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Antiviral effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine against cytomegalovirus infection in a guinea pig model*

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Summary

The antiviral activity of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) against guinea pig cytomegalovirus (GPCMV) was evaluated in guinea pig cell cultures and in Hartley guinea pigs. The 50% effective dose of DHPG against GPCMV replication in cell cultures was 71 μM. Ultrastructural studies revealed that DHPG inhibited the formation of viral cores and the production of nucleocapsids, enveloped virions and dense bodies, but the drug did not prevent the formation of virus induced intranuclear tubular structures. In vivo, guinea pigs inoculated intraperitoneally with GPCMV were treated with DHPG, 25 mg/kg subcutaneously, twice daily. Treatment was initiated 24 h after infection and continued for 7 days. During the acute infection, the average body weights of DHPG-treated, virus infected guinea pigs were approximately 14% lower than the sham-treated counterparts on day 10, 11 and 13 post-virus inoculation. Virus infectivity titers were higher in the lungs of DHPG-treated guinea pigs on day 10 than the sham-treated ones. Although there was no significant difference on histopathologic lesions in the

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spleen, liver and lungs of the drug-treated and the sham-treated guinea pigs, DHPG treated animals appeared to have fewer virus-induced lesions or inclusions in the kidneys and salivary glands than the sham-treated ones. In addition, virus infectivity titers in the salivary gland of DHPG treated guinea pigs were consistently lower than the sham-treated animals.

antiviral agents, chemotherapy, cytomegalovirus, guinea pig

Introduction

The acyclic nucleoside analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) has been shown to effectively inhibit herpes simplex virus infection both in vitro [1,4,5,22,26,27] and in vivo [22,26]. More recently, the antiviral effects of DHPG on cytomegaloviruses (CMV) have also been studied in vitro indicating that DHPG is a potent inhibitor for cytomegaloviruses [15,20,21,28]. Although DHPG has been reported to reduce murine CMV replication in mice [15,25], it has not been adequately determined in other animal models whether this drug is effective in vivo against CMV infection. Guinea pig CMV (GPCMV) infection in guinea pigs have been shown to mimic CMV infections in humans [2,6,12]. Using this animal model of CMV infection, the effect of acyclovir, phosphonoformate (PFA) and 2′-fluoro-5-methylarabinosyluracil (FMAU) against GPCMV have been evaluated [13,19]. All three compounds had adverse effects on guinea pigs when they were used for treatment of acute GPCMV infection. These drug-treated guinea pigs lost more weight, developed disseminated CMV infection and had a higher mortality rate than the infected control animals [13,19]. This communication is concerned with the antiviral effects of DHPG on replication of GPCMV in cell cultures, and its therapeutic activity against infections with GPCMV in guinea pigs.

Materials and Methods

Cell cultures, virus strain and infectivity assay

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 [8]. The cells were initially grown in Eagle's minimal essential medium (EMEM) in Hank's balanced salt solution supplemented with 10% heat inactivated newborn calf serum (NCS). When the cell monolayers were confluent, the growth medium was replaced with maintenance medium which consisted of EMEM in Earle's salt solution supplemented with 5% NCS. GPE cells at passage levels one to three were used in all experiments. For experiments in cell cultures, GPCMV, strain 22122 was passaged in GPE cells. The stock virus infectivity titer was in the range of 10^7 to $10^{7.5}$ $TCID_{50}$ (50% tissue culture infective dose) per ml. For experiments in guinea pigs, GPCMV was passaged in Hartley guinea pigs as

previously described [10]. The supernatant from the salivary gland homogenates of GPCMV infected guinea pigs containing 10^7 to 10^8 $TCID_{50}$/ml of infectious virus was used for animal inoculation.

GPCMV infectivity titers in infected cell cultures or in tissue suspensions obtained from infected guinea pigs were determined in GPE cells grown in 24-well cell culture panels. Virus titers were expressed in $\log_{10}$ $TCID_{50}$/ml or PFU (plaque forming units)/ml. For plaque formation, infected cells were overlaid with EMEM in Earle's salt solution containing 5% NCS and 0.5% methylcellulose and incubated at 37°C. After 14–17 days, cells were fixed with 5% formalin, stained with 0.5% crystal violet solution, and plaques enumerated.

Chemicals

DHPG was kindly supplied by Syntex Research, Inc., Palo Alto, California. For use in cell cultures, DHPG was dissolved in Hank's balanced salt solution (HBSS) at 4 mg/ml, filtered through Millipore filter membranes with a pore size of 0.2 μm, and diluted with EMEM. Because of solubility limitations DHPG had to be used as a suspension in animal experiments. Therefore it was suspended in sterile normal saline at 50 mg/ml and mixed vigorously to produce a homogeneous suspension just prior to use.

Effect of DHPG on virus infection in cell cultures with or without DHPG

Virus yield reduction. GPE cell cultures grown in 25-cm^2 flasks were infected with GPCMV tissue culture passaged virus at approximately one $TCID_{50}$/cell. After virus adsorption, the unadsorbed virus was removed by repeated washing with HBSS. Maintenance media containing 5% NCS and various concentrations of DHPG were then added to the virus-infected or uninfected cell cultures. After 3–4 days incubation at 37°C, when 75% of cells in the monolayers showed cytopathic effect in the virus-infected, drug-free cultures, all infected cell cultures were removed from the incubator and frozen at –70°C. To release the intracellular virus, infected cultures were frozen and thawed twice. Virus infectivity titers were determined in GPE cell cultures.

Plaque reduction. GPE cells grown in 24-well cell culture panels were infected with 20–30 PFU of GPCMV and incubated at 37°C for 1 h. Cells were then overlaid with maintenance medium containing 5% NCS, 0.5% methylcellulose, and various concentrations of DHPG. The infected cultures were incubated at 37°C in a humidified 5% CO_2 and 95% air mixture for 2–3 weeks until plaques were visible with the aid of a microscope in the control cultures.

Electron microscopy

GPE cell monolayers grown in 60-mm petri dishes were infected with GPCMV at approximately one $TCID_{50}$/cell. Following virus adsorption for 1 h at 37°C, EMEM in Earle's salt solution containing 5% NCS and various concentrations of DHPG were added to the infected cell cultures and they were incubated at 37°C

in a humidified 5% CO_2 and 95% air mixture. When approximately 50–75% of the cells in the virus infected drug-free cell cultures showed cytopathic effect (i.e., 2–3 days) all DHPG treated and untreated cultures were fixed in situ with 2% buffered glutaraldehyde for one hour at 4°C. The fixed cell monolayers were scraped from the plastic surface in the fixative fluid and followed by centrifugation. The cell pellets were washed in 0.1 M cacodylate buffer solution and post-fixed in 1.33% osmium tetroxide in s-collidine buffer for one hour. Cell pellets were treated for 4 h with 0.5% uranyl acetate for en bloc staining, followed by dehydration in graded ethanol and embedded in Epon as described previously [14]. Thin sections were stained with uranyl acetate and lead citrate, and examined under a Philips EM 300 electron microscope.

Animal inoculation, DHPG treatment and specimen collection

For experiments in vivo young Hartley guinea pigs (350 ± 20 g) were used. Before virus inoculation, all guinea pigs were bled and the sera collected for GPCMV antibody determination. Only those guinea pigs showing no antibody to GPCMV were included in the experiment. For each experiment a total of 24–30 Hartley guinea pigs were used; 16–20 guinea pigs were each inoculated intraperitoneally with salivary gland passaged stock containing 1.5×10^5 $TCID_{50}$ of GPCMV. One day after virus inoculation, half of the infected guinea pigs were treated with DHPG subcutaneously with a dose of 25 mg/kg, twice daily for 7 days. The other half of the infected guinea pigs were sham-treated with normal saline for 7 days. A total of 8–10 uninfected control guinea pigs were divided into two groups, one was treated with DHPG as described above, and the other sham-treated with normal saline.

Animals were checked daily and their body weights were recorded. Two to four guinea pigs from each group were sacrificed at different time intervals post-inoculation. Guinea pigs were anesthetized with diethyl ether; blood samples were obtained by cardiac puncture, and various organs were removed aseptically for virus isolation and histologic examination as described below.

Histopathological studies

Lungs were inflated by intratracheal instillation of 10% buffered formalin. Parts of liver, spleen, kidneys, and salivary glands were also fixed with 10% buffered formalin. Fixed tissues were processed for routine histology including dehydration through graded alcohols to xylene and embedded in Paraplast II. Sections were cut at 6 μm thickness and stained with hematoxylin and eosin as described previously [12].

Tissue sections from guinea pigs of each experiment were examined and randomly graded by a single individual without the knowledge of the experimental status of each animal. Viral lesions were scored histologically from 0 to 4, based on the lesion severity as reflected by the amount of tissue necrosis, inflammation and the number of lesions.

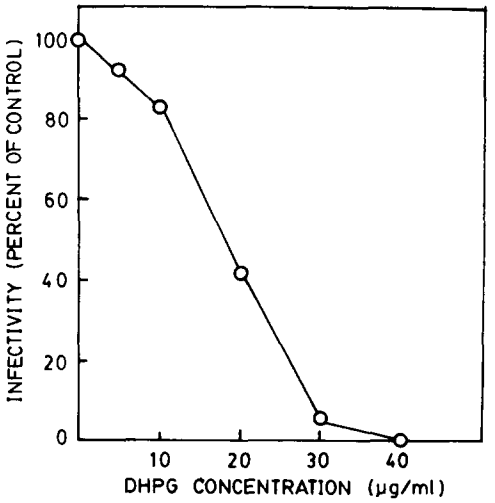

Fig. 1. Dose–response curve of GPCMV to various concentrations of DHPG.

Results

Effect of DHPG on GPCMV replication in cell cultures

The antiviral effect of DHPG on GPCMV replication in GPE cells was determined by virus yield reduction indicating that DHPG, at concentrations of 40 and 80 μg/ml, virus infectivity titers were reduced by 2.5 and 4 $\log_{10}$ $TCID_{50}$, respectively, when compared with the drug-free virus infected controls. The results of a dose–response experiment using plaque reduction is illustrated in Fig. 1. When DHPG was used at concentrations ranging from 20 to 40 μg/ml, plaque formation of GPCMV in GPE cells was inhibited from 58 to 100%. The estimated concentrations of DHPG for 50% and 90% inhibition was 18 μg/ml (or 71 μM), and 29 μg/ml (or 114 μM), respectively. When GPCMV infected cultures were treated with DHPG for two days, and the culture media were removed and replaced with fresh media without DHPG, GPCMV replication resumed promptly and the virus titers at 4 days post-infection were comparable to those obtained in the drug-free control cultures (data not shown).

Effect of DHPG on GPCMV morphogenesis

The effects of DHPG on morphogenic development of GPCMV infected cells were studied at the ultrastructural level. Fig. 2A illustrates events in a drug-free GPE cell 3 days after GPCMV infection. Intranuclear viral inclusions consisting of electron-dense amorphous matrices, tubular structures and viral nucleocapsids at various stages of development were observed. Fig. 2B shows events in a GPE cell infected with GPCMV and exposed to 40 μg DHPG/ml for 3 days during virus replication. Viral inclusions contained predominantly tubular structures. Only a few nucleocapsids without dense cores were seen. When the concentration of DHPG

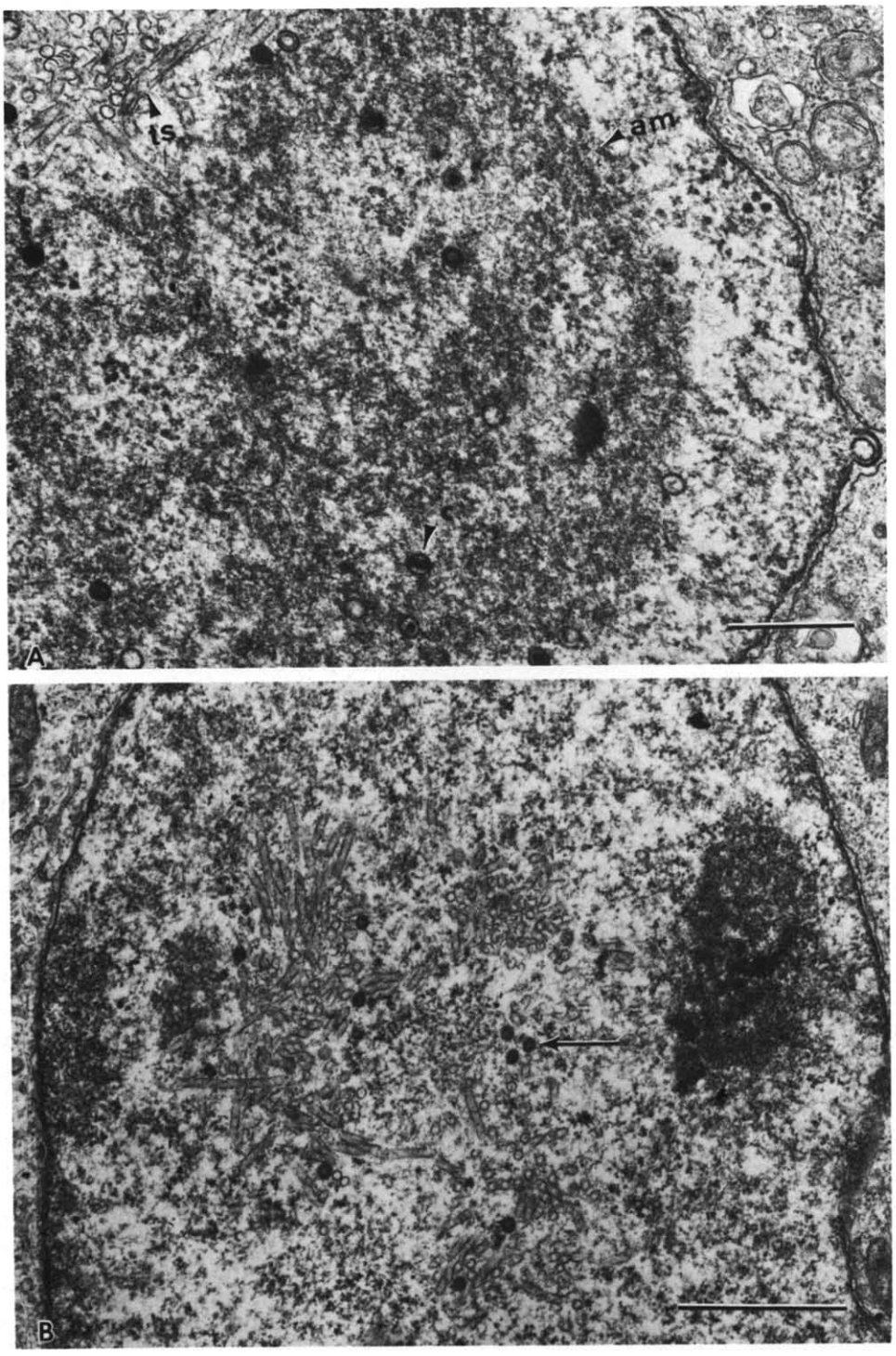

Fig. 2. (A) Electron micrograph of a GPCMV infected GPE cell, 3 days post-infection. An intranuclear viral inclusion containing amorphous matrices (am), nucleocapsids (nc), and tubular structures (ts) is illustrated. Bar = 500 nm. (B) Electron micrograph of a GPCMV infected GPE cell treated with DHPG 40 μg/ml for 3 days. Intranuclear viral inclusion contains predominately tubular structures. A few nucleocapsids (arrow) without dense core are also seen. Bar = 1 μm.

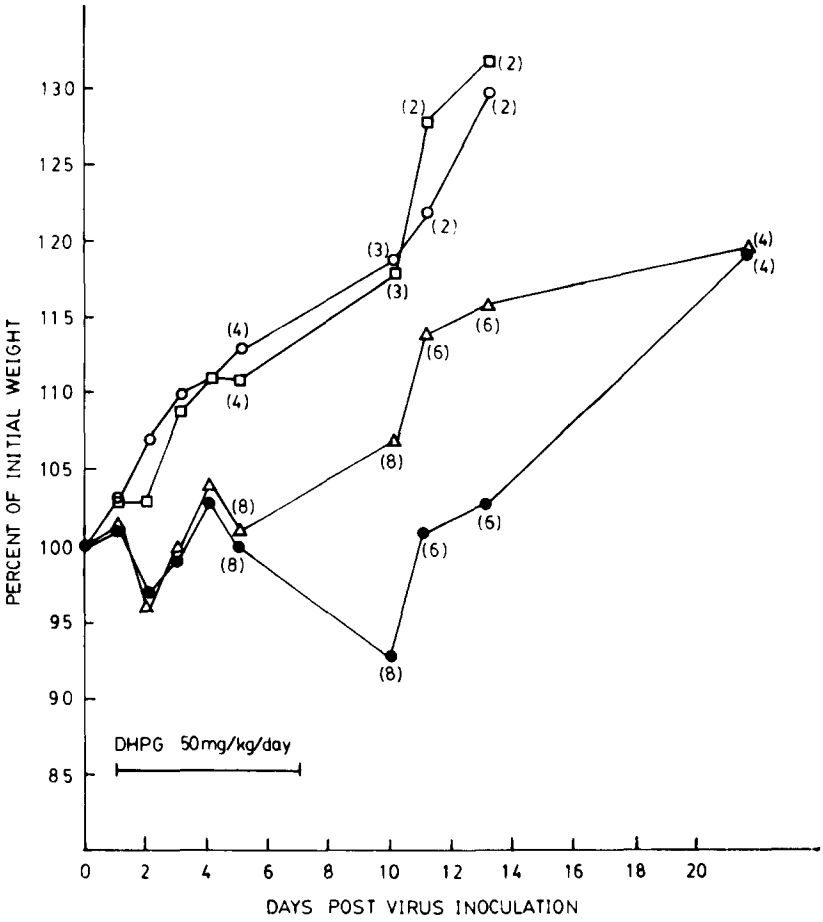

Fig. 3. Effect of DHPG on body weight of guinea pigs during acute GPCMV infection. ○—○, uninfected and sham-treated; □—□, uninfected and DHPG-treated; △—△, GPCMV-infected and sham-treated; ●—●, GPCMV-infected and DHPG-treated. The numbers in parenthesis indicate the number of animals for each data point.

increased to 80 μg/ml, only tubular structures were found in the infected cells but nucleocapsids were rarely seen.

Effect of DHPG on acute and persistent GPCMV infection in guinea pigs

Weight loss. The effect of DHPG treatment on body weight of virus infected and uninfected guinea pigs was evaluated. The results are shown in Fig. 3. It shows that there was no difference in weight changes between the sham-treated and the DHPG-treated uninfected guinea pigs. Both groups gained weight rapidly at similar rates. On the other hand, GPCMV infected, sham-treated guinea pigs had an initial loss of weight on day 2 followed by a slow but gradual increase of body weight

TABLE 1

Comparison of virus infectivity titers in DHPG-treated & sham-treated GPCMV infected guinea pigs

Days post-infection	Treatment	Virus infectivity titer[a] (mean ± S.D.)				Antibody titer [b]
		Blood	Spleen	Lung	Salivary gland	
10	DHPG[c] (4)[d]	1.00	2.38±1.00	2.25±0.52	1.16±0.15	< 5
	Sham (4)	1.00	1.92±1.09	1.46±0.32	1.92±0.35	< 5
	P-value[e]		NS[f]	$P < 0.05$	$P < 0.01$	
13	DHPG (4)	+(1/4)[g]	2.38±0.88	1.79±0.71	1.39±1.00	< 5
	Sham (4)	+(1/4)	1.92±0.17	1.67±0.62	4.67±0.62	10
	p-value		NS	NS	$P < 0.01$	
21	DHPG (2)	–[h]	–	1.50	3.94±0.62	10–20
	Sham (2)	–	–	+(1/2)	6.50±0.00	10–20
	P-value				$P < 0.05$	
28	DHPG (2)	–	–	–	3.13±0.88	10–20
	Sham (2)	–	–	+(1/2)	6.50±0.00	10–20
	P-value				$P < 0.05$	

[a] $Log_{10}TCID_{50}$/ml of 10% cell suspension.
[b] Neutralizing antibody titer, reciprocal value of serum dilution.
[c] DHPG, 25 mg/kg subcutaneously, two times daily for 7 days, started one day after virus inoculation.
[d] Number of animals.
[e] Determined by Student's *t*-test.
[f] Not significant.
[g] Number of animals positive for virus isolation per total number of animal tested.
[h] Virus was not detected.

during the period of acute infection. DHPG-treated GPCMV infected guinea pigs also had an initial weight loss on day 2 and 3, followed by a very slow recovery until day 13. Initially, there was no difference on average weights between the virus-infected drug-treated and the sham-treated guinea pigs during the first 5 days. However, the weights of drug-treated virus-infected guinea pigs were 13–14% lower than the sham-treated counterparts on day 10, 11 and 13. The differences were statistically significant ($P<0.01$). By day 21, both DHPG-treated and sham-treated virus-infected animals had recovered from acute GPCMV infection and the body weight of DHPG-treated animals had reached to the same level as the sham-treated infected controls.

Virus distribution. On day 6 post-infection, virus was detected in blood of all infected guinea pigs; the mean infectivity titer of GPCMV in blood of DHPG-treated and sham-treated animals were 3.35 and 3.15 $\log_{10}$ $TCID_{50}$ respectively. Virus titers in lung, spleen, and salivary gland were determined on days 10, 13, 21 and 28 post-virus inoculation representing acute and persistent infections and these virus titers are shown in Table 1. Virus infectivity titers were generally higher in the spleens and lungs of DHPG-treated GPCMV infected guinea pigs than the sham-treated counterparts during acute infection on day 10 and 13. Except in the lungs at day 10 the differences were not significant. However, virus infectivity titers in salivary glands of DHPG-treated guinea pigs were consistently and significantly lower than that obtained in the sham-treated animals. Similar results were obtained in repeated experiments. Neutralizing antibodies to GPCMV were first detected on day 13 and persisted thereafter. There was no difference in neutralizing antibody titers between the drug-treated and the sham-treated animals.

Histopathologic lesions. The results of the histopathologic examination of lung, spleen, liver, kidney and salivary gland are illustrated in Table 2. The lesion severity in the spleen, lung, and liver were comparable in sham-treated and DHPG-treated infected animals. In contrast, the lesions in the kidney and salivary gland of the DHPG-treated guinea pigs, 21–28 days post-infection, were significantly less severe than those seen in sham-treated guinea pigs.

Characteristic cytomegalic inclusions were seen in the salivary glands of the sham-treated but not in the DHPG-treated infected animals. These differences were consistent and also in agreement with the difference of viral infectivity titers in salivary glands between the DHPG-treated and the sham-treated animal.

Discussion

Patients having organ transplantation with immunosuppressive treatment often suffer from primary or reactivated cytomegalovirus infections which may cause a serious problem of disseminated CMV infection [18,23,24]. Methods of treatment and prevention are needed for control of this viral disease especially in immunocompromised patients. Although CMV belongs to the herpesvirus group, its path-

TABLE 2

Comparison of histopathologic lesions in DHPG-treated and sham-treated GPCMV infected guinea pigs

Days post-infection	Treatment	Lesion score[a] (mean ± S.D.)				
		Spleen	Liver	Lung	Kidney	Salivary gland
10	DHPG (4)[b]	1.13±0.75	2.38±0.75	1.13±0.48	1.25±0.05	0.38±0.75
	Sham (4)	1.63±0.95	1.38±1.11	1.38±0.75	1.13±0.25	1.38±1.60
	P-value[c]	NS[d]	NS	NS	NS	NS
13	DHPG (4)	1.50±1.41 (n=2)	1.00±0.82	1.00±0.82	1.38±0.48	1.00±0.41
	Sham (4)	0.75±0.35 (n=2)	0.88±0.25	1.88±0.63	1.63±0.48	1.88±1.25
	P-value	NS	NS	NS	NS	NS
21–28	DHPG (4)	–	0.25±0.29	1.63±1.65	0.13±0.25	0.38±0.25
	Sham (4)	–	0.38±0.48	1.25±0.65	2.17±0.58	1.50±0.41
	P-value		NS	NS	$P < 0.05$	$P < 0.05$

[a] Lesion scores: 0=No lesion, 1=mild, 2=moderate, 3=moderate to severe, 4=severe.
[b] Number of animals per group unless specified separately.
[c] Determined by Student's *t*-test.
[d] Not significant.

ogenesis, biological properties, and sensitivity to antiviral drugs, differ from that of other herpesviruses. CMV is generally less sensitive to antiviral agents than other human herpesviruses [7] and, there is currently no effective antiviral therapy for CMV infection.

DHPG has been shown to inhibit human CMV (HCMV) infection in cultured human fibroblast cells. The 50% effective dose (ED_{50}) of DHPG for inhibition of HCMV replication in human fibroblast cells is in the range of 0.5–13 μM [15,20,21,28]. Our data indicated that a 50% inhibition of GPCMV replication in GPE cells required DHPG 71 μM, which was significantly higher than that for HCMV. It has been shown for HCMV and herpes simplex virus that drug sensitivity is influenced by both the multiplicity of infection (M.O.I.) and the cell types used in the viral assay [17]. Therefore, the observed difference in drug sensitivity for HCMV and GPCMV may be due to the differences in viral properties and viral input M.O.I. GPCMV at one $TCID_{50}$/cell was used in our viral yield experiment; it was about 10 times higher than that used by other investigators for testing HCMV in human cell lines [15,20,21,28].

The results of the ultrastructural studies indicate that the antiviral effect of DHPG to GPCMV replication was probably due to the inhibition of viral DNA synthesis since most of the nucleocapsids lacked cores. This observation has been repeatedly seen in many other nucleoside analog treated, GPCMV infected cells [9,13]. In the GPCMV replication system, it is a unique characteristic that intranuclear tubular structures were not inhibited by nucleoside analogs which inhibit DNA synthesis [9,13,14]. Although synthesis of viral structural protein(s) is a late event and requires the synthesis of viral DNA, it was frequently observed that small numbers of capsids or empty nucleosides were produced in the presence of inhibitors for DNA synthesis. The latter observation may be due to incomplete inhibition as a result of insufficient amount of inhibitor in the culture medium. When a higher dose of DHPG was used, capsids were rarely found.

In our in vivo experiments, the data on body weight showed that DHPG had no apparent toxic effect to uninfected guinea pigs. Uninfected animals receiving DHPG alone gained weight at the similar rates to those uninfected guinea pigs receiving normal saline (Fig. 3). However, in the GPCMV infected guinea pigs, animals treated with DHPG gain weight slower than those sham treated guinea pigs on day 10, 11 and 13. This phenomenon has been observed repeatedly in other drug-treated guinea pigs [13,19]. It appears that the combination of virus infection and drug treatment may produce an adverse effect on guinea pigs. The exact mechanism of such adverse effect is not clear and cannot be explained at this time.

Based on the data of virus distribution in various organs, DHPG treatment of guinea pigs during acute GPCMV infection did not reduce virus infectivity titers in the blood, spleen and lungs of infected guinea pigs when it was compared to the sham-treated counterparts. The higher virus titers in the lungs of DHPG-treated GPCMV infected guinea pigs compared to the sham-treated ones on day 10 may be due to the reversibility of DHPG inhibitory effect and caused a rapid synchronized growth of GPCMV in this tissue once the drug was removed. However, virus titers in salivary glands were consistently lower in guinea pigs treated with

DHPG than the sham-treated animals. Lower virus infectivity in salivary glands in DHPG-treated animals correlated well with the observation that fewer viral inclusions were seen in salivary glands of DHPG-treated animals as compared to the sham-treated counterparts. During chronic persistent infection of adult guinea pigs, GPCMV can persist in salivary glands and excrete in the saliva for a long time [3,16]. The possibility that DHPG may reduce virus persistency in CMV infection warrants further investigation.

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