Cdk4/6 Inhibition Induces Epithelial–Mesenchymal Transition and Enhances Invasiveness in Pancreatic Cancer Cells

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Abstract

Aberrant activation of Cyclin D-Cdk4/6 signaling pathway is commonly found in pancreatic ductal adenocarcinoma (PDAC). Here, we show that PD-0332991, a highly specific inhibitor for Cdk4 and Cdk6, exerted growth inhibitory effects on three human PDAC cell lines. Microarray analysis revealed that PD-0332991 downregulated cell-cycle-related genes, but upregulated genes implicated in extracellular matrix (ECM) remodeling and pancreatic cancer cell invasion and metastasis. Moreover, PD-0332991 enhanced invasion in TGF-β-responsive PDAC cell lines that harbor a wild-type SMAD4 gene (COLO-357, PANC-1), but not in TGF-β-resistant AsPC-1 cells that harbor a mutated SMAD4. PD-0332991 also induced epithelial–mesenchymal transition (EMT) in COLO-357 and PANC-1, but not in AsPC-1 cells. Inhibition of Cdk4/6 using shRNA mimicked the effects of PD-0332991 on EMT induction. Furthermore, PD-0332991 increased Smad transcriptional activity in luciferase readout assays and activated TGF-β signaling. SB-505124, an inhibitor of the type-I TGF-β receptor (TβRI) kinase, completely blocked EMT induction by PD-0332991. When combined with PD-0332991, SB-505124 inhibited the growth of COLO-357 and PANC-1 cells. Taken together, these data suggest that anti-Cdk4/6 therapy could induce EMT and enhance pancreatic cancer cell invasion by activating Smad-dependent TGF-β signaling, and that combining PD-0332991 and SB-505124 may represent a novel therapeutic strategy in PDAC. Mol Cancer Ther; 11(10); 2138–48. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States, with a median survival of 6 to 7 months and a 5-year survival rate of 6% (1). While resection prolongs survival and offers a potential cure, 80% to 85% of PDACs are unresectable at the time of diagnosis (2). Treatment failure is due, in part, to the fact that PDAC displays a wide range of genetic and epigenetic alterations, resistance to chemotherapy and radiotherapy, and intense desmoplasia, which interferes with the drug delivery (3, 4).

PDAC is characterized by a high frequency of activating K-RAS mutations (5) as well as by the inactivation of the INK4A, p53, and SMAD4 tumor suppressor genes, occurring in conjunction with the overexpression of multiple tyrosine kinase receptors and their ligands (6). Together, these alterations contribute to the increased expression of Cyclin D1 and to the loss of negative control over Cdk4/6, leading to excessive activation of Cyclin D-Cdk4/6 signaling and unchecked proliferation. Cyclin D1 is also the downstream target of other aberrantly activated pathways in PDAC, such as Notch, Sonic Hedgehog, and Wnt (7–9). Moreover, Cyclin D1 overexpression in PDAC correlates with decreased postoperative patient survival (10). Therefore, targeted inhibition of Cyclin D-Cdk4/6 may have a therapeutic potential in PDAC.

PD-0332991 is an orally available, highly specific, and reversible inhibitor for Cdk4 and Cdk6 (11). PD-0332991 exerts antiproliferative effects in breast and ovarian cancer cells, myeloma cells, and glioblastoma cells in vitro (12–15), and significantly inhibits tumor growth in human xenograft models (15, 16). It is currently in phase I/II clinical trials (17). A first-in-human phase I study of PD-0332991 in patients with Rb-positive advanced solid tumors or non-Hodgkin lymphoma showed that PD-0332991 was generally well tolerated (18). Whether PD-0332991 could be used to treat PDAC is currently unknown.

There are 3 mammalian TGF-β ligands that bind to the type II TGF-β receptor homodimer, thereby recruiting and activating TβRI homodimers. Phosphorylated TβRI recruits and phosphorylates receptor-regulated Smad2 and Smad3, which form heteromeric complexes with the common partner Smad4 and translocate into the nucleus where they associate with coactivators and corepressors thereby regulating gene expression (19). In early stages of tumorigenesis, TGF-β functions as a tumor suppressor by inhibiting cancer cell proliferation and activating apoptosis (20–21). During cancer progression, TGF-β assumes...
tumor-promoting functions (19), which explains why elevated TGF-β immunoreactivity in resected PDACs correlates with shorter patient survival (22).

TGF-β signaling intersects with other signaling pathways to fine-tune context-dependent biologic responses (23). Nuclear Cdk4 phosphorylates the linker region of Smad3, and inhibits its transcriptional activity and anti-proliferative functions (24). Inhibition of Smad activity by Cdk4 enables cancer cells to bypass the antiproliferative function of TGF-β signaling (25). However, it is not known whether inhibition of Cdk4 modulates TGF-β signaling in pancreatic cancer cells.

Here, we report that the Cdk4/6 inhibitor, PD-0332991, exerted growth inhibitory effects on 3 human pancreatic cancer cell lines. However, PD-0332991 upregulated multiple genes implicated in the regulation of ECM remodeling and pancreatic cancer invasion and metastasis. Moreover, PD-0332991 induced EMT, and enhanced the invasion of COLO-357 and PANC-1 cells, but not AsPC-1 cells, and increased Smad transcriptional activity. SB-505124, a TβRI kinase inhibitor, completely blocked EMT induced by PD-0332991. Thus, anti-Cdk4/6 therapy may induce EMT and enhance pancreatic cancer cell invasion by activating components of the TGF-β signaling cascade.

Materials and Methods

Cell culture

AsPC-1 and PANC-1 human pancreatic cancer cells were obtained from and authenticated by the American Type Culture Collection. COLO-357 cells were a gift from Dr. R. Metzger at Duke University (Durham, NC), and were originally placed in culture from a patient with metastatic PDAC (26). They were authenticated by chromosomal analysis. AsPC-1 cells were grown in RPMI-1640, and PANC-1, and COLO-357 cells were grown in Dulbecco’s Modified Eagle’s Medium. Media were supplemented with 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (complete medium).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was done as described previously (27).

Clonogenic assay

Clonogenic assay was done as described previously (28).

Cell-cycle analysis

Cells were fixed in 70% ethanol at 4 °C, and resuspended in PI/RNase solution (BD Pharmingen). For each sample, 5 × 10^4 propidium iodide (PI)-labeled cells were collected on a BD FACS Caliber flow cytometer. Data analysis was conducted using FlowJo software.

Immunoblotting

Immunoblotting was done as described previously (27). Antibodies for the following antigens were purchased: phospho-Rb (Ser 807/811), total Rb (4H1), Cyclin D1 (DCS6), and Slug (C1967), from Cell Signaling Technology; Cdk4 (C-22), Cdk6 (C-21), Cdk2 (D-12), Twist (H81), p15 (C-20), and ERK2 (C-14), from Santa Cruz Biotechnology; N-cadherin and E-cadherin from BD Biosciences; and β-catenin (9G10) from Calbiochem. Horseradish peroxidase–conjugated anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad.

Immunocytofluorescence

Cells seeded on LabTek chamber slides (Nalge Nunc) were washed in PBS, and fixed in 10% formalin for 15 minutes at room temperature. Cells were then permeabilized with 0.15% Triton X-100 in PBS for 2 minutes and incubated in blocking buffer [1% bovine serum albumin and 5% FBS in PBS] for 30 minutes. Primary antibodies were added for 1 hour, followed by incubation with Alexa Fluor–labeled secondary antibodies for 30 minutes, and mounted in Prolong Gold antifade reagent with 4’-6-diamino-2-phenylindole (DAPI; Invitrogen). Antibodies directed against N-cadherin, E-cadherin, and vimentin were from BD Biosciences. Cells were scanned with a Zeiss LSM 510 laser-scanning confocal microscope using a ×60 (water) magnification.

Illumina-microarray gene analysis

Microarray analysis was conducted by the Genomics and Microarray Core Facility at Dartmouth Medical School (Hanover, NH). Briefly, RNeasy purification kit (Qiagen) was used to extract total RNA. Each experimental condition had 3 biologic replicates. Biotin-labeled cRNA was generated from total RNA using Illumina TotalPrep Kit (Ambion), and hybridized to Illumina HumanHT-12 BeadChips (version 4.00; Illumina). BeadChips were imaged using the Illumina BeadArray Reader (Illumina, Inc.). Array data analysis was conducted using BRB-Array Tools, version 3.8.1 and the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7). The array data have been registered with GEO (GSE39646) for public access.

Reverse transcription and real-time quantitative PCR

Total RNA was extracted using RNeasy purification kit (Qiagen), and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit. TaqMan quantitative real-time PCR (qRT-PCR) was carried out on an ABI Prism 7300 machine and analyzed using a StepOnePlus Real-Time PCR system, all from Applied Biosystems. All probes were predesigned and obtained from Applied Biosystems. 18S was used as internal control. Gene expression levels were calculated using the relative ΔCt method.

Cell invasion assay

Cell invasion assay was done as described previously (29).

Cdk4/6 knockdown using lentivirus-delivered shRNA

pTRIPZ lentiviral vectors for Cdk4 and Cdk6 were from Open Biosystems. A scrambled control shRNA was...
designed not to target mammalian genes: shCtrl sense TGCTGTTGACCTGAGCGCAACAATACGATTAA-
GATATTAGGCAAGCAGTGAATACTCTTAA-
ATCGTTATTGGCTGCTACTGCGCTGGG and shCtrl antisense AATTCGAGCAGTGAGCGACACAATA-
ACGATATGAGTACATCTGCGTTACACTAATT-
ACTCTTAACTCTATGCCCCTACTGTCAACAGCA.
The sense and antisense oligonucleotides (Sigma) were annealed and cloned into the HpaI/EcoRI sites of the pTRIPZ lentiviral vector. Packaging was conducted using a second generation plasmid transfection system as follows: 293T cells were transfected with 60 μg pTRIPZ lentiviral vector construct, 40 μg packaging plasmid psPAX2 (Addgene), and 20 μg packaging plasmid pMD2.G (Addgene). The supernatant containing the virus particles was collected at 48 and 72 hours after transfection and concentrated using an ultracentrifuge at 25,000 rpm for 1.5 hours. The pellets were suspended in 25 μL Opti-MEM (Invitrogen) overnight, pooled together, and aliquoted. After infecting cells with lentivirus in the presence of 8 μg/mL polybrene (Sigma), cells were selected with 6 μg/mL puromycin, and 2 μg/mL doxycycline was added for 72 hours before Cdk4/6 knockdown was examined by immunoblotting.

**Transient transfection and luciferase assay**

SBE4-Luc and a control reporter construct (pRenilla) were cotransfected at the ratio of 100:1 using Lipofectamine 2000 (Invitrogen). Four hours later, cells were incubated for 20 hours in complete medium in the absence or presence of 1 nmol/L TGF-β, 5 μmol/L PD-0332991, or both TGF-β and the inhibitor. Cells were then lysed and luciferase assays were conducted using the Dual Luciferase Assay Kit (Promega). Luminescence was measured using a TD-20/20 luminometer (Turner Designs).

**3-dimensional culture**

Cells were cultured in a 3-dimensional culture system as previously reported (30).

**Statistical methods**

Statistical analysis was conducted using the GraphPad InStat software (version 3.00; GraphPad Software Inc.). Significance was determined using one-way ANOVA, followed by the Dunnett test to compare all groups against the corresponding control group, and the Benferroni test for specific pairwise comparisons. Statistical significance was taken as \( P \leq 0.05 \).

**Results**

**PD-0332991 inhibits the growth of human PDAC cell lines**

The effects of PD-0332991 on cell growth were evaluated by MTT assay. In all 3 cell lines, PD-0332991 exerted a dose- and time-dependent growth inhibitory effect. AsPC-1 was most resistant to the antiproliferative effect of PD-0332991, with an IC\(_{50}\) of 10 μmol/L at 72 hours. COLO-357 and PAN-1 cells were more sensitive to PD-0332991, with IC\(_{50}\) values of 5 μmol/L at 72 hours (Fig. 1A). PD-0332991 also exerted a dose-dependent inhibition of colony formation (Supplementary Fig. S1). In all 3 cell lines, PD-0332991 induced G\(_{0}\)–G\(_{1}\) cell-cycle arrest in a dose-dependent manner within 24 hours. At a concentration of 10 μmol/L, PD-0332991 exerted cytotoxic effects in COLO-357 and PANC-1 cells and triggered G\(_{2}\)–M arrest in the surviving cells. Following prolonged incubation (up to 72 hours), AsPC-1 cells gradually resumed cell-cycle progression, even in the presence of high concentrations of PD-0332991, whereas COLO-357 and PANC-1 cells were still growth inhibited by 1 and 5 μmol/L PD-0332991 at 72 hours (Fig. 1B).

Cdk4/6 bind to Cyclin D1 and phosphorylate and inactivate retinoblastoma protein (Rb), thus allowing E2F release and cell-cycle progression. Therefore, we next sought to assess the impact of PD-0332991 on Rb and E2F. Cdk4/6-specific Rb phosphorylation at Serine 807/811 was inhibited by PD-0332991, which also decreased the level of the E2F target gene, Cdk2. Moreover, PD-0332991 caused marked downregulation of total Rb protein and significant upregulation of Cyclin D1, Cdk4, and Cdk6 (Fig. 1C). Collectively, these results suggest that PD-0332991 inhibited Cdk4/6 kinase activity, that this effect was associated with loss of Rb function most likely due to enhanced Rb degradation, and that PD-0332991 suppressed the growth of pancreatic cancer cells, but only transiently in AsPC-1 cells.

**PD-0332991 upregulates the expression of genes promoting invasion and metastasis**

Microarray analysis was conducted next using RNA from AsPC-1 and COLO-357 cells that had been incubated in the absence or presence of PD-0332991 for 24 or 72 hours. At 24 hours, 131 genes were downregulated at least 2-fold by PD-0332991 in AsPC-1 cells. These genes were enriched in cell-cycle regulation, DNA metabolic processes and damage response, cytoskeleton organization, intracellular signaling, and ubiquitin-proteasomal degradation pathways. Most of these downregulated cell-cycle–related genes recovered their expression at 72 hours. PD-0332991 also upregulated 64 genes, which encode proteins implicated in the regulation of angiogenesis, ECM remodeling, cell adhesion, cell motility, and inflammatory responses, at both 24 and 72 hours (Fig. 2A). Similar results were obtained in COLO-357 cells, except that these cells displayed more persistent inhibition of cell-cycle–related genes in the presence of PD-0332991, by comparison with AsPC-1 (Fig. 2B).

Gene expression profiles of AsPC-1 and COLO-357 cells revealed 122 common genes with at least a 2-fold change in both cell lines after 24 hours of incubation with PD-0332991, most of which were enriched in cell-cycle regulation (Fig. 2C, Supplementary Table S1). We also noted 16 common genes with at least 2-fold change in both cell lines after a 72-hour incubation with PD-0332991, most of which were involved in regulating angiogenesis, cell...
motility, ECM remodeling, and inflammatory responses (Fig. 2C, Supplementary Table S2).

We next selected 12 representative genes for validation, based on literature mining about their biologic functions in PDAC. Thus, from the 122 common genes with at least 2-fold change after a 24-hour incubation with PD-0332991 in both cell lines, we selected 6 genes for validation that were markedly downregulated and that have been reported to be important in pancreatic cancer cell chemoresistance and growth (31–33), including 3 genes implicated in regulating the cell cycle and cytoskeleton organization (AURKA, AURKB, KIF20A), and 3 genes involved in DNA synthesis and damage repair (RRM1, RRM2, TYMS). Both microarray and qRT-PCR results showed that these cell-cycle–related genes were dramatically suppressed after incubation with PD-0332991 for 24 hours (Fig. 2D), and that their expression recovered to some degree after 72 hours (Supplementary Fig. S2A).

Similarly, among the 16 genes whose expression changed by at least 2-fold in both cell lines following a 72-hour incubation with PD-0332991, 6 genes (LAMC2, LAMA3, Cyr61, SERPINE1, F3, and ABCA1) were selected for validation due to their potential role in PDAC metastasis and EMT (34–38), revealing marked concordance between array and qRT-PCR data (Fig. 2D).

qRT-PCR indicated that PD-0332991 also inhibited the expression of same cell-cycle–related genes in PANC-1 cells, but only upregulated expression of ABCA1 (Supplementary Fig. S2B and S2C).

PD-0332991 induces EMT and enhances invasion in TGF-β-sensitive cells

We next sought to investigate the effects of PD-0332991 on pancreatic cancer cell invasion. Accordingly, PANC-1 and COLO-357 cells were incubated with PD-0332991 for either 72 hours or 8 days, and their invasiveness was determined. In both cell lines, which are wild-type for SMAD4, PD-0332991 significantly increased EGF-mediated invasion (Fig. 3A and 3B). By contrast, PD-0332991 did not alter invasion in AsPC-1 cells (Supplementary Fig. S3), which harbor an inactivating SMAD4 mutation (39).

Cancer cells tend to acquire mesenchymal characteristics before becoming invasive (40). To determine whether PD-0332991 induced EMT, we examined the effects of PD-
0332991 on the expression of epithelial and mesenchymal markers. COLO-357 cells began to display increased expression of mesenchymal markers following a 72-hour incubation with PD-0332991. After 8 days, COLO-357 cells lost their close cell–cell contacts becoming more scattered and assuming a spindle-shaped mesenchymal morphology. Moreover, there was marked induction of β-catenin, Slug, Twist, N-cadherin, and vimentin. Although PD-0332991 did not alter E-cadherin expression, it induced its relocation from the cell membrane and sites of cell–cell contacts into cytosol, suggesting that it caused functional E-cadherin perturbations (Fig. 3C). PANC-1 cells responded faster to PD-0332991 and displayed an invasive phenotype within 72 hours of incubation (Fig. 3D), whereas these changes were not observed in AsPC-1 cells (Supplementary Fig. S3). Thus PD-0332991 activated an EMT program and enhanced the invasion of COLO-357 and PANC-1 cells, but not AsPC-1 cells.

Inhibition of Cdk4/6 using shRNA induces EMT in TGF-β-sensitive cells

To determine whether targeted disruption of Cdk4 and Cdk6 would lead to similar results, cells were stably...
transduced with lentiviruses encoding shRNA against cdk4, cdk6, or a scrambled control. Knockdown of Cdk4/6 was confirmed by immunoblotting. Cdk4/6 knockdown in COLO-357 cells was associated with loss of cell–cell contacts and assumption of a scattered, spindle-shaped mesenchymal morphology, accompanied by induction of β-catenin, Slug, N-cadherin, and vimentin. Although E-cadherin expression was not altered, it relocated from the cell membrane and sites of cell–cell contacts into the cytosol (Fig. 4A and B). Similar results were obtained in PANC-1 cells (Supplementary Fig. S4), but not in AsPC-1 cells. Next, we measured the expression of the 6 representative proinvasion genes found to be upregulated by PD-0332991. Most of these genes were induced following Cdk4/6 knockdown, and this effect was more marked in COLO-357 compared with AsPC-1 cells (Fig. 4C). Thus, silencing of Cdk4/6 with shRNA also activated an EMT program in COLO-357 and PANC-1 cells, but not in AsPC-1 cells.

Cdk4/6 inhibition increases Smad transcriptional activity and activates TGF-β signaling

Nuclear Cdk4 phosphorylates the Smad3 linker region, thereby inhibiting its transcriptional activity (24). Therefore, we next sought to investigate the effects of Cdk4/6 inhibition on TGF-β/Smad signaling by assessing the expression of p15, a canonical target gene for Smad-dependent TGF-β signaling (20). In COLO-357 cells, PD-0332991 induced p15 expression within 24 hours, and this effect was further increased following prolonged
incubation (Fig. 5A). In contrast, PD-0332991 did not induce p15 expression in either AsPC-1 cells (Fig. 5A) or PANC-1 cells (not shown). Similar results were obtained following Cdk4/6 silencing (Fig. 5A). Next, Smad transcriptional activity was assessed using a TGF-β-responsive reporter construct driven by consensus binding sites for Smad3 and Smad4 (SBE4-Luc). AsPC-1 did not respond to either TGF-β or PD-0332991, whereas both COLO-357 and PANC-1 cells exhibited increased TGF-β-induced Smad transcriptional activity in the presence of PD-0332991 (Fig. 5B). Similarly, Cdk4/6 knockdown enhanced TGF-β-mediated Smad transcriptional activity in COLO-357 and PANC-1 cells, but not in AsPC-1 cells (Supplementary Fig. S5).

Cells were next incubated with PD-0332991 in the absence or presence of SB-505124, a TβRI kinase inhibitor. In COLO-357 cells, but not in AsPC-1 cells, SB-505124 completely blocked PD-0332991-mediated induction of p15 and proinvasion and EMT-associated genes, including β-catenin, Slug, and N-cadherin (Fig. 5C and D). Similar results, with respect to the expression of EMT-associated genes, were obtained in PANC-1 cells, even though neither PD-0332991 nor SB-505124 altered p15 expression in these cells (Fig. 5D). Thus, Cdk4/6 inhibition increased Smad transcriptional activity and activated TGF-β signaling, thereby inducing EMT in a manner that was independent of p15 induction.

Combination of PD-0332991 and SB-505124 inhibits COLO-357 and PANC-1 colony growth

We next sought to investigate the effects of PD-0332991 and SB-505124 on colony growth in 3-dimensional culture...
COLO-357 cells grew into large, well-organized colonies, whereas PANC-1 cells formed large disorganized colonies with irregular epithelial budding extensions (Supplementary Fig. S6A). PD-0332991 decreased the size of COLO-357 and PANC-1 colonies, but increased epithelial budding conferring an invasive appearance to the cells. In contrast, SB-505124 did not alter colony growth in either COLO-357 or PANC-1 cells. However, the combination of PD-0332991 and SB-505124 decreased colony number and size in both cell lines without inducing a more invasive phenotype (Fig. 6A and B). Although AsPC-1 cells did not form large colonies in 3-dimensional culture and failed to respond to either drug (Fig. 6A and B), all 3 cell lines were growth inhibited in a dose-dependent manner by PD-0332991 in a clonogenic assay, and the addition of SB-505124 to PD-0332991 resulted in greater...

Figure 5. Effects of PD-0332991 and Cdk4/6 inhibition on Smad transcriptional activity. A, effects of PD-0332991 and Cdk4/6 silencing on p15 levels. AsPC-1 and COLO-357 cells that were incubated in the absence or presence of 5 μmol/L PD-0332991 (left) or stably transduced with shRNA against scrambled control, cdk4, or cdk6 (right) were immunoblotted for p15. B, effects of TGF-β and PD-0332991 on SBE4-Luc activity. Cells cotransfected with SBE4-Luc reporter plasmid and pRenilla control reporter plasmid were incubated with vehicle, 1 nmol/L TGF-β, 5 μmol/L PD-0332991, or both for 20 hours. Results from dual-luciferase reporter assay were normalized to the Renilla levels and then calculated relative to vehicle control. C, expression of proinvasion genes. RNA samples from AsPC-1 and COLO-357 cells incubated for 72 hours with vehicle, 5 μmol/L PD-0332991, 2 μmol/L SB-505124, or both inhibitors were subjected to qRT-PCR. Results were normalized to 18S levels and calculated relative to vehicle control. The structure of SB-505124 is shown next to C. D, expression of EMT markers. COLO-357 and PANC-1 cells incubated for 72 hours with vehicle, 5 μmol/L PD-0332991, 2 μmol/L SB-505124, or both inhibitors. Data are the mean ± SEM of 3 independent experiments, analyzed using one-way ANOVA. *, P < 0.05; **, P < 0.01, compared with vehicle. #, P < 0.05; ##, P < 0.01, compared with TGF-β.
growth inhibition than in the presence of either inhibitor alone (Supplementary Fig. S6B).

Discussion

In the current study, we determined that the Cdk4/6 inhibitor PD-0332991 increased Smad transcriptional activity, induced EMT, enhanced expression of metastasis-associated genes, and promoted invasion in COLO-357 and PANC-1 cells, which are growth inhibited by TGF-β, but not in AsPC-1 cells, which are resistant to TGF-β–mediated growth inhibition due to the presence of a SMAD4 mutation (39). Moreover, knockdown of Cdk4/6 using shRNA mimicked the effects of PD-0332991 on Smad transcriptional activity and EMT induction, whereas SB-505124, a TβRI kinase inhibitor, completely blocked EMT induction by PD-0332991. These findings indicate that PD-0332991 has the capacity to exert deleterious effects in certain pancreatic cancer cells that are mediated, at least in part, by the activation of TGF-β signaling pathways.

COLO-357 cells displayed sustained growth arrest and persistent downregulation of cell-cycle–related genes in the presence of PD-0332991, whereas AsPC-1 cells were only transiently arrested in G0–G1 phase by PD-0332991 and its effects on cell-cycle gene expression was also transient. Moreover, PD-0332991 upregulated genes that promote PDAC invasion, metastasis, and chemoresistance. Thus, LAMC2 encodes laminin-γ2 chain, which together with laminin-α3 (LAMA3) and β3 (LAMB3) chains, comprise laminin-5, the major component of basement membrane and tumor ECM. Pancreatic cancer cells synthesize and deposit laminin-5 in basement membrane, and invading cancer cells adhere to this newly produced basement membrane and migrate on it (34). Laminin-γ2 also increases invasive potential and correlates with distant metastasis in PDAC (35–36). Cyr61 encodes a secreted, cysteine-rich, heparin-binding protein, which plays important roles in cell adhesion, migration, EMT, and angiogenesis (37). Expression of SERPINE1, encoding plasminogen activator inhibitor type-1 (PAI-1), is increased in epithelial cells undergoing EMT and in cancer cells and myofibroblasts at the invasive front (41). Tissue factor (F3) is the initiating cell surface receptor for the extrinsic coagulation cascade. It is abundant in pancreatic cancer cells, and it stimulates tumor invasiveness (38). Moreover, ABCA1 was the only gene among the 6 genes that was expressed at high levels in both COLO-357 and PANC-1 cells. ABCA1 is an ATP-binding cassette (ABC) transporter that enhances chemoresistance by...
increasing drug efflux (42), raising the possibility that PD-0332991 may act to promote chemoresistance in pancreatic cancer cells.

Prolonged incubation with PD-0332991 induces flattened, senescence-like morphologic changes in glioblastoma, melanoma, and breast cancer cells, accompanied by senescence-associated β-galactosidase (SA-βGal) activity (15, 43, 44). However, our findings argue against the induction of senescence by PD-0332991 in pancreatic cancer cells. First, prolonged (8–10 days) incubation of COLO-357 and PANC-1 cells with PD-0332991 markedly enhanced their invasive capacity and increased the expression of mesenchymal markers and EMT-associated transcription factors, indicating that these cells underwent EMT instead of senescence. Second, SA-βGal staining was not evident in these cells even after a 15-day incubation with PD-0332991 (data not shown). Third, Rb protein levels were markedly decreased following prolonged incubation with PD-0332991, and induction of senescence by PD-0332991 is dependent on Rb (15, 44).

PDAC is associated with increased expression of many EMT-triggering factors, such as TGF-β, BMP, Wnt, Hedgehog (8, 9, 22). Furthermore, decreased E-cadherin and increased N-cadherin, vimentin, and fibronectin expression in PDAC correlate with increased neural invasion, liver metastasis, and poor prognosis (45). These observations indicate that EMT contributes to the high metastatic potential of PDAC. The ability of PD-0332991 to upregulate the expression of EMT-inducing transcription factors, and genes associated with PDAC invasion and metastasis that are upregulated by TGF-β (41, 46), raises the possibility that PD-0332991 may exert deleterious effects in patients with PDAC and suggests that PD-0332991 may act, in part, by activating deleterious TGF-β signaling. In support of this possibility, SB-505124, a potent and specific small-molecule inhibitor of TβRI (47), reversed the deleterious actions of PD-0332991. Taken together, these observations suggest that PD-0332991 mimics tumor-promoting actions of TGF-β (19) by suppressing the inhibitory effect of Cdk4/6 on Smad signaling and potentiating TGF-β signaling and that TβRI is required to initiate these signaling events. Conversely, TGF-β1–mediated growth inhibition in COLO-357 and PANC-1 cells occurs in conjunction with EMT induction (48).

SB-505124 did not suppress colony growth in 3-dimensional culture, whereas the combination of PD-0332991 and SB-505124 achieved optimal growth-inhibitory effects and abrogated the EMT effects elicited by PD-0332991. Given that the combination of PD-0332991 and SB-505124 potently inhibited colony growth in 3-dimensional culture of 2 pancreatic cancer cells that harbor a wild-type SMAD4 gene, we propose that combining PD-0332991 and SB-505124 may represent a novel therapeutic strategy in the 45% of patients with PDAC whose cancer does not harbor a SMAD4 mutation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: F. Liu, M. Korc

Development of methodology: F. Liu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Liu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Liu

Writing, review, and/or revision of the manuscript: F. Liu, M. Korc

Study supervision: M. Korc

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