Boswellic Acid Blocks Signal Transducers and Activators of Transcription 3 Signaling, Proliferation, and Survival of Multiple Myeloma via the Protein Tyrosine Phosphatase SHP-1

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Abstract
Activation of signal transducers and activators of transcription-3 (STAT-3) has been linked with survival, proliferation, chemoresistance, and angiogenesis of tumor cells, including human multiple myeloma (MM). Thus, agents that can suppress STAT3 activation have potential as cancer therapeutics. In our search for such agents, we identified acetyl-11-keto-β-boswellic acid (AKBA), originally isolated from Boswellia serrata. Our results show that AKBA inhibited constitutive STAT3 activation in human MM cells. AKBA suppressed IL-6–induced STAT3 activation, and the inhibition was reversible. The phosphorylation of both Jak 2 and Src, constituents of the STAT3 pathway, was inhibited by AKBA. Interestingly, treatment of cells with pervanadate suppressed the effect of AKBA to inhibit the phosphorylation of STAT3, thus suggesting the involvement of a protein tyrosine phosphatase. We found that AKBA induced Src homology region 2 domain–containing phosphatase 1 (SHP-1), which may account for its role in dephosphorylation of STAT3. Moreover, deletion of the SHP-1 gene by small interfering RNA abolished the ability of AKBA to inhibit STAT3 activation. The inhibition of STAT3 activation by AKBA led to the suppression of gene products involved in proliferation (cyclin D1), survival (Bcl-2, Bcl-xL, and Mcl-1), and angiogenesis (VEGF). This effect correlated with the inhibition of proliferation and apoptosis in MM cells. Consistent with these results, overexpression of constitutive active STAT3 significantly reduced the AKBA-induced apoptosis.

Overall, our results suggest that AKBA is a novel inhibitor of STAT3 activation and has potential in the treatment of cancer.


Introduction
Numerous recent reports indicate that multitranslated, rather than monotranslated, anticancer agents have a better chance for success (1). Most natural products are multitranslated “naturally” (2). Boswellia serrata, an Indian frankincense or Salai guggul, has been used in Ayurvedic systems of medicine against a number of inflammatory diseases, including osteoarthritis, chronic colitis, ulcerative colitis, Crohn’s disease, and bronchial asthma but the mechanism is poorly understood. Acetyl-11-keto-β-boswellic acid (AKBA), the active compound isolated from this plant possess inhibitory activities against experimental ileitis (3), experimental colitis (4), autoimmune encephalomyelitis (5), nociception (6), inflammation and atherogenesis (7), bovine serum albumin–induced arthritis (8), and growth of glioma in rats (9). AKBA also inhibited age-associated abnormalities in mice (10). There are also reports that this agent exhibits immunomodulatory effects (11). This triterpenoid can suppress the growth of glioma, colon cancer, prostate, and leukemic cells has also been reported (12-17). In addition, AKBA suppressed the basic fibroblast growth factor–induced angiogenesis in vivo in a Matrigel plug assay (18). Although a number of molecular targets inhibited by AKBA, such as 5-lipoxygenase, cyclooxygenase-2, P-glycoprotein (19), extracellular signal regulated kinase 1 and 2 (13, 20), human leukocyte elastase (21), and human topoisomerases 1 and 2 (22), have been reported, the exact mechanism of its anti-inflammatory and anticancer activities remains elusive. AKBA has been shown to bind directly to 5-lipoxygenase (23), human leukocyte elastase (21), and topoisomerase 2 (15) and to inhibit their enzymatic activity.

Signal transducers and activators of transcription (STAT) is a family of transcription factors that has been associated with inflammation, survival, proliferation, metastasis, angiogenesis, and chemoresistance of tumor cells (24). One of these members, namely STAT3, is constitutively expressed in multiple myeloma (MM), leukemia, lymphoma, squamous cell carcinoma, and other solid tumors, including cancers of the prostate, breast, head and neck, and nasopharynx (24). STAT3 can also be activated by certain interleukins (e.g., IL-6) and growth factors (e.g., epidermal growth factor). Upon activation,
 STAT3 undergoes phosphorylation at serine 727 and at tyrosine 705, dimerization, nuclear translocation, and DNA binding, which in turn leads to transcription of various genes, including those for apoptosis inhibitors (Bcl-xL, Mcl-1, and survivin), cell cycle regulators (cyclin D1 and c-myc), and inducers of angiogenesis (VEGF) and metastasis (TWIST; ref. 25). Because these gene products are closely related to tumor development and growth, agents that can inhibit the activation of STAT3 may have great potential in the treatment of cancer and other inflammatory diseases. The phosphorylation of STAT3 is mediated through the activation of non–receptor protein tyrosine kinases, including Janus-like kinase (JAK)-1, JAK2, JAK3, TYK2, and c-Src kinase. Thus, agents that disrupt this pathway would be good candidates for STAT3 inhibitors.

Because AKBA (see structure in Fig. 1A) has been used to alleviate various inflammatory diseases, we hypothesized that it would inhibit STAT3 activation. We tested this hypothesis using a MM cell line. Our results show that AKBA inhibited both constitutive and inducible STAT3 activation, inhibited JAK and c-Src activation, induced Src homology region 2 domain-containing phosphatase 1 (SHP-1), and down-regulated the expression of STAT3-regulated gene products, thus leading to the suppression of proliferation and induction of apoptosis in MM cells.

Results

The present study was aimed at determining whether AKBA inhibits the STAT3 activation pathway in MM cells and if so through what mechanism. Both constitutive and IL-6–induced STAT3 activation were examined. Whether AKBA affects STAT3-regulated gene products involved in cellular proliferation, survival, and apoptosis was also investigated. We also examined whether AKBA can modulate the growth of MM cells.

AKBA Inhibits Constitutive STAT3 Phosphorylation

Human MM U266 cells are known to express constitutive STAT3 activation (26). Whether AKBA can suppress constitutive STAT3 activation in these MM cells was investigated. As shown in Fig. 1B, AKBA inhibited the constitutive activation of STAT3 in a dose-dependent (left) and time-dependent (right) manner. This triterpene had no effect on the expression of STAT3 protein. The results showed that AKBA completely inhibited constitutive activation of STAT3 at 50 μmol/L concentration after a 4-h exposure. Cells were fully viable under these conditions. Hence, we selected this concentration for our further experiments.

AKBA Suppresses the Nuclear Translocation of STAT3

Because tyrosine phosphorylation causes dimerization of STATs and then nuclear translocation, whether AKBA inhibited nuclear translocation of STAT3 was examined in U266 cells by immunocytochemistry. Our results showed that AKBA suppressed the constitutive phosphorylation of STAT3 in a dose-dependent manner after a 4-h exposure. Cells were fully viable under these conditions. Hence, we selected this concentration for our further experiments.

AKBA Inhibits Binding of STAT3 to the DNA

When STAT3 is translocated to the nucleus, it binds to the DNA, an event that in turn regulates gene transcription (27). Whether AKBA inhibits DNA binding activity of STAT3 was examined by electrophoretic mobility shift assay. Nuclear extracts prepared from U266 cells showed STAT3 DNA-binding activity and AKBA inhibited the binding in a dose-dependent (Fig. 1D, left) and time-dependent manner (Fig. 1D, right). No loss of cell viability was noted under these conditions.

AKBA-Induced Inhibition of STAT3 Phosphorylation Is Reversible

Whether AKBA-induced inhibition of STAT3 phosphorylation was reversible was also examined. Our results showed that AKBA inhibited STAT3 phosphorylation (Fig. 2A, left) and that removal of the compound reversed its effect by 12 hours (Fig. 2A, right). The STAT3 protein levels remained constant under these conditions (Fig. 2A, bottom). Cells were fully viable under this condition.

AKBA Also Inhibits IL-6–Induced STAT3 Phosphorylation

IL-6 is a growth factor for MM cells, is overexpressed in various cancers, and, as Kawano’s group showed, is a potent inducer of STAT3 (28). Hence, we examined whether AKBA could inhibit IL-6–induced STAT3 phosphorylation in MM1.S cells, which lack constitutively active STAT3. IL-6–induced phosphorylation of STAT3 as early as 5 minutes and the pretreatment of MM1.S cells with AKBA for 4 hours suppressed IL-6–induced STAT3 phosphorylation (Fig. 2B).

AKBA Suppresses IL-6–Induced JAK2 Kinase Activity and Constitutive Activation of JAK2

Because STAT3 is activated by soluble tyrosine kinases of the Janus family (JAK; ref. 29) and JAK2 is one of the main kinases involved, we examined the effects of AKBA on JAK2 phosphorylation. U266 cells were treated with different concentrations and time intervals with AKBA and phosphorylation of JAK2 was analyzed by Western blot. The results showed that AKBA inhibited constitutive phosphorylation of JAK2 in a dose-dependent (Fig. 3A, left) and time-dependent (Fig. 3A, right) manner. The levels of total JAK2 remained unchanged under these conditions (Fig. 3A, bottom).

Whether AKBA can inhibit IL-6–induced activation of JAK2 kinase was examined. For this, MM1.S cells were pretreated with different concentrations of AKBA for 4 hours and then treated with IL-6 for 10 minutes; the kinase activity was then analyzed by immunoprecipitation kinase. Our results showed that IL-6 induced JAK2 kinase activity in MM1.S cells and AKBA inhibited its effect in a dose-dependent manner (Fig. 3B).

AKBA Suppresses Constitutive Activation of c-Src

Because activation of Src has also been linked with STAT3 activation (30), we examined the effect of AKBA on constitutive activation of c-Src kinase in U266 cells. We found that AKBA suppressed the constitutive phosphorylation of c-Src kinase in a dose- and time-dependent manner (Fig. 3C). The levels of total c-Src kinase remained unchanged under these conditions (Fig. 3C, bottom).

Tyrosine Phosphatase Inhibitor Abrogates AKBA-Induced Inhibition of STAT3 Phosphorylation

Protein tyrosine phosphatases (PTP) have been implicated in STAT3 activation (31). Therefore, we examined whether
AKBA-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a PTP. Treatment of U266 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate reversed the AKBA-induced inhibition of STAT3 phosphorylation (Fig. 3D). This suggests that PTPs are involved in AKBA-induced inhibition of STAT3 phosphorylation.

**AKBA Induces the Expression of SHP-1**

SHP-1 is a nontransmembrane PTP that has been linked with regulation of STAT3 activation (32). Whether inhibition of STAT3 phosphorylation by AKBA is due to induction of the expression of SHP-1 was examined. As shown in Fig. 4A, AKBA indeed induced the expression of SHP-1 both at a translational level (Fig. 4A, left) and at a transcriptional level (Fig. 4A, right) in a dose-dependent manner.

Whether AKBA modulates any other PTP such as CD45 was examined. We found that AKBA had no effect on the expression of CD45 (Supplementary Fig. S1).

**Induction of SHP-1 by AKBA Is Transient**

Because inhibition of STAT3 phosphorylation by AKBA was found to be reversible by 24 hours (Fig. 2A, right), we

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**FIGURE 1.** A. The structure of AKBA. B. Left, AKBA inhibits constitutive STAT3 activation in U266 cells. U266 cells (1 × 10^6/mL) were treated with the indicated concentrations of AKBA for 4 h, after which whole-cell extracts were prepared and 30 μg of protein were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. Right, AKBA suppresses phospho-STAT3 levels in a timedependent manner. U266 cells (1 × 10^6/mL) were treated with the 50 μmol/L AKBA for the indicated times, after which Western blotting was done as described previously. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. C. AKBA suppresses STAT3 nuclear translocation. U266 cells (1 × 10^6/mL) were treated with the 50 μmol/L AKBA for 4 h. The samples were cytospinned and immunocytochemistry was done with STAT3 antibody. D. AKBA inhibits constitutively active STAT3 in U266 cells. Left, U266 cells (2 × 10^6/mL) were treated with the indicated concentrations of AKBA for 4 h and analyzed for nuclear STAT3 levels by electrophoretic mobility shift assay. Right, U266 cells (2 × 10^6/mL) were treated with 50 μmol/L AKBA for the indicated durations and analyzed for nuclear STAT3 levels by electrophoretic mobility shift assay.
**AKBA Abrogates STAT3 Activation**

**AKBA Down-Regulates the Expression of Antiapoptotic Gene Products**

STAT3 has been shown to regulate the expression of various gene products involved in proliferation and cell survival (33, 34); thus, whether down-regulation of STAT3 activation by AKBA leads to down-regulation of these gene products was examined. The results showed that AKBA inhibited the expression of survivin, bcl-xl, bcl-2, and mcl-1 in a time-dependent manner, with maximum suppression observed at around 12 to 24 hours (Fig. 5A).

**AKBA Down-Regulates the Expression of Angiogenic Gene Products**

Vascular endothelial growth factor (VEGF), a major mediator of angiogenesis, is regulated by STAT3 activation.

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**FIGURE 2.**

A. AKBA-induced inhibition of STAT3 phosphorylation is reversible. U266 cells (1 x 10^6) were treated with 50 μmol/L AKBA for the indicated durations (left) or treated for 1 h and washed with PBS twice to remove AKBA before resuspension in fresh medium (right). Cells were removed at the indicated times and lysed to prepare the whole-cell extract. Thirty micrograms of whole-cell extracts were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for the phosphorylated STAT3 (pSTAT3), and stripped and reprobed for STAT3 antibodies. B. AKBA down-regulates IL-6-induced phospho-STAT3. MM.1S cells (2 x 10^6) were treated with IL-6 (10 ng/mL) for the indicated times, whole-cell extracts were prepared, and phosphorylated STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.

**FIGURE 3.**

A. AKBA suppresses the activation of JAK2 in a dose- and time-dependent manner. U266 cells (2 x 10^6/mL) were treated with AKBA at the indicated doses (left) or with 50 μmol/L AKBA for the indicated time intervals (right). Whole-cell extracts were immunoprecipitated with antibody against JAK2 and analyzed by Western blot. The same samples were analyzed for JAK2 protein. B. AKBA suppresses the IL-6–induced JAK-2 kinase activity. MM.1S cells (4 x 10^6/mL) were preincubated with AKBA at the indicated doses for 4 h and treated with IL-6 for 10 min. Whole-cell lysates were prepared and the JAK-2 activity was analyzed by immunocomplex kinase assay. C. AKBA suppresses phospho-Src levels in a dose- and time-dependent manner. U266 cells (2 x 10^6/mL) were treated with the indicated doses of AKBA (left) or with 50 μmol/L AKBA for the indicated times (right), after which whole-cell extracts were prepared and 30 μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-Src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. D. Pervanadate reverses the phospho-STAT3 inhibitory effect of AKBA. U266 cells (2 x 10^6/mL) were treated with the indicated concentration of pervanadate and 50 μmol/L AKBA for 4 h, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3.

**FIGURE 4.**

A. AKBA-induced inhibition of STAT3 phosphorylation is reversible. U266 cells (1 x 10^6) were treated with 50 μmol/L AKBA for the indicated durations (left) or treated for 1 h and washed with PBS twice to remove AKBA before resuspension in fresh medium (right). Cells were removed at the indicated times and lysed to prepare the whole-cell extract. Thirty micrograms of whole-cell extracts were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for the phosphorylated STAT3 (pSTAT3), and stripped and reprobed for STAT3 antibodies. B. AKBA down-regulates IL-6–induced phospho-STAT3. MM.1S cells (2 x 10^6/mL) were treated with IL-6 (10 ng/mL) for the indicated times, whole-cell extracts were prepared, and phosphorylated STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.

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Therefore, we examined the effect of AKBA on constitutive VEGF expression in U266 cells. Our results show that AKBA inhibited the expression of VEGF in U266 cells in a time- and dose-dependent manner (Fig. 5A).

AKBA Inhibits the Proliferation of MM Cells

Because AKBA suppressed the expression of STAT3-regulated cyclin D1 expression, we examined whether AKBA inhibits the proliferation of MM cells. The results shown in Fig. 5B indicate that AKBA suppressed the proliferation of U266 cells in a time- and dose-dependent manner.

AKBA Causes the Accumulation of the Cells in the Sub-G1 Phase of the Cell Cycle

Because D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to the S phase (35) and a rapid decline in levels of cyclin D1 was observed in AKBA-treated cells, we determined the effect of AKBA on cell cycle phase distribution. We found that AKBA caused significant accumulation of G2-M and of sub-G1 phase after treatment for a maximum of 24 hours (Fig. 5C).

AKBA Activates Caspase-3 and Causes Poly(ADP)Ribose Polymerase Cleavage

Whether suppression of STAT3-regulated antiapoptotic gene products survivin, bel-xl, bcl-2, and mcl-1in U266 cells by AKBA leads to apoptosis was also examined. Cells were treated with AKBA for different times and then examined for caspase activation by Western blot using specific antibodies. We found a time-dependent activation of caspase-3 by AKBA (Fig. 5D, left) and transfection with SHP-1 siRNA reverses AKBA-induced suppression of STAT3 activation. The same whole-cell extracts were subjected to phospho-STAT3 and STAT3 (right). D. Effect of SHP-1 knockdown on AKBA-induced inhibition of pSTAT3. SCC4 cells (1 × 10⁶/mL) were transfected with either scrambled or SHP-1–specific siRNA (50 nmol/L). After 48 h, cells were treated with 50 μmol/L AKBA for 4 h and whole-cell extracts were subjected to Western blot analysis for pSTAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.

SHP-1 siRNA Reduces AKBA-Induced Apoptosis

We showed above that SHP-1 plays a critical role in suppression of STAT3 phosphorylation by AKBA. Whether SHP-1 siRNA also affects AKBA-induced apoptosis was determined. We found that knockdown of SHP-1 significantly decreased the apoptotic effects of AKBA (Fig. 6A).
Enforced Expression of Constitutively Active STAT3 Rescues Cells from AKBA-Induced Apoptosis

Whether the transfection of constitutive active STAT3 can suppress AKBA-induced apoptosis was examined. For this, cells were transfected with constitutively active STAT3 plasmid for 24 hours, then incubated with AKBA for 48 hours and examined for apoptosis by esterase staining assay. The transfection of constitutive active STAT3 plasmid led to the expression of STAT3 protein as indicated by Western blot (Fig. 6B). The results also show that the forced expression of STAT3 significantly reduced the AKBA-induced apoptosis (Fig. 6C).

FIGURE 5. A. AKBA suppresses STAT3-regulated proliferative, survival, and antiangiogenic gene products. U266 cells (2 × 10⁶/mL) were treated with 50 μmol/L AKBA for the indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE; the membrane was sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-xl, Mcl-1, and VEGF antibody. The same blots were stripped and reprobed with h-actin antibody to verify equal protein loading.

B. AKBA suppresses cell proliferation in MM. U266 and MM.1S cells were plated in triplicate, treated with 10 and 50 μmol/L AKBA, and then subjected to MTT assay on days 0 to 3 to analyze proliferation of cells.

C. AKBA causes significant accumulation of cells in the G1 phase. U266 cells (1 × 10⁶/mL) were synchronized by incubation overnight in the absence of serum and then treated with 50 μmol/L AKBA for the indicated times, after which the cell were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

D. Left, AKBA induces caspase-3 activation. U266 cells were treated with 50 μmol/L AKBA for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against caspase-3 antibody. The same blot were stripped and reprobed with h-actin antibody to show equal protein loading. Right, AKBA causes PARP cleavage. U266 cells were treated with 50 μmol/L AKBA for the indicated times and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody. The same blots were stripped and reprobed with h-actin antibody to show equal protein loading. The results shown are representative of three independent experiments.
Discussion

Because STAT3 has been linked with survival, proliferation, chemoresistance, and angiogenesis of tumor cells, its inhibitors have potential for the treatment of cancer. In the present study, we report the identification of a novel inhibitor of STAT3. We found that AKBA inhibited both constitutive and IL-6–induced STAT3 activation in MM cells and that it involved the inhibition of activation of JAK2 and c-Src and the induction of SHP-1. This correlated with suppression of various STAT3-regulated gene products, inhibition of proliferation, and induction of apoptosis of MM cells.

This is the first report to suggest that AKBA can inhibit STAT3 activation. Whether examined by STAT3 phosphorylation at tyrosine 705, by nuclear translocation, or by DNA binding, we found that this triterpene suppressed STAT3 activation. The suppression was, however, fully reversible by 12 to 24 hours, indicating that the inhibition is transitory. We found that AKBA also suppressed STAT3 activation induced by IL-6, one of the many tumor cell growth factors that activate STAT3. A previous study showed that all Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation (36, 37).

How AKBA inhibits activation of STAT3 was investigated in detail. The activation of JAK2 has been closely linked with STAT3 activation and we found that AKBA inhibited the activation of constitutively active as well as IL-6–induced JAK2 kinase activity in MM cells. Besides JAK2, c-Src has also been implicated in STAT3 activation. Again, AKBA also inhibited the c-src activation in MM cells.

We also found that PTP is involved in the down-regulation of STAT3 by AKBA. One of the first evidence that PTP is involved in the action of AKBA is that a broad-acting PTP inhibitor, pervanadate, inhibited the effect of AKBA on STAT3 activation. Several PTPs have been implicated in STAT3 activation.
signaling, including SHP-1 (38), SHP-2 (39), TC-PTP (40), PTEN (41), PTP-1D (42), CD45 (43), and PTP-ε (44). Loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma (31). Our results showed that AKBA is a potent inducer of SHP-1 protein but not CD45. Thus, it is possible that induction of SHP-1 led to inhibition of STAT3 activation. We also found that the knockdown of SHP-1 reversed the inhibitory effect of AKBA on STAT3 and decreased apoptosis. Consistent with suppression of STAT3 activation, AKBA was found to down-regulate the expression of STAT3-regulated genes that are involved in proliferation (cyclin D1) and survival (Bcl-2, Bcl-xl, and Mcl-1) of tumor cells. It is possible that the antiproliferative effects of AKBA in glioma, colon cancer, prostate, and leukemic cells (12-17) are due to suppression of these gene products.

In MM cells that express constitutively active STAT3, we showed that AKBA suppressed the proliferation of these cells and induced accumulation of cells in the G2-M phase and induction of sub-G1. In addition, this triterpene was found to activate caspase-3 and induce apoptosis in MM cells, which is consistent with previous reports (45). We also found that AKBA down-regulated the expression of VEGF needed for angiogenesis of tumor cells. These results are consistent with a report that AKBA inhibits basic fibroblast growth factor–induced angiogenesis (18).

Previously, we and others have reported that AKBA can inhibit nuclear factor-κB (NF-κB) activation through inhibition of IκK activation (46, 47). Whether inhibition of STAT3 by AKBA is connected with suppression of IκK activation is not clear. The p65 subunit of NF-κB has been shown to communicate with STAT3 (48), but activation of STAT3 and NF-κB are dependent on different cytokines and different kinases. Tumor necrosis factor is the major activator of NF-κB, whereas IL-6 is the most potent inducer of STAT3. Interestingly, JAK2 kinase needed for STAT3 activation has been shown to be required for erythropoietin-induced NF-κB activation (49). Thus, it is possible that inhibition of JAK2 activation is the potential link for inhibition of both NF-κB and STAT3 activation by AKBA. Alternatively, transforming growth factor-β–activated kinase 1, which is the p70 subunit of JAK2, may be the link, as it is another kinase involved in both NF-κB and STAT3 activation (50). Transforming growth factor-β–activated kinase 1, however, is known to phosphorylate STAT3 at serine 727 and not tyrosine 705, as was the case in the current study.

Our results clearly show that AKBA inhibits IL-6 signaling quite effectively. Thus, it is possible that the role of AKBA in osteoarthritis, chronic colitis, ulcerative colitis, Crohn’s disease, bronchial asthma, experimental ileitis (3), experimental colitis (4), autoimmune encephalomyelitis (5), nociception (6), inflammation and atherogenesis (7), bovine serum albumin–induced arthritis (8), and immunomodulatory effects (11) are all due to suppression of IL-6 signaling as reported here.

Overall, our results show that AKBA inhibits both inducible and constitutive STAT3 activation through the induction of tyrosine kinase phosphatase, which makes it a potentially effective suppressor of tumor cell survival, proliferation, and angiogenesis. Further in vivo studies may provide important leads for using AKBA as treatment of cancer and other inflammatory diseases.

Materials and Methods

A 50 mM/L solution of AKBA (Fig. 1A), kindly supplied by Sabinsa Corporation, was prepared in 100% DMSO, stored as small aliquots at –20°C and diluted as needed in cell culture medium. RPMI 1640, fetal bovine serum, 0.4% trypan blue vital stain, and an antibiotic-antimycotic mixture were obtained from Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich. Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (tyrosine 705) and Bcl-2, Bcl-xl, Mcl-1, SHP-1, procaspase-3, phospho-PTEN, and PARP were obtained from Santa Cruz Biotechnology. Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad. Antibodies to phosphospecific Src (tyrosine 416), Src, and JAK2 were purchased from Cell Signaling Technology. GST-JAK2 was kindly provided by Dr. Z.J. Zhao (University of Oklahoma Health Sciences Center, Oklahoma, OK). CD45 RA was obtained from BD BioSciences. Goat anti-mouse horseradish peroxidase was purchased from Transduction Laboratories. The siRNA for SHP-1 and the scrambled control were obtained from Ambion. The constitutive active STAT3 construct was kindly supplied by Dr. John DiGiovanni (The University of Texas M. D. Anderson Cancer Center, Smithville, TX).

Cell Lines

Human MM cell lines U266, MM.1S (dexamethasone-sensitive), SCC4, and A293 were obtained from American Type Culture Collection. Cell line U266 (ATCC-TIB-196) is a plasmacytoma of B-cell origin and is known to produce monoclonal antibodies and IL-6. The MM.1S cell line, established from the peripheral blood cells of a patient with IgA myeloma, secretes XL chain, is negative for the presence of the EBV genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens (51). U266 and MM.1S cells were cultured in RPMI 1640 containing 10% fetal bovine serum. A293 and SCC4 cells were cultured in DMEM and DMEM/F12, respectively, supplemented with 10% fetal bovine serum. All media were also supplemented with 100 units/mL of penicillin and 100 μg/mL of streptomycin.

Electrophoretic Mobility Shift Assay for STAT3-DNA Binding

STAT3-DNA binding was analyzed by electrophoretic mobility shift assay using a [32P]-labeled high-affinity siss-inducible element (hSIE) probe (5′-CTTCATTCTCCCCTGTAATCCCTAAGCT-3′) and 5′-AGTTTTAGGATTACGGGAATGTA-3′) as previously described (27). Briefly, nuclear extracts were prepared from AKBA-treated cells and incubated with hSIE probe. The DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels. The dried gels were visualized and the radioactive bands were quantitated with a Storm 820 and Imagequant software (Amersham).

Western Blotting

For detection of STAT proteins, AKBA-treated whole-cell extracts were lysed in lysis buffer [20 mM/L Tris (pH 7.4),...
250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L NaVO₄. Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 10% SDS-PAGE. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1,000) overnight. Immunocomplexes were visualized with a Storm820. To determine the total amounts of JAK2 in each sample, 30 μg of whole-cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with anti-JAK2 antibody.

**Transfection with Constitutive STAT3 Construct**

A293 cells (5 × 10⁵ per well) were plated in six-well plates in DMEM containing 10% fetal bovine serum. After 24 h, the cells were transfected with constitutive STAT3 plasmid (0.5 μg/well) by calcium phosphate method according to the manufacturer’s protocol (Invitrogen). Twenty-four hours after transfection, the cells were harvested and the transfection was confirmed by Western blot. U266 cells, which express constitutively active STAT3, were used as a control for Western blot analysis.

**Transfection with SHP-1 siRNA**

Human squamous cell carcinoma (SCC4) cells were plated in six-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μL HiPerFect transfection reagent (Qiagen) were added to 50 mmol/L SHP-1 siRNA in a final volume of 100 μL culture medium. After 48 h of transfection, cells were treated with AKBA for 4 h and whole-cell extracts were prepared for SHP-1, STAT3, and phospho-STAT3 analysis by Western blot.

**Apoptosis Assay**

To determine apoptosis, we used a Live/Dead assay kit (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium bromide homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells.

**RNA Analysis and Reverse Transcription-PCR**

U266 cells were left untreated or treated with AKBA for various times, washed, and suspended in Trizol reagent. Total RNA was extracted according to the manufacturer’s instructions (Invitrogen, Life Technologies). One microgram of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum Taq polymerase using Superscript One Step reverse transcription-PCR kit (Invitrogen). The relative expression of SHP-1 was analyzed using quantitative reverse transcription-PCR with glyceraldehyde-3-phosphate dehydrogenase as an internal control. The reverse transcription-PCR reaction mixture contained 12.5 μL of 2X reaction buffer, 10 μL each of RNA, 0.5 μL each of forward and reverse primers, and 0.5 μL of RT Platinum Taq in a final volume of 24 μL. The reaction was done at 50°C for 30 min, 94°C for 2 min, 94°C for 30 cycles of 15 s each, 55°C for 30 s, and 72°C for 1 min with extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.
Immunoblot Analysis of PARP Degradation

AKBA-induced apoptosis was examined by proteolytic cleavage of PARP. Briefly, cells (1 × 10⁶/mL) were treated with AKBA for the indicated times at 37°C. The cells were then washed and whole-cell extracts were prepared by incubating for 30 min on ice in 0.1 mL buffer containing 20 mMol/L HEPES (pH 7.4), 2 mMol/L EDTA, 250 mMol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotnin, 1 mMol/L phenylmethylsulfonyl fluoride, 0.5 μg/mL benzamidine, 1 mMol/L DTT, and 1 mMol/L sodium vanadate. The lysate was centrifuged and the supernatant was collected. Cell extract (30 μg) was resolved on 7.5% SDS-PAGE, electro-transferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by chemiluminescence (ECL; Amersham).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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