CTGF antagonism with mAb FG-3019 enhances chemotherapy response without increasing drug delivery in murine ductal pancreas cancer

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Pancreatic ductal adenocarcinoma (PDA) is characterized by abundant desmoplasia and poor tissue perfusion. These features are proposed to limit the access of therapies to neoplastic cells and blunt treatment efficacy. Indeed, several agents that target the PDA tumor microenvironment promote concomitant chemotherapy delivery and increased antineoplastic response in murine models of PDA. Prior studies could not determine whether chemotherapy delivery or microenvironment modulation per se were the dominant features in treatment response, and such information could guide the optimal translation of these preclinical findings to patients. To distinguish between these possibilities, we used a chemical inhibitor of cytidine deaminase to stabilize and thereby artificially elevate gemcitabine levels in murine PDA tumors without disrupting the tumor microenvironment. Additionally, we used the FG-3019 monoclonal antibody (mAb) that is directed against the pleiotropic matricellular signaling protein connective tissue growth factor (CTGF/CCN2). Inhibition of cytidine deaminase raised the levels of activated gemcitabine within PDA tumors without stimulating necroptotic cell killing or decreasing the growth of tumors, whereas FG-3019 increased PDA cell killing and led to a dramatic tumor response without altering gemcitabine delivery. The response to FG-3019 correlated with the decreased expression of a previously described promoter of PDA chemotherapy resistance, the X-linked inhibitor of apoptosis protein. Therefore, alterations in survival cues following targeting of tumor microenvironmental factors may play an important role in treatment responses in animal models, and by extension in PDA patients.

chemoresistance | pancreatic tumor stroma | genetically engineered mouse models

Pancreatic ductal adenocarcinoma (PDA) remains a uniformly lethal disease with a catastrophic 5-y survival rate of less than 5% (1). Despite intensive preclinical and clinical research efforts to tackle this disastrous disease, the oncologic management of PDA patients has hardly changed over the last several decades. The poor responsiveness to standard single and combination chemotherapies is reflected in a median survival of 6–11 mo in advanced disease, and emphasizes the desperate need for novel therapies (2, 3). A striking histological feature of PDA is the extremely dense and highly abundant tumor stroma consisting of activated cancer-associated fibroblasts (CAF\textsuperscript{s}), infiltrating immune cells, and perturbed vascular cells that form a reactive, inflammatory, immunosuppressive, and highly dynamic tumor microenvironment around neoplastic ductal cells. More than in any other solid malignancy, this microenvironmental network of soluble cytokines, growth factors, proteases, and additional extracellular matrix (ECM) components has increasingly been appreciated to support cancer cell proliferation, differentiation, invasion, early metastasis, and therapeutic resistance in PDA (4–7). In contrast to traditional preclinical assays, genetically engineered mouse models (GEMM\textsuperscript{s}) constitute relatively novel tools in preclinical therapeutic testing that elegantly recapitulate the tumor microenvironment in appropriate tissue compartments, thus allowing the evaluation of therapeutic efficacy more accurately (8–10). For pancreatic cancer, the LSL-Kras\textsuperscript{G12D/+}, LSL-Trap3\textsuperscript{R172H/+};Pdx-1-Cre (KPC) mouse model was generated with conditional mutations in both the Kras oncogene and the p53 tumor-suppressor gene analogous to the genetic mutations found in PDA patients, and may represent a more predictive model for preclinical evaluation compared with historical xenograft models. KPC mice develop endogenous pancreatic adenocarcinomas with 100% penetrance and closely mimic many features of human PDA including extensive desmoplasia, occurrence and site of metastases, cachexia, and ascites formation (11).

We previously established a preclinical therapeutics platform using GEMM\textsuperscript{e}s and demonstrated that the pronounced desmoplastic reaction in PDA confers an obstacle to sufficient drug delivery. The combination of Sonic Hedgehog (SHH) inhibition by the semisynthetic cyclopamine derivative IPI-926 and gemcitabine resulted in stromal depletion, significantly increased microvessel density and patency, and improved drug delivery in a GEMM of pancreas cancer (12). In addition, megadalton glycosaminoglycan hyaluronan (HA) is profusely found in the ECM of murine and human PDA and maintains a high interstitial fluid pressure, thus compressing blood vessels (13–15). We and others have recently provided evidence that enzymatic degradation of HA by PEGPH20 significantly increased vessel patency and perfusion without increasing the density of tumor vessels, resulting in increased active gemcitabine levels in the tumor (15, 16). Both the antismoothed and hyaluronidase therapeutic approaches resulted in transient antitumor responses and prolonged survival in the KPC mouse model. However, the aforementioned studies could not address whether the disruption of stromally derived factors also sensitized cancer cells to gemcitabine. Indeed, we also recently published that γ-secretase inhibition synergized with gemcitabine in the same mouse PDA model by cotargeting tumor endothelial cells and neoplastic...
cells, without increasing chemotherapy delivery (17). Therefore, we asked whether increasing chemotherapy concentrations alone is sufficient to elicit improved response rates, or rather that ECM modulation/degradation sensitizes tumors to the antineoplastic properties of chemotherapy. Accordingly, we investigated the function of connective tissue growth factor (CTGF), a protein known to be important in stromal formation. CTGF is a pleiotropic and cysteine-rich matricellular protein that is abundant in many solid malignancies including pancreas, breast, esophageal, glioblastoma, and hepatocellular carcinoma (18–23). CTGF is expressed in both stromal (23, 24) and neoplastic cells (25, 26) of the pancreas, and participates in a variety of signaling pathways that influence pancreatic stellate cell (PSC)-mediated fibrogenesis in pancreatitis and pancreatic cancer. Upon activation of profibrogenic molecules such as TGF-β, CTGF is synthesized and regulates integrin α5β1-dependent adhesion, migration, and collagen I synthesis in PSCs (27, 28). By using an antibody directed against CTGF, we uncouple drug delivery from stromal depletion in KPC mice and propose that CTGF within the tumor microenvironment mediates resistance to gemcitabine in murine PDA.

Results

Isolated Elevation of Active Gemcitabine Triphosphate Does Not Improve Therapeutic Response in Mouse PDA. We have recently shown that pharmacological inhibition of SHH by IPI-926 and the enzymatic degradation of HA by PEGPH20 improved chemotherapy delivery either through increased mean vessel density and stromal depletion or by reexpansion and endothelial fenestration formation of blood vessels, respectively (12, 16). Here we investigated whether increased accumulation of active gemcitabine triphosphate (2′,2′-difluorodeoxycytidine-5′-triphosphate; dFdCTP) without additional modifications of the tumor vasculature or stromal composition would be sufficient to improve therapeutic response in tumor-bearing KPC mice. Gemcitabine is either rapidly phosphorylated inside cells to the active compound dFdC or quickly enzymatically inactivated both inside and outside cells from its native form (2′,2′-difluorodeoxycytidine; dFdC) to the inactive metabolite 2′,2′-difluorodeoxyuridine (dFdU) by the enzyme cytidine deaminase (CDA), and CDA is highly expressed in murine PDA neoplastic cells (29). First, we established the pharmacokinetic and pharmacodynamic profile of gemcitabine metabolites in KPC mice using a highly sensitive LC-MS/MS assay (30). Accordingly, plasma (Fig. S1A), tumor (Fig. S1B and C), and intestine (Fig. S1D) tissue biopsies from KPC mice were obtained following i.p. treatment with gemcitabine. LC-MS/MS analysis was used to measure gemcitabine metabolites, and the immunohistochemical detection of phosphohistone H3 (PH3) and cleaved caspase 3 (CC3) was used to assess cellular proliferation and apoptosis, respectively. This analysis revealed that the peak of apoptotic cell death coincided with the peak of dFdCTP 2 h after gemcitabine administration (Fig. S1 B–D), and that decreases in proliferation were nearly complete by 3 days. Based upon these data, we chose to analyze all subsequent pharmacokinetic and pharmacodynamic parameters of gemcitabine 2 h after the last dose.

To investigate whether increasing chemotherapy concentrations alone is sufficient to elicit improved response rates, we used the CDA inhibitor 3,4,5,6-tetrahydroxuridine (THU) (31, 32) to decrease the degradation and elimination of gemcitabine. Gemcitabine and THU were coadministered in tumor-bearing KPC mice, followed by the analysis of gemcitabine metabolites in plasma and PDA tumor biopsies. Inhibition of CDA by THU significantly increased dFdC and decreased dFdU concentrations in the plasma of mice (Fig. 1A). In tumor biopsies, the active, cytotoxic metabolite dFdCTP was significantly increased by addition of THU compared with single-agent gemcitabine (Fig. 1B). Despite 10-fold higher dFdC plasma concentrations and two to threefold higher concentrations of intratumoral dFdCTP, the mean content of apoptotic cells was not elevated (Fig. 1C). The increase in dFdCTP upon THU treatment was comparable to the results reported with PEGPH20 but cannot be directly compared with the IPI-926/gemcitabine data, as a less sensitive method of gemcitabine quantification was used (12, 16). In addition, although prolonged treatment was precluded by increased systemic toxicity, a randomized two-arm treatment study with gemcitabine or gemcitabine/THU did not show significant responses over a week as determined by tumor volume measurements using high-resolution ultrasound (Fig. 1D). Taken together, these results show that increasing the concentration of active gemcitabine alone does not elicit significant responses in murine PDA.

CTGF Is Highly Expressed in Cancer-Associated Fibroblasts and Tumor Cells in the KPC Model. As increased accumulation of gemcitabine alone did not yield a therapeutic benefit, we reasoned that previously observed antitumor effects with SHH inhibition and HA depletion could partly be attributed to the removal of various, yet unidentified, survival cues within the stromal compartment of murine pancreas tumors. Accordingly, we characterized the

![Fig. 1. Pharmacological inhibition of cytidine deaminase with THU increases intratumoral gemcitabine without altering tumor growth. (A) Plasma dFdU and dFdC concentrations in mice treated once with gemcitabine monotherapy (n = 6) or THU/gemcitabine combination (n = 11; *P < 0.005). (B) Concentration of gemcitabine triphosphate (dFdCTP) in whole-tumor samples from KPC mice treated once with gemcitabine or THU/gemcitabine (n = 5 each cohort; *P < 0.05). (C) Computer-based quantification of apoptosis (cleaved caspase 3) in individual tumors from mice treated once with gemcitabine or THU/gemcitabine (mean: 0.52 vs. 0.76; n = 5 each; P = 0.6). (D) Quantification of tumor volume growth using biweekly 3D high-resolution ultrasound in mice treated for 7 d with gemcitabine or THU/gemcitabine (mean 184.9% vs. 175.2%; n = 7 for both cohorts). All animals in A–D were killed 2 h after the last dose of gemcitabine.](https://www.pnas.org/doi/10.1073/pnas.1300415110)
function of CTGF in murine PDA because CTGF is expressed in human PDA and is known to participate in a large number of neoplastic cell-stromal interactions in cancers (23, 24). We found CTGF to be present in KPC tumor tissue with high expression in plasma and CAFs and lower but detectable levels in neoplastic cells (Fig. S2). Normal and metaplastic pancreata, as well as pancreatic intraepithelial neoplasia, exhibited no or very low CTGF content (Fig. S2A). Quantification of CTGF in tumor and plasma samples of KPC mice by ELISA showed a robust increase (about 50-fold) in CTGF protein compared with normal murine pancreas and plasma samples (Fig. S2B and C). Western blot analysis revealed strong CTGF protein expression in α-smooth muscle actin (SMA)-positive and E-cadherin–negative CAFs, whereas primary KPC tumor cells showed very low CTGF protein (Fig. S2D). Because CTGF is overexpressed in tumor-bearing KPC mice in a similar pattern to what has been described in PDA patients (23, 24), this mouse model represents a tractable experimental platform to interrogate CTGF function in PDA.

CTGF Inhibition and Gemcitabine Reduce Tumor Burden and Induce Apoptosis in KPC Mice. FG-3019 is a therapeutic monoclonal human antibody against CTGF that is currently under clinical investigation in pancreatic cancer patients in a phase 1/2 study [national clinical trial (NCT)01181245]. To test the efficacy of CTGF inhibition in the KPC model, we treated mice with established tumors of comparable size for 9 d with normal human IgG, gemcitabine/IgG, FG-3019, or FG-3019/gemcitabine, and response to treatment was assessed by biweekly abdominal ultrasound examinations (Fig. 2A and Fig. S1 E and F). Consistent with clinical observations, gemcitabine/IgG treatment had only marginal effects on tumor growth. Final tumor volumes in mice treated with single-agent FG-3019 (mean: 244.7% growth, SE: 33.73) did not significantly differ from the gemcitabine/IgG cohort (193.5%, SE: 16.74; P = 0.17). Treatment with FG-3019/ gemcitabine resulted in significantly smaller tumors (117.3%, SE: 10.91) compared with gemcitabine/IgG (P < 0.02) and IgG alone (236.7%, SE: 26.02; P < 0.002) (Fig. 2A). Tumor proliferation was assessed, and no significant differences were observed among the cohorts (Fig. 2B). In contrast, levels of intratumoral apoptosis were significantly and substantially elevated in the combination treatment compared with gemcitabine/IgG treatment (3-fold, P < 0.005), and less substantially but still significantly increased in FG-3019–treated versus IgG-treated tumors (1.8-fold, P < 0.02) (Fig. 2C). This increase in tumor cell apoptosis is consistent with the significantly reduced tumor burden after 9 d of treatment in FG-3019/gemcitabine–treated mice. Therefore, CTGF antagonism with FG-3019 and gemcitabine treatment synergize in murine PDA.

CTGF Inhibition Targets Epithelial Tumor Cells Without Increasing Intratumoral Gemcitabine Concentrations. As gemcitabine is known to elicit its antitumoral effects through induction of apoptosis, we sought to determine whether the enhanced antitumor activity of FG-3019/gemcitabine after 9 d of treatment stemmed in part from increased drug delivery caused by induction of apoptosis in fibroblasts and subsequent stromal collapse or stimulation of tumor angiogenesis. Accordingly, we performed a detailed histological analysis of tumors at the end point of the study (9 d) to assess potential alterations in the cellular and acellular composition of the tumor microenvironment. Interestingly, the number of α-SMA–positive fibroblasts and the amount and composition of collagen as determined by Picrosirius, Trichrome, and Herovici staining were unchanged (Fig. 3A–C). Furthermore, fibronectin and secreted protein acidic and rich in cysteine (SPARC) were present in KPC mice (Fig. 5A) and unchanged by FG-3019 treatment. Also, the number of CD31-positive vessels did not significantly differ among the treatment cohorts (Fig. S3D). Notably, commounfluorescence analyses revealed that the vast majority of apoptotic cells were E-cadherin–expressing neoplastic cells rather than α-SMA–expressing stromal cells, and these apoptotic cells were significantly increased in both FG-3019– and FG-3019/gemcitabine–treated mice (Fig. 2D and Fig. S3E).

We next examined the intratumoral levels of the gemcitabine prodrug dFdC as well as its inactivated and activated metabolites dFdU and dFdCTP, respectively. Interestingly, we found that concomitant treatment with FG-3019 and gemcitabine did not increase the levels of intratumoral gemcitabine metabolites (Fig. S3 F–H). Therefore, we conclude that the antitumor effect observed by antagonism of secreted CTGF in combination with gemcitabine is mediated by induction of apoptosis in neoplastic cells without increasing gemcitabine delivery or metabolism.

FG-3019 Decreases Expression of X-Linked Inhibitor of Apoptosis in PDA. We investigated candidate pathways previously demonstrated to augment pancreatic cancer cell survival, and found that the X-linked inhibitor of apoptosis (XIAP) protein level was markedly reduced in the lysates of FG-3019–treated KPC tumors (Fig. 3A and B). This effect was most pronounced in FG-3019/gemcitabine combination-treated mice, consistent with the ability of Xiap silencing (33) or Xiap chemical inhibition (34) to sensitize PDA cell lines to additional therapies. Xiap mRNA levels were also significantly reduced by FG-3019 in the presence and absence of gemcitabine (Fig. 3C). To identify additional genes that could plausibly participate in the sensitization to PDA

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cell death following FG-3019 treatment, we performed a whole-genome expression analysis using bulk-tumor mRNA. Surprisingly, only a small number of genes were coordinately down-regulated more than 1.5-fold ($P < 0.01$) by FG-3019 and FG-3019/gemcitabine compared with their respective control cohorts (Fig. 3D). Importantly, our analysis confirmed decreased expression of $Xiap$ (two separate probes), and also identified the down-regulation of additional prosurvival transcripts such as $Birc6$, $Psen1$, $Ubqin2$, and $Hif1a$ (Fig. 3D). Therefore, the reduced expression of $Xiap$ and several additional genes correlates with FG-3019 and treatment response in murine PDA.

**CTGF Inhibition in Combination with Gemcitabine Prolongs Survival and Reduces Hemorrhagic Ascites and Liver Metastasis in KPC Mice.** To evaluate the effects of FG-3019 on intratumoral CTGF, we analyzed tumor lysates obtained after 9 d of treatment by immunoblotting. Full-length CTGF (37 kDa) could be detected in bulk-tumor lysates from treated and untreated KPC mice. Additionally, a 15- to 20-kDa protein fragment recognized by the CTGF antibody occurred exclusively in FG-3019- and FG-3019/gemcitabine–treated tumors (Fig. 4A). This fragment represents an amino-terminal cleaved CTGF fragment of uncertain function (35, 36) that is derived from the 37-kDa full-length CTGF protein, and its presence may indicate the intratumoral binding of CTGF by FG-3019 with exposure to extracellular proteases. Because the prior short-term experiments with FG-3019 indicated evidence of antineoplastic effects in PDA, we performed a randomized treatment study to determine whether these effects would translate to a survival advantage. Tumor-bearing KPC mice were enrolled with comparable tumor volumes (Fig. S4A), and tumor growth was monitored once per week by 3D ultrasonography. KPC mice treated with FG-3019 alone showed no survival benefit in comparison with gemcitabine-treated controls (11 versus 8 d). In contrast, combination treatment with FG-3019/gemcitabine extended the median survival of KPC mice to 29 d (Fig. 4B; $P = 0.03$, log-rank test). The majority of FG-3019/gemcitabine–treated KPC mice demonstrated objective slowing of tumor growth without evidence of obvious tumor shrinkage. In addition, FG-3019/gemcitabine treatment resulted in a significant decrease of hemorrhagic ascites (4/10) compared with gemcitabine alone (13/16) at end point (Fig. 4C; $P < 0.05$). Histopathological evaluation revealed no clear differences in tumor morphology, extracellular matrix composition, or vascular density following FG-3019/gemcitabine treatment. In particular, collagen I+III content as determined by polarized light microscopy of Picrosirius red stains did not show significant differences (Fig. S4B). Interestingly, the number and size of metastases throughout the liver were reduced in FG-3019/gemcitabine–treated KPC mice, albeit not in a statistically significant manner compared with gemcitabine monotherapy (Fig. S4C).

**Discussion**

As conventional and targeted treatment approaches have largely failed to achieve substantial treatment responses in pancreatic cancer patients, novel therapies are urgently needed in the clinic. The notion that the pronounced desmoplastic reaction in PDA actively contributes to the unusual refractoriness of PDA to systemic therapies has stimulated this area of research and revealed candidate therapeutic targets. Nonetheless, a potential role for desmoplasia in drug resistance has been only partially addressed, due to the multitude of signaling interactions between tumor cells and the surrounding microenvironment (37–40).

We and others have previously shown that depletion of abundant ECM components improves perfusion of hypovascular...
FG-3019 targets CTGF to prolong survival when combined with gemcitabine in the KPC mouse model. (A) Western blot analysis of whole-tumor lysates from KPC mice treated for 9 d showing a cleaved CTGF fragment (15–20 kDa) in an FG-3019–treated specimen. (B) Survival is extended by the combination of FG-3019/gemcitabine (median survival, gemcitabine 7.5 d vs. 29 d with FG-3019/gemcitabine, *P = 0.03; FG-3019 monotherapy, 11 d). (C) Malignant hemorrhagic ascites was significantly decreased at end point in KPC mice treated with the combination of FG-3019/gemcitabine (n = 10; *P < 0.04, Fisher’s exact test) compared with gemcitabine (n = 16) and FG-3019 (n = 9).
III (GE Healthcare). Images were processed using LabScan 6.0 and ImageQuant TL 9.0 software (GE Healthcare) to quantify signal and normalize to Hsp90.

Quantitative PCR. Quantitative real-time PCR was performed on a 7900HT Real-Time PCR System using relative quantification (ΔΔCt) with TaqMan assays Mm00775805_m1 and 4352341E (Applied Biosystems).

mRNA Expression Profiling. Tumor mRNA profiling was performed using Affymetrix mouse genome 430A 2.0 arrays (Gene Expression Omnibus accession no. GSE46205). GeneChip Robust Multiaarray Averaging (GC-RMA) processed, median normalized data were analyzed using Agilent Genespring GX software.

Statistical Analysis. Statistical analysis was carried out using GraphPad Prism version 5.01 (GraphPad Software). The log-rank test was performed on the Kaplan–Meier survival curves, and the Mann–Whitney nonparametric test was used for all other analyses if not indicated otherwise. Results are presented as mean ± standard error of mean (SEM) if not indicated otherwise. P < 0.05 was considered to be significant.

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