Hydroxyurea Induces Bystander Cytotoxicity in Cocultures of Herpes Simplex Virus Thymidine Kinase–Expressing and Nonexpressing HeLa Cells Incubated with Ganciclovir

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Abstract

Suicide gene therapy with the herpes simplex virus thymidine kinase (HSV-TK) cDNA and ganciclovir can elicit cytotoxicity to transgene-expressing and nonexpressing bystander cells via transfer of ganciclovir phosphates through gap junctions. HeLa cells do not exhibit bystander cytotoxicity, although we showed recently that they transfer low levels of ganciclovir phosphates to bystander cells. Here, we attempted to induce bystander cytotoxicity using hydroxyurea, an inhibitor of ribonucleotide reductase, to decrease the endogenous dGTP pool, which should lessen competition with ganciclovir triphosphate for DNA incorporation. Addition of hydroxyurea to cocultures of HSV-TK-expressing and bystander cells synergistically increased ganciclovir-mediated cytotoxicity to both cell populations while producing primarily an additive effect in cultures of 100% HSV-TK-expressing cells. Whereas HSV-TK-expressing cells in coculture were ~50-fold less sensitive to ganciclovir compared with cultures of 100% HSV-TK-expressing cells, addition of hydroxyurea restored ganciclovir sensitivity. Quantification of deoxynucleoside triphosphate pools showed that hydroxyurea decreased dGTP pools without significantly affecting ganciclovir triphosphate levels. Although hydroxyurea significantly increased the ganciclovir triphosphatedGTP value for 12 to 24 hours in HSV-TK-expressing and bystander cells from coculture (1.4- to 4.9-fold), this value was increased for <12 hours (2.5-fold) in 100% HSV-TK-expressing cells. These data suggest that the prolonged increase in the ganciclovir triphosphatedGTP value in cells in coculture resulted in synergistic cytotoxicity. Compared with enhancement of bystander cytotoxicity through modulation of gap junction intercellular communication, this strategy is superior because it increased cytotoxicity to both HSV-TK-expressing and bystander cells in coculture. This approach may improve clinical efficacy. (Cancer Res 2006; 66(7): 3845-51)

Introduction

Expression of the herpes simplex virus thymidine kinase (HSV-TK) in tumor cells results in multilog cell killing when incubated with the antiviral drug ganciclovir (1–5). Phosphorylation of ganciclovir by HSV-TK and then by endogenous cellular enzymes produces the cytotoxic metabolite, ganciclovir triphosphate, which competes with endogenous dGTP for incorporation into DNA, an event correlated with cytotoxicity (6–11). Although HSV-TK/ganciclovir has produced strong tumor growth inhibition and complete regression in animal tumor models, it has had limited efficacy in patients likely due to the inability to express HSV-TK in a sufficient percentage of cells within a tumor (12). Therefore, this form of enzyme-prodrug gene therapy relies heavily on and benefits from a phenomenon known as the bystander effect or the ability of HSV-TK-expressing cells to cause ganciclovir-mediated cytotoxicity in neighboring cells that do not express the transgene (13–17). Bystander cytotoxicity has been attributed to the transfer of phosphorylated ganciclovir metabolites from HSV-TK-expressing to nonexpressing cells through protein channels known as gap junctions (18–24).

With the importance of gap junction intercellular communication (GJIC) in the efficacy of HSV-TK/ganciclovir therapy (19) combined with the finding that GJIC is usually decreased in malignancy (25), methods for enhancing GJIC in cells with functional channels have been explored. Expression of cDNAs encoding connexins 32 or 43 have successfully increased bystander cell killing with HSV-TK/ganciclovir in vitro in a variety of tumor types (26–28). However, as with HSV-TK, the inability to express connexins in a large percentage of a tumor is likely to limit the effectiveness of this method in vivo. Studies using this approach in animal models have produced augmentation of antitumor activity in some studies (27, 29, 30), whereas others have shown efficacy of this method in vitro but not in vivo (31), possibly reflecting the variable efficiency of connexin gene transfer or expression. Others have used pharmacologic modulation with retinoic acid, cyclic AMP, butyrate, apigenin, or lovastatin to increase connexin expression resulting in greater bystander cytotoxicity in vitro (32, 33) and in vivo (34, 35).

We have described previously a pharmacologic approach to enhancing bystander killing that does not affect GJIC. By decreasing the endogenous dGTP levels with an inhibitor of ribonucleotide reductase, ganciclovir triphosphate incorporation into DNA was increased and resulted in synergistic enhancement of HSV-TK/ganciclovir-mediated bystander cytotoxicity in the U251 glioblastoma and the HT29 and SW620 human colon carcinoma cell lines (36, 37). When this approach was tested in vivo, the combination of ganciclovir and either hydroxyurea or gemcitabine (dFdCyd) resulted in significant tumor growth delay and some complete tumor regressions compared with single-drug treatment (37, 38).

Previous reports have shown that cocultures of HSV-TK-expressing and nonexpressing HeLa cells do not exhibit bystander cytotoxicity (39–41). This has been attributed to the lack of GJIC, as HeLa cells reportedly are devoid of connexin proteins (42). Expression of a cDNA for connexin 26, 32, or 43 enabled GJIC and bystander cytotoxicity with HSV-TK/ganciclovir (19, 39, 40). We have shown that the lack of bystander...
cytotoxicity in HeLa cells was not due to an inability to transfer phosphorylated ganciclovir but resulted from a low level of ganciclovir triphosphate transfer along with a rapid half-life of ganciclovir triphosphate in bystander cells when compared with the HSV-TK-expressing cells (41). The transfer of phosphorylated drug metabolites from HSV-TK-expressing to bystander cells also resulted in an increased survival of HSV-TK-expressing cells from coculture when compared with cultures of 100% HSV-TK-expressing cells. Therefore, simply increasing transfer of phosphorylated ganciclovir through enhancing GJIC would not be advantageous in vivo, as it may induce bystander cytotoxicity while sparing the HSV-TK-expressing cells. We hypothesized that decreasing the endogenous dGTP pools would allow the low levels of ganciclovir triphosphate in bystander cells to compete more effectively for incorporation into DNA, thereby inducing bystander cytotoxicity. In addition, we hypothesized that reduction of dGTP in HSV-TK-expressing cells in coculture may restore cytotoxicity in this population. Here, we have evaluated the ability of hydroxyurea to enhance cytotoxicity to HSV-TK-expressing and bystander HeLa cells in coculture.

Materials and Methods

Cell culture and generation of stable cell lines. The HeLa cell lines were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mmol/L L-glutamine (Fisher Scientific, Pittsburgh, PA) and 10% calf serum (Life Technologies). Exponentially growing cells were maintained in a humidified 37°C incubator with an atmosphere containing 5% CO₂ and 95% air.

Generation of HeLa cell lines that stably express HSV-TK or β-galactosidase (β-gal) was accomplished through transduction with a retrovirus vector containing the corresponding cDNA as described previously (5). Transgene-expressing cells were selected and maintained with 400 µg/mL G418 (Life Technologies).

Cell survival assay. Cytotoxicity in exponentially growing HeLa cells was measured by a colony formation assay in cultures that contained HSV-TK-expressing and/or β-gal cells as described previously (5). Briefly, after treatment with 0.1 to 1000 µmol/L ganciclovir (Cytovene, Syntex, Palo Alto, CA) and/or 0.5 to 2.5 mmol/L hydroxyurea (Sigma, St. Louis, MO) for 24 hours, cells were enumerated and plated at ~100 viable cells per well. Seven to 12 days after plating, cells were stained with 0.2% X-gal (Roche, Indianapolis, IN) and enumerated to determine bystander cell survival. Plates were then stained with 4% crystal violet (Fisher Scientific) and the difference between the total number of colonies and the number of bystander colonies resulted in the survival of the HSV-TK-expressing cells. Cell survival was calculated as a fraction of the plating efficiency for untreated control cells for each cell type. All colony formation assays were done independently at least three times and each condition was plated in triplicate.

Fluorescence-activated cell sorting. Cocultures of HSV-TK-expressing and bystander (β-gal) HeLa cells were separated as described previously (41). Briefly, HeLa bystander cells were stained with 20 µmol/L PKH26 (Sigma; ref. 43) and plated with equal numbers of HSV-TK-expressing HeLa cells to ~50% confluency. Twenty-four hours after plating, cultures were incubated with 100 µmol/L ganciclovir and/or 2.5 mmol/L hydroxyurea. Following drug incubation period, cells were suspended at a concentration of 4.0 × 10⁶/mL and analyzed using fluorescence-activated cell sorting on a flow cytometer, which separated the two populations based on PKH26 fluorescence at 567 nm. Purity of the samples was ~98% as analyzed by flow cytometry and by plating a portion of each sample followed by staining with X-gal.

Analysis of ganciclovir phosphorylated metabolites. Following drug treatment, cells were harvested and nucleotides were extracted with perchloric acid and neutralized with KOH as described previously (5). Ganciclovir triphosphate was separated from endogenous nucleotides and quantitated by strong anion exchange high-performance liquid chromatography (HPLC) as described previously (5). Nucleotides were identified and quantified by comparison of their peak areas with that of known amounts of the appropriate standard at wavelengths 254 and 281 nm.

Results

Effect of hydroxyurea on ganciclovir cytotoxicity in HeLa HSV-TK and bystander cocultures. We have shown previously that incubation of a ribonucleotide reductase inhibitor, hydroxyurea, with ganciclovir produced a synergistic increase in bystander cytotoxicity in cell lines that already exhibited bystander cell killing (36). Although HeLa cells do not exhibit bystander cytotoxicity when incubated with ganciclovir for 24 hours (41), the presence of phosphorylated ganciclovir in bystander cells from coculture with HSV-TK-expressing cells suggested a potential for inducing ganciclovir-mediated cytotoxicity. To that end, cultures of HSV-TK-expressing and/or bystander HeLa cells were incubated with ganciclovir and hydroxyurea alone or in combination for 24 hours and cell survival was measured. In cocultures of HSV-TK-expressing (Fig. 1A) and nonexpressing (Fig. 1B) bystander cells incubated with ganciclovir, addition of hydroxyurea enhanced ganciclovir-mediated cytotoxicity. In HSV-TK-expressing cells from coculture, addition of 0.5 mmol/L (IC₂₀) or 2.5 mmol/L (IC₇₀) hydroxyurea decreased the IC₉₀ (85 and 3.6 µmol/L, respectively) of ganciclovir when compared with cocultures incubated with ganciclovir alone (IC₉₀ 100 µmol/L). In bystander cells, addition of 0.5 or 2.5 mmol/L hydroxyurea decreased the IC₉₀ (220 and 125 µmol/L, respectively) of ganciclovir when compared with cocultures incubated with ganciclovir alone (IC₉₀ 380 µmol/L). Isobologram analysis (Fig. 1C and D) showed that the combination of ganciclovir and hydroxyurea produced synergistic cytotoxicity in each of the populations from coculture. In cultures of 100% HSV-TK-expressing HeLa cells, addition of 2.5 mmol/L hydroxyurea produced a modest increase in ganciclovir-mediated cytotoxicity (IC₉₀ 1.0 µmol/L) compared with cultures incubated with ganciclovir alone (IC₉₀ 1.8 µmol/L; Fig. 1E). Isobologram analysis showed an additive/possibly synergistic effect between ganciclovir and hydroxyurea in cultures of 100% HSV-TK-expressing HeLa cells (Fig. 1F).

Effect of ganciclovir and hydroxyurea on deoxynucleoside triphosphate/ganciclovir triphosphate pools in cultures of HSV-TK-expressing cells. To understand the mechanism for additive cytotoxicity with ganciclovir and hydroxyurea in 100% HSV-TK-expressing cell cultures compared with synergistic cytotoxicity exhibited in cocultures, we measured levels of ganciclovir triphosphate and its endogenous competitor for DNA incorporation, dGTP, under these conditions. Because bystander cells accumulated substantially less ganciclovir triphosphate than 100% HSV-TK-expressing cells, we used 100 µmol/L ganciclovir for these studies to be able to quantitate ganciclovir triphosphate levels in bystander cells. When cultures of 100% HSV-TK-expressing cells were incubated with 100 µmol/L ganciclovir alone or with 2.5 mmol/L hydroxyurea, similar levels of ganciclovir triphosphate accumulated at each time point throughout the 24-hour incubation (Fig. 2). With ganciclovir alone, there was a modest decrease in the dGTP pool (Fig. 3A) and nonexpressing (Fig. 3B) bystander cells incubated with ganciclovir and hydroxyurea alone or in combination for 24 hours and cell survival was measured. In cocultures of HSV-TK-expressing (Fig. 1A) and nonexpressing (Fig. 1B) bystander cells incubated with ganciclovir, addition of hydroxyurea enhanced ganciclovir-mediated cytotoxicity. In HSV-TK-expressing cells from coculture, addition of 0.5 mmol/L (IC₂₀) or 2.5 mmol/L (IC₇₀) hydroxyurea decreased the IC₉₀ (85 and 3.6 µmol/L, respectively) of ganciclovir when compared with cocultures incubated with ganciclovir alone (IC₉₀ 100 µmol/L). In bystander cells, addition of 0.5 or 2.5 mmol/L hydroxyurea decreased the IC₉₀ (220 and 125 µmol/L, respectively) of ganciclovir when compared with cocultures incubated with ganciclovir alone (IC₉₀ 380 µmol/L). Isobologram analysis (Fig. 1C and D) showed that the combination of ganciclovir and hydroxyurea produced synergistic cytotoxicity in each of the populations from coculture. In cultures of 100% HSV-TK-expressing HeLa cells, addition of 2.5 mmol/L hydroxyurea produced a modest increase in ganciclovir-mediated cytotoxicity (IC₉₀ 1.0 µmol/L) compared with cultures incubated with ganciclovir alone (IC₉₀ 1.8 µmol/L; Fig. 1E). Isobologram analysis showed an additive/possibly synergistic effect between ganciclovir and hydroxyurea in cultures of 100% HSV-TK-expressing HeLa cells (Fig. 1F).
Figure 1. Effect of hydroxyurea on ganciclovir cytotoxicity in 50:50 cocultures of HSV-TK-expressing and bystander HeLa cells and cultures of 100% HSV-TK-expressing HeLa cells. Cytotoxicity in 50:50 cocultures of HSV-TK-expressing (A) and bystander (B) cells and cultures of 100% HSV-TK-expressing HeLa cells (E) incubated with ganciclovir (GCV) alone (■) was compared with cultures coincubated with ganciclovir and 0.5 (▲) or 2.5 (◇) mmol/L hydroxyurea (HU). As a control for bystander cytotoxicity, cultures of 100% β-gal-expressing cells (◆) were incubated with ganciclovir. Points, mean of at least three experiments; bars, SE. Data from the clonogenic survival curves were used to generate isobolograms for HSV-TK-expressing cells from coculture (C), bystander cells from coculture (D), and cultures of 100% HSV-TK-expressing HeLa cells (F). The concentration of ganciclovir and hydroxyurea corresponding to 20% (◇), 30% (▲), and 40% (■) surviving fractions was calculated from at least three separate experiments plated in triplicate. Diagonal line, isoeffective line of additivity.
effects on dGTP pools similar to that observed with hydroxyurea alone. Although the increase in dGTP pools in the presence of hydroxyurea may suggest that ribonucleotide reductase was not well inhibited, this does not seem to be the case because dATP was decreased within 4 hours after hydroxyurea addition to <10% of the control value and remained at approximately that level for the duration of the incubation (Fig. 3B). Although incubation with ganciclovir alone resulted in a ~4-fold increase in dATP by 24 hours, in combination with hydroxyurea, the dATP pool remained at <60% of the control value throughout the incubation. Calculation of the ganciclovir triphosphate:dGTP value in HSV-TK-expressing cultures showed that the addition of hydroxyurea increased this ratio 2.5-fold at both 4 and 8 hours when compared with cultures incubated with ganciclovir alone (Table 1). However, by 12 hours, the difference between the ratios was not significant (P > 0.25) and that trend continued through the end of the 24-hour incubation period.

**Figure 3.** Effect of ganciclovir and/or hydroxyurea on dATP and dGTP pools in cultures of 100% HSV-TK-expressing HeLa cells. Cultures of 100% HSV-TK-expressing HeLa cells were incubated with 100 μmol/L ganciclovir alone (■) or with 2.5 mmol/L hydroxyurea (▲). Points, mean of at least three experiments; bars, SE.

Incubation of cocultures with ganciclovir alone or with hydroxyurea decreased dGTP in both HSV-TK-expressing and bystander cells to nondetectable levels by 4 hours after drug addition. The addition of hydroxyurea produced a more prolonged decrease in dGTP as this pool remained at or below control levels for at least 12 hours after drug addition (Table 2). In contrast, dGTP had surpassed control levels by 12 hours after drug addition in both HSV-TK-expressing and bystander cells incubated with ganciclovir alone. It seems that ribonucleotide reductase was inhibited by hydroxyurea in these studies as shown by a sustained decrease in dATP (data not shown) similar to that observed in cultures of 100% HSV-TK-expressing cells (Fig. 3B).

The addition of hydroxyurea to ganciclovir incubation in cocultures increased the ganciclovir triphosphatedGTP value in both HSV-TK-expressing and bystander cells (Table 3). In HSV-TK-expressing cells, hydroxyurea elevated the ganciclovir triphosphatedGTP value by 1.4-fold at 4 hours and 2.7-fold at 12 hours after drug addition compared with the value with ganciclovir alone. However, there was no significant difference in this ratio at the conclusion of drug incubation (P > 0.3). In contrast, the presence of hydroxyurea resulted in a higher ganciclovir triphosphatedGTP value in HSV-TK-expressing cells, hydroxyurea elevated the ganciclovir triphosphatedGTP value by 1.4-fold at 4 hours and 2.7-fold at 12 hours after drug addition compared with the value with ganciclovir alone. It seems that ribonucleotide reductase was inhibited by hydroxyurea in these studies as shown by a sustained decrease in dATP (data not shown) similar to that observed in cultures of 100% HSV-TK-expressing cells (Fig. 3B).

**Effect of ganciclovir and hydroxyurea on deoxynucleoside triphosphate/ganciclovir triphosphate pools in HSV-TK and bystander cells after coculture.** Because the addition of hydroxyurea to ganciclovir incubation produced cytotoxicity in bystander cells, we wished to determine the effect of these drugs on deoxynucleotide triphosphate (dNTP) and ganciclovir triphosphate pools. Before coculture, non-HSV-TK-expressing HeLa cells were labeled with the fluorescent dye PKH26 so that they could be recovered after coculture by fluorescence-activated cell sorting and evaluated for dNTPs and ganciclovir triphosphate. Cocultured HSV-TK-expressing and nonexpressing bystander cells were incubated with the 100 μmol/L ganciclovir alone or with 2.5 mmol/L hydroxyurea. There was no significant difference in accumulation of ganciclovir triphosphate in each of the populations from coculture when incubated for 24 hours with ganciclovir and hydroxyurea when compared with cocultures incubated with ganciclovir alone (Fig. 4; P > 0.17). However, the HSV-TK-expressing cells in coculture accumulated significantly less ganciclovir triphosphate (751 ± 197 pmol/10⁶ cells) compared with the 100% HSV-TK culture (1,824 ± 507 pmol/10⁶ cells; P < 0.05).
triphosphatedGTP ratio in bystander cells at 4 and 12 hours after drug addition (1.8- and 49-fold higher than with ganciclovir alone, respectively) and this increased ratio persisted through the conclusion of drug incubation (2.8-fold higher than ganciclovir alone, respectively) and this increased ratio persisted through the conclusion of drug incubation (2.8-fold higher than ganciclovir alone).

**Discussion**

Bystander cytotoxicity from HSV-TK/ganciclovir suicide gene therapy has been attributed to the transfer of phosphorylated ganciclovir from HSV-TK-expressing to bystander cells via GJIC (20). Ganciclovir triphosphate then competes with endogenous dGTP for incorporation into DNA, an event correlated with cytotoxicity (6–11). Previously, it has been reported that cell lines with high GJIC exhibited bystander cytotoxicity, whereas HeLa cells, which supposedly lack GJIC, exhibited no bystander cytotoxicity (19, 23, 24). However, we have shown recently that HeLa cells accumulated ganciclovir triphosphate from neighboring HSV-TK-expressing cells and that a rapid half-life of ganciclovir triphosphate and low-level transfer to bystander cells accounted for the lack of bystander cytotoxicity (41). In an attempt to induce bystander cytotoxicity in HeLa cells, we decreased the endogenous ganciclovir triphosphate competitor, dGTP, which should allow greater incorporation of ganciclovir triphosphate into DNA resulting in an increase in cytotoxicity (36). The data presented here showed that the addition of hydroxyurea to HeLa HSV-TK-expressing cells cultured with bystander cells significantly enhanced ganciclovir-mediated cytotoxicity. The enhancement of cytotoxicity was not the result of increased levels or increased transfer of phosphorylated ganciclovir metabolites but the result of decreased dGTP pools through inhibition of ribonucleotide reductase.

We have reported previously that the addition of hydroxyurea to ganciclovir incubation in cultures of 100% HSV-TK-expressing SW620 colon carcinoma cells resulted in an additive increase in cytotoxicity (36). In addition, the combination of either hydroxyurea or dFdCyd and ganciclovir in cocultures of HSV-TK-expressing and bystander SW620 cells resulted in additive cytotoxicity in HSV-TK-expressing cells while resulting in a synergistic bystander effect (36, 37). Consistent with our previous reports, the results presented here showed an additive effect with ganciclovir and hydroxyurea in cultures of 100% HSV-TK-expressing HeLa cells and synergistic bystander cytotoxicity in cocultures. However, although we expected only an additive effect in the HSV-TK-expressing HeLa cells from coculture, addition of hydroxyurea produced a strong synergistic increase in ganciclovir-mediated cytotoxicity. This is an important finding because, as we have shown here and previously (41), ganciclovir cytotoxicity is reduced in HSV-TK-expressing HeLa cells in coculture compared with cultures of 100% HSV-TK-expressing cells. With the addition of hydroxyurea, cytotoxicity of ganciclovir in HSV-TK-expressing cells in coculture was similar to that in HSV-TK-expressing cells cultured alone.

Other published methods to enhance bystander cytotoxicity rely primarily on increasing GJIC between tumor cells either through transfer of a connexin cDNA or up-regulation of GJIC (32, 33, 39, 40, 44). Because GJIC-mediated transfer of ganciclovir phosphates from HSV-TK-expressing to bystander cells can reduce cytotoxicity of ganciclovir to HSV-TK-expressing cells, as we and others have shown (24, 41, 45), enhancing GJIC in vivo may not result in a greater antitumor effect (31). Thus, the ability to enhance cytotoxicity to both HSV-TK-expressing and bystander cells through pharmacologic means is an important finding. The importance of this strategy is further illustrated by noting that it was effective not only in HeLa cells but also in several other tumor cell lines, including the HT29 and SW620 colon carcinoma cell lines and the U251 glioblastoma cell line.

**Table 1. Ganciclovir triphosphate:dGTP ratio in cultures of 100% HSV-TK-expressing HeLa cells**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ganciclovir triphosphate:dGTP ratio for ganciclovir alone</th>
<th>Ganciclovir triphosphate:dGTP ratio for ganciclovir and hydroxyurea</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>150 ± 37</td>
<td>375 ± 40</td>
<td>2.5 ± 0.3*</td>
</tr>
<tr>
<td>8</td>
<td>230 ± 110</td>
<td>585 ± 192</td>
<td>2.6 ± 1.1*</td>
</tr>
<tr>
<td>12</td>
<td>209 ± 86</td>
<td>264 ± 52</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>16</td>
<td>299 ± 180</td>
<td>267 ± 84</td>
<td>0.9 ± 1.0</td>
</tr>
<tr>
<td>24</td>
<td>167 ± 44</td>
<td>132 ± 52</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

NOTE: After cultures of HSV-TK-expressing HeLa cells were incubated with 100 μmol/L ganciclovir alone or with 2.5 mmol/L hydroxyurea, cell extracts were analyzed by HPLC for dNTP/ganciclovir triphosphate pools. Data are mean ± SE from at least three experiments.

*p < 0.05, ratio for ganciclovir alone significantly different from ratio for ganciclovir and hydroxyurea.
Furthermore, in the animal models, ganciclovir was combined with nontoxic doses of hydroxyurea or dFdCyd, producing synergistic antitumor activity without increasing host toxicity (37, 38). Taken together, these results encourage translation of this strategy to the clinic.

As expected, hydroxyurea effected a decrease in dGTP pools in HSV-TK-expression and bystander cells. However, the reason for the greater hydroxyurea-mediated decrease in dGTP pools in cells in coculture compared with 100% HSV-TK-expressing cells is not clear. We have reported previously that the half-life of ganciclovir triphosphate was shorter in HSV-TK-expressing and bystander cells in coculture compared with cultures of 100% HSV-TK-expressing cells, suggesting that HSV-TK contributes to the rephosphorylation of ganciclovir phosphates after degradation (41). Similarly, it is possible the half-life for dGTP is shorter in bystander cells compared with HSV-TK-expressing cells, resulting in lower dGTP pools in cocultures compared with cultures of 100% HSV-TK-expressing cells.

The ability of hydroxyurea to decrease dGTP pools resulted in an increase in the ganciclovir triphosphate:dGTP value in each of the cultures incubated with ganciclovir and hydroxyurea compared with cultures incubated with ganciclovir alone. Although hydroxyurea effected a 2.5-fold increase in the ganciclovir triphosphate:dGTP value in cultures of 100% HSV-TK-expressing HeLa cells, this increase lasted for <12 hours resulting in primarily additive cytotoxicity in these cultures. In contrast, in HSV-TK-expressing and bystander cells in coculture, hydroxyurea effected a higher increase in the ganciclovir triphosphate:dGTP value that persisted for 12 to 24 hours. These results suggest that the synergy between hydroxyurea and ganciclovir shown in each of the populations from coculture is the result of a prolonged increase in the ganciclovir triphosphate:dGTP value when compared with the shorter-lived increase in the ratio in cultures of 100% HSV-TK-expressing HeLa cells. In view of these results, it will be important to explore the use of other inhibitors of ribonucleotide reductase that may be able to produce a more prolonged decrease in dGTP.

As we have shown previously in the HT29, SW620, and U251 cell lines, an increased ganciclovir triphosphatedGTP value resulted in higher incorporation of ganciclovir monophosphate into DNA, thus producing greater cytotoxicity (36, 37). In this study, we were unable to evaluate the ganciclovir monophosphate incorporation into DNA in HeLa cells from coculture, as they would not express the single-chain antigen that we have used previously to separate bystander cells (23). Although we could stain the bystander cells and separate them after coculture using fluorescence-activated cell sorting, we could not use radioactivity (necessary to detect ganciclovir monophosphate in DNA) in the fluorescence-activated cell sorting machine. Because our previously published study shows that an increase in the ganciclovir triphosphatedGTP ratio resulted in higher incorporation of ganciclovir monophosphate into DNA (36), we suggest

Table 2. dGTP levels in HeLa cocultures of HSV-TK-expressing and bystander cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drugs incubated</th>
<th>Control*</th>
<th>4 h*</th>
<th>12 h*</th>
<th>24 h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-TK</td>
<td>Ganciclovir</td>
<td>2.5 ± 0.2</td>
<td>ND†</td>
<td>5.1 ± 0.9</td>
<td>6.6 ± 5.9</td>
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<tr>
<td>HSV-TK</td>
<td>Ganciclovir and hydroxyurea</td>
<td>2.5 ± 0.2</td>
<td>ND†</td>
<td>2.3 ± 0.7</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Bystander</td>
<td>Ganciclovir</td>
<td>2.9 ± 1.0</td>
<td>ND†</td>
<td>5.2 ± 3.6</td>
<td>9.9 ± 1.8</td>
</tr>
<tr>
<td>Bystander</td>
<td>Ganciclovir and hydroxyurea</td>
<td>2.9 ± 1.0</td>
<td>ND†</td>
<td>ND†</td>
<td>4.3 ± 1.2</td>
</tr>
</tbody>
</table>

NOTE: Cocultures of HSV-TK-expressing and PKH26-labeled bystander HeLa cells were incubated with 100 μmol/L ganciclovir alone or with 2.5 mmol/L hydroxyurea. After the designated incubation periods, cells were separated based on PKH26 fluorescence by flow cytometry and cell extracts were analyzed by HPLC for dGTP pools. Data are mean ± SD.

*Expressed as pmol dGTP/10⁶ cells.
†Below the limit of detection by HPLC.

Table 3. Ganciclovir triphosphate:dGTP ratio in HeLa cocultures of HSV-TK-expressing and bystander cells

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<th>Fold increase</th>
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<tr>
<td>4</td>
<td>HSV-TK</td>
<td>160 ± 28*</td>
<td>217 ± 65*</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>12</td>
<td>HSV-TK</td>
<td>108 ± 24</td>
<td>288 ± 44</td>
<td>2.7 ± 0.5†</td>
</tr>
<tr>
<td>24</td>
<td>HSV-TK</td>
<td>114 ± 27</td>
<td>108 ± 3</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>Bystander</td>
<td>8 ± 1*</td>
<td>15 ± 0.1*</td>
<td>1.8 ± 0.1†</td>
</tr>
<tr>
<td>12</td>
<td>Bystander</td>
<td>13 ± 5</td>
<td>63 ± 3*</td>
<td>4.9 ± 0.6†</td>
</tr>
<tr>
<td>24</td>
<td>Bystander</td>
<td>25 ± 3</td>
<td>68 ± 8</td>
<td>2.8 ± 0.8†</td>
</tr>
</tbody>
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NOTE: Cocultures of HSV-TK-expressing and PKH26-labeled bystander HeLa cells were incubated with 100 μmol/L ganciclovir alone or with 2.5 mmol/L hydroxyurea. After the designated incubation periods, cells were separated based on PKH26 fluorescence by flow cytometry and cell extracts were analyzed by HPLC for dNTP/ganciclovir triphosphate pools. Data are mean ± SD.

*Calculated with minimal level of dGTP detectable by HPLC (1.2 pmol/10⁶ cells).
†P < 0.01, ratio for ganciclovir alone significantly different from ratio for ganciclovir and hydroxyurea.
that this is the likely mechanism mediating the synergistic cytotoxicity with ganciclovir and hydroxyurea shown here in HeLa cocultures.

Here, we have shown a pharmacologic approach that induces ganciclovir-mediated bystander cytotoxicity in HeLa cells. In comparison with previous reports on induction of bystander cytotoxicity in HeLa cells by increasing connexin expression (19, 39), our approach does not require enhancement of GJIC, which may be difficult to attain in a sufficient number of cells in vivo. Notably, the approach used here enhanced ganciclovir-mediated cytotoxicity in both bystander and HSV-TK-expressing cells. With HeLa cells representing a cell type that is relatively insensitive to HSV-TK/ganciclovir and typically does not display bystander cytotoxicity, the ability of hydroxyurea to induce bystander cytotoxicity suggests that this may be an important approach to increase efficacy of HSV-TK/ganciclovir in patients.

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Hydroxyurea Induces Bystander Cytotoxicity in Cocultures of Herpes Simplex Virus Thymidine Kinase–Expressing and Nonexpressing HeLa Cells Incubated with Ganciclovir

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