

Effect of Shikonin on Human Breast Cancer Cells Proliferation and Apoptosis *In Vitro*

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Shikonin, isolated from the plant *Lithospermum erythrorhizon* Sieb. Et Zucc, has been reported to induce apoptosis in several tumor cells. However, such effect of shikonin on human breast cancer cells has not been reported. Thus, in the present study, whether shikonin could induce MCF-7 human breast cancer cell apoptosis was investigated. The results showed that shikonin (2.5–80 μM) induced MCF-7 cell death in a time- and dose-dependent manner, as measured by MTT assay. The IC_{50} of a 24 h, 48 h and 72 h time course for MCF-7 cells was 7.4 ± 0.4 , 6.3 ± 0.6 and 3.9 ± 0.5 μM , respectively. Cellular morphology observation showed that MCF-7 cells underwent marked apoptotic morphological changes upon treatment with 10 μM shikonin compared with the untreated control. Flow cytometric analysis of shikonin-treated MCF-7 cells showed that the ratio of the apoptotic DNA fragmentation increased in a dose-dependent manner. The present study demonstrated for the first time that the cytotoxic effect of shikonin on MCF-7 cells underwent apoptosis process.

Key words—shikonin; apoptosis; MCF-7 cells

INTRODUCTION

Shikonin (Fig. 1) is a naphthoquinone that has been isolated from the ground rhizome of *Lithospermum erythrorhizon* Sieb. Et Zucc, which has been used as herbal medicine in East Asia.¹⁾ Shikonin has anti-inflammatory,^{2,3)} antifungal,⁴⁾ antitumor effects and inhibits angiogenesis *in vivo* and *in vitro*.^{5,6)} It has been also reported that shikonin induced apoptosis in various tumor cells, such as HL60 human premyelocytic leukemia cells,⁷⁾ SK-Hep-1 human hepatoma cells,⁸⁾ A375-S2 human malignant melanoma cells,⁹⁾ human colorectal carcinoma cells¹⁰⁾ and human cervical epithelial cancer cells.¹¹⁾ However,

such effect of shikonin on human breast cancer cells has not been reported yet. Thus, in the present study, whether shikonin could induce MCF-7 human breast cancer cell apoptosis was investigated.

MATERIALS AND METHODS

Chemicals Shikonin was obtained from the Beijing Institute of Biologic Products (Beijing, China). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China). RPMI 1640 was from Gibco (Grand Island, USA). Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO).

Cell Culture Human breast cancer cells (MCF-7) was obtained from the American Type Culture Collection (ATCC Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

Cell Viability The cytotoxic effect of shikonin on MCF-7 cells was measured by MTT assay as described previously.¹²⁾ In brief, the cells were dispensed in 96-well flat bottom microtiter plates at a density of 5×10^3 cells per well. After 24 h incubation, they were treated with various concentrations of

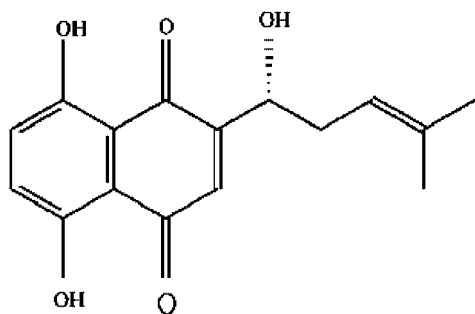


Fig. 1. Molecular Structure of Shikonin

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shikonin for the indicated time periods. After various treatments, the cells were incubated with MTT (0.25 mg/ml) for 3 h at 37 °C. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was determined by measuring its absorbance at the wavelength of 490 nm with a SPECTRA (shell) Reader (TECAN, Austria).

Morphological Changes MCF-7 cells were placed in the wells of a six-well plate. After 24 h cell culture they were treated with shikonin for the indicated time periods. The cellular morphology was observed by means of photomicroscopy (Motic Incorporation Ltd., Hong Kong).

Flow Cytometric Analysis MCF-7 cells (1×10^6 cells) were harvested and washed once in cold PBS. Cell pellets were fixed in 70% ethanol and washed in cold PBS. Then the pellets were suspended in propidium iodide (PI) solution (1 ml) containing 50 $\mu\text{g/ml}$ of PI, 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X. Cell samples were incubated at 4°C in the dark for at least 15 min, and analyzed by a FACScan flow cytometer (Becton Dickinson).¹³⁾

Data Analysis Results were expressed as mean \pm S.D. Statistical significance ($p < 0.05$) was assessed by one-way ANOVA followed by Dunnett's *t*-test (SPSS12.0 software, SPSS, USA).

RESULTS AND DISCUSSION

Inhibitory effect of shikonin on the growth of MCF-7 cells was detected by MTT assay. Shikonin (2.5–80 μM) induced MCF-7 cell death in a time- and dose-dependent manner, as measured by MTT assay (Fig. 2). The IC_{50} of a 24 h, 48 h and 72 h time course for MCF-7 cells was 7.4 ± 0.4 , 6.3 ± 0.6 and $3.9 \pm 0.5 \mu\text{M}$, respectively.

Cell death induced by various stimuli occurs by either of two distinct mechanisms, necrosis or apoptosis. To determine whether the features of MCF-7 cell death induced by shikonin were caused by apoptosis or necrosis, cellular morphology was examined. The cells underwent marked morphological changes (Fig. 3(b)) upon treatment with 10 μM shikonin compared with the untreated control (Fig. 3(a)). The MCF-7 cells became round, and granular apoptotic bodies were observed.

Flow cytometric analysis of 1 and 10 μM shikonin-treated MCF-7 cells showed that the ratio of the

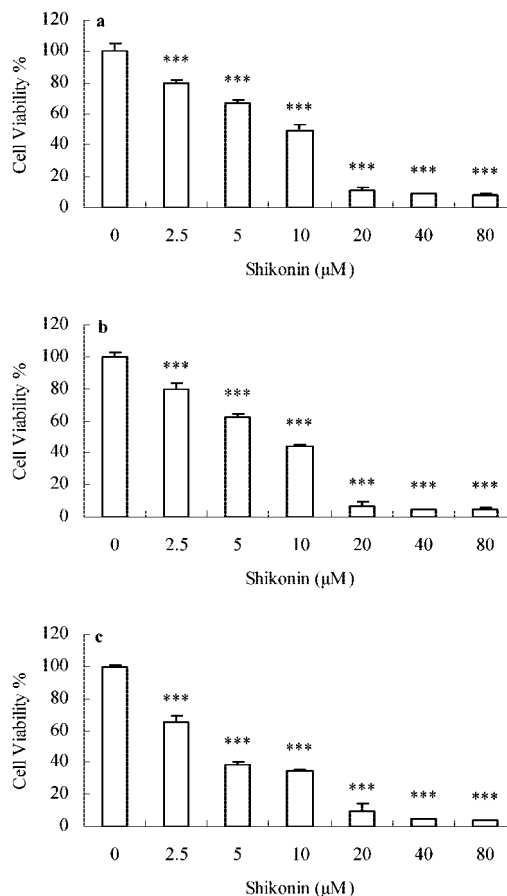


Fig. 2. Effect of Shikonin on MCF-7 Cell Viability

MCF-7 cells were treated with shikonin for 24 h (a), 48 h (b) and 72 h (c). Cell viability was examined by MTT reduction assays and the results were expressed as percentage of surviving cells over control cells. Data are presented as mean \pm S.D. ($n=3$). *** $p < 0.001$ compared with the control group.

apoptotic DNA fragmentation increased in a dose-dependent manner (Fig. 4).

Apoptosis, or programmed cell death, is an important process in biological systems, including normal cell turnover, immune system, embryonic development, metamorphosis and endocrine-dependent tissue atrophy. Apoptosis is characterized by a decrease in cell volume, condensation and fragmentation of nuclear chromatin and dilatation of the endoplasmic reticulum. Many natural products have been reported to exert their antitumor effects through various apoptotic pathways.^{14,15)} For example, induction of apoptosis by shikonin was through coordinative modulation of the Bcl-2 family, p27, and p53, release of cytochrome c, and sequential activation of caspases in human colorectal carcinoma cells.¹⁰⁾ Shikonin regulates HeLa cell death via caspase-3 activation and blockage of DNA synthesis.¹³⁾ Apoptosis

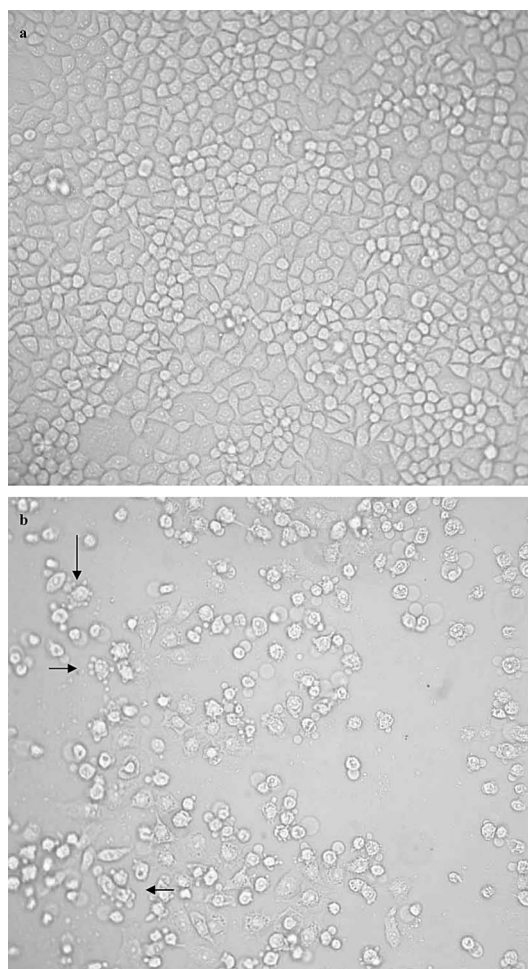


Fig. 3. Shikonin-induced Apoptosis in MCF-7 Cells

Morphological changes of MCF-7 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 indicate multiblebbing cells and apoptotic bodies.

induced by shikonin was through a caspase-9-dependent mechanism in human malignant melanoma A375-S2 cells.⁹⁾ Reactive oxygen species was involved in the apoptotic induction of human SK-Hep-1 hepatoma cells by shikonin.⁸⁾ Shikonin modulates cell proliferation by inhibiting epidermal growth factor receptor signaling in human epidermoid carcinoma cells.¹⁶⁾

In conclusion, the present study demonstrated for the first time that the cytotoxic effect of shikonin on MCF-7 cells underwent apoptosis process. However, the mechanisms underlying shikonin-induced MCF-7 cell apoptosis merits further investigation.

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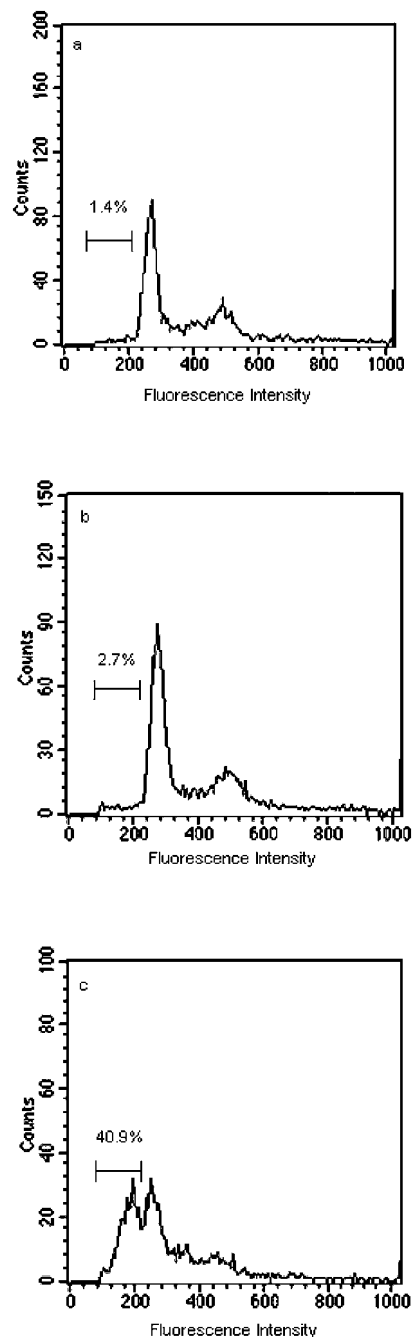


Fig. 4. Effect of Shikonin on the Induction of Apoptosis by DNA Damage in MCF-7 Cells

MCF-7 cells (1×10^6 cells) were treated with medium (a) $1 \mu\text{M}$ shikonin (b) and $10 \mu\text{M}$ shikonin (c) for 24 h. The DNA content after PI staining was analyzed by fluorescence flow cytometry.

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