Polyamine depletion and cell cycle manipulation in combination with HSV thymidine kinase/ganciclovir cancer gene therapy

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Abstract. We have shown earlier that polyamine biosynthesis inhibition is accompanied by cell cycle alterations that can be utilized to enhance the efficacy of herpes simplex virus thymidine kinase - ganciclovir (HSV-TK/GCV) cancer gene therapy. In the present study, we asked 1) can the activated polyamine catabolism instead of biosynthesis inhibition be utilized to enhance the efficacy of HSV-TK/GCV gene therapy, and 2) can other known cell cycle inhibitors be used to make tumor cells more sensitive to this form of gene therapy? We show, using rat (9L) and human (U251-MG) glioma cell populations with 15% of HSV-TK-positive cells that DENSPM-induced activation of polyamine catabolism caused a profound polyamine deprivation in U251-MG cells, but there were no associated cell cycle effects in these cells. Consequently, we did not see any enhancement of the HSV-TK/GCV system. Aphidicolin, hydroxyurea, mimosine and resveratrol, but not lovastatin induced an apparent cell cycle arrest, followed by an intense but transient increase of the S phase cells after removal of the drug. This effect was shown to potentiate the HSV-TK/GCV cytotoxicity to some extent, especially in 9L cells and when the GCV treatment was started 0-24 h before the drug treatment. However, the enhancement was weaker than observed earlier with DFMO-induced cell cycle arrest and a considerable degree of the effect appeared to result from the growth-inhibitory actions of the drugs. In summary, we demonstrate that polyamine deprivation via DENSPM action is not associated with cell cycle effects and is not sufficient to cause enhancement of the HSV-TK/GCV system. Also, drugs with a rapid effect to the cell cycle are weak boosters of the HSVTK/GCV gene therapy, thus being less useful than DFMO for enhancement of this gene therapy form in animal studies and clinical trials.

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Introduction

Different forms of cancer gene therapy have been studied widely during the last 20 years. Especially the feasibility of the so-called suicide gene therapy approach that utilizes prodrugs and their activating enzymes (1), has been demonstrated in cultured cancer cells, in animal tumor models and even in clinical setting. Perhaps the best known form of suicide gene therapy is the system that uses the thymidine kinase gene from herpes simplex virus type I (HSV-TK). Thymidine kinase, a typical prodrug activating enzyme, is capable of efficiently converting non-toxic antiviral drugs like ganciclovir (GCV) into a toxic form that kills the cells upon DNA replication, i.e. during S phase of the cell cycle. Originally this method was developed to control the graft-vs-host disease (reviewed in ref. 2), but it was also realized that it can be exploited in destruction of malignant cells (3). Although HSV-TK/GCV gene therapy has been successfully used in many preclinical studies and in a few highly promising clinical trials (4), the fact remains that this method is not yet efficient enough to completely eradicate malignant cells from the human body. Thus, HSV-TK/GCV gene therapy still needs enhancement to be a useful clinical treatment form.

One approach to intensify the cytotoxicity of this suicide gene therapy system is to make the target tumor cells more vulnerable to toxic effect of phosphorylated GCV by increasing the proportion of S phase cells. In theory, this can be accomplished by arresting the cell population at a certain point of the cell cycle, releasing the block and then destroying the enriched S phase fraction of the cells with HSV-TK/GCV gene therapy. Perhaps the simplest way to introduce transient cell cycle arrest is to use serum deprivation, but our results have shown that increase of the S phase cells is too transient to be synergized with the HSV-TK/GCV gene therapy (5) and certainly this type of approach would not be useful in a clinical setting. Fortunately, there are many drugs available today that can halt cell proliferation at certain point of the cell cycle via different mechanisms. Furthermore, some of the drugs, like hydroxyurea and lovastatin, are clinically approved compounds. Hydroxyurea (HU) is a widely used chemotherapeutic agent that blocks the cells to G1 preventing them from entering the S phase. This effect results from inhibition of ribonucleotide reductase that causes a decrease of cellular

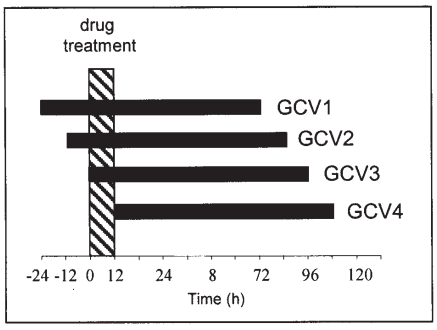

Figure 1. Treatment schedules for cell cycle altering drugs in combination with HSV-TK/GCV gene therapy. The cell lines (9L and U-251 MG) were treated with aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol for 12 h (hatched vertical bar) in all schedules. Four different initiation times for a 5-day ganciclovir treatment period were used (solid horizontal bars): 24 or 12 h before the drug treatment initiation (GCV1 and GCV2), simultaneously with the drug treatment initiation (GCV3) or at the end of the drug treatment (GCV4).

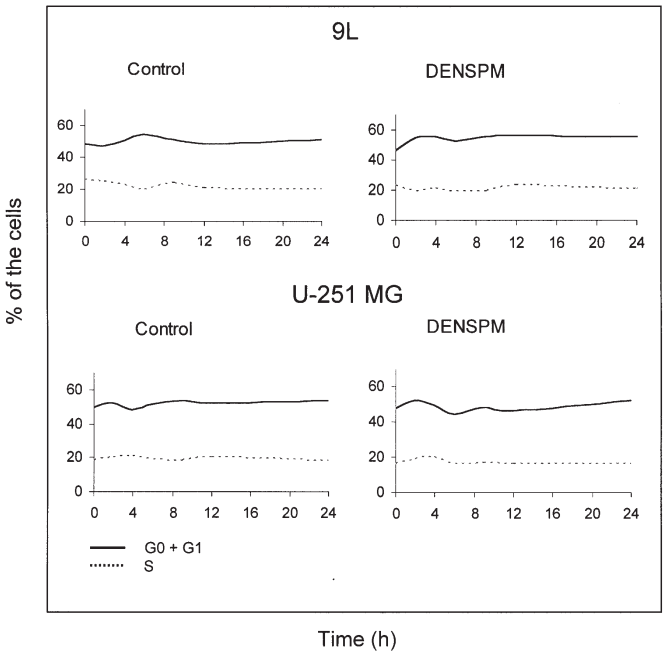

Figure 2. Cell cycle phase distribution in rat and human brain tumor cells after activation of polyamine catabolism with DENSPM. The cell lines (9L and U-251 MG) were treated with 50 μM DENSPM for 12 h. Cell samples were collected at the end of the treatment and 2, 4, 6, 9, 12 and 24 h post-drug removal. Propidium iodide-stained cells were analyzed with flow cytometer and the proportion of the G0+G1 phase (solid black line) and S phase (hatched line) cells were determined. Ten thousand cells were analyzed at each data point.

dATP and dGTP pools; thus, inhibiting DNA replication. HU has been tested as a synergizing agent of HSV-TK/GCV gene therapy and its effect has been demonstrated *in vitro* (6) and *in vivo* (7). Aphidicolin, a tetracyclic diterpenoid is a DNA polymerase inhibitor that allows cells to enter the S phase, but prevents them from proceeding with the DNA replication (8). The cell cycle arresting effect of this drug has been utilized recently to study the features of electropermeabilization (9) and viral gene transfer (10). A tyrosine analog mimosine from *Mimosa* and *Leucaena* (11) is a reversible blocker that has been shown to arrest the cell cycle at G1 (12,13) or early after entry to S phase (14,15); thus, displaying a cell line-dependent mode of action. A recent study has elucidated mimosine's role as a zinc chelator that inhibits transcription of zinc-inducible genes, such as serine hydroxymethyltransferase (12). Resveratrol is a phytoalexin found in many plants where they are produced to fight against fungal infections (16). This compound has been shown to possess many health-promoting properties, including anti-cancer activity (17). Moreover, its function as a cell cycle blocker has been demonstrated. Resveratrol arrests the target cells in G1 (18,19) or S phase (18,20,21), depending on the cell line, and inhibition of DNA synthesis appears to be its central mode of action. Lovastatin, a commonly prescribed drug for treatment of hypercholestremia, is an HMG-CoA reductase inhibitor that prevents the synthesis of mevalonate and consequently reduces the cellular levels of cholesterol as well as affects isoprenylation of several proteins. Lovastatin and other statins have also demonstrated anti-cancer activity in preclinical studies with cell lines, in animal models and in recent clinical trials (reviewed in refs. 22 and 23). The cell cycle-specific effects of lovastatin have been established in several studies with different cell lines, demonstrating that this drug arrests cultured cells to G1 (24-26). On the other hand, it has also been claimed that lovastatin does not exactly synchronize cells to a certain cell cycle phase, but the cytostatic effect is due to general growth retardation and subsequent enrichment of the cells in the G0/G1 phase (27).

As demonstrated in our earlier study, also the 2-difluoromethyl ornithine (DFMO)-induced polyamine biosynthesis inhibition accompanied with a predominant cell cycle arrest can be used to enhance HSV-TK/GCV gene therapy (5). We showed that DFMO caused a long-lasting disturbance to the cell cycle of different tumor cells and a correct timing of DFMO and GCV treatments yielded synergistic cytotoxic effect. Moreover, our results indicated that a more transient cell cycle block induced by serum deprivation did not have any positive effect on the HSV-TK/GCV toxicity. To further characterize the synergy between HSV-TK/GCV gene therapy and cell cycle manipulation, we used two different strategies: 1) to test the effect of polyamine deprivation induced with other drugs than DFMO; 2) to test different cell cycle arresting drugs that have an effect through mechanisms other than inhibition of polyamine biosynthesis. For another strategy to induce polyamine deprivation, we examined the effect of N^1,N^{11}-diethylnorspermine (DENSPM) that is a powerful inducer of spermidine/spermine N^1-acetyltransferase (SSAT). This enzyme catalyzes conversion of higher polyamines to their N^1-acetyl derivatives that are either excreted from the cell or oxidized by polyamine oxidase (28). Thus, DENSPM causes a dramatic polyamine deprivation through a different mechanism than DFMO. Our results show that polyamine depletion that is not accompanied with cell cycle alterations cannot be used to enhance HSV-TK/GCV gene therapy. Also, we demonstrate that aphidicolin, hydroxyurea, mimosine and resveratrol can cause a transient cell cycle arrest that allows some enhancement of the HSV-TK/GCV system, yet in most cases the effect appears to be attributable to the cytotoxic/-static effect of the drug.

Table I. Analysis of polyamine levels and SSAT activity after DENSPM treatment.[a]

	Putrescine[b]	Spermidine[b]	Spermine[b]	SSAT[c]
9L				
Control	3150±51	2487±54	1132±45	7.85±0.33
DENSPM 24 h	3110±120	2305±39	1058±6	7.87±0.28
DENSPM 48 h	2338±36	2022±28	1115±54	10.30±0.49
U251-MG				
Control	2769±119	2021±54	2412±52	4.27±0.4
DENSPM 24 h	18±31	629±40	208±10	538.20±1.97
DENSPM 48 h	0±0	30±11	15±1	817.42±1.83

[a]9L and U-251 MG cells were treated with 50 μM DENSPM and cell samples were collected at 24 and 48 h after initiation of the treatment, followed by analyses for polyamine content and SSAT activity as described in Materials and methods. Controls are untreated cells collected at 24 h after plating. [b]pmol/10^6 cells, mean of triplicate analyses ± SD. [c]pmol/10^6 cells/10 min, mean of triplicate analyses ± SD.

Materials and methods

Cell lines and viral vectors. Rat glioma cell line 9L (ATCC CRL-2200) and human glioma cell line U-251 MG (JCRB IFO50288) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 2 mM Na-pyruvate, 2 mM glutamine and 50 μg/ml of gentamycin. Cells were propagated at 37˚C under 5% CO_2. Lentivirus vector with fusion construct of thymidine kinase and green fluorescent protein (Lenti TK-GFP) was used to generate thymidine kinase positive cell lines as described earlier (29). Transduced cells were analyzed with flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA) and a population containing 15% of TK-GFP positive cells was created by mixing with parental cells.

Drug treatment. Aphidicolin, hydroxyurea, lovastatin (Mevinolin), mimosine and resveratrol were purchased from Sigma. N^1,N^{11}-diethylnorspermine (DENSPM) was synthesized essentially as described earlier (30). Cells were incubated with different cell cycle altering drugs for 12 h using the following concentrations: aphidicolin (1 μg/ml), hydroxyurea (0.5 mM), lovastatin (60 μM), mimosine (0.5 mM) and resveratrol (50 μM). Cells were incubated with 50 μM DENSPM for 24 or 48 h.

SSAT activity and polyamine analyses. The SSAT enzyme activity was determined from 1-2x10^6 cells suspended in 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Triton X-100 as described previously (31). Polyamine concentrations were measured with the aid of HPLC (32).

Cell cycle phase distribution. The cell cycle phases of 9L and U-251 MG cells were analyzed after a 12 h treatment with different drugs (concentrations indicated above). Cell samples were collected immediately after drug removal and 2, 4, 6, 9, 12 and 24 h later, stained with propidium iodide and the DNA content was analyzed with flow cytometer as described before (5).

Ganciclovir sensitivity analysis. To determine the most synergistic schedule for each drug and HSV-TK/ganciclovir (GCV) gene therapy, four different treatment combinations were tested (schematically described in Fig. 1). Treatment (12 h) with cell cycle altering drugs was started 24 or 12 h after GCV treatment initiation (GCV1 and GCV2), simultaneously with GCV initiation (GCV3) or 12 h before the start of GCV treatment (GCV4). The TK-GFP positive cells (6000 cells/well in a 48-well plate) were incubated with 1 μg/ml GCV for 5 days, followed by cell viability analysis with MTT assay (cell proliferation kit II, Roche) as described before (5). Control cells were treated with different drugs for 12 h, followed by incubation without GCV for 60, 72, 84 and 96 h (GCV1, GCV2, GCV3 and GCV4 controls, respectively) and viability analysis with MTT assay.

Statistical analyses. One-way analysis of variance with the Bonferroni's post hoc test for multiple comparisons was used for statistical analyses with aid of GraphPad Prism 3.0 software (GraphPad Software, Inc., San Diego, CA).

Results and Discussion

DENSPM-induced polyamine deprivation is not associated with cell cycle alterations and does not enhance the HSV-TK/GCV cytotoxicity. To examine the effect of polyamine depletion through a mechanism other than inhibition of putrescine biosynthesis (ornithine decarboxylase inhibition with DFMO), we treated 9L and U-251 MG glioma cells with DENSPM, an inducer of the polyamine catabolizing enzyme, SSAT. It turned out that 9L cells were not responsive to this drug, with only marginally elevated SSAT activity and slightly lowered cellular putrescine and spermidine concentrations after a 48 h incubation in the presence of DENSPM (Table I). However, in U-251 MG cells the SSAT activity was induced about 120x and 200x during a 24 h or a 48 h incubation with DENSPM, respectively. This was subsequently manifested as an almost complete absence of all polyamines after a 48 h incubation with the drug.

To verify whether the observed changes in cellular polyamine contents were reflected in the cell cycle phase distribution, as was the case with DFMO-induced deprivation

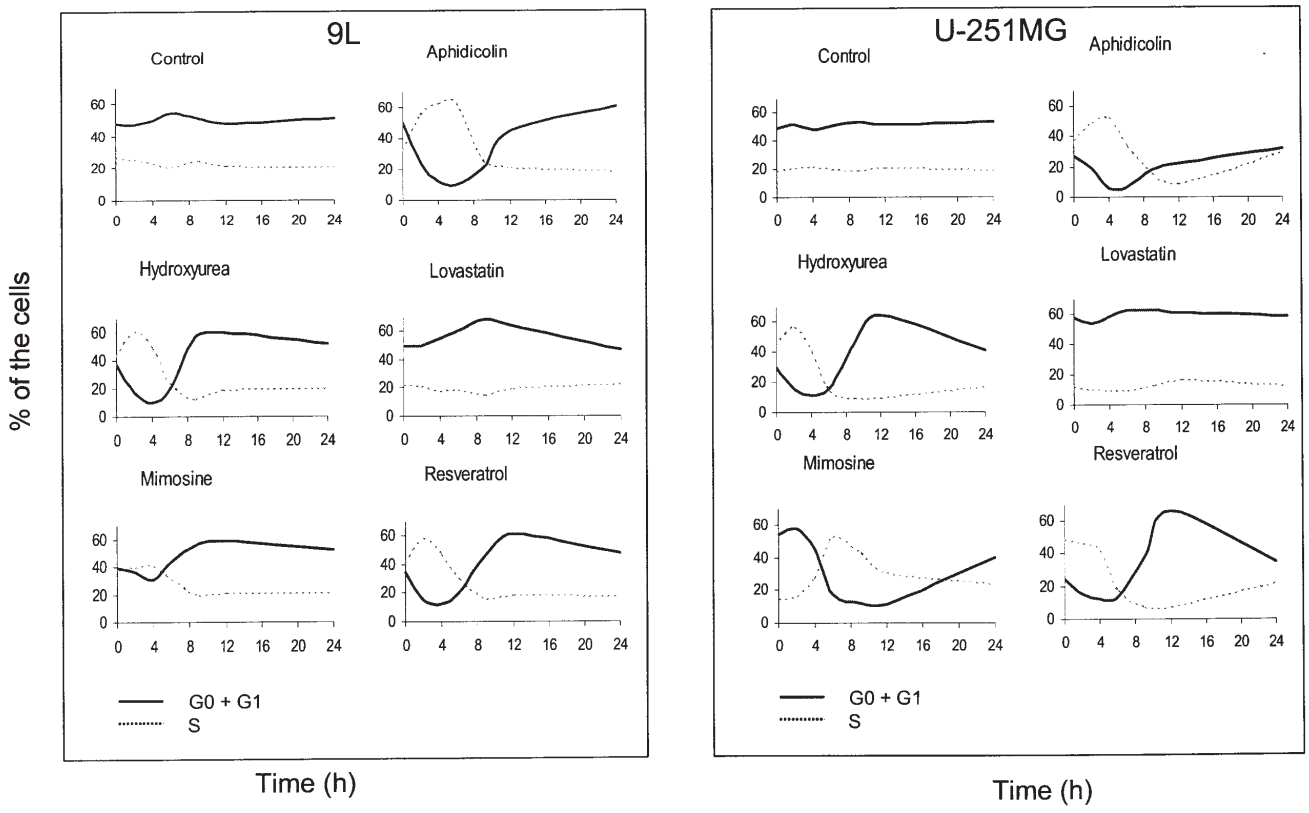

Figure 3. Cell cycle phase distribution in rat and human brain tumor cells after treatment with various cell cycle altering drugs. The cell lines (9L and U-251 MG) were treated with aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol for 12 h using the concentrations indicated in Materials and methods. Cell samples were collected at the end of the treatment and 2, 4, 6, 9, 12 and 24 h post-drug removal. Propidium iodide-stained cells were analyzed with flow cytometer and the proportion of G0+G1 phase (solid black line) and S phase (hatched line) cells were determined. Ten thousand cells were analyzed at each data point.

(5), both cell lines were subjected to 24 h treatment with DENSPM, followed by removal of the drug and monitoring the cell cycle phases for 24 h. We anticipated that due to lack of significant SSAT induction and polyamine deprivation, DENSPM would not cause any dramatic cell cycle changes in 9L cells. This appeared to be the case and the cell cycle phase distribution of the treated cells was identical to the control cell cycle distribution (Fig. 2, upper panel). This also held true with U-251 MG cells that did not display any signs of cell cycle alterations (Fig. 2, lower panel), despite a profound depletion of putrescine, spermidine and spermine after a 24 h incubation with the drug. Alm *et al* (33) have studied the cell cycle effect of DENSPM on hamster CHO cells and found that incubation with 7.5 μM DENSPM for 24 h yielded some accumulation of S phase cells (68 vs. 55% in the control cells). They postulated that the effect was initially due to delay in the S phase, followed by prolongation of other cell cycle phases. Even though Alm and coworkers did not study the events following the drug removal, the lack of increased proportion of S phase U-251 MG cells after a 24 h incubation with 50 μM DENSPM suggests that the cell cycle effect of this drug is cell line-dependent.

Thus, it was unlikely that DENSPM-induced polyamine depletion would be useful for enhancement of HSV-TK/GCV gene therapy because of the non-disturbed cell cycle. However, it cannot be ruled out that polyamine deprivation per se would be sufficient to synergize with the HSV-TK/GCV cytotoxicity and cause enhanced cell death. To investigate this, we subjected both cell lines (with 15% of TK-GFP positive cells) to a combination of 24 h DENSPM treatment and 5 days of incubation with GCV according to different schedules shown in Fig. 1. As expected, none of the combinations of DENSPM and GCV had any enhancing effect, i.e. the cytotoxicity in all cases was not significantly changed compared to the controls that received only GCV (results not shown). Hence, our data demonstrate that polyamine deprivation as such is not sufficient for synergistic effect with the HSV-TK/GCV gene therapy, but changes in the cell cycle phase distribution need to occur.

Cell cycle arrest induced by aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol. Since it was likely that at least some degree of cell cycle phase disturbance would be necessary for a drug to have a synergistic effect with the HSV-TK/GCV gene therapy, we turned our attention to drugs that are known to arrest the cell cycle at a certain point. We selected the drugs for their commercial availability, their well-characterized effects on the cell cycle and in some cases for their clinical relevance (for more background, see Introduction). 9L and U-251 MG cells were treated with each drug for 12 h, using concentrations that were chosen to be the least toxic but still able to induce the characteristic cell cycle effect. When the cell samples were taken during a 24 h period after drug removal (Fig. 3) and analyzed for the cell cycle phase distribution, it was shown that all the compounds except lovastatin were able to induce a prominent cell cycle block in

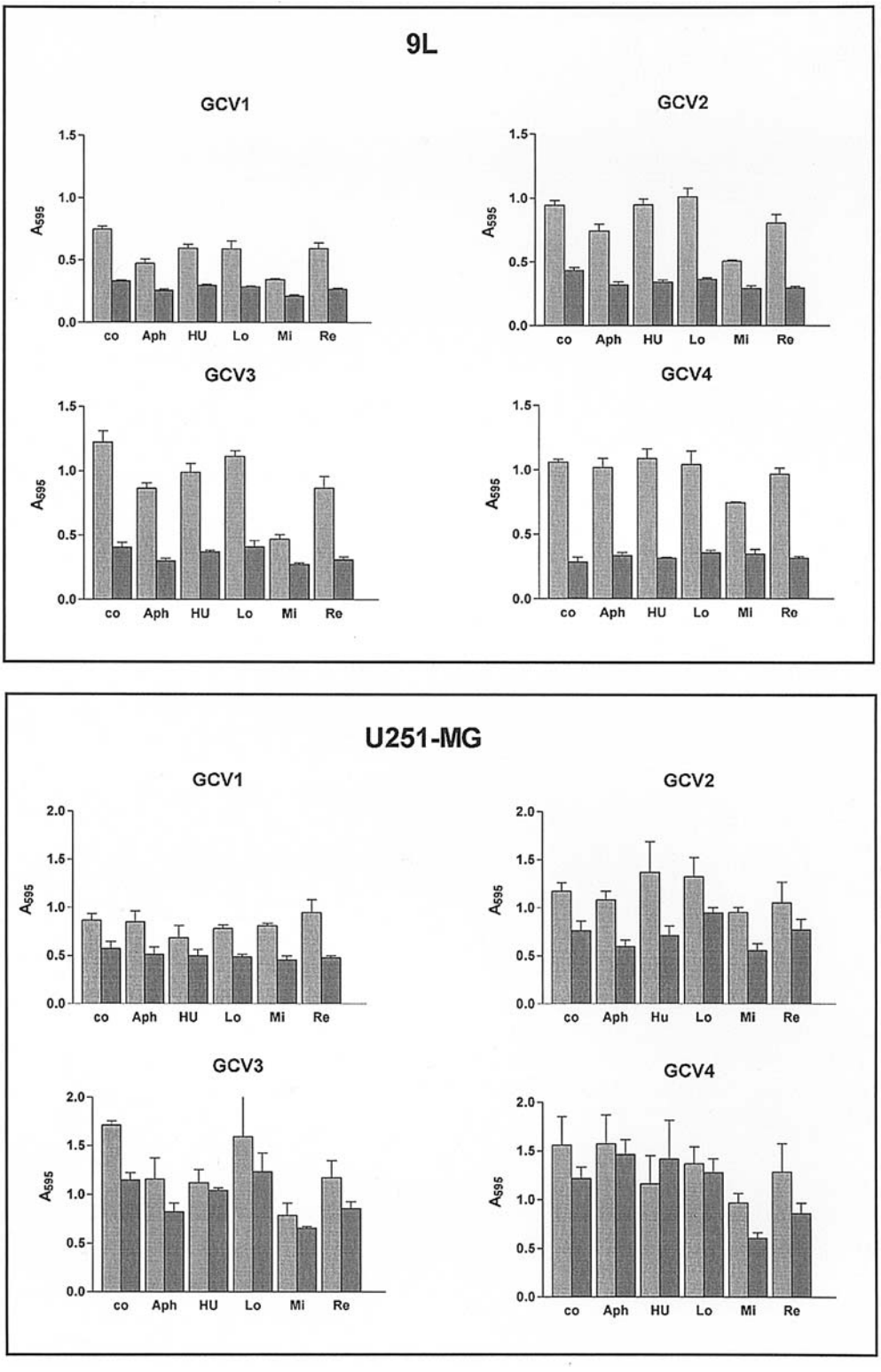

Figure 4. Cell viability of HSV-TK positive cells after treatment with ganciclovir in combination with the cell cycle altering drugs. The cell lines (9L and U-251 MG) were treated with aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol for 12 h, and GCV for 5 days according to 4 schedules (GCV1, GCV2, GCV3 and GCV4) described in Fig. 1. The dark gray bars represent combination-treated cells. Control cells (light gray bars) were treated with the drugs for 12 h and incubated without GCV for 60, 72, 84 and 96 h (GCV1, GCV2, GCV3 and GCV4 controls, respectively). The values shown are results from MTT assays (3 measurements per data point), the error bars indicate standard deviation.

both cell lines. When the block was released, aphidicolin-, hydroxyurea-, mimosine- and resveratrol-treated cells showed a rapid and transient increase of S phases and respective decrease of G0/G1 cells. In 9L cells the proportion of S phase cells peaked before 8 h after the release from all drugs and the cells reached the equilibrium shortly after that, displaying very rapid kinetics that was probably attributable to rapid growth of this cell line.

In U-251 MG cells, hydroxyurea and resveratrol induced similar effect, but there were still signs of synchrony in these

cells at later time points. Namely, the proportion of G0/G1 cells started to fall (and proportion of S phases to rise) again after a 12 h post release, indicating that a marked fraction of the cells were simultaneously entering the S phase for the second time and preparing to divide again. Aphidicolin-treated U-251 MG cells also displayed a rapid increase of S phase cells, but the initial peak was followed by a gradual increase in the percentage of sub-G0/G1 cells (cells that contain less DNA than equivalent of one haploid genome, generally considered late apoptotic or early necrotic, results not shown). The proportion of dying cells was as high as 12.5% at 24 h post drug removal, as opposed to 1.1% in control cells and <4% in cells treated with the other drugs, suggesting a pro-apoptotic effect of aphidicolin in these cells. Aphidicolin induced a comparable effect in 9L cells as well, with 11.3% of sub-G0/G1 cells at 9 h after the release (2.6% in control cells and 4-8% in cells treated with the other drugs, results not shown). In mimosine-treated U-251 MG cells, the S phase peaked later than with the other drugs and remained higher than the control values for almost 24 h, suggesting that this prolonged elevation of dividing cells could facilitate synergy with the HSV-TK/GCV gene therapy better than the other compounds.

The cell cycle effects observed in our study were in accordance with observations by other groups. Boucher *et al* (7) studied the effect of HU on the colon cancer cell line SW620 and found that a 4-h treatment with a 2 mM drug concentration caused complete elimination of DNA synthesis. This block was rapidly reversed after HU was withdrawn and the cells began to grow again 24 h post drug removal. They also tested the effect of aphidicolin in the same and an earlier study (6) and observed that this drug failed to increase GCV triphosphate levels in bystander effect analyses and actually protected the SW620 cells from the cytotoxic effect of GCV. This confirms our observations that the features of aphidicolin are incompatible with the HSV-TK/GCV system. Previous studies with mimosine have suggested that the arrest point can be at G1 or S, depending on the cell line. Similarly, our results showed that U251-MG cell line was arrested at G1, whereas accumulation of S phase cells took place in the 9L cell line. The data regarding resveratrol indicated that both the cell lines were halted at S phase. This has been shown to be the case also with MCF-7 breast (18), SW480 colon (21) and LNCaP prostate cancer cells (20). In contrast to many earlier studies with lovastatin and human cancer cell lines (24-26), we did not detect any cell cycle arrest with this drug in either of the cell lines. It could be suggested that in 9L cells, there was a slight increase of G0/G1 cells around 12 h post drug removal, followed by an increase of the S phase cells. This increase was, however, superficial and did not compare to the clear cell cycle effect caused by the other drugs used in this study. Our results are in accordance with the L1210 cell line study by Cooper (27) who claims that the cell cycle effect of lovastatin is highly dependent on the cell type used and the changes seen after lovastatin treatment in many cases can be explained by the retarded cell growth and resulting increase in the fraction of cells with G1-phase amount of DNA without actual arrest of growth.

Taken together, all the compounds except lovastatin induced a clear cell cycle arrest that was followed by a notable, but transient elevation of the cells replicating their genomes. Only aphidicolin displayed a mild toxic/pro-apoptotic effect in both cell lines, whereas all the other drugs at used concentrations appeared to be relatively harmless to the cells and thus potentially useful for enhancement of gene therapy. However, the rapid by-pass of the elevated S phase fraction is not considered a favorable feature for enhancement of the HSV-TK/GCV system. Our earlier study (5) revealed that elevated proportion of S phase cells induced by serum deprivation was very transient (passed in 24 h after release from the arrest) and could not be used to augment HSV-TK/GCV cytotoxicity. On the other hand, reversal of the DFMO-induced polyamine depletion and the accompanying cell cycle disturbance was a significantly longer process (proportion of S phases remained elevated for 2-3 days) and allowed synergy with the HSV-TK/GCV gene therapy.

Cell cycle altering drugs are weak enhancers of the HSV-TK/GCV gene therapy. To verify whether the rapid disturbance of cell cycle induced by aphidicolin, hydroxyurea, mimosine and resveratrol (and the lack of cell cycle effect with lovastatin) can be used to potentiate the HSV-TK/GCV gene therapy, we treated TK-GFP-positive 9L and U251-MG cells with these compounds in combination with GCV using 4 different schedules (Fig. 1). The viability of the cells was analyzed after a 5-day GCV treatment using the MTT assay. Also, the cells were treated with aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol, and incubated without GCV for 60-96 h (the respective time after the dug treatment as the cells exposed to the combination treatment) to validate the effect of these drugs alone.

As shown in Fig. 4, some of the drugs displayed cytostatic/cytotoxic effect in both cell lines without any GCV treatment. Especially mimosine caused an apparent viability decrease that remained detectable in both cell lines and statistically significant ($P<0.001$) in 9L cells throughout the 96-h incubation period after removal of the drug. This indicates that mimosine has other effects on these target cells than the transient cell cycle block, causing a long-lasting growth inhibition that synergizes poorly with the HSV-TK/GCV gene therapy. This is in accordance with the known toxicity of mimosine and the fact that farm animals fed on mimosine-containing Mimosa plant became sick (34). Furthermore, the mimosine mode of action [inhibition of zinc finger-containing transcription factors through chelation of zinc (12)] is presumably causing a wide range of effects and is likely to cause generally detrimental effects in most cell types.

A weak reduction of viability by aphidicolin, hydroxyurea and resveratrol was detectable in 9L cells at earlier time points but disappeared later (GCV4), whereas in U251-MG cells the effect was detectable but not significant ($P>0.05$) only at one time point after drug treatment (GCV3). Not surprisingly, lovastatin caused a significant ($P<0.001$) effect on the growth of 9L cells only at the first time point, but the effect was not detected later and there was no effect at all in U251-MG cells. These findings are in line with our observations regarding the lack of any cell cycle effects with this drug (Fig. 3).

Combination treatment of HSV-TK/GCV and the drugs (Fig. 4) showed that especially in 9L cells, some enhancement

can be obtained but the degree of the effect remained low even in the best cases. In these cells, all the drugs caused a significantly potentiated HSV-TK/GCV-mediated cytotoxicity ($P<0.005$) when treatment schedules GCV1 and GCV2 were used (i.e. GCV was initiated before the drug treatment). However, in case of mimosine and to some extent also with aphidicolin and resveratrol, at least part of the effect was attributable to the viability-reducing effects of these drugs. This held true also with the treatment scheme GCV3 (mimosine, aphidicolin and resveratrol: $P<0.005$). Hydroxyurea and lovastatin were able to induce a enhancing effect with the treatment schedule GCV2 without any effect on the cell growth. Hydroxyurea was shown to block the cell cycle in these cells, whereas lovastatin did not show any signs of cell cycle arrest (Fig. 3); thus, raising the possibility of yet another unknown mechanism of statins to augment HSV-TK/GCV gene therapy without any apparent cell cycle effects.

In U251-MG cells, a significantly increased cytotoxic effect was detected only with aphidicolin (GCV3, $P<0.005$) and mimosine (GCV3 and GCV4, $P<0.005$), but this effect was again due to toxicity of these compounds and not the synergy with the two treatment forms. Even though cell cycle analyses of U251-MG cells after mimosine treatment suggested potential enhancement of the HSV-TK/GCV system owing to slower release kinetics and lack of pro-apoptotic effect, most (if not all) of the cytotoxic effect appeared to result from the mimosine action alone. Thus, these data revealed that changes in the cell cycle phase distribution after a drug treatment are poor predictors of their utility in enhancement of the HSV-TK/GCV gene therapy and the final judgment can be made only with a combination treatment and viability analyses.

The lack of augmenting effect with HU in U251-MG cells was surprising because Boucher and coworkers have earlier shown a strong additive cytotoxicity of this drug and HSV-TK/GCV gene therapy (6). However, in that approach the aim was to deplete cellular dGTP pools in order to increase the incorporation of GCV monophosphate and their experimental design was different. They incubated a population with 100% TK-positive cells for 24 h in the presence of both HU and GCV, followed by clonogenic survival assay, whereas in our experimental setting with 15% TK^+ cell population was exposed to short incubation with HU and long incubation with GCV. The different results with different experimental conditions suggest that dGTP depletion, but not cell cycle synchronization, is a valid approach to use HU for enhancement of HSV-TK/GCV gene therapy.

In conclusion, the present results reveal more details of the properties that are required for a drug to enhance the HSV-TK/GCV gene therapy. We have shown earlier that polyamine biosynthesis inhibition and the accompanying cell cycle arrest can be used to potentiate the therapy (5), but it was not determined clearly whether polyamine deprivation alone can cause the effect or the cell cycle effect was just a co-existing phenomenon. Herein, we demonstrated that disturbed polyamine homeostasis per se without any cell cycle consequences (as caused by a mechanism other than inhibition of ornithine decarboxylase) is not sufficient to cause any enhancing effect with the HSV-TK/GCV system. Our previous analyses also suggested that to potentiate the HSV-TK/GCV gene therapy, the cell cycle consequences of a drug treatment need to be correctly timed and long-lasting (5). This hypothesis was further confirmed here with two different brain tumor cell lines and five different drugs that are known to arrest the cell cycle via different mechanisms. Studies with aphidicolin, hydroxyurea, lovastatin, mimosine and resveratrol revealed that most of these drugs induced characteristic cell cycle arrest that was followed by a clear but transient increase of S phase cells. This increase was able to enhance the HSV-TK/GCV cytotoxicity when appropriately timed, but the enhancement remained modest at best and some of the effect was shown to be due to the action of the drug alone. Thus, drugs that induce too rapid a cell cycle disturbance are unlikely to be useful in enhancing the HSV-TK/GCV gene therapy in animal models or in clinical trials.

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