Abrogation of Signal Transducer and Activator of Transcription 3 Reactivation after Src Kinase Inhibition Results in Synergistic Antitumor Effects

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Abstract

Purpose: The Src family of kinases (SFKs) regulate multiple signal transduction cascades and influence proliferation, motility, survival, and angiogenesis. Dasatinib inhibits SFKs, which leads to cytotoxicity, cell cycle arrest, apoptosis, and decreased invasion of cancer cells. Signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor that regulates survival and proliferation. Dasatinib results in rapid and durable inhibition of c-Src, whereas STAT3 undergoes only transient inactivation. We hypothesized that the reactivation of STAT3 after dasatinib treatment represents the engagement of a compensatory signal for cell survival that blocks the antitumor effects of SFK inhibition.

Experimental Design: The effects of upstream inhibitors on STAT3 activation were assessed with western blotting and a quantitative bioplex phosphoprotein assay. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the cytotoxicity and propidium iodine/annexin V staining with fluorescence-activated cell sorting (FACS) analysis to evaluate cell cycle change and apoptosis. The combination index was calculated by the Chou-Talalay equation. Cytokines were quantitated using a multiplexed, particle-based FACS analysis.

Results: C-Src and several downstream molecules were rapidly and durably inhibited by dasatinib. However, STAT3 was reactivated by 24 h. The addition of JAK inhibitors during dasatinib incubation resulted in sustained inhibition of STAT3, although JAK activation by dasatinib was not shown. Combined SFK and JAK inhibition resulted in synergistic cytotoxicity due to increased apoptosis.

Conclusions: The reactivation of STAT3 during dasatinib treatment is caused by the engagement of a compensatory pathway that suppresses the antitumor effects of SFK inhibition and allows cancer cell survival. Abrogation of this pathway resulted in synergistic cytotoxicity. Given that STAT3 reactivation occurred in 14 of 15 solid tumor cell lines, dasatinib combined with Janus-activated kinase inhibitors may have widespread application in cancer treatment.
pathways subverts the desired effects of SFK inhibition; this has been observed with other kinase inhibitors (7). In support of this hypothesis, we observed that although SFK inhibition in HNSCC and NSCLC led to initial signal transducer and activator of transcription 3 (STAT3) inhibition, STAT3 inactivation was transient, with prominent reactivation occurring 16 to 24 h after dasatinib incubation (3). A lack of durable STAT3 inhibition has also been observed in prostate cancer cells treated with dasatinib (5), and we have found the same pattern of STAT3 reactivation in mesothelioma (8) and squamous cell skin cancer cell lines, suggesting that STAT3 is important for the survival of multiple cancer cell types after SFK inhibition.

The STAT family of transcription factors, especially STAT3, regulates oncogenic signaling in many different tumor types (9). STAT3 can be activated by growth factor receptors, cytokine receptors, and nonreceptor tyrosine kinases (SFKs or Janus-activated kinases [JAK]). In HNSCC, STAT3 activation is mediated by the epidermal growth factor receptor (EGFR), erythropoietin receptor, and interleukin-6R (IL-6R) via c-Src or JAK2 (10–14). Mitogen-activated protein kinase (MAPK) activation can lead to decreased STAT3 phosphorylation in HNSCC (14). In solid tumors, platelet-derived growth factor receptor and c-Met can activate STAT3 via c-Src (15). Insulin-like growth factor receptor 1 (IGFRI) can activate STAT3 in a JAK-independent manner (16). Cyclooxygenase-2 (COX-2) can activate STAT3 in NSCLC by inducing IL-6 expression (17). STAT3 activation leads to the increased expression of several downstream target genes in HNSCC (Bcl-XL, cyclin D1, and vascular endothelial growth factor) and increased cell proliferation and tumor growth in vivo (18). Inhibition of STAT3 in HNSCC leads to increased apoptosis, decreased proliferation, and decreased tumor size (19, 20).

We hypothesized that the reactivation of STAT3 diminishes the proapoptotic and antiproliferative effects of SFK inhibition. Thus, we first sought to determine the mechanism that underlies the reactivation of STAT3 in cancer cells treated with SFK inhibitors and then assessed the biological effects of inhibiting both SFKs and STAT3. Because EGFR is a major growth factor pathway in epithelial cancers, particularly HNSCC and NSCLC, we investigated this pathway and found that STAT3 reactivation by dasatinib was not mediated by EGFR or MAPK, nor did we find evidence of the involvement of the growth factor receptors.
outlined above. We also found no evidence that SFK inhibition led to the release of a soluble factor or cytokine. Treatment of cells with JAK inhibitors (pyridone 6 or AG490) inhibited both basal STAT3 activation and reactivation. The combination of pyridone 6 and dasatinib was synergistic in all cell lines tested. Given the nearly universal finding of STAT3 reactivation in the cancer cell lines that we tested, this combination may have widespread therapeutic application.

**Materials and Methods**

**Materials.** Dasatinib was provided by Bristol-Myers Squibb and prepared as a 10 mmol/L stock solution in DMSO. Antibodies used in the Western blot analysis included phosphorylated MAPK (Promega); AKT and phosphorylated AKT (New England Biolabs); Src (Santa Cruz Biotechnology); PY419-c-Src, pY705-STAT3, pY594-STAT5, total EGFR, pEGFR (Y845, 992, 1068, and 1148), pSTAT1 (Y701), HIF-1α, cyclin D1, insulin growth factor receptor β (IGFR), JAK1, JAK2, JAK3, and TYK2 (Cell Signaling Technology); pY861-focal adhesion kinase (Biosource); pTyrosine (Upstate Biotechnology); phosphoplatelet-derived growth factor (pPDGFR α Y572/574 and β Y579/581; Biosource); and β-actin (Sigma Chemical). Pyridone 6, AG490, PD98059, and PP1 were purchased from EMD Bioscience. SKI-606 was a gift from Wyeth Pharmaceuticals (Pearl, NY). CAY10404 was purchased from Cayman Chemical.

**Cell culture.** Fifteen human cancer cell lines were used in this study: six HNSCC cell lines (Tu167, MDA1986, Tu686, 686LN, OSC-19, and JMAR, obtained from Drs. Jeff Myers and Gary Clayman of the University of Texas M.D. Anderson Cancer Center, Houston, TX), four NSCLC cell lines (H322, H460, H226, and A549, from the American Type Culture Collection), three mesothelioma cell lines (MSTO-211H, H28, H2052, and H2452, American Type Culture Collection), and three squamous cell skin carcinoma (Colo16, Srb1, Srb12 obtained from Dr. Jeff Myers). Cells were grown in monolayer cultures in DMEM (HNSCC and skin cancer cell lines) or RPMI 1640 medium (NSCLC and mesothelioma cell lines) containing 10% fetal bovine serum and 2 mmol/L glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO2. Transformed human keratinocytes (HOK16b) were...
obtained from the American Type Culture Collection and maintained in keratinocyte medium.

**Western blot analysis.** Detached cells from each cell culture plate were collected by centrifugation, washed in PBS, and added to the cell lysate from their corresponding plates. Adherent cells were rinsed with ice-cold PBS and lysed in the cell culture plate for 20 min on ice in a lysis buffer consisting of 50 mmol/L Trizma base (pH 8; Sigma Chemical), 1% Triton X-100, 150 mmol/L NaCl, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium vanadate. Lysates were spun in a centrifuge at 15,000 rpm for 5 min, and the supernatant was collected. Equal protein aliquots 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 (ECL) reagent (Amer sham Biosciences).

**Immunoprecipitation.** Cells were lysed as described for Western blotting. Equal amounts of protein cell lysates (200–300 μg in lysis buffer) were precleared with Protein G Sepharose beads (Sigma–Aldrich) for 1 h. The precleared lysate was incubated with 5 μg of the indicated antibody (IGFR, JAK2, or TYK2) for 2 h. The JAK2 and TYK2 antibodies were already conjugated with agarose beads. Agarose beads (30 μL) were added to IGFR. The lysate/antibody/bead complex was then incubated for 1 h. The beads were washed four times with immune-complex buffer (0.5% Triton X-100, 0.5% NP40, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonyl fluoride) and resolved by SDS-PAGE. Resolved precipitate was blotted with the indicated antibody (antiphosphotyrosine, IGFR, JAK2, or TYK2), which was detected with the secondary antibody and ECL reagent. The membrane was stripped, reprobed with anti-EGFR antibody, and detected with the secondary antibody and ECL reagent.

**Quantitative Bio-Plex phosphoprotein assay.** Cells (5 × 10^7/mL) were treated with 2.5 μmol/L pyridone 6, 100 nmol/L dasatinib, or both for 24 h. Protein lysates were prepared using cell lysis buffer, with phenylmethylsulfonyl fluoride (Bio-Rad Laboratories) on the samples collected. Phosphorylated proteins were detected with a Bio-Rad phosphoprotein immunooassay kit using the Bio-Plex 100 system and collected. Phosphorylated proteins were detected with a Bio-Rad Array Luminex 100 system (Biosource, Invitrogen Corp.) according to the manufacturer’s instructions. These included IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40, IL-13, IL-15, IL-17, tumor necrosis factor-α (TNFα), IFN-α and IFN-γ, granulocyte macrophage colony-stimulating factor, macrophage chemoattractant protein-1, macrophage inflammatory protein-1α and macrophage inflammatory protein-1β, inducible protein-10, monokine induced by IFN-γ, eotaxin, and RANTES. This is a multiplexed, particle-based, flow-cytometric assay that uses anticytokine monoclonal antibodies linked to microspheres that incorporate distinct proportions of two fluorescent dyes. Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were done using Bio-Plex Manager software version 4.1.1.

**Cell cycle and apoptosis analysis.** Subconfluent cells were treated with 100 nmol/L dasatinib, 2.5 μmol/L pyridone 6, or both for 6 h (to

<table>
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<th>Dasatinib</th>
<th>24 h, ng/mL (fold control)</th>
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Abbreviation: ND, not detectable.
determine the effect on early apoptosis) or 24 and 48 h (to assess cell cycle changes). Cells were also treated with nocodazole as a positive control for G2-M arrest. For the cell cycle analysis, cells were harvested, washed in PBS, fixed in 1% paraformaldehyde, rewashed in PBS, and resuspended in 70% ethanol at -20°C overnight. Cells were washed twice with PBS and stained with 20 μg/mL propidium iodide. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan; Becton Dickinson and Company) using ModFit software (Verity Software House, Turramurra). For the apoptosis analysis, treated cells were harvested, stained with annexin V and propidium iodide, and analyzed on a cytofluorimeter by FACScan using ModFit software.

**Results**

**Src inhibition led to initial STAT3 inhibition and later reactivation in multiple cancer cell types in culture.** Fifteen human cancer cell lines were treated with 100 nmol/L dasatinib for 0, 2, 6, and 24 h. Protein expression and phosphorylation were measured by Western blot analysis. In all cell lines, c-Src phosphorylation at a site associated with its activation (Y419) was rapidly and durably inhibited. Several molecules downstream of Src (AKT, STAT5, and focal adhesion kinase) were also durably inhibited. STAT3 activation was transiently inhibited, but levels of pSTAT3 (Y705) returned to baseline or higher levels by 24 h in all HNSCC, mesothelioma, and squamous cell skin carcinoma cell lines, three of the four NSCLC cell lines, and keratinocytes (Fig. 1A and B; refs. 3, 8). Treatment of one representative cell line (Tu167) with three distinct SFK inhibitors all resulted in rapid (within 15 min, data not shown) and durable c-Src inhibition but reactivation of STAT3 (Y705) by 24 h (Fig. 1C), demonstrating that the transient nature of STAT3 inactivation is not restricted to dasatinib alone. Treatment of oral keratinocytes with dasatinib showed a similar pattern of STAT3 reactivation (Fig. 1B and D).

Dasatinib-mediated reactivation of STAT3 was not dependent on activation of the EGFR pathway. STAT3 can be activated by growth factor or cytokine receptors coupled to the Src or JAK families of kinases (9). Dasatinib has no known direct stimulatory effect on growth factor or cytokine receptors (24). To determine the potential involvement of EGFR in STAT3...
The combination of SFK and JAK inhibition resulted in synergistic antitumor effects in vitro. Tu167 (A), Tu686 (B), A549 (C), H226 (D), and HOK16b (E) cells were treated with pyridone 6 alone, dasatinib alone, or the two agents combined in a fixed ratio at the indicated doses for 72 h. The number of viable cells was determined with an MTT assay and expressed as a fold control (vehicle alone). In all cell lines, the combination of SFK and JAK inhibitors resulted in a significantly higher level of cytotoxicity than did single agents. F, Tu167 cells were treated with dasatinib, pyridone 6, or both for 24 h, and downstream mediators of SFK and STAT3 were assayed by Western blotting with the indicated antibodies. The combination treatment resulted in a higher level of HIF-1α, cyclin D1, and SOCS1 inhibition and p27 up-regulation than did either agent alone.
reactivation, we evaluated the effects of dasatinib on EGFR because EGFR is a key growth factor pathway in several epithelial tumors and because EGFR activation often leads to STAT3 activation in HNSCC (19). Dasatinib treatment at concentrations that suppress c-Src activation (100 nmol/L) for 15 min did not affect EGFR activation in intact cells, with or without EGF (Fig. 2A), which confirmed previous results from in vitro kinase assays (24). We hypothesized that SFK inhibition leads to indirect EGFR stimulation and subsequent STAT3 reactivation on the basis of the observation that MAPK had been transiently activated in HNSCC cells treated with dasatinib (3). Erlotinib, an inhibitor of EGFR, had no effect

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**D**

![Graph D](image)

**E**

![Graph E](image)

**F**

![Graph F](image)

*Fig. 4 Continued.*
on STAT3 reactivation by dasatinib in Tu167 cells (Fig. 2B). Treatment of Tu167 cells with EGF led to only a slight increase in STAT3 activation compared with untreated cells. In contrast, MAPK was markedly activated by EGF. To determine whether MAPK activation leads to STAT3 activation in cells treated with dasatinib, we treated Tu167 cells with an inhibitor of MAP/ERK kinase (MEK; PD98059), alone or in combination with dasatinib; the inhibitor had no effect on STAT3 reactivation, but did completely and durably inhibited MAPK activation (Fig. 2C). COX-2 can activate STAT3 (17); thus, we determined the effect of COX-2 inhibition on STAT3 reactivation. COX-2 inhibitors had no effect on STAT3 baseline activation or reactivation in these cells after 24 h of dasatinib treatment (Fig. 2C). Stimulation of cells with IGF did not lead to significant activation of STAT3, and dasatinib had no effect on IGF-1R activation (Fig. 2D). Dasatinib did not lead to activation of PDFR (Fig. 2E).

**STAT3 reactivation was not mediated by cytokine release.** Cytokines regulate the activation state of STATs by recruiting and phosphorylating these proteins at cytokine receptors through the activation of receptor-associated JAKs. STAT3 reactivation in dasatinib-treated cells may be mediated by changes in the expression or release of tumor-associated cytokines. To determine the effects of SFK inhibition on cytokine production, cells were treated with 100 nmol/L dasatinib, and the level of expression of 25 different cytokines was assayed in both serum-free and complete media for 6 and 24 h. The results were similar for all treatment groups. Only IL-6 and IL-8 levels decreased significantly after treatment with dasatinib. All other cytokines or growth factors were unaffected by incubation with dasatinib (Table 1). Several cytokines were undetectable in any samples, including IL-1Ra, IL-2R, IL-4, IL-5, IL-7, IL-13, IL-17, IFN-γ, granulocyte macrophage colony-stimulating factor, macrophage inflammatory protein-1β, and eotaxin. We transferred conditioned medium from dasatinib-treated Tu167 cells (24 h) to untreated, fresh cells and observed no STAT3 activation (data not shown). Although we cannot fully exclude all potential autocrine or paracrine factors, these data suggest that the reactivation of STAT3 is not mediated by the release of a soluble factor or cytokine.

**JAK family kinase inhibitors blocked STAT3 reactivation.** One major pathway of STAT activation is through the JAK family of kinases, which includes JAK1, JAK2, JAK3, and TYK2. Using Western blot analysis, we detected expression of JAK2 and TYK2 but not JAK1 or JAK3 in Tu167 cells (Fig. 3A-D), which represents an expected expression pattern in an epithelial tumor. To determine whether JAKs were involved in STAT3 reactivation by dasatinib, cells were treated with dasatinib, the JAK inhibitor pyridone 6 (25, 26), or both, and protein expression was assayed by Western blot analysis and the Bio-Plex phosphoprotein assay (Figs. 1B and 2B). Pyridone 6 has been shown to inhibit all JAK family members in vitro, with IC50 of 1 to 15 nmol/L for the isolated enzymes (25). In contrast to the EGFR, MAPK, COX-2, and SFK inhibitors, pyridone 6 completely and durably blocked the basal activation of STAT3 and the reactivation of STAT3 in cells treated with dasatinib. Pyridone 6 did not inhibit the activity of c-Src, AKT, or MAPK; these findings are consistent with published in vitro kinase assay data (25). Similar effects on pSTAT3 reactivation were observed with another JAK inhibitor, AG490 (Fig. 3E).

Pyridone 6 also inhibited STAT1 activation, which was only minimally altered by dasatinib (Fig. 3F). Surprisingly, pyridone 6 did not significantly inhibit JAK2 and only minimally inhibited TKY2 in intact cells at this concentration.

**Blocking STAT3 reactivation led to enhanced cytotoxicity and effects on downstream mediators of proliferation and angiogenesis.** The reactivation of STAT3 likely opposes the antitumor effects of SFK inhibitors; thus, we hypothesized that blocking the reactivation of STAT3 would enhance the antitumor effects of SFK inhibitors. To test this hypothesis, we combined dasatinib with pyridone 6 in HNSCC, NSCLC, and keratinocyte cell lines at various concentrations and assessed the resulting cell viability using the MIT assay (Fig. 4A-E). The combination of pyridone 6 and dasatinib was synergistic in all cancer cell lines tested, regardless of their basal sensitivity to either drug. In many cases, the combination indices were significantly <1 for the drug combination, indicating considerable synergy (Table 2). In contrast, the combination of the EGFR inhibitor erlotinib and dasatinib, which did not lead to sustained inhibition of STAT3, had only additive effects in vitro (Fig. 5). Cell cycle analysis revealed that treatment with dasatinib alone caused an increase in the number of cells in the G0-G1 fraction, with a corresponding decrease in the number of cells in the S and G2-M phases; these findings were consistent with our previous data (3). Treatment with pyridone 6 led to an increase in the number of cells in the G2-M fraction and a corresponding decrease in the number of cells in the G0-G1 fraction. The combination of the two drugs led to a substantial increase in cells in the sub-G0 fraction and a decrease in cells in the G0-G1 fraction (Fig. 6A). The combination also resulted in a higher proportion of apoptotic cells (Fig. 6B). Downstream targets of SFKs and STAT3 [cyc1 D1, hypoxia-inducible factor (HIF)-1α, and suppressors of cytokine signaling 1 (SOCS1)] (27, 28) were also significantly inhibited by the combination, and p27 was up-regulated (Fig. 4F); these results are consistent with the enhanced effects seen in the biological assays.

**Discussion**

In this study, we showed that SFK inhibition initially led to STAT3 inhibition but later STAT3 reactivation in 14 of 15 cancer cell lines tested, including HNSCC, NSCLC, mesothelioma, and squamous cell skin carcinoma cell lines. STAT3 has been reported to be primarily activated by growth factor and cytokine receptors via SFKs or JAK kinases, but other kinases may also play a role. We initially focused on the EGFR pathway because it is known to activate STAT3 in HNSCC and NSCLC and because transient activation of MAPK by dasatinib has been previously shown in these cell lines (3). However, our results do not support the involvement of EGFR or MAPK in dasatinib-mediated STAT3 reactivation. Treatment of cells with JAK inhibitors led to the durable inhibition of STAT3 and prevented its reactivation after dasatinib treatment. The combination of pyridone 6 and dasatinib was synergistic in vitro and led to significantly higher levels of apoptosis. We observed enhanced effects on downstream signaling molecules, including p27, cyclin D1, SOCS1, and HIF-1α, which were consistent with the combined effects of these agents on the inhibition of both SFKs and STAT3 activation and the enhanced biological effects of the combination.
Given the intimate relationship between SFKs and STAT3 in HNSCC (10), the lack of sustained STAT3 inhibition after dasatinib treatment was surprising. The mechanism of STAT3 reactivation has not been fully elucidated. It may be a compensatory pathway activated by c-Src to promote survival in response to sustained SFK inhibition or one of its durably inhibited downstream targets. The effects of both AG490 and pyridone 6 on STAT3 activity suggest that STAT3 is reactivated through a JAK-dependent mechanism, but JAK activation by dasatinib was not observed. In addition, we were unable to identify any growth factor, cytokine, or receptor tyrosine kinase that is associated with JAK activation after dasatinib treatment. An analogous situation may exist with the phosphoinositide-3-kinase (PI3K) pathway. The dual inhibition of PI3K and its downstream mediator mTOR has a more profound effect on proliferative arrest in glioblastoma cells than did inhibitors of PI3K or mTOR alone. This was at least partly due to the inhibition of a compensatory pathway: the mTOR inhibitor activated signaling through PI3K (29, 30). Activation of AKT by mTOR inhibitors has also been observed in endothelial cells (31) and tumors (32). Sharma et al. (33, 34) found that oncogene addiction relies on a transient imbalance between proapoptotic pathways and prosurvival compensatory pathways after kinase inactivation. In tumors that are not driven by a single oncogene, this transient imbalance may not exist, and by preventing the engagement of the compensatory pathway, the proapoptotic pathway may predominate.

It is possible that some STAT3 activation by c-Src is at least partially kinase independent (35), and that by inhibiting only kinase activity of c-Src, we did not completely block the influence of c-Src on STAT3 activation. Alternatively, the reactivation of STAT3 may be due to the effects of dasatinib on other targets. Although this would not be expected given the known targets of dasatinib, unpredictable molecular and biological effects occur with other selective tyrosine kinase inhibitors. For example, imatinib treatment can lead to MAPK activation in chronic myelogenous leukemia cells (36) and to the release of heparin-binding–EGF and the subsequent activation of EGFR and MAPK in HNSCC cells (37, 38). Imatinib also reverses the multidrug resistance of chronic myelogenous leukemia cells by an unknown mechanism that requires prolonged exposure (39). The reactivation of STAT3 after treatment with a distinct SFK inhibitor suggests that this is a target-specific effect; however, none of these inhibitors is completely specific for SFKs.

In this study, we found no evidence that EGFR activation or inhibition significantly altered STAT3 or c-Src activation in HNSCC cells. Modulation of EGFR led to the expected impact on MAPK (extracellular signal-regulated kinase 1 or 2). This was surprising because EGFR activation has been reported to be linked to c-Src and STAT3 activation in other HNSCC cell lines and in patient tissues. STAT3 activation, which is shown by increased dimer formation (STAT3:STAT3 and STAT3:STAT5), increased phosphorylation, is common in HNSCC tissue specimens (19). Abrogation of either EGFR or transforming growth factor-α led to decreased STAT3 activation in HNSCC cell lines, both in vitro and in vivo (11, 19). However, c-Src and STAT3 activation are not always dependent on EGFR. Sriramanpong et al. (40) found that STAT3 activation in HNSCC cells was dependent on IL-6–mediated JAK activation and not EGFR activation. In patients treated with the EGFR inhibitor gefitinib, skin biopsies showed a decrease in EGFR phosphorylation but a concomitant increase in STAT3 phosphorylation (41). In a panel of NSCLC cell lines that included those with mutant and wild-type EGFR, the effect of SFK inhibition (dasatinib) on STAT3 activation was modest to absent (42, 43). Inhibition of EGFR did not significantly affect c-Src or STAT3 activation in EGFR wild-type cell lines, but it did significantly inhibit c-Src activation in the mutant EGFR cell lines (16, 42–44). NSCLC cells that are dependent on the EGFR pathways (mutant EGFR) for survival and proliferation are more sensitive to the proapoptotic effects of SFK inhibition in vitro (42). Synergy between Src and EGFR inhibitors has been observed in these cell lines (16), suggesting that if STAT3 activation is driven by EGFR in patients with NSCLC or HNSCC, the coadministration of EGFR and SFK inhibitors would result in enhanced cytotoxic effects; this is clearly the case in NSCLC cell lines with EGFR mutations (16, 42). In our study, the addition of an EGFR inhibitor had additive-to-modest synergistic effects in HNSCC and NSCLC cell lines. None of the cell lines in this study has a known EGFR mutation.

Although the mechanism of STAT3 reactivation has not been fully elucidated, it is clear that SFK and JAK inhibitors have synergistic antitumor effects. With the combination treatment, we observed durable inhibition of several pathways known to be important for cancer cell survival and proliferation: MAPK, focal adhesion kinase (FAK), STAT5, AKT, c-Src, and STAT3. There were also enhanced effects on cyclin D1, HIF-1α, and p27. The enhanced biological and signaling effects seem to be mediated through the durable inhibition of both SFKs and STAT3.

The use of rationally designed combinations of targeted agents that are based on pathway elucidation is appealing for

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<th>Combination, IC_{50} (μmol/L)</th>
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<td>H226</td>
<td>1.6 ± 1.2*</td>
<td>2.2 ± 1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dasatinib</td>
<td>Pyridone 6</td>
<td></td>
</tr>
<tr>
<td>Tu167</td>
<td>0.015 ± 0.007</td>
<td>0.39 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Tu686</td>
<td>0.005 ± 0.020</td>
<td>0.30 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>0.27 ± 0.11</td>
<td>1.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>H226</td>
<td>0.20 ± 0.06</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fa = 0.5</td>
<td>Fa = 0.75</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data are presented as mean ± SD.
*Extrapolated.
the treatment of tumors that are not driven by a single genetic mutation. There has been a renewed interest in SFK inhibitors recently because of the development of specific, potent agents such as dasatinib, the drug used in the current study.

Dasatinib is orally bioavailable and well tolerated and has recently been approved for the treatment of chronic myelogenous leukemia. STAT3 inhibitors are being developed, but none are yet being studied in clinical trials (20, 45–47). JAK

Fig. 5. The combination of EGFR and SFK inhibition results in additive effects in HNSCC and NSCLC cells in vitro. Tu167 (A), Tu686 (B), A549 (C), and H226 (D) cells were treated with pyridone 6 alone, dasatinib alone, or the two agents combined in a fixed ratio at the indicated doses for 72 h. The number of viable cells was determined with an MTT assay and is expressed as a fold control (vehicle alone).
inhibitors, such as the one used in this study, are being developed for clinical use, but none are yet being used in clinical trials for the treatment of cancer. JAK3 inhibitors, most of which also inhibit JAK2, have been designed to prevent organ allograft rejection, and at least one is in early clinical development in this setting (48, 49). Our data clearly show synergy for STAT3 and SFK inhibitors in the treatment of HNSCC, NSCLC, and possibly other cancer types.

**Fig. 6.** The effects of SFK and JAK inhibition on cell cycle and apoptosis. A, Tu67 cells were treated with dasatinib, pyridone 6, or both for 24 and 48 h before being stained with propidium iodide and analyzed by fluorescence-activated cell sorting to determine the proportion of cells in each phase of the cell cycle. Nocodazole treatment was added as a positive control because it is known to cause G2-M arrest. B, Tu67 cells were treated with dasatinib, pyridone 6, or both for 6 h and stained with propidium iodide and annexin V to estimate the number of necrotic (propidium iodide positive) and early apoptotic (annexin V positive) cells. The combination of SFK and JAK inhibition resulted in higher levels of apoptosis than did either drug alone.

**References**

Abrogation of Signal Transducer and Activator of Transcription 3 Reactivation after Src Kinase Inhibition Results in Synergistic Antitumor Effects

Faye M. Johnson, Babita Saigal, Hai Tran, et al.


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