

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät
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**Antiviral efficacy of nine nucleoside reverse transcriptase
inhibitors against feline immunodeficiency virus in feline
peripheral blood mononuclear cells**

von

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ABBREVIATIONS

3TC	2',3'-dideoxy-3'-thiacytidine, lamivudine
ABC	(-)-(1 <i>S</i> ,4 <i>R</i>)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, abacavir
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AMD3100	plerixafor
AZT	3'-azido-3'-deoxythymidine, zidovudine
CCR5	chemokine (C-C motif) receptor 5
CD	cluster of differentiation
CD134	primary cell receptor for FIV, T cell activation antigen
CD4 ⁺ cells	cells expressing the CD4 receptor
CD8 ⁺ cells	cells expressing the CD8 receptor
CD25 ⁺ cells	cells expressing the CD25 receptor
CD25 ⁻ cells	cells not expressing the CD25 receptor
CRFK	Crandell-Rees feline kidney
CRI	Co-receptor inhibitor
CXCR4	chemokine (C-X-C motif) receptor 4
d4T	2',3'-didehydro-2',3'-dideoxythymidine, stavudine
ddC	2',3'-dideoxycytidine, zalcitabine
ddI	2',3'-dideoxyinosine, didanosine
DNA	deoxyribonucleic acid
EC ₅₀	50% effective concentration
eg	exempli gratia, for example
EI	entry inhibitor
ELISA	enzyme-linked immunosorbent assay

et al.	et alii
FDA	U.S. Food and Drug Administration
FI	fusion inhibitor
fig.	figure
FIV	feline immunodeficiency virus
FTC	(-)-2',3'-deoxy-5-fluoro-3'-thiacytidine
gp	glycoprotein
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HR	heptad repeat
INI	integrase inhibitor
kg	kilogram
K_i	inhibition constant
mg	milligram
ml	milliliter
M184V mutation	methionine-to-valine mutation at codon 184
μg	microgram
μM	micromolar
NA	not assessed
ND	not determined
nM	nanomolar
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
OD	optical density
p	protein

PBL	peripheral blood mononuclear
PCR	polymerase chain reaction
PI	protease inhibitor
PMEA	9-(2-phosphonylmethoxyethyl)adenine, adefovir
PO	per os
RNA	ribonucleic acid
(R)-PMPA	(R)-9-(2-phosphonylmethoxypropyl)adenine, tenofovir
RT	reverse transcriptase
SC	subcutaneously
SIV	simian immunodeficiency virus
SPF	specific pathogen-free
SU	surface
T cell	lymphocyte that matures in the thymus
T-20	enfuvirtide
Tab.	table
TDF	tenofovir disoproxil fumarate

I. INTRODUCTION

Feline immunodeficiency virus (FIV) was first isolated in 1986 (PEDERSEN et al., 1987) and has since been shown to occur worldwide, infecting domestic cats and wild felids (HOKANSON et al., 2000). Like human immunodeficiency virus (HIV), FIV is a member of the retroviridae family of viruses, genus lentivirus, and both viruses cause an acquired immunodeficiency syndrome (AIDS) in their respective hosts (NORTH et al., 1989). The two viruses share many morphologic, genomic, and biochemical characteristics. Of particular importance to chemotherapeutic studies is the close similarity between the reverse transcriptase (RT) of FIV and HIV (GOBERT et al., 1994) which results in *in vitro* susceptibility of FIV to many RT-targeted antiviral compounds used in the treatment of HIV-infected patients.

The nucleoside reverse transcriptase inhibitor (NRTI) zidovudine is the only antiviral drug which has been evaluated thoroughly against FIV and is sometimes used in the treatment of naturally FIV-infected cats. It has been shown that it can have positive effects on the cat's immunologic status and improve clinical condition scores in cats with stomatitis and neurological signs (HARTMANN et al., 1992; HARTMANN et al., 1995a). However, it can also result in dose-dependent anemia and neutropenia (HARTMANN et al., 1995a; ARAI et al., 2002) which can necessitate termination of therapy. Furthermore, it has been shown that drug-resistant mutations can develop in cats treated with zidovudine (MARTINS et al., 2008). To avoid drug resistance development and to achieve optimal suppression of virus replication, multi-agent antiviral chemotherapy, is the mainstay of treatment in HIV-infected humans (CLAVEL and HANCE, 2004). Since combination treatment with most protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) is likely ineffective in cats, it is important to assess other NRTIs with good efficacy against FIV and low toxicity which could be used in the treatment of FIV-infected cats.

The purpose of the present study was to test three NRTIs, which had not been evaluated in feline cells before, together with six NRTIs with known anti-FIV activity, for their cytotoxicity in primary feline peripheral blood mononuclear (PBM) cells and antiviral efficacy against FIV.

II. LITERATURE REVIEW: IN VITRO EFFICACY OF ANTIRETROVIRAL COMPOUNDS AGAINST FIV

Like HIV, FIV is a member of the lentivirus subfamily of the family Retroviridae (BENNETT und SMYTH, 1992) and there are several striking similarities between the two viruses regarding their morphological, physical, and biochemical characteristics as well as their clinical and hematological manifestations (GOBERT et al., 1994; VAHLENKAMP et al., 1995; ARAI et al., 2002; ELDER et al., 2010). Because these similarities allow the evaluation of the effects of antiviral therapy on disease pathogenesis (HAYES et al., 2000) FIV infection of cats has become an important animal model for HIV infection of humans (NORTH et al., 1989; VAHLENKAMP et al., 1995; WILLETT et al., 1997a; HAYES et al., 2000; MCCRACKIN STEVENSON and MCBROOM, 2001; BISSET et al., 2002; DIAS AS et al., 2006).

Since the discovery of FIV in 1986 (PEDERSEN et al., 1987) many different antiviral compounds have been assessed for their efficacy against FIV in *in vitro* and *in vivo* studies. Most of these compounds are derived from human medicine and are used in the treatment of HIV-infected patients (HARTMANN, 2012). Experimental studies with antiviral compounds are generally conducted in three stages. *In vitro* experiments are usually performed first and assess the ability to suppress viral infection in cell or tissue culture. If good antiviral efficacy is demonstrated, *in vivo* studies might follow, such as experimental virus challenge studies. Eventually, promising drugs are administered to animals with well-characterized chronic experimental retroviral infections and various parameters can be monitored and compared to animals from a control group (LEVY, 2000).

Over the last 30 years, the use of combination-antiretroviral therapy has resulted in HIV infection, which previously was rapidly fatal, becoming a chronic but manageable condition (BRODER, 2010). Highly active antiretroviral therapy (HAART), the standard of care for HIV-infected individuals currently consists of three or more anti-HIV drugs. Most commonly, two of these drugs are NRTIs or a NRTI together with a nucleotide reverse transcriptase inhibitor (NtRTI) combined with a third agent from a different drug class (CIHLAR and RAY, 2010; TRESSLER and GODFREY, 2012). Despite this great success in human

medicine, no such therapy is available for cats (ZEINALIPOUR-LOIZIDOU et al., 2007; PALMISANO and VELLA, 2011; MOHAMMADI and BIENZLE, 2012).

All antiviral compounds interfere with one or more steps of the virus replication process. Based upon this, the drugs can be assigned to different drug classes (DE CLERCQ, 1995a; PALMISANO and VELLA, 2011) (fig. 1, tab. 1).

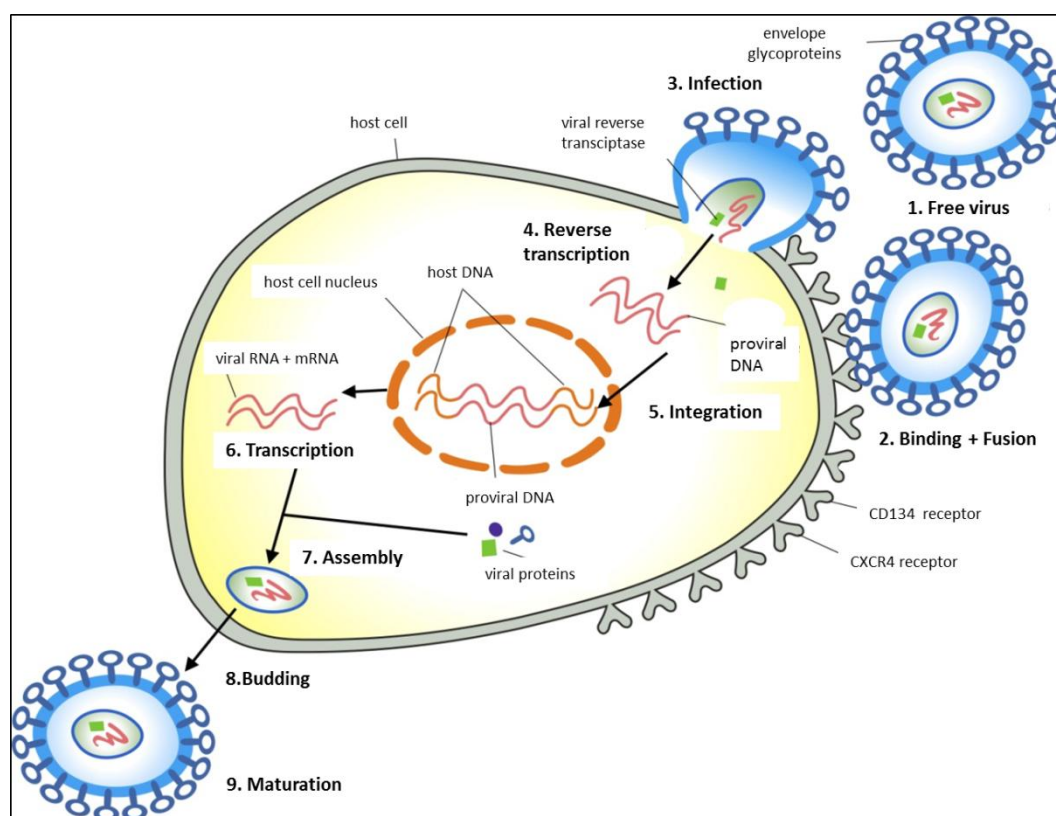


Fig. 1: Replicative cycle of FIV (printed and modified with kind permission of Prof. K. Hartmann)

Potential targets in the retroviral replication process for antiviral drugs include (DE CLERCQ, 1995a; HARTMANN, 2012; MOHAMMADI and BIENZLE, 2012):

- binding of virus to specific cell surface receptors
- entry into the cell and uncoating of the virus
- reverse transcription of viral genome
- integration of proviral DNA into host genome
- viral protein processing
- virion assembly and maturation
- virion release

Currently there are 26 compounds that have been formally approved by the Food and Drug Administration (FDA) for the treatment of AIDS and these drugs are generally divided into the following classes (MOHAMMADI and BIENZLE, 2012; DE CLERCQ, 2013):

- Reverse transcriptase inhibitors
 - Nucleoside reverse transcriptase inhibitors (NRTIs)
 - Nucleotide reverse transcriptase inhibitors (NtRTIs)
 - Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Protease inhibitors (PIs)
- Entry inhibitors (EIs)
 - Fusion inhibitors (FIs)
 - Co-receptor inhibitors (CRIs)
- Integrase inhibitors (INIs)

Several compounds from most of these drug classes have also been assessed against FIV *in vitro*. Table 1 provides an overview of all FDA-approved antiretroviral compounds assessed *in vitro* against FIV. These are the focus of this doctoral thesis.

Table 1: FDA-approved anti-HIV drugs and their efficacy against FIV *in vitro* (NA, not assessed)

Drug class	Compound	Efficacy <i>in vitro</i>	Chapter for references
Nucleoside reverse transcriptase inhibitors	Zidovudine	Yes	1.1.2.1.
	Didanosine	Yes	1.1.2.2.
	Zalcitabine (no longer marketed)	Yes	1.1.2.3.
	Stavudine	Yes	1.1.2.4.
	Lamivudine	Yes	1.1.2.5.
	Emtricitabine	Yes	1.1.2.6.
	Abacavir (licensed as abacavir sulfate)	Yes	1.1.2.7.
Nucleotide reverse transcriptase inhibitors	Tenofovir (licensed as tenofovir disproxil fumarate)	Yes	1.2.2.

Non-nucleoside reverse transcriptase inhibitors	Nevirapine	No	1.3.2.
	Delavirdine	No	
	Efavirenz	No	
	Etravirine	N/A	
	Rilpivirine	N/A	
Protease inhibitors	Saquinavir (licensed as saquinavir mesylate)	No	2.2.
	Indinavir	No	
	Ritonavir	No	
	Nelfinavir (licensed as nelfinavir mesylate)	No	
	Amprenavir (no longer marketed)	No	
	Lopinavir	Yes	
	Atazanavir (licensed as atazanavir sulfate)	Yes	
	Fosamprenavir (licensed as fosamprenavir calcium)	No	
	Tipranavir	Yes	
	Darunavir	No	
Fusion inhibitors	Enfuvirtide	No	3.1.2.
Co-receptor inhibitor	Maraviroc	N/A	3.2.2.
Integrase inhibitors	Raltegravir	Yes	4.2.
	Dolutegravir	N/A	

1. Reverse transcriptase inhibitors

The retroviral enzyme reverse transcriptase transcribes the viral RNA into proviral DNA, which is subsequently integrated into the host cell's genome (MITSUYA and BRODER, 1987). This is an important step in the retroviral replication cycle and the compounds that inhibit this step have become the cornerstone of successful anti-HIV therapy (CIHLAR and RAY, 2010).

Reverse transcriptase inhibitors can be divided into three categories (DECLERCQ, 2009):

- NRTI
- NtRTI
- NNRTI

1.1. Nucleoside reverse transcriptase inhibitors

The first drugs approved for the treatment of HIV were NRTIs and they still represent a vital component of the treatment regimens for HIV-infected patients (TRESSLER and GODFREY, 2012).

Currently there are seven NRTIs approved by the FDA for the treatment of HIV infection. One of these (zalcitabine) is no longer marketed (FDA, 2014).

1.1.1. Mechanism of action

NRTIs are analogues of endogenous 2'-deoxynucleosides (DE CLERCQ, 2009; CIHLAR and RAY, 2010). Nucleosides are the building blocks of nucleic acids and are composed of a nitrogenous base and a five-carbon sugar (ribose or deoxyribose). Like natural nucleosides, NRTIs require intracellular enzymatic activation through three phosphorylation steps to their 5'-triphosphate form (nucleotide) (DE CLERCQ, 2009). In their active form, they compete with the endogenous nucleotides at the catalytic, i.e., substrate-binding, site of RT and are incorporated into the elongating proviral deoxyribonucleic acid (DNA) strand (DE CLERCQ, 2009; CIHLAR and RAY, 2010), thus functioning as competitive substrate inhibitors (DE CLERCQ, 2009). However, in comparison to the natural nucleotides, NRTIs lack the 3'-hydroxyl group on the deoxyribose moiety and this leads to strand termination as the subsequent nucleotide cannot form the next 5'-3' phosphodiester bond necessary to extend the DNA strand (MOHAMMADI and BIENZLE, 2012; TRESSLER and GODFREY, 2012).

1.1.2. Efficacy against FIV

As FIV became an important animal model for HIV infection for the purpose of assessing NRTIs, all currently licensed NRTIs have been tested in feline cell culture for their anti-FIV efficacy. The initial studies were usually performed to assess the drugs' anti-FIV activity and subsequent studies assessed their behavior against different FIV strains or molecular clones, drug-resistant mutants or the influence of different cell culture systems on antiviral efficacy (GOBERT et al., 1994; VAHLENKAMP et al., 1995; SMITH et al., 1997; SMITH et al., 1998; BISSET et al., 2002; VAN DER MEER et al., 2007).

1.1.2.1. Zidovudine

Zidovudine (3'-azido-3'-deoxythymidine, AZT) is a thymidine analogue and was

first synthesized in the 1960ties (HORWITZ et al., 1964) as a potential anticancer agent. Mitsuya and coworkers (1985) showed for the first time its anti-HIV efficacy *in vitro* and Furman and coworkers (1986) elucidated that it acts by inhibiting RT through its triphosphate form (MITSUYA et al., 1985; FURMAN et al., 1986). In 1987 it was the first drug to be approved by the FDA for the treatment of HIV infection and to this day it remains an important component of HAART (EZZELL, 1987; TRESSLER and GODFREY, 2012).

Since the discovery of FIV in 1986 (PEDERSEN et al., 1987), the anti-FIV activity of zidovudine has been assessed in numerous *in vitro* studies. In 1989, North and coworkers showed that zidovudine inhibited FIV replication in Crandell-Rees feline kidney (CRFK) cells (see table 2). The susceptibility of FIV to zidovudine was similar to that of HIV-1 (NORTH et al., 1989). In order to demonstrate the presence or absence of antiviral efficacy most studies report the 50% effective concentration (EC_{50}) which is the concentration of drug required to inhibit virus proliferation in cell culture by 50%. The EC_{50} allows comparison of antiviral activities. Table 2 summarizes the *in vitro* studies investigating the antiviral efficacy of zidovudine against FIV in different cell systems.

Table 2: *In vitro* antiviral efficacy of zidovudine against FIV (FIV, feline immunodeficiency virus; EC_{50} , 50% effective concentration; CRFK, Crandell-Rees feline kidney; RT, reverse transcriptase; PBL, peripheral blood lymphocytes; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; PBM, peripheral blood mononuclear; *pol* RT-PCR, reverse transcription-polymerase chain reaction specific for *pol* region of FIV; ND, not determined; [†], mean \pm standard error; ^{*}, mean \pm standard deviation; [§], not specified)

Study	FIV strain	Cells	Days after inoculation	EC_{50}	Detection method of virus replication
North et al., 1989	FIV Petaluma	CRFK cells	5	0.15 ± 0.02 [§] μ M	RT activity
Remington et al., 1991	FIV Petaluma	CRFK cells	4	1.4 ± 0.4 [*] μ M	Focal infectivity assay
Tanabe-Tochikura et al., 1992	FIV Petaluma	3201 cells	28	0.03 μ M	RT activity

Gobert et al., 1994	FIV Petaluma	CRFK cells	4	$0.32 \pm 0.01^{\dagger}$ μM	Focal infectivity assay
	FIV 34TF10			$0.13 \pm 0.01^{\dagger}$ μM	
Smyth et al., 1994	FIV E77	PBL	5	$0.75 \pm 0.34^*$ μM	p24 Ag ELISA
	FIV-8	CRFK cells	7	$0.19 \pm 0.02^*$ μM	
Remington et al., 1994	FIV Petaluma	CRFK cells	4	$1.4 \pm 0.4^*$ μM	Focal infectivity assay
	FIV 34TF10			$2.0 \pm 0.9^*$ μM	
Medlin et al., 1995	FIV 34TF10	CRFK cells	4	$0.34 \mu\text{M}$	Focal infectivity assay
Vahlenkamp et al., 1995	FIV UT- 113	Thymocytes	6	$0.05 \pm 0.02^{\dagger}$ μM	p24 Ag ELISA
		CRFK cells		$4.0 \pm 0.52^{\dagger}$ μM	
Hartmann et al., 1995	FIV Petaluma	CRFK cells	10	$0.06 \mu\text{M}$	RT activity
				$8.63 \mu\text{M}$	p24 Ag ELISA
		PBL		$0.02 \mu\text{M}$	RT activity
				$0.14 \mu\text{M}$	p24 Ag ELISA
Zhu et al., 1996	FIV 34TF10	CRFK cells	4	$0.3 \pm 0.1^{\dagger}$ μM	Focal infectivity assay
Smith et al., 1997	FIV 34TF10	CRFK cells	4	$1.0 \pm 0.3^{\dagger}$ μM	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	$1.3 \pm 0.2^{\dagger}$ μM	Focal infectivity assay
McCrackin Stevenson and McBroom, 2000	FIV Maxam	PBM cells	7	$0.07 \pm 0.02^{\dagger}$ μM	p24 Ag ELISA
	FIV pPPR			$0.03 \pm 0.02^{\dagger}$ μM	
Bisset et al., 2002	FIV Petaluma	CRFK cells	8	$2.13 \pm 0.83^{\dagger}$ μM	<i>pol</i> RT-PCR

Arai et al., 2002	FIV Glasgow-8	PBM cells from infected cats	9, 12, 15	ND	RT activity
	FIV Bangston	FIV _{Bang} /FeT- J cells			
	FIV Shizuoka	FIV _{Shi} /FeT-J cells			
	FIV Petaluma	FL-4 cells			
	FIV Glasgow-8	T-cell enriched PBM cells			
van der Meer et al., 2007	FIV 113 _{Th}	Thymocytes	6	$0.56 \pm 0.26^*$ μM	p24 Ag ELISA
		Dendrital cell- thymocyte coculture		$3.67 \pm 2.32^*$ μM	

1.1.2.2. Didanosine

Didanosine (2',3'-dideoxyinosine, ddI) is a dideoxynucleoside analogue which is converted to its active form dideoxyadenosin triphosphate by intracellular phosphorylation (PERRY and BALFOUR, 1996). In 1986, shortly after the discovery of zidovudine's anti-HIV activity, Mitsuya and Broder described the antiviral activity of didanosine against HIV (MITSUYA and BRODER, 1986) and in 1991 it was the second antiretroviral drug to be approved by the FAD for the treatment of HIV/AIDS (FDA, 2014). Several studies have investigated its efficacy against FIV *in vitro* (table 3).

Table 3: *In vitro* antiviral efficacy of didanosine against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; RT, reverse transcriptase; PBL, peripheral blood lymphocytes; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; [†], mean ± standard error; *, mean ± standard deviation)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
Remington et al., 1991	FIV Petaluma	CRFK cells	4	2.1 ± 0.7* μM	Focal infectivity assay
Tanabe-Tochikura et al., 1992	FIV Petaluma	3201 cells	28	1.1 μM	RT activity
Gobert et al., 1994	FIV Petaluma	CRFK cells	4	1.9 ± 0.04 [†] μM	Focal infectivity assay
	FIV 34TF10			1.1 ± 0.01 [†] μM	
Smyth et al., 1994	FIV E77	PBL	5	3.25 ± 0.25* μM	p24 Ag ELISA
	FIV 8	CRFK cells	7	0.97 ± 0.34* μM	
Remington et al., 1994	FIV Petaluma	CRFK cells	4	2.1 ± 1* μM	Focal infectivity assay
	FIV 34TF10			1.8 ± 0.6* μM	
Medlin et al., 1995	FIV 34TF10	CRFK cells	4	5.5 ± 0.8 [†] μM	Focal infectivity assay
Zhu et al., 1996	FIV 34TF10	CRFK cells	4	1.1 ± 0.04 [†] μM	Focal infectivity assay
Smith et al., 1997	FIV 34TF10	CRFK cells	4	1.9 ± 0.4 [†] μM	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	3.2 ± 0.5 [†] μM	Focal infectivity assay

1.1.2.3. Zalcitabine

Like didanosine, zalcitabine (2',3'-dideoxycytidine, ddC) is a dideoxynucleoside analogue which is converted intracellularly to its active form 2',3'-dideoxycytidine triphosphate. In 1986, Mitsuya and Broder showed that it is a very potent inhibitor

of HIV (MITSUYA and BRODER, 1986) and after zidovudine it was the second drug to be tested clinically in patients with AIDS (YARCHOAN et al., 1988; MERIGAN et al., 1989). It was approved by the FDA in 1992 but it has not been marketed since December 2006 (FDA, 2014).

Table 4: *In vitro* antiviral efficacy of zalcitabine against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; RT, reverse transcriptase; PBL, peripheral blood lymphocytes; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; PBM, peripheral blood mononuclear; †, mean ± standard error; *, mean ± standard deviation)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
North et al., 1989	FIV Petaluma	CRFK cells	5	>1 µM	RT activity
Gobert et al., 1994	FIV Petaluma	CRFK cells	4	5.7 ± 0.4 † µM	Focal infectivity assay
	FIV 34TF10			2.3 ± 0.05 † µM	
Smyth et al., 1994	FIV E77	PBL	5	0.71 ± 0.24* µM	p24 Ag ELISA
	FIV 8	CRFK cells	7	0.33 ± 0.12* µM	
Remington et al., 1994	FIV Petaluma	CRFK cells	4	6.0 ± 1* µM	Focal infectivity assay
	FIV 34TF10			4.7 ± 2.3* µM	
Medlin et al., 1995	FIV 34TF10	CRFK cells	4	4.9 ± 0.7 † µM	Focal infectivity assay
Zhu et al., 1996	FIV 34TF10	CRFK cells	4	4.4 ± 0.3 † µM	Focal infectivity assay
Smith et al., 1997	FIV 34TF10	CRFK cells	4	1.9 ± 0.3 † µM	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	2.0 ± 0.1 † µM	Focal infectivity assay
McCrackin Stevenson and McBroom, 2000	FIV Maxam	PBM cells	7	0.86 ± 0.07 † µM	p24 Ag ELISA
	FIV pPPR			1.21 ± 0.56 † µM	

1.1.2.4. Stavudine

Like zidovudine, stavudine (2',3'-didehydro-2',3'-dideoxythymidine, d4T) is a thymidine analogue and was first synthesized in the 1960ies (HORWITZ et al., 1966) as a potential anticancer agent. Its efficacy against HIV was first described in 1987 by two working groups (BABA et al., 1987; BALZARINI et al., 1987; LIN et al., 1987; AUGUST et al., 1988). It was approved by the FDA for the treatment of HIV infection in 1994 and was an essential component of initial antiretroviral combination therapy. Only in recent years it has been replaced by better tolerated medications (MARTIN et al., 2010). Several studies have assessed stavudine's anti-FIV efficacy and table 5 provides a summary.

Table 5: *In vitro* antiviral efficacy of stavudine against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; PBL, peripheral blood lymphocytes; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; †, mean ± standard error; *, mean ± standard deviation)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
Remington et al., 1991	FIV Petaluma	CRFK cells	4	12.3 ± 3.5* μM	Focal infectivity assay
Gobert et al., 1994	FIV Petaluma	CRFK cells	4	5.7 ± 0.6 † μM	Focal infectivity assay
	FIV 34TF10			5.9 ± 0.9 † μM	
Smyth et al., 1994	FIV E77	PBL	5	11.60 ± 2.23* μM	p24 Ag ELISA
	FIV 8	CRFK cells	7	8.92 ± 13.38* μM	
Remington et al., 1994	FIV Petaluma	CRFK cells	4	12 ± 4* μM	Focal infectivity assay
	FIV 34TF10			21 ± 9* μM	
Balzarini et al., 1996	FIV UT113	CRFK cells	6	4.0 μM	p24 Ag ELISA
Zhu et al., 1996	FIV 34TF10	CRFK cells	4	9 ± 2 † μM	Focal infectivity assay

Smith et al., 1997	FIV 34TF10	CRFK cells	4	$14.0 \pm 1.9^{\dagger}$ μM	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	$13 \pm 1.8^{\dagger}$ μM	Focal infectivity assay

1.1.2.5. Lamivudine

Like zalcitabine, lamivudine ((-)-2',3'-dideoxy-3'-thiacytidine, 3TC) is a cytidine analogue. It is structurally different in that the 3' carbon of the ribose in zalcitabine is replaced by a sulfur atom (COATES et al., 1992). Lamivudine's anti-HIV activity was first described by Belleau and coworkers in 1989 (BELLEAU et al., 1989) and it was licensed by the FDA for the treatment of HIV/AIDS in 1995 (FDA, 2014). It is a potent and well tolerated anti-HIV drug. The major side effect is the development of a M184V mutation in the HIV *pol* gene (RAVICHANDRAN et al., 2008). It is also active against FIV (table 6).

Table 6: *In vitro* antiviral efficacy of lamivudine against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; RT, reverse transcriptase; PBL, peripheral blood lymphocytes; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; PBM, peripheral blood mononuclear; *pol* RT-PCR, reverse transcription-polymerase chain reaction specific for *pol* region of FIV; ND, not determined; [†], mean \pm standard error; *, mean \pm standard deviation)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
Smyth et al., 1994	FIV E77	PBL	5	$0.17 \pm 0.04^*$ μM	p24 Ag ELISA
	FIV 8	CRFK cells	7	$0.39 \pm 0.13^*$ μM	
Medlin et al., 1995	FIV 34TF10	CRFK cells	4	0.58 μM	Focal infectivity assay
Smith et al., 1997	FIV 34TF10	CRFK cells	4	$1.2 \pm 0.1^{\dagger}$ μM	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	$1.5 \pm 0.3^{\dagger}$ μM	Focal infectivity assay

McCrackin Stevenson and McBroom, 2000	FIV Maxam	PBM cells	7	$0.14 \pm 0.01^{\dagger}$ μM	p24 Ag ELISA
	FIV pPPR			$0.46 \pm 0.04^{\dagger}$ μM	
Bisset et al., 2002	FIV Petaluma	CRFK cells	8	$2.44 \pm 0.64^{\dagger}$ μM	<i>pol</i> RT-PCR
Arai et al., 2002	FIV Glasgow 8	PBM cells from infected cats	9, 12, 15	ND	RT activity
	FIV Bangston	FIV _{Bang} /Fe T-J cells			
	FIV Shizuoka	FIV _{Shi} /Fe T-J cells			
	FIV Petaluma	FL-4 cells			
	FIV Glasgow-8	T-cell enriched PBM cells			

1.1.2.6. Emtricitabine

Emtricitabine ((-)-2',3'-deoxy-5-fluoro-3'-thiacytidine, FTC) is another cytidine analogue and is structurally similar to lamivudine. It was developed after the discovery of the antiviral activity of lamivudine and was found to be very potent and selective against HIV (SCHINAZI et al., 1992). It was shown that treatment with emtricitabine *versus* lamivudine leads to reduced emergence of the M184V/I resistance mutation in antiretroviral naïve patients (MCCOLL et al., 2011). It was licensed by the FDA in 2003 (FDA, 2014).

Table 7: *In vitro* antiviral efficacy of emtricitabine against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; p24 Ag, p24 antigen; ELISA, enzyme-linked immunosorbent assay; PBM, peripheral blood mononuclear; ND, not determined; [†], mean \pm standard error)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
Smith et al., 1997	FIV 34TF10	CRFK cells	4	0.9 \pm 0.2 [†] μ M	Focal infectivity assay
Smith et al., 1998	FIV 34TF10	CRFK cells	4	1.0 \pm 0.1 [†] μ M	Focal infectivity assay
McCrackin Stevenson and McBroom, 2000	FIV Maxam	PBM cells	7	ND	p24 Ag ELISA
	FIV pPPR			0.18 \pm 0.04 [†] μ M	

1.1.2.7. Abacavir

Abacavir ((-)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, ABC) is a guanosine analogue and was first described in 1997 by Daluge and coworkers. It was shown to have potent and selective anti-HIV activity equivalent to that of zidovudine in human blood lymphocyte cultures. It showed minimal cross-resistance with zidovudine and other approved HIV NRTIs and was synergistic with zidovudine and some other compounds (DALUGE et al., 1997). In 1998 it was approved by the FDA (FDA, 2014). Prior to the study reported here, it has been assessed against FIV in only one study, to our knowledge (table 8).

Table 8: *In vitro* antiviral efficacy of abacavir against FIV (FIV, feline immunodeficiency virus; EC₅₀, 50% effective concentration; CRFK, Crandell-Rees feline kidney; *pol* RT-PCR, reverse transcription-polymerase chain reaction specific for *pol* region of FIV; ND, not determined; †, mean ± standard error)

Study	FIV strain	Cells	Days after inoculation	EC ₅₀	Detection method of virus replication
Bisset et al., 2002	FIV Petaluma	CRFK cells	8	3.07 ± 1.39 [†] μM	<i>pol</i> RT-PCR

1.2. Nucleotide reverse transcriptase inhibitors

Currently there is only one FDA approved NtRTI (tenofovir disoproxil fumarate) which was licensed in 2001 (FDA, 2014). Since then it has become one of the most widely used antiretroviral drugs in HIV therapy (DE CLERCQ, 2010; TRESSLER and GODFREY, 2012).

1.2.1. Mechanism of action

Like NRTIs, NtRTIs interact with the catalytic site of RT and are incorporated into the elongating proviral DNA strand, subsequently causing strand termination (RAVICHANDRAN et al., 2008; DE CLERCQ, 2009). They compete with the natural nucleotides and thus also function as competitive substrate inhibitors. However in contrast to NRTIs, NtRTIs contain a phosphate group and therefore need only two intracellular phosphorylation steps to be converted into their active forms (BALZARINI et al., 1997; DE CLERCQ, 2009). This circumvents the first and often rate limiting phosphorylation step (RAVICHANDRAN et al., 2008; CIHLAR and RAY, 2010) and therefore offers an advantage over NRTIs. Currently, the only approved NtRTI for the treatment of HIV infection is tenofovir disoproxil fumarate (TDF), the prodrug of tenofovir ((R)-9-(2-phosphonylmethoxypropyl)adenine, (R)-PMPA), which is a member of the acyclic nucleoside phosphonates (BALZARINI et al., 1997; CIHLAR and RAY, 2010). In acyclic nucleoside phosphonates, the alkyl side chain of purines and pyrimidines is linked to a modified phosphate moiety and a C-P phosphonate linkage replaces the normal O5'-P phosphate linkage (BALZARINI et al., 1997;

CIHLAR and RAY, 2010). This phosphonate bond is non-hydrolyzable which makes it more difficult to cleave off these compounds once they have been incorporated at the 3'-terminal end of the elongating proviral DNA strand (DE CLERCQ, 2009).

1.2.2. Efficacy against FIV

Balzarini and coworkers (1997) showed that tenofovir ((R)-9-(2-phosphonylmethoxypropyl)adenine, (R)-PMPA)) is a potent inhibitor of FIV in both CRFK cells and PBM cells. It was compared to two other antiretroviral compounds, including PMEA (9-(2-phosphonylmethoxyethyl)adenine), the prototype acyclic nucleoside phosphonate (BALZARINI et al., 1996a; BALZARINI et al., 1997). PMEA is a potent inhibitor of FIV and has been investigated more extensively *in vitro* regarding its anti-FIV activity and generally has been shown to be at least equally as potent as zidovudine (GOBERT et al., 1994; REMINGTON et al., 1994; VAHLENKAMP et al., 1995; ZHU et al., 1996; SMITH et al., 1998; VAN DER MEER et al., 2007). In comparison to PMEA, tenofovir (R-PMPA) was less toxic in feline PBM cells and showed greater anti-FIV efficacy (BALZARINI et al., 1997). Vahlenkamp and coworkers (1995) also compared tenofovir to other antiretroviral compounds, including PMEA in feline thymocytes and CRFK cells. The antiviral efficacy against FIV and cytotoxicity of both compounds were comparable, although PMEA was somewhat more toxic in PBM cells (VAHLENKAMP et al., 1995).

1.3. Non-nucleoside reverse transcriptase inhibitors

NNRTIs were the second drug category from which members were approved for the treatment of HIV infection. There are currently five FDA approve NNRTIs: nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine (FDA, 2014).

1.3.1. Mechanism of action

Unlike NRTIs and NtRTIs, which bind to the catalytic site of RT, NNRTIs interact with an allosteric site of the enzyme (DE CLERCQ, 2009) and are not incorporated into the proviral DNA strand (RAVICHANDRAN et al., 2008). They are classified as non-competitive inhibitors of RT and do not require intracellular activation to inhibit the enzyme (RAVICHANDRAN et al., 2008; MOHAMMADI and BIENZLE, 2012). NNRTIs are a group of structurally diverse compounds that all bind a single site in the HIV RT enzyme (XIA et al.,

2007). Five NNRTIs are currently licensed for treatment of HIV infection and these drugs are used in several anti-retroviral therapy regimens. Despite their common use, the molecular mechanism of inhibition by these drugs is not entirely clear (DAS et al., 2012). The interaction with the allosteric site which is located in close proximity to the catalytic site, leads to a number of conformational changes within the RT (XIA et al., 2007; DAS et al., 2012). Amongst other effects, these changes cause a decrease in the interaction between the DNA primer and the polymerase domain of the enzyme and thus, result in inhibition of virus replication. Additionally it has been shown that excision of an incorporated NRTI from the elongating proviral DNA strand is blocked in the presence of the NNRTI nevirapine, indicating that NNRTIs and NRTIs have complementary roles in inhibiting RT (DAS et al., 2012).

Unlike NRTIs, NNRTIs are highly specific inhibitors of HIV-1 and it has been shown that they are not active against HIV-2 or other retroviruses, including FIV (AUWERX et al., 2004; RAVICHANDRAN et al., 2008). This is the case despite close similarity of the NNRTI binding pocket of HIV-1 RT and FIV RT. In fact, only four of the 20 relevant amino acids lining the binding pocket are not present in FIV RT (AUWERX et al., 2004). Only three of the five FDA-approved NNRTIs (nevirapine, delavirdine, efavirenz) have been assessed against FIV *in vitro*. *In vivo* studies have not been performed, presumable because of the lack of *in vitro* efficacy.

1.3.2. Efficacy against FIV

In a study by Auwerx and coworkers (2002) it was shown that nevirapine, delavirine and efavirenz, had no inhibitory activity against FIV RT, even at drug concentrations several orders of magnitude higher than those required for suppression of HIV-1 RT activity. Chimeric RT enzymes were also assessed and it was found that HIVp66/FIVp51 RT retained susceptibilities to all NNRTIs similar to the wild-type HIV-1 enzyme, and FIVp66/HIVp51 RT remained resistant to the NNRTIs, pointing to a minor role of the p51 subunit in terms of susceptibility to these RT inhibitors (AUWERX et al., 2002). These findings differ markedly from those reported in a previous study by Amacker and Hubscher (1998) who found nevirapine to be inhibitory towards both FIV RT as well as the chimeric FIVp66/HIVp51 RT (AMACKER and HUBSCHER, 1998).

In order to map the determinants of lack of susceptibility of FIV RT to NNRTIs effective against HIV, Auwerx and coworkers (2004) conducted another study and designed several chimeric HIV-1/FIV RTs. Similarly to the previous study by Auwerx and coworkers (2002), nevirapine and efavirenz were investigated. In accordance with the findings from that previous study, FIV RT was not inhibited by any NNRTI, even at concentrations as high as 1000 μM , whereas HIV-1 RT was sensitive to both NNRTIs. None of the FIV-derived chimera which contained several amino acid fragments from HIV-1 RT gained susceptibility to the NNRTIs. In a second part of this study, point mutations were introduced into the FIV RT and the susceptibility of the mutant enzymes to nevirapine, efavirenz, and delavirdine was investigated. The amino acids of choice were those that were crucial for HIV-1 RT to maintain full sensitivity to NNRTIs but were different in FIV RT. Neither of the NNRTIs showed any inhibitory activity against any FIV RT mutant (AUWERX et al., 2004).

Etravirine and rilpivirine gained their FDA approval more recently in 2008 and 2011, respectively (FDA, 2014). Neither of these two drugs has been assessed against FIV.

2. Protease inhibitors

Currently, there are ten PIs that have been licensed by the FDA (saquinavir mesylate, ritonavir, indinavir, nelfinavir, lopinavir, atazanavir sulfate, amprenavir [no longer marketed], fosamprenavir, darunavir, tipranavir) (FDA, 2014).

2.1. Mechanism of action

The retroviral protease is responsible for the processing of Gag and Gag-Pol polyproteins into individual structural and enzymatic proteins. These steps take place during virus assembly and maturation and are critical for the production of infectious virions (SLEE et al., 1995; ELDER et al., 2010).

Both FIV and HIV-1 protease are homodimers which have very similar three-dimensional structures; however, each monomer of the FIV protease is comprised of 116 amino acids as opposed to 99 amino acids for the HIV-1 protease and both enzymes share only 23% amino acid identity (ELDER et al., 2008; LIN et al., 2010). Despite similarities between the two enzymes, it has been shown that the

FIV protease is specific to its respective substrates. Most of the currently employed anti-HIV PIs that have been tested were not effective in inhibiting FIV protease.

PIs contain a hydroxyethylene scaffold which mimics the normal peptide linkage that would normally be cleaved within the active site of the enzyme. This linkage however, has been replaced with a nonhydrolyzable one and therefore PIs prevent the protease from carrying out its normal action (DE CLERCQ, 1995b, 2009). This is the mechanism of action for all but one (tipranavir) of the FDA-approved PIs (DE CLERCQ, 2009).

Tipranavir is different in that it is a non-peptidic PI belonging to the class of sulfonamide-containing dihydropyrones. It also binds the active site of the protease; however it is based on a different chemical scaffold than the other PIs (LUNA and TOWNSEND, 2007; MUZAMMIL et al., 2007).

A number of previous studies (POPPE et al., 1997; LARDER et al., 2000; RUSCONI et al., 2000) showed that tipranavir had excellent *in vitro* antiviral activity against HIV-1 laboratory strains and clinical isolates as well as HIV-1 isolates that had become resistant to other PIs. Muzammil and coworkers (2007) demonstrated that tipranavir's mode of action was different to that of other PIs in that it established unique favorable thermodynamic interactions with the protease resulting in a high binding affinity, and structurally it built a very strong hydrogen bond network with regions of the protease that cannot undergo mutation. Therefore this strong network was maintained with the mutant forms of the protease. Additionally, tipranavir relied on fewer water-mediated hydrogen bonds than the other PIs and formed direct hydrogen bond interactions which is considered energetically more favorable (MUZAMMIL et al., 2007).

2.2. Efficacy against FIV

Many compounds, some of which later became the PIs that are now FDA-approved and are used in the treatment of HIV-infected patients today, were assessed during their experimental stage for their activity against the FIV protease. However, no potent inhibitory effect on the FIV protease could be detected. For example Wlodawer and coworkers (1995) assessed four different inhibitors containing hydroxyethyl peptidomimetics for their inhibitory activity on FIV as well as HIV protease. The K_i value (inhibition constant, concentration

required to produce half maximum inhibition of the enzyme) for all four compounds was between 15 to 127 times higher for FIV than HIV, indicating weak inhibition of the FIV protease, while they were strong inhibitors of the HIV protease. The authors also commented that this was in accordance with their previous findings where, despite testing over 30 potent HIV protease inhibitors with a wide variety of different structures, they were not able to identify any compounds that would also potentially inhibit FIV protease (WLODAWER et al., 1995).

Slee and coworkers (1995) described the development of new pyrrolidine-containing α -keto amide and hydroxyethylamine core structures as inhibitors of the HIV protease. None of these structures showed significant inhibitory activity against the mechanistically identical FIV protease. For one of the assessed compounds, their research showed that additional specific residues between the P4 – P4' sites (residues of the substrate at position P4 – P4' which bind to S4 – S4' subsites in the active site of the enzyme) were required before FIV protease was able to recognize it as a substrate (SLEE et al., 1995).

Wlodawer and coworkers (1989) and Schnolzer and coworkers (1996) also showed that FIV PR requires a longer substrate than HIV PR of at least eight amino acids. Since most PIs for HIV-1 PR are based on peptides less than seven amino acids, this offers an explanation for their lack of efficacy against FIV protease (WLODAWER et al., 1989; SCHNOLZER et al., 1996).

Lee and coworkers (1998) proposed a structural explanation for the discrepancy between the inhibition of HIV and FIV protease by anti-HIV protease inhibitors. They showed that there is a severe restriction of P3 and P3' moieties in FIV protease due to a restriction in size of the combined S1/S3 (subsite 1/subsite 3) substrate binding pocket of the FIV protease in comparison to the same site in the HIV-1 protease (LEE et al., 1998; ELDER et al., 2010). Lee and coworkers (1998) showed that saquinavir, a HIV PI with a bulky aromatic group at the P3 position, completely lost potency against FIV protease. Other PIs with large P3 and P3' moieties are e.g. ritonavir, indinavir, and nelfinavir (LEE et al., 1998).

In a study by Lin and coworkers (2003) it was shown that none of the potent HIV-1 protease inhibitors saquinavir, ritonavir and nelfinavir, were good inhibitors of the FIV protease. Their K_i values could not be determined due to their poor

inhibitory activity and their poor solubility at high concentrations (LIN et al., 2003).

It has been shown that FIV protease behaves like the drug-resistant phenotypes of HIV protease (DUNN et al., 1999) as several HIV protease amino acid residues mutate to the structurally aligned residue found in FIV protease (SLEE et al., 1995).

In a recent study by Norelli and coworkers (2008), three second generation FDA-approved PIs (lopinavir, atazanavir, tipranavir) were investigated for their *in vitro* efficacy against FIV. The binding of lopinavir and atazanavir to the FIV protease was limited which was related to the fact that the protease of FIV resembles the protease of HIV drug-resistant mutations. The binding of tipranavir however, was not limited. All PIs resulted in dose-dependent FIV inhibition, however only tipranavir's efficacy against FIV was comparable to that against HIV (NORELLI et al., 2008). As mentioned above, tipranavir's mode of action is different from the other anti-HIV PIs. Norelli and coworkers (2008) showed in molecular docking simulations that tipranavir maintains favorable energetic interactions with the dimeric FIV protease. It establishes a very strong hydrogen bond network with FIV protease involving invariant regions of the enzyme (NORELLI et al., 2008). These findings are in line with those for drug-resistant HIV mutants by Muzammil and coworkers (MUZAMMIL et al., 2007).

In a study by Lin and coworkers (2010) high resistance of the wild-type FIV protease to darunavir and lopinavir was shown, and in an *ex vivo* infectivity assay it was demonstrated that replication of wild-type FIV could not be inhibited by these two HIV-1 PIs at any of the concentrations (up to 800 nM) investigated (LIN et al., 2010). Lin and coworkers (2010) also designed infectious FIV mutants encoding selected FIV/HIV chimeric proteases. In contrast to the FIV wild-type protease, the chimeric proteases showed high sensitivity to both darunavir and lopinavir (LIN et al., 2010).

3. Entry inhibitors

The compounds within this group are fusion inhibitors and co-receptor inhibitors. They interfere with different steps of viral entry into the host cell.

3.1. Fusion inhibitors

Only one fusion inhibitor is currently licensed by the FDA which is enfuvirtide, also known as T-20. It was licensed in 2003 (FDA, 2014).

3.1.1. Mechanism of action

For viral entry and infection, fusion between the target cell membrane and the virus is a critical step in the lentivirus life cycle which in the case of FIV is mediated by the envelope glycoprotein 40 (gp40) (OISHI et al., 2009). Binding of the FIV surface (SU) glycoprotein to the cellular receptors CD134 and CXCR4 results in a conformational change of gp40. Although the detailed mechanism of the virus-cell fusion process of FIV is not known, it is believed to be similar to that of HIV (OISHI et al., 2009). The HIV transmembrane protein gp41 contains functional domains consisting of a fusion peptide, heptad repeats 1 and 2 (HR1 and 2) and a transmembrane domain (MIZUKOSHI et al., 2009). After the attachment of HIV to the CD4 receptor and chemokine co-receptor on the host cell, a conformational change in gp41 allows it to insert its hydrophobic N terminus into the host cell membrane (MATTHEWS et al., 2004). Subsequently, the HR1 and HR2 domains interact with each other which brings the viral and cellular membranes into close contact, allowing membrane fusion and infection of the target cell (MATTHEWS et al., 2004; OISHI et al., 2009).

Fusion inhibitors are synthetic peptides derived from gp41 and block the entry of HIV into the host cell by binding to the HR regions of gp41 (MATTHEWS et al., 2004). Thereby they prevent the necessary interaction between HR1 and HR2 (MEDINAS et al., 2002; MATTHEWS et al., 2004; DE CLERCQ, 2009).

3.1.2. Efficacy against FIV

Currently only one fusion inhibitor (enfuvirtide, T-20) is licensed by the FDA for the treatment of HIV infection which is a 36-amino-acid peptide derived from the HR2 region of HIV gp41 (MATTHEWS et al., 2004). It has been shown that enfuvirtide selectively inhibits HIV-1; neither HIV-2 nor simian immunodeficiency virus (SIV) was susceptible (MATTHEWS et al., 2004). A study by Medinas and colleagues (2002) found that there was also a lack of activity of enfuvirtide against FIV *in vitro* as demonstrated by an 88,000-fold higher EC₅₀ compared to that against HIV-1. Furthermore this study assessed the antiviral activity of 15 peptides derived from the HR2 domain of FIV gp40 and

showed that the peptides examined had varied antiviral activities *in vitro*. Several of the peptides exhibited good activity against FIV as demonstrated by inhibition of syncytium formation in a cell-cell fusion assay and inhibition of RT production in a FIV infectivity assay (MEDINAS et al., 2002). A number of other studies assessed various synthetic peptides for their anti-FIV activity and identified several peptides with anti-FIV activity *in vitro* (LOMBARDI et al., 1996; GIANNECCHINI et al., 2003; D'URSI et al., 2006; MIZUKOSHI et al., 2009; OISHI et al., 2009).

3.2. Co-receptor inhibitors

There is currently only one co-receptor inhibitor (maraviroc) which is FDA-approved for the treatment of HIV/AIDS. It was approved in 2007 (FDA, 2014).

3.2.1. Mechanism of action

Similar to T cell-tropic HIVs, all domestic cat FIVs use the chemokine receptor CXCR4 as one of their receptors for virus entry into the target cell (ELDER et al., 2010). Binding of FIV and HIV to this cell receptor is essential for cell entry of both viruses (MIZUKOSHI et al., 2009). Therefore, the binding of CXCR4 antagonists will prevent binding of the FIV major SU glycoprotein to this receptor and prevent infection of the target cell. In the whole cell entry process of the virus, this step lies between the initial attachment of the virus to the CD134 receptor and the fusion of the virus with the cell membrane mediated by gp41 (DE CLERCQ, 2009; WILLETT and HOSIE, 2013). The amino acid sequence of feline and human CXCR4 is highly similar (94.9% sequence identity) (WILLETT et al., 1997b) and hence CXCR4 antagonists developed against HIV could also be effective against FIV (MOHAMMADI and BIENZLE, 2012).

Macrophage-tropic HIV strains however, use a different co-receptor, namely CCR5 (DE CLERCQ, 2009). Unlike these HIV strains, FIV does not bind to CCR5. CXCR4 is the sole co-receptor of FIV (WILLETT and HOSIE, 2013).

3.2.2. Efficacy against FIV

The currently licensed co-receptor inhibitor (maraviroc) is a CCR5 co-receptor antagonist. As FIV does not bind this cell receptor, this drug has not been assessed against FIV.

Egberink and colleagues (1999) showed that bicyclams, which are selective

inhibitors of the human chemokine receptor CXCR4, potentially inhibit FIV replication. Inhibition of fusion and entry of FIV was clearly demonstrated in the presence of bicyclams. The prototype bicyclam plerixafor (AMD3100) as well as other bicyclam derivatives were assessed in different feline cell lines (EGBERINK et al., 1999). Plerixafor is not licensed as an anti-HIV drug, but is used in humans for stem cell mobilization (LILES et al., 2003).

Joshi and colleagues (2005) investigated the mechanism responsible for the preferential replication of FIV in highly purified CD4⁺ CD25⁺ Treg cells as opposed to their CD4⁺ CD25⁻ counterparts. As part of this study it was shown that AMD3100 in concentrations of 0.01 to 1 µg/ml was able to reduce FIV replication in both cell types, however significantly more so in CD4⁺ CD25⁻ cells (JOSHI et al., 2005).

4. Integrase inhibitors

To date, two integrase inhibitors (raltegravir and dolutegravir) have been approved by the FDA for the treatment of HIV infection (FDA, 2014).

4.1. Mechanism of action

The retroviral enzyme integrase is encoded by the *pol* gene and is first translated as a large component of the polyprotein Gag-Pol. During maturation of the virions, protease cleaves the Gag-Pol polyprotein which results in the release of integrase. Integrase catalyzes the integration of double-stranded viral DNA into host cell DNA (ZEINALIPOUR-LOIZIDOU et al., 2007; DE CLERCQ, 2009). To achieve this, two catalytic reactions of integrase are necessary, 3'-end processing and strand transfer (3'-end joining). 3'-end processing takes place in the cytoplasm after reverse transcription and creates reactive 3'-hydroxyls at both ends of the viral DNA (SAVARINO et al., 2007; DE CLERCQ, 2009). As part of the pre-integration complex, the viral DNA is translocated to the nucleus, where the second reaction catalyzed by integrase, (strand transfer) takes place. During this step integrase catalyzes the insertion of both viral DNA ends into the host chromosome (ZEINALIPOUR-LOIZIDOU et al., 2007; DE CLERCQ, 2009). Once integrated, the provirus persists in the host cell genome and functions as a template for replication of the viral genome, leading to the formation of new

viruses (MOUSCADET and TCHERTANOV, 2009).

4.2. Efficacy against FIV

The structure of FIV integrase is similar to that of HIV-1 integrase and it has been shown that the catalytic site of the integrase of both viruses is almost identical (SAVARINO et al., 2007). Raltegravir and dolutegravir, the two FDA-approved integrase inhibitors, are targeted at the strand transfer reaction and efficiently chelate the magnesium cation required for the activity of integrase, interrupting the final step of strand transfer (MOUSCADET and TCHERTANOV, 2009; RATHBUN et al., 2014). Of these two drugs, only raltegravir has been assessed against FIV *in vitro*. Togami and colleagues (2013) showed that raltegravir had anti-FIV activity in two human cell lines. While FIV was less susceptible to raltegravir than HIV-1, the EC₅₀ against FIV was at the nanomolar level (TOGAMI et al., 2013).

In a previous study, Savarino and colleagues (2007) showed that three investigational integrase inhibitors (L-870,810; CHI1019; CHI1010) were able to inhibit FIV replication in feline lymphoblastoid MBM cell culture as efficiently as HIV-1 replication. The authors concluded that integrase inhibitors might provide a potential treatment option for FIV-infected cats and could potentially be combined with NRTIs active against FIV and therefore offer combination antiretroviral therapy (SAVARINO et al., 2007).

III. STUDY

Antiviral efficacy of nine nucleoside reverse transcriptase inhibitors against feline immunodeficiency virus in feline peripheral blood mononuclear cells

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Antiviral efficacy of nine nucleoside reverse transcriptase inhibitors against feline immunodeficiency virus in feline peripheral blood mononuclear cells

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Objective—To compare cytotoxic effects and antiviral efficacy of 9 nucleoside reverse transcriptase inhibitors (NRTIs) against FIV in feline peripheral blood mononuclear cells.

Sample—Peripheral blood mononuclear cells obtained from 3 specific pathogen-free cats.

Procedures—3 of the 9 NRTIs had not been previously assessed in feline cell lines. Cytotoxic effects were determined by colorimetric quantification of a formazan product resulting from bioreduction of a tetrazolium reagent by viable peripheral blood mononuclear cells; uninfected cells from 1 cat were used in these assays. Cells from all 3 cats were infected with a pathogenic clone of FIV, and in vitro antiviral efficacy of each NRTI was assessed with an FIV p24 antigen capture ELISA.

Results—Cytotoxic effects in feline peripheral blood mononuclear cells were observed only at concentrations > 10 μ M for all 9 NRTIs. Comparison of the cytotoxic effect at the highest concentration investigated (500 μ M) revealed that didanosine and amdoxovir were significantly less toxic than abacavir. All drugs induced a dose-dependent reduction of FIV replication. At the highest concentration investigated (10 μ M), there was no significant difference in antiviral efficacy among the test compounds.

Conclusions and Clinical Relevance—The evaluated NRTIs had low cytotoxicity against feline peripheral blood mononuclear cells and appeared to be safe options for further in vivo evaluation for the treatment of FIV-infected cats. There was no evidence suggesting that the newly evaluated compounds would be superior to the existing NRTIs for reducing FIV burden of infected cats. (*Am J Vet Res* 2014;75:273–281)

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ABBREVIATIONS

CCID ₅₀	Viral dose required to infect 50% of cells in culture
CRFK	Crandell-Rees feline kidney
EC ₅₀	Concentration of a compound at which 50% of its maximal effect is observed
NRTI	Nucleoside reverse transcriptase inhibitor
OD	Optical density
PBM	Peripheral blood mononuclear
RT	Reverse transcriptase
SPF	Specific pathogen-free

Since its first isolation in 1986,¹ FIV has been identified worldwide and FIV infection continues to be a major health problem among cats, especially in countries with large populations of free-roaming cats.^{2–4} In cats, FIV induces an acquired immunodeficiency syndrome similar to that caused by HIV in humans,^{5,6} and both viruses share many immunopathogenic and genomic features.^{7,8} Close similarities exist between the RTs of HIV and FIV, and it has been shown that several RT-targeted antiviral compounds active against HIV are also effective in inhibiting FIV replication in vitro.^{6,9} However, experience with these drugs in vivo

in cats is limited, and clinical use of antiviral drugs is still uncommon in veterinary medicine. To date, treatment of FIV-infected cats is largely based on supportive measures and the management of secondary problems.

In the treatment of humans with AIDS, specific targeting of HIV is important, and it is well documented that clinical outcome is improved when plasma viral burden is reduced.^{3,10,11} Single-agent treatment is no longer recommended for treatment of HIV-infected individuals because mutations of HIV, which are not susceptible to the antiviral agent, can develop. Highly active antiretroviral therapy of HIV-infected patients involves administration of combinations of antiviral drugs from different drug classes, such as 2 NRTIs combined with a protease inhibitor or 2 NRTIs with a non-NRTI.¹⁰ Combination treatment with drugs from different drug classes has not been assessed in FIV-infected cats, to our knowledge. This is mainly due to the fact that despite similarities between the HIV and FIV proteases, all but one of the currently available HIV protease inhibitors failed to inhibit the protease of FIV.^{8,12,13} Similarly, it has been shown that non-NRTIs are highly specific for HIV and are not effective against FIV.^{12,14} This is in contrast to NRTIs that inhibit HIV as well as other lentiviruses, including FIV.¹⁴

Of all antiviral drugs, only the NRTI zidovudine has been assessed thoroughly in cats in terms of *in vitro* inhibition of nonpathogenic¹⁵ and pathogenic¹⁶ FIV, inhibition of virion RT purified from *in vitro*-propagated nonpathogenic¹⁵ and pathogenic¹⁶ FIV, pharmacokinetics in uninfected cats,¹⁷ and clinical response of experimentally¹⁸ and naturally FIV-infected cats treated with the drug.¹⁹ Zidovudine increases the CD4:CD8 ratio and can improve clinical condition scores in FIV-infected cats with stomatitis¹⁹ and neurologic signs.²⁰ Its use, however, can result in adverse effects, such as dose-dependent nonregenerative anemia and neutropenia in cats^{21,22}; moreover, results of an *in vitro* study in cats²³ and reports of humans^{24,25} and cats²⁶ treated with zidovudine have indicated that mutations conferring resistance against the drug can develop. Therefore, it is important to assess the activities of other antiviral drugs and drug combinations for the treatment of FIV-infected cats.

Because a wide range of antiretroviral drugs have been assessed in humans and some of these (at least drugs from the NRTI class) might be of value in FIV-infected cats, the purpose of the study reported here was to compare 9 NRTIs regarding their cytotoxic effects in feline PBM cells and antiviral efficacy against FIV. The drugs included 3 NRTIs that had not been evaluated in feline cell lines before and 6 well-characterized NRTIs.

Materials and Methods

Test compounds—Of the 9 NRTIs assessed in the study, 3 had not been evaluated in feline cell lines previously; these were amdoxovir,^a racivir^a and dextelucitabine^a (Appendix). The other 6 NRTIs were abacavir,^b didanosine,^b emtricitabine,^a lamivudine,^a stavudine,^a and zidovudine.^a Each test compound was dissolved in dimethyl sulfoxide to create a 40mM stock solution, which was stored at -20°C. This solution was then fur-

ther diluted with distilled water to provide working solutions at the concentrations required for the respective experiments.

Cats—Housing and husbandry practices were in accordance with federal guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

Isolation of PBM cells from cats—Peripheral blood mononuclear cells were harvested from blood samples collected from 3 castrated male SPF cats.^c The cats were used on a rotating schedule. Six to 12 blood samples were collected from each cat (cat 1, 12 samples; cat 2, 7 samples; and cat 3, 6 samples) over a period of 16 months. The samples were collected intermittently. No more than 20 mL of blood was collected from an individual cat more frequently than once monthly.

The collection and isolation of PBM cells were performed as previously described by McCrackin Stevenson and McBroom.¹⁶ Briefly, 5 to 20 mL of blood was collected from a jugular vein of each cat into commercial vacuum tubes containing sodium heparin. Anticoagulated blood was diluted with an equal volume of PBS solution and layered over a density gradient^d followed by centrifugation and PBM cell aspiration. Isolated PBM cells were stimulated for 72 hours in medium (RPMI-1640 cell culture medium containing human recombinant interleukin-2 [29 U/mL],^e penicillin [50 U/mL], streptomycin [50 µg/mL], L-glutamine [2mM], HEPES buffer [10mM], β₂-mercaptoethanol [5 × 10⁻³M], sodium pyruvate [1mM], and heat-inactivated 10% fetal bovine serum) containing concanavalin A^f (1 µg/mL) and incubated at 37°C in an atmosphere containing 5% CO₂. Cells were then transferred to medium without concanavalin A for the remainder of the experiments.

Determination of endpoint for the cytotoxicity assay—To determine the ideal endpoint for cytotoxicity assays, a preliminary experiment was conducted that incorporated uninfected PBM cells from 1 of the 3 SPF cats used in the present study. Ten quadruplicates of concanavalin A-stimulated feline PBM cells were seeded in each well of a 96-well plate (5 × 10⁴ cells/well). Then, 200 µL of medium without concanavalin A was added to each well. The plate was incubated at 37°C in an atmosphere containing 5% CO₂. After incubation for 24 hours, 10 µL of suspension was harvested from the first set of quadruplicate wells to determine the cell count per milliliter of cell suspension. The cell counts for replicate wells were averaged to provide a mean value. This step was repeated on 9 consecutive days, and data were used to create a curve representing cell growth kinetics. These data (not shown) suggested that day 5 would be an ideal endpoint for the cytotoxicity assay because day 5 was on the linear portion of the growth curve.

Cytotoxicity assays—All cytotoxicity assays were conducted with PBM cells from only 1 of the 3 SPF cats used in the present study. For a cytotoxicity assay, concanavalin A-stimulated feline PBM cells were seeded in each well of a 96-well plate (5 × 10⁴ cells/well), and 200 µL of medium without concanavalin A

was added to each well. Each test compound was added to quadruplicate wells at serial 1:5 and 1:10 dilutions, resulting in final concentrations ranging from 0.001 to 500 μM . Sterile distilled water was used as the negative control. After incubation at 37°C in an atmosphere containing 5% CO_2 for 5 days, 20 μL of the tetrazolium reagent MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium)⁸ was added to each well, and the plates were incubated under the same conditions for 4 hours. Viable cells are able to reduce the tetrazolium compound to a formazan product, which was quantified colorimetrically with a 96-well microplate reader⁸ at a wavelength of 490 nm. Cell viability was expressed as a percentage of the negative control value. Mean cell viability was calculated from results of replicate wells. Two experiments were performed for each drug, and an overall mean cell viability was calculated.

Determination of the CCID₅₀ for the antiviral assay—To determine the CCID₅₀, virus stock¹ of a pathogenic molecular clone of FIV (FIV-pPPR)²⁷ produced in the continuous interleukin-2-dependent feline T-cell line, 104-C1,²⁸ was titrated in two 24-well plates by culturing quadruplicate replicas of 200 μL of 5-fold serial dilutions of virus starting with either a 1:10 dilution or 1:5 dilution (1 plate for each dilution). To each well, 2×10^5 SPF feline PBM cells and 500 μL of medium were added (day 0).⁸ Three quadruplicate replicas of uninfected tissue culture supernatant were set up in a separate plate as a negative control. The plates were incubated at 37°C in an atmosphere containing 5% CO_2 . On day 3, 500 μL of fresh medium was added to each well. On day 7, 200 μL of supernatant was harvested from each well and stored at -70°C for determination of FIV p24 antigen concentration.

The supernatant samples were tested for FIV p24 antigen by use of an antigen capture ELISA as described by Joshi et al.²⁹ All wells with absorbance readings that exceeded the mean of the absorbance readings of the negative control wells by > 2 SDs were designated positive. The Spearman-Kärber equation³⁰ was used to calculate the virus titers for the 2 plates; the mean of these 2 values was the final CCID₅₀.

Antiviral assay—The pathogenic molecular clone FIV-pPPR was used for the antiviral assay as described by McCrackin Stevenson and McBroom.¹⁰ Concanavalin A-stimulated SPF feline PBM cells were cultured for 3 to 5 days and subsequently exposed at a density of 5×10^6 cells/mL to 1 mL of virus-positive tissue culture supernatant containing $450 \times \text{CCID}_{50}/\text{mL}$ (as determined by assessment of the titer of the virus stock). The cells were inoculated in 2 mL of medium for 2 to 4 hours. One 24-well plate was set up for each test compound and contained quadruplicate replicas of 5 drug dilutions (final concentrations from 0.1 to 10 μM) and sterile distilled water (negative control) in 2 mL of medium. Virus-exposed PBM cells (2×10^5 cells) were then added to each well, and plates were incubated at 37°C in an atmosphere containing 5% CO_2 for 7 days. Three and a half days after the end of inoculation, 1 mL of supernatant was removed from each well without disturbing the cells at the bottom, and fresh medium

containing half of the initial amount of drug was added. Seven days after the end of inoculation, supernatants were harvested. This endpoint was selected in accordance with results from a previous study.¹⁰ Replication of FIV was determined by use of an FIV p24 antigen capture ELISA as described by Joshi et al.²⁹ Production of p24 antigen was expressed as a percentage of the control value generated by FIV-infected, untreated cells in control wells. Optical density readings of the supernatant from uninfected PBM cells were subtracted neither from the OD readings of the negative control wells nor from the OD readings of the drug-containing wells. The mean OD reading was calculated for results of replicate wells. Two experiments were performed for each drug, and an overall mean value was calculated from the results.

Statistical analysis—Results are reported as mean \pm SEM. Differences in toxic effects among the antiviral compounds at the concentration of 500 μM and differences in antiviral efficacy among the antiviral compounds at the concentration of 10 μM were assessed by

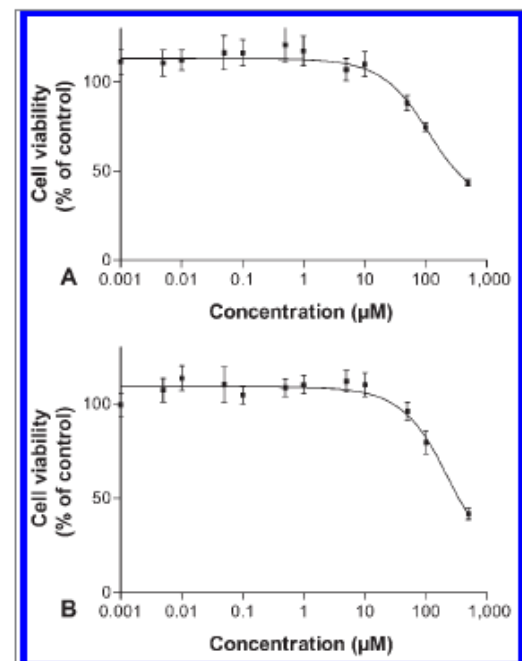


Figure 1—Representative dose-response curves for the cytotoxic effects of the NRTIs emtricitabine (A) and dextelavocitabine (B) on purified PBM cells obtained from a single SPF cat. The PBM cells were seeded in each well of a 96-well plate (5×10^4 cells/well), and 200 μL of medium without concanavalin A was added to each well. Each test compound was added to quadruplicate wells at final concentrations ranging from 0.001 to 500 μM . Sterile distilled water was used as the negative control. After incubation at 37°C in an atmosphere containing 5% CO_2 for 5 days, 20 μL of a tetrazolium reagent was added to each well, and the plates were incubated under the same conditions for 4 hours. Viable cells were quantified colorimetrically at a wavelength of 490 nm. Cell viability was expressed as a percentage of the negative control value. Mean cell viability was calculated from results of replicate wells; 2 experiments were performed for each drug, and an overall mean \pm SEM cell viability was calculated.

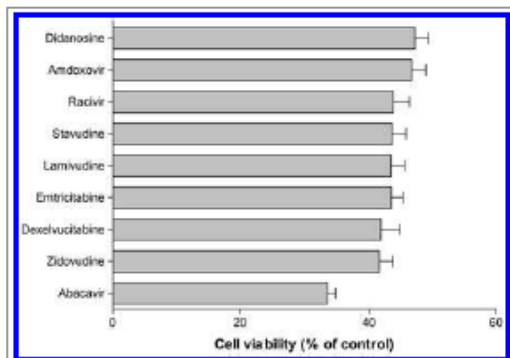


Figure 2—Comparison of the cytotoxic effects of 9 NRTIs (each at a concentration of 500µM) on feline PBM cells from a single SPF cat. The PBM cells were seeded in each well of a 96-well plate (5×10^4 cells/well), and 200 µL of medium without concanavalin A was added to each well. Each test compound was added to quadruplicate wells at a final concentration of 500µM. Sterile distilled water was used as the negative control. After incubation at 37°C in an atmosphere containing 5% CO₂ for 5 days 20 µL of a tetrazolium reagent was added to each well, and the plates were incubated under the same conditions for 4 hours. Viable cells were quantified colorimetrically at a wavelength of 490 nm. Cell viability was expressed as a percentage of the negative control value. The mean \pm SEM cell viability for each drug treatment was derived from 2 experiments, each of which was conducted with 4 replicates for each drug. Viability varied significantly ($P < 0.001$); both didanosine and amdoxovir were significantly ($P < 0.05$) less toxic than abacavir.

use of the Kruskal-Wallis test and Dunn multiple comparisons test. A commercially available software package¹ was used for the analyses. A value of $P \leq 0.05$ was considered significant.

Results

Cytotoxicity of the NRTIs—All 9 NRTIs had cytotoxic effects on feline PBM cells at concentrations $> 10\mu\text{M}$; the severity of the effects increased with increasing drug concentration (Figure 1). The cytotoxic effects induced by each drug at a concentration of 500µM were compared (Figure 2). Toxic effects among the 9 drugs varied significantly ($P < 0.001$) at this concentration; both didanosine and amdoxovir were significantly ($P < 0.05$) less toxic to feline PBM cells than was abacavir.

Antiviral efficacy—The ability of the 9 NRTIs to inhibit FIV replication in feline PBM cells was tested at noncytotoxic concentrations ranging from 0.1 to 10µM. The quantities of FIV p24 antigen (expressed as a percentage of OD readings from FIV-infected, untreated cells in control wells) in the supernatant from PBM cells incubated with 10µM of each drug were compared (Figure 3). Results indicated that emtricitabine, didanosine, and lamivudine were the most potent inhibitors and that dextelucitabine was the weakest inhibitor, although the findings did not differ significantly (Figure 4).

Discussion

The main aim of the study reported here was to evaluate 3 NRTIs (amdoxovir, dextelucitabine, and racivir) in terms of their cytotoxic effects on primary feline PBM cells and efficacy against a pathogenic molec-

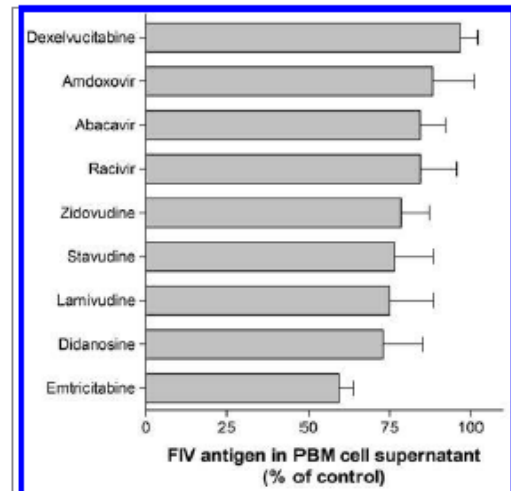


Figure 3—Comparison of the inhibitory effect of 9 NRTIs (each at a concentration of 10µM) on FIV replication in SPF feline PBM cells. The pathogenic molecular clone FIV-pPPR was used for the antiviral assay. Concanavalin A-stimulated feline PBM cells were cultured for 3 to 5 days and subsequently exposed at a density of 5×10^6 cells/mL to 1 mL of virus-positive tissue culture supernatant containing 450 X CCID₅₀/mL (as determined by assessment of the titer of the virus stock). The cells were inoculated in 2 mL of medium for 2 to 4 hours. Virus-exposed PBM cells (2×10^5 cells) were then added to each well of a 24-well plate, and 10µM of 1 of the 9 drugs or sterile distilled water (negative control) was added to separate wells containing 2 mL of medium; plates were incubated at 37°C in an atmosphere containing 5% CO₂ for 7 days. Three and a half days after the end of inoculation, 1 mL of supernatant was removed and refreshed with medium containing a concentration of drug or distilled water equal to that present in the well. Seven days after the end of inoculation, supernatants were harvested. Replication of FIV was determined by use of an FIV p24 antigen capture ELISA. Production of p24 antigen was expressed as a percentage of the control value generated by FIV-infected, untreated cells in control wells. Optical density readings of the supernatant from uninfected PBM cells were not subtracted from the OD readings of the negative control wells or from the OD readings of the drug-containing wells. The mean OD reading was calculated for results of replicate wells. Two experiments were performed for each drug, and an overall mean \pm SEM value was calculated from the results.

ular clone of FIV, with the goal of identifying potential novel treatment options for cats naturally infected with FIV. To this end, the efficacies of these NRTIs were compared with the efficacies of other NRTIs that are either currently used in the treatment of FIV-infected cats in the field (zidovudine) or potential therapeutic options from the NRTI class for which previously published data existed (ie, abacavir, didanosine, emtricitabine, lamivudine, and stavudine). The results indicated that although amdoxovir, dextelucitabine, and racivir appeared to have acceptable cytotoxicity profiles in feline PBM cells, compared with those of other NRTIs used for the treatment of HIV infection in humans, their efficacies were less (albeit not significantly) than those of didanosine, emtricitabine, lamivudine, stavudine, and zidovudine. On the basis of the data obtained in the present study, amdoxovir, dextelucitabine, and racivir appear to be treatment options for future studies investigating their potential use in FIV-infected cats. However, regarding the reduction of the viral burden of FIV-infected cats, there was no evidence to suggest

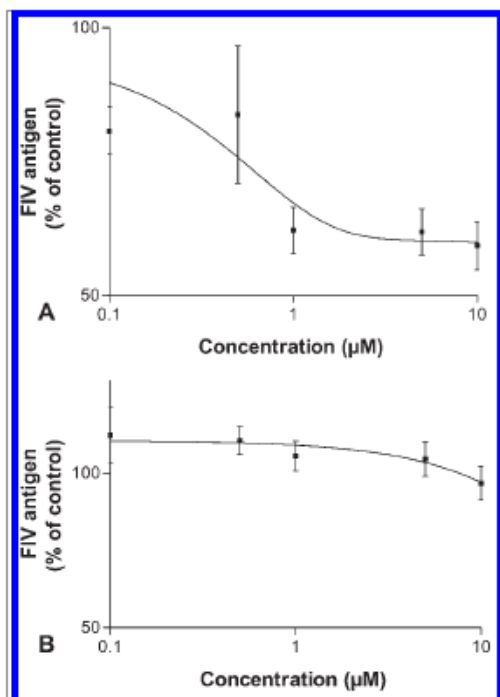


Figure 4—Representative dose-response curves for effects of the NRTIs emtricitabine (A) and dextelvucitabine (B) on FIV replication in SPF feline PBM cells. The pathogenic molecular clone FIV-pPPR was used for the antiviral assay. Concanavalin A-stimulated feline PBM cells were cultured for 3 to 5 days and subsequently exposed at a density of 5×10^6 cells/mL to 1 mL of virus-positive tissue culture supernatant containing $450 \times \text{CCID}_{50}/\text{mL}$. The cells were inoculated in 2 mL of medium for 2 to 4 hours. Virus-exposed PBM cells (2×10^5 cells) were then added to each well of a 24-well plate, and 5 dilutions (final concentrations from 0.1 to 10 µM) of 1 of the 2 drugs or sterile distilled water (negative control) were added to separate wells (quadruplicate replicates) containing 2 mL of medium; plates were incubated at 37°C in an atmosphere containing 5% CO_2 for 7 days. Three and a half days after the end of inoculation, 1 mL of supernatant was removed and refreshed with medium containing a concentration of drug or distilled water equal to that present in the well. Seven days after the end of inoculation, supernatants were harvested. Replication of FIV was determined by use of an FIV p24 antigen capture ELISA. Production of p24 antigen was expressed as a percentage of the control value generated by FIV-infected, untreated cells in control wells. Optical density readings of the supernatant from uninfected PBM cells were not subtracted from the OD readings of the negative control wells or from the OD readings of the drug-containing wells. The mean OD reading was calculated for results of replicate wells. Two experiments were performed for each drug, and an overall mean \pm SEM value was calculated.

that amdoxovir, dextelvucitabine, or racivir is superior to existing NRTIs.

The cytotoxic effects of some of the drugs investigated in the present study, namely lamivudine,^{12,21,31} zidovudine,^{12,21,31–33} abacavir,¹² didanosine,^{9,31} and stavudine,^{31,34} have been previously evaluated in feline cell lines. In those previous studies, the drugs had cytotoxic effects comparable to those detected in the present study; all those drugs were fairly nontoxic in cell culture at clinically relevant plasma concentrations with noticeable cytotoxic effects only at doses $> 10 \mu\text{M}$.

Similar results for zidovudine and lamivudine in feline PBM cells were observed by Arai et al.²¹ Abacavir was the most toxic drug in the present study and was significantly more toxic than didanosine and amdoxovir. Also in a study of the effects of abacavir, zidovudine, and lamivudine in CRFK cells by Bisset et al.,¹² abacavir was the most toxic NRTI. The concentration of drug required to inhibit PBM cell proliferation by 50% was $22.9 \mu\text{M}$ for abacavir, compared with $216.8 \mu\text{M}$ for zidovudine and $170.5 \mu\text{M}$ for lamivudine.¹² In a study by Smyth et al.³¹ that assessed cytotoxic effects of several compounds (including didanosine, lamivudine, stavudine, and zidovudine) in feline lymphocytes, didanosine had the lowest toxicity, which also corresponds to the findings in the present study.

In the present study, dose-response curves of each drug revealed that cytotoxic effects were only observed at concentrations $> 10 \mu\text{M}$. At concentrations of $100 \mu\text{M}$, the investigated drugs had only a mild to moderate toxic effect on cell viability (Figure 1). However, a plasma concentration of zidovudine of $100 \mu\text{M}$ has been associated with acute, transient hemolysis after a single IV infusion of 25 mg/kg in cats; thus, this dose and plasma concentration are too high for clinical use.¹⁷ Cytotoxicity of each drug was tested at concentrations up to $500 \mu\text{M}$ in the present study. This is a very high concentration, which far exceeds the circulating concentrations that will be attained in cats when drugs are given at dosages that are typically administered to cats (as demonstrated for zidovudine,²² which is usually given at a dosage of 5 to 10 mg/kg, PO or SC, q 12 h,²⁰ and results in a serum concentration²² of 20 to $30 \mu\text{M}$). In addition, at high doses of drug, the concentration of dimethyl sulfoxide, the solvent for all NRTIs in the present and previous studies,^{12,31} was at its highest, and dimethyl sulfoxide is known to cause mild cytotoxic changes in feline PBM cells at similar concentrations.³¹ Even at these high concentrations of drug ($500 \mu\text{M}$) and dimethyl sulfoxide, cell viability was not completely suppressed by any of the test compounds in the present study.

Prior to the present study, the NRTIs amdoxovir, dextelvucitabine, and racivir had not been assessed for their cytotoxic effects in feline cells, to our knowledge. The study results indicated that cytotoxicity of these drugs did not differ significantly from that of the other test compounds, except for amdoxovir, which was significantly less toxic than abacavir. Low cytotoxicity in vitro, however, does not necessarily exclude toxicity in vivo. For example, didanosine is a widely used antiretroviral drug with low cytotoxicity against human cells in culture (and in feline PBM cells as demonstrated in the present study), yet it can cause acute pancreatitis and peripheral neuropathy when used at higher doses in HIV-infected patients.^{35,36} It has also been shown that didanosine treatment can cause sensory neuropathy (as detected by sophisticated testing methods) in experimentally FIV-infected cats,³ although the clinical relevance of this finding in cats naturally infected with FIV is not clear. Toxic effects on mitochondria in certain tissues have been associated with many NRTIs, and this mechanism of mitochondrial changes appears to be involved in the development of NRTI-related adverse effects, although other pathophysiologic mechanisms

are likely to contribute as well.^{3,37} Amdoxovir also has little cytotoxicity against human cell lines, which corresponds with the finding for this drug in feline PBM cells in the present study.

From a pharmacokinetic study²² of zidovudine in cats, it is known that when the drug is administered at routinely used dosages of 5 to 10 mg/kg every 12 hours, the highest serum concentration attained is 20 to 30 μ M. Administration of zidovudine at a concentration of 30 μ M does not induce noteworthy cytotoxic changes in feline lymphocytes,³² as confirmed by the results of the present study in feline PBM cells. The other drugs were not significantly more toxic than zidovudine. Therefore, as far as cytotoxic properties are concerned, it can be assumed that all compounds evaluated in the present study could be used in vivo at dosages comparable to that for zidovudine, although pharmacological data from cats are not available for most of the drugs.

Human and feline PBM cells are widely used for cell culture studies involving HIV or FIV^{10,16,21,32,38} because these cell populations contain CD4-positive lymphocytes, which are the primary target of these lentiviruses. Hence, feline PBM cells were used in the present study and FIV-pPPR, a pathogenic molecular clone of FIV, was used for the infection of those cells. It has been shown that in PBM cells, FIV-pPPR behaves similarly to FIV-Maxam, a natural FIV isolate, and that results are therefore applicable to natural FIV infection.¹⁶ However, a first-pass virus derived from a molecular clone is a more homogenous viral population, compared with a natural isolate. McCrackin Stevenson and McBroom¹⁶ showed that FIV-Maxam was more susceptible to lamivudine, compared with findings for FIV-pPPR, and concluded that results of studies of the susceptibility of FIV-pPPR to NRTIs might overestimate the resistance of FIV populations found in naturally infected cats to these drugs. This might in part explain why susceptibility of the virus to the evaluated NRTIs in the present study was lower than previously described.

Because the dose-response curves indicated that there were no observable cytotoxic effects on feline PBM cells for any of the 9 drugs at a concentration of 10 μ M, concentration that is commonly achieved in plasma in cats administered zidovudine at the recommended dosage^{20,22} of 5 to 10 mg/kg, PO or SC, every 12 hours, the 10 μ M concentration was set as the highest dose to be investigated in the part of the present study designed to assess the antiviral efficacy of the test compounds. All drugs induced a concentration-dependent reduction of FIV replication; however, none of the drugs achieved 50% reduction of virus replication at the highest concentration (10 μ M) investigated. No significant difference in antiviral efficacy among the test compounds was detected; therefore, all drugs can be considered comparable in their in vitro antiviral efficacy against FIV. Of the drugs investigated in the present study, lamivudine,^{12,16,21,31} zidovudine,^{9,12,16,21,31–33,39} abacavir,¹² didanosine,^{9,23,31} stavudine,^{9,31,34} and emtricitabine^{16,23} have been assessed previously for their anti-FIV efficacy in different feline cell lines. To our knowledge, the results of the present study have indicated the anti-FIV efficacy of amdoxovir, dextelucitabine, and racivir for the first time.

Among the previous studies of lamivudine, zidovudine, abacavir, didanosine, stavudine, and emtricitabine, antiviral efficacy against FIV was demonstrated despite the use of different cell culture systems. Vahlenkamp et al³³ detected an 80-fold difference in the antiviral efficacy of zidovudine when the drug was used in different cell lines (CRFK cells vs thymocytes), and van der Meer et al³⁸ found a 6-fold difference in the inhibitory potency of zidovudine in thymocytes versus a dendritical cell-thymocyte coculture system. In another study,³² a difference in the EC_{50} (ie, the concentration of drug required to inhibit FIV p24 expression by 50%) for zidovudine between peripheral blood leukocytes and CRFK cell cultures was observed. Thus, the cell culture systems used markedly influence the EC_{50} values achieved. When results of different studies are compared, the cell culture system used has to be taken into consideration, and a comparison of a newly investigated drug with drugs of known in vitro efficacy (eg, zidovudine) is more useful than just comparison of EC_{50} values.

The cell system used in the present study involved primary feline PBM cells. Results of the previous studies that compared PBM cells with other feline cell lines, such as CRFK cells, generally indicated that the test compounds had greater inhibitory potency in PBM cells, compared with findings in CRFK cells. The fact that none of the compounds evaluated in the present study achieved a 50% reduction in virus replication at a concentration of 10 μ M was surprising because in other studies,^{9,12,16,33,34} much lower concentrations were required to induce 50% virus inhibition. In the present study, OD readings generated by supernatants of uninfected PBM cells were not determined. These background OD readings were therefore not subtracted from the readings of the plate wells containing infected cells. This might have led to an underestimation of the percentage reduction in p24 antigen concentration achieved in plate wells treated with the test compounds and might explain, at least in part, why reduction of viral replication by 50% was not achieved. Differences in FIV strains might also partly explain this finding. However, McCrackin Stevenson and McBroom¹⁶ used both the same cell system and virus and found EC_{50} values for zidovudine, lamivudine, and emtricitabine that were much lower than 10 μ M. Gobert et al⁹ made a similar observation; the EC_{50} values for zidovudine against 2 FIV strains detected in a previous study¹³ in their laboratory were higher than the values determined in the later study.⁹ They concluded that these differences might be related to variations in the batches of fetal bovine serum used in the experiments. However, independent of the system used, the outcome of the present study was that the antiviral efficacies of all drugs investigated were comparable.

The fact that reduction of viral load by 50% was not attained in the present study does not preclude the clinical usefulness of the investigated compounds. In a study by Arai et al,²¹ combination treatment of zidovudine and lamivudine administered to chronically FIV-infected cats at a high dosage of 20 mg of each drug/kg, PO, every 12 hours, did not result in a significant decrease in FIV load. However, it is well known that in

cats that are naturally infected with FIV, zidovudine administration at much lower dosages results in beneficial effects, such as improvement of stomatitis and clinical condition scores,^{19,22,40} reduction in severity of neurologic signs,²⁰ and improvement of CD4:CD8 ratios.^{19,22} Ideally, a drug that is considered for in vivo testing should be effective and have very low toxicity. However, the limiting factor as to whether an NRTI other than zidovudine should be considered for in vivo testing might be the drug's associated cytotoxicity rather than its ability to maximally suppress viral replication.

A limitation of the present study was that EC₅₀ values could not be reported for the test compounds because the highest drug dose investigated did not achieve a 50% reduction of virus replication. However, comparison of the test compounds at the highest dose investigated was nevertheless considered useful because it allowed comparison of newly investigated drugs with drugs that had been previously tested in feline cell cultures.

In the present study, examination of dose-response curves for cytotoxic effects and antiviral efficacy of emtricitabine and dextelucitabine revealed that the cell viability (as a percentage of the negative control value) at the lower drug concentrations was just > 100%. This finding was likely attributable to experimental variabilities, which might have led to slightly higher OD readings in individual plate wells of the assay. Similarly, in the antiviral assay, variabilities among individual plate wells might have contributed to the calculated FIV p24 antigen concentrations being slightly > 100% at low dextelucitabine concentrations.

Zidovudine treatment in cats has well-known adverse effects, such as development of nonregenerative anemia and neutropenia,^{19,21,22} which can necessitate cessation or interruption of treatment. Drug-resistant viral mutants have been detected in HIV-infected patients treated with zidovudine.^{23–25} Mutations leading to drug resistance have also been reported for FIV in vitro studies^{15,16,23} and in naturally infected cats treated with zidovudine for ≥ 5 years.²⁶ Therefore, the in vivo investigation of other NRTIs that have demonstrated efficacy against FIV in vitro similar to that of known compounds can result in identification of useful antiviral drugs, which might provide veterinarians with an alternative treatment option for FIV-infected cats.

Although it is difficult to make recommendations about clinical treatment only on the basis of in vitro data, the findings of the present study have suggested that further investigation of didanosine in the treatment of cats naturally infected with FIV is warranted. In a study³¹ assessing cytotoxic effects of 18 antiviral agents on feline lymphocytes, didanosine had the least toxicity, corresponding to the findings in the present study. In addition, the only NRTI tested in the present study that had greater in vitro efficacy than didanosine was emtricitabine. The combined profile of low cytotoxicity and relative efficacy, compared with characteristics of other NRTIs used in the assays performed in the present study, suggests that didanosine might be an interesting candidate drug for further in vivo testing either as a sole agent or in combination with zidovudine. In fact, monotherapy with didanosine (33 mg/kg, PO, q 24 h from 6

to 12 weeks after infection) in neonatal kittens infected with FIV resulted in improvements in multiple variables, compared with findings in untreated kittens, including reduction in plasma viral load, significant improvement in the animals' neurobehavioral performance, and attenuation of neuroinflammation.⁴¹ There is also support for treatment of HIV-infected humans with a combination of zidovudine and didanosine, which resulted in an overall reduction in mortality rate of 32%, compared with results following zidovudine monotherapy.⁴² Logical next candidates for in vivo testing of potential novel treatments for FIV-infected cats would include the 3 newly evaluated NRTIs, amdoxovir, dextelucitabine, and ractivir.

- a. Synthesized in Dr. R. E. Schinazi's laboratory, Emory University, Atlanta, Ga.
- b. Obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institutes of Health and Infectious Diseases, National Institutes of Health, Bethesda, Md.
- c. Liberty Research Inc, Waverly, NY.
- d. Ficoll-Hypaque, Pharmacia, Uppsala, Sweden.
- e. Provided by Dr. Niels C. Pedersen, Center for Companion Animal Health, School of Veterinary Medicine, University of California-Davis, Davis, Calif.
- f. Concanavalin A, Sigma-Aldrich, St Louis, Mo.
- g. CellTiter 96 AQ_{non}, One Solution cell proliferation assay, Promega, Madison, Wis.
- h. VMax, kinetic microplate reader software, SoftMax, Molecular Devices, Sunnyvale, Calif.
- i. Provided by Dr. John H. Elder, Department of Molecular Biology, The Scripps Research Institute, La Jolla, Calif.
- j. Generated by Dr. C. Grant, Custom Monoclonals, West Sacramento, Calif.
- k. Modification of a protocol provided by Dr. Lawrence E. Mathes, Department of Veterinary Biosciences, The Center for Retrovirus Research, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio.
- l. GraphPad Prism, version 5.00 for Windows, GraphPad Software, San Diego, Calif.

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Appendix appears on the next page

Appendix

Nucleoside RT inhibitors evaluated for antiviral efficacy against FIV in feline PBM cells.

NRTI	Chemical name
Abacavir	{(1 <i>S</i> ,4 <i>R</i>)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-enyl}methanol
Amdoxovir	{(2 <i>R</i> ,4 <i>R</i>)-4-(2,6-diaminopurin-9-yl)-1,3-dioxolan-2-yl}methanol
Dexelvucitabine	β -D-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine
Didanosine	2',3'-dideoxyinosine
Emtricitabine	(-)-2',3'-deoxy-5-fluoro-3'-thiacytidine
Lamivudine	(-)-2',3'-dideoxy-3'-thiacytidine
Racivir	(\pm)- β -2',3'-dideoxy-5-fluoro-3'-thiacytidine
Stavudine	2',3'-didehydro-2',3'-dideoxythymidine
Zidovudine	3'-azido-3'-deoxythymidine

Amdoxovir, dexelvucitabine, and racivir had not been previously assessed in feline cell lines, to the authors' knowledge. Abacavir and didanosine were obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Amdoxovir, dexelvucitabine, emtricitabine, lamivudine, racivir, stavudine, and zidovudine were synthesized in the laboratory of one of the authors (RFS).

IV. DISCUSSION

Over the last 30 years since the first description of HIV as the causative agent of AIDS (GALLO et al., 1984), 26 antiretroviral drugs from seven different drug categories have been developed and approved by the FDA for the treatment of HIV infection (DE CLERCQ, 2013; FDA, 2014), two of which are no longer used or marketed (DE CLERCQ, 2010; FDA, 2014).

The ever growing number of antiretroviral drugs has made combination treatment possible and since the mid 1990ties its importance in the treatment of HIV/AIDS has been widely accepted. Highly active antiretroviral therapy (HAART) utilizes combination regimens of classically three antiretrovirals from different drug categories, and this therapy has drastically altered the course of AIDS which previously was an almost uniformly fatal disease to now being a chronic manageable one (BRODER, 2010; DE CLERCQ, 2010). The latest of the FDA-approved antiretrovirals was licensed in 2013 and research into new drugs remains a continuous effort. Despite these achievements there is still a need for new antiretroviral drugs in order to address issues like tolerability, drug-drug interactions and cross-resistance amongst members of a particular drug classes (GHOSH et al., 2011). One example of a novel drug which is currently in advanced phase 2 studies is the NRTI amdoxovir, one of the compounds that was evaluated for its efficacy against FIV for the first time in the present study.

As further drugs are being developed for anti-HIV therapy and given the close similarity between HIV and FIV it is very likely that some of these new drugs would be effective against FIV as well. This is particularly true for NRTIs as there is a striking similarity between the RT of HIV and FIV, whereas drugs from other drug categories such as NNRTIs and most PIs show no anti-FIV activity. This similarity between the two lentiviruses has resulted in FIV becoming a useful *in vitro* and *in vivo* animal model, especially for studying NRTIs (NORTH and LACASSE, 1995; DIAS AS et al., 2006; VAN DER MEER et al., 2007), and all currently FDA approved NRTIs have been assessed against FIV in feline cell culture using various different cell systems. Generally all NRTIs show anti-FIV activity *in vitro* (REMINGTON et al., 1991; GOBERT et al., 1994; SMITH et al., 1998; MCCRACKIN STEVENSON and MCBROOM, 2001; BISSET et al.,

2002).

In the present study we assessed three experimental NRTIs (amdoxovir, dexelvucitabine and racivir) which to our knowledge had not been evaluated against FIV in feline cell culture before. We compared their cytotoxicity in feline PBM cells and antiviral efficacy to the licensed NRTIs with the goal of identifying additional compounds with acceptable cytotoxicity and efficacy profiles which could be potential novel treatment options for naturally FIV-infected cats.

When assessing antiretroviral compounds *in vitro* it has been shown that the cell culture system used can influence the antiviral efficacy of NRTIs (CRONN et al., 1992; HARTMANN et al., 1995b; VAHLENKAMP et al., 1995; BALZARINI et al., 1996b; VAN DER MEER et al., 2007). Commonly, the fibroblastoid cell line CRFK is used for *in vitro* studies involving FIV, as this is a continuous cell line which some isolates of FIV have been adapted to (NORTH and LACASSE, 1995; SIEBELINK et al., 1995; VERSCHOOR et al., 1995; BAUMANN et al., 1998). For example, Vahlenkamp and coworkers (1995) described an 80-fold decrease in the antiviral efficacy of zidovudine when assessed in CRFK cells compared to thymocytes (VAHLENKAMP et al., 1995). Similarly, in another study an approximately 60-fold increase in the EC₅₀ of zidovudine was detected in CRFK cells compared to peripheral blood lymphocytes (HARTMANN et al., 1995b). Van der Meer and coworkers (2007) detected a 6-fold difference in the inhibitory potency of zidovudine in thymocytes *versus* a dendritical cell-thymocyte coculture system. As a consequence, unjustified rejection of candidate antiviral drugs might occur in some cases if the cell culture system used is not taken into account (VAN DER MEER et al., 2007).

Balzarini and coworkers (1988) showed for zidovudine, zalcitabine and stavudine, that differences in drug-modifying cellular enzymes, which are responsible for the conversion of the drug to its active triphosphate form, are likely responsible for the cell-dependent efficacies of these drugs (BALZARINI et al., 1988; BALZARINI et al., 1996b). For stavudine in particular it was shown that the intracellular conversion to its active triphosphate form by either thymidylate kinase and/or nucleoside diphosphate kinase is poor in CRFK cells (BALZARINI et al., 1996b).

In order to increase the predictability of the antiviral efficacy of a certain compound *in vivo*, the *in vitro* model system ideally should mimic the situation in the patient closely (PAUWELS, 2006). The natural cell tropism of FIV which is broader than that of HIV includes CD4⁺ and CD8⁺ lymphocytes, B cells, macrophages, and astrocytes (MIYAZAWA et al., 1992; SIEBELINK et al., 1995; VERSCHOOR et al., 1995; DIAS AS et al., 2006). PBM cells contain lymphocytes and macrophages and are therefore also commonly used for *in vitro* studies involving lentiviruses such as HIV and FIV (HARTMANN et al., 1995b; MCCRACKIN STEVENSON and MCBROOM, 2001; ARAI et al., 2002; HERNANDEZ-SANTIAGO et al., 2007; VAN DER MEER et al., 2007). This cell system was also chosen for the present study.

In the first part of the present study, the cytotoxic effect of all test compounds in feline PBM cells was assessed. When compared at the highest concentration investigated (500 µM), didanosine and amdoxovir were significantly less toxic than abacavir. No further statistically significant differences among the test compounds were found. Dose-response curves showed that noticeable cytotoxicity was only observed at concentrations above 10 µM.

The cytotoxic effects of five of the drugs investigated in the present study, namely lamivudine (SMYTH et al., 1994; ARAI et al., 2002; BISSET et al., 2002) zidovudine (SMYTH et al., 1994; HARTMANN et al., 1995b; VAHLENKAMP et al., 1995; ARAI et al., 2002; BISSET et al., 2002), abacavir (BISSET et al., 2002) didanosine (REMYNGTON et al., 1991; TANABE-TOCHIKURA et al., 1992; GOBERT et al., 1994; REMYNGTON et al., 1994; SMYTH et al., 1994; MEDLIN et al., 1996; ZHU et al., 1996; SMITH et al., 1997; SMITH et al., 1998), and stavudine (SMYTH et al., 1994; BALZARINI et al., 1996b), had been assessed in feline cell lines before. In those previous studies, the drugs showed low cytotoxicity, like in the present study where all investigated drugs were fairly non-toxic in PBM cell culture at clinically relevant plasma concentrations. Noticeable cytotoxic effects were only observed at doses higher than 10 µM. Similar results for zidovudine and lamivudine were observed by Arai and coworkers. In that study toxicity in T-cell enriched PBM cells was observed at concentrations greater than 10 µM for zidovudine and greater than 50 µM for lamivudine (ARAI et al., 2002). Abacavir was the most toxic drug in the present study and was significantly more toxic than didanosine and amdoxovir. In a study

by Bisset and coworkers (2002) investigating the effects of abacavir, zidovudine, and lamivudine in CRFK cells, abacavir was also the most toxic NRTI. The concentration of drug required to inhibit cell proliferation by 50% was 22.9 μM for abacavir, compared with 216.8 μM for zidovudine and 170.5 μM for lamivudine. However, the authors considered this difference and the resulting difference in the selectivity index not clinically relevant and claimed that this drug is suitable for use in cats (BISSET et al., 2002). In a study by Smyth and coworkers that assessed cytotoxic effects of several compounds (including didanosine, lamivudine, stavudine, and zidovudine) in feline lymphocytes, didanosine was the least toxic drug, which also corresponds to the findings in the present study (SMYTH et al., 1994).

Cytotoxicity of each drug was tested at concentrations up to 500 μM in the present study. This is a very high concentration, which far exceeds the circulating concentrations that will be attained in cats when drugs are given at dosages that are typically administered to cats [as demonstrated for zidovudine (HARTMANN et al., 1995a), which is usually given at a dosage of 5 to 10 mg/kg, PO or SC, q 12 h (LEVY et al., 2008) and results in a serum concentration of 20 to 30 μM]. In addition, at high doses of drug, the concentration of dimethyl sulfoxide, the solvent for all NRTIs in the present and previous studies, was at its highest concentration, and is known to cause mild cytotoxic changes in feline PBM cells at similar concentrations (SMYTH et al., 1994). Even at these high concentrations of drug (500 μM) and dimethyl sulfoxide, cell viability was not completely suppressed by any of the test compounds in the present study.

In the present study, the NRTIs amdoxovir, dexelvucitabine, and racivir were investigated for the first time for their cytotoxic effects in feline cells. The results indicated that cytotoxicity of these drugs did not differ significantly from that of the other test compounds, except for amdoxovir, which was significantly less toxic than abacavir. Low cytotoxicity *in vitro*, however, does not necessarily exclude toxicity *in vivo*. In a previous study on the pharmacokinetics of zidovudine in cats it was shown that a single IV infusion of 25 mg/kg, which achieved a plasma concentration of 100 μM , was associated with acute, transient hemolysis (ZHANG et al., 2004). While in the present study only mild to moderate cytotoxicity was seen at concentrations of 100 μM , the study by Zhang and coworkers showed that for zidovudine this plasma concentration is too high

for clinical use. A discrepancy between *in vitro* and *in vivo* toxicity has also been described for didanosine. It has low cytotoxicity against human cells in culture (and in feline PBM cells as demonstrated in the present study), yet it can cause acute pancreatitis and peripheral neuropathy when used at higher doses in HIV-infected patients (DU et al., 1990; LAMBERT et al., 1990). More recently it has also been associated with the development of non-cirrhotic portal hypertension, a rare but increasingly reported serious liver complication of HIV-infected patients (CACHAY et al., 2011) and in January 2010 the FDA issued a safety announcement and updated the drug label for didanosine accordingly (CHANG et al., 2012). It has also been shown that didanosine treatment can cause sensory neuropathy (as detected by sophisticated testing methods) in experimentally FIV-infected cats (ZHU et al., 2007), although the clinical relevance of this finding in cats naturally infected with FIV is not clear. Toxic effects on mitochondria in certain tissues have been associated with many NRTIs and this mechanism of mitochondrial changes appears to be involved in the development of NRTI-related adverse effects, although other pathophysiological mechanisms are likely to contribute as well (WHITE, 2001; ZHU et al., 2007). Amdoxovir also has little cytotoxicity against human cell lines, which corresponds with the finding for this drug in feline PBM cells in the present study.

When compared with zidovudine, the other test compounds were not significantly more toxic. Therefore, it can be assumed that dosages that are tolerated for zidovudine in cats should also be tolerated for the other compounds. Zidovudine is usually used in cats at a dosage of 5 to 10 mg/kg every 12 hours. A pharmacokinetic study of zidovudine in cats showed that this dosage resulted in a maximum serum concentration of 20 to 30 μ M (HARTMANN et al., 1995a). This concentration did not induce noteworthy cytotoxicity in feline peripheral blood lymphocytes. Also in the present study, there was only minimal cytotoxicity in PBM cells at this concentration. Therefore, it can be assumed that this plasma concentration would also be safe for the other compounds evaluated in the present study, although pharmacological data from cats are not available for most of the drugs.

In the second part of the present study, the anti-FIV efficacy of the nine test compounds was investigated in PBM cells. As the results of the first part of this study indicated that no cytotoxic effects were induced by any of the test

compounds up to a concentration of 10 μM , this was set as the maximal concentration to be investigated when assessing the antiviral efficacy of the test compounds. In addition, 10 μM is a concentration which is attained in a cat's plasma when administering zidovudine at the routinely used dosage of 5 to 10 mg/kg every 12 hours PO or SC (HARTMANN et al., 1995a). Therefore it was considered useful to compare the antiviral efficacy of the test compounds at this concentration.

Dose response curves showed that all drugs induced a concentration-dependent reduction of FIV replication; however, none of the drugs achieved 50% reduction of virus replication at the highest concentration (10 μM) investigated. No significant difference in antiviral efficacy among the test compounds was detected when they were compared at the highest concentration of 10 μM ; therefore, all drugs can be considered comparable in their antiviral efficacy against FIV. The FDA-approved NRTIs lamivudine (SMYTH et al., 1994; MEDLIN et al., 1996; SMITH et al., 1997; SMITH et al., 1998; MCCRACKIN STEVENSON and MCBROOM, 2001; ARAI et al., 2002; BISSET et al., 2002) zidovudine (NORTH et al., 1989; REMINGTON et al., 1991; TANABE-TOCHIKURA et al., 1992; GOBERT et al., 1994; REMINGTON et al., 1994; SMYTH et al., 1994; HARTMANN et al., 1995b; VAHLENKAMP et al., 1995; MEDLIN et al., 1996; ZHU et al., 1996; SMITH et al., 1997; SMITH et al., 1998; MCCRACKIN STEVENSON and MCBROOM, 2001; ARAI et al., 2002; BISSET et al., 2002; VAN DER MEER et al., 2007), abacavir (BISSET et al., 2002), didanosine (REMINGTON et al., 1991; TANABE-TOCHIKURA et al., 1992; GOBERT et al., 1994; REMINGTON et al., 1994; SMYTH et al., 1994; MEDLIN et al., 1996; ZHU et al., 1996; SMITH et al., 1997; SMITH et al., 1998), stavudine (REMINGTON et al., 1991; GOBERT et al., 1994; REMINGTON et al., 1994; SMYTH et al., 1994; BALZARINI et al., 1996b; ZHU et al., 1996; SMITH et al., 1997; SMITH et al., 1998) and emtricitabine (SMITH et al., 1997; SMITH et al., 1998; MCCRACKIN STEVENSON and MCBROOM, 2001) have been assessed previously for their anti-FIV efficacy in different feline cell lines. To our knowledge, the present study assessed the anti-FIV activity of amdoxovir, dexelvucitabine and racivir for the first time.

FIV-pPPR, a pathogenic molecular clone of FIV, was used for the infection of PBM cells. This clone was developed by Phillips and coworkers (1990) and is

derived from a FIV isolate from a cat from the San Diego, California area (PHILLIPS et al., 1990). This clone productively infects PBM cells, however not CRFK or G355-5 cells. The immunologic and neurologic abnormalities induced by this molecular clone have been characterized in specific pathogen-free (SPF) cats (PHILLIPS et al., 1996). General clinical signs associated with infection were lymphadenopathy, oral ulceration, rough hair coat, and conjunctivitis. During acute infection, plasma antigenemia, viremia, inversion of the CD4⁺ to DC8⁺ T-cell ratio and reduced CD4⁺ cell counts can be observed. Later in infection, an increase in the antibody titer and decrease in viral load occurs. Specific neurological changes found in experimentally infected cats included hind limb paresis, delayed righting and pupillary light reflexes, behavioral changes, delayed visual and auditory evoked potentials, decreased spinal and peripheral nerve conduction velocities, and altered sleep patterns (PHILLIPS et al., 1996).

McCrackin Stevenson and McBroom (2000) showed that in PBM cells, FIV-pPPR behaves similarly to FIV-Maxam, a natural FIV isolate, and that results are therefore applicable to natural FIV infection (MCCRACKIN STEVENSON and MCBROOM, 2001). However, a first-pass virus derived from a molecular clone is a more homogenous viral population, compared with a natural isolate. McCrackin Stevenson and McBroom (2000) showed that the susceptibility to lamivudine of FIV-Maxam was higher than that of FIV-pPPR and concluded that results of studies on the susceptibility of FIV-pPPR to NRTIs might overestimate the resistance of FIV populations found in naturally infected cats to these drugs (MCCRACKIN STEVENSON and MCBROOM, 2001).

Among the previous studies of lamivudine, zidovudine, abacavir, didanosine, stavudine, and emtricitabine, antiviral efficacy against FIV was demonstrated irrespective of the use of different cell culture systems. As mentioned above, considerable differences in the antiviral potency of certain drugs were observed, depending on the *in vitro* cell system in which they were assessed. When comparing results of different studies, the cell culture system used has to be taken into consideration, and comparison of a newly investigated drug with drugs of known *in vitro* efficacy (eg, zidovudine) is more useful than just comparison of EC₅₀ values.

An unexpected finding in the present study was the failure of all test compounds to achieve a 50% reduction in virus replication at a concentration of 10 µM. In

previous studies, much lower concentrations were required to induce 50% virus inhibition (GOBERT et al., 1994; VAHLENKAMP et al., 1995; BALZARINI et al., 1996b; MCCRACKIN STEVENSON and MCBROOM, 2001; BISSET et al., 2002). There are several possible explanations for this. In the present study, optical density (OD) readings generated by supernatants of uninfected PBM cells were not determined. These background OD readings were therefore not subtracted from the readings of the plate wells containing infected cells. This might have led to an underestimation of the percentage reduction in p24 antigen concentration achieved in plate wells treated with the test compounds and might explain, at least in part, why reduction of viral replication by 50% was not achieved. Differences in FIV strains might also partly explain this finding. However, McCrackin Stevenson and McBroom (2000) used both the same cell system and virus and found EC_{50} values for zidovudine, lamivudine, and emtricitabine that were much lower than 10 μ M (MCCRACKIN STEVENSON and MCBROOM, 2001). Gobert and coworkers (1994) made a similar observation; the EC_{50} values for zidovudine against two FIV strains detected in a previous study in their laboratory (REMLINGTON et al., 1991) were higher than the values determined in the later study (GOBERT et al., 1994). They considered it likely that these differences were related to variations in the batches of fetal bovine serum used in the experiments. As mentioned above, the molecular clone FIV-pPPR is less susceptible to lamivudine and possibly other NRTIs than a natural FIV isolate (FIV-Maxam) (MCCRACKIN STEVENSON and MCBROOM, 2001). This could also, to some degree, account for the need of higher drug concentrations to reach 50% virus inhibition. However, independent of the system used, the outcome of the present study was that the antiviral efficacies of all drugs investigated were comparable.

Ideally, a drug that is considered for *in vivo* testing should be effective and have very low toxicity. However, the limiting factor as to whether a NRTI other than zidovudine should be considered for *in vivo* testing might be the drug's associated cytotoxicity rather than its ability to maximally suppress viral replication.

A limitation of the present study was that EC_{50} values could not be reported for the test compounds because the highest drug dose investigated did not achieve a 50% reduction of virus replication. However, comparison of the test compounds at the highest dose investigated was nevertheless considered useful because it

allowed comparison of newly investigated drugs with drugs that had been previously tested in feline cell cultures at a concentration which is clinically relevant.

As mentioned above, single-agent therapy is no longer recommended for the treatment of HIV/AIDS for a number of reasons, one being the development of drug-resistant mutations of the virus. Mutations as a result of treatment with various NRTIs have been described in HIV-infected individuals (LARDER et al., 1989; FITZGIBBON et al., 1992; RICHMAN, 1995; SCHUURMAN et al., 1995; KURITZKES, 1996). As antiviral therapy in FIV-infected cats is still uncommon, there is much less information available for FIV. Zidovudine is the only antiretroviral drug that has been assessed thoroughly for its anti-FIV activity *in vitro* as well as *in vivo* and it has been shown that drug-resistant mutants develop both *in vitro* (REMYINGTON et al., 1991; SMITH et al., 1998; MCCRACKIN STEVENSON and MCBROOM, 2001) and in naturally FIV-infected cats treated with zidovudine for over five years (MARTINS et al., 2008). It would be advantageous to have additional drugs available which have proven effective and safe *in vitro* and which could be evaluated *in vivo* for their use as an alternative to zidovudine in cats with viral resistance to this drug or for the use in combination with zidovudine in an attempt to delay the development of drug-resistant mutants.

There are well known adverse effects of zidovudine treatment in cats, such as development of dose-dependent non-regenerative anemia and neutropenia, which can necessitate cessation or interruption of zidovudine therapy (HARTMANN et al., 1992; HARTMANN et al., 1995a; ARAI et al., 2002). In the treatment of HIV/AIDS it has been shown that combination treatment allows reduction of individual drug dosages which can limit the toxic side effects (DE CLERCQ, 2009). This could be a further advantage of combination treatment in FIV-infected cats, in addition to delaying the development of drug-resistant viral mutations.

The combination of two or three NRTIs against FIV has been assessed in two studies (ARAI et al., 2002; BISSET et al., 2002). Bisset and coworkers (2002) showed that FIV replication in CRFK cells can be inhibited in a synergistic manner by the combined usage of zidovudine, lamivudine and abacavir (BISSET et al., 2002).

Arai and coworkers (2002) showed that the combination of zidovudine and

lamivudine had additive to synergistic effects against FIV in primary PBM cells, however not in chronically infected cell lines. In a second, *in vivo* part of that study chronically FIV-infected cats were initially treated with a high dose of 20 mg/kg/drug PO every 12 hours which had to be lowered to 10 mg/kg/drug every 12 hours after 4 weeks due to the development of side effects (anemia, fever). The total length of treatment was 8 weeks. Despite the relatively high doses, compared to what is routinely used for zidovudine in the field, this combination treatment regimen did not result in statistically significant differences in either the FIV load in PBM cells, anti-FIV antibody titer, CD4/CD8 ratio, or CD4⁺ and CD8⁺ cell counts compared to the control group. The authors concluded that zidovudine/lamivudine combination treatment in chronically FIV-infected cats is not as effective as in HIV-infected patients (ARAI et al., 2002). It is however known from previous studies of naturally FIV-infected cats that even lower doses of zidovudine monotherapy can have beneficial clinical effects such as improvement of stomatitis, and clinical condition scores (HARTMANN et al., 1992; HART and NOLTE, 1995; HARTMANN et al., 1995a) and reduced severity of neurological signs (LEVY et al., 2008). The experimentally infected cats in the study by Arai and coworkers (2002) however did not show any abnormal clinical signs prior to combination treatment (ARAI et al., 2002) and therefore, it is impossible to draw any conclusions as to whether this drug combination, despite not improving virological or immunological parameters, might have beneficial clinical effects like previously described for zidovudine monotherapy.

Although it is difficult to make recommendations about clinical treatment based only on *in vitro* data, the findings of the present study have suggested that further investigation of didanosine in the treatment of cats naturally infected with FIV is warranted. In a study assessing cytotoxic effects of 18 antiviral agents on feline lymphocytes, didanosine had the least toxicity (SMYTH et al., 1994), corresponding to the findings in the present study. In addition, only one of the other eight NRTIs tested in the present study had greater *in vitro* efficacy than didanosine. The combined profile of low cytotoxicity and relative efficacy, compared with characteristics of other NRTIs used in the assays performed in the present study, suggests that didanosine might be an interesting candidate drug for further *in vivo* testing either as a sole agent or in combination with zidovudine. In

fact, monotherapy with didanosine (33 mg/kg, PO, every 24 hours from 6 to 12 weeks after infection) in neonatal kittens infected with FIV resulted in improvements in multiple variables, compared with findings in untreated kittens, including reduction in plasma viral load, significant improvement in the animals' neurobehavioral performance, and attenuation of neuroinflammation (ZHU et al., 2009). There is also support for treatment of HIV-infected humans with a combination of zidovudine and didanosine, which resulted in an overall reduction in mortality rate of 32%, compared with results following zidovudine monotherapy (DELTA COORDINATING, 2001). Logical next candidates for *in vivo* testing of potential novel treatments for FIV-infected cats would include the three newly evaluated NRTIs, amdoxovir, dexelvucitabine, and racivir.

The results of the present study indicate that amdoxovir, dexelvucitabine, and racivir have acceptable cytotoxicity profiles in feline PBM cells, compared with those of other NRTIs licensed for the treatment of HIV in humans. However, their anti-FIV efficacies were less (albeit not significantly) than those of five of the six FDA-approved NRTIs (didanosine, emtricitabine, lamivudine, stavudine, and zidovudine) that they were compared to. On the basis of the data obtained in the present study, amdoxovir, dexelvucitabine, and racivir appear to be safe treatment options for future studies investigating their potential use in FIV-infected cats.

V. SUMMARY

The purpose of the study reported here was to compare the antiviral efficacy against feline immunodeficiency virus (FIV) and cytotoxicity in feline peripheral blood mononuclear (PBM) cells of 9 nucleoside reverse transcriptase inhibitors (NRTIs), three of which had not been evaluated against FIV in feline cells before. PBM cells were isolated from the blood of three specific pathogen-free (SPF) cats.

The cytotoxic effects of the test compounds were determined by colorimetric quantification of a formazan product resulting from bioreduction of a tetrazolium reagent by viable PBM cells. Each compound was tested in 12 concentrations ranging from 0.001 to 500 μM . Uninfected cells from one SPF cat were used in these assays. PBM cells (from all three SPF cats) were infected with the molecular clone FIV pPPR and the antiviral efficacy of the test compounds was assessed using a FIV p24 antigen capture enzyme-linked immunosorbent assay. Each compound was tested in 5 concentrations ranging from 0.1 to 10 μM .

Cytotoxic effects in feline PBM cells were observed only at concentrations over 10 μM for all 9 NRTIs. Comparison of the cytotoxic effect at the highest concentration investigated (500 μM) revealed that didanosine and amdoxovir were significantly less toxic than abacavir. As no cytotoxicity was noted up to a concentration of 10 μM , this was set as the highest concentration for the second part of this study investigating the anti-FIV efficacy of the test compounds. All drugs induced a dose-dependent reduction of FIV replication. When compared at the highest concentration investigated, there was no significant difference in the antiviral efficacy among the test compounds. The EC_{50} could not be determined as none of the test compounds achieved 50% viral inhibition.

The evaluated NRTIs had low cytotoxicity against feline PBM cells and appear to be safe options for further *in vivo* evaluation for the treatment of FIV-infected cats. There was no evidence suggesting that the newly evaluated compounds would be superior to the existing NRTIs for reducing the FIV burden of infected cats.

VI. ZUSAMMENFASSUNG

Das Ziel der vorliegenden Studie war es, die antivirale Wirksamkeit gegen das feline Immunschwächevirus (FIV) und die Zytotoxizität in feline mononukleären Zellen des peripheren Blutes von neun nukleosidischen Reverse-Transkriptase-Inhibitoren (NRTI) zu vergleichen. Die *in vitro* Wirksamkeit gegen FIV von drei dieser NRTI war zuvor noch nicht in feline Zellen untersucht worden. Die mononukleären Zellen des peripheren Blutes wurden aus dem Vollblut von drei spezifisch-pathogenfreien (SPF) Katzen gewonnen.

Der zytotoxische Effekt der Testmedikamente wurde durch die kolorimetrische Quantifikation eines Formazanprodukts nachgewiesen, das bei der Bio-reduktion eines Tetrazoliumreagenz durch lebende mononukleären Zellen des peripheren Blutes entsteht. Jedes Medikament wurde in zwölf Konzentrationen untersucht, die von 0,001 bis 500 μM reichten. Für diese Versuche wurden nicht-infizierte Zellen einer SPF Katze verwendet. Mononukleären Zellen des peripheren Blutes (aller SPF Katzen) wurden mit dem molekularen Klon FIV pPPR infiziert und die antivirale Wirksamkeit der Testmedikamente mithilfe eines FIV-p24-Antigen Enzyme-Linked Immunosorbent Assay nachgewiesen. Jedes Medikament wurde in fünf Konzentrationen untersucht, die von 0,1 bis 10 μM reichten.

Bei allen neun NRTI wurde ein zytotoxischer Effekt in feline mononukleären Zellen des peripheren Blutes nur bei Konzentrationen über 10 μM festgestellt. Der Vergleich des zytotoxischen Effekts in der höchsten untersuchten Konzentration (500 μM) zeigte, dass Didanosin und Amdoxovir signifikant weniger toxisch waren als Abacavir. Da bis zu einer Konzentration von 10 μM kein zytotoxischer Effekt feststellbar war, wurde diese Konzentration als die höchste für den zweiten Teil dieser Studie festgelegt, in dem die Wirksamkeit gegen FIV untersucht wurde. Alle Medikamente führten zu einer dosisabhängigen Reduktion der FIV-Vermehrung. Bei einem Vergleich der höchsten eingesetzten Konzentrationen aller Testmedikamente konnte kein signifikanter Unterschied in deren antiviraler Wirksamkeit festgestellt werden. Die 50 % effektive Konzentration konnte nicht bestimmt werden, da keines der Testmedikamente die Virusreplikation zu 50 % einschränkte.

Die getesteten NRTI hatten geringe Zytotoxizität in feline mononukleären Zellen

des peripheren Blutes und scheinen sichere Kandidaten für die weitere *in vivo* Untersuchung zur Therapie von FIV-infizierten Katzen darzustellen. Es gab keinen Hinweis darauf, dass die Medikamente, die hier zum ersten Mal untersucht wurden, die Viruslast von FIV-infizierten Katzen besser senken würden als die existierenden NRTI.

VII. REFERENCES

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