ABSTRACT

Many molecular aberrations occur in pancreatic cancer. Although aberrant epidermal growth factor receptor (EGFR), Src, and signal transducer and activator of transcription 3 (Stat3) are implicated in pancreatic cancer, therapies that target only one of these entities are undermined by signaling cross-talk. In the human pancreatic cancer lines, Panc-1 and Colo-357, pY845EGFR, pY1068EGFR, pY1086EGFR, and pY1173EGFR levels and pY416c-Src are concurrently elevated with aberrantly active Stat3 in a complex signaling cross-talk. Thus, understanding the signaling integration would facilitate the design of effective multiple-targeted therapeutic modalities. In Panc-1 and Colo-357 lines, pY845EGFR, pY1068EGFR, and pY1086EGFR levels are responsive to c-Src inhibition in contrast to pY1173EGFR, which is EGFR kinase-dependent. Constitutively active Stat3 is sensitive to both EGFR and Src inhibition, but the early suppression of aberrantly active Stat3 in response to the inhibition of EGFR and Src is countered by a Janus kinase (Jak)-dependent reactivation, suggesting that Jak activity is a compensatory mechanism for Stat3 induction. The inhibition of EGFR, Src, or Stat3 alone induced weak biological responses. By contrast, the concurrent inhibition of Stat3 and EGFR or Src induced greater viability loss and apoptosis and decreased the migration/invasion of pancreatic cancer cells in vitro. Significantly, the concurrent inhibition, compared with monotargeting modality, induced stronger human pancreatic tumor growth inhibition in xenografts. We infer that the tumor growth inhibition in vivo is caused by the simultaneous suppression of the abnormal functions of Stat3 and EGFR or Src. These studies strongly suggest that the concurrent targeting of Stat3 and EGFR or Src could be a beneficial therapeutic approach for pancreatic cancer.
mic transcription factors. As with the other STATs, Stat3 requires extrinsic tyrosine phosphorylation to become activated, and this event is induced by growth factor receptors and cytoplasmic tyrosine kinases, such as Src and Janus kinase (Jaks) families (Darnell, 2005). In contrast to normal STAT signaling that is transient in accordance with the requirements for normal biological processes, tumor cells harbor aberrant Stat3 activation, which compelling evidence indicates dysregulates cell growth and survival, promotes tumor angiogenesis and tumor cell migration and invasion, and induces tumor immune tolerance (Turkson, 2004; Yue and Turkson, 2009).

The concurrence of the hyperactive EGFR and Src tyrosine kinases, together with aberrant Stat3, in pancreatic cancer raises key questions about the contributory role of each entity to the disease. Deregulated signal transduction provides the framework for signaling cross-talk and functional cooperation that would not only support the malignant phenotype and the disease progression but also would influence drug responsiveness. Thus, a potential collaboration among hyperactive EGFR, Src, and Stat3 in support of the malignant phenotype and in regulating the response to monotherapeutic therapy is a reasonable model to propose. It is also a concept that would support the recent approval of the combined gemcitabine and EGFR inhibitor erlotinib for the treatment of pancreatic cancer patients (Saif, 2008). An increased understanding of the integration, functional relationship, and the collective roles of the EGFR, Src, and Stat3 in supporting pancreatic cancer is needed to derive effective, multiple-targeted therapeutic modalities for this disease. We provide evidence that the concurrent inhibition of aberrant Stat3 and EGFR or Src is effective in inducing antitumor cell responses in vitro and in inhibiting human pancreatic tumor growth in xenograft models.

Materials and Methods

Cells and Reagents. v-Src-transformed mouse fibroblasts (NH3T3/v-Src), human pancreatic cancer (Panc-1), and leukemic (K562) lines have been described (Turkson et al., 1998; Garcia et al., 2001; Huang et al., 2002). The human pancreatic cancer lines Colo-357 and Mia-PaCa-2 were gifts from Drs. J. M. Lancaster and M. P. Malafa (Moffitt Cancer Center, Tampa, FL). The immortalized human pancreatic duct epithelial cell (HPDEC) line was obtained from Dr. M. S. Teas (Ontario Cancer Institute, University Health Network/Princess Margaret Hospital, Toronto, ON, Canada) (Ouyang et al., 2000). Except for the HPDEC line that was grown in keratinocytes/serum-free medium supplemented with 0.2 ng of EGF, 30 μg/ml bovine pituitary extract and containing antimycol, and the K562 line cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin, all other cell lines were grown in Dulbecco’s modified Eagle’s medium containing 5% iron-supplemented bovine calf serum and 100 units/ml penicillin/streptomycin. Recombinant human EGF is from Bioxgen (Port Jefferson Station, NY), and gemcitabine is from Ely Lilly & Co. (Indianapolis, IN). Erlotinib, ZD1839 (ZD/Iressa), and dasatinib (Das) were purchased from ChemieTek (Indianapolis, IN).

Nuclear Extract Preparation and Gel-Shift Assays. Nuclear extract preparation and DNA binding with electrophoretic mobility-shift assay (EMSA) were carried out as reported previously (Turkson et al., 1998; Garcia et al., 2001). The 32P-labeled oligonucleotide probes used were high affinity sis-inducible element from the c-fos gene (m67 variant, 5’-AGCTTCATTTCCCTGA-ATCCCTA) that binds Stat1 and Stat3 (Wagner et al., 1990) and the mammary gland factor element (from the bovine β-casein gene promoter, 5’-AGATTCTAGGATTCGAA-3’) that binds Stat1 and Stat5 (Gouilleux et al., 1995; Seidel et al., 1995).

SDS-Polyacrylamide Gel Electrophoresis/Western Blot Analysis. Western blot analysis was performed as described previously (Turkson et al., 1998; Zhang et al., 2000). Primary antibodies used were anti-Stat3 (C20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-pY416Src (Millipore Corporation, Billerica, MA), and antibodies against pY705Stat3, Stat3, pY1068EGFR, pY1086EGFR, pY1175EGFR, EGFR, pY416Src, Src, c-Myc, and β-actin (Cell Signaling Technology Inc., Danvers, MA).

Small-Interfering RNA Transfection. Small-interfering RNA (siRNA) for EGFR and Src were ordered from Dharmacon RNAi Technologies, Thermo Scientific (Lafayette, CO). Sequences used were: EGFR sense strand, 5’-GAAGGGAAACUGAAUUCUAAUU-3’; EGFR antisense strand, 5’-pUUUGAАACAGUUUСССUCUU-3’; control siRNA sense strand, 5’-AGAUAУААААСГСГААГАУУ-3’; and control siRNA antisense strand, 5’-pUCUUАААСCUU-3’. The c-Src SMARTpool siRNA reagent (NM-005417; Millipore Corporation) was used for Src. Transfection into cells was performed using 20 nM EGFR siRNA or 25 nM Src siRNA and 8 μl of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in OPTI-MEM culture medium (Invitrogen).

Cell Proliferation Assay and Propidium Iodide Staining or Annexin V Binding with Flow Cytometry. Proliferating cells in 6- or 96-well plates were treated once with 0.1 to 1 μM Iressa, 100 nM Das, 50 to 100 μM SSI-201, 1 μM gemcitabine, or combinations of inhibitors for up to 96 h. Viable cells were counted by trypan blue exclusion/phase-contrast microscopy or assessed by the CyQuant cell proliferation assay according to the manufacturer’s instructions (Invitrogen), or cells were stained with propidium iodide for 30 min or subjected to annexin V binding (BD Biosciences, San Jose, CA) and analyzed by flow cytometry for cell cycle distribution or apoptosis, respectively.

Colony Survival Assay. Single-cell suspension of Panc-1 and Colo-357 cells were seeded in 6-cm dishes (500 cells/dish) and assayed as reported previously (Zhao et al., 2008). Briefly, single-cell suspension of Panc-1 and Colo-357 cells were seeded in 6-cm dishes (500 cells/well), treated the next day with inhibitors for 48 h, and allowed to grow until large colonies were visible. Colonies were stained with crystal violet for 4 h and counted under a phase-contrast microscope.

Cell Migration and Matrigel Invasion Assays. Cell migration and invasion experiments were carried out and quantified as described previously (Siddique et al., 2007b) using Bio-Coat migration chambers (BD Biosciences, Franklin Lakes, NJ) of 24-well companion plates with cell culture inserts containing 8-μm pore size filters, according to the manufacturer’s protocol.

Mice and in Vivo Tumor Studies. Studies with mice were performed under approved Institutional Animal Care and Use Committee procedures and guidelines. Six-week-old female athymic nude mice were purchased from Harlan (Indianapolis, IN) and maintained in the institutional animal facilities approved by the American Association of Accreditation of Laboratory Animal Care. Athymic mice were inoculated (subcutaneously) in the left flank area with Colo-357 cells (3×10⁶) in 100 μl of 1× phosphate-buffered saline or Panc-1 cells (8×10⁶) in 100 μl of 1:1 (v/v) DMEM/Matrigel (Basement Membrane Matrix; BD Biosciences) suspension. Animals were monitored daily after inoculation. For Colo-357 cells, tumors of 60 mm³ were established after 4 to 6 days, whereas tumors developed in Panc-1 cells took 10 to 15 days to reach 100 mm³. Animals were grouped so that mean tumor sizes in all the groups were nearly identical. For the xenografts developed with the Colo-357 line, tumor-bearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), SSI-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days for the first 2 weeks and daily for 5 days each week for the next 3 weeks. Mice bearing tumors developed using Panc-1 cells were given ZD (75 mg/kg i.v.) alone or in combination every 2 or 3 days for the first 2 weeks and daily for 5 days each week for the next 3 weeks.
mg/kg i.v.), Das (15 mg/kg i.v.), or S3I-201 (5 mg/kg i.v.) alone or in combination every 3 to 5 days, or erlotinib (5 mg/kg i.v., every day) in combination with gemcitabine (100 mg/kg i.v., every day) for 48 days. The doses and the treatment schedules used were inferred from literature reports. Tumor sizes were measured by calipers every 2 or 3 days and converted to tumor volumes by the formula \( V = \frac{a^2 \times b}{b^2} \), where \( a \) is the smallest superficial diameter and \( b \) is the largest superficial diameter. In addition, at the conclusion of the studies, weights of mice were recorded. For each treatment group, the tumor volumes for each set of measurements were statistically analyzed and compared with the control (nontreated) group.

**Statistical and Data Analyses.** Statistical analysis was performed on mean values using Prism software (GraphPad Software, Inc., San Diego, CA). Cell proliferation data and effect of drug combinations were analyzed with CalcuSyn software (Biosoft, Cambridge, UK) to determine additive or synergistic effects. The significance of differences between groups was determined by the paired \( t \) test at *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \).

**Results**

Aberrant EGFR, Src, and Stat3 in Pancreatic Cancer Lines. Consistent with published reports (DeArmond et al., 2003; Scholz et al., 2003), Stat3 activity, per DNA binding with EMSA analysis in nuclear extract preparations, is constitutive in Panc-1 and Colo-357, low in Mia-Paca-2, and undetectable in the normal HPDEC compared with aberrant levels in NIH3T3/v-Src (Turkson et al., 1998) (Supplemental Fig. 1Ai). Per supershift analysis, the DNA–protein complex contains Stat3 (Supplemental Fig. 1Ai, lane 3). No Stat5 activity is detectable in pancreatic cancer cells (Supplemental Fig. 1Aii) compared with aberrant levels in the K562 leukemic cells (Huang et al., 2002). Similarly, immunoblotting analysis showed pY416c-Src levels are moderate in Mia-Paca-2 but elevated in Panc-1 and Colo-357 cells as reported previously (Korc et al., 1986; Trevino et al., 2006) and similar to the levels in NIH3T3/v-Src compared with low levels in HPDEC (Supplemental Fig. 1B, top). The elevated pY416Src levels parallel the enhanced levels of the Src-sensitive pY845EGFR motif (Tice et al., 1999) in Panc-1 and Colo-357 cells compared with low levels of the same in HPDEC (Supplemental Fig. 1B, bottom). The total Src or EGFR protein remained unchanged. Immunoblotting analysis further showed enhanced EGFR autophosphorylation motifs (Downward et al., 1984), pY1068EGFR (Fig. 1Ai, lanes 2 and 7), pY1086EGFR (Fig. 1Aii, lanes 2 and 7), and pY1173EGFR (Fig. 1Aii, lanes 2 and 7) in Panc-1 and Colo-357 compared with basal levels of the same in HPDEC (Fig. 1Aii–iii, lane 1).

**Functional Integration of EGFR and Src in Pancreatic Cancer Cells.** We examined the functional relationship between the activated EGFR and Src. Immunoblotting analysis showed treatment of cells with Das inhibited Src activity (pY416Src) (Nam et al., 2005) and induced an early (1 h) and a sustained (24 h) decrease in pY845EGFR levels (Fig. 1B). By contrast, no detectable changes in pY416Src and pY845EGFR levels were induced by treatment with the pan-ErbB inhibitor, PD169540 (PD169) (Mahtouk et al., 2005) (data not shown) or the selective EGFR inhibitor, ZD (Wakeling et al., 2002) (Fig. 1B). In confirmation, the siRNA knockdown of c-Src abrogated pY845EGFR levels (Fig. 1Biii, Src siRNA), whereas the EGFR knockdown by siRNA had minimal effect on pY416Src levels (Fig. 1Biii, EGFR siRNA). Scrambled siRNA (con siRNA) has no effect (Fig. 1B, ii and iii, con siRNA). Thus, elevated pY845EGFR levels in pancreatic cancer cells are sensitive to Src activity.

Immunoblot analysis further showed that the treatment of Panc-1 and Colo-357 cells with ZD decreased pY1173EGFR levels (Fig. 1Aiii, lanes 3, 4, 8, and 9) by as early as 1 h and up to 24 h, with no effect on pY1068EGFR (Fig. 1Ai, lanes 3, 4, 8, and 9) or pY1086EGFR levels (Fig. 1Aii, lanes 3, 4, 8, and 9), suggesting that the EGFR kinase is essential for the induction of pY1173EGFR but not of pY1068EGFR or pY1086EGFR. By contrast, Das treatment decreased pY1068EGFR and pY1086EGFR levels (Fig. 1A, i and ii, lanes 5, 6, 10, and 11) with minimal effect on pYEGFR1173 levels (Fig. 1Aiii, lanes 5, 6, 10, and 11). Thus, Src activity additionally promotes pY1068EGFR and pY1086EGFR in pancreatic cancer cells.

**EGFR and Src Promote Aberrant Stat3 Activation but Do Not Induce ErkMAPK or Akt Activity.** Both pY1068EGFR and pY1086EGFR levels are binding sites for Stat3 (Shao et al., 2003). Given the concurrent EGFR and Src activation in Panc-1 and Colo-357 cells, we sought to define the regulation of aberrant Stat3 activation. By in vitro DNA-binding assay with EMSA analysis of nuclear extract preparations, we observed an early repression (in the first 30 min to 1 h of treatment) of constitutively active Stat3 by the pan-ErbB inhibitor, PD169, the ErbB2-selective inhibitor, AG879 (DeArmond et al., 2003), ZD, or Das (Fig. 2 Ai, lanes 4, 5, 7, and 8, Aii, lanes 2, 4, 6, and 11, and B, 1 h), or by a
combined PD169 and Das (Fig. 2Ai, lanes 10 and 11 and Aii, lane 8). However, the Stat3 activity in Panc-1 cells consistently rebounded after 24-h treatments with Das, ZD, or PD169 (Fig. 2Ai, aii, and ii, 24 h), even though EGFR or Src activity remained inhibited (Fig. 1, 24 h). Twenty-four-hour treatment with the AG879 moderately inhibited Stat3 activity (Fig. 2Aii, lane 12), which we speculate may be because of its widespread activity as a pan-ErbB inhibitor. By contrast, treatment with the Jak inhibitor AG490 for 1 h had no effect on constitutive Stat3 activity but abolished Stat3 activity at 24 h (Fig. 2Aii, lane 12), which we speculate may be because of some degree of inhibition/activation of Stat3 activity (Fig. 2D), with no effect of scrambled siRNA (con). We confirm that treatment of Panc-1 cells with S3I-201 for 24 h inhibits aberrant Stat3 activity in a dose-dependent manner (Fig. 2Ai, lanes 13–16).

In contrast to the effects on Stat3 activity, immunoblotting analysis showed elevated pErk1/pErk2 and pAkt activities in Panc-1 and Colo-357 cells, which were not responsive to ZD or Das treatment (Supplemental Fig. 2), suggesting that aberrant EGFR and Src kinases do not promote the baseline elevated Erk and Akt activities in pancreatic cancer cells. Thus, Erk and Akt are not key mediators of EGFR and Src functions in pancreatic cancer.

Inhibition of Stat3 Sensitizes Pancreatic Cancer Cells to Effects of EGFR and Src Inhibitors. We investigated the biological and therapeutic implications of the unique inter-relationship between EGFR, Src, and Stat3 activation. Das and ZD were used at 100 nM and 0.1 to 1 nM, respectively, as in the literature reports (Mahtouk et al., 2005; Nam et al., 2005), whereas the Stat3 inhibitor S3I-201 was used at the suboptimum, 50 nM, or at the 100 nM required to inhibit Stat3 activation (Siddiquee et al., 2007a). Cell growth was measured as viable cell count by trypan blue exclusion/phase-contrast microscopy. Cells treated with 1 nM ZD, 100 nM Das, or 50 nM S3I-201 alone for 24 h showed minimal effect on growth (Fig. 3A, day 1). Treatment for 48 to 96 h with Das or S3I-201 alone appeared to slow down cell growth, whereas treatment for the same period with ZD showed minimal effect (Fig. 3A, i and ii, days 2–4). The concurrent treatment with both S3I-201 and ZD or S3I-201 and Das for 24 h seemed to decrease growth (Fig. 3A, day 1). By contrast, the concurrent treatment with both S3I-201 and ZD or S3I-201 and Das for 48 to 96 h induced larger decreases in the number of viable cells (Fig. 3A, days 2–4). These data were confirmed by the CyQuant cell proliferation assay (Invitrogen). The CyQuant assay showed that, to some extent, the 48-h treatment with each inhibitor alone decreased cell growth in a dose-dependent manner (Fig. 3B, ZD, Das, and S3I-201). However, the observed effects of any single agent were significantly weaker than the effects of the concurrent treatment with a Stat3 inhibitor and an inhibitor of EGFR or Src. Thus, treatment with 50 nM S3I-201 increased the sensitivity of Panc-1 and Colo-357 cells to ZD and Das, shifting the dose-response curves to the left (Fig. 3B, ZD + S3I-201 and Das + S3I-201; insert shows the effect of 50 nM S3I-201 alone on growth). Cotreatment with S3I-201 significantly decreased the IC50 values from 17 to 0.4 µM and 100 to 6 nM, respectively, for ZD and Das against Panc-1 growth (Fig. 3B, i and ii), and from 6.5 to 2.4 µM and 90 to 8 nM, respectively, for ZD and Das against Colo-357 growth (Fig. 3B, iv and v). Analysis, per Chou and Talalay equation, using CalcuSyn ( Biosoft) indicated synergism for the combination of S3I-201 and ZD and S3I-201 and Das, with combination indexes of <1. The CyQuant cell proliferation assay also showed that Das but not ZD increased the sensitivity of both cell lines to S3I-201, decreasing the IC50 value from 40 to 15 µM and 45 to 20 µM, respectively, for effects on Panc-1 and Colo-357 cells (Fig. 3B, iii and iv). Thus, treatment with S3I-201 sensitized pancreatic cancer cells to ZD and Das.

Given the potential that the concurrent treatment with agents slowed down growth of pancreatic cancer cells, pro-
pidium iodide staining with flow cytometry was performed for cell cycle profile analysis. Treatment of Panc-1 or Colo-357 cells with ZD, Das, or S3I-201 alone for 24 h had no significant impact on the cell cycle distribution, with nearly identical distribution at G0/G1, S, and G2/M phases as in the control, dimethyl sulfoxide-treated cells (Fig. 4A, ZD, Das, and S3I-201). Similarly, the combined treatment with S3I-201 and ZD or S3I-201 and Das for 24 h appeared to have no dramatic effect on the cell cycle distribution of Colo-357 cells (Fig. 4Ai). For Panc-1, a noticeable decrease in the population in the S phase, with no dramatic increase in G0/G1, and an increased sub-G0 population occur in response to the combined treatment with ZD and S3I-201 or Das and S3I-201 (Fig. 4Ai, ZD + S3I and Das + S3I). We deduce that the combined treatment with the inhibitors of Stat3 and Src or EGFR induces some degree of apoptosis in Panc-1 cells at 24 h, with no dramatic change in the cell cycle profile in Colo-357 cells.

Given the clinical implications of these findings, we extended these studies to examine the effect of inhibitors of the EGFR, Src, and Stat3 pathway on the sensitivity of pancreatic cancer lines to gemcitabine, the antimetabolite agent used for treating pancreatic cancer. The rationale for this study is the approval of erlotinib and gemcitabine combination therapy for locally advanced metastatic pancreatic cancer. CyQuant cell proliferation studies showed that inhibition of EGFR, Src, or Stat3 alone did induce some sensitization of Panc-1 and Colo-357 cells to gemcitabine (Fig. 4B). More importantly, the combined inhibition of Stat3 and EGFR or Src induced a higher sensitization of cells to gemcitabine (Fig. 4B), further supporting the ability to substantially suppress the malignant phenotype by the combined inhibition of Stat3 and EGFR or Src.

To further explore the sensitization potential of Stat3 inhibitors, we performed colony survival assay (Zhao et al., 2008). Generally, colonies were formed by day 21 after the single, 48-h treatment, and there were no differences between control (untreated) and treated samples in the length of time to form colonies and the sizes of colonies. Panc-1 cells showed decreased sensitivity to inhibitors, and the treatment with single inhibitors had minimal effect on colony numbers compared with the combined treatment with S3I-201 (S3I) and Das or S3I-201 and ZD (Fig. 4Ci). Colo-357 cells were less sensitive to Das or ZD but showed a high sensitivity to S3I-201 and the combined S3I-201 and Das or ZD (Fig. 4Cii). Except for the Colo-357 response to S3I-201, overall the results indicate that Panc-1 and Colo-357 cells are more sensitive to the concurrent treatment with S3I-201 and Das or S3I-201 and ZD. Furthermore, annexin V binding/flow cytometric analysis showed higher percentages of Panc-1 and Colo-357 cells undergo apoptosis in response to the concurrent inhibition of Stat3 and EGFR or Src than to the treatment with any single agent alone. Treatment of Panc-1 and Colo-357 cells with S3I-201, ZD, or Das alone induced lower percentage of apoptosis (Fig. 4D, ii and iii). Panc-1 cells showed higher sensitivity to the combined treatment with S3I-201 and ZD or Das and S3I-201 and ZD induced apoptosis in both Panc-1 and Colo-357 cells at 24 h and a higher percentage of apoptosis at 48 h (Fig. 4D, ii and iii). Similar results were obtained for the concurrent treatments with AG490 and ZD or Das (Fig. 4D, ii and iii), whereas similar treatments of normal HPDEC cells showed no significant apoptosis in response to combination treatments (Fig. 4Di). Altogether, our findings indicate that pancreatic cancer cells have a higher sensitivity to the combined inhibition of Stat3 and EGFR or Stat3 and Src than to the inhibition of a single entity.

EGFR, Src, and Stat3 Together Promote Pancreatic Cancer Cell Migration and Invasion. Consistent with roles for Src and Stat3 in tumor cell motility, migration, invasion, and metastasis (Nam et al., 2005; Trevino et al., 2005;...
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Panc-1 cells, cell cycle analysis revealed some evidence of growth of Panc-1 and Colo-357 cells (Fig. 3), although for Panc-1 migration (Fig. 5A). At the 24-h treatment when invasion, except for the similar effect of Src inhibition on induced stronger effects on Colo-357 migration and Panc-1 alone suppresses migration and invasion (Fig. 5A). However, 2006), in vitro Matrigel assay showed that Das or S3I-201 alone (Fig. 5B). These data raise the potential that Stat3, responsive to the 24-h treatment with both S3I-201 and ZD and S3I-201 or Das and S3I-201 (Fig. 4Ai), which may contribute to the reduced number of migrating Panc-1 cells.

EGFR, Src, and Stat3 Module Regulates c-Myc Overexpression in Pancreatic Cancer Cells. For insight into the underlying molecular mechanisms, we studied the regulation of key cancer-relevant genes and observed that c-Myc is overexpressed in pancreatic cancer lines compared with normal HPDEC, and its expression was consistently more responsive to the 24-h treatment with both S3I-201 and ZD or S3I-201 and Das than the inhibition of Stat3, Src, or EGFR alone (Fig. 5B). These data raise the potential that Stat3, EGFR, and Src functionally cooperate to induce c-Myc overexpression in pancreatic cancer cells.

**Concurrent Inhibition of Stat3 and EGFR or Src Inhibits Human Pancreatic Tumor Growth in Xenografts.** Subcutaneous xenografts of Colo-357, a metastatic pancreatic adenocarcinoma line, and the epithelioid carcinoma, Panc-1, line were used to study the therapeutic implication of the Stat3, EGFR, and Src inter-relationships and evaluate the in vivo antitumor effects of concurrent inhibition of Stat3 and EGFR or Src. Data showed that, in general, xenografts of Colo-357 cells showed low responsiveness to treatment with inhibitor of EGFR, Src, or Stat3 alone, although as the therapy progressed, tumors treated with only one inhibitor alone appeared to show reduced growth, which was statistically not significant from control, nontreated tumors (Fig. 6A). On the other hand, xenografts of Panc-1 cells showed greater sensitivity to the Src or the Stat3 inhibitor, which induced significant (p < 0.05) inhibition of tumor growth (Fig. 6B, Das and S3I-201). Importantly, for both Colo-357 and Panc-1 lines, xenograft tumors were much more sensitive to the combined inhibition of Stat3 and EGFR or
Stat3 and Src than to the inhibition of either one alone, with tumors consistently showing reduced growth and smaller sizes in response to the combined treatment throughout the entire study (Fig. 6, ZD + S3I-201 and Das + S3I-201). Results also showed that xenograft tumors of Panc-1 showed increased sensitivity to the concurrent treatment with the approved combination therapy, gemcitabine and erlotinib, similarly to the treatment with Das and S3I-201 (Fig. 6B, gemcitabine + erlotinib compared with Das + S3I-201). Studies also showed that xenografts of Panc-1 were more sensitive to drug combinations than xenografts derived from Colo-357 cells (Fig. 6B, compare with Fig. 6A), which is consistent with the in vitro cell growth/viability and apoptosis data (Figs. 3 and 4D). Thus, significant differences ($p < 0.05$) between the tumor volumes (sizes) for Colo-357 tumors in mice treated with the combination inhibitors and the control tumors occurred at day 20 and onward posttreatment, compared with the significant differences that occurred at day 10 posttreatment between the tumor volumes for xenografts of Panc-1 that were treated with the combination of Das and S3I-201 or gemcitabine and erlotinib and the tumor volumes for control tumors (Fig. 6). The in vivo antitumor effects of the combination treatment with S3I-201 and Das or S3I-201 and ZD are consistent with the in vitro antitumor cell data and together indicate that aberrant Stat3 cooperates with hyperactive EGFR or Src to sustain human pancreatic cancer.

**Fig. 5.** Concurrent inhibition of Stat3 and EGFR or Src inhibits migration and invasion and suppresses c-Myc expression. A, effects of 24-h treatment with ZD, Das, and/or S3I-201 (S3I) on migration and invasion. B, immunoblotting analysis of whole-cell lysates for c-Myc and β-actin expression in Panc-1 cells after treatment with inhibitors for 24 h. Values, mean and S.D. $n = 3$ or 4 experiments each in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

**Fig. 6.** In vivo study of growth inhibition of human pancreatic cancer xenografts. Human pancreatic Colo-357 tumor-bearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), and S3I-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days for the first 2 weeks and then daily for 5 days each week for the next 3 weeks (A), or Panc-1 tumor-bearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), and S3I-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days, or given erlotinib (5 mg/kg i.v., every day) in combination with gemcitabine (100 mg/kg i.v., every 3 days) for 48 days (B). Tumor sizes, measured at 2- to 5-day intervals, were converted to tumor volumes and plotted against days. Values, mean and S.E.M. $n = 7$ or 8 mice per group. *, $p < 0.05$; **, $p < 0.001$. 

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For detailed analysis and interpretation of the graphs and figures, please refer to the original paper as the visual aids are not fully described in this text.
cancer. There were no obvious signs of toxicity of any of the drugs or drug combinations, and the average weights of mice for control and treated mice were 23 to 26 mg at the end of the studies.

Discussion

Pancreatic cancer is a lethal disease that has remained a challenge to treatments. The lack of understanding of the disease has also limited the chances of designing effective therapeutic modalities. We present evidence from in vitro and in vivo studies that the multiple targeting of Stat3 and EGFR or Stat3 and Src has the potential to induce strong antitumor responses in pancreatic cancer. Our study shows that the concurrent treatment with the Stat3 inhibitor, S3I-201, and the EGFR inhibitor, ZD, or S3I-201 and the Src inhibitor, Das, induced an enhanced tumor growth inhibition, with reduced tumor sizes in xenograft models developed from the metastatic pancreatic cancer line, Colo-357, and the epithelioid carcinoma line, Panc-1. The robust antitumor effects of the combination therapy contrast the weaker antitumor responses to the inhibition of EGFR, Src, or Stat3 alone in the xenografts of Colo-357 and Panc-1, suggesting the monotargeting of EGFR, Src, or Stat3 alone might not be sufficient to suppress the malignant phenotype and induce significant antitumor effects. These findings are generally consistent with the reports of dismal responses in molecular targeted monotherapy, such as the anti-EGFR monotherapy (Philip, 2008; Saif, 2008). Although the combined therapy of gemcitabine and the EGFR inhibitor, erlotinib, for locally advanced/metastatic pancreatic cancer (Senderowicz et al., 2007; Burris and Rocha-Lima, 2008) may be justified by the same argument as the multiple-targeted therapy in our study, it is not without toxicities (Saif, 2008). In our study, the treatment with Das and S3I-201 showed a similar antitumor efficacy. Given that the Das and S3I-201 combination therapy is based on molecular targeting and is mechanism-based, this therapeutic modality presents advantages over the approved gemoetabine and erlotinib combination. We also note from our in vitro study that the concurrent inhibition of Stat3 and EGFR or Src promotes a higher sensitization of pancreatic cancer cells to gemoetabine than the inhibition of EGFR or Src alone. The present in vitro data also provide evidence that the combined inhibition of Stat3 and Src or EGFR induces stronger cell growth inhibition and apoptosis, mechanisms that would contribute to the decreased tumor growth in vivo. Molecularly, the enhanced antitumor effects of the concurrent inhibition of Stat3 and EGFR or Src against pancreatic cancer could in part be the result of the increased potential to down-regulate critical tumor-relevant genes. Altogether, the present study shows the in vivo efficacy of the concurrent targeting of aberrant Stat3 and EGFR or Src and provides support for the use of this multiple-targeting modality as a therapeutic approach for pancreatic cancer.

In vitro studies provide the biochemical and biological insights into the enhanced antitumor effects in vivo of the multiple targeting of Stat3 and EGFR or Src in pancreatic cancer. Our study provides strong evidence for a complex signaling cross-talk and a functional cooperation (Fig. 7), which would undercut the biological response of targeting only the EGFR, Src, or Stat3 protein. In the context of this signal integration (Fig. 7), data presented reveal a strong role for Src in increasing the aberrant EGFR activity in pancreatic cancer cells by promoting the phosphorylation of multiple tyrosine residues, including the Y1068EGFR and Y1086EGFR motifs. The induction of aberrant Stat3 activation is promoted by both hyperactive EGFR and Src activities. In this context, the induction of pY1068EGFR and pY1086EGFR by Src would serve to further promote Stat3 activation, given that those two EGFR tyrosine motifs are essential sites for the binding and the activation of Stat3 (Shao et al., 2003). We also note that apart from the baseline, constitutive Stat3 activation in pancreatic cancer cells, which is caused primarily by EGFR and Src, there is a contributory role for Jak tyrosine kinases in the induction of Stat3 activation (Fig. 7). However, Jak3 are unlikely to be the predominant mediators of the baseline, constitutive Stat3 activity, given that their inhibition by AG490 did not abolish aberrant Stat3 activation at the earliest time point, whereas the EGFR or Src inhibition completely attenuated the baseline, aberrant Stat3 activity. Instead, Jak3 could provide a secondary, compensatory mechanism for Stat3 activation, as a part of a two-phase model for the induction of Stat3 activity in pancreatic cancer cells. A similar secondary induction of Stat3 activation via Jak activities has previously been observed in the head and neck squamous cell carcinoma line (Johnson et al., 2007) and is suggested to be caused by the growth-stimulatory factors that are released from tumor cells (Salomon et al., 1995), which in turn induce Jak3 activity and thereby promote Stat3 activation. In this complex signaling structure (Fig. 7), the inhibition of EGFR or Src alone is unlikely to abolish the functional effects of abnormal Stat3 signaling. Instead, combining the direct inhibition of aberrant Stat3 with the EGFR or Src inhibition serves to provide the
framework for substantially thwarting the oncogenic events with measureable beneficial outcome. Our data would suggest that the inhibition of Stat3 enhances the sensitivity of pancreatic cancer cells to the antitumor effects of ZD and Das.

Additional in vitro biological data further indicate a collective role of EGFR, Src, and Stat3 in supporting pancreatic cancer. Although the inhibition of the activity of each of the three proteins induced some degree of antitumor cell response in vitro, albeit minimal, data are convincing that the simultaneous targeting of Stat3 and EGFR or Src has a higher potential to be more effective in inducing diverse antitumor cell effects against pancreatic cancer cells than the inhibition of EGFR or Src alone. The hyperactivation of EGFR signaling has been deemed a prognostic indicator of low survival among pancreatic cancer patients (Dong et al., 1998; Ueda et al., 2004). In addition, there is evidence to indicate the concurrence of hyperactive Src signaling potentiates the effects of aberrant EGFR (Tice et al., 1999). Such increase of effect or the cooperation of activities and/or functions between the critical molecular entities that supports the pancreatic cancer phenotype can undermine the efficacy of a targeted monotherapy in pancreatic cancer. Indeed, the present data that the simultaneous inhibition of Stat3 and EGFR or Src induced greater antitumor cell effects and a higher sensitization to gemcitabine strongly supports this anticipation. We infer from our study that the more robust tumor growth inhibition in vivo by simultaneous inhibition of Stat3 and EGFR or Src is the result of the concomitant suppression of the abnormal functions of both Stat3 and EGFR or Stat3 and Src, suggesting that the concurrent targeting of Stat3 and EGFR or Stat3 and Src could be a more effective, multitargeted therapeutic modality for pancreatic cancer than the inhibition of EGFR, Src, or Stat3 alone.

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References


DeArmond D, Brattain MG, Jessup JM, Kreisberg J, Malik S, Zhao S, and Freeman JW (2004) Mutations in the human amphiregulin gene increase the potential to be more effective in inducing diverse antitumor cell effects against pancreatic cancer cells than the inhibition of EGFR or Src alone. The hyperactivation of EGFR signaling has been deemed a prognostic indicator of low survival among pancreatic cancer patients (Dong et al., 1998; Ueda et al., 2004). In addition, there is evidence to indicate the concurrence of hyperactive Src signaling potentiates the effects of aberrant EGFR (Tice et al., 1999). Such increase of effect or the cooperation of activities and/or functions between the critical molecular entities that supports the pancreatic cancer phenotype can undermine the efficacy of a targeted monotherapy in pancreatic cancer. Indeed, the present data that the simultaneous inhibition of Stat3 and EGFR or Src induced greater antitumor cell effects and a higher sensitization to gemcitabine strongly supports this anticipation. We infer from our study that the more robust tumor growth inhibition in vivo by simultaneous inhibition of Stat3 and EGFR or Src is the result of the concomitant suppression of the abnormal functions of both Stat3 and EGFR or Stat3 and Src, suggesting that the concurrent targeting of Stat3 and EGFR or Stat3 and Src could be a more effective, multitargeted therapeutic modality for pancreatic cancer than the inhibition of EGFR, Src, or Stat3 alone.