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Multi-Log Cytotoxicity of Carbocyclic 2′-Deoxyguanosine in HSV-TK-Expressing Human Tumor Cells

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ABSTRACT

Ganciclovir (GCV) is widely used as a prodrug for selective activation in tumor cells expressing herpes simplex virus thymidine kinase (HSV-TK) because of its ability to induce multi-log cytotoxicity to HSV-TK-expressing as well as nonexpressing bystander cells. We now report that another substrate for HSV-TK, D-carbocyclic 2′-deoxyguanosine (CdG), induces multi-log cytotoxicity in HSV-TK-expressing and bystander cells at concentrations ≤3 μM. We have compared the cytotoxicity and cell cycle effects of CdG to that observed with GCV in two human tumor cell lines. The results demonstrated that cytotoxicity of CdG was similar to that of GCV in both U251 glioblastoma and SW620 colon carcinoma cells that stably expressed HSV-TK. In addition, CdG induced a potent bystander effect in both cell types in co-cultures consisting of HSV-TK-expressing and nonexpressing bystander (lacZ-expressing) cells at ratios of 50:50 or 10:90. Selectivity for HSV-TK-expressing compared to lacZ-expressing cells was similar for CdG and GCV in the U251 cells, however CdG was less selective than GCV in the SW620 cell lines. Despite their ability to induce multi-log cytotoxicity at similar concentrations, CdG and GCV exhibited differential effects on cell cycle progression. Cells incubated with 1 μM CdG for 24 hr accumulated in S-phase and G_2/M after drug washout, and the majority of cells died prior to cell division. This contrasts with the delayed effects of 1 μM GCV that were not evident until after cell division when cells attempted S-phase for the second time. Thus, CdG is a potent cytotoxic agent that merits further investigation to determine whether it will be therapeutically effective in enzyme-prodrug therapy with HSV-TK.

OVERVIEW SUMMARY

We have evaluated D-carbocyclic 2′-deoxyguanosine (CdG) as a prodrug for activation by the herpes simplex virus thymidine kinase (HSV-TK) stably expressed in human tumor cells. Similar to previous findings with ganciclovir (GCV), CdG elicited multi-log cell killing at micromolar concentrations in two different cell lines. CdG also displayed potent bystander cytotoxicity even when HSV-TK was expressed in only 10% of the cells in the culture. HSV-TK-expressing cells incubated with CdG accumulated in G_2/M, in contrast to the delayed S-phase arrest observed previously with GCV. Thus, CdG is a potent cytotoxic agent for enzyme-prodrug cancer therapy with HSV-TK that merits further investigation.

INTRODUCTION

TRANSFER OF A FOREIGN ENZYME into tumor cells to activate a nontoxic prodrug has been proposed as a more selective approach to cancer chemotherapy (Moolten, 1994). One of the more promising of these gene therapy approaches uses transfer of the herpes simplex virus thymidine kinase (HSV-TK) cDNA followed by treatment with the antiviral nucleoside analog ganciclovir (GCV). The HSV-TK phosphorylates GCV to its monophosphate, and subsequently cellular kinases phosphorylate it further to the di- and triphosphate derivatives (Keller *et al.*, 1981; Cheng *et al.*, 1983; Field *et al.*, 1983; Boehme, 1984; Biron *et al.*, 1985). GCV triphosphate then competes with dGTP for incorporation into DNA to elicit cytotoxicity (Cheng *et al.*, 1983; St.Clair *et al.*, 1987; Reid *et al.*, 1988; Reardon, 1989;

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Ilsley *et al.*, 1995). Because GCV is not a good substrate for any of the endogenous mammalian kinases, this approach should be more selective than traditional nucleoside analogs which can be phosphorylated in many normal host tissues. The combination of HSV-TK/GCV has produced complete tumor regressions in animal models (Moolten, 1986; Culver *et al.*, 1992). In addition, even when only 10% of the tumor expressed the activating HSV-TK, complete tumor regressions have been obtained (Culver *et al.*, 1992; Ram *et al.*, 1993; Link *et al.*, 1997). The ability to kill neighboring non-HSV-TK-expressing cells, termed the bystander effect (Freeman *et al.*, 1996; Pope *et al.*, 1997), is important because current methods for gene transfer will result in transgene expression in only a small percentage of the tumor cells (Roth and Cristiano, 1997).

Despite the excellent antitumor activity of GCV in preclinical models, HSV-TK/GCV has not shown efficacy in human trials (Shand *et al.*, 1999; Rainov, 2000; Trask *et al.*, 2000). This has been attributed in part to the low delivery of the HSV-TK cDNA to tumors using currently available methodologies. In the absence of improved delivery techniques, investigators have attempted to improve HSV-TK enzyme-prodrug therapy by enhancing the affinity of HSV-TK for GCV (Black *et al.*, 1996; Kokoris *et al.*, 1999; Qiao *et al.*, 2000), improving bystander cell killing (Park *et al.*, 1997; Touraine *et al.*, 1998; Carystinos *et al.*, 1999; Boucher *et al.*, 2000; Robe *et al.*, 2000), combining it with other modalities (Castleden *et al.*, 1997; Aghi *et al.*, 1998; Freytag *et al.*, 1998; Wildner *et al.*, 1999), or identifying better substrates for HSV-TK (Degreve *et al.*, 1999). We and others have reported previously that GCV was significantly more cytotoxic to cells expressing HSV-TK than other substrates (Rubsam *et al.*, 1998; Degreve *et al.*, 1999). Our studies have demonstrated that GCV produced multi-log cell killing at submicromolar concentrations (Shewach *et al.*, 1994; Boucher *et al.*, 1998; Rubsam *et al.*, 1998). GCV triphosphate was shown to be highly potent, in which an intracellular concentration of 5–10 μM was sufficient to kill >3 logs of cells (Rubsam *et al.*, 1998; Boucher *et al.*, 1998). Other substrates could achieve higher triphosphate concentrations but produced <1.5 logs of cell death. Mechanistically, GCV differed from classical nucleoside analogs in that cells divided once after incubation with GCV, then arrested permanently during the S-phase following cell division (Rubsam *et al.*, 1998). The high potency of GCV triphosphate appears to explain the ability of GCV to kill non-HSV-TK-expressing bystander cells adjacent to HSV-TK-expressing cells. Transfer of phosphorylated GCV from HSV-TK-expressing to nonexpressing bystander cells resulted in low but highly cytotoxic levels of GCV triphosphate (Bi *et al.*, 1993; Ishii-Morita *et al.*, 1997; Boucher *et al.*, 1998; Rubsam *et al.*, 1999).

On the basis of the unique toxicity of GCV, we have searched for other similarly effective analogs. We have noted that the experimental antiviral drug D-carbocyclic 2′-deoxyguanosine (CdG) was also capable of inducing multi-log cell killing at low concentrations. Similar to GCV, CdG is a guanine nucleoside analog with therapeutic activity in herpes virus infections due to its ability to be phosphorylated by the herpes-encoded thymidine kinase and subsequently become incorporated into DNA (Bennett *et al.*, 1990, 1993; Parker *et al.*, 1992). We hypothesized that the unique multi-log cell killing observed with these drugs may share a common pathway. Thus, we undertook an evaluation of the cytotoxicity and cell cycle effects for CdG. We also assessed the ability of CdG to induce killing of non-HSV-TK-expressing bystander cells, because this is an important requirement for drugs in a gene transfer enzyme-prodrug approach to cancer chemotherapy. A preliminary account of a portion of these results has been reported (Murphy *et al.*, 1999).

MATERIALS AND METHODS

Cell culture

U251 human glioblastoma cells were cultured in RPMI-1640 medium supplemented with 2 m*M* L-glutamine and 10% bovine calf serum (Life Technologies Inc., Grand Island, NY). SW620 human colon carcinoma cells were cultured in McCoy's 5a medium supplemented with 2 m*M* glutamine and 10% fetal calf serum (Life Technologies Inc., Grand Island, NY). Cell cultures were maintained in exponential growth at 37°C in a humidified atmosphere containing 5% CO_2. Each cell line had previously been stably transduced with a retroviral vector containing the cDNA for herpes simplex virus type 1 thymidine kinase or β-galactosidase (β-Gal) and monoclonal sublines were developed for each transgene (Boucher *et al.*, 1998; Rubsam *et al.*, 1998).

Cell survival assays

Exponentially growing cells were incubated with drug for 4–24 hr and cell survival was measured by a colony formation assay as previously described (Boucher *et al.*, 1998; Rubsam *et al.*, 1998). Briefly, cultures of 100% HSV-TK-expressing or 100% β-Gal-expressing cells were harvested at the conclusion of the drug incubation period and diluted to plate approximately 10–100 viable cells in a 35-mm-diameter well in 6-well culture plates. Three to six wells were plated with the same number of cells to estimate viability at each drug concentration. After 10–14 days, the resulting colonies were fixed and stained with 0.4% crystal violet, and colonies of at least 50 cells each were enumerated. For coculture experiments with HSV-TK- and β-Gal-expressing cells, cells were plated as described, and β-Gal-expressing (bystander) colonies were detected by staining with 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Boehringer Mannheim, Indianapolis, IN). After enumeration of the β-Gal-positive colonies, the culture dish was stained with crystal violet and all colonies enumerated. The difference between the number of crystal violet-stained colonies and β-Gal-expressing colonies yielded the number of HSV-TK-expressing colonies. Cell survival was expressed as a fraction of plating efficiency for control (no drug treatment) cells. All colony formation assays were performed at least twice.

Cell cycle analysis

Flow cytometric analysis was performed essentially as described (Ostruszka and Shewach, 2000). Control or drug-treated cells were incubated with bromodeoxyuridine for 15 min, harvested, washed and fixed with 70% ethanol. Within 6 hr prior to flow cytometric analysis, fixed cells were washed, and incubated with RNase A for 30 min at 37°C. DNA was denatured by treatment with 0.1 *N* HCl containing 0.7% Triton X-100, then resuspended in dH_2O and incubated at 95°C for 15 min.

Cells were washed and resuspended in phosphate-buffered saline (PBS) with 0.5% Tween 20 and 5% calf serum, followed by the addition of anti-bromodeoxyuridine mouse immunoglobulin G_1 (IgG_1) antibody (PharMingen, San Diego, CA). After centrifugation, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma Chemical Co., St. Louis, MO) was added for detection of the primary antibody, and propidium iodide was added to label total DNA. Trout erythrocyte nuclei (Biosure, Grass Valley, CA) were added as an internal standard. Cells were analyzed using a Coulter EPICS Elite ESP flow cytometer, and the cell cycle distribution was determined using WinMDI software provided by Joseph Trotter of The Scripps Research Institute. For measurement of apoptosis, cells were fixed and treated with propidium iodide and RNase A only, and analyzed by flow cytometry for cells with sub-G_1 content.

RESULTS

Cytotoxicity of CdG in HSV-TK- or LacZ-expressing U251 cells

U251 cells were incubated with a broad range of CdG concentrations for 24 hr and survival was determined using a colony formation assay. As illustrated in Fig. 1A, U251 cells expressing HSV-TK (U251tk) exhibited similar sensitivity to CdG and GCV, with the IC_{50} value for each drug approximately 0.06 μM. In addition, CdG and GCV produced multi-log cell killing in the U251tk cells, with approximately 3 logs of cell killing at 1 μM. Less toxicity was evident in U251 cells expressing β-Gal (U251lacZ) , with IC_{50} values of 40 and 60 μM for CdG and GCV, respectively, and at 1000 μM both drugs induced approximately 1–1.5 log decreases in cell survival (Fig. 1B). Shorter incubation periods with CdG also induced multi-log cytotoxicity with U251tk cells, with 10 μM CdG producing 2 to 3 logs of cell death following incubation periods of 4–12 hr (Fig. 2). At a concentration of 1 μM, the 4-hr CdG incubation reduced cell survival by 70%, and a 24-hr exposure decreased survival to $<0.5\%$. The corresponding L-isomer of CdG did not show significant cytotoxicity in these cell lines, with $>95\%$ cell survival following a 24 hr incubation with 100 μM L-CdG in U251tk cells (data not shown).

Cytotoxicity of CdG in U251 Co-cultures of HSV-TK-expressing and nonexpressing bystander cells

HSV-TK-expressing U251 cells were co-cultured with U251lacZ bystander cells to evaluate the ability of CdG to induce bystander cell killing. When 50% of the cells in the co-culture expressed HSV-TK, the effect of CdG in the HSV-TK-expressing cells was similar to that in the 100% HSV-TK-expressing culture (Fig. 3; $IC_{50} = 0.04\ \mu M$). Although CdG was somewhat less toxic to the bystander cells, killing was still impressive with an IC_{50} value of 0.2 μM. When only 10% of the co-culture expressed HSV-TK, the sensitivity to CdG was reduced approximately 10-fold in both the HSV-TK-expressing and bystander cell populations, with IC_{50} values of 0.4 and 3 μM, respectively. Notably, CdG was able to induce ≥ 1 log reduction in cell survival in HSV-TK-expressing and bystander cells when present in co-culture at a ratio of 50:50 ($IC_{90} = 0.25$ and 1.0 μM, respectively) or 10:90 ($IC_{90} = 5$ and 7 μM, respectively).

Cell cycle progression

With the similarity in cytotoxicity for CdG and GCV, we wished to determine whether CdG killed cells by the novel mechanism proposed for GCV. Previously, we observed that cells incubated with GCV were slowed in their ability to progress through the cell cycle, but they doubled following a 24-hr GCV incubation and then arrested in the subsequent S phase (Rubsam *et al.*, 1998). We performed dual-parameter (bromodeoxyuridine incorporation and propidium iodide staining) flow cytometry on U251tk glioblastoma cells following incubation with 1 μM CdG for 24 hr. As indicated in Table 1, after a 24-hr incubation with CdG the percentage of cells in G_1 decreased by almost four-fold, with a nearly two-fold increase in the percentage of cells in S and G_2/M. By 48 hr after drug washout, the S-phase percentage decreased with a concomitant increase in G_1, but the G_2/M population remained high. There was a slight increase in cell number at 24 hr after drug washout; however, the cell number decreased over the next 48 hr. These data indicate that at best only a small portion ($<27\%$) of the cell population divided. Some of the cells appeared to die in S-phase as evidenced by the two- to four-fold increase in the percentage of cells in S-phase that did not incorporate bromodeoxyuridine (S_{NI}). CdG was able to induce apoptosis; however, this was a delayed effect that appeared at least 48 hr after drug washout. Thus, most cells exposed to CdG died without going through cell division.

Cytotoxicity in SW620 colon carcinoma cells

We wished to determine whether effects of CdG in the SW620 human colon carcinoma cells were similar to those that we observed previously with GCV. SW620 cells that stably expressed either HSV-TK (SW620tk) or β-Gal (SW620lacZ) were used for these studies. As shown in Table 2, CdG was less potent than GCV, but CdG was able to induce ≥ 1 log cytotoxicity at micromolar concentrations. CdG showed similar potency to GCV in killing bystander cells co-cultured with 10% or 50% HSV-TK-expressing SW620 cells. Furthermore, the bystander killing ability of CdG could be augmented synergistically by the addition of hydroxyurea at concentrations ranging from 0.5 to 4 mM (data not shown), as we have demonstrated previously for GCV (Boucher *et al.*, 2000).

The major distinction between CdG and GCV in these studies was in the cytotoxicity to 100% cultures of lacZ-expressing SW620 bystander cells. The SW620lacZ cells were approximately five-fold more sensitive to CdG than GCV based on relative IC_{50} concentrations. In addition, the relative IC_{50} values for CdG in 100% cultures of SW620lacZ and SW620tk cells differed by 100-fold, whereas there was a >4500-fold difference in the corresponding values for GCV. Thus, CdG was not as selective as GCV for HSV-TK-expressing SW620 cells; however, selectivity was similar between CdG and GCV in the U251 cell lines.

DISCUSSION

For enzyme-prodrug gene therapy for cancer to be effective clinically, the prodrug must be able to induce the killing of not only the enzyme-expressing cells, but also the neighboring by-

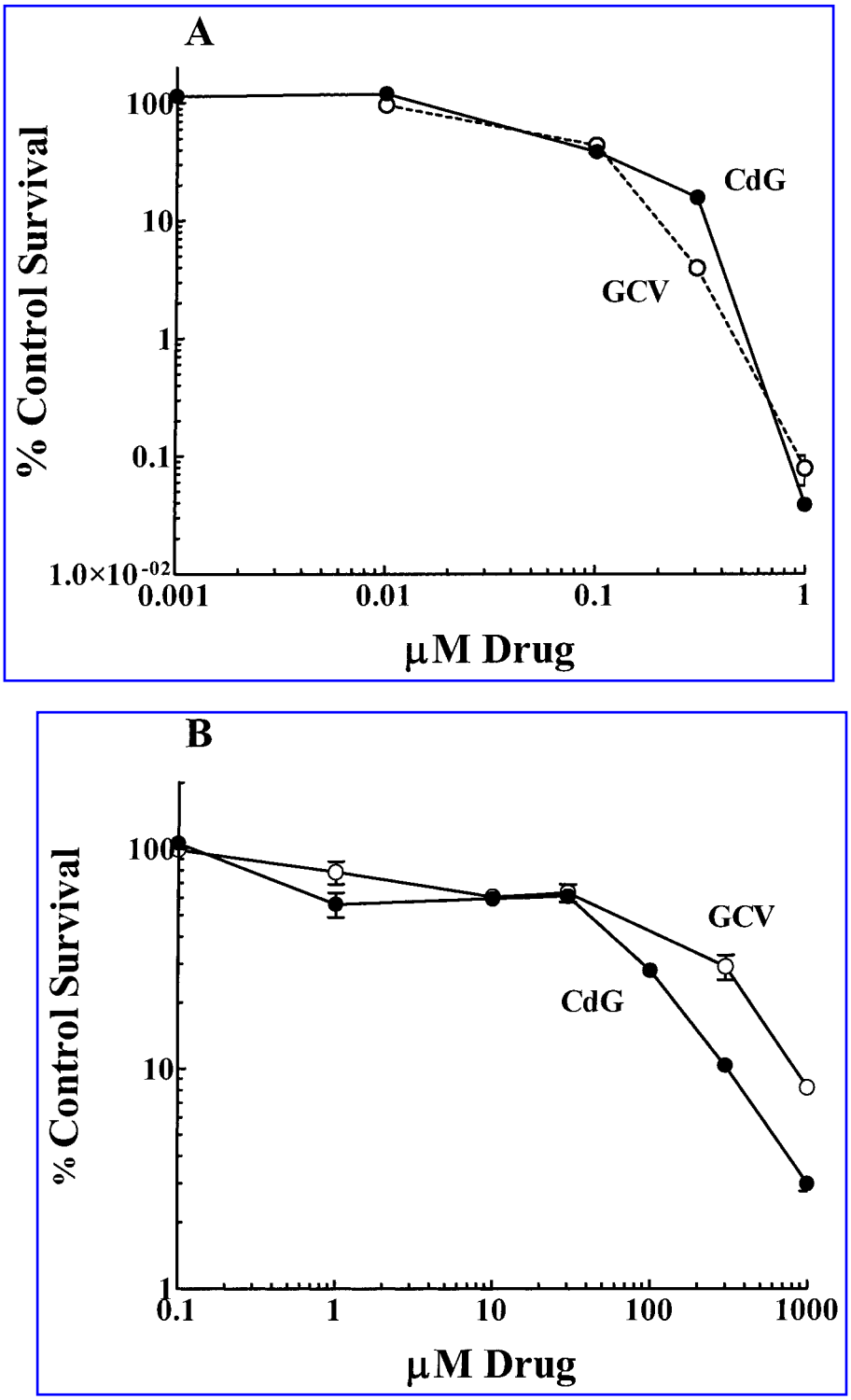

FIG. 1. Comparison of the cytotoxicity of CdG and GCV in U251 cells. U251 cells that express either HSV-TK (U251tk, *panel* **A**) or β-Gal (U251lacZ, **B**) were incubated with the indicated concentrations of CdG or GCV for 24 hr. Survival was determined at the conclusion of the drug incubation period using a colony formation assay. Each experiment was performed at least three times, and a representative experiment is shown. Values represent average ± SE of triplicate determinations.

stander (non-transgene-expressing) cells because gene transfer *in vivo* is estimated to result in gene expression in fewer than 10% of cells within a tumor (Roth and Cristiano, 1997). Previously, we and others have demonstrated that substrates for HSV-TK differed significantly in their potency of cytotoxicity to HSV-TK-expressing cells, and that GCV exhibits strong bystander killing ability (Balzarini *et al.*, 1993; Rubsam *et al.*, 1998). Here we report on another antiherpesvirus drug, D-CdG,

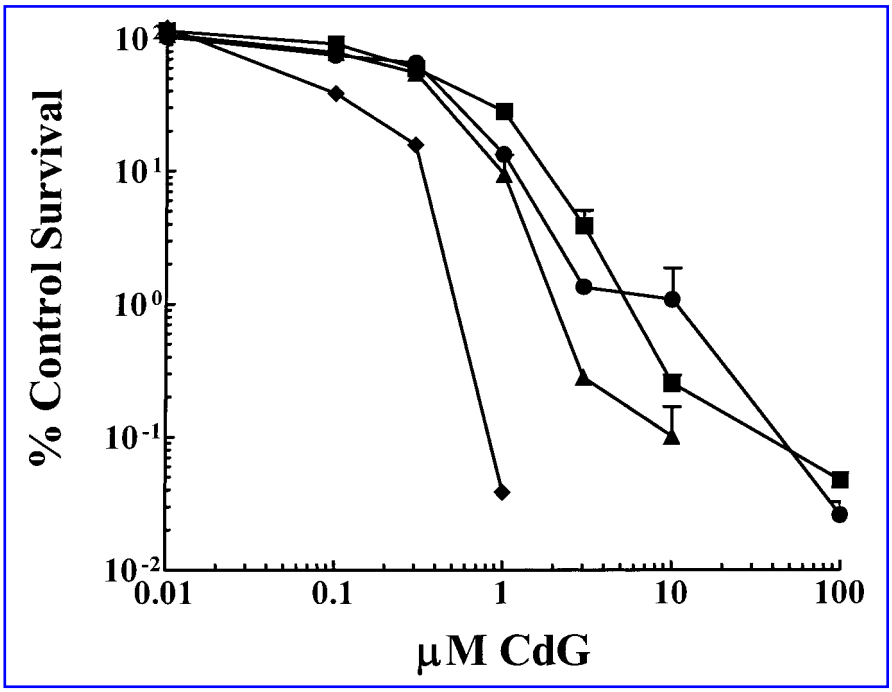

FIG. 2. Effect of length of incubation on cytotoxicity of CdG. U251tk cells were incubated with CdG for 4 (■), 8 (●), 12 (▲) or 24 (◆) hr. Cell survival was determined by a colony formation assay. Values represent the average ± SE of triplicate determinations.

with similar potency to the highly cytotoxic GCV in HSV-TK-expressing cells, the ability to induce multi-log cell killing, and excellent bystander cytotoxicity. Although non-HSV-TK-expressing SW620 cells appeared more sensitive to CdG than to GCV, the strong cytotoxicity of CdG in HSV-TK-expressing and bystander cells in two different cell lines suggests that this drug may have efficacy *in vivo*.

In the U251 cells, cytotoxicity with CdG was similar to that

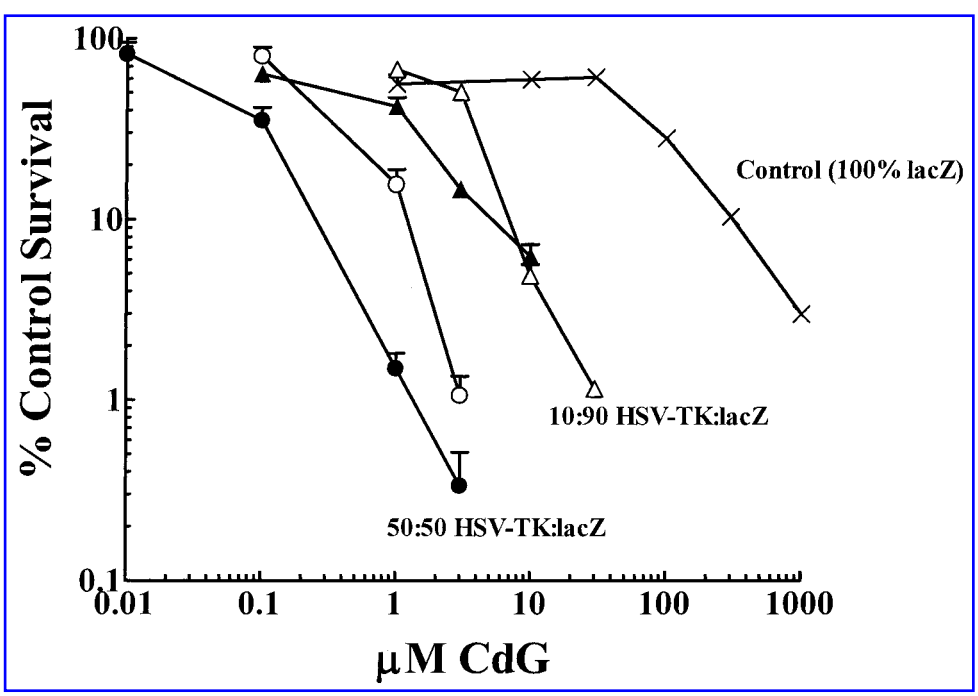

FIG. 3. Cytotoxicity of CdG to HSV-TK-expressing or bystander cells. U251tk cells (filled symbols) and U251lacZ (bystander) cells (open symbols) were co-cultured at ratios of 50:50 (circles) or 10:90 (triangles) with CdG at the indicated concentrations. Control cells (100% U251lacZ culture) (×) is shown for comparison. This is a representative experiment performed at least three times. Each value represents the average ± SE of triplicate determinations.

TABLE 1. CELL CYCLE DISTRIBUTION OF U251tk CELLS AFTER CdG INCUBATION

Treatment	Time after drug washout (hr)	Percentage of cells in: G_1	S	G_2/M	S_{NI}[a]	Apoptosis	Cell number
Control cells	0	58.2	26.6	9.2	3.3	2.8	1.45×10^6
1 μM CdG	0	15.0	46.5	16.4	5.7	5.7	0.94×10^6
	24	25.7	40.4	20.1	7.8	6.0	1.19×10^6
	48	29.8	20.0	20.8	13.4	16.0	1.02×10^6
	72	29.5	26.0	22.2	9.8	12.7	0.70×10^6

U251tk cells were incubated with 1 μM CdG for 24 hr. Drug-containing medium was replaced with fresh medium (drug washout) and cells were analyzed periodically for cell cycle distribution using dual-parameter (bromodeoxyuridine and propidium iodide) flow cytometry as described in Materials and Methods. Results are from a single representative experiment repeated twice.

[a]S_{NI}, Cells with S-phase DNA content by propidium iodide staining with no significant incorporation of bromodeoxyuridine.

observed with GCV in the HSV-TK-expressing as well as non-HSV-TK-expressing (lacZ) bystander cells, cultured alone or together. However, cell cycle progression differed between these two drugs. Whereas cells incubated with 1 μM GCV were able to complete one cell division 24–48 hr after drug washout (Rubsam *et al.*, 1998), following treatment with 1 μM CdG the majority of cells did not divide but instead showed an accumulation in S-phase and G_2/M with a decrease in cell number. Although we did not observe a large increase in G_2/M with GCV in our cell lines, a report on B16 murine melanoma cells stably expressing HSV-TK indicated that a high percentage of cells accumulated in G_2/M after a 48–72-hr incubation with a cytotoxic concentration of GCV (Halloran and Fenton, 1998; Wei *et al.*, 1998). We observed only a doubling of the percentage of cells in G_2/M after treatment with 1 μM CdG. In addition, the murine melanoma cells did not undergo an apoptotic cell death, whereas in our cell lines both GCV and CdG induced apoptosis, although the onset occurred at approximately 48 hr following drug washout. Thus, multi-log cell killing may occur by different mechanisms depending upon the drug and cell line.

CdG exhibited similar selectivity as GCV for U251tk cells, but was less selective in the SW620 cell lines. It is difficult to translate these *in vitro* results to *in vivo* selectivity. For example, it is not known whether CdG will be as toxic as GCV to bone marrow cells, and drug metabolism and disposition *in vivo* can affect the therapeutic index. Further studies are needed to determine whether selectivity *in vivo* differs for these two drugs.

It is not clear why CdG exhibited higher toxicity than GCV in the SW620lacZ cells. It is likely that toxicity in the non-HSV-TK-expressing cell lines was due to minimal phosphorylation by an endogenous enzyme. Previously, it has been demonstrated that deoxycytidine kinase, deoxyguanosine kinase, and 5′-nucleotidase can phosphorylate L- and D-CdG in uninfected mammalian cells (Bennett *et al.*, 1998). Although L-CdG was a better substrate than was D-CdG for deoxycytidine kinase and deoxyguanosine kinase, it accumulated in intact cells primarily as the monophosphate (Bennett *et al.*, 1993), which may explain the low toxicity of L-CdG to U251 cells. Cytosolic 5′-nucleotidase has also been implicated in the phosphorylation of GCV in non-HSV-TK-expressing MOLT-4 T-lymphoblastic leukemia cells (Agbaria *et al.*, 1994). Differential expression of these enzymes in the two cell lines used in the studies presented here may lead to greater activation of CdG in

TABLE 2. CYTOTOXICITY OF CdG AND GCV IN HUMAN COLON CARCINOMA AND GLIOBLASTOMA CELLS

Cell line	[CdG] (μM) IC_{50}	IC_{90}	[GCV] (μM) IC_{50}	IC_{90}
SW620tk	0.9	4.5	0.07	5
SW620lacZ	90	265	450	>1000
50% SW620lacZ + 50% SW620tk[a]	2.4	8.7	1.5	14
90% SW620lacZ + 10% SW620tk[a]	12	>100	55	260
U251tk	0.06	0.3	0.06	0.2
U251lacZ	40	300	60	800

U251 glioblastoma and SW620 colon carcinoma cells expressing either HSV-TK or β-Gal were incubated as indicated with CdG or GCV for 24 hr. Cell survival was determined using a colony formation assay.

[a]IC_{50}, IC_{90} values represent survival of bystander cells after co-culture with HSV-TK-expressing cells at the indicated ratios.

the SW620 cells, resulting in higher cytotoxicity. Previous reports in the literature indicate that CdG is not highly toxic to normal cells. Human hepatoma cells treated for 10 days with CdG at 5 μg/ml did not show evidence of toxicity (Price *et al.*, 1989). Similarly, no toxicity was observed in primary duck hepatocyte cultures treated with 10 ng/ml CdG for 8 days (Fourel *et al.*, 1994b). Furthermore, *in vivo* studies in ducks treated with CdG showed only mild liver abnormalities (hepatocyte vacuolization, mild disruption of lobular architecture) after prolonged administration (100 μg/kg CdG administered every other day for 5 weeks) (Fourel *et al.*, 1994a). These results are encouraging, and suggest that CdG may have low toxicity in humans.

Of all the substrates tested for cytotoxicity in tumor cells expressing HSV-TK, purine analogs have shown higher cytotoxicity compared to the pyrimidine analogs, with good bystander killing (Balzarini *et al.*, 1993; Rubsam *et al.*, 1998; Degreve *et al.*, 1999). An evaluation of six guanine analogs that varied in the moiety attached to the base showed a wide variation in cytotoxicity. GCV, its elaidic acid derivative, and a cyclobutyl dervative showed good bystander cell killing, whereas other acyclic analogs such as penciclovir, buciclovir, and acyclovir displayed poor or no bystander cell killing (Degreve *et al.*, 1999). Here we have demonstrated that a carbocyclic guanine analog has high cytotoxicity to HSV-TK-expressing cells with good bystander activity. With so few analogs available that can efficaciously eliminate HSV-TK-expressing as well as nonexpressing bystander cells, further investigation is warranted to compare their antitumor effects *in vivo*. The drugs that have shown the highest bystander cell killing also show the highest toxicity to non-HSV-TK-expressing cells. Therefore, it will be important to determine whether these analogs have an advantage over the commonly used GCV, such as reduced toxicity to bone marrow cells.

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