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Short communication

In vitro characterization of the anti-human cytomegalovirus activity of PMEA (Adefovir)

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Abstract

PMEA {9-[2-(phosphonomethoxy)ethyl]adenine; adefovir} has shown anti-cytomegalovirus activity in animal models and in preliminary human trials. PMEA diphosphate (PMEApp), the active antiviral metabolite of PMEA, is a potent inhibitor of human cytomegalovirus (HCMV) DNA polymerase. PMEA is efficiently taken up and phosphorylated to PMEApp in numerous human cell lines. In vitro replication of wild type and drug resistant HCMV clinical isolates is effectively inhibited by PMEA. PMEA in combination with other anti-HCMV agents shows additive inhibition of HCMV replication. © 1997 Elsevier Science B.V.

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1. Introduction

PMEA is a potent inhibitor of retrovirus, hepadnavirus and herpesvirus replication in vitro and in vivo (De Clercq, 1994; De Clercq et al., 1986, 1987; Pauwels et al., 1988). Adefovir dipivoxil (the oral prodrug of PMEA) is currently under clinical evaluation for the treatment of diseases caused by human immunodeficiency virus (HIV), hepatitis B virus (HBV) and human cytomegalovirus (HCMV). Preliminary data from a phase I/II clinical study of adefovir dipivoxil for the treatment of HIV infection, showed that this therapy also demonstrated anti-HCMV activity (decrease in the amount of HCMV DNA in the semen, measured by bDNA assay) in this patient population (Deeks et al., 1997). Based on these data, the anti-HCMV activity of adefovir dipivoxil is being actively investigated in two ongoing adefovir dipivoxil phase II/III HIV clinical trials.

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Table 1
Kinetic constants of PMEApp for HCMV DNA polymerase[a]

Substrate	K_m (μM)	V_{max} (pmol/min/U)	V_{max}/K_m (pmol/min/U/μM)	Catalytic efficiency[b]
dATP	0.9 ± 0.3	0.1 ± 0.02	0.1	1
PMEApp	5.7 ± 0.9	0.2 ± 0.02	0.03	0.2

[a] All kinetic constants are the average of at least three separate experiments. K_m and V_{max} values are means $\pm$ S.D.
[b] Catalytic efficiency equals (V_{max}/K_m analog)/(V_{max}/K_m normal substrate).

HCMV encoded DNA polymerase is the target of all three compounds approved for the treatment of HCMV retinitis in AIDS patients: cidofovir, ganciclovir and foscarnet. Foster et al. (1991) demonstrated that herpes simplex virus (HSV) DNA polymerase is a target for the anti-HSV action of PMEApp. Accordingly, the HCMV encoded DNA polymerase is likely to be the target for the anti-HCMV action of PMEA.

PMEA differs from GCV in two important respects. First, like the related compound cidofovir, PMEA is a nucleotide monophosphate analog. Therefore, unlike ganciclovir, which requires initial phosphorylation by the phosphotransferase encoded by the viral UL97 gene, phosphorylation of PMEA is carried out by cellular kinases (Biron et al., 1985; Smee et al., 1985; Balzarini and De Clercq, 1991; Robbins et al., 1995). This initial phosphorylation can limit the effectiveness of nucleoside antivirals in multiple cell types and can also be a site of resistance development (Lurain et al., 1992; Sullivan et al., 1992; Metzger et al., 1994). Second, PMEA is an obligate DNA chain terminator when incorporated into DNA. In contrast, ganciclovir triphosphate and cidofovir diphosphate each require incorporation of more than one molecule to cause termination of viral DNA synthesis (Reid et al., 1988; Xiong et al., 1997).

Several approaches were undertaken to further characterize the activity of PMEA versus HCMV. Enzymatic experiments were performed to determine the mechanisms of inhibition of HCMV DNA polymerase by PMEApp. The metabolism of PMEA was measured in numerous cell lines that are representative of cell and tissue types infected with HCMV in vivo. The in vitro antiviral activity of PMEA against wild type and drug resistant HCMV clinical isolates was evaluated, as was the activity of PMEA in combination with other anti-HCMV agents.

Using previously described methods (Xiong et al., 1996), PMEApp was found to be a potent competitive inhibitor of HCMV DNA polymerase with respect to the natural substrate dATP with an inhibition constant (K_i) of 0.45 μM. The K_i value of PMEApp is more than 10 times lower than the K_i value (6.6 μM) of cidofovir diphosphate (CDVpp) (Xiong et al., 1996) and similar to that of ganciclovir triphosphate (GCVTP) which has reported K_i values of 0.02–1.7 μM for HCMV DNA polymerase (Biron et al., 1985; Mar et al., 1985; Duke et al., 1986).

To further explore the mechanisms of inhibition, the kinetic constants (K_m and V_{max}) for HCMV DNA polymerase-catalyzed incorporation of PMEApp and dATP were measured using a gel based primer extension method (Xiong et al., 1996). The primer SX1 (5′ TGA-CCA-TGT-AAC-AGA-GAG 3′) was 5′ end labeled with [γ-^{32}P] and annealed to the template (3′ ACT-GGT-ACA-TTG-TCT-CTC-TCA-ACA-CAG-CCA-ACA-CAG-CAA-CAC-GCA-CAA-CGA 5′), allowing for the measurement of the incorporation of a single dATP or PMEApp molecule in a standing start primer-template system. As shown in Table 1, the K_m value for PMEApp is 5.7 μM, lower than that of GCVTP (14.2 μM; Xiong and Chen, 1996) and CDVpp (18.7 μM; Xiong et al., 1996). These data indicate that PMEApp has higher affinity for HCMV DNA polymerase than does GCVTP or CDVpp; the K_m values of HCMV DNA polymerase for dATP, dCTP and dGTP are comparable to each other (Xiong and Chen, 1996; Xiong et al., 1996). HCMV DNA polymerase is able to incorporate

Table 2
Intracellular concentration of PMEA and cidofovir metabolites in different cell lines

Cell line	Total PMEA[b] (pmol/10^6 cells)	PMEApp (pmol/10^6 cells)	Total CDV[c] (pmol/10^6 cells)	CDVpp (pmol/10^6 cells)
Caco-2[a]	5.2	0.5	3.8	0.4
MRC-5[a]	3.4	1.1	1.1	0.2
NHDF[a]	12.5	4.7	1.1	0.2
NHBE[a]	2.2	0.7	1.7	0.4
NHEK[a]	4.2	0.6	1.5	0.6
RPTEC[a]	0.8	0.3	0.4	0.1
MRC-5[d]	n.d.	n.d.	3.1	0.7
A3.01[d]	4.0	1.0	0.6	0.1

[a] Cells were labeled with 5 μM [^{3}H]PMEA or [^{3}H]CDV for 24 h.
[b] Total intracellular concentration of PMEA, PMEAp and PMEApp.
[c] Total intracellular concentration of CDV, CDVp, CDVpp and CDVp-choline.
[d] Cells were labeled with 10 μM [^{3}H]PMEA or [^{3}H]CDV for 24 h.
n.d., not determined.

PMEApp with 18% the efficiency of dATP (Table 1). In contrast, incorporation of GCVTP and CDVpp by HCMV DNA polymerase proceeds with only 2–3% the efficiency of their corresponding natural substrates (Xiong and Chen, 1996; Xiong et al., 1996).

The K_i values of PMEApp for the human DNA polymerases α, β, and γ as determined in previous experiments are 1, 70 and 1 μM, respectively (Cherrington et al., 1995). The K_i value of GCVTP for DNA polymerase α was reported to be 2.5–17 μM (St. Clair et al., 1984; Duke et al., 1986), similar to the K_i value of GCVTP for HCMV DNA polymerase. The specificity of the inhibition for HCMV DNA polymerase compared to the mammalian enzymes as measured by the selectivity indices (CC_{50}/IC_{50}) of PMEA and GCV in tissue culture experiments are 40 and >20, respectively (Mulato et al., 1996). Furthermore, in phase I/II clinical trials adefovir dipivoxil has been very well tolerated, with minimal gastrointestinal side effects (Barditch-Crovo et al., 1997; Deeks et al., 1997).

Since HCMV causes a myriad of end organ diseases, the metabolism of PMEA as well as the accumulation of the active metabolites in the potential target cells is an important consideration. The cell lines investigated here were chosen in an attempt to mimic in vitro the cell and tissue types HCMV expected to infected by HCMV in vivo (Alford and Britt, 1990). Therefore, the metabolism of PMEA was investigated in human cell lines derived from organs (lung, kidney, colon, and skin) which are common sites of HCMV disease, and in fibroblasts (permissive for HCMV replication in vitro) and a human T cell line. MRC-5 human embryonic lung fibroblast cells and Caco-2 human colorectal carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Normal human bronchial epithelial cells (NHBE), normal human dermal fibroblast cells (NHDF), renal proximal tubule epithelial cells (RPTEC), and normal human epidermal keratinocytes (NHEK) were from Clonetics (San Diego, CA). A3.01 cells, a human T-cell derivative of CEM cells, were a gift from Thomas Folks (National Cancer Institute). All cell lines were cultured as suggested by the manufacturer; A3.01 cells were grown in RPMI plus 10% fetal bovine serum.

The intracellular concentrations of PMEA and its phosphorylated metabolites were measured in the seven cell lines according to previously described procedures (Chen et al., 1992; Bischofberger et al., 1994). As shown in Table 2, PMEA was efficiently taken up and converted to PMEApp in all six fibroblast and epithelial cell lines under confluent growth conditions. Similar

results were obtained under proliferating growth conditions and at 48 h of incubation rather than 24 h (data not shown). PMEA was also efficiently taken up and metabolized to PMEApp in the human T cell line, A3.01. While the C_{max} of PMEA has been measured to be approximately 0.5–2 μM in vivo (Barditch-Crovo et al., 1997), a more relevant determinant of antiviral activity may be the intracellular concentration of the diphosphate, which is between 0.3 and 4.7 μM in the cell lines investigated in vitro (Table 2). These in vitro derived values are equivalent to or above the K_i value of PMEApp for HCMV DNA polymerase. Additionally, the intracellular half life of PMEApp is 18 h, which may contribute to its long duration of action in vivo (Balzarini et al., 1991).

For comparison, metabolism experiments were also performed with cidofovir, which is an approved therapy for the treatment of HCMV retinitis and like PMEA, is a nucleotide analog. Cidofovir was taken up and phosphorylated efficiently by all of the fibroblast and epithelial cell lines tested but was taken up approximately 5-fold less efficiently in A3.01 cells as compared to human MRC-5 fibroblasts. Srinivas et al. (1997) also recently reported that cidofovir was taken up 3 to 10-fold less efficiently by MT2 cells (a T cell line) compared to HeLa CD4+ cells (an epithelial cell line). Experiments to quantitate the metabolites of GCV were not performed here since the initial phosphorylation of GCV is dependent on a virally encoded enzyme and therefore the amount of GCVTP in uninfected cells is minimal (Biron et al., 1985).

The in vitro antiviral activity of PMEA was determined for HCMV laboratory strains and clinical isolates using a previously described plaque assay in NHDF cells (Lurain et al., 1992; Mulato et al., 1996). The Towne and AD169 strains of HCMV were purchased from ATCC. Clinical isolates were obtained from the Mt. Zion Medical Center, University of California, San Francisco (gift from L. Drew) and from the University of California, San Diego (gift from I. Smith and S. Spector). In vitro wild type antiviral susceptibility values were set at <2, <8.5 and <324 μM for cidofovir, GCV and PFA, respectively, as previously described (Smith et al., 1997). Results shown in Table 3 demonstrate that PMEA had a mean IC_{50} value of 70 μM for the laboratory strains and wild type clinical isolates. These IC_{50} values are lower than for foscarnet but higher than for cidofovir and ganciclovir. Low level ganciclovir resistant viruses remained susceptible to PMEA. The triple drug resistant viruses had markedly increased IC_{50} values for ganciclovir, foscarnet and cidofovir, while PMEA showed no significant change in IC_{50} values for these drug resistant viruses. There was however, a modest increase (<3-fold) in IC_{50} value for PMEA versus the two foscarnet only resistant viruses. This finding is in agreement with that of Snoeck et al. (1996) who showed that foscarnet resistant HCMV selected in vitro showed a slightly reduced susceptibility (4-fold) to PMEA.

Since therapy for the treatment of HCMV retinitis may involve drug combinations, the in vitro activity of PMEA in combination with ganciclovir and foscarnet was determined according to described methods (Mulato et al., 1996). The data from these experiments was analyzed by the MacSynergy program (Prichard et al., 1993) as previously published (Mulato et al., 1996). As defined by the MacSynergy program, in vitro synergy or antagonism values >25 $\mu M^2\%$ may potentially be significant in vivo; previous work has shown a close agreement between synergy results obtained using the MacSynergy program and the isobologram method (Mulato et al., 1996). In past work, PMEA and cidofovir in combination showed additive rather than synergistic inhibition of HCMV replication in vitro (Mulato et al., 1996). The data shown in Table 4 demonstrate that PMEA shows additive rather than synergistic inhibition of HCMV replication in vitro in combination with ganciclovir or foscarnet. Importantly, no significant antiviral antagonism was measured for any of the combinations (Table 4). Also, none of the combinations showed increased cytotoxicity in comparison with each drug alone (data not shown).

PMEApp is a potent competitive inhibitor of HCMV DNA polymerase, and is efficiently incorporated into DNA by this enzyme, functioning as an obligate chain terminator of viral DNA syn-

Table 3
Antiviral effects of PMEA, cidofovir, ganciclovir and foscarnet against HCMV laboratory strains and clinical isolates[a]

	HCMV virus	IC_{50} (μM)[b]			
		PMEA	Cidofovir	Ganciclovir	Foscarnet
Laboratory strains	AD169	60	0.5	3	100
	Towne	70	0.4	3.2	80
Wild type clinical isolates[c]					
	1	80	0.8	4	100
	2	93	1	3	130
	3	50	1.5	2	110
	4	65	0.8	4	85
	5	70	1.5	3	120
Drug resistant clinical isolates[c]					
Ganciclovir resistant	6	65	0.5	15	132
	7	30	0.4	17	190
Ganciclovir, cidofovir and foscarnet resistant	8	40	8	90	450
	9	50	15	100	600
	10	65	5	44	434
Foscarnet resistant	11	120	1	3	600
	12	180	0.8	4	650

[a] Antiviral susceptibilities were performed as previously described (Lurain et al., 1992; Mulato et al., 1996).
[b] IC_{50} values are the mean of greater than two separate determinations. The standard deviations were in no case $>20\%$ of the mean.
[c] Clinical isolates were obtained pretherapy or after ganciclovir and/or foscarnet therapy.

thesis. PMEA is efficiently metabolized in numerous human cell lines, including those that represent sites of end organ HCMV infection in vivo and in T cells. The anti-HCMV activity of PMEA in tissue culture is less than that of cidofovir and ganciclovir but greater than that of foscarnet. PMEA retains full activity against the majority of drug resistant HCMV clinical isolates tested. PMEA has well documented anti-CMV activity in animal models also (DeCastro et al., 1991; Neyts et al., 1993). Clearly, a single, well tolerated agent that demonstrates both antiretroviral and antiherpesvirus activity would be of significant clinical benefit. Importantly, preliminary data from a phase I/II clinical trial investigating the use of adefovir dipivoxil for the treatment of HIV, showed anti-HCMV activity in these patients (Deeks et al., 1997). Based on these results and the in vitro properties described here, adefovir dipivoxil is currently undergoing clinical evaluation for the potential treatment of diseases caused by HCMV as well as HIV and HBV.

Table 4
Volumes of antiviral synergy and antagonism for drug combinations

Drug combination	Cell type	Volume ($\mu M^2\%$)[a]	
		Synergy	Antagonism
PMEA + GCV	NHDF	11 ± 5.5	16 ± 5
PMEA + PFA	NHDF	6.6 ± 3.8	9 ± 2

[a]Volumes of synergy and antagonism were computed by the MacSynergy II program (Prichard et al., 1993): values <25 $\mu M^2\%$ indicate insignificant synergy or antagonism.
Means and standard deviations are shown from at least two independent experiments.

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