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Abstract

Purpose: Near equal rates of incidence and mortality emphasize the need for novel targeted approaches for better management of patients with pancreatic cancer. Inflammatory molecules NF-κB and STAT3 are overexpressed in pancreatic tumors. Inhibition of one protein allows cancer cells to survive using the other. The goal of this study is to determine whether targeting STAT3/NF-κB crosstalk with a natural product Nexrutine 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, cyclooxygenase-2 (COX-2), NF-κB, and STAT3 level in response to Nexrutine treatment. Transient expression, gel shift, chromatin immunoprecipitation assay was used to examine transcriptional regulation of COX-2. STAT3 knockdown was used to decipher STAT3/NF-κB crosstalk. Histopathologic and immunoblotting evaluation was performed on BK5–COX-2 transgenic mice treated with Nexrutine. In vivo expression of prostaglandin receptor E-prostanoid 4 (EP4) was analyzed in a retrospective cohort of pancreatic tumors using a tissue microarray.

Results: Nexrutine treatment inhibited growth of pancreatic cancer cells through induction of apoptosis. Reduced levels and activity of STAT3, NF-κB, and their crosstalk led to transcriptional suppression of COX-2 and subsequent decreased levels of prostaglandin E2 (PGE2) and PGF2. STAT3 knockdown studies suggest STAT3 as negative regulator of NF-κB activation. Nexrutine intervention reduced the levels of NF-κB, 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.

Conclusions: Dual inhibition of STAT3–NF-κB by Nexrutine may overcome problems associated with inhibition of either pathway. Clin Cancer Res; 20(5); 1259–73. ©2014 AACR.

Introduction

Pancreatic cancer 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). In addition, numerous phase III trials of Gemcitabine, the first-line therapeutic agent for pancreatic cancer, 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 pancreatic cancer.

The underlying etiology and pathophysiology of pancreatic cancer is poorly understood. Available evidence suggests that malignant progression from pancreatic intraepithelial neoplasia (PanIN) 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 proinflammatory molecule cyclooxygenase-2 (COX-2) has been observed in approximately 46% to 70% and 47% to 66% of pancreatic cancer, respectively (5). Recently it has been suggested that deregulation of chemokines, cytokines including interleukin (IL)-6, and activation of downstream effectors such as STAT3 and NF-κB are key events in the initiation and progression of pancreatic cancer (6). Intense fibrosis or desmoplasia surrounding the tumoral glands is a unique feature of pancreatic cancer (7). Such tumor–stromal interactions contribute...
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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, a natural product isolated from Phellodendron amurense bark inhibited the growth of multiple pancreatic cancer cell lines by dual-targeting inflammatory mediators Stat3/NF-kB to induce apoptosis. Inhibition of Stat3/NFkB is correlated with reduced fibrosis in the pancreas from animals in response to Nexrutine intervention. Given the published data showing increased activity of NF-kB/cyclooxygenase-2 (COX-2) in the presence of oncogenic Ras, our observations reporting modulation of NF-kB, Stat3, and the downstream target Cox-2 by Nexrutine supports the relevance of these studies to human pancreatic cancer. Dual inhibition of Stat3–NF-kB and downstream target Cox-2 by Nexrutine may be able to overcome cardiotoxic problems associated with specific Cox-2 inhibitors. Furthermore, expression of Cox-2 and the prostaglandin E2 (PGE2) receptor, E-prostanoid 4 (EP4) was significantly elevated in human pancreatic tumors compared with benign tissue suggesting modulation of EP4 could have potential therapeutic benefit.

not only to tumor progression, but also poor drug delivery and chemoresistance. Therefore, drugs that can inhibit desmoplastic stroma in pancreatic cancer might expand the limited pool of therapeutic options against pancreatic cancer, 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 pancreatic cancer.

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 using multiple pancreatic cancer cell lines and a preclinical animal model that develops pancreatitis and fibrosis. Nexrutine 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 (5, 8, 9). Although it has not been extensively studied in terms of its anticancer activity, studies from our laboratory demonstrated that Nexrutine inhibits prostate tumor growth both in vitro and in vivo by targeting multiple signaling pathways including Akt, NF-kB, CAMK response element-binding protein, COX-2, and cyclin D1 (8, 9). However, its effects on pancreatic cancer have not been explored. In this study, we demonstrate that Nexrutine (i) significantly inhibits the growth of multiple pancreatic cancer cell lines with minimal effect on immortalized nontumorigenic HPNE cells; and (ii) inhibits STAT3 and NFkB activation leading to transcriptional suppression of COX-2, modulation of prostaglandin receptor E-prostaglandin 4 (EP4) and apoptosis. Our findings also show EP4 overexpression in human pancreatic tumors compared with adjacent benign pancreatic tissue. In addition, Nexrutine 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, Nexrutine had no effect on COX-2-induced lesions under these experimental conditions. Our results are the first to demonstrate that STAT3/NF-kB/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. 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. Furthermore, 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 pancreatic ductal adenocarcinoma (PDAC; refs. 10 and 11). Therefore, HPNE cells provide an excellent model system to examine the effect of Nexrutine. All cell lines were grown in Roswell Park Memorial Institute medium (Mediatech, Inc.) supplemented with 10% FBS, 100 µg/mL penicillin–streptomycin, and 100 µg/mL Amphotericin in a humidified incubator at 37°C and 5% CO2. A stock solution of Nexrutine (5 mg/mL) was prepared by dissolving Nexrutine powder in 50% dimethyl sulfoxide (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 Nexrutine dose (150 µg/mL). Prostaglandin E2 (PGE2) was obtained from Sigma. Monoclonal antibodies (p65, stat3, pstat3, p50, IkBα) and STAT3 inhibitor V were purchased from Santa Cruz Biotechnology (Santa Cruz). Next Pharmaceuticals Inc. supplied Nexrutine manufactured by Cortex Scientific under pharmaceutical good manufacturing practices. The quality of Nexrutine was tested for quality control by high-performance liquid chromatography based on the berberine content for reproducibility. A single batch of Nexrutine 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. H. 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 semiquantitatively as described previously by a pathologist (A.P. Kumar), who was blinded to the clinicopathologic and follow up data, based on the proportion (percent) and intensity (negative, 1+ for low, 2+ for medium, and 3+ for high; refs. 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 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. S.M. Fischer, The University of Texas MD Anderson Cancer Center (Smithville, TX). Breeding and genotyping was performed essentially as described in Colby and colleagues 2008 (13). After confirming the genotype, BK5–COX-2 mice were randomized into 2 groups each of 23 animals each. A group of animals fed AIN93G-based pelleted diet supplemented with Nexrutine (300 mg/kg diet obtained from Dyets, Inc.) for 6 weeks. Intervention with Nexrutine was initiated immediately after weaning. Mice fed AIN93G-based diet without Nexrutine 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 pathologic changes including inflammation, fibrosis, and mPanIN lesions (R. Reddick). 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 to 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) and CytoSelect 96-well soft agar colony formation assay (Cell Biolabs), respectively, according to the manufacturer’s directions as described previously (8, 9). Experiments were conducted using 3 different doses of Nexrutine for each cell line: low, medium, and high (50, 100, and 150 µg/mL for Capan-2; 20, 40, and 60 µg/mL for BxPC-3; and 20, 50, and 80 µg/mL for HPNE-Ras; Table 1). Apoptosis was measured using FITC-Annexin assay (Medical and Biological Laboratories) and DNA fragmentation using cellular DNA fragmentation ELISA Kit (Roche Applied Science) 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.). Immunoblot analysis, real-time PCR and transient expression assays and confocal microscopy were conducted as described previously (8, 9).

Electrophoretic mobility shift assay

Nuclear extracts prepared from Capan-2 and BxPC3 cells treated with Nexrutine at low ~IC₅₀ (50 and 20 µg/mL for Capan-2 and BxPC3, respectively), medium ~IC₁₀ (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 24 hours were incubated with ³²P-labeled oligonucleotides containing STAT3 or NF-κB binding sites and DNA binding activity was measured. Briefly, the double-stranded NF-κB or STAT3 oligonucleotide (20 ng) was end labeled with γ-p-³²-ATP using T4 polynucleotide kinase. Twelve micrograms of nuclear extract was incubated with the radiolabeled probe in binding buffer containing 4 mmol/L Tris-HCl, 12 mmol/L Hepes, pH 7.9, 60 mmol/L KCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, and 12% glycerol for 25 minutes at room temperature in a final volume of 20 µL. Following this incubation, samples were fractionated on a 4% polyacrylamide gel in 0.25× 1 ris-borate EDTA 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 NF-κB or STAT3 oligonucleotide (homologous) and AP1 (heterologous) for 5 minutes before the addition of nuclear extract. For super-shift experiments, nuclear extracts were preincubated with 1 µg each of STAT3 or p65 or combination of both STAT3 and p65 antibodies for 30 minutes on ice before use in electrophoretic mobility shift assay (EMSA).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) was performed using ChIP-it Express Kit (Active Motif) as described previously (12). Briefly, logarithmically growing cells treated with Nexrutine for 24 hours were cross-linked with 1%
formaldehyde for 10 minutes at room temperature followed by termination with 125 mmol/L 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 immunoglobulin G (IgG; as negative control) and immune complexes were absorbed with protein G magnetic beads. Ten percent 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 (NF-κB binding site 1), −363/−343 (NF-kB binding site 2), and −266/−250 (STAT3 binding site) in the COX-2 promoter by real-time PCR. Primers used are shown in Supplementary Fig. S1. Duplicate PCR reactions were performed for each sample and the expression data were normalized to respective input values. Data are presented as the average ± SD.

Statistical analysis

All in vitro experiments were conducted at least 3 times and the data presented were 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 versus normal tissues were compared with a Wilcoxon rank sum test. P values < 0.05 were considered significant. Contingency tables with a 2-tailed Fisher exact test were also used to compare the presence of staining in normal versus 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

Nexrutine inhibits proliferation and induces apoptosis in human pancreatic cancer cells

The antiproliferative effect of Nexrutine was examined using immortalized normal pancreatic cell lines (HPNE) and 3 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 KRasG12D (HPNE-Ras). As shown in Fig. 1A, incubation of pancreatic cancer cell lines with different concentrations of Nexrutine for 24 hours 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 Nexrutine. 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 3 different doses of Nexrutine: low, medium, and high corresponding to IC10, IC32, and IC50 (50, 100, and 150 μg/mL for Capan-2; 20, 40, and 60 μg/mL for

Figure 1.  Nexrutine inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Capan-2, BxPC-3, HPNE, and HPNE-Ras cells were treated with the indicated concentration of Nexrutine. The concentrations used were 50, 100, and 150 μg/mL for Capan-2; 20, 40, and 60 μg/mL for BxPC-3; and 20, 50, and 80 μg/mL for HPNE-Ras. A, cell proliferation (Capan-2, BxPC-3, and HPNE-Ras) measured 24 hours after treatment using the Cell Titer 96 Aqueous One solution assay. B, cell proliferation measured 24 hours after treatment using the Cell Titer 96 Aqueous One solution assay in nontumorigenic HPNE cells. C, soft agar colony formation assay performed over 7 days. D, measurement of apoptosis by FITC-Annexin staining 24 hours after treatment.
Nexrutine Inhibits Pancreatic Cancer via STAT3/NF-κB

BxPC-3; and 20, 50, and 80 μg/ml for HPNE-Ras; Table 1). Because HPNE cells do not form colonies on soft agar, we examined the effect of Nexrutine 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 anti-proliferative effects of Nexrutine, this cell line was relatively resistant to Nexrutine-induced anchorage-independent growth on soft agar. The observed differences could be related to the differential effects of Nexrutine 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 Nexrutine compared with nontumorigenic HPNE cells. As it was previously reported that the anti-proliferative activity of Nexrutine is mediated by induction of programmed cell death including apoptosis in prostate cancer cells (8, 9), we measured apoptosis in the pancreatic cancer cells in response to Nexrutine 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 Nexrutine treatment (Fig. 1D). Histograms of FITC-Annexin staining are shown in Supplementary Fig. S2A. Additional approaches including morphologic assessment (data not shown) and cellular DNA fragmentation showed similar results (Supplementary Fig. S2B). Taken together, these data suggest that Nexrutine inhibits both anchorage dependent and independent growth of human pancreatic cancer cell lines possibly through induction of apoptosis.

Nexrutine inhibits NF-κB/STAT3 signaling

Constitutive activation of inflammatory mediators NF-κB 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 Nexrutine on protein levels and activity of these transcription factors. As shown in Fig. 2A, Nexrutine treatment caused a significant reduction in the protein levels of pSTAT3 and tyrosine kinase Janus-activated kinase 1 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 nontumorigenic Ras-transformed HPNE-Ras cells (Supplementary Fig. S3A). Next we performed EMSAs with STAT3 binding site oligonucleotide as radiolabeled probe. As shown in Fig. 2B, nuclear extracts prepared from Capan-2 (Fig. 2B, left) and BxPC-3 cells (Fig. 2B, right) 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 asterisk). Nuclear extracts prepared from both Capan-2 (Fig. 2B, left) and BxPC-3 cells (Fig. 2B, right) treated with increasing doses of Nexrutine showed dose-dependent decrease in the observed DNA–protein complex (compared lines 2–5). Lower exposure blot is shown in Supplementary Fig. S3B. To determine the components of this DNA–protein complex, we performed gel super-shift experiments using antibodies specific for STAT3 and p65 for NF-κB. We observed enhanced intensity of the observed specific DNA–protein complex when nuclear extracts prepared from Capan-2 cells preincubated with anti-STAT3 antibody, but not with IgG, were used in these experiments (compare lines 11 with 8). However, 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 preincubated 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). Taken together, these data suggest the presence of both STAT3 and p65 in the observed STAT3 oligo–protein complex from Capan-2 cells and that Nexrutine treatment reduces the observed DNA binding activity.

To determine the role of NF-κB signaling, we measured the protein levels of p65 using immunoblot analysis and NF-κB activity using EMSA. Immunoblot analysis revealed reduced levels of p65 in Nexrutine-treated extracts in Capan-2 and BxPC-3 cells (Fig. S2B). Although absolute levels of p50 seem to decrease at high dose of Nexrutine, quantification data from multiple experiments indicated no statistically significant change in the levels of p50 (Fig. 2C). Similar results were observed using nontumorigenic HPNE-Ras cells albeit p50 was more prominent (Supplementary Fig. S3A). As shown in Fig. 2D and S3B (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 (NF-κB) and heterologous (AP1) unlabeled oligonucleotide probes (indicated with an asterisk). Nexrutine-treatment reduced the formation of observed DNA–protein complex. Furthermore, 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). Preincubation with both antibodies further reduced the mobility of the super-shifted band, indicating the presence of both p65 and STAT3 in the observed NF-κB–oligo protein complex (compare lines 10 with 11). However, 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
Figure 2. Nexrutine inhibits NF-κB/STAT3 signaling. A, whole cell extracts prepared from Capan-2 or BxPC-3 cells treated with a low, medium, or high dose of Nexrutine for 24 hours 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 Nexrutine for 24 hours 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-κB oligonucleotide as the radiolabeled probe in the presence of 100-fold excess of cold competitor (NF-κB oligonucleotide) and heterologous competitor (AP1 oligo), preincubation with anti-STAT3, anti-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 Nexrutine. Capan-2 and BxPC-3 cells treated with Nexrutine were stained with immunofluorescent antibodies for STAT3 (green) and p65 (red) and cellular localization of proteins observed by confocal microscopy.
formed with the NF-κB probe. Furthermore, the presence of p65 in STAT3 DNA–protein complexes and STAT3 in the NF-κB complex led us to examine the potential crosstalk between STAT3 and p65 using STAT3 knockdown pancreatic cancer cells. As shown in Supplementary Fig. S4A(top), knockdown of STAT3 resulted in approximately 80% reduction in its expression compared with control nontargeting shRNA in both Capan-2 and BxPC-3 cells. Cells transfected with scrambled shRNA showed colocalization 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 Fig. S4B). Knockdown of STAT3 resulted in nuclear accumulation of p65 in Capan-2 cells. Furthermore, nuclear accumulation decreased following treatment with Nexrutine in Capan-2 cells. Interestingly Nexrutine 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 NF-κB and STAT3 interact with each other in the cytoplasm and that Nexrutine 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 NF-κB reporter activity. We found that Nexrutine treatment significantly reduced the STAT3 and NF-κB 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 crosstalk between NF-κB and STAT3 in STAT3 knockdown cells. Surprisingly STAT3 knockdown resulted in significant elevation of NF-κB 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 Nexrutine-induced proliferation. Scrambled control and STAT3 knockdown Capan-2 and BxPC3 cells were treated with different doses of Nexrutine for 24 hours. As shown in Supplementary Fig. S4A(bottom), Nexrutine 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 Nexrutine albeit statistically significant in
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Capan-2 cells. In contrast, lack of STAT3 in BxPC-3 cells further sensitized the cells to Nexrutine-induced proliferation inhibition. This may be because of 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 Fig. S4C). It should be mentioned that despite the observed antiproliferative effects of Nexrutine 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 antiproliferative effects of Nexrutine irrespective of K-Ras of both Capan-2 and BxPC3 cell lines (Supplementary Fig. S5A). We also measured the levels of PGE2 and PGF2α in BxPC-3 cells (Supplementary Fig. S5F).

In Capan-2 cells, although low-dose Nexrutine decreased but higher doses increased PGE2 levels (Supplementary Fig. S5E). Several possibilities exist for the observed increase in PGE2 levels despite decreased COX-2 expression in Capan-2 cells following Nexrutine treatment. We believe that this may be because of presence of compensatory feedback 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). Furthermore, 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 Nexrutine treatment in Capan-2 cells could be because of compensatory feedback mechanism or because of 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 transmembrane receptors. Four E-prostanoid receptors (EP1, EP2, EP3, and EP4) mediate distinct signaling pathways (24–27). We investigated the effect of Nexrutine on modulation of E-prostanoid and Prostaglandin F (FP) receptor expression in pancreatic cancer cell lines. We were able to detect expression of all 4 receptors (EP1 to EP4) using real-time PCR in these cell lines. However, EP4 was expressed at higher levels compared with 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). Furthermore, a recent study demonstrated that although pancreatic stellate cells express all 4 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 pancreatic cancer samples compared with 29% (31/106) in adjacent benign pancreatic tissue (P = 0.001; Fig. 4B). A representative immunohistochemical staining of TMA is shown in Fig. 4C. It is noteworthy to mention that although both membranous and cytoplasmatic staining has been reported for EP4, we observed mostly cytoplasmatic staining that could be related to the antibody used (http://www.proteinatlas.org). Furthermore, 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, Nexrutine 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 Nexrutine 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 Nexrutine-mediated biologic effects. However, the precise mechanism of such differential effects is unclear. Furthermore, these data need to be validated in additional cell lines with oncogenic Ras activation.

Nexrutine 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 pancreatic cancer. 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 upregulated in precancerous conditions including pancreatitis and pancreatic cancer (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 to 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–COX-2 mouse model, we assessed the effect of Nexrutine on pancreatic pathology, STAT3 and p65 activation. A total of 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. Furthermore, 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. However, only 2 of the animals in the Nexrutine intervention group (8.6%) had such features. Although 9 of animals on control and 11 on Nexrutine 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 Nexrutine-treated pancreas are shown in Fig. 5A. Pathologic quantification of the data is presented in Fig. 5B. Nexrutine treatment had no significant effect on the body weight of these animals, indicating nontoxic nature (Supplementary Fig. S6A). We also did not observe any significant change in the food consumption between control and experimental group of animals (Supplementary Fig. S6B). 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 suggest the potential for Nexrutine to inhibit pancreatic fibrosis. Although, additional studies using isolated stromal and epithelial compartments are required to precisely define the cellular targets of Nexrutine. These data warrant additional investigations including using large number of animals and dose–response studies to see impact of Nexrutine on tumor development.

The Nexrutine-induced reduction in fibrosis is associated with reduced STAT3 levels

We analyzed pancreatic tissue using 3 animals per group for changes in the expression of STAT3, NF-κB, and COX-2. As shown in Fig. 5C, we detected STAT3, p65, and COX-2 in all 3 samples from control mice. However, only 1 of the samples from the treatment group showed strong STAT3 and p65 expression and the other 2 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 (Fig. 5D). The observed changes in the levels of COX-2 in response to Nexrutine 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 Nexrutine-mediated effects are associated with deregulation of STAT3/NF-κB signaling. Expression analysis and functional assays using isolated stromal and epithelial cells and their potential crosstalk would help define the precise cellular target of Nexrutine.

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 2 critical risk factors for pancreatic cancer (7). Notably, desmoplasia not only impedes drug delivery but also contributes to therapeutic resistance (7). Activation of inflammatory signaling molecules including STAT3, NF-κB, and COX-2 has been shown to be involved in the proliferation of pancreatic stellate cells (PSC) 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 pancreatic cancer. Therefore, drugs targeting critical molecular abnormalities such as desmoplasia and inflammation would be ideal candidates for effective management of pancreatic cancer.

By virtue of their anti-inflammatory and anti-oxidant properties, a vast majority of phytochemicals has been reported to possess antitumorigenic potential (34). Furthermore, 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 article, we investigated the potential of Nexrutine, 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 Nexrutine inhibits the activity of both STAT3 and NF-κB, and possibly their crosstalk, 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 an alternate approach to overcome COX-2 inhibition (using traditional COX-2 inhibitors) that is generally associated with cardiotoxicity.

The transcription factors NF-κB and STAT3 are constitutively activated in a variety of tumors including pancreatic cancer to regulate the initiation and progression of the tumorigenic process (15–18). Therefore, interference with NF-κB or STAT3 activation can inhibit pancreatic carcinogenesis. Interestingly, our results demonstrated the

| Table 3. 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+            |                  |                  |                |                |
| Control         | PK12 116        | 0.5+            | 0.5+            |                  |                  |                | FOCAL           |
| Control         | PK12 123        | 0.5+            | 0.5+            |                  |                  |                |                |
| Control         | PK12 124        | 0.5+            | 0.5+            |                  |                  |                |                |
| Control         | PK12 126        | 0.5+            | 0.5+            |                  |                  |                |                |
| Control         | PK12 127        | 0.5+            | 0.5+            |                  |                  |                |                |
| Control         | PK12 128        | 0.5+            | 0.5+            |                  |                  |                |                |
| Control         | PK12 114        | 0               | 0               |                  |                  |                |                |
| 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               |                  |                  |                |                |
| Nx              | PK12 129        | 0               | 0               |                  |                  |                |                |
| Nx              | PK12 72         | 0.5+            | 0.5+            |                  |                  |                |                |
| Px              | PK12 73         | 0.5+            | 0.5+            |                  |                  |                |                |
| Px              | PK12 83         | 0               | 0               |                  |                  |                |                |
| Px              | PK12 84         | 0.5+            | 0.5+            |                  |                  |                |                |
| Px              | PK12 88         | 0.5+            | 0.5+            |                  |                  |                | Y               |
| Px              | PK12 89         | 0.5+            | 0.5+            |                  |                  |                |                |
| Px              | PK12 118        | 0.5+            | 0.5+            |                  |                  |                | FOCAL           |
| Px              | PK12 120        | 0               | 0               |                  |                  |                |                |
| Px              | PK12 122        | 0.5             | 0.5             |                  |                  |                |                |
| Px              | PK12 130        | 0.5             | 0.5             |                  |                  |                |                |
| Px              | PK12 131        | 0               | 0               |                  |                  |                |                |
| Px              | PK12 74         | 1+              | 1+              |                  |                  |                |                |
| Px              | PK12 77         | 1+              | 1+              |                  |                  |                |                |
| Px              | PK12 85         | 0.5+            | 1+              |                  |                  |                | Y               |
| Px              | PK12 87         | 1+              | 1+              |                  |                  |                | Y               |
| Px              | PK12 133        | 1+              | 1+              |                  |                  |                | Y               |
| Px              | PK12 79         | 1+              | 2+              |                  |                  |                | Y               |
| Px              | PK12 132        | 3+              | 3+              |                  |                  |                | Y               |
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 NF-κB for therapeutic benefit. Although the exact molecular mechanism that led to activation of NF-κB by STAT3 knockdown is unclear, given the recent reports showing activation of NF-κB signaling by K-Ras (15, 39), we suggest that the observed activation of NF-κB–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 NF-κB with reduced NF-κB activity, sug- gesting a negative regulation (Fig. 3). These data suggest that inhibition of STAT3 signaling alone may even promote tumor growth by activating NF-κB 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 NF-κB–inducible genes by STAT3 and activation of NF-κB–regulated genes involved in antitumor immunity when STAT3 is inhibited (37); and (iii) activation of NF-κB pathway by STAT3 inhibitor in human Glioblastoma cells (38). Because both STAT3 and NF-κB 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 NF-κB–STAT3 albeit with limited efficacy (because complete inhibition may not be optimal for growth and survival of normal cells) might have the advantage of avoiding some of

following: (i) the presence of both NF-κB and STAT3 in the DNA–protein complexes obtained with either NF-κB or STAT3 (Fig. 2); and (ii) both NF-κB 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 NF-κB activation, suggesting a negative regulation (Fig. 3). These data suggest that inhibition of STAT3 signaling alone may even promote tumor growth by activating NF-κB 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 NF-κB–inducible genes by STAT3 and activation of NF-κB–regulated genes involved in antitumor immunity when STAT3 is inhibited (37); and (iii) activation of NF-κB pathway by STAT3 inhibitor in human Glioblastoma cells (38). Because both STAT3 and NF-κB 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 NF-κB–STAT3 albeit with limited efficacy (because 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 NF-κB for therapeutic benefit. Although the exact molecular mechanism that led to activation of NF-κB by STAT3 knockdown is unclear, given the recent reports showing activation of NF-κB signaling by K-Ras (15, 39), we suggest that the observed activation of NF-κB–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 NF-κB could function toward tumor growth suppression. These scenarios need to be examined. Furthermore, these observations need to be validated in additional cell lines with oncogenic Ras activation. Moreover, Nexrutine intervention reduced fibrosis in the pancreas of BK5-COX-2 in association with reduced levels of NF-κB and STAT3 with no significant changes in the levels of COX-2. Importantly Nexrutine intervention had no significant effect on the body weight indicating nontoxic nature.

The observed inhibition of STAT3/NF-κB by Nexrutine was associated with reduced mRNA expression and protein levels of COX-2. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin H2 that is subsequently
converted to prostanoids including PGE2 by PGE synthase. PGE2 transmits downstream signaling by binding to G-protein–coupled receptors with 7 transmembrane domains including EP1, EP2, EP3, and EP4. Upon ligand binding, each of these receptors transmits downstream 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; refs. 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 with other 3 receptors in both Capan-2 and BxPC-3 cells. Furthermore, Nexrutine 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 Nexrutine treatment. Although several possibilities exist for the observed increase in PGE2 levels, we believe that this may be because of presence of compensatory feedback mechanisms (22, 23). In addition, both agonistic and antagonistic role for EP4 has been reported (42, 43). Both pro- and anti-apoptotic role for EP2-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). Furthermore, 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 gastrointestinal models support the view that EP4 agonist such as Nexrutine could reduce fibrosis possibly by suppressing the production of extracellular matrix proteins or profibrotic cytokines such as platelet-derived growth factor or TGF-β1. 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 Nexrutine 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 with adjacent benign pancreatic tissue. We also observed significantly decreased expression of EP4 in histologically poorly differentiated pancreatic tumors. Furthermore, 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 Nexrutine 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 2 critical inflammatory mediators and activation of EP4, Nexrutine has enormous potential as a novel strategy for pancreatic cancer 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, Nexrutine has never been tested. The advantages of Nexrutine are that it targets (i) signaling pathways mediated by 2 critical transcription factors and their crosstalk; and (ii) desmoplasia, a critical player in the development of drug-resistant pancreatic cancer. Along these lines, in additional unpublished studies we found that Nexrutine treatment inhibited proliferation of PSCs, which are critical players in the development of desmoplasia (D. Chakravarty, Huang, and A.P. Kumar, unpublished data). 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 Nexrutine decreased the extent of fibrosis in the mouse model has tremendous clinical significance because combination of Nexrutine with existing standards of care could enhance therapeutic efficacy. Being a complex mixture, one of the advantages of Nexrutine is its inherent ability to target multiple signaling pathways. Second, Nexrutine 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...
Nexrutine may circumvent some of these toxicity-associated problems. In addition, we are currently evaluating the potential of Nexrutine in patients with prostate cancer and we found no toxicity-associated problems. Future studies with increasing doses of Nexrutine could reveal its potential to inhibit COX-2–induced lesions in addition to promoting anti-inflammatory and antifibrotic effects in vivo. These data warrant further studies to test the potential of Nexrutine either alone or in combination with current standards of care in the management of pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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