

A small-molecule compound identified through a cell-based screening inhibits JAK/STAT pathway signaling in human cancer cells

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Abstract

Inappropriate activation of JAK/STAT signaling occurs with high frequency in human cancers and is associated with cancer cell survival and proliferation. Therefore, the development of pharmacologic STAT signaling inhibitors has therapeutic potential in the treatment of human cancers. Here, we report 2-[(3,5-bis-trifluoromethyl-phenyl)-hydroxy-methyl]-1-(4-nitro-phenylamino)-6-phenyl-1,2,4a,7a-tetrahydro-pyrrolo[3,4-*b*]-pyridine-5,7-dione (AUH-6-96) as a novel small-molecule inhibitor of JAK/STAT signaling that we initially identified through a cell-based high-throughput screening using cultured *Drosophila* cells. Treatment of *Drosophila* cells with AUH-6-96 resulted in a reduction of Unpaired-induced transcriptional activity and tyrosine phosphorylation of STAT92E, the sole *Drosophila* STAT homologue. In human cancer cell lines, AUH-6-96 inhibited both constitutive and interleukin-6-induced STAT3 phosphorylation. Specifically, in Hodgkin lymphoma L540 cells, treatment with AUH-6-96 resulted in reduced levels of tyrosine phosphorylated STAT3 and of the STAT3 downstream target gene *SOCS3* in a dose- and time-dependent manner. In addition, AUH-6-96-treated L540 cells showed decreased expression of persistently activated JAK3, sug-

gesting that AUH-6-96 inhibits the JAK/STAT pathway signaling in L540 cells by affecting JAK3 activity and subsequently blocking STAT3 signaling. Importantly, AUH-6-96 selectively affected cell viability only of cancer cells harboring aberrant JAK/STAT signaling. In support of the specificity of AUH-6-96 for inhibition of JAK/STAT signaling, treatment with AUH-6-96 decreased cancer cell survival by inducing programmed cell death by down-regulating the expression of STAT3 downstream target antiapoptotic genes, such as Bcl-xL. In summary, this study shows that AUH-6-96 is a novel small-molecule inhibitor of JAK/STAT signaling and may have therapeutic potential in the treatment of human cancers harboring aberrant JAK/STAT signaling. [Mol Cancer Ther 2008; 7(9):2672–80]

Introduction

The JAK/STAT cascade has emerged as an essential reutilized facet of vertebrate signaling through a large number of cytokines and growth factors, which induce proliferation or differentiation and are crucial for the proper growth and development of mammalian tissues (1). In mammals, four JAK and seven STAT genes have been identified, and more than 40 different cytokines and growth factors have been shown to activate specific combinations of JAK or STAT proteins (2, 3). The vital role of these JAK and STAT molecules in the biological response to these extracellular signals has been shown through the generation of gene-targeted mice of JAK or STAT family members (4–9). Interestingly, in addition to the critical role of this JAK/STAT pathway in blood cell formation and immune response, recent studies suggest that constitutive activation of JAK/STAT signaling correlates with tumorigenesis through its intimate connection to growth factor signaling (10, 11).

One of the earliest clues that STAT signaling contributes to oncogenesis was the finding that STAT3 is constitutively activated in Src-transformed mouse fibroblasts and that interrupting STAT3 signaling blocks the cell transformation by the Src oncoprotein (12). In humans, extensive surveys of primary tumors and tumor-derived cell lines indicate that inappropriate activation of STAT signaling occurs with surprisingly high frequency in human cancers, including a wide variety of solid tumors, as well as many leukemias and lymphomas (13). Several chemical compounds including S3I-201, Stattic, STA-21, and a JAK kinase inhibitor AG490 have been reported to successfully inhibit JAK/STAT signaling (14–17). Importantly, studies with these small-molecule inhibitors indicate that the abrogation of JAK/STAT signaling is sufficient to induce growth arrest and apoptosis in various types of

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tumors. The therapeutic potential of pharmacologic JAK/STAT signaling inhibitors has also been shown in recent studies. For example, blocking constitutively activated STAT3 signaling by the compound inhibitors can sensitize chemotherapeutic drug-resistant cancer cells and induce apoptosis, suggesting a role for small-molecule inhibitors of STAT3 signaling as novel class of chemotherapeutic sensitizing agents capable of reversing the drug-resistant phenotype of tumor cells (18, 19). Given that persistently active JAK/STAT signaling participates in the pathogenesis of cancers and that inhibition of JAK/STAT signaling can lead to successful suppression of tumor progression, future therapeutic strategies based on JAK/STAT signaling hold great potential for the treatment of human cancers.

As a tool for drug discovery, the fruit fly *Drosophila* has several major advantages, including the reduced redundancy of *Drosophila* genome compared with mammalian genomes. Importantly, *Drosophila* contains a conserved yet streamlined JAK/STAT pathway that consists of only three highly related activating ligands of the Unpaired (Upd) family, one receptor, one JAK, and one STAT called STAT92E (20, 21). To identify novel small-molecule inhibitors of JAK/STAT signaling, we have conducted a cell-based high-throughput chemical genetics screening using a combinatorial library of polysubstituted imidopiperidines (22) and a cultured *Drosophila* cell line that stably expresses a STAT92E reporter gene. We identified 2-[(3,5-bis-trifluoromethyl-phenyl)-hydroxy-methyl]-1-(4-nitrophenylamino)-6-phenyl-1,2,4a,7a-tetrahydro-pyrrolo[3,4-*b*]pyridine-5,7-dione (AUH-6-96) as a potent inhibitor of JAK/STAT signaling in both *Drosophila* and mammalian cells. Importantly, AUH-6-96 affected the growth and survival only of human cancer cells with aberrant JAK/STAT signaling, suggesting that this compound selectively blocks JAK/STAT signaling. We also show that treatment of Hodgkin lymphoma L540 cells with AUH-6-96 blocked their growth and caused induction of programmed cell death by down-regulating the expression of antiapoptotic genes, known STAT3 downstream targets.

Materials and Methods

Drosophila Cell Line, Transfection, and a Cell-Based Luciferase Assay

Parental macrophage-like *Drosophila* Schneider cells (S2-NP) were maintained in Schneider's medium supplemented with L-glutamine, penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (Gemini Bio-Products) in an incubator at 25°C. To generate a *Drosophila* cell line that stably expresses both a STAT92E reporter gene and a PolIII-*Renilla* gene as an internal control, S2-NP cells were cotransfected with plasmids of both 10×STAT92E-*luciferase* (23) and a RNA polymerase III promoter-driven *Renilla luciferase* expression vector (PolIII-*Renilla*), using Effectene Transfection Reagent (Qiagen) according to the manufacturer's protocol. For examining the effect of AUH-6-96 on Upd-induced STAT92E transcriptional activity, S2-NP-

STAT92E cells were cocultured for 24 h with Upd-producing cells, which are parental S2-NP cells transiently transfected with actin promoter-driven *Upd*, in the presence of AUH-6-96 at various concentrations. The reporter activity was quantified by measuring relative luciferase units, which equaled the ratio of the absolute activity of firefly *luciferase* to *Renilla luciferase*. For analyzing the effect of AUH-6-96 on Upd-induced STAT92E phosphorylation, S2-NP cells transiently transfected with an expression plasmid for HA-tagged STAT92E were cocultured with Upd-producing cells in the presence of AUH-6-96 (40 μmol/L) for 24 h. Whole-cell extracts were processed for Western blot analysis using antibodies specific for phospho-STAT92E (Cell Signaling Technology) and HA (Roche Applied Science).

Human Cancer Cell Lines and Culture Conditions

Hodgkin lymphoma L540 cells, chronic myeloid leukemia EM-3 cells, and Burkitt's lymphoma DG-75 cells were obtained from the German Collection of Microorganisms and Cell Cultures. Breast cancer cell lines MDA-MB-468 and MCF-7, a prostate cancer cell line DU145, and a multiple myeloma cell line RPMI 8226 were purchased from the American Type Culture Collection. L540 cells were grown in RPMI 1640 containing penicillin/streptomycin and 20% fetal bovine serum, and DG-75, EM-3, and RPMI 8226 cells were grown in RPMI 1640 with penicillin/streptomycin and 10% fetal bovine serum. MDA-MB-468, MCF-7, and DU145 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO₂.

Western Blot Analysis and Antibodies

Cell pellets were lysated with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1% NP-40, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktails] on ice. Protein concentration was determined using the Lowry method (Bio-Rad). Whole-cell extracts were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed with appropriate antibodies. In brief, membranes were blocked in 5% skim milk in TBS (pH 7.4) containing 0.1% Tween 20 for 1 h and subsequently probed with primary antibodies at 4°C for overnight. Membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and then visualized by Enhanced Chemiluminescence Reagent (GE Healthcare). Antibodies specific for phospho-STAT3, phospho-STAT5, phospho-JAK2, JAK2, phospho-Src, Src, phospho-Lyn, phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2, poly(ADP-ribose) polymerase, caspase-3, Bcl-xL, Bcl-2, survivin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology. Anti-STAT3, anti-STAT5, anti-phospho-JAK3, anti-JAK3, anti-Lyn, and anti-SOCS3 antibodies were purchased from Santa Cruz Biotechnology.

Immunohistochemistry for Phospho-STAT3 Localization

L-540 cells were exposed to either DMSO alone or 40 $\mu\text{mol/L}$ AUH-6-96 for 24 h. Cells were then fixed with 100% methanol for 15 min and subsequently permeabilized with 0.1% Triton X-100 in PBS (pH 7.4). After blocking with 2% bovine serum albumin in PBS, cells were incubated with an antibody specific for phospho-STAT3 at 4°C for overnight. Cells were then washed with PBS and incubated with FITC-conjugated secondary antibody (Jackson ImmunoResearch) at room temperature for 1 h. Cells were counterstained with 4',6-diamidino-2-phenylindole and then imaged using an inverted fluorescence microscopy (Carl Zeiss).

Cell Viability, Morphology, and Apoptosis Assay

For cell viability assay, cancer cells (5×10^4 cells/mL) were treated with either vehicle (DMSO) alone, AUH-6-96 (40 $\mu\text{mol/L}$), or the JAK kinase inhibitor AG490 (150 $\mu\text{mol/L}$) and incubated for the indicated time periods. Trypan blue exclusion assay was conducted to count viable cells. For the morphology analysis, L540 cells were cultured in the presence of either vehicle (DMSO) alone, AUH-6-96 (40 $\mu\text{mol/L}$), or AG490 (150 $\mu\text{mol/L}$) for 24 h. Cells were then fixed with ice-cold 80% ethanol, washed with PBS, and stained with 4',6-diamidino-2-phenylindole (2.8 $\mu\text{g/mL}$) and sulforhodamine 101 (20 $\mu\text{g/mL}$; Sigma-Aldrich). For performing a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, L540 cells (1.0×10^6 cells/mL) were either treated or untreated with AUH-6-96 (40 $\mu\text{mol/L}$) for 48 h, stained using an APO-BRDU kit, according the manufacturer's protocol (Phoenix Flow Systems), and subsequently subjected to Elite ESP flow cytometry (Coulter).

Compound Synthesis

A compound library of novel polysubstituted imidopiperidines was synthesized using a tandem three-component aza[4+2]cycloaddition/allylboration multicomponent reaction between 1-aza-4-boronobutadienes, maleimides, and aldehydes. The library was generated by a parallel synthetic strategy, in solution phase, with compound purification by high-performance liquid chromatography with mass-based fraction collection, which provided the compounds in a high degree of purity suitable for biological screening (22).

Results**Identification of AUH-6-96 through a Cell-Based High-Throughput Screening in *Drosophila***

Drosophila STAT92E is most similar to STAT3 and STAT5 and is thought to regulate transcription in a manner similar to that observed for mammalian STATs, thus making STAT92E a useful model to identify small molecules that inhibit JAK/STAT transcriptional output. To find such molecules, we conducted a cell-based high-throughput screening using a cultured *Drosophila* cell line that stably expresses both a STAT92E transcriptional reporter and a PolIII-*Renilla* gene. These cells were cocultured for 24 h with Upd (cytokine)-producing cells in the presence of a library

of approximately 1,000 polysubstituted imidopiperidines (ref. 22; see Materials and Methods). The reporter activity was quantified by measuring relative luciferase units. We monitored the activity of *Renilla* luciferase by the mean of measuring the cytotoxicity of compounds tested and discarded those that resulted in more than 20% decrease in the activity compared with that of control. We conducted this high-throughput screening in triplicates and identified AUH-6-96 (Fig. 1C) as a potential inhibitor of STAT92E signaling. AUH-6-96 showed ability to inhibit STAT92E transcriptional activity in a dose-dependent manner. In fact, 10 $\mu\text{mol/L}$ AUH-6-96 reduced the reporter activity by more than 50%, whereas 40 $\mu\text{mol/L}$ blocked STAT92E transcriptional activity back to the level observed with vehicle (DMSO) alone (Fig. 1A). Tyrosine phosphorylation is a key step in STAT activation on cytokine/receptor stimulation. To assess if AUH-6-96 blocked this critical event, we next examined the effect of AUH-6-96 on Upd-induced phosphotyrosine STAT92E levels. We found treatment with 40 $\mu\text{mol/L}$ AUH-6-96 almost completely abrogated STAT92E phosphorylation (Fig. 1B). These findings suggest that AUH-6-96 is a novel small-molecule inhibitor of STAT92E signaling in *Drosophila*.

AUH-6-96 Suppresses Constitutive and Interleukin-6-Induced STAT3 Phosphorylation in Human Cells

We next tested whether AUH-6-96 can also block STAT signaling in human cells. Because STAT3 is the most common form found in human cancers, we monitored the effect of AUH-6-96 on its activity. We used a breast cancer cell line MDA-MB-468, a prostate cancer cell line DU145, and a Hodgkin lymphoma cell line L540, which all express constitutively active STAT3 (Fig. 2A). We examined phospho-STAT3 levels using an antibody specific for STAT3 that is phosphorylated on Y705. In all cancer cell lines tested, treatment with AUH-6-96 led to a dramatic decrease in the levels of phospho-STAT3, whereas total STAT3 levels remained unchanged (Fig. 2A). This suggests that the mechanism of action of AUH-6-96 is conserved between *Drosophila* and humans and that AUH-6-96 disrupts STAT3 signaling in a cell type-independent manner. STAT3 was initially identified as an interleukin-6 (IL-6)-dependent transcription factor that promotes acute-phase gene expression. Thus, we next attempted to examine the effect of AUH-6-96 on IL-6-induced STAT3 phosphorylation, using a multiple myeloma cell line RPMI 8226, for which IL-6 is a critical growth factor. RPMI 8226 cells were treated with AUH-6-96 at different concentrations for 6 h and then subsequently stimulated by IL-6 for 15 min. A dramatic induction of phosphotyrosine STAT3 was observed in untreated cells in response to IL-6 stimulation. However, the enhanced STAT3 phosphorylation was gradually decreased in AUH-6-96-treated cells in a dose-dependent manner and was completely blocked by 40 $\mu\text{mol/L}$ AUH-6-96 (Fig. 2B).

AUH-6-96 Affects STAT3 Phosphorylation in a Dose- and Time-Dependent Manner

We next examined the dose effect of AUH-6-96 on STAT3 phosphorylation using various cancer cells. In

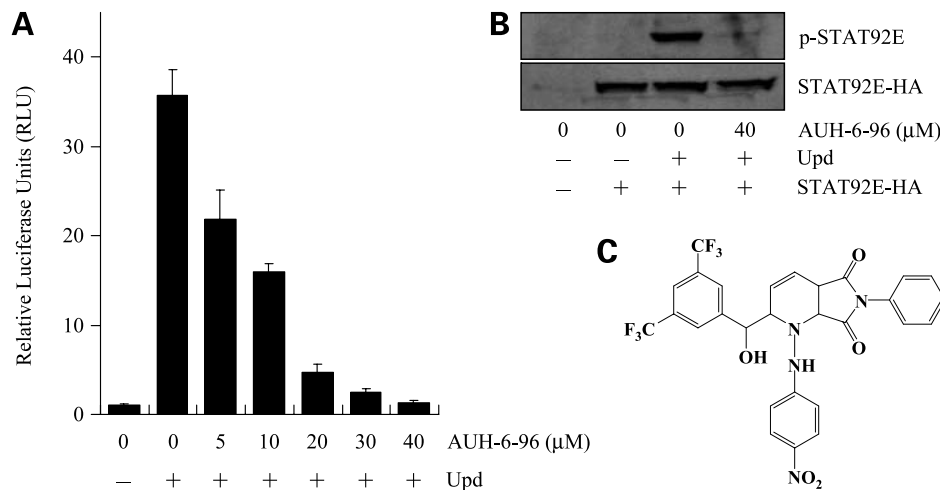


Figure 1. AUH-6-96 inhibits transcription and phosphorylation of STAT92E in *Drosophila*. **A**, dose effect of AUH-6-96 on Upd-induced STAT92E reporter activity. Cultured *Drosophila* cells that stably express a STAT92E reporter gene were cocultured for 24 h with Upd-producing cells in the presence of different concentrations of AUH-6-96. The STAT92E reporter activity was normalized as the ratio of firefly luciferase to *Renilla*. Note that the reporter activity without Upd stimulation was set to 1. Columns, mean of three independent experiments; bars, SD. **B**, effect of AUH-6-96 on Upd-induced tyrosine phosphorylation of STAT92E. S2-NP cells transiently transfected with an expression plasmid for STAT92E-HA were cocultured for 24 h with Upd-producing cells in the presence of either vehicle (DMSO) alone or AUH-6-96 (40 μmol/L). Western blot analysis was done with antibodies specific for phospho-STAT92E and HA. STAT92E-HA serves as a loading control. **C**, chemical structure of AUH-6-96 (C₂₈H₂₀F₆N₄O₅; molecular weight, 606.5).

MDA-MB-468 and DU145 cells, we detected a dramatic reduction of STAT3 phosphorylation at a 40 μmol/L concentration of AUH-6-96 (Fig. 3A and B), whereas in L540 cells treatment with 10 μmol/L AUH-6-96 blocked phosphorylated STAT3 levels by more than 50%, and

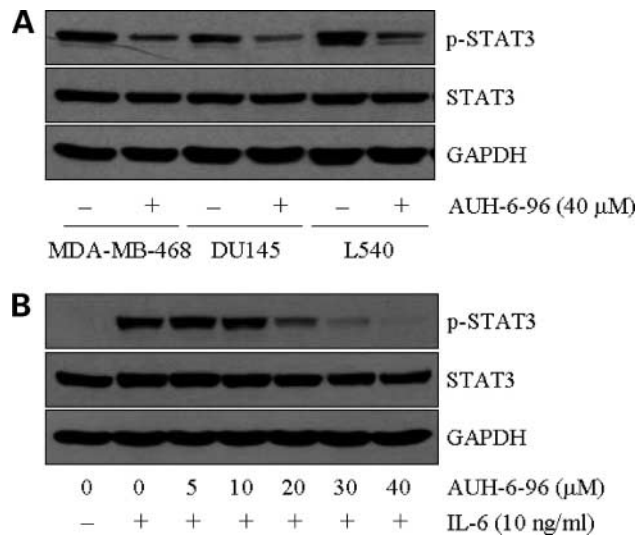


Figure 2. AUH-6-96 suppresses both constitutive and IL-6-induced STAT3 phosphorylation. **A**, human cancer cells that express constitutively active STAT3 were incubated with or without AUH-6-96 for 24 h. Whole-cell extracts were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies specific for phospho-STAT3 and STAT3. GAPDH serves as a loading control. **B**, human multiple myeloma RPMI 8226 cells were incubated with AUH-6-96 at different concentrations for 6 h and subsequently stimulated with IL-6 (10 ng/mL) for 15 min.

40 μmol/L AUH-6-96 almost completely inhibited phospho-STAT3 levels (Fig. 3C). In either case, the levels of total STAT3 protein remained unchanged. The survival and proliferation of Hodgkin lymphoma cells are affected by many cytokines, including IL-6 (24). Because STATs play an important role in cytokine signaling and AUH-6-96 efficiently inhibits phospho-STAT3 levels in L540 cells, we further analyzed the role of AUH-6-96 in JAK/STAT signaling using L540 cells. To assess if AUH-6-96 also blocks transcriptional activity of STAT3 in a dose-dependent manner, we monitored the expression of the STAT3 target gene *SOCS3*. Treatment with AUH-6-96 suppressed the expression of *SOCS3* in a dose-dependent manner as well that parallels the inhibition of phospho-STAT3 (Fig. 3C). We next examined the time effect of AUH-6-96 on phosphorylated STAT3 levels. The effect of AUH-6-96 on phosphorylated STAT3 levels occurred between 4 and 6 h of treatment (Fig. 3D). After 6 h, phospho-STAT3 levels remain nearly undetectable. Similarly, 6 h after treatment with AUH-6-96, the expression of *SOCS3* was significantly decreased (Fig. 3D).

AUH-6-96 Inhibits Constitutively Activated JAK3 but not ERK1/2

To gain more insights into the mechanisms of STAT3 inhibition, we next examined whether AUH-6-96 can affect upstream regulators of STAT3 in L540 cells. JAK kinases have been shown to be key upstream regulators of STAT activity. Therefore, AUH-6-96 may inhibit JAK kinase activity, which subsequently leads to a block in STAT3 activity. To test this possibility, we investigated the ability of AUH-6-96 to inhibit constitutively active JAK3, which is preferentially expressed in leukocytes. Western blot analysis showed that AUH-6-96 effectively reduced phosphotyrosine JAK3

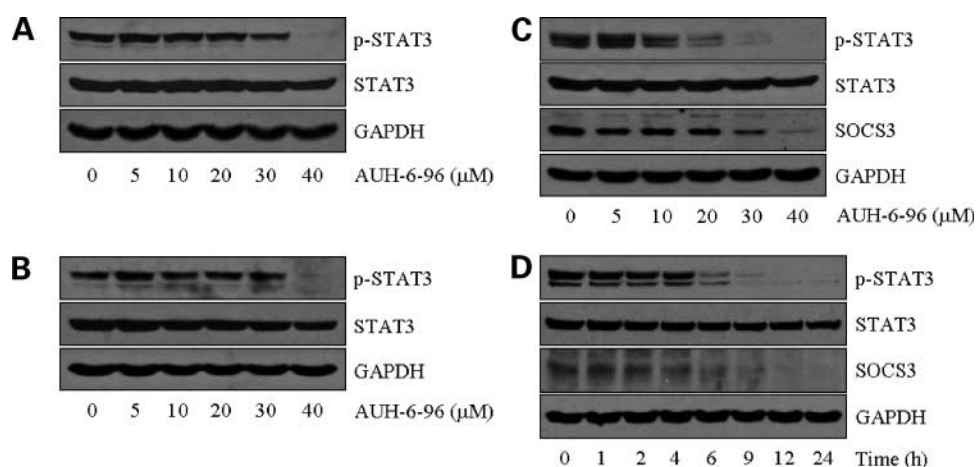


Figure 3. AUH-6-96 inhibits constitutively active STAT3 in a dose- and time-dependent manner. **A** and **B**, dose effects of AUH-6-96 on persistently active STAT3 in human cancer cell lines. MDA-MB-468 (**A**) and DU145 (**B**) cells were incubated for 24 h with AUH-6-96 at different concentrations ranging from 5 to 40 μmol/L. Whole-cell extracts were processed for Western blot analysis using anti-phospho-STAT3 and STAT3 antibodies. GAPDH serves as a loading control. **C**, dose effects of AUH-6-96 on persistently active STAT3 and the STAT3 transcriptional target gene *SOCS3* in L540 cells. An antibody specific for *SOCS3* was used for Western blot analysis. **D**, time effects of AUH-6-96 on the levels of phosphorylated STAT3 and *SOCS3* in L540 cells. Cells were exposed to 40 μmol/L AUH-6-96 and cultured for the indicated periods.

levels in a dose-dependent manner, whereas total JAK3 levels remained unchanged (Fig. 4A, lanes 1 and 2). The known inhibitors of JAK kinase, such as AG490, can inhibit JAK1, JAK2 and JAK3. To assess if this is the case for AUH-6-96, we turned to another human cancer cell line MDA-MB-468 because persistently active JAK2 is not detected in L540 cells (data not shown). In MDA-MB-468 cells, JAK2 has

sustained activity as evidenced by a phosphospecific JAK2 antibody. AUH-6-96 did decrease levels of phospho-JAK2 without effecting total JAK2 levels (Fig. 4B, lanes 1 and 2). However, AUH-6-96 seemed to more potently inhibit JAK3 than JAK2 because at 20 to 30 μmol/L concentrations phospho-JAK3 was almost completely absent, whereas phospho-JAK2 levels were only partially inhibited. Because

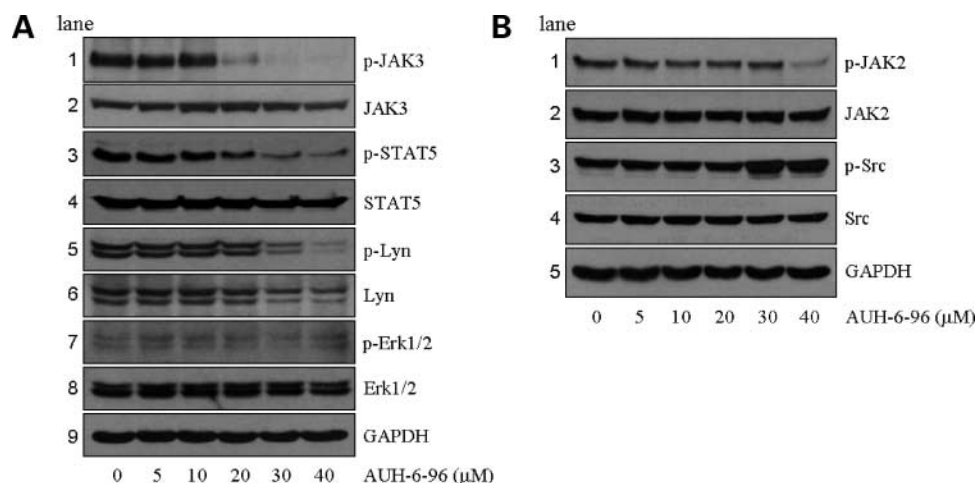


Figure 4. AUH-6-96 decreases the levels of tyrosine phosphorylated JAK3 but not ERK1/2. **A**, L540 cells were treated with AUH-6-96 at different concentrations for 24 h. Whole-cell extracts were processed for immunoblotting analysis using antibodies specific for the molecules indicated. AUH-6-96 decreased the levels of tyrosine phosphorylated JAK3 in a dose-dependent manner (lane 1), whereas total JAK3 levels remained unchanged (lane 2). AUH-6-96 also inhibits phospho-STAT5 levels in a dose-dependent manner (lane 3). Total STAT5 levels remained unchanged (lane 4). Treatment of L540 cells with high levels of AUH-6-96 (30-40 μmol/L) inhibited the expression of phosphotyrosine Lyn (lane 5) but also lowered total Lyn levels (lane 6). Note that AUH-6-96 at a concentration of 20 μmol/L that significantly abrogated constitutively active JAK3 did not affect Lyn phosphorylation. Neither constitutively active ERK1/2 (lane 7) nor total ERK1/2 (lane 8) levels were altered after AUH-6-96 treatment. GAPDH serves as a loading control (lane 9). **B**, MDA-MB-468 cells were treated with AUH-6-96 at different concentrations for 24 h. Whole-cell extracts were processed for Western blotting analysis using antibodies specific for the molecules indicated. Note that treatment with 40 μmol/L AUH-6-96 induced a significant reduction of phospho-JAK2 (lane 1) but had no effect on Src phosphorylation levels (lane 3). In both cases, the levels of total JAK2 and Src remained unchanged (lanes 2 and 4). GAPDH serves as a loading control (lane 5).

our data suggest that AUH-6-96 blocks JAK kinases, we assessed if this reagent can also inhibit other STAT family members, such as STAT5. As expected, AUH-6-96 inhibited the levels of phospho-STAT5 in a dose-dependent manner (Fig. 4A, lanes 3 and 4). Some Src family tyrosine kinases have also been shown to promote STAT3 activity via phosphorylating Y705 in STAT3. To address if our compound inhibits Src family kinases, we monitored the tyrosine phosphorylation state of Lyn, a Src family member that is preferentially expressed in B lymphocytes (25). Treatment of L540 cells with high levels of AUH-6-96 lowered Lyn tyrosine phosphorylation levels but also affected total Lyn levels (Fig. 4A, lanes 5 and 6). In addition, our time-course analysis showed that 40 $\mu\text{mol/L}$ AUH-6-96 induced a significant reduction of both phosphorylated JAK3 and STAT3 levels between 4 and 6 h after the treatment, whereas no significant effect on Lyn phosphorylation was observed up to 8 h (Fig. 3D; Supplementary Fig. S1).⁵ These results suggest that in L540 cells the abrogation of STAT3 signaling by AUH-6-96 is primarily caused by its inhibitory effect on JAK3 but not Lyn and that the effect of AUH-6-96 on Lyn is indirect. We also found that AUH-6-96 does not alter Src kinase phosphorylation levels. Treatment of breast cancer cells MDA-MB-468 with 40 $\mu\text{mol/L}$ AUH-6-96 significantly abrogated both phospho-JAK2 and STAT3 (Figs. 3A and 4B, lane 1) but had no inhibitory effect on the levels of Src tyrosine phosphorylation (Fig. 4B, lane 3). We further examined whether AUH-6-96 can affect other oncogenic signaling pathway components, such as mitogen-activated protein kinase. L540 cells have low but detectable levels of phospho-mitogen-activated protein kinase (in this case, ERK1/2), suggesting that these cells have constitutive mitogen-activated protein kinase activity. However, neither the phosphorylation of ERK1/2 nor the total levels of ERK1/2 protein were altered after the treatment of AUH-6-96 at the concentrations up to 40 $\mu\text{mol/L}$ (Fig. 4A, lanes 7 and 8).

AUH-6-96 Selectively Inhibits Cancer Cell Viability

The inhibition of STAT3 signaling was reported to reduce cancer cell survival (26, 27). We next assessed if AUH-6-96 can decrease cancer cell viability by down-regulating STAT3 activity. We used five cancer cell lines: L540, MDA-MB-468, Burkitt's lymphoma DG-75, chronic myeloid leukemia EM-3, and breast cancer MCF-7. Western blot analysis revealed that persistently active STAT3 is present in L540 and MDA-MB-468 cells (Fig. 5A). We detected very low levels of phospho-STAT5 and JAK2 in MCF-7 cells, but we did not detect constitutively active JAKs and STATs in DG-75 and EM-3 cells (data not shown). For cell viability assay, the cells were treated with either vehicle alone, 40 $\mu\text{mol/L}$ AUH-6-96, or 150 $\mu\text{mol/L}$ AG490, which was included as a positive control. As predicted, we found that the viability of L540 and MDA-MB-468 cells was decreased significantly by AUH-6-96 and AG490, whereas that of

DG-75, EM-3, and MCF-7 cells was unaffected (Fig. 5B-F). In addition, we found that AUH-6-96 does not show ability to affect the viability of normal breast cells MCF-10A (data not shown). Together, these data indicate that AUH-6-96 selectively decreased cell viability only in cancer cell lines harboring aberrant JAK/STAT signaling and suggest that AUH-6-96 affects cancer cell survival by down-regulating STAT3 activity.

AUH-6-96 Induces Apoptosis

To show that the decreased viability observed in AUH-6-96-treated L540 cells resulted from the induction of apoptosis, we examined the morphology of L540 cells treated with AUH-6-96 or AG490. In vehicle-treated cells, no change in cell morphology was detected (Supplementary Fig. S2A-C).⁵ In contrast, AUH-6-96- or AG490-treated cells showed distinct morphologic features associated with apoptosis, such as membrane blebbing and chromatin condensation (Supplementary Fig. S2D-I).⁵ To further confirm that compound-treated cells did indeed undergo programmed cell death, we did a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Cells were either treated or untreated for 48 h. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells were increased more than 20-fold in AUH-6-96-treated cells compared with controls (Fig. 6A). Because the cleavage of poly(ADP-ribose) polymerase and caspase-3 are hallmarks of apoptosis, we examined the size of these molecules in cells treated with AUH-6-96. As expected, treatment with the compound resulted in an increase in both poly(ADP-ribose) polymerase and caspase-3 cleaved fragments in a dose-dependent manner (Fig. 6B).

AUH-6-96 Induces Apoptosis via Down-Regulating the Expression of Antiapoptotic Genes

The abrogation of STAT3 signaling induces apoptosis by down-regulating the expression of antiapoptotic gene (28–31). To obtain more insights into the molecular mechanisms of apoptosis induced by AUH-6-96, we examined the effect of this reagent on the expression of antiapoptotic proteins, which are known STAT3 targets. L540 cells were exposed to AUH-6-96 at various concentrations for 48 h. Whole-cell extracts were processed for Western blot analysis using antibodies specific for Bcl-xL, Bcl-2, and survivin. As expected, treatment with AUH-6-96 decreased the expression of these antiapoptotic factors in a dose-dependent manner, wherein the levels of GAPDH remained unchanged (Fig. 6C). These data suggest that AUH-6-96 induces programmed cell death via suppressing the expression of antiapoptotic genes in L540 cells.

Discussion

The fruit fly *Drosophila* is often used as a simple model organism for evaluating the *in vivo* efficacy of known chemical compounds in models of various human diseases, such as neurodegenerative disorders (32), longevity (33), and sleep disorders (34). However, the contribution of *Drosophila* to drug discovery has thus far been modest and untapped (35). This is despite several major advantages of

⁵ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

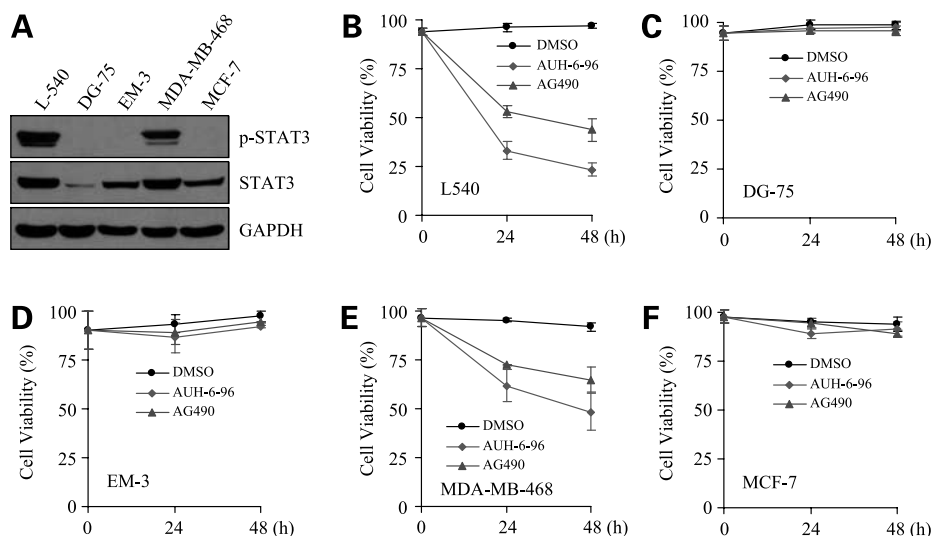


Figure 5. AUH-6-96 selectively affects cell viability only of cancer cells harboring aberrant JAK/STAT signaling. **A**, expression of constitutively active STAT3 was examined in various human cancer cell lines. Note that persistently active STAT3 are present only in L540 and MDA-MB-468 cells. **B to F**, cancer cells were treated with either vehicle (DMSO) alone, AUH-6-96 (40 $\mu\text{mol/L}$), or the JAK kinase inhibitor AG490 (150 $\mu\text{mol/L}$) as a positive control and incubated for the indicated periods. Trypan blue exclusion assay was conducted to count viable cells. *Points*, mean of three independent experiments; *bars*, SD. Note that the viability of L540 and MDA-MB-468 cells was decreased significantly by AUH-6-96 and AG490, whereas that of DG-75, EM-3, and MCF-7 cells was unaffected.

Drosophila, including (a) the reduced redundancy of *Drosophila* genome compared with mammalian genomes; (b) the versatility and ease of compound validation in whole animals by oral administration; and (c) the rapid identification of the cognate targets for compounds through genetic manipulation. Importantly, *Drosophila* contains an evolutionarily well-conserved and yet simplified JAK/STAT pathway that consists of only one JAK and one STAT. In addition, a variety of tools that can monitor STAT

activation both *in vitro* and *in vivo* are available. In this study, we have finally begun to tap into *Drosophila*'s potential for drug discovery. Using cultured *Drosophila* cells and high-throughput screening of a chemical library, we identified AUH-6-96 as a novel small-molecule inhibitor of JAK/STAT signaling in *Drosophila* and in human cancer cells. AUH-6-96 inhibited STAT92E transcriptional activity and phosphorylation induced by cytokine stimulation in *Drosophila* (Fig. 1A and B). Because the JAK/STAT

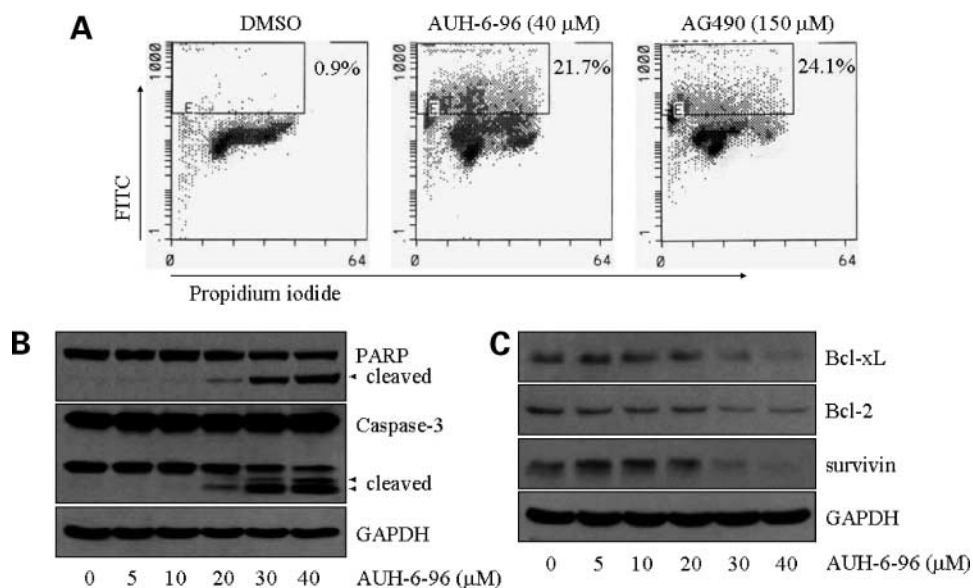


Figure 6. AUH-6-96 induces apoptosis via down-regulating the expression of antiapoptotic genes in L540 cells. **A**, a representative flow cytometry analysis for programmed cell death. Cells were cultured for 48 h in the presence of DMSO alone, 40 $\mu\text{mol/L}$ AUH-6-96, or 150 $\mu\text{mol/L}$ AG490. Cells were then harvested, stained with FITC-conjugated bromodeoxyuridine antibody and propidium iodide, and subsequently subjected to flow cytometry. The percentages of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells are indicated. **B**, cells were treated with AUH-6-96 at various concentrations for 24 h. Whole-cell extracts were resolved on SDS-PAGE and then immunoblotted with antibodies specific for poly(ADP-ribose) polymerase and caspase-3. Treatment with AUH-6-96 results in an increase in both poly(ADP-ribose) polymerase and caspase-3 cleaved fragments in a dose-dependent manner. GAPDH serves as a loading control. **C**, cells were treated with AUH-6-96 at different concentrations ranging from 5 to 40 $\mu\text{mol/L}$ for 24 h. Whole-cell extracts were processed for Western blot analysis using antibodies specific for the molecules indicated. Note that abrogation of STAT3 signaling by AUH-6-96 decreases the expression of Bcl-xL, Bcl-2, and survivin that are known STAT3 downstream target genes.

signal transduction pathway is conserved such that true structural and functional homologues of components originally identified in vertebrate systems are also present in *Drosophila* (36), we predicted that AUH-6-96 may function in other species in an expected manner. Indeed, we found that AUH-6-96 can also inhibit STAT signaling in human cells. AUH-6-96 effectively inhibited both constitutive and cytokine (IL-6)-induced STAT3 phosphorylation in various human cancer cell lines (Fig. 2) and induced the redistribution of phospho-STAT3 (Supplementary Fig. S3).⁵ It also suppressed STAT3 transcriptional activity as evidenced by decrease in the expression of STAT3 target gene *SOC53* (Fig. 3C and D).

Our study suggests that in L540 cells the inhibition of transcription and phosphorylation of STAT3 by AUH-6-96 is at least in part due to its inhibitory effect on constitutively active JAK3. In L540 cells, JAK3 may play a critical role in STAT3-mediated signaling. In support of this, the disruption of JAK3 activity by RNA interference in L540 cells resulted in the inhibition of STAT3 DNA-binding activity and induced programmed cell death through disrupting the expression of cell survival genes that are known targets of STAT3 (37). It is notable that AUH-6-96 strongly inhibits phospho-JAK3 levels, whereas it can moderately inhibit phospho-JAK2. For example, when used at 20 to 30 $\mu\text{mol/L}$ concentrations, AUH-6-96 almost completely inhibited phospho-JAK3 in L540 cells (Fig. 4A, lane 1). In contrast, at the same concentrations, phospho-JAK2 was partially suppressed in MDA-MB-468 cells (Fig. 4B, lane 1). These are consistent with the finding that AUH-6-96 blocks phospho-STAT3 more efficiently in L540 cells with constitutively active JAK3 than in cells without the persistent activation of JAK3, such as MDA-MB-468 or DU145 (Fig. 3A-C). However, it cannot be ruled out that the potency of AUH-6-96 to each JAK family member is to some extent dependent on cell types tested of which have different genetic backgrounds. Therefore, the IC_{50} values of AUH-6-96 against all JAK kinases need to be determined in cells that express all these JAK kinases. Regardless, AUH-6-96 shows ability to block JAK2 and JAK3 activity. STAT3 activity is also shown to be regulated by several other upstream kinases, such as nonreceptor Src family, phosphatidylinositol 3-kinase/Akt, Ras/Raf/MEK/ERK, and epidermal growth factor receptor (38–41). A Src family member, Lyn is shown to be required for B-cell antigen receptor-mediated STAT3 activation in B-cell signaling (42) and for stem cell factor-induced activation of STAT3 in bone marrow master cells (43). Treatment of L540 cells with AUH-6-96 at a 40 $\mu\text{mol/L}$ concentration showed comparable inhibition of persistently active Lyn, suggesting a role for Lyn in STAT3 inhibition. However, our data suggest that AUH-6-96 affects Lyn kinase indirectly and that the inhibition of STAT3 signaling by this reagent is not due to the abrogation of Lyn activity. First, AUH-6-96 at a 40 $\mu\text{mol/L}$ concentration also lowered total Lyn levels, suggesting its effect on transcription and/or degradation of Lyn (Fig. 4A, lane 6). Second, a 20 $\mu\text{mol/L}$ concentration of AUH-6-96

that induced a dramatic reduction of both constitutively active STAT3 and JAK3 did not affect the levels of Lyn phosphorylation (Figs. 3C and 4A, lanes 1 and 5). Third, treatment with AUH-6-96 at a 40 $\mu\text{mol/L}$ concentration induced a dramatic reduction of both phosphorylated JAK3 and STAT3 levels between 4 and 6 h, whereas at the same concentration no significant effect on both phosphorylated and total Lyn levels was observed up to 8 h (Fig. 3D; Supplementary Fig. S1).⁵ Notably, we also found that AUH-6-96 did not alter Src kinase phosphorylation levels: treatment of breast cancer cells MDA-MB-468 with 40 $\mu\text{mol/L}$ AUH-6-96 profoundly decreased both phospho-STAT3 and phospho-JAK2 levels (Figs. 3A and 4B, lane 1) but had no effect on the levels of Src tyrosine phosphorylation (Fig. 4B, lane 3). Our study also showed that treatment with AUH-6-96 neither altered levels of phosphorylated ERK1/2 nor total ERK1/2 (Fig. 4A, lanes 7 and 8). Together, our study suggests that AUH-6-96 has selectivity for JAK/STAT signaling. This has been further supported by the findings that AUH-6-96 reduces cell viability only of cancer cells harboring aberrant JAK/STAT signaling (Fig. 5) and that AUH-6-96 induces apoptosis through down-regulation of the expression of antiapoptotic genes, such as Bcl-2, Bcl-xL, and survivin that are known STAT3 downstream targets (Fig. 6C).

In summary, we have identified a novel chemical compound that selectively inhibits JAK/STAT signaling in both *Drosophila* and humans using *Drosophila* as a model organism. Given that higher concentrations of AUH-6-96 require inhibiting JAK/STAT signaling, it should be emphasized that AUH-6-96 is an early lead compound identified from a rather modestly sized library of diverse polysubstituted piperidines. The next phase of development will examine a more focused second-generation library to optimize the inhibitory effect and obtain a detailed structure-activity relationship that will also aid on designing more efficient and potent probes. Therefore, AUH-6-96 can be used as a starting point to develop a new class of anticancer drugs to target cancer cells harboring aberrant JAK/STAT signaling. Finally, this study also provides an effective method to identify low molecular weight inhibitors of JAK/STAT signaling using *Drosophila* as a tool for drug discovery.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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