Synergistic Anticancer Effects of Ganciclovir/Thymidine Kinase and 5-Fluorocytosine/Cytosine Deaminase Gene Therapies

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Background: A bacterial enzyme, Escherichia coli cytosine deaminase, which converts the prodrug 5-fluorocytosine into the toxic drug 5-fluorouracil, and a viral enzyme, herpes simplex virus thymidine kinase, which converts ganciclovir from an inactive prodrug to a cytotoxic agent by phosphorylation, are being actively investigated for use in gene therapy for cancer. The purpose of this study was to determine whether combining these prodrug-activating gene therapies might result in enhanced anticancer effects. Methods: Rat 9L gliosarcoma cells were transfected with plasmids containing the E. coli cytosine deaminase gene (9L/CD cells), with plasmids containing the herpes simplex virus thymidine kinase gene (9L/TK cells), or with both expression plasmids (9L/CD-TK cells). The drug sensitivities of the cell lines were evaluated; in addition, the sensitivities of 9L and 9L/CD-TK cells mixed in varied proportions were measured. The effects of prodrug treatment on 9L/CD-TK tumor growth (i.e., size and volume) in nude mice were monitored. The isobologram method of Loewe and the multiple drug-effect analysis method of Chou–Talalay were used to measure the interaction between the two prodrug-activating gene therapies. To elucidate the mechanism of interaction, the phosphorylation of ganciclovir in 9L/CD-TK cells after varying prodrug treatments was studied. Results and Conclusions: The presence of transfected cytosine deaminase and thymidine kinase genes in 9L gliosarcoma cells reduced cell survival, both in vitro and in vivo, following treatment with the relevant prodrugs; the effects of the two components appeared to be synergistic and related mechanistically to the enhancement of ganciclovir phosphorylation by thymidine kinase following 5-fluorouracil treatment. [J Natl Cancer Inst 1998;90:370–80]

Gene therapy represents a rapidly evolving approach to cancer treatment. While many types of transgenes have potential tumoricidal effects [reviewed in (1,2)], genes encoding for prodrug-activating enzymes have been the most thoroughly investigated [reviewed in (3)]. After genetically modifying tumor cells to express such enzymes, systemic prodrug treatment leads to the selective killing of tumor cells. Two well-characterized prodrug-activating enzymes are the herpes simplex virus type-1 thymidine kinase (HSV-TK) enzyme (4), which phosphorylates antiviral nucleoside analogues such as ganciclovir, and the Escherichia coli cytosine deaminase (CD) enzyme, which deaminates the prodrug 5-fluorocytosine, forming 5-fluorouracil (5).

The mechanisms of action of the drugs generated by these enzymes are well established. Ganciclovir phosphate is further phosphorylated by cellular kinases, forming ganciclovir triphosphate, which inhibits cellular DNA polymerase and gets incorporated into DNA in place of guanosine-5’-triphosphate. Ganciclovir incorporated into DNA forms a poor substrate for continued chain elongation (6). 5-Fluorouracil is metabolized into 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP), 5-fluoro-2’-deoxyuridine-5’-triphosphate (FdUTP), and 5-fluorouridine-5’-triphosphate (FUTP). FdUMP inhibits the enzyme thymidylate synthetase, which converts deoxyuridylic acid (dUMP or 2’-deoxyuridine-5’-monophosphate) into thymidylate (dTMP or 2’-deoxythymidine-5’-monophosphate). Because thymidylate synthetase is the only source for de novo synthesis of thymidylate, a cell treated with 5-fluorouracil ultimately becomes deficient in deoxythymidine-5’-triphosphate (dTTP), leading to the incorporation of both uridine triphosphate and FdUTP into DNA. Attempts at DNA repair by uracil-N-glycosylases fail because of the lack of dTTP. The nicked DNA strands cannot be replicated, leading to cell death. 5-Fluorouracil also targets RNA via incorporation of FUTP into RNA, leading to inhibition of messenger RNA polyadenylation, transfer RNA methylation, and processing of ribosomal RNA precursors (7).

Short-term therapeutic efficacy of these prodrug-activating gene therapies has been shown in animal models (8,9). However, in this study, tumors expressing HSV-TK or CD displayed recurrent growth within a few weeks, despite uninterrupted prodrug treatment. Recurrent tumor growth can arise either from the development of drug resistance (a challenge also faced in conventional chemotherapy) or from loss of transgene expression (a challenge unique to gene therapy). Strategies aimed at overcoming these limitations must be developed. Limitations in conventional chemotherapy can be partially overcome by combining chemotherapy agents, and it stands to reason that the same benefits could arise from combining prodrug-activating gene therapies. We have thus combined the CD and HSV-TK prodrug-
activating gene therapies in an experimental brain tumor model. The interaction between the two gene therapies was analyzed quantitatively through the use of two methods—the Loewe isobologram method (10) and the multiple drug-effect analysis of Chou–Talalay (11,12).

Materials and Methods

Expression Plasmids

The plasmid pTLKRNL-1 contains the HSV-TK gene under the control of the Moloney murine leukemia virus long terminal repeat (LTR) promoter and the neomycin phosphotransferase gene under the control of the Rous sarcoma virus promoter (8). The plasmid pCD2 (obtained from R. Michael Blaese, National Cancer Institute, Bethesda, MD) contains the CD gene under the control of the Moloney murine sarcoma virus LTR promoter and the neomycin phosphotransferase gene under the control of the simian virus 40 early promoter (5). The plasmid pBabe-Puro (13) contains the gene for puromycin resistance under the control of the simian virus 40 early promoter.

Cell Lines

All cell lines were grown in a 5% CO2-95% air atmosphere in Dulbecco’s modified Eagle medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies, Inc.), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Chemical Co., St. Louis, MO). The 9L rat gliosarcoma cell line has been described previously (14). The 9L/TK cells were cloned with 9L cells at varying percentages such that the total number of cells was 2×10^5 per 10-cm-diameter plate. Cells were plated in medium containing varying concentrations of the prodrug(s) for 5 days, after which they were removed by brief trypsin treatment and counted with a Coulter apparatus.

For cell survival studies, 9L/CD-TK cells were plated in triplicate at 2×10^5 cells per 10-cm-diameter plate. Cells were plated in medium containing varying concentrations of ganciclovir (Cytovene; Hoffmann-La Roche, Inc., Nutley, NJ), 5-fluorocytosine (Sigma Chemical Co.), or both. Cells were grown in the presence of the prodrug(s) for 5 days, after which they were removed by brief trypsin treatment and counted with a Coulter apparatus. For the determination of bystander effects, 9L/CD-TK cells were plated with 9L cells at varying percentages such that the total number of cells was 2×10^5 per 10-cm-diameter plate. Cells were plated in triplicate in medium containing prodrug concentrations that had previously been shown to be completely cytotoxic to 9L/CD-TK cells but nontoxic to 9L cells. Five days later, cells were removed by trypsin treatment and counted with a Coulter apparatus.

Mitotic Arrest of Cells

To mitotically arrest 9L/CD-TK cells, we plated the cells in triplicate with 1.5×10^5 cells per 10-cm-diameter dish. The following day, the medium was replaced with medium containing 10 µg/mL aphidicolin (Sigma Chemical Co.). Mitotic arrest of the cells was confirmed by the measurement of tritiated thymidine incorporation. Aphidicolin-treated cells were incubated with 2 µCi/mL [3H]thymidine (23.2 Ci/mmol; Du Pont NEN, Boston, MA) for 30 minutes, then washed twice with phosphate-buffered saline before being lysed in 1 mL of lysis buffer (100 mM Tris–HCl [pH 8], 50 mM EDTA, and 0.5% sodium dodecyl sulfate). One milliliter of cold 10% trichloroacetic acid was added to each cell lysate. The lysates were kept on ice for 20 minutes, and then precipitates were collected by filtration on Whatman G/F filters. The filters were then put in scintillation vials. We first added 50 µL 1 N NaOH to neutralize acid and then added 200 µL Tris–HCl (pH 7). Scintillation fluid (ScintiVerse II; Fisher Scientific Co., Pittsburgh, PA) was added to each vial, and radioactivity incorporation was assayed by scintillation counting. We verified that, within 1 hour of exposure to aphidicolin, 9L/CD-TK cells did not incorporate [3H]thymidine more than background. Therefore, after 1 hour of exposure of the cells to aphidicolin, the medium was replaced with medium containing aphidicolin and varying concentrations of ganciclovir or 5-fluorocytosine. Mitotically arrested cells were exposed to prodrug for 72 hours before they were removed by trypsin treatment and counted with a Coulter apparatus.

Pharmacologic Analyses of Synergy

Two methods, the Loewe isobologram method (10) and the multiple drug-effect analysis of Chou–Talalay (11,12), were used to quantify the interaction between the two gene therapies because any enhanced effects (i.e., greater than additive effects) that are indicated by both types of analysis may represent biochemical synergy. Each method starts by defining the additive effect, the combined cytotoxicity expected if the two treatments do not alter each other’s individual tumoricidal effects. Each method then determines how much the experimental data differ from what would be expected with additivity, providing a quantitative assessment of whether the interaction is additive, synergistic, or antagonistic. The Loewe method determines what combinations produce a given effect and is usually limited to the case where the effects of two drugs are mutually exclusive (i.e., the drugs possess similar modes of action). The Chou–Talalay method is a more generalized method that determines the expected effect of a given combination if the agents are additive and quantifies synergy or antagonism by determining how much the experimental effect differs from the effect expected with additivity. The Chou–Talalay analysis applies to mutually exclusive and nonexclusive (i.e., drugs with distinct modes of action) interactions and takes into account the shape of each dose–effect curve.

For the Chou–Talalay analysis, the data from Figs. 2, A, and 4, A, and Table 1 were employed for calculations with the use of a computer program (16). The stepwise calculations of this program are described below:

**Step 1.** To relate dose and effect for a single drug, the following median-effect equation (17) was used:

\[ f_{50} = \frac{D}{D_{m}} \]

where \( D \) is the dose; \( f_{50} \) and \( D_{m} \) are the fractions of cells affected and unaffected, respectively, by dose \( D \); \( D_{m} \) is the dose required to produce the median effect, i.e., the dose at which 50% of the cells are affected; and \( m \) is a coefficient signifying the shape of the dose–effect curve (i.e., \( m = 1 \) for hyperbolic/first-order/ Michaelis–Menten-type curves, \( m > 1 \) for sigmoidal curves, and \( m < 1 \) for negatively sigmoidal curves). The median-effect plots for single-drug treatments are generated through the following logarithmic form of the median-effect equation:

\[ \log \left( \frac{f_{50}}{1-f_{50}} \right) = m \log D - m \log D_{m} \]

The median-effect plot is \( y = \log \left( \frac{f_{50}}{1-f_{50}} \right) \) versus \( x = \log D \). The values of \( m \) and \( D_{m} \) are obtained from the slope and the ordinate of the x intercept, respectively. The conformity of the data to the median-effect principle can be verified by calculation of the linear correlation coefficient \( r \) of the median-effect plot.

**Step 2.** Drugs 1 and 2 are then combined at varying doses; each combination has a ratio of the concentration of drug 1 to the concentration of drug 2 approximately equal to the \( D_{m} \) of drug 1 divided by the \( D_{m} \) of drug 2. For experimental reasons, this ratio often can be approximated without affecting the analysis because the degree of synergism is affected more by the mechanisms of the drugs or the drug schedule than by the precise combination ratio. Median-effect plots are then generated for drug 1, drug 2, and their combination in order to obtain the parameters \( m \) and \( D_{m} \) for each treatment. These values are then substituted into the following rearrangement of equation 1:

\[ D_{1} = D_{m} \times \left[ \frac{D_{2}}{D_{2}-D_{m}} \right]^{m} \]

In other words, equation 3 is used to calculate the dose \( D_{1} \) of each treatment regimen (drug 1, drug 2, or drug 1 + drug 2) required to achieve \( f_{e} \) values ranging from 0.05 to 0.95 (5% of system affected up to 95% of system affected).

**Step 3.** For mutually exclusive drugs, the combination index (CI) at a particular \( f_{e} \) (11) is as follows:

\[ CI = \left( \frac{D_{1}}{D_{1}(f_{e})} \right) \left( \frac{D_{2}(f_{e})}{D_{2}} \right) \left( \frac{D_{1}(f_{e})}{D_{1}} \right) \left( \frac{D_{2}}{D_{2}(f_{e})} \right) \]

where \( D_{1} \) and \( D_{2} \) are the doses of drug 1 and drug 2, respectively, that must be used together to achieve effect \( f_{e} \) (\( D_{1} \)) is the dose of drug 1 that must be used
alone to achieve effect fa; and (Dx)3 is the dose of drug 2 that must be used alone to achieve effect fa. For mutually exclusive drugs, the third term in equation 4 should be eliminated (12). In all cases, a CI–1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism. The data are presented as an fCI plot with CI on the y axis and fa values between 0 and 1 on the x axis.

The Chou–Talalay analysis is based on the mass-action law, which encompasses systems that adhere to Michaelis–Menten first-order kinetics (hyperbolic curves) as well as systems with sigmoidal and negatively sigmoidal curves. Chou–Talalay analysis cannot be applied to systems that do not adhere to the mass-action law, such as biphasic or multiphasic dose-effect curves and mutually depleting, titrating, or precipitating systems (i.e., antigen–antibody complexes). But such systems will be revealed by their poor linear correlation coefficient (r value) in the median-effect plot. In these experiments, the r values were all close to 1, suggesting adherence to the mass-action law.

The isobologram method of Loewe (10), an additional means of analyzing drug interactions, was also employed. In an isobologram, the x and y axes represent doses of drugs 1 and 2. A curve is then drawn for any fa value of interest representing doses of drug 1 + drug 2 that would be required to achieve the given fa value if the two drugs were additive. In the original Loewe isobologram method, this curve was a straight line. Chou–Talalay showed that the Loewe analysis was limited to mutually exclusive drugs and was defined by the mutually exclusive version of equation 4 with CI = 1. Chou–Talalay showed that the mutually nonexclusive version of equation 4 with CI = 1 could be used to generate a concave curve (11) representing the additive effect on an isobologram used to analyze mutually nonexclusive drugs. The final step in generating any isobologram is to plot the observed experimental concentrations at which combined treatment generated the given fa value—if these points lie to the lower left of the curve/straight line defining additivity at that fa value, synergism is indicated; if the experimental points lie on the curve/straight line, additivity is indicated; if they lie to the upper right of the curve/straight line, antagonism is indicated.

An initial experiment with 9L/CD-TK cells treated with 5-fluorocytosine or ganciclovir alone was performed to determine the range and ratio of prodrug concentrations to employ in combined treatment. From this initial experiment (see Fig. 1), Da values of 0.90 μg/mL 5-fluorocytosine and 0.03 μg/mL ganciclovir were estimated. Therefore, for combination treatment, prodrug doses were selected such that the ratio of the concentration (micrograms per milliliter) of 5-fluorocytosine to ganciclovir was 25:1, approximating the ratio of the median-effect doses. The 9L/CD-TK cells were treated with varying doses of 5-fluorocytosine, ganciclovir, and 5-fluorocytosine + ganciclovir (at a 25:1 ratio) by use of triplicate dishes. This experiment was then independently repeated two more times. Results from these three experiments were then averaged (see Fig. 2, A, and Table 1; Fig. 2, A, and Table 1 reveal slightly different Da values than Fig. 1, but the variability can be attributed to the different concentrations and wider range of concentrations employed in the Fig. 1 experiment compared with the Fig. 2 experiment).

For the bystander experiments, initial experiments (data not shown) provided Da values of 34.06 μg/mL 5-fluorocytosine and 6.15 μg/mL ganciclovir. Based on these Da values, combined treatment was performed by use of a ratio of 5-fluorocytosine to ganciclovir of 10:1. This ratio was selected for three reasons: 1) It approximated the median-effect dose ratio of 5.5:1; 2) 5-fluorocytosine’s enhancement of ganciclovir phosphorylation (discussed later in the experiments depicted in Fig. 7) provided an intuitive rationale for adding more 5-fluorocytosine in the mixture; and 3) bystander effect experiments had to be performed in the range of prodrug concentrations that were toxic to 9L/CD-TK cells but nontoxic to 9L cells (1–9 μg/mL ganciclovir and 10–90 μg/mL 5-fluorocytosine), and a 10:1 ratio allowed us to utilize both prodrugs at all concentrations in these ranges, whereas a 5.5:1 ratio would have precluded the use of 5-fluorocytosine doses in the upper half of the usable range. Combined treatment at a 10:1 ratio was performed alongside single-prodrug treatment (see Fig. 4 and Table 1 for results). The single-prodrug median-effect doses obtained during this second experiment (60.77 μg/mL 5-fluorocytosine; 16.19 μg/mL ganciclovir) differed slightly from the single-prodrug median-effect doses obtained during the first experiment (34.06 μg/mL 5-fluorocytosine; 6.15 μg/mL ganciclovir), but the difference is within the range of interexperimental error and also illustrates the importance of simultaneously performing single and combined treatments, even if an initial experiment with single treatment was already performed.

For both the cell culture and bystander experiments described above and for the in vivo experiments described below, the interaction between the two treatments was analyzed first by use of equations appropriate for the assumption of mutual exclusivity and then by use of those appropriate for the assumption of mutual nonexclusivity. For purposes of space, the latter analyses are not shown.

In Vivo Experiments

The 9L/CD-TK cells (106 in 200 μL phosphate-buffered saline supplemented with 1 mM MgCl2, 1 mM CaCl2, and 1% glucose) were injected subcutaneously into the flanks of 6-week-old female nude mice (NCr/Sed, nu/nu, 20 g; Massachusetts General Hospital breeding colony). After 7 days, when the tumors had reached 10 mm in longest dimension, the mice were randomly divided into experimental groups of five mice per group. Intraperitoneal injections of 2-mL solutions of prodruk(s) dissolved in 0.9% NaCl were administered daily. Tumor size was measured by use of calipers. Tumor volume was calculated as length × width × height, as described previously (18).

For analysis of possible enhanced (i.e., synergistic) effects of combined-prodrug treatment in the animal model, the mean tumor volumes after 2 weeks of daily treatment were calculated. The average fold growth (in volume) of tumors from prodruk-treated mice (FGP) was divided by the average fold growth of saline-treated tumors (FGS) to generate the growth ratio FGP/FGS. A ratio of 5-fluorocytosine-to-ganciclovir dose of 100:1 (below the median-effect ratio of 500:1) was selected, because 5-fluorocytosine was toxic to animals at doses
equal to 1000 mg/kg body weight per day (data not shown) and thus the higher ratio would have impaired the ability to escalate the dose level. Both Chou–Talalay and Loewe isobologram analyses were performed.

**Ganciclovir Phosphorylation Activity**

Phosphorylation of ganciclovir by 9L/CD-TK cells was measured as described previously (19). First, $8 \times 10^5$ 9L/CD-TK cells were plated in triplicate, by use of 6-cm-diameter dishes containing normal medium or medium containing 60 μg/mL 5-fluorocytosine. Three days later, the cells were removed by brief trypsin treatment, suspended in 25 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 2 μg/mL aprotinin (Sigma Chemical Co.), and then sonicated for 15 seconds at half-maximal power (550 Sonic Dismembrator; Fisher Scientific Co.). Sonicated cells were centrifuged at 10,000g for 2 minutes at 4°C. Protein concentration was calculated by use of the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). The reaction mixture consisted of 13 mM adenosine triphosphate, 50 mM Tris–HCl (pH 7.5), 1 μCi $^3$H]ganciclovir (1 mCi/mL; Moravek Biochemicals, Inc., Brea, CA), 10 mM MgCl₂, and 40 μL cell extract in a total volume of 100 μL. Varying concentrations of thymidine (Sigma Chemical Co.) were added to some reaction mixtures. The reaction was carried out at 37°C for 5 hours. The reaction was terminated by the addition of 20 μL of the reaction mixture to 4 μL cold 50% trichloroacetic acid. Then 20 μL of the terminated reaction was spotted onto Whatman DE-81 paper disks. The disks were washed twice with 1.5 mM ammonium formate, once with distilled water, and once with 100% ethanol. Disks were dried under a vacuum, and incorporated radioactivity was measured with a scintillation counter.

**Results**

**Single-Prodrug Treatment in Culture**

To functionally characterize the activity of the CD and HSV-TK enzymes of the clonal cell lines 9L/CD, 9L/TK, and 9L/CD-TK and of the parental 9L cell line, we evaluated the sensitivity of these lines to ganciclovir and 5-fluorocytosine in parallel. The TK and of the parental 9L cell line, we evaluated the sensitivity to ganciclovir and 5-fluorocytosine was as amplified in doubly transfected 9L/CD-TK cells as it was in singly transfected 9L/TK or 9L/CD cells and thus that the activity of the CD drug-activating enzyme did not appear to substantially alter the activity of the other.

**Combined Treatment in Culture and Analysis of Prodrug Interaction**

The 9L/CD-TK cells were then treated with each prodrug alone and with prodrug combinations such that the ratio of the concentrations (micrograms per milliliter) of 5-fluorocytosine to ganciclovir was 25:1, approximating their median-effect dose ratio (Fig. 2, A). A cursory inspection of Fig. 2, A, suggests that combined treatment produced an effect greater than one would expect if the prodrugs interacted in an additive fashion, since the $D_m$ for 5-fluorocytosine alone was approximately 1.64 μg/mL, that for ganciclovir alone was 0.049 μg/mL, and that for the combination was 0.27 μg/mL. To determine if the enhanced cytotoxicity observed in combined treatment reflected synergism, two methods were employed. First, by use of the Chou–Talalay analysis (11,12), the $CI$ values were computed. The $f_C$–$CI$ plot (Fig. 2, B) indicated that the $CI$ was well below 1 over the entire range of $f_C$ values, suggesting strong synergism at all effect levels. The $f_C$–$CI$ plot was also relatively flat, indicating that the extent of synergism was dose independent. Second, the Loewe isobologram method (10) confirmed synergism at $f_C$ values of 0.5, 0.7, and 0.9 (Fig. 2, C), as well as at other $f_C$ values not shown.

The advantage of combined treatment can also be appreciated by the calculation of the dose-reduction index of each drug. This index represents the fold reduction in dose allowed by combining the drug with another treatment. In other words, the dose-reduction index is the concentration of a drug required to achieve a given level of cell death divided by the concentration of the drug required when combined treatment is used to achieve the same level of cell death. The index is calculated by the rearrangement of the median-effect equation (11). The dose-reduction index for 5-fluorocytosine decreased as cell death increased, from 10 to 3.6 (mean values determined from three

![Fig. 1](https://academic.oup.com/jnci/article-figures/90/5/370/979119)
independent experiments) as cell death increased from 5% to 95%. The dose-reduction index for ganciclovir increased slightly with cell death, from 3.7 to 5.3 (mean values determined as above) as cell death increased from 5% to 95%. Synergy can also be verified by examination of the improvement in cytotoxicity achieved over the additive effect. For example, if these prodrug-activating systems were additive, the median-effect dose for combined treatment would be 0.70 mg/mL 5-fluorocytosine + 0.028 mg/mL ganciclovir. But, in reality, this dose produced 30% cell survival, a substantial improvement over the 50% survival expected with additivity.

As a control, combined treatment of 9L cells was performed by use of extremely high prodrug doses, which are toxic to cells regardless of the level of prodrug-activating enzyme expression. 5-Fluorocytosine and ganciclovir proved to be slightly antagonistic (CI values of 1.2) in their high-dose cytotoxicity to cells lacking activating enzymes (median-effect doses in Table 1; fa–CI plot not shown).

Taken together, these studies demonstrate that strong synergy (as defined by both the Loewe isobologram method and by the Chou–Talalay analysis) occurred when tumor cells expressing CD and HSV-TK were treated in cell culture with combinations of the prodrugs 5-fluorocytosine and ganciclovir.

**Bystander Effect Synergy**

Both the CD and HSV-TK prodrug-activating systems exhibit a bystander effect. The bystander effect describes the ability of tumor cells expressing a prodrug-activating enzyme to eradicate neighboring nontransduced tumor cells following prodrug treatment (20,21). In culture, the bystander effects of these prodrugs probably result from transfer of the active metabolites of the prodrugs from enzyme-expressing cells to nontransduced tumor cells, either by facilitated diffusion in the case of 5-fluorouracil (20) or through gap junctions or apoptotic vesicles in the case of ganciclovir phosphate (21). The interaction between the bystander effects of these prodrug-activating systems was evaluated in culture for synergism.

Initially, cocultures of 9L and 9L/CD-TK cells were exposed to the highest concentration of either prodrug that is nontoxic to 9L cells (9 μg/mL ganciclovir or 90 μg/mL 5-fluorocytosine).
At low cell densities, the bystander effect of 5-fluorocytosine was stronger than that of ganciclovir, presumably because the bystander effect of ganciclovir requires more cell-to-cell contact than that of 5-fluorocytosine. However, even in a coculture containing only 2% 9L/CD-TK cells, ganciclovir killed 53% of the coculture and 5-fluorocytosine killed 70% of the coculture (Fig. 3). Therefore, cocultures containing 2% 9L/CD-TK cells were used to generate single-prodrug dose–response curves for the bystander effect. The median-effect doses for single-prodrug bystander killing of 9L cells were 60.77 μg/mL 5-fluorocytosine and 16.19 μg/mL ganciclovir (Table 1).

Cocultures containing 2% 9L/CD-TK cells were then treated with prodrug combinations whose ratio of concentrations (micrograms per milliliter) of 5-fluorocytosine to ganciclovir was 10:1; this ratio was chosen for reasons stated in the ‘‘Materials and Methods’’ section. All single and combined doses were nontoxic to 9L cells plated alone (data not shown). The improvement in the bystander effect achieved by combined treatment can be estimated by a comparison of the bystander effect versus the prodrug dose curves for 2% 9L/CD-TK cells in ganciclovir, 5-fluorocytosine, or ganciclovir + 5-fluorocytosine (Fig. 4, A). The median-effect dose for the combined bystander effect was 12.49 μg/mL 5-fluorocytosine + 1.25 μg/mL ganciclovir (Table 1), significantly less than the 44 μg/mL 5-fluorocytosine + 4.4 μg/mL ganciclovir expected with additivity. The improvement in the bystander effect achieved by combined treatment can also be appreciated by a comparison of the minimum percentage of 9L/CD-TK cells in coculture required for complete coculture death (<1% survival). While complete coculture death after treatment with 90 μg/mL 5-fluorocytosine required 25% 9L/CD-TK cells in the coculture and complete coculture death was impossible with 9 μg/mL ganciclovir, complete coculture death was achievable with only 5% 9L/CD-TK cells when these two doses were combined (data not shown).

Again, to provide more quantitative analysis, the Chou–Talalay method was used. The fEC50–CI plot (Fig. 4, B) indicated that CI was well below 1 over the entire range of fEC50 values, suggesting strong synergism at all effect levels. In addition, the Loewe isobologram method was employed to confirm the synergism between the bystander effects of these gene therapies (Fig. 4, C).

These experiments thus indicated that synergistic interactions between the 5-fluorocytosine/CD and the ganciclovir/HSV-TK gene therapies occurred even with mixed, heterogeneous cell populations in which only 2% of cells expressed both transgenes.

Synergism In Vivo

The in vivo response to prodrug therapy was evaluated in subcutaneous tumors generated by injection of 9L/CD-TK cells into the flanks of athymic mice. To evaluate the therapeutic efficacy achievable with combined-prodrug therapy, we examined the effect on tumor growth of daily intraperitoneal treatment with 500 mg 5-fluorocytosine/kg body weight per day and/or 5 mg ganciclovir/kg body weight per day (close to the highest tolerable dose of each). Treatment was administered for 4 weeks, at which time saline-treated mice had to be killed because of excessive tumor growth. Fig. 5, A, reveals that single-prodrug treatment produced transient inhibition of tumor growth for approximately 18 days. However, despite uninterrupted prodrug treatment, the growth of these tumors then resumed. On the other hand, combined-prodrug treatment completely inhibited tumor growth for the entire 4 weeks.

Next, multiple, single-prodrug doses were administered daily for 2 weeks, so that a more quantitative analysis of the in vivo interactions between the two treatments could be performed. After 2 weeks of daily intraperitoneal treatment, the D50 values (the doses required to achieve a 50% growth ratio, with the growth ratio measured as described in the ‘‘Materials and Methods’’ section) for single-prodrug treatment of 9L/CD-TK tumors in vivo were 31.13 mg 5-fluorocytosine/kg body weight per day and 0.060 mg ganciclovir/kg body weight per day (Table 1).

Combined, daily treatment in vivo was then performed for 2 weeks with various prodrug combinations, each having a dose ratio of 5-fluorocytosine to ganciclovir of 100:1 for reasons discussed in the ‘‘Materials and Methods’’ section. The D50 for combined treatment at this ratio was 0.79 mg 5-fluorocytosine + 0.0079 mg ganciclovir/kg body weight per day (Table 1), substantially less than the 5.03 mg 5-fluorocytosine + 0.050 mg ganciclovir/kg body weight per day expected with additivity. Chou–Talalay analysis produced CI values below 0.45 (fEC50–CI plot not shown), suggesting synergism at all effect levels studied. Once again, the Loewe isobologram confirmed synergism (Fig. 5, B).

Role of Inhibited RNA Processing

5-Fluorouracil inhibits both DNA synthesis and RNA processing, while ganciclovir triphosphate inhibits only DNA synthesis (4,5). Since inhibition of sequential metabolic steps can lead to synergism (22), the hypothesis that the synergy stemmed from the ability of 5-fluorouracil to inhibit RNA processing complementing the ability of ganciclovir triphosphate to inhibit DNA synthesis was investigated. The relative importance of the effects of 5-fluorouracil on RNA and DNA varies in different cell lines (23). Thus, the first step in investigating this proposed...
Fig. 4. Synergistic interaction between the HSV-TK and CD bystander effects in culture as shown by A) 5-day survival of 2% 9L/CD-TK cells + 98% 9L cells in varying concentrations of 5-fluorocytosine (———), ganciclovir (——□——), or 5-fluorocytosine plus ganciclovir (——■——) with doses being in units of 0.5 μg/mL ganciclovir and/or 5 μg/mL 5-fluorocytosine. B) \( f_a - CI \) plot (mutually exclusive assumption) from the same experiment—the \( f_a - CI \) curve (solid line) is derived as described in the ‘Materials and Methods’ section; five actual experimental points are plotted alongside the \( f_a - CI \) curve. \( CI \) values are below 1 for all \( f_a \) values (as indicated by the solid \( f_a - CI \) curve falling below the dotted line at \( CI = 1 \)), indicating synergism at all effect levels. C) Loewe isobologram from the same experiment. Lines representing \( f_a \) values of 0.5 (open circle), 0.6 (open square), and 0.7 (open triangle) are shown. In all three cases, the points representing combined treatments generating these \( f_a \) values occurred at dose combinations to the lower left of the corresponding line, indicating synergism.

Fig. 5. Effect of combined-prodrug treatment on subcutaneous tumor growth in vivo. A) Fold growth of subcutaneous 9L/CD-TK tumors as a function of number of days treated with saline (———), 500 mg 5-fluorocytosine/kg body weight per day (——□——), 5 mg ganciclovir/kg body weight per day (——■——), or 500 mg 5-fluorocytosine + 5 mg ganciclovir/kg body weight per day (——□——). B) Loewe isobologram generated by 2 weeks of daily treatment with varying doses (data pooled from two separate experiments). Lines shown represent \( f_a \) values of 0.5 (open circle), 0.75 (open square), and 0.8 (open triangle). In all three cases, the points representing combined treatments generating these \( f_a \) values occurred at dose combinations to the lower left of the corresponding line, indicating synergism.
mechanism for the synergy was to determine the role of 5-fluourouracil’s inhibition of RNA processing in the cytotoxicity of 5-fluorocytosine to 9L/CD-TK cells. This determination was done by mitotically arresting 9L/CD-TK cells with the DNA polymerase inhibitor aphidicolin, which inhibits DNA synthesis but not RNA synthesis (24). Mitotically arrested 9L/CD-TK cells were then treated with 5-fluorocytosine or ganciclovir. If the effect of 5-fluorouracil on RNA contributed to the cytotoxicity of 5-fluorocytosine to 9L/CD-TK cells, we would expect arrested 9L/CD-TK cells to be substantially more sensitive to 5-fluorocytosine than to ganciclovir.

While arrested 9L/CD-TK cells displayed somewhat more prodrug sensitivity than arrested or dividing 9L cells, their sensitivity was substantially less than that of dividing 9L/CD-TK cells (Fig. 6). Thus, the cytotoxicity of both prodrugs to 9L/CD-TK cells required DNA synthesis. Most importantly, the slight 5-fluorocytosine and ganciclovir sensitivities of the mitotically arrested cells were comparable, suggesting that the unique ability of 5-fluorouracil to target RNA does not contribute substantially to the cytotoxicity of 5-fluorocytosine to 9L/CD-TK cells. This renders the targeting of RNA by 5-fluorouracil less likely to play a role in the synergism observed between the two prodrug-activating systems.

Ganciclovir Phosphorylation Activity After 5-Fluorocytosine Administration

Another potential mechanism for the observed synergy is that 5-fluorouracil may enhance phosphorylation of ganciclovir. This hypothesis was investigated by a comparison of the ganciclovir phosphorylation activities of 9L, 9L/CD, 9L/TK, and 9L/CD-TK cells grown with or without 60 mg/mL 5-fluorocytosine. After the cells grew for 3 days, the ganciclovir phosphorylation activities of the cell extracts were measured. These activities fell into three categories (Fig. 7). 1) The highest activity level was present in 9L/CD-TK cells that had been treated with 5-fluorocytosine. 2) The group with intermediate ganciclovir phosphorylation activity consisted of untreated 9L/CD-TK cells and 9L/TK cells grown with or without 5-fluorocytosine. The ganciclovir phosphorylation activity of this group was fourfold lower than that of 9L/CD-TK cells treated with 5-fluorocytosine. 3) The group of cells with the lowest ganciclovir phosphorylation activity consisted of 9L cells grown with or without 5-fluorocytosine and 9L/CD cells grown with or without 5-fluorocytosine. The ganciclovir phosphorylation activity of this group was fourfold lower than cells in the group with intermediate ganciclovir phosphorylation activity. Thus, HSV-TK expression raises ganciclovir phosphorylation activity fourfold. Growing cells expressing HSV-TK in the presence of high 5-fluorouracil concentrations raises ganciclovir phosphorylation activity an additional fourfold. The increased ganciclovir phosphorylation activity seen in 9L/CD-TK cells treated with 5-fluorocytosine could not be due to unactivated 5-fluorocytosine in the medium because 5-fluorocytosine-treated 9L/TK cells did...
not display increased ganciclovir phosphorylation activity. The increased ganciclovir phosphorylation activity seen in 9L/CD-TK cells treated with 5-fluorocytosine was partially eliminated by the addition of 0.25 μM thymidine to the cell extract and was almost completely eliminated by the addition of 1 μM thymidine (data not shown). These results suggest that the observed synergy stems from the ability of CD-generated 5-fluorouracil to increase phosphorylation of ganciclovir by HSV-TK.

Discussion

This study demonstrates that simultaneous prodrug activation by HSV-TK and CD in a homogeneous population in culture, in a heterogeneous cell population in culture, and in tumors established in vivo yields an anticancer effect that is enhanced compared with the effect observed with single-prodrug treatment and that at first glance appears greater than the effect expected by simple summation of the two prodrugs' individual effects. To obtain a more quantitative analysis, two widely published methods were then employed to determine if the two prodrug-activating systems interacted to generate an enhanced effect (i.e., in a synergistic manner): the Loewe isobologram method (10) and the CI method of Chou–Talalay (11,12). Various analyses have been described to study drug interactions; each has its advantages and disadvantages. No method can take into account every conceivable factor (e.g., drug absorption, intracellular steric constraints, and metabolic activation/inactivation) that may affect drug interactions. However, quantitative analyses and combined dose–effect models remain useful because they permit comparisons of treatment regimens and provide the impetus to search for mechanisms of interaction between treatments. The methods selected for this study have been used extensively to identify synergistic interactions in combination regimens of traditional anticancer chemotherapy (12,25–27). The identification of synergy made by these two methods was then confirmed mechanistically when the ganciclovir phosphorylation activity was enhanced in 9L/CD-TK cells after 5-fluorocytosine treatment.

Synergy between prodrug-activating systems could result from interactions between the prodrugs themselves, one prodrug being activated more in the presence of the other, or an interaction between the active metabolites of the prodrugs. A synergistic interaction between ganciclovir and 5-fluorocytosine themselves is unlikely because combined treatment of 9L cells with very high prodrug doses produced slight antagonism, suggesting that the prodrugs synergize only in the presence of their activating enzymes. The possibility of synergism between the active metabolites could be investigated by the treatment of nontransduced tumor cells with combinations of 5-fluorouracil and ganciclovir phosphate. However, such treatment is not feasible because of the instability of ganciclovir phosphate. One mechanism for synergism between the active metabolites involves the targeting of RNA processing by 5-fluorouracil complementing the targeting of DNA synthesis by ganciclovir phosphate. We refute this mechanism by demonstrating that the RNA-directed effects of 5-fluorouracil contribute minimally to the cytotoxicity of 5-fluorocytosine to 9L/CD-TK cells. The possibility that the synergy results from increased activation of one of the prodrugs is strongly supported by our observation that treatment of 9L/CD-TK cells with 5-fluorocytosine enhances their ganciclovir phosphorylation activity.

The above observation is consistent with previous studies of antiviral nucleoside analogues. Methotrexate and 5-fluoro-2'-deoxyuridine (antineoplastic agents that are also antitherpetic) potentiate the antitherpetic effect of acyclovir, a guanosine analogue similar to ganciclovir (28). One effect that both of these agents have is to reduce intracellular dTTP. It has also been shown that acyclovir has a stronger antiviral effect in tissues with lower levels of intracellular thymidine (29). The importance of thymidine in nucleoside analogue antiviral therapy probably results from thymidine’s ability to outcompete nucleoside analogues for phosphorylation by HSV-TK. The Michaelis constant (Km) of thymidine for HSV-TK is 0.2 μM (30), considerably less than intracellular thymidine concentration, which ranges from 1 to 20 μM (29). Ganciclovir is a guanosine analogue and, although it binds to the same site on HSV-TK as thymidine, its Km is considerably higher, i.e., 100 μM, typical of guanosine (30). Antiviral ganciclovir treatment generates peak plasma concentrations of 45 μM (6); intracellular ganciclovir concentration is presumably below this value. Because the intracellular concentration of thymidine exceeds its Km for HSV-TK, whereas that of ganciclovir is below its Km for HSV-TK, thymidine normally outcompetes ganciclovir for binding to the active site of HSV-TK. In fact, phosphorylation of ganciclovir by HSV-TK is the rate-limiting step in ganciclovir-mediated cytotoxicity (31).

Because 5-fluorouracil inhibits de novo synthesis of dTMP by thymidylate synthetase, treating cells with 5-fluorouracil ultimately reduces intracellular dTTP by 90% (32). Reduced dTTP relieves feedback inhibition of mammalian thymidine kinase (33). Thymidine kinase mediates salvage synthesis of dTMP from thymidine, and increased thymidine kinase activity reduces intracellular thymidine concentration, which cannot be restored by the low thymidine levels in circulation (0.1–1 μM). Therefore, treating 9L/CD-TK cells with 5-fluorocytosine could reduce the intracellular thymidine concentration enough to substantially lower the fraction of HSV-TK active sites occupied by thymidine. This effect could liberate enough HSV-TK active sites for ganciclovir to bind, thus favoring the rate-limiting reaction in ganciclovir treatment, i.e., prodrug phosphorylation by HSV-TK. This interpretation is consistent with our observation that adding 1 μM thymidine to extracts of 9L/CD-TK cells that had been treated with 5-fluorocytosine eliminated the increased ganciclovir phosphorylation activity seen in these cells. An alternative interpretation of the importance of dTTP in ganciclovir therapy is that reduced dTTP concentrations may relieve feedback inhibition of HSV-TK. However, this hypothesis is unlikely because, unlike mammalian thymidine kinase, HSV-TK is extremely resistant to feedback inhibition by dTTP (34).

A mutant HSV-TK with a fivefold lower Km for ganciclovir and a twofold higher Km for thymidine (35) has been engineered, making ganciclovir phosphorylation more favorable. However, the mutant’s Km for thymidine is still below the intracellular thymidine concentration. Since 5-fluorouracil may lower intracellular thymidine concentration to below the Km of HSV-TK for thymidine, combined gene delivery of wild-type CD and HSV-TK followed by combined-prodrug treatment may enhance ganciclovir phosphorylation more than delivery of mutant HSV-
TK followed by ganciclovir treatment. In fact, combined gene delivery of CD and mutant HSV-TK would probably produce an even greater tumoricidal effect and, mechanistically, could ensure that phosphorylation of ganciclovir by HSV-TK is no longer the rate-limiting step in ganciclovir-mediated cytotoxicity.

The mechanism suggested for the synergy observed in the treatment of a homogeneous 9L/CD-TK population is consistent with strong bystander synergism. In other words, had the mechanism been reversed (i.e., ganciclovir phosphate enhancing 5-fluorocytosine activation), the enhanced 5-fluorocytosine activation in the bystander effect could occur only when ganciclovir phosphate diffused into CD-expressing cells, which would have been unlikely because charged ganciclovir phosphate diffuses across membranes less readily than 5-fluorouracil. Thus, other mechanisms explaining the synergy observed in the prodrugs' cytotoxicity to homogeneous enzyme-expressing cells are less consistent with the strong bystander effect synergism observed in this study. In addition, the observed bystander effect synergism is consistent with the augmented combined bystander effects observed between other pairs of prodrug-activating systems in which one bystander effect relies on gap junctions and the other relies on transfer of a metabolite through the medium (36,37).

The synergy between the CD and HSV-TK bystander effects is important for in vivo gene therapy. The bystander effect is important because, during gene delivery using viral vectors in vivo, the gene transfer efficiency is low enough that most cells do not end up expressing the transgene. During phase I clinical trials of gene therapy in cancer patients, less than 1% of cells in the tumor incorporated the transgene (38,39). The bystander effect thus seems responsible for any observed anticancer effect. The benefit of combined gene therapy utilizing dual prodrug-activating systems will depend heavily on the individual bystander effects as well as on the extent of synergy between the bystander effects. Importantly, we observed that, while complete coculture death after 5-fluorocytosine treatment required 25% enzyme-expressing cells and was impossible with ganciclovir treatment, complete coculture elimination required only 5% enzyme-expressing cells with combined treatment. It has been suggested that the in vivo bystander effect may depend on an immune response against the prodrug-activating enzyme leading to a diffuse immune response against the entire tumor regardless of transgene expression (21). Even if this mechanism is more important in the bystander effect than transfer of the prodrugs' active metabolites, combining two foreign antigens like viral HSV-TK and bacterial CD could generate an augmented, synergistic antitumor immune response against nontransduced tumor cells.

We observed comparable synergism when 5-fluorocytosine was used with the nucleoside analogues acyclovir and 1-beta-D-arabinofuranosylthymine (data not shown), which confirms that the synergism between the HSV-TK and CD prodrug-activating systems depends on the mechanism of nucleoside analogue activation or cytotoxicity and does not rely on any structural property specific to ganciclovir. The synergism between acyclovir and 5-fluorocytosine is important because these agents can be given orally, while ganciclovir must be given intravenously. Although acyclovir has been less effective than ganciclovir in HSV-TK gene therapy, its synergism with 5-fluorocytosine may make it effective enough to allow the clinician to take advantage of combined oral administration of acyclovir and 5-fluorocytosine, should combined HSV-TK and CD gene therapy be attempted clinically.

Besides possible synergism, another potential advantage of combined gene therapy is that, as with combined conventional chemotherapy, combined gene therapy may diminish the impact of acquired drug resistance. 5-Fluorouracil resistance can result from altered binding affinity of thymidylate synthetase forFdUMP and from amplification of the thymidylate synthetase gene (7). Ganciclovir resistance can arise from altered binding affinity of DNA polymerase for ganciclovir triphosphate (6). The impact of acquired prodrug resistance might be diminished through combination gene therapy followed by combined-prodrug administration because the acquisition of simultaneous resistance to the active metabolites of both prodrugs would be less likely than the acquisition of resistance to the active metabolite of a single prodrug. Similarly, loss of expression of two transgenes encoding for prodrug-activating enzymes may be less likely than loss of expression of a single transgene, particularly if the transgenes were delivered by separate vectors.

A recent study (40) showing enhanced cytotoxicity during combined treatment of cells expressing a CD/HSV-TK fusion gene reaches a conclusion that is consistent with this study, but there are important differences between the approaches. Our study not only shows synergy in cell culture on a homogeneous population, but also shows synergy in the bystander effect and in vivo. Another unique aspect of our study is the use of the Chou–Talalay and the Loewe isobologram methods, two methods that offer quantitative analyses of drug interactions by first defining the additive effect. Our study also offers additional insight by demonstrating that the synergy probably arises because CD-generated 5-fluorouracil enhances phosphorylation of ganciclovir by HSV-TK. Furthermore, expression of a fusion protein would be driven by a single promoter, and loss of expression from this single promoter would simultaneously destroy the therapeutic efficacy of both prodrug-activating systems. However, with two genes driven by separate promoters either in the same or in different vectors, the impact of loss of transgene expression becomes diminished, as mentioned above.

In conclusion, we have demonstrated synergism between the CD and HSV-TK prodrug-activating gene therapies. This finding should provide a basic framework for investigating the combined delivery into tumors of the genes encoding for these two prodrug-activating enzymes by use of a variety of viral and nonviral vectors.

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Notes
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