Signal Transducer and Activator of Transcription 3, Mediated Remodeling of the Tumor Microenvironment Results in Enhanced Tumor Drug Delivery in a Mouse Model of Pancreatic Cancer

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BACKGROUND & AIMS: A hallmark of pancreatic ductal adenocarcinoma (PDAC) is the presence of a dense desmoplastic reaction (stroma) that impedes drug delivery to the tumor. Attempts to deplete the tumor stroma have resulted in formation of more aggressive tumors. We have identified signal transducer and activator of transcription (STAT) 3 as a biomarker of resistance to cytotoxic and molecularly targeted therapy in PDAC. The purpose of this study is to investigate the effects of targeting STAT3 on the PDAC stroma and on therapeutic resistance. METHODS: Activated STAT3 protein expression was determined in human pancreatic tissues and tumor cell lines. In vivo effects of AZD1480, a JAK/STAT3 inhibitor, gemcitabine or the combination were determined in Ptf1a−/−;LSL-KrasG12D/+;Tgfbr2fl/fl; pSTAT mice and in orthotopic tumor xenografts. Drug delivery was analyzed by matrix-assisted laser desorption/ionization imaging mass spectrometry. Collagen second harmonic generation imaging quantified tumor collagen alignment and density. RESULTS: STAT3 activation correlates with decreased survival and advanced tumor stage in patients with PDAC. STAT3 inhibition combined with gemcitabine significantly inhibits tumor growth in both an orthotopic and the PTK mouse model of PDAC. This combined therapy attenuates in vivo expression of SPARC, increases microvessel density, and enhances drug delivery to the tumor without depletion of stromal collagen or hyaluronan. Instead, the PDAC tumors demonstrate vascular normalization, remodeling of the tumor stroma, and down-regulation of cytidine deaminase. CONCLUSIONS: Targeted inhibition of STAT3 combined with gemcitabine enhances in vivo drug delivery and therapeutic response in PDAC. These effects occur through tumor stromal remodeling and down-regulation of cytidine deaminase without depletion of tumor stromal content.

Keywords: Drug Delivery; Tumor Microenvironment; Stroma; Pancreatic Cancer.

Pancreatic ductal adenocarcinoma (PDAC) remains a major therapeutic challenge. Five-year survival rate is approximately 5% and has not changed significantly in the past 40 years.1 The poor response of PDAC patients to targeted and systemic therapies may be due to impedance of drug delivery to the tumor by its dense desmoplastic stroma, a hallmark of both mouse and human PDAC.2,3 This marked fibrosis (desmoplasia) is characterized by a poorly functioning vasculature that has variable blood flow through leaky, immature vessels, resulting in increased interstitial fluid pressure.4,5 Targeted inhibition of the Hedgehog pathway has been shown to deplete the tumor stroma, resulting in a transient increase in intratumoral perfusion, enhanced delivery of gemcitabine to the tumor and stabilization of disease in a mouse model of PDAC.6 Recent studies, however, have demonstrated that tumor stroma might also play an important role in restraining PDAC.7,8 Therefore, therapeutic efficacy and improved survival hinge on treatments that enhance drug delivery without incurring the negative consequences of stromal depletion.

Pancreatic stellate cells and myeloid-derived suppressor cells in the PDAC tumor microenvironment (TME) produce factors to create a pro-tumorigenic environment. Within the TME, pancreatic stellate cells are known to secrete several factors, including interleukin 6, which can activate JAK-mediated signal transducer and activator of transcription (STAT) 3 signaling and induce stromally mediated local immunosuppression through the expansion of myeloid-derived suppressor cells.9 We have recently demonstrated a mechanistic rationale for activated STAT3 as a biomarker of therapeutic resistance in PDAC.10 Targeted inhibition of STAT3 may therefore directly impact the TME to improve therapeutic response.

Genetically engineered mouse models (GEMM) of PDAC progression are invaluable tools to study tumor stromal interactions.11 Ijichi et al12 developed the Ptf1a−/−;
LSL-KrasG12D/þ;Tgfbr2fl/ox (PKT) GEMM of PDAC that develops autochthonous well-differentiated PDAC with abundant stroma. Of the PDAC GEMMs, the PKT mouse represents the closest stromal approximation to human PDAC.12–14 Additionally, this model displays constitutive STAT3 activation in both the epithelial and stromal components of the TME.15 Therefore, the PKT GEMM provides a clinically and molecularly relevant tool to probe the role of STAT3 in the PDAC TME.

In this study, we demonstrate that STAT3 activation increases with the stepwise progression from precancerous lesions to PDAC in human and mouse tumors. PDAC patients with tumors that have high levels of activated STAT3 expression exhibit higher tumor grades, more advanced stages of disease, and decreased overall survival (OS). To target JAK-mediated activation of STAT3, we used AZD1480, a JAK-selective small molecule inhibitor. STAT3 inhibition combined with gemcitabine results in significantly increased tumor microvessel density, enhanced in vivo drug delivery, and improved survival in both xenograft mouse models and PKT mice. These effects are seen without depletion of collagen or hyaluronan content within the tumor, but rather through remodeling of the tumor stroma and down-regulation of cytidine deaminase (Cda) within PDAC tumors. Taken together, these results suggest that combining STAT3 inhibition with gemcitabine is a promising therapeutic strategy for PDAC.

**Materials and Methods**

**Mice**

Female athymic nude mice—Foxn1 nu/nu (4–5 weeks old)—were purchased from Harlan Sprague Dawley, Inc.

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**Figure 1.** STAT3 expression is associated with chemoresistance and overall survival in PDAC patients. (A, B) Representative tissue sections from a TMA constructed from patient samples are shown (left panels). The tissues were grouped by diagnosis and grade, and percentage of cells positively stained for total STAT3 are shown. Scale bar = 0.5 mm. (A) Total and (B) pSTAT3 expression increased in a stepwise fashion from normal pancreas to chronic pancreatitis and through advancing grades of PDAC (right panels). (C) Kaplan-Meier survival curve comparing overall survival for patients with PDAC stratified by level of pSTAT3 staining. Patients with high pSTAT3 expression have significantly decreased overall survival compared with patients with low pSTAT3 expression. (D) Expression of pSTAT3 and total STAT3 in pancreatic cell lines generated from pancreatic intraepithelial neoplasia (PanIN), primary PDAC (PDA) and liver metastatic (LMP) lesions from LSL-KrasG12D/þ; Pdx1Cre/þ (PanIN) and LSL-KrasG12D/þ; Trp53R172H/þ; Pdx1Cre/þ (PDA and LMP) mice (left panel), and in human PDAC cell lines (right panel) are demonstrated. tSTAT3, total STAT3; pSTAT3, phosphorylated STAT3; *P < .05; **P < .01; ***P < .001.
**Figure 2.** Combined AZD1480 and gemcitabine treatment decreases tumor growth and alters the tumor microenvironment in PDAC xenograft models. (A) AZD1480 with gemcitabine (Gem) induces in vivo tumor regression. Growth rate of PANC1 flank xenografts in Fox1-nu/nu mice treated with vehicle, AZD1480, Gem, or AZD1480/Gem treatment. Error bars indicate SD of mean; n = 5 per group. **P < .01. (B) Orthotopic pancreatic injections of luciferase tagged PANC1 cells were treated with vehicle, AZD1480, Gem, or AZD1480/Gem treatment. Tumor xenograft tissues (n = 3) were analyzed for total STAT3, pSTAT3, and SPARC expression by immunoblot analysis. AZD1480/Gem treatment significantly inhibits expression of pSTAT3 and SPARC. (C) Immunohistochemistry of orthotopic pancreatic tumor xenograft tissues show decreased SPARC expression with AZD1480 and AZD1480/Gem treatment. Left panels show representative examples of tumor tissues stained with SPARC (scale bar = 0.5 mm) and the quantified graph in the right panel. *P < .05; ***P < .001. (D) Analysis of tumor lysates of orthotopic pancreas injections of sh-scrambled and sh-STAT3 PANC1 cells show that sh-STAT3 PANC1 tumors have significantly decreased expression of pSTAT3 and SPARC. (E) Orthotopic pancreatic tumor xenograft tissues were stained with CD31 antibody to measure microvessel density. Left panels show representative examples of tumor tissues stained with CD31 (scale bar = 0.5 mm) and the quantified graph in the right panel. CD31 staining showed markedly increased microvessel density with AZD1480/Gem combined therapy. *P < .05.

Tissue Microarray of Pancreatic Tissues

A tissue microarray (TMA) was constructed as described previously. Eight normal pancreas, 7 chronic pancreatitis, 11 well-differentiated PDAC, 24 moderately differentiated PDAC, and 11 poorly differentiated PDAC tissue cores were used. TMA slides were concurrently evaluated by 2 of the authors (C.S. and N.B.M.). Nuclear and cytoplasmic staining was scored as follows: the staining index was considered as the sum of the intensity score (0, no staining; 1+, weak; 2+, moderate; 3+, strong) and the distribution score (0, no staining; 1+, staining of <33% of cells; 2+, between 33% and 66% of cells; and 3+, staining of >66% of cells). Staining indices were classified as follows: 3+ or higher, strong staining; 2+ to 3+, weak staining; and 0, negative staining. STAT3 and phosphorylated STAT3 (pSTAT3) were scored as positive if any detectable nuclear or cytoplasmic staining was present. For survival analysis, low pSTAT3 staining was defined as a staining index of 0 to 2+. High pSTAT3 staining was defined as an intensity score of 3+. 

(Indianapolis, IN). Ptf1a<sup>cre/+;</sup>Tgfb1<sup>2lox/flox</sup> and LSL-Kras<sup>G12D/+;</sup>Tgfb2<sup>2lox/flox</sup> mice were provided by Dr Hal Moses (Vanderbilt University Medical Center, Nashville, TN). These 2 lines were intercrossed to generate Ptf1a<sup>cre/+;</sup>LSL-Kras<sup>G12D/+;</sup>Tgfb2<sup>2lox/flox</sup> mice (PKT) on a C57Bl/6 background. Genotyping of alleles was performed using oligonucleotide primers as described previously.16–18
Treatment of \( \text{Ptf1a}^{\text{cre}+/} \cdot \text{LSL-Kras}^{G12D/+} \cdot \text{Tgfb}^{\text{2}^{\text{fl}x/\text{fl}x}} \) Mice

PKT mice were treated with vehicle, gemcitabine, AZD1480, or a combination of gemcitabine and AZD1480. Mice received twice-weekly intraperitoneal injections of vehicle or 20 mg/kg of gemcitabine, starting at 4 weeks of age. Mice in the AZD1480 arm received 30 mg/kg/d by oral gavage 5 days/week, starting at 3 weeks of age. Mice were euthanized and dissected after 3 weeks unless they were part of the survival arm. Due to the irregularity of the tumor dimensions, size was determined by weighing the entire tumor. Tumor tissue was processed for further immunohistochemical and second harmonic generation (SHG) examination. Overall survival was determined by log-rank analysis using STATA software (version 13.1, StataCorp, College Station, TX).

Second Harmonic Generation Imaging

Collagen SHG imaging was performed on a custom-built multiphoton microscope (Prairie Technologies, Middleton, WI) as described previously. Briefly, excitation and emission light were coupled through a 40× oil immersion objective (1.3 NA) within an inverted microscope (TII; Nikon, Tokyo, Japan). A titanium:sapphire laser (Coherent Inc, Santa Clara, CA) was tuned to 900 nm (mean power 8.4–8.6 mW) for SHG imaging. A dichroic mirror, 500 nm, and a bandpass filter, 450/35 nm, isolated SHG emission light. A pixel dwell time of 4.8 microseconds was used to acquire 1024×1024 pixel images. Each image was frame averaged 8 times to reduce noise. Collagen alignment was quantified from the SHG images using the curvelet-based alignment analysis software CurveAlign;
Collagen density was quantified by Hessian filter vessel segmentation.

Statistical Analysis

Descriptive statistics were calculated using Microsoft Excel (Redmond, WA) and Prism software (GraphPad, San Diego, CA). Results are shown as mean ± SD unless otherwise indicated. Statistical analyses of immunohistochemistry data were performed using the analysis of variance followed by Tukey’s multiple comparisons test to determine P values. The 2-sided Student t test was used for statistical analysis; P < .05 was considered significant except when indicated otherwise. Kaplan-Meier survival analysis was performed, and survival differences between groups were assessed with the log-rank test.

Study Approval

All animal experimental protocols were approved by the Institutional Animal Care and Use Committees of Vanderbilt University and conducted according to the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines.

See the Supplementary Materials for cell lines, immunohistochemistry, Western blot analysis, xenograft models, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, magnetic resonance imaging (MRI), in vivo bioluminescence imaging, STAT3 gene knockdown by short hairpin (sh) RNA, and orthotopic injections.

Results

Total and Activated Signal Transducer and Activator of Transcription 3 Expression in Human Pancreas Tissues and Cell Lines

A TMA of patient samples was examined for total and activated STAT3 (pSTAT3) expression in order to determine the expression of STAT3 in normal pancreatic and PDAC tissue. Analysis confirmed a stepwise increase of both total (Figure 1A) and pSTAT3 expression (Figure 1B) from normal pancreas to chronic pancreatitis through advancing grade and stage of PDAC, indicating that STAT3 expression and activation increases with the progression of pancreatic neoplasia. Furthermore, patients with PDAC tumors that expressed high levels of pSTAT3 had significantly higher tumor grade, stage, and lower OS when compared with patients with tumors that had low pSTAT3 expression (median survival of 14 months vs 22 months,
respectively; \( P = .029 \), Figure 1C and Supplementary Table 1).

Expression levels of total and pSTAT3 were characterized in 9 human PDAC cell lines, a pancreatic intraepithelial neoplasia cell line derived from the \( LSL-Kras^{G12D/+}; Pdx1^{Cre/+} \) GEMM and primary PDAC (PDA) and liver metastasis (LMP) cell lines derived from the \( LSL-Kras^{G12D/+}; Trp53^{R172H/+}; Pdx1^{Cre/+} \) GEMM. We have previously characterized the

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F
sensitivity of these 9 human PDAC cell lines to various therapeutic agents, including AZD1480 (Supplementary Table 2). The resistant human cell lines (PANC1, MiaPaCa2, and CFPAC), as well as the murine metastatic cell line (LMP), were found to have the highest baseline expression of pSTAT3, while the highly sensitive human cell lines (BxPC3, HPAC) and mouse pancreatic intraepithelial neoplasia cells had little or no baseline expression of pSTAT3 (Figure 1D). Expression of other isoforms of STAT, such as STAT5, did not show variable expression in these cell lines (data not shown).

Pharmacologic JAK-STAT3 inhibition with AZD1480 revealed a dose-and time-dependent down-regulation of pSTAT3 levels in the resistant PANC1 and MiaPaCa2 cell lines (Supplementary Figure 1A and B). Stattic, a small molecule that specifically inhibits the SH2 domain of STAT3, also significantly decreased pSTAT3 levels in the resistant MiaPaCa2 and LMP cell lines (Supplementary Figure 1C). Knockdown of STAT3 in PANC1 cells (sh-STAT3 PANC1) also resulted in significantly decreased colony formation (Supplementary Figure 2).

STAT3 Inhibition Combined With Gemcitabine Effectively Inhibits Pancreatic Ductal Adenocarcinoma Tumor Growth In Vivo

We next sought to determine the efficacy of STAT3 inhibition in vivo. Mice were inoculated subcutaneously (n = 5/group) with PANC1 cells. Tumor-bearing mice were treated with AZD1480 and gemcitabine either individually or in combination. Compared with vehicle-treated mice, tumor volume was significantly decreased in mice treated with AZD1480 (P < .001) or gemcitabine alone (P < .001). The combination of AZD1480 and gemcitabine, however, resulted in the optimal treatment effect and tumor regression and was superior to both vehicle and monotherapy (P < .001, Figure 2A). The combination therapy was well tolerated and did not result in any significant in vivo toxicity (Supplementary Figure 3A).

Orthotopic tumors were induced with direct pancreatic injections of luciferase-tagged PANC1 cells and bioluminescence imaging was utilized to monitor orthotopic tumor growth and treatment response. Tumor-bearing mice treated with the same regimens underwent bioluminescence imaging before initiation of treatment and then weekly thereafter. There was a trend toward the mean photon emission over the treatment interval being lowest in the groups that received either AZD1480 or the combination of AZD1480 and gemcitabine, but this did not achieve statistical significance (Supplementary Figure 4). This decline in signal correlated with decreased tumor growth (data not shown). Immunoblotting of orthotopic xenograft tumor lysates demonstrated significant inhibition of STAT3 phosphorylation (Figure 2B).

STAT3 Inhibition Combined With Gemcitabine Alters the Pancreatic Ductal Adenocarcinoma Tumor Microenvironment

To determine the mechanism responsible for the synergistic antitumor activity of JAK-STAT3 inhibition and gemcitabine treatment, we focused on stromal effectors of STAT3 activation. STAT3-specific inhibition with Stattic resulted in decreased SPARC expression levels in the resistant MiaPaCa2 and PANC1 cell lines (Supplementary Figure 1D). Orthotopic and flank tumors treated with AZD1480 or AZD1480 and gemcitabine had significantly decreased pSTAT3 and epithelial SPARC expression (Figure 2B and C and Supplementary Figure 5A). Immunoblotting of STAT3 shRNA PANC1 flank xenografts confirmed that these findings were specifically related to STAT3 inhibition and not an off-treatment effect of AZD1480 (Figure 2D).

Combined treatment with AZD1480 and gemcitabine also resulted in significantly increased CD31 staining and microvessel density in both orthotopic and flank tumor xenografts when compared with vehicle control and monotherapy-treated tumors (Figure 2E, Supplementary Figure 5B). Therefore, this combined therapy alters stromal composition by attenuating the expression of SPARC and increasing microvessel density within the TME.

Signal Transducer and Activator of Transcription 3 Inhibition Combined With Gemcitabine Inhibits Tumor Growth and Improves Survival in Ptf1acr+/−;LSL-KrasG12D+/−, Tgfbr2lox/lox Mice

To further explore the effect of STAT3 inhibition on the tumor stroma, we utilized the PKT mouse model. These mice develop autochthonous PDAC with full penetrance that reliably recapitulates the clinical and histopathologic features of the human disease. They consistently develop...
pancreatic intraepithelial neoplasia lesions at 3.5 weeks of age and progress to invasive cancer by 4.5 weeks of age. Median overall survival of PKT mice is consistently around 59 days.12 Treatments were initiated at 4 weeks of age and mice were allowed to progress with PDAC until they died or became moribund. Tumor weight was significantly reduced in mice treated with AZD1480 and gemcitabine compared with vehicle-treated mice (Figure 3A) without significant additive toxicity (as indicated by change in mouse body weight, Supplementary Figure 3B). Immunoblot analyses of whole-tumor lysates showed decreased expression of pSTAT3 and SPARC with either AZD1480 or combined AZD1480 and gemcitabine treatment (Figure 3B). CD31 staining and microvessel density were also significantly enhanced in mice treated with the combination therapy (Figure 3C). PKT mice treated with either AZD1480 or gemcitabine alone showed no survival benefit compared with vehicle-treated mice (Supplementary Figure 6). In contrast, treatment with AZD1480 and gemcitabine significantly extended the median survival of PKT mice (52 vs 60 days; P = .033, log-rank test) (Figure 3D).

Treatment With AZD1480 and Gemcitabine Results in Significantly Enhanced In Vivo Drug Delivery

MALDI imaging mass spectrometry, an excellent tool for visualizing small molecules in tissue sections, was utilized to determine the presence and localization of AZD1480 and gemcitabine (Supplementary Figure 7A and B) in subcutaneous (Figure 4A) and orthotopic (Figure 4B) xenograft tumors. Although AZD1480 was present in tumors of mice treated with AZD1480 monotherapy, there was no evidence of gemcitabine penetration in the tumors of mice treated with gemcitabine alone. Mice treated with AZD1480 and gemcitabine demonstrated significantly enhanced tumor penetration of both AZD1480 and gemcitabine to the tumor (Supplementary Figure 6A and B). These results show that in vivo tumor drug delivery is significantly enhanced with combined AZD1480 and gemcitabine treatment.

Increased Tumor Drug Delivery Is Not Due to Depletion of the Tumor Stroma

We next sought to determine whether the enhanced antitumor effect and drug delivery with AZD1480 and gemcitabine treatment was due to depletion of the tumor stroma. In addition to collagen, hyaluronan is an important constituent of the stroma and has been implicated as a potential barrier to drug delivery in PDAC. Immunohistochemical quantification of hyaluronan and collagen in tissues stained for hyaluronan binding protein, collagen IV, and trichrome blue, respectively (Figure 5A and B), demonstrated no significant changes in stromal content between treatment groups. Analysis of H&E and α-smooth muscle actin stained slides failed to demonstrate any difference in the inflammatory cell infiltrate or myofibroblast composition of the tumor stroma of untreated vs treated tumors (data not shown). Furthermore, there was no difference in the collagen content of STAT3-shRNA PANC1 tumor xenografts when compared with sh-scrambled control PANC1 tumors (Figure 5C).

Magnetization transfer (MT)-MRI provides a correlation of the level of fibrosis within tissues. No significant difference in the longitudinal assessment of MT ratio signal between vehicle vs monotherapy or the combination treatment in PKT mice was seen, despite a slower rate of tumor growth (Figure 5D, E, and F). Contrary to our expectations, treatment with AZD1480 and gemcitabine did not deplete collagen or hyaluronan content, despite the decrease in tumor size, decreased expression of the stromal effector SPARC, and enhanced drug delivery to the tumor.

Improved Therapeutic Response Is Mediated by Stromal Remodeling and Down-Regulation of Cytidine Deaminase

To further define the effect of AZD1480 and gemcitabine treatment on the tumor stroma, SHG images of PKT mouse tumors were obtained and analyzed (Figure 6A). Multiphoton SHG imaging allows specific imaging of collagen fibers, as well as analysis of fiber orientation and quantification within tissue samples, making it a potent tool to investigate collagen organization, quantity, and remodeling. Analysis of collagen density revealed no difference between treatment groups, further confirming the lack of collagen depletion. However, the coefficient of collagen fiber alignment was significantly decreased and the angle variance was significantly increased with the combination therapy, indicating less parallel fiber alignment and increased variation in fiber direction, respectively. These data indicate that treatment with AZD1480 and gemcitabine results in tumor stroma that has significantly fewer parallel collagen fibers than vehicle- or monotherapy-treated tumors. Analysis of the angle distribution showed that the tumors treated with the combined therapy also had significantly lower kurtosis, indicating a less-skewed distribution of collagen fiber rearrangement. Thus, treatment with AZD1480 and gemcitabine results in significant stromal remodeling with greater collagen fiber disorganization, rather than collagen depletion, while still significantly decreasing tumor size.

Gemcitabine can be enzymatically inactivated by Cda, which is highly expressed in murine PDA neoplastic cells. Due to the enhanced delivery of gemcitabine when combined with AZD1480, we analyzed PKT mouse lysates for Cda expression. There was significant down-regulation of Cda levels with AZD1480 treatment, as well as combined AZD1480 and gemcitabine treatment (Figure 6B). Monotherapy with gemcitabine actually led to increased tumor Cda expression. Immunoblotting of Cda in STAT3 shRNA PANC1 cells confirmed that the change in Cda expression is specifically related to STAT3 inhibition and not off-target effects of AZD1480 (Supplementary Figure 9). These findings are consistent with the lack of drug delivery seen on MALDI imaging mass spectrometry of gemcitabine-treated tumors. These results show that the treatment effect of AZD1480 combined with gemcitabine results in
remodeling of the tumor stroma without depletion of collagen content and down-regulation of Cda expression, leading to enhanced drug delivery to the tumor (Figure 6C and D).

**Discussion**

The focus of this study was to analyze the effect of STAT3 inhibition on the tumor stroma in PDAC. Our previous work has provided compelling evidence establishing
STAT3 as a key mediator of chemo-resistance in PDAC. Further confirming these results, we now show a clear association between pSTAT3 expression levels in patient PDAC tumors and poor prognosis. Interestingly, total STAT3 expression also rises with advancing grades of tumor. Recent studies have demonstrated that non-phosphorylated STAT3 may translocate to the nucleus and stimulate expression of pro-inflammatory genes in a distinct manner from pSTAT3. Activated STAT3 signaling in tumor cells acts as a crucial oncogenic mediator and is critical to the interplay between stromal and preneoplastic pancreatic cells. Taken together, these findings provide strong evidence that disrupting STAT3 signaling blocks a key interface of tumor-stromal interaction in PDAC.

Targeting JAK/STAT3 exerts a significant anti-tumor effect in vivo and has a direct impact on the TME. Our findings indicate that STAT3 inhibition consistently down-regulates SPARC expression and, when combined with gemcitabine, leads to a significant increase in microvessel density of the tumor. Pancreatic cancer is notable for its dysfunctional vasculature and hypoxic TME, and although anti-angiogenic-targeted therapy has been ineffective in clinical trials, the ability to induce vascular normalization may result in significantly improved clinical outcomes through increased drug delivery and anti-tumor effects. Recent findings suggest that depletion of the tumor stroma through genetic ablation or pharmacologic inhibition of Sonic hedgehog leads to a substantial increase in tumor vascularity and enhanced drug delivery in LSL-KrasG12D/; Trp53R172H/+; Pdx1Cre/+ mice. In human PDAC tumors, gemcitabine delivery is impeded due to increased stromal density. We initially hypothesized that enhanced microvessel density was the result of stromal depletion and alleviation of solid stress, given the well-described role of the stroma in PDAC tumor promotion and progression. Using MALDI mass spectrometry, we showed that the combined treatment with AZD1480 and gemcitabine significantly enhances delivery of both drugs to the tumor. Interestingly, however, we found that this enhanced drug delivery was not due to depletion of tumor stromal collagen or hyaluronan content. This finding was consistently seen when assessing stromal density with both MT-MRI and SHG imaging. Although preclinical studies in LSL-KrasG12D/; Trp53R172H/+; Pdx1Cre/+ mice have linked enhanced delivery of gemcitabine to tumor stromal depletion with Sonic hedgehog inhibition, we demonstrate that enhanced drug delivery is not necessarily dependent on depletion of the tumor stroma. Failure to deplete stromal bulk may actually be a key factor in the improved therapeutic efficacy of the combination therapy. Özdemir et al found that depletion of myofibroblasts in the PKT mouse model led to reduced OS and an increase in immunosuppressive T- regulatory cells in the TME. Rhim et al also found that depletion of stromal cells with Sonic hedgehog inhibition resulted in formation of more aggressive and undifferentiated tumors and decreased OS. These studies clearly demonstrate that myofibroblasts and desmoplasia also play key roles in restraining PDAC, and that depletion of the stroma may be an undesirable outcome. Analysis of the stromal organization in Pkt mice revealed that STAT3 inhibition combined with gemcitabine resulted in significantly increased disorganization and remodeling of the stromal collagen architecture, despite retaining its bulk. The high interstitial pressure in PDAC tumors may result most directly from solid stress induced by the solid components of the tumor stroma, such as collagen and hyaluronan, and stromal remodeling may allow for alleviation of this solid stress without incurring the negative impact of stromal depletion.

Another key mechanism of PDAC tumor resistance to gemcitabine is through expression of Cda, which metabolizes gemcitabine, leading to decreased intra-tumor levels of the drug. Frese et al found that the synergistic effect of protein-bound paclitaxel is due to reduction of Cda levels through reactive oxygen species-mediated degradation and increased stabilization of gemcitabine. Our results demonstrate that STAT3 inhibition, pharmacologic or shRNA knockdown, significantly down-regulates expression of Cda. This represents a novel finding of STAT3 inhibition that has not been described previously. Therefore, AZD1480-mediated stromal remodeling and down-regulation of Cda in the tumor play critical roles in the enhanced delivery of gemcitabine to the tumor, as well as enhanced activity of gemcitabine within the tumor, although the relative contribution of each is yet to be determined.

Collectively, our results demonstrate that elevated STAT3 expression correlates with poor prognosis in patients with PDAC. Combining STAT3 inhibition with gemcitabine results in enhanced drug delivery of both agents and improved OS in an aggressive GEMM of PDAC. The mechanism of this synergistic response is not mediated by stromal depletion, but rather through stromal remodeling and down-regulation of Cda. These findings indicate that combining pharmacologic blockade of JAK/STAT3 signaling with cytotoxic gemcitabine may be an effective therapeutic strategy for patients with PDAC.

Supplementary Material
To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.07.058.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials

Cell Lines/Culture and Reagents

Murine PanIN, primary PDAC (PDA) and liver metastasis (LMP) cell lines were derived from the LSL-KrasG12D/+; Pdx1Cre/+ and LSL-KrasG12D/+; Trp53R172H+/+; Pdx1Cre/+ mouse models of PDAC1 (kindly provided by Dr Andrew Lowy) and maintained as described previously.2 The immortalized human pancreatic ductal cell line HPDE6-E6E7 (H6c7) was kindly provided by Dr M. S. Tsao3 and was maintained in in keratinocyte growth media (Invitrogen, Carlsbad, CA) supplemented with human epidermal growth factor and bovine pituitary extract. Human pancreatic cancer cell lines MiaPaCa2, PANC1, SW1990, AsPC1, Capan2, Capan1, CFPAC, HPAC, and BxPc3 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Tumor cells were maintained according to the ATCC guidelines. PANC1-Luc cells (kindly provided by Dr Thiru Arumugam (MD Anderson Cancer Center, Houston, TX). Stattic was purchased from Calbiochem (Bi- erica, MA). AZD1480 was provided by AstraZeneca (London, UK), and gemcitabine was purchased from Eli Lilly and Company (Indianapolis, IN). For in vitro experiments, AZD1480 was dissolved in 100% dimethyl sulfoxide to prepare a 10-mM stock and stored at −20°C. For in vivo experiments, AZD1480 was formulated daily in purified, sterile water supplemented with 0.5% hydroxypropyl methyl cellulose (Acros Organics, Geel, Belgium) and 0.1% Tween 80 (Fisher Scientific, Logan, UT).

Cell Viability Assay

Cells were treated with dimethyl sulfoxide or AZD1480 (0-5000 nmol/L) for 48 hours and cell viability was determined by MTT (Sigma, St Louis, MO) assay according to the manufacturer’s direction. The 50% inhibitory concentration was calculated using Prism software package (GraphPad). Each condition was assayed in triplicate.

Immunohistochemistry

Tissues were fixed and immunostained using antibodies against pSTAT3 (Tyr705), trichrome blue, and collagen IV (Abcam, Cambridge, MA), SPARC (Invitrogen), hyaluronic acid binding protein (Calbiochem), and CD31 (Bianova, Hamburg, Germany). Stained tissues were evaluated by an expert pathologist (C.S). Immunostained slides were scanned and stained was quantified using the Ariol SL-50 platform (Leica Camera AG, Wetzlar, Germany). Digital slide images were adjusted to exclude areas containing obvious histologic artifacts, such as tissue folds or nonorganic material, from the digital image. Computer-based image analysis was performed with Ariol Review Software (Leica Camera AG). After threshold levels were established for each stain, the resulting color recognition algorithm was applied to all digital images. Calculated percentage positive cells stained relative to total area analyzed with a scale for relative intensity and reported as relative expression of protein staining.

Western Blot Analysis

Western blot analyses were performed using standard methods described previously.

Xenograft Models

Subcutaneous tumors were established by injecting 2 × 10⁶ PANC1 cells into the flank of 6-week-old Fox1- nu/nu mouse (n = 5 in each group). Treatment was initiated when the subcutaneous tumors reached 75–100 mm³ size. Orthotopic tumors were established by injecting a single-cell suspension of 250,000 PANC1-Luc cells (infected with lentivirus-expressing luciferase) into the pancreas of 8-week-old Fox1- nu/nu mice (n = 3 in each group). Bioluminescent imaging of anesthetized animals was performed every 3 days starting 15 days after injection until the end of the study to assess tumor growth. Drug treatment was initiated at the same time point. AZD1480 (30 mg/kg/d) or vehicle (hydroxypropyl methyl cellulose/ Tween 80) was administered by oral gavage; gemcitabine (20 mg/kg/3 d) was administered intraperitoneally for 50 (subcutaneously) or 40 (orthotopically) days. The subcutaneous tumor volume (V) was determined by caliper measurements obtained every 2 days and calculated by the equation V = L × W² × 0.5, where L is length and W is width of a tumor. The percent body weight change for each mouse was calculated with the following formula: [(Wₙ – W₀)/W₀] × 100%, where Wₙ is the mouse weight on day n, and W₀ is the mouse weight at the start of treatment. Growth curves for tumors were plotted as the mean volume ± SD of tumors of mice from each group. At the end of the study, animals were sacrificed and their primary tumors were removed for further analysis.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Tumors from animals were snap-frozen in liquid N₂ and stored at −80°C. Frozen tissues were cut into 12-μm-thick sections on a cryostat (Leica Microsystems, Buffalo Grove, IL). These sections were transferred and thawed onto gold-coated stainless-steel MALDI target plates. For imaging applications, matrix solution (2,5-dihydroxybenzoic acid, 30 mg/mL in 1:1 acetonitrile:water with 0.1% trifluoroacetic acid) was manually spray-coated over the tissue using a glass nebulizer until there was a relatively homogeneous layer of matrix crystals over the surface as detailed previously.1 MALDI mass spectra were acquired on a LTQ XL mass spectrometer (Thermo Scientific) in MS/MS mode. MS/MS was performed on the protonated parent ions and full product ion spectra were obtained, allowing AZD1480 and gemcitabine to be effectively analyzed by selected reaction monitoring. The following transitions were used for each compound: for AZD1480, m/z 349/351 → m/z 225/227 and for gemcitabine, m/z 264→m/z 112 (Supplementary Figure 8). Spectra were acquired over each tissue section at 150 or 200 μm spatial resolution. Images were created in ImageQuest software (Thermo Scientific) by plotting the intensity of the main fragment ions as a function of position over the tissue surface.

Magnetic Resonance Imaging

Mice were anesthetized via inhalation of 2%/98% isoflurane/oxygen. Animals were secured in a prone position and placed in a 38-mm inner diameter radiofrequency coil. A rigid bite-bar and head restraint were used to secure the animal’s head, as well as to reduce motion-induced artifacts in the images. Animals were then placed in a Varian 7T horizontal bore imaging system (Varian, Palo Alto, CA) for data collection.
Respiration rate and internal body temperature were continuously monitored, and a constant body temperature of 37°C was maintained using heated airflow.

For each animal, multi-slice scout images were collected in all 3 imaging planes (axial, sagittal, and coronal) using a gradient echo sequence with repetition time $= 75$ milliseconds, echo time $= 5$ milliseconds, slice thickness $= 2$ mm, flip angle $= 35$ degrees, and a mean of 4 acquisitions. Additional parameters include field of view $= 50 \text{ mm} \times 50 \text{ mm}$ and data matrix $= 128 \times 128$. Following localization of the pancreas, T2-weighted fast-spin echo images were acquired over 22 slices in the axial and coronal planes, with field of view $= 25.6 \text{ mm} \times 25.6 \text{ mm}$, slice thickness $= 1.0 \text{ mm}$, and data matrix $= 256 \times 256$. Additional parameters included repetition time $= 2$ seconds, effective echo time $= 36$ milliseconds, echo train length $= 8$, echo spacing $= 9$ milliseconds, and number of experiments $= 12$. Additionally, MT ratio data were acquired using a multi-slice spoiled gradient echo sequence over the same field of view and axial slices as the anatomical (fast spin-echo) images acquired earlier. Additional parameters included repetition time $= 500$ milliseconds, echo time $= 3.2$ milliseconds, matrix $= 256 \times 256$, flip angle $= 30$ degrees, number of experiments $= 6$, and off-resonance frequency $= 1500$ Hz. An additional scan, without MT saturation, was also acquired.

After acquisition, data were transferred to Matlab 2013a (MathWorks), and voxel-wise MT ratio maps were calculated as follows: $100 \times (1 - M_{\text{sat}}/M_0)$, where $M_{\text{sat}}$ represents the signal intensity of the image after application of the off-resonance MT pulse, and $M_0$ is the signal intensity without MT saturation. MT ratio data matrices were then written to ASCII-delimited files for further analysis in ImageJ (National Institutes of Health, Bethesda, MD). Once the data was imported into ImageJ, a region of interest circumscribing the pancreas was determined for each imaging slice, and the mean MTR within the region of interest and volume were calculated and reported.

**In Vivo Bioluminescence Imaging**

Mice were injected once intraperitoneally with 150 mg/kg (10 mg/mL in phosphate-buffered saline) VivoGlo luciferin (Promega, Madison, WI). Mice were anesthetized with 1.5% isoflurane and placed in the imaging chamber of an IVIS 200 CCD camera (Caliper Life Sciences, Hopkinton, MA). Bioluminescence images were captured with 1-minute integration time for 10 minutes after the luciferin injection. Images and measurements of bioluminescent signals were acquired and analyzed using Living Image software (Xenogen, Alameda, CA). Image data are displayed in photons/s/cm²/sr. Camera settings, such as integration time, binning, f/stop, and field of view were kept constant during all measurements. Mice were initially imaged before treatment and thereafter imaged weekly.

**Signal Transducer and Activator of Transcription 3 Gene Knockdown by Short Hairpin RNA**

Lentiviral pS1H1-puro-STAT3 shRNA (#26596) and pS1H1-puro-control shRNA (#26597) were obtained from Addgene (Cambridge, MA). Transfection was performed as described previously. Lentiviral particles were prepared by co-transfecting 3 plasmids into 293T cells, including pMD2.G, psPAX2, and lentivectors. Supernatants were collected every 12 hours between 36 and 96 hours after transfection, pooled together, and concentrated using ultracentrifugation. PANC1 cells were transduced with lentiviral vectors at a multiplicity of infection of 20 supplemented with polybrene (6 µg/mL) for 18 hours, and green fluorescent protein expression was confirmed by FACS Caliber flowcytometer (BD Biosciences, San Jose, CA). Cells were then selected for 7 days with puromycin (1.5 µg/mL), and when cultures reached near confluency, cells were trypsinized and processed by fluorescence-activated cell sorting analysis to separate cells with highest green fluorescent protein expression. To generate stable knockdown clones, these cells were plated at high dilutions in 10-cm Petri dishes and colonies obtained from single cells were screened for the expression of STAT3 by Western blot analysis.

**Orthotopic Injections**

Mice were anesthetized via inhalation of 2%/98% isoflurane/oxygen. Animals were secured in a supine position on a 37°C heating pad and the abdomen was sterilized with a 10% povidone iodine solution. All animals were given analgesia before surgery. Sterile surgical instruments were utilized to make a 1.5-cm transverse incision in the left upper quadrant of the abdomen. The tail of the pancreas was isolated and a 29-gauge 0.3-µL syringe was used to inject 250,000 cells in 25 µL phosphate-buffered saline. After injection, the site was monitored to ensure no leakage or active bleeding. The spleen and pancreas were then internalized and the abdominal wall was closed in 2 layers with absorbable suture. Mice were monitored every 15 minutes for 2 hours, and then every 12 hours over 48 hours. Forty-eight hours of postoperative analgesia was provided to the mice.

**Soft Agar Assays**

Then $5 \times 10^5$ cells were suspended in media containing 0.33% Select Agar (Invitrogen) and plated on a bottom layer of media containing 0.5% select agar. Plates were incubated at 37°C for 2–3 weeks before imaging. Colonies were photographed and quantified using ImageJ software and analysis was performed with Prism software (Graphpad Software Inc).

**References**


4. Schwartz SA, Reyzer ML, Caprioli RM. Direct tissue analysis using matrix-assisted laser desorption/


Supplementary Figure 1. Effect of STAT3 inhibition on PDAC cell lines. (A) Immunoblot of total and pSTAT3 expression in PANC1 and MiaPaCa2 cells treated with AZD1480 (0–500 nmol/L) for 4 hours shows a dose-dependent decrease in pSTAT3. (B) Immunoblot of total and pSTAT3 expression in PANC1 cells treated with 100 nmol/L AZD1480 for up to 8 hours shows a time-dependent decrease in pSTAT3. (C) Immunoblot of total and pSTAT3 expression in MiaPaCa2 and LMP cells treated with Stattic (0–100 μmol) for 4 hours shows a dose-dependent decrease in pSTAT3. (D) Immunoblot of SPARC expression in MiaPaCa2 and PANC1 cells treated with Stattic (25 micromolar, 4 hours).

Supplementary Figure 2. STAT3 knockdown decreases number of colonies. sh-Scrambled (sh-Scram) and sh-STAT3 PANC1 cells were analyzed for colonies as detailed in Materials and Methods. Each experiment was performed in triplicate. ***P < .001.
Supplementary Figure 3. Mouse weight during therapeutic intervention. (A) Orthotopic pancreatic injections of luciferase tagged PANC1 cells in Fox1-nu/nu mice were treated as indicated. Weights were obtained weekly, and combination treated mice actually had significantly more weight gain during therapy than mice treated with gemcitabine or vehicle (2-way analysis of variance with Tukey’s multiple comparison test). (B) PKT mice were treated as indicated and weights were recorded weekly. There was no significant difference between the weights of any treatment group. **P < .01.

Supplementary Figure 4. Bioluminescence imaging (BLI) of orthotopic xenograft tumors. Luciferase tagged PANC1 cells (PANC1-Luc) were injected orthotopically into the pancreata of Fox1-nu/nu mice. BLI was performed weekly for 6 weeks after initial injections. Representative mice from each treatment group (n = 2 for control, AZD1480, gemcitabine; n = 3 for combination) are shown in the upper panels, with measured mean photon emission of the corresponding mouse in the lower panel. There was a trend toward decreased mean photon emission with AZD1480 and the combination therapy when compared with gemcitabine treatment (P = .073 and P = .016, respectively; Benjamini-Hochberg corrected significance level q = .014). Mean photon intensity was expressed in units of photons/sec/cm²/steradian (p/s/cm²/sr).
Supplementary Figure 5. Immunohistochemical analysis of flank tumor xenografts. Athymic nude mice were injected with PANC1 cells, and harvested tumors were analyzed for (A) SPARC and (B) CD31 expression. SPARC expression was significantly decreased with AZD1480 treatment and AZD1480/Gem treatment compared with Gem or vehicle treatment. CD31 expression was significantly increased with AZD1480/Gem treatment compared with all other treatments. *P < .05; **P < .01; ***P < .001.

Supplementary Figure 6. Overall survival of PKT mice. AZD1480/Gem treatment led to significantly longer OS compared with vehicle-treated controls (P = .033, log-rank test). There was no significant difference in OS compared with Gem or AZD1480 monotherapy. *P < .05.
Supplementary Figure 7. MALDI imaging mass spectrometry (MS) detection of therapeutic compounds. (A) Structure of AZD1480 and the resulting spectra of standard AZD1480 in MS and MS/MS mode, using 2,5-dihydroxybenzoic acid as matrix. The fragment ions at m/z 225/227 likely result from cleavage of the amine bond as shown in the figure. (B) Structure of gemcitabine and the resulting spectra in MS/MS mode, using 2', 4', 6'-trihydroxyacetophenone (THAP) as matrix.
Supplementary Figure 8. Quantification of MALDI mass spectrometry signal intensity. (A) Quantification of average signal intensity over the middle region of each flank tumor xenograft (n = 3) demonstrates significantly enhanced delivery of AZD1480 and Gem in only the combination therapy group. (B) The same process demonstrates that the combination therapy enhances delivery of both AZD1480 and Gem in orthotopic tumor xenografts (n = 2). ***P < .001.

Supplementary Figure 9. STAT3 knockdown in PDAC cells and expression of Cda. (A) Multiple sh-STAT3 PANC1 clones were generated, and Western blot confirmed attenuated expression of total and pSTAT3 compared with sh-Scrambled (sh-Scram) controls. (B) sh-STAT3 PANC1 cell lysate was analyzed for Cda expression by immunoblot.
### Supplementary Table 1. Patient Information

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### Supplementary Table 2. Pancreas Cancer Cell Lines Characteristics and Their IC₅₀ Values for AZD1480

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IC₅₀, 50% inhibitory concentration; ND, not determined; wt, wild type.