Reciprocal Regulation of c-Src and STAT3 in Non-Small Cell Lung Cancer

Lauren Averett Byers,1,2 Banibrata Sen,2 Babita Saigal,2 Lixia Diao,3 Jing Wang,3 Meera Nanjundan,7 Tina Cascone,2 Gordon B. Mills,4,6 John V. Heymach,2,5 and Faye M. Johnson2,6

Abstract

Purpose: Signal transducer and activator of transcription-3 (STAT3) is downstream of growth factor and cytokine receptors, and regulates key oncogenic pathways in non-small cell lung cancer (NSCLC). Activation of STAT3 by cellular Src (c-Src) promotes tumor progression. We hypothesized that c-Src inhibition could activate STAT3 by inducing a homeostatic feedback loop, contributing to c-Src inhibitor resistance.

Experimental Design: The effects of c-Src inhibition on total and phosphorylated STAT3 were measured in NSCLC cell lines and in murine xenograft models by Western blotting. c-Src and STAT3 activity as indicated by phosphorylation was determined in 46 human tumors and paired normal lung by reverse phase protein array. Modulation of dasatinib (c-Src inhibitor) cytotoxicity by STAT3 knockdown was measured by MTT, cell cycle, and apoptosis assays.

Results: Depletion of c-Src by small interfering RNA or sustained inhibition by dasatinib increased pSTAT3, which could be blocked by inhibition of JAK. Similarly, in vivo pSTAT3 levels initially decreased but were strongly induced after sustained dasatinib treatment. In human tumors, phosphorylation of the autoinhibitory site of c-Src (Y527) correlated with STAT3 phosphorylation \((r = 0.64; P = 2.5 \times 10^{-6})\). STAT3 knockdown enhanced the cytotoxicity of dasatinib.

Conclusions: c-Src inhibition leads to JAK-dependent STAT3 activation in vitro and in vivo. STAT3 knockdown enhances the cytotoxicity of dasatinib, suggesting a compensatory pathway that allows NSCLC survival. Data from human tumors showed a reciprocal regulation of c-Src and STAT3 activation, suggesting that this compensatory pathway functions in human NSCLC. These results provide a rationale for combining c-Src and STAT3 inhibition to improve clinical responses. (Clin Cancer Res 2009;15(22):6852–61)

Lung cancer accounts for 29% of all cancer deaths in the United States, with a 5-year overall survival rate of 15% for all stages (1). Although chemotherapy remains the standard treatment for advanced or metastatic non–small cell lung cancer (NSCLC), response rates do not exceed 35% with frontline therapies and are even lower in the second-line setting (2). Improving our understanding of the signaling pathways that drive tumor behavior is essential for improving clinical outcomes.

One potential therapeutic target in NSCLC for which clinical inhibitors have been developed is cellular Src (c-Src; ref. 3). The Src family consists of nonreceptor tyrosine kinases involved in signal transduction in both normal and cancer cells (4). c-Src is the best characterized and most often involved in cancer progression. c-Src overexpression has been shown in multiple tumor types, in which its activation correlates with shorter survival (reviewed in ref. 3). In NSCLC, c-Src is expressed and...
Translational Relevance

Cellular Src (c-Src) inhibitors represent an exciting new class of targeted drugs that have shown clinical activity in several disease types. However, despite the fact that c-Src overexpression and activation are associated with worse prognosis in non–small cell lung cancer (NSCLC) and c-Src inhibition leads to a universal and profound inhibition of NSCLC invasion, the cytotoxic effects of c-Src inhibition are variable. Understanding the mechanisms of resistance to these drugs is of critical importance so that they can be used most effectively. In these studies, we show that sustained c-Src inhibition in NSCLC leads to phosphorylation of its downstream target STAT3 and that knockdown of STAT3 increases cytotoxicity induced by c-Src inhibition. These findings support assessment of combinations of drugs that inhibit STAT3, such as JAK kinase inhibitors, with c-Src inhibitors in NSCLC.

activated in both adenocarcinomas and squamous cell carcinomas (5, 6).

c-Src participates in several normal cellular functions during development and adulthood, including cell cycle progression, immune recognition, adhesion, spreading, migration, apoptosis regulation, and differentiation (reviewed in refs. 3, 7). In cancer cells, constitutive activation of c-Src disregulates many of these processes. Inhibition of c-Src activity using both molecular approaches and pharmacologic inhibitors in multiple cancer cell types has been found to lead to reduced anchorage-independent growth (8), decreased proliferation (9), cell cycle arrest (10), decreased tumor growth in vivo (11, 12), apoptosis (9), increased susceptibility to anoikis (13), diminished in vitro invasion and migration (14, 15), decreased in vivo metastasis (12, 16), and decreased in vivo vascularity (17). In NSCLC specifically, c-Src inhibition leads to decreased hypoxia-induced vascular endothelial growth factor expression (18). Inhibition of c-Src with a pharmacologic inhibitor (dasatinib) leads to profound and universal in vitro inhibition of migration and invasion of NSCLC cells. However, its effect on viability and proliferation is more variable and occurs at concentrations of dasatinib that are near or above the peak plasma concentrations possible in humans (14).

c-Src has multiple downstream substrates that mediate its biologic functions in cancer cells. The interaction between c-Src and its substrate focal adhesion kinase (FAK) is essential for normal cell migration and invasion (19). c-Src also regulates downstream proliferation induced by growth factor receptors. Following activation by growth factor receptors, c-Src promotes survival via phosphorylation of the p85 subunit of phosphatidylinositol 3 kinase (PI3K) and thus the AKT pathway, signal transducer and activator of transcription-3 (STAT3), STAT5, and Shc, and thus the Ras/mitogen-activated protein kinase pathway (13, 20, 21). The STAT family of transcription factors, especially STAT3, regulates oncogenic signaling in many different tumor types (22). Indeed STAT3 is required for viral Src-mediated transformation (23). STAT3 can be activated by growth factor receptors or cytokine receptors, usually via non-

receptor tyrosine kinases such as c-Src or janus-activated kinase (JAK) proteins. STAT3 activation leads to the increased expression of downstream targets (e.g., Bcl-XL, cyclin D1, survivin) and increased cell survival, proliferation, and tumor growth in vivo (24). Inhibition of STAT3 results in increased apoptosis, decreased proliferation, and decreased tumor size (25, 26). STAT3 activation can also contribute to angiogenesis (27). Hypoxia-induced vascular endothelial growth factor expression is dependent on c-Src activation; this activation of c-Src leads to the downstream activation of STAT3, which binds to the vascular endothelial growth factor promoter with hypoxia-inducible factor-1α (HIF-1α).

Although targeting growth factors and signal transduction pathways is a successful strategy in several tumor types, feedback and parallel signaling pathways can limit the efficacy of this approach. Despite c-Src expression in epithelial tumors, including NSCLC, and robust inhibition of c-Src with clinically relevant agents (e.g., dasatinib), the effect of c-Src inhibition on cell survival and proliferation has been modest (14). Defining mechanisms that limit the cytotoxic effects of c-Src inhibitors may result in the development of combinations of therapeutic agents for NSCLC that inhibit metastasis and enhance cytotoxicity. Because c-Src mediates its effects on cancer cell survival and proliferation via diverse substrates, including STATs, in this study we tested our hypothesis that STAT3 may not be inhibited sufficiently by c-Src inhibition in NSCLC to result in clinical effects. We determined that STAT3 was not durably inhibited in NSCLC cell lines and xenografts following c-Src inhibition, making it a candidate pathway for resistance to chemotherapy. Consistent with this result, we found that depletion of STAT3 enhanced the cytotoxicity of c-Src inhibition. In addition, we observed an inverse correlation between c-Src and STAT3 activation levels in untreated primary lung tumors by reverse-phase protein array. The results of these studies support a model in which c-Src and STAT3 are reciprocally regulated in NSCLC tumors, allowing for cancer cell survival following c-Src inhibition.

Materials and Methods

Pharmacologic inhibitors. Dasatinib for in vitro studies was provided by Bristol-Myers Squibb and was prepared as a 10 mmol/L stock solution in DMSO. Dasatinib for animal studies was purchased from The University of Texas M. D. Anderson Cancer Center pharmacy. Pyridone 6 was purchased from Calbiochem.

Cell line selection and culture. Human NSCLC cell lines A549 and H226 were obtained from the American Type Culture Collection. H1299, H2009, and H1792 were gifts from Dr. John Minna (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas). Cell lines with wild-type epidermal growth factor receptor (EGFR) were selected for these studies because EGFR mutations can profoundly affect the response of NSCLC to c-Src inhibition. Three KRAS mutant cell lines were included (A549, H2009, and H1792) because this mutation is common in patients with NSCLC. Cells were grown in monolayer cultures in RPMI 1640 medium containing 10% fetal bovine serum (A549, H226, H1299, and H1792) or RPMI 1640 medium supplemented with hydrocortisone, insulin, transferrin, estadiol, and selenium (HTES) containing 5% fetal bovine serum (H2009) at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Western blot analysis. Western blot analysis was done to measure protein expression and phosphorylation at 30 min and 7 h after inhibition or specific knockdown of c-Src, STAT3, and/or JAK. Protein levels in treated cells were compared with those in untreated cells at these
same times to control for the effect of confluence or vehicle effects on STAT3 activation. Antibodies used in the Western blot analysis included c-Src and pSTAT3 S727 (Santa Cruz Biotechnology); pS6 Y241, pSTAT3 Y705, STAT3, pFAK Y861, Lyn, Yes, Bcl-XL, survivin, and STAT5 (Cell Signaling Technology); and β-actin (Sigma Chemical Company).

For the Western blot analysis, cells were rinsed and lysed as previously described (28). Equal protein aliquots from cleared lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with primary antibody, and detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reagent (Amersham Biosciences).

Transfection with small interfering RNA. To knock down STAT3, the NSCLC cells were harvested, washed, and suspended at a density of 1 million cells/100 μL of Nucleofector-V solution (Amaxa Corp.). Small interfering RNA (siRNA; 200 pmol/100 μL) was added to the cell suspension and electroporated using the U-31 Nucleofector program (Amaxa). Immediately after electroporation, 500 μL of premature RPMI medium were added to the cuvette, and the cells were transferred to 6-well plates. The medium was changed after 16 h. STAT3 and c-Src siRNA were predesigned by siGenome Smartpool (Dharmacon, Inc., a part of Thermo Fisher Scientific) and obtained from Ambion. Controls included cells that were mock-transfected (i.e., without siRNA) and those transfected with a nontargeting (scrambled) siRNA.

MTT, cell cycle, and apoptosis assays. The MTT assay was used to assess cytotoxicity as previously described (28). For each cell line, eight wells were treated with 0, 1, 2, 4, or 8 μmol/L dasatinib and the IC50. Cell cycle analysis was done as described previously (29). Briefly, cells were fixed and stained with propidium iodide. DNA content was analyzed by flow cytometry (Becton Dickinson) using FlowJo software (TreeStar Software House). Apoptosis was measured using terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling staining using the manufacturer’s protocol (APO-BrdU kit, Phoenix Flow Systems) as described previously (30). Briefly, fixed cells were incubated with terminal deoxynucleotidyl transferase and Br-dUTP and subsequently incubated with a fluorescein-labeled anti-BrdU antibody and analyzed by fluorescence-activated cell sorting analysis.

Xenograft nude mouse models. All animal procedures were done in accordance with the policies of M.D. Anderson's Institutional Animal Care and Use Committee. Ten female Swiss nu/nu strain, 6-week-old mice were used for each xenograft model. Each athymic nude mouse was injected s.c. with 4 million A549 or H226. When visible tumors had developed, dasatinib was administered by oral gavage at a dose of 20 mg/kg for 5 d. The mice were euthanized 2 h after the last dose of dasatinib, tumors were dissected, and the mice were examined for distant metastases. The tumors were homogenized and subjected to Western blotting as previously described (30).

Human NSCLC Tumors. Forty-six paired normal lung and NSCLC tumor samples were obtained from surgical specimens in the M. D. Anderson Cancer Center Thoracic Tissue Bank (Table 1). Of the tumors, 22 were squamous cell carcinomas and 24 were adenocarcinomas. The median age of the patients from whom the tumors had been excised was 67 y (range, 48-81 y); 22 (48%) were women, and 44 (96%) were former or current smokers. Six (13%) patients had stage IA cancer, 14 (30%) stage IB, 2 (4%) stage IIA, 13 (28%) stage IIB, 3 (7%) stage IIIA, 6 (13%) stage IIIB, and 2 (4%) stage IV.

Reverse-phase protein array. Protein lysate was prepared from pellets from tumor tissues as previously described (31). Briefly, lysis buffer [1% Triton X-100, 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L NaPiP, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, and 10 μg/mL aprotinin] was added to the samples, followed by microcentrifugation at 14,000 rpm for 10 min. Clear supernatants were collected, followed by protein quantitation using the BCA reaction kit (Pierce Biotechnology, Inc.). The cell lysates were mixed with SDS sample buffer without bromophenol blue (three parts cell lysate plus one part 4× SDS sample buffer, which contained 35% glycerol, 8% SDS, 0.25 mol/L Tris-HCl (pH 6.8)]. Before using the cell lysate, 10% β-mercaptoethanol was added. The samples were boiled for 5 min. Then, the samples (each in duplicate) were serially diluted (1:2-1:16) with SDS sample buffer. To each of the diluted samples an equal amount of 800 μg/mL/2 PBS solution (8 mL of glycerol mixed with 2 mL of 10× PBS without Ca2+ and Mg2+ ) was added, after which the diluted samples were transferred to 384-well plates. Reverse-phase protein arrays (RPPA) were produced and analyzed as described (52), with slight modifications. Protein arrays were printed on nitrocellulose-coated glass FAST Slides (Sleicher & Schlue BioScience Inc.) by a GeneTAC G3 arrayer (Genomic Solutions) with 48 200-mm-diameter pins arranged in a 4 × 12 format. Forty-eight grids were printed at each slide with each grid containing 24 dots. Protein dots were printed in duplicate with five concentrations. Arrays were produced in batches of 15, and occasional low-quality arrays (e.g., with many spot dropouts) were discarded.

Antibody staining of each array was done using an automated BioGenex autostainer. Briefly, each array was incubated with a specific primary antibody: pS6 Y241, pSTAT3 Y705, pSrc Y416 (which binds the pSrc Y419 activation site in humans), pFAK Y576, p-p38α (Y239), and pPaxillin Y118; Cell Signaling). c-Src and STAT3 (Upstate). The signal was detected using the catalyzed signal amplification (CSA) system according to the manufacturer’s recommended procedure (DakoCyto- mation California, Inc.). In brief, the RPPA slides were blocked for endogenous peroxidase, avidin, and biotin protein activity with 1× block (Applied Biosystems) at room temperature for 15 min. After the blocking procedure, the slides were incubated with primary antibody and secondary antibody and diluted in DAKO antibody diluent with a background-reducing compound at room temperature for 20 min each. The slides were then incubated with streptavidin-biotin complex and biotinyl-tyramide (for amplification) for 15 min each, streptavidin-peroxidase for 15 min, and 3,3-diaminobenzidine tetrahydrochloride chromogen for 5 min. Between steps, each slide was washed with TBS containing 0.1% Tween-20 (TBST). Spot images were quantified using imaging analysis with an HP Scanjet 8200 scanner (Hewlett Packard) with a 256-shade gray scale at 600 dots per inch.

RPPA data processing and statistical analysis. RPPA data were quantified using a SuperCurve method which detects changes in protein level by Microvogene software (VigeneTech) and an R package developed in-house (32). Briefly, the SuperCurve method generates a common logistic curve by pooling data from all samples on the slide. Individual dilution series numbers for each sample are then mapped onto the SuperCurve for quantification. After quantification, data were logarithm-transformed (base 2) for further processing and analyses. Then median-control normalization was applied on the dataset. The statistical analyses were done using R (version 2.7.0). All samples were done in duplicate, and average values were used for analysis. Two sample t-tests were used to compare protein levels between normal and tumor tissue; Pearson correlations were used to analyze the association between protein levels in the samples. In all analyses, P ≤ 0.05 was considered significant.

Results

c-Src inhibition fails to durably inhibit STAT3 in NSCLC cell lines. Western blot analysis of five NSCLC cell lines showed that c-Src phosphorylation was rapidly (30 minutes) and durably (7 hours) inhibited at a site essential for c-Src activation (pS6 Y241 in human c-Src; Fig. 1A). Total c-Src levels were not changed by dasatinib treatment. In contrast to pSrc Y419, in which inhibition was prolonged, STAT3 activation (as shown by pSSTAT3 Y705 levels) was transiently inhibited at 30 minutes in A549 and H226 (0.60 and 0.57 times the control value, respectively), followed by reactivation by 7 hours (1.25 and 1.18 times control). In H2009, H1299, and H1792 activation was...
seen beginning at 30 minutes (range, 1.20-1.78 times control) and persisted at 7 hours (1.19-1.63 times control; Fig. 1B).

c-Src inhibition leads to initial STAT3 inhibition then reactivation in vivo. After 1, 5, or 7 hours and after 5 days of dasatinib treatment, tumors from mouse xenografts of NSCLC cells were grossly dissected and examined by immunohistochemistry. Tumors were confirmed to consist primarily of NSCLC cells (>90%) with no distant metastases (data not shown). Protein expression and phosphorylation were then measured by Western blot analysis and compared with control (vehicle-treated mice sacrificed at 7 hours or 5 days; Fig. 2). As expected, dasatinib treatment resulted in pSrc Y419 inhibition at all times. In A549 xenografts, pFAK inhibition was seen beginning at 5 hours. In contrast, but consistent with the in vitro data, pSTAT3 Y705 was inhibited at early times (0.58-fold at 5 hours after treatment) but was strongly induced by 7 hours (3.1-fold). pSTAT3 Y705 levels returned to baseline after 5 days of continuous daily treatment. Unlike the in vitro studies, the in vivo studies showed that total STAT3 levels were also elevated at 7 hours after treatment but were not significantly different from

Table 1. Clinicopathologic characteristics of patients included in RPPA analysis of NSCLC tumors

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Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma.
control levels. In H226 xenografts, pSTAT3 Y705 was inhibited between 5 and 7 hours (0.32- and 0.44-fold at 5 and 7 hours, respectively), but rose above baseline by 5 days (1.7-fold).

**c-Src depletion leads to STAT3 activation.** To determine whether STAT3 reactivation was downstream of c-Src and not caused by an off-target effect of dasatinib, we examined the effect of c-Src depletion by siRNA at 72 and 96 hours in the NSCLC cell lines A549 and H226. Cell lines were transfected with either scrambled siRNA or c-Src-specific siRNA. In A549 cells, c-Src knockdown decreased total and activated c-Src levels but increased pSTAT3 Y705 by 6.7- and 7.2-fold at 72 and 96 hours, respectively (Fig. 3). In H226 cells, total and activated c-Src levels were suppressed at both time points; pSTAT705 was strongly induced by 72 hours (3.5-fold) and returned near baseline by 96 hours (1.5-fold; Fig. 3). Other Src family members, such as Yes and Lyn, were not affected by c-Src knockdown in either cell line.

**STAT3 reactivation is JAK-dependent.** To determine whether STAT3 reactivation was mediated by JAK proteins, we tested the effect of JAK inhibition on STAT3 activation. NSCLC cell lines were treated with the JAK inhibitor pyridone 6 alone and in combination with dasatinib (Fig. 1A). JAK inhibition alone had no effect on pSrc Y419 but led to complete inhibition of pSTAT3 Y705 and its downstream targets survivin and Bcl-XL at 7 hours. When combined with dasatinib, JAK inhibition by pyridone 6 prevented STAT3 phosphorylation.

**c-Src and STAT3 are reciprocally regulated in NSCLC patient tumors.** To evaluate the relationship between c-Src and STAT3 in clinical samples, we used reverse-phase protein array, due to its sensitivity and the small amount of protein required, to quantify the levels of total and phosphorylated Src and STAT3 as well as downstream targets of Src in paired samples of NSCLC tumors and normal lung tissue. Paired t-test of tumor and normal tissue from the same patients showed significantly higher c-Src activity in tumors as illustrated by decreased levels of auto- and inactivated c-Src (pSrc Y527; \( P = 1.09 \times 10^{-9} \)) in tumor tissue as compared with normal lung (Fig. 4A). In contrast, activated STAT3 (pSTAT3 Y705) levels were significantly lower in tumor tissue (\( P = 0.006 \); Fig. 4A).

Among the tumor specimens, there was a statistically significant correlation between levels of pSrc Y527 and pSTAT3 Y705, with a Pearson correlation coefficient of 0.32 (\( P = 0.03 \); Fig. 4B). This correlation was also seen when the ratio of pSrc Y527 to total c-Src in tumors was compared with the ratio of...
In addition, the decrease in pSrc Y527 from normal to tumor correlated directly with the decrease in pSTAT3 Y705 from normal to tumor tissue \( (r = 0.33; P = 0.03) \).

Downstream targets of Src (FAK, p130Cas, and paxillin) were also evaluated by RPPA. As expected, activation of these targets (as measured by phosphorylation) was inversely correlated with pSTAT3 Y705 and pSrc Y527. Specifically, correlation coefficients between pSTAT3 Y705 and phosphorylated FAK, p130Cas, and paxillin were \( r = -0.50 \) \((P = 0.005)\), \(-0.30 \) \((P = 0.04)\), and \(-0.42 \) \((P = 0.004)\), respectively \((Fig. 4C)\).

Despite testing several antibodies for RPPA, there is not yet a validated antibody for this assay for the activated form of c-Src \((human \text{ pSrc Y419})\). However, using the best performing antibody available, we observed a 1.6-fold higher level of pSrc Y419 in tumors compared with normal tissue \((P = 0.06)\). The ratio of pSrc Y419 to total Src was also correlated with the ratio of pSTAT3 Y705 to total STAT3, although this was not statistically significant \((P = 0.67)\).

**Inhibition of STAT3 with siRNA enhances the cytotoxicity of dasatinib.** Because of its role in mediating survival and proliferation, STAT3 induction following c-Src inhibition may represent a mechanism of drug resistance. To examine the biological effects of STAT3 reactivation in NSCLC cells, we evaluated the effect of STAT3 knockdown on cytotoxicity when combined with dasatinib. A549 and H226 cells were transfected with STAT3 siRNA, scrambled (nontargeting) siRNA, or mock-transfected. A549 and H226 cells transfected with STAT3 siRNA showed an 87% and 79% decrease in STAT3 protein levels (respectively) at 48 hours after transfection with STAT3 siRNA as compared with scrambled siRNA \((Fig. 5A and B)\). To determine the biological effect of c-Src inhibition combined with specific depletion of STAT3, cells were treated with dasatinib 48 hours after transfection. A MTT assay was then used to estimate the number of living cells remaining after 72 hours of treatment. Cells with depleted STAT3 were significantly more sensitive to dasatinib than those transfected with scrambled siRNA. In A549 cells, the IC\(_{50}\) values were 0.7 and 4 \(\mu\)mol/L in control and STAT3 siRNA–transfected cells, respectively, for A549 \((Fig. 5C)\), and 5 and 38 \(\mu\)mol/L for H226 \((Fig. 5D)\). Unlike dasatinib alone, the combination of STAT3 knockdown with dasatinib strongly induced apoptosis in both cell lines \((Fig. 5E)\). Dasatinib alone induced cell cycle arrest in H226 cells, but this was not significantly affected by the addition of STAT3 depletion.

**Discussion**

In this study, we found that c-Src and STAT3 activation, as shown by phosphorylation status, were reciprocally regulated in NSCLC cell lines, xenografts, and human tumors. Despite an initial inhibition of STAT3 phosphorylation in the A549 and H226 models, prolonged c-Src inhibition resulted in an increase in STAT3 phosphorylation in all NSCLC cell lines tested both *in vitro* and *in vivo*. STAT3 reactivation was JAK-dependent, as illustrated by the observation that reactivation was inhibited by the addition of a JAK inhibitor, pyridone 6. Finally, we showed that inhibition of STAT3 reactivation \((by \text{STAT3} \text{siRNA})\) enhanced the cytotoxicity of dasatinib, showing that this pathway has biological significance. Taken together, these results suggest that STAT3 reactivation may be an important mechanism of resistance to c-Src inhibitors in NSCLC and may be a clinically relevant target for combination therapy.

Previous studies have shown that c-Src is overexpressed in NSCLC and that increased c-Src activity is associated with worse clinical outcome. Clinical investigators are enthusiastic about c-Src inhibitors because specific and potent kinase inhibitors are well tolerated in humans \((33)\). Two such approved anticancer drugs, imatinib and erlotinib, use ATP-competitive kinase
inhibition to inhibit Bcr-Abl (34) and EGFR (35), respectively, proving that kinase inhibition of signal transduction molecules can lead to profound tumor responses. However, thus far c-Src inhibitors have shown limited activity in NSCLC patients. Understanding the mechanisms of resistance to c-Src inhibition in NSCLC will be extremely important for understanding how these drugs can be used more effectively in this disease.

In NSCLC, resistance to tyrosine kinase inhibitors, such as those targeting EGFR, is often due to either activation of the signaling pathway downstream to the drug target (e.g., k-Ras mutations) or signaling through alternate pathways (e.g., c-Met; refs. 36, 37). Therefore, because STAT3 activity is an important downstream target of c-Src and necessary for c-Src signal transduction, characterization of its relationship to c-Src activity and response to c-Src inhibition was of particular interest. Interestingly, these studies did show reactivation of STAT3 in the setting of c-Src inhibition. In cell lines from head and neck squamous cell cancers (30), squamous cell carcinoma of the skin, and mesothelioma (38), sustained c-Src inhibition also resulted in STAT3 re-activation. This suggests that reciprocal c-Src-STAT3 regulation

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**Fig. 4.** Phosphorylated c-Src, STAT3, and downstream Src targets were measured in paired normal lung and NSCLC tumor tissues from patients who had previously undergone resection. **A,** tumors had higher mean c-Src activity, as indicated by decreased levels of inactive c-Src (pSrc Y527). Conversely, mean activated STAT3 (pSTAT3 Y705) was lower in tumor tissue than in normal lung. **B,** levels of inactive c-Src (pSrc Y527) correlated directly with activated STAT3 (pSTAT3 Y705) when analyzed as total levels of phosphorylated protein or ratio of phosphorylated protein to total protein. **C,** levels of phosphorylated FAK, p130Cas, and paxillin were inversely correlated with pSTAT3 Y705.

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^{8} Unpublished data.
exists in multiple tumor types. However, this is the first study to show reciprocal regulation of c-Src and STAT3 in patient tumors.

Three negative feedback loops regulate STAT function after cytokine signaling: SH-2–containing phosphatases, which inactivate JAK by dephosphorylation; protein inhibitors of activated STAT, which are negative regulators of STAT-induced transcription (i.e., downstream of STATs); and suppressors of cytokine signaling, which inhibit JAK activity, facilitate proteosomal degradation of JAK, and compete with STATs for binding to cytokine receptors (39). Although there are no known positive feedback loops leading to STAT3 activation after its inhibition, loss of a negative feedback loop could play the same role. For example, v-Abl leads to JAK/STAT activation via its disruption of suppressor of cytokine signaling 1 function (SOCS-1) (40).

The concentration (100 nmol/L) of dasatinib was chosen for these studies because it has been shown to completely inhibit c-Src and is relatively specific (41). For example, in intact cells, we observed ∼90% reduction in phosphorylated

![Depletion of STAT3 enhances cytotoxicity of dasatinib. A549 (A) and H226 (B) cells were transfected with STAT3-specific siRNA, scrambled siRNA, or mock-transfected. Forty-eight hours after transfection, cells were treated with various concentrations of dasatinib. The number of viable A549 (C) and H226 (D) cells after 72 h was evaluated by MTT assay. The percentage of cells in S phase and in apoptosis were then measured under the conditions shown. The control sample was compared with each treatment group and significant differences (P < 0.05) are marked with an asterisk (E).](image-url)
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c-Src with >40 nmol/L dasatinib. In patients treated with dasatinib, plasma levels of approximately 400 nmol/L were reached with sustained levels of 40 to 100 nmol/L (42, 43), supporting the clinical relevance of our selected drug concentration. However, at these concentrations dasatinib also inhibits Abl, PDGFR, Btk, and EphA2 (44, 45). To determine that STAT3 reactivation is downstream of c-Src specifically and not due to an off-target effect of dasatinib, we showed that when c-Src was depleted by siRNA, the levels of pSrc decreased significantly, but pSTAT3 levels increased.

Although we showed an inverse correlation between c-Src and STAT3 activity in human tumors, human NSCLC samples were not available from the post-dasatinib setting to confirm a reactivation of STAT3 in patients following c-Src inhibition. Nevertheless, we believe that our in vitro and murine models sufficiently support a rationale for combining c-Src and STAT inhibition in the clinical setting. Three ATP-competitive c-Src inhibitors are being studied in clinical trials: dasatinib, AZD0530 (AstraZeneca), and SKI-606 (Wyeth). A non–ATP-competitive c-Src inhibitor is also in clinical trial (Kinex pharmaceuticals). Preclinical studies of c-Src inhibitor AZD0530 also showed a reactivation of STAT3 in A549 cells 24 hours after treatment with this drug, further supporting that this may be an important mechanism of resistance across this class of drugs (46). Many JAK inhibitors are being studied in the laboratory and several are in early clinical trial (47–50). Our long-term goal is to use the results of these studies to design clinical trials of these or other more specific c-Src and JAK inhibitors, as available, to improve the survival of patients with NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Doris R. Siwak for her contribution to the RPPA experiments.

References


Reciprocal Regulation of c-Src and STAT3 in Non-Small Cell Lung Cancer

Lauren Averett Byers, Banibrata Sen, Babita Saigal, et al.


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