Antiviral Research, 20 (1993) 1–12

AVR 00595

Efficacy of ganciclovir in combination with other antimicrobial agents against cytomegalovirus in vitro and in vivo

Vicki R. Freitas, Elizabeth B. Fraser-Smith and Thomas R. Matthews

Syntex Research, Palo Alto, CA 94304, USA

(Received 31 March 1992; accepted 9 July 1992)

Summary

In MRC-5 cell cultures, the efficacy of the acyclic nucleoside ganciclovir (GCV) against human cytomegalovirus (CMV) was unaffected when combined with either amphotericin B (AMP B), ketoconazole (KCZ), dapsone (DAP), or trimethoprim/sulfamethoxazole (TMP/SMX). When differences in 3-dimensional plots for antiviral activity and cytotoxicity of GCV alone and in combination were compared, the anti-CMV activity of GCV (IC_{50} 8 μM, 5–9 μM range) was not affected by concentrations of up to 10 μM AMP B, 1000 μM KCZ, 100 μM DAP or 320 μM TMP/SMX (higher concentrations could not be tested due to cytotoxicity). In Swiss Webster mice, the anti-CMV activity of GCV was also unaffected when administered in combination with any of the four other antimicrobial agents against murine CMV. GCV (s.c.) alone had an ED_{50} of 7 mg/kg (2–12 mg/kg range) which was unaffected by daily doses of 1 mg/kg AMP B (i.p.), 60 mg/kg KCZ (i.p.), 32 mg/kg DAP (p.o.) or 80/400 mg/kg TMP/SMX (p.o.). These results suggest that GCV can be administered in combination with these other drugs for treatment of various opportunistic infections in AIDS patients without compromising the efficacy of GCV against CMV.

Human cytomegalovirus; Murine cytomegalovirus; Ganciclovir; Amphotericin B; Ketoconazole; Dapsone; Trimethoprim/sulfamethoxazole; Combination study

Correspondence to: V.R. Freitas, Syntex Research, 3401 Hillview Avenue, Palo Alto, CA 94304, USA.

Introduction

Numerous opportunistic infections may afflict patients with the acquired immunodeficiency syndrome (AIDS). Since such infections often occur concurrently, patients may require treatment with more than one drug to alleviate multiple disease states. Before using multiple drug therapies, studies should be undertaken to ensure that detrimental drug interactions do not occur.

In this paper, we report the results of a series of in vitro and in vivo studies conducted to determine if the efficacy of ganciclovir (GCV) against cytomegalovirus (CMV) is affected when combined with either amphotericin B (AMP B), ketoconazole (KCZ), dapsone (DAP), or trimethoprim/sulfamethoxazole (TMP/SMX). AMP B and KCZ are antifungals used against *Candida*, and in addition, AMP B is also used against *Cryptococcus* (Fazio, et al., 1983; Walsh, 1988). TMP/SMX by itself and DAP in combination with other agents are presently used for treatment of *Pneumocystis carinii* (Fischl et al., 1988; Leoung et al., 1986; Mills et al., 1988).

For the present studies, an attempt was made to mimic the drug levels which would be achieved in patients, understanding that both in vitro and in vivo models have limitations. With the in vitro assays, the concentrations of GCV which were chosen bracketed the range achieved in the clinic, while the concentrations of AMP B, DAP, KCZ, and TMX/SMX were limited to the highest levels that could be achieved without significant cell toxicity. With the in vivo tests, the various routes employed in the clinic were used where possible, and in all cases, the daily doses were either equivalent to or greater than the doses used in the clinic.

Materials and Methods

Compounds

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine, GCV] and ketoconazole (KCZ) were synthesized by Syntex Research, Palo Alto, CA. Amphotericin B (AMP B) was obtained from Sigma Chemical Company, St. Louis, MO. Dapsone (DAP) was obtained from Jacobus Pharmaceuticals, Princeton, N.J. Trimethoprim/sulfamethoxazole (TMP/SMX) was obtained from Roche Laboratories, Nutley, NJ.

Cells and virus

Human embryonic lung (MRC-5) cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cells were maintained and passaged in Eagle's minimum essential medium with Earle's salts (EMEM, JRH Bioscience) containing 10% fetal calf serum (FCS, Hyclone), 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer, and 0.75 mg/ml $NaHCO_3$. No antibiotics were used. Human cytomegalovirus

(HCMV, strain AD169) was plaque titered in MRC-5 cells. Murine CMV (strain Smith) for the in vivo studies was plaque titered in primary mouse embryo fibroblast (MEF) cells. Both viruses were obtained from the ATCC.

Plaque assays

Confluent monolayers of MRC-5 cells in 12-well plates were infected with 100 pfu/well of HCMV. After a 1.25-h adsorption period, the fluid containing the virus was aspirated, and an overlay was applied consisting of EMEM supplemented with 2% FCS, 0.75 mg/ml $NaHCO_3$, 0.5% Sea Plaque agarose (FMC Bio-Products), and various concentrations of GCV alone or in combination. Six 3-fold dilutions of each drug were tested in duplicate assays, using two wells per drug combination and four wells of placebo (cell growth control) per test. Incubation was for 8 days at 37°C in air containing 5% CO_2, with a fresh overlay being added after 4 days. The overlay was then removed, the cells fixed in methanol for 15 min, and the monolayer stained with 0.05% methylene blue for 15 min. All plaques were counted using a Bellco plaque viewer.

Cytotoxicity assays

Two different methods were employed for cytotoxicity assays. In the first, a crystal violet assay (Gillies et al., 1986) was used. Briefly, 96-well plates were seeded with 1×10^5 MRC-5 cells/ml, and drugs were added to cells 24 h after seeding. After 72 h of incubation at 37°C in air containing 5% CO_2, cells were fixed in 1% glutaraldehyde (Sigma) and then stained for 30 min with 0.1% crystal violet (Sigma). Plates were destained for 15 min in deionized water, allowed to air dry, and then absorbance was read at a wave length of 570 nm on a Flow Titertek Multiskan reader. In the second procedure (Alley et al., 1988; Mosmann, 1983), 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT, from Sigma) at 1 mg/ml was added to each well after the 72-h incubation period. Plates were then incubated for 4 h at 37°C in air containing 5% CO_2. Drug and MTT were removed and replaced with DMSO (Sigma). After vigorous pipetting to dissolve the formazan crystals, absorbance was read at 570 nm on the Multiskan reader. Duplicate tests were run for each type of assay, using 4 wells per drug combination and 16 wells of placebo per test. In these tests, the two assays gave the same results regarding cell cytotoxicity, even though the crystal violet assay stains all cells following cell proliferation while the formation of formazan measures only viable cells.

Animal studies

Female Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA) weighing 10–12 g were used for the in vivo studies. All mice were infected intraperitoneally with 3.2×10^4 pfu/mouse of murine CMV. For all studies, GCV was administered subcutaneously twice daily, AMP B was given either intraperitoneally twice daily or orally once daily, KCZ was given intraperitoneally once daily, and DAP and TMP/SMX were given orally twice daily.

All treatments were given for 5 days starting 6 h after infection. The various treatment regimes were based on the clinical use of each drug (Physicians' Desk Reference, 1991). Studies continued for 14 days after infection. Remaining animals were healthy at that time.

Data analysis

For the in vitro studies, the lowest inhibitory concentration of test agent which reduced viral replication by 50% (IC_{50}) or 90% (IC_{90}) was determined using probit analysis (Finney, 1971). In addition, 3-dimensional plots were prepared for (a) percent reduction in plaques compared to virus control, (b) percent reduction in cell growth compared to cell control, and (c) the difference between reductions in plaques and cell growth. Then, stepwise linear regression coupled with analysis of covariance (ANCOVA) (Dixon et al., 1969; SAS User's Guide, 1989) was used on (a) plaque counts, (b) cytotoxicity values, and (c) percent differences between reductions in plaques and cytotoxicity values in order to determine the combined effects of the drugs on efficacy and cell toxicity. Overall differences as well as pairwise comparisons of both slopes and *Y*-intercepts from each GCV concentration curve alone and in combination with the other agent were analyzed for synergy, antagonism, or lack of

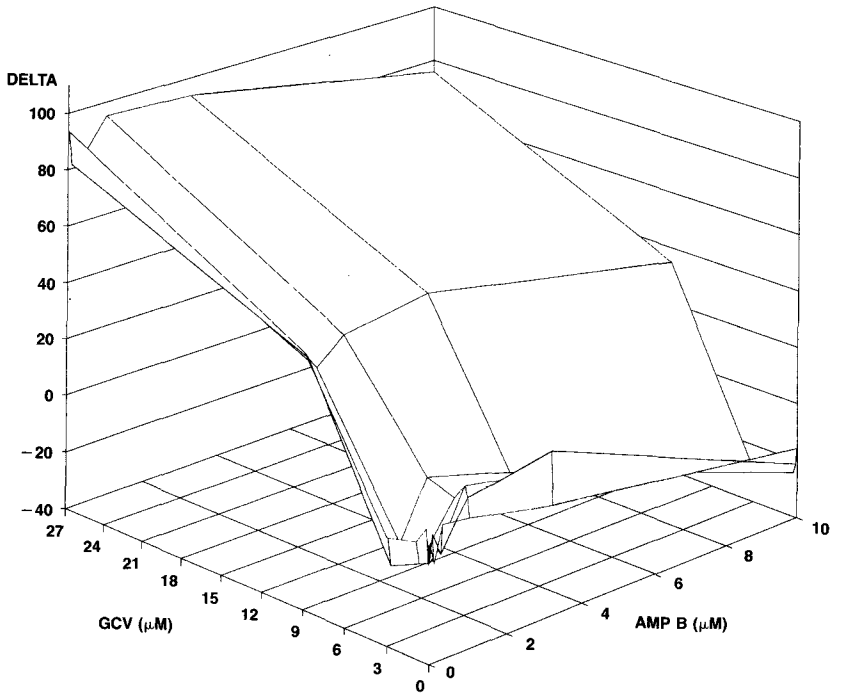

Fig. 1. Antiviral minus cell toxicity effects of GCV and AMP B alone and in combination against human CMV. DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

interaction (Chiu et al., 1992).

For the in vivo studies, Fisher exact probability (Maxwell, 1961) was used to evaluate either an increase or decrease in the number of survivors with GCV alone or in combination with the other test agent. In addition, the effective dose at which 50% of the mice survived (ED_{50}) was determined for GCV alone or in combination using probit analysis (Finney, 1971).

Results

In vitro tests

In MRC-5 cell culture, the mean IC_{50} for GCV alone was 8 μM (5–9μM range) against human CMV in the 4 plaque reduction studies, while the mean IC_{90} was 12 μM (data not shown). AMP B, KCZ and DAP were inactive against human CMV, with IC_{50} and IC_{90} values greater than the highest concentration tested (i.e., >10 μM AMP B, >1000 μM KCZ, >100 μM DAP). The activity of TMP/SMX against human CMV could not be determined from the plaque assay alone due to the cytotoxicity of this drug against the MRC-5 cells.

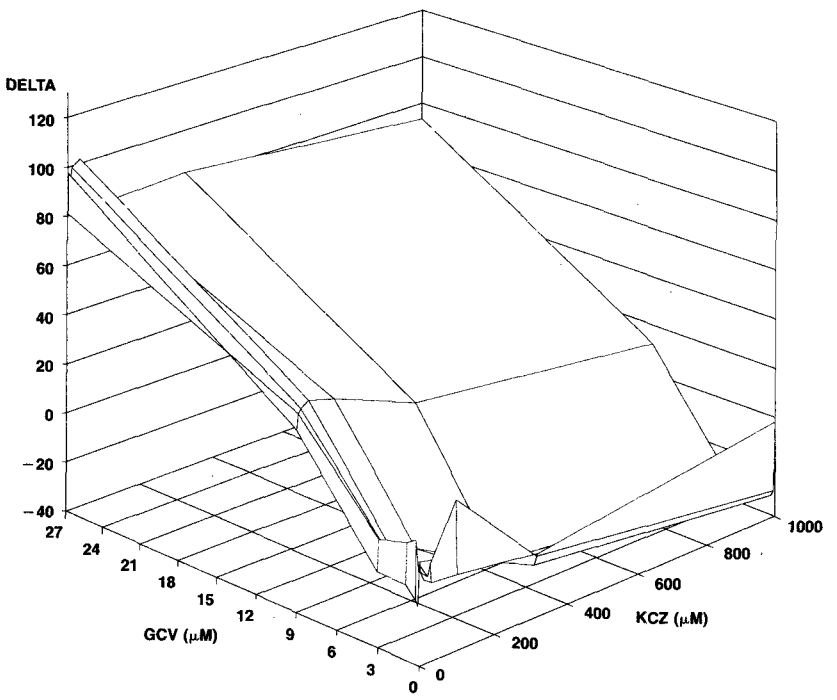

Fig. 2. Antiviral minus cell toxicity effects of GCV and KCZ alone and in combination against human CMV. DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

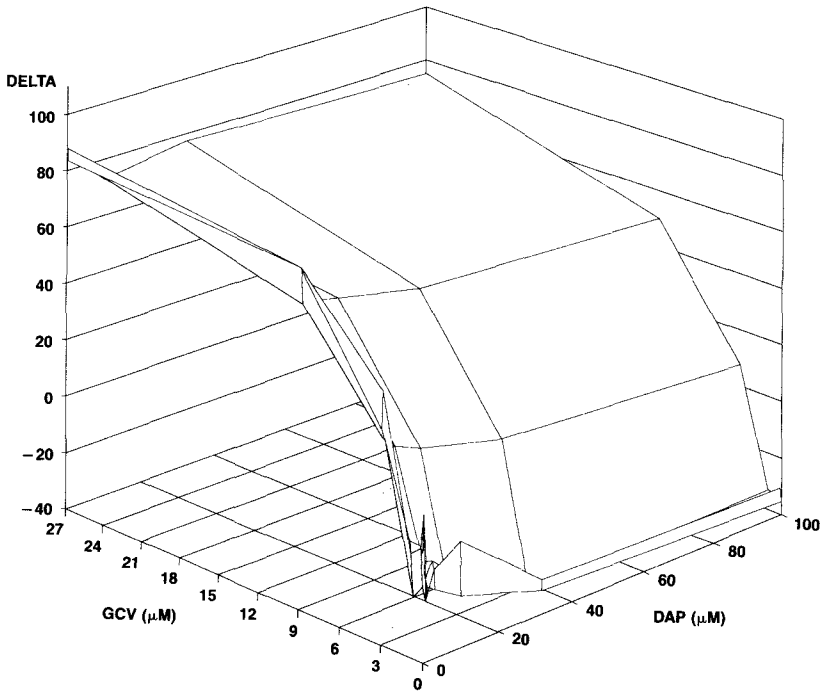

Fig. 3. Antiviral minus cell toxicity effects of GCV and DAP alone and in combination against human CMV. DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

GCV was only slightly toxic against proliferating MRC-5 cells, with 17% reduction in cellular metabolism at 27 μM, the highest concentration tested (data not shown). AMP B, KCZ and DAP were mildly toxic, with 27, 28, and 30% reductions in cellular metabolism at the highest concentrations, respectively. TMP/SMX was moderately toxic with a 40% reduction at 320 μM. In combination, the cytotoxicity of these 4 drugs did not affect the GCV toxicity profile. Neither synergy nor antagonism was detected ($P > 0.1$, linear regression analysis coupled with ANCOVA).

When differences in antiviral activity and cytotoxicity were compared, the anti-CMV activity of GCV was not affected by either AMP B, KCZ, DAP, or TMP/SMX (Figs. 1–4). The estimated slopes and Y-intercepts of the regression lines for GCV alone at each concentration of AMP B, DAP, KCZ or TMP/SMX were not significantly different overall ($P > 0.1$). In addition, pairwise comparisons of the slopes and Y-intercepts from each GCV curve alone and in combination also revealed no significant differences ($P > 0.1$).

In vivo tests

In mice, GCV alone had ED_{50}s of 2, 5, 12, and 9 mg/kg in the 4 murine CMV

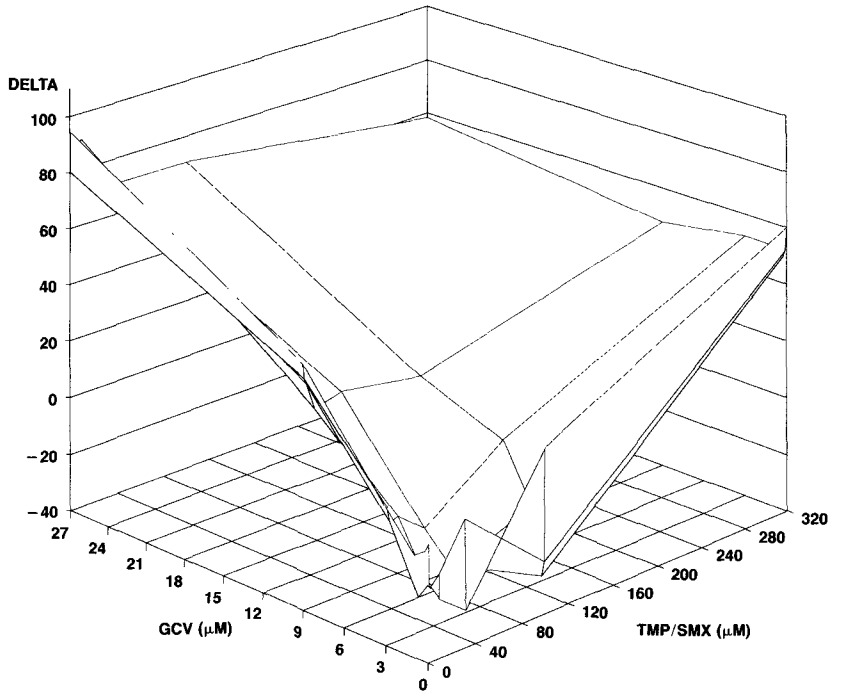

Fig. 4. Antiviral minus cell toxicity effects of GCV and TMP/SMX alone and in combination against human CMV. DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

studies with AMP B, KCZ, DAP and TMP/SMX, respectively (Tables 1–4). When various doses of GCV were used in combination with these 4 test agents, the ED_{50} for GCV remained essentially unchanged, being 2, 4, 10, and 9 mg/kg, respectively. In addition, none of the 4 test agents had protective activity against murine CMV either alone or in combination with a marginally effective dose of GCV. Consequently, the ED_{50}s were all greater than the highest dose administered (i.e., >3, >60, >32, and $>80/400$ mg/kg, respectively). When AMP B was administered intraperitoneally, some toxicity was seen at the highest dose (3 mg/kg, Table 1), and higher doses caused acute toxicity in mice (data not shown).

When AMP B was given orally, the ED_{50} for GCV alone was 13 mg/kg against murine CMV (data not shown). In combination with 20 mg/kg AMP B, the ED_{50} for GCV was unchanged at 16 mg/kg. AMP B alone did not have protective activity ($ED_{50} > 20$ mg/kg, the highest dose tested). In combination with a marginally effective dose of GCV (3 mg/kg in this test) the ED_{50} for AMP B remained >20 mg/kg. When AMP B was given orally, no signs of toxicity were seen even at the highest dose of 20 mg/kg.

TABLE 1

Survival of mice treated with different doses of ganciclovir or AMP B or both against a murine CMV infection[a]

Treatment regimen[b]		Response to treatment	ED_{50} alone or in combination		
Ganciclovir (mg/kg)	AMP B (mg/kg)	No. survivors/total[c]	Ganciclovir (mg/kg)		AMP B (mg/kg)
Saline control		1/20			
1	0	4/20			
3	0	10/20[d]	2		
9	0	20/20[d]			
27	0	20/20[d]			
1	1	5/20			
3	1	13/20[d]	2	+	1
9	1	20/20[d]			
27	1	20/20[d]			
0	0.3	1/20			
0	1	1/20			>3
0	3	1/20			
3	0.3	14/20[f]			
3	1	12/20[d]	3	+	>3
3	3	4/20[e]			

[a]Mice were treated with various concentrations of ganciclovir or AMP B to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 3 mg/kg; AMP B, 1 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination.

[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously twice daily in two equal doses. AMP B was given intraperitoneally twice daily in two equal doses. All treatments started 6 h after challenge and continued for 4 more days.

[c]Animals were held for 14 days after infection, and remaining mice were healthy at that time.

[d]$P < 0.05$, compared with saline-treated control, Fisher exact probability.

[e]$P < 0.05$, compared with same dose of GCV alone, Fisher exact probability.

Discussion

Both the present in vitro and in vivo combination studies indicate that the efficacy of GCV against CMV will not be changed when administered concurrently with normal doses of either AMP B, KCZ, DAP, or TMP/SMX. Three dimensional plots of the differences in antiviral activity and cytotoxicity appeared to be the most accurate method for studying combination effects. Including cytotoxicity analyses in statistical equations dealing with combination studies has the advantage of reducing false-positive results. For example, TMP/SMX appeared to increase the efficacy of GCV when the 3-dimensional antiviral plaque assay by itself was analyzed, whereas TMP/SMX was actually seen to have no effect on the anti-CMV activity of GCV when cytotoxicity was factored into the analysis. Similarly, TMP/SMX did not affect the activity of GCV in the in vivo murine CMV infection model.

TABLE 2

Survival of mice treated with different doses of ganciclovir or KCZ or both against a murine CMV infection[a]

Treatment regimen[b]		Response to treatment	ED_{50} alone or in combination		
Ganciclovir (mg/kg)	KCZ (mg/kg)	No. survivors/total[c]	Ganciclovir (mg/kg)		KCZ (mg/kg)
Saline Control		2/20			
1	0	5/20			
3	0	9/20[d]	5		
9	0	16/20[d]			
1	60	5/20			
3	60	12/20[d]	4	+	60
9	60	16/20[d]			
0	10	0/20			
0	30	8/20			>60
0	60	8/20			
3	10	5/20			
3	30	6/20	3	+	>60
3	60	5/19			

[a]Mice were treated with various concentrations of ganciclovir or KCZ to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 3 mg/kg; KCZ, 60 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination.

[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously twice daily in two equal doses. KCZ was given intraperitoneally once daily. All treatments started 6 h after challenge and continued for 4 more days.

[c]Animals were held for 14 days after infection, and remaining mice were healthy at that time.

[d]$P < 0.05$, compared with saline-treated control, Fisher exact probability.

[e]$P < 0.05$, compared with same dose of GCV alone, Fisher exact probability.

AMP B was given to mice by two routes of administration, intraperitoneally and orally. The drug had some toxicity at 3 mg/kg when given intraperitoneally; none was seen when given orally at 20 mg/kg. AMP B is presently administered intravenously. In the future, oral AMP B may have application in treating *Candida* gastroenteritis, an opportunistic infection of AIDS patients, since it may have sufficient efficacy against *Candida* in the gut and would be relatively non-toxic. The present tests indicate that the concomitant administration of either oral or systemic AMP B at the appropriate doses should not reduce the protective activity of GCV against murine CMV.

GCV, AMP B, KCZ, DAP, and TMP/SMX all have notable manifestations of toxicity when given for long periods of time or when higher than normal doses need to be used in patients (Collaborative DHPG Treatment Study Group, 1986; Fazio et al., 1983; Fischl et al; 1988; Mills et al., 1988; Walsh, 1988). Since the mechanisms of toxicity of each test agent are different from

TABLE 3

Survival of mice treated with different doses of ganciclovir or DAP or both against a murine CMV infection[a]

Treatment regimen[b]		Response to treatment	ED_{50} alone or in combination		
Ganciclovir (mg/kg)	DAP (mg/kg)	No. survivors/total[c]	Ganciclovir (mg/kg)		DAP (mg/kg)
Saline control		4/20			
1	0	5/20			
3	0	4/20	12		
9	0	9/20			
27	0	20/20[d]			
1	32	5/20			
3	32	12/20[d,e]	10	+	32
9	32	10/20			
27	32	19/20[d]			
0	3.2	0/20			
0	10	8/20			>32
0	32	3/20			
3	3.2	3/20			
3	10	6/20	3	+	>32
3	32	4/20			

[a]Mice were treated with various concentrations of ganciclovir or DAP to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 3 mg/kg; DAP, 32 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination.

[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously twice daily in two equal doses. DAP was given orally twice daily in two equal doses. All treatments started 6 h after challenge and continued for 4 more days.

[c]Animals were held for 14 days after infection, and remaining mice were healthy at that time.

[d]$P < 0.05$, compared with saline-treated control, Fisher exact probability.

[e]$P < 0.05$, compared with same dose of GCV alone, Fisher exact probability.

those of GCV, it is less likely that combination treatments of these agents with GCV will exert interactive toxic effects. A possible exception could be the combination of GCV with TMP/SMX, since both drugs alone can cause neutropenia in patients (Fischl, et al., 1988; Physician's Desk Reference, 1991; Collaborative DHPG Treatment Study Group, 1986). In addition, the nephrotoxic potential of AMP B could increase GCV blood levels and thereby contribute to its toxicity under certain treatment conditions.

The present results suggest that GCV can be used in combination with either AMP B, KCZ, DAP, or TMP/SMX for treatment of various opportunistic infections of AIDS patients without compromising the efficacy of GCV against CMV. As such, these data extend previous information in which no detrimental interactions on antiviral efficacy were found between GCV in combination with KCZ against HSV-2 (Pecyk et al., 1989) or between GCV in combination with PFA against CMV and HSV-2 (Freitas et al., 1989).

TABLE 4

Survival of mice treated with different doses of ganciclovir or TMP/SMX or both against a murine CMV infection[a]

Treatment regimen[b]		Response to treatment	ED_{50} alone or in combination		
Ganciclovir (mg/kg)	TMP/SMX (mg/kg)	No. survivors/total[c]	Ganciclovir (mg/kg)		TMP/SMX (mg/kg)
Saline control		4/20			
1	0	0/20			
3	0	2/19	9		
9	0	12/20[d]			
27		18/20[d]			
1	80/400	4/20			
3	80/400	5/20	9	+	80/400
9	80/400	12/20[d]			
27	80/400	20/20[d]			
0	9/44	7/20			
0	27/133	1/20			>80/400
0	80/400	2/20			
3	9/44	3/20			
3	27/133	2/20	3	+	>80/400
3	80/400	6/20			

[a]Mice were treated with various concentrations of ganciclovir or TMP/SMX to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 3 mg/kg; TMP/SMX, 80/400 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination.
[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously twice daily in two equal doses. TMP/SMX was given orally twice daily in two equal doses. All treatments started 6 h after challenge and continued for 4 more days.
[c]Animals were held for 14 days after infection, and remaining mice were healthy at that time.
[d]$P < 0.05$, compared with saline-treated control, Fisher exact probability.
[e]$P < 0.05$, compared with same dose of GCV alone, Fisher exact probability.

Acknowledgements

We thank Judy Haller, Rick Roberts, Yong Kim, and Pat Lowry for technical assistance.

References

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 48, 589–601.

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

Chiu, S., Michelson, S., Freitas, V.R., Fraser-Smith, E.B. and Schatzman, R.C. (1992) Use of stepwise linear regression combined with analysis of covariance to analyze drug combination interactions. Antivir. Res. 17S (1), 98.

Dixon, W.J. and Massey, F.J. Jr. (1969) Introduction to Statistical Analysis, 3rd edn., pp. 222–236, McGraw Hill, San Francisco.

Fazio, R.A., Wickremesinghe, P.C. and Arsura, E.L. (1983) Ketoconazole treatment of Candida esophagitis – a prospective study of 12 cases. Amer. J. Gastro. 78, 261–264.

Finney, D.J. (1971) Probit Analysis, 3rd edn., pp. 333. Cambridge University Press, London.

Fischl, M.A., Dickinson, G.M. and LaVoie, L. (1988) Safety and efficacy of sulfamethoxazole and trimethoprim chemoprophylaxis for pneumocystis carinii pneumonia in AIDS. JAMA 259, 1185–1189.

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. Antivir. Res. 12, 205–212.

Gillies, R.J., Didier, N. and Denton, M. (1986) Determination of cell number in monolayer cultures. Anal. Biochem. 159, 109–113.

Leoung, G.S., Mills, J., Hopewell, P.C., Hughes, W. and Wofsy, C. (1986) Dapsone-trimethoprim for pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. Ann. Int. Med. 105, 45–48.

Maxwell, A.E. (1961) Analyzing Quantitative Data, pp. 13–23, Ballantyne, London.

Mills, J., Leoung, G., Medina, I., Hopewell, P.C., Hughes, W.T. and Wofsy, C. (1988) Dapsone treatment of pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. Antimicrob. Agents Chemother. 32, 1057–1060.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65, 55–63.

Pecyk, R.A., Fraser-Smith, E.B. and Matthews, T.R. (1989) Lack of antiviral activity of ketoconazole alone or in combination with the acyclic nucleoside ganciclovir against a herpes virus type 2 infection in mice. Acta. Virol. 33, 569–572.

Physicians' Desk Reference (1991) 45th edn., pp. 1107–1108, 1118–1119, 1828–1830, 2147–2148, Barnhart, E.R. (publisher), Medical Economics Data, Oradell, New Jersey.

SAS User's Guide: Statistics (1989) 6th edn., SAS Institute Inc., Cary, North Carolina.

Walsh, T.J. (1988) Recent advances in the treatment of systemic fungal infections: a brief review. ASM News. 54, 240–243.