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 cylcin 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*.

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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*.[6–9] 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

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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.[20,21] There is a strong correlation between *STAT3* activation and tumorigenesis, and suppression of *STAT3* by genetic or pharmaceutical modalities has antitumor effects in vivo and in vitro.[22] 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-1[25–27] 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–*cyclin 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 (2.5×10^3 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 450 nm 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 antirabbit 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]

