Ursolic Acid Inhibits STAT3 Activation Pathway Leading to Suppression of Proliferation and Chemosensitization of Human Multiple Myeloma Cells

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Abstract

The activation of signal transducers and activators of transcription 3 (STAT3) has been linked with the proliferation of a variety of human cancer cells, including multiple myeloma. Agents that can suppress STAT3 activation have potential for prevention and treatment of cancer. In the present report, we tested an agent, ursolic acid, found in basil, apples, prunes, and cranberries, for its ability to suppress STAT3 activation. We found that ursolic acid, a pentacyclic triterpenoid, inhibited both constitutive and interleukin-6–inducible STAT3 activation in a dose- and time-dependent manner in multiple myeloma cells. The suppression was mediated through the inhibition of activation of upstream kinases c-Src, Janus-activated kinase 1, Janus-activated kinase 2, and extracellular signal–regulated kinase 1/2. Vanadate treatment reversed the ursolic acid–induced down-regulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Indeed, we found that ursolic acid induced the expression of tyrosine phosphatase SHP-1 protein and mRNA. Moreover, knockdown of SHP-1 by small interfering RNA suppressed the induction of SHP-1 and reversed the inhibition of STAT3 activation, thereby indicating the critical role of SHP-1 in the action of this triterpene. Ursolic acid down-regulated the expression of STAT3-regulated gene products such as cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and vascular endothelial growth factor. Finally, ursolic acid inhibited proliferation and induced apoptosis and the accumulation of cells in G_1-G_0 phase of cell cycle. This triterpenoid also significantly potentiated the apoptotic effects of thalidomide and bortezomib in multiple myeloma cells. Overall, these results suggest that ursolic acid is a novel blocker of STAT3 activation that may have a potential in prevention and treatment of multiple myeloma and other cancers. (Mol Cancer Res 2007;5(9):943–55)

Introduction

Signal transducer and activator of transcription (STAT) proteins have been shown to play an important role in tumor cell survival and proliferation (1). One STAT family member, STAT3, is often constitutively active in many human cancer cells including multiple myeloma, leukemia, lymphoma, and solid tumors (2, 3). STAT3 can also be activated by certain interleukins [e.g., interleukin-6 (IL-6)] and growth factors (e.g., epidermal growth factor). On activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription. The phosphorylation is mediated through the activation of nonreceptor protein tyrosine kinases called Janus-activated kinases (JAK). JAK1, JAK2, JAK3, and TYK2 have been implicated in the activation of STAT3 (4, 5). In addition, the role of c-Src kinase has been shown in STAT3 phosphorylation (6). Besides tyrosine phosphorylation, there is evidence that STAT3 can undergo serine phosphorylation as well, which is mediated through the activation of kinases of the mitogen-activated protein kinase family (7).

The major phosphorylation sites in STAT3 include tyrosine and serine residues at positions 705 and 727, respectively, located in the transactivation domain. The activation of STAT3 results in expression of numerous gene products required for tumor cell survival (e.g., survivin, Bcl-xL, Bcl-2, and Mcl-1), proliferation (e.g., cyclin D1), and angiogenesis [e.g., vascular endothelial growth factor (VEGF); ref. 8]. Thus, agents that suppress STAT3 activation have a potential in prevention and therapy of cancer (9, 10).

One potential source of STAT3 inhibitors is natural dietary components. Numerous animal studies and epidemiologic studies in humans suggest that fruits and vegetables can prevent cancer (11). We describe here the identification of a compound derived from rosemary (*Rosemarinus officinalis*), apples (*Malus domestica*), cranberries (*Vaccinium macrocarpon*), makino (*Perilla frutescens*), pears (*Pyrus pyrifolia*), prunes (*Prunus domestica*), bearberries (*Arctostaphylos*

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alpina), loquat (*Eriobotrya japonica)*, scotch heather (*Calluna vulgaris*), basil (*Ocimum sanctum*), and jamun (*Eugenia jumbolana*; ref. 12), called ursolic acid, which has a potential in prevention of cancer. Ursolic acid is a pentacyclic triterpenoid that has been shown to suppress the growth of various hematopoietic tumors (13, 14), inhibit tumor promotion (15, 16), and inhibit tumor angiogenesis (17). We have recently shown that ursolic acid is a potent inhibitor of nuclear factor-κB (NF-κB) activation pathway (18), which is activated by inflammatory agents, carcinogens, and tumor promoters.

Because of the critical role of STAT3 in tumor cell survival, proliferation, and angiogenesis, we hypothesized that ursolic acid mediates its effects, in part, through suppression of the STAT3 pathway. We tested this hypothesis in multiple myeloma cells. In our experiments, ursolic acid indeed suppressed both constitutive and inducible STAT3 activation. This inhibition decreased cell survival and down-regulated expression of proliferative and angiogenic gene products, leading to suppression of proliferation, induction of apoptosis, and enhancement of the response to the cytotoxic effects of thalidomide (an inhibitor of tumor necrosis factor expression) and bortezomib (a proteasome inhibitor; also called PS341) in multiple myeloma cells.

Results

We have previously shown that ursolic acid is a potent inhibitor of activation of NF-κB (18), a transcription factor that plays a critical role in the survival and proliferation of tumor cells. The present study was undertaken to determine the effect of ursolic acid on STAT3 signaling pathway. We investigated the effect of ursolic acid on both constitutive and IL-6–inducible STAT3 activation in multiple myeloma cells. We also evaluated the effect of ursolic acid on various mediators of cellular proliferation, cell survival, and apoptosis. The structure of ursolic acid is shown in Fig. 1A. The dose and duration of ursolic acid used for STAT3 experiments had no effect on cell viability (data not shown).

Ursolic Acid Inhibits STAT3 DNA Binding Activity in Multiple Myeloma Cells

Because tyrosine phosphorylation causes dimerization of STATs and their translocation to the nucleus, where they bind to DNA and regulate gene transcription (19), we determined whether ursolic acid suppresses DNA binding activity of STAT3. Electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts prepared from U266 cells showed that ursolic acid caused a decrease in STAT3 DNA-binding activity in a dose- (Fig. 1B) and time-dependent manner (Fig. 1C). Supershift analysis indicated that the binding of STAT3 to the DNA was blocked by cold competitor oligonucleotide and anti–STAT-3 antibody, thus confirming that the protein/DNA complex observed actually contained STAT3. No constitutive activation of STAT3 could be detected in human myeloid KBM-5 cells (Fig. 1D). These results show that ursolic acid abrogates DNA binding ability of STAT3.

Ursolic Acid Inhibits Constitutive STAT3 Phosphorylation in Multiple Myeloma Cells

Whether ursolic acid can modulate the constitutive STAT3 activation in multiple myeloma cells was investigated. U266 cells were incubated with different concentrations of ursolic acid for 4 h and whole-cell extracts were prepared and examined for phosphorylated STAT3 by Western blot analysis with antibody that recognizes STAT3 phosphorylated at Tyr^{705}. As shown in Fig. 1E, ursolic acid inhibited the constitutive activation of STAT3 in U266 cells in a dose-dependent manner, with maximum inhibition occurring at 50 to 100 μmol/L. Ursolic acid had no effect on the expression of STAT3 protein (Fig. 1E, *bottom*). AG490 is a well-characterized inhibitor of JAK2-STAT3 phosphorylation (20). Similar to ursolic acid, exposure of cells to 50 μmol/L of AG490 was needed to inhibit STAT3 phosphorylation (Fig. 1F).

We also determined the incubation time with ursolic acid required for suppression of STAT3 activation in U266 cells. As shown in Fig. 1G, the inhibition was time dependent, with maximum inhibition occurring at ~4 h, again with no effect on the expression of STAT3 protein (Fig. 1G, *bottom*). In comparison, 8 h was required to suppress STAT3 activation by AG490 (data not shown).

Effect of Ursolic Acid on STAT3 Phosphorylation Is Specific

Whether ursolic acid affects the activation of other STAT3 proteins in U266 cells was also investigated. Under the conditions where ursolic acid completely inhibited STAT3 phosphorylation, it altered neither the levels of constitutively phosphorylated STAT5 nor the expression of STAT5 proteins (Fig. 1H).

Ursolic Acid Depletes Nuclear Pool of STAT3 in Multiple Myeloma 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 (21, 22), we determined whether ursolic acid suppresses nuclear translocation of STAT3. Figure 1I clearly shows that ursolic acid inhibited the translocation of STAT3 to the nucleus in U266 cells.

Ursolic Acid Inhibits Inducible STAT3 Phosphorylation in Human Multiple Myeloma Cells

Because IL-6 is a growth factor for multiple myeloma and induces STAT3 phosphorylation (23, 24), we determined whether ursolic acid could inhibit IL-6–induced STAT3 phosphorylation. MM1.S cells, which lack constitutively active STAT3, were treated with IL-6 for different time points and then examined for phosphorylated STAT3. IL-6 induced phosphorylation of STAT3 as early as 5 min, but phosphorylation began to decline at 30 min (Fig. 2A). In MM1.S cells incubated with ursolic acid for different time points, IL-6–induced STAT3 phosphorylation was suppressed by ursolic acid in a time-dependent manner. Exposure of cells to ursolic acid for 8 h was sufficient to completely suppress IL-6–induced STAT3 phosphorylation (Fig. 2B).

Ursolic Acid Inhibits Inducible JAK2 Phosphorylation in Human Multiple Myeloma Cells

In view of the prominent role of IL-6 in multiple myeloma and because JAKs are a key component of gp130-mediated signaling (25-27), we next determined whether IL-6 induces

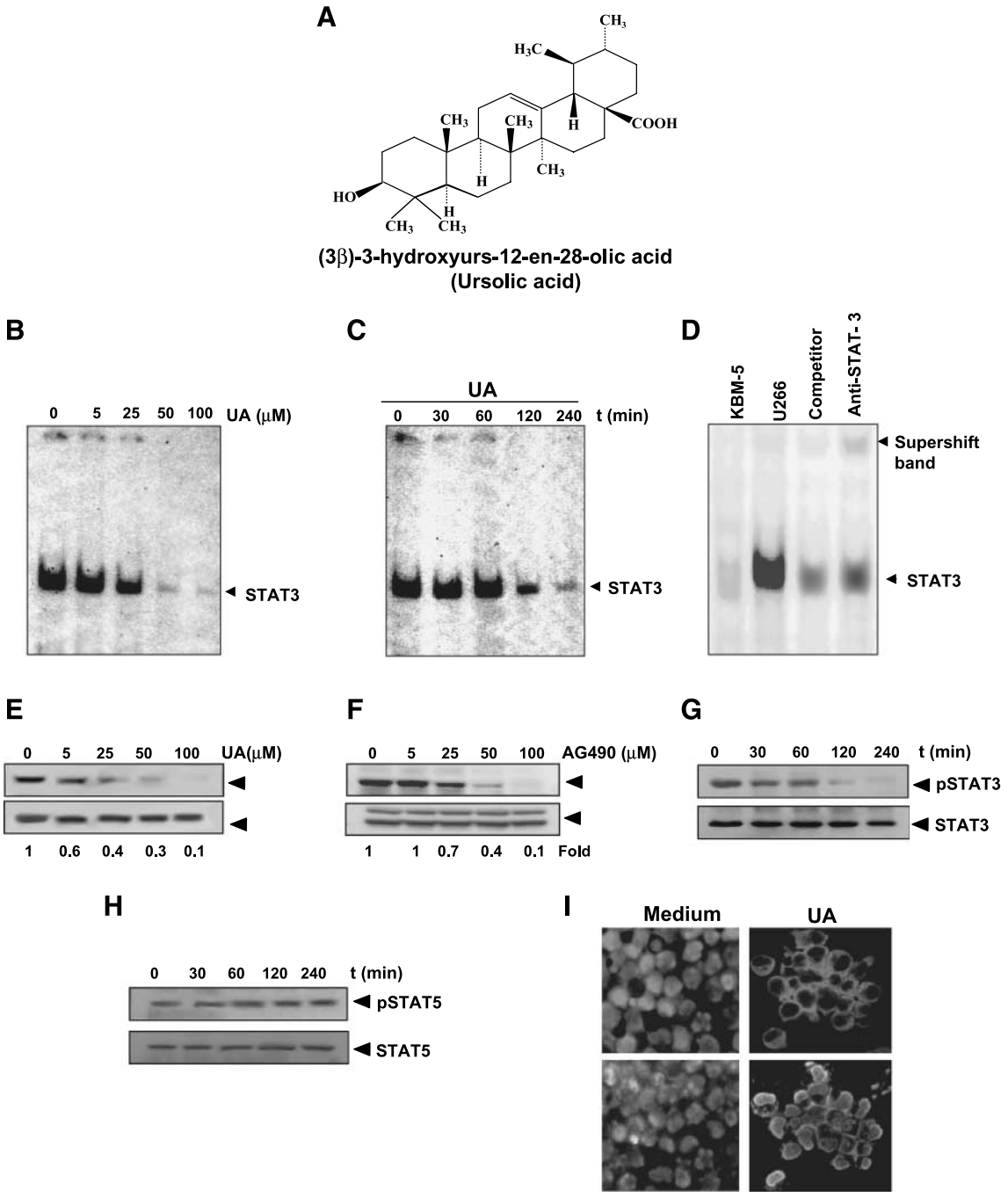

FIGURE 1. Ursolic acid inhibits constitutively active STAT3 in U266 cells. **A.** The structure of the pentacyclic triterpenoid ursolic acid. **B.** U266 cells (2×10^6/mL) were treated with the indicated concentrations of ursolic acid for 4 h and analyzed for nuclear STAT3 levels by EMSA. **C.** U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated durations and analyzed for nuclear STAT3 levels by EMSA. **D.** Nuclear extracts from U266 cells were incubated with STAT3 antibody and an unlabeled STAT3 oligo probe. Nuclear extracts from untreated myeloid leukemia (KBM-5) cells were taken alone. They were then assayed for STAT3 DNA binding by EMSA. **E.** Ursolic acid suppresses phospho-STAT3 levels in a dose-dependent manner. U266 cells (2×10^6/mL) were treated with the indicated concentrations of ursolic acid for 4 h, after which whole-cell extracts were prepared and 30 μg of protein were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. **F.** Effect of AG490 on STAT3 phosphorylation in U266 cells. U266 cells (2×10^6) were treated with indicated concentrations of AG490 for 8 h and whole-cell extracts were prepared. Thirty micrograms of whole-cell extract were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for phospho-STAT3 (*top*), stripped, and reprobed for STAT3 (*bottom*). **G.** Ursolic acid suppresses phospho-STAT3 levels in a time-dependent manner. U266 cells (2×10^6/mL) were treated with the 50 μmol/L ursolic acid for the indicated time points, after which Western blotting was done as described for (**E**). The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. **H.** Ursolic acid had no effect on phospho-STAT5 and STAT5 protein expression. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time points. Whole-cell extracts were prepared, fractionated on SDS-PAGE, and examined by Western blotting with antibodies against phospho-STAT5 and STAT5. **I.** Ursolic acid causes inhibition of translocation of STAT3 to the nucleus. U266 cells (1×10^5/mL) were incubated with or without 50 μmol/L ursolic acid for 4 h and then analyzed for the intracelullar distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with Hoechst (50 ng/mL) for 5 min.

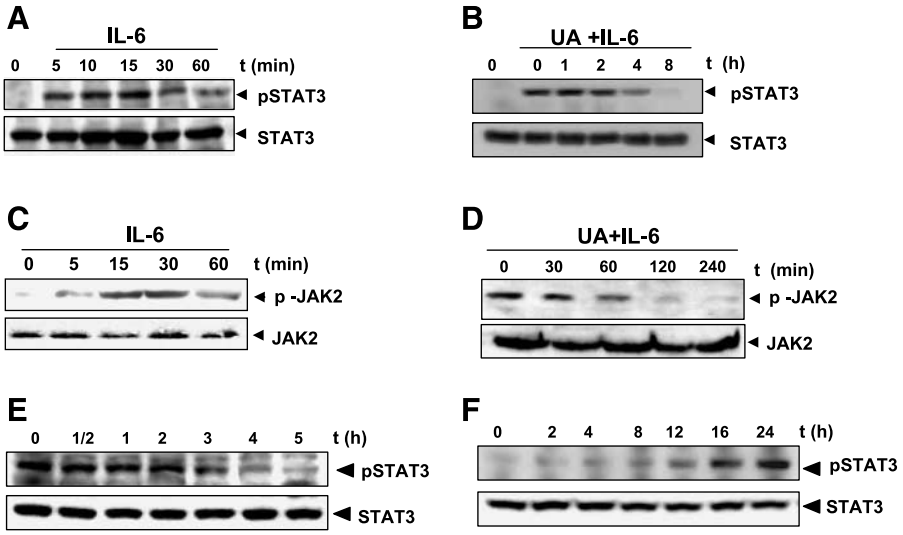

FIGURE 2. Ursolic acid down-regulates IL-6–induced phospho-STAT3. **A.** MM1.S cells (2×10^6/mL) were treated with IL-6 (10 ng/mL) for the indicated time points, whole-cell extracts were prepared, and phospho-STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. **B.** MM1.S cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time points and then stimulated with IL-6 (10 ng/mL) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blotting. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. Representative of three independent experiments. **C.** MM1.S cells (4×10^6/mL) were treated with IL-6 (10 ng/mL) for the indicated time points. Whole-cell extracts were prepared and 500 μg of sample were incubated with JAK2 antibody overnight. Immunocomplex was precipitated with protein A/G-agarose beads and then fractionated on 10% SDS-PAGE. Western blot analysis was done with anti–phospho-JAK2 antibody. **D.** MM1.S cells (4×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time points and then stimulated with IL-6 (10 ng/mL) for 15 min. Whole-cell extracts were prepared and 500 μg of sample were incubated with JAK2 antibody overnight. Immunocomplex was precipitated with protein A/G-agarose beads and then fractionated on 10% SDS-PAGE. Western blot analysis was done with anti–phospho-JAK2 antibody. The same samples were analyzed for JAK2 protein. Ursolic acid–induced inhibition of STAT phosphorylation is reversible. U266 cells (2×10^6) were treated with 50 μmol/L ursolic acid for the indicated durations (**E**) or treated for 1 h and washed with PBS twice to remove ursolic acid before resuspension in fresh medium. Cells were removed at indicated time points and lysed to prepare the whole-cell extract (**F**). Thirty micrograms of whole-cell extracts were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for the phosphorylated-STAT3, and stripped and reprobed with STAT3 antibodies. Representative of three independent experiments.

JAK2 phosphorylation in multiple myeloma cells and whether ursolic acid modulates this activation. For this, IL-6–treated whole-cell lysates were immunoprecipitated with JAK2 antibodies followed by Western blot with the anti–phospho-JAK2 antibody. As shown in Fig. 2C, IL-6 induced the phosphorylation of JAK2 in a time-dependent manner with maximum activation occurring at 15 to 30 min. Pretreatment with ursolic acid suppressed IL-6–induced phosphorylation of JAK2 in a time-dependent manner with maximum suppression observed at 120 to 240 min after treatment (Fig. 2D).

Ursolic Acid–Induced Inhibition of STAT3 Phosphorylation Is Reversible in Human Multiple Myeloma Cells

We further examined whether ursolic acid–induced inhibition of STAT3 phosphorylation is reversible. U266 cells were first treated for 60 min with ursolic acid, and then the cells were washed twice with PBS to remove ursolic acid. The cells were then cultured in fresh medium for various durations, and the levels of phosphorylated STAT3 were measured. Ursolic acid induced the suppression of STAT3 phosphorylation (Fig. 2E), and the removal of ursolic acid resulted in a gradual increase in phosphorylated STAT3 (Fig. 2F). The reversal was complete by 24 h and did not involve changes in STAT3 protein levels (Fig. 2F, *bottom*).

Ursolic Acid Suppresses Constitutive Activation of c-Src

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (6). Hence, we determined the effect of ursolic acid on constitutive activation of Src kinase in U266 cells. We found that ursolic acid suppressed the constitutive phosphorylation of c-Src kinase (Fig. 3A). The levels of nonphosphorylated c-Src kinase remained unchanged under the same conditions.

Ursolic Acid Suppresses Constitutive Activation of JAK1 and JAK2

STAT3 has been reported to be activated by soluble tyrosine kinases of the JAK family (4); thus, we determined whether ursolic acid affects constitutive activation of JAK1 in U266 cells. We found that ursolic acid suppressed the constitutive phosphorylation of JAK1 (Fig. 3B). The levels of nonphosphorylated JAK1 remained unchanged under the same conditions (Fig. 3B, *bottom*). To determine the effect of ursolic acid on JAK2 activation, untreated and ursolic acid–treated whole-cell lysates were immunoprecipitated with anti-JAK2 antibodies followed by Western blot with the anti–phospho-JAK2 antibody. As shown in Fig. 3C, JAK2 was constitutively active in U266 cells and pretreatment with ursolic acid suppressed this phosphorylation in a time-dependent manner.

Ursolic Acid Inhibits Extracellular Signal–Regulated Kinase

Apart from tyrosine phosphorylation, STAT3 also undergoes phosphorylation at serine residues (4). IL-6 can activate the Ras/mitogen-activated protein kinase pathway for STAT3

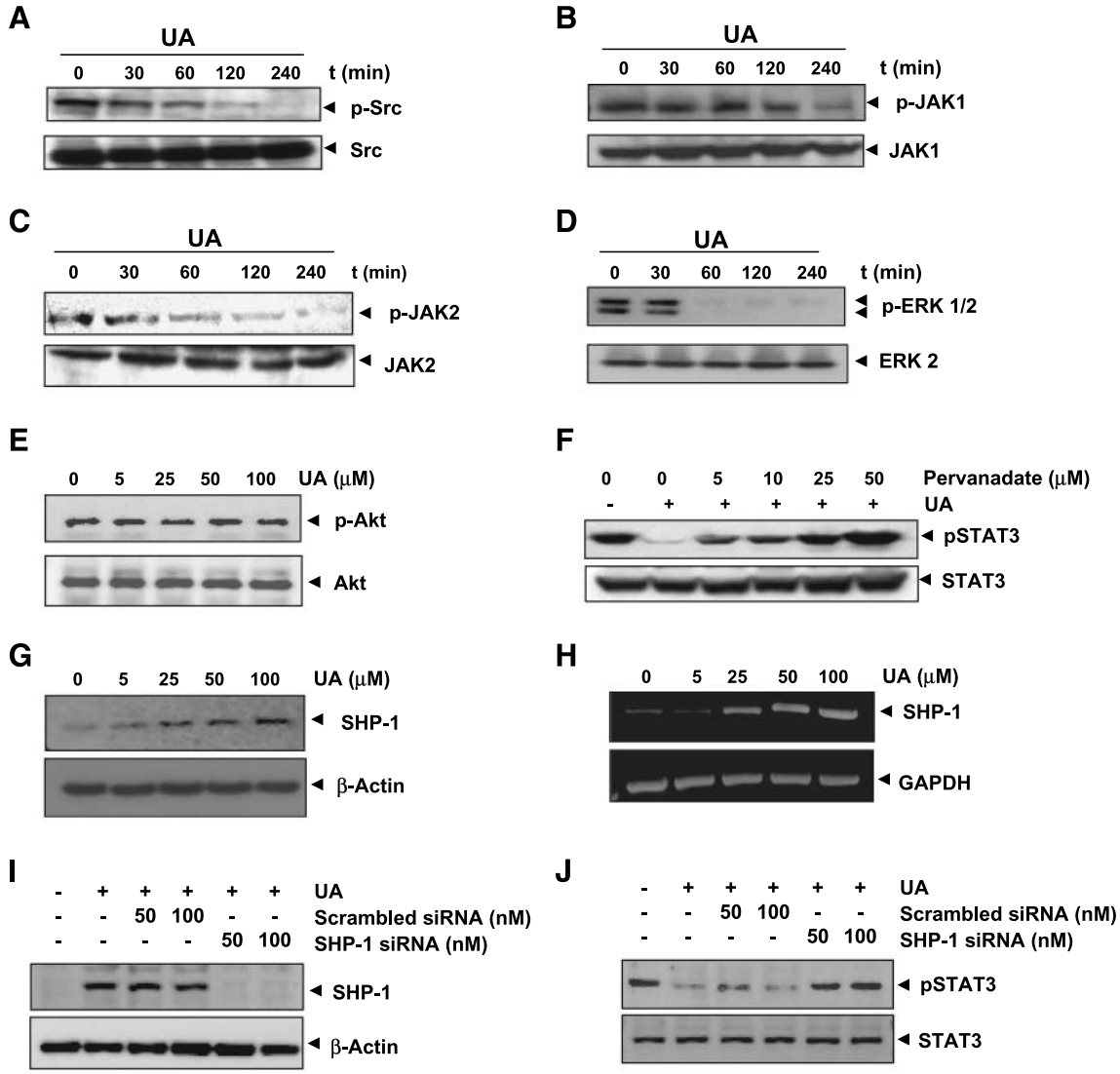

FIGURE 3. **A.** Ursolic acid suppresses phospho-Src levels in a time-dependent manner. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid, after which whole-cell extracts were prepared and 30-μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with phospho-Src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. **B.** Ursolic acid suppresses phospho-JAK1 expression in a time-dependent manner. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with phospho-JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. **C.** Ursolic acid suppresses phospho-JAK2 expression in a time-dependent manner. U266 cells (4×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time intervals. Whole-cell extracts were prepared and 500 μg of sample were incubated with JAK2 antibody overnight. Immunocomplex was precipitated with protein A/G-agarose beads and then fractionated on 10% SDS-PAGE. Western blot analysis was done with anti–phospho-JAK2 antibody. The same samples were analyzed for JAK2 protein. **D.** U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid 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, electrotransferred onto nitrocellulose membranes, and probed with phospho-ERK1/2 antibody. The same blots were stripped and reprobed with ERK1/2 antibody to verify equal protein loading. **E.** U266 cells (2×10^6/mL) were treated with indicated concentrations of ursolic acid, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with phospho-Akt antibody. The same blots were stripped and reprobed with Akt antibody to verify equal protein loading. **F.** Pervanadate reverses the phospho-STAT3 inhibitory effect of ursolic acid. U266 cells (2×10^6/mL) were treated with the indicated concentration of pervanadate and 50 μmol/L ursolic acid for 4 h, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. **G.** Ursolic acid induces the expression of SHP-1 protein in U266 cells. U266 cells (2×10^6/mL) were treated with indicated concentrations of ursolic acid for 4 h, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with SHP-1 antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **H.** Ursolic acid induces *SHP-1* gene expression. U266 cells (4×10^6/mL) were treated with indicated concentrations of ursolic acid for 4 h, and total RNA was extracted and examined for expression of *SHP-1* by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to show equal RNA loading. **I.** Effect of SHP-1 knockdown on ursolic acid–induced expression of SHP-1. U266 cells (2×10^6/mL) were transfected with either SHP-1 siRNA or scrambled siRNA (50 and 100 nmol/L). After 24 h, cells were treated with 50 μmol/L ursolic acid for 4 h and whole-cell extracts were subjected to Western blot analysis for SHP-1. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **J.** Transfection with SHP-1 siRNA reverses ursolic acid–induced suppression of STAT3 activation. U266 cells (2×10^6/mL) were transfected with either SHP-1 siRNA or scrambled siRNA (50 and 100 nmol/L). After 24 h, cells were treated with 50 μmol/L ursolic acid for 4 h and whole-cell extracts were subjected to Western blot analysis for phosphorylated STAT3. The same blots were stripped and reprobed with STAT3 antibody.

activation (7). We therefore investigated whether ursolic acid affects constitutive activation of extracellular signal–regulated kinase (ERK)-1/2 kinases in U266 cells. We found that ursolic acid suppressed the constitutive phosphorylation of ERK1/2 kinase within 60 min (Fig. 3D). The levels of nonphosphorylated ERK1/2 remained unchanged under the same conditions.

Ursolic Acid Does Not Affect Constitutive Activation of Akt

One of several signals activated by IL-6 in multiple myeloma cells is the phosphatidylinositol 3-kinase/Akt pathway (28, 29). We therefore investigated whether ursolic acid modulates constitutive activation of Akt in U266 cells. We found that ursolic acid did not affect the constitutive phosphorylation of Akt in U266 cells (Fig. 3E). The levels of nonphosphorylated Akt remained unchanged under these conditions.

Tyrosine Phosphatases Are Involved in Ursolic Acid–Induced Inhibition of STAT3 Activation

Because protein tyrosine phosphatases (PTP) have also been implicated in STAT3 activation (30), we determined whether ursolic acid–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 prevented the ursolic acid–induced inhibition of STAT3 activation (Fig. 3F). This suggests that tyrosine phosphatases are involved in ursolic acid–induced inhibition of STAT3 activation.

Ursolic Acid Induces the Expression of SHP-1 in Multiple Myeloma Cells

SHP-1 is a nontransmembrane PTP expressed most abundantly in hematopoietic cells (31, 32). PTP is important in the negative regulation of Jak/STAT signaling and has been shown to be frequently silenced by methylation in leukemias and lymphomas (33, 34). We therefore examined whether ursolic acid can modulate expression of SHP-1 in U266 cells. Cells were incubated with different concentrations of ursolic acid for 4 h and whole-cell extracts were prepared and examined for SHP-1 protein by Western blot analysis. As shown in Fig. 3G, ursolic acid induced the expression of SHP-1 protein in U266 cells in a dose-dependent manner, with maximum expression at 50 to 100 μmol/L. This stimulation of SHP-1 expression by ursolic acid correlated with down-regulation of constitutive STAT3 activation in U266 cells (Fig. 1B).

We also examined the effect of ursolic acid on the transcription of SHP-1. For this, cells were incubated with different concentrations of ursolic acid for 4 h, and then total RNA was extracted, converted to cDNA, and examined for *SHP-1* gene expression by reverse transcription-PCR (RT-PCR) analysis. As shown in Fig. 3H, ursolic acid induced the expression of SHP-1 mRNA, with maximum expression at 50 to 100 μmol/L.

SHP-1 Small Interfering RNA Down-Regulated the Expression of SHP-1 Induced by Ursolic Acid

Whether the suppression of SHP-1 expression by small interfering RNA (siRNA) abrogates the ursolic acid–induced SHP-1 expression was also investigated. As observed by Western blot analysis, ursolic acid–induced SHP-1 expression was effectively abolished in the cells transfected with SHP-1 siRNA but not in those treated with scrambled siRNA (Fig. 3I).

SHP-1 siRNA Reversed the Inhibition of STAT3 Activation by Ursolic Acid

We next determined whether the suppression of SHP-1 expression by siRNA abrogates the inhibitory effect of ursolic acid on STAT3 activation. We found that ursolic acid failed to suppress STAT3 activation in the cells transfected with SHP-1 siRNA (Fig. 3J). However, in cells transfected with scrambled siRNA, ursolic acid caused down-regulation of STAT3 activation. Thus, these results with siRNA show the critical role of SHP-1 in the suppression of STAT3 phosphorylation by ursolic acid.

Ursolic Acid Increases Expression of Bax and Bak Proteins in Multiple Myeloma Cells

The Bcl-2 family proteins have emerged as critical regulators of the mitochondria-mediated apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (35). Once activated, Bax and Bak permeabilize the mitochondrial outer membrane, resulting in the release of cytochrome *c* and other proapoptotic factors that induce caspase activation and cell death (36). Therefore, we determined the effect of ursolic acid on the expression of Bax and Bak. Increased accumulation of proapoptotic proteins Bax and Bak was seen in a time-dependent manner on treatment of U266 cells with ursolic acid (Fig. 4A).

Ursolic Acid Down-Regulates the Expression of Cyclin D1, Bcl-2, Bcl-xL, Survivin, Mcl-1, and VEGF

STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, and angiogenesis. We found that expression of the cell cycle regulator protein cyclin D1; antiapoptotic proteins Bcl-2, Bcl-xL, and survivin; and the angiogenic gene product VEGF, all reported to be regulated by STAT3 (9, 10, 22, 37), was modulated by ursolic treatment. Ursolic acid treatment down-regulated expression of these proteins in a time-dependent manner, with maximum suppression observed at ~24 h (Fig. 4B). We also determined the effect of ursolic acid on Mcl-1, an antiapoptotic protein that is highly expressed in hematopoietic cells (38, 39). Ursolic acid dramatically inhibited the expression of Mcl-1 protein in U266 cells (Fig. 4C).

Ursolic Acid Modulates Gene Expression

To determine whether ursolic acid affects the transcription, the mRNA expression of *cyclin D1, Bcl-2*, and *Bcl-xL* was examined. The mRNAs of all these genes were constitutively expressed and ursolic acid treatment suppressed their expression in a time-dependent manner (Fig. 4D). These results suggest that ursolic acid modulates the expression of genes at transcription level.

Ursolic Acid Inhibits Proliferation of Multiple Myeloma Cells

Because ursolic acid suppressed the activation of STAT3 and STAT3-regulated gene products, whether it modulates proliferation of cells was examined. Results in Fig. 5A show that ursolic acid suppressed the increase in proliferation of

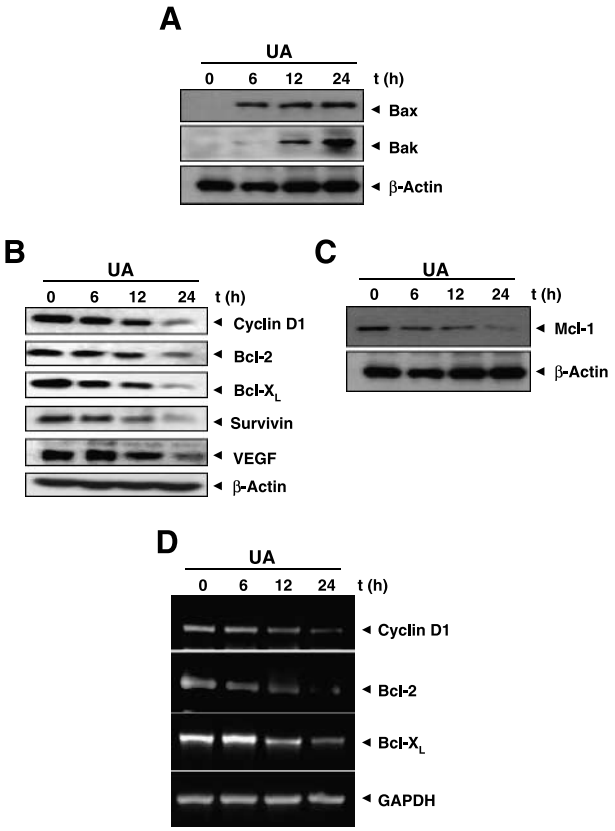

FIGURE 4. **A.** Ursolic acid enhances the expression of proapoptotic Bax and Bak proteins. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time intervals, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 10% SDS-PAGE and probed against Bax and Bak antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **B.** Ursolic acid suppresses STAT3 regulated antiapoptotic gene products. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid 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, membrane sliced according to molecular weight, and probed against cyclin D1, Bcl-2, Bcl-X_L, survivin, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **C.** Ursolic acid suppresses the expression of Mcl-1 protein. U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid 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 and probed against Mcl-1 antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. **D.** Ursolic acid inhibits gene expression. U266 cells (4×10^6/mL) were treated with ursolic acid (50 μmol/L) for the indicated time points, and total RNA was extracted and examined for expression of *cyclin D1, Bcl-2*, and *Bcl-x_L* by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control to show equal RNA loading.

U266 MM1.S and RPMI 8826 cells in a time-dependent manner. The results of trypan blue exclusion assay also indicated that treatment with ursolic acid for 12, 24, 36, and 48 h reduced the cell viability by 22%, 41%, 64%, and 74%, respectively, in U266 cells.

Ursolic Acid Causes the Accumulation of the Cells in the G_1 Phase of the Cell Cycle

Because D-type cyclins are required for the progression of cells from the G_1 phase of the cell cycle to S phase (40) and rapid decline in levels of cyclin D1 was observed in ursolic acid–treated cells, we determined the effect of ursolic acid on cell cycle phase distribution. We found that ursolic acid caused significant accumulation of cell population in G_1 phase after treatment for 24 h. However, after 36 h, 20% of cell population accumulated in sub-G_1 phase, which is indicative of apoptosis (Fig. 5B).

Ursolic Acid Does Not Modulate Expression of IL-6 Receptor α-Chain Expression in Multiple Myeloma Cells

U266 cells are known to express abundant levels of IL-6 receptor α chain (IL-6Rα; refs. 41, 42). Hence, we also examined whether the effect of ursolic acid on inhibition of STAT3 signaling pathway could be due to modulation of IL-6Rα. We examined the expression of IL-6Rα with flow cytometry in untreated and ursolic acid–treated U266 cells. No difference was found in the expression of the IL-6Rα in untreated and ursolic acid–treated cells (Fig. 5C).

Ursolic Acid Induces Apoptosis of Multiple Myeloma Cells in a Time-Dependent Manner

To further confirm the apoptosis of U266 cells induced by ursolic acid, we used Annexin V staining, which detects an early stage of apoptosis. The results indicate that ursolic acid induces apoptosis of these cells in a time-dependent manner. Results show 55% apoptosis at 48 h after treatment with ursolic acid (Fig. 5D).

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

Whether suppression of constitutively active STAT3 in U266 cells by ursolic acid leads to apoptosis was also investigated. In U266 cells treated with ursolic acid, there was a time-dependent activation of caspase-3 (Fig. 5E). Activation of downstream caspases led to the cleavage of a 116-kDa poly(ADP-ribose) polymerase (PARP) protein into an 87-kDa fragment (Fig. 5F). These results clearly suggest that ursolic acid induces caspase-3–dependent apoptosis in U266 cells. Moreover, the treatment with broad-spectrum caspase inhibitor zVAD-FMK prevented ursolic acid–induced apoptosis as examined by Annexin V staining (Fig. 5G). These results further confirm the involvement of caspase-3 in ursolic acid–induced apoptosis.

Ursolic Acid Potentiates the Apoptotic Effect of Bortezomib and Thalidomide in Multiple Myeloma Cells

Bortezomib, an inhibitor of proteasome, and thalidomide, an inhibitor of tumor necrosis factor expression, have been approved for the treatment of multiple myeloma patients (43, 44). Whether ursolic acid can potentiate the effect of these drugs was examined. For this, U266 cells were treated with ursolic acid together with either thalidomide or bortezomib, and then examined for apoptosis by live/dead assay, which determines plasma membrane stability using esterase staining. As shown in Fig. 6A and B, ursolic acid significantly enhanced the apoptotic effects of thalidomide from 20% to 70% and of bortezomib from 25% to 80%.

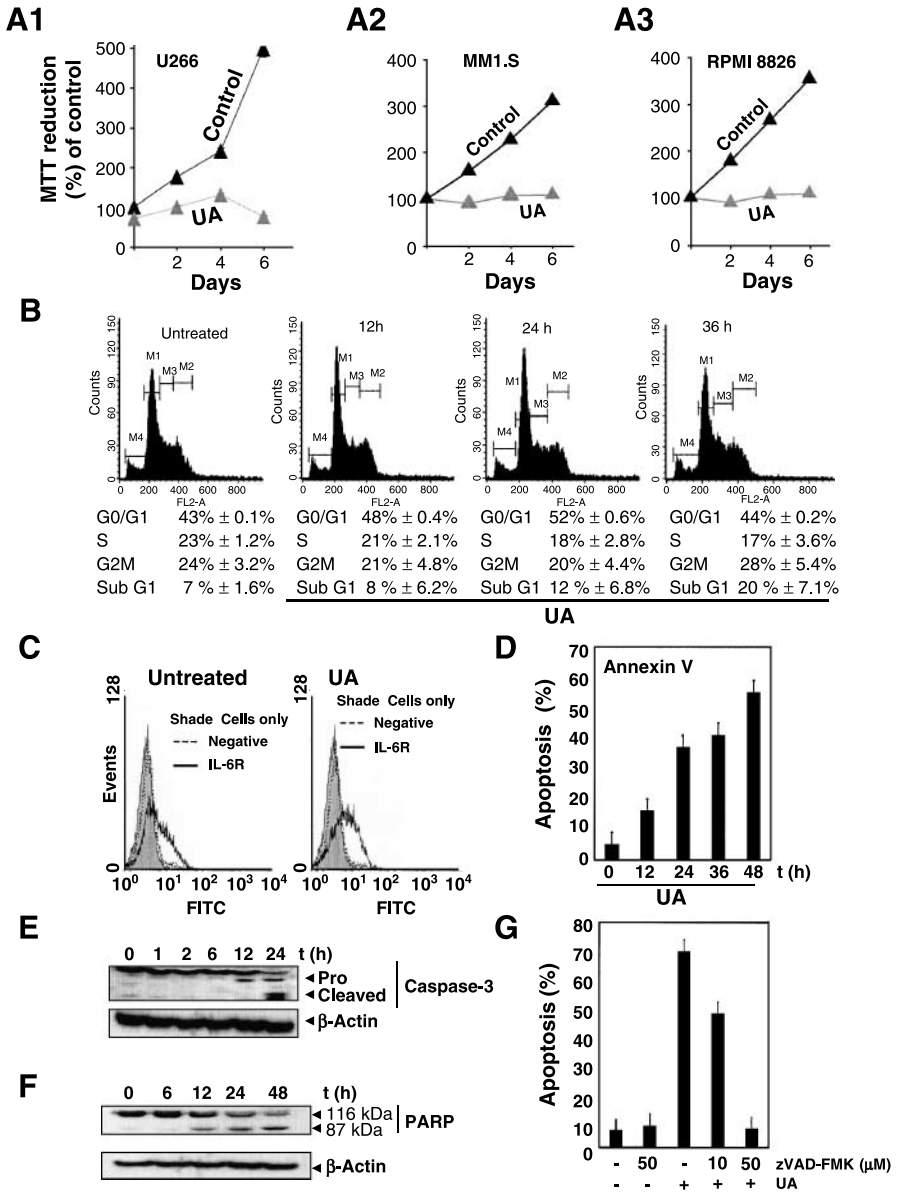

FIGURE 5. Ursolic acid suppresses proliferation, causes accumulation of cells in G_0-G_1 phase, does not affect IL-6Rα expression, and activates caspase-3. **A1** to **A3.** U266, MM1.S, and RPMI 8826 cells were plated in triplicate, treated with 25 μmol/L ursolic acid, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on day 2, 4, or 6 to analyze proliferation of cells. **B.** U266 cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with 50 μmol/L ursolic acid for the indicated time points, after which the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. **C.** Ursolic acid does not modulate cell-surface expression of IL-6Rα in U266 cells. Cells were harvested and labeled with mouse anti-human IL-6Rα FITC-conjugated antibody and analyzed by flow cytometry. **D.** U266 cells (1×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time intervals at 37°C. Cells were incubated with anti–Annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. **E.** U266 cells were treated with 50 μmol/L ursolic acid for the indicated time points and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against caspase-3 antibody. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. **F.** U266 cells were treated with 50 μmol/L ursolic acid for the indicated time points and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody. The same blot was stripped and reprobed with β-actin antibody to show equal protein loading. Representative of three independent experiments. **G.** Caspase inhibitor suppresses the ursolic acid–induced apoptosis of U266 cells. U266 cells (1×10^6/mL) were preincubated with 50 μmol/L ursolic acid and 10 and 50 μmol/L zVAD-FMK alone or in combination for 48 h at 37°C. Cells were incubated with anti–Annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects.

Discussion

The goal of this study was to determine whether ursolic acid exerts its anticancer effects through the abrogation of the STAT3 signaling pathway in multiple myeloma cells. We found that this dietary factor suppressed both constitutive and IL-6–inducible STAT3 activation in parallel with the inhibition of c-Src, JAK1, JAK2, and ERK1/2 activation. Ursolic acid stimulated the expression of nontransmembrane PTP SHP-1 in U266 cells. The knockdown of SHP-1 by siRNA suppressed the induction of SHP-1 and reversed the inhibition of STAT3 activation, thereby indicating the critical role of SHP-1 in the action of this triterpene. Ursolic acid also down-regulated the expression of STAT3-regulated gene products, including cyclin D1, survivin, Bcl-2, Bcl-xL, Mcl-1, and VEGF. It induced the inhibition of proliferation, accumulation of cells in G_1-G_0 phase, and apoptosis, and it significantly potentiated the apoptotic effects of bortezomib and thalidomide in multiple myeloma cells.

We found for the first time that ursolic acid could suppress both constitutive and inducible STAT3 activation in multiple myeloma cells and that these effects were specific to STAT3 because ursolic acid had no effect on STAT5 phosphorylation. We found that exposure of cells to 50 μmol/L ursolic acid for 4 h was needed to fully abolish STAT3 activation. In comparison, exposure of cells to 50 μmol/L AG490 (a rationally designed inhibitor of JAK2) for 8 h was required to suppress STAT3 activation (2, 20). Similar to our results, a recent report showed the suppression of constitutive and IL-6–inducible STAT3 activation by a synthetic triterpenoid, CDDO-imidazolide (45). Unlike our results, however, Liby et al. (45) also showed inhibition of STAT5 phosphorylation.

The effects of ursolic acid on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases JAK1 and c-Src. Although ursolic acid also blocked the activation of ERK1/2, it is unlikely that this kinase is directly

involved because it is a serine kinase. We also observed that ursolic acid suppressed nuclear translocation and DNA binding activity of STAT3. STAT3 phosphorylation plays a critical role in transformation and proliferation of tumor cells (9, 10). All Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation (21, 22). Dominant-negative STAT3 has also been shown to induce apoptosis in cells with constitutively active STAT3 (46). Other forms of cancer, including head and neck cancers (47), hepatocellular carcinoma (48), lymphomas, and leukemia (49), also have constitutively active STAT3. The suppression of constitutively active STAT3 in multiple myeloma cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of cancer cells.

We also found evidence that the ursolic acid–induced inhibition of STAT3 activation involves a PTP. Ursolic acid has been shown to inhibit PTP-1B (50, 51), thus suggesting that PTP-1B is not involved in the dephosphorylation of STAT3. Numerous PTPs have been implicated in STAT3 signaling, including SHP-1, SHP-2, T-cell PTP, PTEN, PTP-1D, CD45, PTPε, and low molecular weight PTP (52-60). The type of PTP involved in down-regulation of STAT3 phosphorylation is not clear. Loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in anaplastic lymphoma kinase–positive anaplastic large-cell lymphoma (30). Indeed, we found for the first time that ursolic acid stimulates the expression of SHP-1 protein and mRNA in U266 cells, which correlated with down-regulation of constitutive STAT3 phosphorylation in these cells. Transfection with SHP-1 siRNA reversed the STAT3 inhibitory effect of ursolic acid, thereby further implicating a critical role of this phosphatase in ursolic acid–induced down-regulation of STAT3 activation. Moreover, the synthetic triterpenoid CDDO-imidazolide has been shown to induce the expression of SHP-1 (45).

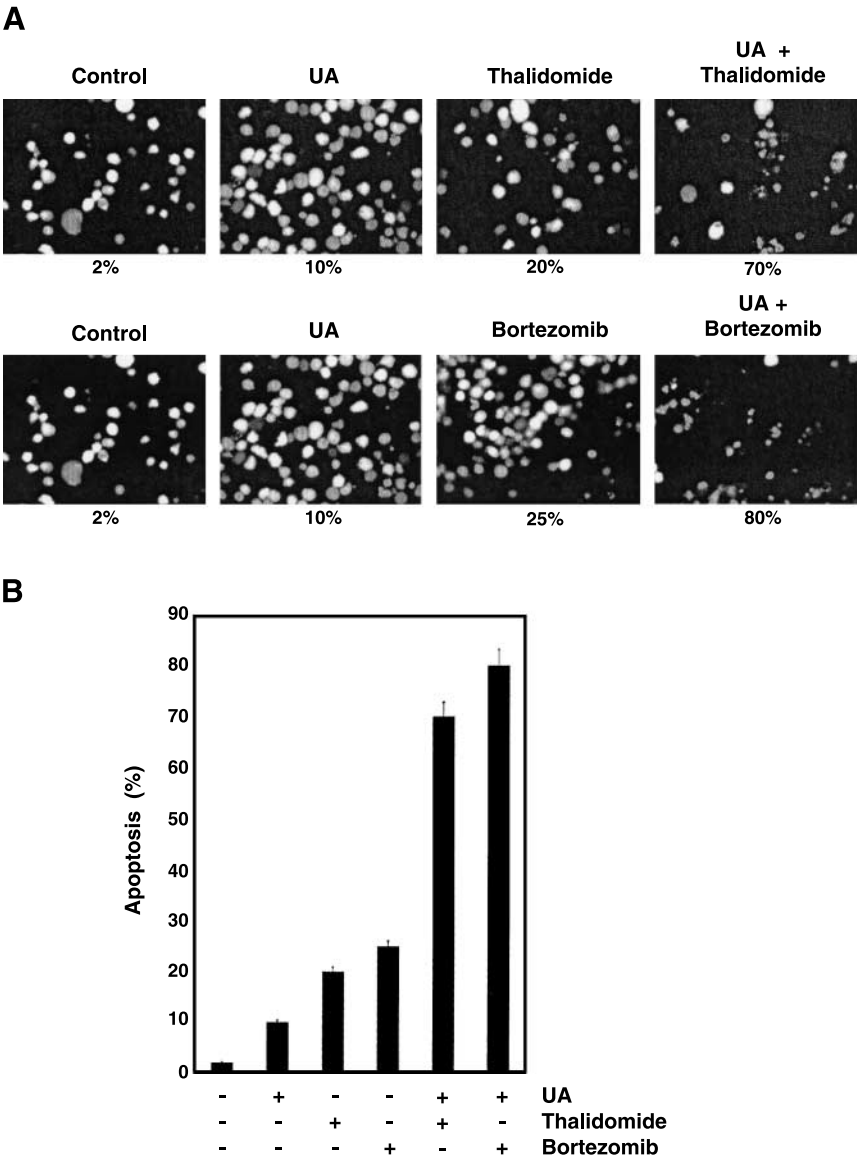

FIGURE 6. **A.** Ursolic acid potentiates the apoptotic effect of thalidomide and bortezomib. U266 cells (1×10^6/mL) were treated with 25 μmol/L ursolic acid and 10 μg/mL thalidomide or 20 nmol/L bortezomib alone or in combination for 24 h at 37°C. Cells were stained with a live/dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. The results shown are percent apoptosis and are representative of three independent experiments. **B.** Quantitative analysis of live/dead assay done in (**A**). Bars, SD between the triplicates.

Previously, we have shown that ursolic acid can also suppress NF-κB activation (18). Whether suppression of STAT3 activation by ursolic acid is linked to inhibition of NF-κB activation is not clear. The p65 subunit of NF-κB has been shown to interact with STAT3 (61). STAT3 and NF-κB, however, are activated in response to different cytokines. Whereas IL-6 is a major activator of STAT3, tumor necrosis factor is a potent activator of NF-κB. Interestingly, erythropoietin has been shown to activate both NF-κB and STAT3 through the activation of JAK2 kinase (62). Thus, it is possible that suppression of JAK activation is the critical target for inhibition of both NF-κB and STAT3 activation by ursolic acid.

We also report for the first time that ursolic acid suppresses the expression of several STAT3-regulated genes, including proliferative (cyclin D1) and antiapoptotic gene products (survivin, Bcl-2, Bcl-xL, and Mcl-1) and angiogenic gene product (VEGF). Constitutively active STAT3 can contribute to oncogenesis by protecting cancer cells from apoptosis; this implies that suppression of STAT3 activation by agents such as ursolic acid could facilitate apoptosis. Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis (46), possibly through the expression of Bcl-2 and cyclin D1 (63, 64). The down-regulation of cyclin D1 expression by ursolic acid correlated with suppression in proliferation and accumulation of cells in G_1 phase of cell cycle, which is consistent with the requirement of cells for cyclin D1 if they are to advance from the G_1 to S phase of the cell cycle. Expression of Bcl-xL is regulated by STAT3 (65) and is overexpressed in multiple myeloma cells (66). Bcl-xL can also block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance (67). The down-regulation of the expression of Bcl-2, Bcl-xL, and survivin is likely linked with the ability of ursolic acid to induce apoptosis in multiple myeloma cells. We further observed that ursolic acid induced the down-regulation of Mcl-1 protein in U266 cells. Indeed, it has been shown that inhibition of Src or STAT3 by a Src inhibitor (PD180970) results in down-regulation of expression of the *Mcl-1* gene in melanoma cells (68).

Recently, a proteasome inhibitor (PS341; also called bortezomib) and a tumor necrosis factor inhibitor (thalidomide) were approved for the treatment of multiple myeloma (43, 44). We found that ursolic acid potentiates the apoptotic effect of bortezomib and thalidomide in multiple myeloma cells. Ursolic acid has been shown to be well tolerated in animal studies, with little toxicity (12, 69, 70). We contend that the apparent pharmacologic safety of ursolic acid and its ability to down-regulate the expression of several genes involved in cell survival and chemoresistance provide a sufficient rationale for testing ursolic acid further in patients with multiple myeloma.

Multiple myeloma that has relapsed after conventional-dose therapy or stem cell transplantation is typically treated with high-dose corticosteroids, thalidomide, or bortezomib. However, disease in significant proportions 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 ursolic acid and its ability to suppress STAT3 activation; inhibit IL-6–induced STAT3 and JAK2 phosphorylation; down-regulate the expression of cyclin D1, Bcl-2, Bcl-xL, Mcl-1, and VEGF; inhibit cell proliferation; induce apoptosis; and potentiate the effect of bortezomib and thalidomide warrant further preclinical studies preceding human trials.

Materials and Methods

Reagents

Ursolic acid, Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich. Ursolic acid was dissolved in DMSO as a 10 mmol/L stock solution and stored at 4°C. Further dilution was done in cell culture medium. RPMI 1640, fetal bovine serum, 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Life Technologies. Rabbit polyclonal antibodies to STAT3 and STAT5 and mouse monoclonal antibodies against phospho-STAT3 (Tyr^{705}) and phospho-STAT5 (Tyr^{694}/Tyr^{699}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK, Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, SHP-1, cyclin D1, survivin, Akt, procaspase-3, and PARP were obtained from Santa Cruz Biotechnology. Annexin V staining kit was obtained from Santa Cruz Biotechnology. IL-6Rα FITC-conjugated monoclonal antibody was purchased from Chemicon International. Goat anti-rabbit-horseradish peroxidase conjugate was purchased from Bio-Rad. Anti-VEGF was purchased from NeoMarkers. Antibodies to phospho-specific Src (Tyr^{416}), Src, phospho-specific JAK1 ($Tyr^{1022/1023}$), phospho-specific Jak2 ($Tyr^{1007/1008}$), phosphorylated Akt (Ser^{473}), JAK1, and JAK2 were purchased from Cell Signaling Technology. Goat anti-mouse horseradish peroxidase was purchased from Transduction Laboratories, and goat anti-rabbit Alexa 594 was purchased from Molecular Probes. AG490 was obtained from Calbiochem. Bacteria-derived recombinant human IL-6 was kindly provided by Novartis Pharmaceuticals. Bortezomib (PS-341) was obtained from Millennium. Thalidomide was obtained from Tocris Cookson. Broad-spectrum caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) was obtained from R&D Systems. The siRNA for SHP-1 and the scrambled control were obtained from Ambion.

Cell Lines

Human multiple myeloma cell lines U266, RPMI 8226, and MM1.S (melphlan sensitive) were obtained from the American Type Culture Collection (Manassas, VA). Cell line U266 (ATCC TIB-196) is a plasmacytoma of B-cell origin and is known to produce monoclonal antibodies and IL-6 (23, 71). RPMI 8226 produces only immunoglobulin light chains, with no evidence of heavy chain or IL-6 production. The MM1.S cell line, established from the peripheral blood cells of a patient with immunoglobulin A myeloma, secretes light chain, is negative for the presence of the EBV genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens (72). U266 and MM1.S cells were cultured in RPMI 1640 containing 1× antibiotic-antimycotic solution with 10% fetal bovine serum. Human myeloid leukemia (KBM-5) cells were obtained from American Type Culture Collection and cultured in Iscove's modified

Dulbecco's medium with 15% fetal bovine serum, containing 1× antibiotic-antimycotic solution.

EMSA for STAT3-DNA Binding

STAT3-DNA binding was analyzed by EMSA using a ^{32}P-labeled high-affinity sis-inducible element (hSIE) probe (5′-CTTCATTTCCCGTAAATCCCTAAAGCT-3′ and 5′-AGCTTTAGGGATTTACGGGAAATGA-3′) as previously described (19). Briefly, nuclear extracts were prepared from ursolic acid–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 with Storm 820 and the radioactive bands quantitated with ImageQuant software (Amersham).

Western Blotting

For detection of STAT proteins, ursolic acid–treated whole-cell extracts were lysed in lysis buffer [20 mmol/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 $NaVO_4$]. Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% SDS gel. 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 at 4°C. The blot was washed, exposed to horseradish peroxidase–conjugated secondary antibodies for 1 h, and finally examined by enhanced chemiluminescence (Amersham Pharmacia Biotech).

To detect STAT3-regulated proteins and caspase-3, U266 cells (2×10^6/mL) were treated with 50 μmol/L ursolic acid for the indicated time points. The cells were then washed and extracted by incubation for 30 min on ice in 0.05-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 aprotinin, 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. Whole-cell extract protein (50 μg) was resolved on 12% SDS-PAGE; electrotransferred onto a nitrocellulose membrane; blotted with antibodies against survivin, Bcl-2, Bcl-xL, cyclin D1, VEGF, and caspase-3; and then detected by enhanced chemiluminescence (Amersham).

Immunocytochemistry for STAT3 Localization

Ursolic acid–treated multiple myeloma cells were plated on a glass slide by centrifugation using Cytospin 4 (Thermoshendon), air-dried for 1 h at room temperature, and fixed with cold acetone. 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 overnight incubation, the slides were washed and then incubated with goat anti-rabbit immunoglobulin G-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).

Immunoprecipitation for JAK2 Activation

Cells were lysed for 30 min on ice in whole-cell lysis buffer [20 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.5 mmol/L phenylmethanesulfonyl fluoride, and 2 mmol/L sodium orthovanadate]. Lysate containing 500 μg of proteins in lysis buffer was incubated with 1 μg/mL of JAK2 antibody overnight. Immunocomplex was precipitated using protein A/G-agarose beads for 1 h at 4°C. Beads were washed with lysis buffer and resuspended in SDS sample buffer, boiled for 5 min, subjected to SDS-PAGE, and blotted with phospho-JAK2 antibody.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The antiproliferative effect of ursolic acid against multiple myeloma cell lines was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye uptake method as described earlier (73).

Transfection with SHP-1 siRNA

U266 cells were transfected with siRNA as previously described (74). Briefly, cell suspension (2×10^6 per 100 μL of solution V; Nucleofection kit V, Amaxa Biosystems) was mixed with 50 or 100 nmol siRNA for SHP-1, and the mixture was transferred to cuvettes and subjected to electroporation according to the manufacturer's instructions. The cell suspension was then mixed with 500 μL of prewarmed RPMI 1640 and transferred to 24-well plates. After 24 h, cells were treated with ursolic acid for 4 h and whole-cell extracts were prepared to examine the expression of SHP-1 and phosphor-STAT3 by Western blot analysis.

Flow Cytometric Analysis

To determine the effect of ursolic acid on the cell cycle, U266 cells were first synchronized by serum starvation and then exposed to ursolic acid for the indicated time intervals. Thereafter, cells were 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 μg/mL propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Immunoblot Analysis of PARP Degradation

Ursolic acid–induced apoptosis was examined by proteolytic cleavage of PARP. Briefly, cells (2×10^6/mL) were treated with ursolic acid for indicated time points at 37°C. The cells were then washed and extracted by incubation for 30 min on ice in 0.05-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 aprotinin, 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 collected. Cell extract protein (50 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by enhanced chemiluminescence (Amersham).

Live/Dead Assay

Viability of cells was also determined by live/dead assay (Molecular Probes) that measures intracellular esterase activity and plasma membrane integrity as previously described (75).

RNA Analysis and RT-PCR

U266 cells were left untreated or treated with ursolic acid, 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 RT-PCR kit (Invitrogen). The relative expression of SHP-1, cyclin D1, Bcl-2, and Bcl-xL was analyzed using quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase as an internal control. The RT-PCR reaction mixture contained 12.5 μL of 2× reaction buffer, 10 μL each of cDNA, 0.5 μL each of forward and reverse primers, and 1 μL of RT-Platinum Taq in a final volume of 50 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, 60°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.

Cell-Surface Expression of IL-6R

For analysis of cell-surface expression of IL-6Rα, untreated or ursolic acid–treated U266 cells were harvested and suspended in Dulbecco's PBS containing 1% fetal bovine serum and 0.1% sodium azide. The cells were preincubated with 10% goat serum for 20 min and washed, and then mouse anti-human FITC-conjugated (IL-6Rα) antibody was added. Following a 12-h incubation at 4°C, the cells were washed and analyzed with a flow cytometer (FACSCalibur, BD Biosciences) and acquisition and analysis programs (CellQuest, BD Biosciences).

Annexin V Assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by utilizing the binding properties of Annexin V. Briefly, 1×10^6 U266 cells were pretreated with ursolic acid for various time points or treated with combination of ursolic acid and broad-spectrum caspase inhibitor zVAD-FMK, and then subjected to Annexin V staining. Cells were washed, stained with FITC-conjugated anti–Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

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