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 pharmaco-logically 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; Cdkn2a<lox/lox>; 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, 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 with control or gemcitabine-treated tumors. These results highlight the potent antitumor 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% to 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-VEGF monoclonal antibody (4, 5); marimastat, a matrix metalloproteinase inhibitor (6); and various tyrosine kinase inhibitors (TKI; ref. 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 antiangiogenic strategies slow primary tumor growth but can promote metastasis in animal models of cancer, including PDAC (9–11). Therefore, future studies on antiangiogenic 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 that 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, 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 patients with PDAC 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 who express high levels of heparanase in their resected tumors have a worse postoperative survival (34 vs. 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 mimic that has antiangiogenic and antimetastatic properties. In preclinical studies, PG545 induces antitumor and antimetastatic effects in breast and hepatocellular cancers (24, 25). In the present study, the antitumor 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 (HUVEC) were all purchased from the American Type Culture Collection. Colo357 cells were obtained from the UT MD Anderson Cancer Center (Houston, TX). The murine pancreatic cancer cell line Pan02 was obtained from the National Cancer Center (Houston, TX). The murine pancreatic cancer cell line P20 was obtained from the National Cancer Institute (Bethesda, MD). Cell lines were confirmed to be pathogen-free, and human cell lines were authenticated to confirm origin before use. Cells were cultured in Dulbecco’s Modified Eagles Media (DMEM; Invitrogen) or RPMI (Invitrogen) containing 10% FBS and maintained at 37°C in a humidified incubator with 5% CO₂ and 95% air.

Western blot analysis

Subconfluent monolayers of cells were lysed, supernatants were recovered by centrifugation at 13,000 rpm. Protein concentrations were measured, and equal amounts of total protein were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) 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). The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnologies) for 1 to 2 hours. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences) 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 nmol/L to 10 μmol/L) 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 conducted in 12-well plates. Monolayers of Pan02 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 (2,000 cells per well) were resuspended in 0.375 mol/L for PG545 and photographed at 0, 6, 12, 24, and 48 hours after wounding. Wound closure was measured as a percentage of original wound width.

Animal studies

All animals were housed in pathogen-free facility with access to food and water ad libitum. Experiments were approved and carried out 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 × 10⁵ AsPC-1 cells as described (26). Mice were randomized and 2 weeks posttumor cell injection animals were injected i.p. with saline (control), Gem (100 mg/kg twice weekly), or PG545 (5 mg/kg for the first week and 5 mg/kg/week thereafter; n = 6–7 animals/group) for 2 weeks. Animals were weighed twice weekly and euthanized when moribund.

**Genetic model of PDAC.** LSL-KrasG12D; Cdkn2aΔexons; p48Cre (mPDAC) mice (27, 28) were genotyped shortly after birth and randomized to receive saline (control), Gem (12.5 mg/kg 3 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 1 × 10⁶ MiaPaca-2 cells. Mice were randomized 3 weeks posttumor cell injection to receive saline (control), Gem (12.5 mg/kg 3 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 5 × 10⁵ 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 conducted 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 (PhosphoSolution, 2105-VIM), F4/80 (Santa Cruz, sc-26642), CD205-MMR FITC-conjugated (Biogenex, 123006), CD11b-FITC conjugated (Biogenex, 101206), and Gr1-PE conjugated (Biogenex, 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 Ds1200mc camera and ACT1 software (Universal Imaging Corporation). Pictures were analyzed using NIS Elements (Nikon).

**In vivo vascular studies**

Before euthanasia, mice bearing MiaPaca-2 tumors were injected i.v. with fluorescein isothiocyanate (FITC) dextran (50 mg/mL, 2 × 10⁵ MW; Sigma, FD20005-IG) and rhodamine dextran (25 mg/mL, 1.0 × 10⁴ 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 optimum cutting temperature (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) before 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**

Lysates (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). 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 conducted 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) forms 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, 10, and 20 µmol/L PG545. In HUVECs, a 23% and 38% delay in wound closure was observed by 1 and 10 µmol/L 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**

Anticancer 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 2 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 with 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, whereas 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 with 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 with 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). In addition, hematoxylin and eosin (H&E) 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 (\( n = 8 \)), whereas 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 in Gem-treated animals (data not shown). These data suggest that PG545 delays local intrapancreatic progression of primary PDACs.

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 in Gem (\(^{\cdot\cdot}\), \(P < 0.005\)). In the mPDAC model, liver micrometastases (Supplementary 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- than in 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 with 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. 5A and B) and vessel permeability (FITC-labeled high molecular weight dextran, 2M MW; Fig. 5A and 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 with tumors from control- and Gem-treated animals (Fig. 5D and 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 with control or Gem treatment (Fig. 5F and 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 2 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 and 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 and E) and decreased vimentin expression (Fig. 6C and E) despite elevating hypoxia. In addition, \(\beta\)-catenin localization to the membrane was increased after PG545 therapy (Fig. 6D and E). These data suggest that the prosurvival 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 and 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+ / Gr-1+) (Fig. 7B and E), which have been implicated in immune suppression, angiogenesis, and resistance to anti-VEGF strategies (40).

Taken together, our results show that PG545 has potent antimetastatic effects in murine models of pancreatic cancer and contend that the efficacy of this compound is due to direct antitumor cell and anti-stromal cell activity.

**Discussion**

Angiogenesis inhibitors have been evaluated extensively in pancreatic cancer. While these agents showed anti-tumor 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 drives tumor progression. ECM components, HS and heparanase, are highly expressed 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 HS by heparanase promotes tumor angiogenesis and metastasis resulting in poor prognosis.
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 MVD (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.
In the present study on PDAC, we evaluated the efficacy of PG545, an antiangiogenic and antimetastatic 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 antiangiogenic 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 antimetastatic activity, which resulted in prolonged survival in an implant model of human pancreatic cancer. This finding suggests that 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 of 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 heparan sulfate proteoglycans (HSPG) 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.

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 and 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 before 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 and 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.
Consistent with our findings, PG545 has been shown to inhibit angiogenesis (25, 46). We found that PG545 had little effect on HUVEC proliferation \textit{in vitro} (Fig. 1B) but suppressed MVD and vascular function \textit{in vivo}. Potential mechanistic explanations for the lack of effect of PG545 \textit{in vitro} include an elevated level of heparanase in HUVECs and a slower doubling time than cells that were sensitive to PG545 \textit{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 HS inhibitors have also shown potent activity alone or in combination with chemotherapy in models of breast cancer and melanoma (47). Recently, HS-\(\alpha\)-glucosaminyl-3-O-sulfotransferase-3B1 (3-OST3B1), a gene encoding the final modifications of HSPGs, has been shown to promote pancreatic cancer cell lines to undergo EMT \textit{in vitro} and \textit{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 \textit{in vivo}. In addition, 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 PNETs and PDACs 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; ref. 50), cells that are affected by targeting VEGF (37). These immune cells [e.g., myeloid-derived suppressor cells (MDSC)] 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 antimetastatic 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 antiproliferative, 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. Because of a switch in the clinical route of administration (from subcutaneous to intravenous infusion), PG545 is currently being reassessed in definitive nonclinical safety studies and is anticipated to enter phase I trials for patients with advanced cancer by late-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.

References

Disclosure of Potential Conflicts of Interest
K. Dredge is employed as the Director of Preclinical Development in Progen Pharmaceuticals Ltd. K. Dredge is a consultant/advisory board member of Progen Pharmaceuticals. No potential conflicts of interest were disclosed.

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Conception and design: K.T. Ostapoff, K. Dredge, R.A. Brekken
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.T. Ostapoff, B.K. Cenik, S. Hinz
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