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ORIGINAL ARTICLE

Beta-elemene inhibits melanoma growth and metastasis via suppressing vascular endothelial growth factor-mediated angiogenesis

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Abstract
Purpose It was to assess antiangiogenic effect of β-elemene in vitro and in vivo, and it was involved in inhibiting melanoma growth and metastasis, as well as to elucidate its intrinsic mechanism.
Methods Inhibitive effect of β-elemene on B16F10 cells was performed by cell proliferation assay. Angiogenesis assays in vitro including rat aortic ring and chick embryo chorioallantoic membrane were used, as well as melanoma growth and metastasis assay in C57BL/6 mice. Vascular endothelial growth factor (VEGF) expression in vitro and in vivo was measured respectively by western blot analysis and enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry analysis of CD34 and VEGF expression in primary melanoma was also presented.
Results β-Elemene inhibited B16BF10 cell proliferation starting from 200 μM, but VEGF from 20 μM. Both 20 and 50 μM β-elemene in vitro inhibited VEGF-induced sprouting vessel of rat aortic ring and microvessel formation of chick embryo chorioallantoic membrane. In vivo, tumor size of primary melanoma in mice intraperitoneally treated with β-elemene was significantly smaller than that of the control; CD34 expression of primary melanoma was also suppressed; and the metastatic melanoma colonies and content of melanin in lung were detected obviously decreased in mice of β-elemene-treated groups. Furthermore, results of VEGF expressing in primary melanoma, serum and lung of mice also disclosed that VEGF was inhibited in vivo.
Conclusions β-Elemene inhibited melanoma growth and metastasis through suppressing VEGF-mediated angiogenesis. It is a natural potential antiangiogenic agent.

Keywords β-Elemene · Melanoma growth · Metastasis · Vascular endothelial growth factor · Angiogenesis

Introduction

Melanoma is a malignant tumor of melanocytes that are found predominantly in skin but also in the bowel and the eye. It is one of the less common types of skin cancer but causes the majority of skin cancer–related deaths [1]. Furthermore, melanoma is also a high-metastatic tumor causing nearly all patients with metastasis and a lot of general symptoms like loss of appetite, nausea, vomiting and fatigue [2]. In clinic, surgical excision, adjuvant treatment [3], chemotherapy [4], immunotherapy [4] and radiation therapy [5] are the major therapeutic methods for melanoma.

Metastasis, the spread of malignant cells from a primary tumor to distant sites, is an extremely complicated process [6, 7]. It was regarded as the most intractable problem for cancer therapy and the main cause of death for cancer patients [7]. It occurs in a series of consecutive steps, which typically is divided into four phases including separation from the primary tumor, invasion through surrounding tissues and basement membranes, entry and survival in the circulation, lymphatic space and colonization in other distant organs [8]. Angiogenesis is a fundamental process required for a number of physiological and pathological processes [9].

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Specially, it was considered a key step in tumor growth, invasion and metastasis. Both expansion of the primary tumor and metastasis to other organs are dramatically dependent on angiogenesis [10]. Many growth factors playing a critical role in tumor growth and metastasis such as acidic fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and others secreted by tumors and endothelial cells enhance the process of angiogenesis [11]. In addition, VEGF had been implicated as the major prognostic factor in tumor growth, metastasis and angiogenesis. It expresses high in response to hypoxia, oncogenes and other cytokines [9]. Thus, recently, much attention has been focused on how to inhibit angiogenesis to prevent melanoma cells transferring to other sites in early stage in laboratory investigation [12].

β-Elemene (Fig. 1), purified from the essential oil, which was the main effective constituents of *Curcuma zedoaria*, had been reported a multi-effect compound including antimicrobial activity [13], antifibrotic [14] and antitumor [15]. Specially, much attention has been paid to its antitumor activity and intrinsic mechanism. It was reported that β-elemene could inhibit tumor growth in glioblastoma cells depending on activation of p38 MAPK pathway; inhibiting p38 MAPK could reverse β-elemene-mediated antiproliferation effect [16]. Antiproliferative effect of β-elemene in chemoresistant ovarian carcinoma cells was mediated by arresting cell cycle in G2/M phase, which resulted in suppressing non-small-cell lung cancer combined with inducing cell apoptosis [15, 23]. However, whether there is another mechanism involving in antitumor effect of β-elemene or not.

In this study, antiproliferative effect of β-elemene on B16F10 cells was first determined, then its antiangiogenic activity in vitro was determined. Subsequently, melanoma growth and metastasis assay in mice were further defined to verify whether β-elemene could inhibit melanoma growth, metastasis and angiogenesis in vivo. Concurrently, measurement of VEGF expression in vitro and in vivo enabled us to know its primary mechanism. Finally, a new mechanism of β-elemene on antitumor was confirmed. Dependent on inhibition of VEGF and VEGF-mediated angiogenesis, β-elemene inhibited not only melanoma growth, but melanoma metastasis. It should be a promising angiogenesis inhibitor.

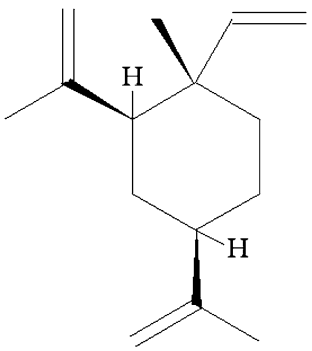

Fig. 1 Chemical structure of β-elemene

Materials and methods

Chemicals and bioreagents

β-Elemene (97% purity) was obtained from Jingang Pharmaceuticals (Dalian, China). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA) and trypsin from Invitrogen (Grand Island, NY, USA). MTS, fibrinogen and thrombin were from Promega (Madison, WI, USA). CD34 antibody was purchased from Boster Biological Technology (Wuhan, China) and VEGF polyclonal antibody from Santa Cruz Biotech (Santa Cruz, CA, USA).

Cell lines, cell culture and animals

B16F10 melanoma cells were cultured in DMEM with 10% FBS and grown in a humidified atmosphere, containing 5% CO_2 at 37°C. Male C57BL/6 mice and Sprague-Dawley rats (6 weeks old) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch. Six-week-old female mice were used for metastasis experiments. Mice were housed under specific pathogen-free conditions and handled in a laminar flow air cabinet. Experiments were approved by the Animal Ethics Committees of Nanjing University of Chinese Medicine and strictly performed according to the NIH guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats were used for aortic ring assay.

Cell proliferation assay

B16F10 cells dispersed evenly in medium and were seeded in a 96-well plate with a density of 1×10^4 cells/well. Next day, cells were treated with various concentration of β-elemene (0–1,000 μM) for 48 h or treated with indicated concentrations for 120 h with 6 replicates of each treatment. After incubation, each well was added 20 μl of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent and incubated for 3 h. Cell viability was determined by measuring the optical density at 490 nm using a SpectraMax 199 microplate reader (Molecular Device, Sunnyvale, CA, USA).

Rat aortic ring assay

The rat aortic ring assay was performed following the modified methods as described [17]. Thoracic aortas were excised from Sprague-Dawley male rats and cut into cross-section, 1-mm-wide rings and flushed with DMEM/F12 medium (Hyclone, USA). Rings were immediately placed into the wells of a 48-well plate containing 400 μl fibrinogen/thrombin solution and then incubated at 37°C until the fibrinogen/thrombin solution polymerized. The wells were then overlaid with 600 μl DMEM/F12 medium containing 10 ng/ml VEGF and various concentrations of β-elemene. On the seventh day, vascular vessels sprouting from each ring were examined using a Leica inverted phase-contrast microscope (Leica, Germany) equipped with the quick imaging system to take photos and to facilitate counting.

Chick embryo chorioallantoic membrane assay

Angiogenic activity was assayed using chick embryo chorioallantoic membranes (CAMs) as described [18]. A window (~3 cm in diameter) was cut into the egg shell of 3-day-old embryos, resealed with transparent film and incubated for further 5 days. For the CAM assay, β-elemene was pipetted onto a bacteriological-grade gelatin sponge (4 × 4 × 4 mm), air-dried for 1 h, and the resulting sponge were placed onto the 8-day-old CAMs. Control sponges contained the appropriate volume of physiological saline. Evaluation of the CAMs was performed after 4-day treatment of β-elemene. To better visualize the vascular system of the CAM, 10% formaldehyde was injected into a vitelline vein using glass capillaries. Photographs were taken using a canon digital camera. The angiogenic index was defined as the mean number of visible microvessel branch within the defined area of the gelatin sponge. Ten eggs were used for replicates in each test.

Melanoma growth and metastasis assay

According the described methods [19], 2.5×10^5 B16F10 cells were injected subcutaneously into the left footpad of each 6-week-old female C57BL/6 mice. The mice were evenly divided in three groups (6 mice/group). Furthermore, five additional mice without any treatment (no injection of B16F10 cells and treatment with β-elemene) but raised at the same environment were added as the untreated group. Except mice of the control and untreated groups with 0.1 ml of saline, other two groups were injected intraperitoneally with β-elemene (20 and 50 mg/kg) once a day, starting from the day after injection of the B16F10 cells into the footpad. This continued until the mice were killed. The length and width of the tumor were measured every day. Approximately 3 weeks later, when tumors in control group reached a diameter of 1 cm, the tumor-bearing legs were amputated along with the left popliteal lymph node. The primary melanoma was carefully separated from the legs for size analysis and then fixed with formalin for immunohistochemistry assay. The volume of the tumor was calculated from the formula length × width^2 × 0.52 as described [20]. After an additional 21 days, the mice were killed and autopsied to count the metastatic colonies in the bilateral lungs, and the lung of mice was stored at −80°C refrigerator for melanin measurement. In addition, the serum was prepared before the mice were killed and stored at −80°C refrigerator.

Immunohistochemistry staining

Immunohistochemistry staining was performed as described. Briefly, the paraffin-embedded primary melanoma tissues were sectioned with a microtome, deparaffined with xylene, rehydrated and stained immunohistochemically. The slides were incubated with 3% hydrogen peroxide for 10 min at room temperature and then performed antigen-retrieval using hot water bath (95–99°C). After complete washing, the slides were incubated with the appropriate primary antibody including CD34 and VEGF antibody at 4°C overnight. Next day, the tissue sections were incubated in the corresponding secondary antibody at room temperature for 15 min. After washing with PBS, immunohistoreactivity was visualized using diaminobenzidine (DAB) and photographed by a light microscope at the indicated magnification. Microvessel density (MVD) represented by CD34 molecule staining was measured according to the methods [21].

Melanin measurement and ELISA

The lung tissue of mice was put in a glass tube with 3.0 ml of redistilled water and homogenized using a homogenizer (IKA, Germany). Melanin was isolated from the homogenate and measured following the described methods [22]. The content of VEGF in serum and lung of mice was determined using the ELISA (Enzyme-linked immunosorbent assay) kit following the manufacturer's protocol (Raybiotech, Norcross, GA, USA).

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% SDS; 1% Triton-X 100; 10 mM NaF; 1 mM Na_3VO_4; protease inhibitor). Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as

standard (Pierce, Rockford, IL, USA). Aliquots of each cytosolic extract containing 40 μg of protein were separated by SDS–PAGE (12%), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) by electroblotting. Membranes were incubated with 5% non-fat milk solution containing 0.05% Tween-20 for blocking non-specific binding and were incubated with primary antibodies overnight at 4°C cold room, then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence reagent.

Statistical analysis

All values were expressed as means ± SD. Statistical analysis was conducted by ANOVA. Group differences resulting in $P < 0.05$ were considered statistically significant.

Results

β-Elemene indicated antiproliferative effect on B16F10 cells

Many reports have reported that β-elemene inhibited proliferation on many types of cancer cells including A549, H460, U251 [15, 16, 23]. The dose of β-elemene with antiproliferative effect started from about 200 μM and at 1 mM caused almost all of cancer cells dead [15]. In this study, B16F10 cells were treated for 48 h with a wide range of dose of β-elemene. The results shown in Fig. 2A indicated that β-elemene from 0.01 to 100 μM showed no inhibition on B16F10 cells, because cell viability varied near 100%, but from 200 μM, the cell viability had an obvious dose-dependent decrease and at 800 μM reached nearly zero, which meant that β-elemene had antiproliferative effect at the dose more than 200 μM. The IC_{50} value in B16F10 cells was about 468.8 μM. Concurrently, B16F10 cells were treated with indicated concentrations for a time-course study. The data showed that β-elemene at 50 μM had no inhibitive effect on B16F10 cells even for 120 h, but just contrarily, induced cell death obviously at 400 μM by a time-dependent manner (Fig. 2B).

β-Elemene inhibited the expression of VEGF protein in B16F10 cells in vitro

It was well known that VEGF played a pivotal role in angiogenesis, tumor growth and metastasis [9, 10, 24]. And its mechanism revealed that VEGF could regulate cell proliferation, cell migration, cell survival, cell invasion and so on through binding VEGF receptor and then activating a

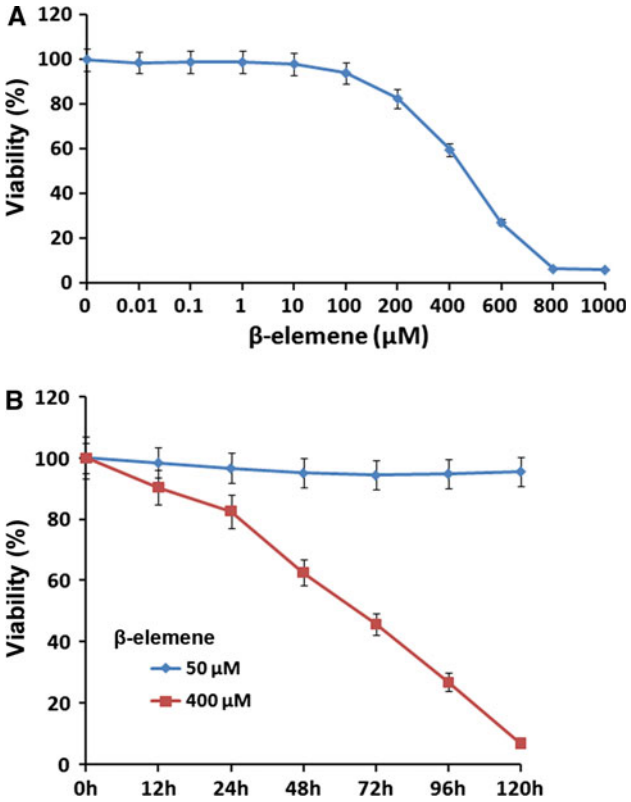

Fig. 2 β-Elemene inhibited B16F10 cell proliferation. B16F10 cells were seeded in 96-well plates with a density of 1×10^4 cells/well, then **A** treated with β-elemene (0–1,000 μM) for 48 h with 6 replicates of each treatment; **B** treated with 50 and 400 μM for 120 h with 6 replicates of each treatment. Cell proliferation was evaluated by MTS reagent using a SpectraMax 199 microplate reader. Results were presented as mean ± SD ($n = 6$)

series of intracellular responses. Since β-elemene could inhibit B16F10 cell proliferation, it was necessary to know how β-elemene regulated VEGF protein. The expression of VEGF protein was in vitro tested by western blot analysis in B16F10 cells. We found that β-elemene inhibited expression of VEGF in a dose- and time-dependent way in B16F10 cells. At the low concentration of 4 μM, the expression of VEGF showed no change, whereas significant decrease when treated with β-elemene 20 μM and more (Fig. 3A). For the time-dependent study, 50 μM β-elemene inhibited VEGF starting from 4-h treatment (Fig. 3B).

β-Elemene inhibited sprouting vessels of rat aortic ring and microvessels formation of chicken embryo chorioallantoic membrane

To investigate β-elemene's effect on angiogenesis, two angiogenic evaluation models were used. Aortic vessels of rats always grow slowly and few vessels will be sprouted, but in a condition with growth factors such as VEGF, the plenty of vessels from the aortic vessel would sprout and

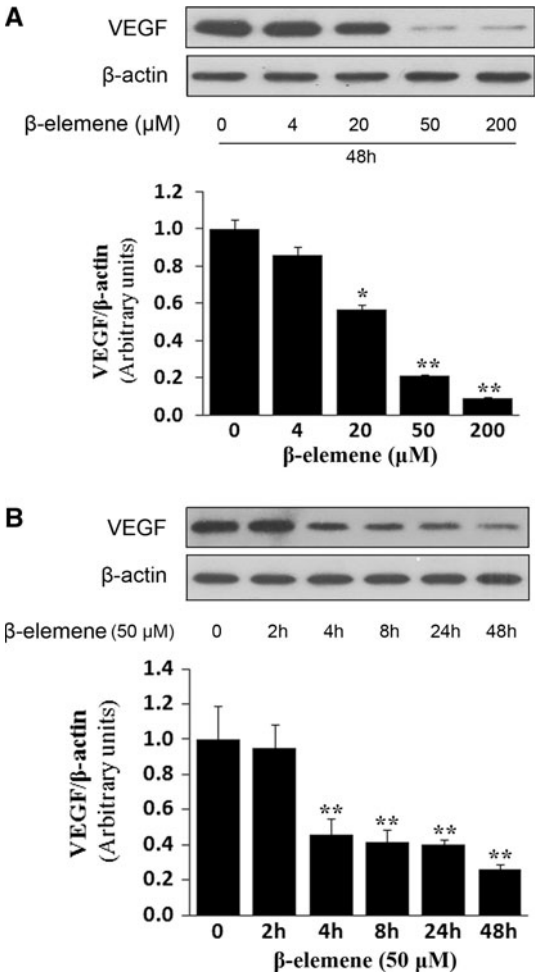

Fig. 3 β-Elemene inhibited VEGF expression in vitro. B16F10 cells were seeded in 6-well plates with a density of 1×10^5 cells/well, then **A** treated with a series dose of β-elemene for 48 h; **B** treated with 50 μM β-elemene for the indicated time. Cellular lysates were subjected to western blot analysis with antibodies against VEGF, β-actin (loading control). Densitometry analysis of VEGF bands was normalized to β-actin using NIH ImageJ from three independent experiments, expressed as arbitrary units. $^*P < 0.05$, $^{**}P < 0.01$ versus control (β-elemene 0 μM)

even gradually inosculate to form rings like tubes. So, it is a good model in vitro for screening the angiogenic inhibitors. Compared with the control pretreated with or without VEGF (10 ng/ml), β-elemene 20 μM and 50 μM both showed significant inhibition on sprouting vessels and no vessel rings formed (Fig. 4A). Another model for angiogenesis is chicken embryo chorioallantoic membrane assay. β-Elemene was added to the chicken embryo chorioallantoic membrane where angiogenesis is very abundant to treat it for 4 days. As shown in Fig. 4B–C, 10 ng/ml VEGF could induce much neovascularization of chicken embryo chorioallantoic membrane, while β-elemene inhibited it by the indication of less new microvessels formation at 20 and 50 μM β-elemene-treated groups. β-elemene inhibited angiogenesis in vitro.

β-Elemene inhibited primary melanoma growth and angiogenesis

To further investigate whether β-elemene inhibited angiogenesis in vivo, animal model of melanoma growth and metastasis was used. B16F10 melanoma spontaneous metastasis model was specially designed for the research on tumor metastasis, but also suitable for the solid tumor growth because B16F10, a high metastatic cell line, not only spontaneously transferred to other sites, also accelerated expanded in primary sites [25]. After a 21-day treatment with 20 and 50 mg/kg β-elemene a day in mice, the growth curve of the primary melanoma indicated that tumor size of β-elemene-treated groups was significantly smaller than that of the control from about 10 to 21-day treatment (Fig. 5A). The photos of separated solid melanoma presented the similar results (Fig. 5B). CD34 was an endothelial antigen used to highlight the microvasculature vessel density as a direct marker for the degree of angiogenesis in vivo [26, 27]. Based on the immunohistochemistry staining results (Fig. 5C–D), microvessel density (MVD) of the control was clearly more than that of the β-elemene-treated, which suggested that angiogenesis was inhibited by β-elemene.

β-Elemene inhibited B16F10 melanoma lung metastasis

Since β-elemene could inhibit melanoma growth in primary site, plus with the importance of angiogenesis in metastasis, we checked whether it had the same effect on metastasis. As shown in Fig. 6A, melanoma metastatic colonies had distinctly transferred to and widely distributed in whole lung of the mice in the control, but the less could be checked in other two groups. Similarly, further statistic data on metastatic colonies disclosed that less metastatic colonies appeared in the lung of the mice treated with 20 or 50 mg/kg β-elemene for 42 days (Fig. 6B). Melanin, a type of substance produced by melanocytes, could be measured in various melanoma models (B16 in C57 mice, Harding-Passey in BALB/c mice, and KHDD in C3H mice), as well as in selected normal tissues [22]. Thus, melanoma metastasis could also be judged through determining the content of melanin in specific organ. As shown in Fig. 6C, the melanin content of lung of two β-elemene-treated groups was respectively only about one-second or one-fourth of the control. Collectively, β-elemene inhibited melanoma metastasis toward lung.

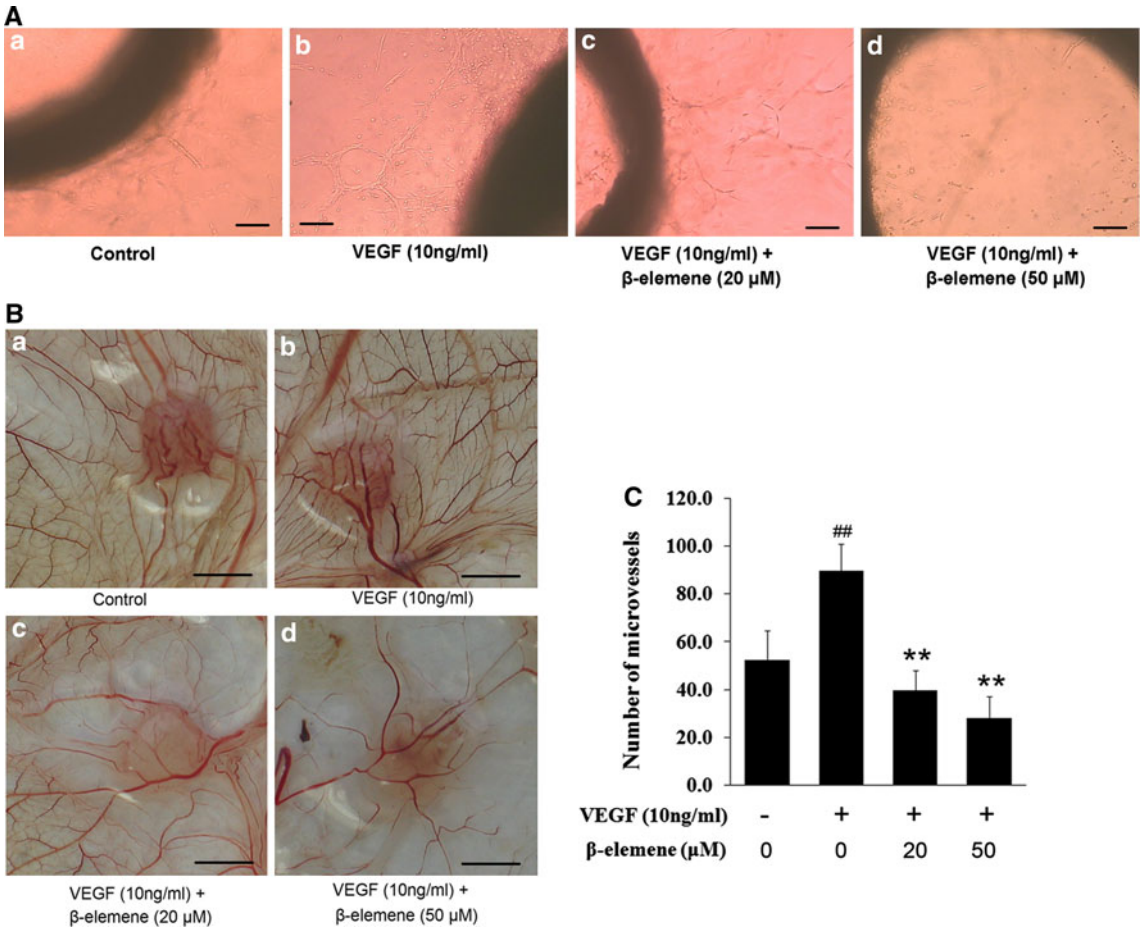

Fig. 4 β-Elemene inhibited VEGF-induced angiogenesis in vitro. **A** Vascular vessels sprouting from rat aortic *ring* was photographed at a 200× magnification using a Leica inverted phase-contrast microscope equipped with the quick imaging system (*a–d scale bar* 1 mm). **B** The chick embryo chorioallantoic membranes after 96 h treatment with β-elemene or saline were photographed. Representative photos show the CAMs of different groups (*a–d scale bar* 5 mm). **C** Quantification of newly formed microvessels of chick embryo chorioallantoic membranes. Results were presented as mean ± SD ($n = 6$). $^{\#\#}P < 0.01$, difference versus control; $^{**}P < 0.01$, difference versus VEGF (10 ng/ml)

β-Elemene inhibited VEGF in vivo

VEGF was found to be inhibited in vitro (Fig. 3), so it was necessary to investigate whether in vivo also done. VEGF expressed highly not only in tumor tissues, but also in serum, plasma, urine and the organ with tumor metastasis in patients with malignant tumors. Here, VEGF expression in primary melanoma, serum and lung of mice was measured by different assays. Upon the semi-quantification data of immunohistochemistry staining (Fig. 7A), VEGF expressing in primary melanoma had a significant decrease through comparing the control with the β-elemene-treated groups. And upon the ELISA data shown in Fig. 7B–C, VEGF level of serum and lung of mice in the untreated group respectively was 56.7 ± 7.9 ng/l and 103.4 ± 21.8 ng/g protein then got a significant increase to 241.3 ± 62.4 ng/l and 357.1 ± 79.5 ng/g protein in the control group treated with B16F10 melanoma cells. However, its level in serum, respectively, 139.2 ± 38.1 and 96.8 ± 18.7 ng/l in 20 and 50 mg/kg β-elemene-treated groups, was significantly inhibited compared with the control. The data of VEGF level in lung showed the similar statistic results. According to these, β-elemene also inhibited VEGF in vivo.

Discussion

Since Folkman first demonstrated the importance of angiogenesis in tumor initiation, development and metastasis [28], it was thought of one of promising targets for tumor therapy [9, 10, 12]. So much attention had been paid to

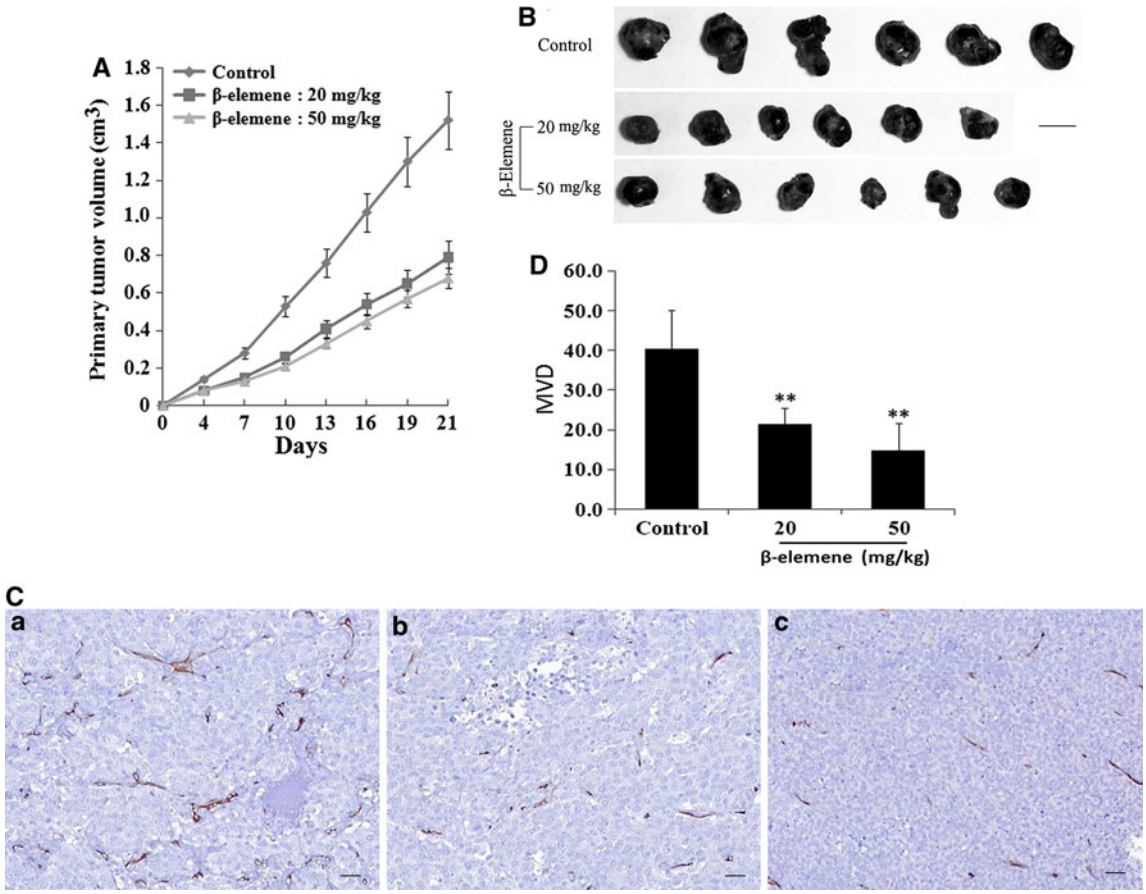

Fig. 5 *β*-Elemene inhibited primary melanoma growth and angiogenesis. B16F10 cells were injected subcutaneously into the footpad of the mice. After 3-week treatment with *β*-elemene (20 and 50 mg/kg d), the left leg of the mice was cut, and the primary melanoma was separated carefully from the left footpad for analysis. **A** Size of the primary melanoma before it was excised; **B** photos of the primary melanoma (*scale bar* 1 cm); **C** CD34 expresses in primary melanoma by immunohistochemistry assay; photos were taken using the inverted microscope (200×, *scale bar* 50 μm). (*a* control, *b* *β*-elemene—20 mg/kg, *c* *β*-elemene—50 mg/kg); **D** quantification of microvessel density (MVD). Results were presented as mean ± SD ($n = 6$). $^{**}P < 0.01$, difference versus control

invent or look for antiangiogenic agents [29]. At present, the main way to find antiangiogenic agents has been focused on biological antibodies such as Bevacizumab, a kind of humanized monoclonal antibody directed against VEGF [9, 30], getting approved by the Food and Drug Administration (FDA). Certainly, synthesizing compounds against angiogenesis was another way. Two antiangiogenic drugs, sorafenib and sunitinib, targeting multiple receptor tyrosine kinases including VEGF receptors and platelet-derived growth factor (PDGF) receptors had been used in clinic [9, 24, 31]. However, we were also interested in whether it was possible to find an antiangiogenic agent from the plant or wide-used Chinese medicine. Based on the preliminary and reported data on Curcuma zedoaria, we found that *β*-elemene containing antitumor effect also maybe a potential antiangiogenic agent that was worthy to be further investigated.

Recent studies showed that *β*-elemene inhibiting cell proliferation was correlated with G2-M phase arrest in human non-small-cell lung cancer H460 and A549 cells [15]. In addition, *β*-elemene was found to trigger apoptosis in glioma SHG-44 cells and leukemia K562 cells [32], and the apoptosis was associated with reduction of Bcl-2 protein expression [33]. Our antiproliferative results indicated that *β*-elemene did not show inhibitive effect on B16F10 cells at a low range of dose (0–100 μM), but did starting from about 200 μM. This data is basically consistent with the reported. Then VEGF expression was measured in B16F10 cells treated with from 4 to 200 μM *β*-elemene for 48 h. *β*-Elemene could inhibit expression of VEGF in B16F10 cells at dose of 20 μM and more, which suggested that dose of *β*-elemene (≥20 μM) showing antiangiogenic effect was lower than that (≥200 μM) with antiproliferative effect. So, to partially exclude its cytotoxicity on cells,

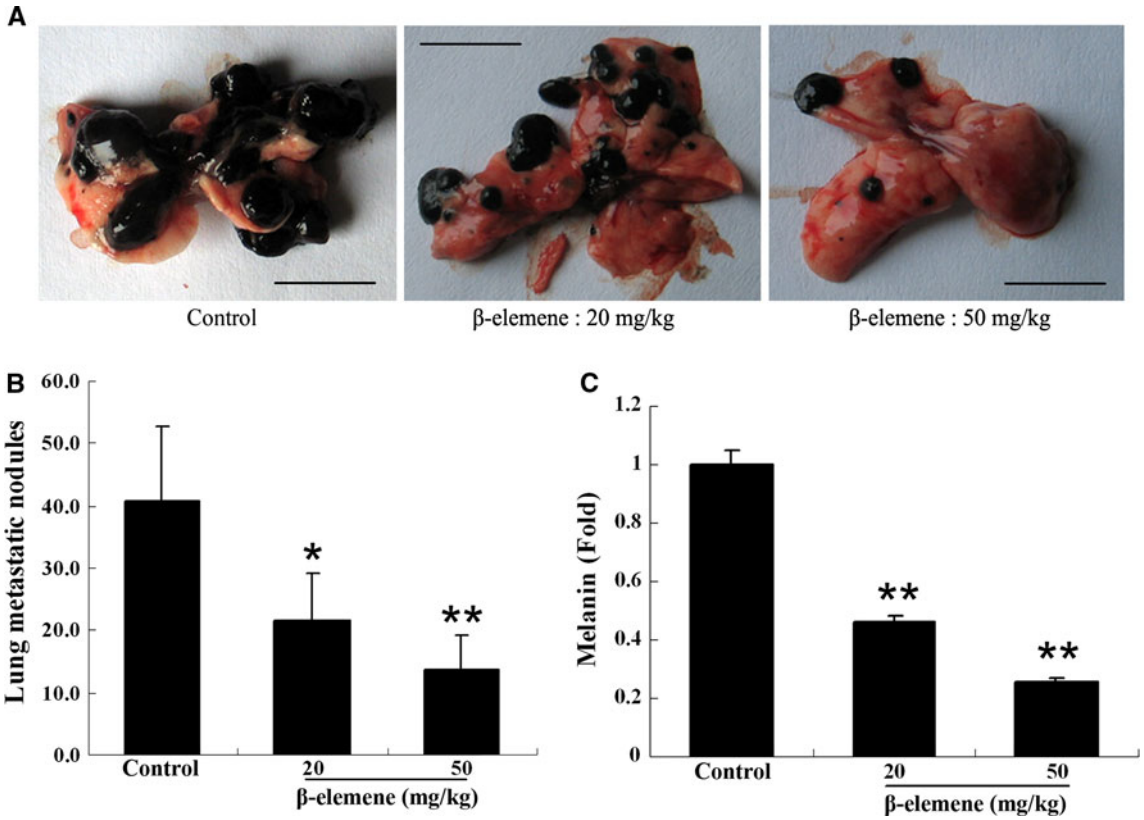

Fig. 6 β-Elemene inhibited B16F10 melanoma lung metastasis. B16F10 cells were injected subcutaneously into the footpad of the mice. After 6-week treatment with β-elemene (20 and 50 mg/kg d), the lung of mice was taken out for analysis of metastatic colonies. **A** Representative photos of the lung with the metastatic colonies (*scale bar* 1 cm); **B** statistical metastatic nodules in lung of mice of different treatment groups; **C** quantification of melanin of the whole lung. Results were presented as mean ± SD ($n = 6$). $^{**}P < 0.01$, difference versus control

20 and 50 μM β-elemene were used in the assay of rat aortic ring and chicken embryo chorioallantoic membrane, and both indicated the obvious inhibition on angiogenesis induced by VEGF. Similarly, animal experimental data also revealed β-elemene's antiangiogenic action. The growth of B16F10 melanoma in primary site—footpad was significantly inhibited, the metastatic colonies in lung also suppressed by β-elemene. Additionally, CD34, an important molecular marker for angiogenesis, expresses less in primary melanoma of β-elemene-treated groups. VEGF, an initiator of angiogenesis and controlling the progress of tumor growth and metastasis, was not only inhibited in vitro, but also downregulated in primary melanoma, serum and lung of mice in vivo. Taken together, it was confirmed that β-elemene plays inhibitive role in angiogenesis, which resulted in suppressing melanoma growth and metastasis. More importantly, this course was dependent on inhibition of VEGF, suggesting that β-elemene like sorafenib maybe an inhibitor of tyrosine protein kinases, but from natural Chinese medicine.

Although we found that β-elemene inhibiting melanoma growth and metastasis was mainly attributed to mediating VEGF, many other growth factors such as bFGF, aFGF could not be excluded, because they also played an important role in inducing angiogenesis. As such, VEGF was regulated by many upstream molecular signaling pathways including estrogen [34], mTOR [35] and AKT [36] and controlled downstream signaling pathways related with cell proliferation, cell motility and cell adhesion [37]. Additionally, VEGF receptors were an indispensable factor in initiating and introducing angiogenesis [37]. All of them should be considered to completely elucidate its complicated molecular mechanism. However, it is unclear at present that β-elemene inhibiting VEGF-mediated angiogenesis is through directly targeting VEGF or competitively binding VEGF receptors or affecting its upstream or downstream signaling pathway. Hence, a lot of works should be conducted based on this study in the future, which is under investigation.

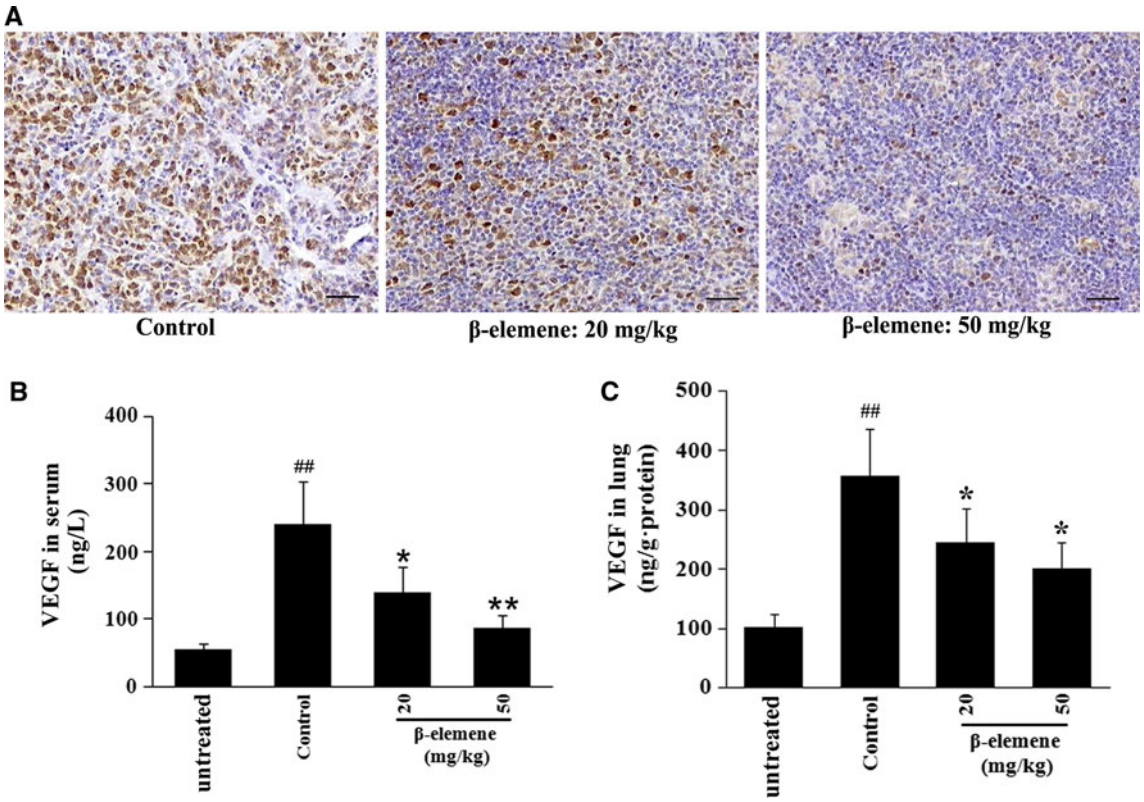

Fig. 7 β-Elemene inhibited VEGF expression in vivo. B16F10 cells were injected subcutaneously into the footpad of the mice. After 3-week treatment with β-elemene (20 and 50 mg/kg d), the left leg of the mice was cut and the primary melanoma was separated carefully from the left footpad for Immunohistochemistry analysis of VEGF (**A**), photos were taken using the inverted microscope (200× *scale bar* 50 μm); After 6-week treatment with β-elemene (20 and 50 mg/kg d), samples of serum and lung of mice were prepared and stored in −80°C. VEGF content in serum (**B**) and lung (**C**) was measured by ELISA. Results were presented as mean ± SD ($n = 6$). $^{\#\#}P < 0.01$, difference versus untreated; $^{*}P < 0.05$, $^{**}P < 0.01$, difference versus control

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Conflict of interest statement None.

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