Intralesional injection of Rose Bengal augments the efficacy of gemcitabine chemotherapy against pancreatic tumors

Patrick Innamarato[1,2], Jennifer Morse[1], Amy Mackay[1], Sarah Asby[1], Matthew Beatty[1], Jamie Blauvelt[1], Scott Kidd[1], John E Mullinax[1,4], Amod A. Sarnaik[1,3], and Shari Pilon-Thomas[1]

[1]Department of Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA; [2]Cancer Biology Ph.D. Program, University of South Florida, Tampa, Florida, USA; [3]Department of Cutaneous Oncology, H. Lee Moffitt Cancer Center, Tampa, Florida, USA; [4]Sarcoma Department, H. Lee Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL 33606, USA.

Abstract

Chemotherapy regimens that include the utilization of gemcitabine are the standard of care in pancreatic cancer patients. However, most patients with advanced pancreatic cancer die within the first 2 years after diagnosis, even if treated with standard of care chemotherapy. In this study, we demonstrate that the injection of PV-10, a 10% solution of rose bengal, into mouse pancreatic tumors caused lesion-specific ablation. We show that the combination of intralesional PV-10 with the systemic administration of gemcitabine caused lesion-specific ablation and delayed the growth of untreated distal tumors. We observed that this treatment strategy was markedly more successful in immunogenic tumors that express the neoantigen, ovalbumin (OVA), suggesting that the combination therapy enhanced the immune clearance of tumors. Moreover, the regression of tumors in mice that received PV-10 in combination with gemcitabine was associated with increases in damage associated molecular patterns (DAMPs), HMGB1, S100A8, and IL-1α. Together, these results demonstrate that intralesional therapy with PV-10 can enhance the efficacy gemcitabine against pancreatic tumors.

Methods

Cell lines and cell culture

Panc02 pancreatic cancer (obtained from ATCC), were cultured in RPMI media supplemented with 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 50 mg/ml gentamicin, 0.5 mg/ml fungizone (all from Life Technologies, Rockville, MD), and 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, MO). To generate the ovalbumin (OVA) expressing fluorescent Panc02 cell line, cells were exposed to supernatants containing a lentiviral vector comprised of a fluorescent ZsGreen (ZsG) protein and OVA. Upon successful transfection, ZsGreen^{hi} tumor cells were subjected to FACS using BD FACSAria. OVA-ZsGreen^{hi} tumor cells were passaged *in vitro* 4 times whereby OVA expression was validated by staining for H2-K^{b} bound to SIINFEKL peptide (25-D1.16, BioLegend). CFPAC1, MiaPaca2, Panc-1, and SU8686 cells (obtained from ATCC) were grown and maintained in culture according to supplier guidelines. The cell lines tested negative for mycoplasma contamination. All cell lines were passaged less than 10 times after initial revival from frozen stocks. All cell lines were validated in core facilities prior to use.

Apoptosis and cell death detection

Human and murine pancreatic tumor cells were cultured in 12 well plates and grown to ~60% confluency. Then, the indicated concentrations of PV-10 were added to media and cells were cultured for 24hrs. Adherent cells were collected by gentle scraping and pooled with non-adherent cells. Cells were washed 3 times in PBS to remove excess PV-10. Washed cells were then stained with Annexin-V APC and DAPI (both from BioLegend) and analyzed on a BD FACSCelesta to determine the frequency of apoptotic and dead cells.

Mouse models and treatment

Female C57BL/6 mice (6–8 weeks old) were purchased from Harlan Laboratories. Panc02 and Panc02OVA-ZsGreen tumor cells (5x10^4) were implanted subcutaneously into one flank of a mouse to establish a single tumor. To establish a bilateral tumor model, tumor cells were implanted in the opposite flanks. On day 7, a single tumor was treated with intralesional PV-10. Gemcitabine (60mg/kg) was injected intraperitoneally twice per week for 2 weeks. Mice were housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute. Mice were humanely euthanized by CO_2 inhalation and cervical dislocation according to the American Veterinary Medical Association Guidelines. Mice were observed daily and were humanely euthanized if a solitary subcutaneous tumor exceeded 300 mm^2 in area, evidence of ulceration, or mice showed signs referable to metastatic cancer. All animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the U.S. Public Health Service policy and National Research Council guidelines.

Detection of DAMPs in mouse serum

Blood was collected at the termination of experiments. Blood specimens were centrifuged at 2000xg for 10 minutes at room temperature to separate serum from other blood content. The abundance of HMGB1 was determined by HMGB1 ELISA (IBL International); HSP70 and IL-1α were determined by Human/Mouse/Rat Total HSP70/HSPA1A DuoSet IC ELISA and Mouse IL-1 alpha/IL-1F1 Quantikine ELISA Kit (both from R&D Systems, a Biotechne brand); S100A8 and S100A9 were determined by Mouse Magnetic Luminex Assay (R&D Systems, a Biotechne brand) and analyzed on the Luminex 100 (LuminexCorp).

Statistical Analysis

Graphs were generated using GraphPad Prism software. Graphs represent mean values with SEM. P values were calculated in each respective figure where statistical tests were indicated. For mouse-tumor growth studies, tumor growth curves are shown as mean with SEM and significance was determined by 2-way ANOVA and Sidak's multiple comparison's test. Mice were randomized after tumor cell implantation into respective treatment groups. For all other experiments, data were compared using either an unpaired 2-tailed Student's t-test corrected for multiple comparisons by a Bonferroni adjustment or Welch's correction. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001; ns= not significant.

Acknowledgements

This work was supported in part by the H. Lee Moffitt Cancer Center Support Grant P30 CA076292. We also thank the Comparative Medicine Department of University of South Florida. This work was supported by a sponsored research agreement from Provectus Inc. to the H Lee Moffitt Cancer Center and Research Institute. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Results

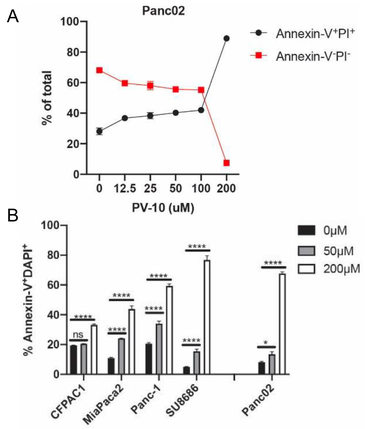

Figure 1. PV-10 kills mouse and human pancreatic tumor cells *in vitro*. (A) (B) Percentage of Annexin-V$^+$PI$^+$ human or mouse pancreatic tumor cells after 24hr incubation with PV-10.

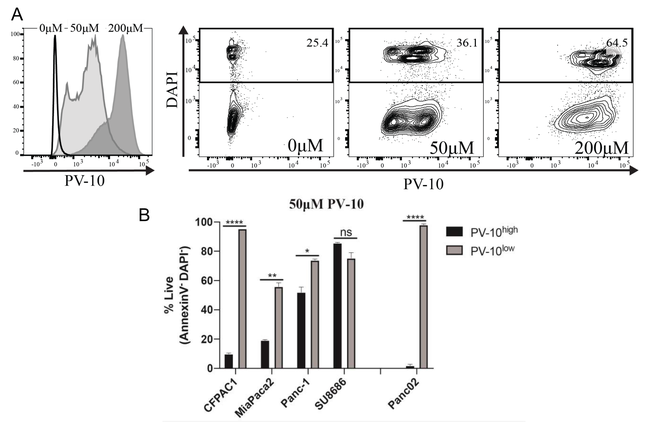

Figure 2. PV-10 saturation is an indicator of cell death (A) Staining intensity of PV-10 on Panc-1 cells (histogram on far left) and the frequency of DAPI$^+$ dead cells (dot plots to the right of the histogram) after incubation with 50μM or 200μM PV-10. (B) Percentage of live PV-10$^{high/low}$ cells treated with 50μM PV-10.

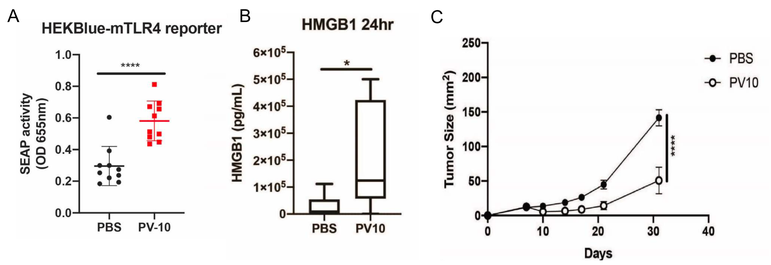

Figure 3. Effect of PV-10 against murine Panc02 tumors *in vivo*. (A) Serum collected from mice treated with i.l. PBS or i.l. PV-10 were collected 24hrs after treatment. Serum was cultured with HEKBlue-mTLR4 reporter cells overnight. (B) HMGB1 in the sera of mice treated with i.l. PBS or i.l. PV-10. (C) Tumor growth of treated tumors with i.l. PBS or i.l. PV-10.

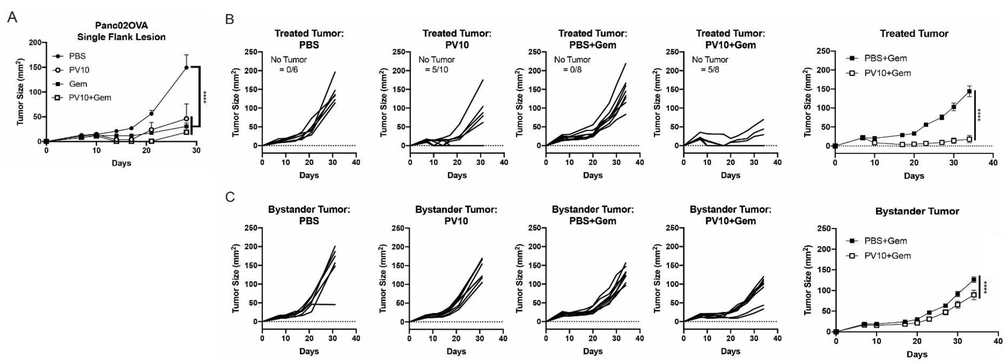

Figure 4. Panc02OVA tumors are responsive to PV-10, gemcitabine combination therapy. (A) Tumor growth in mice bearing a single Panc02OVA tumors treated with i.l. PV-10 ± i.p. gemcitabine (Gem). (B-C) Tumor growth curves of individual mice with bilateral Panc02OVA tumors. (B) Tumor growth curves for mice treated with i.l. PBS or i.l. PV-10 ± i.p. Gem. Summary of growth among treated tumors with i.l. PBS or i.l. PV-10 in combination with Gem (far right). (C) Growth curves for uninjected contralateral tumors among individual mice. Summary of growth among contralateral tumors in mice that received i.l. treatment in the opposite flank in combination with i.p. Gem (far right). Sample size is indicated on each graph in (B). The rate of complete regression is also indicated on each graph in (B).

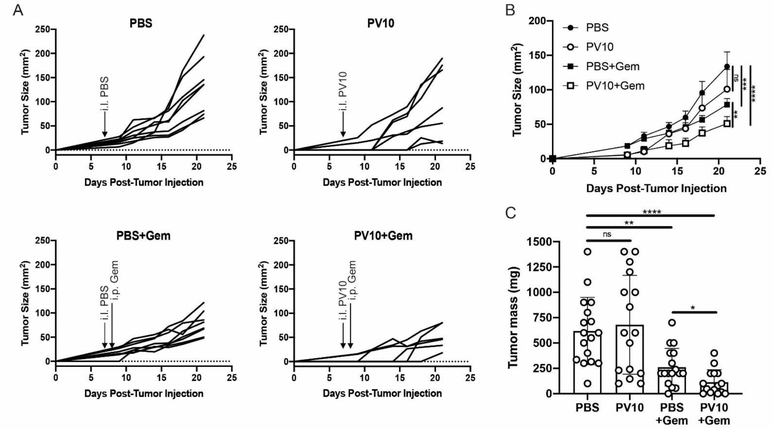

Figure 5. Combination therapy with PV-10 and gemcitabine induces regression of Panc02 tumors. (A) Individual tumor growth curves in mice that received i.l. PBS (top left), i.l. PV-10 (top right), i.l. PBS + i.p. Gem (bottom left), i.l. PV-10 + i.p. Gem (bottom right). (B) Summary of tumor growth curves from (A) (n=6-8 per group). Data are representative of 2 independent experiments (C) The mass of tumors at the termination of the experiment. Data are a compilation of 2 independent experiments (n=16-17 per group).

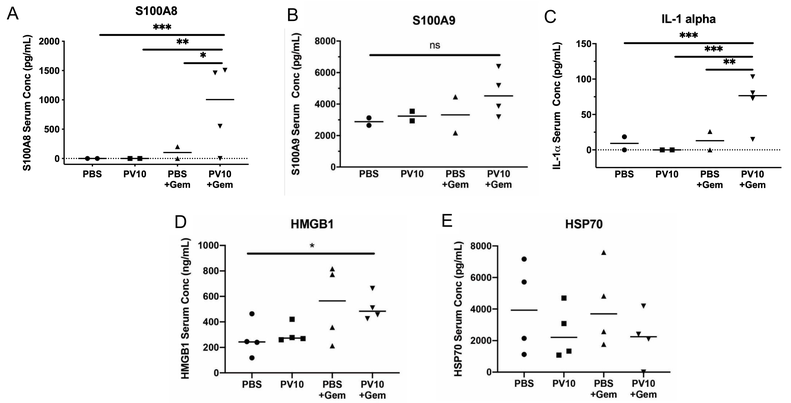

Figure 6. PV-10 and gemcitabine combination therapy increases the abundance of DAMPs in circulation. (A) S100A8, (B) S100A9, (C) IL-1α, (D) HMGB1, and (E) Hsp70 were measured in the sera of mice 9 days after the initiation of treatment. (n=2-4 mice per group).

Conclusions

- PV-10 kills human and murine pancreatic tumor cells *in vitro*
- PV-10 & gemcitabine combination therapy reduces the growth rate of murine Panc02 tumors
- Immunogenic Panc02OVA tumors are more sensitive to PV-10 monotherapy
- PV-10 & gemcitabine combination therapy reduce the growth of uninjected, bystander Panc02OVA tumors
- Reduced tumor growth after PV-10 treatment is associated with the release of DAMPs