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Reciprocal regulation of c-Src and STAT3 in non-small cell lung cancer

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Abstract

Purpose—STAT3 (signal transducer and activator of transcription-3) is downstream of growth factor and cytokine receptors and regulates key oncogenic pathways in non-small cell lung cancer (NSCLC). Activation of STAT3 by 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 siRNA 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

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allows NSCLC survival. Data from human tumors demonstrated 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.

Keywords

c-Src; STAT3; non-small cell lung cancer; dasatinib; drug resistance

Introduction

Lung cancer accounts for 29% of all cancer deaths in the United States, with a five-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 front-line 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) (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 demonstrated in multiple tumor types, where its activation correlates with shorter survival (reviewed in (3)). In NSCLC, c-Src is expressed and 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 (3,7)). In cancer cells, constitutive activation of c-Src dysregulates 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 (VEGF) expression (18). Inhibition of c-Src with a pharmacological 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 biological 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 (GFRs). Following activation by GFRs, c-Src promotes survival via phosphorylation of the p85 subunit of phosphatidylinositol 3 kinase (PI3K) and thus the AKT pathway, STAT3 (signal transducer and activator of transcription-3), STAT5, and Shc and thus the Ras/MAPK 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 GFRs or cytokine receptors, usually via nonreceptor tyrosine kinases such as c-Src or janus-activated kinase (JAK) proteins. STAT3 activation leads to the increased expression of downstream target genes (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 VEGF expression is dependent on c-

Src activation; this activation of c-Src leads to the downstream activation of STAT3, which binds to the VEGF promoter with hypoxia-inducible factor-1 α (HIF-1 α).

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 (New York, NY) and prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Dasatinib for animal studies was purchased from The University of Texas M. D. Anderson Cancer Center pharmacy. Pyridone 6 was purchased from Calbiochem (San Diego, CA).

Cell line selection and culture

Human NSCLC cell lines A549 and H226 were obtained from the American Type Culture Collection (Manassas, VA). H1299, H2009, and H1792 were gifts from Dr. John Minna. Cell lines with wild type EGFR receptors 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) since 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 HITES medium 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 performed to measure protein expression and phosphorylation at 30 min and 7 hours after inhibition or specific knockdown of c-Src, STAT3, and/or JAK. Protein levels in treated cells were compared to 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, Santa Cruz, CA); pSrc Y419, pSTAT3 Y705, STAT3, pFAK Y861, Lyn, Yes, Bcl-XL, survivin, and STAT5 (Cell Signaling Technology, Danvers, MA); and β -actin (Sigma Chemical Company, St. Louis, MO).

For the Western blot analysis, cells were rinsed and lysed as previously described (28). Equal protein aliquots from cleared lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, immunoblotted

with primary antibody, and detected with horseradish peroxidase–conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) and ECL reagent (Amersham Biosciences, Piscataway, NJ).

Transfection with siRNA

To knock down STAT3, the NSCLC cells were harvested, washed, and suspended at a density of 1 million cells/100 μ L of Nucleofector-V solution (Amaxa Corp., Gaithersburg, MD). Small interfering RNA (siRNA) (200 pmol/100 μ L) was added to the cell suspension and electroporated using the U-31 Nucleofector program (Amaxa). Immediately after electroporation, 500 μ L of prewarmed RPMI medium was added to the cuvette, and the cells were transferred to six-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, Lafayette, IN) and obtained from Ambion (Austin, TX). 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 μ M dasatinib and the 50% inhibitory concentration (IC₅₀) calculated. Cell cycle analysis was performed as described previously (29). Briefly, cells were fixed and stained with propidium iodide. DNA content was analyzed by fluorescence-activated cell sorting analysis (FACS) (Becton Dickinson, San Jose, CA) using ModFit software (Verity Software House, New South Wales, Australia). Apoptosis was measured using terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining using the manufacturer's protocol (APO-BRDU kit; Phoenix Flow Systems, San Diego, CA) as described previously (30). Briefly, fixed cells were incubated with terminal deoxynucleotidyl transferase and Br-dUTP and subsequently incubated with a fluorescein-labeled anti-Br-dUTP antibody and analyzed by FACS.

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 weeks old mice were used for each xenograft model. Each athymic nude mice was injected subcutaneously with four million A549 or H226. When visible tumors had developed, dasatinib was administered by oral gavage at a dose of 20 mg/kg/day for 5 days. 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). 22 of the tumors were squamous cell carcinomas and 24 were adenocarcinomas. The median age of the patients from whom the tumors had been excised was 67 years (range 48–81); 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 lysate array (RPPA)

Protein lysate was prepared from pellets from tumor tissues as previously described (31). Briefly, lysis buffer (1% Triton X-100, 50 mM HEPES [pH 7.4], 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, 100 mM NaF, 10 mmol/L NaPPi, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 , and 10 $\mu\text{g/mL}$ 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., Rockford, IL). The cell lysate was mixed with SDS sample buffer without bromophenol blue (three parts cell lysate plus one part 4 $\times$ SDS sample buffer, which contained 35% glycerol, 8% SDS, 0.25 M 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 80% glycerol/2 $\times$ phosphate buffered saline (PBS) solution (8 ml of glycerol mixed with 2 ml of 10 $\times$ PBS without Ca^{2+} and Mg^{2+}) was added, after which the diluted samples were transferred to 384-well plates. RPPA was produced and analyzed as described (52), with slight modifications. Protein lysate arrays were printed on nitrocellulose-coated glass FAST Slides (Schleicher & Schuell BioScience Inc., Sanford, ME) by a GeneTAC G3 arrayer (Genomic Solutions, Ann Arbor, MI) with forty-eight 200- μm -diameter pins arranged in a 4 $\times$ 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 performed using an automated BioGenex autostainer (San Ramon, CA). Briefly, each array was incubated with a specific primary antibody: pSrc Y527, pSTAT3 Y705, pSrc Y416 (which binds the pSrc Y419 activation site in humans), pFAK Y576, p-p130Cas Y249, and pPaxillin Y118) (Cell Signaling, Danvers, MA); c-Src and STAT3 (Upstate, Lake Placid, NY). The signal was detected using the catalyzed signal amplification (CSA) system according to the manufacturer's recommended procedure (DakoCytomation California, Inc., Carpinteria, CA). In brief, the microarray slides were blocked for endogenous peroxidase, avidin, and biotin protein activity with I-block (Applied Biosystems, Foster City, CA) 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 TBST. Spot images were quantified using imaging analysis with an HP Scanjet 8200 scanner (Hewlett Packard, Palo Alto, CA) with a 256-shade gray scale at 600 dots per inch.

RPPA Data Processing and Statistical Analysis

RPPA data was quantified using a SuperCurve method which detects changes in protein level by Microvigene software (VigeneTech, Carlisle, MA) 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 was logarithm transformed (base 2) for further processing and analyses. Then median-control normalization was applied on the dataset. The statistical analyses were performed using R (version 2.7.0). All samples were done in duplicate and average values 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. P-value of ≤ 0.05 was considered significant in all analyses.

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 min) and durably (7 h) inhibited at a site essential for c-Src activation (pSrc Y419 in human c-Src) (Fig. 1A). Total c-Src levels were not changed by dasatinib treatment. In contrast to pSrc Y419 where inhibition was prolonged, STAT3 activation (as demonstrated by pSTAT3 Y705 levels) was transiently inhibited at 30 min in A549 and H226 (0.60 and 0.57 times the control value, respectively), followed by reactivation by 7 h (1.25 and 1.18 times control). In H2009, H1299, and H1792 activation was seen beginning at 30 min (range 1.20-1.78 times control) and persisted at 7 h (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 h 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 to control (vehicle treated mice sacrificed at 7h or 5d) (Fig. 2). As expected, dasatinib treatment resulted in pSrc Y419 inhibition at all times. In A549 xenografts, pFAK inhibition was seen beginning at 5 h. In contrast, but consistent with the in vitro data, pSTAT3 Y705 was inhibited at early times (0.58 fold at 5 h after treatment) but was strongly induced by 7 h (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 h after treatment but were not significantly different from control levels. In H226 xenografts, pSTAT3 Y705 was inhibited between 5-7 h (0.32 and 0.44 fold at 5 and 7 h, respectively), but rose above baseline by 5 d (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 h and 96 h 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 h and 96 h, respectively (Fig. 3). In H226 cells, total and activated c-Src levels were suppressed at both time points; pSTAT Y705 was strongly induced by 72 h (3.5 fold) and returned near baseline by 96 h (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 h. 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 autophosphorylated and inactive c-Src (pSrc Y527) ($p=1.09\times 10^{-9}$) in tumor tissue as compared to 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 to the ratio of pSTAT3 Y705 to total STAT3 ($r=0.56$, $p=5.25\times 10^{-5}$) (Fig. 4B). 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 $r = -0.50$ ($p=0.0005$), -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 pSrc Y419). However, using the best performing antibody available, we observed a 1.6 fold higher level of pSrc Y419 in tumors compared to 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 demonstrated an 87% and 79% decrease in STAT3 protein levels (respectively) at 48 h after transfection with STAT3 siRNA as compared to scrambled siRNA (Fig. 5A and 5B). To determine the biologic effect of c-Src inhibition combined with specific depletion of STAT3, cells were treated with dasatinib 48 h after transfection. An MTT assay was then used to estimate the number of living cells remaining after 72 h of treatment. Cells with depleted STAT3 were significantly more sensitive to dasatinib than those transfected with scrambled siRNA. In A549 cells, the 50% inhibitory concentration (IC₅₀) values were 0.7 and 4 μ M in control and STAT3 siRNA-transfected cells, respectively, for A549 (Fig. 5C) and 5 and 38 μ M 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 demonstrated 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 demonstrated that inhibition of STAT3 reactivation (by STAT3 siRNA) enhanced the cytotoxicity of dasatinib, demonstrating 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 anti-cancer drugs, imatinib and erlotinib, use ATP-competitive kinase inhibition to inhibit Bcr-Abl (34) and epithelial growth factor receptor (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) (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. Interesting, these studies did demonstrate 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 (unpublished data), and mesothelioma (38) sustained c-Src inhibition also resulted in STAT3 reactivation. This suggests that reciprocal c-Src-STAT3 regulation exists in multiple tumor types. However, this is the first study to demonstrate reciprocal regulation of c-Src and STAT3 in patient tumors.

Three negative feedback loops regulate STAT function after cytokine signaling: SH-2-containing phosphatases (SHPs), which inactivate JAK by de-phosphorylation; protein inhibitors of activated STAT (PIAS), which are negative regulators of STAT induced transcription (i.e., downstream of STATs); and suppressors of cytokine signaling (SOCS), 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 SOCS1 function (40).

The concentration (100 nM) 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 c-Src with ≥ 40 nM dasatinib (unpublished data). In patients treated with dasatinib, plasma levels of approximately 400 nM were reached with sustained levels of 40 to 100 nM (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 demonstrated when c-Src was depleted by siRNA, the levels of pSrc decreased significantly, but pSTAT3.

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, London, UK), and SKI-606 (Wyeth,

Madison, NJ, USA). A non-ATP-competitive c-Src inhibitor is also in clinical trial (Kinex pharmaceuticals, Buffalo, NY). 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.

Statement of Translational Relevance

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 is 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 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.

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Fig. 1. c-Src inhibition fails to durably inhibit pSTAT3 Y705 in vitro. Activation of pSTAT3 Y705 following dasatinib treatment is inhibited by the addition of the JAK inhibitor pyridone 6 (P6). (A) NSCLC cells were incubated with 100 nM dasatinib, 2.5 μ M pyridone 6, both drugs, or vehicle alone (control) for the indicated times, lysed, and analyzed by Western blotting with the indicated antibodies. (B) Changes in pSTAT3 Y705 were quantified using densitometry. pSTAT3 Y705 levels were normalized to total β -actin and the degree of change was measured between dasatinib treated cells and control cells corresponding to the same time point (30 min or 7 h).

Fig. 2. c-Src inhibition results in STAT3 reactivation in vivo. A549 and H226 xenografts were treated daily with dasatinib at 20 mg/kg/day or with vehicle. Tumors were lysed at the times shown and analyzed by Western blotting with the indicated antibodies.

Fig. 3. c-Src depletion results in STAT3 reactivation in vitro. A549 and H226 cells were transfected with c-Src-specific siRNA or nontargeting (scrambled) siRNA. Control cells were mock-transfected. Cells were lysed at 72 h or 96 h and analyzed by Western blotting.

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.

Fig. 5.

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 to each treatment group and significant differences ($p < 0.05$) marked with an asterisk (E).

Table 1
Clinicopathological characteristics of patients included in RPPA analysis of NSCLC tumors

Specimen Number	Histology	Age at surgery	Gender	Race	Smoking Status	T Stage	N Stage	M Stage	Overall Stage
700	SQ	79	M	Caucasian	Former	T2	N1	M0	IB
708	SQ	61	F	African American	Never	T2	N1	M0	IB
719	SQ	76	M	Caucasian	Former	T2	N0	M0	IB
720	SQ	67	F	Caucasian	Current	T1	N1	M0	IIA
739	AD	76	F	Caucasian	Former	T2	N2	M1	IV
746	AD	67	F	Caucasian	Former	T4	N0	M0	IIIB
759	AD	60	M	Caucasian	Current	T2	N2	M0	IIIB
763	AD	60	F	Caucasian	Current	T2	N2	M0	IIIA
764	AD	67	F	Caucasian	Current	T2	N0	M0	IB
767	AD	53	F	African American	Current	T1	N2	M0	IIIA
771	SQ	67	M	Caucasian	Former	T2	N1	M0	IB
773	AD	77	F	Caucasian	Former	T4	N2	M1	IV
774	SQ	67	M	African American	Former	T4	N2	M0	IIIB
781	SQ	80	F	Caucasian	Former	T1	N0	M0	IA
782	AD	67	M	Caucasian	Former	T4	N0	M0	IIIB
786	SQ	81	M	Caucasian	Former	T2	N1	M0	IB
795	AD	64	M	Caucasian	Never	T1	N0	M0	IA
799	SQ	61	F	Caucasian	Current	T2	N0	M0	IB
801	AD	63	F	Caucasian	Current	T2	N1	M0	IB
803	AD	65	F	African American	Current	T2	N1	M0	IB
804	SQ	77	M	Caucasian	Former	T2	N0	M0	IB
811	SQ	62	M	Caucasian	Current	T2	N1	M0	IB
813	SQ	70	M	Caucasian	Current	T4	N0	M0	IIIB
816	AD	73	M	Caucasian	Former	T2	N0	M0	IB
833	AD	72	M	Caucasian	Current	T1	N0	M0	IA
844	SQ	67	M	Caucasian	Former	T2	N1	M0	IB
847	AD	60	F	Caucasian	Current	T2	N0	M0	IB
848	AD	48	F	Caucasian	Current	T2	N0	M0	IB
849	AD	57	M	Caucasian	Current	T1	N1	M0	IIA

Specimen Number	Histology	Age at surgery	Gender	Race	Smoking Status	T Stage	N Stage	M Stage	Overall Stage
852	SQ	67	M	Caucasian	Current	T2	N0	M0	IB
857	SQ	71	M	Caucasian	Current	T2	N0	M0	IB
870	SQ	53	M	Caucasian	Current	T2	N1	M0	IIB
877	AD	68	M	Caucasian	Former	T2	N1	M0	IIB
879	SQ	67	M	Caucasian	Current	T2	N1	M0	IIB
883	AD	58	F	Caucasian	Former	T2	N1	M0	IIB
884	AD	70	F	Caucasian	Former	T1	N0	M0	IA
890	AD	68	M	Caucasian	Former	T2	N0	M0	IB
896	AD	49	F	Caucasian	Current	T1	N0	M0	IA
899	AD	65	F	Caucasian	Current	T1	N0	M0	IA
905	SQ	80	M	Caucasian	Former	T2	N1	M0	IIB
910	AD	74	F	Caucasian	Former	T2	N0	M0	IB
911	SQ	65	F	Caucasian	Former	T2	N0	M0	IB
913	SQ	61	M	Caucasian	Former	T2	N2	M0	IIIA
920	AD	54	F	Caucasian	Current	T2	N0	M0	IB
930	SQ	66	M	Caucasian	Former	T2	N1	M0	IIB
931	SQ	71	F	Caucasian	Current	T4	N0	M0	IIB

(AD=adenocarcinoma, SQ=squamous cell carcinoma)