Preclinical Development

Inhibition of Constitutive Activation of STAT3 by Curcurbitacin-I (JSI-124) Sensitized Human B-Leukemia Cells to Apoptosis

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Abstract
Phosphorylation of STAT3 on serine 727 regulates gene expression and is found to be elevated in many B-leukemia cells including chronic lymphocytic leukemia (CLL). It is, however, unclear whether targeting STAT3 will be an effective antileukemia therapy. In this study, we assessed in vitro antileukemia activity of the STAT3 inhibitor JSI-124 (cucurbitacin I). JSI-124 potently induces apoptosis in 3 B-leukemia cell lines (BJAB, I-83, and NALM-6) and in primary CLL cells and was associated with a reduction in serine 727 phosphorylation of STAT3. Similarly, knockdown of STAT3 expression induced apoptotic cell death in these leukemia cells. In addition, we found that JSI-124 and knockdown of STAT3 decreased antiapoptotic protein XIAP expression and overexpression of XIAP blocked JSI-124-induced apoptosis. Furthermore, we found that combined treatment of JSI-124 and TRAIL increased apoptosis associated with an increase in death receptor 4 expression. Besides apoptosis, we found that JSI-124 also induced cell-cycle arrest prior to apoptosis in B-leukemia cells. This corresponded with reduced expression of the cell-cycle regulatory gene, cdc-2. Thus, we present here for the first time that JSI-124 induced suppression of serine 727 phosphorylation of STAT3, leading to apoptosis and cell-cycle arrest through alterations in gene transcription in B-leukemia cells.

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Introduction
STAT proteins comprise a family of transcription factors involved in normal cellular events such as differentiation, proliferation, apoptosis, and regulation of hematopoietic cell function (1). However, inappropriate activation of STAT signaling gives rise to various pathologic events contributing to cancer progression. Specifically, activated STAT3 increases tumor cell proliferation, survival, and invasion while suppressing antitumor immune responses (1, 2).

STAT proteins are activated in response to a number of cytokines, growth factors, and hormones (3). The activation of the STAT proteins involves phosphorylation of specific tyrosine and serine residues, leading to dimerization and subsequent translocation to the nucleus, where STATs bind to specific target gene promoters to regulate gene transcription. STAT1 and STAT3 are phosphorylated on serine 727, allowing for increased transcription. Several reports showed that the level of STAT1 and STAT3 phosphorylation on serine 727 are elevated in B-leukemia cells, although the biologic importance of activated STAT proteins is unknown (4, 5).

Using a natural compound screen, it was discovered that the JSI-124 (cucurbitacin I) is a selective dual inhibitor of phospho-JAK2 and phospho-STAT3 levels in many cancer cells, such as human breast cancer, lung cancer, neuroblastoma, and murine melanoma cells (6–8). This inhibitor has been shown to exert antiproliferative and antitumor activity both in vivo and in vitro. Specifically, treating xenograft tumors with JSI-124 effectively reduced tumor growth (9). This indicates that targeting the STAT pathway with JSI-124 could be an effective treatment of cancers.

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia and is characterized by CD5+ /CD19+ mature B cells that accumulate gradually in lymphoid organs, bone marrow, and peripheral blood (10, 11). Despite initial responses to therapy, relapse and eventual drug resistance usually occur, demonstrating the need for novel drug therapies. It has been shown in CLL cells that STAT3 phosphorylation on serine 727 is increased, leading to transcription activation suggesting that STAT3 could be an important target for CLL therapy (3, 4).

In this study, we treated the 3 lymphoma and leukemia cell lines (BJAB, I-83, and NALM-6) and primary CLL cells with JSI-124. Our results indicated that JSI-124 can efficiently induce apoptosis in B-leukemia cells including...
CLL cells by blocking serine 727 phosphorylation of STAT3, leading to the downregulation of antiapoptotic gene, XIAP, and cell-cycle regulatory gene, cdc2, and upregulation of death receptor 4 (DR4) gene expression. These findings collectively indicated that JSI-124 could be a therapeutic agent against B-leukemias.

Materials and Methods

Materials
JSI-124 was purchased from Calbiochem Inc. and dissolved in DMSO initial stock concentration at 10 mmol/L. Rabbit polyclonal anti-STAT3/1, XIAP, and cdc2, caspase-8, and caspase-3 antibodies were purchased from Cell Signaling Technology; siRNA-STAT3, siRNA control, and monoclonal Mcl-1 antibody were purchased from Sigma. DR4 antibody was purchased from Abcam, and HDAC1 were purchased from Santa Cruz Biotechnology. Anti-DR5 antibody was purchased from Medi-corp. Soluble super-TRAIL was purchased from Alexis. Annexin-V–FITC and propidium iodide (PI) were acquired from Pharmingen (BD Biosciences).

Cells
Burkitt lymphoma cell line (BJAB) was obtained from the American Type Culture Collection (Cedarlane Laboratory) and pre-acute lymphoblastic leukemia cell line, NALM-6, was obtained from DSMZ. Cells were cultured and frozen down from the first passage for future. Cells were characterized by the cell bank by staining with CD3+ or CD10+, CD19+, CD37+, cyCD79a+, CD80+, CD138+, HLA-DR+, sm/cyIgG+, cylgM+, smlgM+, sm/cykappa+, and sm/cylambda. I-83 was a kind gift from Dr. Panasci (McGill University, Montreal, Quebec, Canada) and characterized by with CD19+, CD5+ surface staining. I-83 cells were last tested in June 2010. All cell lines were grown in RPMI 1640 medium (HyClone ThermoScientific) containing 10% fetal calf serum (HyClone ThermoScientific) and characterized by with CD19+, CD5+ surface staining. I-83 cells were last tested in June 2010. All cell lines were grown in RPMI 1640 medium (HyClone ThermoScientific) containing 10% fetal calf serum (HyClone ThermoScientific) in a humidified atmosphere (37°C, 5% CO2). Primary B-CLL cells were isolated from patients and maintained as previously described (12). The use of primary CLL cells for this project was approved by the Research Ethics Board at the University of Manitoba.

Immunoprecipitation and immunoblot analysis
Lysates were prepared from BJAB, I-83, NALM-6, and primary CLL cells, stimulated with JSI-124, immunoprecipitated with STAT3/1 antibodies, and immunoblotted for the expression of STAT3/1 proteins as previously described (12), with antibodies to p-STAT3 ser-727/tyr-705, p-STAT1 (serine 727) or STAT3/1. Immunoblotting was carried out with appropriate antibodies for the expression of Mcl-1, XIAP, caspase 3/8, and cdc2 proteins.

Chromatin immunoprecipitation assay
The chromatin immunoprecipitation assay (ChIP) assay was done as described by Ishidorj et al. (12), with some modifications. BJAB and NALM-6 cells were treated with JSI-124 and lysed and prepared as previously. The lysate was precipitated with the primary antibody to STAT3 at 1:50 dilution overnight at 4°C. The DNA was purified using the QIA-AMP DNA purification system (Qiagen). PCR products were detected, using primers specific for XIAP promoter region for STAT binding (forward primer, 5’-TTTCTACATAGCAGAGG-GAGT-3’; and reverse primer, 5’-CGCGGCTAATAAGG-TAACTGAG-3’), for 33 cycles with cycling conditions as follows: 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. DR4 (forward primer, 5’-TCTCCCGTGTATTAAAGACTTTCAG-3’; and reverse primer, 5’TCAAGCGATTCTTCTGCTCA-3’), for 30 cycles with cycling conditions as follows: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. For each treatment point, 1% of the total DNA was assayed for equal loading (input). ChIP with rabbit control IgG–ChIP grade antibody (Abcam) was conducted for each condition tested.

Transfection of BJAB and I-83 cells with siRNA
A total of 2.5 × 10⁶ cells were used for electrophoresis (Nucleofector I Device; Lonza), with Cell Line Nucleofector Kit-T-program-O17 (Lonza) according to the manufacturer’s instruction. siRNA (40 nmol/L) against STAT3 and universal negative control were used. After 48 hours of transfection, the cells were analyzed by FACS for cell-cycle analysis or cell lysate was prepared for immunoblotting as described earlier. Transfection efficiency was checked by transfecting 2 μg of pmax-GFP with same conditions as described earlier.

Transfection of BJAB cells with XIAP-cDNA
Four micrograms of plasmid containing the cDNA-XIAP or empty vector pCMV-XL5 (Origene) was transfected into BJAB cells by using DreamFect cold transfection reagent (BD Biosciences) according to the manufacturer’s instructions. After 2 days of transfection, the cells were treated with JSI-124 for additional 24 hours.

Real-time PCR
Real-time PCR was used to assess mRNA level of XIAP in cells that had been treated with JSI-124 for various times. Total RNA was isolated using the RNaseq RNA Isolation Kit (Qiagen) and 2 μg of total cellular RNA was used as a template for reverse transcription PCR with random hexamers (Invitrogen). CDNA was then used as a template for real-time PCR using predesigned primer sets and SYBR Green-PCR Master Mix (Bio-Rad) according to the manufacturer’s instructions. The following primers were used for the real-time PCR reaction: mRNA-XIAP, forward primer 5’-GCACCGATCTTACCTTGCGG-3’; reverse primer 5’-TGCAACCCTGATACCACTT-3’. Following primers to housekeeping gene, GAPDH, were used to standardize results: forward primer 5’-GCCATGACAACTTTGGTATCGTGG3’ and reverse primer 5’-GACGACTCCCCTCACCATCTCT-3’.
Cell death detection and annexin-V/7-AAD staining

The cell death assay was done as described by Ishdorj et al. (12).

Cell-cycle analysis

Cells were fixed for at least 1 hour with 70% (w/v) ice-cold ethanol at 4°C. Cells were washed with PBS and resuspended in 1 mL of PBS containing 50 μg/mL of PI and 500 U/mL of RNase A. Following incubation for 15 minutes in the dark at room temperature, cells were analyzed by flow cytometer using the CellQuest software (Becton Dickinson). The PI fluorescence signal at FL2A peak versus the count was used to discriminate G2-M cells from G0-G1 doublets.

Assessment of cell surface receptor expression

The assay was done as described by Ishdorj et al. (12).

MTT assay

Cell viability was assessed by the MTT colorimetric assay. Lymphocytes were incubated with varying concentrations of JSI-124 for 18 hours at 37°C in a 5% CO2 environment. Cell dilution was then done in quadruplicate in 96-well microtiter plates for each drug concentration including nontreated control cells, and the cells were incubated at 37°C with 5% CO2 for 72 hours. Fifty microliters of MTT (1 mg/mL) in RPMI was added to each well, and plates were incubated at 37°C for 2 to 6 hours in 5% CO2. The microtiter plates were then spun for 10 minutes at 1,500 rpm, and 150 μL of DMSO was added to each well to dissolve the dark blue crystals and the absorbance was read at 540 nm with a microplate reader.

Immunostaining

Cells were treated with Mitotracker CMXRos (1:10,000, stock 1 μg/mL; Molecular Probes) for 15 minutes. Cells were then cytospun onto glass slides at a density of 5 × 10⁵ cells/mL followed by fixation in 3.7% paraformaldehyde/PBS for overnight at 4°C. Fixed cells were then washed 3 times in PBS and permeabilized in 100% methanol for 10 minutes at −20°C. Cells were blocked for 30 minutes in 5% BSA/PBS and probed with a 1:75 dilution of anti-cytochrome c and STAT3 serine 727 (Cell Signaling) for 2 hours at room temperature. Cells were washed 3 times for 5 minutes in 0.1% NP-40/PBS followed by probing for 1 hour with dilutions 1:200 of biotinylated anti-rabbit (Vector Labs.) and 1:500 of Alexafluor 488 (Molecular Probes). Immunostained cells were washed 3 times and imaged by sequential scanning using Fluoview 4.3 software, FV-500 Confocal Laser Scanning microscope (Olympus).

Statistical analysis

All experiments were repeated at least 3 times, and each experiment was done at least in duplicate. The data were expressed as means ± SE (standard error). Statistical analysis was done by using Student’s t test. The criterion for statistical significance was P < 0.05.

Results

JSI-124 suppresses the levels of serine-phosphorylated STAT3 in B-leukemia cell lines and B-CLL primary cells

To identify the molecular target of JSI-124 (Supplementary Fig. 1) treatment in I-83 (CLL-like cell line), Burkitt lymphoma cell line (BJAB) and human pre–acute lymphocytic leukemia cell line (NALM-6), we examined the effect of JSI-124 treatment on STAT3 phosphorylation. Cells were treated with 1 μmol/L of JSI-124 over a 24-hour time course. Cell extracts were immunoprecipitated with antibodies to STAT1, STAT3, and STAT5. The immunoprecipitates were then immunoblotted with antibodies against serine 727 phosphorylation of STAT3 or STAT1 or with antibodies against tyrosine 705/694 phosphorylation of STAT3 or STAT5. As a control, total protein for STAT3, STAT1, and STAT5 was determined. Total STAT3 protein levels in cells treated with JSI-124 failed to show significant change; however, serine 727 phosphorylation levels were inhibited in time-dependent fashion. Significant decrease in the amount of serine 727 phosphorylation of STAT3 was also observed in BJAB, I-83, and NALM-6 cells treated for 6 hours with JSI-124 and was barely detectable at the 24-hour time point (Fig. 1A). Isotope-matched control antibody was used as a negative control. We failed to detect tyrosine 705 phosphorylation of STAT3 in BJAB, I-83, and NALM-6 cells (Fig. 1A). No significant change was observed in the tyrosine 694 phosphorylation of STAT5 or in serine 727 phosphorylation of STAT1 in all cells treated with same condition as described earlier (Fig. 1A). Consistent with Fig. 1A, we failed to observe any tyrosine 705 phosphorylation of STAT3 in BJAB cells (Supplementary Fig. 2A). As a positive control, lysate from A341 cell treated with EGF showed tyrosine 705 phosphorylation of STAT3 (Supplementary Fig. 2A). Similar results were also observed in I-83 and NALM-6 cells (data not shown). In primary CLL cells, JSI-124 reduced serine 727 phosphorylation of STAT3 significantly at 6 hours and blocked phosphorylation completely after 24 hours (Fig. 1B). Recent studies reported that STAT3 is localized within mitochondria (13), so we next studied the localization of STAT3 in B-leukemia cells. As illustrated in Fig. 1C, phosphorylated STAT3 was found mainly in cytoplasm and not in mitochondria, as denoted by a lack of overlap staining between Mitotracker and phosphorylated STAT3. Significant decrease in the staining of serine 727 phosphorylation of STAT3 was also observed in the cells treated with JSI-124 (Fig. 1C).

Because JSI-124 is also a dual inhibitor for Janus kinase (JAK) and STAT3 pathway, it is possible that JAK was a target for JSI-124 treatment. To address this question, we used a selective inhibitor of JAK, AG490. BJAB, I-83, and NALM-6 cells were treated with AG490 (25 μmol/L) for...
24 hours and the amount of apoptosis was determined using flow cytometry. The accumulation of sub-G1 phase cells (indicator of apoptosis) failed detection in BJAB, I-83, and NALM-6 cells (Supplementary Fig. 2B). In addition, AG490 had no effect on serine 727 phosphorylation of STAT3 in these cells (data not shown). This indicates that serine 727 phosphorylation of STAT3 is the likely target of JSI-124 treatment in B-leukemias.
JSI-124-induced apoptosis in B-leukemia cells

To investigate the effect of JSI-124 on apoptosis, I-83, BJAB, and NALM-6 cells were treated with various doses of JSI-124 for 24 hours. Apoptotic cell population was measured using sub-G1 peak analysis. The results indicated that JSI-124 induced apoptosis in a dose-dependent manner (Fig. 2A). As little as 100 nmol/L of JSI-124 could induce apoptosis in the cells, 1 μmol/L of JSI-124 increased accumulation of sub-G1 phase cells to a greater extent (from 35% to 45%; Fig. 2A).

It is well known that cytochrome c accumulates in the cytosol in response to various apoptotic stimuli (14). Next, we determined whether cytochrome c was released by JSI-124 in B-leukemia cells. In Fig. 2B, immunostaining in mitochondrial and cytosolic fractions showed that cytochrome c in the corresponding mitochondrial fraction decreased and sequentially increased in the cytosolic fraction. GAPDH was used as the loading control for the cytosolic fraction in the blot (Fig. 2B). This was also observed in immunostaining in which cytochrome c in the cytosol fraction increased significantly after 6 hours by JSI-124 compared with control, as indicated by a lack of punctate staining (Fig. 2B).

In addition, primary CLL cells from 6 different patients were analyzed by annexin-V/PI staining for apoptosis after 24 hours of treatment with JSI-124. JSI-124 induced apoptosis from 3.0% to 81% (Fig. 2Ci, left panel). All analyzed CLL patient samples showed significant apoptosis after JSI-124 treatment (Fig. 2Ci, right panel). JSI-124-induced apoptosis in primary CLL cells was confirmed by time course accumulation of sub-G1 phase, although 24 hours of treatment with JSI-124 could induce apoptosis in the cells by 18.7%, 72 hours of treatment increased apoptosis to 73% (Fig. 2Cii). The sensitivities of the CLL cells to JSI-124 or fludarabine, which is a standard treatment, were measured by MTT assay, and the concentrations of drug required to decrease cell viability by 50% (IC50) varied from 2.9 to 10.5 μmol/L for JSI-124 (median, 5 μmol/L) and 2.2 to 40 μmol/L (median, 14 μmol/L) for fludarabine. Cells with IC50 that were greater than the median were considered drug resistant for the group, and for sensitive cells, an IC50 that was less than the median. As shown in Fig. 2Ciii, of 4 patients, only 2 showed fludarabine toxicity whereas in all 6 different patient samples only 1 required a high dose of JSI-124 (10.5 μmol/L) to achieve an IC50. Similar results were found using sub-G1 peak analysis (Supplementary Fig. 3). In addition, JSI-124 induced apoptosis regardless of mutational status of the immunoglobulin variable region (a biomarker for aggressive disease; data not shown).

JSI-124 inhibition of STAT3 activation contributes to cell-cycle arrest in B-leukemia cell lines

STAT3 activation has been implicating in cell-cycle regulations and JSI-124 has been shown to have antiproliferative effects on cancer cells (6, 7). We determined the effect of JSI-124 on cell-cycle progression. Cell-cycle arrest was observed in all 3 cell lines (BJAB, I-83, and NALM-6; Fig. 2Di) following treatment with JSI-124. The percentage of BJAB cells in G2-M phase elevated from 15.6% to 32.2%; in I-83 cells, the G2-M fraction increased from 14.9% to 35.5% (Fig. 2Di). Similarly, JSI-124 induced NALM-6 cell accumulated in G2-M phase from 16.7% to 38.1% (Fig. 2Di). These results showed that JSI-124 inhibited BJAB, I-83, and NALM-6 cells growth, at least in part, via cell-cycle arrest at G2-M phase. Because cdc2 represents critical checkpoints in G2-M transition, we analyzed protein level of cdc2 in BJAB, I-83, and NALM-6 cells exposed to JSI-124 in various time points. As shown in Fig. 2Dii, cdc2 levels were downregulated in all 3 cell lines at 12 and 24 hours of treatment with JSI-124.

JSI-124-induced apoptosis was dependent on downregulation of XIAP

We studied whether JSI-124-induced apoptosis is associated with the changes in the expression levels of antiapoptotic proteins. Cells were treated with 1 μmol/L of JSI-124 and Mcl-1, and Bcl-xL, Bcl-2, and XIAP, a member of IAP family, protein levels were determined by immunoblot analysis. Although JSI-124 is known to inhibit antiapoptotic proteins Mcl-1, Bcl-2, and Bcl-xL, these protein levels were not changed with same treatment (Supplementary Fig. 3; data not shown). However, we found that JSI-124 treatment led to decreased expression of the XIAP protein level in a time-dependent manner in all B-cell lines and primary B-CLL cells (Fig. 3Ai and ii). In addition, XIAP downregulation was confirmed by mRNA level (Fig. 3Aiii). The functional consequence of JSI-124-induced downregulation of XIAP was determined by overexpression of XIAP, with a cDNA vector encoding for full-length XIAP protein in BJAB cells. As a negative control, BJAB cells were transfected with empty vector. After 48 hours of XIAP overexpression, cells were treated with JSI-124 for an additional 24 hours. As shown in Fig. 3Bi, significant inhibition of drug-induced apoptosis (17.2%) was observed in the cells expressing XIAP, comparing to the untransfected cells (35.5%). No protection was observed in the cells expressing empty vector (41%; Fig. 3Bi). The overexpression of XIAP was confirmed by Western blotting, in which JSI-124 inhibits XIAP expression in cells not expressing XIAP, but cells expressing XIAP had high-level XIAP even after JSI-124 treatment (Fig. 3Bii). We also elucidated the mechanisms involved in the regulation of XIAP transcription. STAT3 activation has been shown to increase expression of XIAP (15). Because JSI-124 acts as an inhibitor of STAT3 activity, STAT3 might also be involved in XIAP expression. To this end, we did a ChiP assay, using extracts from BJAB and NALM-6 cells exposed to JSI-124 treatment for a 24-hour time course. As shown in Fig. 3C, JSI-124 significantly reduced association of STAT3 with the XIAP promoter.

To address the significance of caspase activation in JSI-124–induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk. As shown in Fig. 3Di, JSI-124-induced apoptosis was significantly
Figure 2. JSI-124–induced apoptosis in B-leukemia cells. A, BJAB, I-83, and NALM-6 cells were treated with various concentration of JSI-124 for 24 hours. Apoptotic cell population was measured using flow cytometric analysis for the accumulation of sub-G1 phase. Data represent 3 independent experiments. Standard error was determined on the basis of 3 independent experiments. Bi and ii, I-83 cells were treated with JSI-124 for 6 hours and cells were stained with cytochrome c as described in the Materials and Methods section. Ci, primary CLL cells were analyzed by annexin-V/PI staining for apoptosis after 72 hours of treatment by JSI-124. DMSO-treated cells were taken as control. Experiments were done in primary cells from 6 different patients with CLL. Cells that were 7-AAD–negative and annexin-V–positive were undergoing apoptosis. The percentage of cells in each quadrant is indicated in the quadrant. Representative original data are shown in left panel and mean values are shown in right panel. Cii, primary CLL cells were treated with JSI-124 for a 72-hour time course and apoptotic cell population was measured by DNA accumulation of sub-G1 phase. Ciii, sensitivity of primary CLL cells to JSI-124 and fludarabine were measured by the MTT assays. All cells from 6 different CLL patients indicated an IC50 value between 2.9 and 10.5 μmol/L for JSI-124, whereas 2 of 4 patients showed toxicity to fludarabine. Di, cells were treated with 1 μmol/L of JSI-124 for overnight and were analyzed for cell cycle by FACS as described in the Materials and Methods section. Experiments were done at least in 3 times and representative original data are shown. Dii, cells were treated with 1 μmol/L of JSI-124 for 0- to 24-hour time periods and cell lysates were subjected to Western blot analysis.
Figure 3. JSI-124 downregulates activation of XIAP. Ai–iii, decreased level of XIAP protein levels were detected by Western blotting and mRNA levels by real-time PCR in I-83, BJAB, NALM-6, and primary CLL cells after treatment with JSI-124 for indicated time course. Representative original data from 4 independent experiments are shown. Bi, BJAB cells were overexpressed with either cDNA-XIAP or pCMV-XL5 vector for 48 hours following JSI-124 treatment for an additional 24 hours. Apoptosis measured by flow cytometry following annexin-V and 7-AAD staining. Cells that were 7-AAD–negative and annexin-V–positive were undergoing apoptosis. The percentage of cells in each quadrant is indicated in the quadrant. Bii, Western blotting indicates that effective overexpression of XIAP by plasmid cDNA. C, cells treated by JSI-124 over a time course, and ChIP assays were carried out with anti-STAT3 antibody. DNA was analyzed by PCR using primers specific for the XIAP promoter region for STAT3 binding sites (C0793 to 1,335). Samples with antibody for rabbit IgG1 were used as a negative control and total DNA was used as the input control. Di, BJAB, I-83, and NALM-6 cells were incubated with 50 μmol/L of z-VAD-fmk or solvent for 1 hour before treatment with 1 μmol/L of JSI-124 for 24 hours. Apoptosis was measured as a sub-G1 fraction by FACS. Standard error was determined on the basis of 3 independent experiments, and an asterisk represents significant difference (P < 0.005) between JSI-124 alone or pretreated with z-VAD-fmk. Dii, the same cells were used to determine protein levels of XIAP and caspase-3 by Western blotting. Equal loading of the protein samples were confirmed by the Western blotting of β-actin for the same membrane.
inhibited by pretreatment with z-VAD-fmk. Because XIAP has been reported to be a substrate of caspases (16), we next examined whether the decreased protein levels of XIAP was mediated by JSI-124 treatment because of caspase activity. Although pretreatment with z-VAD-fmk completely blocked JSI-124 activation of caspase-3, it did not affect downregulation of XIAP (Fig. 3Dii).

**JSI-124 sensitizes TRAIL-induced apoptosis and upregulates DR4 expression**

To investigate the effect of JSI-124 on DR-mediated apoptosis, we determined the IC50 of TRAIL or JSI-124 or in combination with 10% of TRAIL IC50 in vitro. The IC50 ranged from 1 to 15 μmol/L and 1 to 2 μg/mL for JSI-124 and TRAIL, respectively (Supplementary Table 1). As shown in Fig. 4A, cotreatment of cells with JSI-124 and TRAIL resulted in a significant increased accumulation of sub-G1 phase cells compared with the treatment with JSI-124 or TRAIL alone and was synergistic with an I value of less than 1 (Supplementary Table 1). We then determined whether JSI-124 modulated the expression of DR4, DR5, and c-FLIP, which are key components in the TRAIL-mediated apoptotic pathway (17). The cells were treated with 1 μmol/L of JSI-124 in various times and DR4/DR5, and c-FLIP protein levels were determined. The expression of DR4 protein was significantly increased by this treatment (Fig. 4B); however, DR5 level remained unchanged with the same treatment (Fig. 4B). We also observed decreases in c-FLIP protein levels. In addition, we analyzed whether treatment of cells with JSI-124 caused caspase-8 activation, as it is often activated by the inhibition of c-FLIP. As shown in Fig. 4B, the cleavage of caspase-3 and -8 into their active forms was observed at 6 hours of JSI-124 treatment. In Fig. 4B, primary CLL cells also showed the same increased expression of DR4 protein levels and caspase-8 activation but failed to increase DR5 expression. Treatment with JSI-124 resulted in a statistically significant elevation of DR4 mRNA levels, supporting JSI-124-induced DR4 gene activation (Fig. 4C). We also determined the expression of DR4 on the cell surface of NALM-6 and primary CLL cells, using a specific monoclonal antibody against DR4. The expression of DR4 on the cell surface was significantly higher in cells treated with JSI-124 than in untreated control (Fig. 4D).

It has been previously shown that STAT3 repressed DR4 expression in endothelial cells (18), and we have previously shown that HDAC1 represses DR4 expression (12). We, therefore, did coimmunoprecipitation experiments to determine whether STAT3–HDAC1 complex formation after JSI-124 treatment. STAT3 was precipitated from extracts from all above-indicated cell experiments to determine whether STAT3–HDAC1 complex formation was reduced association of HDAC1 to STAT3 after JSI-124 treatment (Fig. 5Ai). Isotope-matched control antibody was used for specificity of immunoprecipitation. Similarly, primary CLL cells showed reduced association of HDAC1 to STAT3 after JSI-124 treatment (Fig. 5Aii). These data imply that JSI-124 reduced the complex formation of STAT3–HDAC1. To further study the regulation of STAT3 on DR4 gene expression, we did re-ChIP experiments. HDAC1 was immunoprecipitated from isolated chromatin extracts and purified DNA was analyzed by semiquantitative PCR using primers specific for the DR4 promoter. Extracts form HDAC1 immunoprecipitation were used for re-ChIP experiment with a STAT3 antibody. Equal amount of extract before each immunoprecipitation (input) was taken as the loading control. In JSI-124–treated I-83 cells, the binding of STAT3–HDAC1 complex to the promoter of DR4 gene was reduced by JSI-124 in a time-dependent manner (Fig. 5B). This indicates that STAT3 represses DR4 expression by recruiting HDAC1 to the DR4 promoter. To further confirm the involvement of STAT3 in JSI-124–reduced DR4 expression, STAT3 was knocked down by using siRNA (siRNA-STAT3). DR4 and DR5 protein levels were determined by immunoblot analysis in siRNA-STAT3 or siRNA-control transfected cells. Significant increase in DR4 protein was found in siRNA-STAT3–treated cells, whereas no significant change was observed in DR5 expression levels. Efficiency of knockdown of STAT3 was confirmed by immunoblotting of STAT3 levels. β-Actin was used as the loading control for the blot (Fig. 5C). Taken together, these results imply that JSI-124 induced apoptosis through activation of the TRAIL apoptotic pathway.

**siRNA knockdown of STAT3 induced apoptosis and cell-cycle arrest in B-leukemia cell lines**

To confirm that JSI-124 effects apoptosis through STAT3 inhibition, STAT3 knockdown with siRNA and cells was examined for cell survival. Apoptotic cell population was measured using sub-G1 peak analysis. siRNA STAT3 caused significant cytotoxicity of BJAB and I-83 cells (~25%) comparing control siRNA (~12%; Fig. 6A). To confirm transfection efficiency, the STAT3 level was assessed by immunoblot analysis in transfected and nontransfected cells and this confirmed a significant decrease in STAT3 in siRNA-treated cells (Fig. 6B). Furthermore, the requirement for STAT3 to increase XIAP expression was also evaluated. Treatment of BJAB and I-83 cells with siRNA against STAT3 resulted in a suppression of XIAP expression compared with nontransfected or cells transfected with control siRNA (Fig. 4B). Mcl-1 protein level was unaffected in cells transfected with siRNA-STAT3 and nontargeting control siRNA (Fig. 6B).

Knockdown of STAT3 resulted in significant downregulation of cdc2 protein levels (Fig. 6B) but also induced accumulation of cells at G2-M phase. In Fig. 6C, G2-M phase fractions of BJAB and I-83 cells increased from 8.9% to 10.5% (untreated) and 22.9% to 23.8% (siRNA-STAT3), respectively. Cell transfected with nontargeting control siRNA showed no increase in
accumulation at the G2-M checkpoint (BJAB, 10.9; I-83, 12.6) (Fig. 6C). This indicates that JSI-124 has antiproliferative effects mediated by STAT3-dependent cdc2 inhibition.

**Discussion**

STAT3 is aberrantly activated in many human solid and hematologic cancers and plays a role in oncogenesis.
Although tyrosine phosphorylation is a hallmark of STAT3 activation in many tumors, we and others confirmed that in B-leukemia cells, STAT3 is constitutively phosphorylated on serine 727 whereas tyrosine 705 is barely detectable (4, 5). Recent study of the peripheral blood cells of 106 patients with CLL showed that serine 727 phosphorylation of STAT3 was constitutively elevated in all patients studied regardless of blood count, disease stage, or treatment status (19).

Recent studies suggested that JAK1 and JAK2 function as part of integrated signaling (20). JAK1-mediated STAT3 activation was reported in ovarian cancer cells and hepatocytes (20, 21). Moreover, it has been shown that Src family kinases mediate constitutive STAT3 activation rather than JAK2 in several cancer cells (22, 23) and growth factor receptors such as epidermal growth factor receptor activate STAT3 through Src family kinases (24), in which AG490 failed to inhibit the constitutive activation of STAT3 (24). Similarly, we also showed here that AG490 failed to inhibit activation of STAT3 in B-leukemia cells. Whether JAK1 mediates STAT3 activation in B-leukemia cells is not known. In addition, a role of MAPK and IL-6 signaling pathways has been implicated in serine 727 phosphorylation of STAT3 (25–29). The upstream mediators of STAT3 in B-leukemia cell remain to be determined.

Several target genes of STAT3, including Bcl-xl, Bcl-2, Mcl-1, and XIAP (30–33) that are essential for cell survival, have been identified. We here found that JSI-124 selectively downregulated the expression of the antiapoptotic gene XIAP in B-leukemia cells compared with other STAT3-regulated antiapoptotic genes. For example, curcumin inhibits STAT3 serine phosphorylation and reduces the expression of Mcl-1 and Bcl-xl as well as XIAP. Unlike curcumin, JSI-124 reduces FLIP expression and increases DR4 expression. Taken together, this indicates that XIAP is a critical downstream target for STAT3 activation leading to cell survival in B-leukemia cells.

Several studies have shown that abrogation of STAT3 signaling sensitized cancer cells to chemotherapeutic agents (34, 35). For example, STAT3-inactivated keratinocytes become sensitive to UV-induced apoptosis (36). In addition, TRAIL has been known as a tumor-specific death ligand; however, several cancer cells including CLL cells are resistant to TRAIL-induced apoptosis (37, 38).
Figure 6. Knockdown of STAT3 increased apoptosis and decreased XIAP similar to JSI-124 treatment. A, cells were transfected with siRNA-STAT3 or siRNA nontarget control (siRNA-neg/cnt). Apoptotic cells were determined for accumulation of sub-G1 population. B, cell extract was assayed for Western blotting for XIAP, cdc2, and Mcl-1. Knockdown of STAT3 was confirmed by Western blotting with antibody to STAT3. C, the same transfected cells were used for cell-cycle analysis of G1- and G2-phase fractions in the cells transfected with siRNA-STAT3 or siRNA nontargeting control (siRNA-neg/cnt).
Recent studies suggested that abrogation of STAT3 activity sensitized human hepatoma and melanoma cells to TRAIL-mediated apoptosis (39, 40). Curcumin treatment inhibited STAT3 activity and decreased the expression of prosurvival proteins in B-CLL cells, sensitizing the cells to epigallocatechin-3 gallate (34). In the present study, we showed for the first time that the combination of JSI-124 and TRAIL increased apoptosis in B-leukemia cells and components of the TRAIL apoptotic pathway are upregulated in B-leukemia cells. This suggests that JSI-124 combined with TRAIL will increase apoptosis in B-leukemia cells.

Previously, we and others have shown that HDAC inhibitors potentiate TRAIL-induced apoptosis and preferentially activate DR4 apoptotic pathway, sensitizing CLL cells to TRAIL (12, 38). This sensitization requires class I HDAC enzymatic activity (12). HDAC1 is a nuclear protein and can repress gene transcription either directly or as part of these multiprotein complexes when recruited by a variety of transcriptional regulators. More recently, it has been shown that STAT3 binds to HDAC1 and interferes with human angiotensinogen gene expression and HDAC1 controls STAT3 nucleocytoplasmic distribution (41). Moreover, transcriptional repressor STRA13 blocks STAT1 transcription in an HDAC1-dependent mechanism (42). These reports agree with our findings, showing that HDAC1 interacts with STAT3 and the complex is recruited to the DR4 promoter repressing DR4 expression. JSI-124 reversed this repression and blocked HDAC1–STAT3 recruitment to the DR4 promoter.

In many tumor cells, CDK-cyclin complex are expressed aberrantly at high levels and thus make the normal control at G1/S or G2–M transition point ineffective (43, 44). Recently, it has been shown that JSI-124 induced cell-cycle arrest in primary astrocytes and neural stem cells, mouse glioma cells, and human malignant peripheral nerve sheath tumor cells through inhibition of serine 727 phosphorylation of STAT3 (45, 46). Moreover, it is known that JSI-124 exhibits antiproliferative effects and induces apoptosis through suppressing tyrosine and serine phosphorylation of STAT3 in human lung adenocarcinoma, breast carcinoma, glioblastoma, neuroblastoma, and multiple melanoma cells (6–8, 47, 48). We found that JSI-124 induces cell-cycle arrest at the G2-M checkpoint and downregulates STAT3-dependent cdc2 expression, indicating that JSI-124 could also effectively inhibit B-leukemia cell growth.

In conclusion, we have shown that abrogation of constitutive activation of STAT3 by JSI-124 causes cell-cycle arrest and induces apoptosis in human B-leukemia cells through alteration in gene expression. These results suggest that STAT3 activation is a potential target for therapy and the combination of JSI-124 and TRAIL may have therapeutic potential in the treatment of human B-leukemia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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