

Inhibition of oncogenic Pim-3 kinase modulates transformed growth and chemosensitizes pancreatic cancer cells to gemcitabine

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer with a 5-year survival rate of only 6%. Although the cytosine analog gemcitabine is the drug commonly used to treat PDAC, chemoresistance unfortunately renders the drug ineffective. Thus, strategies that can decrease this resistance will be essential for improving the dismal outcome of patients suffering from this disease. We previously observed that oncogenic Pim-1 kinase was aberrantly expressed in PDAC tissues and cell lines and was responsible for radioresistance. Furthermore, members of the Pim family have been shown to reduce the efficacy of chemotherapeutic drugs in cancer. Therefore, we attempted to evaluate the role of Pim-3 in chemoresistance of PDAC cells. We were able to confirm upregulation of the Pim-3 oncogene in PDAC tissues and cell lines vs. normal samples. Biological consequences of inhibiting Pim-3 expression with shRNA-mediated suppression included decreases in anchorage-dependent growth, invasion through Matrigel and chemoresistance to gemcitabine as measured by caspase-3 activity. Additionally, we were able to demonstrate that Pim-1 and Pim-3 play overlapping but non-identical roles as it relates to gemcitabine sensitivity of pancreatic cancer cells. To further support the role of Pim-3 suppression in sensitizing PDAC cells to gemcitabine, we used the pharmacological Pim kinase inhibitor SGI-1776. Treatment of PDAC cells with SGI-1776 resulted in decreased phosphorylation of the proapoptotic protein Bad and cell cycle changes. When SGI-1776 was combined with gemcitabine, there was a greater decrease in cell viability in the PDAC cells vs. cells treated with either of the drugs separately. These results suggest combining drug therapies that inhibit Pim kinases, such as Pim-3, with chemotherapeutic agents, to aid in decreasing chemoresistance in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the predominant cancer of the pancreas with an estimated 43,920 new cases and 37,390 deaths in 2012.¹ It ranks fourth in cancer-related deaths in the US and has a relative 1-year survival rate of 26% and a 5-year survival rate of only 6%.¹ Due to the intrinsic chemoresistance of pancreatic cancer cells, chemotherapy increases median overall survival of no more than 6 mo.² Therefore, in order to improve this dismal outcome, there is a need to identify and validate novel molecular targets that have a significant impact on the aberrant growth and resistance of PDAC cells.

Pim-3, a serine/threonine kinase, is a member of the proviral integration site for the Moloney murine leukemia virus (Pim) family and belongs to the Ca²⁺/calmodulin-dependent protein kinase group.³ It was originally identified as a novel kinase induced by

depolarization (KID)-1 in PC12 cells (a rat pheochromocytoma cell line).⁴ Due to having high sequence homology with the Pim family of kinases, KID-1 was renamed Pim-3. The Pim-3 gene is located on chromosome 22q13 in the human genome and encodes a protein of 326 amino acids with a molecular weight of approximately 35 kD.^{5,6} Other members of the Pim kinase family include Pim-1 and Pim-2 with 77 and 61% homology to Pim-3, respectively.⁵ Although the crystal structure of the Pim-3 protein has not been generated, structures have been obtained for both Pim-1 and Pim-2 with the kinase site maintained in an active conformation.⁷⁻⁹ It is expected that Pim-3 will also be constitutively active and share similar substrates, too.

Previous research has provided ample evidence in which Pim kinases, including Pim-3, are aberrantly expressed in various types of malignancies and help to induce tumorigenicity.^{3,6-9} Overexpression of Pim-3 mRNA has been found in a panel of

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human Ewing family tumor cell lines and nasopharyngeal carcinoma cell lines.¹⁰ Additionally, Pim-3 overexpression was found in the premalignant and malignant lesions in the liver, stomach and colon compared with the normal tissues.^{6,9} Moreover, mice studies have demonstrated that Pim-3 can promote EWS/FLI-mediated NIH 3T3 tumorigenesis as well as hepatocellular carcinoma.^{6,10} Recently, Pim-3 was found to be aberrantly expressed in PDAC cells and to phosphorylate the pro-apoptotic protein Bad.⁹ Also, Pim-3 was shown to be regulated by transcription factors such as ETS-1 and serve as a positive regulator of STAT3 signaling in pancreatic cancer cells.^{11,12} Past studies demonstrate a correlation between persistent expression of activated STAT3 and drug resistance in tumors including breast, ovarian, head and neck and multiple myeloma.¹³⁻¹⁶ Additionally, studies by Chen et al.¹⁷ showed that the upregulation of Pim-1 is important in hypoxia-mediated cisplatin resistance in a pancreatic cancer cell line. In our studies, we focus on elucidating the role of Pim-3 in transformed growth and chemoresistance of PDAC cells. We demonstrated upregulation of Pim-3 protein expression in PDAC patient tumor tissues and cell lines compared with the normal state. Also, we found downregulation of Pim-3 via shRNA resulted in a decrease in phosphorylation of the downstream substrate Bad, anchorage-dependent growth and invasion of PDAC cells through Matrigel. Additionally, inhibition of Pim-3 expression led to an increase in gemcitabine-induced apoptosis. Finally, the Pim kinase inhibitor SGI-1776 provided by Astex Pharmaceuticals increased chemosensitivity to gemcitabine in PDAC cells. Overall, our results provide additional roles for Pim-3 in PDAC and help to validate Pim-3 as an important modulator of chemoresistance in pancreatic cancer.

Results

Pim-3 is aberrantly expressed in PDAC tumor tissues and cell lines. Using immunohistochemical staining, we compared Pim-3 protein expression between normal and tumor tissue samples of the pancreas of paraffin-embedded patient specimens. A total of 8–10 slides for each tissue type were reviewed and scored by pathologists blinded to the actual tissue diagnosis. The values for intensity of staining and percentage of positive cells were collected and recorded. Pim-3 protein was found to be overexpressed in the PDAC tissues compared with the normal pancreas (Fig. 1A). Next, we compared Pim-3 expression in matched pairs of normal and tumor tissues of the pancreas. Lysates were prepared from PDAC tumor and adjacent normal tissues from two patients and analyzed by western blot analysis for Pim-3 protein expression. Densitometry was performed to quantitate band intensity of normal and tumor tissue samples. Pim-3 was overexpressed in both tumor tissues compared with the matched normal tissues (Fig. 1B). To further support our observations, we measured Pim-3 protein expression in a panel of human PDAC cell lines compared with the immortalized HPNE (human pancreatic nestin-expressing) epithelial cell line. We found variable overexpression of Pim-3 protein in all PDAC cell lines (Fig. 1C). Thus, Pim-3 is constitutively overexpressed in PDAC tumor tissues and cell lines.

Inhibition of endogenous Pim-3 kinase decreases phosphorylation of the pro-apoptotic protein Bad. Next, we attempted to stably knock down Pim-3 in two human PDAC cell lines, PANC-1 and MIA PaCa-2, using lentiviruses-expressing shRNAs targeting Pim-3 kinase. We were able to confirm suppression of endogenous Pim-3 at the mRNA and protein level in both cell lines, respectively (Fig. 2A and B; Fig. 2C and D). Studies by MacDonald et al.¹⁸ have demonstrated that Pim-3 kinase is able to phosphorylate the Bad protein at multiple phosphorylation sites, including Ser¹¹². This site is known for being critical for promoting apoptosis. More specifically, studies by Li et al.⁹ showed that Pim-3 phosphorylates Bad on Ser¹¹² in pancreatic cancer cells. Therefore, to confirm these results in our PDAC cells, we evaluated the phosphorylation status of Bad and demonstrated that, as expected, suppression of Pim-3 resulted in decreased phospho-Ser¹¹² Bad in each cell line compared with the scrambled shRNA controls (Fig. 2C and D). The decrease in Pim-3 expression was most likely the main contributing factor for the observed decrease in phosphorylated Bad, based on the fact that the suppression of Pim-3 not only decreased total Bad but also decreased the ratio of phosphorylated to total Bad. Thus, inhibition of Pim-3 expression led to a reduction of phospho-Ser¹¹² Bad in PDAC cell lines.

Pim-3 contributes to anchorage-dependent growth of PDAC cells. Pim kinases have been shown to promote cell cycle progression and proliferation in solid tumors. To investigate the role of Pim-3 kinases on growth transformation phenotypes of PDAC cells, we used PANC-1 and MIA PaCa-2 cells stably-expressing Pim-3 shRNAs from our previous experiments. Cells were grown on plastic plates for a limited number of days and counted each day during the study. We observed a decrease in cell proliferation of both Pim-3 knock down cell lines compared with the scrambled shRNA cell lines at the latter timepoints (Fig. 3A and B). To determine if Pim-3 plays a role in anchorage-independent growth, we used the soft agar colony-forming assay. Neither cell line expressing the Pim-3 shRNA showed significant changes in colony number nor size in soft agar compared with the control scramble shRNA cells (data not shown). Therefore, our results demonstrate that Pim-3 has a role in anchorage-dependent growth and is not required for anchorage-independent growth in PDAC cell lines.

Pim-3 is important for PDAC cell invasion. Most pancreatic cancer patients are diagnosed at advance stages in which the cancer has invaded locally and/or metastasized to distant sites.¹ Additionally, PDAC cell lines have been shown to be highly invasive through Matrigel matrices.^{19,20} To determine if Pim-3 plays an important role with the invasive properties of PDAC cell lines, PANC-1 and MIA PaCa-2 cells stably expressing Pim-3 shRNAs were plated on Matrigel. Suppression of Pim-3 resulted in a significant decrease in the number of cells able to invade through the gel matrix (Fig. 3C and D). To our knowledge, these findings provide the first evidence that Pim-3 is important for PDAC cells to invade through Matrigel.

Suppression of Pim-3 increases gemcitabine-induced apoptosis in PDAC cells. Although the nucleoside-analog drug gemcitabine is the main treatment for pancreatic cancer, many

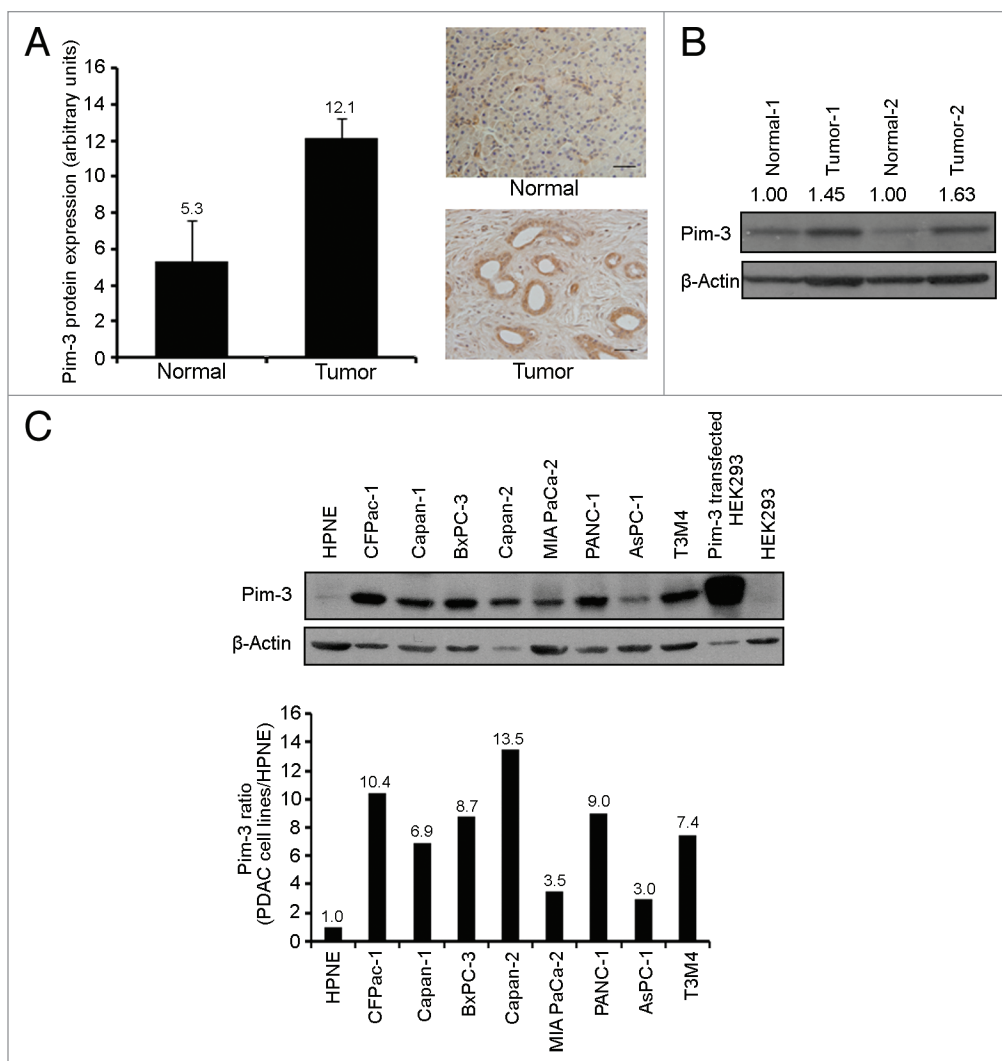


Figure 1. Pim-3 protein is upregulated in human pancreatic cancer tissues and cell lines. **(A)** Paraffin-embedded patient specimens of normal pancreas and pancreatic adenocarcinoma were immunohistochemically stained with an anti-Pim-3 antibody. Slides were scored for staining intensity and percentage of positive cells by pathologists blinded to the actual tissue diagnosis. These values were multiplied to provide a combined score that ranged from 0–24. Representative IHC results for the pancreatic normal and tumor tissues are shown. **(B)** Lysates from two sets of matched normal (N) and tumor (T) pancreatic tissues were isolated and immunoblotted with an anti-Pim-3 antibody. The numerical values represent relative band intensities for Pim-3 expression normalized to β -actin. **(C)** Lysates were prepared from a panel of PDAC cell lines and immunoblotted for Pim-3 protein expression as in **(B)**. Pim-3 expression was quantitated by densitometry and normalized to HPNE cells. HEK293 cells transfected with human Pim-3 cDNA were used as a positive control. β -Actin served as a loading control. Data shown are representative of three independent experiments.

patients are resistant to this drug. Initially, we treated parental PDAC cell lines with varying doses of gemcitabine in the MTT assay (Fig. 4A). MIA PaCa-2 was found to have more decreased growth than PANC-1 cells. To determine if suppression of Pim-3 could enhance gemcitabine-induced apoptosis, we compared PANC-1 and MIA PaCa-2 cells expressing Pim-3 shRNAs with scrambled shRNAs control cells (Fig. 4B). Both groups were treated with two concentrations (1 and 5 μ M) of gemcitabine. Apoptosis was evaluated in the cells using the caspase-3 activity assay. In each of the treatment groups, we observed an increase in caspase-3 activity in the Pim-3 shRNA cells compared with the scrambled control cells, especially in the MIA PaCa-2 cells (Fig. 4B). These results are novel in that they provide direct evidence that inhibition of Pim-3 expression

can sensitize PDAC cells to apoptosis after treatment with gemcitabine.

As previously discussed, Pim-3 is a member of the Pim kinase family and shares 77% functional homology with Pim-1. In order to address the distinct roles of Pim-3 in comparison to Pim-1 in PDAC cells, we grew MIA PaCa-2 cells stably expressing either Pim-3 shRNAs or Pim-1 shRNAs compared with scrambled shRNA cells in varying doses of gemcitabine in the MTT assay (Fig. 4C). Pim-3 shRNA cells had less proliferation than the Pim-1 shRNA cells. Additionally, we were able to demonstrate that MIA PaCa-2 cells stably expressing Pim-3 shRNAs grew slower on plastic than cells expressing Pim-1 shRNAs over a 6-day time course (data not shown). We were not able to stably express Pim-1 shRNAs in PANC-1 cells. These results are novel

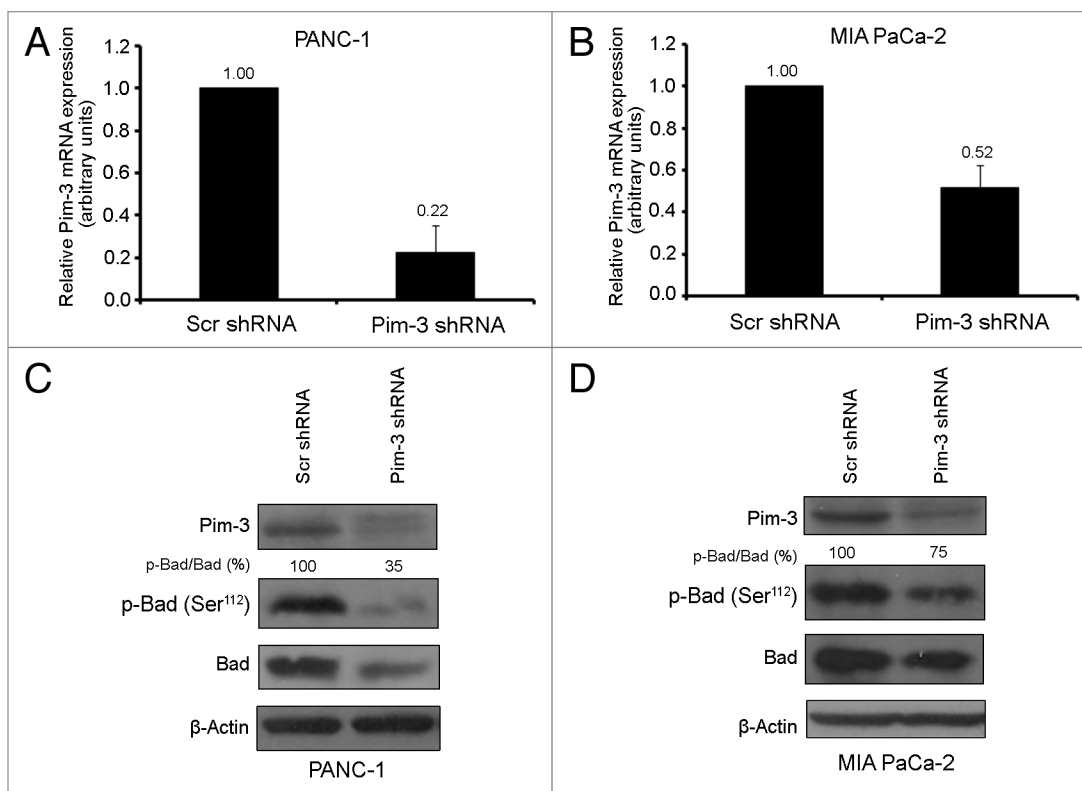


Figure 2. Inhibition of endogenous Pim-3 expression and downregulation of phospho-Ser¹¹² Bad in PDAC cell lines. To determine Pim-3 mRNA expression from PANC-1 (A) or MIA PaCa-2 (B) cells stably expressing scramble (scr) or Pim-3 shRNAs, total RNA were isolated and analyzed using quantitative RT-PCR. To evaluate the effect of downregulating Pim-3 on its immediate substrate, Bad, PANC-1 (C) and MIA PaCa-2 (D) cells stably expressing scramble or Pim-3 shRNAs were immunoblotted with antibodies for Pim-3, p-Bad (Ser¹¹²) and total Bad. The ratio of phosphorylated Bad to total Bad was determined. β -Actin served as a loading control. Data shown are representative of three independent experiments.

and provide evidence that Pim-3 and Pim-1 have overlapping but non-identical roles as it relates to anchorage-dependent growth and sensitivity of PDAC cells to gemcitabine.

SGI-1776 inhibits phosphorylation of Bad and causes cell cycle changes in PDAC cells. In vitro kinase assays described by Mumenthaler et al.²¹ have demonstrated that the small molecule Pim inhibitor SGI-1776 is most potent against Pim-1 and Pim-3, respectively. To investigate whether SGI-1776 was capable of disrupting substrates of Pim kinases, such as Bad, in pancreatic cancer cells, PANC-1 and MIA PaCa-2 parental cells were treated with SGI-1776 over a 48-h period with selected doses before being lysed for immunostaining. Western blot analysis revealed a significant decrease in phospho-Ser¹¹² Bad in all 3 doses (1, 5 and 10 μ M) of the Pim inhibitor for PANC-1 compared with the untreated cells (Fig. 5A). Also, we observed decreased levels of total Bad protein in the treatment groups. For MIA PaCa-2, we observed a dose-dependent decrease in phospho-Ser¹¹² Bad between cells treated with the Pim inhibitor at the 5 μ M dose and the untreated cells. These results are consistent with the decreased phospho-Ser¹¹² Bad observed in our PDAC cells stably expressing Pim-3 shRNA (Fig. 2). Studies by Mumethaler et al.²¹ demonstrated that SGI-1776 induced G₁ cell cycle arrest in prostate cancer cells. To determine the influence of SGI-1776 on the cell cycle in PDAC cells, we stained cells with propidium iodide and used flow cytometry on the PDAC cells after being incubated

with SGI-1776 for 72 h. Although there was no observable difference at 1 μ M, an accumulation of cells in the G₁ phase of the cell cycle compared with the untreated cells at 5 μ M of SGI-1776 was discovered (Fig. 5B). For the PANC-1 cells, there were no significant changes observed with the cell cycle (data not shown). Taken together, these data demonstrate that SGI-1776 inhibits phosphorylation of Bad and elicits cell cycle changes, such as cells arresting in the G₁ phase of the cell cycle.

SGI-1776 chemosensitizes PDAC cells to gemcitabine treatment. It has been demonstrated that members of the Pim kinase family are responsible for the resistance of cancer cells to chemotherapy due to being a survival factor.²² Considering our previous results with the Pim inhibitor, we were interested in determining whether SGI-1776 was capable of sensitizing PDAC cells to gemcitabine treatment. We treated cells with selected doses of SGI-1776 and gemcitabine as single agents and in combination for 72 h. Higher doses of gemcitabine and SGI-1776 had to be used for the PANC-1 cells due to the lack of sensitivity at lower doses (Fig. 6A). As single agents, both gemcitabine and SGI-1776 inhibited cell growth as compared with the untreated cells (Fig. 6A and B). When SGI-1776 was combined with gemcitabine, we observed a significantly, greater decrease in growth of the PDAC cells. This observation was more apparent in the MIA PaCa-2 cells in which we saw a synergistic decrease in cell proliferation with the drug combination

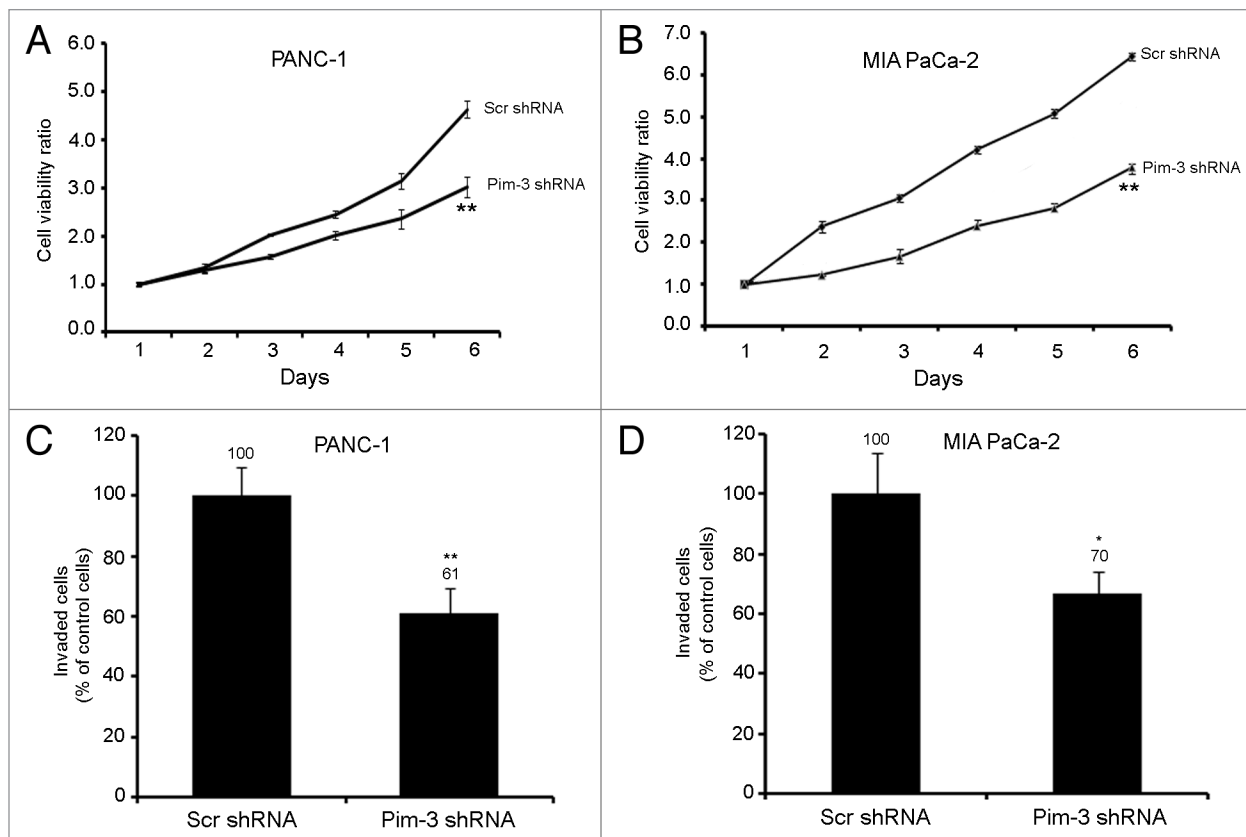


Figure 3. Pim-3 has a role in anchorage-dependent growth and invasion of pancreatic cancer cell lines. To evaluate anchorage-dependent growth, PANC-1 (A) and MIA PaCa-2 (B) cells stably expressing scramble (scr) or Pim-3 shRNAs were grown in 96-well microplates with the initial time point designated as day 0. Cells were counted daily for a total of 4–6 d as described in Materials and Methods. Data are representative of at least three independent experiments and based on O.D. readings. To determine the contribution of Pim-3 in invasion, PANC-1 (C) and MIA PaCa-2 (D) cells stably expressing scramble (scr) or Pim-3 shRNAs were plated in the Matrigel invasion assay as described in the Materials and Methods. Numerical values represent percentage of invasion of Pim-3 shRNA-expressing cells compared with scr shRNA control cells. Results are representative of three independent assays. (A and B) p value of < 0.05 was considered statistically significant and displayed as p < 0.05 (*) and p < 0.001 (**). (C) p value of < 0.01 (**) and for (D) p value < 0.05 (*) was used to show statistical significance.

compared with the single agents alone (Fig. 6B). These data indicate that SGI-1776, in addition to inhibiting PDAC cell growth and altering the cell cycle, chemosensitizes PDAC cells to gemcitabine treatment.

Discussion

Pancreatic cancer is a lethal cancer with minimal survival for patients due to lack of early detection and limited success with treatment strategies.¹ Thus, there is an urgent need for identification and validation of novel molecular targets in PDAC that are druggable and can provide effective therapy to patients. Members of the oncogenic Pim family of serine/threonine kinases are upregulated in many cancers and have been shown to support cell growth, survival and are potential molecular targets for therapeutics.^{23,24} Pim-3, the least studied of the Pim kinase family, has been shown to be aberrantly expressed in various cancers including gastric,⁸ nasopharyngeal,²⁵ colon,⁷ hepatocellular⁶ and the pancreas.⁹ We demonstrate that Pim-3 protein can modulate growth transformation phenotypes and sensitize cancer cells to chemotherapy. Additionally, Pim-3 was found to have overlapping but

non-identical roles to Pim-1 kinase in PDAC cells which could provide novel insights in developing better treatments against pancreatic cancer.

After confirmation of Pim-3 expression in PDAC tissues and cell lines, we decided to investigate the role of Pim-3 in PDAC growth transformation. Using shRNAs made against Pim-3, we observed a decrease in anchorage-dependent, but not anchorage-independent growth in PDAC cell lines compared with the controls. Studies by Li et al.⁹ showed a similar decrease in anchorage-dependent growth of PDAC cells transfected with Pim-3 shRNA. Despite the inhibition of endogenous Pim-3 expression, cells continued to grow throughout the time course in our studies. This observation might be explained by the compensatory ability of the other Pims, such as Pim-1, since there is a high sequence similarity between Pim-3 and Pim-1.^{5,21} Also, we demonstrated that Pim-3 is important for invasion of PDAC cells through Matrigel. Zheng et al.⁸ showed a positive correlation of Pim-3 expression with the lymphatic and venous invasion of gastric cancer cells. Future animal studies should be conducted to determine the correlation between Pim-3 expression and the invasion of PDAC cells.

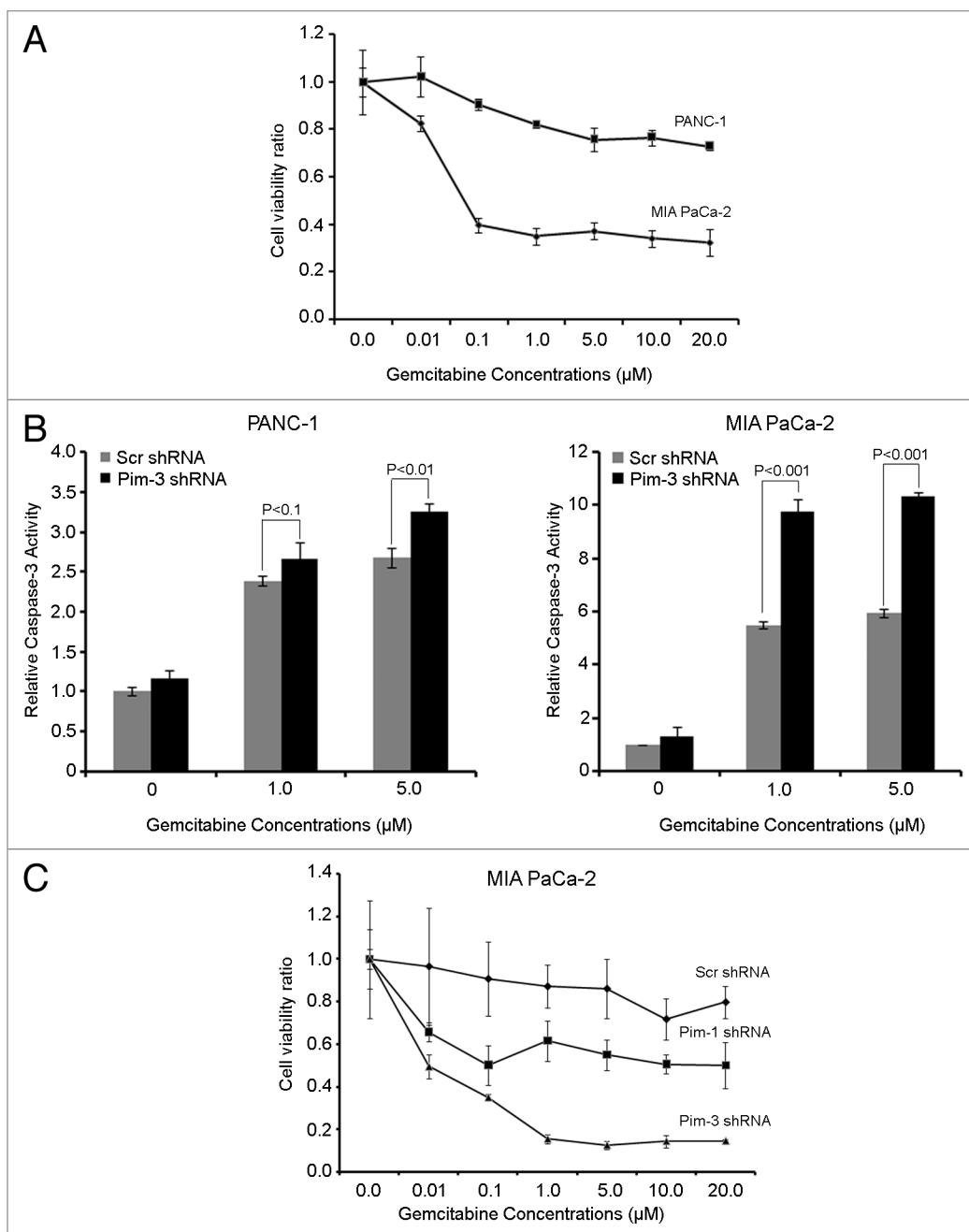


Figure 4. Inhibition of Pim-3 increases gemcitabine-induced apoptosis in pancreatic cancer cell lines. **(A)** To determine sensitivity to gemcitabine, parental PANC-1 and MIA PaCa-2 cells were treated with various concentrations of gemcitabine in the MTT assay for 72 h. Data are representative of at least three independent experiments and based on O.D. readings. **(B)** To evaluate the effects of Pim-3 inhibition on gemcitabine-induced apoptosis, PANC-1 and MIA PaCa-2 cells stably-expressing Pim-3 shRNA or scrambled shRNA were treated with 1.0 μM and 5.0 μM gemcitabine for 72 h. Afterwards, lysates were analyzed for apoptosis using the caspase-3 activity assay. Data shown are representative of three independent experiments. **(C)** To determine the sensitivity of MIA PaCa-2 cells stably-expressing Pim-1 and Pim-3 shRNA to gemcitabine, cells were treated with various concentrations of gemcitabine in the MTT assay for 72 h. Data are representative of at least three independent experiments and based on O.D. readings.

The lack of significant clinical response observed in patients treated with gemcitabine warrants the need for discovering new ways to overcome chemoresistance in pancreatic cancer.^{1,28} MIA PaCa-2 and PANC-1 are both gemcitabine-resistant PDAC cell lines³³ that were used to suppress Pim-3 protein expression in our studies. We observed that suppression of endogenous Pim-3 protein increased caspase-3 activity of PDAC cells treated with the

gemcitabine, especially in the MIA PaCa-2 cells, which is indicative of increased apoptosis (Fig. 4B). In order to determine if this sensitivity to gemcitabine was due to Pim-3 or a combination of Pim-1 and Pim-3, we compared the growth of Pim-1 and Pim-3 shRNA expressing cells treated with gemcitabine. We found that Pim-3 was more important than Pim-1 in making cells more sensitive to gemcitabine (Fig. 4C). To our knowledge, this is the first

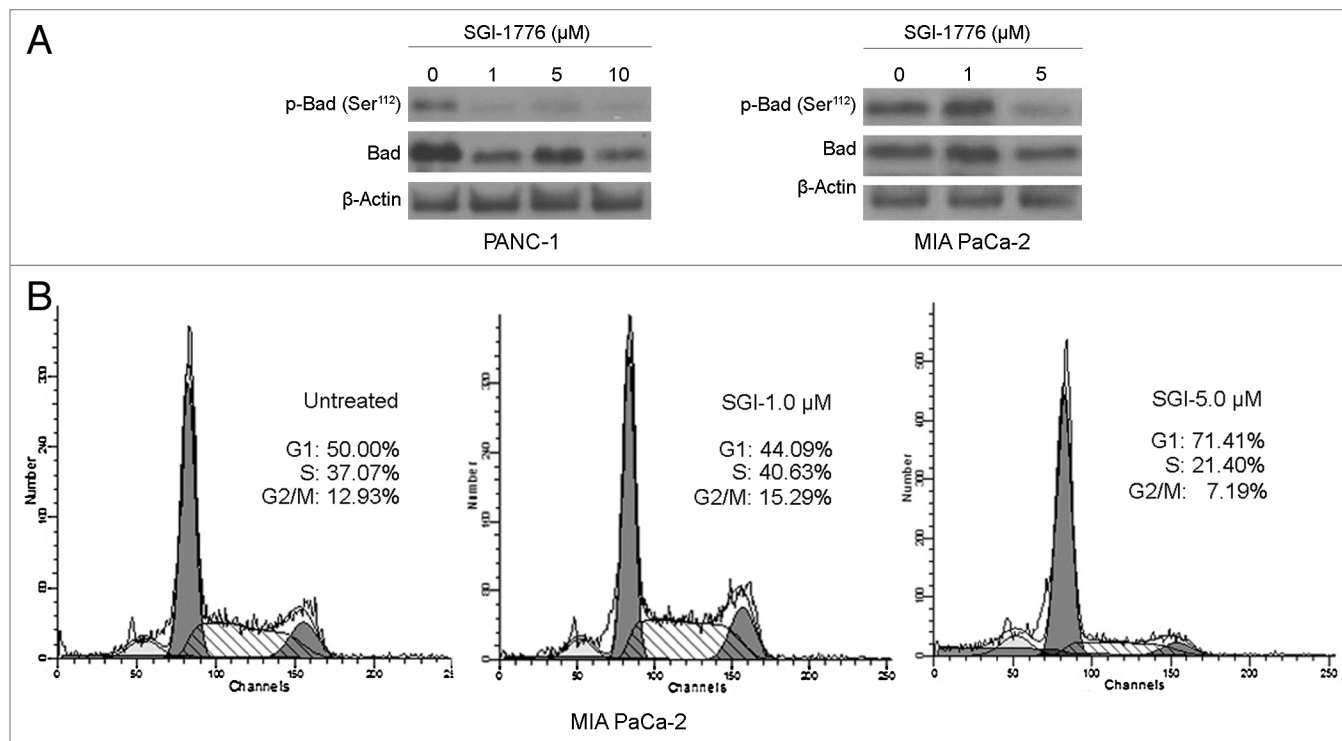


Figure 5. Pim inhibitor SGI-1776 downregulates phospho-Ser¹¹² Bad and elicits cell cycle changes in pancreatic cancer cell lines. **(A)** PANC-1 and MIA PaCa-2 cells were treated with selected concentrations of SGI-1776. Afterwards, lysates were immunoblotted with antibodies against P-Bad (Ser¹¹²) and total Bad. β-Actin served as a loading control. **(B)** Lysates from MIA PaCa-2 cells treated with selected concentrations of SGI-1776 for 72 h were collected, stained with propidium iodide and analyzed by flow cytometry as described in Materials and Methods. Data shown are representative of three independent experiments.

time that Pim-1 and Pim-3 have been shown to have overlapping, but non-identical roles in PDAC cell lines as it relates to chemosensitivity.

A number of research groups have developed over 50 different Pim kinase inhibitors for potential anticancer therapies.^{23-29,30} The majority of Pim inhibitors that have been developed primarily target Pim-1 with a few inhibitors being reported to target Pim-2.²⁹ To our knowledge, there are no selective Pim-3 inhibitors available that have been described in the literature. The pan-Pim inhibitor SGI-1776 (developed by Astex Pharmaceuticals) was at one time evaluated in phase 1 clinical trials for prostate cancer or non-Hodgkin lymphoma before being terminated due to off-target effects. In our studies, we were able to demonstrate that SGI-1776 in combination with gemcitabine in PDAC cells resulted in decreased proliferation compared with the single drug treatments, especially with the MIA PaCa-2 cells (Fig. 6). Our results are in agreement with studies where SGI-1776 was able to re-sensitize prostate cancer cell lines to taxane-based therapies.³¹ Additionally, Song et al.³² demonstrated the sensitizing of prostate cancer cells to apoptosis after treatment with a Pim inhibitor and a Bcl-2 family inhibitor.

Recently, studies by Wang et al.³⁴ identified a phenanthrene derivative that was very potent against Pim-3 and Pim-1 and decreased in vitro and in vivo growth of a pancreatic cancer cell line. Our studies along with others provide evidence for the development of Pim inhibitors selective against Pim-3. Treatment of

solid tumors having aberrant expression of Pim-3, such as pancreatic cancer, with Pim-3 inhibitors either as single agents or in combination with traditional chemotherapy may help provide a safe and novel drug regimen. Finally, past studies have demonstrated Pim-1 being a prognostic marker in pancreatic cancer.³⁵ It will be of interest to determine whether Pim-3 has the same capability of becoming a prognostic or diagnostic biomarker in pancreatic cancer. Future studies to better understand the distinct roles of each member of the Pim kinase family in pancreatic cancer could be instrumental for developing inhibitors that can potentially treat this deadly disease.

Materials and Methods

Patient tissues. Eight to ten paraffin-embedded patient specimens of normal pancreas and pancreatic adenocarcinoma from the University of North Carolina Chapel Hill School of Medicine were immunohistochemically stained with institutional review board approval. After deparaffinization, the slides were submerged in methanol containing 0.3% hydrogen peroxide for 15 min at RT to inhibit endogenous peroxidase activity. Antigen retrieval was done for Pim-3 by incubating the sections in 0.01 mol/L citrate buffer (pH 6) in a microwave oven for 18 min. Sections were incubated with a goat polyclonal anti-Pim-3 antibody (C-18, sc-49485) (1:50–1:500) overnight at 4°C. Tissue sections were then washed again in PBS and incubated

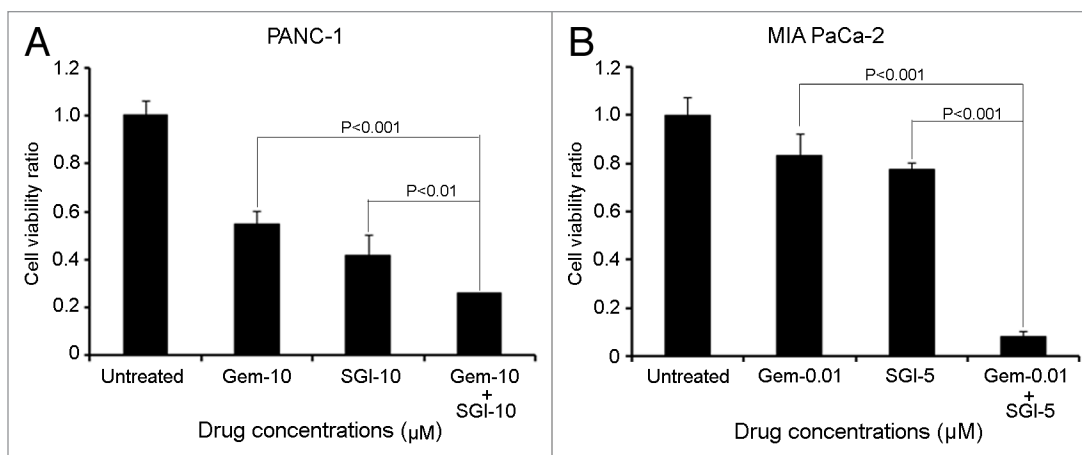


Figure 6. Alterations in cell viability using SGI-1776 combination therapy with gemcitabine-based chemotherapy in pancreatic cancer cells. To evaluate the combination of SGI-1776 and gemcitabine on cell viability, PANC-1 (A) and MIA PaCa-2 (B) parental cells were treated with selected concentrations of gemcitabine, SGI-1776 or the combination of gemcitabine and SGI-1776 for 72 h in the MTT assay. Treated cells were compared with untreated cells. Data are representative of at least three independent experiments and based on relative O.D. readings.

with suitable peroxidase-conjugated secondary antibodies for 30 min at 37°C. Detection of the antibody complex was done by the streptavidin-peroxidase reaction kit using DAB as a chromogen. To ensure the specificity of the primary antibody, control tissue sections were incubated in the absence of primary antibody. Histological features were used to identify pancreatic ductal epithelium and associated stromal fibroblasts. The slides were reviewed and scored by pathologists blinded to the tissue diagnosis. Each specimen was assigned a score for staining intensity (values 1–4, with 4 representing the most intense stain) and a score for percentage of positively stained cells (values 0–6, with 6 representing greater than 75%). The values for intensity of staining and percentage of positive cells were then multiplied to yield a combined score for each sample (ranging from 0 to 24). For the western blot analysis of the tissues, de-identified matched normal and primary pancreatic tumor samples from two PDAC patients were harvested in a NP-40-based lysis buffer containing a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich and Roche, respectively) and used for western blotting to check pim-3 expression.

Cell culture and reagents. The human PDAC cell lines CFPac-1, Capan-1, BxPC-3, Capan-2, MIA PaCa-2, PANC-1, AsPC-1 and T3M4 were obtained from the American Type Culture Collection and maintained in culture with RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Cellgro) and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. Lysates isolated from immortalized human pancreatic nestin-expressing (HPNE) vector cell lines and HEK 293 cells were kindly provided by Dr Channing Der at University of North Carolina Lineberger Comprehensive Cancer Center. The full length human Pim-3 cDNA that was used to transfect HEK 293 cells was kindly provided by Dr Naofumi Mukaida at the Cancer Research Institute at Kanazawa University in Japan. The Pim kinase inhibitor SGI-1776 (Astex Pharmaceuticals) was dissolved in DMSO at a concentration of 20 mM as a stock solution

and used at various concentrations. Gemcitabine (Santa Cruz Biotechnology) was dissolved in Milli-Q water at a concentration of 20 mM.

Lentiviral shRNA and creation of stable cell lines. The shRNA constructs directed against Pim-3 kinase or scrambled sequences were purchased from Open Biosystems through the University of North Carolina Chapel Hill Lentiviral Core Facility. Lentiviral particles were generated using a three-plasmid system as described previously.³⁶ Twenty-four hours after infection, cells were treated with 2 µg/ml puromycin for at least 4 d to eliminate uninfected cells and thus yield mass populations of puromycin-resistant cells expressing the shRNAs.

Preparation of cell lysates and western blotting analysis. Cells were collected from 10 cm plates and lysed in 200 µl freshly made lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1.5 µg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM Na₃VO₄, 1 mM NaF and 1 mM dithiothreitol) for 5 min on ice. Twenty-five micrograms of protein, as determined by a modified Bradford protein assay (Bio-Rad), were loaded per well onto a 10% sodium dodecyl sulfate-PAGE gel. Proteins were separated by sodium dodecyl sulfate-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) and incubated with the following primary antibodies diluted according to the manufacturer's recommendation: β-actin (A5316) (Sigma-Aldrich); Pim-3 (C-18) from Santa Cruz Biotechnology; Phospho-Bad (Ser112) (#9291) and Bad (#9292) from Cell Signaling Technology. After incubation with primary antibody, the membranes were washed in tris-buffered saline supplemented with 0.1% Tween 20 and incubated with the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) followed by development with the chemiluminescent substrate WestDura (Pierce Biotechnology) and exposure to X-ray film (Denville Scientific). After staining for primary antibodies, membranes were stripped and re probed for β-actin.

Cell proliferation (MTT) assay. Cell lines were trypsinized so that single-cell suspensions could be generated. 3.0×10^3 cells/well were seeded in a 96-well plate. The cell proliferation was determined every day for 4–6 d by CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega).

Cell proliferation assay in monolayer culture. Cell lines were trypsinized (0.5% trypsin/ethylene-diaminetetraacetic acid) to generate single-cell suspensions. Between 1.0×10^3 and 2.0×10^3 cells per well (96-well plates) were seeded and cells were counted using a cell hemacytometer daily for up to 6 d. Assays were performed a total of three times in triplicate, independently.

Reverse transcription and quantitative real-time PCR validation. Total RNA was extracted from a monolayer cell culture using TriZol (Invitrogen) extraction according to manufacturer's protocol. This was followed by RQ1 DNase (Promega) treatment followed by RNA cleanup using again TriZol extraction according to Invitrogen's protocol. PCR analysis using GAPDH primers and RNA sample (200 ng) as template was performed to check for chromosomal contamination. The total RNA was quantified using a Nano Drop ND-1000 Spectrophotometer. All RNA samples were kept at -80°C . The reverse transcription was performed according Applied Biosystems' protocol using a High Capacity cDNA Reverse Transcription Kit, (#4368814). Quantitative PCR analysis of Pim3 mRNA expression was performed on a 7500 Real Time PCR System (Applied Biosystems). PCR products were generated from cDNA samples using Absolute Blue QPCR SYBR low ROX Mix, (AB-4323/A) (Thermo Fisher Scientific Inc., Epsom). Assays were run on 96-well plates so that each sample was run in triplicate for the target gene and for the endogenous control. Primers were used to a final concentration of 160 nM per reaction. A concentration of 480 ng per well of cDNA template was used for each assay. All results were normalized to GAPDH levels. Two pair of primers was designed for real time PCR analysis of Pim3 expression. The sense and antisense primers for Pim3 mRNA were Pim3aFP: 5'-GCC AAG GCG GAC AAG GAG AG-3'; Pim3aRP: 5'-GAA CCA GTC CAG CAG GCG G-3'; Pim3bFP: 5'-ACG TGG TGA AGG AGC GGG TGA-3'; Pim3bRP: 5'-CCA GCA CCA GCA GGA AGC C-3'; which yielded, respectively, 255-bp and 162-bp PCR products. Cycle conditions were as follows: After an initial stage 1 at 50°C for 2 min and stage 2 at 95°C for 15 min, samples were cycled 40 times at 95°C for 15 sec and at 60°C for 60 sec.

Cell cycle analysis. Stably infected cells or parental cells with drug treatments were harvested and fixed in ice cold 70% ethanol at 4°C for at least 2 h. Fixed cells were washed in phosphate-buffered saline and then treated with a staining solution in

phosphate-buffered saline composed of 20 $\mu\text{g/ml}$ propidium iodide (Molecular Probes), 200 $\mu\text{g/ml}$ RNase A and 0.1% Triton X-100. Samples were equilibrated for 30 min at room temperature prior to data collection at 575 nm on a FACSAria flow cytometer (Becton Dickinson). The distribution of cells in each cell-cycle phase was determined by using cell ModFit LT Software (Verity Software House) following pulse width-based gating for doublet discrimination.

In vitro invasion assay. Invasion assay was performed according to Xu et al.²⁷ Briefly, log-phase cell lines were incubated in serum-free medium for 24 h. Growth-factor-reduced Matrigel invasion chambers (BD BioCoat BD Matrigel Invasion 24-well Chamber, 8 μm pore, BD Biosciences) were then rehydrated for 2 h at 37°C with serum-free medium and immediately prior to the addition of dissociated cells to the upper chamber (5.0×10^3 cells/well). 10% v/v FBS was added to the lower chamber. After 24 hr, Matrigel and uninvaded cells were removed from the upper chamber with a Q-tip. Invaded cell on the bottom of the membrane were fixed with methanol and stained with 0.1% methylene blue. After drying overnight, stained cells were counted under light microscopy.

Analysis of apoptosis. Apoptosis was determined by measuring caspase-3 activity using the Caspase-3 Colorimetric Assay (Promega). Data were normalized to untreated samples and displayed as relative caspase-3 activity.

Statistical analysis. All obtained data were calculated and expressed as mean \pm standard deviation. The differences were analyzed statistically by a one-way analysis of variance based on the actual mean values. For all analyses, a p value of < 0.05 was considered statistically significant and displayed as $p < 0.05$ (*) and $p < 0.001$.

Disclosure of Potential Conflict of Interest

No potential conflict of interest was disclosed.

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