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ABBS

# Dihydrotanshinone I inhibits angiogenesis both *in vitro* and *in vivo*

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**Dihydrotanshinone I (DI), a naturally occurring compound extracted from *Salvia miltiorrhiza Bunge*, has been reported to have cytotoxicity to a variety of tumor cells. In this study, we investigated its anti-angiogenic capacity in human umbilical vein endothelial cells. DI induced a potent cytotoxicity to human umbilical vein endothelial cells, with an $IC_{50}$ value of approximately 1.28 μg/ml. At 0.25–1 μg/ml, DI dose-dependently suppressed human umbilical vein endothelial cell migration, invasion, and tube formation detected by wound healing, Transwell invasion and Matrigel tube formation assays, respectively. Moreover, DI showed significant *in vivo* anti-angiogenic activity in chick embryo chorioallantoic membrane assay. DI induced a 61.1% inhibitory rate of microvessel density at 0.2 μg/egg. Taken together, our results showed that DI could inhibit angiogenesis through suppressing endothelial cell proliferation, migration, invasion and tube formation, indicating that DI has a potential to be developed as a novel anti-angiogenic agent.**

*Keywords* dihydrotanshinone I; angiogenesis; human umbilical vein endothelial cell

Angiogenesis is a process of new blood vessel formation by endothelial cells that plays a critical role in normal physiology, such as development and wound healing [1]. At the pathological level, angiogenesis is regulated by numerous pro-angiogenic factors leading to the induction of several diseases such as spreading of tumor, diabetic retinopathy, and rheumatoid arthritis [2]. Most primary solid tumors are dependent on angiogenesis for survival, growth, invasion, and metastasis. Therefore, targeting the angiogenesis process has become one of the important strategies in treating tumors [3]. In the angiogenesis process, vascular endothelial cells are activated to migrate out from the parental vessels, invade through the matrix, proliferate, and get together to format capillary tubes. Each step is tightly controlled by pro- and anti-angiogenic factors [4]. In tumor tissues, vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and other pro-angiogenic factors are usually overexpressed [5], whereas anti-angiogenic factors such as interleukin-12 (IL-12) and γ-interferon have few functions [6]. Thus, the anti-angiogenic therapy strategy is to suppress the functions of pro-angiogenesis factors and/or promote the functions of anti-angiogenesis factors. Currently, there are a variety of angiogenesis inhibitors being used in clinical trials [6, 7], many of which are natural products. Drug development from natural products has become a rapidly emerging and highly promising strategy to identify novel anti-angiogenic and anticancer agents.

Dihydrotanshinone I (DI) is a tanshinone extracted from a well-known traditional Chinese medicinal plant, *Salvia miltiorrhiza Bunge*. The dry root of this plant, called Danshen, has been widely used in China to treat haematological abnormalities, heart disease, hepatitis, hemorrhage, menstrual abnormalities, and collagen-induced platelet aggregation [8,9]. DI was reported to possess the function of cytotoxicity *in vitro* [10] and showed inhibitory effects on mast cell degranulation [11], lipopolysaccharide-induced nitric oxide generation [12], osteoclast differentiation [13], and production of IL-12 and γ-interferon in immune cells [14]. However, its anti-angiogenic capacity has not been well studied. Considering IL-12 and nitric oxide both play important roles in mediating angiogenesis [15,16], we hypothesized that DI might have an anti-angiogenic action.

In this study, we used different assays to identify and characterize whether DI could induce inhibitory effects

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on angiogenesis *in vitro* and *in vivo*.

## Materials and Methods

### Drugs and reagents

DI was bought from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). It was dissolved with ethanol for the stock concentration of 5 mg/ml, and stored at –20 ºC. The stock solution was further diluted immediately before use. Dimethyl thiazolyl-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, USA). Dulbcco's modified Eagle's medium (DMEM), Medium 199, fetal bovine serum, and bovine calf serum were obtained from Gibco (Rockville, USA).

### Isolation of human umbilical vein endothelial cells (HUVECs) and cell cultures

HUVECs were isolated from fresh human umbilical veins by established methods as described previously [17]. Briefly, endothelial cells were harvested from human umbilical veins by adding 0.1% collagenase (Gibco) for 30 min. The cells were grown to confluence in Medium 199 supplemented with 20% heat-inactivated fetal bovine serum, 100 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 ng/ml acidic fibroblast growth factor (aFGF) (Sigma), and 5 U/ml heparin. Cells were assessed for endothelial cell phenotype by morphology, the typical monolayer cobblestone growth pattern, and the expression of von Willebrand factor antigen. Cells between passages 1 and 2 and cell populations with more than 95% purity were used in all experiments.

The hepatocellular carcinoma SMMC-7721 cell line was obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM supplemented with 10% bovine calf serum, incubated in a humidified atmosphere of 5% $CO_2$ at 37 ºC, and subcultured every 2 d.

### MTT assay

Cells were seeded in 96-well tissue culture plates (4000 cells/well), incubated for 24 h, then treated with various concentrations of DI for another 24 h. Then 20 µl MTT was added to each well (final concentration of 0.5 mg/ml) for another 4 h incubation. The medium was pipetted out from each well, 200 µl dimethyl sulfoxide was added and the optical density was read at 570 nm. The $IC_{50}$ values were calculated and defined as the concentration of drug causing 50% inhibition in absorbance compared with control cells [18].

### Wound healing assay

The wound healing assay was carried out according to previous studies [19]. Briefly, HUVECs were seeded on 24-well tissue culture plates. When the cells reached 90% confluence, they were wounded with a tip from the center of each well and marked at the injury line. After wounding, the cultures were washed with serum-free medium and further incubated with different concentrations of DI for 24 h. Then cells in each well were stained with Wright-Giemsa, and photographed under an inverted microscope. The total migrated cells in each group were quantified by counting the number of cells that moved beyond the reference lines in five views.

### Cell invasion assay

Cell invasion assay was carried out using Transwell chambers (Corning, New York, USA) with an 8 µm pore polycarbonate filter insert (Corning) according to a previous report [20]. Briefly, the upper side of every insert was coated with 10 µl Matrigel (3 mg/ml; Becton Dickinson, Mountain View, USA). HUVECs ($3\times10^4$ cells/well) were seeded in the upper inserts, and SMMC-7721 cells were seeded in the lower chamber 24 h beforehand. After 6 h of incubation, the medium in the inserts was pipetted out and 150 µl medium with different concentrations of DI was added. Chambers were incubated for 24 h. Cells on the lower surface of the filters were fixed and stained with Wright-Giemsa and sealed on slides. Stained filters were photographed under a microscope. Invasiveness was determined by counting the cell number. Five visual fields were chosen randomly for each filter. The average number of the invaded cells in the five fields was taken as the mean of cell invasion numbers of the group.

### *In vitro* tube formation assay

Unpolymerized Matrigel (17 mg/ml) was placed in a 96-well plate (0.32 $cm^2$/well) at 50 µl/well and polymerized for 1 h at 37 ºC. HUVECs ($2\times10^4$ cells/well) in 200 µl medium, as well as in the presence or absence of DI (0.25, 0.5, and 1 µg/ml) were layered onto the Matrigel surface. After 24 h of incubation, cell growth and 3-D organization were observed under a microscope [21].

### Chick embryo chorioallantoic membrane (CAM) assay

The modified CAM assay was used to evaluate the inhibitory effects on angiogenesis *in vivo* on a chicken embryo model according to a previous report [22].

Fertilized chick eggs were incubated at 37 °C and with 60% humidity for 6 d. Then a square window was opened on the egg's shell, exposing the CAM and ensuring that the yolk sac membrane remained intact and the embryo was viable. A methylcellulose membrane loaded with various concentrations of DI was placed in areas between vessels of the 6-d-old egg but never onto any large vessels. Then the window was covered with tape. After another 48 h of incubation, the CAMs were fixed *in situ*, excised from the eggs, placed on slides, and left to air dry. Pictures were taken through a stereoscope equipped with a digital camera. Local vessel density was measured and the inhibitory effects on CAM angiogenesis were evaluated. Assays were repeated three times and each experiment group included 10 eggs.

### Statistical analysis

All of the experiments were carried out at least in triplicate. The results are expressed as mean±SD. The statistical differences between means were evaluated using Student's *t*-test. Differences between two groups were determined using a *P*-value. A value of $P<0.05$ was regarded as being statistically significant.

## Results

### DI induced cytotoxicity in HUVECs and SMMC-7721 cells

Cells were treated with increasing concentrations of DI for 24 h, then cell viability was determined by MTT assay. The cell viability was shown as the DI dose-dependent reduction in both HUVECs and SMMC-7721 cells (**Fig. 1**). The $IC_{50}$ values were 1.28 and 5.02 μg/ml for HUVECs and SMMC-7721 cells, respectively. We set the working concentrations of DI as 0.25, 0.5, and 1 μg/ml for the following experiments.

### DI inhibited HUVEC migration

The wound healing migration assay is an established and widely-used procedure that allows an examination of cell migration in response to an artificial wound produced on a cell monolayer. DI showed a dose-dependent inhibitory effect on the wound healing ability of HUVECs (**Fig. 2**). The control group produced a distinguished cell migration in the wound area 24 h after wounding, whereas DI-treated groups (0.25, 0.5 and 1 μg/ml) showed dose-dependent inhibitory effects on wound healing under the same conditions [**Fig. 2(A)**]. The mean migratory cell number in five fields in the control group was 504±23, but in experimental groups treated with DI at 0.25, 0.5,

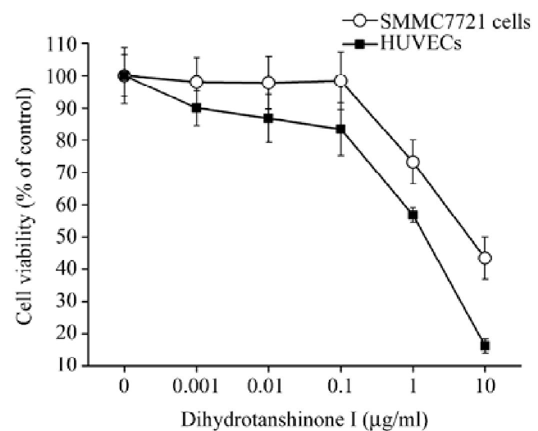


**Fig. 1 Cytotoxicity effects of dihydrotanshinone I (DI) on human umbilical vein endothelial cells and hepatocellular carcinoma SMMC-7721 cells** Cells were treated with various concentrations of DI for 24 h, then cell viability was detected by dimethyl thiazolyl-2,5-diphenyltetrazolium bromide assay.

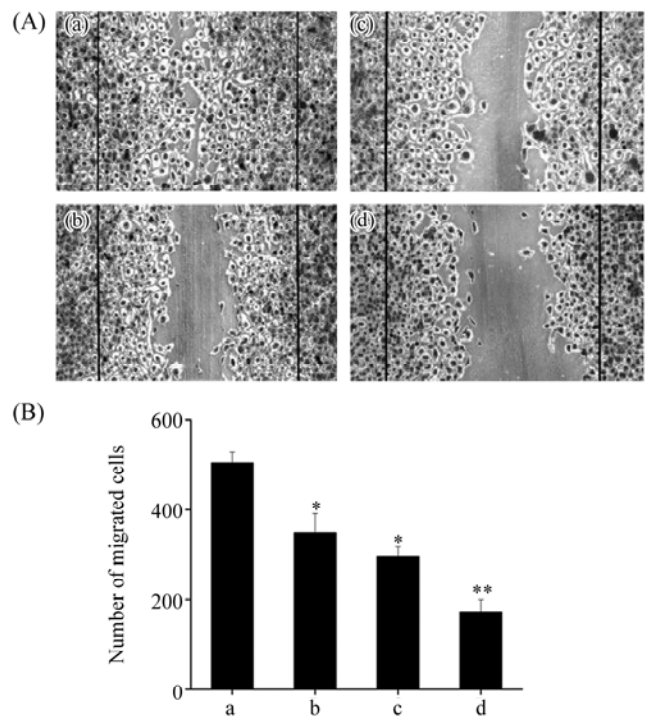


**Fig. 2 Effects of dihydrotanshinone I (DI) on cell migration** Human umbilical vein endothelial cells (HUVECs) were wounded then treated with tested doses of DI for 24 h. The number of cells migrated from the reference lines were counted. (A) Inhibitory effects of DI on HUVECs motility in a wound healing model. a, control (solvent of DI); b, 0.25 μg/ml DI; c, 0.5 μg/ml DI; d, 1 μg/ml DI. (B) Cell migration ability, determined by counting the total number of cells moving into the wound area in five views under a microscope (Magnification, 400×). Areas between the parallel lines represent the artificial wounds. *$P<0.05$ versus control; **$P<0.01$ versus control.

or 1 μg/ml, the cell numbers were 349±42, 295±22, and 172±28, respectively, with inhibitory rates of 30.75% ($P$<0.05), 41.81% ($P$<0.05), and 65.87% ($P$<0.01), respectively. These results indicated that sub-cytotoxicity of DI inhibited the motility of HUVECs.

### DI suppresses HUVEC invasion in a dose-dependent way

The invasion of endothelial cells is also one of the critical features in the formation of new blood vessels and in the repair of injured vessels. We investigated the invasion ability of HUVECs through Transwell inserts. Results showed that the average number of invaded cells in the control was 159.33±5.68, and the numbers were 135.33±4.04, 82.00±10.54, and 41.00±3.61 in 0.25, 0.5, and 1 μg/ml groups, respectively, with inhibitory rates of 15.06% ($P$<0.05), 48.53% ($P$<0.05), and 74.27% ($P$<0.01), respectively. In agreement with the results of the wound healing assay, DI at all three tested doses significantly inhibited HUVEC invasion ($P$<0.05 or $P$<0.01). The inhibitory effect was dose-dependent (**Fig. 3**).

### DI inhibited *in vitro* tube formation on Matrigel

The production of tubular structures is another important step in angiogenesis. We therefore investigated the effects of DI on HUVEC tube formation. As shown in **Fig. 4**, HUVECs plated on Matrigel and incubated with control medium aligned to form lumen-like structures and anastomotic tubes with multicentric junctions [**Fig. 4(A)**]. When HUVECs were treated with various concentrations

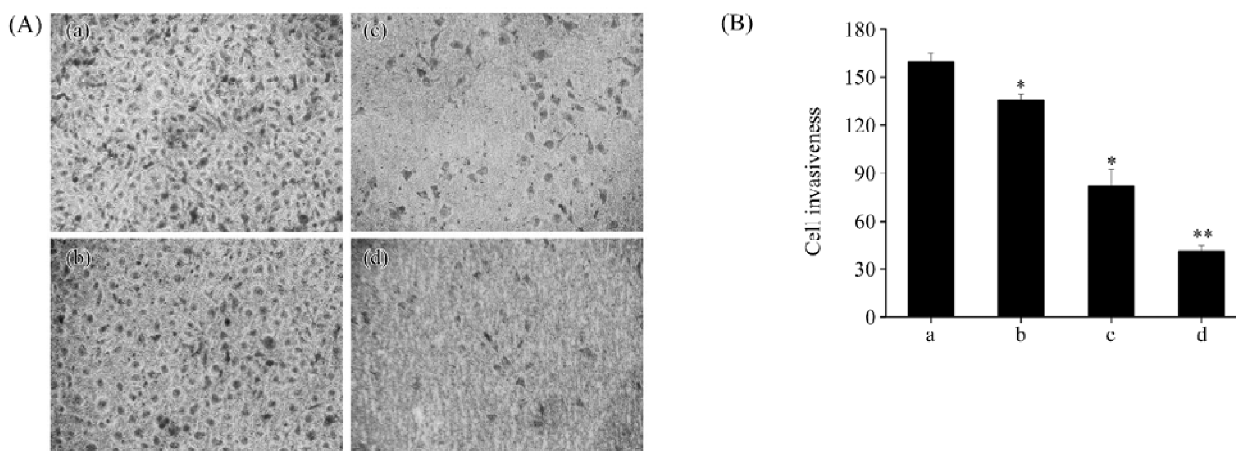


**Fig. 3 Inhibitory effects of dihydrotanshinone I (DI) on cell invasion** Human umbilical vein endothelial cells (HUVECs) on the upper surface of a transwell (Corning, New York, USA) were treated with dose of DI for 24 h. The number of cells that moved to the lower surface of the transwell was counted. (A) a, control (solvent of DI); b, 0.25 μg/ml DI; c, 0.5 μg/ml DI; d, 1 μg/ml DI. HUVECs were on the lower surface of the transwell filters. (B) The number of cells that invaded through the filters was counted under a microscope (Magnification, 400×) in five views. *$P$<0.05 versus control; **$P$<0.01 versus control.

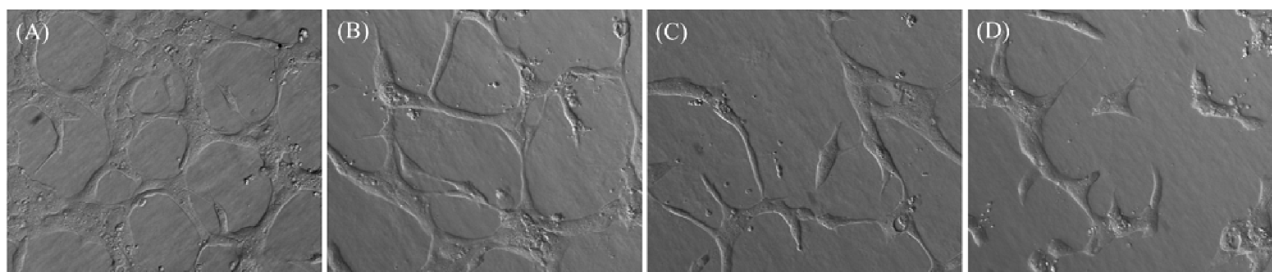


**Fig. 4 Effects of dihydrotanshinone I (DI) on tube formation of human umbilical vein endothelial cells** Cells cultured on Matrigel (Becton Dickinson, Mountain View, USA) and exposed to tested doses of DI for 24 h, then photographed under a microscope (Magnification, 200×). (A) Control (solvent of DI); (B) 0.25 μg/ml DI; (C) 0.5 μg/ml DI; (D) 1 μg/ml DI.

of DI for 24 h, the cells formed fewer tubes, as well as fewer and weaker anastomoses, in a dose-dependent manner [**Fig. 4(B–D)**].

### DI reduced *in vivo* angiogenesis in CAM model

CAM assay is a widely-used model to determine angiogenesis *in vivo*. The average vessel numbers under the methylcellulose membrane in the four groups (vehicle, ethanol, 0.1 μg DI/egg, and 0.2 μg DI/egg) were 69.25±29.64, 70.00±36.23, 31.17±11.94, and 27.20±9.09, respectively (**Fig. 5**). The vessel density between the vehicle and ethanol groups had no significant difference ($P>0.05$). Experimental groups both represented distinguished differences from the vehicle and ethanol groups ($P<0.05$). This result suggested that DI had an inhibitory effect on *in vivo* angiogenesis in CAM model.

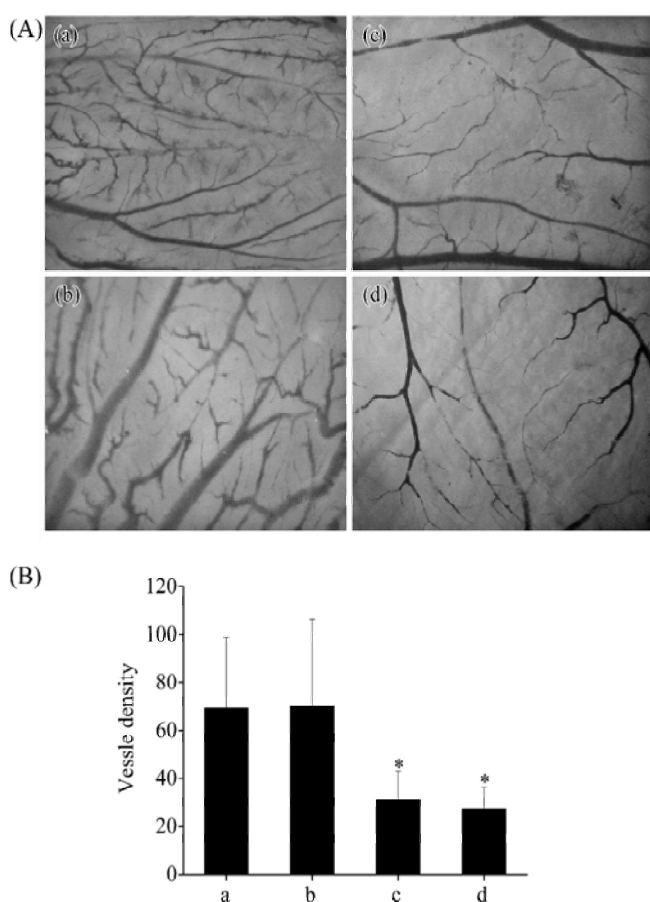


**Fig. 5 Inhibitory effects of dihydrotanshinone I (DI) on *in vivo* chick embryo chorioallantoic membrane (CAM) model** (A) CAMs were treated with different concentrations of DI for 48 h, which showed an inhibitory effect on angiogenesis. a, methylcellulose; b, control (solvent of DI); c, 0.1 μg/egg DI; d, 0.2 μg/egg DI. The density of CAM microvessels was observed under a microscope (Magnification, 200×) and photographed with a digital camera. (B) Vessel density in the scale of the CAM was evaluated by determining the vessel number. *$P<0.05$ versus control.

## Discussion

Angiogenesis plays a critical role in tumor invasion and metastasis [23]. In the initial process of tumor invasion, angiogenesis is activated by the pro-angiogenesis factors produced by environmental cells. Notably, VEGF plays a key role in the whole angiogenesis processes. VEGF can be expressed and secreted by various solid tumor cells mediated by hypoxia inducible factor 1 (HIF-1) [4]. With a high invasion and metastasis capacity, human hepatocellular cells secrete a certain level of VEGF and express VEGF receptors [5]. Based on this and other studies, VEGF monoclonal antibodies have been used in the clinic to treat metastasis cancers [24]. Other angiogenesis inhibitors suppress angiogenesis by blocking signal transduction induced by VEGF [25].

In the HUVEC invasion assay in this study, we put SMMC-7721 cells on the lower chamber of the Transwell, and found DI treated groups significantly suppressed the invasion of HUVECs to the lower chamber (**Fig. 3**), suggesting DI might block the signal transduction of VEGF produced by SMMC-7721 cells. In addition, DI directly induced cytotoxicity in HUVECs, with the $IC_{50}$ 3.92-fold less than that in SMMC-7721 cells (**Fig. 1**). This result indicates that, in treating solid tumors like hepatocellular carcinoma, DI might preferentially target vascular endothelial cells to tumor cells.

The anti-angiogenesis capacity of DI might be underlined by its chemical structure. The anti-angiogenesis capacity of cryptotanshinone was determined by the double bond at the C-15 position of the dihydrofuran ring [26]. DI is another tanshinone with such a key structure [27]. In our previous study, we found both cryptotanshinone and DI could inhibit HIF-1 activity by HIF-1 receptor using the screening method (data not shown), and this finding was verified by Dat *et al* [28]. Previous studies have shown that cryptotanshinone could inhibit vascular endothelia cell invasion, tube formation *in vitro*, and CAM angiogenesis *in vivo* [22,29]. In this study, we illustrated that DI (0.25–1 μg/ml) possessed the capacity to suppress HUVEC migration (**Fig. 2**), invasion (**Fig. 3**), and tube formation (**Fig. 4**). The mechanism might be that DI blocked the signal transduction of the effects of some growth factors, like VEGF, produced by tumor cells, but this remains to be further investigated. Also, DI could suppress microvessel density *in vivo* in the CAM model

in a dose-dependent way (**Fig. 5**). These results lay the critical foundation for further research into the antitumor ability of DI, and suggest that DI has a potential to be developed as a novel anti-angiogenic agent.

## References


1 Folkman J. Tumor angiogenesis: Therapeutic implications. N Engl J Med 1971, 285: 1182–1186

2 Carmeliet P. Angiogenesis in health and disease. Nat Med 2003, 9: 653–660

3 Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 2000, 52: 237–268

4 Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003, 9: 677–684

5 Zhou XD. Recurrence and metastasis of hepatocellular carcinoma: Progress and prospects. Hepatobiliary Pancreat Dis Int 2002, 1: 35–41

6 Masiero L, Figg WD, Kohn EC. New anti-angiogenesis agents: Review of the clinical experience with carboxyamido-triazole (CAI), thalidomide, TNP-470 and interleukin-12. Angiogenesis 1997, 1: 23–35

7 Pandya NM, Dhalla NS, Santani DD. Angiogenesis – a new target for future therapy. Vascul Pharmacol 2006, 44: 265–274

8 Yasumasa I, Izumi M, Yutaka T. Abietane type diterpenoids from *Salvia miltiorrhiza*. Phytochem 1989, 28: 3139–3141

9 Zhi HB, Alfermann AW. Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. Phytochem 1993, 32: 699–702

10 Mosaddik MA. *In vitro* cytotoxicity of tanshinones isolated from *Salvia miltiorrhiza Bunge* against P388 lymphocytic leukemia cells. Phytomedicine 2003, 10: 682–685

11 Choi HS, Kim KM. Tanshinones inhibit mast cell degranulation by interfering with IgE receptor-mediated tyrosine phosphorylation of PLCγ2 and MAPK. Planta Med 2004, 79: 178–180

12 Choi HS, Cho DI, Choi HK, Im SY, Ryu SY, Kim KM. Molecular mechanisms of inhibitory activities of tanshinones on lipopolysaccharide-induced nitric oxide generation in RAW 264.7 cells. Arch Pharm Res 2004, 27: 1233–1237

13 Lee SY, Choi DY, Woo ER. Inhibition of osteoclast differentiation by tanshinones from the root of *Salvia miltiorrhiza bunge*. Arch Pharm Res 2005, 28: 909–913

14 Kang BY, Chung SW, Kim SH, Ryu SY, Kim TS. Inhibition of interleukin-12 and interferon-gamma production in immune cells by tanshinones from *Salvia miltiorrhiza*. Immunopharmacology 2000, 49: 355–361

15 Airoldi I, Di Carlo E, Cocco C, Taverniti G, D'Antuono T, Ognio E, Watanabe M *et al*. Endogenous IL-12 triggers an antiangiogenic program in melanoma cells. Proc Natl Acad Sci USA 2007, 104: 3996–4001

16 Roberts DD, Isenberg JS, Ridnour LA, Wink DA. Nitric oxide and its gatekeeper thrombospondin-1 in tumor angiogenesis. Clin Cancer Res 2007, 13: 795–798

17 Grobmyer SR, Kuo A, Orishimo M, Okada SS, Cines DB, Barnathan ES. Determinants of binding and internalization of tissue-type plasminogen activator by human vascular smooth muscle and endothelial cells. J Biol Chem 1993, 268: 13291–13300

18 He Q, Yang B, Lou YJ, Fang RY. Contragestazol (DL111-IT) inhibits proliferation of human androgen-independent prostate cancer cell line PC3 *in vitro* and *in vivo*. Asian J Androl 2005, 7: 389–393

19 Lee MS, Moon EJ, Lee SW, Kim MS, Kim KW, Kim YJ. Angiogenic activity of pyruvic acid *in vivo* and *in vitro* angiogenesis models. Cancer Res 2001, 61: 3290–3293

20 Zheng ZZ, Liu ZX. CD151 gene delivery increases eNOS activity and induces ECV304 migration, proliferation and tube formation. Acta Pharmacol Sin 2007, 28: 66–72

21 Podar K, Tai YT, Davies FE, Lentzsch S, Sattler M, Hideshima T. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood 2001, 98: 428–435

22 Bian WP, Xu Y, Wang J, Chen F. The antiangiogenesis effect of cryptotanshinone on chick embryo chorioallantoic membrane. J Chinese Microcirculation 2007, 1: 23–26

23 Risau W. Mechanisms of angiogenesis. Nature 1997, 386: 671–674

24 Magdelaine-Beuzelin C, Kaas Q, Wehbi V, Ohresser M, Jefferis R, Lefranc MP, Watier H. Structure-function relationships of the variable domains of monoclonal antibodies approved for cancer treatment. Crit Rev Oncol Hematol 2007, 64: 210–225

25 Bai X, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B, Der CJ *et al*. Honokiol, a small molecular weight natural product, inhibits angiogenesis *in vitro* and tumor growth *in vivo*. J Biol Chem 2003, 278: 35501–35507

26 Don MJ, Liao JF, Lin LY, Chiou WF. Cryptotanshinone inhibits chemotactic migration in macrophages through negative regulation of the PI3K signaling pathway. Br J Pharmacol 2007, 151: 638–646

27 Dittmann K, Gerhauser C, Klimo K, Hamburger M. HPLC-based activity profiling of *Salvia miltiorrhiza* for MAO A and iNOS inhibitory activities. Planta Med 2004, 70: 909–913

28 Dat NT, Jin X, Lee JH, Lee D, Hong YS, Lee K, Kim YH *et al*. Abietane diterpenes from *Salvia miltiorrhiza* inhibit the activation of hypoxia-inducible factor-1. J Nat Prod 2007, 70:1093–1097

29 Hur JM, Shim JS, Jung HJ, Kwon HJ. Cryptotanshinone but not tanshinone IIA inhibits angiogenesis *in vitro*. Exp Mol Med 2005, 37: 133–137