—Regular Articles—

Shikonin, Acetylshikonin, and Isobutyroylshikonin Inhibit VEGF-induced Angiogenesis and Suppress Tumor Growth in Lewis Lung Carcinoma-bearing Mice

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Lithospermum erythrorhizon has been used for treatment of inflammatory diseases and cancer as a folk remedy. Based on the evidences that anti-inflammatory agents frequently exert antiangiogenic activity, thus we examined comparatively the antiangiogenic activities of three naphthoquinone derivatives (shikonin, acetylshikonin, and isobutyroylshikonin) isolated from the plant. Three derivatives exhibited weak cytotoxicity against human umbilical vein endothelial cells (HUVECs) with IC_{50} of over 20 μM. Shikonin had more specific inhibitory effects on proliferation and vascular endothelial growth factor (VEGF) production by VEGF compared with different derivatives. All of derivatives significantly suppressed the migration of VEGF treated HUVECs at different optimal concentrations. Also, shikonin and acetylshikonin significantly disrupted VEGF-induced tube formation. Furthermore, three derivatives effectively downregulated the expression of urokinase-type plasminogen activator (uPA), but not its receptor uPAR. Additionally, shikonin significantly inhibited tumor growth in LLC-bearing mice, whereas its derivatives had relatively mild effects. Taken together, our findings suggest that shikonin and its derivatives exhibit the antiangiogenic and antitumorigenic effects by suppressing proliferation and angiogenic factors.

Key words——*lithospermum erythrorhizon*; shikonin derivative; angiogenesis; vascular endothelial growth factor (VEGF)

INTRODUCTION

Lithospermum erythrorhizon Siebold & Zucc. has been used for treating tumors, inflammation, and burns. Shikonin, a pigment ingredient isolated from the *Lithospermum erythrorhizon*, has been reported to exhibit a variety of biological activities such as accelerating tissue granulation proliferation[1] and wound healing,[2] exerting antibacterial,[3] anti-inflammatory,[4] and antitumor effects.[5] Shikonin has also been shown to possess a high level of growth inhibitory activity against sarcoma-180 ascite cells,[5] suggesting its potent application as an antitumor reagent, and induce apoptosis in HL60 cells. Shikonin also inhibited topoisomerase I and II activities by inducing the topoisomerase-mediated cleavage of DNA *in vitro*.[6,7] Shikonin derivatives such as acetylshikonin and isobutyroylshikonin were also isolated from the same plant, had a similar structure, and gave similarly biological activity compared with shikonin.[8–11] However, there is no comparison between shikonin and its derivatives on the effect of antiangiogenesis and antitumorigenesis that have not yet investigated. Thus, the purpose of this study was to evaluate the antiangiogenic and antitumorigenic activities of shikonin, acetylshikonin, and isobutyroylshikonin comparatively.

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MATERIALS AND METHODS

Chemical Reagents Dulbecco's modified eagle medium (DMEM), M199 medium RPMI 1640 medium and antibioticantimycotics were purchased from GIBCO (Grand Island, NY). Vascular endothelial growth factor (VEGF) and Basic fibroblast growth factor (bFGF) was from R & D (Minneapolis, MN). Fetal bovine serum (FBS) was from JRH (Lenexa, KS). Heparin, gelatin, BSA, sodium bicarbonate, 2,3-bis [2-methoxy-4-nitro-5-sulfo] -2H-tetrazolium-5- carboxanilide (XTT), PMS were from Sigma Chemical (St. Louis, MO). DiffQuick was purchased

from Dade Behring (Newark, DE). Matrigel was purchased from Becton Dickinson (Bedford, MA). Trizol was purchased from Life Techologies (Grand Island, NY). Oligo poly dT was purchased from Roche Diagnostics (Indianpolis, IN). Taq polymerase and deoxynucleotide triphosphate (dNTP) were bought from Takara. Murine moloney lentivirus (MMLV) reverse transcriptase was purchased from Promega (Madison, WI). Urokinase type plasminogen activator (uPA), Urokinase type plasminogen activator receptor (uPAR) glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers were purchased from GenoTech (Yusung, TJ).

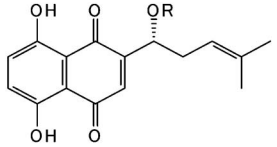
Plant Materials The roots of *Lithospermum erythrorhizon* were purchased from the Oriental drug store, Gyeong Dong (Seoul, Korea). A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University.

Extraction and Isolation The plant material (2 kg) was extracted three times with MeOH at room temperature, and then filtered. The filtrate was evaporated *in vacuo* to give a dark red residue. The resultant methanolic extract (450 g) was subjected to successive solvent partitioning to give $CHCl_3$ (25 g), EtOAc (7 g), *n*-BuOH (43 g) and H_2O soluble fractions. Silica gel column chromatography of the $CHCl_3$-soluble fraction, with *n*-hexane-$CHCl_3$-MeOH (20 : 6 : 1), gave six fractions (C1-C6). Fraction C2 was chromatographed by silica gel with *n*-hexane-$CHCl_3$-MeOH (25 : 6 : 1), and purified by Sephadex LH-20 (MeOH) to give compound **1** (23 mg). Fraction C3 and C4 were chromatographed by Lobar A column with *n*-hexane-$CHCl_3$-MeOH (20 : 6 : 1), and purified by Sephadex LH-20 (MeOH) to give compound **2** (120 mg) and **3** (21 mg), respectively (Fig. 1).

(1) Isobutyroylshikonin -^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 12.55, 12.41 (each 1H, s, OH), 7.15 (2H, s, H-6,7), 6.93 (1H, s, H-3), 5.21 (1H, m, H-13), 5.12 (1H, m, -CH=C<), 2.50 (2H, m, H-11), 1.69 (3H, s, H-16), 1.58 (3H, s, H-15).

(2) Acetylshikonin -^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 12.54, 12.38 (each 1H, s, OH), 7.15 (2H, s, H-6,7), 6.99 (1H, s, H-3), 6.02 (1H, dd, J= 7.6, 4.4, H-11), 5.13 (1H, m, H-13), 2.62, 2.47 (each 1H, m, H-12), 2.16 (3H, s, $COCH_3$), 1.70 (3H, s, H-16), 1.59 (3H, s, H-15).

(3) Shikonin -^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 12.57, 12.48 (each 1H, s, OH), 7.18 (2H, s, H-6,7), 7.14 (1H, s, H-3), 4.90 (1H, dd, J=7.7, 4.3, H-11), 5.20 (1H, m, H-13), 2.64, 2.36 (each 1H, m, H-12), 1.75 (3H, s, H-16), 1.62 (3H, s, H-15).



R = H (Shikonin)

R = $COCH_3$ (Acetylshikonin)

R = $COCH(CH_3)_2$ (Isobutyroylshikonin)

Fig. 1. Structures of Shikonin and Its Derivatives

Cell Culture Mouse LLC cells (Lewis lung carcinoma cells) were kindly provided by Dr. I. Saiki (Toyama Medical and Pharmaceutical Univ., Toyama, Japan) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml antibiotic-antimycotic and 2.2 g/l sodium bicarbonate. A549 cells (human lung adenocarcinoma cells) were purchased from Korean cell line bank. A549 cells were cultured in RPMI1640l medium containing 10% heat-inactive FBS, 100 U/ml antibiotic-antimycotic and 2.2 g/l sodium bicarbonate. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins according to a published protocol[12] and cultured in M199 supplemented with 20% heat-inactivated FBS, 3 ng/ml bFGF, 5 U/ml heparin and 100 U/ml antibiotic-antimycotic in 0.1% gelatin-coated flasks. HUVECs were used in passages three to six. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

Proliferation Assay HUVECs (5×10^3) were seeded onto 0.1% gelatin-coated 96-well microplates and incubated in a humidified incubator for 24 h. Cells were starved for 6 h in M199 containing 5% heat-inactivated FBS and then treated with various concentrations of shikonin and its derivatives in M199 containing 5% heat-inactivated FBS, 10 ng/ml VEGF and 5 U/ml heparin. After 48 h incubation, XTT working solution was added and then the optical density was measured using microplate reader (Molecular Devices) at 450 nm.

Migration Assay Confluent HUVECs (3×10^5) monolayer was seeded onto 0.1% gelatin coated 6-well plates and ''scratch'' wounded using the tip of a universal 200 μl pipette tip. Then cells were treated with various concentrations of shikonin and its derivatives in M199 with 5% FBS, 10 ng/ml VEGF

and 5 units/ml heparin. After 17 h incubation, rinsed with PBS and cells were stained Diff Quick solution and randomly chosen fields were photographed under a light microscope at 100× magnification. The number of migration was counted.

Tube Formation Assay *In vitro* differentiation assay of HUVECs on Matrigel into capillary-like tubes was performed as described by Grant *et al.*[13] HUVECs (3×10^4) were seeded onto Matrigel-coated 24-well plates, and shikonin and its derivatives were added in M199 with 1% FBS, 10 ng/ml VEGF and 5 U/ml heparin. After 7 h, cells were fixed with 4% paraformaldehyde, and randomly chosen fields were photographed under an Axiovert S 100 light microscope (Carl Zeiss, USA) at 100× magnification. The number of tube formation was counted.

Measurement of hVEGF The level of hVEGF in the supernatant of HUVECs was measured with a commercially available ELISA kit (R&D systems Inc., Minneapolis, MN). Briefly, HUVECs were starved for 6 h in M199 containing 5% FBS and then treated with VEGF (10 ng/ml) containing shikonin (2.5, 5 μM) and its derivatives (10, 20 μM) for 24 h. After 24 h incubation, the supernatant was individually collected and measured by ELISA kit.

RT-PCR Analysis mRNA levels of uPA, and uPAR were determined by an internal-based semi-quantitative reverse transcriptase-polymerase chain reaction assay (RT-PCR), as previously described.[14] A549 cells were plated at 5×10^5 cells/well in 6 well plates for 24 h. The cells were treated with various concentrations of shikonin and its derivatives for 24 h. Total RNA was prepared from A549 cells by using Trizol reagent. Total RNA (1.0 mg) was reverse transcribed by using oligo poly (dT) and MMLV reverse transcriptase. The mixture was incubated for 10 min at 25℃, then for 60 min at 42℃ and finally for 5 min at 99℃. Specific primers were used to amplify cDNAs:

uPA sense was 5′ CTGCCTGCCCTGGAACTCTG-3′, uPA antisense was 5′-CCTTGCGTGTTGGAG-TTAAG-3′ (expected product 477 bp).

uPAR sense was 5′-CATGCAGTGTAAGACCCA-ACGGGGA-3′, uPAR antisense was 5′-AATAG-GTGACAGCCCGGCCAGAGT-3′ (expected product 253 bp).

GAPDH sense was 5′-GTGGATATTGTTGCCAT-CA-3′, GAPDH antisense was 5′-ACTCATACAG-CACCTCAG-3′ (expected product 700 bp). GAPDH was used in each experiment as an internal control. 30 cycles consisting of 30 s at 94℃, 30 s at 59℃ and 30 s at 72℃ were used, and then followed by 5 min incubation at 72℃. Agarose gel electrophoresis was performed and the ethidium bromide-stained cDNAs were photographed under a U.V. transilluminator by using Polaroid positive/negative instant films. The amount of mRNA transcripts was analyzed by densitometry.

LLC Tumor Model Five-week-old female C57BL/6 mice were purchased from Daehan Biolink, (Chungbuk, Korea) and given food and water ad libitum. Mice were housed in a room maintained at 25± 1℃ with 55% relative humidity. One week later, mice were anesthetized and each was inoculated with 3×10^5 LLC cells by subcutaneous (s.c.) injection in 100 ml phosphate-buffered saline (PBS) on the right flank. Eight days after LLC inoculation, mice were each given a daily intraperitonial (i.p.) injection of shikonin and its derivatives at 2 mg/kg. Shikonin and its derivatives were dissolved in PBS. Control mice were administered with PBS. Tumor volumes were measured every other day with a caliper, and calculated according to the formula $[(\text{length}\times\text{width}^2)/2]$, where length represents the larger tumor diameter and width represents the smaller tumor diameter.[15–17] All mice were killed 21 days after inoculation with LLC cells and the tumors were removed.

Statistical Analysis All data are presented as means±standard deviation S.D. or S.E. The statistically significant differences between control and sample groups were calculated by the Student's *t*-test.

RESULTS

Shikonin and Its Derivatives Inhibit VEGF-induced Proliferation To determine whether shikonin and its derivatives exhibit the growth inhibitory effect of HUVECs (Fig. 2A) and A549 (Fig. 2B) cells were determined by XTT assay. Shikonin had no cytotoxicity within 5 μM in human endothelial cells, while shikonin derivatives exhibited no cytotoxicity at 5–20 μM, but human lung carcinoma A549 cells exhibited very sensitive to low concentrations of shikonin. Based on these data, we next determined whether shikonin and its derivatives possess the antiangiogenic and antitumorigenic activities at different concentrations. To elucidate antiangiogenic activity of shikonin derivatives, we investigated VEGF-induced proliferation of HUVECs *in vitro*. VEGF treated HUVECs

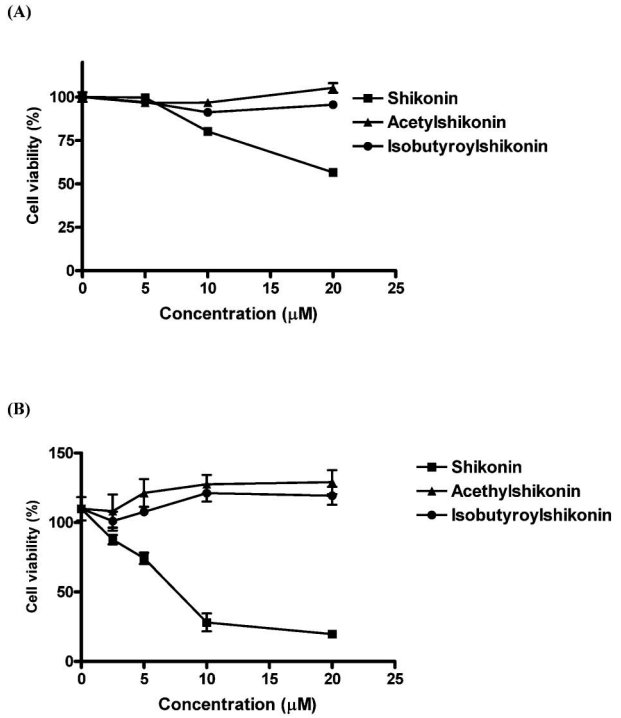


Fig. 2. Cytotoxicity Effect of Shikonin and Its Derivatives on Viable Cell Number of HUVECs Cells and A549 Cells as Evaluated by XTT Sssay

All data are presented as means±S.D. ($n=6$). (A) HUVECs. (B) A549 cells.

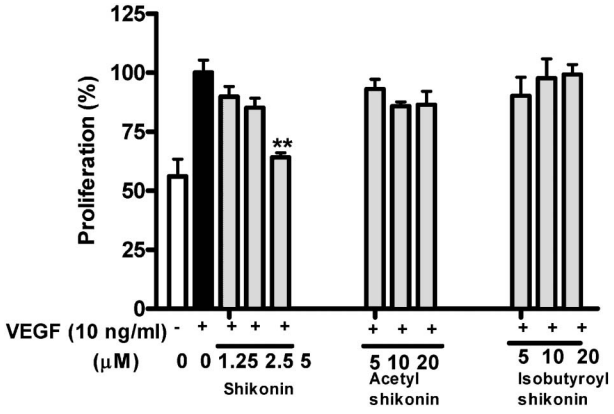


Fig. 3. Shikonin and Its Derivatives Inhibit VEGF-induced Proliferation of HUVECs *In Vitro*

Values represent means±S.D. $^{**}p<0.01$ *versus* VEGF positive control.

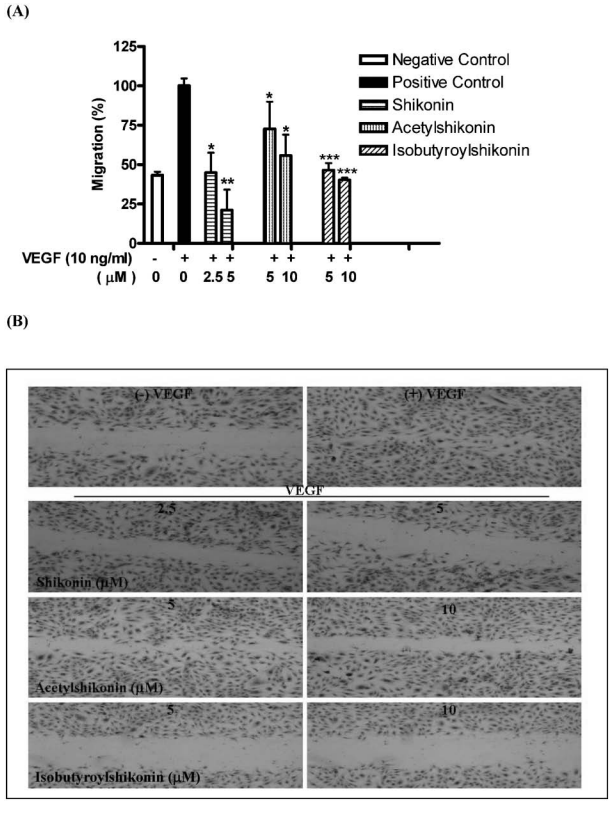


Fig. 4. Shikonin and Its Derivatives Inhibit VEGF-induced Migration of HUVECs *In Vitro*

(A) The percentage of migration cells was determined as described in Materials and Methods. Values represent means±S.D. $^{**}p<0.01$ and $^{***}p<0.001$ *versus* VEGF positive control. (B) Several randomly chosen fields were photographed under a light microscope at ×100 magnification.

was exposed to various concentrations of shikonin and its derivatives for 48 h. Shikonin inhibited VEGF-induced proliferation of HUVECs by 40% of untreated control at 5 μM. Similarly, acetylshikonin was mildly growth inhibitory at 10 and 20 μM. Isobutyroylshikonin did not have significantly effects on the proliferation of HUVECs under these conditions (Fig. 3). These results indicated that shikonin and its derivatives were required for their optimal concentration to exhibit anti-proliferative activity.

Shikonin and Its Derivatives Suppress VEGF-induced Migration Endothelial cell migration is critical events for angiogenesis. To examine whether shikonin and its derivatives have antiangiogenic activity *in vitro*, VEGF-induced migration assay was carried out. The results showed that VEGF significantly enhanced migratory activity and cotreatment with shikonin (5 μM), acetylshikonin (10 μM) and isobutyroylshikonin (10 μM) for 17 h resulted in 75, 50 and 60% inhibition of VEGF-induced migration, respectively (Fig. 4A). We photographed that shikonin and its derivatives produced comparable inhibitory effects on VEGF-induced HUVECs migration under a light microscope (Fig. 4B).

Shikonin and Its Derivatives Suppress VEGF-induced Tube Formation Stimulation of HUVECs seeded on Matrigel by angiogenic factors such as VEGF and bFGF promotes differentiation to form capillary-like tubes.[18] We performed the tube forma-

tion assay to assess any specific effect of shikonin and its derivatives on this process. The results showed that VEGF significantly increased the number of tube formation and cotreatment with shikonin (5 μM) and acetylshikonin (10 μM) for 5 h resulted in 45 and 50 % inhibition of VEGF-induced tube formation, respectively (Fig. 5A). We photographed that shikonin and its derivatives produced comparable inhibitory effects on VEGF-induced HUVECs tube formation under a light microscope (Fig. 5B).

Shikonin and Its Derivatives Reduce VEGF Production in VEGF-induced HUVECs The levels of secreted VEGF were measured by ELISA in VEGF-induced HUVECs. Shikonin (2.5 μM), acetylshikonin (10, 20 μM) and isobutylroylshikonin (10, 20 μM) significantly decreased secreted VEGF level in a concentration-dependent manner compared with untreated control in HUVECs (Fig. 6). From these results, shikonin has the most specific inhibitory effect on the production of VEGF in VEGF-induced cells.

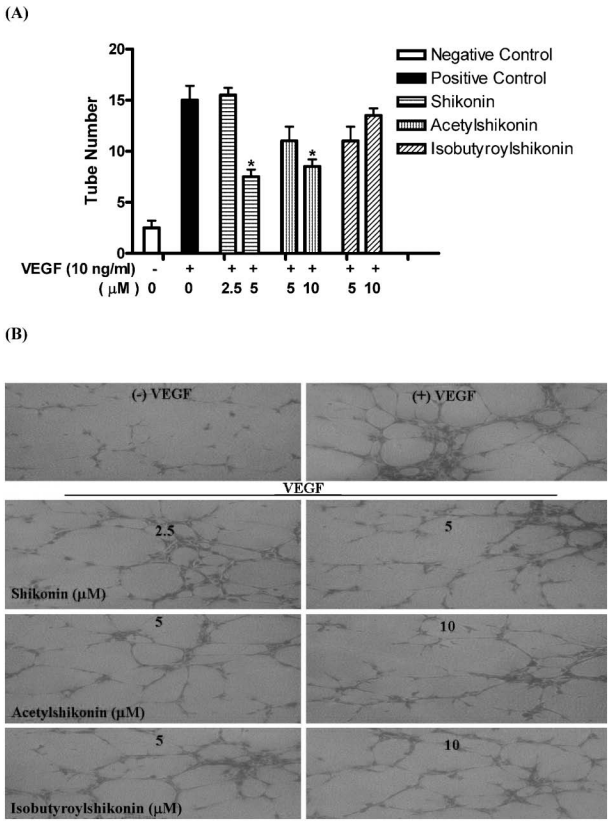


Fig. 5. Shikonin Derivatives Inhibit VEGF-induced Tube Formation of HUVECs *In Vitro*

(A) Capillary-like tube formation was examined 7 h after cell seeding as described in Materials and Methods. Values represent means±S.D. $^{*}p<0.05$ *versus* VEGF Positive control. (B) Several randomly chosen fields were photographed under a light microscope at ×100 magnification.

Shikonin and Its Derivatives Suppress the Expression of uPA, but Not uPAR To determine the effect of shikonin and its derivatives on the expression of uPA and uPAR in A549 cells, RT-PCR was performed. The mRNA level of uPA was effectively down-regulated, while the expressions of uPAR were not significantly affected by shikonin and its derivatives compared with untreated control (Fig. 7).

Shikonin and Its Derivatives Inhibit Tumor Growth in LLC-bearing Mice To compare the antitumor efficacy of shikonin and its derivatives *in vivo*, we inoculated C57BL/6 mice with an s.c. injection of LLC cells, and starting 8 days after tumor in-

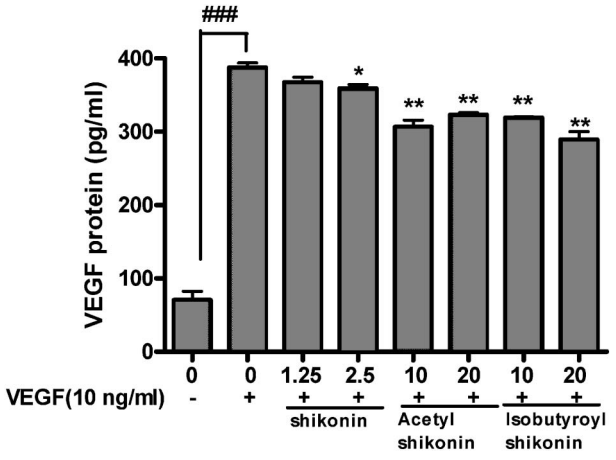


Fig. 6. Shikonin Derivatives Reduce the Secretion of VEGF in VEGF-induced HUVECs

HUVECs was exposed to shikonin derivatives for 24 h, the level of VEGF in conditioned medium was measured by ELISA. Values represent means±S.D. $^{\#\#\#}p<0.001$ *versus* unstimulated control; $^{*}p<0.05$, and $^{**}p<0.01$ *versus* control.

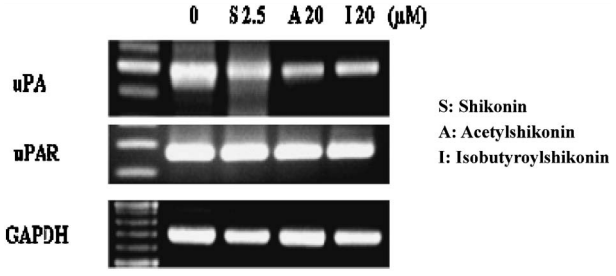


Fig. 7. Effect of Shikonin Derivatives on the Expression of uPA and uPAR mRNA in A549 Cells

Total RNA (1.0 mg) was reverse transcribed by using oligo poly (dT) and MMLV reverse transcriptase. The mixture was incubated for 10 min at 25℃, then for 60 min at 42℃ and finally for 5 min at 99℃. Primers of uPA, and uPAR were used to amplify cDNAs. After 25 cycles of PCR amplification, 10 ml aliquots were taken and separated on a 1% (w/v) agarose gel containing ethidium bromide (1 mg/ml). The PCR products were quantified densitometrically by a laser scanner. Markers: lane 1: 100 bp marker, lane 2: control, lane 3: shikonin 2.5 μM, lane 4: acetylshikonin 20 μM, lane 5: isobutyroylshikonin 20 μM.

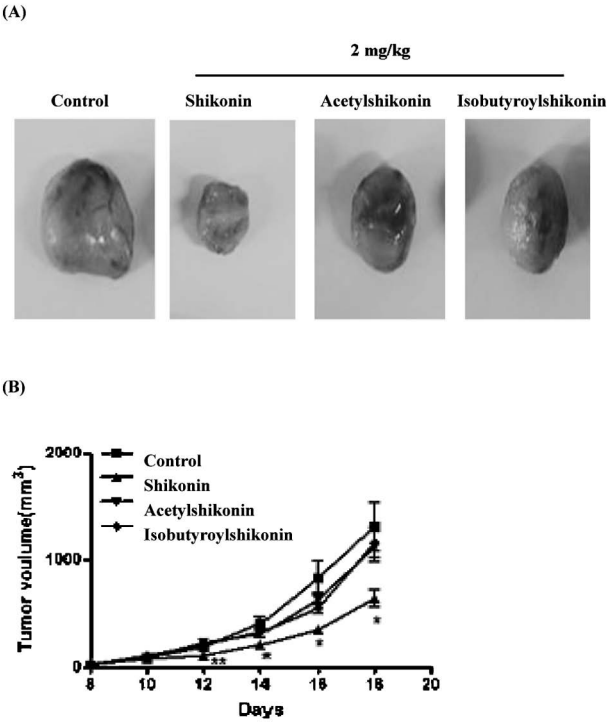


Fig. 8. Shikonin Derivatives Inhibit Tumor Growth in LLC-bearing Mice

(A) Excised tumors (from B) on day 18 of treatment. (B) Tumor volumes were measured every other day. $n=5$. Values represent means± S.E. $^{*}p<0.05$ *versus* control.

oculation we administered shikonin derivatives by daily i.p. injection. Tumor growth was monitored every other day for 3 weeks. Although two derivatives tumor volume inhibition was lesser than shikonin, they also exhibited tumor volume to reduce mildly when compared with control group (Fig. 8A). The tumor volume was decreased to 50% of control mice by 2 mg/kg of shikonin and to 17% by 2 mg/kg of shikonin derivatives respectively (Fig. 8B). However, shikonin (2 mg/kg) decreased weight of mouse by side effect, while shikonin derivatives (acetylshikonin and isobutyroylshikonin) inhibited tumor growth without side effect in same dose (2 mg/kg) (data not shown).

DISCUSSION

Angiogenesis plays an essential role in tumor growth, intravasation, and metastasis fundamental concepts of the angiogenic process[19] involves the proliferation, migration, and tube formation of endothelial cells and can be initiated by angiogenic cytokines such as bFGF and VEGF. Neo-angiogenesis is now considered essential for solid tumor growth and progression. Any significant increase in tumor mass beyond 2–3 mm must precede by an increase in the vascular supply to deliver nutrients and oxygen to the tumor cells and the neo-vascularization of endothelial cells.[20] Thus, angiogenesis inhibitors, which are not directly cytotoxic to tumor cells, can suppress tumor growth by inhibiting endothelial proliferation and migration and/or by inducing endothelial apoptosis in the vascular bed of tumors.[21] Our data significantly showed that shikonin, acetylshikonin, and isobutyroylshikonin decreased VEGF-induced migration in a concentration manner in HUVECs (Fig. 4A). Isobutyroylshikonin did not affect anti-proliferative and capillary-like formation activities (Figs. 3 and 5A), though it decreased VEGF-induced HUVECs migration. This result was similar to the *in vitro* and *in vivo* antiangiogenic activity of shikonin in B16 melanoma-induced HUVECs.[22] Enhanced angiogenesis can lead to accelerated growth of the primary tumor, and also facilitate the process of metastasis. It is well established that the primary mediator of this process in tumor is the cytokine, vascular endothelial growth factor (VEGF).[23] VEGF has been shown to promote endothelial cell migration and acts as mitogen for endothelial cells. It has been suggested that VEGF expression correlates with the degree of angiogenesis and plays a major role in non-small lung cancer prognosis.[24] Results from the ELISA indicated shikonin and its derivatives inhibit VEGF production in VEGF-treated HUVECs indicating that these compounds could inhibit vascular endothelial cells migration and capillary-like formation. Thus shikonin and its derivatives have the optimal concentrations showed an antitumor and antiangiogenic activity through inhibiting the VEGF production.

Antiangiogenic activity of shikonin and its derivatives predicts antitumor activity *in vivo*, which we demonstrated with LLC cells inoculated into the flank of C57BL/6 mice. Actually the tumor volume was decreased to 50% of control mice by 2 mg/kg of shikonin and to 17% by 2 mg/kg of shikonin derivatives (Fig. 8A and B), indicating that antiangiogenic activity of shikonin and its derivates can mediate the *in vivo* antitumor activity. Thus, these *in vivo* data support the *in vitro* antiangiogenic effects of shikonin and its derivatives. However, shikonin decreased the body weight drastically as a side-effect whereas the shikonin derivatives suppressed the tumor weight without any adverse effect of body weight loss, even

when the animals were treated with shikonin derivatives for prolonged period of time (data not shown). Interestingly the reductions of tumor size were also observed irrespective of time in shikonin derivative treated animals (data not shown). Additionally, the acetylshikonin and isobutyroylshikonin showed a potent inhibitory effect on VEGF-induced angiogenesis *in vitro* and tumor growth *in vivo*. These results suggest that the acetylshikonin and isobutyroylshikonin inhibits the migration of endothelial cells induced by VEGF, which may explain its antiangiogenic properties. Although shikonin showed the strong cytotoxicity and body weight loss in mice as a side-effect compared with its derivatives, it possessed the most potent antitumor activity and its derivatives also exhibited mild antitumor and antiangiogenic activities. However, to improve these biological activities by shikonin and its derivatives contained in *Lithospermum erythrorhizon*, the combination therapy between these compounds is recommended to synergize the anticarcinogenic activity by them as well as overcome the side effects during cancer treatment.

On the other hand, tumor-associated proteolytic factors enable tumor cells to disintegrate the stroma in their immediate surrounding area, intravasate into lymphatic or blood vessels, and then spread systemically. Among the key players in the proteolytic cascade leading tumor invasion and metastasis are factors of the plasminogen activation system.[23] The urokinase type plasminogen activator (uPA) plays a pivotal role in the regulation of cell adhesion and migration during tissue remodeling. uPA not only specifically cleaves plasminogen and converts it into plasmin but also activates intracellular signaling upon binding to certain receptors on the cell surface.[25] The overexpression of proteases, such as urokinase-type plasminogen activator (uPA), its receptor (uPAR), and matrix metalloproteinases (MMP), is correlated with the progression of cancer.[26] The expression of uPA and uPAR are known to be highly expressed in A549 cells. To evaluate the efficacy of shikonin derivatives on the expression of uPA and uPAR in A549 cells, RT-PCR was performed. Shikonin and its derivatives showed antimetastatic activity through downregulating the expression of uPA in A549 cells, (Fig. 7). Consequently, shikonin and its derivatives inhibit tumor metastasis and angiogenesis *via* regulation of uPA.

In conclusion, these results demonstrate that shikonin and its derivatives can be a potent inhibitor of metastasis and angiogenesis *via* regulation of uPA and inhibition of VEGF production and we also suggest that shikonin and its derivatives (acetylshikonin and isobutyroylshikonin) can be a novel cancer chemopreventive agent. However, it is still necessary to further investigate the potential antiangiogenic and antitumorigenic activity by combination of shikonin and its derivatives in future.

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