Antiviral Research, 12 (1989) 205–212
Elsevier

AVR 00382

Increased efficacy of ganciclovir in combination with foscarnet against cytomegalovirus and herpes simplex virus type 2 in vitro and in vivo

Vicki R. Freitas, Elizabeth B. Fraser-Smith and Thomas R. Matthews
Syntex Research, Palo Alto, CA 94304, U.S.A.

(Received 13 February 1989; accepted 22 August 1989)

Summary

In tissue culture, efficacy against either murine CMV or HSV-2 was increased 27-fold for the acyclic nucleoside ganciclovir and 3-fold for foscarnet (trisodium phosphonoformate) when the 2 drugs were combined; whereas against human CMV, efficacy was increased 3-fold for both drugs. In mice, efficacy was increased 2-fold for ganciclovir and 4- to 5-fold for foscarnet when used in combination against either murine CMV or HSV-2. These results suggest an additive interaction between the two drugs in vivo.

Cytomegalovirus, human, murine; HSV-2; Ganciclovir; DHPG, Foscarnet; PFA; Combination study

Introduction

The triphosphate of ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine, DHPG] and foscarnet (trisodium phosphonoformate, PFA) are both inhibitors of human cytomegalovirus (CMV) and herpes simplex virus (HSV) DNA polymerase (Frank and Cheng, 1985; Oberg, 1983; Tyms et al., 1987) and show encouraging results in the treatment of life-threatening CMV infections (Reed and Myers, 1987). In this process, the triphosphate of ganciclovir is initially produced by viral and/or cellular kinases (Reed and Myers, 1987).

In this paper, we report the results of a series of in vitro and in vivo studies conducted to determine if the combination of ganciclovir and foscarnet exhibit en-

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

hanced activity against human or murine CMV, or against herpes simplex virus type 2 (HSV-2). In previous in vitro studies, acyclic nucleosides were reported to have synergy with either foscarnet or phosphonoacetic acid (PAA) against HSV-1 and -2 (Crumpacker et al., 1984; Smith et al., 1982).

Materials and Methods

Compounds

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine, DHPG] was obtained from Syntex Research, Palo Alto, CA. Foscarnet (trisodium phosphonoformate, PFA) was obtained from Sigma, St. Louis, MO.

Cells and viruses

Human embryonic lung (MRC-5) cells (M.A. Bioproducts, Walkersville, MD) were used for all assays involving human CMV (strain AD169), primary mouse embryo fibroblast (MEF) cells for assays involving murine CMV (strain Smith), and African green monkey kidney (VERO) cells (American Type Culture Collection, Rockville, MD) for assays involving HSV-2 (strain G). All viruses used were obtained from the ATCC.

Plaque assays

Plaque assays were done using the procedures previously described (Freitas et al., 1985) with the following modifications: 12-well microplates were used for all assays, and eight dilutions of each drug were run either in combination or alone against each virus, using two wells per drug combination and four wells of placebo per test. Duplicate assays were run, and the same IC_{100}s were obtained for each virus.

Cytotoxicity assays

Two different methods were employed for cytotoxicity assays. In the first, a crystal violet assay (Gillies et al., 1978) was used. Briefly, 96-well plates were seeded with 1×10^5 MRC-5 cells/ml, 5×10^4 MEF cells/ml, or 1×10^4 VERO cells/ml. Drugs were added to cells 24 h after seeding. After 72 h of incubation at 37°C in 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; Mossmann, 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 four hr at 37°C in 5 percent 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 four wells per drug combination and sixteen wells of placebo per test. In these tests, the two assays gave the same results regarding cell cytotoxicity, even though the crystal violet assay measures cell proliferation and the formation of formazan measures cell viability.

Animal studies

Female Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA) weighing 10–12 g for the murine CMV studies or 14–17 g for the HSV-2 studies were used. All mice were infected intraperitoneally, either with 3.2×10^4 PFU/mouse of murine CMV, or else with 1.5×10^4 PFU/mouse of HSV-2. For the murine CMV studies, ganciclovir was administered subcutaneously twice daily, while foscarnet was given intraperitoneally three times daily for 5 days, both starting 6 h after infection. For the HSV-2 studies, ganciclovir was administered once daily for 5 days starting 24 h after infection, while foscarnet was given intraperitoneally twice daily for 5 days starting 6 h after infection. Differences in treatment regimes reflect differences in efficacy of the two drugs against either virus. Studies continued for either 14 (murine CMV) or 21 (HSV-2) days after infection. Remaining animals were healthy at that time.

Data analyses

The lowest inhibitory concentration of each drug, alone or in combination, that suppressed plaque formation by 100% (IC_{100}) and the effective dose at which 50% of the mice survived (ED_{50}) were calculated using probit analysis (Finney, 1971). The IC_{100} value was chosen for the plaque assays since it provides for a more rigorous evaluation of possible combination effects. The Fisher exact probability (Maxwell, 1961) was used to evaluate an increase in the number of survivors in the animal studies. The possibility of enhanced efficacy was analyzed by calculating the fractional inhibitory concentration (FIC) index (Dougherty et al., 1977) for in vitro studies, or the fractional protective dose (FPD) index (Berenbaum, 1978) for in vivo studies as follows: (IC_{100} or ED_{50} of drug A in combination/IC_{100} or ED_{50} of drug A alone) + (IC_{100} or ED_{50} of drug B in combination/IC_{100} or ED_{50} of drug B alone). In these experiments, FIC or FPD values of <0.5 were considered synergistic, and values between 0.5 and 1.5 were considered additive. For the cytotoxicity assays, the percent reduction in either viable or total cells was determined by comparing the optical densities of drug versus control wells as follows: [(absorbance value of untreated cells – absorbance value of treated cells)/absorbance value of untreated cells]100.

Results

In vitro

For ganciclovir, the IC_{100} for the drug alone was 27 μM against all three viruses tested (Table 1). In combination with 100 μM foscarnet, the IC_{100} for ganciclovir was reduced to 9 μM against human CMV, and to 1 μM against murine CMV. Against HSV-2, the IC_{100} for ganciclovir was reduced to 1 μM in combination with 320 μM foscarnet. This 3 to 27-fold reduction in activity by ganciclovir appeared to be directed against the viruses, since at these concentrations neither drug alone or in combination showed evidence of cytotoxicity against the cells. In addition, ganciclovir had no cytotoxic effect at 27 μM, the highest dose tested.

For foscarnet, the IC_{100} for the drug alone was 320 μM against human and murine CMV, while against HSV-2, the IC_{100} was 1000 μM. In combination with 1 μM ganciclovir, the IC_{100} for foscarnet was reduced to 100 μM against murine CMV and to 320 μM against HSV-2. Against human CMV, the IC_{100} for foscarnet was reduced to 100 μM in combination with 9 μM ganciclovir. Again, these 3-fold reductions in activity by foscarnet appeared to be directed against the viruses, since at these concentrations, neither drug alone or in combination showed evidence of cytotoxicity against the cells.

Against human CMV, the calculated FIC index was 0.64, which suggests an additive effect between the two drugs (Table 1). Enhanced efficacy between the two drugs against murine CMV and HSV-2 was confirmed, with FIC values of 0.34 and 0.36, respectively, where values <0.5 indicate potentiation (Table 1).

In vivo

When given alone, foscarnet at 300 mg/kg per day had little protective activity against murine CMV (Table 2). Only 20% of the foscarnet-treated mice survived, compared with no survivors in the saline treated controls ($P > 0.1$). When this

TABLE 1

Antiviral efficacy of ganciclovir and foscarnet alone or in combination against either human CMV, murine CMV, or HSV-2 in tissue culture

Virus	IC_{100} (μM)			
	Alone		Combination	FIC Index
	Ganciclovir	Foscarnet	Ganciclovir + Foscarnet	
Human CMV[a]	27	320	9 + 100	0.64
Murine CMV[b]	27	320	1 + 100	0.34
HSV-2[c]	27	1000	1 + 320	0.36

[a]Grown in MRC-5 cells.
[b]Grown in MEF cells.
[c]Grown in Vero cells.

TABLE 2

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

Treatment regimen[b]		Response to treatment	ED_{50} alone or in combination		
Ganciclovir (mg/kg)	Foscarnet (mg/kg)	No. of survivors/total[c]	Ganciclovir (mg/kg)		Foscarnet (mg/kg)
Saline control		0/15			
1	0	4/15			
3	0	4/14	3.5		
9	0	15/15			
1	300	6/13			
3	300	7/13	2	+	300
9	300	15/15			
0	96	0/15			
0	300	3/15			242
0	960	10/14			
3	96	8/15			
3	300	6/13	3	+	57
3	960	14/15			

[a]Mice were treated with various concentrations of ganciclovir or foscarnet to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 3 mg/kg; foscarnet, 300 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination. FPDI = 2/3.5 + 57/242 = 0.8 (additive).

[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously twice daily in two equal doses. Foscarnet was given intraperitoneally three times daily in three equal doses. All treatments started at six h after challenge and continued for four more days.

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

marginally effective dose was used in combination with various doses of ganciclovir, foscarnet increased the activity of ganciclovir 2-fold against murine CMV as measured by a reduction in ED_{50} from 4 to 2 mg/kg. Likewise, using a 3 mg/kg dose of ganciclovir alone, only 28% of the mice survived ($P > 0.1$). When this marginally effective dose was used in combination with various doses of foscarnet, ganciclovir increased the activity of foscarnet by 4-fold as measured by a reduction in ED_{50} from 242 to 57 mg/kg.

An additive interaction between these two drugs was confirmed by calculating the FPD index, which was 0.8. Values between 0.5 and 1.5 are interpreted as an additive interaction.

When given alone, foscarnet at 32 mg/kg per day had little protective activity against a HSV-2 infection (Table 3). Only 22% of the foscarnet treated mice survived, compared with 10% survivors for the untreated controls ($P > 0.1$). When this marginally effective dose was used in combination with various doses of ganciclovir, foscarnet increased the activity of ganciclovir 2-fold against HSV-2 as measured by a reduction in ED_{50} from 8 to 4 mg/kg. Likewise, using a 5 mg/kg

TABLE 3

Survival of mice treated with different doses of ganciclovir or foscarnet or both against an HSV-2 infection

Treatment regimen[b]			Response to treatment	ED_{50} alone or in combination		
Experiment No.	Ganciclovir (mg/kg)	Foscarnet (mg/kg)	No. of survivors/total[c]	Ganciclovir (mg/kg)		Foscarnet (mg/kg)
1	Untreated control		2/20			
	0	32	4/18			
	1.5	0	4/20			
	5	0	8/20	7.5		
	14	0	17/20			
	1.5	32	10/20			
	5	32	9/20	3.7	+	32
	14	32	19/19			
2	Untreated control		0/20			
	5	0	6/20			
	0	32	6/20			
	0	100	10/20	170		
	0	320	14/20			
	5	32	15/20			
	5	100	15/20	5	+	<32
	5	320	19/20			

[a]Mice were treated with various concentrations of ganciclovir or foscarnet to determine the ED_{50} alone and with the marginally effective dose of each agent (ganciclovir, 5 mg/kg; foscarnet, 32 mg/kg) combined with various concentrations of the other to determine the ED_{50} in combination. FPDI = 3.7/7.5 + <32/170 = >0.5, <0.68 (additive).

[b]Doses are expressed in mg/kg per day. Ganciclovir was given subcutaneously once daily for five days starting 24 hr after challenge. Foscarnet was given intraperitoneally twice daily in two equal doses for five days starting 6 hr after challenge.

[c]Animals were held for 21 days after infection, and remaining mice were healthy at that time.

dose of ganciclovir alone, only 30% of the mice survived ($P > 0.1$). When this marginally effective dose was used in combination with various doses of foscarnet, ganciclovir increased the activity of foscarnet >5-fold as measured by a reduction in ED_{50} from 170 to <32 mg/kg.

An additive interaction between these two drugs was confirmed by calculating the FPD index, which was between 0.5 and 0.68.

Discussion

In tissue culture, efficacy against either murine CMV or HSV-2 was increased 27-fold for ganciclovir and 3-fold for foscarnet when the two drugs were combined. With a calculated FIC index of ≤0.36, these results suggest a synergistic interaction between the two drugs against either virus. This result supports previous reports of synergy with ganciclovir and foscarnet against HSV-2 in human

fetal fibroblast cells (Smith et al., 1982) and between other acyclic nucleosides and foscarnet or PAA (Crumpacker et al., 1984). On the other hand, against human CMV, efficacy was increased only 3-fold for both drugs in combination. With an FIC index of 0.6, these results suggest additive efficacy rather than true synergism.

In mice, however, protective activity against either murine CMV or HSV-2 increased only 2-fold for ganciclovir and 4- to 5-fold for foscarnet. With an FPD index of 0.8 for murine CMV and 0.5 to 0.68 for HSV-2, these results are interpreted as indicating additive efficacy against either virus. Interestingly, an additive effect for these two drugs in combination was predicted, based on recent dual inhibition studies by Frank and Cheng (1985). In their studies, inhibition of HSV DNA polymerase by ganciclovir triphosphate prevented simultaneous inhibition by foscarnet. This mutually exclusive inhibition pattern suggested that binding sites on the DNA polymerase molecule for these two compounds were functionally overlapping and, thus, additive rather than synergistic activity might occur.

In the present experiments, both in vitro and in vivo activities seen with either drug alone closely matched that seen in previous publications (Freitas et al., 1985; Kern et al., 1978; Smith et al., 1982). In all cases, ganciclovir exhibited much greater antiviral activity than did foscarnet. The persistence of ganciclovir triphosphate in virus-infected cells after drug removal may contribute to the greater influence in vivo of ganciclovir over foscarnet (Smee et al., 1985). In such a situation, virus replication may be inhibited even after blood levels have declined. This potent activity may be a reason why, in the in vivo tests, a marginally effective dose of ganciclovir reduced the ED_{50} of foscarnet by 4- to 5-fold, while a marginally effective dose of foscarnet only reduced the ED_{50} of ganciclovir 2-fold.

Acknowledgements

We thank Judy Haller, Pat Lowry, Rick Roberts and Jose Rosete 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.

Berenbaum, M.C. (1978) A method for testing for synergy with any number of agents. J. Infect. Dis. 137, 122–130.

Crumpacker, C.S., Kowalsky, P.N., Oliver, S.A., Schnipper, L.E. and Field, A.K. (1984) Resistance of herpes simplex virus to 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine: physical mapping of drug synergism within the viral DNA polymerase locus. Proc. Natl. Acad. Sci. U.S.A. 81, 1556–1560.

Dougherty, P.F., Yotter, D.W. and Matthews, T.R. (1977) Microdilution transfer plate technique for determining in vitro synergy of antimicrobial agents. Antimicrob. Agents Chemother. 11, 225–228.

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

Frank, K.B. and Cheng, Y.-C. (1985) Mutually exclusive inhibition of herpesvirus DNA polymerase

by aphidicolin, phosphonoformate, and acyclic nucleoside triphosphates. Antimicrob. Agents Chemother. 27, 445–448.

Freitas, V.R., Smee, D.F., Chernow, M., Boehme, R. and Matthews, T.R. (1985) Activity of 9-(1,3-dihydroxy-2-propoxymethyl)guanine compared with that of acyclovir against human, monkey, and rodent cytomegaloviruses. Antimicrob. Agents Chemother. 28, 240–245.

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

Kern, E.R., Glasgow, L.A., Overall, J.C. Jr., Reno, J.M. and Boezi, J.A. (1978) Treatment of experimental herpesvirus infections with phosphonoformate and some comparisons with phosphonoacetate. Antimicrob. Agents Chemother. 14, 817–823.

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

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

Oberg, B. (1983) Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmac. Ther. 19, 387–415.

Reed, E.C. and Myers, J.D. (1987) Treatment of cytomegalovirus infection. In: W.L. Drew (Ed.), Clinics in Laboratory Medicine, Vol. 7, No. 4, pp. 838–845. W.B. Saunders, Philadelphia.

Smee, D.F., Boehme, R., Chernow, M., Binko, B.P. and Matthews, T.R. (1985) Intracellular metabolism and enzymatic phosphorylation of 9-(1,3-dihydroxy-2-propoxymethyl)guanine and acyclovir in herpes simplex virus-infected and uninfected cells. Biochem. Pharm. 34, 1049–1056.

Smith, K.O., Galloway, K.S., Ogilvie, K.K. and Cheriyan, U.O. (1982) Synergism among Biolf-62, phosphonoformate, and other antiherpetic compounds. Antimicrob. Agents Chemother. 22, 1026–1030.

Tyms, A.S., Davis, J.M., Clarke, J.R. and Jeffries, D.J. (1987) Synthesis of cytomegalovirus DNA is an antiviral target late in virus growth. J. Gen. Virol. 68, 1563–1573.