Cancer Chemother Pharmacol (2002) 50: 65–70
DOI 10.1007/s00280-002-0438-2

ORIGINAL ARTICLE

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Suicide gene therapy using *E. coli* β-galactosidase

Received: 10 September 2001 / Accepted: 18 January 2002 / Published online: 8 June 2002

Abstract *Purpose*: Suicide gene therapy offers the potential to increase the selective toxicity of antitumor agents by intratumoral expression of exogenous enzymes that convert nontoxic prodrugs to toxic products. The use of herpes simplex virus thymidine kinase with ganciclovir, and *E. coli* cytosine deaminase with 5-fluorocytosine are well-known examples of this approach. The purpose of this study was to investigate a novel suicide gene therapy using *E. coli* β-galactosidase (β-gal) as the prodrug-activating enzyme. Advantages of this approach include: (1) the ability to use prodrugs that are cleaved by β-gal to agents that are known to possess activity against human solid tumors, and (2) the extensive experience gained with targeting β-gal to specific tumors in experimental animals and in humans. *Methods*: Two different structural types of anthracycline prodrugs, *N*-[4″-(β-D-galactopyranosyl)-3″-nitrobenzyloxycarbonyl]daunomycin (Daun02) and *N*-[(4″*R,S*)-4″-ethoxy-4″-(1‴-*O*-β-D-galactopyranosyl) butyl]daunorubicin (gal-DNC4) were investigated. The prodrugs were evaluated as substrates for β-gal. Cytotoxicity studies of Daun02 were conducted against a murine tumor (Panc02), two human breast tumors (MCF-7 and T47D), and three human prostate tumors (PC3, DU145 and LNCAP) that had been transduced to express β-gal. Antitumor studies of Daun02 were conducted against mouse tumor Panc02 xenografts implanted subcutaneously. *Results*: Daun02 was a good substrate for β-gal. By comparison, gal-DCN4 was a poor substrate. Except for PC3, the β-gal-transduced tumors showed 3- to 60-fold increased sensitivity to Daun02 compared with mock-transduced control cells. Daunomycin was formed from Daun02 in tissue culture medium containing β-gal-transduced cell lines but was not observed in the medium from mock-transduced controls. In vivo therapeutic studies of Daun02 against the Panc02 tumor in athymic mice showed no significant inhibition of tumor growth. Pharmacokinetic studies showed limited distribution of the prodrug beyond the vascular space. *Conclusions*: *E. coli* β-gal may be useful as a prodrug-activating enzyme in suicide gene therapy and has the potential to increase the selective toxicity of conventional antitumor agents. Although this approach worked well against tumor cells in vitro, it was not effective against a xenograft model in vivo, apparently because of poor drug-tissue distribution.

Keywords Gene therapy · Prodrug · Anthracycline
β-galactosidase · Antitumor · Mouse

Abbreviations *β-gal:* β-galactosidase from *E. coli* · *CD:* cytosine deaminase · *Daun02: N*-[4″-(β-D-galactopyranosyl)-3″-nitrobenzyloxycarbonyl]daunomycin · EC_{50}*:* concentration of drug or prodrug that produced a 50% reduction in cell viability · *FBS:* fetal bovine serum · *gal-DNC4: N*-[(4″*R,S*)-4″-ethoxy-4″-(1‴-*O*-β-D-galactopyranosyl)butyl] daunorubicin · *HPLC:* high-performance liquid chromatography · *HSVtk:* herpes simplex virus thymidine kinase · *ONPG: o*-nitrophenyl-β-D-galactopyranoside · *PBS:* phosphate-buffered saline

Introduction

It is generally accepted that the failure of many tumors to respond to chemotherapy is due to poor drug selectivity. One way to improve selectivity is to insert "suicide genes" into drug-refractory tumors [1]. In theory, this modality should lead to the release of toxic drugs in or near tumor cells that express the appropriate "suicide gene." The best-studied examples of this approach are ganciclovir and 5-fluorocytosine, compounds that are activated by herpes simplex virus thymidine kinase (HSVtk) and *E. coli* cytosine deaminase (CD), respec-

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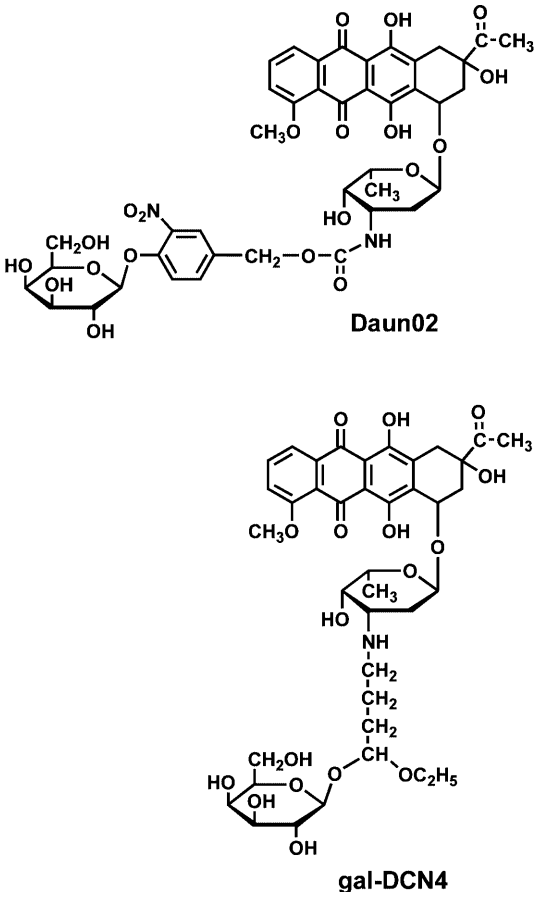

Fig. 1. The structures of Daun02 and gal-DNC4

tively. Significant shortcomings of these prodrug strategies are the narrow substrate specificities of HSVtk and CD, and the low sensitivity of many human solid tumors to antimetabolites.

We report here initial results of a suicide gene therapy approach in which antitumor prodrugs are activated by β-galactosidase (β-gal). Potential advantages of this approach are: (1) the enzyme is substrate permissive and thus should activate a broad structural variety of prodrugs, and (2) extensive experience has been gained with targeting of β-gal to specific tumors and tissues. Two different structural types of anthracycline prodrugs (Fig. 1) were selected to investigate this strategy. One of these, Daun02, contains an *o*-nitrobenzyl linker group, a moiety reminiscent of the classical β-gal substrate, ONPG. Cleavage of the galactoside sugar by β-gal should yield a labile intermediate that should spontaneously lose a quinone methide, followed by carbon dioxide, to generate daunomycin (Fig. 2) [2]. The second prodrug, gal-DNC4, gives rise to a hemiacetal upon cleavage of the galactoside sugar. The hemiacetal should eliminate ethanol to yield an aldehyde that should spontaneously cyclize to an aminal. Loss of hydroxyl anion from the aminal yields an intensely cytotoxic iminium derivative. Preliminary reports of some of these data have appeared [2, 3].

Materials and methods

Drugs

Daun02 and gal-DNC4 were synthesized in our laboratories as described previously [3, 4]. Daunomycin hydrochloride was purchased from Sigma-Aldrich. All drugs were checked for purity by thin-layer chromatography and HPLC before use.

Transduced tumor cells

The murine pancreatic tumor cells (Panc02) used in this study, originally established by Wilkoff and Dulmadge [5], were obtained from the American Type Culture Collection (Rockville, Md.). The human breast (MCF-7, T47-D) and prostate (PC3, DU145,

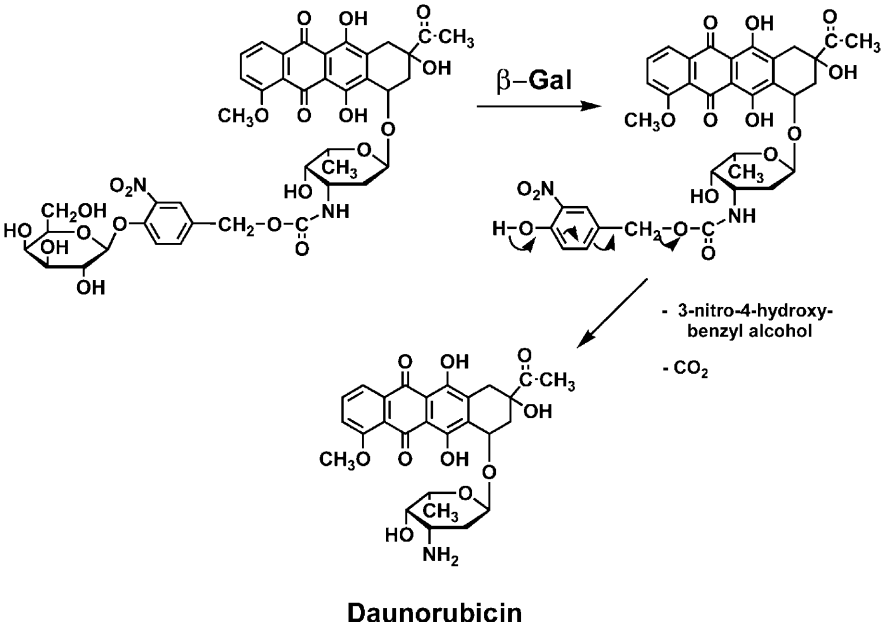

Fig. 2. Mechanism of activation of Daun02 by β-gal

LNCaP) tumor cell lines were obtained from the M. D. Anderson Cancer Center Tumor Bank Repository. These cell lines were transduced to express *E. coli* β-gal using a modified LNL6 retrovirus [6]. LNL6 refers to transduced cells that express a neomycin phosphotransferase gene promoted by the retroviral LTR. β-Gal transductants express the LacZ gene promoted by the retroviral LTR and the neomycin phosphotransferase gene promoted by the SV40 early promoter. Selection for transduced cells utilized G418 (neomycin-resistance) and, with the exception of Panc02/β-gal, the transduced populations were not cloned. Expression of the enzymes of interest was confirmed by enzyme measurement and increased sensitivity of the transductants toward Daun02 in tissue culture. β-Gal activity was measured spectrophotometrically in terms of the increase in absorbance at 405 nm upon incubation of the enzyme at 37°C with ONPG [7]. In the case of Daun02 or gal-DNC4, β-gal activity was measured by analysis of the substrate and product by HPLC, as described below.

Partition coefficient

n-Octanol (3 ml), previously equilibrated with 0.05 *M* PBS, pH 7.4, was added to 3 ml of a solution of the prodrug (10^{-4} *M*) in PBS, pH 7.4, previously equilibrated with *n*-octanol. The mixture, contained in a 15-ml capped centrifuge tube, was agitated for 5 min on a Vortex shaker and then centrifuged at 2000 rpm for 5 min. The octanol and aqueous layers were separated, and the concentration of prodrug in each was determined by UV spectrometry at a wavelength of 480 nm. Each result was read in duplicate. The experiment was repeated three times.

Plasma protein drug binding

The binding of Daun02 and daunomycin to mouse plasma proteins was determined by ultrafiltration. Frozen mouse plasma was thawed at room temperature, shaken, and 1-ml aliquots were transferred to each of three microcentrifuge tubes. Stock solutions (10^{-3} *M*) of Daun02 and daunomycin hydrochloride in 0.05 *M* PBS (pH 7.4)/DMSO (19:1) were added to each microcentrifuge tube to give a final drug concentration of 10^{-5} *M*. The mixtures were shaken in an incubator bath at 37°C for 5 min then transferred to Amicon protein filtration cones (MW of filtration 30 kDa; Millipore Catalog J]. Samples were centrifuged at 3000 rpm at 4°C for 30 min, and 200-μl aliquots of the filtrate were analyzed by HPLC on a C18 reverse-phase column using 0.05 *M* NH_4OAc buffer/acetonitrile (65:35) as mobile phase. To correct for drug bound to the filtration cones, similar studies were done substituting 0.05 *M* PBS for plasma. Standard curves were established by preparing a series of concentrations for each agent in 0.05 *M* PBS, pH 7.4, over the range 1 to 100 μg/ml, and plotting the HPLC peak areas against the known drug concentrations. All samples were assayed in duplicate.

Cell cytotoxicity assays

Murine Panc02 cells were maintained as exponentially growing monolayer cultures in DMEM/F12 or RPMI-1640 medium supplemented with 10% FBS,1% glutamine, penicillin, and streptomycin at 37°C as previously described [8]. For cytotoxicity assay, the cells were seeded into 96-well microplates and incubated overnight. Initial experiments indicated that FBS contained low levels of intrinsic β-gal activity as evidenced by the slow conversion of Daun02 to daunomycin; however, this was not evident for human serum. Therefore, prior to addition of Daun02, the FBS concentration was reduced from 10% to 1% for Panc02 cells. Human serum (10%) was used for the transduced human cell lines. The cells were incubated for 24 h and then MTT was added. Lysis buffer (20% SDS dissolved in 50% DMF) was added 4 h after the addition of MTT and the cells were incubated overnight [9]. The optical density at 570 nm was determined using a BIO-RAD (Hercules, Calif.) microplate reader. Cytotoxicity is expressed as the concentration of drug or prodrug that produced a 50% (EC_{50}) reduction in cell viability.

Antitumor evaluation in vivo

Male athymic BALB/c mice (nu/nu genotype, 18–20 g) were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and housed in sterile filter-capped microisolator cages in a barrier facility. Tissue-cultured Panc02 cells (1.5×10^7) were harvested and resuspended in 750 μl serum-free DMEM/F12 and 750 μl Matrigel (Collaborative Research, Bedford, Mass.). Panc02 cells (10^6) in 100 μl vehicle were implanted subcutaneously into the axillary region of groups of five mice. Drug treatment was initiated when the tumors reached a size of approximately 100 mg (about 2 weeks after tumor inoculation). Daunomycin was administered at a dose of 20 mg/kg in 100 μl normal saline solution into the tail vein. Daun02 was formulated in normal saline containing 10% DMSO and 10% Emulfur, and was administered intraperitoneally at a dose of 200 mg/kg in 200 μl vehicle. (This route was selected because the volume of drug solution, 200 μl, was too great for tail vein administration.) Tumor volume was determined by caliper measurement in two dimensions and converted to tumor mass as described previously [8]. Tumor growth was monitored over a period of 30 days or until the tumors had reached a mass of 5% of body weight (about 1 g). The animals were then killed by carbon dioxide asphyxiation.

Statistical analysis was performed using the Wilcoxon rank-order test [8]. The protocol for this study was approved by the Animal Care and Use Committee of the M. D. Anderson Cancer Center, and conducted in facilities accredited by the American Association for Accrediting Laboratory Animal Care in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23).

Drug tissue distribution studies

At 30, 60 and 180 min after Daun02 or daunomycin treatment, groups of three randomly selected mice were killed by carbon dioxide asphyxiation, decapitated, and exsanguinated into heparin-coated microcentrifuge tubes. Plasma was obtained by centrifugation at 12,000 *g* for 2 min at 25°C. The plasma was separated, frozen in a dry-ice/acetone bath, and stored at –70°C until use. Tumors were excised under sterile conditions in a laminar flow containment hood. After weighing, the tumors were cut into small fragments and homogenized in a mixture of MeOH and $CHCl_3$ (3:2). Plasma and red blood cells were extracted similarly by vortex mixing. After centrifugation at 3000 rpm, the supernatants were dried under nitrogen at 37°C. The samples were redissolved in 1 ml of the HPLC mobile phase buffer. After passage through 0.2 μm filters, the samples were analyzed by HPLC on a Waters (Milford, Mass.) C_{18} reverse-phase column using the same mobile phase buffer at a flow rate of 1 ml/min. The column eluent was monitored at 480 nm using a Model 600 E Waters Control System with a 484 Tunable Absorbance Detector. Similar analytical methods were used to assay for the formation of daunomycin in the tissue culture medium of β-gal-transduced cells exposed to Daun02.

Results

As shown in Table 1, Daun02 was a good substrate for β-gal with Km and Vmax values comparable to those of ONPG. By contrast, the daunomycin derivative gal-DNC4 [4] (which does not possess an *o*-nitrophenyl group) was a poor substrate. Although the binding affinity of gal-DNC4 for the enzyme (Km 0.14 m*M*) was comparable to that of ONPG, the Vmax was three orders of magnitude less. Because of the poor substrate

Table 1. Kinetic constants for β-gal substrates

Substrate	Km (m*M*)	Vmax (μmol/min/mg protein)
ONPG[a]	0.15	54.1
Daun02[b]	0.37	8.6
Gal-DNC4[b]	0.14	0.0027

[a]Enzyme activity determined spectrophotometrically [9]
[b]Enzyme activity determined by HPLC as described in Materials and methods

properties of gal-DNC4 for the enzyme, no further mechanistic or therapeutic studies were conducted with this compound.

The β-gal activity in various transduced tumor cell lines ranged from 0.012 to 0.319 U/mg protein (Table 2). When 0.01 β-gal unit equivalents of homogenates from these cells were incubated with Daun02 for 24 h, the prodrug was converted to daunomycin in 30 to 85% yield. The "activation" of Daun02 in these cell lines was also apparent from the cytotoxicity data (Table 2). With the exception of PC3 cells (which had the lowest β-gal activity), the prodrug was 3- to 60-fold more cytotoxic (i.e. lower EC_{50} values) to β-gal transduced cells than to LNL6-transduced controls. The conversion of Daun02 to daunomycin in the tissue culture medium of β-gal-expressing cells was confirmed by HPLC (Fig. 3).

In vivo therapeutic studies were conducted with β-gal-transduced Panc02 cells implanted subcutaneously in athymic mice. This tumor was selected because it had shown excellent conversion of Daun02 to daunomycin in cell culture studies and was fairly sensitive to daunomycin in vitro (EC_{50} 0.7 μ*M*). However, none of the tumors responded to treatment with either intravenous daunomycin at a dose of 20 mg/kg or intraperitoneal Daun02 at a dose of 200 mg/kg (data not shown). The dose of daunomycin is approximately the LD_{10} in mice [10], and the dose for Daun02 was the highest tested due to the limited water solubility of the compound. At this dose of Daun02, the animals did not appear to experience any toxicity as evidenced by normal physical and behavioral characteristics and continued weight gain.

The basis for the lack of response to Daun02 treatment was further explored by measuring the amount of prodrug and drug in the Panc02 tumor at various time intervals after drug administration (Table 3). The distribution of Daun02 to the tumor at a given plasma concentration was much less than that observed with daunomycin. Specifically, daunomycin demonstrated a propensity to enter and remain within the tumor as a function of time whereas the distribution of Daun02 to the tumor was roughly equivalent to the "extracellular space" (about 20% of tumor weight). We [8] and others [11] have previously noted that the distribution of antitumor and other small molecules to Panc02 is not significantly different from that observed in model "drug-sensitive" tumors. The distributions of Daun02 and daunomycin to red blood cells were approximately the same as to tumor tissue, confirming the poor penetrability of Daun02 across cell membranes. No measurable formation of daunomycin from Daun02 was observed in these experiments, consistent with both the lack of antitumor response and the absence of overt toxicity.

The partition coefficient for Daun02 in octanol/PBS was 4.1 compared to 3.5 for daunomycin. At a concentration of 10^{-5} *M*, Daun02 was 79% bound to plasma protein compared to 94% for daunomycin.

Discussion

In general, epithelial-derived tumors (carcinomas) are resistant to chemotherapy with antimetabolites. It is assumed that the poor selective toxicity of such agents accounts for therapeutic failure. One way to increase drug selectivity is to insert exogenous "suicide" genes into tumors such that nontoxic prodrugs are converted to their active forms. In the current study, we used *E. coli* LacZ as the suicide gene (β-gal is the gene product) and an anthracycline derivative, Daun02, as the prodrug.

Table 2. β-Gal activity in transduced tumors and their abilities to convert Daun02 to daunomycin (*n.d.* not determined)

Cell line	β-Gal activity (U/mg protein)[a]	Conversion (%) by 0.01 U equivalent of cell homogenate (β-Gal-transduced line)[b]	EC_{50} for Daun02 (μ*M*)[c]	
			LNL6	β-Gal
Panc02	0.103	85	>10	1.5
MCF-7	0.021	30	200	3.5
T47-D	0.170	65	8	0.5
PC3	0.012	32	5	5.0
DU145	0.031	45	15	5.5
LNCap	0.319	n.d.	50	5.0
E. coli β-gal	54.1	51	–	–

[a]Enzyme activity was measured using ONPG as substrate as described in Methods and Table 1
[b]Conversion of 100 μ*M* Daun02 to daunomycin during 24 min incubation at 37°C was determined by HPLC as described in Methods and Table 1. There was no conversion of Daun02 by control (LNL6-transduced) cell homogenates under these conditions
[c]The concentration of Daun02 producing 50% decrease in cell viability as described in Methods. LNL6 and β-gal are the vector (control) and β-gal-transduced cells, respectively. The EC_{50} values for daunomycin under these conditions were 0.1–0.4 μ*M* for the human cell lines and 0.7 μ*M* for the Panc02 cell lines

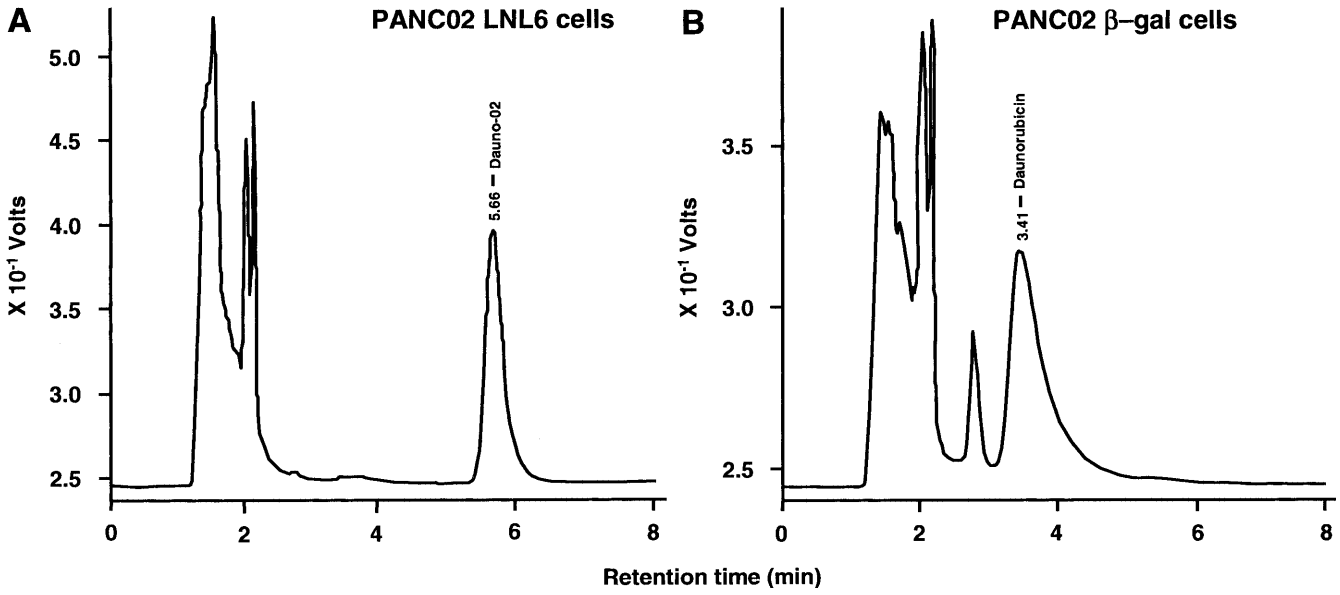

Fig. 3A, B. HPLC chromatograms of the formation of daunomycin from Daun02 by Panc02/β-gal cells in tissue culture. Samples of tissue culture medium of cells exposed to 10 μ*M* Daun02 were subjected to HPLC analysis as described in Materials and methods. **A** Chromatogram obtained for aliquot taken from Panc02/LNL6 cell medium indicating the presence of Daun02, retention time 6 min. **B** Chromatogram obtained for aliquot taken from Panc02/β-gal cell medium indicating conversion of Daun02 (retention time 6 min) to daunomycin (retention time 4 min)

The idea is to prepare galactoside prodrugs of antitumor agents that can revert to the parent drug in the presence of β-gal. Since this enzyme is extensively used as a "marker" for the expression of exogenous genes, a large number of vector constructs are available to evaluate the concept clinically. Of the two potential prodrugs that were investigated in this study, only one, Daun02, was a good substrate for β-gal (Tables 1 and 2). This is consistent with the good "leaving group" properties of the *o*-nitrobenzyl linker group, a structural moiety that resembles the *o*-nitrophenyl benzyl group present in the classical β-gal substrate, ONPG. The other prodrug investigated, gal-DNC4, was a poor substrate for the enzyme, presumably due to the poor "leaving group" properties of the butylacetal moiety which links the 1″-*O* position of the galactose sugar to the 3′-amino group of daunomycin.

Except for one cell line, in which β-gal expression was low, the sensitivity to Daun02 was increased compared with mock-transduced controls. The magnitude of the increased sensitivity, however, was lower than that usually observed with the HSVtk-GCV or CD-FC suicide gene systems [6, 12]. Although the β-gal suicide gene therapy approach was effective against tumor cells in culture, it was ineffective against the murine tumor Panc02 grown in nude mice. This may have been due to the inability to administer sufficient prodrug to achieve a therapeutic effect (because of the limited aqueous solubility of the agent) or, more likely, to the observed limited penetration of Daun02 into tumor cells in vivo.

The differences in tissue distribution of the Daun02 and daunomycin cannot be accounted for in terms of differences in lipophilicity because the partition coefficients in octanol/buffer were similar. Neither can the differences be explained in terms of plasma protein binding since the percentage of free drug present in samples of mouse plasma treated with Daun02 was

Table 3. Distribution of daunomycin and Daun02 to Panc02/β-gal tumors and red blood cells. The results obtained from two separate experiments in which daunomycin or Daun02 were administered intravenously to mice bearing Pan02/β-gal tumors (> 200 mg) are summarized. Plasma and tumor samples were harvested, extracted, and daunomycin and Daun02 measured by HPLC as described in Materials and methods (*n.d.* not determined)

Experiment	Treatment	Sampling time (min)	RBC (μg/ml)	Tumor (μg/g)	Plasma (μg/ml)	Tumor/plasma ratio
1	Daunomycin 50 mg/kg	60	n.d.	5.0	1.2	4.2
	Daun02 50 mg/kg	60	n.d.	< 1	1.8	< 0.6
2	Daunomycin 20 mg/kg	30	4.8	5.1	1.0	5.0
		60	2.1	5.1	< 1	> 5
		180	< 1	4.2	< 1	> 4
	Daun02 200 mg/kg	30	8.2	5.0	41	0.12
		60	1.8	3.9	14	0.27
		180	< 1	< 1	< 1	–

greater than that observed for daunomycin. It is not established whether poor tissue distribution is a feature unique to Daun02 or is common to most galactose derivatives. Further studies are required to address this consideration.

In summary, *E. coli* β-gal may be useful as a prodrug-activating enzyme in suicide gene therapy and has the potential to increase the selective toxicity of conventional antitumor agents. Although this approach worked well in the present study against tumor cells in vitro, it was not effective in vivo, apparently because of poor drug-tissue distribution.

Acknowledgements This work was supported by grant R01 CA71527, Cancer Center Support Grant CA16672, and a grant from the Keck Foundation.

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