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## Liposomal insulin promoter–thymidine kinase gene therapy followed by ganciclovir effectively ablates human pancreatic cancer in mice

James X. Wu<sup>a</sup>, Shi-He Liu<sup>a</sup>, John J. Nemunaitis<sup>b</sup>, and F. Charles Brunicardi<sup>a,\*</sup>

<sup>a</sup>David Geffen School of Medicine, UCLA, Los Angeles, CA, USA

<sup>b</sup>Mary Crowley Cancer Research Centers, Dallas, TX, USA

### Abstract

PDX1 is overexpressed in pancreatic cancer, and activates the insulin promoter (IP). Adenoviral IP–thymidine kinase and ganciclovir (TK/GCV) suppresses human pancreatic ductal carcinoma (PDAC) in mice, but repeated doses carry significant toxicity. We hypothesized that multiple cycles of liposomal IP-TK/GCV ablate human PDAC in SCID mice with minimal toxicity compared to adenoviral IP-TK/GCV. SCID mice with intraperitoneal human pancreatic cancer PANC-1 tumor implants were given a single cycle of 35 µg iv L-IP-TK, or four cycles of 1, 10, 20, 30, or 35 µg iv L-IP-TK (n = 20 per group), followed by intraperitoneal GCV. Insulin and glucose levels were monitored in mice treated with four cycles of 35 µg iv L-IP-TK. We found that four cycles of 10–35 µg L-IP-TK/GCV ablated more PANC-1 tumor volume compared to a single cycle with 35 µg. Mice that received four cycles of 10 µg L-IP-TK demonstrated the longest survival ( $P < 0.05$ ), with a median survival of 126 days. In comparison, mice that received a single cycle of 35 µg L-IP-TK/GCV or GCV alone survived a median of 92 days and 68.7 days, respectively. There were no significant changes in glucose or insulin levels following treatment. In conclusion, multiple cycles of liposomal IP-TK/GCV ablate human PDAC in SCID mice with minimal toxicity, suggesting non-viral vectors are superior to adenoviral vectors for IP-gene therapy.

### Keywords

Pancreatic cancer; Gene therapy; PDX1; Insulin promoter; Thymidine kinase

### Introduction

Pancreatic cancer remains a terrible and challenging disease, representing only 2.8% of new cancers but ultimately responsible for 6.8% of all cancer deaths [1]. Pancreatic cancer has demonstrated remarkable resistance to conventional therapies. Only a quarter of patients with localized disease survive 5 years beyond diagnosis [1].

\*Corresponding author. Tel.: +1 310 562 1635. CBrunicardi@mednet.ucla.edu. .

**Conflict of interest** The authors declare that there is no conflict of interest.

Pancreatic duodenal homeobox 1 (PDX1) is a gene specific to pancreatic tissue, is aberrantly expressed in pancreatic cancer, and facilitates oncogenesis [2,3]. Taking advantage of PDX1-mediated stimulation of the insulin promoter, we previously developed a novel recombinant gene therapy that targets PDX1 expressing cells: a recombinant rat insulin promoter–viral thymidine kinase (IP-TK) gene [4]. Aberrant PDX1 expressed by pancreatic cancer cells would bind and activate the insulin promoter, driving the expression of viral thymidine kinase, sensitizing them to ganciclovir (GCV) [4,5]. Previously, we demonstrated adenoviral IP-TK/GCV therapy ablates human pancreatic cancer in vitro and in vivo in SCID mice [4,6–9].

Adenoviral IP-TK/GCV therapy is limited by several factors. These include toxicity to healthy tissue, resulting in hyperglycemia with repeat dosing, as well as reduced efficacy of repeat doses due to induction of viral antigen targeting antibodies [8]. Even a single virus infusion will result in antibody development, which has led to myriad strategies to circumvent this therapeutic obstacle [10–13]. Given that a number of studies have demonstrated successful gene delivery to cancer cells using non-viral liposomal vectors [14–16], we hypothesized that multiple cycles of liposomal naked IP-TK DNA followed by GCV can efficiently ablate human PDAC in mice with less toxicity and retain efficacy with repeat dosing.

## Materials and methods

### Cell lines, plasmid vectors and antibodies

The human pancreatic cancer cell line PANC-1 was purchased from the American Type Culture Collection (ATCC, Bethesda, MD), and was maintained in DMEM medium (Invitrogen, MD) supplemented with 100,000 units/l of penicillin, 100,000 µg/l of streptomycin and 10% fetal bovine serum. Plasmid DNA IP-lacZ and IP-TK were constructed as described previously [7,17]. Rabbit and goat anti-HSV-TK antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cy3 conjugated anti-rabbit IgG antibodies were purchased from Sigma (St. Louis, MO).

### Preparation of liposomal IP-TK complex (L-IP-TK)

L-IP-TK was prepared fresh at room temperature. Formulated liposomes (Gradalis Inc, at Dallas, TX) (DOTAP or DOTAP:chol) were diluted to a final concentration of 4 mM in 300 µl final volume with 5% dextrose in water. Plasmid DNA was diluted to a concentration of 1 µg/µl, and then mixed with an equal volume of 4 mM DOTAP to give a final concentration of 50 µg/100 µl.

### Animals and gene delivery

SCID mice were housed in a BL-4 facility and cared for under the guidelines in *The Care and Use of Laboratory Animals* manual prepared by the Institute of Laboratory Animal Resources, the Commission on Life Science, the National Research Council, and the Animal Research Committee of Baylor College of Medicine.

To investigate the maximally tolerated dose of liposomal empty vector DNA, male SCID mice 8–10 weeks of age ( $n = 5$ ) were given one cycle of 20, 30, 35, 40, 50, 60, 70, or 80  $\mu\text{g}$  of liposomal empty vector DNA. Mice were observed for 26 days.

To simulate intraperitoneal metastatic pancreatic cancer, male SCID mice 8–10 weeks of age were inoculated with  $0.5 \times 10^5$  PANC-1 cells by intraperitoneal injection. This model of metastatic pancreatic cancer has been used in our previous studies [7]. These mice were randomized to seven groups ( $n = 20$ ): (1) 1 cycle of 35  $\mu\text{g}$  iv L-IP-TK/GCV, (2) 4 cycles of 1  $\mu\text{g}$  iv L-IP-TK/GCV, (3) 4 cycles of 10  $\mu\text{g}$  iv L-IP-TK/GCV, (4) 4 cycles of 20  $\mu\text{g}$  iv L-IP-TK/GCV, (5) 4 cycles of 30  $\mu\text{g}$  iv L-IP-TK/GCV, (6) 4 cycles of 35  $\mu\text{g}$  iv L-IP-TK/GCV, and (7) 40 mg/kg iv GCV without IP-TK. Each cycle consisted of liposomal IP-TK iv injection on the first day followed by 2 weeks of GCV (40 mg/kg body weight twice daily) and 1 week of rest.

### Tumor evaluation and survival analysis

Necropsy and tumor evaluation were performed at 77 and 120 days after treatment. At least five mice were sacrificed at each time point. Tissues were saved for further immunohistochemical analyses. Tumor volume was evaluated at each time point. Peritoneal tumors were evaluated macroscopically and microscopically and the larger (A) and smaller (B) diameters measured and recorded. Tumor volume (V; a rotational ellipsoid) was calculated according to the formula:  $V (\text{mm}^3) = A (\text{mm}) \times B^2 (\text{mm}^2) \times 0.5$ . Mice were classified according to presence or absence of tumor. Mouse survival was measured from the date of initial treatment to date of death or sacrifice.

### Immunohistochemistry

Pancreata and tumors were removed and fixed in 4% paraformaldehyde at 4 °C for 4 hours at the time of necropsy. Tissues were embedded in paraffin and tissue sections were prepared. For immunostaining, sections were deparaffinized in xylene and hydrated gradually through graded alcohol. Slides were then placed in a humidified chamber, overlaid with 1:100 diluted antibody against HSV-TK, and incubated overnight at 4 °C. After washing with PBS, slides were incubated with Cy3-conjugated rabbit antibody for 1 hour at room temperature. Slides were then washed with PBS and mounted with cover slides.

### Detection of apoptosis in tumor xenografts and the islet cells of mice

Apoptosis in tumor and pancreatic specimens was determined with TUNEL assay (FragEL DNA Fragmentation Detection Kit, Colorimetric-TdT Enzyme; Calbiochem, La Jolla, CA) according to the manufacturer's protocol and expressed as the ratio of apoptotic cancer cells to the total number of endothelial cells in 10 fields at 100 $\times$  magnification. To evaluate the effect of L-IP-TK/GCV on the endocrine pancreas, at least 10 islets per specimen were evaluated.

### Insulin and glucose measurements

For the four cycles of 35  $\mu\text{g}$  iv L-IP-TK treatment group, 50  $\mu\text{l}$  whole blood samples were collected from each mouse and spun to separate the serum at days 14, 35, 56, and 77. This group was selected for serum insulin and glucose measurements because these mice

received the greatest amount of L-IP-TK therapy. Serum samples were stored at  $-20^{\circ}\text{C}$  until completion of experiments. Glucose levels and insulin levels were measured as reported previously [18].

### Statistical analysis

The unpaired Student's *t*-test was used for statistical analyses of tumor volume, glucose, and insulin levels, with  $P < 0.05$  indicating significance. The  $\chi^2$  test was used for rate comparison. Log rank test was used to compare the mice survival data. Kaplan–Meier in SPSS 15.0 for Windows was used to plot survival curves.

## Results

### Cytotoxicity of empty vector liposomal DNA

SCID mice that received 35  $\mu\text{g}$  or less of liposomal empty vector plasmid DNA had a 100% survival rate at 26 days. Mice that received 40  $\mu\text{g}$  of liposomal empty vector DNA had a 50% survival rate at 26 days, and doses 50  $\mu\text{g}$  and higher resulted in 0% survival at 26 days.

### Multiple doses of L-IP-TK/GCV reduce or ablate xenograft pancreatic tumor volume in mice

Four cycles of L-IP-TK/GCV, 10–35  $\mu\text{g}$ , significantly reduced more PANC-1 tumor volume compared to a single cycle of 35  $\mu\text{g}$ . Four cycles of 35  $\mu\text{g}$  iv L-IP-TK/GCV resulted in the smallest remaining tumor volume among all treatment groups (versus four cycles of 30  $\mu\text{g}$  L-IP-TK, next smallest mean tumor volume,  $P = 0.017$ ). See Table 1, Fig. 1. There was no significant difference in residual tumor volume among 10, 20, or 30  $\mu\text{g}$  L-IP-TK treatment groups after 4 cycles. Complete tumor ablation was noted in 50% of the 10 and 20  $\mu\text{g}$  L-IP-TK treatment groups, and 57.1% of the 30  $\mu\text{g}$  and 35  $\mu\text{g}$  L-IP-TK treatment groups.

### Multiple doses of L-IP-TK/GCV improve survival in mice

Four cycles of 10  $\mu\text{g}$  L-IP-TK/GCV resulted in the longest survival in study mice, for a median survival of 126 days (versus all treatment groups  $P < 0.05$ ; the  $P$  values of four cycles of 10  $\mu\text{g}$  iv L-IP-TK versus a single cycle of 35  $\mu\text{g}$ , as well as four cycles of 1  $\mu\text{g}$ , 10  $\mu\text{g}$ , 20  $\mu\text{g}$ , and 30  $\mu\text{g}$  of L-IP-TK were 0.035, 0.017, 0.028, 0.029, and 0.018, respectively) (Fig. 2). For median survival of each treatment group, see Table 1.

### Multiple doses of L-IP-TK did not significantly affect pancreatic islets

Following multiple treatments with 35  $\mu\text{g}$  iv L-IP-TK, there was no significant alteration in serum glucose or insulin levels (Fig. 3). TUNEL assay revealed extensive apoptosis in tumor tissue following L-IP-TK/GCV therapy, but no evidence of apoptosis in pancreatic islets. When control liposomal empty vector DNA/GCV therapy was given, no apoptosis was noted in tumor tissue (Fig. 4).

## Discussion

Multiple doses of liposomal IP-TK/GCV successfully ablated human pancreatic cancer in SCID mice and prolonged survival. The longest median survival was seen in a lower dose of L-IP-TK, 10  $\mu\text{g}$ , with 50% of tumors completely ablated. Improved survival in the 10  $\mu\text{g}$

group versus higher doses indicates toxicity, albeit minimal, and begins to outweigh the benefit of greater tumor-killing efficacy with higher doses. Tumor killing efficacy was maintained with repeated dosing and was more effective than a single dose. All treatment groups that received multiple cycles of 10, 20, 30 or 35  $\mu\text{g}$  of L-IP-TK therapy were able to reduce tumor volume significantly more than a single dose of 35  $\mu\text{g}$ . Finally, there was also no alteration in serum glucose or insulin levels even at the highest dose of 35  $\mu\text{g}$  of L-IP-TK. TUNEL assay demonstrated apoptosis of tumor tissue with sparing of the murine pancreatic islets following therapy.

Compared to our prior adenoviral IP-TK/GCV therapy, L-IP-TK/GCV was able to effectively treat human pancreatic cancers in mice with significantly less toxicity [4]. After four doses of adenoviral IPTK, mice developed severe hyperglycemia and hypoinsulinemia with evidence of islet cell apoptosis [17]. Thus, the main drawback of adenoviral IP-TK was the off-target effect of therapy on pancreatic islets. While adenoviral IP-TK therapy caused an increase in serum glucose to 200–400 mg/dl, serum glucose did not exceed 140 mg/dl during L-IP-TK therapy [7]. The sparing of pancreatic islet function by L-IPTK therapy is likely partially due to decreased transfection efficiency of liposomal compared to adenoviral gene delivery, which has been demonstrated previously [19–21]. Nonetheless, L-IP-TK still maintained the ability to ablate human PDAC in mice. The residual tumor volume following adenoviral IP-TK DNA was 3.2 mm<sup>3</sup> [7], whereas four cycles of 10  $\mu\text{g}$  of L-IP-TK DNA resulted in a residual tumor volume of 5.7 mm<sup>3</sup>. Thus, these findings represent a significant advancement in delivery of targeted gene therapy to the pancreas, and support the use of non-viral vectors for targeting gene therapy to the pancreas with the insulin promoter.

Liposomal naked DNA gene delivery also avoids another obstacle of adenoviral gene delivery, which is the formation of anti-adenoviral antibodies. Adenoviral IP-TK induces dose-dependent increases in anti-adenoviral antibodies in immune competent mice with a subsequent blunting of therapeutic efficacy; when adenoviral CMV-LacZ was given to mice previously exposed to the adenoviral vector, study mice only demonstrated 0.4% of the initial response [8]. Even with liposomal encapsulation, a second dose of adenoviral CMV-LacZ in sensitized mice only achieved 3% of the initial response [8]. Thus, even though liposomal encapsulation significantly increases the effect of repeat adenoviral gene therapy, there is still a significant reduction in efficacy despite liposomal protection. In stark contrast to adenoviral vectors, multiple doses of 10  $\mu\text{g}$  of naked DNA liposomal IP-TK was more effective than a single dose of 35  $\mu\text{g}$  L-IP-TK, suggesting subsequent doses of liposomal RIP-TK maintain therapeutic efficacy.

The lack of effect of L-IP-TK on pancreatic islets would suggest its biodistribution is different from that of adenoviral IP-TK. Previous studies established the specificity of insulin promoter-driven gene delivery to pancreatic cells [4,17]. However, when the insulin promoter was combined with a LacZ reporter gene, staining was also observed to a much lesser degree in the liver, gut, spleen, lungs, salivary gland, and brain [17]. Further studies are required to investigate whether liposomal IP-TK has a similar distribution when delivered intravenously. This mouse model also demonstrated that L-IPTK has the ability to ablate peritoneal tumor implants of human pancreatic cancer in mice. However, the mice were not inspected for distant metastases in other organs. Further studies are required to

determine whether intravenous L-IP-TK can successfully transfect distant metastases, and whether the level of transfection is sufficient for therapeutic efficacy.

Multiple cycles of L-IP-TK/GCV significantly improved the therapeutic effect of reducing human PDAC tumors in SCID mice. Most importantly, L-IP-TK allows repeat doses with no significant effect on islet function, which were two significant drawbacks to using adenoviral-IP-TK. Further studies are indicated to evaluate the biodistribution of L-IP-TK in mice and whether L-IP-TK can treat *de novo* pancreatic cancer lesions.

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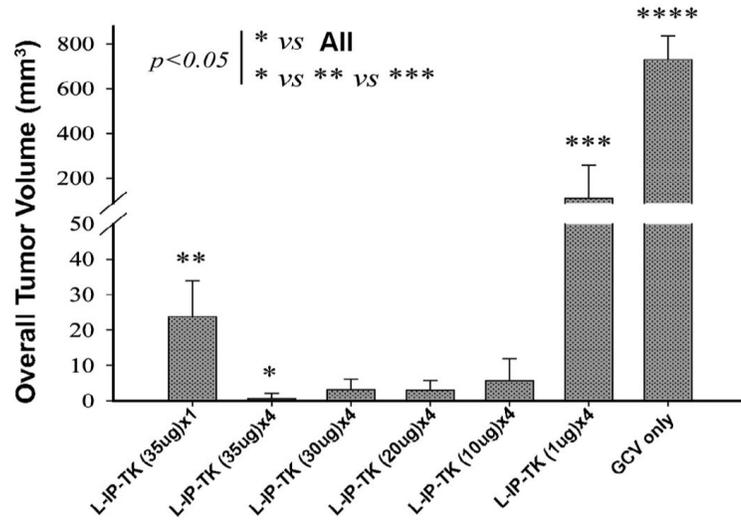
## Abbreviations

<b>IP</b>	insulin promoter
<b>TK</b>	thymidine kinase
<b>GCV</b>	ganciclovir
<b>PDAC</b>	pancreatic ductal adenocarcinoma

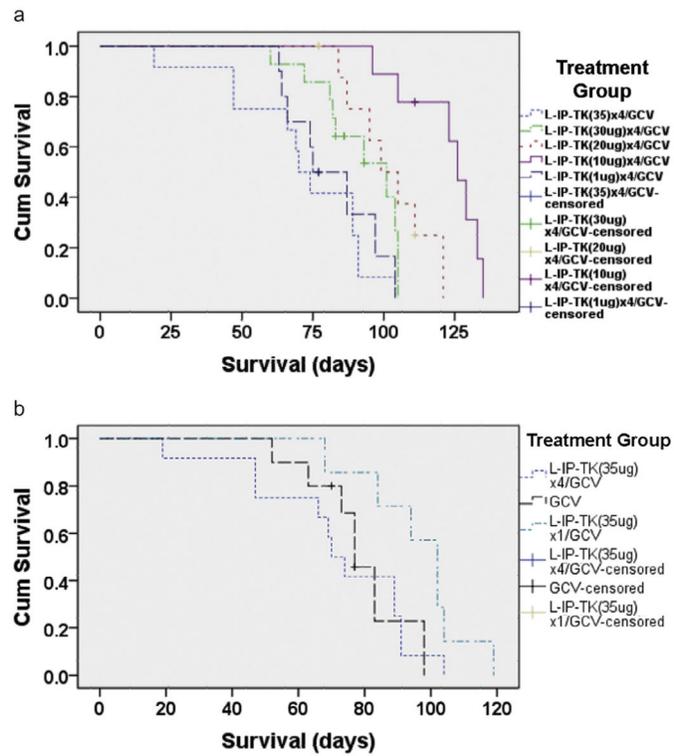
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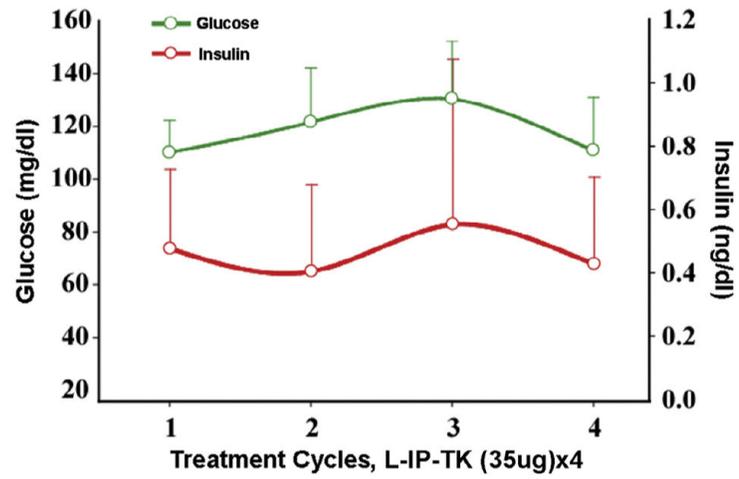
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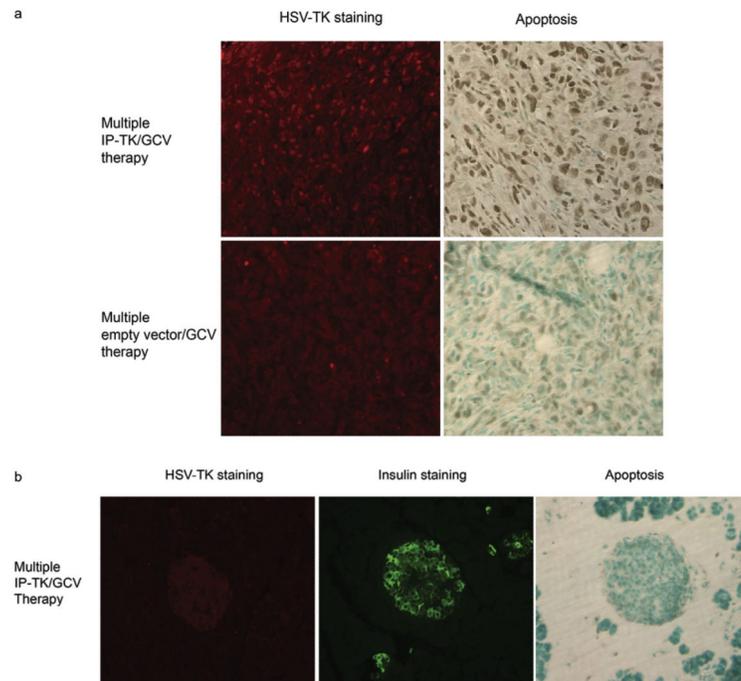
**Fig. 1.**  
Effect of L-IP-TK dose on tumor volume.



**Fig. 2.**  
(a, b) Kaplan–Meier survival curves per treatment group.



**Fig. 3.**  
Glucose and insulin levels per treatment cycle with IP-TK ( $35 \mu\text{g} \times 4$ ).



**Fig. 4.** TUNEL assay of mouse pancreata following L-IP-TK/GCV therapy. (a) Cells at 200× magnification. Left: HSV-TK staining demonstrating expression of HSV-TK following L-IP-TK therapy (red), and no expression with empty vector DNA; right: TUNEL assay demonstrating apoptosis of tumor cells with L-IP-TK therapy (brown), and none with empty vector DNA. (b) Cells at 200× magnification. Left: HSV-TK staining demonstrating no expression in pancreatic islet cells; center: insulin expression noted in pancreatic islet cells (green); right: TUNEL assay demonstrating absence of apoptosis in pancreatic islet cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**

Mean tumor volume and median survival after L-IP-TK/GCV therapy.

<b>Treatment group</b>	<b>Mean final tumor volume (mm<sup>3</sup>)</b>	<b>Median survival (days)</b>
GCV 40 mg/kg, without L-IP-TK	730.14 ± 107.01	68.7 ± 5.1
L-IP-TK 35 µg, single cycle	23.78 ± 10.19	92 ± 4.4
L-IP-TK 1 µg, four cycles	109.40 ± 148.51	75 ± 7.7
L-IP-TK 10 µg, four cycles	5.70 ± 6.25	126 ± 3.7
L-IP-TK 20 µg, four cycles	3.00 ± 2.78	99 ± 7.0
L-IP-TK 30 µg, four cycles	3.12 ± 2.97	101 ± 11.8
L-IP-TK 35 µg, four cycles	0.56 ± 1.62	70 ± 4.3