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# Efficacy of ganciclovir in combination with zidovudine against cytomegalovirus in vitro and in vivo

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## Summary

In cultured MRC-5 cells, ganciclovir (GCV) alone had good activity against both the established AD169 strain ($IC_{50}$ 8 and 9 $\mu$M) and a clinical isolate ($IC_{50}$ 14 $\mu$M) of human cytomegalovirus (CMV), while 3′-azido-3′-deoxythymidine (AZT) was relatively inactive [$IC_{50}$ 508 and >800 (AD169 strain); >800 $\mu$M (clinical isolate)]. When reductions in plaques were compared against reductions in the cellular metabolism of MTT at all GCV and AZT combination concentrations using an improved 3-dimensional linear regression analysis, AZT had an additive effect on the antiviral activity of GCV against the AD169 strain and potentiated the antiviral activity of GCV against the clinical isolate. Calculations showed that, in the presence of 50 $\mu$M AZT, the anti-CMV activity of GCV was unchanged for the AD169 strain, whereas the activity of GCV was increased approximately 5–10-fold for the clinical isolate. An increase in GCV efficacy for the AD169 strain first became apparent at 100 $\mu$M AZT with an approximately 3-fold increase in activity. In Swiss-Webster mice, the anti-CMV activity of GCV against murine CMV was unaffected when administered in combination with AZT. GCV given alone subcutaneously had an $ED_{50}$ of 6 mg/kg which was unaffected by daily intraperitoneal doses of 320 mg/kg AZT. These results suggest that AZT will not adversely affect the efficacy of GCV against CMV in HIV-positive, non-neutropenic patients.

Human and murine cytomegalovirus; Ganciclovir; GCV; Zidovudine; AZT; Combination studies

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## Introduction

The majority of patients that are infected with human immunodeficiency virus (HIV) eventually get cytomegalovirus (CMV) disease (Armstrong et al., 1985). Under these conditions, it is common practice for HIV patients to undergo multiple drug therapy for both the HIV infection and other opportunistic diseases such as CMV. When using multiple drug therapies, studies should be undertaken to ensure that detrimental drug interactions either do not occur or can be managed successfully.

Ganciclovir (GCV) is presently used for treatment of CMV retinitis and gastrointestinal disease (Buhles et al., 1988; Collaborative DHPG Treatment Study Group, 1986). Zidovudine (azidothymidine, AZT) is effective in the treatment of HIV, the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (Fischl et al., 1987). Previous in vitro studies have suggested that GCV and AZT are synergistically cytotoxic to human cells (Prichard et al., 1991; Medina et al., 1992) and that AZT reduces the antiviral activity of GCV against human CMV (Tian et al., 1991). More recently, Medina et al. (1992), have suggested that GCV antagonizes the anti-HIV activity of AZT and dideoxyinosine in vitro.

In this paper, we report the results of a series of in vitro and in vivo studies designed to determine whether the efficacy of GCV against CMV is affected when combined with AZT. An improved three-dimensional analytical approach including stepwise linear regression coupled with analysis of covariance was used to determine the combined effects of the drugs on both efficacy and cell toxicity. In all studies, the drug levels which would be achieved in patients were either bracketed or exceeded.

## Materials and Methods

*Compounds.* Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine, GCV] was synthesized by Syntex Research, Palo Alto, CA. Zidovudine (3′-azido-3′-deoxythymidine, AZT) was obtained from Pharmatec, Alachua, FL.

*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) from ATCC as well as a clinical isolate of HCMV (T8618) from Dr. Lawrence Drew, Mt. Zion Hospital, San Francisco, CA, were plaque titered in MRC-5 cells. Murine CMV (strain Smith) from ATCC for the in vivo studies was plaque titered in primary mouse embryo fibroblast (MEF) cells.

*Plaque assay.* 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 of $NaHCO_3$, 0.5% Sea Plaque agarose (FMC Bio-Products), and various concentrations of GCV alone or in combination with AZT. Six dilutions of each drug were tested in duplicate assays, using two wells per drug combination and four wells of cell growth control (vehicle alone) 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 were fixed with methanol for 15 min, and the monolayer was stained with 0.05% methylene blue for 15 min. All plaques were counted using a Bellco plaque viewer.

*MTT assay.* Plates containing either confluent or proliferating uninfected cells were incubated with various concentrations of GCV and/or AZT for 4 days at 37°C in air containing 5% $CO_2$. The medium was EMEM with 5% FCS and 0.75 mg/ml $NaHCO_3$. A standard MTT assay was then run in which the uptake and metabolism of 3-[4,5-dimethylthiazol-2-yl] 2,5-dephenyltetrazolium bromide (MTT, Sigma) at 1 mg/ml by the cells was measured (Alley et al., 1988; Mosmann, 1983). The amount of formazan produced from MTT in 3 h was determined by dissolving the product in isopropanol and then measuring absorbance at 570 nm as well as at 650 nm to correct for light scattering of the cell layer (Thermomax spectrophotometer, Molecular Devices). Duplicate tests were run for each assay, using four wells per drug combination and sixteen wells of cell control per test.

The MTT assay is a measure of the metabolic capacity of the mitochondria. As such, a decrease in the amount of formazan produced from MTT can represent either a decrease in the number of mitochondria (fewer cells) or a decreased intrinsic capacity of the mitochondria to metabolize MTT. In the latter case, the effect can be either cytostatic or cytocidal in nature; neither can be distinguished. For the purposes of this report, cytotoxicity refers to the decreased metabolic conversion of MTT, keeping in mind the possible interpretations. Since AZT is selectively toxic to mitochondria and the MTT assay measures mitochondrial metabolism, the use of this assay for cytotoxicity studies is particularly appropriate.

*Animal studies.* Weanling female Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA) weighing 10–12 g were used for in vivo studies. All mice were infected intraperitoneally with $3.2 \times 10^4$ pfu/mouse of murine CMV. GCV was administered subcutaneously twice daily 6 h apart, while AZT was given intraperitoneally three times daily 3 h apart for 5 days, both starting 6 h after infection. Studies continued for 14 days after infection. Surviving animals were healthy at that time.

*Data analyses.* For the in vitro studies, the possibility of either enhanced or

reduced efficacy of GCV in combination with AZT was analyzed in several ways. Initially, 3-dimensional representations of (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 were prepared as a function of the various drug combination pairs. Then, stepwise linear regression combined with analysis of covariance (ANCOVA) (Dixon et al., 1969; SAS User's Guide, 1989) was performed on (a) plaque counts, (b) cytotoxicity values, and (c) differences between percent reductions in plaques and cytotoxicity values in order to determine whether drug effects were additive, synergistic, or antagonistic. Overall differences as well as pairwise comparisons of both slopes and y-intercepts from each GCV concentration curve alone and in combination with AZT were analyzed. A brief description of the statistical analysis can be found at the end of the Materials and Methods. In addition, a complementary analysis using nonlinear regression (Greco et al., 1990) was performed for validation. In every instance, the two analyses agreed. 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).

For the in vivo studies, Fisher's 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 AZT. In addition, the effective dose at which 50% of the mice survived ($ED_{50}$) was determined for GCV alone or in combination with AZT using probit analysis (Finney, 1971).

*Stepwise linear regression combined with analysis of covariance to analyze drug combination interactions.* In the statistical model used for the in vitro assays, an overall test for consistent deviation from additivity across the entire dose-response surface is first evaluated using stepwise linear regression. This phase of the analysis must be completed before a point-by-point analysis is performed. If evidence for consistent supra-additivity exists, the point-by-point comparisons can be performed directly, i.e., at an alpha level of 0.05. If a point-by-point analysis is desired, even in the face of no evidence for supra-additivity, then a Bonferroni correction for multiple comparisons is carried out using Dunn's Procedure and Fisher's LSD strategy (Kirk, 1982). These procedures adjust the alpha level downward, i.e., 10 point-by-point comparisons would adjust the alpha down to 0.005 to be significant; all values above 0.005 would be accounted for by noise. In addition, the present statistical method contains a logic branch point to analyze the effects of drugs which on their own are ineffective. These additional analyses are also carried out over the entire dose-range, and are based upon ANCOVA and Dunn's procedure for multiple comparisons.

Briefly, the procedure is as follows (Fig. 1): (i) Determine linearity and scales: The relationship between the responses and doses for each drug is determined. If it is not linear, an appropriate transformation for doses, for example, $\log_{10}$ dose, etc., is made for linearity. (ii) Determine the activity level of each drug

alone: The complete dose-response curve for each drug alone is examined to see if the drug is active by itself within the tested dose-range. (iii) If both drugs are active: A Stepwise Linear Regression is used for the additive model of drug–drug interaction and is compared to a synergy model which also includes a term

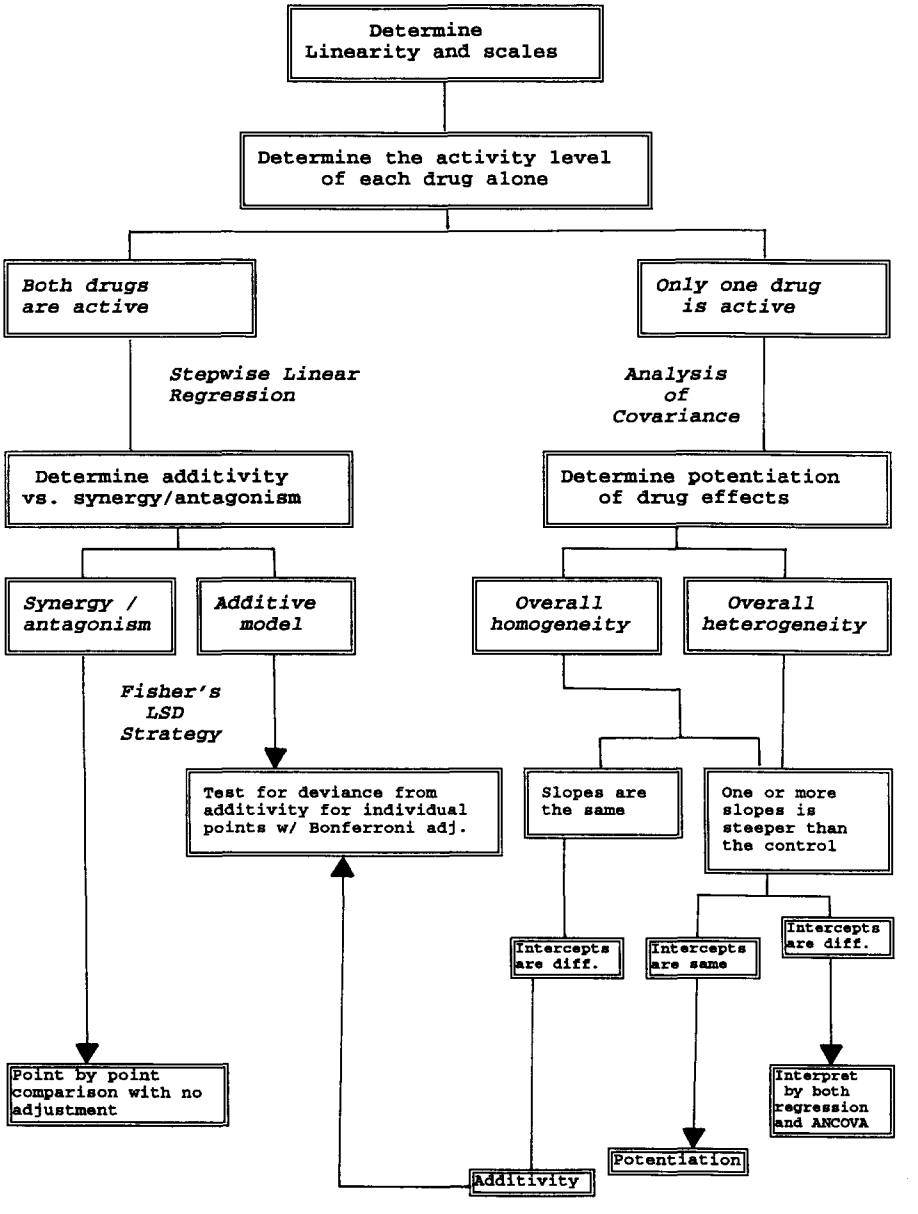


Fig. 1. Flow chart for stepwise linear regression combined with analysis of covariance to analyze drug combination interactions. See Materials and Methods for further information.

to account for combination effects; a consistent supra-additive effect across the majority of the active range indicates an evidence of synergy; (a) if the additive model is not rejected in favor of the synergy model, individual points within the combination space can be tested for deviance from additivity, but the post hoc analysis must be adjusted using a Bonferroni Strategy to account for the problem of multiple comparisons; (b) if the additive model is rejected and the synergy or antagonism model is accepted, point-by-point comparisons may then be made without adjustment (Dunn's Procedure and Fisher's LSD strategy). (iv) If only one drug is active: The model can still be used to test for potentiation of drug effects by performing an ANCOVA to test for heterogeneity of slopes; (a) if one or more active drug slopes is significantly steeper than the control slope and if no differences in intercepts exist, then evidence of potentiation exists; (b) if the slopes are the same and if the intercepts are different, then evidence of additivity exists; (c) if both slopes and intercepts are different, the results must be interpreted within the structure of the regression and ANCOVA.

## Results

*In vitro tests.* GCV alone had activity against human CMV (AD 169). The $IC_{50}$ was 8 $\mu$M, and the $IC_{90}$ was 18 $\mu$M. AZT alone was essentially inactive, with an $IC_{50}$ and $IC_{90}$ of $>800$ $\mu$M. When the two drugs were combined, AZT had an additive effect on the reduction of plaques by GCV (Fig. 2a, $P>0.1$, where this lack of significance meant an additive interaction rather than synergism or antagonism). The same additive results were seen in a second test, in which the GCV $IC_{50}$ and $IC_{90}$ were 9 $\mu$M and 19 $\mu$M, respectively, and the AZT $IC_{50}$ and $IC_{90}$ were 508 and $>800$ $\mu$M, respectively (data not shown).

Both GCV and AZT alone were slightly toxic to confluent MRC-5 cells, with $\leq 18\%$ reduction in the cellular metabolism of MTT at the highest concentrations. In combination, additive effects on cell toxicity were observed at the higher concentrations (Fig. 2b, $P>0.1$, where this lack of significance meant an additive interaction). The same additive results were seen in a second test, with $\leq 28\%$ reduction in the cellular metabolism of MTT. In neither test were synergistic effects on cell toxicity apparent. Cell toxicity was determined in confluent cells in order to compare each drug's effect directly with its antiviral efficacy which was also determined in confluent monolayers.

In order to minimize any experimental bias, the plaque test was compared with the confluent cytotoxicity test (Fig. 2c). When differences in antiviral activity and cytotoxicity using confluent cells were compared, the results were consistently biphasic. With GCV alone and in combination with the 25–200 $\mu$M concentrations of AZT, a large increase in the slope of the difference curve was seen with increasing doses, indicating that the inhibition of human CMV (AD169) increased rapidly with a minimal effect on cell toxicity. With GCV in combination with the 200–800 $\mu$M concentrations of AZT, a slight decrease in

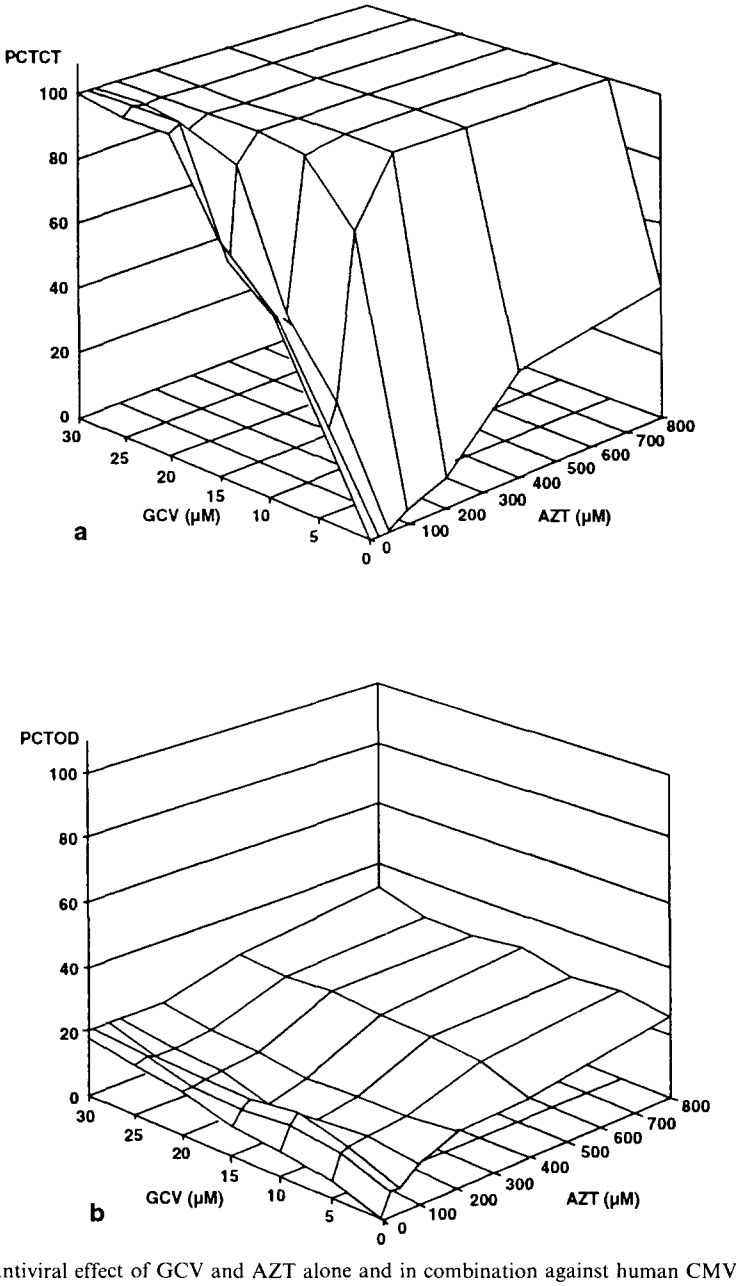


Fig. 2. (a) Antiviral effect of GCV and AZT alone and in combination against human CMV (AD169) in MRC-5 cells, PCTCT = % reduction in plaque count compared to virus control; (b) Cell toxicity effect of GCV and AZT alone and in combination on confluent MRC-5 cells, PCTOD = % reduction in optical density of formazan compared to cell control.

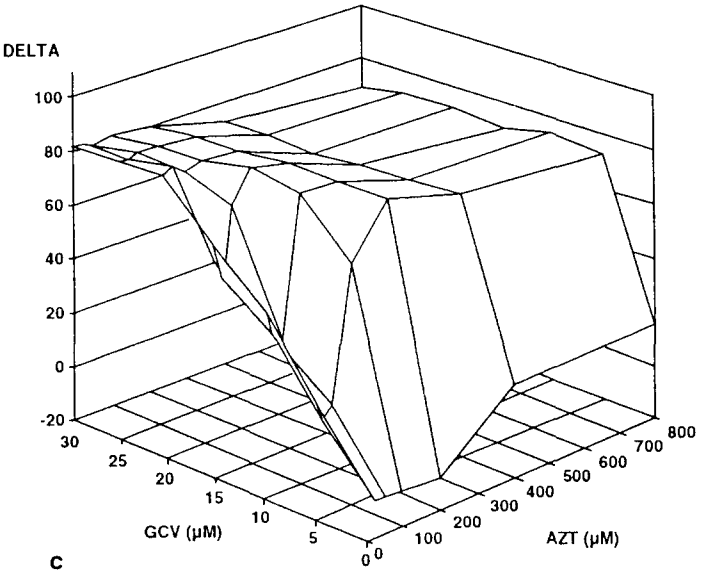


Fig. 2. (c) Antiviral minus cell toxicity effects of GCV and AZT alone and in combination against human CMV (AD169) in confluent MRC-5 cells, DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

slope with a large offset at the intercept was seen, indicating that all of the virus had been inhibited and that only small reductions in the cellular metabolism of MTT had occurred. These results were consistent when the second antiviral test was compared with the second cell toxicity test (data not shown). In all cases *P*-values were $<0.05$ when the slope or intercept for the reduction in plaques was compared with the slope or intercept for cell toxicity. Again, the results of the analysis generated from the difference between antiviral activity and cell toxicity data indicated that the anti-CMV effects of the drug combinations were additive ($P>0.1$). The activity of GCV alone was increased approximately 3-fold in the presence of $\geq$100 $\mu$M AZT.

Cell toxicity was determined in proliferating cells in addition to confluent cells since rapidly dividing cells are often more sensitive to the deleterious effects of a drug. When examined in proliferating MRC-5 cells, additive effects were observed between the two drugs (Fig. 3). GCV was slightly toxic to proliferating MRC-5 cells, with $\leq$16% reduction in cellular metabolism when a 5–30 $\mu$M concentration range was tested. AZT was moderately toxic with $\leq$ 46% reduction (25–800 $\mu$M range tested). Neither synergy nor antagonism was detected ($P>0.1$).

In an effort to ensure that the results obtained with CMV strain AD169 were not intrinsic to that strain, we obtained a primary clinical isolate of CMV (T8618) for testing. GCV alone was also active against this isolate of CMV (T8618). The $IC_{50}$ was 14 $\mu$M, while the $IC_{90}$ was 22 $\mu$M. AZT alone was not active against the HCMV isolate with both the $IC_{50}$ and $IC_{90}>800$ $\mu$M. The

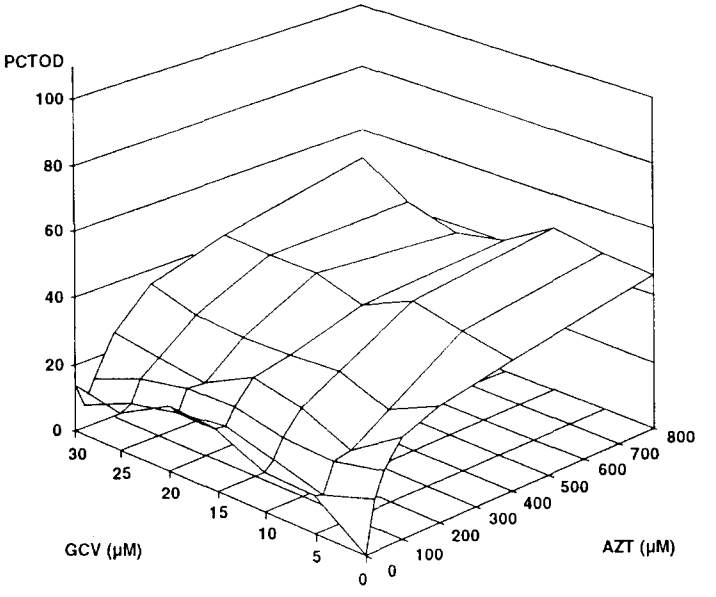


Fig. 3. Cell toxicity effect of GCV and AZT alone and in combination on proliferating MRC-5 cells, PCTOD = % reduction in optical density of formazan compared to cell control.

results of the analysis generated from the plaque reduction assay as well as from the difference between antiviral activity and confluent cell toxicity indicated that the antiviral activity of GCV was potentiated when combined with AZT (Figs. 4a, b and c, $P<0.05$ where this significance indicated potentiation rather than antagonism or additivity). The activity of GCV alone was increased approximately 5–10-fold in the presence of $\geq 50$ $\mu$M AZT.

*In vivo tests.* In order to examine the effect of the two drugs in vivo, mice were infected with murine CMV and the anti-CMV effects of GCV and AZT combinations examined. GCV alone had an $ED_{50}$ of 6 mg/kg against murine CMV (Table 1). When various doses of GCV were used in combination with AZT, the $ED_{50}$ for GCV remained essentially unchanged (5 mg/kg). In addition, AZT had no protective activity against murine CMV either alone or in combination with a marginally effective dose of GCV, with the $ED_{50}$ for AZT remaining at $>320$ mg/kg.

## Discussion

In the in vitro combination studies, AZT had an additive effect on the antiviral activity of GCV against human CMV (strain AD169) and potentiated the activity of GCV against the clinical isolate of HCMV. No indication of antagonism was apparent in any of the assays. In addition, AZT did not

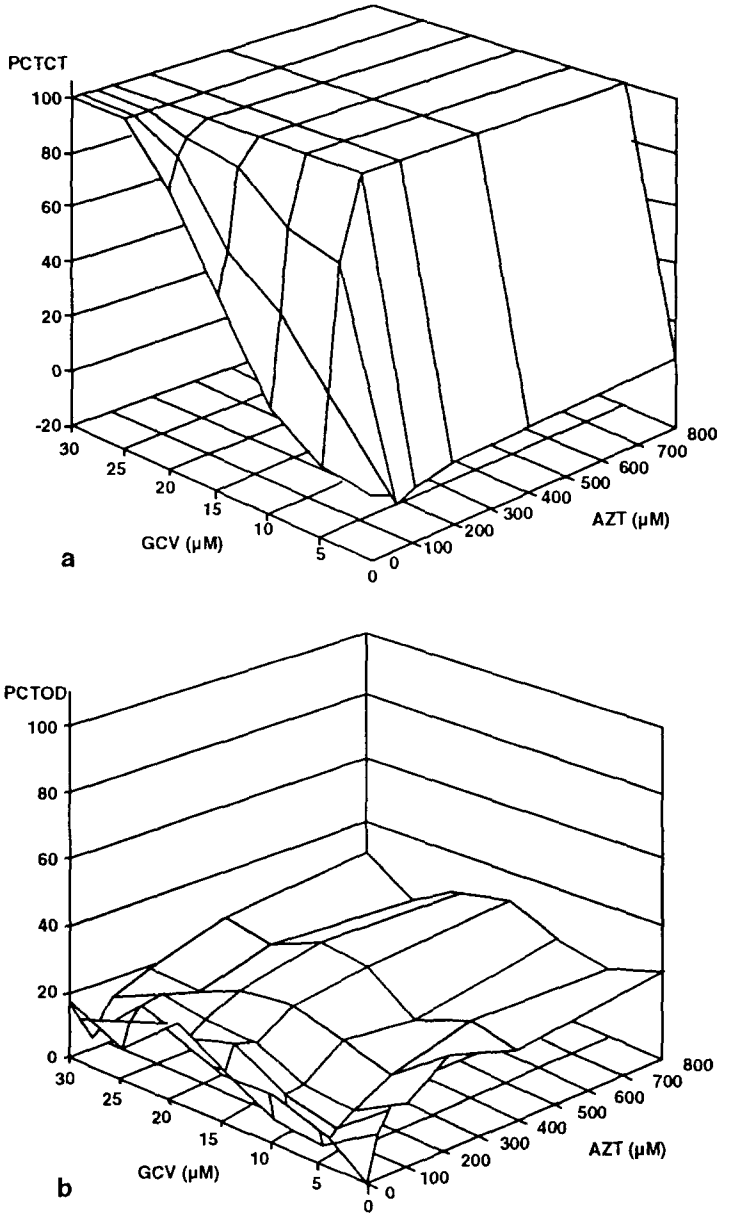


Fig. 4. (a) Antiviral effect of GCV and AZT alone and in combination against a clinical isolate of human CMV (T8618) in MRC-5 cells, PCTCT = % reduction in plaque count compared to virus control; (b) Cell toxicity effect of GCV and AZT alone and in combination on confluent MRC-5 cells, PCTOD = % reduction in optical density of formazan compared to cell control.

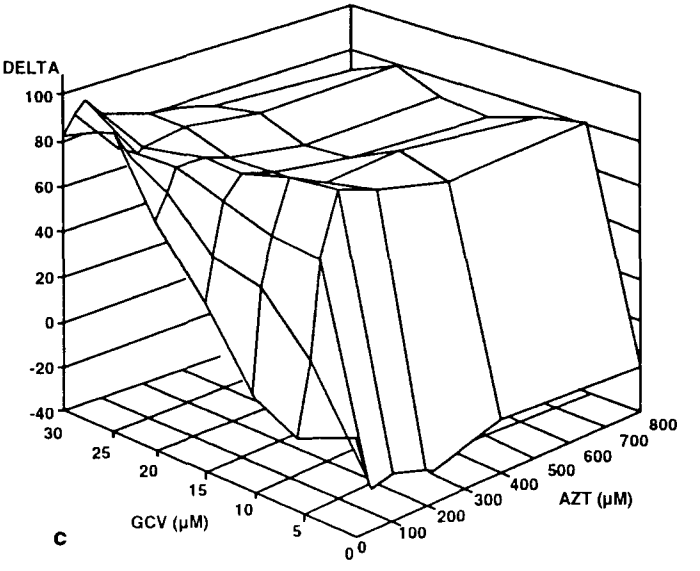


Fig. 4. (c) Antiviral minus cell toxicity effects of GCV and AZT alone and in combination against a clinical isolate of human CMV in confluent MRC-5 cells, DELTA = % reduction in number of plaques minus % reduction in cellular metabolism of MTT.

adversely affect the in vivo antiviral activity of GCV against murine CMV at the doses tested. The similar results regarding lack of antagonism with both the laboratory strain of CMV (AD169) and the primary clinical isolate (T8618) demonstrate that these results generalize to more than just a single strain of human CMV. The reason that an additive effect was seen for the laboratory strain versus potentiation for the clinical isolate may be due to the fact that the laboratory strain was carried in culture; selective changes may have occurred from pass to pass.

These in vitro results are in contrast to those of Prichard et al. (1991) and Tian et al. (1991). Using a limited three-dimensional method of analysis, Prichard et al. (1991) reported synergistic cytotoxicity between GCV and AZT. In addition, Tian et al. (1991) described certain individual combinations of GCV and AZT as being cytotoxically antagonistic. However, the methods utilized by Prichard et al. (1991) and Tian et al. (1991) did not take into account the entire drug combination response surface in their analyses. The Prichard and Shipman method first calculates the theoretical additive surface, which is subtracted from the experimental surface to reveal regions of greater (or smaller) than expected additivity. This theoretical additive surface is calculated based on a series of assumptions which must be made about additivity of the two drugs, and has to be predicted beyond the $IC_{50}$ by using the equations in Prichard and Shipman's model. Even if one were to assume non-additive drug–drug effects, the method still requires extrapolation. Such extrapolation will severely impact the adequacy of the analysis. By using the present linearized

TABLE 1

Survival of mice treated with different doses of GCV or AZT alone and in combination against a murine CMV infection[a]

| Treatment regimen[b] | | Response to treatment | $ED_{50}$ alone or in combination | |
|---|---|---|---|---|
| GCV (mg/kg) | AZT (mg/kg) | No. survivors/total[c] | GCV (mg/kg) | AZT (mg/kg) |
| Untreated Control | | 4/20 | | |
| 1 | 0 | 3/20 | | |
| 3 | 0 | 8/20 | 6 | |
| 9 | 0 | 17/20[d] | | |
| 27 | 0 | 20/20[d] | | |
| 1 | 320 | 11/20[d,e] | | |
| 3 | 320 | 7/20 | 5 | |
| 9 | 320 | 16/20[d] | | |
| 27 | 320 | 20/20[d] | | |
| 0 | 32 | 7/20 | | |
| 0 | 100 | 0/20 | | >320 |
| 0 | 320 | 1/20 | | |
| 3 | 32 | 5/19 | | |
| 3 | 100 | 9/19 | | >320 |
| 3 | 320 | 8/20 | | |

[a]Mice were treated with various concentrations of GCV or AZT to determine the $ED_{50}$ alone and with the marginally effective dose of each agent (GCV, 3 mg/kg; AZT, 320 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. GCV was given subcutaneously twice daily in two equal doses 6 h apart. AZT was given intraperitoneally three times daily in three equal doses 3 h apart. 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.

regression model as well as a nonlinear regression model (Greco et al., 1990) across the entire dose-response surface in our analysis, our results suggest that no antagonism between GCV and AZT exists. In every instance, both the linearized and non-linearized regression models gave the same conclusions. By avoiding the assumptions made by Prichard and Shipman (1990), as well as by Tian et al. (1991), the present analyses are necessarily more conservative and consequently reduce the false-positive rate.

Other current techniques for analyzing drug combination interactions such as the isobologram (Loewe, 1953) and the combination index method (Chou and Talalay, 1981) usually look at drug–drug combination therapy on a point-by-point basis or across a range of given dose ratios, without first verifying an overall consistent response across the entire drug–drug response surface. Comparing such techniques with a non-linear regression model has shown flaws in these methods. For example, Syracuse and Greco (1986) did a Monte-Carlo simulation study to compare the method of Chou and Talalay (1981) with Greco's nonlinear Universal Response Surface method. In this simulation

study, different amounts of true additivity with varying amounts of true noise were pre-programmed to generate test data and the results were then analyzed by both methods. The results of this simulation study showed that Greco's nonlinear regression model was correct a majority of the time, while Chou and Talalay's method often gave ambiguous results, yielding a mixed conclusion of both antagonism and synergism. The only case where the Chou and Talalay method performed as well as the Syracuse and Greco method was for the model in which the dose-response curves for drug 1 and drug 2 were parallel (same slopes). Finding mixed results in the face of a single (but noisy) true result means that in practice the false-positive rate is inflated, probably as a result of selective sampling and system noise.

Including cytotoxicity analyses in statistical equations dealing with combination studies can also reduce false-positive results. Certain drugs appeared to increase the efficacy of GCV when the 3-dimensional antiviral plaque assay by itself was analyzed, whereas they were seen to have no effect when cytotoxicity was factored into the analysis. The effect of GCV in combination with trimethoprim/sulfamethoxazole (TMP/SMX) against CMV is a good example (Freitas et al., 1993).

In addition to the different statistical analyses used in the various drug combination studies, discrepancies in the results may also be related to the different cytoxicity assays used. For example, Prichard et al. (1991) used total protein stains in exponentially growing cells and plating efficiency studies, while we chose the MTT assay, which measures mitochondrial metabolism.

Several researchers have noticed hematological toxicity problems when GCV and AZT were given concurrently to AIDS patients (Jacobson et al., 1988; Hochster et al., 1990; Millar et al., 1990). Hochster et al. (1990) and Jacobson et al. (1988), both concluded that this toxicity was the result of the myelosuppressive activity of each drug rather than being due to any pharmacodynamic interaction. Even though pharmacokinetic measurements were not performed directly in the in vivo test, the additive results seen in the in vitro drug combination studies suggest that no change in pharmacodynamic variables of either drug has occurred with coadministration.

The myelosuppressive effects of both GCV and AZT make them difficult to use concurrently in the clinic. Recently, a new approach to combination therapy has been successful (Causey, 1991). The treatment involved an individual patient approach that monitored absolute granulocyte counts to provide a flexible dosing regimen for both GCV and AZT based on dose modification and interruption. In addition, ongoing clinical trials suggest that granulocyte-macrophage colony-stimulating factor (GM-CSF) combined with GCV against CMV retinitis leads to reduced neutropenia over patients receiving GCV alone (Grossberg et al., 1989; Hardy, 1991). If additional clinical studies demonstrate these types of regimens to be effective, the concomitant use of GCV and AZT would be possible in a larger patient population.

The present results suggest that GCV can be used in combination with AZT,

while treating an opportunistic CMV infection of AIDS patients without compromising the efficacy of GCV against CMV. These data extend previous reports in which no detrimental interactions on antiviral efficacy were found between GCV in combination with ketoconazole against herpes simplex virus type 2 (HSV-2) (Pecyk et al., 1989) or between GCV in combination with foscarnet, amphotericin B, ketoconazole, dapsone, or TMP/SMX against CMV and/or HSV-2 (Freitas et al., 1989; Freitas et al., 1993).

More recently, in an in vitro study by Medina et al. (1992), GCV was reported to reduce the anti-HIV activity of AZT. Unlike the present studies on CMV, Medina utilized fixed molar ratios of AZT and GCV and the method of analysis consisted solely of combination indices; such limited studies could adversely impact the results as described previously for the combination index method. In addition, Medina et al. (1992) used trypan blue viability and total cellular protein assays to measure cytotoxicity. We are currently performing more detailed analysis of combinations of AZT and GCV on HIV in an effort to clarify these observations.

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