Signal Transducer and Activator of Transcription 3 as Molecular Therapy for Non–Small-Cell Lung Cancer

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Introduction: Targeting signal transducer and activator of transcription 3 (STAT3), a transcription factor that modulates survival–directed transcription, is often persistently activated in epidermal growth factor receptor (EGFR) wild-type non–small–cell lung cancer (NSCLC). The aim of this study was to determine whether sorafenib and its derivative can inhibit EGFR wild-type NSCLC via STAT3 inactivation.

Methods: EGFR wild-type NSCLC cell lines (A549 H292 H322 H358 and H460) were treated with sorafenib or SC-1, a sorafenib derivative that closely resembled sorafenib structurally but was devoid of kinase inhibitory activity. Apoptosis and signal transduction were analyzed. In vivo efficacy was determined in nude mice with H460 and A549 xenograft.

Results: SC-1 had better effects than sorafenib on growth inhibition and apoptosis in all tested EGFR wild-type NSCLC lines. SC-1 reduced STAT3 phosphorylation at tyrosine 705 in all tested EGFR wild-type NSCLC cells. The expression of STAT3–driven genes, including cyclin D1 and survivin, was also repressed by SC-1. Ectopic expression of STAT3 in H460 cells abolished apoptosis in SC-1–treated cells. Sorafenib and SC-1 enhanced Src homology-2 containing protein tyrosine phosphatase-1 (SHP-1) activity, whereas knockdown of SHP-1, but not SHP-2 or protein-tyrosine phosphatase 1B (PTP-1B), by small interference RNA reduced SC-1–induced apoptosis. SC-1 significantly reduced H460 and A549 tumor growth in vivo through SHP-1/STAT3 pathway.

Conclusions: SC-1 provides proof that targeting STAT3 signaling pathway may be a novel approach for the treatment of EGFR wild-type NSCLC.

Key Words: Sorafenib, Cyclin D1, Survivin, Non–small-cell lung cancer, STAT3.

*Lung cancer is the leading cause of cancer–related deaths worldwide. More effective therapies are needed because of the poor survival rates. Lung cancer is broadly classified into two major categories: small-cell lung cancer and non–small–cell lung cancer (NSCLC). Clinical reports show that 80% of lung cancers are diagnosed with NSCLC.1 Platinum-based chemotherapy has become the standard treatment for NSCLC based on the results from a number of clinical trials.2–4 Presently, target therapies with little or minor side effects may compensate for the incompleteness of conventional chemotherapies. Epidermal growth factor receptor (EGFR) mutations are identified in 10% to 15% of white patients, and even higher in Asian patients.5 Patients with certain EGFR mutations have a higher response rate to an EGFR–targeted drug, gefitinib (Iressa; AstraZeneca, Wilmington, DE), than those with wild-type EGFR.6,6 However, carrying EGFR mutations does not assure NSCLC patients of sensitivity to EGFR inhibitors.10–12 Thus, it is important to develop more effective therapeutic strategies for treating NSCLCs that are resistant to current therapies.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates cell growth and survival by modulating the expression of target genes.13 It acts as an oncogene that is constitutively active in many cancers, including liver, lung, head and neck, prostate, and breast cancer, as well as myeloma and leukemia.14–18 A key factor that regulates STAT3 activity is Src homology-2 containing protein tyrosine phosphatase-1 (SHP-1). From a mechanistic perspective, SHP-1 exhibits protein phosphatase activity that reduces the level of phospho-STAT3 (p-STAT3) and subsequently blocks the dimerization of p-STAT3. Thus, the expression of target genes, such as cyclin D1 and survivin, are significantly reduced. Moreover, extensive studies of SHP-1 protein and...
SHP-1 mRNA show that the expression level of SHP-1 is low in most cancer cells. The increased SHP-1 gene expression in cancer cells results in the suppression of cell growth, suggesting that the SHP-1 gene acts as a tumor suppressor.19

Dysregulation of STAT3 activity is associated with the pathogenesis of numerous types of cancers including breast, colon, cervical, and prostate cancers.18 Several lines of evidence also indicate the correlation between STAT3 activity and pathogenesis of lung cancer.20,22 There is a strong correlation between STAT3 activation and tumorogenesis, and suppression of STAT3 by genetic or pharmaceutical modalities has anti-tumor effects in vivo and in vitro.23 Sorafenib (BAY43-9006; Nexavar; Bayer, West Haven, CT) is used clinically for renal carcinoma and hepatocellular carcinoma. It targets the c-Raf and b-Raf kinases and prolongs survival. Studies show that sorafenib can reduce the phosphorylation level of STAT3 in medulloblastoma and esophageal carcinoma.23,24 Further studies show that sorafenib reduces p-STAT3 by activating SHP-1 phosphatase activity in hepatocellular carcinoma cells.25 Because of the ability of sorafenib to reduce the p-STAT3 level, a novel sorafenib analogue, SC-1, was synthesized.25-27 SC-1 retains the ability to reduce cell survival but does not affect Raf kinase activity. This study aims to provide evidence that SC-1 mediates apoptosis via decrease of STAT3 phosphorylation rather than Raf activity.

MATERIALS AND METHODS

Cell Lines and Culture

Five EGFR wild-type NSCLC cell lines were used in this study. The A549 (bronchioloalveolar carcinoma [BAC], mutant v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog [KRAS], cyclin-dependent kinase inhibitor 2A [CDKN2A], and serine/threonine kinase 11 [STK11]), H322 (BAC, mutant TP53 and CDKN2A), H292 (squamous cell carcinoma, wild-type KRAS), and H358 (BAC, mutant KRAS) cell lines were obtained from the American Type Culture Collection (Manassas, VA) whereas the H460 (large-cell lung cancer, mutant KRAS, PIK3CA, STK11, and CDKN2A) cell line was from the Buoresource Collection and Research Center (Hsinchu, Taiwan). The NSCLC cell lines were kept in RPMI1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate in a 37°C humidified incubator with 5% carbon dioxide in air.

Reagents and Antibodies

Sorafenib (Nexavar) was provided by Bayer Pharmaceuticals (West Haven, CT). For in vitro studies, sorafenib and SC-125-27 at various concentrations were dissolved in dimethyl sulfoxide (DMSO) and then added to cells in serum-free RPMI1640. Sodium vanadate, SHP-1, SHP-2, and protein-tyrosine phosphatase 1B (PTP1B) inhibitors were purchased from Merck (Calbiochem). Antibodies for immunoblotting, such as anti–SHP-1, were purchased from Santa Cruz Biotechnology (San Diego, CA) and anti-Flag (M2) were from Sigma-Aldrich (St. Louis, MO). Other antibodies, such as anti–cycdin D1, phospho-STAT3 (Tyr705), STAT3 poly(ADP-ribose) polymerase (PARP) SHP-2, PTP-1B, survivin, and caspase-9, were from Cell Signaling (Danvers, MA).

Cell Viability Assay and Apoptosis Analysis

Five EGFR wild-type NSCLC cells were seeded in 96-well plate (2.5 × 103 cells/well). To determine cell viability and proliferation, 10% WST-1 (water-soluble tetrazolium monosodium salt; Cell Proliferation Reagent WST-1; Roche Applied Science, Indianapolis, IN) agent was added to the cell suspension in each well for 0.5 to 3 hours of incubation. This colorimetric assay was based on the cleavage of the membrane-permeable tetrazolium salt WST-1 to produce a formazan-class dye. The reaction was catalyzed by a mitochondrial reductase in active cells and the amount of formazan dye was quantified by measuring the absorbance at 450nm by using the Bio-Rad ELISA Reader (Bio-Rad, Hercules, CA) to calculate optical density values (A450nm–A655nm). Statistical analysis was done using Student’s t test, with p value less than 0.05 considered statistically significant. Apoptotic cells were measured by flow cytometry (sub-G1) and cell death detection was by Western blot.

Immunocytochemistry for STAT3 Localization

Sorafenib and SC-1–treated cells were plated on a glass slide and fixed in 4% formaldehyde and ice acetone. After brief washing in phosphate-buffered saline, the slides were blocked with 5% normal goat serum for 1 hour and incubated with rabbit polyclonal antihuman STAT3 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-FITC (1:200) for 1 hour and counterstained for nuclei with DAPI for 10 minutes. The stained slides were mounted and analyzed under an epifluorescence microscope (Leica). Pictures were captured with the use of a Leica Photometrics CoolSNAP EZ (high-performance charge-coupled device [CCD] and electron-multiplying CCD [EMCCD] cameras) and MetaMorph version software (Molecular Devices, Wetzlar, Germany).

Ectopic Expression of STAT3

The STAT3 cDNA plasmid was described previously.25 In brief, after transfection, the cells were incubated in the presence of G418 (0.78 mg/ml). After 8 weeks of selection, surviving colonies, that is, those arising from stably transfected cells, were selected and individually amplified. H460 cells with stable expression of STAT3 were then treated with sorafenib or SC-1, harvested, and processed for Western blot analysis.

SHP-1 and Raf-1 Activity Assay

After sorafenib or SC-1 treatment, H460 protein extract were incubated with anti–SHP-1 antibody in immunoprecipitation buffer overnight. Protein A/G sepharose fast flow (GE Healthcare, Madison, WI) was added to each sample, followed by incubation for 3 hours at 4°C with rotation. SHP-1 activity assay kit (DuoSet IC activity assay, DYC2808; R&D systems, Minneapolis, MN) was used for SHP-1 tyrosine phosphatase activity. The Raf-1 kinase assay kit (Upstate-Millipore, Billerica, MA) was used to examine the Raf-1 kinase activity.

Gene Knockdown Using siRNA

Smart-pool siRNA, including control (D-001810-10), SHP-1, SHP-2, and PTP-1B were all purchased from Dharmacon Inc. (Chicago, IL). The procedure has been described previously.25-27
Xenograft Tumor Growth

Male NCr nude mice (5–7 weeks of age) were used. All experimental procedures using these mice were performed according to protocols approved by the Institutional Laboratory Animal Care and Use Committee of Cardinal Tien Hospital. Each mouse was inoculated subcutaneously in the dorsal flank with 1 × 10⁷ of H460 cells and 5 × 10⁶ A549 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 100 to 200 mm³, the mice were given sorafenib tosylate (10 mg/kg) by mouth once daily, or SC-1 (10 mg/kg) by mouth once daily. The controls received vehicle. The tumors were measured twice weekly by using calipers and their volumes calculated using the following standard formula:

\[ \text{Width} \times \text{length} \times \text{height} \times 0.523^{35} \]

Immunohistochemistry

Personal identities were encrypted and all data were analyzed anonymously. Because information that could be used to identify beneficiaries was scrambled, the Institutional Review Board of Cardinal Tien Hospital approved this study protocol and waived the requirement of informed consent. Immunohistochemical (IHC) stains were performed, using the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). In brief, 4-μm thick sections would be cut consecutively from formalin-fixed, paraffin-embedded human tissue. Sections would be mounted and allowed to dry overnight at 37°C. After deparaffinization and rehydration, slides would be incubated with 3% hydrogen peroxide solution for 5 minutes. After a washing procedure with the supplied buffer, tissue sections were repaired for 40 minutes with ethylenediamine tetra-acetic acid. The slides would be again incubated with the primary antibody for 60 minutes at 37°C and then overnight at 4°C. The primary antibody, p-STAT3 (1:50; Cell Signaling) and stat3 (1:200; Cell Signaling) were performed. After three rinses in buffer, the slides were incubated with the secondary antibodies (unbiotinylated antibody; EnVisionTM System; HRP, anti-mouse/rabbit, DakoCytomation; Dako, Glostrup, Denmark). Tissue staining will be visualized with a 3,3-diaminobenzidine (DAB) substrate chromogen solution (DakoCytomation). Slides will be counterstained with hematoxylin, dehydrated, and mounted. Each run included, for each patient, phosphate-buffered solution used as the primary antibody for the negative controls, whereas samples known to express these markers strongly served as the positive controls.

Statistical Analysis

Statistical analysis was performed using a two-tailed Student’s t test. The results were expressed as mean ± standard error of the mean. Differences were considered significant at p value less than 0.05 and highly significant at p value less than 0.01.

RESULTS

SC-1 Caused Cell Death Effect in EGFR Wild-Type NSCLC Cell Lines

In terms of the relationship between SC-1 and sorafenib Raf-1 activity, there was a significant reduction in Raf-1 kinase activity in the presence of sorafenib, but no reduction in Raf-1 kinase activity in the presence of SC-1, suggesting that SC-1 was not a kinase inhibitor like sorafenib (Fig. 1A). After examining the antiproliferation effects of sorafenib and SC-1, both SC-1 and sorafenib decreased the viability of various EGFR wild-type NSCLC cells, including A549, H292, H332, H358, and H460 in a dose-dependent manner for 24 hours (Fig. 1B).

To determine whether SC-1 induced cell death, apoptosis was measured by propidium iodide (PI) staining of DNA. SC-1 induced apoptosis from 20% to 50% in SC-1-treated cells within 24 hours at 10 μM, especially at H460 cells (Fig. 1C). Apoptosis-related gene activation of caspase-9 and PARP cleavage was also measured (Fig. 1D). SC-1 cleaved caspase-9, which led to the appearance of caspase-9, and cleaved PARP. Taken together, all of these suggested that SC-1 could induce apoptosis in human NSCLC cells, indicating that SC-1 was highly cytotoxic to tumor cells.

STAT3 Was An Important Candidate of Sorafenib and SC-1 in EGFR Wild-Type NSCLC Cell Line

To verify whether the phosphorylation of STAT3 was regulated by SC-1, the STAT3-related signaling pathway was assayed in SC-1–treated NSCLC cells (i.e., A549, H332, H292, H358, and H460). STAT3 showed reduced phosphorylation by SC-1, which resulted in the induction of cell death, as well as apoptosis-related molecules like cyclin D1 and survivin. The phosphorylation of STAT3 at tyrosine 705 was critical for STAT3 transactivation. SC-1 and sorafenib decreased phosphorylation of STAT3 at the Y705 residue and suppressed cyclin D1 in NSCLC cells (Fig. 2A). Moreover, SC-1 and sorafenib reduced STAT3 phosphorylation in a dose- and time-dependent manner (Fig. 2B, top and lower).

The activation status of p-STAT3 was measured by STAT3 ELISA, human DuoSet ELISA development kit (R&D Systems, Minneapolis, MN). The results showed that sorafenib and SC-1 significantly decreased the activity of p-STAT3. SC-1 and sorafenib affected STAT3 phosphorylation activity (Fig. 2C). Using STAT3-overexpressing stable clones of NSCLC cells, it was observed that both sorafenib and SC-1 suppressed the expression of p-STAT3 in STAT3-overexpressed H460 cells, suggesting that STAT3 was a major mediator of sorafenib- and SC-1–induced apoptosis (Fig. 2D). STAT3 was dominantly localized to the nuclear compartment of untreated cells by immunofluorescence. On the contrary, sorafenib and SC-1 treatment lead to STAT3 predominantly cytoplasmic localization so the drugs prevent STAT3 nuclear accumulation (Fig. 2E).

SC-1 Inhibition of STAT3 Activation Involved a Protein Tyrosine Phosphatase

Several protein phosphatases involved in regulating p-STAT3 were examined. Sodium vanadate decreased apoptosis and restored STAT3 phosphorylation (Fig. 3A, left lane, 5 and 6). Furthermore, SHP-1 phosphatase-specific inhibitor, but not SHP-2 inhibitor (Fig. 3A, right), reversed the SC-1– and sorafenib-induced cell death and inhibition of p-STAT3 (Fig. 3A, middle). To further verify the role of SHP-1 in SC-1– and sorafenib-induced inhibition of p-STAT3, siRNA strategy specific to SHP-1 was applied to examine the influence of sorafenib and SC-1. Silencing of SHP-1 reversed the sorafenib- or SC-1–induced apoptosis and inhibition of p-STAT3 (Fig. 3B).
In addition, both sorafenib and SC-1 increased SHP-1 activity compared with controls \( (p<0.05) \). Sorafenib- or SC-1–treated H460 cells were immunoprecipitated by SHP-1–specific antibody. The SHP-1–containing complex then underwent fluorescence-based phospho-group assay. Neither sorafenib nor SC-1 affected the interaction of STAT3 and SHP-1, as evidenced by SHP-1 immunoprecipitation (Fig. 3C), suggesting that sorafenib induced cell
FIGURE 2. SC-1 reduced STAT3 phosphorylation in NSCLC cells. A, Effects of sorafenib or SC-1 on STAT3-related proteins. Cells were exposed to sorafenib or SC-1 at the indicated doses for 24 hours. B, Dose-escalation (top) and time-dependent (lower) effects of sorafenib or SC-1 on phospho-STAT3 in H460 cells. Cells were treated with sorafenib or SC-1 at 10 μM for 24 hours. C, Effects of sorafenib and SC-1 on STAT3 activity, phospho-STAT3 enzyme-linked immunosorbent assay (ELISA). Points, man; bars, SD (n=3) **p < 0.01. D, Protective effects of STAT3 on apoptosis induced by sorafenib or SC-1 in H460 cells. Cells were exposed to sorafenib or SC-1 at 10 μM for 24 hours. Apoptotic cells were analyzed by flow cytometry. Columns, mean; bars, SD (n=3) **p < 0.01. E, Untreated and two drugs-treated cells were analyzed by fluorescence microscopy. DAPI staining and overlay demonstrated nuclear accumulation of STAT3 in untreated, but not in sorafenib- or SC-1–treated cells. STAT3, signal transducer and activator of transcription 3; DAPI, 4′,6-diamidino-2-phenylindole.
death through SHP-1–dependent STAT3 inactivation. The effects of sorafenib on apoptosis and p-STAT3 were not reversed by silencing SHP-2 or PTP-1B (data not shown) (Fig. 3D), suggesting that neither SHP-2 nor PTP-1B played a role in mediating the effects of sorafenib or SC-1 on p-STAT3.

**Therapeutic Evaluation of Effect of SC-1 and Sorafenib on H460 or A549-Bearing Mice**

To verify the therapeutic effect of SC-1, SC-1 was applied to the NSCLC xenograft to evaluate its significance in vivo. SC-1 treatment significantly inhibited H460 or A549 xenograft tumor growth and SC-1 treated animals had tumor...
sizes that were less than half of those of control mice (Fig. 4A and D). There were no apparent differences in body weight or toxicity in any mice (data not shown).

To further correlate the molecular mechanism of sorafenib with its anticancer effect in vivo, tumor extract from vehicle, SC-1–treated, and sorafenib-treated mice were immunoblotted for p-STAT3. p-STAT3 was decreased in SC-1–treated tumor (Fig. 4B and E). SHP-1 activity in SC-1–treated and sorafenib-treated H460 or A549 xenografts were also examined. SC-1–treated tumor showed significant induction of SHP-1 activity in vivo (Fig. 4C and F). Taken together, these results confirmed that SC-1 could increase SHP-1 activity to repress p-STAT3 involved in tumor inhibition in the EGFR wild-type NSCLC xenograft model.

Expression of STAT3 and p-STAT3 by Immunohistochemistry in EGFR Wild-Type NSCLC Clinical Tissue Specimen

Immunohistochemistry was performed in EGFR wild-type NSCLC specimen in which STAT3 and p-STAT3 were overexpressed in tumor samples. The STAT3 and p-STAT3 proteins were expressed in both cytoplasmic and nuclear components of tumor cells with stronger signal (Fig. 5). However, the adjacent normal bronchial epithelia exhibited less STAT3 and p-STAT3 staining.

DISCUSSION

This study demonstrates that SC-1, a sorafenib derivative that lacks hydrogen donor ability because of its pyridine ring and amide functional group being replaced by phenyl cyanide, has antitumor effects that equal those shown by sorafenib on EGFR wild-type NSCLC cells. Despite showing no Raf-1 kinase inhibition activity in EGFR wild-type NSCLC cells, SC-1 still induces significant apoptosis and triggers p-STAT3 inhibition in the same manner as sorafenib in EGFR wild-type NSCLC cells both in vitro and in vivo. On the basis of the results, SC-1, a novel compound that affects STAT3 in the same manner as sorafenib, has an anticancer effect on EGFR wild-type NSCLC cells. This anticancer effect is not correlated with repression of Raf-1 kinase.
STAT3 activation is present in a substantial number of NSCLC cell lines and NSCLC tumor specimens, especially EGFR mutation NSCLC. Gao et al. pointed that p-STAT3 is found in primary adenocarcinomas and cell lines harboring somatic-activating mutations in the tyrosine kinase domain of EGFR. Jiang et al. detected p-STAT3 expression in lung cancer by immunohistochemistry in tissue from 127 lung carcinomas (100 adenocarcinomas and 27 squamous cell carcinomas). Among the 127 cases of NSCLC, p-STAT3 immunoreactivity is significantly correlated with sex (p=0.004), smoking history (p=0.006), EGFR mutation status (p=0.003), clinical stage (p=0.034), and lymph node metastasis (p=0.009). However, the role of STAT3 in EGFR wild-type NSCLC is nearly not mentioned. Data in the present study prove that STAT3 is also crucial in EGFR wild-type NSCLC and thus is a promising potential target for anticancer therapy.

This study demonstrates that the novel compound SC-1, as well as sorafenib, induces EGFR wild-type NSCLC cell death and represses p-STAT3 in NSCLC xenograft models through SHP-1–dependent STAT3 inactivation. Thus, STAT3 seems to be a promising biological target for new therapeutic strategies for the treatment of EGFR wild-type NSCLC.

Clinically, the phase II trials of sorafenib in previously treated advanced NSCLC have failed. However, sorafenib has better effects in EGFR wild type and EGFR fluorescence in situ hybridization–negative patients with better progression-free survival and overall survival. The present study shows that sorafenib has an anticancer effect on EGFR wild-type NSCLC cells through SHP-1–dependent STAT3 inactivation instead of Raf-1 kinase. Sorafenib should also have an effect on wild-type NSCLC if the patient group is carefully selected. Moreover, STAT3 pathway screening maybe an important condition for whether sorafenib has anticancer effect on EGFR wild-type NSCLC patients. Aside from EGFR mutation, there are many gene abnormalities in NSCLC, such as echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (EML4-ALK) fusions, human epidermal growth factor receptor 2 (HER2), PIK3CA, protein kinase B or AKT (PKB/AKT), v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), mitogen-activated protein kinase kinase 1 (MAP2K1), mesenchymal-epithelial transition (MET), and KRAS mutations. STAT3 pathway screening will be a promising method to screen candidates for sorafenib or SC-1.

In conclusion, STAT3, rather than Raf-1, is critical in the anticancer effect of sorafenib and its novel analogue SC-1 on NSCLC. Blocking STAT3 signaling by SC-1 may be a novel therapeutic approach for lung cancer.

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