

5-Hydroxy-2-Methyl-1,4-Naphthoquinone, a Vitamin K3 Analogue, Suppresses STAT3 Activation Pathway through Induction of Protein Tyrosine Phosphatase, SHP-1: Potential Role in Chemosensitization

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Abstract

The activation of signal transducers and activators of transcription 3 (STAT3) has been linked with carcinogenesis through survival, proliferation, and angiogenesis of tumor cells. Agents that can suppress STAT3 activation have potential not only for prevention but also for treatment of cancer. In the present report, we investigated whether 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), an analogue of vitamin K, and isolated from chitrak (*Plumbago zeylanica*), an Ayurvedic medicinal plant, can modulate the STAT3 pathway. We found that plumbagin inhibited both constitutive and interleukin 6–inducible STAT3 phosphorylation in multiple myeloma (MM) cells and this correlated with the inhibition of c-Src, Janus-activated kinase (JAK)1, and JAK2 activation. Vanadate, however, reversed the plumbagin-induced downregulation of STAT3 activation, suggesting the involvement of a protein tyrosine phosphatase. Indeed, we found that plumbagin induced the expression of the protein tyrosine phosphatase, SHP-1, and silencing of the SHP-1 abolished the effect of plumbagin. This agent also downregulated the expression of STAT3-regulated cyclin D1, Bcl-xL, and vascular endothelial growth factor; activated caspase-3; induced poly (ADP ribose) polymerase cleavage; and increased the sub-G₁ population of MM cells. Consistent with these results, overexpression of constitutive active STAT3 significantly reduced the plumbagin-induced apoptosis. When compared with AG490, a rationally designed STAT3/JAK2 inhibitor, plumbagin was found more potent in suppressing the proliferation of cells. Plumbagin also significantly potentiated the apoptotic effects of thalidomide and bortezomib in MM cells. Overall, these results suggest that the plumbagin inhibits STAT3 activation pathway through the induction of SHP-1 and this may mediate the sensitization of STAT3 overexpressing cancers to chemotherapeutic agents. *Mol Cancer Res*; 8(1); 107–118. ©2010 AACR.

Introduction

The modern era of cancer therapy began in 1941 with the introduction of nitrogen mustard, a chemical warfare agent, as an effective treatment for cancer, and then chemotherapeutic agents in 1971. The search for specificity and safety led to the discovery of targeted cancer therapies. First introduced in 1991, targeted therapies are also toxic, lack efficacy, and are highly expensive. The use of anticancer agents derived from traditional therapies (sometimes

called alternate or complementary therapies) provides a novel opportunity to improve the existing standard of care for cancer and other diseases. 5-Hydroxy-2-methyl-1,4-naphthoquinone (plumbagin, an analogue of vitamin K3) is one such agent. Plumbagin is a naturally occurring yellow pigment found in the plants of the Plumbaginaceae, Droseraceae, Anastrocladaceae, and Dioncophyllaceae families. The root of chitrak (*Plumbago zeylanica*), a major source of plumbagin, has been used in Indian medicine since the period of Charaka as an antiatherogenic, cardioprotective, hepatoprotective, and neuroprotective agent (1). The active principle, plumbagin, is also present along with a series of other structurally related naphthoquinones in the roots, leaves, bark, and wood of *Juglans regia* (variously known as the English walnut, Persian walnut, and California walnut), *Juglans cinerea* (butternut and white walnut), and *Juglans nigra* (black walnut; refs. 2, 3).

Plumbagin has been shown to exert anticancer activities against a wide variety of tumor cells, including breast cancer (4), lung cancer (5, 6), ovarian cancer (7), acute promyelocytic leukemia (8), melanoma (9), and prostate cancer (10, 11). How plumbagin mediates these anticellular effects

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is not fully understood. It has been shown to induce G₂-M cell cycle arrest through the downregulation of cyclin B1, cyclin A, CDC2, and CDC25C (4, 6, 8, 9, 12). Plumbagin, regarded as a redox recycling quinone and induces superoxide radicals (13), inhibits AKT (4, 6), NF- κ B (14), and topoisomerase II (13); downregulates the expression of survivin and epidermal growth factor receptor (6); and induce p21 (4, 12), p53 (5), and c-Jun-NH₂-kinase (5, 9). Plumbagin has been shown to bind NADPH oxidase (15), an estrogen receptor- α (7), and multidrug resistance-linked ATP-binding cassette drug transporter (ABCG2; ref. 16), and inhibit their activity. In animals, plumbagin has shown to exhibit anticancer (5, 10, 17, 18), radiosensitizer of tumor cells (19), antibacterial (20), and antiarthritic potential (21). The latter was mediated through the inhibition of neutrophil activation, collagenase activation, and angiogenesis (21). Plumbagin can also radiosensitize melanoma and cervical cancer cells (22). Because several of these effects require the activation of the transcription factor signal transducer and activators of transcription 3 (STAT3), we postulated that plumbagin mediates its effects through the modulation of this pathway.

STAT proteins are known to play an essential role in tumorigenesis (23). STAT3, one member of the STAT family, is often constitutively active in many human cancer cells, including multiple myeloma (MM), lymphomas, leukemia, breast cancer, prostate cancer, head and neck squamous cell carcinoma, brain tumor, colon cancer, Ewing's sarcoma, gastric cancer, esophageal cancer, ovarian cancer, nasopharyngeal cancer, and pancreatic cancer (24, 25).

Because of the critical role of STAT3 in tumor cell survival, proliferation, and angiogenesis, we hypothesized that plumbagin mediates its effects in part through the modulation of the STAT3 pathway. We tested this hypothesis in MM cells. In our experiments, plumbagin indeed suppressed both constitutive and inducible STAT3 activation. This inhibition decreased the gene products linked to cell survival, proliferation, and angiogenesis. This correlated with suppression of proliferation, induction of apoptosis, and enhancement of the response to the cytotoxic effects of thalidomide [an inhibitor of tumor necrosis factor (TNF) expression] and bortezomib (a proteasome inhibitor) in MM cells.

Materials and Methods

Reagents

Plumbagin (with a purity of >97%) was purchased from Sigma-Aldrich. A 100-mmol/L solution of plumbagin was prepared in DMSO, stored as small aliquots at -70°C, and then diluted as needed in cell culture medium. Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich. RPMI 1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen. Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr705

and Ser727), Bcl-xL, SHP-1, cyclin D1, procaspase-3, JAK2, and PARP were obtained from Santa Cruz Biotechnology. Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad. Antibodies to phospho-Src (Tyr416), Src, phospho-JAK1 (Tyr1022/1023), phospho-JAK2 (Tyr1007/1008), and JAK1 were purchased from Cell Signaling Technology. Goat anti-mouse horseradish peroxidase was purchased from Transduction Laboratories, and goat anti-rabbit Alexa 594 was from Invitrogen. AG490 was obtained from Calbiochem. Bacteria-derived recombinant human IL-6 was kindly provided by Novartis Pharmaceuticals. The siRNA for SHP-1 and the scrambled control were obtained from Ambion. Bortezomib (Velcade, PS-341) was obtained from Millennium, and thalidomide was from Tocris Cookson. GST-JAK2 substrate was kindly provided by Dr. Zhizhuang Joe Zhao (Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). The constitutively active STAT3 construct was kindly provided by Dr. John DiGiovanni from The University of Texas M.D. Anderson Cancer Center (Smithville, TX).

Cell Lines

Human MM cell lines U266 and MM.1S (dexamethasone sensitive) were obtained from the 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 λ L chain, is negative for the presence of the EBV genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens. U266, MM.1S cells were cultured in RPMI 1640 containing 1 \times antibiotic-antimycotic solution with 10% FBS. Human embryonic kidney (A293) and human head and neck (SCC4) cells were maintained in DMEM containing 1 \times antibiotic-antimycotic solution with 10% FBS.

EMSA for STAT3-DNA Binding

STAT3-DNA binding was analyzed by EMSA using a ³²P-labeled high-affinity sis-inducible element probe (5'-CTTCATTTCCCGT AAATCCCT AAA GCT-3' and 5'-AGCTTTAGGGATTTACGGGAAATGA-3') as described previously (26). Briefly, nuclear extracts were prepared from plumbagin-treated cells and incubated with the ³²P-labeled high-affinity sis-inducible element probe. The resultant DNA-protein complex was separated from free oligonucleotide on 5% native polyacrylamide gels. For super shift assays, nuclear extracts prepared from U266 cells were incubated with antibodies against either phospho-STAT3 or STAT3 for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum was used as control. The dried gels were visualized, and the radioactive bands were quantified with the Storm 820 imaging system and Image quant software (GE Healthcare).

Western Blotting

To detect various proteins, U266 cells (2×10^6) were treated with plumbagin. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 mL of buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μ g/mL leupeptin, 2 μ g/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 μ g/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged and the supernatant was collected. Whole-cell extract protein (30 μ g) was resolved on 7.5% to 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with antibodies, and then detected by enhanced chemiluminescence (GE Healthcare).

Immunocytochemistry for STAT3 Localization

Plumbagin-treated MM cells were plated on a glass slide by centrifugation using a Cytospin 4 (Thermoshendon), air-dried for 1 h at room temperature, and fixed in 4% formaldehyde. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After an overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/mL) for 5 min. Stained slides were mounted with mounting medium (Sigma-Aldrich) and analyzed under an epifluorescence microscope (Labophot-2; Nikon). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

STAT3 Luciferase Reporter Assay

A293 cells were plated in six-well plates with 5×10^5 per well in DMEM containing 10% FBS. The STAT3-responsive elements linked to a luciferase reporter gene was transfected with wild-type or dominant-negative STAT3-Y705F (STAT3F). Transfections were done according to the manufacturer's protocols using Fugene-6 (Roche). At 24 h after transfection, cells were pretreated with plumbagin for 4 h and then induced by IL-6 for additional 24 h before being washed and lysed in luciferase lysis buffer (Promega). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega) and was normalized to β -galactosidase activity. All luciferase experiments were done in triplicate and were repeated three or more times. The data show the mean and the SD of the mean of the experiments.

Transfection with SHP-1 siRNA

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

MTT Assay

The antiproliferative effect of plumbagin and AG490 against the MM cell lines was determined by the MTT dye uptake method.

Live/Dead Assay

Viability of cells was also determined by using the Live/Dead assay (Invitrogen), which measures intracellular esterase activity and plasma membrane integrity.

Flow Cytometric Analysis

To determine the effect of plumbagin on the cell cycle, U266 cells were first synchronized by serum starvation and then exposed to plumbagin. The cells were then washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μ g/mL propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Kinase Assay

To determine the effect of plumbagin on JAK2 activation, we performed an immunocomplex kinase assay using GST-JAK2 as the substrate. Briefly, the JAKs complex from whole-cell extracts was precipitated with antibody against JAK2 and was treated with protein A/G-agarose beads (Pierce). After 2 h, the beads were washed with whole-cell extract buffer and then resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl₂, 2 mmol/L DTT, 20 μ Ci of [³²P]ATP, 10 μ mol/L unlabeled ATP, and 2 μ g of substrate GST-JAK2. After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with the Storm 820 imaging system. To determine the total amounts of JAK2 in each sample, 30 μ g of whole-cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with anti-JAK2 antibody.

Transfections with Constitutive STAT3 Construct

A293 cells were plated in chamber slides in DMEM containing 10% FBS. After 24 h, the cells were transfected with constitutive STAT3-plasmid by FuGene 6 according to the manufacturer's protocol (Roche). Cells were treated with plumbagin for 24 h, and the viability of the cells was determined by Live/Dead assay.

Statistical Analysis

The statistical analysis was done using Student's *t* test with Microsoft excel software.

Results

The present study was undertaken to determine the effect of plumbagin on the STAT3 signaling pathway. We investigated the effect of plumbagin on both constitutive

and interleukin (IL)-6-inducible STAT3 activation in MM cells. We also evaluated the effect of plumbagin on the various mediators of cellular proliferation, cell survival, and apoptosis. The structure of plumbagin is shown in Fig. 1A. The dose and duration of plumbagin used to modulate STAT3 activity did not affect cell viability, indicating that downregulation of STAT3 was not due to cell killing (data not shown).

Plumbagin Inhibits Constitutive STAT3 Phosphorylation in MM Cells

Whether plumbagin can modulate the constitutive STAT3 activation in MM cells was investigated. U266 cells were first incubated with different concentrations of plumbagin for 4 hours. After incubation, whole-cell extracts were prepared and examined for phosphorylated STAT3 by Western blot analysis using an antibody that recognizes STAT3 that is phosphorylated at tyrosine 705. As shown in Fig. 1B (*left*), plumbagin inhibited the constitutive phosphorylation of STAT3 in U266 cells, with maximum inhibition occurring at 5 $\mu\text{mol/L}$. We also determined the

incubation time required for plumbagin to suppress STAT3 activation in U266 cells. The inhibition was time dependent, with maximum inhibition occurring at 4 hours (Fig. 1B, *right*).

Whether plumbagin modulates the phosphorylation of STAT3 at serine 727 residue was also examined. We found that plumbagin inhibited the serine phosphorylation of STAT3 in a dose-dependent manner (Fig. 1B, *third panel*). This quinone had no effect on the expression of STAT3 protein under these conditions.

Plumbagin Inhibits the Binding of STAT3 to DNA in MM Cells

Tyrosine phosphorylation causes dimerization of STAT3, leading to its translocation to the nucleus, where it binds to DNA and regulates gene transcription (26). We, therefore, determined whether plumbagin suppresses DNA-binding activity of STAT3. Electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts prepared from plumbagin-treated U266 cells showed that it caused a decrease in STAT3 DNA-binding activity in

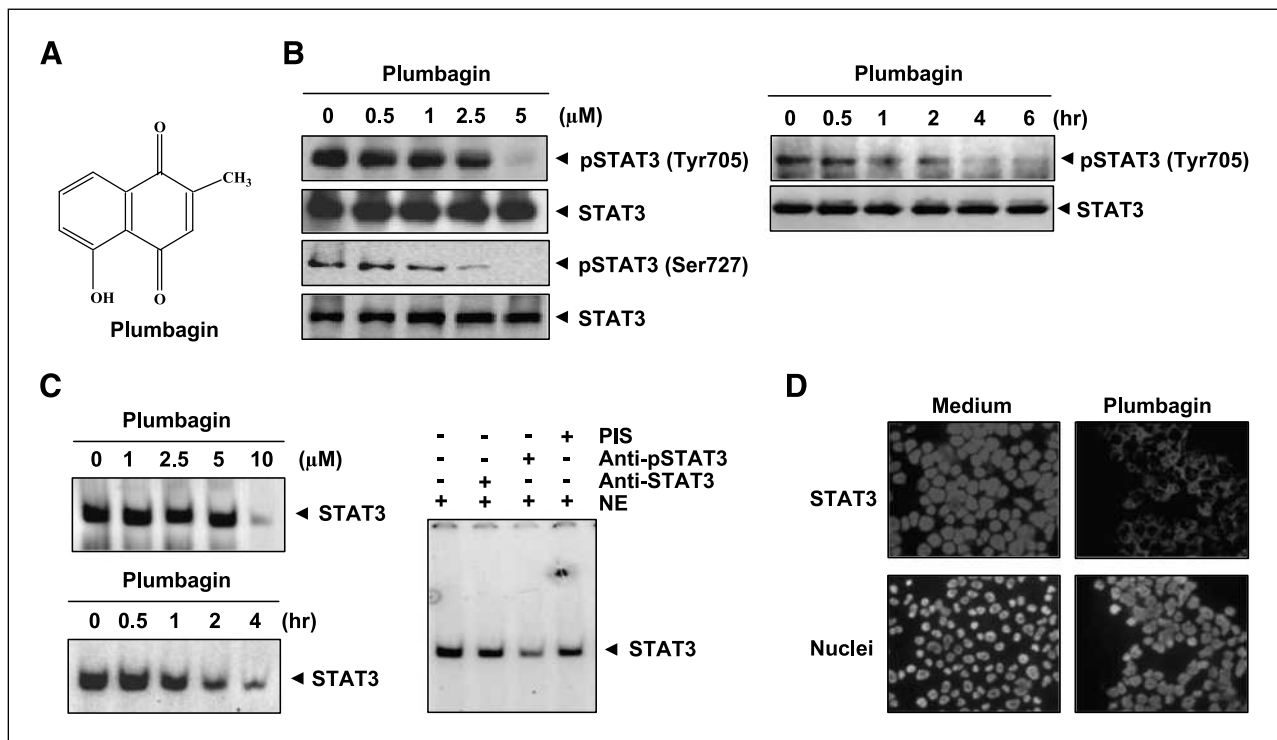


FIGURE 1. Plumbagin inhibits constitutively active STAT3 in U266 cells. **A.** The structure of plumbagin. **B.** Plumbagin suppresses phospho-STAT3 levels in a dose and time-dependent manner. U266 cells (2×10^5) were first treated with the indicated concentrations of plumbagin for 4 h after incubation; whole-cell extracts were prepared and analyzed for phospho-STAT3 through Western blot. For time-dependent assessment, U266 cells (2×10^6) were treated with 5.0 $\mu\text{mol/L}$ plumbagin for the indicated times and analyzed for phospho-STAT3 levels. **C.** Plumbagin suppresses STAT3 DNA binding in a dose- and time-dependent manner. U266 cells (2×10^6) were treated with the indicated concentrations of plumbagin for 4 h and nuclear extracts were analyzed for STAT3 DNA binding by EMSA. For time-dependent assessment, cells were treated with 10 $\mu\text{mol/L}$ plumbagin for the indicated times and nuclear extracts were examined for STAT3-DNA binding by EMSA. For supershift analysis, nuclear extracts (NE) from U266 cells were incubated with phospho- and total STAT3 antibody and preimmune serum (PIS). They were then assayed for STAT3 DNA binding by EMSA. **D.** Plumbagin causes the inhibition of translocation of STAT3 to the nucleus. U266 cells (1×10^5) were incubated with or without 5 $\mu\text{mol/L}$ plumbagin for 4 h and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with Hoechst (50 ng/mL) for 5 min. The results shown are representative of two independent experiments.

a dose-dependent (Fig. 1C, *left*) and time-dependent manner (Fig. 1C, *middle*). These results show that plumbagin abrogates the DNA-binding ability of STAT3. Supershift analysis indicated that the binding of STAT3 to the DNA was blocked by an anti-phospho-STAT3 antibody, thus confirming that the protein/DNA complex observed is indeed STAT3 (Fig. 1C, *right*).

Plumbagin Depletes Nuclear Pool of STAT3 in MM Cells

Because nuclear translocation is central to the function of transcription factors and because it is not certain whether phosphorylation is mandatory for nuclear transport of STAT3 and its oncogenic functions (27), we investigated whether plumbagin suppresses the nuclear retention of STAT3. Figure 1D clearly shows that plumbagin inhibited the translocation of STAT3 to the nucleus in U266 cells.

Plumbagin Inhibits IL-6-Induced STAT3 Phosphorylation in Human MM Cells

Because IL-6 is a growth factor for MM cells and this effect is mediated through the activation of STAT3 (28), we determined whether plumbagin could inhibit IL-6-induced STAT3 phosphorylation. MM.1S cells, which lack the constitutively active STAT3, were treated with IL-6 at different concentrations and then examined for phosphorylated STAT3. IL-6 induced the phosphorylation of STAT3 at a concentration of 10 ng/mL within 5 minutes (Fig. 2A). Pretreatment of cells with plumbagin suppressed this IL-6-induced STAT3 phosphorylation. Exposure of cells to plumbagin for 1 hour was sufficient to completely suppress IL-6-induced STAT3 phosphorylation (Fig. 2B).

Plumbagin Suppresses IL-6-Induced STAT3-Dependent Reporter Gene Expression

Our results showed that plumbagin inhibited the phosphorylation, nuclear translocation, and DNA-binding activity of STAT3. We next determined whether plumbagin affects STAT3-dependent gene transcription. We used A293 cells for this study because these cells do not constitutively express STAT3 and are easy to transfect. When cells transiently transfected with the STAT3-Luc construct were stimulated with IL-6, STAT3-mediated luciferase gene expression significantly increased. Dominant-negative STAT3 blocked this increase, indicating specificity. When the cells were pretreated with plumbagin, IL-6-induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 2C).

Plumbagin Suppresses Constitutive Activation of c-Src

How plumbagin inhibits STAT3 activation in MM cells was investigated. STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (29). Hence, we determined the effect of plumbagin on constitutive activation of Src kinase in U266 cells. We found that plumbagin suppressed the constitutive phosphorylation of c-Src kinase at tyrosine 416 (Fig. 3A).

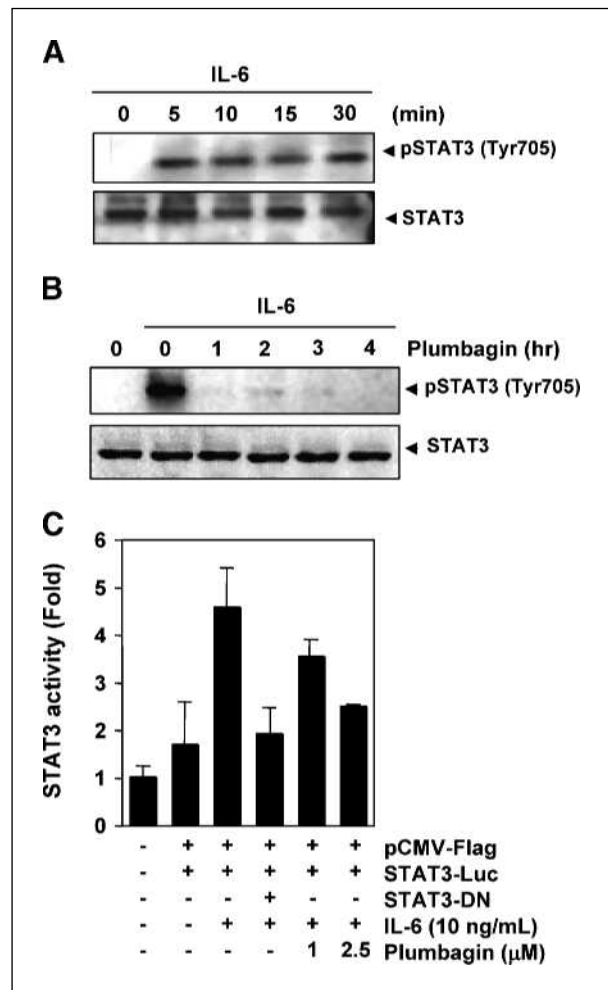


FIGURE 2. Plumbagin downregulates IL-6-induced phospho-STAT3. **A.** MM1.S cells (2×10^6) were treated with IL-6 (10 ng/mL) for the indicated times. Whole-cell extracts were prepared, and phospho-STAT3 level was detected by Western blot. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading. **B.** MM1.S cells (2×10^6) were treated with 5 μ mol/L plumbagin for the indicated times and then stimulated with IL-6 (10 ng/mL) for 30 min. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blotting. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading. **C.** A293 cells (5×10^5) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with 1 and 2.5 μ mol/L plumbagin for 4 h and then stimulated with IL-6 (10 ng/mL) for 24 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. Cells were cotransfected with β -galactosidase and the data were normalized with β -galactosidase assay (data not shown). The results shown are representative of two independent experiments.

The levels of total nonphosphorylated c-Src kinase protein remained unchanged under the same conditions.

Plumbagin Suppresses Constitutive Activation of JAK1 and JAK2

STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAK; ref. 30), so we determined whether plumbagin affects constitutive activation

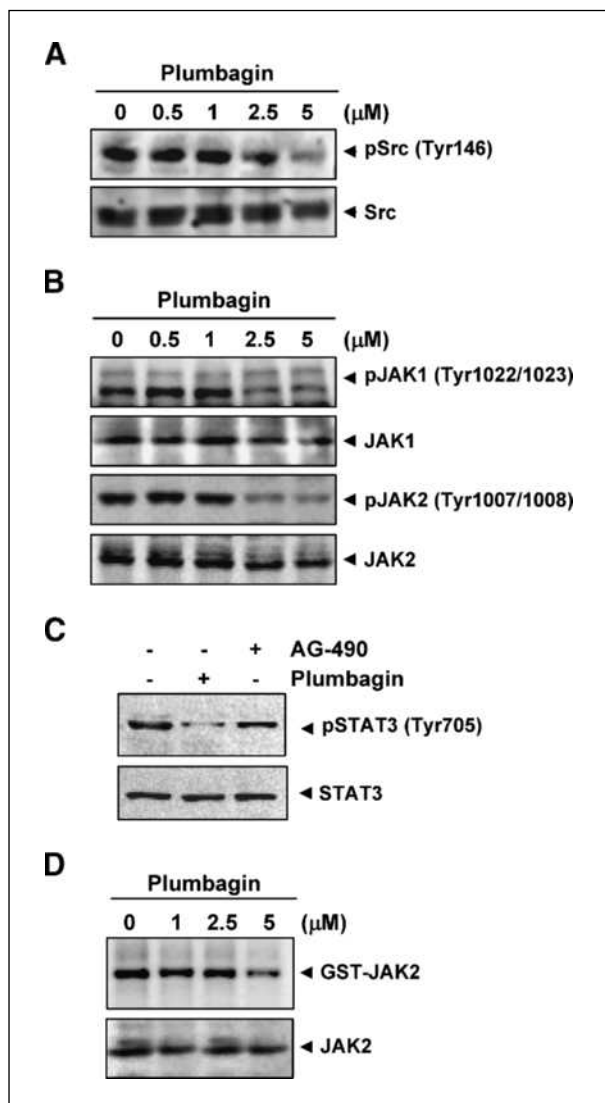


FIGURE 3. A. Plumbagin suppresses phospho-Src levels. U266 cells (2×10^6) were treated with the indicated concentrations of plumbagin for 4 h, and whole-cell extracts were prepared. Aliquots (30 μg) of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-Src antibody. The same blot was stripped and reprobed with Src antibody to verify equal protein loading. **B.** Plumbagin suppresses phospho-JAK1 and JAK2 expression. U266 cells (2×10^6) were treated with the indicated concentrations of plumbagin for 4 h, and whole-cell extracts were prepared. Aliquots (30 μg) of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-JAK1 and phospho-JAK2 antibody. The same blots were stripped and reprobed with JAK1 and JAK2 antibody to verify equal protein loading. **C.** To compare the effects of plumbagin and AG490 on phospho-STAT3, U266 cells were treated with 5 $\mu\text{mol/L}$ concentration of plumbagin for 4 h and 100 $\mu\text{mol/L}$ AG490 for 4 h; whole-cell extracts were prepared; and 30 μg portions of those extracts were resolved on 10% SDS-PAGE and probed against phospho-STAT3 and STAT3. **D.** Plumbagin suppresses JAK2 activity. U266 cells (2×10^6) were treated with the indicated concentrations of plumbagin for 4 h; whole-cell extracts were prepared; and the kinase assay was done. The results shown are representative of two independent experiments.

of JAK1 and JAK2 in U266 cells. We found that plumbagin suppressed the constitutive phosphorylation of JAK1 at tyrosine 1022/1023 and JAK2 at tyrosine 1007/1008 (Fig. 3B). The levels of nonphosphorylated JAK1 and JAK2 protein remained unchanged under the same conditions (Fig. 3B, *bottom*).

Plumbagin Is More Effective than AG490 in Inhibiting the Activation of STAT3

AG490 is the best known rationally designed inhibitor of JAK2 kinase linked to STAT3 activation (30). How the relative activity of plumbagin compares with that of AG490 for the suppression of phosphorylation of STAT3 was examined. As shown in Fig. 3C, plumbagin at 5 $\mu\text{mol/L}$ was found to be more effective than AG490 at 100 $\mu\text{mol/L}$.

Plumbagin Suppresses Constitutive JAK2 Activity

JAK2 has also been linked with STAT3 phosphorylation. We therefore investigated whether plumbagin affects JAK2 activity in U266 cells, by using the immune complex kinase assays. We found that plumbagin also suppressed the activity of JAK2 (Fig. 3D).

Tyrosine Phosphatases Are Involved in Plumbagin-Induced Inhibition of STAT3 Activation

Because protein tyrosine phosphatases (PTPase) have been implicated in the inactivation of STAT3 (31), we determined whether plumbagin-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a PTPase. Treatment of U266 cells with the broad-acting PTPase inhibitor sodium pervanadate prevented the plumbagin-induced inhibition of STAT3 activation (Fig. 4A). This suggests that PTPases are involved in plumbagin-induced inhibition of STAT3 activation.

Plumbagin Induces the Expression of SHP-1 in MM Cells

SHP-1 is a nontransmembrane PTPase expressed most abundantly in hematopoietic cells (32). This PTPase is an important negative regulator of JAK/STAT signaling in leukemias and lymphomas (33). We therefore examined whether plumbagin can modulate the expression of SHP-1 in U266 cells. Cells were incubated with different concentrations of plumbagin for 4 hours; whole-cell extracts were prepared and examined for SHP-1 protein by Western blot analysis. As shown in Fig. 4B, plumbagin induced the expression of SHP-1 protein in U266 cells.

SHP-1 Small Interfering RNA Downregulate the Expression of SHP-1 and Reverses the Inhibition of STAT3 Activation by Plumbagin

We determined whether the suppression of SHP-1 expression by small interfering RNA (siRNA) would abrogate the inhibitory effect of plumbagin on STAT3 activation. Western blotting showed that plumbagin-induced SHP-1 expression was effectively abolished in the cells treated with SHP-1 siRNA; treatment with

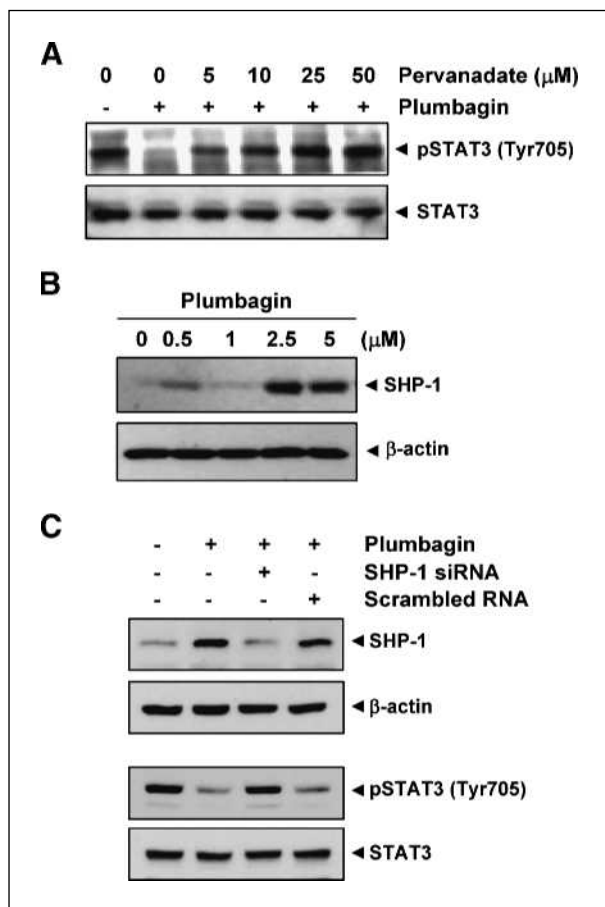


FIGURE 4. A. Pervanadate reverses the phospho-STAT3 inhibitory effect of plumbagin. U266 cells (2×10^5) were treated with the indicated concentrations of pervanadate and 5 $\mu\text{mol/L}$ plumbagin for 4 h, and whole-cell extracts were prepared. Thirty micrograms of protein extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. **B.** Plumbagin induces the expression of SHP-1 protein. U266 cells (2×10^5) were treated with the indicated concentrations of plumbagin for 4 h. After incubation, whole-cell extracts were prepared, and 30 μg of protein were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for SHP-1 antibody. The same blots were stripped and re-probed with β -actin antibody to verify equal protein loading. **C.** Effect of SHP-1 knockdown on plumbagin-induced expression of SHP-1. SCC4 cells (1×10^5) were transfected with either SHP-1-specific or scrambled siRNA (50 nmol/L). After 48 h, cells were treated with 5 $\mu\text{mol/L}$ plumbagin for 4 h, and whole-cell extracts were subjected to Western blot analysis for SHP-1. The same blots were stripped and re-probed with β -actin antibody to verify equal protein loading. Transfection with SHP-1 siRNA reverses plumbagin-induced suppression of STAT3 activation. The same whole-cell extracts were subjected to phospho-STAT3 and STAT3. The results shown are representative of two independent experiments.

scrambled siRNA had no effect (Fig. 4C). We also found that plumbagin failed to suppress STAT-3 activation in cells treated with SHP-1 siRNA (Fig. 4C, *bottom*). These siRNA results corroborate our earlier evidence of the critical role of SHP-1 in suppression of STAT-3 phosphorylation by plumbagin.

Plumbagin Inhibits the Proliferation of MM Cells and Is More Effective than AG490

Because plumbagin downregulated the expression of cyclin D1, the gene critical for cell proliferation, we investigated whether plumbagin inhibits the proliferation of MM cells by using the MTT method. Plumbagin inhibited proliferation of cells in a dose-dependent manner (Fig. 5A).

To determine how the potency of plumbagin compares with that of well-established and rationally designed inhibitors of the STAT3 pathway, we examined the antiproliferative activity of AG490 and plumbagin side by side. As shown in Fig. 5A, plumbagin was more potent than AG490 in suppressing the proliferation of MM cells. These results coincide with their relative effects on STAT3 phosphorylation.

Plumbagin 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 S phase (34), and because a rapid decline in levels of cyclin D1 was observed in plumbagin-treated cells, we determined the effect of plumbagin on cell cycle phase distribution. We found that plumbagin caused a significant accumulation of the cell population in the sub-G₁ phase. After 48 hours, 22% of the cell population had accumulated in the sub-G₁ phase, which is indicative of apoptosis (Fig. 5B).

Plumbagin Downregulates the Expression of Cyclin D1, Bcl-xL, and Vascular Endothelial Growth Factor

STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, and angiogenesis. We investigated whether the expressions of the antiapoptotic proteins Bcl-xL, cell cycle regulator protein, cyclin D1, and vascular endothelial growth factor (VEGF), which are all reported to be regulated by STAT3 (24, 25, 35), were modulated by plumbagin. Plumbagin treatment downregulated the expression of these proteins in a time-dependent manner, with maximum suppression observed at 36 hours followed by 48 hours of treatment (Fig. 5C).

Plumbagin Activates Caspase-3 and Causes Poly ADP ribose Polymerase Cleavage

We examined whether the suppression of constitutively active STAT3 in U266 cells by plumbagin leads to poly ADP ribose polymerase (PARP) cleavage. Treatment of U266 cells with plumbagin induced a caspase3-dependent cleavage of a 118-kDa PARP protein into an 87-kDa fragment (Fig. 5D).

Overexpression of Constitutively Active STAT3 Rescues Plumbagin-Induced Apoptosis

We assessed whether the overexpression of constitutive active STAT3 can rescue plumbagin-induced apoptosis. We used A293 cells to furnish this experiment because these cells are easily transfectable compared with U266 and do not express STAT3 constitutively. A293 cells were

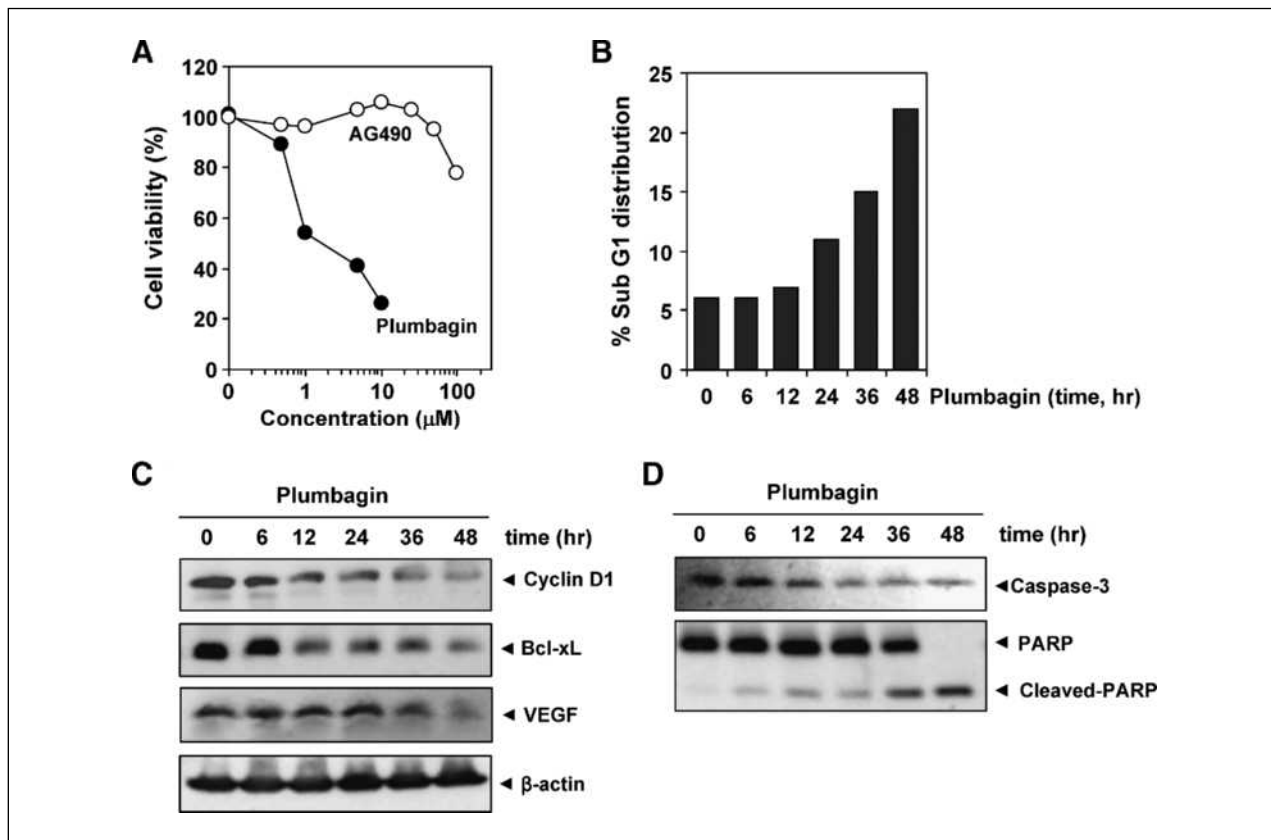


FIGURE 5. A. Effects of AG490 and plumbagin on the proliferation of cells. U266 cells were plated in triplicate, treated with the indicated concentrations of plumbagin and AG490 for 24 h, and then subjected to the MTT assay. **B.** Plumbagin causes accumulation of cells in sub-G₁ phase. U266 cells (2×10^6) were synchronized by incubation overnight in the absence of serum and then treated with 2.5 μmol/L plumbagin for the indicated times, after which the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. **C.** Plumbagin downregulates the expression of cell proliferative, antiapoptotic, and angiogenic proteins. U266 cells (2×10^6) were treated with 2.5 μmol/L plumbagin for the indicated time, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE and probed against Bcl-xL, cyclin D1, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **D.** Plumbagin induces caspase-3-dependent PARP cleavage. U266 cells were treated with 2.5 μmol/L plumbagin for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against caspase-3 and PARP antibodies. The results shown are representative of two independent experiments.

transfected with constitutively active STAT3 plasmid for 24 hours, and cells were incubated with plumbagin for the next 24 hours and examined for apoptosis by esterase staining assay. The results show that the forced expression of STAT3 significantly reduces the plumbagin-induced apoptosis (Fig. 6A).

Plumbagin Potentiates the Apoptotic Effect of Bortezomib and Thalidomide in MM Cells

Bortezomib, an inhibitor of proteasome, and thalidomide, an inhibitor of TNF expression, have been approved for the treatment of MM patients (36). We investigated whether plumbagin can potentiate the effect of these drugs. For this, U266 cells were treated with plumbagin together with either thalidomide or bortezomib. The cells were then examined for apoptosis by using the Live-Dead assay, which determines plasma membrane stability using esterase staining. As shown in Fig. 6B, plumbagin significantly enhanced the apoptotic effects of thalidomide from 12% to 36% and of bortezomib from 12% to 40%.

Discussion

The goal of this study was to determine whether plumbagin exerts its anticancer effects through the modulation of the STAT3 signaling pathway in MM cells. We found that this quinone suppressed both constitutive and IL-6-inducible STAT3 activation in parallel with the inhibition of c-Src and JAK1 and JAK2 activation. Plumbagin stimulated the expression of the nontransmembrane PTPase SHP-1. Plumbagin also downregulated the expression of STAT3-regulated gene products, including Bcl-xL, cyclin D1, and VEGF. It induced inhibition of proliferation, induced apoptosis, and significantly potentiated the apoptotic effects of bortezomib and thalidomide in MM cells.

We found that plumbagin could suppress both constitutive and inducible STAT3 activation in MM cells. Exposure of cells to 5 μmol/L plumbagin for 4 hours was needed to fully abolish STAT3 activation. In comparison, exposure of cells to 100 μmol/L AG490 (a rationally designed inhibitor of JAK2) for 8 hours was required

to suppress STAT3 activation (37). Because we used an antibody that recognizes the phosphorylation of serine and tyrosine located at position 727 and 705, respectively, in the STAT3 protein, plumbagin seems to inhibit this phosphorylation. Constitutive activation of STAT3 has been

ported in a large variety of tumors, including breast cancer, prostate cancer, head and neck squamous cell carcinoma, lymphomas and leukemia, brain tumor, colon cancer, Ewing's sarcoma, gastric cancer, esophageal cancer, ovarian cancer, nasopharyngeal cancer, and pancreatic cancer (24).

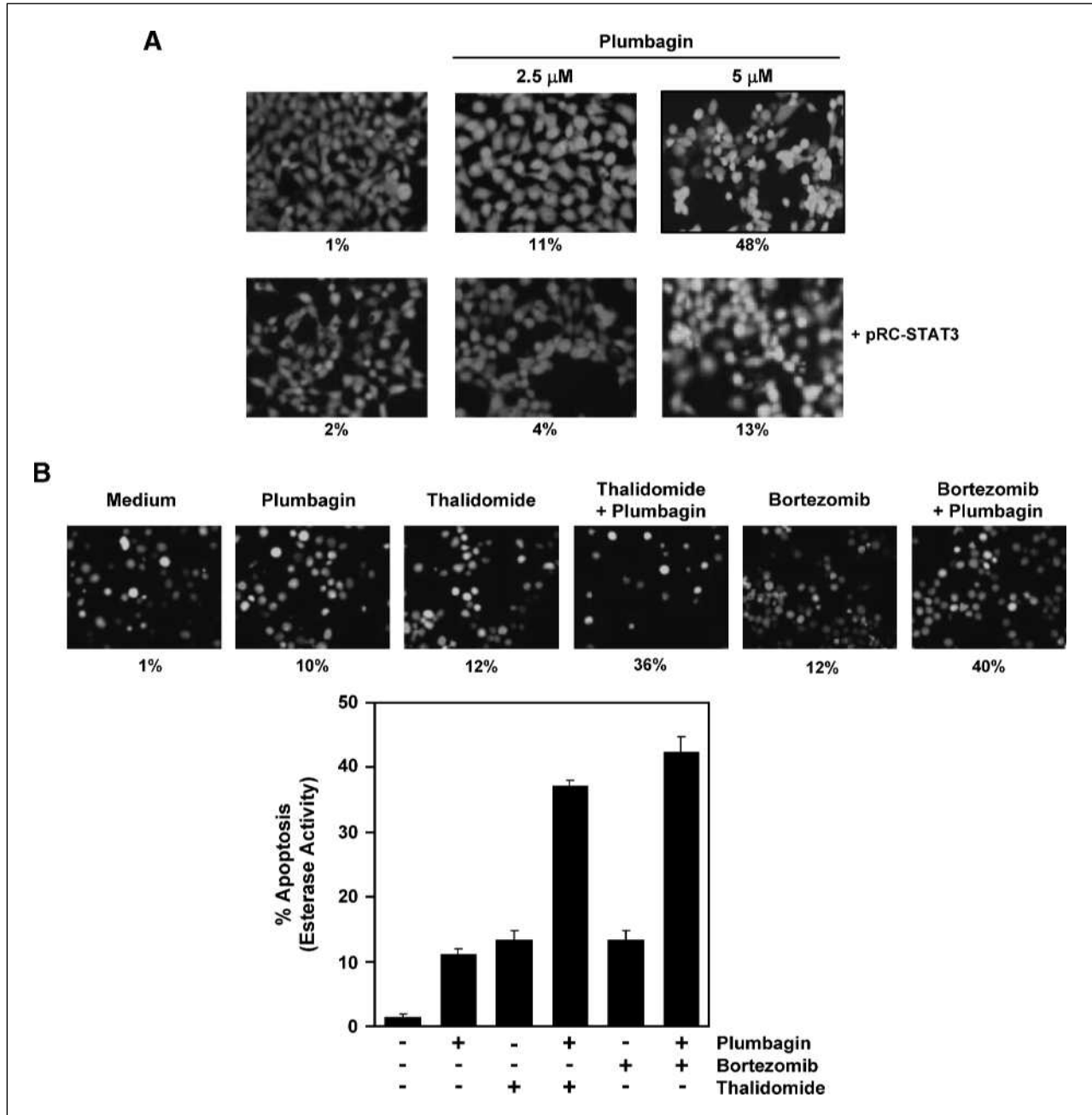


FIGURE 6. A. Overexpression of constitutive STAT3 rescues A293 cells from plumbagin-induced cytotoxicity. First, A293 cells were transfected with constitutive STAT3 plasmid. After 24 h of transfection, the cells were treated with 2.5 μ mol/L plumbagin for 24 h, the cytotoxicity was determined by Live/Dead assay, and 20 random fields were counted. **B.** Plumbagin potentiates the apoptotic effect of thalidomide and bortezomib. U266 cells (1×10^6) were treated with 2.5 μ mol/L plumbagin, and 10 μ g/mL thalidomide or 20 nmol/L bortezomib alone or in combination for 24 h at 37°C. Cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. The results shown are representative of two independent experiments.

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Hence, the suppression of constitutively active STAT3 in MM cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of cancer cells. It is possible that the antiproliferative effects of plumbagin reported previously against breast cancer, lung cancer, ovarian cancer, acute promyelocytic leukemia, melanoma, and prostate cancer cells (4-11) could be due to the downregulation of the STAT3 pathway. Furthermore, we observed that overexpression of STAT3 also rescues the apoptotic effects of plumbagin, strengthening our hypothesis that the antiproliferative effects of plumbagin are mediated through the abrogation of the STAT3 signaling pathway.

We also observed that plumbagin suppressed the nuclear translocation and DNA-binding activity of STAT3. However, the dose of plumbagin required to suppress DNA binding is higher than the dose required in suppressing phosphorylation. We speculate that phosphorylation of STAT3 is an early event and nuclear translocation is a late event. These results suggested that plumbagin may be affecting the early event at 5 $\mu\text{mol/L}$ but not the late event. It has been recently recognized that nonphosphorylated forms of STATs also shuttle between nucleus and cytoplasm at all times in a constitutive manner (38), suggesting that the inhibition of phosphorylation of STAT3 is not sufficient to inhibit its nuclear translocation. STAT3 phosphorylation plays a critical role in the transformation and proliferation of tumor cells (35). All Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation (27). The effects of plumbagin on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases JAK1, JAK2, and c-Src. Dominant-negative STAT3 has also been shown to induce apoptosis in cells with constitutively active STAT3 (39). Our results agree with a recent report that plumbagin can inhibit phosphorylation of JAK2 and STAT3 in prostate cancer cells (10), and suggest the potential contribution of JAK1 and c-Src in downregulation of STAT3 activation by plumbagin.

We also found evidence that the plumbagin-induced inhibition of STAT3 activation involves a PTPase. Numerous PTPases have been implicated in STAT3 signaling, including SHP-1, SHP-2, TC-PTP, PTEN, PTP-1D, CD45, and PTP- ϵ (40-42). The type of PTPase involved in the downregulation of STAT3 phosphorylation is not clear. We found that plumbagin induced both the protein and mRNA for SHP-1 (data not shown). Loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma (31).

Previously, we have shown that plumbagin can also suppress NF- κB activation (14). Whether the suppression of STAT3 activation by plumbagin is linked to the inhibition of NF- κB activation is not clear. The p65 subunit of NF- κB has been shown to interact with STAT3 (43). STAT3 and NF- κB , however, are activated in response to different cytokines: whereas IL-6 is a major activator of STAT3, TNF is a potent activator of NF- κB . Interestingly, erythropoietin has been shown to activate both NF- κB through

the activation of JAK2 kinase (44). Thus, it is possible that the suppression of JAK activation is the critical target for the inhibition of both NF- κB and STAT3 activation by plumbagin.

We also found that plumbagin suppressed the expression of STAT3-regulated genes, including cyclin D1, and the antiapoptotic gene products. Constitutively active STAT3 can contribute to oncogenesis by protecting cancer cells from apoptosis; this implies that suppression of STAT3 activation by agents such as plumbagin could facilitate apoptosis. Expression of Bcl-xL is regulated by STAT3 (45) and is overexpressed in MM cells (46). Bcl-xL can also block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance (47). The downregulation of the expression of Bcl-xL is likely linked with the ability of plumbagin to induce apoptosis in MM cells. VEGF is a critical growth factor for angiogenesis of the tumor and is also regulated by STAT3. Plumbagin also downregulated the expression of this growth factor.

Recently, a proteasome inhibitor (bortezomib) and a TNF inhibitor (thalidomide) were approved for the treatment of MM (36, 48). We found that plumbagin potentiates the apoptotic effect of bortezomib and thalidomide in MM cells. We argue that the pharmacologic safety of plumbagin and its ability to downregulate the expression of several genes involved in cell survival and chemoresistance provide sufficient rationale for further testing in patients with MM.

In addition to plumbagin, we have shown previously that juglone and 1,4-naphthoquinone but not menadione (vitamin K3) can modulate NF- κB activation (14). Whether these analogues can also modulate STAT3 activation is not clear. Unlike plumbagin, shikonin (49), and compound 5 (50), which are analogues of vitamin K, are known to inhibit protein tyrosine phosphates. Thus, it is possible that not all analogues of vitamin K exhibit similar effects on STAT3 activation.

The downregulation of epidermal growth factor receptor signaling, the inhibition of expression of survivin (6), the suppression of angiogenesis (21), and the radiosensitization of tumors (22) attributed to plumbagin could be due to the modulation of the STAT3 pathway as described here. Various animal studies have indicated that plumbagin is well tolerated (5, 17-21). In addition, doses of plumbagin used in the present study are achievable *in vivo* as supported by the studies of Hsieh et al. (51) that maximal plasma concentration of plumbagin in rat following treatment with 3 mg/kg i.v. or 100 mg/kg orally was shown to be about 1.0 and 1.85 $\mu\text{mol/L}$ with corresponding half-life of about 108 and 1,028 minutes, respectively.

MM that has relapsed after conventional dose therapy or stem cell transplantation is typically treated with high-dose corticosteroids, thalidomide, or bortezomib. However, significant numbers of patients does not respond to these agents. Moreover, prolonged exposure leads to the development of resistance and toxicity, and progression-free and overall survival times are short. Collectively, the lack of toxicity of plumbagin and its ability to suppress STAT3

activation inhibit IL-6-induced STAT3 and JAK1 phosphorylation; downregulate the expression of cyclin D1, Bcl-xL, and VEGF; inhibit cell proliferation; induce apoptosis; and potentiate the effect of bortezomib and thalidomide indicate the need for further preclinical studies preceding human trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Tilak JC, Adhikari S, Devasagayam TP. Antioxidant properties of *Plumbago zeylanica*, an Indian medicinal plant and its active ingredient, plumbagin. *Redox Rep* 2004;9:219–27.
- Binder RG, Benson ME, Flath RA. Eight 1,4-Naphthoquinones from *Juglone*. *Phytochemistry* 1989;28:2799–801.
- Hedin PA, Collum DH, Langhans VE, Graves CH. Distribution of *Juglone* and related compounds in pecan and their effects on *Fusicladium effusum*. *J Agric Food Chem* 1980;28:340–2.
- Kuo PL, Hsu YL, Cho CY. Plumbagin induces G₂-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. *Mol Cancer Ther* 2006;5:3209–21.
- Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH₂-terminal kinase-mediated phosphorylation at serine 15 *in vitro* and *in vivo*. *J Pharmacol Exp Ther* 2006;318:484–94.
- Gomathinayagam R, Sowmyalakshmi S, Mardhatillah F, Kumar R, Akbarsha MA, Damodaran C. Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells. *Anticancer Res* 2008;28:785–92.
- Thasni KA, Rakesh S, Rojini G, Ratheeshkumar T, Srinivas G, Priya S. Estrogen-dependent cell signaling and apoptosis in BRCA1-blocked BG1 ovarian cancer cells in response to plumbagin and other chemotherapeutic agents. *Ann Oncol* 2008;19:696–705.
- Zhao YL, Lu DP. Effects of plumbagin on the human acute promyelocytic leukemia cells *in vitro*. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2006;14:208–11.
- Wang CC, Chiang YM, Sung SC, Hsu YL, Chang JK, Kuo PL. Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells. *Cancer Lett* 2008;259:82–98.
- Aziz MH, Dreckschmidt NE, Verma AK. Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone-refractory prostate cancer. *Cancer Res* 2008;68:9024–32.
- Powolny AA, Singh SV. Plumbagin-induced apoptosis in human prostate cancer cells is associated with modulation of cellular redox status and generation of reactive oxygen species. *Pharm Res* 2008; 25:2171–80.
- Jaiswal AS, Bloom LB, Narayan S. Long-patch base excision repair of apurinic/aprimidinic site DNA is decreased in mouse embryonic fibroblast cell lines treated with plumbagin: involvement of cyclin-dependent kinase inhibitor p21Waf-1/Cip-1. *Oncogene* 2002;21: 5912–22.
- Kawiak A, Piosik J, Stasilojc G, et al. Induction of apoptosis by plumbagin through reactive oxygen species-mediated inhibition of topoisomerase II. *Toxicol Appl Pharmacol* 2007;223:267–76.
- Sandur SK, Ichikawa H, Sethi G, Ahn KS, Aggarwal BB. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF- κ B activation and NF- κ B-regulated gene products through modulation of p65 and I κ B α kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J Biol Chem* 2006;281:17023–33.
- Rossary A, Arab K, Steghens JP. Polyunsaturated fatty acids modulate NOX 4 anion superoxide production in human fibroblasts. *Biochem J* 2007;406:77–83.
- Shukla S, Wu CP, Nandigama K, Ambudkar SV. The naphthoquinones, vitamin K3 and its structural analogue plumbagin, are substrates of the multidrug resistance linked ATP binding cassette drug transporter ABCG2. *Mol Cancer Ther* 2007;6:3279–86.
- Singh UV, Udupa N. Reduced toxicity and enhanced antitumor efficacy of β -cyclodextrin plumbagin inclusion complex in mice bearing Ehrlich ascites carcinoma. *Indian J Physiol Pharmacol* 1997;41: 171–5.
- Tiwari SB, Pai RM, Udupa N. Temperature sensitive liposomes of plumbagin: characterization and *in vivo* evaluation in mice bearing melanoma B16F1. *J Drug Target* 2002;10:585–91.
- Devi PU, Rao BS, Solomon FE. Effect of plumbagin on the radiation induced cytogenetic and cell cycle changes in mouse Ehrlich ascites carcinoma *in vivo*. *Indian J Exp Biol* 1998;36:891–5.
- Abdul KM, Ramchender RP. Modulatory effect of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) on macrophage functions in BALB/c mice. I. Potentiation of macrophage bactericidal activity. *Immunopharmacology* 1995;30:231–6.
- Jackson JK, Higo T, Hunter WL, Burt HM. Topoisomerase inhibitors as anti-arthritis agents. *Inflamm Res* 2008;57:126–34.
- Nair S, Nair RR, Srinivas P, Srinivas G, Pillai MR. Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. *Mol Carcinog* 2008;47:22–33.
- Al Zaid Siddiquee K, Turkson J. STAT3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res* 2008;18:254–67.
- Aggarwal BB, Sethi G, Ahn KS, et al. Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. *Ann N Y Acad Sci* 2006; 1091:151–69.
- Aggarwal BB, Kunnumakkara AB, Harikumar KB, et al. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci* 2009;1171:59–76.
- Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 1995;269:81–3.
- Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. *J Interferon Cytokine Res* 2005;25:733–44.
- Kawano M, Hirano T, Matsuda T, et al. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature* 1988; 332:83–5.

29. Schreiner SJ, Schiavone AP, Smithgall TE. Activation of STAT3 by the Src family kinase Hck requires a functional SH3 domain. *J Biol Chem* 2002;277:45680–7.
30. Ihle JN. STATs: signal transducers and activators of transcription. *Cell* 1996;84:331–4.
31. Han Y, Amin HM, Franko B, Frantz C, Shi X, Lai R. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteasome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. *Blood* 2006;108:2796–803.
32. Wu C, Sun M, Liu L, Zhou GW. The function of the protein tyrosine phosphatase SHP-1 in cancer. *Gene* 2003;306:1–12.
33. Oka T, Ouchida M, Koyama M, et al. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Res* 2002;62:6390–4.
34. Matsushime H, Rousset MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 1991;65:701–13.
35. Yu H, Jove R. The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer* 2004;4:97–105.
36. Cavo M. Proteasome inhibitor bortezomib for the treatment of multiple myeloma. *Leukemia* 2006;20:1341–52.
37. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. *J Immunol* 2003;171:3863–71.
38. Sehgal PB. Paradigm shifts in the cell biology of STAT signaling. *Semin Cell Dev Biol* 2008;19:329–40.
39. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 1999;10:105–15.
40. Tenev T, Bohmer SA, Kaufmann R, et al. Perinuclear localization of the protein-tyrosine phosphatase SHP-1 and inhibition of epidermal growth factor-stimulated STAT1/3 activation in A431 cells. *Eur J Cell Biol* 2000;79:261–71.
41. Woetmann A, Nielsen M, Christensen ST, et al. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. *Proc Natl Acad Sci U S A* 1999;96:10620–5.
42. Kim H, Baumann H. Dual signaling role of the protein tyrosine phosphatase SHP-2 in regulating expression of acute-phase plasma proteins by interleukin-6 cytokine receptors in hepatic cells. *Mol Cell Biol* 1999;19:5326–38.
43. Yu Z, Zhang W, Kone BC. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor κ B. *Biochem J* 2002;367:97–105.
44. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF- κ B signalling cascades. *Nature* 2001;412:641–7.
45. Niu G, Heller R, Catlett-Falcone R, et al. Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor *in vivo*. *Cancer Res* 1999;59:5059–63.
46. Tu Y, Renner S, Xu F, et al. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. *Cancer Res* 1998;58:256–62.
47. Simonian PL, Grillot DA, Nunez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood* 1997;90:1208–16.
48. Glasmacher A, Hahn C, Hoffmann F, et al. A systematic review of phase-II trials of thalidomide monotherapy in patients with relapsed or refractory multiple myeloma. *Br J Haematol* 2006;132:584–93.
49. Nigorikawa K, Yoshikawa K, Sasaki T, et al. A naphthoquinone derivative, shikonin, has insulin-like actions by inhibiting both phosphatase and tensin homolog deleted on chromosome 10 and tyrosine phosphatases. *Mol Pharmacol* 2006;70:1143–9.
50. Markovits J, Wang Z, Carr BI, et al. Differential effects of two growth inhibitory K vitamin analogs on cell cycle regulating proteins in human hepatoma cells. *Life Sci* 2003;72:2769–84.
51. Hsieh YJ, Lin LC, Tsai TH. Measurement and pharmacokinetic study of plumbagin in a conscious freely moving rat using liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006;844:1–5.