

Discovery of drugs that possess activity against feline leukemia virus

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Feline leukemia virus (FeLV) is a gammaretrovirus that is a significant cause of neoplastic-related disorders affecting cats worldwide. Treatment options for FeLV are limited, associated with serious side effects, and can be cost-prohibitive. The development of drugs used to treat a related retrovirus, human immunodeficiency virus type 1 (HIV-1), has been rapid, leading to the approval of five drug classes. Although structural differences affect the susceptibility of gammaretroviruses to anti-HIV drugs, the similarities in mechanism of replication suggest that some anti-HIV-1 drugs may also inhibit FeLV. This study demonstrates the anti-FeLV activity of four drugs approved by the US FDA (Food and Drug Administration) at non-toxic concentrations. Of these, tenofovir and raltegravir are anti-HIV-1 drugs, while decitabine and gemcitabine are approved to treat myelodysplastic syndromes and pancreatic cancer, respectively, but also have anti-HIV-1 activity in cell culture. Our results indicate that these drugs may be useful for FeLV treatment and should be investigated for mechanism of action and suitability for veterinary use.

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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 leukaemias and lymphomas, as well as secondary infections related to FeLV-induced immune suppression. Infected cats transmit the virus through saliva, nasal secretions and coitus, as well as vertically from queen to kitten (Cattori *et al.*, 2009; Essex *et al.*, 1971; Rickard *et al.*, 1969). 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 *et al.*, 1975). Despite its prevalence and its associated morbidity and mortality, there are limited treatment options for FeLV infection. Currently, 3'-azido-3'-deoxythymidine (AZT; also known as zidovudine) is the only antiviral drug used routinely to treat FeLV, but its use is associated with significant side effects (Haschek *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 (Gingerich, 2008; Hartmann *et al.*, 1998; Tavares *et al.*, 1987). Due to the lack of effective treatment

options, many owners choose to euthanize or provide palliative care to infected cats.

Among retroviruses, human immunodeficiency virus type 1 (HIV-1) is the most extensively studied, particularly in terms of drug development. According to the US Food and Drug Administration (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 these two retroviruses to indicate that anti-HIV-1 drugs may also inhibit replication of FeLV.

In order to expand the treatment options for feline leukaemia, here we describe the anti-FeLV activity of four FDA-approved drugs: tenofovir, raltegravir, decitabine and gemcitabine. Whilst tenofovir and raltegravir are FDA-approved for AIDS chemotherapy, decitabine (5-aza-2'-deoxycytidine) and gemcitabine (2',2'-difluoro-2'-deoxycytidine) are cytidine analogues used for the treatment of myelodysplastic syndromes (Garcia *et al.*, 2010) and pancreatic cancer (Cerqueira *et al.*, 2007; Wang *et al.*, 2009), respectively. We recently demonstrated the ability of decitabine and gemcitabine to inhibit HIV-1 replication

(Clouser *et al.*, 2010, 2011). In addition, recent studies have shown that tenofovir and raltegravir exhibit antiviral activity against a related gammaretrovirus, xenotropic murine leukemia-related virus (Paprotka *et al.*, 2010; Singh *et al.*, 2010; Smith *et al.*, 2010). 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, an FeLV construct was designed to express GFP from an internal ribosomal entry site (IRES) element that was inserted into the *env* gene. As this vector, FeLV-GFP (Fig. 1), lacks a functional *env* gene, vector replication is limited to one round of replication. The single-cycle assay allows for the detection of agents that possess antiviral activity by looking at the drug–virus interaction for one cycle of virus replication. It also eliminates compounding factors like reinfection and drug resistance that can be seen with *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 that has been used clinically to treat FeLV. To do this, 293T cells were cotransfected with FeLV-GFP and a vesicular stomatitis virus glycoprotein G (VSV-G) envelope expression plasmid. Cell-culture supernatants were harvested and used to infect Crandell–Rees feline kidney (CRFK) cells that had been pre-treated with the AZT concentrations indicated in Fig. 2(a). The percentage of infected cells was determined by flow cytometry using GFP expression as a marker for infection. Fig. 2(a) 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.

FeLV infectivity is inhibited in cell culture

To examine the ability of decitabine, gemcitabine, tenofovir and raltegravir to inhibit FeLV replication, the single-cycle assay with FeLV-GFP was used as described. Fig. 2(b–e) shows that all 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 by Cattori *et al.* (2011). The concentrations required to reduce infection by 50% (IC_{50}) are shown in Table 1 and demonstrate that decitabine, gemcitabine and raltegravir have potent (nanomolar) anti-FeLV activity, whilst tenofovir has micromolar anti-FeLV activity.

Antiviral activity at concentrations that are not toxic in CRFK cells

Each drug was examined to determine whether the concentrations that exert antiretroviral activity were also 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 10- and 280-fold 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 1).

DISCUSSION

FeLV is responsible for significant mortality in cats worldwide and, despite both its prevalence and its

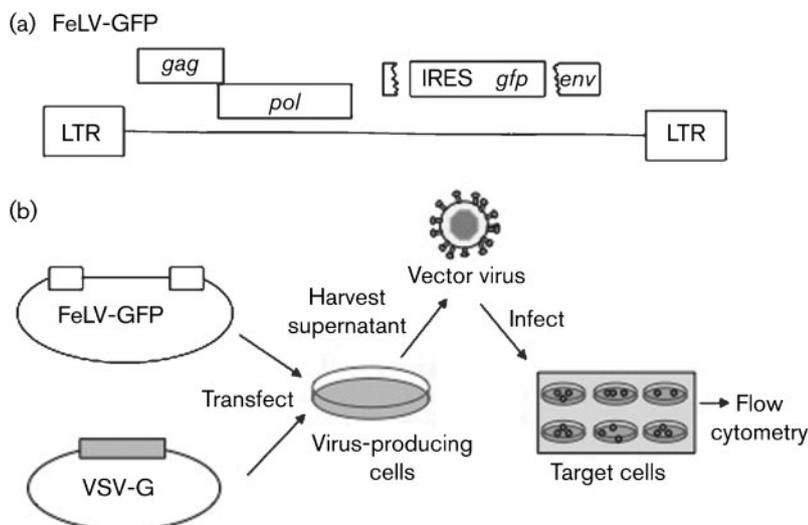


Fig. 1. FeLV-GFP vector and model system for monitoring virus infectivity. (a) The FeLV vector, FeLV-GFP, is shown with the *gfp* gene inserted into the *env* gene. The LTRs, *gag* and *pol* genes are indicated. (b) Single-replication-cycle assay performed with permissive CRFK target cells. FeLV-GFP was cotransfected into 293T cells with a VSV-G envelope expression plasmid. Cell-culture supernatants were harvested and used to infect CRFK target cells. Virus infectivity was monitored by measuring GFP expression using flow cytometry.

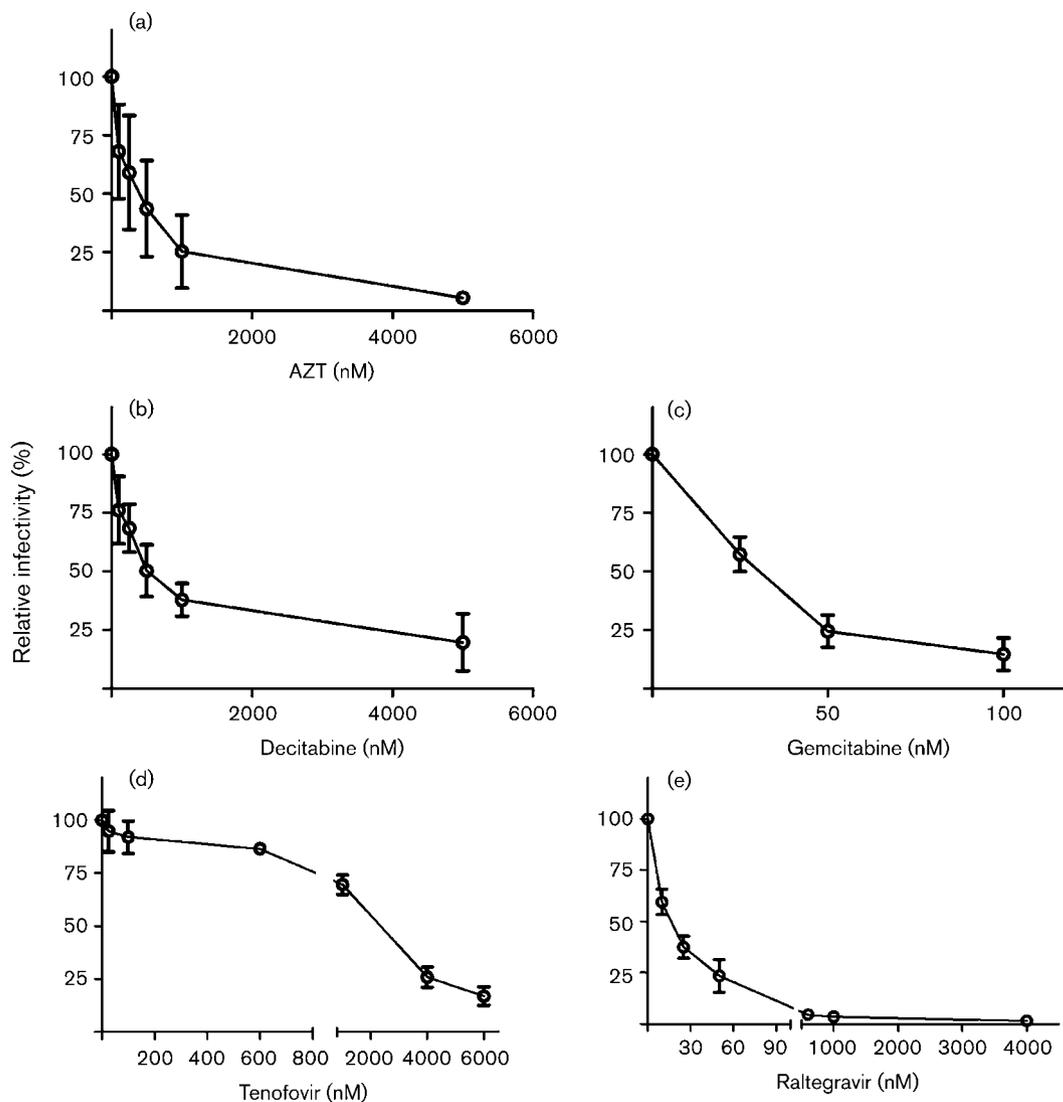


Fig. 2. Dose–response of FeLV infectivity to decitabine, gemcitabine, tenofovir and raltegravir. CRFK cells were exposed to the indicated drug 2 h prior to infection with FeLV. Infected cells were analysed by flow cytometry. Each data point represents the mean of three independent replicates with the SD indicated. The molarity of the drug-concentration range analysed is indicated along the x -axis. (a) Validation of assay using AZT. (b–e) Analysis of decitabine, gemcitabine, tenofovir and raltegravir, respectively.

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 extremely active. As FeLV and HIV-1 are both retroviruses, 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, whereas 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 an alternative mechanism of action. Previous studies have shown that differences in reverse transcriptase (RT) active sites among different retroviruses can affect susceptibility to nucleoside reverse transcriptase inhibitors (NRTIs). For example, the YXDD motif in the active site of all retroviral RTs plays a significant role in susceptibility to NRTIs. For example, differences in the X position of this motif affect the ability

Table 1. *In vitro* cytotoxicity of decitabine, gemcitabine, tenofovir and raltegravir in CRFK cells

CRFK cells were incubated in the presence of drug for 48 h. The IC₅₀ values represent the concentration at which 50 % of virus replication was inhibited, as determined from the data in Fig. 2 with the 95 % confidence intervals indicated. CC₅₀ values were determined using the Cell Titre-Glo Luminescent Cell Viability Assay (see Methods for details).

Drug	Inhibition activity, IC ₅₀ (nM)	<i>In vitro</i> cytotoxicity, CC ₅₀ (μM)	SI (CC ₅₀ /IC ₅₀)	95 % confidence interval (μM)
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

of RT to incorporate nucleoside analogues containing modified sugars (Boyer *et al.*, 2001; Jamburuthugoda *et al.*, 2008; Operario *et al.*, 2005; Poch *et al.*, 1989). HIV-1 and other lentiviruses have a methionine at the X position that enables incorporation of a nucleoside analogue with modified sugars. In contrast, structural studies show that the valine present at the X residue in FeLV and other oncoretroviruses restricts access of nucleoside analogues with modified ribose. Although gemcitabine has a modified ribose (fluorinated on the 2' carbon), it is a potent inhibitor of FeLV replication (Fig. 2). However, the antiviral activity of gemcitabine may not necessitate its corresponding triphosphate to be a substrate of RT. 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 *et al.*, 1992).

As decitabine has an unmodified ribose, it is not expected that the 'Val' present in the FeLV active site would exclude its incorporation into viral DNA by RT. Clinically, decitabine is used for its ability to be incorporated in place of dCTP into DNA, 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 RT (Clouser *et al.*, 2010). Once incorporated, it induces G-to-C mutations and this increase in mutation 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 RT has a higher fidelity than HIV-1 RT suggests that decitabine could act by a mechanism distinct from that used against HIV-1 (Operario *et al.*, 2005).

The anti-FeLV mechanisms of tenofovir and raltegravir are probably 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 that is an analogue of adenosine 5'-monophosphate. Once converted to the active diphosphate form, tenofovir is incorporated by RT into viral DNA, where it acts as a chain terminator to inhibit further elongation of the viral DNA (Kearney *et al.*, 2004; Robbins *et al.*, 1998). In contrast, raltegravir inhibits integration of the viral dsDNA that is

produced by reverse transcription of the viral RNA genome (Beck-Engeser *et al.*, 2009; Buzón *et al.*, 2010; Malet *et al.*, 2008; Reigadas *et al.*, 2010).

In summary, we have demonstrated the anti-FeLV activity of four FDA-approved drugs whose anti-HIV-1 activity has been described previously. All four drugs exerted antiviral activity at concentrations examined with SIs 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 actions, as well as their suitability as treatments for FeLV. 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.

METHODS

Materials, cells and reagents. CRFK cells were obtained from Dr Richard Van Deusen (National Veterinary Services Laboratory, Ames, IA, USA) and 293T cells were obtained from the ATCC. Gemcitabine was obtained from Carbosynth and decitabine from Moravik Biochemicals. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: tenofovir (cat. # 10199), raltegravir (cat. # 11680), zidovudine (AZT; cat. # 3485) and pEECC-FeLV (cat. # 105). pEECC-FeLV is a replication-competent FeLV clone. The IRES and the EGFP gene sequence used to create the FeLV-GFP vector were from the plasmid pHIG (Clouser *et al.*, 2010) and were originally derived from pIRES2-eGFP (Clontech). The VSV-G protein (pL-VSV-G) envelope expression plasmid was obtained from Dr Jane C. Burns (University of California, San Diego, CA, USA).

CRFK and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech) supplemented with 10 % fetal clone 3 (FC3) serum (HyClone) and penicillin/streptomycin (100 μg ml⁻¹ and 100 U ml⁻¹, respectively) at 37 °C in 5 % CO₂. 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 sequence was amplified by PCR using the following primers: 5'-ATGCATACCATGGTGCCAGGCTAGGG-3' (forward) and 5'-CCATGCATTTACTTGTACAGCTCGTCCATG-3' (reverse), which included the *Nsi*I restriction site (underlined). The pEECC-FeLV

plasmid was digested with *Nsi*I, removing a 615 bp sequence from *env*. The linear pEECC-FeLV backbone was dephosphorylated with antarctic phosphatase (New England Biolabs). The pEECC-FeLV backbone was gel-purified using the Wizard SV Gel and PCR Clean-up system (Promega) 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:DNA insert) molar ratio. The ligation product was transformed into DH5 α (*Escherichia coli*) cells (Invitrogen) and plated onto Luria-Bertani (LB) agar (Becton Dickinson) containing 50 μ g ampicillin ml⁻¹. Individual colonies were grown in LB broth containing 50 μ g ampicillin ml⁻¹ and the DNA from these cultures was purified using an Invitrogen PureLink Quick Plasmid Miniprep kit. DNA sequencing (Functional Biosciences) was done to verify the correct construction of the vector.

Transfection of 293T cells. The FeLV-GFP plasmid (10 μ g) and pLVSV-G plasmid (1 μ g) were cotransfected into 293T cells using the calcium phosphate coprecipitation method. Twenty-four hours after transfection, medium was removed and replaced with 6 ml fresh medium. Supernatant containing infectious virions was collected from cells 48 h post-transfection and passed through a 0.2 μ m 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 in each well of a 12-well dish 24 h 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. Two hours after initiating drug treatments, cells were infected and then incubated at 37 °C for 24 h. Twenty-four hours post-infection, medium was removed and replaced with fresh DMEM and the cells were then incubated for an additional 24 h at 37 °C. To analyse FeLV-infected cells, 48 h post-infection cells were trypsinized, centrifuged at 800 g for 5 min and the cell pellets were resuspended in 2% paraformaldehyde in PBS containing 2% FC3. Samples were analysed by flow cytometry using a Becton Dickinson FACSscan. 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 were 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 in each well of a 96-well plate 24 h prior to the initiation of drug treatment. Cells were treated with drug (AZT, decitabine, gemcitabine, tenofovir or raltegravir) for 24 h, at which time the medium was replaced with fresh DMEM containing 10% FC3. Cell viability was examined using a Promega CellTiter-Glo kit according to the manufacturer's instructions. Briefly, cell viability was assessed 48 h 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.

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