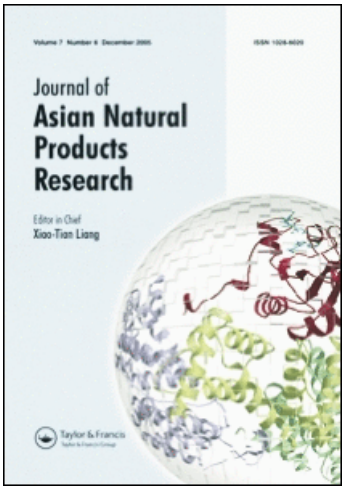

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EN-LONG Ma[a]; YAN-CHUN Li[a]; HIROSHI Tsuneki[b]; JIN-FANG Xiao[a]; MING-YU Xia[a]; MIN-WEI Wang[a]; IKUKO Kimura[b]
[a] Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang, China [b] Department of Clinical Pharmacology, Toyama Medical and Pharmaceutical University, Toyama, Japan

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β-Eudesmol suppresses tumour growth through inhibition of tumour neovascularisation and tumour cell proliferation

EN-LONG MA†*, YAN-CHUN LI†, HIROSHI TSUNEKI‡, JIN-FANG XIAO†, MING-YU XIA†, MIN-WEI WANG† and IKUKO KIMURA‡

†Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China
‡Department of Clinical Pharmacology, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

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In the present study, we investigated the potential anti-angiogenic mechanism and anti-tumour activity of β-eudesmol using *in vitro* and *in vivo* experimental models. Proliferation of human umbilical vein endothelial cells (HUVEC) stimulated with vascular endothelial growth factor (VEGF, 30 ng/ml) and basic fibroblast growth factor (bFGF, 30 ng/ml) was significantly inhibited by β-eudesmol (50–100 μM). β-Eudesmol (100 μM) also blocked the phosphorylation of cAMP response element binding protein (CREB) induced by VEGF (30 ng/ml) in HUVEC. β-Eudesmol (10–100 μM) inhibited proliferation of HeLa, SGC-7901, and BEL-7402 tumour cells in a time- and dose-dependent manner. Moreover, β-eudesmol treatment (2.5–5 mg/kg) significantly inhibited growth of H_{22} and S_{180} mouse tumour *in vivo*. These results indicated that β-eudesmol inhibited angiogenesis by suppressing CREB activation in growth factor signalling pathway. This is the first study to demonstrate that β-eudesmol is an inhibitor of tumour growth.

Keywords: Angiogenesis; Growth factor; Tumour; CREB; HUVEC

1. Introduction

Angiogenesis is a process by which new capillaries are formed from pre-existing blood vessels. Angiogenesis is a complex process that includes degradation of extracellular matrix, migration and proliferation of endothelial cells, tube formation, and sprouting of new capillary branches [1]. This process is tightly regulated by a net balance between pro- and anti-angiogenic factors under physiological conditions [2,3]. Yet once the net balance is destroyed in the angiogenic state, unregulated angiogenesis occurs, which causes various diseases, especially cancer. Tumour require capillaries to provide oxygen and nutrients for their survival, and the new vessels allow tumours to escape into the circulation and lodge in other organs (tumour metastases). This means that without blood vessels, tumours cannot grow beyond a critical size or metastasise to another organ [4]. Therefore, the blockade of angiogenesis is a promising strategy to prevent cancer.

*Corresponding author. Email: maenlong@hotmail.com

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β-Eudesmol is a sesquiterpenoid isolated from the rhizome of *Atractylodes lancea*. It was reported that β-eudesmol can inhibit angiogenesis *in vitro* and *in vivo* by blocking extracellular signal-regulated kinase (ERK1/2) signalling pathway [5]. β-Eudesmol is also known to have various unique effects on the nervous system. For example, β-eudesmol induces neurite outgrowth in rat pheochromocytoma PC12 cells via an activation of mitogen-activated protein (MAP) kinase [6]. However, the detailed mechanism of β-eudesmol on anti-angiogenesis is not clear and the direct effect of β-eudesmol on tumour growth has not yet been tested. In the present study, we investigated the potential anti-angiogenic mechanism and anti-tumour activities of β-eudesmol using *in vitro* and *in vivo* experimental models.

2. Results and discussion

2.1 Inhibitory effect of β*-eudesmol on proliferation of HUVEC stimulated with VEGF and bFGF*

When human umbilical vein endothelial cells (HUVECs) were cultured with vascular endothelial growth factor (VEGF) (30 ng/ml) and basic fibroblast growth factor (bFGF) (30 ng/ml) for 4 days, both VEGF and FGF significantly stimulated proliferation of HUVEC. However, in the presence of β-eudesmol (50 and 100 μM), increases in HUVEC numbers induced by VEGF and bFGF were significantly decreased (figure 1).

2.2 Inhibition of the CREB phosphorylation by β*-eudesmol in the endothelial cells*

The influence of β-eudesmol on response element binding protein (CREB) phosphorylation was investigated in the serum-starved HUVEC. CREB were markedly phosphorylated 10 min after the stimulation with VEGF (30 ng/ml). If HUVEC was pretreated with β-eudesmol (30–100 μM) for 3 h, the VEGF-stimulated CREB phosphorylation was blocked, while total CREB expression was not affected (figure 2). Significant reduction of the phosphorylation levels of CREB was obtained in the presence of β-eudesmol (100 μM).

2.3 Inhibitory effect of β*-eudesmol on proliferation of human tumour cells* in vitro

Various tumour cells, HeLa, SGC-7901, and BEL-7402, were chosen to investigate THE effect of β-eudesmol on human tumour cells *in vitro*. β-Eudesmol (10–100 μM) inhibited proliferation of HeLa, SGC-7901, and BEL-7402 cells in a time- and dose-dependent manner, and maximal inhibition rates were 87.5%, 50.3% and 39.8%, respectively (figure 3A–C).

2.4 Inhibitory growth effect of β*-eudesmol on the mice H_{22} and S_{180} solid tumour* in vivo

To further evaluate the effect of β-eudesmol on tumour growth, mice tumour models were created by implanting H_{22} and S_{180} mice tumour cells into oxters of 7-week-old KM mice. β-eudesmol treatment (2.5–5 mg/kg) significantly inhibited H_{22} and S_{180} mouse tumour growth when compared to the control group (tables 1 and 2). Meanwhile, β-eudesmol (2.5–5 mg/kg) had no obvious influence on the weight increase of mice.

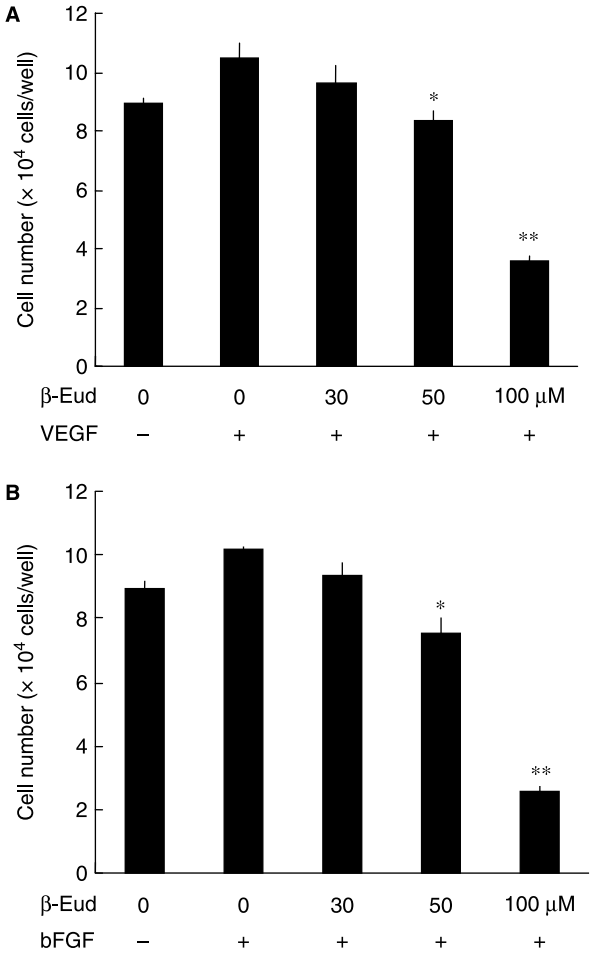

Figure 1. Inhibitory effect of β-eudesmol on the bFGF- and VEGF-stimulated proliferation of human umbilical endothelial cells (HUVEC). HUVEC (1.5×10^4 cells per dish) were incubated with β-eudesmol (β-Eud, 0–100 μM) for 2 h and then stimulated with bFGF (30 ng/ml) or VEGF (30 ng/ml). Concentration-dependent inhibition by β-eudesmol on bFGF-stimulated (A) or VEGF-stimulated (B) proliferation activity was assessed by the number of HUVEC at day 6 in culture. As negative controls, HUVEC were treated with only 0.1% DMSO (vehicle). As a positive control, cells were treated with bFGF or VEGF. Data values are mean ± S.E.M., $n = 6$ per group. $P < 0.05$ and $P < 0.01$ *vs* positive control.

2.5 Inhibitory effect of β*-eudesmol on angiogenesis in mice implanted with H_{22} tumour cells*

The inhibitory effect of β-eudesmol on angiogenesis was investigated in H_{22} mouse tumour models. β-Eudesmol treatment (2.5–5 mg/kg) obviously inhibited vascular index when compared to the control group (table 3). As a positive control, thalidomide (Tha, 200 mg/kg) treatment also inhibited the formation of new blood vessels in the tumour tissues.

Formation of new blood vessels is essential to proliferation and metastasis of tumours. Among processes of angiogenesis, proliferation of endothelial cells (EC) plays a crucial role [10]. Therefore, EC usually is considered as a target to investigate angiogenesis. Some compounds that can inhibit proliferation of endothelial cells are promising as angiogenesis inhibitors. Under normal conditions, endothelial cells are in static condition and only a few

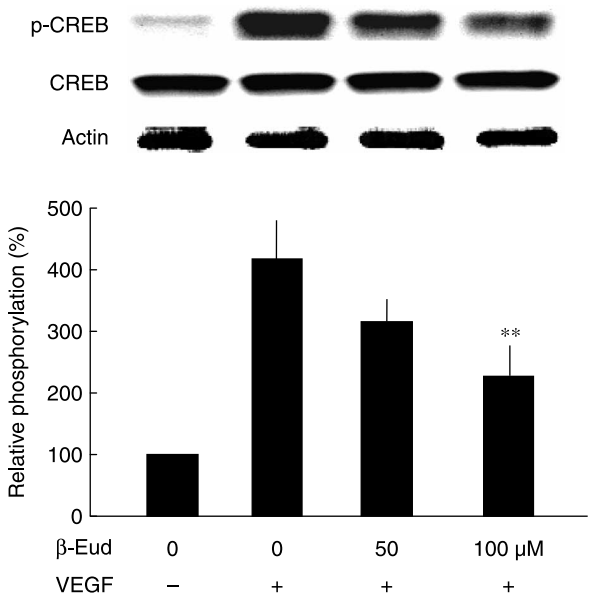

Figure 2. β-Eudesmol blocks VEGF-induced CREB activation in endothelial cells. HUVEC were preincubated with β-eudesmol (β-Eud, 50, 100 μM) for 3 h and then stimulated with VEGF (10 ng/ml) for 10 min. The phosphorylation of CREB and their expression levels in HUVEC were analysed by Western blotting. Blots shown are representatives of four independent experiments. The histograms represent the relative intensity of phosphorylated CREB levels as determined by densitometric analysis. Data values are mean ± S.E.M., $n = 4$ per group. $^{**}P < 0.01$ *vs* the phosphorylated CREB levels increased by VEGF in the absence of β-eudesmol.

cells can proliferate. When a tumour occurs, tumour cells can secret the growth factors (VEGF and bFGF) to stimulate proliferation of endothelial cells, and the process of angiogenesis is switched on [11]. Our results showed that both VEGF and bFGF obviously stimulated HUVEC towards proliferation, and this proliferation-stimulated effect was blocked in the presence of eudesmol (10–100 μM). This suggested that β-eudesmol can produce an angiogenesis-inhibited effect by blockade of growth factor signalling pathway.

VEGF and its receptors play a crucial role in angiogenesis [12]. Inhibition of VEGF signalling pathway was considered as a strategy to block angiogenesis [13]. We have proved that β-eudesmol inhibits the growth factor signalling pathway by depressing activation of ERK-MAPK. As CREB, a transcription factor responsive to cAMP, plays an important role in promoting proliferation and cellular adaptive responses, a mechanism has been defined through which VEGF/KDR may permit endothelial cells to respond to changes in their environment through alteration of gene expression important to angiogenesis. VEGF induces CREB phosphorylation and transactivation, and activated CREB mediates endothelial cell proliferation and blood vessel development [14]. Our experiments indicate that β-eudesmol blocked activation of CREB induced by VEGF in HUVEC (figure 3). Therefore, we think that inhibition of phosphorylation of CREB may explain, at least in part, the mechanism of anti-angiogenic effect by β-eudesmol (figure 4).

In this study, β-eudesmol inhibited obviously the proliferation of some tumour cells *in vitro*. Previous data showed that β-eudesmol inhibited vascular endothelial cell proliferation by inhibiting ERK-MAPK activation, but did not inhibit proliferation and ERK-MAPK activation of both smooth muscle cells and astrocytes in rat [5]. Therefore, we presume that inhibition of tumour cell proliferation *in vitro* by β-eudesmol may be explained as blockade

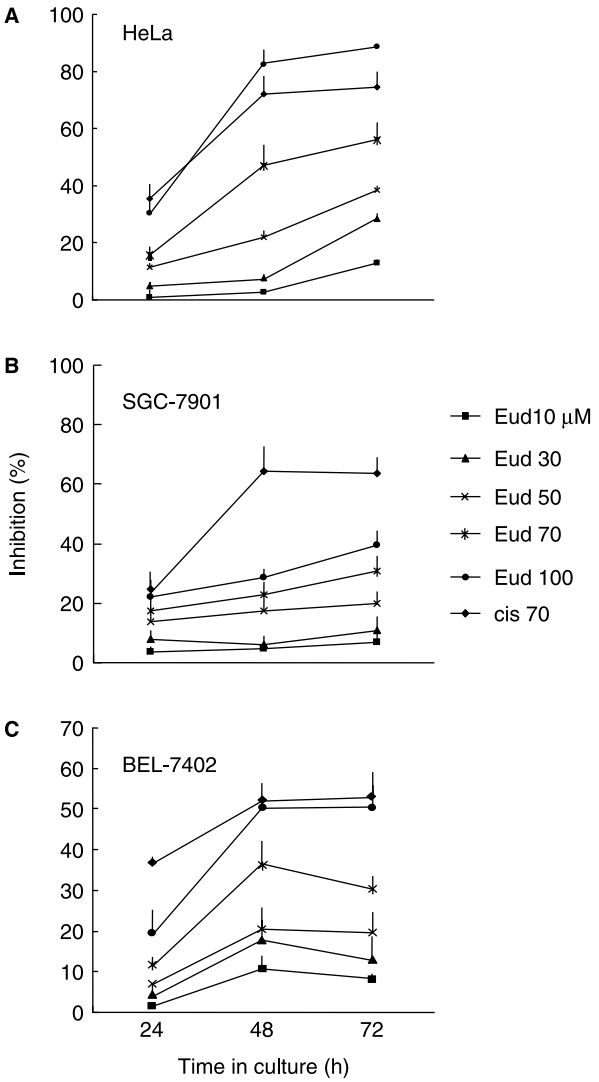

Figure 3. Inhibitory effect of β-eudesmol on the proliferation of tumour cells. HeLa, BEL-7402 or SGC-7901 tumour cells were plated at 5×10^3 cells per well in the absence and presence of β-eudesmol (1–100 μM) or cisplatin (cis, 70 μM). Time-dependent and concentration-dependent inhibitory effects by β-eudesmol on HeLa (A), BEL-7402 (B) or SGC-7901 (A) are shown. Data values are mean ± S.E.M., $n = 6$ per group.

Table 1. Anti-tumour activity of β-eudesmol in mice implanted with H_{22} tumour cells.

			Body weight (g)			
Group	*Dose (mg/kg)*	*n*	*d 0*	*d 7*	*Tumour weight (g)*	*Inhibition (%)*
Control	–	16	19.2 ± 0.5	26.9 ± 1.1	1.21 ± 0.36	–
	1.25	12	19.0 ± 0.6	26.3 ± 2.3	0.76 ± 0.29*	37.2
β-Eud	2.5	13	19.1 ± 0.5	24.9 ± 1.8	0.73 ± 0.30**	39.9
	5	14	18.8 ± 0.6	24.1 ± 1.6	0.62 ± 0.13**	49.3
Cis	15	12	19.3 ± 0.5	22.9 ± 0.9	0.68 ± 0.45**	43.8

Data are shown as means ± S.E.M. d 0 means the body weight of mice before treatment, and d 7 means the body weight of mice after 7 days of treatment. *n*, number of data. $*P < 0.05$; $**P < 0.01$ compared with the control group.

Table 2. Anti-tumour activity of β-eudesmol in mice implanted with S_{180} tumour cells.

			Body weight (g)			
Group	*Dose (mg/kg)*	*N*	*d 0*	*d 7*	*Tumour weight (g)*	*Inhibition (%)*
Control	–	10	19.0 ± 0.5	25.1 ± 1.4	1.53 ± 0.37	–
	1.25	10	19.2 ± 0.4	25.0 ± 1.9	1.39 ± 0.57	8.8
β-Eud	2.5	10	18.9 ± 0.6	24.1 ± 2.1	1.18 ± 0.29*	22.4
	5	10	19.0 ± 0.5	23.2 ± 2.3	0.88 ± 0.27**	42.2
Cis	15	10	18.8 ± 0.3	21.1 ± 1.9	0.64 ± 0.14**	58.4

Data are shown as means ± S.E.M. d 0 means the body weight of mice before treatment, and d 7 means the body weight of mice after 7 days of treatment. *n*, number of data. $*P < 0.05$; $**P < 0.01$ compared with the control group.

Table 3. Anti-angiogenic effect of β-eudesmol in mice implanted with H_{22} tumour cells.

			Body weight (g)			
Group	*Dose (mg/kg)*	*N*	*d 0*	*d 7*	*Vascular index (mg. g^{-1})*	*Inhibition (%)*
Control	–	10	19.3 ± 0.7	26.1 ± 0.9	0.378 ± 0.051	–
	1.25	10	19.0 ± 0.6	25.6 ± 1.7	0.263 ± 0.043**	30.4
β-Eud	2.5	10	18.9 ± 0.4	24.7 ± 1.4	0.248 ± 0.051**	34.4
	5	10	18.9 ± 0.5	23.9 ± 1.9	0.211 ± 0.027**	44.3
Tha	200	10	19.1 ± 0.6	25.1 ± 2.1	0.260 ± 0.038**	31.2

Data are shown as means ± S.E.M. d 0 means the body weight of mice before treatment, and d 7 means the body weight of mice after 7 days of treatment. *n*, number of data. $*P < 0.05$; $**P < 0.01$ compared with the control group.

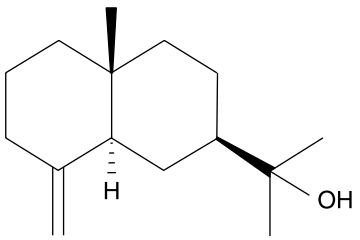

Figure 4. Structure of β-eudesmol.

of the ERK-MAPK pathway. More importantly, growth of mouse H_{22} and S_{180} tumour, and formation of new blood vessels in tumour tissues *in vivo*, were also obviously inhibited by β-eudesmol. These results suggested that β-eudesmol may be a promising anti-tumour compound. The inhibitory effect of β-eudesmol on tumour growth *in vivo* may be attributed to two distinct and complementary mechanisms, namely the inhibitory effect on tumour neovascularisation and tumour cell proliferation.

In conclusion, β-eudesmol inhibits angiogenesis by suppressing CREB activation in the growth factor signalling pathway. This is the first study to demonstrate that β-eudesmol is an inhibitor of tumour growth. This study provides new insight into the biological function of β-eudesmol as a tumour inhibitor.

3. Experimental

3.1 Materials

All reagents used were purchased from Sigma (St. Louis, MO, USA), unless indicated. β-Eudesmol was provided by Dr. M. Yoshizaki (Herbal garden, Toyama Medical and

Pharmaceutical University, Japan). Recombinant human basic fibroblast growth factor (bFGF) and recombinant human vascular endothelial growth factor (VEGF) were purchased from Pepro Tech EC (London, U.K.). A rabbit anti-CREB antibody and a rabbit anti-phospho-specific CREB kinase antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). A rabbit anti-Actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Culture of cells

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Cambrex Bio Science, Walkersville, MD, USA), and cultured in endothelial cell basal medium-2 (EBM-2, Clonetics) containing growth supplements and 2% heat inactivated foetal calf serum (Bio Wittaker).

HeLa, human cervical cells, were obtained from the American Type Culture Collection (ATCC, #CRL, 1872, MD, USA); BEL-7402, human liver cancer cell, and SGC-7901, human gastric cancer cell, were obtained from the Chinese Academy of Medical Sciences. Cancer cells were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% heat-inactivated foetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), L-glutamine 2 mmol/L (Gibco, USA), penicillin 100 kU/L, and streptomycin 100 g/L (Gibco) at 37°C in 5% CO_2.

3.3 Proliferation assay

The endothelial cells were plated at 1.5×10^4 cells per 35-mm-diameter dish coated with collagen type IV, and incubated for 2 h at 37°C to allow the cells to adhere. β-Eudesmol (1–100 μM) and VEGF (30 ng/ml) or bFGF (30 ng/ml) was then added to growth medium (5% FBS-containing EGM-2). The medium was exchanged every other day. For counting the cell numbers, all the cells were detached from dishes by treating with endothelial-cell trypsin. Trypan blue was added into the cell suspension to differentiate dead cells from living cells, and the number of living cells was counted using the image analysis software (Win Roof).

HeLa, SGC-7901, and BEL-7402 tumour cells were plated at 5×10^3 cells per 96-well cell culture plate, and incubated for 12 h at 37°C to allow the cells to adhere. β-Eudesmol (10–100 μM) or Cisplatin (70 μM) was then added to growth medium. As a control group, DMSO (end-concentration of 0.1%) was added to growth medium. The medium was exchanged every day. Cell viability was determined by MTT assay. The percentage of cell growth inhibition was calculated as the following equation:

$$\text{Inhibition}(\%) = [A_{492(\text{control})} - A_{492(\text{drug})}]/A_{492(\text{control})} \times 100.$$

3.4 Western blotting

Phosphorylation of CREB was analyzed by Western blotting as described previously [5,7]. In brief, HUVEC were starved for 48 h in serum-free DMEM at 60–70% confluence, and treated with β-eudesmol at 37°C for 3 h, and then stimulated with VEGF for 10 min. The cells were suspended in lysis buffer and centrifuged. Proteins in the supernatant were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membranes were treated with specified

antibodies, and then incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin, Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Blots were detected using ECL enhanced chemiluminescence kit (Amersham). The density of protein bands was quantified with Fluor-S Multi-Imager (Bio-Rad, Hercules, CA, USA). Phosphorylated level of CREB was calculated by comparing the value obtained for phosphorylated CREB with the value obtained for expression level of total CREB in each sample.

3.5 *Anti-tumour assay* in vivo

The experiment was carried out according to the method described by Xu *et al.* [8]. Male KM mice [18–20 g, Animal Research Certificate: SCXK (LIAO) 2003–008] were obtained from Shenyang Pharmaceutical University Animal Research Centre (Shenyang, China). The mice were housed in a temperature- and light-controlled environment, and were allowed free access to food and water. Mouse hepatic tumour cells H_{22} (4×10^6 cells) or sarcoma cell S_{180} (4×10^6 cells) in 0.1 ml sterilised saline were injected subcutaneously into oxters of 7-week-old KM mice. Twenty-four hours after implantation, animals were treated with β-eudesmol (1.25–5 mg/kg) or cisplatin (15 mg/kg) by intraperitoneal injection once a day for 7 days. The control group was treated with the vehicle (0.5% CMC-Na containing 0.5% ethanol). At the end of the experiment, animals were euthanised under ether anaesthesia. Then the tumour tissues were isolated and weighed to determine the anti-tumour effect of β-eudesmol. The percentage of tumour growth inhibition was calculated as the following equation:

$$\text{Inhibition}(\%) = [\text{tumour weight(control)} - \text{tumour weight(drug)}]/\text{tumour weight(control)} \times 100\%.$$

3.6 *Anti-angiogenesis assay* in vivo

H_{22} tumour cells were injected subcutaneously into oxters of 7-week-old KM mice. Twenty-four hours after implantation, animals were treated with β-eudesmol (1.25–5 mg/kg) or thalidomide (Tha, 200 mg/kg) by intraperitoneal injection once a day for 7 days. The control group was treated with the vehicle (0.5% CMC-Na containing 0.5% ethanol). At the end of the experiment, animals were euthanised by the injection of 1 ml 10% carmine solution. The tumour tissues were isolated, weighed, and homogenised in 10 ml of 3 M NaOH. After centrifugation, the supernatant was filtered. The carmine content in the supernatant was determined as described by Kobayashi *et al.* [5,9]. Finally, vascular index was calculated as the following equation to investigate the newly formed blood vessels in tumour tissues:

$$\text{Vascular index} = \text{Carmine content in the tumour tissues}/\text{tumour tissue weight}.$$

3.7 *Statistical analysis*

The difference between multiple groups was assessed by one-way analysis of variance (ANOVA) followed by the Scheffé's multiple range tests. Values of P less than 0.05 were considered to be significant.

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