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Novel Acyclic Adenosine Analogs Inhibit Epstein-Barr Virus Replication

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The effect of three new acyclic adenosine analogs, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], on Epstein-Barr virus (EBV) replication was studied. Both (*S*)-HPMPA and PMEA but not (*S*)-DHPA effectively inhibited EBV DNA replication in virus-producer P3HR-1 cells and in latently infected Raji cells superinfected with P3HR-1 virus, as determined by cRNA-DNA hybridization and density gradient centrifugation. The 50% effective doses for inhibiting virus replication were 0.08 and 1.1 μM for (*S*)-HPMPA and PMEA, respectively. Both drugs were cytostatic but not cytotoxic to the cells at a concentration as high as 100 μM. These results indicate that (*S*)-HPMPA and PMEA are potent and selective anti-EBV agents in vitro.

In recent years, we have shown that several nucleoside analogs selectively inhibit the replication of Epstein-Barr virus (EBV) (7–10). Most of these drugs are toxic and do not exhibit a high therapeutic index. A new compound, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], has recently been shown to have potent and selective activity against a broad spectrum of human herpesviruses, such as herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus, and cytomegalovirus (3). (*S*)-HPMPA is also active against seal, monkey, pig, bovine, and equine herpesviruses; African swine fever virus; vaccinia virus; human adenoviruses; and retroviruses (3). Furthermore, thymidine kinase-deficient (TK^-) mutants of HSV and varicella-zoster virus are also susceptible to the drug. In view of these striking effects against a variety of viruses, we decided to evaluate the effects of (*S*)-HPMPA and its close congener, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), as well as the parental (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] (2), on EBV replication (Fig. 1).

To determine the effects of the drugs on EBV DNA replication, we used the virus-producing cell line P3HR-1. Exponentially growing P3HR-1 cells were treated for 14 days (9) with various concentrations of the drugs in RPMI 1640 medium. The cells were harvested, and EBV genome copy numbers were determined by complementary RNA-DNA hybridization with an EBV-specific cRNA probe (9).

Table 1 shows the dose-dependent inhibition of EBV genome replication by PMEA and (*S*)-HPMPA. (*S*)-DHPA did not show any inhibitory effect at the concentrations used. (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU), a drug known to be active against EBV replication (7, 10), was included in the assays as a positive control. EBV genome copy numbers decreased with increasing concentrations of PMEA, (*S*)-HPMPA, and BVdU. The 50% effective dose for viral inhibition was determined from the semilogarithmic plot of drug concentrations against viral genome copies per cell, assuming the residual genome level (~30 copies per cell) achieved by an effective drug concentration (100 μM) as zero and the viral genome level in the no-drug control as 100 (9). We have shown previously that the residual EBV copy number of ~30 per cell is due to episomal forms, which are insensitive to antiviral drugs (9). The 50% effective doses thus obtained were 1.1, 0.08, and 0.06 μM for PMEA, (*S*)-HPMPA, and BVdU, respectively. The results obtained here with BVdU are identical to the data reported previously (10). Interestingly, (*S*)-DHPA, the parental compound of (*S*)-HPMPA, which has been shown to be effective against a variety of RNA viruses, such as rabies virus, vesicular stomatitis virus, parainfluenza virus, measles virus, reoviruses, and rotaviruses (2), was not active against EBV (Table 1).

Since the inhibitory effects of these drugs on EBV could be the consequence of selective killing of the productive cells in the P3HR-1 population, we monitored cell growth and viability during drug treatment. At a drug concentration of 10 μM, approximately 23 and 34% reductions in cell number were observed for the PMEA- and (*S*)-HPMPA-treated cells (Table 1). At a drug concentration of 100 μM, a further reduction (50%) in cell number was observed for the PMEA-treated cells, compared with an 80% decrease for the (*S*)-HPMPA-treated cells; however, the viability of the cells was not significantly affected in either case at drug concen-

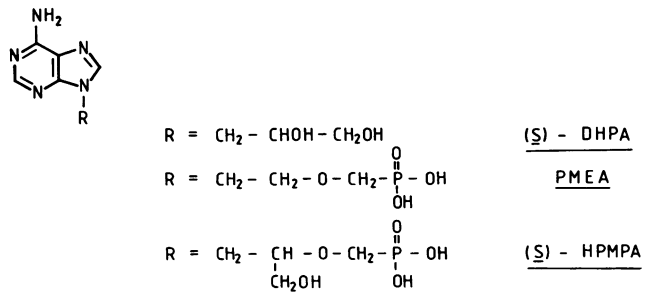

FIG. 1. Structures of (*S*)-DHPA, PMEA, and (*S*)-HPMPA.

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TABLE 1. Effect of PMEA, (*S*)-DHPA, (*S*)-HPMPA, and BVdU on EBV replication

Drug	Drug concn (μM)	Cells/ml (10^6)[a]	% Viability	EBV genome (copies/cell)[b]
PMEA	0.01	2.7	95	185
	0.1	2.6	96	170
	1.0	2.8	96	150
	10.0	2.1	95	50
	100	1.4	92	30
	Control	2.8	96	220
(*S*)-DHPA	0.01	3.1	97	216
	0.1	2.8	95	210
	1.0	2.6	96	213
	10.0	2.3	94	196
	100	2.0	96	208
	Control	2.8	96	220
(*S*)-HPMPA	0.01	2.7	96	208
	0.1	2.3	93	108
	1.0	2.1	93	60
	10.0	1.8	95	35
	100	0.6	89	ND[c]
	Control	2.8	96	220
BVdU	0.01	2.8	96	214
	0.1	2.9	97	101
	1.0	2.5	94	78
	10.0	2.7	96	56
	100	2.4	95	32
	Control	2.8	96	220

[a] Cell density was determined after the cells were grown for 4 days. The original cell density was 5×10^5/ml.

[b] Virus-producing cells (P3HR-1) were exposed to various concentrations of drug for 14 days, and EBV genome copies per cell were determined by EBV-specific cRNA-DNA hybridization. Results were based upon the average values for two determinations.

[c] ND, Not determined.

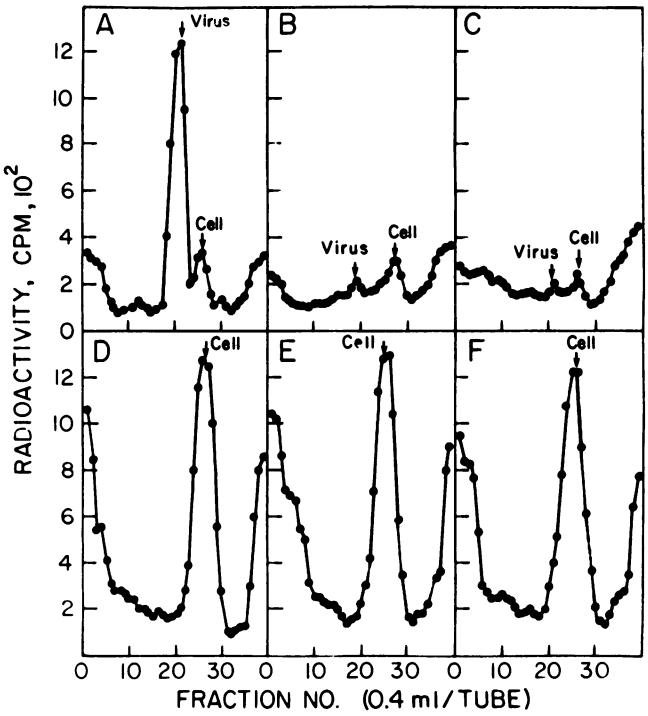

FIG. 2. Inhibition of EBV DNA replication by PMEA and (*S*)-HPMPA in superinfected Raji cells. The effect of drugs on DNA synthesis was determined by measuring the incorporation of ^{32}P into both viral and cellular DNA which were analyzed by cesium chloride density gradient centrifugation (9). Shown are superinfected Raji cells without drug (A), with 5 μM PMEA (B), and with 5 μM (*S*)-HPMPA (C); and mock-infected Raji cells without drug (D), with 5 μM PMEA (E), and with 5 μM (*S*)-HPMPA (F).

trations as high as 100 μM. These results indicate that the drugs are cytostatic but not cytotoxic to P3HR-1 cells.

To assess whether the inhibitory effect of PMEA and (*S*)-HPMPA on EBV DNA synthesis is a general phenomenon and not limited to a particular cell system, we evaluated the drugs in latently infected Raji cells after superinfection with P3HR-1 virus (Fig. 2A). The anti-EBV activity of PMEA (Fig. 2B) and (*S*)-HPMPA (Fig. 2C) was confirmed. At concentrations of 5 μM, PMEA and (*S*)-HPMPA completely inhibited the synthesis of viral DNA in P3HR-1 virus-superinfected Raji cells (cf. Fig. 2B and C with Fig. 2A). Neither PMEA (Fig. 2E) nor (*S*)-HPMPA (Fig. 2F) had a significant effect on cell DNA synthesis in Raji cells which had not been superinfected with the P3HR-1 virus (cf. Fig. 2E and F with Fig. 2D).

The molecular mechanism of inhibition of EBV replication by PMEA and (*S*)-HPMPA remains to be established. The inhibition of EBV DNA replication at a drug concentration that is not cytotoxic to the cells suggests that a virus-specific function is affected. Studies with a number of nucleoside analogs have clearly indicated that the initial phosphorylation which occurs preferentially in virus-infected cells is a prerequisite for their selective activity against virus replication (4; for a review, see reference 6). Subsequently, the nucleotide analogs are further converted by host cellular kinases to triphosphates which, in turn, act as either inhibitors or substrates for the virus-specific DNA polymerase (7). With PMEA and (*S*)-HPMPA, virus-specified TK does not appear to be required for phosphorylation, since both TK^+ and TK^- HSV strains and TK^+ and TK^- varicella-zoster virus strains are equally susceptible to the drugs (3). In addition, (*S*)-HPMPA is active against the DNA polymerase-based HSV-1 mutants PAA^r-1 and PAA^r-5 (3). Thus, an alteration in viral TK or DNA polymerase activity does not necessarily result in resistance to (*S*)-HPMPA. (*S*)-HPMPA may be taken up by the cells in its native form since it cannot be enzymatically dephosphorylated. Subsequently, the drug may be converted by cellular kinase(s) to its active form, the diphosphoryl derivative, which may be targeted at the viral DNA polymerase.

Our recent studies indicate that several congeners of (*S*)-HPMPA and PMEA are quite effective against human immunodeficiency virus replication in cell cultures (unpublished data). EBV has been suggested as one of the cofactors which determine whether human immunodeficiency virus infection leads to acquired immune deficiency syndrome (5). Since opportunistic virus infections, in particular herpesvirus (e.g., EBV, cytomegalovirus, HSV, and varicella-zoster virus) infections, are prevalent in acquired immune deficiency syndrome (1, 11), the broad activity of (*S*)-HPMPA and its congeners may have an additional benefit if the drugs come to be used in treatment.

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In Vitro Activity of Cefmetazole, Cefotetan, Amoxicillin-Clavulanic Acid, and Other Antimicrobial Agents against Anaerobic Bacteria from Endometrial Cultures of Women with Pelvic Infections

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The MICs of the new antimicrobial agents cefmetazole, cefotetan, and amoxicillin-clauvulanic acid were compared with the MICs of other antimicrobial agents against anaerobic bacteria from endometrial cultures from women with pelvic inflammatory disease or endometritis. The activity of cefmetazole was similar to that of cefoxitin and generally greater than that of cefotetan. Amoxicillin-clavulanic acid was generally more active than all cephamycins tested.

Several of the newer antimicrobial agents, among them cefmetazole, cefotetan, and amoxicillin-clavulanic acid, are being considered for use in the treatment of upper genital tract soft tissue infections in women. Since a wide variety of anaerobic bacteria, including many beta-lactamase-producing *Bacteroides* spp., are frequently involved in these infections, it is important that these antimicrobial agents be effective against such organisms. Reports of in vitro activity of these agents against clinical isolates of anaerobic bacteria are limited mainly to reports of activity against the *Bacteroides fragilis* group (1, 3, 8, 10, 11). We determined the MICs of some of the newer antimicrobial agents (see Table 1) against anaerobic bacteria isolated from endometrial cultures from women with pelvic inflammatory disease or postpartum endometritis because anaerobic bacteria other than the *B. fragilis* group are more commonly isolated from cultures from such patients. These MICs were compared with those of other agents commonly used to treat obstetric and gynecologic infections (see Table 1). Beta-lactamase production by organisms was also determined.

Endometrial cultures were obtained by using a double-catheter-enclosed brush as described by Knuppel et al. (5). The device was inserted through a speculum to the cervical os, which had been wiped clean with a large sterile swab. The inner catheter was extended into the endometrial cavity, and the brush was extended out of the inner catheter. After the specimen was obtained, the brush and inner catheter were retracted into the outer catheter before the device was removed from the patient.

All of the organisms, which were isolated between May 1984 and May 1986, were recovered from infected women who had not received antimicrobial treatment for at least 2 months. The organisms were identified by using gas-liquid chromatography and prereduced anaerobically sterilized media (2). Organisms which had been frozen in 10% skim milk at −70°C were subcultured at least twice before susceptibility testing was done.

Standard powders of the antimicrobial agents were supplied by their manufacturers. The amoxicillin-clavulanic acid mixture was supplied in combined form by Beecham Laboratories, Bristol, Tenn., with the concentration of amoxicillin being twice that of clavulanic acid. The amount of powder weighed out to make the initial solution was based on the concentration of amoxicillin.

MICs were determined by the agar dilution method, with doubling dilutions of the antimicrobial agents incorporated into brucella agar supplemented with vitamin K and 5% sheep blood (9). Overnight growth of organisms in supplemented thioglycolate broth was adjusted to the turbidity of a 0.5 McFarland standard and inoculated to the agar plates with a Steers replicator. The plates were read after being incubated at 35°C in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) for 48 h. Control organisms (*B. fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Peptococcus magnus* ATCC 29328) were inoculated with each batch of organisms tested, and the MICs were within control limits for each antibiotic for each batch.

Beta-lactamase production was determined by the Cefinase disk (BBL) method.

Table 1 shows the range of MICs of each antimicrobial agent for each group of organisms tested and the MICs for 50 and 90% of the organisms. The activity of cefmetazole was very similar to that of cefoxitin. All but 5 of the 270 isolates tested were susceptible to ≤8 μg of either antibiotic per ml. For four isolates of *Peptostreptococcus anaerobius*, MICs were equal to 16 μg/ml. For one isolate of *Bacteroides distasonis*, MICs were equal to 64 μg/ml. Cefotetan was less active than cefmetazole or cefoxitin, with MICs for 50 and 90% of the isolates generally 1 to 2 dilutions higher. Nine isolates of *P. anaerobius*, in addition to the *B. distasonis* isolate, had cefotetan MICs ≥32 μg/ml. Mezlocillin showed activity greater than or equal to that of the cephamycins against anaerobic gram-positive cocci and *Fusobacterium* spp. and activity somewhat less than that of cefoxitin or cefmetazole against the *Bacteroides* spp.

All isolates were susceptible to amoxicillin-clavulanic acid, imipenem, and metronidazole. One isolate of *Peptostreptococcus asaccharolyticus* and one isolate of *Bacteroides bivius* were resistant to clindamycin. These isolates were both from one patient, and nothing in the medical history of the patient indicated that she had received prior treatment with clindamycin, erythromycin, tetracycline, or any other antimicrobial agent.

Organisms which were inhibited by higher concentrations of the cephamycins and mezlocillin were generally those in which beta-lactamase production was detected (Table 2).

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