Discovery of JSI-124 (Cucurbitacin I), a Selective Janus Kinase/Signal Transducer and Activator of Transcription 3 Signaling Pathway Inhibitor with Potent Antitumor Activity against Human and Murine Cancer Cells in Mice

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ABSTRACT

Constitutively activated, tyrosine-phosphorylated signal transducer and activator of transcription (STAT) 3 plays a pivotal role in human tumor malignancy. To discover disrupters of aberrant STAT3 signaling pathways as novel anticancer drugs, we developed a phosphotyrosine STAT3 cytoblot. Using this high throughput 96-well plate assay, we identified JSI-124 (cucurbitacin I) from the National Cancer Institute Diversity Set. JSI-124 suppressed the levels of phosphoSTAT3 in v-Src-transformed NIH 3T3 cells and human cancer cells potently (IC50 value of 500 nM in the human lung adenocarcinoma A549) and rapidly (complete inhibition within 1–2 h). The suppression of phosphoSTAT3 levels resulted in the inhibition of STAT3 DNA binding and STAT3-mediated but not serum response element-mediated gene transcription. JSI-124 also decreased the levels of tyrosine-phosphorylated Janus kinase (JAK) but not those of Src. JSI-124 was highly selective for JAK/STAT3 and did not inhibit other oncogenic and tumor survival pathways such as those mediated by Akt, extracellular signal-regulated kinase 1/2, or c-Jun NH2-terminal kinase. Finally, JSI-124 (1 mg/kg/day) potently inhibited the growth in nude mice of A549 tumors, v-Src-transformed NIH 3T3 tumors, and the human breast carcinoma MDA-MB-468, all of which express high levels of constitutively activated STAT3, but it did not affect the growth of oncogenic Ras-transformed NIH 3T3 tumors that are STAT3 independent or of the human lung adenocarcinoma Calu-1, which has barely detectable levels of phosphoSTAT3. JSI-124 also inhibited tumor growth and significantly increased survival of immunologically competent mice bearing murine melanoma with constitutively activated STAT3. These results give strong support for pharmacologically targeting the JAK/STAT3 signaling pathway for anticancer drug discovery.

INTRODUCTION

STATs4 are key signal transduction proteins that play a dual role of transducing biological information from cell surface receptors to the cytoplasm and translocating to the nucleus, where, as transcription factors, they regulate gene expression (reviewed in Refs. 1–4). Mammalian cells express seven different STATs (1, 2, 3, 4, 5a, 5b, and 6). Gene knockout and other experiments implicated STATs in many important physiological functions such as immune modulation, inflammation, proliferation, differentiation, development, cell survival, and apoptosis (1–4).

STAT tyrosine phosphorylation is required for the biological function of STATs. This occurs when cytokines such as interleukin 6 and IFN or growth factors such as platelet-derived growth factor and epidermal growth factor bind their respective receptors, which results in STAT protein recruitment to the inner surface of the plasma membrane in the vicinity of the cytoplasmic portion of the receptors (5, 6). Tyrosine kinases that are known to phosphorylate STATs are non-RTKs such as Src and the JAKs, JAK1 and JAK2. Other possible tyrosine kinases that can phosphorylate STATs are peptide growth factor receptors such as platelet-derived growth factor receptor and EGFR. The cellular levels of STATs that are tyrosine phosphorylated could also be regulated by phosphotyrosine STAT phosphatases such as SHP-1 and SHP-2 (7–9). Once tyrosine phosphorylated, STAT monomers dimerize via reciprocal phosphotyrosine-SH2 interactions and translocate to the nucleus, where they bind DNA and regulate gene transcription (10). Whereas tyrosine phosphorylation of STATs regulates dimerization, nuclear translocation and DNA binding, serine/threonine phosphorylation is believed to regulate the transcriptional activity of STATs (11).

Several lines of evidence have implicated some STAT family members in malignant transformation and tumor cell survival (12, 13). STAT3 involvement in oncogenesis is the most thoroughly characterized. First, STAT3 is found constitutively tyrosine phosphorylated and activated in many human cancers (12–14). This abnormal activation of STAT3 is prevalent in breast, pancreatic, ovarian, head and neck, brain, and prostate carcinomas, as well as in melanomas, leukemias, and lymphomas. In those tumors investigated, aberrant STAT3 activation is required for growth and survival (12–14). Second, many known oncogenes, especially those belonging to the non-RTK family such as src, induce constitutive activation of STAT3 (15). Third, expression of a constitutively activated mutant of STAT3, for which stable dimerization was forced through disulfide covalent linkage, was shown to be sufficient to induce cell transformation and tumor growth in nude mice (16). Finally, perhaps the most compelling evidence for the requirement of STAT3 for oncogenesis and its validation as an anticancer drug target comes from experiments in which a dominant negative form of STAT3 was used in cultured cells and in gene therapy animal experiments to show that blocking aberrant activation of STAT3 results in inhibition of tumor growth and survival and induction of apoptosis with few side effects to normal cells (17, 18).

On the basis of the above observations, we and others have proposed to target STAT3 for the development of novel anticancer drugs (reviewed in Refs. 12–14). Depending on the aberrant genetic alterations that result in constitutively tyrosine-phosphorylated, activated STAT3, several approaches could be undertaken including blocking.

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7 The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; EGFR, epidermal growth factor receptor; NCI, National Cancer Institute; TBS, Tris-buffered saline; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; PI3k, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; SHP, SH2-containing phosphatase; DAPI, 4′-6-diamidino-2-phenylindole.

8 The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; EGFR, epidermal growth factor receptor; NCI, National Cancer Institute; TBS, Tris-buffered saline [10 mM Tris (pH 7.4), 150 mM NaCl]; PMSF, phenylmethylsulfonyl fluoride; MEK, mitogen-activated protein/extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; SRE, serum response element; RIPA, radioimmunoprecipitation assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; EMSA, electrophoretic mobility shift analysis; ERK, extracellular signal-regulated kinase; PI3k, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; SHP, SH2-containing phosphatase; DAPI, 4′-6-diamidino-2-phenylindole.
ligating phosphotyrosine STAT3 phosphates, and blocking STAT3 dimerization, nuclear translocation, DNA binding, and gene transcription. In addition, gene therapy, antisense, or RNA interference approaches can also be attempted. One approach that we have taken, the results of which are described in this study, is to identify small molecules capable of interfering with the signaling events leading to the abnormally elevated levels of tyrosine-phosphorylated STAT3 in many human cancer cells.

MATERIALS AND METHODS

Cell Lines. All human and murine tumor cell lines used were obtained from the American Type Culture Collection (Manassas, VA). Stably transfected v-Src, oncogenic H-Ras, and vector NIH 3T3 cell lines have been described previously (11, 19).

NCI Diversity Set. The NCI Structural Diversity Set is a library of 1,992 compounds selected from the approximately 140,000-compound NCI drug repository. These compounds were selected based on various criteria including availability, drug-like structure, uniqueness of pharmacophore, and anticancer activity as determined by cell growth inhibition assays against a panel of human tumor cell lines. In-depth data on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program website.

Cytoblot Screening for Phospho-STAT3 Inhibition. NIH 3T3 cells stably transfected with v-Src or NIH 3T3 vector control cells (11) were plated into sterile, opaque, 96-well tissue culture plates at 25,000 cells/well. After overnight growth at 37°C, the cells were treated for 4 h in the presence of either vehicle control or 10 nM of NCI Diversity Set compounds. After treatment, cells were washed in 100 μl of cold TBS and then fixed for 1 h at 4°C with cold 3% formaldehyde in TBS (100 μl/well) as described previously for a similar cytoblot for phospho-nucleolin (20). Membranes were permeabilized during a 5-min incubation in ice-cold methanol at 4°C. Cells were washed with 3% milk in TBS (180 μl/well) and then rocked overnight at 4°C with 3% milk in TBS (50 μl/well) containing a 1:1,000 dilution of anti-phospho-STAT3 (P-Tyr 705; Cell Signaling Technology, Beverly, MA) and a 1:2,000 dilution of horseradish peroxidase-conjugated goat antiriabbit IgG (Jackson ImmunoResearch, West Grove, PA). Antibodies were aspirated, and then plates were washed twice with TBS (180 μl/well). Results were visualized by adding Western blot chemiluminescence reagent directly to the wells of the plates, incubating at room temperature for 5 min, and then placing X-ray film directly on top of the plate in a dark room for 1–5 min. Quantification of results was done using a GS-700 scanning densitometer (Bio-Rad Laboratories, Hercules, CA).

Western Blotting. Treated cell samples were lysed in 30 mM HEPES (pH 7.5), 10 mM NaCl, 5 mM MgCl2, 25 mM NaF, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, 25 μg/ml leupeptin, 2 mM PMSF, and 6.4 mg/ml p-nitrophenyl phosphate. Phospho-STAT3, phospho-Akt, phospho-MEK, and phospho-p42/p44 mitogen-activated protein kinase antibodies were obtained from Cell Signaling Technologies (Cambridge, MA). Antibodies to STAT3, JAK2, and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-JAK2 antibody came from Upstate Biotechnology (Lake Placid, NY). Membranes were blocked in either 5% milk in PBS (pH 7.4) containing 0.1% Tween 20 or 1% BSA in TBS (pH 7.5) containing 0.1% Tween 20. Phospho-specific antibodies (except phospho-mitogen-activated protein kinase and phospho-JNK) were incubated in 1% BSA in TBS (pH 7.5) containing 0.1% Tween 20, whereas all other antibodies were diluted in 5% milk in PBS (pH 7.4) containing 0.1% Tween 20 for either 2 h at room temperature or overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) were diluted in 5% milk in either PBS (pH 7.4) containing 0.1% Tween 20 or TBS (pH 7.5) containing 0.1% Tween 20 at a 1:1000 dilution for 1 h at room temperature. Western blots were visualized using enhanced chemiluminescence as described previously (21).

Immunoprecipitation of STAT3. A549 cells were treated for 4 h with vehicle or JSI-124 and then lysed in 150 μM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 10% glycerol, 5 mM NaF, 1 mM DTT, 1 mM PMSF, 2 mM sodium orthovanadate, and 5 μg/ml leupeptin. Sample lysates were collected and cleared, and then 500 μg of lysate were immunoprecipitated with 50 ng of STAT3 antibody overnight at 4°C and rocked with 25 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 1 h at 4°C. Samples were washed four times with lysis buffer and then boiled in 2× SDS-PAGE sample buffer and run on 10% SDS-PAGE gel. Protein was transferred to nitrocellulose and then blotted as described above for both phospho-specific STAT3 and STAT3.

DNA Binding and Transcription. The STAT3 reporter, pLucTKS3, driving expression of firefly luciferase has been described previously (11). The pLucTKS3 plasmid harbors seven copies of a sequence corresponding to the STAT3-specific binding site in the promoter of the human C-reactive protein gene (22). The STAT3-independent plasmid, pRLSRE, contains two copies of the SRE from the c-fos promoter (23), subcloned into Renilla luciferase reporter, pRL-null (Promega Corp., Madison, WI).

Transfection and Generation of Stable Clones. NIH 3T3/v-Src/pLucTKS3 and NIH 3T3/v-Src/pRLSRE are stable clones that were generated by transfecting NIH 3T3/v-Src fibroblasts with pLucTKS3 or pRLSRE and selecting for G418-resistant and zeocin clones, respectively (11, 24). In the case of NIH 3T3/v-Src/pLucTKS3/pRLSRE, pRLSRE was transfected into NIH 3T3/v-Src/pLucTKS3 cells, and stable G418-resistant clones were selected. Transfections were carried out with LipofectAMINE Plus (Invitrogen Corp., Carlsbad, CA), according to the manufacturer’s protocol. Concerning treatment of cells with inhibitors, Src-transformed NIH 3T3 cells stably expressing reporter constructs pLucTKS3, pRLSRE, or both were treated with JSI-124 (10 μM) for 24–48 h before harvesting cells for cytotoxic and nuclear extract preparation and luciferase assay.

Preparation of Cytosolic Extracts. Cytosolic extracts were prepared from fibroblasts as described previously (11). Briefly, after two washes with PBS and equilibration for 5 min with 0.5 ml of PBS-0.5 mM EDTA, cells were scraped off the dishes, and the cell pellet was obtained by centrifugation (4,500 × g for 2 min at 4°C). Cells were resuspended in 0.4 ml of low-salt HEPES buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM MgCl2, 0.1 mM EDTA, 1 mM PMSF, and 1 mM DTT] for 15 min, lysed by the addition of 20 μl of 10% NP40, and centrifuged (10,000 × g for 30 s at 4°C) to obtain the cytosolic supernatant, which was used for luciferase assays (Promega Corp.) measured with a luminometer.

Nuclear Extract Preparation and Gel Shift Assay. Nuclear extract preparation and EMSA were carried out as described previously (11). The 32P-radiolabeled oligonucleotide probe is hSIE (high affinity sis-inducible element, m67 variant, 5′-AGCTTTTCTCCGTTAATCCTCA-3′) that binds STAT1 and STAT3.

JAK Kinase Assays. A549, MDA-MB-468, and v-Src-transformed NIH 3T3 cells were harvested, washed three times in PBS [10 mM sodium phosphate (pH 7.4), 137 mM NaCl, and 1 mM sodium orthovanadate], and then lysed for 30 min on ice in JAK kinase lysis buffer [25 mM HEPES (pH 7.4), 0.1% Triton X-100, 0.5 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Samples were spun at high speed to clear, and 800-1000 μg of protein were immunoprecipitated per treatment condition with 50 ng of either JAK1 or JAK2 antibody (Santa Cruz Biotechnology) with rocking overnight at 4°C. Twenty-five μl of protein A/G PLUS-agarose were then added, and rocking continued for 1 h at 4°C. Samples were spun to collect agarose pellet, and pellet was washed twice in wash buffer [50 mM HEPES (pH 7.4), 0.1% Triton X-100, 0.5 mM DTT, and 150 mM NaCl] and once in phosphorylation buffer [50 mM HEPES (pH 7.4), 0.1% Triton X-100, 0.5 mM DTT, 6.25 mM manganese chloride, and 100 mM NaCl]. Kinase reactions were performed at 30°C for 15 min in a final volume of 100 μl of phosphorylation buffer. Samples were pretreated with DMSO control, JSI-124, and control compounds [AG490 (100 μM) and PD189079 (2 μM)] before addition of 20 μCi/sample (γ-32P)ATP. The reaction was halted using stop buffer (wash buffer + 10 mM EDTA), samples were spun to collect pellet, and then pellet was washed once with stop buffer and twice with wash buffer. Samples were then placed in 2× SDS-PAGE sample buffer, boiled at 100°C.

and run on 8% SDS-PAGE gels to separate proteins. Autophosphorylation results were visualized by autoradiography.

Src Kinase Assay. A549, MDA-MB-468, and v-Src cells were harvested and lysed for 30 min on ice in RIPA buffers (10 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml antipain). Samples were spun at high speed to clear, and then 1000 μg of protein per treatment condition were immunoprecipitated with 2 μg of v-Src antibody (Ab-1; Oncogene Research Products, San Diego, CA) with rocking overnight at 4°C. Twenty-five μl of protein A/G PLUS-agarose were then added, and rocking continued for 4 h at 4°C. Samples were spun to collect agarose pellet, and pellet was washed three times in RIPA150 buffer, twice in RIPA10 buffer (10 mM Tris (pH 7.5), 10 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml antipain), and three times in 40 mM Tris (pH 7.4). Pellet was then resuspended in 30 μl of kinase reaction buffer (20 mM Tris (pH 7.4) and 5 mM MgCl2) containing 10 μCi of [γ-32P]ATP. Inhibitors were preincubated for several minutes before the addition of ATP. kinase reactions were carried out at room temperature for 15 min. Reaction was stopped with the addition of 2× SDS-PAGE sample buffer. Samples were boiled and run on 10% SDS-PAGE gels. Autoradiography results were visualized by autoradiography.

**In Vitro Cellular Proliferation and TUNEL Assays.** Subconfluent A549, MDA-MB-468, Calu-1, v-Src-transformed NIH 3T3, H-Ras-transformed NIH 3T3, and vector NIH 3T3 cells were grown in the presence of 10 μM JSI-124 or DMSO vehicle control. After 24 h, cells were harvested by trypsinization and counted via trypan blue exclusion assay to determine cellular viability. Cells (75,000–150,000, depending on cell line) were then spun onto glass slides using a Cytospin 3 centrifuge (Thermo Shandon Inc., Pittsburgh, PA). After fixing the cells to the slides with 4% paraformaldehyde in PBS (pH 7.5) for 1 h at room temperature, cells were labeled for apoptotic DNA strand breaks by TUNEL reaction using an in situ cell death detection kit (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s instructions, and then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI) to counterstain DNA. Fluorescein-labeled DNA strand breaks (TUNEL-positive cells) were then visualized using a fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL), and pictures were taken with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). TUNEL-positive nuclei were counted and compared with DAPI-stained nuclei to determine the percentage induction of apoptosis by 10 μM JSI-124.

**Antitumor Activity in the Nude Mouse Tumor Xenograft Model.** Nude mice and C57 BL-6 mice (NCI, Bethesda, MD) were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. nude mice and C57 BL-6 mice (NCI, Bethesda, MD) were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Subconfluent A549, MDA-MB-468, and Calu-1 cells were harvested, resuspended in PBS, and injected s.c. into the right and left flank (10 × 10⁶ cells/flank) of 8-week-old female nude mice as reported previously (25). Similarly, murine B16-F10 melanoma cells were injected s.c. into the right and left flank (10⁶ cells/flank) of C57 black mice. When tumors reached about 150 mm³, animals were randomized (5 animals/group; 2 tumors/animal) and dosed i.p. with 0.2 ml vehicle of drug once daily. Control animals received DMSO (20%) vehicle, whereas treated animals were injected with JSI-124 (1 mg/kg/day) in 20% DMSO in water. The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume (V = l/2w) as described previously (25). Statistical significance between control and treated animals was evaluated by using Student's t test.

**Antitumor Activity in Immuno-Competent Mouse Model.** For the mouse survival experiments, C57 BL-6 mice were given s.c. injections of B16-F10 cells (10⁶–10⁷ cells/flank). On day 16 after implantation, the mice were randomized (6 animals/group) and treated with either vehicle or JSI-124 (1 mg/kg/day) for 25 days. The percentage of surviving mice was determined by monitoring the death of mice until all mice died. Two experiments of 12 animals each (6 control mice and 6 mice treated with JSI-124) were carried out. For each of the two experiments, mice receiving JSI-124 were compared with those receiving vehicle control with respect to survival using the permutation log-rank test as implemented in the statistical software package, ProcStatXact (P values are two sided and exact). The results of both experiments were then pooled in a stratified analysis and resulted in a P of 0.01.

**RESULTS**

Development of Phosphotyrosine STAT3-specific Cytoblot High Throughput Assay and Identification of JSI-124. STAT3 is found tyrosine phosphorylated and constitutively activated in many human cancer types. Blockade of this aberrant activation using dominant negative STAT3 was shown previously to result in inhibition of tumor growth and induction of tumor cell apoptosis, giving strong support to the validation of STAT3 as a cancer drug discovery target (reviewed in Refs. 12–14). In an attempt to identify novel anticancer drugs based on interference with the aberrant activation of STAT3, we have developed a 96-well plate high throughput cytoblot assay in which the levels of activated tyrosine-phosphorylated STAT3 are determined by an antibody specific to tyrosine-phosphorylated STAT3. Using this cytoblot assay, we have evaluated a 1992-compound library from the NCI (the NCI Diversity Set) for agents capable of blocking v-Src activation of STAT3 in NIH 3T3 cells as described in “Materials and Methods.” Analysis of the cytoblot results indicated that several compounds inhibited activation of STAT3 to various degrees. The most potent of these compounds, JSI-124 (NCI identifier: NSC 521777), suppressed v-Src-activated STAT3 at a concentration of 10 μM. Fig. 1a shows the structure of JSI-124, which is also known as cucurbitacin I (26–28). Fig. 1b shows an example of a 96-well plate cellular assay where the effects of 88 compounds from the NCI Diversity Set on phosphotyrosine STAT3 levels were evaluated. JSI-124 (10 μM) at position D6 on the plate reduced phosphotyrosine STAT3 to barely detectable levels.

**JSI-124 Suppresses Phosphotyrosine STAT3 Levels in Human Cancer Cell Lines.** Fig. 1 identifies JSI-124 as an inhibitor of v-Src activation of STAT3 in NIH 3T3 cells. To determine whether JSI-124...
suppresses phosphotyrosine STAT3 levels in human cancer cell lines, we first evaluated several human cancer cell lines and identified those with high levels of tyrosine-phosphorylated STAT3 (Fig. 2a). Among the human cancer cell lines evaluated, A549 (a lung adenocarcinoma), MDA-MB-468 and MDA-MB-231 (two breast carcinomas), and Panc-1 (a pancreatic carcinoma) contained high levels of tyrosine-phosphorylated STAT3. These human cancer cell lines, along with the positive control cell line (v-Src-transformed NIH 3T3 cells), were treated with either vehicle or JSI-124 (10 μM) for 4 h, and the cell lysates were processed for Western blotting with antiphosphotyrosine STAT3 antibody as described in “Materials and Methods.”

Fig. 2b shows that JSI-124 was very effective at reducing the levels of tyrosine-phosphorylated STAT3. These results confirm those of the cytoblot and demonstrate the ability of JSI-124 to suppress the levels of constitutively activated, tyrosine-phosphorylated STAT3 not only in v-Src-transformed NIH 3T3 murine fibroblasts but also in human cancer cells of epithelial origin.

Because the phosphotyrosine STAT3 antibody could possibly cross-react with other tyrosine-phosphorylated proteins, we confirmed that JSI-124 suppresses phosphotyrosine STAT3 levels by first immunoprecipitating STAT3 with an anti-STAT3 antibody and then Western blotting with antiphosphotyrosine STAT3 antibody as described in “Materials and Methods.” Fig. 2c shows that JSI-124 was very effective at reducing the levels of tyrosine-phosphorylated STAT3. These results confirm those of the cytoblot and demonstrate the ability of JSI-124 to suppress the levels of constitutively activated, tyrosine-phosphorylated STAT3 not only in v-Src-transformed NIH 3T3 murine fibroblasts but also in human cancer cells of epithelial origin.

JSI-124 Inhibits STAT3 Signaling by Disrupting STAT3 DNA-binding Activity and STAT3-mediated Gene Expression. Tyrosine phosphorylation of STAT3 is required for its biological activity (reviewed in Refs. 1–4). We therefore reasoned that the suppression by JSI-124 of the phosphotyrosine levels of STAT3 should lead to disruption of STAT3 DNA-binding activity and STAT3-mediated gene expression. To this end, we first evaluated the effect of JSI-124 on STAT3 DNA-binding activity by EMSA. v-Src-transformed NIH 3T3 cells and A549 cells were treated with vehicle or JSI-124, and nuclear extracts containing activated STAT3 were incubated with 32P-labeled hSIE oligonucleotide probe for EMSA as described in Fig. 2.

Fig. 3. JSI-124 inhibits STAT3 DNA-binding activity and STAT3-mediated transcription. a, v-Src-transformed NIH 3T3 cells and A549 cells were treated with vehicle or JSI-124 and then harvested and processed for EMSA as described in “Materials and Methods.” Samples in Lanes 1–4 and 6–9 are from cells that were treated with vehicle control, whereas samples from Lanes 5 and 10 are from cells treated with JSI-124. Lanes 2, 3, 7, and 8 are from samples supershifted with anti-STAT1 (Lanes 2 and 3) or anti-STAT3 antibodies (Lanes 7 and 8). b, v-Src-transformed NIH 3T3 cells transfected with either pLucTKS3- or pRLSRE-dependent luciferase reporters were treated with either vehicle or JSI-124 and processed for luciferase assays as described in “Materials and Methods.” Data are representative of two independent experiments.
Materials and Methods. Fig. 3a shows that STAT3 DNA-binding activity was greatly reduced in nuclear extracts from v-Src/NIH 3T3 and A549 cells treated with JSI-124 compared with extracts from vehicle-treated cells (Fig. 3, Lanes 4 versus 5 and Lanes 9 versus 10). To confirm that the band seen in the gel contains STAT3-DNA complexes, the nuclear extracts were preincubated with anti-STAT3 or anti-STAT1 antibodies. The anti-STAT3 antibody, but not the anti-STAT1 antibody, supershifted or blocked the complex, demonstrating that the protein-DNA complex contains STAT3, not STAT1 (Fig. 3a, Lanes 1, 2, 3, 6, 7, and 8). These results demonstrate that JSI-124, by reducing the levels of tyrosine-phosphorylated STAT3, inhibits STAT3 signaling, resulting in disruption of STAT3 DNA binding.

We next determined whether this suppression of STAT3 activation results in inhibition of STAT3-mediated gene expression. To this end, v-Src/NIH 3T3 fibroblasts that stably express pLucTKS3 or that stably express pRLSRE were treated with either vehicle or JSI-124, and cytosolic extracts were prepared for luciferase assays as described in “Materials and Methods.” Fig. 3b shows that JSI-124 significantly suppresses induction of the STAT3-dependent pLucTKS3 luciferase reporter without affecting the pRLSRE reporter. Because v-Src activates pLucTKS3 in a STAT3-dependent manner and activates pRLSRE in a STAT3-independent manner in v-Src-transformed NIH 3T3 cells, the results shown in Fig. 3b demonstrate that JSI-124 is specific to STAT3-mediated transcription. Thus, JSI-124 inhibits STAT3 signaling by suppressing phosphotyrosine levels of STAT3, inhibiting STAT3 DNA binding and STAT3-mediated gene expression.

JSI-124SuppressesPhosphotyrosineLevels of STAT3 and JAK2 but not Src in A549 and MDA-MB-468 Cells. The ability of JSI-124 to suppress phosphotyrosine levels of STAT3 suggests that this agent may interfere with the function of the upstream tyrosine kinases JAK and Src that are known to phosphorylate STAT3. We therefore evaluated the effects of JSI-124 on the phosphotyrosine levels of JAK2 and Src in whole cells and the ability of JSI-124 to inhibit Src, JAK1, and JAK2 kinase activities in vitro. Fig. 4a shows that treatment of A549 and MDA-MB-468 cells with JSI-124 results in reduction of the levels of tyrosine-phosphorylated STAT3, with A549 cells being more sensitive than MDA-MB-468 cells. Furthermore, JSI-124 was also effective at suppressing the levels of tyrosine-phosphorylated JAK2, but not the levels of tyrosine-phosphorylated JAK2.

Fig. 4. Effects of JSI-124 on phosphotyrosine levels and kinase activities of JAK and Src kinases. a, JSI-124 suppresses phosphotyrosine levels of STAT3 and JAK2 but not Src. A549 and MDA-MB-468 cells were treated with various concentrations of JSI-124 and processed for immunoblotting with antibodies specific for either phosphotyrosine STAT3, phosphotyrosine JAK2, or phosphotyrosine Src as described in “Materials and Methods.” The membranes were also rebotted with antibodies to STAT3 and JAK2. b, suppression by JSI-124 of phosphotyrosine STAT3 and JAK2 is rapid. A549 and MDA-MB-468 cells were treated with JSI-124 (10 μM) for various lengths of time (0–240 min) and processed as described above. c, JSI-124 does not inhibit JAK1, JAK2, and Src kinase activities. Lysates from v-Src-transformed cells, A549 cells, and MDA-MB-468 cells were immunoprecipitated with antibodies against JAK1, JAK2, and Src. Autophosphorylation kinase assays were then performed as described in “Materials and Methods.” Immunoprecipitates were incubated either with vehicle control (C), JSI-124 (J), the JAK kinase inhibitor AG490 (A), or the Src kinase inhibitor PD180970 (P). Data are representative of three independent experiments.
Src. JSI-124 has no effect on the protein levels of STAT3 and JAK2 in both cell lines (Fig. 4a).

The effects of JSI-124 on JAK/STAT3 signaling described above were determined after 4 h of JSI-124 treatment. To ascertain the length of treatment time required for JSI-124 to suppress phosphotyrosine STAT3 and JAK levels, we carried out a time course experiment. Fig. 4b shows that treatment of A549 and MDA-MB-468 cells with JSI-124 for as little as 60 min was effective, and in both cell lines the suppression was complete after 2 h. Thus, the suppression by JSI-124 of the levels of tyrosine-phosphorylated STAT3 and JAK2 is rapid.

We next evaluated the ability of JSI-124 to inhibit the kinase activities of Src, JAK1, and JAK2 in vitro. To this end, we immunoprecipitated Src, JAK1, and JAK2 from either A549, MDA-MB-468, or v-Src/NIH 3T3 cells and incubated the immunoprecipitates with either vehicle control, JSI-124, the JAK tyrosine kinase inhibitor AG490, or the Src kinase inhibitor PD180970 and followed autophosphorylation of Src, JAK1, and JAK2 as described in “Materials and Methods.” Fig. 4c shows that, as expected, in all three cell lines PD180970 inhibits Src but not JAK1 or JAK2 activities (A549 did not have JAK1 kinase activity). Similarly, AG490 inhibited JAK1 and JAK2 but not Src kinase activities. In contrast, all three kinase activities were not affected by JSI-124 (Fig. 4c). Therefore, although in whole cells JSI-124 is very effective at suppressing the levels of tyrosine-phosphorylated STAT3 and JAK2, it is unable to directly inhibit Src, JAK1, or JAK2 kinase activities in vitro.

**JSI-124 Is Highly Selective for the JAK/STAT3 Signaling Pathway Over the Akt, ERK, and JNK Signaling Pathways.** To determine whether the effects of JSI-124 were selective to the JAK/STAT3 pathway over other oncopgenic and survival pathways, we treated A549 and MDA-MB-468 cells with various concentrations of JSI-124 and processed the lysates for Western blotting with antibodies specific for phospho-STAT3, phospho-ERK1/2, phospho-JNK, and phospho-Akt as described in “Materials and Methods.” Fig. 5 shows that A549 and MDA-MB-468 have constitutively phosphorylated ERK1/2, JNK, and Akt in addition to phospho-STAT3. Treatment with JSI-124 resulted in suppression of phospho-STAT3 levels in both cell lines. In contrast, treatment with JSI-124 had no inhibitory effect on phospho-Akt, phospho-ERK1/2, or phospho-JNK with a concentration as high as 10 μM. With ERK1/2, not only did JSI-124 not inhibit the levels of phosphorylation, it actually increased the levels of phosphorylation. Thus, these results demonstrate that JSI-124 suppressive effects are highly selective for the JAK/STAT3 pathway over the ERK, JNK, and Akt tumor survival and oncogenic signaling pathways.

**JSI-124 Inhibits Cellular Proliferation and Induces Apoptosis.** The ability of JSI-124 to selectively inhibit the JAK/STAT3 pathway suggested that it may inhibit proliferation and/or induce apoptosis preferentially in those tumors that express constitutively activated STAT3. To this end, we treated cells that express constitutively activated STAT3 (A549, MDA-MB-468, and v-Src-transformed NIH 3T3) and cells that do not (Calu-1, H-Ras-transformed NIH 3T3, and vector NIH 3T3) with JSI-124 as described in “Materials and Methods.” Fig. 6 shows that 10 μM JSI-124 inhibited cellular proliferation by 73% (A549), 80% (MDA-MB-468), 90% (v-Src/3T3), 68% (H-Ras/3T3), 71% (vector/3T3), and 70% (Calu-1). Fig. 6 also shows that JSI-124 induced apoptosis by 18-fold (A549), 15-fold (MDA-MB-468), 11-fold (v-Src/3T3), 1.4-fold (H-Ras/3T3), 1.2-fold (vector/3T3), and 1.3-fold (Calu-1). Thus, JSI-124 induces apoptosis only in cells that express constitutively activated tyrosine-phosphorylated STAT3. In contrast, the ability of JSI-124 to inhibit cellular proliferation is independent of STAT3 activation status.

**JSI-124 Inhibits Growth in Mice of Tumors with High Levels of Constitutively Activated STAT3.** Previous studies have shown that interfering with STAT3 signaling using a gene therapy approach with a dominant negative variant of STAT3 (STAT3−β) resulted in induction of apoptosis and inhibition of the growth of melanoma cells in mice (17, 18). Because JSI-124 inhibits aberrantly activated STAT3 signaling, DNA binding, and STAT3-mediated gene expression, and because it induced apoptosis only in cancer cells with constitutively activated STAT3, we reasoned that the growth in nude mice of tumors with constitutively activated STAT3 should be more sensitive to JSI-124 than that of tumors with low or without constitutively activated STAT3. To this end, we s.c. implanted A549, MDA-MB-468, and Calu-1 cells in nude mice. When the tumors reached an average size of about 150 mm³, the animals were randomized and treated i.p. with either vehicle or JSI-124 (1 mg/kg/day) as described in “Materials and Methods.” Fig. 7 shows that A549 and Calu-1 tumors from animals treated with vehicle grew to about 500 mm³ 26 days after tumor implantation. MDA-MB-468 tumors treated with vehicle control grew to about 300 mm³ 60 days after tumor implantation. JSI-124 inhibited A549 and MDA-MB-468 tumor growth by 76% and 86%, respectively. In contrast, JSI-124 had little effect on the growth of Calu-1 cells in nude mice. Treatment of mice bearing A549 cells with a reduced dose of JSI-124 (0.5 mg/kg/day) for 23 days also inhibited tumor growth by 52% (data not shown). At both doses, 1 and 0.5 mg/kg/day, JSI-124 had no effects on body weight, activity, or food intake of mice. However, at the local site of drug injection, the peritoneal cavity, JSI-124 at the 1 mg/kg/day dose caused edema. A
similar observation was made by the NCI Developmental Therapeutics Program, where edema was observed at the s.c. site of injection. The results from A549, Calu-1, and MDA-MB-468 xenograft studies suggest that human cancer cells that express constitutively activated STAT3 should be sensitive to JSI-124. We further reasoned that if the ability of JSI-124 to inhibit tumor growth in nude mice depends on constitutively activated STAT3, v-Src-transformed NIH 3T3 cells that require constitutively activated STAT3 for malignant transformation (15) should be sensitive to JSI-124, whereas oncogenic Ras-transformed NIH 3T3 cells, in which STAT3 is not constitutively activated, should be resistant. Fig. 7 shows that, in the absence of JSI-124, the growth of both v-Src- and Ras-transformed NIH 3T3 tumors was highly aggressive, and tumors reached average sizes of about 2500 and 1000 mm$^3$, respectively, within 9 days of tumor cell implantation. Fig. 7 also shows that JSI-124 (1 mg/kg/day) inhibited the growth of v-Src/NIH 3T3 tumors by 64%. In contrast, the growth of Ras/NIH 3T3 tumors was resistant to JSI-124. These results, coupled with those from the human tumor xenografts, strongly suggest that JSI-124 selectively targets tumors with constitutively activated STAT3 signaling.

The above results were obtained from experiments with immune-deficient nude mice. Furthermore, the studies did not investigate the effects of JSI-124 on the survival of mice bearing death-inducing tumors. We therefore evaluated the ability of JSI-124 to inhibit tumor growth and increase survival of immunologically competent C57 BL-6 mice that had been s.c. implanted with murine B16-F10 melanoma that expresses constitutively activated STAT3 (17). Fig. 7 shows that B16-F10 tumors from control mice given injections with vehicle grew to an average size of 1194 ± 141 mm$^3$, whereas those treated with JSI-124 (1 mg/kg/day) grew only to an average size of 588 ± 94 mm$^3$. Thus, JSI-124 treatment inhibited tumor growth by 56%. To determine the effect of JSI-124 on mouse survival, we s.c. implanted B16-F10 melanoma and followed mouse survival over time. Fig. 7 shows that mice treated with vehicle begin to die on day 19 after B16-F10 implantation. By day 21, half of the mice were dead, and by day 35, all six mice were dead. In contrast, none of the JSI-124-treated mice were dead by day 23, half of the mice died on day 34, and all of the mice died by day 42. Fig. 7 also shows that 50% of vehicle-treated mice survived up until day 21 ($T_{50} = 21$), whereas the JSI-124-treated group of mice had a longer $T_{50}$ of 34 days. Thus, treatment with JSI-124 significantly increased the life span ($T_{50}$ increase of 13 days) of immunologically competent mice that were implanted with B16-F10 melanoma.
**DISCUSSION**

Many modern anticancer drug discovery approaches have focused on targeting signal transduction pathways involving RTKs (e.g., ErbB2 and EGFR), farnesylated proteins (e.g., Ras), and nonreceptor cytosolic kinases (e.g., Raf, MEK, PI3k, and Akt; Ref. 29). These important efforts resulted in several novel agents such as RTK monoclonal antibodies; RTK, farnesyltransferase, Raf, and MEK inhibitors that are presently in clinical trials; and some such as the Bcr-Abl tyrosine kinase inhibitor STI-571 (Gleevec), which has recently been approved by the Food and Drug Administration for chronic myelogenous leukemia. In contrast to this heavily exploited area, little has been done to target the STAT3 signaling pathway. However, experiments in animal models using gene therapy with a dominant negative form of STAT3 and a constitutively active mutant of STAT3, as well as the prevalence of constitutively activated STAT3 in many human cancers, strongly suggest STAT3 as having a causal role in oncogenesis (12–14). Furthermore, the fact that constitutive activation of STAT3 induces genes such as cyclin D1, c-myc, and bcl-xl that are intimately involved in oncogenesis and tumor survival, coupled with the fact that constitutively activated STAT3 is required for survival of some human cancer cells, further validates the STAT3 signaling pathway as a selective cancer drug discovery target (12–14).

In this study, we have identified JSI-124, a selective JAK/STAT3 signaling pathway inhibitor with potent antitumor activity against human tumors in nude mice, from the NCI Diversity Set of 1,992 compounds. JSI-124 is a plant natural product identified previously as cucurbitacin I, a member of the cucurbitacin family of compounds that are isolated from various plant families such as the Cucurbitaceae and Cruciferae and have been used as folk medicines for centuries in countries such as China and India. However, little was known about the biological activities of the various cucurbitacins until recently. Some cucurbitacins have been shown to have anti-inflammatory and analgesic as well as cytotoxic effects. Furthermore, cucurbitacins were also found to inhibit DNA, RNA, and protein synthesis in HeLa cells (30) and to inhibit proliferation of HeLa cells (30), endothelial cells (31), and T lymphocytes (32). Finally, some cucurbitacins were shown to suppress skin carcinogenesis (33), inhibit cell adhesion (34), and disrupt the actin and vimentin cytoskeleton in prostate carcinoma cells (27, 31). Although the reports on cucurbitacin biological activity suggest antiproliferative activity and possible antitumor activity, no data are available concerning the oncogenic and tumor survival pathways that are targeted by cucurbitacins.

In this report, we demonstrate that cucurbitacin I (JSI-124) reduced the levels of phosphotyrosine of constitutively activated STAT3 in many human cancer cell lines including pancreatic, lung, and breast carcinomas. This suppression in the levels of constitutively activated STAT3 resulted in blockade of STAT3 DNA-binding activity and STAT3-mediated gene transcription. JSI-124 was highly selective for disrupting STAT3 signaling over other pivotal oncogenic and tumor survival pathways. For example, in two cell lines, the human lung adenocarcinoma A549 and the human breast carcinoma MDA-MB-468, JSI-124 did not inhibit the constitutive activation of the Ser/Thr protein kinase B, PKB/Akt, indicating that the PI3k/Akt survival pathway is not a target for JSI-124. Similarly, the Ras/Raf/MEK/ERK oncogenic signaling pathway was not inhibited by JSI-124 in these two cell lines. Finally, the constitutive activation of JNK in A549 and MDA-MB-468 was not affected by JSI-124, indicating that the stress-activated protein kinase signaling pathway is not targeted by JSI-124.

Many human cancers rely on the PI3k/Akt and Ras/Raf/MEK/ERK pathways to induce malignant transformation and tumor survival. For example, the great majority of tumors overexpress the ErbB family of receptors such as EGFR and ErbB2 and contain mutant forms of Ras. These RTK and Ras genetic alterations result in constitutive activation of the PI3k/Akt and Ras/Raf/MEK/ERK pathways. The fact that JSI-124 inhibits tumor growth and blocks STAT3 signaling without inhibiting the constitutive activation of Akt and ERK1/ERK2 suggests that its ability to inhibit the growth of A549 and MDA-MB-468 in nude mice does not depend on blocking Akt and ERK activation. This also suggests that JSI-124 may be more selective toward inhibiting the growth of tumors with constitutively activated STAT3. Consistent with this, JSI-124 induces apoptosis in A549, MDA-MB-468, and v-Src/NIH 3T3 cells that express constitutively activated STAT3, but...
it does not induce apoptosis in Calu-1, H-Ras/NIH 3T3, and vector/NIH 3T3 cells that do not express constitutively activated STAT3. Interestingly, the ability of JSI-124 to inhibit proliferation in vitro did not depend on STAT3 activation status. However, inhibition of tumor growth in animals did depend on STAT3 activation status. Indeed, consistent with the apoptosis data, but not the proliferation data, we found v-Src-transformed NIH 3T3 tumors that depend on constitutively activated STAT3 for malignant transformation to be sensitive to JSI-124 antitumor activity in nude mice. In contrast, oncogenic Ras-transformed NIH 3T3 tumors, in which STAT3 is not constitutively activated, were resistant to JSI-124. Furthermore, the fact that JSI-124 inhibited the growth in mice of the human lung adenocarcinoma (A549), human breast carcinoma (MDA-MB-468), and murine melanoma (B16-F10), all of which express constitutively activated STAT3, but did not inhibit growth of the human lung adenocarcinoma (Calu-1) that has very low levels of tyrosine-phosphorylated STAT3 gives further support to the notion that the ability of JSI-124 to inhibit tumor growth depends on an aberrantly activated STAT3 signaling pathway. Importantly, JSI-124 also significantly increased the survival of immunologically competent mice in which B16-F10 murine melanoma was implanted.

The ability of JSI-124 to suppress the cellular levels of phosphorytrosine-STAT3 but not phospho-ERK1/2, phospho-JNK, and phospho-Akt suggested that a STAT3 tyrosine kinase is a possible molecular target for JSI-124. Consistent with a direct inhibition of the enzymatic activity of a tyrosine kinase is the fact that suppression of the STAT3 phosphotyrosine levels was rapid (observed as early as 30 min and complete after only 2 h of treatment). There are two well-characterized STAT3 tyrosine kinases: JAK and Src kinase. Because phosphorytrosine JAK2 levels were also reduced by JSI-124, this suggested that JAK2 is likely not the target. This was confirmed by in vitro kinase assays where JAK2 and JAK1 enzymatic activities were inhibited by AG490, a known JAK inhibitor, but were not inhibited by JSI-124. Similarly, Src kinase activity was inhibited in vitro by the known Src kinase inhibitor PD180970 but was not inhibited by JSI-124, indicating that Src kinase is not a target. The RTK EGFR, which is also believed to phosphorylate STAT3, is most likely not a target either because epidermal growth factor stimulation of EGFR tyrosine phosphorylation in breast cell line MCF-10A and EGFR-overexpressing NIH 3T3 cells was inhibited only minimally by JSI-124 (data not shown).

Reduction in phosphorysine levels could be a result of either inhibition of protein tyrosine kinases or activation of protein phosphotyrosine phosphatases. STAT3 is known to be phosphorytrosine dephosphorylated by two protein phosphotyrosine phosphatases, SHP-1 and SHP-2 (7–9), and JSI-124 could down-regulate phosphorytrosine-STAT3 levels by promoting the protein phosphatase activities of SHP-1 and SHP-2. Alternatively, JSI-124 could also activate physiological inhibitors that are known to directly or indirectly down-regulate STAT3 activation. These include suppressors of cytokine signaling, STAT-induced STAT inhibitor, JAK-binding protein, and STAT3-interacting protein (13).

In summary, we have developed a phosphorysine STAT3-specific cytoblot that led us to the discovery of a JAK/STAT3 signaling inhibitor with potent antitumor activity. JSI-124 blocked activation of STAT3 in several human cancer cell lines that contain high levels of constitutively activated tyrosine-phosphorylated STAT3 and subsequently inhibited STAT3 DNA-binding activity and STAT3-dependent gene expression. This JAK/STAT3 signaling disrupter is highly selective in that other oncogenic and tumor survival pathways were not affected. The ability of JSI-124 to increase mouse survival, to inhibit growth in mice of human and murine tumors and oncogene-transformed NIH 3T3 tumors with high levels of constitutively activated STAT3, and to not inhibit the growth of those tumors with low levels of activated STAT3 further validates interference with STAT3 signaling as a sound approach to cancer chemotherapy. Present studies are geared toward evaluating the antitumor efficacy of JSI-124 in a broader spectrum of human cancer cell lines and identifying the biochemical target of JSI-124.

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Discovery of JSI-124 (Cucurbitacin I), a Selective Janus Kinase/Signal Transducer and Activator of Transcription 3 Signaling Pathway Inhibitor with Potent Antitumor Activity against Human and Murine Cancer Cells in Mice

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