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.

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′-deoxy-cytidine) 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% (IC50) 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 IC50 for antiviral activity. Similarly, tenofovir and raltegravir failed to induce toxicity at 10- and 280-fold their IC50 values, respectively. In contrast, gemcitabine induced cytotoxicity with a cytotoxic concentration 50 (CC50) of 230 nM, giving a selectivity index [SI (=CC50/IC50)] of 9.2 (Table 1).

**DISCUSSION**

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

![Graphs showing dose-response of FeLV infectivity to decitabine, gemcitabine, tenofovir and raltegravir.](image-url)
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 ribose. 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 monophosphate 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. Gencitabine was obtained from CarboSynth and decitabine from Moravek 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 (pLVSV-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 units/ml). 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 and 100 U/ml, 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’-ATGCACTACATTGGTGCCAGGCTAGG-3’ (forward) and 5’-CCATGCACTTTACCTTACAGCTGTCCATG-3’ (reverse), which included the NsiI restriction site (underlined). The pEECC-FeLV

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition activity, IC₅₀ (nM)</th>
<th>In vitro cytotoxicity, CC₅₀ (μM)</th>
<th>SI (CC₅₀/IC₅₀)</th>
<th>95% confidence interval (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decitabine</td>
<td>437</td>
<td>&gt;158</td>
<td>&gt;361.6</td>
<td>0.14–1.4</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>25</td>
<td>0.23</td>
<td>9.2</td>
<td>0.02–0.03</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>2000</td>
<td>&gt;20</td>
<td>&gt;10</td>
<td>1.0–3.0</td>
</tr>
<tr>
<td>Raltegravir</td>
<td>14</td>
<td>&gt;4</td>
<td>&gt;285.7</td>
<td>0.011–0.017</td>
</tr>
</tbody>
</table>
plasmid was digested with NsiI, 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 DH5α (Escherichia coli) 
cells (Invitrogen) and plated onto Luria-Bertani (LB) agar (Becton Dickinson) 
containing 50 μg ampicillin ml⁻¹. Individual colonies were grown in LB 
broth containing 50 μg 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 μg) and pL-
YSV-G plasmid (1 μg) 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 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 in each 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 concentra-
tions 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% FCS. 
Samples were analysed 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 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 × 10⁵) 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% FCS. 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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# 10199), zidovudine (cat. # 3485) and pEECC-FeLV (cat. # 105).

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**REFERENCES**

dNTP pool imbalances on frameshift fidelity during DNA replication. 

Beck-Engeser, G. B., Eilat, D., Harrer, T., Jäck, H. M. & Wabl, M. 
(2009). Early onset of autoimmune disease by the retroviral integrase 

virus type 1 and Moloney murine leukemia virus reverse transcriptase 
are resistant to lamivudine triphosphate (3TCTP) in vitro. *J Virol* 75, 
6321–6328.

Buzón, M. J., Massanella, M., Llibre, J. M., Esteve, A., Dahl, V., Puertas, 
M. C., Gatell, J. M., Domingo, P., Paredes, R. & other authors (2010). 
HIV-1 replication and immune dynamics are affected by raltegravir 
intensification of HAART-suppressed subjects. *Nat Med* 16, 
460–465.

Cattori, V., Tandon, R., Riond, B., Pepin, A. C., Lutz, H. & Hofmann-
Lehmann, R. (2009). The kinetics of feline leukemia virus shedding in 
experimentally infected cats are associated with infection outcome. 

virus replication by the integrase inhibitor raltegravir. *Vet Microbiol* 
152, 165–168.

ribonucleotide reductase inactivation by gemcitabine. *Chemistry* 13, 
8507–8515.

Clouser, C. L., Patterson, S. E. & Mansky, L. M. (2010). Exploiting 
drug repositioning for discovery of a novel HIV combination therapy. 
*J Virol* 84, 9301–9309.

Clouser, C. L., Holtz, C. M., Mullett, M., Crankshaw, D. L., Briggs, J. E., 
Chauhan, J., VanHoutan, I. M., Patterson, S. E. & Mansky, L. M. 
(2011). Analysis of the ex vivo and in vivo antiretroviral activity of 

between humoral antibody and regression of tumours induced by 

and efficacy of decitabine in the treatment of myelodysplastic 

Gingerich, D. A. (2008). Lymphocyte T-cell immunomodulator (LTCI): view of 
the immunopharmacology of a new veterinary biologic. *Intern J Appl Res Vet Med* 6, 
61–68.

Hartmann, K., Block, A., Ferk, G., Vollmar, A., Goldberg, M. & Lutz, H. 
(1998). Treatment of feline leukemia virus-infected cats with 

Haschek, W. M., Weigel, R. M., Scherba, G., DeVera, M. C., Feinmehl, R., 
toxicity to cats infected with feline leukemia virus. *Fundam Appl Toxicol* 
14, 764–775.

Jamburuthugoda, V. K., Santos-Velazquez, J. M., Skasko, M., 
Operario, D. J., Purohit, V., Chugh, P., Szymanski, E. A., Wedekind, 
J. E., Bambara, R. A. & Kim, B. (2008). Reduced dNTP binding affinity 
of 3Tc-resistant M184I HIV-1 reverse transcriptase variants responsible 

fumarate: clinical pharmacology and pharmacokinetics. *Clin Pharma-
cokinet* 43, 595–612.


