Full Paper

p53-Mediated Cell Cycle Arrest and Apoptosis Induced by Shikonin via a Caspase-9-Dependent Mechanism in Human Malignant Melanoma A375-S2 Cells

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Received November 4, 2003; Accepted December 22, 2003

Abstract. Natural products regulate cell growth in response to oncogene activation that induces cell cycle arrest and apoptosis in tumor cell lines. We investigated the mechanisms of caspase activation in human malignant melanoma, A375-S2 cells, by the natural product shikonin, which was isolated from the plant *Lithospermum erythrorhizon* SIEB. et ZUCC. Shikonin inhibited cell growth in a time- and dose-dependent manner, which might be mediated through up-regulation of p53 and down-regulation of cyclin-dependent protein kinase 4. Caspase activation was detected in shikonin-induced cell apoptosis, which involved in a post-mitochondrial caspase-9-dependent pathway. Decreased Bcl-2 protein levels and increased Bax protein levels were positively correlated with elevated expression of p53 protein. Apoptosis-inducing factor, another apoptotic protein of mitochondria, partially contributed to shikonin-induced release of cytochrome *c*. Taken together, shikonin-induced DNA damage activates p53 and caspase-9 pathways.

Keywords: shikonin, cell cycle arrest, apoptosis, caspase, p53

Introduction

Shikonin, a red naphthoquinone pigment isolated from the ground rhizome of *Lithospermum erythrorhizon* SIEB. et ZUCC., which has been used in East Asia for treating burns, has anti-inflammatory (1, 2) and antitumor effects (3). It was reported that shikonin induced apoptosis in human premyelocytic leukemia HL60 cells via a caspase-3-dependent mechanism (4). Shikonin induced mammalian topoisomerase II-mediated DNA cleavage in vitro (5). Topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription (6). The DNA damage response, caused by a variety of stimuli including UV and epotocide, arrests the cell cycle to allow damage repair or direct cell apoptosis (7). An imbalance between DNA damage and DNA repair activities may affect the cell's viability.

After DNA damage, the cell cycle was arrested at the transition from the G1 to S phase or from the G2 to M phase of the cell cycle. Growing evidence indicates a central role for p53 in mediating cell cycle arrest or apoptosis (8, 9).

Apoptosis is a biological process that removes cancerous or virally infected cells. Aberrant apoptosis is the major cause for tumor development and progression. The development of cancer is associated with several key events, including deregulated cell growth in response to oncogene activation that sensitizes cells to apoptosis. Apoptosis is executed by caspases, a family of intracellular aspartate-specific cysteine proteases, which amplify the apoptotic signal and proteolytically process numerous cellular target molecules with different functions (10). A hallmark of DNA damage-triggered apoptosis is reduced Bcl-2 expression, which was followed by caspase-9/caspase-3 activation and DNA degradation. Bcl-2 protein family members including Bcl-2 and Bax function either to inhibit or

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promote apoptotic cell death (11). Once apoptosis was initiated, Bcl-2 was cleaved by caspase-3 to attenuate its anti-apoptotic effect (12). The subtle balance of the Bcl-2/Bax complex might modulate the anti- or pro-apoptotic effect. AIF, apoptosis-inducing factor, is a mitochondrial flavoprotein that is released in response to death stimuli. It is reported that activation of poly (ADP-ribose) polymerase-1 (PARP-1) is required for translocation of AIF from the mitochondria to the nucleus and AIF is necessary for PARP-1-dependent cell death (13).

In the present study, we demonstrated that activated p53 contributed to shikonin-induced cell cycle arrest and apoptosis, and up-regulation of Bax and down-regulation of Bcl-2 amplified the activation of caspase cascade, resulting in apoptosis in A375-S2 cells.

Materials and Methods

Materials

Shikonin was obtained from Beijing Institute of Biologic Products (Beijing, China). Caspase-8 apoptosis detection kit, caspase-3 apoptosis detection kit, and rabbit polyclonal antibodies against inhibitor of caspase-activated deoxyribonuclease (ICAD), Bcl-2, and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against p53 was purchased from Oncogene Research Products (Boston, MA, USA). Ac-Val-Ala-Asp-CMK (Ac-YVAD-CMK), Z-Ile-Glu (OMe)-Thr-Asp (OMe)-FMK (Z-IETD-FMK), and Z-LEHD-FMK were purchased from ICN (Aurora, OH, USA). Benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) were purchased from Calbiochem (La Jolla, CA, USA). Hoechst 33258, propidium iodide (PI), RNase A, proteinase K, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,4-Dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), rabbit polyclonal antibodies against cytochrome *c* and AIF, mouse polyclonal antibodies against cyclin-dependent protein kinase (cdk) 4, and rabbit polyclonal antibodies anti-PARP were obtained from Medical & Biological Laboratories (Osaka).

Cell lines and cell culture

Human malignant melanoma cells (A375-S2 #CRL-1872) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine

(2 mM, Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml), and maintained at 37°C with 4% CO₂ in a humidified atmosphere.

Viability assay

The inhibitory effect of shikonin on the growth of A375-S2 cells was measured by MTT assay as described (14). The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 5×10^3 cells per well. After a 12-h incubation, they were treated with various concentrations of shikonin for the indicated time periods. The cells were incubated with inhibitors for 1 h prior to the administration of shikonin. Cell growth was measured with an ELISA reader (Tecan Spectra, Wetzlar, Germany) by MTT assay at the indicated time points.

$$\text{Viability (\%)} = 100 - [(A_{490, \text{control}} - A_{490, \text{shikonin}}) / A_{490, \text{control}} \times 100]$$

Flow cytometric analysis

A375-S2 cells (1×10^6 cells) were harvested and washed once in cold PBS. Cell pellets were fixed in 70% ethanol and washed in cold phosphate buffered saline (PBS). Then the pellets were suspended in 1 ml of PI solution containing 50 µg/ml of PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) Triton X-100. Cell samples were incubated at 4°C in the dark for at least 15 min and analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cycle analysis

A375-S2 cells (1×10^6 cells) were incubated with 2.5 mM thymidine (cell cycle blocker from G1 to S phase) for 18 h and then collected by centrifugation at $100 \times g$ for 5 min. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and cultured for 15 h. The cells were treated with 2.5 mM thymidine for 15 h again, centrifuged at $100 \times g$ for 5 min, washed by PBS, stained with PI, and analyzed by fluorescence flow cytometry. The cells were further cultured with different concentrations of shikonin for 12 h, collected, and centrifuged at $100 \times g$ for 5 min, and analyzed by fluorescence flow cytometry after staining with PI.

Western blot analysis

A375-S2 cells were harvested and lysed for 1 h on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)], supplemented with proteinase inhibitors: 100 µg/ml aprotinin, 10 µg/ml leupeptin, and 100 µg/ml pepstatin. Protein concentra-

tion was determined by the Folin assay. The lysate was centrifuged at $16,000 \times g$ at 4°C for 10 min. Equal amounts of total protein were mixed in $2 \times$ loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue], heated at 100°C for 5 min, and then analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes. After being blocked with Tween 20-Tris-buffered saline [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.02% Tween 20] containing 5% nonfat milk at room temperature, the membranes were incubated for 2 h at room temperature with the primary antibodies at 1:500 dilution in blotting buffer. After being washed three times for 10 min each in Tris-buffered saline, the membrane was incubated with a diluted horseradish peroxidase-labeled secondary antibody (1:500) in blotting buffer at room temperature for 1 h. After three more washes, proteins were detected by chemiluminescence, according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

The apoptotic effect of shikonin on A375-S2 cells was analyzed by observation of morphological changes, DNA staining, and DNA fragmentation assay. A375-S2 cells were placed on glass coverslips in the wells of a 6-well plate. After 12-h cell culture, they were treated with shikonin for the indicated time periods. The cellular morphology was observed by means of photomicroscopy (Motic, Hong Kong, China). After cells were treated with shikonin, they were washed with PBS and fixed in 3.7% formaldehyde overnight. The cells were centrifuged and washed, stained with Hoechst 33258, and fixed on glass microscope slides. Apoptotic cells were identified as cells with condensed and fragmented nuclei.

A375-S2 cells (1×10^6 cells) were collected by centrifugation at $150 \times g$ for 5 min and then washed with Ca^{2+} - and Mg^{2+} -free PBS. The cells were pelleted and suspended in 10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100, and kept at 4°C for 10 min. The supernatant was incubated with 20 mg/ml RNase A (2 μl) and 20 mg/ml proteinase K (2 μl) at 37°C for 1 h, kept in 0.5 M NaCl (20 μl) and isopropanol (120 μl) at -20°C overnight, and then centrifuged at $15,000 \times g$ for 15 min. DNA was dissolved in TE buffer [10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0)] and subjected to 2% agarose gel electrophoresis at 50 V for 40 min and stained with ethidium bromide.

Caspases activity assay

A375-S2 cells (1×10^6 cells) were incubated with or

without shikonin. Harvested cells at various time points were washed with PBS and centrifuged at $150 \times g$ for 5 min. The supernatant was aspirated off and cell lysis buffer (provided) was added to an Eppendorf centrifuge at 0.5 ml per 1×10^6 cells. Cells in lysis buffer were incubated on ice for 10 min. Reaction buffer containing 5 μl DDT, 5 μl of IETD-AFC (or DEVD-AFC) substrate, and 380 μl H_2O was added to each aliquot of cell lysate. Mixtures were incubated at 37°C for 1 h. The fluorescence of the cleaved substrates was determined with a spectrofluorometer set at 400 nm excitation wavelength and at 505 nm emission wavelength. The unit of enzyme activity corresponds to the activity that cleaves the respective substrate in 1 min/mg protein at 37°C .

LDH activity-based cytotoxicity assay

LDH activity was measured in both floating dead cells and viable adherent cells (15). One hundred microliters (5×10^4 cells/ml) of a cell suspension was placed in the wells of 96-well plates. They were cultured in the incubator at 37°C for 12 h. After treatment with shikonin, floating cells were collected from culture medium by centrifugation at $240 \times g$ at 4°C for 10 min. The LDH released in the culture medium was used as an index of necrotic cell death (referred to as LDHdn). The pellets were lysed by 1% NP40 in RPMI 1640 (100 μl) at 37°C for 30 min and centrifuged at $240 \times g$ at 4°C for 10 min. The supernatant was transferred to other 96-well plates. The LDH content from the supernatant was used as an index of apoptotic cell death (referred to as LDHda). The viable adherent cells were lysed by 1% NP40 in RPMI 1640 (100 μl) at 37°C for 30 min and then centrifuged at $240 \times g$ at 4°C for 10 min. The LDH present in the adherent viable cells was used as an index of viable cells (LDHv). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\% \text{ apoptosis} = \text{LDHda} / (\text{LDHdn} + \text{LDHda} + \text{LDHv}) \times 100$$

$$\% \text{ necrosis} = \text{LDHdn} / (\text{LDHdn} + \text{LDHda} + \text{LDHv}) \times 100$$

Statistical analyses

All data represent at least three independent experiments and are expressed as the mean $\pm$ S.D. unless otherwise indicated. Statistical comparisons were made by Students's *t*-test. Significance was considered as $P < 0.05$.

Results

Inhibitory effects of shikonin on the growth of A375-S2 cells

To evaluate the inhibitory effects of shikonin on the

Fig. 1. Inhibitory effects of shikonin on the growth of A375-S2 cells. A375-S2 cells were treated with various concentrations of shikonin for 6, 12, 24, and 48 h. The viability was determined by the MTT assay. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments.

growth of A375-S2 cells, the cells were treated with 2.5–80 μ M of shikonin for 6, 12, 24, and 48 h; and the cell viability was determined by MTT assay. Shikonin inhibited the growth of A375-S2 in a time- and dose-dependent manner. IC_{50} of the 48-h time course was $7.1 \pm 0.9 \mu$ M (Fig. 1).

Shikonin-induced cell cycle arrest at G1 phase in A375-S2 cells

To investigate further the features of cell growth inhibition by shikonin, flow cytometric analysis was performed. After treatment with increasing concentrations of shikonin, cells were accumulated in the G1 phase of the cell cycle, suggesting that cell cycle did not enter the S phase (Fig. 2A). The cell cycle was examined to further determine the molecular features associated with the induction of prolonged arrest in A375-S2 cells cultures. When A375-S2 cells were treated with 2.5 mM thymidine (an agent that blocks cell cycle transition from the G1 to S phase) for indicated time periods, the cells were effectively blocked at the G1 phase (Fig. 2Ba). After further treatment with 0, 0.05, 5, and 5 μ M shikonin for 12 h, the numbers of G2/M phase cells were detected by fluorescence flow cytometry. Shikonin at 5 μ M significantly blocked the transition from G1 to S (Fig. 2Be). The cells at the G2/M phase were not observed in treatment with 5 μ M shikonin; however, the proportion of cells at G2/M was 38.33% of the shikonin-free culture (Fig. 2Bb). Shikonin effectively induced cell cycle arrest in a dose-dependent manner (Fig. 2Bc–e). It was suggested that shikonin inhibited the cell growth of A375-S2 cells by arresting the A375-S2 cell cycle.

Fig. 2. Effects of shikonin on cell cycle in A375-S2 cells. A: A375-S2 cells (1×10^6 cells) were treated with 0, 0.05, 0.5, and 5 μ M shikonin for 24 h and DNA content after PI staining was analyzed by fluorescence flow cytometry. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments. B: After treatment with 2.5 mM thymidine for the indicated time periods, A375-S2 cells were further treated with 0 (b), 0.05 (c), 0.5 (d), and 5 μ M (e) shikonin for 12 h. The cells were washed by PBS and stained with PI followed by flow cytometric analysis. Arrows indicate cells at the G2/M phase. The cells were blocked at the G1 phase after thymidine treatment (a).

p53 activation required for cell cycle arrest upon DNA damage

p53 plays an important role in both induction of G1 arrest (8) and maintenance of G2/M arrest in response to DNA damage (9). Western blot analysis was performed to examine the expression of p53, cdk 4, Bcl-2, and Bax during this apoptotic process. The expression level of p53 increased in 9 h after treatment with shikonin. In contrast, the expression of cdk 4 decreased after treatment with this drug for 12 h (Fig. 3). Exposure of the cells to shikonin for 9 h significantly reduced the expression of Bcl-2, whereas the Bax expression level slightly increased in a time-dependent manner. The results suggested that activation of p53 were involved in the process in response to DNA damage by shikonin-induced A375-S2 cell cycle arrest and the Bcl-2/Bax ratio was also related to this apoptotic process.

Shikonin-induced apoptotic cell death in A375-S2 cells

After DNA damage, activation of p53 resulted in cell apoptosis of tumor cell lines (16). To determine the features of A375-S2 cell death, apoptosis assessment assay and DNA fragmentation analysis were performed. The cells underwent marked morphological changes such as becoming round in shape and apoptotic bodies were observed (Fig. 4b) by treatment with 10 μ M shikonin, compared with the untreated control (Fig. 4a). Hoechst 33258-stained A375-S2 cells showed that the

apoptotic cells had characteristic condensed nuclei (Fig. 4d). Treatment with 5 and 10 μ M shikonin for 24 h resulted in typical DNA fragmentation in agarose electrophoresis that is a hallmark of apoptosis (Fig. 4e). These results demonstrate that some cause of A375-S2 cell death induced by shikonin was apoptosis.

To further confirm whether cause of A375-S2 cell death induced by shikonin was by apoptosis or necrosis, LDH activity-based cytotoxicity assay was performed. After exposure to 0, 5, 10, 20, and 40 μ M shikonin for the indicated time periods, the proportion of necrotic cells increased time dependently (Fig. 5). Higher doses of shikonin caused earlier induction of apoptosis, and then necrotic cells increased. Apoptosis induced by lower doses of shikonin occurred later than apoptosis induced by high doses. These results indicated that in the early stages, the major cause of A375-S2 cell death was apoptosis; however, after 48 h, necrosis played greater roles in the cell death.

Shikonin-induced caspase-dependent apoptosis in A375-S2 cells

To investigate the signal transduction pathways underlying shikonin-induced apoptosis in A375-S2 cells, five caspase inhibitors were applied and the enzyme activities were measured. Shikonin-induced apoptosis was blocked by a pan-caspase inhibitor (20 μ M Z-VAD-FMK), indicating that caspase family proteinases play a

Fig. 3. p53 activation in response to DNA damage is required in the cell cycle arrest. The cells were treated with 10 μ M shikonin for 0, 3, 6, 9, 12, and 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis; and p53, Bcl-2, Bax, and cdk 4 protein bands were detected by Western blot analysis.

Fig. 4. Shikonin-induced apoptosis in A375-S2 cells. Morphological changes of A375-S2 cells treated with shikonin were observed in the absence (a) and presence (b) of shikonin ($10 \mu\text{M}$) at 24 h with $\times 200$ magnification. Arrows indicating multiblebbing cells and apoptotic bodies; Morphological changes of A375-S2 cell nuclei was observed by fluorescence microscopy. c: Medium control. d: Treated with $10 \mu\text{M}$ shikonin for 24 h. The cells were stained with Hoechst 33258 to identify apoptotic cells with $\times 400$ magnification. Arrows indicate fragmented nuclei. e: Internucleosomal DNA fragmentation induced in A375-S2 cells by shikonin. A375-S2 cells were treated with shikonin for 24 h (lane A, marker; lane B, $0 \mu\text{M}$; lane C, $5 \mu\text{M}$; lane D, $10 \mu\text{M}$).

role in shikonin-induced A375-S2 cell apoptosis. Treatment with $20 \mu\text{M}$ caspase-9 inhibitor (Z-LEHD-FMK), $20 \mu\text{M}$ caspase-3 inhibitor (Z-DEVD-FMK), and $20 \mu\text{M}$ caspase-8 inhibitor (Z-IETD-FMK) significantly

reversed $10 \mu\text{M}$ shikonin-induced apoptosis, whereas caspase-1 inhibitor (Ac-YVAD-CMK) at any doses failed to inhibit cell death (Fig. 6A). Upon treatment of A375-S2 cells with $10 \mu\text{M}$ shikonin, caspase-3 and

Fig. 5. Characterization of cell death induced by shikonin in A375-S2 cells. A375-S2 cells were treated with 0, 5, 10, 20, and 40 μ M shikonin for 12, 24, and 48 h. The extent of cell death was assessed by LDH activity-based assays. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments.

caspase-9 activities increased markedly within 6 h after the drug treatment, and then they continued to increase, reaching maximum by 24 h (Fig. 6B). Simultaneously, the shikonin-treated A375-S2 cells underwent moderate activation of caspase-8. It was suggested that shikonin induced cell apoptosis via a caspase-dependent mechanism.

Shikonin-induced mitochondrial pathway-dependent apoptosis

To investigate whether the cytochrome *c*/Apaf-1/caspase-9 'apoptosome' was required for shikonin-induced cell apoptosis (17), Western blot analysis was performed to examine AIF and cytochrome *c* expression during cell apoptosis. The expression level of AIF significantly increased within 6 h and then started to decline. Cytochrome *c* dissipated much later, 12 to 24 h

after shikonin treatment (Fig. 7). In view of these observations, AIF played an important role in this apoptosis at an earlier stage. This apoptotic pathway was involved in mitochondrial function.

Effects of shikonin on the PARP and ICAD expression in A375-S2 cells

The two classical caspase substrates PARP (18) and ICAD (19) were applied to investigate the role of caspase-3, caspase-6, or caspase-7. As shown in Fig. 8, the PARP inhibitor DPQ effectively reduced shikonin-induced A375-S2 cell apoptosis. Western blot analysis showed that by treatment with shikonin for 6 h, PARP protein was cleaved to a 85-kDa fragment, which was inhibited by 20 μ M DPQ. ICAD protein decreased with culturing time periods, and this decline was also reversed by a caspase-3 inhibitor (Z-DEVD-FMK) in 24 h. These data indicated that caspase-3 activation and cleavage of its substrates, PARP and ICAD, were involved in shikonin-induced cell apoptosis.

Discussion

In this study, we have demonstrated that shikonin inhibited the cell growth, arrested the cell cycle, and induced apoptosis in A375-S2 cells. These biochemical events were possibly associated with the p53 tumor suppressor gene. The p53 protein is a regulator of cell cycle progression and mediator of apoptosis in many cell lines, and the cell cycle progression was accelerated by cdk and decelerated by p53 and cdk inhibitors including p21 (9). A large body of data documented the key role of p53 in the G1/S checkpoint in response to DNA damage, which was associated with cyclin-dependent kinases including cdk 4, along with its cyclin partner, cyclin D (8, 20). cdk 4 and its cyclin D/cdk 4 complex play a role of in regulating either G1 cell cycle progression or cellular growth (21–23). The p53 gene is often mutated in many tumor cells and the mutations contribute to clonal cellular expansion and genomic instability because of diminished regulation of cell cycle checkpoints, DNA repair, and apoptosis (8, 9, 24, 25). It was reported that human melanoma cells A375-C6 did not show mutation in the p53 gene (26). It has not been reported that the p53 gene is mutated in A375-S2 cells. In addition, topoisomerase II, a target for anti-tumor drugs, is part of the cellular response to different types of DNA damage. Many Topoisomerase II inhibitors have been widely used to couple DNA damage to apoptosis. Etoposide, a topoisomerase II inhibitor, enhanced mammalian topoisomerase II-mediated DNA cleavage activity and induced apoptosis involved in p53 activation in mouse fibroblasts L929 cells (5, 16).

Fig. 6. Effects of various caspase inhibitors on shikonin-induced A375-S2 cell death and activities of caspase proteases. A: A375-S2 cells (5×10^4 cells) were pretreated with Z-VAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, Ac-YVAD-CMK, and Z-LEHD-FMK for 1 h and then treated with 10 μ M shikonin for 24 h. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments (* $P < 0.05$, ** $P < 0.01$ vs shikonin alone group). B: A375-S2 cells (1×10^6 cells) were incubated with or without shikonin (10 μ M) and caspase-3, -9, and -8 activities (units/mg protein) were measured. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments.

Fig. 7. Shikonin-induced mitochondria-dependent apoptotic cell death. The cells were treated with 10 μ M shikonin for 0, 3, 6, 9, 12, and 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and AIF and cytochrome *c* protein bands were detected by Western blot analysis.

Fig. 8. Effects of shikonin on the expression of PARP and ICAD. A: A375-S2 cells (5×10^4 cells) were pretreated with DPQ for 1 h, and then treated with $10 \mu\text{M}$ shikonin for 24 h. The data are presented as the mean $\pm$ S.D. of the results for three independent experiments (* $P < 0.05$, ** $P < 0.01$ vs shikonin alone group). B: Western blot analysis for PARP and ICAD protein expression in A375-S2 cells treated with shikonin ($10 \mu\text{M}$) for various time periods. The cells were incubated with $20 \mu\text{M}$ DPQ and $20 \mu\text{M}$ Z-DEVD-FMK for 1 h prior to the administration of shikonin.

Topoisomerase II forms molecular complexes with cell cycle regulators including p53 (27). Shikonin might to be one of the topoisomerase II inhibitors. The present results indicated that shikonin caused cell cycle arrest in the G1 phase through up-regulation of p53 and down-regulation of cdk 4. No change was observed in protein expression of cdk 2 and cdk 6 (data not shown). Activated p53, after DNA damage by shikonin, might either trigger the onset of DNA repair, leading to the completion of the cell cycle or alternatively, leading to exit from the cell cycle, apoptosis. It was possible that p53 activation contributed to either cell cycle arrest or apoptosis in response to low or high intensity DNA insults.

Caspases play a crucial role in the apoptotic progression (28, 29). It was reported that in HL60 cells, shikonin induced apoptosis by activation of caspase-3, which was prevented by the pan-caspase inhibitor Z-VAD-FMK (4), but the initiation pathways of shikonin-induced apoptosis remain unclear. Morphological changes and DNA fragmentation suggested that shikonin induced

A375-S2 cell death involved in a mechanism of apoptosis. We found that this apoptosis was blocked by pan-caspase inhibitor, indicating that caspase family proteinases play a role in this apoptotic process. Since the caspase-9 inhibitor Z-LEHD-FMK, caspase-3 inhibitor Z-DEVD-FMK, and caspase-8 inhibitor Z-IETD-FMK effectively reduced the shikonin-induced A375-S2 cell apoptosis, initiator caspase-8 and caspase-9 are indicated to be involved in the apoptotic cell death.

Bax, a proapoptotic protein, acts in the mitochondria to cause the release of cytochrome *c*, leading to the activation of caspase-9, and subsequently, downstream caspase-3 is activated (30). Bax and Bcl-2 are known to function upstream of caspase-3 to regulate apoptosis induced by various stimuli. Bax expression is regulated by p53 and the protein products of the target genes of p53 including Bcl-2 are involved in this process (31, 32). An increased expression of p53 can be apoptotic, although the mechanisms of p53-induced cell death are not well known. The present study showed that activation of caspase-8, -9, and -3 contributed to apoptosis

in response to shikonin administration. Decreased levels of Bcl-2 expression and elevated levels of Bax expression were associated with increased level of p53 expression and activation of caspase-3. In addition, an increase in the level of p53 protein expression occurred at 6 h after the induction of shikonin; at this time, the release of cytochrome *c* from mitochondria to cytosol was not observed. Simultaneously, the cells already exhibited several apoptotic changes: caspases were activated and their substrates were processed. It was suggested that the increased p53 protein expression at the early time point was a response to DNA damage and was required to mediate the apoptotic signal. AIF activation led to chromatin condensation and triggered phosphatidylserine exposure on the cell surface and mitochondrial membrane depolarization. Cytosolic AIF acted on the mitochondrial membrane potential and initiated the release of cytochrome *c*, which activated down-stream caspases (33, 34). Expectedly, AIF activation was observed at an earlier stage in shikonin-induced A375-S2 cell apoptosis, and it was possible that activation of AIF was responsible for the cleavage of PARP, leading to A375-S2 cell death.

PARP is a 116-kDa nuclear enzyme at the downstream of caspase-3. It detects and binds to DNA strand breaks and functions in transcription and DNA repair (18). ICAD is expressed as two isoforms, ICAD-L/DFF45 and ICAD-S/DFF35. The activation of CAD is attributed to the cleavage of ICAD, which is cleaved by caspase-3 or caspase-7 (35). Our studies demonstrated that the PARP inhibitor DPQ effectively reversed shikonin-induced cell death, indicating that caspase-3 or -7 was activated during apoptosis. It was further confirmed by down-regulation of PARP and ICAD, which was prevented by their respective inhibitors. In view of these data, these events constitute a sequence that proceeds from DNA damage through p53 activation to cell cycle arrest and regulation of Bax and AIF. It results in the release of cytochrome *c* and caspases activation, and ultimately, apoptosis of the cells. In addition, the activation of caspases was earlier than the release of cytochrome *c*, suggesting that it was possible that other substances were involved in the process.

In summary, shikonin inhibited A375-S2 cell growth and arrested cell cycle through activation of p53 in response to DNA damage and decreased expression of cdk 4 was involved in the apoptotic progression. Simultaneously, shikonin induced cell apoptosis mediated by p53, through up-regulation of AIF and Bax and down-regulation of Bcl-2 to release of cytochrome *c*, which contributed to the activation of caspase-8 and -9, leading to the activation of downstream caspase-3 in the process.

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