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(*R*)-9-(2-Phosphonylmethoxypropyl)-2,6-Diaminopurine Is a Potent Inhibitor of Feline Immunodeficiency Virus Infection

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The antiviral efficacy of acyclic nucleoside phosphonates, including 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine [(R)-PMPDAP] against feline immunodeficiency virus (FIV) infection was determined. (*R*)-PMPDAP showed the highest selectivity index (>2,000) in vitro. Treatment of experimentally FIV-infected asymptomatic cats with PMEA or (*R*)-PMPDAP had no effect on the CD_4^+/CD_8^+ ratio. However, mean plasma viral RNA concentrations decreased significantly in the (*R*)-PMPDAP-treated cats. Our data show that, in comparison to PMEA, (*R*)-PMPDAP is a more potent and less toxic inhibitor of FIV replication both in vitro and in vivo.

Feline immunodeficiency virus (FIV) resembles human immunodeficiency virus (HIV) in its morphological, physical, and biochemical characteristics. FIV infection of cats, like HIV type 1 (HIV-1) infection of humans, leads after an asymptomatic period to AIDS and is used as an animal model (25, 30). Similarities between the reverse transcriptases (RTs) of FIV and HIV-1 have been demonstrated with respect to template specificity and magnesium requirement (9, 23). Zidovudine (AZT [3′-azido-3′-deoxythymidine]) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) have shown to be effective inhibitors of FIV replication in vitro (13) and have been used successfully as prophylactic (13, 20, 26) and therapeutic (13, 14, 17) agents in experimentally and naturally FIV-infected cats. In these experiments PMEA was found to be more effective than AZT, an observation that had also been made in mice (4). Toxic effects of these drugs, however, limit their therapeutic use. We therefore tested the derivatives of PMEA (enantiomers *S* and *R*) (*R*)-9-(2-phosphonylmethoxypropyl)adenine, (*S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine, and (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine [(*R*)-PMPDAP] in vitro and PMEA and (*R*)-PMPDAP in experimentally FIV-infected, asymptomatic cats.

The anti-FIV activity of the drugs was tested in primary feline thymocytes isolated from specific-pathogen-free cats and in Crandell feline kidney (CrFK) cells. CrFK cells grown in 96-well plates to confluent monolayers or feline thymocytes (5×10^5 cells per well) were preincubated for 1 h with fivefold dilutions of the antiviral drugs (ranging from 200 μM to 0.1 nM) and subsequently infected with 100 50% tissue culture infective doses of FIV UT-113. After 2 h, CrFK cells were rinsed twice with phosphate-buffered saline (PBS), and the cultures were maintained in the presence of the different dilutions of the antiviral drugs. FIV replication was determined after 6 days in the supernatants by an FIV p24 enzyme-linked immunosorbent assay utilizing the monoclonal antibody 11C7 for p24 antigen capture and the biotinylated monoclonal antibody 5E6 for p24 detection (15). The concentration of the antiviral drug required to reduce the antigen production by 50% (compared with that in the untreated control) was defined as the 50% effective concentration (EC_{50}). This was calculated with the statistical computer program NCSS (NCSS, Kaysville, Utah). Cytotoxicity of the drugs was measured with an MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] assay (24) with the use of dimethyl sulfoxide as a lysis buffer for formazan. Cytotoxicity in feline thymocytes was determined by trypan blue exclusion staining. The concentration of the drug reducing the number of viable uninfected cells by 50% (compared with that in the untreated control) was defined as the 50% cytotoxic concentration (CC_{50}). In feline thymocytes, (*R*)-PMPDAP (EC_{50}, 0.07 μM) was almost as active as AZT (EC_{50}, 0.05 μM), whereas PMEA (EC_{50}, 0.5 μM), the prototype of the acyclic nucleoside phosphonates, was less active (Table 1). PMEA was also shown to be the most toxic drug. All nucleoside analogs were less active in CrFK cells, which was most obvious with AZT (EC_{50}, 4.0 μM). Differences in the activity of drug-modifying enzymes are thought to be responsible for the cell-dependent efficacies of the drug (6). (*R*)-PMPDAP had the highest selectivity index (>2,000) in both cell systems. In studies on the effect of nucleoside phosphonates on HIV-1 infection of MT-4 cells (3, 4), (*R*)-PMPDAP emerged as the most active nucleoside phosphonate. Similar efficacies were found in our study with FIV.

On the basis of the in vitro results and its activity against Moloney sarcoma virus infection in mice (3), (*R*)-PMPDAP was chosen for testing in cats and was compared with the parent drug PMEA. Pharmacokinetic studies of (*R*)-PMPDAP were performed in two cats injected subcutaneously with 10 or 50 mg/kg of body weight (Fig. 1). Plasma elimination was similar for both doses used, with total body clearance of 0.13 and 0.1 liter/kg/h, respectively. The half-life in plasma was

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TABLE 1. Antiviral activities of the test compounds[a]

Compound[b]	Avg concn (μM) ± SE in:				Selectivity index[c] in:	
	Thymocytes		CrFK cells			
	EC_{50}	CC_{50}	EC_{50}	CC_{50}	Thymocytes	CrFK cells
PMEA	0.5 ± 0.09	80 ± 7.3	1.5 ± 0.11	90 ± 10.2	160	60
(*R*)-PMPA	0.8 ± 0.11	165 ± 9.1	0.9 ± 0.20	>200	206	>200
(*S*)-FPMPA	1.4 ± 0.21	155 ± 12.8	1.5 ± 0.15	>200	110	>133
(*R*)-PMPDAP	0.07 ± 0.02	190 ± 5.1	0.1 ± 0.03	>200	2,714	>2,000
AZT	0.05 ± 0.02	110 ± 7.7	4.0 ± 0.52	>200	2,200	>50

[a] Indicated are the average values from three independent tests.
[b] (*R*)-PMPA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine; (*S*)-FPMPA, (*S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine.
[c] CC_{50} divided by EC_{50}.

similar to the values previously found for PMEA (12) and in rhesus monkeys (5).

The cats used in this study were clinically healthy and had been experimentally infected with the Dutch isolate FIV UT-48 for 3.5 to 4 years. Cats [four with 20 mg of (*R*)-PMP DAP/kg of body weight, two with 20 mg of PMEA per kg of body weight, and three with PBS] were injected subcutaneously three times a week for 6 weeks. Blood was collected at the beginning of treatment and after 2, 4, 6, and 9 weeks and was used to determine packed cell volume and hemoglobin values. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque gradient centrifugation.

Toxic side effects of anemia were observed only in the PMEA-treated group. Over the 6-week period, average packed cell volumes and hemoglobin values decreased significantly ($P < 0.05$; the Student *t* test) compared with those in the placebo- and (*R*)-PMPDAP-treated groups from 0.31 ± 0.02 liter/liter and 6.3 ± 0.39 mM to 0.15 ± 0.03 liter/liter and 2.9 ± 0.65 mM, respectively. One PMEA-treated cat also showed an increase of alanine aminotransferase activity from 26 to 313 U/liter and lost 300 g of body weight. Hemoglobin values and packed cell volumes had increased again 3 weeks after the treatment to 4.8 ± 0.2 mM and 0.24 ± 0.01 liter/liter, respectively.

Enumeration of CD_4^+ and CD_8^+ lymphocytes was done as described elsewhere (28). The CD_4^+/CD_8^+ ratios of the treated cats were low (0.9 ± 0.27) compared with published ratios (1.2 to 2.6) of healthy uninfected cats (7, 10). However, the CD_4^+/CD_8^+ ratios of the treated cats did not change within the 6-week period. The mean CD_4^+ lymphocyte counts were 217 ± 31 CD_4^+ cells per μl. Lymphocyte stimulation assays were performed by a method slightly modified from that of Barlough et al. (7). PBMC (10^5) were stimulated in triplicate with 5 μg of concanavalin A per ml. The cultures were pulsed after 72 h with 1 μCi of [*methyl*-^{3}H]thymidine (Amersham; 5 Ci/mmol) per well and harvested 18 h later. No increase in responsiveness of PBMC to concanavalin A mitogen stimulation was detected; mean stimulation index values were 55.7 ± 14.5.

Cell-associated virus was quantified by endpoint dilution cultures (18). At 0, 2, 4, 6, and 9 weeks after starting the treatment, serial 10-fold dilutions starting at 10^5 PBMC were

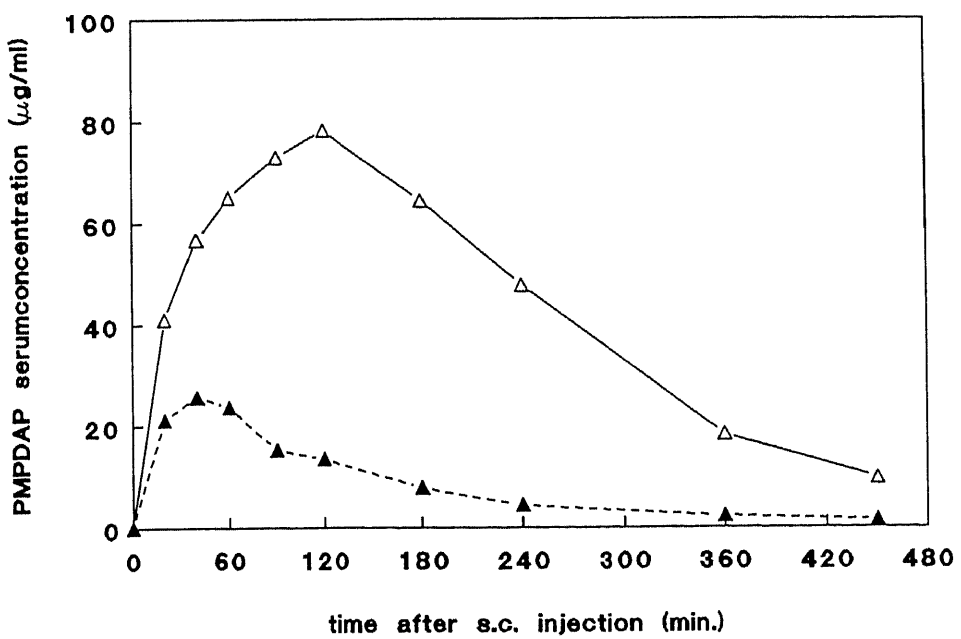

FIG. 1. Pharmacokinetics of (*R*)-PMPDAP determined in two cats following subcutaneous (s.c.) injection of the drug at 10 (▲) and 50 (△) mg/kg of body weight. Concentrations of (*R*)-PMPDAP in serum were determined from blood samples by the chloroacetaldehyde fluorescence method (22).

TABLE 2. Amount of FIV RNA in plasma of infected cats

Cat	Compound	No. of viral RNA copies (10^3)[a]/ml of plasma	
		Before treatment	After treatment
320	PMEA	8.5	7.2
330	PMEA	10	9.9
308	(*R*)-PMPDAP	14	3.2
326	(*R*)-PMPDAP	5.6	6.2
336	(*R*)-PMPDAP	43	3.0
833	(*R*)-PMPDAP	9.2	<1.6
322	Placebo	7.4	6.9
340	Placebo	15	14
831	Placebo	10	8.6

[a] Determined by a quantitative competitive RT-PCR (29).

cocultivated with 10^5 concanavalin A-stimulated thymocytes from specific-pathogen-free cats. Most cultures containing 10^4 PBMC and some containing 10^3 PBMC became p24 positive after 7 to 11 days, numbers which did not change during treatment.

Proviral DNA content in PBMC reflects the number of both productively and latently infected cells in the peripheral blood (past and present status). The viral load present in plasma provides a more actual picture of viral replication. However, titration of plasma from FIV-infected cats on PBMC from specific-pathogen-free cats can lead to diminished cell viability and reduced infection rates (21, 28a). The outcome of infections in vitro may be influenced by neutralizing antibodies, by virus unable to replicate in PBMC, or by interference of antiviral drugs still present in plasma. The changes in plasma viral load were therefore investigated by a quantitative competitive RT-PCR (29), which was developed for FIV similarly to the method described previously for HIV-1 (27). The RT-PCR was performed twice for each sample, resulting in a variation of values of less than 15%. Viral RNA concentrations in plasma ranged between $<1.6 \times 10^3$ and 4.3×10^4 copies per ml (Table 2). The concentration of viral RNA between the treatment groups was not significantly different before therapy. The amount of viral RNA in plasma did not alter in the placebo- or PMEA-treated group, whereas it decreased in three of four (*R*)-PMPDAP-treated cats. The mean plasma RNA concentration in the (*R*)-PMPDAP-treated cats decreased significantly ($P < 0.05$; the Student *t* test) compared with that in the placebo- and PMEA-treated cats.

Our data are in agreement with those found in HIV-infected patients in which PBMC virus titers (18) and levels of HIV proviral copy numbers did not significantly decrease within the first 6 weeks of therapy with AZT (12), AZT and interleukin-2 (8), dideoxyinosine (ddI) (1), or AZT and ddI (19). However, a significant reduction of the virus concentration in plasma could be observed in AIDS patients within 4 weeks of treatment with AZT (27) or ddI (2) and upon combination therapy with AZT and ddI (19).

Toxic side effects of anemia were observed only in the PMEA-treated group, which is in agreement with the relatively low CC_{50}s determined in vitro. PMEA treatment is known to delay the onset of viremia after primary infection (13) as well as to reduce gingivitis and stomatitis symptoms seen in naturally FIV-infected cats (13, 14, 17). This favorable effect may have been due to a more pronounced effect of RT inhibitors in the acute phase of infection, to differences in treatment regimen, and to other reported activities of PMEA. These include the induction of cytokines (11) or inhibition of herpesvirus replication (16); feline herpesvirus is a notorious cause of oral cavity disease in cats.

In conclusion, (*R*)-PMPDAP proves, as compared with PMEA, to be a more potent and less toxic inhibitor of FIV replication both in vitro and in vivo. By the quantitative competitive RT-PCR, asymptomatic FIV-infected cats can be monitored for the effects of antiviral drugs in a short treatment regimen.

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