Shikonin Induces Apoptosis through Reactive Oxygen Species/Extracellular Signal-Regulated Kinase Pathway in Osteosarcoma Cells

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Shikonin, a major ingredient in the Chinese traditional herb Lithospermum erythrorhizon, exhibits multiple biological functions including antimicrobial, anti-inflammatory, and antitumor effects. In this study, we delineated the molecular mechanisms of shikonin in the apoptosis of 143B osteosarcoma cells. Shikonin reduced the cell viability of 143B cells in a dose- and time-dependent manner. The IC₅₀ at 24 h and 48 h for 143B cells was 4.55 and 2.01 μM, respectively. A significantly elicited hypodiploid cell population was found in cells treated with 2, 4, and 8 μM shikonin for 24 h. Moreover, treatment with shikonin induced reactive oxygen species (ROS) generation, increased extracellular signal-regulated kinase (ERK) phosphorylation, decreased B-cell lymphoma-2 (Bcl2) expression, and was accompanied by poly(ADP-ribose) polymerase (PARP) cleavage. Pretreatment with the antioxidant agent N-acetyl cysteine (NAC) not only reversed shikonin-induced ROS generation but also significantly attenuated the cytotoxic effects of shikonin in 143B cells. Furthermore, NAC attenuated shikonin-induced ERK phosphorylation. Taken together, our results reveal that shikonin increased ROS generation and ERK activation, and reduced Bcl2, which consequently caused the cells to undergo apoptosis. Therefore, shikonin may be a promising chemotherapeutic agent for osteosarcoma treatment.

Key words apoptosis; extracellular signal-regulated kinase; osteosarcoma; shikonin

It is well documented that extracts from dietary foods or medical plants can function as chemopreventive agents and affect the processes of tumorigenesis, including the initiation and promotion of several human tumors. Shikonin, one of the major naphtoquinone pigments isolated from the Chinese plant Lithospermum erythrorhizon, has been used as an ointment for wound healing. Now, extensive reports have demonstrated that this compound exhibits many biological functions such as antimicrobial, anti-inflammatory, and antitumor effects. Shikonin contains stronger antiyeast-like activity against the fungi Candida krusei and Saccharomyces cerevisiae than fluconazole. Shikonin has also been shown to inhibit the replication of human immunodeficiency virus (HIV) in human monocyties.

The antitumor effect of shikonin was first evidenced by its activity against ascite sarcoma 180 at a dose of 5—10 mg/kg/d. It has also been demonstrated that the administration of shikonin reduces the volume of intestinal neoplasms induced by azoxymethane. Recently, shikonin has been shown to induce apoptosis in several human tumors. The treatment of HL-60, HeLa, bladder carcinoma cells, and melanoma cells with shikonin induced apoptosis through increased caspase-3 activity. Gao et al. showed that shikonin significantly decreased total thiols, protein thiols, and glutathione levels but elevated lipid peroxidation in HL-60 human leukemia cells. In addition, shikonin enhanced the production of intracellular reactive oxygen species (ROS) and subsequently triggered apoptosis in human SK-Hep-1 hepatoma cells and K562 leukemia cells. It also has been demonstrated that diminished ROS production by scavengers blocked shikonin-induced apoptosis. In human colorectal carcinoma cells, treatment with shikonin increased p53, p27, and Bad expression, decreased Bcl-2 and Bcl-2L expression, enhanced caspase activities, and consequently triggered apoptosis. Shikonin inhibited mitogen-activated protein kinase (MAPK) and protein tyrosine kinase activities and increased apoptosis-related protein kinase activity, which caused epidermoid carcinoma cells to undergo apoptosis.

Osteosarcoma is the most frequent primary bone tumor and is typically characterized by spindle cells that produce osteoid. Osteosarcoma can be diagnosed at any age, with a higher incidence in children and young adults. When patients received surgery alone, the survival rate is under 20%, but with surgery and chemotherapy, the overall survival rate is between 50 and 60%. Due to the high motility and increasing drug resistance of osteosarcoma, emerging studies have focused on extracts of traditional medicine for its treatment. Jang et al. showed that flavonoids isolated from Rhus verniciflua Stokes inhibited its proliferation and triggered apoptosis in human osteosarcoma cells. These compounds induced p53 and Bax expression, decreased the Bcl2 level, and subsequently induced apoptosis in osteosarcoma cells. Recently, flavonoids such as genistein and quercetin have been shown to trigger apoptosis in osteosarcoma cells that were accelerated by the e-Jun N-terminal kinase (JNK) inhibitor or down-regulation of JNK by siRNA. Green tea polyphenol also induced apoptosis of osteosarcoma cells through the down-regulation of nuclear factor-κB (NF-κB) and activation of the caspase cascade. Shikonin has been shown to trigger apoptosis in several human cancers; however, its role in osteosarcoma remains a puzzle. In this study, shikonin induced apoptosis through generation of ROS, decreased Bcl2 expression, activated caspase3, and was accompanied by poly(ADP-ribose) polymerase (PARP) cleavage. Pretreatment with an antioxidant agent attenuated shikonin-induced apoptosis. To our knowledge, this is the first report to delineate the molecular mechanisms of shikonin in the...
apoptosis of osteosarcoma cells.

MATERIALS AND METHODS

Chemicals All chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Anti-phospho-ERK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-extracellular signal-regulated kinase (ERK), anti-Bcl2 and anti-β-actin were purchased from Cell Signaling (Boston, MA, U.S.A.). Shikonin was obtained from Calbiochem (San Diego, CA, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from GIBCO (NY, U.S.A.).

Cell Culture Human osteosarcoma 143B cells were obtained from American Type Culture Collection (ATCC) and were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 4 mM l-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% CO2.

MTT Assay Human 143B cells were seeded in 24-well plates at a density of 1×10^5/ml and were treated with the indicated concentration of shikonin for 24 or 48 h. The cells were treated with a medium containing 5.0 g/l 3-(4,5-dimethylthiazol-2)-2 and 5-diphenyltetrazolium bromide (MTT), and incubated at 37 °C for 2 h. After washing with phosphate-buffered saline (PBS), dark blue formazan was dissolved in 1 ml isopropanol, and the absorbance was measured at 563 nm. The relative cell viability was determined by the absorbance of OD 563 in each well.

Flow Cytometric Analysis The 143B cells were treated with the indicated concentrations of shikonin for 24 or 48 h. The cells were washed with PBS, detached by trypsinization, and fixed in 70% ethanol at -20 °C overnight. The cells were then washed with ice-cold PBS and stained with 50 µg/ml propidium iodine (PI) in the dark for 15 min. The distribution of cell cycle phases was detected using the BD Biosciences FACScan system with CellQuest™ Pro software (BD Biosciences, San Jose, CA, U.S.A.). The percentage of apoptosis was quantified by counting the fraction of cells with DNA contents at the sub-G1 phase.

4,6-Diamidino-2-phenylindole Dihydrochloride Hydrate (DAPI) Staining The 143B cells were seeded in 100 mm Petri dishes at a density of 2×10^5/ml. The cells were then treated with shikonin or dimethyl sulfoxide (DMSO) for 48 h, fixed in PBS containing 4% paraformaldehyde for 30 min, and incubated with DAPI (1 mg/ml) for 30 min. The apoptotic nuclei characterized as intensively stained were detected using Nikon fluorescent microscopy.

Detection of ROS The intracellular ROS was evaluated by determining the level of hydrogen peroxide (H2O2) using a 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescent probe. In the presence of H2O2, DCFDA was converted into 2',7'-dichlorofluorescein (DCF), which can be detected by flow cytometry. Briefly, 5×10^4 cells were plated in six-well plates and allowed to attach overnight. After being treated with 2, 4, and 8 µM shikonin for 24 h, the cells were incubated with 10 µM DCFDA for an additional 30 min, followed by washing and resuspending in PBS. The fluorescence was detected using the BD Biosciences FACScan system.

Western Blot Analysis The cell lysates from shikonin-treated 143B cells were obtained, and protein concentrations were measured using a Bradford protein assay kit (Bio-Rad, U.S.A.). Twenty-five micrograms of protein was separated by 10% polyacrylamide gel and transferred into a nitrocellulose membrane. The membrane was blocked by PBS containing 0.5% nonfat milk for 1 h at room temperature. Afterwards, the membrane was incubated with primary antibodies at room temperature for 1 h or at 4 °C overnight. The membrane was washed with PBS containing 0.1% Tween-20, followed by reaction with horseradish peroxidase (HRP)-conjugated bovine anti-goat immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology, U.S.A.; 1 : 5000 dilution). The membrane was then extensively washed with PBST, and the reactive signal was detected by enhanced chemiluminescence (ECL commercial kit, Amersham Pharmacia Biotech, U.K.). The β-actin expression was used as the internal control.

Statistical Analysis Data reported are the mean±standard deviation of three independent experiments. They were evaluated by one-way analysis of variance (ANOVA). Significant differences were established at p<0.05.

RESULTS

Cytotoxicity of Shikonin on 143B Osteosarcoma Cells We first sought to determine the cytotoxic effects of shikonin on osteosarcoma cells. Human osteosarcoma 143B cells were treated with different concentrations of shikonin (0.5 to 8 µM) for 24 and 48 h. As shown in Fig. 1, the reduced viability of cells treated with 0.5, 1, 2, 4, and 8 µM shikonin was 98.86±0.39, 98.35±0.99, 86.54±1.77, 53.46±5.00, and 28.16±9.27%, respectively, at 24 h compared with the vehicle-treated cells. For the 48-h treatment, cell viability was 95.06±2.31, 89.4±3.07, 50.24±7.12, 11.16±4.3, and 2.33±1.35%. The IC50 of shikonin was 4.55 and 2.01 µM for 24 and 48 h, respectively. Our data indicated that shikonin exhibited dose- and time-dependent inhibitory effects on the viability of 143B cells.

Shikonin-Induced Apoptosis and Cell Cycle Arrest It
is well known that alternation of cell morphology, apoptotic body formation, and DNA condensation are the hallmarks of apoptosis. To confirm that shikonin induced apoptosis, shikonin-treated 143B cells were subjected to morphology analysis and DAPI staining. In the presence of shikonin, the cell number was reduced and the cell shape became round up, and apoptotic bodies were found (Fig. 2a). Shikonin also induced nuclear condensation, as evidenced by an intensive

![Figure 2](image1.png)

**Fig. 2. The Effects of Shikonin on Cell Morphology and DNA Condensation of 143B Cells**

143B cells were treated with different concentrations of shikonin for 24 h (a) and 48 h (b). Cell morphology (left panels) was detected by using a phase contrast microscope with 100× magnification. DNA condensation (right panels) was measured by DAPI stain and analyzed under a Nikon fluorescent microscope. Scale bar indicates 10 μm.

![Figure 3](image2.png)

**Fig. 3. Shikonin Induced Apoptosis in 143B Cells**

(a) Cells were treated with the indicated concentrations of shikonin for 24 (left panels) and 48 (right panels) h. Cell cycle distribution was analyzed by flow cytometry using propidium iodide staining. The G0/G1 and G2/M phases are indicated with an arrow and arrow head respectively. The apoptotic cell (sub-G1 population) content (b) was counted and presented as the mean±S.D. obtained from three independent experiments. *p<0.05; **p<0.001.
ERK, Bcl2, and caspase 3 were quantified and compared to the vehicle treated group.

To address whether shikonin changed the cell cycle distribution in 143B cells, shikonin-treated 143B cells were subjected to apoptosis assay by propidium iodide staining. The percentage of apoptotic cells was 4.01±1.20, 3.27±0.65, 5.09±1.55, 7.15±2.77, and 8.25±2.63% in response to the vehicle, 1, 2, 4, and 8 μM shikonin, respectively, for 24 h (Fig. 3). In treatment with 1, 2, 4, and 8 μM shikonin for 48 h, the apoptotic cells increased to 6.48±0.5, 12.90±1.19, 18.60±2.54, and 9.05±3.88%, respectively. Moreover, cells treated with 4 and 8 μM shikonin for 24 h obviously caused G2/M phase arrest (21.73±2.68% in 4 μM and 24.01±3.25% in 8 μM compared to 17.28±2.35% in vehicle treated cells) and reduced the G0/G1 cell population (39.52±6.19% in 4 μM and 33.05±3.10% in 8 μM compared to 51.93±3.07% in vehicle treated cells) (Table 1). On the other hand, a decreased G0/G1 phase and increased S phase was found in 2, 4, and 8 μM shikonin treated for 48 h (Table 2). Overall, our results indicate that the number of apoptotic cells was markedly increased in response to shikonin treatment in a dose- and time-dependent manner.

The Effects of Shikonin on Apoptosis-Related Molecules

To verify the mechanism by which shikonin caused

Table 1. The Cell Cycle Distribution of 143B Cell Treated with Different Concentration of Shikonin with or without 1 mM NAC for 24 h

<table>
<thead>
<tr>
<th>Sub-G1 (%)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.01±1.02</td>
<td>51.93±3.07</td>
<td>13.03±4.92</td>
</tr>
<tr>
<td>Shikonin 1 μM</td>
<td>3.27±0.65</td>
<td>52.98±3.44</td>
<td>12.38±4.31</td>
</tr>
<tr>
<td>Shikonin 2 μM</td>
<td>5.09±1.55</td>
<td>51.69±3.73</td>
<td>12.41±4.54</td>
</tr>
<tr>
<td>Shikonin 4 μM</td>
<td>7.15±2.77</td>
<td>39.52±6.19</td>
<td>16.85±2.26</td>
</tr>
<tr>
<td>Shikonin 8 μM</td>
<td>8.25±2.63</td>
<td>33.05±3.10</td>
<td>17.89±2.25</td>
</tr>
<tr>
<td>NAC alone</td>
<td>2.67±0.77</td>
<td>45.06±9.06</td>
<td>21.53±3.22</td>
</tr>
<tr>
<td>Shikonin 4 μM+NAC</td>
<td>3.05±0.33</td>
<td>43.69±3.42</td>
<td>22.24±0.55</td>
</tr>
<tr>
<td>Shikonin 8 μM+NAC</td>
<td>4.31±0.39</td>
<td>45.69±3.42</td>
<td>20.28±1.62</td>
</tr>
</tbody>
</table>

Table 2. The Cell Cycle Distribution of 143B Cell Treated with Different Concentration of Shikonin with or without 1 mM NAC for 48 h

<table>
<thead>
<tr>
<th>Sub-G1 (%)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.83±1.94</td>
<td>49.00±2.24</td>
<td>11.07±0.56</td>
</tr>
<tr>
<td>Shikonin 1 μM</td>
<td>6.84±0.50</td>
<td>43.00±2.65</td>
<td>11.09±2.15</td>
</tr>
<tr>
<td>Shikonin 2 μM</td>
<td>12.90±1.19</td>
<td>38.38±4.17</td>
<td>14.23±1.04</td>
</tr>
<tr>
<td>Shikonin 4 μM</td>
<td>18.60±2.54</td>
<td>27.41±2.89</td>
<td>17.48±2.16</td>
</tr>
<tr>
<td>Shikonin 8 μM</td>
<td>9.05±3.88</td>
<td>39.82±2.97</td>
<td>14.41±1.39</td>
</tr>
<tr>
<td>NAC alone</td>
<td>2.58±0.94</td>
<td>49.84±7.45</td>
<td>18.60±5.69</td>
</tr>
<tr>
<td>Shikonin 4 μM+NAC</td>
<td>3.25±1.12</td>
<td>54.19±1.12</td>
<td>16.26±1.80</td>
</tr>
<tr>
<td>Shikonin 8 μM+NAC</td>
<td>5.42±1.12</td>
<td>48.88±6.62</td>
<td>15.32±0.48</td>
</tr>
</tbody>
</table>

Fig. 4. The Effects of Shikonin on Apoptosis-Related Protein Expression

143B cells were treated with the indicated concentrations of shikonin for 24 (a) and 48 (b) h. Cell lysates were harvested and subjected to Western blot analysis using antibodies as indicated. The Western blot data represent one of the three independent experiments. β-Actin was used as a loading control. The intensity of cleavage PARP, Phospho-ERK, ERK, Bcl2, and caspase 3 were quantified and compared to the vehicle treated group.
apoptosis, the expression of apoptosis-related proteins was measured by Western blot analysis. Previous reports have shown that ERK signaling is involved in several events of flavonoid and/or ROS-induced apoptosis.25) As shown in Fig. 4, phosphorylated ERK was apparently increased in response to shikonin treatment for 24 and 48 h. It has been documented that Bcl-2 family proteins play a pivotal role in apoptosis.26) Shikonin treatment significantly reduced Bcl2 expression. In addition, decreased pro-caspase 3 was detected in proportion to shikonin concentration. PARP cleavage, another well-known characteristic of apoptosis, was also found in shikonin-treated cells.

**Shikonin-Induced Accumulation of ROS** A previous report has shown that shikonin increased ROS production in SK-Hep-1 cells.15) To address whether shikonin enhanced ROS generation in 143B cells, the intracellular H2O2 concentration was detected by DCFDA-based flow cytometric analysis. As shown in Fig. 5a, a significantly elevated ROS concentration was found in cells treated with 4 and 8 μM shikonin for 24 h.

To determine whether ROS was involved in shikonin-induced cell death, 1 mM of the ROS scavenger N-acetyl cysteine (NAC) was co-incubated with shikonin 4 and 8 μM for 24 h. After 24-h exposure to 4 and 8 μM shikonin combined with 1 mM NAC, cell viability significantly increased from 37.45±5.34% and 22.45±6.47% to 92.40±2.95 and 79%, respectively (Fig. 5b). Similarly, co-treatment of 4 or 8 μM shikonin with 1 mM NAC obviously increased the cell viability from 5.25±0.96% and 2.98±1.22% in shikonin treated alone to 97.50±0.93% and 90.73±6.18%, respectively. Moreover, cell morphology was changed from round to fibroblast-like phenotype and accompanied with decreased DAPI staining in the presence of 1 mM NAC (Figs. 5c, d).

To investigate whether NAC also reduced shikonin-induced ERK phosphorylation, cell lysates derived from shikonin alone and shikonin combined with NAC were subjected to Western blot analysis. As expected, 1 mM NAC significantly inhibited shikonin-induced activation of ERK (Fig. 5e). Overall, our results reveal that NAC significantly protected cells from shikonin-induced death by attenuating ROS generation.

**ERK Inhibitor Has No Effects on Shikonin Inhibited Cell Proliferation** To address the role of ERK phosphorylation of shikonin-induced cell apoptosis, cell viability of 143B cells co-treated with different concentrations of the ERK inhibitor PD98059 with 4 or 8 μM shikonin for 24 h was measured by MTT assay. Treatment with 4 and 8 μM shikonin decreased cell viability to 23.84±5.22% and 20.69±4.34% compared to the vehicle treated group. Conversely, reduced cell viability was observed when treated with 20 (90.64±9.66%), 30 (76.21±7.13%), or 50 (77.60±6.13%) μM PD98059 alone. Co-treatment of 4 μM shikonin with 20, 30, or 50 μM PD98059 reduced the cell vi-

![Image](http://example.com/image.png)

**Fig. 5.**
Fig. 5. Effects of N-Acetyl Cysteine (NAC) on Shikonin-Induced ROS Generation (a), Cell Death (b—d), and ERK Phosphorylation (c)

143B cells were pretreated or not pretreated with 1 mM NAC for 1 h and were incubated with 0, 4, or 8 μM shikonin for an additional 24 h. The ROS concentration was detected by DCFDA stain (a). The Y and X axis indicate the cell counts and fluorescent intensity of DCFDA, respectively. The dashed line denotes an intensity above $10^2$. Cell viability was measured by MTT assay (b). The cell morphology and DAPI stain for 24 (c) and 48 (d) are shown. Cell morphology (left panels) was detected by using a phase contrast microscope with $100\times$ magnification. DNA condensation (right panels) was measured by DAPI stain and analyzed under a Nikon fluorescent microscope. Scale bar indicates 10 μm.

143B cells were pretreated with or not pretreated with 1 mM NAC for 1 h, and were incubated with 0, 2, or 4 μM shikonin for an additional 24 h; cell lysates were harvested and subjected to Western blot analysis (e). The Western blot data represent one of the three independent experiments. β-Actin was used as a loading control. The intensity of Phospho-ERK and ERK were quantified and compared to the vehicle treated group.
ability to 17.68±6.68%, 15.76±6.17%, and 15.29±1.11%, respectively. Co-treatment of 8 mM shikonin with 20, 30, or 50 mM PD98059 reduced the cell viability to 13.51±3.87%, 12.83±3.75%, and 11.44±1.54%, respectively (Fig. 7). Compared to treatment with 4 and 8 mM shikonin, co-treatment with PD98059 slightly decreased cell viability, although statistical significance was not reached.

**DISCUSSION**

Due to the often-acquired drug resistance and metastasis, the overall survival rate of patients with osteosarcoma is around 50—65%\(^{19}\); therefore, a more effective, less toxic osteosarcoma treatment would be of value. Recently, the utilization of natural bioactive compounds extracted from dietary foods, vegetables, or traditional Chinese herbs for cancer chemoprevention has received much attention.\(^{27,28}\) Shikonin, a naphthoquinone derivative compound identified in *Lithospermum erythrorhizon*, has been shown to exert various biological properties such as anti-inflammation\(^{5}\) and antitumor\(^{29}\) effects. In this study, we investigated the molecular mechanisms of shikonin on the apoptosis of 143B osteosarcoma cells.

Previous reports have demonstrated the antitumor effects of shikonin. Shikonin-rendered cell apoptosis through activation of a caspase-dependent pathway was found in HL60 human leukemia cells,\(^{10}\) malignant melanoma cells,\(^{13}\) Hela cells,\(^{11}\) colorectal cancer,\(^{17}\) and breast cancer.\(^{30}\) Indeed, shikonin diminished the cell proliferation of 143B osteosarcoma cells in a dose- and time-dependent manner. Shikonin-induced apoptosis in 143B osteosarcoma cells was evidenced by an increased sub-G1 population and enhanced DNA condensation. Recently, shikonin triggered necrosis in drug-resistant MCF-7 breast cells through a caspase-independent pathway.\(^{13}\) Wu *et al.* showed that high-dose shikonin induced apoptosis in the early stages, but necrosis played a major role after treatment for 48 h in A375-S2 melanoma cells.\(^{15}\) Of interest is that 143B cells treated with 8 \(\mu M\) shikonin for 48 h showed reduced cell viability of nearly 98%, but the percentage of cells in the sub-G1 phase was only 9.05%. The low percentage of apoptotic cells with this treatment implied that...
cells might undergo necrosis, as evidenced by the round-up cells with a condensed nucleus under phase contrast microscopy. Moreover, decreased G0/G1 phase was detected in cells treated with shikonin 24 (4, 8 μM) and 48 h (2, 4, 8 μM). A previous report has shown that shikonin induced p53 expression, which in turn rendered malignant melanoma A375-S2 cells to undergo arrest in the G2/M phase of the cell cycle.13) Wu and co-workers also demonstrated that HeLa cells treated with 10 μM shikonin resulted in the prevention of G1 to S phase transition and blocked DNA synthesis.11) Very recently, G0/G1 arrest due to increased p21 and decreased cyclin E, cdk4 and cdk6 protein expression was found in human bladder cancer cells exposed to shikonin for 72 h.32) Our results suggest that shikonin may induce different cell cycle arrest in different cell types.

ROS mainly generated from cellular metabolism can damage DNA, protein, and lipid. Several studies have demonstrated that bioactive food compounds manifest their chemopreventive effects through the generation of ROS, leading to the development of oxidative stress and subsequent apoptosis.25,33,34) Chen et al. demonstrated that bioactive food compounds manifest their chemopreventive effects through the generation of ROS, leading to apoptosis.26) Reports have shown that modulation of the Bax/Bcl-2 family proteins, including anti-apoptotic agents such as Bax, Bad, and Bak, played a critical role in apoptosis by regulating mitochondrial membrane integrity.26) Decreased Bcl-2 and/or increased Bax, which reduced the Bcl-2/Bax ratio, led to mitochondria dysfunction, triggered activation of the caspase cascade, and consequently, caused cells to undergo apoptosis.40) Reports have shown that modulation of the Bax and/or Bcl-2 protein level and increased caspase 3 activity were involved in the apoptosis induced by shikonin and its derivatives.13,35) In this study, treatment with shikonin also decreased Bcl-2 expression, and decreased the pro-caspase 3 expression which accompanied the cleavage of PARP, but there was no effect of Bax expression (data not shown) on osteosarcoma cells. Consistent with our observation, shikonin had little effect on Bax expression, but it apparently decreased the level of Bcl-2 in colorectal cancer cells.15) Taken together, modulation of the Bcl-2 family protein expression level and caspase 3 activation was involved in shikonin-mediated apoptosis.

The MAPK family proteins are composed of three major groups: ERK, JNK, and p38.36) These proteins are activated in response to various stimuli, such as growth hormones, cytokines, and environmental stresses.35) Emerging evidence suggests that the activation of ERK by increasing the ROS level contributes to apoptosis. Kim et al. showed that kaempferol caused MCF-7 breast cancer cells to undergo apoptosis through sustained ERK activation and elevated ROS generation, which could be abrogated by PD98059 (ERK inhibitor) or NAC (ROS scavenger).37) Similarly, kaempferol triggered apoptosis in glioma cells through an ERK-dependent pathway. Wu et al. showed that shikonin enhanced ERK activity and resulted in the triggering of apoptosis in HeLa cells.11) In agreement with previous reports, shikonin apparently induces the activation of ERK, but when pretreated with NAC, a ROS scavenger, shikonin diminishes the activation of ERK. Intriguingly, co-treatment with shikonin and ERK inhibitor (PD98059) enhanced but did not reduce shikonin-induced apoptosis. Generally, ERK is viewed as a cell proliferative and a survival mediator involved in hormone signals against cell death. It was reasonable to provide the hypothesis that elevated ERK activity may also function as a survival signal to overcome shikonin-induced cell death. Inhibition of the survival signal induced more apoptosis. Moreover, treatment with shikonin for 24 h reduced ERK expression, indicating that shikonin not only alters the protein level but also the phosphorylation status of ERK. Overall, our data suggest that ROS plays a critical role in shikonin-elicited apoptosis, and ERK may act as one of the downstream signaling molecules of ROS.

In summary, our results indicate that shikonin induced cell cycle arrest and apoptosis in 143B osteosarcoma cells. Shikonin triggered ROS production, activated ERK, decreased Bcl-2 expression, activated caspase 3, and accompanied PARP cleavage, consequently resulting in apoptosis in osteosarcoma cells. Our results also suggest that shikonin is a promising chemotherapeutic agent for treating osteosarcoma.

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