Inhibitory Effects of Acyclic Nucleoside Phosphonate Analogs, Including (S)-1-(3-Hydroxy-2-Phosphonylmethoxypropyl)Cytosine, on Epstein-Barr Virus Replication

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(S)-9-(3-Hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA], (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine [(S)-HPMPDAP], cyclic (S)-HPMPA [(S)-cHPMPA], 9-(2-phosphonylmethoxyethoxyethyl)-2,6-diaminopurine (PMEDAP), and (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(S)-HPMPC] were examined for their inhibitory effects on Epstein-Barr virus (EBV) replication. The 50% effective concentrations for inhibition of viral DNA replication were 0.16, 0.03, 2.0, 1.5, and 0.08 μ M for PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA, respectively. The relative efficacies based on the *in vitro* therapeutic index was (S)-HPMPC (5,000) > PMEDAP (1,000) = (S)-HPMPA (1,000) > (S)-cHPMPA (136) > (S)-HPMPDAP (78). Certain ratios of combinations of (S)-HPMPC with 3'-azido-3'-deoxythymidine produced a synergistic inhibitory effect on EBV genome replication, but others exhibited an antagonistic effect. These results indicate that this series of acyclic nucleoside phosphonate analogs, and in particular (S)-HPMPC, are potent and selective anti-EBV agents *in vitro*.

A new class of adenine analogs, with (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA] as the prototype, has potent and selective activity against a broad spectrum of DNA viruses, including, in some cases, Epstein-Barr virus (EBV) (4, 7). (S)-HPMPA and its close congener 9-(2-phosphonylmethoxyethyl)adenine (PMEA), but not (S)-9-(2,3-dihydroxypropyl)adenine, are potent inhibitors of EBV replication (7). The new acyclic nucleoside phosphonate analogs (5) that have been synthesized recently [(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine [(S)-HPMPDAP], (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(S)-HPMPC], cyclic (S)-HPMPA [(S)-cHPMPA], and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP)] provide the opportunity to evaluate the structure-activity relationships involved in the inhibition of EBV replication, disclose aspects of the molecular mechanisms underlying anti-EBV activity, and guide the further development of new antiviral drugs.

To determine the effects of the drugs on EBV DNA replication, exponentially growing P3HR-1(LS) cells were treated for 14 days with various concentrations of the drugs. The cells were harvested, and the EBV genome copy numbers were determined (9). Table 1 shows the concentration-dependent inhibition of EBV genome replication by PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA. All these compounds strongly inhibited EBV replication in a concentration-dependent manner. The number of EBV genome copies per cell decreased with increasing drug concentrations in all cases, but with different slopes.

We monitored cell growth and viability during treatment. At a drug concentration of 10 μ M, in comparison with the control, there was an approximately 23% reduction in cell

number with all drugs tested. At a drug concentration of 100 μ M, a further reduction (33 to 45%) in cell number was observed. However, cell viability was not significantly affected at a drug concentration of 5 μ M and was only slightly reduced at a drug concentration of 10 μ M.

The 50% effective concentration for virus inhibition (EC_{50}) was determined from the semilogarithmic plot of drug concentrations against the number of viral genome copies per cell, assuming the residual genome level (30 copies per cell) achieved by an effective drug concentration (5 to 100 μ M, depending on the individual drug) as 0% and the viral genome level in the controls with no drug as 100% (9). We have shown previously (9) that the residual EBV copy number of 30 per cell is due to episomal forms, which are insensitive to nucleoside analogs. The EC_{50} s thus obtained from the plots were 0.16, 0.03, 2.0, 1.5, and 0.08 μ M for PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA, respectively (Table 2). Similarly, the EC_{90} s were 1.85, 1.76, 9.0, 7.0, and 3.42 μ M for PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA, respectively. The values obtained from analysis of the data by using the Concentration-Effect Analysis Program (3) were in close agreement with those obtained by the graphical method (9). As shown in Table 2, the relative efficacy of the compounds based on their therapeutic index (EC_{50}/IC_{50} , where IC_{50} is the 50% inhibitory concentration for cell growth) was (S)-HPMPC (5,000) > PMEDAP (1,000) = (S)-HPMPA (1,000) > (S)-cHPMPA (136) > (S)-HPMPDAP (78).

The anti-EBV activities of these compounds appeared to be selective, in that they all inhibited virus replication at drug concentrations which were not significantly cytotoxic or cytostatic *in vitro*. Despite the effective inhibition of the production of linear forms of EBV DNA, which are replicated by a virus-specified DNA polymerase (8), these drugs had no effect on the EBV episomal forms of DNA (approximately 30 copies per cell in the productive cell line P3HR-1

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TABLE 1. Concentration-dependent inhibition by PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA of replication of EBV genomes

Drug	Concn (μ M)	Viability (%) ^a	No. of EBV genome copies/cell ^b
PMEDAP	0.1	82	325
	1.0	81	159
	5.0	80	31
	10.0	75	31
	100.0	71	ND
(S)-HPMPC	0.1	84	200
	1.0	84	94
	5.0	81	34
	10.0	79	26
	100.0	78	ND
(S)-HPMPDAP	0.1	87	595
	1.0	85	412
	5.0	85	157
	10.0	80	57
	100.0	78	ND
(S)-cHPMPA	0.1	88	598
	1.0	88	323
	5.0	85	99
	10.0	80	47
	100.0	77	ND
(S)-HPMPA	0.1	85	245
	1.0	84	120
	5.0	82	98
	10.0	80	36
	100.0	76	ND
Control	0.0	87	600

^a Cell viability was determined after the cells were grown for 4 days.

^b Virus-producing cells, P3HR-1(LS), were exposed to various concentrations of drug for 14 days, and the number of EBV genome copies per cell was determined by EBV-specific DNA-DNA hybridization. Results were the average of two separate experiments with triplicate determinations. ND, not determined because of a cytostatic effect.

(LS) and 60 copies per cell in non-virus-producing Raji cells), which are presumed to be replicated by host DNA polymerase.

Since (S)-HPMPC was the most potent drug among the series of analogs tested, we decided to determine the kinetics of inhibition and reversibility of (S)-HPMPC in parallel with (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU). Figure 1 indicates that for both drugs, 14 days of drug treatment was needed to reduce the viral genome copy numbers (30 copies per cell) to the residual level because of EBV episomes. However, (S)-HPMPC exhibited a faster reduction of viral

FIG. 1. Kinetics of inhibition and reversibility of EBV DNA replication in P3HR-1 (LS) cells treated with 10-fold the EC₉₀ of (S)-HPMPC (18 μ M) or BVdU (50 μ M). Values are means $\pm$ standard deviations for two separate experiments with triplicate determinations. (S)-HPMPC-treated cells exhibited a significantly slower kinetics of reversibility than BVdU-treated cells did ($P < 0.001$). The results of Fig. 1 were the average of two separate experiments with triplicate determinations. The data were statistically analyzed by using the two-way analysis of variance for randomized blocks (14).

genome copy numbers than BVdU did. Upon removal of BVdU, the number of viral genome copies per cell remained at low levels (18% of the control level) for 12 days, becoming gradually restored to 38% of the control level by 19 days. (S)-HPMPC-treated cells exhibited a significantly slower kinetics of reversibility than BVdU-treated cells did ($P \leq 0.001$). The viral genome numbers were restored to 15% of the control level by 12 days. After 19 days of drug removal, the viral genome copies per cell remained at a low level, approximately 26% of the control level.

To assess whether the combination of 3'-azido-3'-deoxythymidine (AZT) and (S)-HPMPC may have a synergistic effect on the inhibition of EBV genome replication, we treated P3HR-1(LS) cells with various combinations of the two drugs as well as with each drug alone. The effects on EBV genome copy numbers were determined after 14 days of treatment (Table 3). For example, the combined effect of 0.1 μ M (S)-HPMPC plus 1 μ M AZT (EBV genome copy numbers at 27% of the control level) was greater than that of 0.1 μ M (S)-HPMPC alone (32% of control) or 1 μ M AZT alone (67% of control). The analysis of the data for (S)-HPMPC alone and for AZT alone by the median-effect plot resulted in linear correlation coefficients (r) of 0.976 and 0.998, respectively. On the basis of the numbers of EBV genome copies, the two drugs were evaluated for synergy by applying the multiple drug effect analysis. Synergy calculations were performed for all datum points that resulted in less than 99% inhibition by combination indices (CIs) (Table

TABLE 2. Inhibitory effects of PMEDAP, (S)-HPMPC, (S)-HPMPDAP, (S)-cHPMPA, and (S)-HPMPA on cell growth and EBV replication

Drug	IC ₅₀ (μ M)	EC ₅₀ (μ M) ^a	EC ₉₀ (μ M) ^a	IC ₅₀ /EC ₅₀
PMEDAP	160	0.16 (0.154)	1.85 (2.73)	1,000
(S)-HPMPC	150	0.03 (0.0314)	1.76 (1.89)	5,000
(S)-HPMPDAP	155	2.0 (2.197)	9.0 (9.46)	78
(S)-cHPMPA	150	1.5 (1.94)	7.0 (7.64)	136
(S)-HPMPA	80	0.08 (0.09)	3.42 (3.78)	1,000

^a Values in parentheses were determined by using the dose-effect analysis program of Chou and Chou (3), as described in the text.

TABLE 3. CIs for combined effects of AZT and (S)-HPMPC on EBV genome copy numbers in P3HR-1(LS) cells

(S)-HPMPC concn (μ M)	AZT concn (μ M)	Ratio ^a	Concn (μ M) ^b	No. of EBV genome copies/cell ^c	Fraction affected ^d	CI ^e	
						$\alpha = 0$	$\alpha = 1$
0	0			445			
0	1			300	0.326		
0	5			150	0.663		
0	10			110	0.753		
0.1	0			140	0.685		
1.0	0			80	0.820		
5.0	0			30	0.933		
0.1	1	1:10	1.1	120	0.730	0.63	0.69
1.0	1	1:1	2.0	65	0.854	1.01	1.06
5.0	1	1:0.2	6.0	30	0.933	0.76	0.77
0.1	5	1:50	5.1	125	0.719	1.23	1.61
1.0	5	1:5	6.0	62	0.861	1.07	1.26
5.0	5	1:1	10	28	0.937	0.72	0.77
0.1	10	1:100	10.1	114	0.744	1.56	2.06
1.0	10	1:10	11	60	0.865	1.22	1.56
5.0	10	1:2	15	25	0.944	0.62	0.69

^a Molar ratio of (S)-HPMPC and AZT in the mixture.^b Sum of the concentrations of (S)-HPMPC and AZT.^c The numbers of EBV genome copies per cell were determined by EBV-specific DNA-DNA hybridization.^d The fraction affected for EBV genome copies per cell was calculated as $[1 - (\text{EBV genome copy numbers of drug-treated culture} / \text{EBV genome copy numbers of no-drug culture})]$.^e CIs were calculated as described previously (1, 2); the values are for the mutually exclusive drug effect ($\alpha = 0$) and for mutually nonexclusive effect ($\alpha = 1$).

3) and by a classical isobologram (data not shown) constructed from the data. As seen in Table 3, four of nine combinations [ratio of (S)-HPMPC:AZT, 1:10, 1:0.2, 1:1, and 1:2] showed synergistic inhibitory effects ($CI < 1$) on EBV genome replication, and the other five ratios produced antagonistic effects ($CI > 1$). The equipotency ratio based on the EC_{50} s for (S)-HPMPC (0.03 μ M) and AZT (2.38 μ M) was 1:79. There appeared to be no specific ratio which tended to favor synergism. Furthermore, the (S)-HPMPC and AZT effects, whether they were mutually exclusive ($\alpha = 0$) or mutually nonexclusive ($\alpha = 1$), yielded similar CIs and, thus, similar degrees of synergism or antagonism.

The molecular basis for the differences in the anti-EBV activities of these compounds is not known. The present results indicate that substitution of the 9-(2-phosphonylmethoxyethyl) group in PMEDAP by a 9-(3-hydroxy-2-phosphonylmethoxypropyl) group, as in (S)-HPMPDAP, results in a 13-fold decrease in anti-EBV potency. Similarly, when the 2-amino group of PMEDAP is substituted by a hydrogen, as in PMEAP ($EC_{50} = 1.1 \mu$ M, according to our previously published data [7]), a sevenfold reduction in anti-EBV activity was observed. Interestingly, substitution of the 2-amino group of (S)-HPMPDAP by a hydrogen, as in (S)-HPMPA, resulted in a 25-fold increase in anti-EBV activity [EC_{50} for (S)-HPMPA, 0.08 μ M]. With the purine ring remaining intact, replacement of the linear 3-hydroxy-2-phosphonylmethoxypropyl side chain of (S)-HPMPA by a cyclic phosphonylmethoxyethyl group, as in (S)-cHPMPA, resulted in an approximately 19-fold decrease in potency. Furthermore, with the 3-hydroxy-2-phosphonylmethoxypropyl side chain unchanged, substitution of cytosine, as in (S)-HPMPC, for adenine, as in (S)-HPMPA, gave an almost threefold increase in anti-EBV activity. (S)-HPMPC thus emerged as the most potent EBV inhibitor of the compounds examined here.

In studies of the structure-activity relation of acyclic pyrimidine nucleosides (2), we have found that modifications in the pyrimidine ring influence the selective activity against EBV. Furthermore, studies with acyclic adenosine analogs

have indicated that modification of the 2,3-dihydroxypropyl of (S)-9-(2,3-dihydroxypropyl)adenine to 2-phosphonylmethoxyethyl, as in PMEAP, or 3-hydroxy-2-phosphonylmethoxypropyl, as in (S)-HPMPA, significantly enhances antiviral activity (7).

This series of acyclic nucleoside phosphonate analogs containing a phosphorus atom attached via a P-C bond were designed such that hydrolysis by phosphomonoesterases could be prevented. The ether linkage between the alkyl residue and the phosphonylmethyl group cannot be metabolized to phosphonoacetate or phosphonoformate, which is known to inhibit human immunodeficiency virus (HIV) (12); further evidence for this lack of metabolism stems from the activity of (S)-HPMPA against two DNA polymerase mutants of herpes simplex virus type 1 that are resistant to phosphonoacetate (4). In addition, antiviral activity resides primarily with the (S)-enantiomer, which means that the antiviral activity of the compound is based on its chirality. Also, the drugs are not thought to interact with S-adenosylhomocysteine hydrolase (4), be degraded by deaminases (11), or require phosphorylation by a virus-specified thymidine (deoxycytidine) kinase (TK) for activity. An important feature that may have therapeutic implications is the long-lasting antiviral effect of (S)-HPMPC, as observed in reversibility experiments (Fig. 1). (S)-HPMPC, (S)-HPMPA, and their congeners may be taken up by the cells in their native form, since they cannot be enzymatically dephosphorylated, and this may account for the longer activity against EBV replication.

Several different ratios of combinations of AZT with (S)-HPMPC were found to have synergistic inhibitory effects on EBV genome replication, whereas some ratios exhibited an antagonistic effect. Previously, we reported (10) that combinations of interferon alpha, interferon gamma, and AZT are also synergistic in preventing cellular microfocus formation of EBV-infected umbilical cord blood lymphocytes. These results have implications for the treatment of EBV-induced lymphoproliferation. Here we presented indications that some combination of drugs may also be syner-

gistic, although not strikingly so, in inhibiting EBV replication itself. These results may provide a lead to the further development and use of drug combinations that may produce fewer side effects through lower dosages and a reduced likelihood of emergence of drug-resistant virus strains.

Our previous findings (13) indicated that some of the acyclic nucleoside phosphonate analogs (i.e., PMEA) are quite effective against HIV replication in cell cultures (13). EBV has been suggested as one of the cofactors which determines whether HIV infection leads to AIDS (6). Since opportunistic virus infections, in particular herpesvirus (EBV, herpes simplex virus, varicella zoster virus, human cytomegalovirus, and human herpesvirus 6) infections, are prevalent in patients with AIDS (1, 15), the broad-spectrum activities of these series of compounds may be of additional benefit if the compounds are used in the treatment of patients with AIDS.

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