Abstract

Dysregulated Notch signaling plays an important role in the progression of cancer. Notch signaling affects tumor growth and angiogenesis through the actions of its ligand Jagged-1. In this study, we developed a novel compound 3,5-bis(2,4-difluorobenzylidene)-4-piperidone (DiFiD) and determined that it inhibits cancer cell growth and its effects on Notch signaling. Intraperitoneal administration of DiFiD significantly suppressed growth of pancreatic cancer tumor xenografts. There was a reduction in CD31-positive blood vessels, suggesting that there was an effect on angiogenesis. In vitro, DiFiD inhibited the proliferation of various human and mouse pancreatic cancer cells while increasing activated caspase-3. Cell-cycle analyses showed that DiFiD induced G2–M arrest and decreased the expression of cell-cycle–related proteins cyclin A1 and D1 while upregulating cyclin-dependent kinase inhibitor p21WAF1. We next determined the mechanism of action. DiFiD reduced Notch-1 activation, resulting in reduced expression of its downstream target protein Hes-1. We further determined that the reduced Notch-1 activation was due to reduction in the ligand Jagged-1 and two critical components of the γ-secretase enzyme complex presenilin-1 and nicastrin. Ectopic expression of the Notch intracellular domain rescued the cells from DiFiD-mediated growth suppression. DiFiD-treated tumor xenografts also showed reduced levels of Jagged-1 and the γ-secretase complex proteins presenilin-1 and nicastrin. Taken together, these data suggest that DiFiD is a novel potent therapeutic agent that can target different aspects of the Notch signaling pathway to inhibit both tumor growth and angiogenesis.

Introduction

Pancreatic cancer is the fourth leading cause of adult cancer-related death associated with a high mortality rate (1). The American Cancer Society estimated that 43,140 new cases and 36,800 deaths would have occurred during 2010 (2). Despite the advances in molecular pathogenesis, pancreatic cancer remains a major unsolved health problem in the United States (3, 4). Pancreatic cancer is a rapidly invasive, metastatic tumor that is resistant to standard therapies (5, 6). At present, single-agent–based chemotherapy (e.g., gemcitabine) is the mainstay treatment of metastatic adenocarcinoma of pancreas. Gemcitabine treatment has a tumor response rate of less than 10%; similarly, none of the available current chemotherapeutic agents has objective response rate of more than 10% (3, 5). The magnitude of this problem mandates the need for novel therapeutic agents.

Curcumin, an active ingredient of the spice turmeric, has been used to treat a number of ailments. Recent preclinical and clinical studies have shown the antitumor, antiangiogenic properties of curcumin (7–9). Pilot clinical trials have shown that curcumin is safe when consumed at a daily dose of 12 g for 3 months (10–12). However, poor intestinal absorption and bioavailability have limited its use (12, 13). Consequently, analogues of curcumin with similar safety profiles but increased anticancer activity and solubility are being developed. We and others have shown that EF24, a fluorinated curcumin analogue, has greater biological activity and better bioavailability but no increased toxicity (14–16). More importantly, EF24 had better pharmacokinetic profile than curcumin (14, 15). On the basis of this compound, we further developed a novel derivative 3,5-bis(2,4-difluorobenzylidene)-4-piperidone (DiFiD) and determined the effect of this compound on tumor growth.

Notch signaling plays a critical role in maintaining the balance between cell proliferation and apoptosis and in the development of pancreatic cancer (17). Interaction of
Jagged-1/2 with the Notch-1 receptor promotes a γ-secretase–dependent cleavage of the receptor and release of the Notch-1 intracellular domain (NICD), which translocates to the nucleus and activates transcription of target genes such as Hes-1 and Hey1 (18). Increased expression of Notch genes and their ligands has been detected in human pancreatic cancer tissues (19). Overexpression of NICD accelerates the formation of oncogenic KRas-induced pancreatic intraepithelial neoplasia (PanIN) lesions (20). Oral administration of γ-secretase inhibitor (GSI) in mice blocks the progression of PanIN to ductal adenocarcinoma (21). γ-Secretase is a multiprotein intramembrane-cleaving protease with a growing list of protein substrates including the Notch receptors. The 4 components of γ-secretase complex—presenilin, nicas- trin, Pen2, and Aph1—are all thought to be essential for activity (19). The catalytic domain resides within presenilin; nicas- trin has been suggested to be critical for substrate recognition. In this article, we have determined the effect of DiFiD on pancreatic cancer cells and identified 1 mechanism of action in the inhibition of the Notch signaling pathway.

Materials and Methods

Cells and reagents
AsPC-1, MiaPaCa-2, PanC-1, and BxPC-3 human and mouse embryonic fibroblast cell lines were obtained from American Type Culture Collection at passage 4. Pan02 mouse pancreatic cancer cell line was obtained from the National Cancer Institute DCTD tumor repository and previously published (9). All cells were grown in RPMI-1640 or Dulbecco’s Modified Eagle’s Medium containing 10% heat-inactivated FBS (Sigma-Aldrich) and 1% antibi- otic-antimycotic solution (Mediatech Inc.) at 37°C in a humidified atmosphere of 5% CO2. All cells used in this study were within 20 passages after receipt or resuscit- ation (~3 months of noncontinuous culturing). The cell lines were not authenticated as they came from national repositories. DiFiD was synthesized by Dr. Awasthi. N-[N-(3,5-Difluorophenacetyl)-l-allyl]-S-phenylglycine t-butyl ester (DAPT) was purchased (Sigma-Aldrich).

Proliferation and apoptosis assays
To assess proliferation, cells were seeded on to 96-well plates and grown overnight before treatment with increasing doses of DiFiD. Cell proliferation was determined by enzymatic hexosaminidase assay as described previously (22). For apoptosis, caspase-3/7 activity was measured with the Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega).

Colony formation assay
Briefly, 500 cells were incubated with DiFiD for 24 hours and then incubated for an additional 10 days in complete medium to allow colonies to form. The colonies were fixed in 10% formalin, followed by staining with hematoxylin. Experiments were done in triplicate.

Cell-cycle analyses
Cells were treated with DiFiD for 24 hours and subsequently trypsinized and suspended in PBS. Single-cell suspensions were fixed with 70% ethanol for 2 hours and subsequently permeabilized with PBS containing 1 mg/mL propidium iodide (PI; Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 2 μg DNase-free RNase (Sigma-Aldrich) at room temperature. Flow cytometry was done with a FACScalibur analyzer (Becton Dickinson), capturing 50,000 events for each sample. Results were analyzed with ModFit LT software (Verity Software House).

Real-time reverse transcriptase PCR analysis
Total RNA isolated from MiaPaCa-2 or Pan02 cells and tumor xenograft with TRIzol reagent was reverse transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (Invitrogen). Real-time PCR (RT-PCR) was carried out with JumpStart Tag DNA polymerase (Sigma-Aldrich) and SYBR Green nucleic acid stain (Molecular Probes). Crossing threshold values for individual genes were normalized to β-actin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β-actin: 5′-CTTGATCCACATCTGTGGC-3′ and 5′-ATCACCCCTTCTCCTAAC-3′; COX-2: 5′-GAATCCATTCAACAGGAAATTG-3′ and 5′-TCTGTTACCTGCTTCAAGC-3′; interleukin-8 (IL-8): 5′-CTCTTGCAACCTCTCTCTGAT-3′ and 5′-TCTGTTACCTGCTTCAAGC-3′; cyclin D1: 5′-AATGACCCCCGACAGATITC-3′ and 5′-TCAGGTTACCTGGTCCAC-3′; Notch-1: 5′-CAGCCTGGGATTCC-3′ and 5′-GTTGATTGGTCTGCGCAC-3′; Hes-1 5′-AGGGCAATGTCTTCTCCTCAGG-3′ and 5′-CGGTACTTCCCCAGCAGC-3′; nicastrin 5′-TCTGTTACCTGCTTCAAGC-3′ and 5′-TCAGGTTACCTGGTCCAC-3′; presenilin-1 5′-ATCATGCTTCTGCTTCCAC-3′ and 5′-TCTGTTACCTGCTTCAAGC-3′ and nicastrin 5′-CAGGTTGCTTCCAC-3′ and 5′-CTCCAGCAGAAC-3′.

Western blot analysis
Cell lysates were subjected to PAGE and blotted onto Immobilon polyvinylidene difluoride membranes (Milli- pore). Antibodies were purchased from Cell Signaling Technology, Abcam Inc., and Santa Cruz Biotechnology Inc., and specific proteins were detected by the enhanced chemiluminescence system (GE Healthcare).

Immunofluorescence staining
The cells were grown on coverslips and treated with DiFiD for 24 hours. After formalin fixing, the cells were then incubated with anti-Notch-1, anti-Jagged-1, and anti-Hes-1 antibody followed by fluorescein isothio-cyanate–conjugated secondary antibody. Cell images were observed under a fluorescent microscope.

Plasmids and transfections
MiaPaCa-2 cells were transfected with plasmid EF. hiCN1.CMV.GFP encoding the NICD or the empty vector.
Tumor, DiFiD (200 μmol/L) cells and after observing the presence of a palpable tumor, we were allowed to form tumors. One week following planting the compounds, DiFiD cells in the left and right flanks and allowed to form tumors. One week following planting the cells and after observing the presence of a palpable tumor, DiFiD (200 μg/kg body weight) in 5% Na₂HCO₃ buffer was administered intraperitoneally daily for 23 days. Tumor size was measured weekly. At the end of treatment, the animals were euthanized, and the tumors were removed and weighed for use in histology and gene expression studies.

Results

DiFiD inhibits pancreatic cancer cell proliferation

Curcumin is known to induce apoptosis of cancer cells, but the need for the high dose raises the question of in vivo bioavailability (23). Accordingly, we generated a novel compound, DiFiD. We first determined the effect of DiFiD on proliferation of 4 pancreatic cancer cell lines (Fig. 1A). DiFiD significantly suppressed the proliferation of these pancreatic cancer cells in a dose- and time-dependent manner. This antiproliferation effect on tumor cells was seen within 24 hours at a dose of 1 μmol/L, which continued to significantly increase over the next 72 hours (Fig. 1B). Similar results were obtained with colon, breast, lung, esophageal, and gastric cancer cells (data not shown). Furthermore, the compound had much higher potency in inhibiting proliferation than the parent compound. In contrast, DiFiD did not affect the proliferation of normal mouse embryonic fibroblasts (MEF) even when treated at 5 μmol/L (Fig. 1C). As a positive control, hydrogen peroxide, a known inducer of apoptotic cell death, was used. Treatment with hydrogen peroxide significantly affected the proliferation of the MEFs (Fig. 1C). These data suggest that DiFiD is not toxic to normal cells. To determine the long-term effect of DiFiD treatment, cells were treated with DiFiD for 24 hours, following which they were allowed to grow in normal medium. DiFiD treatment suppressed colony formation in all the pancreatic cancer cells (Fig. 1D), suggesting that DiFiD-mediated effects on the tumor cells are irreversible.

DiFiD induces cell-cycle arrest and apoptosis

Given its effects on cell proliferation, we next conducted cell-cycle analysis to further characterize the effects of DiFiD. At 24 hours, DiFiD (1 and 2.5 μmol/L) induced growth arrest of MiaPaCa-2 cells at the G₂-M and S-phase (Fig. 2A). At 48 hours, there was a significant increase of cells in the G₀ hypodiploid/fragemented DNA stage (data not shown). Similar results were observed in Pan02 cells (data not shown). Suppression of colon formation following treatment suggested that the compound was killing the cells. We therefore determined whether cell death was occurring through the apoptotic pathway. Caspase-3 and caspase-7 are key effector proteins in the apoptosis pathway involved in amplifying the signal from initiator caspsases, such as caspase-8 and caspase-9 (24, 25). Increased activation of caspase-3 and caspase-7 was observed within 24 hours in BxPC-3 and MiaPaCa-2 cells treated with 1 μmol/L DiFiD (Fig. 2B). This was further confirmed by Western blot analyses of MiaPaCa-2 cell lysates, which showed a significant increase in activated caspase-3 in cells treated with 1μmol/L DiFiD (Fig. 2C). In addition, 1μmol/L DiFiD inhibited the expression of antiapoptotic genes Bcl-2 and Bcl-xL protein while increasing the expression of apoptosis-promoting Bax protein (Fig. 2D). These data suggest that even at a dose of 1 μmol/L, DiFiD is a potent inducer of apoptosis of pancreatic cancer cells.

DiFiD affects cell-cycle-related proteins

To further characterize the S-phase arrest, we examined the level of expression of several known S-phase cell-cycle regulatory factors. Consistent with cell-cycle arrest, the expression of cyclin A and D1 was found to be decreased whereas p21 expression was increased (Fig. 3A and B), suggesting the mechanistic roles of these molecules during DiFiD-induced cell-cycle progression and cell-cycle arrest by DiFiD. This observation suggests that the S-phase arrest by DiFiD is, in part, due to profound alterations in the expression of positive and negative regulatory cell-cycle–related proteins. Cyclin D1 overexpression has been linked to the development and progression of cancer. It is a cell-cycle regulatory protein that regulates the G₁ to S-phase transition of the cell cycle and functions as a cofactor for several
transcription factors (26). However, MiaPaCa-2 cells treated with DiFiD resulted in reduced cyclin D1 expression at 24 hours (Fig. 3A and B). Furthermore, cyclin A2, which regulates S–G2 progression, was downregulated potentially slowing progression of cells out of S-phase.

DiFiD inhibits the expression of cancer-promoting genes

COX-2, a key rate-limiting enzyme in prostaglandin synthesis, is overexpressed in many cancers. It plays a significant role in carcinogenesis, including increased invasiveness, promotion of angiogenesis, and resistance...
Previous studies have shown increased COX-2 levels in pancreatic adenocarcinomas (28). Therefore, we next determined the effects of DiFiD treatment on COX-2 expression. DiFiD treatment significantly reduced COX-2 mRNA and protein levels in MiaPaCa-2 cells (Fig. 3A). Prostaglandins and the other tumor promoters are known to induce the expression of VEGF and IL-8 in epithelial cells, thereby promoting angiogenesis and hence tumor growth (29). VEGF and IL-8 are known potent inducers of capillary growth into the tumor, and without angiogenesis, tumor growth normally stops at a diameter of about 1 to 2 mm (30, 31). Hence, we also determined the effect of DiFiD on the expression of these 2 genes. Both VEGF and IL-8 mRNA expression and protein expression were significantly reduced in MiaPaCa-2 cells (Fig. 3C and D). Similar results were obtained with other pancreatic cancer cells (data not shown).

**DiFiD inhibits Notch activation by downregulating the γ-secretase complex**

Notch-1 is a cell membrane–associated protein. Ligand engagement causes NICD to be cleaved from the membrane through the action of the γ-secretase complex. NICD translocates to the nucleus, where it associates with a family of DNA-binding proteins to activate transcription of Notch target genes such as hairy and enhancer-of-split 1 (Hes1; ref. 32). We determined the effect of DiFiD on Notch-1, its ligand Jagged-1, and Hes-1 in MiaPaCa-2 cells. Both Notch-1 and Jagged-1 were downregulated by DiFiD in MiaPaCa-2 cells within 24 hours at the mRNA and protein levels (Fig. 4A and B). Protein levels were confirmed by immunofluorescence staining, where significantly lower levels of nuclear Notch-1 and cytoplasmic Jagged-1 were observed in the DiFiD-treated cells. Lysates from MiaPaCa-2 cells incubated with 1 μmol/L DiFiD were analyzed by Western blotting for caspase-3 protein levels, using rabbit anti-caspase-3 antibody. DiFiD-treated cells show cleaved (activated) caspase-3, whereas untreated cells have no cleaved caspase-3. D, DiFiD reduces expression of antiapoptotic proteins Bcl-2 and Bcl-xL in treated cells when compared with untreated cells. Lysates from MiaPaCa-2 cells incubated with 1 μmol/L DiFiD were analyzed by Western blotting for Bcl-2, Bcl-xL, and Bax proteins. Bcl-2 and Bcl-xL expression was reduced, whereas Bax expression was increased following DiFiD treatment.
nicastrin, Pen2, and Aph1, are all thought to be essential for activity (19, 33, 34). The catalytic domain resides within presenilin, whereas nicastrin has been suggested to be critical for substrate recognition (35). DiFiD treatment resulted in significant downregulation in the expression of 2 γ-secretase complex proteins, presenilin and nicastrin (Fig. 4D). This was at both the mRNA and protein levels. In addition, treatment with combination of DiFiD with a γ-secretase complex inhibitor DAPT further inhibits proliferation and induces apoptosis (Supplementary Fig. S1B). Effect of the combination on the inhibition of Notch activity was confirmed by the reduced expression of Hes-1 protein (Supplementary Fig. S1A). These data suggest that DiFiD-mediated downregulation of the Notch signaling pathway occurs in part through the inhibition of the γ-secretase complex.

**Ectopic expression of NICD protects DiFiD-mediated inhibition of proliferation and induction of apoptosis**

We next determined whether lack of Notch-1 activation is the reason for reduced growth of pancreatic cells, we expressed the intracellular domain NICD in MiaPaCa-2 and BxPC-3 cells. Western blot analyses of extracts from MiaPaCa-2 cells showed increased expression of Hes-1 following ectopic expression of NICD (Supplementary Fig. S1C). Furthermore, although DiFiD alone inhibited the basal levels of Hes-1 expression, NICD rescued this inhibition resulting in increased Hes-1 expression. Moreover, ectopic expression of NICD reversed DiFiD-mediated inhibition of MiaPaCa-2 cell proliferation and induction of apoptosis (Supplementary Fig. S1D). Similar results were obtained in BxPC-3 cells (data not shown).

**DiFiD inhibits tumor growth and angiogenesis**

To evaluate the role of DiFiD on tumor growth in vivo, we next examined the ability of the compound in suppressing the growth of mouse pancreatic cancer cell xenografts. Pancreatic cancer cell xenografts were allowed to develop and grow for 1 week, following which DiFiD was administered intraperitoneally daily for 3 weeks. Treatment with DiFiD significantly inhibited the growth of the tumor xenografts (Fig. 5A). The excised tumors from control animals ranged from 700 to 800 mg, whereas those treated with DiFiD weighed less than 300 mg (Fig. 5B). In addition, tumor volume was significantly decreased (Fig. 5C). There was no apparent change in liver weight, spleen weight, or body weight in the animals (data not shown). These data imply that DiFiD is a potential therapeutic agent for treating
pancreatic cancers but is relatively nontoxic to the mice. We also determined the effect of DiFID on tumor vascularization by staining for the endothelial-specific antigen CD31. As shown in Fig. 5D, DiFID treatment leads to a significant reduction in CD31 staining and to the obliteration of the normal vasculature that is associated with tumor angiogenesis. We also calculated the microvessel density and found it to be significantly decreased following DiFID treatment (Fig. 5D).

DiFID inhibits the expression of cancer and angiogenesis-related genes and Notch-1
Given that DiFID affected COX-2 expression in cells in culture, we next determined the effects of treatment on COX-2 expression. Both COX-2 mRNA expression and protein expression were significantly lower in DiFID-treated tumor xenografts than in the control tumors (Fig. 6A and B). Immunohistochemistry showed diffuse cytoplasmic staining for COX-2 in the epithelial cells of the control tumors, with accentuated staining in...
subepithelial myofibroblasts (Fig. 6C). COX-2 staining was significantly reduced in both the epithelial cells and myofibroblasts in DiFiD-treated tumors.

We also determined the effect of DiFiD on VEGF and cyclin D1 expression. Both VEGF mRNA expression and protein expression were significantly lower in DiFiD-treated tumor xenografts than in controls (Fig. 6A and B). Immunohistochemistry further showed that DiFiD treatment significantly reduced VEGF staining (Fig. 6C). DiFiD treatment also resulted in decreased cyclin D1 expression in the tumor xenografts (Fig. 6A–C). To further investigate whether DiFiD could downregulate Notch-1 in vivo, we examined the Notch-1 expression in tumor tissues obtained from control and DiFiD-treated tumor mice. DiFiD treatment resulted in significantly lower levels of Notch-1 than in control untreated tumors, suggesting that DiFiD could downregulate Notch-1 in vivo (Fig. 6A–C). There was also a significant reduction in the expression of γ-secretase complex proteins, presenilin and nicastrin, as well as the downstream target gene Hes-1 (Fig. 6B).

**Discussion**

Our results indicate that DiFiD possesses great potential as a promising antipancreatic cancer therapeutic agent. Pancreatic cancer is one of the most lethal cancers and has emerged as a leading cause of cancer-related deaths in the Western world, with most patients dying within 1 year of diagnosis. The significant morbidity, apparent toxicity, and poor response rates of current chemotherapy regimens have led to searches for less toxic alternative therapies. The data presented in the article show that DiFiD inhibits the proliferation of pancreatic cancer cells and induces cell-cycle arrest and apoptosis, resulting in reduced colony formation. These results were also replicated in vivo, where DiFiD decreased tumor growth and microvessel formation. Consistent with these findings, we observed reduced expression of the angiogenesis-inducing proteins VEGF and IL-8.

DiFiD treatment resulted in downregulation of Notch signaling through the inhibition of the γ-secretase complex. DiFiD also inhibited the expression of a downstream target for Notch-1, the Hes-1 gene. Recently, it has...
been reported that the Notch pathway plays a critical role in the processes of tumor cell proliferation and apoptosis in pancreatic cancer (18). Therefore, DiFiD-mediated cell growth inhibition could be partly mediated via inactivation of Notch-1 activity. This was further confirmed by the combination of a GSI and DiFiD, which further inhibited proliferation and induced apoptosis. However, ectopic expression of NICD reversed the effects of DiFiD and partially restored cell growth. Similarly, although the combination of DiFiD with a GSI further inhibited Hes-1 expression, the ectopic NICD partially rescued Hes-1 expression. However, Notch-1 is not the only pathway active in pancreatic cancer, as many other cellular pathways are activated (36, 37). It would be interesting to determine whether DiFiD is equally potent in inhibiting these other signal transduction pathways.

Although curcumin can effectively inhibit the growth of pancreatic cancer cells, its low bioavailability in vivo means higher doses are required for effective treatment or prevention (38, 39). Hence, more potent and soluble curcumin analogues are being developed (14–16). DiFiD is a novel derivative that interferes with the progression of cancer by disrupting many of the characteristic cancer-promoting events. Moreover, in cell-cycle analyses, DiFiD was observed to increase the number of cells in the G2–M phase after 24 hours. At the same time, there are significantly higher levels of apoptosis. These data imply that DiFiD treatment leads to mitotic catastrophe in which the proliferating cancer cells undergo cell death but not necessarily an arrest in the G2–M phase of the cell cycle. This was further supported through our observation that the majority of cells were present in

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Figure 6. DiFiD suppresses cancer-promoting genes and Notch-1. A, total RNA from Pan02 tumor xenografts were subjected to RT-PCR. DiFiD treatment resulted in reduced expression of COX-2, VEGF, cyclin D1, and Notch-1 mRNA levels when compared with control Pan02 tumor xenografts (*, P < 0.05). B, Western blot analysis showed that DiFiD-treated animals have significantly lower levels of COX-2, VEGF, cyclin D1, cleaved Notch-1, Jagged-1, Hes-1, presenilin 1, and nicastrin proteins. C, immunohistochemistry showed that DiFiD treatment results in significantly reduced expression of COX-2, VEGF, cyclin D1, and Notch-1 in the tumor xenografts. Representative photographs, magnification ×400.
the sub-G₀ phase at 48 hours following treatment. Additional studies are of course necessary to determine whether indeed there is mitotic catastrophe happening as a result of DiFiD treatment. This would include effects on cyclin B1 and cdc2, proteins that are abnormally activated during mitotic catastrophe (40). The role of checkpoint kinases Chk1/Chk2 and microtubule assembly also needs to be determined, and these studies are in progress.

In our studies, we observed marked suppression of tumor growth in mouse xenografts with DiFiD treatment. Further studies are needed to extend these findings before initiating clinical trials for pancreatic cancer. Specifically, absorption and pharmacokinetic activity are needed; nevertheless, preliminary studies in this article suggest that DiFiD does not have any toxicity in liver, kidney, and spleen at the levels tested and allows the mice to maintain normal weight gain (data not shown). In addition, DiFiD seems to mediate its actions through multiple molecular targets, including COX-2, VEGF, IL-8, and Notch-1. Because COX-2 overexpression during pancreatic carcinogenesis causes resistance to apoptosis (41), treatment of pancreatic cancer cells with DiFiD may potentially restore susceptibility to apoptosis. Furthermore, overexpression of IL-8 plays an important role in tumor angiogenesis and contributes significantly to the aggressive biology of human pancreatic cancer (42, 43), so treatment with DiFiD may also potentially inhibit angiogenesis and decrease the aggressive behavior of the pancreatic cancer. Finally, VEGF is important in angiogenesis and promotion of tumor growth in many cancers including pancreatic cancer. VEGF and its receptors are overexpressed in pancreatic cancer (44). The ability of DiFiD to inhibit VEGF expression is yet another molecular mechanism by which DiFiD may function to prevent pancreatic cancer. In fact, previous studies have shown that downregulation of Notch-1 or Jagged-1 leads to decreased expression and the activity of NF-κB transcription factor (37). Indeed, COX-2, VEGF, and IL-8 are targets for NF-κB–mediated transcription. Furthermore, the Notch ligand Jagged-1 has been shown to be a proangiogenic regulator (45, 46). DiFiD treatment reduced Jagged-1 expression. Therefore, DiFiD-mediated suppression of cell growth may, in part, be due to loss of Notch-1–mediated activation of COX-2, VEGF, and IL-8 expression through NF-κB–mediated transcriptional activity or through downregulation of Jagged-1 expression. Further studies in these directions are currently being explored.

In conclusion, our studies show that DiFiD treatment of pancreatic cancer cells results in growth inhibition in vitro and in vivo. It should be noted, however, that the drug seems to do multiple things and is not clear which one is key for the antitumor effects. Although Notch is a target, there are also cell-cycle blockade and inhibition of angiogenesis. Clearly, more detailed mechanistic work is needed. Given the broad effects, this may be an agent for which system biology approaches such as gene expression profiling before and after treatment with pathway analyses may provide clues, which is a focus of our future studies. Nevertheless, given the observation that DiFiD does not affect proliferation of normal cells strongly suggests that DiFiD has promising potential for use as a therapeutic or chemopreventive agent for pancreatic cancer as well as other cancers and inflammatory disease states.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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