Positive selection of gene-modified cells increases the efficacy of pancreatic cancer suicide gene therapy

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Abstract
Thymidine kinase (TK)-mediated suicide gene therapy has been considered for the treatment of pancreatic cancer. However, despite a bystander effect, the proportion of transduced tumor cells has proven too low to result in efficacy. We propose the use of a drug-selectable marker (MDR1) to enrich TK-expressing cells using chemotherapy. This enrichment or positive selection phase may increase the efficacy of suicide gene therapy. To test this strategy, we generated stable NP18MDR/TK-GFP transfecants and showed docetaxel resistance in vivo. Mixed tumors of MDR/TK-expressing cells and parental NP18 cells were established and docetaxel was used to increase the proportion of TK-expressing cells. After this positive selection phase, suicide gene therapy with ganciclovir was applied. Upon positive selection, the proportion of TK-expressing cells increased from 4% to 22%. Subsequent suicide gene therapy was more effective compared with a control group without positive selection. Starting with 10% of TK-expressing cells the positive-negative selection strategy completely inhibited tumor growth. Taken together, these results suggest that a positive-negative selection strategy based on MDR and TK genes represents an efficient way to increase the proportion of TK-expressing cells in the tumor and the efficacy of TK-mediated suicide gene therapy. [Mol Cancer Ther 2009;8(11): 3098–107]

Introduction
Pancreatic cancer is the fourth leading cause of cancer-related death in most industrialized countries, with an average survival after diagnosis of 3 to 6 months (1). Conventional treatments for pancreatic cancer are scarcely effective, and the development of new therapeutic approaches is of major interest. Suicide or prodrug-converting cancer gene therapy may represent a good approach if potent inducers of cell death and efficient gene transfer systems are found. The herpes simplex virus thymidine kinase (HSV-TK) gene is the prodrug-converting gene therapy system most widely used in suicide gene therapy (2). Expression of HSVTK within tumor cells selectively sensitizes them to the antiviral agent ganciclovir. The basis of the selective sensitivity is caused by preferential monophosphorylation of nontoxic ganciclovir by the viral thymidine kinase gene (TK). Cellular kinases further phosphorylate the drug into triphosphates forms that become incorporated into nascent DNA, inhibiting DNA synthesis and leading eventually to cell death (3). In addition to selective sensitization, a further advantage of this system is the bystander cytotoxic effect on nontransduced cells associated with intercellular transfer of phosphorylated ganciclovir to surrounding cells (4). This suicide gene therapy has shown therapeutic efficacy in preclinical studies (5), but poor gene transfer efficiency has limited clinical efficacy (6). Better delivery vectors (3) or cell carriers (7, 8) may allow the transfer of enough suicide genes to eliminate all tumor cells. A new approach to improve suicide therapy efficacy is based on conferring a selective advantage to transduced cells (9, 10).

Conventional chemotherapy is the standard treatment for most types of advanced cancers. Docetaxel has been widely used in the treatment of cancers, including advanced pancreatic adenocarcinoma (11). Unfortunately, the initial response to this agent may be hampered by the development of multidrug-resistant cells (12). Multidrug resistance was first described in 1970 after selection in Chinese hamster ovarian cancer cells exposed to increasing concentrations of actinomycin D (13). The resistance to chemotherapy has been termed “intrinsic” when the tumor initially fails to respond to anticancer agents and “acquired” when failure occurs after exposure to chemotherapeutic agents to which they are initially sensitive. Both intrinsic and acquired drug resistance can be obstacles to pancreatic adenocarcinoma chemotherapy (12). The most important cause of chemoresistance is the intrinsic or acquired overexpression of multidrug resistance gene1 (MDR1; ref. 12). MDR1 encodes the membrane-bound P-glycoprotein, which was associated
with poor prognosis (14). However, negative \textit{MDR1} expression in a tumor does not necessarily guarantee the success of chemotherapy, because during tumor progression, or due to the effect of treatment itself, the P-glycoprotein may be induced (15). On the other hand, multidrug resistant genes have been used in chemoprotective gene therapy or as selectable markers \textit{in vitro}. Human hematopoietic cells have been transferred with chemoresistant genes to protect bone marrow from the adverse effects of high-dose chemotherapy (16). To confer biosafety to this strategy, hematopoietic cells were transfected with a vector coexpressing \textit{MDR1} and \textit{TK} genes, which offers the possibility to kill undesirable \textit{MDR}-transfected tumor cells (17).

Pancreatic carcinoma cell lines, like other neoplasms, are heterogeneous with regard to their sensitivity to chemotherapeutic agents and may contain different expressions of drug-resistant phenotypes (intrinsic chemoresistant cells). Moreover, malignant cells continuously change their phenotype (15), and better adapted tumor cells gain selection advantages (acquired chemoresistant cells). Some experimental data show that an originally \textit{MDR1}-negative tumor may induce overexpression of \textit{MDR1} and become resistant after chemotherapeutic treatments (18). The acquired resistance usually arises several weeks after treatment. In this time-window tumor cells that have intrinsic \textit{MDR1} overexpression or that induce it faster show a proliferative advantage against sensitive tumor cells.

We aimed to use the \textit{MDR1} gene as a drug selectable marker to confer a selective advantage to \textit{TK}-expressing cells to increase their proportion in the tumor. Under chemotherapy, \textit{MDR}/\textit{TK} expressing cells should be enriched, and a later ganciclovir treatment should be more efficacious. To apply this strategy in a poor prognostic tumor type, a pancreatic adenocarcinoma model sensitive to docetaxel was used. Starting with tumors with 10\% of \textit{TK}-expressing cells, after chemotherapy treatment, modified cells colonized the entire tumor and suicide therapy eradicated tumors.

\section*{Materials and Methods}

\subsection*{Cell Culture and Reagents}

The human pancreatic adenocarcinoma cell line NP18 was established in our laboratory (19) and grown in DMEM containing 5\% fetal bovine serum and 1\% of penicillin and streptomycin (P/S). Cells were maintained at 37°C and 5\% CO₂ in a humidified atmosphere and were periodically tested for mycoplasma. Taxotere (docetaxel) was provided by Sanofi Aventis S.A. A 10 μg/mL stock solution was prepared in a polysorbate buffer according to the manufacturer’s protocols and selected with 0.625 μmol/L of methotrexate (MTX) and 4 μg/mL of docetaxel. To label NP18MDR/TK with green fluorescent protein (GFP), the packaging cell line PA317DsGFP (kindly provided by Fernando Larcher, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, CIEMAT, Madrid, Spain) was used to produce a Moloney leukemia vector expressing GFP. Cells were grown in DMEM 10\% fetal bovine serum, and medium was replaced when cells reached 70\% to 80\% of confluence and PA317DsGFP conditioned medium was used to infect NP18MDR/TK cells in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich) and DMEM 5\% fetal bovine serum. Twelve hours after infection, medium was replaced with fresh medium and cells were seeded in a 96-well plate at 2 to 3 cells/well and incubated with 0.625 μmol/L of MTX and 4 μg/mL of docetaxel. After 21 d of selection and under fluorescent microscopy we obtained NP18MDR/TK-GFP clones.

\subsection*{In vitro Characterization of NP18MDR and NP18MDR/TK-GFP Cells}

To examine the expression of human P-glycoprotein on the cell surface of N18, NP18MDR and NP18MDR/TK-GFP cells, fluorescence activated cell sorting (FACS) analysis was used in which cells were incubated with a human P-glycoprotein–specific monoclonal antibody MRK16 (21). Cells (5 × 10⁵) were trypsinized, washed, and incubated with MRK16 (10 μg/mL in a volume of 50 μL). After 30 min of incubation cells were washed twice with PBS (+Ca²⁺/Mg²⁺)/1\% bovine serum albumin and incubated with Alexa Fluor 647 goat anti-mouse IgG for 1 h (1:300 dilution in a volume of 100 μL; Invitrogen Molecular Probes). After washing twice with PBS (+Ca²⁺/Mg²⁺)/1\% bovine serum albumin, cells were resuspended in 300 μL of PBS (+Ca²⁺/Mg²⁺)/1\% bovine serum albumin and a minimum of 50,000 cells were analyzed by flow cytometry (Becton Dickinson, FACSCalibur system) using 488 nm and 635 nm lasers and CellQuest Pro software. MDR expression was determined in quadruplicates.

To determine docetaxel sensitivity of NP18, NP18MDR, and NP18MDR/TK-GFP transfectants, cells were plated in 96-well plates (5 × 10³ cells/well) and incubated with serial dilutions of docetaxel (0, 0.8, 1.5, 3, 6, 12, 24, 49, 97.5, 195, 391, 781, 1,562, 3,125, 6,250, 12,500, 25,000, and 50,000 pg/mL). Cell viability was measured by the MTT assay kit (Sigma-Aldrich) 7 d after treatment according to the manufacturer’s protocol. The percentage of cell viability was defined by the equation \textit{T}/\textit{NT} \times 100, where \textit{T} is the cell viability of treated cells and \textit{NT} is the cell viability of nontreated cells.
In vivo Docetaxel Sensitivity of NP18 and NP18MDR/TK-GFP Cells

To check in vivo docetaxel sensitivity of NP18 and NP18MDR/TK-GFP cells, s.c. xenografts were established into the flanks of 4- to 8-week-old male BALB/c nu/nu mice by inoculation of 2 to 4 × 10^6 NP18 cells or NP18MDR/TK-GFP cells. The animals were hosted in the animal facility of the Institut d’Investigacions Biomèdiques de Bellvitge (IDIBELL), in Barcelona, Spain (Association for Assessment and Accreditation of Laboratory Animal Care International, AAALAC unit 1155) and were kept and manipulated in accordance with recommendations of IDIBELL’s Ethical Committee of Animal Experimentation. Once tumors reached 100 to 200 mm^3, the animals started docetaxel treatment (25 mg/kg ip once every week for 2 wk) or PBS treatment (n = 8–10). They were measured two or three times weekly. Tumor volume was calculated according to the equation V = π/6 WL^2, where L and W are length and width of the tumor, respectively, +SE. The significance of differences in tumor volume rate between treatment group and control group was assessed by the unpaired Student’s t test.

In vivo Positive Selection with Docetaxel

To allow for in vivo positive selection two different initial proportions of NP18MDR/TK-GFP cells were used. S.c. xenografts were established into the flanks of 4- to 8-week-old male BALB/c nu/nu mice by inoculation of a mixture of 2 to 4 × 10^6 cells containing 4% or 10% of NP18MDR/TK-GFP cells mixed with 96% or 90% of parental NP18 cells, respectively. Once tumors reached 100 to 200 mm^3, the animals were randomly distributed in two groups (docetaxel and PBS; n = 14). Then the animals were treated with PBS or with docetaxel (25 mg/kg ip once every week) for 2 wk in mice with tumors with an initial proportion of 4% NP18MDR/TK-GFP cells or 3 wk in mice with 10% NP18MDR/TK-GFP tumors. Tumors were measured two or three times weekly until they began to regrow. Tumor volume was calculated as above. Percentage of tumor growth was defined by the equation \((V-V_0)/V_0\) × 100, where \(V_0\) is the tumor volume on day 0 after starting the treatment. The significance of differences in tumor growth rate between treatment group and control group was assessed by the unpaired Student’s t test. At the beginning
of docetaxel treatment \((n = 4–6)\) and after the positive selection phase \((n = 14)\) animals were sacrificed and tumors were excised and fixed with 4% paraformaldehyde in PBS overnight. After washing with PBS, the tumors were incubated with sucrose 5% in PBS (2 h) and sucrose 30% in PBS (overnight). Then the tumors were cryopreserved with OTC embedding medium (Miles Laboratories, Elkhart, IN) and cryosectioned at 5 μm with a cryotome (Global Medical Instrumentation, Shandon Cryotome Cryostat). Sections were mounted with mounting medium with 4′,6-diamidino-2-phenylindole (Vectorshied, Vector Laboratories, Inc.) and GFP expression was viewed using a fluorescent microscope (Olympus BX51). GFP quantification was done on three fields \((×100)\) of each section by an external observer blind to the source of the samples. Representative images \((×100)\) were taken from different tumors of each group.

**In vitro Ganciclovir-Sensitivity of NP18 and NP18MDR/TK-GFP Cells**

NP18 and NP18MDR/TK-GFP cells were plated in 96-well plates \((5 \times 10^3 \text{ cells/well})\) and were incubated with serial dilutions of ganciclovir \((0, 0.2, 0.6, 1.3, 2.5, 5, 10, 20, 40, \text{ and } 80 \mu \text{g/mL})\). Cell viability was measured by the MTT assay kit 6 d after treatment according to the manufacturer’s protocol. The percentage of cell viability was defined as in the previous experiments.

**In vitro and in vivo Bystander Effect of NP18MDR/TK-GFP Cells with Ganciclovir**

To allow for negative selection, in vitro and in vivo bystander experiments were carried out. NP18 and NP18MDR/TK-GFP cells were plated in 96-well plates at different proportions of transfected cells \((0, 0.8, 1.5, 3.1, 6.25, 12.5, 25, 50, \text{ and } 100%; 5 \times 10^3 \text{ cells/well})\) and were incubated with 10 μg/mL of ganciclovir. Cell viability was measured by MTT as above 6 d after treatment and the percentage of cell viability was defined.

To do a bystander experiment in vivo, s.c. xenografts were established into the flanks of 4- to 8-week-old male BALB/c nu/nu mice by inoculation of 2 to 4 \times 10^6 NP18 cells, NP18MDR/TK-GFP cells, or a mixture containing 25% or 50% NP18MDR/TK-GFP cells mixed with NP18 cells. Once tumors reached 100 to 200 mm³, the animals were treated with ganciclovir \((50–75 \mu \text{g/Kg i.p. daily for } 14 \text{ d}; n = 8)\). Tumors were measured two or three times weekly.

**In vivo Positive Selection with Docetaxel and Negative Selection with Ganciclovir**

The combination of positive and negative selection in vivo was done using the same proportions of NP18MDR/TK-GFP cells as in the previous positive selection experiments. S.c. xenografts with 4% or 10% of NP18MDR/TK-GFP cells were established. Once tumors reached 100 to 200 mm³, the animals were randomly distributed in four groups (PBS, ganciclovir, docetaxel plus PBS, or docetaxel plus ganciclovir; \(n = 10\)). Then the animals were treated with PBS or with docetaxel \((25 \mu \text{g/kg i.p. once every week})\) for 2 wk in mice with tumors with an initial proportion of 4% NP18MDR/TK-GFP cells or for 3 wk in mice with tumors containing an initial proportion of 10% of modified cells. A control group that did not receive previous docetaxel treatment was treated with ganciclovir \((50–75 \mu \text{g/kg i.p. daily for } 18 \text{ d})\). Tumors were measured two or three times weekly, and when they began to regrow and reached 200 to 300 mm³ the animals were also treated with ganciclovir \((50–75 \mu \text{g/kg i.p. daily for } 18 \text{ d})\). At the end of the experiment some tumors of the docetaxel plus ganciclovir group were excised, and tumor cells were extracted by mechanical disruption and plated. GFP-expressing cells were quantified for each tumor in a fluorescence microscope. Then the cells were plated in 96-well plates \((5 \times 10^3 \text{ cells/well})\) and incubated with ganciclovir \((10 \mu \text{g/mL})\). Cell viability was measured by MTT 6 d after treatment and the percentage of cell viability was determined.

**Results**

**In vitro Characterization of NP18MDR and NP18MDR/TK-GFP Cells**

We generated the NP18MDR/TK-GFP cell line to achieve positive-negative selection of transfected cells. The MDR gene was transfected to allow for positive selection with docetaxel and TK to confer sensitivity to ganciclovir. To

![Figure 2. In vivo docetaxel sensitivity of NP18 and NP18MDR/TK-GFP cells. A, in vivo docetaxel sensitivity in NP18 tumors. S.c. xenografts were established by inoculating NP18 cells in nude mice. Once tumors were established, the animals started docetaxel \((\text{black squares})\) or PBS \((\text{white triangles})\) treatment \((n = 8–10)\). Arrows, days of docetaxel treatment. Tumor volumes of the docetaxel group and the PBS group are shown. Bars, + SE. B, in vivo docetaxel sensitivity in NP18MDR/TK-GFP tumors. S.c. xenografts were established as in the experiment above by inoculation with NP18MDR/TK-GFP cells. Once tumors were established, animals started docetaxel \((\text{black squares})\) or PBS \((\text{white triangles})\) treatment \((n = 8–10)\). Tumor volumes of the experimental groups are shown. *, statistically significant differences \((P < 0.05)\). Bars, + SE.](image)
facilitate the follow-up of the selected cells, NP18MDR/TK cells were transduced with GFP recombinant retrovirus (NP18MDR/TK-GFP). We first determined the expression of human P-glycoprotein on the cell surface of N18, NP18MDR, and NP18MDR/TK-GFP cells. FACS analysis shows P-glycoprotein overexpression in NP18MDR and NP18MDR/TK-GFP cells compared with NP18 cells (Fig. 1A).

Figure 3. In vivo positive selection with docetaxel. A, a mixture containing 4% or 10% of NP18MDR/TK-GFP cells and 96% or 90% parental NP18 cells, respectively, were injected s.c. in nude mice. Once tumors were established, the animals were randomly distributed in two groups (n = 14): docetaxel (DOCE; black squares) and PBS (white triangles). Docetaxel treatment started when tumors were established. Arrows, days of docetaxel treatment. Tumor growth of the experimental groups is shown. *, statistically significant differences with control group (P < 0.00001). Bars, +SE. B, increase of NP18MDR/TK-GFP cells in tumors after positive selection with docetaxel. At the beginning of the treatment (n = 4–6) and after positive selection with docetaxel (n = 14) the animals were sacrificed and tumors were processed for fluorescent microscope. Sections were analyzed and GFP expression was viewed and quantified using a fluorescent microscope. The proportion of GFP cells of experimental groups is shown. Bars, +5D. C, three images (×100) of three representative tumors from each group.
To determine if this overexpression of P-glycoprotein conferred docetaxel resistance, an *in vitro* sensitivity experiment to docetaxel was done with parental and modified cell lines and IC₅₀ was calculated. The IC₅₀ of NP18 and NP18MDR/TK-GFP cells are indicated at the bottom of the figure. Bars, + SD. **A**, *in vitro* ganciclovir sensitivity. NP18 (white bars) and NP18MDR/TK-GFP cells (black bars) were plated in 96-well plates and were incubated with serial dilutions of ganciclovir (0–80 μg/mL). Six days after treatment, cell viability was measured by MTT. The IC₅₀ of NP18 and NP18MDR/TK-GFP cells are indicated at the bottom of the figure. Bars, + SD. **B**, *in vitro* bystander effect of NP18MDR/TK-GFP cells with ganciclovir. NP18 and NP18MDR/TK-GFP cells were plated in 96-well plates at different proportions of transfected cells (0, 0.8, 1.5, 3.1, 6.25, 12.5, 25, 50, and 100%) and were incubated with ganciclovir. Six days after treatment, cell viability was measured by MTT. Bars, + SD. **C**, *in vivo* bystander effect of NP18MDR/TK-GFP cells with ganciclovir. S.c. xenografts were established by inoculation of NP18 cells, NP18MDR/TK-GFP cells, or a mixture containing 25% or 50% of NP18MDR/TK-GFP cells and NP18 cells. Once tumors were established, the animals were treated with ganciclovir (n = 8). Tumor volume is shown. *, statistically significant differences (P < 0.05). Bars, + SE.

To assess the selective pressure achieved *in vivo* with docetaxel in our NP18 model, we first determined the docetaxel sensitivity of NP18 cells *in vivo*. Mice with NP18-implanted tumors were treated with 25 mg/kg of docetaxel i.p. once every week for 2 weeks and showed statistically significant reduction of tumor size (P < 0.05; Fig. 2A). During docetaxel treatment weight loss did not...
exceed 10% of body weight, no mice died, and there were no gross signs of toxicity.

To do a preliminary test of the growth advantage of the NP18MDR/TK-GFP cells under selective pressure, we determined whether NP18MDR/TK-GFP cells were resistant to docetaxel in vivo. Mice with NP18MDR/TK-GFP tumors were treated with docetaxel, and no reduction of tumor size was observed (Fig. 2B). These preliminary experiments showed that NP18 cells were sensitive to docetaxel and that NP18MDR/TK-GFP cells were resistant. These results suggested that positive selection might be done in mixed tumors with NP18 and NP18MDR/TK-GFP cells.

**In vivo Positive Selection with Docetaxel**

To allow for positive selection in vivo, we established NP18 s.c. tumors containing 4% or 10% of NP18MDR/TK-GFP cells. The animals were distributed randomly in two groups and were given docetaxel or PBS. Tumor growth in the docetaxel-treated group was statistically lower compared with the control group (Fig. 3A; \( P < 0.00001 \)). The positive selection phase was considered as the period between the beginning of the docetaxel treatment and the time the tumors regrew. During this period, NP18MDR/TK-GFP cells should have a proliferative advantage compared with parental NP18 cells resulting in tumor repopulation by MDR/TK-expressing cells. To evaluate the enrichment of NP18MDR/TK-GFP cells during the positive selection, tumor sections were analyzed by fluorescent microscopy and the percentage of GFP cells was estimated in the tumors of the docetaxel or PBS group (Fig. 3B). A clear enrichment of GFP-positive modified cells upon docetaxel treatment from 4% and 10% until more than 20% and 80%, respectively, was observed, whereas tumors in the PBS group maintained the initial proportion of GFP-expressing cells. Figure 3C shows three images (×100) of three representative}

![Graph](image-url)
tumors from the pretreated group (animals before docetaxel treatment) compared with three tumors from the docetaxel group (tumors after positive selection) and from the PBS group.

**Negative Selection with Ganciclovir**

Once positive selection was achieved, the *in vitro* ganciclovir sensitivity of NP18 and NP18MDR/TK-GFP cells was assessed. The NP18MDR/TK-GFP cells were sensitive to ganciclovir (IC$_{50} < 0.3 \mu$g/mL) whereas the parental NP18 cells were not (IC$_{50} = 54 \mu$g/mL; Fig. 4A). We observed a slight decrease in cell viability of parental NP18 cells incubated with ganciclovir (concentrations between 0.3 and 20 $\mu$g/mL). To evaluate the potency of the TK/ganciclovir system a bystander effect experiment was done *in vitro*. More than 60% of the cell culture was eliminated with only 3.1% of NP18MDR/TK-GFP cells in the monolayer; with 12.5% of the modified cells, 90% of the cells were dead (Fig. 4B). These *in vitro* results indicate that NP18MDR/TK-GFP cells could be used for negative selection with ganciclovir. To assess if the proportion of NP18MDR/TK-GFP cells achieved after positive selection (22% and 80% from 4% and 10% initial modified cells, respectively) could eliminate tumors *in vivo*, a bystander effect experiment was done. Mice with implanted tumors containing a mixture of different proportions of NP18MDR/TK-GFP and NP18 cells were treated with ganciclovir. A clear bystander effect was observed in all mixed tumors (50% and 25% NP18MDR/TK-GFP cells; $P < 0.05$; Fig. 4C). During ganciclovir treatment, weight loss did not exceed 10% of body weight, no mice died, and there were no gross signs of toxicity.

This *in vivo* bystander effect experiment showed that the proportion of NP18MDR/TK-GFP cells achieved after positive selection was enough to induce a strong bystander effect on nonmodified NP18 cells and to abrogate tumor growth. We then did a therapy based on positive-negative selection of modified cells.

**In vivo Positive and Negative Selection Strategy**

To assess the potency of our positive-negative selection therapy we carried out an experiment with the two different proportions of gene-modified cells used in the positive selection experiments. We established NP18 s.c. tumors containing 4% or 10% of NP18MDR/TK-GFP cells. The animals were distributed randomly in groups and were given docetaxel or PBS. As in the positive selection experiments, tumor volume in the docetaxel group was statistically lower compared with the control group (Fig. 5A and B; $P < 0.0004$). After this phase animals were treated with ganciclovir. Ganciclovir treatment should eliminate NP18MDR/TK-GFP cells and induce a bystander effect that leads to the death of surrounding NP18 cells (negative selection). Ganciclovir treatment without a previous positive selection period (ganciclovir group) did not have any therapeutic effect in tumors with an initial proportion of 4% of modified cells. In the experiment with an initial proportion of 10% of modified cells, the tumor volume of the ganciclovir group was statistically different compared with the control group ($P < 0.0006$; Fig. 5B). Despite this early therapeutic effect, tumors of this group began to regrow at day 22, and the animals were sacrificed at day 32 for ethical reasons. This indicates that 10% of modified cells homogenously distributed in the tumor were not enough to eliminate all the tumor cells in the absence of a positive selection period. As we expected, the positive selection phase increased the proportion of modified cells in tumors. Upon ganciclovir treatment, this enrichment resulted in a higher therapeutic efficacy (Fig. 5A; $P < 0.04$), allowed an almost complete regression of tumors, and avoided tumor relapse (Fig. 5B; $P < 0.0005$). No additional toxicity was observed associated with combined treatment with docetaxel plus ganciclovir. To understand why tumors did not disappear completely, the remaining cells from tumors after positive and negative selection were extracted and plated from remaining tumors. The proportion of GFP-expressing cells was determined by fluorescence microscopy, and tumors with different proportions of GFP-expressing cells were analyzed. The sensitivity to ganciclovir was tested for different disaggregated tumors, and the results showed that GFP-modified cells remained ganciclovir sensitive (data not shown). Tumors without GFP-expressing cells were also analyzed, and the morphology of the remaining cells suggested that they were fibroblasts (data not shown).

**Discussion**

Suicide gene therapy efficacy depends on the transduction of a high proportion of tumor cells and a strong bystander effect *in vivo*. Although many groups are improving the delivery vectors [adenovirus (3), retrovirus and cell carriers (22)] and bystander effect of suicide genes (23) to increase their therapeutic effect, the results of clinical trials indicate a need for greater efficacy (24).

We provide proof of principle that a selectable marker such as MDR1 could be used to compensate the poor gene transfer of suicide genes *in vivo*, allowing an enrichment of modified tumor cells and improving suicide therapy efficacy. In this way, chemoresistant genes could be used for positive selection of suicide gene sensitive cells *in vivo*. Our selectable marker was P-glycoprotein, the MDR1 gene product, that confers resistance to a variety of chemotherapeutic agents like anthracyclines, vinca alkaloids, epipodophytoxins, and taxane, but not to gemcitabine and 5-fluorouracil, which are the only agents clinically useful against pancreatic cancer (25). On the contrary, P-glycoprotein increases sensitivity to gemcitabine, which is the current first-line therapy for pancreatic cancer (26). Thus, the use of MDR1 as a drug selectable marker in this type of cancer may be a reasonable option from a clinical point of view. In addition, multidrug-resistant cells do not show cross-resistance to the TK/ganciclovir system (17), so the MDR1/TK combination is safe.

The positive-negative selection strategy was tested in a pancreatic adenocarcinoma model because this tumor type shows a very bad prognosis and new therapeutic strategies need to be explored. The NIH18 model (19) represents a tumor that is sensitive to docetaxel treatment. Classic chemotherapies such as docetaxel have been in disuse in...
pancreatic cancer because they did not show any benefit in patient survival. However, we expect that the cytotoxic effect of these chemotherapies is sufficient to induce a replacement of tumor cells (9). Our hypothesis is based on the evidence that under chemotherapy, resistant tumor cells replace sensitive cells very efficiently. In a clinical setting, this quick replacement prevents any patient survival improvement, but in our positive selection with docetaxel we aimed to replace chemosensitive tumor cells with ganciclovir-sensitive cells. Moreover, the NP18 tumor model was also sensitive to doxorubicin and etoposide, whereas MDR1/TK cells were resistant to these agents (data not shown). These results suggest that in our NP18 model different chemotherapies could be used to carry out positive selection with the MDR1 genes. This approach only requires tumors to be sensitive to a drug that is not used in the standard clinical treatment. Hence, this strategy could be done in other types of cancer such as colon, ovarian, lung, prostate, breast, and liver cancers to eliminate chemoresistant tumor cells. Recently, in our laboratory, the positive-negative strategy was also done with dihydrofolate reductase (DHFR) as a selectable marker and thymidine kinase as a suicide gene in a colon cancer model (HCT116) partially sensitive to MTX (10).

Upon positive selection with docetaxel starting with 4% and 10% of TK-modified cells, the proportion of TK-modified cells increased 6- to 8-fold, respectively, whereas no enrichment occurred in PBS-treated controls. According to in vivo bystander effect experiments mixing cells, tumor growth could be abrogated with a proportion of only 25% of TK-expressing cells in the tumor. After positive selection, we achieved a proportion of 22% of TK-expressing cells from the 4% initial proportion. However, tumor growth could not be abrogated with ganciclovir with such a 22% of modified cells. One explanation could be that the enrichment of TK-expressing cells was not homogeneous in all the tumors compared with the mixing experiments. The enrichment of TK-expressing cells was more homogeneous starting with 10% of NP18MDR/TK-GFP cells than with 4%. In this experimental setting and after positive and negative selection we could abrogate tumor growth of all the tumors upon ganciclovir treatment.

The positive-negative selection strategy has a precedent in the work of Unger and collaborators (9). A fusion gene of cytosine deaminase (CD) and uracil phosphoribosyl transferase (UPRT; FCU1 fusion gene) was used along with N-(phosphonacetyl)-L-aspartate (PALA) and cytosine to enrich the transduced cells. 5-Fluorocytosine was used as a prodrug for negative selection of transduced cells. Starting from s.c. tumors with a 6% proportion of modified cells, the administration of PALA and cytosine to mice allowed an enrichment of 5-fluorocytosine-sensitive cells and a decreased tumor growth after 5-fluorocytosine treatment. Some differences between our work and such strategy are worth discussing. To confer positive selection the PALA-cytosine strategy required PALA and cytosine to reach the tumor mass. Although high levels of cytosine plasma levels were obtained in vivo they decreased very fast. Therefore, the time frame in which both agents are available limits the ability of cytosine to rescue PALA-induced toxicity. On the other hand, our approach only used one agent for positive selection. Furthermore, although modified cells grew clonally, our strategy resulted in an almost complete replacement of tumor cells by fibroblasts may induce a misleading interpretation of the response to the treatment.
We have proved here the concept that a drug resistance gene can be used to enrich suicide gene–expressing cells in a tumor. Our next main challenge is to transfer the MDR1/TK construct to tumor cells and transduce them stably at a level of 10% of transfection. Reasonable options to transport these genes are episomal plasmids (33), adenovirus, or adeno-associated vectors.

In conclusion, the current study proposes a novel strategy to enrich suicide gene–expressing cells in the tumor mass and improve the efficacy of suicide therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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