CDK-4 Inhibitor P276 Sensitizes Pancreatic Cancer Cells to Gemcitabine-Induced Apoptosis

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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/Akt and mTOR signaling pathways are frequently dysregulated in pancreatic cancer. Gemcitabine 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 gemcitabine-induced apoptosis, a mechanism-mediated through inhibition of Akt-mTOR signaling. In vitro, the combination of P276 and gemcitabine 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 showed that this combination downregulated Akt-mTOR signaling pathway, which was confirmed by Western blot analyses. There was also a downregulation of 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. Mol Cancer Ther; 11(7); 1598–608. ©2012 AACR.

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 has 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 that is resistant to standard therapies (5, 6). At present, single-agent based chemotherapy (e.g., gemcitabine) is the mainstay treatment for metastatic adenocarcinoma of pancre-

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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 mTOR that 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 FBS (Sigma-Aldrich) and 10 ng/mL bFGF and 50 μg/mL gentamicin. All the cell lines used in this study were within 20 passages after receipt or resuscitation (~3 months of noncontinuous 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 sulfoxide (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 gemcitabine (0–100 nmol/L) or P276 (0–1 μmol/L) and their combinations in 10% FBS containing RPMI 1640 or DMEM with 4.5 g/L glucose, L-glutamine, and Sodium Pyruvate (Mediatech Inc) with 5% FBS, 1 × N2, 10 ng/mL bFGF, and 50 μg/mL gentamicin. All the cell lines used in this study were within 20 passages after receipt or resuscitation (~3 months of noncontinuous 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 sulfoxide (Sigma-Aldrich).

Colony formation assay

Briefly, 6-well dishes were seeded with 500 viable cells and allowed to grow for 24 hours. The cells were then incubated in the presence or absence of gemcitabine, P276, and their combinations for 24 hours. The compound containing the medium was then removed and the cells were washed in PBS and incubated for an additional 10 days in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 minutes 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 gemcitabine, P276, and its combinations for 12 and 24 hours and subsequently trypsinized and suspended in PBS. Single-cell suspensions were fixed using 70% ethanol for 2 hours 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 PCR 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 reagent and reverse transcription was expressed as fold change relative to control. The scanned slides were gridded using the GeneTAC robotic arrayer from Genomic Solutions Inc. Fluorescent labeling of total RNA was carried out using the Chipshot Indirect labeling kit (Promega). Hybridization was carried out 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 PAGE 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 used 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 4 groups. Group 1: Control, group 2: Gem-20 mg/kg body weight, group 3: P276-10 mg/kg body weight, and group 4: P276-10 mg/kg body weight + gemcitabine-20 mg/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 µ were cut, deparaffinized, and treated with antigen decloaker for 15 minutes (Biocare Medical). Then, they were blocked with Avidin/Biotin for 20 minutes (Invitrogen). The slides were incubated with anti-VEGF, cyclin D1, CD31, pAKT, p90S6 kinase antibodies for overnight at 4°C. Next, the slide was treated with broad-spectrum secondary antibody (Invitrogen) and horse radish peroxidase conjugate for one hour and then developed with 3,3-diaminobenzidine (Invitrogen). Finally, the slides were counterstained with hematoxylin and mounted. The slides were examined in Nikon Eclipse Ti microscope under a 40× objective.

Statistical analysis

All values are expressed as the mean ± SEM. Data were analyzed using an unpaired 2-tailed t test. A *P* value of less than 0.05 was considered statistically significant. In addition, one-way ANOVA was done using Banferroni 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 shown 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 gemcitabine. First, we determined the effect of the combination on proliferation of normal human pancreatic ductal epithelial cells. The combination of up to 1 µmol/L P276 and 100 nmol/L gemcitabine 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, top and bottom panel). This antiproliferation effect on tumor cells was seen within 24 hours, which continued to significantly increase over the next 72 hours (Fig. 1C, top and bottom panel). To determine the long-term combination effect of P276-Gem treatment, cells were incubated with P276 and gemcitabine for 24 hours, washed with media, and allowed to grow in normal medium for a week. There were lower numbers of colonies in all 4 of the MiaPaCa-2, PanC-1, AsPC-1, and BxPC-3 cells in the combination treatment when compared with cells treated with either P276 or gemcitabine alone (Fig. 1D, left and right panel). These data suggested that combination of P276 and gemcitabine treatment suppresses growth of pancreatic cancer cells in culture.

The P276-Gem combinations induces apoptosis

Given its effects on cell proliferation, we next carried out cell-cycle analysis to further characterize P276-Gem’s combination effect. At 12 hours, P276-Gem combination induced cell death in both PanC-1 and MiaPaCa-2 cells (Fig. 2A, top and bottom panel). At 24 hours, 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 hours 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 gemcitabine (Fig. 2C, right and left panel). In addition, P276 and gemcitabine 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 suggested that combination of P276 and gemcitabine 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 carried out a microarray analysis using an oligonucleotide array. The combination of P276 and gemcitabine showed an effect on the Akt and mTOR pathways while inducing apoptosis pathway transcripts (Fig. 3A and 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 suggested 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 was 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 hours (Fig. 4A).
The P276-Gem combination inhibits expression of cancer-promoting genes

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 shown increased COX-2 levels in pancreatic adenocarcinomas (24). Therefore, we next determined the effects of the P276-Gem combination on COX-2 expression. Although 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 in which COX-2 expression was not observed following the combination treatment (Fig. 4C).

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 either P276 or gemcitabine alone and its combination was administered intraperitoneally every other day for up to the 7th week (Fig. 5A). Although treatment with either P276 or gemcitabine inhibited the growth of the tumor xenografts, there was an enhanced effect in response to P276-Gem combination treatment caused significant downregulation in the phosphorylation of Akt, mTOR, and p70 S6 kinase. In addition, total eIF4E levels were reduced. Gem, gemcitabine.
weighed approximately 2,000 mg. Tumors from animals treated with either P276 or gemcitabine alone weighed approximately 1,000 and 800 mg, respectively. However, the tumors from animals treated with the P276-Gem combination weighed less than 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 implied that the P276-Gem is a potential therapeutic combination for treating pancreatic cancers but is relatively nontoxic 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 showed a reduction in normal vasculature, when compared with either P276 or gemcitabine alone (Fig. 5D).

**P276 and gemcitabine 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 gemcitabine, when compared with controls or either P276 or gemcitabine 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, gemcitabine 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 with 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 suggested 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 gemcitabine 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 show that the combination of P276 and gemcitabine 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 gemcitabine 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 shown that PI3K inhibition enhances gemcitabine-induced apoptosis in human pancreatic cancer cells (30) and improves gemcitabine 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 show that P276 inhibits phosphorylation of Akt and 4E-BP1 and abrogated expression of HIF-1-inducible gene viz. VEGF (11). Our studies show that P276-Gem combination treatment effectively downregulated Akt (Ser473) and mTOR (Ser2481) phosphorylation. Expression of downstream mTORC1 target proteins phospho-p70 S6K (Thr389) and phospho-4E-BP1 (Thr37/46) was also significantly decreased by the P276-Gem combination. These findings suggest that 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 antiangiogenic 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). In addition, P276-Gem combination treatment

![Figure 5. Combination of P276 and gemcitabine 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 days. Subsequently either P276 or gemcitabine was injected every other day intraperitoneally (i.p.) 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 with controls or either P276 or gemcitabine alone (\( * \), \( P < 0.05 \)). C, tumor volumes in P276 or gemcitabine 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×). Gem, gemcitabine.](https://www.aacrjournals.org/molcanther/article-pdf/11/7/1605/10135914/1605.pdf)
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 because of 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. Although 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-Gem 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.

Disclosure of Potential Conflicts of Interest

D. Chakrabarti has been a senior research scientist at Piramal Healthcare Limited; M. Padigaru has been an associate director at pharmaceutical research for Piramal Life Sciences Limited; and S. Sharma has been a chairman and has ownership interest (including patents) in Piramal Life Sciences.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Subramaniam, D. Chakrabarti

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): D. Subramaniam, G. Peryasamy, S. Ponnurangam, A. Sugumar, A. Balakrishnan, S. Anant

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