

Shikonin causes apoptosis by disrupting intracellular calcium homeostasis and mitochondrial function in human hepatoma cells

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Abstract. Shikonin is known to suppress proliferation and induce apoptosis in a variety of cancer cell lines. In the present study, SMMC-7721 human hepatocellular carcinoma cells were treated with shikonin (1, 2 or 4 μ M) for 12-48 h. Cell morphological alterations and DNA damage were determined. Furthermore, changes in cell cycle, mitochondrial transmembrane potential, calcium homeostasis and levels of reactive oxygen species were measured. Shikonin-treated SMMC-7721 cells exhibited morphological changes and DNA damage. Shikonin inhibited cell proliferation causing cell cycle arrest at the G₀/G₁ phase and induced apoptosis in a dose- and time-dependent manner. Shikonin-induced apoptosis was associated with activation of caspases-3, -8 and -9, elevated levels of intracellular Ca²⁺ and reactive oxygen species, reduced mitochondrial membrane potential and enhanced efflux of Ca²⁺ and K⁺. Gene expression B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), p53 and caspase-3 was up-regulated, whereas Bcl-2 expression was downregulated. Shikonin caused apoptosis by inhibiting cell cycle progression, disrupting Ca²⁺ homeostasis, inducing oxidative stress and triggering mitochondrial dysfunction. Activation of caspases-3, -8 and -9, K⁺ efflux, and regulation of Bax, Bcl-2,

p53 and caspase-3 expression are involved in the process. These results provide in-depth insight into the mechanisms of action of shikonin in the treatment of cancer.

Introduction

Human hepatocellular carcinoma (HCC) has a poor prognosis and is the fifth most frequent type of cancer and the third leading cause of cancer-associated mortalities globally, being accountable for >600,000 deaths per year (1). Despite monitoring efforts, most tumors, including HCC, are diagnosed in late stages. As uncontrolled cell proliferation and disrupted apoptosis are among the most important mechanisms underlying tumorigenesis, the focus of common tumor therapies is inhibition of cell division and induction of apoptosis. Apoptosis is the programmed cell death, regulated by numerous intra- and extracellular signals and governed by several genes, several of which are mutated or dysregulated in various human tumors (2). Drug-induced apoptosis of malignant cells is a promising anti-tumor strategy with emerging evidence supporting its effectiveness against hepatoma and other cancer types. However, currently available HCC therapies have proven unsatisfactory.

To reduce HCC mortality, development of efficient drugs with minimal cytotoxicity against normal, healthy cells is important. Traditional Chinese Medicine is a source of such compounds and is attracting increasing attention in cancer therapy.

Shikonin, whose chemical structure is displayed in Fig. 1A, is a natural naphthoquinone isolated from the traditional Chinese medical herb Zi Cao (*Lithospermum erythrorhizon*, Siebold & Zucc. of the Boraginaceae family, also known as purple gromwell), which has been used as an herbal medicine in East Asia for centuries (3). It has anti-tumor, anti-oxidant, anti-bacterial, antiangiogenic and anti-inflammatory properties (4). The anti-tumor activity of shikonin has been demonstrated in various cancer cell lines (5-7). The anti-tumor potential of shikonin is associated with inhibition of malignant cell growth and induction of cancer cell apoptosis by intracellular oxidative stress (8,9), as well as caspase-3-dependent apoptosis, topoisomerase-mediated DNA cleavage and cell cycle arrest (10). Shikonin accumulates in mitochondria and deregulates cellular Ca²⁺ and the level of reactive oxygen

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species (ROS), leading to breakdown of the mitochondrial membrane (11). In addition, shikonin inhibits cancer cell proliferation, as well as glucose and lactate metabolism by targeting tumor pyruvate kinase M2 (12).

Several cellular processes have been implicated in the anti-cancer effects of shikonin; however, the precise underlying mechanisms remain elusive and require further research. The SMMC-7721 hepatoma cell line is primarily used for drug screening and investigation of apoptotic mechanisms. The present study investigated the antitumor effects of shikonin in the SMMC-7721 hepatoma cell line, focusing on apoptotic effects, cell proliferation, DNA damage, mitochondrial function, calcium and potassium homeostasis, caspase activity and gene expression. Understanding the underlying mechanisms of shikonin's anti-tumor potential may provide an experimental foundation for the clinical use of shikonin in the treatment of hepatocellular carcinoma.

Materials and methods

Materials. Shikonin (98% purity), MTT, acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Propidium iodide (PI), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Annexin V-FITC Apoptosis Detection kit I was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Fluo3-AM was from Thermo Fisher Scientific, Inc.

Cell culture. The SMMC-7721 human hepatoma cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The SMMC-7721 cells were cultured in high-glucose DMEM supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO₂. The culture medium was changed every 2-3 days.

Shikonin treatment. Shikonin was dissolved in dimethyl sulfoxide (DMSO) to generate a 4-mM stock solution (stored at -20°C) and subsequently diluted in culture medium to the final concentration used in each experiment. The final concentration of DMSO was 0.1%. Controls were treated with an equivalent amount of DMSO. Cells were in the logarithmic phase when treated with shikonin (0, 1, 2 or 4 μM) for predetermined periods (12, 24, 36 and 48 h).

Cell viability assay. Cell viability was analyzed using the MTT assay. Cell suspensions (200 μl) were seeded in 96-well plates at a density of 1x10⁵ cells/well. Cells in the logarithmic phase were exposed to 180 μl fresh complete medium containing different concentrations of shikonin. MTT (20 ml, 5 mg/ml) was added, and the cells were incubated for another 4 h. After discarding the supernatant, the precipitate was dissolved in DMSO (200 μl) and the absorbance was measured at 490 nm using a microplate reader (Elx 800; BioTek Instruments, Winooski, VT, USA).

DNA fragmentation assay. In brief, cells were harvested, lysed with 200 μl lysis buffer (1 mM EDTA, 0.2% Triton X-100 and 10 mM Tris-HCl, pH 7.5) and centrifuged at 12,000 x g and 4°C for 5 min, the supernatant for further used. DNA samples were

precipitated in 12.5% trichloroacetic acid (TCA) and quantified using diphenylamine after hydrolysis in 5% TCA, the fragmented and total DNA was measured with a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, NC, USA). The DNA samples were electrophoresed on 2% agarose gel and visualized under UV light with ethidium bromide.

Morphological observation. Cells were seeded in 6-well plates at a density of 1x10⁵ cells/well, when cells were in the logarithmic phase, they were treated with shikonin (1, 2 and 4 μM) at 37°C for 24 h, and then examined under a phase contrast microscope at a magnification of x100.

Transmission electron microscopy (TEM). Cells were collected, fixed with 2.5% (m/v) glutaraldehyde and washed with 0.1 mol/l PBS prior to serial dehydration with 30, 50, 70, 80, 90 and 100% acetone (v/v). The samples were embedded with epoxy resin (Spurr) for polymerization, sliced with an ultramicrotome (LEICAUC6i), and stained with uranyl acetate and lead citrate prior to TEM analysis (JEM-2000Ex; JEOL Ltd., Tokyo, Japan).

AO/EB double staining. Cells were treated with 1, 2 or 4 μM shikonin for 24 h, after which cell suspensions were harvested and stained with AO and EB solutions (both AO and EB in ethanol, the respective concentration was 2 mg/ml). After incubation at 25°C in the dark for 5 min, the samples were observed using confocal microscopy (Nikon TE-2000-E; Nikon, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. The TUNEL assay was performed as previously described (13). In brief, cells were stained with TUNEL reagent (Roche Diagnostics, Basel, Switzerland), and the number of TUNEL-positive cells was counted in 20 randomly selected microscopic fields at 40x magnification.

Annexin V-fluorescein isothiocyanate (FITC)/PI double-labeling. Apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection kit per the manufacturer's instructions. In brief, cells were stained with FITC and PI and analyzed via flow cytometry (BD FACSCalibur; BD Biosciences) to detect early (FITC-positive and PI-negative) and late (FITC- and PI-positive) apoptotic cells.

Cell cycle analysis. To estimate the proportion of cells in different phases of the cell cycle and the apoptotic effects of shikonin, cell DNA contents were analyzed using flow cytometry. Cell samples were harvested by centrifugation, gently fixed with 70% ethanol on ice overnight and stained with PI solution (0.05 mg/ml PI, 0.02 mg/ml RNase, 0.585 g/ml NaCl, 1 mg/ml sodium citrate, pH 7.2-7.6) in the dark for 15 min. The cells were analyzed by flow cytometry (BD FACSCalibur).

Analysis of mitochondrial membrane potential. The cells were collected and washed twice with prewarmed PBS, and adjusted to the concentration of 1x10⁶ cells/sample. After the addition of JC-1 working solution (5 μg/ml, 0.5 ml/sample), the cells were incubated at 37°C in the dark for 10 min and washed twice with prewarmed PBS. After centrifugation at 1,000 x g (25°C

for 5 min), the supernatant was discarded. Samples were resuspended with 0.5 ml PBS and analyzed using flow cytometry (BD FACSCalibur).

Analysis of intracellular calcium homeostasis. Cells were collected and the concentration adjusted to 2×10^6 cells/ml prior to loading with Fluo3-AM, final concentration, $6 \mu\text{M}$; (stock solution of Fluo3-AM was 2 mM, 1 mg Fluo3-AM was dissolved in $442 \mu\text{l}$ DMSO). Cells were incubated at 37°C with 5% CO_2 in the dark for 40 min and then resuspended in 0.5 ml calcium-free PBS prior to analysis using flow cytometry (BD FACSCalibur).

Extracellular Ca^{2+} and K^+ flux measurements. A microelectrode ion flux estimation (MIFE) technique was used to monitor Ca^{2+} and K^+ flux during shikonin-induced apoptosis in SMMC-7721 human hepatoma cells, as previously described (14).

ROS measurement. Cells ($2 \times 10^6/\text{ml}$) were loaded with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) at a final concentration of $8 \mu\text{M}$, incubated at 37°C with 5% CO_2 for 30 min, washed and analyzed using flow cytometry (FC500; Beckman Coulter, Brea, CA, USA). At least 10,000 events were analyzed at excitation and emission wavelengths of 488 and 525 nm, respectively.

Caspase activity assay. Activities of caspase-3, -8 and -9 were evaluated using a caspase activity kit (Beyotime Institute of Biotechnology, Haimen, China) per the manufacturer's instructions and as previously described (15).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured cells and used to synthesize the first complementary DNA (cDNA) strand using the PrimerScript RT reagent kit (Takara, Dalian, China) per the manufacturer's instructions. Specific primers for B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase-3, p53 and GAPDH (housekeeping gene used as an internal control) were designed by Primer Premier 5.0 software and synthesized by Shanghai Biotechnology Co., Ltd. (Shanghai, China). Primer sequences are listed in Table I. Composition of the PCR mixture is listed in Table II. PCR conditions were as follows: Denaturation at 94°C for 5 min, 32 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at $52\text{--}55^\circ\text{C}$ and elongation at 72°C for 30 sec, and a final elongation at 72°C for 10 min. PCR products were identified by gel electrophoresis on 1.5% agarose as previously described (16).

Statistical analysis. Assays were performed in triplicates of three independent experiments. Values are expressed as the mean \pm standard deviation. Data were analyzed using Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC, USA) and compared using 2-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Shikonin reduces the viability of hepatocellular carcinoma cells. SMMC-7721 cell viability was evaluated using the MTT assay following exposure to increasing concentrations of shikonin (0–4 μM) for 12, 24, 36 or 48 h. The population of viable cells

decreased significantly with increasing shikonin concentrations and duration of treatment in a dose- and time-dependent manner (Fig. 1B). These results suggested that shikonin is an effective inhibitor of SMMC-7721 cell proliferation.

DNA fragmentation assay. Agarose gel electrophoresis of SMMC-7721 cell samples at 24 h after shikonin treatment revealed a DNA ladder, indicating DNA fragmentation and demonstrating that shikonin damages the DNA and is a DNA-binding agent with cytotoxic activity on SMMC-7721 cells (Fig. 1C).

Morphological observation. Under normal circumstances, adherent cells are elliptical. During the logarithmic growth phase, passaged cells gradually adhere and grow. In the present study, control cells were closely arranged, with a uniform size, as well as good vitality and refractivity (Fig. 1D). Shikonin-treated cells displayed atrophy and vacuoles, shrinkage of the cytoplasm, decreased cell number, cell fragmentation, blurred contours, and reduced cell connections; certain cells were lysed into small pieces. Apoptotic cells detached from adjacent normal cells, displaying obvious differences in morphology (Fig. 1D).

TEM. The effects of shikonin on the microstructure of SMMC-7721 cells were assessed using TEM (Fig. 1E). Cells in the control group displayed a homogenous cytoplasm, microvilli on the cell surface, large, round nuclei with intact nuclear membrane and subcellular structures, clear and regular nucleoli, and loose chromatin. Cells in the treatment group exhibited vesiculation of cytoplasmic organelles, membrane blebbing, pyknosis, fragmentation, shrinkage or disappearance of the nucleus, cytoskeletal degradation, rupture of the plasma and nuclear membrane and reduced microvilli on the cell surface.

AO/EB double staining. AO and EB differ in permeability and fluorescence, distinguished cells in early (yellow) and late apoptosis (orange fluorescence), as well as cells that were normal (green) and necrotic (red). AO/EB double staining demonstrated that shikonin caused apoptosis of SMMC-7721 cells in a dose-dependent manner (Fig. 1F).

TUNEL assay. Shikonin treatment for 24 h triggered apoptosis in SMMC 7721 cells in a dose-dependent manner as determined by the TUNEL assay (Fig. 1G).

Annexin V-FITC/PI double-staining. The apoptotic effects of shikonin on SMMC-7721 cells were quantified using flow cytometry after Annexin V-FITC/PI staining, which is based on phosphatidylserine externalization. Shikonin was demonstrated to exert dose- and time-dependent apoptotic effects (Fig. 2A and B).

Shikonin inhibits the cell cycle of hepatocellular carcinoma cells. Shikonin treatment resulted in a dose-dependent accumulation of cells in the G_0/G_1 phase of the cell cycle, with a concomitant decrease in the proportion of cells in the S phase (Fig. 2C and D), indicating cell cycle arrest in G_1 phase. A dose-dependent increase in the proportion of apoptotic cells in the hypodiploid DNA peak (sub- G_1 population) was identified (Fig. 2C and D).

Table I. Primer sequences of apoptosis-associated genes and their melting temperatures.

Gene	Primer	Length (bp)	T _m (°C)
GAPDH	F, 5'-AGAAGGCTGGGGGCTCATTG-3'	258	55
	R, 5'-AGGGGCCATCCACAGTCTTC-3'		
Bax	F, 5'-CAGGATGCGTCCACCAAGAA-3'	765	55
	R, 5'-GTGTCCCGAAGGAGGTTTATT-3'		
Bcl-2	F, 5'-TGGGAGAACAGGGTACGATAA-3'	275	53
	R, 5'-ACAGCCAGGAGAAATCAAACA-3'		
Fas	F, 5'-CGCTTCTGGGGAGTGAGGGA-3'	454	52
	R, 5'-GGTGTGCTGGTGTAGTGTGC-3'		
P53	F, 5'-AGGGGAGACCAGAACCTCTC-3'	661	55
	R, 5'-GCACCACCTTCCTTCCAAAA-3'		
Caspase-3	F, 5'-GAAGAACTTAGGCATCTGTGGGC-3'	661	55
	R, 5'-GTATGGAGAAATGGGCTGTAGGC-3'		

F, forward; R, reverse; bp, base pairs; T_m, melting temperature; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

Shikonin reduces the mitochondrial membrane potential of hepatocellular carcinoma cells. To observe changes in mitochondrial membrane potential after shikonin treatment, cells were stained with JC-1 and examined using flow cytometry. JC-1 is a lipophilic, cationic dye that selectively enters mitochondria and reversibly changes color from green to red as the membrane potential increases. The JC-1 dye aggregates and fluoresces red in normal cells, whereas at a low mitochondrial membrane potential, JC-1 monomers fluoresce green. Reduced red fluorescence and increased green fluorescence in shikonin-treated cells, compared to the control group, indicated a reduced mitochondrial membrane potential (Fig. 3A and B).

Shikonin affects intracellular Ca²⁺ homeostasis. Intracytoplasmic esterases hydrolyze Fluo3-AM into Fluo3, which binds to intracytoplasmic free Ca²⁺ to form Fluo3-Ca²⁺ complexes. The Ca²⁺ concentration in shikonin-treated cells was significantly higher than that in control cells, indicating a positive association between Ca²⁺ release and shikonin treatment (Fig. 3C and D).

Shikonin increases extracellular Ca²⁺ and K⁺ flux. Extracellular Ca²⁺ and K⁺ flux prior to and after shikonin treatment (4 μM) in SMMC-7721 cells was monitored using the MIFE method (Fig. 3E and F). The results indicated that the efflux of Ca²⁺ and K⁺ increased after shikonin treatment.

Shikonin increases ROS in hepatocellular carcinoma cells. DCFH-DA is hydrolyzed by cellular esterases to form the nonfluorescent DCFH, which is then oxidized by a variety of ROS to form the highly fluorescent DCF. DCFH-DA staining was used to determine whether ROS have a role in shikonin-induced apoptosis in SMMC-7721 cells. Shikonin treatment significantly and dose-dependently enhanced ROS production in SMMC-7721 cells (Fig. 4A).

Shikonin causes caspase activation in hepatocellular carcinoma cells. To elucidate the mechanisms of shikonin-induced

Table II. Composition of the polymerase chain reaction mixture.

Components	Volume (μl)
Takara Ex-Taq	0.25
10X Ex-Taq Buffer	5
dNTP (2.5 mM)	4
cDNA	2
Primer (F, 10 μM)	2
Primer (R, 10 μM)	2
ddH ₂ O	35
Total	50

F, forward; R, reverse.

apoptosis in SMMC-7721 cells, the activation of caspase-3, -8 and -9 was monitored. Caspase-3 and -9 activities in the 2- and 4-μM shikonin treatment groups were significantly higher than those in the controls (P<0.01), as was caspase-8 activity in the 4 μM shikonin treatment group (P<0.01; Fig. 4B). Collectively, these results indicate that shikonin activates caspases-3, -8 and -9 in SMMC-7721 cells.

Shikonin induces the expression of apoptotic genes. Bax, Bcl-2, p53 and caspase-3 mRNA expression levels were determined using RT-PCR to assess the involvement of corresponding proteins in shikonin-mediated apoptosis. Exposure of SMMC-7721 cells to shikonin significantly increased Bax, p53 and caspase-3 gene expression (P<0.01) and significantly decreased Bcl-2 gene expression (P<0.01), resulting in an increase in the Bax/Bcl-2 ratio (Fig. 4C and D).

Discussion

Growth inhibition and induction of apoptosis are two key mechanisms by which chemotherapeutic agents induce

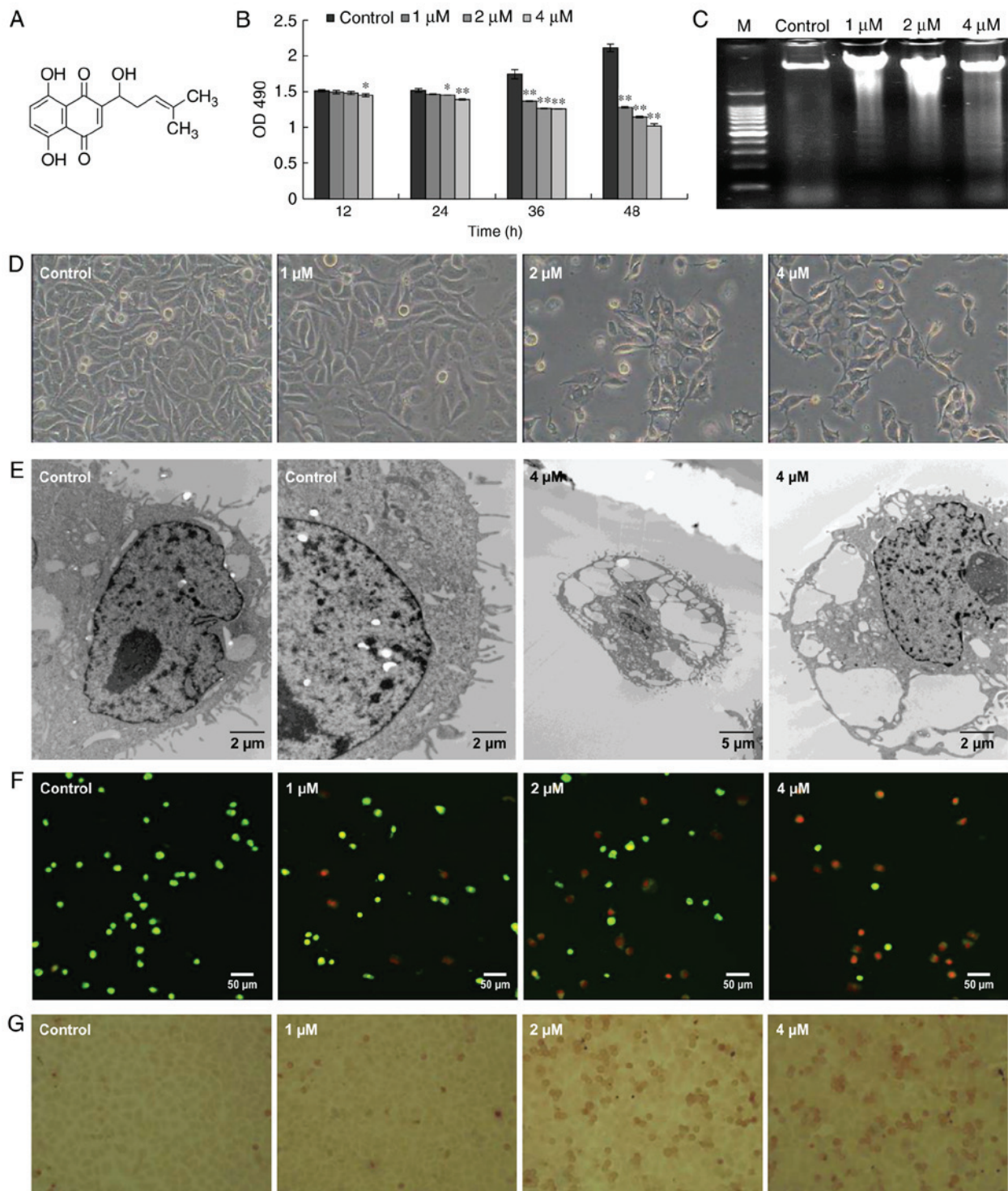


Figure 1. Apoptotic effects of shikonin on SMMC-7721 cells. (A) Chemical structure of shikonin. (B) Cytotoxicity analysis via the MTT assay. Values are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01 vs. corresponding control. (C) DNA fragmentation analysis of cells treated with shikonin for 24 h. (D) Morphological changes of SMMC-7721 cells treated with shikonin for 24 h (magnification, $\times 100$). (E) Transmission electron microscopy images of changes in subcellular structures after 24 h (scale bar, 2 or 5 μ m). (F) Morphological observation of nuclear damage in SMMC-7721 cells after 24 h (scale bar, 50 μ m). (G) Apoptosis analysis of cells treated with shikonin for 24 h using a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay (magnification, $\times 40$). OD 490, optical density at 490 nm; M, marker.

cytotoxic effects in cancer cells (17). Therefore, chemical agents with potent differentiation- or apoptosis-inducing activity and acceptable toxicity have potential as anticancer drugs. Shikonin inhibits cell proliferation and induces apoptosis in various human cancer cell types (18). Induction of apoptosis is associated with caspases (19), the Bcl-2 family (4)

and mitogen-activated protein kinase (20). Furthermore, shikonin has been reported to induce autophagy in the BEL7402 and Huh7 human hepatocellular carcinoma cell lines (21).

In the present study, shikonin caused typical apoptosis-associated morphological changes and DNA fragmentation,

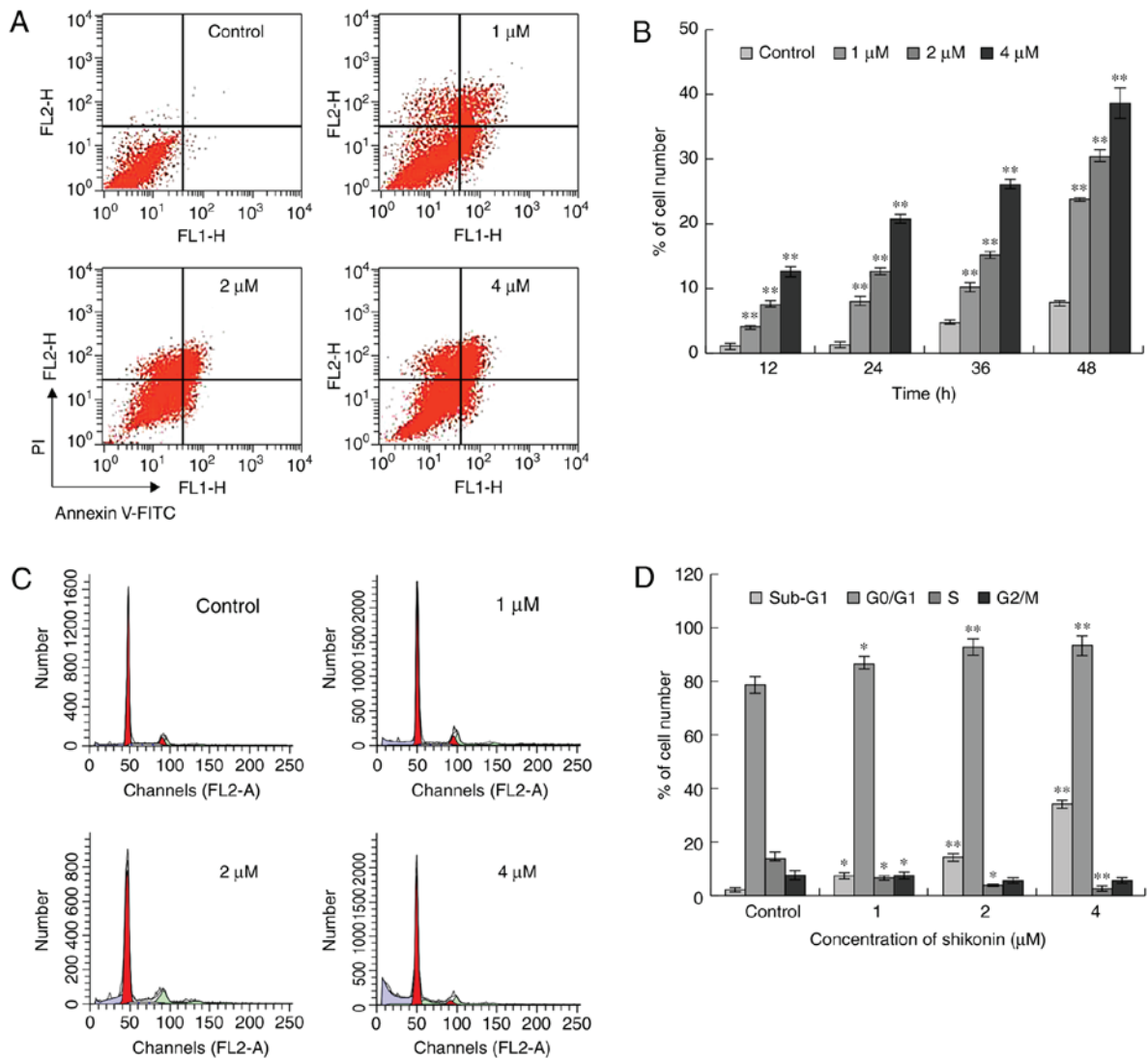


Figure 2. Effect of shikonin on apoptotic rates and cell cycle. (A) Apoptotic rates of SMMC-7721 cells treated with shikonin for 24 h (scatter plot data at 12, 36 and 48 h not shown). (B) Apoptotic rates of SMMC-7721 cells treated with shikonin for 12, 24, 36 and 48 h. (C) Cell cycle progression and sub-G₁ content of SMMC-7721 cells after shikonin treatment for 24 h. (D) Distribution of cells in the sub-G₁, G₀/G₁, S and G₂/M phases of the cell cycle. Values are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding control. PI, propidium iodide; FITC, fluorescein isothiocyanate.

inhibited cell proliferation and induced apoptosis of SMMC-7721 cells in a dose- and time-dependent manner. It was further demonstrated that shikonin induces apoptosis of SMMC-7721 cells associated with increased caspase activity and ROS generation, mitochondrial dysfunction, K⁺ efflux, disturbed Ca²⁺ homeostasis and altered Bax, Bcl-2, p53 and caspase-3 gene expression.

Physiological or pathological apoptotic stimuli are correlated with cell cycle progression. In the present study, it was demonstrated that shikonin inhibits cell proliferation by inducing apoptosis and G₀/G₁ phase arrest of SMMC-7721 cells *in vitro*. Shikonin caused a dose- and time-dependent loss of plasma membrane integrity, as indicated by the increased proportion of PI-stained cells. In addition, shikonin-treated cells exhibited apoptotic DNA fragmentation and dose-dependent increases in the sub-G₁ cell population, in agreement with the TUNEL assay results. The anti-proliferative effects of shikonin associated with the induction of cell cycle arrest in G₀/G₁ phase were previously reported for various cancer cell lines (11,22). However,

shikonin-induced G₂/M cell cycle arrest was reported in Ewing sarcoma cells (23). Induction of cell cycle arrest in different cell cycle phases under different biological conditions and simultaneous cell cycle arrest at two checkpoints have been previously described (24,25). Further research is required to elucidate shikonin-induced cell cycle arrest and the factors associated with it.

Decreased mitochondrial membrane potential is a relatively early event of apoptotic signaling, occurring prior to nuclear apoptotic changes. The importance of mitochondria for apoptosis is well recognized (26). Shikonin induced a decrease in mitochondrial membrane potential that correlated with the loss of plasma membrane integrity. Shikonin treatment was previously demonstrated to lead to apoptosis of cells characterized by reduced mitochondrial membrane potential and positive PI staining (27). Decreased mitochondrial membrane potential was also observed in shikonin-treated human SK-Hep-1 cells (28) and HepG2 cells (29). In the present study, the decreased mitochondrial membrane potential detected by JC-1

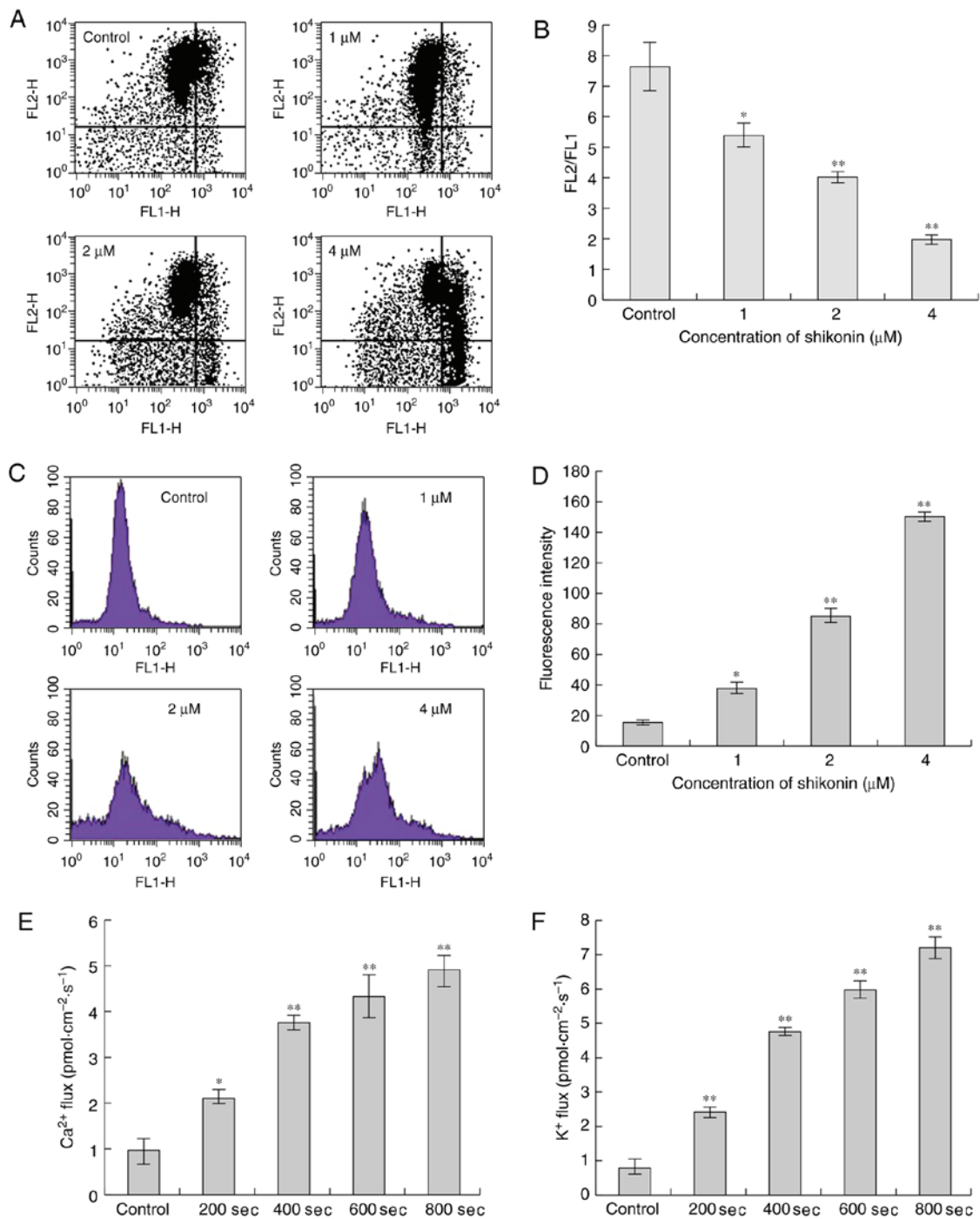


Figure 3. Effect of shikonin on cell mitochondrial and intracellular Ca^{2+} . (A and B) Mitochondrial membrane potential of SMMC-7721 cells at 24 h after treatment with shikonin. (C and D) Intracellular Ca^{2+} homeostasis of SMMC-7721 cells treated with shikonin for 24 h. A rightward-moving peak indicates an increase in intracellular free Ca^{2+} . (E) Ca^{2+} flux and (F) K^{+} flux in SMMC-7721 cells prior to and after treatment with $4 \mu\text{M}$ shikonin. Values are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding control.

staining confirmed the association between mitochondrial dysfunction and shikonin-induced apoptosis.

Shikonin was reported to induce ROS generation and subsequently trigger apoptosis in human HCC cells (1). It also enhanced ROS generation in chronic myelogenous leukemia (8), osteosarcoma cells (30) and human liver cancer cells, including the HepG2 hepatoblastoma, as well as the Huh7 and BEL7402 hepatocellular carcinoma cell lines (1). The results of the present study indicated that shikonin increases ROS generation in a dose-dependent manner, triggering mitochondrial dysfunction.

The release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm is a key signaling event, sensitizing mitochondria in numerous models of apoptosis (31). Mitochondrial calcium overload leads to mitochondrial damage, release of cytochrome *c*, activation of caspases and subsequent apoptosis (32). The present results indicated that during shikonin-induced apoptosis of SMMC-7721 cells, the free cytosolic Ca^{2+} concentration increased, disturbing Ca^{2+} homeostasis. Disturbed Ca^{2+} homeostasis, combined with a decrease in mitochondrial membrane potential, implies that

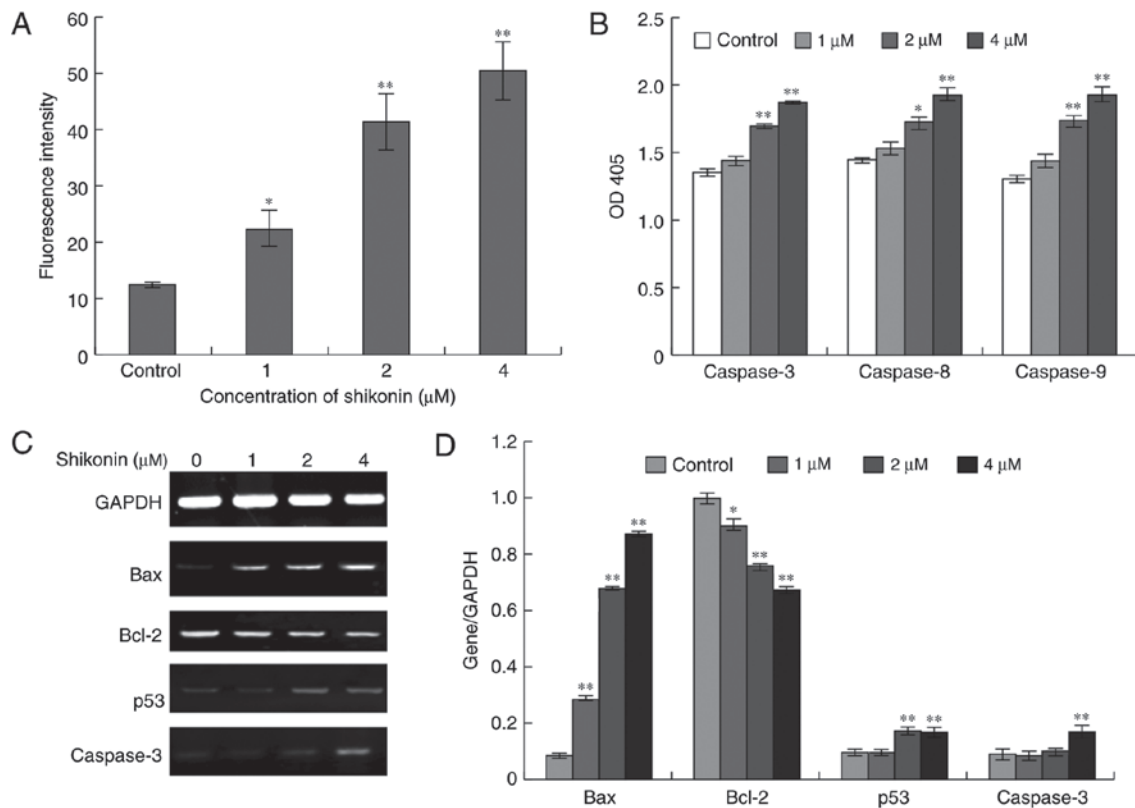


Figure 4. Analysis of shikonin-induced apoptosis of SMMC-7721 cells. (A) Generation of intracellular reactive oxygen species in SMMC-7721 cells treated with shikonin for 24 h. (B) Caspase-3, -8, and -9 activities were determined in cytosolic extracts of SMMC-7721 cells at 24 h after treatment with shikonin. (C and D) Analysis of Bax, Bcl-2, p53 and caspase-3 mRNA expression levels at 24 h (normalized to GAPDH). Values are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. corresponding control. OD 405, optical density at 405 nm; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

Ca^{2+} is released from the endoplasmic reticulum and mitochondria. Ca^{2+} release from intracellular calcium stores and extracellular Ca^{2+} influx are two possible causes of an increase in intracellular free Ca^{2+} . The present analysis of Ca^{2+} flux indicated that in the presence of shikonin the flow rate of the Ca^{2+} efflux increased with time. These results implied that the increase in intracellular free Ca^{2+} was caused by a release of endogenous Ca^{2+} stores rather than the absorption of extracellular Ca^{2+} .

Loss of intracellular K^+ is a characteristic of apoptosis, alongside cell shrinkage, nuclear condensation, DNA fragmentation and formation of apoptotic bodies (33). In the present K^+ flux analysis, efflux was observed prior to and after treatment, with the flow rate increasing with time. K^+ efflux has been documented in the early stages of apoptosis, with high extracellular K^+ concentrations and K^+ channel blockers inhibiting the process (34).

Shikonin has been reported to trigger apoptosis through caspase-dependent pathways in HL60 human promyelocytic leukemia (35) and HeLa human cervical cancer cells (36). In the present study, shikonin increased the activity of caspases-3, -8 and -9, as well as the gene expression of caspase-3. Regulation of gene expression is important in directing apoptosis (37). The present study demonstrated that shikonin treatment affects the gene expression of apoptosis-associated proteins, including the downregulation of Bcl-2 and upregulation of Bax, p53 and caspase-3. These results are in agreement with those of previous studies

indicating that shikonin treatment downregulates the expression of Bcl-2 and stimulates the activation of pro-caspase-3 to caspase-3 (38).

In conclusion, the present study demonstrated that shikonin suppresses the growth of SMMC-7721 human hepatocellular carcinoma cells by inhibiting DNA synthesis, increasing ROS generation, triggering mitochondrial dysfunction, interfering with calcium homeostasis and altering apoptosis-associated gene expression. The present study supports the potential of shikonin as an efficient anti-cancer drug. Further study on hepatocellular carcinoma will contribute to the development of more effective and sophisticated treatment strategies.

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