Targeted Inhibition of Src Kinase Signaling Attenuates Pancreatic Tumorigenesis

Nagathihalli S. Nagaraj1, J. Joshua Smith1,2, Frank Revetta3, M. Kay Washington3,4, and Nipun B. Merchant1,4

Abstract

Elevated Src expression correlates with malignant potential and metastatic disease in many tumors including pancreatic cancer. We sought to characterize the molecular effects of Src kinase inhibition with dasatinib (BMS-354825), a novel, multitargeted kinase inhibitor that targets Src family kinases in pancreatic ductal adenocarcinoma (PDA). We identified sensitive and resistant PDA cell lines to dasatinib treatment and tested the molecular effects of Src inhibition in vitro and in vivo. We show for the first time that cellular localization of Src expression affects survival in patients with PDA. Pancreatic tumors with increased membranous expression of Src resulted in decreased survival compared with tumors that had increased cytoplasmic Src expression. Src kinase inhibition with dasatinib markedly inhibits cell proliferation, migration, invasion, cell cycle progression and anchorage-independent growth, and stimulates apoptosis. This was accompanied by decreased phosphorylation of Src, focal adhesion kinase, paxillin, AKT, signal transducers and activators of transcription 3 (STAT3), extracellular signal–regulated kinase, and mitogen-activated protein kinase (MAPK), as well as decreased cyclin D1 expression in a time- and concentration-dependent manner. Furthermore, small interfering RNA to Src results in a significant decrease in cell proliferation, invasion, and migration of pancreatic cancer cells. Dasatinib treatment also inhibits in vivo pancreatic tumor growth. Mechanisms of resistance to Src inhibition seem to be related to a lack of inhibition of STAT3 and MAPK signaling. These results establish a mechanistic rationale for Src inhibition with dasatinib as a therapeutic target in the treatment of pancreatic cancer and identify potential biomarkers of resistance to Src inhibition. Mol Cancer Ther; 9(8); 2322–32. ©2010 AACR.

Introduction

Pancreatic cancer remains one of the most lethal forms of human cancer, with a 5-year survival rate of only 3% to 5% (1). Cytotoxic chemotherapy based on the purine analogue, gemcitabine, remains the standard approach in the adjuvant and palliative setting, but results in minimal responses in the majority of patients. The failure of conventional chemotherapeutic regimens to produce any meaningful effect on survival in patients with pancreatic cancer highlights a desperate need for novel treatment strategies.

The proto-oncogene c-Src (Ssrc) encodes a nonreceptor tyrosine kinase, the expression and activity of which are correlated with cancer progression, advanced malignancy, and poor prognosis in a variety of human cancers including pancreatic cancer (2–6). Src family kinases are involved in regulating important mechanisms of receptor tyrosine kinases, G protein–coupled receptors, and focal adhesion kinase (FAK), thereby influencing many aspects of tumor cell behavior, including proliferation, survival, angiogenesis, adhesion, invasion, and metastasis (7–11).

Src is an integrator of divergent signals, facilitating the action of other signaling proteins, making it an attractive target for the treatment of human tumors. It is able to channel phosphorylation signals through Ras/Raf/extracellular signal–regulated kinase 1/2, and in certain cells, phosphatidylinositol 3-kinase/AKT pathways (3). Phosphorylation of FAK Tyr992 creates a binding site for Src, indicating that Src may also regulate cell adhesion (12). Paxillin is a substrate for the FAK-Src complex that functions as an adaptor molecule for various signaling and structural proteins in adhesions (13, 14). The mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase cascade is a well-known target of FAK-Src signaling (15), and its activation can be facilitated by association with paxillin (16). Therefore, Src transmits multiple signals including phosphatidylinositol 3-kinase/AKT, c-Myc/cyclin D1, and FAK/p130CAS/paxillin to induce tumor growth and survival.

Several lines of evidence suggest that Src inhibition is effective as a therapeutic target in preclinical models of pancreatic cancer. The well-characterized Src inhibitor pyrazolopyrimidine (PP2) and the quinazoline compound AZM475271 have shown activity in orthotopic
models for pancreatic cancer (4, 5). Src inhibition has also been shown to decrease proliferation, microvessel density, and decrease metastasis in an orthotopic implant mouse model (5, 17). However, most Src family kinases have been shown to have limited single-agent activity in the clinical setting, therefore, understanding their molecular mechanisms of action remains critical to the successful clinical implementation of these inhibitors.

This study provides the first characterizations of the molecular effects of Src kinase inhibition with dasatinib (BMS-354825), a multitargeted kinase inhibitor of Bcr-Abl and Src family kinase signaling (18) in pancreatic ductal adenocarcinoma (PDA) in vitro and in vivo. We show for the first time that cellular localization of Src expression affects survival in patients with pancreatic cancer. We have identified sensitive and resistant pancreatic cancer cell lines to Src inhibition, allowing us to further elucidate the signaling pathways affected by dasatinib treatment and identify potential biomarkers of resistance to Src inhibition.

Materials and Methods

Materials

Mouse monoclonal antibodies against signal transducers and activators of transcription 3 (STAT3) and Src; rabbit monoclonal antibodies against MAPK, pSrc (Y418), pAkt (Ser473), and pSTAT3 (Ser727); rabbit polyclonal antibodies against pFAK (Tyr925), pPaxillin (Yr118), pAKT (Ser473), pMAPK (Thr202/Tyr204), AKT, paxillin, and FAK were purchased from Cell Signaling Technology. Mouse polyclonal antibodies directed against phosphorylated c-jun-NH2-kinase (JNK) and cytoplasmic D1 were obtained from Santa Cruz Biotechnology, Inc. The rabbit polyclonal antibody against pFAK (Y397) was purchased from Abcam, Inc. The secondary antibodies for Western blots (anti-mouse and anti-rabbit IgG antibodies) were obtained from Santa Cruz Biotechnology. Dasatinib (BMS-354825) was kindly provided by Richard Smykla from Bristol-Myers Squibb Oncology (Princeton, NJ).

Cell culture and animals

Human pancreatic cancer cell lines BxPC3, PANC1, MiaPaca2, AsPC1, CEPAC, Capan1, Capan2, SW1990, and HPAC were obtained from American Type Culture Collection; tumor cells were maintained according to the instructions of the manufacturer. Female athymic nude mice, Foxn1 nu/nu (4–5 weeks old), were purchased from Harlan Sprague-Dawley, Inc., and maintained at the Vanderbilt University School of Medicine animal facility under protocols approved by the Vanderbilt Institutional Animal Care and Use Committee.

Tissue microarray

Tissue microarrays (TMA) were constructed as previously described (19). TMA slides were concurrently evaluated by two of the authors (M.K. Washington and N.B. Merchant). Nuclear and cytoplasmic staining was scored as follows: the staining index was considered as the sum of the intensity score (0, no staining; 1+, weak; 2+, moderate; 3+, strong) and the distribution score (0, no staining; 1+, staining of <33% of cells; 2+, between 33% and 66% of cells; and 3+, staining of >66% of cells). Staining indices were classified as follows: 3+ or higher, strong staining; 1+ to 2+, weak staining; and 0, negative staining. c-Src was scored as positive if any detectable membranous or cytoplasmic staining was present.

Src gene knockdown by short hairpin RNA

Open Biosystems pGIPZ-based short hairpin RNA (shRNA) lentiviral vectors were used to deplete Src expression. Human pancreatic cancer cell line BxPC3 was cultured in RPMI containing 10% fetal bovine serum. Lentiviral shRNA vector pGIPZ with either targeting sequences for knocking down human Src (clone IDs: V2LHS_262793 and V2LHS_70230) or nonsilencing control sequence was obtained from Vanderbilt University Microarray Core and transfected into BxPC3 cells with FuGENE 6 transfection reagent (Roche) following the instructions of the manufacturer.

Proliferation assay

Cells were treated with DMSO or dasatinib (0–5,000 nmol/L) for 48 hours, and cell viability was determined by MTT (Sigma) assay according to the directions of the manufacturer. IC50 was calculated using Prism software package.

Apoptosis assay

Apoptosis was determined using a luminescence assay method that quantifies caspase 3 and 7 activity. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay kit (Promega) according to the protocols of the manufacturer and as previously described (20).

Cell cycle analysis

Cells were harvested, washed, and resuspended as described previously (21). Cells were stained with propidium iodide for 30 minutes. Cell fluorescence signals were determined using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with its CellQuest software. The percentages of cells present in different phases of the cell cycle were measured and analyzed.

Migration and invasion assay

The upper chamber of 8 mmol/L pore transwells were overlaid with 50 μL (~100 μg) of diluted matrigel (BD Biosciences) solution for invasion. Cells (3 × 106) were seeded into the upper chamber of 8 mmol/L pore transwells coated with collagen for migration. Medium containing 10% fetal bovine serum and DMSO or dasatinib (1–1,000 nmol/L) was used as a chemoattractant in the lower chamber as indicated. The vector control and Src-shRNA cells were also plated as above with or without...
DMSO or dasatinib. Cells were allowed to migrate for 5 hours or invade the matrigel for 24 hours. Migrated or invaded cells were fixed with 4% paraformaldehyde, stained with 1% crystal violet, and counted from six random fields for each membrane at x20 magnification and averaged.

**Soft agar colony formation**

BxPC3 and PANC1 cells (1.5 × 10^4) were suspended in 1 mL of 0.4% sea plaque agarose containing 10% fetal bovine serum and then plated on top of 1 mL of semisolid 0.8% agarose in 35 mm plates. Cells were treated with DMSO or dasatinib (1–1,000 nmol/L) for every 48 hours for 2 weeks. Colonies grown on soft agarose were counted in 10 random fields per well.

**Western blot analysis**

Western blot analyses were done using standard methods (20). Cells were grown in complete medium overnight and then treated with dasatinib as required in each assay. Membranes were probed with total and phosphorylated antibodies as detailed above in Materials and Methods.

**Immunofluorescence assay**

Cells were grown and treated with dasatinib (5 nmol/L) for 12 hours, fixed, and stained with anti–cyclin D1 or c-myc. The anti–cyclin D1 staining was detected with a Cy3-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) and c-myc with FITC-green. Slides were prepared using Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.), and imaged with a Zeiss Axiophot microscope (Carl Zeiss, Inc.). Images were merged using NIH ImageJ software.

**In vivo tumorigenicity assay**

Tumors were established by injecting 5 × 10^6 BxPC3 or PANC1 cells into the flank of 6-week-old female athymic nude mice Fox1 nu/nu mice (n = 5, in each group). Dasatinib (25 mg/kg/d) or citrate buffer (vehicle) was administered by oral gavage and tumor volume (V) was determined by caliper measurements obtained every 2 days and calculated by the equation V = L × W^2 × 0.5, where L is the length and W is the width of a tumor. At the end of the study, animals were sacrificed and their primary tumors were excised for further analyses. Growth curves for tumors were plotted as the mean volume ± SD of tumors of mice from each group. All experiments were done in compliance with the Vanderbilt Institutional Animal Care and Use Committee guidelines.

**Immunohistochemistry**

Mice were euthanized and tumor tissues were collected for immunohistochemical analysis. Tissues were fixed and immunostained using antibodies against cleaved caspase 3, pSrc (Tyr416), pAKT (Ser473), and Ki67 (Biocare). Cleaved caspase 3, Ki67, pSrc, and pAKT were evaluated by an expert pathologist (M.K. Washington).

For Ki67, caspase 3, pSrc, and pAKT staining quantification, positive staining was quantified by using NIH image analysis software, ImageJ, and is reported as a percentage of the area of positive staining.

**Statistical analysis**

Statistics including mean values and SD were calculated using Microsoft Excel and Prism software (GraphPad). All data represent at least three independent experiments and are expressed as the means ± SD unless otherwise indicated. ANOVA was used to assess the differences between experimental groups unless otherwise indicated.

Survival analysis was done by using SPSS PC package (SPSS, Inc.). Overall survival time was calculated from the date of diagnosis until death or the last follow-up contact. The effect of Src expression on survival was assessed using the Kaplan-Meier method and compared using the log rank test. Multivariate analysis (Cox model) was done using tumor grade with survival time and median Src expression score (>1 or ≤1).

**Results**

**Src expression in human pancreas tissues**

A TMA consisting of cores from 25 normal pancreatic ductal epithelia and 13 chronic pancreatitis, and PDAs from 13 well-differentiated, 25 moderately differentiated, and 30 poorly differentiated tumors were stained for Src expression.

The staining indices of cytoplasmic Src and membranous Src varied significantly between normal pancreas, chronic pancreatitis, and PDA tissues. Analysis confirmed a stepwise progression of cytoplasmic and membranous staining of Src from normal pancreas to chronic pancreatitis to advancing tumor grade of PDAs (Fig. 1A and B). These results indicate that Src expression increases with the progression of pancreatic neoplasia.

Although increased Src expression and activity are correlated with cancer progression and advanced malignancy, these results also suggest that increased Src expression also occurs at an earlier neoplastic stage associated with inflammation in patients with chronic pancreatitis.

Furthermore, an increase in Src expression with increasing tumor grade of PDA was also seen on an independent analysis of Src staining of tumor grade compared with normal ducts (P < 0.01, Fisher's exact test).

Of the 65 PDAs represented on the TMA (median patient age was 66 years; range, 37–84 years), there were 36 American Joint Committee on Cancer stage IIB, 25 stage IIA, 2 stage III, and 1 case each was stage IA and IB. Median duration of follow-up was 19 months, during which time 51 patients died (Supplementary Table S1). Median cytoplasmic and membranous Src expression scores were determined and overall survival analysis was done for scores above and below the median expression score.
Analysis of the effect of low or high Src expression composite score on overall survival by cytoplasmic or membranous Src expression is shown in Fig. 1C. Increased membranous Src expression resulted in a significantly lower overall survival ($P = 0.001$), whereas survival outcomes were significantly improved when Src expression was higher in the cytoplasmic compartment ($P = 0.001$).

**Src inhibition attenuates pancreatic cancer cell proliferation, induces cell cycle arrest, and enhances apoptosis**

Nine human pancreatic cancer cell lines were tested for sensitivity to dasatinib in vitro using a proliferation assay. These cell lines were derived from different stages of the tumor and different genetic backgrounds including the oncogenic K-ras mutations (in >95% of analyzed tumors), the loss of the tumor suppressor p53 (≈75%), and DPC4/smad4 (50%). The IC$_{50}$ was calculated after treatment in complete medium for 48 hours (Table 1). In general, dasatinib showed antiproliferative activity at low nanomolar concentrations in all cell lines studied. BxPC3 and HPAC (IC$_{50}$, 2.7–2.8 nmol/L) cell lines were markedly more sensitive than any other cell line studied. SW1990, Capan1, and Capan2 (IC$_{50}$, 4.8–9.6 nmol/L) were less sensitive when compared with BxPC3 and HPAC cells, but more sensitive compared with MiaPaca2, PANC1, AsPC1, and CEPAC (IC$_{50}$, 24.9–51.2 nmol/L) cell lines which were least sensitive.

BxPC3 and PANC1 cells were treated with dasatinib, resulting in a decreased percentage of cells in G$_{2}$M and S phases and an increased percentage of cells in the sub-G$_{0}$ population, consistent with cell cycle blockade in the G$_{2}$-S transition and induction of apoptosis. Dasatinib treatment resulted in a decreased percentage of cells in S phase in both BxPC3 (most sensitive) and PANC1 (least sensitive) cell lines (Fig. 2A). This concentration-dependent decrease was significant ($P < 0.001$) starting at 10 nmol/L of dasatinib in BxPC3 cells and starting at 50 nmol/L in PANC1 cells, consistent with the sensitivity of BxPC3 cells to dasatinib and the resistant nature of PANC1 cells to dasatinib.

To further investigate the mechanism of growth inhibition by dasatinib, we examined its effect on the induction of apoptosis (Fig. 2B). BxPC3 cells were highly apoptotic at any concentration studied when compared with more resistant PANC1 cells.
Src inhibition attenuates the metastatic potential of pancreatic cancer cells

There was a concentration-dependent reduction in cell motility (Supplementary Fig. S1A) and migration (Fig. 3A) in pancreatic cancer cells treated with dasatinib. BxPC3 cells showed a significant decrease in motility and migration starting at dasatinib concentrations as low as 1 to 2 nmol/L. BxPC3 cells were significantly more sensitive to dasatinib when compared with more resistant PANC1 cells. Cells were allowed to recover by removal of dasatinib after wounding. PANC1 cells recovered faster than BxPC3 cells at any concentration of dasatinib treatment (Supplementary Fig. S1B).

Inhibition of BxPC3 cell migration increased with the concentration of dasatinib to a maximum at 10 nmol/L, whereas in PANC1 cells, the decrease was significant starting at 100 nmol/L (Fig. 3A). There was also a concentration-dependent decrease in cell invasion with dasatinib treatment in BxPC3 and PANC1 cells (Fig. 3B). BxPC3 cell invasion was significantly inhibited with as little as 5 nmol/L of dasatinib ($P < 0.05$). Dasatinib at 100 nmol/L showed a 22-fold decrease in BxPC3 cell invasion compared with untreated cells, whereas only a 1.5-fold decrease was observed compared with untreated cells in PANC1 cell invasion at the same dose.

To determine whether dasatinib affects cell transformation, we did a soft agar colony formation assay. Dasatinib inhibited anchorage-independent growth of BxPC3 and PANC1 cells (Fig. 3C). With the addition of dasatinib, BxPC3 and PANC1 cells formed fewer colonies in soft agar compared with DMSO control (BxPC3 control, 96.53 ± 12.76 colonies versus 100 nmol/L of dasatinib, Table 1).

**Table 1. Pancreatic cancer cell lines characteristics and their dasatinib IC$_{50}$**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>Genetic background</th>
<th>IC$_{50}$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiaPaca2</td>
<td>Primary</td>
<td>Mutant K-ras, mutant p53, wt smad4</td>
<td>51.28</td>
</tr>
<tr>
<td>PANC1</td>
<td>Primary</td>
<td>Mutant K-ras, mutant p53, wt smad4</td>
<td>45.68</td>
</tr>
<tr>
<td>AsPC1</td>
<td>Metastasis (ascites)</td>
<td>Mutant K-ras, wt p53, wt smad4</td>
<td>44.97</td>
</tr>
<tr>
<td>CEPAC1</td>
<td>Metastasis (liver)</td>
<td>Mutant K-ras, wt p53, mutant smad4</td>
<td>24.99</td>
</tr>
<tr>
<td>Capan1</td>
<td>Metastasis (liver)</td>
<td>Mutant K-ras, ND p53, mutant smad4</td>
<td>9.60</td>
</tr>
<tr>
<td>SW1990</td>
<td>Metastasis (spleen)</td>
<td>Mutant K-ras, wt p53, ND smad4</td>
<td>5.11</td>
</tr>
<tr>
<td>Capan2</td>
<td>Primary</td>
<td>Mutant K-ras, wt p53, wt smad4</td>
<td>4.87</td>
</tr>
<tr>
<td>HPAC</td>
<td>Primary</td>
<td>Mutant K-ras, wt p53, wt smad4</td>
<td>2.79</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Primary</td>
<td>Wt K-ras, mutant p53, mutant smad4</td>
<td>2.81</td>
</tr>
</tbody>
</table>

Abbreviations: WT, wild-type; ND, not determined.
6.37 ± 2.83 colonies, \( P < 0.01 \); PANC1 control, 76.32 ± 10.3 colonies versus 100 nmol/L of dasatinib, 57.68 ± 5.92 colonies, \( P < 0.05 \).

Because dasatinib is a multitargeted kinase inhibitor, we sought to confirm that these effects of dasatinib were specific to its activity on Src kinase inhibition. We stably inhibited the protein expression levels of Src in BxPC3 cells using small interfering RNA (lentiviral-shRNA vector, Supplementary Fig. S2A). Blocking Src expression resulted in a significant reduction in proliferation (\( P < 0.001 \); Supplementary Fig. S2B), migration (\( P < 0.001 \)), and invasion (\( P < 0.01 \); Fig. 3D) compared with control cells. These data further confirm that dasatinib inhibits migration and invasion through Src signaling regardless of the antiproliferative or apoptotic effects of the drug.

**Src inhibition blocks the activation of multiple downstream signaling pathways in pancreatic cancer cells**

To explore the potential regulatory mechanisms mediating the growth-inhibitory effects of dasatinib, we tested its ability to inhibit kinases important in survival, angiogenesis, proliferation, migration, and invasion in a concentration- and time-dependent manner in BxPC3 (sensitive) and PANC1 (least sensitive) cells. Src kinase activity was significantly inhibited in all cell lines, whereas
total Src levels were not affected. Phosphorylation of Src, FAK, paxillin, AKT, STAT3 and MAPK were decreased in BxPC3 cells treated with dasatinib compared with control cells in a concentration-dependent manner (Fig. 4A). There was a similar inhibition of Src, FAK, and AKT phosphorylation, but at much higher concentrations of dasatinib in PANC1 cells, whereas no significant effects were noted in phosphorylation of paxillin, STAT3, or MAPK (Fig. 4A). STAT3 and MAPK are involved in cell survival and angiogenesis, whereas JNK and paxillin are involved in motility/migration and invasion of cancer cells. The lack of inhibition of phosphorylation of paxillin, STAT3, and MAPK even at higher concentrations of dasatinib in PANC1 cells, suggests that some of the resistance of dasatinib in certain pancreatic cancer cell lines is related to its limited activity in targeting certain pathways related to cell survival, angiogenesis (STAT3 and MAPK), and cell adhesion (paxillin). Dasatinib did markedly inhibit AKT activation, suggesting a role for dasatinib in targeting both cell survival and proliferation pathways in pancreatic cancer cells.

The effect of dasatinib on G1-S transition prompted us to study its effect on c-Myc, a Src target gene (22) and cyclin D1, the rate-limiting factor for cellular proliferation (23). Both c-Myc and cyclin D1 were downregulated with dasatinib treatment (Fig. 4A, B, and C). A more striking correlation was found between the cell cycle regulatory effect of dasatinib and the modulation of cyclin D1 expression.

**Dasatinib inhibits pancreatic tumor progression in vivo**

Having shown the biological effects and the cellular responses to Src inhibition with dasatinib in vitro, we sought to determine its efficacy in vivo using a mouse xenograft model implanting BxPC3 (Fig. 5A) and PANC1 (Fig. 5B) cells. Mice were treated with citrate buffer (control or vehicle) or dasatinib (25 mg/kg) by oral gavage.
for 14 days. Tumor volumes of untreated BxPC3 xenografts increased 62% compared with a 17% (1.96-fold) decrease in tumor volume in dasatinib-treated animals at 10 days of treatment ($P < 0.001$). Tumor volumes of untreated PANC1 xenografts increased 174% at 10 days, compared with only a 50% increase in tumor volume of dasatinib-treated animals ($P < 0.001$). These in vivo data show tumor growth inhibition even in less sensitive PANC1 cells, indicating that dasatinib is a promising agent for inhibiting pancreatic tumor progression.

**Inhibition of Src activity correlates with in vivo inhibition of proliferation and increased apoptosis in pancreatic tumors**

To correlate our *in vitro* findings with the *in vivo* data, immunohistochemical analyses of dasatinib-treated
tumor xenografts of BxPC3 and PANC1 relative to vehicle-treated controls at the end of the treatment period were measured. Dasatinib-treated tumors exhibited increased apoptosis (cleaved caspase 3) and decreased proliferation (Ki67) as well as a significant inhibition of pSrc Tyr416 and pAKT Ser473 relative to controls (Fig. 5C and D).

Discussion

The role of the proto-oncogene c-Src in the progression of many tumors is well documented (18). However, overexpression of wild-type Src is weakly oncogenic on its own (24) and activating mutations are rare, limiting our understanding of Src in the development, maintenance, and progression of cancer (25–29). Our results show that Src inhibition in pancreatic cancer cells treated with dasatinib results in inhibition of cell proliferation, migration, invasion, and anchorage-independent growth, which directly correlate with a significant reduction in tumor growth in vivo. We identified sensitive and more resistant pancreatic cancer cell lines to dasatinib treatment to further elucidate the molecular mechanisms involved with Src inhibition. Src kinase inhibition with dasatinib blocks the activation of multiple downstream signal transduction pathways known to promote survival, angiogenesis, proliferation, motility, migration, and invasion of tumor cells (30, 31) including AKT, STAT3, MAPK, FAK, and paxillin in sensitive cell lines (BxPC3).

Targeting signal transduction pathways is an effective strategy in many tumor types. However, activation of parallel signaling pathways could limit the efficacy of this approach, therefore, understanding the mechanisms of resistance to targeted agents remains critical to enhance their clinical usefulness. Resistance to dasatinib treatment seems to be related to lack of inhibition of STAT3 and MAPK signaling. STAT3 phosphorylation is inhibited with dasatinib treatment in sensitive pancreatic cancer cell lines, but not in the more resistant PANC1 cell line. Consistent with this finding, reactivation of STAT3 signaling after Src inhibition has recently been shown in head and neck cancer (32) and non–small cell lung cancer (33). Somewhat selective to Src family kinases is their ability to activate STAT3, which leads to the activation of downstream targets including Bcl-XL, c-Myc (34, 35), and cyclin D1 (36), resulting in increased cell survival, proliferation, and tumor growth. Our results suggest that this compensatory pathway might account for the resistance to Src inhibition and implicates elevated STAT3 expression as a potential biomarker of this resistance. Furthermore, this data provides the rationale to combine STAT3 inhibitors with dasatinib to improve clinical response in pancreatic cancer.

Additionally, basal pAKT levels were also noted to be markedly elevated in the more resistant PANC1 cell line, as well as other less sensitive cell lines such as MiaPaca2 and AsPC1 (data not shown), when compared with BxPC3-sensitive cells. Although, dasatinib treatment resulted in significant inhibition of pAKT activation in both PANC1 and BxPC3 cells, this increased basal pAKT levels might be another contributing factor towards the resistant nature of PANC1 cells and requires further investigation.

We also show for the first time that Src expression increases with the progression of pancreatic neoplasias from normal pancreas to chronic pancreatitis to PDA. In addition, the increase in Src expression with increasing tumor grade of PDAs is consistent with the role of Src activity playing an important role in cancer progression and metastasis (18, 37). Our finding that survival outcomes are significantly improved when Src expression is lower in the membranous compartment compared with the cytoplasmic compartment suggests that cytoplasmic Src is weakly oncogenic and requires membranous localization to become activated and regulate important mechanisms of specific receptor pathways, stimulating oncogenesis. We have previously shown that activation of Src results in its translocation from the cytoplasm to the cell membrane, where it activates tumor necrosis factor-α–converting enzymes to induce shedding epidermal growth factor receptor ligands from the cell membrane and activate epidermal growth factor receptor signaling (20, 38).

Interestingly, we also found that Src expression is increased in patients with chronic pancreatitis. Increased Src expression and activity are generally correlated with cancer progression and advanced malignancy. However, these results implicate the activation of Src at an earlier stage of pancreatic neoplasia and might suggest that patients with chronic pancreatitis may have a higher propensity toward malignant transformation depending on their levels of Src activation.

Dasatinib is a multita rgeted kinase inhibitor and it is likely that not all of its biological and molecular effects are due to Src inhibition alone. However, several lines of evidence suggest that the effects of dasatinib in pancreatic cancer are related to Src kinase inhibition. Phosphorylation of Tyr416 in the kinase domain is a critical activation step in the regulation of Src tyrosine kinase activity (10). We show that Src phosphorylation at Tyr416 is inhibited with the addition of dasatinib. In addition to our results with Src shRNA cells, these findings suggest that the predominant effects of dasatinib treatment is related to inhibition of Src kinase activity.

Inhibition of Src signaling has been shown to restore sensitivity to gemcitabine in cell lines derived from pancreatic tumors. Similarly, silencing of FAK expression in PANC1 cells restores sensitivity to gemcitabine treatment (4, 5, 39). Furthermore, inhibition of Src also has been shown to revert chemoresistance against 5-fluorouracil in human pancreatic carcinoma cells (40). Therefore, these results clearly implicate Src inhibition in restoring chemosensitivity and suggest a novel approach using dasatinib in combination with gemcitabine for the treatment of pancreatic cancer.

There were no known genetic mutations that influenced cell sensitivity to dasatinib (Table 1). Inactivating
p53 mutations, activating Ras mutations (predominantly K-ras), and Smad4/DPC4 mutations are commonly found in PDAs. We did not find any relationship between p53 or K-ras or smad4 status and sensitivity to dasatinib, which is consistent with other studies in head and neck squamous cell carcinoma and non–small cell lung carcinoma cells (41).

In conclusion, we show that dasatinib inhibits pancreatic tumor growth both in vitro and in vivo. Our results, for the first time, show that dasatinib is effective in inhibiting multiple signaling pathways involved in tumor growth, proliferation, angiogenesis, survival, motility, migration, and invasion in pancreatic cancer, whereas resistance to Src inhibition seems to be related to activation of parallel signaling pathways including STAT3. Furthermore, dasatinib inhibits the metastatic potential of pancreatic cancer by inhibiting cell migration, invasion, and anchorage-independent growth. The in vivo effects of dasatinib on pancreatic tumor growth correlate with significant inhibition of Src and AKT phosphorylation, demonstrating biological and translational relevance.

References


Molecular Cancer Therapeutics

Targeted Inhibition of Src Kinase Signaling Attenuates Pancreatic Tumorigenesis

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