CDK-4 inhibitor P276 sensitizes Pancreatic Cancer cells to Gemcitabine induced Apoptosis

Dharmalingam Subramaniam$^{1,2}$, Giridharan Periyasamy$^3$, Sivapriya Ponnurangam$^1$, Debarshi Chakrabarti$^4$ Aravind Sugumar$^{2,5}$, Muralidhara Padigaru$^4$, Scott J. Weir$^{2,6}$, Arun Balakrishnan$^3$, Somesh Sharma$^{3,4}$ and Shrikant Anant$^{1,2,5}$

Departments of $^1$Molecular and Integrative Physiology, the $^2$KU Cancer Center, $^5$Division of Gastroenterology, Department of Internal Medicine, and $^6$Department of Pharmacology and Toxicology, University of Kansas Medical Center, Kansas City, KS 66160, USA. $^3$Department of High Throughput Screening, and the $^4$Department of Translational Research, Piramal Life Sciences Limited, Mumbai, 400 063, India.

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2. Full name, mailing address, and e-mail address (optional) of the person to whom reprint requests should be sent: Shrikant Anant, University of Kansas Medical Center, 3901 Rainbow Boulevard, Mail Stop 3040, Kansas City, KS 66160. Fax: 913-945-6327; E-mail: sanant@kumc.edu

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Abstract

Despite advances in molecular pathogenesis, pancreatic cancer remains a major unsolved health problem. It is a rapidly invasive, metastatic tumor that is resistant to standard therapies. The phosphatidylinositol-3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) signaling pathways are frequently dysregulated in pancreatic cancer. Gemcitabine (Gem) is the mainstay treatment for metastatic pancreatic cancer. P276 is a novel CDK inhibitor that induces G2/M arrest and inhibits tumor growth in vivo models. Here, we determined that P276 sensitizes pancreatic cancer cells to Gem induced apoptosis, a mechanism mediated through inhibition of Akt-mTOR signaling. In vitro, the combination of P276 and Gem resulted in a dose- and time-dependent inhibition of proliferation and colony formation of pancreatic cancer cells but not with normal pancreatic ductal cells. This combination also induced apoptosis, as seen by activated caspase 3 and increased Bax/Bcl2 ratio. Gene profiling studies demonstrated that this combination downregulated Akt-mTOR signaling pathway, which was confirmed by western blot analyses. There was also a downregulation of vascular endothelial growth factor (VEGF) and interleukin-8 expression suggesting effects on angiogenesis pathway. In vivo, intraperitoneal administration of the P276-Gem combination significantly suppressed the growth of pancreatic cancer tumor xenografts. There was a reduction in CD31 positive blood vessels, and reduced VEGF expression, again suggesting an effect on angiogenesis. Taken together, these data suggest that P276-Gem combination is a novel potent therapeutic agent that can target the Akt-mTOR signaling pathway to inhibit both tumor growth and angiogenesis.
Introduction

Pancreatic cancer is one of the fourth leading cause of adult cancer related deaths associated with a high mortality rate (1). The American Cancer Society have estimated that 43,920 new cases and 37,390 deaths would occur during 2012 (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 which is resistant to standard therapies (5, 6). At present, single agent based chemotherapy (e.g. Gemcitabine) is the mainstay treatment for metastatic adenocarcinoma of pancreas. Gemcitabine (Gem) is a deoxycytidine nucleoside analog that requires intracellular phosphorylation to get converted into its active triphosphate form. Gem exerts its cytotoxic effects by being incorporated into the DNA strand and inhibits DNA synthesis (7). Gem treatment has a tumor response rate of below 10%; similarly none of the available current chemotherapeutic agents have objective response rates of over 10% (3, 5). The magnitude of this problem mandates the need for novel therapeutic agents.

P276 is a flavone that inhibits cyclin-dependent kinases that has been recently reported as a novel antineoplastic agent (8, 9). The in vitro cellular potency, together with in vivo antitumor activity has been demonstrated for multiple myelomas (10). The mechanism of inhibition in multiple myeloma cells was determined to be through inhibition of cdk9 and RNA polymerase II dependent transcription (11, 12). P276 has also been demonstrated to inhibit transcriptional activation of HIF-1 and phosphorylation of Akt and 4E-BP1 in prostate cancer cells (12).

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays an important role in many biological processes including cell proliferation, differentiation and survival (13).
Deregulation of this pathway is a prominent characteristic of pancreatic cancer, and appears to play an important role in the aggressive nature of this disease including its resistance to chemotherapy (14-17). PI3K is activated upon growth factors binding to their cognate receptors. Activated PI3K leads to the activation of Akt by phosphorylation at Ser473 and Thr308 (17). Akt activates several downstream targets including mammalian target of rapamycin (mTOR) which plays a central role in cell proliferation. Deregulation of mTOR signaling occurs in several human tumors including pancreatic cancer (18, 17). mTOR associates with Raptor (mTORC1 complex) to phosphorylate p70 S6 kinase, which in turn phosphorylates 4E-BP1, leading to increased cell proliferation (19). In addition, mTOR associates with Rictor (mTORC2 complex) and functions in a feedback loop to phosphorylate and activate AKT at Ser473 (17).

In this article, we have determined the effect of P276-Gem combination on pancreatic cancer cells and identified that at least one mechanism of action for the compounds is through the inhibition of the Akt-mTOR signaling pathway.
Materials and Methods

Cells and reagents

Human pancreatic cancer cells PanC-1, MiaPaCa-2, AsPc-1 and BxPC-3 (all the cell lines obtained from American Type Culture Collection, at passage 4) were grown in RPMI 1640 containing 10% heat inactivated fetal bovine serum (Sigma-Aldrich) and 1% antibiotic and antifungal solution (Mediatech Inc) at 37°C in a humidified atmosphere of 5% CO2. HPNE cells were kindly provided by Dr. Anirban Maitra, Johns Hopkins University School of Medicine and grown in DMEM with 4.5g/L glucose, L-glutamine and Sodium Pyruvate (Mediatech Inc) with 5% FBS, 1X N2, 10ng/ml bFGF and 50 μg/ml Gentamycin. All the cell lines used in this study were within 20 passages after receipt or resuscitation (~3 months of non-continuous culturing). The cell lines were not authenticated as they came from national repositories. Gemcitabine-HC; was purchased from Eli Lilly Pharmaceuticals (Fig. 1A right panel). Piramal Life Sciences Limited, Mumbai, India, synthesized the P276 compound (Fig. 1A left panel), purity of 98.8% and dissolved in dimethyl sulphoxide (Sigma-Aldrich).

Proliferation and apoptosis assays

To assess proliferation, cells were seeded onto 96 well plates and grown overnight. Then, the cells were treated with increasing doses of Gem (0-100 nM) or P276 (0-1 μM) and their combinations in 10% FBS containing RPMI 1640 or DMEM with 4.5g/L glucose, L-glutamine and sodium pyruvate (Mediatech Inc) with 5% FBS, 1X N2, 10 ng/ml bFGF and 50 μg/ml Gentamycin. Analysis of cell proliferation was performed by hexoseaminidase enzymatic assay as described previously (20). For apoptosis, caspase 3/7 activity was measured using the Apo-one Homogeneous Caspase-3/7 Assay kit (Promega).
Colony formation assay

Briefly, 6 well dishes were seeded with 500 viable cells and allowed to grow for 24 h. The cells were then incubated in the presence or absence of Gem, P276 and their combinations for 24 h. The compound-containing the medium was then removed and the cells were washed in PBS and incubated for an additional 10 d in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min at room temperature and then washed with PBS followed by staining with Crystal violet. The colonies were counted and compared with untreated cells.

Cell cycle analyses

Cells were treated with Gem, P276 and its combinations for 12- and 24-h and subsequently trypsinized and suspended in phosphate buffered saline (PBS). Single-cell suspensions were fixed using 70% ethanol for 2 h, and subsequently permeabilized with PBS containing 1 mg/ml propidium iodide (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 10,000 events for each sample. Histograms were analyzed for cell cycle compartments and the percentage of cells at each phase of the cell cycle was calculated using CellQuest (Becton Dickinson) analysis software.

Real time reverse-transcription polymerase chain reaction analysis

Total RNA isolated from MiaPaCa-2 cells and tumor xenograft tissues using TRIZOL reagent and reverse transcribed with Superscript II reverse transcriptase in the presence of random hexanucleotide primers (Invitrogen). Complementary DNAs were then used for Real
Time PCR using Jumpstart Taq 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 are shown in Supplementary Table 1.

Microarray analysis

Aminosilane slides were procured from ArrayIT. A 36,685 70-meroligonucleotide library was purchased from Operon Biotechnologies. The oligonucleotides were robotically spotted on to the aminosilane slides using a robotic arrayer from Genomic Solutions Inc. Fluorescent labeling of total RNA was performed using the Chipshot Indirect labeling kit (Promega). Hybridization was performed using the GeneTAC Hybstation. The slides were scanned using the dual laser scanner (GeneTAC UC4) for Cy3 and Cy5 at constant power with auto PMT settings. The scanned slides were gridded using the GeneTAC Integrator software for generating the signal intensities for each spot. The array data were deposited into GEO (GSE36703).

Western blot analysis

Cell lysates were subjected to polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore). Antibodies were purchased from Cell Signaling Technology., Abcam Inc., Thermo Scientific., Upstate., and Santa Cruz Biotechnology Inc., and specific proteins were detected by the enhanced chemiluminescence system (GE Healthcare).
PanC-1 xenograft tumors in mice

Five-week-old male athymic nude mice (Charles River Laboratory) were utilized for in vivo experiments. They were maintained with water and standard mouse chow ad libitum, as per the approved protocol by the University’s Animal Studies Committee. Animals were injected with $1 \times 10^6$ PanC-1 cells in the left and right flank and allowed to form tumors. One week following implanting the cells, the animals were divided into four groups. Group 1: Control, Group 2: Gem-20mg/Kg body weight, Group 3: P276-10mg/Kg body weight, Group 4: P276-10mg/Kg body weight + Gem-20mg/Kg body weight. After observing the presence of a palpable tumor, these compounds were administered intraperitoneally every other day for up to the 7th week. Tumor size was measured weekly. On the day after the last administration of the drugs, the animals were euthanized and tumors were removed and weighed. The tissues were used for histology and gene expression studies.

Immunocytochemistry

Tissues were fixed in 10% buffered formalin and subsequently embedded in paraffin. For angiogenesis-related staining with CD31, the tissues were fixed in a zinc fixative instead of buffered formalin. Sections of 4 μM were cut, deparaffinized and treated with antigen decloaker for 15 minutes (Biocare Medical). Then, they were blocked with Avidin/Biotin for 20 min (Invitrogen). The slides were incubated with anti-VEGF, cyclin D1, CD31, pAKT, pmTOR or p70S6Kinase antibodies for overnight at 4 °C. Next, the slide was treated with broad-spectrum secondary antibody (Invitrogen) and HRP- conjugate for one hour and then developed with DAB (Invitrogen). Finally, the slides were counterstained with hematoxylin and mounted. The slides were examined in Nikon Eclipse Ti microscope under a 40X objective.
Statistical analysis

All values are expressed as the mean ± SEM. Data was analyzed using an unpaired 2-tailed t test. A $P$ value of less than 0.05 was considered statistically significant. In addition, one-way analysis of variance (ANOVA) was performed using Bonferroni corrections for multiple comparisons. To be considered significant, the $P$ value must be less than 0.017. The SPSS V17 statistical software was used for these analyses.
Results

Combination of P276 and Gem inhibits pancreatic cancer cell proliferation

Previous studies have demonstrated that P276 is a cyclin dependent kinase inhibitor inhibiting cancer cell growth (9). Given that P276 can reduce the growth of cancer cells, we sought to determine its effect on pancreatic cancer cells when combined with Gem. First, we determined the effect of the combination on proliferation of normal human pancreatic ductal epithelial cells. The combination of up to 1 μM P276 and 100 nM Gem did not affect proliferation of these normal cells (Fig. 1B). In contrast, the P276-Gem combination significantly suppressed proliferation of pancreatic cancer cell lines in a dose and time dependent manner (Fig. 1C upper and lower panel). This anti-proliferation effect on tumor cells was seen within 24 h, which continued to significantly increase over the next 72 h (Fig. 1C upper and lower panel). To determine the long-term combination effect of P276-Gem treatment, cells were incubated with P276 and Gem for 24 h, washed with media and allowed to grow in normal medium for a week. There were lower numbers of colonies in all four of the MiaPaCa-2, PanC-1, AsPC-1 and BxPC-3 cells in the combination treatment when compared to cells treated with either P276 or Gem alone (Fig. 1D left and right panel). These data suggest that combination of P276 and Gem treatment suppresses growth of pancreatic cancer cells in culture.

The P276-Gemcitabine combinations induces apoptosis

Given its effects on cell proliferation, we next performed cell cycle analysis to further characterize P276-Gem’s combination effect. At 12 h, P276-Gem combination induced cell death in both PanC-1 and MiaPaCa-2 cells (Fig. 2A upper and lower panel). At 24 h, there was a significant increase of cell death in both MiaPaCa-2 and PanC-1 cells (data not shown).
Suppression of colony formation following treatment further confirmed that the compounds were killing the cells. We therefore determined the mechanism of cell death. Caspase-3 and caspase-7 are key effector proteins in the apoptosis pathway involved in amplifying the signal from initiator caspases, caspase-8 and caspase-9 (21, 22). Increased activation of caspase-3/7 was observed within 24 h in both PanC-1 and MiaPaCa-2 cells treated with P276-Gem combination (Fig. 2B). This was further confirmed by western blot analyses of PanC-1 and MiaPaCa-2 cell lysates, which showed a significant increase in activated caspase-3 in cells treated with combinations of P276 and Gem (Fig. 2C right and left panel). In addition, P276 and Gem combination inhibited the expression of anti-apoptotic genes Bcl-2 and Bcl-xL protein while increasing the expression of apoptosis-promoting Bax protein (Fig. 2D right and left panel). These data suggest that combination of P276 and Gem is a potent inducer of apoptosis of pancreatic cancer cells.

**The P276-Gem combination affects Akt/mTOR signaling**

To further characterize the mechanism of cell death, we performed a microarray analysis using a oligonucleotide array. The combination of P276 and Gem showed an effect on the Akt and mTOR pathways while inducing apoptosis pathway transcripts (Fig. 3A, B). We further confirmed that the Akt-mTOR pathway is affected by western blot analyses. The P276-Gem combination downregulated phosphorylation of both Akt and mTOR proteins (Fig. 3C). Furthermore, the P276-Gem combination reduced TSC1/2 and eIF4E protein levels (Fig. 3C). These data suggest that P276-Gem combination significantly affects Akt and mTOR pathway thereby decreasing the growth of pancreatic cancer cells.
The P276-Gem combination affects cell cycle related proteins

To further characterize the cell death in the cell cycle, we examined the level of expression of several known cell cycle regulatory factors. Consistent with cell cycle arrest, the expression of cyclin D1 and CDK4 were decreased in both MiaPaCa-2 and PanC-1 cell lines (Fig. 4A). Cyclin D1 overexpression has been linked to the development and progression of many cancers. 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. However, MiaPaCa-2 and PanC-1 cells treated with P276-Gem combination resulted in reduced cyclin D1 expression at 24 h (Fig. 4A).

The P276-Gem combination inhibits expression of cancer-promoting genes

Cyclooxygenase-2 (COX-2), a key rate-limiting enzyme in prostaglandin synthesis is overexpressed in many cancers. COX-2 plays a significant role in increased invasiveness, promotion of angiogenesis and resistance to apoptosis (23). Previous studies have demonstrated increased COX-2 levels in pancreatic adenocarcinomas (24). Therefore, we next determined the effects of the P276-Gem combination on COX-2 expression. While both compounds were able to reduce expression of COX-2, there was further reduction in the combination treatment in MiaPaCa-2 cells (Fig. 4B). This was even more pronounced in western blot analysis where COX-2 expression was not observed following the combination treatment (Fig. 4C). Prostaglandins and the other tumor promoters are known to induce the expression of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in epithelial cells, thereby promoting angiogenesis and hence tumor growth (25). 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 (26). Previous studies have demonstrated that prostaglandins and
the other tumor-promoting mediators are known to induce the expression of VEGF and IL-8 in epithelial cells (27). Hence, we also determined the effect of P276-Gem combination on the expression of these two genes. Both VEGF and IL-8 mRNA and protein expression were significantly reduced in MiaPaCa-2 cells (Fig. 4B, C). Similar results were obtained with other pancreatic cancer cells (data not shown).

The P276-Gem combination inhibits tumor growth and angiogenesis

To evaluate the role of P276-Gem combination on tumor growth in vivo, we next examined its effects on growth of pancreatic cancer cell xenografts. PanC-1 pancreatic cancer xenograft tumors were allowed to develop and grow for one week following which either P276 or Gem alone and its combination was administered intraperitoneally every other day for up to the 7th week (Fig. 5A). While treatment with either P276 or Gem inhibited the growth of the tumor xenografts, there was an enhanced effect in response to P276-Gem combination (Fig. 5C). The excised tumors from control animals weighed ~2000 mg. Tumors from animals those treated with either P276 or Gem alone weighed ~1000 mg and ~800 mg, respectively. However, the tumors from animals treated with the P276-Gem combination weighed <500 mg suggesting a significant effect when the animals were treated with the combination of drugs (Fig. 5B). In addition, tumor volume was significantly decreased (Fig. 5C). There was no apparent change in liver, spleen, or body weight in the animals (data not shown). These data imply that the P276-Gem is a potential therapeutic combination for treating pancreatic cancers but is relatively non-toxic to the animals. Given the effect of the combination on VEGF expression in vitro, we also determined the effect of the combination on tumor vascularization. Treatment with the P276-Gem combination leads to a significant reduction in endothelial-specific antigen CD31 (Fig. 5D).
Moreover, the P276-Gem combination shows a reduction in normal vasculature, when compared with either P276 or Gem alone (Fig. 5D).

**P276 and Gem combination inhibits the expression of cancer and angiogenesis-related genes and Akt-mTOR signaling proteins**

Given that VEGF expression was inhibited in the cells and the xenografts also had lower levels of microvasculature when they were treated with the combination of compounds, we investigated whether VEGF expression is affected in the tumor xenograft tissues. VEGF levels were significantly reduced in the tumor tissues treated with the combination of P276 with Gem, when compared to controls or either P276 or Gem alone (Fig. 6A). Cyclin D1 protein levels were also significantly lower in P276-Gem combination treated tumor xenografts (Fig. 6A). We also examined the P276, Gem effects on Akt and mTOR signaling in the tumor tissues. Treatment with the P276-Gem combination also resulted in significant downregulation in the phosphorylation of Akt and mTOR when compared to control untreated tumors (Fig. 6B). Further confirmation of the downregulation of the proteins was obtained by immunohistochemistry for the proteins in the xenograft tissues (Fig. 6C). These data suggest that the P276-Gem combination significantly affects expression of cancer promoting genes and phosphorylation of Akt and mTOR, which potentially contributes to the inhibitory effects of the treatment.
Discussion

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 one 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. Occurrence of several genetic abnormalities with very high frequency in pancreatic cancer includes: K-Ras mutation, loss of p16, p53 and DPC4 (deleted in pancreatic cancer, locus 4) function, and overexpression of multiple receptor tyrosine kinases (28, 29). These alterations also upregulate PI3K/AKT activity, a process that has been shown to stimulate proliferation, enhance survival-related response including drug resistance. Therefore, therapeutic targeting of the PI3K pathway with its downstream targets Akt and mTOR at multiple molecular levels may provide better antitumor effects than selective inhibition of only one component of the pathway. Our results indicate that the P276-Gem combination possesses potential as a promising therapeutic agent against pancreatic cancers.

Combining conventional cytotoxic drugs such as Gem with novel targeted agents that specifically interfere with key operational pathways responsible for pancreatic cancer progression has recently gained much attention in an effort to identify novel and effective treatments for pancreatic cancer. Studies presented here demonstrate that the combination of P276 and Gem can lead to significantly inhibiting proliferation of pancreatic cancer cells, while inducing cell death and apoptosis, resulting in reduced colony formation. More importantly, these results were also replicated in vivo, where the combination of P276 and Gem decreased tumor growth and microvessel formation. Consistent with these findings, we observed reduced
expression of the angiogenesis-inducing proteins VEGF and IL-8 which makes such a combination approach with these agents a plausible choice for future clinical applications.

The high frequency of aberrant PI3K/AKT signaling occurs in pancreatic cancer, previous studies have demonstrated that PI3K inhibition enhances Gem induced apoptosis in human pancreatic cancer cells (30), and improves Gem activity in orthotopic human pancreatic cancer xenografts (31,32). Recent studies have also reported that the therapeutic targeting of the PI3K/AKT/mTOR network at multiple molecular levels rather than selective inhibition of only one component of the pathway will avoid PI3K/AKT pathway reactivation (17, 33). Most recent studies in prostate cancer demonstrated that P276 inhibits phosphorylation of Akt and 4E-BP1 and abrogated expression of HIF-1-inducible gene viz. vascular endothelial growth factor (11). Our studies demonstrate that P276-Gem combination treatment effectively downregulated Akt (Ser473) and mTOR (Ser248) phosphorylation. Expression of downstream mTORC1 target proteins phospho-p70 S6K (Thr389) and phospho-4E-BP1 (Thr37/46) were also significantly decreased by the P276-Gem combination. These findings suggest that P276-Gem combination downregulates the functions of Akt, mTORC1 and mTORC2. P276-Gem combination treatment decreased cell proliferation and induced apoptosis in pancreatic cancer cell lines indicating that the in vivo antitumor effects of P276-Gem combination may be due to induction of apoptosis, in addition to its anti-angiogenic properties.

In our studies, we observed marked suppression of tumor growth in mouse xenografts with P276-Gem combination treatment and no apparent toxicity in liver, kidney, and spleen. Also, the treatment allowed the mice to maintain normal weight gain (data not shown).
addition, P276-Gem combination treatment seems to mediate its actions through multiple molecular targets, including COX-2. Because COX-2 overexpression during pancreatic carcinogenesis causes resistance to apoptosis (34, 35), treatment of pancreatic cancer cells with P276-Gem combination may potentially restore susceptibility to apoptosis due to downregulation of COX-2. Furthermore, overexpression of IL-8 plays an important role in tumor angiogenesis and contributes significantly to the aggressive biology of human pancreatic cancer (36-39). Therefore, treatment with P276-Gem combination 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 (40, 41). The ability of P276-Gem combination to inhibit VEGF expression is yet another molecular mechanism by which P276-Gem combination may function to prevent pancreatic cancer.

In conclusion, our studies show that treatment of pancreatic cancer cells with the P276-Gem combination results in growth inhibition in vitro and in vivo. However, the drug seems to have multiple targets and is not clear which one is key for the antitumor effect. While PI3K/Akt is a target, there are also cell cycle blockade and inhibition of angiogenesis. Clearly, more detailed mechanistic work is needed. The fact that the P276-gemictabine combination does not affect proliferation of normal pancreatic ductal epithelial cells strongly suggests that the combination has promising potential for use as a therapeutic option for metastatic pancreatic cancer as well as other cancers.
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Figure legends

Figure 1. Combination of P276 and Gem inhibits pancreatic cancer cell proliferation. A, molecular structures of P276 (Ar → generic structure) (left panel), and gemcitabine (right panel). B, proliferation of human normal pancreatic ductal epithelial cells is not affected by the combination of P276 and Gem (Gem). Cells were incubated with increasing doses of P276 (0-1 μM) and Gem (0-100 nM) for up to 48 h and analyzed for cell proliferation. Data shows that the combination of these two compounds do not affect proliferation of normal cells. C, P276-Gem combination inhibits proliferation of pancreatic cancer cells. PanC-1, MiaPaCa-2, AsPC-1 and BxPC-3 cells were incubated with increasing doses of P276 (0-1 μM) and Gem (0-100 nM) for up to 72 h. Treatment with the combination of P276 and Gem resulted in a significant dose- and time-dependent decrease in cell proliferation in all four cell lines when compared with untreated controls (*P <0.05). D, P276-Gem combination inhibits clonogenicity. PanC-1, MiaPaCa-2, AsPC-1 and BxPC-3 cells were incubated with P276 (500 nM) or Gem (10 nM) alone or in combination for 24 h and allowed to grow into colonies for 10 d. The combination treatment completely inhibits colony formation. Results are representative of three independent experiments.

Figure 2. The P276/Gem combination induces cell death by apoptosis. A, Cell cycle analyses of the combination of P276 and Gem treated cells. Panc-1 and MiaPaCa-2 cells were treated with either P276 (1 μM) or Gem (100 nM) alone and its combination for 12 h and examined by flow cytometry following propidium iodide staining for DNA content. The P276-Gem combination treatment induced cell death. Graphs are representative of data collected from three experiments. B, The P276-Gem combination induces caspase 3/7 activity. PanC-1 and
MiaPaCa-2 cells were incubated with P276 (1μM) and Gem (100 nM) for 24 h and analyzed for caspase 3/7 activity. P276-Gem combination increased the number of apoptotic cells compared to either P276 or Gem alone and untreated controls (*P <0.05). C, The P276-Gem combination activates caspase 3, an apoptosis mediator. Lysates from MiaPaCa-2 and PanC-1 cells incubated with the P276-Gem combination were analyzed by western blotting for caspase 3 protein levels using a rabbit anti-caspase 3 antibody. P276 and the combination treated cells show cleaved (activated) caspase 3. D, P276-Gem combination reduces expression of anti-apoptotic proteins Bcl-2 and BclXL in treated cells when compared to untreated cells. Lysates from MiaPaCa-2 and PanC-1 cells were analyzed by western blotting for Bcl-2, Bcl-xL, and Bax proteins. Both Bcl-2 and Bcl-xL were reduced while Bax expression was increased following P276-Gem combination treatment.

Figure 3. Combination of P276 and Gem affects Akt and mTOR pathway. A, microarray analysis. Total RNA from PanC-1 cells treated with either P276 (1 μM) or Gem (100 nM) alone and its combination for 12 h was hybridized with a 33,631 oligonucleotide array. B, Pathway analysis of microarray data. Data shows significant effect on the PI-3 kinase (PI3K) pathway. C, Lysates from PanC-1 cells was analyzed by western blotting for the PI3K/Akt-mTOR pathway. P276-gemictabine combination treatment caused significant downregulation in the phosphorylation of Akt, mTOR, and p70 S6 kinase. In addition, total eiF4E levels were reduced.

Figure 4. Combination of P276 and Gem affects expression of cell cycle related proteins and cancer-promoting genes. A, western blotting. Lysates from MiaPaCa-2 and PanC-1 cells were analyzed by western blotting for CDK4, CDK6 and cylinD1 expression levels. P276-Gem
combination caused a significant reduction CDK4 and cyclinD1 levels, there is no significant change in CDK6 expression. A, Real Time PCR analysis. Total RNA from MiaPaCa-2 cells treated with P276-Gem combination for 24 h was subjected to Real Time PCR analyses. The P276-Gem combination showed significant reduction in the expression of COX-2, VEGF, and IL-8 mRNA (*P <0.05). Data from three independent experiments. D, Western Blotting. Lysates from MiaPaCa-2 cells were analyzed by western blotting for COX-2, VEGF and IL-8 expression levels. P276-Gem combination caused a significant reduction of all three proteins.

Figure 5. Combination of P276 and Gem inhibits growth of PanC-1 tumor xenografts. A, Experimental Plan: PanC-1 cells were injected in to the flanks of nude mice and palpable tumors were allowed to develop for 7 d. Subsequently either P276 and Gem were injected every other day intraperitoneally for up to 7 weeks. Tumor size was measured every week. On the day after the final injection, mice were euthanized and tumors were excised. B, P276-Gem combination resulted in significantly lower tumor weight when compared to controls or either P276 or Gem alone (*P <0.05). C, Tumor volumes in P276 or Gem administered mice were smaller than that of control mice (*P <0.05). D, Tumor sections were stained for CD31, an endothelial cell specific surface marker and the vessel areas were counted. A representative figure is presented showing significant reduction in microvessels (400 X).

Figure 6. Combination of P276 and Gem suppresses cancer promoting genes and Akt/mTOR pathway. A, Western blot analyses show that P276-Gem treated animals have significantly lower levels of VEGF and cyclin D1 proteins. B) Western blot analyses show that P276-Gem treated tumors have significantly down-regulated the phosphorylation of Akt, mTOR,
and P70S6 kinase. C, Immunohistochemistry demonstrates that P276-Gem treatment results in significantly reduced expression of VEGF, cyclin D1 and phosphorylated Akt, mTOR and p70S6 kinase in the tumor xenografts. Representative photographs are the magnification of X 400.
Figure 4

A

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<td>Gem</td>
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Cyclin D1  CDK4  CDK6  Actin
1 2 3 4  1 2 3 4

B

![Graph showing mRNA expression relative to control for COX-2, VEGF, and IL-8 with treatments P276, Gem, and P276+Gem.

C

COX-2  VEGF  IL-8  Actin
1 2 3 4

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Figure 5

A

Experimental plan

PanC-1 Cells

P276, Gem, P276+Gem

Every other day (i.p.)

Euth

(WEEKS) 1 2 3 4 5 6 7

B

Tumor weight (g)

<table>
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<tr>
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<tr>
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<td>1.5 *</td>
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<tr>
<td>Gem</td>
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C

Tumor volume (1 x 10^{2} mm^{3})

Treatment in weeks

D

Control  P276  GEM  P276+GEM

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Figure 6

(A) P276, Gem

- - + +

VEGF
Cyclin D1
Actin

1 2 3 4

(B) P276, Gem

- + + +

pAkt
Akt
pmTOR
mTOR
pP70S6 Kinase
P70S6 Kinase

1 2 3 4

(C) VEGF
Cyclin D1
pAkt
pmTOR
p70S6 Kinase

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Molecular Cancer Therapeutics

CDK-4 inhibitor P276 sensitizes Pancreatic Cancer cells to Gemcitabine induced Apoptosis

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