Shikonin Induced Apoptosis and Inhibited Angiogenesis on HSE Cells

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Previously we have shown that shikonin has strong anti-tumor activities via inducing apoptosis and suppressing metastasis on LLC cells *in vivo* and *in vitro*. Here we have investigated anti-angiogenic potential of shikonin and its possible mechanism of action in HSE cells. Shikonin inhibited the proliferation of HSE cells in a concentration-dependent manner. It was shown that this proliferation inhibition was caused by apoptosis induced by shikonin *via* BrdU incorporation and Western blotting analysis. Shikonin treatment was caused that decrease of activation of caspases and cleavage of PARP. And shikonin 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. Moreover, shikonin showed anti-angiogenic activities inhibiting tube-like formation of HSE cells in vitro and vascular formation of LLC cells *in vivo*. These findings suggest that shikonin may a possible candidate not only anti-metastatic agent but also anti-angiogenic agent.

Key words : Shikonin, angiogenesis, apoptosis, HSE cells

Introduction

Angiogenesis, the formation of new capillaries is essential during development, inflammatory disorders, wound healing, and cancer[1-2]. Tumors require vascular network to supply nutrition and oxygen as well as to remove waste for tumor growth. Moreover, the high degree of neovascularization increases the development of metastasis[3-4] and the absence of angiogenesis caused tumor to necrotic or apoptotic stage[5]. Therefore, inhibition of angiogenesis has been the attractive and powerful target to treat cancer and metastasis.

Apoptosis is an essential component of animal development establishing and maintaining functional tissue architecture[6]. The most dramatic cases are nervous and immune systems in which, after extensive proliferation and differentiation, apoptosis has an important role in cell selection[7]. Several recent reports have demonstrated that apoptosis and growth reduction of endothelial cells is the major mechanism for inhibition of angiogenesis[6,8-9]. It was reported that tumors induce regression of the preexisting host vasculature via apoptosis, leading to massive tumor cell loss[10-11].

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Shikonin exhibited a variety of abilities such as accelerating tissue granulation proliferation[12] and would healing[5], exerting antibacterial[13], anti-inflammatory[14] and anti-tumor effects[15]. Shikonin induced apoptosis in HL60 cells via mitogen-activated protein kinases (MAPK) activation[16] and also inhibited angiogenesis induced by tumor necrosis factor (TNF)-α[17] and decrease mRNA levels of insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF)[18].

However, the activities of shikonin against endothelial cells have not yet investigated. Therefore, we have examined the anti-angiogenic and apoptosis-inducing activities of shikonin using Murine hepatic sinusoidal endothelial (HSE) and Lewis lung carcinoma (LLC) cells and demonstrated that shikonin was very effective target to inhibit tumor-induced angiogenesis and tumor growth.

Materials and Methods

1. Chemicals

Shikonin was isolated at College of Pharmacy, Chungnam University, Taejon, Korea[19]. 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.

2. Cell cultures

HSE cells were kindly provided by Dr. G L Nicolson

(The University of Texas, MD Anderson Cancer Center, TX, USA)[20]. HSE cells were maintained in Attachment Factor (Cell Systems, Kirkland, WA, USA)-coated culture flasks in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 5% FBS and 100 ㎍/㎖ endothelial mitogen (Biomedical Technologies, Inc., MA, USA). Lewis lung carcinoma (LLC) cell lines were maintained as monolayer cultures in Eagle's Minimal Essential Medium (EMEM; GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% FBS. 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 Japan SLC Inc. (Hamamatsu, Japan). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

4. Cell proliferation assay

Log-phase cell cultures of LLC (1×10^4 cells/well) were seeded in 100 ㎖ of complete medium on 96-well culture plates. After preincubation for 24 hr, the medium was replaced with new medium supplemented with 0.1 % bovine serum albumin (BSA) containing with various concentration of shikonin. The cultures were incubated for a various time period and then cytotoxic activity was determined by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzen e disulfonate (WST-1) colorimetric method25). Briefly, before 2 hr of the termination of incubation, 10 ㎖ of the working solution containing WST-1 reagent (WST-1 Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) was added to each well. After the termination of the culture, the absorbance was measured at 450 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon InterMed K.K., Tokyo, Japan).

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. 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 ㎎/㎖ aprotinin, and 10 ㎎/㎖ 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℃ and probed with anti-caspase-3, anti-poly(ADPPA-ribose) polymerase (PARP), anti-phospho-c-Jun N-terminal kinase (JNK), anti-phospho-p38, anti-phospho-extracellular signal-regulated kinase (ERK) (Cell Signaling Technology Inc., MA, USA), anti-JNK, anti-p38, anti-ERK, anti-proliferating cell nuclear antigen (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 wasreprobed to indicate evenness of loading of protein extract from each treatment.

6. Tube formation assay

Tube-like formation assay was performed as described previously[21] with some modifications. Briefly, assessment of *in vitro* capillary formation used growth factor-reduced Matrigel (BD Biosciences, MA, USA). Matrigel (100 ㎖/well of 10 ㎎/㎖) was plated in 48-well culture plates after thawing on ice, and allowed to polymerize at 37℃ for 30 min. Endothelial cells (3×10^4 cells/500 ㎖ in DMEM/F-12 medium containing 0.1% FBS) were added to each well, in the absence or presence of shikonin and the plates were then incubated for 5 hr at 37℃ in a 5% CO_2/95% O_2 atmosphere. After the end of incubation, cells were photographed five fields per well at ×50 magnification and the length of tube formation was measured using digital curvimeta (Uchida Yoko Co., Ltd., Japan). Briefly, a connecting branch between two discreet endothelial cells was measured as a tube-like structure. The extent of inhibition of tube-like formation induced by shikonin was estimated by comparing the length of the tube-like structures formed in the control treatment.

7. Tumor-induced angiogenesis assay

Tumor-induced angiogenesis was assessed according to the previously described methods[22] with some modifications. Mice were inoculated intradermally with LLC cells (5×10^5 cells/50 ㎖) on the back. shikonin was administered by *i.p.* injections of 5 ㎎/㎏per day starting on 1 day after tumor cell

inoculation for 5 days and Doxorubicin (7 ㎎/㎏) was administered by *i.v.* on 4 days after tumor cell inoculation. Six days after the tumor inoculation, the mice were sacrificed and the tumor-inoculated skin was separated from the underlying tissues. Angiogenesis was quantified by counting only the vessels directly supplying the tumor under a dissecting microscope. Tumor growth was assessed by measuring with a caliper square along the longer axes (a) and the shorter axes (b). Tumor volumes (㎣) were calculated by the following formula. Tumor volume (㎣) $=ab^2/2$.

8. Statistical Analysis

Representative data from each experiment are presented as mean values SD, as described in the figure legends. 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. Effect of shikonin on HSE cells proliferation

Shikonin inhibited the growth of HSE cells in a concentration-dependent manner (Fig. 1). Treatment with shikonin for less than 12 hr, at concentrations ranging from 1 to 10 μM, did not significantly inhibit the growth of HSE cells (Fig. 1A). Therefore, we used shikonin at concentrations of less than 10 μM for the following experiments for developing anti-angiogenic properties of shikonin.

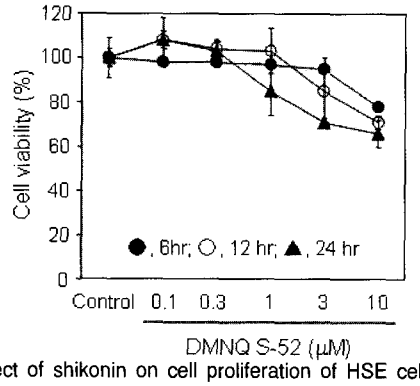

Fig. 1. Effect of shikonin on cell proliferation of HSE cells. HSE cells were seeded in 96-well culture plates. After a 24-hr preincubation, various concentrations of shikonin were added to the cultures and the cultures were employed a further incubation for a various time period. After the end of incubation, WST-1 colorimetric method (A) or BrdU incorporation (B) 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.

2. Effect of shikonin on HSE cell apoptosis

As shown in Fig. 2, shikonin treatment caused the decrease of procaspase-3 in a concentration and time-dependent manner. These results showed that shikonin caused that the stimulation of procaspase-3 to the active form of caspase-3. Shikonin also caused the proteolytic cleavage of PARP with disappearance of the full-length 116 kDa protein in a concentration and time-dependent manner. These data showed that shikonin induced apoptosis of endothelial cells *via* activation of caspase-3 and cleavage of PARP (Fig. 2).

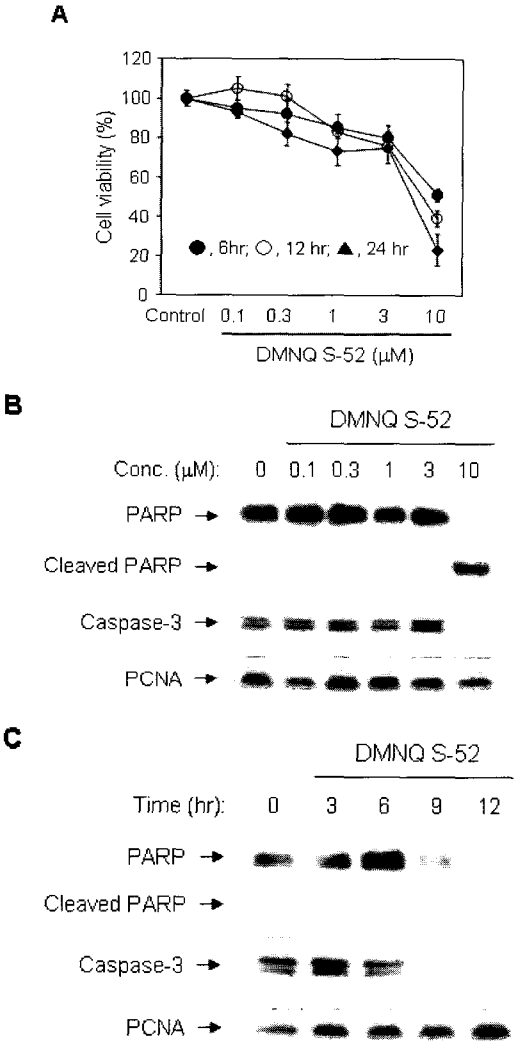

Fig. 2. Western blotting analysis of apoptosis induced by shikonin. A. HSE cells were stimulated with various concentrations of shikonin and the expression of PARP, caspase-3 and PCNA was assessed by Western blot analysis. B. Time-course analysis of apoptosis-related molecules induced by shikonin. HSE 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.

The balance between the MAP kinases, such as ERK, JNK and p-38, has been involved the upstream of apoptosis and then played an pivotal role in the regulation of apoptosis[23-24]. As shown in Fig. 3, western blotting analysis revealed that JNK, and p38 MAPK were activated by shikonin. However, ERK was reached the peak activation at 4 hr incubation and after then the phosphorylation was decreased.

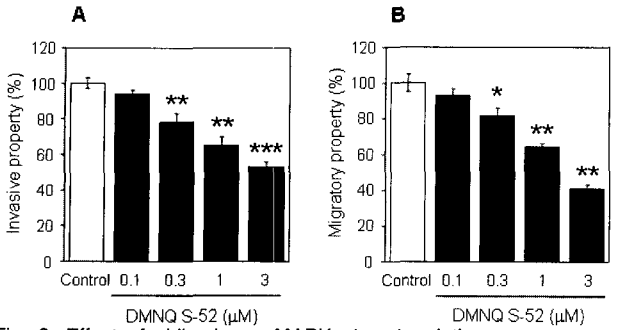

Fig. 3. Effect of shikonin on MAPK phosphorylation. HSE cells were stimulated with shikonin for various time periods and the expression of MAP kinases and phosphor-MAP kinases was assessed by western blot analysis.

3. Effect of shikonin on tube-like formation of HSE cells

We next investigated that the effect of DMNQ S-52 against in vitro tube-like formation by endothelial cells plated on a basement matrix. The incubation of HSE cells in Matrigel-coated wells caused tube-like formations within 5 hr. However, incubation with shikonin led to the suppression the tube-like formation of HSE with concentrations higher than 0.1 μM (Fig. 4).

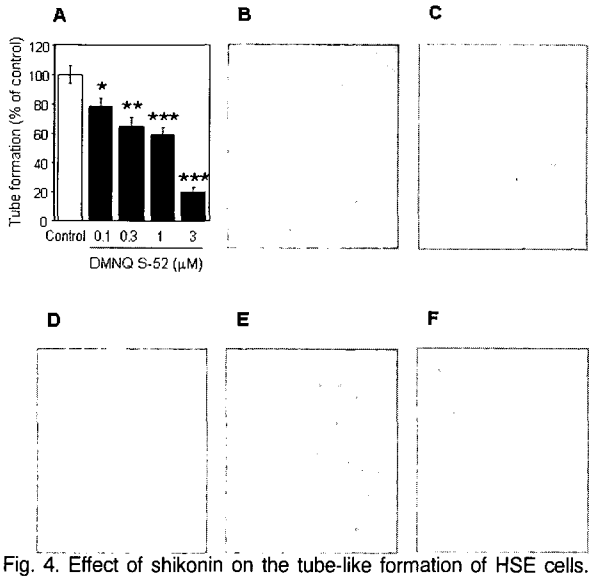

Fig. 4. Effect of shikonin on the tube-like formation of HSE cells. HSE cells were seeded in Matrigel-coated wells and incubated in the absence or presence of shikonin. The tube-like formation was monitored every 1 hr, over 5 hr. A. The extent of inhibition of tube-like formation induced by shikonin was estimated by comparing the length of tube-like structures formed in the control treatment. A. Data are represented as the mean S.D. *, $p<0.05$; **, $p<0.01$. B~F, the microscopic observation of tube-like formation in shikonin-treated wells (50). B, control; C, D, E, and F, 0.1, 0.3, 1, and 3 mM of shikonin-treated wells.

4. Effect of shikonin on the tumor growth and tumor-induced angiogenesis

To confirm the anti-angiogenic activity of shikonin in vivo, we employed an angiogenesis model by orthotopic implantation of LLC cells. Angiogenesis was induced by the implantation of LLC cells into the dorsal side of mice and shikonin (5 mg/kg/day, i.p.) was administered for 5 days from the next day of tumor cell inoculation (Fig. 5). As shown in Fig. 5B, shikonin treatment caused the reduction of the vessel number oriented to the tumor mass, indicating the inhibition of angiogenesis. Moreover, shikonin inhibited the primary tumor growth in a dose-dependent manner (Fig. 5A). Shikonin did not show any side effects including reduced body weight during the in vivo experiments (data not shown).

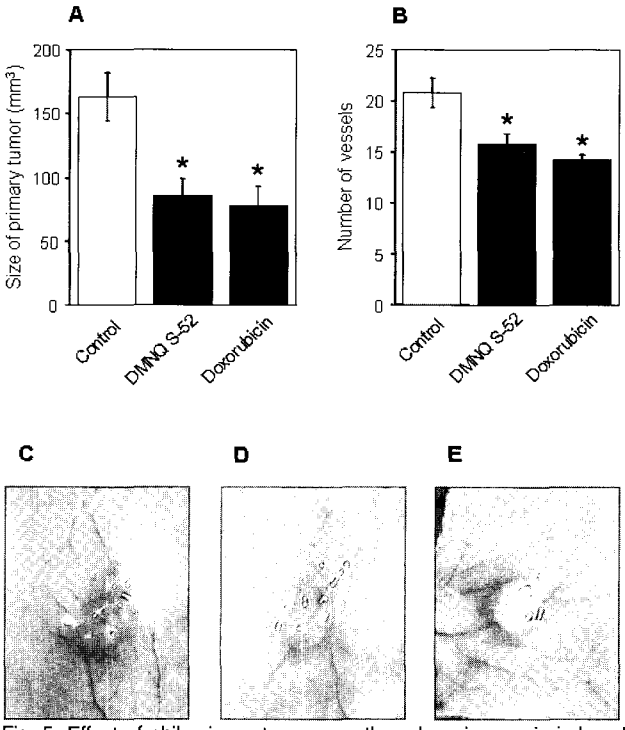

Fig. 5. Effect of shikonin on tumor growth and angiogenesis induced by HSE cells implanted on the back skin of mice. Mice were intradermally inoculated with HSE cells and subcutaneously administered shikonin, doxorubicin or vehicle for 5 days from the next day of tumor inoculation. Tumor-inoculated sites were isolated from vehicle- or sample-treated mice 6 days after the tumor inoculation. The macroscopic observation is shown in Fig. 5. The tumor size was measured (A) and tumor-supplying vessels were counted (B). Data are represented as the mean S.E. of 7 mice in each group. *, $p<0.05$. C, control; D, shikonin-treated group; E, doxorubicin-treated group.

Discussion

Identification of mutations in tumors that lead to decreased apoptosis is not only of academic interest but rather an important goal in the cancer therapy. Clearly, mutations in cell death control do affect sensitivity of tumor cells to anti-cancer therapy which in most cases functions by inducing apoptosis[25].

Much of current knowledge have demonstrated the activities and mechanism of shikonin on apoptosis and angiogenesis[5,12,14-18]. We also reported that shikonin induced apoptosis in LLC cells[26]. However, it was not fully examined that the effect of shikonin on endothelial cells. Therefore, in

this report, we investigated that the effect of shikonin on angiogenesis and apoptosis against murine endothelial cells.

Shkonin induced apoptosis and inhibited proliferation in HSE cells (Fig. 1). The increased apoptosis seemed to be related to cleavage of PARP, activation of caspase-3 and phosphorylation of MAPK (Fig. 2-3). 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[27]. Various agents have been found to mediate the induction of apoptosis through the activation of p38 and JNK[27-29]. The phosphorylationn of p38 and JNK induced by shikonin in HSE cells was associated with proliferation inhibition and an increase of apoptotic cells. On the other hand, ERK phosphorylation decreased by shikonin treatment showing that the shikonin-activated ERK stimulated cell survival[28,30].

The growth of a tumor and its ability to metastasize is dependent on angiogenesis[31]. Tumor cells can escape from the primary site by entering existing vessels or new vessels actively recruited into the primary tumor. The generation of blood vessels is an essential step in the transition of a tumor from a small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body[32]. Tumor cells invade either the blood or lymphatic vessels to access the general circulation and then establish themselves in other tissues. The role of angiogenesis in facilitating the growth of solid tumors has been well established.

In the present study, the effect of shikonin on the endothelial function, such as tube-like formation, was investigated. These functions are essential for neovessel sprouting[33] and angiogenesis[34-35], which is an important biological process involved in metastasis formation. The tube-like formation of endothelial cells were significantly inhibited by shikonin concentration-dependently(Fig. 4). These results were further demonstrated in the *in vivo* tumor-induced angiogenesis model implanted with LLC cells in the dorsal skin of mice. In advance of the tumor growth in this system, the implanted tumor cells produce the formation of vessels by inducing the angiogenic actions of the host endothelial cells. This study showed that shikonin prevented *in vivo* angiogenesis and reduced primary tumor growth(Fig. 5). The possibility cannot rule out that the inhibition of tumor-induced angiogenesis by shikonin might be because of inhibition of tumor growth like as lrthotopic implanted lymph node metastasis model[26]. However, shikonin specifically inhibited angiogenic potentials of cultured and host endothelial cells. Therefore, inhibition of neovessel formation by shikonin was associated with both anti-angiogenic activity and anti-tumor activity.

Collectively, we have investigated that shikonin not only induced apoptosis but also suppressed angiogenic properties of endothelial cells *in vitro* and *in vivo*. According to this report, the biological activities of shikonin are very diverse, therefore shikonin may a useful anti-tumor drug candidate suppressing primary tumor growth and angiogenesis. However, it was not fully understood the detailed mechanism of angiogenesis and apoptosis during tumorigenesis. The characterization of the detailed biological and molecular mechanism will provide new insights into the relationship between angiogenesis and apoptosis related to shikonin.

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References

1. Jones, M.K., Wang, H., Peskar, B.M., Levin, E., Itani, R.M., Sarfeh, I.J., Tarnawski, A.S. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 5(12):1418-1423, 1999.
2. Varet, J., Vincent, L., Mirshahi, P., Pille, J.V., Legrand, E., Opolon, P., Mishal, Z., Soria, J., Li, H., Soria, C. Fenofibrate inhibits angiogenesis in vitro and in vivo. Cell Mol Life Sci 60(4):810-819, 2003.
3. Woodhouse, E.C., Chuaqui, R.F., Liotta, L.A. General mechanisms of metastasis. Cancer 80(8 Suppl), pp 1529-1537, 1997.
4. Saaristo, A., Karpanen, T., Alitalo, K. Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. Oncogene 19(53):6122-6129, 2000.
5. Folkman, J., Shing, Y. Angiogenesis. J Biol Chem 267(16):10931-10934, 1992.
6. Jacobson, M.D., Weil, M., Raff, M.C. Programmed cell death in animal development. Cell 88(3):347-354, 1997.
7. Meier, P., Finch, A., Evan, G. Apoptosis in development. Nature 407(6805):796-801, 2000.
8. Folkman, J. Angiogenesis and apoptosis. Semin Cancer Biol 13(2):159-167, 2003.
9. Segura, I., Serrano, A., De Buitrago, G.G., Gonzalez, M.A., Abad, J.L., Claveria, C., Gomez, L., Bernad, A., Martinez, A.C., Riese, H.H. Inhibition of programmed cell death impairs in vitro vascular-like structure formation and

reduces in vivo angiogenesis. Faseb J 16(8):833-841, 2002.

10. Holash, J., Maisonpierre, P.C., Compton, D., Boland, P., Alexander, C.R., Zagzag, D., Yancopoulos, G.D., Wiegand, S.J. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284(5422):1994-1998, 1999.
11. Holash, J., Wiegand, S.J., Yancopoulos, G.D. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene 18(38):5356-5362, 1999.
12. Ozaki, Y., Ohno, A., Saito, Y., Satake, M. Accelerative effect of shikonin, alkannin and acetylshikoninon the proliferation of granulation tissue in rats. Biol Pharm Bull 17(8):1075-1077, 1994.
13. Tanaka, Y., Odani, T. Pharmacodynamic study on "Shiunko.": I. Antibacterial effect of "Shiunko". Yakugaku Zasshi 92(5):525-530, 1972.
14. Tanaka, S., Tajima, M., Tsukada, M., Tabata, M. A comparative study on anti-inflammatory activities of the enantiomers, shikonin and alkannin. J Nat Prod 49(3):466-469, 1986.
15. Sankawa, U., Ebizuka, Y., Miyazaki, T., Isomura, Y., Otsuka, H. Antitumor activity of shikonin and its derivatives. Chem Pharm Bull 25(9):2392-2395, 1977.
16. Yoon, Y., Kim, Y.O., Lim, N.Y., Jeon, W.K., Sung, H.J. Shikonin, an ingredient of Lithospermum erythrorhizon induced apoptosis in HL60 human premyelocytic leukemia cell line. Planta Med 65(6):532-535, 1999.
17. Hisa, T., Kimura, Y., Takada, K., Suzuki, F., Takigawa, M. Shikonin, an ingredient of Lithospermum erythrorhizon, inhibits angiogenesis in vivo and in vitro. Anticancer Res 18(2A):783-790, 1998.
18. Gaddipati, J.P., Mani, H., Shefali, Raj, K., Mathad, V.T., Bhaduri, A.P., Maheshwari, R.K. Inhibition of growth and regulation of IGFs and VEGF in human prostate cancer cell lines by shikonin analogue 93/637 (SA). Anticancer Res 20(4):2547-2552, 2000.
19. Song, G.Y., Kim, Y., You, Y.J., Cho, H., Ahn, B.Z. Naphthazarin derivatives (VII): antitumor action against ICR mice bearing ascitic S-180 cells. Arch Pharm Res 24(1):35-38, 2001.
20. Belloni, P.N., Carney, D.H., Nicolson, G.L. Organ-derived microvessel endothelial cells exhibit differential responsiveness to thrombin and other growth factors. Microvasc Res 43(1):20-45, 1992.
21. Rodrigues, S., Van Aken, E., Van Bocxlaer, S., Attoub, S., Nguyen, Q.D., Bruyneel, E., Westley, B.R., May, F.E., Thim, L., Mareel, M., Gespach, C., Emami, S. Trefoil peptides as proangiogenic factors in vivo and in vitro: implication of cyclooxygenase-2 and EGF receptor signaling. Faseb J 17(1): 7-16, 2003.
22. Murata, J., Saiki, I., Makabe, T., Tsuta, Y., Tokura, S., Azuma, I. Inhibition of tumor-induced angiogenesis by sulfated chitin derivatives. Cancer Res 51(1):22-26, 1991.
23. Yue, T.L., Wang, X., Louden, C.S., Gupta, S., Pillarisetti, K., Gu, J.L., Hart, T.K., Lysko, P.G., Feuerstein, G.Z. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces apoptosis in endothelial cells and inhibits angiogenesis: possible role for stress-activated protein kinase signaling pathway and Fas expression. Mol Pharmacol 51(6):951-962, 1997.
24. Shaheen, R.M.,Davis, D.W., Liu, W., Zebrowski, B.K., Wilson, M.R., Bucana, C.D., McConkey, D.J., McMahon, G., Ellis, L.M. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. Cancer Res 59(21):5412-5416, 1999.
25. Zornig, M., Hueber, A., Baum, W., Evan, G. Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 1551(2):F1-37, 2001.
26. Lee, S.J., Kim, S.H. Shikonin modulates cell proliferation by inducing apoptosis in LLC cells via MAPK regulation and caspase activation. J Oriental Physiol Pathol 19(2):501-507, 2005.
27. Lewis, T.S., Shapiro, P.S., Ahn, N.G. Signal transduction through MAP kinase cascades. Adv Cancer Res 74. 49-139, 1998.
28. Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270(5240):1326-1331, 1995.
29. Chuang, S.M., Wang, I.C., Yang, J.L. Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. Carcinogenesis 21(7): 1423-1432, 2000.
30. Wang, X., Martindale, J.L., Liu, Y., Holbrook, N.J. The cellular response to oxidative stress:influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 333(Pt 2), pp 291-300, 1998.
31. Folkman, J., Tumor angiogenesis: therapeutic implications. N Engl J Med 285(21):1182-1186, 1971.
32. Zetter, B.R., Angiogenesis and tumor metastasis. Annu Rev Med 49, pp 407-424, 1998.
33. Kubota, Y., Kleinman, H.K., Martin, G.R., Lawley, T.J. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107(4):1589-1598, 1988.

34. Weidner, N., Semple, J.P., Welch, W.R., Folkman, J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 324(1):1-8, 1991.

35. Liotta, L.A., Steeg, P.S., Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64(2):327-336, 1991.