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Toxicity associated with high dosage 9-[(2*R*,5*R*-2,5-dihydro-5-phosphonomethoxy)-2-furanyl]adenine therapy and attempts to abort early FIV infection

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Abstract

9-[(2*R*,5*R*-2,5-dihydro-5-phosphonomethoxy)-2-furanyl]adenine, or D4API, was tested in the feline immunodeficiency virus (FIV) infection model and found to be significantly more inhibitory in vitro than its parent compound 9-phosphonylmethoxethyl adenine (PMEA). Cytotoxicity was less than for PMEA or azidothymidine (AZT) for culture periods of 7 days, but more toxic after 10 days. D4API was rapidly absorbed by cats following subcutaneous inoculation, with a plasma half-life of less than 1 h after intravenous inoculation and between 2 and 3 h after subcutaneous injection. Peripheral blood mononuclear cells collected from cats given a single dose of D4API were refractory, however, to FIV infection in vitro for up to 24 h. Given its prolonged intracellular phase and high selectivity index, high dose D4API therapy was tested for its ability to abort an acute (i.e. 2 week) FIV infection. A divided daily dose of D4API, which was one-fourth the toxic dose and 125 times the concentration that would totally inhibit virus replication in vitro, completely abrogated the anticipated viremia and antibody responses. Unfortunately, a majority of treated/uninfected and treated/infected test cats died acutely of drug toxicity after 47 days of treatment. Toxicity in vivo mirrored what was observed in vitro, being precipitous and cumulative in nature. Toxic signs included widespread hepatic and lymphoid necrosis. A surviving treated/FIV infected cat remained healthy to day 175 when the study was terminated; antibodies appeared 2 months later than in untreated/infected cats and virus was only detectable at low levels on day 175. In contrast, untreated/infected cats were viremic and antibody positive from 3 to 4 weeks post-infection onwards. Therefore, it was possible to alter, but not abort, an early FIV infection with prolonged, high-dose D4API treatment. © 1997 Elsevier Science B.V.

Keywords: 9-[(2*R*,5*R*-2,5-dihydro-5-phosphonomethoxy)-2-furanyl]adenine; Feline immunodeficiency virus; Antiviral toxicity

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1. Introduction

A number of derivatives of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) possess decreased toxicity and increased antiviral effect when compared to their parent compound. One of these derivatives, 9-[(2*R*,5*R*-2,5-dihydro-5-phosphonomethoxy)-2-furanyl]adenine (D4API) is two to five fold more effective against HIV-1 in vitro and much less toxic to cultured cells than PMEA (Kim et al., 1991a,b; Balzarini et al., 1994). The efficacy of D4API against murine sarcoma virus (MSV) was two to ten fold higher than that of PMEA (Balzarini et al., 1994). D4API also showed higher efficacy against HIV-1 and Rauscher murine leukemia virus (R-MuLV) in vitro than 2′,3′-dideoxy-2′,3′-didehydrothymidine (D4T) (Kim et al., 1991a,b). In addition to its high degree of efficacy and safety, D4API reaches very high concentrations within cells and its biologically active diphosphate form has a prolonged intracellular half-life of 13 h in CEM cells and 30 h in human peripheral blood mononuclear cells (PBMCs) (Balzarini et al., 1994).

Although D4API had not been tested in lentivirus infection models prior to this study, in vitro and in vivo experiments with a closely related compounds, 9-(2-phosphonyl methoxypropyl)adenine (PMPA) and PMEA, have been conducted in the immunodeficiency virus infection models. A conventional dose (3–6 mg/kg per day) of PMEA inhibited, but did not prevent, FIV infection in vivo when given 1–49 days after exposure (Philpott et al., 1992). A similar, but more beneficial, effect was observed in cats treated with PMEA at a dose of 20 mg/kg per day 0–35 days post-infection (Egberink et al., 1990). In contrast, a conventional dose of a more potent analogue, PMPA, prevented 100% of SIV infections when started within the first 24 h after experimental infection of rhesus monkeys (Tsai et al., 1995). When this same PMPA therapy was delayed until 3 weeks post-infection, subsequent virus replication was greatly decreased but not eliminated (Van Rompay et al., 1996). The question remains as to whether high dose phosphonate therapy might be even more effective.

The favorable selectivity index of compounds like D4API lend themselves to high dose therapy with the possible goals of aborting early infections, clearing established infections, or delaying or preventing antiviral drug resistance or its effects. Nevirapine, which also has a high safety index, was tested at three to four fold the normal therapeutic dose (Havlir et al., 1995). Viral resistance, which normally occurred at 1–2 weeks at normal dosage was delayed until 8–12 weeks. The present study also attempted to use high dose anti-retroviral drug therapy, not for delaying drug resistance, but rather to increase the antiviral effect. Since a similar drug (PMPA) has been effective in preventing very early lentivirus infections in primates (Tsai et al., 1995), the design of these experiments was to treat infection at a somewhat later stage, when provirus was well integrated into host cells but before detectable levels of virus could be measured in plasma or PBMC.

In order to minimize cost, feline immunodeficiency virus (FIV) infection of domestic specific pathogen free cats was chosen as the antiviral drug treatment model system (Barlough et al., 1993; North et al., 1989) rather than SIV infection in rhesus macaques. FIV infection of laboratory cats is less virulent than SIV infection of macaques but is closer to HIV-1 infection of people in its clinical stages and their timing (George et al., 1993; Dua et al., 1994). FIV has also been shown to be very susceptible in vitro to PMEA (Hartmann, 1995), so it was anticipated that D4API would also be inhibitory. The amount of D4API administered to cats in this study was calculated to produce average daily plasma levels that were about one-fourth of the toxic dose and 125 times the minimum virus inhibitory dose. The objective was to continue high dose D4API therapy for as long as it was tolerated, thus maintaining maximal intracellular levels for as long as possible in the hopes of aborting the infection at an early stage.

2. Materials and methods

2.1. Antiviral drug (D4API)

D4API was obtained from Gilead Science (Foster City, CA) and courtesy of Dr N. Bischofberger. The comparison compound for in vitro investigation, PMEA, was obtained from Prof. J. Balzarini, Rega-Instituut, Katholieke Universiteit Leuven, Belgium. AZT was commercially available from Burroughs Wellcome, Burgwedel, Germany.

2.2. FIV inoculum for in vitro studies

Peripheral blood mononuclear cells (PBMCs) from cat # 5000 were isolated from 5 ml of heparinized whole blood by density gradient centrifugation with Ficoll/Hypaque lymphocyte separation medium. Cells were counted, brought to a concentration of 1×10^6 PBMC/ml in lymphocyte stimulation medium [RPMI medium with 10% fetal bovine serum, 5 μg/ml concanavalin A (ConA) and 100 IU human rIL-2/ml (IL-2 courtesy of Cetus, Emeryville, CA)]. Stimulated PBMC cultures were split weekly and cocultivated in lymphocyte maintenance medium (stimulation medium without ConA) with an equal number of freshly stimulated PBMCs from a noninfected donor cat. Culture supernatants were monitored twice weekly for FIV-p24 antigen expression. When antigen levels peaked, usually after 3–4 weeks, culture supernatants were harvested, pooled and filtered through 0.45 μM filters. Aliquots of the culture supernatants were prepared and stored at -70°C. An aliquot of the virus stock was titrated for infectivity on stimulated primary PBMC cultures and the tissue culture infectious dose-50% ($TCID_{50}$) calculated.

2.3. Antigen capture ELISA for FIV nucleocapsid (p24) protein

FIV-p24 was measured in tissue culture supernatants by an antigen capture ELISA (Dandekar et al., 1992). A specific virus supernatant with known FIV-p24 concentration was assayed in triplicate and the mean values used to construct a standard curve relating OD values to FIV-p24 concentration.

2.4. Cell culture assays for antiviral drug activity

Antiviral drug activity was measured in vitro in stimulated primary PBMC cultures as previously reported (Hartmann et al., 1994). Cells were infected with 100 $TCID_{50}$ of virus/well and FIV-p24 antigen measured after 4, 7, 10 and 14 days. Virus replication in untreated control cultures rose after day 4 and plateaued by days 10–14. The mean FIV-p24 concentration of infected control cells cultured in triplicate without compound were defined as 100% virus replication. Identical cultures, in triplicate, were treated with various concentrations of antiviral drugs. The effective concentration-50% (EC_{50}) was defined as the concentration of the compound which reduced FIV-p24 concentration of the control cells by 50%.

2.5. Cell culture assays for antiviral drug cytotoxicity

The cytotoxicity of the compounds was compared by trypan blue vital staining (Hartmann et al., 1994; Philpott et al., 1992). Prestimulated PBMCs were seeded in 96-well plates at 10^5 cells per well and cultured in 200 μl maintenance medium and containing 0, 0.04, 0.2, 1, 5, 25, 125 and 625 μM dilutions of AZT, PMEA or D4API in duplicate. After an incubation period of 4, 7, and 10 days, 100 μl of the cell suspension was collected from each well and mixed with 100 μl trypan blue. Dead, blue-colored, PBMCs and healthy uncolored cells, were counted in a Neubauer hemocytometer (five squares/well). The mean percentage cytotoxicity (colored/uncolored cells $\times$ 100) was calculated from values of duplicate cultures. Duplicate control wells were cultured without the addition of drug and included in each assay. The compound concentrations reducing the number of surviving cells by 50% over parallel untreated control cultures were defined as cytotoxic dose-50% (CC_{50}). The selectivity index (SI) was represented as the ratio of CC_{50}/EC_{50}.

2.6. Experimental animals

A total of 11 male neutered 6 month old SPF cats were obtained from the breeding colony of the Nutrition Research Laboratory, University of California (UC), Davis (courtesy of Drs James Morris and Quinton Rogers) and housed in facilities monitored by the Animal Resources Services, UC Davis. The cats were separated into four groups, A–D. Group A contained two cats (#1, 2), which served as untreated/uninfected controls. Group B contained three cats (#3–5) that were treated but not infected. A total of six cats were infected with FIV at day 0 by intraperitoneal injection of 1 ml fresh heparinized whole blood taken from a cat with chronic FIV (Petaluma strain) infection; three of these animals (#6–8) were placed in group C and three (#9–11) in group D.

2.7. Pharmacokinetic studies

Serum drug levels were measured after intravenous and subcutaneous injection in two independent experiments, each with the same two SPF cats. In the first experiment, cat #1 was treated with 10 mg/kg D4API intravenously (jugular vein) and cat #2 with 10 mg/kg D4API subcutaneously. In the second experiment, which was conducted 8 weeks later, cat #1 was injected subcutaneously and cat #2 intravenously. A 2 ml volume of whole blood (into heparin) was taken from the jugular vein at 0, 10, 20, 30, 60, 90, 180 and 360 min after injection. Plasma was separated and stored at −70°C. The plasma concentrations of D4API were determined by HPLC (assays courtesy of Gilead Sciences, Foster City, CA). The D4API elimination curve was derived from the mean values from both experiments.

2.8. Duration of intracellular antiviral activity following a single dose of D4API

A single adolescent cat was injected subcutaneously with 20 mg/kg of D4API. Blood was then taken 5 h later and gradient purified PBMCs adjusted to a concentration of 1×10^6 cells/ml in lymphocyte stimulation medium. PBMCs harvested from the same cat just prior to D4API injection were used for the negative control, while the positive control consisted of a portion of these same PBMCs treated with 134 μM D4API in vitro for 5 h. The level of drug used for the positive control was extrapolated from pharmacokinetic studies; 20 mg/kg subcutaneously to the PBMC donor cat would result in a peak serum level of 40 μg/ml, or 134 μM (twice that achieved by the 10 mg/kg dosage in the pharmacokinetic study). All PBMC cultures were washed twice at time zero with PBS to remove any extracellular D4API, whether exposed in vitro or in vivo, and then resuspended to a concentration of 1×10^6 cells/ml. A total of seven culture flasks, containing 10 ml of cell suspension, were set up for each of the four cell preparations.

PBMC cultures, set up in triplicate, were infected with 100 $TCID_{50}$ of FIV-Petaluma at 0, 2, 24, 48, 120 h time points. Virus remained in contact with cells for 2 h, after which the culture supernatant was removed and the cells washed twice with PBS. After washing, the cells were resuspended to a concentration of 5×10^5 PBMC/ml medium. A 200 μl volume of cell suspension were placed in each well of a 96 well culture plate. At day 4, 7, 10 and 14 after virus exposure, 125 μl supernatant was removed and stored at −20°C; this was replaced by fresh maintenance medium. The mean concentration of FIV-p24 antigen was calculated from triplicate antigen capture ELISA values of triplicate culture supernatants harvested on days 4, 7, 10 and 14. Viral antigen was first detected at day 4, increased rapidly through days 7 and 10 and plateued thereafter (data not shown).

2.9. Calculation of drug dosage used for in vivo safety and efficacy studies

Data from in vitro and in vivo studies were used to calculate a drug dosage for the in vivo safety and efficacy studies that would provide maximum average plasma levels with a margin of safety. Based on pharmacokinetic studies in cats and extrapolation from areas under the curve (plasma drug level versus time), a dosage of D4API of 10 mg/kg, q12 h, subcutaneously, was

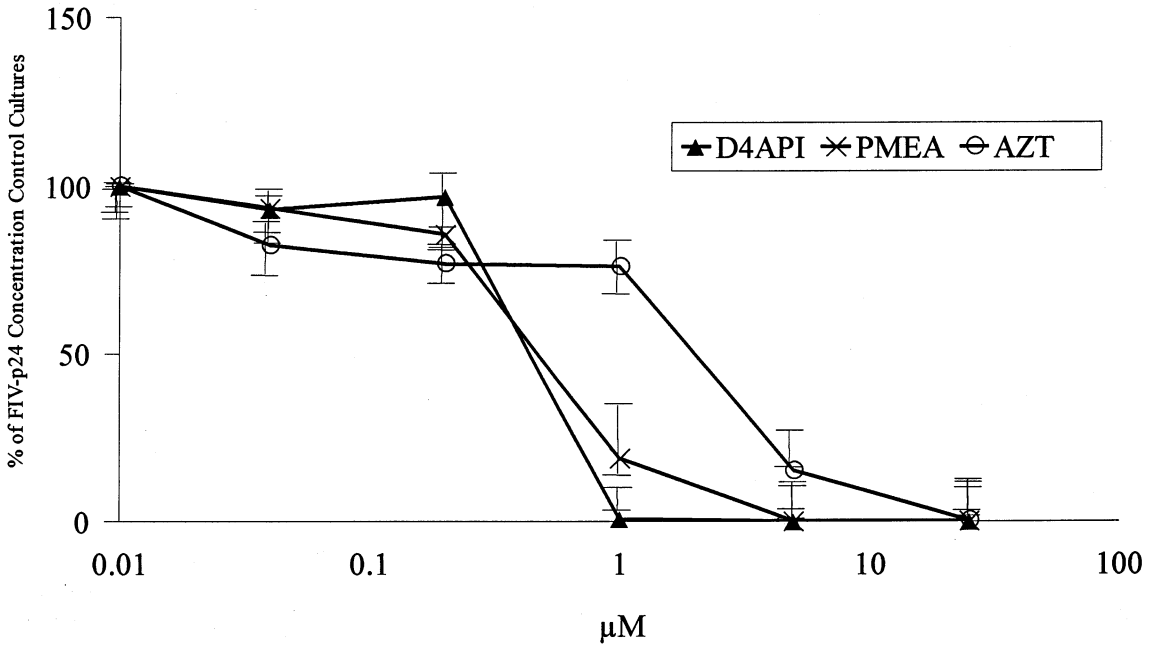

Fig. 1. FIV-p24 antigen concentration (mean ± 1 S.D.) in supernatants from FIV infected PBMCs by 0, 0.04, 0.2, 1, 5 and 25 μM concentrations of D4API, PMEA and AZT following a 10 day incubation.

calculated to achieve an average daily plasma level of 3.8 μg/ml (125 μM) of drug. This was 125 times greater than the dose (1 μM) required to completely inhibit virus replication in vitro on day 10 of culture (Fig. 1) and less than one-fourth of the dose that was 100% cytotoxic after this same time (580 μM) (Fig. 2).

2.10. FIV inoculum for in vivo studies

Cats were infected with the fourth cat-to-cat passage of the Petaluma strain of FIV (FIV-Petaluma). The inoculum was in the form of 1 ml of fresh whole blood from a chronically infected cat (# 5000) with low CD4+ T-cell levels as described elsewhere (Dua et al., 1994).

2.11. Monitoring cats for signs of drug toxicity

A cursory gross physical examination was carried out every day and a complete physical examination following a strict protocol was performed weekly. Complete blood cell counts, including absolute leukocyte, erythrocyte, neutrophil, lymphocyte, monocyte, eosinophil and platelet numbers, red blood cell indices and total plasma protein and a 16 channel blood chemistry profile, were carried out biweekly (Dua et al., 1994).

2.12. FIV antibody assay

Antibodies to the p24 nucleocapsid protein of FIV were measured weekly using an ELISA based on recombinant FIV p24-antigen (George et al., 1993; Reid et al., 1991).

2.13. Virus isolation

Virus isolation was carried out every second week. Peripheral blood mononuclear cells were isolated from 5 ml of heparinized whole blood by density gradient centrifugation with Ficoll/Hypaque lymphocyte separation medium. Cells were counted, brought to a concentration of 1×10^6 PBMC/ml of lymphocyte stimulation medium and cultured for 4 weeks. At the time of weekly medium change, an aliquot of culture supernatant from each sample was retained and stored at

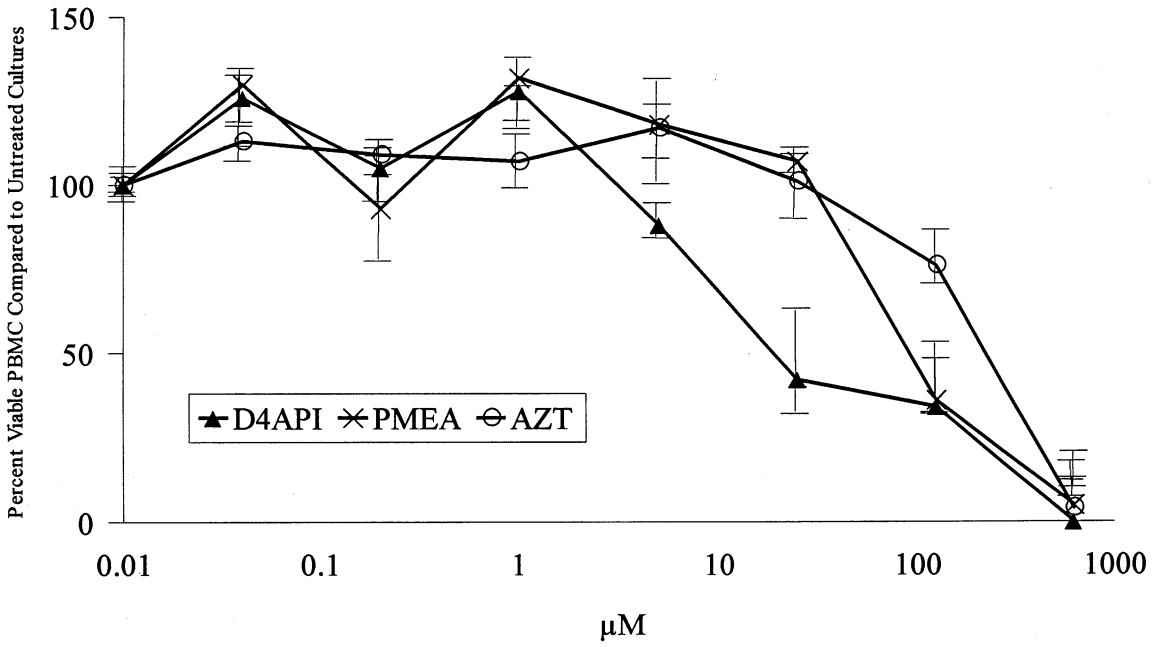

Fig. 2. Percentage (mean ± 1 S.D.) of viable PBMCs following a 10 day cell incubation by D4API, PMEA and AZT in different concentrations (0, 0.04, 0.2, 1, 5, 25, 125, 625 μM) compared to control cells cultured without compound.

− 20°C. The concentration of FIV p24-antigen in the stored supernatants was measured by antigen capture ELISA.

2.14. Polymerase chain reaction for FIV p24

A nested PCR in the FIV-p24 gene was used to detect proviral DNA in fresh or cultured PBMC (Reubel et al., 1994). The proportion of infected PBMC that were infected in vivo at various time points following infection was quantitated by the procedure of Dua et al. (1994). The PCR was capable of detecting 1 in 10^6 provirus containing cells.

2.15. Gross and histologic examination of necropsy material

All cats dying on project were examined for gross and microscopic evidence of disease. Routine formalin-fixed, paraffin embedded, hematoxylin and eosin stained tissue sections were read without knowledge of virus or treatment status by Dr J. Woo, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, UC, Davis.

2.16. Statistical analyses

Group differences in the presence or absence of virus isolation from PBMC cultures were evaluated using a Fisher's exact test. Repeated measures analysis of variance was used to evaluate the effects of time, group and time-by-group interaction on log FIV-infected cell counts per million PBMC; the functional form of trend across time was tested using orthogonal polynomials. Post-hoc tests were performed using Student's two-sample t-test heteroscedasticity. One-tailed P-values (reflecting the alternative to the null hypothesis that treatment would reduce, not raise, the level of FIV infection) of 0.05 or less were considered to be statistically signficant. Since it is impossible to obtain a logarithm of zero, 0.001 was added to all values.

3. Results

3.1. Comparative in vitro drug efficacy and toxicity studies

The antiviral efficacy of D4API was compared to that of PMEA and AZT in primary feline PBMC cultures at days 7, 10, and 14 after FIV infection (Table 1). No virus replication was detected in control cultures at day 4, so earlier time points were not listed. The EC_{50} values at all three time points of measurement were very low ($<$ 0.64 μM), with the exception of AZT at day 14 (2.71 μM).

Complete virus suppression was achieved with all three drugs, as measured on day 10 (Fig. 1). Cultures that were exposed to 1 μM D4API and 5 μM of PMEA prior to infection demonstrated no signs of virus replication, whereas AZT fully inhibited virus growth only at a concentration of 25 μM.

Cytotoxicity to primary PBMC cultures was examined using trypan blue vital staining at days 4, 7 and 10 post-treatment with 0, 0.04, 0.2, 1, 5, 25, 125 and 625 μM of the various compounds (Table 2). The CC_{50} for all three drugs was very high on day 4 of culture. This began to change, however, on day 7; PMEA decreased cell viability by more than 50% following 7 days of exposure at a concentration of 107 μM, whereas D4API and AZT produced a similar degree of toxicity only at around 580 μM. The most dramatic differences were seen at day 10. The CC_{50} of D4API at day 10 was 22 μM at day 10, whereas PMEA and AZT exhibited comparable toxicity only at concentrations of 105 and 306 μM, respectively. Cytotoxicity at day 10 was only evident at concentrations above 1 μM for D4API, while PMEA and AZT were cytotoxic only at levels above 10 μM (Fig. 2). The selectivity indexes ($SI = CC_{50}/EC_{50}$) of each compound at day 7 and 10 are given in Table 3. AZT and D4API had the highest SI values on day 7 ($\approx$ 19 000) and were the least toxic, while D4API and PMEA had the lowest SI values at day 10 ($\approx$ 200) and were the most toxic.

Table 1
Antiviral activity of the compounds D4API, PMEA and AZT in feline PBMCs, based on monitoring FIV-p24 antigen production in the cell culture supernatants

Compound	Cell type	EC_{50} (μM) of drug after various times in culture		
		Day 7	Day 10	Day 14
D4API	PBMC	0.03	0.12	0.59
PMEA	PBMC	0.03	0.52	0.63
AZT	PBMC	0.03	0.14	2.71

EC_{50}, compound concentration required to inhibit the p24 antigen expression in the cell culture supernatants by 50%.

Table 2
Cytotoxicity of D4API, PMEA and AZT in feline peripheral blood lymphocytes (PBL), based on the decrease of cell viability

Compound	Cell type	CC_{50} (μM) Time of exposure		
		Day 4	Day 7	Day 10
D4API	PBMC	517	580	22
PMEA	PBMC	447	108	105
AZT	PBMC	>625	582	306

CC_{50}, drug concentration reducing the percentage of viable cells by 50%.

3.2. In vivo pharmacokinetics

Elimination curves of D4API in plasma after intravenous and subcutaneous injection are shown in Fig. 3. The plasma half-life was less than 1 h after intravenous inoculation and between 2 and 3 h after subcutaneous injection.

Table 3
Selectivity indexes (SI) of the compounds D4API, PMEA and AZT

Compound	Cell type	SI—Time of exposure	
		Day 7	Day 10
D4API	PBMC	19333	183
PMEA	PBMC	3600	202
AZT	PBMC	19400	2186

$SI = CC_{50}/EC_{50}$.

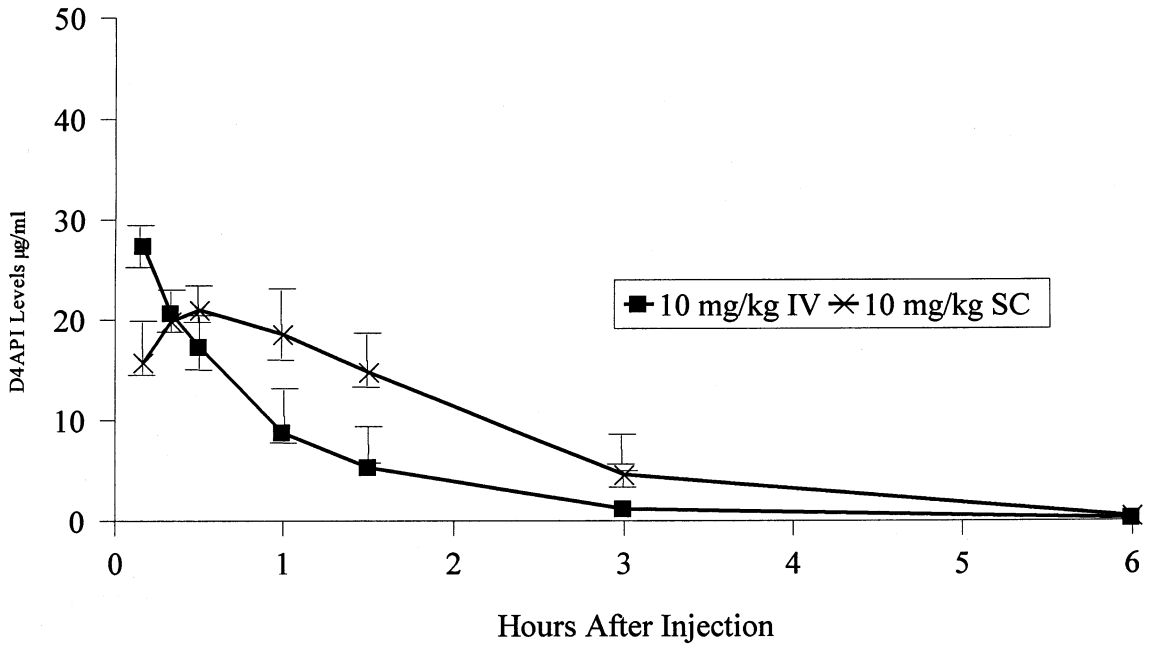

Fig. 3. Serum concentration (mean ± 1 S.D.) of D4API (μg/ml) in cats following intravenous (IV) and subcutaneous (SC) bolus injection of 10 mg/kg body weight.

3.3. Intracellular pharmacokinetics

Cultures made up of cells that were never exposed to D4API, cells that had been treated only in vitro, or cells exposed to drug entirely in vivo were infected with FIV in vitro at various time points after treatment and assayed for virus production after 2 weeks (Table 4). PBMCs that were never exposed to D4API in vitro or in vivo could be infected with FIV at all time points. PBMCs collected 5 h after in vivo exposure to D4API were resistant to infection at hours 0, 2, 4, and 24 following in vitro culture but sensitive thereafter. In contrast, PBMCs exposed to D4API in vitro were not infectable at any time during the 5 day test period.

3.4. In vivo toxicity and efficacy studies

Cats were divided into four groups depending on whether they were FIV-infected or uninfected and D4API treated or untreated. Detectable FIV viremia in the infection model employed in this study regularly appears by 3–4 weeks post-infection (Dua et al., 1994); therefore, cats were infected with FIV and treatment commenced at 14 days post-infection. The dosage regimen was as described in Section 2. Since this dose of D4API would produce plasma levels that were near the CC_{50}, the treatment period was meant to last only as long as the animals remained grossly, hematologically and biochemically normal; the objective being to avert mortality by withdrawing treatment at the earliest signs of toxicity.

3.4.1. Toxicity studies

No gross, biochemical, or CBC abnormalities were noted during the first 6 weeks of treatment in either treated or untreated groups (data not shown). Signs of toxicity appeared abruptly in two of three treated/FIV-infected cats (group D) and three out of three treated/uninfected cats (group B) at day 47 post-treatment (61 days post-infection) (Table 5). Physical signs of toxicity included serosanguinous ocular discharge, photophobia, depression, anorexia, rough hair coats, occasional vomiting and profound dehydration; biochemical signs of toxicity consisted mainly of a

sudden elevation of liver enzymes (AST and ALT) in the serum (Fig. 4). Severe hemoconcentration and a degenerative left shift in blood neutrophils was also evident in all affected animals during the terminal stage of illness and prothrombin and partial thromboplastin times were greatly prolonged (data not shown). In spite of immediate cessation of drug treatment and vigorous supportive fluid/electrolyte therapy, four of the affected cats had to be euthanatized within 1–3 days. The remaining animal from group B (cat #4) appeared to recover several days after the drug treatment was discontinued, but became moribund 5 weeks later and euthanatized. A surviving cat from group D, cat #11, recovered rapidly from acute signs of toxicity and remained healthy throughout the remainder of the study.

Gross necropsy changes in the four cats that died acutely consisted of pulmonary congestion and reddening and occasional hemorrhages on the surfaces of abdominal organs. The only gross lesion in cat #4, the animal that survived for 5 weeks following initial toxicity, was a small, irregularly shaped, fibrotic liver. Histopathologic examination of tissue sections taken from multiple organs of cats dying acutely demonstrated severe lymphoid depletion with cytolysis in spleen, tonsils and lymph nodes and pronounced vacuolar degeneration in the liver. More variable lesions included renal vascular congestion with proteinaceous casts in glomeruli and tubules; hemorrhage, edema and alveolar protein accumulation in the lungs; ischemic and myofibriller necrosis of the myocardium; necrosis and regeneration of crypt epithelium in the small intestine and pancreatic necrosis.

Table 4
FIV-p24 antigen expression by PBMCs exposed at various time points after being collected and put into culture

Time of infection (h)	Nontreated PBMCs	Treated PBMCs	
		In vitro	In vivo
0	+	−	−
2	+	−	−
4	+	−	−
24	+	−	−
48	+	−	+
120	+	−	+
Uninfected	−	−	−

In vivo treated cells were harvested 5 h after the cat had been given a single subcutaneous injection of 20 mg/kg D4API. Nontreated and in vitro treated control PBMCs were collected from the same donor cat immediately prior to in vivo drug treatment; in vitro treatment was with 134 μM D4API for 5 h. Cells from each of the same batches were left uninfected as controls.

3.4.2. Antiviral efficacy studies

None of the noninfected cats in control groups A (nontreated) and B (treated) were ever positive by either virus isolation (Table 5) or PCR (data not shown).

All three group C (infected/untreated) animals were viremic by virus isolation from day 21 post-infection onward (Table 5). The three FIV-infected/treated cats in group D were FIV negative by virus culture during the treatment period from 14 through 49–63 days post-infection. With respect to Table 5, the distribution of positive and negative cats in groups C compared to D were signficantly different ($P = 0.050$) at days 21, 35, 45 and 63. Day 63 being the time all treatment was stopped.

Proviral DNA was first detected in PBMCs by PCR at days 34–62 post-infection in the three cats in group C (uninfected/treated); infected cells were undetectable at day 14 and increased 1–1000 fold over the following weeks (Table 6). Among group D (infected/treated) cats, only cat #9 was PCR positive for proviral DNA at the time of death and only at the level of 1 in 10^6 cells. Differences in the log number of FIV-infected cells per million PBMC between groups C and D were found to be linearly dependent on the days post-infection ($P = 0.049$). Notably, by day 62 there were signficantly more FIV infected cells in group C (infected/untreated) than group D (infected/treated) cats ($P = 0.024$), thus reconfirming the in vivo efficacy of D4API treatment demonstrated by classical virus isolation.

Cat #11 from group D, the sole long-term survivor of drug treatment, remained virus negative by virus isolation until day 175 post-infection. However, 1 in 100 000 PBMCs from this animal contained proviral DNA on day 124. PBMCs

Table 5
Virus isolation from PBMC cultures of groups A–D cats

Group/cat #	Days following FIV infection										
	21	28	35	42	49	56	63	77	105	133	175
Group A											
1	−	nt	−	nt	−	nt	−	−	nt	nt	nt
2	−	nt	−	nt	−	nt	−	−	nt	nt	nt
Group B[a]											
3	−	nt	−	nt	−	nt	−	−	Died day 97		
4	nt	−	nt	−	nt	Died day 62					
5	−	nt	−	nt	−	nt	Died day 64				
Group C											
6	+	nt	+	nt	+	nt	+	+	+	+	+
7	+	nt	+	nt	+	nt	+	+	+	+	+
8	+	nt	+	nt	+	nt	+	+	+	+	+
Group D											
9	−	nt	−	nt	−	nt	Died day 63				
10	−	nt	−	nt	−	nt	Died day 63				
11	−	nt	−	nt	−	nt	−	−	−	−	+[b]

Virus positive (+), virus negative (−), not tested (nt).
[a] D4API treatment was started at day 14 post-infection and stopped at day 61.
[b] Positive only when 5×10^6, rather than 1×10^6, of test PBMC were cocultivated with an equal number of normal PBMC and tested 4 weeks later for FIV-p24.

collected from day 175 yielded virus, but only after culturing 5×10^6 rather than 1×10^6 cells for 4 weeks.

Anti-FIV antibodies were evaluated weekly (Fig. 5). The untreated cat # 6 of group C had the first positive antibody response at day 35 post infection, followed by the other two cats of the group 2 weeks later. Infected cats of the treated groups had no detectable FIV-p24 antibodies up to the time when drug toxicity occurred and treatment was halted. The surviving cat # 11 of group D had detectable levels of serum antibodies from day 105 post-infection onward, which was 44 days after treatment was discontinued.

4. Discussion

D4API demonstrated lower EC_{50} values and therefore higher efficacy in vitro against FIV than AZT and PMEA, confirming the results obtained with the drug on HIV-1 infected cell lines (Balzarini et al., 1994). Antiviral potency of D4API was especially high on day 14 in culture; only D4API completely inhibited virus production at a concentration of 1 μM. Initial tests indicated that D4API was comparatively non-toxic, because the CC_{50} values on day 4 and 7 were higher than the values for PMEA or AZT. However, D4API turned out to be more toxic than AZT and PMEA following 10 days in culture. This delayed toxicity was thought to result from a progressive accumulation of drug within the intracellular compartment. In contrast, Kim and coworkers (Kim et al., 1991a,b) did not find any signs of toxicity for D4API at concentrations as high as 600 μM in human MT-4 cells, presumably after 5 days in culture. This difference could be explained by possible variations in the drug sensitivity between primary feline PBMCs and MT-4 cells or to the time after treatment when toxicity was measured.

D4API was quickly distributed throughout the body following intravenous injection, and the half-life was less than 1 h and total clearance was 0.4 l/h per kg. In contrast, peak plasma levels of

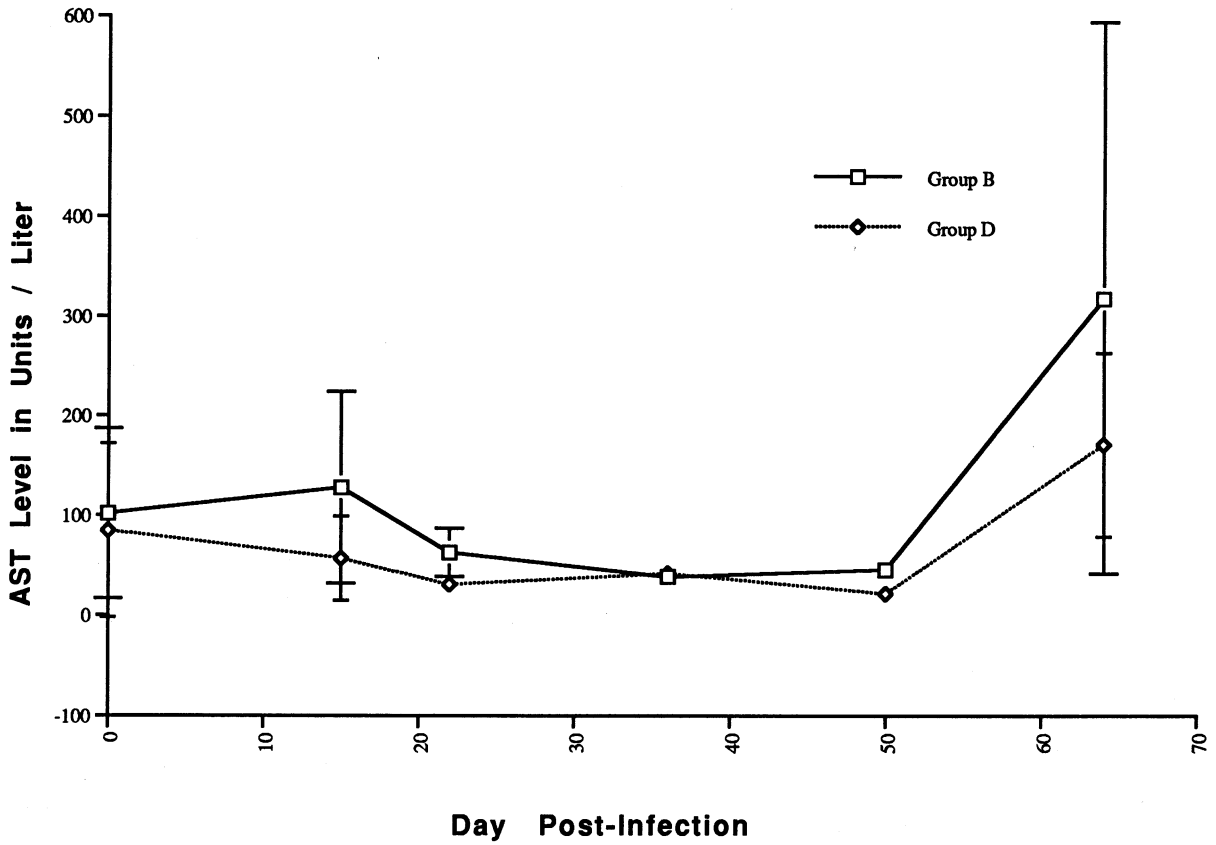

Fig. 4. Serum aspartate transaminase levels (mean ± 1 S.D.) in groups B (noninfected/treated) and D (infected/treated) cats.

D4API were only slightly lower after subcutaneous injection but remained elevated for several hours; the bioavailability after subcutaneous injection reached 163%. The elimination curve in blood was similar to that previously shown for PMEA (Egberink et al., 1990; Naesens, 1993). However, in the present study, higher maximum concentrations (20–30 μg/ml) were observed after injection of 10 mg/kg D4API than for a similar dose of PMEA; studies of Egberink and coworkers (Egberink et al., 1990) and Naesens (1993) reported peak plasma concentrations for PMEA lower than 20 μg/ml. Although the half lives of both D4API and PMEA were almost 1 h, the area under the curve for D4API in cats was approximately five-times higher than that of PMEA administered at the same dosage (Naesens, 1993).

Although the plasma half-life of D4API was short, antiviral concentrations within the intracellular compartment were surprisingly long. Peripheral blood mononuclear cells taken 5 h after injection of 20 mg/ml D4API were found to be protected from in vitro FIV infection for up to 24 h. Interestingly, PBMCs treated with D4API in vitro were protected for up to 120 h. Therefore, the drug may be taken up and metabolized somewhat differently by cells in vivo than in vitro substances or factors present within the animal may inhibit or preclude resorption of D4API into the cells, or some intracellular D4API may be lost during isolation of in vivo treated PBMC. This is the first time that intracellular antiviral activity has been tested in cells collected from treated animals. The prolonged intracellular antiviral ef-

Table 6
Level of PBMC-associated infection as determined by PCR in groups C (not treated) and D (treated) cats following infection

Group #	Cat #	Days post-infection	Days post-treatment	FIV-infected cells per million PBMC
C	6	14	na	0
		34	na	0
		48	na	0
		62	na	1
		124	na	1
C	7	14	na	0
		34	na	0
		48	na	1
		62	na	1000
		124	na	100
C	8	14	na	0
		34	na	100
		48	na	100
		62	na	1000
		124	na	100
D	9	14	0	0
		34	20	0
		48	34	0
		62 (died day 63)	48	1
D	10	14	0	0
		34	20	0
		48	34	0
		62 (died day 63)	48	0
D	11	14	0	0
		34	20	0
		48	34	0
		62	48 (drug stopped)	0
		124	110	10

na, not applicable.

fect of D4API is supported by in vitro studies of Ho et al. (1992), who showed an intracellular half life of 13–30 h in human cell lines for D4API by radioactive labeling.

Attempts to abort an early FIV infection with toxic to near-toxic levels of D4API failed, but a great deal was learned about the antiviral properties of the drug and its pattern of toxicity. When treatment was commenced just prior to the time that viremia was to occur, virus replication was strongly inhibited for the duration of treatment, as measured by virus isolation from PBMC, measurements of proviral DNA in PBMC by PCR and the failure to detect antibodies. This was in contrast to infected control animals that were untreated; virus was detected in the blood from 3 weeks onwards and seroconverstion by weeks 5–7. This finding was similar to that reported earlier for PMEA in a similar infection model (Egberink et al., 1990).

Because of the suddenness and severity of toxicity, only one cat in the D4API treated/FIV infected group D (cat #11) survived in good health. The surviving cat remained antibody negative until day 105 after infection, which was 8 weeks after finishing treatment. Repeated attempts to isolate virus from PBMC cultures during this period failed, and virus was finally isolated with difficulty on day 175. This was much different than what has been observed with the three untreated/FIV-infected group C control cats and with numerous similar historical controls in-

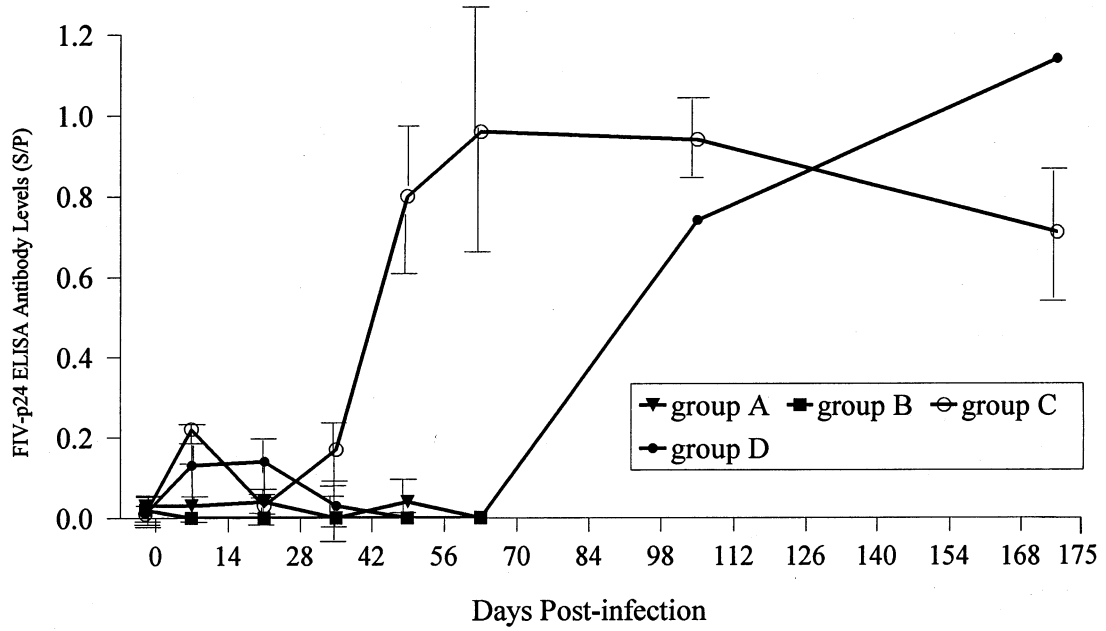

Fig. 5. Anti-FIV-p24 antibody concentration (mean ± 1 S.D.) of the cats of groups A, B, C, and D during the study, represented as S/P ratio (test well absorption/background absorption). Antibodies for group D represent mean values for all three cats up to day 63; following time points are for cat # 11 only.

fected in the same manner (Dua et al., 1994; George et al., 1993); virus and antibodies are easily isolated detected from days 3–4 weeks post-infection onwards. The delay in appearance of viremia and antibodies was identical to that shown previously for PMEA (Egberink et al., 1990). Cats receiving 20 mg/kg per day of PMEA from day 0–35 post infection did not become viremic until days 42–56 and were not antibody positive until days 56–70; infected but untreated cats were viremic from day 14 onward and antibody positive after day 21. It can be concluded, therefore, that early immunodeficiency virus infection cannot be aborted once significant amounts of proviral DNA have become integrated into host cells, even using an antiviral drug that accumulates within cells at high levels and given at toxic levels for 7 weeks. However, it appears that drug treatment under these conditions may have a beneficial effect on the subsequent course of infection once treatment is stopped. Virus levels were very low following drug cessation, as witnessed by the prolonged period before seroconversion and impossibility to isolate virus by conventional means. This conclusion was identical to that of Philpott and colleagues (Philpott et al., 1992), who treated cats with a more conventional dose of PMEA from 1 to 49 days after FIV infection; infection was not prevented, but the levels of replicating virus at 1 year was significantly lower than untreated and infected control animals.

The present study also helped delineate toxicities of PMEA-related drugs such as D4API, especially when used at high dosages. The unique feature of the toxicity was that it appeared without warning and with devastating effects. Severe clinical signs appeared in nearly all treated cats between days 49 and 51 without timely gross, hematologic, or biochemical prodrome. The disseminated intravascular coagulopathy associated with the toxicity and evidence of pulmonary edema and congestion, were suggestive of a widespread loss of vascular integrity. The liver was also heavily damaged, although pronounced pathology was also observed in lymphoid tissues, kidney, intestine, heart and lungs. The nature,

severity and acuteness suggested that toxicity occurred only when a threshhold intracellular accumulation of drug was surpassed. A hint of this cumulative threshold type of toxicity was seen in the in vitro studies; toxicity at days 4 and 7 were very low compared to PMEA and AZT, while the toxicity at day 10 was significantly greater than the latter drugs. Balzarini et al. (1987) studied the cytotoxic activity of various 2′,3′-unsaturated purine dideoxynucleoside analogues; an adenine containing compound released the highest level of free base upon incubation at 37°C (50% hydrolysis in less than 24 h). One possibility is that free adenine, which can be cardiotoxic, was the toxic principal. PMEA related compounds inhibit mitochondrial polymerases and therefore damage redox systems of the liver (J. Balzarini, personal communication). Because D4API has similar pharmacokinetics to PMEA (Hartmann, 1995), an accumulation of the phosphonate in the liver would be expected. Finally, it is noteworthy that D4API demonstrated lower toxicity than PMEA in vitro for the first 7 days in culture, but that its true and greater toxicity was only apparent after 10 days. This suggests the need to conduct toxicity studies over a longer time period both in vitro and in vivo for compounds of this nature.

In conclusion, the use of high dosages of antiviral drugs will inevitably increase as newer compounds with higher margins of safety to toxicity are developed. The potential advantages of high dose therapy in delaying drug resistance, increasing the degree of viral inhibition and even for curing infection are obvious. The present study was only the second anti-lentivirus experiment with one of these objectives in mind; the first being with high-dose nevirapine in humans to delay resistance (Havlir et al., 1995). Although attempts to abort a newly established immunodeficiency virus infection failed, several conclusions could still be made from the study. The cumulative and abrupt toxicity of D4API is of interest, especially as it might apply to related compounds such as PMPA or PMEA, which are in early human trials. Certain types of drugs that accumulate within cells for long periods of time and at high levels may demonstrate unique chronic toxicities that may be both unique and catastrophic. The low level of viremia in the single surviving FIV infected/D4API treated cat was also noteworthy because it suggested that a transient therapeutic intervention with a potent viral inhibitory drug, early in an immunodeficiency virus infection, might lead to a much milder chronic infection. An identical conclusion was obtained from a study with a larger number of cats treated with conventional levels of PMEA (Philpott et al., 1992). This suggested that it was not the high dose of D4API used in this study that altered the infection, but rather its application early after exposure.

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