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# Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization

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## Summary

**Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors that transmit signals from the cell membrane to the nucleus. One family member, STAT3, is constitutively activated by aberrant upstream tyrosine kinase activities in a broad spectrum of cancer cell lines and human tumors. Screening of chemical libraries led to the identification of Stattic, a nonpeptidic small molecule shown to selectively inhibit the function of the STAT3 SH2 domain regardless of the STAT3 activation state in vitro. Stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 and increases the apoptotic rate of STAT3-dependent breast cancer cell lines. We propose Stattic as a tool for the inhibition of STAT3 in cell lines or animal tumor models displaying constitutive STAT3 activation.**

## Introduction

Upon activation of cytokine receptors or growth factor receptors, STATs are recruited via their SH2 domains and phosphorylated on a tyrosine residue adjacent to the SH2 domain by receptor-associated tyrosine kinases or the intrinsic kinase activity of growth-factor receptors [1]. STATs can also be phosphorylated by constitutively active nonreceptor tyrosine kinases (e.g., v-Src). Tyrosine-phosphorylated STATs form homo- or heterodimers via reciprocal phosphotyrosine (pTyr)-SH2 interactions and translocate to the nucleus, where they bind to their respective DNA-binding motifs within the promoter elements of target genes and induce transcription. Since the SH2 domain is required for both tyrosine-phosphorylation and dimerization of STATs, the most logical approach toward inhibition of any STAT, including STAT3, would impair the function of its SH2 domain [2]. This should not only inhibit STAT3 activation but also prevent dimerization of any STAT3 molecules that escape inhibition of activation (Figure 1). Direct inhibition of STAT3 itself is also less likely to result in unintentional inhibition of additional signaling pathways than the targeting of upstream molecules. While the principle feasibility to inhibit STAT3 in cells by a ligand for its SH2 domain has been demonstrated with fusion peptides carrying hydrophobic or basic peptide sequences to achieve cell permeability [3, 4] and a peptide-derived molecule [5], the nature of these agents required their use at relatively high concentrations in tissue culture, and their conversion to nonpeptidic molecules is likely to be difficult.

STAT3 has received particular attention among the seven members of the STAT family since it is considered a candidate target for the treatment of human tumors [6, 7]. It is constitutively activated by aberrant upstream tyrosine kinase activities in a broad spectrum of cancer cell lines and human tumors [8, 9]. Inhibition of STAT3 signaling by a dominant-negative mutant [10, 11], antisense approaches [12], decoy oligonucleotides [13–15], siRNAs [16–18], or G-quartet oligonucleotides [19, 20] has been demonstrated to suppress tumor growth and to induce apoptosis in cancer cells. While numerous small molecules have been reported to inhibit STAT3 signaling, the vast majority of them act on targets other than STAT3. STAT3 signaling inhibitors with unknown targets [21, 22] include members of the cucurbitacin family of natural products [23–25]. A recent study suggested that the JAK/STAT3 pathway inhibitor cucurbitacin I also affected the actin cytoskeleton in nontumor cells [26]. Curcumin, another indirect natural product inhibitor of STAT3 signaling [27], has also been identified as an inhibitor of numerous additional signaling pathways [28]. Similarly, magnolol was shown to inhibit signaling via STAT3 and other pathways [29]. In addition to these inhibitors with unknown targets, a number of natural products have been shown to inhibit kinases upstream of STAT3. They include indirubin, a constituent of a Chinese herbal prescription used for treatment of chronic myelogenous leukemia [30], and a known inhibitor of cyclin-dependent kinases [31], which was shown to inhibit STAT3 signaling in breast cancer cells by inhibiting upstream kinase activity, presumably that of c-Src [32]. A similar mechanism of action was suggested for the natural product Resveratrol [33]. Flavopiridol, a cytotoxic compound with several identified targets, was shown to inhibit STAT3 signaling, presumably by intercalation into DNA [34]. Among the small molecules that are thought to bind to STAT3 directly, galiellalactone, a natural product with weak activity against the de novo synthesis of $\alpha$-amylases, proteases, and phosphatases in embryoless halves of wheat seeds [35], was reported to inhibit interleukin-6 (IL-6)-mediated STAT3 signaling [36]. The compound was assumed to bind to the DNA-binding domain of dimeric STAT3, possibly by covalently modifying a cysteine residue in the STAT3 DNA-binding domain. A similar mechanism of action was discussed for a platinum complex that was reported to bind to STAT3 and to inhibit STAT3 signaling [37]. Other platinum complexes were also reported to inhibit STAT3 signaling [38].

To date, the only report of a nonpeptidic small-molecule reported to inhibit STAT3 by direct binding to its SH2 domain used virtual screening to identify candidate compounds with an increased likelihood of binding to the STAT3 SH2 domain. One of these compounds,

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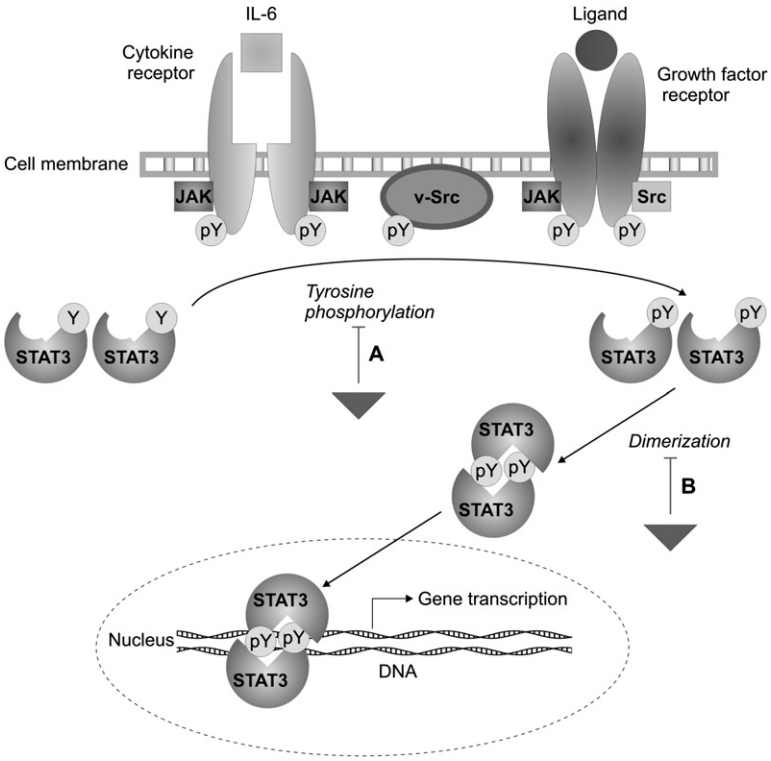


**Figure 1. Inhibition of the Function of the STAT3 SH2 Domain Would Selectively and Effectively Inhibit STAT3 Functions**

A substance that blocks the function of the STAT3 SH2 domain would inhibit binding of STAT3 to activated cell surface receptors (e.g., the IL-6 receptor) and constitutively activated tyrosine kinases (e.g., v-src) (inhibition mechanism A, inhibition is indicated by ⊥) and also inhibit dimerization of STAT3 molecules that escape inhibition of phosphorylation (inhibition mechanism B) and could therefore be a particularly efficient inhibitor.

STA-21, was shown to inhibit DNA binding of prephosphorylated STAT3 and to display STAT3-dependent cellular effects [39]. While STA-21 could be docked into the STAT3 SH2 domain [40] by computational methods, biochemical experiments that demonstrate its effectiveness against the function of the SH2 domain of unphosphorylated STAT3 in vitro, or its effect on STAT3 phosphorylation of serum-deprived cells in response to cytokine stimulation, were not provided. Here, we report the discovery of Stattic, to our knowledge the first nonpeptidic small molecule demonstrated to inhibit the function of the STAT3 SH2 domain regardless of the STAT3 phosphorylation state in vitro. Stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 and induces apoptosis in STAT3-dependent cancer cell lines.

## Results and Discussion

Screening of chemical libraries consisting of a diverse collection of 17,298 substances in a fluorescence polarization-based binding assay [41] resulted in the identification of 144 substances that inhibited binding of a fluorescein-labeled, phosphotyrosine-containing peptide to the STAT3 SH2 domain by more than 60%. One of these substances was able to inhibit interleukin-6 (IL-6)-induced nuclear translocation of STAT3 as assessed by immunolocalization and to inhibit DNA binding of prephosphorylated STAT3 in electrophoretic mobility shift assays (EMSA) with selectivity over STAT1. This compound, 6-nitro-benzo[*b*]thiophene-1,1-dioxide **1** (Figure 2A), was dubbed Stattic (for *STAT t*hree *i*nhibitory *c*ompound, and based on its ability to retain STAT3 in the cytosol). Stattic inhibited binding of a phosphotyrosine-containing peptide derived from the gp130 receptor to the STAT3 SH2 domain in a strongly temperature-dependent manner: while only weak activity was observed at 22°C, the compound displayed moderate activity at 30°C and high potency at the physiologically relevant temperature of 37°C (apparent $IC_{50}$ value after 1 hr of incubation: 5.1 ± 0.8 μM) (Figure 2B). Stattic only had a very weak effect on binding of a tyrosine-phosphorylated peptide to the SH2-domain of the tyrosine kinase Lck (Figure 2C). Dimerization of two other dimeric transcription factors (c-Myc/Max and Jun/Jun) was not significantly inhibited [42]. Further specificity analysis involving the protein family members STAT1 and STAT5b was performed at an intermediate temperature of 30°C since the limited thermal stability of the STAT1 and STAT5b proteins expressed from *E. coli* prohibited analysis at 37°C. Reduced inhibition of the SH2 domains of STAT1 (78% similar) and STAT5b (59% similar) by Stattic correlated well with the degree of similarity in the SH2 domains between the respective proteins and STAT3 (Figure 2D). Analysis of structure-activity relationships of Stattic and three related compounds performed at 37°C revealed the importance of the nitro group since its exchange by an $NH_2$-group in compound **2** or by hydrogen in compound **3** (Figure 2A) resulted in loss of activity in the fluorescence polarization assay (**2**, 8% ± 4%; **3**, 18% ± 5% inhibition at 100 μM after 1 hr of incubation). Both compounds **2** and **3** were part of the screening library but were not registered as hits due to their low activities. A similar loss in activity was observed when the double bond of the vinyl sulfone moiety was saturated (compound **4**, 12% ± 2% inhibition at 100 μM after 1 hr of incubation) (Figure 2A).

The temperature dependence of the inhibition, in combination with the observed importance of the double bond conjugated to the sulfonyl group, led us to

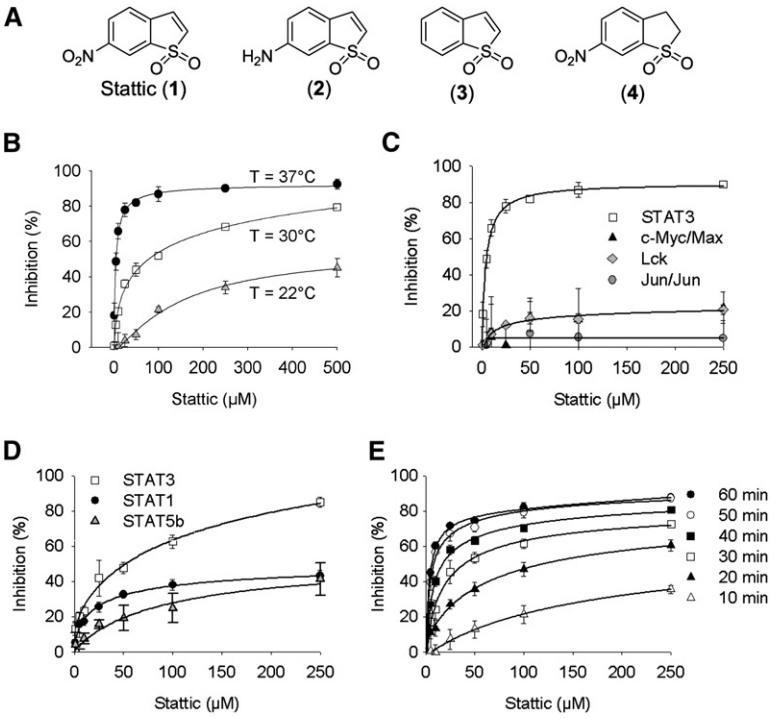


Figure 2. Stattic Selectively Inhibits STAT3 In Vitro

(A) Structure of Stattic (**1**) and inactive derivatives **2–4**.

(B) Temperature dependence of the inhibition of binding between 5-carboxyfluorescein-GY($PO_3H_2$)LPQTV-$NH_2$ and STAT3 by Stattic assayed by fluorescence polarization. Error bars represent standard deviations (SD).

(C) Inhibition of binding of fluorescein-labeled phosphopeptides to the SH2 domains of STAT3 and Lck and of fluorescein-labeled oligonucleotides to c-Myc/Max and Jun/Jun dimers, by Stattic at 37°C assayed by fluorescence polarization. The inhibition curve for c-Myc/Max is masked by the inhibition curve for Lck. Error bars represent SD.

(D) Inhibition of binding of fluorescein-labeled phosphopeptides to the SH2 domains of STAT3, STAT1, and STAT5b by Stattic at 30°C assayed by fluorescence polarization. Error bars represent SD.

(E) Time dependence of the inhibition of binding between 5-carboxyfluorescein-GY($PO_3H_2$)LPQTV-$NH_2$ and STAT3 by Stattic at 37°C assayed by fluorescence polarization. Error bars represent SD.

investigate whether inhibition by Stattic was irreversible. Inhibition of Stat3 was in fact time dependent, which pointed toward an irreversible mode of action (Figure 2E). The observation that Stattic's activity was strongly reduced in the presence of 2 mM dithiothreitol (DTT) in the fluorescence polarization assay buffer can be explained by a nucleophilic attack of DTT on Stattic (data not shown). The presence or absence of DTT in the fluorescence polarization assay buffer had no significant effect on the STAT3 assay (see Figure S1 available with this article online). Final evidence for a covalent modification of STAT3 by Stattic could for example arise from the identification of modified residues via mass spectrometry-based approaches. While it is tempting to speculate that such modified residues are nucleophilic amino acids within or near the STAT3 SH2 domain, which are covalently modified in a Michael addition, initial attempts to identify such an amino acid by mass spectrometry have remained inconclusive.

Electrophoretic mobility shift assays (EMSA) performed at 37°C using nuclear extracts from EGF-stimulated cells, which contain prephosphorylated STAT3 and STAT1, demonstrated inhibition of DNA binding of STAT3 homodimers by Stattic at a concentration of 10 μM, while binding of STAT1 homodimers to the same DNA probe was not significantly inhibited at concentrations of up to 200 μM (Figure 3, left panel). Control compounds **2–4** did not significantly inhibit DNA binding of STAT dimers at concentrations of 100 and 200 μM (Figure 3, right panel). A potential explanation for the significantly enhanced selectivity of Stattic for STAT3 over STAT1 in EMSA, as compared to the fluorescence polarization assay (see Figure 2D), is that the observed activity profile in the fluorescence polarization assay at 30°C does not reflect the activity profile at the physiologically relevant temperature of 37°C.

Specific cellular activity is a key requisite for the suitability of any interfering agent as a research tool. To

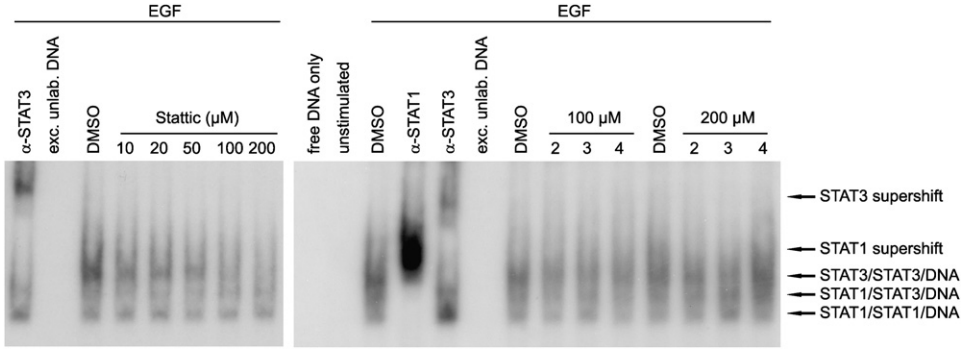


Figure 3. Stattic Inhibits STAT3 Dimerization and DNA Binding In Vitro

Inhibition of STAT3 homodimerization and DNA binding, but not STAT1 homodimerization and binding to the same DNA probe, in EMSA by Stattic at 37°C (left panel). Control compounds **2–4** do not significantly inhibit DNA binding of STAT dimers at 100 and 200 μM (right panel).

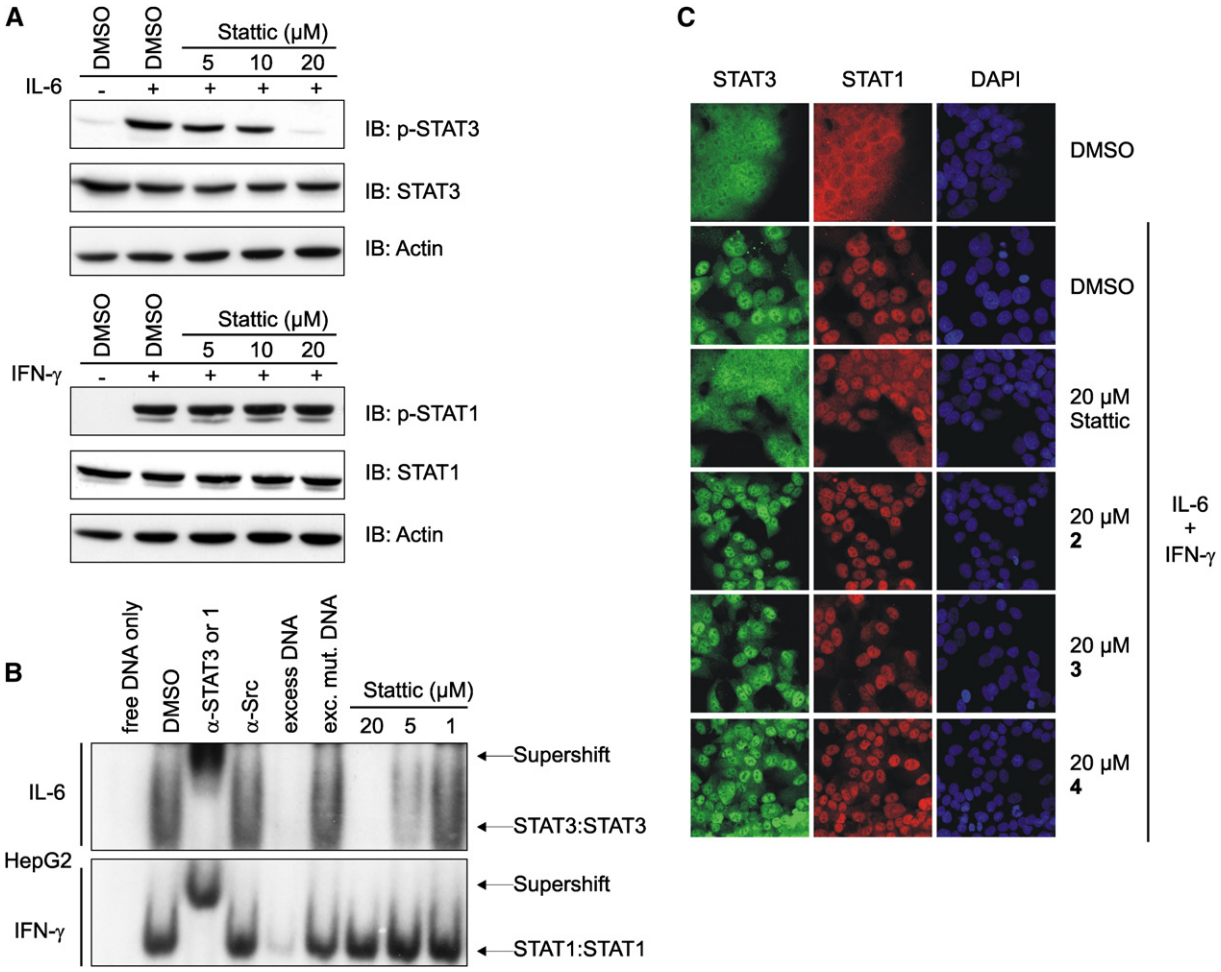


Figure 4. Stattic Selectively Inhibits STAT3 Activation and Nuclear Translocation in HepG2 Cells

(A) Stattic was added to the media of serum-deprived HepG2 cells 1 hr prior to stimulation by IL-6 (for STAT3) or IFN-γ (for STAT1). Upon cell lysis, phosphorylation of STAT3 Tyr705 and STAT1 Tyr701 was analyzed by western blot.

(B) Cells were treated as in (A), but instead of whole-cell lysates, nuclear lysates were prepared and analyzed in EMSA by using the same DNA probe for both STAT3 and STAT1.

(C) Serum-deprived HepG2 cells grown on 16-well Nunc LabTek slides were incubated with Stattic and compounds **2–4** for 1 hr prior to costimulation by IL-6 and IFN-γ. Cells were fixed, permeabilized, and stained for STAT3 and STAT1.

investigate Stattic's suitability as a tool for the analysis of STAT3 biological functions in vivo, we tested it in two unrelated cellular assay systems. HepG2 liver carcinoma cells were induced with either IL-6, leading to selective phosphorylation of STAT3 at Tyr705, or IFN-γ, for selective activation of STAT1 at Tyr701, as a stringent specificity control. Analysis of the tyrosine phosphorylation states revealed that preincubation of serum-starved HepG2 cells with 20 μM of Stattic led to a selective reduction of phosphorylation of STAT3 Tyr705, while activation of STAT1 Tyr701 remained unchanged (Figure 4A). The inactivity of the derivatives **2–4** in this assay correlated well with their lack of activity in vitro (data not shown). EMSA performed on nuclear lysates obtained from starved HepG2 cells preincubated with 20 μM of Stattic and subsequently stimulated with IL-6 and IFN-γ showed that STAT3 DNA-binding activity had been lost, while STAT1 DNA binding was not inhibited (Figure 4B). Since tyrosine phosphorylation and dimerization of STATs is a prerequisite for their cytokine-induced nuclear translocation, we would expect a selective STAT3 SH2 domain inhibitor to inhibit IL-6-induced STAT3 nuclear translocation, but not IFN-γ-induced STAT1 translocation. Accordingly, immunofluorescence microscopy showed that cytokine-induced nuclear translocation of STAT3 was inhibited after preincubation with 20 μM of Stattic, while the corresponding STAT1 translocation was only minimally affected. Consistent with the previous results, derivatives **2–4** were inactive in this assay (Figure 4C).

The breast cancer cell lines MDA-MB-231 and MDA-MB-435S display constitutive phosphorylation of STAT3 Tyr705 and have been reported to undergo apoptosis upon inhibition of STAT3 signaling by small molecules [25, 37, 38]. In contrast, MDA-MB-453 breast cancer cells do not show elevated STAT3 pTyr705 levels and do not enter apoptosis by inhibition of STAT3 signaling. Western blot analysis revealed that phosphorylation of STAT3 Tyr705 is reduced in MDA-MB-231 and MDA-MB-435S cells in the presence of 10 or 20 μM Stattic (Figure 5A). The activation state of the tyrosine kinases JAK1, JAK2, and c-Src, which are considered to be

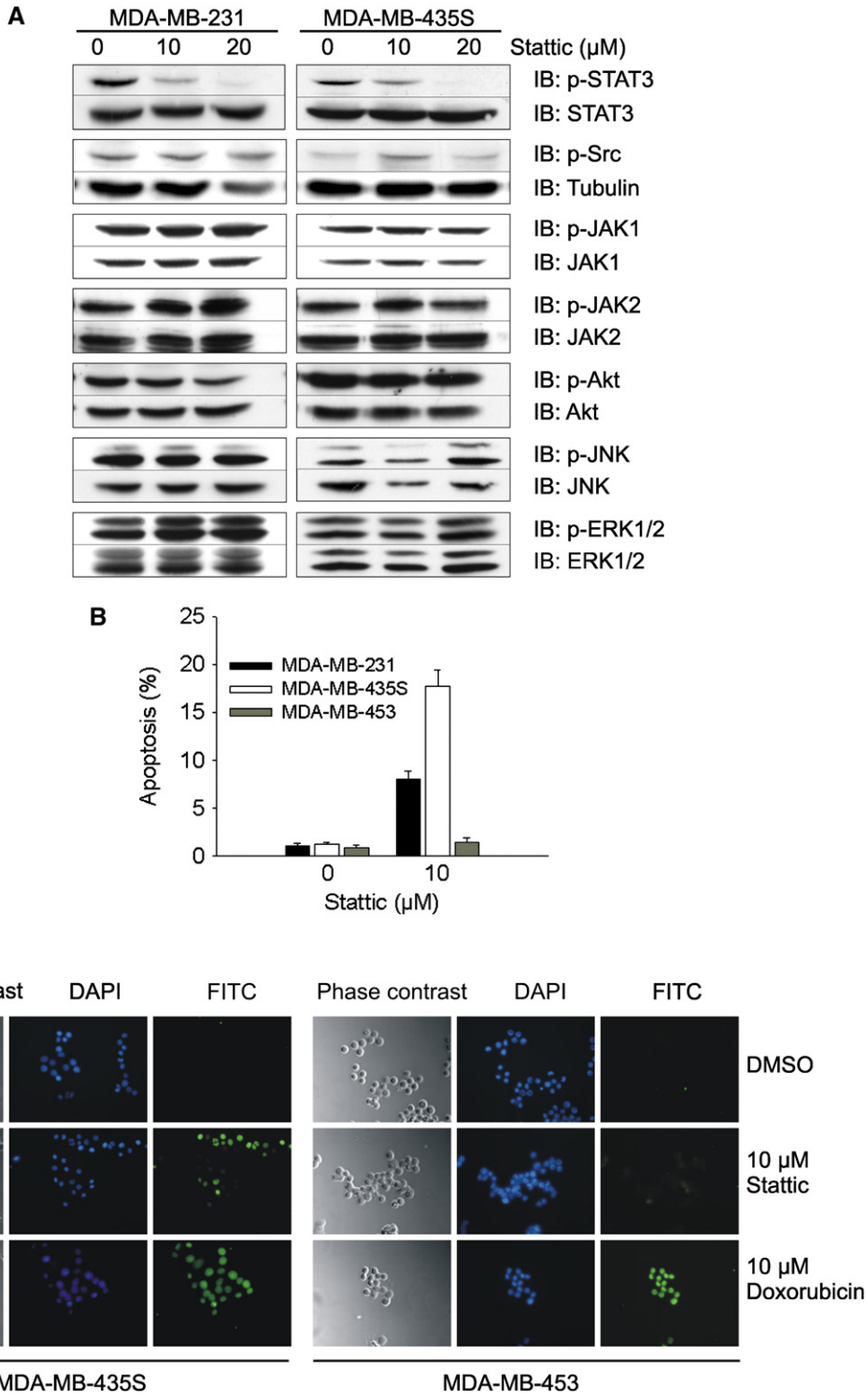


Figure 5. Stattic Selectively Induces Apoptosis in STAT3-Dependent Breast Cancer Cell Lines

(A) MDA-MB-231 and MDA-MB-435S cells were treated with Stattic for 2 hr at the indicated concentrations. The activation state of STAT3, STAT3-activating kinases, and other signaling molecules was analyzed by western blot analysis of whole-cell lysates with phospho-specific antibodies.

(B) Incubation with 10 μM Stattic for 24 hr induces apoptosis in STAT3-dependent breast cancer cell lines MDA-MB-231 and MDA-MB-435S, but not in STAT3-independent MDA-MB-453 cells, as judged by the sub-G1 peak obtained by flow cytometry. Error bars represent SD.

(C) Incubation with 10 μM Stattic for 24 hr induces apoptosis in the STAT3-dependent breast cancer cell line MDA-MB-435S, but not in STAT3-independent MDA-MB-453 cells, as judged by TUNEL assay.

responsible for phosphorylation of STAT3 Tyr705, was not significantly inhibited by the presence of 10 or 20 μM Stattic, as judged by western blot analysis using phospho-specific antibodies against tyrosine residues that are thought to be autophosphorylated by the respective kinases themselves. This is consistent with the idea that the compound exerts its cellular effects by preventing the activating kinases from binding to the STAT3 SH2 domain, without interfering with their enzymatic activities. The basal activation states of other signaling pathways were not significantly inhibited. Flow cytometry-based analysis of cell lysates treated with 10 μM of Stattic showed a 7.6-fold increase in the sub-G1 population induced in the STAT3-dependent MDA-MB-231 cells, and even a 14.4-fold increase in MDA-MB-435S cells. In contrast, only a slight increase (1.7-fold) in the sub-G1 population was induced in the STAT3-independent MDA-MB-453 cells, suggesting the induction of apoptosis in a STAT3-dependent manner (Figure 5B). The minor induction of apoptosis in MDA-MB-453 cells might perhaps be due to reactive oxygen species (ROS), which have been reported to be caused by some related benzo[*b*]thiophenesulphonamides [43, 44]. Consistent with these results, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) revealed significant apoptosis in MDA-MB-435S cells after treatment with 10 μM Stattic for 24 hr (Figure 5C) and, to lesser extent, in MDA-MB-231 cells (data not shown), whereas no significant change in the apoptotic rate could be observed in MDA-MB-453 cells (Figure 5C).

The tight temporal control of the inhibition of STAT3 by Stattic (see Experimental Procedures for the immunofluorescence assay shown in Figure 4) could make Stattic an attractive tool for the analysis of STAT3 functions that were hitherto impossible to perform in genetically unmodified systems. For example, Stattic could be used for the analysis of STAT3 functions within a given phase of the cell cycle of synchronized cell populations or for the analysis of immediate-early responses in gene regulation of STAT3-dependent cancer cells upon inactivation of STAT3. Hence, inhibition of STAT3 by Stattic could complement siRNA-based approaches toward depletion of STAT3, the temporal control of which is limited by the half-life time of the pre-existing STAT3 molecules (8.5 hr) [45]. Moreover, Stattic should be widely applicable in studying the functions of STAT3 in cellular systems and animal models, especially those that display constitutive activation of STAT3.

## Significance

**The transcription factor STAT3 is constitutively activated in most human tumors. Inhibition of STAT3 signaling has been demonstrated to increase the apoptotic rate of STAT3-dependent tumor cells. Since the function of the STAT3 SH2 domain is crucial for both STAT3 activation and nuclear translocation, STAT3 signaling can be inhibited by small molecules that impair the function of the STAT3 SH2 domain. Using high-throughput screening of diverse chemical libraries, we have identified Stattic, to our knowledge the first nonpeptidic small molecule demonstrated to selectively inhibit the function of the STAT3 SH2 domain regardless of the STAT3 activation state in vitro. Stattic inhibits binding of a physiologically relevant tyrosine-phosphorylated peptide motif to the STAT3 SH2 domain and inhibits dimerization and DNA binding of STAT3 with selectivity over the inhibition of its family member STAT1. It potently and selectively inhibits STAT3 activation and nuclear translocation, and selectively induces apoptosis of STAT3-dependent cancer cell lines. Stattic thus reveals itself as a potentially useful tool for the inhibition of STAT3 activation and activity and for the disruption of aberrantly high STAT3 activity in cancer cell lines and tumor models. The data presented should encourage the scientific community to explore the development of cancer drugs that act by inhibition of the SH2 domain of STAT3. The current perception that "druggable" proteins are generally restricted to enzymes and receptors should thus be reconsidered.**

## Experimental Procedures

### Antibodies

The following antibodies were used: rabbit anti-STAT3 pTyr705, rabbit anti-STAT1 pTyr701, rabbit anti-STAT1 (for western blots), rabbit anti-JAK2 (pTyr1007/pTyr1008), rabbit anti-p44/p42 MAP kinase (Thr202/Tyr204), rabbit anti-JNK (pThr183/pTyr185), and rabbit anti-pAkt (pSer473) from Cell Signaling; rabbit pJAK1 (pTyr1022/pTyr1023) from BioSource; rabbit anti-STAT3, rabbit anti-src, rabbit anti-JNK1, rabbit anti-ERK1/2, and rabbit anti-Akt1/2 from Santa Cruz; rabbit anti-JAK2 from Upstate; mouse anti-JAK1 and mouse anti-STAT1 (for immunofluorescence) from BD Biosciences; and rabbit anti-actin and mouse anti-tubulin from Sigma. Secondary horseradish peroxidase (HRP)-conjugated antibodies were from DakoCytomation. Secondary Alexa Fluor 488 goat anti-rabbit antibody and Alexa Fluor 546 goat anti-mouse $IgG_1$ antibody were from Molecular Probes.

### Chemical Substances

Compounds **1**–**3** were purchased from Maybridge. Compound **4** was synthesized by catalytic hydrogenation of **1** as described in the Supplemental Data available online.

### High-Throughput Screening and Fluorescence Polarization Assays

8,298 compounds from Chemical Diversity and 9,000 compounds from Maybridge were tested in a fluorescence polarization assay that analyzes binding of small molecules to the STAT3 SH2 domain. Screening was performed at approximately 30°C. The specificity of screening hits was validated in analogous assays for binding of the test compounds to the SH2 domains of STAT1 [46], STAT5, and Lck [41]. The final concentration of buffer components used for all FP assays was 10 mM HEPES (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 50 mM NaCl, and 10% DMSO. The absence of dithiothreitol is essential for inhibitory activity. The sequences of the peptides were: STAT3, 5-carboxyfluorescein-GY($PO_3H_2$)LPQTV-$NH_2$; STAT1, 5-carboxyfluorescein-GY($PO_3H_2$)DKPHVL; STAT5, 5-carboxyfluorescein-GY($PO_3H_2$)LVLDKW; and Lck, 5-carboxyfluorescein-GY($PO_3H_2$)EEIP. Peptides were >95% pure. For specificity analysis at 30°C, proteins were used at 150 nM (STAT1, STAT3, and STAT5). For specificity analysis at 37°C, proteins were used at 370 nM (STAT3) or 100 nM (Lck). Proteins were incubated with test compounds in Eppendorf tubes at the indicated temperatures for 60 min prior addition of the respective 5-carboxyfluorescein labeled peptides (final concentration: 10 nM). Analysis of c-Myc/Max and Jun/Jun dimerization and DNA binding at 37°C was performed as described [42], but in the absence of DTT. Before measurement at room temperature, the mixtures were allowed to equilibrate for at least 30 min. Test compounds were used at the indicated concentrations diluted from a 20× stock in DMSO. Binding curves and inhibition curves were fitted with SigmaPlot (SPSS Science Software GmbH). All competition curves were repeated three times in independent experiments. For

the analysis of time dependence of the inhibition, the components were mixed from stock solutions kept at 0°C and then incubated at 37°C. Aliquots were taken at the indicated time points.

### Electrophoretic Mobility Shift Analysis

To obtain the prephosphorylated STAT proteins used in Figure 3, NIH3T3 cells stably transformed with the EGF receptor were stimulated with 100 ng/ml EGF for 10 min, and nuclear lysates were prepared essentially as described [47]. Three microliters of nuclear protein (approximately 8 μg/μl) was added to 2 μl of 5× binding buffer (65 mM HEPES [pH 7.9], 0.75 mM EDTA, 40% glycerol), 1 μl test compound dissolved in DMSO from a 10× stock, and 1 μg of poly(dI-dC) (Roche Applied Science). For competition binding assays, unlabeled oligonucleotide was added to the reaction in 1000-fold molar excess. For a supershift control, 1 μg anti-STAT3 antibody or 1 μg anti-STAT1 antibody was added to the reaction mixtures. Total reaction volume was 10 μl. All reactions were incubated at 37°C for 2 hr prior to addition of the [$\gamma$-$^{32}$P]ATP-labeled oligonucleotides (upper strand sequence: [hSIE], 5′-AGCTTCATTTCCCGTAAATCCCTA-3′) for 20 min at room temperature. Protein-DNA complexes were resolved on a 4% acrylamide gel (45 mM Tris-borate, 1 mM EDTA), and gels were dried before autoradiography. To assess the effect of the test compounds on STAT phosphorylation as shown in Figure 4B, HepG2 cells were treated with compound for 1 hr (final DMSO concentration of 1%) and then stimulated with 50 ng/ml IL-6 for 30 min (for STAT3) or 50 ng/ml IFN-$\gamma$ for 15 min (STAT1). Nuclear lysates were prepared essentially as described [36] and incubated with the hSIE oligonucleotides as outlined above. As controls, 1 μg anti-STAT3 antibody, 1 μg anti-STAT1 antibody, 1 μg anti-Src antibody, five molar equivalents of unlabeled hSIE oligonucleotides or mutated hSIE oligonucleotides (5′-AGCTTCATTTCCCTTAAATCCC TA-3′) were added to the respective reaction mixtures.

### Immunofluorescence Assay

HepG2 cells were grown in 16-well LabTek slides (Nunc) and subjected to serum-free MEM medium for 24 hr. After incubation with the indicated concentration of compound for 1 hr at a final DMSO concentration of 1%, cells were stimulated with 50 ng/ml IL-6 and 50 ng/ml IFN-$\gamma$ for 40 min. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized for 10 min with 1% Triton X-100 in PBS. After blocking with 1% goat serum, simultaneous incubation with antibodies against STAT3 and STAT1 for 1 hr was followed by simultaneous incubation with both secondary antibodies for 1 hr. Cells were counterstained with DAPI. Cells were imaged by confocal microscopy at 40× magnification.

### Analysis of Apotosis by Flow Cytometry

MDA-MB-231, MDA-MB-435S, and MDA-MB-453 cells were seeded at $5 \times 10^4$ cells in 6-well plates (Corning), grown for 24 hr before adding DMSO or Stattic (final DMSO concentration 0.1%), and then incubated with the inhibitor for 24 hr. All cells were collected and resuspended in buffer (0.1% sodium citrate, 0.1% Triton X-100, 20 μM propidium iodide) and incubated for 3 hr before $10^4$ cells per sample were analyzed by flow cytometry with a FACSCalibur (BD Biosciences) equipped with a 488 nm laser.

### Analysis of Apotosis by TUNEL

MDA-MB-231, MDA-MB-435S, and MDA-MB-453 cells were seeded in 16-well LabTek slides (NUNC) at 4,000 cells per well and allowed to grow for 24 hr. The cells were then incubated with either 1% DMSO, 10 μM Stattic, or 10 μM doxorubicin for an additional 24 hr. Cells were washed with PBS, fixed with 4% paraformaldehyde in PBS at room temperature for 60 min, and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 2 min on ice. DNA strand breaks were visualized by using the In Situ Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer's instructions. Cells were then stained with DAPI. The slides were mounted with a coverslip by using Fluoromount G. Pictures were taken on a Zeiss Axioplan 2 imaging microscope with 40× magnification.

### Supplemental Data

Supplemental Data describing the synthesis of compound **4**, one experimental figure, tissue culture conditions, and western blot analysis are available online at http://www.chembiol.com/cgi/content/full/13/11/1235/DC1/.

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