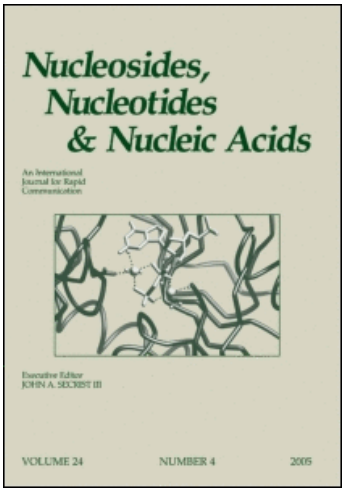

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Nelson, J. Arly , Pan, Bih-Fang , Marini III, Frank C. , Farquhar, David and Mullen, Craig A.(1997) 'Suicide Gene Therapy for Intrinsically Drug Resistant Solid Tumors', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1251 — 1259
To link to this Article: DOI: 10.1080/07328319708006167
URL: http://dx.doi.org/10.1080/07328319708006167

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1251-1259 (1997)

SUICIDE GENE THERAPY FOR INTRINSICALLY DRUG RESISTANT SOLID TUMORS

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ABSTRACT The failure of available anticancer drugs to cure the common human solid tumors is thought to be caused by their poor selective toxicity. As demonstrated herein, suicide gene therapy offers the potential to improve selective toxicity.

INTRODUCTION

It is widely accepted that the failure of available antitumor agents to cure common solid tumors in humans is caused by their poor selective toxicity, i.e., low chemotherapeutic indexes. Using gene-therapy techniques, it is possible to insert genes into drug-resistant tumors in such a manner that they become susceptible to otherwise nontoxic prodrugs, a process commonly referred to as suicide gene therapy[1]. This methodology provides a means for testing the hypothesis that the drug failure is due to poor selectivity. Herein we have modified two drug-resistant solid tumors of mice to express either herpes simplex virus thymidine kinase *(HSVtk)* or *Escherichia coli* cytosine deaminase*(CD)*. *HSVtk* renders the cells sensitive to the antiviral nucleoside analog, ganciclovir (GCV), whereas *CD* catalyzes the conversion of 5-fluorocytosine (FC) to toxic 5-fluorouracil. The improved selective toxicity, chemotherapeutic responses, and a novel means to exploit this approach, clinically, are illustrated herein.

METHODS

Tumor models. The CT-26 colon and PANC02 pancreatic carcinomas were derived from a BALB/c and C57/BL/6 mouse, respectively[2,3]. The cells were grown in tissue culture as described below. In each case, a modified LNL6 retrovirus was used to transduce the cells to express *HSVtk*[4], *CD*[5],*β-galactosidase (βgeo*[6]) and G418 (neomycin) resistance. For in vivo experiments, the cells were implanted subcutaneously on the flank regions of syngeneic host mice or, where indicated, immune-deficient, nude mice. The tumor mass was estimated by caliper measurement in two directions[3,7]. To assess the bystander effect in vivo, *HSVtk*-expressing cells were injected directly into the tumor mass as previously described[8], and GCV was administered intraperitoneally at a dose of 100 mg/kg daily for 7 days.

Tissue culture. The CT-26 cells were grown in Dulbecco's modified Eagles medium. The tissue culture line of PANC02 cells was a generous gift from Dr. Lee Wilkoff, Southern Research Institute, Birmingham, AL[9]. They were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Viability of the cells was assessed by clonogenic assay[10] or trypan blue dye exclusion. *βgeo* cells were determined histochemically after staining the cells with X-gal[11]. Where indicated, cells were lethally irradiated as previously described[7].

GCV nucleotides. GCV nucleotides were determined in neutralized, acid-soluble extracts of the cells as follows. Cells were grown in the presence of ^{3}H-GCV (Moravek Biochemicals, Brea, CA) for various times at which the cells were harvested and extracted with 0.5 M perchloric acid. The acid-soluble material collected by centrifugation was neutralized with potassium hydroxide. An aliquot was used to determine "total radioactivity" and another aliquot was placed on a DE-81 (Whatman, Inc., Fairfield, NJ) disc. The dried discs were washed twice in 50% ethanol for 15 min at room temperature, dried, and placed in liquid scintillation vials. Nucleotides were solubilized by the addition of 1 ml of 1 N HCl. After adding 10 ml of liquid scintillation fluid, the radioactivity was determined by liquid scintillation spectrometry.

FACS separation. Fluorescence activated cell sorting (FACS) was performed to separate the individual *βgeo*$^-$ and *βgeo*$^+$ populations after coculture. Flow cytometry of the mixture was performed on a Becton-Dickson FACSTARplus instrument. After incubating the cells in C_{12}-FDG (a FITC conjugated, β-gal substrate[12]) the two populations were sorted with the argon laser set at 488nm. Green fluorescence was detected at 530/30. The negative control was performed first and the threshold was set at which < 1% of the cells were positive. FITC fluorescence vs FSC was set and only cells in the high positive window were accepted as *βgeo*$^+$. Cells exhibiting fluorescence below the negative threshold were collected as *βgeo*$^-$. Efficiency of sorting was determined by repetitive flow cytometry and confirmed by X-gal staining of the separated populations.

RESULTS

Suicide genes increase the sensitivity of drug-resistant solid tumors to prodrugs. The expression of *HSVtk* in PANC02 cells increased the sensitivity of the cells to GCV over 100-fold, i.e., ED_{50} value of 0.14 vs. 39-110 μM in wild-type or other transduced controls (Table 1). Likewise, expression of *CD* enhanced the sensitivity of PANC02 cells to FC by a similar order of magnitude. We have previously reported the similar increase in in vitro sensitivity of CT-26 cells to GCV following transduction to express *HSVtk*[13]. Although not shown, the growth rates and cloning efficiences of PANC02 or CT-26 cells in vitro were not changed by transduction with the modified retroviruses.

Transduced PANC02 cells exhibit bystander effect killing. As shown in Table 2, the growth of either the *HSVtk*-expressing or *βgeo*-expressing PANC02 cells is unchanged when one population is grown in the presence of the other. That is, when the cells are seeded at equal numbers on day 0, the numbers of both populations of cells at day 4 are essentially the same, 4.2 and 4.4 x 10^5 cells. Irradiating the *HSVtk* cell population with 18 Gy prevented the growth of that population; however, the growth of the *βgeo* cells was unimpaired in the presence of the lethally irradiated *HSVtk* cells, 5.2 vs. 4.2 x 10^5 cells on day 4 in the experiment shown. In a separate experiment that enumerated the *βgeo* cell population only, these results were confirmed (Table 3). That is, at day 4, the number of *βgeo* cells that grew in the presence of viable *HSVtk*-expressing cells was the same, i.e., 16 x 10^4 cells in the experiment shown. The presence of irradiated *HSVtk*-expressing cells (5 days after 4000 rads) reduced the growth of the *βgeo* cells

TABLE 1. Cellular cytotoxicity of prodrugs against PANC02 cells transduced to express suicide genes.

Cell line[a]	ED_{50} [b]		
	GCV, μM	FC, μM	G418, mg/ml
PANC02	110	> 8000	0.08
PANC02/LNL6	39	> 8000	4.4
PANC02/*tk*	0.14	> 8000	3.1
PANC02/*CD*	45	90	2.2

[a]PANC02 = nontransduced parent line[9]; PANC02/LNL6 = transduced with modified retrovirus only expressing the neomycin-resistance gene; PANC02/*tk* = transduced cell line that expresses *HSVtk*; PANC02/*CD* = transduced to express *E. coli* cytosine deaminase.
[b]Concentration of drug shown that reduced cloning efficiency by 50% of controls[10].

TABLE 2. Lethally irradiated PANC02/*tk* cells do not alter the growth of PANC02/*βgeo* cells.

Cell line(s) Seeded on Day 0	Cell Number x 10^5, day 4[d]	
	X-gal$^+$	X-gal$^-$
PANC02/*βgeo*[a]	9.3	0.1
PANC02/*βgeo* + PANC02/*tk*[b]	4.2	4.4
PANC02/*βgeo* + irradPANC02/*tk*[b,c]	5.2	0.2
irradPANC02/*tk*[a,c]	0	0.4

[a]0.5 x 10^5 cells cultured alone
[b]0.25 x 10^5 cells from each population cultured together
[c]PANC02/*tk* cells were irradiated with 18 Gy immediately prior to culture
[d]The numbers of *βgeo*$^+$ and *βgeo*$^-$ cells were determined by X-gal staining after 4 days incubation at 37° as described in Methods.

only by about 40% (10 x 10^4 cells on day 4). Both populations of cells were killed by GCV at a dose that normally has no effect on the *βgeo* cells (10 μM). Perhaps more importantly, this "bystander effect" occured whether or not the *HSVtk* cells were lethally irradiated. In the experiment shown, the irradiated cells exhibited a greater bystander effect than did the nonirradiated *HSVtk* cells, 0.1 vs. 2.3 x 10^4 cells. Similar results with irradiated and nonirradiated effector cells have been observed by us using the CT-26/*tk* and CT-26/*βgeo* cell lines[13]. Although not shown, the viable PANC02 cells that express *CD* also produced a marked bystander effect toward *βgeo* target cells in the presence of FC.

The observation that lethally irradiated PANC02/*tk* cells could produce the bystander effect killing suggests that such cells are capable of forming GCV nucleotides and transferring them to

TABLE 3. PANC02/*tk* cells and GCV produce a potent bystander effect even after irradiation of the PANC02/*tk* cells.

Culture Conditions[a]	Number x 10^4 of *βgeo* + Cells 4 Days After Incubation[b]
PANC02/*βgeo*	16
PANC02/*βgeo* + PANC02/*tk*	16
PANC02/*βgeo* + PANC02/*tk* + GCV	2.3
PANC02/*βgeo* + irradPANC02/*tk*	10
PANC02/*βgeo* + irradPANC02/*tk* + GCV	0.1

[a]PANC02/*βgeo* cells were seeded alone (1 x 10^4 cells) or with an equal number (1 x 10^4 each) of PANC02/*tk* cells that had or had not been irradiated (40 Gy) 5 days earlier. Where indicated, GCV was added at the time of seeding the cells to a final concentration of 10 μM.

[b]The numbers of *βgeo* + cells (x 10^4) were determined by X-gal staining after 4 days incubation at 37° as described in Methods.

target cells. This exchange has been hypothesized to occur either via gap junctions, referred to as metabolic cooperation[14-16], or apoptotic vesicles[17-19]. PANC02/*tk* and PANC02/*CD* cells remain viable for several days following lethal irradiation (Table 4). By trypan blue dye exclusion, 20-30% of the cells remain viable even 6 days following irradiation. The formation of GCV nucleotides from GCV is apparent in the PANC02/*tk* cell population even 10 days after irradiation. The apparent discrepancy between the number of viable cells and the amount of GCV nucleotides is perhaps due to the cellular enlargement ("giant cells") produced by irradiation.

Chemotherapy experiments. When implanted into syngeneic C57/BL/6 mice, transduced PANC02 cells either failed to grow, grew slowly or grew and regressed compared with nontransduced PANC02 cells (Table 5). Consequently, we were unable to perform chemotherapy experiments with initial implants of the transduced PANC02 cells. When the tumors that grew were harvested and serially-implanted into second, third, fourth and fifth generations, the tumors grew reproducibly and approached the growth of the nontransduced cell line (Table 5).

Unlike the transduced PANC02 cells, the CT-26/*tk* cell line grew as well as its nontransduced counterpart in the syngeneic host, BALB/c mice, and was used for the chemotherapy experiments described or shown herein. Treatment of advanced CT-26 tumors in mice with GCV (100 mg/kg/day, i.p. for 7 days) produced complete regressions when as few as 10% of the cell population was comprised of *HSVtk*-expressing cells[20]. Further, as shown herein with PANC02 cells, this bystander effect was the same in the CT-26/*HSVtk*/GCV system whether viable or lethally irradiated cells were used in vitro and in vivo[13]. The bystander effect in the CT-26 system probably does not require an immune component because a similar bystander effect was produced whether immune-competent or immune-deficient (nude) mice were used (Figure 1). Approximately 4 x 10^4 CT-26 cells were required to establish tumors in the BALB/c mice (experiments not shown). Thus, mice bearing approximately 10^8 such cells (100 mg mass when therapy is begun, Figure 1) experience a 3-4 log cell kill by the GCV treatment. Regrowth of GCV-treated tumors occurred in 3/10 nude mice and in 0/10 syngeneic hosts (Figure 1) during the

TABLE 4. Viability and GCV nucleotide formation in lethally irradiated PANC02 cells.

Days after irradiation[a]	Viability[b] %		GCV Nucleotides[c] pmole/T-75 culture dish	
	PANC02/*tk*	PANC02/*CD*	PANC02/*tk*	PANC02/*CD*
0	100	100	235	1.0
2	61	40	263	1.6
4	48	44	253	3.1
6	30	22	224	2.3
8	15	14	128	1.7
10	19	10	145	1.1

[a]Cells were irradiated with 40 Gy on day 0. Day "0" cells were not irradiated (controls).
[b]Viability was assessed on the days shown by trypan blue dye exclusion.
[c]GCV nucleotides were determined after 4 hr incubation in the total cell populations on the days shown as described in Methods.

TABLE 5. Failure of transduced PANC02 cells to grow in syngeneic hosts.

Passage[a]	Days required for tumors to reach 1000 mg[b]			
	PANC02	PANC02/LNL6	PANC02/*tk*	PANC02/*CD*
1st	33	--	--	--
2nd	29	76	80	80
3rd	11	41	55	78
4th	17	16	52	25
5th	--	16	27	22

[a]The tissue cultured cells (1×10^7) were implanted subcutaneously as a suspension of cells on day 0 for the 1st passage. Subsequent passages employed trocar implantation of tumor fragments (approximately 50 mg) obtained from a donor mouse when the tumor was approximately 1000 mg[10]. In each case, 5 mice per group were used.
[b]Tumor size was estimated by caliper measurement 3 times per week. The first passage of transduced cells grew poorly or not at all such that it was not possible to estimate the time required to reach 1000 mg. A fifth passage of control PANC02 cells was not performed.

period of observation. This finding suggests that there may be a small contribution of the immune system to total tumor cell erradication in this model.

The bystander effect in the CT-26 tumors appears to require that the two cell populations grow in proximity since a bystander effect was not observed when the target cells were implanted at a remote site from that of the effector cells, i.e., contralateral flank (data not shown). Taken together, these data suggest that the immune system can not fully account for the bystander effect observed in the CT-26/*HSVtk*/GCV system, in vivo. The requirement that the cells be in close

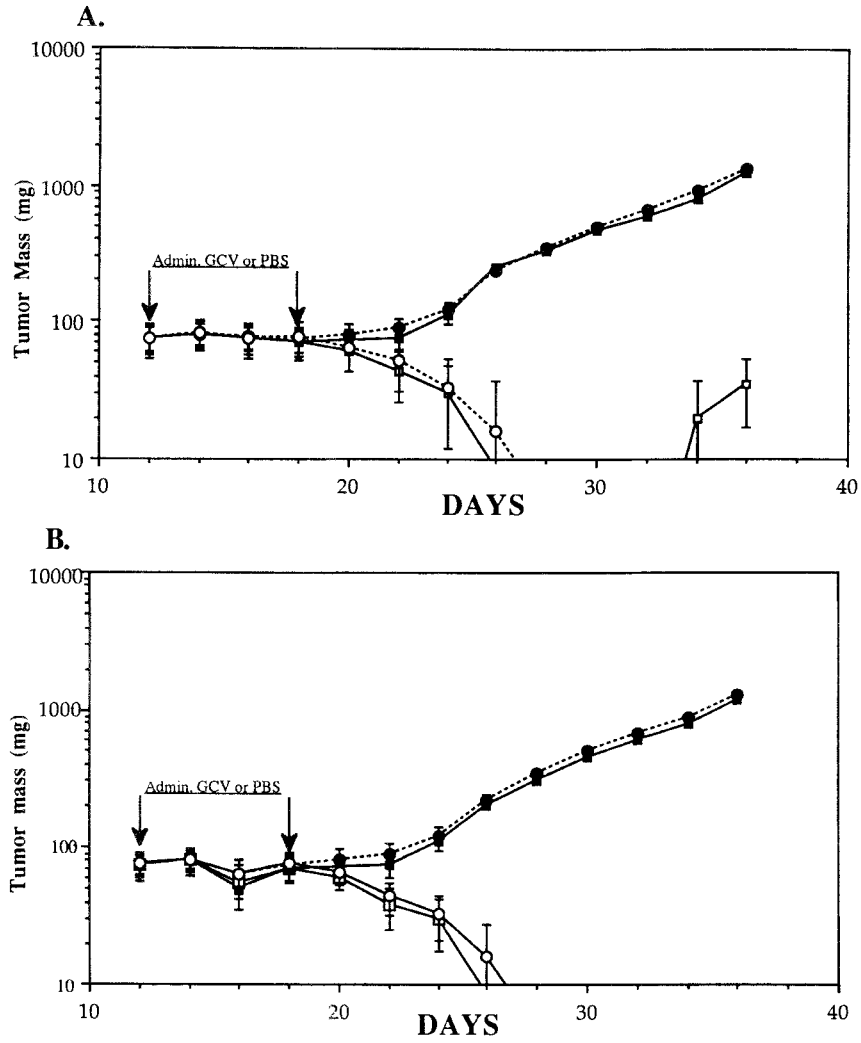

FIG 1. The bystander effect produced by GCV and CT-26/*tk* cells in BALB/c mice. CT-26/*tk* cells (10^7; Panel A) or an equal number (5 x 10^6 each; Panel B) of CT-26 and CT-26/*tk* cells were implanted s.c. on the flank region of BALB/c (circles) or nude (squares) mice on day 0. Beginning on day 12 and for 7 consecutive days, the mice were treated with GCV, 100 mg/kg/day, i.p. Tumor mass was estimated by caliper measurement on the days shown. Closed symbols = saline-treated (control) group; open symbols = GCV-treated group.

proximity for a bystander effect to occur suggests that perhaps GCV nucleotides are being transferred from the effector to target cells. To directly address this possibility, we grew the effectors (CT-26/*tk*) in the presence of targets (CT-26/*βgeo*) and separated them by FACS. As shown in the Table 6, after 2 days incubation, the GCV nucleotides in the acid-soluble pool or in nucleic acids was approximately equal in the two cell populations. Although not shown, the efficiency of the separation was such that the βgeo^+ population contained only 7% βgeo^- cells.

Table 6. Transfer of GCV nucleotides from CT-26/*tk* cells (effectors) to CT-26/*βgeo* cells (targets) in vitro

Treatment	GCV Nucleotides (acid-soluble)	GCV in DNA and RNA (acid-insoluble)
Grown separately:		
βgeo$^+$	127	15
βgeo$^-$(tk$^+$)	1069	102
Grown together (50/50 mix):		
βgeo$^+$	264	35
βgeo$^-$(tk$^+$)	258	32

CT-26/*βgeo* and CT-26/*tk* cells were grown separately or as a 50/50 mix for 1 day. They were then treated with ^{3}H-labeled GCV (1 μCi/ml; 10 μM) for 2 days, at which time they were harvested. Those populations grown together as a 50/50 mix were subjected to fluorescence activated cell sorting as described in Methods. GCV nucleotides were determined in the acid-soluble fraction by DE-81 disc anion exchange and in the acid-insoluble fraction (DNA and RNA) after redissolving in alkali[21]. The results shown are pmoles equivalent GCV per 10^6 cells.

DISCUSSION

A major finding reported herein is the ability of lethally irradiated cells that express the *HSVtk* suicide gene to produce a "bystander effect". The effect is as potent as that seen with nonirradiated effector cells in the case of PANC02/*tk* in vitro (Table 3) and with CT-26/*tk* cells in vitro and in vivo[13]. The bystander effect observed with both PANC02/*tk* and CT-26/*tk* cells requires treatment with GCV, i.e., dying, irradiated cells fail to produce a bystander effect in the absence of GCV (Tables 2 and 3; reference 13). The effect persists in irradiated cells perhaps because they maintain their ability to form GCV nucleotides for several days following irradiation (Table 4; reference 13). The significance of these observations is that one should be able to exploit the bystander effect in a chemotherapeutic strategy. That is, after removing a patient's tumor, the cells could be transduced to express HSV*tk*, irradiated, and reimplanted perhaps at nonresectible tumor sites. GCV could then be administered to take advantage of the bystander effect. The attractiveness of the approach is that it does not require the administration of viral vectors to the patient. A number of clinical studies have been performed or are currently underway that use a patient's own tumor in a similar fashion in an effort to produce an immune response. That is, tumor cells are removed, irradiated, and given to the patient in an attempt to "vaccinate" the host. These studies suggest that the approach will have negligible or minimal toxicity. In short, the bystander effect is a promising means to use suicide gene therapy for the treatment of drug-refractory solid tumors while more efficient, tumor-specific vectors are being developed for the future.

The observations reported herein bear on the possible mechanism(s) for the bystander effect observed in the *HSVtk*/GCV system. Namely, a bystander effect occurs in vitro with either the PANC02 or CT-26 cell lines, indicating that the effect can occur in the absence of an immune system. In the case of CT-26 cell lines, the bystander effect was essentially the same whether

immune-competent or immune-deficient mice were used as hosts (Figure 1). The failure of dying, lethally irradiated transduced cells to influence the growth of nontransduced target cells indicates the absolute requirement for GCV treatment, i.e., the release of "toxic factors" from dying effector cells does not appear to be an adequate explanation for the effect in PANC02 cells in vitro (Table 2 and Table 3) or for CT-26 cells in vitro or in vivo[13]. Preliminary experiments shown in Table 6 suggest a role for "metabolic cooperation" in the bystander effect; however, additional experiments need to be performed to determine whether the apparent exchange of GCV nucleotides is an adequate explanation for the bystander effect observed. Also, it would be informative to determine whether GCV nucleotide exchange occurs independent of apoptosis.

The PANC02 tumor is perhaps the most drug refractory solid tumor known in mice[3]. Of 38 known active, antitumor agents representing each class of cytotoxic compounds tested for activity toward this tumor, none produced regressions of advanced disease or cure of early disease[3]. Unfortunately, the transduced PANC02 cell lines grew poorly in syngeneic hosts (Table 5), rendering it difficult to perform meaningful chemotherapeutic experiments. The basis for the poor growth is currently unknown; however it is possible that the transduced cells elicit an immune response because this has been reported for other transduced cell lines[22-24]. We have not determined whether the expression of the foreign proteins was lost during serial transplant in these experiments. The CT-26 tumor does respond to certain alkylating agents and 5-fluorouracil, but fails to respond to doxorubicin, actinomycin D, vincristine, arabinosylcytosine, and an antifol (NSC127755; Table 6 of reference 3). Thus, CT-26 does not provide as rigorous a test of the potential use of suicide gene therapy to "cure" refractory solid tumors as does PANC02. Clearly, we would like to perform chemotherapeutic experiments with the transduced PANC02 cells in nude mice, but have not had the opportunity to date.

In other experimental tumor systems, evidence for a contribution by the immune system to the in vivo bystander effect has been reported[24-26]. Also, the immunogenicity of cells transduced to express suicide genes has been substantiated with tumor-specific cytotoxic lymphocytes[22]. It appears from these observations that some tumor cells are rendered more immunogenic as a consequence of their expressing foreign proteins, intracellularly. It can be inferred that the processing of these foreign proteins (i.e., neomycin resistance, HSVtk, CD, etc.) to present peptides in the context of MHC class I antigens enhances the cellular immunity toward the tumor cell in a specific fashion. If true, the enhanced immunogenicity generated by transducing solid tumors may play to the advantage of the patient in the application of suicide gene therapy as described above.

ACKNOWLEDGEMENTS

The work reported herein was supported in part by Grant CA71527 from the National Cancer Institute, National Institutes of Health and by a Career Development Award to Craig A. Mullen from the American Cancer Society.

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