Studies of feline leukemia virus drug susceptibility and antiviral mechanism of action

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Dedication

To my parents Willie Mayfield and Mary Eliza Greggs Jr., whose encouragement and lessons in personal discipline has taught me that the greatest things have simple beginnings.

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Abstract

Antiretroviral drugs have saved and extended the lives of millions of individuals infected with human immunodeficiency virus type 1 (HIV-1). The major classes of anti-HIV-1 drugs include reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors and entry/fusion inhibitors. While antiretroviral drug regimens are commonly used to treat other types of retroviral infections, there are instances where there is a perceived need for re-evaluation of the benefits of new antiretroviral therapy. One case in point is that of feline leukemia virus (FeLV), an infection of domesticated felines. While vaccines exist to prevent FeLV infection and spread, they have not eliminated FeLV infection. For FeLV-infected felines and their human companions, antiretroviral therapy would be desirable and of practical importance if good options were available.

The goal of this dissertation was to 1) determine the susceptibility of FeLV to drugs that could be amendable to clinical translation, and 2) explore the anti-FeLV mechanism of action of these drugs. FeLV was found to be susceptible to two anticancer drugs (i.e., decitabine and gemcitabine) as well as two anti-HIV-1 drugs (raltegravir and tenofovir). FeLV, but not HIV-1, was also found to be susceptible to cyclopentenyl cytosine. Mechanism of action studies suggested that decitabine and gemcitabine did not enhance FeLV mutagenesis, which is contrary to previous observations of enhanced HIV-1 mutagenesis observed with these drugs. Cyclopentenyl cytosine did not enhance viral mutagenesis, was observed to reduce dCTP levels in the Crandell-Rees feline kidney cell line, and

FeLV susceptibility to cyclopentenyl cytosine was enhanced by a mutation in a conserved region of reverse transcriptase. These studies 1) support the further exploration of the clinical translation of decitabine, gemcitabine and cyclopentenyl cytosine for the treatment of FeLV infection, and 2) suggest differences in the antiviral mechanisms of action of decitabine, gemcitabine and cyclopentenyl cytosine between FeLV and HIV-1.

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List of Acronyms

CPEC – cyclopentenyl cytosine
U373-MAGI – HIV-1 indicator cell line expressing beta-galactosidase gene under control of HIV-1 long terminal repeat; cells express CD4 linked to neo^r and $CXCR_4$ expression linked to $hygro^r$
CRFK – Crandell-Rees feline kidney cell line
GFP - green fluorescent protein
YVDD – tyrosine-valine-aspartic acid-aspartic acid conserved protein sequence in reverse transcriptase
YMDD – tyrosine-methionine-aspartic acid-aspartic acid conserved protein sequence in reverse transcriptase
3TC – lamivudine, 2',3'-dideoxy-3'-thiacytidine
DMSO – dimethyl sulfoxide
LCMS-MA – liquid chromatography mass spectrometry-tandem mass spectrometry

List of Publications

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CHAPTER I

GENERAL INTRODUCTION:

Broadening the use of antiretroviral therapy: the case for feline leukemia virus

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Background

Retroviruses are a large group of RNA viruses that are found in all vertebrates. They share many common features, such as similarities in genetic organization and mechanism of replication – and in particular for their encoding for a reverse transcriptase. These viruses are a significant source of morbidity and mortality in both humans and animals. In humans, human immunodeficiency virus (HIV) is responsible for a pandemic that continues to be a significant cause of morbidity and mortality. In animals, feline leukemia virus (FeLV) represents a significant source of fetal mortality and cancerous diseases in cats. Besides its role in feline mortality, FeLV has played an important role in advancing the understanding of retroviruses in general. In fact, the characterization of FeLV as well as other animal retroviruses such as bovine leukemia virus and Rous sarcoma virus, led to the concepts and techniques that later enabled the discovery and characterization of human retroviruses including HIV(Levy 1993). Additionally, FeLV was 1) the first retrovirus in which a vaccine was developed, 2) the first retrovirus for which a practical diagnostic test was developed, and 3) the first retrovirus that elicited the development of a program whose goal was to control its spread (Levy 1993). Despite the significant achievements and understanding of FeLV biology, FeLV is still a significant source of morbidity and mortality in felines and the treatment options for infected cats are ineffective, toxic, or cost-prohibitive. Here, we discuss FeLV epidemiology, pathogenicity,

and current treatments as well as future drug targets that may advance the field of FeLV treatment.

Epidemiology

FeLV is highly transmissible through saliva and nasal secretions as well as through coitus and vertical transmission from queen to kitten. Because the primary mode of transmission is through the oronasal route, its prevalence is often dictated by the extent of animal-to-animal contact. For example, the high prevalence of FeLV in the stray cat population (43%) increases the risk of infection for indoor-outdoor cats compared to indoor only cats. Similarly, animal-to-animal contact contributes to the different rates of FeLV infection in single cat households (4-11%) compared to multicat households whose prevalence has been reported to be as high as 70% (Rogerson, Jarrett et al. 1975; Ettinger 2005; Côté 2007).

Containment of FeLV is difficult due to transmission routes, the time between infection and the onset of symptoms, and the ability of latently-infected cats to become viremic. In fact, exposure of cats to FeLV usually leads to one of three outcomes, two of which can contribute to the spread of disease (Essex, Cotter et al. 1973; Rogerson, Jarrett et al. 1975; Sparkes 1997; Ettinger 2005; Côté 2007; Levy, Crawford et al. 2008; Lutz, Addie et al. 2009). The first outcome is accounted for by the 10% of exposed cats that become latently infected without a detectable viremia. While these cats would not seem to be a

source of infection, they can become viremic, and subsequently shed virus into the environment. The second outcome is represented by 40% of cats exposed to FeLV, and is characterized by persistent viremia and antigenemia. These cats are chronically infected, and therefore represent a significant source of viral shedding in the environment. The third outcome includes cats that become infected with FeLV, but are then able to clear the virus to the point where it is undetectable by standard testing methods. Approximately 50% of cats exposed to FeLV fall into this last group and these cats are not considered to be a reservoir for viral spread (Ettinger 2005; Côté 2007).

FeLV replication

FeLV was first described by Jarrett et al in 1964 who isolated viral particles from lymphomas obtained from infected cats (Jarrett, Crawford et al. 1964). Using electron microscopy, Jarrett et al described the infectious agent as being similar in appearance to murine leukemia virus (MuLV). Later studies confirmed FeLV to be a retrovirus. FeLV is a “simple” retrovirus, in that it encodes for three genes common to all retroviruses (*gag*, *pol*, and *env*), but lacks many of the additional genes found in complex retroviruses such as HIV (Chen, Bechtel et al. 1998; Levy, Crawford et al. 2008; Lutz, Addie et al. 2009). As with other retroviruses, the *gag* gene encodes for structural proteins while the *pol* gene encodes for the enzymatic proteins necessary for reverse transcription of the FeLV genome, integration of its DNA into the host genome, and processing

of viral proteins. Finally, *env* encodes for two envelope proteins that determine cellular tropism, including p15E, a transmembrane protein, and the associated external envelope protein, gp70 (Levy 1993). Amino acid variation in the virus envelope protein has led to the division of FeLV into four different subtypes that defines their cell tropism: A, B, C and T (Table 1-1) (Sarma and Log 1973; Jarrett, Hardy et al. 1978; Jarrett 1980). Subtype A is considered to be the founder, transmitted form of FeLV with all other subtypes arising through mutations in FeLV A Env or by recombination events with one of the endogenous FeLVs (enFeLV) contained within the cat genome (Levy 1993; Kahn 2005). The enFeLV has an incomplete genome, is not replication competent, and is theorized to have originated hundreds of thousands of years ago when a cat ate a mouse that was viremic with murine leukemia virus (MuLV). Such an event enabled the incorporation of the MuLV genome into the genome of the cat's germ line cells (Benveniste and Todaro 1973; Benveniste, Sherr et al. 1975).

Cell-free FeLV gains entry into target cells when the envelope protein binds to the appropriate host receptor that is dependent on the FeLV subtype (Table 1-1). Once inside of the target cell, the RNA genome is reverse transcribed into viral DNA by the viral protein reverse transcriptase (Coffin 1979; Coffin 1992; Coffin 1992; Coffin 1996). The viral DNA is then transported into the nucleus where it integrates into the host genome through the enzymatic action of viral integrase. The integrated viral DNA is then transcribed to produce RNA that serves as both viral progeny as well as mRNA for the translation of viral

proteins. Translated Gag and Pol proteins are then trafficked to the cell membrane where the new virions bud from the cell membrane. As the virion buds, proteolytic cleavage of viral proteins causes structural changes in the virion that are necessary for viral maturation and the formation of infectious virus particles (Coffin 1979; Coffin 1992; Coffin 1992; Coffin 1996).

Pathobiology

FeLV usually enters the feline host through the oronasal route either through mutual grooming, biting, or a shared food source (Rickard, Post et al. 1969; Essex, Klein et al. 1971; Cattori, Tandon et al. 2009). In the pharynx, FeLV infects the tonsorial B-lymphocytes and monocytes which can enter the draining lymph nodes (Rojko JL 1979). The draining lymph nodes serve as a site of replication and as an entry point for the virus to enter the bloodstream. Once in the bloodstream, the virus can gain access to and infect cells in the bone marrow (Levy 1993). This represents a critical point in the infection process as it is thought that a persistent infection can be avoided if the immune system can mount an appropriate response before cells in the bone marrow are infected (Rojko, Hoover et al. 1979). Once the virus becomes systemic, it infects epithelial cells in the intestines, stomach, trachea and salivary glands and becomes shed into the environment. Persistently-infected cats can demonstrate symptoms of disease anywhere from weeks to years after infection. FeLV-

mediated disease typically falls into one of two major categories – cytoproliferative or immunosuppressive (Levy 1993).

Cytoproliferative diseases associated with FeLV include leukemias, lymphomas, fibrosarcomas, and associated myeloproliferative disorders (Hardy, Hess et al. 1976; Ettinger SF 2004). Most cytoproliferative diseases are attributed to insertional mutagenesis, a process where FeLV DNA integrates at a site in the cat's genome that disrupts or deregulates expression of proteins involved in the regulation of cell cycle, cell survival or apoptosis (Levy, Lobelle-Rich et al. 1993; Tsujimoto, Fulton et al. 1993; Levy, Starkey et al. 1997; Fujino, Satoh et al. 2003; Fujino, Ohno et al. 2008; Fujino, Liao et al. 2009). In contrast, FeLV plays an indirect role in the formation of feline fibrosarcoma. Specifically, fibrosarcoma is caused by dual infection by both FeLV and feline sarcoma virus (FSV). FSV is a replication-defective virus that encodes an oncogene that drives cellular transformation (Snyder and Theilen 1969; Naharro, Dunn et al. 1983; Naharro, Tronick et al. 1983; Naharro, Robbins et al. 1984). Therefore, FeLV serves as a helper virus by providing FSV with proteins that are necessary for its replication, which allows for expression of the FSV oncogene (Snyder and Theilen 1969; Naharro, Dunn et al. 1983; Naharro, Tronick et al. 1983; Naharro, Robbins et al. 1984).

Besides having cytoproliferative effects, FeLV also mediates a significant loss of immune function. This immune suppression is due to a progressive loss of T and B lymphocytes as well as neutrophils. Immune suppression leads to

secondary infections such as bacterial or fungal infections that would not be a significant source of morbidity in an otherwise healthy cat (Perryman, Hoover et al. 1972; Hoover, Perryman et al. 1973; Hardy, McClelland et al. 1981; Trainin, Wernicke et al. 1983). Although the exact mechanism of FeLV-mediated immunosuppression is not clear, evidence supports three distinct mechanisms of action. First, immune suppression may be a result of FeLV-mediated myeloproliferative disorder (Kahn 2005; Côté 2007). This disorder leads to an over-proliferation of incompetent mature or immature white blood cells within the bone marrow that eventually overcrowd hematopoietic cells, thereby decreasing red blood cell production and leading to a hindrance of the immune system. Second, the virus may be cytopathic or induce cellular apoptosis, although most evidence indicates that this may be specific for FeLV subtype T. Third, it has been suggested that the transmembrane envelope protein, p15E, may have immunosuppressive properties (Hebebrand, Olsen et al. 1979; Mathes, Olsen et al. 1979; Lafrado, Lewis et al. 1987; Quackenbush, Mullins et al. 1989). For example, p15E has been reported to inhibit production of mitogenic lymphokines in T cells and has been shown to inhibit lymphocyte function without affecting receptor function (Mathes, Olsen et al. 1979; Lafrado, Lewis et al. 1987). Additionally, while p15E is not known to be cytotoxic, lymphocyte populations that are exposed to FeLV demonstrate a decline in size (Hebebrand, Olsen et al. 1979; Mathes, Olsen et al. 1979; Lafrado, Lewis et al. 1987; Quackenbush, Mullins et al. 1989). FeLV-mediated immunosuppression allows for secondary

infections such as bacterial, parasitic and other viral infections. One example includes the blood borne parasitic infection, hemobartonellosis, which is seen with the subgroup C infections and results in anemia with a hemolytic aspect (Levy 1993).

Other diseases are associated with FeLV, however the mechanisms remain unclear. For example, neurological diseases and infertility are seen in FeLV-infected cats, though it is not clear how FeLV replication causes these disorders. Also, FeLV-C is known to cause a non-regenerative aplastic anemia. While the mechanism is not clear, it has been suggested that the anemia may be due to FeLV's use of the heme export receptor, FLVCR1, leading to a toxic accumulation of heme in erythroid progenitor cells and decreasing their numbers (Tailor, Willett et al. 1999; Quigley, Burns et al. 2000). Figure 1-1 provides a summary of the FeLV pathogenesis.

Therapeutics: vaccines and limitations

Besides containment of infected cats, commercially available vaccines for FeLV such as Pfizer's Leukocell® or Merial's Purevax® are marketed and may significantly reduce FeLV spread and viral reservoir development. These particular vaccines are derived from chemically-inactivated antigens or are of recombinant viral origin, respectively, and do not offer sterilizing immunity (i.e., these vaccines essentially prime the cat's immune system enabling it to clear the virus upon exposure) (Sparkes 1997; Sparkes 2003; Hofmann-Lehmann, Tandon

et al. 2006). The American Association of Feline Practitioners reserves the vaccination regimen for high risk populations such as indoor-outdoor cats and catteries rather than including it as part of the core vaccines.

There are several reasons why these FeLV vaccines are likely reserved strictly for the high-risk populations. The link between FeLV vaccine and feline sarcoma is likely to play an important role in why these vaccines are not part of the core vaccines. Vaccine-associated sarcoma (VAS) is an aggressive malignant cancer, which requires aggressive surgery and often chemotherapy to treat (Madewell, Gieger et al. 2004; Kahn 2005; Côté 2007). The association of VAS is a significant reason why many owners and breeders choose not to vaccinate their cats against FeLV. A second reason why the FeLV vaccine is not part of the core vaccines is that a "closed" population of indoor only cats, meaning no introduction or contract with new cats, is not susceptible to FeLV, making the risk of VAS greater than the risk of infection. A third factor that might limit the use of the FeLV vaccine is that its efficacy is still not known and is difficult to determine. Further complicating the issue is that the biological response to FeLV exposure differs significantly among cats. For example, 50% of cats clear the virus, 40% become persistently infected and another 10% become latently infected. Finally, efficacy may need to be determined by using age-matched groups given that younger cats are more susceptible to a pathogenic infection compared to older individuals (Flynn, Hanlon et al. 2000; Kahn 2005; Côté 2007). Analyzing the results from studies that have examined

efficacy is difficult because of the differences in the study design, viral strain used, and age at which cats were challenged with virus.

Many owners choose not to vaccinate their cats for fear of VAS and because the efficacy of the vaccine is not clear. The unvaccinated cat population, as well as stray cat population remains at risk for FeLV and is a significant source for FeLV transmission. The prevalence of FeLV in the cat population is evident in veterinary care where there is a significant demand for the treatment of FeLV.

Therapeutics: antiviral drug targets and opportunities

The lack of effective treatment options leads most owners to choose palliative care for FeLV-infected cats. Palliative care may include medications to treat infections, pain management, nutritional support, or any other care with the goal of keeping the cats comfortable and improving their quality of life.

Although some antivirals and immune modulators have been reported to improve the quality or quantity of life for FeLV infected cats, no studies have convincingly shown that any antivirals or immune modulators actually improve the quality or quantity of life for FeLV-infected felines in a clinically useful form. Among antivirals used to treat FeLV, azidothymidine (AZT) was the first antiretroviral used to treat HIV. AZT is a nucleoside analog that gets incorporated into the viral DNA during reverse transcription of the RNA genome to double-stranded DNA. AZT lacks the 3'-hydroxyl group necessary for DNA

polymerization, which results in chain termination of viral DNA synthesis. Although AZT is the primary antiviral used clinically to treat FeLV, there is little, if any, literature to support its ability to improve the course of the disease once infection is established. Some studies have suggested that treatment of cats 24 hours prior to infection up until 24 hours after infection, might delay or minimize infection (Tavares, Roneker et al. 1987; Nelson, Sellon et al. 1995), but there have been no well-designed studies to address the efficacy of AZT to prolong the lives of cats with established infection. The studies that indicate that AZT might be efficacious demonstrated that even minor improvements in disease indicators were associated with drug-related toxicities. For example, animals treated with doses of 30 and 60 mg/kg/day had elevated antibody titers suggesting that their immune system was better able to respond to infection, although all animals receiving these doses demonstrated drug related toxicities. (Haschek, Weigel et al. 1990) Thus, the main limitation for the use of AZT is its lack of apparent efficacy at tolerable doses.

Similar to antivirals, studies reporting the efficacy of immune modulators have small numbers, lack of appropriate controls, and/or have not been independently verified. One of the immune modulators with little to no support for efficacy is lymphocyte T-cell immune modulator (LTCI). LTCI is a protein produced by a thymic stromal epithelial cell line whose manufacturers claim that this protein induces cytokines that activate CD8 cytotoxic T cells to attack virally-infected cells. It is also claimed that LTCI led to clinical improvement in FeLV-

infected cats. These claims are not documented in the peer-reviewed literature and the data supplied by the manufacturers do not argue strongly for improvements that are likely to improve the quality of life or length of life for FeLV infected animals (Gingerich 2008).

Another immune modulator, inactivated parapoxvirus ovis, strain D1701 (Baypamun®, Bayer, Leverkusen, Germany), is reported to non-specifically activate the immune system and improve or cure FeLV infected cats. Baypamun is a preparation of chemically that is reported to increase neutrophil counts and increase the production of interferon, interleukins, and tumor necrosis factor. While, initial reports by Horber and Mayr reported that Baypamun cured 80 to 100% of FeLV infected cats (Hörber D 1992; Mayr B 1992), numerous independent studies have failed to find a difference in the clinical response between cats treated with Baypamun and those treated with placebo.(Hartmann, Block et al. 1999)

SPA is perhaps the only immune modulator with evidence to support its ability to improve FeLV infected cats. SPA is a bacterial polypeptide purified from the cell walls of *Staphylococcus aureus* Cowan I. Although its mechanism of action is not clear, it has been shown to bind preferentially to IgG in the form of an immune complex rather than its monomeric form. It has been speculated that SPA may bind to IgG that is bound to a “blocking factor” associated with antigen-antibody complexes and that this blocking factor may facilitate a tumor or viruses escape from immunological control. Therefore, SPA removes antigen-antibody-

blocking factor complexes, allowing the immune system to react to viral invasion. Others have suggested that SPA may stimulate the immune system by inducing antibody synthesis, expression of interferon, and by potentiating the natural killer activity of lymphocytes. Although some studies have shown that SPA can improve the life expectancy of FeLV-infected cats, the treatment regimens described in these studies are too cost prohibitive and demanding to be clinically useful (Day, Engelman et al. 1984). For example, the treatment regimens required whole body irradiation followed by treatments given twice weekly. Additionally, it took 14 to 45 treatments to clear the virus and even this many treatments did not elicit a response from all of the cats (Operario, Reynolds et al. 2005). Given the conflicting results on treatment efficacy in addition to the cost and time associated with the treatments, SPA is not a clinically feasible treatment option.

Treatment options, drug targets, and the need for FeLV-based antiretrovirals

The significant morbidity and mortality associated with FeLV combined with the significant knowledge of FeLV biology and availability of antiretrovirals developed for HIV should facilitate the identification and development of new treatment options for FeLV. Perhaps one of the main problems with treating FeLV is that early treatment is almost a prerequisite for success. Since insertional mutagenesis is responsible for FeLV-mediated lymphomas and

leukemias, a quick decrease in viral loads would ensure fewer integration events thereby decreasing the likelihood of oncogenesis. Given the success in anti-HIV drugs at quickly decreasing viral loads, it is reasonable to assume that some of these drugs could be repositioned for the treatment of FeLV. The most likely classes of anti-HIV drugs that could be repositioned include the nucleoside reverse transcriptase inhibitors (NRTIs). Many of the other classes of anti-HIV drugs are structure-based small molecule inhibitors and would therefore be unlikely to possess anti-FeLV activities. Although there are a number of NRTIs that could be repositioned to treat FeLV, in *vitro* and *in vivo* studies indicate that NRTIs may not be readily repositioned to treat FeLV infections. Biochemical studies have shown that reverse transcriptase (RT) from oncoretroviruses such as FeLV have a higher fidelity and a significantly lower susceptibility to certain nucleoside analogs when compared to lentiviruses.(Day, Engelman et al. 1984) The differences in susceptibility to nucleoside analogs between lentiviral RTs and oncoretroviral RTs suggest that nucleoside analogs used to treat HIV will have different susceptibilities for FeLV.

Conclusion

In human medicine, improvement in treatment options is driven by the demand of those affected by the disease. A prominent example of this demand and supply is seen with conditions like cancer and HIV, where the impact on society fueled the funding and motivation necessary to create a rapid expansion

of treatment options in a short time. Although diseases of companion animals do not have the same effect on society as those that affect humans, there is an increasing awareness of the benefits of companion animals in improving the health and well-being of human and a trend for demanding better treatment options at whatever cost. Antiretroviral drugs and molecular tools are readily available to determine if drugs used to treat HIV could be repositioned to treat FeLV. An important factor in assessing and instituting these potential treatments is adequate funding of well-designed studies that have sufficient numbers and controls to clearly define the treatment's efficacy and potential toxicity.

The goal of this dissertation is to identify and characterize compounds that may be useful in the treatment of FeLV. To do this, a FeLV vector system was used to identify compounds with anti-FeLV activity. Any compounds that demonstrated anti-FeLV activity were further characterized for the antiviral mechanism of action. Since some of the compounds identified also demonstrate anti-HIV activity, comparative studies were performed to compare the anti-HIV and anti-FeLV activities. The results described in this dissertation should help lead to the development of novel drugs that can be used to treat FeLV infection in domestic cats.

Table 1-1 Description of feline leukemia virus (FeLV) subtypes, their tropism, and prevalence in infected cats.

FeLV subtypes[a]	Tropism	Receptor used for entry	Prevalence in infected cats	Others
A	Kidney, liver, T-cells, small intestine	THTR1 (thiamine transport protein)	100%	Considered original transmitted FeLV
B	Wide range of tissues	Pit 1 and Pit 2	Occurs with FeLV A	Arose through recombination of FeLV A and endogenous sequences
C	Erythroid progenitor cells	FLVCR1 (heme exporting protein)	1%	Arose through mutation in FeLV A env
T	T cells	Pit 1 in combination with co-receptor, FeLIX	Unknown	Arose from evolution of FeLV A (mutation and recombination)

Notes: [a]The four subtypes of FeLV arose through mutation and recombination. Each subtype uses different host receptors for cell entry, resulting in different tissue tropism. For more information on FeLV subtypes, see text and references therein, pages 5 -9.

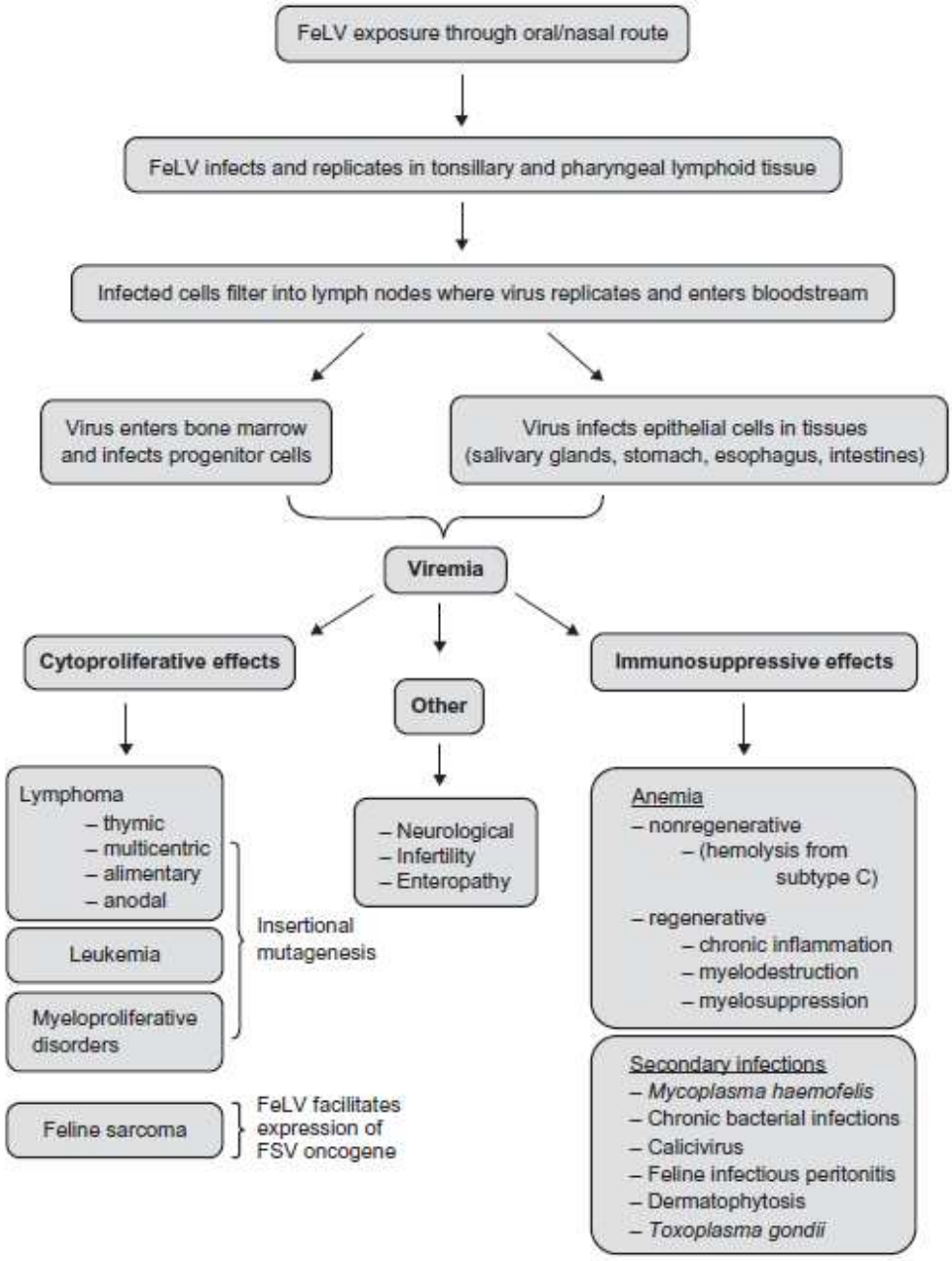


Figure 1-1. Pathogenesis of FeLV showing common entry route of virus, sites of viral replication and pathological consequences of infection.

Chapter II

Discovery of drugs that possess activity against feline leukemia virus

Reprinted with permission from: Greggs WM III, Clouser CL, Patterson SE, Mansky LM. Discover of drugs that possess activity against feline leukemia virus. Journal of General Virology. 2012; 93:900-905. Epub 2012 Jan 18.

Introduction

Feline leukemia virus (FeLV) is a highly transmissible retrovirus that causes significant morbidity and mortality in felids worldwide. FeLV-infected cats succumb to diseases such as leukemias and lymphomas as well as secondary infections related to FeLV-induced immune suppression. Infected cats transmit the virus through saliva, nasal secretions, coitus, as well as vertically from queen to kitten (Rickard, Post et al. 1969; Essex, Klein et al. 1971; Cattori, Tandon et al. 2009). These routes of transmission lead to a high prevalence of infected cats, especially in the stray cat population where the prevalence may be as high as 43% (Rogerson, Jarrett et al. 1975). Despite its prevalence and its associated morbidity and mortality, there are limited treatment options for FeLV infection. Currently, AZT is the only antiviral drug routinely used to treat FeLV, but its use is associated with significant side effects (Haschek, Weigel et al. 1990) including aplastic manifestations of FeLV. Besides AZT, immunomodulators are used as a treatment for FeLV, however, these drugs are often cost-prohibitive and the efficacy of these drugs is limited (Tavares, Roneker et al. 1987; Hartmann, Block et al. 1998; Gingerich 2008). Due to the lack of effective treatment options, many owners choose to euthanize or provide palliative care to infected cats.

Among retroviruses, HIV-1 is the most extensively studied in terms of drug development. According to the FDA, there are approximately 25 drugs currently

approved for the treatment of HIV-1. Although HIV-1 is a lentivirus and FeLV is a gammaretrovirus, there are enough similarities between the mechanisms of replication of the two viruses to indicate that anti-HIV-1 drugs may also inhibit replication of FeLV.

In order to expand the treatment options for feline leukemia, here we describe the anti-FeLV activity of four FDA-approved drugs: tenofovir, raltegravir, decitabine, and gemcitabine. We hypothesize that While tenofovir and raltegravir are FDA approved for AIDS chemotherapy, decitabine (5-aza-2'-deoxycytidine) and gemcitabine (2',2'-difluoro-2'-deoxycytidine), shown in Figure 3-1, are cytidine analogs used for the treatment of myelodysplastic syndrome (Garcia, Jain et al. 2010) and pancreatic cancer (Cerqueira, Fernandes et al. 2007; Wang, Lohman et al. 2009), respectively. We recently demonstrated the ability of decitabine and gemcitabine to inhibit HIV-1 replication (Clouser, Patterson et al. 2010; Clouser, Holtz et al. 2011). In addition, recent studies have shown that tenofovir and raltegravir exhibit antiviral activity against, a related gammaretrovirus xenotropic murine leukemia related virus (XMRV) (Paprotka, Venkatachari et al. ; Singh, Gorzynski et al. ; Smith, Gottlieb et al.). Here we show that these drugs also inhibit FeLV replication in cell culture.

Results

Validation of single cycle infectivity assay using GFP-tagged FeLV. A single cycle assay was used to examine the potential anti-FeLV activity of decitabine, gemcitabine, raltegravir, and tenofovir. The relevance of this model is to act as a "first pass" assay to identify potential agents with anti-FeLV activity. To do this, a FeLV construct was designed to express GFP from an internal ribosomal entry site (Schafer and Squires) element that was inserted into the *env* gene. Since this vector FeLV-GFP (Figure 2-1) lacks a functional *env* gene, vector replication is limited to one round of replication. The single cycle aspect of this assay allows for the detection of agents that posses antiviral activity and eliminates compounding factors like re-infection and drug resistance that can be seen *in vivo* models. To validate the activity of the FeLV-GFP vector and the ability of the assay to detect antiviral activity, the single cycle assay was performed using AZT, an antiretroviral used clinically to treat FeLV. To do this, 293T cells were cotransfected with FeLV-GFP and a vesicular stomatitis virus glycoprotein (VSV-G) envelope expression plasmid. Cell culture supernatants were harvested and used to infect Crandell-Rees feline kidney (CRFK) cells that had been pretreated with the AZT concentrations indicated in Figure 2-2A. The percent of infected cells was determined by flow cytometry using GFP expression as a marker for infection. Figure 2-2A shows that AZT led to a concentration-dependent decrease in the percentage of cells infected with FeLV, thereby validating the use of the FeLV-GFP assay to detect anti-FeLV activity.

Decitabine, gemcitabine, tenofovir, and raltegravir inhibit FeLV infectivity in cell culture. To test they hypothesis that decitabine, gemcitabine, tenofovir, and raltegravir can inhibit FeLV replication, the single cycle assay with FeLV-GFP was performed as described. Figure 2-2B-2-2E show that individual treatment of target cells with each of the four drugs led to a concentration-dependent decrease in FeLV infection, with raltegravir demonstrating the greatest antiretroviral potency. Our observation that raltegravir has anti-FeLV activity confirms a recent observation (Cattori, Weibel et al.). The concentrations required to reduce infection by 50% (IC_{50}) are shown in Table 2-1 and demonstrate that decitabine, gemcitabine, and raltegravir have potent (nanomolar) anti-FeLV activity while tenofovir has micromolar anti-FeLV activity.

Tenofovir, raltegravir, decitabine, and gemcitabine have antiviral activity at concentrations that are not toxic in CRFK cells. Each drug was examined to test the hypothesis that the concentrations that exert antiretroviral activity were not cytotoxic. None of the drugs induced cytotoxicity at the concentrations required to exert antiviral activity. In fact, decitabine failed to induce toxicity even at concentrations 360-fold greater than the IC_{50} for antiviral activity. Similarly, tenofovir and raltegravir failed to induce toxicity at concentrations that were 10- and 280-fold higher than their IC_{50} values, respectively. In contrast, gemcitabine induced cytotoxicity with a cytotoxic concentration 50 (CC_{50}) of 230 nM, giving a selectivity index ($SI = CC_{50}/IC_{50}$) of 9.2 (Table 2-1).

Discussion

FeLV is responsible for significant mortality in cats worldwide and despite both its prevalence and associated morbidity and mortality, treatment options for FeLV are extremely limited, associated with significant side effects, and can be cost-prohibitive for many cat owners. In comparison to the development of novel therapeutics for FeLV, the development of drugs to treat HIV-1 has been more active. Since both FeLV and HIV-1 have similar mechanisms of replication, drugs used to treat HIV-1 may be useful in the treatment of FeLV and therefore may be used to expand the available treatment options for FeLV.

In this study, we developed a construct that was used to identify four FDA-approved drugs that inhibit FeLV in cell culture. Two of the drugs, tenofovir and raltegravir are used clinically to treat HIV-1 while the other two drugs, decitabine and gemcitabine, are used for the treatment of myelodysplastic syndromes and pancreatic cancer, respectively. Furthermore, the antiviral activity of all four drugs was achieved at concentrations that were not cytotoxic.

Although both FeLV and HIV-1 are retroviruses, there is a possibility that the drugs may possess alternative mechanisms of action. Previous studies have shown that differences in reverse transcriptase active sites among different retroviruses can affect susceptibility to NRTIs. For example the YXDD motif in the active site of all retroviral reverse transcriptases (RT) plays a significant role in susceptibility to NRTIs. Specifically, differences in the “X” position of this motif affect the ability of RT to incorporate nucleoside analogs containing modified

sugars (Poch, Sauvaget et al. 1989; Boyer, Gao et al. 2001; Operario, Reynolds et al. 2005; Jamburuthugoda, Santos-Velazquez et al. 2008). HIV-1 and other lentiviruses have a methionine at the "X" position that enables incorporation of nucleoside analogs with modified sugars. In contrast, structural studies show that a valine present at the "X" residue in FeLV and other oncoretroviruses restricts access of nucleoside analogs with a modified ribose. Although gemcitabine has a modified ribose (fluorinated on the 2' carbon), it is a potent inhibitor of FeLV replication (Figure 2-2). However, the antiviral activity of gemcitabine may not necessitate its corresponding triphosphate to be a substrate of reverse transcriptase. Instead, gemcitabine's mechanism of action may be related to its ability to alter dNTP pools. In support of this, previous studies have shown that retrovirus replication may be especially sensitive to changes in dNTP pools such that replication is inhibited prior to inhibition of cell cycle progression (Bebenek, Roberts et al. 1992).

Since decitabine has an unmodified deoxyribose, it is not expected that the "Val" present in the FeLV active site would exclude its incorporation into viral DNA by reverse transcriptase. Clinically, decitabine is used for its ability to be incorporated into DNA in place of dCTP where it binds to and irreversibly inhibits DNA methyltransferase. Its anti-HIV-1 activity has been attributed to its incorporation into viral DNA in place of dCTP by reverse transcriptase (Clouser, Patterson et al. 2010). Once incorporated, it induces G-to-C mutations and this increase in mutant frequency correlates with its anti-HIV-1 activity. However,

whether decitabine has the same mechanism of action in FeLV is not clear. The fact that FeLV reverse transcriptase has a higher fidelity than HIV-1 RT suggests that decitabine could act by a mechanism distinct from HIV-1 (Operario, Reynolds et al. 2005).

The anti-FeLV mechanisms of tenofovir and raltegravir are likely to be similar to what has been described for HIV-1. Clinically tenofovir is given in the form of a prodrug where it is converted to an acyclic nucleoside phosphate which is an analog of adenosine 5'-monophosphate. Once converted to the active diphosphate form, tenofovir is incorporated into viral DNA by reverse transcriptase where it acts as a chain terminator to inhibit further elongation of the viral DNA (Robbins, Srinivas et al. 1998; Kearney, Flaherty et al. 2004). In contrast, raltegravir inhibits integration of the double stranded viral DNA that is produced by reverse transcription of the viral RNA genome (Beck-Engeser, Eilat et al. 2009; Reigadas, Andreola et al. 2010).

In summary, we have demonstrated the anti-FeLV activity of four FDA-approved drugs whose anti-HIV-1 activity has been previously described. All four drugs exerted antiviral activity at concentrations examined with selectivity indexes of > 361.6 (decitabine), 9.2 (gemcitabine), > 10 (tenofovir), and > 285.7 (raltegravir). None of the drugs exhibited cytotoxicity within the therapeutic range tested, thereby warranting further investigation into their mechanisms of action as well as their suitability as treatments for FeLV (e.g., pharmacokinetics and pharmacodynamics studies). Expansion of the available treatments for FeLV is

expected to significantly impact the morbidity and mortality of infected cats since current treatments have limited efficacy and are associated with serious side effects.

Materials and Methods

Materials, cells and reagents. Crandell-Rees feline kidney (CRFK) cells were obtained from Dr. Richard Van Deusen, National Veterinary Services Laboratory (Ames, IA), and 293T cells were obtained from American Tissue Type Culture (Manassas, VA). DMEM was obtained from Mediatech (Manassas, VA). Gemcitabine was from Carbosynth (Berkshire, U.K), and decitabine was obtained from Moravek Biochemicals (Brea, CA). The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (tenofovir, cat #: 10199) from (Division of AIDS, NAID); (raltegravir, cat #: 11680) from (Merck & Company, Inc); and (pEECC-FeLV, cat #: 105) from (Dr. James I. Mullins). The pEECC-FeLV is a replication competent feline leukemia virus clone. The internal ribosomal internal entry site (IRES) and the enhanced green fluorescent protein (GFP) gene sequence used to create the FeLV-GFP vector was from the plasmid pHIG (Clouser, Patterson et al. 2010) and was originally derived from pIRES2-eGFP (Clontech, Mountain View, CA). The vesicular stomatitis virus G protein (pL-VSV-G) envelope expression plasmid was obtained from Dr. Jane C. Burns (UC-San Diego). CRFK cells and the 293T cells were maintained in DMEM supplemented with 10% fetal clone 3 (FC3)

serum from HyClone (Logan, VT) and penicillin/streptomycin (100 ug/mL and 100 units/mL) at 37 ˚C in 5% CO_2. During drug treatment, cells were maintained in DMEM supplemented with 10% FC3 without penicillin/streptomycin.

Design and construction of the FeLV-GFP vector. The FeLV-GFP vector was created by inserting the IRES-GFP sequence into the *env* gene of FeLV from the pEECC-FeLV plasmid. To do this, the IRES-GFP sequenced was amplified by PCR using the following primers:

5’ ATGCATACCATGGTGGCCAGGCTAGGG 3’ (forward) and

5’ CCATGCATTTACTTGTACAGCTCGTCCATG 3’ (reverse),

which included the Nsi I restriction site (underlined). The pEECC-FeLV plasmid was digested with Nsi I restriction enzyme, removing a 615 bp sequence from *env*. The linear pEECC-FeLV backbone was dephosphorylated with antarctic phosphatase. The pEECC-FeLV backbone was gel purified using the Wizard SV Gel and PCR Clean-up system from Promega (Madison, WI) following the manufacturer’s instructions. The ligation of the pEECC-FeLV backbone with the IRES-GFP insert was done at a 1:5 (plasmid backbone to DNA insert) molar ratio. The ligation product was transformed into DH5α (E. coli) cells from Invitrogen (Carlsbad, CA) and plated onto Luria-Bertani (LB) agar containing 50 μg/mL ampicillin. Individual colonies were grown in LB broth containing 50 μg/mL ampicillin and the DNA from these cultures was purified using Invitrogen’s PureLink Quick Plasmid Miniprep Kit (Carlsbad, CA). DNA sequencing

(Functional Biosciences, Madison, WI) was done to verify the correct construction of the vector.

Transfection of 293T cells. FeLV-GFP plasmid (10 µg) and pL-VSV-G plasmid (1 µg) were cotransfected into 293T cells using the calcium phosphate coprecipitation method. Twenty four hours after transfection, media was removed and replaced with 6 mL of fresh media. Supernatant containing infectious virions was collected from cells 48 hours post-transfection and passed through a 0.2 µm filter. The filtered supernatant was stored at -80 ˚C for later use.

Drug treatment and infection of CRFK cells. CRFK cells (65,000) were plated per well of a 12-well dish 24 hours prior to drug treatments. Twenty-four hours later, the cells were treated with drug (decitabine, gemcitabine, tenofovir, or raltegravir) at the concentrations indicated in Fig. 2-2. Two hours after initiating drug treatments, cells were infected and then incubated at 37 ˚C for 24 hours. Twenty-four hours post-infection, the media was removed and replaced with fresh DMEM and the cells were then incubated for an additional 24 hours at 37 ˚C. To analyze FeLV-infected cells, 48 hours post-infection, cells were trypsinized, centrifuged at 800 x g for 5 minutes and the cell pellets were resuspended in 2% paraformaldehyde in PBS containing 2% FC3. Samples were analyzed by flow cytometry using a Becton Dickinson FACScan. Samples were gated based on FSC vs. SSC to eliminate dead cells and GFP-expressing cells were detected in the FL1 channel. The raw data was converted to relative

GFP expression by setting non-treated cells (0 nM) to 100% and multiplying the data for each individual treatment by the factor used to convert the non-treated group to 100. Uninfected cells were used to account for any non-specific signal in the FL1 channel.

Analysis of cytotoxicity. CRFK cells (1.5×10^3) were plated per well of a 96 well plate 24 hours prior to the initiation of drug treatment. Cells were treated with drug (AZT, decitabine, gemcitabine, tenofovir, or raltegravir) for 24 hours at which time the media was replaced with fresh DMEM containing 10% FC3. Cell viability was examined using Promega's CellTiter-Glo kit according to the manufacturer's instructions. Briefly, cell viability was assessed 48 hours post drug treatment by adding a substrate that emits light at 570 nm in the presence of ATP. Background luminescence was subtracted from the value obtained from each well. The data were converted to relative luminescence by setting non-treated cells (0 nM) to 100 and multiplying the data for each individual treatment by the factor used to convert the non-treated group to 100.

Drug	Inhibition activity, IC_{50} (nM)	In vitro cytotoxicity, CC_{50} (mM)	SI (CC_{50}/IC_{50})	95% confidence interval (mM)
Decitabine	437	>158	>361.6	0.14–1.4
Gemcitabine	25	0.23	9.2	0.02–0.03
Tenofovir	2000	>20	>10	1.0–3.0
Raltegravir	14	>4	>285.7	0.011–0.017

Table 2-1. In vitro cytotoxicity of decitabine, gemcitabine, tenofovir, and raltegravir in Crandell-Rees feline kidney (CRFK) cells. CRFK cells were

incubated in the presence of drug for 48 hours. The IC_{50} values represent the concentration at which 50% of virus replication was inhibited, as determined for the data in Fig 2-2 with the 95% confidence interval indicated. CC_{50} values were determined using the Cell Titer-Glo Luminescent Cell Viability Assay (see Methods for details).

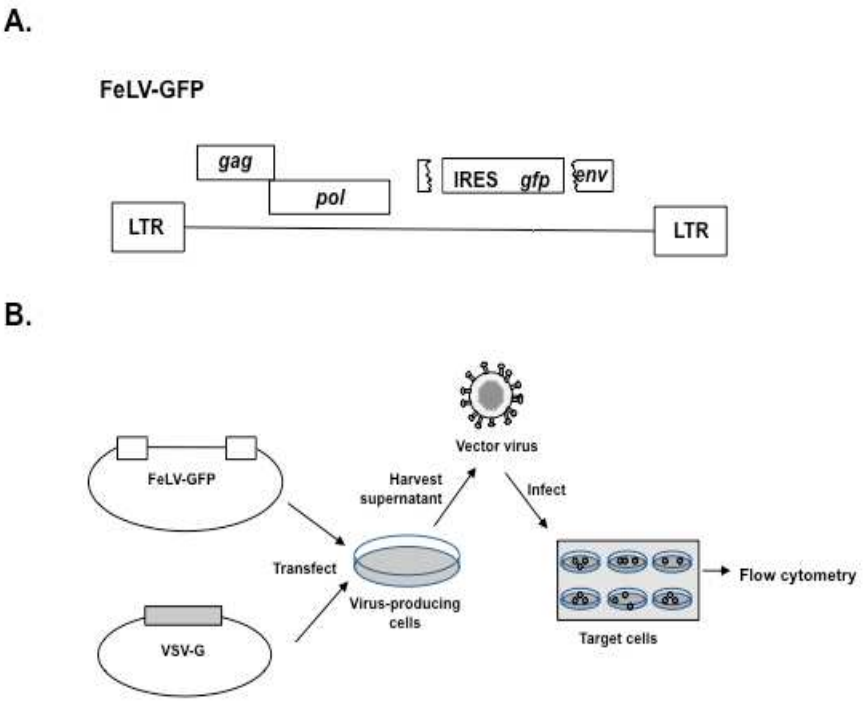
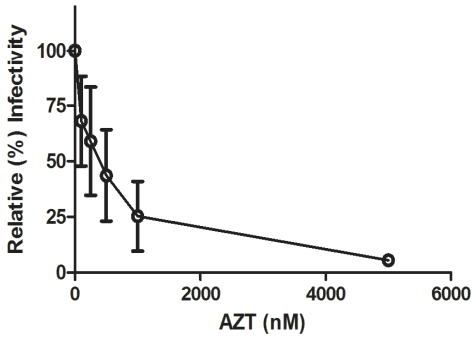


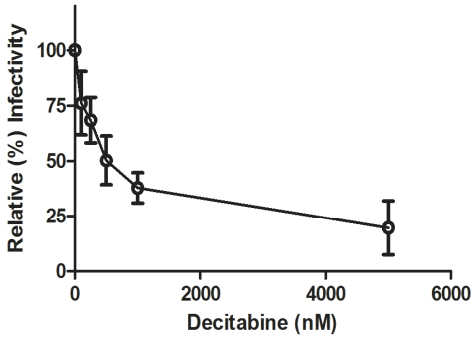
Figure 2-1. FeLV-GFP vector and model system for monitoring viral infectivity. (A) The FeLV vector, FeLV-GFP, is shown with the *gfp* gene inserted into the *env* gene. The long terminal repeats (LTRs), *gag* and *pol* genes are indicated. (B) Single replication cycle assay performed with permissive Crandell-Rees feline

kidney (CRFK) target cells. FeLV-GFP was co-transfected into 293T cells with a vesicular stomatitis virus glycoprotein G (VSV-G) envelope expression plasmid. Cell culture supernatants were harvested and used to infect CRFK target cells. Viral infectivity was monitored by measuring GFP expression using flow cytometry.

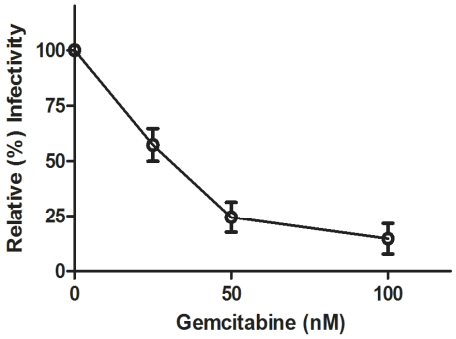
A


Relative (%) Infectivity
100
75
50
25
0
0
2000
4000
6000
AZT (nM)

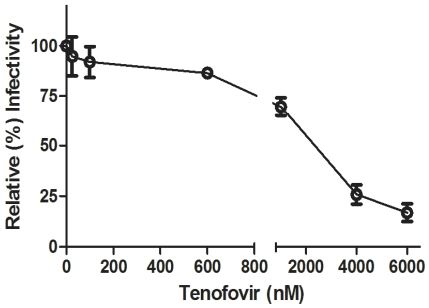
B


Relative (%) Infectivity
100
75
50
25
0
0
2000
4000
6000
Decitabine (nM)

C


Relative (%) Infectivity
100
75
50
25
0
0
50
100
Gemcitabine (nM)

D


Relative (%) Infectivity
100
75
50
25
0
0
200
400
600
800
2000
4000
6000
Tenofovir (nM)

E

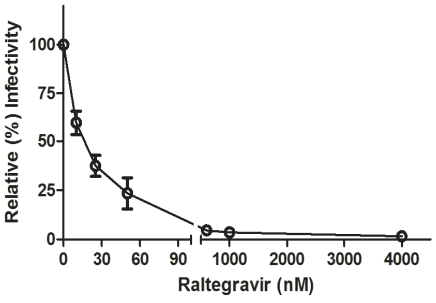

Relative (%) Infectivity
100
75
50
25
0
0
30
60
90
1000
2000
3000
4000
Raltegravir (nM)

Figure 2-2. Dose response of FeLV infectivity to decitabine, gemcitabine, tenofovir, and raltegravir. CRFK cells were exposed to the indicated drug 2 hours prior to infection with FeLV. Infected cells were analyzed by flow cytometry. Each data point represents the average of 3 independent replicates with the standard deviation indicated. The molarity of the drug concentration range analyzed is indicated along the x-axis. A) Validation of assay using AZT. B) - E). Analysis of decitabine, gemcitabine, tenofovir and raltegravir, respectively.

CHAPTER III

INVESTIGATING THE MECHANISM OF ACTIVITY OF DECITABINE, GEMCITABINE AND CYCLOPENTENYL CYTOSINE AGAINST FELINE LEUKEMIA VIRUS

Introduction

Over the years, there has been improvement in the treatment of hematological malignancies, solid tumors, and viral infections, such as human immunodeficiency virus type 1 (HIV-1), with the development of a specific class of drugs known as nucleoside analogs. This special class of drugs is characterized for its ability to mimic natural nucleosides. Like naturally occurring nucleosides, nucleoside analogs are transported, activated, and incorporated into DNA. Of the four natural nucleosides, dCTP is generally the lowest in abundance (de Korte, Haverkort et al. 1985), implicating that cytosine analogs could be an effective target for cancer therapy. Some nucleoside analogs that have anti-cancer activity can also show antiviral activity at non-toxic concentrations however in some cases the antiviral mechanism of action may differ from the anti-cancer mechanism. Two such cytosine analogs, gemcitabine and decitabine were previously shown to have anti-HIV-1 activity at non-cytotoxic concentrations (Clouser, Patterson et al. 2010; Clouser, Holtz et al. 2012). Data suggested that these analogs worked by inducing viral mutations. Decitabine is phosphorylated to its triphosphate form in cells and is recognized by HIV-1 reverse transcriptase and incorporated into viral DNA. Once incorporated, it undergoes a conformational change creating a G-to-C mutation (Dapp, Clouser et al. 2009). Gemcitabine is also proposed to act as a viral mutagen, but instead of being incorporated into the viral genome by reverse transcriptase (RT), it may act as a ribonucleotide reductase inhibitor (Clouser, Patterson et al.). This

enzyme is vital for the generation of deoxynucleotide triphosphates (dNTPs). Studies have shown that altering dNTP pool levels can lead to genomic mutations in retroviruses (Bebenek, Roberts et al. 1992; Mezei and Minarovits 2006). Similarly, we previously reported the susceptibility of FeLV to gemcitabine and decitabine (Greggs, Clouser et al. 2012).

In this study, the anti-FeLV activity of decitabine, gemcitabine, and a third cytosine analog, cyclopentenyl cytosine (CPEC) were examined (Figure 3-1). CPEC is a cytosine analog that inhibits deoxycytosine triphosphate (dCTP) synthetase, the enzyme responsible for synthesis of cytosine triphosphate (CTP) from uridine triphosphate (UTP), a key process involved in the generation of dCTPs, (Kang, Cooney et al. 1989; Verschuur, Van Gennip et al. 2000). Unlike the broader activity of gemcitabine that acts on multiple dNTP pools, CPEC specifically targets the dCTP pool. In this study, comparative analysis with HIV-1 was done to investigate the antiviral activity and action of mechanism of decitabine, gemcitabine and CPEC.

Results

CPEC possesses antiviral activity against FeLV. CPEC is a cytosine analog that has been shown to selectively decrease the levels of dCTP. Its ability to inhibit CTP pools has led to its use for the treatment of cancer. Additionally, CPEC has been shown to have a broad-spectrum antiviral activity against viruses, including cytomegalovirus, influenza, parainfluenza, and

coronavirus, (De Clercq, Murase et al. 1991). CPEC was hypothesized to possess anti-FeLV activity. Since retroviruses such as HIV-1 and FeLV have been shown to be susceptible to cytosine analogs, we examined the ability of CPEC to inhibit HIV-1 and FeLV replication using the single replication cycle assay (Figure 3-2A and 3-2B). Specifically, CPEC-treated or DMSO-treated MAGI cells were infected with vesicular stomatitis virus protein-G (VSV-G) psuedotyped FeLV and flow cytometry was used to detect GFP expression, which is used as an indicator of the percentage of infected cells.

The data revealed a concentration-dependent decrease in FeLV infectivity (Figure 3-4A). Furthermore, this antiviral activity was unique to FeLV since CPEC failed to demonstrate anti-HIV-1 activity even at concentrations as high as 1 µM. While FeLV demonstrated an inhibitory concentration 50% (IC_{50}) of 872 nM (Figure 3-4A), this value could not be calculated for HIV-1. Consistent with our previous studies, decitabine and gemcitabine demonstrated antiviral activity against FeLV with IC_{50} values of 1301 nM (Figure 3-11C) and 68 nM (Figure 3-11E), respectively. These IC_{50} values are higher than previously shown for decitabine and gemcitabine, which could be due to drug stability or drug lot variability.

The cytosine analogs decitabine, gemcitabine, and CPEC are active during reverse transcription. To test the hypothesis that the antiviral target of decitabine, gemcitabine, and CPEC was reverse transcriptase, the time-of-addition assay was performed using a single cycle replication assay (Hayouka,

Levin et al. ; Daelemans, Pauwels et al. 2011; Lara, Ixtepan-Turrent et al. 2011). In order to perform the time-of-addition assay, CRFK cells were infected and then treated with decitabine, gemcitabine or CPEC at specific time points post-infection. Infectivity was then monitored by flow cytometry, which was used to detect GFP expression.

In the time-of-addition assay, drugs that act at the point of reverse transcription should possess antiviral activity until 2-6 h post-infection. Compounds that target viral integration are expected to possess antiviral activity until about 8-10 h post-infection, beyond which there is a loss of activity.

Two control drugs, AZT, a nucleoside analog that inhibits reverse transcriptase (RT) and raltegravir, an integrase inhibitor were included in the analyses. Consistent with other published data, AZT was less potent after 3 h post-infection while raltegravir had less activity after 8 h post-infection (Figure 3-3). The antiviral activity of decitabine declined after 3 h post-infection while gemcitabine's antiviral activity declined after 2 h post-infection (Figure 3-3). The antiviral activity of both drugs decreased at a time that is consistent with inhibitors of reverse transcription activity. In contrast, the antiviral activity of CPEC declined as early as 2 h post-infection (Figure 3-3). This early time point could suggest reverse transcription as a target, but is also in range for what has been seen for fusion inhibitors.

Antiviral activity of CPEC is not due to cytotoxicity. The therapeutic mechanism of CPEC is known to target cellular factors, which could induce

cellular toxicity and lethality at high drug concentrations. To test the hypothesis that the antiviral activity observed was not due to drug cytotoxicity, the cytotoxic effects of CPEC in both MAGI and the CRFK cell lines were examined. As shown in Figure 3-5, only minimal cytotoxicity was seen at the highest concentration tested for antiviral activity (1 µM), where only 7% and 15% of the cells shown signs of cytotoxicity to CPEC in MAGI and CRFK cell lines, respectively. At the 1µM concentration, an antiviral activity of IC_{52} was observed as determined in MAGI cells (Figure 3-4A). CPEC was found to have a cytotoxic concentration 50% (CC_{50}) of 52 µM in MAGI cells and 6 µM in CRFK cells (Figure 3-5A and 3-5B, respectively). The selectivity index (CC_{50}/IC_{50}), the cell culture equivalent to a therapeutic index, for CPEC with FeLV in MAGI cells was determined to be 59.6. Given this, the observed antiviral activity of CPEC could not be attributed to cellular toxicity.

CPEC dependent potentiation of cytosine analogs is not evident against FeLV infectivity. The cytosine triphosphate synthetase inhibitor, CPEC, decreases dCTP levels in cancer cells (Ford, Cooney et al. 1991; Verschuur, Van Gennip et al. 2000). Since the cytosine analogs decitabine and gemcitabine have potent antiviral activity that appears to be due to their ability to compete with dCTP, we hypothesized that a CPEC-mediated decrease in dCTP levels would potentiate decitabine and gemcitabine's antiviral activity. As shown in Figure 3-5C, CPEC did not potentiate the anti-HIV-1 activity of decitabine. Although there was an increase in antiviral activity with increasing concentrations

of CPEC, this increase was not statistically significant. In contrast, CPEC significantly increased the anti-HIV-1 activity of gemcitabine. The data demonstrated a significant effect of 800 nM CPEC on the potency of 20 nM gemcitabine (Figure 3-6E) with a $p < .05$ [$F(2, 6) = 6.005$, $p = .0370$]. Post-hoc comparison using the Tukey test indicated that the mean infectivity for gemcitabine 20 nM alone was significantly different from the CPEC 800 nM – gemcitabine 20 nM combination (M diff = 38.56, 95% CI = 4.418 – 72.70). While CPEC potentiated the anti-HIV-1 activity of gemcitabine, it had no effect on the anti-FeLV activity of gemcitabine. (Figure 3-6F). As expected, CPEC had no effect on the antiviral activity of AZT, a thymine analog that competes with TTP, not CTP for incorporation into the viral DNA for both HIV-1 (Figure 3-6A) and FeLV (Figure 3-6B).

Gemcitabine minimally affects dNTP pool levels. Since both gemcitabine and CPEC are reported to effect dNTP levels (Kang, Cooney et al. 1989; Heinemann, Schulz et al. 1995; Verschuur, Van Gennip et al. 2000; Cerqueira, Fernandes et al. 2007; van Bree, Rodermond et al. 2008), we hypothesized that the dNTP levels would be imbalanced in cells that were treated with either gemcitabine or CPEC. To test this, dNTPs were extracted from 5×10^6 CRFK or 3×10^6 MAGI cells that were exposed to gemcitabine or CPEC for 4 h. The extracted dNTPs were then examined using liquid chromatography mass spectrometry. The data revealed that 100 nM gemcitabine decreased dATP pools by 4.3-fold ($p \leq 0.001$) in MAGI cells, whereas 800 nM CPEC, led to a 1.9-

fold increase ($p \leq 0.001$) in dATP pools (Figure 3-7A). No significant changes in the dCTP, dTTP or dGTP pools were detected in MAGI cells (Figure 3-7B – 3-7D). In CRFK cells, 500 nM and 5 μM of decitabine increased dATP pools by 1.5- and 1.4-fold, $p \leq 0.05$ and $p \leq 0.001$, respectively (Figure 3-7E). Additionally, 500 nM and 800 nM of CPEC decreased dCTP pools by 2.3- and 10.7-fold, respectively (Figure 3-7F), while no changes in dTTP or dGTP pools were detected in CRFK cells after treatment with any drug (Figures 3-7G and 3-7H).

Decitabine, gemcitabine, and CPEC have no affect on the mutation frequency or mutation spectra of FeLV. Previous studies have shown that decitabine and gemcitabine act as viral mutagens to decrease viral replication, a process known as lethal mutagenesis (Penn 1987; Clouser, Patterson et al. 2010; Clouser, Holtz et al. 2011; Clouser, Holtz et al. 2012). Additionally, the ability of CPEC to alter dNTP pools suggests that it may induce mutations in the retroviral genome. These observations let to the hypothesis that decitabine, gemcitabine and CPEC can increase FeLV mutation frequency and spectra by incorporation and induction of G-to-C mutations (i.e., decitabine) or by causing imbalances in cellular dNTP pools (i.e., gemcitabine and CPEC). To examine the effect of decitabine, gemcitabine, and CPEC on the mutation frequency of FeLV, we used Illumina® next-generation DNA sequencing to sequence integrated FeLV proviral DNA from cells that had been treated with or without decitabine, gemcitabine, or CPEC. Approximately 17.5 million sequences were

examined in order to detect drug-induced changes in the viral mutation frequency. Unexpectedly, none of the drugs increased the FeLV mutation frequency (Figure 3-8A) [$F(3,8,) = 0.6368$, $p = 0.6121$]. Furthermore, results from our mutation spectra analysis revealed no significant changes in the types of mutations that occurred when comparing no drug to each of the drug treatments (Figure 3-8B).

Decitabine, gemcitabine, and CPEC do not affect the late stages of the viral life-cycle. While the time-of-addition assay indicated that decitabine, gemcitabine, and CPEC target viral replication at the point of reverse transcription, it is possible that the compounds could also have antiviral activity at other steps in the life cycle. To test the hypothesis that the late steps in the FeLV replication cycle (i.e., viral RNA transcription, translation, viral RNA and protein trafficking, virus assembly and release) are not affected by decitabine, gemcitabine and CPEC, we examined if these drugs could also target viral replication at a post-integration step. Briefly, the assay involves the production of virus in the presence or absence of drug (decitabine, gemcitabine, or CPEC) at approximately the IC_{80} values, with the exception of CPEC where the IC_{50} was used because this was the highest IC value attainable with CPEC without significant cytotoxic effects. The virus was then used to infect target CRFK cells and flow cytometry was used to examine the percentage of infected cells. Figure 3-9 shows that virus produced in the presence of decitabine, gemcitabine, and CPEC led to the same percentage of infected cells as virus produced in the

presence of the DMSO control. This finding indicates that each of these drugs exerts antiviral activity prior to viral production. This finding is consistent with the data generated with the time-of-addition assay (Figure 3-3), which indicated that each of these drugs act at or before reverse transcription. As a negative control, we used AZT, a nucleoside reverse transcriptase inhibitor whose antiviral activity is limited to the reverse transcription phase of the viral life cycle. As expected, when compared to no drug (DMSO solvent only), AZT did not have antiviral activity in the target cells when infected with virus that was produced in the presence of drug (Figure 3-9). Comparable levels of GFP expression were observed in all cells regardless of the drug treatment, indicating that the transfection efficiency were relatively consistent (Figure 3-10).

The retroviral YXDD motif does not dictate susceptibility of viral replication to decitabine. Previous studies have demonstrated that specific amino acid motifs within the active site of reverse transcriptase affect the susceptibility of viral replication to nucleoside analogs. For example, the “X” position of the YXDD motif, a motif that is present in all retroviruses, plays a significant role in the ability of RT to incorporate nucleoside analogs that contain modified sugars (Poch, Sauvaget et al. 1989; Boyer, Gao et al. 2001; Operario, Reynolds et al. 2005; Jamburuthugoda, Santos-Velazquez et al. 2008). HIV-1 and other lentiviruses have a methionine (M) in the “X” position and are susceptible to the cytosine analog lamivudine (3TC). In contrast, oncoviruses like FeLV, and some drug resistance HIV-1 mutants, have a valine (V) in the “X” position, are less

susceptible to 3TC. For example, wild type HIV-1 which has the Y[M]DD motif is susceptible to the nucleoside analog 3TC. However, the M184V (Tisdale, Kemp et al. 1993; Gallant 2006; Jamburuthugoda, Santos-Velazquez et al. 2008) mutation, which changes the Y[M]DD motif to Y[V]DD, is resistant to 3TC. Since the YXDD motif is known to affect susceptibility of retroviruses to nucleoside analogs, we examined if the "X" position within the YXDD motif affected susceptibility of viral replication to decitabine, gemcitabine, and CPEC. To do this, we compared wild type FeLV, which has a valine in the "X" position of the YXDD motif, to a mutant FeLV where the valine was replaced by a methionine, making the YXDD motif resemble wild-type HIV-1.

Consistent with previous reports, wild type FeLV demonstrated resistance to 3TC (Operario, Reynolds et al. 2005). However, the FeLV-YMDD mutant displayed susceptibility to 3TC, with an IC_{50} value of 8.3 μM (Figure 3-11A and 3-11B, respectively). A cytotoxic analysis of 3TC confirmed that the apparent antiviral activity was not due to cytotoxicity in the CRFK cells (Figure 3-12). We next examined the effect of the YXDD motif on the susceptibility of FeLV to decitabine and gemcitabine, two cytosine analogs with potent anti-HIV-1 activity (Clouser, Patterson et al. 2010; Clouser, Holtz et al. 2012). It was observed that the wild-type FeLV and the FeLV-YMDD mutant displayed comparable susceptibilities to decitabine. (Figure 3-11C and 3-11D, respectively). Figures 3-11E and 3-11F show that the wild-type and Y[M]DD mutant had similar susceptibility to gemcitabine. In contrast, wild-type and the Y[M]DD mutant

demonstrated a significant difference in susceptibility to CPEC. Specifically, the Y[M]DD mutant displayed a 1.8-fold decrease in the IC_{50} value compared to wild-type (Figures 3-11G and 3-4A, respectively).

Discussion

The *Retroviridae* is a diverse family that shares similarities in genetic organization, mechanism of replication, and all utilize a high mutation rate to generate genomic diversity. These similarities give rise to the potential for the use of one drug to inhibit the replication of more than one retrovirus. This is particularly useful when examining potential treatments for FeLV, a virus that is not as well characterized as HIV-1.

The feline gammaretrovirus, feline leukemia virus (FeLV), is associated with morbidity and morality of companion felines (Hosie, Robertson et al. 1989; Bandecchi, Matteucci et al. 1992; Lutz, Addie et al. 2009) and wild felines (Gardner and Luciw 1989; Brown, Cunningham et al. 2008). In companion felines, once infected there are limited treatment options and the ones that are available are often cost prohibitive and/or ineffective. Preventatively, the option of vaccination is an affordable option, but the vaccines do not offer sterile immunity, so vaccinated cats that become infected can pass the infection to another susceptible cat. Furthermore, it is general practice that the vaccine is usually reserved for cats thought to be at high risk, such as multi-cats household and catteries.

There is only limited knowledge involving the treatment of FeLV with the use of antiretrovirals. Being related to HIV-1, it is plausible that drugs used to treat HIV-1 will be useful against FeLV. Previously, we were able to identify the anti-FeLV activity of two anti-cancer nucleoside analogs decitabine and gemcitabine, and two anti-HIV-1 drugs tenofovir (nucleotide analog) and raltegravir (integrase inhibitor) (Greggs, Clouser et al. 2012). In this study, we examined the mechanism by which they elicit this antiviral activity. In addition to decitabine and gemcitabine, we also examined the anti-FeLV activity of CPEC, a cytosine analog that we predicted may have anti-FeLV activity. Although both FeLV and HIV-1 belong to the same family, it is possible for these drugs to inhibit viral infectivity through different mechanisms. For example, nucleoside analogs have been shown to inhibit HIV-1 replication by at least three distinct mechanisms: 1) binding directly to the active site of reverse transcriptase to inhibit the enzyme, 2) inhibiting RT by binding to hydrophobic pockets distant to the active site, and 3) enhancement of viral mutagenesis.

The data in this study, particularly the results from the time-of-addition assay, support that decitabine, gemcitabine and CPEC exhibit potent anti-FeLV activity that targets the reverse transcription phase of the retroviral life cycle. Specifically, the data generated in the time-of-addition assay supported that each drug inhibited viral replication at the point of reverse transcription or earlier (Figure 3-3). While gemcitabine did demonstrate a reduction in infectivity an hour earlier than the AZT control, the difference in the timing of activity could be

a result of differences in the amount of time required for the drug to be converted into its active form (Daelemans, Pauwels et al. 2011) or that gemcitabine has more than one mechanism of action. Furthermore, the drugs had no inhibitory activity on virus production (Figures 3-9, and 3-10 and Tables 3-1 and 3-2). In contrast, the cytosine analog CPEC began to lose antiviral activity as early as 2 h. This is generally within the time range observed with early phase inhibitor like fusion or entry inhibitors (Hayouka, Levin et al. ; Daelemans, Pauwels et al. 2011). However, there is no known mechanism that would suggest that nucleoside analogs have the structural capability to act as either an entry or fusion inhibitor. Further investigation may be needed to completely elucidate the implications of the data. The findings from the time-of-addition assay are supported by the producer cell assay that showed none of the compounds possess any activity on the late phase (post integration) of the retroviral life cycle (Figures 3-9, and 3-10 and Tables 3-1 and 3-2).

Based on structure of the drugs, known antiviral and/or anticancer activities, and the results from the time-of-addition assay, we hypothesized that the drugs inhibited reverse transcription. There are two potential ways that the drugs can target reverse transcription: 1) induce lethal mutagenesis, 2) inhibit production of double stranded viral DNA either through direct inhibition of RT or through the inhibition of cellular dNTP production.

The DNA sequencing data presented here do not support lethal mutagenesis as the primary antiviral mechanism of action. Although gemcitabine

induced changes in dNTP levels, the changes were not large enough to suggest that the dNTP levels were too low to support reverse transcription. Thus, the data presented here is most consistent with direct inhibition of reverse transcriptase. The sequencing data for decitabine also suggests that its mechanism of activity may primarily involve a direct inhibition of FeLV reverse transcriptase. In contrast, the changes induced by CPEC treatment do suggest that this drug acts by limiting the availability of dCTP for reverse transcription. Due to its structure, CPEC is not believed to behave as a substrate for reverse transcriptase. CPEC resembles cytidine in the RNA synthesis pathway, which is evident in the phosphorylation and activation of CPEC (Bierau, van Gennip et al. 2006; Schimmel, Gelderblom et al. 2007). Examination of the late phase of the viral life cycle suggests that RNA synthesis/processing and virus production was not directly targeted.

To further examine the mechanism by which the drugs inhibit FeLV, we examined if the retroviral motif YXDD affected susceptibility of viral replication to the cytosine analogs, based on increase in susceptibility to the cytosine analog 3TC seen with HIV-1 that has the phenotype Y[M]DD; and a decrease in susceptibility to 3TC when the methionine is mutated to a valine. With the valine being found in the wild-type FeLV, the data show that mutation of this well-known resistance phenotype did not affect FeLV's susceptibility to decitabine nor gemcitabine. These data suggest that this particular residue is not a primary participant in drug susceptibility of FeLV to decitabine or gemcitabine. In

contrast, susceptibility to CPEC does seem to be affected by mutation in this residue. Knowing that CPEC is an unlikely candidate for reverse transcriptase (RT), it is probable that the lower fidelity seen in RTs that posses the Y[M]DD phenotype could explain the shift in susceptibility to CPEC.

In summary, the anti-FeLV activity of three cytosine analogs – i.e., decitabine, gemcitabine, and CPEC – has been demonstrated. We found that CPEC had anti-FeLV, but not anti-HIV-1 activity. It is important to mention that as nucleoside analogs, all three of the nucleoside analogs examined were active during the early phase of the FeLV retroviral life cycle, with reverse transcription being the most likely target. This suggests that CPEC is effective in reducing dCTP pool levels in the CRFK cell lines, and did not demonstrate a significant decrease in dCTP in the MAGI cell line. The data also suggests that these cytosine analogs, though shown to have mutagenic properties in HIV-1, may possess a unique mechanism for inhibiting FeLV replication.

Material and Methods

*Cell lines, plasmids, and compounds***.** Crandell-Rees feline kidney (CRFK) cells were obtained from Dr. Richard Van Deusen (National Veterinary Services Laboratory, Ames IA, USA), U373-MAGI-$CXCR_4$ (MAGI) cells were obtained from the AIDS Reagent Program (from M. Emerman), and the 293T cells were obtained from the ATCC. Decitabine (catalog # M1774) was obtained from Moravek and gemcitabine (product # ND04237) was obtained from Carbosynth.

Cyclopentenyl cytosine (CPEC; NSC. # 375575-R) was obtained from the National Cancer Institute. Raltegravir (catalog # 11680, from Merck & Company, Inc), zidovudine (AZT; catalog # 3485), lamivudine (3TC; catalog # 8146), and pEECC-FeLC (catalog # 105) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAIDS. pEECC-FeLV is a replication-competent FeLV clone. The IRES and the EGFP gene sequence in the FeLV-GFP vector were from the plasmid pHIG (Clouser et al., 2010) and were originally derived from pIRES2-EGFP from Clontech (Mountain View, CA). The VSV-G protein (pL-VSV-G) envelope expression plasmid was obtained from Dr. Jane C. Burns (University of California, San Diego, CA, USA). The HIV-1 vector contains the IRES and the EGFP gene sequence, derived from pIRES2-EGFP and a HSA gene sequence, derived from pNL4-3.HSA-R+.E-; and has been previously described (Dapp, Clouser et al. 2009).

Cell Culture. CRFK and 293T cells were maintained in Dulbecco's Modified Eagle's Medium from Mediatech, and were supplemented with 10% fetal clone 3 (FC3) serum (HyClone) and penicillin/streptomycin (100 mg/ml and 100 U/ml, respectively). U373-MAGI$_{CXCR4}$ were maintained in Dulbecco's Modified Eagle's Medium from Mediatech, and was supplemented with 10% FC3 (HyClone), 0.2 mg/ml neomycin, 0.1 mg/ml hygromycin B, and 1.0 ug/ml puromycin. All cells were maintained at 37ºC in 5% CO_2.

Transfection of 293T cells. The FeLV-GFP plasmid (10 µg) or the HIV-1 plasmid (10 µg) was cotransfected with VSV-G plasmid (1 µg) on to 293T cells

using the calcium phosphate coprecipitation method. Twenty-four hours after transfection, the medium was removed and replaced with fresh medium (6 ml). The supernatant containing infectious virions was collected from cells 48 h post-transfection and passed through a 0.2 µm filter to remove cellular contaminants. The filtered supernatant was stored at -80ºC for later use.

Flow cytometry. Paraformaldehyde-fixed cell samples were analyzed by flow cytometry using a Becton Dickinson FACScan. Gating based on FSC vs SSC was used to eliminate dead cells, and GFP-expressing cells were detected in the FL1 channel. Relative GFP expression was calculated by setting non-treated cells (0 nM) to 100% and multiplying the data for each treatment by the factor used to convert the non-treated group to 100. Background signal was accounted for by subtracting out the data from uninfected cells.

Analysis of cytotoxicity. To examine the cytotoxic activity of drug (CPEC, 3TC, or CPEC in combination with decitabine, gemcitabine, or AZT), CRFK cells (1.5×10^3) or MAGI cells (4.5×10^3) were plated in each well of a 96-well plate 24 h prior to the initiation of drug treatment. Cells were treated with drug for 24 h, after which the cell medium was removed and replaced with fresh DMEM containing 10% FC3. For the CPEC only treatments, the cells were incubated with drug for 48 h prior to the media change. For CPEC-drug (AZT, decitabine, gemcitabine) combination, the cells were pre-incubated with CPEC 24 h prior to drug treatment. Promega CellTiter-Glo kit was used according to the manufacturer's instructions to monitor cell viability. Background luminescence

was subtracted from the value obtained from each well. The data was converted to relative luminescence by setting the non-treated cells (0 nM) to 100 and multiplying the data for each individual treatment by the factor used to convert the non-treated group to 100.

Time-of-addition assay. CRFK cells (3.5×10^4) were plated into each well of a 24-well plate 24 h prior to viral infection. At time point zero (t=0), the cells were infected with a titer of virus to yield approximately 20% ± 10% infectivity. Drug (decitabine, gemcitabine, CPEC, raltegravir, or AZT) was added to designated wells corresponding to a specific time point (0, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 24 h). The medium was then removed and replaced with fresh DMEM 24 h post-infection. After the media change, the 24 h time point was treated with drug. Each sample was trypsinized, centrifuged at 1,200 g for 5 min and the cell pellets were resuspended in 2% paraformaldehyde in PBS with 2% FC3 48 h post-infection. These samples were then analyzed by flow cytometry for GFP expression. The data is interpreted by looking for the time point where the drug starts to decrease potency. This was interpreted by a trending rebound of infectivity above baseline, which is illustrated by the point of inflection of the data points. As a standard of measure, two characterized control drugs, AZT and raltegravir, were included. AZT is a nucleoside analog that inhibits RT, and raltegravir is a integrase inhibitor. Other investigators have found the point of inflection for drugs that act during reverse transcription to range from 2 - 6 h and drug acting as integrase inhibitors ranging from 8 - 10 h (Hayouka, Levin et al. ;

De Clercq, Yamamoto et al. 1992; Daelemans, Pauwels et al. 2011; Lara, Ixtepan-Turrent et al. 2011). In this assay, the time point of decline of antiviral activity was defined by the time point of inflection where the virus increased above baseline.

Drug potentiation assay. MAGI cells (3.5×10^4) were plated into each well of a 24-well plate 48 hours prior to viral infection, after which the cells began a 24 h pretreatment with CPEC. After the pretreatment, the cells were exposed to drug (decitabine, gemcitabine, AZT) for 2 h, after which they were infected with FeLV-GFP with a titer of virus to yield approximately 20% ± 10% infectivity. The medium was then removed and replaced with fresh DMEM 24 h post-infection. After the media change, the 24 h time point was treated. Each sample was trypsinized, centrifuged at 1,200 g for 5 min and the cell pellets were resuspended in 2% paraformaldehyde in PBS with 2% FC3 48 h post-infection. The samples were then analyzed for GFP expression by flow cytometry. Statistical analysis was done using the GraphPad Prism program. For each drug, statistical significance was determined using a one-way ANOVA with a Tukey post-test comparing the drug only to each CPEC – drug combination.

Assay for late phase antiviral activity. The FeLV-GFP plasmid (10 µg) and VSV-G plasmid (1 µg) were cotransfected into 293T cells using the calcium phosphate coprecipitation method. Twenty-four hours after transfection, the medium was removed and replaced with fresh medium, with or without drug (AZT, decitabine, gemcitabine, or CPEC). The drug concentration used was

approximately the IC_{80} – IC_{90}, with the exception of CPEC, where the concentration used was approximately the EC_{50}. The supernatant was collected 24 h post treatment and 25 µl was used to infect target CRFK cells that were plated 24 h prior to a density of 6.5 x 10^4 cells per well on a 12-well plate. The transfected 293T cells were trypsinized, centrifuged at 1,200 g for 5 min and the cell pellets were resuspended in 2% paraformaldehyde in PBS with 2% FC3. These samples were then analyzed by flow cytometry for GFP expression. The target cells underwent a medium change 24 h post-infection, and 48 h post infection they were also trypsinized, pelleted, and resuspended in 2% paraformaldehyde in PBS with 2% FC3. These samples were then analyzed by flow cytometry for GFP expression. Transfected 293T (producer) cells that were used to produce FeLV in the presence of drug were analyzed by flow cytometry. Flow cytometry was also used to analyze the infected CRFK (target) cells. The relative GFP expression for both the transfected producer (293T) cells and the infected target (CRFK) cells sample were calculated by setting no drug to 100% and multiplying the data for each individual treatment by the factor used to convert the non-treated group to 100%.

Analysis of FeLV mutation spectra by Illumina DNA sequencing. CRFK cells (2 x 10^5) were plated into each well of a 6-well plate 24 h prior to viral infection. The cells were pretreated with drug (decitabine or gemcitabine) for 2 h prior to viral infection; CPEC had a 24 h pretreatment time. After the 2 h or 24 h pretreatment, the cells were infected with FeLV-GFP to a MOI of 2. The virus

used for infections was pretreated with *DNAse I* (20 U/ml) for 1.5 h at 37ºC to eliminate plasmid contamination. The medium was removed and replaced with fresh DMEM 24 h post infection. The cells were trypsinized and centrifuged at 1,200 g for 5 min. Cell pellets were used for genomic DNA extraction according to the manufacturer's instructions of ZymoBead^{TM} Genomic DNA Kit (Zymo Research). PCR was then used to amplify proviral regions of *gag*, *pol*, *env*, and two segments of *gfp* genes. These primers were specifically designed for Illumina® next-generation sequencing. The primers contained 2 degenerate bases to normalize reaction initiation and a barcode (underlined) on the forward primer only:

5'NNTTTGGATCAACCAACCTGGGACGACT3' and

5'NNAATGACATTGGGCAGCTGGGTT3' (gag, p27),

5'NNTTTGGTCCAGAAATAGGACTGTCAGGGCA3' and

5'NNAGCCAGCAAGAGGTCATCTACA3' (pol, reverse transcriptase),

5'NNTTTGGTCCCGACAATCTCAAACAGGGT3' and

5'NNTCGGTGGCATTTAAGGCTAGGT3' (env, gp 70),

5'NNTTTGGTTCTTCAAGGACGACGGCAACT3' and

5'NNGGCCATGATATAGACGTTGTGGCT3' (gfp 1),

5'NNTTTGGAGGTGAACTTCAAGATCCGCCA3' and

5'NNATGTGATCGCGCTTCTCGTT3' (gfp, 2).

The entire primer set is listed in Table I-1 of Appendix I. A plasmid control also underwent PCR to monitor PCR generated background mutation. PCR

reactions were done in triplicate for each amplicon, with respect to treatment and internal replication using the high fidelity *Phusion Hot Start II polymerase* (Finnzymes). Each internal PCR replication was pooled for each treatment and gel purified to remove primers and genomic DNA. The gel band was excised and cleaned using the Wizard SV Gel and PCR Clean-up System (Promega). The PCR amplicons were ligated into pGEM-T vector using the pGEM®-T and pGEM®-T Easy Vector Systems (Promega), according to the manufacturer's instructions, and transformed into DH5α (*Escherichia coli*). Individual colonies were isolated, expanded, and the DNA plasmid recovered by Invitrogen PureLink Quick Plasmid Miniprep kit. DNA samples for each amplicon was quantified by Quant-iT™ PicoGreen® dsDNA Assay kit (Invitrogen) for an equimolar pooling between samples to a total concentration of 3 µg.

The samples were submitted to University of Minnesota BioMedical Gemonics Center where the quality of the DNA was assessed using a fluorimetric PicoGreen assay. In order to pass quality control, prior to library construction, the sample must have a quantity greater than 1 µg. Then 1 µg of the sample was used for library creation using the Illumina's Truseq DNA Sample Preparation Kit (cat#: FC-121-2001, see www.illumina.com for kit content and methods). Library generation involved fragmenting 1 µg of genomic DNA using Covaris ultrasonic shearing and repairing the ends prior to ligating adaptor and index sequences. Caliper XT (see www.perkinelmer.com for methods) was used to selects fragments of an average of 400 base pairs. The library was then

amplified using 10 cycles of PCR and validated using capillary electrophoresis and quantified by the fluorimetric PicoGreen assay and/or Kapa Q-PCR.

For cluster generation, the library was hybridized to a paired end flow cell where individual fragments are clonally amplified by bridge amplification onboard the MiSeq system. After clustering, sequencing was conducted using Illumina's SBS chemistry. After read 1 was performed, a 6 base pair index read was performed. Lastly, the library fragments were re-synthesized in the reverse direction and sequenced from the opposite end of the read 1 fragment. For primary analysis and de-multiplexing, Base call (.bcl) files for each cycle of sequencing are generated by Illumina Real Time Analysis (RTA) software. The base call files are de-multiplexed and then converted to index specific fastq files using the MiSeq Reporter software on instrument (Please visit www.illumina.com for further details), after which the data was process for bioinformatics.

Data analysis began with pre-processing and running quality control with the *Fastqc* program. The adapter sequencing attached to the amplicons were trimmed using the *Fastq-mcf* program. Further processing involved demultiplexing the data, leaving more than half (9,641,870 of 17,711,281) of the reads unmatched to any barcode. The presence of a PhiX spike accounted for some to this loss of sequencing depth. Demultiplexing was performed using *Fastq-multx* program. Paired end reads (3' forward and 5' reverse complement) were joined into a single read using *Fastq-join* program. Two-thirds of each sample were joinable, and the reads that did not overlap were excluded. A 6 bp

overlap and a 5% maximum difference between the two reads were the constraints for joining. The *Bowtie2* program was used to align each viral loci to the reference sequence at an overall acceptable rate of 99.45 - 93.04%. The extreme edge base-pair of each loci were excluded due to a significant degradation in mapping quality. The *Samtools* were used to produce pileup files that detailed the nature of the alignment for each read in accordance to the reference database. These pileup files were used to compute mutations frequency and mutation spectra.

Mutation spectra were analyzed by computing the types of mutations that occurred within each viral loci per drug treatment. The primary computation was custom-designed with the *Phython* program. The strategy was to quantify mutations as a matrix of 'starting nucleotide' and 'ending nucleotide.' For the preliminary analysis the R was used to compute whether the spectra of mutations were different between drug treatments versus the no drug control. The mutation matrix was converted to a proportion matrix, and the distance score between each pair of matrices was computed. For the final analysis, the number for each type of base pair change was quantified and compared to no drug. The final statistical analysis was done using the GraphPad Prism program. For each drug, statistical significance was determined using a one-way ANOVA with a Tukey post-test comparing the no drug to each drug treatment.

Mutation frequency was analyzed by computing mutation per base-pair sequenced using the same custom-designed program used for the mutation

spectra. The mutation frequency of each viral loci for each drug treatment were compared to that of no drug control. The R was used to compute whether mutation rates per locus (mutations/read mapped) were higher in drug versus control. The data was preliminarily analyzed using a T-test one-tailed. For the final analysis, the number for each base pair change was quantified and divided by the number of base pair sequenced for each drug treatment to calculate the mutation frequency. The frequency of each drug treatment was compared to that of to no drug control. The final statistical analysis was done using the GraphPad Prism program. For each drug, statistical significance was determined using a one-way ANOVA with a Tukey post-test comparing the no drug to each drug treatments.

dNTP Pool analysis. CRFK cells (6×10^6) or MAGI cells (3.5×10^6) were split between two 10 cm culture dishes per treatment group 16 - 24 h prior to drug treatment. The cells were treated with drug (AZT, decitabine, or gemcitabine) for 4 h to simulate the 2 h pretreatment of the infection assay and 2 h for reverse transcription to take place (Daelemans, Pauwels et al. 2011). The CPEC treatments underwent a 24 h pretreatment prior to dNTP extraction. After the 4 h or 24 h drug incubation, the cells were trypsinized and centrifuged at 1,200 g for 5 min.

The cell pellet was resuspended in 1 ml of ice cold PBS. They were counted and 5×10^6 (CRFK) or 3×10^6 (MAGI) cells were removed for dNTP extraction. These cells were centrifuged at 1.2 g for 5 min and PBS removed

from pellet. The pellet was then resuspended in 500 µL 65 % methanol containing 10 µM of the internal standard 5'-Iodo-2'dCTP (IdCTP), purchased from TriLink BioTechnologies (San Diego, CA). The cell suspension was vigorously vortexed for 2 min and stored at -80C overnight to precipitate proteins. After the 24 h incubation, the cells were centrifuged at 14,000 g for 3 min. The supernatant was collected and underwent speed-vacuum centrifugation to remove the methanol. The dried pellet was submitted for ion-pair based liquid chromatography-tandem mass spectrometry (LCMS-MS), utilizing the hexylamine ion-pair agent system.

The LCMS-MS was performed using an Agilent 1260 HPLC couple with an AB SCIEX QTRAP 5500 mass spectrometer. To analyze the samples, an electrospray source was operating in positive and negative mode polarity switching. The source and parameters were set to the following: curtain gas: 20; CAD gas: medium; ion spray voltage: 4500; temperature: 600°C; gas1: 45; and gas 2: 45. Deoxynucletides and internal standard (IS) were analyzed using multiple reaction monitoring (MRM) using the parameters listed in Table II-1 (Appendix II). Each dNTP was distinguishable by its unique mass post fragmentation. Figure II-1 illustrates the fragmentation sites for each dNTP and IS (Appendix II).

The samples were separated using a 150mm × 3.9 mm Waters XTerra MS C-18 column (5 µm) 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) with a flow rate of 0.3 ml/min (Zhang, Tan et al. 2011). Analytes were eluted using a linear gradient of 0% to 30% B over 30 min. The column was then washed with mobile phase B (95%) for 3 min and re-equilibrated with mobile phase A (100%) for 15 min. Each LCMS-MS run was exported into MultiQuant (AB SCIEX), which was used to calculate peak areas. An example chromatograms for dATP, dCTP, dTTP, and the IS are illustrated in Figure II-2 A of Appendix II. Because dGTP has a lower peak than the other dNTP, a separate chromatogram is usually illustrated (Figure II-2 B of Appendix II). Data was interpreted as integrated peak area, and normalized to the internal standard, 5-iodo-2'-dCTP (IdCTP) for mass spectrometry calibration, then normalized to no drug (DMSO only control). This data was analyzed using one-way ANOVA, with a Tukey post-hoc test (Graphpad Prism).

Generation of the FeLV YXDD reverse transcriptase mutant. The FeLV-GFP wild type was the pEECC-FeLV replication competent construct (NIH AIDS Reagent Program) that has the *ires-gfp* gene inserted into the *env* gene, previously described (Greggs, Clouser et al. 2012). The YMDD RT mutant was constructed by site directed mutating the valine of the normal Y[V]DD motif to a methionine to resemble the Y[M]DD motif of HIV-1. To synthesize this construct, a segment of RT was subcloned, using restriction enzyme *MfeI* (New England Biolabs). Primers: CCTCCTACAATATATGGATGACCTCTT (forward) CCAGCAAGAGGTCATCCATATATTGT (reverse); were used to alter the Y[X]DD motif using site directed mutagenesis. The amplification conditions were 95 ºC for

1 min, followed by 18 cycles of 95ºC for 50 s,60 ºC for 50s, and 68 ºC for 6 min; and 68 ºC for 7 min. The RT segment was digest out of the pGEM-T vector with restriction enzymes *MfeI* and *BspII*. The RT segment was gel purified along with the original FeLV-GFP construct that was digested with the same restriction enzymes. These were gel purified using the wizard SV Gel and PCR Clean-Up Kit system (Promega). The FeLV-GFP plasmid was digested with Mfe I to linearize, and dephosphorylated with antarctic phosphatase (New England Biolabs).

The RT sequence and the FeLV-GFP backbone segment was ligated at 4ºC in a 1:3 (plasmid backbone: DNA insert) molar ratio. The ligation product was transformed into DH5α (*Escherichia coli*) cells (Invitrogen) and plated onto Luria-Bertani (LB) agar (Becton Dickinson) containing 50µg ampicillin ml^{-1}. Individual colonies were selected and cultured in LB broth containing 50 µg ampicillin ml^{-1}, which were purified using PureLink Quick Plasmid Miniprep kit (Invitrogen). Plasmid DNAs were sequenced to verify the creation of the desired mutant.

	t	df	p
AZT	.9474	13	.3607
Decitabine	.7944	13	.4412
Gemcitabine	.4456	13	.6632
CPEC	.1585	12	.8767

Table 3-1. Statistical analysis of the effects of decitabine, gemcitabine, and cyclopentenyl cytosine on the late phase of FeLV replication. The data represents the statistical analysis of target (CRFK) cells infected with FeLV produced in the presence or absence of drug. Data analyzed using two-tailed student t-test.

	t	df	p
AZT	.00245	14	.9981
Decitabine	.1953	14	.8480
Gemcitabine	.1417	15	.8892
CPEC	.4476	14	.6613

Table 3-2. Statistical analysis of the effects of decitabine, gemcitabine, and cyclopentenyl cytosine on viral transfection efficiency. The data represents the statistical analysis of producer (293T) cell cotransfected with FeLV-GFP and VSV-G envelope in the presence or absence of drug. Data analyzed using two-tailed student t-test.

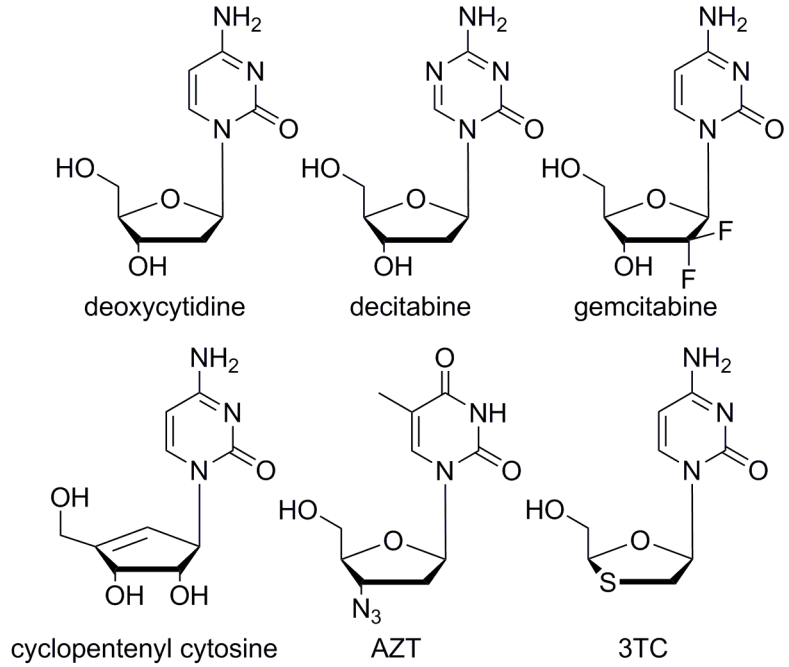


Figure 3-1. Illustration of the chemical structure of cytosine analogs. Shown are the chemical structures of deoxycytidine and the cytosine analogs decitabine, gemcitabine, and cyclopentenyl cytosine (CPEC), as well as azidothymidine (AZT) and the cytosine analog lamivudine (3TC). Figures were drawn using ChemBioDraw Ultra 13.0 software.

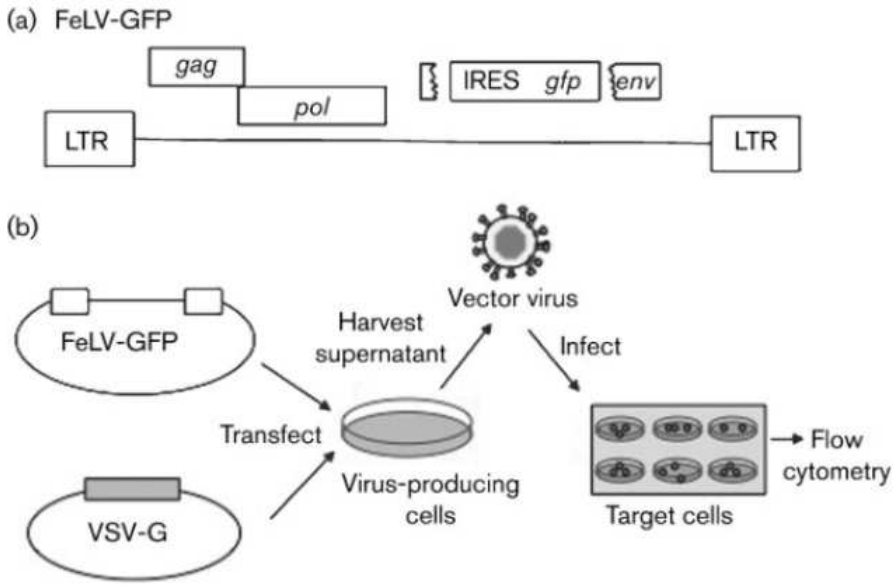


Figure 3-2. FeLV-GFP vector and model system for monitoring virus infectivity. (a) The FeLV vector, FeLV-GFP, shown with the *gfp* gene inserted in *env* gene. (b) Single-replication-cycle assay performed by infecting CRFK target cells. Infectious viral particles were created by the cotransfection of the FeLV-GFP vector with the VSV-G envelope into 293T cells. Viral particles were harvested and used to infect CRFK target cells. Viral infectivity was monitored by flow cytometry.

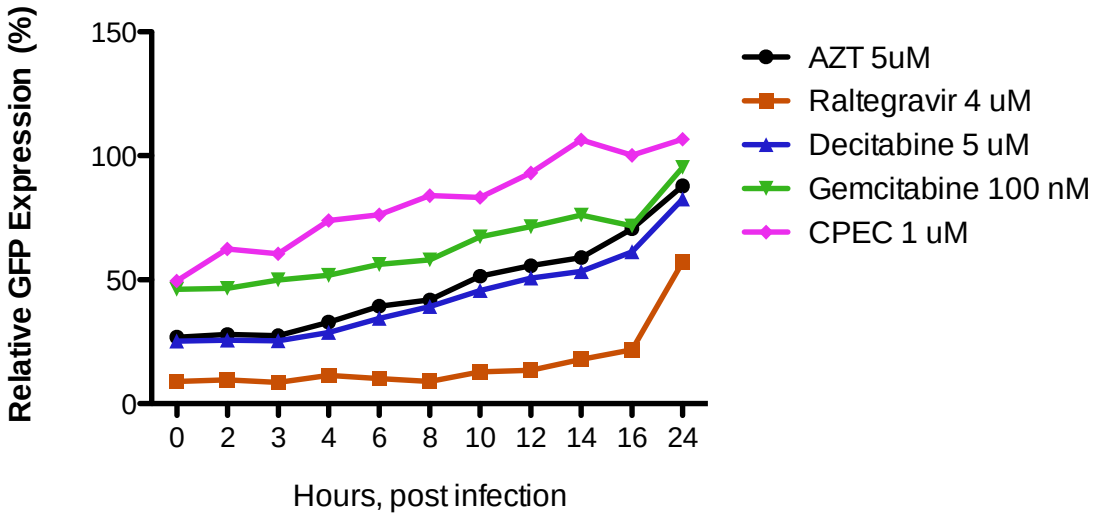
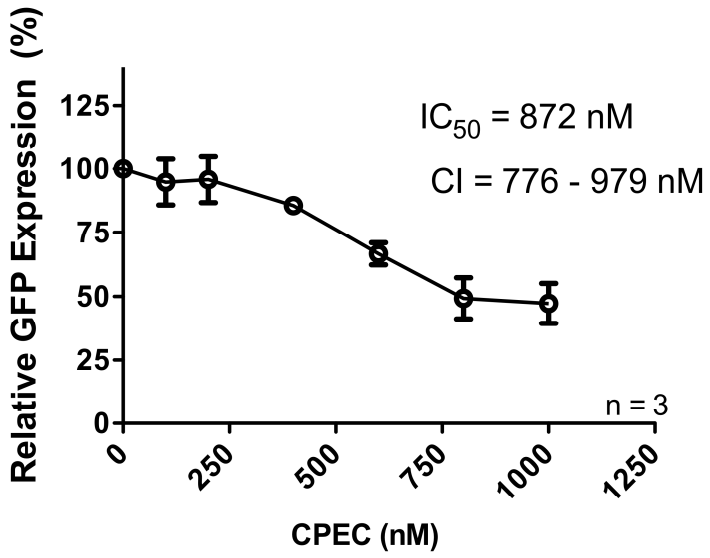


Figure 3-3. Time-of-addition assay identifies reverse transcription as the antiviral target of decitabine, gemcitabine, and cyclopentenyl cytosine (CPEC). CRFK cell were infected with FeLV-GFP pseudotyped with VSV-G envelope. Cells were treated with the indicated drug for the indicated times post-infection. Infectivity was measured by flow cytometry. Loss of infectivity was determined by a trending increase in GFP expression, which correlates to loss of drug activity (rebound in infection).

A)



B)

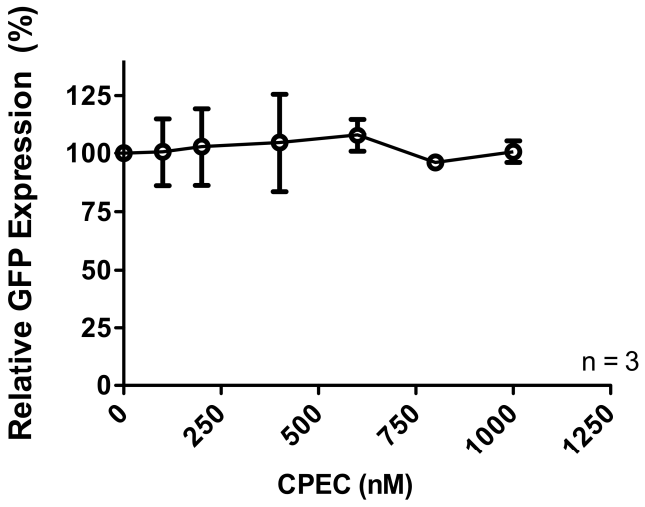
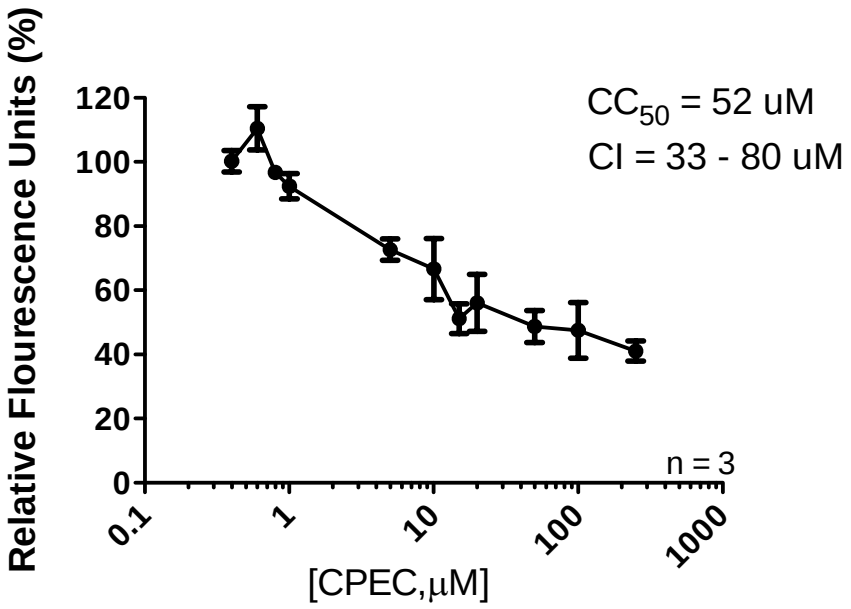


Figure 3-4. Antiviral activity of cyclopentenyl cytosine. MAGI cells pretreated for 24 h with the indicated concentrations of cyclopentenyl cytosine (CPEC), after

which they were infected with the pseudotyped (A) FeLV-GFP or (B) HIV-GFP viral stock. Flow cytometry was used to monitor infectivity. Data was normalized to the no drug control. IC_{50} values were calculated using GraphPad Prism. Data represents the mean with ± the standard error mean of 3 independent replicates.

A)



B)

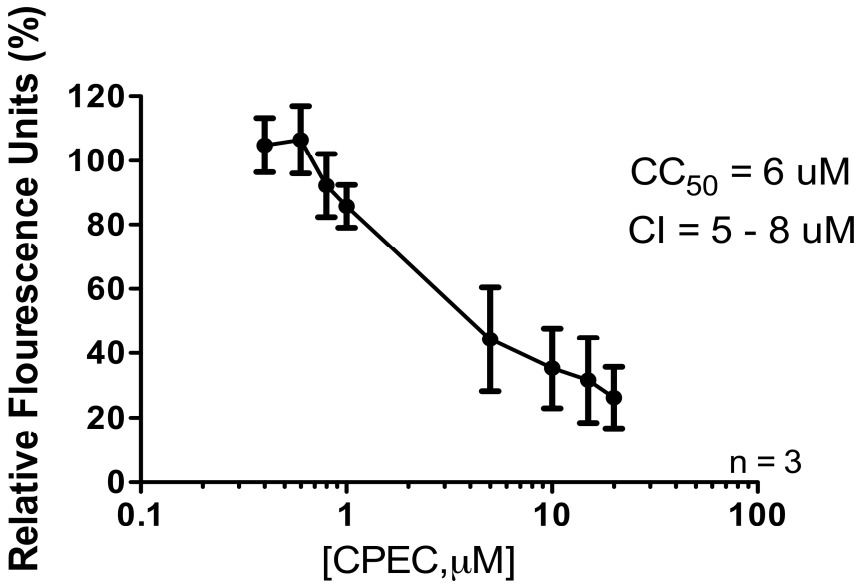
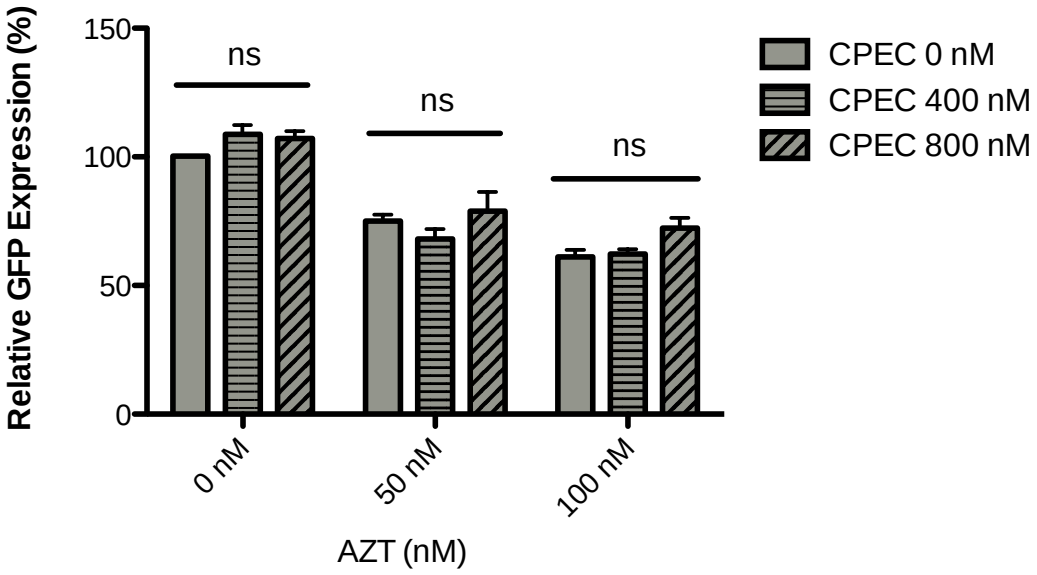
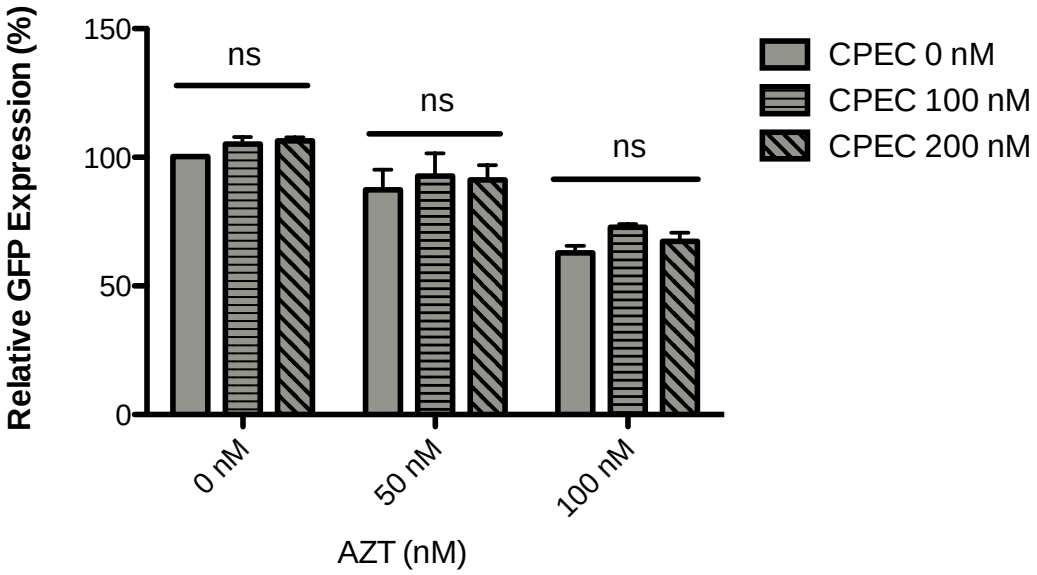


Figure 3-5. Cytotoxic effects of cyclopentenyl cytosine in MAGI and CRFK cell lines. A) MAGI and B) CRFK cells were exposed to the indicated concentration of CPEC for 72 h. After which, they were tested for cell viability using the Promega CellTiter-Glo kit by adding a luciferase based substrate that emits light at 570 nm in the presence of ATP. Data was normalized to the no drug control. Data represents the mean with ± the standard error mean of 3 independent replicates.

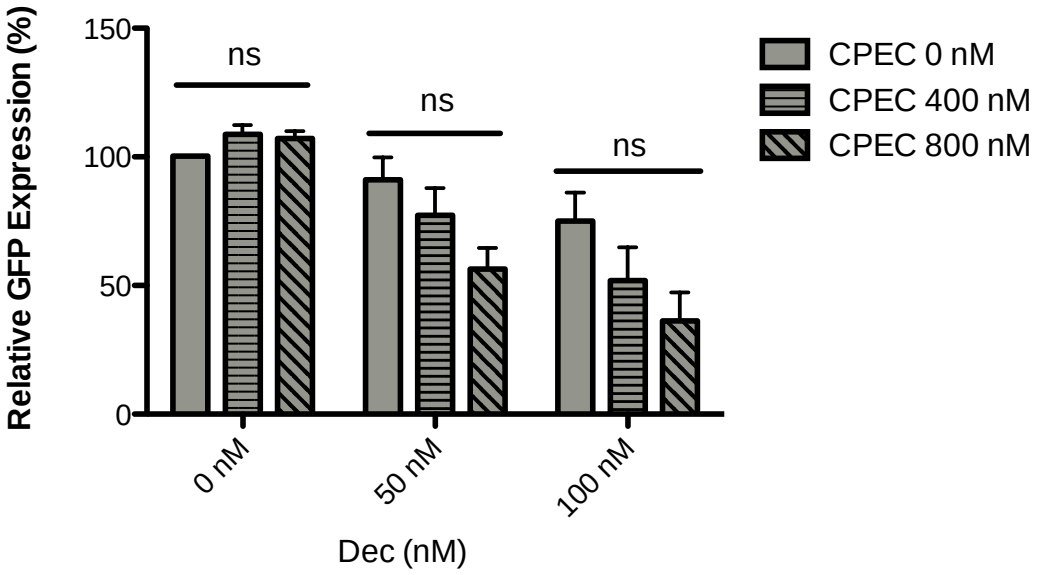
A)


Relative GFP Expression (%)
150
100
50
0
ns
ns
ns
0 nM
50 nM
100 nM
AZT (nM)
CPEC 0 nM
CPEC 400 nM
CPEC 800 nM

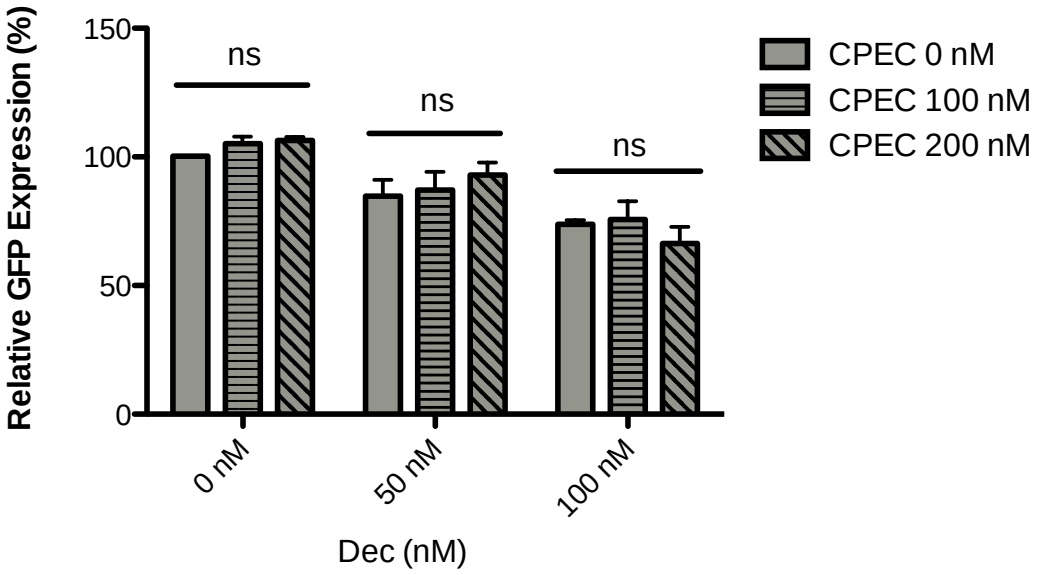
B)


Relative GFP Expression (%)
150
100
50
0
ns
ns
ns
0 nM
50 nM
100 nM
AZT (nM)
CPEC 0 nM
CPEC 100 nM
CPEC 200 nM

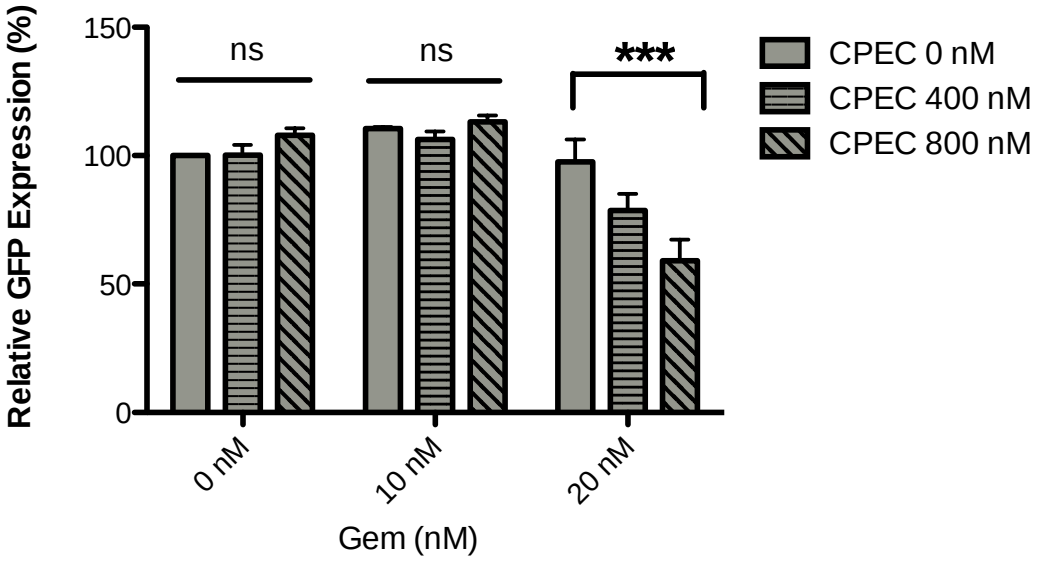
C)


Relative GFP Expression (%)
150
100
50
0
ns
ns
ns
0 nM
50 nM
100 nM
Dec (nM)
CPEC 0 nM
CPEC 400 nM
CPEC 800 nM

D)


Relative GFP Expression (%)
150
100
50
0
ns
ns
ns
0 nM
50 nM
100 nM
Dec (nM)
CPEC 0 nM
CPEC 100 nM
CPEC 200 nM

E)



F)

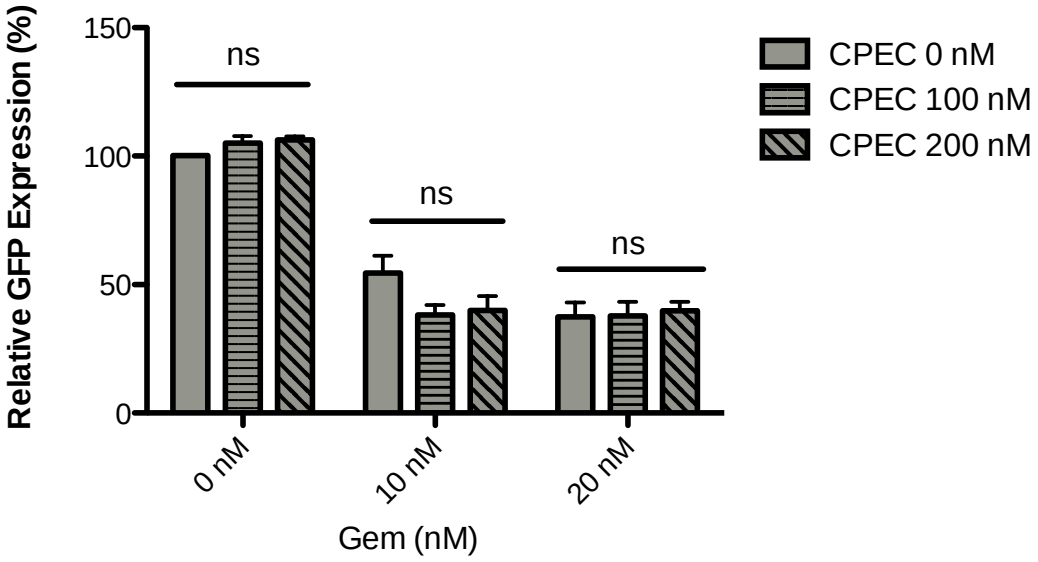
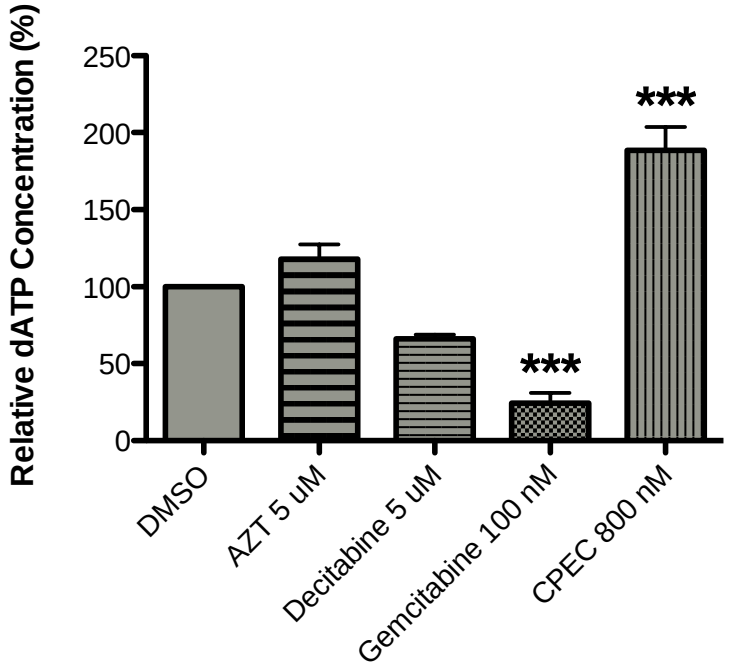


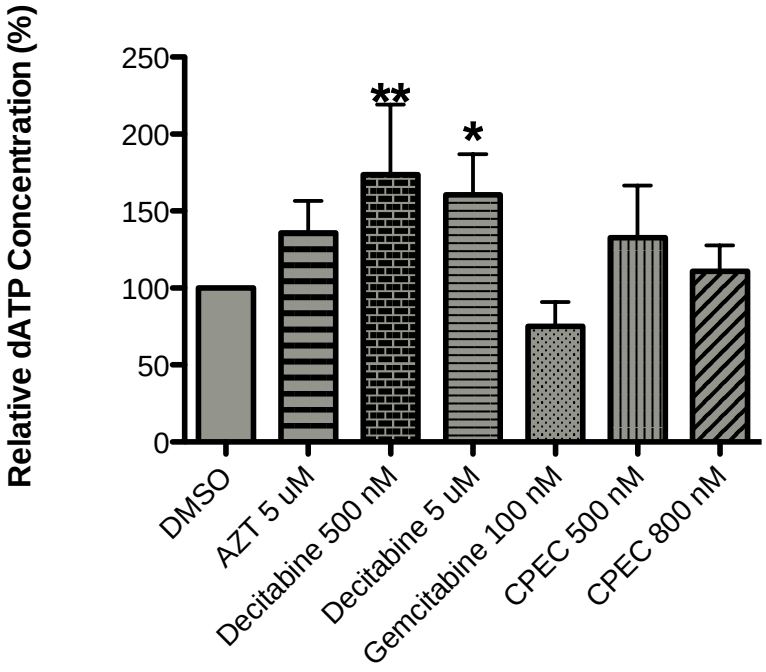
Figure 3-6. The potentiating effects of cyclopentenyl cytosine in combination with other cytosine analogs. MAGI cells were pretreated for 24 h with cyclopentenyl cytosine (CPEC) and the treated with AZT, with A) HIV-1 or B)

FeLV; decitabine with C) HIV or D) FeLV; or gemcitabine with E) HIV-1 or F) FeLV; 2 h prior to viral infection, at the concentrations indicated. Flow cytometry was used to monitor infectivity. Each treatment was normalized to no drug. Data represents the mean with ± the standard error mean of 3 independent replicates. Data was analyzed using a one-way ANOVA with a Tukey post test. *** $p \leq 0.001$.

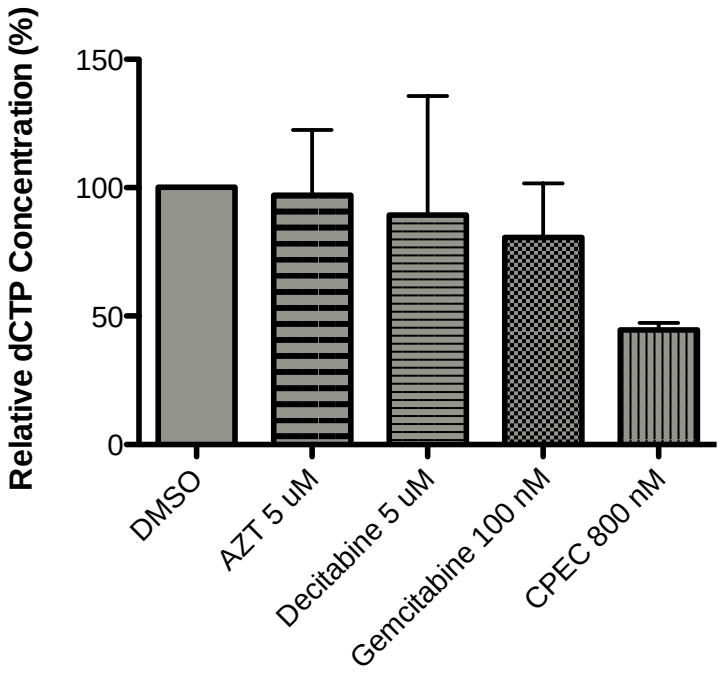
A)



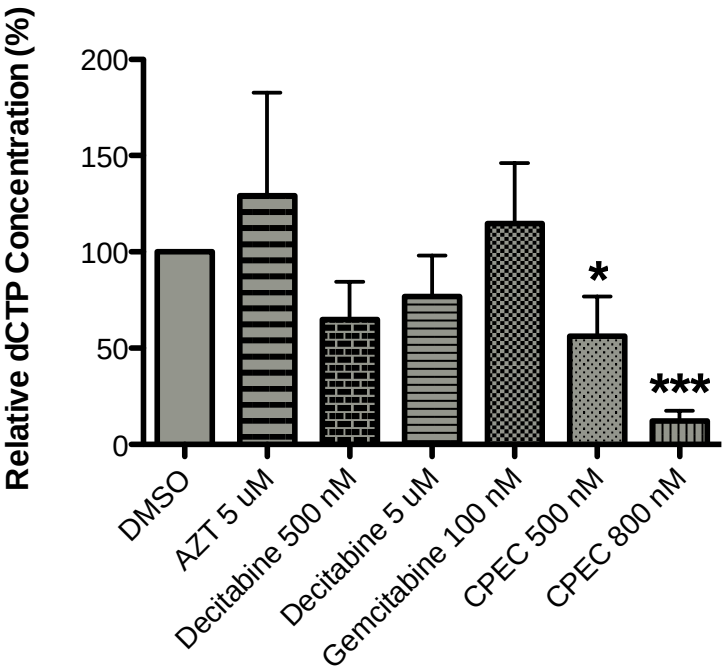
B)


Relative dATP Concentration (%)
250
200
150
100
50
0
**
*
DMSO
AZT 5 uM
Decitabine 500 nM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 500 nM
CPEC 800 nM

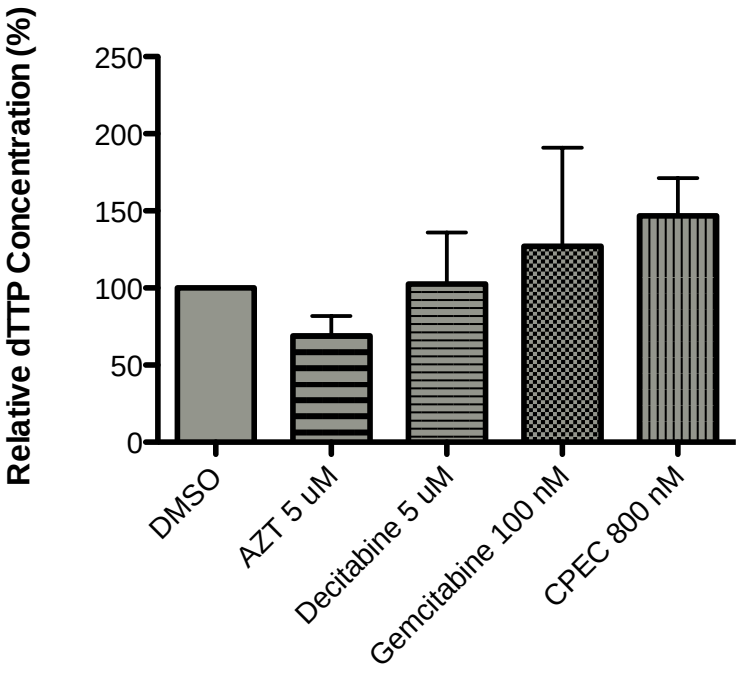
C)


Relative dCTP Concentration (%)
150
100
50
0
DMSO
AZT 5 uM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 800 nM

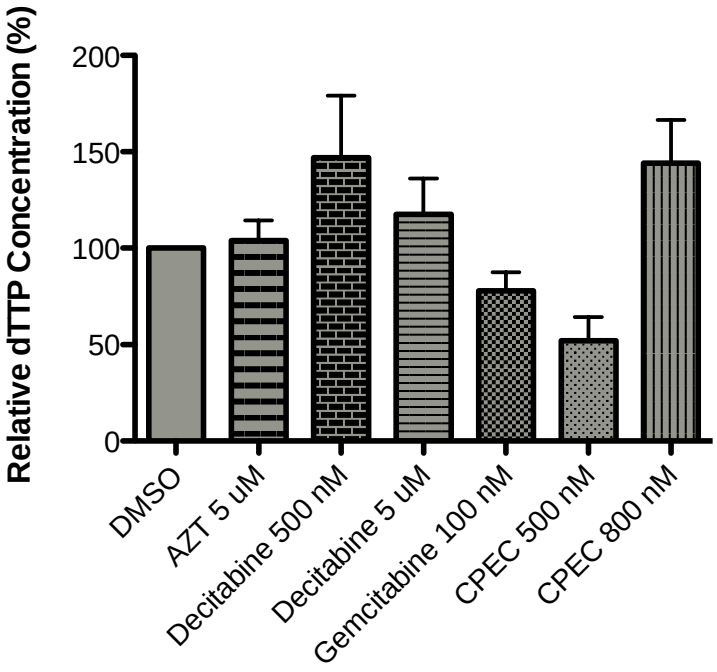
D)


Relative dCTP Concentration (%)
200
150
100
50
0
*
***
DMSO
AZT 5 uM
Decitabine 500 nM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 500 nM
CPEC 800 nM

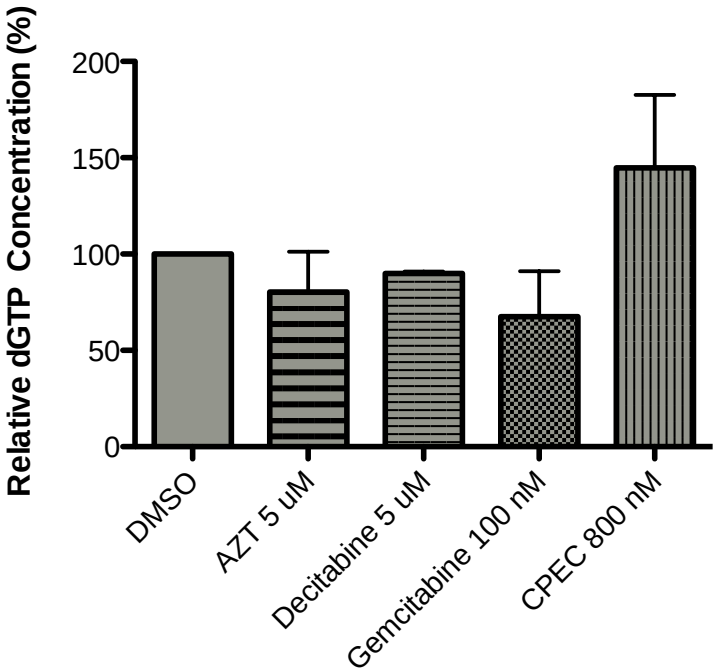
E)


Relative dTTP Concentration (%)
250
200
150
100
50
0
DMSO
AZT 5 uM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 800 nM

F)


Relative dTTP Concentration (%)
200
150
100
50
0
DMSO
AZT 5 uM
Decitabine 500 nM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 500 nM
CPEC 800 nM

G)


Relative dGTP Concentration (%)
200
150
100
50
0
DMSO
AZT 5 uM
Decitabine 5 uM
Gemcitabine 100 nM
CPEC 800 nM

H)

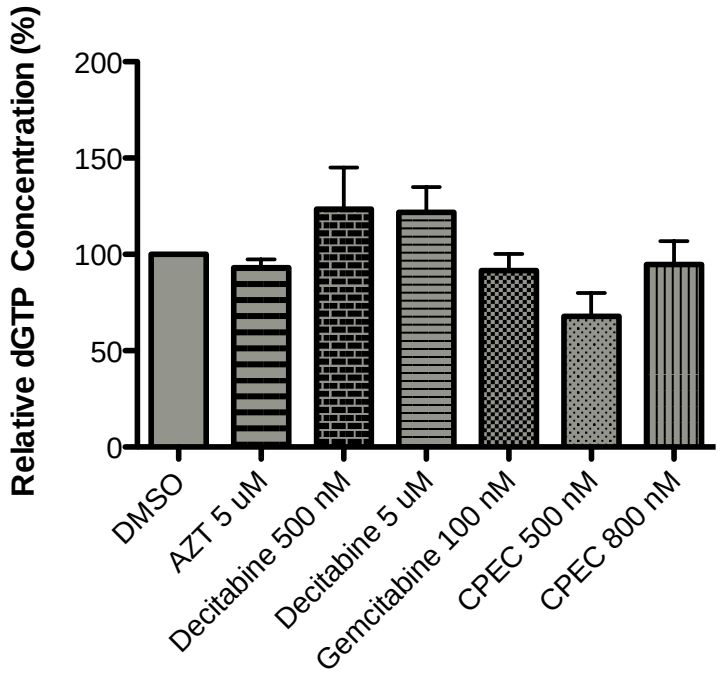
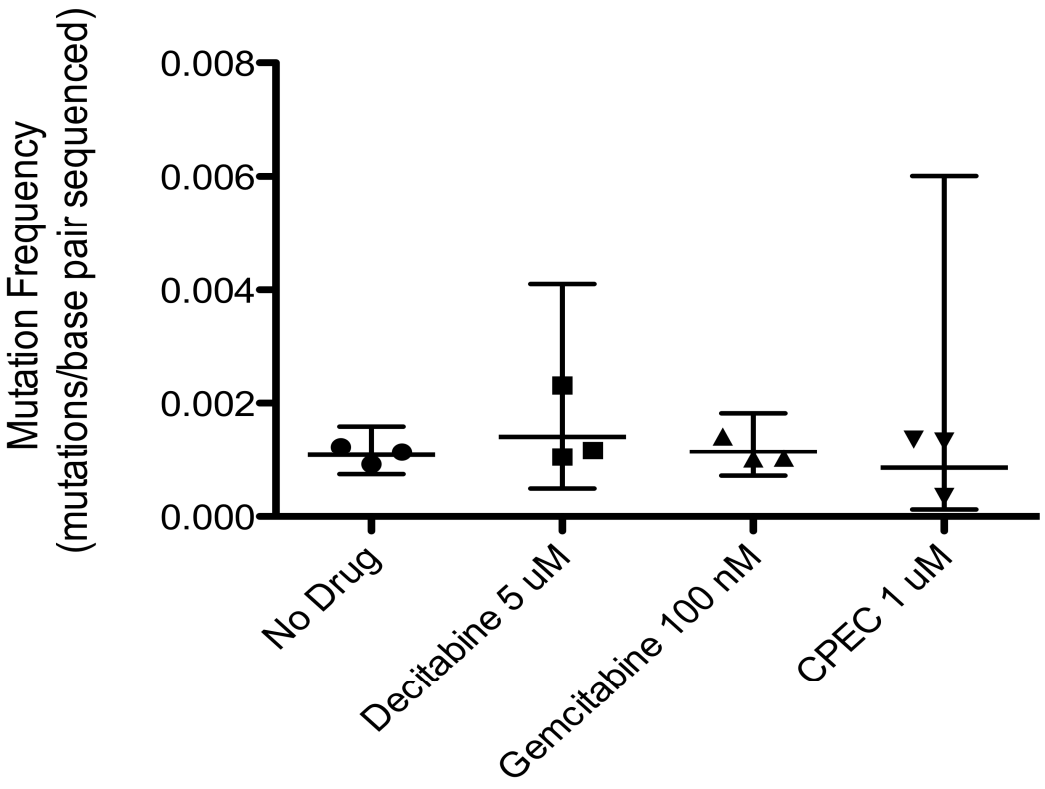


Figure 3-7. Decitabine, gemcitabine, and cyclopentenyl cytosine effects on the dNTP pools of MAGI and CRFK cell lines. MAGI or CRFK cells were treated with the indicated concentrations of AZT, decitabine, gemcitabine, or cyclopentenyl cytosine (CPEC). Twenty-four hours post-treatment, 3 x 10^6 cells were used for dNTP extraction in the MAGI cell line and 5 x 10^6 cells were used for dNTP extraction in the CRFK cell line. Liquid chromatography-tandem mass spectrometry (LCMS-MS) was used to monitor concentration of dATP A) MAGI, B) CRFK; dCTP C) MAGI, D) CRFK; dTTP E) MAGI F) CRFK; and dGTP G) MAGI H) CRFK. The LCMS-MS readings were normalized to an internal standard. Data was normalized to no drug (DMSO control). Data represents the

mean with ± the standard error mean of 3 independent replicates. Data was analyzed using a one-way ANOVA with a Tukey post test. * p ≤ 0.05, *** p ≤ 0.001.

A)



B)

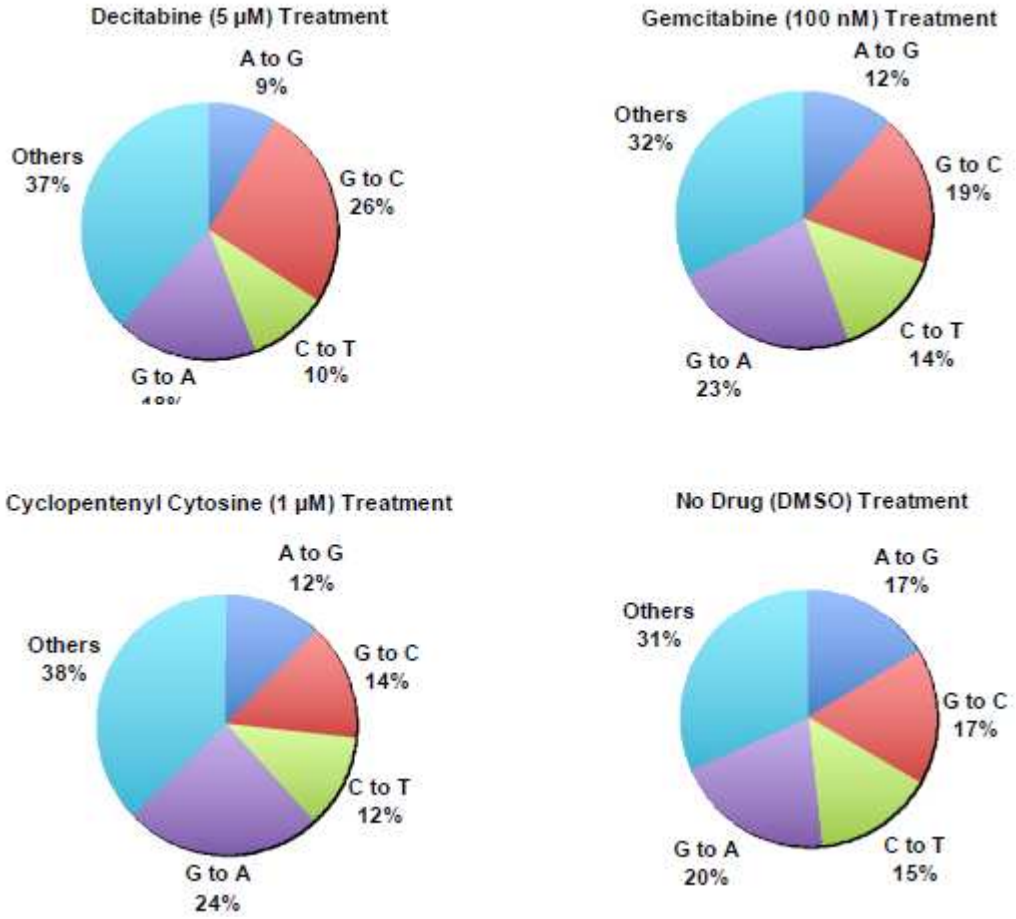


Figure 3-8. Mutation frequency and spectra of FeLV in the presence of decitabine, gemcitabine, and cyclopentenyl cytosine. CRFK cells were pretreated with either decitabine 5 µM, gemcitabine 100 nM, or cyclopentenyl cytosine (CPEC) 1 µM for 2 h prior to infection. After 48 h post infection, the genomic DNA was extracted from the cells and the proviral DNA, as indicated in the Materials and Methods, was PCR amplified. The sequenced using Illumina DNA sequencing. A) The mutation frequency was calculated by the number of mutation per base sequenced. Data represents the mean with ± the 95 % confidence interval of 3 independent replicates. Data was analyzed using a one-

way ANOVA with a Tukey post test. B) The FeLV mutation spectra was calculated by dividing the number of mutations for each mutation type by the total number of mutations observed for each drug treatment. For decitabine: A to T = 285/3294, G to C = 844/3294, C to T = 324/3294, G to A = 587/3294, all other mutation types = 1254/3294. For gemcitabine, A to T = 256/2204, G to C = 415/2204, C to T = 308/2204, G to A = 513/2204, all other mutation types = 712/2204. For cyclopentenyl cytosine, A to G = 297/2456, G to C = 356/2456, C to T = 287/2456, G to A = 601/2456, all other mutation types = 915/2456. For no drug (DMSO), A to G = 370/2242, G to C = 379/2242, C to T = 334/2242, G to A = 448/2242, all other mutations = 711/2242. The calculated values were normalized and then multiplied by 100 to determine the percentage of the total. Data was analyzed using a one-way ANOVA with a Tukey post test.

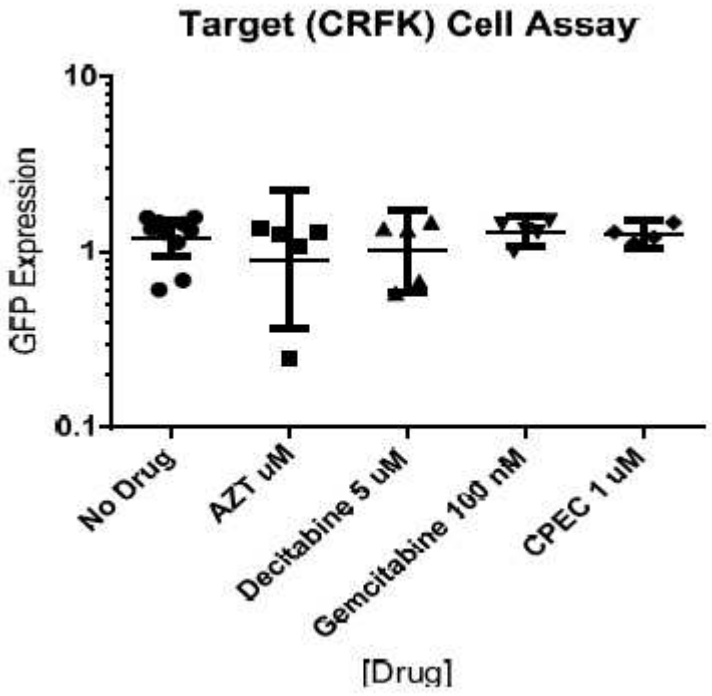


Figure 3-9. Analysis of the effects of decitabine, gemcitabine, and cyclopentenyl cytosine on the late phase of FeLV replication. CRFK cells were infected with FeLV produced in the presence of drug. The post-integration antiviral effects of these drugs were evaluated by measuring changes in infectivity of drug treatments in comparison to no drug. Infectivity was monitored by flow cytometry. Data represents the mean with ± the 95 % confidence interval of 5 or 9 independent replicates.

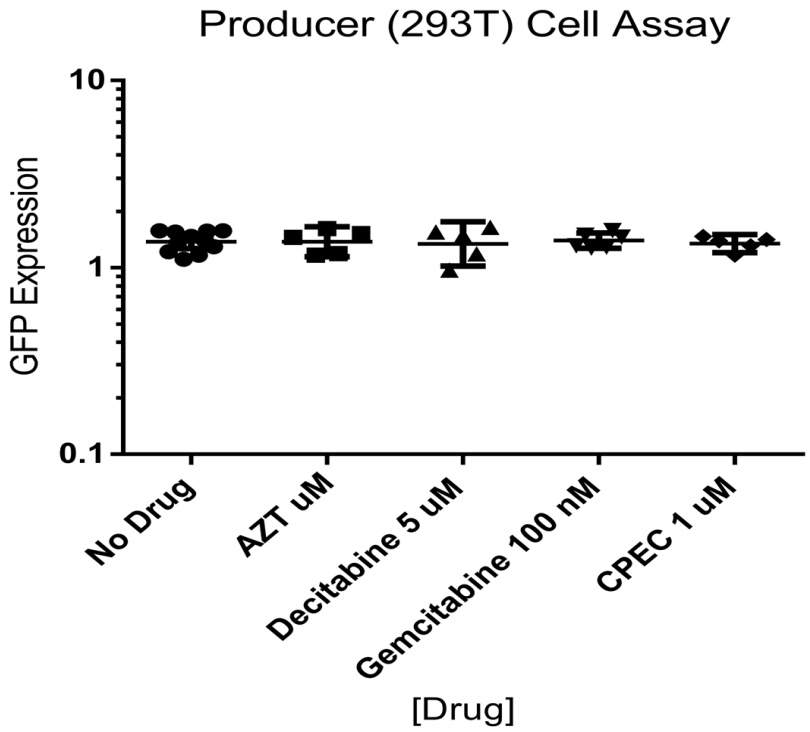
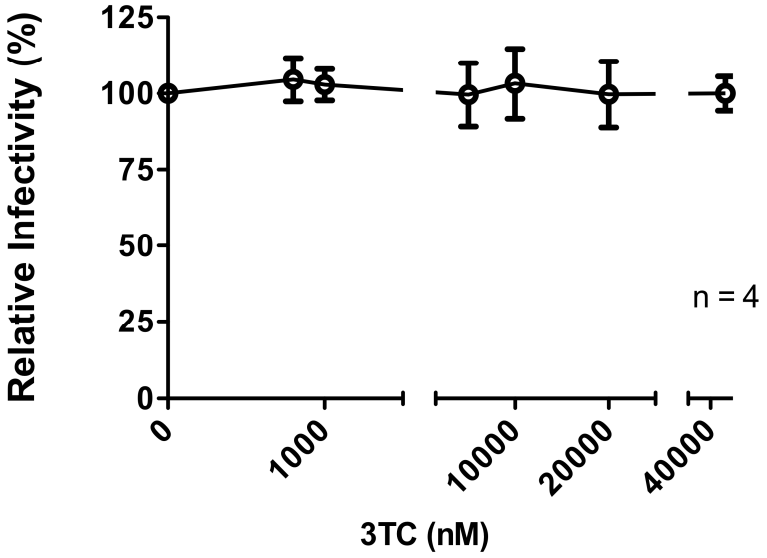
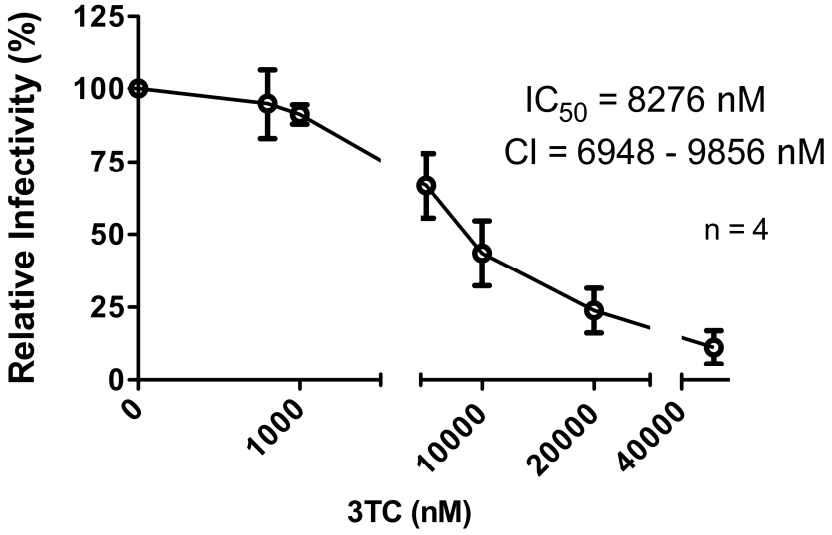


Figure 3-10. Analysis of the effects of decitabine, gemcitabine, and cyclopentenyl cytosine on viral transfection efficiency. 293T cells were cotransfected with FeLV-GFP and VSV-G envelope in the presence or absence of drug, as illustrated. The virus was harvested 48 h post transfection and immediately used to infect target cells. The producer (293T) cells were collected and analyzed for post-integration effects of these compounds by using flow cytometry to monitor changes in GFP expression. Data represents the mean with ± the 95 % confidence interval of 5 or 9 independent replicates.

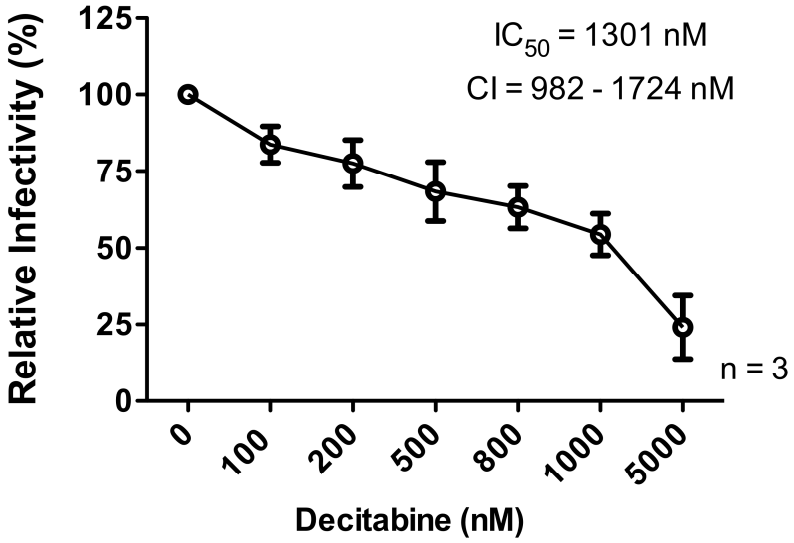
A)


Relative Infectivity (%)
125
100
75
50
25
0
n = 4
0
1000
10000
20000
40000
3TC (nM)

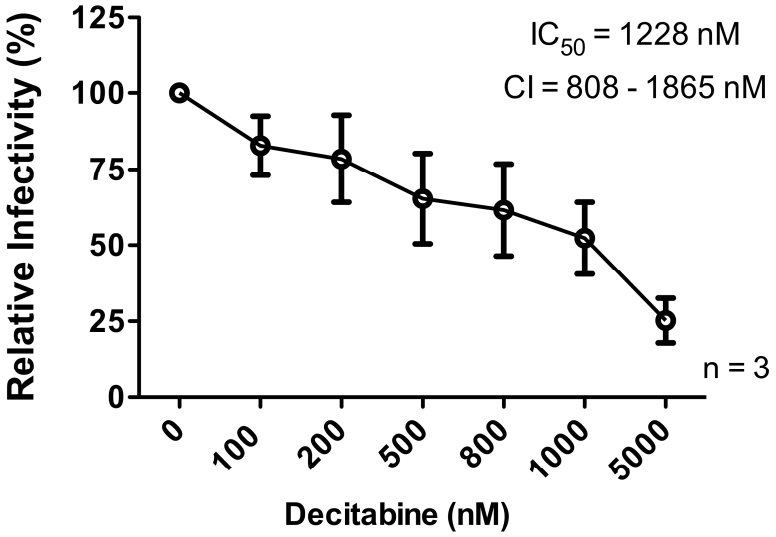
B)


Relative Infectivity (%)
125
100
75
50
25
0
IC50 = 8276 nM
CI = 6948 - 9856 nM
n = 4
0
1000
10000
20000
40000
3TC (nM)

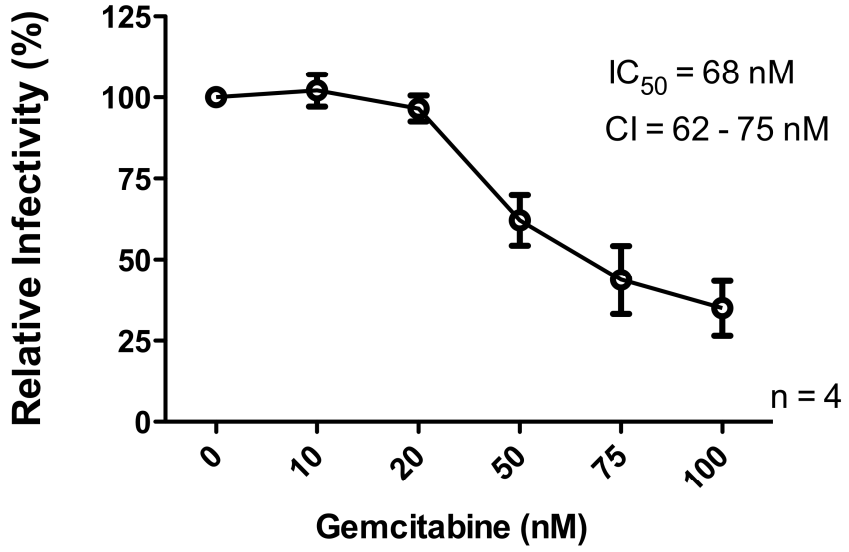
C)


Relative Infectivity (%)
125
100
75
50
25
0
IC50 = 1301 nM
CI = 982 - 1724 nM
n = 3
0
100
200
500
800
1000
5000
Decitabine (nM)

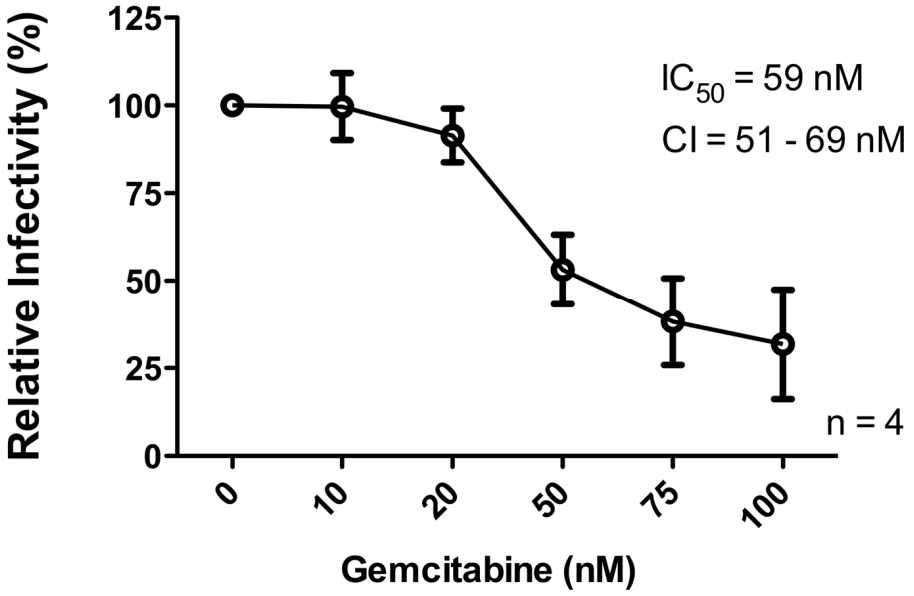
D)


Relative Infectivity (%)
125
100
75
50
25
0
IC50 = 1228 nM
CI = 808 - 1865 nM
n = 3
0
100
200
500
800
1000
5000
Decitabine (nM)

E)


Relative Infectivity (%)
125
100
75
50
25
0
IC50 = 68 nM
CI = 62 - 75 nM
n = 4
0
10
20
50
75
100
Gemcitabine (nM)

F)


Relative Infectivity (%)
125
100
75
50
25
0
IC50 = 59 nM
CI = 51 - 69 nM
n = 4
0
10
20
50
75
100
Gemcitabine (nM)

G)

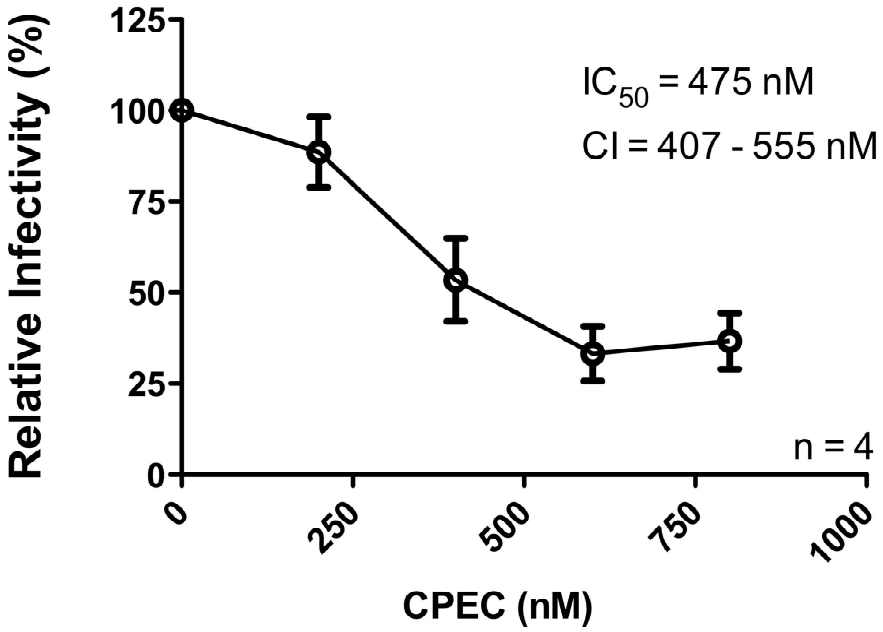


Figure 3-11. The effects of a YVDD to YMDD reverse transcriptase phenotype on the susceptibility to cytosine analogs decitabine, gemcitabine, and cyclopentenyl cytosine. CRFK cells were infected with virus after a pretreated for 2 h with 3TC A). wild-type FeLV or B) YMDD mutant; decitabine C) wild-type FeLV or D) YMDD mutant; gemcitabine E) wild-type FeLV or F) YMDD mutant; or cyclopentenyl cytosine (CPEC) G) YMDD-mutant. Flow cytometry was used to monitor infectivity 48 h post infection. Data normalized to no drug. IC_{50} values were calculated using GraphPad Prism. Data represents the mean with ± the standard error mean of 3 or 4 independent replicates.

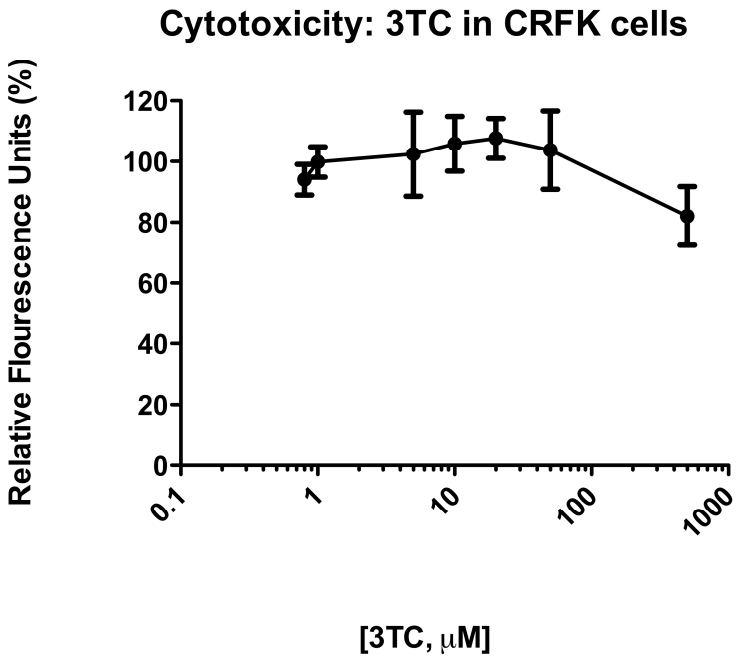


Figure 3-12. Cytotoxic effects of 3TC in the CRFK cell line. CRFK cells were exposed to the indicated concentration of cyclopentenyl cytosine (CPEC) for 72 h. Cells were then tested for cell viability using the Promega CellTiter-Glo kit by adding a luciferase based substrate that emits light at 570 nm in the presence of ATP. Data was normalized to no drug (see Materials and Methods). Data represents the mean with ± the standard error mean of 3 independent replicates.

Chapter IV

Dissertation Summary and Final Discussion

Study summary

The content of this dissertation was inspired by the need for antiretrovirals for the treatment the feline leukemia virus (FeLV). FeLV is the most common cause of cancerous disease and immunodeficiencies in cats world wide. The need for new antiretrovirals for treatment also presented the opportunity to exploit FeLV as a model for understanding the breadth of the antiviral spectrum of activity for these cancer related compounds; decitabine, gemcitabine, and cyclopentenyl cytosine (CPEC), while understanding the mechanism by which they can elicit their antiviral activity. This dissertation outlines that decitabine, gemcitabine, and CPEC possess antiviral activity against FeLV in the nanomolar range. Unlike what was seen for HIV-1, my studies suggest that these drugs may not enhance FeLV mutagenesis. Further research is needed to confirm this observation and to investigate other plausible mechanisms of action (e.g., inhibition of viral DNA synthesis).

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Evaluation of chain-termination activity

In order to evaluate if decitabine, gemcitabine, or CPEC are acting by inhibiting RT production, it is important to utilize quantitative PCR (qPCR) to monitor changes in late RT products when the virus is exposed to drug. If the drug elicits chain-terminating activity, as you would see with AZT or tenofovir, one would expect a decrease in late RT products detectable by qPCR. Because our data thus far suggests that these cytosine analogs have activity during the

reverse transcription phase of the retroviral life cycle, this is a likely candidate for the antiviral mechanism that should be further investigated.

Confirmation of viral DNA sequencing

To address the issue with elevated background that occurs with Illumina DNA sequencing, Sanger DNA sequencing could be done using the fluorescence protein gene, *gfp*. This would allow independent evaluation the mutagenic potential using a different DNA sequencing methodology. In the case of decitabine, it is believed that in HIV-1 its mutation activity is based on drug incorporation into the viral genome. This could be directly investigated by utilizing radio-labeling the compounds to visualize drug incorporation. This *in vitro* assay would involve negating cellular dependant mechanisms like drug phosphorylation and transport, allowing for addressing the simple question of whether these drugs could be substrates for FeLV reverse transcriptase.

Evaluation of the potential for clinical translation

Ultimately, clinical studies should be done to address the pharmacokinetic, pharmacodynamics, and cytotoxicity of decitabine, gemcitabine, and CPEC as antiretrovirals for FeLV. Though my study investigated cellular toxicity in a feline cell line, in order to get an accurate evaluation of toxicity, it is necessary to analyze this *in vivo*. This would address issues of drug activation, accumulation in tissues, targeted organ or cell type toxicity, etc. This would also assess the

clinical relevance of these compounds as antiretrovirals against FeLV. Since gemcitabine is currently approved as a cancer therapeutic in cats, there exist some information for the pharmacokinetics, and pharmacodynamics of this drug in felines. The use of gemcitabine for anti-FeLV therapy would involve significantly lower dosing, as indicated by this dissertation. Other drugs would need to have acceptable therapeutic indices. Currently, these drugs are given intravenously. A more compliant treatment regimen would be *per os*, so attempts have to be made to make these compounds orally available. Studies have been done to testing orally available derivative of decitabine and gemcitabine described by Clouser et al (2013); and so an extension of that study for FeLV infection would be important. Clinically, some cats that become infected can clear the infection. This mechanism for viral clearance is not completely understood. In practice, if safe antiretroviral can be given during early infection, it is possible to aid the newly infected cats with overcoming viral infection. The animal study should extend into exploring the role of antiretrovirals in latent and rebound infections in order to increase the probability of natural clearance and immunity in FeLV infected cats.

The use of anti-FeLV drugs would have the greatest clinical impact during the early stages of viral infection and prior to the onset of clinical pathology. A limitation of using nucleoside analogs is an active viral infection. Latent viral infections in the bone marrow would not be expected to be susceptible.

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APPENDIX I

IIlUMINA® NEXT-GENERATION SEQUENCING BARCODE LIST

Primer Set	Primer Name	Barcode	Sequence	Location	Ordered Primer	Sample	Rep
Pol 2	Pol-2F B145	TTTGG	TCCAGAAATAGGACTGTCAGGGCA	RT	NNTTTGGTCCAGAAATAGGACTGTCAGGGCA	Plasmid Ctrl	1
	Pol-2R B145		AGCCAGCAAGAGGTCATCTACA	RT	NNAGCCAGCAAGAGGTCATCTACA	Plasmid Ctrl	1
Env 1	Env-1F B145		TCCCGACAATCTCAAACAGGGT	gp70	NNTTTGGTCCCGACAATCTCAAACAGGGT	Plasmid Ctrl	1
	Env-1R B145		TCGGTGGCATTTAAGGCTAGGT	gp70	NNTCGGTGGCATTTAAGGCTAGGT	Plasmid Ctrl	1
Gag 1	Gag-1F B145		ATCAACCAACCTGGGACGACT	p27	NNTTTGGATCAACCAACCTGGGACGACT	Plasmid Ctrl	1
	Gag-1R B145		AATGACATTGGGCAGCTGGGTT	p27	NNAATGACATTGGGCAGCTGGGTT	Plasmid Ctrl	1
Gfp 1	Gfp-1F B145		TTCTTCAAGGACGACGGCAACT	gfp	NNTTTGGTTCTTCAAGGACGACGGCAACT	Plasmid Ctrl	1
	Gfp-1R B145		GGCCATGATATAGACGTTGTGGCT	gfp	NNGGCCATGATATAGACGTTGTGGCT	Plasmid Ctrl	1
Gfp 2	Gfp-2F B145		AGGTGAACTTCAAGATCCGCCA	gfp	NNTTTGGAGGTGAACTTCAAGATCCGCCA	Plasmid Ctrl	1
	Gfp-2R B145		ATGTGATCGCGCTTCTCGTT	gfp	NNATGTGATCGCGCTTCTCGTT	Plasmid Ctrl	1
Pol 2	Pol-2F B133	TGTAA	TCCAGAAATAGGACTGTCAGGGCA		NNTGTAATCCAGAAATAGGACTGTCAGGGCA	No Rx	1
Env 1	Env-1F B133		TCCCGACAATCTCAAACAGGGT		NNTGTAATCCCGACAATCTCAAACAGGGT	No Rx	1
Gag 1	Gag-1F B133		ATCAACCAACCTGGGACGACT		NNTGTAAATCAACCAACCTGGGACGACT	No Rx	1
Gfp 1	Gfp-1F B133		TTCTTCAAGGACGACGGCAACT		NNTGTAATTCTTCAAGGACGACGGCAACT	No Rx	1
Gfp 2	Gfp-2F B133		AGGTGAACTTCAAGATCCGCCA		NNTGTAAAGGTGAACTTCAAGATCCGCCA	No Rx	1
Pol 2	Pol-2F B134	TGTGC	TCCAGAAATAGGACTGTCAGGGCA		NNTGTGCTCCAGAAATAGGACTGTCAGGGCA	No Rx	2
Env 1	Env-1F B134		TCCCGACAATCTCAAACAGGGT		NNTGTGCTCCCGACAATCTCAAACAGGGT	No Rx	2
Gag 1	Gag-1F B134		ATCAACCAACCTGGGACGACT		NNTGTGCATCAACCAACCTGGGACGACT	No Rx	2
Gfp 1	Gfp-1F B134		TTCTTCAAGGACGACGGCAACT		NNTGTGCTTCTTCAAGGACGACGGCAACT	No Rx	2
Gfp 2	Gfp-2F B134		AGGTGAACTTCAAGATCCGCCA		NNTGTGCAGGTGAACTTCAAGATCCGCCA	No Rx	2
Pol 2	Pol-2F B135	TGTTT	TCCAGAAATAGGACTGTCAGGGCA		NNTGTTTTCCAGAAATAGGACTGTCAGGGCA	No Rx	3
Env 1	Env-1F B135		TCCCGACAATCTCAAACAGGGT		NNTGTTTTCCCGACAATCTCAAACAGGGT	No Rx	3
Gag 1	Gag-1F B135		ATCAACCAACCTGGGACGACT		NNTGTTTATCAACCAACCTGGGACGACT	No Rx	3
Gfp 1	Gfp-1F B135		TTCTTCAAGGACGACGGCAACT		NNTGTTTTTCTTCAAGGACGACGGCAACT	No Rx	3
Gfp 2	Gfp-2F B135		AGGTGAACTTCAAGATCCGCCA		NNTGTTTAGGTGAACTTCAAGATCCGCCA	No Rx	3
Pol 2	Pol-2F B140	TTGAT	TCCAGAAATAGGACTGTCAGGGCA		NNTTGATTCCAGAAATAGGACTGTCAGGGCA	Dec 5 uM	2
Env 1	Env-1F B140		TCCCGACAATCTCAAACAGGGT		NNTTGATTCCCGACAATCTCAAACAGGGT	Dec 5 uM	2
Gag 1	Gag-1F B140		ATCAACCAACCTGGGACGACT		NNTTGATATCAACCAACCTGGGACGACT	Dec 5 uM	2
Gfp 1	Gfp-1F B140		TTCTTCAAGGACGACGGCAACT		NNTTGATTTCTTCAAGGACGACGGCAACT	Dec 5 uM	2
Gfp 2	Gfp-2F B140		AGGTGAACTTCAAGATCCGCCA		NNTTGATAGGTGAACTTCAAGATCCGCCA	Dec 5 uM	2
Pol 2	Pol-2F B141	TTGCG	TCCAGAAATAGGACTGTCAGGGCA		NNTTGCGTCCAGAAATAGGACTGTCAGGGCA	Dec 5 uM	3
Env 1	Env-1F B141		TCCCGACAATCTCAAACAGGGT		NNTTGCGTCCCGACAATCTCAAACAGGGT	Dec 5 uM	3
Gag 1	Gag-1F B141		ATCAACCAACCTGGGACGACT		NNTTGCGATCAACCAACCTGGGACGACT	Dec 5 uM	3
Gfp 1	Gfp-1F B141		TTCTTCAAGGACGACGGCAACT		NNTTGCGTTCTTCAAGGACGACGGCAACT	Dec 5 uM	3
Gfp 2	Gfp-2F B141		AGGTGAACTTCAAGATCCGCCA		NNTTGCGAGGTGAACTTCAAGATCCGCCA	Dec 5 uM	3
Pol 2	Pol-2F B142	TTGGC	TCCAGAAATAGGACTGTCAGGGCA		NNTTGGCTCCAGAAATAGGACTGTCAGGGCA	Gem 100 nM	1
Env 1	Env-1F B142		TCCCGACAATCTCAAACAGGGT		NNTTGGCTCCCGACAATCTCAAACAGGGT	Gem 100 nM	1
Gag 1	Gag-1F B142		ATCAACCAACCTGGGACGACT		NNTTGGCATCAACCAACCTGGGACGACT	Gem 100 nM	1
Gfp 1	Gfp-1F B142		TTCTTCAAGGACGACGGCAACT		NNTTGGCTTCTTCAAGGACGACGGCAACT	Gem 100 nM	1
Gfp 2	Gfp-2F B142		AGGTGAACTTCAAGATCCGCCA		NNTTGGCAGGTGAACTTCAAGATCCGCCA	Gem 100 nM	1
Pol 2	Pol-2F B143	TTGTA	TCCAGAAATAGGACTGTCAGGGCA		NNTTGTATCCAGAAATAGGACTGTCAGGGCA	Gem 100 nM	2
Env 1	Env-1F B143		TCCCGACAATCTCAAACAGGGT		NNTTGTATCCCGACAATCTCAAACAGGGT	Gem 100 nM	2
Gag 1	Gag-1F B143		ATCAACCAACCTGGGACGACT		NNTTGTAATCAACCAACCTGGGACGACT	Gem 100 nM	2
Gfp 1	Gfp-1F B143		TTCTTCAAGGACGACGGCAACT		NNTTGTATTCTTCAAGGACGACGGCAACT	Gem 100 nM	2
Gfp 2	Gfp-2F B143		AGGTGAACTTCAAGATCCGCCA		NNTTGTAAGGTGAACTTCAAGATCCGCCA	Gem 100 nM	2
Pol 2	Pol-2F B144	TTTCA	TCCAGAAATAGGACTGTCAGGGCA		NNTTTCATCCAGAAATAGGACTGTCAGGGCA	Gem 100 nM	3
Env 1	Env-1F B144		TCCCGACAATCTCAAACAGGGT		NNTTTCATCCCGACAATCTCAAACAGGGT	Gem 100 nM	3
Gag 1	Gag-1F B144		ATCAACCAACCTGGGACGACT		NNTTTCAATCAACCAACCTGGGACGACT	Gem 100 nM	3
Gfp 1	Gfp-1F B144		TTCTTCAAGGACGACGGCAACT		NNTTTCATTCTTCAAGGACGACGGCAACT	Gem 100 nM	3
Gfp 2	Gfp-2F B144		AGGTGAACTTCAAGATCCGCCA		NNTTTCAAGGTGAACTTCAAGATCCGCCA	Gem 100 nM	3
Pol 2	Pol-2F B130	TGCTG	TCCAGAAATAGGACTGTCAGGGCA		NNTGCTGTCCAGAAATAGGACTGTCAGGGCA	CPEC 1 uM	1
Env 1	Env-1F B130		TCCCGACAATCTCAAACAGGGT		NNTGCTGTCCCGACAATCTCAAACAGGGT	CPEC 1 uM	1
Gag 1	Gag-1F B130		ATCAACCAACCTGGGACGACT		NNTGCTGATCAACCAACCTGGGACGACT	CPEC 1 uM	1
Gfp 1	Gfp-1F B130		TTCTTCAAGGACGACGGCAACT		NNTGCTGTTCTTCAAGGACGACGGCAACT	CPEC 1 uM	1
Gfp 2	Gfp-2F B130		AGGTGAACTTCAAGATCCGCCA		NNTGCTGAGGTGAACTTCAAGATCCGCCA	CPEC 1 uM	1
Pol 2	Pol-2F B131	TGGCT	TCCAGAAATAGGACTGTCAGGGCA		NNTGGCTTCCAGAAATAGGACTGTCAGGGCA	CPEC 1 uM	2
Env 1	Env-1F B131		TCCCGACAATCTCAAACAGGGT		NNTGGCTTCCCGACAATCTCAAACAGGGT	CPEC 1 uM	2
Gag 1	Gag-1F B131		ATCAACCAACCTGGGACGACT		NNTGGCTATCAACCAACCTGGGACGACT	CPEC 1 uM	2
Gfp 1	Gfp-1F B131		TTCTTCAAGGACGACGGCAACT		NNTGGCTTTCTTCAAGGACGACGGCAACT	CPEC 1 uM	2
Gfp 2	Gfp-2F B131		AGGTGAACTTCAAGATCCGCCA		NNTGGCTAGGTGAACTTCAAGATCCGCCA	CPEC 1 uM	2
Pol 2	Pol-2F B132	TGGTC	TCCAGAAATAGGACTGTCAGGGCA		NNTGGTCTCCAGAAATAGGACTGTCAGGGCA	CPEC 1 uM	3
Env 1	Env-1F B132		TCCCGACAATCTCAAACAGGGT		NNTGGTCTCCCGACAATCTCAAACAGGGT	CPEC 1 uM	3
Gag 1	Gag-1F B132		ATCAACCAACCTGGGACGACT		NNTGGTCATCAACCAACCTGGGACGACT	CPEC 1 uM	3
Gfp 1	Gfp-1F B132		TTCTTCAAGGACGACGGCAACT		NNTGGTCTTCTTCAAGGACGACGGCAACT	CPEC 1 uM	3
Gfp 2	Gfp-2F B132		AGGTGAACTTCAAGATCCGCCA		NNTGGTCAGGTGAACTTCAAGATCCGCCA	CPEC 1 uM	3

Table I-1. List of the Illumina® barcodes and primers used in next-generation sequencing.

APPENDIX II

Liquid Chromatography-Tandem Mass Spectrometry Parameters

Analyte	ESI mode	Q1	Q3	DP[a]	EP[b]	CE[c]	CXP[d]
dCTP	–	466.1	159.0	–55	10	–32	–14
dTTP	–	481.1	159.0	–52	10	–32	–15
dATP	–	490.1	159.0	–35	10	–34	–14
IS	–	591.8	511.8	–47	10	–36	–22
dGTP	+	581.0	152.1	39	10	34	17

Table II-1. LCMS-MS parameters for detection of deoxynucleotides and internal standard. [a]Declustering potential; [b]Entrance potential; [c]Collision energy; [d]Collision cell exit potential.

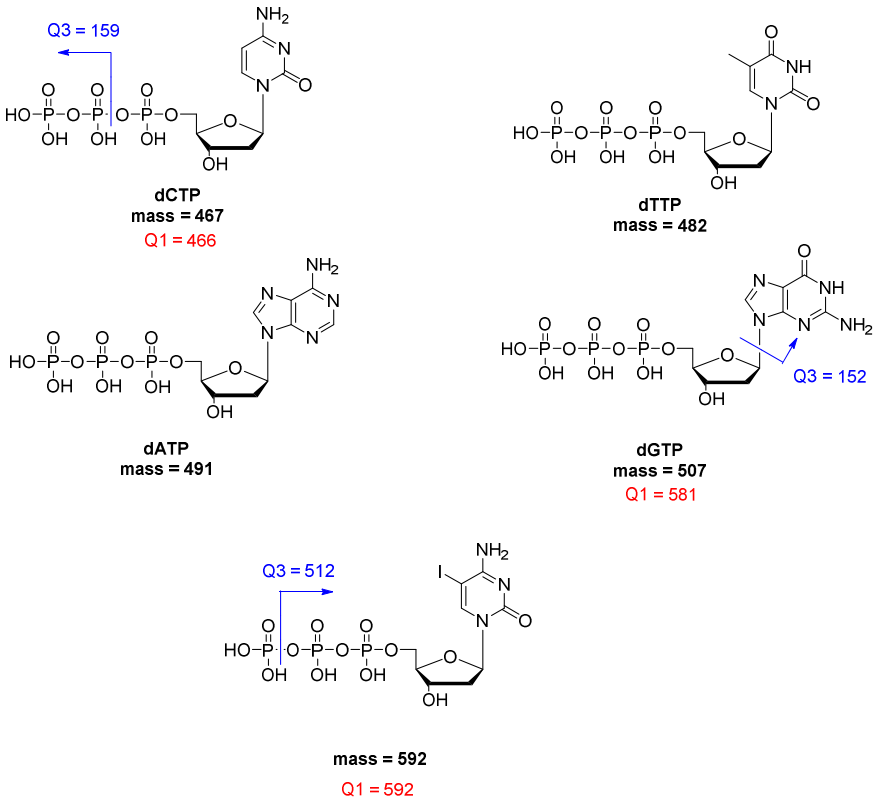
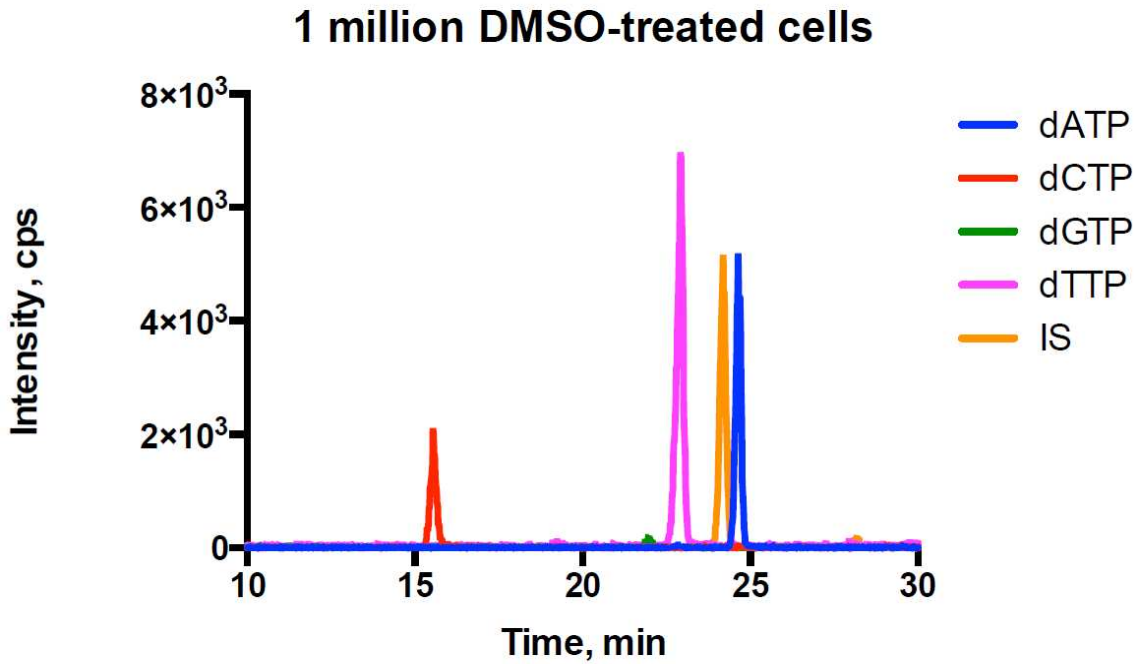


Figure II-1. Illustration of the dNTP fragmentation pattern. dCTP, dTTP, and dATP all have the same fragmentation pattern. dGTP has a unique fragmentation pattern to distinguish it from ATP.

A)


1 million DMSO-treated cells
Intensity, cps
8×10^3
6×10^3
4×10^3
2×10^3
0
10
15
20
25
30
Time, min
dATP
dCTP
dGTP
dTTP
IS

B)

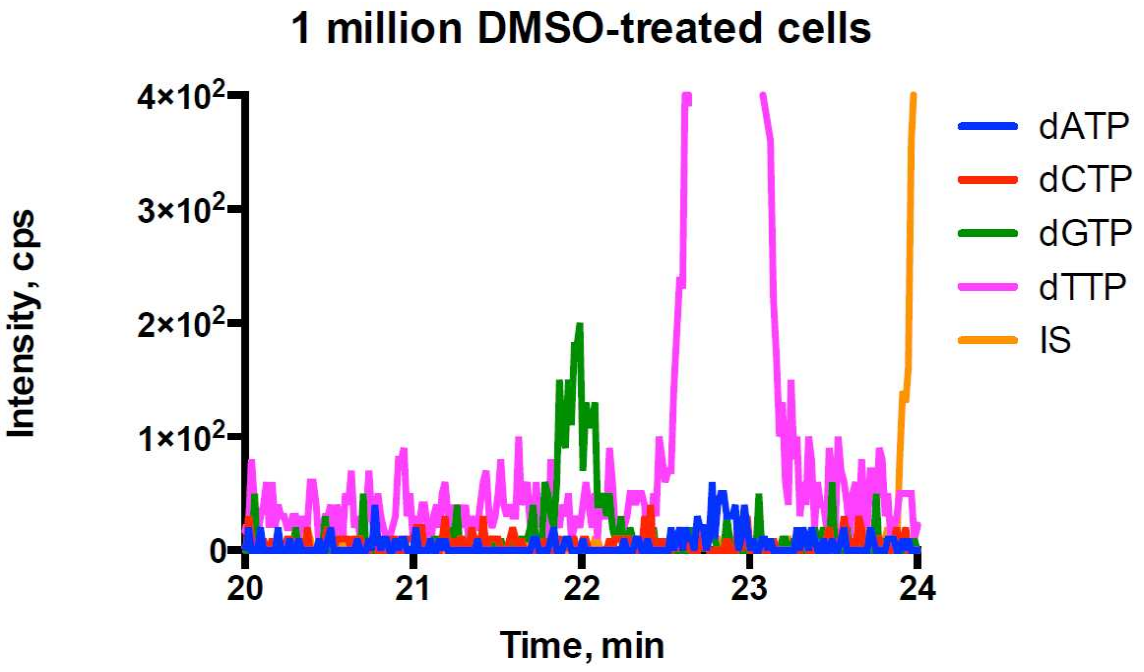

1 million DMSO-treated cells
Intensity, cps
4×10^2
3×10^2
2×10^2
1×10^2
0
20
21
22
23
24
Time, min
dATP
dCTP
dGTP
dTTP
IS

Figure II-2. Example of LCMS-MS chromatogram with DMSO (no drug) control. Cells were plated on a 10 cm dish and treated with DMSO for 4 hours. Treated cells are collected, counted, and methanol treated to extract dNTPs. The methanol treatment was spiked with 5'-Iodo-2'dCTP (internal standard, IS) and incubated overnight. dNTP were collected from the supernatant and LCMS-MS was conducted to calculate peak areas for A) dATP, dCTP, dTTP, and B) dGTP (see Materials and Methods for details).