

CHINESE MEDICAL SCIENCES JOURNAL

ORIGINAL ARTICLE

The Inhibitory Effects of Arresten Protein on Tumor Formation[△]

Yi Lv and Jin-ping Zheng*

Department of Health Toxicology, School of Public Health in Shanxi
Medical University, Taiyuan 030001, China

Key words: arresten; prokaryotic expression; purification; tumor

Objective To examine the inhibitory effects of recombinant purified arresten on tumor formation.

Methods Purified arresten protein was incubated with human umbilical vein endothelial cells (HUVECs) and HeLa cells *in vitro*. The effect on proliferation of HUVECs and HeLa cells was examined using 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide assay, and apoptosis of these cells monitored by flow cytometry. The effect on migration of HUVECs and HeLa cells was examined by Boyden chamber. Twenty colon carcinoma-bearing C67BL/6 mice were used to investigate the antitumor effects of arresten protein. The mice were randomly divided into arresten treatment group ($n=10$) and control group ($n=10$). The microvessel densities of the tumors were measured by immunohistochemical staining with anti-CD31 monoclonal antibody.

Results Arresten inhibited the proliferation and migration of HUVECs in a dose-dependent manner while promoting apoptosis. However, arresten had no significant effects on the proliferation and apoptosis of HeLa cells. The migration of HeLa cells was modestly inhibited by arresten. The arresten treatment group of mice showed no weight loss or unusual behavior during the course of treatment, and the tumor growth was significantly decreased; in contrast, the control group of mice exhibited rapidly growing tumors and cachexia. A dramatically decreased microvessel density in tumor tissues was found in arresten-treated mice compared with that in the control mice.

Conclusion Arresten can inhibit tumor growth through inhibition of tumor angiogenesis.

Chin Med Sci J 2012; 27(1):11-17

ANGIOGENESIS is a complex process that involves extracellular matrix (ECM) remodeling, endothelial cells (ECs) migration, proliferation, and functional maturation of new ECs into

mature blood vessels.^{1,2} The development of the ECM, a complex structure comprising of different proteins, is a crucial event in the evolution of multicellular organisms.^{2,3} During angiogenesis, new capillaries sprout from preexisting blood vessels by an invasive process called "neovascularization" that occurs physiologically during embryonic development, reproduction, and wound healing, and pathologically during tumor growth, metastasis, arthritis onset, and development of age-related macular degeneration.^{4,5} It has been proposed that tumor growth and metastasis are mediated by angiogenesis.⁶ Prevention

Received for publication March 6, 2012.

*Corresponding author Tel/Fax: 86-351-4135779, E-mail: zhengzheng200101@yahoo.com.cn

△Supported by Key Scientific and Technological Project of Shanxi Province (042082) and Technological and Engineering Project of the Department of Education of Shanxi Province (20080017).

of angiogenesis has been found to inhibit the growth of tumors.⁷⁻¹⁰ Solid tumors can not grow beyond 2-3 mm in diameter without recruitment of their own blood supply and nutrients.^{5,6} Tumor growth thus depends on the balance between circulating endogenous pro-angiogenic factors and angiogenesis inhibitors.^{7,8} Some non-collagenous C-terminal domains from various collagens have been identified to be anti-angiogenic and anti-tumorigenic.^{11,12} ECM-derived endogenous anti-angiogenic peptides control tumor cell proliferation, apoptosis, and tumor angiogenesis, which are critical for the growth of primary tumors and metastases. Given their crucial role in anti-angiogenesis, those peptides have been the focus of much research work with the aim of finding potential novel therapies for tumors.^{12,13}

Arresten, or human non-collagenous domain 1 of the $\alpha 1$ chain of type IV collagen, is derived from the carboxy terminal of type IV collagen. It is an effective endogenous angiogenesis inhibitor, which can specifically prevent the growth of solid tumor.¹² The administration of arresten can suppress the growth of primary and metastatic lesions in multiple murine tumor models and appears not to induce acquired drug resistance. Arresten protein has been applied as a primary agent in anti-angiogenic treatment, and the results in tumor treatment have been optimistic.¹²⁻¹⁶ In one of our previous studies, a novel arresten gene was identified and deposited in the GenBank database (accession number: DQ414683). After expression from bacterial cells, the arresten protein (accession number: ABE73157) exhibited significant anti-angiogenic effect.¹⁵ In this study, the arresten protein was purified and further examined for its inhibitory effects on tumor formation.

MATERIALS AND METHODS

Arresten protein expressed in prokaryotic system

The sequence encoding arresten was amplified by reverse transcription-polymerase chain reaction using total RNA isolated from human placental tissue that was provided by a healthy mother (age, 27 years; weight, 75 kg; first baby birth; Caesarean section in the Second Affiliated Hospital of Shanxi Medical University). The study was approved by Hospital Ethics Committee. The forward primer (5'-GC-CGAATTCATGTCTGTTGATCACGGC-3') and reverse primer (5'-GCGTCGACCTATTAGTGGTGGTGGTGGTGGTGTCTCTCTC-3') were designed to amplify the cDNA encoding the arresten protein. The amplified product was digested with *EcoR* I and *Sal* I, ligated into a pMD19-T transfer vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) that was predigested with the same restriction enzymes, and sub-

sequently ligated into a pBV221 expression vector, which was preserved in our laboratory, forming pBV221-arresten. The recombinant clones were sequenced using a high-throughput DNA sequencing facility. The sequences were further confirmed by a BLAST search. Clones expressing the 6×His tagged version of arresten were transformed in *E.coli* competent JM109 cells. The expressed arresten protein was purified using a Ni^{2+} -NTA Column (QIAGEN GmbH, Hilden, Germany) under denaturing conditions, and the purity was over 95% after purification. The purified products were dissolved in phosphate-buffered saline (PBS) and filtrated with a 0.22- μm micro-pore filter membrane to produce arresten protein solution.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai BioLeaf Biotech Co., Ltd. The HeLa cell line was kindly donated by Dr. Wu (First Affiliated Hospital of Shanxi Medical University). The murine colon carcinoma CT26 cell line was kindly donated by Dr. Chen (Molecular Biology Research Institute of Shanxi Medical University). The cells were grown in high/low glucose DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials, China), 100 U/mL penicillin, and 100 U/mL streptomycin, and maintained at 37°C in a 95% humidified atmosphere with 5% CO_2 . The cells were subcultivated every 2 or 3 days.

Cell proliferation assay

When reaching 70%-80% confluence, the HUVECs and HeLa cells were harvested by trypsinization (Life Technologies, Inc., Shanghai, China) at 37°C for 3 minutes. A suspension of cells ($1 \times 10^5/\text{mL}$) in DMEM with 0.5% FBS was added to each well of a 96-well plate. After a 12-hour incubation, the cells were treated with arresten at 0, 0.4, 0.8, 1.6, and 3.2 $\mu\text{g}/\text{mL}$ for 24, 48, and 72 hours. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/mL, AMRESCO, Solon, OH, USA) was added to cultured cells 4 hours before ending the experiment to produce formazan with light purple and the formazan was subsequently dissolved with 100 μL dimethyl sulfoxide. The absorbance (A) was detected at 490 nm, and the proliferation inhibitory rate was calculated.

Cell apoptosis assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine, was

used to detect cell apoptosis. HUVECs or HeLa cells were added to a 6-well plate in DMEM supplemented with 10% FBS and incubated for 36 hours. The media was then replaced by fresh DMEM containing arresten at different concentrations (0, 0.4, 0.8, 1.6, and 3.2 $\mu\text{g/mL}$). Cells incubated in PBS at the same volume served as control. After 24 hours of treatment, the cells were digested with 0.25% trypsin, collected, and resuspended in 500 μL binding buffer. Annexin V-FITC and PI (5 μL of each, KeyGEN Biotech, Nanjing, China) were added, and the cells were incubated in darkness for 15 minutes. Annexin V-FITC/PI-labeled cells were counted using a Becton-Dickinson FACSsan flow cytometer (Franklin Lakes, NJ, USA). These data were analyzed using Cell Quest software (Becton-Dickinson).

Migration assay

Migration of HUVECs and HeLa cells was assessed with Boyden chamber chemotaxis assay (Kylin-bell, Haimen, China). Chambers with 8- μm pore filters were used. The cells were cultured until reaching the exponential phase of growth, resuspended to a final concentration of $2 \times 10^6/\text{mL}$ by trypsinizing in serum-free medium with 0.05% FBS, and seeded into the wells of the upper compartment of the Boyden chamber. Serum-free medium containing different concentrations of arresten was added to the wells of the bottom compartment of the chamber. The control group received an equal volume of PBS. The Boyden chamber was incubated at 37°C in a 10% CO_2 atmosphere for 6 hours. The filter was removed from the chamber, and the non-migratory cells on the upper side of the filter were scraped off with a rubber policeman. The migratory cells on the lower side of the filter were fixed and stained with hematoxylin and counted under microscope in high power fields of view ($\times 400$). Migration experiment was repeated at least three times unless otherwise indicated.

Animal study

A total of 20 C57BL/6 colon carcinoma-bearing mice (6-8 weeks, weighing about 20 g) were used. The procedures were conducted according to the National Institute of Health (USA) guidelines for the care and use of laboratory animals. In a sterile environment, after injection with $2 \times 10^7/\text{mL}$ CT26 cells to establish a transplanted mice tumor model, the mice were divided randomly into arresten treatment group ($n=10$) and control group ($n=10$). Injection of arresten was performed every other day for the 10 mice in the arresten treatment group, while injection of physiological saline for the 10 mice in the control group. The mice were observed for viability, weighed, and the

diameters of the tumors were measured. Ten days later, the tumor volume (TV), relative tumor volume (RTV), and relative tumor inhibition ratio (RTVR) were determined according to the following formulas: $\text{TV} = 1/2 \times a \times b^2$ [a , width (mm); b , length (mm)]; $\text{RTV} = V_t/V_0$ (V_0 , initial tumor volume; V_t , final tumor volume); $\text{RTVR} = T/C \times 100\% = \text{TRTV}/\text{CRTV} \times 100\%$ (T , arresten treatment group; C , control group; TRTV , RTV of arresten treatment group; CRTV , RTV of control group).

The blood vessel density in tumor tissues was measured by immunohistochemistry using a CD31 polyclonal antibody (Wuhan Boster Bio-engineering Limited Company, China). Microvessel density was calculated as previously reported.¹⁷ After the area with the highest neovascularization was identified at 200 $\times$ magnification under a light microscope, the number of blood vessels in 10 regions at 200 $\times$ magnification was counted, and the average number of microvessels was recorded.

Statistical analysis

SPSS 13.0 software was employed for statistical analysis. The data were presented as means $\pm$ SD. Comparisons were performed with analysis of variance (ANOVA) with one-tailed Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of arresten protein

The sequence for arresten is diagrammatically represented in Figure 1. Sodium dodecyl sulfonate polyacrylamide gel electrophoresis detected a single protein with a molecular mass of 26 kDa and that the purity of the protein was at least 95% (Fig. 2).

Impact of arresten on the growth of HUVECs and HeLa cells

Arresten (0.8 $\mu\text{g/mL}$) inhibited the proliferation of HUVECs ($P < 0.05$) in a dose-dependent manner to some extent (Fig. 3). However, arresten had no effect on the proliferation of HeLa cells (Fig. 4).

Arresten significantly induced apoptosis in HUVECs, which was dose-dependent within the range of 0.4-3.2 $\mu\text{g/mL}$ ($P < 0.01$). No such effect of arresten was observed in HeLa cells (Fig. 5).

Arresten protein inhibited HUVECs migration ($P < 0.01$) in a dose-dependent manner within the range of 0.8-3.2 $\mu\text{g/mL}$ (Fig. 6). Arresten protein could also inhibit the migration of HeLa cells, yet the extent of inhibition was lower than that observed in HUVECs (Fig. 6).

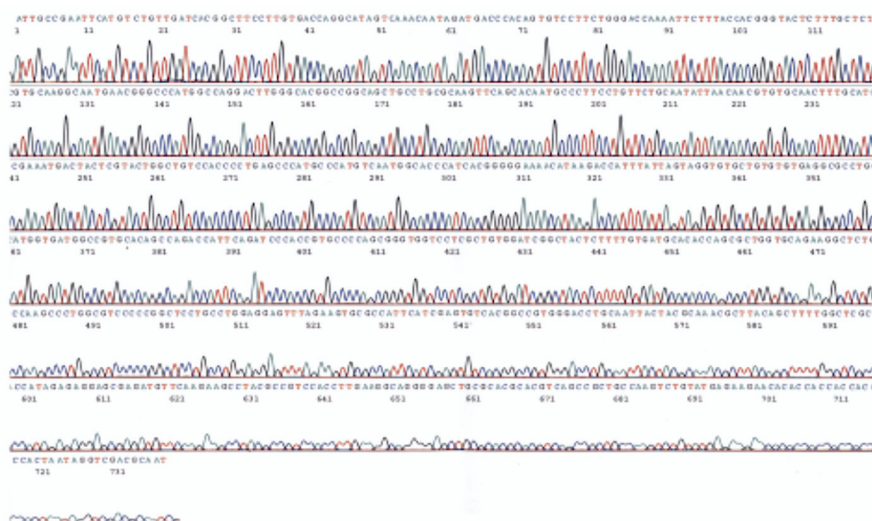


Figure 1. Nucleotide sequence of arresten.
The sequencing result is consistent with the sequence for arresten in GenBank.

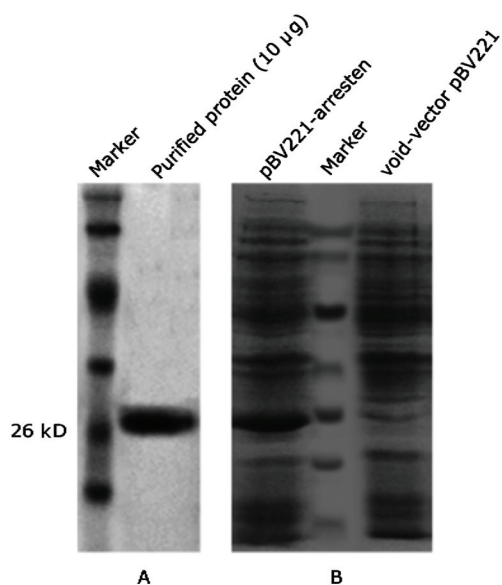


Figure 2. Expression and purification of recombinant human arresten.

The expressed arresten proteins were prepared and purified through an affinity matrix (Ni^{2+} -NTA agarose) column. The figure is a Coomassie-stained 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel.

A. The left lane is the molecular weight marker, and the right lane represents approximately 10 μg purified protein;

B. The left lane represents approximately 10 µg expression product from recombinant pBV221-arresten, the middle lane is the molecular weight marker, and the right lane contains approximately 10 µg expression product from the empty pBV221 vector.

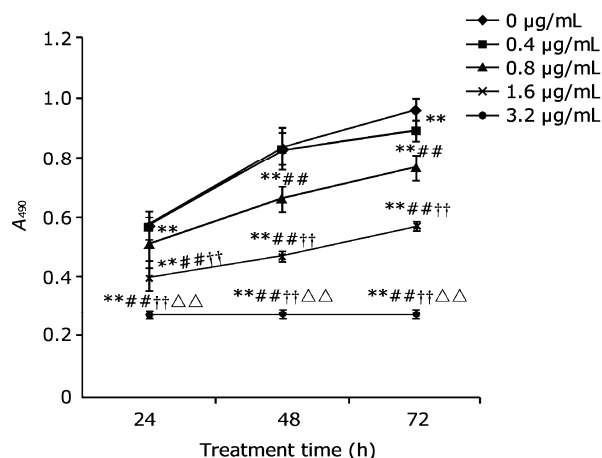


Figure 3. The effect of arresten on the proliferation of human umbilical vein endothelial cells (HUVECs). The absorbance (*A*) was detected at 490 nm.

** $P < 0.01$ compared with 0 $\mu\text{g/mL}$; ## $P < 0.01$ compared with 0.4 $\mu\text{g/mL}$; ++ $P < 0.01$ compared with 0.8 $\mu\text{g/mL}$; $\Delta\Delta P < 0.01$ compared with 1.6 $\mu\text{g/mL}$.

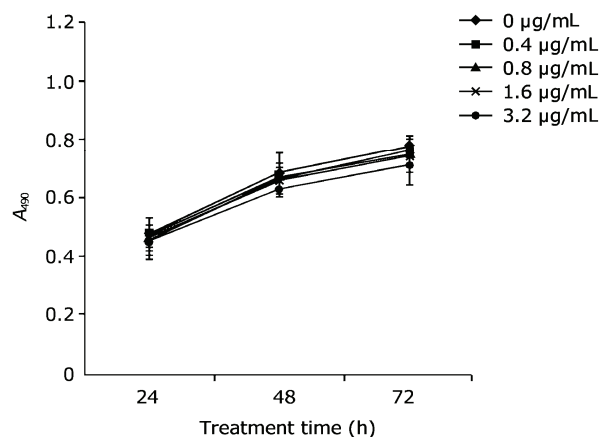


Figure 4. Arresten protein had no obvious effect on the growth of HeLa cells.

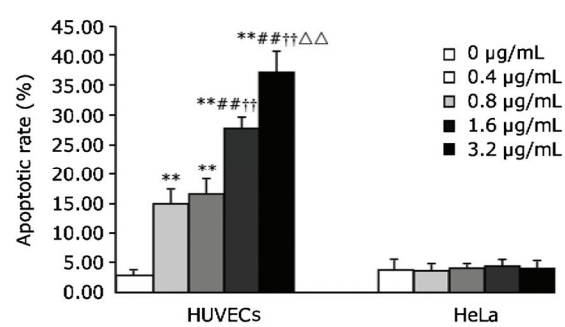


Figure 5. The effect of arresten on the apoptosis of HUVECs and HeLa cells. Arresten significantly induced apoptosis of HUVECs, which was dose-dependent within the range of 0.4-3.2 µg/mL ($P<0.01$); yet exhibited no effect on HeLa cells apoptosis. $**P<0.01$ compared with 0 µg/mL; $##P<0.01$ compared with 0.4 µg/mL; $††P<0.01$ compared with 0.8 µg/mL; $\Delta\Delta P<0.01$ compared with 1.6 µg/mL.

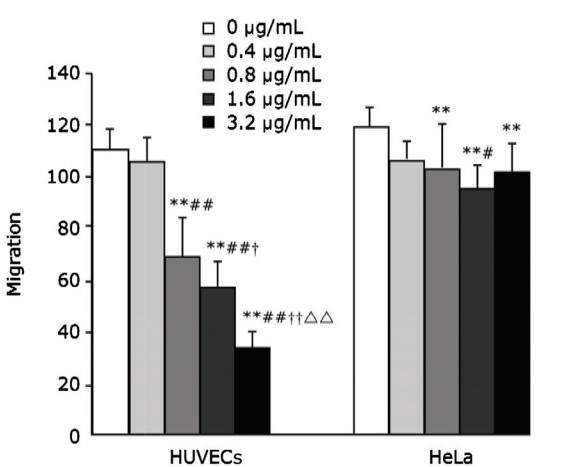


Figure 6. The effect of arresten on the migration of HUVECs and HeLa cells. Arresten effectively inhibited the migration of HUVECs, which was dose-dependent within the range of 0.8-3.2 µg/mL. It modestly inhibited the migration of HeLa cells, but to a lesser extent than that observed in HUVECs. $**P<0.01$ compared with 0 µg/mL; $#P<0.05$, $##P<0.01$ compared with 0.4 µg/mL; $†P<0.05$, $††P<0.01$ compared with 0.8 µg/mL; $\Delta\Delta P<0.01$ compared with 1.6 µg/mL.

Therapeutic effect of arresten on the tumor-bearing mice

Mice in the control group exhibited progressive tumor growth with a decline in diet, activity, and response, which developed into cachexia and one mouse died eventually. In contrast, mice in the arresten treatment group exhibited normal diet behavior both before and after treatment. The tumor volume and weight in the arresten treatment group was significantly lower than those in control group

($P<0.01$). It was found that the growth of tumor was inhibited with a relative inhibition ratio of 80.81%. The therapeutic effect of arresten on the tumor-bearing mice is shown in Table 1.

The prepared tumor tissues were stained with CD31 (1:100) immunohistochemistry by Strept-Avidin-Biotin-Complex (SABC) method. Tumor tissue in the control group demonstrated a wide distribution of vascular lumen formation, while in the arresten treatment group, significantly reduced angiogenesis was noted in the tumors ($P<0.01$, Fig. 7).

Table 1. Inhibitory effect of arresten on tumor in tumor-bearing mice[§]

Group	TW (g)	TV (mm ³)	RTV	RTVR (%)
Control (n=10)	2.16±0.37	3461.20±927.53	9.54±5.43	
Arresten Treatment (n=10)	0.88±0.25*	765.19±249.41*	1.83±0.99*	80.81

§: Plus-minus values are means±SD.
TW: tumor weight; TV: tumor volume; RTV: relative tumor volume; RTVR: relative tumor inhibition ratio
* $P<0.01$ compared with the control group.

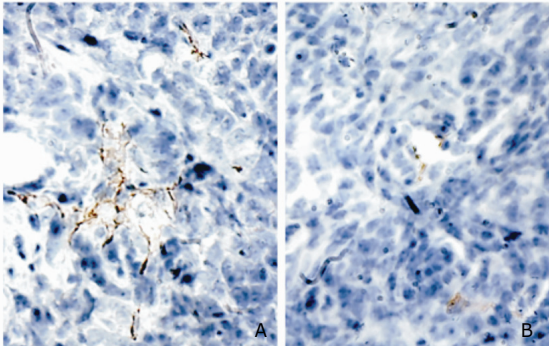


Figure 7. Inhibitory effect of arresten on tumor angiogenesis in tumor-bearing mice. hematoxylin ×200
A. In the control group, a wide distribution of vascular lumen formation is observed;
B. The arresten treatment group displays significantly lower vessel quantity ($P<0.01$).

DISCUSSION

The malignant growth and metastasis of tumors rely upon angiogenesis to receive an adequate supply of oxygen and nutrients.¹⁷ Inhibiting neovascularization may efficiently inhibit tumor cell growth and lead to tumor dormancy.^{17,18} Arresten, an endogenous angiogenesis inhibitory factor, has been found to have no toxic side effects and

is more stable than endostatin,^{12,15} thus may be a potential therapeutic agent in tumor therapy. Lu et al¹⁹ have shown that transfected arresten could inhibit the growth of an SGC-7901 tumor xenograft model in nude mice and prevent hepatic metastasis of colorectal cancer. However, the gene transfection method in that study was difficult to perform clinically and might lead to mutation and carcinogenesis. In addition, the cost of gene transfection therapy restricts its application for tumor patients. Recombinant arresten production by prokaryotic expression is an attractive method for producing a large amount of the protein, and the dosage can be adjusted according to the different needs of individual patients. Additionally, bacterial production of recombinant arresten avoids the danger of the rapid growth of recombinant cells into a new tumor. Chemotherapy based on prokaryotic expression of arresten is a safe, highly efficient, and low-cost tumor therapy, which can be combined with surgery and radiotherapy. Thus, this method could be readily applied in clinical practice.¹⁵

In the animal study in this research, we found the dose of arresten we used safe for animals and effective in inhibiting tumor growth. These results show that the pure arresten prepared in our laboratory has a satisfactory therapeutic effect, thus could be further considered as a potential novel drug in the treatment for solid tumors. As shown by the results of the present study, arresten inhibited the proliferation and migration of HUVECs while promoting apoptosis of these cells; however, it had no such significant effect on the proliferation or viability of HeLa cells. These findings confirm that arresten plays the inhibitory role through suppressing angiogenesis. This conclusion is supported by the decrease in tumor vessel density in arresten-treated tumor-bearing mice. We hypothesize that the mechanism underlying the effects of arresten may be as follows: arresten binds to $\alpha 1\beta 1$ integrin, competing with type IV collagen binding to $\alpha 1\beta 1$ integrin.¹³ Additionally, arresten pretreatment could inhibit the FAK/c-Raf/MEK/ERK1/2/p38 MAPK activation in ECs and inhibits the migration, proliferation, and vessel formation by ECs.^{14,20} However, the present study demonstrates that the migration of tumor cells per se can also be inhibited by arresten, although to a lesser extent. The relevant mechanism is not clear; study in this area may shed some light on how to therapeutically inhibit the metastasis of cancer.

ACKNOWLEDGMENT

We sincerely thank Prof. Jun Xie, Prof. Xian-jiu Chen, Prof. Hai-ying Tang, and other administrative personnel for

helping in organizing and facilitating the research. We would also like to thank the Department of Biochemistry and Molecular Biology as well as the Second Affiliated Hospital of Shanxi Medical University for their support in the project. More importantly, we feel grateful for the mother who voluntarily donated the placental tissue for this study.

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