Clinical Cancer Research

Impaired Synthesis of Stromal Components in Response to Minnelide Improves Vascular Function, Drug Delivery, and Survival in Pancreatic Cancer

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Abstract

Purpose: Pancreatic cancer stromal microenvironment is considered to be the major reason for failure of conventional and targeted therapy for this disease. The desmoplastic stroma, comprising mainly collagen and glycosaminoglycans like hyaluronan (HA), is responsible for compression of vasculature in the tumor resulting in impaired drug delivery and poor prognosis. Minnelide, a water-soluble prodrug of triptolide currently in phase I clinical trial, has been very effective in multiple animal models of pancreatic cancer. However, whether Minnelide will have efficacious delivery into the tumor despite the desmoplastic stroma has not been evaluated before.

Experiment Design: Patient tumor-derived xenografts (PDX) and spontaneous pancreatic cancer mice were treated with 0.42 and 0.21 mg/kg body weight for 30 days. Stromal components were determined by IHC and ELISA-based assays. Vascular functionality and drug delivery to the tumor were assessed following treatment with Minnelide.

Result: Our current study shows that treatment with Minnelide resulted in reduction of ECM components like HA and collagen in the pancreatic cancer stroma of both the spontaneous KPC mice as well as in patient tumor xenografts. Furthermore, treatment with Minnelide improved functional vasculature in the tumors resulting in four times more functional vessels in the treated animals compared with untreated animals. Consistent with this observation, Minnelide also resulted in increased drug delivery into the tumor compared with untreated animals. Along with this, Minnelide also decreased viability of the stromal cells along with the tumor cells in pancreatic adenocarcinoma.

Conclusions: In conclusion, these results are extremely promising as they indicate that Minnelide, along with having anticancer effects is also able to deplete stroma in pancreatic tumors, which makes it an effective therapy for pancreatic cancer. *Clin Cancer Res; 22(2); 415–25. ©2015 AACR.*

Introduction

Pancreatic cancer is among the most devastating of all cancers with a dismal survival rate. In United States alone, the estimated case for pancreatic cancer is almost 48,000 (www.cancer.gov). Five-year survival in patients with pancreatic cancer is about 5% and this figure has remained relatively unchanged over the past 25 years (1, 2). Pancreatic cancer stromal microenvironment is considered to be the major reason behind the failure of conventional and targeted therapy for this disease (3). It has been observed that the dense stroma comprising mostly of proteoglycans like hyaluronan (HA) and collagen forms a physical barrier for drugs targeting the tumor cells (4, 5). Recent efforts directed toward development of new therapies have thus been targeted toward depletion of stroma in order to improve drug delivery (6, 7).

A molecular analysis of the pancreatic ductal adenocarcinoma (PDAC) stroma revealed that it was extremely heterogeneous, comprising cellular and acellular components, like fibroblasts, myofibroblasts, pancreatic stellate cells, immune cells, blood vessels, extracellular matrix (ECM) made of collagen and HA, and soluble proteins such as cytokines and growth factors. As a result of accumulation of ECM components, the normal architecture of the pancreatic tissue is distorted resulting in an abnormal configuration of blood and lymphatic vessels in the tumor (4, 5, 8–10). On the basis of this observation, it was hypothesized that the desmoplastic stroma acted as a barrier to drug delivery in the tumor (5, 10). HA and collagen are the two major components of the pancreatic tumor stroma. HA is a high-molecular weight glycosaminoglycan comprising a polymer of N-Acetyl Glucosamine and glucuronic acid, that retains water due to its high colloid osmotic pressure (11). Excessive HA accumulation in solid tumors has been reported to raise interstitial fluid pressure and compress blood vessels (3, 6, 7). Collagen, particularly type IV collagen, is the main component of the basement membrane of the ECM that provides a scaffold for its assembly and mechanical stability. Along with providing an extensive support, the collagen

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Translational Relevance

The prevalence of stroma in pancreatic tumors is the primary reason behind impaired drug delivery in pancreatic cancer. The desmoplastic stroma constricts the blood vessels in the tumor, leading to inefficient vasculature and poor drug delivery. Thus there is an urgent need to develop and evaluate therapy that along with having anticancer effect will also ensure better drug delivery into the tumors. Minnelide, a potent anticancer compound, is currently in phase I clinical trial. In this study, we have evaluated the effect of Minnelide on stromal lysis in pancreatic cancer. Our studies show that Minnelide depletes the extracellular matrix components by depleting hyaluronan and collagen in the stroma, leading to better drug delivery and improved survival in a spontaneous animal model of pancreatic cancer. This is translationally relevant as it suggests that Minnelide can be developed as an effective therapy against pancreatic cancer.

network in the stroma also plays a significant role in cell adhesion, migration, survival, proliferation, and differentiation (12, 13). Sonic hedgehog (SHH) signaling has been shown to be restricted to the stromal compartment (10, 14). Thus, pharmacologic inhibition of the Shh pathway was thought to have a positive impact on gemcitabine delivery, by reducing the desmoplastic stroma. Though a study involving combination of the Smoothened inhibitor (IPI-926) and gemcitabine caused depletion of tumor stroma and resulted in increased microvessel density (5), the compound failed to offer significant survival benefit in a clinical trial.

In another study, HA degradation by hyaluronidase PEGPH20 decreased interstitial fluid pressure in murine PDAC. Consequently, increased vessel patency, drug delivery, and survival were also observed (3, 7). Even though "anti-stromal" therapy has been emerging as a therapeutic option, the role of stroma in pancreatic cancer has remained controversial. It is also proposed that the stroma is more "restraining" in nature for the tumor, thereby acting to prevent metastasis by constricting blood vessels (15). Thus, an ideal therapy for pancreatic cancer would be one that on one hand will have a potent anticancer effect and at the same time will ensure its efficacious delivery into the tumor.

Triptolide, an active compound isolated from a Chinese herb has been used as an anticancer compound since the 1990s. Its efficacy in reducing a number of tumors including pancreatic tumors has been reported over last few years (16–21). Owing to its limited solubility in aqueous medium, triptolide had restricted use in clinical and preclinical studies. Recently, a water-soluble analog of triptolide, Minnelide has been effectively used as an anticancer therapy (22–27). Minnelide is currently being evaluated in a phase1 clinical trial against gastrointestinal cancers. Our preclinical studies with Minnelide have shown that this compound is able to increase survival in a number of mouse models both orthotopic as well as patient-tumor derived xenografts (PDX). Minnelide, apart from reducing the primary tumor burden, also decreases metastasis in pancreatic as well as other cancers (24). Our previous studies have shown that Minnelide acts by inhibiting the transcriptional activity of Sp1, by altering its glycosylation status (22). Though there have been a number of studies on triptolide/Minnelide, its effect on the stromal architecture has not been studied till date.

In the current study, we show that Minnelide, apart from having a profound anticancer effect as seen in our earlier studies, also effectively depletes the stromal architecture in pancreatic cancer by inhibiting collagen stabilization and HA synthesis. This eventually results in improved vascular function and better drug delivery, leading to an increased survival of treated $KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre (KPC) mice.

Materials and Methods

qRT-PCR

qRT-PCR for indicated primers was carried out using primers procured from Qiagen (Quantitect Primer Assay, Qiagen). PCR array for ECM and Adhesion pathway (Qiagen, SA Biosciences) was used to analyze transcripts of genes involved in the ECM. RNA was isolated from the tumor samples according to manufacturer's instructions using TRIzol (Invitrogen). Total RNA (1 μg) was used to perform real-time PCR using the Quantitect Sybr green PCR kit (Qiagen) according to the manufacturer's instructions using an Applied Biosystems 7300 real-time PCR system. All data were normalized to the housekeeping gene 18S (18S Quantitect Primer Assay; Qiagen).

Estimation of HA in tumors, HA synthase, and hyalouronidase assay

For estimating HA, hyaluronidase, and HA synthase activity, tumor tissue (from control animals and animals treated with Minnelide) was washed several times in ice-cold PBS to remove traces of blood. The tissue was then homogenized in the assay buffer (20 mmol/L sodium phosphate buffer, 77 mmol/L sodium chloride) in the presence of protease inhibitors and used for assay. HA was estimated in the tumor samples using the ELISA-based Hyaluronan Duo Set (R&D system) according to manufacturer's instruction. For Hyaluronidase assay, ELISA-based hyaluronidase kit (AMS Biotech) was used according to manufacturer's instruction. HA synthase assay was done by slightly modifying the protocol established by Itano (28). Briefly, the membrane fraction of the tumor tissue was incubated at 37°C for 1 hour in 0.2 mL of 25 mmol/L Hepes-NaOH, pH 7.1, 5 mmol/L dithiothreitol, 15 mmol/L $MgCl_2$, 0.1 mmol/L UDP-GlcNAc (Sigma), and 2 μmol/L UDP-GlcA (Sigma). Reactions were terminated by adding SDS at 2% (w/v). For negative control, the total reaction mix was heat inactivated before incubation. HA formed was estimated by the ELISA-based Hyalurona Duo set as described before. Results were expressed as the amount of HA formed per μg protein per minute.

Collagen isolation and hydroxyproline and hydroxylysine estimation

Tumor tissue from Minnelide-treated and untreated mice were minced into 1 to 2 mm pieces and extracted in 0.5 mol/L Acetic Acid for 72 hours at 4°C. The supernatant containing acid soluble collagen was dialyzed against 0.05 M Na_2HPO_4. Hydroxyproline assay was performed on this extracted collagen using a hydroxyproline assay kit (Sigma) and results were expressed as hydroxyproline formed per ng collagen. Hydroxylysine in the collagen was estimated using hydroxylysine assay kit (Biosource) according to manufacturer's instruction. Results were expressed at hydroxylysine produced per ng of collagen.

IHC and immunofluorescence

For IHC, paraffin tissue sections were deparaffinized in xylenes and hydrated through graded ethanol. Hematoxylin and eosin (H&E) staining was used for evaluation of histologic features. Sirius Red staining was used to visualize the collagen in the tumor stroma. Slides were steamed with Reveal Decloaker (Biocare Medical) to minimize background staining. Sniper Universal Blocking Sera (Biocare Medical) were used throughout the protocol. Primary antibodies for α SMA, CD31 (Abcam), HABP-Biotin (Sigma) were diluted according to vendor's instruction and incubated overnight at 4°C. The primary antibody was omitted for the negative controls. For immunofluorescence, fluorescent antibody conjugates were used after primary antibody staining. Slides were counterstained with DAPI and visualized in a Nikon fluorescent microscope. Tissue samples were incubated with mouse IgG1 isotype controls (BD Biosciences) and did not demonstrate any specific staining.

Human xenograft pancreatic cancer tumor model

PDXs were established by implanting deidentified human pancreatic tumors subcutaneously into NOD-SCID mice (Jackson Laboratories). Upon reaching a volume of 500 mm^3, tumors were dissected and cut into 10 mm^3 pieces, which were then subcutaneously implanted into both flanks of additional SCID mice ($n = 20$ animals). Animals were randomized and tagged before treatment. Treatment with Minnelide (0.42 mg/kg body weight) was started when the tumors were approximately 500 mm^3 in size. Animals were treated for 7 days before collecting tissues for histology, vascular function assay, or drug delivery assay.

Transgenic mouse model of spontaneous pancreatic cancer

$KRas^{G12D}$; $Trp53^{R172H}$; Pdx-1Cre animals were generated by crossing Lox Stop Lox (LSL) $KRas^{G12D}$; LSL $Trp53^{R172H}$ animals with Pdx-1 Cre animals. Minnelide treatment with 0.42 mg/kg body weight/day was started when animals were 4 to 6 weeks of age. Animals in saline and treatment groups were age matched. Animals were weighed every week to monitor for weight loss during experiment and treatment doses were adjusted accordingly. Animals were treated for 30 days before collecting tissues for histology, vascular function assay, or drug delivery assay.

Experiments were performed and animals sacrificed in accordance with animal care committee regulations at the University of Minnesota (Minneapolis, MN). For survival study, the survival data were represented as a Kaplan–Meier plot using GraphPad Prism 6.

Vascular function assay

Four to five mice were evaluated for each experimental arm. Twenty-four hours after the final dose of Minnelide, mice received an intravenous infusion of fluorophore-labeled tomato lectin. Thirty minutes later, mice were terminally perfused sequentially with 50 mL saline followed by 30 mL of 4% paraformaldehyde/PBS (pH 7.4). Perfused tissues were harvested, fixed for 16 to 24 hours in paraformaldehyde and transferred to 70% ethanol before paraffin embedding. Sections were deparaffinized, rehydrated and, for vascular patency assays, immunostained for CD31. All sections were counterstained with DAPI. Visualization was performed on a Nikon Ti confocal microscope using standardized settings, and background signal intensities were established against unlabelled terminally perfused samples. The open/compressed vessels were counted for 10 nonadjacent 20× fields for each slide.

Drug delivery assay

For the doxorubicin delivery assay, a minimum of 5 mice from saline and Minnelide were included for analysis. Mice received an intravenous infusion of doxorubicin 24 hours after the final dose of Minnelide. Mice were euthanized and tissues processed as above. Sections were deparaffinized, rehydrated, and counterstained with DAPI. Doxorubicin fluorescence was visualized under a Nikon Ti confocal microscope using standardized settings, and background signal intensities were established against samples without doxorubicin stain.

Viability of CAF and KPC tumor cells

Cancer-associated fibroblasts were isolated from five to seven tumors from KPC mice as described in Sharon and colleagues (29). The purity of the fibroblasts was checked by flow cytometry after staining isolated fibroblasts with FSP antibody and CK19 antibody. Population with FSP+CK19 staining was used for viability experiments.

Both isolated CAFs from KPC tumors and mouse epithelial PDAC cell line KPC001 and Panc-02 were seeded on 96-well plates and allowed to grow for 48 hours prior to treating them with indicated doses of triptolide for 24 and 48 hours. Cell viability was measured using an MTT-based assay.

Statistical analysis

Values are expressed as mean ± SEM. All *in vitro* experiments were performed at least three times. The significance of the difference between any two samples was analyzed by Student *t* test using GraphPad Prism 6; values of $P < 0.05$ were considered statistically significant.

Results

Molecular components of desmoplastic stroma are upregulated in human and murine PDAC compared with normal pancreas

Pancreatic cancer is characterized by an intense desmoplastic stroma. The molecular components of the stroma include the activated stellate cells in the pancreas, the myofibroblasts, proteoglycans, and glycosaminoglycans. Synthesis of this complex ECM involves a number of very tightly regulated pathways (4, 30, 31). PCR array of genes responsible for the extracellular matrix or ECM showed several fold increased expression in PDAC tissues (from KPC tumors) compared with normal ductal epithelial cells (Supplementary Fig. S1). The genes that were significantly upregulated or downregulated are tabulated in Supplementary Table S1. Consistent with this, the histologic sections of pancreatic tumors resected from patients or from the KPC mice showed presence of an extensive stroma as seen by H&E staining (Fig. 1A) when compared with normal pancreas tissue (Fig. 1C). Both PDX as well as tumors from KPC mice also stained heavily with collagen as visualized by Sirius Red staining (Fig. 1B).

Patient tumor xenografts and KPC spontaneous tumors show reduced stroma in response to Minnelide treatment

Minnelide has been extremely efficient in regressing tumor both in human tumor-derived xenografts as well as in spontaneous KPC mice ($KRAS^{G12D}$, TP53-PdxCre mice) for pancreatic cancer (24). To see whether Minnelide was also effective in reducing the stromal components, the animals were treated with Minnelide and the histologic sections were stained with stromal markers and

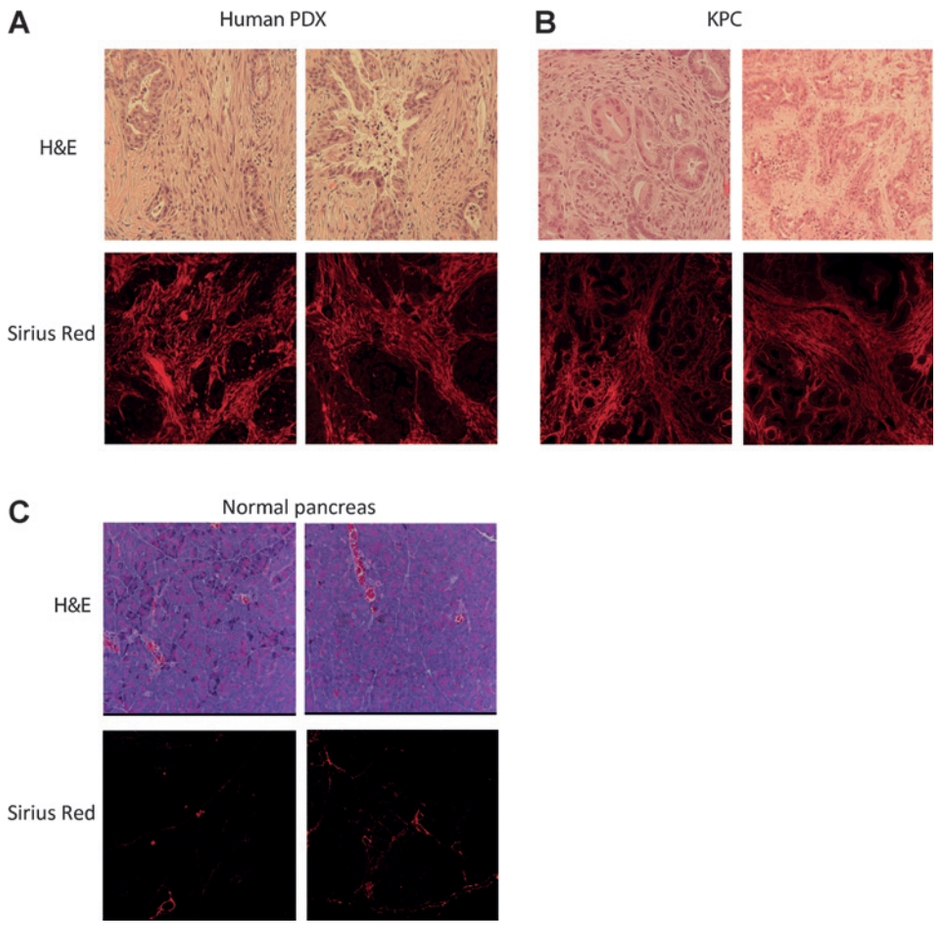

Figure 1.
Figure showing presence of extensive stroma in PDAC in (A) PDX and (B) spontaneous KRasG12D, TP53,Pdx-Cre (KPC) mice in H&E stain, picrosirius red stain. Normal pancreas tissue showed very little staining with picrosirius red (C).

quantitated. HA is one of the major constituents on the pancreatic tumor stroma, which is typically visualized in tissue sections by staining with HABP (HA-binding protein) staining. HABP staining was significantly less in the Minnelide treated animals in both PDX (Fig. 2A) and KPC models (Fig. 2B). Minnelide also decreased α SMA staining in tissues indicating that activated stellate cells were also reduced (Fig. 2A and B). Quantitation of the α SMA staining indicated that this was significantly less than untreated tumors (Supplementary Fig. S2). Minnelide treatment reduced fibrosis in the stroma (as visualized by Sirius red staining) in both PDX and spontaneous KPC model as well (Fig. 2C and D). Quantitation of staining showed that the fibrosis (collagen) was significantly decreased in the Minnelide-treated tissues (Supplementary Fig. S2).

To further confirm whether the HA in the treated tumor is indeed decreased by Minnelide, we quantitated the total HA in the tumors treated with Minnelide. Consistent with the histologic observation, HA was decreased in the Minnelide-treated tumors derived from both KPC (Fig. 2E) and PDX models (Fig. 2F).

Reduction in fibrosis is caused by impaired collagen synthesis and modification

Since our histological sections revealed that Minnelide treatment significantly reduced fibrosis in the two models (as seen decreased Sirius Red staining), we studied the pathways involved in synthesis of collagen. RNA analysis revealed that the transcription of genes involved in the synthesis of collagen (*ColE1-5*), or stabilization of collagen (*PLOD1-4, P4H*), were not affected by Minnelide (Supplementary Fig. S3). Our results show that *PLOD1-4* (procollagen lysine 5 dioxygenase) enzymes are very highly expressed in pancreatic tumors (Supplementary Fig. S3). These genes catalyze the hydroxylation of lysine to hydroxylysine, a key step in stabilization and formation of collagen. Because Minnelide did not alter the expression of *PLOD* genes, we measured the total hydroxylysine in the total collagen from treated tissues. Hydroxylysine is a rare amino acid only present in collagen, thus a measure of total hydroxylysine in tumors is a measure of the enzyme activity of the PLOD genes. Both KPC and PDX tumors treated with Minnelide showed a decrease in the total hydroxylysine content indicating that Minnelide prevented stabilization of collagen in the tumor thereby leading to depletion of stromal architecture (Fig. 3A and B).

The other class of enzymes responsible for the stabilization of collagen is procollagen hydroxylases that are overexpressed in pancreatic tumors. Similar to the procollagen lysine dioxygenase, prolyl hydroxylase predominantly catalyzes hydroxylation of proline residues in collagen. Minnelide did not reduce the expression of P4H enzymes in KPC or PDAC tumors (Supplementary Fig. S3). However, treatment with Minnelide did decrease the total

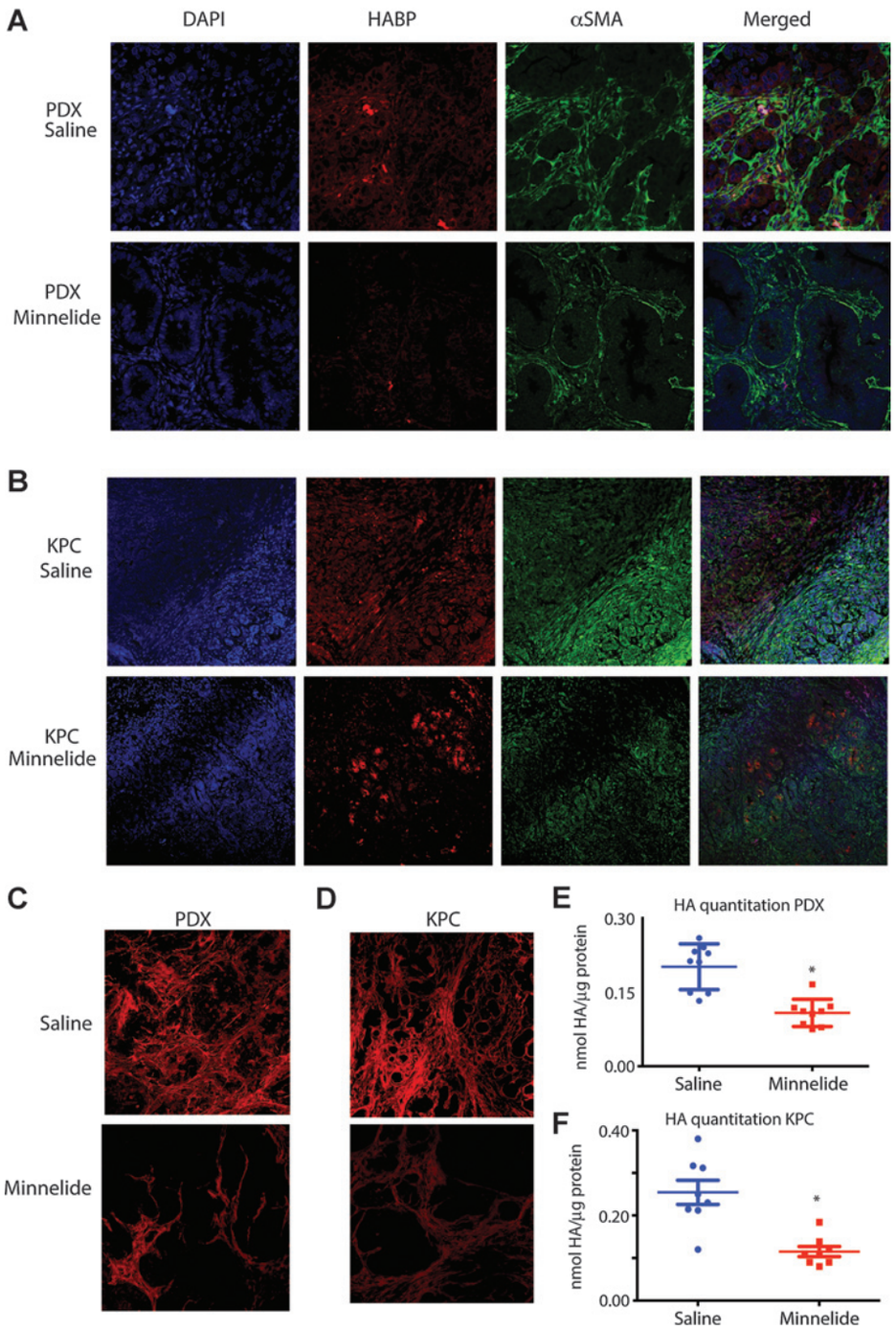

Figure 2.
Minnelide reduces stromal component α SMA and HA in (A) PDX as well as (B) in KPC tumors. C, picrosirius red staining is also decreased in both models. D, quantitation of HA from the PDX tumors (E) and KPC tumor (F) also shows decreased HA in the tumors. *, $P < 0.05$.

hydroxyproline in the collagen of tumor tissues treated with Minnelide (Fig. 3C and D).

Because both PLOD enzymes as well as P4H enzymes are oxygen dependent and require α ketoglutarate as the primary cofactor in their reaction, we assayed for the total α KG produced in response to Minnelide treatment. Our results indicated decreased α KG in response to Minnelide treatment in both KPC and PDX models (Fig. 3E and F).

Decreased hyaluronan in stroma results from reduced HAS activity

Because Minnelide decreased HA in the tumors, we next evaluated whether this was due to decreased HA synthesis or enhanced HA degradation in the tumors. HA is synthesized by the tumor cells by HA Synthase enzymes (HAS1-3). Analysis of mRNA showed that HAS1, HAS2, and HAS3 genes were decreased in response to Minnelide treatment in both models (Fig. 4A).

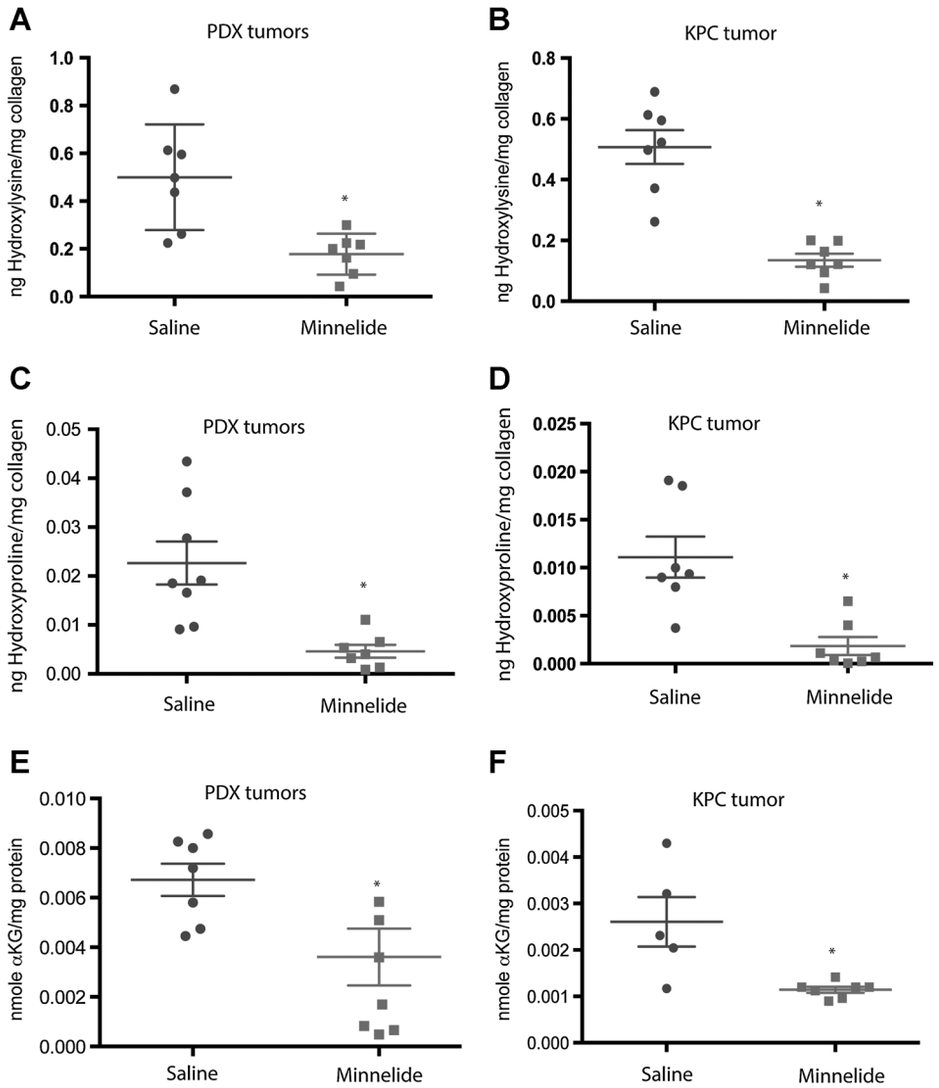

Figure 3.
Minnelide decreased hydroxylysine in (A) PDXs and (B) KPC tumors; hydroxyproline in PDXs (C) and (D) KPC tumors; and α ketoglutarate in (E) PDXs and (F) KPC tumors. *, $P < 0.05$.

Consistent with this observation, the HAS enzyme activity was significantly decreased in the tumors treated with Minnelide (Fig. 4B) in both KPC mice and PDX tumor models (Supplementary Fig. S4). HA degradation is orchestrated by the hyaluronidase enzymes (HYAL1-5, PH20). Of these, HYAL1, HYAL2, and PH20 were present in KPC and PDX tumors. Minnelide did not decrease the mRNA of these genes (Fig. 4C). As expected, hyaluronidase activity also remained unaltered in the tumor tissues after treatment with Minnelide (Fig. 4D) in both KPC mice and PDX tumor models (Supplementary Fig. S4).

This indicated that decrease in HA in response to Minnelide was in response to decreased synthesis and not increased degradation.

Decreased stromal component result in improved drug delivery and survival

Inefficient drug delivery is a hallmark of pancreatic tumors. This phenomenon is often attributed to compression of tumor vasculature by dense stroma. To see whether the physical depletion of stroma translated to an improved drug delivery by improving vascular function, we injected doxorubicin to study drug delivery in these animals. Doxorubicin fluorescence was observed inside the tumors of KPC animals treated with Minnelide (0.42 mg/kg/d), whereas negligible doxorubicin fluorescence was present in the saline animals (Fig. 5A). Compressed vasculature in the pancreatic tumors prevents drug delivery to the tumor cells in pancreatic cancer. To see whether Minnelide-induced stromal depletion relieved this vascular compression, we tested for vascular functionality in the tumors. Treatment with Minnelide resulted in greater number of open blood vessels in the tumor compared with the untreated tumors (Fig. 5B and C).

Our previous studies have shown that Minnelide improved survival and caused tumor regresssion of PDX model. To see whether Minnelide indeed improved survival of the KPC model, we treated age-matched KPC animals with 0.42 mg/kg Minnelide and plotted their survival. Minnelide indeed increased the median survival of these animals by 58 days following treatment (Fig. 5D).

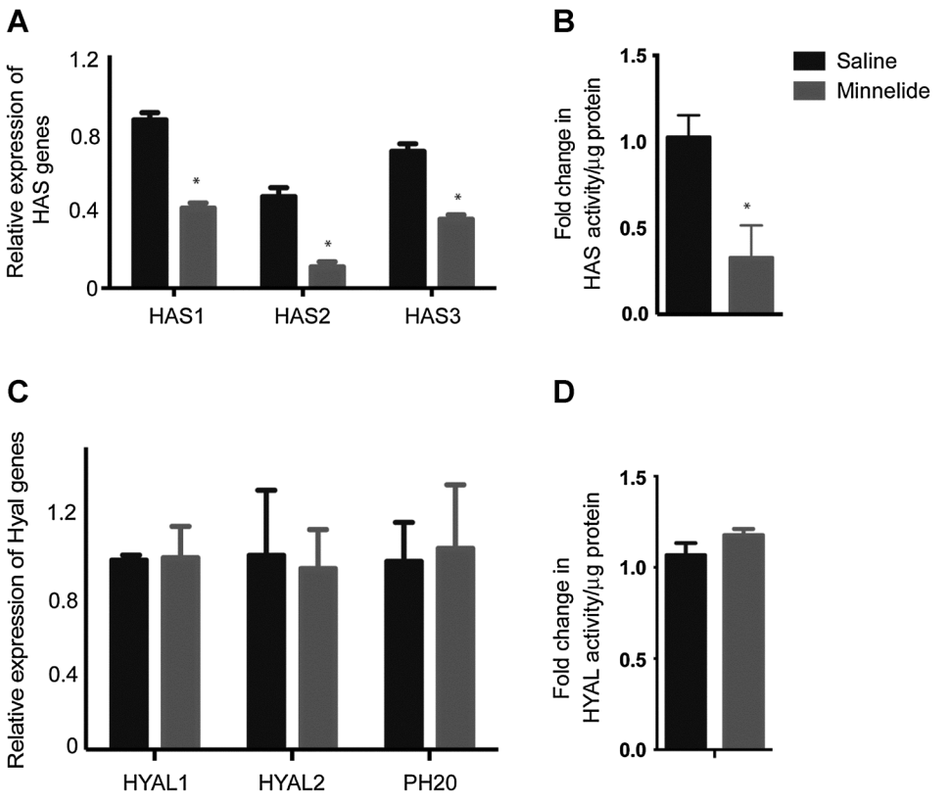

Figure 4.
Minnelide decreased (A) HA synthase expression along with (B) the activity in KPC tumors. C, hyaluronidase expression as well as (D) activity was found to be unaltered in these tumors following treatment. *, $P < 0.05$.

Minnelide decreased viability of both tumor cells and stromal cells

Our previous studies have shown that Minnelide decreases viability of tumor cells in a number of animal models and pancreatic cancer cell lines, while having no effect on the normal pancreatic ductal cells. To study whether it also reduced the viability of cancer-associated fibroblasts (CAF) as efficiently as the cancer epithelial cells, we compared the cell viability of CAFs and cancer epithelial cells isolated from KPC animals. Our results showed that Minnelide indeed decreased the viability of CAFs (Fig. 6A and B) as efficiently as it decreased viability of mouse epithelial PDAC cells (Fig. 6C and D), indicating that it not only depleted the stromal ECM but also induced stromal cell death. The IC_{50} values for tumor and stromal cells are indicated in Supplementary Table S2.

Discussion

Pancreatic cancer stromal microenvironment is considered to play a major role in chemoresistance of the tumor by preventing efficient drug delivery into the tumor. The stroma has received a lot of focus in pancreatic cancer research over the last 6 to 8 years. There are conflicting evidences as to whether targeting stroma and its components are beneficial for regression of the tumor. On one hand, relieving the vascular compression in the tumor results in better drug delivery to the tumor (3, 7, 32), while on the other hand, it is also argued that stroma is actually restraining the tumor by preventing metastasis (15, 33, 34). Thus, an ideal antipancreatic tumor drug would thus be the one that will cause stromal depletion to ensure efficacious drug delivery into the tumor and at the same time be able to inhibit metastasis by being tumoricidal.

The KrasG12D; TP53-mutated mice (KPC) has been extensively used to understand this phenomenon pancreatic tumor microenvironment (7, 35). A molecular analysis of the PDAC stroma in these mice revealed that it was extremely heterogeneous, comprising cellular and acellular components, like fibroblasts, myofibroblasts, pancreatic stellate cells, immune cells, blood vessels, extracellular matrix (ECM), and soluble proteins such as cytokines and growth factors. Similar stromal architecture is also found in the resected pancreatic tumors from patients. Our studies showed that PDXs in SCID mice also show similar elaborate stromal architecture in the first 2 to 3 passages (Fig. 1). Similar to the KPC mice, these tumors had extensive fibrosis (as visualized by Sirius red staining), cancer-associated fibroblasts, and activated stellate cells and an extensive extracellular matrix comprising of HA (Fig. 1). HA is a glycosaminoglycan made of N-acetyl glucosamine and glucuronic acid (36). Apart from being instrumental in fluid retention and wound healing in normal tissues, HA is also involved in mediating a number of signaling in human body, one of which is the sperm-ova recognition (37). Interestingly, triptolide, the active drug of Minnelide, derived from a Chinese herb, has been used as a male contraceptive in China and has been shown to influence HA synthesis (38).

Previous studies from our group have shown that Minnelide, the water soluble prodrug of triptolide, is extremely effective as an anticancer compound in a number of animal models for pancreatic cancer, including the KPC model (24). Minnelide is currently under phase I clinical trial at the University of Minnesota. In that context, it was imperative to test if treatment with Minnelide was

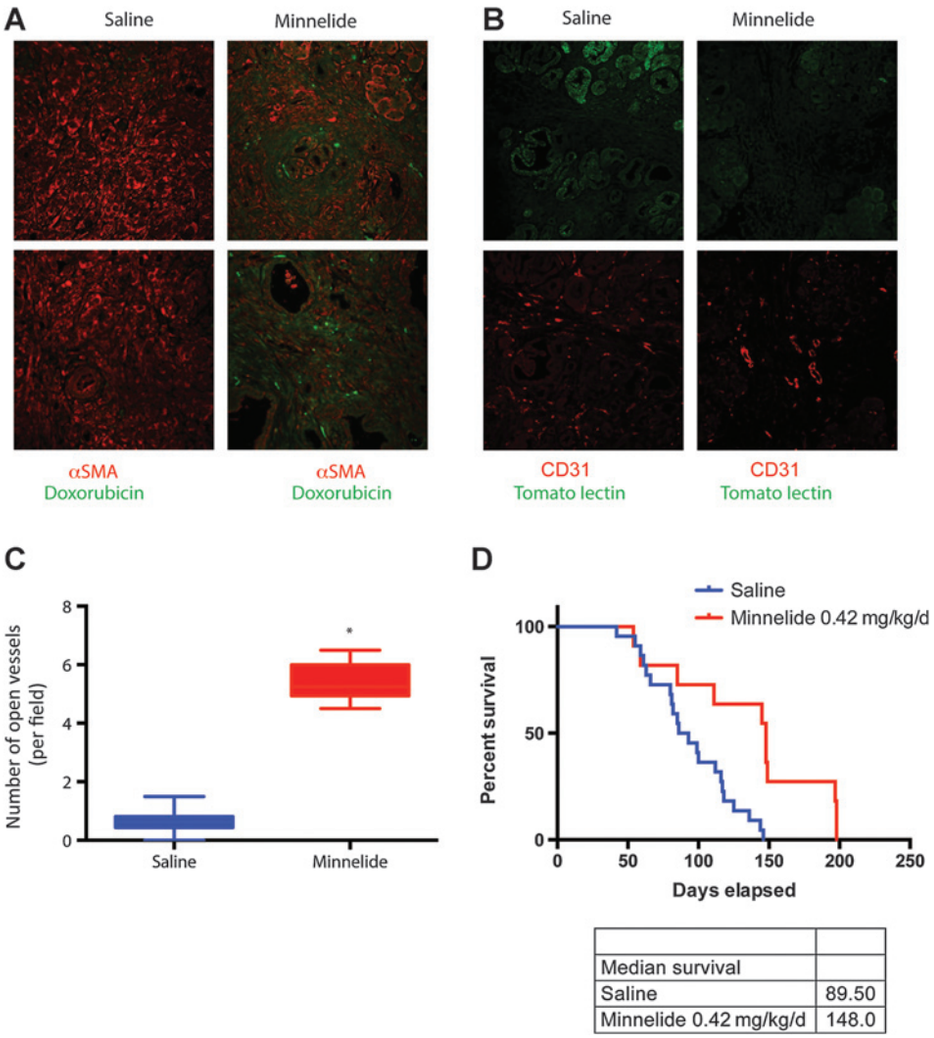

Figure 5.
Minnelide improved (A) drug delivery as seen by doxorubicin fluorescence in both KPC and PDX tumors. Minnelide also reduced vascular compression (B) and resulted in (C) more "open" blood vessels compared with untreated tumors. This resulted in a (D) greater survival of the KPC mice receiving Minnelide compared with the untreated mice. *, $P < 0.05$.

able to breach the stromal barrier. Our histologic studies showed that Minnelide significantly reduced both total collagen and HA in both the PDX and the KPC tumor models (Fig. 2).

The extracellular matrix in pancreatic tumor is predominantly made of collagen. Synthesized as a pro-collagen molecule, collagen assembly and cross-linking into tertiary structure provides the structural strength to the matrix. Interestingly, our studies revealed that the expression of the genes involved in synthesis or stability of collagen (for e.g. ColE genes, PLOD, genes and P4H genes remained unchanged in response to Minnelide (Supplementary Fig. S3). The stability of the procollagen into cross-linked collagen fibrils is mediated by a group of two enzymes: Procollagen lysine 2-oxoglutarate dioxygenase (PLOD1-4) and Prolyl 4 hydroxylase (P4H). PLOD1-4 is a group of enzymes that catalyzes the transfer of a hydroxyl group to the lysine residues in the collagen. The addition of hydroxyl groups is essential for collagen molecules to form stable interactions, called cross-links, with one another. Cross-links between these molecules allow collagen to form networks of strong, slender fibrils that eventually constitute the stroma. Because hydroxylysine is a rare amino acid only present in collagen, a measure of the total hydroxylysine is a measure of the total PLOD1-4 activity. P4H, on the other hand catalyze the formation of 4-hydroxyproline, that is essential to the proper three-dimensional folding of newly synthesized procollagen chains. Once again, because the major source hydroxyproline is collagen, estimation of total hydroxyproline is a measure of the activity of P4H in the tumors. Minnelide decreased total hydroxylysine and hydroxyproline in the tumor tissues (Fig. 4), indicating that the activity of these enzymes was inhibited by this compound.

Our results showed that Minnelide decreased total HA in the tumor tissues. We hypothesized this could either be due to increased degradation of HA or decreased synthesis of HA. To address this, we perform activity assays for both HA synthase (HAS) enzymes as well as hyaluronidase (HYAL1-4, PH20) enzymes. Our results showed that Minnelide decreased activity as well as expression of HAS genes, were as HYAL genes remained unaffected by Minnelide treatment (Fig. 4). Review of literature showed that activity of HA synthase was dependent on the availability of UDP-GlcNAc in the cell (39). Previous results from our group showed that triptolide/Minnelide downregulates the hexosamine biosynthesis pathway that results in production of

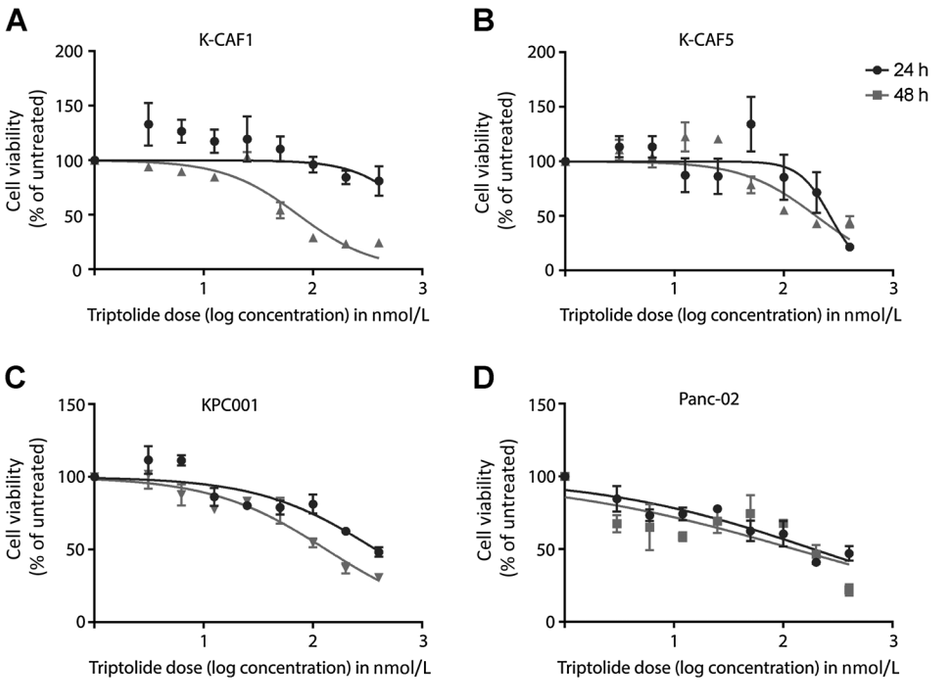

Figure 6.
Triptolide decreased viability of cancer-associated fibroblasts as well as epithelial tumor cells. Representative CAFs from KPC tumors, K-CAF1 (A), K-CAF5 (B), and epithelial mouse PDAC cell lines KPC001 (C) and Panc02 (D) were treated with indicated doses of triptolide for 24 and 48 hours. Viability was plotted as percentage of untreated control. The IC_{50} values are represented in Supplementary Table S2.

UDP-GlcNAc (22). Furthermore, this downregulation of hexosamine pathway by Minnelide also leads to inhibition of O-GlcNAc transferase that glycosylates Sp1, a transcription factor that is responsible for transcription of HAS genes (22, 39, 40). This suggested that Minnelide resulted in decreased synthesis of HA by (i) depleting the substrate pool of UDP-GlcNAc in the cells resulting in decreased activity of the HA synthase, as well as (ii) downregulating activity of Sp1, which resulted in decreased transcription of HAS genes.

Initial studies on Minnelide indicated that this compound downregulated HSP70 in pancreatic cancer cells and as a result induced cell death by triggering multiple cell death pathways (19, 21). Follow-up studies in this line have indicated that the downregulation of HSP70 is mediated by inhibition of transcriptional activity of Sp1 (22). Interestingly, Sp1 has been reported to be instrumental in regulating HA synthase genes as well (40, 41). Thus, downregulation of the HA in the stroma of the pancreatic tumor by HA may be a result of inhibition of Sp1 transcriptional activity.

Because the stromal "compression" was relieved following treatment with Minnelide, the next question was to determine whether it translated to better intratumoral drug delivery. Our results showed that treatment with Minnelide resulted in more number of "open" blood vessels in the tumor (Fig. 5A). This further facilitated a better delivery of doxorubicin (a fluorescent compound that is studied routinely to determine efficacy of drug delivery) in the tumors (Fig. 5B). The current statistics show that the presence of the abundant stroma in pancreatic tumor models like KPC, results in hypo-vascularized tumors with very poor drug perfusion. Our study showed that Minnelide not only decreased the stromal components, but also improved the functional vasculature in the tumor and increased drug delivery into the tumor that resulted in a better median survival of the KPC mice (Fig. 5C).

The understanding of the role of stroma in the pancreatic carcinogenesis, progression, and treatment is under evolution. Studies have shown that stromal elements can not only enhance cancer cell proliferation and invasion, but can also help cancer evade immune system (42). Furthermore, studies suggest that desmoplastic stroma of pancreatic cancer may also impede deliver of chemotherapeutics. In a study by Olive and colleagues, treatment of mice with pancreatic cancer in KPC genetically engineered mouse model of pancreatic cancer with gemcitabine in combination with a Shh inhibitor led to decrease stromal content and better delivery of gemcitabine into the tumor, as compared with gemcitabine treatment alone. This was ascribed to presence of increased functional vessel on depletion of stroma. However, recent studies from Rhim and colleagues (15) have shown that stroma may have a restraining effect on the tumor. In this study, chronic depletion of stroma by embryonic deletion of Shh in pancreatic epithelial cells or by chronic administration of smoothened inhibitor to KPC mice led to aggressive tumors with increased vascularity, heightened proliferation, and increased metastases. Furthermore, this effect was reversed, at least partially by VEGFR blocking antibodies, suggesting that the stroma may also restraint tumor by suppressing tumor angiogenesis.

In our study, we have shown that Minnelide is very effective in reducing stromal content of tumors as well as in improving survival of mice in KPC model of pancreatic cancer. Though this seems contrary to previous study by Rhim and colleagues where stromal depletion led to worse survival of KPC mice, we believe that this effect is due to a combination of antitumor and antistromal effect of Minnelide. We have shown in multiple previous studies that Minnelide is very effective in killing tumor cells. Thus Minnelide's stromal depletion properties ensure the intratumoral delivery of Minnelide, and of another antitumor agent when used in combination with Minnelide, for enhanced killing of tumor cells and possibly increased survival. These data support use of

stroma depletion strategy only in combination with a strong anticancer agent.

Conclusion

Minnelide has shown tremendous promise in the preclinical studies. Since August 2013, it is being evaluated in a phase1 clinical trial against GI cancers. In this respect, evaluating its effect on stroma in pancreatic cancer is extremely relevant and timely. This study shows that Minnelide is not only tumoricidal at the given doses as we have seen before, but also depletes the stromal architecture and induces stromal cell death, thus increasing the intratumoral concentration of the drug and finally eradicating them. Pancreatic cancer has extremely very poor prognosis owing to very poor drug delivery into the tumor. In this context, our study is a step forward in addressing the current challenges that exist in conquering this devastating disease.

Disclosure of Potential Conflicts of Interest

S. Banerjee is a consultant/advisory board member for Minneamrita Therapeutics. A.K. Saluja reports receiving commercial research grants from, holds ownership interest (including patents) in, and is a consultant/advisory board member for Minneamrita Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Banerjee, S. Modi, O. McGinn, V. Dudeja, A.K. Saluja
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