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Dasatinib can enhance paclitaxel and gemcitabine inhibitory activity in human pancreatic cancer cells

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ABSTRACT

SRC and its activated form, phospho-SRC (pSRC), are aberrantly activated in pancreatic cancer and SRC represents a potential target for pancreatic cancer therapy. In this paper, we examined the inhibitory effect of dasatinib, a potent SRC inhibitor in combination with paclitaxel or gemcitabine on human and murine pancreatic cancer cell lines. The results showed that p-SRC can be highly expressed in most human and mouse pancreatic cancer cell lines compared with normal human cell lines and can be induced by paclitaxel or gemcitabine in HPAC cells. Dasatinib can enhance the efficacy of paclitaxel or gemcitabine by reducing the cell viability and inhibiting the cell proliferation. Dasatinib with paclitaxel combination exhibits statistically greater inhibition of the cell migration ability than single agent alone, paclitaxel with gemcitabine or FOLFIRINOX (combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin) in HAPC, PANC-1, and BXPC-3 human pancreatic cancer cell lines as well as 8–285 APR and 8–365 APR mouse pancreatic cancer cell lines. In addition, dasatinib with gemcitabine combination also showed statistically greater inhibition of cell migration than single agent alone, paclitaxel with gemcitabine, or FOLFIRINOX in HAPC, PANC-1 and 8–285 APR cells. The combination of dasatinib with paclitaxel or gemcitabine also showed greater inhibition of the colony formation ability of pancreatic cancer cells compared with single-agent monotherapy or FOLFIRINOX. Dasatinib with paclitaxel or gemcitabine combination also inhibits p-SRC, p-STAT3, p-AKT, and/or p-ERK in these pancreatic cancer cells. Therefore, our results support that combined dasatinib and paclitaxel or gemcitabine therapy may be a viable therapeutic approach for human pancreatic cancer.

Introduction

Pancreatic cancer is the third leading cause of cancer death in the United States .[1] While curative resection and chemotherapy remains the standard of care in pancreatic cancers, the 5-y overall survival for pancreatic cancer is still less than 10% .[2,3] High resistance to traditional chemo- and radiation therapy, including both intrinsic (*de novo*) and acquired (therapy induced) is regarded as a major reason for the limited benefit of most pancreatic cancer therapies .[4]

Single-agent gemcitabine, a pyrimidine antimetabolite and analog of deoxycytidine, the most commonly used chemotherapeutic drug for pancreatic cancer, has modest clinical benefit and may not improve overall survival (OS) to a clinically meaningful degree. The lack of significant clinical response to monotherapy of gemcitabine is likely due to the inherent or acquired chemoresistance.[5,6] Although recent study shows that newer combination chemotherapy regimen, FOLFIRINOX (combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin) is a superior option for patients with advanced pancreatic cancer, the disease still rapidly develops resistance to the drug and no apparent improvement in OS rate was achieved.[7] Another regimen, consisting of nab-paclitaxel plus gemcitabine, was introduced to increase the

available options for the refractory advanced pancreatic adenocarcinoma, the OS or progression-free survival (PFS) was still comparatively low.[8] Taken together, the failure of conventional chemotherapeutic regimens highlights a desperate need for novel treatment strategy.

Src-family kinases are non-receptor tyrosine kinases with a critical role in cellular proliferation. Src, one of the nine members of the Src-family kinases, was reported to be overexpressed with the progression of pancreatic neoplasia and correlated with poor prognosis.[9,10] Hyper-activation of Src has been known to confer therapeutic resistance to a few current anticancer therapies and indicated poor prognosis in pancreatic cancer patients. It involves in a few of signaling pathways. Src is also known to be crucial during tumor metastasis, mainly as a result of its role in regulating the cytoskeleton, cell migration, adhesion, and invasion in a variety of human cancers including pancreatic cancer.[11] Given the essential role of Src in tumor development and extensive pre-clinical evidence of metastasis suppression by Src inhibition, inhibition of Src therefore represents a promising oncologic therapeutic target for pancreatic cancer. Dasatinib is a potent oral Src inhibitor which was approved in the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic

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leukemia.[12] It not only shows promising results in hematological malignancy with well tolerance and limited toxicity but can also go across the blood–brain barrier.[13] Previously, several papers found that dasatinib can inhibit pancreatic tumor growth in xenograft mouse model but not in clinical practice.[10,14] Its monotherapy shows no clinical benefit for small-cell-lung cancer and for metastatic colorectal cancer and should be combined with other drugs.[15,16] Src activation appears to be a facilitator but may not necessarily a driving force for tumor progression as shown in some clinical models.[11] Preclinical data have demonstrated that dasatinib has additive or synergistic activity in combination with several cytotoxic and biologic agents, providing rationale for combination therapy.[17] Therefore, we hypothesize that Src inhibitors may provide better outcome if it combines with conventional therapeutic drugs and its combination may provide additional clinical benefit for pancreatic patients.

Paclitaxel is an anticancer agent used in pancreatic cancers as well as many other types of solid cancer. It may exert its role by stabilizing polymerized microtubules and enhances microtubule assembly, which arrests the cell cycle in G0/G1 and G2/M phases, and leads to cell death.[18] The combination efficacy of dasatinib and paclitaxel in pancreatic cancer has not been reported before. Gemcitabine is another single agent commonly used in pancreatic cancer for many years. Although several reports have confirmed the inhibitory effects of gemcitabine with dasatinib in pancreatic cancer cells,[19,20] the exact role in overcoming the resistance of pancreatic cancers has not been investigated. Here, we investigated the combination efficacy of dasatinib with paclitaxel or gemcitabine on human and mice pancreatic cancer cells to find a better treatment strategy for pancreatic cancer treatment.

Materials and methods

Cell lines and reagents

Human pancreatic cancer cell lines HPAC, PANC-1, Capan-1, AsPC-1, and BxPC-3 (KRAS wild-type); human chorionic mesenchymal stromal cells (HCMSC); and normal human lung fibroblasts (NHLF) were purchased from the American Type Culture Collection (Manassas, VA, USA). Short-term cultured primary murine pancreatic cancer cell lines (8–365 APR, 8–285 APR and 22–614 APR) from a clinically relevant p16$^{lox/lox}$; LSL-Kras^{G12D}; Pdx1-Cre genetically engineered mouse model were provided by Dr Gloria H. Su at Columbia University Medical Center. All cell lines were routinely propagated in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) as well as 1% penicillin/streptomycin and then grown in a humidified 37°C incubator with 5% CO_2. Routinely, they were assessed microscopically for the expected morphologies.

All reagents in the study were purchased as follows: dimethyl sulfoxide (DMSO) (Sigma, USA), dasatinib (LC Laboratories, Woburn, MA, USA), paclitaxel (LC Laboratories), gemcitabine (LC Laboratories), oxaliplatin (LC Laboratories), Irinotecan (LC Laboratories), leucovorin calcium (Sigma), and 5-fluorouracil (Sigma). All the drugs were stored at −20°C with 20 mM stock solution.

Cell viability and cell proliferation assay

Human pancreatic cancer cell lines (HPAC, PANC-1, Capan-1, and ASPC-1) were seeded in 96-well plates at a density of 3,000 cells per well and cultured in the presence of 10% FBS at 37°C. The next day, the culture medium in each well was not only maintained at 100 μL as the final volume, but also respectively supplemented with either various concentrations of dasatinib (0.25 or 0.5 μM), paclitaxel (0.1 or 0.5 μM), and gemcitabine (0.25 or 0.5 μM) or in combination under the same cultivation condition. After 72 h, 25 μl of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well. After incubation for 4 h, 150 μl of N,N-dimethylformamide (Sigma) solubilization solution were added to each well. The absorbance was read at a wavelength of 595 nm. Combination index (CI) was determined using data obtained from the MTT assay with CompuSyn software.[21] CI values indicate a synergistic effect when <1, an antagonistic effect when >1, and an additive effect when equal to 1.

To evaluate the combination effects on cell growth, HAPC, PANC-1, and Capan-1 PDAC cells were incubated in the six-well plates (3×10^5 cells per hole) in triplicate. The next day, the cells were treated with 0.25 μM dasatinib, 0.1 μM paclitaxel, 0.1 μM gemcitabine, 0.25 μM dasatinib with 0.1 μM paclitaxel, or 0.25 μM dasatinib with 0.1 μM gemcitabine. The culture of cell proliferation was measured, cell in 2 ml medium, counted manually after 2, 4, 6, and 8 d using a hemacytometer (Hawksley, West Sussex, UK) and a bright field microscope. It combined with Trypan blue staining method to determine the growth state of dispersed cells.

Wound healing assay assessed cell migratory ability

Human pancreatic cancer cells (HAPC, PANC-1, and BXPC-3) and mouse pancreatic cancer cells (8–285 APR and 8–365 APR) were seeded in six-well plates. When the cells reached 100% confluence, the monolayer was scratched in a uniform width using a pipette tip. After washing, cells were treated with DMSO, dasatinib alone, paclitaxel alone, gemcitabine alone, dasatinib with paclitaxel, dasatinib with gemcitabine, gemcitabine with paclitaxel, and FOLFIRINOX. When the DMSO group was completely healed after 24 h to 6 d after scratching, images were captured by inverted microscope (Nikon, Eclipse TS100, Japan). The percentage of wound healing was measured by software ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and calculated by the equation: percent wound healing = average of (gap area before treatment – gap area after treatment)/gap area before treatment.

Colony formation assay

Human pancreatic cancer cells (HPAC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3 cells) were seeded in six-well plates at a density of 1,000 cells per well. On the next day, cells were incubated with vehicle DMSO, or treated with dasatinib alone (0.1–1 μM), paclitaxel alone (0.005–0.01 μM), gemcitabine alone (0.01 μM), dasatinib with paclitaxel, dasatinib with gemcitabine, and FOLIFOX. After treatment for 72 h, cells were replenished with fresh growth medium comprising the same

drug ingredient. One two weeks later when the assay ended, cells were washed twice with PBS and fixed with cold methanol for 30 min at 4°C followed by a staining with 1% crystal violet dye (dissolved in 25% methanol) at room temperature for 1 h. After staining, the plates were washed with distilled water, dried, and then quantified by colorimetric scanning.

Caspase-3/7 activity

Cells were cultured in the respective media and treated with 0.1 μM dasatinib, 0.1 μM paclitaxel, 0.1 μM gemcitabine, 0.1 μM dasatinib with 0.1 μM paclitaxel or 0.1 μM dasatinib with 0.1 μM gemcitabine. Caspase-3/7 activity was measured using the Caspase-3/7 Fluorescence Assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instruction. The fluorescence intensity of each well was read using excitation at 485 nm and emission at 535 nm.

Western blotting analysis

Five human pancreatic cancer cell lines (HPAC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3), two normal human cell lines (HCMSC and NHLF), and three pancreatic mouse cancer cell lines (8–365 APR, 8–285 APR, and 22–614 APR) were harvested when cells were cultured to reach 80–90%. In addition, human pancreatic cancer cell lines (HPAC and PANC-1) and mouse pancreatic cancer cell line (8–285 APR) cultured to reach 80–90% confluence were subjected to treatments either with vehicle DMSO or with dasatinib alone (0.1 μM), paclitaxel alone (0.1 μM), gemcitabine alone (0.1 μM), dasatinib with paclitaxel (0.1 μM respectively), and dasatinib with gemcitabine (0.1 μM respectively). Twenty-four hours later, treated cells were harvested and lysed in the ice-cold RIPA buffer (Cell Signaling Technology) comprising cocktail inhibitors to block protease and phosphatase activities. The lysates were subjected to an electrophoresis on a 10% SDS-PAGE gel and the resolved proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare). Membranes were further incubated with 1,000-fold diluted primary antibody of interest for an overnight followed by with 10,000-fold diluted horse radish peroxidase-conjugated secondary antibody for 1.5 h. Primary antibodies employed in this study, respectively, recognized phosphorylated Src (Tyr416), p-SRC (Tyr527), phospho-specific extracellular signal-regulated kinase (p-ERK) 1/2 (Threonine 202/Tyrosine 204), phosphorylated STAT3 (Tyr705, p-STAT3^{Y705}), pan STAT3, phosphorylated AKT (Ser473), SRC, ERK GAPDH, and secondary antibody (all from Cell Signaling Technology, USA). Membranes were analyzed using enhanced chemiluminescence plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech Inc., USA)

Statistical analysis

Significance of correlations was assessed using GraphPad Prism software 7.0 (GraphPad Software, Inc., USA). Unpaired *t*-tests were used for analyses, assuming Gaussian populations with a 95% confidence interval. Data are presented as mean ± standard deviation. Differences were analyzed with the Student's *t*-test, and significance was set at $p < 0.05$. *, **, and *** indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Results

p-SRC was highly expressed in pancreatic cancer cells and can be activated by paclitaxel or gemcitabine

To determine the expression of SRC activation in pancreatic cancer cells, we have performed Western blotting analysis in five human pancreatic cancer cell lines (HPAC, BXPC-3, ASPC-1, PANC-1, and CAPAN-1) and three mouse pancreatic cancer cell lines (22–614 APR, 8–285 APR, and 8–365 APR). The results showed that all these pancreatic cancer cell lines express p-SRC (Y416 and Y527, Figure 1a) and the relative ratio level of p-SRC (Y416 to Y527) was higher than the NHLF and HCSMC normal tissue cell lines (Figure 1b).

Since paclitaxel and gemcitabine are first-line chemotherapeutic drugs currently used in pancreatic chemotherapy, we then tested if these two drugs can reduce the SRC/STAT3 pathway. The results, however, showed that paclitaxel can induce the activation of p-SRC (Y416 and Y527) and p-ERK1/2$^{T202/Y204}$ in HPAC cells. It did not reduce the expression of p-STAT3^{Y705} and p-AKT^{S473}. Gemcitabine can also slightly induce the expression of p-SRC (Y416) and p-ERK1/2$^{T202/Y20}$. It did not reduce the expression of p-AKT^{S473} and p-STAT3^{Y705} (Figure 1c–d). p-AKT^{T308} was not detectable in HPAC cells.

Dasatinib can enhance the efficacy of paclitaxel or gemcitabine by reducing the cell viability and inhibiting the cell proliferation

Since Src protein was highly expressed and can be induced by paclitaxel or gemcitabine, we then tested whether dasatinib can enhance the efficacy of paclitaxel or gemcitabine. The MTT array showed that dasatinib with paclitaxel can reduce the cell viabilities than single drug alone in all four human pancreatic cancer cells (HPAC, PANC-1, CAPAN-1, and ASPC-1, Figure 2a, CI<1). Similarly, dasatinib with gemcitabine can reduce the cell viabilities than single drug alone in all four human pancreatic cancer cells (HPAC, PANC-1, CAPAN-1, and ASPC-1, Figure 2b, CI<1).

To assess whether dasatinib with paclitaxel or dasatinib with gemcitabine plays a suppressive role in pancreatic cancer cells development, we then evaluated the effect of these drugs on cell proliferation using the Trypan blue staining method in HAPC, PANC-1, and Capan-1 cells. The results showed that dasatinib with paclitaxel exhibited significantly lower growth rates compared with dasatinib or paclitaxel alone in HAPC, PANC-1, and Capan-1 cells (Figure 2c). Dasatinib with gemcitabine also exhibited significantly lower growth rates compared with dasatinib or gemcitabine alone in HAPC, PANC-1, and Capan-1 cells (Figure 2d).

Dasatinib inhibited the cell migration of pancreatic cancer cells when combined with paclitaxel or gemcitabine

Cell migration ability is an important process for cancer cell metastasis. Wound healing assay was also performed to see the additive effect of dasatinib with paclitaxel or gemcitabine. We chose three pancreatic cancer cell lines (HPAC, PANC-1,
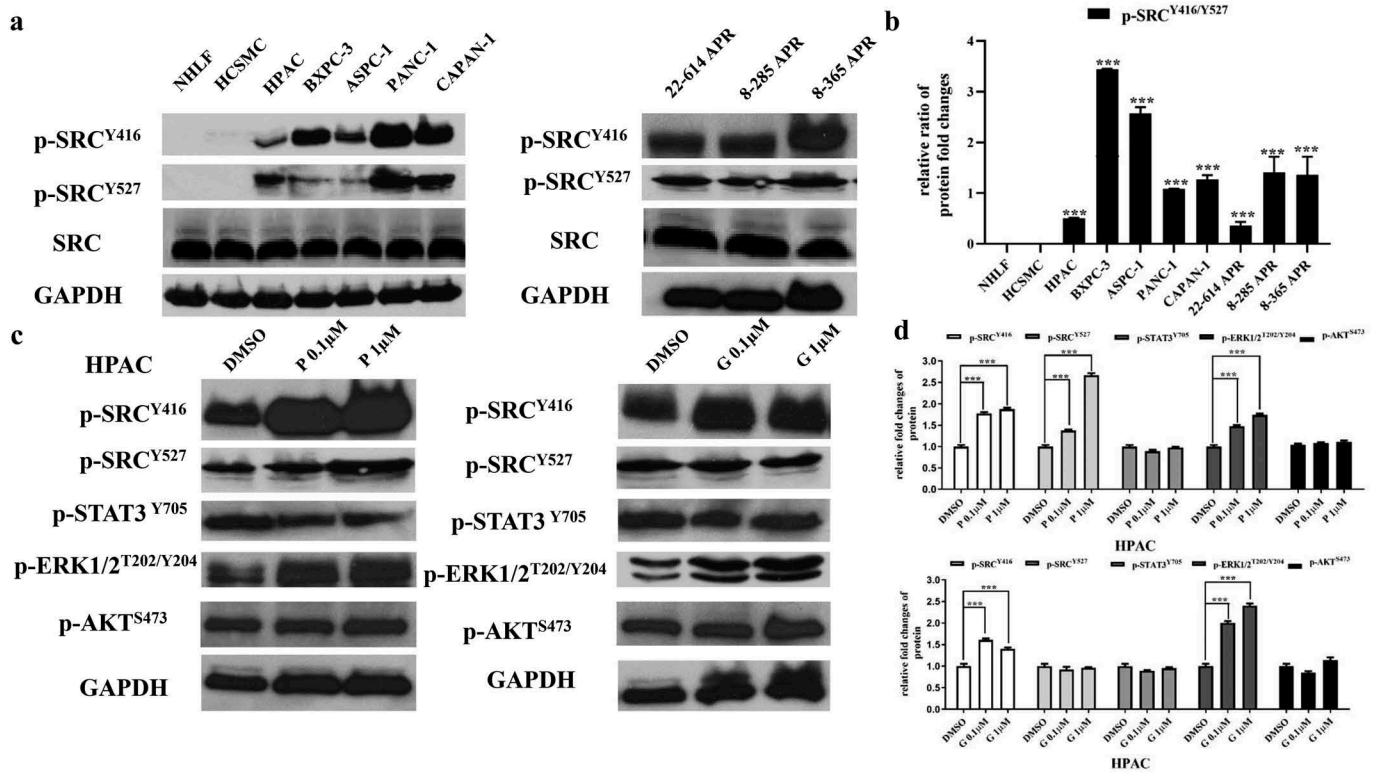
Figure 1. p-SRC was highly expressed in pancreatic cancer cell lines and can be induced by paclitaxel or gemcitabine.

a: The expression of p-SRC (Y416 and Y527) and total SRC was evaluated in five human pancreatic cancer cells (HPAC, BXPC-3, ASPC-1, PANC-1, and CAPAN-1), three mouse pancreatic cancer cells (22–614-APR, 8–285-APR, and 8–365-APR), and two normal human cells (NHLF and HCMSC). Cells were harvested, and the protein expression was detected by Western blot. GAPDH was served as loading control. b: The relative fold change of protein expression level was normalized to GAPDH and quantified by ImageJ. The relative ratio of protein fold changes (Y416/Y527) was presented in all cell lines (***, $p < 0.001$ compared with NHLF or HCSMC). c: HPAC human pancreatic cancer cells were treated with paclitaxel (0.1 and 1 μM) or with gemcitabine (0.1 and 1 μM) for 24 h. The protein expression of p-SRC (Y416 and Y527), p-STAT3[Y705], p-AKT[S473], and p-ERK1/2[T202/Y204] were detected by western blots with GAPDH as loading control. d: The relative fold change of protein expression level was quantified by ImageJ. (***, $p < 0.001$)

and BXPC-3) and two mouse pancreatic cancer cell lines (8-285 APR and 8-365 APR) to test. The results showed that in HPAC and PANC-1 cells (Figure 3a–b), dasatinib with pacli-taxel or gemcitabine inhibited cell migration ability better than single agent alone, paclitaxel with gemcitabine and FOLFIRINOX under same concentration. In BXPC-3 cells (Figure 3c), dasatinib with paclitaxel was better than paclitaxel with gemcitabine or FOLFIRINOX at the same concentration.

In 8–285 APR pancreatic cancer cell lines (Figure 4a and c), dasatinib with paclitaxel or gemcitabine can inhibit cell migra-tion ability better than single agent alone as well as paclitaxel and gemcitabine or FOLFIRINOX at the same concentration. In 8–365 APR pancreatic cancer cell lines (Figure 4b and d), dasatinib with paclitaxel can inhibit cell migration ability better than single agent alone as well as paclitaxel and gemcitabine or FOLFIRINOX at the same concentration.

Dasatinib inhibited colony formation of pancreatic cancer cells when combined with paclitaxel or gemcitabine

We next sought to investigate whether these combinations might inhibit the colony formation capability than single agent alone. Since FOLFIRINOX proved to be the current most effective treatment strategy in the pancreatic chemother-apy, the same concentration of these combinations was used

to compare the dasatinib with paclitaxel or gemcitabine. As shown in Figure 5, the cells showed a decreased ability to recover and form colonies following treatment with dasatinib and paclitaxel or gemcitabine. The less colonies were formed compared with FOLFIRINOX at the same concentration.
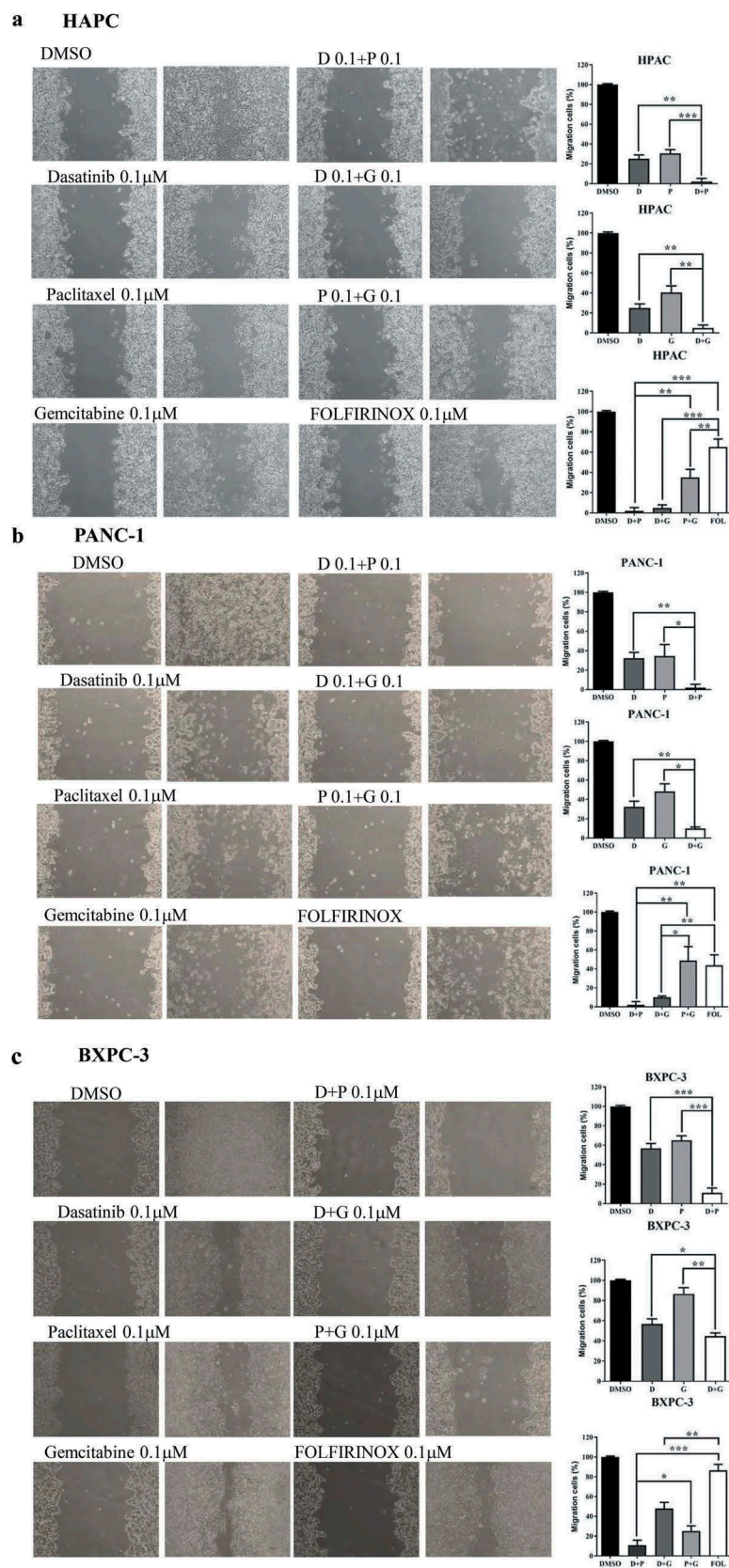
Combination of dasatinib with paclitaxel or gemcitabine can induce apoptosis and inhibit the activation of SRC/STAT3 pathway

We then tested whether the combination of dasatinib with paclitaxel or gemcitabine can induce pancreatic cancer cell apoptosis. The results showed that the level of cleaved cas-pase-3/7 was elevated with drug combination (dasatinib plus paclitaxel) compared with levels for either drug alone in HPAC, PANC-1, and 8–285-APR cells (Figure 6a). Since p-SRC was highly expressed in all pancreatic cancer cell lines and activation of SRC or ERK could occur through paclitaxel, we then assessed the phosphorylation of STAT3, ERK1/2, AKT, and SRC after treatment of pancreatic cancer cells with dasatinib and paclitaxel. The results showed that p-SRC (Y416 and Y527) and p-ERK1/2[T202/Y204] induced by paclitaxel in HPAC pancreatic cancer cell lines were demol-ished with the combination use of dasatinib with paclitaxel in HAPC, PANC-1, and 8–285 APR cells. Dasatinib with
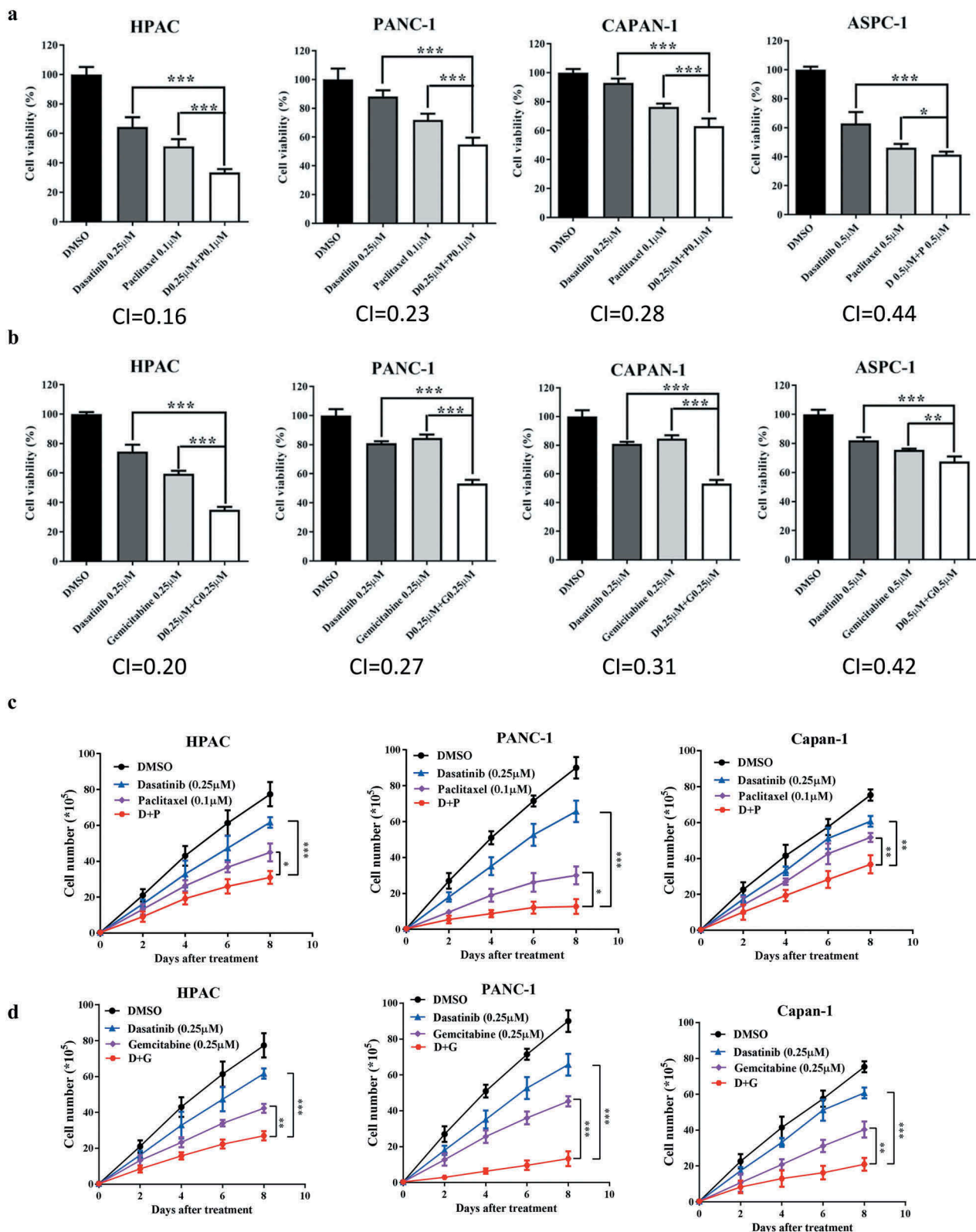
Figure 2. Dasatinib shows synergistic effects when combined with paclitaxel or gemcitabine.

a: Human pancreatic cancer cells (HAPC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3) were seeded in 96-well plates at a density of 3,000 cells per well for 24 h and then treated with dasatinib, paclitaxel or dasatinib and paclitaxel combined at the indicated doses for 72 h. Cell viability was performed by MTT. The CI values of all the combination treatments were calculated by CompuSyn software. b: Human pancreatic cancer cells (HAPC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3) were seeded in 96-well plates at a density of 3,000 cells per well for 24 h and then treated with dasatinib, gemcitabine or dasatinib and gemcitabine combined at the indicated doses for 72 h. Cell viability was performed by MTT. The CI values of all the combination treatments were calculated by CompuSyn software. c: Human pancreatic cancer cells (HAPC, PANC-1, and CAPAN-1) were seeded in six-well plates at a density of 50,000 cells per well for 24 h and then treated with paclitaxel or dasatinib and paclitaxel combined at the indicated doses for 2, 4, 6, and 8 days using Trypan blue staining method. d: Human pancreatic cancer cells (HAPC, PANC-1, and CAPAN-1) were seeded in six-well plates at a density of 50,000 cells per well for 24 h and then treated with dasatinib, gemcitabine, or dasatinib and gemcitabine combined at the indicated doses for 2, 4, 6, and 8 days using Trypan blue staining method. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 3. Combination of dasatinib with paclitaxel or gemcitabine can greatly inhibit the cell migration of human pancreatic cancer cells.

Wound healing assay was performed in HPAC (a), PANC-1 (b), and BXPC-3 (c) human pancreatic cancer cells. Representative pictures are shown in each cell line. It was conducted by scratching the cells with yellow pipette tip when HPAC, PANC-1, and BXPC-3 cells grew into the monolayer. Then, cells were treated with dasatinib, paclitaxel, and gemcitabine alone and in combination of dasatinib and paclitaxel or dasatinib and gemcitabine. The same concentration of paclitaxel plus gemcitabine and FOLFIRINOX was used to compare the effects at the same time. Cells were then allowed to migrate into the scratched area for 24 h (HPAC and PANC-1 cells) or 6 days (BXPC-3 cells) when the DMSO group heals completely. Red arrows indicate gap of scratched area. The percentage of migrating area in wound healing assay was quantified in HPAC cells. (***, $p < 0.001$).
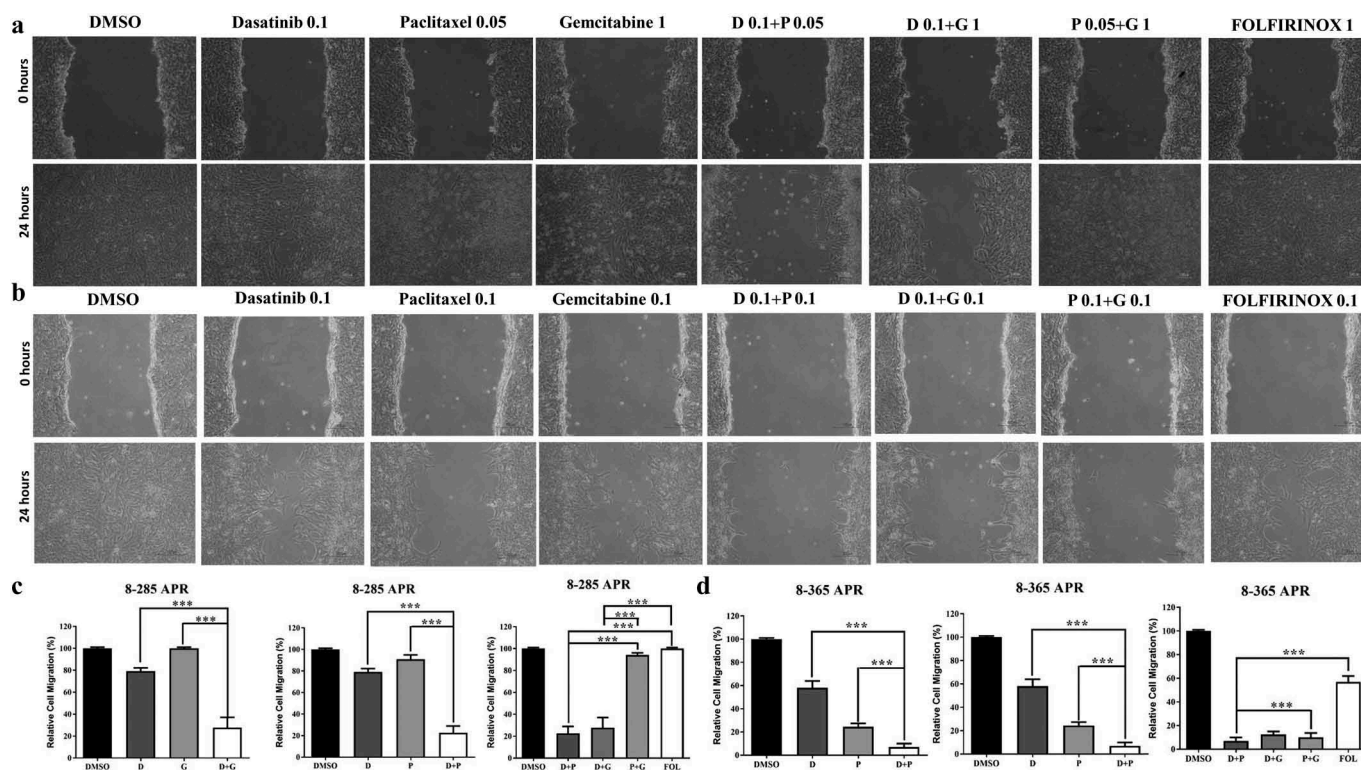
Figure 4. Combination of dasatinib with paclitaxel or gemcitabine can greatly inhibit the cell migration of mouse pancreatic cancer cells.

Wound healing assay was performed in 8–285 APR and 8–365 APR mice pancreatic cancer cells. Representative pictures are shown in 8–285 APR (a) and 8–365 APR (b) mice pancreatic cancer cells. It was conducted by scratching the cells with yellow pipette tip when 8–285 APR and 8–365 APR cells grew into the monolayer. Then, cells were treated with dasatinib, paclitaxel, and gemcitabine alone and in combination of dasatinib and paclitaxel or dasatinib and gemcitabine. The same concentration of paclitaxel plus gemcitabine and FOLFIRINOX was used to compare the effects at the same time. Cells were then allowed to migrate into the scratched area for 24 h when the DMSO group heals completely. Red arrows indicate gap of scratched area. The percentage of migrating area in wound healing assay was quantified in 8–285 APR (c) and 8–365 APR cells (d). (***, $p < 0.001$).

paclitaxel can also reduce the level of p-STAT3^{Y705} and p-AKT^{S473} in HAPC, PANC-1, and 8–285 APR cells (Figure 6b). Similarly, the level of cleaved caspase-3/7 was elevated with dasatinib and gemcitabine compared with levels for either drug alone in HPAC, PANC-1, and 8–285-APR cells (Figure 6c). p-SRC (Y416) induced in HAPC, PANC-1, and 8–285 APR cells was demolished with the combination use of dasatinib with gemcitabine. This combination can also reduce the level of p-SRC (Y527), p-STAT3^{Y705} and p-AKT^{S473}, and p-ERK1/2$^{T202/Y204}$ in HAPC, PANC-1, and 8–285 APR cells, respectively (Figure 6d).

Discussion

Due to resistance to cytotoxic chemotherapy, pancreatic cancer is still an incurable disease with poor outcomes.[20] Overexpression of Src is known to be crucial during tumor metastasis by promoting dissociation of cell–cell adherents junction and facilitating cell mobility,[11] and this can cause insensitive to most conventional chemotherapeutic drugs. Hence, there is considerable interest in exploiting Src kinase as a novel therapeutic target in malignant disease and in inhibiting the development of metastatic disease.[22] The Src's role in the migration and invasion steps of the metastatic cascade might imply the potential for Src inhibitors in the early adjuvant setting to reduce the risk of metastatic recurrence during or after definitive therapies.[11] As our results indicated, overexpression of Src can

be observed in many pancreatic cancer cell lines. Although exact mechanisms of Src overexpression remain complex and elusive, it was likely associated with their deregulated upstream regulators and signaling like EGFR, HER2, and/or c-MET.[23–25]

Interestingly, our results found that the overexpressed p-Src can be further induced by paclitaxel and gemcitabine in HPAC and PANC-1 cells and this suggests us Src may be a potential target with combination of these two drugs in the treatment of pancreatic cancer. The major downstream signaling upon Src activation includes activation of Akt, enhancement of cell proliferation, STAT3 activation, and transcriptional upregulation of secretary factors involved in metastasis and angiogenesis.[11] Inhibition of Src signaling has been shown to restore the sensitivity to gemcitabine and 5-flourouracil in human pancreatic cancer cells.[10,26] This implicates that Src inhibition in restoring the chemosensitivity and suggests a novel approach using dasatinib in combination with gemcitabine for the treatment of pancreatic cancer.

Gemcitabine, which was confirmed as the standard drug for advanced pancreatic cancer in 1997, showed little effects on improving survival outcomes.[27,28] Many gemcitabine-based combinations demonstrated no more efficacy than gemcitabine alone, aside from when administered in patients with good performance status. Previously, one data showed that gemcitabine resistance correlates with increased Src activity, and Src inhibition can overcome this resistance.[29] There is one report indicating that dasatinib synergized with gemcitabine in MIA-PaCa-2 by decreasing the levels of ALDH1A1.[19] In consistent of these findings, our
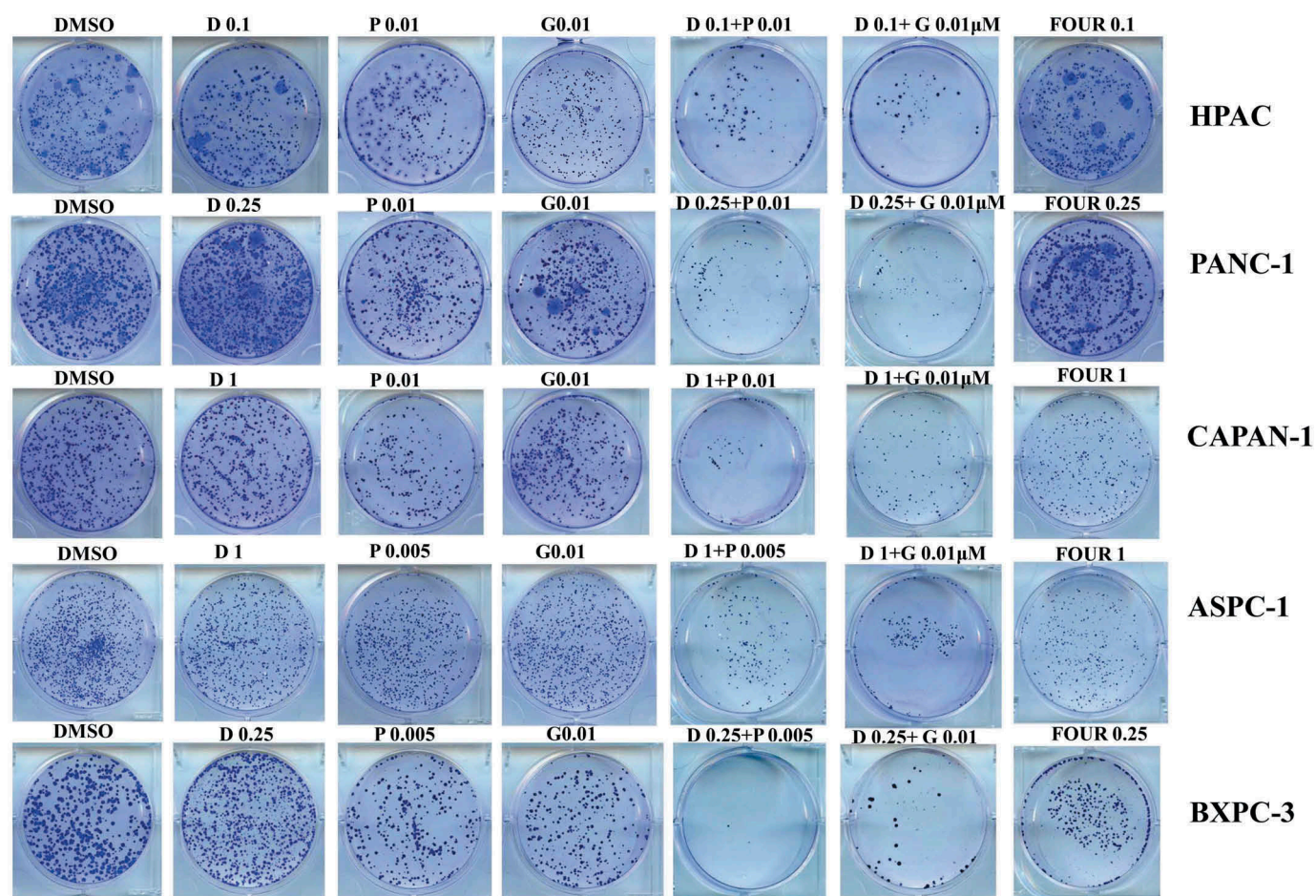
Figure 5. Dasatinib with paclitaxel or with gemcitabine can greatly reduce the cell colony formation ability of human pancreatic cancer cells.

Colony formation assay was conducted in HAPC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3 cells as described in the "Material and methods" section. HAPC, PANC-1, CAPAN-1, ASPC-1, and BXPC-3 human pancreatic cells were treated with dasatinib, paclitaxel, and gemcitabine alone and in combination of dasatinib and paclitaxel or dasatinib and gemcitabine at the indicated doses for 72 h. Same concentration of folfirinox was used simultaneously. After treatment, the same number of cells were seeded and cultured in a drug-free medium for 1–2 weeks. Colonies were fixed by ice-cold methanol and stained by 1% crystal violet.

results showed that dasatinib can enhance the effects of gemcitabine in five pancreatic cancer cell lines and three mouse pancreatic cancer cell lines. STAT3 is a key oncogenic factor in many human cancers and is required for oncogenesis in the mouse model of cancers. Hyperactivation of STAT3 in Tyr705 has been reported in many pancreatic cancer cell lines. Its activation form can regulate oncogenic signaling and increase the tumor cell survival, proliferation and tumor growth.[30] The combination uses of dasatinib and gemcitabine not only reduce the level of Src but also target multiple tumor-associated pathways including STAT3, ERK, and p-AKT and may overcome resistance to monotherapy by multiple mechanisms.

As a microtubule-stabilizing agent, paclitaxel possesses both mitosis-dependent and mitosis-independent activities against cancer cells.[31] Resistance of paclitaxel limits its use in pancreatic cancer treatment. Some has tried dasatinib with paclitaxel in advanced breast cancer, uterine cervical adenocarcinoma cells, ovarian cancer, and lung adenocarcinoma.[32–35] In these studies, the mechanism underlying the interaction of Src inhibitor enhanced paclitaxel sensitivity include enhancing apoptosis, inducing autophagic death, enhancing microtubule stability, and/or targeting tumor neovasculature. Our investigation is the first report to combine dasatinib with paclitaxel in pancreatic

cancer cell lines. Our primitive results showed great efficacy when combining these two drugs *in vitro*. Similar to dasatinib with gemcitabine, it may enhance the efficacy of paclitaxel through inhibition of induced SRC expression.

Our results showed elevated cleaved caspase-3/7 was observed with dasatinib and gemcitabine or dasatinib and paclitaxel. Several mechanisms of apoptosis and autophagy induced by dasatinib were investigated in a certain type of solid tumors or CML. It induces autophagic cell death in ovarian cancer that partially depends on Beclin-1, AKT, and Bcl-2.[36] In gastric cancers, it promoted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis via upregulation of CCAAT/enhancer-binding protein homologous protein (CHOP)-dependent death receptor 5 (DR5) expression.[37] In CML model (K562R cells), dasatinib promoted apoptosis through downregulation of AKT/mTOR activities, while preventing exosomal release and inhibiting autophagy by reducing expression of beclin-1 and Vps34.[38] MEK/ERK and p38 MAPK were found to control dasatinib/valproic acid-induced apoptosis as upstream regulators.[39]

There are two clinical trials investigating the efficacy of dasatinib and gemcitabine, but the results are quite different. One phase I results showed an encouraging clinical activity in
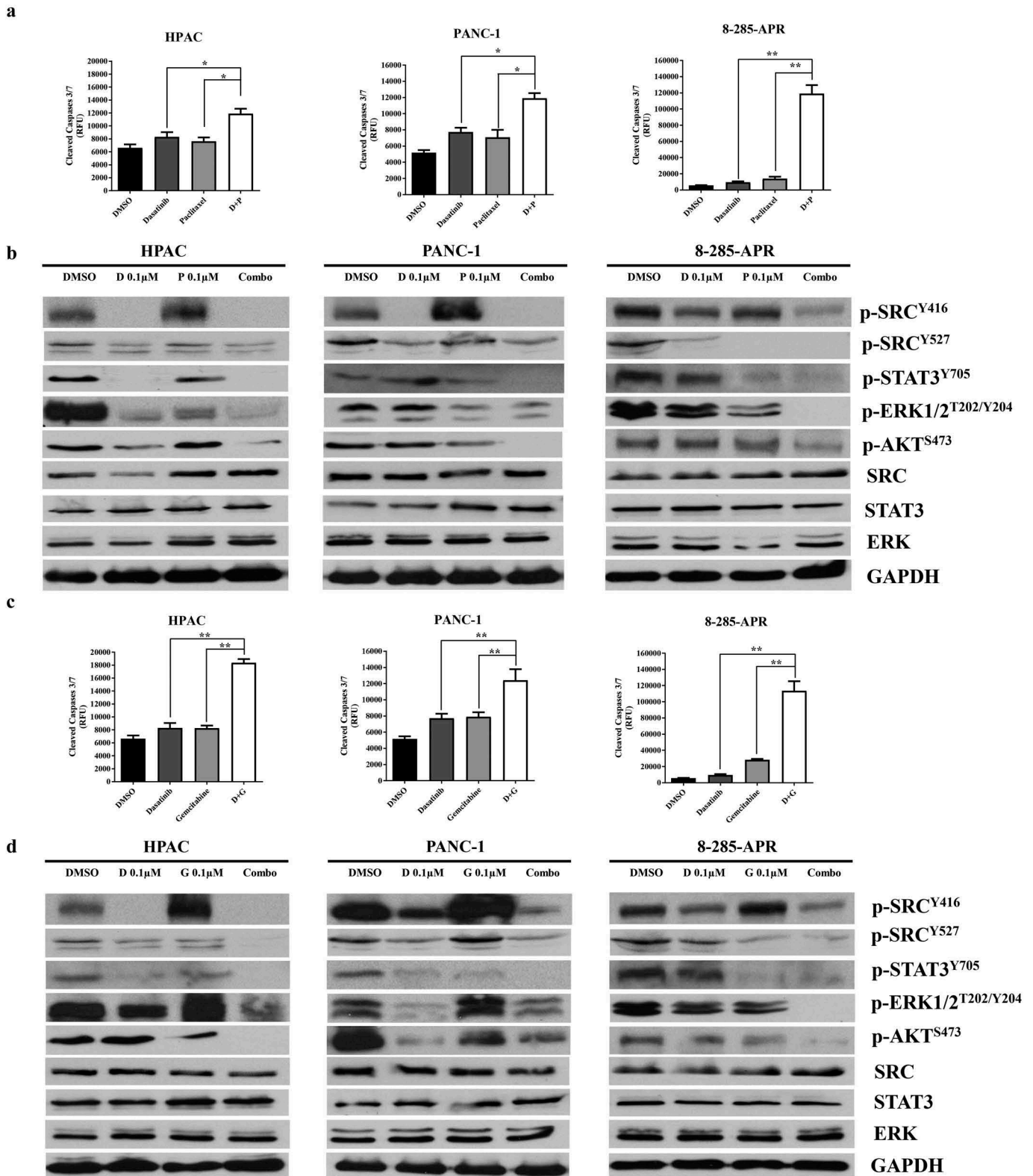
Figure 6. Combination of dasatinib with paclitaxel or gemcitabine can induce apoptosis and inhibit the activation of SRC/STAT3 pathway.

a: 8,000 cells per well of HAPC, PANC-1 and 8–285-APR cells were treated with 0.1 μM dasatinib, 0.1 μM paclitaxel and a combination of dasatinib and paclitaxel for 6 h. The level of cleaved caspase-3/7 (RFU) was measured using Caspase-3/7 Fluorescence Assay kit. b: HPAC, PANC-1and 8–285 APR cells were treated with dasatinib (0.1 μM), paclitaxel (0.1 μM) alone or in combination for 24 h. The protein expression of p-Src (Y416 and Y527), p-STAT3^{Y705}, p-AKT, p-ERK1/2, and STAT3 were detected by Western blots with GAPDH as loading control. c: 8,000 cells per well of HAPC, PANC-1 and 8–285-APR cells were treated with 0.1 μM dasatinib, 0.1 μM gemcitabine and a combination of dasatinib and gemcitabine for 6 h. The level of cleaved caspase-3/7 (RFU) was measured using Caspase-3/7 Fluorescence Assay kit. d: HPAC, PANC-1and 8–285 APR cells were treated with dasatinib (0.1 μM), gemcitabine (0.1 μM) alone or in combination for 36 h. The protein expression of p-Src (Y416 and Y527), p-STAT3^{Y705} p-AKT^{S473}, p-ERK1/2$^{T202/Y204}$, STAT3, ERK, and SRC were detected by Western blots with GAPDH as loading control.

advanced and metastatic pancreatic cancer.[40] Another clinical trial however showed that dasatinib failed to show increased OS or PFS in patients with locally advanced PDCA .[41] Specific biomarkers have not yet been identified that predict the ability of Scr inhibitors to potentiate gemcitabine or paclitaxel treatment. There are no any clinical trials for dasatinib and paclitaxel.

Taken together, our results clearly show that dasatinib can greatly enhance the efficacy of paclitaxel and gemcitabine activity in human pancreatic cancer cells through inhibition of paclitaxel- and gemcitabine-induced activation of Src and ERK. More clinical trials are needed to further confirm these combination effects.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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