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Single-dose administration of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) in the prophylaxis of retrovirus infection in vivo

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Summary

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) are selectively inhibitory to human immunodeficiency virus and other retroviruses. We have now investigated the effects of different PMEA and PMEDAP treatment schedules in newborn mice infected with Moloney murine sarcoma virus (MSV). Administration of a single dose of PMEA or PMEDAP on the day of MSV inoculation conferred a greater protective effect against MSV-induced tumor formation than when this dose was divided over two, four or seven injections per week. Also, the therapeutic index of PMEA and PMEDAP was increased if administered as a single dose. Furthermore, PMEA and PMEDAP afforded a marked antiviral protection if administered within one day before MSV infection. Thus, single doses of PMEA or PMEDAP, when administered shortly before or after MSV infection, appear to be effective in preventing the manifestations of the retroviral disease.

Human immunodeficiency virus, HIV; Moloney murine sarcoma virus, MSV; Single dose administration; 9-(2-phosphonylmethoxyethyl)adenine, PMEA; 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine, PMEDAP

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Introduction

The replicative cycle of human immunodeficiency virus (HIV) contains several possible targets for antiviral therapy (De Clercq, 1986). However, most substances that have been used in the treatment of acquired immunodeficiency syndrome (AIDS) belong to the class of the 2′,3′-dideoxynucleoside analogues and act as inhibitors of the viral reverse transcriptase. 3′-Azido-2′,3′-dideoxythymidine (AZT, zidovudine) has been shown to decrease the morbidity and mortality of AIDS patients (Yarchoan et al., 1986; Fischl et al., 1987). Initial clinical studies also point to the anti-HIV efficacy of 2′,3′-dideoxyinosine (DDI), as evidenced by an increase in $CD4^+$ cell counts and decrease in p24 antigen levels (Yarchoan et al., 1989; Cooley et al., 1990; Lambert et al., 1990). The clinical benefits of AZT and DDI are limited by toxic side-effects, i.e. bone-marrow suppression (Richman et al., 1987) and pancreatitis/peripheral neuropathy (Cooley et al., 1990; Lambert et al., 1990), respectively. Moreover, prolonged treatment with AZT has led to the emergence of HIV mutants that show reduced sensitivity to AZT (Larder et al., 1989, 1990). To overcome toxicity problems with AZT and reduce the risk of virus drug-resistance development to AZT, clinical studies are now performed with lower doses of AZT. There is, however, an urgent need for other, more effective and/or less toxic anti-HIV drugs.

We have recently reported on the selective anti-HIV activity of a new class of phosphonylmethoxyalkylpurine derivatives (Pauwels et al., 1988). The lead compound 9-(2-phosphonylmethoxyethyl)adenine (PMEA) has been shown to inhibit the replication of a number of animal retroviruses both in vitro and in vivo, i.e. murine retroviruses [Moloney murine sarcoma virus (MSV; Balzarini et al., 1989, 1990a,b), Friend leukemia virus (FLV; Naesens et al., 1990); LP-BM5 (murine AIDS) virus (Gangemi et al., 1989)]; feline immunodeficiency virus (FIV; Egberink et al., 1990) and simian immunodeficiency virus (SIV; Balzarini et al., 1990c,1991a). Another member of this class of nucleotide analogues, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), has also proven to be a potent inhibitor of HIV replication in vitro, as well as MSV and FLV infection in vivo (Naesens et al., 1989, 1990).

We have now investigated the inhibitory effects of different treatment regimens, including prophylactic single-shot administration of PMEA and PMEDAP, on MSV infection. Such single-shot administration may hold several advantages. Apart from its practicability, it may minimize the risk of toxicity as well as the risk of emergence of drug-resistant virus strains.

Materials and Methods

Compounds

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmeth-

oxyethyl)-2,6-diaminopurine (PMEDAP) were kindly provided by Dr. A. Holý and Dr. I. Rosenberg. Their synthesis has been described previously (Holý and Rosenberg, 1987).

Virus

MSV stocks were prepared from tumors induced by MSV infection of 3-day-old NMRI mice (De Clercq and Merigan, 1971).

Inhibitory effect of PMEA and PMEDAP on MSV-induced transformation of murine C3H/3T3 embryo fibroblast cells

In vitro activity against MSV was assessed as described previously (Balzarini et al., 1987, 1989). In brief, murine C3H/3T3 embryo fibroblast cells were seeded at 5×10^5 cells per ml into 1-cm^2 wells of a 48-well microplate and incubated at 37°C. In the preincubation experiments, cell culture medium was removed at either 40, 24 or 16 h before MSV infection and replaced by medium containing various concentrations of PMEA or PMEDAP. At the time of virus infection, the cell culture medium containing the test compounds was removed and the cell cultures were infected with 80 focus-forming units of MSV for 60–90 min at 37°C. After removal of the virus, fresh medium was added and cells were further incubated at 37°C for six days. In another set of experiments, PMEA and PMEDAP were added after the virus had been removed from the cell cultures and remained in contact with the cells for six days. At that time, transformation of the cells was examined microscopically. The EC_{50} was defined as the compound concentration that inhibited MSV-induced cell transformation by 50%.

Effect of different treatment schedules of PMEA and PMEDAP in newborn NMRI mice infected with MSV

Three-day-old NMRI mice (weighing $\pm$ 2 g) were inoculated intramuscularly in the left hind leg with 50 μl of an MSV suspension containing 100 focus-forming units (as determined on the basis of MSV-induced transformation of murine C3H/3T3 embryo fibroblast cells). On day 4–5 after infection, tumors appeared and mice died within ten to twelve days after virus infection. PMEA was administered in total doses of 100, 50, 25 or 12.5 mg/kg, whereas PMEDAP was administered in total doses of 35, 8.75 or 3.5 mg/kg. The total doses were given intraperitoneally, as a single injection on day 0 (3 h prior to virus inoculation), or spread over two injections (on days 0 and 3), or over four injections (on days 0, 2, 4 and 6) or over seven injections (on days 0, 1, 2, 3, 4, 5 and 6). Tumor appearance and animal death was recorded daily until day 20. Data shown are the results of two individual experiments, performed with ten to twelve mice per treatment group. In another set of experiments, PMEA and PMEDAP were administered intraperitoneally as a single dose at 50, 42, 27, 19

or 3 h before virus inoculation. The doses used were 50 or 20 mg/kg for PMEA, and 20 or 10 mg/kg for PMEDAP.

Results

Preincubation of C3H/3T3 cells with PMEA and PMEDAP before infection with MSV

Murine C3H/3T3 cells were treated with PMEA or PMEDAP for varying times before the cells were infected with MSV. PMEA and PMEDAP were almost equally effective against MSV-induced cell transformation, whether they were exposed to the cells for 40, 24 or 16 h before MSV infection or added to the cell cultures immediately after virus infection (EC_{50} = 7.8–8.4 μM and 1.3–4.4 μM, respectively; Table 1). When PMEA and PMEDAP were exposed to the cells before MSV infection, no visible toxicity was noted [minimal cytotoxic concentration (MCC) > 100 μM]. When PMEA and PMEDAP were exposed to the cell cultures for six days starting immediately after virus infection, their MCCs were 100 μM and 50 μM, respectively (data not shown).

Different treatment schedules of PMEA and PMEDAP in newborn NMRI mice infected with MSV

MSV-infected NMRI mice were subjected to different treatment schedules of PMEA or PMEDAP. The total doses that were administered ranged from 12.5 to 100 mg/kg for PMEA, and from 3.5 to 35 mg/kg for PMEDAP. The inhibitory effect of PMEA and PMEDAP on MSV-induced tumor formation appeared highly dependent on the total dose and frequency of administration. A single administration of PMEA at 100 mg/kg on day 0 (3 h before virus inoculation) completely suppressed MSV-induced tumor formation; none of the mice of this group showed tumor development within the 20-day observation period. Following treatment with a single dose of 50, 25 or 12.5 mg PMEA per kg, respectively 18%, 25% or 71% of the mice showed tumor development within a mean period of 14, 16 or 15 days (data not shown). Under the same experimental conditions, 100% of the control mice developed

TABLE 1

Anti-MSV activity (EC_{50}; μM) of PMEA and PMEDAP in murine C3H/3T3 embryo fibroblasts following different preincubation times of the cells with the compounds

Compounds	Preincubation time (h)			
	40	24	16	0[a]
PMEA	8.4	8.4	8.4	7.8
PMEDAP	2.8	3.7	4.3	1.3

[a]Compounds added immediately after virus infection.

tumors, and the mean period of tumor initiation for this group was 4.9 days. When the total PMEA dose was spread over two, four or seven administrations, its anti-MSV efficacy significantly decreased, the decrease being more pronounced the more the PMEA dose was fractionated. For example, when given a single PMEA dose of 50 mg/kg, only 18% of the MSV-infected mice developed a tumor (mean period of tumor initiation: 14 days). However, when this PMEA dose was spread over two (days 0 and 3), four (days 0, 2, 4 and 6) or seven daily administrations, 20%, 73% or 100% of the mice developed a tumor, the mean periods of tumor initiation being 23.5, 14.6 and 10.7 days, respectively (data not shown).

Similar observations were made with PMEDAP (Table 2). With PMEDAP at a dose of 8.75 mg/kg, no tumors appeared in 67% of the mice following a single administration schedule, whereas the same dose divided over seven daily injections protected only 20% of the mice against tumor formation. A single dose of 3.5 mg/kg on day 0 delayed the mean day of tumor initiation from 4.7 days (untreated control mice) to 11.2 days, and 86% of the mice developed a tumor within 20 days. When the total dose of 3.5 mg/kg of PMEDAP was spread over two, four or seven daily injections, the anti-MSV efficacy decreased: all mice now developed a tumor, and mean tumor initiation was at 9.5, 7.3 and 7.2 days, respectively.

We also determined the protective effect of different treatment schedules of PMEDAP on death associated with MSV-induced tumor formation (Table 2). Again, the protective effect of PMEDAP was less pronounced when a more frequent treatment schedule was used. For example, single administration of PMEDAP at a dose of 8.75 mg/kg resulted in 90% survivors; this survival rate

TABLE 2

Inhibitory effect of different treatment schedules of PMEDAP on MSV-induced tumor formation and associated death in newborn NMRI mice

Total dose of PMEDAP (mg/kg)	Time (days) at which compound was administered	Mean day of tumor initiation	Mean day of animal death
35	0	15.2 (20%)[a]	19.0 (8%)[b]
8.75	0	12.9 (33%)	17.5 (10%)
3.5	0	11.2 (86%)	15.6 (57%)
35	0,3	12.8 (47%)	17.2 (30%)
8.75	0,3	11.6 (38%)	16.4 (24%)
3.5	0,3	9.5 (100%)	14.0 (55%)
35	0,2,4,6	14.7 (39%)	15.4 (25%)
8.75	0,2,4,6	9.3 (50%)	14.2 (40%)
3.5	0,2,4,6	7.3 (100%)	13.4 (95%)
35	0,1,2,3,4,5,6	16.7 (29%)	18.2 (25%)
8.75	0,1,2,3,4,5,6	10.3 (80%)	15.8 (65%)
3.5	0,1,2,3,4,5,6	7.2 (100%)	13.1 (90%)
0	0	4.7 (100%)	10.7 (100%)

[a] In parentheses, percentage of mice developing tumor.
[b] In parentheses, percentage of dead mice by day 20.

decreased to 76, 60 and 35%, when the total dose of 8.75 mg/kg was spread over two, four or seven injections, respectively.

Prophylactic administration of PMEA or PMEDAP in newborn NMRI mice infected with MSV

Newborn mice were treated with a single dose of PMEA or PMEDAP at various times before the mice were infected with MSV. Administration of PMEA at 50 mg/kg or PMEDAP at 20 mg/kg at 19 h before MSV infection led

TABLE 3

Inhibitory effect of prophylactic administration of PMEA or PMEDAP on MSV-induced tumor formation and associated death in newborn NMRI mice

Compound/dose (mg/kg)	Time (h) at which compound was administered prior to MSV infection	Mean day of:	
		Tumor initiation	Animal death
PMEA			
50	50	6.6 (100%)[a]	13.5 (90%)[b]
20	50	5.0 (100%)	11.7 (100%)
PMEDAP			
20	50	6.7 (100%)	12.5 (100%)
10	50	5.5 (100%)	11.1 (100%)
PMEA			
50	42	7.6 (95%)	13.7 (86%)
20	42	5.3 (100%)	11.5 (100%)
PMEDAP			
20	42	6.9 (100%)	13.2 (100%)
10	42	5.6 (100%)	11.5 (100%)
PMEA			
50	27	8.7 (70%)	14.0 (50%)
20	27	9.2 (76%)	14.5 (48%)
PMEDAP			
20	27	10.6 (86%)	14.9 (52%)
10	27	10.6 (80%)	13.5 (20%)
PMEA			
50	19	10.6 (65%)	14.3 (30%)
20	19	11.5 (52%)	15.2 (19%)
PMEDAP			
20	19	12.8 (68%)	15.8 (38%)
10	19	9.6 (62%)	18.0 (12%)
PMEA			
50	3	10.5 (11%)	17.0 (6%)
20	3	14.2 (38%)	16.0 (5%)
PMEDAP			
20	3	13.0 (18%)	16.0 (6%)
10	3	13.8 (36%)	17.0 (18%)
Control	3	4.6 (100%)	11.2 (100%)

[a] In parentheses, percentage of mice developing tumor.
[b] In parentheses, percentage of dead mice by day 20.

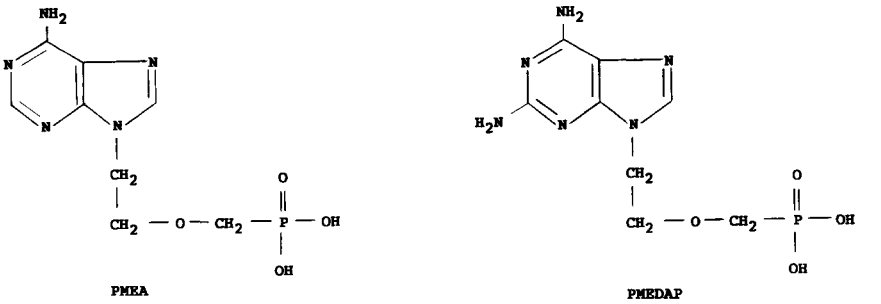

Fig. 1. Structural formulae of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP).

to a marked inhibition of MSV-induced tumor formation (mean period of tumor initiation: 10.6 and 12.8 days, respectively, compared with 4.6 days for the untreated control group; Table 3). The percentages of mice that did not show tumor development within 20 days were 35% and 32%, respectively, compared with 0% for the untreated control group. Even if PMEA (50 mg/kg) or PMEDAP (20 mg/kg) were administered as early as 50 h before infection, there was a marked increase in the mean period of tumor initiation (6.6 and 6.7 days, compared with the untreated control group's 4.6 days; $P < 0.0005$, Student's t test). Thus, although PMEA and PMEDAP became less effective in suppressing MSV-induced tumor formation with increasing time between administration of the test compounds and MSV infection, they exhibited marked anti-retrovirus activity if administered (as single doses) at one or two days before virus infection.

Also, the protective effect of prophylactic administration of PMEA and PMEDAP on death associated with MSV-induced tumor formation was determined (Table 3). Again, PMEA and PMEDAP were more protective the shorter the time between PMEA or PMEDAP administration and MSV infection. When administered at 27 h before MSV infection, PMEA at 50 mg/kg and PMEDAP at 20 mg/kg significantly delayed death of the mice (means 14.0 and 14.9 days, respectively, compared with 11.2 days for the untreated control group). This prophylactic drug regimen also resulted in a markedly increased survival rate (percentage of survivors on day 20: 50% and 48%, respectively, compared with 0% for the untreated control group).

Discussion

From the clinical studies with AZT and DDI, it appears that prolonged administration will be necessary for any anti-retrovirus agent to be effective in the treatment of AIDS. To meet the requirements of long-term treatment, potential anti-HIV drugs should achieve a long-acting antiviral response, together with minimal toxicity. The recent report of Cooley et al. (1990) on the

prominent anti-HIV activity and low toxicity of DDI upon once daily administration in AIDS or ARC patients suggests that infrequent administration schedules may be compatible with therapeutic effectiveness in the treatment of retrovirus infections.

We have examined the anti-retrovirus activity of PMEA upon infrequent dosing, to assess whether less frequent administration via the parenteral route would compensate for the low oral bioavailability of these phosphonate derivatives (Balzarini et al., 1990b). We found that infrequent dosing of PMEA led to a marked increase in antiretroviral activity and higher therapeutic selectivity. In this paper, we demonstrate that the increased anti-MSV activity of PMEA upon infrequent administration also extends to other acyclic nucleoside phosphonate derivatives (i.e. PMEDAP). It should be noted that no such phenomenon is observed when 3′-azido-2′,3′-dideoxythymidine (AZT) is administered under similar experimental conditions. AZT did not show increased antiviral efficacy when the frequency of administration was reduced (Balzarini et al., 1990b).

Toxicity experiments in newborn NMRI mice indicated that, for both PMEA and PMEDAP, the degree of toxicity is not influenced by the treatment schedule. Indeed, we found that PMEA at a total dose of 350 mg/kg, or PMEDAP at a total dose of 140 mg/kg, were equally toxic to newborn mice with the four treatment schedules used (one, two, four or seven injections; data not shown). For example, at 350 mg/kg PMEA proved lethal to about 75% of the mice, irrespective of the treatment schedule. At a total dose of 175 mg PMEA/kg, the compound did not cause any casualty, irrespective of whether it had been given as a single dose or fractionated into repeated doses (Balzarini et al., 1990b). Since administration of PMEA and PMEDAP as a single dose markedly increases anti-MSV activity without increasing toxicity in newborn mice, PMEA and PMEDAP thus become therapeutically more selective following reducing the frequency of their administration.

The inhibitory effect of PMEA (and PMEDAP) on MSV-induced tumor initiation and associated mortality cannot be attributed to a direct antitumor action of the compound (Balzarini et al., 1989; Naesens et al., 1989). Indeed, when administered seven days after MSV inoculation, PMEA had no substantial effect on the further tumor development or life-span of the mice and no tumor shrinkage was observed.

Recent studies point to the clinical benefit of the prophylactic use of AZT in asymptomatic HIV carriers (Volberding et al., 1990). Whether AZT is also effective when administered immediately after HIV exposure (Lange et al., 1990; Puro and Ippolito, 1990) is as yet unclear. However, high doses of an antiviral agent administered during a relatively short time at an early stage after viral exposure may be expected to significantly reduce the total virus load. Our present findings point to the marked anti-MSV efficacy of PMEA and PMEDAP when administered as a single prophylactic dose to newborn mice infected with MSV. This suggests that PMEA and PMEDAP may be particularly useful in post-exposure prophylaxis of retrovirus infections.

Anti-MSV activity in our prophylaxis studies was observed with doses of 20–50 mg/kg (PMEA) or 10–20 mg/kg (PMEDAP). These doses are at least ten times lower than the doses causing toxicity in newborn mice, i.e. 350 mg/kg (PMEA) and 140 mg/kg (PMEDAP). Thus, complete suppression of retrovirus infection at an early stage after viral exposure may be achieved at drug doses that are far below the toxicity threshold.

An interesting question arising from our findings pertains to the mechanism that is responsible for the marked antiviral activity of PMEA and PMEDAP following a single prophylactic administration. PMEA and PMEDAP need to be converted intracellularly to their diphosphorylated form (i.e. PMEApp and PMEDAPpp), before they can act as inhibitors of the viral reverse transcriptase (Balzarini et al., 1991b). PMEApp and PMEDAPpp have long intracellular half-lives (18 h in the case of PMEApp), and, therefore, intracellular accumulation of these active metabolites may be held responsible for the sustained antiviral activity of PMEA and PMEDAP in vivo.

The reason for the decreasing antiviral efficiency of PMEA at a more frequent treatment schedule is not clear. Possibly, high-peak levels of the antivirally active diphosphorylated PMEA metabolite (i.e. PMEApp) may be more important than continuous low-peak levels of PMEApp in the eventual expression of the antiretroviral activity of PMEA. In this respect, PMEA clearly differs from AZT (Balzarini et al., 1990b), an observation that may suggest that the dose-response relationship of these drugs in their interaction with their retroviral target (i.e. reverse transcriptase) may be different. Additional experiments are required to elucidate the molecular basis of this phenomenon.

The marked anti-MSV effect of PMEA and PMEDAP, which has been noted in vitro upon pretreatment of murine fibroblast cells as early as 40 h before MSV-infection, may also be explained by the intracellular accumulation of the active metabolites of PMEA and PMEDAP. Prolonged antiviral protection, both in vitro and in vivo, has also been observed with (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), a potent anti-CMV agent that is structurally related to PMEA and PMEDAP (Kern and Vogt, 1989; Neyts et al., 1990). HPMPCpp has also been reported to have a long intracellular half-life (18 h; Hitchcock et al., 1990). Thus, intracellular accumulation of the active metabolite, resulting in prolonged antiviral activity, may therefore be considered as a general feature of the acyclic nucleoside phosphonate analogues.

In conclusion, our findings on the marked antiviral activity of PMEA and PMEDAP upon administration of a single prophylactic dose in MSV-infected mice add a new dimension to the clinical use of PMEA and PMEDAP in the therapy/prophylaxis of retroviral diseases. According to our data, PMEA (or PMEDAP) should be considered for prophylactic use in the control of retrovirus infections, which means that they could be administered shortly after exposure to the virus or during the asymptomatic carrier stage.

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