Shikonin Modulates Cell Proliferation by Inducing Apoptosis in LLC Cells *via* MAPK Regulation and Caspase Activation

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Shikonin is a chemically characterized component of traditional herbal medicine, the root of *Lithospermum erythrorhizon* and has been shown to possess antitumor activities. Here we investigated anticancer potential of shikonin and its possible mechanism of action in LLC cells. Shikonin inhibited the proliferation of LLC cells in a concentration-dependent manner. It was also demonstrated that shikonin induced apoptosis in LLC cells by Annexin V staining and TUNEL staining analysis. Shikonin treatment was caused that decrease of Bcl-2, activation of caspases and cleavage of PARP. And shikonin also induced that the activation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Interestingly, the cell proliferation inhibition induced by shikonin was recovered by specific inhibitors of JNK and p38 but the inhibitor of MEK, the upstream kinase of ERK, did not recover. Additionally, shikonin administration at doses of 5 mg/kg in C57BL/6 mice strongly inhibited the primary tumor growth of LLC. Taken together, these results suggest that shikonin may suppress LLC cell proliferation by inducing an apoptotic process via activation of caspases and MAPKs

Key words : Shikonin, apoptosis, MAP kinase, caspases, LLC cells

Introduction

Apoptosis, programmed cell death, is a physiological process and is critical for normal development process, tissue homeostasis, development of nervous system and the regulation of the immune system in multicellular organisms and so on[1-2]. Cells are continuously exposed to multiple opposing death and survival triggers. Dysregulation of the delicate balance between death and life is associated with the pathogenesis of a wide variety of diseases including cancer, viral infections, neurodegenerative disorders, and some autoimmune disorders[2-3]. Apoptosis is a strictly controlled mechanism of cell suicide triggered by certain internal or external signals[4]. Because many chemotherapeutic drugs have been shown to induce apoptosis in malignant cells, the therapeutic application of apoptosis has currently been used as a model for developing antitumor drugs[5-6]. For example, most inducers of differentiation, such as camptothecin[7-8] and VP16[7], also induce apoptosis in cancer cells. Various chemotherapeutic agents, such as cisplatin[9], adriamycin[10], and taxol[11], have been reported to have apoptosis-inducing activity.

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Protein kinases are involved in various intracellular signaling pathways. Protein kinases and other associated signaling proteins are perfectly suited to regulated life and death decisions made in response to extracellular signals. The mitogen-activated protein kinase (MAPK) cascade is evolutionarily well conserved in eukaryotic cells and is typically composed of three kinases that establish a sequential activation pathway comprising a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAP kinase[3,12].

. JNK, p38 and ERK are well-characterized subgroups of a large MAP kinase family. These kinase pathways are structurally similar, but functionally distinct[12]. While ERK is rapidly activated by a variety of cell growth and differentiation stimuli and plays a central role in mitogenic signaling, JNK and p38 are primarily activated by various environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, proinflammatory cytokines such as tumor necrosis factor (TNF-a) and interleukin (IL)-1[3].

Shikonin, a napthaquinone is the active ingredient isolated from the *Lithospermum erythrorhizon* SIEB. et ZUCC. and it has been shown to possess a high level of growth inhibitory activity against ascite sarcoma 180[13]. Shikonin also

has been reported to exhibit a variety of abilities such as accelerating tissue granulation proliferation[14] and would healing[15], exerting antibacterial[16], and anti-inflammatory[15].

Additionally, shikonin has been associated with apoptosisin HL60 cells, as well as expression of c-jun protooncogene[17-18]. Shikonin also inhibited topoisomerase I and II activities by inducing the topoisomerase-mediated cleavage of DNA in vitro[19-20]. b-Hydroxyisovalerylshikonin, a shikonin-related compound, also exhibited apoptosis-inducing ability viaactivating mitogen activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK) 2, c-Jun N-terminal kinase (JNK), and p38, which are involved in the upstream regulation of apoptosis[21]. Lately, shikonin has been shown to modulate cell proliferation by inhibiting epidermal growth factor receptor (EGFR) signaling[22]. Non small lung cancer is the very common primary malignant tumor of the lung. Clinical observations in later-stage lung cancer patients who were not candidates for surgery, radiotherapy or chemotherapy showed that the shikonin mixture inhibited the growth of lung tumors and improved the immune function of the body[23].

In the present study, anti-proliferate potential of shikonin was evaluated using LLC cells, non small lung cancer cells. The molecular mechanisms of shikonin-induced apoptotic cell death were also investigated by flow cytometric analysis and western blot analysis. We also assessed the in vivo ability of shikonin to suppress tumor growth.

Materials and methods

1. Chemicals and Reagents

Shikonin was isolated at College of Pharmacy, Chungnam University, Taejon, Korea[24]. shikonin was dissolved in dimethylsulfoxide (DMSO) for the in vitro study and dissolved in saline and adjusted to pH 7.0 for the in vivo study.N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) was purchased from Medical and Biological Laboratories (Nagoya, Japan). SP600125, SB203580 and U0126 were purchased from Calbiochem (CA, USA).

2. Cell cultures

Lewis lung carcinoma (LLC) cells, originated spontaneously from mouse lung, were kindly provided by Dr. K Takeda (Tohoku University, Tohoku, Japan). They were maintained as monolayer cultures in Eagle's Minimal Essential Medium (EMEM; GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS; INC Biomedicals Inc., CA, USA). LLC cells were collected by brief treatment with EDTA, and then used for the experiments. All cultures were maintained at 37℃ in a humidified atmosphere of a 5% CO_2/95% O_2 air.

3. Animals

Six-week old, specific-pathogen-free female C57BL/6 mice were purchased from Daehan Biolink (Chungbuk, Korea). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

4. Cell proliferation BrdU assay

5-bromo-2'-deoxyuridine (BrdU) incorporation was determined by Cell Proliferation Enzyme Linked Immunosorbent Assay (ELISA) BrdU (Roche Diagnostics., Mannheim Germany) according to the manufacturer's instructions.

5. Annexin V binding analysis by flow cytometry

Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured by flow cytometer by using Annexin V-enhanced green fluorescent protein (EGFP) Apoptosis Detection Kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) according to the manufacturer' instructions. The cells were stained simultaneously with propidium iodide (PI). Flow cytometric analysis was conducted with a Becton Dickinson FACS Calibur (BD Biosciences, NJ, USA), with application of Cell Quest software.

6. TUNEL assay

For flow cytometric detection of Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-positive cells, LLC cells were stimulated with shikonin and cells were washed twice and harvested after 12 hr. TUNEL staining was performed using the MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) according to the manufacturer's instructions, and conducted flow cytometric analysis with a Becton Dickinson FACS Calibur (BD Biosciences, NJ, USA), with application of Cell Quest software.

7. Western blot analysis

LLC cells were cultured in complete medium for 24 hr. The cells were then incubated with shikonin for various time periods. After indicated treatment, the cells were then rinsed with ice-cold PBS and then whole cell lysates were prepared with lysis buffer (25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylemethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Cell lysates

were subjected to SDS-polyacrylamide gel and transferred to Immobilion-P membranes (Millipore, MA, USA). The membrane was treated with Block Ace (Dainipponseiyaku, Osaka, Japan) overnight at 4 C and probed with anti-caspase-3, anti-PARP, anti-phospho-JNK, anti-phospho-ERK, anti-phospho -p38, anti-b-actin (Cell Signaling Technology Inc., MA, USA), anti-JNK, anti-ERK, anti-p38 and anti-PCNA (Santa Cruz Biotechnology Inc., CA, USA) antibodies for 2 hr. The primary antibodies were detected using horseradish peroxidase-conjugated rabbit anti-mouse IgG, goat anti-rabbit IgG, rabbit anti-mouse IgG (DAKO A/S Denmark), and visualized by enhanced chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK). PCNA and b-actin were reprobed to indicate evenness of loading of protein extract from each treatment.

8. In vivo tumor growth model

LLC cells were harvested by brief exposure to EDTA, washed and resuspended in PBS. Mice were given subcutaneous injection in the flank region with LLC cells (2×10^4/50 ml). shikonin (5 mg/kg) was i.padministered for 5 days from day 3 after tumor cell inoculation, whereas doxorubicin was i.v. administered for 7 days after tumor cell inoculation. Tumor sizes were monitored every three days for eighteen days after tumor inoculation. The mice were sacrificed and the tumor-inoculated skin was separated from the underlying tissues. Tumor growth was assessed by measuring with a caliper square along the longer axes (a) and the shorter axes (b). Tumor volumes (mm^3) were calculated by the following formula. Tumor volume (mm^3) = $ab^2/2$.

9. Statistical Analysis

Representative data from each experiment are presented as mean values SD or SE, as described in the figure legends. The statistical differences between the groups were determined by applying the Student's two-tailed t-test. The Dunnett's test was performed to decrease the multiplicity in comparisons of drug-treated groups with control group. Statistical significance was defined as a P value < 0.05.

Results

1. Inhibitory effects of shikonin on the proliferation of LLC cells

Previous report, we demonstrated the cytotoxicity of shikonin on LLC cells using WST-1 assay (in press). Shikonin, at concentrations of more than 1 mM, showed direct cytotoxicity against LLC cells for 24, 48 hr incubation, For example, IC_{50} of 24 hr incubation was 6.5 8.5 mM on shikonin. Next, the inhibitory effect of shikonin on the BrdU incorporation was examined. As shown in Fig. 1, shikonin may inhibit cell proliferation even though in the non-cytotoxic concentrations via inhibiting the BrdU incorporation.

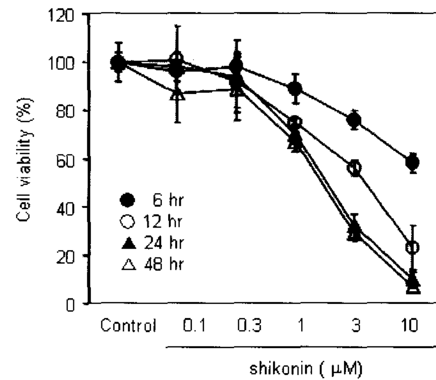

Fig. 1. Effect of shikonin on the proliferation of LLC cells. LLC cells were seeded in 96-well culture plates. After a 24-hr preincubation, various concentrations of shikonin were added to the cultures and the cultures were employed a further incubation. After the end of incubation, BrdU incorporation wasaccomplished according to manufacturer's instructions. The absorbance was measured at 450 nm. The data were expressed as the mean SD of triplicate cultures.

2. Flow cytometric analysis of apoptosis induced by shikonin

To examine whether the inhibitory effect of the shikonin on LLC cell proliferation was due to the induction of apoptosis, Annexin V and TUNEL staining analysis were carried out. As shown in Fig. 2A, shikonin increase the number of annexin V positive LLC cells in a concentration-dependent manner, and at the indicated concentrations of shikonin (1 to 10 mM), cells were not stained with PI (data not shown). Actinomycin D was used as positive control (Fig. 2B) and PI staining was performed for the purpose of excluding necrotic cells. To further confirm the aspect of cell death induced by shikonin, TUNEL staining analysis was performed. We observed that shikonin-treated LLC cells increased the fluorescence intensity of TUNEL positive cells, suggesting shikonin may induce apoptosis (Fig. 2C).

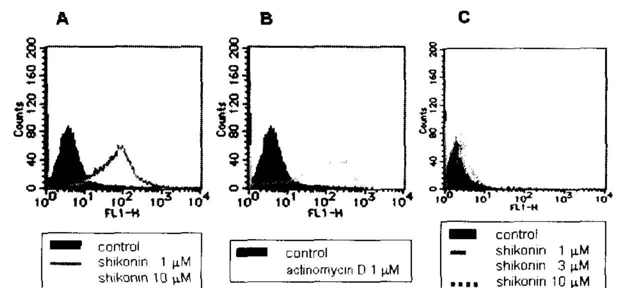

Fig. 2. Annexin V and TUNEL analysis of apoptosis induced by shikonin. A and B show the Annexin V staining of shikonin and Actinomycin D, respectively. C shows the TUNEL staining of shikonin

3. Analysis of the changes of apoptosis-related proteins induced by shikonin

To investigate the mechanism of shikonin-induced apoptosis, the expression levels of Bcl-2, caspases and PARP cleavage were examined by western blot analysis. The decrease in Bcl-2, the anti-apoptotic protein, or the inhibition of Bcl-2 activity might provoke apoptosis or at least sensitize cells to apoptotic death[26]. As shown in Fig. 3A, the shikonin treatment for 12 hr dramatically decreased the level of Bcl-2 in a concentration-dependent manner. It is interesting that the expression of active forms of caspase-3 were increased at concentrations of shikonin 1 ㎛ and 10 ㎛ (Fig. 3A). In a time-course analysis, caspase-3 was significantly activated from 6 to 12 hr culturewith shikonin (Fig. 3B). In order to further understand the molecular pathway of shikonin-induced apoptotic cell death, the effect of shikonin on the expression of PARP was investigated. Shikonin caused the proteolytic cleavage of PARP with accumulation of the 89 kDa fragmentsand disappearance of the full-length 116 kDa protein in a concentration-dependent manner (Fig. 3A). Time-course analysis also showed that PARP were significantly cleaved by 6 to 12 hr culturewith shikonin (Fig. 3B).

To certify the involvement of caspases in shikonin-induced apoptosis, we used Z-VAD-FMK, a broad spectrum inhibitor of the caspase family. LLC cells were incubated with 10 mM of shikonin in the absence or presence of Z-VAD-FMK, and then the BrdU incorporation assay was performed. Treatment with Z-VAD-FMK significantly reduced the shikonin-induced inhibition of cell proliferation (Fig. 5A). These results suggest that caspases was involved the induction of apoptosis by shikonin.

4. Effects of shikonin on the activities of MAP kinases

The balance between the MAP kinases, such as ERK, JNK and p-38, has been proposed to play an important role in the regulation of apoptosis3[12]. As shown in Fig. 4. Western blotting analysis revealed that the level of phospho-JNK and phosphor-p38 MAPK were increased by shikonin. However, the level of ERK reached the peak activation at 6 hr incubation and then the phosphorylation level was decreased.

To confirmthe contributions of MAP kinases to the apoptosisinduced by shikonin, LLC cells were incubated with 10 mM of shikonin in the absence or presence of their specific inhibitors and then the BrdU incorporation assay employed. As shown in Fig. 5, the addition of SP600125, a specific inhibitor of JNK, and SB203580, a specific inhibitor of p38, significantly recovered the decreased cell proliferation inhibited by shikonin (Fig. 5B and C). On the contrary, the specific inhibitor of MEK, U0126, additionally inhibited the decreased cell proliferation (Fig. 5D). These results suggest that p38 and JNK are associated with the induction of apoptosis by shikonin, whereas ERK activation is associated with the cell survival[12].

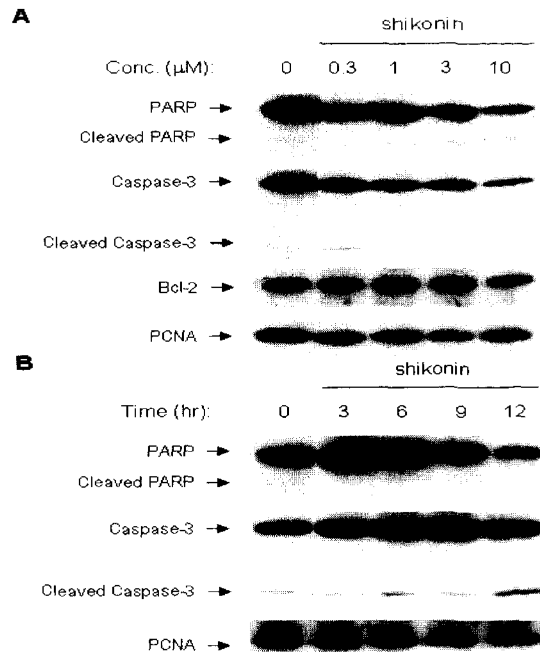

Fig. 3. Analysis of apoptosis-related molecules induced by shikonin. A. LLC cells were stimulated with various concentrations of shikonin and expression of PARP, caspase-3, Bcl-2 and PCNA was assessed by Western blot analysis. B. Time-course analysis of apoptosis-related molecules induced by shikonin. LLC cells were stimulated with 10 mM of shikonin for various time periods. Expression of PARP, caspase-3 and PCNA was assessed by Western blot analysis.

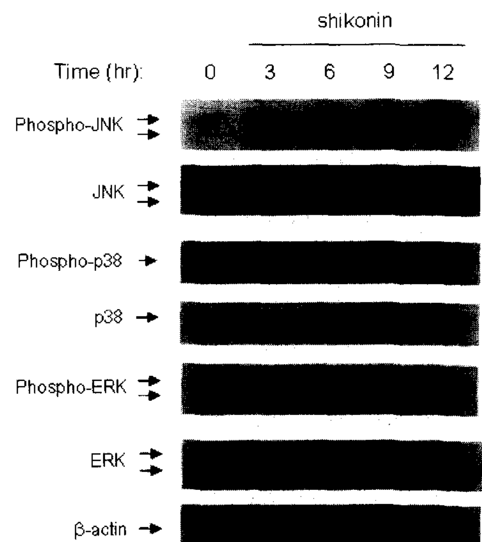

Fig. 4. MAPKs phosphorylation induced by shikonin against LLC cells. LLC cells were stimulated with shikonin for various time periods. MAPKs and phospho-MAPKs expression was assessed by Western blot analysis.

5. Effects of shikonin on tumor growth in LLC tumor-bearing mice

Next, we investigated that the anti-tumor activity of

shikonin in vivo using a tumor growth model by orthotopic implantation of LLC cells. LLC cells were subcutaneously injected in the flank region and shikonin (5 mg/kg/day, i.p.) or doxorubicin (7 mg/kg/day, i.v.) were administered for 5 days from 3 day after tumor cell inoculation. As shown in Fig. 6, treatment with shikonin significantly suppressed the tumor growth in a dose-dependent manner. shikonin did not show any side effects including body weight loss during the in vivo experiments (data not shown). These results suggest that shikoninhas a potent inhibitory activity against the tumor growth in vivo.

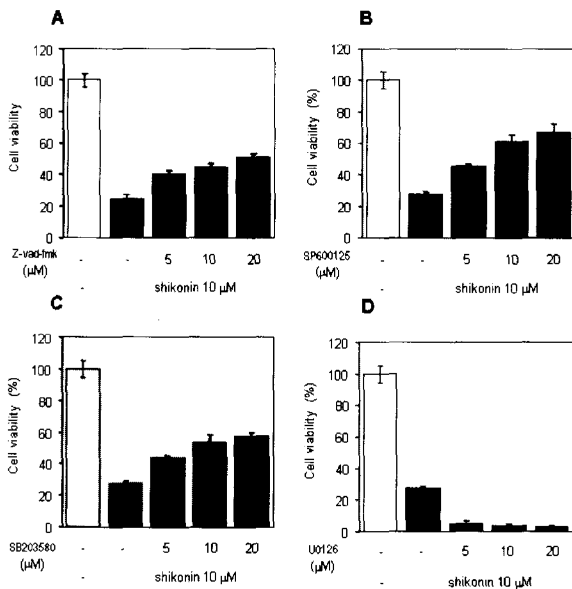

Fig. 5. Effect of caspases and MAPKs inhibitors on shikonin-induced apoptosis. LLC cells were seeded in 96-well culture plates. After a 24-hr preincubation, both inhibitors and test-compounds were added to the cultures and the cultures were employed a further 12-hr incubation. After the end of incubation, BrdU incorporation was accomplished according to manufacturer's instructions. The absorbance was measured at 450 nm. The data were expressed as the mean SD of triplicate cultures. A, treated with Z-VAD-FMK. B, treated with SP600125. C, treated with SP203580. D, treated with U0126.

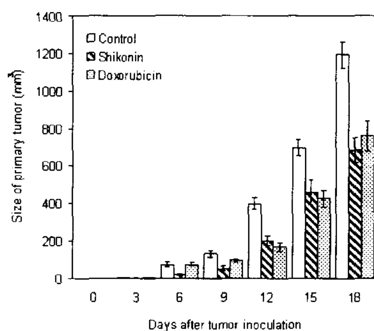

Fig. 6. Effect of shikonin on tumor growth induced by LLC cells inoculated in the flank region of mice. Mice were subcutaneously inoculated with LLC cells and administered shikonin, doxorubicin or vehicle for 5 days from the 3 days after tumor cell inoculation. Tumor-inoculated sites were isolated from vehicle- or sample-treated mice 18 days after the tumor inoculation. The tumor size was measured every three days. Data are represented as the mean SE of 7 mice in each group.

Discussion

Cancer cells from a wide variety of human malignancies have shown decreased apoptosis in response to at least some physiologic stimuli. Therefore, mutations in cell death control do affect sensitivity of tumor cells to anti-cancer therapy and increasing apoptotic activities would be useful tip to develop anti-cancer drugs 2. In the present study, we have demonstrated that shikonin, a natural compound acts as a chemotherapeutic agent by modulating the MAPK signaling in LLC cells. Shikonin preferentially inhibited proliferation of LLC cells in a concentration- and time-dependent manner(Fig. 1). We also reported that the inhibition of proliferation induced by shikonin was proved to be apoptosisby Annexin V and TUNEL staining analysis (Fig. 2). This apoptotic cell death was mediated by the induction of caspases activities, cleavage of PARP and decrease of Bcl-2 proapoptotic proteins (Fig. 3) in a concentration- and time-dependent manner. Heterodimerization with Bcl-2 leads to the inhibition of its apoptotic function[27] and shikonin significantly decreased the level of Bcl-2. The caspase family of cysteine proteases is emerging as the central executioner of apoptosis and caspase-3 is activated in a variety of cell types during apoptosis[28]. Also the cleavage of PARP is another hallmark of apoptosis although PARP is not essential for cell death. This study has presented that the active form of caspase-3 was shown and intact form of 116 kDa of PARP was decreased in a concentration-dependent manner. To confirm the connection between caspases and shikonin-induced apoptosis, we employed the inhibitor of broad spectrum of caspases, Z-VAD-FMK. It was confirmed that shikonin-induced apoptosis was caspases-dependent because Z-VAD-FMK recovered the inhibition of cell proliferation caused by shikonin in a concentration-dependent manner(Fig. 5A).

MAP kinases regulate cell fate, such as growth, differentiation or apoptosis, in response to environmental changes, through phosphorylation of a variety of downstream substrates[3]. One interesting feature of shikonin was that MAP kinases, such as JNK and p38, were activated within 1 hr after treatment of LLC cells with 10 mM of shikonin and the phosphorylation of JNK and p38 was continued over 12 hr (Fig. 4). However, the phosphorylation of ERK was decreased after 6 hr of shikonin treatment (Fig. 4). Several studies have indicated that the stress-related kinase p38 and JNK are mainly associated with the induction of responses such as cytokine release and apoptosis[29]. Various agents have been found to mediate the induction of apoptosis through the activation of p38 and JNK[12,29,30]. The activation of p38 and JNK induced by shikonin in LLC cells was associated with proliferation

inhibition and an increase of apoptotic cells. Moreover, specific inhibitor of p38 (SP203580) or specific inhibitor of JNK (SP600125) recovered the proliferation inhibition induced by shikonin in a concentration-dependent manner (Fig. 5B and C). In contrast, specific inhibitor of MEK (U0126) was not only effective for recovering, but accelerated the inhibition of cell proliferation caused by shikonin (Fig. 5D). These results have shown that shikonin-activated JNK/p38 induced apoptosis and shikonin-activated ERK apparently stimulated survival, while U0126 accelerated the proliferation inhibition induced by shikonin.

Finally, we investigated the anti-cancer effect of shikonin against in vivo tumor-bearingmice model. As shown in Fig. 6, shikonin suppressed the primary tumor growth via induction of apoptosis and inhibition of cancer cell proliferation (Fig. 6). This in vivo study hasshown that shikonin may be a possible candidate for suppression of tumor growth.

In conclusion, our study has demonstrated that shikonin may have a chemotherapeutic potential for controlling tumor growth on its proliferation inhibitory activity and apoptosis-inducing effect. Characterization of the detailed biological mechanism and further animal experiments will provide new insights into the future clinical evaluation of this compound.

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