Targeting Inhibitors of the Tumor Suppressor PP2A for the Treatment of Pancreatic Cancer

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Abstract

Pancreatic cancer is a deadly disease that is usually diagnosed in the advanced stages when few effective therapies are available. Given the aggressive clinical course of this disease and lack of good treatment options, the development of new therapeutic agents for the treatment of pancreatic cancer is of the utmost importance. Several pathways that have shown to contribute to pancreatic cancer progression are negatively regulated by the tumor suppressor protein PP2A. Here, the endogenous inhibitors of PP2A, SET (also known as I2PP2A) and cancerous inhibitor of PP2A (CIP2A), were shown to be overexpressed in human pancreatic cancer, contributing to decreased PP2A activity and overexpression and stabilization of the oncoprotein c-Myc, a key PP2A target. Knockdown of SET or CIP2A increases PP2A activity, increases c-Myc degradation, and decreases the tumorigenic potential of pancreatic cancer cell lines both in vitro and in vivo. Moreover, treatment with a novel SET inhibitor, OP449, pharmacologically recapitulates the phenotypes and significantly reduces proliferation and tumorigenic potential of several pancreatic cancer cell lines, with an accompanying attenuation of cell growth and survival signaling. Furthermore, primary cells from patients with pancreatic cancer were sensitive to OP449 treatment, indicating that PP2A-regulated pathways are highly relevant to this deadly disease.

Implications: The PP2A inhibitors SET and CIP2A are overexpressed in human pancreatic cancer and are important for pancreatic cancer cell growth and transformation; thus, antagonizing SET and/or CIP2A may be an innovative approach for the treatment of human pancreatic cancer. Mol Cancer Res; 12(6); 924–39. ©2014 AACR.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and is associated with a high rate of mortality, as it is generally diagnosed in the advanced stages when no successful treatments are available. The 5-year survival rate for patients with pancreatic cancer remains at 6%; moreover, the incidence of pancreatic cancer is increasing and is estimated to be the second leading cause of cancer deaths in the United States by 2020 (1, 2). Current approved therapies include gemcitabine, a nucleoside analog, and gemcitabine/erlotinib, a small molecule that inhibits EGF receptor (EGFR; ref. 3). Newer combination chemotherapy regimens such as FOLFIRINOX and gemcitabine/Abraxane have shown improved efficacy but cause profound toxicity (4). Even so, there are no curative treatment options for advanced or metastatic pancreatic cancer with most patients dying within 1 year (5). Given the aggressive nature of this disease and lack of good treatment options, the development of new targeted therapies with less toxicity and greater efficacy is critically important.

Several studies have analyzed the gene and protein expression profiles in pancreatic tumor samples. These studies have revealed a number of common oncogenic alterations, including activating mutations in KRAS, loss of SMAD4 and INK4A expression, and loss or mutation of TP53, with resulting activation of multiple oncogenic signaling pathways (6). Interestingly, many of these cancerous signaling pathways are negatively regulated by PP2A. PP2A, the major serine/threonine-specific phosphatase in mammalian cells (7), helps control a number of cellular processes, including cell-cycle progression, and it is a critical tumor suppressor, the inhibition of which is required to fully transform human cells (8, 9). Key signaling pathways that are negatively regulated by PP2A include members of the Raf/MEK/ERK and PI3K/AKT pathways, NF-κB, c-Myc, and WNT signaling (10).
Regarding c-Myc, PP2A specifically dephosphorylates serine 62 (S62), a key residue in c-Myc that is phosphorylated in response to mitogen stimulation and Ras/Raf/MEK/ERK signaling (11). Phosphorylation at S62 increases c-Myc transactivation of pro-growth genes and slows c-Myc protein degradation (12, 13). Thus, increasing PP2A activity toward c-Myc suppresses its oncogenic activity and stimulates its ubiquitination catalyzed by SCF^Hoe7, leading to degradation by the proteasome (14).

The catalytic activity of PP2A is regulated by holoenzyme formation, including a structural A subunit and a variable B subunit that directs substrate specificity. In addition, the catalytic C subunit is regulated by phosphorylation at Y307 (15) and methylation at the C-terminus (16). Inhibition of PP2A in cancer can occur through its inactivation by viral oncoproteins, mutation of specific subunits, or by overexpression of endogenous inhibitors (7, 9). Endogenous inhibitors of PP2A include SET (I2PP2A) and cancerous inhibitor of PP2A (CIP2A). CIP2A was initially identified to interact with PP2A and c-Myc to stabilize c-Myc (17), and its overexpression cooperates with Ras and c-Myc to transform primary mouse embryo fibroblasts (7). Reports indicate that CIP2A is frequently overexpressed in several tumor types, including head and neck squamous cell carcinoma, colon cancer, gastric cancer, breast cancer, and most recently, pancreatic cancer (18, 19). SET is also reported to be upregulated in multiple cancer types, including chronic myelogenous leukemia (CML), Wilms tumor, malignant brain tumors, tumors of the head and neck, and testicular cancers (9, 20). Furthermore, SET expression levels have been correlated with more aggressive disease in ovarian cancer (21), acute myelogenous leukemia (AML; ref. 22), and chronic lymphocytic leukemia (CLL; 23). While SET contributes to tumorigenesis at least in part by inhibiting PP2A activity (24), it also forms an inhibitory complex with the metastasis suppressor nm23-H1 (25).

Because PP2A is a negative regulator of many of the signaling pathways that provide cell growth, survival, and therapeutic resistance to pancreatic cancer (26), we investigated the role of SET and CIP2A in pancreatic cancer and the potential of therapeutically targeting these inhibitors for the treatment of pancreatic cancer. We show here that SET and CIP2A are frequently overexpressed in human pancreatic cancer and this overexpression is (i) associated with decreased PP2A activity and (ii) important for pancreatic cancer cell growth and transformation. In addition, we show that the pharmacologic antagonism of SET with a novel compound decreases pancreatic cancer cell tumorigenic potential, suggesting that antagonizing SET and/or CIP2A may be an innovative approach for the treatment of human pancreatic cancer.

**Materials and Methods**

**Cell culture**

HPAF, CD18, Panc1, and CFPAC1 cells were maintained in Dulbecco’s Modified Eagle Media (DMEM) with 10% FBS. MiaPaCa2 cells were maintained in DMEM with 10% FBS and 2.5% horse serum. As-PC1, CAPAN1, and Colo357 were maintained in RPMI with 10% FBS. CAPAN2 cells were maintained in McCoy’s 5a with 10% FBS. hTERT-HPNE (DT) cells (27) were maintained in medium D (1 V Medium M3, 3 V glucose-free DMEM, 5% FBS, 5.5 mmol/L glucose, 10 ng/mL EGF, and 50 μg/mL gentamycin). All cell lines were obtained from Michel Ouellette (University of Nebraska Medical Center, Omaha, NE). They have not been tested or authenticated.

**Quantitative reverse transcription-PCR**

RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was generated using the High Capacity cDNA Reverse Transcription kit (ABI). Quantitative RT-PCR (qRT-PCR) analysis was performed using the indicated primers and SYBR Green reagent (Invitrogen) for Myc (F: 5′-CAGTGGAAGCTG-GAGGAGGTTT-3′, R: 5′-CAGGCTCTGGCCTAAGAGT-3′) and TaqMan reagents for CIP2A and SET on a Step-One Real-Time PCR machine (Applied Biosystems, Invitrogen). Myc primers were validated by performing a standard melt curve analysis. For qRT-PCR, the cycle numbers for each run were used to calculate fold change using the ddt(Ct) method between each sample and the average of the control sample (set as 1) in gene expression as follows:

\[
dt(C_t) = \text{gene cycle} \# - 18S \text{cycle} \#\]

\[
ddt(C_t) = \text{dt}(C_t) - \text{average dt}(C_t) \text{ of normal samples}\]

**Fold change = power(2,ddt(C_t))**

**Antibodies and Western blotting**

The following antibodies were used: Myc antibody Y69 (Abcam) for Western blotting, Myc antibody N262 (Santa Cruz) for quantitative chromatin immunoprecipitation (qChIP), pS62-Myc (Abcam) antibody for Western blotting, PP2Ac antibody from Millipore, and SET antibody from Bethyl Laboratories. The CIP2A antibody was a kind gift from Dr. Jukka Westermarck. Total and phospho-IκB-α (Ser32/Ser36), total ERK1/2 and phospho-ERK (Thr183/Tyr202), Mcl-1, phospho-Bcl2 (Ser70), p53, and phospho-AKT1 (Thr308) were from Santa Cruz. The total Bcl2 and AKT antibodies were from Cell Signaling. Cell lysates were run on SDS-PAGE gels and transferred to Immobilon-FL membrane (Millipore). Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences). Primary antibodies were diluted in 1:1 Odyssey Blocking Buffer:PBS with 0.05% Tween20. Primary antibodies were detected with secondary antibodies IRDye800 (Rockland) and Alexa Fluor 680 (Molecular Probes), diluted 1:10,000 in 1:1 Odyssey Blocking Buffer:PBS with 0.05% Tween20. Blots were scanned with a LI-COR Odyssey Infrared Imager. When indicated in the figure, protein levels were quantified using the Li-COR Odyssey to measure fluorescence intensity.

For pS62-Myc immunoprecipitation—Western blots, immunoprecipitation (IP) buffer was prepared on ice (20 mmol/L Tris pH 7.5, 50 mmol/L NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid (DOC), 0.5% SDS, 1 mmol/L EDTA), with protease and phosphatase inhibitors (Roche). Cells were washed with 5 mL cold PBS before lysis with 1 mL
of IP buffer. Cells were carefully scraped and pipetted up and down 10× to break up clumps and then transferred to 1.5-mL tubes. Suspensions were sonicated with 10 pulses at 3 output and 30% duty and then incubated on ice for 15 minutes. Lysates were precleared with 50 μL of 50% PA beads slurry (Repligen) for 1 hour at 4°C. Beads were removed, and 1% to 2% of lysate was aliquoted for input samples before overnight incubation at 4°C with 1.5 μg Myc antibody (N262, Santa Cruz Biotechnology Inc.). About 15 μL of pA beads were added and incubated for 1 hour at 4°C. Beads were washed 3 times with 500 μL of IP buffer with 1-minute rotations between washes. Beads were resuspended in SDS sample buffer and Western blotting was done as above.

**Quantitative PCR arrays**

Quantification of SET and CIP2A expression levels in patient samples was carried out using the TissueScan Pancreatic Cancer qPCR Panel 1 (PNRT301) array (Origene), which contains dried cDNAs from 23 human pancreas samples, including 4 normal and 19 cancer samples in 5 identical replicate plates. Individual plate runs were performed for SET, CIP2A, and 18s using probes and master mix purchased from Applied Biosystems (SET Hs00053870_g1, CIP2A Hs00405413_m1, 18S Hs03003631_g1, and 2× TaqMan Master Mix 00558004344-01). The recommended protocols for TaqMan quantitative PCR (qPCR) were followed using an Applied Biosystems Prism 7300 instrument. After each run, the cycle number for each run was used to calculate fold change using the ddc(T) method between each cancer sample and the average of the normal samples (set as 1) in expression of SET or CIP2A as follows:

\[
dt(C_T) = \text{SET or CIP2A cycle} \# - 185 \text{ cycle} \\
ddt(C_T) = \frac{dt(C_T)}{\text{average dt}(C_T)} \text{ of normal samples} \\
\text{Fold change} = \text{power(2, ddt}(C_T))
\]

**Immunofluorescence**

Serial paraffin sections from patients with matched normal and tumor formalin-fixed human pancreatic cancer tissues (obtained from archives of OHsu Department of Pathology, IRB #2086) were incubated with rabbit polyclonal pS62-Myc or pT58-Myc; ref. 28), CIP2A, SET, CK8/18 (Fitzgerald), or Ki67 (Novacstra)-specific antibodies overnight at 4°C. Sections were then incubated with Alexa Fluor 594 donkey anti-rabbit IgG (1:1000) and mounted using anti-fade containing 4',6-diamidino-2-phenylindole (DAPI). Images were taken with a Hamamatsu digital camera mounted on a fluorescence microscope. Immunofluorescence density was analyzed using the Measure Density tool in OpenLab 5.5 software. Staining intensity was measured in 10 random individual cells in each of 5 to 10 random fields of view and averaged for each sample.

**PP2A activity assay**

PP2A activity was measured using the PP2A Immuno- precipitation Assay Kit (Millipore). Cells were lysed and protein concentration determined using the BCA Assay (Pierce). About 300 μg lysate was mixed with 4 μg PP2A antibody and 40 μL protein A slurry. The volume was brought to 500 μL with assay buffer and samples incubated for 2 hours at 4°C with rotation. Beads were washed 3 times with 700 μL TBS and one time with 500 μL assay buffer. About 60 μL phosphopeptide and 20 μL assay buffer was then added and incubated for 10 minutes with rocking at 30°C. After a brief spin, 25 μL was added to each well of a 96-well plate, 100 μL of Malachite green solution was added, and reactions were incubated at room temperature for 15 minutes. Absorbance was read at 650 nm.

**Myc protein half-life determination**

Cells were treated with 20 μg/mL cycloheximide for 0 to 90 minutes and harvested in AB lysis buffer (1 mol/L Tris, pH 7.5, 5 mol/L NaCl, 10% Triton X-100, 10% DOC, 20% SDS, and 0.5 mol/L EDTA). Myc protein levels were determined by Western blotting and half-life was calculated.

**Short hairpin RNA–mediated knockdown and selection of stable clones**

Cells were transfected using FuGENE 6 (Roche). Cells were seeded into 10-cm dishes the day before transfection. On the day of transfection, 8 mL of fresh medium was added to the cells. About 18 μL of FuGENE 6 reagent was mixed with 600 μL OPTI-MEM. About 6 μg of each targeted or scramble short hairpin RNA (shRNA) was added to the FuGENE 6 mixture and incubated for 15 minutes at room temperature. The transfection mix was added to the cells, incubated for 24 hours, and cells were expanded into 150-mm plates and selected with 2 μg/mL puromycin. Stable targeted knockdown clones were picked, verified, and expanded. Stable scramble control cells were pooled.

**siRNA transfection**

Transient SET (M-019586-01) and CIP2A (M-014135-00) knockdowns were performed using siRNAs (Dharmacon) and DharmaFECT I transfection reagent. Nontargeting siRNA (D-001206-14) was used as a control. Briefly, cells were plated in 10-cm dishes in antibiotic-free medium and allowed to adhere overnight. About 5 μmol/L siRNA stock solution was prepared in water and siRNAs were diluted to a final concentration of 25 nmol/L in 600 μL serum-free medium. About 12 μg DharmaFECT I was diluted in 588 μL serum-free medium. The siRNA and DharmaFECT mixes were then combined, incubated at room temperature for 20 minutes, and added to cells. Cells were incubated with transfection mix for 24 hours and then cells were seeded for additional experiments.

**qChIP**

Cells were cross-linked with formaldehyde to a final concentration of 1% in media at room temperature for 10 minutes. Cells were washed with PBS and collected in 700 μL ChIP lysis buffer [0.1% SDS, 0.5% Triton X-100, 20 mmol/L Tris-HCl (pH 8.1), and 150 mmol/L NaCl]. Cell lysates were sonicated 6× (output = 3.5, 30% duty, 10 pulses) and cleared by centrifugation at 14,000 rpm for 15 minutes at 4°C. Cell lysates were precleared with 50 μL...
Xenografts and the total number of colonies counted. were stained with 0.5 ml 0.005% crystal violet for 2 hours containing carrier or 2 agar layer. Plates were fed twice a week with 2 agar in 1.5 were harvested and 24,000 cells were mixed with 0.6% noble plates, with 24,000 cells per well. The base agar layer were transferred to new tubes and 5 mol/L NaCl was added to a final concentration of 0.2 mol/L and samples incubated at 65°C overnight. DNA was purified with the QIAquick PCR purification Kit (Qiagen) and used for qPCR analysis with specified primers. For qChIP experiments, primers to the promoter regions of the c-Myc target genes nucleolin (F: 5'-ACTGACCGGGAAACTGGGTCT-3', R: 5'-TG GCCCCAGTCCAGGAAGTATC-3'), E2F2 (F: 5'-AC AAGGCCCAAAAGAGGCTG-3', R: 5'-TCTA GTCTGTTGGCAGCTTC-3'), and p21 (F: 5'-CG CTAAT-GGCGGGCTG-3', R: 5'-CGGTGACACAAGTG CAGT- TCC-3') were used in qPCR to measure signals in 1% of the input material as well as each IP. The percentage of input was calculated for each IP (control IgG and specific) as the IP signal above the input signal using the formula: 100 × (Input Ct – IP Ct). Relative level of bound DNA was then graphed as the percent input of the specific IP relative to the percent input of the mock IgG control using GraphPad Prism. About 1% of the input was boiled for 30 minutes in SDS sample buffer before electrophoresis to detect the indicated proteins by Western blotting.

Migration assays
Cells were plated in media with 5% serum at 25,000 (As-PC1) or 50,000 (CPA1) cells per well in a 96-well Essen ImageLock plate for 24 hours. Two hours before wounding, cells were treated with 10 μg/mL Mitomycin C. Cells were scratched with a 96-pin WoundMaker, washed with PBS, and fresh media were added with vehicle (PBS), 1.25 or 0.625 μmol/L OP449. Cells were incubated in an IncuCyte Zoom (Essen Biosciences) for 36 hours, with wound images taken every 2 hours. The relative wound density was calculated by dividing the density inside the wound by the density outside the wound using IncuCyte software.

Colony-forming assays
Soft agar assays were carried out in triplicate in 6-well plates, with 24,000 cells per well. The base agar layer contained 0.8% noble agar in 1.5× growth medium. Cells were harvested and 24,000 cells were mixed with 0.6% noble agar in 1.5× growth medium and plated on top of the base agar layer. Plates were fed twice a week with 2× medium containing carrier or 2 μmol/L OP449. After 4 weeks, plates were stained with 0.5 ml 0.005% crystal violet for 2 hours and the total number of colonies counted.

Xenografts
About 1 × 10^6 cells in 25% Matrigel/75% growth medium were injected into the flanks of 6- to 8-week-old NOD/SCID γ-chain null mice. Once the tumors became palpable, mice were randomized to receive OP449 treatments (5 mg/kg), administered via i.p. injection 3 times per week, or vehicle control (PBS), and continued until sacrifice (when a tumor became 2.0 cm or ulcerated in any group).

Cell proliferation assays
For WST assays (cell proliferation assay kit, Millipore), cells were cultured in 96-well plates with or without OP449 (1 μmol/L) in a final volume of 100 μL for 72 hrs. Ten microliters of WST solution was added to each well and incubated for 4 hours at 37°C. After shaking the plate for 1 minute, absorbance was read at 440 nm.

For Incucyte cell growth assays, 5,000 cells were seeded into each well of a 96-well plate. After 24 hours, OP449 was added as indicated, and plates were placed in the Incucyte Zoom. Images were taken every 3 hours for the indicated amount of time. Percent confluence over time was then calculated using the Incucyte Zoom software.

Growth and treatment of primary patient material
For the treatment of primary patient material, tissue was obtained from surgical patients with pancreatic adenocarcinoma through the Oregon Pancreas Tissue Registry with informed consent, and placed in DMEM/F12 with 100 μg/mL soybean trypsin inhibitor. Tissue was minced and disassociated at 37°C for 30 to 40 minutes in disassociation buffer (1.25 mg/mL collagenase IV and DNase1 in HBSS). Cells were passed through a 40-μm filter and plated on collagen-1-coated plates. After 24 hours, colonies were imaged and cells treated with 1.25 or 2.5 μmol/L of OP449 or PBS. Fresh drug was added after 48 hours. Individual cell colonies were imaged over 4 days, and the area of each colony was quantified using ImageJ.

Data and statistics
Three or more independent biologic replicate experiments were performed in all cases, except when primary patient materials were used. With the exception of immunofluorescence data where SE was calculated from quantification across multiple tissue regions, all SEs were calculated from biologic replicates. P values were calculated using a standard Student t-test analysis (2-tailed distribution and 2-sample unequal variance) to determine statistical significance as indicated in the graphs. Correlation coefficients were calculated using Microsoft Excel. P values for relevant comparisons are given. If no P value is shown, the comparison is not relevant or not significant. One asterisk (*) indicates a P value of 0.05 to 0.001, whereas 2 asterisks (**) indicate a P value of less than 0.001.

Results
CIP2A and SET are frequently overexpressed in human pancreatic cancer cell lines and primary patient samples
To begin investigating a potential role for CIP2A and SET in pancreatic cancer, we examined their expression in both pancreatic cancer cell lines and primary patient...
Figure 1. CIP2A and SET are frequently overexpressed in human pancreatic cancer. A, qRT-PCR for CIP2A in a normal pancreatic ductal epithelial cell line (DT) and 9 pancreatic cancer cell lines was performed and graphed relative to the DT cells. B, qRT-PCR for SET as described in A. C, Western blot analyses were performed for CIP2A, SET, PP2A, and GAPDH protein expression in normal DT cells and 9 pancreatic cancer cell lines. D, protein levels for CIP2A (left) and SET (right) were quantified from immunoblots shown in C and biologic replicates using the Li-COR Odyssey to measure fluorescence intensity. Data are represented relative to the normal DT cells (set as 1). E, CIP2A and SET mRNA levels are increased in pancreatic cancer tumors relative to normal tissue (NML, shown as an average of 4, with SE). The TissueScan Pancreatic Cancer qPCR Panel 1 (PNRT301) array (Origene) was run as described in Materials and Methods. Dashed line represents 1 SE above the mean expression in normal tissue. F, CIP2A and SET protein levels are increased in pancreatic cancer tissues relative to adjacent normal tissue. Immunofluorescence for CIP2A and SET was performed in matched tumor (T) and adjacent normal (N) pancreatic patient (Pt) samples. Immunofluorescence for CIP2A and SET in a representative matched normal and tumor pair is shown. G, average staining intensity from F (n = 9) was determined as described in Materials and Methods. Figure statistics: statistical analysis was carried out as described in the Materials and Methods. Error bars represent SE. *, P = 0.05-0.001; **, P < 0.001.
samples. For analysis of the pancreatic cancer cell lines, we used hTERT-immortalized pancreatic ductal epithelial cells (DT) as a nontransformed control (27). Relative to the DT cells, CIP2A and SET mRNA expression was significantly increased in 33% and 66.7% of the pancreatic cancer cell lines, respectively. Overexpression of CIP2A and SET was even more evident at the protein level, with nearly 66.7% of cell lines overexpressing CIP2A and 77.8% overexpressing SET (Fig. 1C and D). PP2Ac levels were similar in this panel of cell lines and did not appear to be affected by changes in CIP2A or SET expression (Fig. 1C).
Fold enrichment

**p21**

p<0.001

p=0.004

p=0.06

**SCR**

shCIP2A-1

shCIP2A-2

**shSET-1**

shSET-2

**Myc**

**qChIP**

**CIP2A**

**SET**

**GAPDH**

**Nucleolin**

**E2F2**

**p21**

**CAPAN1 cells:**

**Panc1 cells:**

**pAKT**

**AKT**

**pS62-Myc**

**Myc**

**GAPDH**

**IP**

**Inputs**

**Relative PP2A activity**

**Panc1 Cells**

**CAPAN1 Cells**

**siNT**

siSET

siCIP2A

**CIP2A**

**GAPDH**

**SET**

**Myc**

**pS62-Myc**

**pAKT**

**AKT**

**GAPDH**

**Rel. SET level:**

1 0.26 0.93 1 0.34 0.89

**Rel. CIP2A level:**

1 0.83 0.11 1 0.89 0.05

**Rel. pS62-Myc level:**

1 0.52 0.15 1 0.82 0.27

**Rel. pAKT level:**

1 0.59 0.30 1 0.93 0.20

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To examine the clinical relevance of our cell line findings, we measured the expression of CIP2A and SET in primary human pancreatic cancer samples. We initially used a commercially available pancreatic qPCR array and found that expression of CIP2A was elevated in 55.6% and SET expression was increased in 61% of pancreatic cancer specimens relative to normal pancreatic tissue (Fig. 1E). As CIP2A expression was recently shown to be a poor prognostic indicator in pancreatic cancer (19), this 55.6% overexpression rate for CIP2A is likely to be clinically relevant. At this point, it is unclear whether SET overexpression correlates with poor patient outcome in pancreatic cancer as it does in other tumor types (21–23). This frequent overexpression of CIP2A and/or SET was confirmed by qRT-PCR in a smaller set of primary patient pancreatic cancer material relative to benign pancreatic lesions (Supplementary Fig. S1A and S1B). In addition, we measured CIP2A and SET protein expression in primary patient tissue using immunofluorescence. Relative to patient-matched adjacent normal tissue, CIP2A was overexpressed in 88.9% and SET was overexpressed in 77.8% of the pancreatic cancer samples examined (Fig. 1F and G). Thus, CIP2A and SET are frequently overexpressed in primary human pancreatic cancer, suggesting that PP2A inhibition may be important for pancreatic cancer development and that inhibitors of PP2A might be relevant therapeutic targets.

PP2A activity is decreased in pancreatic cancer associated with increased expression of stabilized pS62-Myc

We next examined PP2A enzymatic activity in the pancreatic cancer cell lines we had analyzed for SET and CIP2A expression. PP2A activity was reduced in all of the cancer cells relative to the normal DT cells (Fig. 2A). Analysis of the correlation between CIP2A and SET expression and PP2A activity trended toward higher inhibitor expression and lower PP2A activity, although this did not reach statistical significance (Supplementary Fig. S2A). This is not surprising given the multiple modes of PP2A regulation. Prior work has demonstrated that c-Myc is a key target for tumor suppressor function of PP2A as the requirement to inhibit PP2A for human cell transformation can be overcome by expression of stabilized c-Myc that is resistant to PP2A-mediated dephosphorylation (29). We have previously shown that PP2A removes the stabilizing phosphorylation at S62 in c-Myc, allowing c-Myc to be targeted for degradation by the proteasome, and inhibition of PP2A or knockdown of PP2Ac slows c-Myc turnover (11, 13, 30). Thus, we examined c-Myc protein levels and S62 phosphorylation in the pancreatic cancer cell lines to determine whether reduced PP2A activity is associated with increased total c-Myc levels and S62 phosphorylation levels. As shown in Fig. 2B, c-Myc protein levels and S62 phosphorylation were upregulated in many of the pancreatic cancer cell lines, compared with DT cells. Correlation analysis indicated that decreased PP2A activity trended toward increased c-Myc and pS62-Myc protein levels (Supplementary Fig. S2B). In addition, increased total c-Myc and pS62-Myc protein levels trended with increased CIP2A and SET protein levels, although these correlations were not quite statistically significant (Supplementary Fig. S2C). c-Myc mRNA levels were also increased in most of the cancer cell lines analyzed (Supplementary Fig. S2D). As c-Myc is posttranslationally regulated by PP2A (11, 29), we measured c-Myc half-life in each of the cancer cell lines as well as the DT cells. Like other nontransformed cells where c-Myc protein is quickly turned over (28, 31), c-Myc half-life in the DT cells was approximately 23 minutes (listed in Fig. 2B, see Supplementary Fig. S2E for half-life measurements). In contrast, the half-life of c-Myc was increased in all of the pancreatic cancer cell lines relative to DT cells (Fig. 2B and Supplementary Fig. S2E), demonstrating that c-Myc is stabilized in these cancer cell lines with reduced PP2A activity. Thus, SET and/or CIP2A overexpression commonly associates with reduced PP2A activity, which can contribute to increased c-Myc expression, S62 phosphorylation, and protein stability in pancreatic cancer cell lines.

We extended these studies to primary pancreatic cancer patient samples. Our previous work demonstrated that increased c-Myc expression and stability in human breast cancer is associated with increased S62 phosphorylation and decreased T58 phosphorylation, which provides the docking site for the Fbw7 E3 ubiquitin ligase (28). Using matched adjacent normal and tumor tissue from patients with pancreatic cancer, we performed immunofluorescence using antibodies specific for pS62-Myc or pT58-Myc (28). A representative example of staining in these samples is shown in Fig. 2C. We examined 9 pairs of patient-matched normal and tumor tissue and found increased pS62-Myc staining with decreased pT58-Myc staining in tumor cells in nearly all samples (Fig. 2C). These are the same samples analyzed for SET and CIP2A expression in Fig. 1F and G, indicating that high SET and/or CIP2A trends with elevated pS62-Myc levels in primary human pancreatic cancer.

Figure 3. Knockdown of CIP2A or SET in pancreatic cancer cell lines increases PP2A activity to inhibit downstream targets. A, generation of stable CIP2A and SET knockdown cells. CIP2A was stably knocked down in Panc1 cells (left) and SET was stably knocked down in CAPAN1 cells (right). Two different shRNAs were used for CIP2A (shCIP2A) and SET (shSET) and stable expression of a scrambled shRNA (SCR) pool was established as a negative control in each cell line. B, CIP2A and SET knockdown increases PP2A activity. Relative PP2A activity was measured in stable CIP2A and SET knockdown Panc1 and CAPAN1 cells and is represented relative to SCR control cells. C, CIP2A and SET knockdown reduces AKT and c-Myc phosphorylation. Left, Total and phospho-AKT levels were measured by Western blotting in stable CIP2A and SET knockdown Panc1 and CAPAN1 cells. Right, c-Myc was immunoprecipitated using a total c-Myc antibody and IPs were blotted for total c-Myc and pS62-Myc. Inputs were blotted for GAPDH. D, CIP2A and SET knockdown reduces Myc DNA binding. Myc chromatin mapping to the nucleolin, E2F2 (top graph), and β2I promoters (bottom graph) was determined by ChIP in stable CIP2A and SET knockdown Panc1 and CAPAN1 cells. Western blot analyses show ChIP input material. E, transient SET and CIP2A knockdown in Panc1 and CAPAN1 cells reduces AKT and c-Myc phosphorylation. CAPAN1 and Panc1 cells were transfected twice with a nontargeting siRNA (siNT) or SET/CIP2A targeting siRNAs (siSET and siCIP2A). Relative ( Cel kinase protein levels were quantified from immunoblots as the ratio of each protein to GAPDH band intensity. Data are represented relative to the siNT cells (set as 1). Figure statistics: indicated statistical analyses were carried out as described in Materials and Methods. Error bars represent SE. P values are shown.
Figure 4. Knockdown of CIP2A or SET reduces tumorigenic potential of pancreatic cancer cell lines. A, stable CIP2A and SET knockdown reduces the population growth rates of pancreatic cancer cell lines. Stable shSCR, shCIP2A-2, and shSET-2 (see Western blots in Fig. 3A) clones were analyzed using an Incucyte Zoom to measure relative change in confluence over time. B, transient CIP2A and SET knockdown reduces the population growth rates of pancreatic cancer cell lines. CIP2A (siCIP2A) and SET (siSET) were transiently knocked down in CAPAN1 and Panc1 cells with siRNA, and change in confluence over time was measured using an Incucyte Zoom. A nontargeting siRNA (siNT) was used as a control. Western blot analyses for these cells are shown in Fig. 3E. C, CIP2A and SET knockdown reduces tumorigenic potential of pancreatic cancer cell lines. Soft agar assays were performed with the indicated stable shRNA clones (see Fig. 3A) and the number of colonies formed is shown. D, the indicated stable shRNA clones (see Fig. 3A) were xenografted into immunocompromised mice (n = 6 tumors per group). Tumor growth, as measured by repeated caliper measurements, is shown. Figure statistics: statistical analysis was carried out as described in Materials and Methods. Error bars represent SE. P values are shown: *, P < 0.001.
Knockdown of CIP2A or SET increases PP2A activity and decreases the tumorigenic potential of human pancreatic cancer cell lines

To determine whether targeting CIP2A or SET might be effective for the treatment of human pancreatic cancer, we examined the effects of CIP2A or SET knockdown on pancreatic cancer cells. We initially generated stable lines with knockdown of CIP2A in Panc1 and SET in CAPAN1 cells, as these cell lines had high expression of CIP2A and SET, respectively. In each case, we used a scrambled control shRNA (SCR) and 2 different CIP2A or SET targeting shRNAs (Fig. 3A). Knockdown of CIP2A in Panc1 cells and SET in CAPAN1 cells increased PP2A activity (Fig. 3B) and subsequently decreased phosphorylation of AKT and c-Myc, 2 downstream targets of PP2A (Fig. 3C). Stable depletion of CIP2A in Panc1 and SET in CAPAN1 cells also reduced the binding of c-Myc to 3 of its target gene promoters (Fig. 3D). We also transiently knocked down SET and CIP2A in CAPAN1 or Panc1 cells using siRNAs. Interestingly, while CAPAN1 cells showed decreased AKT and c-Myc phosphorylation with knockdown of SET or CIP2A, Panc1 cells appeared less sensitive to SET knockdown with respect to these downstream phosphorylation events (Fig. 3E), suggesting that these cells might be more dependent on CIP2A-mediated PP2A inhibition than on SET-dependent PP2A inhibition. Together, these data indicate that loss of CIP2A and/or SET can activate PP2A and attenuate its oncogenic targets.

We next determined the effects of CIP2A and SET knockdown on the growth and tumorigenic potential of these pancreatic cancer cells. We measured cell growth using the Incucyte Zoom, which performs live cell kinetic imaging. As shown in Fig. 4A, stable knockdown of SET in the CAPAN1 cell line and CIP2A in the Panc1 cell line reduced cell population growth. In addition, transient knockdown of CIP2A and SET in Panc1 or CAPAN1 cells also decreased their growth (Fig. 4B). Interestingly, similar to our findings regarding AKT and c-Myc phosphorylation changes after transient knockdown of CIP2A and SET (see Fig. 3E), CAPAN1 cells were sensitive to both CIP2A and SET knockdown, whereas Panc1 cells appeared less sensitive to SET knockdown (Fig. 4B). To determine the effects of CIP2A and SET knockdown on tumorigenic potential, we performed soft agar colony-forming assays and xenograft experiments. Panc1 clones with stable knockdown of CIP2A or CAPAN1 clones with stable knockdown of SET (Fig. 3A) showed significantly decreased growth in soft agar relative to their respective controls (Fig. 4C). Furthermore, these clones also showed significantly reduced tumor growth compared with scrambled controls when xenografted into immunocompromised mice (Fig. 4D). These data suggest that targeting CIP2A or SET could be beneficial in the treatment of human pancreatic cancer.

Inhibition of SET with a novel SET antagonist, OP449, decreases the tumorigenic potential of human pancreatic cancer cell lines

Unfortunately, there are currently no pharmacologic means to inhibit CIP2A. However, our collaborators have developed a novel SET inhibitor, OP449, which shows excellent bioavailability in vitro and in vivo and strongly reactivates PP2A in cancer cells (23, 32, 33). As several of the pancreatic cancer cell lines express elevated levels of SET, and knockdown of SET reduced the tumorigenic activity of CAPAN1 cells, we screened 7 of our pancreatic cancer cell lines as well as the nontransformed DT cells for sensitivity to OP449. As shown in Fig. 5A, DT cells were relatively insensitive to OP449 treatment, with an IC50 near 10 μmol/L. Four of the pancreatic cancer cell lines (CFPAC1, CAPAN1, HPAF, and As-PC1) were sensitive to OP449 treatment with IC50 values of less than 2.5 μmol/L, whereas the other 3 (Panc1, MiaCaPa2, and CAPAN2) were less sensitive (Fig. 5A). Interestingly, Panc1 cells were less sensitive to OP449 treatment, consistent with their reduced response to SET knockdown (Figs. 3E and 4B). To explore the relationship between SET levels and OP449 sensitivity, we analyzed the correlation between SET levels and OP449 sensitivity. As shown in Supplementary Fig. S3A, we observed that pancreatic cancer cell lines expressing higher levels of SET were generally less sensitive to OP449, with a correlation coefficient of 0.78 (P = 0.06). This supports the specificity of OP449 for SET, as increased SET expression pushes the SET:PP2A equilibrium toward the SET inhibited form of PP2A and should require a higher dose of OP449 to compete with the interaction.

To determine whether OP449 treatment reactivated PP2A activity in the pancreatic cancer cell lines, cells were treated with OP449 and PP2A activity was assayed. As shown in Fig. 5B, treatment with 1 μmol/L OP449 increased PP2A activity in the 3 sensitive cell lines (CFPAC1, CAPAN1, and As-PC1). Cell lines that showed less sensitivity in the cell viability assays (Fig. 5A, MiaPaCa2 and Panc1) did not show reactivation of PP2A activity when treated with this concentration of OP449 (Fig. 5B). To follow this up, we analyzed several important PP2A targets in the OP449-treated versus -untreated cell lines. In the sensitive CFPAC1 and CAPAN1 cells, we observed decreased expression of proteins known to be destabilized by PP2A (c-Myc and Mcl-1; refs. 11, 34, 35) and dephosphorylation of the PP2A targets AKT, Bcl2, and IKBα (36, 37) upon OP449 treatment (Fig. 5C). Little effect was observed in the less sensitive MiaPaCa2 and Panc1 cells (Fig. 5C). Furthermore, OP449 treatment reduced c-Myc S62 phosphorylation (Fig. 5D) and DNA-binding activity (Fig. 5E and Supplementary Fig. S3B) in the sensitive CAPAN1 and CFPAC1 cells but not in the less sensitive cells. To verify that OP449 was decreasing c-Myc protein levels through increased protein turnover, we measured c-Myc half-life in treated versus untreated cells and found that c-Myc protein half-life was reduced upon OP449 treatment (Supplementary Fig. S3C). Thus, OP449 treatment of pancreatic cancer cell lines increases PP2A activity and suppresses PP2A targets involved in cell survival and proliferative signaling.

To test the specificity of OP449 for SET, we treated stable SET knockdown cells with OP449 and measured population growth rates. As shown in Supplementary Fig. S3D, stable SET knockdown CAPAN1 cells were significantly less...
Figure 5. Several pancreatic cancer cell lines are sensitive to OP449 treatment associated with increased PP2A activity and inhibition of downstream signaling. A, OP449 reduces the viability of pancreatic cancer cell lines. WST cell viability assays were performed with nontransformed DT cells and 7 pancreatic cancer cell lines treated with increasing amounts of OP449. B, OP449 treatment increases PP2A activity in sensitive pancreatic cancer cell lines. CFPAC1, CAPAN1, As-PC1, MiaPaCa2, and Panc1 pancreatic cancer cell lines were treated with PBS or 1 μmol/L OP449 for 24 hours and relative PP2A activity was measured. PP2A activity is shown relative to PBS-treated cells (set as 1). C, OP449 treatment reduces levels and phosphorylation of several PP2A targets. CFPAC1, CAPAN1, As-PC1, MiaPaCa2, and Panc1 pancreatic cancer cell lines were treated as in B and immunoblots for various PP2A targets were performed. D, OP449 treatment reduces c-Myc S62 phosphorylation. (Continued on the following page.)
sensitive to OP449 than the scrambled shRNA control cells. In addition, transient knockdown of SET reduced CAPAN1 cell sensitivity to OP449 when compared with cells transfected with a nontargeting control siRNA (Fig. 5F). To further test the specificity of OP449, we transiently knocked down the catalytic subunit of PP2A (PP2Ac) in CAPAN1 cells and monitored cell population growth after OP449 treatment. As shown in Fig. 5G, PP2A-knockdown cells were significantly less sensitive to OP449 than control cells. Thus, these data suggest that OP449 specifically reactivates PP2A by antagonizing SET.

Several PP2A targets have been shown to regulate cell migration; therefore, we sought to determine whether OP449 treatment affects migration of pancreatic cancer cells. As shown in Supplementary Fig. S4A and S4B, Mitomycin C–treated CAPAN1 and As-PC1 cells exposed to sublethal doses of OP449 had reduced migration through a layer of Matrigel as compared with PBS controls. Furthermore, OP449 treatment significantly reduced anchorage-independent growth of CAPAN1 and CFPAC1 pancreatic cancer cell lines but not the less sensitive Panc1 cells (Fig. 6A). To test the effects of OP449 in vivo, we xenografted CAPAN1 or CFPAC1 cells into the flanks of NOD/SCID mice. Once tumors became palpable, mice were randomized into 2 groups and treated 3 times per week by i.p. injection of PBS or OP449. Once any tumor reached a diameter of 2.0 cm or the mice became moribund, all mice for each cell line were sacrificed and final tumor weight was determined. As shown in Fig. 6B, growth of CAPAN1 and CFPAC1 tumors in mice was slowed by OP449 treatment, and final tumor weight was significantly reduced with OP449 treatment (Fig. 6C). In addition, proliferation was decreased (Fig. 6D and Supplementary Fig. S4C) at study endpoint in OP449-treated xenograft tumors. Importantly, OP449 treatment was well-tolerated by the mice, with little to no observed side effects, consistent with previous reports (32, 33). These results indicate that SET inhibition and reactivation of PP2A has antitumor activity in pancreatic cancer, and OP449 could represent potential new therapeutics for pancreatic cancer.

**OP449 reduces growth of primary patient-derived PDAC cells**

Finally, to examine whether primary human pancreatic cancer cells are sensitive to OP449 treatment, surgical tissue from a patient with pancreatic adenocarcinoma was dissociated and grown on collagen-1–coated plates. Epithelial cell colonies that grew out were treated with increasing doses of OP449 and cell expansion was assessed. To quantify colony expansion, individual colonies were imaged over 4 days using an EVOS digital inverted microscope and the area of each colony was measured. As shown in Fig. 7A and B, OP449 dramatically reduced expansion of these colonies. In addition, immunofluorescent staining of cancer cell colonies with antibodies to cytokeratin 8/18 verified that these cells were epithelial (Fig. 7C). Thus, similar to pancreatic cancer cell lines, OP449 is also cytotoxic to primary pancreatic epithelial cancer cells. Therefore, OP449 warrants further preclinical investigation as a potential pancreatic cancer therapeutic.

**Discussion**

Several studies have demonstrated that inhibition of the PP2A tumor suppressor protein is critical for cell transformation (7, 9, 10, 38) and therapeutically targeting PP2A to increase its activity has shown promise. Specifically, FTY-720, a structural analog of sphingosine, has been shown to activate PP2A in AML (39), CML (40), acute lymphoblastic leukemia (ALL; ref. 40), and CLL (41), leading to reduced tumor cell proliferation. FTY-720 has also been shown to reduce tumorigenic properties and/or increase apoptosis in hepatocellular carcinoma (42), lung cancer (43), breast cancer (44), bladder cancer (45), and prostate cancer (46). Ceramide, which also activates PP2A, has growth-inhibitory effects in CML and prostate cancer cells (47–51). As many signaling pathways altered in pancreatic cancer are negatively regulated by PP2A (6), we sought to determine whether PP2A inhibition is important in pancreatic cancer, whether this involves upregulation of endogenous PP2A inhibitors, and whether targeting PP2A-inhibitory proteins could be a promising strategy for the treatment of this devastating disease.

We report here that PP2A activity is reduced in many pancreatic cancer cell lines relative to normal pancreatic epithelial cells and that 2 endogenous PP2A inhibitors, CIP2A and SET, are frequently overexpressed in human pancreatic cancer cell lines and patient samples; and CIP2A expression was recently shown to be a poor prognostic indicator in pancreatic cancer (19). Using molecular techniques, we also report that knockdown of these endogenous inhibitors can recover PP2A activity and suppress pancreatic cancer cell growth. Furthermore, we have demonstrated that overexpression of SET and CIP2A in pancreatic cancer contributes to increased stability, levels, and activation state of several important PP2A targets that are involved in promoting cell growth, proliferation, and survival. Thus, these results suggest a new therapeutic strategy aimed at...
antagonizing PP2A inhibitors to permit reactivation of a tumor-suppressive phosphatase to suppress pancreatic cancer growth.

Our previous work has demonstrated that the c-Myc oncoprotein is a key target for the tumor-suppressive activity of PP2A (29). PP2A inactivates c-Myc by dephosphorylating serine 62, which decreases the transcriptional activity of c-Myc and increases its degradation by ubiquitin-dependent proteolysis (11, 13, 30). We show here, for the first time, that c-Myc protein stability is increased in human pancreatic cancer cells, contributing to its overexpression in pancreatic cancer, and this is associated with increased levels of SET and/or CIP2A and decreased PP2A activity. These results are highly relevant to the human disease as c-Myc is found to be overexpressed in at least 40% to 70% of pancreatic tumors (52–54). Moreover, similar to what we have seen in other tumor types, including leukemia (31) and human breast cancer (28), this stabilization of c-Myc correlates with increased phosphorylation at S62 and decreased phosphorylation at T58, which can be observed in primary pancreatic tumor samples. Furthermore, our recent work has demonstrated that stabilized c-Myc with increased S62 phosphorylation has enhanced oncogenic activity associated with selective activation of target genes driving ribosome biogenesis and glycolytic metabolic pathways (12). Thus, high pS62-Myc expression in pancreatic cancer is likely to play an important role in supporting the high bioenergetic state of this disease. Importantly, we show that antagonism of SET with subsequent activation of PP2A reduces S62 phosphorylation of c-Myc, c-Myc levels, and c-Myc target gene promoter-binding capabilities in several pancreatic cancer cell lines.

Given that pancreatic cancer is an aggressive disease with few effective treatment options, the development of novel...
therapeutic approaches is critically important. Our data suggest that targeting inhibitors of PP2A that are frequently upregulated in pancreatic cancer to reactivate PP2A could be an effective treatment for pancreatic cancer. Here, we show that knockdown of CIP2A or SET or treatment with OP449, a novel SET inhibitor, reactivates PP2A and this has potent tumor-suppressive functions. Specifically, knockdown or inhibition of CIP2A or SET reduces pancreatic cancer cell proliferation, decreases cell migration, decreases growth in soft agar, and suppresses tumor formation in vivo. Importantly, SET inhibition alone, by knockdown or with OP449, can substantially inhibit pancreatic cancer cell growth, even with expression of CIP2A. This suggests that if one can even modestly elevate PP2A activity, it can have potent cell growth-suppressing activity. This also helps provide a therapeutic window, as we are indirectly targeting PP2A through its upregulated inhibitors, and thus are only trying to restore PP2A activity toward normal levels. Furthermore, given that long-term OP449 treatment did not show adverse effects in vivo while continuing to suppress tumor growth, our data suggest that the increased PP2A activity is only deleterious in the cancer cells that are addicted to activated signaling pathways driving cancerous growth.

To translate our discovery of the overexpression of PP2A inhibitors and reduced PP2A activity in pancreatic cancer, we have chosen to focus on the novel SET antagonist, OP449. Two reasons for this focus are the lack of available pharmacologic inhibitors for CIP2A and studies indicating that OP449 is more efficacious in activating PP2A relative to other known SET inhibitors, including FTY720 (33). Furthermore, sphingosines, such as FTY-720, display adverse immunologic and cardiac events and FTY-720 has been linked to several deaths of patients with multiple sclerosis (55, 56). In addition, it was recently reported that the phosphorylated form of FTY-720 generated by sphingosine kinase modification of FTY-720 actually inhibits PP2A through a shingosine-1-phosphate receptor–mediated mechanism (57). In contrast, OP449 was well-tolerated by mice for treatment periods as long as 60 days (Christensen and colleagues, unpublished data). In addition, OP449 is stable in plasma, is largely cleared from blood through uptake into tissues, is taken up by cancer cells, and activates PP2A in the tumors of mice with orthotopic breast tumors (Janghorban and colleagues, manuscript under review). These findings suggest that the pharmaceutical properties of OP449 are sufficient for eventual progression to human clinical trials.

Furthermore, because SET has been shown to be overexpressed in other tumor types, including B-cell CLL, non–Hodgkin lymphoma (NHL; ref. 23), human breast cancer (Janghorban and colleagues, manuscript under revision), CML (58), and ALL (22), and PP2A negatively regulates multiple signaling pathways important for cancer growth.
progression, including Akt, NF-xB, Bcl2, and c-Myc (20), the re-activation of PP2A could represent an effective therapeutic approach for multiple tumor types. In support of this idea, SET inhibition using OP449 provided selective cytotoxicity for CLL and NHL cells in vitro and in a xenograft model of NHL (23). Furthermore, we have shown that SET inhibition with OP449 reduces the tumorigenicity of human breast cancer cell lines (Janghorban and colleagues, manuscript under review). In this report, we have focused on pancreatic cancer and provided evidence that inhibition of PP2A, which regulates c-Myc phosphorylation and subsequent degradation, as well as many other oncogenic pathways, contributes to pancreatic cancer and this suggests that reactivation of PP2A may have use in treating this aggressive and devastating disease.

Disclosure of Potential Conflicts of Interest
D.J. Christensen and M.P. Vitek are shareholders of Oncotide Pharmaceuticals and employed as President and Chief Scientific Officer and CEO, respectively. M.P. Vitek is an Associate Professor at Duke and his potential individual and institutional conflict of interest has been reviewed and is managed by the DUMC Conflict of Interest Committee. J. Oddo is a former employee of Oncotide Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Targeting Inhibitors of the Tumor Suppressor PP2A for the Treatment of Pancreatic Cancer


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