Identification of an Agent Selectively Targeting DPC4 (Deleted in Pancreatic Cancer Locus 4)–Deficient Pancreatic Cancer Cells

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Abstract

One of the most common types of genetic alterations in cancer is the loss-of-function mutations in tumor-suppressor genes. Such mutations are usually very specific to cancer cells and present attractive and unique opportunities for therapeutic interventions. However, for various reasons, antitumor agents that target loss-of-function mutations have not been readily identified. In this report, using the *deleted in pancreatic cancer locus 4* (*DPC4*) gene in pancreatic cancer as an example, we show the feasibility of a novel screening strategy, which we have named Pharmacological Synthetic Lethal Screening, for the identification of agents that selectively target cancer cells with loss-of-function mutations. We created *DPC4* isogenic cell lines through the restoration of wild-type *DPC4* in a pancreatic cancer cell line, BxPC-3, whose *DPC4* gene was homozygously deleted. The isogenic cell lines were then used in the Pharmacological Synthetic Lethal Screening to evaluate compound libraries for antiproliferative activity and selectivity against DPC4 deficiency. After screening 19,590 compounds, we identified one lead compound, UA62001, which showed 4.6-fold selectivity against DPC4 deficiency in the *DPC4* isogenic cell lines. UA62001 selectivity was also seen in another set of *DPC4* isogenic cell lines generated by small interfering RNA knockdown. In addition, UA62001 was evaluated in commonly used pancreatic cancer cell lines. A fairly good correlation between DPC4 deficiency and UA62001 sensitivity was observed. Cell cycle analysis indicates that UA62001 arrests cells in S and G_2-M phases. The results of microarray gene expression profiling and quantitative real-time reverse transcription-PCR suggest that cyclin B/CDC2 and minichromosome maintenance complexes might be the downstream cellular targets of UA62001. (Cancer Res 2006; 66(19): 9722-30)

Introduction

Pancreatic cancer is the fourth leading cause of cancer death among adults in the United States (1, 2). In the year 2005 alone, an estimated 32,180 new cases of pancreatic cancer will be diagnosed in the United States and 31,800 patients with pancreatic cancer will die (2). The 5-year survival rate is ~4% (1, 2). This is largely due to the lack of symptoms and diagnostic tools for the detection of this disease in the early stages as well as a deficiency of effective therapeutics for later-stage disease. In addition, pancreatic cancer is a highly chemoresistant malignancy. The most effective first-line drug for patients with advanced pancreatic cancer is gemcitabine, which provides only a modest improvement in survival (3). Thus, the discovery of new treatments for patients with pancreatic cancer is critically important.

One of the main genetic alterations in pancreatic cancer is the loss-of-function mutation of the *deleted in pancreatic cancer locus 4* (*DPC4*) tumor-suppressor gene. The *DPC4* gene is located on chromosome 18q21, a region that is homozygously deleted in 30% to 37% pancreatic ductal adenocarcinomas (4–6). Also, intragenic inactivating mutations, such as nonsense, misssense, and frame-shift, occur commonly in the *DPC4* gene, accompanied by a loss of the other allele and the loss of heterozygosity (5, 7). In total (both homozygous deletion and loss of heterozygosity), the *DPC4* gene is inactivated in ~55% of tumors taken directly form patients (1, 8). The loss of *DPC4* gene is thought to be associated with the progression and malignancy of pancreatic cancer, as it occurs only in PanIN3 and pancreatic adenocarcinomas (9, 10). DPC4 deficiency is also associated with poor survival of patients with pancreatic cancer. Patients with pancreatic cancer with normal DPC4 protein expression have a significantly longer survival. The unadjusted median survival was 19.2 months in patients with pancreatic cancers with DPC4 protein expression compared with 14.7 months in patients with pancreatic cancers lacking DPC4 protein (11). In summary, loss of DPC4 has been considered a predicator of decreased survival in pancreatic cancer (11, 12). Of additional interest is that loss of DPC4 is associated with progression and malignancy in other types of tumor. Miyaki et al. (13) reported that among 176 colorectal tumors at varying stages, DPC4 is lost in 0% (0 of 40) of adenomas, in 10% (4 of 39) of intramucosal carcinomas, in 7% (3 of 44) of invasive carcinomas without distant metastases, in 35% (6 of 17) of primary invasive carcinomas with distant metastases, and in 31% (11 of 36) of carcinomas metastasized to the liver or distant lymph nodes. These findings were further supported by Maitra et al. (14).

With its high frequency in pancreatic cancer, DPC4 deficiency presents a target for therapeutic intervention. The underlying promise of targeting DPC4 deficiency in pancreatic cancer is the hypothesis that molecular targets that are critical to the viability of cancer cells, in combination with DPC4 deficiency (synthetic lethality), exist in the genome of cancer cells and can be explored using small molecular weight compounds. It has been previously reported that some cancer cells with a specific loss-of-function mutation in tumor-suppressor genes can be selectively killed by certain antitumor agents. For example, some myeloma cells with *PTEN* mutations are remarkably sensitive to CCI-779, a rapamycin analogue than those with wild-type *PTEN* (15). Erastin, a quinazoline analogue, has been shown to specifically kill transformed tumorigenic human foreskin fibroblast cells expressing SV40 small T antigen and the RAS^{v12} oncoprotein (16). DNA cross-linking agents, such as mitomycin C and cisplatin, have higher activity in pancreatic cancer cells with BRCA2 deficiency (17). Most

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interestingly, it has been reported that breast cancer cells with the deficiency of *BRCA1* or *BRCA2* gene are extremely sensitive to the inhibitors of poly(ADP-ribose)polymerase in cell culture and in xenograft models (18, 19).

In this report, we describe a pharmacologic synthetic lethal screening (PSLS) method for the identification of antitumor agents that have genotype selectivity against cancer cells with specific patterns of loss-of-function mutations/deletions. We show the feasibility and efficiency of this method through the identification of an antitumor agent, which showed selectivity against tumor cells with a DPC4 deficiency.

Materials and Methods

Cell culture. The human pancreatic adenocarcinoma cell lines (BxPC-3, AsPC-1, Capan-1, Capan-2, CFPAC-1, Hs 766T, MIA PaCa-2, PANC-1, SU.86.86, and the human embryonic lung fibroblast cell line, IMR-90) were purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI except for IMR-90 (Mediatech, Herndon, VA) with 10% fetal bovine serum (FBS; GEMINI, Woodland, CA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). IMR-90 was grown in Eagle's MEM with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, 10% FBS (GEMINI), and 1% penicillin-streptomycin (Invitrogen; ref. 20). The normal human foreskin fibroblast cell line, Forf, was obtained from the Cell Culture Shared Services at the Arizona Cancer Center (21) and grown in DMEM with 10% FBS (GEMINI) and 1% penicillin-streptomycin (Invitrogen). The immortalized human pancreatic ductal epithelial cell line, HPDE6, was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada; refs. 22, 23). HPDE6 cells were maintained in keratinocyte serum-free medium supplemented with epidermal growth factor (0.2 ng/mL) and bovine pituitary extract (30 μg/mL; Invitrogen).

Expression vector construction and retrovirus based delivery. The full-length cDNA of *DPC4* was amplified using reverse transcription-PCR (RT-PCR) with DPC4 forward (5′-CACGAATTCATGGACAATATGTCTATT-3′) and DPC4 reverse (5′-ATTCTCGAGTCAGTCTAAAGGTTGTGG-3′) primers containing *Eco*RI and *Xho*I restriction enzyme sites, respectively. The PCR product was unilaterally cloned into *Eco*RI and *Xho*I sites of the multiple cloning site of pMSCVneo, a retrovirus vector (Clontech, Palo Alto, CA), to generate a DPC4 expression construct, pMSCVneoDPC4. To establish a cell line that constitutively expresses the *DPC4* gene, pMSCVneoDPC4 was introduced into BxPC-3 cells whose *DPC4* gene was homozygously deleted using the retrovirus delivery system following the protocol of the manufacturer (Clontech). Briefly, the retrovirus containing the *DPC4* gene was produced by cotransfecting the packaging cell line GP2-293 with pVSV-G (Clontech) and pMSCVneoDPC4 using the Lipofectin reagent (Invitrogen). As a negative control, the retrovirus containing the pMSCVneo empty vector was also produced. The retrovirus was filtered through a 0.45 μmol/L cellulose acetate membrane syringe filter (Nalgene, Lima, OH) and concentrated for 90 minutes by ultracentrifugation at 50,000 × *g*. BxPC-3 cells seeded in six-well plates were infected by the retrovirus in the presence of polybrene (8 μg/mL; Sigma-Aldrich, St. Louis, MO) for 12 hours and then replaced with fresh growth medium. Thirty-six hours after infection, the cells were trypsinized and grown in RPMI medium containing neomycin (G418 at 350 μg/mL) to select positive clones. Ten individual clones were isolated using serial dilution of the cells in 96-well plates. DPC4 expression in individual clones was evaluated using Western blot analysis.

DPC4 small interfering RNA gene silencing. To generate a cell line that has the DPC4 expression constantly suppressed by small interfering RNA (siRNA), we cloned the H1 promoter into the pMSCVpuro retrovirus vector to create a siRNA expression vector (pMSCVpuroH). The oligonucleotides for siRNA expression consist of a 19-nucleotide sequence selected from *DPC4* gene, separated by a short spacer from the reverse complement of the same 19-nucleotide sequence (Supplementary Fig. S1; ref. 24). Several oligonucleotide sequences targeting different regions of the *DPC4* gene were designed and evaluated for their suppression ability using the transient transfection. The expression construct that showed the highest suppression of DPC4 expression was chosen for the subsequent stable knockdown. The construct was delivered into the immortalized normal pancreatic ductal epithelial cell line HPDE6 through a retrovirus delivery system. The procedure for retrovirus production and infection was the same as described earlier for the reexpression of DPC4. After retrovirus infection, the HPDE6 cells were selected with puromycin at 3 μg/mL. Individual clones were isolated using serial dilution of the cells in 96-well plates. The suppression of DPC4 expression in stable clones was evaluated using Western blotting. Individual clones containing empty vector were also produced to serve as controls.

For the transient knockdown of DPC4 expression in BxPC-3-DPC4 cells, the siRNA duplex, which targets the position of 562 to 581 bp (5′-AATCGTGCATCGACAGAGA-3′) in DPC4 coding region, was acquired from Qiagen (Valencia, CA). DPC4 and GFP (control) siRNA duplexes were transfected into BxPC-3-DPC4 cells using LipofectAMINE 2000 (Invitrogen) based on the protocol of the manufacturer. DPC4 knockdown efficiency was evaluated in six-well plate format through LipofectAMINE 2000 transfection of siRNA duplexes into BxPC-3-DPC4 cells. The cytotoxicity test was done in 96-well plates. The working concentration of siRNA duplexes in both 6- and 96-well plate formats is 25 nmol/L. In brief, 1 day before transfection, BxPC-3-DPC4 cells were seeded into 96-well plates (2,000 per well/80 μL RPMI). The siRNA oligomer-LipofectAMINE 2000 complexes were prepared as follows. Twelve microliters of 20 μmol/L siRNA and 24 μL LipofectAMINE 2000 were dissolved in 5 mL Opti-MEM medium, respectively, for 5 minutes. Then, the diluted siRNA and LipofectAMINE 2000 were combined and incubated for 20 minutes at room temperature. Ten microliters of siRNA oligomer-LipofectAMINE 2000 complexes were added into each well of 96-well plates. After 24 hours of incubation, 10 μL of serially diluted UA62001 in RPMI medium was added into the well. The 96-well plate with siRNA and UA62001 was incubated for 96 hours. The cell viability was measured by 3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium, inner salt (MTS) assay.

Western blot analysis. Whole cell extracts were prepared using NP40 lysis buffer [1% IGEPAL CA-630, a substitute for NP40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), and 2 mmol/L EDTA]. For DPC4 protein localization analysis, BxPC-3-vector, BxPC3-DPC4, and PANC-1 were seeded into six-well plate (1×10^5 per well) and grown overnight. The next day, the cells were treated with transforming growth factor-β (TGF-β; 10 ng/mL) for 6 hours and then harvested by trypsinization. The nuclei and cytoplasmic fractions of the lysates were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Protein concentrations of the lysates or fractionates were determined using BCA reagents (Pierce). Aliquots of the cell lysates or fractionates with equal amounts of total protein were resolved on SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then incubated with the primary and horseradish peroxidase (HRP)–conjugated secondary antibodies under the conditions recommended by the manufacturers. The DPC4 protein was detected by a monoclonal antibody (Oncogene Research Products, Boston, MA). The p21 protein was detected by a polyclonal antibody (Cell Signaling Technology, Beverly, MA). Both the active and proto- forms of caspase-3 were detected by a polyclonal antibody obtained from BD Biosciences (San Jose, CA). β-Actin, which served as a loading control, was detected by a monoclonal antibody (Sigma). The protein was detected by adding LumiGLO reagent (chemiluminescent substrate of HRP) and peroxide (Cell Signaling Technology).

Anchorage-independent growth assay. BxPC-3-vector cells or BxPC-3-DPC4 cells (3,000 per Petri dish) were mixed with Difco agar (final concentration 0.26%; Clontech) and RPMI medium containing 10% FBS and 1% penicillin-streptomycin, and were overlaid onto an underlayer of 0.45% Difco agar containing RPMI medium, 10% FBS, and 1% penicillin-streptomycin in a 35-mm-grid Petri dish. The cells were allowed to grow for 19 days at 37°C before counting the number of colonies (≥8 cells) under a light microscope. The assay was done in triplicate. The *P* values were calculated using a *t* test.

MTS-based cell proliferation assay. The inhibition of cell proliferation by library compounds was evaluated using the CellTiter 96 Non-Radioactive

Cell Proliferation Assay following the protocol recommended by the manufacturer (Promega, Madison WI). Briefly, 2,000 cells were seeded in each well of 96-well plates in 90 μL growth medium. Cells were allowed to attach overnight followed by addition of 10 μL of serially diluted compounds into the plates. After incubation for 4 days at 37°C in a humidified incubator, 20 μL of a 20:1 mixture of MTS (2 mg/mL) and an electron coupling reagent, phenazine methosulfate (0.92 mg/mL), were added to each well and incubated for 3 hours at 37°C. Absorbance was then measured using the Wallace Victor microplate reader at 490 nm (Perkin-Elmer, Wellesley, MA). Data were expressed as the percentage of cell growth calculated from the absorbance corrected for background absorbance. The surviving fraction of cells was determined by dividing the mean absorbance values of the treated samples by the mean absorbance values of the DMSO control.

Compound libraries. Compound libraries used in the pharmacologic synthetic lethal screens consist of an National Cancer Institute (NCI) diversity set (1,990 compounds), NanoSyn Pharm II (8,800 compounds), and Nanosyn Pharm IV (8,800 compounds). All the compounds were prepared as 200 μmol/L in DMSO from the stock concentration (1 mmol/L in DMSO for NCI library, 2 mmol/L in DMSO for NanoSyn Pharm libraries) and stored at −20°C. Five microliters of 200 μmol/L compounds (the final concentration 10 μmol/L) were added into 95 μL of cell growth medium for the antiproliferative assay.

Cell cycle distribution analysis. Cells (5×10^5) were plated in a T-25 tissue culture flask 24 hours before the drug treatment. The DPC4 reexpression isogenic cells were treated with UA62001 at 3.2 and 15 μmol/L (close to the IC_{50} values) for 24, 48, 72, and 96 hours. At each time point, the cells were harvested by trypsinization and centrifugation and then stained with propidium iodide (Sigma) in a modified Krishan buffer (25) for 1 hour at 4°C. The propidium iodide–stained samples were then analyzed with a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Histograms were analyzed for cell cycle compartments and the percentage of cells at each phase of the cell cycle was calculated using CellQuest (BD Immunocytometry Systems) analysis software.

DNA oligonucleotide microarray analysis. BxPC-3-vector and BxPC-3-DPC4 cells (5×10^5) were seeded in T-25 cm^2 tissue culture flasks 24 hours before the drug treatment. The cells were then treated with UA62001 (15 μmol/L) and harvested by trypsinization after 3 and 24 hours of treatment. To avoid the circadian influence on the gene expression, treatment and control samples were harvested at the exact same time. Total RNA of the control and treated samples was isolated using the NucleoSpin RNA II isolation kit (BD Biosciences, Palo Alto, CA). The microarray analysis, including target labeling and chip hybridization and processing, was carried out by following the protocols recommended by the chip manufacturer (Agilent Technologies, Palo Alto, CA). Briefly, 1 μg total RNA was used to generate cyanine 3 (control) or cyanine 5 (drug treated) cRNA targets using the Agilent low-input RNA fluorescent linear amplification kit. The concentration and integrity of fluorescent cRNA as well as the incorporation efficiency of cyanine dyes were analyzed using the Agilent Spectrophotometer. Equal amount of labeled cRNA targets from the paired control and treated samples were hybridized onto Agilent Human 1A (V2) oligonucleotide array, which contains 21,073 features representing over 21,000 individual human genes and transcripts. The hybridization signals were acquired and normalized using Agilent's Feature Extraction Image Analysis software (v. 7.1). To identify genes whose expression was changed significantly after the drug treatment, the following criteria were used to filter the genes: (*a*) ≥1.5-fold change upon drug treatment; (*b*) *P* value of the log-transformed ratio ($P_{LogRatio}$) ≤ 0.01; and (*c*) the dye normalized signal intensity (DyeNormSignal) ≥ 150. Venn diagram analysis (Supplementary Fig. S2) was done for the uniqueness of regulated genes in each of the isogenic cell lines and the overlap of regulated genes between the isogenic cell lines using GeneSpring 7.2 (Agilent Technologies).

Validation of array data by quantitative real-time RT-PCR. Real-time PCR primers for each of the tested genes were designed using the Primer3 software[1] and listed in the Supplementary Table. Two micrograms of total RNA isolated from the control and drug-treated samples (15 μmol/L) were used in a 20 μL reverse transcription reaction to generate cDNA using the First Strand cDNA Synthesis kit (Fermentas, Hanover, MD). To perform real-time PCR, 1.5 μL of the reverse transcription reaction was mixed with 1 μL primer mixture (5 μmol/L), 10 μL water, and 12.5 μL of 2× QuantiTect SYBR PCR Master Mix (Qiagen). Each sample was subjected to 40 cycles of amplification on an Opticon I real-time thermocycler (Bio-Rad Laboratories, Hercules, CA). The transcript abundance of each gene was calculated and normalized to β-actin using the algorithm provided by the thermocycler manufacturer.

Results

Ectopic expression of *DPC4* gene in BxPC3 cells. To target the DPC4 deficiency in pancreatic cancer cells through PSLS, we established the *DPC4* isogenic cell lines through ectopical reexpression approach. It has been known that three pancreatic cancer cell lines, BxPC-3, CFPAC-1, and Hs 766T, have the homozygous deletion of *DPC4* gene (4, 6). We chose BxPC-3 cell line because restorations of DPC4 function in CFPAC-1 and Hs 766T cells produced cells lacked TGF-β–stimulated growth inhibition (26, 27). We first subcloned *DPC4* cDNA into a retrovirus-based vector, pMSCVneo, to create the DPC4 expression vector, pMSCVneoDPC4. To establish a stable cell line that constitutively expresses DPC4, pMSCVneoDPC4 was introduced into BxPC-3 cells through the retrovirus delivery system (See Materials and Methods). Ten individual clones were isolated using serial dilution of the cells in 96-well plates under G418 selection (350 μg/mL). DPC4 expression in individual clones was evaluated by Western blot using a DPC4 monoclonal antibody. The DPC4 protein expression level of an individual clone, named BxPC-3-DPC4, as shown in Fig. 1*A*, has a DPC4 expression level that is comparable with that in the immortalized HPDE6 cells. As a negative control, the pMSCVneo empty vector was also delivered into BxPC-3 cells by the retrovirus to create the BxPC-3-vector cell line. Thus, BxPC-3-vector and BxPC3-DPC4 are considered as a pair of DPC4 reexpression isogenic cell lines.

Characterization of the DPC4 reexpression isogenic cell lines. To verify that the ectopic expression of DPC4 has functionally restored the TGF-β pathway, we carried out a series experiments to evaluate the DPC4 function in BxPC-3-DPC4 cells. First, the cell morphology of BxPC-3-DPC4 was inspected under a light microscope and was found to be very similar to that of the parental cell line BxPC-3. In an examination of the proliferation of BxPC-3-DPC4 cells versus BxPC-3-vector cells, BxPC-3-DPC4 cells proliferate slightly slower than that of BxPC-3-vector cells (Fig. 1*D*). Second, we investigated the localization of DPC4 protein in the BxPC-3-DPC4 cells upon the treatment of TGF-β cytokine. We observed that DPC4 protein was localized to the nucleus upon TGF-β treatment in the BxPC-3-DPC4 cells but not in the BxPC-3-vector cells (Fig. 1*B*), which is consistent with previous reports (26, 27). Third, we examined the protein levels of p21 in the isogenic cell lines. p21 is a cyclin-dependent kinase inhibitor whose expression was shown to be up-regulated when DPC4 function was restored (27). As expected, the p21 protein level was increased significantly in the BxPC-3-DPC4 cells compared with the BxPC-3-vector cells (Fig. 1*C*). Finally, we evaluated the tumorigenicity of the BxPC-3-DPC4 cells using a soft-agar growth assay. As shown in Fig. 1*E*, the ectopic expression of DPC4 in the BxPC-3-DPC4 cells dramatically suppressed the anchorage-independent growth of the cells (>70% reduction in colony numbers, $P = 0.0025$). These data alerted us to be careful in any conclusions regarding compounds,

[1] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

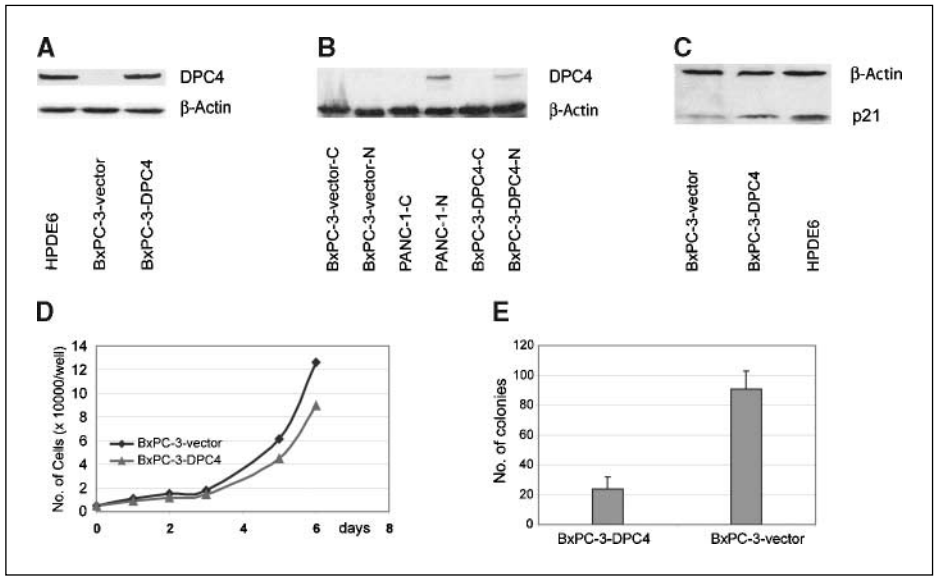

Figure 1. Ectopic expression of DPC4 in pancreatic cancer cell line BxPC-3. *A,* ectopic expression of DPC4 in BxPC-3 cells detected by Western blot. Each lane was loaded with 20 μg total protein. *B,* DPC4 nuclear localization upon the activation of TGF-β signaling pathway. The cells of BxPC-3-vector, BxPC3-DPC4, and PANC-1 were treated with TGF-β (10 ng/mL) for 6 hours. *BxPC-3-vector-C,* cytoplasmic fraction of BxPC-3-vector cell extracts; *BxPC-3-vector-N,* nucleic fraction of BxPC-3-vector cell extracts; *PANC-1-C,* cytoplasmic fraction of PANC-1 cell extracts; *PANC-1-N,* nucleic fraction of PANC-1 cell extracts; *BxPC-3-DPC4-C,* cytoplasmic fraction of BxPC-3-DPC4 cell extracts; *BxPC-3-DPC4-C,* nucleic fraction of BxPC-3-DPC4 cell extracts. *C,* up-regulation of p21 protein in BxPC-3-DPC4 cells. BxPC-3-vector, BxPC-3-DPC4, and HPDE6 cells were treated with 10 ng/mL TGF-β for 12 hours. *D,* growth curves of BxPC-3-vector and BxPC-3-DPC4. Cells were seeded in six-well plates and allowed to grow under normal conditions. Two wells of cells for each cell line were harvested daily for 6 consecutive days and counted with a hemocytometer. *E,* anchorage-independent growth of BxPC-3-DPC4 cells. Each cell line was done in triplicates.

which selectively affect the growth of one of the isogenic cell lines although a cell cycle–specific agent would most likely have a greater inhibition effect on the more rapidly proliferating DPC4-deleted BxPC3 cells than DPC4-restored BxPC3 cells. In addition, we investigated the growth response of BxPC-3-DPC4 cells to TGF-β cytokine and did not observe any apparent TGF-β–induced cell growth inhibition in BxPC-3-DPC4 (data not shown). Although this observation is different from what has been seen in the PANC-1 cell line, which has wild-type DPC4, it is consistent with reported data from studies that dealt with DPC4 restoration in other pancreatic cancer cell lines, such as CFPAC-1 and Hs 766T (26, 27). Together, these results showed that the ectopic expression of DPC4 has at least partially restored the TGF-β signaling pathway in the BxPC-3-DPC4 cells.

Identification of genotype-selective compounds against the DPC4 deficiency through PSLS. Once we had generated the isogenic cell lines for the *DPC4* gene, we then carried out a screening process using the cell lines with the aim to identify compounds that selectively kill cells with deficient DPC4 function. The screening process, which we named pharmacologic synthetic lethality screening (PSLS), consists of three rounds of screening, namely, toxicity screen, synthetic lethality screen, and selectivity

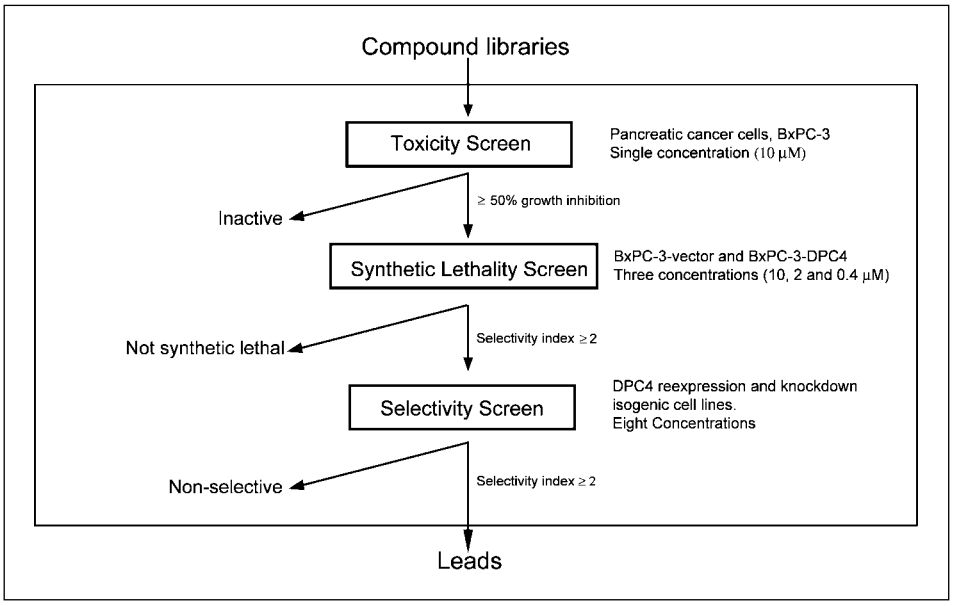

Figure 2. Schematic depiction of the PSLS.

screen (Fig. 2). In each round, the antiproliferative activity of compounds was evaluated in different cell lines using the CellTiter 96 nonradioactive cell proliferation assay (Promega). In the toxicity screen, compounds were evaluated in the BxPC-3 parental cell line at a single concentration of 10 μmol/L. Generally, cancer cell lines, especially highly chemoresistant pancreatic cancer cell lines, have significant variation in their sensitivities to antitumor agents. To ensure that lead compounds to be identified had decent antitumor activity, we used this screen against the parental line to eliminate compounds that have low antiproliferative activity. After screening three compound libraries, the NCI diversity set (1,990 compounds), NanoSyn Pharm II (8,800 compounds), and Nanosyn Pharm IV (8,800 compounds), we identified a total of 200 compounds that showed ≥50% growth inhibition against the BxPC-3 cells at 10 μmol/L. These compounds were then subjected to the second round of screening, the synthetic lethality screen. In this round of screening, each compound was tested against both of the DPC4 reexpression isogenic cell lines (BxPC-3-vector and BxPC-3-DPC4) at three different concentrations (10, 2, and 0.4 μmol/L; if 0.4 μmol/L was too high for a compound, lower testing concentrations were used) in triplicate. The activities of a compound in the DPC4 reexpression isogenic cell lines were calculated and a selectivity index was determined for each concentration (selectivity index = percentage cell survival in BxPC-3-DPC4 cells / percentage cell survival in BxPC-3-vector cells). To select compounds for further evaluation in the third round of screening, we set the selection criteria as percentage inhibition in the BxPC-3-vector cells ≥50 and selectivity index >2 at least at one of the three concentrations. Among the 200 compounds selected from toxicity screening, 11 compounds were selected based on these criteria. These 11 compounds were further evaluated in a third round of screening, the selectivity screen, at eight different concentrations in triplicate (concentration range was determined based on the compound potency from the synthetic lethality screening). In this screening, compounds were tested against a panel of cell lines, including the DPC4 reexpression and siRNA knockdown isogenic cell lines. IC_{50} values of the cell lines tested were calculated and selectivity index was determined (selectivity index = IC_{50} of DPC4 wild-type cells/IC_{50} of corresponding DPC4 mutated cells). The criterion of this screening is that selectivity index is >2. After three rounds of PSLS screening, a compound, 2-phenyl-1,2-dihydro-naphtho[2,3-*g*]indazole-3,6,11-trione (UA62001), was identified to have DPC4 genotype selectivity.

The activity profile of UA62001 in DPC4 reexpression isogenic cell lines is shown in Fig. 3. UA62001 has an IC_{50} value of 3.2 ± 0.5 μmol/L in BxPC-3-vector cells and an IC_{50} value of 14.7 ± 4.3 μmol/L in BxPC-3-DPC4 cells (selectivity index = 4.6, Table 1). To exclude the possibility that such sensitivity difference is a result of random clonal variability, we tested UA62001 in another stable clone (BxPC-3-DPC4F9) that reexpresses the wild-type *DPC4* gene. The IC_{50} value of UA62001 in BxPC3-DPC4F9 is 10.6 ± 1.2 μmol/L (selectivity index = 3.3). As predicted, transient knockdown of DPC4 expression in BxPC-3-DPC4 cells (Supplementary Fig. S3) reverses the sensitivity to UA62001 (UA62001 IC_{50}: 6.2 ± 0.5 and 11.7 ± 1.3 μmol/L in BxPC-3-DPC4 cells transiently transfected with DPC4 or GFP siRNAs respectively; Table 1). In addition, UA62001 was evaluated in a second pair of isogenic cell lines (HPDE6-vector and HPDE6-siDPC4) generated by stable RNA interference knockdown. The DPC4 gene expression in HPDE6-siDPC4 cells was knockdown by 70% (Supplementary Fig. S1). UA62001 showed a similar DPC4 genotype selectivity in this pair of isogenic cell lines (selectivity index = 3; Table 1).

To see if UA62001, identified from the engineered isogenic cell lines, has the differential killing effect against a panel of pancreatic cancer cell lines that vary by DPC4 status, we tested this lead compound among commonly used pancreatic cancer cell lines. Initially, we did not expect a good correlation of UA62001 sensitivity and DPC4 status among the pancreatic cancer cell lines tested, because of the high aneuploidity and diverse genetic context. However, the results showed a fairly good correlation between UA62001 sensitivity and DPC4 expression status (see Discussion; Table 2).

Potential mechanism of action of the lead compound. Although the lead compound identified from the PSLS selectively kills cancer cells harboring DPC4 deficiency, the lethality screening does not tell us how the agent might exert its killing effects. To elucidate the possible mechanism of action of the lead compound, we investigated the cellular responses of BxPC-3 cells to UA62001 treatment using cell cycle analysis and caspase-3 activation through pro-caspase-3 and active caspase-3 Western blot.

In the cell cycle distribution analysis, BxPC-3-vector and BxPC4-DPC4 cells were treated with UA62001 at 3.2 and 15 μmol/L (IC_{50} values of UA62001 in BxPC-3-vector and BxPC-3-DPC4, respectively) in a time course up to 96 hours. As shown in Table 3, BxPC-3-vector and BxPC-3-DPC4 cells treated with UA62001 at 15 μmol/L had a significant increase in S-phase population (46.3% and 60.4% for treated samples versus 31.7% and 36.8% for untreated controls at 24 and 48 hours, respectively; 40.3% and 50.2% for treated samples versus 14.8% and 17.2% for untreated controls at 72 and 96 hours, respectively). The cell population in the G_2-M phase was

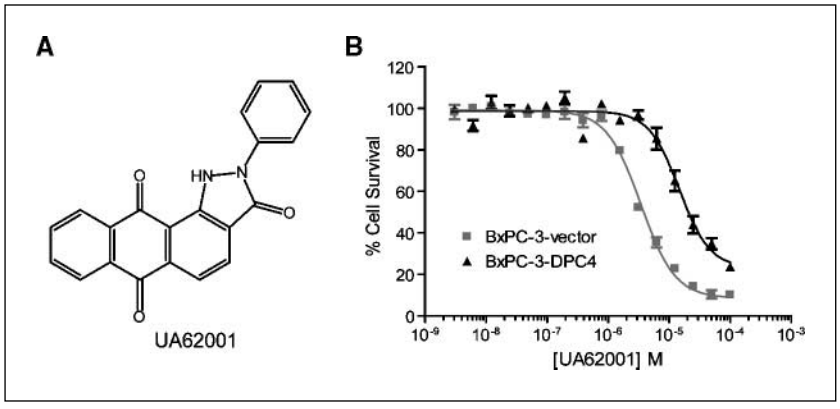

Figure 3. UA62001 activities in the DPC4 reexpression isogenic cell lines. Structure of UA62001, 2-phenyl-1,2-dihydro-naphtho[2,3-*g*]indazole-3,6,11-trione, and its activity profile in the DPC4 reexpression isogenic cell lines.

Table 1. IC_{50} values of UA62001 in DPC4 reexpression and siRNA knockdown isogenic cell lines

Cell lines	BxPC-3-vector	BxPC-3-DPC4	BxPC-3-DPC4F9	BxPC-3-DPC4 DPC4 siRNA	BxPC-3-DPC4 GFP siRNA	HPDE6-siDPC4	HPDE6-vector
IC_{50} (μmol/L)	3.2 ± 0.5	14.7 ± 4.3	10.6 ± 1.2	6.2 ± 0.5	11.7 ± 1.3	7.0 ± 1.1	21.3 ± 4.6
Selectivity index	—	4.6	3.3	—	1.9	—	3.0

NOTE: The table lists the average μmol/L concentration required to achieve 50% inhibition in each cell line from three MTS assays (IC_{50} values were calculated by GraphPad Prism 4, GraphPad Software, Inc., San Diego, CA). To obtain an accurate IC_{50} value, UA62001 was tested at 16-point 2-fold dilution in these cell lines in triplicate.

also much higher for UA62001-treated samples at 72 and 96 hours (19.5% and 30.8% for treated samples versus 3.2% and 6.7% for untreated controls; Table 3). At the lower concentration (3.2 μmol/L), UA62001 treatment (72 and 96 hours) caused a significant increase in S and G_2-M cell population in BxPC-3-vector cells compared with untreated control, whereas no significant difference in cell cycle distribution was observed in BxPC-3-DPC4 cells upon the treatment (Table 3).

To check if the cell cycle arrest induced by UA62001 results in the caspase-3-dependent programmed cell death (apoptosis), we examined the activation of caspase-3 upon UA62001 treatment using Western blotting. No caspase-3 activation was observed in either isogenic cell line treated with UA62001 at 15 μmol/L for up to 96 hours (data not shown), indicating that caspase-3-dependent apoptosis may not be the main mechanism responsible for UA62001 antiproliferative effect and the differential sensitivity of DPC4 isogenic cell lines.

Differential gene expression changes in DPC4 reexpression isogenic cell lines after UA62001 treatment. To explore the molecular bases of the genotype selectivity of UA62001, we compared the gene expression profiles of DPC4 reexpression isogenic cell lines at 3 and 24 hours after UA62001 treatment (15 μmol/L) using an Agilent oligonucleotide microarray. Based on the selection criteria (see Materials and Methods), much fewer genes responded to UA62001 treatment at the 3-hour time point compared with those at 24-hour time points (Supplementary Fig. S2). At the 3-hour time point, 39 genes in BxPC-3-DPC4 cells and 36 genes in BxPC-3-vector cells are differentially regulated, whereas only 8 genes are overlapped (Supplementary Fig. S2*C*). At the 24-hour time point, 297 genes in BxPC-3-DPC4 cells and 371 genes in BxPC-3-vector cells are differentially regulated and 152 genes are overlapped (Supplementary Fig. S2*D*). Eleven genes in BxPC-3-DPC4 cells and 23 genes in BxPC-3-vector cells were differentially regulated at both two time points (Supplementary Fig. S2*A* and *B*).

To find the targets or pathways whose gene expression was regulated by UA62001 treatment and might contribute to the genotype selectivity, we analyzed the genes uniquely regulated in each of the isogenic cell lines using the BioRag informatics tool developed by Dr. David Mount and his coworkers at the University of Arizona.[2] One pathway that emerged from this analysis is the cell cycle regulatory pathway. We identified that *CCNB1* gene expression changed only in BxPC-3-vector cells, and not in BxPC-3-DPC4 cells, after UA62001 treatment at the 24-hour time point. However, closer examination of the microarray data showed that CCNB1 was also down-regulated in the BxPC-3-DPC4 cells, but to a lesser degree (it therefore did not pass our selection criteria). Interestingly, CDC2, which formed the complex with CCNB1, had the same pattern as CCNB1 in response to UA62001 treatment, down-regulated in both DPC4 reexpression isogenic cell lines, but by different degrees. In addition, cyclin B2 (CCNB2) showed the same pattern (Table 4). The minichromosome maintenance complex (MCM) also showed the similar pattern of gene expression changes in the isogenic cell lines as the cyclin B/CDC2 complex after UA62001 treatment. All six members of the hexameric MCM complex (MCM2-7) were differentially down-regulated in both DPC4 reexpression isogenic cell lines (Table 4), and the degree of down-regulation was always higher in the BxPC-3-vector cells than in BxPC-3-DPC4 cells. The differential down-regulation of these genes in DPC4 reexpression isogenic cell lines has been confirmed by quantitative real-time RT-PCR analysis (Table 4).

Discussion

In recent years, target-based drug discovery has played a greater role in cancer therapeutics development. It largely relies on rational drug design based on a protein/DNA structure of target molecules (28–31) or a mechanism-based screening with various readouts (32–35). These approaches mainly focus on gain-of-function alterations in oncogenes in cancer cells (30, 31). Gleevec, an inhibitor of BCR-ABL oncoprotein and Herceptin, a monoclonal antibody of HER2/NEU oncoprotein, are two successful examples developed using these target-based approaches (36–38). Loss-of-function mutations of tumor-suppressor genes are usually neglected for drug discovery and development, mainly because it is much easier to design small molecules to inhibit the gain of functions of oncogenes than to restore the function of tumor suppressor genes, which are lost in tumorigenesis. However, loss-of-function mutations or deletions of tumor-suppressor genes can also play important roles in the tumorigenicity (1).

Synthetic lethal screening is a genetic technique that has been used for years in genetic model systems to identify mutations that are lethal in combinations (39, 40). Synthetic lethal screening was proposed as a drug development tool by Hartwell et al. (41) and then further defined by Friend et al. (42, 43). The systematic analysis of synthetic lethal interaction in yeast is likely to produce novel targets for cancer drug development. Unfortunately, many of genetic defects in pancreatic ductal adenocarcinomas do not have good structural or functional orthologues in the yeast genome. Here, we have described a cell-based screening approach, PSLS,

[2] http://www.biorag.org.

Table 2. IC_{50} values of UA62001 in pancreatic cancer cell lines and primary cells

Cell line	BxPC-3	AsPC-1	Capan-1	Capan-2	CFPAC-1	Hs 766T	MIA PaCa-2	PANC-1	SU.86.86	Forf	IMR-90
IC_{50} (μmol/L)	$3.2^{a} \pm 0.2$	$15.0^{d} \pm 1.4$	$10.8^{c} \pm 2.1$	$4.7^{a} \pm 0.4$	$6.4^{b} \pm 0.7$	$3.7^{a} \pm 0.4$	$3.5^{a} \pm 0.2$	$18.7^{d} \pm 3.7$	$6.5^{b} \pm 0.3$	⩾200	⩾200
DPC4 status	HD	—	Mut (343Stop)	— (Low protein level)	HD	HD	— (Deficient in TGF-β receptor)	—	—	—	—
References	(4)	(45, 46)	(6, 45)	(45)	(4)	(4)	(44)	(4, 45)	(45, 46)	(21)	(20)

NOTE: The table lists the average μmol/L concentration required to achieve 50% inhibition in MTS assay in each cell line (IC_{50} values were calculated by GraphPad Prism 4) from three different cytotoxicity tests. —, absence of the genetic alteration. The statistical analysis was done with one-way ANOVA. Statistically significant differences among UA62001 IC_{50} values of pancreatic cancer cell lines are indicated in superscript letters, a, b, c, and d ($P < 0.05$). Abbreviations: HD, homozygous deletion; Mut, mutations (amino acids involved were indicated in the parenthesis).

that uses small molecular weight compounds as probes to explore differences in sensitivity of pancreatic cancer cells with a specific loss-of-function mutation/deletion of a tumor suppressor gene.

The genotype selectivity of the lead compound identified from engineered isogenic cell lines using PSLS may be overshadowed by the heterozygous genetic background among different cancer cells or various cancer types (43). Pancreatic cancer cells are known for their high levels of aneuploidity and complexity of genetic alterations and we predicted that UA62001 might only work in a subset of pancreatic cancer cell lines that have deficient DPC4. However, our data showed that the correlation between the UA62001 sensitivity and DPC4 status is fairly good among the cell lines tested (Table 2). Two of the most sensitive cell lines (BxPC-3 and Hs 766T with an IC_{50} value of 3.2 and 3.7 μmol/L, respectively) have homozygous deletion in the *DPC4* gene (4). Although MIA PaCa-2 (IC_{50} 3.5 μmol/L) has a wild-type DPC4, its TGF-β signaling pathway is compromised due to the lack of TGF-β receptor expression (44). Capan-2 (IC_{50} 4.7 μmol/L) has a wild-type DPC4; however, it has been reported that this cell line expresses a very low level of DPC4 protein (45). In addition, we were either not able to detect any DPC4 protein in Capan-2 cells (data not shown). The two least sensitive cell lines were PANC-1 (IC_{50} 18.7 μmol/L) and AsPC-1 (IC_{50} 15.0 μmol/L). Both cell lines have been reported to have wild-type DPC4 (4, 45, 46). Two cell lines with deficient DPC4 (CFPAC-1 and Capan-1) showed moderate sensitivity to UA62001 (IC_{50} 6.4 and 10.8 μmol/L, respectively; refs. 4, 6), whereas SU.86.86, which has a wild-type DPC4, also showed moderate sensitivity (IC_{50} 6.5 μmol/L; refs. 45, 46). For the human normal cell lines tested, both Forf and IMR-90 are not sensitive to UA62001 (IC_{50} ⩾ 200 μmol/L).

Table 3. Cell cycle distribution of DPC reexpression isogenic cell lines after UA62001 treatment

Cell lines	Treatment	Cell cycle (%)	24 h	48 h	72 h	96 h
BxPC3-vector	Control	S	35.8	32.3	16.3	17.2
		G_0-G_1	47.3	57.7	77	79.6
		G_2-M	16.9	10	6.7	3.2
	Treatment (3.2 μmol/L)	S	43.2	39.1	28.1	26.9
		G_0-G_1	38.1	45.1	54.3	56.4
		G_2-M	18.8	15.9	17.6	16.7
	Treatment (15 μmol/L)	S	57.5	60.4	47.7	50.2
		G_0-G_1	28.4	21.9	21.5	21
		G_2-M	14.1	17.7	30.8	28.8
BxPC3-DPC4	Control	S	36.8	31.7	15.6	14.8
		G_0-G_1	47.3	57.7	77.6	79.7
		G_2-M	15.9	10.5	6.8	5.5
	Treatment (3.2 μmol/L)	S	39.6	32.9	19.2	9.6
		G_0-G_1	43.5	53.3	68.6	81.9
		G_2-M	16.9	13.8	12.2	8.5
	Treatment (15 μmol/L)	S	47.9	46.6	40.6	40.3
		G_0-G_1	36.4	38	38	40.2
		G_2-M	15.7	15.4	21.4	19.5

NOTE: DPC4 isogenic cell lines were treated with UA62001 at two concentrations (3.2 and 15 μmol/L) in a time course up to 96 hours. Cells were harvested and analyzed by fluorescence-activated cell sorter.

Table 4. Comparison between Agilent microarray data and quantitative real-time RT-PCR data

Functional complexes	Gene name	Accession no.	Oligonucleotide array (ratio, treatment/control, 15 μmol/L)		QRT-PCR (ratio, treatment/control, 15 μmol/L)	
			BxPC-3-vector	BxPC-3-DPC4	BxPC-3-vector	BxPC-3-DPC4
Cyclin B/CDC2	*CCNB1*	NM_031966	0.29 (62.7%)	0.77	0.17 (15%)	0.20
	CCNB2	NM_004701	0.26 (53.8%)	0.57	0.11 (28.6%)	0.14
	CDC2	NM_001786	0.36 (20.6%)	0.52	0.08 (42.8%)	0.14
MCM	*MCM2*	NM_004526	0.41 (34.1%)	0.62	0.25 (3.8%)	0.26
	MCM3	NM_002388	0.32 (20.8%)	0.60	0.25 (37.5%)	0.40
	MCM4	NM_005914	0.43 (42.1%)	0.75	0.28 (28.2%)	0.39
	MCM5	NM_006739	0.26 (51.1%)	0.53	0.22 (37.1%)	0.35
	MCM6	NM_005915	0.30 (32.9%)	0.44	0.28 (9.7%)	0.31
	MCM7	NM_005916	0.23 (56.5%)	0.53	0.28 (12.5%)	0.32

NOTE: Quantitative real-time RT-PCR was done to validate the transcript changes of the genes of cyclin B/CDC2 and MCM complexes, measured by Agilent microarray analysis, in DPC4 reexpression isogenic cell lines in response to UA62001 treatment at 15 μmol/L for 24 hours. The percentage values in parentheses represent the ratio (treatment/control) difference of each gene involved in cyclin B/CDC2 or MCM complex between the DPC4 reexpression isogenic cell lines.

PSLS may be a potentially powerful approach for the identification of genotype-selective antitumor agents, given ~1% of compounds in chemical libraries to inhibit human cancer cell growth at the concentration tested in a typical cell-based, high-throughput screening (43). However, the molecular target(s) of the lead compound identified from PSLS is unknown and needs to be identified subsequently. In some cases, the protein target(s) of a lead compound can be identified through affinity chromatography or photoaffinity labeling (47, 48). The search for the molecular target(s) of lead compounds may be expedited by the simultaneous phenotypic RNA interference knockdown screening for identifying synthetic lethal targets in the isogenic cell lines used for PSLS. In addition, the analysis of gene expression pattern using DNA microarray between the isogenic cell lines after the treatment with the lead compound could give some clues on the perturbation of signaling pathways that might be responsible for the selectivity. Here, our data of DNA oligonucleotide microarray and quantitative real-time RT-PCR suggest that the cyclin B/CDC2, which controls the cell cycle transition from G_2 to M phase (49, 50), and the MCM complexes, involving the initiation of DNA replication (51, 52), might be the downstream targets of UA62001 and that the differential down-regulation of these complexes in DPC4 reexpression isogenic cell lines may contribute their different sensitivity to UA62001. Obviously, obtaining a detailed understanding of the target(s) of UA62001 will require a substantial amount of additional investigation.

In summary, although the *in vivo* activity and clinical outcome of the lead compound, UA62001, remain to be seen, our results have shown that it is feasible to use PSLS for the identification of an agent that selectively targets tumor cells with a loss-of-function mutation in tumor-suppressor genes.

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