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Kinome-wide siRNA screening identifies molecular targets mediating the sensitivity of pancreatic cancer cells to Aurora kinase inhibitors

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ABSTRACT

Aurora kinases are a family of mitotic kinases that play important roles in the tumorigenesis of a variety of cancers including pancreatic cancer. A number of Aurora kinase inhibitors (AKIs) are currently being tested in preclinical and clinical settings as anti-cancer therapies. However, the antitumor activity of AKIs in clinical trials has been modest. In order to improve the antitumor activity of AKIs in pancreatic cancer, we utilized a kinome focused RNAi screen to identify genes that, when silenced, would sensitize pancreatic cancer cells to AKI treatment. A total of 17 kinase genes were identified and confirmed as positive hits. One of the hits was the platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), which has been shown to be overexpressed in pancreatic cancer cells and tumor tissues. Imatinib, a PDGFR inhibitor, significantly enhanced the anti-proliferative effect of ZM447439, an Aurora B specific inhibitor, and PHA-739358, a pan-Aurora kinase inhibitor. Further studies showed that imatinib augmented the induction of G2/M cell cycle arrest and apoptosis by PHA-739358. These findings indicate that PDGFRA is a potential mediator of AKI sensitivity in pancreatic cancer cells.

1. Introduction

Due to the lack of early diagnosis and effective therapeutic modalities, pancreatic cancer remains a devastating disease with a 5-year survival of less than 5% [1]. Gemcitabine, a nucleoside analog which was approved for the treatment of patients with locally advanced or metastatic pancreatic cancer, only has moderate therapeutic effects with an average median survival of 6 months. The FDA approved erlotinib plus gemcitabine combination treatment for locally advanced, inoperable or metastatic pancreatic cancer only demonstrated a moderate survival benefit in a Phase III study (median 6.24 months vs. 5.91 months) [2]. Most recently, a Phase I/II clinical trial showed promising activity of the gemcitabine plus nab-paclitaxel combination in patients with advanced pancreatic cancer [3]. This regimen is currently being evaluated in a randomized Phase III trial. In addition, the FOLFIRINOX (5-FU/leucovorin, irinotecan, and oxaliplatin) regimen was shown to have improved survival compared to gemcitabine alone in a Phase III trial, albeit, with more toxicity [4]. To further improve the treatment outcome and increase the survival rate of pancreatic cancer patients, better tumor markers for diagnosis and new therapeutics are urgently needed.

Aurora kinases are serine-threonine kinases that play important, yet distinct, roles in mitosis [5,6]. There are three Aurora kinases, Aurora A, B, and C in mammals. Since its identification in the late 1990s [7,8], the human Aurora A kinase gene has been reported to be overexpressed and/or amplified in many malignant diseases including breast, colon, bladder, ovarian, melanoma, and pancreatic cancers [9,10]. Deregulation of Aurora A and Aurora B has been linked to advanced tumor stages and poor prognosis of patients (reviewed in [9]). Aurora A is shown to be oncogenic and play an important role in cancer initiation and progression [11]. Although the role of Aurora B in tumorigenesis is less clear, many studies support an association between Aurora B and malignant transformation [11,12]. In pancreatic cancer, we and others have shown that both Aurora A and Aurora B kinases are highly expressed in tumor tissues and the Aurora A gene is amplified in tumor cells [13–15]. In recent years, several small-molecule Aurora kinase inhibitors have been developed and shown to exhibit antitumor activity in both pancreatic cancer cell lines and xenograft models [16,17]. A number of Aurora kinase inhibitors including VX-680 (MK-0457) [18,19], AZD1152 [20,21], MLN8237 [22,23], PHA-739358 [24,25], either have been or are currently in Phase I/II clinical development. Although some of the AKIs have shown evidence of clinical activity, the overall patient response has been modest. For instance, the clinical activity of PHA-739358, a pan-Aurora kinase inhibitor with a dominant Aurora B kinase inhibition related cellular phenotype [24], has largely been consistent with cytostatic effects, with the best

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response so far being stable disease in about 23.7% of evaluable patients [26]. Recently, a Phase I study of PHA-739358 in patients with advanced solid tumors showed that one patient with refractory small cell lung cancer had an objective response lasting 23 weeks [27]. Although the reason for the modest clinical activity of AKIs could be multifaceted, one of the most plausible possibilities is that patient tumors may harbor additional genetic changes (i.e. *context of vulnerability*) that may affect the sensitivity of tumor cells to AKI therapies. For example, it has been shown that Aurora A protects ovarian cancer cells from cisplatin-induced apoptosis by activating the Akt pathway in p53 wildtype cells [28]. This indicates that cisplatin might increase the activity of AKIs in p53 wildtype cells and combining inhibitors of the Akt pathway and AKIs might be synergistic. We hypothesize that similar contexts of vulnerability might also exist in pancreatic cancer cells. By identifying such contexts of vulnerability we will be able to develop either new biomarkers for selecting patient populations for AKI therapies or new AKI-based combination therapies that increase patient response. With the advances in genome-based techniques, particularly in the area of high-throughput RNAi screening, it is possible to carry out systematic searches for the context of vulnerabilities for individual targeted therapies.

As kinases are major control points in cellular signaling and are considered to be highly druggable, the kinome has been the target of large scale functional genomics with RNAi screens and of drug discovery efforts, especially in cancer therapeutics [29]. The aim of this study was to identify kinases that, when inhibited, sensitize pancreatic cancer cells to the treatment of AKIs. To achieve this aim, we carried out a screen using the Human Validated Kinase siRNA Set from Qiagen in combination with an Aurora kinase inhibitor (AKI-1) previously reported by Lampson et al. [30] in pancreatic cells. Positive hits were further subjected to confirmation/validation studies using multiple AKIs in multiple pancreatic cell lines. Using this approach we identified a list of 17 genes that, when silenced by siRNA oligonucleotides, sensitize pancreatic cancer cells to the treatment of AKIs. These genes present potential new targets against which agents that enhance the antitumor activity of AKIs can be developed.

2. Materials and methods

2.1. Chemicals and reagents

VX-680 (MK-0457), sorafenib, and imatinib were purchased from ChemieTek, LLC (Indianapolis, IN). ZM447439 was purchased from Tocris Bioscience (Ellisville, MI). Aurora kinase inhibitor-1 (AKI-1) and MP235 were synthesized in our lab [16,31]. PHA-739358 was purchased from Selleck Chemicals (Houston, TX). Etopside was purchased from Sigma–Aldrich (St. Louis, MO). The chemical structures of the Aurora kinase inhibitors used in this study are shown in Supplementary Figure S1.

The Human Validated Kinase siRNA Set (HVKS) V2 was purchased from Qiagen (Valencia, CA). This siRNA library contains two validated siRNA oligonucleotides for each of 588 kinase and kinase related genes (a total of 1176 siRNA oligonucleotides; see www.qiagen.com). Additional siRNA oligonucleotides targeting individual genes or negative (non-targeting) siRNA oligonucleotides were also purchased from Qiagen. The siRNA oligonucleotides were dissolved in a DNase/RNase free siRNA buffer containing 100 mM KOAc, 30 mM HEPES-KOH, and 2 mM MgOAc at 10 μM stock concentration and stored at −80 °C until use.

2.2. Cell culture

BxPC-3, Mia PaCa-2, AsPC-1, CFPAC-1, PANC-1 and SU.86.86 pancreatic cancer cell lines were purchased from American Type Tissue Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA).

Cell line identities were verified by STR profiling [32] using the AmpFISTR Identifiler PCR amplification kit (Applied Biosystems, Foster City, CA). This method simultaneously amplifies 15 STR loci and Amelogenin in a single tube, using 5 dyes, 6-FAM™, JOE™, NED™, PET™, and LIZ™ which are then separated on a 3100 Genetic Analyzer (Applied Biosystems). GeneMapper ID v3.2. Software was used for analysis (Applied Biosystems). AmpFISTR control DNA and the AmpFISTR allelic ladder were run concurrently. Results were compared to published STR sequences from the ATCC. The STR profiling is repeated once a cell line has been passaged more than 6 months after previous STR profiling.

2.3. Optimization of transfection conditions for HT-siRNA screen

To find the most optimal transfection reagent and conditions for pancreatic cancer cells, we first tested a panel of transfection reagents with two siRNA oligonucleotides, a non-silencing negative control siRNA (Qiagen) and a positive control siRNA (UBB1, Qiagen) [33] in a panel of pancreatic cancer cell lines, including AsPC-1, BxPC-3, CFPAC-1, Mia PaCA-2, PANC-1, and SU.86.86. The panel of transfection reagents includes Lipofectamine 2000 (Invitrogen), Lipofectamine RANiMax (Invitrogen), siLentFect (Bio-Rad, Hercules, CA), Oligofectamine (Invitrogen). The siRNA (9 ng in 2 μl) was first printed onto solid white 384-well plates using a Biomek FX liquid handling system (Beckman Coulter, Fullerton, CA). The transfection reagents were diluted in OptiMEM (Invitrogen) at five different ratios (1:2, 1:3, 1:5, 1:7, and 1:8) from 200 nl/well. The final volumes of the transfection reagents tested were therefore 100, 66.7, 40, 28.6, and 25 nl/well. Diluted transfection reagents (20 μl) were added to the 384-well plates containing siRNA oligonucleotides and were allowed to complex for 30 min. Equal volume of cells was added in growth media resulting in 1000–1200 cells per well depending on growth characteristics of the cell lines (determined in separate experiments). The cells were then incubated in a CO_2 incubator at 37 °C for 96 h at which point 25 μl of CellTiter-Glo® reagent (Promega, Madison, WI) was added to each well to determine cell viability. The luminescence intensities were obtained for each plate using an Analyst GT microplate reader (Molecular Devices, Sunnyvale, CA). Percent viability values were calculated by comparing the intensity units from each treatment condition with that of the untreated controls. The transfection reagent and conditions that give the highest difference in cell viability between the Non-silencing siRNA (negative control purchased from Qiagen) and the lethal siRNA (positive control) were then chosen for the subsequent HT RNAi screening in combination with AKIs.

2.4. Selection of cell lines and AKIs for HT-siRNA screening

To select a cell line and an AKI that would maximize our chances of finding siRNA hits that are specific to Aurora kinase inhibition, we first evaluated three different AKIs in a panel of pancreatic cancer cells, including AsPC-1, BxPC-3, CFPAC-1, Mia PaCa-2, PANC-1, and SU.86.86, using the same growth and assay conditions as those for the siRNA transfection. The three AKIs were VX-680 [34], MP235 [35,36], and AKI-1 [30]. All three AKIs have been shown to inhibit Aurora kinases in cell-free assays with nM IC_{50}s and induce phenotypes in cancer cells that are consistent with the inhibition of Aurora kinases [30,34,36]. The cells were treated with varying concentrations of AKIs. Cell viability was determined 96 h after adding the drug using CellTiter-Glo® Assay. The cell line that provided the most consistent dose–response results with a modest

sensitivity crossing all the AKIs tested was selected as the screening cell line (BxPC-3). The AKI showing relatively smooth dose–response curves with modest activity crossing all cell lines tested would be selected as the screening compound (AKI-1).

2.5. High-throughput RNAi screening

The siRNA library was printed onto 384-well cell culture plates (Corning, Lowell, MA) at 2 μl/well (the final concentration of siRNA in the screening assay was 16 nM). A set of control siRNA oligonucleotides including GFP siRNA, All Star Negative Control siRNA, Non-silencing siRNA, and the UBB1 positive control siRNA (all purchased from Qiagen) were also included. Each of the library siRNA sequences was printed in duplicates and the control siRNAs were printed in quadruplicates. A set of siRNA buffer only wells were also printed for inclusion of negative controls such as buffer and transfection reagent only controls. For each compound concentration one set of the plates printed with siRNA library were used. The assay procedure is shown in Supplementary Figure S2. Briefly, on Day 1, 20 μl of siLentFect diluted in serum free medium was added onto the pre-printed siRNA library plates and incubated for 30 min at room temperature. 20 μl of BxPC-3 cells (1200 cells/well) were then added to each well of the plates. Following an overnight (16 h) incubation in a CO_2 incubator, 5 μl of the Aurora kinase inhibitor, AKI-1, at appropriate concentrations was added into each well of the plates. To ensure the quality of the positive hits to be identified, we designed a screening scheme with five different AKI-1 drug concentrations, EC_{10}, EC_{20}, EC_{30}, EC_{50} (concentrations required to achieve 10%, 20%, 30% and 50% of the maximal growth inhibitory effect, respectively), as well as a vehicle control, all of which were calculated based on the non-regression curve fitting equations of the dose–response curves using the Prism 5 software (GraphPad Inc., La Jolla, CA) in the screening cell line. The zero, EC_{10} and EC_{30} concentration sets were performed in duplicate to ensure the screening quality (this makes a total of nine screening sets of the siRNA library). After adding drugs, the cells were further incubated for 96 h in a CO_2 incubator. On Day 6, the viability of cells in each well was measured by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) as per manufacturer's instructions.

2.6. Hit selection

Hit selection was based on the detection of changes between the drug-dose–response curves (DDRCs) generated from individual siRNA and the negative siRNA control. In general, the following steps were involved: data normalization, filtration of toxic siRNA and outliers, IC_{50} calculation, and ranking. The data normalization was done by fixing the DDRC of plate median and shifting the sample DDRC so that both curves have identical origins at drug dose 0 (siRNA only control). Such normalization allowed us to calculate the size of enclosed area formed by two DDRCs (sample and plate median). Toxic siRNA oligonucleotides (percentage cell survival for siRNA only control is less than 50%) were removed from further analysis. EC_{50}s and EC_{30}s (drug concentration required to achieve 50% and 30% of the maximal cell growth inhibitory effect) for plate median and each siRNA were calculated by fitting the data to a sigmoid dose–response model using nonlinear regression with the Matlab software (2007a, The MathWorks Inc.). The EC_{30} and EC_{50} shift between sample DDRC and the DDRC of plate median was then used to rank the siRNA. For the subsequent confirmation/validation experiments, since more potential sensitizer hits were tested, we used a negative siRNA control (Non-silencing siRNA from Qiagen) as a reference instead of plate median in data normalization.

2.7. Confirmation screening

From primary screening, we identified kinase genes targeted by siRNA that mediate sensitivity of AKI-1 in the BxPC-3 cell line. To exclude the possibility of siRNA with biological off-target effects, we performed a confirmation screen using four siRNA sequences per gene in combination with AKI-1 in the BxPC-3 cell line and defined confirmed hits as those kinases whose inhibition was synthetically lethal with AKIs in pancreatic cancer cells with concordant results from two or more unique siRNAs.

2.8. Drug combination treatment

Cells were seeded at 2000 cells/well in 96-well plates and allowed to grow overnight. On the second day, a serial dilution of the Aurora kinase inhibitors (ZM447439 or PHA-739358) combined with fixed concentrations of the second drug (imatinib or sarofenib) as indicated in the figures was added to cells and incubated for 96 h. At the end of drug incubation, cell viability was determined using the SRB (Sulforhodamine B) assay.

2.9. Sulforhodamine B (SRB) assay

After drug treatment, culture media were removed from the 96-well plate and the cells were fixed by adding 65 μl of 10% trichloroacetic acid (TCA) solutions and incubating for 30 min at 4 °C. Cells were then rinsed five times with deionized water and stained with 0.04% SRB solution (40 μl/well) for 30 min at room temperature. Cells were then washed five times with 1% acetic acid to remove unbound dye, and left to air dry. The bound SRB dye was then solubilized by adding 50 mM Tris-base solution (100 μl/well), and plates were incubated at room temperature for 40 min with shaking. Plates were finally read at OD 564 nm using a BioTek plate reader (BioTek, Winooski, VT). Cell viability was calculated by dividing the average of the reading number for the drug treated wells by the average of the reading number for vehicle treated wells. The IC_{50} values (concentration required to achieve 50% growth inhibition) were determined using the Prism 5 software (GraphPad Software).

2.10. Cell cycle analysis using flow cytometry

Cells were seeded in T-25 tissue culture flasks (6×10^5 cells per flask) (Corning, Lowell, MA) and grown overnight before drug treatment. For cell cycle analysis, AsPC-1 cells were treated with PHA-739358 (3 μM), imatinib (15 μM), or PHA-739358 (3 μM) plus imatinib (15 μM) for 24, 48, and 72 h. The drug-treated cells and untreated control samples were harvested by trypsinization and stained with propidium iodide (Sigma–Aldrich, St. Louis, MO) in a modified Krishan buffer for 1 h at 4 °C. The propidium iodide-stained samples were then analyzed with a FACSCalibur 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 Pro Software (BD Immunocytometry Systems).

2.11. Caspase 3/7 activity based apoptosis assay

Cells were seeded in 6-well plates (0.3×10^6 cells/well) and incubated for 24 h at 37 °C to allow attachment. Then cells were treated with various concentrations of drugs as indicated in the figure legends. Culture media were collected at 72 h after drug treatment. After washing with phosphate buffer saline (PBS) solution, the cells were detached by trypsinization and combined with the culture media for each sample. The cell suspension was pelleted by centrifugation at 1000 rpm for 5 min. 200 μl of NP40

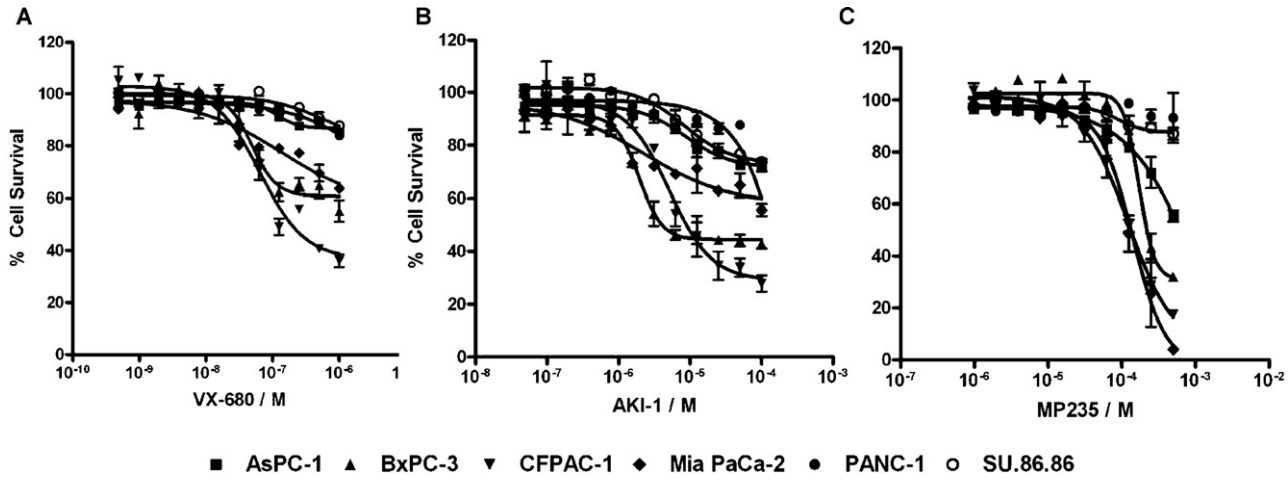

Fig. 1. Inhibition of pancreatic cancer cell growth by AKIs. AsPC-1, BxPC-3, CFPAC-1, Mia PaCa-2, PANC-1, and SU.86.86 pancreatic cancer cells were treated with a serial dilution of VX-680 (A), AKI-1 (B), or MP235 (C). Cell viability was measured at 96 h after drug treatment by CellTiter-Glo® Assay.

lysis buffer (10 mM Tris–Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40) was then added into the cell pellet and mixed by pipetting and incubated on ice for at least 30 min. The lysed cell mixture was then spun down at 13,000 × *g* for 10 min to remove cell debris. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay kit (Promega) according to the manufacture instructions. Briefly, an equal volume (100 μl) of Caspase-Glo® 3/7 reagent was added to each cell lysate sample (100 μl) in a 96-well assay plate with a final assay volume of 200 μl. Samples were incubated at room temperature for 1 h (protected from light) with shaking, and the luminescence of each sample is measured using a Veritas™ Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). The Caspase 3/7 activity was normalized to the amount of total protein contained in the cell lysate as determined by the BCA protein assay (Thermo Scientific, Rockford, MI).

2.12. Western blotting analysis

The cells were treated with AKIs, imatinib, or AKIs plus imatinib at concentrations indicated in the figures, for 72 h and then harvested by trypsinization. The cell lysates were prepared as described for the Caspase 3/7 activity assay. Cell lysates containing equal amount of protein (20 μg) were resolved on 4–12% SDS-PAGE (polyacrylamide gel electrophoresis) gels. The separated proteins were transferred to nitrocellulose membranes. Membranes were then probed with primary antibodies against Phospho-PDGFRA, Bcl-xL, Bcl-2, PI3K, Phospho-PI3K, ERK, Phospho-ERK and β-actin (All from Cell Signaling Technology Inc., Danvers, MA). β-Actin was included to serve as a protein loading control. The bound primary antibodies were detected using peroxidase-conjugated secondary antibodies (Cell Signaling) and chemiluminescence by the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) according to manufacturer's instructions. The luminescent signal of the membrane was then detected by photographic film.

3. Results

3.1. Optimization of conditions for HT-siRNA screening

3.1.1. Selection of cell lines and AKIs

To select an AKI that would maximize our chances of finding siRNA hits that are specific to Aurora kinase inhibition, we first evaluated 3 different AKIs, VX-680, MP235, and AKI-1, in a panel of pancreatic cancer cells, including AsPC-1, BxPC-3, CFPAC-1, Mia PaCa-2, PANC-1 and SU.86.86, using the same growth and assay conditions as described in Section 2. As shown in Fig. 1, the three AKIs showed different levels of cell growth inhibition in pancreatic cancer cell lines. VX-680 was the most potent with EC_{50}s (concentration required to achieve 50% of the maximal cell growth inhibition) below 100 nM; AKI-1 had modest EC_{50}s (low μM); and MP235 was the least potent with EC_{50}s over 100 μM. Although the reason for the different cellular potency of the AKIs is potentially complex, we believed that AKI-1 would be a good compound for HT-siRNA due to its modest activity and relatively smooth dose–response curves in the cell lines. BxPC-3 is one of the cell lines that gave the most consistent dose responses to all three AKIs and its sensitivity to the AKIs is modest among the cell lines (Fig. 1). We therefore decided to carry out the HT-siRNA screen with AKI-1 in the BxPC-3 cell line.

3.1.2. Optimization of transfection conditions

Efficient delivery of siRNA into cells is critical to the success of a HT-RNAi screen. To find the best transfection reagent and conditions for pancreatic cancer cells, we first tested a panel of 4 transfection reagents (Lipofectamine 2000, RNAiMax, siLentFect, and Oligofectamine) with two siRNA oligonucleotides, a negative control siRNA control (Non-silencing siRNA, Qiagen) and a positive control siRNA (UBB1, Qiagen) which was found to be lethal in all cell lines tested (data not shown). Among the 4 transfection reagents, siLentFect (SLF) showed the most consistent highly transfection efficiency across different pancreatic cancer cell lines (data not shown). The transfection conditions were further optimized by evaluating the transfection efficiency at different SLF dilutions. The optimal SLF dilutions for 6 pancreatic cancer cell lines are shown in Supplementary Figure S3A. For BxPC-3 cells, the optimal transfection reagent is SLF with a dilution rate at 1:5 (SLF to serum free medium, Figure S3B).

3.1.3. Identification of siRNAs sensitizing pancreatic cancer cells to AKIs

We first performed an RNAi screen with the Human Validated Kinase Set (HVKS) siRNA library from Qiagen (two siRNA sequences/gene), in combination with AKI-1 in the BxPC-3 cell line. The screen was performed in duplicates. From this initial screen, a total of 172 siRNAs targeting 152 different kinase or kinase related genes showed greater than 1.5 fold decrease in the EC_{50} or EC_{30} of the AKI-1 dose–response curves compared to the

Table 1
Confirmed gene hits from the HT-RNAi screen.

HUGO symbol	# of siRNAs	Entrez ID	Description
BMPR2	3	659	Bone morphogenetic protein receptor, type II
CSNK1A1	3	1452	Casein kinase 1, alpha 1
LIMK2	2	3985	LIM domain kinase 2
MAP3K10	2	4294	Mitogen-activated protein kinase kinase kinase 10
MET	2	4233	met proto-oncogene (hepatocyte growth factor receptor)
NEK2	2	4751	NIMA (never in mitosis gene a)-related kinase 2
NME2	2	4831	Non-metastatic cells 2, protein (NM23B)
PAK4	2	10298	p21(CDKN1A)-activated kinase 4
PAK7	2	57144	p21(CDKN1A)-activated kinase 7
PDGFRA	2	5156	Platelet-derived growth factor receptor, alpha polypeptide
PIM2	2	11040	pim-2 oncogene
PINK1	3	65018	PTEN induced putative kinase 1
PIP5K2B	2	8396	Phosphatidylinositol-4-phosphate 5-kinase, type II, beta
PTK2B	2	2185	PTK2B protein tyrosine kinase 2 beta
STK32B	2	55351	Serine/threonine-protein kinase 32B
STK39	2	27347	Serine threonine kinase 39
TESK2	2	10420	Testis-specific kinase 2

plate median and were selected as positive hits. We then obtained four different siRNA sequences for each of the 152 gene hits and performed a confirmation screen using the same procedure as the initial screen. A total of 17 different kinase genes were confirmed to have at least 2 out of 4 siRNA oligonucleotides to show greater than 1.5 fold decrease in EC_{50} or EC_{30} values. Table 1 lists those 17 genes and the drug dose–response curves in the presence of the positive siRNAs are shown in Supplementary Figure S4. Many of the 17 gene hits have been previously reported to be involved in tumorigenesis or progression of various tumor types including pancreatic cancer. For instance, PDGFRA (platelet-derived growth factor receptor alpha) has been shown to be overexpressed in human pancreatic cancer and PDGFR inhibitors such as imatinib reduce the growth and metastasis of pancreatic tumors in mouse xenograft models [37–39]. Our analysis of DNA microarray gene expression profiling datasets of pancreatic normal and cancerous tissues deposited in the oncomine database (www.oncomine.com) also showed overexpression of PDGFRA in pancreatic tumor tissues (Supplementary Figure S5).

3.2. Inhibition of PDGFRA by small molecule inhibitors sensitizes pancreatic cancer cells to AKIs

To further validate PDGFRA as a sensitizing target for AKIs in pancreatic cancer, we examined the anti-proliferation activity of combination treatment of PDGFR inhibitors (imatinib and sorafenib) and different AKIs.

Various concentrations of imatinib combined with a serial dilution of two AKIs (ZM447439 and PHA-739358) were first evaluated in three pancreatic cancer cell lines (AsPC-1, BxPC-3, and SU.86.86). As shown in Fig. 2, addition of 9 or 13 μM of imatinib to ZM447439 (a selective Aurora B inhibitor) resulted in a left shift of the dose–response curves in all 3 cell lines (Fig. 2). Imatinib at 13 μM reduced the IC_{50} values of ZM447439 by 2 and 3 fold in the AsPC-1 and SU.86.86 cell lines, respectively (Fig. 2A and C). Addition of imatinib to PHA-739358 (a pan-Aurora kinase inhibitor) also increased the sensitivity of two of the cell lines. Imatinib (20 μM) reduced the IC_{50} of PHA-739358 by 2 fold in AsPC-1 and 9 fold in SU.86.86 (Fig. 2D and F). In addition to the IC_{50}

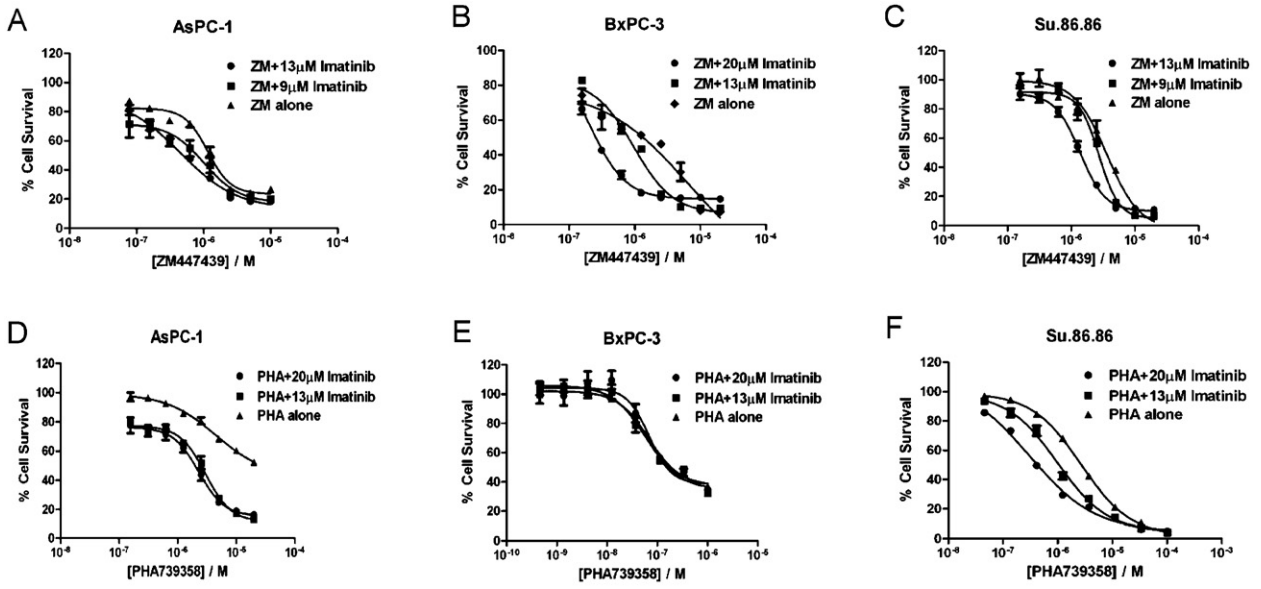

Fig. 2. Imatinib sensitizes pancreatic cancer cells to the treatment of ZM447439 and PHA-739358. AsPC-1, BxPC-3, and SU.86.86 cells were treated with a serial dilution of ZM447439 (A–C) or PHA-739358 (D–F) in combination with different fixed concentrations of imatinib. Each of the drug dose–response curves was normalized to the percentage cell survival of imatinib only treatment at the indicated concentration based on the Bliss independence drug interaction model. ZM: ZM447439; PHA: PHA-739358.

Table 2
IC_{50} of AKIs in combination with imatinib in pancreatic cancer cell lines.

Cell line	Treatments	AKI IC_{50} (mM)[a]	IC_{50} ratio to AKI alone
BxPC-3	PHA alone	0.07	–
	PHA + Imatinib (20 μM)	0.07	1.00
	PHA + Imatinib (13 μM)	0.06	0.88
	ZM alone	10.59	N/A
	ZM + Imatinib (20 μM)	1.81	0.17
	ZM + Imatinib (13 μM)	2.92	0.28
AsPC-1	PHA alone	4.49	–
	PHA + Imatinib (20 μM)	1.83	0.41
	PHA + Imatinib (13 μM)	1.95	0.43
	ZM alone	12.00	–
	ZM + Imatinib (13 μM)	4.46	0.37
	ZM + Imatinib (9 μM)	5.31	0.44
SU.86.86	PHA alone	2.66	–
	PHA + Imatinib (20 μM)	0.31	0.12
	PHA + Imatinib (13 μM)	0.99	0.37
	ZM alone	3.32	–
	ZM + Imatinib (13 μM)	1.30	0.39
	ZM + Imatinib (9 μM)	2.32	0.70

[a] IC_{50} values for the combination treatments were normalized to imatinib only treatment. PHA: PHA-739358; ZM: ZM447439.

Table 3
Induction of G2/M arrest by the combination treatment of PHA-739358 and imatinib in AsPC-1 pancreatic cancer cells.

Treatment	Cell cycle	24 h	48 h	72 h
Untreated control	G0-G1 (%)	52.1	60.7	72.7
	S (%)	21.0	12.8	9.1
	G2-M (%)	19.4	19.8	12.9
	Polyploidy (%)	5.8	4.8	3.8
	Debris (%)	0.3	0.3	0.4
PHA alone	G0-G1 (%)	33.6	55.4	41.1
	S (%)	19.1	14.4	12.0
	G2-M (%)	30.6	22.7	24.7
	Polyploidy (%)	15.6	22.4	16.9
	Debris (%)	0.9	0.2	3.0
Imatinib alone	G0-G1 (%)	57.5	31.4	68.0
	S (%)	22.1	11.3	11.5
	G2-M (%)	12.8	31.7	15.3
	Polyploidy (%)	5.6	4.1	2.9
	Debris (%)	0.4	1.3	0.4
PHA + Imatinib	G0-G1 (%)	34.5	31.4	31.2
	S (%)	12.3	8.5	8.6
	G2-M (%)	45.7	48.8	48.3
	Polyploidy (%)	5.4	9.8	10.4
	Debris (%)	0.2	0.2	0.2

decrease in the AsPC-1 cell line, this combination enhanced the cytotoxicity effect at the higher concentration of PHA-739358 (Fig. 2D). Table 2 summarizes the IC_{50} values of the AKIs in combination with imatinib after normalization with the imatinib only treatment (assuming the Bliss independence criterion [40]) and their ratios to the IC_{50} values of AKI only treatments in the three pancreatic cancer cell lines. A ratio of less than 1 indicates a synergistic interaction between the AKIs and imatinib at the concentrations tested.

Since imatinib is known to inhibit other kinases besides PDGFR, to further confirm that the synergism observed is specific to PDGFR inhibition we tested another known small molecule inhibitor of PDGFR, sorafenib. Similar to imatinib, sorafenib (2 μM and 4 μM) caused a left shift of PHA-739358 dose–response curves in AsPC-1 and SU.86.86 cell lines but not in BxPC-3 (Supplementary Figure S6).

3.3. Effects of imatinib and PHA-739358 combination on cell cycle progression

Since Aurora kinase inhibition has been shown to induce cell cycle arrest we examined the effects of the combination treatment of imatinib and PHA-739358 on cell cycle progression in AsPC-1 cells. As expected, PHA-739358 alone induced significant G2/M arrest and polyploidy. PHA-739358 (3 μM) significantly increased the G2/M population from 19.37% to 30.56% and the population of polyploidy cells from 5.80% to 15.61% within 24 h (Table 3). Imatinib (15 μM) does not affect the cell cycle distribution of at 24 h. However, the combination treatment of both drugs resulted in further induction of G2/M arrest (45.72% of total cell population) compared to PHA-739358 alone (30.56%). Similar synergistic effect was observed at both 48 and 72 h time points where the combination treatment significantly increased G2/M arrest when compared to either drug alone (Table 3). Interestingly, the addition of imatinib to PHA-739358 reduced the polyploidy (>4N DNA content) population induced by PHA-739358 at all 3 time points (Table 3). For instance, at the 24 h time point, the cell population with >4N DNA increased from 5.8% in untreated control and 5.6% in imatinib only treatment to 15.6% in PHA-739358 only treatment, and reduced back to 5.4% in the imatinib plus PHA-739358 combination treatment.

3.4. Induction of apoptotic cell death by the combination treatment of imatinib and PHA-739358 in pancreatic cancer cells

Consistent with its inhibitory activity against both Aurora A and B, PHA-739358 (3 μM) as a single agent reduced the proliferation of AsPC-1 cells and increased the formation of multinucleated cells (top right panel in Fig. 3A). Imatinib (15 μM), as a single agent, did not significantly affect the growth of AsPC-1 cells (bottom left panel in Fig. 3A). However, combination treatment of PHA-739358 and imatinib induced dramatic cell death (bottom right panel in Fig. 3A). Caspase 3/7 activity assays indicated that PHA-739358 (3 μM) alone significantly induced apoptosis at 72 h compared to vehicle control ($P < 0.001$) whereas imatinib (15 μM) did not (Fig. 3B). When the two drugs were combined, the induction of apoptosis further increased significantly when compared to PHA-739358 only treatment ($P = 0.00003$, Fig. 3B), indicating that PHA-739358 and imatinib act synergistically in inducing apoptosis. Furthermore, combination of another AKI, ZM447439, and imatinib also showed a significant increase in the induction of caspase activity in comparison to either drug alone in the BxPC-3 cell line (Supplementary Figure S7). To explore the mechanism of action of the increased apoptotic effect of the combination treatment, the expression of the two anti-apoptotic proteins, Bcl-2 and Bcl-xL, were examined by Western blotting. As shown in Fig. 3C, treatment with either PHA-739358 or imatinib alone did not significantly affect the level of either protein whereas the combination treatment reduced the expression of Bcl-2 and Bcl-xL by 73% and 68%, respectively, compared to the untreated control, indicating that the increased anti-apoptotic effect of the combination treatment might be a result of the synergistic down-regulation of Bcl-2 and Bcl-xL expression by the two drugs.

3.5. Combination treatment of imatinib and AKIs decreased the phosphorylation of PI3K but not ERK

Two major effector pathways of PDGF/PDGFR signaling are the Ras/Erk pathway and the PI3K/Akt pathway. To investigate the effect of combination treatment of imatinib and AKI on these two pathways, we examined the phosphorylation of PI3K and Erk1/2 upon the drug treatment. As shown in Fig. 4, AsPC-1 cells treated with single agent PHA-739358 (3 μM) or imatinib (15 μM) did not significantly affect the phosphorylation of either Erk1/2 or PI3K.

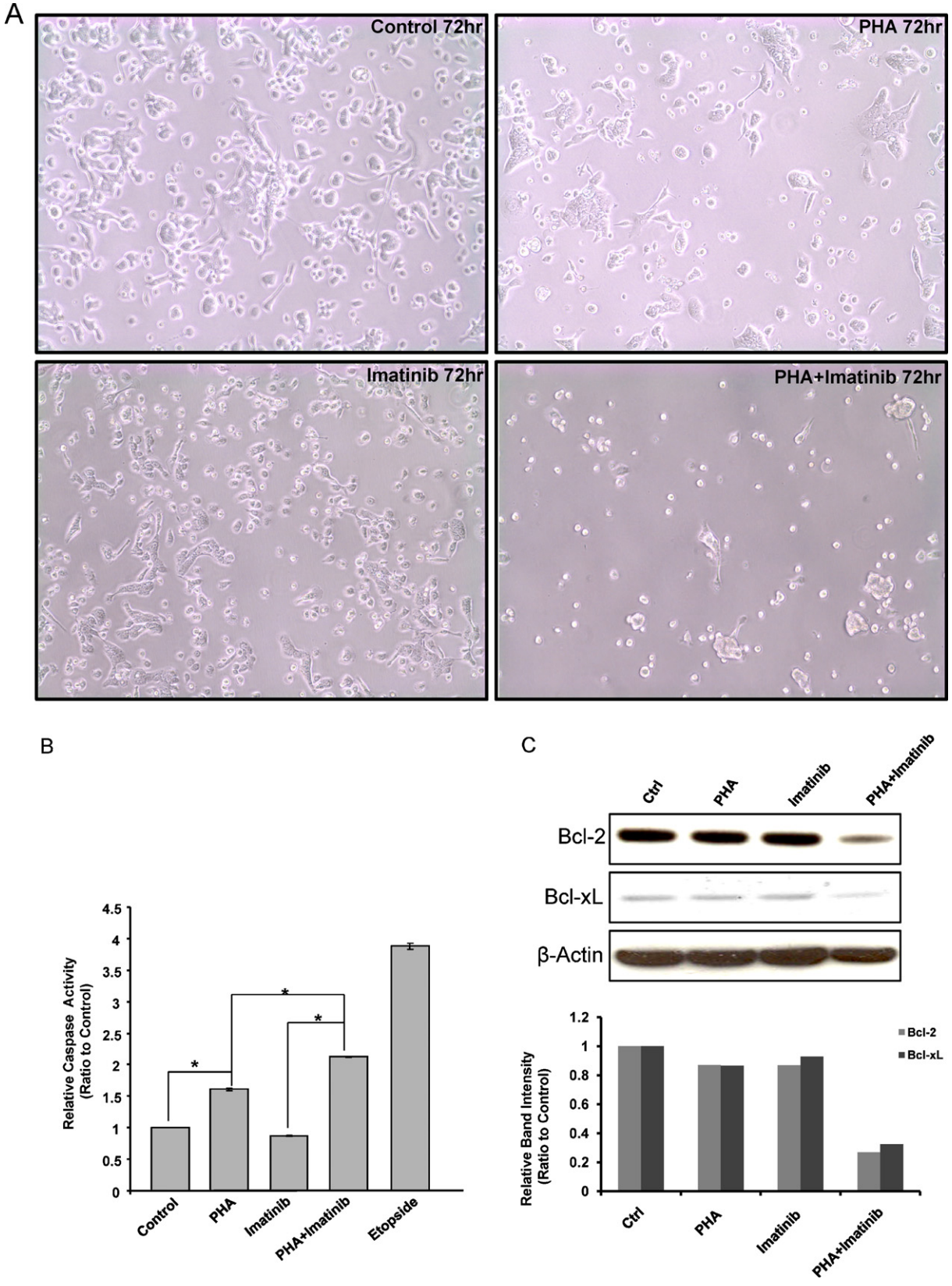

Fig. 3. Increased apoptotic cell death induced by the combination treatment of PHA-739358 and imatinib. AsPC-1 cells were treated with PHA-739358 (PHA) (3 μM), imatinib (15 μM), or combination of PHA and imatinib for 72 h and then subjected to evaluation of cell morphology changes by microscope (A), caspase activity levels by the Caspase 3/7 Glo® Assay (B), and Bcl-2 and Bcl-xL expression levels by Western blotting (C). Etopside (100 μM) was used as a positive control for the Caspase 3/7 assay in B. The intensities of the Western blotting bands (top panel in C) were quantified using the ImageJ software [61] and each band was normalized with their corresponding β-actin loading controls (bottom panel in C). * indicates significant differences between the two treatments ($P < 0.001$).

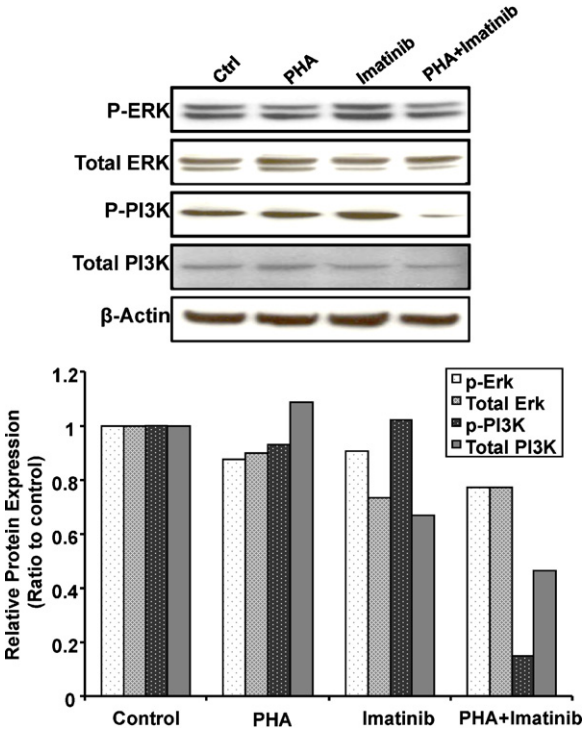

Fig. 4. Combination of PHA-739358 and imatinib inhibits the phosphorylation of PI3K but not ERK. AsPC-1 cells were treated with PHA-739358 (PHA) (3 μM), imatinib (15 μM), or the combination of PHA and imatinib. Cells were harvested 72 h after drug treatment and 20 μg of whole cell lysates were used in Western blotting detection of the proteins (top panel). The intensities of the bands were quantified using the ImageJ software and normalized with their corresponding β-actin loading controls (bottom panel).

However, combination treatment of PHA-739358 and imatinib resulted in decreased phosphorylation of PI3K but not the ERK1/2 kinases. Similarly, combination of ZM447439 and imatinib resulted in a significant decrease of PI3K phosphorylation level, but not the phosphorylation of Erk kinase in the BxPC-3 cell line (Supplementary Figure S8). These results suggest that AKIs and imatinib might act synergistically in inhibiting the PI3K/Akt induced cell survival in pancreatic cancer cells.

4. Discussion

Over the past decade, more than a dozen of small molecule Aurora kinase inhibitors have been developed and entered into clinical studies. Many of these inhibitors were reported to show impressive *in vitro* and *in vivo* activities in a variety of tumor types including colon, breast, ovarian and pancreatic cancers [41–43]. Phase I and early Phase II results reported for some of the AKIs are promising with stable disease observed in about 20% of the patients. Hence, combining with other agents might be needed to further enhance the efficacy of AKIs [44]. In this study, we utilized high throughput RNAi (HT-RNAi) screening to identify genes that can potentiate AKI response in pancreatic cancer cells. Using HT-RNAi screening as a tool to identify drug sensitizing targets has gained wide attraction in recent years [45–50]. However, the majority of those screens use one or two drug concentrations in combination with RNAi. Since the synergism between siRNA and drug is often drug concentration dependent, using only one or two drug concentrations could miss a significant number of potential positive hits. In our study we used 5-dose serial dilutions of the drugs, which allowed us to generate drug dose–response curves for comparison of growth inhibitory effects. This approach not only significantly reduces the impact of experimental variations among different drug concentrations but also provides activity data on the combination of RNAi and multiple drug concentration, therefore, reducing false positive and negative rates.

Among the 17 kinase gene targets we identified, some are involved in cell cycle regulation. For instance, NEK2 is a centrosomal resident protein that regulates centrosome separation and mitotic spindle assembly. Overexpression of NEK2 has been shown to cause centrosome missegregation and aneuploidy [51]. Both NEK2 and Aurora A kinase have been reported to interact with protein phosphatase 1 (PP1) and regulate cell cycle progression [52,53]. Another gene hit, the c-Met oncogene, is known for signaling the invasive growth of tumor cells. Recently, overexpression of c-Met is shown to induce centrosome amplification and chromosomal instability (CIN) via the PI3K–Akt pathway in a p53-dependent manner [54]. In pancreatic cancer, we and others have shown that c-Met is overexpressed in cancer cells and tumor tissues [14,55,56]. Besides c-Met and PDGFRA, a number of the other gene targets have also been associated with pancreatic cancer. For instance, BMPR2 is reported to be overexpressed by 8 fold in pancreatic cancer tissues in comparison to normal pancreas [57]. Knockdown of LIMK2 expression is shown to reduce the invasiveness and metastatic capabilities of pancreatic cancer cells in a zebrafish xenograft metastasis assay [58]. The p21-activating kinase 4 (PAK4) gene is amplified in pancreatic tumors and is shown to promote the motility and invasion of pancreatic ductal carcinoma cells [59,60]. Although the mechanisms of the synergistic effect between the knockdown of these genes and the AKIs remain to be investigated, it is possible that the signaling pathways involving these genes may crosstalk with one or more of Aurora kinases and act in augmentation to promote pancreatic cancer progression and/or metastasis. Molecules that modulate the activity/expression of these gene targets may hence enhance the antitumor activity of AKIs. In this study, we demonstrated that the multi-targeted kinase inhibitor (PDGFR, ABL, and c-Kit), imatinib, synergize with AKIs in inhibiting pancreatic cancer cell growth. It has been reported that imatinib treatment reduced the level of phosphorylated PDGFRA in a pancreatic cancer mouse xenograft model [38]. We also observed the inhibition of PDGFRA autophosphorylation by imatinib in AsPC-1 pancreatic cancer cell line (data not shown). Furthermore, a second PDGFR inhibitor, sorafenib, also showed synergistic effect in combination with the pan-Aurora kinase inhibitor PHA-739358 in pancreatic cancer cells. These results further support the conclusion that PDGFR inhibition can sensitize pancreatic cancer cells to the treatment of Aurora kinase inhibitors. However, further studies are needed to test whether or not the inhibition of other cellular targets of imatinib and sorafenib (e.g. ABL and RAF) also contributes to the synergism. Although our study was performed in pancreatic cancer cells, considering the fact that both Aurora kinases and PDGFR have been implicated in multiple tumor types, it is plausible that agents targeting these kinases may also show synergist effects in other cancer types. In fact, a recent study reported that the combination of PHA-739358 and sorafenib showed significantly increased antitumor activity compared to single drug treatments in a mouse xenograft model for hepatocellular carcinoma [27].

PHA-739358 is among the few AKIs that have entered Phase II clinical trials for patients with solid tumors (www.clinicaltrials.gov). *In vitro* studies have shown that PHA-739358 causes a failure of cell division, resulting in polyploidy and reduction in viability [27]. In agreement with these results, our study shows PHA-739358 induces G2/M arrest and polyploidy (Fig. 3), and inhibited proliferation in pancreatic cancer cell lines (Fig. 2). We further showed that imatinib and sorafenib could sensitize pancreatic cancer cells to the treatment of PHA-739358 (Fig. 2). Imatinib

further enhances the G2/M arrest and apoptosis induced by PHA-739358 (Table 3). Such synergistic effect is potentially mediated through inhibition of PI3K activation but not ERK activation (Fig. 4).

In conclusion, this is the first report describing the use of kinome-wide siRNA library to functionally screen for sensitizer targets of AKIs in pancreatic cancer cells. The findings from this study further demonstrated the power of high-throughput RNAi screening identifying sensitizers for existing therapeutic agents. The genes identified from this study present new opportunities for the development of rational combination regimens that include Aurora kinase inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.11.005.

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