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Combined treatment with 2′-nor-cGMP and ganciclovir against cytomegalovirus infection in a guinea pig model

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Summary

The combination 2′-nor-cGMP/DHPG at fixed ratios 1:5, 1:10 and 1:20 showed synergistic antiviral effects against GPCMV replication in vitro with CI value < 1. In vivo, a fixed ratio of 1:10 at three different dosage levels of 1.25/12.5 mg, 2.5/25 mg and 5/50 mg/kg/day 2′-nor-cGMP/DHPG combination showed only additive results when compared with each drug alone. However, synergistic antiviral effects were obtained when infected guinea pigs were treated with 2′-nor-cGMP/DHPG combination 2.5/10 mg/kg/day (1:4). A significantly lower GPCMV infectivity titer was noted in the salivary gland, lung and spleen of infected guinea pigs treated with the combination of 2′-nor-cGMP/DHPG 2.5/10 mg/kg/day, as compared to animals treated with a corresponding dose of each drug alone. In addition, GPCMV-infected animals treated with the latter combination showed increased body weight than when either drug was used alone. Histopathologically, each drug alone reduced the viral induced changes in the lung and spleen, but the combination therapy reduced these changes still further. Toxic changes seen in the kidney and bone marrow of infected animals treated with 2′-nor-cGMP, 2.5 mg/kg/day were not significantly increased when DHPG 10 mg/kg/day was added to the regimen. Therefore, combined treatment with 2′-nor-cGMP/DHPG in appropriate concentration is more helpful for acute cytomegalovirus infection in guinea pigs than when either drug was used alone.

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Combined treatment; 2′-nor-cGMP, DHPG; Cytomegalovirus; Guinea pig

Introduction

Combination chemotherapy has been widely explored as a therapeutic approach to many diseases, including infectious diseases and cancer. For viral infections, application of combination chemotherapy using drugs which have different antiviral mechanisms and/or drugs which have similar mechanisms but different sites of toxicity have been examined. This approach may enhance efficacy, minimize drug toxicity and retard the emergence of drug-resistant variants (Hayashi et al., 1990).

Ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) has been approved for the treatment of human cytomegalovirus (HCMV) infection; however, its bone marrow toxicity at high doses (Shepp et al., 1985) and the emergence of drug-resistant HCMV strains (Biron et al., 1986) limits its clinical use. A derivative of DHPG, 2′-nor-cGMP, 9-[(2-hydroxy-1,3,2-dioxaphosphorinan-5-yl)oxymethyl]-guanine P-oxide, has a different mode of action from DHPG. Unlike DHPG, its action against herpes virus is not dependent upon virus-specific thymidine kinase nor does it have any correlation with the amount of DHPG triphosphate accumulated in the cell (Germershausen et al., 1986). Furthermore, 2′-nor-cGMP was shown to be 10-fold more potent than DHPG against guinea pig cytomegalovirus (GPCMV) infection in guinea pigs but was toxic to the proximal renal tubules as well as the bone marrow in drug-treated animals (Yang et al., 1989). Previously, we reported a synergistic reaction between 2′-nor-cGMP and DHPG at several fixed ratios against GPCMV replication in cultured guinea pig embryo cells by plaque reduction assay (Yang et al., 1990). Therefore, the combined antiviral effects of 2′-nor-cGMP and DHPG at various dosages against GPCMV infection were examined in cultured cells and in guinea pigs.

Materials and Methods

Cell culture and virus strain

Primary guinea pig embryo (GPE) cells were prepared from 30- to 40-day-old embryos of Hartley guinea pigs (Camm Research Institute, Wayne, NJ) as described previously (Hsiung, 1982). The cells were initially grown in Eagle's minimal essential medium in Hanks' balanced salt solution (MEMH) supplemented with 10% heat-inactivated newborn bovine serum (NBS). When the cell monolayers were confluent, the growth medium was replaced with maintenance medium which consisted of Eagle's medium in Earle's balanced salt solution (MEME) supplemented with 2% NBS. GPE cells at passage levels 1–3 were used in all experiments. For in vitro examinations,

GPCMV strain 22122 was routinely prepared in GPE cells and the stock virus infectivity titer was within the range of 6.5–7.0 $\log_{10}$ 50% tissue culture infective dose per 0.1 ml ($TCID_{50}$/0.1 ml).

Plaque reduction assay

Confluent monolayers of GPE cells in 24-well panels were infected with GPCMV at approximately 50–70 PFU per well. After 2 h adsorption at 36°C, infected cells were overlaid with MEME containing 5% newborn bovine serum, 0.5% methylcellulose, and various concentration of drugs (2′-nor-cGMP, DHPG each alone or 2′-nor-cGMP/DHPG combinations at fixed ratios 1:5, 1:10 and 1:20). Ten days later, the overlay medium was removed, the monolayers were fixed and stained with a fixative fluid containing 5% formalin and 1.3% crystal violet and plaques were enumerated. Using the number of virus plaques counted in the control as a reference (infected and untreated cultures), the drug concentration required to reduce the number of plaques by 50% (ED_{50}) was calculated by dose–effect analysis with a microcomputer (Chou and Chou, 1987).

Animal inoculation

Young female Hartley guinea pigs approximately one month old (300 ± 20 g) were purchased from Camm Research Institute, Wayne, NJ. Before virus inoculation, blood samples were obtained by cardiac puncture from all animals, and their sera were collected for guinea pig cytomegalovirus (GPCMV) neutralizing antibody determination. Only those animals showing no antibody to GPCMV were included in the experiment. Stock virus of GPCMV, strain 22122, was prepared from the salivary glands of infected guinea pigs as described previously (Fong et al., 1987). For each experiment, a total of 40–60 Hartley guinea pigs were used, 4–6 guinea pigs per group. Each guinea pig received intraperitoneally GPCMV salivary gland suspension containing 6.7 $\log_{10}$ $TCID_{50}$ of virus.

Antiviral drugs

2′-nor-cGMP was prepared by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ, and dissolved in phosphate-buffered saline (PBS). DHPG (Ganciclovir sodium 'CYTOVENE') was purchased from Syntex Laboratories, Inc., Palo Alto, CA. The drugs were dissolved in sterile water and diluted with lactated Ringer's solution, adjusted to pH 7.0 by the addition of 6 N HCl.

Drug treatment

All infected animals were treated through intraperitoneal injection with 2′-nor-cGMP/DHPG combination and each drug alone once daily for 7 consecutive days, starting 24 h post-viral inoculation. In the first set of in vivo experiments, 2′-nor-cGMP/DHPG combinations at a fixed ratio of 1:10 at 3 dose levels (2′-nor-cGMP at 1.25, 2.5 and 5 mg/kg/day; DHPG at 12.5, 25 and

50 mg/kg/day; and the combination of 2′-nor-cGMP/DHPG at 1.25/12.5, 2.5/25 and 5/50 mg/kg/day) were tested. In the second set of in vivo experiments, variable ratios of 1:2, 1:4 and 1:8 of 2′-nor-cGMP/DHPG combinations, i.e. 5/10 mg, 2.5/10 mg and 2.5/20 mg/kg/day, were chosen to treat GPCMV-infected guinea pigs. Sham-treated animals were injected intraperitoneally with sterile lactated Ringer's solution using the same dosing schedule. Uninfected animals treated with lactated Ringer's solution were included as controls. Body weight of all animals were checked and recorded daily.

Virus isolation and histopathological studies

During acute infection, infected animals treated with 2′-nor-cGMP or DHPG alone and in combination at various drug concentration levels were sacrificed on days 9 and 10 post-GPCMV inoculation. At autopsy, spleens were removed, weighed and spleen indices (spleen weight/body weight $\times 10^4$) were calculated. Portions of spleen, lung and salivary gland were removed aseptically, finely minced, homogenized and suspended (10% weight/volume) in Hanks' balanced salt solution (HBSS) for virus isolation. Serial 10-fold dilutions of tissue suspension were prepared and 0.1 ml of each dilution was inoculated into quadruplicate wells containing GPE cell monolayers. Inoculated cell cultures were incubated for 2–3 weeks and observed for the appearance of virus-induced CPE and virus infectivity titers were calculated. Data analysis of virus-infection with or without the drug-treatment groups were performed using Student's *t*-test.

For histopathological examination, lungs were inflated by intratracheal instillation of 10% buffered formalin. Portions of salivary gland, spleen, kidney, lung and bone marrow were removed and fixed in 10% buffered formalin, processed, stained with hematoxylin and eosin and examined microscopically for evidence of histopathological lesions and virus-induced inclusions.

Results

Combined effects of 2′-nor-cGMP and DHPG on GPCMV replication in cultured guinea pig embryo cells

Table 1 summarizes the ED_{50}, ED_{70} and ED_{90} of 2′-nor-cGMP and DHPG each alone or in combination at 3 fixed ratios (1:5, 1:10 and 1:20) on GPCMV replication in GPE cells by plaque reduction assay. Their combination index (CI) values are illustrated in Fig. 1. The results showed that the ED_{50-90} of 2′-nor-cGMP in the 3 combinations ratios with DHPG were lower than those of 2′-nor-cGMP alone. Similarly the ED_{50-90} of DHPG in the 3 combination ratios were lower than those of DHPG alone. In addition, the CI values of all 3 combination ratios were <1 (Fig. 1). These data indicate that synergistic reactions occurred between 2′-nor-cGMP and DHPG at fixed ratios 1:5, 1:10 and 1:20 against GPCMV replication in cultured GPE cells.

TABLE 1

Combined effects of 2'-nor-cGMP and DHPG on GPCMV replication in GPE cells

Treatment (drug ratio)	Antiviral activity (μM)[a]								
	ED_{50}			ED_{70}			ED_{90}		
	cGMP	DHPG	cGMP + DHPG	cGMP	DHPG	cGMP + DHPG	cGMP	DHPG	cGMP + DHPG
2'-nor-cGMP	1.84	NA[b]	NA	2.27	NA	NA	3.17	NA	NA
DHPG	NA	81.68	NA	NA	<100	NA	NA	<100	NA
2'-nor-cGMP/ DHPG (1:5)	1.08	5.43	6.51	1.38	6.92	8.30	2.03	10.16	12.19
2'-nor-cGMP/ DHPG (1:10)	0.89	8.97	9.86	1.16	11.60	12.76	1.73	17.39	19.12
2'-nor-cGMP/ DHPG (1:20)	0.93	18.68	19.61	1.13	22.69	23.82	1.55	30.92	32.47

[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.
[b]NA = not applicable.

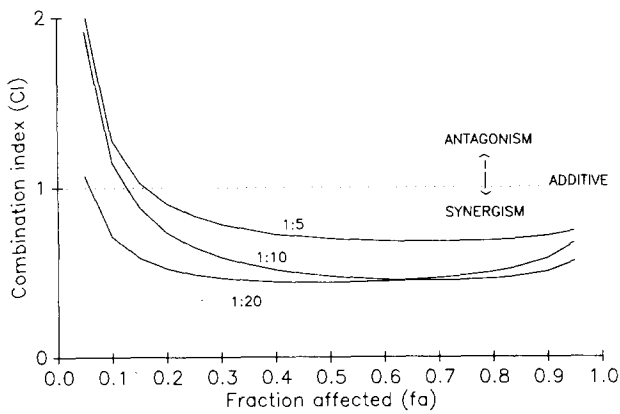

Fig. 1. Combination index (CI) values for 2'-nor-cGMP/DHPG combinations at 3 fixed ratios. CI < 1, synergy; CI = 1, additivity; CI > 1, antagonism; fa, fractional inhibition of virus replication. CI values were determined using mutually non-exclusive assumptions.

Antiviral effects of 2'-nor-cGMP and DHPG each alone or in combination at 1:10 fixed ratio on acute GPCMV infection in guinea pigs

In order to determine whether synergistic antiviral effect of 2'-nor-cGMP and DHPG against GPCMV infection in guinea pigs exist, the following experiments were performed. In the first set of in vivo experiments, GPCMV-infected guinea pigs were treated once daily for 7 consecutive days with 2'-nor-cGMP (1.25, 2.5 and 5 mg/kg/day) and DHPG (12.5, 25 and 50 mg/kg/day) each alone and in combination at 1:10 fixed ratio at 3 dosage levels, i.e. 1.25/12.5, 2.5/25 and 5/50 mg/kg/day. The results of one representative experiment are summarized in Table 2. During acute infection, guinea pigs treated with 2'-

TABLE 2

Virus infectivity titers and spleen indices of guinea pigs treated with 2′-nor-cGMP and DHPG each alone or in combination during acute GPCMV infection (9–10 days post-inoculation)[a] at a fixed ratio 1:10

Drug dosage (mg/kg/day)	No. of animals	Virus infectivity titers[b] (mean ± S.D.)			Spleen index (spleen wt/body wt × 10^4)
		Salivary gland	Lung	Spleen	
Virus control (sham-treated)	8	4.5 ± 0.8	2.8 ± 0.4	4.4 ± 0.4	71.91 ± 73.94
Lactated Ringer's solution control	4	–	–	–	25.65 ± 2.60
2′-nor-cGMP alone					
1.25	4	4.0 ± 0.6	2.5 ± 0.7	4.2 ± 0.2	62.75 ± 2.61
2.50	6	3.9 ± 0.5	2.1 ± 0.4 ($P<0.01$)[c]	4.3 ± 0.5	70.67 ± 29.50
5.00	6	3.4 ± 0.5 ($P<0.05$)[c]	2.6 ± 0.8	4.1 ± 0.2	49.97 ± 22.66
DHPG alone					
12.5	4	2.5 ± 0.9 ($P<0.01$)[c]	2.2 ± 0.4 ($P<0.05$)[c]	4.2 ± 0.3	66.23 ± 19.53
25.0	6	2.2 ± 0.4 ($P<0.01$)[c]	2.0 ± 0.6 ($P<0.05$)[c]	4.2 ± 0.4	79.77 ± 22.89
50.0	6	2.3 ± 0.3 ($P<0.01$)[c]	1.9 ± 0.5 ($P<0.01$)[c]	4.0 ± 0.5	48.68 ± 11.54
2′-nor-cGMP/DHPG					
1.25/12.5 (1:10)	4	2.9 ± 0.9 ($P<0.05$)[c]	2.1 ± 0.4 ($P<0.05$)[c]	3.9 ± 0.3	68.73 ± 20.76
2.5/25 (1:10)	6	3.2 ± 0.8 ($P<0.05$)[c]	2.4 ± 0.5	3.9 ± 0.5	57.43 ± 19.19
5/50 (1:10)	6	2.4 ± 0.9 ($P<0.01$)[c]	2.3 ± 0.6	3.9 ± 0.3 ($P<0.05$)[c]	59.45 ± 9.81

[a]Each animal was inoculated intraperitoneally with GPCMV salivary gland suspension containing 6.7 $\log_{10}$ $TCID_{50}$ and treated for 7 days, 24 h post-GPCMV infection. Statistical analysis was performed using Student's *t*-test.

[b]$\log_{10}$ $TCID_{50}$/0.1 ml of 10% wt./vol. tissue suspension.

[c]*P*-value between virus infectivity titers of virus control (sham-treated) and corresponding tissues of drug-treated animals.

nor-cGMP 1.25 mg/kg/day alone showed no antiviral effect in the target organs when compared with the virus control group (GPCMV-infected sham-treated animals). Variable results were obtained when 2′-nor-cGMP 2.5 mg and 5.0 mg/kg/day were used. All 3 groups of infected guinea pigs treated with DHPG alone at 3 dose levels (12.5 mg, 25 mg, and 50 mg/kg/day showed significantly lower virus infectivity titers in both salivary gland and lung compared with the virus control group. Although lower virus infectivity titers were observed in the salivary gland of GPCMV-infected guinea pigs treated with 2′-nor-cGMP/DHPG combinations as compared to infected sham-treated animals, no significant difference in virus infectivity titers could be found in animals treated with the 3 combinations and those treated with corresponding doses of DHPG alone.

Antiviral effects of 2′-nor-cGMP and DHPG each alone or in combination at variable ratios on acute GPCMV infection in guinea pigs

In the second set of in vivo experiments, 2′-nor-cGMP (2.5 and 5 mg/kg/day) and DHPG (10 and 20 mg/kg/day) each alone and in combination at 3 variable ratios, i.e. 1:4 (2.5/10 mg/kg/day), 1:8 (2.5/20 mg/kg/day) and 1:2 (5/10 mg/kg/day), were used to determine whether there is any evidence of synergistic antiviral effects. These results are summarized in Table 3. Guinea pigs treated with 2′-nor-cGMP 2.5 mg or 5.0 mg/kg/day did not show significant reduction in virus infectivity titers in the target organs of GPCMV-infected animals when compared to the virus control group (infected, sham-treated group). Significantly lower virus infectivity titers were demonstrated in the salivary gland and lung but not in the spleen of infected guinea pigs receiving DHPG 10 mg or 20 mg/kg/day alone. On the other hand, significantly lower GPCMV infectivity titers in the salivary gland, lung and spleen of infected guinea pigs treated with 2′-nor-cGMP/DHPG combination 2.5/10 mg (1:4) were noted as compared with those guinea pigs treated with 2′-nor-cGMP 2.5 mg/kg/day alone or DHPG 10 mg/kg/day alone, whereas the drug combination 2.5/20 mg/kg/day (1:8) showed no significant difference as compared with guinea pigs treated with DHPG 20 mg/kg/day alone. GPCMV infectivity titers in the salivary gland, lung and spleen of infected guinea pigs treated with 2′-nor-cGMP/DHPG at 5.0/10 mg/kg/day were not significantly reduced as compared with each drug alone. These results suggest that only 2′-nor-cGMP/DHPG combination 2.5/10 mg/kg/day (1:4) showed convincing evidence of enhanced antiviral effects on GPCMV infection in guinea pigs as compared with infected guinea pigs treated with each drug alone. Spleen index of GPCMV-infected animals treated with the combinations or each drug alone were essentially the same as those of the virus control.

Body weights

During the 7–10 days period after infection, the average body weight of all 3 groups of GPCMV-infected animals treated with 2′-nor-cGMP/DHPG combination at 1:10 ratio at 3 dose levels were lower than those animals

TABLE 3

Virus infectivity titers and spleen indices of guinea pigs treated with 2′-nor-cGMP and DHPG each alone or in combination during acute GPCMV infection (9–10 days post-inoculation)[a] at variable ratios

Drug dosage (mg/kg/day)	No. of animals	Virus infectivity titers[b] (mean ± S.D.)			Spleen index (spleen wt/body wt × 10^4)
		Salivary gland	Lung	Spleen	
Virus control (sham-treated)	2	4.15 ± 0.21	3.15 ± 0.21	3.60 ± 0.14	70.18 ± 23.33
Lactated Ringer's solution control	4	–	–	–	26.35 ± 9.76
2′-nor-cGMP					
2.5	4	4.05 ± 0.53	2.68 ± 0.24	3.78 ± 0.15	57.56 ± 14.22
5.0	4	3.30 ± 0.85	2.38 ± 0.30	3.73 ± 0.21	46.78 ± 13.83
DHPG					
10.0	4	3.18 ± 0.39 ($P<0.05$)[c]	2.50 ± 0.16 ($P<0.05$)[c]	4.08 ± 0.43	68.99 ± 13.22
20.0	4	2.73 ± 0.21 ($P<0.01$)[c]	2.33 ± 0.24 ($P<0.05$)[c]	3.30 ± 0.36	66.91 ± 9.48
2′-nor-cGMP/DHPG					
2.5/10.0 (1:4)	4	2.60 ± 0.12 ($P<0.01$)[c,d] ($P<0.05$)[e]	2.13 ± 0.25 ($P<0.05$)[c,d,e]	2.95 ± 0.53 ($P<0.05$)[d,e]	59.00 ± 15.74
2.5/20.0 (1:8)	4	2.50 ± 0.16 ($P<0.01$)[c,d]	2.08 ± 0.15 ($P<0.01$)[c,d]	3.45 ± 0.10($P<0.05$)[d]	59.26 ± 11.31
5.0/10.0 (1:2)	4	3.05 ± 0.33 ($P<0.05$)[c]	2.48 ± 0.33	3.63 ± 0.63	66.28 ± 20.55

[a]Each animal was inoculated intraperitoneally with GPCMV salivary gland suspension containing 6.7 $\log_{10}$ $TCID_{50}$ and treated for 7 days, 24 h post-GPCMV infection. Statistical analysis was performed using Student's *t*-test.

[b]$\log_{10}$ $TCID_{50}$/0.1 ml of 10% wt./vol. tissue suspension.

[c]*P*-value between virus infectivity titers of virus control (sham-treated) and corresponding tissues of drug-treated animals.

[d]*P*-value between virus infectivity titers of animals treated with 2′-nor-cGMP/DHPG combinations 2.5/10 or 2.5/20 mg/kg/day and animals treated with 2′-nor-cGMP 2.5 mg/kg/day alone.

[e]*P*-value between virus infectivity titers of animals treated with 2′-nor-cGMP/DHPG combinations 2.5/10 mg/kg/day and animals treated with DHPG 10 mg/kg/day alone.

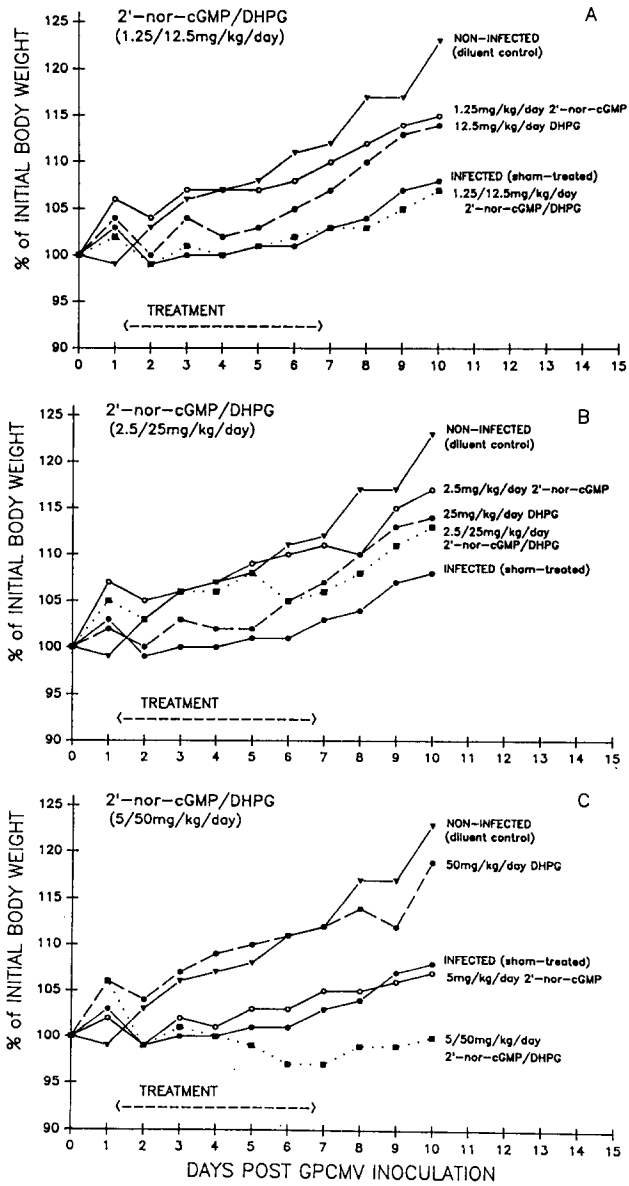

Fig. 2. Effects of 2'-nor-cGMP and DHPG each alone or in combination on body weights of GPCMV-infected guinea pigs. The combination 2'-nor-cGMP/DHPG at 1:10 fixed ratio at 3 dose levels were used: (A) 1.25/12.5 mg/kg/day; (B) 2.5/25 mg/kg/day; (C) 5/50 mg/kg/day. The drugs were administered daily for 7 days by intraperitoneal injection, starting 24 h post-infection. Four to six animals per group were used.

treated with a corresponding dose of 2'-nor-cGMP or DHPG alone (Fig. 2A–C). Similar results were observed in uninfected guinea pigs treated with the above combinations at 3 dose levels (data not shown); thus, in terms of body

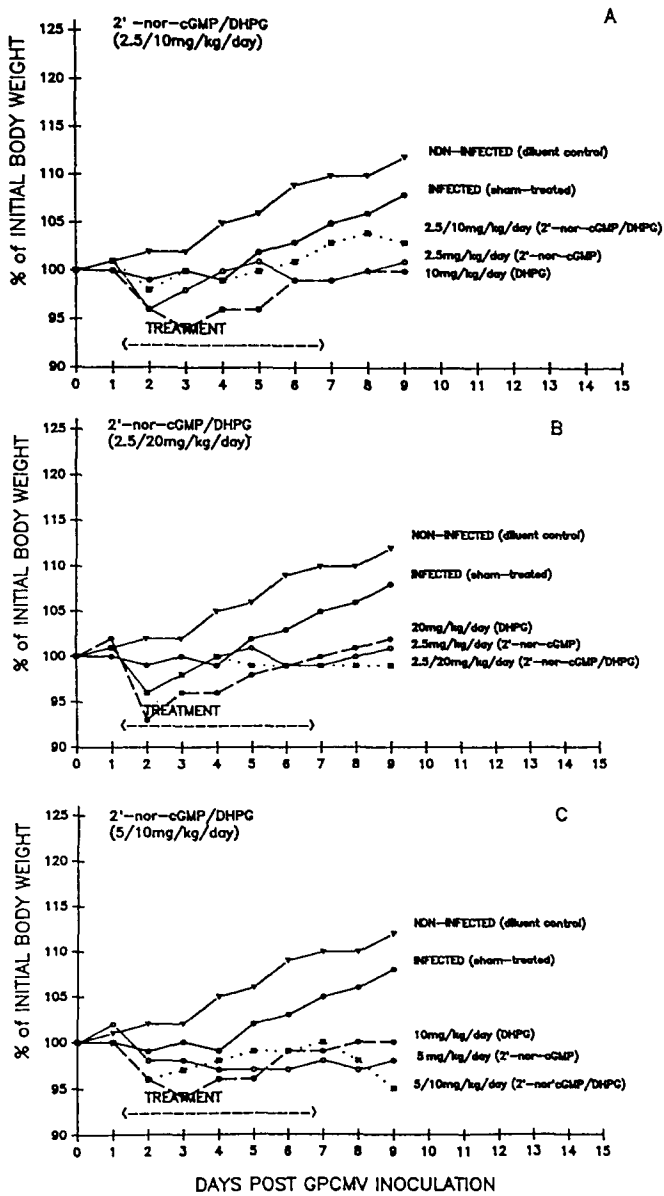

Fig. 3. Effects of 2'-nor-cGMP/DHPG each alone and in combination on body weights of GPCMV-infected guinea pigs. The combination 2'-nor-cGMP/DHPG at variable ratios were used: (A) 2.5/10 mg/kg/day; (B) 2.5/20 mg/kg/day; (C) 5/10 mg/kg/day. The drugs were administered daily for 7 consecutive days by intraperitoneal injection, starting 24 h post-infection. Four animals per group were used.

weight, combined synergistic toxicity of the drug combination at 3 dose levels using 1:10 fixed ratio was noted. However, body weights of GPCMV-infected guinea pigs treated with 2'-nor-cGMP/DHPG combination at 2.5/10 mg/kg/

TABLE 4

Histopathological findings in GPCMV-infected guinea pigs treated with 2′-nor-cGMP and DHPG each alone or in combination during acute infection (9–10 days post-inoculation)

Drug Dosage (mg/kg/day)	Measurement of antiviral efficacy		Measurement of drug toxicity	
	Spleen[a] (virus-induced inclusions & necrosis)	Lung[b] (Pneumonia)	Kidney[c] (Toxicity)	Bone marrow[d] (Toxicity)
Virus infected (sham-treated)	+ + + +	+ + +	−	−
2′-nor-cGMP alone				
2.5	+ + + +	+ + +	+	±
5.0	+	+	+ +	+
DHPG alone				
25.0	+ +	+ +	−	+
50.0	+	+	−	+ +
2′-nor-cGMP/DHPG 1:10				
2.5/25	+	−	+ +	+
5/50	+	−	+ + +	+ +

[a]Spleen + + + +: large patches of necrosis, many inclusions; + +: patches of necrosis, with inclusions; +: small foci to smaller areas of necrosis, a few to fewer inclusions.
[b]Lung + + +: interstitial pneumonia; + +: focal pneumonia; +: mild to some pneumonia; −: clear.
[c]Kidney (atypical tubular necrosis) + + +: moderate toxic changes; + +: mild to moderate toxicity; +: mild toxicity; −: no toxic changes.
[d]Bone marrow (decrease in cellularity, replacement of active bone marrow with fat and areas of necrosis) + +: moderate toxicity, 45–65% cellularity; +: some to mild toxicity, 65–85% cellularity; −: no toxic changes, 85–100% cellularity.

day (1:4 ratio) were higher than those of animals treated with a corresponding dose of 2′-nor-cGMP or DHPG alone (Fig. 3A). Animals treated with the drug combinations 2.5/20 mg/kg/day (1:8) or 5/10 mg/kg/day (1:2) showed less body weight gain than animals treated with each drug alone (Fig. 3B and C).

Histopathological findings

In the first set of experiments, the results of histopathological examinations of the spleen, lung, kidney and bone marrow of infected guinea pigs treated with 2′-nor-cGMP, and DHPG each alone or in combination are summarized in Table 4. Evidence of pneumonia was not observed in the lungs and only a small foci of necrosis and a few virus-induced inclusions were found in the spleen of animals treated with 2.5/25 mg/kg/day and 5/50 mg/kg/day of the 2′-nor-cGMP/DHPG combination. Conversely, interstitial or mild pneumonia was noted in animals treated with 2.5 or 5.0 mg/kg/day of 2′-nor-cGMP alone, and focal pneumonia or some pneumonia was observed in animals treated with 25 or 50 mg/kg/day of DHPG alone. Toxic effects were mild to moderate in the kidneys of infected animals treated for 7 days with 2′-nor-cGMP/DHPG

combination, 2.5/25 mg/kg/day. These toxicities were more evident in animals treated with 5/50 mg/kg/day of the drug combination at a fixed ratio of 1:10. On the other hand, no toxic effects were observed in the kidneys of animals treated with DHPG alone at doses as high as 50 mg/kg/day. In the bone marrow, mild to moderate toxicities were observed in animals treated with the drug combination at 1:10 fixed ratio 2.5/25 mg and 5.0/50 mg/kg/day for 7 days as well as with DHPG alone (25 or 50 mg/kg/day), but lesser toxicity was observed in infected animals treated with 2′-nor-cGMP alone (2.5 mg or 5.0 mg/kg/day). These data further suggest that combined treatment with 2′-nor-cGMP/DHPG at 3 dosage levels (1:10 fixed ratio) showed increased toxicity in the kidney and bone marrow of GPCMV-infected guinea pigs when compared to each drug alone. In the second set of experiments, when animals received 2.5/10 mg, 2.5/20 mg, and 5.0/10 mg/kg/day of 2′-nor-cGMP/DHPG, comparatively, viral-induced changes were less than those seen in animals receiving either drug alone at the same dosages. Only the combination of 2′-nor-cGMP/DHPG (2.5/10 mg/kg/day) did not show notable increase in toxicity either to the kidney or to the bone marrow as compared to animals treated with each drug alone.

Discussion

Since 2′-nor-cGMP/DHPG combination at ratios of 1:5, 1:10 and 1:20 showed synergistic antiviral effects against GPCMV replication in cultured guinea pig embryo cells, it was very challenging to test such synergistic effects in guinea pigs. In the first set of in vivo experiments (Table 2), 3 dose levels of the drug combination at a fixed ratio of 1:10 (1.25/12.5 mg, 2.5/25 mg and 5/50 mg/kg/day) were chosen to treat GPCMV-infected guinea pigs, but did not show impressive results. Although lower virus infectivity titers were noted in animals treated with the drug combinations when compared with sham-treated animals, no significant reductions of virus infectivity titers were noted when compared with animals treated with a corresponding dose of DHPG alone. Although some evidence of enhanced antiviral effect was suggested by the reduced pneumonia and few viral inclusions in the spleen of guinea pigs treated with the drug combination at 2.5/25 and 5.0/50 mg/kg/day, as compared to animals treated with either drug alone, such combinations were more toxic to the renal tubules and the bone marrow of GPCMV-infected animals than when either drug was used alone as noted in histopathological studies (Table 4).

Body weights of guinea pigs treated with the drug combinations at 1:10 fixed ratio at 3 dose levels were lower than those treated with each drug alone, suggesting synergistic toxic effects of the drug combination using the same fixed ratios. In the second set of in vivo experiments (Table 3), we modified the combination ratios and the concentration of 2′-nor-cGMP and DHPG, i.e. 1:4 (2.5/10), 1:8 (2.5/20) and 1:2 (5/10), to treat GPCMV-infected guinea pigs. It was then that an enhanced antiviral effect was noted using modified

concentrations specifically when the 1:4 combination ratio of 2′-nor-cGMP/DHPG (2.5/10 mg/kg/day) was used (Table 3). However, no significant difference in spleen index could be found among GPCMV-infected guinea pigs treated with either drug alone or in combination. When body weights of guinea pigs treated with the drug combinations at variable ratios (Fig. 3) were compared with those treated with the drug combinations at fixed ratios (Fig. 2), it was noted that animals treated with the drug combination 2.5/10 mg/kg/day (1:4) had a higher body weight than those guinea pigs treated with each drug alone. However, guinea pigs treated with the drug combinations 2.5/20 mg/kg/day (1:8) or 5/10 mg/kg/day (1:2) remained to have a lower body weight than those animals treated with a corresponding dose of each drug alone. These data suggest that in terms of body weight, the toxic effects of the drug combinations can be reduced after a thorough evaluation of the concentration of each drug used in the combination is done.

In previous studies (Freitas et al., 1989), the drug combination DHPG with foscarnet (PFA) against murine cytomegalovirus showed synergistic effects in vitro, but in vivo only additive effects was noted. Our data indicates that 2′-nor-cGMP/DHPG combinations showed synergistic antiviral effects in vitro at 1:10 fixed ratio but such combinations did not show any synergistic antiviral effects in vivo using the same fixed ratios. Only when concentration or dosage modification was done specifically using the 1:4 ratio (2.5/10 mg/kg/day) were we able to clearly demonstrate such synergistic antiviral effect in vivo. Thus, drug concentrations and combination ratios used in in vivo experiments require considerable test designs. It appears that in vitro data can only be used as a guideline in so far as their combination effects are concerned. During in vitro experiments, each drug probably remains constant throughout the test period, with respect to drug concentration etc., but in vivo, metabolism and excretion of the drugs complicate matters, and the tissue toxicity and physiological conditions of animals may vary considerably. Concentration and combination ratios must be taken into consideration when using the drug combination in vivo guided by in vitro results. Thus our data suggest that results obtained with combination therapy in vitro should be followed by in vivo testing whenever it is feasible.

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