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Activation of protein phosphatase 2A tumor suppressor as potential treatment of pancreatic cancer

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ABSTRACT

We utilized three tiers of screening to identify novel therapeutic agents for pancreatic cancers. First, we analyzed 14 pancreatic cancer cell lines against a panel of 66 small-molecule kinase inhibitors and dasatinib was the most potent. Second, we performed RNA expression analysis on 3 dasatinib-resistant and 3 dasatinib–sensitive pancreatic cancer cell lines to profile their gene expression. Third, gene profiling data was integrated with the Connectivity Map database to search for potential drugs. Thioridazine was one of the top ranking small molecules with highly negative enrichment. Thioridazine and its family members of phenothiazine including penfluridol caused pancreatic cancer cell death and affected protein expression levels of molecules involved in cell cycle regulation, apoptosis, and multiple kinase activities. This family of drugs causes activation of protein phosphatase 2 (PP2A). The drug FTY-720 (activator of PP2A) induced apoptosis of pancreatic cancer cells. Silencing catalytic unit of PP2A rendered pancreatic cancer cells resistant to penfluridol. Our observations suggest potential therapeutic use of penfluridol or similar agent associated with activation of PP2A in pancreatic cancers.

1. Introduction

Pancreatic ductal adenocarcinoma has a dismal prognosis (5%, 5 year survival rate) because patients are usually diagnosed at a late stage, and non-surgical therapies are not very effective (Siegel et al., 2013). This year more than 45,000 new cases and 38,000 deaths from pancreatic cancer are estimated to occur in the United States. Successful surgical therapy can occur in

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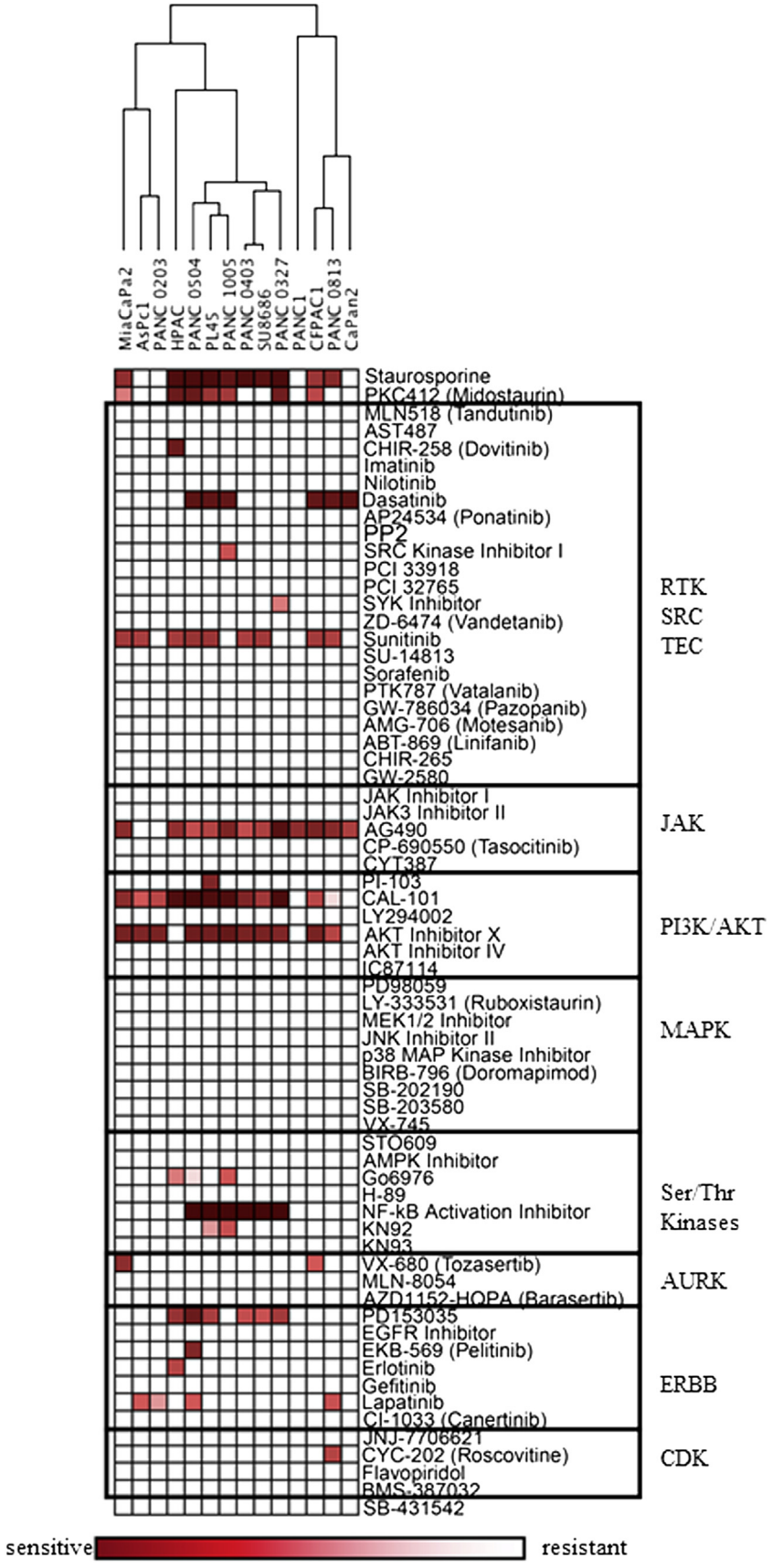


Figure 1 – Activity analysis of multiple families of kinase inhibitors in 14 human pancreatic cancer cell lines. The IC50s of 14 human pancreatic cancer cell lines for 66 small-molecule kinase inhibitors are shown as a heat map. To estimate hypersensitivity, each IC50 is expressed as a percent of the median IC50 for the same drug tested against a large cohort of hematologic malignant specimens as described in Material and methods. Cell

a minority of patients because the disease has often spread beyond the confines of the pancreas. Conventional chemotherapeutic treatments with either gemcitabine or 5-flurouracil alone or in combination with a second cytotoxic agent such as capecitabine, oxaliplatin, or cisplatin only marginally provide short term survival (Heinemann et al., 2006; Mukherjee et al., 2013; O'Reilly et al., 2014). Recently a gemcitabine-free combination therapy regimen using the FOLFIRINOX protocol (folinic acid, fluorouracil, irinotecan and oxaliplatin) showed improved overall survival, increasing from 6.8 to 11.1 months when compared to gemcitabine treatment (Conroy et al., 2011). Additionally, investigators are attempting to overcome the massive stromal environment of pancreatic cancer. A pre-clinical study in human pancreatic cancer xenografts which employed nanoparticle albumin-bound (nab)-paclitaxel in combination with gemcitabine disrupted the pancreatic desmoplastic stroma and increased intratumoral concentrations of gemcitabine (Frese et al., 2012; Von Hoff et al., 2011). Pancreatic cancer patients receiving nab-paclitaxel plus gemcitabine had improved progression-free and overall survival (Von Hoff et al., 2013).

Frequent mutations of KRAS, TP53, CDKN2A/p16, and SMAD4/DPC4 occur in pancreatic cancers. Somatic KRAS mutations occur early in progression of the disease, and the frequency rises to more than 95% in advanced pancreatic cancers. The KRAS oncogene and its signaling downstream molecules are the focus of therapies. Small molecules have been designed to interfere with membrane localization of KRAS, such as farnesyltransferase inhibitors (Appels et al., 2005). More recently, biochemical screening identified small molecules targeting PDEδ (PDE6D), a regulator of KRAS localization and signaling (Zimmermann et al., 2013). Two major RAS signaling pathways have been implicated in pancreatic cancer (RAF-MEK-ERK and PI3K/AKT). A number of pharmacologic agents have been developed to target these pathways (Courtney et al., 2010; Friday and Adjei, 2008). Also, activated tyrosine kinases are essential for cell proliferation of pancreatic cancer, and various kinase inhibitors have shown modest promise in various types of cancers; but drug resistance presents an ongoing challenge (Wong and Lemoine, 2009). To date, successful therapy of pancreatic cancer not cured by surgery remains only a wishful dream. In this study by three tiers of screening, we sought to identify novel therapeutic agents and potential biomarkers of response to assist in the treatment of pancreatic cancer.

2. Material and methods

2.1. Cell culture

Pancreatic cancer cell lines were purchased from ATCC and maintained in either DMEM or RPMI-1640 supplemented with 10% fetal calf serum. Normal pancreatic epithelial cell line, HPDE, was a generous gift from Dr. Ming-Sound Tsao of Ontario Cancer Institute, Ontario, Canada. HPDE was cultured in keratinocyte SFM supplemented with human recombinant EGF and bovine pituitary extract (Life Technologies, Carlsbad, CA).

2.2. Kinase inhibitor screening

Inhibitors were generously provided by and/or purchased from several manufacturers (Supplementary Table 1); and detailed protocols were described previously (Tyner et al., 2013). Briefly graded concentrations of the kinase inhibitors were applied to 96-well plates containing 5000 cells per well and incubated for 3 days at 37 °C, 5% CO_2. Relative numbers of viable cells were assayed with a tetrazolium-based cell viability assay (CellTiter Aqueous One Solution Cell Proliferation Assay, Promega). All cell viability values were normalized to cells cultured in the absence of any drug, and a second order polynomial curve fit was used to calculate IC50 values for each drug. Wherever an IC50 value was not achieved, the maximum tested concentration was reported. The IC50 for each drug for each individual cell line was compared with the median IC50 observed from a cohort of 151 clinical specimens obtained from patients with a diversity of hematologic malignancies (Tyner et al., 2013) to estimate whether the pancreatic cancer cells were uniquely hypersensitive to any given drug.

2.3. Chemicals and reagents

Dasatinib was purchased from LC Laboratories (Woburn, MA). Thioridazine, chlorpromazine, perphenazine, penfluridol, pimethixene, and fingolimod (FTY-720) were from Sigma–Aldrich (St. Louis, MO).

2.4. MTT assays

For IC50 determination of drugs, 5000 cells were seeded in 96-well plates on day 0. On day 1, drugs in a series of 10-fold dilutions were added to the cells. For dasatinib and FTY-720, the doses were from 10 μM to 10 pM; For phenothiazines, the concentration was from 100 μM except pimethixene (5 mM). On day 4, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich) based assays were performed and absorbance was measured at 595 nm with 630 nm as reference. Data were calculated based on 3 independent experiments, each time with triplicate or quadruplicate wells. IC50 values were calculated by nonlinear regression model using GraphPad Prism (La Jolla, CA).

2.5. Cell cycle analysis and annexin V apoptosis assay

Human pancreatic cancer cells treated either with or without penfluridol were fixed with 70% ethanol and stained with propidium iodide. DNA contents were analyzed by flow cytometry. For apoptosis analysis after treatment with penfluridol cells were stained with FITC-conjugated annexin V and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's instructions. Stained cells were analyzed with flow cytometer (LSRII, BD Biosciences).

lines with IC50s at or above 100% of this median value were designated as completely resistant (white), and those with IC50s below 10% of the median value designated as most sensitive (darkest red). The heat map was constructed from IC50s shown in Supplementary Table 2.

2.6. RNA array expression analysis and gene ontology analysis

Total RNA was isolated using Qiagen RNAeasy kit (Qiagen, Valencia, CA) according to manufacturer's protocols. Three dasatinib-resistant (MiaPaCa2, Panc1, SU8686) and three dasatinib-sensitive (Panc0504, Panc0403, Panc1005) pancreatic cancer cell lines were used. Biological triplicates were employed for each cell line. Complementary DNA microarray analysis was performed using Illumina Human HT-12 v4 BeadChip (Illumina, San Diego, CA) at the National University of Singapore Core Facility following the manufacturer's instructions. Microarray data were deposited in Gene Expression Omnibus (www.ncbi.nlm.nih.giv/geo/) under accession number GSE59357. Raw data with subtraction of background were obtained using Illumina BeadStudio v3. Based on the detection p value ($p = 0.05$ as cutoff), pre-processing for those with either negative or low intensities were performed for each probe. Pre-processed data were normalized using the Cross-Correlation method (Chua et al., 2006). Normalized data were used to identify differentially expressed genes based on average fold change (fold change = 1.5 as cutoff) of resistant versus sensitive cell lines and two-tailed student test cross the replicated samples ($p = 0.05$ as cutoff). WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) (Zhang et al., 2005) was used to perform pathway analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 was used to discover gene enrichment and functional groups (Huang DW, 2009a, b).

2.7. Connectivity Map (CMAP)

The differentially up- and down-regulated genes from the microarray data were fed as up-tag and down-tag signatures to the online software Connectivity Map from the Broad Institute (Cambridge, MA) to identify a set of drugs with similar effects in a treatment-control pair to the genotypic differences between dasatinib-resistant and -sensitive cell lines. The gene set enrichment analysis generated groups of drug with an enrichment score between −1 and +1. The score represented the correlation between the gene signature of the query and a treatment-control pair in CMAP. A positive enrichment score represents positive connectivity, indicating that the compound induced the expression of the query signature. Compounds with negative enrichment scores represent negative connectivity between the compound and the phenotype of dasatinib-resistant cell lines (the query).

2.8. Colony formation assays

For colony formation on plastic, 800 cells were seeded in 6-well plates and exposed to drug treatment for 14 days. Cells were stained with crystal violet (0.2%) and washed with PBS. For clonogenic growth in soft agar, 3000 or 5000 cells/well in top layer of soft agar plates (top layer of 0.35% low melting agarose and bottom layer of 0.5% agarose) were cultured either with or without drugs until the colonies were large enough for enumeration. Colonies were stained with 1:50 Gentin Violet and rinsed with PBS until the colonies were easily detected. Colonies were photographed and counted with ImageJ (http://rsbweb.nih.gov).

2.9. Combination index analysis

MTT assay was performed on various combinations of drugs and the results were analyzed with Calcusyn (Biosoft, UK). The analysis on the dose effect produces a combination index (CI) which indicates synergism (<1), additive effect (=1), or antagonism (>1) (Chou, 2006).

2.10. Western blot analysis

Protein lysates were prepared with ProteoJet mammalian cell lysis reagent plus Proteoblock Protease Inhibitor cocktail (Thermo Fisher, Waltham, MA). Fifty microgram of protein was analyzed with SDS-PAGE followed by Western transfer to PVDF membranes. Antibodies were from Cell Signaling (Danvers, MA), Sigma–Aldrich (St. Louis, MO), or Santa Cruz (Santa Cruz, CA).

2.11. PP2A phosphatase assay

Cell lysates were immunoprecipitated with PP2A-C antibody in assay buffer from PP2A Immunoprecipitation Phosphatase Assay Kit (Millipore, Temecula, CA, USA). The immunoprecipitates were incubated with phosphopeptide substrate and enzyme activity of phosphatase was detected by reading at 650 nm according to Manufacturer's instructions. Immunoprecipitates pretreated with okadaic acid (1 nM) before incubation with phosphopeptide substrate was used as internal control to detect specific PP2A activity. Relative PP2A activity was compared to negative controls without enzyme.

Table 1 – IC50 (nM) of dasatinib in pancreatic cancer cell lines.

Pancreatic cancer cell line	Library screen	MTT
CaPan2	68	50
Panc0504	71	54
Panc0403	1000	103
PANC 0813	94	22
HPAC	459	243
Panc1005	95	272
CFPAC1	97	R
PL45	85	R
Panc0327	671	R
Panc0203	1000	R
SU8686	931	R
Panc1	1000	R
MiaCaPa2	1000	R
AsPc1	1000	R
BxPc3	n.a.	R

Highest dosage used was 1 mM in library screen and 10 mM in MTT assays; IC50 was determined as described in Material and Methods; Gray shaded areas indicate discrepant results between library screen and MTT assays; R: resistant (IC50 ≥ 1 uM); n.a.: not done.

Table 2 – Compounds that reverse gene expression pattern of dasatinib resistant pancreatic cancer cell lines using Connectivity Map.

Compound	Enrichment score	*p* value
Thioridazine	−0.422	0.00081
Metoclopramide	−0.696	0.00187
PHA-00851261E	−0.611	0.00221
Loxapine	−0.808	0.00265
Fendiline	−0.868	0.00459
Minocycline	−0.691	0.00637
Iopamidol	−0.738	0.00937
Prestwick-864	−0.717	0.01313
Suloctidil	−0.693	0.01880
Trimethylcolchicinic acid	−0.671	0.02616

The differentially up- and down-regulated genes between dasatinib-resistant and -sensitive cell lines from the microarray data were analyzed using Connectivity Map. Negative enrichment scores indicate reversal of expression of the query gene signature by the compound.

2.12. *Lentivirus production and gene knockdown of PP2A*

Scramble shRNA SHC002 was purchased from Sigma (St. Louis, MO). Sequences used for gene knockdown were shPPP2CA: (1) CCGGTGGAACTTGACGATACTC-TAACTCGAGTTAGAGTATCGTCAAGTTCCATTT TG (2) CCGGCCCATGTTGTTCTTTGTTATTCTCGAGAATAACAAA-GAACAACATGGG TTTTTG. Oligonucleotides were annealed and cloned into pLKO.1. Lentivirus was prepared using Sigma MISSION lentiviral packaging mix (SHP001) together with shRNA and transfected into 293T cells. Virus supernatants were harvested 48 h after transfection, filtered with a 0.45 μm filter; and together with polybrene, used to infect pancreatic cancer cells. Stable knockdown cells were selected with puromycin.

2.13. *Statistical analysis*

For comparison between two groups, Graphpad Prism (La Jolla, CA) was used to calculate statistical significance with two-tailed *t*-test. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

3. Results

3.1. *Activity analysis of multiple families of kinase inhibitors against pancreatic cancer cells*

A large panel of 66 kinase inhibitors (collectively targeting SRC/TEC/RTK, JAK, ERBB, PI3K/AKT, MAPK, other Ser/Thr kinases including AMPK, PKA, PKC, IκK (NFKBIA), CAMK, AURK, and CDK families) was tested for their ability to suppress the proliferation of 14 human pancreatic cancer cell lines (AsPc1, MiaPaCa2, HPAC, Panc0203, Panc0403, SU8686, Panc0504, PL45, Panc1005, Panc0327, Panc1, CFPAC1, Panc0813, CaPan2). These cell lines showed diverse sensitivity to the kinase inhibitors and the data (IC50) are summarized as a heat map (Figure 1). Overall, the pancreatic cancer cell lines were resistant to most of the kinase inhibitors relative to effects of these drugs on large cohorts of other cell types, such as hematologic malignancy cells (Tyner et al., 2013). Nevertheless, many cell lines were sensitive to the broad-spectrum kinase inhibitor staurosporine as well as its analog midostaurin (PKC-412), the RTK/SRC/TEC inhibitors dasatinib and sunitinib, the JAK inhibitor AG490, the PI3K/AKT inhibitor CAL-101 (GS-1101/idelalisib), as well as an NFkB (NFKB1) inhibitor that targets IκK (Figure 1).

Because of its activity, we focused on dasatinib. Six cell lines (AsPc1, MiaPaCa2, Panc0203, Panc0403, Panc1, SU8686) were resistant to dasatinib, not reaching an IC50 even at the highest concentration tested (1 μM); two (HPAC1 and Panc0327) were marginally sensitive (IC50 around 500 nM); and six (Panc0504,

Table 3 – IC50 of phenothiazines in pancreatic cancer cell lines.

	Pancreatic cancer cell lines	Perphenazine (μM)	Chlorpromazine (μM)	Penfluridol (μM)	Pimethixene (mM)	Thioridazine (μM)
1	MiaCaPa2	R	R	36.9	R	76.1
2	Panc1	–	–	12.0	–	–
3	SU8686	44.4	73.8	9.3	R	19.0
4	Panc0504	–	–	21.0	–	–
5	Panc0403	–	–	35.8	–	–
6	Panc1005	R	R	R	R	24.8
7	Panc0203	26.2	64.6	8.9	R	24.4
8	Panc0327	31.4	50.1	10.5	0.44	18.2
9	AsPc1	23.9	43.9	R	R	17.9
10	BxPc3	16.7	48.2	16.2	1.83	18.9
11	HPDE	–	–	54.4	–	4.4

R: Resistant.
–: Not tested.
Rows 1 to 3: Dasatinib-resistant cells used in microarray analysis.
Rows 4 to 6: Dasatinib-sensitive cells used in microarray analysis.
IC50s were calculated from the results of MTT assays as described in Materials and Methods.

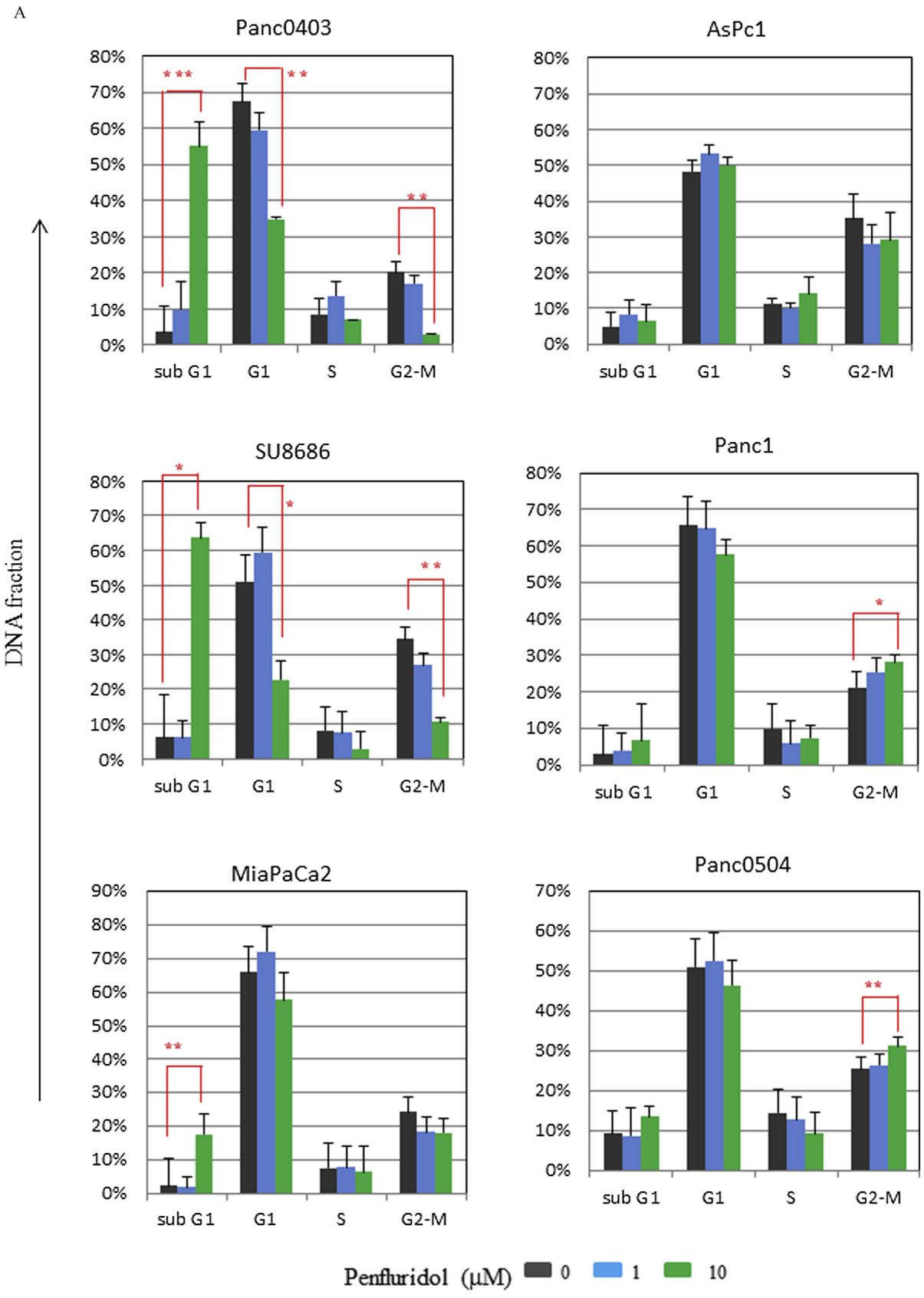


Figure 2 – Effects of phenothiazines on cell cycle and apoptosis of pancreatic cancer cell lines. (A) Cell cycle analysis. Six pancreatic cancer cell lines (Panc0403, SU8686, MiaPaCa2, Pan1, Panc0504) were treated with penfluridol (1, 10 μM; 36 h) and analyzed by flow cytometry after staining with propidium iodide. (B) Cell cycle analysis. Three pancreatic cancer cell lines (Panc0403 MiaPaCa2, AsPc1) were treated with penfluridol (1, 3 μM; 18 h) and analyzed for DNA content by flow cytometry. (C) Apoptosis analysis by annexin V assay. Three pancreatic cancer cell lines

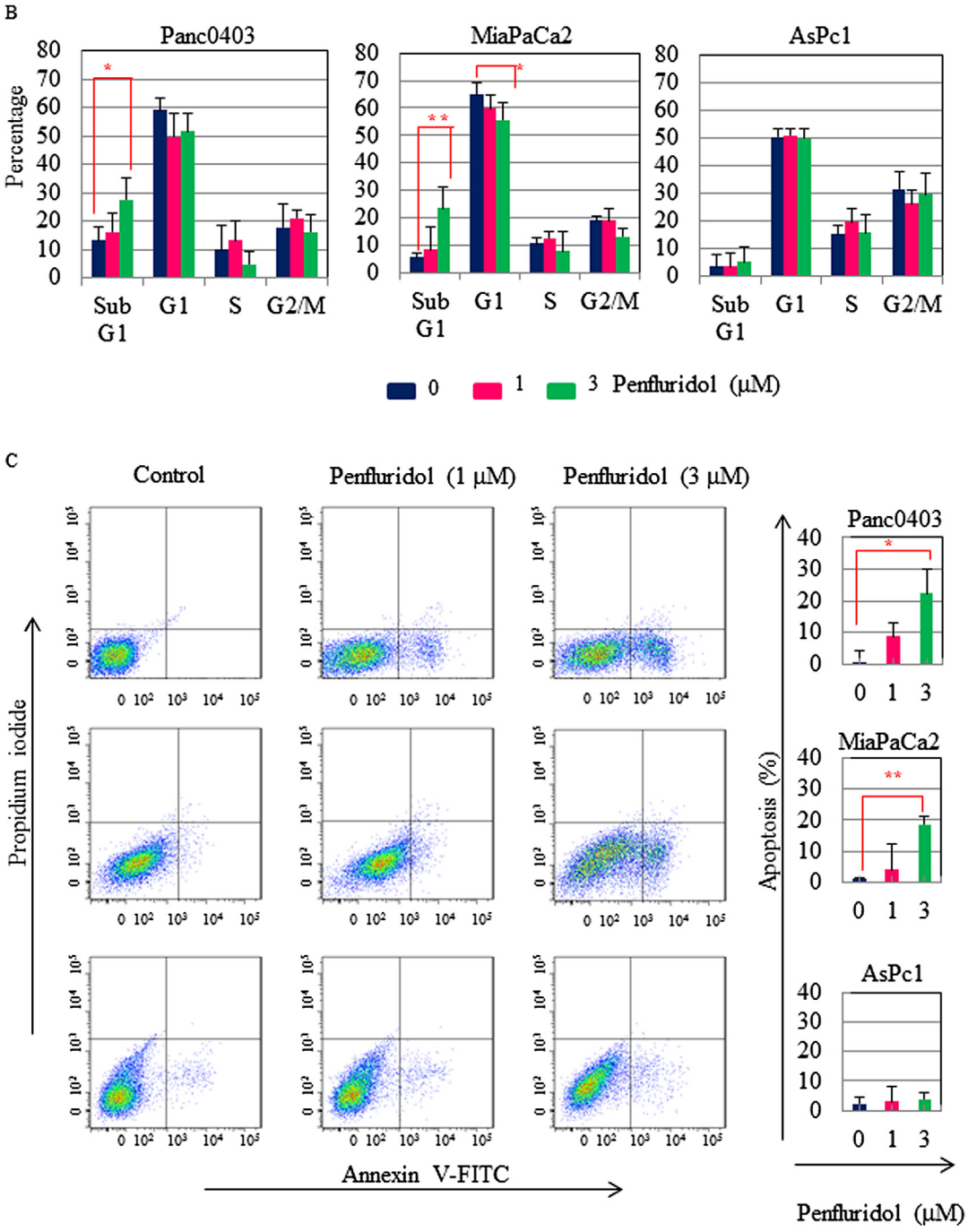
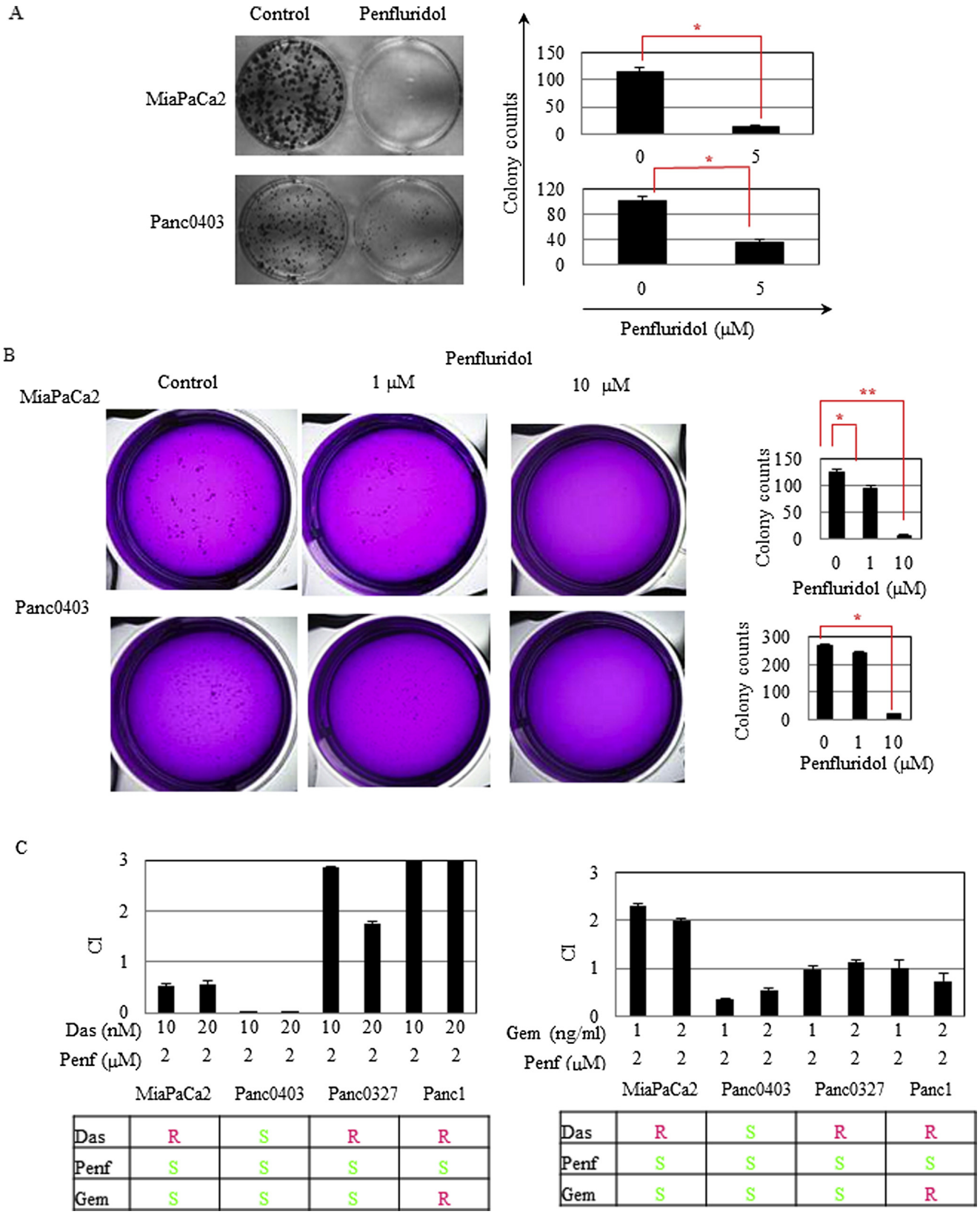


Figure 2 – (Continued)

PL45, Panc1005, CFPAC1, Panc0813, CaPan2) were sensitive to dasatinib (IC50 < 100 nM). To validate the sensitivities of the pancreatic cancer cells to dasatinib, we tested a more finely tuned series of concentrations for 72 h and evaluated proliferation by MTT assays for the 15 pancreatic cancer cell lines. Cell lines were classified as either dasatininb resistant (BxPc3, AsPc1, MiaPaCa2, Panc1, SU8686, Panc0203, Panc0327, PL45, CFPAC) when the IC50s were ≥1 μM or dasatinib-sensitive (CaPan2, Panc0504, Panc0403, Panc0813, HPAC, Panc1005) when IC50s were in the 20–250 nM range (Table 1).

(MiaPaCa2, Panc0403, AsPc1) were treated with penfluridol (1, 3 μM; 18 h) and analyzed with flow cytometer after staining with FITC-conjugated annexin V and propidium iodide. Percentage apoptotic cells is shown by a bar graph (right panel). Data are representative of three experiments. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. No p values indicates nonsignificant.



Das	R	S	R	R
Penf	S	S	S	S
Gem	S	S	S	R

Das	R	S	R	R
Penf	S	S	S	S
Gem	S	S	S	R

Figure 3 – Anti-proliferative properties of penfluridol in pancreatic cancer cell lines. Colony formation assays on (A) plastic and (B) soft agar. Two pancreatic cancer cell lines (MiaPaCa2, Panc0403) were grown either with or without penfluridol at indicated concentrations. Clonogenic growth was counted on day 14 of culture. Representative images of colonies are shown. Right panels are bar charts of number of colonies counted by ImageJ. Colony counts represent the mean ± SD of two experiments done in triplicates. *: $p < 0.05$; **: $p < 0.01$. No p values indicates nonsignificant (C) Combination index (CI) of penfluridol either with dasatinib or gemcitabine. Four pancreatic cancer cell lines (MiaPaCa2,

3.2. Discovery of anti-pancreatic cancer drugs by in silico screening with CMAP

Dasatinib is an FDA-approved tyrosine kinase inhibitor for pancreatic cancer, but similar to conventional therapeutic drugs, pancreatic cancer cells have either intrinsic resistance or quickly become resistant to dasatinib. To address this problem, we analyzed 3 dasatinib-resistant (MiaPaCa2, Panc1, SU8686) and 3 dasatinib–sensitive (Panc0504, Panc1005, Panc0403) pancreatic cancer cell lines by RNA expression array. A total of 1754 unique genes were differentially (fold change ≥ 2, $p < 0.05$) expressed in dasatinib-resistant cell lines compared to the dasatinib-sensitive cell lines (Supplementary Table 3). Heatmap of gene expression levels distinguished differences between these two groups of cell lines and within each group the gene profiles were comparably similar (Supplementary Figure 1). Gene-Ontology Analysis of microarray data indicated that dasatinib-resistant cells had enrichment of genes related to regulation of cell proliferation, apoptosis, signal transduction and metabolic processes of small molecules and macromolecules, as well as regulation of kinase activity (Supplementary Table 4). Pathway analysis identified an association of resistance to dasatinib with signal transduction of MAPK and p38MAPK (MAPK14) and metabolisms of sphingolipid and glutathione (Supplementary Table 5).

To search for novel therapeutic drugs that sensitize pancreatic cancer cells to dasatinib, we analyzed the microarray data using the Connectivity Map database (CMAP). A negative enrichment indicates negative association between the compound and the phenotype of the "query signature", in this case, the up-regulated genes in the dasatinib-resistant cells. In the list of top ten small molecules of negative enrichment (Table 2), thioridazine is an FDA approved drug for psychosis. Previous studies showed it sensitizes renal carcinoma cells to TRAIL-induced apoptosis (Min et al., 2014) and kills activated B-cell like diffuse large B-cell lymphoma cells *in vitro* and *in vivo* (Nagel et al., 2012). Therefore, the potential of thioridazine was further pursued as a therapeutic drug either alone or with dasatinib in pancreatic cancers.

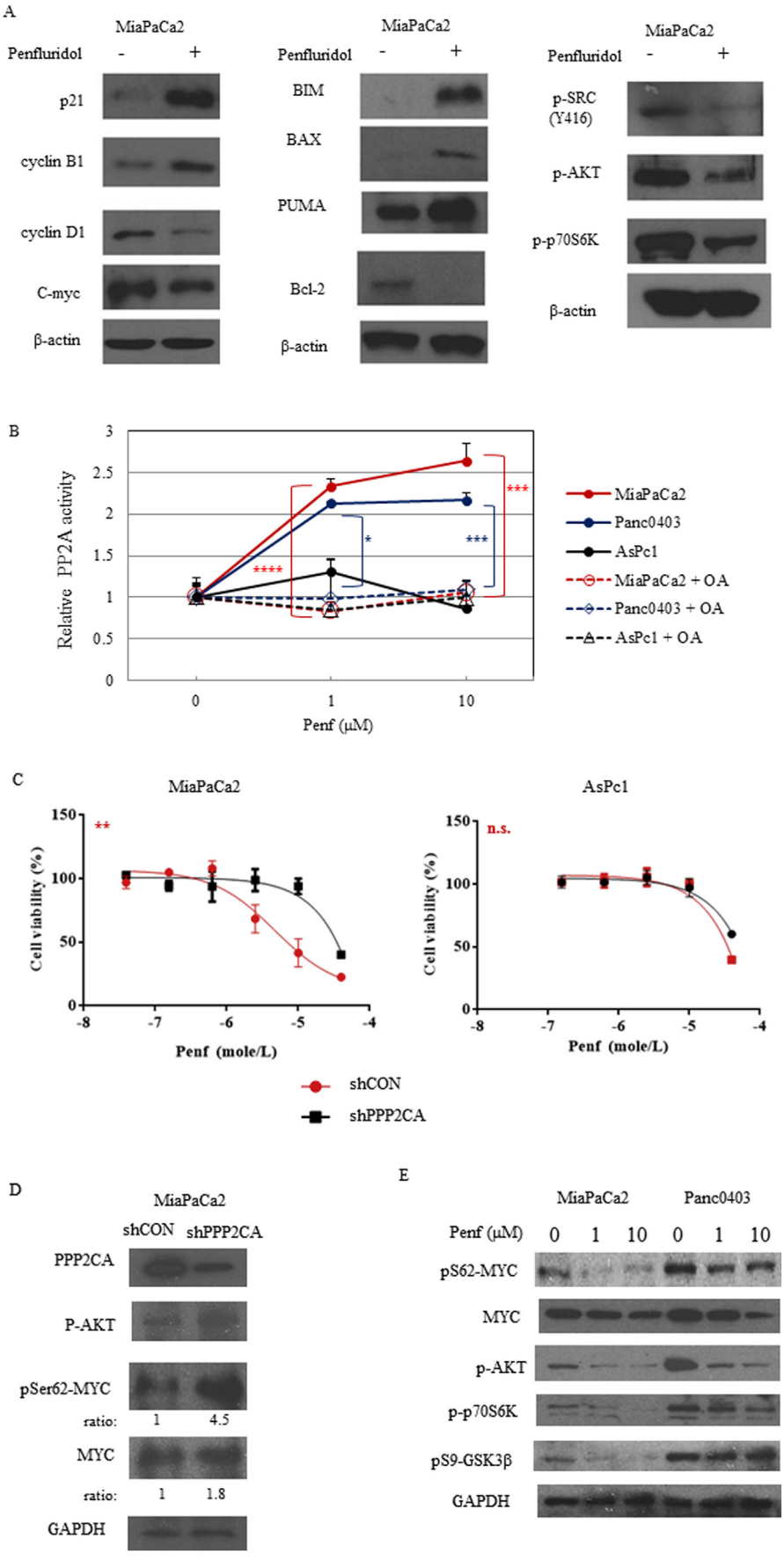
3.3. Antiproliferative properties of phenothiazines

Thioridazine belongs to a family of drugs known as phenothiazines, which also include chlorpromazine, perphenazine, penfluridol, and pimethixene. We evaluated the ability of this group of drugs to inhibit cell proliferation of 10 pancreatic cancer cell lines, plus one normal pancreatic epithelial cell line, HPDE. The potency of drugs as determined by MTT assays varied between cell lines (Table 3). Pimethixene showed the least potency against these pancreatic cancer cell lines with an IC50 up to a millimolar concentration (Table 3). Penfluridol and thioridazine were the two more potent drugs, with the IC50 of penfluridol ranging between 8.9 and 36.9 μM and thioridazine ranging between 17.9 and 76.1 μM. The normal pancreatic epithelial cell line HPDE cells had an IC50 for penfluridol and thioridazine of 54.4 and 4.4 μM, respectively. We selected penfluridol for further investigation of its effect on pancreatic cancer cells. Six pancreatic cancer cell lines (Panc1, Panc0504, Panc0403, SU8686, MiaPaCa2, AsPc1) were treated with penfluridol (1, 10 μM; 36 h) and DNA content was analyzed. Cell cycle analysis showed an increase in the G2-M phase in 2 representative cell lines (Panc1, Panc0504) and an increase in the pre-G0 population in 3 representative cell lines (Pan0403, SU8686, MiaPaCa2) (Figure 2A). No change was observed in penfluridol-resistant cell line AsPc1 (Figure 2A). These results indicate penfluridol has an impact on both cell cycle progression and apoptosis in the context of different cell lines. Two penfluridol-sensitive cell lines (MiaPaCa2 [IC50: 36.9 μM], Panc0403 [IC50: 35.8 μM]) and one penfluridol-resistant cell line (AsPc1) were treated with penfluridol at lower concentration and shorter incubation time (1 or 3 μM, 18 h). Cell cycle analysis showed a dose-dependent increase of sub-G1 population in MiaPaCa2 (2%–18%) and Panc0403 (10%–55%) but no change in AsPc1 (Figure 2B). These cells were further evaluated by annexin V assay. In the two penfluridol sensitive cell lines (MiaPaCa2, Panc0403), increase in the fraction of annexin V-positive/propidium iodide-negative cells indicated these cells underwent apoptosis after 18 h treatment with 1 μM penfluridol. The apoptoic fraction increased as the concentration of penfluridol increased from 1 to 3 μM (Panc0403: 8.6%–22.4%; MiaPaCa2: 3.8%–18.5%) (Figure 2C). In contrast, the penfluridol-resistant cell line AsPc1 did not undergo apoptosis (Figure 2C). Clonogenic growth of the pancreatic cancer cell lines (MiaPaCa2, Panc0403) on both plastic (Figure 3A) and soft agar (Figure 3B) was decreased by penfluridol. Moreover, the cytotoxicity of the drug combination of penfluridol and dasatinib was evaluated with the combination index analysis based on the MTT assay. Penfluridol augmented the activity of dasatinib in 2 pancreatic cancer cell lines (MiaPaCa2, Panc0403), but not in the two dasatinib-resistant pancreatic cancer cell lines (Panc0327, Panc1) (Figure 3C, left panel). In addition, penfluridol sensitized three cell lines (Panc0403, Panc0327, Panc1) to gemcitabine treatment (Figure 3C, right panel).

3.4. Mechanistic studies of penfluridol in pancreatic cancer cells

Effect of penfluridol on key proteins in pancreatic cancer cells was evaluated by Western blots. Exposure to penfluridol (10 μM, 24 h) caused accumulation of proteins involved in the G2-M phase of cell cycle arrest (cyclin B1 and p21 [CDKN1A]) and suppression of proteins associated with cell growth (cyclin D and MYC) (Figure 4A). Expression levels of pro-apoptotic proteins (BIM, BAX, and PUMA) were markedly up-regulated, while the anti-apoptotic Bcl-2 was down-

Pan0403, Panc0327, Panc1) were incubated with drugs for 48 h at concentrations indicated in the Figure. CIs were calculated with CalcuSyn software as described in the Materials and methods. CI: <1 (synergism); = 1 (additive effect); >1 (antagonism). Das: dasatinib; Penf: penfluridol; Gem: gemcitabine; R: resistant; S: sensitive.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MiaPaCa2
Penfluridol - +
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C-myc
β-actin
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BAX
PUMA
Bcl-2
β-actin
MiaPaCa2
Penfluridol - +
p-SRC (Y416)
p-AKT
p-p70S6K
β-actin
B
Relative PP2A activity
Penf (μM)
MiaPaCa2
Panc0403
AsPc1
MiaPaCa2 + OA
Panc0403 + OA
AsPc1 + OA
C
MiaPaCa2
AsPc1
Cell viability (%)
Penf (mole/L)
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E
MiaPaCa2 Panc0403
Penf (μM) 0 1 10 0 1 10
pS62-MYC
MYC
p-AKT
p-p70S6K
pS9-GSK3β
GAPDH

regulated in penfluridol-treated pancreatic cancer cells (Figure 4A). Multiple signaling pathways (SRC, PI3K/AKT, mTOR/p70S6K [MTOR/RPS6KB1]) play essential roles in pancreatic tumorigenesis; and penfluridol treatment decreased phosphorylation levels of SRC, AKT, and p70S6K (Figure 4A).

Both p70S6K and AKT are substrates of PP2A (Clerkin et al., 2008; Wainszelbaum et al., 2012) and a recent study demonstrated the anti-leukemic activity of perphenazine by targeting PP2A in T cell acute lymphoblastic leukemia (Gutierrez et al., 2014). Our observation suggests a potential role of PP2A protein phosphatase in pancreatic cancer cell death induced by penfluridol. This was confirmed by increase of PP2A phosphatase activity in cells treated with penfluridol (Figure 4B). Penfluridol induced PP2A activity in penfluridol-sensitive cells (MiaPaCa2, Panc0403), but not in penfluridol-resistant cells (AsPc1) (Figure 4B). On the other hand, knockdown of the catalytic subunit of endogenous PP2A (shPPP2CA) inMiaPaCa2 cells rendered these cells more resistant to killing by penfluridol. In contrast, in the phenothiazine resistant AsPc1 cells shPPP2CA showed no significant effect on growth (Figure 4C). Knockdown of PPP2CA in MiaPaCa2 calls increased expression levels of phosphorylated AKT and MYC. Furthermore, phosphorylation at Ser 62 of MYC was relatively increased compared to increase of total protein (4.5 versus 1.8) (Figure 4D). On the other hand, loss of the phosphate at Ser62 occurred in pancreatic cancer cells treated with penfluridol (Figure 4E). Two pancreatic cancer cell lines (MiaPaCa2, Panc0403) were treated with penfluridol (0, 1, 10 μM, 24 h). Expression of Ser62-phosphorylated MYC was decreased with increasing concentration of penfluridol (Figure 4E). Dephosphorylation of three other substrates of PP2A (AKT, p70S6K, GSK3β) was also observed (Figure 4E). These results demonstrate PP2A activity was induced by penfluridol and subsequently resulted in cell death in these pancreatic cancer cells.

3.5. *The role of PP2A protein phosphatase in cell proliferation of pancreatic cancer*

FTY-720 (fingolimod) is an FDA-approved drug that is used for multiple sclerosis; and it appears to have multiple modes of action including activation of PP2A (Matsuoka et al., 2003). It has antileukemic activity in myeloproliferative neoplasms (Oaks et al., 2013) and selectively eliminates tyrosine kinase inhibitor-resistant chronic myeloid leukemic stem cells (Neviani et al., 2007). FTY720 had potent anti-proliferative activity and produced IC50 values in the nanomolar range against dasatinib-resistant cell lines (Panc1, Panc0203, Panc0327, AsPc1, BxPc3, MiaPaCa2, PL45, SU8686) (Table 4). Clonogenic growth in soft agar was suppressed after FTY-720 treatment in the pancreatic cancer cell lines (MiaPaCa2, Panc0403) (Figure 5A). Phosphorylation levels of proteins in two pancreatic cancer cell lines (MiaPaCa2, Panc0403) treated with FTY-720 (0, 10 nM, 24 h) were examined. Decreased phosphorylation of AKT and p70S6K was observed in cells after incubation with FTY-720 (Figure 5B). In addition relative phosphorylation at Ser62 of MYC was suppressed compared to decrease in total MYC (0.3 versus 0.6 and 0.2 versus 0.4 in MiaPaCa2 and Panc0403, respectively, Figure 5B). These results demonstrate similar activity of FTY-720 to that of penfluridol. MiaPaCa2 cells with PPP2CA knockdown (shPPP2CA) became less sensitive to FTY-720 compared to control cells (shCON) (Figure 5C). Co-

Table 4 – IC50 values of gemcitabine, dasatinib, penfluridol, and FTY-720 in human pancreatic cancer cells.

Cell Line	Gem (ng/mL)	Dasatinib (nM)	Penfluridol (mM)	FTY-720 (nM)
Panc1	R	R	12	343
PANC0203	R	R	9	41
PANC0327	26	R	11	23
PANC0403	2	103	36	152
PANC0504		54	2	
PANC0813	R	22		
PANC1005		272	R	22
AsPc1	R	R	R	68
BxPc3	46	R	16	33
MiaPaCa2	1	R	37	53
CaPan1				
CaPan2		50		
CFPac1	25	R		
HPAC		243		
HPAFII				
PL45	R	R		15
SU8686	68	R	9	24
HPDE	1		54	53

IC50 was calculated from results of MTT assays as described in Materials and methods. Mean ± SD of 2 experiments was performed in quadruplicates, and the calculated IC50 values are shown. IC50s for gemcitabine, dasatinib, and penfluridol are compiled together with FTY-720 for Reference. Gem: gemcitabine. Blacked shaded columns: not determined. R: resistant.

Figure 4 – Exploration of mechanisms by which penfluridol inhibits growth of pancreatic cancer cells. (A) Western blot analysis of pancreatic cancer cell lines following exposure to penfluridol treatment. SU8686 and MiaPaCa2 cell lines were treated with penfluridol (10 uM, 24 h), and cell lysates were analyzed by immunoblotting with the indicated antibodies. (−) control; (+) penfluridol. (B) Activation of PP2A phosphatase activity by penfluridol. Three pancreatic cancer cell lines (MiaPaCa2, Panc0403, AsPc1) were treated with penfluridol (1, 10 μM) for 24 h, and PP2A activity was measured as described in Materials and methods. Relative PP2A activity was normalized to cells without treatment. Data are mean ± standard deviation from two individual experiments. OA: Okadaic acid. (C) Assessment of penfluridol sensitivity after knockdown of PPP2CA (catalytic subunit of PP2A). After PPP2CA knockdown by shRNA (shPPP2CA) in two pancreatic cancer cell lines (MiaPaCa2, AsPc1). Cells were treated with Penfluridol and dose-response was examined by MTT assay. Control cells were infected with scrambled shRNA (shCON). Mean ± SD of 2 experiments done in quadruplicates. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; n.s.: non-significant. (D) Western blot analysis of MiaPaCa2 stably expressing shPPP2CA versus control shCON. Multiple protein expression levels were examined in MiaPaCa2 cells after knockdown of endogenous catalytic subunit of PP2A. (E) Western blot analysis of cells treated with penfluridol. Phosphorylation levels of PP2A substrates in pancreatic cancer cells (MiaPaCa2, Panc0403) treated with penfluridol and analyzed by Western blot.

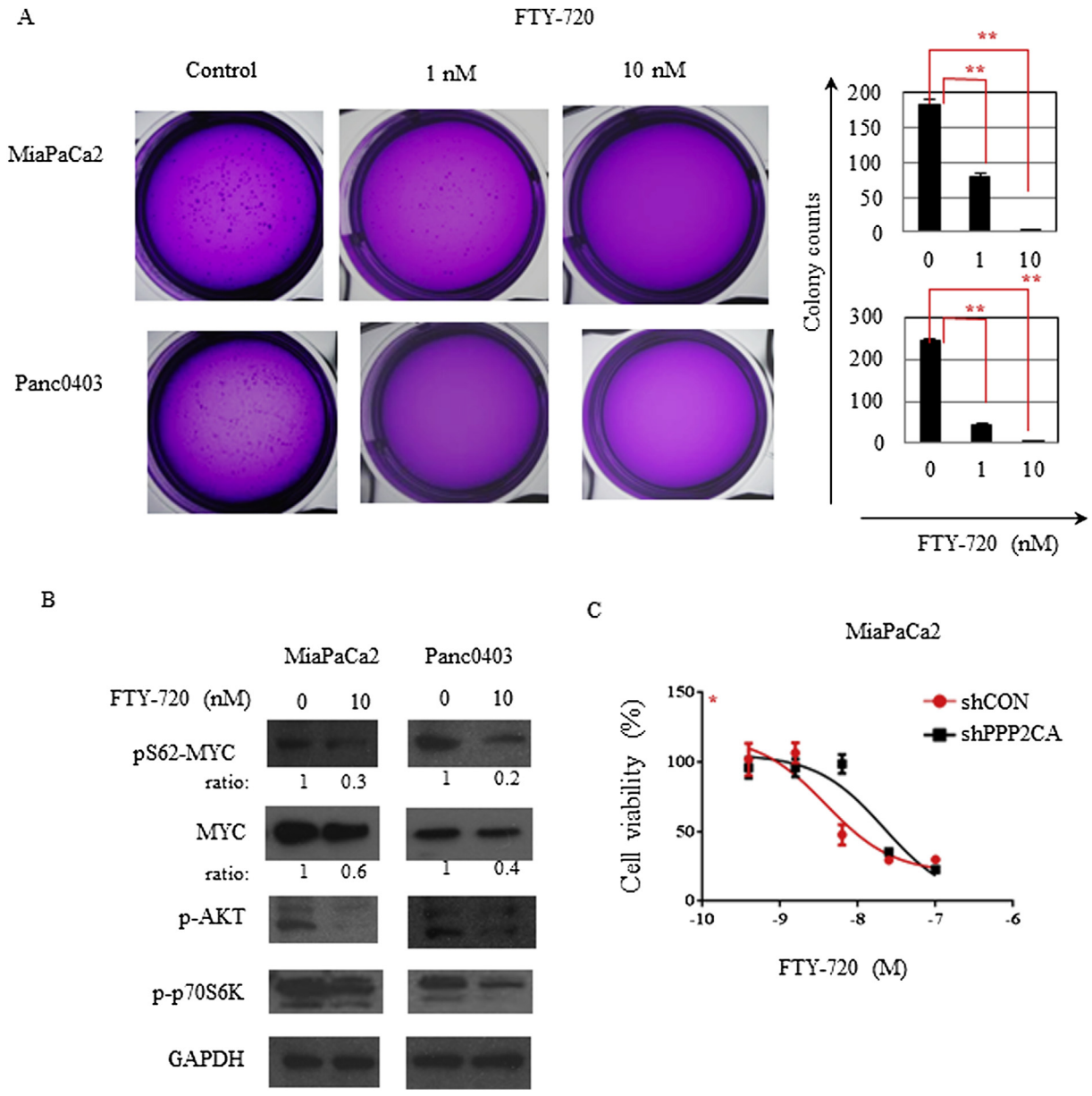
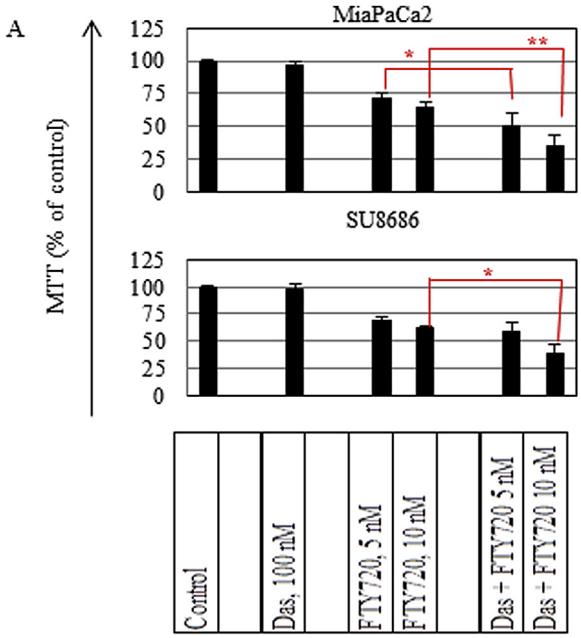


Figure 5 – Anti-proliferative activity of FTY-720 in pancreatic cancer cells. (A) Colony formation assay. Two pancreatic cancer cell lines (MiaPaCa2, Panc0403) were grown in soft agar and treated with FTY-720 for 14 days. Representative wells were from two independent assays with triplicate wells for each experiment. Number of colonies was measure by Image J and shown in bar graph on right panels. **: $p < 0.01$; No p values indicates nonsignificant. (B) Western blot analysis of pancreatic cancer cells treated with FTY-720. Phosphorylation levels of PP2A substrates in pancreatic cancer cells (MiaPaCa2, Panc0403) treated with FTY-720 and detected by Western blot. Ratio shown was estimated using Bio-Rad Quantity One software. (C) Assessment of sensitivity to FTY-720 in PPP2CA knockdown pancreatic cancer cells. PPP2CA was silenced by shRNA (shPPP2CA) in MiaPaCa2 pancreatic cancer cells, cultured with FTY-720 and dose-response was examined by MTT assay. Control cells were infected with scrambled shRNA (shCON). Mean ± SD of 2 experiments done in quadruplicates. *: $p < 0.05$.

treatment with FTY-720 and dasatinib in dasatinib-resistant pancreatic cancer cell lines (MiaPaCa2, SU8686) showed that FTY-720 potentiated dasatinib cytotoxicity (Figure 6A). In additional studies, two dasatinib-resistant cell lines (AsPc1, PL45) were treated with FTY-720 for 24 h before incubation with dasatinib for 48 h. Calculation of the combination index demonstrated synergy at many concentrations of dasatinib and FTY-720 (Figure 6B).

4. Discussion

Targeted therapies have shown benefits in a variety of cancers. In pancreatic cancer, overexpression of either VEGFR, EGFR, IGF1R, or MMPs are some of the molecular targets being evaluated with either small molecule inhibitors or antibodies against these proteins. Overall, the response rate is not high.



B

AsPc1

FTY-720 (nM)	Dasatinib (nM)	CI
20	50	1.0381
20	100	0.9123
20	200	1.1605
50	50	0.3023
50	100	0.4747
50	200	1.9404

PL45

FTY-720 (nM)	Dasatinib (nM)	CI
20	50	0.2261
20	100	0.4202
20	200	2.1270
50	50	0.6252
50	100	0.6498
50	200	0.7407

Figure 6 – Potentiation of dasatinib cytotoxicity by FTY-720. (A) Cell proliferation of dasatinib-resistant pancreatic cancer cell lines (MiaPaCa2, SU8686) treated with increasing concentrations of dasatinib either with or without FTY720 for 48 h. Results were from two independent experiments done in triplicates. *: $p < 0.05$; **: $p < 0.01$. (B) Combination index of FTY-720 with dasatinib in two dasatinib-resistant cell lines (AsPc1, PL45) as calculated by CalcuSyn (Chou, 2006). CI < 1, $= 1$, > 1 indicate synergism, additive effect, and antagonism, respectively. Cells were treated with FTY-720 for an initial 24 h alone, followed by an additional 48 h together with dasatinib. Gray shaded area: CI > 1.

Furthermore, drug resistance often occurs rapidly. Thus, discovery of new anti-proliferative cancer drugs is needed. Previously, screening of human primary leukemias with a panel of 66 kinase inhibitors identified a correlation between drug sensitivity and clinical response (Tyner et al., 2013). We have applied the same panel of kinase inhibitors to explore the therapeutic potential of these compounds against a panel of 14 pancreatic cancer cell lines. Some of the pancreatic cancer cell lines (10/14, 71%) were sensitive to the broad-spectrum kinase inhibitor staurosporine, which has anti-proliferative activity against a variety of cancer types. Clinical studies with the staurosporine analog, midostaurin (PKC-412) in NSCLC patients had notable results (Monnerat et al., 2004), and other related analogs including Sotrastaurin are in phase I and II clinical trials (https://clinicaltrials.gov/show/NCT01801358, 2013). The JAK inhibitor AG490 was moderately potent in 12/14 (85%) pancreatic cancer cell lines. An earlier study shows that AG490 inhibited the growth of the pancreatic cancer cell line, Capan-2, and decreased invasive potential of another pancreatic cancer cell line (SW1990) (Chen et al., 2010). Also, we noted that a fair number of pancreatic cancer cell lines (11/14, 75%) were sensitive to the PI3K/AKT inhibitor CAL-101 (GS-1101/idelalisib). This small molecule is effective in treatment of B-cell malignancies by targeting PI3Kδ (PIK3CD) pathway (Brown et al., 2014).

The RTK/SRC/TEC inhibitor, dasatinib, was the most potent small molecule to decrease the growth of pancreatic cancer cell lines (9/14, 64%). The drug is FDA-approved for pancreatic cancer treatment. Increased activation of SRC, one of the targets of dasatinib, correlates with poor survival in pancreatic cancer patients (Morton et al., 2010), and dasatinib in combination with gemcitabine resulted in stable disease and partial response in these patients (Hong et al., 2013). Although in preclinical studies dasatinib has shown potential in the

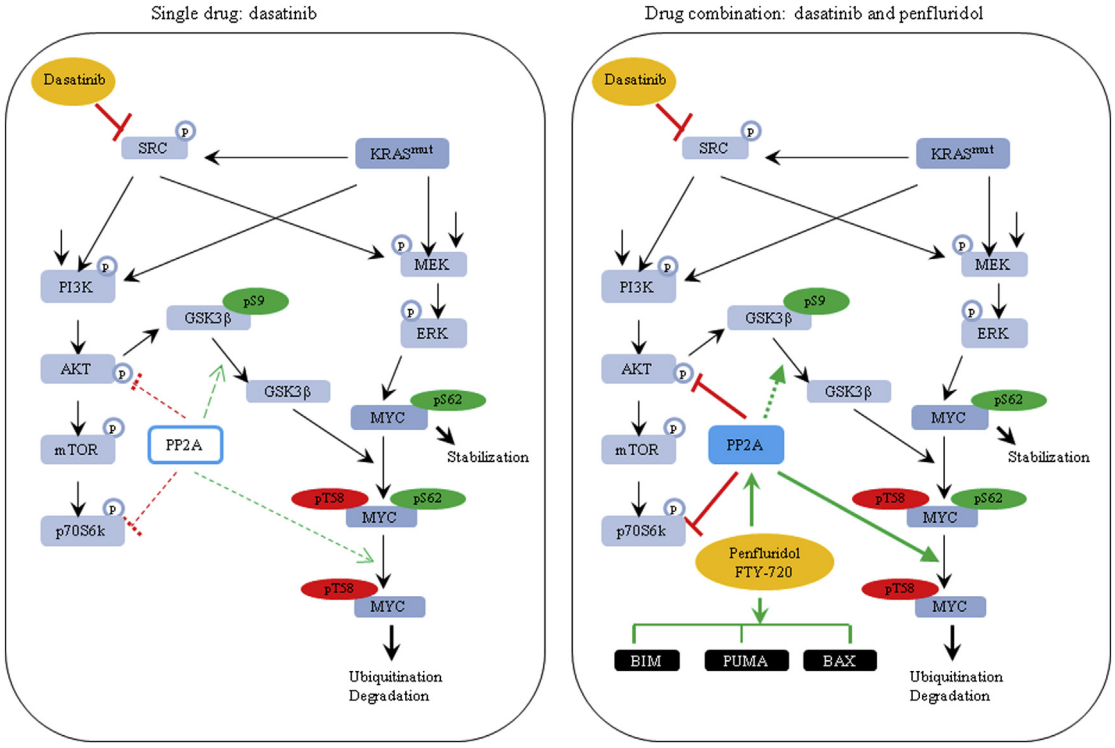


Figure 7 – Scheme representing inhibition of cell proliferation in pancreatic cancer cells by penfluridol and FTY-720. Modulation of PI3K/AKT and MEK/ERK signaling pathways mediated through PP2A. Phosphorylation of AKT, p70S6K, and GSK3β are suppressed by PP2A. Phosphoserine 62 of dual-phosphorylated MYC is removed by PP2A and Thr-58-phosphorylated MYC is ubiquitinated and degraded. Moreover, penfluridol activates pro-apoptotic molecules (BIM, PUMA, BAX) and promotes cell death.

treatment of pancreatic cancer, it has not demonstrated significant results in clinical trials (Chee et al., 2013). KRAS mutation is the driver in pancreatic tumorigenesis and it initiates PI3K/AKT signal transduction promoting cancer growth. In addition, KRAS activates RAF/MEK/ERK cascade that enhances tumor progression. Simultaneous inhibition of targets downstream of KRAS may improve the efficacy of dasatinib in pancreatic cancer. In the current study, a gene signature was identified which was present in three dasatinib-resistant pancreatic cancer cell lines but absent in three dasatinib-sensitive pancreatic cancer cell lines as identified by cDNA microarray analysis. Among the up-regulated genes in the dasatinib-resistant cell lines was VIM, which is an epithelial to mesenchymal transition marker associated with aggressive pancreatic cancer (Shah et al., 2007). Another upregulated gene in the resistant cells was HK2 (hexokinase 2) which encodes a rate-limiting enzyme in aerobic glycolysis (Ying et al., 2012; Zhou et al., 2011). This is consistent with displaying the Warburg effect in which cancer cells abundantly utilize aerobic glycolysis; This has been shown to be associated with drug resistance in multiple cancer types (Tamada et al., 2012; Xu et al., 2005). Among the down-regulated genes in the dasatinib-resistant cells, MTAP (methylthioadenosine phosphorylase) was of interest. MTAP is frequently homozygously deleted together with p16INK/CDKN2A in invasive pancreatic adenocarcinoma (Hustinx et al., 2005). Furthermore, BIK was down-regulated in dasatinib-resistant cells. Frequent degradation of BIK is driven by ERK1/2 (MAPK1/3) in SRC-, KRAS- or BRAF-activated cancer cells (Lopez et al., 2012). In addition, expression of several kinases and phosphatases were differentially expressed. Of these, up-regulated kinase PAK4 (p21-activated kinase 4) is recurrently amplified in pancreatic cancer (Chen et al., 2008), and several inhibitors of this kinase have been synthesized and appear to have activity against various cancer cells (Senapedis et al., 2014). Another down-regulated gene in the resistant pancreatic cancer cells is the phosphatase INPPL1 (inositol polyphosphate phosphatase-like 1) which is a negative regulator of endocytosis of the EphA2 receptor (Leone et al., 2008).

By employing CMAP, we identified compounds that can potentially reverse the gene expression profile associated with dasatinib resistance in pancreatic cancer cells. One of the drugs that we identified is fendiline, an antianginal drug, which is a calcium channel blocker that can trigger cell death in human oral cancer cells (Huang et al., 2009). A recent study demonstrated fendiline specifically inhibited K-RAS, not H-RAS nor N-RAS, plasma membrane targeting and signaling (van der Hoeven et al., 2013). Because K-RAS is frequently aberrant in pancreatic cancer, further studies of fendiline and related molecules should be explored. A second drug noted in our screen is minocycline, a tetracycline antibiotic. It is able to inhibit cell growth of human prostate cancer cell lines (Regen et al., 2014) and to decrease cell migration and adhesion in ovarian cancer cells through suppression of IL6

and TGFB-NFkB signaling (Ataie-Kachoie et al., 2013a, 2013b). A third drug identified in our screen is loxapine, a P-glycoprotein inhibitor, which sensitizes doxorubicin-resistant human myelogenous leukemia (K562/DOX) to killing by doxorubicin (Palmeira et al., 2011). Each of these novel leads for therapeutic approaches to pancreatic cancer deserves further study.

Another family of molecules identified by our CMAP screen was thioridazine, a member of the phenothiazine family. We chose to do additional studies with these drugs and focused on penfluridol. Notably, penfluridol-sensitive pancreatic cancer cells had a dramatic increase in expression levels of p21 and the pro-apoptotic proteins BAX and PUMA when cultured with penfluridol (10 μM, 24 h). These changes occurred in the absence of p53 induction in almost all the cell lines (SU8686 being the single exception, data not shown). A recent study suggested that induction of PUMA is critical for cell killing by gemcitabine, and this drug rarely induces PUMA in pancreatic cancers (Hill et al., 2013). We observed penfluridol synergized with gemcitabine in both gemcitabine-resistant Panc1 and gemcitabine-sensitive Panc0327 cells. This suggests PUMA induction by penfluridol augmented killing of pancreatic cancer cells by gemcitabine. With the combination of dasatinib and penfluridol, penfluridol potentiated the effect of dasatinib in dasatinib-resistant MiaPaCa2 cells but not in the other two dasatinib-resistant cells (Panc1, Panc0327). A recent study found that sequential exposure of triple negative breast cancer cells to anti-tumor drugs rather than simultaneous enhanced drug efficacy (Lee et al., 2012). This may have occurred by one drug rewiring the signaling network of tumor cells making them more susceptible to DNA-damaging chemotherapy. Nonetheless, sequential or simultaneous exposure of Panc1 and Pan0327 to penfluridol and dasatinib gave similar results (Supplementary Figure 2).

The phenothiazine compounds are known to activate PP2A (Gutierrez et al., 2014). Silencing of the catalytic subunit (PPP2CA) caused the pancreatic cancer cells to be more resistant to penfluridol treatment. Our observation is similar to a recent study in which knockdown of endogenous inhibitors (SET or CIP2A) of PP2A increased the activity of PP2A and decreased tumorigenic potential of pancreatic cancer *in vitro* and *in vivo* (Farrell et al., 2014). Stimulation of PP2A activity is essential for deceleration of pancreatic tumorigenesis and it may be accomplished by either activation of PP2A itself as shown in our study or targeted inhibition of endogenous inhibitors of PP2A (Farrell et al., 2014). FTY-720 is a small molecule activator of PP2A, and we found that it had robust antiproliferation activity in pancreatic cancer cells including even those resistant to dasatinib.

We propose a scheme showing how penfluridol or FTY-720 may mediate pancreatic cancer death and augment the antitumor activity of dasatinib (Figure 7). Dasatinib is a SRC inhibitor and suppresses signaling of PI3K/AKT and MEK/ERK. Activated PP2A may inhibit these pathways by dephosphorylating its targets including AKT, p70S6K, MYC, GSK3β (Figure 7, left panel). However, 95% of pancreatic cancers have a mutant KRAS whose stimulation pathway may not be inhibited by dasatinib. Increased enzyme activity of PP2A by penfluridol or FTY-720 down-regulates activities of AKT, p70S6K, GSK3 β. Also, removal of phosphor-Ser62 of MYC by PP2A causes ubiquitination and degradation of MYC (Figure 7, right panel). Thus, simultaneous inhibition of targets downstream of KRAS pathway as well as MYC degradation may improve the anti-pancreatic cancer efficacy of dasatinib in pancreatic cancer.

Conflict of interest

The authors have no conflicts of interest to declare.

Authorship contribution

WC, QYS, JT, and HPK lead design, performance and interpretation of the experiments.
KLL, LWD, PW, HY contributed to design of experiments, data collection and analysis.
LP supervised data collection and analysis.
SM contributed to design of experiments.
LATF, SZT, IT, and NZ carried out experimental procedures.
JT and HPK are joint senior authors.
WC and HPK wrote the manuscript with contribution from all co-authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molonc.2015.01.002.

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