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# Immunotoxic potential of antiviral drugs: effects of ganciclovir and (*S*)-1-(3-hydroxy-2-phosphonylmethoxy propyl) cytosine on lymphocyte transformation and delayed-type hypersensitivity responses

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## Summary

In the present studies, we examined the in vitro and in vivo effects of ganciclovir (DHPG) and a relatively new nucleoside analogue, (*S*)-1-(3-hydroxy-2-phosphonylmethoxy propyl) cytosine (HPMPC), on lymphocyte responses to T cell mitogens and delayed-type hypersensitivity (DTH) responses to dinitrofluorobenzene (DNFB). Initially, responses of mouse splenic mononuclear cells and human peripheral blood mononuclear cells to PHA and con A were evaluated in vitro in the presence of each drug. Both drugs inhibited the responses to each mitogen; however, DHPG had a greater inhibitory effect on con A responses of human and mouse lymphocytes than did HPMPC. Also, spleen cells from mice treated for 7 days with DHPG responded less well to PHA stimulation than cells from untreated or HPMPC-treated mice. No effect of either drug was observed on con A responses. Treatment of mice with either drug decreased the development of DTH responses, with HPMPC having a greater inhibitory effect than DHPG. The results from the present studies suggest that both DHPG and HPMPC may have inhibitory effects on the development of certain immune functions at high dosages, but at drug concentrations that were therapeutic in animal model studies, little inhibitory effects were observed.

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## Introduction

Antiviral drugs, that have activity against herpesviruses, particularly cytomegalovirus (CMV), are often used in patients that are immunocompromised due either to an immunosuppressive agent prior to transplantation or to an underlying disease process such as AIDS. Thus, in the development and evaluation of a new antiviral drug, it is important to determine whether drug therapy alters the normal immune responses as this may be an important side effect in patients with only a partially intact immune system.

Ganciclovir (DHPG) is fairly effective in treatment of CMV infections. DHPG therapy reduces the severity of CMV retinitis, gastrointestinal disease and to a lesser extent, pneumonia in AIDS and organ or bone marrow transplant patients (Buhles Jr. et al., 1988; Crumpacker et al., 1988; Dieterich, 1988; Mills et al., 1988; Snydman, 1988). However, at least for retinitis, the beneficial effect of the therapy is often lost after drug treatment is discontinued, and long term maintenance therapy is needed (Mills et al., 1988). Also, hematologic toxicity has been noted in patients treated with DHPG (Buhles Jr. et al., 1988; Dieterich, 1988; Snydman, 1988). In addition, DHPG has been reported to have a detrimental effect on immune responses in vitro (Bowden et al., 1987). Thus, there is still a need for more effective and non-toxic drugs for the treatment of CMV infections in patients with AIDS and organ and bone marrow transplant recipients.

A relatively new nucleoside analogue, (*S*)-1-(3-hydroxy-2-phosphonylmethoxy propyl) cytosine (HPMPC), is highly effective against human CMV replication in vitro and murine CMV infections in vitro and in vivo (Bronson et al., 1989, 1990; Kern, 1991). Treatment with HPMPC increased the survival of mice after infection and was about 10-fold more potent than DHPG. Treatment of a chronic murine CMV infection with HPMPC decreased the titer of virus in kidney, salivary glands, spleens, livers and lungs of infected mice (Kern, 1991). At comparable dosages, HPMPC was considerably more active than DHPG. HPMPC also has activity against human CMV and varicella-zoster virus in tissue culture cells (Bronson et al., 1989; DeClercq et al., 1987; Snoeck et al., 1988) and is as effective as acyclovir in experimental models of herpes simplex virus (HSV-1 and HSV-2) infections (Bronson et al., 1989, 1990). Thus, HPMPC is potentially a new effective antiviral drug for herpesvirus infections, but further evaluation of its toxicity is needed.

The purpose of the following studies was to compare the effects of DHPG and HPMPC on lymphocyte responses to mitogens and delayed type hypersensitivity (DTH) responses as measures of cellular responses in vitro and in vivo.

## Materials and Methods

### *Animals*

Eight-week-($\pm$2 weeks)-old, pathogen-free C3H/HeN mice, reared and maintained in Trexler-type plastic film isolators, were used in these experiments (Parker et al., 1987). Pathogen-free status of the mouse colony, from which these mice were obtained, is monitored as follows: All retired breeders from the colony are examined for the presence of mycoplasmas by enzyme-linked immunosorbent assay [ELISA) for serum IgG and IgM antibodies to *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. The presence of all other murine pathogens are detected by fecal cultures, necropsy and histological examination and serologic tests for viruses. Sera from mice were tested by either hemagglutination inhibition, complement fixation or ELISA by Charles River Biotechnical Services (Wilmington, MA) for Sendai virus, pneumonia virus of mice, polyoma virus, minute virus of mice, ectromelia, mouse hepatitis virus, reovirus-3, Theiler's GD-VII virus, lymphocytic choriomeningitis virus, mouse adenovirus. No murine pathogens have been detected in this animal colony for over the past five years. Animals used for experiments were matched by age and sex. Experimental mice were maintained in micro-isolators with sterile bedding, and sterile food and water ad libitum.

Prior to sensitization of animals or collection of tissues, mice were anesthetized with a combination of Ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) and Rompun (Haver-Lockhart, Shawnee, KS).

### *Drugs and animal treatments*

HPMPC was provided by Bristol Myers through the Antiviral Substances program (NIAID/NIH). Ganciclovir sodium (DHPG (Cytovene®, Syntex Laboratories, Palo Alto, CA)) was purchased from the University of Alabama at Birmingham hospital pharmacy. Both DHPG and HPMPC were diluted with media (RPMI 1640 (GIBCO Laboratories), 10 mM Hepes, pH 7.4) for both in vitro and in vivo studies. For in vivo treatment, drugs were injected intraperitoneally in 1-ml volumes once a day for a total of 7 days. Control mice were treated similarly with media alone.

### *Isolation of lymphocytes*

Mouse spleen and human peripheral blood mononuclear cells were isolated using Lympho-Paque (Nyegaard, Oslo, Norway) and Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradients, respectively (Davis et al., 1985). Lymphocytes from spleens of 3 or 4 mice were pooled for these assays, and in the lymphocyte transformation experiments, two pools of cells were used in most experiments. All cell washes were done using RPMI 1640 (Gibco Laboratories) supplemented with 10 mM Hepes and 5% fetal bovine serum

(FBS, Hyclone Laboratories, Logan, UT). Viability of cells was determined by trypan blue exclusion (Mishell and Shiigi, 1980)

*Lymphocyte transformation assay*

In initial experiments, we determined the optimal conditions for mouse and human lymphocyte transformation after in vitro stimulation with mitogens. These conditions, including cell density, media supplementation and time for culture, were used in the present studies and are as follows: Cells ($4 \times 10^5$ cells/well) were cultured in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) in a humidified atmosphere containing 95% air and 5% $CO_2$ for a total of 48 h. The cultures, containing drug and/or mitogen, were at a final volume of 0.2 ml/well. The mitogens used were PHA-M (0.2 mg/ml, Difco Laboratories, Detroit, MI) and Con A (5 μg/ml, Sigma, St. Louis, MO). The culture medium was RPMI 1640 (Gibco) supplemented with 10 mM Hepes, gentamycin and 5% FBS. Twenty-four hours prior to cell harvest, 0.5 μCi of [*methyl*-$^3$H]thymidine (Amersham Searle) was added to each well. The cells were collected on glass fiber strips, lysed and washed with deionized water in a cell harvester (Skatron, Sterling, VA). The strips were allowed to dry and then counted using Econofluor (New England Nuclear, Boston, MA) with a Betatrac 6895 scintillation counter (TM Analytic, Elk Grove, IL).

All experimental treatments were tested in triplicate. The results were expressed as either the geometric mean of degradations per minute ($\pm$ SD) or percent of the control response. The percent control response was calculated as follows: The net response (DPM with mitogen − DPM without mitogen) of drug treated cultures was divided by the net response of untreated cultures; this was multiplied by 100 to get the final result.

*DTH response*

Shaved bellies of mice were painted on days 0 and 1 with 25 μl of 0.5% dinitrofluorobenzene (DNFB) suspended in an 1:4 mixture of olive oil and acetone. The earlobes were measured for thickness and challenged (painted) with 10 μl of 0.2% DNFB on day 5. The ears were remeasured for swelling 24 h later.

For DTH, the percent control response was calculated as follows. The increase in ear thickness of drug treated mice (difference between that at one day after challenge and prior to challenge) was divided by the net increase of ear thickness in untreated nice; this was multiplied by 100 to get the final result.

*Statistical analysis*

Statistical analysis was done using SYSTAT version 5.1 (Systat, Evanston, IL) on a Macintosh SE. The data were analyzed by Analysis of Variance (ANOVA) after logarithmic transformation of the data (Armatradge, 1977)

followed by post-hoc tests for multigroup comparisons. A probability (P) of less than 0.05 was accepted as significant.

## Results

*In vitro effect of antiviral drugs on human and mouse lymphocyte responses*

To compare their immunotoxic potential on mouse and human cells, DHPG and HPMPC were examined using in vitro stimulation of lymphocytes. Human peripheral blood mononuclear cell and mouse splenic mononuclear cell responses to PHA and con A were evaluated in the presence of these drugs. Both DHPG or HPMPC inhibited the responses by human and mouse lymphocytes to each of the mitogens ($P < 0.05$). DHPG had a greater inhibitory effect on human lymphocyte responses to con A ($P = 0.01$) (Fig. 1) and PHA ($P < 0.001$) (Fig. 2) than HPMPC. In mouse lymphocyte cultures stimulated with con A, DHPG also had a greater inhibitory effect than HPMPC ($P = 0.001$) (Fig. 3), but no difference between the drugs was seen when mouse lymphocytes were stimulated with PHA (Fig. 4).

To determine if the inhibitory effect of DHPG and HPMPC on lymphocyte responses was due to loss of cell viability, mouse and human lymphocytes were cultured for 24 and 48 h in the presence of each of the drugs or media alone. As shown in Table 1, the viability of cells recovered from culture was determined at each of these times. There was no significant effect on cell viability due to the presence of drug. Thus, the immunotoxic effect of HPMPC and DHPG cannot be simply accounted for by cell death.

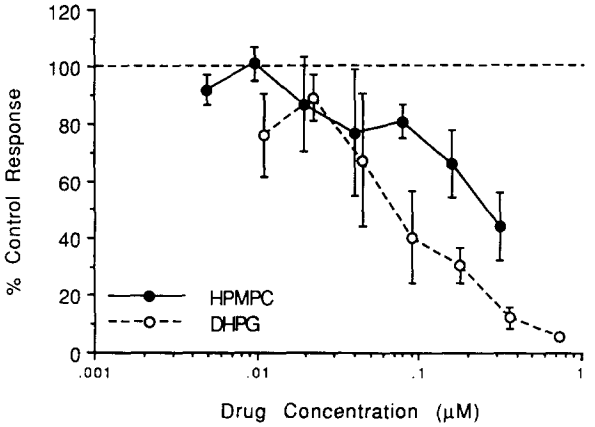


Fig. 1. Effects of drugs on human lymphocyte responses to con A in vitro. Human peripheral blood lymphocytes were cultured in the presence of different concentrations of DHPG and HPMPC and stimulated with con A. The results are displayed as the mean and standard error of mean (SEM) of 3 separate experiments. The dash line at 100% represents the lymphocyte response in cultures without drug.

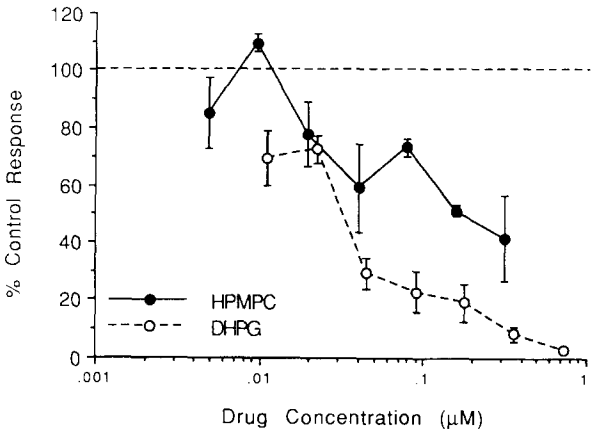


Fig. 2. Effects of drugs on human lymphocyte responses to PHA in vitro. Human peripheral blood lymphocytes were cultured in the presence of different concentrations of DHPG and HPMPC and stimulated with PHA. The results are displayed as the mean and standard error of mean (SEM) of 3 separate experiments. The dash line at 100% represents the lymphocyte response in cultures without drug.

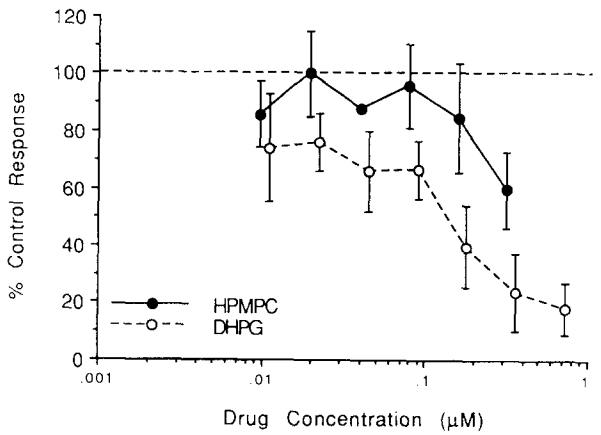


Fig. 3. Effects of drugs on mouse lymphocyte responses to con A in vitro. Mouse splenic lymphocytes were cultured in the presence of different concentrations of DHPG and HPMPC and stimulated with con A. The results are displayed as the mean and standard error of mean (SEM) of 2 separate experiments. The dash line at 100% represents the lymphocyte response in cultures without drug.

### *In vivo effect of antiviral drugs on mouse lymphocyte responses*

To evaluate the in vivo effects of the drugs, mice were given daily i.p. injections of drug at doses of 5, 15, or 50 mg/kg per day for 7 days. Splenic lymphocytes from these mice were then collected and stimulated immediately in vitro with PHA or con A in the absence of drug. Groups of mice, which were either untreated or given media alone, were included as controls. Because there was no difference in the results from the two control groups, they were combined into one control group.

Treatment of mice with HPMPC had no effect on the ability of their splenic

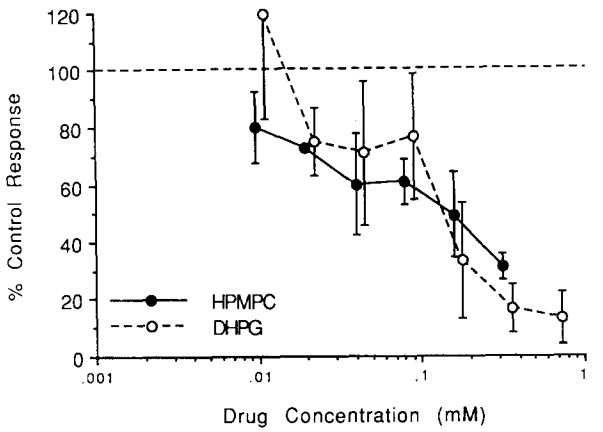


Fig. 4. Effects of drugs on mouse lymphocyte responses to PHA in vitro. Mouse splenic lymphocytes were cultured in the presence of different concentrations of DHPG and HPMPC and stimulated with PHA. The results are displayed as the mean and standard error of mean (SEM) of 2 separate experiments. The dash line at 100% represents the lymphocyte response in cultures without drug.

lymphocytes to respond to either con A and PHA stimulation in vitro (Fig. 5). Although no detrimental effect was seen with DHPG treatment on responses to con A, DHPG did inhibit PHA responses ($P < 0.05$) (Fig. 6). Splenic lymphocytes, from mice which were treated with 15 or 50 mg/kg per day DHPG, responded significantly less to PHA stimulation than control mice ($P = 0.01$), while there was no difference between control and mice treated with 5 mg/kg per day.

TABLE 1

The effect of HPMPC and DHPG on the viability of lymphocytes in culture

| Lymphocytes | Culture time (h)[a] | Viability of cells | |
|---|---|---|---|
| | | HPMPC[b] | DHPG |
| Mouse | 24 | 82(7.1)[c] | 91(12.5) |
| | 48 | 80(2.8) | 82(0.6) |
| Human | 24 | 111(16.7) | 103(8.6) |
| | 48 | 110(14.9) | 112(2.1) |

[a] Mouse and human lymphocytes, at $2 \times 10^6$ cells/ml, were cultured for 24 or 48 h in the presence of each of the drugs.

[b] HPMPC or DHPG were added to lymphocyte cultures at concentrations ranging from 0.39 to 100 μg/ml. The results from cultures, supplemented with 100 μg/ml of each drug, are shown in table; however, no significant differences in the numbers of viable cells were seen at any dose of drug.

[c] Mean (SD) of the percentage of viable cells relative to cultures without drugs recovered from culture after the incubation period. The results are from 2 separate experiments. Peripheral blood lymphocytes were collected from two individuals while splenic cells for each experiment were from a pool from 3 mice. In mouse cell cultures, there was a loss of about 50% of cells from 24 to 48 h in culture, regardless of the addition of drugs or not, but few cells were lost during culture of human lymphocytes.

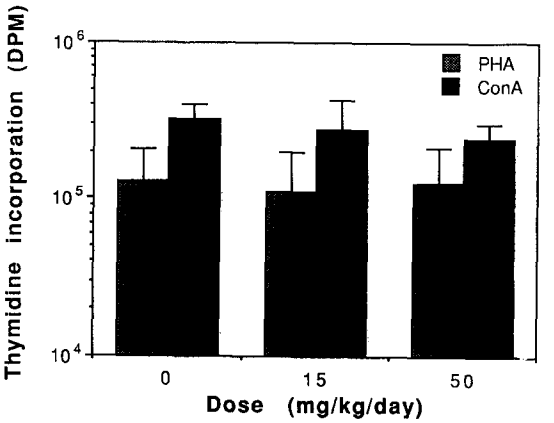


Fig. 5. Effects of HPMPC treatment of mice on in vitro splenic lymphocyte responses. Spleen cells from mice treated with HPMPC were collected and stimulated, in the absence of drug, with PHA or con A. The results are displayed as the mean and standard error of mean (SEM) of 4 separate experiments. There was no significant effect due to HPMPC treatment.

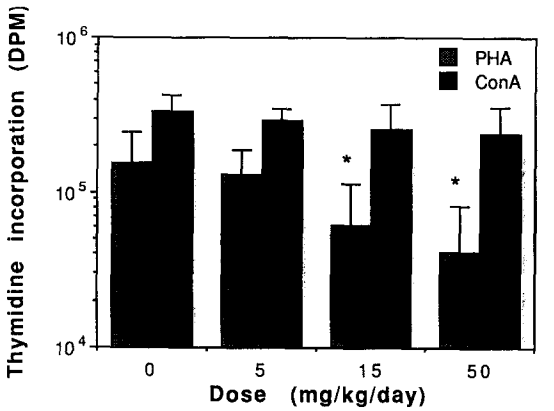


Fig. 6. Effects of DHPG treatment of mice on in vitro splenic lymphocyte responses. Spleen cells from mice treated with DHPG were collected and stimulated, in the absence of drug, with PHA or con A. The results are displayed as the mean and standard error of mean (SEM) of 4 separate experiments. Asterisk (*) indicates a significant difference ($P < 0.05$) from control.

## *Effect of antiviral drugs on DTH response*

To evaluate the effect of DHPG and HPMPC on the development of DTH responses, mice were given daily i.p. injections of drug at doses of 5, 15, or 50 mg/kg per day for 7 days. On the second and third days of drug treatment, the mice were sensitized to DNFB, while on the last day of drug treatment, the ears of the mice were challenged with the sensitizing agent.

Treatment of mice with either drug decreased the development of DTH responses as measured by increased ear thickness 24 h after challenge (Fig. 7).

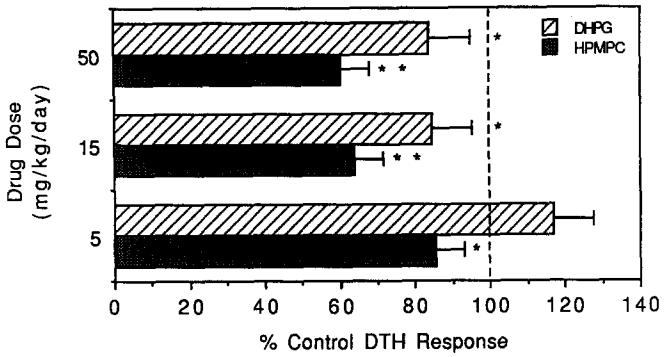


Fig. 7. The inhibitory effects of drug treatment on the development of DTH responses in mice. The results are displayed as the mean and standard error of mean (SEM) of 2 separate experiments with a total of 12 animals per experimental group. Single asterisk (*) denotes a significant difference ($P < 0.05$) from control while double asterisk (**) indicates a difference for the treatment groups without double asterisk, including control animals.

DHPG treatment at doses of 15 or 50 mg/kg per day significantly decreased DTH responses below that of control or 5 mg/kg per day treated mice ($P < 0.05$). All doses of HPMPC treatment significantly decreased DTH responses ($P = 0.001$) with 15 and 50 mg/kg per day having the greatest effect ($P < 0.001$). Also, HPMPC treatment had a greater inhibitory effect than DHPG treatment ($P = 0.01$). For example, HPMPC-treated mice had 60–75% of the response of control mice while DHPG-treated mice had 70–95% of the control response at a dose of 15 mg/kg per day.

## Discussion

As part of the preclinical evaluation of a new antiviral drug, the potential side effects of the drug on the immune system need to be examined. Although DHPG has been reported as immunotoxic in vitro (Bowden et al., 1987), there is no report of its potential effect in vivo. Also, HPMPC has not been evaluated for its immunotoxic potential. In the present studies, we compared the effects of DHPG with HPMPC on lymphocyte transformation and DTH responses.

The presence of either DHPG or HPMPC in lymphocyte cultures in vitro has a pronounced inhibitory effect on the response of both human and mouse lymphocytes to PHA and con A. However, human lymphocyte responses to con A and PHA were inhibited to a greater extent by the presence of DHPG than HPMPC. Mouse lymphocyte responses to con A were also more sensitive to DHPG than HPMPC, but there was no difference in the inhibition of PHA responses. Thus, PHA responses of human lymphocytes were more sensitive to DHPG than those of mouse lymphocytes. This suggests that DHPG may have a greater inhibitory effect on some immunologic functions in man than indicated by in vivo studies in mice; however, further studies are required.

Each of the drugs also had no effect on lymphocyte viability, indicating that the inhibitory effect by DHPG and HPMPC was not due to toxicity resulting in cell death. Previous studies, which examined toxicity of DHPG and HPMPC to human foreskin fibroblasts (Kern, 1991), also demonstrated that cell death did not occur at drug concentrations up to 100 $\mu$g/ml. Cellular proliferation was also inhibited by these drugs, but in contrast to the present studies, HPMPC inhibited fibroblast proliferation to a greater extent than DHPG.

DHPG may have a more prolonged effect on lymphocyte function than HPMPC. We found that treatment of mice with DHPG decreased the effectiveness of their lymphocytes to respond to PHA stimulation in vitro, while no effect was seen after HPMPC treatment. As the lymphocyte cultures were established in the absence of drug in these experiments, the effect of DHPG was exerted only in the animal and not during the actual stimulation or activation of the cells as in the previous studies. It is unlikely that drugs were carried over into the cultures as the cells were isolated by density gradient and washed with media, prior to culture. Therefore, the depressed response due to DHPG treatment was due to the prior exposure of the lymphocytes to the drug in vivo.

The development of DTH responses in vivo were also inhibited by drug treatment of the animals. Mice treated with 15 or 50 mg/kg per day of DHPG had lower DTH responses as compared to control mice and those treated with 5 mg/kg per day. However, at all doses of HPMPC tested (5, 15, 50 mg/kg per day), there was a depression of DTH response, with the higher two doses having the greatest effect. In addition, HPMPC treatment of mice had the greater inhibitory effect on the development of DTH responses than similar treatment with DHPG.

These drugs may not have a large effect at doses effective in patients. In previous animal studies (Kern, 1991), HPMPC, at doses as low as 1 to 5 mg/kg per day, was able to effectively reduce mortality in mice inoculated with MCMV whereas DHPG required doses of 10–15 mg/kg per day. Thus, the present studies indicate that the immunotoxic potential of HPMPC is not greater, and in many cases less, than that of DHPG when the drugs are compared at equivalent effective doses. The major effects on immune responses were at high doses which are clearly greater than those generally used in the treatment of viral infection in humans. In fact, neither drug in vivo had a large effect on lymphocyte blastogenesis or DTH responses in mice, suggesting that the immunotoxic potential for DHPG and HPMPC is small during treatment of patients; however, it is possible that these effects may be important in patients with partially intact immune systems.

In summary, these studies demonstrate that both DHPG and HPMPC can have an inhibitory effect on the development of normal immunity, and the level of these effects can differ between the drugs. Lymphocyte transformation was affected more by DHPG than HPMPC, while the reverse was true with DTH responses. Also, DHPG treatment had a prolonged effect on lymphocyte function whereas a similar effect was not seen with HPMPC. Lastly, the overall

immunotoxic potential of HPMPC is less than that of DHPG when the drugs are compared at equivalent therapeutic dosages.

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