EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 37, No. 2, 133-137, April 2005

Cryptotanshinone but not tanshinone IIA inhibits angiogenesis *in vitro*

Jong Moon Hur[1], Joong Sup Shim[1], Hye Jin Jung[1] and Ho Jeong Kwon[1,2]

[1]Chemical Genomics National Research Laboratory
Department of Bioscience and Biotechnology
Institute of Bioscience
Sejong University
98 Kunja-dong, Kwangjin-gu
Seoul 143-747, Korea
[2]Corresponding author: Tel, 82-2-3408-3640;
Fax, 82-2-3408-3334; E-mail, kwonhj@sejong.ac.kr

Accepted 6 March 2005

Abbreviations: BAEC, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide

Abstract

In the course of screening of angiogenesis inhibitor from natural products, cryptotanshinone from *Salvia miltiorrhiza* was isolated as a potent small molecule inhibitor of angiogenesis. Cryptotanshinone inhibits bFGF-induced angiogenesis of BAECs at ten micromolar ranges *in vitro* without cytotoxicity. Tanshinone IIA, another tanshinone isolated from *S. miltiorrhiza*, which is structurally very similar to cryptotanshinone except C-15 position of dihydrofuran ring does not inhibit angiogenesis induced by bFGF. These results demonstrate that cryptotanshinone is a new anti-angiogenic agent and double bond at C-15 position of the dihydrofuran ring plays a crucial role in the activity.

Keywords: abietane; angiogenesis; cryptotanshinone; *Salvia miltiorrhiza*; tanshinone

Introduction

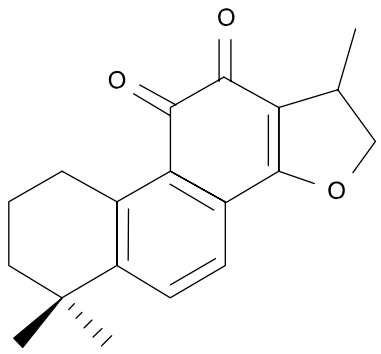
Angiogenesis is a physiological process of new blood vessel formation by endothelial cells, which is critical for normal physiology such as development and wound healing (Folkman, 1971; Carmeliet, 2003). In pathological states, angiogenesis is deregulated by numerous pro-angiogenic factors leading to induce several diseases such as diabetic retinopathy, rheumatoid arthritis and spreading of cancer (Folkman, 1985; Walsh, 1999; Martin *et al.,* 2003). In particular, angiogenesis is crucially required for blood supply and metastasis of most types of solid tumors. Accordingly, abrogation of angiogenic process and enhancement of anti-angiogenic factors have been considered as potential targets for cancer therapy (Cao, 2001; Madhusudan and Harris, 2002; Tosetti *et al.,* 2002; Kwon, 2003).

Based on this idea, a number of angiogenesis inhibitors have been developed from various sources, including endogenous protein fragments (O'Reilly *et al.,* 1994; 1997; Kim *et al.,* 2003), monoclonal antibodies (Brekken *et al.,* 2000) and small molecules originated from natural products and organic synthesis (Ingber *et al.,* 1990; Shim *et al.,* 2003). As a result of our continuing search for new anti-angiogenic agents from chemical spheres, we found cryptotanshinone from the root of *Salvia miltiorrhiza* Bunge (Labiatae) exhibits a potent anti-angiogenic activity. Dried roots of *S. miltiorrhiza* have been used in traditional Chinese medicine for the treatment of several pathologies, including disorders caused by poor blood supply such as coronary artery disease and angina pectoris, hepatitis, menstrual disorder and miscarriage (Chang and But, 1986; Zhu, 1998). As major chemical constituents, more than 25 tanshinones which impart the reddish orange pigments, have been isolated from the plant (Ryu *et al.,* 1997; Lin and Chang, 2000; Lin *et al.,* 2001), and a variety of biological activities, including antioxidant, antibacterial, anti-inflammatory, neuroprotective activity have been reported (Lee *et al.,* 1999; Ng *et al.,* 2000; Kim *et al.,* 2002; Lam *et al.,* 2003). However, there has been no report related with their activity on angiogenesis. In this report, the isolation and anti-angiogenic activity of cryptotanshinone, one of tanshinones from *S. miltiorrhiza*, are described.

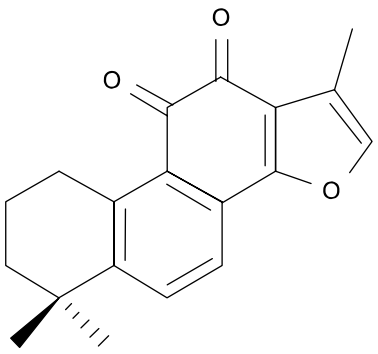
Materials and Methods

Active compound isolation

Dried roots of *S. miltiorrhiza* (11 kg) were extracted with CH_2Cl_2 at room temperature for 7 days and the solvent was evaporated to obtain crude extract (93.11 g). CH_2Cl_2 extract (90 g) was applied to the silica gel column chromatography eluted by *n*-Hexane-EtOAc mixture and CH_2Cl_2-EtOAc as mobile phases to obtain 6 fractions (SMC1-6), based on the TLC pattern. According to the bioactivity-guided fraction method, SMC-5 fraction (13.7 g) was rechromatographed over a silica gel column using Hexane-CH_2Cl_2 mixture (5 : 1, 3 : 1, 1 : 1, 1 : 3, successively) to obtain 5 fractions (SMC5A-5E). Then, SMC5E fraction (2.3 g) was separated using ODS column chromatography with a



Cryptotanshinone



Tanshinone IIA

Figure 1. The structure of cryptotanshinone and tanshinone IIA from *S. miltiorrhiza*.

solvent system of water-CH_3CN mixture (6:1) to gain 3 fractions. Cryptotanshinone was separated as SMC5E2 fraction (0.53 g). Likewise, SMC-3 fraction (15.3 g) was separated under a silica gel column chromatography with a solvent system of Hexane-EtOAc mixture (30 : 1, 10 : 1, 7 : 1, respectively) to gain 3 fractions (SMC3A-C), and tanshinone IIA (3 g) was isolated from the SMC3B fraction. Cryptotanshinone and tanshinone IIA were identified by comparison of NMR and MS spectral data with reference values (Kang *et al.,* 1997).

Cell culture and growth assay

Early passages (4-8 passages) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. Jo at KNIH. BAECs were grown in MEM supplemented with 10% fetal bovine serum (FBS, Life Technology, Grand Island, NY). CHANG (immortalized hepatocyte derived from normal human liver), HeLa (cervical carcinoma), and HT1080 (fibrosarcoma) cells were maintained in DMEM, and HT29 (colon carcinoma) cells in RPMI1640 containing 10% FBS. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cell growth assay was carried out using MTT colorimetric assay. Cells were inoculated at a density of 5×10^3 cells per well in 96-well culture plates and incubated for 24 h for stabilization. Various concentrations of compounds were added to each well and the incubation was continued for 2 days. Fifty microliter of MTT (2 mg/ml stock solution, Sigma, St. Louis, MO) was added and the plate was incubated for an additional 4 h. After removal of medium, DMSO (100 μl) was added. The plate was read at 540 nm by universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Chemoinvasion assay

The invasiveness of BAECs was examined *in vitro* using a Transwell chamber system with 8.0 μm pore-sized polycarbonate filter inserts (Corning Costar, Cambridge, MA). The lower side of the filter was coated with 10 μl of gelatin (1 mg/ml), whereas the upper side was coated with 10 μl of Matrigel (3 mg/ml). Cells (1×10^5 cells) were placed in the upper part of the filter and compounds were added in lower parts in the presence of bFGF (30 ng/ml, Upstate Biotechnology, Lake Placid, NY). The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting the whole cell numbers in a lower side of filter using optical microscopy at a ×100 magnification.

Table 1. IC_{50} values (μM) of cryptotanshinone (1) and tanshinone IIA (2) on various cell lines.

Compound	Chang	BAECs	HT1080	HT29	HeLa	HepG2
1	30	10	10	>30	25	>30
2	>30	20	10	>30	>30	>30

Tube formation assay

Matrigel (150 μl, 10 mg/ml, Collaborative Biomedical Products, Bedford, MA) was coated in a 48-well culture plate and polymerized for 2 h at 37°C. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Then, compounds were added and incubated for 6-18 h. The morphological changes of cells were observed under microscope and photographed at ×100 magnification using JVC digital camera (Victor, Yokohama, Japan). Cytotoxicity of tube-forming endothelial cells was evaluated by trypan blue staining.

Results and Discussion

Two tanshinones were obtained as active principles from the CH_2Cl_2 extract of root of *S. miltiorrhiza* based on bioactivity-guided isolation of the inhibitory activity against the proliferation of BAECs. These active compounds were identified as cryptotanshinone and tanshinone IIA (Figure 1), which belong to abietane diterpenes from the comparison of spectral data with published values (Kang *et al.,* 1997). As shown in

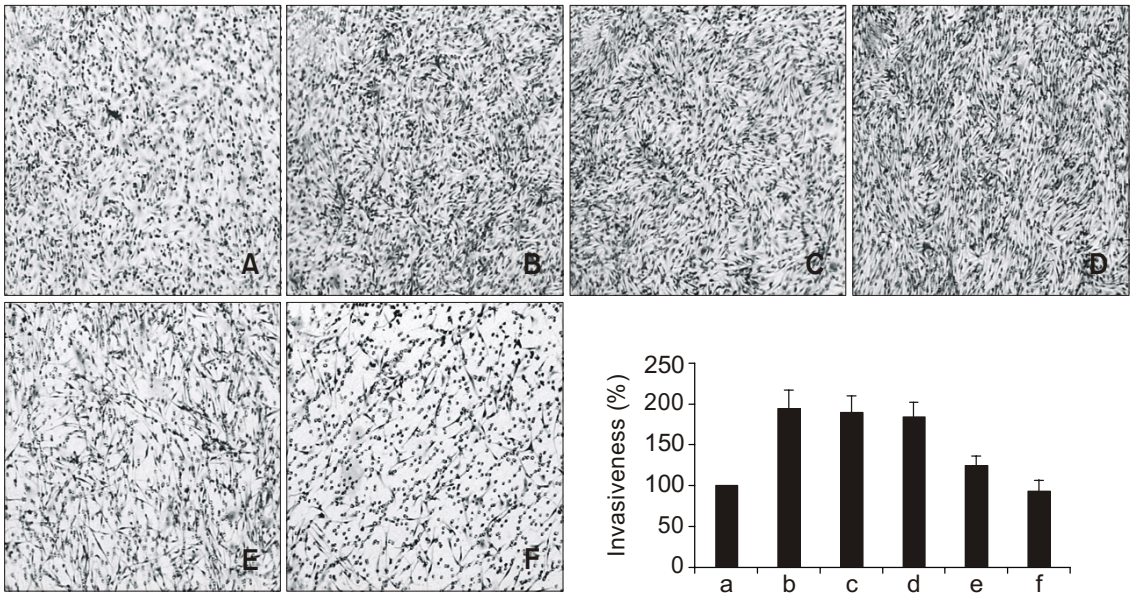


Figure 2. Effects of cryptotanshinone and tanshinone A on angiogenic phenotypes of BAECs. Microscopic observation of invaded cells (×100 magnification). (A) Control; (B) bFGF alone; (C) bFGF+tanshinone IIA (10 μM); (D) bFGF+tanshinone IIA (20 μM); (e) bFGF+cryptotanshinone (10 μM); (F) bFGF+cryptotanshinone (20 μM). Bar graph represents the quantitative analysis of the invasion assay from three independent experiments.

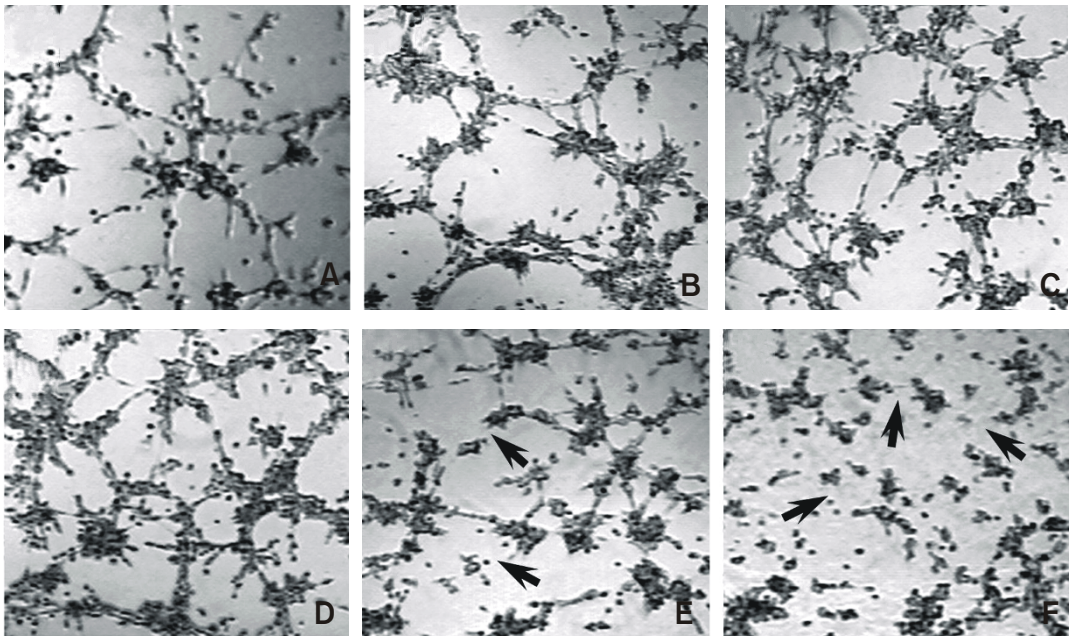


Figure 3. Effects of cryptotanshinone and tanshinone IIA on tube forming ability of BAECs. (A) Control; (B) bFGF alone; (C) bFGF+tanshinone IIA (10 μM); (D) bFGF+tanshinone IIA (20 μM); (E) bFGF+cryptotanshinone (10 μM); (F) bFGF+cryptotanshinone (20 μM). Arrows indicate the inhibition of tube formation by cryptotanshinone.

Figure 1, the structural difference between cryptotanshinone and tanshinone IIA is only the presence of double bond at C-15 position of furan ring.

We first investigated the effects of two tanshinones on the proliferation of various cell lines using MTT colorimetric assay. Both tanshinones inhibited the pro-

liferation of various cell lines tested with a different inhibitory spectrum. IC_{50} values of cryptotanshinone and tanshinone IIA against cell lines tested are shown in Table 1. Interestingly, BAECs and HT1080, human fibrosarcoma cells having metastatic activity, are highly sensitive to both tanshinones.

We, next, conducted the cell invasion and tube formation assay using the BAECs to investigate the inhibitory effects of two tanshinones on angiogenesis *in vitro*. Basic fibroblast growth factor (bFGF) was used as a pro-angiogenic factor stimulating the spreading and migration of endothelial cell invasion. As shown in Figure 2, bFGF greatly increased cell invasion through the filter coated with Matrigel than that of the control. Cryptotanshinone dose-dependently blocked the invasion of BAECs into the filter induced by bFGF. Interestingly, tanshinone IIA did not inhibit bFGF-induced invasion of BAECs at the same concentration range. Moreover, cryptotanshinone dose-dependently inhibited the tube formation of BAECs induced by bFGF, whereas tanshinone IIA did not, either (Figure 3). The cytotoxicity was not observed at the concentration ranges up to 20 μM of the compounds examined by trypan blue staining (data not shown). These results demonstrate that cryptotanshinone is a new small molecule angiogenesis inhibitor and can be used as a chemical probe for studying the regulatory mechanism of angiogenesis.

The mechanism of angiogenesis inhibition by cryptotanshinone is currently not understood. However, the present study provides a clue for structure-activity relationship of anti-angiogenic activity of cryptotanshinone. The only structural difference between two tanshinones is double bond at C-15 position of the dihydrofuran ring. This double bond of cryptotanshinone may play a critical role in angiogenesis inhibition by the compound. Moreover, the anti-angiogenic activity of cryptotanshinone may be due to a specific inhibition of angiogenic differentiation of endothelial cells rather than anti-proliferative activity against the cells, because tanshinone IIA also inhibits the proliferation of the endothelial cells. We currently investigate several mechanistic studies of anti-angiogenic activity of cryptotanshinone and attempt to identify the cellular target protein of the compound. The identification of the target protein of cryptotanshinone will help to elucidate the anti-angiogenic mechanism of the compound and provide a new therapeutic target for angiogenesis-related diseases.

Acknowledgement

This work was supported by the National Research Laboratory Grant from the Ministry of Science and Technology, Republic of Korea and the Brain Korea 21 Project.

References

Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, Thorpe PE. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res 2000;60:5117-24

Cao Y. Endogenous angiogenesis inhibitors and their therapeutic implications. Int J Biochem Cell Biol 2001;33:357-69

Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653-60

Chang HM, But PP. Pharmacology and Applications of Chinese Materia Medica, 1986, World Scientific, Singapore.

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182-6

Folkman J. Tumor angiogenesis. Adv Cancer Res 1985;43: 175-203

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 1990; 348:555-7

Kang HS, Chung HY, Jung JH, Kang SS, Choi JS. Antioxidant effect of Salvia miltiorrhiza. Arch Pharm Res 1997;20: 496-500

Kim KS, Hong YK, Lee Y, Shin JY, Chang SI, Chung SI, Joe YA. Differential inhibition of endothelial cell proliferation and migration by urokinase subdomains: amino-terminal fragment and kringle domain. Exp Mol Med 2003;35:578-85

Kim SY, Moon TC, Chang HW, Son KH, Kang SS, Kim HP. Effects of tanshinone I isolated from Salvia miltiorrhiza Bunge on arachidonic acid metabolism and *in vivo* inflammatory responses. Phytother Res 2002;16:616-20

Kwon HJ. Chemical genomics-based target identification and validation of anti-angiogenic agents. Curr Med Chem 2003; 10:717-36

Lam BY, Lo AC, Sun X, Luo HW, Chung SK, Sucher NJ. Neuroprotective effects of tanshinones in transient focal cerebral ischemia in mice. Phytomedicine 2003;10:286-91

Lee DS, Lee SH, Noh JG, Hong SD. Antibacterial activities of cryptotanshinone and dihydrotanshinone I from a medical herb, Salvia miltiorrhiza Bunge. Biosci Biotech Biochem 1999;63:2236-9

Lin HC, Chang WL. Diterpenoids from Salvia miltiorrhiza. Phytochemistry 2000;53:951-3

Lin HC, Ding HY, Chang WL. Two new fatty diterpenoids from Salvia miltiorrhiza. J Nat Prod 2001;64:648-50

Madhusudan S, Harris AL. Drug inhibition of angiogenesis. Curr Opin Pharmacol 2002;2:403-14

Martin A, Komada MR, Sane DC. Abnormal angiogenesis in diabetes mellitus. Med Res Rev 2003;23:117-45

Ng TB, Liu F, Wang ZT. Antioxidative activity of natural products from plants. Life Sci 2000;66:709-23

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277-85

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA,

Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315-28

Ryu SY, No ZS, Kim SH, Ahn JW. Two novel abietane diterpenes from Salvia miltiorrhiza. Planta Med 1997;63:44-6

Shim JS, Kim JH, Cho HY, Yum YN, Kim SH, Park HJ, Shim BS, Choi SH, Kwon HJ. Irreversible inhibition of CD13/aminopeptidase N by the antiangiogenic agent curcumin. Chem Biol 2003;10:695-704

Tosetti F, Ferrari N, Flora SD, Adriana A. Angiogenesis: anigiogenesis is a common and key target for cancer chemopreventive agents. FASEB J 2002;16:2-14

Walsh DA. Angiogenesis and arthritis. Rheumatology 1999; 38:103-12

Zhu, YP. Chinese Materia Medica, 1998, Harwood Academic Publisher, Amsterdam, The Netherlands.