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Combined targeting of Stat3/NFκB/Cox-2/EP4 for effective management of pancreatic cancer

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Abstract

Purpose—Near equal rates of incidence and mortality emphasize the need for novel targeted approaches for better management of pancreatic cancer patients. Inflammatory molecules NFκB and Stat3 are overexpressed in pancreatic tumors. Inhibition of one protein allows cancer cells to survive using the other. The goal of the present study is to determine whether targeting Stat3/NFκB cross talk with a natural product Nexrutine (Nx) can inhibit inflammatory signaling in pancreatic cancer.

Experimental design—HPNE, HPNE-Ras, BxPC3, Capan-2, MIA PaCa-2 and AsPC-1 cells were tested for growth, apoptosis, Cox-2, NFκB and Stat3 level in response to Nx treatment. Transient expression, gel shift, ChIP was used to examine transcriptional regulation of Cox-2. Stat3 knockdown was used to decipher Stat3/NFκB cross talk. Histopathological and immunoblotting evaluation was performed on BK5-Cox2 transgenic mice treated with Nx. *In vivo* expression of prostaglandin receptor EP4 was analyzed in a retrospective cohort of pancreatic tumors using a TMA.

Results—Nx treatment inhibited growth of pancreatic cancer cells through induction of apoptosis. Reduced levels and activity of Stat3, NFκB and their cross talk led to transcriptional suppression of *Cox-2* and subsequent decreased levels of *PGE2* and *PGF2*. Stat3 knockdown studies suggest Stat3 as negative regulator of NFκB activation. Nx intervention reduced the levels of NFκB, Stat3 and fibrosis *in vivo*. Expression of prostaglandin receptor EP4 that is known to play a role in fibrosis was significantly elevated in human pancreatic tumors.

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Disclosure of potential conflicts of interest:

Authors have no conflicts of interest.

Conclusions—Dual inhibition of Stat3-NFκB by Nx may overcome problems associated with inhibition of either pathway.

Keywords

Pancreatic cancer; Nexrutine; Stat3; NFκB; Prostaglandin receptor EP4; Cox-2

INTRODUCTION

Pancreatic cancer (PanCA) is a formidable medical and public health challenge because of the difficulties in early diagnosis, its aggressive behavior and resistance to conventional therapy (1). The best hope for cure is complete surgical resection. However, only a minority (approximately 20%) of patients is eligible for surgical resection at the time of diagnosis (1). Additionally, numerous phase 3 trials of Gemcitabine, the first-line therapeutic agent for PanCA, in combination with other cytotoxic or molecularly targeted agents showed no substantial clinical benefit over the use of Gemcitabine alone (1–3). These data clearly warrant additional studies to identify and develop novel compounds for more effective treatment of PanCA.

The underlying etiology and pathophysiology of pancreatic cancer is poorly understood. Available evidence suggests that malignant progression from pancreatic intraepithelial neoplasia (PanINs) to invasive and metastatic disease is accompanied by mutations in the KRAS oncogene, which occur in more than 90% of cases (4). Increased expression of the active form of the cell survival signaling kinase Akt and the pro-inflammatory molecule cyclooxygenase-2 (Cox-2) has been observed in approximately 46–70% and 47–66% of PanCA respectively (5). Recently it has been suggested that deregulation of chemokines, cytokines including interleukin-6 (IL-6), and activation of downstream effectors such as Stat3 and NFκB are key events in the initiation and progression of PanCA (6). Intense fibrosis or desmoplasia surrounding the tumoral glands is a unique feature of PanCA (7). Such tumor-stromal interactions contribute not only to tumor progression, but also poor drug delivery and chemoresistance. Therefore, drugs that can inhibit desmoplastic stroma in PanCA might expand the limited pool of therapeutic options against PanCA, either alone or in combination with chemotherapeutic agents, by enhancing drug delivery (7). These data suggest that therapeutic strategies targeting molecular abnormalities such as inflammation and desmoplasia that are implicated in pancreatic tumor growth, invasion, metastasis, and apoptotic resistance have enormous potential in the management of PanCA.

Natural compounds provide abundant potential for the development of novel drugs for cancer management. Here we investigated the anticancer activity and the underlying molecular mechanism of Nexrutine (Nx) using multiple pancreatic cancer cell lines and a pre-clinical animal model that develops pancreatitis and fibrosis. Nx is a bark extract from *Phellodendron amurense* that has been used as an anti-diarrheal and anti-inflammatory agent for centuries in traditional Chinese medicine (8, 9). Although it has not been extensively studied in terms of its anticancer activity, studies from our laboratory demonstrated that Nx inhibits prostate tumor growth both *in vitro* and *in vivo* by targeting multiple signaling pathways including Akt, nuclear factor kappa B (NFκB), cAMP response element-binding protein (CREB), Cox-2, and Cyclin D1 (8, 9). However, its effects on PanCA have not been explored. In this study we demonstrate that Nx (i) significantly inhibits the growth of multiple pancreatic cancer cell lines with minimal effect on immortalized non-tumorigenic HPNE cells; and (ii) inhibits Stat3 and NFκB activation leading to transcriptional suppression of Cox-2, modulation of prostaglandin receptor EP4 and apoptosis. Our findings also show EP4 overexpression in human pancreatic tumors compared to adjacent benign pancreatic tissue. In addition, Nx intervention studies using a BK5-Cox-2 transgenic mouse

model showed a reduction in the number of animals that develop intense fibrosis associated with reduced levels of p65 and Stat3. However, Nx had no effect on Cox-2-induced lesions under these experimental conditions. Our results are the first to demonstrate that Stat3/NF κ B/Cox-2/EP4 signaling as a promising therapeutic target for pancreatic cancer with a natural extract.

MATERIALS AND METHODS

Cell Culture and Chemicals

Pancreatic cancer cell lines Capan-2, MIA PaCa-2 and AsPC-1 (K-ras mutation) and BxPC3 (wild type K-Ras) were obtained from American Type Cell Culture (ATCC, Rockville, MD). The hTERT-immortalized (hTERT-HPNE) nestin-expressing (marker of developmental precursors of the exocrine pancreas) human pancreatic ductal progenitor cells; hTERT-HPNE cell line modified to express E6/E7 alone in conjunction with oncogenic K-Ras (referred to as HPNE-Ras), Capan-2 and BxPC3 cells with Stat3 stably knocked down were generous gifts from Dr. James Freeman (The University of Texas Health Science Center at San Antonio, TX). HPNE cells share properties similar to that of intermediary cells produced during acinar-to-ductal metaplasia including undifferentiated phenotype, expression of nestin, and ability to differentiate to pancreatic ductal cells in addition to their mesenchymal properties. Further, it is known that (i) mesenchymal cells express extracellular matrix remodeling enzymes that have increased capacity for migration and invasion contribute to fibrosis; and (ii) association of acinar-to-ductal metaplasia with PanIN lesions implicate them as putative precursor lesions for PDAC (10, 11). Therefore, HPNE cells provide an excellent model system to examine the effect of Nx. All cell lines were grown in Roswell Park Memorial Institute medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum, 100 μ g/ml penicillin-streptomycin, and 100 μ g/ml Amphotericin in a humidified incubator at 37°C and 5% CO₂. A stock solution of Nx (5 mg/ml) was prepared by dissolving Nx powder in 50% DMSO and was further diluted with the media to obtain required concentrations. In parallel cells also received 50% DMSO as solvent control. The final concentration of DMSO was 0.015% in cells receiving the maximum Nx dose (150 μ g/ml). PGE₂ was obtained from Sigma (St. Louis, MO). Monoclonal antibodies (p65, stat3, pstat3, p50, I κ B α) and STAT3 inhibitor V were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Next Pharmaceuticals Inc. supplied Nx manufactured by Cortex Scientific under pharmaceutical good manufacturing practices. The quality of Nx was tested for quality control by HPLC based on the berberine content for reproducibility. A single batch of Nx was used in the study.

Immunohistochemical staining for EP4

Tissue microarray (TMA) containing 133 pancreatic tumor specimens along with 106-paired adjacent normal tissue were obtained from Dr. Huamin Wang (Department of Pathology, The University of Texas at MD Anderson Cancer Center, Houston, TX). Immunohistochemical staining was performed on TMA sections using EP4 polyclonal antibody (Cayman) at a dilution of 1:500. The staining results were scored semi-quantitatively as described previously by a pathologist (A.P.), who was blinded to the clinicopathological and follow up data, based on the proportion (percent) and intensity (negative, 1+ for low, 2+ for medium and 3+ for high; 9 and 12). Final score for EP4 staining was calculated as a product of proportion and intensity of staining. Only representative tissue cores containing at least 30% of tumor cells were scored. The institution review board approved the use of human tissue samples.

Animal experiments

Animal studies were conducted in accordance with the Institutional Animal Care and Use Committee approved protocol. These studies were carried out in an animal facility at the University of Texas Health Science Center, San Antonio, TX accredited by the American Association for the Assessment and Accreditation of Laboratory Animal Care. Breeder pairs of BK5-Cox-2 mice were obtained from Dr Susan M Fischer, The University of Texas M.D Anderson Cancer Center, Smithville, TX. Breeding and genotyping was performed essentially as described in Colby et al 2008 (13). After confirming the genotype BK5-Cox-2 mice were randomized into two groups each of 23 animals each. A group of animals fed AIN93G based pelleted diet supplemented with Nx (300 mg/kg diet obtained from Dyets, Inc.) for 6-weeks. Intervention with Nx was initiated immediately after weaning. Mice fed AIN93G based diet without Nx was served as control. Food intake and body weights were measured weekly. At the end of this period, mice were sacrificed by cervical dislocation. Necropsy was conducted and pancreas was evaluated for pathological changes including inflammation, fibrosis and mPanIN lesions (RR). Briefly we evaluated tissues for extent of fibrosis (0 for no ductal alterations); 0.5 for fibrosis surrounding rare to few to ductal alterations; 1 for easily seen ductal alterations involving a greater number of ducts coupled with some proliferation; and 2–3 for prominent ductal proliferation and abundant fibrosis with few dominant nodules. Inflammatory changes usually accompanied ductal alterations.

Biochemical experiments

Cell proliferation and anchorage-independent growth were measured using CellTiter 96 Aqueous One solution assay (Promega Corporation, Madison, WI) and CytoSelect™ 96-well soft agar colony formation assay (Cell Bio labs, San Diego, CA) respectively according to the manufacturer's directions as described previously (8, 9). Experiments were conducted using three different doses of Nx for each cell line: low, medium, and high (50, 100, 150 µg/ml for Capan-2; 20, 40, 60 µg/ml for BxPC-3; and 20, 50, 80 µg/ml for HPNE-Ras; Table I). Apoptosis was measured using FITC-Annexin assay (Medical and Biological Laboratories, Watertown, MA) and DNA fragmentation using cellular DNA fragmentation ELISA kit (Roche Applied Science, Indianapolis, IN) essentially as per manufacturer's instructions. PGE2 and PGF2α concentrations were determined in the media by PGE2 enzyme Immunoassay Kit and PGF2α enzyme Immunoassay Kit according to the manufacturer's directions (Assay Designs, Inc., Ann Arbor, MI). Immunoblot analysis, Real-Time PCR and transient expression assays and confocal microscopy were conducted as described previously (8, 9).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts prepared from Capan-2 and BxPC3 cells treated with Nx at low ~IC₁₆ (50 and 20 µg/ml for Capan-2 and BxPC3 respectively), medium ~IC₃₂ (100 and 40 µg/ml for Capan-2 and BxPC3 respectively) and high dose IC₅₀ (150 and 60 µg/ml for Capan-2 and BxPC3 respectively) for 24h were incubated with ³²P-labeled oligonucleotides containing Stat3 or NFκB binding sites and DNA binding activity was measured. Briefly the double stranded NFκB or Stat3 oligonucleotide (20 ng) was end labeled with γ-p³²-ATP using T4 polynucleotide kinase. 12 µg nuclear extract was incubated with the radiolabeled probe in binding buffer containing 4 mM Tris-HCl, 12 mM Hepes, pH 7.9, 60 mM KCl, 0.5 mM EDTA, 1 mM DTT and 12% glycerol for 25 min at room temperature in a final volume of 20 µl. Following this incubation, samples were fractionated on a 4% polyacrylamide gel in 0.25× TBE at 4°C. Following electrophoresis, the gel was dried and autoradiographed. For competition experiments, the radiolabeled probe was mixed with 100-fold molar excess of unlabeled double-stranded synthetic NFκB or Stat3 oligonucleotide (homologous) and AP1 (heterologous) for 5 min prior to the addition of nuclear extract. For super-shift experiments,

nuclear extracts were pre-incubated with 1 µg each of Stat3 or p65 or combination of both Stat3 and p65 antibodies for 30 minutes on ice prior to use in EMSA.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP was performed using ChIP-it express kit (Active Motif, Carlsbad, CA) as described previously (12). Briefly logarithmically growing cells treated with Nx for 24h were cross-linked with 1% formaldehyde for 10 min at room temperature followed by termination with 125 mM glycine for 5 minutes and nuclei were isolated. Isolated nuclei were sonicated on ice to break chromatin DNA to an average length of ~300 bp. Soluble chromatin was used in immunoprecipitation with Stat3 or p65 antibody and IgG (as negative control) and immune complexes were absorbed with protein G magnetic beads. 10% of the input extract was saved as input control for normalization before adding antibody for immunoprecipitation. Following reversing the cross-links and Proteinase K digestion, immunoprecipitated DNA was amplified by a primer pair corresponding to -587/-567 (NFκB binding site 1); -363/-343 (NFκB binding site 2) and -266/-250 (Stat3 binding site) in the Cox-2 promoter by real-time PCR. Primers used are shown in supplementary figure SF1. Duplicate PCR reactions were performed for each sample and the expression data was normalized to respective input values. Data are presented as the average ± standard deviation.

Statistical analysis

All *in vitro* experiments were conducted at least three times and the data presented was average±sd. Statistical significance among different treatments was determined by ANOVA followed by t-test and p values <0.05 was considered significant. For statistical analysis, the expression of EP4 was categorized as negative or low (0) and high (1) based on the median score for EP4. The mean staining scores of EP4 in tumor vs. normal tissues were compared with a Wilcoxon rank sum test, p values < 0.05 were considered significant. Contingency tables with a two tailed Fisher exact test were also used to compare the presence of staining in normal vs. tumor with similar results (not shown). The relationship of EP4 staining expression and tumor differentiation was explored with crosstabs reporting Fisher exact. The analysis was carried out in STATA version 9.2 (STATA corporation).

RESULTS

Nx inhibits proliferation and induces apoptosis in human PanCA cells

The anti-proliferative effect of Nx was examined using immortalized normal pancreatic cell lines (HPNE) and three different human pancreatic cancer cell lines that differ in K-Ras status: Capan-2 with mutant K-Ras; BxPC-3 with wild type K-Ras; and HPNE cells stably expressing KRas^{G12D} (HPNE-Ras). As shown in Fig 1A, incubation of pancreatic cancer cell lines with different concentrations of Nx for 24 h significantly inhibited their proliferation, albeit to different levels. BxPC-3 cells were most sensitive. The concentration required to achieve 50% proliferation inhibition (IC₅₀) was 150 µg/ml in Capan-2 cells, 60 µg/ml in BxPC-3, and 80 µg/ml in HPNE-Ras cells (Fig 1A). Remarkably, HPNE cells showed modest decrease in proliferation that reached significance only at higher doses of Nx. There was no significant decrease in proliferation at doses lower than 100 µg/ml (Fig 1B). Based on these data, subsequent mechanistic investigations were conducted using three different doses of Nx: low, medium, and high corresponding to IC₁₆, IC₃₂ and IC₅₀ (50, 100, 150 µg/ml for Capan-2; 20, 40, 60 µg/ml for BxPC-3; and 20, 50, 80 µg/ml for HPNE-Ras; Table I). Since HPNE cells do not form colonies on soft agar, we examined the effect of Nx on the ability of HPNE-Ras, Capan-2 and BxPC-3 cells to form colonies on soft agar using an anchorage-independent growth assay. Consistent with the cell proliferation data, we observed significant reduction in their colony forming ability (Fig 1C). It is noteworthy to mention that although BxPC-3 cells were found to be most sensitive toward antiproliferative

effects of Nx; this cell line was relatively resistant to Nx-induced anchorage independent growth on soft agar. The observed differences could be related to the differential effects of Nx on genes associated with proliferation vs anchorage independent growth. Nevertheless, taken together these data suggest that the Ras-transformed cancer cell lines were particularly sensitive to anti-proliferative effects of Nx compared to non-tumorigenic HPNE cells. As it was previously reported that the antiproliferative activity of Nx is mediated by induction of programmed cell death including apoptosis in prostate cancer cells (8, 9), we measured apoptosis in the PanCA cells in response to Nx treatment. All cell lines showed dose-dependent induction of apoptosis and a moderate degree of necrosis as evidenced by FITC-Annexin staining in response to Nx treatment (Fig 1D). Histograms of FITC-Annexin staining are shown in supplementary data SF2A. Additional approaches including morphological assessment (data not shown) and cellular DNA fragmentation showed similar results (supplementary data SF2B). Taken together, these data suggest that Nx inhibits both anchorage dependent and independent growth of human pancreatic cancer cell lines possibly through induction of apoptosis.

Nx inhibits NFκB/Stat3 signaling

Constitutive activation of inflammatory mediators NFκB and Stat3 has been reported in pancreatic cancer specimens (14–17). Both of these factors are involved not only in the regulation of a plethora of cellular processes including proliferation, apoptosis, angiogenesis, and metastasis, but also in the development of therapeutic resistance (17, 18). Therefore, we examined the effect of Nx on protein levels and activity of these transcription factors. As shown in Fig 2A, Nx treatment caused a significant reduction in the protein levels of pStat3 and tyrosine kinase Janus kinase 1 (JAK1) in Capan-2 and BxPC-3 cell lines with no change in the total levels of Stat3. Although we did not detect total Stat3, we observed decreased pStat3 levels in non-tumorigenic Ras transformed HPNE-Ras cells (supplementary figure SF3A). Next we performed electrophoretic mobility shift assays (EMSAs) with Stat3 binding site oligonucleotide as radiolabeled probe. As shown in Fig 2B, nuclear extracts prepared from Capan-2 (Fig 2B left panel) and BxPC-3 cells (Fig 2B right panel) formed a specific DNA-protein complex as demonstrated by competition experiments in the presence of unlabeled homologous Stat3 and heterologous AP1 oligonucleotides (indicated with an asterick). Nuclear extracts prepared from both Capan-2 (Fig 2B left panel) and BxPC-3 cells (Fig 2B right panel) treated with increasing doses of Nx showed dose-dependent decrease in the observed DNA-protein complex (compared lines 2 to 5). Lower exposure blot is shown in supplementary figure SF 3B. To determine the components of this DNA-protein complex, we performed gel super-shift experiments using antibodies specific for Stat3 and p65 for NFκB. We observed enhanced intensity of the observed specific DNA-protein complex when nuclear extracts prepared from Capan-2 cells pre-incubated with anti-Stat3 antibody but not with IgG were used in these experiments (compare lines 11 with 8). On the other hand, when anti-p65 antibody but not with IgG was used, we detected super-shift (indicated by SS, compare lines 11 with 9) suggesting the presence of p65 in the observed DNA-protein complex (Fig 2B). Similar super shift was observed when nuclear extracts were pre-incubated with combination of both stat3 and p65 antibodies (compare lines 11 with 10). However we could not detect any super-shift with either anti-Stat3 or anti-p65 or combination of both antibodies in BxPC-3 cells (Fig 2B, right panel). Taken together these data suggests the presence of both Stat3 and p65 in the observed Stat3 oligo-protein complex from Capan-2 cells and that Nx treatment reduces the observed DNA binding activity.

To determine the role of NFκB signaling, we measured the protein levels of p65 using immunoblot analysis and NFκB activity using EMSA. Immunoblot analysis revealed reduced levels of p65 in Nx-treated extracts in Capan-2 and BxPC-3 cells. Although

absolute levels of p50 appear to decrease at high dose of Nx, quantification data from multiple experiments indicated no statistically significant change in the levels of p50 (Fig 2C). Similar results were observed using non-tumorigenic HPNE-Ras cells albeit p50 was more prominent (supplementary figure SF3A). As shown in Fig 2D and SF3B (lower exposure blot), nuclear extracts from both Capan-2 and BxPC-3 cells formed a specific DNA-protein complex as evidenced by competition experiments using homologous (NFκB) and heterologous (AP1) unlabeled oligonucleotide probes (indicated with an asterisk). Nx-treatment reduced the formation of observed DNA-protein complex. Further, to determine the identity of this DNA-protein complex, we performed super-shift experiments using antibodies specific for Stat3 and p65. As shown in Fig 2D, preincubation of nuclear extracts of Capan-2 cells with anti-Stat3 antibody but not with IgG reduced the binding of the DNA-protein complex (compare lines 8 with 11) whereas incubation with anti-p65 resulted in a super-shifted band (compare lines 9 with 11). Pre-incubation with both antibodies further reduced the mobility of the super-shifted band indicating the presence of both p65 and Stat3 in the observed NFκB-oligo protein complex (compare lines 10 with 11). On the other hand, when gel super-shift experiments were conducted using nuclear extracts from BxPC-3 cells, we observed a super-shift with both anti-Stat3 and anti-p65 antibodies (compare lines 8 and 9 with 11). These data suggest the presence of both p65 and Stat3 in the DNA-protein complexes formed with the NFκB probe. Further the presence of p65 in Stat3 DNA-protein complexes and Stat3 in the NFκB complex led us to examine the potential cross talk between Stat3 and p65 using Stat3 knockdown pancreatic cancer cells. As shown in supplementary data SF4A top panel, knockdown of Stat3 resulted in approximately 80% reduction in its expression compared with control non-targeting shRNA in both Capan-2 and BxPC-3 cells. Cells transfected with scrambled shRNA showed co-localization of Stat3 and p65 in cytoplasm and to some extent in the nucleus from untreated Capan-2 cells as indicated by yellow pixels in the merged image (Fig 2E). Similar observations were made using wild type untransfected cells (supplementary data SF4B). Knockdown of Stat3 resulted in nuclear accumulation of p65 in Capan-2 cells. Furthermore, nuclear accumulation decreased following treatment with Nx in Capan-2 cells. Interestingly Nx treatment reduced fluorescence of p65 with no significant effect on fluorescence of total Stat3. Although similar results were obtained using BxPC-3 cells; we did not detect nuclear accumulation of p65 following Stat3 knockdown (Fig 2F). These data suggest that NFκB and Stat3 interact with each other in the cytoplasm and that Nx reduces p65 levels thereby inhibiting its nuclear localization and reducing its interaction with Stat3 in the cytoplasm.

To demonstrate the functional relevance of these observations, we measured Stat3 and NFκB reporter activity. We found that Nx treatment significantly reduced the Stat3 and NFκB reporter activity in both Capan-2 and BxPC-3 cells (Fig 3A and B). Based on our data showing nuclear accumulation of p65 following Stat3 knockdown, we also examined cross talk between NFκB and Stat3 in Stat3 knockdown cells. Surprisingly Stat3 knockdown resulted in significant elevation of NFκB promoter activity specifically in Capan-2 but not in BxPC-3 cells (Fig 3C). These results are consistent with our observation that knockdown of Stat3 resulted in accumulation of p65 in the nucleus (Fig 2E). These observations prompted us to examine the impact of Stat3 knockdown on Nx-induced proliferation. Scrambled control and Stat3 knockdown Capan-2 and BxPC3 cells were treated with different doses of Nx for 24 h. As shown in supplementary data SF4A bottom panel, Nx treatment inhibited proliferation of both Capan-2 and BxPC-3 cells transfected with scrambled shRNA. However knockdown of Stat3 partially reduced the inhibitory effect of Nx albeit statistically significant in Capan-2 cells. In contrast, lack of Stat3 in BxPC-3 cells further sensitized the cells to Nx-induced proliferation inhibition. This may be due to accumulation of p65 in the nucleus following Stat3 silencing in Capan-2 but not in BxPC-3 cells. To further validate the role of Stat3 in cell growth, we used stable Stat3 knockdown lines of Capan-2 and BxPC-3. Silencing Stat3 significantly inhibited growth of both Capan-2 and BxPC3 cell lines

(supplementary data SF4C). It should be mentioned that despite the observed antiproliferative effects of Nx irrespective of K-Ras status, BxPC-3 with wild type K-Ras is relatively more sensitive than the mutant K-Ras cells tested. For example the IC₅₀ for BxPC-3 with wild type K-Ras is ~60 µg/ml vs. 150 µg/ml for Capan-2 with mutant K-Ras. However, although K-Ras mutation is one of the most common genetic alteration (90%) observed in pancreatic ductal adenocarcinoma (PDAC), other genetic alterations including Her-2, p16, p53 and Smad4 have been identified. Therefore it remains unknown to what extent the observed differential regulation of Stat3/NFκB axis and Nx-mediated antiproliferative effects might have been due to oncogenic Ras. Overall these data suggest that K-Ras status could be a contributing factor but may not be the sole reason for the observed differential sensitivity. Taken together, these data suggest that Nx treatment inhibits activation of Stat3 and NFκB; and possibly their cross talk to mediate its biological effects. However, the relevance of this inhibition or their cross talk in the regulation of downstream target gene expression is currently unclear.

Cox-2 is one of the downstream targets of the oncogenic transcription factors NFκB and Stat3 and overexpression of Cox-2 has been observed in ~46–70% of pancreatic tumors (19–21). Analysis of the Cox-2 promoter identified Stat3 binding site located at –266 and two NFκB binding sites located at –587 and –363 (supplementary data SF1). Chromatin immunoprecipitation assay was performed to examine the observed Nx-induced modulation of Stat3/NFκB cross talk in the regulation of Cox-2. As shown in Fig 3D, E and F, we detected occupancy of both Stat3 and NFκB on the endogenous Cox-2 promoter. Treatment with Nx reduced occupancy of these factors. Based on the observed reduced occupancy of Stat3 and NFκB on the Cox-2 promoter, we evaluated the mRNA expression, protein levels, and enzymatic activity of Cox-2 in PanCA cell lines. HPNE-Ras, Capan-2 and BxPC-3 cells expressed Cox-2 protein and mRNA (supplementary data SF5A). Nx treatment (i) reduced the Cox-2 protein levels; and (ii) RNA expression in Capan-2 and BxPC-3 with no significant change in HPNE-Ras (supplementary data SF5A right panel). We did not detect Cox-2 protein or mRNA in MIA PaCa-2 or AsPC-1 cells; yet, Nx treatment inhibited their proliferation and reduced the levels of pStat3 (supplementary data SF5B and C).

We also measured the levels of PGE₂ and PGF₂α in the medium as a measure of Cox-2 enzymatic activity. BxPC-3 cells express constitutively higher levels of both PGE₂ and PGF₂ α compared to Capan-2 cells (supplementary data SF5D). Treatment with Nx also resulted in a marginal but statistically significant decrease in the levels of secreted PGE₂ and PGF₂ α in BxPC-3 cells (supplementary data SF5F). In Capan-2 cells while low dose Nx decreased but higher doses increased PGE₂ levels (supplementary data SF5E). Several possibilities exist for the observed increase in PGE₂ levels despite decreased Cox-2 expression in Capan-2 cells following Nx treatment. We believe that this may be due to presence of compensatory feed-back regulatory mechanisms. For example it has been reported that COX-deficient cells overexpress alternate COX isoforms, show increased cPLA₂ expression and significantly elevated levels of PGE₂ (22). Further, despite the undetectable levels, expression and activity of Cox-2 in MIA PaCa-2 and AsPC-1 mutant K-Ras cells, levels of mPGES2 and cPGES were equal to Cox-2 expressing cells (23). Therefore it is possible that the observed increase in PGE₂ following Nx treatment in Capan-2 cells could be due compensatory feedback mechanism or due to activation of PGE synthases given the elevated expression of PGE synthases and cPLA₂ in human pancreatic tumors and cells.

Cox-2 generated prostaglandins including PGE₂ transmit signaling by binding to its specific E-prostanoid (EP) trans-membrane receptors. Four EP receptors (EP1, EP2, EP3 and EP4) mediate distinct signaling pathways (24–27). We investigated the effect of Nx on modulation of EP and FP receptor expression in pancreatic cancer cell lines. We were able

to detect expression of all four receptors (EP1 to EP4) using real-time PCR in these cell lines. However EP4 was expressed at higher levels compared to other prostaglandin receptors in both BxPC-3 and Capan-2 but not in Cox-2 negative MIA PaCa-2 cells (Fig 4A and data not shown). Further, a recent study demonstrated that although pancreatic stellate cells express all four receptors, blocking EP4 receptor inhibits PGE₂-mediated stellate cell activation (28). Although our present study is not related to stellate cells, these observations implicate a potential role for EP4 in pancreatic cancer management. To the best of our knowledge no prior studies published studies have examined the expression of EP4 in human pancreatic tissues. Therefore we analyzed *in vivo* expression of EP4 protein by immunohistochemistry in a retrospective cohort of 133 human pancreatic ductal carcinoma samples and 106 adjacent benign pancreatic tissues. We observed expression of EP4 in 95% (126/133) human PanCA samples compared to 29% (31/106) in adjacent benign pancreatic tissue ($p=0.001$; Fig 4B). A representative immunohistochemical staining of TMA is shown in Figure 4C. It is noteworthy to mention that although both membranous and cytoplasmic staining has been reported for EP4, we observed mostly cytoplasmic staining which could be related to the antibody used (<http://www.proteinatlas.org>). Further, although, we found no correlation between EP4 staining with survival (data not shown), a statistically significant inverse relationship between EP4 expression with tumor differentiation was observed in this cohort ($p=0.01$; Table 2). These data suggest the association of EP4 negativity with aggressive tumor phenotype. To the best of our knowledge, this is the first report showing relationship between EP4 and human pancreatic tumors. Surprisingly, Nx treatment showed increase in the mRNA expression (7.4-fold; $p=0.006$) and protein levels of EP4 in Capan-2 cells (Fig 4D and E). We also observed marginal (1.4-fold), albeit statistically significant ($p=0.0001$) increase in EP4 expression, however, protein levels of EP4 were consistently decreased following treatment with Nx in BxPC-3 cells (Fig 4D and E). These data suggest differential regulation of EP4 possibly by oncogenic Ras and involvement of Cox-2-mediated signaling in Nx-mediated biological effects. However the precise mechanism of such differential effects is unclear. Further these data need to be validated in additional cell lines with oncogenic Ras activation.

Nx treatment reduces fibrosis in pancreatic tumors

Chronic inflammatory changes in the pancreas in response to alcohol and tobacco use and diabetes have been linked to development of PanCA. This is also exemplified by the susceptibility of individuals with heritable or sporadic chronic pancreatitis (29, 30). Prostaglandin production mediated by Cox-2 is frequently associated with the development and maintenance of chronic inflammation. It is also known that Cox-2 is up regulated in precancerous conditions including pancreatitis and PanCA (20). Transgenic mice with overexpression of Cox-2 in the pancreas driven by the bovine keratin-5 promoter (BK5-Cox-2 mice) have been shown to develop inflamed pancreatic ductal lesions by 3 months of age and high-grade PanIN resulting from chronic pancreatitis by 6–8 months (13). Therefore, these mice provide an excellent model for evaluating strategies to prevent progression from chronic pancreatitis to pancreatic cancer. Using the BK5-Cox2 mouse model, we assessed the effect of Nx ($n=23$) on pancreatic pathology, Stat3 and p65 activation. 17% (4/23) mice in the control group showed chronic pancreatitis and prominent ductal proliferation associated with abundant fibrosis (2+) and obvious ductal alterations involving a greater number of ducts together with mucinous properties. Further, increased fibrosis was accompanied by increased inflammation and ductal changes including reduced exocrine glands in the area of normal fibrosis and inflammation. In addition, animals with ductal alterations also displayed mPanIN 1B and 2 lesions. On the other hand only two of the animals in the Nx intervention group (8.6%) had such features. While 9 of animals on control and 11 on Nx intervention group had lower fibrosis (0.5+) surrounding rare ductal alterations. Five animals in each group either did not develop any fibrosis or had 1+ fibrosis

(Table 3). Representative images showing the extent of fibrosis and inflammation in both control and Nx-treated pancreas are shown in Fig 5A. Pathological quantification of the data is presented in Fig 5B. Nx treatment had no significant effect on the body weight of these animals indicating non-toxic nature (supplementary data SF6A). We also did not observe any significant change in the food consumption between control and experimental group of animals (supplementary data SF6B). Despite heterogeneity in the extent of fibrosis and lack of statistical significance, cumulative analysis of these studies although not necessarily imply an impact on fibrosis, these data suggests the potential for Nx to inhibit pancreatic fibrosis. Though, additional studies using isolated stromal and epithelial compartments are required to precisely define the cellular targets of Nx. These data warrant additional investigations including using large number of animals and dose-response studies to see impact of Nx on tumor development.

The Nx-induced reduction in fibrosis is associated with reduced Stat3 levels

We analyzed pancreatic tissue using three animals per group for changes in the expression of Stat3, NF κ B and Cox-2. As shown in Fig 5C, we detected Stat3, p65, and Cox-2 in all three samples from control mice. However, only one of the samples from the treatment group showed strong Stat3 and p65 expression and the other two showed relatively weak expression. Normalization of the observed changes with respect to β -actin indicated an approximately 50% decrease in the levels of both Stat3 and p65 with no significant change in the levels of Cox-2. The observed changes in the levels of Cox-2 in response to Nx treatment *in vivo* could be related to Cox-2 expression from the transgene. Although we cannot make any firm conclusions based on the small sample size and sample heterogeneity including presence of both stromal and epithelial components, we speculate that Nx-mediated effects are associated with deregulation of Stat3/NF κ B signaling. Expression analysis and functional assays using isolated stromal and epithelial cells and their potential crosstalk would help define the precise cellular target of Nx.

Discussion

The near equal rates of incidence and mortality emphasize the need for novel molecularly targeted approaches for the successful management of pancreatic cancer (1–3). Chronic inflammation and extensive fibrosis in the pancreas are two critical risk factors for PanCA (7). Notably, desmoplasia not only impedes drug delivery but also contributes to therapeutic resistance (7). Activation of inflammatory signaling molecules including Stat3, NF κ B, and Cox-2 has been shown to be involved in the proliferation of pancreatic stellate cells (PSCs) that play a vital role in desmoplasia (31–33). These data indicate that cooperative interactions between inflammation and desmoplasia could play a major role in the development and progression of PanCA. Therefore, drugs targeting critical molecular abnormalities such as desmoplasia and inflammation would be ideal candidates for effective management of PanCA.

By virtue of their anti-inflammatory and anti-oxidant properties, a vast majority of phytochemicals has been reported to possess antitumorigenic potential (34). Further, majority of approved anticancer drugs are either (i) natural products, (ii) derived semi-synthetically from natural products or (iii) synthetic products based on natural products (34–35). Although some of the currently used chemotherapeutic agents including paclitaxel are derived from natural products, natural products have not been comprehensively studied for their potential therapeutic benefits. Lack of thorough characterization, quality control including lack of understanding their mechanism of action limits their potential clinical utility (34–35). However, natural products or complex botanicals offer an added advantage by targeting multiple signaling pathways, deregulation of which is a characteristic feature of

cancer (34, 35). In this paper, we investigated the potential of Nx, a bark extract of *Phellodendron amurense* that is used as an anti-inflammatory agent in traditional Chinese medicine, to inhibit pancreatic cancer growth using *in vitro* and *in vivo* model systems and mechanism of action. We found that Nx inhibits the activity of both Stat3 and NFκB, and possibly their cross talk, leading to transcriptional suppression of Cox-2 and inhibition of pancreatic cell growth through induction of apoptosis. Inhibition of Cox-2 through transcriptional suppression may be alternate approach to overcome Cox-2 inhibition (using traditional Cox-2 inhibitors) that is generally associated with cardiotoxicity.

The transcription factors NFκB and Stat3 are constitutively activated in a variety of tumors including PanCA to regulate the initiation and progression of the tumorigenic process (15–18). Therefore, interference with NFκB or Stat3 activation can inhibit pancreatic carcinogenesis. Interestingly, our results demonstrated the following: (i) the presence of both NFκB and Stat3 in the DNA-protein complexes obtained with either NFκB or Stat3 (Fig 2); and (ii) both NFκB and Stat3 bound to the endogenous Cox-2 promoter (Fig 3). Surprisingly our data also show that genetic inactivation of Stat3 using shRNA in K-Ras mutant (Capan-2) but not with wild type cell line (BxPC-3) led to increased NFκB activation suggesting a negative regulation (Fig 3). These data suggest that inhibition of Stat3 signaling alone may even promote tumor growth by activating NFκB signaling under certain conditions, therefore limiting the clinical utility of Stat3-targeted agents. Consistent with these observations recent reports show (i) tumor suppressor role for Stat3 in thyroid tumors by activating hypoxia-inducible factor 1-α target genes (36); (ii) suppression of NFκB-inducible genes by Stat3 and activation of NFκB-regulated genes involved in antitumor immunity when Stat3 is inhibited (37); and (iii) activation of NFκB pathway by Stat3 inhibitor in human Glioblastoma cells (38). Since both Stat3 and NFκB regulate a plethora of genes involved in cell survival, systemic inhibition of either of these factors could be associated with toxicity. Therefore dual inhibitors of NFκB-Stat3 albeit with limited efficacy (since complete inhibition may not be optimal for growth and survival of normal cells) might have the advantage of avoiding some of the side effects associated with inhibition of either of these pathways alone. To the best of our knowledge, no published studies have shown dual targeting of Stat3 and NFκB for therapeutic benefit. Although the exact molecular mechanism that led to activation of NFκB by Stat3 knockdown is unclear, given the recent reports showing activation of NFκB signaling by K-Ras (15, 39), we suggest that the observed activation of NFκB-reporter activity in Capan-2 cells under Stat3 knockdown conditions could be related to oncogenic Ras. Alternatively, when K-Ras is mutated (chronic condition) lack of Stat3 could trigger nuclear retention of p65 keeping it active. It is also possible that activated NFκB could function towards tumor growth suppression. These scenarios need to be examined. Further these observations need to be validated in additional cell lines with oncogenic Ras activation. Moreover, Nx intervention reduced fibrosis in the pancreas of BK5-Cox-2 in association with reduced levels of NFκB and Stat3 with no significant changes in the levels of Cox-2. Importantly Nx intervention had no significant effect on the body weight indicating non-toxic nature.

The observed inhibition of Stat3/NFκB by Nx was associated with reduced mRNA expression and protein levels of Cox-2. Cox-2 catalyzes the conversion of arachidonic acid to prostaglandin H₂ (PGH₂) that is subsequently converted to prostanoids including prostaglandin E₂ (PGE₂) by PGE synthase. PGE₂ transmits downstream signaling by binding to G-protein coupled receptors with seven transmembrane domains including EP1, EP2, EP3 and EP4. Upon ligand binding, each of these receptors transmits down stream signaling by (i) increasing levels of intracellular calcium (EP1); (ii) increasing levels of cyclic AMP (cAMP; EP2 and EP4); or (iii) by decreasing cAMP levels (EP3); (40–42). A recent study reported potential role for EP4 in mediating stellate cell activation implicating an important role for EP4 in pancreatic cancer management (28). We found significantly

elevated expression of EP4 compared to other three receptors in both Capan-2 and BxPC-3 cells. Further Nx treatment increased expression of EP4 in Capan-2 with oncogenic Ras activation but not in BxPC-3 cell line with wild type K-Ras. Interestingly, we also observed increased Cox-2 enzymatic activity as evidenced by elevated PGE₂ levels in Capan-2 cells but decrease in BxPC-3 cells following Nx treatment. Although several possibilities exist for the observed increase in PGE₂ levels, we believe that this may be due to presence of compensatory feedback mechanisms(22, 23). Additionally, both agonistic and antagonistic role for EP4 has been reported (42, 43). Both pro and anti-apoptotic role for PGE₂-EP4 was reported. For example in lung fibroblasts, it induces apoptosis. EP4 deficient mice have increased interstitial fibrosis and that treatment of wild type but not EP4 deficient mice with EP4 agonist reduced fibrosis suggesting a role for EP4 in fibrosis (41). Further, EP4 receptor deficient mice develop colitis and administration of EP4 selective agonist inhibits symptoms of severe colitis (43). EP4 agonist reduced the expression of α -smooth muscle actin and Twist, a marker of epithelial to mesenchymal transition in tubular cells (40). Although we did not find any published evidence for agonistic role of EP4 specifically in the pancreatic model, published evidence in these gastro-intestinal models support the view that EP4 agonist such as Nx could reduce fibrosis possibly by suppressing the production of extracellular matrix proteins or profibrotic cytokines such as platelet-derived growth factor or transforming growth factor- β 1. Studies to test this hypothesis are in progress in our laboratory. A hypothetical model is presented in Fig 6.

Given these data and our observation showing elevated expression of EP4 and its modulation with Nx in pancreatic cancer cells, we evaluated the expression of EP4 in clinical samples. For the first time our findings show EP4 overexpression in human pancreatic tumors compared to adjacent benign pancreatic tissue. We also observed significantly decreased expression of EP4 in histologically poorly differentiated pancreatic tumors. Further our data showing inverse association of EP4 expression with tumor differentiation highlights the importance of EP4 in tumor progression. Therefore activation of EP4 could potentially prevent progression to poorly differentiated tumors.

To our knowledge, this is the first demonstration that Nx can inhibit the growth of pancreatic cancer *in vitro* and *in vivo* targeting fibrosis and inflammation. Because of its low toxicity and its ability to target two critical inflammatory mediators and activation of EP4, Nx has enormous potential as a novel strategy for PanCA treatment. The concept of using natural compounds for cancer management is not new and has been applied in a wide variety of tumor models including pancreatic cancer. Several natural compounds including Curcumin show promise for pancreatic cancer treatment (44–47), albeit not for their ability to target desmoplasia and inflammation *in vivo*. However, Nx has never been tested. The advantages of Nx are that it targets (i) signaling pathways mediated by two critical transcription factors and their crosstalk; and (ii) desmoplasia, a critical player in the development of drug resistant PanCA. Along these lines, in additional unpublished studies we found that Nx treatment inhibited proliferation of PSCs, which are critical players in the development of desmoplasia (Chakravarthy, Huang and Kumar, unpublished observations). It is known that pancreatic tumors are extremely resistant to various therapeutic strategies because of the presence of extensive fibrosis in the stromal component of the tumor environment. Therefore the fact that Nx decreased the extent of fibrosis in the mouse model has tremendous clinical significance since combination of Nx with existing standards of care could enhance therapeutic efficacy. Being a complex mixture, one of the advantages of Nx is its inherent ability to target multiple signaling pathways. Secondly Nx is being sold over the counter as an anti-inflammatory with no significant toxicity associated problem reported. It is noteworthy to mention that use of traditional Cox-2 inhibitors is associated with cardiac and gastrointestinal problems limiting their clinical utility, therefore use of Nx may circumvent some of these toxicity-associated problems. In addition we are currently

evaluating the potential of Nx in prostate cancer patients and we found no toxicity-associated problems. Future studies with increasing doses of Nx could reveal its potential to inhibit Cox-2 induced lesions in addition to promoting anti-inflammatory and anti-fibrotic effects *in vivo*. These data warrant further studies to test the potential of Nx either alone or in combination with current standards of care in the management of PanCA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

Near equal incidence and mortality rates underscore the need to develop new therapeutic modality for pancreatic cancer. In this regard, Nexrutine (Nx), a natural product isolated from *Phellodendron amurense* bark inhibited the growth of multiple pancreatic cancer cell lines by dual-targeting inflammatory mediators Stat3/NFκB to induce apoptosis. Inhibition of Stat3/NFκB is correlated with reduced fibrosis in the pancreas from animals in response to Nx intervention. Given the published data showing increased activity of NFκB/Cox-2 in the presence of oncogenic Ras, our observations reporting modulation of NFκB, Stat3 and the downstream target Cox-2 by Nx supports the relevance of these studies to human PanCA. Dual inhibition of Stat3-NFκB and downstream target Cox-2 by Nx may be able to overcome cardiotoxic problems associated with specific Cox-2 inhibitors. Further, expression of Cox-2 and the prostaglandin PGE₂ receptor, EP4 was significantly elevated in human pancreatic tumors compared to benign tissue suggesting modulation of EP4 could have potential therapeutic benefit.

Figure 1. Nx inhibits proliferation and induces apoptosis in human PanCA cells

Capan-2, BxPC-3, HPNE and HPNE-Ras cells were treated with the indicated concentration of Nexrutine (Nx). The concentrations used were 50, 100 and 150 $\mu\text{g/ml}$ for Capan-2; 20, 40 and 60 $\mu\text{g/ml}$ for BxPC-3 and 20, 50 and 80 $\mu\text{g/ml}$ for HPNE-Ras).

A. Cell proliferation (Capan-2, BxPC-3 and HPNE-Ras) measured 24 h after treatment using the Cell Titer 96 Aqueous One solution assay.

B. Cell proliferation measured 24 h after treatment using the Cell Titer 96 Aqueous One solution assay in non-tumorigenic HPNE cells.

C. Soft agar colony formation assay performed over 7 days.

D. Measurement of apoptosis by FITC-Annexin staining 24 h after treatment.

Figure 2. Nx inhibits NFκB/Stat3 signaling

A. Whole cell extracts prepared from Capan-2 or BxPC-3 cells treated with a low, medium, or high dose of Nx for 24 h were subjected to immunoblot analysis with the indicated antibodies.

B. EMSA was conducted with nuclear extracts prepared from Capan-2 or BxPC-3 cells using Stat3 oligonucleotide as the radiolabeled probe in the presence of 100-fold excess of cold competitor (Stat3 oligonucleotide) and heterologous competitor (AP1 oligo), and preincubation with anti-Stat3, anti-p65, both antibodies, or IgG as a negative control. Specific DNA-protein complex formed was labeled with an *. Unbound probe is labeled UB.

C. Whole cell extracts prepared from Capan-2 or BxPC-3 cells treated with low, medium, and high dose of Nx for 24 h were subjected to immunoblot analysis with the indicated antibodies.

D. EMSA was conducted with nuclear extracts prepared from Capan-2 or BxPC-3 cells using NF κ B oligonucleotide as the radiolabeled probe in the presence of 100-fold excess of cold competitor (NF κ B oligonucleotide) and heterologous competitor (AP1 oligo), preincubation with anti-Stat3, ant-p65 or both antibodies, and IgG as a negative control. Specific DNA-protein complex formed was labeled with an *. Unbound probe is labeled UB.

E and F. Cellular localization of Stat3 (green) and p65 (red) was observed by confocal microscopy after immunofluorescent staining of stable scrambled and Stat3 knockdown transfectants of Capan-2 (E) and BxPC-3 (F) cells that were treated with Nx. Capan-2 and BxPC-3 cells treated with Nx were stained with immunofluorescent antibodies for Stat3 (green) and p65 (red) and cellular localization of proteins observed by confocal microscopy.

Figure 3. NFκB/Stat3 regulates Cox-2

A and B. Stat3 (A) or NFκB (B) reporter constructs containing the firefly luciferase reporter were transfected into Capan-2 and BxPC-3 cells together with Renilla luciferase. After 24 h transfection, cells were treated with Nx (high dose for 6 h) and luciferase activity in cell lysates was measured. The normalized luciferase/renilla activity was calculated with respect to untreated control. The data shown are representative of three independent experiments conducted in triplicate.

C. Effect of knocking down Stat3 on NFκB promoter activity. Capan-2 or BxPC-3 cells stably expressing Stat3-specific shRNA or scrambled ShRNA were transfected with NFκB luciferase reporter plasmid and Renilla luciferase. After 24 h of transfection luciferase activity was measured in cell lysates. The normalized luciferase/renilla activity was calculated with respect to scrambled shRNA control cells.

D, E and F. DNA from Stat3- and p65-immunoprecipitated lysates from Capan-2 and BxPC-3 cells following Nx treatment for 24 h was amplified by real-time PCR using primers for the Stat3 binding site (D), NFκB site 1 (E) and NFκB site 2 (F) on the Cox-2 promoter. DNA binding was calculated (in arbitrary units) by normalizing to input DNA. IgG was used as a negative control. The data shown are representative of three independent experiments conducted in triplicate.

Figure 4. EP4 expression in human pancreatic tumors

A. RNA isolated from Capan-2 and BxPC-3 cells was used in real-time PCR for analysis of prostaglandin receptors as described in methods. Basal expression of EP receptors normalized to β -actin expression is shown. Primers used for determining expression of EP receptors are shown in supplementary data SF 1B.

B. Tissue microarray (TMA) containing pancreatic tumor specimens along with adjacent normal tissue were stained using EP4 polyclonal antibody (Cayman) at a dilution of 1:500. TMAs were scored semi-quantitatively based on the proportion (percent) and intensity (negative, 1+ for low, 2+ for medium and 3+ for high) as described in methods. Final score

for staining was obtained by product of proportion and intensity of staining. Box plot showing differential expression of EP4 in normal and pancreatic tumors.

C. Representative immunohistochemical staining of EP4 expression at different magnifications in different human pancreatic tumors.

D and E. Alteration in the expression (mRNA expression) and levels (immunoblotting) of EP4 was analyzed in RNA and lysates prepared from Capan-2 and BxPC-3 cells treated with Nx (high dose) for 24h using real-time PCR (D) and immunoblotting (E) respectively as described in methods.

Figure 5. Nx treatment reduces fibrosis in pancreatic tumors

A and B. The effect of Nx on development of pancreatic ductal lesions was assessed by the presence or absence of atrophy, inflammation, fibrosis, and mPanIN lesions. The extent of fibrosis was scored in a blinded fashion (RR). Briefly, no fibrosis indicates no evidence of fibrosis; grade 0.5 indicates fibrosis surrounding rare to few ducts; grade 1 indicates easily seen periductal fibrosis that involves a greater number of ducts and mild epithelial proliferation; grade 2-3 indicates prominent ductal epithelial proliferation and abundant fibrosis usually with a few dominant nodules in addition to small proliferation. A is a representative H&E picture. Graphical representation of the histopathological evaluation data is shown in panel B.

C. Immunoblot analysis of Stat3, p65, and Cox-2 in pancreatic tissue from three different animals. Normalization was performed using β-actin as a loading control. Quantification of the data is presented in panel below.

Figure 6. Hypothetical model showing Nx-mechanism of action

Activation of Stat3/NFκB/Cox-2/EP4 signaling contributes to fibrosis and tumor differentiation leading to development of PanCA. Treatment with Nx reduces (i) levels and DNA-binding activity of both Stat3 and NFκB in pancreatic cancer cells; (ii) recruitment of Stat3 and NFκB to the endogenous Cox-2 promoter leads to its transcriptional suppression. Nx treatment increases secreted levels of PGE₂ and expression of EP4 receptor possibly via Cox-independent regulatory pathways.

Table I

Doses of Nexrutine ($\mu\text{g/ml}$) used in the study.

	Low	Medium	High
Capan-2	50	100	150
BxPC3	20	40	60
HPNE-Ras	20	50	80

Table II

Negative association of EP4 with poorly differentiated human pancreatic tumors

	Well to moderately differentiated	Poorly differentiated	Total
0	41	28	67
1	54	12	66
Total	95	38	133

Fisher's exact two sided test $p=0.001$

Table III

Effect of Nexrutine intervention on inflammation, fibrosis in BK5-Cox-2 mice.

Treatment	Animal ID#	Inflammation	Fibrosis	mPan 1A	mPan 1B	mPan 2	mPan 3
Control	PK12 100	0	0				
Control	PK12 103	0	0				
Control	PK12 104	0	0				
Control	PK12 117	0	0				
Control	PK12 125	0	0				
Control	PK12 101	0.5+	0.5+				
Control	PK12 102	0.5+	0.5+		FOCAL		
Control	PK12 116	0.5+	0.5+	Y			
Control	PK12 123	0.5+	0.5+				
Control	PK12 124	0	0.5+				
Control	PK12 126	0	0.5+				
Control	PK12 127	0.5	0.5+				
Control	PK12 128	0.5+	0.5+				
Control	PK12 114	0	0.5A				
Control	PK12 62	1+	1+				
Control	PK12 63	1+	1+				
Control	PK12 64	1+	1+				
Control	PK12 80	1+	1+				
Control	PK12 115	0.5+	1+	FOCAL			
Control	PK12 66	2+	2+		Y		
Control	PK12 78	1+	2+			Y	
Control	PK12 91	0.5+	2+				
Control	PK12 99	1+	2+			Y	
Nx	PK12 86	0	0				
Nx	PK12 90	0	0				
Nx	PK12 119	0	0				
Nx	PK12 121	0	0				

Treatment	Animal ID#	Inflammation	Fibrosis	mPan 1A	mPan 1B	mPan 2	mPan 3
Nx	PK12 129	0	0				
Nx	PK12 72	0.5+	0.5+				
Nx	PK12 73	0.5+	0.5+				
Nx	PK12 83	0	0.5+				
Nx	PK12 84	0.5+	0.5+				
Nx	PK12 88	0.5+	0.5+		Y		
Nx	PK12 89	0.5+	0.5+				
Nx	PK12 118	0.5+	0.5+	FOCAL			
Nx	PK12 120	0	0.5+				
Nx	PK12 122	0.5	0.5+				
Nx	PK12 130	0.5+	0.5+				
Nx	PK12 131	0	0.5+				
Nx	PK12 74	1+	1+				
Nx	PK12 77	1+	1+				
Nx	PK12 85	0.5+	1+			Y	
Nx	PK12 87	1+	1+			Y	
Nx	PK12 133	1+	1+		Y		
Nx	PK12 79	1+	2+			Y	
Nx	PK12 132	3+	3+		Y		