

Synergistic Enhancement of Herpes Simplex Virus Thymidine Kinase/Ganciclovir-mediated Cytotoxicity by Hydroxyurea¹

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ABSTRACT

We have previously demonstrated (L. Z. Rubsam *et al.*, *Cancer Res.*, 59: 669–675, 1999) that low ganciclovir (GCV) triphosphate (TP) levels similar to cellular deoxynucleotide concentrations can induce multilog killing in cells stably expressing herpes simplex virus thymidine kinase (HSV-TK). In this study, we evaluated whether reducing the endogenous competitor of GCV-TP, dGTP, enhanced GCV-mediated cytotoxicity. In SW620 human colon carcinoma cells stably expressing HSV-TK, the addition of the ribonucleotide reductase inhibitor, hydroxyurea (HU), decreased cellular dGTP pools and simultaneously increased the accumulation of GCV-TP levels. The amount of GCV nucleotide transfer from HSV-TK-expressing to nonexpressing (bystander) cells was quantitated in physically separated pHook-expressing bystander cells. Elevation of the GCV-TP:dGTP ratio by HU resulted in increased levels of GCV nucleotides transferred from HSV-TK-expressing to bystander cells during a 24 h drug incubation and enhanced GCV monophosphate incorporation into DNA after drug removal. Isobologram analysis demonstrated that the combination of GCV and HU was additive in 100% HSV-TK cultures and synergistic in HSV-TK/bystander mixtures. IC₅₀ values for GCV in 1:1 cocultures of HSV-TK-expressing and nonexpressing SW620 cells were reduced from 1.5 μM to 0.07 μM with 2 mM HU. A similar reduction was also observed with HT-29 cells and U251 cells. With 2 mM HU, IC₅₀ values for GCV in 10:90, 5:95, and 1:99 SW620 HSV-TK-expressing and nonexpressing cocultures were reduced from 55 μM to 0.3 μM, 71 μM to 0.8 μM, and 118 μM to 7 μM, respectively. These results demonstrate the ability to pharmacologically enhance HSV-TK/GCV-mediated bystander killing and may have an important therapeutic impact.

INTRODUCTION

Several approaches to cancer treatment have been used in which either a viral or a bacterial enzyme (suicide gene) is introduced into tumor cells that allows them to selectively sensitize them to a drug that is normally nontoxic to host cells (1–3). One widely used strategy involves the transfer of the cDNA for the *HSV-TK*³ gene into tumor cells; the gene then sensitizes the cells to the antiviral drug GCV (4). The antitumor activity is the result of HSV-TK-expressing tumor cells activating GCV to its cytotoxic triphosphate derivative. This acyclic dGTP analogue competes with endogenous dGTP pools for incorporation into DNA in which it interferes with cellular DNA synthesis (5, 6). This form of therapy also benefits from the ability of a small percentage of HSV-TK-expressing cells to cause GCV-mediated cell death to tumor cells that do not express the transgene—a phenomenon also known as the “bystander effect” (4, 7, 8). HSV-TK/GCV cancer gene therapy has been successful *in vitro* with numerous types of

tumor cells and has resulted in marked tumor regression in several animal models (8–15). This success has prompted protocols for clinical trials for the treatment of brain and ovarian tumors (16).

Previous work in this laboratory with glioblastoma cells established that GCV elicits a unique 4- to 5-log cell kill compared with less than a 2-log cell kill with other nucleoside analogues (17). In subsequent studies, this multilog kill was shown to be accompanied by substantial bystander killing (18). Two proposed theories that explain the observed bystander killing *in vitro* include (a) transfer of phosphorylated GCV via GJIC and (b) phagocytosis by non-HSV-TK-expressing cells of apoptotic vesicles containing GCV metabolites from HSV-TK-expressing tumor cells (7, 11, 19). We have also quantitated the transfer of phosphorylated GCV from HSV-TK-expressing cells to bystander cells over time in both human glioblastoma and colon carcinoma cell lines (18, 20). With human U251 glioblastoma cells, the transfer of phosphorylated GCV was observed as soon as 4 h after a drug addition and without apoptotic vesicle formation (18). Although GJIC was considerably lower, bystander killing was also observed in the SW620 human colon carcinoma cell line and was dependent on the level of HSV-TK expression, the number of cells expressing HSV-TK, and the overall confluency of the cells (20). After a 24 h exposure to GCV, the amount of GCV nucleotides transferred to bystander cells was 2-fold greater in U251 cells compared with SW620 cells with a corresponding 5-fold increase in bystander cell cytotoxicity (18, 20).

Because current methodologies for transducing genes *in vivo* usually allow only a small proportion (≤ 10%) of a tumor to actually express HSV-TK, bystander killing is critical for clinical success and the eradication of tumors (16). Enhancing the efficiency of bystander killing may offer new strategies for improving the clinical application of HSV-TK/GCV gene therapy. On the basis of our previous reports that low GCV-TP levels can induce multilog kills and that these levels are similar to cellular deoxynucleotide concentrations, we hypothesized that decreasing its endogenous competitor, dGTP, would enhance the cytotoxicity of GCV. In this study, the ability of the ribonucleotide reductase inhibitor HU to enhance GCV cytotoxicity was evaluated in both HSV-TK-expressing and cocultures of HSV-TK-expressing and nonexpressing (bystander) cells. Isobologram analysis demonstrated that the combination of GCV and HU are additive in HSV-TK-expressing cultures and synergistic in mixtures of HSV-TK and bystander cells, even when only 1% of the cocultures express HSV-TK. These results demonstrate the ability to pharmacologically enhance HSV-TK/GCV-mediated bystander killing and may have an important therapeutic effect in tumors with low efficiencies of gene transfer or low levels of GJIC.

MATERIALS AND METHODS

Cell Culture and Generation of Stable Cell Lines. Human colon carcinoma cell lines, SW620 and HT-29, were cultured in McCoy's 5A medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The U251 human glioblastoma cell line was grown in RPMI with 2 mM L-glutamine and 10% calf serum (Life Technologies, Inc., Grand Island, NY). Cells were maintained in exponential growth in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air.

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³ The abbreviations used are: HSV, herpes simplex virus; TK, thymidine kinase; GCV, ganciclovir; GCV-TP, GCV triphosphate; GCV-MP, GCV monophosphate; GJIC, gap junction/junctional intercellular communication; HU, hydroxyurea HPLC, high-performance liquid chromatography; phOx, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; CD, cytosine deaminase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil.

SW620, HT-29, and U251 clonal cell lines that stably expressed HSV-TK were developed from their parental cell line using a retrovirus vector containing the cDNA for HSV-TK under the control of the 5' long terminal repeat sequence and have been characterized previously (20). Clonal cell lines stably expressing β -galactosidase were developed similarly (20). SW620 cells stably expressing the pHoek single-chain antibody were produced by transfection with the pHoek-2 vector (Invitrogen, Carlsbad, CA; Ref. 20).

Clonogenic Survival Assays. Cytotoxicity was measured after 24 h exposure to GCV in the presence or absence of HU. Exponentially growing cells were treated with 0.01–100 μ M GCV (Cytovene, Syntex, Palo Alto, CA) and/or 0.5–5 mM HU (Sigma Chemical C., St. Louis, MO) for 24 h and were trypsinized, counted with a Coulter electronic particle counter, and diluted to approximately 100 viable cells per 35-mm-diameter well in 6-well culture dishes. After 10–14 days, the resulting cell colonies from 100% HSV-TK cultures were fixed in methanol:glacial acetic acid (3:1, v/v), stained with 0.4% crystal violet, and visually counted. In experiments with mixtures of HSV-TK- and *Lac Z*-expressing cells, bystander cell survival was measured by staining with 0.2% 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal, Boehringer Mannheim, Indianapolis, IN). Cell survival was expressed as a fraction of plating efficiency for untreated cells. All of the colony formation assays were performed independently at least twice, and each point was plated in triplicate. The cytotoxic activity of GCV and HU combinations based on the dose-response cell-survival curves was evaluated by isobologram analysis (21).

Analyses of Cellular GCV Nucleotides. Cellular deoxyribonucleotides and GCV-TP levels were measured as described previously (20). Briefly, SW620 cells were treated with 1 μ M [3 H]GCV (Moravak Biochemicals Inc., Brea, CA) and harvested by trypsinization; nucleotides were extracted with ice-cold 0.4 N perchloric acid. The acid-insoluble cell pellets were washed with 0.4 N perchloric acid and solubilized overnight in 1 N KOH. Incorporation of [3 H]GCV into DNA was then quantitated by liquid scintillation spectrometry. Cellular nucleotides and GCV-TP were separated and quantitated by strong anion exchange HPLC using a Waters (Milford, MA) gradient system controlled by Millennium 2010 software. Samples were loaded onto a 5- μ m Partisphere 4.6- \times 250-mm SAX column (Whatman, Hillsboro, OR), and nucleoside triphosphates were eluted with a linear gradient of ammonium phosphate buffer ranging from 0.15 M (pH 2.8) to 0.6 M (pH 3.8). Fractions containing radiolabeled GCV nucleotides were collected and quantitated by liquid scintillation spectrometry based on the known specific activity of the tritiated GCV.

Magnetic Separation of pHoek-expressing (Bystander) Cells. pHoek-expressing cells produce an extracellular single-chain antibody directed toward pHoek-coated beads, which allows the physical separation of these cells with a strong magnet (22–24). Equal amounts of SW620 cell lines that express HSV-TK and pHoek were cocultured on 35-mm-diameter 6-well culture dishes at a density of 1×10^6 cells per dish and were grown for 24–48 h. After a 2- to 24-h exposure to 1 μ M [3 H]GCV, cells were harvested with PBS/3 mM EDTA and pipetted to achieve a single-cell suspension. Cells were incubated with 3×10^6 pHoek-coated beads (Capture-Tec Beads, Invitrogen, Carlsbad, CA) for 30 min in 1 ml of complete medium. Tubes containing the cells were placed in a magnetic stand and mixed for 5 min. The bound cells were washed extensively, resuspended in 1 ml of complete medium, and counted. Approximately 20–30% of the pHoek-expressing cells were recovered for analysis by HPLC. We have previously demonstrated that this technique was capable of separating pHoek-expressing cells from HSV-TK-expressing cells with $\geq 97\%$ purity, with no artifactual carryover of radioactivity (20).

RESULTS

Effect of HU on GCV-TP and dNTP Levels. To determine the effect of HU (a ribonucleotide reductase inhibitor) on cellular nucleotide pools, SW620 cells that stably expressed HSV-TK were incubated for 24 h with 1 μ M GCV and 0.05–5.0 mM HU. As illustrated in Fig. 1, the dGTP pool was unaffected by the addition of HU at concentrations of HU ≤ 0.5 mM. At higher HU concentrations, the amount of dGTP began to decrease and was 25% of its original level with the addition of 5 mM HU. The level of dATP steadily declined (from 11 to 1.0 pmol/ 10^6 cells) with increasing amounts of HU and reached 10% of its control value at 5 mM HU. The dTTP pools

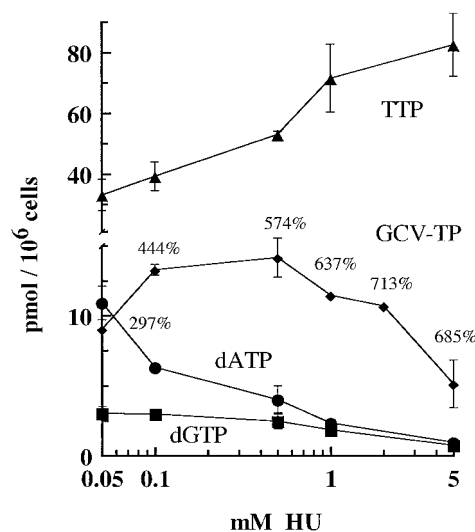


Fig. 1. Levels of GCV-TP (\blacklozenge), dGTP (\blacksquare), dATP (\bullet), and dTTP (\blacktriangle) in GCV-treated SW620 HSV-TK-expressing cells after the addition of HU. Exponentially growing cells were harvested after a 24-h incubation in the presence of 1 μ M GCV with 0.05–5 mM HU. Cellular nucleotides were extracted with 0.4 N perchloric acid, subjected to a boronate affinity column to reduce cellular ribonucleotides, and analyzed and quantitated by strong anion exchange HPLC. Levels of dCTP were not included in this graph because dCTP coeluted with CTP. The percentages represent the ratio of GCV-TP:dGTP at the corresponding HU concentration. Results are expressed as the average \pm SE from two separate experiments.

increased 2.5-fold (from 33 to 83 pmol/ 10^6 cells) with increasing amounts of HU. A similar pattern of deoxynucleotide pool alteration was also observed with HU treatment alone in HSV-expressing cells or in *Lac Z*-expressing cells (data not shown). In the absence of HU, the level of GCV-TP measured 5.5 pmol/ 10^6 cells (data not shown). The accumulation of GCV-TP increased with increasing concentrations of HU to a maximum of 2.5-fold at 0.5 mM HU; the accumulation decreased slightly at 2 mM and returned to control (without HU) levels with 5 mM HU. The combination of an increase in the level of GCV-TP and a decrease in cellular dGTP pools resulted in a 3- to 7-fold increase in the ratio of GCV-TP:dGTP with HU (Fig. 1).

Analysis of GCV-TP Levels and DNA Incorporation in Bystander Cells. To investigate whether the levels of GCV-TP are also elevated in bystander cells with HU treatment, we used a technique that we had previously developed to physically separate cocultures of HSV-TK and bystander cells, and we analyzed GCV nucleotide levels (20). Cocultures consisting of 1:1 mixtures of HSV-TK-expressing and pHoek-expressing (bystander) cells, treated with 1 μ M GCV in the absence or presence of 2 mM HU, were incubated over a 24-h period, separated using a pHoek/magnetic system and analyzed by HPLC. The results, presented in Fig. 2, demonstrate an increase over time in both the accumulation of GCV-TP in HSV-TK-expressing cells and the transfer of phosphorylated GCV to bystander cells. Consistent with the previous experiment, almost twice as much GCV-TP was present in HSV-TK-expressing cells after the concurrent addition of 2 mM HU. This increase is also reflected in bystander cells, in which a similar and simultaneous increase in GCV-TP levels is observed. With HU, GCV-TP concentrations in bystander cells approached levels detected in HSV-TK-expressing cells in the absence of HU. Because HU induced a similar reduction of cellular dGTP levels in bystander cells, the elevation of GCV-TP levels with 2 mM HU corresponds to a 63% increase in the GCV-TP:dGTP ratio in bystander cells.

We have demonstrated previously that, in human colon carcinoma HSV-TK-expressing cells, the amount of GCV-MP in DNA corresponded to the degree of cytotoxicity (20). To investigate whether a

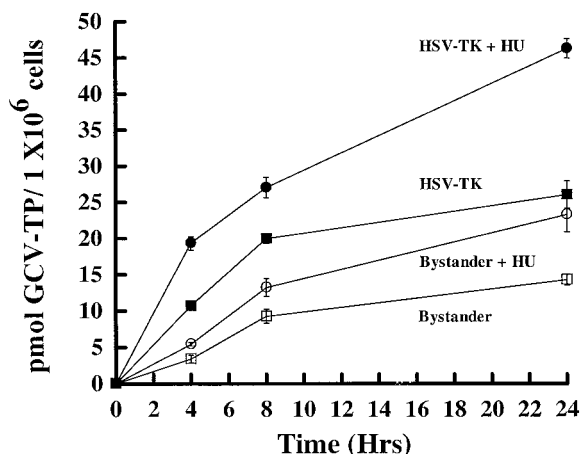


Fig. 2. Transfer of GCV nucleotides between HSV-TK-expressing (● and ■) and bystander cells (○ and □) in the absence (■ and □) or presence (● and ○) of HU. An equal number of SW620 HSV-TK- and pHoK-expressing clones were cocultured and incubated with $1 \mu\text{M}$ [^3H]GCV \pm 2 mM HU for the indicated times. Bystander cells were physically separated from HSV-TK-expressing cells with a magnet, and the level of GCV-TP was determined by HPLC and liquid scintillation spectrophotometry. Values represent the mean \pm SE from at least two separate experiments.

corresponding increase in the amount of GCV-MP present in DNA is also observed with the addition of HU, we measured the incorporation of radiolabeled GCV-MP into DNA after a 24-h drug incubation and 48 h after the drug washout. With the addition of 2 mM HU, the level of GCV-MP incorporation into DNA in SW620 HSV-TK-expressing cells was reduced to <40% of cells treated with GCV alone (Fig. 3A, time 0). In contrast, the level of GCV-MP present in the DNA of bystander cells was not significantly altered by HU addition (Fig. 3B, time 0). After the removal of exogenous GCV and HU, GCV was rapidly incorporated into DNA during the initial 12 h, and the level of GCV-MP in DNA from HU-treated cells at this and subsequent time points exceeded the levels in cells treated with GCV alone (Fig. 3A). In bystander cells, the addition of HU resulted in an increase in GCV incorporation into DNA as early as 4 h after drug washout and continued to be elevated for 48 h (Fig. 3B). This level of incorporation was similar to what we observed in HSV-TK-expressing cells without HU treatment (Fig. 3A).

Cytotoxicity with a Combination of GCV and HU. To determine whether the concurrent addition of GCV and HU had an effect on the level of GCV-mediated cell death, we measured the clonogenic survival of *Lac Z*-expressing bystander cells using a chromogenic assay. Three doses of GCV or two doses of HU were administered at concentrations that resulted in 20–80% cell kill when either drug was given alone. An equal number of HSV-TK- and *Lac Z*-expressing SW620 cells grown in coculture were incubated 24 h with drug(s). In Fig. 4, the addition of 0.5 mM or 2 mM HU decreased the IC_{50} of GCV in SW620 cells from $1.5 \mu\text{M}$ to 0.37 or $0.07 \mu\text{M}$, respectively. This effect was not limited to this cell type because a similar reduction in the IC_{50} of GCV was also observed in another human colon carcinoma cell line, HT-29, and in the human U251 glioblastoma cell line (Fig. 4, middle and right).

To determine whether the interaction between the two drugs exhibited synergistic cytotoxic effects, the data from the above dose-response curves was examined by constructing isobolograms. The isobolograms in Fig. 5 demonstrate that the combination of GCV and HU had synergistic cytotoxic effects in bystander cells cocultured with an equal number of HSV-TK cells (Fig. 5B) with all of the points lying to the left of the line of additivity, but the effects in cultures of 100% HSV-TK-expressing cells were only additive (Fig. 5A). These effects were observed with all of the three cell lines using 30, 40, and

50% surviving fractions for 1:1 mixtures and 20, 30, and 40% surviving fractions for HSV-TK cultures.

Sensitivity of SW620 Bystander Cells in Coculture to GCV and HU. We have demonstrated that HU enhanced bystander killing in 1:1 SW620 HSV-TK:bystander cocultures. To investigate whether the addition of HU would increase GCV cytotoxicity in mixtures containing more clinically relevant levels of HSV-TK-expressing cells, bystander killing was again measured using a chromogenic assay in cocultures containing 10, 5, and 1% HSV-TK-expressing cells. The IC_{50} of GCV in SW620 cells that did not express HSV-TK was approximately $450 \mu\text{M}$ (data not shown). Concentrations of GCV or HU that achieved 20–80% cell killing individually were added simultaneously to these SW620 cocultures and incubated for 24 h. In the absence of HU, the IC_{50} of GCV ranged from 55 to $118 \mu\text{M}$ in cocultures with 10 to 1% HSV-TK cells, respectively. As illustrated in Fig. 6, the addition of 2 mM HU to these GCV-treated cocultures decreased the IC_{50} of GCV from $55 \mu\text{M}$ to $0.30 \mu\text{M}$ (Fig. 6, left), from $71 \mu\text{M}$ to $0.80 \mu\text{M}$ (Fig. 6, middle) or from $118 \mu\text{M}$ to $7.25 \mu\text{M}$ (Fig. 6, right) in cocultures containing 10, 5, or 1% HSV-TK cells, respectively. The isobologram analysis in Fig. 7 using 30, 40, and 50%

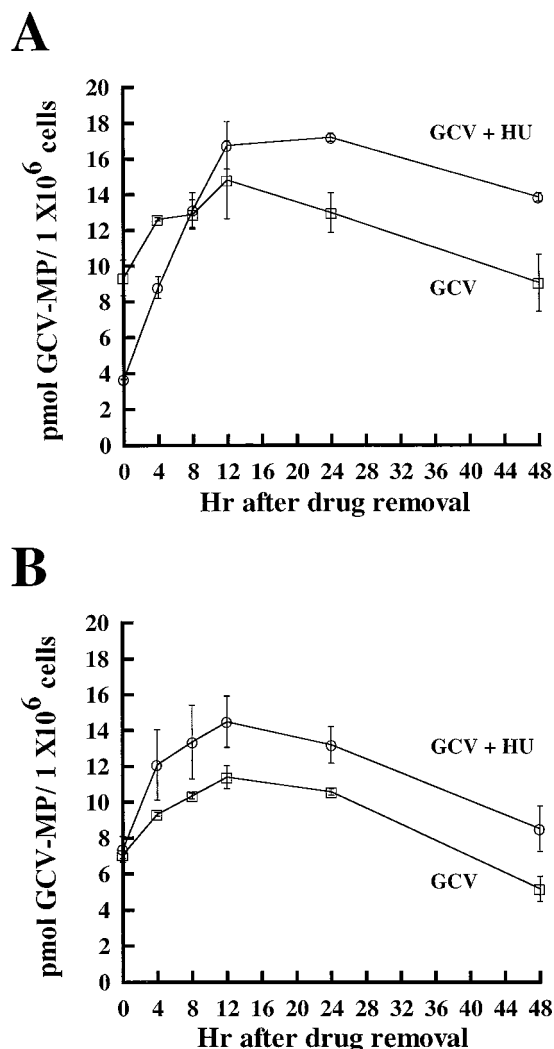


Fig. 3. Incorporation of [^3H]GCV-MP into DNA in HSV-TK-expressing (A) and bystander cells (B). The level of GCV-MP in DNA from HSV-TK and bystander cells was determined after a 24 h incubation with $1 \mu\text{M}$ [^3H]GCV \pm 2 mM HU. Nucleic acids from HSV-TK-expressing and physically separated (bystander) cells were precipitated with perchloric acid and quantitated by liquid scintillation spectrophotometry. Data are expressed as the mean \pm SE from at least two separate experiments.

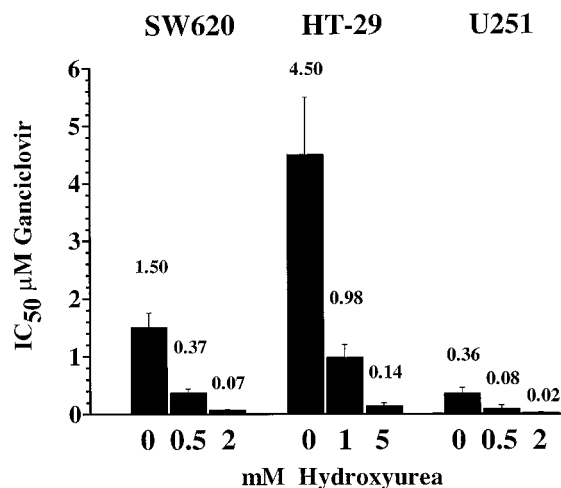


Fig. 4. Effect of HU and GCV on the sensitivity of SW620, HT-29, and U251 clones. HSV-TK-expressing clones were cocultured with an equal number of β -galactosidase-expressing cells. Exponentially growing cocultures were incubated with concentrations of HU or GCV, which produced between 20 and 80% cell kill when given alone. Clonal cell survival of *Lac Z*-expressing (bystander) cells was determined by a chromogenic assay after a 24-h incubation with GCV, HU, or the combination of the two drugs. Cytotoxicity curves were constructed, and the IC₅₀ of GCV was calculated in the presence or absence of HU from these curves. Bar, the mean \pm SE from two separate experiments with at least six determinations.

surviving fractions indicates that the combination of GCV and HU in cocultures containing low amounts of HSV-TK-expressing cells is also synergistic. These data demonstrate that the addition of HU lowers the sensitivity of SW620 cocultures to GCV from concentrations of $>50 \mu\text{M}$ to clinically achievable doses.

DISCUSSION

We have evaluated the ability of HU to enhance cytotoxicity with the HSV-TK/GCV enzyme-prodrug strategy. Many groups have demonstrated the excellent antitumor activity of GCV in a variety of different types of tumor cells engineered to express HSV-TK, but few reports have described effective modulation of GCV-mediated cytotoxicity *in vitro*. Samejima and Meruelo (25) used forskolin, verapamil, and the protein kinase C inhibitor, H7, in conjunction with GCV in C6 rat glioma cells expressing HSV-TK. Of these agents, only the adenylate cyclase activator, forskolin, had an effect resulting in a dose-dependent decrease in bystander killing. The mechanism for this inhibition was not identified, but forskolin may participate in signal transduction, phagocytosis, or gap junctional intercellular communication. Others have attempted to enhance the bystander killing by overexpressing connexin proteins, the basic components of gap junction channels (26, 27). More efficient GCV-mediated cell killing was observed in connexin transfectants compared with their parental lines, which differed only in their GJIC capacity. Although effective in tissue culture with stable cell lines, this strategy suffers from the inability to transduce the majority of cells in a tumor with present gene-therapy techniques. To be advantageous, both HSV-TK and nontransduced cells must: (a) express the product of the connexin transgene; (b) align properly; and (c) form gap junction channels (28). In addition, connexin expression and GJIC have been shown to vary greatly between tumor types and even within a particular tumor (29, 30). Yet another approach to augment the efficacy of the HSV-TK/GCV gene therapy is the addition of a second suicide gene, *CD*. The *Escherichia coli* *CD* converts the nontoxic prodrug 5-FC to the antitumor drug 5-FU and has been used independently as an enzyme-prodrug gene-therapy strategy (3). When both the *HSV-TK* and *CD* genes are coexpressed, the coadministration of GCV and 5-FC has

been shown to produce a synergistic effect (31, 32). The mechanism suggested for this synergy involved the enhancement of GCV phosphorylation due to 5-FU effects on lowering intracellular thymidine and relieving the competition for GCV to bind to HSV-TK (32). In this study, we have demonstrated a pharmacological elevation of GCV phosphorylation without the necessity of additional transgene expression.

The observation that HU can render cocultures of HSV-TK-expressing and -nonexpressing cells more sensitive to GCV in an additive or synergistic fashion suggests that this drug combination may have promising clinical implications. Indeed, HU already has a proven history of clinical application and has been shown to be a good biochemical modulator in combination with other chemotherapeutic agents including antimetabolites such as 1- β -D-arabinofuranosylcytosine, fludarabine, and 5-FU (33). The primary site of action for HU is the inhibition of ribonucleotide reductase, the enzyme responsible for *de novo* production of deoxyribonucleotides and a rate-limiting reaction in the regulation of DNA synthesis (34).

We have previously reported (20) that accumulation of less than 20 pmol GCV-TP/10⁶ cells was sufficient to induce more than a 2-log decrease in cell survival in SW620 cells, and differences of approximately 2-fold in GCV-TP levels and incorporation into DNA corresponded to a 10-fold difference in cytotoxicity. For these reasons, we hypothesized that a reduction of the endogenous competitor (dGTP) for GCV-TP incorporation into DNA would result in enhanced GCV-mediated cytotoxicity. The data presented here demonstrate that HU reduced the endogenous dGTP and dATP pools, whereas dTTP pools

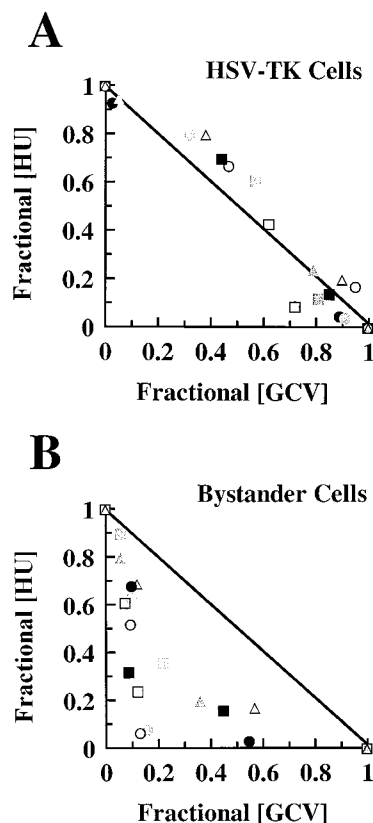


Fig. 5. Isobologram analysis of SW620 (circles), HT-29 (squares), and U251 (triangles) HSV-TK-expressing clones (A) or 1:1 HSV-TK- to *Lac Z*-expressing cocultures (B). Data from dose-response clonogenic survival curves generated in Fig. 4 were used to determine the concentration of the combinational treatment corresponding to 20% (\circ , \square , \triangle), 30% (shaded symbols), and 40% (\bullet , \blacksquare , \blacktriangle) surviving fractions for HSV-TK cultures or 30% (\circ , \square , \triangle), 40% (shaded symbols), and 50% (\bullet , \blacksquare , \blacktriangle) surviving fractions for 1:1 mixtures. Diagonal line, the isoeffect line of additivity. Each point, the mean from at least two separate experiments performed in triplicate.

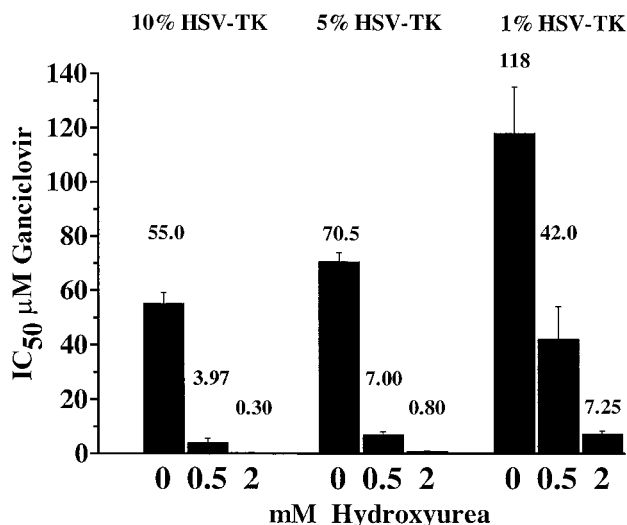


Fig. 6. Effect of HU and GCV on the sensitivity of SW620 cocultures containing 10% or less HSV-TK-expressing cells. Mixtures of 10:90, 5:95, and 1:99 HSV-TK- to Lac Z-expressing cells were grown together. Exponentially growing cocultures were incubated with concentrations of HU or GCV that produced between 20 and 80% cell kill when given alone. Clonogenic survival of Lac Z-expressing (bystander) cells was determined by a chromogenic assay after a 24-h incubation with GCV, HU, or the combination of the two drugs. Survival curves were constructed and the IC₅₀ for GCV was calculated in the presence or absence of HU from these curves. Bars, the mean \pm SE from at least six determinations over two individual experiments.

increased. This pattern of depleted purine dNTP pools and increased dTTP levels with HU treatment was also observed in other mammalian cells in culture and may be caused by stimulated uptake of pyrimidine deoxyribonucleotides from the medium (35). GCV-TP levels were also elevated 2-fold with the addition of HU, which may also be the result of enhanced uptake of GCV from the medium. Alternatively, inhibition of DNA synthesis could increase the accumulation of GCV-TP. However, blocking DNA synthesis with aphidicolin at a dose that produced equivalent levels of growth inhibition (data not shown), failed to increase GCV-TP levels, which suggests that the perturbations in dNTP pools produced by HU may be important for this enhancement.

The decrease in endogenous dGTP (Fig. 1) and an elevation of GCV-TP in both HSV-TK-expressing and bystander cells (Fig. 2) resulted in an increase in the GCV-TP:dGTP ratio, which should favor incorporation of GCV-MP into DNA. However, GCV-MP incorporation was decreased 60% in HSV-TK-expressing cells with the addition of HU (Fig. 3A). Flow cytometry analysis of BrdUrd incorporation in these cells under the same conditions demonstrated that the combination of GCV and HU decreased the mean DNA synthesis to approximately 20% of control compared with 82% with GCV alone (data not shown). Four h after exogenous drug removal, DNA synthesis in cells treated with both drugs returned to control levels. GCV-MP incorporation into DNA also increased after drug removal and surpassed levels in cells that were treated with GCV only. This increase could be the result of a higher GCV-TP:dGTP ratio in cells treated with GCV and HU. A decrease in GCV-MP incorporation into DNA was not evident in bystander cells that were incubated with both GCV and HU. However, the lower GCV-TP levels observed in bystander cells may lead to less DNA synthesis inhibition during drug treatment. Whereas the increase in GCV-MP in DNA was relatively modest for both HSV-TK and bystander cells (1.5-fold), we have shown previously (20) that less than a 2-fold increment in DNA incorporation produced a 10-fold decrease in cell survival.

We hypothesize that the increase in GCV-TP accompanied by the decrease in dGTP in bystander cells allows cytotoxic levels of GCV

incorporation to occur in bystander cells. In the presence of HU, levels of both GCV-TP (Fig. 2) and GCV-MP in DNA (Fig. 3) in bystander cells were similar to HSV-TK-expressing cells that were not treated with HU. Synergy may occur in bystander cells because increased GCV-TP levels in HSV-TK-expressing cells lead to a greater number of bystander cells receiving cytotoxic levels of GCV-TP. Additive cytotoxicity may occur in HSV-TK-expressing cells because the entire population produces rapid and sustained levels of GCV-TP in the absence of HU that are high enough to compete with endogenous dGTP, and a cytotoxic insult is achieved. Alternatively, the degree of DNA synthesis inhibition may contribute to the observed additive effects of these two drugs in HSV-TK-expressing cells compared with the synergistic effects in bystander cells. Previously (36), we have demonstrated that the cytotoxicity with GCV does not require potent inhibition of DNA synthesis, and the absence of a strong block in bystander cells may be advantageous. HU could also be eliciting its effects through dNTP pool perturbations or alterations in DNA repair. Imbalances in dNTP pools have been implicated as a trigger for the activation of a cellular endonuclease that produces DNA strand breaks resulting in programmed cell death (37, 38). HU also has been proposed to inhibit the repair of DNA lesions or delay the resynthesis at damaged sites (39). The number of damaged sites may be augmented by the presence of increased GCV-MP in the DNA.

The ability to enhance GCV-mediated bystander killing in this therapeutic approach is important, given the low efficiencies of gene transfer currently obtained *in vivo*. During Phase I clinical trials for cancer gene therapy, the percentage of tumor cells reported to incorporate or express transgene were very low (16). In this study, we have demonstrated synergy between HU and GCV even when as few as 1% of the cells express HSV-TK. Growing evidence in the literature suggests that bystander killing with GCV is mediated by the transfer of GCV nucleotides from HSV-TK-expressing to HSV-TK-nonexpressing cells through gap junctional channels (26, 27, 30). Synergistic enhancement of bystander killing by HU seems to be independent of the level of GJIC. The degree of enhancement is similar between U251 cells (which are capable of transferring dye to greater than 80% of surrounding cells) and SW620 cells (which communicate with fewer than 3% of neighboring cells; Ref. 20). Considering the importance of bystander killing in antitumor therapy with HSV-TK/GCV *in vivo*, complete identification and characterization of the process by which HU enhances GCV-mediated cytotoxicity and the extension of these studies in an animal model are warranted.

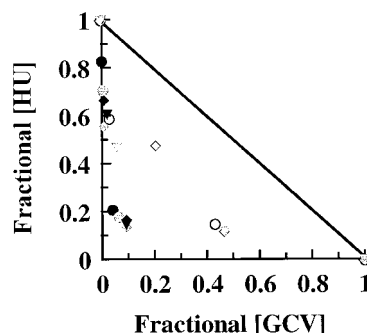


Fig. 7. Isobologram analysis of SW620 cocultures containing 10% (circles), 5% (diamonds), and 1% (inverted triangles) HSV-TK-expressing clones. Data from colony survival curves generated in Fig. 6 were used to determine the concentrations of GCV and HU combinations that resulted in 30% (open symbols), 40% (shaded symbols), and 50% (closed symbols) surviving fractions. Diagonal line, the isoeffect line of additivity. Each point, the mean from triplicate determinations from at least two separate experiments.

REFERENCES

- Moolten, F. L. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. *Cancer Gene Ther.*, *1*: 279–287, 1994.
- Bridgewater, J. A., Springer, C. J., Knox, R. J., Minton, N. P., Michael, N. P., and Collins, M. K. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur. J. Cancer*, *31A*: 2362–2370, 1995.
- Mullen, C. A., Kilstrup, M., and Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. Natl. Acad. Sci. USA*, *89*: 33–37, 1992.
- Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.*, *46*: 5276–5281, 1986.
- Balzarini, J., Bohman, C., and DeClercq, E. Differential metabolism of cytostatic of (E)-5-(2-bromovinyl)-2'-deoxyuridine, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, and other antiherpetic drugs on tumor cells transfected by the thymidine kinase gene of herpes simplex virus type 1 or type 2. *J. Biol. Chem.*, *269*: 6332–6337, 1993.
- Ilsley, D. D., Lee, S.-H., Miller, W. H., and Kuchta, R. D. Acyclic guanosine analogs inhibit DNA polymerases α , δ , and ϵ with very different potencies and have unique mechanisms of action. *Biochemistry*, *34*: 2504–2510, 1995.
- Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.*, *53*: 5274–5283, 1993.
- DiMaio, J. M., Clary, B. M., Via, D. F., Coveney, E., Pappas, T. N., and Lyster, H. K. Directed enzyme pro-drug gene therapy for pancreatic cancer *in vivo*. *Surgery*, *116*: 205–213, 1994.
- Ezzeddine, Z. D., Martuza, R. L., Platika, D., Short, M. P., Malick, A., Choi, B., and Breakfield, X. O. Selective killing of glioma cells in culture and *in vivo* by retrovirus transfer of the herpes simplex virus thymidine kinase gene. *New Biol.*, *3*: 608–614, 1991.
- Caruso, M., Panis, Y., Gagandeep, S., Houssin, D., Salzmann, J.-L., and Klatzmann, D. Regression of established macroscopic liver metastases after *in situ* transduction of a suicide gene. *Proc. Natl. Acad. Sci. USA*, *90*: 7024–7028, 1993.
- Ram, Z., Culver, K. W., Walbridge, S., Blaese, R. M., and Oldfield, E. H. *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res.*, *53*: 83–88, 1993.
- Vile, R. G., and Hart, I. R. Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res.*, *53*: 3860–3864, 1993.
- Ido, A., Nakata, K., Kato, Y., Nakao, K., Murata, K., Fujita, M., Ishii, N., Tamaoki, T., Shiku, H., and Nagataki, S. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human α -fetoprotein gene promoter. *Cancer Res.*, *55*: 3105–3109, 1995.
- O'Malley, B. W., Jr., Chen, S.-H., Schwartz, M. R., and Woo, S. L. C. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res.*, *55*: 1080–1085, 1995.
- Yoshida, K., Kawami, H., Yamaguchi, Y., Kuniyasu, H., Nishiyama, M., Hirai, T., Yanagihara, K., Tahara, E., and Toge, T. Retrovirally transmitted gene therapy for gastric carcinoma using herpes simplex virus thymidine kinase gene. *Cancer (Phila.)*, *75*: 1467–1471, 1995.
- Roth, J. A., and Cristiano, R. J. Gene therapy for cancer: what have we done and where are we going? *J. Natl. Cancer Inst.*, *89*: 21–39, 1997.
- Denning, C., and Pitts, J. D. Bystander effects of different enzyme-prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern clinical choice of appropriate regimens. *Hum. Gene Ther.*, *8*: 1825–1835, 1997.
- Rubsam, L. Z., Boucher, P. D., Murphy, P. J., KuKuruga, M., and Shewach, D. S. Cytotoxicity and accumulation of ganciclovir triphosphate in bystander cells cocultured with herpes simplex virus type 1 thymidine kinase-expressing human glioblastoma cells. *Cancer Res.*, *59*: 669–675, 1999.
- Bi, W. L., Parysek, L. M., Warnick, R., and Stambrook, P. J. *In vitro* evidence that metabolic cooperation is responsible for the bystander effect observed with *HSVtk* retroviral gene therapy. *Hum. Gene Ther.*, *4*: 725–731, 1993.
- Boucher, P. D., Ruch, R. J., and Shewach, D. S. Differential ganciclovir-mediated cytotoxicity and bystander killing in human colon carcinoma cell lines expressing herpes simplex virus thymidine kinase. *Hum. Gene Ther.*, *9*: 801–814, 1998.
- Machado, S. G., and Robinson, G. A. A direct, general approach based on isobolograms for assessing the joint action of drugs in pre-clinical experiments. *Stat. Med.*, *13*: 2289–2309, 1994.
- Griffiths, G. M., Berek, C., Kaartinen, M., and Milstein, C. Somatic mutation and maturation of immune response to 2-phenyl-oxazolone. *Nature (Lond.)*, *312*: 271–275, 1984.
- Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chriswell, D. J., Hudson, P., and Winter, G. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res.*, *19*: 4133–4137, 1991.
- Coloma, M. J., Hastings, A., Wims, L. A., and Morrison, S. L. Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J. Immunol. Methods*, *152*: 89–104, 1992.
- Samejima, Y., and Meruelo, D. 'Bystander killing' induces apoptosis and is inhibited by forskolin. *Gene Ther.*, *2*: 50–58, 1995.
- Mesnif, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc. Natl. Acad. Sci. USA*, *93*: 1831–1835, 1996.
- Elshami, A. A., Saavedra, A., Zhang, H., Kucharczuk, J. C., Spray, D. C., Fishman, G. I., Amin, K. M., Kaiser, L. R., and Albelda, S. M. Gap junctions play a role in the "bystander effect" of the herpes simplex virus thymidine kinase/ganciclovir system *in vitro*. *Gene Ther.*, *3*: 85–92, 1996.
- Yeager, M., and Nicholson, B. J. Structure of gap junction intercellular channels. *Curr. Opin. Struct. Biol.*, *6*: 183–192, 1996.
- Fick, J., Barker, F. G. L., Dazin, P., Westphale, E. M., Beyer, E. C., and Israel, M. A. The extent of heterocellular communication mediated by gap junctions is predictive of bystander tumor cytotoxicity *in vitro*. *Proc. Natl. Acad. Sci. USA*, *92*: 11071–11075, 1995.
- Yang, L., Chiang, Y., Lenz, H., Danenberg, K. D., Spears, C. P., Gordon, E. M., Anderson, W. F., and Parekh, D. Intercellular communication mediates the bystander effect during herpes simplex thymidine kinase/ganciclovir-based gene therapy of human gastrointestinal tumor cells. *Hum. Gene Ther.*, *9*: 719–728, 1998.
- Rogulski, K. R., Kim, J. H., Kim, S. H., and Freytag, S. O. Glioma cells transduced with an *Escherichia coli CD/HSV-1 TK* fusion gene exhibit enhanced metabolic suicide and radiosensitivity. *Hum. Gene Ther.*, *8*: 73–85, 1997.
- Aghi, M., Kramm, C. M., Chou, T., Breakfield, X. O., and Chiocca, E. A. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J. Natl. Cancer Inst.*, *90*: 370–380, 1998.
- Gwilt, P. R., and Tracewell, W. G. Pharmacokinetics and pharmacodynamics of hydroxyurea. *Clin. Pharmacokinet.*, *34*: 347–358, 1998.
- Donehower, R. C. Hydroxyurea. *In*: B. A. Chabner, and D. L. Longo (eds.), *Cancer Chemotherapy and Biotherapy*, pp. 253–261. Philadelphia: Lippincott-Raven Publishers, 1996.
- Bianchi, V., Pontis, E., and Reichard, P. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J. Biol. Chem.*, *261*: 16037–16042, 1986.
- Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. Superior cytotoxicity with ganciclovir compared with acyclovir and 1- β -D-arabinofuranosylthymine in herpes simplex virus-thymidine kinase-expressing cells: a novel paradigm for cell killing. *Cancer Res.*, *58*: 3873–3882, 1998.
- Yoshioka, A., Tanaka, S., Osamu, H., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C., and Wataya, Y. Deoxyribonucleoside triphosphate imbalance. *J. Biol. Chem.*, *262*: 8235–8241, 1987.
- Wadler, S., Horowitz, R., Zhang, H., and Schwartz, E. L. Effects of perturbations of pools of deoxyribonucleoside triphosphates on expression of ribonucleotide reductase, a G_1/S transition state enzyme, in p53-mutated cells. *Biochem. Pharmacol.*, *55*: 1353–1360, 1998.
- Collins, A., and Oates, D. J. Hydroxyurea: effects on deoxyribonucleotide pool sizes correlated with effects on DNA repair in mammalian cells. *Eur. J. Biochem.*, *169*: 299–305, 1987.

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Synergistic Enhancement of Herpes Simplex Virus Thymidine Kinase/Ganciclovir-mediated Cytotoxicity by Hydroxyurea

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