PG545, an angiogenesis and heparanase inhibitor, reduces primary tumor growth and metastasis in experimental pancreatic cancer

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Abstract

Aggressive tumor progression, metastasis and resistance to conventional therapies lead to an extremely poor prognosis for pancreatic ductal adenocarcinoma (PDAC). Heparanase, an enzyme expressed by multiple cell types including tumor cells in the tumor microenvironment, has been implicated in angiogenesis and metastasis, and its expression correlates with decreased overall survival in PDAC. We evaluated the therapeutic potential of PG545, an angiogenesis and heparanase inhibitor, in experimental PDAC. PG545 inhibited the proliferation, migration and colony formation of pancreatic cancer cells in vitro at pharmacologically relevant concentrations. Heparanase inhibition also reduced the proliferation of fibroblasts but had only modest effects on endothelial cells in vitro. Furthermore, PG545 significantly prolonged animal survival in intraperitoneal and genetic models (mPDAC: LSL-KrasG12D; Cdkn2aΔlox/Δlox; p48Cre) of PDAC. PG545 also inhibited primary tumor growth and metastasis in orthotopic and genetic endpoint studies. Analysis of tumor tissue revealed that PG545 significantly decreased cell proliferation, increased apoptosis and reduced microvessel density, disrupted vascular function and elevated intratumoral hypoxia. Elevated hypoxia is a known driver of collagen deposition and tumor progression; however tumors from PG545 treated animals displayed reduced collagen deposition and a greater degree of differentiation compared to control or gemcitabine treated tumors. These results highlight the potent anti-tumor activity of PG545 and support the further exploration of heparanase inhibitors as a potential clinical strategy for the treatment of PDAC.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is characterized by rapid tumor growth, late presentation, early metastasis and significant resistance to conventional treatments. Surgical resection provides a potential cure; however, 80% of PDAC cases are unresectable at the time of diagnosis and the majority of resected patients succumb to recurrent disease (1). Intense desmoplasia, an exuberant deposition of extracellular matrix (ECM) interferes with drug delivery and is one of the causative factors in treatment failure in PDAC (2). Gemcitabine (Gem), a deoxycytidine nucleoside analog is currently a standard treatment for advanced and metastatic PDAC. However, gemcitabine is only modestly effective in 20-30% of patients with a median progression-free survival of 5.7 months (3). Therefore, recognizing novel targets and exploring new therapeutic strategies are needed to improve outcome for patients diagnosed with PDAC.

A vascular supply is essential for the progression of primary and metastatic solid tumors including PDAC. Targeting tumor angiogenesis is a well-established strategy for the treatment of cancer. Numerous anti-angiogenic agents including bevacizumab, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody (4, 5); marimastat, a matrix metalloproteinase inhibitor (6); and various tyrosine kinase inhibitors (TKIs) (7) have been tested clinically in PDAC with limited survival benefit (8). Furthermore, the strategy of inhibiting angiogenesis has been questioned due to recent studies that have shown that anti-angiogenic strategies slow primary tumor growth but can promote metastasis in animal models of cancer, including PDAC (9-11). Therefore, future studies on anti-angiogenic approaches in PDAC should include agents that can combat metastasis as well as blood vessel growth. Heparanase is an enzyme found in the tumor microenvironment that has been implicated in angiogenesis and metastasis (12, 13). Heparan sulfate (HS) proteoglycans are key components of the ECM, which regulate several aspects of cancer biology, including angiogenesis, tumor progression and
metastasis (14, 15). Cleavage of HS by the endo-β-glucuronidase heparanase facilitates inflammation, angiogenesis and metastasis (12). This occurs through the regulation of several heparin bound growth factors such as VEGF, fibroblast growth factor (FGF)-1, FGF-2, epidermal growth factor (EGF) and hepatocyte growth factor (HGF), all of which are known to promote carcinogenesis (16, 17). Heparanase is known to participate in the progression of PDAC and elevated levels of heparanase in PDAC patients correlate with worse overall survival (18, 19). Heparanase expression is elevated in patients with dedifferentiated histology of the primary tumor and correlated with increased incidence of lymph node metastasis suggesting the importance of heparanase in tumor progression (20). Also, despite negative margins after potentially curative resection, patients that express high levels of heparanase in their resected tumors have a worse post-operative survival (34 versus 17 months), again suggesting that heparanase expression confers a more aggressive tumor phenotype (21).

HS mimetics such as PG545 function by simultaneously blocking HS-mediated growth factor signaling leading to angiogenesis (22) and inhibiting heparanase (23) from facilitating metastasis. PG545 is a synthetic, fully sulfated HS mimetic that has anti-angiogenic and anti-metastatic properties. In preclinical studies PG545 induces anti-tumor and anti-metastatic effects in breast and hepatocellular cancers (24, 25). In the present study, the anti-tumor activity of PG545 was evaluated in robust mouse models of PDAC where it induced potent reductions in ECM deposition, angiogenesis and metastasis.
Materials and Methods:

Cell lines

Human pancreatic cancer cell lines (AsPC-1, MiaPaca-2, HPAF-II, BxPC-3), human fibroblast cell line WI-38 and human umbilical vein endothelial cells (HUVECs) were all purchased from the American Type Culture Collection. Colo357 cells were obtained from the UT MD Anderson Cancer Center. The murine pancreatic cancer cell line Pan02 (Panc02) was obtained from the National Cancer Institute. Cell lines were confirmed to be pathogen-free and human cell lines were authenticated to confirm origin before use. Cells were cultured in DMEM (Invitrogen) or RPMI (Invitrogen) containing 10% fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO2 and 95% air.

Western blot analysis

Sub-confluent monolayers of cells were lysed, supernatants were recovered by centrifugation at 13000 rpm, protein concentrations were measured and equal amounts of total protein were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA) followed by blockade for 1 hour in 5% milk in TBS-T. The membranes were incubated overnight at 4°C with heparanase 1 antibody (Cell Sciences, Canton, MA). The membranes were then incubated with the corresponding HRP-conjugated secondary antibody (Pierce Biotechnologies, Santa Cruz, CA) for 1 to 2 hour. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film.

Cell viability assay

Cell viability was evaluated by the colorimetric WST-1 assay. The measurement is based on the ability of viable cells to cleave the sulfonated tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases. Cells (4,000 cells per well) were
plated in a 96-well plate in regular growth medium, and after 16 hours the medium was replaced with low serum containing medium. After 5 hours of incubation, the cells were treated with PG545. The range of concentrations used (500 nM to 10 μM) for PG545 was comparable to clinically achievable concentrations. After 72 hours, 10 μl WST-1 reagent was added in each well followed by additional incubation for 2 hours. The absorbance at 450 nm was measured using a microplate reader.

**Scratch assay**

Wound closure (scratch) assays were performed in 12-well plates. Monolayers of PanO2 cells or HUVECs in low serum media were scratched once per well with a P20 pipette tip to create an artificial wound. Cells were treated with different concentrations of PG545 and photographed at 0, 6, 12, 24 and 48 hours after wounding. Wound closure was measured as a percentage of original wound width.

**Anchorage-independent growth assay**

Twelve well plates were coated with 0.75% agar. Tumor cells (2000 cells/well) were resuspended in 0.375% agar with increasing concentrations of PG545 (0.5 – 10 μM) and added to the bottom agar layer. Fresh media with or without PG545 was added weekly and colonies were measured by light microscopy after 4 weeks by staining with 0.005% crystal violet.

**Animal Studies**

All animals were housed in pathogen-free facility with access to food and water ad libitum. Experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. C57BL/6 mice and NOD-SCID mice were purchased from an on campus supplier. At the time of sacrifice, orthotopic tumors were removed en bloc to determine primary tumor burden. Gross metastatic events were identified through visual
inspection of lymph node basins, liver, peritoneum, diaphragm and spleen. Samples were fixed in 10% formalin or snap frozen in liquid nitrogen for further studies.

**Intraperitoneal survival model.** Six to 8 week old NOD/SCID mice were injected intraperitoneally (i.p.) with 7.5 x 10^5 AsPC-1 cells as described (26). Mice were randomized and two weeks post tumor cell injection animals were injected i.p. with saline (control), Gem (100 mg/kg twice weekly) or PG545 (10 mg/kg weekly) (n=6-7 animals/group) for two weeks. Animals were weighed twice weekly and euthanized when moribund.

**Genetic model of pancreatic ductal adenocarcinoma.** LSL-Kras^{G12D}; Cdkn2a^{lox/lox}, p48^{cre} (mPDAC) mice (27, 28) were genotyped shortly after birth and randomized to receive saline (control), Gem (12.5 mg/kg three times weekly) or PG545 (5 mg/kg twice weekly) via i.p. injection at 4 weeks of age. Tumor incidence at 4 weeks of age in this GEMM is 100%. To assess survival, mice were treated and monitored daily and euthanized when moribund (n=8-9/group). For the endpoint study, mice were treated with the same therapies starting at 4 weeks of age and the experiment was terminated after 4 weeks of treatment (n=6-8/group).

**Orthotopic models.** NOD/SCID mice (6 to 8 weeks old) were injected orthotopically with 1x10^6 MiaPaca-2 cells. Mice were randomized 3 weeks post tumor cell injection to receive saline (control), Gem (12.5 mg/kg three times weekly) or PG545 (5 mg/kg twice weekly) via i.p. injection for 8 weeks (n=12-14/group). Eight to 10 week old C57BL/6 mice were injected orthotopically with 5x10^5 Pan02 cells and randomized 1 week after tumor cell injection to receive previously mentioned drug treatments (n=6-10/group) for 4 weeks of treatment. Mice in each orthotopic model were monitored for tumor growth by ultrasound.

**Histology**
Formalin-fixed tissues were embedded in paraffin and stained with Masson’s trichrome by the Molecular Pathology Core (UT Southwestern). Immunohistochemistry was performed using the following antibodies: phospho-Histone H3 (Millipore, 06-570), cleaved caspase-3 (Cell Signaling, 9664), Endomucin (Santa Cruz, sc-65495), E-cadherin (Santa Cruz, SC-7870), vimentin (PhosphoSsolution, 2105-VIM), F4/80 (Santa Cruz, sc-26642), CD205-MMR FITC-conjugated (Biolegend, 123006), CD11b-FITC conjugated (Biolegend, 101206) and Gr1-PE conjugated (Biolegend, 108408). Fluorescent images were captured with a Photometric Coolsnap HQ camera using NIS Elements AR 2.3 Software (Nikon). Color images were obtained with a Nikon Eclipse E600 microscope (Nikon) using Nikon Digital Dx1200me camera and ACT1 software (Universal Imaging Corporation). Pictures were analyzed using NIS Elements (Nikon).

In vivo vascular studies

Prior to euthanasia, mice bearing MiaPaca-2 tumors were injected i.v. with FITC dextran (50 mg/ml, 2x10^6 MW; Sigma, FD20005-IG) and rhodamine-dextran (25 mg/ml, 1.0x10^4 MW; Invitrogen, D1824) in 0.9% sterile saline (200 μL). After a 10 minute circulation interval, tumors were isolated, snap frozen in liquid nitrogen, embedded in OCT, sectioned and visualized by fluorescence microscopy. A minimum of 6 images per animal (n=3/treatment group) were obtained. Pictures were analyzed using NIS Elements (Nikon) and results were recorded as mean percent thresholded area.

Mice bearing MiaPaca-2 tumors (n=3/group) were injected i.v. with Pimonidazole (60 mg/kg; HPI, Hydroxyprobe-1) prior to termination. After 90 minutes, tumors were harvested and snap frozen in liquid nitrogen and processed as above and stained with rat anti-mouse CD31 (Dianova, DIA310) and FITC-conjugated mouse anti-pimonidazole (Chemicon). Images were captured and analyzed as described above (29).

Mouse VEGF-A ELISA
Lysate (20 µg) from Pan02 and MiaPaca-2 tumors in the endpoint experiments were prepared and VEGF-A levels were measured using an AlphaLISA mVEGFA kit (Perkin Elmer, AL520 C/F) according to manufacturer’s instructions.

**Statistics**

Data were analyzed using GraphPad software (GraphPad Software, San Diego, CA). Results are expressed as mean +/- SEM. Data were analyzed by one way ANOVA and unpaired-t test, with differences considered significant at p<0.05. Survival analysis was performed using Kaplan-Meier statistics and log-rank comparisons.
Results

**PG545 inhibits tumor cell proliferation and migration in vitro**

Heparanase participates in tumor invasion, metastasis and angiogenesis. Analysis of heparanase protein levels revealed robust expression of the latent precursor (65 kDa) and the active (50 kDa) form of the enzyme (Fig. 1A) in human pancreatic cancer cell lines, HUVECs and WI-38 cells, supporting the concept of heparanase inhibition as a therapeutic strategy. Furthermore, PG545 treatment reduced the proliferation of AsPC-1, MiaPaca-2, Pan02 and WI-38 cells in vitro at pharmacologically relevant concentrations but had little effect on endothelial cell proliferation (Fig. 1B). A wound closure (scratch) assay was used to evaluate the potential effect of PG545 on cell migration. In Pan02 cells, a 20%, 24% and 47% delay in wound closure was observed after treatment with 5 μM, 10 μM and 20 μM PG545. In HUVECs, a 23% and 38% delay in wound closure was observed by 1 μM and 10 μM PG545 (Fig. 1C). PG545 also reduced colony formation of AsPC-1 cells in a dose-dependent manner (Fig. 1D).

**PG545 prolongs survival and inhibits primary tumor growth and metastasis in mouse models of PDAC**

Anti-cancer agents are often efficacious in preclinical animal models but fail to show improvement in survival in clinical trials (30). As a result, we tested the efficacy of PG545 in two survival models of pancreatic cancer that mirror early clinical studies. In the AsPC-1 intraperitoneal model, mice with known peritoneal disease were treated with vehicle (control), Gem or PG545. In this model, PG545 treated animals showed prolonged median survival compared to control (36 vs. 22 days, p=0.006) or Gem therapy (29 days, p=0.012) (Fig. 2A). In the mPDAC genetic mouse model, therapy was initiated when the mice were 4 weeks of age. Median survival of control-treated animals was 54 days while PG545 or Gem therapy prolonged animal survival comparably, 66 and 68 days respectively (Fig. 2B).
Following identification that PG545 prolongs overall survival in murine models of PDAC, its effect on primary tumor growth and metastasis was evaluated in local tumor tissue-based endpoint studies. In MiaPaca-2 tumor bearing mice, PG545 and Gem reduced primary tumor weight by 50% and 25%, respectively compared to control treated mice. In this setting PG545 was more effective than Gem (p<0.01 vs. control, p<0.05 vs Gem) (Fig. 3A). In the Pan02 model, PG545 and Gem significantly reduced primary tumor weight by 60% and 52%, respectively, compared to control-treated tumors (each, p<0.01 vs. control). In the mPDAC model, mean tumor weight was significantly reduced by Gem and PG545 (each, p<0.001, Fig. 3A). Additionally, hematoxylin and eosin analysis of tumors revealed that PG545 treatment group retained more normal pancreatic tissue than Gem treatment group (Fig. 3B), although evidence of local invasion persisted in PG545 treated animals. Importantly, 100% of PG545 treated mice had areas of normal pancreas (n=8) while only 60% of the Gem treated animals had areas of remaining normal pancreas (n=8). α-Amylase reactivity in the pancreas was also higher in PG545 than Gem-treated animals (data not shown). These data suggest that PG545 delays local intrapancreatic progression of primary PDAC.

To further assess tumor progression we evaluated metastatic burden in each tumor model. In the Pan02 and MiaPaca-2 models total gross metastases identified on necropsy was reduced significantly by PG545 treatment (Fig. 3C). Importantly, PG545 was a more effective anti-metastatic agent in the MiaPaca-2 model than Gem (^^^, p<0.005). In the mPDAC model, liver micrometastases (Fig. S1) were identified by H&E and were also reduced significantly by PG545 (p<0.01 vs. control) in contrast to Gem, which did reduce metastases although not significantly (Fig. 3C).

Consistent with its effect on primary tumor size and metastasis, PG545 potently inhibited tumor cell viability as assessed by phospho-histone H3 (Fig. 4A) and cleaved caspase-3 (Fig. 4B) immunoreactivity.
Importantly, inhibition of cell proliferation and induction of apoptosis was greater in PG545 compared to Gem-treated animals in each model.

**PG545 reduces vascular function in tumors**

Tumor cell surface expression of heparanase can drive tumor angiogenesis, increase vessel density and enhance vessel functionality (31). We assessed the effect of PG545 on vascular parameters in multiple PDAC models. Microvessel density (MVD) was determined by expression of endomucin (Fig. 4C) and was reduced significantly by PG545 in Pan02, MiaPaca-2, and mPDAC models. Furthermore, in contrast to the in vitro results with HUVECs (Fig. 1C), in vivo PG545 reduced the number of endothelial cells expressing phospho-histone H3 compared to control or Gem treatment (data not shown).

We also evaluated the effect of PG545 on vascular function within tumors using labeled dextran. In MiaPaca-2 tumors PG545 treatment caused a significant reduction in tumor perfusion (rhodamine-low molecular weight dextran, 10,000 MW, Fig. 5 A, B) and vessel permeability (FITC-labeled high molecular weight dextran, 2M MW) (Fig. 5A, C). The striking reduction in MVD and vascular perfusion and permeability predict that PG545 therapy induces tumor hypoxia. Therefore, we assessed MiaPaca-2 tumors treated with control, Gem or PG545 for hypoxia using pimonidazole (Fig. 5D). PG545 therapy resulted in a striking induction of hypoxia in tumor tissue compared to tumors from control and Gem treated animals (Fig. 5D, E). Hypoxia can drive VEGF expression and secretion. Therefore, we examined the level of VEGF protein in MiaPaca-2 and Pan02 tumors and found that PG545 reduced the level of mouse VEGF-A compared to control or Gem treatment (Fig. 5F, G).

**PG545 promotes tumor cell differentiation**

A key characteristic of metastatic cells is the ability to invade the surrounding ECM and intravasate into vessels (blood and lymphatic) for dissemination systemically. ECM remodeling and tumor cell epithelial
to mesenchymal transition (EMT) are two processes that are induced by hypoxia and facilitate the metastatic cascade (32). We evaluated collagen deposition as a measure of ECM remodeling. mPDAC tumors from animals treated with PG545 had a significant reduction in collagen deposition as depicted by Masson’s trichrome staining (Fig. 6A, B). Similar results were observed in Pan02 tumors (data not shown).

Antiangiogenic therapies induce hypoxia, which can promote tumor cell metastasis and invasion (9, 10) and loss of tumor cell differentiation (33). Given that hypoxia is a known driver of EMT (34-36) and the fact that PG545 therapy resulted in a significant increase in tumor hypoxia, we evaluated the effect of PG545 on tumor cell phenotype in the mPDAC model. Unexpectedly, PG545 treatment increased Ecad expression (Fig. 6C, E) and decreased vimentin expression (Fig. 6C, E) despite elevating hypoxia. Additionally, β-catenin localization to the membrane was increased after PG545 therapy (Fig. 6D, E). These data suggest that the pro-invasive effects of hypoxia can be abrogated by inhibition of heparanase and are consistent with our findings that PG545 reduced metastasis in multiple models of pancreatic cancer.

**PG545 inhibits immune cell infiltration**

Immune cells are critical participants in the metastatic cascade. Antiangiogenic therapy can target the immune cell microenvironment and inhibit macrophage infiltration into breast and pancreatic tumors (37, 38). Furthermore, heparanase promotes macrophage tumor infiltration by removing barriers to tumor cell invasion and drives the heparanase expression in cancer cells (39). We found that PG545 reduced the number of F4/80+ (Fig. 7A, C) and M2 or anti-inflammatory (MMR+) macrophages (Fig. 7D) in MiaPaca-2 tumors. Furthermore, we found that PG545 reduced the number of tumor associated myeloid derived suppressor cells (MDSCs, CD11b+/Gr1+) (Fig. 7B, E), which have been implicated in immune suppression, angiogenesis and resistance to anti-VEGF strategies (40).
Taken together, our results demonstrate that PG545 has potent anti-metastatic effects in murine models of pancreatic cancer and contend that the efficacy of this compound is due to direct anti-tumor cell and anti-stromal cell activity.

Discussion

Angiogenesis inhibitors have been evaluated extensively in pancreatic cancer. While, these agents showed antitumor effects in clinical studies, only a modest benefit was observed on overall patient survival, likely due to hypoxic induction of tumor escape mechanisms (9-11). A complex interaction among tumor cells, immune cells, endothelial cells, ECM and stromal components drive tumor progression. ECM components, heparan sulfate and heparanase are expressed highly in pancreatic cancer (18, 19, 41) and correlate with increased HIF-α expression in patients (20). Furthermore, radiation, a known hypoxia inducer, increases heparanase expression in PDAC cells in vitro (42). Degradation of heparan sulfate by heparanase promotes tumor angiogenesis and metastasis resulting in poor prognosis (13, 23). In the present study on PDAC, we evaluated the efficacy of PG545, an anti-angiogenic and anti-metastatic agent with a unique mechanism of action. We observed that PG545 inhibited primary tumor growth and metastasis and prolonged survival in models of PDAC. Anti-tumor effects of PG545 were accompanied with inhibition of vascular function within the tumor and increased tumor hypoxia. Also, unlike many other anti-angiogenic therapies, PG545 inhibited collagen deposition and promoted tumor cell differentiation in the setting of hypoxia.

The effects of PG545 on tumor growth inhibition were comparable to Gem but PG545 had better anti-metastatic activity, which resulted in prolonged survival in an implant model of human pancreatic cancer. These findings suggest combining Gem with PG545 might be more efficacious than prior strategies that target the vascular compartment. Our initial exploration of combination studies resulted
in toxicity when gemcitabine was given at doses 100 mg/kg twice weekly. Subsequent studies with lower metronomic dosing of Gem appear to be better tolerated and open up the possibility of combination studies in the future. It is important to note that combination of small molecules that target HSPGs with chemotherapy resulted in improved tumor control (43). Given promising early clinical and preclinical results from nab-paclitaxel plus gemcitabine trials in pancreatic cancer (44), combination of dual angiogenesis and heparanase inhibitors such as PG545 with cytotoxic agents represents a promising therapeutic strategy for PDAC.

Our study also focused on the effect of PG545 on vascular function. PG545 is known to inhibit VEGF, FGF-1 and FGF-2 (24), which are the primary stimulants of angiogenesis in the majority of human cancers. In fact, compensation for VEGF blockade by the FGF family has been suggested as a mechanism of resistance to anti-VEGF strategies (45). Thus, PG545 or other strategies to block heparanase activity have the potential to provide greater long term suppression of angiogenesis in tumors than pathway specific inhibitors.

Consistent with our findings, PG545 has been shown to inhibit angiogenesis (25, 46). We found that PG545 had little effect on HUVEC proliferation in vitro (Fig. 1B) but suppressed microvessel density and vascular function in vivo. Potential mechanistic explanations for the lack of effect of PG545 in vitro include an elevated level of heparanase in HUVECs and a slower doubling time compared to cells that were sensitive to PG545 in vitro. Importantly, PG545 inhibited primary tumor growth and metastasis in multiple subcutaneous models of melanoma, breast, prostate, liver, colon and head and neck cancer as a single agent (25, 46). Other heparan sulfate inhibitors have also shown potent activity alone or in combination with chemotherapy in models of breast cancer and melanoma (47). Recently, heparan sulfate D-glucosaminyl 3-O-sulfotranserase-3B1 (3-OST3B1), a gene encoding the final modifications of HSPGs, has been shown to promote pancreatic cancer cell lines to undergo EMT in vitro and in vivo (48).
This suggests that HSPGs participate in pancreatic cancer metastasis via EMT induction. Our study shows that inhibition of heparanase by PG545 induces tumor cell differentiation in vivo. Additionally, work in neuroendocrine pancreatic tumors (PNET) suggests that anti-VEGF therapy induces an invasive tumor phenotype. Several groups have shown that targeting alternate angiogenic pathways such as FGF (with Brivanib) or c-met/HGF (with Crizotinib) inhibit metastasis and tumor cell EMT in pancreatic neuroendocrine transgenic Rip-Tag2 model (11, 49). Although PNET and PDAC are separate histologic and biologic entities, our data suggest that by inhibiting other angiogenic growth factors, PG545 prevents the development of a more aggressive tumor phenotype in PDAC.

The contribution of the innate immune system is also considered in this study. Previously in Rip-Tag2 tumors the majority of heparanase expression was identified in cells from the Gr1+/Mac1+ cell lineages (i.e. Infiltrating immune cells) (50), cells which are affected by targeting VEGF (37). These immune cells (e.g., MDSCs) have also been implicated in mediated or participating in resistance to anti-VEGF therapy (40). This suggests that PG545 by targeting heparanase, decreases MDSC infiltration and macrophage infiltration which may contribute to its anti-metastatic effects.

In summary, challenges to the treatment of pancreatic cancer lie in the lack of efficacious therapy, significant amount of desmoplasia within tumors and early metastasis. To combat this aggressive disease, agents or therapeutic strategies that are anti-proliferative, pro-apoptotic and target the tumor microenvironment are necessary. Our data suggest that PG545 by targeting tumor cells and stromal cells inhibits primary tumor growth, metastasis and increases survival by inhibiting several angiogenic heparanase-dependent pathways. Due to a switch in the clinical route of administration (from subcutaneous to intravenous infusion), PG545 is currently being re-assessed in definitive nonclinical safety studies and is anticipated to enter Phase I trials for advanced cancer patients by mid-2013. These
preclinical studies support further evaluation of PG545 alone or in combination with cytotoxic agents as a potentially efficacious clinical strategy for pancreatic cancer therapy.

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References


**Figure Legends**

**Figure 1.** Expression of heparanase and effects of PG545 on in vitro cell proliferation and migration in human pancreatic cancer cell lines, endothelial cells (HUVEC) and fibroblasts (WI-38) cells. A, Lysates from cells cultured for 24 hours were subjected to Western Blot analysis of heparanase expression. B, In vitro cell proliferation after 72 hours was assessed using WST-1 reagent in tumor (AsPC-1, MiaPaca-2, and Pan02), HUVECs and WI-38 cells. C, In vitro wound closure assay to analyze the effect of PG545 on cell migration in Pan02 cells and HUVECs. D, Anchorage independent growth of AsPC-1 cells in the presence of PG545. Bar graphs represent mean +/- SEM, except for panel C which displays mean +/- SD. Data is representative of single experiment performed in triplicate independently. ***, p<0.001; ****, p<0.0001 vs. Control.

**Figure 2.** PG545 enhances survival in two murine models of pancreatic cancer. A, 7.5x10^5 AsPC-1 cells were injected intraperitoneally into SCID mice and treatment initiated two weeks after tumor cell injection. Mice were treated with gemcitabine (Gem, 100 mg/kg twice weekly) or PG545 (10 mg/kg for 1 week and then 5 mg/kg twice weekly) for two weeks. B, LSL-Kras^G12D; Cdkn2a^lox/lox; p48Cre (mPDAC) mice were randomized at 4 weeks of age to receive Gem (12.5 mg/kg three times weekly) or PG545 (5 mg/kg twice weekly). Treatment was given until the mice were sacrificed due to morbidity.

**Figure 3.** PG545 inhibits primary tumor growth and metastasis. A, Orthotopic Pan02 (n=6-10/group) and MiaPaca-2 (n=12-14/group) tumors were established and mice were treated with gemcitabine (Gem, 12.5 mg/kg three times weekly) and PG545 (5 mg/kg twice weekly). LSL-Kras^G12D; Cdkn2a^lox/lox; p48Cre (mPDAC, n=6-8/group) were randomized at 4 weeks of age to receive Gem or PG545 treatment at the
doses indicated above. Tumor weight was determined at the time of sacrifice. B, H&E histology of pancreatic tissue from mPDAC mice treated with control, Gem or PG545. NP, normal pancreas. Scale bar, 100 µm. C, Total gross metastasis was determined at necropsy in Pan02 and MiaPaca-2 models. The % of mPDAC mice displaying liver micrometastases was determined by hematoxylin and eosin staining of liver sections. *, p<0.05; **, p<0.01; ***, p<0.001 vs. Control. ^, p<0.05; ^^^, p<0.0001 vs. Gem.

Figure 4. PG545 inhibits proliferation and increases apoptosis in vivo. A-C, Sections of MiaPaca-2, Pan02 and mPDAC tumors from mice treated with control, gemcitabine (Gem) or PG545 were analyzed by immunofluorescence for proliferation (A, phospho-histone H3), apoptosis (B, cleaved caspase-3) and microvessel density (C, endomucin). For all analyses a minimum of 6 images from 3 tumors/group were evaluated. Bar graphs represent mean +/- SEM. *, p<0.05; ** p<0.01; ***, p<0.001 vs. Control. ^ P<0.05; ^^, p<0.01; ^^^, p<0.001 vs. Gem.

Figure 5. PG545 inhibits vascular function in vivo. Mice bearing MiaPaca-2 orthotopic tumors were injected with Rhodamine-labeled low molecular weight dextran and FITC-labeled high molecular weight dextran. Mice were sacrificed and tumors snap frozen for analysis. A, Representative images of Rhodamine (red, 10 kDa) and FITC (green, 2 million Da) dextran are shown. B-C, Quantitation of signal intensity of Rhodamine (B) and FITC (C) dextran in tumor tissue from mice treated with control, gemcitabine (Gem) and PG545. Scale bar= 100 µM. D, 90 minutes prior to sacrifice, mice were injected with pimonidazole and tumors snap frozen. Tumor sections were stained with CD31 (red) and hypoxia was quantified using FITC conjugated anti-pimonidazole probe. Representative images are shown. Scale bar = 100 µM. E, Quantification of pimonidazole detection (n=3 animals/treatment group). F-G, Lysates (20 µg in 5 µl) from MiaPaca-2 (F) and Pan02 (G) tumors were analyzed for mouse VEGF-A by ELISA. Bar
graphs represent mean +/- SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 vs. Control. ^, p<0.05; ^^^, p<0.001 vs. Gem.

Figure 6. PG545 reduces collagen deposition. A-B, Representative images of Masson’s trichrome analysis of mPDAC tumors are displayed. Scale bar, 10 μM (A); 5 μm (B). C&D, mPDAC tumors were stained for ECAD (red) and vimentin (green) (C) and β-catenin (D). Scale bar= 50 μM. E, Quantitation of collagen and % area fraction of immunofluorescent staining is shown. Bar graphs represent mean +/- SEM. **, p<0.01; ****, p<0.0001 vs. Control. ^^^, p<0.001; ^^^^, p<0.0001 vs. Gem.

Figure 7. PG545 reduces immune cell infiltration into tumors. MiaPaca-2 tumors were analyzed by immunohistochemistry for macrophage and MDSC cell infiltrates. Representative images of immunofluorescence for macrophages (A, F4/80 (red)) and MDSCs (B, Gr1 (red) and CD11b+ (green)) in tumors from control, Gem and PG545 treated animals. C-E, Analysis of F4/80+ (C), MMR+ (D) and GR1+/CD11b+ (E) cells by area fraction. Scale bar = 100 μM. Bar graphs represent mean +/- SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 vs. Control. #, p<0.05 vs. Gem.
Ostapoff KT et al Figure 2

A

AsPC-1

Days After Treatment

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<th>Treatment</th>
<th>Median Survival (days)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (−) n=7</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Gem (−−−) n=6</td>
<td>29</td>
<td>&lt;0.012 vs Control</td>
</tr>
<tr>
<td>PG545 (•••) n=6</td>
<td>36</td>
<td>&lt;0.0006 vs Control, &lt;0.012 vs Gem</td>
</tr>
</tbody>
</table>

B

mPDAC

Days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median Survival (days)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (−) n=9</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Gem (−−−) n=8</td>
<td>68</td>
<td>&lt;0.05 vs Control</td>
</tr>
<tr>
<td>PG545 (•••) n=8</td>
<td>67</td>
<td>&lt;0.002 vs Control, ns vs Gem</td>
</tr>
</tbody>
</table>

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Ostapoff KT et al Figure 4

A

% Area pH3

MiaPaca-2
Pan02
mPDAC

Control
Gem
PG545

B

% Area Caspase 3

MiaPaca-2
Pan02
mPDAC

Control
Gem
PG545

C

# of Vessels / 200x

MiaPaca-2
Pan02
mPDAC

Control
Gem
PG545

Endomucin
Ostapoff KT et al Figure 5

A

B

MiaPaca-2

% Area Rhodamine

Control Gem PG545

Control Gem PG545

C

% Area FITC

D

MiaPaca-2

Control Gem PG545

CD31/Pimonidazole DAPI

Control Gem PG545

E

MiaPaca-2

% Area Pimonidazole

Control Gem PG545

F

MiaPaca-2

mVEGF-A (pg/mg of tissue)

Control Gem PG545

G

Pan02

VEGF-A (pg/mg of tissue)

Control Gem PG545
Molecular Cancer Therapeutics

PG545, an angiogenesis and heparanase inhibitor, reduces primary tumor growth and metastasis in experimental pancreatic cancer

Katherine T Ostapoff, Niranjan Awasthi, Bercin Kutluk Cenik, et al.

Mol Cancer Ther Published OnlineFirst May 21, 2013.

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