Bystander Killing of Melanoma Cells Using the Human Tyrosinase Promoter to Express the Escherichia coli Purine Nucleoside Phosphorylase Gene

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ABSTRACT

We used a gene transfer-based system to generate highly toxic purine bases in tumor cells transfected with the *Escherichia coli* purine nucleoside phosphorylase (PNP) gene. Because these toxic purines are membrane permeant, they mediate effective killing of neighboring cells that do not express *E. coli* PNP ("bystander" toxicity). In mixed cultures containing increasing percentages of cells with gene expression, 100% cancer cell growth arrest and total population killing was demonstrated when as few as 1–2% of cells expressed *E. coli* PNP. We used *E. coli* PNP to test bystander killing of human melanoma cells. A 529-bp region upstream of the human tyrosinase gene start site was shown to direct melanoma-specific expression in human cell lines. When this human tyrosinase regulatory region was used to control *E. coli* PNP expression, profound toxicity was observed in melanoma cells after treatment with the relatively nontoxic substrate 6-methylpurine-deoxyriboside, which is converted by *E. coli PNP* into the highly toxic purine base 6-methylpurine. Bystander toxicity was estimated as at least 100 cells killed for each cell expressing *E. coli PNP*, a level substantially higher than that of other tumor sensitization genes currently being used in clinical trials. These results suggest that the high bystander activity of the system could lead to significant antimalanoma responses in vivo.

INTRODUCTION

The use of gene transfer to sensitize tumors to chemotherapeutic agents requires selective expression of therapeutic genes within tumor cells. Several approaches have been studied for targeting toxin genes to tumors. Retroviral integration into dividing tumor cells in the central nervous system (1, 2), herpes virus tropism to neural tissue (3), cationic liposomal delivery (4), and receptor-mediated gene targeting (5, 6) have been useful in certain settings. A flexible, complementary approach to tumor targeting is the application of tissue- or tumor-specific promoters to drive the expression of toxin genes within a particular target tissue. Tissue-specific promoters have been used, for example, to target gene expression in liver cells (7–9) and neurons (3), as well as in melanoma (10), lung (11–13), and breast (14) tumors.

In addition to cancer cell specificity, a second required property of a gene toxin-based approach to therapy is bystander toxicity, *i.e.*, the ability to kill neighboring cells not expressing the therapeutic gene. It is not likely that *in vivo* gene transfer efficiencies approaching 100% could be achieved for any human tumor with the use of current gene delivery vehicles. In addition to cellular barriers, gene transfer after regional or systemic administration is compromised by reticuloendothelial clearance of the vector and by diffusion barriers, such as endothelium. Tumor-selective expression of enzymes capable of activating nontoxic prodrugs to toxic form has led to considerable progress in this context (7, 15–21). For example, the expression of HSV-tk mediates tumor regression *in vivo* after ganciclovir administration but requires that at least 10% of the tumor cells be transduced with the HSV-tk gene (1, 2, 13–15, 22–24). Human trials based on the same principle have not yet shown comparable tumor regression, a finding which might be attributable to inadequate bystander toxicity mediated by HSV-tk/ganciclovir (25). In addition, a component of the bystander effect noted *in vivo* is likely due to immunological clearance of tumor cells (26, 27). It is not known whether similarly pronounced immunological clearance of tumor cells can be achieved in humans after HSV-tk expression.

Both tumor targeting and bystander killing have been applied to experimental models of melanoma. In studies using the mouse tyrosinase promoter, melanoma specificity and some measure of bystander killing with the use of HSV-tk have been demonstrated *in vitro* and *in vivo* (10, 22). Further development of this strategy for human-based melanoma therapy is likely to require (a) evidence of melanoma tissue selectivity using human tyrosinase-regulatory elements (a finding which has been difficult to establish in the past); and (b) inclusion of a bystander killing mechanism substantially greater than that which can be obtained with the use of HSV-tk. We have shown previously that expression of the *E. coli* Deo D gene *PNP* in eukaryotic cells leads to production of a functional enzyme capable of generating the diffusible cellular toxin MeP from the prodrug MeP-dR (28). The PNP/MeP-dR combination has a pronounced bystander effect. In the present study, we demonstrate that even when a small fraction of tumor cells in culture (*e.g.*, 1–2%) express the *E. coli PNP* enzyme, the addition of MeP-dR leads to nearly 100% cell death in breast and melanoma tumor cells. Furthermore, we demonstrate that melanoma specificity and drug sensitization with a profound bystander effect is conferred when *E. coli PNP* expression is under the regulatory control of the human tyrosinase promoter.

MATERIALS AND METHODS

Generation of Stable Cell Lines Expressing *E. coli PNP*. High level bystander killing of cancer cells in *vitro* was evaluated with the use of stable, PNP-expressing cell lines. The *E. coli PNP* gene was cloned into the *HindIII* and *SalI* sites of pSV40 (29), a retroviral vector in which the neomycin resistance gene is driven by the long terminal repeat, and the SV40 early promoter regulates *E. coli PNP* expression. Cloning was accomplished by excising the *E. coli PNP* gene from SV-PNP with *HindIII* and *SalI* (28) and directionally cloning the fragment into LNSX. Correct recombinants were verified with the use of five restriction digests with enzymes that cut in both vector and insert, and by PCR reamplification of the full-length PNP cDNA from the recombinant plasmid. Correct constructs were then transfected with the use of the lipofectin reagent (GIBCO-BRL, see below) into eukaryotic 3T3-based packaging cell lines (q2). Supernatants were collected from transduced q2 cells 48–60 h after transfection. To obtain a higher retroviral titer, supernatants from the initial viral infection were used to transduce fresh q2...
This product was extracted with phenol/chloroform, precipitated with ethanol, elongation with the use of 1 ng template; 100 ng of each primer in a 100-μl single PCR product of the predicted size (553 bp) was obtained [94°C for 1.3' ends of the desired product. Template DNA was prepared from whole cells. These cells were passaged to 100-mm plates 24 h after transduction and containing the E. coli PNP gene under regulatory control of the SV40 early selected. A control vector (SV-Luc) containing the SV40 virus early promoter reverse orientation (Rev-Tyr-Luc), for use as a negative control, was also clone was identified (Tyr-Luc). A plasmid with the tyrosinase promoter in any promoter (pGL2 Basic vector, Promega) was cut with MfeI, and the above digested with NheI, and gel purified. A luciferase reporter gene vector lacking HIMKC1, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 0.01% gelatin (w/v) reaction mixture containing 2.5 units Taq polymerase, 200 μM each dNTP, 50 μM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 0.01% gelatin (w/v). This product was extracted with phenol/chloroform, precipitated with ethanol, digested with NheI, and gel purified. A luciferase reporter gene vector lacking any promoter (pGL2 Basic vector, Promega) was cut with NheI, and the above PCR product was ligated immediately upstream of the luciferase gene (Fig. 1). Recombinants were screened by restriction mapping, and a correctly oriented clone was identified (Tyr-Luc). A plasmid with the tyrosinase promoter in reverse orientation (Rev-Tyr-Luc), for use as a negative control, was also selected. A control vector (SV-Luc) containing the SV40 virus early promoter and SV40 enhancer region driving the expression of firefly luciferase (pGL2 Control vector, Promega) was used to verify successful transfection of cells.

Construction of Plasmids Containing the E. coli PNP Gene. A plasmid containing the E. coli PNP gene under regulatory control of the SV40 early promoter (SV-PNP) was described previously (28). To create a plasmid in which the tyrosinase promoter controlled PNP expression, the PNP gene was substituted for luciferase in the Tyr-Luc. This was accomplished with the use of a XhoI/Sall digest to excise the full-length PNP gene from SV-PNP, followed by insertion of this fragment into the XhoI/Sall sites remaining after a XhoI/Sall digest to remove the luciferase gene from Tyr-Luc.

Cell Lines and Cell Culture. B16 and 16/C are of murine origin and were a gift of Dr. W. Waud (Southern Research Institute, Birmingham, AL); all other cell lines (described below) are of human derivation. Mel-1 (melanoma) was provided by Thomas Carey (University of Michigan) as UMC-Mel-1. Mel-21 (melanoma) was provided by M. B. Khazaeli (University of Alabama, Birmingham, AL). GP6F2 (prostate) was a gift of M. Moore (Grady Memorial Hospital, Atlanta, GA). U-373 (glioma) was provided by Yancey Gillespie (University of Alabama, Birmingham, AL). HeLa (cervical carcinoma), Hep G2 (hepatocellular carcinoma), and T-84 (colon carcinoma) were obtained from the American Type Culture Collection. Mel-1, Mel-21, Hep G2, and HeLa cells were cultured in Eagle’s MEM containing Earle’s salts and 1% L-glutamine (GIBCO-BRL), with 10% fetal bovine serum and 1% nonessential amino acids. T-84 and GP6F2 cells were cultured in a 1:1 mixture of DMEM and Ham’s F-12 nutrient mixture (GIBCO-BRL) with 15% FBS, 1% L-glutamine, and 10% fetal bovine serum. B16, 16/C, and RPMI 8226 cells were cultured in RPMI 1640 with 1% L-glutamine (GIBCO-BRL) and 10% fetal bovine serum. All cells were cultured at 37°C with 85% humidity and 5% CO2.

Lipofectin Transfections. The lipofectin transfection protocol was used for all luciferase reporter gene experiments. Cells were seeded at 50% confluence in 6-well plates and allowed to grow overnight. Immediately before transfection, each well was washed three times with sterile PBS. A single well of a 6-well plate was transfected with a 10 μg liposomes:10–20 μg plasmid DNA ratio, depending on the cell line. Liposome/DNA complexes were prepared according to manufacturer’s instructions. The liposome/DNA complexes were mixed with serum-free media, and a total volume of 700 μl was placed in a single well of a 6-well plate. After incubation at 37°C for 14–16 h, the transfection mixture was aspirated, and 2 ml of complete media was added. The cells were harvested after 48 h, and luciferase activity was determined.

DOTAP/DOPE Transfections. To eliminate possible toxicity associated with the nonhydrolyzable cationic lipid component of lipofectin, we chose an alternative liposome transfection vehicle for the transfections used in the killing experiments and transfection efficiency experiments (Fig. 4; see “Results”). A liposome vehicle consisting of a 1:1 mixture of the cationic lipid DOTAP and the neutral lipid DOPE (Avanti Polar Lipids) has been shown in our laboratory to display transfection properties similar to lipofectin but with less toxicity. DOTAP/DOPE liposomes were prepared by mixing 0.5 mg of DOTAP and 0.5 mg of DOPE and evaporating the chloroform solvent. After the addition of 500 μl of cyclohexane, the mixture was placed on dry ice and lyophilized. One ml of sterile water was added to the powdered lipids, and the solution was vortexed every 5 min for 30 min. Cells were seeded at 30%
confluency in 24-well plates and allowed to grow overnight. Immediately before transfection, each well was washed three times with sterile PBS. To transfect a single well of a 24-well plate, 7.5 μg of DOTAP/DOPE was mixed with 1.875 μg of plasmid DNA and incubated for 15 min. After a 15-min incubation, the lipoamine/DNA complexes were mixed with 266 μl of serum-free media and added to a single well of a 24-well plate. The plates were incubated for 4 h at 37°C, and then the transfection mixture was aspirated and replaced with 500 μl of complete media. With the use of this protocol, no significant toxicity due to transfection was observed. 5-Bromo-4-chloro-3-indoyl-β-D-galactoside staining for transfection efficiency with the use of LacZ constructs was as described previously (28).

**Luciferase Assays.** Each plate was washed three times with PBS, and 100 μl of lysis buffer (Luciferase Assay System, Promega) was added to each well of a 6-well plate. After 15-min incubation at 37°C, the lystate and cell debris were collected. Forty μl of the lystate was added to 100 μl of luciferase assay substrate (Promega) in a clear polystyrene 12 x 75 mm tube and immediately placed in a luminometer (Analytical Luminescence Laboratory model 2010), and light production was measured for 15 s. Some lystate samples were stored at −70°C and could be read up to 7 days later without loss of signal.

**Killing and Proliferation Assays.** Two days after transfection (using DOTAP/DOPE protocol), media was changed and MeP-dR (obtained from Dr. J. A. Secrist, III, Southern Research Institute) was added at a final concentration of 20–30 μg/ml, as described below. Four days later, the cells were fed by adding fresh media with MeP-dR to the wells without removing the old media. Two days later (day 6), the cells were removed with trypsin, washed once with PBS, resuspended, and counted in a 20% solution of trypan blue reagent (trypan blue stain, 0.4%, Gibco-BRL) with the use of a hemacytometer. In some studies, cellular toxicity (percentage of dead cells) was measured by LDH release from dying cells. Proliferation assay (living cell number/well) was performed with the use of a measurement of tetrazolium conversion to formazin during cell growth. Both the LDH release and tetrazolium conversion assays are commercially available as kits designed for these purposes [Cytotox 96 (toxicity) and Cell Titer 96 (proliferation) kits, Promega]. Because these two assays are designed to study approximately 10,000 cells/condition (with the use of 96-well trays), measurements of bystander effects below approximately 1% (100 transduced cells) was effectively limited by difficulty in accurately counting very small numbers of transduced, viable cells (Fig. 2).

**RESULTS**

**Demonstration of Bystander Effect with the Use of Cell Lines with Stable E. coli PNP Expression**

We have demonstrated previously that transient E. coli PNP expression in a human colon cancer cell line is capable of mediating total cell population killing in vitro, even when only 1% of cells express the E. coli PNP gene (28). To titrate the bystander effect in vitro, we developed two melanoma cell lines with stable PNP expression. In initial experiments, growth characteristics were found to be identical for the wild-type and transduced B16 cells in the absence of MeP-dR; the same conclusion was reached with the use of wild-type and transduced 16/C cells (not shown). In Fig. 2, a dose of MeP-dR (20 μg/ml), which is nontoxic in untransduced B16 melanoma or 16/C breast cancer cells, was added to mixed cultures containing an increasing population of transduced, E. coli PNP-expressing cells. Effects on both cell proliferation and cell survival were evaluated in the presence or absence of MeP-dR. In these experiments, concentrations of MeP-dR that had no effect on untransduced (wild-type) B16 or 16/C tumor cells completely eliminated cell proliferation, even when as few as 2% of cells in culture expressed the E. coli PNP gene (Fig. 2). On the basis of an LDH release assay, total population cell killing required that 10% of B16 cells and ≤1% of 16/C cells expressed the gene (not shown). When E. coli PNP activity in the transduced B16 and 16/C cells was assayed by direct enzymatic measurement with the use of cell-free extracts, the activity measured in transduced 16/C cells was approximately 4-fold higher than in B16 cells. [16/C, 10.7 nmol MeP-dR converted/mg cell protein/h (n = 6 measurements on six independent cultures of transduced cells); B16, 2.4 nmol MeP-dR converted/mg cell protein/h (n = 2); background activity in non-transduced 16/C and B16 cells was 0 (n = 4 measurements for each cell line).]

**Demonstration of Tissue Specificity of the Human Tyrosinase Promoter**

A 529-bp segment of the 5′-untranslated region of the human tyrosinase promoter immediately upstream of the start of translation (−451 to +78) was inserted upstream of a luciferase reporter gene (Tyr-Luc; Fig. 1). Luciferase activity in Mel-1 and Mel-21 cells transfected with the Tyr-Luc construct was comparable to luciferase activity generated by transfection with a plasmid utilizing the SV40 early promoter to control luciferase gene expression (SV-Luc) (Fig. 3A). Both negative controls [luciferase without promoter (basic) and luciferase with tyrosinase promoter sequences inserted in the reverse orientation (Rev-Tyr-Luc)] gave the anticipated negative results. Negligible Tyr-Luc activity was seen in five additional human cell lines (T84-colon cancer, U373-glial, HeLa-cervical carcinoma, RPMI 8226-myeloma, GP6F2-prostate), which all showed substantial SV40 driven reporter gene activity (Fig. 3B). In a sixth cell line (Hep G2, derived from human liver) the SV-Luc was 28-fold more active than the Tyr-Luc. However, the Tyr-Luc vector had activity above background in the Hep G2 cells. Because the promoterless luciferase vector resulted in similar luciferase activity, luciferase activity in Hep G2 cells is likely to be nonspecific and due to cryptic promoters or enhancers present within the vector itself, rather than nonspecific regulation by the human tyrosinase promoter.

**High Level Bystander Toxicity with the Use of the E. coli PNP Gene to Generate Toxic Purines**

Use of Tyr-PNP to Kill Melanoma Cells in a Tissue-specific Manner with High Bystander Effect. We tested the relative toxicity of the prodrug MeP-dR and the product MeP on wild-type melanoma cell viability. Mel-1 cells were incubated in various concentrations of MeP-dR and MeP for 5 days. The Mel-1 cells were unaffected by concentrations of MeP-dR as high as 50 μg/ml, whereas concentrations of the MeP as low as 0.5 μg/ml were nearly 100% lethal. Similar results have been obtained in T-84, B16, and 16/C cells. Both MeP-dR and MeP are stable under tissue culture conditions as measured by HPLC analysis of supernatants.

E. coli PNP vectors under the regulatory control of the SV40 promoter (SV-PNP) or the human tyrosinase promoter (Tyr-PNP) were transfected into Mel-1 and T-84 cells (Fig. 4). Forty-eight h after transfection, selected cultures were given 30 μg/ml of MeP-dR. After 6 days of treatment with MeP-dR, the cells were harvested and counted. In the T-84 colon cells (Fig. 4A), over 99% of those cells transfected with the SV-PNP and treated with MeP-dR were killed. MeP-dR had no toxic effect on T-84 cells after either Tyr-PNP or Tyr-Luc transfections, indicating that the Tyr-PNP failed to generate sufficient PNP enzyme in T-84 cells to convert MeP-dR to toxic levels of MeP. In contrast, nearly 100% of Mel-1 cells transfected with either SV-PNP or Tyr-PNP were killed after treatment with MeP-dR (Fig. 4B). No toxicity was observed in either untransfected Mel-1 cells or in cells transfected with Tyr-Luc after treatment with MeP-dR. Cell death under these conditions correlates with the amount of MeP generated by the action of recombinant E. coli PNP on MeP-dR (28).

The transfection of plasmid containing either a cytoplasmic or a nuclear targeted β-galactosidase gene under the same conditions yielded no background luciferase activity in non-transduced 16/C and B16 cells (Fig. 2). Luciferase activity in transduced 16/C cells was approximately 4-fold higher than in B16 cells. [16/C, 10.7 nmol MeP-dR converted/mg cell protein/h (n = 6 measurements on six independent cultures of transduced cells); B16, 2.4 nmol MeP-dR converted/mg cell protein/h (n = 2); background activity in non-transduced 16/C and B16 cells was 0 (n = 4 measurements for each cell line).]
Fig. 2. Effect of MeP-dR and MeP on transduced cells with stable E. coli PNP expression. A, action of the E. coli PNP enzyme on MeP-dR forms the diffusible toxin MeP. B and C, mixing experiments in which transduced and wild-type B16 (B) or 16/C (C) cells were cocultured. Complete abrogation of cellular proliferation was observed when as few as 2% of the cultured cells expressed E. coli PNP under the regulatory control of an SV-40 promoter. A high-level bystander effect also was observed when either B16 or 16/C cells expressed E. coli PNP, as measured by a standard cellular LDH release assay ("Materials and Methods"). Growth characteristics of transduced and wild-type (nontransduced) B16 cells were identical in the absence of drug; the same was true of the wild-type and transduced 16/C cell lines.
DISCUSSION

The experiments described in the present report using murine melanoma and breast cancer cells with stable PNP expression establish that much higher level bystander killing is possible in vitro with the use of E. coli PNP than can be obtained with HSV-tk. Some measure of bystander killing mediated by HSV-tk has already been demonstrated in vivo with the use of established brain, melanoma, sarcoma, pancreas, colon, ovarian, and mesothelial tumors (1, 2, 13-18, 22, 23, 26, 33). Experimental human protocols using HSV-tk are in progress for the treatment of primary brain tumors, central nervous system tumor metastases, and ovarian carcinoma; human trials for treatment of mesothelioma and head and neck cancer using ganciclovir activation by HSV-tk are also under development (34-40). Whereas substantial progress has been made concerning the characterization of bystander killing by HSV-tk in vitro and in vivo, studies from our own laboratory (12) and many others (1, 2, 13-18, 22-25, 33-41) indicate that at least 10-50% of tumor cells must express the viral gene product to establish a pronounced bystander effect. The importance of the development of gene toxin strategies capable of mediating much higher levels of bystander killing is evident because current in vivo gene delivery vehicles (including retrovirus and/or retroviral producer cell line inoculation, liposomes,
replication-deficient adenovirus or adeno-associated virus, and receptor-mediated endocytosis pathways) for most applications are not capable of mediating expression of foreign genes in 10–50% of tumor target cells after either regional or systemic administration (42, 43).

Several transcriptional regulatory elements have been proposed for the purpose of selective gene expression within solid tumors (3, 7–14). Although none of these has yet been shown to be completely tumor restricted, each is believed to provide a significant measure of tumor selectivity. We evaluated a segment of the human tyrosinase promoter for expression within melanoma cells because: (a) the murine tyrosinase regulatory region has been shown previously to mediate melanoma specific expression in vitro and in vivo (10, 22); (b) the human tyrosinase regulatory region has not been characterized in this regard but differs in several predicted regulatory elements from those present in the mouse (45–47); and (c) substantial differences in tissue expression may exist when comparing otherwise homologous human and nonhuman gene-regulatory sequences (48–52). Although regulatory sequences identified by transfection into cultured cells do not always predict in vivo tissue responsiveness, the finding that a short (529-bp) segment of the 5′-untranslated region of the human tyrosinase regulatory region confers tissue specificity in human cell lines is in agreement with the conclusions reached with the use of the murine promoter both in vivo and in vitro. The use of the human tyrosinase promoter to drive the transient expression of the E. coli PNP gene resulted in tissue-specific killing. Although 100% of Mel-1 melanoma cells were killed after transient transfection with Tyr-PNP and treatment with 30 μg/ml MeP-dR, the T-84 colon cells transfected with Tyr-PNP were unaffected by the same concentration of produg.

Poor bystander toxicity using HSV-tk is due to phosphorylation of the ganciclovir produg, which results in a membrane-impermeant toxin. Freeman et al. (15) have suggested a requirement of direct cell contact for the bystander effect to occur with the HSV-tk/ganciclovir system. Generation of a membrane-permeant toxin as described here with E. coli PNP obviates the need for direct cell contact (Figs. 2 and 4). In addition, at least a portion of the bystander effect observed with the use of HSV-tk in vivo appears to depend on immunological clearance of tumor cells not expressing the thymidine kinase gene, utilizing mechanisms that are not well understood (26, 27). Although immune clearance of tumors in vivo may be difficult to predict and/or augment, the pronounced bystander effect observed with the use of E. coli PNP in vitro suggests that efficient in vivo tumor cell killing using E. coli PNP might be accomplished even in the absence of a substantial immune component. On the other hand, the use of membrane-permeant toxins also introduces the possibility of enhanced systemic toxicity after produg activation. Although it seems less likely that local, intratumoral generation of membrane permeant (MeP or other) toxins in vivo would result in toxicity greater than that which is observed after standard, systemic administration of chemotherapeutic agents, issues concerning both in vivo tumor regression and safety can now be addressed with the use of the retroviral lines described in this report.

E. coli cytosine deaminase, which converts 5-fluorocytosine to 5-fluorouracil, has been suggested as a gene superior to HSV-tk for the purpose of enhanced bystander killing (21, 53, 54). Because cytosine deaminase generates a membrane permeant toxin (5-fluorouracil), this approach is anticipated to demonstrate bystander killing. However, substantial bystander killing has been difficult to establish in vitro in mixed cultures, where at least 10–30% of cells must express cytosine deaminase to produce a strong bystander effect (21, 53, 54). One recent study reported tumor regressions in vivo in human colon cancers composed of as little as 2–4% cytosine deaminase-expressing cells, lending support to the general strategy of enzyme/prodrug activation of membrane-permeant toxins within established tumors (21).

A broad range of useful purine nucleoside produgs suitable for the purpose of toxin generation further distinguishes the approach discussed here from the use of HSV-tk. Expression of E. coli PNP within malignant cells could be used to selectively produce a variety of toxic adenine analogues, such as 2-fluorodeoxyadenine, 2-azadenedine, 4-amino- pyrazolo[3,4-d]pyrimidine, etc. Arabinofuranosyl-2-fluorodeoxyadenine, a compound already approved for treatment of human malignancies, is also converted by E. coli PNP to the highly toxic adenine analogue 2-fluorodeoxyadenine. We have recently demonstrated substantial sensitization of tumor cells to arabinofuranosyl-2-fluorodeoxyadenine after the expression of recombinant E. coli PNP. In any case, our present results indicate that even with conventional methods of gene expression, in vitro tumor sensitization with E. coli PNP may be substantially greater than with HSV-tk, and support the development of in vivo models for tumor sensitization to chemotherapeutic drugs using E. coli PNP.

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