Sustained Src Inhibition Results in Signal Transducer and Activator of Transcription 3 (STAT3) Activation and Cancer Cell Survival via Altered Janus-Activated Kinase–STAT3 Binding

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Abstract

Locoregional and distant recurrence remains common and usually fatal for patients with advanced head and neck squamous cell carcinoma (HNSCC). One promising molecular target in HNSCC is the Src family kinases (SFK). SFKs can affect cellular proliferation and survival by activating the signal transducer and activator of transcription (STAT) family of transcription factors, especially STAT3. Surprisingly, sustained SFK inhibition resulted in only transient inhibition of STAT3. We investigated the mechanism underlying STAT3 activation and its biological importance. Specific c-Src knockdown with small interfering RNA (siRNA) resulted in STAT3 activation showing specificity, which was inhibited by Janus-activated kinase (JAK; TYK2 and JAK2) depletion with siRNA. Sustained SFK inhibition also resulted in recovered JAK-STAT3 binding and JAK kinase activity after an initial reduction, although JAK phosphorylation paradoxically decreased. To determine the biological significance of STAT3 activation, we combined specific STAT3 depletion with a pharmacologic SFK inhibitor and observed increased cell cycle arrest and apoptosis. Likewise, the addition of STAT3- or JAK-specific siRNA to c-Src-depleted cells enhanced cytotoxicity relative to cells incubated with c-Src siRNA alone. These results show that reactivation of STAT3 after sustained, specific c-Src inhibition is mediated through altered JAK-STAT3 binding and JAK kinase activity and that this compensatory pathway allows for cancer cell survival and proliferation despite durable c-Src inhibition. To our knowledge, this novel feedback pathway has never been described previously. Given that pharmacologic SFK inhibitors are currently being evaluated in clinical trials, these results have potential clinical implications for cancer therapy. [Cancer Res 2009;69(5):1958–65]

Introduction

The estimated annual worldwide incidence of head and neck cancer is 500,000 (1). Head and neck cancers are particularly difficult to treat because both the tumor and the treatment can impair essential functions, such as speech and swallowing, and can also severely alter facial appearance, leading to social isolation. Although novel approaches have improved locoregional control in patients with advanced head and neck squamous cell carcinoma (HNSCC), recurrence remains common and almost always fatal. There is a great need to improve systemic therapy for HNSCC. One promising set of molecular targets for which new agents have been developed is the Src family kinases (SFK; ref. 2). SFKs are nonreceptor tyrosine kinases that regulate multiple signaling cascades. Aberrant activation of c-Src occurs in many solid tumors and contributes to properties of progression and metastasis (3, 4). c-Src is expressed in areas of hyperproliferation in HNSCC and in dysplastic epithelium (5). Several SFKs are expressed and activated by the epidermal growth factor receptor (EGFR) ligand, transforming growth factor-α, in HNSCC cell lines (6). Expression of activated c-Src is higher in HNSCC tumor tissue than in normal mucosa, and it correlates with an invasive, poorly differentiated phenotype and advanced nodal stage (7). Interest in SFKs as targets for cancer therapy has increased recently because of the development and low toxicity of pharmacologic SFK inhibitors. c-Src can affect cellular proliferation and survival by activating signal transducer and activator of transcription 3 (STAT3; ref. 8). The STAT family of transcription factors, especially STAT3, regulates oncogenic signaling in many tumor types (9). Activation of STAT3 is required for viral Src-mediated transformation (10). STAT3 activation leads to the increased expression of downstream target genes (e.g., Bel-XL, cyclin D1, and survivin) and to increased cell proliferation, angiogenesis, and tumor growth in vivo (11). Inhibition of STAT3 in HNSCC leads to increased apoptosis, decreased proliferation, and decreased tumor size (12, 13). We found that SFK inhibition using pharmacologic inhibitors led to an initial and expected inhibition of STAT3, but surprisingly, this was followed by STAT3 reactivation despite durable SFK inhibition (14). No defined pathway explains this reactivation of STAT3 in the face of sustained SFK inhibition. STAT3 can be activated by growth factor and cytokine receptors via nonreceptor tyrosine kinases [SFKs or Janus-activated kinases (JAK); ref. 9], although growth factor receptors can activate STAT3 independent of JAK (15). We previously evaluated the effects of dasatinib, an SFK/Abl inhibitor currently in clinical trial, on several pathways known to lead to STAT activation in HNSCC and other epithelial tumors. EGFR, mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase (ERK) 1/2], cyclooxygenase 2, insulin-like growth factor-I receptor (IGF-IR), platelet-derived growth factor receptor (PDGFR), and c-Met were not mediators of dasatinib-induced STAT3 reactivation. None of the 25 cytokines or growth factors that we measured were up-regulated by dasatinib. The addition of pharmacologic JAK inhibitors (16) during dasatinib incubation resulted in the sustained inhibition of STAT3. However, JAK activation by dasatinib (as measured by phosphorylation) was not demonstrated (14).

In this study, we investigated both the mechanism underlying the activation of STAT3 that occurs after sustained SFK inhibition.
and its biological consequences. We determined that STAT3 reactivation occurs after specific c-Src depletion and is JAK dependent. Our data show that STAT3 reactivation is mediated through JAK kinase activity and JAK-STAT3 binding but is independent of JAK phosphorylation. Inhibition of STAT3 reactivation via specific knockdown of JAK or STAT3 enhanced the apoptosis and cell cycle arrest caused by c-Src depletion. Because STAT3 reactivation occurs in multiple cancer cell types (14, 17), our findings have implications for a broad range of tumors for which SFK inhibitors are currently in clinical trial.

Materials and Methods

Materials. Dasatinib was provided by Bristol-Myers Squibb and prepared as a 10 mmol/L stock solution in DMSO. Dasatinib for animal studies was purchased from M. D. Anderson Cancer Center pharmacy. Antibodies used included c-Src (Santa Cruz Biotechnology); pY419-c-Src, pY705-STAT3, pS473 AKT (S473), total AKT, pERK1/2, total ERK, BcL-XL, survivin, cleaved poly(ADP-ribose) polymerase (PARP), cyclin E, pRb (S780), p27, p21, JAK2, and TYK2 (Cell Signaling Technology); phosphotyrosine (Upstate Biotechnology); and h-actin (Sigma Chemical Co.). Pyridone 6 was purchased from Calbiochem.

Cell culture. Human HNSCC cell lines used in this study were obtained from Drs. Jeffrey Myers and Gary Clayman (M. D. Anderson Cancer Center, Houston, TX) and maintained as previously described (3).

Western blot analysis and immunoprecipitation. Western blot analysis was performed as previously described (14). For immunoprecipitation, cells were lysed [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1% glycerol, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L sodium vanadate]. Equal amounts of protein were precleared with protein A G-Sepharose beads (Sigma Chemical) for 1 h. The precleared lysate was incubated with 5 μg of the indicated antibody for 2 h. The beads were washed four times with immunocomplex buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% glycerol, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L PMSF, 1 mmol/L sodium vanadate] and resolved by SDS-PAGE. Following transfer to nitrocellulose membranes, immunoblots were probed with primary antibody and detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reagent (Amersham Biosciences).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cytotoxicity as previously described (14). Eight wells were treated for each experimental condition.

Cell cycle and apoptosis assays. Cell cycle analysis was performed as previously described (18). Briefly, fixed cells were stained with propidium iodide and DNA content was analyzed by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson) using ModFit software (Verity Software House).

For the apoptosis analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using the manufacturer’s protocol (APO-BRDU kit, Phoenix Flow Systems). Briefly, fixed cells were washed and then incubated with DNA labeling solution that contained terminal deoxynucleotidyl transferase and bromodeoxyuridine triphosphate (Br-dUTP) for 1 h. Subsequently, cells were incubated with a fluorescein-labeled anti-Br-dUTP antibody and then analyzed by FACS.

Measurement of STAT3 DNA binding. Cells were incubated with 100 nmol/L dasatinib or vehicle (DMSO). Nuclear proteins were extracted

Figure 1. SFK inhibition results in transient STAT3 inhibition followed by STAT3 reactivation in vitro and in vivo. A, six HNSCC cell lines were incubated with 100 nmol/L dasatinib for the indicated times and analyzed by Western blotting. B, Western blots for activated STAT3 (pSTAT3, Y705) were analyzed using densitometry and normalized to h-actin. Bars, SD. *, P < 0.05 versus control. C, cells were incubated with 100 nmol/L dasatinib for the indicated times and analyzed by Western blotting with pSTAT3 (Ser727). D, mice bearing human HNSCC orthotopic xenografts were treated with dasatinib and euthanized at the indicated times. Tumors were lysed and subjected to Western blotting.
using the manufacturer’s protocol (Nuclear Extraction kit, Chemicon International). Cells were harvested using trypsin, washed with PBS, lysed, and disrupted with a small-gauge needle. Disrupted cells were spun in a centrifuge at 8,000 g for 20 min. The nuclear pellet was resuspended in nuclear extraction buffer containing protease and phosphatase inhibitors. The nuclear suspension was then spun in a centrifuge at 16,000 g for 5 min. The supernatant (nuclear extract) was incubated with a biotinylated, double-stranded oligonucleotide containing a consensus STAT3 binding site. STAT3/oligonucleotide complexes were captured by an immobilized STAT3 antibody and detected using streptavidin-horseradish peroxidase (R&D Systems). Absorbance was read and wavelength correction was performed by subtracting the A570 from the A450. Controls included a sample with no cell lysate, one with an unlabeled oligonucleotide, and interleukin-6 (IL-6)–stimulated cells.

Transfection with small interfering RNA. Cells were harvested, washed, and suspended (106/100 μL) in Nucleofector V solution (Amaxa). Small interfering RNA (siRNA; 200 pmol/100 μL) was added and electroporated using the U-31 Nucleofector program (Amaxa). Immediately after electroporation, 500 μL of prewarmed RPMI 1640 were added, and the cells were transferred to six-well plates. The medium was changed after 16 h. All siRNAs were predesigned (siGENOME SMARTpool, Dharmacon). Controls included cells that were mock transfected (i.e., no siRNA) and interleukin-6–stimulated cells.

STAT3 transfection assay. The acute-phase response element (APRE)-luciferase reporter gene construct, which has four copies of APREs in front of the minimal JunB promoter-luciferase gene, was provided by Dr. Shuo Dong (Baylor College of Medicine, Houston, TX; ref. 19). Tu167 cells were transfected with 500 ng of APRE-luciferase reporter gene. Transfected cells were incubated with dasatinib, vehicle control, IL-6 (positive control), or pyridone 6 (negative control). Cell lysates were analyzed for luciferase activity.

Orthotopic nude mouse models. All animal procedures were done in accordance with the policies of M. D. Anderson’s Institutional Animal Care and Use Committee. Tu167 cells were injected submucosally into the tongues of athymic nude mice as described elsewhere (20). When visible tumor developed, dasatinib was administered by oral gavage at a dosage of 20 mg/kg/d. Mice were euthanized, tumors were dissected, and mice were examined for regional and distant metastases. The tongue tumors were homogenized and subjected to Western blotting.

In vitro kinase assay. Tu167 cells were incubated with 100 nmol/L dasatinib, vehicle control, or 10 μmol/L pyridone 6. Cells were then lysed and immunoprecipitated with the JAK2 antibody as described above. The immunocomplexes were washed thrice with wash buffer (20 mmol/L Tris-Cl, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5% glycerol) and resuspended in kinase assay reaction buffer (20 mmol/L HEPES/100 μmol/L sodium orthovanadate (pH 7.4), 3 mmol/L MgCl2, 3 mmol/L MnCl2, 10 μCi [γ-32P]ATP (3,000 Ci/mmol; 1 Ci = 37 GBq)). During the kinase assay, 100 nmol/L dasatinib or 10 μmol/L pyridone 6 was added in their respective samples. Acid-denatured rabbit muscle enolase (10 μg; Sigma Chemical) was added as an exogenous substrate and incubated at room temperature for 15 min. The reaction was terminated with sample buffer [2% SDS, 5% β-mercaptoethanol, 0.125 mol/L EDTA, 1% Triton X-100, 2.5% glycerol] and resuspended in kinase assay reaction buffer (20 mmol/L HEPES/100 μmol/L sodium orthovanadate (pH 7.4), 3 mmol/L MgCl2, 3 mmol/L MnCl2, 10 μCi [γ-32P]ATP (3,000 Ci/mmol; 1 Ci = 37 GBq)). During the kinase assay, 100 nmol/L dasatinib or 10 μmol/L pyridone 6 was added in their respective samples. Acid-denatured rabbit muscle enolase (10 μg; Sigma Chemical) was added as an exogenous substrate and incubated at room temperature for 15 min. The reaction was terminated with sample buffer [2% SDS, 5% β-mercaptoethanol, 0.125 mol/L EDTA, 1% Triton X-100, 2.5% glycerol] and resuspended in kinase assay reaction buffer (20 mmol/L HEPES/100 μmol/L sodium orthovanadate (pH 7.4), 3 mmol/L MgCl2, 3 mmol/L MnCl2, 10 μCi [γ-32P]ATP (3,000 Ci/mmol; 1 Ci = 37 GBq)). After incubation, the reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min. The reaction was terminated with sample buffer and incubated at room temperature for 15 min.
STAT3 activation, we examined the effect of the SFK inhibitor dasatinib at early time points in a panel of HNSCC cells using Western blot analysis (Fig. 1A). Because confluence can affect STAT3 activation, vehicle controls were included at 0 and 7 hours. In all cell lines, c-Src phosphorylation was rapidly and durably inhibited at a site associated with its activation (pSrc, Y419 in human c-Src). STAT3 activation was transiently inhibited at 30 minutes to 0.4 ± 0.3 times the control levels, but levels of phospho-STAT3 (pSTAT3; Y705) began to recover by 2 hours (1.1 ± 0.5 times control) and returned to baseline levels or above by 4 and 7 hours (1.5 ± 0.8 and 2.6 ± 1.5 times control, respectively; Fig. 1B).

Because phosphorylation of STAT3 at Ser727 can also influence STAT3 transcriptional activity, we examined the effect of SFK inhibition on this site (Fig. 1C). In contrast to tyrosine phosphorylation, dasatinib did not affect STAT3 serine phosphorylation at early time points; however, serine phosphorylation increased at 7 hours (2.3 ± 0.9 times the control level), consistent with STAT3 reactivation.

To determine whether STAT3 reactivation occurred in vivo, we injected Tu167 cells into the tongues of nude mice. Once tumors formed, dasatinib (20 mg/kg/d) was administered, and the mice were euthanized at 1, 5, and 7 hours and at 14 days (Fig. 1D). On immunohistochemical staining, the grossly dissected tumors were primarily HNSCC (>80%) and no nodal or distant metastases were discovered (data not shown). Dasatinib treatment resulted in durable Src and focal adhesion kinase inhibition at all time points. Consistent with our in vitro data, STAT3 activation (Y705) was inhibited at the early time points but its activity recovered after chronic exposure to dasatinib. Although the pattern was identical between the in vivo and in vitro models, STAT3 reactivation occurred later in the in vivo model, likely the result of differences in drug exposure.

To show that the phosphorylation of STAT3 actually reflected increased STAT3 activity, we also measured STAT3 DNA binding in cellular nuclear extracts (Fig. 2A) and observed a similar pattern of early STAT3 inhibition (30 minutes, 0.5 ± 0.1 times the control level), but STAT3 activity began to recover by 4 hours (0.8 ± 0.2 times control) and returned to baseline levels or above by 7 hours (1.3 ± 0.2 times control). To measure STAT3 transcriptional activity, cells were transfected with the APRE-luciferase reporter gene and incubated with dasatinib, vehicle control, IL-6 (positive control), or pyridone 6 (JAK inhibitor, negative control). Again, we...
observed a similar pattern of decreased STAT3 activity at early time points with reactivation by 4 to 7 hours (Fig. 2B).

STAT3 reactivation is c-Src specific. To determine whether STAT3 reactivation is downstream of c-Src and is not caused by an off-target effect of dasatinib, we transfected HNSCC cells with siRNA specific to c-Src and examined the effect on STAT3 activation (Fig. 2C). When c-Src was depleted, the levels of pSrc decreased, but pSTAT3 increased by 2.1 ± 0.3 times the control levels (Fig. 2D).

STAT3 reactivation depends on JAK2 and TYK2. We hypothesized that JAK mediates STAT3 reactivation during sustained SFK inhibition, so we examined the effect of JAK depletion on STAT3 activation. Tu167 cells express two of the four known JAK family members: TYK2 and JAK2 (14). Incubation of Tu167 cells with TYK2-specific siRNA led to decreased STAT3 activation; TYK2 depletion also decreased STAT3 reactivation after dasatinib exposure. JAK2 depletion alone did not affect STAT3 activation, but it did decrease STAT3 activation after SFK inhibition. The combination of TYK2 and JAK2 depletion completely abrogated the reactivation of STAT3 after dasatinib exposure (Fig. 3A). To show specificity, we combined c-Src–specific siRNA with TYK2/JAK2 siRNA. c-Src depletion led to STAT3 activation, which was inhibited by specific knockdown of JAK2 and TYK2 (Fig. 3B and C).

Sustained c-Src inhibition results in recovery of JAK activity and JAK-STAT3 binding despite decreased JAK phosphorylation. Given that our results show that the reactivation of STAT3 is mediated by JAK2 and TYK2, we hypothesized that SFK inhibition leads to JAK2 and TYK2 activation; we next examined phosphorylation of JAK2 and TYK2 after SFK inhibition at sites shown to be important for kinase activity in other systems. Incubation with dasatinib resulted in prompt and sustained decreases in both JAK2 and TYK2 phosphorylation (Fig. 4A) that corresponded with the time course during which we observed both STAT3 inhibition and reactivation (Fig. 1). In other words, there was no correlation between the effects of dasatinib on STAT3 activation and its effects on the phosphorylation of JAK (JAK2 or TYK2). Dasatinib has no direct effects on JAK proteins.4 Likewise, specific c-Src knockdown using siRNA resulted in decreased JAK2 and TYK2 phosphorylation (Fig. 4B).

To investigate this, we performed an in vitro kinase assay in which HNSCC cells were incubated with vehicle, dasatinib, or pyridone 6 followed by immunoprecipitation with the JAK2 antibody. A kinase assay was then performed on the immunoprecipitate. In cells incubated with pyridone 6 for 7 hours or with dasatinib for 15 minutes, JAK2 activity was profoundly inhibited. However, incubation with dasatinib for 7 hours resulted in the recovery of JAK activity; this correlated with the reactivation of STAT3 observed in these cells after sustained SFK exposure (Figs. 1A and 2A and B).

Figure 4. The effects of c-Src inhibition on JAK phosphorylation, kinase activity, and STAT3 binding. A, Tu167 cells were incubated with 100 nmol/L dasatinib and analyzed by Western blotting. B, Tu167 cells were transfected with c-Src–specific siRNA or controls and then analyzed by Western blotting. C, JAK2 was immunoprecipitated from Tu167 cells treated with the indicated agents and subjected to an in vitro kinase assay. D, Tu167 cells were incubated with 100 nmol/L dasatinib or 2.5 μmol/L pyridone 6 and immunoprecipitated with either the TYK2 or JAK2 antibodies, as indicated. The immunoblots were then probed with the indicated antibodies.

4 Unpublished data.
We next examined the effect of c-Src inhibition on interactions between JAK and STAT3. Cells were treated with dasatinib or pyridone 6, lysed, and immunoprecipitated with JAK2 or TYK2 antibodies to identify JAK-binding proteins that were affected by c-Src or JAK inhibition. When blotted with an anti-phosphotyrosine antibody, only two phosphoproteins were observed, of approximately 80 and 135 kDa (data not shown), which were subsequently shown to be STAT3 and JAK2/TYK2 (Fig. 4D). At the 1-hour time point, dasatinib incubation resulted in decreased JAK-STAT3 binding. Conversely, at the 7-hour time point, JAK-STAT3 binding recovered to baseline levels. The JAK inhibitor did not affect JAK phosphorylation but did inhibit JAK-STAT3 binding. In contrast to the effects on phosphorylated JAK, the effects of dasatinib and pyridone 6 on JAK-STAT3 binding corresponded to their effects on STAT3 activation.

**Specific depletion of STAT3 enhances cytotoxicity, cell cycle arrest, and apoptosis induced by SFK inhibition.** STAT3 mediates survival and proliferation in HNSCC (12), and its activation likely opposes the antitumor effects of SFK inhibition. We next examined the biological effects of STAT3 reactivation on HNSCC cells. To specifically knock down STAT3, we transfected HNSCC cell lines with STAT3 siRNA. Tu167 and Tu686 cell lines were chosen because of their distinct baseline sensitivities to dasatinib. We observed that STAT3 protein levels decreased by 45% at 24 hours, by >80% at 48 hours, and by >95% at 72 to 120 hours (Fig. 5A; data not shown). Dasatinib or vehicle was added 48 hours after transfection, and an MTT assay was used to estimate the number of living cells remaining 72 hours later. HNSCC cells with depleted STAT3 were significantly more sensitive to dasatinib than control cells. IC_{50} values for Tu167 were 23, 19, and 4 nmol/L in mock-transfected, scrambled siRNA–transfected, and STAT3 siRNA–transfected cells, respectively; IC_{50} values for Tu686 were 616, 558, and 205 nmol/L in the same respective transfections.

To determine the effects of combined STAT3 and SFK inhibition on cell cycle and apoptosis, HNSCC cells were transfected with STAT3 siRNA and incubated with dasatinib. In Tu167 cells, STAT3 depletion alone had no effect on the cell cycle but enhanced the degree of arrest induced by dasatinib (Fig. 5B). The percentage of Tu167 cells in G1 at 48 hours was approximately equal in all samples treated with vehicle control (~40%), approximately equal in cells treated with 40 nmol/L dasatinib alone (~66%), and markedly increased in cells treated with STAT3 siRNA and dasatinib together (89%). Tu686 cells were treated with higher concentrations of dasatinib (200 nmol/L) because of their decreased sensitivity to the drug. As it did in Tu167 cells, dasatinib resulted in a significant decrease in the number of cells in the S phase, although this was only minimally enhanced by the knockdown of STAT3 (Fig. 5B). Dasatinib alone resulted in apoptosis in both cell lines that was enhanced by the depletion of STAT3 siRNA (Fig. 5C). These data show that SFKs and STAT3 cooperate to maintain cell survival and proliferation.

**Depletion of STAT3 enhances cytotoxicity of specific c-Src depletion.** To determine whether STAT3 knockdown enhances the cytotoxicity of c-Src depletion, we transfected HNSCC cells with scrambled siRNA, STAT3 siRNA, c-Src siRNA, or both c-Src and STAT3 siRNA and subsequently measured cytotoxicity using the MTT assay (Fig. 6A). Unlike dasatinib, c-Src siRNA inhibits only one SFK family member, although HNSCC cell lines express seven SFKs (data not shown); c-Src siRNA is also a less potent c-Src inhibitor than dasatinib (Fig. L4 versus Fig. 2C). Despite these limitations, c-Src depletion still resulted in cytotoxicity that was enhanced by the addition of STAT3 siRNA. Likewise, the addition of TYK2 and JAK2 depletion to c-Src depletion resulted in increased cytotoxicity (Fig. 6B). These data are consistent with the results for STAT3 siRNA combined with dasatinib, but they showed that this effect is specific to c-Src.
STAT3 depletion enhances the proapoptotic and antiproliferative cell signaling pathways downstream of SFK inhibition.

We examined the pathways known to be downstream of STAT3 and SFKs in HNSCC cells incubated with dasatinib and transfected with STAT3 siRNA (Fig. 6C and D). Consistent with the biological effects of combined STAT/SFK inhibition, we observed increased expression of proapoptotic markers (cleaved PARP) and decreased expression of antiapoptotic molecules (BcL-XL and survivin) in cells with combined SFK inhibition and STAT3 knockdown. The decreased expression of cyclin E and pRb and increased expression of p27 and p21 are consistent with the observed cell cycle arrest in G1. As previously shown, use of dasatinib alone led to inhibition of AKT and MAPK in the sensitive cell line (Tu167; Fig. 6C) but not in the resistant cell line (Tu686; Fig. 6D; ref. 3).

Discussion

In this study, we found that during sustained c-Src inhibition, STAT3 is activated, as shown by increased levels of STAT3 phosphorylation, DNA binding, and transcriptional activity. The recovery of STAT3 phosphorylation was reproduced in all six HNSCC cell lines. We showed that this pathway is c-Src specific and JAK dependent. However, JAK phosphorylation after c-Src or JAK inhibition did not correlate with effects on STAT3 activation. In contrast, both acute c-Src inhibition and pyridone 6 led to decreased JAK-STAT3 binding and JAK kinase activity; sustained c-Src inhibition resulted in the restoration of both JAK-STAT binding and JAK activity. Inhibition of this compensatory pathway with either STAT3 or JAK2/TYK2 siRNA enhanced the cytotoxic, antiproliferative, and proapoptotic effects of c-Src inhibition.

The means by which sustained c-Src inhibition affects JAK-STAT3 interaction and JAK kinase activity remains undefined. In epithelial tumors, STAT3 activation is mediated by EGFR, erythropoietin receptor, IL-6 receptor, PDGFR, c-Met, and IGF-IR (6, 15, 21–25). We previously showed that inhibition of these receptors does not inhibit STAT3 reactivation after incubation with dasatinib (14). Three negative feedback loops regulate JAK/STAT function after cytokine signaling: SH-2–containing phosphatases (SHP), which inactivate JAK; protein inhibitors of activated STAT (PIAS), which are negative regulators of STAT3-mediated transcription; and suppressors of cytokine signaling (SOCS). SOCS proteins regulate JAK-STAT signaling by inhibiting JAK kinase activity, facilitating proteosomal degradation, and competing with STATs for binding to cytokine receptors (26). No known positive feedback loops lead to STAT3 activation after its inhibition (27).

Given that there was no change in the protein levels of JAK or STAT3, JAK phosphorylation decreased, and STAT3 phosphorylation and transcription recovered, it is unlikely that proteosomal
degradation, SHPs, or PIAS mediate STAT3 reactivation. However, reactivation of STAT3 after its initial inhibition does suggest a feedback loop, possibly via the loss of a negative regulator that is transcribed by the basally activated STAT3. The most likely regulators are SOCS proteins that can compete for STAT binding and inhibit JAK kinase activity.

We showed recovery of JAK-STAT3 binding and JAK kinase activity despite decreased JAK phosphorylation. JAK is activated after ligand binding leads to cytokine receptor dimerization and JAK transphosphorylation. JAKs contain two conserved phosphorylation sites adjacent to the activation site (Y1007/1008 in JAK2 and Y1054/1055 in TYK2) that regulate kinase activity. The activated JAK molecules then phosphorylate multiple sites on the cytokine receptor that allow for STAT binding and phosphorylation by JAK (9). However, the molecular mechanisms leading to JAK activation are not fully understood, and this model may be an oversimplification. For example, activation of JAK2 can occur in the absence of Y1007/1008 phosphorylation, which theoretically obviates the need for JAK activation by an upstream kinase (28). Our data clearly reinforce the finding that JAK2 kinase can be activated in the absence of Y1007/1008 phosphorylation. Several other JAK phosphorylation sites exist; for example, Y221 (JAK2) and Y570 (JAK2) have been reported to increase and decrease kinase activity, respectively.

Although both HNSCC cell lines we tested showed increased cytotoxicity with the combination of dasatinib and STAT3 siRNA, the intrinsically resistant line (Tu686) still had IC50 values for dasatinib that are above the concentrations needed to inhibit c-Src and that are barely achievable in humans. This is consistent with additional resistance mechanisms existing in Tu686 cells that are not affected by STAT3 knockdown. We previously showed that these additional mechanisms result in the persistent activation of AKT and MAPK in resistant cells after SFK inhibition (14).

To our knowledge, this is the first study to show that JAK-STAT3 binding and JAK kinase activity are initially inhibited and then recover, leading to STAT3 reactivation after sustained, specific c-Src inhibition. We have observed STAT3 reactivation in multiple tumor types (14, 17), showing that this pathway is not restricted to HNSCC. STAT3 reactivation diminishes the antitumor effects of SFK inhibition by affecting cancer cell survival and proliferation. Given that STAT3 activation can also increase angiogenesis (29) and that SFK inhibitors are currently being evaluated in clinical trials, defining the role of this compensatory pathway in patients treated with SFK inhibitors is essential and planned for the future. Studies that combine SFK and JAK inhibitors in vivo are also planned.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Disclose of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Banibrata Sen, Babita Saigal, Nila Parikh, et al.


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