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Isolation and characterization of Fuantai-03 from *Dasyatis akajei* and its inhibitory effects on migration and proliferation of human umbilical vein endothelial cells, and angiogenesis and tumor-induced angiogenesis

Rundi Ma[1], Lijian Yu[1]*, Weiming Su[1], Mingneng Liao[1], Laizhen Huang[1], Haiying Xu[1], Tingxi Yu[1,2]

[1]Key Laboratory of Marine Materia Medica, Guangdong Ocean University, Zhanjiang 524025, (PEOPLE'S REPUBLIC OF CHINA)

[2]Cell Biology Group, Department of Surgery, Department of Pathology, University of Maryland School of Medicine and Baltimore Veterans Affairs Medical Center, Baltimore, MD 21201, (USA)

E-mail: ywyj9578@sohu.com; yutingxi@yahoo.com

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ABSTRACT

A novel inhibitor of angiogenesis (Fuantai-03, FAT-03) was isolated and characterized from Dasyatis akajei, SDS-PAGE analysis and silver staining determined the purified FAT-03 as a single band at 43.0 kDa. Its purity was also confirmed by the finding of a unique NH_2-terminal amino acid sequence. To determine whether this protein was capable of inhibiting angiogenesis in vitro and in vivo, migration assay was performed using a Transwell model with polycarbonate membrane; the effect of FAT-03 on the growth of human umbilical vein endothelial cells (HUVECs) was measured by MTT; apoptotic induction of HUVECs by rFAT-03 was determined by fluorescence microscopy and flow cytometry; the effects of FAT-03 on angiogenesis in vivo were assayed in chick chorioallantoic membrane (CAM) and Lewis lung carcinoma (LLC). Immunohistochemistry assay was used to analyzed the effect of FAT-03 on intratumoral microvessel density (iMVD). The results showed that the eluted chromatographic fractions at the accorded molecular weght demonstrated an anti-angiogenic activity. This study is the first demonstration that both the cartilage and soft tissues of Dasyat akajei can produce a inhibitor of angiogenesis having an Mr of 43.0 kDa.

KEYWORDS

Fuantai-03;
Inhibitor of angiogenesis;
Human umbilical vein endothelial cells;
Chick chorioallantoic membrane;
Lewis lung carcinoma.

INTRODUCTION

Substantial effort has been made in the past more than thirty years to identify, purify, and synthesize angiogenic inhibitory molecules. Initially, antiangiogenics were identified in extracts from naturally avascular tissues such as cartilage and vitreous humour of the eye[1,2]. Relatively little research has been directed at exploiting vascular soft tissues as a source of potentially useful angiogenesis inhibitors.

Dasyatis akajei (Müller & Henle) (Family: Dasyatidae/Stingrays; Order: Rajiformes/skates and rays; Class: Elasmobranchii/sharks and rays; FishBase name: Red stingray) are found in coral reefs and estuarine areas, on sand and mud bottoms, and their foods are small fishes and crustaceans. Dasyatis akajei are mainly distributed over Western Pacific: from the East China Sea to the South China Sea, from southern Ja-

pan to Thailand[3].

Dasyatis akajei is a fish having food and medicinal values, and is employed, for example, in the treatment of gastric cancer, esophageal cancer, lung cancer, mastitis, pharyngitis, malaria, and toothache, in the folk remedy. The caudal spine stabbed with a variety of toxins, has an important role in pharmacology[4]. Our group has been engaging in the isolation and characterization of antitumor-active components from Dasyatis akajei and the investigation into the underlying mechanisms of the antitumor effects since 1998. Firstly, we found that the extracts from caudal spine of Dasyatis akajei showed anti-tumor effect[5]. Subsequently, we proven that Fuantai (FAT), the crude extracts precipitated by salting-ou, having molecular weights from 14.4 kDa to approximately 97.4 kDa, exhibited anti-tumor and anti-angiogenesis activities in mice[6,7]. Here we show the isolation and characterization of Fuantai-03 (FAT-03) from FAT with inhibitory effects on migration and proliferation of human umbilical vein endothelial cells, angiogenesis and tumor-induced angiogenes, and tumor growth and metastasis.

MATERIALS AND METHODS

Chemicals

HiPrep 26/10 Desalting, Sephadex G-25 Superfine, 17-5087-01£»XK50/30, Q Sepharose Fast Flow, 17-0510-01; XK26/20, Butyl Sepharose 4 Fast Flow, 17-0980-01£»and SOURCE 5RPC ST 4.6/150, 17-5116-01, 5 μm SOURCE were purchased from amersham pharmacia biotech (Sweden). Newborn calf serum (NCS) was purchased from Sijiqing Organism Engineering Materials Co. Ltd (Hangzhou, China). RPMI-1640 medium was purchased from Hyclone Co.. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co.. Trypsin, Triton-X-100, tris(hydroxymethy)aminomethane (Tris), Sodiun dodecyl sulfate (SDS), Coomassie brilliant blue R250, and molecular weight marker were purchased from Sigma Chemical Co. . All other chemicals used were of reagent grade.

Capture of Dasyatis akajei

Dasyatis akajei was captured in Zhanjiang coastal waters, and identified by Professor Cai Y.(Fisheries College of Guangdong Ocean University, China). A voucher specimen (No. 099) has been deposited in the Key Laboratory of Marine Materia Medica, Guangdong Ocean University, Zhanjiang 524025, China.

Homogenizing and salting-out

The cartilage or skin-free soft tissues of Dasyatis akajei were cut into small pieces and homogenized using an homogenizer. A saturated ammonium sulfate solution was slowly added to the homogenized mixture to bring up the salt concentration of the mixture. After removing the precipitate by filtration or centrifugation, the desired protein can be precipitated by altering the salt concentration to the level at which the desired protein becomes insoluble; unwanted proteins can be removed from a protein solution mixture by salting out. The precipitated protein is collected and categorized according to the concentration of the salt solution at which it is formed. This partial collection of the separated product is called fractionation. The fraction of the precipitated protein was collected at 60% of ammonium sulfate saturation in the experiment.

Chromatography

To isolate FAT-03 from FAT, media of the protein sample FAT precipitated by salting-out were exhaustively dialyzed against distilled water and equilibrated with 0.015 mol/L Tris-HCl (pH8.0) before chromatography. The equilibrated samples were diluted in 0.015 mol/L Tris-HCl (pH 8.0), containing 0.015% sodium azide. Samples of FAT were then applied to a XK 50/30 Ion Exchange Chromatography column filled with Q Sepharose Fast Flow medium and equilibrated with the appropriate buffers at a flow rate of 10 ml/min and eluted with A solution (0.015 mol/L Tris-HCl, pH 8.0) and B solution (0.015 mol/L Tris-HCl, pH 8.0, containing 0-100% of 1 mol/L NaCl) for salt gradient formation.10-ml fractions were collected. The 10-ml fractions enriched in angiogenesis inhibitory activity were collected for the next step.

The fractions enriched in angiogenesis inhibitory activity from ion exchange chromatography were pooled and concentrated, and then applied to a XK 26/20 Hydrophobic Interaction Chromatography column filled with Butyl Sepharose Fast Flow medium (Pharmacia, Uppsaia, Sweden) and equilibrated with the appropriate

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buffers at a flow rate of 5 ml/min and eluted with A solution (0.015mol/L Tris-HCl, pH 8.0) and B solution (0.015mol/L Tris-HCl, pH 8.0, containing 100-0% of 3 mol/L NaCl) for salt gradient formation.5-ml fractions were collected. The 5-ml fractions enriched in angiogenesis inhibitory activity were collected for the next step.

The Revered Phase Chromatography column was equilibrated with (10%ACE, 1%TFA) before chromatography. The fractions enriched in angiogenesis inhibitory activity from Hydrophobic Interaction Chromatography were pooled and concentrated, and then applied to a Revered Phase Chromatography and equilibrated with the appropriate buffers at a flow rate of 0.5ml/min and eluted with A solution (10%ACE, 0.1%TFA) and B solution (100%ACE, 0.1%TFA) for salt gradient formation. 1ml fractions were collected. The 0.5-ml fractions enriched in angiogenesis inhibitory activity were collected for analysis of purify by SDS-PAGE.

Protein assays

Protein concentration was determined by the Lowry method using bovine serum albumin as a standard[8].

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[9] 0.75-mm 15% gels were used to monitor the purification of FAT-03. Samples were reduced with mercaptoethanol prior to application unless otherwise specified. Silver staining of gels was carried out by a modification of the method of Wray et al.[10]. Gels were fixed for 30 min in 50% methanol, 10% acetic acid for 30 min followed by an overnight incubation in 5% methanol, 7% acetic acid. The next morning gels were placed in 10% glutaraldehyde for 30 min and then washed with four changes of water over a period of 2 h. Gels were then silver-stained for 15 min and developed as described by Wray et al[10].

NH_2-terminal sequence analysis

NH_2-terminal sequence determination was carried out in the Centre of Protein Structure Analysisr, Mayo Clinic (Rochester, USA).

Cell culture

The human nasopharyngeal carcinoma CNE-2Z cells (CNE-2Z) cell line from a Cantonese patient established by Gu et al.[11] was obtained from Guangdong Medical College. human umbilical vein endothelial cells (HUVECs), LLC cells, mouse B16 meloanoma (B16) cells, and all the other human malignant tumor cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 or DMEM media supplemented with 10% (v/v) heat-inactivated NCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2g/L NaHCO3 at 37ºC in humidified air containing 5% CO_2 in monolayer. Cells in log phase growth were used in the experiments.

Migration assay

A migration assay was performed using a 24-well transwell insert with polycarbonate membrane (Corning Incorporated). The lower chamber was filled with 600 μl of RPMI 1640 containing 50 ng/ml VEGF. The upper chamber was seeded with 2.0×10^4 HUVECs (300 μl cell suspension) treated by FAT-03 (160 μg/ml) for 24 h. Cells were allowed to migrate for 4 h at at 37 0C. The cells on the upper surface of the membrane were removed with a cell scraper; the migrated cells on the lower surface were fixed in 3% formaldehyde, stained in PBS containg 50μg/ml propidium iodide (PI), and then counted. The rate of migratory inhibition of treated ECs was calculated as percent of control values. The experiment was repeated 3 times under identical conditions.

MTT assay

The mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble blue formazan was used for enumerating cells to assess the anti-proliferative effects of FAT-03. Briefly, Single-cell suspensions were prepared and seeded into 96 well microculture plates with 1.0×10^4 cells/ml (90 μl/well). Cells were cultured for 12 h before addition of FAT-03. FAT-03 was diluted into RPMI-1640 medium and added to each well in a volume of 10 μl. Cells were incubated at 37ºC for the time indicated. MTT solution (5 mg/ml) was aliquoted to each well in a volume of 20 μl, and 5 h later 100 μl of the solubilization solution [10% SDS–5% isobutyl alcohol–0.012 M HCl (w/v/v)] was added into each well. The plates were allowed to stand

overnight in the incubator in a humidified atmosphere. Absorbance at 570 nm (A570) was determined for each well using an ELISA reader. Control wells contained all of the agents presented in the treated wells except FAT-03. Each experimental point was performed in three replicates. The 50% inhibitory concentration (IC_{50}) was determined from dose-response data from at least three experiments.

Morphological analysis by fluorescence microscopy

FAT-03-induced apoptosis was analyzed by acridine orange/ethidium bromide (AO/EB, Sino-American Biotechnology Co.) or Hoechst-33342/propidium iodide (Hoechst-33342-PI, Sigma) double fluorescent staining. 1×10^5 cells/well were seeded in a 24-well plate and treated with 100μl of 160 μg/ml FAT-03l for 72 h at 37ºC. For the AO/EB procedure, cells were washed with phosphate buffered saline (PBS), rinsed in 92 μl PBS, and then 8 μl of AO/EB solution (one part of 100 μg/ml AO in PBS, one part of 100 μg/ml EB in PBS) was added. For the Hoechst-33342-PI procedure, cells were collected by centrifugation, and 0.5 ml of fresh growth medium was added to each tube and mixed. Then Hoechst-3334 dye was added to the solution to a final concentration of 50 μg/ml. Cells were waterbathed at 30 0C for 15 min. Medium was aspirated, followed by resuspension in growth medium containig 10 μg/ml PI dye and icebath for 15 min. The cells were then spun and resuspended in 100 μl of PBS. The cells stained by AO/EB or Hoechst-33342-PI were analyzed in a fluorescence microscope (DMIRB, Leica) using a fluorescence filter.

Annexin V-fluorescein isothiocyanate (Annexin V-FITC) staining by flow cytometry analysis

HUVECs were seeded at a density of 5×105 cells of medium in 6-well culture plates. FAT-03-treated and control cells were harvested after the indicated time. Harvested cells were washed twice with PBS, counted, and suspended in binding buffer at a density 1×106 cells/ml (Annexin V-FITC staining Kit,BD Biosciences Pharmingen). Hundred microliters of this cell suspension were stained with 5 μl of annexin V conjugated to fluorescein isothiocyanate (FITC) and 10 μl of propidium iodide (PI, 20 μg/ml) for 15 min in the dark followed by the addition of 400 μl of PBS. Cells were immediately analyzed by flow cytometry (Epics XL, Coulter Co.). Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for annexin and PI, respectively.

Angiogenesis in CAM assay

The CAM assay was performed by a modification of the method described as Taraboletti et al.12 and Yu et al..13 Briefly, fertilised White Wenchang (Wenchang County, Hainan province, China) chicken eggs (8 per group) were incubated at 37^0C at constant humidity. On day 6 of incubation, a round window 1 cm in diameter was opened in the centre of egg shell of air chamber, and then a sterilised filter disc was placed on the top of the CAMs of individual embryos. Then, the filters were loaded with: (a) 80μl of FAT-03 solution (20, 40, 80 μg/embryo), (b) 80μl of PBS as control, respectively; once daily, for three days. The window was sealed with a cellophane and the eggs were returned to the incubator. On day 11, CAMs were fixed with acetone and alcohol and examined under a stereoscope for the formation of avascular zones.

CNE-2Z cells-induced angiogenesis in CAM assay

The CAM assay was performed as above mentioned. Briefly, fertilised White Wenchang chicken eggs (8 per group) were incubated at 37 oC at constant humidity. On day 7 of incubation, a round window 1 cm in diameter was opened in the centre of egg shell of air chamber, and then CNE-2Z cells (1.9×10^6 cells /embryo) were seeded on the CAMs of individual embryos. The window was sealed with a cellophane and the eggs were returned to the incubator. On day 11, the sterilised filter discs with a window in the centre (for the clumps of CNE-2Z cells) were placed on the top of the CAMs of individual embryos surrounding the clumps of CNE-2Z cells. Then, the filters were loaded with: (a) FAT-03 (40, 80,160 μg/embryo), (b) 80 μl of PBS as control, respectively, once daily, for five days. On day 17, CAMs were fixed with acetone and alcohol and examined under a stereoscope for the formation of vascular and avascular zones.

Assay for angiogenesis, growth and metastasis of tumors

Subconfluent B16 and LLC cells were harvested with EDTA trypsin in PBS. The scruff of the mice was inoculated subcutaneous (s.c.) with a suspension of

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4×10^6 LLC cells, and the tail vein of the mice was inoculated with a suspension of 2×10^5 B16 cells. In the experiment of LLC, FAT-03 was administered ip daily at the doses of 10, 20, 30 mg/kg/day for 14 days from 7th day after inoculation to one day before the date of sacrifice. In the experiment of B16, FAT-03 was administered ip daily at the doses of 10, 20, 30 mg/kg/day for 14 days from the first day after inoculation to one day before the date of sacrifice. The mice were sacrificed 15 (for B16) and 22 (for LLC) days after tumor inoculation. The primary LLC and the livers (for LLC) and lungs (for B16) were excised and weighted, and the number of the metastatic foci in the liver and lung was scored[14].

The primary LLC specimens (n=4) from unselected mice in FAT-03-treated and control groups were excised, fixed in 10% formalin and routinely embeded in paraffin. Paraffin sections (μm) were baked, deparaffinized and rehydrated. Sections were probed with rabbit anti-CD31 antibody (Dako, Carpinteria, CA) overnight at 4 °C, followed by treatment with a biotinylated secondary antibody (Dako)[15-17]. Microvessels were counted in high-power fields (×40) in five randomly selected fields of tumor specimen from four mice of each group[16].

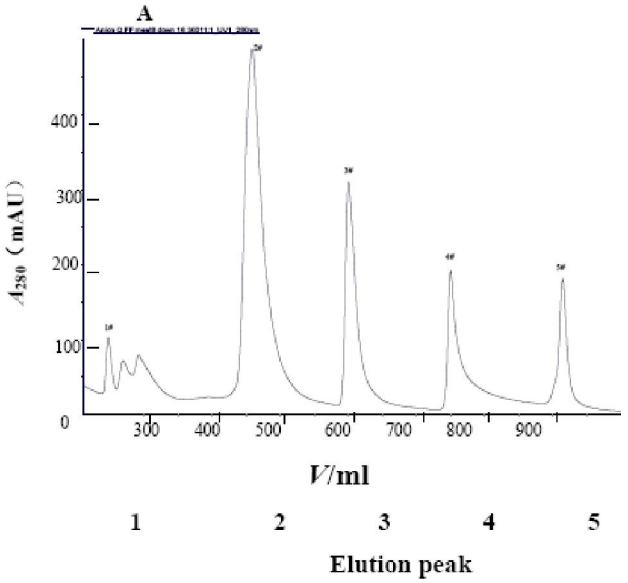

Figure 1A : Ion exchange chromatography elution profile

Media of the protein sample (Fuantai, FAT) precipitated by salting-out were exhaustively dialyzed against distilled water and equilibrated with 0.015 mol/L Tris-HCl (pH 8.0) before chromatography. The equilibrated samples were diluted in 0.015 mol/L Tris-HCl (pH 8.0), containing 0.015% sodium azide. Samples of FAT were then applied to a XK 50/30 Ion Exchange Chromatography column filled with Q Sepharose Fast Flow medium and equilibrated with the appropriate buffers at a flow rate of 10 ml/min and eluted with A solution (0.015 mol/L Tris-HCl, pH 8.0) and B solution (0.015 mol/L Tris-HCl, pH 8.0, containing 0-100% of 1 mol/L NaCl) for salt gradient formation.10-ml fractions were collected.

Statistical analysis of data

All values obtained were expressed as means±SEM or means±SD. Statistical processing was performed using the Student's t test with SPSS v. 10.0. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Purification and characterization of FAT-03

The cartilage or skin-free soft tissues of Dasyatis akajei were cut into small pieces and homogenized. The fraction of the precipitated protein collected at 60% of ammonium sulfate saturation had molecular weights from 14.4 kDa to approximately 97.4 kDa (Figure 1B), for which a term Fuantai (FAT) was coined and brought into the following usage.

Ion exchange chromatography elution patterns showed five peaks (Figure 1A). When the eluate frac-

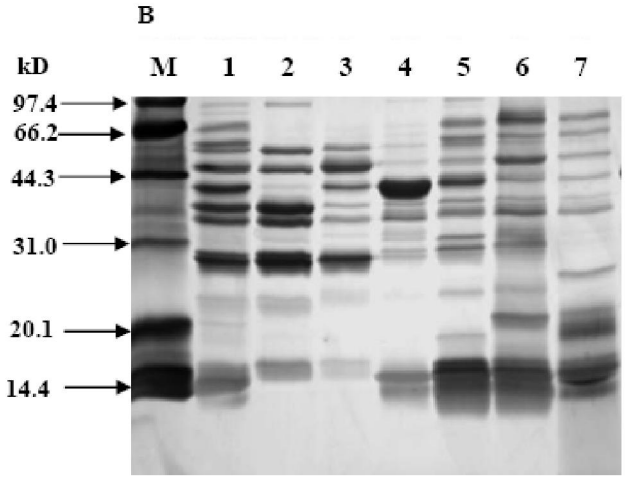

Figure 1B : SDS-PAGE analysis of ion exchange chromatography eluate

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[7]. 0.75-mm 15% gels were used to monitor the purification of FAT-03. Samples were reduced with mercaptoethanol prior to application. Silver staining of gels was carried out by a modification of the method of Wray et al.[8].
M: Protein marker; 1: FAT (Protein sample precipitated by salting-ou); 2: Penetration peak; 3: Eluation peak 1; 4: Eluation peak 2; 5: Eluation peak 3; 6: Eluation peak 4; 7: Eluation peak 5

tions were placed in Rochelle salt solution containing copper sulfate at alkaline pH, a purplish-blue colored complex formed, namely, the eluate fractions gave the biuret reaction. SDS-PAGE analysis revealed fewer bands on the 2nd peak (Figure 1B, lane 4),and the 2nd peak contained the highest protein content and showed anti-angiogenic activity.

Hydrophobic interaction chromatography elution patterns showed two peaks (Figure 1C). SDS-PAGE analysis revealed a main band and a few delicate shallow bands on the 2nd peak (Figure 1D, lane 2),and the 2nd peak contained more protein and showed anti-angiogenic activity.

Revered phase chromatography elution patterns exhibited a single peak (Figure 1E). SDS-PAGE analysis and silver staining revealed a single band at 43.0 kDa (Figure 1F), named as Fuantai-03 (FAT-03), which possessed potent anti-angiogenic activity.

Analysis of densitometry of the bands suggested that the purification procedure yielded the purity of of FAT-03 was >98.0% (Figure 1EF). Its purity was also confirmed by the finding of a unique NH_2-terminal amino acid sequence that read as follows: PFGNTHNKWKLNYSAEQEFP. The close correlation between the molecular weight determined by SDS-PAGE and anti-angiogenic activity measured in CAM is strong presumptive evidence that 43.0-kDa protein band is a Dasyatis akajei-derived protein having inhibitory effect on angiogenesis.

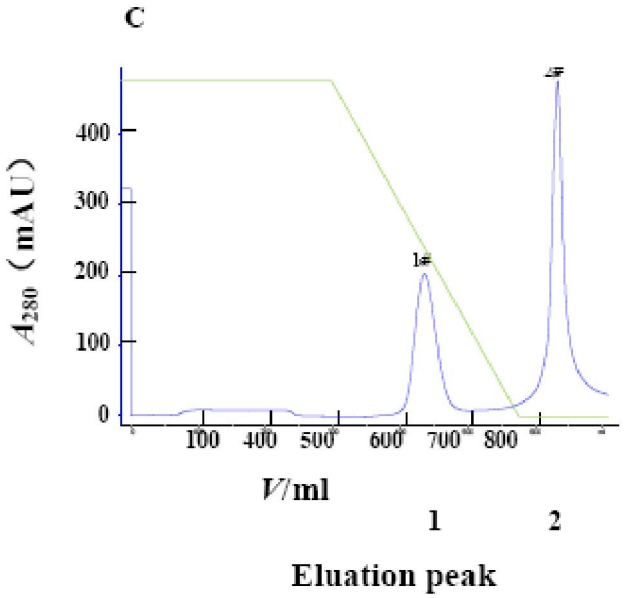

Figure 1C : Hydrophobic interaction chromatography elution profile

The fractions enriched in angiogenesis inhibitory activity from ion exchange chromatography were pooled and concentrated, and then applied to a XK 26/20 Hydrophobic Interaction Chromatography column filled with Butyl Sepharose Fast Flow medium (Pharmacia, Uppsaia, Sweden) and equilibrated with the appropriate buffers at a flow rate of 5 ml/min and eluted with A solution (0.015mol/L Tris-HCl, pH 8.0) and B solution (0.015mol/L Tris-HCl, pH 8.0, containing 100-0% of 3 mol/L NaCl) for salt gradient formation. 5-ml fractions were collected.

Inhibitin of migration of HUVECs by FAT-03

After cells were treated with 160 μg/ml FAT-03 for 24 h, a 57.9% inhibition of HUVEC migration was observed as compared to the control ($P<0.01$) (Figure 2). The results showed that FAT-03 was capable of inhibiting the migration of HUVECs in vitro.

Effect of FAT-03 on the growth of HUVECs and other human malignant tumor cell lines

Inhibitory effect of FAT-03 on growth of HUVECs was dose- and time-dependent (Figure 3), but FAT-03 did not show significant effects on several human malignant tumor cell lines, such as CNE-2Z cells, HeLa cells, HL-60 cells, K562 human erthroleukemia cells, human 0varian cancer HO-8910PM cells, and human highly metastatic giant lung carcinoma cell line PGCL3 (data not shown).

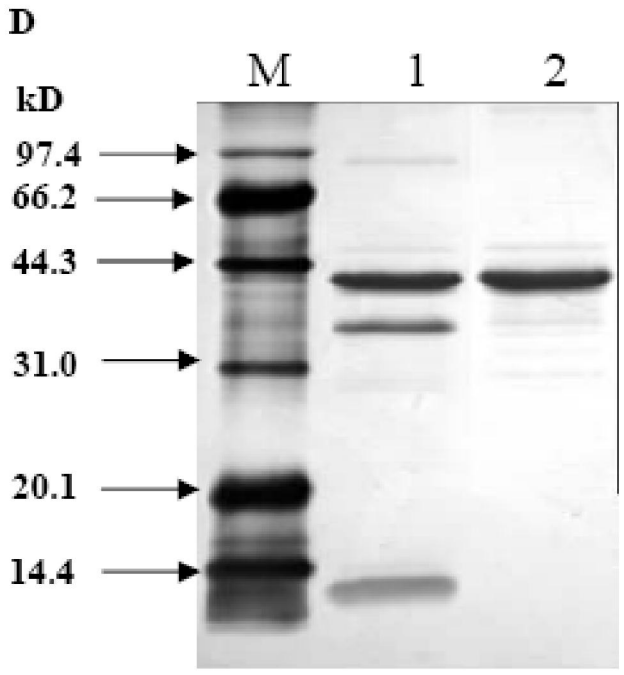

Figure 1D : SDS-PAGE analysis of hydrophobic interaction chromatography eluate

M: Protein marker; 1: Eluation peak 1; 2: Eluation peak 2

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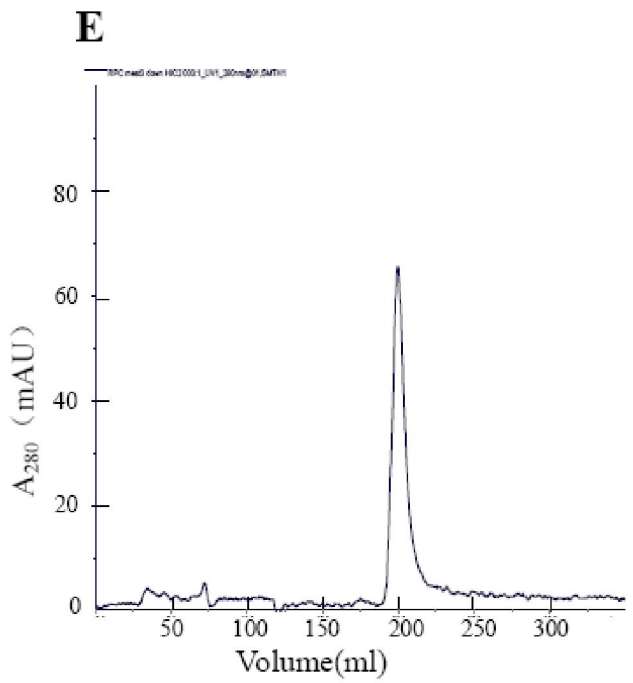

Figure 1E : Revered phase chromatography elution profile

The Revered Phase Chromatography column was equilibrated with (10%ACE, 0.1%TFA) before chromatography. The fractions enriched in angiogenesis inhibitory activity from Hydrophobic Interaction Chromatography were pooled and concentrated, and then applied to a Revered Phase Chromatography and equilibrated with the appropriate buffers at a flow rate of 0.5 ml/min and eluted with A solution (10%ACE, 0.1%TFA) and B solution (100%ACE, 0.1%TFA) for salt gradient formation. 1ml fractions were collected.

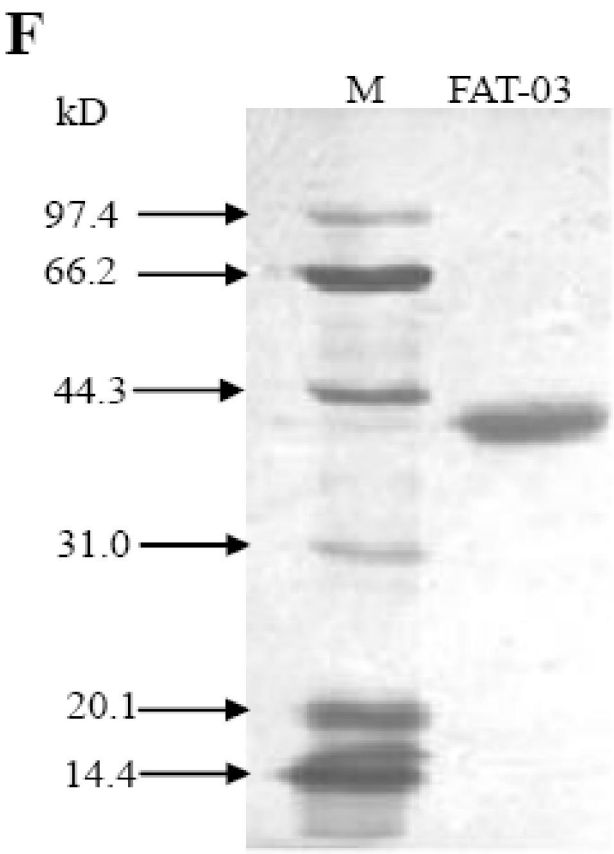

Figure 1F : SDS-PAGE analysis of purified FAT-03

The fractions enriched in angiogenesis inhibitory activity purified by Revered Phase Chromatography were collected for SDS-PAGE analysis.

Figure 1 : Purification and identification of FAT-03

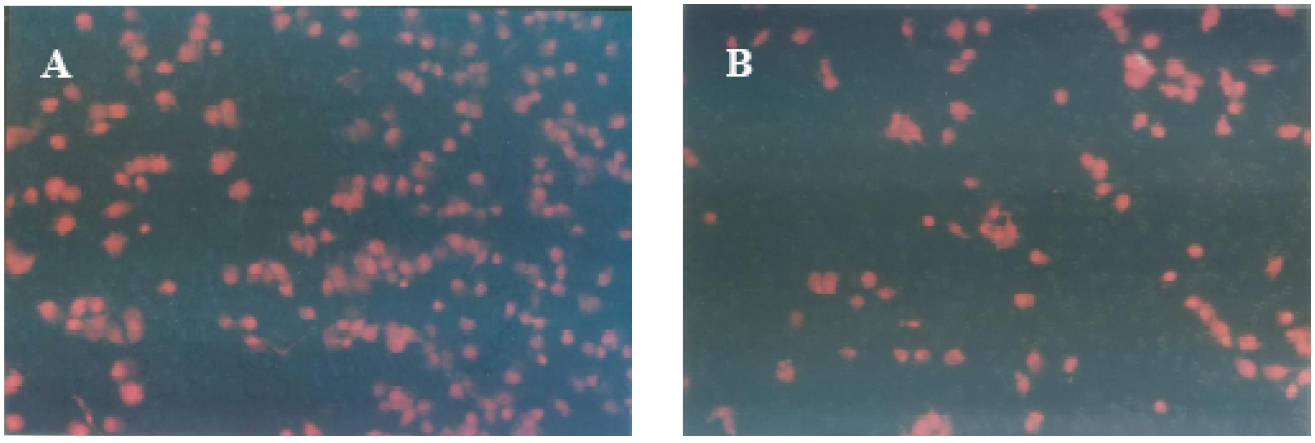

Figure 2 : Inhibitory effect of FAT-03 on migration of HUVECs

A migration assay was performed using 24-well transwell insert. The lower chamber was filled with 600 μl of RPMI 1640 containing 50 ng/ml VEGF. The upper chamber was seeded with 2.0×10^4 HUVECs (300 μl cell suspension) treated by FAT-03 (160 μg/ml) for 24 h. Cells were allowed to migrate for 4 h at at 37 ^{0}C, and then fixed and stained in PBS containg 50μg/ml PI. The number of the migrated cells on the lower surface was calculated as percent of control values. The experiment was repeated 3 times under identical conditions.

A: Control; B: FAT-03 (160 μg/ml, 24 h)

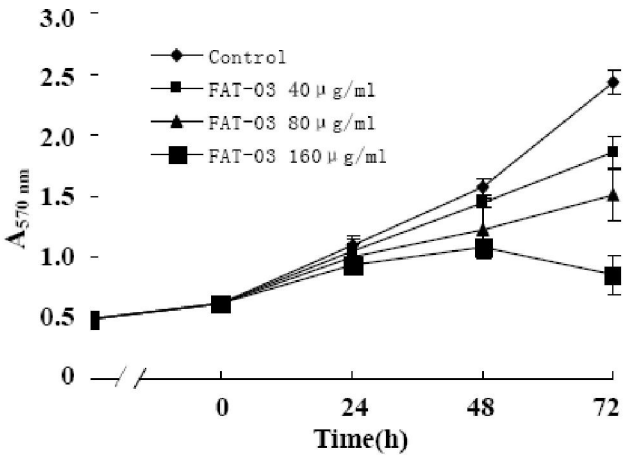

Figure 3 : Dose- and time-response of FAT-03 on growth of HUVECs cells.

Cells were treated with various concentration of FAT-03 for different time intervals. The cell proliferation was determined by MTT assay. The values are expressed as mean ± SEM of three independent experiments.

Apoptosis detected by fuorescence microscopy

Obvious differences were observed in the nuclei of FAT-03-treated and untreated HUVECs after staining with Hoechst/PI or AO/EB dyes. Hoechst/PI or AO/EB dyes stained morphologically normal nuclei bluish (Figure 4A) or green (Figure 4C), whereas 160 μg/ml FAT-03-treated cells demonstrated reddish (Figure 4B) or yellow and yellow-red (Figure 4D), smaller and shrunken nuclei (Figure 4BD). These changes in nuclear morphology, which were observed after 72 h of 160 μg/ml FAT-03 treatment, reflected chromatin condensation and nuclear shrinkage.

Loss of plasma membrane asymmetry during apoptosis

Changes in plasma membrane phospholipids, such as externalization of phosphatidylserine residue in the outer plasma membrane, are characteristic marker of early apoptotic events. Phosphatidylserine externalization can be conveniently detected by fluoresceinated annexin V binding. Counterstaining with PI, which detects cells with compromised cell membrane integrity, allows one to distingguish among necrotic, early-apopotic, and late-apopotic cells. Representative flow cytometric histograms (Figure 5) illustrated obvious shifts in annexin V and PI signals in HUVECs following FAT-03 treatment for 24 h. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis[18].

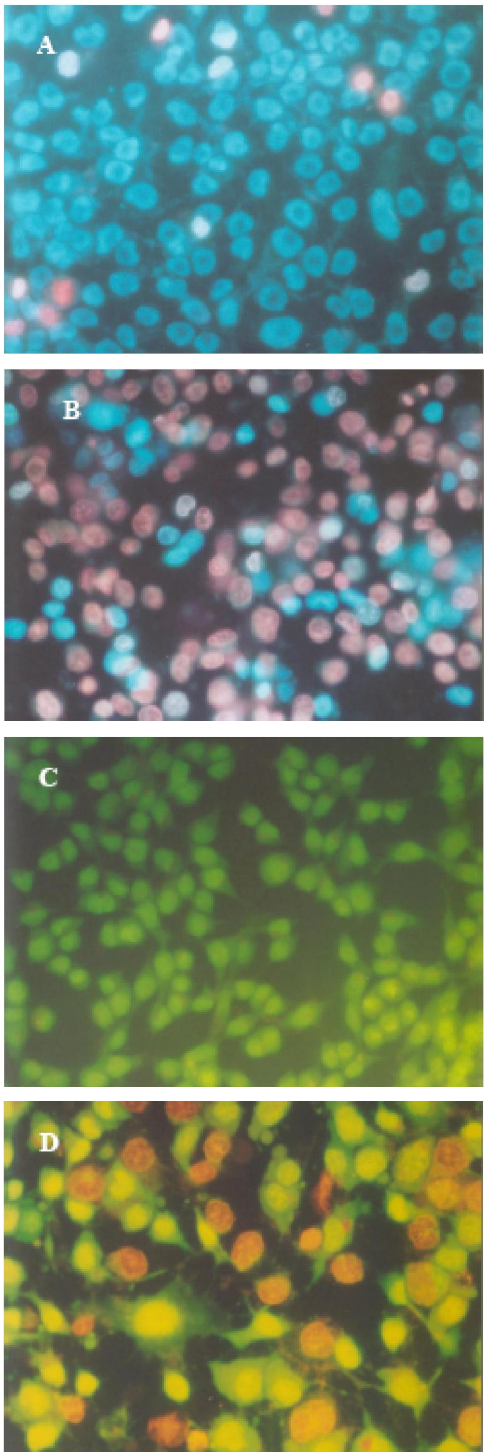

Figure 4 : FAT-03-induced apoptosis in HUVECs

Cells were treated with 160 μg/ml FAT-03 for 72 h and stained with Hoechst33342-PI or AO/EB as described in "Materials and methods". Cells in which nuclei were reddish (Hoechst33342-PI staining) or yellow and yellow-red (AO/EB staining) indicate apoptotic cells (original magnification, ×200).
A: Control ; B: 160 μg/ml FAT-03, 72 h; C: Control : D: 160 μg/ml FAT-03, 72 h.

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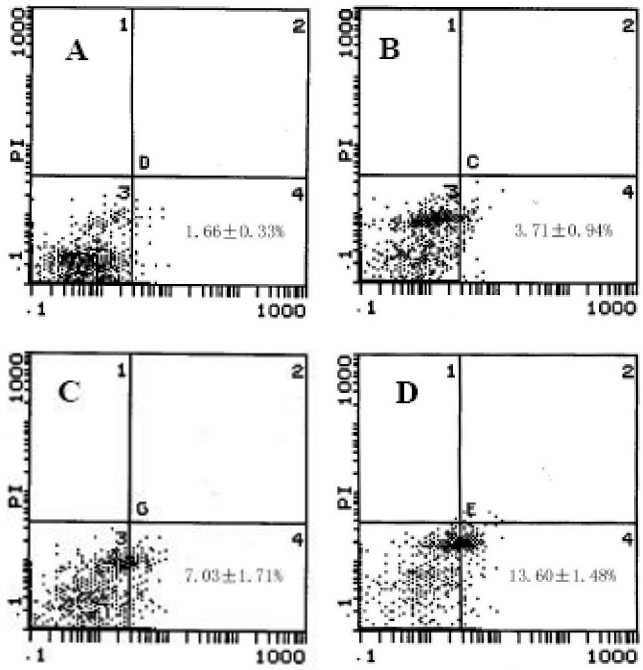

Figure 5 : Flow cytometric analysis of phosphatidylserine externalization (annexin stainign) with PI counterstaining.

Representative examples of flow cytometric histograms after of FAT-03 treatment. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis. A: Control; B:FAT-03 (40 μg/ml); C: FAT-03 (80 μg/ml£©;D: FAT-03 (160 μg/ml)

Inhibition of angiogenesis in CAM assay

Figure 6 ABCD shows the significant inhibition of embryonic neovascularization as evidenced by large avascular zone caused by FAT-03 placed in filter discs. Treatment with FAT-03 (20, 40, 80 μg/embryo, once-daily, for 3 days) resulted in notable suppression of microvessel formation, with inhibition rates of 23.6%, 33.1%, and 50.8% ($P<0.05$), respectively (TABLE 1). This effect was observed in about 90% of the eggs tested. This observation was reproduced in four separate sets of CAM assay. These results showed that FAT-03 was capable of inhibiting angiogenesis in CAM in a dose-dependent manner.

TABLE 1 : Inhibitory effect of FAT-03 on the angiogenesis in CAM (n = 5)

Group	Dosage (μg/embryo×d)	Number of vessel ramification ($\bar{x}$±SEM)	Inhibitory rate (%)
Control	--	165.6±7.6	--
FAT-03	20×3	126.5±5.7	23.6
FAT-03	40×3	110.8±7.6	33.1
FAT-03	80×3	81.5±1.6	50.8*

***P<0.05, Student's t-test versus control**

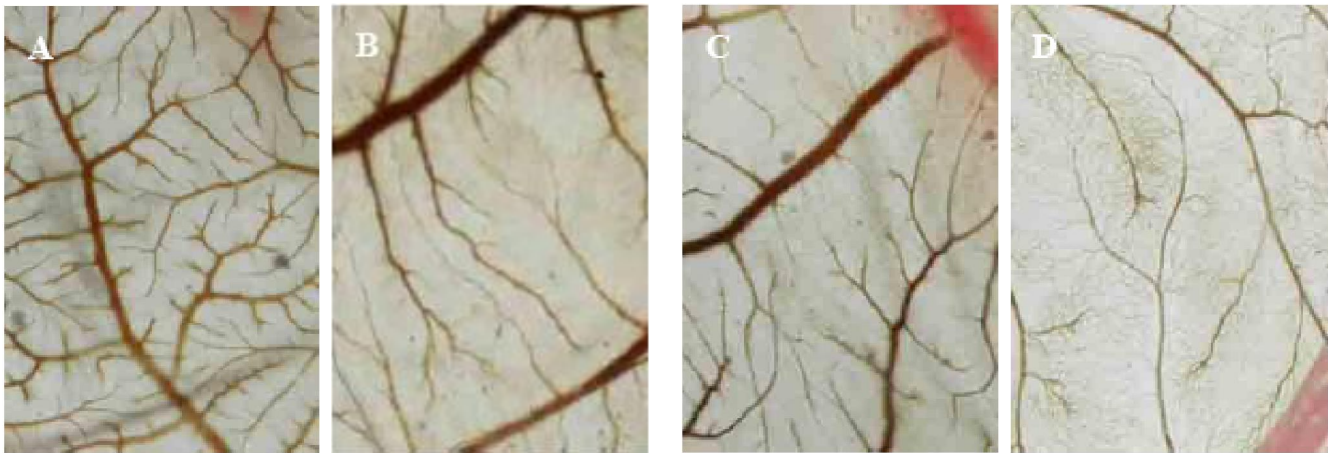

Figure 6 : Inhibitory effect of FAT-03 on angiogenesis in CAM (20×)

Fertilised White Wenchang chicken eggs (5 per group) were incubated at 37^{0}C at constant humidity. On day 6 of incubation, a round window 1 cm in diameter was opened in the centre of egg shell of air chamber, and then a sterilised filter disc was placed on the top of the CAMs of individual embryos. Then, the filters were loaded with: (a) 80μl of FAT-03 (20, 40, 80 μg/embryo), (b) 80μl of PBS as control, respectively; once daily, for three days. The window was sealed with a cellophane and the eggs were returned to the incubator. On day 9, CAMs were fixed with acetone and alcohol, and examined under a stereoscope for the formation of avascular zones.

A: Control; B: FAT-03 (20 μg/embryo/d×3); C: FAT-03 (40 μg/embryo/d×3); D: FAT-03 (80 μg/embryo/d×3)

Inhibition of CNE-2Z cell-induced angiogenesis in CAM assay

Figure 7 AB shows the significant promotion of embryonic neovascularization as evidenced by large vascular zone caused by CNE-2Z cells, and Figure 7 BC shows the significant inhibition of CNE-2Z cell-induced

embryonic neovascularization as evidenced by marked avascular zone surrounding the clump of CNE-2Z cells, caused by FAT-03 (80.0 μg/embryo, once-daily, for 5 days) placed in filter discs. Treatment with FAT-03 (40, 80, 160 μg/embryo, for 5 days) resulted in notable suppression of tumor-induced microvessel formation, with inhibition rates of 34.3%, 43.3%, and 46.2%, rerspectively. This effect was observed in about 90% of the eggs tested. This observation was reproduced in four separate sets of CAM assay. These results showed that FAT-03 was capable of inhibiting tumor-induced angiogenesis in CAM in a dose-dependent manner.

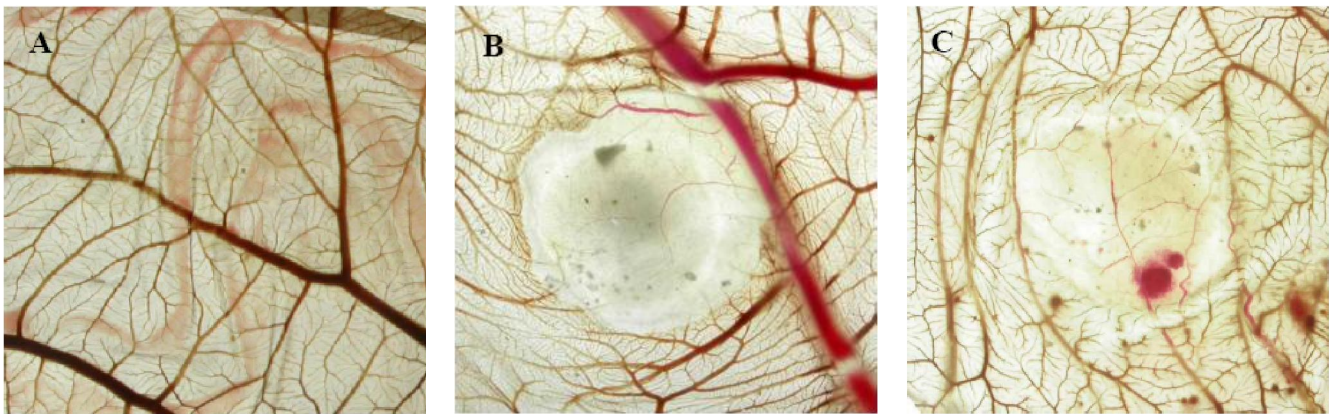

Figure 7 : Inhibitory effect of FAT-03 on angiogenesis induced by CNE-2Z cells in CAM (20×)

Fertilised chicken eggs (n=8) were incubated at 37^{0}C at constant humidity. On day 7 of incubation, a round window 1 cm in diameter was opened in the centre of egg shell of air chamber, and then CNE-2Z cells (1.9×10^6 cells /embryo) were seeded on the CAMs of individual embryos. The window was sealed with a cellophane and the eggs were returned to the incubator. On day 11, the sterilised filter discs with a window in the centre (for the clumps of CNE-2Z cells) were placed on the top of the CAMs of individual embryos surrounding the clumps of CNE-2Z cells. Then, the filters were loaded with (a) 80μl of FAT-03 (40, 80,160 μg/embryo), (b) 80μl of PBS as control, respectively; once daily, for five days. On day 17, CAMs were fixed and then examined under a stereoscope for the formation of vascular and avascular zones.
A: Control; B: CNE-2Z; C: CNE-2Z+FAT-03 (80 μg/embryo/d×5)

Inhibition of angiogenesis, growth and metastasis of LLC and B16 melanoma

LLC is a spontaneous metastasis model of LLC, and B16 is an experimental metastasis model. FAT-03 (10, 20, 30mg/kg/day) was administered ip daily for 14 days. The FAT-03-treated primary LLC were smaller than control tumors, and the growth inhibition rates were 21.1%, 47.7% (P<0.05), 63.3% (P<0.01), (TABLE 2). The liver metastasis inhibition rates (MIRs) of LLC by FAT-03 were 25.5%, 57.6%(P<0.05), 81.7(P<0.01), respectively (TABLE 3, Figure 8AB). The lung MIRs of B16 by FAT-03 (10, 20, 30mg/kg/day) were 42.0%, 64.2%(P<0.05), 89.1% (P<0.01), respectively (TABLE 4, Figure 8CD). These results showed that FAT-03 significantly inhibited the growth and metastasis of LLC, and the metastasis of B16.

TABLE 2 : Inhibitory effect of FAT-03 on the growth of primary tumor in LLC-inocutaled mice

Groups	Average body weight ($\bar{x}$ ±SD)		Tumor weight ($\bar{x}$ ±SD, g)	Inhibition (%)
	before treatment	after treatment		
Control	21.5±1.2	20.1±1.2	1.28±0.18	—
FAT-03 (10.0mg/kg/d×14)	21.6±.09	20.8±0.8	1.01±0.12	21.1
FAT-03 (20.0mg/kg/d×14)	21.3±0.8	21.9±1.2	0.67±0.11*	47.7
FAT-03 (30.0mg/kg/d×14)	21.2±1.2	21.6±1.1	0.47±0.15**	63.3

***P<0.05,**P<0.01, Student's t-test versus control**

TABLE 3 : Inhibitory effect of FAT-03 on the spontaneous metastasis of LLC

Groups	Metastasis rate	Number of metastatic foci in livers ($\bar{x}$ ±SD)	MIR (%)
Control	6/6	98.2±43.1	
FAT-03 (10.0mg/kg/d×14)	6/6	73.2±35.3	25.5
FAT-03 (20.0mg/kg/d×14)	4/6	41.6±28.6*	57.6
FAT-03 (30.0mg/kg/d×14)	3/6	18.0±16.9**	81.7

***P<0.05,**P<0.01, Student's t-test versus control**

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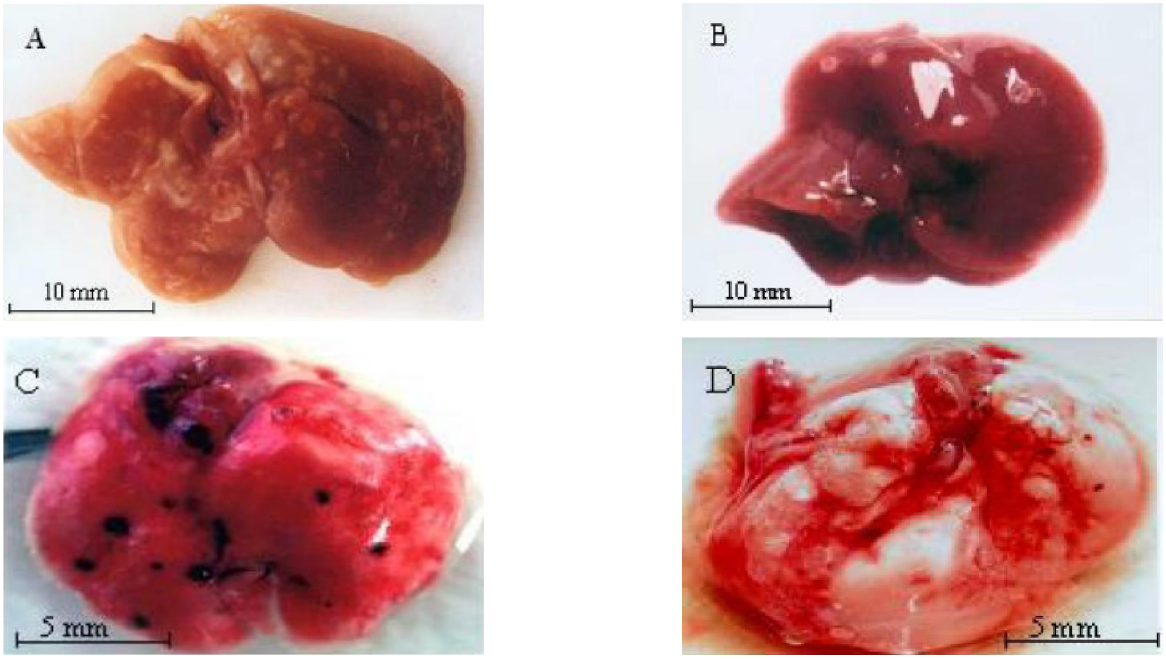

Figure 8 : Inhibitory effects of FAT-03 on the liver metastasis of LLC and the lung metastasis of B16 cells

Subconfluent LLC or B16 cells were harvested with EDTA trypsin in PBS. The scruff of the mice was inoculated s.c. with a suspension of 4×10^6 LLC cells; the tail vein of the mice was inoculated with a suspension of 3×10^6 B16 cells. For LLC, FAT-03 was administered ip daily at the doses of 10.0, 20.0, 30.0 mg/kg/day for 14 days from 7th day after inoculation to one day before the date of sacrifice. For B16, FAT-03 was administered ip daily at the doses of 10.0, 20.0,30.0 mg/kg/day for 14 days from the first day after inoculation to one day before the date of sacrifice. The mice were sacrificed 22 (for LLC) or 15 (for B16) days after tumor inoculation. The livers (for LLC) or lungs (for B16) were excised, and the number of the metastatic foci in the livers or lungs scored.
Liver: A. Control, B. FAT-03 (30.0 mg/kg/d×14, ip); Lung: C. Control, D. FAT-03 (30.0 mg/kg/d×14, ip).

TABLE 4 : Inhibitory effect of FAT-03 on the experimental metastasis of B16 cells

Groups	Metastasis rate	Number of metastatic foci in lungs ($\bar{x}$ ±SD)	MIR (%)
Control	6/6	151.5±41.2	
FAT-03 (10.0mg/kg/d×14)	5/6	87.8±28.0	42.0
FAT-03 (20.0mg/kg/d×14)	4/6	54.3±22.2*	64.2
FAT-03 (30.0mg/kg/d×14)	4/6	16.5±7.5**	89.1

***P<0.05,**P<0.01, Student's t-test versus control**

Immunohistochemical analysis with anti-CD31 antibody were performed to investigate the effect of FAT-03 on tumor angiogenesis. Gross and microscopic examinations showed that primary LLC treated with FAT-03 (10 mg/kg/day, once daily, ip, for 14 days) demonstrated extensive necrosis. The treated tumors had fewer vessels and their iMVD was 9.8±1.2 (vs 20.2±2.2 of control), and the observed vessels were malformed (Figure 9B) compared to those of control group (Figure 9A). Treatment with FAT-03 resulted in notable suppression of tumor-induced microvessel formation with inhibition rate of 51.5% ($P<0.05$). These results indicated that FAT-03 significantly inhibited angiogenesis in LLC.

DISCUSSION

The targeting of tumor-induced angiogenesis as a means of blocking tumor progression has generated a growing interest in recent years. This interest stems from several facts that tumor cells cannot grow significantly in the absence of blood vessels; until tumor angiogenesis occurs, tumors grow no larger than 2–4 mm in diameter; also, tumor angiogenesis is necessary at the beginning and at the end of the metastatic cascade of events[19-22]; and molecules interfering with angiogenesis have potent antitumor properties in animal models[23]. Moreover, endothelial cells (ECs) are genetically stable, inhibitors specific to these cells should not induce resistance in tumors in contrast to cytotoxic compounds (antimitotic, antimetabolites and akylating agents) for which resistance is commonly observerd[24].

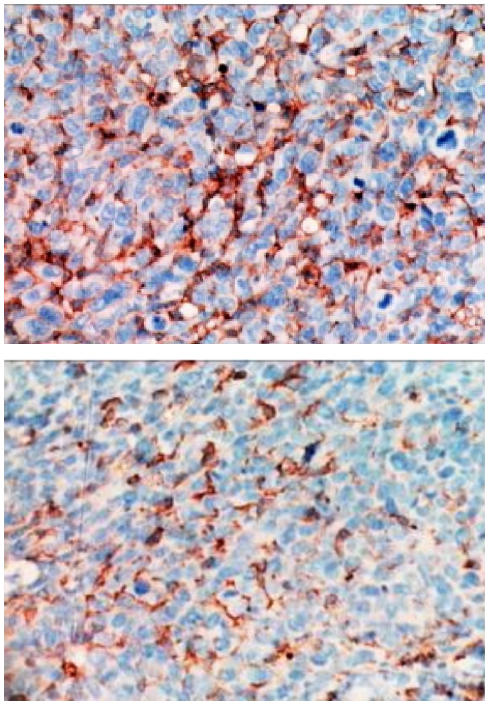

Figure 9 : Inhibitory effect of FAT-03 on angiogenesis in LLC

The primary LLC specimens (n=4) from unselected mice in FAT-03 (10.0 mg/kg/d×14)-treated and control groups were excised, fixed in 10% formalin and routinely embeded in paraffin. Paraffin sections (μm) were baked, deparaffinized and rehydrated. Sections were probed with rabbit anti-CD31 antibody overnight at 4°C, followed by treatment with a biotinylated secondary antibody.
A: Control; B: FAT-03 (10.0 mg/kg/d×14, ip)

Vigorously pursued as a novel anticancer strategy[25-28], the idea of antiangiogenesis is now widely considered to be a promissing approach to the treatment of a range of pathologies of which uncontroled vascular proliferation is a component. One of recent advances in the field of antiangiogenesis and vascular targeting is in vitro and in vivo selection of peptides that bind to endothelium in an organ-specific and tumor-selective fashion[29,30].

The ocean is teeming with unique organisms. More than half the organisms in the ocean don't even occur on land. The diversity and specificity of the marine species and their biological substances contained cause the marine organism to become huge treasure trove of drugs, and finding new drugs in ocean has great development potential[31]. At the present time, more than 20 angiogenesis inhibitors of marine origin have been identified, such as neovastat (AE-941)[32], aplidine (APLD)[33], fucoidan)[34], salinosporamide A[35], bastadin 6[36], SargA[37], puupehenone[38], psammaplin A (PsA)[39], philinopside A[40] and E[41], oligomannurarate sulfate (JG3)[42], grateloupia longifolia polysaccharide (GLP)[43], etc, and Luo et al.[44] reported that Dasyatis akajei cartilage guanidine hydrochloride extract (DCGE) of molecular weights from 3 kDa to approximately 300 kDa was obtained from the Dasyatis akajei cartilage, and Dasyatis akajei cartilage angiogenesis inhibitor from 20%-30% acetone precipitation of DCGE was found to have the strongest angiogenesis inhibitory effect.

FAT-03 is an antiangiogenic protein isolated from the fish Dasyatis akajei. This study is the first demostration that Dasyatis akajei can produce a inhibitor of angiogenesis, tumor growth and metastasis having an Mr of 43.0 kDa. Moreover, the experimental facts demonstrated that angiogenesis inhibitor could be also isolated and identified from vascular soft tissues of Dasyatis akajei. Anyhow, at the present time, FAT-03 as a natural product anti-tumor agent adds to the growing list of agents with the mechanism of antiangiogenesis, not to mention the impression that we obtain from this study is that FAT-03 is a potent angiogenesis inhibitor, any new natural product that has the mechanism of action has no doubt inherent interest.

Finally, our data have confirmed that FAT-03 obviously inhibited the migration and proliferation of human umbilical vein endothelial cells (HUVECs) in a dose- and time-dependent manner, and FAT-03-treated HUVECs showed typical morphologic and cellular evidences of apoptosis, but FAT-03 did not show significant effects on several human malignant tumor cell lines. The expressions of vascular endothelial growth factor (VEGF) and bcl-2 in the FAT-03-treated HUVECs were evidently down-regulated, and expression of bax was obviously up-regulated (data will be published elsewhere). However, other, still unknown, mechanisms are possibly involved in anti-angiogenesis and antitumor effects of FAT-03. Further investigation of the details of the cell and molecular mechanisms of FAT-03-mediated anti-angiogenesis and antitumor effects are currently in progress in our laboratory.

ABBREVIATIONS

FAT-03 : Fuantai-03
HUVECs : Human umbilical vein endothelial cells

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MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CAM : Chick chorioallantoic membrane
LLC : Lewis lung carcinoma
iMVD : Intratumoral microvessel density

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