Combined Blockade of Src Kinase and Epidermal Growth Factor Receptor with Gemcitabine Overcomes STAT3-Mediated Resistance of Inhibition of Pancreatic Tumor Growth

Nagathihalli S. Nagaraj1, M. Kay Washington2,3, and Nipun B. Merchant1,3

Abstract

Purpose: We previously established a mechanistic rationale for Src inhibition as a novel therapeutic target in pancreatic cancer and have identified activated STAT3 as a potential biomarker of resistance to Src inhibition. The purpose of this study was to translate the current understanding of complementary activated tyrosine kinase signaling pathways by targeting Src kinase and epidermal growth factor receptor (EGFR).

Experimental Design: IC50 values for dasatinib, a Src kinase inhibitor, erlotinib, an EGFR tyrosine kinase inhibitor and gemcitabine were determined and sensitive and resistant pancreatic cancer cell lines were identified. The in vitro and in vivo effects of these agents on multiple signaling pathways and tumorigenicity in pancreatic cancer were investigated.

Results: The combination of dasatinib, erlotinib, and gemcitabine resulted in cooperative inhibition of cell migration and invasion of both sensitive and resistant pancreatic cancer cells as well as cooperative inhibition of multiple signaling pathways including FAK, AKT, ERK, JNK, MAPK, and STAT3 at concentrations that were ineffective as individual agents or as double combinations of agents. The triple combination of agents was also most effective at inhibiting the growth of xenografts of both sensitive and resistant pancreatic cancer cells in vivo without increasing toxicity. Furthermore, combined inhibition of Src and EGFR with gemcitabine inhibited constitutively activated STAT3 in vitro and in vivo.

Conclusions: These results provide evidence that combined targeted biological therapy in addition to cytotoxic chemotherapy can overcome treatment resistance. Such treatment strategies may be used to tailor therapy based on identified biomarkers of resistance to targeted monotherapy. Clin Cancer Res; 17(3); 1–11.

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Introduction

Pancreatic cancer remains a major therapeutic challenge. Five-year survival remains abysmal, around 5%, and has not changed over the past 30 years (1). Cytotoxic chemotherapy based on the pyrimidine analogue, gemcitabine, remains the standard approach in the adjuvant and palliative setting, but results in minimal responses. The failure of conventional chemotherapeutic regimes to produce any meaningful impact on survival in patients with pancreatic cancer highlights a desperate need for novel treatment strategies.

Over the past decade, a large number of studies have shown that strategies targeting specific molecular abnormalities implicated in pancreatic oncogenesis may result in inhibition of pancreatic tumor growth in preclinical studies. However these results have failed to translate into clinical benefit in numerous Phase III trials of molecularly targeted therapies in patients with advanced pancreatic cancer even when combined with gemcitabine. Recently, a detailed, global, genomic analysis identified pancreatic tumors to be highly heterogeneous, containing a large number of genetic alterations (average of 63) that affect a core set of 12 signaling pathways and processes that are genetically altered in 67% to 100% of cases (2). This suggests that treatment for pancreatic cancer should target these complex and overlapping signaling pathways, rather than just the products of a single gene. We sought to translate the current understanding of activated tyrosine kinase signaling pathways in pancreatic cancer into improved patient therapies by targeting two tyrosine kinases, the nonreceptor Src kinase and epidermal growth factor receptor (EGFR), which acts in complementary pathways. We hypothesized that...
targeting multiple tumor-associated pathways will enhance the therapeutic effects of cancer treatment by overcoming the inherent and acquired resistance associated with targeted monotherapy.

The rationale for targeting Src and EGFR signaling is based on the fact that both EGFR and Src kinase specific activity are elevated in the majority of pancreatic cancers and involved in cancer progression and metastasis (3, 4). Moreover, high EGFR and Src activity is also an indicator of poor clinical prognosis (5, 6). EGFR and Src family members are involved in numerous signaling pathways involved in proliferation, migration, invasion, tumor adhesion, and angiogenesis (7, 8). Several current paradigms in tumor cell biology attribute growth promotion to EGFR activity, while Src activity is thought to promote invasion. As Src and EGFR are both commonly activated in pancreatic cancer, these two tyrosine kinases may act in concert to regulate tumor growth (9–11).

Erlotinib, a tyrosine kinase inhibitor (TKI) of EGFR, was recently approved for patients advanced stage, nonresectable, and metastatic pancreatic tumors based on a modest but significant survival benefit in combination with gemcitabine therapy (12). This limited clinical utility further emphasizes the heterogenous and complex nature of tumors that makes it extremely unlikely that all tumors within a particular subtype will harbor the same activating mutation, or that the tumor will be entirely dependent on the de-regulation of one particular signaling pathway. Preclinical studies have shown resistance to targeting the EGFR pathway, and hence failure of such therapies is likely due to cross-talk between pathways that would circumvent any inhibitory effects on downstream signaling (13).

Src, is one of nine members of the Src family of non-receptor protein tyrosine kinases. We have previously shown that Src kinase is over expressed with the progression of pancreatic neoplasia from normal pancreas to chronic pancreatitis to increasing grade of pancreatic ductal adenocarcinoma (PDAC) (14). Under normal conditions, Src is a cytoplasmic protein that is maintained in an inactive form. It is not activated by a mutation, but plays a critical role in mediating multiple signal transduction pathways via its interactions with multiple proteins including G-protein coupled receptors, receptor tyrosine kinases, such as EGFR and integrins, making it an ideal target for therapeutic intervention (15).

Src directly modulates EGFR function through phosphorylation of tyrosine residues on EGFR that allows for coupling to downstream signaling events (16). Other interaction partners include signal transducers and activators of transcription (STATs), heterotrimeric G proteins, the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and focal adhesion kinase (FAK; refs. 17, 18).

We and others have previously established a mechanistic rationale for Src inhibition as a therapeutic target in the treatment of pancreatic cancer (14, 19–21). In addition, we have identified that one of the mechanisms of resistance to Src inhibition appear to be related to a lack of inhibition of activated STAT3 signaling. This study focuses on a novel approach that shifts away from conventional chemotherapy combinations and instead focuses on combining targeted therapies to Src and EGFR in pancreatic cancer that can have direct clinical impact by overcoming the limited antitumor activity seen with individual targeted therapy.

Materials and Methods

Materials

Mouse monoclonal antibodies against STAT3 and Src; rabbit monoclonal antibodies against MAPK, pSrc (Tyr416), pSTAT3 (Ser277), and pSTAT3 (Tyr705); rabbit polyclonal antibodies against pFAK (Tyr925), pEGFR (Tyr845), pEGFR(Tyr1173), pAKT (Ser473), pMAPK (Thr202/Tyr204), AKT, STAT3, and FAK were purchased from Cell Signaling Technology, Inc. EGFR antibody was obtained from Upstate. Mouse polyclonal antibodies directed against pJNK, pERK1/2, and also Rabbit polyclonal antibodies directed against JNK and ERK were obtained from Santa Cruz Biotechnology, Inc. Dasatinib (BMS-354825) was kindly provided by Richard Smykla from Bristol-Myers Squibb Oncology. For in vitro study, stock solution of dasatinib or erlotinib in 100% DMSO was diluted directly into the medium to indicated concentrations and stored at −20°C. For in vivo oral gavage, dasatinib was prepared freshly as a suspension in 80 mM sodium citrate/citric acid buffer, pH 3.0. Erlotinib hydrochloride was purchased from LC laboratories and formulated as a fine suspension with sodium carboy methylcellulose and Tween 80 in water for oral gavage in...
Migration and invasion assay

Wound.

Media with 10% FBS. Days were counted to close the second day removed the old media and changed to fresh to 15 days and observed for recovery postwounding. Every cell were washed and treated with regular media for up

nmol/L); PANC1 cells were treated with DMSO or dasatinib (100 nmol/L) and/or gemcitabine (200 nmol/L) and/or erlotinib (1000 nmol/L) for 40 hours. Phase contrast expression was observed in all BxPC3 cells. Expression of Src

mL and after 3 to 4 weeks of culture, vector encoded GFP expression was obtained from Vanderbilt University Microarray Core and transfected into BxPC3 cells with FuGENE 6 transfection reagent (Roche) following the manufacturer’s instruction. After 48 hours, cells were cultured in 0.5 mg/mL puromycin containing media for selecting pGIPZ vector expressing cells. By gradually increasing the concentration of puromycin to 3 mg/mL and after 3 to 4 weeks of culture, vector encoded GFP expression was observed in all BxPC3 cells. Expression of Src and EGFR in the above vector transfected cells were characterized by Western Blot analysis.

Western blot analysis

Western blot analyses were done using standard methods (22). Cells were grown in complete media overnight and then treated with dasatinib and/or erlotinib and/or gemcitabine as required in each assay. Membranes were probed with total and phosphorylated antibodies as detailed above in materials.

Src and EGFR gene knockdown

We used Open Biosystems pGIPZ-based short hairpin RNA (shRNA) lentiviral vectors to deplete Src and EGFR expression. Human pancreatic cancer cell line BxPC3 was cultured in RPMI containing 10% FBS. Lentiviral shRNA vector pGIPZ with either targeting sequences for knocking-down human Src (Clone IDs: V2LHS_262793 and V2LHS_70230) or EGFR (Clone IDs: V2LHS_200678 and V2LHS_201187) or nonsilencing control sequence was obtained from Vanderbilt University Microarray Core and cultured in RPMI containing media as described above. Cells were allowed to invade the matrigel for 24 hours, fixed, stained and calculated as indicated above. Each data point represents the average number from three wells.

In vivo tumorigenicity assay

Tumors were established by injecting 5\times10^6 cells of BxPC3 or PANC1 into the flank of 6-week-old female athymic nude mice Fox1-nu/nu mice (n = 5, in each group). Treatment was initiated when the tumors reached approximately 100 mm^3 size. Dasatinib (25 mg/kg) or erlotinib (50 mg/kg) or vehicle was administered by oral gavage; gemcitabine (20 mg/kg/3 days) was administered intraperitoneal (IP). We administered dasatinib daily twice and erlotinib daily based on the substantially longer half-life of erlotinib (36 hours; ref. 23) relative to dasatinib (3–5 hours; ref. 24). The combination of dasatinib and/or gemcitabine and/or erlotinib was treated and tumor volume (V) was determined from caliper measurements obtained every two days and calculated by the equation V = L \times W^2 \times 0.5, where L is length and W is width of a tumor. The percent body weight change for each mouse was calculated with the following formula: \[ (((W_n - W_0)/W_0) \times 100\% \) (in which W_0 is the mouse weight on day_0). At the end of the study, animals were sacrificed and their primary tumors were removed for further analysis. Growth curves from six random fields for each membrane at ×20 magnifications and averaged. The control vector shRNA, Src shRNA, EGFR shRNA and STAT3 shRNA cells were treated with or without erlotinib (100 nmol/L) or dasatinib (5 nmol/L) as described above. Cells were allowed to invade the matrigel for 24 hours, fixed, stained and calculated as indicated above. Each data point represents the average number from three wells.

Cell viability assay

Cells were treated with DMSO or dasatinib (0–5,000 nmol/L) (14) or gemcitabine (0–300 nmol/L) or erlotinib (0–5,000 nmol/L) for 48 hours and cell viability was determined by MTT (Sigma) assay according to the manufacturer’s direction. IC_{50} was calculated using Prism software package. The control vector shRNA, Src shRNA and EGFR shRNA cells were treated with or without erlotinib (100 nmol/L) or dasatinib (3 nmol/L) for 48 hours and the cell viability was determined as detailed above. Each condition was assayed in triplicate.

Wound-healing assay

Cells were treated with mitomycin C (0.5 μg/mL) for four hours prior to wounding. Wounds were made across the cell monolayer by a sterile pipette tip. After wounding, BxPC3 cells were treated with DMSO or dasatinib (5 nmol/L) and/or gemcitabine (50 nmol/L) and/or erlotinib (100 nmol/L); PANC1 cells were treated with DMSO or dasatinib (100 nmol/L) and/or gemcitabine (200 nmol/L) and/or erlotinib (1000 nmol/L) for 40 hours. Phase contrast images were taken. After 40 hours of wound healing study, the cells were washed and treated with regular media for up to 15 days and observed for recovery postwounding. Every second day removed the old media and changed to fresh media with 10% FBS. Days were counted to close the wound.

Migration and invasion assay

3 \times 10^4 cells were seeded into the upper chamber of 8-
mM pore transwells coated with collagen for migration and 50 μL (~100 μg) of diluted matrigel (BD Biosciences) for invasion assay. BxPC3 cells were treated with DMSO or dasatinib (5 nmol/L) and/or gemcitabine (50 nmol/L) and/or erlotinib (100 nmol/L); PANC1 cells were treated with DMSO or dasatinib (50 nmol/L) and/or gemcitabine (100 nmol/L) and/or erlotinib (500 nmol/L) was added to the lower chambers as a chemo attractants as indicated with medium containing 10% FBS. 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.
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 IACUC 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 (Tyr527), pAKT (Ser473), pEGFR (Tyr1173), pSTAT3 (Tyr705), and Ki67 (Biocare). Cleaved caspase-3, Ki67, pSrc, pAKT, pEGFR, and pSTAT3 were evaluated by an expert pathologist (M.K.W). For Ki67, caspase-3, pSrc, and pAKT staining quantification, positive staining was quantified by using NIH image analysis software, Image J, and reported as percentage area of staining.

Statistical analysis
Descriptive 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.

Results

Determination of sensitivity to dasatinib, erlotinib, and gemcitabine in pancreatic cancer cell lines
We screened nine human pancreatic cancer cell lines derived from different stages of tumor and with varied genetic backgrounds. IC_{50} values of dasatinib, erlotinib, and gemcitabine treatment were determined in these cell lines and identified both sensitive and resistant lines (Table 1; ref. 14). All three drugs showed antiproliferative activity at nanomolar concentrations in all cell lines studied. BxPC3 and HPAC cell lines, which showed the greatest sensitivity to dasatinib treatment, were also markedly more sensitive to both erlotinib and gemcitabine. Furthermore, MiaPaca2, PANC1, and AsPC1 cells which are least sensitive to dasatinib treatment were also more resistant to erlotinib or gemcitabine treatment. We assessed the expression of pSrc, Src, pEGFR, and EGFR protein in all nine cell lines (Fig. 1A). pSrc and pEGFR expression was lower in MiaPaca2, when compared to PANC1 cells.

We therefore sought to determine if combining these targeted therapies with gemcitabine would overcome the resistance seen with these individually agents alone. The combination of dasatinib, erlotinib and gemcitabine inhibited cell viability (Supplementary Fig. S1) and increased apoptotic activity (Supplementary Fig. S2) of pancreatic cancer cells in vitro to a greater degree than individual or double combination of agents.

Src and EGFR inhibition with gemcitabine augments attenuation of cell motility, migration, and invasion of pancreatic cancer cells
We investigated the functional effects of dasatinib, erlotinib, and gemcitabine on cell motility, migration and invasion (Fig. 1). The effect of these drugs on cell motility was evaluated initially using a monolayer wound-healing assay. Cells were treated with IC_{50} doses of these drugs for 40 hours. Optimal inhibition of wound closure was seen with the combined treatment of dasatinib, erlotinib and gemcitabine as compared with individual and two-agent combinations. Complete wound closure was seen in 30 to 35 hours in all of the untreated cells (Fig. 1B). Removal of the agents resulted in remigration of cells into the wound (Supplementary Fig. S3).

Single agent treatment weakly affected both cell migration and invasion of BxPC3 and PANC1 cells. Double combination therapy of dasatinib, erlotinib, or gemcitabine augmented the effect of cell migration and invasion.

Table 1. Pancreatic cancer cell lines characteristics and their IC_{50} values for dasatinib, erlotinib, and gemcitabine

<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></td>
<td></td>
<td></td>
<td>Dasatinib*</td>
</tr>
<tr>
<td>MiaPaca2</td>
<td>Primary</td>
<td>Mutant K-ras, mutant p53, wt smad4, wt EGFR</td>
<td>51.28</td>
</tr>
<tr>
<td>PANC1</td>
<td>Primary</td>
<td>Mutant K-ras, mutant p53, wt smad4, mutant EGFR</td>
<td>45.68</td>
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<tr>
<td>AsPC1</td>
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<td>Mutant K-ras, wt p53, wt smad4, wt EGFR</td>
<td>44.97</td>
</tr>
<tr>
<td>CEPAC1</td>
<td>Metastasis (liver)</td>
<td>Mutant K-ras, wt p53, mutant smad4, ND EGFR</td>
<td>24.99</td>
</tr>
<tr>
<td>Capan1</td>
<td>Metastasis (liver)</td>
<td>Mutant K-ras, ND p53, mutant smad4, mutant EGFR</td>
<td>9.6</td>
</tr>
<tr>
<td>SW1990</td>
<td>Metastasis (spleen)</td>
<td>Mutant K-ras, wt p53, ND smad4, ND EGFR</td>
<td>5.1</td>
</tr>
<tr>
<td>Capan2</td>
<td>Primary</td>
<td>Mutant K-ras, wt p53, wt smad4, ND EGFR</td>
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<tr>
<td>HPAC</td>
<td>Primary</td>
<td>Mutant K-ras, wt p53, wt smad4, mutant EGFR</td>
<td>2.79</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Primary</td>
<td>Wt K-ras, mutant p53, mutant smad4, wt EGFR</td>
<td>2.8</td>
</tr>
</tbody>
</table>

wt = wild type; ND = not determined.
*Data from Nagaraj and colleagues (14).
compared with treatment of individual agents. However, the addition of gemcitabine to the combination of dasatinib and erlotinib resulted in the optimal inhibition of cell migration and invasion in both sensitive BxPC3 as well as resistant PANC1 cells (Fig. 1C and D). These data indicate that combined treatment of dasatinib and erlotinib with gemcitabine elicits the greatest antiinvasive properties of pancreatic cancer cells.

Cooperative effects of Src and EGFR inhibition

Since dasatinib is a multitargeted kinase inhibitor, and erlotinib can affect other kinase pathways besides EGFR, we...
sought to confirm that the effects of dasatinib and erlotinib were specific to their activity on Src kinase and EGFR inhibition, respectively. We stably inhibited the expression levels of Src and EGFR in BxPC3 cells using small interfering RNA (Lentiviral-shRNA vector) as previously described (Supplementary Fig. S4) (14). Cell viability (Fig. 2A) and invasion assays (Fig. 2B) were performed in nonsilencing vector stably transfected control cells, and Src or EGFR knockdown cells. Src shRNA cells were treated with erlotinib and EGFR shRNA cells were treated with dasatinib to determine the cooperative effects of Src and EGFR inhibition. Erlotinib treatment resulted in a 54% growth inhibition at 48 hours in vehicle treated control (nonsilencing) shRNA cells ($P < 0.05$). The antiproliferative effects of erlotinib were significantly increased when added to Src shRNA cells (44.2% compared with erlotinib treated control shRNA cells, $P < 0.05$). Dasatinib treatment resulted in a 24% growth inhibition at 48 hours in vehicle treated control shRNA cells ($P < 0.01$). The antiproliferative effects of dasatinib were increased significantly when combined with EGFR shRNA cells (62.2% compared with dasatinib treated control shRNA cells, $P < 0.001$) (Fig. 2A). Similarly, Src shRNA and EGFR shRNA significantly reduced BxPC3 cell invasion when combined with erlotinib (78.2% compared with erlotinib treated control shRNA cells, $P < 0.01$) or dasatinib (67.8% compared with dasatinib...
treated control shRNA cells, \( P < 0.05 \)), respectively, when compared with control shRNA cells treated with these agents alone (Fig. 2B). These results confirm that dasatinib and erlotinib augment inhibition of cell viability and invasion through Src and EGFR signaling.

**Cooperative effect of dasatinib, erlotinib and gemcitabine on signal transduction in pancreatic cancer**

We have previously shown that Src inhibition results in decreased phosphorylation of FAK, paxillin, AKT, STAT3, ERK, JNK, and MAPK in a time and concentration-dependent manner in pancreatic cancer cell lines sensitive to dasatinib, whereas one of the mechanisms of resistance to Src inhibition appear to be related to a lack of inhibition of STAT3 signaling in resistant PANC1 cells (14). As revealed by immunoblotting of total cell lysates (Fig. 2C), at IC\(_{50}\) concentrations of dasatinib, erlotinib and gemcitabine for each cell line, there is minimal effect on cellular signaling in both BxPC3 and PANC1 cells. The combination of dasatinib and erlotinib enhances the inhibition of several signaling pathways, more so in sensitive BxPC3 cells compared with PANC1 cells. However, the addition of gemcitabine to dasatinib and erlotinib results in inhibition of pSrc, pEGFR, pFAK, pAKT, pERK, and pJNK activity in both sensitive BxPC3 and resistant PANC1 cells. More importantly, the combination of these three agents overcomes the constitutive activation of STAT3-mediating signaling seen in PANC1 cells as previously described (ref. 14; Fig. 2C).

**Combined inhibition of Src kinase and EGFR with gemcitabine attenuates pancreatic tumor growth in vivo**

BxPC3 and PANC1 tumor bearing mice were treated with dasatinib (25 mg/kg), erlotinib (50 mg/kg), and gemcitabine (20 mg/kg) either individually or in double or triple combinations. Relatively low doses of these drugs were selected to enable us to determine a cooperative effect in combination. In BxPC3 xenografts (Fig. 3A), treatment with dasatinib, erlotinib, or gemcitabine alone and with the combination of two agents inhibited tumor growth and caused some tumor regression. Compared with vehicle treated control tumors, tumor volume of BxPC3 xenografts treated with dasatinib and erlotinib treatment was decreased 79.8\% \( (P < 0.001) \), 77.3\% \( (P < 0.001) \) with dasatinib and gemcitabine treatment, and 81.1\% \( (P < 0.001) \) with gemcitabine and erlotinib treatment. Optimal tumor regression, however, was seen with the triple combination of dasatinib and erlotinib and gemcitabine (93.6\% compared with vehicle treated xenografts, \( P < 0.001 \)). The addition of dasatinib and erlotinib to gemcitabine produced minimal weight loss in BxPC3 and PANC1 tumor bearing mice, suggesting that these combinations did not produce significant in vivo toxicity (Supplementary Fig. S5). The length of time of the in vivo studies was limited due to the tumor-related morbidity of the control animals.

**Combined inhibition of Src kinase and EGFR with gemcitabine correlates with in vivo inhibition of proliferation and increased apoptosis in pancreatic tumors**

To correlate our in vitro finding of Src and EGFR inhibition with gemcitabine in vivo, immunohistochemical analysis of treated tumor xenografts of BxPC3 and PANC1 cells relative to vehicle-treated controls was measured. Tumor tissues were evaluated for Ki67, cleaved caspase 3 and phosphorylated Src and AKT immunoreactivity (Fig. 4A). Percent area of positive staining was analyzed by Image J software (Fig. 4B). BxPC3 and PANC1 tumors treated with dasatinib, erlotinib individually or in combination and with gemcitabine all showed a decrease in cell proliferation (Ki67). Compared with individual or combined targeted therapy of dasatinib and erlotinib, the addition of gemcitabine to this
combination exhibited the optimal increase in apoptosis (cleaved caspase-3) and optimal downregulation of pSrc Tyr527 and pAKT Ser473. The reduction in Ki67 staining with the combination of dasatinib, erlotinib, and gemcitabine appears to be greater in PANC1 xenografts. Caspase 3, pAKT and pSrc staining are similar in BxPC3 and PANC1 xenografts. Taken together, these results show that in pancreatic cancer, the combination of dasatinib and erlotinib with gemcitabine results in optimal in vivo effects of apoptosis and inhibited tumor growth.

**Combined inhibition of Src and EGFR with gemcitabine inhibits constitutively activated STAT3**

We have previously shown that activated STAT3 signaling is associated with resistance to Src inhibition in resistant PANC1 pancreatic cancer cells (14). As analyzed by Western blot, treatment with dasatinib, erlotinib, or gemcitabine as individual agents or in double combinations showed no decrease in pSTAT3 (both Ser727 and Tyr705) levels. Only the combination of dasatinib, erlotinib, and gemcitabine showed substantially decreased inhibition of activated STAT3, even in resistant PANC1 xenografts tissues (Fig. 5A), suggesting that combining multitargeted agents with cytotoxic agents can overcome resistance to targeted monotherapy. Both cytoplasmic and nuclear expression of phosphorylated STAT3 was seen in the control tumors and this expression was significantly decreased in tumors treated with dasatinib, erlotinib and gemcitabine (Fig. 5B).

**Discussion**

Treatment for pancreatic cancer remains a therapeutic challenge due to the lack of any effective therapy. Despite a greater understanding of the molecular pathways involved in pancreatic tumorigenesis, the use of individual targeted agents, have failed to provide meaningful improvements in survival. We investigated a strategy of targeting multiple tumor-associated pathways, namely, activated EGFR and Src tyrosine kinase signaling, which act as complementary pathways in pancreatic cancer to promote tumor growth. Nine human pancreatic cancer cell lines were characterized for their expression of Src and EGFR, phosphorylated Src and EGFR (Fig. 1A) and sensitivity to dasatinib, erlotinib, and gemcitabine, identifying cell lines that were sensitive and resistant to these agents (Table 1). Interestingly, pancreatic cancer cell lines that were sensitive to dasatinib were also sensitive to erlotinib and gemcitabine, identifying cell lines that were sensitive and resistant to these agents (Table 1). Interestingly, pancreatic cancer cell lines that were sensitive to dasatinib were also sensitive to erlotinib and gemcitabine treatment, while cell lines that were more resistant to dasatinib also showed greater resistance to erlotinib and gemcitabine therapy, suggesting an inherent resistance to individual cytotoxic or targeted therapies.

Numerous reasons exist to expect that targeting multiple tumor-associated pathways will enhance the therapeutic effects of cancer treatment. As stated above, even though the genetic alterations associated with pancreatic cancer can be associated with a core set of 12 signaling pathways, the processes that are genetically altered vary amongst each individual patient (2). In addition, the interplay between
tumor cells and surrounding supportive cells such as vascular endothelial cells and pericytes, fibroblasts and immune cells adds to the complexity of altered cellular signaling to stimulate tumor growth, clearly suggesting that targeting a single component will not result in sustained inhibition of tumor growth. Moreover, even with the use of cytotoxic or targeted therapies, parallel and reciprocal signaling pathway activation can promote resistant cell clones to continue to expand, resulting in refractory tumor response to a given agent that affects a single mechanism of action. Therefore, targeting multiple signaling pathways involved in tumor growth has the potential to overcome either primary or acquired resistance to targeted monotherapy and increase the likelihood of sustained response by affecting different mechanisms of action associated with cancer development and enhance the effects of conventional cytotoxic chemotherapy. Our results clearly indicate that combined inhibition of Src and EGFR signaling results in improved inhibition of pancreatic tumorigenesis and potentiates the effect of gemcitabine chemotherapy, the addition of which further enhances the antitumor effects of EGFR and Src inhibition.

Resistance to cytotoxic chemotherapy has remained a major factor in the poor outcomes seen in patients with pancreatic cancer. Inhibition of Src signaling has been shown to restore sensitivity to gemcitabine and 5-flourouracil in human pancreatic cancer cells (19, 25, 26). Similarly, silencing of FAK expression in PANC1 cells restores sensitivity to gemcitabine treatment (27). In addition, Src inhibition has also been shown to increase sensitivity to other cytotoxic agents such as oxaliplatin in colon cancer (28). This implicates Src inhibition in restoring chemosensitivity and suggests a novel approach using dasatinib in combination with gemcitabine for the treatment of pancreatic cancer. However, we show that at IC50 doses of dasatinib, erlotinib, and gemcitabine as individual agents or as double combinations of agents have minimal effects on inhibiting signaling of multiple tumor-associated pathways in both BxPC3 and PANC1 cells, whereas only the triple combination dasatinib and erlotinib with gemcitabine resulted in cooperative inhibition of these pathways (Fig. 2C). These results support the idea that modulation of chemoresistance may require the inhibition of both Src and EGFR signaling, requiring targeting of different pathways at multiple levels.

The heterogeneity of genetic alterations associated with pancreatic cancer, as with most solid tumors for that matter, makes it highly unlikely that a "one size fits all" approach will be effective and the need to identify biological markers that can predict treatment response (or lack of response) will be essential to optimize targeted treatment strategies. We have previously shown that activated STAT3 is a biomarker of resistance to Src inhibition with dasatinib in resistant PANC1 cells (14). STAT3 is activated by a variety of growth factors receptors such as EGFR via non-receptor tyrosine kinases such as Src family kinases (SFKs; ref. 29). STAT3 activation regulates oncogenic signaling in many tumor types and leads to increased cell survival, proliferation and tumor growth (30). Constitutively active STAT3 is sensitive to both EGFR and Src inhibition (31). However, despite early suppression of aberrant STAT3 signaling, sustained EGFR and Src inhibition can result in Janus kinase (JAK)-mediated reciprocal activation of STAT3 signaling (30). This type of feedback and parallel signaling limits the effectiveness of individual targeted therapies as seen in our results in which dasatinib or erlotinib, either individually or in combination did not result in inhibition of activated STAT3 signaling. Only the combination of dasatinib and erlotinib with gemcitabine overcame STAT3-mediated resistance of EGFR and Src inhibition both in vitro and in vivo. These results implicate treatment strategies not only attacking multiple targets, but combining these multitargeted approaches with cytotoxic chemotherapies.

Figure 5. Effect of Src and EGFR inhibition with gemcitabine on constitutively activated STAT3. (A) BxPC3 and PANC1 tumor tissue lysates were analyzed for pSTAT3 (Ser727 and Tyr705) and total STAT3. Only the combined treatment of dasatinib and erlotinib with gemcitabine inhibits constitutively activated STAT3 in both BxPC3 and PANC1 tumor xenografts in vivo. (B) Representative examples of immunohistochemical analysis of BxPC3 and PANC1 tumor tissues of vehicle (control) and dasatinib (DTB)þ-erlotinib (ERL)þ-gemcitabine (GEM) treated, and were stained for pSTAT3 (positive staining in brown is indicated by arrows).
Although our results show that this triple combination of agents optimizes pancreatic cancer therapy, the addition of multiple targeted or cytotoxic drugs can also add potentially toxicity when administered to patients in combination. This emphasizes the importance of identifying molecular markers of resistance, such as activated STAT3, to tailor therapies targeting specific resistant pathways. We confirmed the role of activated STAT3 as a biomarker of resistance to Src inhibition.

Our results clearly suggest that the combination of dasatinib and erlotinib with gemcitabine may be a potent treatment regimen for pancreatic cancer and overcomes STAT3 mediated resistance of inhibition of pancreatic tumorigenesis. Taken together, our findings provide compelling evidence establishing the role of combined targeted therapy with cytotoxic chemotherapy as a paradigm to overcome resistance associated with reciprocal and parallel signaling seen with biologically targeted monotherapy. In addition, identifying biomarkers of resistance to targeted therapy, such as activated STAT3, can result in tailoring treatment to target specific resistant pathways, thereby limiting the toxicity associated with delivery of multiple agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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