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Susceptibility of Human Immunodeficiency Virus Type 1 Replication In Vitro to Acyclic Adenosine Analogs and Synergy of the Analogs with 3′-Azido-3′-Deoxythymidine

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The replication of human immunodeficiency virus in vitro is inhibited by some acyclic adenosine derivatives, such as 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine [(*S*)-HPMPDAP], as well as by 3′-azido-3′-deoxythymidine (AZT). In a human T-lymphocyte cell line, C3, at 6 days postinfection, the 50% effective concentration (EC_{50}) of AZT was 0.02 μM and the 90% effective concentration (EC_{90}) was 0.33 μM; for PMEA, the EC_{50} was 1.9 μM and the EC_{90} was 27 μM. For (*S*)-HPMPDAP, the EC_{50} was 2.3 μM and the EC_{90} was 36 μM. Most combinations of AZT and PMEA produced a synergistic effect. In the T-cell line C3, the combination indices for 50 to 90% inhibition of virus replication ranged from 0.25 to 1.25. Combinations of PMEA (or other members of this group) with AZT appear to be worth further study for the possible treatment of acquired immunodeficiency syndrome.

The search for effective therapy for acquired immunodeficiency syndrome (AIDS) has uncovered various classes of compounds which show activity in vitro against human immunodeficiency virus type 1 (HIV-1), the causative agent. Among these are suramin (18), nucleoside analogs (17, 19), pyrophosphate analogs (28), ribavirin (16), alpha interferon (10), sulfated polysaccharides (11, 23), inhibitors of glycoprotein processing (9, 30), CD4-binding competitors (8), antisense or other oligonucleotides (15, 20, 33), and glycyrrhizin (12). Among the nucleoside analogs, a new class, phosphonylmethoxyalkylpurine and pyrimidine derivatives, has shown activity against herpesviruses and other DNA viruses (5, 6, 14), as well as in vitro activity against transformation of murine embryo fibroblasts by the Moloney murine sarcoma virus and against HIV infection of MT4 cells (25).

Clinical trials with 3′-azido-3′-deoxythymidine (AZT) in patients with AIDS show beneficial results in some clinical parameters and in decreasing or delaying mortality (7); however, AZT toxicity develops in a sizable portion of the treated population (27). Combinations of drugs which would result in an equivalent antiviral effect while reducing overall toxicity would be of great value. We confirm here that some of the acyclic adenosine derivatives inhibit HIV, and we demonstrate that the combination of 9-(2-phosphonylmethoxyethyl) adenine (PMEA) with AZT results in a synergistic effect.

(Preliminary results of parts of this work were presented at the Second International Conference on Antiviral Research, 10 to 14 April 1988, Williamsburg, Va.)

MATERIALS AND METHODS

Virus and cells. The LAV strain of HIV was obtained from L. Montagnier, and stocks were prepared in H9 or C3 cells. The human T-cell line H9 was grown in RPMI 1640 with 8% fetal calf serum. C3, a human T-lymphocyte line harboring latent human T-cell lymphotropic virus type II (26) obtained from William M. Mitchell, was grown in the same medium. Cells were infected for drug experiments with 600 50% tissue culture infective doses of the LAV strain in the presence of 2 μg of DEAE-dextran per ml for 1 h and then washed twice in medium to remove excess virus. After this washing, aliquots of 5×10^4 cells were set up in the presence or absence of drugs in 48-well plates. For C3 cells, samples of supernatant medium were taken at day 6 or 7, when the positive control was at its peak. H9 cultures in drug experiments were sampled on day 6 or 7.

Test compounds. The phosphonylmethoxyalkylpurines and pyrimidines were obtained from A. Holý and I. Rosenberg, from the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. The structures are shown in Fig. 1. The compounds and their abbreviations were as follows: 9-(2-phosphonylmethoxyethyl)adenine (PMEA), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine [(*S*)-HPMPDAP], (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC], cyclic phosphonate of (*S*)-HPMPA [(*S*)-cHPMPA], and 9-(2-phosphonylmethoxyethyl)-2,6,diaminopurine (PMEDAP). 3′-Azido-3′-deoxythymidine (AZT) was kindly provided by S. Lehrman Nusinoff of Burroughs Wellcome Co., Research Triangle Park, N.C.

Drug toxicity. Cell viability was determined by exclusion of trypan blue dye. For growth curves, several aliquots of cells were set up on day 0 in multiwell plates in each different drug concentration and counted daily by Coulter counter on days 1 through 4, the time during which the cells were in logarithmic growth.

RT assay. Virus-associated reverse transcriptase (RT) activity was assayed in duplicate 50-μl reactions containing 30 μl of clarified unconcentrated supernatant medium and 5 μCi of [^{3}H]dTTP (specific activity, 60 to 80 Ci/mmol; Amersham Corp.) with the addition (final concentrations) of 10 mM Tris hydrochloride (pH 7.5), 8 mM $MgCl_2$, 10 mM dithiothreitol, 0.5% Triton X-100, 50 μg of poly(A) per ml,

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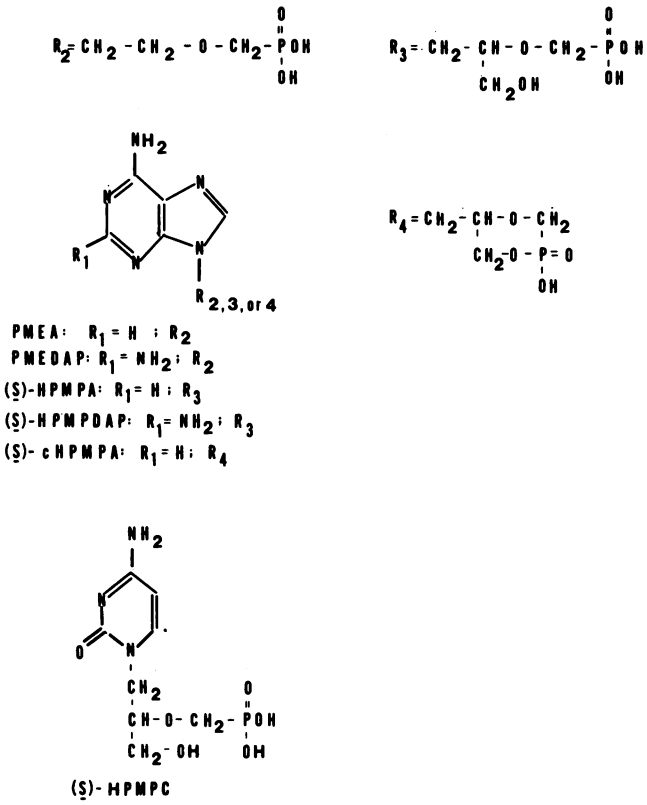

FIG. 1. Structures of the acyclic adenosine derivatives.

and 10 μg of oligo(dT)$_{12-18}$ (Pharmacia, Inc.) per ml. After incubation at 37°C for 45 min, 40 μl of each duplicate reaction was spotted onto DE81 paper (Whatman, Inc.), washed, and counted as described previously (29).

Synergy calculations. The multiple drug effect analysis of Chou and Talalay (2) was used to calculate combined drug effects with the use of computer software (1). Combination indices were calculated on the basis of both mutually nonexclusive and mutually exclusive effect equations by the following equation:

$$CI = \frac{X_{combo}}{X_{alone}} + \frac{Y_{combo}}{Y_{alone}} + \frac{\alpha(X_{combo} \cdot Y_{combo})}{X_{alone} \cdot Y_{alone}}$$

where α is 0 for the mutually exclusive drug effect and 1 for the mutually nonexclusive drug effect, X_{alone} is the concentration of drug X which gives a certain percentage of RT inhibition, and X_{combo} is the concentration of drug X which, in combination with drug Y, gives a certain percentage of RT inhibition. CI values of <1 indicate synergy, CI values of >1 indicate antagonism, and a CI value of 1 indicates the additive effect of the two drugs. The classical isobologram for 90% effective concentration (EC_{90}) levels was also used to depict synergism of combined drug effects. For this graph, the concentration of one drug giving the desired effect (e.g., 90% reduction of virus production) is plotted on one axis and the concentration of the other drug giving the same percentage reduction is plotted on the other axis. A line is drawn between these two points; the concentrations of combinations of the two drugs giving the same percentage effect are also plotted. If these points fall under the line, the combination is considered synergistic for mutually exclusive drugs; if they fall above the line, they are considered antagonistic; and if they are on the line, they are considered additive.

TABLE 1. Effect of acyclic adenosine congeners on HIV replication and growth of human T-cell lines

Drug	C3 EC_{50} (μM)[a]	C3 EC_{90} (μM)[b]	H9 EC_{90} (μM)	C3 IC_{50} (μM)[c]	H9 IC_{50} (μM)	C3 IC_{50}/EC_{50} (μM)
PMEA	1.9	27	8	>200	51	>100
(*S*)-HPMPDAP	2.3	36	64	>200	>100	>83
PMEDAP	0.2	3	0.1	>200	17	>1,000
AZT	0.02	0.33	0.3	10	30	500
AZT:PMEA (1:50)[d]	0.24	ND[e]	ND	>51	ND	>212

[a] EC_{50} is the concentration which decreased the RT level by 50% compared with that in the control infection.
[b] EC_{90} is the concentration which decreased the RT level by 90% compared with that in the control infection.
[c] IC_{50} is the concentration which decreased the total cell number by 50% after 4 days in the drug.
[d] The concentrations shown are the sum of the two drug concentrations at a 1:50 ratio.
[e] ND, Not determined.

RESULTS

Effect of acyclic adenosine derivatives on HIV replication. Six acyclic adenosine derivatives were tested for the ability to inhibit HIV replication in cultures of human T-cell lines (H9 or C3). The drug, (*S*)-HPMPA, reported to inhibit transformation of mouse embryo fibroblasts by Moloney murine sarcoma virus (5), did not inhibit HIV replication at nontoxic doses (data not shown). The other drugs all showed some effect in H9 and C3 cells at levels of drug not causing significant reduction in cellular growth rate. The levels of drug capable of causing a 50 or 90% reduction in RT level (EC_{50} and EC_{90}) are shown in Table 1. Dose-response curves for AZT were performed for comparison. PMEA and (*S*)-HPMPDAP showed similar activity in C3 cells, whereas the PMEDAP produced an EC_{90} at a 10-fold-lower concentration in C3 cells and was almost as effective as AZT on a molar basis in H9 cells.

Growth inhibition by acyclic adenosine derivatives. Growth rates of uninfected cells (C3 and H9) in the presence of various concentrations of PMEA, (*S*)-HPMPDAP, and PMEDAP were determined. The concentrations resulting in 50% reduction in total cell number on day 5 are shown in Table 1. After 4 days of exposure to drug, H9 cell viabilities remained above 90% with levels of PMEA and (*S*)-HPMPDAP below 10 μM, and H9 cell viabilities dropped to 86 and 60% in 100 μM levels of the two drugs, respectively. For the synergy experiments, the toxicity of the combined drugs AZT and PMEA was tested on the uninfected C3 cell line. The combination of 1 μM AZT plus 10 μM PMEA resulted in 90% viable cells after 4 days in the presence of drug. Thus, at effective antiviral levels, PMEA and (*S*)-HPMPDAP had only a slightly cytostatic effect which did not increase in the combination of PMEA with a low concentration of AZT.

Combination of AZT and PMEA. Various combinations of low levels of AZT and PMEA as well as each drug alone were added to C3 cells immediately after infection and removal of excess virus in two separate experiments. The virus production was determined by RT assay at day 6 or 7. Virus production in the presence of each combination is shown in Fig. 2, where it can be seen, for example, that the effect of 0.01 μM AZT plus 0.1 μM PMEA (virus production at 60% of control infection) was greater than that of 0.01 μM AZT alone (76% of control) or of 0.1 μM PMEA alone (88% of control). The fitting of the data for AZT alone and for PMEA alone to the median-effect plot (log F_a/F_u versus log

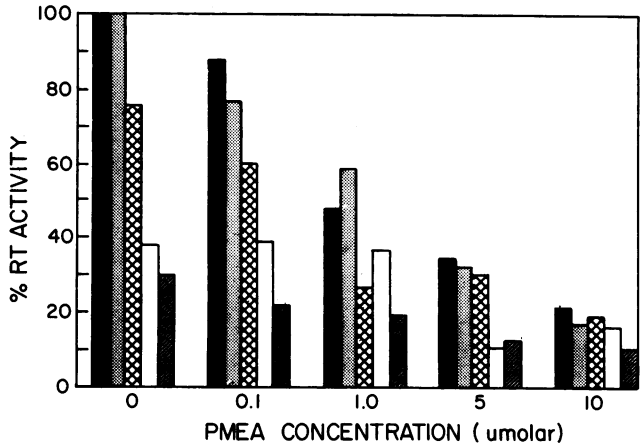

FIG. 2. Effect of combinations of PMEA and AZT on HIV replication in C3 cells. Virus production was determined by RT assay, shown here as percent of the positive control culture. AZT concentrations: ■, 0; ▨, 0.002 μM; ▩, 0.01 μM; □, 0.05 μM; ▨, 0.1 μM. umolar, Micromolar.

concentration, where F_a is the fraction affected, or 1 − percent inhibition, and F_u is the fraction unaffected, or 1 − F_a) resulted in linear correlation coefficients (r) of 0.995 and 0.992, respectively. The measure of virus production was analyzed for synergy of the two compounds by applying the multiple drug effect analysis to the RT data. Synergy calculations were performed for all datum points resulting in less than 99% inhibition by combination indices (Table 2) and by a classical isobologram derived from the data in Fig. 2 (Fig. 3). Both methods are derived from the same set of equations, and both indicated synergism between these drugs. As seen in Table 2, a few of the combinations give rise to combination indices of >1; these may be due to experimental variation, as they are the same points that appear to deviate from a perfect progression of inhibition in Fig. 2. As shown in Table 2, all combinations (eight of eight datum points) of AZT and PMEA producing in excess of 68.1% inhibition showed synergistic inhibitory effects on HIV-1 replication in RT assays, whereas at levels producing less than 68.1% inhibition, three of six datum points showed synergism and the other three datum points showed antagonism. This trend of synergism is favorable since in actual therapy one would expect the inhibitory effect shown to be 90% or more. The equipotency ratio based on EC_{50}s for AZT (0.02 μM) and PMEA (1.9 μM) is 1:95 (Table 1). The combinations emphasizing AZT effect (or concentration) or de-emphasizing PMEA effect around this ratio tend to favor synergism, whereas the combinations de-emphasizing AZT effect or emphasizing PMEA effect tend to yield additive or antagonistic effects (Table 2). Also, AZT and PMEA effects, whether they are mutually exclusive (α = 0) or mutually nonexclusive (α = 1), yield similar combination indices and thus similar degrees of synergism or antagonism (Table 2).

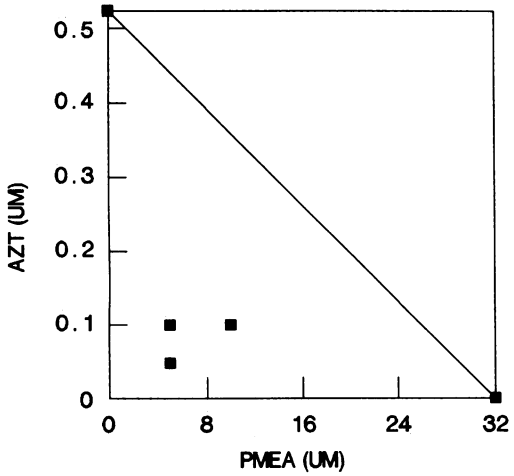

FIG. 3. Isobologram for ED_{90} level of PMEA and AZT. Individual points represent the concentrations of a combination of the two drugs giving the same degree of inhibition (90%) of virus production, based on the data in Fig. 2. UM, Micromolar.

TABLE 2. Combination indices for treatments with AZT and PMEA on RT in C3 cells

[AZT]	[PMEA]	Ratio[a]	Dose[b]	Fraction affected[c]	Combination index[d]	
					α = 0	α = 1
0.01	0			0.245		
0.05	0			0.625		
0.1	0			0.701		
1	0			0.934		
0	0.1			0.122		
0	1			0.524		
0	5			0.663		
0	10			0.791		
0	50			0.930		
0.002	0.1	1:50	0.102	0.236	0.63	0.72
0.002	1	1:500	1.002	0.415	1.25	1.36
0.002	5	1:2500	5.002	0.681	1.24	1.27
0.002	10	1:5000	10.002	0.833	0.74	0.75
0.01	0.1	1:10	0.11	0.403	0.60	0.66
0.01	1	1:100	1.01	0.738	0.25	0.26
0.01	10	1:1000	10.01	0.813	0.94	0.98
0.05	0.1	1:2	0.15	0.613	0.86	0.89
0.05	1	1:20	1.05	0.638	1.04	1.27
0.05	5	1:100	5.05	0.898	0.26	0.28
0.1	0.1	1:1	0.2	0.785	0.60	0.60
0.1	1	1:10	1.1	0.810	0.57	0.62
0.1	5	1:50	5.1	0.878	0.47	0.53
0.1	10	1:100	10.1	0.897	0.53	0.60

[a] Molar ratio of AZT and PMEA in the mixture.
[b] Sum of the concentrations of AZT and PMEA.
[c] Fraction affected for RT is calculated as [1 − (RT of drug-treated culture − background/RT of no-drug culture − background)].
[d] Combination indices are calculated as described in references 1 and 2; the values shown are for the mutually exclusive drug effect (α = 0) and for mutually nonexclusive effect (α = 1).

Lack of effect of PMEA on in vitro RT activity. PMEA was tested in in vitro RT assays to verify that residual drug (a nucleoside monophosphate analog) from supernatant medium would not directly inhibit the RT activity being used as a measure of virus production in the drug experiments. Final concentrations of PMEA ranging from 0.1 to 50 μM were included in the RT assay of a high-titer stock and resulted in no decrease in incorporated [^{3}H]dTTP (data not shown).

DISCUSSION

The biochemical mechanism by which PMEA and its congeners achieve their antiviral effect against HIV replica-

tion remains the subject of further study. These drugs were designed with the phosphorus atom attached via a P-C bond, such that hydrolysis by phosphomonoesterases could be prevented. One of these drugs, (*S*)-HPMPA, it taken up by cells and subsequently phosphorylated to its mono- and diphosphoryl derivatives, presumably by cellular nucleotide kinases (32). The ether linkage between the alkyl residue and the phosphonylmethyl group cannot be metabolized to phosphonoacetate (PAA) or to phosphonoformate, which is known to inhibit HIV (28); this lack of metabolism is further documented by the activity of (*S*)-HPMPA against two DNA polymerase PAA^r mutants of HSV-1 (5). In addition, only the (*S*)-enantiomer has antiviral activity; thus, the antiviral effect is based on its chirality. Also, the drugs are not thought to interact with *S*-adenosylhomocysteine hydrolase (5), nor are they degraded by deaminases (25).

In addition to inhibiting several DNA viruses (4, 5, 14, 32), these drugs (i.e., PMEA and PMEDAP) have been shown recently to inhibit HIV-induced cytopathology and antigen expression in MT-4, ATH8, and H9 T-lymphocyte cells in vitro, as well as inhibiting transformation of mouse fibroblasts by Moloney murine sarcoma virus (25). Our results for the 50% inhibitory concentration (IC_{50}) and EC_{50} of PMEA and PMEDAP agree well with those published by Pauwels et al. (25), despite the differences in the systems used; Pauwels et al. assessed the 50% effective dose (ED_{50}) by cytopathic effect and immunofluorescence in H9 and MT4 cells, whereas we have monitored virus production by RT assay in C3 and H9 cells. Recently, the toxicity and prophylactic antiviral activity of PMEA was tested in the feline retrovirus system (L. E. Mathes, C. L. Swenson, P. A. Polas, R. Sams, K. Hayes, and G. Kociba, Abstr. 5th Int. Conf. AIDS, abstr. no. MCP69, p. 553, 1989). At a dosage of 12.5 mg/kg per day, and a level in plasma of 1 to 2 μg/ml (approximately 5 μM), 3 weeks of infusion prevented or delayed the onset of infection in six of six challenged animals, whereas seven of seven control cats developed chronic viremia by 3 weeks postchallenge.

Another combination of nucleosides (ribavirin and AZT) has produced a decrease in the level of AZT triphosphate in cells, resulting in an antagonistic effect (31; J. A. Fyfe, P. Furman, M. Vogt, and P. Sherman, Abstr. 4th Int. Conf. AIDS, abstr. no. 3617, book 2, p. 170, 1988). We do not believe that PMEA is decreasing dTTP pools in the treated cells. PMEA (at levels up to 500 μM) does not affect the incorporation of [*methyl*-^{3}H]deoxythymidine into DNA in MT-4 cells (J. Balzarini and E. De Clercq, unpublished results). We can infer that the dTMP, dTDP, and dTTP levels are probably not decreased, since [*methyl*-^{3}H]deoxythymidine has to go through these steps in order to be incorporated into DNA, although inhibition of de novo derived pools of dTMP, dTDP, and dTTP is not ruled out. In addition, the anti-HIV activity of PMEA in MT-4 cells was not reversed upon the addition of 200 μM adenosine, 200 μM 2′-deoxyadenosine, 200 μM cytidine, 200 μM 2′-deoxycytidine, 25 μM thymidine, or 2 μM guanosine, suggesting that altered nucleotide pools are not responsible for the antiviral effect.

Treatment of AIDS patients with AZT alone has resulted in side effects such as anemia and neutropenia; some patients are forced to reduce the dosage or discontinue its use altogether (27). AZT alone (most probably like most nucleoside analogs) is unable to stop production of virus from already infected lymphocytes in vitro (24) or to completely prevent new infection of susceptible lymphocytes in vitro (29). The combination of AZT, a thymidine analog, with an acyclic adenosine analog may allow an effective antiviral dosage of the combination while still using a low dose of AZT, perhaps alleviating the side effects of both drugs. Bone marrow toxicity was observed in cats with PMEA at 12.5 mg/kg per day; lower dosages are currently being tested for toxicity and prophylactic activity (Mathes et al., Abstr. 5th Int. Conf. AIDS, 1989). Another possible clinical advantage may be that these drugs are active in vitro against several human herpesviruses, including herpes simplex virus types 1 and 2, varicella-zoster virus, cytomegalovirus, and Epstein-Barr virus, all of which are prevalent in acquired immunodeficiency syndrome and all of which may affect HIV transcription (3, 13, 21). If these drugs prove to be well tolerated in vivo, they may show an additional indirect benefit because of their broad activity against these other viruses (22).

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