ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

A Small Molecule Compound Targeting STAT3 DNA-Binding Domain Inhibits Cancer Cell Proliferation, Migration, and Invasion

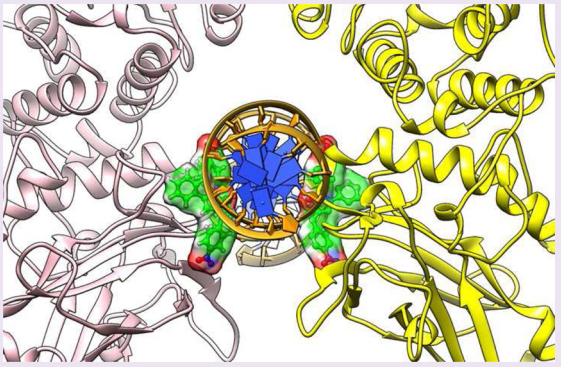
Wei Huang,[†,∥] Zizheng Dong,[†,∥] Fang Wang,[†,∥] Hui Peng,[†] Jing-Yuan Liu,*[,†,§] and Jian-Ting Zhang*[,†,‡]

[†]Department of Pharmacology and Toxicology and [‡]IU Simon Cancer Center, Indiana University School of Medicine, 980 W. Walnut Street, Indianapolis, Indiana 46202, United States

[§]Department of Computation and Information Science, Indiana University Purdue University Indianapolis, 723 W. Michigan St., Indianapolis, Indiana 46202, United States

Supporting Information

ABSTRACT: Signal transducer and activator of transcription 3 (STAT3) plays important roles in multiple aspects of cancer aggressiveness including migration, invasion, survival, self-renewal, angiogenesis, and tumor cell immune evasion by regulating the expression of multiple downstream target genes. STAT3 is constitutively activated in many malignant tumors and its activation is associated with high histological grade and advanced cancer stages. Thus, inhibiting STAT3 promises an attracting strategy for treatment of advanced and metastatic cancers. Herein, we identified a STAT3 inhibitor, inS3-54, by targeting the DNA-binding domain of STAT3 using an improved virtual screening strategy. InS3-54 preferentially suppresses proliferation of cancer over non-cancer cells and inhibits migration and invasion of malignant cells. Biochemical analyses show that inS3-54 selectively inhibits STAT3 binding to DNA without affecting the activation and dimerization of STAT3. Furthermore, inS3-54 inhibits expression of STAT3 downstream target genes and STAT3 binding to chromatin in situ. Thus, inS3-54 represents a novel probe for development of specific inhibitors targeting the DNA-binding domain of STAT3 and a potential therapeutic for cancer treatments.



Signal transducers and activators of transcription 3 (STAT3), a member of the Janus kinase (JAK)/STAT signaling pathway, is a central transcription factor that is activated by phosphorylation of a conserved tyrosine residue (Tyr705) in response to extracellular cytokines and growth factors.[1] Once activated, STAT3 dimerizes and translocates into the nucleus to induce transcription of downstream target genes.[2] Overexpression and/or constitutive activation of STAT3 has been detected in a number of human malignancies including lung and breast cancers.[3−5] Subcutaneous injection of cells harboring constitutively activated STAT3 (STAT3c) resulted in tumor formation.[6] STAT3c overexpression in mouse alveolar type II epithelial cells led to lung inflammation and consequently spontaneous lung bronchoalveolar adenocarcinoma.[7] Furthermore, inhibition of STAT3 expression using antisense oligonucleotides significantly impaired the growth of human and mouse nucleophosmin-anaplastic lymphoma kinase tumors in xenograft models.[8] Thus, STAT3 is an attractive target for anticancer drug discovery.[9]

Various STAT3 inhibitors have been identified in the past[10−12] including peptidomimetics[13,14] and small molecule compounds designed from the peptidomimetics[15] or via high-throughput[16,17] and virtual screening.[18,19] Some of these inhibitors suppressed tumor growth in vivo.[20] Most of these STAT3 inhibitors were designed to target Src Homology 2 (SH2) domain, where the pTyr705 residue binds for activation and dimerization, which may not be able to inhibit STAT3 completely considering that monomeric[21,22] and unphosphorylated dimeric[23] STAT3 may also be functional. Thus, inhibiting the DNA-binding activity of STAT3 regardless of its phosphorylation and dimerization status may represent a better approach. However, disrupting protein−DNA interactions with small molecules targeting DNA-binding domains (DBDs) of transcription factors is challenging due to potentially limited selectivity.[24,25]

In this study, we demonstrate that inhibition of STAT3 function by targeting its DBD is a viable approach using an improved in-silico screening of a virtual compound database in combination with biochemical and cell biology analyses. We identified a small molecule compound that selectively inhibits the DNA-binding activity of STAT3 and expression of STAT3 downstream target genes and suppresses cancer cell proliferation, migration, and invasion. Together, we conclude that the DBD of STAT3 can be targeted for drug discovery.

Received: August 8, 2013
Accepted: March 12, 2014
Published: March 12, 2014

   dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188−1196

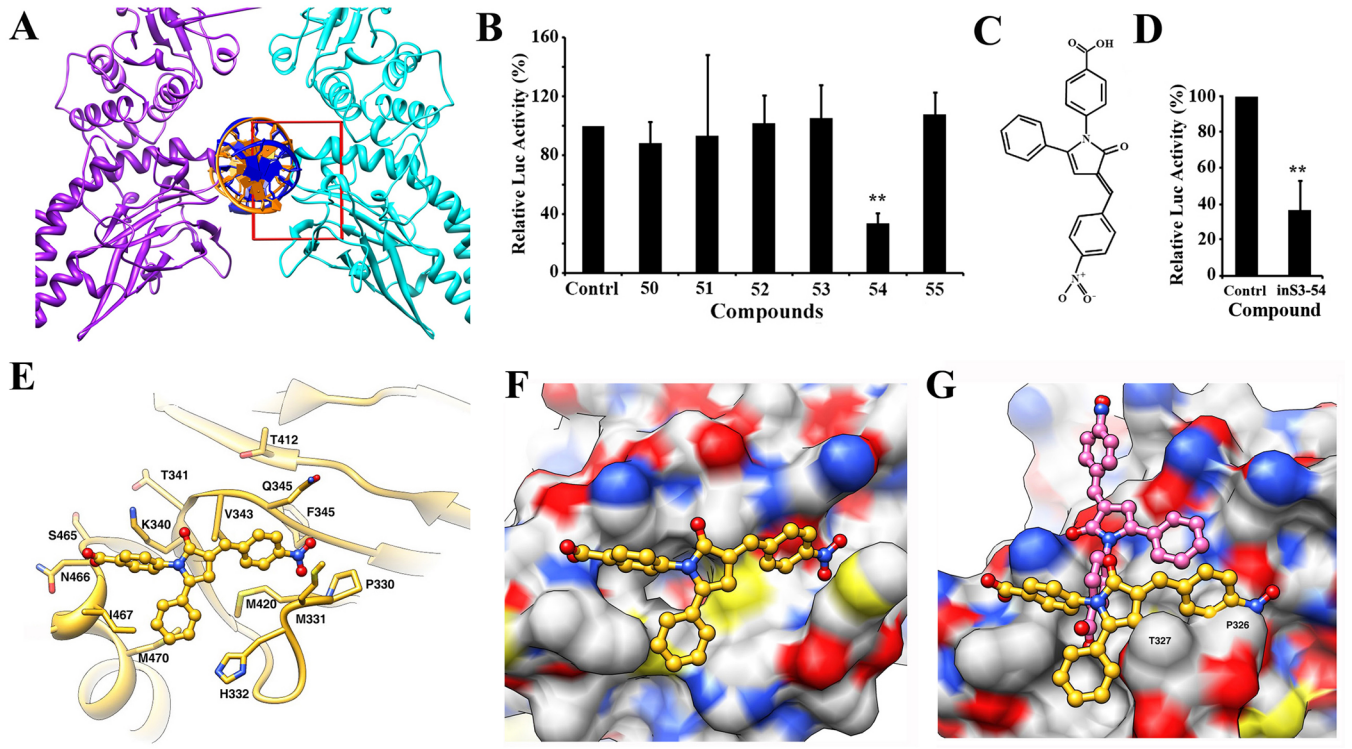


Figure 1. Schematic diagram and identification of inS3-54 by virtual screening. (A) DNA-STAT3 complex structure (PDB code: 1BG1). The red box shows the site for docking in one of the STAT3 subunits. (B) Luciferase activity assay of MDA-MB-231 cells harboring stable STAT3-dependent luciferase reporter following treatment with DMSO control or 20 μM compounds for 48 h. (C) Structure of inS3-54. (D) Luciferase activity assay of H1299 cells transiently transfected with STAT3-dependent luciferase reporter following treatment with DMSO control or 20 μM inS3-54 for 48 h. (E) Simulated average complex structure of inS3-54 in the DBD of STAT3. (F, G) Molecular surface of STAT3 (F) and STAT1 (G) complexed with inS3-54 from MD simulation with orientation shown in gold for STAT3 and pink for STAT1. Molecular surface is colored with gray for carbon, blue for nitrogen, red for oxygen, and yellow for sulfur. (**$p < 0.01$)

Table 1. inS3-54 Binding Free Energies and Energy Components in STAT1 and STAT3

	ΔE_{solute} ± SE (kcal/mol)		ΔG_{solv} ± SE (kcal/mol)			
	ΔE_{ele}	ΔE_{vdw}	ΔG_{es}	ΔG_{nes}	ΔE_{tot_ele} ± SE (kcal/mol)	ΔG_{bind} ± SE (kcal/mol)
STAT1	−139.6 ± 3.4	−23.1 ± 1.0	149.6 ± 2.4	−4.0 ± 0.1	10.1 ± 0.4	−17.1 ± 1.0
STAT3	−144.3 ± 4.4	−27.5 ± 0.9	148.0 ± 2.8	−4.6 ± 0.1	3.6 ± 0.8	−28.4 ± 0.9

RESULTS AND DISCUSSION

Identification of a STAT3 Inhibitor Targeting Its DBD.

To identify compounds that can directly block the interaction between STAT3 and its DNA substrate, we first examined the crystal structure of STAT3β-complexed with DNA and performed virtual docking of approximately 200,000 compounds to the DBD (Figure 1A). Top-scoring compounds with phosphate groups functioning similarly as phosphates in DNA were eliminated due to their potential inability to permeate into cells. The remaining 1000 top-scoring compounds were then docked onto the DBD of STAT1 to eliminate compounds that also bind to STAT1. The final list was shortened to 100 potentially specific candidates.

Of these 100 structurally diversified compounds, 57 chemical samples were obtained and tested for their ability to inhibit STAT3-dependent luciferase reporter expression in MDA-MB-231 cells. One of the compounds, no. 54, exhibited significant inhibitory activity (Figure 1B) in a dose- and time-dependent manner (Supplemental Figure S1A,B) with an IC_{50} of 13.8 ± 0.4 μM and the time required for 50% inhibition at 29.2 ± 4.7 h. This compound, 4-[(3*E*)-3-[(4-nitrophenyl)-methylidene]-2-oxo-5-phenylpyrrol-1-yl] benzoic acid (Figure 1C), was named inS3-54 and used to search the PubChem database of high-throughput screening for STAT3 inhibitors. No compound with the same structure was found.

To confirm the activity of the compound using resupplied material, to eliminate any issues of using a single stable clone harboring the reporter gene, and to ensure that inS3-54 is not cell-line-specific, we tested newly synthesized inS3-54 using H1299 cells transiently transfected with the STAT3-driven luciferase reporter gene. Figure 1D shows that a new batch of inS3-54 also significantly inhibits STAT3-dependent luciferase reporter expression in H1299 cells harboring transient reporter construct. Thus, the activity of inS3-54 is not derived from potential contamination in the original supply and is not dependent on cell line or transfection method used. Furthermore, inS3-54 did not inhibit the reporter expression driven by a p27 promoter[26] without STAT3-binding site (Supplemental Figure S1C), suggesting that inS3-54 inhibition of reporter expression is unlikely due to nonspecific effect on the reporter gene or due to cell death induced by inS3-54. Together, these observations suggest that inS3-54 is a good chemical probe.[27]

dx.doi.org/10.1021/cb500071v | *ACS Chem. Biol.* 2014, 9, 1188−1196

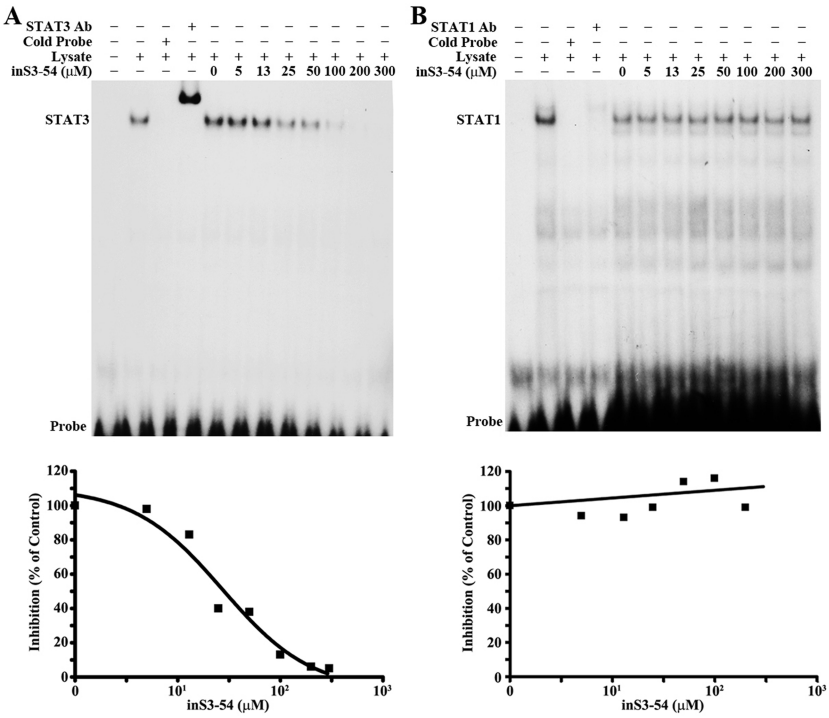


Figure 2. InS3-54 inhibits the DNA-binding activity of STAT3 but not STAT1. The effect of inS3-54 on the DNA binding activity of STAT3 (A) and STAT1 (B) was determined using EMSA and [^{32}P]-labeled double strand DNA probe and whole cell lysate from H1299 cells transiently transfected with FLAG-STAT3c or STAT1 cDNA.

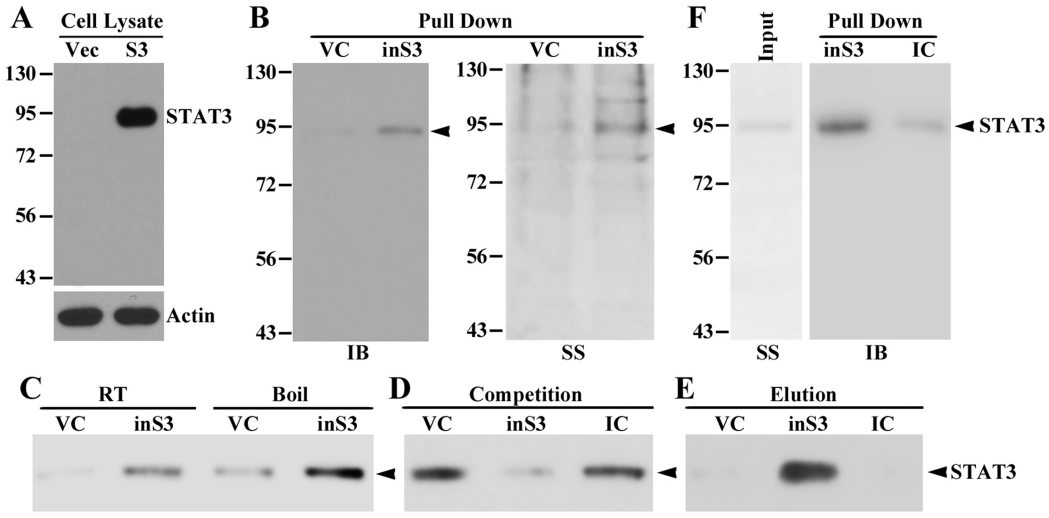


Figure 3. Binding of inS3-54 to STAT3. (A) Ectopic overexpression of FLAG-STAT3 (S3) in H1299 cells. Vec = vector control. (B) Pull-down assay of STAT3 from lysate of FLAG-STAT3-transfected H1299 cells using EAH-Sepharose 4B-conjugated without (vehicle control, VC) or with inS3-54 (inS3). Pull-down materials were separated using SDS-PAGE and Western blot analysis probed with STAT3 antibody (IB) or stained with silver (SS). (C) Pull-down materials using EAH-Sepharose 4B-conjugated without (VC) or with inS3-54 (inS3) were treated at room temperature (RT) or by boiling before separation on SDS-PAGE for Western analysis. (D) Competition of STAT3-binding to inS3-54-conjugated EAH-Sepharose 4B by excess free inS3-54 (inS3), an irrelevant compound (IC) control, or vehicle control (VC). (E) Elution of STAT3 bound to inS3-54-conjugated EAH-Sepharose 4B by inS3-54 (inS3), an irrelevant compound (IC) control, or vehicle control (VC). (F) Pull-down assay of purified STAT3 with input shown by silver staining (SS).

InS3-54 Selectively Inhibits the DNA-Binding Activity of STAT3. To determine the selectivity of inS3-54, we first performed molecular dynamics (MD) simulation and generalized born surface area (GBSA) analyses for the binding free energy (ΔG_{bind}) of inS3-54 docked in the DBD of STAT3 and STAT1. Table 1 shows that both STAT molecules have favorable electrostatic (ΔE_{ele}) and van der Waals (ΔE_{vdw}) interaction energy, although they are more favorable for STAT3 than STAT1. The energy from solvation (ΔG_{solv}) reverses these favorable energies for both proteins. However, the reversal effect is less for STAT3 than for STAT1. Consequently, the total ΔG_{bind} is much more favorable for STAT3 (−28.4 kcal/mol) than STAT1 (−17.1 kcal/mol). Considering the omitted

dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188−1196

entropy term, which is always unfavorable, inS3-54 may not bind to STAT1 or have a very low affinity.

Examination of the average simulated structures (Figure 1E) of inS3-54-bound STAT3 and STAT1 agrees with the calculated ΔG_{bind}. Contribution of hydrophobic interactions from STAT3 to inS3-54 binding is mainly from residues Met331, Val343, Met420, Ile467, and Met470. The amino groups of Lys340 and Asn466 stabilize the carboxyl group of inS3-54 by favorable electrostatic interactions. However, the orientation of inS3-54 docked in STAT1 (Figure 1G) is very different (Figure 1F). This binding mode in STAT1 likely results in an unfavorable ΔG_{bind}. Forcing inS3-54 to adopt the same orientation in STAT1 as in STAT3 results in clashes between inS3-54 and residues Pro326 and Thr327 of STAT1 (Figure 1G). Thus, it is unlikely that inS3-54 can bind to STAT1.

To verify the above findings and to determine the inS3-54 inhibition of the DNA-binding activity of STAT3 or STAT1, we performed electrophoretic mobility shift assay (EMSA) using a [^{32}P]-labeled probe and H1299 cells transiently transfected with FLAG-STAT3c or STAT1. As shown in Figure 2A, the specific binding of the DNA probe to STAT3 was demonstrated using supershift and competition analyses. InS3-54 inhibited the DNA-binding activity of STAT3 in a dose-dependent manner with an IC_{50} of ~20 μM, which is consistent with the cell-based reporter assay (see above). The specific binding of DNA probe to STAT1 as shown by interference of binding using cold probe and STAT1 antibody that is known to interfere DNA-binding activity of STAT1,[28,29] however, was not affected by inS3-54 up to 300 μM (Figure 2B). Thus, inS3-54 selectively inhibits the DNA-binding activity of STAT3 over STAT1.

Binding of inS3-54 to STAT3. To verify that inS3-54 can bind to STAT3, we took advantage of the carboxyl group of inS3-54 and conjugated it to EAH Sepharose. InS3-54-conjugated beads were then used to pull down STAT3 from FLAG-STAT3-transfected H1299 cells followed by Western blot analysis or silver staining. Figure 3A shows the expression of FLAG-STAT3. Figure 3B shows that inS3-54-conjugated beads successfully pull down STAT3, whereas the vehicle control beads do not. It is noteworthy that STAT3 bound to the inS3-54-conjugated beads was solubilized equivalently well by SDS sample buffer with or without heating (Figure 3C). Furthermore, pretreatment of cell lysates using excess free inS3-54, but not vehicle or an irrelevant compound, inhibited STAT3 pull-down by inS3-54-conjugated beads (Figure 3D). STAT3 bound to the inS3-54-conjugated beads could be eluted by excess inS3-54 but not by vehicle control or the irrelevant compound (Figure 3E). Finally, inS3-54-conjugated beads could also pull down purified STAT3 in the absence of other proteins (Figure 3F). On the basis of these findings, we conclude that inS3-54 can bind directly and noncovalently to STAT3.

InS3-54 Is Not an Alkylating Agent. Recently, it was found that Cys468 in DBD of STAT3 can be alkylated by and covalently linked to a small molecule inhibitor, C48.[30] The findings from pull-down assays show that inS3-54 can bind, but unlikely covalently, to any residues (e.g., Cys or Lys) in STAT3, suggesting that inS3-54 did not alkylate STAT3. To further eliminate the possibility that inS3-54 has alkylating activity, we performed luminescence-based glutathione alkylation assay. As shown in Supplemental Figure S2, inS3-54, unlike the known alkylating agent iodoacetamide, did not significantly reduce glutathione level in both A549 and MDA-MB-231 cells. Thus, inS3-54 unlikely possesses any alkylating activity.

InS3-54 Does Not Inhibit STAT3 Dimerization. The SH2 domain of STAT3 has previously been shown to be susceptible for targeting (see beginning paragraphs). To eliminate the possibility that inS3-54 works by off-targeting to the SH2 domain, we tested if inS3-54 inhibits STAT3 dimerization using FLAG-STAT3c, which forms spontaneous homodimers via formation of intermolecular disulfide bond.[6] Supplemental Figure S3A shows that STAT3c is successfully expressed in H1299 cells in both dimeric and monomeric forms separated by nonreducing SDS-PAGE. However, inS3-54 had no effect on production of dimeric STAT3c separated using nonreducing SDS-PAGE or non-denaturing PAGE (Supplemental Figure S3B) while S3I-201, a STAT3 inhibitor that binds to the SH2 domain,[19] inhibited STAT3c dimerization (Supplemental Figure S3B).

To confirm this observation, we performed a co-immunoprecipitation analysis of HA- and FLAG-tagged STAT3. Supplemental Figure S3C shows that HA- and FLAG-tagged STAT3 can be co-expressed and co-immunoprecipitated successfully in H1299 cells. InS3-54 had no effect, while S3I-201 inhibited the co-immunoprecipitation between HA- and FLAG-tagged STAT3 (Supplemental Figure S3D). Thus, inS3-54 likely does not inhibit STAT3 dimerization or bind to the SH2 domain.

InS3-54 Favorably Inhibits Cancer Cell Survival by Inducing Apoptosis. Next, we determined whether inS3-54 inhibits growth and survival of cancer cells using two lung (A549 and H1299) and two breast (MDA-MB-231 and MDA-MB-468) cancer cell lines, as well as non-cancer lung fibroblast (IMR90) and mammary epithelial cell line (MCF10A1). As shown in Figure 4A, all cancer cells had constitutively activated STAT3 as assessed by its phosphorylation status at Tyr705, compared to the non-cancer cells, consistent with previous findings.[4,18,19] The cancer cells are also more sensitive to inS3-54 with IC_{50}'s significant lower than those of the non-cancer cells (3.2−5.4 vs 10−12 μM, see Figure 4B,C). This finding of ~2−4-fold difference in IC_{50} is consistent with the differential status of constitutively activated STAT3 between cancer and non-cancer cells and suggests that there may be a therapeutic window for inS3-54.

To determine if apoptosis contributes to inS3-54 suppression of cancer cell survival, we performed apoptosis analysis of exponentially growing cells using ELISA following inS3-54 treatment for 72 h. As shown in Figure 4D, inS3-54 induced apoptosis of both A549 and MDA-MB-231 cells in a dose-dependent manner. InS3-54 treatment also induced cleavage of PARP in breast cancer cell line MDA-MB-468 (data not shown), a target of activated caspases during execution of apoptosis, confirming that 72-h treatments with inS3-54 induce apoptosis.

InS3-54 Inhibits Cancer Cell Migration and Invasion. STAT3 also plays an important role in controlling cell migration and invasion by regulating the expression of genes such as MMP-1, -2, -9, -10, Twist, and VEGF important for these cellular processes.[31−36] To determine if inS3-54 inhibits migration and invasion, we first performed a wound-healing assay using A549 and MDA-MB-231 cells. Figure 5A,B shows that inS3-54 inhibits migration of both A549 and MDA-MB-231 cells in dose- and time-dependent manners. At 24 h, about 57% and 95% of wounds were healed in the control vehicle-treated A549 and MDA-MB-231 cells, respectively. However,

dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188−1196

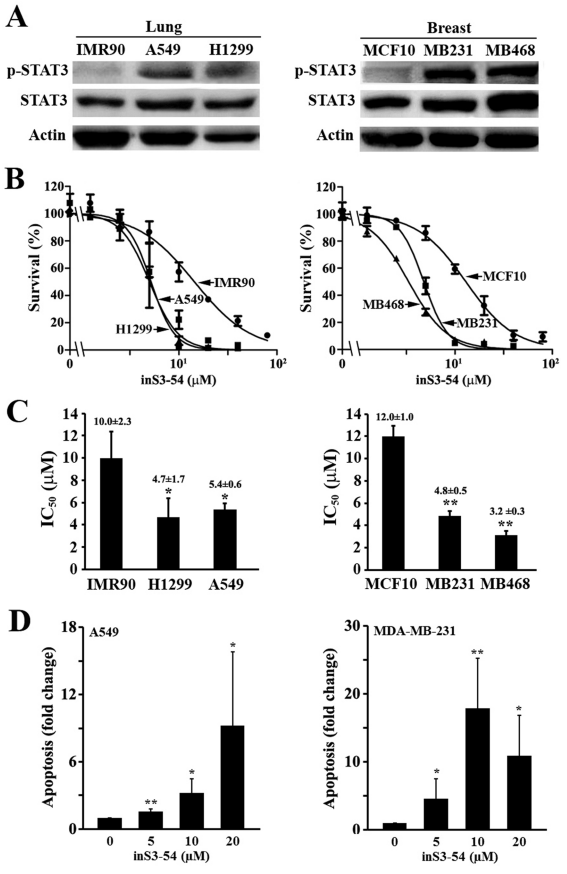


Figure 4. InS3-54 inhibits cancer cell proliferation. (A) Level of STAT3 expression and activation in different cells. Actin was used as a loading control. (B, C) Cytotoxicity assay. IC_{50} of inS3-54 for each cell line was derived, and dose−response curves were created using PrismPad program. (D) Apoptosis assay of exponentially growing A549 and MDA-MB-231 cells. (**$p < 0.01$; *$p < 0.05$)

only 42% and 77% of the wounds were healed for these cells at 24 h following treatment with 10 μM inS3-54. The wound healing further reduced to 23% and 39% after treatment with 20 μM inS3-54.

We then performed a Matrigel invasion assay. Figure 5C,D shows that both A549 and MDA-MB-231 cells exhibit significantly decreased invasion in the presence of inS3-54 than vehicle. At 6 h of treatment with 10 and 20 μM inS3-54, the invasion was reduced to 67% and 49% for A549 cells and to 52% and 24% for MDA-MB-231 cells, respectively. At 24 h of treatment with 10 μM inS3-54, the invasion of A549 and MDA-MB-231 cells was about 71% and 24% of controls, respectively. These numbers were further reduced to 33% and 5% in the presence of 20 μM inS3-54.

Although we used 100% confluent cells and short time incubation in the above assays, inhibition of proliferation may still contribute to the observed outcomes. To eliminate this possibility, we analyzed cell proliferation and apoptosis under the same condition as wound-healing and Matrigel invasion assays with confluent cultures and found that treatment with 20 μM inS3-54 for 24 h had no significant effect on proliferation (Supplemental Figure S4A) and apoptosis (Supplemental Figure S4B) of confluent A549 cells, although it decreased the proliferation and increased apoptosis of MDA-MB-231 cells. However, 10 μM inS3-54 did not significantly decrease proliferation or increase apoptosis of MDA-MB-231 cells (Supplemental Figure S4), under which condition it significantly reduced the migration and invasion activity of these cells (Figure 5). Furthermore, no apoptosis was observed at 6 h of treatment with 20 μM inS3-54. Thus, we conclude that inS3-54 inhibition of migration and invasion is unlikely due to its effect on apoptosis and cell proliferation.

InS3-54 Inhibits STAT3 Downstream Target Gene Expression and STAT3 Binding to Genomic DNA. To validate inS3-54 effect on STAT3 in cells, we determined the expression of STAT3 downstream target genes. Figure 6A shows that the expression of cyclin D1, survivin, VEGF, MMP-2, MMP-9, and Twist are all decreased following inS3-54 treatment in both A549 and MDA-MB-231cell lines at protein level. This observation was confirmed by quantitative RT-PCR analysis of mRNAs (Supplemental Figure S5).

InS3-54, however, had no effect on the level of total STAT3 or basal level of Tyr705-phosphorylated STAT3 (Figure 6A), indicating that inS3-54 does not affect the expression or activation of STAT3. To further determine if inS3-54 inhibits STAT3 activation and phosphorylation, serum-starved A549 cells were pretreated with inS3-54 followed by IL-6 stimulation and analysis of phosphorylated STAT3. Figure 6B shows that IL-6 stimulates phosphorylation of Tyr705 of STAT3 and expression of survivin in serum-starved A549 cells. Pretreatment with inS3-54 had no effect on IL-6-stimulated phosphorylation of STAT3 but inhibited IL-6 stimulated expression of survivin. Thus, inS3-54 does not affect IL-6 stimulated phosphorylation/activation of STAT3 but inhibits STAT3 activity.

The EMSA data (Figure 2) show that inS3-54 inhibits the DNA-binding activity of STAT3 in vitro. To further demonstrate that inS3-54 inhibits the DNA-binding activity of STAT3 in situ, we treated A549 and MDA-MB-231 cells with inS3-54 followed by isolation of cytosol, soluble nuclear, and chromatin-bound fractions and determined STAT3 level in these fractions. Figure 6C shows that STAT3 in the chromatin-bound fraction decreases while the STAT3 level in soluble nuclear fraction increases with the increasing concentration of inS3-54. Furthermore, inS3-54 dramatically decreased the binding of STAT3 to the promoters of Twist and cyclin D1 as determined using ChIP assay (Figure 6D). Taken together, we conclude that inS3-54 inhibits STAT3 binding to endogenous promoters on genomic DNA, resulting in reduced transcription of its downstream target genes.

In summary, with the aid of structure-based virtual screening, we successfully identified a human STAT3 inhibitor targeting its DBD. This study represents one of the first successful attempts in targeting the prevailing "undruggable" DBD of transcription factors. InS3-54 is selective to STAT3 over STAT1 as demonstrated using EMSA. In-silico analysis shows that inS3-54 could not bind to STAT1 due to physical hindrance from residue Pro326 and Thr327 and, thus, has a much lower affinity to STAT1. The finding that inS3-54 does not inhibit the promoter activity of p27 is also consistent with its selectivity. Finally, the less cytotoxic effect of inS3-54 on non-cancer compared to cancer cells further confirms that inS3-54 is likely selective to STAT3.

As expected, inS3-54 inhibits the DNA-binding activity of STAT3 both in vitro and in situ. Although inS3-54 likely binds to STAT3 and is selective for STAT3 over STAT1, it is unknown if it is specific only to STAT3. In fact, other proteins were pulled down together with STAT3 by inS3-54-conjugated beads. While these proteins may have been pulled down

dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188−1196

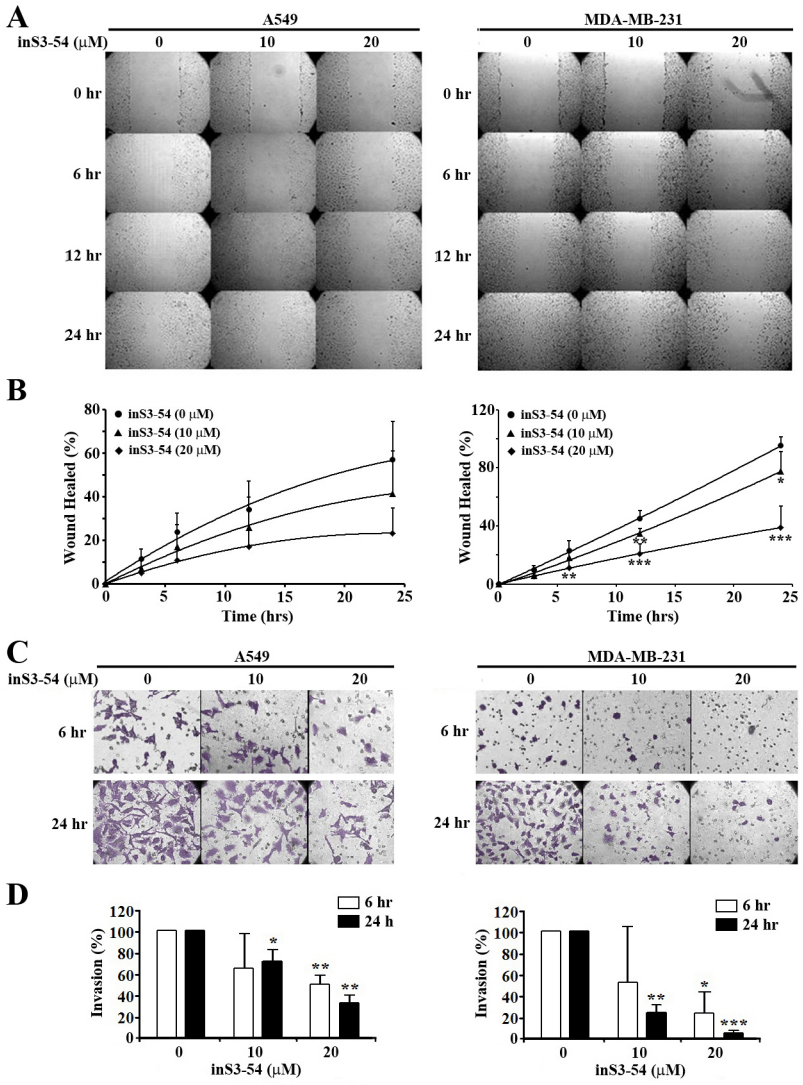


Figure 5. InS3-54 inhibits cancer cell migration and invasion. (A, B) Effect of inS3-54 on migration. Panel B shows quantification analysis of wound healing assay from triplicate measurements of three independent experiments shown in panel A. (C, D) Effect of inS3-54 on cell invasion. Panel D shows quantification of invasion from measurement of 10 random views each of three independent experiments shown in panel C. (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$)

indirectly by interacting with STAT3, they may also interact directly with inS3-54. More studies are needed to differentiate these possibilities. Furthermore, since inS3-54 does not inhibit STAT3 activation and phosphorylation and unlikely binds to the SH2 domain, it likely binds to the DBD of STAT3 and directly inhibits its DNA-binding activity, although more studies are needed to show its direct binding to the DBD of STAT3.

It is noteworthy that inS3-54 has an IC_{50} of ~20 μM in inhibiting DNA-binding activity in the EMSA assay and an IC_{50} of ~15.8 μM in the luciferase reporter assay. However, the IC_{50} of inS3-54 in the cytotoxicity assay ranges from ~3.2−5.4 μM in cancer to ~10−12 μM in non-cancer cells. Currently, it is unknown why inS3-54 is more effective in inhibiting cell survival than inhibiting DNA binding and luciferase reporter expression. However, inS3-54 may have off-target effects that can impact on cell survival, making it more effective in suppressing cancer cell survival. Future studies of transcriptome change due to inS3-54 inhibition of gene expression may help address the potential off-target effect.

METHODS

Structure-Based Virtual Screening. The DNA in the DBD of STAT3β-DNA complex structure (PDB code: 1BG1) was removed, and the protein chain was prepared for docking. The DNA-binding groove consisting of residues 329−332, 340−346, 406−412, and 465−468 was chosen as the targeting site for docking (Figure 1A). Molecular surface was calculated using the DMS (Distributed Molecular Surface) program. Partial charges and protons were added to the protein by the UCSF Chimera Dock Prep module.[37] In-silico dock screening of 200,000 compounds from the ChemDiv library was performed using the UCSF DOCK 6.0 program.[38] The docking of each compound was first scored with the DOCK GRID scoring function.[39] The top-scoring 1000 compounds were analyzed again and rescored using the AMBER scoring function of the DOCK 6.0 package.[40]

To improve in-silico screening for a STAT3-selective inhibitor, the top-scoring compounds from above screening were then docked onto the DBD of STAT1 (PDB code: 1BF5) in the same way as to STAT3. Both STAT3 and STAT1 bind to very similar 9-bp core consensus sequences with minor differences in flanking sequences.[41] However, there are minor differences in DBD between STAT3 and STAT1 (e.g., Met331, Thr412, Ile467 in STAT3 replace Thr327, Gln408, and

dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188−1196

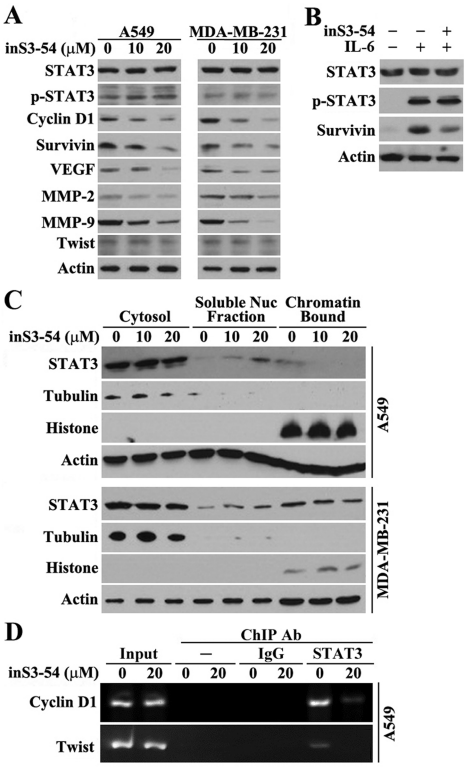


Figure 6. InS3-54 inhibits the expression of STAT3 downstream target genes and STAT3 binding to chromatin. (A) Effect of inS3-54 on the expression of STAT3 downstream target genes in A549 and MDA-MB-231 cells. (B) Inhibition of IL-6 (25 ng/mL) stimulated STAT3 activation in serum-starved A549 cells. Actin was used as a loading control. (C, D) InS3-54 inhibition of STAT3 binding to chromatin in situ as determined using subcellular fractionation and Western blot analysis (C) or ChIP assay of Twist and cyclin D1 promoters (D).

Val461 in STAT1, respectively), which may help distinguish selective compound inhibitors. Compounds that scored well with STAT1 were eliminated, and the remaining ones were clustered using the MOE (Molecular Operating Environment) program and visually examined using the UCSF Chimera ViewDock function. A final 100 compounds were selected on the basis of the combination of GRID and AMBER score, drug likeness (Lipinski's rule of five), and consideration of maximizing compounds from different clusters.

Molecular Dynamics Simulation and Calculation of Binding Free Energy (ΔG_{bind}). ΔG_{bind} determinations of inS3-54 to STAT3 and STAT1 were performed by 3-ns MD simulations followed by energy analysis using GBSA method[42] as we previously described.[43] Briefly, a total of 20 snapshots were collected from the production trajectory for molecular mechanic (MM)-GBSA free energy calculations using the formula $\Delta G_{bind} = G_{complex} - G_{STAT} - G_{inS3\text{-}54}$, where $G = G_{solute} + G_{solvent}$.

STAT3-Dependent Luciferase Assay. In this and all following assays, candidate compounds were dissolved and completely soluble in DMSO at 20 mM as a stock solution. MDA-MB-231 stably transfected or H1299 cells transiently transfected with STAT3-dependent luciferase reporter were incubated with candidate compounds at different concentrations for various times, and luciferase activity was measured using a luciferase assay kit (Promega), following manufacturer's instructions. The final DMSO concentration in this and following assays was 0.1% (v/v).

Cytotoxicity and Apoptosis Assay. Cytotoxicity of inS3-54 was determined using a sulphorhodamine colorimetric assay as described previously.[44] Photometric enzyme immunoassay using a Cell Death Detection ELISA Plus kit (Roche Diagnostics, Indianapolis, IN) was performed for quantitative in vitro determination of cytoplasmic histone-associated DNA fragments and apoptosis as previously described.[44]

Electrophoretic Mobility Shift Assay (EMSA). H1299 cells were transiently transfected with FLAG-tagged STAT3c or STAT1 expression construct. Forty-eight hours following transfection, cells were harvested and lysed with 3 cycles of freeze and thaw. Then 10–20 μg of lysate was mixed with 2 μg of poly(dI-dC), 1 μg BSA in binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 10% glycerol, 0.2 mg mL^{-1} BSA, 1 mM DTT, and 0.2 mM PMSF), and 4×10^4 cpm [^{32}P]-labeled SIE probe. The mixture was incubated for 20 min at RT and separated on 6% non-denaturing PAGE. The signal was detected by autoradiography. For supershift and competition, 2 μL of specific antibodies against STAT3 or STAT1 or 100-fold cold SIE probe (5′-AGCTTCATTTCCCGTAAATCCCTA-3′) was added to the reaction mixture and incubated for 30 min before adding labeled SIE probe. To determine the effect of inS3-54 on STAT3 or STAT1 binding to SIE probe, inS3-54 was first diluted with DMSO, and equal volume of diluted inS3-54 was added to the reaction mixture followed by incubation at RT for 30 min before incubating with the labeled SIE probe.

Conjugation of inS3-54 and Pull-down Assay. EAH-Sepharose 4B containing free amino groups with 11-atom spacer arms was used to couple inS3-54 with the carbodiimide coupling method according to manufacturer's instructions. Control EAH-Sepharose was prepared exactly the same way without inS3-54. Since inS4–54 is orange in color, the conjugation of inS3-54 to EAH-Sepharose was verified by monitoring the color change of the EAH beads.

For the pull-down assay, inS3-54-conjugated and control beads equilibrated with binding buffer (10 mM MES/NaOH, pH 6.5, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 0.5% NP-40) were blocked with 10% milk in the binding buffer containing 0.2 mM PMSF and protease inhibitor cocktails followed by incubation with 60 μg of lysate of H1299 cells harboring FLAG-STAT3 or 1 μg of purified STAT3 (Sigma) at RT for 1 h. The unbound proteins were removed by washing 7 times, and the bound proteins were separated by SDS-PAGE followed by analysis using Western blot or silver staining. For competition, cell lysate was preincubated with 10 μM inS3-54, DMSO vehicle or an irrelevant compound control at RT for 1 h prior to the pull-down assay. For elution, following binding STAT3 using inS3-54-conjugated beads as described above, the protein–bead complex were eluted using vehicle control, 300 μM inS3-54 or the irrelevant compound in binding buffer containing 20% DMSO.

Migration and Invasion Assay. For the wound-healing assay, 1×10^5 cells per well were plated in 6-well plates followed by introduction of a wound and monitoring of the healing process of the wound over a 24-h period. The healing of the wound was determined by measuring the remaining gap between two migrating edges at different times. Cell invasion assay was performed using Matrigel-coated Boyden Chambers (BD Biosciences) according to manufacturer's instructions. At different times, invading cells were stained with crystal violate and counted.

Subcellular Fractionation. Subcellular fractionation was performed as previously described.[45] Briefly, cells were lysed in 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1% Triton X-100, 1 mM DTT, 10 μM leupeptin, and protease inhibitor cocktail and centrifuged at 4,200*g* for 5 min to collect supernatant as cytosolic fraction. The pellet (nuclei) was resuspended in 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10 μM leupeptin, protease inhibitor cocktail and incubated on ice for 30 min followed by centrifugation at 5,000*g* for 5 min. The supernatant was collected as soluble nuclear fraction. The pellet was resuspended in 50 mM Tris/HCl, pH7.4, 150 mM NaCl, 0.5% NP-50, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% SDS, 1 mM DTT, 10 μM leupeptin, protease inhibitor cocktail and sonicated to release proteins from chromatins.

ChIP Assay. H1299 cells were first treated with 20 μM inS3-54 followed by treatment with 1% formaldehyde for 10 min and ChIP assay using a kit (EMD Millipore). Immunoprecipitated DNA was then subjected to PCR with primer pairs specific for promoters of cyclin D1 (5′-AACTTGCACAGGGGTTGTGT-3′/5′-GAGACCAC-

dx.doi.org/10.1021/cb500071v | ACS Chem. Biol. 2014, 9, 1188–1196

GAGAAGGGGTGACTG-3′) and twist (5′-AGTCTCCTCCGA-CCGCTTCCTG-3′/5′-CTCCGTGCAGGCGGAAAGTTTGG-3′).

Quantitative RT-PCR. Quantitative RT-PCR analysis was performed using primers shown in Supplemental Table S1 as previously described.[46] The threshold cycles (Ct) were determined and normalized against that of GAPDH internal control. The relative mRNA levels were shown as the value of $2^{-\Delta Ct}$.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: jliu2@iupui.edu.

*E-mail: jianzhan@iupui.edu.

Author Contributions

‖These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank J. Lin at Ohio State University for the generous gift of MDA-MB-231-STAT3 cells with the STAT3-dependent luciferase reporter, G. Stark at Cleveland Clinic for STAT1 and STAT3c cDNAs, and IU Big Red supercomputers for the CPU time. S3I-201 (NSC74859) was obtained from DTP at NCI.

■ REFERENCES

(1) Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science 264*, 1415−1421.

(2) Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science 264*, 95−98.

(3) Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000) STATs in oncogenesis. *Oncogene 19*, 2474−2488.

(4) Song, L., Turkson, J., Karras, J. G., Jove, R., and Haura, E. B. (2003) Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. *Oncogene 22*, 4150−4165.

(5) Garcia, R., Yu, C. L., Hudnall, A., Catlett, R., Nelson, K. L., Smithgall, T., Fujita, D. J., Ethier, S. P., and Jove, R. (1997) Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ. 8*, 1267−1276.

(6) Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) Stat3 as an oncogene. *Cell 98*, 295−303.

(7) Li, Y., Du, H., Qin, Y., Roberts, J., Cummings, O. W., and Yan, C. (2007) Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung. *Cancer Res. 67*, 8494−8503.

(8) Chiarle, R., Simmons, W. J., Cai, H., Dhall, G., Zamo, A., Raz, R., Karras, J. G., Levy, D. E., and Inghirami, G. (2005) Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. *Nat. Med. 11*, 623−629.

(9) Costantino, L., and Barlocco, D. (2008) STAT 3 as a target for cancer drug discovery. *Curr. Med. Chem. 15*, 834−843.

(10) Yue, P., and Turkson, J. (2009) Targeting STAT3 in cancer: how successful are we? *Expert Opin. Invest. Drugs 18*, 45−56.

(11) Debnath, B., Xu, S., and Neamati, N. (2012) Small molecule inhibitors of signal transducer and activator of transcription 3 (Stat3) protein. *J. Med. Chem. 55*, 6645−6668.

(12) Deng, J., Grande, F., and Neamati, N. (2007) Small molecule inhibitors of Stat3 signaling pathway. *Curr. Cancer Drug Targets 7*, 91−107.

(13) Ren, Z., Cabell, L. A., Schaefer, T. S., and McMurray, J. S. (2003) Identification of a high-affinity phosphopeptide inhibitor of Stat3. *Bioorg. Med. Chem. Lett. 13*, 633−636.

(14) Turkson, J., Kim, J. S., Zhang, S., Yuan, J., Huang, M., Glenn, M., Haura, E., Sebti, S., Hamilton, A. D., and Jove, R. (2004) Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. *Mol. Cancer Ther. 3*, 261−269.

(15) Siddiquee, K. A., Gunning, P. T., Glenn, M., Katt, W. P., Zhang, S., Schrock, C., Sebti, S. M., Jove, R., Hamilton, A. D., and Turkson, J. (2007) An oxazole-based small-molecule Stat3 inhibitor modulates Stat3 stability and processing and induces antitumor cell effects. *ACS Chem. Biol. 2*, 787−798.

(16) Schust, J., and Berg, T. (2004) A high-throughput fluorescence polarization assay for signal transducer and activator of transcription 3. *Anal. Biochem. 330*, 114−118.

(17) Schust, J., Sperl, B., Hollis, A., Mayer, T. U., and Berg, T. (2006) Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem. Biol. 13*, 1235−1242.

(18) Song, H., Wang, R., Wang, S., and Lin, J. (2005) A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A. 102*, 4700−4705.

(19) Siddiquee, K., Zhang, S., Guida, W. C., Blaskovich, M. A., Greedy, B., Lawrence, H. R., Yip, M. L., Jove, R., McLaughlin, M. M., Lawrence, N. J., Sebti, S. M., and Turkson, J. (2007) Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc. Natl. Acad. Sci. U.S.A. 104*, 7391−7396.

(20) Zhang, X., Yue, P., Page, B. D., Li, T., Zhao, W., Namanja, A. T., Paladino, D., Zhao, J., Chen, Y., Gunning, P. T., and Turkson, J. (2012) Orally bioavailable small-molecule inhibitor of transcription factor Stat3 regresses human breast and lung cancer xenografts. *Proc. Natl. Acad. Sci. U.S.A. 109*, 9623−9628.

(21) Yang, J., Liao, X., Agarwal, M. K., Barnes, L., Auron, P. E., and Stark, G. R. (2007) Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. *Genes Dev. 21*, 1396−1408.

(22) Timofeeva, O. A., Chasovskikh, S., Lonskaya, I., Tarasova, N. I., Khavrutskii, L., Tarasov, S. G., Zhang, X., Korostyshevskiy, V. R., Cheema, A., Zhang, L., Dakshanamurthy, S., Brown, M. L., and Dritschilo, A. (2012) Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA. *J. Biol. Chem. 287*, 14192−14200.

(23) Nkansah, E., Shah, R., Collie, G. W., Parkinson, G. N., Palmer, J., Rahman, K. M., Bui, T. T., Drake, A. F., Husby, J., Neidle, S., Zinzalla, G., Thurston, D. E., and Wilderspin, A. F. (2013) Observation of unphosphorylated STAT3 core protein binding to target dsDNA by PEMSA and X-ray crystallography. *FEBS Lett. 587*, 833−839.

(24) Caboni, L., and Lloyd, D. G. (2012) Beyond the ligand-binding pocket: Targeting alternate sites in nuclear receptors. *Med. Res. Rev. 33*, 1081−1118.

(25) Leung, C. H., Chan, D. S., Ma, V. P., and Ma, D. L. (2013) DNA-binding small molecules as inhibitors of transcription factors. *Med. Res. Rev. 33*, 823−846.

(26) Liu, Z., Dong, Z., Han, B., Yang, Y., Liu, Y., and Zhang, J. T. (2005) Regulation of expression by promoters versus internal ribosome entry site in the 5′-untranslated sequence of the human cyclin-dependent kinase inhibitor p27kip1. *Nucleic Acids Res. 33*, 3763−3771.

(27) Frye, S. V. (2010) The art of the chemical probe. *Nat Chem. Biol. 6*, 159−161.

(28) Lee, Y. W., Hennig, B., and Toborek, M. (2003) Redox-regulated mechanisms of IL-4-induced MCP-1 expression in human vascular endothelial cells. *Am. J. Physiol.: Heart Circ. Physiol. 284*, H185−192.

(29) Connett, J. M., Hunt, S. R., Hickerson, S. M., Wu, S. J., and Doherty, G. M. (2003) Localization of IFN-gamma-activated Stat1 and IFN regulatory factors 1 and 2 in breast cancer cells. *J. Interferon Cytokine Res. 23*, 621−630.
(30) Buettner, R., Corzano, R., Rashid, R., Lin, J., Senthil, M., Hedvat, M., Schroeder, A., Mao, A., Herrmann, A., Yim, J., Li, H., Yuan, Y. C., Yakushijin, K., Yakushijin, F., Vaidehi, N., Moore, R., Gugiu, G., Lee, T. D., Yip, R., Chen, Y., Jove, R., Horne, D., and Williams, J. C. (2011) Alkylation of cysteine 468 in Stat3 defines a novel site for therapeutic development. *ACS Chem. Biol. 6*, 432−443.
(31) Sehgal, G., Hua, J., Bernhard, E. J., Sehgal, I., Thompson, T. C., and Muschel, R. J. (1998) Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. *Am. J. Pathol. 152*, 591−596.
(32) Wei, D., Le, X., Zheng, L., Wang, L., Frey, J. A., Gao, A. C., Peng, Z., Huang, S., Xiong, H. Q., Abbruzzese, J. L., and Xie, K. (2003) Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene 22*, 319−329.
(33) Xie, T. X., Wei, D., Liu, M., Gao, A. C., Ali-Osman, F., Sawaya, R., and Huang, S. (2004) Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. *Oncogene 23*, 3550−3560.
(34) Itoh, M., Murata, T., Suzuki, T., Shindoh, M., Nakajima, K., Imai, K., and Yoshida, K. (2006) Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. *Oncogene 25*, 1195−1204.
(35) Cheng, G. Z., Zhang, W. Z., Sun, M., Wang, Q., Coppola, D., Mansour, M., Xu, L. M., Costanzo, C., Cheng, J. Q., and Wang, L. H. (2008) Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function. *J. Biol. Chem. 283*, 14665−14673.
(36) Zhang, X., Yin, P., Di, D., Luo, G., Zheng, L., Wei, J., Zhang, J., Shi, Y., and Xu, N. (2009) IL-6 regulates MMP-10 expression via JAK2/STAT3 signaling pathway in a human lung adenocarcinoma cell line. *Anticancer Res. 29*, 4497−4501.
(37) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem. 25*, 1605−1612.
(38) Lang, P. T., Brozell, S. R., Mukherjee, S., Pettersen, E. F., Meng, E. C., Thomas, V., Rizzo, R. C., Case, D. A., James, T. L., and Kuntz, I. D. (2009) DOCK 6: combining techniques to model RNA-small molecule complexes. *RNA 15*, 1219−1230.
(39) Meng, E. C., Shoichet, B. K., and Kuntz, I. D. (1992) Automated docking with grid-based energy evaluation. *J. Comput. Chem. 13*, 505−524.
(40) Graves, A. P., Shivakumar, D. M., Boyce, S. E., Jacobson, M. P., Case, D. A., and Shoichet, B. K. (2008) Rescoring docking hit lists for model cavity sites: predictions and experimental testing. *J. Mol. Biol. 377*, 914−934.
(41) Horvath, C. M., Wen, Z., and Darnell, J. E., Jr. (1995) A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev. 9*, 984−994.
(42) Onufriev, A., Bashford, D., and Case, D. A. (2000) Modification of the generalized Born model suitable for macromolecules. *J. Phys. Chem. B 104*, 3712−3720.
(43) Liu, J. Y., Li, Z., Li, H., and Zhang, J. T. (2011) Critical residue that promotes protein dimerization: a story of partially exposed phe(25) in 14−3-3sigma. *J. Chem. Inf. Model. 51*, 2612−2625.
(44) Liu, H., Liu, Y., and Zhang, J. T. (2008) A new mechanism of drug resistance in breast cancer cells: fatty acid synthase over-expression-mediated palmitate overproduction. *Mol. Cancer Ther. 7*, 263−270.
(45) Mendez, J., and Stillman, B. (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol. Cell. Biol. 20*, 8602−8612.
(46) Liu, Y., Peng, H., and Zhang, J. T. (2005) Expression profiling of ABC transporters in a drug-resistant breast cancer cell line using AmpArray. *Mol. Pharmacol. 68*, 430−438.