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

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Running Title: Cdk4/6 inhibition in pancreatic cancer

Keywords: Pancreatic cancer; Cdk4/6; PD-0332991; EMT; TGF-β

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The authors declare that they do not have any conflicts of interest with respect to this study.
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 down-regulated cell-cycle-related genes, but up-regulated genes implicated in extracellular matrix (ECM) remodeling and pancreatic cancer cell invasion and metastasis. Moreover, PD-0332991 enhanced invasion in transforming growth factor-beta (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.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the US, with a median survival of 6-7 months and a five-year survival rate of 6% (1). While resection prolongs survival and offers a potential cure, 80-85% of PDAC 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 drug delivery (3, 4).

PDAC is characterized by a high frequency of activating K-RAS mutations (5) as well as by 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 loss of negative control over Cdk4/6, leading to excessive activation of CyclinD-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’s lymphoma showed that PD-0332991 was generally well tolerated (18). Whether PD-0332991 could be used to treat PDAC is currently unknown.
There are three mammalian TGF-β ligands which bind to the type II TGF-β receptor homodimer, thereby recruiting and activating TβRII homodimers. Phosphorylated TβRII 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 co-activators and co-repressors 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 biological 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 anti-proliferative 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 three human pancreatic cancer cell lines. However, PD-0332991 up-regulated 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 TβRII 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 American Type Culture Collection (Manassas, VA). COLO-357 cells were a gift from Dr. R. Metzger at Duke University, 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 DMEM. Media were supplemented with 5% fetal bovine serum (FBS), 100 units/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, San Diego, CA). For each sample, 5×10⁴ PI-labeled cells were collected on a BD FACS Caliber flow cytometer. Data analysis was performed using FlowJo software.

Immunoblotting. Immunoblotting was done as described previously (27). Antibodies for the following antigens were purchased: phosho-Rb (Ser 807/811), total Rb (4H1), Cyclin D1 (DCS6), and Slug (C1967), from Cell Signaling Technology, Danvers, MA; Cdk4 (C-22), Cdk6 (C-21), Cdk2 (D-12), Twist (H81), p15 (C-20) and ERK2 (C-14), from Santa Cruz Biotechnology, Santa Cruz, CA; N-cadherin, and E-cadherin from BD Biosciences, Franklin Lakes, NJ; β-catenin (9G10) from Calbiochem, San Diego, CA. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from BioRad, Hercules, CA.
Immunocytofluorescence. Cells seeded on LabTek chamber slides (Nalge Nunc, Naperville, IL) were washed in PBS, and fixed in 10% formalin for 15 min at room temperature. Cells were then permeabilized with 0.15% Triton X-100 in PBS for 2 min, and incubated in blocking buffer (1% BSA and 5% FBS in PBS) for 30 min. Primary antibodies were added for 1 h, followed by incubation with Alexa Fluor-labeled secondary antibodies for 30 min, and mounted in Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Antibodies directed against N-cadherin, E-cadherin, and vimentin were from BD Biosciences, Franklin Lakes, NJ. Cells were scanned with a Zeiss LSM 510 laser-scanning confocal microscope using a 60X (water) magnification.

Illumina-microarray gene analysis. Microarray analysis was performed by the Genomics and Microarray Core Facility at Dartmouth Medical School. Briefly, RNeasy purification kit (Qiagen, Valencia, CA) was used to extract total RNA. Each experimental condition had three biological replicates. Biotin-labeled cRNA was generated from total RNA using Illumina TotalPrep Kit (Ambion, Austin, TX), and hybridized to Illumina HumanHT-12 BeadChips (version 4.00; Illumina, San Diego, CA). BeadChips were imaged using the Illumina BeadArray Reader (Illumina, Inc., San Diego, CA). Array data analysis was performed 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, Valencia, CA), and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit. Taqman quantitative real-time PCR (Q-PCR) was carried out on an ABI Prism 7300 machine and analyzed using a StepOnePlus Real-Time PCR system, all form Applied Biosystems, (Carlsbad, CA). All probes were pre-designed 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, Huntsville, AL. A scrambled control shRNA was designed not to target mammalian genes: shCtrl sense TGCTGTTGACAGTGAGCGGCACAATAACGATTAAGAGTATTAGTGGAAGCCACAGAT GTAATACTCTTTAATCGTTATTGTGCTGCTACTGCCTCGG and shCtrl antisense AATTCCGAGGCCAGTAGGCACACACAATAACGATTAAGAGTATTACATCTGTGGCTTCA CTAATACTCTTTAATCGTTATTGTGCGGCTCACTGTCAACAGCA. The sense and antisense oligonucleotides (Sigma, St Louis, MO) were annealed and cloned into the Hpal/EcoRI sites of the pTRIPZ lentiviral vector. Packaging was performed using a second generation plasmid transfection system as follows: 293T cells were transfected with 60 μg shRNA lentiviral vector construct, 40 μg packaging plasmid psPAX2 (Addgene, Cambridge, MA), and 20 μg packaging plasmid pMD2.G (Addgene, Cambridge, MA). The supernatant containing the virus particles was collected at 48 and 72 h post-transfection and concentrated using an ultracentrifuge at 25,000 rpm for 1.5 h. The pellets were suspended in 25 μL Opti-MEM (Invitrogen, Carlsbad, CA) overnight, pooled together, and aliquoted. After infecting cells with lentivirus in the presence of 8 μg/ml polybrene (Sigma, St Louis, MO), cells were selected with 6 μg/ml puromycin, and 2 μg/ml doxycycline was added for 72 h before Cdk4/6 knockdown was examined by immunoblotting.

**Transient transfection and luciferase assay.** SBE4-Luc and a control reporter construct (pRenilla) were co-transfected at the ratio of 100:1 using Lipofectamine 2000 (Invitrogen,
Four hours later, cells were incubated for 20 h in complete medium in the absence or presence of 1 nM TGF-β, 5 µM PD-0332991, or both TGF-β and the inhibitor. Cells were then lysed and luciferase assays were performed using the Dual Luciferase Assay Kit (Promega, Madison, WI). Luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

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

**Statistical methods.** Statistical analysis was performed using the GraphPad InStat software (version 3.00; GraphPad Software Inc., San Diego, CA). 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≤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 three cell lines, PD-0332991 exerted a dose- and time-dependent growth inhibitory effect. AsPC-1 was most resistant to the anti-proliferative effect of PD-0332991, with an IC50 of 10 µM at 72 h. COLO-357 and PANC-1 cells were more sensitive to PD-0332991, with IC50 values of 5 µM at 72 h (Fig 1a). PD-0332991 also exhibited a dose-dependent inhibition of colony formation (Supplemental Fig S1). In all three cell lines, PD-0332991 induced G0/G1 cell cycle arrest in a dose-dependent manner within 24 h. At a concentration of 10 µM, PD-0332991 exerted cytotoxic effects in COLO-357 and PANC-1 cells and triggered G2/M arrest in the surviving cells. Following prolonged incubation (up to 72 h), 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 µM and 5 µM PD-0332991 at 72 h (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 up-regulates the expression of genes promoting invasion and metastasis**
Microarray analysis was performed 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 h. At 24 h, 131 genes were down-regulated at least two 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 down-regulated cell-cycle-related genes recovered their expression at 72 h. PD-0332991 also up-regulated 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 h (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 h of incubation with PD-0332991, most of which were enriched in cell cycle regulation (Fig 2c, Supplemental Table 1). We also noted 16 common genes with at least 2-fold change in both cell lines after a 72 h incubation with PD-0332991, most of which were involved in regulating angiogenesis, cell motility, ECM remodeling, and inflammatory responses (Fig 2c, Supplemental Table 2).

We next selected 12 representative genes for validation, based on literature mining about their biological functions in PDAC. Thus, from the 122 common genes with at least 2-fold change after a 24 h incubation with PD-0332991 in both cell lines, we selected 6 genes for validation that were markedly down-regulated 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 Q-PCR results showed that these cell-cycle-related genes were dramatically suppressed after incubation with PD-0332991 for 24 h (Fig 2d), and that their expression recovered to some degree after 72 h (Supplemental Fig S2a). Similarly, among the 16 genes whose expression changed by at least 2-fold in both cell lines following a 72 h 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 Q-PCR data (Fig 2d).

Q-PCR indicated that PD-0332991 also inhibited the expression of same cell-cycle-related genes in PANC-1 cells, but only up-regulated expression of ABCA1 (Supplemental Fig S2b, c).

**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 h 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, 3b). By contrast, PD-0332991 did not alter invasion in AsPC-1 cells (Supplemental Fig S3), which harbor an inactivating SMAD4 mutation (39).

Cancer cells tend to acquire mesenchymal characteristics prior to 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 h 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 h of incubation (Fig 3d), whereas these changes were not observed in AsPC-1 cells (Supplemental 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, b). Similar results were obtained in PANC-1 cells (Supplemental Fig. S4), but not in AsPC-1 cells. Next, we measured the expression of the 6 representative pro-invasion 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 (Figure 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 h, and this effect was further increased following prolonged incubation (Fig 5a). By 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 (Supplemental 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 pro-invasion and EMT-associated genes, including β-catenin, Slug, and N-cadherin (Fig 5c, 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-D culture (30). COLO-357 cells grew into large, well-organized colonies, while PANC-1 cells formed large disorganized colonies, with irregular epithelial budding extensions (Supplemental 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. By 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 (Figure 6a, b). Although AsPC-1 cells did not form large colonies in 3-D culture and failed to respond to either drug (Figure 6a, 6b), all three 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 growth inhibition than in the presence of either inhibitor alone (Supplemental 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 activation of TGF-β signaling pathways.

COLO-357 cells displayed sustained growth arrest and persistent down-regulation 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 six 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 morphological 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 to 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 PDAC patients 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 TGF-β’s tumor-promoting actions (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-D 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-D culture of two 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 PDAC patients whose cancer does not harbor a SMAD4 mutation.

Acknowledgements

We thank the Indiana Center for Biological Microscopy for their assistance with confocal microscopy.

Grant Support

NIH Grant CA-R37-075059 to M. Korc
References


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Figure Legends

Figure 1. PD-0332991 exerts growth inhibitory effects on human pancreatic cancer cells. AsPC-1, COLO-357, and PANC-1 cells were incubated in the absence or presence of varying PD-0332991 concentrations. MTT assays (a), cell cycle analysis (b), and immunoblotting (c) were performed. Data are the means ± SEM of 3 independent experiments. ** p< 0.01, compared with control. The structure of PD-0332991 is shown in panel (a).

Figure 2. Effects of PD-0332991 on gene expression. (a, b) Array analysis following incubation of AsPC-1 and COLO-357 cells in the absence or presence of 5 μM PD-0332991 for 24 h and 72 h. (c) Venn Diagrams of the number of genes with a fold change ≥2 following incubation with PD-0332991. (d) Q-PCR of 6 representative genes from the 122 common genes at 24 h (upper panel) and 6 representative genes from the 16 common genes at 72 h (lower panel). Results were normalized to 18S levels and calculated relative to vehicle control. Data are the means ± SEM of 3 independent experiments.

Figure 3. Effects of PD-0332991 on invasion and EMT. (a, b) Cell invasion. Cells were incubated in the absence or presence of 5 μM PD-0332991 for 72 h (PANC-1 cells) or 8 d (COLO-357 cells), prior to being plated on the top wells of Matrigel-coated chambers with or without 1 nM EGF in the lower compartments. After 16-18 h, cells that had invaded through the Matrigel layer were fixed and stained. (c, d) EMT. Cells were incubated in the absence or presence of 5 μM PD-0332991 for either 72 h (PANC-1 cells) or 8 d (COLO-357 cells) before being subjected to immunoblotting or immunofluorescent staining. Scale, 20 μm. Data are the means ± SEM of 3 independent experiments, analyzed using one-way ANOVA. # p < 0.05,
compared with control in serum-free medium; ** p < 0.01, compared with control in the presence of EGF.

Figure 4. Effects of cdk4 and cdk6 knockdown on EMT. (a) Immunoblotting. Lysates from AsPC-1 and COLO-357 cells stably expressing shRNA against scrambled control, cdk4 or cdk6 were subjected to immunoblotting. (b) Immunofluorescent staining for DAPI and EMT markers in COLO-357 cells stably expressing shRNA against scrambled control, cdk4 or cdk6. (c) Q-PCR analysis of the 6 representative pro-invasion genes. Results were normalized to 18S levels and calculated relative to scrambled control. Scale, 20 µm. Data are the means ± SEM. of 3 independent experiments, analyzed using one-way ANOVA. * p < 0.05; ** p < 0.01, compared with control.

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 µM PD-0332991 (left panel), or stably transduced with shRNA against scrambled control, cdk4, or cdk6 (right panel), were immunoblotted for p15. (b) Effects of TGF-β and PD-0332991 on SBE4-Luc activity. Cells co-transfected with SBE4-Luc reporter plasmid and pRenilla control reporter plasmid were incubated with vehicle, 1 nM TGF-β, 5 µM PD-0332991, or both for 20 h. Results from dual-luciferase reporter assay were normalized to the Renilla levels and then calculated relative to vehicle control. (c) Expression of pro-invasion genes. RNA samples from AsPC-1 and COLO-357 cells incubated for 72 h with vehicle, 5 µM PD-0332991, 2 µM SB-505124, or both inhibitors were subjected to Q-PCR. Results were normalized to 18S levels and calculated relative to vehicle control. The structure of SB-505124 is shown next to panel (c). (d) Expression of EMT markers. COLO-357 and PANC-1 cells incubated for 72 h with vehicle, 5 µM PD-0332991, 2 µM SB-505124, or both inhibitors.
Data are the means ± 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.

Figure 6. Effects of PD-0332991 and SB-505124 on colony growth in 3-D culture. Cells were seeded in 3% MatrigelTM/soft agar, and incubated with vehicle, 5 μM PD-0332991, 2 μM SB-505124, or both inhibitors. (a) Representative images of epithelial structures captured after a 14 day incubation. (b) Quantitative analysis. Total area of MTT-positive colonies was quantified 12-14 days after incubation with the additions indicated above. Data are the means ± SEM of 3 independent experiments, analyzed using one-way ANOVA. ** p < 0.01, compared with vehicle. ## p<0.01, compared with PD-0332991.
Fig 3
**Fig 4**

**a.**

<table>
<thead>
<tr>
<th></th>
<th>AsPC-1</th>
<th>COLO-357</th>
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<tbody>
<tr>
<td>shRNA-scramble</td>
<td>+ - -</td>
<td>+ - -</td>
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<tr>
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</tr>
<tr>
<td>shRNA-cdk6</td>
<td>- - +</td>
<td>- - +</td>
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</tbody>
</table>

**Immunoblotting**

- Cdk4
- Cdk6
- β-catenin
- Slug
- N-cad
- E-cad
- Erk2

**b.**

**Immunofluorescence**

- Phase Contrast
- N-cad/DAPI
- E-cad/DAPI
- vimentin/DAPI

**c.**

**Graphs**

- **AsPC-1**
- **COLO-357**

- LAMC2
- SERINE1
- APCAL
- AMA1
- F3
- YR61

- Fold Change

- shRNA-scramble
- shRNA-cdk4
- shRNA-cdk6

*Significant differences*
Fig 5
Fig 6
Molecular Cancer Therapeutics

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

Fang Liu and Murray Korc

Mol Cancer Ther Published OnlineFirst August 6, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0562

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/08/06/1535-7163.MCT-12-0562.DC1

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