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Activity of Different Antiviral Drug Combinations against Human Cytomegalovirus Replication in Vitro

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The effects of different antiviral drug combinations on the replication of various human cytomegalovirus (CMV) strains in human embryonic lung (HEL) fibroblasts were evaluated. HPMPC combined with either ganciclovir, foscarnet or acyclovir showed additive to synergistic inhibition of CMV replication. Combinations of zidovudine with HPMPC, ganciclovir, foscarnet or acyclovir also resulted in additive to synergistic inhibition of CMV replication. Synergism tended to be higher for the clinical CMV isolates than for the reference strains AD-169 and Davis. Suppression of CMV replication was obtained at lower drug concentrations when the drugs were combined than when the drugs were used alone. At the highest drug concentrations used in the antiviral activity experiments, neither drug alone nor its combination suppressed host cell growth. If higher drug concentrations were used, zidovudine increased the inhibitory effects of ganciclovir, acyclovir and foscarnet but not of HPMPC, on cell proliferation. Use of combinations in the therapy of CMV infections may be considered to enhance drug efficacy, to reduce toxicity and, possibly, to diminish the risk of emergence of drug-resistant virus strains.

Human cytomegalovirus (CMV) is an ubiquitous human pathogen that affects 50 % to 90 % of adults. In healthy individuals CMV infections are usually asymptomatic. However, under certain clinical conditions CMV infections can be quite severe. Primary exposure to CMV during pregnancy may result in intra-uterine infection causing a wide spectrum of syndromes in the newborn, such as prematurity, mental retardation, sensory neural deafness, hepatosplenomegaly and thrombocytopenia (1, 2). CMV infections are often observed following kidney, heart or liver transplantation, and are particularly problematic in allogeneic bone-marrow transplant recipients (3, 4). In these patients, CMV can cause life-threatening interstitial pneumonitis (5). In addition, CMV is one of the most common infections in patients suffering from the acquired immune deficiency syndrome (AIDS), retinitis and colitis being the most frequent CMV manifestations (6, 7).

Only two drugs, ganciclovir and foscarnet are currently used in the treatment of CMV infections. In AIDS patients, ganciclovir can prevent CMV replication, leading to suppression of retinitis and improvement in the outcome of colitis (8, 9). Although ganciclovir has shown some efficacy in the treatment of CMV pneumonitis in AIDS patients, it has not improved the outcome of this disease in bone-marrow transplant patients in spite of suppression of viral replication in the lungs (10, 11). Recently, ganciclovir in combination with hyperimmune anti-CMV immunoglobulins has demonstrated some efficacy in reducing mortality due to CMV pneumonitis in bone-marrow transplant patients (12, 13). A drawback associated with the clinical use of ganciclovir is that prolonged treatment can lead to severe neutropenia and/or thrombocytopenia, necessitating cessation of ganciclovir treatment in some cases (8, 9). Another factor causing problems in the long-term use of ganciclovir is the emergence of virus resistance to the drug (14, 15).

Foscarnet treatment can lead to suppression of CMV replication in kidney transplant recipients (16, 17). In AIDS patients, foscarnet is effective in the treatment of interstitial pneumonitis and retinitis, including ganciclovir-resistant CMV retinitis (18–20). However, it has been documented that foscarnet may lead to reversible renal impairment with increased serum creatinine levels (16–19). In a recent study com-

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paring ganciclovir and foscarnet in the treatment of CMV retinitis in AIDS patients, foscarnet was less well tolerated than ganciclovir (21). Recently, Knox et al. (22) described the isolation of a human CMV strain resistant to both foscarnet and ganciclovir.

Acyclovir is the drug of choice for the treatment of herpes simplex virus (HSV-1 and HSV-2) infections. Although it has only weak activity against CMV, acyclovir may have some efficacy in the prevention of CMV disease in kidney and bone marrow allograft recipients (23, 24).

Considering the severity of CMV infections in immunocompromised patients, more effective and/or less toxic drugs for treatment are required. The use of combination chemotherapy is an interesting approach to enhance drug efficacy and decrease drug toxicity. In addition, such combination chemotherapy may also diminish the risk of development of virus resistance to the drug.

In recent years, various phosphonylmethoxyalkylpurine and -pyrimidine derivatives have been shown to exhibit activity against a broad-spectrum of DNA viruses (i.e. adenoviruses, herpesviruses, iridoviruses, pox viruses and hepadnaviruses) as well as retroviruses including human immunodeficiency virus (HIV) (25–27). We have also reported that (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine (HPMPA) and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine (HPMPC) are potent and selective inhibitors of CMV replication in vitro (28, 29). In this study we tested the anti-CMV activity of the acyclic nucleoside phosphonates HPMPA and HPMPC in combination with ganciclovir, foscarnet and acyclovir. Considering the fact that treatment of CMV infections is often required in HIV-infected patients under therapy with zidovudine, we also examined the effects of zidovudine combined with either HPMPA, HPMPC, ganciclovir, foscarnet or acyclovir on CMV replication in vitro.

Materials and Methods

Cells. Human embryonic lung (HEL) fibroblasts (ATCC CCL 137) were used at low passages. Cells were propagated in minimum essential medium (MEM) supplemented with 10 % inactivated fetal calf serum (Integro, The Netherlands), 1 % L-glutamine and 0.3 % sodium bicarbonate.

Viruses. The CMV reference strains AD-169 (ATCC VR 538) and Davis (ATCC VR 807) were provided by Dr. S. Michelson (Institut Pasteur, Paris, France). The source of the clinical CMV strains and virus stock preparations has been described previously (29).

Compounds. The drugs tested and their sources were as follows: HPMPA and HPMPC (Institute of Organic Chemistry and Biochemistry, Czechoslovakia); ganciclovir (Syntex, USA); acyclovir (Wellcome Research Laboratories, USA); foscarnet and zidovudine (Sigma, USA).

Antiviral Assay. Confluent HEL cells were grown in 96-well microtiter plates and infected with the different virus strains at a concentration of 100 plaque forming units (PFU) per well. After a 2 h incubation period, residual virus was removed and the infected cells were further incubated with MEM supplemented with 2 % inactivated fetal calf serum, 1 % L-glutamine and 0.3 % sodium bicarbonate containing different concentrations of the test compounds (in duplicate). After incubation for seven days at 37 °C in 5 % CO_2 atmosphere, viral cytopathic effect was measured as described previously (28, 30). Antiviral activity was expressed as the IC50, i.e. the inhibitory concentration required to reduce the viral cytopathicity by 50 %.

Virus Yield Assay. Confluent HEL cells grown in two-chamber slides (Nunc, USA) were infected with CMV (AD-169 strain) at a multiplicity of infection of approximately 0.05 PFU/cell. After a 2 h adsorption period, residual virus was discarded and the cell cultures were incubated with fresh medium containing different concentrations of the test compounds. After incubation for seven days at 37 °C, supernatants were harvested and stored at -80 °C. Virus titers were determined by a plaque assay in HEL cell monolayers.

Analysis of Combinations. The combined inhibitory effects of the different drugs on CMV-induced cytopathicity were examined by checkerboard combinations of various concentrations of the test compounds. The combination effects were analyzed by the isobologram method (31). In this analysis, the IC50 was used for calculation of the fractional inhibitory concentration (FIC). The FIC for each drug combination, i.e. compound x plus compound y, was calculated as follows: FIC_x = IC50 of compound x in combination/IC50 of compound x alone, and FIC_y = IC50 of compound y in combination/IC50 of compound y alone. When the FIC index, which corresponds to the FIC of the compounds combined ($FIC_x + FIC_y$), is equal to 1.0 the combination is additive; when it is between 1.0 and 0.5, the combination is partially synergistic; when it is < 0.5 the combination is synergistic; when it is between 1.0 and 2.0 the combination is partially antagonistic and when it is > 2.0 the combination is antagonistic.

Cytotoxicity Assay. Cytotoxicity measurements were based on the inhibition of HEL cell growth, as described previously (27, 28). Briefly, 3 x 10^3 cells/well were seeded in a 96-well microtiter plate and allowed to proliferate for 24 h in MEM containing 10 % fetal calf serum. Different concentrations of the test compounds were added (in duplicate), and after a three-day incubation period at 37 °C in 5 % CO_2 atmosphere, the cell number was determined with a Coulter counter. Cytotoxicity was expressed as the CC50, i.e. the concentration required to reduce cell growth by 50 %. Cytotoxicity was also

Table 1: In vitro inhibition of CMV replication by combination of foscarnet with HPMPC.

CMV strain	IC50 of foscarnet (μg/ml)[a]					IC50 of HPMPC (μg/ml)[a]					FIC index[b]
	Alone	With HPMPC at given concentration				Alone	With foscarnet at given concentration				
		0.004 μg/ml	0.01 μg/ml	0.04 μg/ml	0.1 μg/ml		0.4 μg/ml	1 μg/ml	4 μg/ml	10 μg/ml	
AD-169	20.0	15.0	8.0	3.25	1.45	0.2	0.15	0.105	0.045	0.008	0.37
Davis	17.5	14.0	12.5	12.0	6.0	0.45	0.425	0.375	0.23	0.052	0.56
CM-15	25.0	14.0	14.0	7.0	1.0	0.2	0.14	0.10	0.07	0.014	0.47
CM-F	20.0	40.0	10.0	16.0	2.0	0.28	0.18	0.14	0.084	0.01	0.45

[a] The 50 % antiviral inhibitory concentration (IC50) was calculated from the dose-dependent inhibitory curves. Data represent mean values for two separate experiments.
[b] FIC index = $FIC_x + FIC_y$, whereby FIC = fractional inhibitory concentration.

Table 2: In vitro inhibition of CMV replication by combination of ganciclovir with HPMPC.

CMV strain	IC50 of ganciclovir (μg/ml)[a]					IC50 of HPMPC (μg/ml)[a]					FIC index[b]
	Alone	With HPMPC at given concentration				Alone	With ganciclovir at given concentration				
		0.004 μg/ml	0.01 μg/ml	0.04 μg/ml	0.1 μg/ml		0.04 μg/ml	0.1 μg/ml	0.4 μg/ml	1 μg/ml	
AD-169	1.0	0.4	0.15	0.1	< 0.04	0.064	0.07	0.04	0.004	< 0.004	0.31
Davis	1.45	1.2	0.7	0.65	0.425	0.19	0.35	0.27	0.0875	0.022	0.54
CM-15	1.6	1.0	0.64	0.64	0.04	0.1	0.1	0.08	0.064	0.004	0.50
CM-F	0.58	0.5	0.4	0.4	0.2	0.5	0.2	0.14	0.04	< 0.004	0.45

[a,b] See footnotes to Table 1.

Table 3: In vitro inhibition of CMV replication by combination of acyclovir with HPMPC.

CMV strain	IC50 of acyclovir (μg/ml)[a]					IC50 of HPMPC (μg/ml)[a]					FIC index[b]
	Alone	With HPMPC at given concentration				Alone	With acyclovir at given concentration				
		0.004 μg/ml	0.01 μg/ml	0.04 μg/ml	0.1 μg/ml		1 μg/ml	4 μg/ml	10 μg/ml	40 μg/ml	
AD-169	60	32.5	12.5	5.75	3.5	0.135	0.2	0.0575	0.0175	0.004	0.30
Davis	15.0	12.0	8.0	7.0	6.5	0.3	0.3	0.12	0.022	<0.004	0.59
CM-15	20.0	23.0	20.0	14.0	6.4	0.23	0.2	0.14	0.064	<0.004	0.75
CM-F	64.0	17.0	10.0	7.4	<1	0.14	0.085	0.064	0.01	<0.004	0.26

[a,b] See footnotes to Table 1.

measured by a dye exclusion test based on the use of trypan blue. The procedure is similar to that used to determine the cell number by cell counter: confluent HEL cells in 96-well microtiter plates were incubated with different concentrations of the test compounds (each point of the combination was tested in quadruplicate). After seven days of incubation at 37 °C in 5 % CO_2 atmosphere, the cells were trypsinized with 100 μl of trypsin and diluted in an equal volume of a 1 % solution of trypan blue. Ten microliters of the cell suspension were then transferred to Burker chambers and the living cells were counted under a microscope.

Results

Combinations of HPMPC or HPMPA with Foscarnet, Ganciclovir or Acyclovir. The effects of HPMPC alone and in combination with foscarnet, ganciclovir or acyclovir on the replication of CMV in HEL cells are shown in Tables 1 to 3. The IC50 values obtained for the drugs alone were consistent with previously reported data (29, 30). Thus, the IC50 ranged from 0.06 to 0.5 μg/ml for HPMPC, 0.5 to 1.6 μg/ml for ganciclovir, 17.5 to 20 μg/ml for foscarnet and 15 to 64 μg/ml for acyclovir. The FIC indexes obtained showed that all combinations had partially synergistic activity against CMV. No significant differences were noted between the reference CMV strains (AD-169 and Davis) and the clinical CMV isolates. Mean FIC indexes for combinations of HPMPC with either foscarnet, ganciclovir or acyclovir (Tables 1 to 3) were 0.46, 0.45 and 0.475, respectively. Similar results were obtained when HPMPA was used in combination with foscarnet, ganciclovir or acyclovir (data not shown). The mean FIC indexes for combinations of HPMPA with either foscarnet, ganciclovir or acyclovir were 0.57, 0.54 and 0.52, respectively.

Neither alteration of HEL cell morphology nor inhibition of cell proliferation was observed with any of the drugs alone or in combination at the highest concentrations used (100 μg/ml for acyclovir and foscarnet, 10 μg/ml for ganciclovir and 1 μg/ml for HPMPC and HPMPA). However, when the combined inhibitory effects of HPMPC and acyclovir, ganciclovir or foscarnet on the growth of infected HEL cells were evaluated at higher concentrations (200 μg/ml was used as highest concentration for each compound), the combinations had an additive to partially antagonistic effect on cell growth (Figure 1). FIC values based on the CC50 ranged from 0.94 to 1.7, which means that cytotoxicity was not enhanced following combination of HPMPC with either foscarnet, ganciclovir or acyclovir.

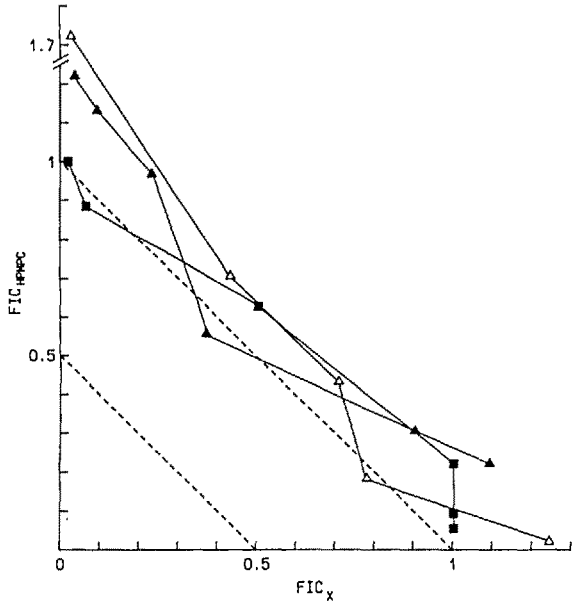

Figure 1: Isobologram representation of the inhibitory effects on HEL cell proliferation of combinations of HPMPC with ganciclovir (Δ—Δ), acyclovir (■—■) or foscarnet (▲—▲). Broken lines represent the unity lines for FIC equal to 1 and 0.5.

Combinations of Zidovudine with HPMPC, HPMPA, Foscarnet, Ganciclovir and Acyclovir. The effects of zidovudine alone and in combination with HPMPC, foscarnet, ganciclovir or acyclovir on the replication of CMV in HEL cells are shown in Tables 4 to 7. Whereas zidovudine did not inhibit CMV cytopathicity up to a concentration of 200 μg/ml, a concentration-dependent inhibition of CMV cytopathicity was observed for all the other compounds. The IC50 ranged from 0.074 to 0.62 μg/ml for HPMPC, 2 to 6.4 μg/ml for ganciclovir, 14 to 74 μg/ml for foscarnet, and 10 to 70 μg/ml for acyclovir. Combination of zidovudine with either HPMPC, foscarnet, ganciclovir or acyclovir resulted in a significant diminution of the IC50 of these drugs. The FIC indexes obtained (Tables 4 to 7) showed that all combinations had additive to synergistic inhibition of CMV, synergism being more evident with the clinical CMV strains. Thus, for the reference CMV strains, the combinations in decreasing order of synergism (based on the mean FIC indexes) were HPMPC/zidovudine (0.255), ganciclovir/zidovudine (0.395), acyclovir/zidovudine (0.485) and foscarnet/zidovudine (0.495). The mean FIC indexes for the clinical CMV isolates, which were lower than for the reference CMV strains pointing to more pronounced synergism of the different combinations against the clinical strains, were as follows: ganciclovir/zidovudine (0.18), HPMPC/zidovudine (0.2), acyclovir/ zidovudine (0.24) and foscarnet/zidovudine (0.41). Similar results were obtained when zidovudine was combined with HPMPA (data not shown), the mean FIC index being 0.33 and 0.2 for the reference and clinical CMV strains, respectively.

To confirm that combinations of zidovudine with HPMPC, ganciclovir, acyclovir or foscarnet really resulted in synergistic inhibition of CMV replication, virus yield assays were performed with combinations of the drugs at different concentrations and a fixed concentration (200 μg/ml) of zidovudine. As shown in Figure 2, concentration-dependent inhibition of the CMV yield was obtained with HPMPC, ganciclovir, acyclovir and foscarnet. Although zidovudine did not affect virus cytopathicity at a concentration of 200 μg/ml, at this concentration it produced a 1 $\log_{10}$ reduction in virus yield. In all cases, at 0.02 and 0.05 μg/ ml for HPMPC (Figure 2), at 0.2 and 0.5 μg/ml for HPMPA (data not shown), at 0.5 μg/ml for ganciclovir (Figure 2) and at 5 and 20 μg/ml for both acyclovir and foscarnet (Figure 2), the differences between the virus yield obtained with the anti-CMV agents in the presence and absence of zidovudine were higher than 1 $\log_{10}$. Neither alteration of HEL cell morphology nor inhibition of cell growth was observed with any of the compounds at the concentrations used in the experiments described in Tables 4 to 7 and Figure 2. If however higher concentrations were employed (200 μg/ml was used as the highest concentration for each compound), inhibition of cell growth was observed. As shown in Figure 3, combinations of zidovudine with acyclovir or ganciclovir resulted in a partially synergistic inhibitory effect on host cell growth. Similarly, combination of zidovudine with HPMPA led to a synergistic inhibitory effect on HEL cell growth (data not shown). Also, slight synergism was noted for the combination of zidovudine with foscarnet (Figure 3). In contrast, the combination of zidovudine with HPMPC resulted in a partially antagonistic inhibitory effect on HEL cell growth. Results obtained by the trypan blue exclusion test on confluent HEL cells for ganciclovir, HPMPC and foscarnet when combined with high concentrations of zidovudine are reported in Table 8. No toxicity could be demonstrated when this method was applied with the different drug combinations. Similar results were obtained when the same method was used to evaluate toxicity of the same combinations on growing cells (data not shown).

Table 4: In vitro inhibition of CMV replication by combination of zidovudine with HPMPC.

CMV strain	IC50 of HPMPC (μg/ml)[a]					IC50 of zidovudine (μg/ml)[a]					FIC index[b]
	Alone	With zidovudine at given concentration				Alone	With HPMPC at given concentration				
		2 μg/ml	5 μg/ml	20 μg/ml	50 μg/ml		0.01 μg/ml	0.04 μg/ml	0.1 μg/ml	0.4 μg/ml	
AD-169	0.5	0.14	0.14	0.072	0.05	>200	>200	200	10	<0.5	0.25
Davis	0.4	0.27	0.15	0.064	0.058	>200	>200	>200	7.5	1.4	0.26
CM-6	0.1	0.025	0.02	0.016	0.01	>200	>200	0.5	<0.5	<0.5	0.25
CM-7	0.14	0.064	0.064	0.064	0.064	>200	>200	0.7	<0.5	<0.5	0.15
CM-16	0.4	0.074	0.062	0.062	0.062	>200	>200	200	0.5	<0.5	0.26
CM-21	0.04	0.0064	0.0064	0.0064	0.0064	>200	0.8	0.8	<0.5	<0.5	0.17
CM-24	0.62	0.2	0.062	0.02	0.01	>200	50	10	3.2	<0.5	0.13
CM-F	0.074	0.074	0.04	0.01	0.01	>200	200	5	1	<0.5	0.24

[a,b] See footnotes to Table 1.

Table 5: In vitro inhibition of CMV replication by combination of zidovudine with ganciclovir.

CMV strain	IC50 of ganciclovir (μg/ml)[a]					IC50 of zidovudine (μg/ml)[a]					FIC index[b]
	Alone	With zidovudine at given concentration				Alone	With ganciclovir at given concentration				
		2 μg/ml	5 μg/ml	20 μg/ml	50 μg/ml		0.1 μg/ml	0.4 μg/ml	1 μg/ml	4 μg/ml	
AD-169	2	1.4	1.0	0.64	0.4	>200	>200	50	5	<0.5	0.42
Davis	3.4	3.6	3.5	2.6	0.4	>200	117	105	16	1.5	0.37
CM-6	4	1	0.4	0.04	0.04	>200	5	5	2	<0.5	0.11
CM-7	6.4	1.4	1.4	0.7	0.5	>200	>200	>200	7	<0.5	0.21
CM-15	4	1.6	1	0.8	0.4	>200	>200	50	5	0.5	0.28
CM-16	5	4	2	0.4	0.4	>200	>200	50	10	2	0.18
CM-21	2	2	0.2	0.074	0.074	>200	10	3.7	<0.5	<0.5	0.13
CM-24	5	2	0.72	0.25	0.14	>200	>200	12	3.2	1.0	0.14
CM-F	2.5	0.78	0.54	0.25	0.25	>200	>200	8	<0.5	<0.5	0.2

[a,b] See footnotes to Table 1.

Table 6: In vitro inhibition of CMV replication by combination of zidovudine with acyclovir.

Isolate	IC50 of acyclovir (μg/ml)[a]					IC50 of zidovudine (μg/ml)[a]					FIC index[b]
	Alone	With zidovudine at given concentration				Alone	With acyclovir at given concentration				
		2 μg/ml	5 μg/ml	20 μg/ml	50 μg/ml		2 μg/ml	5 μg/ml	20 μg/ml	50 μg/ml	
AD-169	70	32	28	25	14	>200	>200	>200	200	<0.5	0.43
Davis	32.5	34	33.5	17.5	10.5	>200	>200	200	15	0.5	0.54
CM-6	36	20	5	2	2	>200	50	5	2	<0.5	0.15
CM-7	70	34	25	12	7	>200	>200	100	7	0.5	0.27
CM-15	32	25	11.5	9	7.4	>200	>200	>200	2.7	0.5	0.38
CM-16	32	14	10	3.2	3.2	>200	>200	10	1	<0.5	0.20
CM-21	50	35	25	7	3.2	>200	200	32	7	0.5	0.24
CM-24	20	25	5	1.2	1.2	>200	10	5	2.5	<0.5	0.15
CM-F	10	20	5	2	1	>200	20	5	2	<0.5	0.3

[a,b] See footnotes to Table 1.

Table 7: In vitro inhibition of CMV replication by combination of zidovudine with foscarnet.

CMV strain	IC50 of foscarnet (μg/ml)[a]					IC50 of zidovudine (μg/ml)[a]					FIC index[b]
	Alone	With zidovudine at given concentration				Alone	With foscarnet at given concentration				
		2 μg/ml	5 μg/ml	20 μg/ml	50 μg/ml		1 μg/ml	4 μg/ml	10 μg/ml	40 μg/ml	
AD-169	28	14	14	7.0	4.7	>200	>200	>200	8.0	<0.5	0.35
Davis	36	35.6	32	24	14.7	>200	150	105	100	16	0.64
CM-6	40	40	40	6.4	4.0	>200	200	200	5.0	5.0	0.26
CM-7	50	64	58	20	14	>200	>200	>200	>200	12.5	0.50
CM-15	14	14	10	6.4	6.4	>200	>200	>200	5.0	<0.5	0.56
CM-21	64	64	64	20	14	>200	>200	>200	>200	11.5	0.41
CM-24	20	7.3	5.5	2.0	0.62	>200	32	10	0.5	0.5	0.2
CM-F	74	64	54	30	25	>200	>200	>200	>200	10	0.5

[a,b] See footnotes to Table 1.

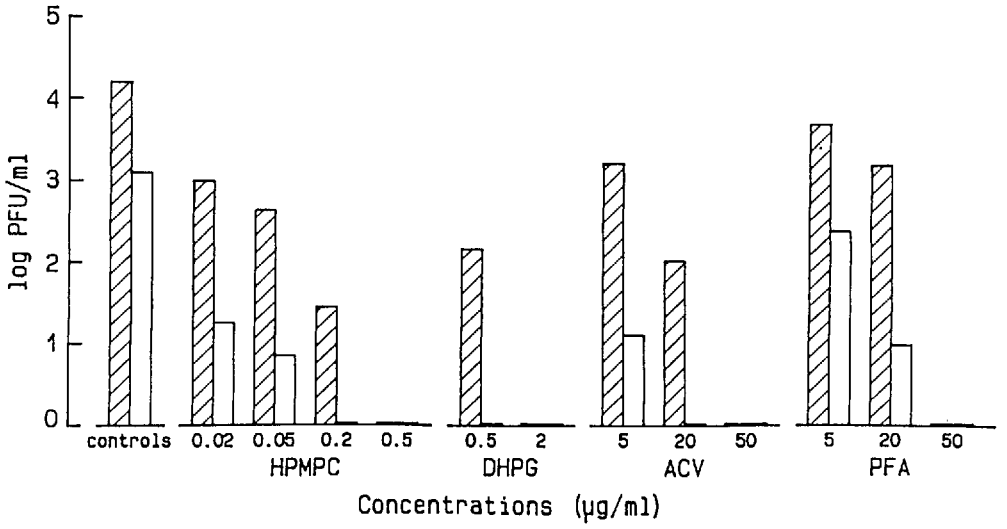

Figure 2: Combined inhibitory effects of various anti-CMV drugs and zidovudine on virus yield production in HEL cells. HEL cell cultures were infected with CMV (AD-169 strain) and, after virus adsorption, cells were incubated in the presence of different concentrations of either HPMPC, ganciclovir, acyclovir or foscarnet without zidovudine (▨) or with zidovudine at 200 µg/ml (□). Virus yield was determined after 7 days of incubation. Mean values for two separate experiments are shown.

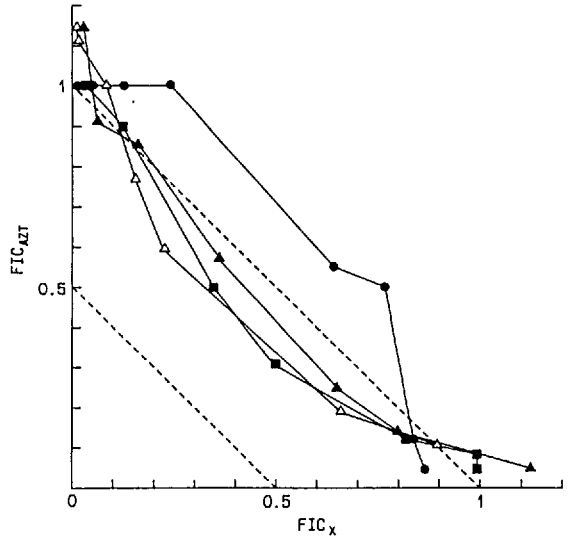

Figure 3: Isobologram representation of the inhibitory effects on HEL cell proliferation of combinations of zidovudine with HPMPC (●—●), ganciclovir (Δ—Δ), acyclovir (■—■) or foscarnet (▲—▲). Broken lines represent the unity lines for FIC equal to 1 and 0.5.

Discussion

Combination of antiviral drugs against CMV is an interesting approach to maximize their antiviral efficacy and minimize their toxicity for the host. In addition, combination chemotherapy may also reduce the possible risk of emergence of drug-resistant virus. Combination chemotherapy is widely used in the treatment of bacterial infections and malignant diseases. Except for the combination of zidovudine and ribavirin that results in antagonistic inhibitory effect on HIV replication (32, 33), other combinations of antiviral agents generally lead to increased antiviral activity (33–35). We therefore evaluated the inhibitory effects on CMV of the phosphonylmethoxyalkyl derivatives HPMPC and HPMPA in combination with the currently available antiviral drugs ganciclovir, acyclovir or foscarnet. In view of the high incidence of CMV infections in AIDS patients, we also evaluated the inhibitory effects on CMV of combinations of these drugs with the anti-HIV agent zidovudine.

Combination of HPMPC with foscarnet, ganciclovir or acyclovir led to an additive to partially synergistic inhibitory effect on CMV replication in HEL fibroblasts. With these combinations suppression of CMV replication was achieved at lower drug concentrations than when the drugs were used individually. None of the drugs alone or in combination affected proliferation of the HEL cells at the highest concentrations used in the antiviral activity experiments. When the concentrations were increased to levels affecting host cell growth, no increased cytotoxicity was observed with any of the combinations (HPMPC/

Table 8: Toxicity of three anti-CMV drugs when combined with high concentrations of zidovudine as measured by the trypan blue exclusion test.

	CC50 (μg/ml)		
	Ganciclovir	HPMPC	Foscarnet
Zidovudine (100 μg/ml)	40	>100	100
Zidovudine (25 μg/ml)	>100	>100	100
Alone	>100	>100	150

CC50: Concentration required to decrease cell viability by 50 %.

foscarnet, HPMPC/ganciclovir or HPMPC/acyclovir). The combination of HPMPA with either foscarnet, ganciclovir or acyclovir also resulted in a partially synergistic inhibitory effect on CMV replication, but it should be recognized that HPMPA is about five to ten times more toxic for HEL cell growth than its congener HPMPC.

Synergistic in vitro anti-CMV effects have been reported previously for combinations of foscarnet with ganciclovir (36), whereas studies in vitro suggest that the interaction is additive (37). The fact that combinations of anti-CMV drugs may result in additive to synergistic antiviral effects is particularly relevant in the prevention of development of drug-resistant CMV strains. Ganciclovir-resistant CMV strains appear to arise with an appreciable frequency in the hospital environment (14, 15), and recently a foscarnet-resistant CMV strain has been isolated from a patient on foscarnet treatment (21). Both foscarnet and ganciclovir interfere with CMV DNA synthesis at the viral DNA polymerase step (38, 39). Similarly, inhibition of CMV replication by HPMPC also results from selective suppression of viral DNA synthesis (40). Whereas virus resistance to foscarnet and acyclovir appears to be based on mutations in the DNA polymerase gene (38), CMV resistance to ganciclovir is mainly associated with a decrease in the phosphorylation rate (41). Drug sensitivity studies have indicated that foscarnet-resistant mutants are also resistant to acyclovir, but still sensitive to ganciclovir, HPMPC and HPMPA (42), whereas ganciclovir-resistant CMV strains remain sensitive to foscarnet and acyclovir (41), as well as HPMPC (43). Whether the use of different drug combinations can modulate the emergence of drug-resistant virus strains in vivo is an interesting issue that remains to be explored.

The in vivo efficacy of HPMPC has been established in several animal models for CMV infection (44–46). A remarkable feature of the phosphonylmethoxyalkyl derivatives is that they generate prolonged antiviral activity (27). The long-lasting activity of HPMPC against CMV has been demonstrated in both cell culture (40, 47) and animal models for CMV infection (44–46). This makes HPMPC an excellent candidate for future clinical trials, since it would seem feasible to generate an antiviral response with infrequent dosing or even a single dose of the compound. Combination of HPMPC with other anti-CMV drugs may potentiate its efficacy, allow lower dosage regimens (thus reducing toxicity), and diminish the risk of development of resistance.

The finding that combinations of zidovudine with either HPMPC, HPMPA, ganciclovir, acyclovir or foscarnet result in an additive (for the reference strains) to synergistic (for the clinical strains) inhibitory activity against CMV may have important implications in view of the high frequency of CMV infections in AIDS patients. A flow cytometry assay has proved useful for evaluating the anti-CMV activity of selected antiviral compounds in vitro (28, 48). However, this method did not prove applicable to the study of drug combinations (e.g. zidovudine combined with anti-CMV compounds), possibly because interactions of the drugs at different stages of the viral cycle led to incomplete fluorescence.

In an attempt to demonstrate that synergism in these combinations could be due to zidovudine-induced deoxynucleoside triphosphate (dNTP) pool depletion, as already demonstrated in human cells (49–51), we conducted virus yield experiments on days 3, 4 and 7 post-infection. The purpose was to see whether an eventual drop of these dNTP pools in HEL-infected cells could lead after one virus replicative cycle to an anti-CMV activity of zidovudine. However, zidovudine by itself did not significantly affect virus yield, while combination with anti-CMV compounds showed a trend towards synergism

after one viral replication cycle (data not shown). Synergism was evident on day 7 (Figure 2). The well known metabolic stimulation with increased production of some enzymes after CMV infection (52) makes interpretation of this synergism rather problematic.

In terms of toxicity, combinations of zidovudine with HPMPA, ganciclovir or acyclovir led to a slight increase in the inhibition of HEL cell proliferation, whereas combination of zidovudine with HPMPC led to a slight decrease in the inhibition of host cell growth. Similar results have been found when these combinations were evaluated for their inhibitory effect on bone marrow cell proliferation (granulocyte-macrophage colony formation) (53).

Eriksson and Schinazi (54) reported a moderate synergistic inhibitory effect for the combination of zidovudine with foscarnet against HIV in vitro. From the present results it appears that this combination also has an additive anti-CMV effect, which thus lends further credence to the potential usefulness of this combination in the treatment of CMV infections in HIV-infected individuals. Our results support the concept that HPMPC may be useful in the treatment of CMV infections in AIDS patients while the latter are under zidovudine treatment for their underlying HIV disease. Furthermore, HPMPC may be useful in combination with other anti-CMV drugs (such as ganciclovir and foscarnet) for maximizing antiviral activity while minimizing toxic side effects and, possibly, reducing the risk of development of drug-resistant virus strains.

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References

1. **Fowler KB, Pass RF:** Sexually transmitted diseases in mothers of neonates with congenital cytomegalovirus infection. Journal of Infectious Diseases 1991, 164: 259–264.
2. **Britt W, Pass R, Stagno S, Alford C:** Pediatric cytomegalovirus infection. Transplantation Proceedings 1991, 23, Supplement 3: 115–117.
3. **Betts R:** Cytomegalovirus infection in transplant patients. Progress in Medical Virology 1982, 73: 241–248.
4. **Schmidt G:** Treatment of CMV infections and disease in transplantation. Transplantation Proceedings 1991, 23, Supplement 3: 126–130.
5. **Meyers JD, Flournoy N, Thomas ED:** Risk factors for cytomegalovirus infection after human marrow transplantation. Journal of Infectious Diseases 1986, 153: 478–488.
6. **Macher A, Reichert C, Straus S, Longo D, Parrillo J, Lane H, Fauci A, Rook A, Manischewitz J, Quinnan G:** Death in the AIDS patients: role of cytomegalovirus. New England Journal of Medicine 1983, 309: 1454.
7. **Tyms A, Taylor D, Parkin J:** Cytomegalovirus and the acquired immunodeficiency syndrome. Journal of Antimicrobial Chemotherapy 1989, 23, Supplement A: 89–105.
8. **Laskin OL, Stahl-Baylis CM, Kalman CM, Rosecran LR:** Use of ganciclovir to treat serious cytomegalovirus infections in patients with AIDS. Journal of Infectious Diseases 1987, 155: 323–327.
9. **Masur H, Lane HC, Palestine A, Smith PD, Manischewitz J, Stevens G, Fujikawa L, Macher AM, Nussenblatt R, Baird B, Megill M, Wittek A, Quinnan GV, Parrillo JE, Rook AH, Eron LJ, Poretz DM, Goldenberg RI, Fauci AS, Gelman EP:** Effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine on serious cytomegalovirus disease in eight immunosuppressed homosexual men. Annals of Internal Medicine 1986, 104: 41–44.
10. **Erice A, Jordan M, Chace B, Fletcher C, Chinnock B, Balfour H:** Ganciclovir treatment of cytomegalovirus disease in transplant recipients and other immunocompromised hosts. Journal of the American Medical Association 1987, 257: 3082–3087.
11. **Shepp DH, Dandliker PS, de Miranda P, Burnett TC, Cederburg DM, Kirk LE, Meyers JD:** Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine in the treatment of cytomegalovirus pneumonia. Annals of Internal Medicine 1985, 103: 368–373.
12. **Reed EC, Bowden RA, Dandliker PS, Lilleby KE, Meyers JD:** Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. Annals of Internal Medicine 1988, 109: 783–788.
13. **Emanuel D, Cunningham I, Jules-Elysee K, Brochstein JA, Kernan NA, Laver J, Stover D, White DA, Fels A, Polsky B, Castro-Malaspina H, Peppard JR, Bartus P, Hammerling U, O'Reilly RJ:** Cytomegalovirus pneumonia after bone marrow transplantation successfully treated with the combination of ganciclovir and high-dose intravenous immune globulin. Annals of Internal Medicine 1988, 109: 777–782.
14. **Drew WL, Miner RC, Busch DF, Follansbee SE, Gullett J, Mehalko SG, Gordon SM, Owen WF, Matthews TR, Buhles WC, DeArmond B:** Prevalence of resistance in patients receiving ganciclovir or serious cytomegalovirus infection. Journal of Infectious Diseases 1991, 163: 716–719.
15. **Erice A, Chou S, Biron KK, Stanat SC, Balfour HH, Jordan MC:** Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. New England Journal of Medicine 1989, 320: 289–293.
16. **Klintmalm G, Lonnqvist B, Öberg B, Gahrton G, Lernestedt JO, Lundgren G, Ringden O, Robert KH, Wahren B, Groth CG:** Intravenous foscarnet for the treatment of severe cytomegalovirus infection in allo-

graft recipients. Scandinavian Journal of Infectious Diseases 1985, 17: 157–163.
17. **Ringden O, Lonnqvist B, Paulin T, Ahlmen J, Klintmalm G, Wahren B, Lernestedt JO:** Pharmacokinetics, safety, and preliminary clinical experiences using foscarnet in the treatment of cytomegalovirus infections in bone marrow and renal transplant recipients. Journal of Antimicrobial Chemotherapy 1986, 17: 373–387.
18. **Farthing C, Anderson MG, Ellis ME, Gazzard G, Chanas AC:** Treatment of cytomegalovirus pneumonitis with foscarnet (trisodium phosphonoformate) in patients with AIDS. Journal of Medical Virology 1987, 22: 157–162.
19. **Walmsley S, Chew E, Read SE, Vellend H, Salit I, Rachlis A, Fanning MM:** Treatment of cytomegalovirus retinitis with trisodium phosphonoformate hexahydrate (foscarnet). Journal of Infectious Diseases 1988, 157: 569–572.
20. **Jacobson MA, Drew WL, Feinberg J, O'Donnell JJ, Whitmore PV, Miner RD, Parenti D:** Foscarnet therapy for ganciclovir-resistant cytomegalovirus retinitis in patients with AIDS. Journal of Infectious Diseases 1991, 163: 1348–1351.
21. **Studies of Ocular Complications of AIDS Research Group, in Collaboration with the AIDS Clinical Trials Group:** Mortality in patients with the acquired immunodeficiency syndrome treated with either foscarnet or ganciclovir for cytomegalovirus retinitis. New England Journal of Medicine 1992, 326: 213–220.
22. **Knox K, Dobryski W, Carrigan D:** Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet 1991, 337: 1292–1293.
23. **Balfour HH, Chace BA, Stapleton JT, Simmons RL, Fryd DS:** A randomized, placebo-controlled trial of oral acyclovir for the prevention of cytomegalovirus disease in recipients of renal allografts. New England Journal of Medicine 1989, 320: 1381–1387.
24. **Meyers JD, Reed EC, Shepp DH, Thornquist M, Dandliker PS, Vicary CA, Flournoy N, Kirk LE, Kersey JH, Thomas ED, Balfour HH:** Acyclovir for prevention of cytomegalovirus infection and disease after allogeneic marrow transplantation. New England Journal of Medicine 1988, 318: 70–75.
25. **De Clercq E, Holy A, Rosenberg I, Sakuma T, Balzarini J, Maudgal PC:** A novel selective broad-spectrum anti-DNA virus agent. Nature 1986, 323: 464–467.
26. **De Clercq E, Sakuma T, Baba M, Pauwels R, Balzarini J, Rosenberg I, Holy A:** Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Research 1987, 8: 261–272.
27. **De Clercq E:** Broad-spectrum anti-DNA virus and antiretrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. Biochemical Pharmacology 1991, 42: 963–972.
28. **Snoeck R, Sakuma T, De Clercq E, Rosenberg I, Holy A:** (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrobial Agents and Chemotherapy 1988, 32: 1839–1844.
29. **Snoeck R, Schols D, Andrei G, Neyts J, De Clercq E:** Antiviral activity of anti-cytomegalovirus agents (HPMPC, HPMPA) assessed by a flow cytometric method and DNA hybridization technique. Antiviral Research 1991, 16: 1–9.
30. **Andrei G, Snoeck R, Schols D, Goubau P, Desmyter J, De Clercq E:** Comparative activity of selected antiviral compounds against clinical isolates of human cytomegalovirus. European Journal of Clinical Microbiology & Infectious Diseases 1992, 10: 1026–1033.
31. **Baba M, Ito M, Shigeta S, De Clercq E:** Synergistic antiviral effects of antiherpes compounds and human leukocyte interferon on varicella-zoster virus in vitro. Antimicrobial Agents and Chemotherapy 1984, 25: 515–517.
32. **Vogt MW, Hartshorn KL, Furman PA, Chou TC, Fyfe JA, Coleman LA, Crumpacker C, Schooley RT, Hirsch SS:** Ribavirin antagonizes the effect of azidothymidine on HIV replication. Science 1987, 235: 1376–1379.
33. **Baba M, Pauwels R, Balzarini J, Herdewijn P, De Clercq E, Desmyter J:** Ribavirin antagonizes inhibitory effects of pyrimidine 2',3'-dideoxynucleosides but enhances inhibitory effects of purine 2',3'-dideoxynucleosides on replication of human immunodeficiency virus in vitro. Antimicrobial Agents and Chemotherapy 1987, 31: 1613–1617.
34. **Baba M, Ito M, Shigeta S, Tanaka H, Miyasaka T, Ubasawa M, Umezu K, Walker RT, De Clercq E:** Synergistic inhibition of human immunodeficiency virus type 1 replication by 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU) and azidothymidine in vitro. Antimicrobial Agents and Chemotherapy 1991, 35: 1430–1433.
35. **Johnson VA, Merrill DP, Videler JA, Chou TC, Byington RE, Eron JJ, D'Aquila RT, Hirsch MS:** Two-drug combinations of zidovudine, didanosine, and recombinant interferon-α A inhibit replication of zidovudine-resistant human immunodeficiency virus type 1 synergistically in vitro. Journal of Infectious Diseases 1991, 164: 646–655.
36. **Manischewitz JF, Quinnan GV, Lane HC, Wittek AE:** Synergistic effect of ganciclovir and foscarnet on cytomegalovirus replication in vitro. Antimicrobial Agents and Chemotherapy 1990, 34: 373–375.
37. **Freitas VR, Fraser-Smith EB, Matthews TR:** Increased efficacy of ganciclovir in combination with foscarnet against cytomegalovirus and herpes simplex virus type 2 in vitro and in vivo. Antiviral Research 1989, 12: 205–212.
38. **Matthews T, Boehme R:** Antiviral activity and mechanisms of action of ganciclovir. Reviews of Infectious Diseases 1988, 10: Supplement: 490–494.
39. **Eriksson B, Öberg B, Wahren B:** Pyrophosphate analogues as inhibitors of DNA polymerases of cytomegalovirus, herpes simplex and cellular origin. Biochimica et Biophysica Acta 1982, 696: 115–123.
40. **Neyts J, Snoeck R, Schols D, Balzarini J, De Clercq E:** Selective inhibition of human cytomegalovirus DNA synthesis by (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC] and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Virology 1990, 179: 41–50.
41. **Biron KK, Fyfe JA, Stanat SC, Leslie LK, Sorrell JB, Lambe CU, Coen DM:** A human cytomegalovirus mutant resistant to the nucleoside analog 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine (BW B759U) induces reduced levels of BW B759U triphosphate. Proceedings of the National Academy of Sciences of the USA 1986, 83: 8769–8773.
42. **Sullivan V, Coen DM:** Isolation of foscarnet-resistant human cytomegalovirus patterns of resistance and sen-

sitivity to other antiviral drugs. Journal of Infectious Diseases 1991, 164: 781–784.

43. **Stanat SC, Reardon JE, Erice A, Jordan MC, Drew WL, Biron KK:** Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrobial Agents and Chemotherapy 1991, 35: 2191–2197.
44. **Snoeck R, De Clercq E:** (*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), a potent and selective inhibitor of cytomegalovirus infection in vitro and in vivo. In: Touraine JL, Traeger J, Beutel H, Dubernard JM, Revillard JP, Dupuy C (ed): Transplantation and clinical immunology. Volume 23. Elsevier Science, Amsterdam, 1991, p. 169–176.
45. **Stals F, De Clercq E, Bruggeman C:** Comparative activity of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine against rat CMV infection in vitro and in vivo. Antimicrobial Agents and Chemotherapy 1991, 35: 2262–2266.
46. **Neyts J, Balzarini J, Naesens L, De Clercq E:** Efficacy of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine and 9-(1,3-dihydroxy-2-propoxymethyl)-guanine for the treatment of murine cytomegalovirus infection in severe combined immunodeficiency mice. Journal of Medical Virology 1992, 37: 67–71.
47. **Neyts J, Snoeck R, Balzarini J, De Clercq E:** Particular characteristics of the anti-human cytomegalovirus activity of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) in vitro. Antiviral Research 1991, 16: 41–52.
48. **Schols D, Snoeck R, Neyts J, De Clercq E:** Detection of immediate early, early and late antigens of human cytomegalovirus by flow cytometry. Journal of Virological Methods 1989, 26: 247–254.
49. **Frick LW, Nelson DJ:** Effects of 3'-azido-3'-deoxythymidine on the deoxynucleoside triphosphate pools of cultured human cells. In: Mikanagi K, Nishioka K, Kelley WN (ed): Purine and Pyrimidine Metabolism in Man VI. Part B. Plenum Press, New York, 1989, p. 389–394.
50. **Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff Lehrman S, Bolognesi DP, Broder S, Mitsuya H, Barry JW:** Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proceedings of the National Academy of Sciences of the USA 1986, 83: 8333–8337.
51. **Frick LW, Nelson DJ, St. Clair MH, Furman PA, Krenitsky TA:** Effects of 3'-azido-3'-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. Biochemical and Biophysical Research Communications 1988, 154: 124–129.
52. **Ho M:** Characteristics of cytomegalovirus. In: Ho M (ed): Cytomegalovirus. Biology and Infection. Plenum, New York, 1991, p. 57–73.
53. **Snoeck R, Lagneaux L, Delforge A, Bron D, Van der Auwera P, Stryckmans P, Balzarini J, De Clercq E:** Inhibitory effects of potent inhibitors of human immunodeficiency virus and cytomegalovirus on the growth of human granulocyte-macrophage progenitor cells in vitro. European Journal of Clinical Microbiology and Infectious Diseases 1990, 9: 615–619.
54. **Eriksson BFH, Schinazi RF:** Combinations of 3'-azido-3'-deoxythymidine (Zidovudine) and phosphonoformate (Foscarnet) against human immunodeficiency virus type 1 and cytomegalovirus replication in vitro. Antimicrobial Agents and Chemotherapy 1989, 33: 663–669.