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ORIGINAL ARTICLE

Fascaplysin, a selective CDK4 inhibitor, exhibit anti-angiogenic activity in vitro and in vivo

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Abstract
Purpose This study was to evaluate the correlation of two important strategies, namely, cell cycle proliferation arrest and anti-angiogenesis. We chose fascaplysin, a marine natural product with selective CDK4 selective inhibition activity, to study its potential anti-angiogenesis effects in vivo and in vitro.
Methods Chorioallantoic membrane (CAM) assay was initially used as an in vivo approach to evaluate anti-angiogenic activity of fascaplysin. In addition, human umbilical vein endothelial cell (HUVEC) line was used to further confirm the anti-angiogenic activity of fascaplysin in vitro. To explore the mechanism of anti-angiogenesis, we examined the effect of fascaplysin on vascular endothelial growth factor (VEGF) expression and secretion by hepatocarcinoma cells BeL-7402.
Results The results of CAM assay suggested fascaplysin inhibited capillary plexus formation in a dose-dependent manner and suppressed VEGF in cross section. Moreover, the in vitro assay also confirmed that fascaplysin provided selective inhibition of endothelial cells proliferation towards tumor cells in low concentration. The immunocytochemical staining and ELISA verified fascaplysin could inhibit VEGF expression and secretion by BeL-7402.
Conclusions These findings strongly suggest that fascaplysin is a natural angiogenesis inhibitor.

Keywords Fascaplysin · Anti-angiogenesis · CAM · HUVEC · VEGF

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Introduction

The cell cycle is regulated by the interplay of many molecules; key among these are the cyclins which are expressed and then degraded in a concerted fashion to drive the stages of the cell cycle. Cyclins combine with cyclin dependent kinases (cdks) to form activated kinases that phosphorylate targets leading to cell cycle regulation. The most important checkpoint in the cell cycle regulation is the restriction point which determines the G1/S transition controlling the passage of eukaryotic cells from the first interphase (G1) into the DNA synthesis phase (S). It is initiated by the activity of Cdk4–cyclin D1 complex phosphorylating the retinoblastoma protein pRb, and growth factor dependent. Misregulation of Cdk4 activity caused either by over expression of its activating partner cyclin D1, loss of the Cdk4 specific inhibitor p16 or mutation(s) in its catalytic subunit can cause deregulated cell growth resulting in tumor formation, as observed in majority of human cancers [1, 2]. In fact, the G1/S transition is misregulated in 60–70% of human cancers. Therefore, cyclin D-dependent kinases have been considered for many years a prime target for cancer chemotherapy [3, 4].

One of the significant cyclin-dependent kinase inhibitor is fascaplysin (Fig. 1). Fascaplysin, a red pigment originally isolated from Fijian marine sponge *Fascaplysinopsis* sp. in 1988, represents the first naturally occurring member of the pentacyclic ring system 12H-pyrido [1, 2-a: 3,4-b′]diindole [5]. This carboline class alkaloid showed highly selective CDK4/D1 and CDK6/D1 inhibition in the high nanomolar range [6]. Because of its easy availability through practical large scale synthesis and its cell cycle regulation bioactivity, it has become the anti-tumor drug lead structure for intensive studies

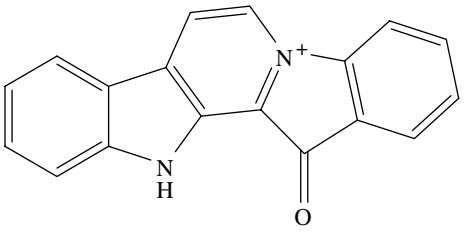

Fig. 1 Chemical structure of fascaplysin

on CDKs mechanistic study and structural optimization [7–9].

On the other hand, angiogenesis, the formation of new blood vessels from preexisting vessels, has been shown to be essential for tumor growth and metastasis. The central concept that tumor growth is "angiogenesis dependent" was firstly perceived by Folkman in 1971 and is well accepted today. Safely speaking, every increment of tumor growth requires an increment of vascular growth. Inhibition of angiogenesis is emerging as a promising strategy for treating cancer. At molecular level, it is now recognized that the tumor cells produces growth factors that stimulate the proliferation of the endothelial cells, among which vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are most important regulators [10].

In the course of our study to search the marine bioactive natural product candidates for hepatocarcinoma, which is the major cause of cancer death in China [11], we are considering the correlations and balance between these two key therapeutic strategies, namely, tumor cell cycle proliferation arrest and anti-angiogenesis. Hence, we decide to study the potential anti-angiogenesis effects of fascaplysin, a marine natural product with selective CDK4 selective inhibition activity. In our present study, we firstly applied chicken chorioallantoic membrane (CAM) model to assay the anti-angiogenesis effect of fascaplysin in vivo [12]. In addition, human umbilical vein endothelial cell (HUVEC) line was used to further confirm the anti-angiogenic activity of fascaplysin in vitro. Our study has shown that fascaplysin could inhibit VEGF production in CAMs in vivo and VEGF produced by human hepatocarcinoma in vitro.

Methods and materials

Cell lines and reagents

HUVEC, human umbilical vein endothelial cells and BeL-7402, human hepatocarcinoma cells were obtained from China Center for Type Culture Collection (Wuhan, China). Fertilized eggs were from the Yida Poultry Farm (Zhejiang, China). Anti-VEGF antibody and Ultra sensitive S-P kit were purchased from Maxim Biotechnology (Fuzhou, China). Minimum essential medium (MEM) was purchased from Gibco invitrogen corporation (Australia), and fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Austria). 3-[4, 5-dimethylthylthiazol-2-yl]-2, 5-diphenynyltetrazoliumbromide (MTT) was purchased from Sheng-Gong Biology Company (Shanghai, China). All other reagents were of highest analytical grade.

Chorioallantoic membrane assay

Fertilized eggs of the white Leghorn were incubated after cleaning at 37.8°C and 85–90% relative humidity throughout the experiment. On day 3 of development, chick embryos were removed from their shells, placed into sterile glass dishes. The eggs were then incubated until day 5, when 5 μl of the samples to be tested (or the same volume of phosphate buffered saline (PBS) as control) were applied to sterile gelatin sponge [13] (3 mm × 3 mm × 1 mm) and placed on the surface of the growing CAM.

Quantification of the capillary plexus

After 48 h exposure of samples to the CAMs, the zone around the sponge was observed directly and carefully, and all blood vessels within a 100 mm^2 area surrounding the applied sponge were captured using a binocular microscope (BX 60, Olympus Tokyo, Japan) and analyzed at 40× magnification. All the evaluations reported here were carried out by at least two experienced investigators.

Histological assessment and immunohistochemistry

CAM surface were fixed with 10% formaldehyde in PBS, pH 7.4. The fixed CAM was carefully cut, and appropriate samples of CAMs were processed for embedding in paraffin wax by standard techniques. After dehydration and embedding in paraffin, CAM was sectioned at 5 μm for histological assessment and immunohistochemistry. A minimum of five sections stained with hematoxylin and eosin (H&E) from each CAM were selected for evaluating subtle changes in CAM matrix and capillary plexus formation. Histological sections were digitized with a spot camera and the numbers of capillary plexus that had formed beneath the ectoderm were observed.

The presence of VEGF in CAMs was revealed by using a Streptavidin–Peroxidase system that uses

3,3-*p*-diaminobenzidine (DAB) as a substrate. Then CAMs were counterstained with hematoxylin. Positive immunostaining, which appeared as a brown color, was visualized under binocular microscope (BX 60, Olympus Tokyo, Japan).

Cell culture and proliferation assays

HUVEC and BeL-7402, were maintained in MEM, supplemented with 10% FBS, 100 U ml^{-1} penicillin and streptomycin, at 37°C under 5% CO_2 in the air. Cell proliferation was determined by standard MTT assay in HUVEC and BeL-7402 [14]. Cells were inoculated in 96-well plates at the density of 2×10^5 cells per well. Attached cells were incubated for 24 h prior to fascaplysin addition at different concentration and then the incubation period was extended for another 48 h. The cell survival fraction was determined with 20 μl MTT (5 mg ml^{-1} in PBS) per well. Cells were incubated further for 4 h at 37°C. Finally the formazan crystals formed were dissolved by the addition of DMSO and measured by the absorbance at 492 nm.

Immunocytochemical staining of VEGF in BeL-7402

Immunocytochemical staining was performed to determine whether fascaplysin could block BeL-7402 expressing VEGF in cytoplasm. BeL-7402 cells were plated on glass coverslips in 24-well plates at a density of 5×10^4 cells ml^{-1}. Subsequently, cells were incubated for 24 h with or without fascaplysin, and fixed in methanol for 5 min at −10°C. The Ultra sensitive S-P kit was carried out according to the manufactures protocol. Then cells were incubated with anti-VEGF antibody for 12 h at 4°C. As a negative control, the primary antibody was omitted. The cell nuclei were slightly counterstained with hematoxylin. The immunostaining results on the slides were examined under an Olympus BX 60 microscope.

Enzyme-linked immunosorbent assay for VEGF secretion

The enzyme-linked immunosorbent assay (ELISA) kit specific for human VEGF (Boster, China) was used to quantify VEGF secretion in the growth media of BeL-7402. Cells were cultured for 24 h with fascaplysin ranging from 0.5×10^{-6} to 2×10^{-6} M as previously described, and medium was collected and centrifuged at 2,000 rpm for 10 min at 4°C. Conditioned medium (100 μl) was placed in each well of a 96-well plate, which was previously coated with antibody for VEGF, and incubated for 90 min at 37°C. Subsequent to aspirating unbound substances, a secondary antibody was added to each well, and incubated for additional 60 min at 37°C. After aspirating and washing the wells again, incubation for 30 min with a chromogenic substrate solution was performed and the reaction was stopped adding the stop solution and measured by the absorbance at 450 nm. VEGF protein levels were normalized to the number of cells.

Results

Inhibition of CAM angiogenesis

Our attempt to correlate the cell cycle arrest with anti-angiogenesis was initially attempted by the evaluation of in vivo effects on CAM angiogenesis model for fascaplysin, a selective CDK4 inhibitor derived from marine sponge. The CAM is a densely vascularized and rapidly growing extra-embryonic membrane that has been used for many years to investigate the effect of a variety of substances on the formation of new blood vessels [15]. In our experiment, we demonstrated in the first time that fascaplysin exhibit strong anti-angiogenesis effects in a dose dependent manner. Fascaplysin start to show CAM angiogenesis inhibition at the concentration of 0.1 μM (5 μl/egg) (Fig. 2), 0.3 μM and 0.6 μM treatment of fascaplysin reduced the number of newly formed microvessels about 40.87% ± 0.35 and 81.98% ± 2.31, respectively (Fig. 3).

Histological assessment and immunohistochemistry in CAM

The CAM capillary plexus provides an excellent model to study capillary formation in vivo. In histological sections stained with H&E, extensive capillary plexus formation and migration of blood vessels towards ectoderm was observed in control group (Fig. 4a). Consistent with macroscopic evaluation, application of fascaplysin caused significant decrease in number of capillary plexus formation according to concentrations (Fig. 4b) in cross section. Dramatic changes were seen in Fig. 4c, where capillary plexus were almost obliterated and have less extracellular matrix than the mesoderm of control CAMs. Since it is reported that the extracellular matrix in the CAM is important in influencing blood vessel development [16], this phenomena indicate a facet of significant action on angiogenesis of CAM by fascaplysin.

VEGF immunohistochemistry was applied to explore the mechanism of fewer capillary plexus formation induced by fascaplysin in CAMs. The control

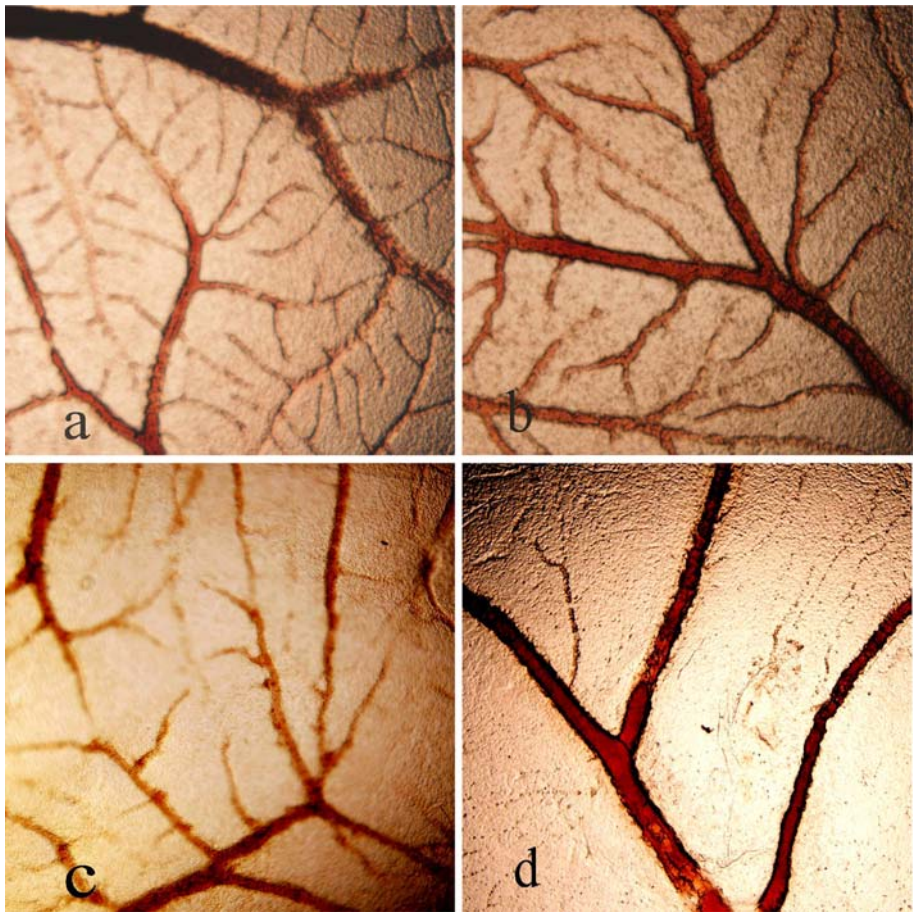

Fig. 2 Chicken chorioallantoic membrane (*CAM*) assay. Typical *pictures* showing effect of fascaplysin on angiogenesis in CAM of day 7 fertilized egg. Five microliter fascaplysin of different concentration were added on sterilized sponge and placed on the chick chorioallantoic membrane of 5-days fertilized egg. After incubation continuously for another 48 h, CAMs was taken pictures. **a** Control, **b** fascaplysin 0.1×10^{-3} M, **c** fascaplysin 0.3×10^{-3} M, **d** fascaplysin 0.6×10^{-3} M (magnification ×40)

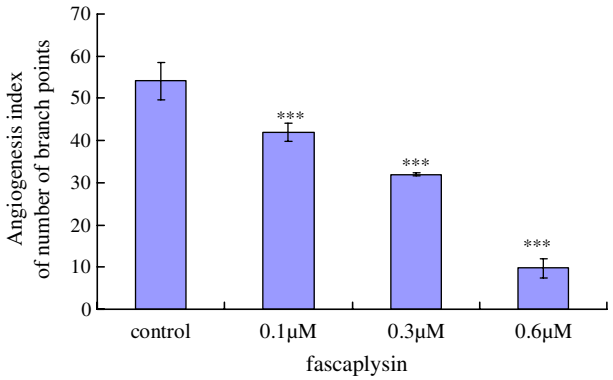

Fig. 3 Macroscopic assessment of vascular density induced by fascaplysin. Macroscopic assessment of vascular density conducted by counting the number of branch points within a 100 mm^2 area surrounding sponges. The number of branch points was markedly decreased with fascaplysin compared to PBS control ($n = 10$; mean ± SEM); ***$P < 0.001$ compared to PBS control

group has shown strong brown colors, which indicates expressing excessive VEGF (Fig. 5a). However, VEGF expression in Fig. 5b is much weaker, indicating large decrease of VEGF in capillary plexus formation.

Inhibition of cell proliferation

To confirm the results of anti-angiogenesis activity in CAM, we further studied the effect of fascaplysin on endothelial cell. Fascaplysin was shown to have remarkable anti-proliferative responses in a concentration-dependent manner, with an EC_{50} of 1.33×10^{-6} M in HUVEC. However, fascaplysin had slighter cytotoxicity effects against BeL-7402 cells compared to HUVEC at concentrations ranging from 0.5×10^{-6} to 4×10^{-6} M (Fig. 6), which indicated that fascaplysin provided selective inhibition of endothelial cells proliferation towards tumor cells in lower concentration (0.5×10^{-6} to 4×10^{-6} M).

Immunocytochemical analyses of VEGF in BeL-7402

BeL-7402 cells in the control groups showed strong expression of VEGF in cytoplasm, which was symbolized by staining the dark brown color (Fig. 7a). On the contrast, the staining of VEGF in experimental groups treated with fascaplysin was weaker in a dose-dependent manner (Fig. 7b, c), which suggest that VEGF

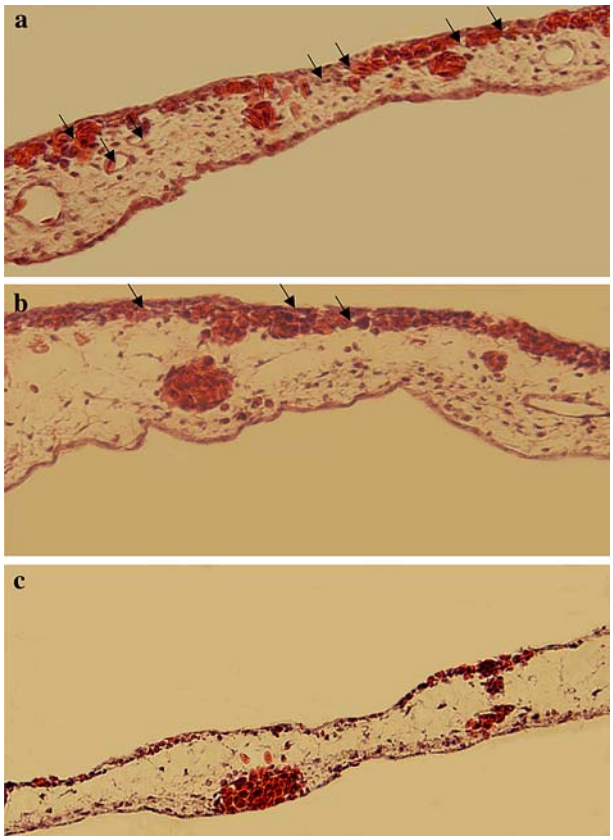

Fig. 4 Cross sections stained with H&E. Cross sections stained with H&E showing variable capillary plexus formation in CAMs. **a** Control: *arrows* pointing numerous formed vessels plexuses formed along the ectoderm (*arrows* indicate new vessels). **b** Fascaplysin 0.3×10^{-3} M: formation of scanty capillary plexus with retarded migration of blood vessel towards the ectoderm. **c** Fascaplysin 0.6×10^{-3} M: distorted CAM matrix with sparse capillary plexus formation beneath the ectoderm(magnification ×200)

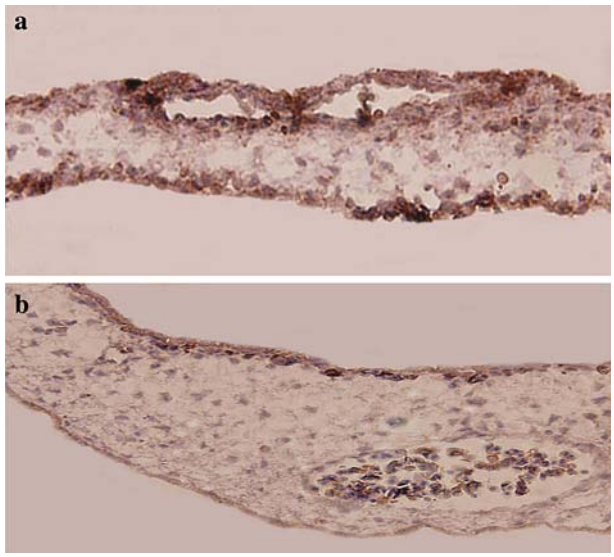

Fig. 5 Immunohistochemical analysis of fascaplysin-induced vessels of CAMs. **a** Immunostaining of untreated CAMs with anti-VEGF antibody. **b** 0.3×10^{-3} M fascaplysin treated CAMs with anti-VEGF antibody (magnification ×200)

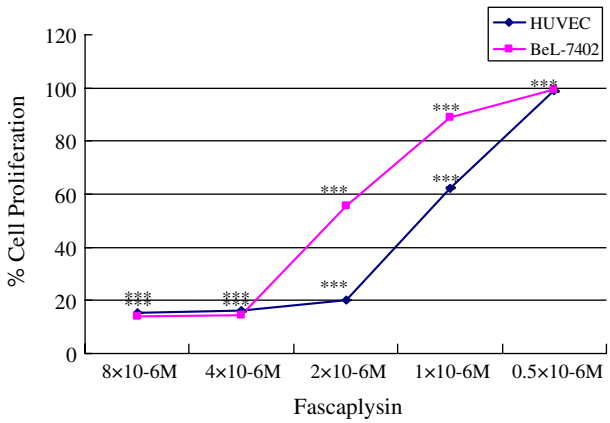

Fig. 6 Fascaplysin inhibited HUVEC cell and BeL-7402 cell proliferation. Cell proliferation was determined by MTT assay. No treatment control sample was defined as 100% cell proliferation, and the decrease in cell proliferation relative to the control was calculated for each sample ($n = 3$; mean ± SEM). ***$P < 0.001$ compared to control

expressed less due to fascaplysin. The results of immunocytochemical analyses BeL-7402 is in accordance with VEGF expression induced by fascaplysin in CAMs [17].

Down-regulated secretion of VEGF

The secretion of VEGF into medium was determined by ELISA. In Fig. 8, the amounts of VEGF secreted over the 24 h period treated by 0.5×10^{-6} to 2×10^{-6} M fascaplysin are illustrated compared to the control. 1×10^{-6} to 2×10^{-6} M fascaplysin reduced VEGF secretion in a dose-dependent manner.

Discussion

Developing novel cancer chemotherapeutic agents that have a well-defined mechanism of action is still an emerging field of oncology where researchers are facing great challenges. In this direction, diverse marine organisms provide prolific source for new molecules that can curtail cancer growth [18–20]. It is known that fascaplysin, isolated from marine sponges, is a significant cyclin-dependent kinase inhibitor. The primary focus of our study is to expose the potential anti-angiogenesis mechanism of fascaplysin contributing to anticancer property.

Angiogenesis is one of the most important factors involved in the development and progression of human tumors [21, 22]. To evaluate the anti-angiogenic

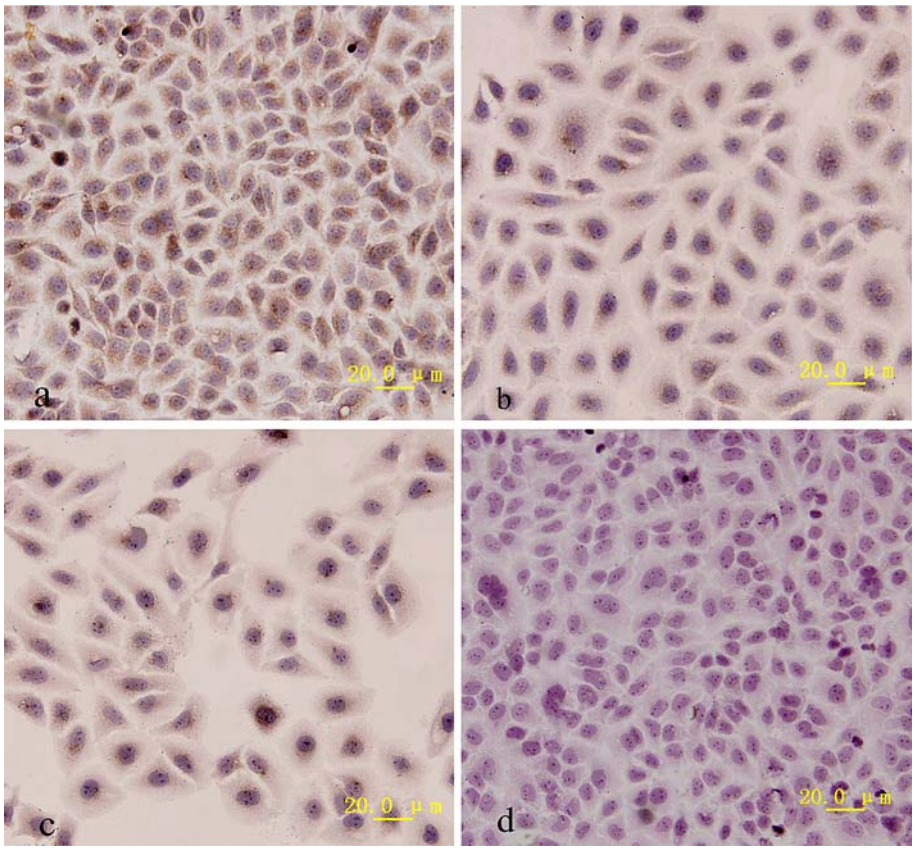

Fig. 7 Immunocytochemical staining of VEGF in human hepatocarcinoma BeL-7402. **a** Control, **b** fascaplysin 0.5×10^{-6} M, **c** fascaplysin 1.5×10^{-6} M, **d** negative control: the primary antibody was omitted (magnification ×200)

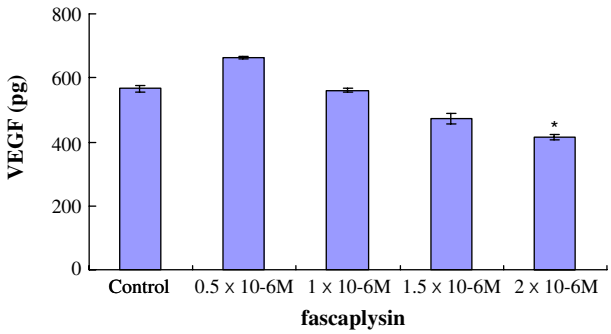

Fig. 8 Secretion of VEGF by BEL-7402 cells following exposure to 0.5×10^{-6} to 2×10^{-6} M fascaplysin for 24 h. VEGF in the culture medium was analyzed by ELISA ($n = 3$; mean ± SEM). *$P < 0.05$ compared to control

activity of fascaplysin in vivo, we used CAM model, which is reported to have advantages of low cost, ease of experimentation and visualization of the quasi-2D vascular tree within the transparent membrane compared to other established assays of angiogenesis in vivo. Fascaplysin caused significant decrease in number of capillary plexus formation according to concentrations in CAM (Fig. 2), which is consistent with the result of microscopic cross section (Fig. 4). VEGF is the most important regulator for angiogenesis. Our study also revealed that fascaplysin could suppress VEGF expression according to concentration in vivo (Fig. 5). Therefore, these findings suggest blockade of VEGF expression is involved in the anti-angiogenesis activity in vivo.

Endothelial cell is the source of new blood vessel, and therefore we focused on the effect of fascaplysin on the endothelial cell in vitro. Endothelial cells are inherently stable and exhibit lower mutagenesis rate than tumor cells, thereby reducing chances of multi-drug resistance [23]. So it is more advisable for fascaplysin to target endothelial cells other than tumor cell for anti-cancer activity. Our data (Fig. 6) showed that fascaplysin provided selective inhibition of endothelial cells proliferation towards tumor cells in low concentration (0.5×10^{-6} to 4×10^{-6} M).

It is well known that VEGF is a potent endothelial cell mitogen for the endothelial cell proliferation. Our study has demonstrated that 1×10^{-6} to 2×10^{-6} M fascaplysin could reduce the secretion of VEGF in tumor cells BeL-7402 (Fig. 8). Thus, the levels of VEGF activity in the proximity between tumor cells and endothelial cells would be expected to inhibited by fascaplysin in vivo. The effect of fascaplysin may be even more significant in inhibition of endothelial cells proliferatin in vivo than in vitro due to a specific response of down-regulating VEGF secretion in vivo.

In summary, our present data point, in the first time, to a possible role of fascaplysin in preventing cancers from becoming malignant, presumably by selective inhibition of new blood vessel formation at the tumor site other than killing tumor cells directly with selective CDK4 inhibition activity.

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