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Shikonin, a naphthoquinone compound, rapidly induces cell death of human myeloma cells via production of reactive oxygen species

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Shikonin is a naphthoquinone derivative extracted from a traditional Chinese medical herb, *Lithospermum erythrorhizon*. It has been found to posses a variety of biological activities including strong wound healing, anti-bacterial, anti-inflammatory, and anti-tumor effects. Recently, it has been reported that shikonin induces cell death of various cancer cells. However, the mechanisms responsible for these activities remain unknown. In addition, no studies have addressed the effects of shikonin on multiple myeloma cells. In this study, we investigated the effects of shikonin on cell proliferation, cell cycle progression, and mechanism of apoptotic cell death in human multiple myeloma cells. Shikonin markedly suppressed the cellular growth of various myeloma cell lines via induction of apoptosis. Shikonin-induced cell death was in association with the loss of mitochondrial transmembrane potentials (ΔΨm), the release of cytocrome *c* and Smac/DIABLO. Elevation of intracellular oxygen species (ROS) production was shown during shikonin-induced cell death in myeloma cells. Shikonin markedly inhibited IL-6-induced phosphorylation of STAT3 in myeloma cells. The antioxidants, NAC, ameliorated shikonin-induced cell death and inhibition of STAT3 phosphorylation in myeloma cells. Our results indicate that shikonin induced cell death in myeloma cells, and that this was mediated through inhibition of mitochondrial damage and IL-6/STAT pathways by production of ROS.

Key words: shikonin, cell death, caspase, STAT3, multiple myeloma, reactive oxygen species (ROS)

Introduction

Multiple myeloma is a plasma cell malignancy that often remains fatal despite the use of high dose chemotherapy with hematopoietic stem cell transplantation[1)]. Recently, novel agents such as thalidomide, lenalidomide, and the proteasome inhibitor bortezomib have been introduced for the treatment of this disease and have remarkably improved patient outcome[2–4)]. However, adverse events and complications of these agents are often problematic in the clinical setting. In addition, prolonged use and repeated disease relapse may lead to the development of drug resistance in myeloma cells[5)]. Therefore, novel effective and less toxic therapeutic strategies are desired in order to improve clinical outcomes.

Shikonin is a natural naphthoquinone derivative compound that is present in the root tissues of *Lithospermum erythrorhizon*. This herb has previously been reported to have many medicinal properties including anti-bacterial, anti-inflammatory, wound-healing, and anti-tumor effects[6)]. Several studies have shown that shikonin induces p53-mediated cell cycle arrest and cell death in various human malignant cancer cells, inhibits epidermal growth factor receptor signaling in human epidermoid carcinoma cells, and induces cell death in HL60 human myeloid leukemia cell line[7–9)]. However, the mechanisms responsible for these activities of shikonin remain unknown.

It has been suggested that the production of reactive oxygen species (ROS) is a common mechanism in one of the representative pathways of apoptotic cell death[10)]. These oxidant and their products are capable of depleting glutathione (GSH) or damaging the cellular antioxidant defense system, and they can directly induce cell death[11)]. Recently, we and other groups

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have found that intracellular production of ROS is important in inducing apoptotic cell death in human myeloma cells [12,13].

In the present study, we conducted an *in vitro* study to assess the effects of shikonin on myeloma cell proliferation, and the underlying mechanisms.

Materials and Methods

Cells and cell culture

The human myeloma cell lines, IM9, RPMI8226, HS-Sultan and U266, were cultured in RPMI1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO) in a humidified atmosphere with 5% CO_2. These cell lines were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). The morphology was evaluated on cytospin slide preparations with Giemsa staining, and the viability was assessed by trypan blue dye exclusion.

Cell proliferation assay

Cell proliferation was measured using an MTS proliferation assay kit (Roche Molecular Biochemicals, Mannheim, Germany). Cells were plated on 96-well culture plates at 5×10^4 cells/ml in a total volume of 100 μM with the indicated reagents. After a 2-day incubation, cellular proliferation was measured using the MTS assay. Mean and standard deviation were calculated from triplicate experiments.

Reagents

Shikonin was purchased from BIOMOL International LP (Plymouth Meeting, PA) (Fig. 1A). N-acetyl-L-cystein (NAC) and L-buthionine sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). These agents were dissolved in PBS.

Assays for apoptotic cell death

Cell death was determined by morphological change as well as by staining with annexin V-FITC and PI labeling. Apoptotic cells were quantified by annexin V-FITC and PI double staining using a staining kit purchased from PharMingen (San Diego, CA). In addition, induction of apoptosis was detected by DNA fragmentation assay. Cells (1×10^6) were harvested and incubated in a lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton-X) at 4°C. After centrifugation, supernatants were collected and incubated with RNase A (Sigma) at 50 μg/ml and proteinase K (Sigma) for 1 h at 37°C. The DNA samples were subjected to 2% agarose gel and were visualized by ethidium bromide staining. The mitochondrial membrane potential (ΔΨm) was determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA). Briefly, cells were washed twice with PBS and incubated with 1 μg/ml Rhodamine 123 (Sigma) at 37°C for 30 min. Rhodamine 123 intensity was determined by flow cytometry as a measure of ΔΨm.

Cell cycle analysis

Cells (1×10^5) were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1 mM EDTA) and stained with 50 μg/ml of PI. The DNA content was analyzed by flow cytometry. The population of cells in each cell cycle phase was determined using Mod FIT software (Becton Dickinson).

Caspase assays

The activation of caspase-3 was analyzed using a caspase-3 assay kit from BD Biosciences (San Jose, CA). Briefly, the FITC-conjugated antibody against the active form of caspase-3 provided in the kit was used for FACS analysis according to the manufacturer's instructions (BD Biosciences).

Measurement of intracellular superoxide production

To assess the production of superoxide, control and shikonin-treated cells were incubated with 5 μM dehydroxyethidium (DHE) (Molecular Probes, Eugene, OR), which is oxidized to ethidium, a fluorescent intercalator, by cellular oxidants, particularly superoxide radicals. Cells (1×10^5) were stained with 5 μM DHE for 30 min at 37°C, and were then washed and resuspended in PBS. The oxidative conversion of DHE to ethidium was measured by flow cytometry (Becton Dickinson).

Cell lysate preparation and western blotting

Cells were collected by centrifugation at 700 g for 10 min and then the pellets were resuspended in lysis buffer (1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Tris-HCl (pH 8.0), and 150 mM NaCl) at 4°C for 15 min. Mitochondrial and cytosolic fractions were prepared with digitonin-nagarse treatment. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA). Cell lysates (20 μg protein per lane) were fractionated in 12.5% SDS-polyacrylamide gel prior to transfer to the membranes (Immobilon-P membranes, Millipore, Bedford, MA) using a standard protocol. Antibody binding was detected by using an enhanced chemiluminescence kit for Western blotting detection with hyper-ECL film (Amersham, Buckinghamshire, UK). Blots were stained with Coomassie brilliant blue to confirm equal amounts of protein extract on each lane. The following antibodies were used in this study: anti-caspase 3, -cytochrome *c* (PharMingen, San Diego, CA), -Bcl-2, -Bcl-X_L, -β-actin (Santa Cruz Biotech, Santa Cruz, CA), -Bax, -Bid and -Smac/DIABLO (MBL, Nagoya, Japan).

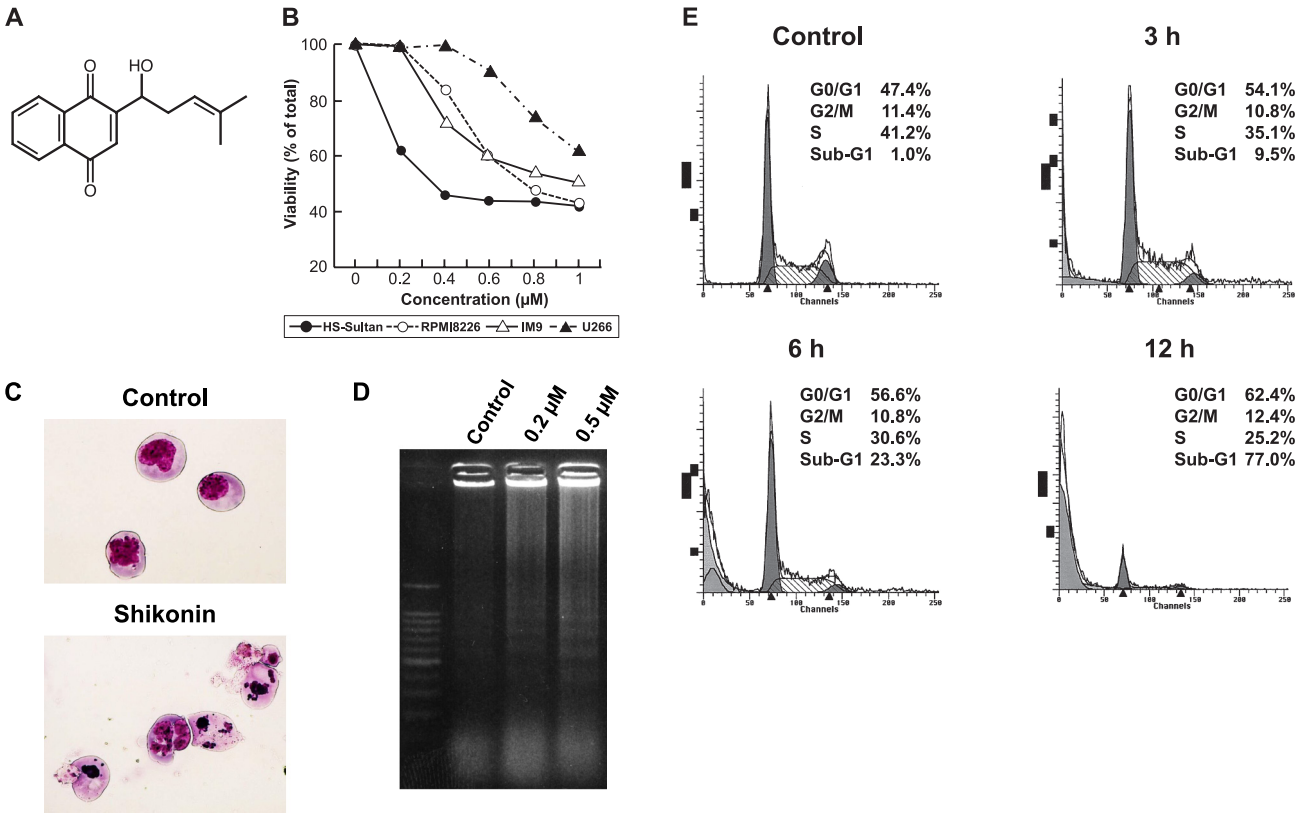


Figure 1. Effects of shikonin on cell growth and apoptotic cell death in myeloma cells. (A) Chemical structure of shikonin. (B) Various human myeloma cell lines (HS-Sultan, RPMI8226, IM9, and U266) were treated with various concentrations (0–1 μM) of shikonin for 24 h, and cell proliferation was determined by MTS assay. (C) Morphological changes characteristic of apoptotic cell death in RPMI8226 myeloma cells. RPMI8226 cells were treated with 0.5 μM shikonin for 24 h, and cytospin slides were then prepared and stained with Giemsa. Original magnification, ×1000. (D) Agarose gel electrophoresis demonstrating DNA fragmentation in RPMI8226 cells treated with 0.2 and 0.5 μM shikonin for 6 h. (E) Cell cycle analysis. RPMI8226 cells were treated with 0.5 μM shikonin for the indicated times (0–12 h), and subsequently stained with PI. DNA content refers to the population of apoptotic cells. Similar results were obtained in three independent experiments.

Results

Shikonin inhibited cellular proliferation of various human myeloma cells

We first examined whether shikonin inhibited the growth of various myeloma cells, including IM9, RPMI8226, HS-Sultan, and U266 cells. Shikonin inhibited the cellular growth of all myeloma cells in a dose (0–1 μM)- and time (0–24 h)-dependent manner (Fig. 1B and data not shown). Cell growth was suppressed from as early as 6 h, and the typical morphological appearance of apoptotic cell death was observed, including condensed chromatin and fragmented nuclei with apoptotic bodies (Fig. 1C, D).

Shikonin induced G1 cell cycle arrest and subsequent cell death

Cultivation with shikonin increased the population of cells in the G0/G1 phase with a reduction of cells in the S phase (Fig. 1E). We confirmed the induction of apoptotic cell death by shikonin by means of DNA ladder formation and annexin V/PI staining. DNA ladder formation was confirmed at as early as 6 h by electrophoresis of genomic DNA extracted from myeloma cells treated with 0.5 μM shikonin (Fig. 1D). RPMI8226 and IM9 cells cultured with shikonin at various doses (0–1 μM) and for various durations (0–18 h) exposure were stained with annexin V/PI to detect externalization of phosphatidyl serine on the cell membrane. Numbers of annexin V-positive and PI-negative cells were increased as early as 6 h, indicating that shikonin rapidly induced cell death in myeloma cells (Fig. 2).

Shikonin decreased expression of caspase by myeloma cells

Caspases are believed to play a central role in mediating various apoptotic responses. To address the apoptotic pathway in shikonin-treated myeloma cells, we next examined the activation of caspases. To clarify the activation of caspase-3, the percentage of cells expressing the active form of caspase-3 was analysed by FACS. After incubation with 0.5 μM shikonin for 6 h, the percentage of myeloma cells expressing the active

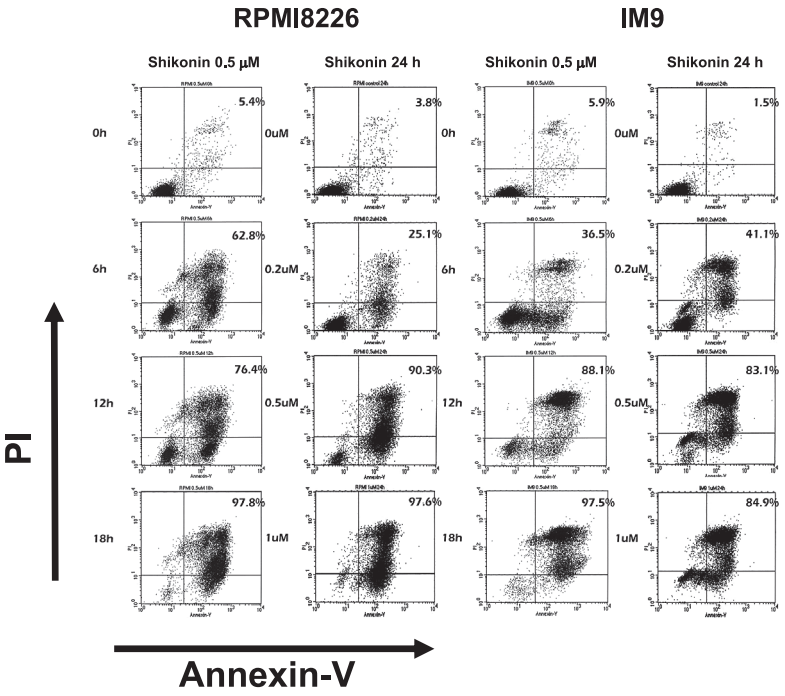


Figure 2. Detection of apoptotic cells by annexin V-FITC and PI double staining. RPMI8226 and IM9 cells were cultured with various concentrations (0–1 μM) of shikonin for indicated times (0–18 h), stained with annexin V-FITC and PI labeling, and analyzed using flow cytometry. Three independent experiments were performed and all gave similar results.

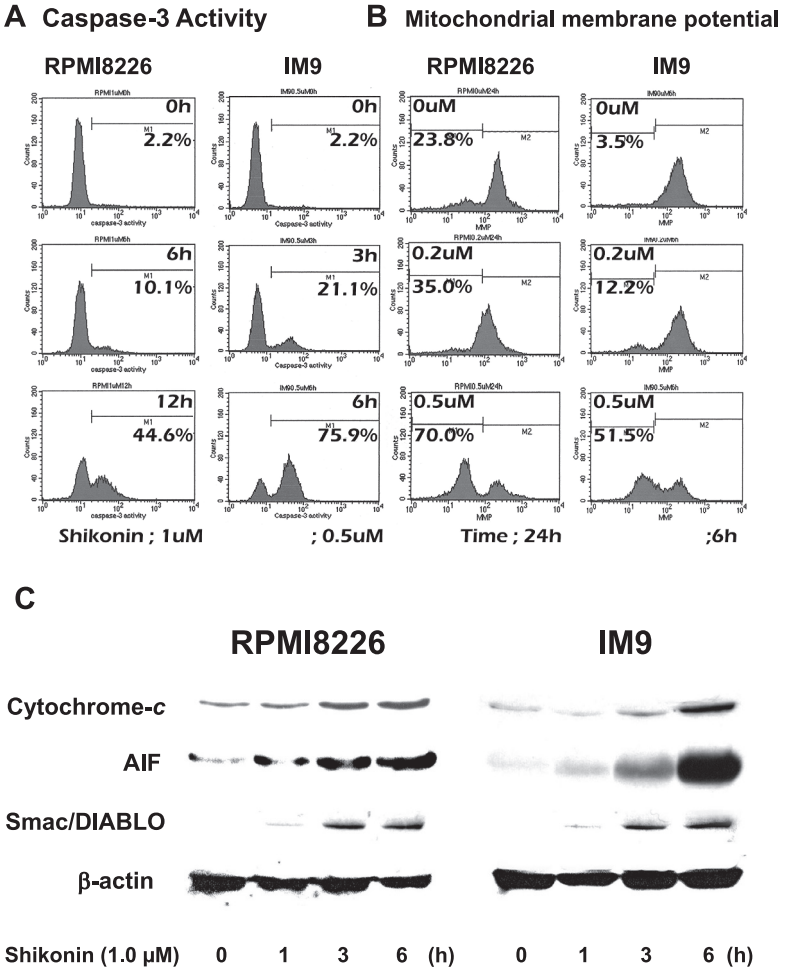


Figure 3. Effects of shikonin on caspase-3 activity and mitochondrial pathway via the mitochondrial membrane potential (ΔΨm). (A) Detection of the active form of caspase-3 by FACS. PRPMI8226 and IM9 cells were treated with 0.5 or 1 μM shikonin for indicated times (0–12 h). The percentage of cells expressing the active form of caspase-3 was analyzed using the specific antibody by FACS. (B) ΔΨm as estimated by Rhodamine 123 intensity on flow cytometric analysis. RPMI8226 and IM9 myeloma cells were cultured with various concentrations (0–0.5 μM) of shikonin for 6 h, and Rhodamine 123 fluorescence was analyzed by flow cytometry. (C) Western blot analysis of mitochondrial apoptogenic proteins in shikonin-treated RPMI8226 and IM9 cells. Cells were incubated with 1 μM shikonin for various times (0–6 h). The cytosolic and mitochondrial proteins were analyzed by Western blotting with anti-cytochrome *c*, Smac/DIABLO, and AIF antibodies. These findings indicated that shikonin-induced cell death occurred via the mitochondrial pathway.

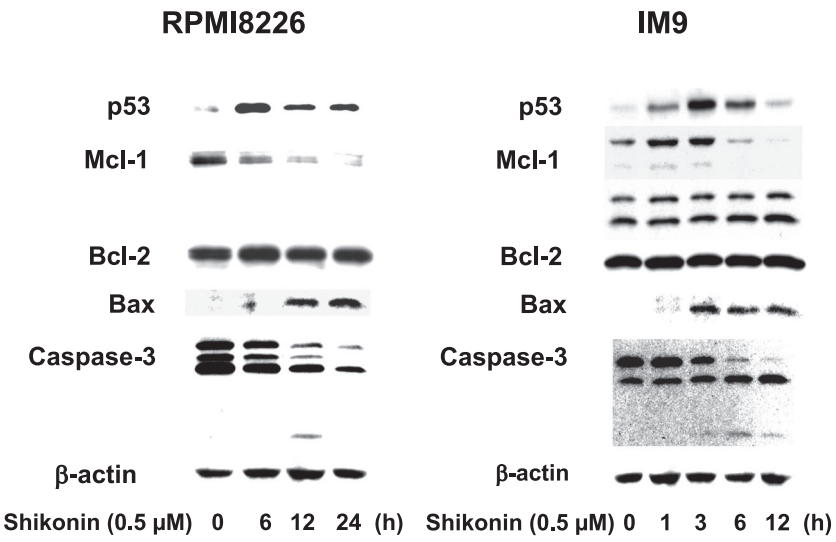


Figure 4. Expression of apoptotic cell death-associated proteins. RPMI8226 and IM9 cells were treated with 0.5 μM shikonin for the indicated time. Cell lysates (20 μg each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against p53, Mcl-1, BCL-2, Bax and β-actin proteins. Protein levels of caspase-3 were detected by Western blot analysis using antibody against caspase-3. Shikonin-induced processing of caspase-3 (32 kDa) was indicated by the appearance of a 17 kDa cleaved active form.

form of caspase-3 were increased (Fig. 3A). In response to shikonin, the expression levels of caspase-3 in RPMI8226 and IM9 cells were decreased (Fig. 4), indicating that shikonin-induced cell death in myeloma cells is mediated through the activation of caspase-3.

Shikonin-induced death signaling is mediated through the mitochondrial pathway

Recent studies have suggested that mitochondria play an essential role in death signal transduction [14,15]. Mitochondrial changes, including permeability transition pore opening and the collapse of the mitochondrial ΔΨm, result in the release of cytochrome *c* into the cytosol, which subsequently causes cell death by the activation of caspases. After treatment with 0.5 μM shikonin for 3 h, low Rh123 staining in myeloma cells indicated an increase in the loss of mitochondrial ΔΨm (Fig. 3B). The loss of ΔΨm appeared in parallel with the activation of caspase-3, as well as with induction of cell death. In addition, shikonin induced a substantial release of various mitochondrial apoptogenic proteins (e.g., cytochrome *c*, Smac/DIABLO and AIF) from the mitochondria into the cytosol in myeloma cells (Fig. 3C). These findings suggest that mitochondrial dysfunction caused the release of cytochrome *c*, Smac/DIABLO and AIF into the cytosol, and that caspase-3 was then activated, thereby propagating the death signal.

Shikonin down-regulated the expression of Mcl-1 and up-regulated that of Bax and p53

To investigate the molecular mechanisms of shikonin-induced cell death in myeloma cells, the expression of several cell death-associated proteins was examined (Fig. 4). Mcl-1, a critical survival factor for myeloma cells, was down-regulated in shikonin-treated myeloma cells. The pro-apoptotic protein Bax and the tumor suppressor, p53, were up-regulated. In contrast, shikonin did not modulate the levels of anti-apoptotic protein Bcl-2 in myeloma cells (Fig. 4).

Shikonin inhibited IL-6-inducible STAT3 phosphorylation in myeloma cells

Numerous reports suggest that IL-6 promotes survival and proliferation of multiple myeloma cells through the phosphorylation of STAT3. We therefore examined whether shikonin could inhibit IL-6-induced STAT3 phosphorylation. As shown in Fig. 5, IL-6-induced STAT3 phosphorylation was clearly inhibited by shikonin in a time-dependent manner.

Shikonin-induced cell death was triggered by ROS production

To determine the intracellular concentration of ROS, IM9 myeloma cells were cultured with DHE, and the fluorescence was measured by flow cytometry (Fig. 6A). Treatment with shikonin 1 μM caused notable ROS production and cell death in myeloma cells. Shikonin-induced ROS production in myeloma cells was completely blocked by the antioxidant NAC (Fig. 6A, B). BSO, an inhibitor of glutathione (GSH) synthesis, is reported to increase ROS levels, accompanied by a reduction in GSH [16]. As shown in Fig. 6C, BSO enhances shikonin-induced cell death in myeloma cells via production of intracellular ROS. Interestingly, NAC decreased the phosphorylation of STAT3 in IL-6/shikonin-treated myeloma cells (Fig. 6D). These results indicate that the modulation of molecules involved in the redox system may determine the sensitivity of myeloma cells to shikonin.

Discussion

Shikonin is a naphthoquinone compound extracted from *Lithospermum erythrorhizon*. It has been shown to have a variety of biological activities such as anti-bacterial, anti-

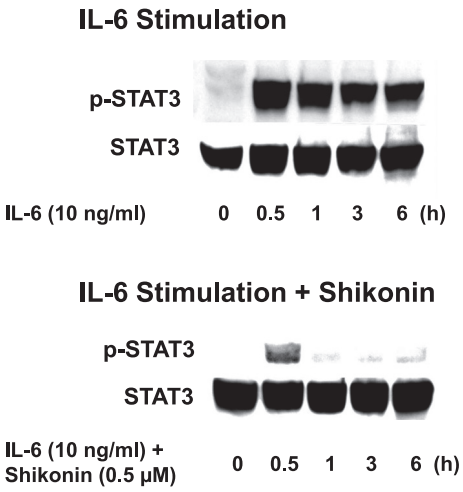
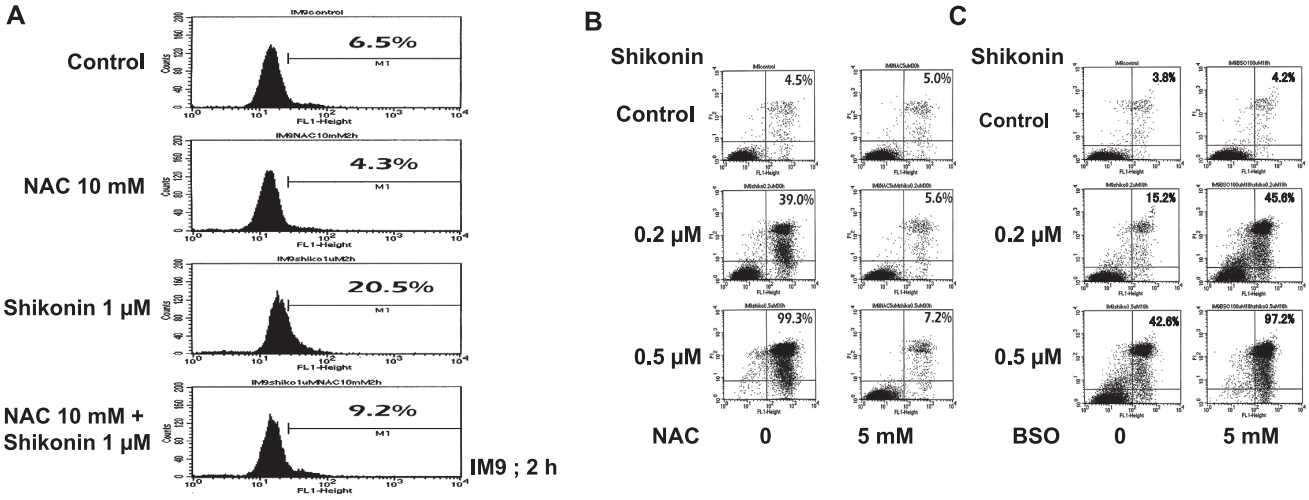


Figure 5. Shikonin induced cell death through by blocking the JAK/STAT signaling pathway. RPMI8226 cells were cultured with 10 mg/ml IL-6 +/– 0.5 μM of shikonin for the indicated time. RPMI8226 cells were treated with 10 ng/ml IL-6 for various times (0–6 h), and cell lysates (20 μg/lane) were fractionated on 12.5% SDS-polyacrylamide gels and Western blotting with antibodies against phospho-STAT3 and STAT was the performed (upper panel). RPMI8226 cells were then pretreated with 0.5 μM of shikonin for the indicated time, and cells were stimulated with IL-6 (10 ng/ml) for 15 min. Western blotting was performed to assess the phosphorylated states or the expression of STAT3 was performed (lower panel).



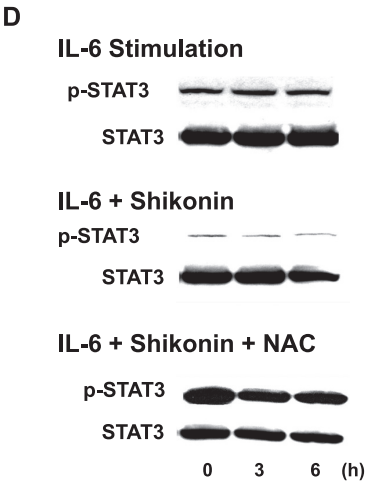


Figure 6. ROS generation by shikonin in myeloma cells. (A) To determine the intracellular concentration of ROS, IM9 myeloma cells were cultured with DHE, and the fluorescence was measured by flow cytometry. DHE-derived fluorescence in untreated IM-9 cells and in cultures treated for 3 h with 1 μM shikonin is shown. Treatment with 10 mM of antioxidant NAC completely inhibited production of intracellular ROS in IM9 cells. (B) The antioxidant, NAC, blocked shikonin-induced cell death in IM9 cells. IM9 cells were treated with various concentrations (0–0.5 μM) of shikonin for 24 h following preincubation with 5 mM NAC. Annexin V-FITC/PI cells were measured using a FACSCalibur flow cytometer. (C) BSO to decrease intracellular levels of reduced GSH enhanced shikonin-induced cell death in IM9 cells. Cells were pretreated with 100 μM BSO for 4 h, and were then treated with or without 0.2 and 0.5 μM shikonin for 24 h. Annexin V-FITC/PI cells were measured using a FACSCalibur flow cytometer. (D) Effects of the antioxidant NAC on the phosphorylation of IL-6/shikonin-treated RPMI8226 cells. Cells were treated with 5 mM NAC, and then cultured with shikonin and IL-6 for the indicated time. Western blotting to assess the phosphorylation stated and performed.

inflammatory, and anti-tumor effects[10]. Shikonin ointment also has the ability to accelerate wound healing[10]. Recently, it has been reported that shikonin inhibited growth of osteosarcoma and Ewing sarcoma cells[17,18]. In addition, shikonin has been associated with cell death and inhibition of angiogenesis in studies that showed increased DNA fragmentation and caspase-3 substrate cleavage in HL60 and K562 leukemic cells[19]. It is also reported to inhibit transcription factor IID binding to the TNF-α promoter and block TNF-α pre-mRNA splicing, resulting in suppression of TNF-α[20,21].

Multiple myeloma is a plasma cell neoplasm derived from clonal B cell lineage cells. The development of new agents

such as the proteasome inhibitor, bortezomib, and the immunomodulatory drugs, thalidomide and lenalidomide, has led to improved outcomes in patients with multiple myeloma [22,23]. However, a higher proportion of patients cannot expect long-term remission due to drug-resistant disease, minimal residual disease, or complications such as severe infections [1–4]. Therefore, new potent therapeutic agents and substantial therapeutic advances are needed for the treatment of multiple myeloma.

In this study, we have demonstrated that shikonin rapidly induces apoptotic cell death in various myeloma cell lines in association with the down-regulation of anti-apoptotic protein, Mcl-1 and up-regulation of pro-apoptotic protein Bax, the loss of mitochondrial membrane potentials (ΔΨm), the release of mitochondrial apoptogenic proteins such as cytochrome *c*, Smac/DIABLO and AIF from mitochondria into the cytosol, and the activation of caspase-3. Bax is a pro-apoptotic member of the Bcl-2 family that resides in the cytosol and translocates to mitochondria during induction of cell death [24]. Compared with chemosensitive myeloma cells, chemoresistant myeloma cells are also reported to express higher levels of the anti-apoptotic proteins, Bcl-2 and Bcl-xL [25,26]. Previous studies have found that shikonin inhibits the expression of Bcl-2 during induction of apoptotic cell death in myeloma cells. Recent reports suggest that alterations in the ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family, rather than the absolute expression level of any single Bcl-2 member, can determine apoptotic sensitivity, which would interfere with the availability and translocation of the Bax protein from the cytosol to mitochondria [27]. We also found that Smac, which stands for second mitochondria-derived activator of caspases, was released from mitochondria to the cytosol during shikonin-induced cell death. Smac binds to XIAP and eliminates its inhibitory effect on caspase-9. Various anti-myeloma agents trigger the loss of mitochondrial ΔΨm and the release of mitochondrial apoptogenic proteins cytochrome *c* and Smac/DIABLO [24,28].

Elevation of intracellular ROS production was also demonstrated during shikonin-induced cell death in myeloma cell lines. Various studies have shown that stress-induced changes in mitochondrial transmembrane potential correlate with an increase in ROS and the release of mitochondrial cytochrome *c* and Smac/DIABLO. The role of ROS in mediating apoptotic cell death in various cancer cells is well established [29,30]. The generation of ROS has also been linked to the release of Smac or cytochrome *c* from mitochondria to the cytosol during apoptosis [31]. The present study found that the antioxidant, NAC, significantly blocked ROS production and shikonin-induced cell death via inhibition of phosphorylation of STAT3 in myeloma cells. These results suggest that ROS is a key an upstream mediator during shikonin-induced apoptotic cell death in myeloma cells, as well as in leukemic cells as previously reported [32]. Previous investigations have reported that shikonin is an inhibitor of tumor proteasome activity *in vitro* and *in vivo*, and it might be one of the important molecular mechanism in shikonin-induced anti-tumor effect [6]. Although specific molecular targets for shikonin has not been discovered, further studies to examine the accumulation of various ubiquitinated proteins in shikonin-treated myeloma cells for assessing proteasome activity are also needed.

In conclusion, our results indicate that shikonin induces apoptotic cell death of human multiple myeloma cells via production of ROS, resulting in mitochondrial injury and caspase activation in addition to inhibiting the phosphorylation of STAT3. Natural compounds might have multiple targets for their biological effects on cancer cells. Therefore, shikonin has a potential as a novel agent for the treatment of multiple myeloma with less toxic effects than existing drugs.

Acknowledgements

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Conflict of Interest

The authors have no conflicts of interest.

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