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Combined antiviral effects of paired nucleosides against guinea pig cytomegalovirus replication in vitro

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Summary

Several promising antiviral nucleosides have been tested in paired combinations against guinea pig cytomegalovirus (GPCMV) replication in guinea pig embryo (GPE) cells by plaque reduction assay; these are [9-(2-hydroxy-1-3-2-dioxaphosphorinan-5-yl)oxymethyl]-guanine P-oxide (2′nor-cGMP, compound 164), [4-amino-5-bromo-7-(2-hydroxyethoxymethyl)-pyrrolo(2,3-d)pyrimidine] (compound 102), (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), 9-(2-hydroxyethoxymethyl)-guanine (acyclovir, ACV) and 3′-azido-3′-deoxythymidine (zidovudine, AZT). Various degrees of interactions were observed; i.e. synergistic reactions were noted in the presence of compound 164/compound 102 and compound 164/DHPG combinations at all concentrations tested. HPMPC/DHPG combinations were synergistic at relatively lower concentrations of DHPG, but became antagonistic as the concentration of DHPG increased. Combinations of compound 164/ACV and DHPG/AZT were antagonistic.

Combined antiviral effect; Guinea pig cytomegalovirus; Nucleoside; Dose-effect analysis

Introduction

Human cytomegalovirus (HCMV) infections can produce serious consequences, including birth defects in newborns and life- and sight-threatening diseases in im-

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munosuppressed individuals, such as organ transplantation and cancer chemotherapy recipients as well as patients with acquired immunodeficiency syndrome (Peterson et al., 1980; Preiksaitis et al., 1983; Reichert et al., 1983; Myers, 1984; Stagno et al., 1985).

Currently, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, (DHPG), also known as ganciclovir, is the only drug that is licensed for the treatment of HCMV infections in the United States. Clinical trials using interferon alone, adenine arabinoside, cytosine arabinoside, acyclovir or fluoroiodoaracytosine have shown that these agents are unsuccessful for treating HCMV infection or preventing its complications (Meyers et al., 1980, 1982; Marker et al., 1980; Balfour et al., 1982; Wade et al., 1982). DHPG has been shown to be much more potent against HCMV in vitro than is ACV (Cheng et al., 1983; Field et al., 1983; Biron et al., 1985; Freitas et al., 1985) and has been clinically used for the treatment of HCMV infection in immunocompromised patients (Collaborative DHPG Treatment Group, 1986; Keay et al., 1987; Laskin et al., 1987a,b). Unfortunately, DHPG did not prevent recrudescence after therapy was stopped and produced marrow toxicity with higher dosages (Shepp et al., 1985; Collaborative DHPG Treatment Group, 1986; Laskin et al., 1987b). In addition, the emergence of drug-resistant strains of HCMV following DHPG treatment has been reported (Biron et al., 1986).

Combination therapy has been shown to be effective against bacterial infections and certain cancers allowing a reduction in dosage and duration of treatment, thus minimizing drug-toxicity and incidence of drug-resistant strains. Numerous in vitro studies have examined the types of interaction (synergistic, additive, or antagonistic) between pairs of various anti-herpetic compounds against HSV replication (Lerner and Bailey, 1974; Bryson and Kronenberg, 1977; Stanwick et al., 1981; Schinazi et al., 1986). Soike et al. (1987, 1990) showed a synergistic effect of DHPG or FEAU in combination with human recombinant beta interferon against simian varicella infection in monkeys. A few studies using paired combinations of antiviral agents against cytomegalovirus infections have been reported (Eriksson and Schinazi, 1989; Freitas et al., 1989).

Recently, our laboratory evaluated the antiviral effects of three new promising antiherpetic compounds; compound 164, compound 102 and HPMPC against CMV infection in vitro and in vivo in a guinea pig model (Wang, 1988; Yang et al., 1989; Li et al., 1990). Our results indicate that these agents are much more potent against CMV infection than DHPG. However, some of these drugs are of limited use due to their toxic effects at higher dosage levels (Yang et al., 1989; Li et al., 1990). Thus, the use of paired combinations of these compounds with different modes of action is an attractive and logical approach for reducing drug toxicity and enhancing drug efficacy. In the present study, the effects of different paired combinations among compound 164, compound 102, DHPG, HPMPC, ACV and AZT on GPCMV replication in cultured guinea pig cells were examined.

Materials and Methods

Cell culture and virus stock

Primary guinea pig embryo (GPE) cell cultures were prepared from 30- to 40-day old embryos of Hartley guinea pigs as previously described (Hsiung, 1982). Cells were grown in Eagle's minimal essential medium in Hanks balanced salt solution (MEMH) supplemented with 10% heat inactivated newborn bovine serum and maintained in MEM-Earle's balanced salt solution (MEME) with 2% newborn bovine serum when the cell monolayers were confluent. Primary and secondary GPE cell cultures at passage levels 2 to 4 were used for all experiments. The prototype strain of GPCMV (Strain 22122; American Type Culture Collection, Rockville, MD) was used. For all experiments, cell culture-passaged virus stock containing virus infectivity titers of $10^{5.5}$ to $10^{6.5}$ 50% tissue culture infective dose ($TCID_{50}$) per 0.1 ml was used. Virus infectivity titers were determined in GPE monolayer cells grown in 24-well cell culture panels by plaque formation assay. Virus infectivity titers were expressed in PFU/0.1 ml or $\log_{10}$ $TCID_{50}$ per 0.1 ml.

Antiviral agents

Compound 164 and DHPG were kindly supplied by Merck Sharp & Dohme Research Laboratories, Rahway, NJ; ACV from Burroughs Wellcome Co, Research Triangle Park, NC; Compound 102 was kindly supplied by Drs J.C. Drach and L.B. Townsend of the University of Michigan, Ann Arbor; HPMPC was obtained from Bristol Myers Co; and AZT was supplied by Dr S.H. Chu of Brown University. All compounds were dissolved directly in the culture medium and filtered through Millipore filter membranes with pore size of 0.2 μm, and stored at $-20°C$ as stock solutions.

Plaque reduction assay

Confluent monolayers of GPE cells in 24-well cell culture panels were infected with approximately 50–70 PFU of GPCMV per well. After two hours of adsorption at 7°C, the overlay medium consisting of MEME with 5% newborn bovine sera and 0.5% methylcellulose, and each of the antiviral agents alone or in paired combinations was added to each well. Four duplicate wells were used for each drug concentration and for each set of controls. The infected cell cultures were incubated for 10 to 12 days. The monolayers were then fixed with 10% buffered formalin and stained with crystal violet. The number of plaques was counted using an inverted microscope and the percentage of inhibition by the compounds of virus plaque formation was calculated.

Analysis of effect of paired combinations of antiviral agents

To determine quantitatively whether synergistic, additive or antagonistic antiviral effect was achieved in GPCMV-infected cell cultures when treated with different

TABLE 1

Combined effects of compounds 164 and 102 on GPCMV replication and their CI values

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}		ED_{70}		ED_{90}		CI[b] at fa		
	Drug 1	Drug 2	Drug 1	Drug 2	Drug 1	Drug 2	0.5	0.7	0.9
Compound 164 alone	1.54	NA[c]	2.28	NA	4.25	NA	NA	NA	NA
Compound 102 alone	NA	165.43	NA	>400	NA	>400	NA	NA	NA
164/102 (1:20)	0.84 +	16.75	1.05 +	20.90	1.48 +	29.75	0.64 (0.70)	0.47 (0.48)	0.35 (0.35)
164/102 (1:40)	0.62 +	24.89	0.86 +	34.40	1.43 +	57.58	0.55 (0.61)	0.40 (0.41)	0.34 (0.34)
164/102 (1:80)	0.63 +	50.50	0.99 +	79.38	2.04 +	163.21	0.71 (0.83)	0.49 (0.51)	0.48 (0.48)

[a] ED_{50}, ED_{70} and ED_{90} are 50, 70 and 90% effective doses by plaque reduction assay in GPE cells. Virus input was 50–70 PFU per well. Drug 1, compound 164; drug 2, DHPG.

[b] CI <1, synergy; CI=1, additive effect; CI >1, antagonism. fa is the fraction of the viral replication affected, (e.g., 0.5 means the CI at 50% inhibition of virus replication). CI values for GPCMV-infected cells were determined using both the mutually exclusive (first entries) and the mutually nonexclusive (second entries in parentheses) assumptions which are based on the classical isobologram and the conservative isobologram equations, respectively (Chou and Talalay, 1981, 1984).

[c] NA, not applicable.

paired combinations of test compounds by plaque reduction assay, the multiple drug dose-effect analysis developed by Chou and Talalay (1981, 1984) was used. Dose-effect curves for each compound alone and in pairs were evaluated using fixed ratios of the drugs tested in multiply diluted combinations (i.e. 1:10, 1:20 etc). Interactions between tested paired drug combinations were determined using the Combination Index (CI) for both mutually exclusive (similar modes of action) and mutually nonexclusive (different modes of action) assumptions. The classical isobologram equation conforms with the mutually exclusive assumption, whereas the conservative isobologram conforms with the mutually nonexclusive assumption (Chou and Talalay, 1981,1984). Since uncertainty may exist as to whether the drugs acted in similar or independent manners, CI values were calculated under both assumptions and compared. Values of CI less than 1 indicated synergy, a CI equal to 1 indicated an additive effect, and CI values greater than 1 indicated antagonism. A computer program developed for IBM microcomputers (Chou and Chou, 1986) was used for automated analysis of all dose-effect data reported in this study. Additional details of the method have been reported previously (Schinazi et al., 1986; Hartshorn et al., 1987; Vogt et al., 1987,1988; Johnson et al., 1989a,b; Lin et al., 1989; Hayashi et al., 1990).

Determination of cell cytotoxicity

Various concentrations of each test compound either alone or in combinations were added to confluent GPE cell monolayer cultures in 24-well panels and maintained for 3 days. Viable cell counts of each culture in duplicates were determined by Trypan blue exclusion method using a hemacytometer. Prior to cell counts, monolayer cultures of the drug treated and untreated controls were removed from the plastic surface by a mixture of 0.25% trypsin and 0.02% EDTA for 0.5-1 minute. Cells were resuspended in culture medium and number of viable cells counted. The drug concentrations required to reduce the viable cell counts to 50% that of the control cultured cells were calculated using dose-effect analysis with microcomputer (Chou and Chou, 1986).

Results

Combined effects of compounds 164 and 102 on GPCMV replication

The combined effects of compounds 164 and 102 on GPCMV replication in GPE cells by plaque reduction assay are shown in Table 1. Fifty percent inhibition of GPCMV replication (i.e. 50% effective dose, ED_{50}) was observed at 1.54 μM and 165 μM when compound 164 and compound 102 were tested alone, respectively, and at 0.84, 0.62 and 0.63 μM when combinations of compound 164 and compound 102 were tested at ratios of 1:20, 1:40 and 1:80, respectively. ED_{70} and ED_{90} (70% and 90% inhibition of virus replication) were also determined from dose-effect plots. The CI values were calculated using both the mutually exclusive and

mutually nonexclusive assumptions. As shown in Table 1, CI values of the three combinations of compounds 164 and 102 at ED_{50}, ED_{70} and ED_{90} were all less than 1, strongly suggesting synergistic effects. Computer-generated CI values for the studies on three paired combinations of compounds 164 and 102 tested produced similar synergistic effect patterns and are shown in Fig. 1A.

Combined effects of compound 164 and DHPG against GPCMV replication

The ED_{50}, ED_{70} and ED_{90} of compound 164 and DHPG tested alone or in paired

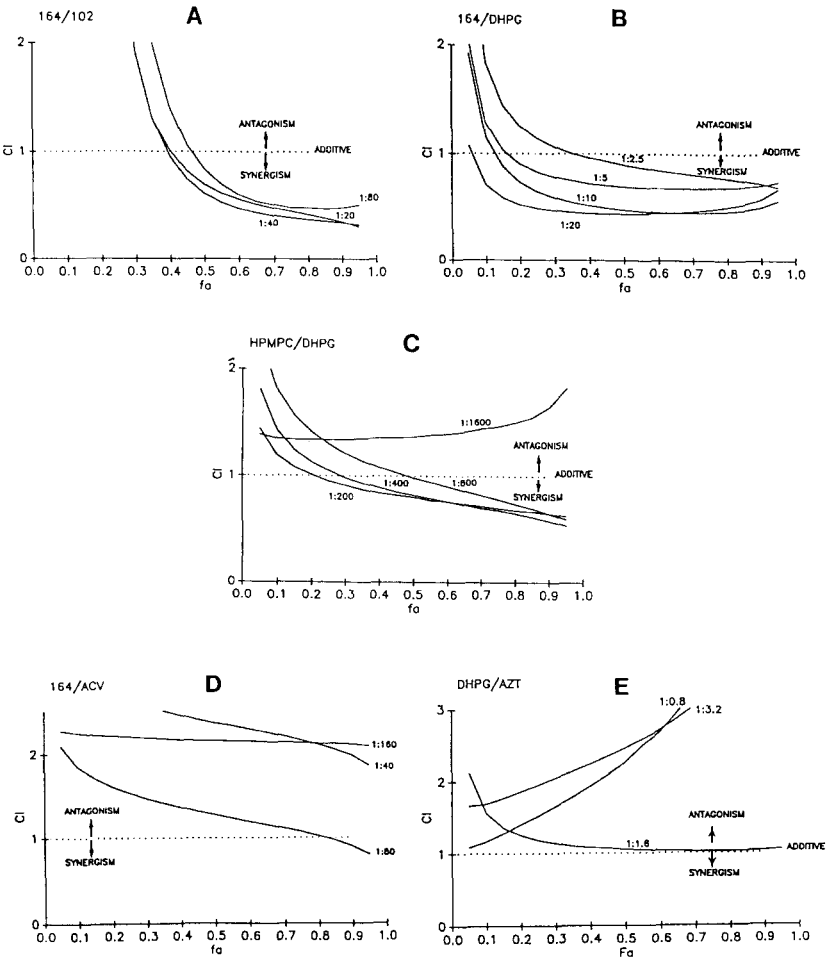

Fig. 1. Examples of computer-generated presentation of the combination indices (CI) with respect to the fraction affected (fa) for the inhibition of GPCMV replication in cultured GPE cells at different molar ratios of each combination. Conservative isobologram equations (mutually non-exclusive assumptions) were used for CI calculations: (A) compound 164 and compound 102, (B) compound 164 and DHPG, (C) HPMPC and DHPG, (D) compound 164 and ACV and (E) DHPG and AZT.

combinations at four different molar ratios (1:2.5, 1:5, 1:10 and 1:20) on GPCMV replication, and their combination index values giving 50, 70 or 90% inhibition of virus replication are shown in Table 2. The ED_{50}, ED_{70} and ED_{90} of compound 164 in all four combinations were 1- to 3-fold lower than that of compound 164 when tested alone at 1.29 (ED_{50}), 1.92 (ED_{70}), and 3.60 (ED_{90}) μM. Concentrations of compound 164 required for the combination increased relative to the increase in inhibition of virus replication. For DHPG, the ED_{50}, ED_{70}, and ED_{90} in all four different combinations were also much lower than that of the drug used alone. The CI values obtained are shown in Table 2. Synergistic effects for all combinations of compound 164 and DHPG were demonstrated. A more synergistic effect was noted with increasing ratio of combination of the two compounds suggesting that the effective contribution of compound 164 was more emphasized in the observed antiviral effects as illustrated in Fig. 1B.

Combined effects of HPMPC and DHPG against GPCMV replication

Although antiviral activities of both HPMPC and DHPG are achieved by inhibition of viral DNA polymerase, the two drugs act independently on different steps of the virus replication cycle. As summarized in Table 3, DHPG was found to inhibit 50% virus plaque formation with an observed ED_{50} of 58.77 μM. For HPMPC, the corresponding ED_{50} of 0.08 μM was approximately 700 times lower than that of DHPG. Therefore, the interactions between the two drugs were investigated at four different combinations with ratios of 1:200, 1:400, 1:800 and 1:1600. In all combinations, the concentrations of HPMPC required for 50–90% inhibition of virus replication were significantly lower than when HPMPC was used alone. Similar results were noted for DHPG. Three combinations of HPMPC and DHPG at molar ratios of 1:200, 1:400 and 1:800 demonstrated synergistic effects which were concentration dependent when virus replication was inhibited between 50 and 90% (Table 3). At a ratio of 1:1600, concentrations of the two drugs HPMPC/DHPG, at ED_{50}, ED_{70} and ED_{90} were still lower than when tested alone, but produced CI values greater than 1 indicating an antagonistic effect as shown in Fig. 1C.

Combined effects of compound 164 and ACV against GPCMV replication

Since compound 164 and ACV are structurally different and presumably inhibit CMV replication by different mechanisms, the interaction of the two drugs was studied at three different combinations with molar ratios of 1:40, 1:80 and 1:160. Table 4 shows that compound 164 alone inhibited GPCMV replication with ED_{50}, ED_{70} and ED_{90} values of 1.37, 2.05, and 3.87 μM, respectively, but only slightly lower or higher ED_{50}, ED_{70} and ED_{90} values of compound 164 were observed in all three combinations. Even though ACV values for ED_{50}, ED_{70} and ED_{90} in all combinations were lower than when tested alone (Table 4), most combinations with compound 164 produced CI values greater than 1 when virus replication was inhibited between 50 and 90% strongly suggesting an antagonistic effect (Fig. 1D).

TABLE 2

Combined effects of compound 164 and DHPG on GPCMV replication and their CI values

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}		ED_{70}		ED_{90}		CI[b] at fa		
	Drug 1	Drug 2	Drug 1	Drug 2	Drug 1	Drug 2	0.5	0.7	0.9
Compound 164 alone	1.29	NA[c]	1.92	NA	3.60	NA	NA	NA	NA
DHPG alone	NA	62.29	NA	>100	NA	>100	NA	NA	NA
164/DHPG (1:2.5)	1.06 +	2.64	1.49 +	3.73	2.59 +	6.48	0.86 (0.89)	0.79 (0.81)	0.72 (0.73)
164/DHPG (1:5)	0.77 +	3.84	1.21 +	6.07	2.50 +	12.58	0.66 (0.69)	0.66 (0.68)	0.71 (0.71)
164/DHPG (1:10)	0.44 +	4.40	0.79 +	7.92	2.02 +	20.19	0.41 (0.44)	0.45 (0.46)	0.57 (0.58)
164/DHPG (1:20)	0.40 +	7.91	0.69 +	13.89	1.70 +	34.02	0.43 (0.47)	0.43 (0.45)	0.49 (0.50)

[a] ED_{50}, ED_{70} and ED_{90} are 50, 70 and 90% effective doses by plaque reduction assay in GPE cells. Virus input was 50–70 PFU per well, drug 1, compound 164; drug 2 DHPG.

[b] $CI < 1$, synergy; $CI = 1$, additivity; $CI > 1$, antagonism. fa means the fractional inhibition of virus replication. CI values for GPCMV-infected cells were determined using both mutually exclusive (first entries) and the mutually nonexclusive (second entries in parentheses) assumptions which are based on the classical isobologram and the conservative isobologram equations, respectively (Chou and Talalay, 1981, 1984).

[c] NA, not applicable.

TABLE 3

Combined effects of HPMPC and DHPG on GPCMV replication and their CI values

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}		ED_{70}		ED_{90}		CI^b at fa		
	Drug 1	Drug 2	Drug 1	Drug 2	Drug 1	Drug 2	0.5	0.7	0.9
HPMPC alone	0.08	NA[c]	0.11	NA	0.16	NA	NA	NA	NA
DHPG alone	NA	58.77	NA	108.65	NA	289.24	NA	NA	NA
HPMPC/DHPG (1:200)	0.05 +	9.49	0.06 +	11.59	0.08 +	15.96	0.73 (0.81)	0.65 (0.70)	0.56 (0.58)
HPMPC/DHPG (1:400)	0.04 +	14.66	0.05 +	19.59	0.08 +	31.09	0.69 (0.79)	0.64 (0.72)	0.06 (0.65)
HPMPC/DHPG (1:800)	0.03 +	25.52	0.04 +	33.42	0.06 +	51.35	0.82 (0.98)	0.70 (0.82)	0.58 (0.65)
HPMPC/DHPG (1:1600)	0.03 +	45.44	0.05 +	75.73	0.11 +	170.89	1.11 (1.37)	1.14 (1.44)	1.26 (1.65)

[a] ED_{50}, ED_{70} and ED_{90} are 50, 70, and 90% effective doses by plaque reduction assay in GPE cells. Virus input was 50–70 PFU per well, drug 1, HPMPC; drug 2, DHPG.

[b] $CI < 1$, synergy; $CI = 1$, additivity; $CI > 1$, antagonism. fa means the fractional inhibition of virus replication. CI values for GPCMV-infected cells were determined using both mutually exclusive (first entries) and the mutually nonexclusive (second entries in parentheses) assumptions which are based on the classical isobologram and the conservative isobologram equations, respectively (Chou and Talalay, 1981, 1984).

[c] NA, not applicable.

TABLE 4

Combined effects of compound 164 and ACV on GPCMV replication and their CI values

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}		ED_{70}		ED_{90}		CI[b] at fa		
	Drug 1	Drug 2	Drug 1	Drug 2	Drug 1	Drug 2	0.5	0.7	0.9
Compound 164 alone	1.37	NA[c]	2.05	NA	3.87	NA	NA	NA	NA
ACV alone	NA	236.84	NA	320.58	NA	519.29	NA	NA	NA
164/ACV (1:40)	2.06 +	82.34	2.81 +	112.61	4.63 +	185.39	1.85 (2.36)	1.73 (2.21)	1.55 (1.98)
164/ACV (1:80)	0.98 +	78.26	1.25 +	100.13	1.85 +	148.29	1.04 (1.28)	0.92 (1.1)	0.76 (0.90)
164/ACV (1:160)	1.11 +	177.68	1.56 +	250.32	2.70 +	432.18	1.56 (2.16)	1.54 (2.14)	1.53 (2.11)

[a] ED_{50}, ED_{70} and ED_{90} are 50, 70 and 90% effective doses by plaque reduction assay in GPE cells. Virus input was 50–70 PFU per well, drug 1, compound 164; drug 2, ACV.

[b] CI <1, synergy; CI= 1, additivity; CI >1, antagonism; fa means the fractional inhibition of virus replication. CI values for GPCMV-infected cells were determined using both the mutually exclusive (first entries) and the mutually nonexclusive (second entries in parentheses) assumptions which are based on the classical isobologram and the conservative isobologram equations, respectively (Chou and Talalay, 1981, 1984).

[c] NA, not applicable.

Combined effects of DHPG and AZT against GPCMV replication

Opportunistic HCMV infection is one of the major problems in individuals with AIDS. DHPG has been used with some success as possible treatment against HCMV infection. At present, AZT is the only drug that proved to be of use for the treatment of patients with AIDS. To study whether AZT would interfere with the anti-CMV effect of DHPG, combined effects of three combinations (1:0.8, 1:1.6 and 1:3.2) of the two drugs on GPCMV plaque formation were studied. AZT was found to be virtually without significant inhibitory effect on GPCMV replication even at concentrations up to 320 μM (data not shown). A dose-dependent inhibition was obtained for DHPG with an ED_{50} of 26.74 μM (Table 5). Although AZT did not significantly inhibit GPCMV replication, an ED_{50} of 675.1 μM was extrapolated from the dose-effect curve in order to calculate the CI values for the studies on the combinations. In all combinations, ED_{50}, ED_{70} and ED_{90} values of DHPG were about equal or greater as compared with that of DHPG alone. These data indicated that AZT probably interfered with the anti-GPCMV effect of DHPG to some degree. CI values obtained were all greater than 1.0 clearly indicating an antagonistic effect for all combinations (Fig. 1E).

Cytotoxicity effect on uninfected GPE cells

Results of cytotoxicity tests and calculated CyD_{50} values for each compound alone or in combination are listed in Table 6. CyD_{50} concentrations were calculated using dose effect analysis. There was no evidence of toxicity on confluent monolayers of cultured GPE cells at the concentrations tested in each experiment since all ED_{50} values were markedly lower than their corresponding CyD_{50} values.

Discussion

Several promising new antiviral agents, compound 164, compound 102, and HPMPC, were evaluated and compared with DHPG for their antiviral effects against CMV infections in the guinea pig model in vitro and in vivo (Fong et al., 1987; Wang, 1988; Yang et al., 1989; Li et al., 1990). From these studies, compound 164 was shown to be 20-fold more active but only 10-fold more selective (therapeutic index) than DHPG as an inhibitor of GPCMV replication in vitro; it is 8- to 10-fold more potent than DHPG against GPCMV infection in vivo. However, severe toxic effects on kidneys of guinea pigs limit its further use (Yang et al., 1989). One approach to solve this problem has been the application of a combination of chemotherapeutic agents. Therefore, experiments in the present study were designed to test antiviral effects of compound 164 in combination with compound 102, DHPG and ACV in GPE cell cultures. The results presented here demonstrated that when compound 164 was combined with compound 102 or DHPG at all ratios tested, clear synergistic antiviral effects were obtained with all CI values less than 1.0 (Figure 1, A and B). In these experiments, concentrations of compound 164 at

TABLE 5

Combined effects of DHPG and AZT on GPCMV replication and their CI values

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}		ED_{70}		ED_{90}		CI[b] at fa		
	Drug 1	Drug 2	Drug 1	Drug 2	Drug 1	Drug 2	0.5	0.7	0.9
DHPG alone	26.74	NA[c]	48.91	NA	123.21	NA	NA	NA	NA
AZT alone	NA	675.09*	NA	>320	NA	>320	NA	NA	NA
DHPG/AZT (1:0.8)	59.72 +	47.78	138.51 +	110.81	529.17 +	423.34	2.3 (2.46)	2.9 (3.04)	4.3 (4.40)
DHPG/AZT (1:1.6)	25.08 +	40.13	46.56 +	74.50	124.77 +	199.64	1.0 (1.05)	1.0 (1.02)	1.02 (1.03)
DHPG/AZT (1:3.2)	45.12 +	144.39	128.74 +	411.95	684.04 +	2188.94	1.9 (2.48)	2.83 (3.73)	5.65 (7.56)

[a] ED_{50}, ED_{70} and ED_{90} are 50, 70 and 90% effective doses by plaque reduction assay in GPE cells; virus input was 50–70 PFU per well, drug 1, DHPG; drug 2, AZT.

[b] CI <1, synergy; CI=1, additivity; CI >1, antagonism; fa means the fractional inhibition of virus replication. CI values for GPCMV-infected cells were determined using both the mutually exclusive (first entries) and the mutually nonexclusive (second entries in parentheses) assumptions which are based on the classical isobologram and the conservative isobologram equations, respectively (Chou and Talalay, 1981, 1984).

[c] NA, not applicable.

* Extrapolated value from dose-effect curve. Highest concentration tested was 320 μM.

TABLE 6

Cytotoxicity tests of each drug alone or in combination on uninfected confluent GPE cell monolayers and their therapeutic indices

Experiment No.[a]	Compound tested	CyD_{50} (μM)[b]			Therapeutic index[c]
		Drug 1		Drug 2	
1	Compound 164 alone	189		NA[f]	122
	Compound 102 alone	NA		896	5
	164/102 (1:20)[d]	40	+	795	47
2	Compound 164 alone	189		NA	146
	DHPG alone	NA		746	11
	164/DHPG (1:10)	67	+	667	151
3	HPMPC alone	153		NA	1821
	DHPG alone	NA		746	12
	HPMPC/DHPG (1:200)	10	+	2046	216
4	Compound 164 alone	189		NA	137
	ACV alone	NA		656	3
	164/ACV (1:40)	54	+	2186	27
5	DHPG alone	746		NA	27
	AZT alone	NA		395	[e]
	DHPG/AZT (1:0.8)	865	+	659	14

[a]Experiment numbers and ED_{50} values of each drug or in combinations are listed in Tables 1–5.
[b]CyD_{50} concentrations were calculated using dose-effect analysis for each drug alone and in combination. CyD_{50} of Compound 164 and DHPG each was an average of three experiments.
[c]Therapeutic index = CyD_{50}/ED_{50} (For the combinations, CyD_{50} and ED_{50} values are combined concentrations of drug 1 and drug 2.
[d]Ratios were chosen based on drug solubility at high molarities required by the assay and availability of each drug tested.
[e]ED_{50} of AZT was an extrapolated value from dose-effect curve; therefore, therapeutic index cannot be calculated.
[f]NA, not applicable.

50, 70 and 90% inhibition of virus replication were about 1- to 3-fold lower than when the drug was used alone. The ED_{50}, ED_{70} and ED_{90} of either compound 102 or DHPG in combination with compound 164 were also lower than when they were tested alone and were not toxic to uninfected GPE cells. In contrast, compound 164 in combination with ACV consistently exhibited additive to antagonistic effects for the three ratios tested (Table 4). The reason for the antagonism due to the combination of compound 164/ACV is not known.

The exact mechanism by which the combination of compound 164 with compound 102 or DHPG produced a synergistic antiviral effect is also unknown. Previous studies have suggested that compound 164 inhibits viral DNA synthesis while compound 102 seems to be both a protein and DNA inhibitor on GPCMV replication (Yang et al., 1990). Although the antiviral activities of both compound 164 and DHPG against CMV replication may be due to the inhibition of viral DNA synthesis, their inhibitory effects are achieved by different pathways (Oliver et al.,

1985; Tolman et al., 1985; Germershausen et al., 1986). Therefore, the synergistic antiviral effects of combinations of compound 164/compound 102 or compound 164/DHPG may include inhibitory effects on viral replication by two or more different pathways.

Another antiviral agent, HPMPC, has been shown to be approximately 5- and 35-fold more potent than DHPG against HCMV and GPCMV in vitro, respectively (Li et al., 1990). Similarly, HPMPC is highly potent against murine CMV in vitro and in vivo as demonstrated by other investigators (Snoeck et al., 1988; Bronson et al., 1989). Although our data indicated that HPMPC was highly effective against GPCMV infection in cultured cells, this compound was highly toxic to guinea pigs when tested at dosages of 5 mg/kg/day or greater. Since combination therapy allows decreased dosage and duration of treatment, thus reducing toxicity (Lerner and Bailey, 1974; Bryson and Kronenberg, 1977; Stanwick et al., 1981; Schinazi and Nahmias, 1982), we investigated the combined use of HPMPC and DHPG against GPCMV replication. Our results demonstrated that HPMPC combined with DHPG at three ratios (1:200, 1:400 and 1:800) had a synergistic antiviral effect whereas an antagonistic effect was observed at a ratio of 1:1600 (Fig. 1C). This suggests that the drug ratio of each compound concentration can be an important factor in determining whether agents have potential synergistic chemotherapeutic value.

Among the herpes viruses, HCMV is recognized as one of the major causes of opportunistic infections in patients with AIDS. It is not without reason therefore, that a concomitant treatment with DHPG against HCMV in HIV-infected individuals undergoing AZT therapy is a logical chemotherapeutic approach. Thus, it makes it important to determine the type of interaction produced by the combination of DHPG and AZT. A similar approach has been investigated using a combination of AZT and Foscarnet against HIV-1 and HCMV in vitro (Eriksson and Schinazi, 1989). Our results indicated that the combination of DHPG and AZT at a ratio of 1:1.6 produced an additive antiviral effect, while ratios of 1:0.8 and 1:3.2 had an antagonistic effect, with ED_{50}s approximately 2-fold greater than when DHPG was tested alone (Fig. 1E). Why AZT interferes with the antiviral effect of DHPG against GPCMV when used in combination is not known. Because both AZT and DHPG are phosphorylated intracellularly by cellular enzymes (Matthews and Boehme, 1988, Koshida et al., 1989), we surmised that AZT, or in one of its phosphorylation steps, might competitively inhibit the phosphorylation of DHPG.

Results obtained from our investigations of paired combinations of compounds in the treatment of GPCMV infection in vitro demonstrated that three different paired combinations, such as compound 164/compound 102, compound 164/DHPG and HPMPC/DHPG, produced significant synergistic antiviral effects. These findings suggest that combined antiviral therapy might be useful in the treatment of HCMV infections.

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References

Balfour, H.H. Jr., Bean, B., Mitchell, C.D., Sachs, G.W., Boen, J.R. and Edelman, C.K. (1982) Acyclovir in immunocompromised patients with cytomegalovirus disease. A controlled trial at one institution. Am. J. Med. Acyclovir Symposium, 73 (Suppl. 1A), 241–248.

Biron, K.K., Stanat, S.C., Sorrell, J.B., Fyfe, J.A., Keller, P.M., Lambe, C.U. and Nelson, D.J. (1985) Metabolic activation of the nucleoside analog 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)-guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. USA, 82, 2473–2477.

Biron, K.K., Fyfe, J.A., Stanat, S.C., Leslie, L.K., Sorrell, J.B., Lambe, C.U. and Coen, D.M. (1986) A human cytomegalovirus mutant resistant to the nucleoside analog 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine (BW B759U) induces reduced levels of BW B759U triphosphate. Proc. Natl. Acad. Sci. USA, 83, 8769–8773.

Bronson, J.J., Ghazzouli, I., Hitchcock, M.J., Webb, R.R., II and Martin, J.C. (1989) Synthesis and antiviral activity of nucleotide analogue (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl] cytosine. J. Med. Chem. 32, 1457–1463.

Bryson, Y.J. and Kronenberg, L.H. (1977) Combined antiviral effects of interferon, adenine arabinoside, hypoxanthine arabinoside, and adenine arabinoside-5′-monophosphate in human fibroblast cultures. Antimicrob. Agents Chemother. 11, 299–306.

Cheng, Y.C., Huang, E.S., Lin, J.C., Mar, E.C., Pagano, J.S., Dutschman, G.E. and Grill, S.P. (1983) Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine against herpesviruses in vitro and its mode of action against herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 80, 2767–2770.

Chou, J. and Chou, T.C. (1987) Dose-effect analysis with microcomputers: Quantitation of ED_{50}, LD_{50}, synergism, antagonism, low dose risk, receptor-ligand binding and enzyme kinetic. Computer software for IBM PC Series. Elsevier-Biosoft, Cambridge, U.K.

Chou, T.C. and Talalay, P. (1981) Generalized equations for the analysis of inhibitors of Michaelis-Menten and higher order kinetic systems with two or more mutually exclusive and non-exclusive inhibitors. Eur. J. Biochem. 115, 207–216.

Chou, T.C. and Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.

Collaborative DHPG Treatment Study Group (1986) Treatment of serious cytomegalovirus infections with 9-(1,3-dihydroxy-2-propoxymethyl)guanine in patients with AIDS and other immunodeficiencies. N. Engl. J. Med. 314, 801–805.

Eriksson, B.F.H. and Schinazi, R.F. (1989) Combinations of 3′-Azido-3′-Deoxythymidine (Zidovudine) and Phosphonoformate (Foscarnet) against human immunodeficiency virus type 1 and cytomegalovirus replication in vitro. Antimicrob. Agents Chemother. 33, 663–669.

Field, A.K., Davies, M.E., DeWitt, C., Perry, H.C., Liou, R., Germershausen, J., Karkas, J.D., Ashton, W.T., Johnston, D.B. and Tolman, R.L. (1983) 9-([2-Hydroxy-1-(hydroxymethyl)ethoxy]methyl)-guanine: a selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. USA 80, 4139–4143.

Fong, C.K.Y., Cohen, S.D., McCormick, S. and Hsiung, G.D. (1987) Antiviral effect of 9(1,3-dihydroxy-2-propoxymethyl)guanine against cytomegalovirus infection in a guinea pig model. Antiviral Res. 7, 11–23.

Freitas, V.R., Smee, D.F., Chernow, M., Boehme, R. and Matthews, T.R. (1985) Activity of 9(1,3-

dihydroxy-2-propoxymethyl)guanine compared with that of acyclovir against human, monkey, and rodent cytomegaloviruses. Antimicrob. Agents Chemother. 28, 240–245.

Freitas, V.R., Fraser-Smith, E.B. and Matthews, T.R. (1989) Increased efficacy of ganciclovir in combination with foscarnet against cytomegalovirus and herpes simplex virus type 2 in vitro and in vivo. Antiviral Res. 12, 205–212.

Germershausen, J., Bostedor, R., Liou, R., Field, A.K., Wagner, A.F., MacCoss, M., Tolman, R.L. and Karkas, J.D. (1986) Comparison of the modes of antiviral action of 2′-nor-deoxyguanosine and its cyclic phosphate, 2′-nor-cyclic GMP. Antimicrob. Agents Chemother. 29, 1025–1031.

Hartshorn, K.L., Vogt, M.W., Chou, T.C., Blumberg, R.S., Byington, R., Schooley, R.T. and Hirsch, M.S. (1987) Synergistic inhibition of human immunodeficiency virus in vitro by azidothymidine and recombinant interferon alpha-A. Antimicrob. Agents Chemother. 31, 168–172.

Hayashi, S., Fine, R., Chou, T.C., Currens, M.J., Broder, S. and Mitsuya, H. (1990) In vitro inhibition of the infectivity and replication of human immunodeficiency virus type-1 by combination of anti-retroviral 2′,3′-dideoxynucleosides and viral binding inhibitors. Antimicrob. Agents Chemother. 34, 82–88.

Hsiung, G.D. (1982) Diagnostic Virology, p. 249. Yale University Press, New Haven, Connecticut.

Johnson, V.A., Barlow, M.A., Chou, T.C., Fisher, R.A., Walker, R.D., Hirsch, M.S. and Schooley, R.T. (1989a) Synergistic inhibition of human immunodeficiency virus type 1 (HIV-1) replication in vitro by recombinant soluble CD4 and 3′-azido-3′-deoxythymidine. J. Infect. Dis. 159, 837–844.

Johnson, V.A., Walker, B.D., Barlow, M.A., Paradis, T.J., Chou, T.C. and Hirsch, M.S. (1989b) Synergistic inhibition of human immunodeficiency virus type 1 and type 2 replication in vitro by castanospermine and 3′-azido-3′-deoxythymidine. Antimicrob. Agents Chemother. 33, 53–57.

Keay, S., Bissett, J. and Merigan, T.C. (1987) Ganciclovir treatment of cytomegalovirus infections in iatrogenically immunocompromised patients. J. Infect. Dis. 156, 1016–1021.

Koshida, R., Vrang, L., Gilljam, G., Harmenberg, J., Oberg, B.O. and Wahren, B. (1989) Inhibition of human immunodeficiency virus in vitro by combinations of 3′-azido-3′-deoxythymidine and foscarnet. Antimicrob. Agents Chemother. 33, 778–780.

Laskin, O.L., Cederberg, D.M., Mills, J., Eron, L.J., Mildvan, D. and Spector, S.A. (1987a) Ganciclovir for the treatment and suppression of serious infections caused by cytomegalovirus. Am. J. Med. 83, 201–207.

Laskin, O.L., Stahl-Bayliss, C.M., Kalman, C.M. and Rosecan, L.R. (1987b) Use of ganciclovir to treat serious cytomegalovirus infections in patients with AIDS. J. Infect. Dis. 155, 323–327.

Lerner, A.M. and Bailey, E.J. (1974) Synergy of 9-β-D-arabinofuranosyladenine and human interferon against herpes simplex virus type 1. J. Infect. Dis. 130, 549–552.

Li, S.B., Yang, Z.H., Feng, J.S., Fong, C.K.Y., Lucia, H.L. and Hsiung, G.D. (1990) Activity of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC) against guinea pig cytomegalovirus infection in cultured cells and in guinea pigs. Antiviral Res. 13, 237–252.

Lin, J.C., Zhang, Z.X., Chou, T.C., Sim, I. and Pagano, J.S. (1989) Synergistic inhibition of Epstein-Barr virus transformation of B-lymphocytes by alpha and gamma interferon and 3′-azido-3′-deoxythymidine. J. Infect. Dis. 159, 248–254.

Marker, S.C., Howard, R.J., Groth, K.E., Mastri, A.R., Simmons, R.L. and Balfour, H.H., Jr (1980) A trial of vidarabine for cytomegalovirus infection in renal transplant patients. Arch. Intern. Med. 140, 1441–1444.

Matthews, T. and Boehme, R. (1988) Antiviral activity and mechanism of action of ganciclovir. Rev. Infect. Dis. 10, S490–S493.

Meyers, J.D., McGuffin, R.W., Neiman, P.E., Singer, J.W. and Thomas E.D. (1980) Toxicity and efficacy of human leukocyte interferon for treatment of cytomegalovirus pneumonia after marrow transplantation. J. Infect. Dis. 141, 555–562.

Meyers, J.D., McGuffin, R.W., Bryson, Y.J. Cantell, K. and Thomas, E.D. (1982) Treatment of cytomegalovirus pneumonia after marrow transplant with combined vidarabine and human leukocyte interferon. J. Infect. Dis. 146, 80–84.

Meyers, J.D., (1984) Cytomegalovirus infection following marrow transplantation: risk, treatment and prevention. In: S.A. Plotkin, S. Michelson, J.S. Pagano and F. Rapp (eds), CMV: Pathogenesis and Prevention of Human Infection, pp. 101–117. Alan R. Liss, New York.

Oliver, S., Bubley, G. and Crumpacker, C. (1985) Inhibition of HSV-transformed murine cells by

nucleoside analogs 2′-NDG and 2′-nor-cGMP: Mechanism of inhibition and reversal by exogenous nucleosides. Virology 145, 84–93.

Peterson, P.K., Balfour, H.H., Jr., Marker, S.C., Fryd, D.S., Howard, R.J. and Simmons, R.L. (1980) Cytomegalovirus disease in renal allograft recipients: a prospective study of the clinical features, risk factors and impact on renal transplantation. Medicine 59, 283–300.

Preiksaitis, J.K., Rosno, S. Grumet, C. and Merigan, T.C. (1983) Infections due to herpesviruses in cardiac transplant recipients: role of the donor heart and immunosuppressive therapy. J. Infect. Dis. 147, 974–981.

Reichert, C.M., O'Leary, T.J., Levens, D.L., Simrell, C.R. and Macher, A.M. (1983) Autopsy pathology in the acquired immune deficiency syndrome. Am. J. Pathol. 112, 357–382.

Schinazi, R.F., Chou, T.C., Scott, R.T., Yao, X. and Nahmias, A.J (1986) Delayed treatment with combinations of antiviral drugs in mice infected with herpes simplex virus and application of the median-effect method of analysis. Antimicrob. Agents Chemother. 30, 491–498.

Schinazi, R.F. and Nahmias, A.J. (1982) Different in vitro effects of dual combinations of anti-herpes simplex virus compounds. Am. J. Med. 73, 40–48.

Shepp, D.H., Dandliker, P.S., DeMiranda, P., Burnette, T.C., Cederberg, D.M., Kirk, E. and Meyers, J.D. (1985) Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine in the treatment of cytomegalovirus pneumonia. Ann. Intern. Med. 103, 368–373.

Snoeck, R., Sakuma, T., De Clercq, E., Rosenberg, I. and Holy, A. (1988) (S)-1-(3-hydroxy-2-phosphonylmethoxpropyl)cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. 32, 1839–1844.

Soike, K.F., Chou, T.-C., Fox, J.J., Watanabe, K.A. and Gloff, C.A. (1990) Inhibition of simian varicella virus infection of monkeys by 1-(2-deoxy-2-fluoro-1-B-D-arabinofuranosyl)-5-ethyluracil (FEAU) and synergistic effects of combination with human recombinant interferon-β. Antiviral Res. 13, 165–174.

Soike, K.F., Eppstein, D.A., Gloff, C.A., Cantrell, C., Chou, T.C. and Gerone, P.J. (1987) Effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine and human recombinant beta interferon alone and in combination on simian varicella infection in monkeys. J. Infect. Dis. 156, 607–614.

Stagno, S. and Whitley, R.J. (1985) Herpesvirus infections of pregnancy. Part I. Cytomegalovirus and Epstein-Barr Virus infections. N. Engl. J. Med. 313, 1270–1274.

Stanwick, T.L., Schinazi, R.F., Campbell, D.E. and Nahmias, A.J. (1981) Combined antiviral effect of interferon and acyclovir on herpes simplex virus types 1 and 2. Antimicrob. Agents Chemother. 19, 672–674.

Tolman, R.L., Field, A.K., Karkas, J.D., Wagner, A.F., Germershausen, J., Crumpacker, C. and Scolnick, E.M. (1985) 2′-Nor-cGMP: a seco-cyclic nucleotide with powerful anti-DNA-viral activity. Biochem. Biophys. Res. Commun. 128, 1329–1335.

Vogt, M.W., Durno, A.G., Chou, T.C., Coleman, L.A., Paradis, T.J., Schooley, T.T., Kaplan, J.C. and Hirsch, M.S. (1988) Synergistic interaction of 2′,3′-dideoxycytidine (ddCyd) and recombinant interferon alpha A on replication of human immunodeficiency virus type 1. J. Infect. Dis. 158, 378–385.

Vogt, M.W., Hartshorn, K.L., Furman, P.A., Chou, T.C., Fyte, J.A., Coleman, L.A., Crumpacker, C., Schooley, R.T. and Hirsch, M.S. (1987) Ribavirin antagonizes the effect of azidothymidine on HIV replication. Science 235, 1376–1379.

Wade, J.C., Hintz, M., McGuffin, R.W., Springmeyer, S.C., Connor, J.D. and Meyers, J.D. (1982) Treatment of cytomegalovirus pneumonia with high-dose acyclovir. Am. J. Med. Acyclovir Symposium 73 (Suppl. 1A), 249–256.

Wang, F. (1988) Antiviral effect of compound 102 against guinea pig cytomegalovirus infection. M.P.H. thesis, Dept. of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT.

Yang, Z.H., Klein, S. and Hsiung, G.D. (1990) Comparative effects of two acyclonucleoside derivatives on the ultrastructure and replication of guinea pig cytomegalovirus in cultured cells. Intervirology, in press.

Yang, Z.H., Lucia, H.L., Tolman, R.L., Colonno, R.J. and Hsiung, G.D. (1989) Effect of 2′Nor-cyclic GMP against guinea pig cytomegalovirus infection. Antimicrob. Agents Chemother. 33, 1563–1568.