Activation of the NF-κB Pathway by the STAT3 Inhibitor JSI-124 in Human Glioblastoma Cells

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Abstract

Glioblastoma tumors are characterized by their invasiveness and resistance to therapies. The transcription factor signal transducer and activator of transcription 3 (STAT3) was recently identified as a master transcriptional regulator in the mesenchymal subtype of glioblastoma (GBM), which has generated an increased interest in targeting STAT3. We have evaluated more closely the mechanism of action of one particular STAT3 inhibitor, JSI-124 (cucurbitacin I). In this study, we confirmed that JSI-124 inhibits both constitutive and stimulus-induced Janus kinase 2 (JAK2) and STAT3 phosphorylation, and decreases cell proliferation while inducing apoptosis in cultured GBM cells. However, we discovered that before the inhibition of STAT3, JSI-124 activates the nuclear factor-κB (NF-κB) pathway, via NF-κB p65 phosphorylation and nuclear translocation. In addition, JSI-124 treatment induces the expression of IL-6, IL-8, and suppressor of cytokine signaling (SOCS3) mRNA, which leads to a corresponding increase in IL-6, IL-8, and SOCS3 protein expression. Moreover, the NF-κB–driven SOCS3 expression acts as a negative regulator of STAT3, abrogating any subsequent STAT3 activation and provides a mechanism of STAT3 inhibition after JSI-124 treatment. Chromatin immunoprecipitation analysis confirms that NF-κB p65 in addition to other activating cofactors are found at the promoters of IL-6, IL-8, and SOCS3 after JSI-124 treatment. Using pharmacological inhibition of NF-κB and inducible knockdown of NF-κB p65, we found that JSI-124–induced expression of IL-6, IL-8, and SOCS3 was significantly inhibited, showing an NF-κB–dependent mechanism. Our data indicate that although JSI-124 may show potential antitumor effects through inhibition of STAT3, other off-target proinflammatory pathways are activated, emphasizing that more careful and thorough preclinical investigations must be implemented to prevent potential harmful effects. Mol Cancer Res; 11(5); 494–505. ©2013 AACR.
Importantly, STAT3 also induces the prosurvival genes including IL-6, vascular endothelial growth factor, and c-Myc (13). Cytokine binding to the corresponding extracellular receptor leads to activation of the receptor and in turn causes auto- and trans-phosphorylation of intracellular JAK2 (11). STAT3 then becomes phosphorylated by JAK2, dimerizes, translocates to the nucleus, and binds to DNA. STAT3 induces the transcription of several prosurvival genes including IL-6, vascular endothelial growth factor, and c-Myc (13). Importantly, STAT3 also induces the expression of suppressor of cytokine signaling (SOCS) proteins, specifically SOCS3, which functions as a negative regulator preventing prolonged STAT3 activation (14).

Numerous studies have shown that the activities of STAT3 and NF-kB are elevated in many cancers, including GBM (10, 13, 15, 16). We and others have observed increased activity and protein levels of both NF-kB and STAT3 in GBM (17–20). Moreover, STAT3 has been determined to be a master regulator of the mesenchymal subtype of GBM (6, 7), and loss of IKKβ has also been linked to GBM (21). Because of the increased interest in these 2 pathways, numerous pharmacological inhibitors have been developed targeting NF-kB and STAT3 with the hopes of benefiting patients with various cancers including GBM (22, 23).

JSI-124 (cucurbitacin I), a chemical compound belonging to the cucurbitacin family, was first discovered as a potent STAT3 inhibitor in multiple cancer cell lines (24). Inhibition of STAT3 activity was attributed to a disruption in STAT3 DNA binding activity and gene expression, and a reduction in subcutaneous lung and breast xenograft tumors was observed with JSI-124 treatment (24), showing antitumor potential. Furthermore, JSI-124 has been shown to be a strong activator of apoptosis in multiple tumor cell lines including GBM and has a synergistic effect with other antitumor agents (20, 25–27). However, the exact mechanism of STAT3 inhibition by JSI-124 remains unclear. In this study, we set out to examine more closely the effect of JSI-124 treatment on GBM tumor cells in vitro. Consistent with other findings, we found that JSI-124 inhibited both constitutive and stimulus-induced JAK2 and STAT3 phosphorylation, decreased cell proliferation, and induced apoptosis in human GBM cells. However, before the inhibition of STAT3, time course analysis revealed activation of the NF-kB pathway starting at 15 minutes after JSI-124 treatment. In addition, we found that JSI-124–induced mRNA expression of IL-8, IL-6, and SOCS3. This increase in mRNA expression translated to protein expression as determined by both IL-8 and IL-6 secretion and cellular SOCS3 protein levels. Pharmacological inhibition and inducible knockdown of NF-kB p65 significantly inhibited JSI-124–induced gene expression. These data reveal that in addition to inhibition of STAT3, additional off-target pathways, including NF-kB, are activated in response to JSI-124 treatment.

Materials and Methods
Reagents and cells
Antibodies (Ab) to phosphorylated STAT3 (Y705), phosphorylated IKKα/β (S176/180), and phosphorylated p65 (S276) were from Cell Signaling Technologies; total IKKα/β, total IKKβ, SOCS3, phosphorylated JAK2 (Y1007/1008), total JAK2, and total p65 from Santa Cruz; total STAT3, Caspase 3, and PARP from BD Transduction Labs; and GAPDH from AbCam. For chromatin immunoprecipitation (ChIP) experiments, Ab to p65 (Abcam), phosphorylated p65 (Cell Signaling), phospho-Pol II (S5; Covance), and STAT3 (Santa Cruz) were used. JSI-124 and BAY-11-7085 were purchased from Calbiochem. OSM was purchased from R&D Systems. U87-MG and U251-MG cells were maintained as previously described (19). U251-MG cells were authenticated and are the same as the parent line of Dr Darrell Bigner (Duke University, Durham, NC). U87-MG cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and are authentic and consistent with the short tandem repeat profile in the ATCC database. U251-MG cells that stably express the tetracycline repressor (TetR) protein and inductively express short hairpin RNA (shRNA) specific for NF-kB p65 (sh-p65) were generated as previously described (19). Briefly, the plasmid carrying shRNA specific for p65 was generated by annealing double-stranded oligonucleotide specific for a 19-bp stretch of the p65 ORF (AA CTG TTC CCC CTC ATC TT) into the pBABE-HI-TetO plasmid, which is under dual control of the tetracycline operator and the HI polymerase (Pol) III promoter. The pBABE–HI–TetO plasmid was a generous gift of Dr Xinbin Chen (University of California at Davis, CA). Primary astrocyte cultures from C57BL/6 mice were established from neonatal cerebra as described (28).

Immunoblotting
Cells were harvested and lysed in radioimmunoprecipitation assay buffer lysis buffer with protease inhibitors. Protein concentration was determined using the Pierce BCA Assay. Equivalent amounts of protein were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred onto a nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline (0.01% Tween) for 1 hour, primary Ab were incubated overnight at 4°C followed by 1 hour with biotinylated horseradish peroxidase secondary Ab, and developed with chemiluminescent enhanced detection system. Immunoblotting was initially probed with antibodies directed toward the phosphorylated protein followed by stripping and reprobing with antibodies directed against the total protein levels.

Subcellular fractionation
Nuclear and cytosolic fractions were isolated as previously described (30). Brieﬂy, cells were washed twice with cold PBS followed by the addition of 0.05% NP-40 lysis solution to each sample. Cells were collected and centrifuged at 2,700 × g for 10 minutes at 4°C. Supernatant (cytosol) was removed, centrifuged at 20,000 × g for 15 minutes, and the top 100 μL saved as the pure cytosolic fraction. The pellet (nuclei) was resuspended in wash buffer and centrifuged at 2,700 × g for 5 minutes. The supernatant was discarded, pellet resuspended in wash...
buffer, layered on top of 1 mL of 1M sucrose and centrifuged at 2,700 × g for 10 minutes. The supernatant was discarded, and the pellet was washed in 0.05% NP-40 lysis solution and centrifuged at 2,700 × g for 5 minutes. The pellet was resuspended in nuclear extraction buffer, incubated on ice for 30 minutes, and centrifuged at 20,000 × g for 15 minutes. The supernatant was saved as pure nuclear extract. To verify purity, samples were immunoblotted with caspase 3 and PARP Ab for cytosolic and nuclear fractions, respectively.

Cell proliferation assay

Cells were plated in 96-well plates at a density of 1 × 10⁴ cells/well, and the WST-1 Cell Proliferation Assay (Roche) was conducted as previously described (31).

ELISA

Cells were treated with JSI-124 for the indicated times and supernatants collected. Human IL-6 and IL-8 ELISA kits were purchased from BioLegend and conducted as previously described (32).

Quantitative RT-PCR

Cells were washed with PBS, and total RNA was isolated using TRIzol (Invitrogen) as previously described (28). Approximately 1 μg of RNA per sample was used to generate cDNA by reverse transcription for PCR. Predesigned Taqman primers (Applied Biosystems) were used to obtain quantitative PCR results using the Applied Biosystems StepOnePlus Real-Time PCR System Thermal Cycling Block and corresponding software analysis for data quantification (StepOneSoftware v2.1; Applied Biosystems). The following Taqman primers and the corresponding Gene Ref# were used: human IL-6 (Hs00174131_m1), human IL-8 (Hs00174103_m1), human NFKBIA (Hs00153283_m1), and human SOCS3 (Hs02330328_s1). Eukaryotic 18s rRNA (Hs99999901_s1) was used as an endogenous control.

Chromatin immunoprecipitation

ChIP assays were conducted as previously described (19, 32). Immunoprecipitation was done with 5 μg of the appropriate Ab, and immune complexes absorbed with protein A beads or protein G beads blocked with bovine serum albumin and salmon sperm DNA. Immunoprecipitated DNA was subjected to semiquantitative PCR and analyzed by gel electrophoresis using the following primers:

- IL-8 Forward 5'-gggcc cca tca gta gca aat c-3'; IL-8 Reverse 5'-ttc cct ccc ggg gtt tct tc-3'; IL-6 Forward 5'-gga tgg gag tca gga ac-3'; IL-6 Reverse 5'-ttg gag ggg tag cgc taa gaa gc-3'; SOCS3 Forward 5'-ggt ccc ctc ccc tct gga atc tcg-3'; SOCS3 Reverse 5'-ccc cca aac ttc tca ttc aca-3'.

Statistical analysis

Student t tests were conducted for comparison of 2 values, and analysis of variance (ANOVA) analysis was conducted on appropriate multivariable analyses. P < 0.05 was considered statistically significant.

Results

JSI-124 inhibits constitutive and stimulus-induced JAK2 and STAT3 activation in GBM cells

JSI-124 was originally discovered as a STAT3 inhibitor (24). First, we validated the inhibition of STAT3 in cultured human GBM cells. U251-MG cells have basal JAK2 and STAT3 phosphorylation, and JSI-124 (1 μmol/L) inhibited the constitutive phosphorylation of both JAK2 and STAT3 (Fig. 1A). JSI-124 pretreatment also prevented OSM-induced phosphorylation of JAK2 and STAT3 in a dose-dependent manner (Fig. 1B). Because of the greatly enhanced phosphorylation of STAT3 after OSM stimulation, we have provided an appropriately exposed blot revealing the constitutive STAT3 phosphorylation (Supplemental Fig. 1A). Both constitutive and stimulus-induced JAK2 and STAT3 phosphorylation were also inhibited in U87-MG cells (Supplementary Figs. S1B and S1C). These results verify the previously established literature regarding JSI-124 inhibition of STAT3 (24).

JSI-124 decreases cell proliferation and induces cell death in GBM cells

It has been shown that in addition to inhibition of STAT3, JSI-124 decreased cell proliferation and induced apoptosis (24, 25). It was previously shown that this decrease in cell proliferation was due in part to a JSI-124–induced G2/M arrest and reduction of cyclin D1 (25), but the exact mechanism remains unclear. We also observed a reduction in cell proliferation with JSI-124 treatment of both U251-MG and U87-MG cells (Fig. 1C and Supplementary Fig. S1D). Dose–response analysis revealed that 0.1 μmol/L JSI-124 was sufficient to significantly inhibit proliferation by 24 hours. Furthermore, a cytotoxic drug dose–response curve yields an IC₅₀ value of approximately 28 nmol/L (Fig. 1D). This reduction in cell proliferation was accompanied by an increase in cell death as measured by the presence of cleaved (c) caspase 3 and poly-ADP ribose polymerase (PARP; Fig. 1E).

JSI-124 treatment activates the NF-κB pathway

To evaluate other potential effects of JSI-124 treatment, U251-MG cells were incubated with JSI-124 (1 μmol/L) for various times. Time course analysis revealed phosphorylation of NF-κB p65, starting as early as 15 minutes after JSI-124 treatment and decreasing after 1 hour (Fig. 2A). Moreover, the JSI-124–induced phosphorylation of NF-κB was accompanied by an increase in the expression of IkBα (Fig. 2B). In addition, both JNK and p38 MAPK, 2 pathways commonly activated during stress, were also found to be activated (data not shown), which has also recently been shown in leukemia cells treated with JSI-124 (33). Activation of the NF-κB pathway involves nuclear translocation of NF-κB p65, where binding of DNA and transcriptional regulation occurs. Under basal conditions, NF-κB p65 is found sequestered in the cytosol with minimal to no detection in the nucleus (Fig. 2C, Lanes 1 and 5). As a positive control, we observed the
presence of nuclear NF-κB p65 after TNF-α treatment in U251-MG cells (Fig. 2C, Lane 6). Moreover, we found that JSI-124 treatment also induced nuclear translocation of NF-κB p65 within 30 minutes (Fig. 2C, Lane 8). These results indicate that JSI-124 treatment results in the phosphorylation of NF-κB p65 as well as nuclear translocation.

The NF-κB pathway is activated in response to stimuli such as TNF-α, which leads to phosphorylation of IKK and the degradation of IκBα by the proteasome (8, 9). Using
TNF-α as a positive control, we observed IKK phosphorylation and IκBα degradation within 5 minutes of TNF-α treatment (Fig. 2D). However, we did not observe phosphorylation of IKK in response to JSI-124 treatment. This indicates that activation of the NF-κB pathway in response to JSI-124 is not mediated through IKK phosphorylation, which will be further explained in the discussion. Modest degradation of IκBα by JSI-124 was observed by 15 minutes (Fig. 2D, Lane 8), which is necessary to allow NF-κB p65 translocation into the nucleus. Overall, these results confirm that JSI-124 treatment activates the NF-κB pathway.

JSI-124 treatment induces IL-6, IL-8, and SOCS3 expression

As JSI-124 activates intracellular signaling cascades including NF-κB, we evaluated the induction of several potential downstream genes. We found that JSI-124 treatment induced mRNA expression of IL-6 and IL-8 in both U251-MG (Fig. 3A and B) and U87-MG cells (Supplementary Fig. S2) as measured by quantitative RT-PCR. Both IL-6 and IL-8 are known targets of NF-κB p65 (13). We also observed an increase in the mRNA expression of SOCS3, an endogenous negative regulator of the JAK/STAT3 pathway, which is most often induced by JAK/STAT3 activation (ref. 14; Fig. 3C; Supplementary Fig. S2). The JSI-124–induced gene expression was also validated in human GBM neurospheres (X1066 cells) as well as murine primary astrocytes (Supplementary Fig. S3).

To verify the induction of IL-6 and IL-8 translation to protein, we treated U251-MG cells with JSI-124 for various times, collected supernatants, and measured secreted IL-6 and IL-8 by ELISA. We found a significant increase in the amount of secreted IL-6 and IL-8 after JSI-124 treatment (Fig. 3D and E). In addition, beginning at 2 hours after JSI-124 treatment, SOCS3 protein expression was increased (Fig. 3F).

Phosphorylated p65 and RNA polymerase (Pol) II are found at the promoters of IL-6, IL-8, and SOCS3 in cells treated with JSI-124

To further characterize the role of NF-κB p65 in JSI-124–induced gene expression, the presence of several transcription factors at the promoters of IL-6, IL-8, and SOCS3 was evaluated. Analysis reveals the presence of total...
and phosphorylated p65 as well as phosphorylated RNA Pol II at the promoters of IL-6, IL-8, and SOCS3 after JSI-124 treatment (Fig. 4A–C). Phosphorylation of RNA Pol II at Serine 5 (S5) is necessary and indicative of transcriptional initiation and activation (34). STAT3 was not at the promoters of the genes analyzed upon JSI-124 treatment, indicating that STAT3 is not activated or responsible for the increase in expression of IL-6, IL-8, or SOCS3 observed after JSI-124 treatment (Fig. 4D). This confirms that in response to JS1-124 treatment, activated NF-κB p65 is recruited to the promoters of IL-6, IL-8, and SOCS3, and is responsible for the increase in gene expression.

**Blockade of the NF-κB pathway inhibits JSI-124–induced gene expression**

To verify the role of the NF-κB pathway in JSI-124–induced gene expression, we used a pharmacological inhibitor of the NF-κB pathway, BAY-11-7085 (BAY-11; Supplementary Fig. S4A; ref. 35). U251-MG cells were pretreated with BAY-11 for 2 hours followed by treatment with JSI-124 for 1 hour, and quantitative RT-PCR was conducted. JSI-124 treatment induced mRNA expression of IL-6, IL-8, and SOCS3, which was inhibited by pretreatment with BAY-11 (Fig. 5A and B). This suggests that the NF-κB pathway is necessary for JSI-124 induction of IL-6, IL-8, and SOCS3. To examine the necessity of NF-κB
p65 in JSI-124–induced gene expression, we used an inducible p65 knockdown U251-MG–derived cell line, U251-TR/sh-p65 (19). U251-TR/sh-p65 cells were grown in the absence or presence of Tetracycline (Tet) for 48 hours to effectively decrease p65 levels before treatment with JSI-124 (Supplementary Fig. S4B). U251-TR/sh-p65 cells treated with Tet exhibit significantly decreased levels of p65 mRNA (Fig. 5C). Furthermore, loss of p65 significantly inhibited JSI-124–induced IL-8, IL-6, and SOCS3 expression (Fig. 5D–F). Similar to the inhibitor results in Fig. 5A and B, JSI-124 gene expression requires the presence of NF-κB p65.

Blockade of the NF-κB pathway does not inhibit JSI-124–induced phenotypic changes or STAT3 inhibition

Next, we used functional analyses to better understand the relationship between NF-κB signaling and the effects of JSI-124 treatment. First, we evaluated a dose–response of BAY-11 in GBM cells and observed a dose-dependent decrease in proliferation at 24 hours (Fig. 6A). Using 5 μmol/L BAY-11, we tested the combination using 2 doses of JSI-124 (low and high) to evaluate any possible additive or synergistic effects (Fig. 6B). We observed no enhanced decrease in proliferation with the combination of JSI-124 and BAY-11. In addition, pretreatment with BAY-11 does not prevent the dramatic decrease in cell proliferation with the higher dose of JSI-124 (0.1 μmol/L; Fig. 6B). In the NF-κB p65 knockdown cells (U251-TR/sh-p65), there is no significant decrease in proliferation with knockdown of NF-κB p65 (Fig. 6C). Using this information, we combined knockdown (+Tet) with 2 doses of JSI-124 (low and high). Similar to the pharmacological inhibition results, we observed that combination treatment does not enhance or prevent the decrease in proliferation with JSI-124 treatment (Fig. 6D). Furthermore, pharmacological inhibition or shRNA knockdown of NF-κB p65 does not prevent JSI-124–induced cell death (Supplementary Fig. 5A and B). Therefore, JSI-124 efficiently inhibits proliferation and induces cell death in the absence of NF-κB.

In this report, the induction of SOCS3, a negative regulator of STAT3, via JSI-124–induced NF-κB activation could provide a potential mechanism of the observed STAT3 inhibition. To test this, we evaluated whether blockade of NF-κB would prevent JSI-124–induced STAT3 inhibition. U251-MG as well as U251-TR/sh-p65 cells were treated with JSI-124 and a noticeable inhibition of STAT3 was achieved (Fig. 6E and F, Lane 2). However, we observed that blockade (BAY-11) or downregulation of NF-κB p65 (U251-TR/sh-p65 cells) does not prevent JSI-124–induced inhibition of STAT3 (Fig. 6E and F, Lanes 4 and 3, respectively).

Discussion

In this report, we have determined that the STAT3 inhibitor JSI-124 has the ability to activate signaling
pathways in addition to the inhibition of STAT3 in human GBM cells. A signaling schematic illustrating the observed effects of JSI-124 treatment is provided in Fig. 7. JSI-124 was originally discovered as a STAT3 inhibitor in multiple cancer cells (24). We first verified the previously established literature regarding the effects of JSI-124, and observed an inhibition of constitutive and stimulus-induced JAK2 and STAT3 phosphorylation in cultured human GBM cells. Inhibition of proliferation and induction of apoptosis was also observed after JSI-124 treatment. These data confirm the literature regarding inhibition of STAT3 signaling, decreased proliferation, and induction of apoptosis in cells in vitro (24–26).

However, we observed that the time needed to inhibit STAT3 phosphorylation was much longer than other known JAK/STAT inhibitors. For example, we and others have shown that AZD1480, a specific JAK1/2 inhibitor, decreases constitutive STAT3 phosphorylation as early as 30 minutes.
complex. It involves the phosphorylation and activation of the IKK. We found that the JSI-124 targets IκB, leading to dissociation and proteasomal degradation (Fig. 7). Similarly, a recent study by Nefedova and colleagues (37) found that JSI-124 treatment of dendritic cells led to the activation of NF-κB, independent of IKK activation (40–42). Therefore, we suspect other kinases may also become activated after JSI-124 treatment that might lead to the activation of NF-κB p65, independent of classical IKK activation.

After JSI-124 treatment and NF-κB activation, we measured the expression of inflammatory NF-κB–regulated genes including IL-6 and IL-8. This expression was translated into protein as measured by secreted IL-6 and IL-8 in the conditioned medium of cells treated with JSI-124. Both IL-6 and IL-8 expression have been shown to be upregulated posttreatment (31, 36). Because the exact mechanism of JSI-124 remains unknown, this suggested that the JSI-124 mechanism of STAT3 inhibition was less specific and could be exerting effects on other signaling pathways. Moreover, we observed that before the inhibition of STAT3, the NF-κB pathway becomes activated, as observed via phosphorylation of NF-κB p65 as well as nuclear translocation and IκBα degradation (Fig. 7). Similarly, a recent study by Nefedova and colleagues (37) found that JSI-124 treatment of dendritic cells led to the activation of NF-κB. However, in this study, the activation of NF-κB was observed after the inhibition of STAT3 signaling, was IκBα degradation independent, and was attributed to the loss of a dominant negative effect of STAT3 binding to NF-κB family members in these cells.

There are several mechanisms that ultimately lead to the activation of NF-κB (9, 22). Activation of NF-κB through the canonical pathway, via stimuli such as TNF-α or LPS, involves the phosphorylation and activation of the IKK complex. IκBα is then phosphorylated by IKK, which targets IκBα for degradation via the proteasome, and allows NF-κB p65 to translocate to the nucleus. Interestingly, we found that the JSI-124–induced activation of NF-κB p65 was through an IKK-independent mechanism, also known as the atypical pathway (9). In the atypical pathway, reports have suggested that other kinases can also phosphorylate IκBα, leading to dissociation and proteasomal degradation independent of IKK activation (9). For example, in response to UV light or expression of the Her2/Neu oncogene, casein kinase II (CK2) phosphorylates IκBα leading to its degradation and subsequent activation of NF-κB that is dependent on p38 MAPK pathway activation (38, 39). Interestingly, we did observe modest activation of the p38 MAPK pathway in response to JSI-124 treatment (data not shown). Other kinases, including Syk, c-Src, and Ribosomal S6 Kinase 1 (RSK1) have also been linked to activation of NF-κB, independent of IKK activation (40–42). Therefore, we suspect other kinases may also become activated after JSI-124 treatment that might lead to the activation of NF-κB p65, independent of classical IKK activation.

Figure 6. Inhibition of NF-κB signaling does not prevent JSI-124–induced phenotypic changes or inhibition of STAT3. A and B, U251-MG cells were plated in 96-well plates and incubated with various concentrations of JSI-124 and/or BAY-11 for 24 hours, and the WST-1 cell proliferation assay was conducted. Data represent mean ± SD, replicates of 3 (*P < 0.001; ANOVA). C and D, U251-TR/sh-p65 cells were plated in 96-well plates and incubated with Tet for the indicated times (C) or pretreated with Tet for 48 hours before plating and treatment with various concentrations of JSI-214 for the indicated times (D), and the WST-1 cell proliferation assay was conducted. Data represent mean ± SD, replicates of 3 (*P < 0.001; ANOVA). E and F, U251-MG cells were incubated with BAY-11 (5 μmol/L) for 2 hours before treatment with JSI-124 (1 μmol/L) for 2 hours (E), or U251-TR/sh-p65 cells were pretreated with or without Tet for 48 hours before treatment with JSI-124 (1 μmol/L) for 8 hours (F). Cells were lysed and immunoblotted with the indicated Ab.
in GBM and the corresponding tumor microenvironment both in vitro and in vivo (43–45), and IL-6 gene amplification has been observed in 40% to 50% of GBM patients and is associated with decreased patient survival (46). Although the role of IL-8 is less characterized in GBM biology, studies have shown that IL-8 acts predominantly as an inflammatory chemoattractant and proangiogenic factor (45). This indicates that JSI-124 induction of IL-6 and IL-8 could have potentially counter-productive effects with regards to inhibiting tumor growth and progression in GBM.

We also observed the induction and expression of SOCS3 after JSI-124 treatment. Upon typical JAK/STAT activation, SOCS3 is expressed to provide a mechanism of negative feedback. Once expressed, SOCS3 interacts with various cytokine receptors and/or JAKs to prevent subsequent STAT3 phosphorylation. In this study, we found the SOCS3 induction was driven by NF-κB, not STAT3. Although NF-κB–induced SOCS3 is uncommon, we and others have shown that under certain conditions, SOCS3 expression can be induced by NF-κB activation (47–49). We found that the expression of SOCS3 was induced by NF-κB as observed by the presence of phosphorylated NF-κB p65 at the promoter of SOCS3 (Fig. 4A). The requirement for NF-κB in SOCS3 expression was validated using pharmacological inhibition and shRNA knockdown of NF-κB. The induction of SOCS3, a negative regulator of STAT3, via JSI-124–induced inhibition of STAT3 (Fig. 6E and F). This could be due, in part, to the fact that NF-κB inhibition before JSI-124 treatment significantly reduces, but does not completely abrogate SOCS3 expression (Fig. 5B and F). Therefore, after JSI-124 treatment, the NF-κB–driven SOCS3 expression acts as a negative regulator of STAT3, abrogating any subsequent STAT3 activation and provides a mechanism of STAT3 inhibition (Fig. 7).

In conclusion, there have been several papers evaluating combination therapies with other anticancer agents and JSI-124. Premkumar and colleagues (27) illustrated that Dassatinib, a small molecule tyrosine kinase inhibitor, synergizes with JSI-124 to increase apoptosis and inhibit growth and migration of GBM cells. However, several chemotherapeutic agents including doxorubicin and cisplatin have also been shown to activate NF-κB, similar to JSI-124 (50). Overall, this report reveals additional off-target effects of JSI-124 treatment, in addition to the inhibition of STAT3. Given the link between NF-κB, inflammation, and cancer progression, continued research of these preclinical therapeutic agents must be conducted to fully understand and anticipate potential harmful effects.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: B. C. McFarland
Development of methodology: B. C. McFarland, S. E. Nozell
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. C. McFarland, S. E. Nozell, S. W. Hong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. C. McFarland, S. E. Nozell
Writing, review, and/or revision of the manuscript: B. C. McFarland, G. K. Gray, E. N. Benveniste
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