future studies will continue to characterize the molecular mechanism of the anti-HIV-1 activity of clofarabine. In particular, these studies will investigate if HIV-1 RT can incorporate clofarabine triphosphate as well as the comparative contributions reductions in viral DNA synthesis and lethal mutagenesis make to clofarabine's anti-HIV effects.

Materials and Methods

Cell Lines, Plasmids and Reagents

The Magi-U373-CXCR4$_{CEM}$ and human embryonic kidney (HEK 293T) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CEMGFP cells, the HIV-1 molecular clone pNL4-3, and the pCXCR4 plasmids were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. pHCMV-G was a kind gift from J. Burns (University of California, San Diego). The HIV-1 vector plasmid encoding EGFP-IRES-mCherry, also known as pNL43-MIG, has been previously described (Rawson *et al.*, 2013). The CellTiter-Glo cell proliferation kit was purchased from Promega

(Madison, WI). Clofarabine was obtained from the National Cancer Institute and from Alfa Aesar (Ward Hill, MA). 5-iodo-dCTP was purchased from TriLink BioTechnologies (San Diego, CA). Dimethylsulfoxide (DMSO) was from ThermoFisher Scientific (Waltham, MA). For transfections, poly-L-lysine was from Newcomer Supply (Middleton, WI), and polyethylenimine (PEI) was from Polysciences, Inc. (Warrington, PA). DNase was from Invitrogen (Carlsbad, CA). DpnI was from New England Biolabs (Ipswich, MA). The genomic DNA from Magi-U373-$CXCR_{CEM}$ cells was extracted using Zymo Research Corporation's Zymobead Genomic DNA Extraction Kit (Irvine, CA). Miniprep kits and Platinum PCR Supermix were from Invitrogen (Carslbad, CA). Sanger sequencing was performed by Functional Biosciences (Madison, WI) using the T7 Forward Promoter sequencing primer, TAA TAC GAC TCA CTA TAG GG. PCR products were ligated into PGEM-T using Promega's (Madison, WI) PGEM-T Kit.

Cell Culture

Magi-U373-$CXCR4_{CEM}$ cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) (Life Technologies; Carlsbad, CA) containing 10% HyClone FetalClone III (FC3) serum from Thermo Scientific (Waltham, MA), 1% penicillin/ streptomycin from Invitrogen (Carlsbad, CA), 1 µg/ml puromycin, 0.2 mg/ml neomycin and 0.1 mg/mL hygromycin. HEK 293T cells were maintained in DMEM containing 10% FC3 and 1% penicillin/streptomycin. CEMGFP cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) (Mediatech, Inc.; Manassas, VA) containing 10% FC3 and 1 mg/mL neomycin. Cell lines were

split every 2-7 days as needed using trypsin from Invitrogen. When splitting, cell lines were washed using Dulbecco's Phosphate Buffered Saline (PBS). All cells were maintained at 37°C in 5% CO_2.

Production of Virus Stocks

Virus was produced by co-transfecting either pNL43-MIG and pHCMV-G or pNL43-MIG and pCXCR4 via the PEI method (Boussif *et al.*, 1995). PEI stocks were prepared at 1 mg/mL by dissolving PEI in water, adjusting the pH to 7.0, and filtering through a 0.2 μM filter. On the day before transfection, 4 million 293T cells were plated onto 10 cm dishes that were pre-coated with 1x poly-L-lysine. Twenty-four hours later, the media was removed and 5 mL of fresh 293T media were added. One mL of serum-free and antibiotic free DMEM containing 10 μg pNL4-3 MIG + 1 μg of envelope protein (either pHCMV-G or pCXCR4) + 33 μL 1 mg/mL PEI that had incubated for 15-20 minutes at room temperature was added dropwise to each plate. Five hours after transfection, the media was replaced, and virus was collected 24 hours later by spinning down the supernatant for 5 minutes at 1200g, following by filtration through a 0.22 μm filter. Viral stocks were aliquoted and frozen at -80°C.

Viral stocks were titered in U373-MAGI-$CXCR4_{CEM}$ cells. The day before infection, 1.5 X 10^4 cells were plated in a 96 well dish. The next day the media was replaced and increasing amounts of either HIV1 vector virus was added, in biological duplicate. The media was replaced again 24 hours post-infection and cells were collected 48 hours post-infection for analysis by flow cytometry.

Drug Treatments and Vector Virus Infection

The day before infection, Magi-U373-CXCR4$_{CEM}$ cells (3.125 X 10^4 per well) were seeded onto 24 well plates. The next day, the medium was removed, and 1 µL of drug or DMSO and 499 µL of medium was added to each well. Each treatment was performed in duplicate. Two hours after drug treatment, HIV-1 CXCR4 vector virus diluted in Magi medium was added to each well for a final volume of 1 mL. Twenty-four hours after infection, the medium was changed. Twenty-four hours after the medium change, cells were trypsinized, centrifuged for 5 minutes at 1200 x *g* and the pellet was resuspended in 2% FC3 in Dulbecco's PBS. Flow cytometry to detect EGFP and MCherry was performed the same day. The resulting flow data was analyzed using FlowJo version 7.6.1 (Ashland, Oregon).

The infectivity observed for each drug concentration was calculated by averaging the biological replicates in Excel (Microsoft; Redmond Washington). Afterwards, for each experiment, the average infectivity for each drug concentration was normalized to DMSO (no drug) infectivity, by dividing the average percent infection observed for each drug concentration by the average percent infection observed for DMSO treatment. DMSO treatment was then set to one and all values were multiplied by 100 to obtain normalized percentages. The experiments were performed on three different days with three independent viral stocks. The EC_{50} value for clofarabine was determined by entering the normalized infectivity for the three independent experiments into GraphPad

Prism and plotting the infectivity against the log-transformed drug concentrations. The nonlinear regression model of log(inhibitor) vs. response - variable slope (four parameters) was used to calculated clofarabine's EC_{50} value and the 95% confidence interval. Replicates analysis indicated that this was the best fit nonlinear regression model to use to analyze this data.

Mutant Frequency Analysis

Mutant frequency values were determined by adding the total number of $EGFP^{+}/mCherry^{-}$ and $EGFP^{-}/mCherry^{+}$ cells and dividing by the total number of infected $EGFP^{+}/mCherry^{-}$, $EGFP^{-}/mCherry+$ and $EGFP^{+}/mCherry^{+}$ cells. These values were then normalized to the DMSO no-drug control mutant frequency values and entered into GraphPad Prism. A linear regression was performed and plotted with the EC 95% confidence intervals (CI). The mutant frequency was calculated at the EC_{50} of clofarabine, along with the 95% CI. Only experiments where the no-drug infection was 13% to 25% were used to calculate the mutant frequency.

Live Virus Infection and Drug Treatments

In 24 well plates, 100,000 CEMGFP cells/well were infected at an MOI of .01 in a spreading infection using four independent HIV-1 NL4-3 viral stocks that had been previously titered using the classical method of Reed and Muench. In duplicate, wells were treated with DMSO or varying concentrations of clofarabine. Every 2 days, cells were removed for flow cytometry and fresh media and drug was added. Infection levels were monitored by quantifying the percentage of cells

that were EGFP positive, using flow cytometry. The experiment was run until the infectivity level peaked, which typically occurred between 8 and 12 days. The EC_{50} value for clofarabine was calculated as describe above in the, “Drug Treatments and Vector Virus Infection,” using the infectivity levels reported during the peak of viral infection.

Cytotoxicity Analysis

The cytotoxicity of clofarabine was examined using the CellTiter-Glo Luminescent Cell Viability Assay kit from Promega, according to the manufacturer's instructions. U373-MAGI-$CXCR4_{CEM}$ cells (5,000/well), 293T cells (5,000/well), or CEMGFP cells (50,000/well) were plated in a 96-well dish 24 hours prior to drug treatment. Cells were treated with clofarabine for 24 hours and 24 hours later, cellular proliferation (an indication of the level of cytotoxicity) was analyzed using an Orion microplate luminometer from Berthold Detection Systems (Huntsville, AL). Dimethyl sulfoxide (DMSO) was used as a control for the no-drug-treated cells. For each experiment, values obtained from wells without cells were subtracted from all samples and the clofarabine-treated values were normalized against the DMSO treated values of the appropriate volumes that were used to add drug. The normalized values from each experiment were then entered into GraphPad Prism version 6.0 (GraphPad Software, Inc.; La Jolla, CA), drug concentrations were log transformed, and a nonlinear regression analysis of log(inhibitor) vs. three parameters was performed to generate IC_{50} values and 95% confidence intervals. Replicates tests indicated that this model

generated an appropriate EC_{50} fit for the data. The data represents a total of 3 experiments for all cell lines analyzed.

Sequencing of Proviral DNA

Magi-U373-CXCR4$_{CEM}$ cells were plated 24 hours before treatment with 5 μM clofarabine or with an equivalent volume of DMSO. Two hours after drug treatment, cells were infected with DNase-treated pNL43-MIG. To confirm that the clofarabine was working as expected, infectivity controls were performed in parallel. The anti-viral activity of clofarabine was confirmed by flow cytometry 48 hours later. Twenty-four hours after infection, genomic DNA from the clofarabine-treated or DMSO-treated cells was isolated using Zymo Research Corporation's ZymoBead DNA Extraction Kit (Irvine, CA). The extracted DNA was treated with DpnI from New England Biolabs (Ipswich, MA). Using the genomic DNA as a template, PCR was performed to amplify the EGFP gene, using Invitrogen's Platinum PCR Supermix (Carlsbad, CA). The primers used in the PCR reaction were 5'-CAAGCGTATTCAACAAGG-3' and 5'-GCAATACAGCAGCTAACAATG-3'. The PCR fragment containing EGFP was ligated into PGEM-T (Promega; Madison, WI) and transformed into chemically competent DH5α *E. coli*. Individual colonies were grown, plasmid DNA was recovered using the Invitrogen PureLink® Quick Plasmid Miniprep Kit (Carlsbad, CA), and the EGFP gene was sequenced using Sanger sequencing. Sequences were aligned to the reference and mutations identified using Lasergene (DNASTAR, Inc., Madison, WI). Any sequences with the same mutations were assumed to be identical, and therefore

only mutations from one of these sequences were counted. Sequencing was performed on genomic DNA extracted from three different infections using three different pNL43-MIG VSV-G stocks. In total, 83 unique mutations from 133 DMSO-treated EGFP sequences and 107 unique mutations from 69 clofarabine-treated EGFP sequences were obtained. Statistical differences between the mutant spectra of the clofarabine-treated cells and the DMSO-treated cells were determined using Fisher's exact test.

dNTP Pool Analysis

The day before dNTP pools were extracted, 3 million Magi-U373-$CXCR4_{CEM}$ cells were plated in a 10 cm^2 dish. Cells were then treated with DMSO, 0.5 μM clofarabine or 5.0 μM clofarabine for 3, 6 or 24 hours. Each plate was trypsinized, washed in PBS to remove dead cells, and cells were counted using a hemocytometer. Three million cells from each treatment were resuspended in 200 μL of 65% ice cold methanol containing 10 μM 5'-Iodo-2'dCTP from TriLink BioTechnologies (San Diego, CA), then lysed by vigorous vortexing. The mixture was stored at -20Cº overnight to precipitate proteins. The next day, samples were centrifuged at 14,000g for 10 minutes. The supernatant was collected and the methanol was removed by speed vacuuming.

The lyophilized samples were submitted for dual ion pair high performance liquid chromatography-tandem mass spectrometry (LCMS-MS). LCMS-MS was performed using an Agilent 1260 HPLC. A 150 mm – 3.9 mm Waters XTerraMS C-18 column (5 μM) was used to separate the nucleotides, with a mobile phase

of 5 mM hexylamine containing 0.5% diethylamine in water (pH 10, adjusted with acetic acid; mobile phase A) and 50/50 water/acetonitrile (mobile phase B). The flow rate was 0.3 ml/min. A linear gradient of 0% to 30% of mobile phase B was used to elute analytes over 30 minutes. Mobile phase B (95%) was used to clean the column for 3 minutes and the column was then re-equilibrated for 15 minutes with mobile phase A (100%).

The Agilent 1260 HPLC was coupled to an AB SCIEX QTRAP 5500 mass spectrometer. The samples were run using an electrospray source operating in positive/negative polarity switching mode, utilizing the following settings: curtain gas: 20;CAD gas: medium; ion spray voltage: 4500; temperature: 600°C; gas1: 45; and gas 2: 45. Multiple reaction monitoring (MRM) was used to analyze deoxynucleotides and internal standard (IS), using the parameters listed in Appendix II. The fragmentation sites for each dNTP are depicted in Figure II-1. Each LCMS-MS run was exported into MultiQuant (AB SCIEX), to calculate peak areas. Figure II-2A of Appendix II illustrates representative chromatograms for dATP, dCTP, dTTP, and IS from a typical run. Due to the overlap of ATP and dGTP peaks, a less efficient mass transition was used to monitor dGTP than for the other dNTP pools. Comparatively, therefore, dGTP has a much lower peak height than the other dNTP peaks in our assay. For ease of analysis, the dGTP peak is illustrated in a separate chromatogram, in Figure II-2B of Appendix II.

In Figure 4, the dNTP pool levels are expressed as integrated peak areas normalized to the internal standard. For quality control purposes, data from dNTP

experiments was only used if the standard of variance for the internal standard was less than 20%. The mean and the standard error of the mean (SEM) were calculated and plotted for the IS normalized data. One-way analysis of variance (ANOVA) with Tukey's post-test was used to assess differences among all treatment groups. A *P* value of <0.05 was considered statistically significant, as indicated by an asterisk. Two asterisks indicate a P-value of less than .01. Three asterisks indicate a P-value of less than .001.

Chapter 4

General summary and future directions

Since 1981, the human immunodeficiency virus (HIV) has been reported to infect seventy-five million people and has claimed the lives of over thirty-six million people worldwide (UNAIDS, 2013), making HIV/AIDS one of the most devastating global infectious disease epidemics the world has ever seen. Though the advent of highly active antiretroviral therapy (HAART) has extended the lives of many HIV infected individuals, there remains a pressing need for the development of new drugs. HIV drug resistance, both primary and transmitted, undermines the effectiveness of existing antiviral treatments. Worse, some viral types and subtypes have naturally occurring resistance polymorphisms that make the use of some or all classes of anti-HIV drugs partially or totally ineffective.

Unlike HIV-1, HIV-2 is resistant to the first generation NNRTIs (Witvrouw *et al.*, 2004). HIV-2 is also partially resistant to some protease inhibitors and to enfuvirtide (Campbell-Yesufu & Gandhi, 2011; Witvrouw *et al.*, 2004). The restricted treatment options for HIV-2 infection and the comparative lack of study of HIV-2 present a serious barrier for the effective treatment of HIV-2 infected individuals. In this dissertation, the anti-HIV-2 activity of ribonucleotide reductase inhibitors was explored, to determine if members of this drug class could expand the treatment options for HIV-2. It was hypothesized that because HIV-2 appears to require higher cellular dNTP concentrations to complete reverse transcription compared to HIV-1, lower doses of RNRIs could be used to inhibit this virus.

The findings of these dissertation studies reveal the RNRIs clofarabine and resveratrol as well as the mutagen 5-azacytidine exert comparatively more potent antiviral activity against HIV-2 than HIV-1 during reverse transcription. The results also indicate gemcitabine is at least as effective at inhibiting HIV-2 replication as HIV-1 replication. Notably, these studies report for the first time the anti-HIV1 and HIV-2 activity of clofarabine.

The remainder of this dissertation presents the first investigation of the anti-HIV-1 mechanism of action of clofarabine. To date, mechanistic studies have revealed the antiviral activity of clofarabine is associated with an increase in HIV-1 mutational burden and the depletion of dATP and dGTP pools in a vector virus assay. This dissertation, while reporting the correlation between increased mutational burden and the antiviral effects of clofarabine, does not contain a definitive analysis of the relative contribution of increased mutation compared to other potential anti-HIV mechanisms of action. Chapter 1 discusses how a reduction in one or more dNTP pools often leads both to increased viral mutation and to decreases in viral DNA synthesis. Reductions in viral DNA synthesis may complicate mechanistic studies that seek to evaluate the exclusive contribution of enhanced mutagenesis to reductions in HIV infectivity.

Although the studies in this dissertation do indicate that clofarabine enhances HIV-1 mutagenesis, they do not prove conclusively that lethal mutagenesis is the primary mechanism of action of clofarabine against HIV-1. Several other mechanisms could also contribute to the antiviral effect of

clofarabine. Based on clofarabine's observed ability to reduce dNTP pools in the Magi-U373$_{CEM}$ cell line, it is likely clofarabine's RNRI activity accounts for at least part of its antiviral mechanism. A reduction in dNTPs could reduce infectivity in several ways. For example, a reduction in dNTP concentrations below the Km of RT for dNTPs would likely reduce viral DNA synthesis (Diamond *et al.*, 2004). This mechanism could be examined through qPCR. Second, alterations in dNTPs have been shown to increase the mutation frequency of HIV, thereby leading to a reduction in infectivity (Mansky, 2003). The results in this dissertation are consistent with enhanced mutagenesis contributing to the antiviral activity of clofarabine.

Besides acting as a RNRI, clofarabine may also inhibit HIV infectivity through direct incorporation into the viral genome after its conversion to the triphosphorylated form. Such incorporation could lead to a pause or delay or non-canonical termination of reverse transcription, which would lead to reduced infectivity. Studies with cellular DNA polymerases indicate multiple polymerases can incorporate both clofarabine triphosphate and clofarabine monophosphate, and that this incorporation limits DNA elongation (Parker *et al.*, 1991; Xie & Plunkett, 1995; Xie & Plunkett, 1996). In vitro RT incorporation assays could be used to examine this potential mechanism.

Other experiments that could contribute to investigations of whether increased mutation or decreased viral DNA synthesis were primarily responsible for the antiviral activity of clofarabine include selecting for clofarabine resistant

HIV-1 and determining if RNR mutants resistant to clofarabine inhibition still retained antiviral activity (Aye & Stubbe, 2011).

The successful selection of HIV-1 drug resistance mutants in cell culture can be used to examine the mechanisms of action of antiviral drugs. If clofarabine's primary mechanism of action is to act as a non-canonical DNA chain terminator due to drug incorporation, resistance mutations would likely be similar to mutations that confer resistance to NRTIs used to treat HIV-1. If, however, clofarabine acts primarily as a viral mutagen, resistance may be difficult to select. However, such a resistance mutation would be expected to increase the fidelity of RT.

Experiments investigating the impact of RNR mutants resistant to clofarabine on HIV infectivity would test if RNR inhibition is required for clofarabine's antiviral effect. Interestingly, the D57N mutant of RRM1 is resistant to allosteric inhibition by clofarabine triphosphate (Aye & Stubbe, 2011). If the antiviral activity of clofarabine persisted even without the drug's ability to decrease dNTP pools, this finding would suggest inhibition of viral DNA synthesis would be clofarabine's primary mode of action against HIV.

Overall, this dissertation contains the first description of the anti-HIV-1 and HIV-2 activity of clofarabine. More experiments are needed to fully characterize the mechanism of clofarabine's antiviral activity. Future pre-clinical testing in humanized mouse models, non-human primate models as well as eventual clinical trials in human subjects would be necessary to evaluate the feasibility of

the clinical translation of clofarabine for use as an antiretroviral agent. To date, neither the long-term side effects nor the antiretroviral efficacy of low-dose clofarabine have been studied. The development, optimization, and approval of RNRIs for use against HIV could provide life-saving salvage therapy options for patients living with HIV-1 and HIV-2. Additionally, RNRIs like clofarabine could likely be used to boost the efficacy of NRTI-containing HAART regimens for patients harboring NRTI drug resistant HIV infections. If drug resistance mutations affect the efficacy of PrEP, RNRIs could also be potentially used to boost the efficacy of PrEP, though this application would require extensive future investigation.

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Appendix I

Live virus activity of clofarabine

The anti-HIV-1 activity of clofarabine was analyzed in CEMGFP cells by Michael Dapp, as described in the, “Live virus infection and drug treatment,” section of the Materials and Methods of Chapter 3. This work revealed clofarabine possessed an EC_{50} of 15.8 nM, with a 95% confidence interval ranging from 9.92 to 25.2 nM, in a live HIV-1 immortalized t-cell line model system (Figure I-1).

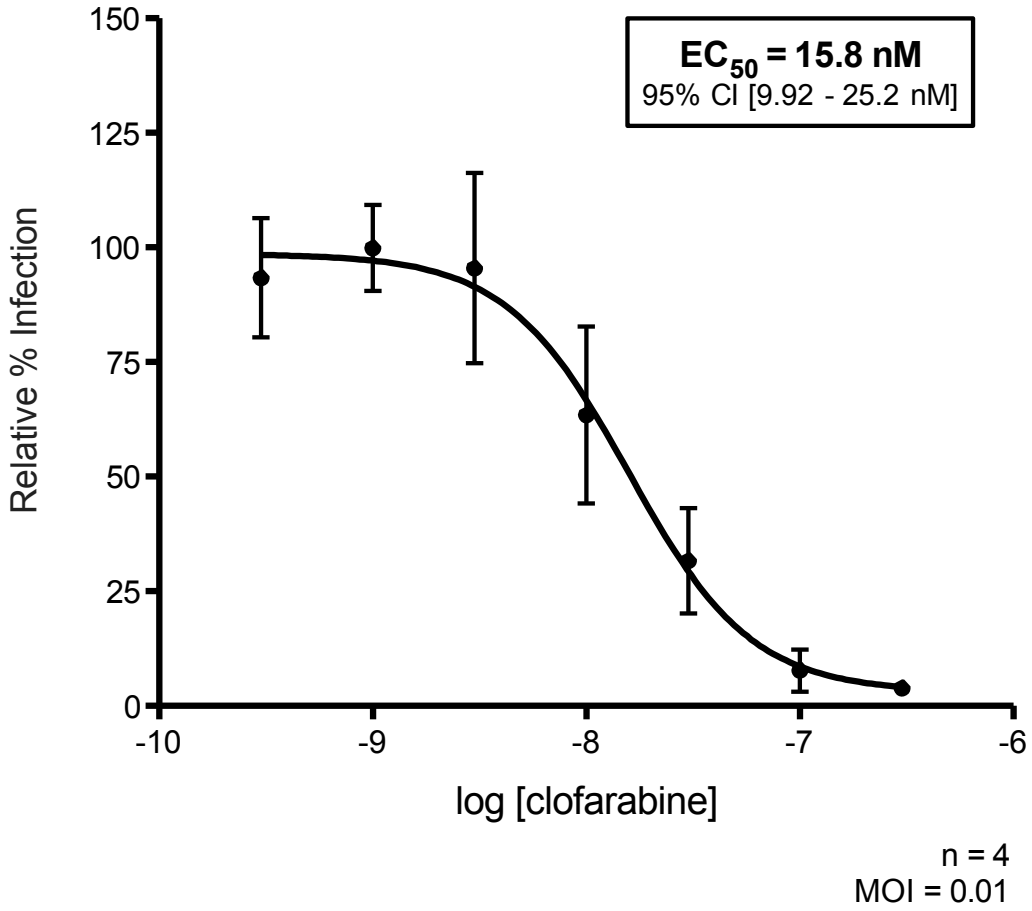

Figure I-1 Clofarabine inhibits HIV-1 in a Spreading Infection. CEMGFP cells were infected with pNL4-3 at an MOI of .01 and treated in duplicate with concentrations of clofarabine ranging from 300 pM to 30 nM. Every two days, cells were split, and fresh drug and media were added. The EC50 values were calculated from the peak day of infection for each experiment (n=4).

Additionally, I performed spreading infections with pNL4-3 using an identical method as described in the Materials and Methods of Chapter 3, with the exception that an MOI of .05 instead of .01 was used. The results from the replication kinetics from these spreading infections are shown in Figure I-2. The EC_{50} value of clofarabine was calculated to be 9.475 nM, placing this value very close to the bottom range of the 95% confidence interval defined by Michael Dapp.

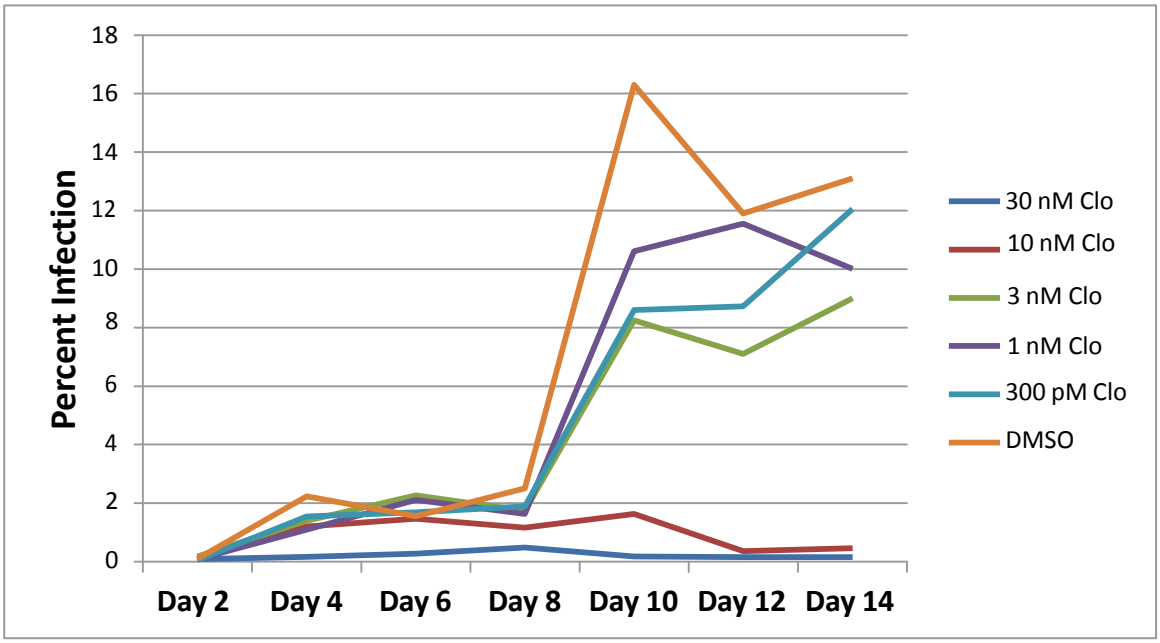

Figure I-2 Clofarabine inhibits HIV-1 in a Spreading Infection. CEMGFP cells were infected with pNL4-3 at an MOI of .05 and treated in duplicate with concentrations of clofarabine ranging from 300 pM to 30 nM. Every two days, cells were split, and fresh drug and media were added. The EC50 values were calculated from the peak day of infection (n=1).

A comparison of the EC_{50} value of 15.8 nM for clofarabine that was calculated by Michael Dapp and the TC_{50} concentration of 567 nM that I determined in the CEMGFP cell line demonstrates that clofarabine possesses a selectivity index of 35.9 in this cell line. This selectivity index (SI) is lower than

the SI of FDA approved anti-HIV-1 drugs, but higher than the SI of many anti-cancer drugs. Further work will need to be done to determine if the toxicity profile of clofarabine warrants additional investigation of clofarabine as a therapeutic agent to treat HIV-1 and HIV-2 infection.

Appendix II

dNTP pool methods

Figure II-1 and Figure II-2 below depict the dNTP fragmentation patterns and sample chromatograms of the different dNTP pools analyzed in the LSMS-MS experiments. The parameters of the LCMS-MS experiments were designed and performed by Dr. Benjamin Duckworth of the Center for Drug Design at the University of Minnesota, with input from Dr. Steven Patterson. The samples analyzed by LCMS-MS were prepared by Lauren Beach.

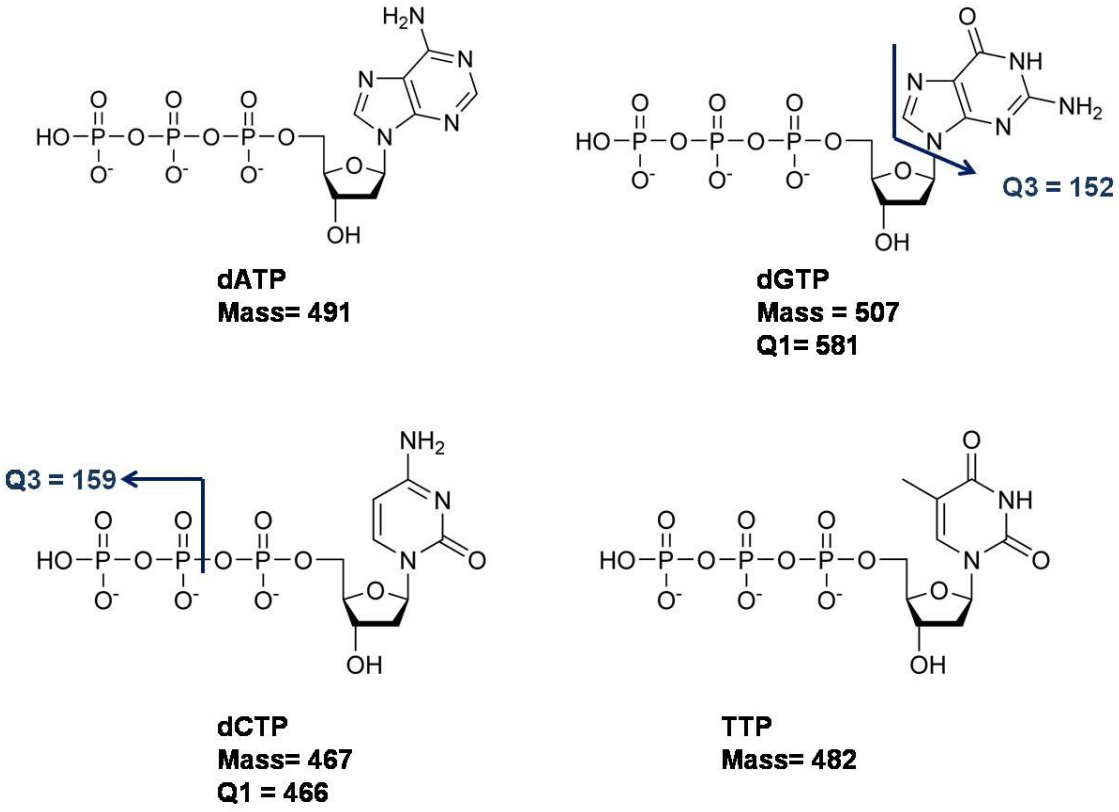

Figure II-1 Schematic of dNTP Fragmentation Pattern from LCMS-MS Experiments.

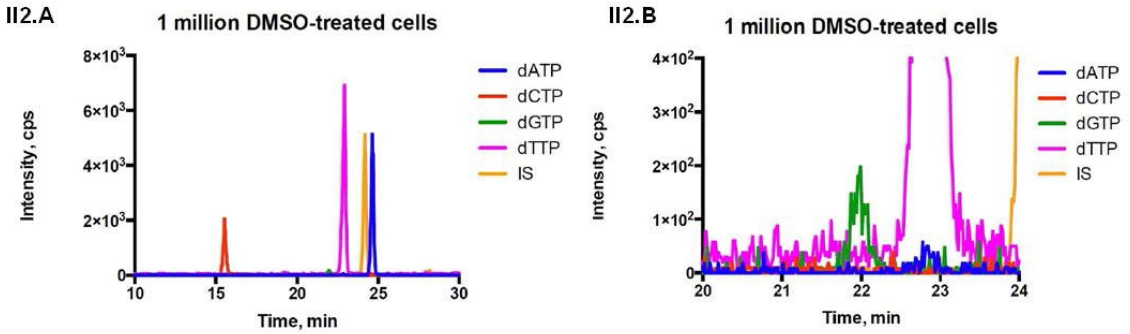

Figure II-2. Representative chromatograms of dNTP pool peaks from LCMS-MS assay. II2.A shows peaks from dATP, dCTP, TTP, and IS pools. II2.B shows the dGTP pool peak using a different scale bar, because of the much less robust detection of the dGTP pool in our assay.

Appendix III

Clofarabine vs. DMSO comparative mutant spectra analysis without G-to-A hypermutants included

To determine the effect of the G to A hypermutations on the overall mutant spectra of HIV exposed to clofarabine or DMSO (refer to Chapter 3, Table 1 and Figure 3.5), the G-to-A mutations from EGFP sequences with 2 or more G-to-A mutations were removed from the mutant spectra analysis. The Fisher's Exact Test was used to compare the different types of transition mutations or compiled transversion mutations. After removal of the G-to-A mutants in the context of hypermutated sequences, no significant differences in transition mutations remained (Table III-1). Total transversion mutations were comparatively higher for the EGFP sequences exposed to clofarabine than to DMSO. However, no significant differences were detected in the 8 possible transversion mutations when comparing genomes exposed to clofarabine or DMSO.

Table III-1 Statistical Analysis of Clofarabine and DMSO Mutant Spectra with G-to-A Mutations from G-to-A Hypermutated Sequences Removed.

Mutation	P-Value	Significance by Fisher's Exact Test
G-to-A	0.3098	ns
A-to-G	0.3134	ns
T-to-C	0.2309	ns
C-to-T	0.7871	ns
All Transversions	0.0477	* significant

[a]Fisher's Exact test was used to compare different mutation types from *egfp* HIV-1 proviral sequences exposed to clofarabine or DMSO, without considering G-to-A transition mutations found in G-to-A hypermutated sequences. *P<0.05

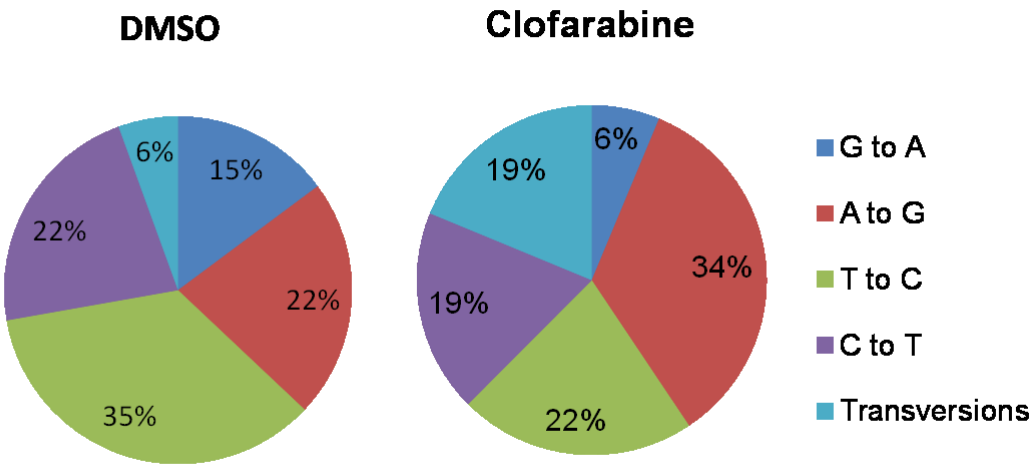

Figure III-1. Comparative Mutant Spectra of Clofarabine vs. DMSO, without G-to-A Hypermutated Sequences. VSV-G pseudotyped pNL4-3 infected cells were treated with 5 μM clofarabine or with DMSO. Genomic DNA was extracted and the *egfp* gene was amplified and Sanger sequencing was performed. Mutations were compiled, and the G-to-A transition mutations from sequences containing 2 or more G-to-A mutations were removed from the analysis. For DMSO, 132 sequences were analyzed, yielding 54 mutations and for clofarabine, 69 sequences were analyzed, yielding 32 mutations. The figure depicts the percent of each type of mutation that was identified.

Appendix IV

The late phase effects of clofarabine

Clofarabine is FDA approved for the intended use of treating relapsed or refractory acute pediatric leukemia (Gandhi & Plunkett, 2006). The well-established cytotoxic molecular mechanisms of action of clofarabine include direct inhibition of DNA synthesis and the inhibition of RNR as reviewed in (Bonate *et al.*, 2006; Zhenchuk *et al.*, 2009). Translating clofarabine's anti-cancer effects to its potential anti-HIV applications indicates these mechanisms would inhibit the early (i.e. pre-integration) phase of the HIV-1 life cycle, assuming an anti-viral selectivity index greater than 1. Chapter 3 of this dissertation demonstrates that decreases in the infectivity of clofarabine are accompanied by an increase in HIV-1 mutant frequency.

At high concentrations, clofarabine has also been reported to contribute to cytotoxicity by being incorporated into RNA and interfering with translation (Bonate *et al.*, 2006). The ability of clofarabine to inhibit the late phase of HIV-1 replication without cytotoxicity was previously unexplored. Accordingly, a single cycle producer cell assay was used to test the ability of clofarabine to inhibit the post-integration, or "late phase" of HIV-1 replication. The method was optimized to eliminate drug carryover concerns, to ensure that the assay would not be influenced by any early-phase inhibitory effects of the drugs tested in the assay.

Briefly, 205,000 293T cells per well were plated onto poly-L-lysine-coated 12 well tissue culture dishes. The next day, cells were transduced with pNL43-MIG and a plasmid containing the VSV envelop protein, pHCMV-G, using the PEI method. Five hours after transduction, the media was changed and increasing

amounts of clofarabine, nelfinavir, zidovudine or DMSO (vehicle) were added to each well. One day later, the media was changed to prevent drug carryover. After 16 hours, the supernatant containing VSV-G pseudotyped HIV-1 vector virus was harvested and centrifuged to remove cellular debris. 293T cells were harvested and analyzed by flow cytometry to ensure equal transduction efficiencies.

An equal ratio by volume of viral supernatant was added to a solution of 4ºC 30% PEG/0.5M NaCl. The mixture was vortexed to homogeneity, stored at 4ºC for 24 hours, and then centrifuged at maximum speed for 10 minutes. Following this, the supernatant was removed, the pellet was washed in a 50/50 mixture of media and PEG solution and centrifuged at top speed for 10 minutes. The pellet was resuspended in 1.1 mL of media and incubated at 4ºC for 2 hours. The pellet was pipetted one time and used to infect 62,500 Magi-U373-$CXCR4_{CEM}$ cells/well of a 12-well plate that were plated the preceding day. Twenty four hours after infection, the media was changed, and 24 hours later, cells were harvested and flow cytometry was performed to determine HIV-1 infectivity. Infectivity was normalized to DMSO and the data was analyzed in GraphPad as described in the "Drug Treatments and Vector Virus Infection," section of Materials and Methods from Chapter 3, with the notable exception that, following a replicates analysis, the log(inhibitor) vs. three parameters was determined to be the best nonlinear regression model used to calculate EC_{50} values for all drugs. The results from the producer cell experiments indicate that clofarabine has low micromolar anti-HIV-1 activity (Table IV-1 and Figure IV-1)

that maps to the late phase of the retroviral life cycle.

Toxicity assays were performed to determine if toxicity of clofarabine in 293T cells could account for clofarabine's late-phase anti-HIV-1 effect using, the CellTiter-Glo Luminescent Cell Viability Assay kit from Promega (Madison, WI), according to the manufacturer's instructions and as outlined in the Materials and Methods of Chapter 3. The results from three independent experiments indicate that in the 293T cell line, clofarabine has a TC_{50} of 245.2 μM, with a 95% confidence interval of 41.9 μM to 1.44 mM (Table IV-1 and Figure IV-2). Clofarabine's producer cell antiviral activity and its 293T cell toxicity result in a selectivity index (SI) of 36.1. This SI is much lower than the SI found for HIV-1 in the Magi-U373-CXCR4$_{CEM}$ cell line, but mirrors closely the anti-HIV-1 SI calculated for the CEMGFP cell line.

Unlike the results from the EC_{50} and TC_{50} calculations for the CEMGFP and Magi-U373-CXCR4$_{CEM}$ cell lines, however, the confidence intervals for the EC_{50} and TC_{50} for the producer cell antiviral activity and toxicity experiments overlap. This finding suggests that at least some of clofarabine's late-phase activity could be due to cytotoxicity in the 293T cell line, rather than to antiviral activity.

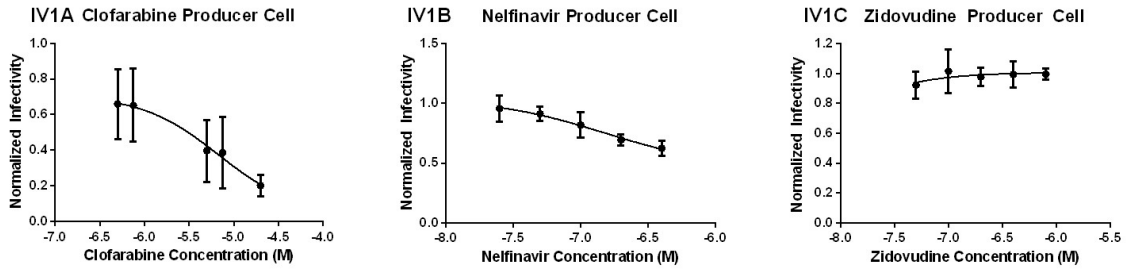

Figure IV-1 Late Phase Effects of Clofarabine, Nelfinavir and Zidovudine. Producer cell experiments were performed using a modified version of the vector virus target cell assay to determine if clofarabine possessed late phase anti-HIV-1 activity. The protease inhibitor nelfinavir (NFV) and the RT inhibitor zidovudine (AZT) were included as positive and negative controls, respectively. As expected, NFV showed potent anti-HIV-1 activity, while AZT did not show any. The error bars represent the standard error of the mean from three independent experiments. The EC_{50} values and 95% confidence intervals are reported in Table IV.1.

Table IV-1 Late Phase EC50 Values of Clofarabine, Nelfinavir, and Zidovudine

Drug	EC50	95% Confidence Interval
Clofarabine	6.79 µM	423 nM to 109 µM
Nelfinavir	154 nM	19.6 nM to 1.2 µM
Zidovudine	No Convergence	N/A

[a] This table reports the EC_{50} and 95% confidence intervals for clofarabine, nelfinavir (NFV) and zidovudine (AZT) from the producer cell vector virus assay, calculated from three independent experiments.

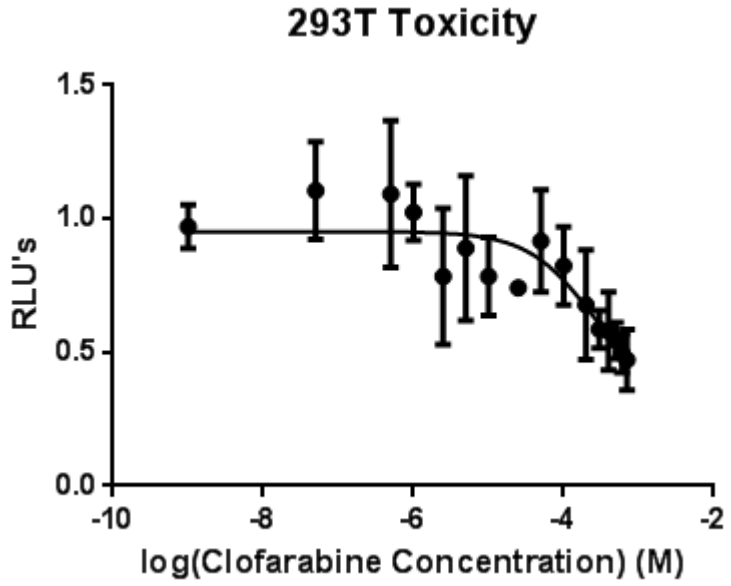

Figure IV-2 Toxicity of Clofarabine in 293T Cells. Toxicity analysis of clofarabine was performed using the Promega CellTiter-Glo Luminescent Cell Viability Assay Kit according to the manufacturer's instructions. All samples were normalized to the relative light units (RLUs) of the no drug DMSO control. The TC_{50} of clofarabine was determined to be 245 μM, with a confidence interval of 41.9 μM to 1.44 mM. Experiments were performed three independent times. Error bars represent the standard deviation.

Appendix V

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Appendix VI

Formatted Adaptation of

"5,6-Dihydro-5-aza-2'-deoxycytidine potentiates the anti-HIV-1 activity of ribonucleotide reductase inhibitors"

Jonathan M. Rawson, Richard H. Heineman, Lauren B. Beach, Jessica L. Martin, Erica K. Schnettler, Michael J. Dapp, Steven E. Patterson, and Louis M. Mansky

Bioorg Med Chem ***21****, 7222-7228.*

Contributions

This appendix is the attachment of a paper on which I was a co-author, "5,6-Dihydro-5-aza-2'-deoxycytidine potentiates the anti-HIV-1 activity of ribonucleotide reductase inhibitors" (Rawson *et al.*, 2013). My contributions to this paper were that I cloned the mCherry-IRES-EGFP cassette, which Jonathan Rawson subsequently inserted into the HIV-1 molecular clone pNL4-3 background, to make the single cycle HIV-1 vector, pNL43-MIG. Additionally, I performed the toxicity experiments shown in Figure 4 of the paper that demonstrate the reason 5 μM KP1212 decreases gemcitabine infectivity is not synergistic toxicity.

Abstract

The nucleoside analog 5,6-dihydro-5-aza-2'-deoxycytidine (KP-1212) has been investigated as a first-in-class lethal mutagen of human immunodeficiency virus type-1 (HIV-1). Since a prodrug monotherapy did not reduce viral loads in Phase II clinical trials, we tested if ribonucleotide reductase inhibitors (RNRIs) combined with KP-1212 would improve antiviral activity. KP-1212 potentiated the activity of gemcitabine and resveratrol and simultaneously increased the viral mutant frequency. G-to-C mutations predominated with the KP-1212-resveratrol combination. These observations represent the first demonstration of a mild anti-HIV-1 mutagen potentiating the antiretroviral activity of RNRIs and encourage the clinical translation of enhanced viral mutagenesis in treating HIV-1 infection.

Introduction

Human immunodeficiency virus type-1 (HIV-1) infects about 34 million individuals globally and resulted in the deaths of 1.7 million in 2011 alone (UNAIDS, 2012). Despite great strides in antiretroviral therapy, current drugs are still limited by toxicity, cross-resistance, and the transmission of drug-resistant viral variants (Kim, 2013). HIV-1 has a high mutation rate (3 to 9 x 10-5 mutations/base pair/cycle), (Gao *et al.*, 2004; Mansky & Temin, 1995; O'Neil *et al.*, 2002) and it has been postulated to replicate near the error threshold—a theoretical upper limit to the mutation rate beyond which genetic information cannot be maintained (Anderson *et al.*, 2004; Dapp *et al.*, 2013; Smith *et al.*, 2005). Thus, it is conceivable to exceed the error threshold with small molecule viral mutagens. This antiviral strategy, called lethal mutagenesis, was first investigated using mutagenic ribonucleoside analogs and RNA-damaging agents in poliovirus and vesicular stomatitis virus by Holland and colleagues in the 1990s (Holland *et al.*, 1990; Lee *et al.*, 1997). It was later demonstrated in HIV-1 using 5-hydroxy-2'-deoxycytidine, (Loeb *et al.*, 1999) and subsequently explored in several other RNA viruses (Crotty *et al.*, 2001; Crotty *et al.*, 2000; Ruiz-Jarabo *et al.*, 2003; Sierra *et al.*, 2000). HIV-1 lethal mutagenesis has not been clinically exploited, but a prodrug of 5,6-dihydro-5-aza-2'-deoxycytidine (KP-1212) has advanced to clinical trials. KP-1212 potently inhibited HIV-1 replication in culture (EC50 of ~10 nM) with low cellular, mitochondrial, and genome toxicities, as well as activity against both wildtype (wt) and drug resistant strains (Harris *et al.*,

2005). KP-1212 was shown to induce primarily G-to-A and A-to-G (and to a lesser extent T-to-C and C-to-T) transition mutations within HIV-1. The prodrug of KP-1212 (KP-1461) was well tolerated in clinical trials, but was not effective in reducing viral loads despite adequate drug bioavailability (Clay *et al.*, 2011; Hicks *et al.*, 2013). Nonetheless, there was a detectable increase in the number of private mutations (i.e., those most likely to have occurred in the latest round of replication) and evidence of an altered mutation spectrum in clinical samples (Mullins *et al.*, 2011).

We have previously demonstrated that combinations of nucleoside analogs that act as viral mutagens and ribonucleotide reductase inhibitors (RNRIs) can result in synergistic decreases in viral infectivity (Clouser *et al.*, 2012; Clouser *et al.*, 2010). Similarly, combining nucleoside reverse transcriptase inhibitors (NRTIs) with RNRIs results in potentiation of anti-HIV-1 activity (Gao *et al.*, 1994; Heredia *et al.*, 2000; Lori *et al.*, 1994). RNRIs likely have a threefold antiretroviral mechanism in such combinations: 1) inhibition of retroviral DNA synthesis via depletion of deoxynucleoside triphosphate (dNTP) pools; (Bianchi *et al.*, 1986; Gao *et al.*, 1993; Heinemann *et al.*, 1990; Lori *et al.*, 1994) 2) many RNRIs do not inhibit synthesis of all four natural dNTPs equally, thus generating dNTP pool imbalances that increase the viral mutation rate; (Bianchi *et al.*, 1994; Gao *et al.*, 1994; Heinemann *et al.*, 1990; Julias & Pathak, 1998) and 3) decreases in cellular dNTP concentrations result in the upregulation of nucleotide kinases, leading to more efficient activation of nucleoside

analogues such as KP-1212 (Clouser *et al.*, 2012; Clouser *et al.*, 2010; Gao *et al.*, 1994; Heredia *et al.*, 2000; Lori *et al.*, 1994). Given this, the combination of an RNRI with KP-1212 or a KP-1212 prodrug should result in potent inhibition of HIV-1 infectivity.

We report here that while KP-1212 alone had minimal antiviral activity in our single round replication assay, it potentiated the activity of the RNRIs gemcitabine and resveratrol. The combination of KP-1212 with resveratrol, in particular, reduced the EC50 by 1.9-fold relative to resveratrol alone (i.e., from 99.6 μM to 52.8 μM). The KP-1212-RNRI combinations led to corresponding increases in the viral mutant frequency in the absence of cell cytotoxicity relative to that of RNRIs alone. Taken together, these observations represent the first demonstration of a mild anti-HIV-1 mutagen having the ability to potentiate the antiretroviral activity of RNRIs. These findings may improve the potential for clinical translation of KP-1212 in the treatment of HIV-1 infection.

Results & discussion

Development of an mCherry/GFP dual reporter HIV-1 vector

In order to efficiently assess both HIV-1 infectivity and mutant frequency, we constructed a HIV-1 vector expressing two fluorescent proteins—mCherry and the green fluorescence protein (GFP) (Figure VI-1A). The resulting vector expresses all viral proteins except Env and Nef. Infectious vector virus was produced by trans-complementing with the vesicular stomatitis virus G protein

(VSV-G) via co-transfection with the vector virus plasmid. This strategy limits virus replication to a single cycle of replication. The utility of the construct for detecting drug-induced increases in virus mutant frequency was confirmed using 5-azacytidine (5-AZC), a ribonucleoside analog previously shown to have antiretroviral activity against HIV-1 (Dapp *et al.*, 2009). We found that treatment of target cells with 5-AZC reduced HIV-1 infectivity in a concentration-dependent manner, with an EC50 of 53.0 μM, comparable to the previously reported value of 57.0 μM (Figure VI-1B) (Dapp *et al.*, 2009). We also observed a concentration-dependent elevation of the viral mutant frequency to a maximum of 19.8-fold relative to the no drug control, confirming previous findings with 5-AZC (Dapp *et al.*, 2009).

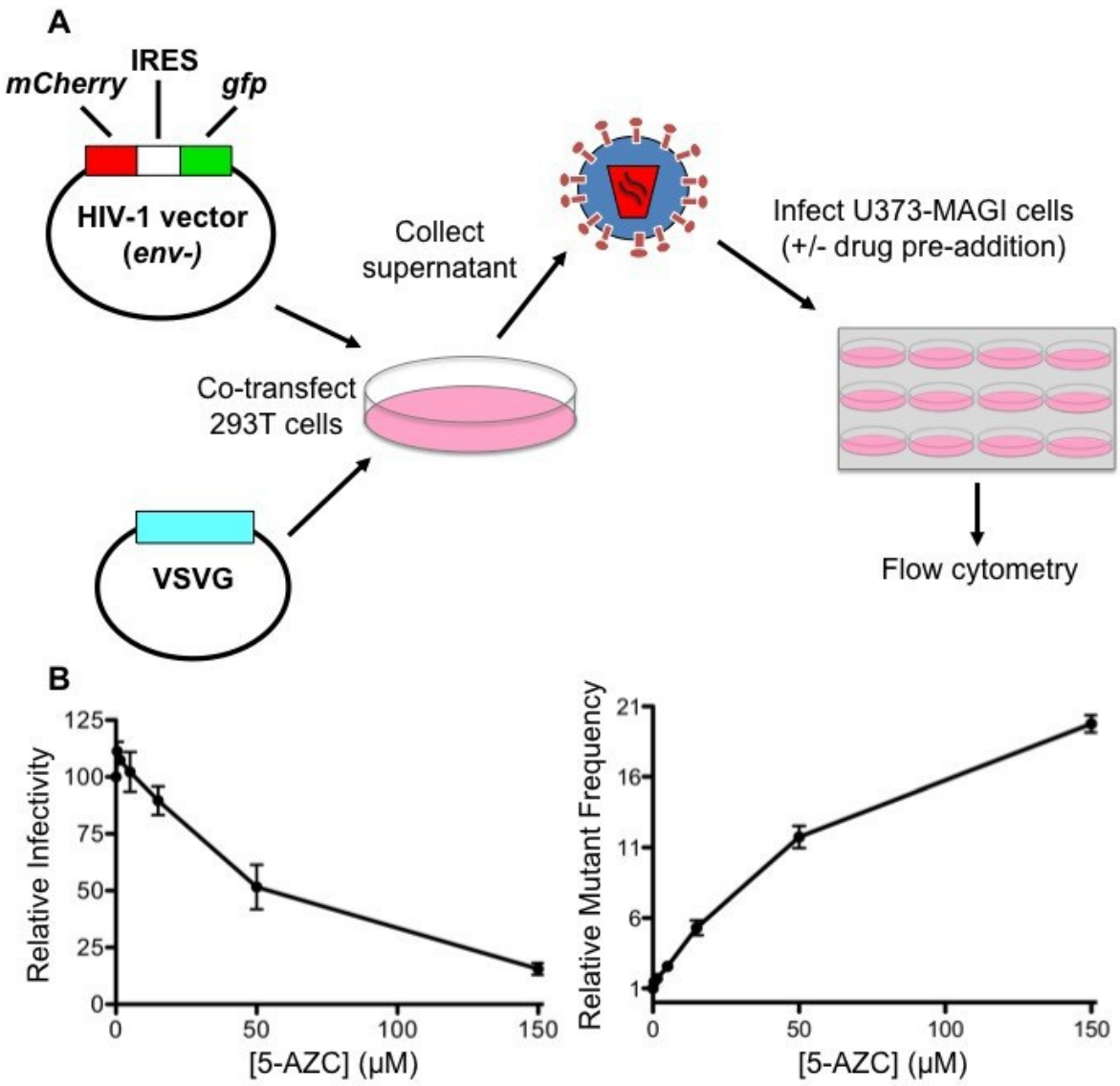

Figure VI-1 Vector and assay design for rapid determination of HIV-1 infectivity and mutant frequency. (A) A dual-reporter (mCherry/GFP) HIV-1 vector was created and co-transfected with VSVG to produce infectious virus. Target cells were pre-incubated with drugs for 2 h before infection. Drug was removed 24 h post-infection and cells were collected 72 h post-infection for determination of infectivity and mutant frequency *via* flow cytometry, as described in Section 3.5. (B) The utility of the dual reporter HIV-1 vector for detecting alterations in infectivity and mutant frequency was validated using 5-azacytidine, a previously characterized potent mutagen of HIV-1.(Dapp *et al.*, 2009) The data were normalized to the no drug (DMSO only) control and represent the mean ± sd of three independent experiments.

Individual effects of RNRIs and KP-1212 on HIV-1 infectivity & mutant frequency

We initially combined KP-1212 at 100 μM with a panel of several anti-metabolites and found that KP-1212 potentiated the antiretroviral activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC), resveratrol, and deferoxamine (data not shown). These three compounds have all been previously reported to have RNRI activity as well as antiretroviral activity, though inhibition of ribonucleotide reductase has not been clearly demonstrated to be their primary mechanism of action against HIV-1 (Baker *et al.*, 1991; Clouser *et al.*, 2012; Clouser *et al.*, 2010; Fontecave *et al.*, 1998; Georgiou *et al.*, 2000; Heinemann *et al.*, 1990; Heredia *et al.*, 2000; Nyholm *et al.*, 1993). Resveratrol, in particular, has been reported to interact with a wide variety of cellular targets in exerting its anti-inflammatory, anti-tumorigenic, and antiviral effects (Smoliga *et al.*, 2011). We next characterized the individual effects of each compound on HIV-1 infectivity and mutant frequency. We found that gemcitabine, resveratrol, and deferoxamine all reduced HIV-1 infectivity (Figure VI-2A & Table VI-1) and elevated the viral mutant frequency in a concentration-dependent fashion (Figure VI-2B).

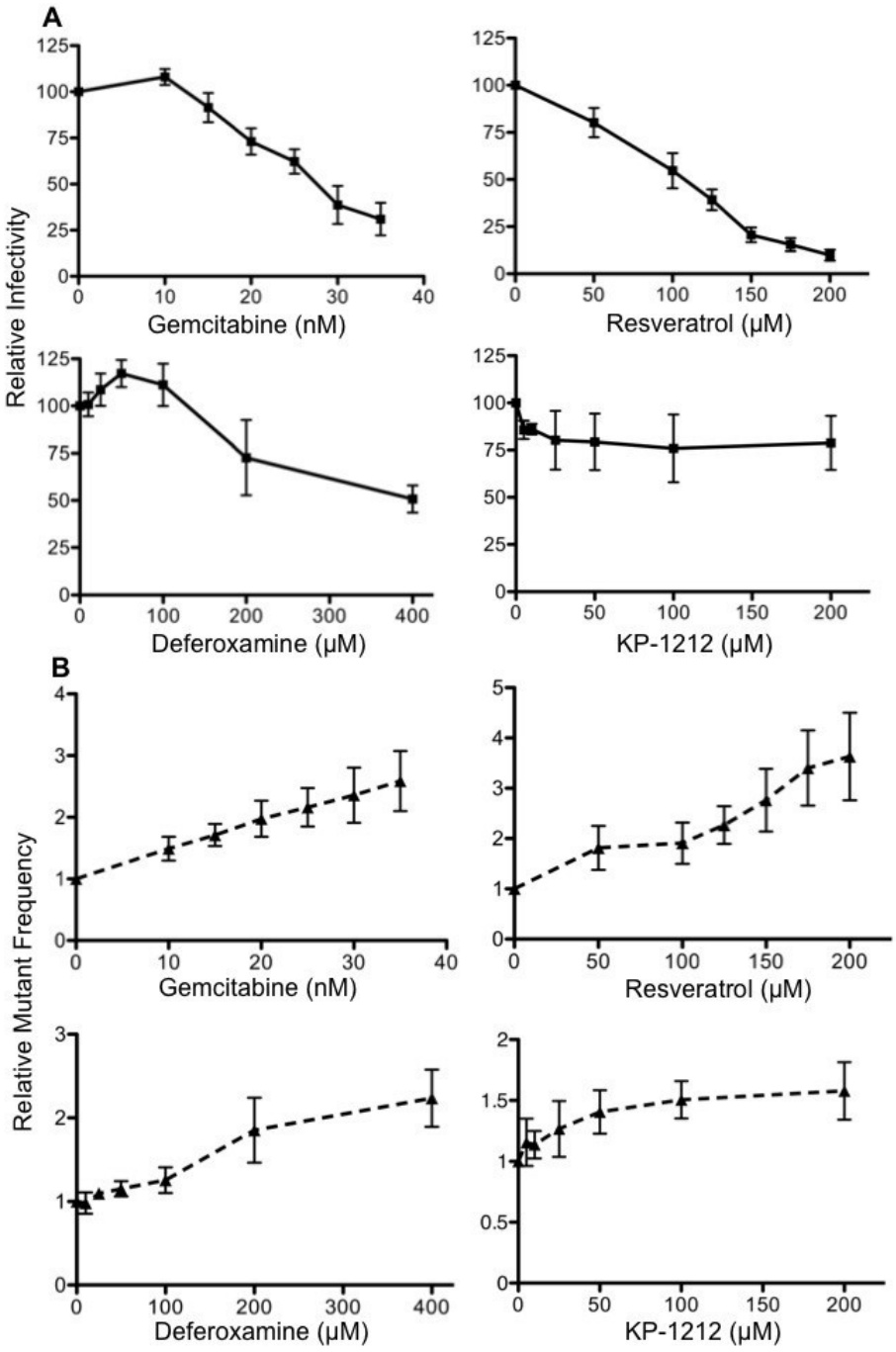

Figure VI-2 Effect of KP-1212 and RNRIs on HIV-1 infectivity and mutant frequency. Cells were infected with HIV-1 after being treated with KP-1212 or selected ribonucleotide reductase inhibitors (RNRIs) for 2 h. Treatments were concluded at 24 h post-infection by replacing the media, and cells were collected for analysis by flow cytometry at 72 h post-infection. The effects of the drugs on infectivity (A) and mutant frequency (B) were determined and normalized to the no drug control. The data shown are the mean ± sd of three independent experiments.

We observed that KP-1212 exerted only a mild antiviral effect alone, without concentration dependence in the concentration range of 5 to 200 μM (Figure VI-2A). However, we observed a slight dose-dependent increase in mutant frequency (up to ~1.6-fold) (Figure VI-2B). Our observations using this assay are somewhat different than previous findings of KP-1212 being a potent inhibitor of HIV-1 replication (Harris *et al.*, 2005). We tested multiple batches of KP-1212 to rule out the possibility of degradation of our liquid drug stocks (data not shown). Additionally, 1H NMR and combustion analysis confirmed the purity and stability of KP-1212 in our hands (data not shown). The differences in observed activity of KP-1212 may be due to the previous study using a very low multiplicity of infection (MOI), ranging from 1:1000 to 1:5000 (Harris *et al.*, 2005). These authors found that higher MOIs led to higher EC50 values, and were not able to obtain a reproducible EC50 at MOIs of 1:300 or greater. In line with this, we used a much higher MOI (~1:3 to 1:5) and were unable to obtain an EC50. Additionally, the previous study used a different cell line (MT-2) and centrifuged the drug onto cells, potentially altering the uptake and/or phosphorylation of KP-1212. Finally, the virus was likely not limited to a single round of replication, which potentially enhanced the observed effects of KP-1212. Despite the apparent discrepancies in antiretroviral potency, our findings are in relatively good agreement with the Phase II clinical trials on KP-1212, which found no clinically significant decline in viral loads but some evidence of mutagenicity

(increases in private mutations and altered mutation spectra) (Hicks *et al.*, 2013; Mullins *et al.*, 2011).

KP-1212 potentiates the effects of RNRIs on HIV-1 infectivity & mutant frequency

To further investigate the interaction between RNRIs and KP-1212, we combined a fixed low concentration of each RNRI with varying concentrations of KP-1212 (i.e., ranging from 5 to 200 μM). For the gemcitabine and KP-1212 drug combination, we observed an unexpected trend: low concentrations of KP-1212 (5 to 50 μM) enhanced the antiviral activity of gemcitabine, whereas high concentrations partially (100 μM) or fully (200 μM) antagonized the antiviral activity (Figure VI-3A). Similarly, the corresponding mutant frequencies increased to a maximum of 4.8-fold at 25 μM KP-1212 and then decreased to levels lower than for gemcitabine alone (Figure VI-3B). KP-1212 likely antagonized gemcitabine at high concentrations because they are both deoxycytidine analogs that require transport and metabolic conversion to their active forms. Specifically, they may utilize the same transporter proteins to breach the plasma membrane (either through facilitated diffusion or active transport), and they are both phosphorylated by deoxycytidine kinase to become active within cells (Heinemann *et al.*, 1988; Huang *et al.*, 1991; Mackey *et al.*, 1998; Murakami *et al.*, 2005). Furthermore, the drugs were simultaneously added to cells, and KP-1212 was used at a much higher concentration than gemcitabine (10,000-fold higher for 200 μM KP-1212). Our observation of potential drug

interference could likely be circumvented by 1) using a more potent mutagenic deoxycytidine analog, 2) using a mutagenic nucleoside analog that is not phosphorylated by deoxycytidine kinase, 3) applying the drugs sequentially rather than simultaneously, or 4) using an RNRI that is not a nucleoside analog.

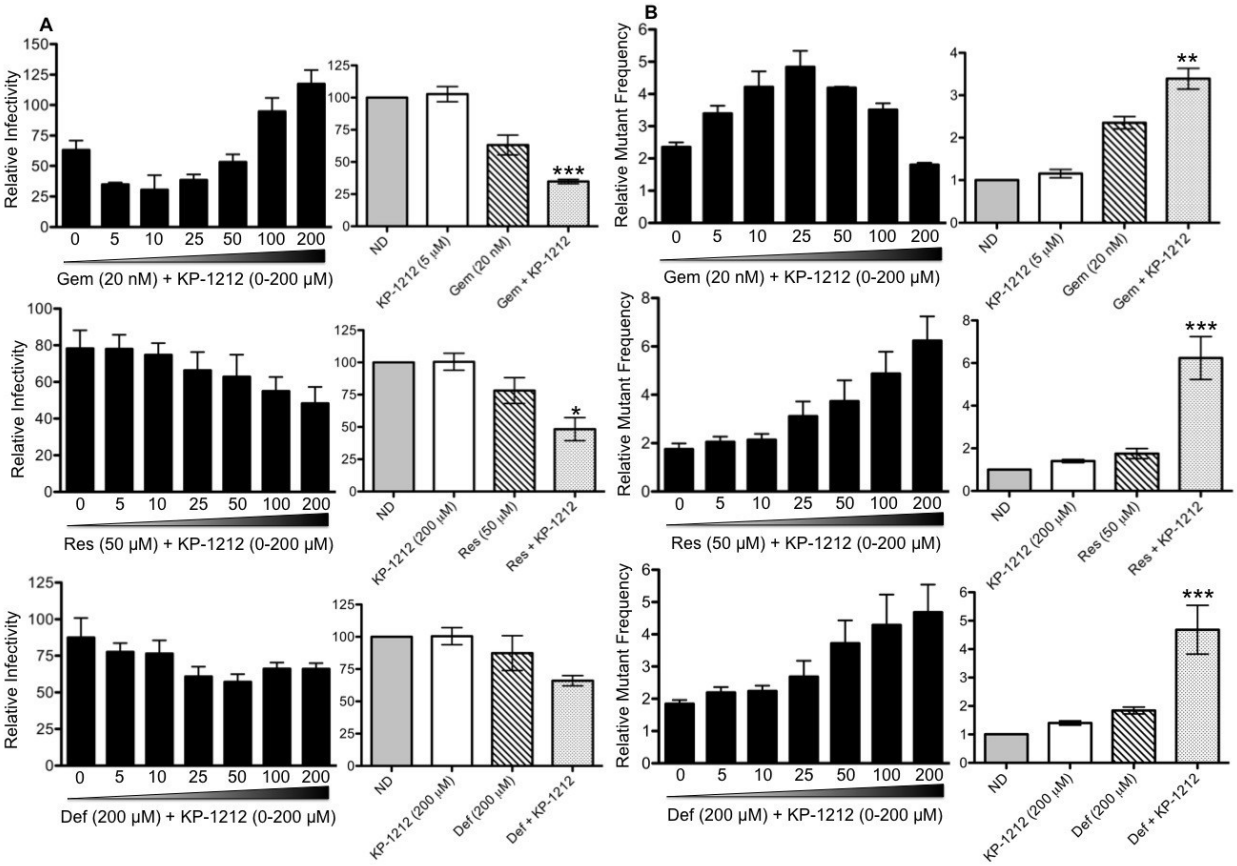

Figure VI-3 KP-1212 potentiates the antiviral and mutagenic activities of RNRIs against HIV-1. Cells were pre-treated with a set amount of each ribonucleotide reductase inhibitor (RNRI) and varying amounts of KP-1212 (from 0 to 200 μM) as described in Section 3.4. Flow cytometry was used to determine HIV-1 infectivity (A) and mutant frequency (B), which were plotted relative to no drug. Statistical significance of drug combinations relative to both single drugs was assessed using a two-way ANOVA. * p-value < 0.05, ** p-value < 0.01, *** p-value <0.001. The data shown represent the mean ± sd of three independent experiments.

We therefore examined the interactions of KP-1212 with two non-nucleoside RNRIs—resveratrol and deferoxamine. The addition of KP-1212 (5 to

200 μM) to resveratrol (50 μM) resulted in a concentration-dependent decline in viral infectivity, with the greatest potentiation observed at 200 μM KP-1212 (Figure VI-3A). We observed a similar, though less concentration-dependent, trend for the combination of KP-1212 with deferoxamine. For both of these combinations, we also observed increases in the viral mutant frequencies (to a maximum of 6.2-fold for resveratrol and 4.7-fold for deferoxamine relative to that of no drug) (Figure VI-3B). These results indicate, in contrast to gemcitabine, high concentrations of KP-1212 were superior compared of that of low concentrations for potentiating the antiviral activity of resveratrol and deferoxamine. The elevation of the viral mutant frequency by the drug combinations is consistent with an increase in virus mutational load as a major mechanism of antiretroviral activity.

KP-1212 potentiates the anti-HIV-1 activity of RNRIs without enhancing cytotoxicity

To further characterize these drug combinations, we investigated the extent to which KP-1212 can augment the potency of RNRIs. We found that the addition of KP-1212 at a fixed amount (5 μM for gemcitabine or 200 μM for resveratrol) enhanced the potency of both gemcitabine and resveratrol, as evidenced by the significant decrease in EC50 values upon addition of KP-1212 (Table VI-1). The greatest enhancement was observed for resveratrol, with the EC50 approximately halved. The effect was much less for gemcitabine, as only low concentrations of KP-1212 could be used due to antagonism. The effect of

KP-1212 in combination with deferoxamine was not statistically significant compared to deferoxamine alone. We then examined the effect of the drugs (alone and in combination) on cell viability in order to determine whether drug cytotoxicity could explain the antiviral effects observed. For gemcitabine, some degree of cytotoxicity was observed at all concentrations tested, but the CC50 was higher than the EC50 (62.0 μM vs. 27.5 nM), indicating that the antiviral activity of gemcitabine cannot be explained by cytotoxicity (Figure VI-4 & Table VI-1). We also performed combustion analysis to confirm high purity of drug stocks, and conducted a separate cytotoxicity assay (trypan blue staining) to confirm cytotoxicity results (data not shown). Taken together, we conclude that gemcitabine has low cytotoxicity in this cell line, possibly due to inactivation by rapid deamination of gemcitabine to its major metabolite, 2',2'-difluoro-2'-deoxyuridine (dFdU) (Abbruzzese *et al.*, 1991; Heinemann *et al.*, 1992). Unlike gemcitabine, KP-1212 was non-toxic up to 200 μM (data not shown), and the addition of KP-1212 (5 μM) did not alter the cytotoxicity of gemcitabine (Figure VI-4 & Table VI-1). These results indicate that enhanced cytotoxicity cannot explain the potentiation of the antiviral activity of gemcitabine by KP-1212. Similarly, the antiviral activity of resveratrol also could not be explained by cytotoxicity, as the CC50 was markedly higher than the EC50 (>400 μM; Figure VI-4 & Table VI-1). Furthermore, the addition of KP-1212 (200 μM) did not significantly alter the cytotoxicity of resveratrol. In summary, KP-1212 potentiates the antiviral activity of gemcitabine and resveratrol without

augmenting cytotoxicity, which improves their selectivity indices against HIV-1 (Table VI-1).

Table VI-1 KP-1212 improves the antiretroviral potency of RNRIs without altering cytotoxicity.

	- KP-1212			*+ KP-1212*		
Drug	**EC_{50}**	**CC_{50}**	**SI**	**EC_{50}**	**CC_{50}**	**SI**
Gemcitabine	27.5 nM (26.0-29.1)	62.0 μM (59.0-65.2)	2250	21.6 nM (20.9-22.3)	64.2 μM (60.8-67.8)	2970
Resveratrol	99.6 μM (92.5-107.4)	> 400 μM	> 4.0	52.8 μM (39.1-71.2)	> 400 μM	> 7.6

[a] The EC_{50} and CC_{50} values for ribonucleotide reductase inhibitors (RNRIs) in the presence or absence of KP-1212 (5 μM for gemcitabine; 200 μM for resveratrol/deferoxamine) in U373-MAGI cells were calculated as described in the Materials and Methods.

[b] Selectivity indices were determined by dividing the CC_{50} by the respective EC_{50}. The 95% confidence intervals are indicated in parentheses. The data shown represent the mean ± sd of at least three independent experiments.

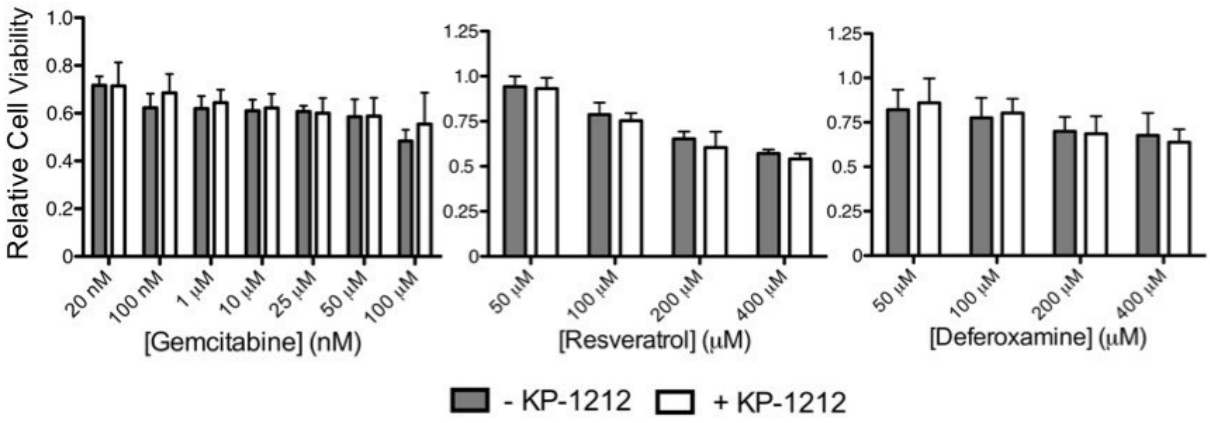

Figure VI-4 The ability of KP-1212 to potentiate RNRIs is not due to enhanced cytotoxicity. U373-MAGI cells were treated with ribonucleotide reductase inhibitors (RNRIs) ± KP-1212 (5 μM for gemcitabine, 200 μM for resveratrol and deferoxamine) for 24 h, then washed and replenished with fresh media. Cell viability was assessed 24 h after molecule removal using a luminescent ATP-based kit, and the data were normalized to the no drug control. The data shown are the mean ± sd of four independent experiments.

Characterization of viral mutation spectra in the presence of KP-1212 and RNRIs

To determine the types of mutations resulting from these drugs (i.e., individually and in combination), we sorted out mCherry+/GFP- cells infected in the presence or absence of drug. We then amplified and performed Sanger sequencing of the gfp gene. We chose not to include deferoxamine in this analysis due to its poor potency against HIV-1 (Table VI-1). We obtained a total of 376 unique mutations across all treatments, but several G-to-A hypermutants (likely a result of APOBEC3 activity) were excluded from further analysis (see "characterization of mutation spectra" in the materials and methods section for details), lowering the total number of mutations to 303. In the absence of drug, G-to-A mutations were the most common, followed by A-to-G, T-to-C, and C-to-T transitions, with the remainder representing transversions (Figure VI-5). In the

presence of only gemcitabine or resveratrol, no statistically significant ($p < 0.05$; 2-way Fisher's exact test) change in the relative appearance of each mutation type within the HIV-1 mutation spectrum was observed compared to the no drug control, suggesting that these drugs did not cause an increase in any particular mutation type. KP-1212, despite having only a modest ability to elevate the mutant frequency of HIV-1, caused a significant increase (p-value = 0.004) in the frequency of G-to-C mutations (from 1% to 22% of all mutations identified, relative to that of no drug), a transversion mutation rarely observed during HIV-1 replication. Strikingly, the combination of KP-1212 with resveratrol led to an even greater increase in the frequency of G-to-C mutations (up to 41% of all substitutions identified), which is significantly different from either resveratrol alone (p-value < 0.0001) or KP-1212 alone (p-value = 0.048). The combination of KP-1212 and gemcitabine did not result in a significant increase in the frequency of G-to-C mutations (p-value = 0.130), likely due to a lower concentration of KP-1212 used in this combination (i.e., 5 μM versus 200 μM) due to drug antagonism observed at higher concentrations of KP-1212.

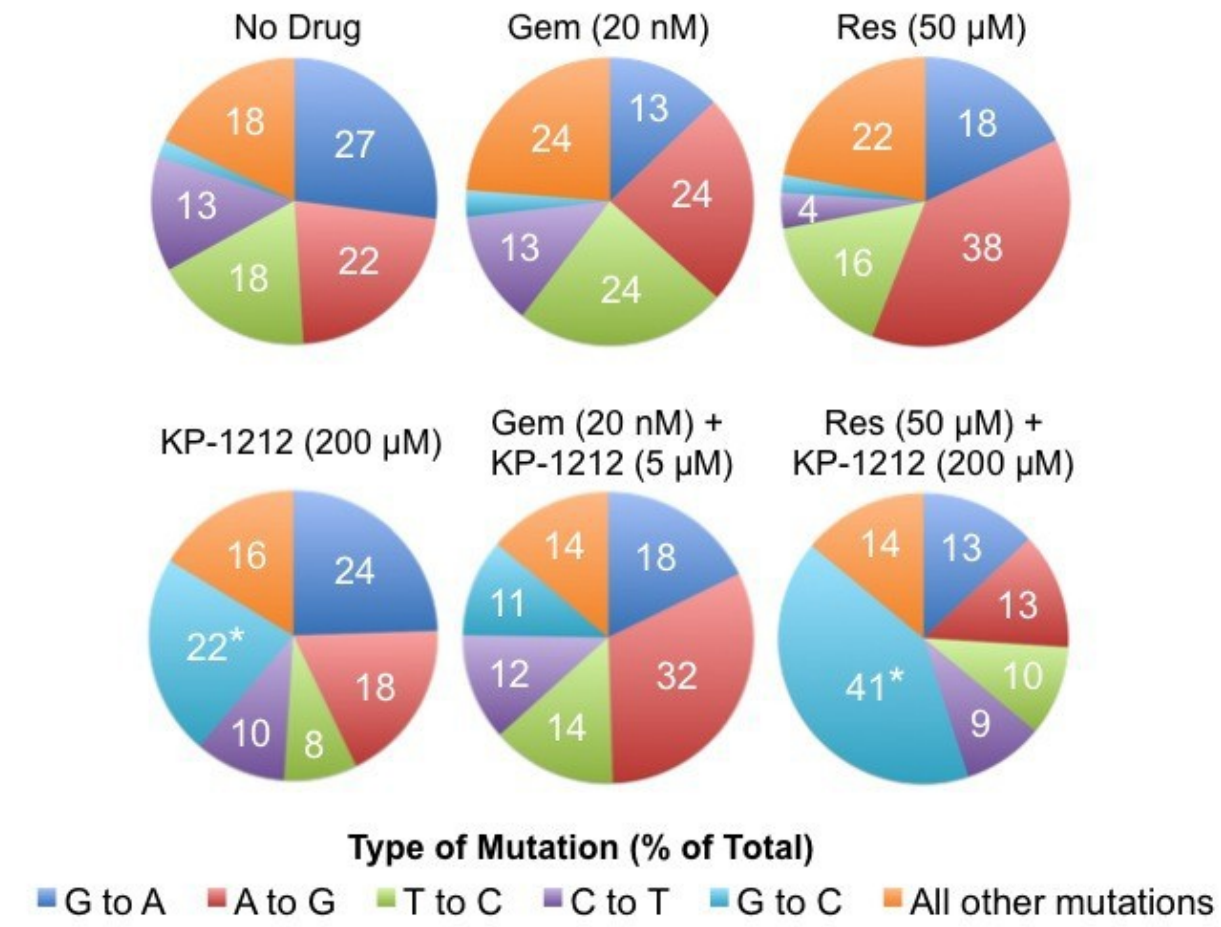

Figure VI-5 Characterization of viral mutation spectra in the presence of KP-1212 and RNRIs. U373-MAGI cells were pre-treated with KP-1212 and/or ribonucleotide reductase inhibitors (RNRIs) and infected with HIV-1. mCherry+/GFP- cells were sorted to enrich for mutants, and the *gfp* gene was amplified and sequenced. Each number in a wedge of a pie chart represents the specific corresponding mutational type, expressed as the percentage of the total mutations observed for that treatment. The total number of mutations sequenced were: no drug, 45, gemcitabine (Gem), 38; resveratrol (Res), 45; KP-1212, 49; gemcitabine + KP-1212, 57; resveratrol + KP-1212, 69 (303 mutations in total). Statistically significant (p-value < 0.05) changes relative to the no drug control are indicated with an asterisk.

KP-1212 has not been previously found to induce G-to-C transversions, but 5-aza-2'-deoxycytidine (decitabine) and its ribonucleoside counterpart (5-AZC), have been reported to cause G-to-C transversions in HIV-1 and cellular genomic DNA (Clouser *et al.*, 2010; Dapp *et al.*, 2009; Jackson-Grusby *et al.*, 1997). These molecules decompose in water at physiological temperature and

pH by hydrolytic opening and deformylation of the triazine ring (Beisler, 1978; Chan *et al.*, 1979; Lin *et al.*, 1981; Liu *et al.*, 2006; Rogstad *et al.*, 2009; Yoo *et al.*, 2007). The resulting ring-opened guanylurea derivatives are thought to pair with cytosine leading to G-to-C transversions (Jackson-Grusby *et al.*, 1997). It is unclear how KP-1212 might induce G-to-C transversions, as KP-1212 should be resistant to hydrolysis.

Conclusions

While KP-1212 exerted minimal antiviral and mutagenic activity against HIV-1 in our assay, it was able to enhance the antiviral activities of the RNRIs gemcitabine and resveratrol, leading to robust reductions in HIV-1 infectivity and improvement of their selectivity indices. We found that the combinations simultaneously elevated the mutant frequency of HIV-1, suggesting enhancement of virus mutational loads as a major mechanism of the drug combinations. While the RNRIs gemcitabine and resveratrol do not appear to cause a specific type of mutation, the addition of KP-1212 to resveratrol led to a pronounced increase in G-to-C transversion mutations. Further studies of the antiretroviral mechanisms of these combinations are warranted, as are efforts to identify prodrug forms and derivatives exhibiting reduced toxicity, improved potency, and prolonged stability. Our findings suggest that the enhancement of viral mutant frequency is a major contributor to these antiviral mechanisms, but do not preclude other activities – such as inhibition of reverse transcription. Resveratrol, while well-tolerated, has low bioavailability due to rapid and

extensive metabolism in a number of clinical trials (Patel *et al.*, 2011) but may serve as a lead for further development. It would also be of great interest to investigate the interplay of RNRIs and KP-1212 with conventional anti-HIV-1 inhibitors. Previous studies have shown that these inhibitors can accelerate mutagen-driven viral extinction. This has been demonstrated for HIV-1 using AZT and 5-hydroxy-2'-deoxycytidine in combination and in foot-and-mouth disease virus, in which case sequential treatments were superior to combination therapy (Perales *et al.*, 2009; Tapia *et al.*, 2005). Overall, our findings are the first to demonstrate that a mild anti-HIV-1 mutagen can significantly potentiate the antiretroviral activity of RNRIs. These observations may enhance the potential for clinical translation of KP-1212 for the treatment of HIV-1 infection.

Materials and methods

Plasmids, cell lines, and reagents

The molecular clone of HIV-1 we used to produce virus, pNL4-3 MIG, was created from pNL4-3 HIG, which has been previously described (Clouser *et al.*, 2010). The virus encoded by this plasmid contains mCherry, an internal ribosome entry site (IRES), and enhanced green fluorescent protein (GFP). We pseudotyped virions with VSV-G expressed from pHCMV-G, a kind gift from J. Burns (University of California, San Diego). The human embryonic kidney (HEK 293T) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) from Cellgro (Manassas, VA) with 10% HyClone FetalClone III (FC3) from Thermo

Scientific (Waltham, MA) and 1% penicillin/streptomycin from Invitrogen (Carlsbad, CA). U373-MAGI-CXCR4CEM cells were obtained from Michael Emerman through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Vodicka *et al.*, 1997). U373-MAGI cells were maintained similarly to 293T cells but with addition of 1.0 µg/mL puromycin, 0.1 mg/mL hygromycin B, and 0.2 mg/mL neomycin to the medium. The chemicals investigated were obtained from Sigma-Aldrich (deferoxamine and resveratrol; St. Louis, MO), Carbosynth (5-AZC and gemcitabine; Compton, UK), and Berry & Associates (KP-1212; Dexter, MI), dissolved in the appropriate solvent, and stored at -20 ºC. For cloning, PCR was performed using the Platinum PCR Supermix from Invitrogen, while restriction enzymes, Klenow fragment, and T4 DNA ligase were from New England Biolabs (Ipswich, MA). For transfections, poly-L-lysine was from Newcomer Supply (Middleton, WI), and polyethylenimine (PEI) was from Polysciences, Inc. (Warrington, PA).

Construction of an mCherry/GFP HIV-1 vector for rapid mutation detection

The mCherry gene was swapped with the mouse heat stable antigen (hsa) in pNL4-3 HIG to generate pNL4-3 MIG as follows. From NL4-3 HIG, an IRES-GFP fragment was amplified via PCR and cloned into a pEGFP-N1-based mCherry vector using NotI and XbaI. The NotI site was then destroyed by fill-in with large Klenow fragment. The entire MIG cassette was then amplified using primers that added a NotI site to the 5' end and a XhoI site to the 3' end of the cassette. The PCR product was directly digested with NotI and XhoI and ligated

into NotI and XhoI-digested pNL4-3 HIG using T4 DNA ligase. The resulting plasmid, pNL4-3 MIG, expresses mCherry and GFP as well as all viral proteins except Env and Nef.

Virus production and titering

Virus was produced by co-transfecting pNL4-3 MIG and pHCMV-G into 293T cells via the PEI method (Boussif *et al.*, 1995). PEI stocks were prepared at 1 mg/mL by dissolving PEI in water, adjusting the pH to 7.0, and filtering through a 0.2 μM filter. Stocks were divided into 1 mL aliquots and frozen at -80 ºC. On the day before transfection, 4 million 293T cells were plated on each 10 cm plate (pre-coated with poly-L-lysine) to be transfected. For each plate, 10 μg pNL4-3 MIG + 1 μg pHCMV-G + 33 μL 1 mg/mL PEI were mixed in a 1.6 mL microcentrifuge tube to a final volume of 1 mL using serum-free DMEM. After 20 minutes of incubation, the medium on the 293T cells was replaced and the DNA-PEI mixture was added. The medium was replaced the morning after transfection, and virus was collected ~48 hours post-transfection by filtering the supernatant through a 0.2 μm filter. Viral stocks were divided into 1 mL aliquots and frozen at -80 ºC.

Prior to any drug treatments, viral stocks were first titered in U373-MAGI cells. The day before infection, 62,500 cells/well were plated in 12-well plates. The next day the media was replaced and varying amounts of virus ranging from 1.25 to 40 μL were added. The media was replaced again 24 hours post-infection and cells were collected at 72 hours post-infection for analysis by flow

cytometry. The infectious titer was calculated from the flow data by adding all positive quadrants (mCherry+ only, GFP+ only, and mCherry+/GFP+) to determine infectivity and then plotting the volume of virus against infectivity.

Drug treatments and infections

Drug treatments were performed using U373-MAGI cells in 12-well plates similar to the way in which viral stocks were titered (Section 3.3). Two hours prior to infection, drugs (1-2 µL) were added to a final volume of 0.5 mL media in each well. Uninfected cells and no drug (DMSO only-treated cells) were included as controls. After two hours, virus and additional media were added, resulting in a final volume of 1 mL in each well. Drug concentrations were based on the final volume of 1 mL. The amount of virus added was targeted to achieve 15-30% infection (for the no drug control) based on the previously determined viral titer. Drugs were removed by replacing the media 24 hours post-infection. The cells were collected 72 hours post-infection for flow cytometry by treating with trypsin, pelleting at 500xg for 5 minutes, resuspending in 200 µL Dulbecco's Phosphate-Buffered Saline (DPBS) + 2% FC3, and transferring to 96-well plates.

Infectivity and mutant frequency determination by flow cytometry

Cells were analyzed for mCherry and GFP expression on a BD LSR II flow cytometer (BD Biosciences; San Jose, CA). Cells were first gated based on forward scatter and side scatter, and a minimum of 10,000 gated cells were analyzed per sample. GFP was excited with a blue 488 nm laser and emission detected using 505LP and 525/50 filters. mCherry was excited by a custom

green 561 nm laser and emission detected using 595LP and 610/20 filters. It should be noted that early on in our work we determined that very little fluorescent compensation was necessary with this particular combination of fluorophores, lasers, and filters (data not shown). These control experiments were performed by transfecting mCherry or GFP into 293T cells and then analyzing the transfected cells (either kept separately or mixed together) by flow cytometry. Flow cytometry data were examined in FlowJo v. 7.6.1 (Ashland, OR). Infectivity was determined by adding all three positive populations: mCherry+ only, GFP+ only, and mCherry+/GFP+. Mutant frequency was calculated by dividing the single positive populations (mCherry+ only and GFP+ only) by all infected cells. Double negative (mCherry-/GFP-) cells were excluded due to the difficulty in detecting these rare events; mutant frequency calculations are therefore underestimates. Infectivity and mutant frequency data were normalized to the no drug controls.

Cellular toxicity analysis

The cytotoxicity of drugs was assessed using the CellTiter-Glo Luminescent Cell Viability Assay from Promega (Madison, WI) following the manufacturer's instructions. 5000 U373-MAGI cells/well were plated in 96-well plates the day before drug treatment. The following day the medium was replaced and drugs (alone or in combination) were added to a final volume of 200 µL/well. No drug (DMSO only) and no cell wells were included as controls. The luminescence was recorded using an Orion microplate luminometer from

Berthold Detection Systems (Huntsville, AL). The data were analyzed by first subtracting the no cell control value and then normalizing to the no drug control.

Characterization of mutation spectra in HIV-1

Infected mCherry+/GFP- U373-MAGI cells were sorted at the Masonic Cancer Center at the University of Minnesota using a BD FACSAria II. A minimum of 3000 cells were collected per sample. Genomic DNA was isolated by the High Pure PCR Template kit from Roche (Indianapolis, IN). GFP was amplified in a single PCR reaction using the Platinum PCR Supermix from Invitrogen and the primers 5'-CAAGCGTATTCAACAAGG-3' and 5'-GCAATACAGCAGCTAACAATG-3'. The PCR product was ligated into pGEM-T (Promega) and the gfp gene was sequenced at Functional Biosciences, Inc. (Madison, WI) using the T7 promoter primer. Sequences were aligned to the reference and mutations identified using Lasergene (DNASTAR, Inc., Madison, WI). Duplicate mutations, presumably arising from PCR amplification, were excluded from analyses. Several G-to-A hypermutants (multiple G-to-A mutations within the same sequence) were observed, and these likely resulted from APOBEC3 activity, as they occurred in a GA or GG dinucleotide context and were present in both the no drug as well as drug treatment samples. These rare events were excluded from the analyses due to their potential for creating false differences in the rate of G-to-A mutations between samples.

Statistical analyses

All graphs were created in Microsoft Excel v 12.3.5 (Redmond, WA) or GraphPad Prism v 5.0 (GraphPad Software, Inc.; La Jolla, CA). EC_{50} values of drugs (drug concentration at which there is a 50% reduction in viral infectivity) were determined by plotting normalized infectivity against the log-transformed drug concentrations and fitting a non-linear regression curve to the data. Likewise, CC_{50} values of drugs (drug concentration at which there is 50% cytotoxicity) were determined by plotting normalized cell viability against the log-transformed drug concentrations and fitting a non-linear regression curve to the data. Two-way ANOVA was performed using the statistical package R to compare the impact of drug combinations against single drugs on infectivity and mutant frequency. All analyses were performed on the log-transformed data without normalization. Differences in mutation spectra were assessed using Fisher's exact test in GraphPad Prism.

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