

Research Paper

PE, a New Sulfated Saponin from Sea Cucumber, Exhibits Anti-Angiogenic and Anti-Tumor Activities In Vitro and In Vivo

Fang Tian^{1,2}

Xiongwen Zhang¹

Yunguang Tong^{1,2}

Yanghua Yi³

Shilong Zhang³

Ling Li³

Peng Sun³

Liping Lin¹

Jian Ding^{1,*}

¹Division of Anti-Tumor Pharmacology; State Key Laboratory of Drug Research; Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Science; Chinese Academy of Sciences; Shanghai P.R. China.

²Graduate School of Chinese Academy of Sciences; Beijing P.R. China.

³Research Center for Marine Drugs; School of Pharmacy; Second Military Medical University; Shanghai 200433 P.R. China

*Correspondence to: Jian Ding; Division of Anti-tumor Pharmacology; State Key Laboratory of Drug Research; Shanghai Institute of Materia Medica; Shanghai Institutes for Biological Sciences; Chinese Academy of Sciences; 555 Zu Chong Zhi Road; Zhangjiang Hi-Tech Park; Shanghai, 201203 P.R. China; Fax: 86.21.50806722; Email: jding@mail.shnc.ac.cn

Received 05/12/05; Accepted 06/14/05

Previously published online as a *Cancer Biology & Therapy* E-publication: <http://www.landesbioscience.com/journals/cbt/abstract.php?id=1917>

KEY WORDS

PE, marine, angiogenesis, endothelial cells, VEGFR2, apoptosis, anti-tumor

ACKNOWLEDGEMENTS

High Tech Research and Development Program (No. 2002AA2Z346A; 2001AA624100); the Knowledge Innovation Program of Chinese Academy of Sciences (No. KSCX2-SW-202, No. KSCX2-3-07-8) and the National Natural Science Foundation (No. 30228032).

ABSTRACT

Here, we examined the in vitro and in vivo anti-angiogenesis and anti-tumor activities of PE, a new marine-derived compound. Inhibition of angiogenesis was assessed in vitro using proliferation, migration, adhesion, tube-formation and apoptosis assays in PE-treated HMECs and HUVECs. In vivo, CAM assays were used to assess inhibition effect of PE on physiological angiogenesis, and immunofluorescent microscopy was used to examine tumor microvessel density and apoptosis in PE-treated mouse tumor models. Finally, Western blotting analyses were performed to examine the effect of PE on VEGF signaling in HMECs. The results showed that PE inhibited proliferation of HMECs and HUVECs with IC₅₀ values of 2.22 ± 0.31 μM and 1.98 ± 0.32 μM, induced endothelial cell apoptosis at concentrations <2 μM, induced dose-dependent suppression of cell migration, cell adhesion and tube formation in HMECs and HUVECs, and showed anti-proliferative activities against several tumor cell lines (IC₅₀ values of ~4 μM). In vivo, PE (5 nM/egg) suppressed spontaneous angiogenesis in our CAM assay, and induced marked growth inhibition in mouse sarcoma 180 and hepatoma 22 models. Specifically, PE treatment reduced mouse sarcoma 180 tumor volume by triggering apoptosis of both tumor and tumor-associated endothelial cells, preferentially targeting on endothelial cells comparable with tumor cells. Finally, PE treatment suppressed the active (phosphorylated) forms of VEGFR2, Akt, ERK, FAK and paxillin, which are involved in endothelial cell survival, proliferation, adhesion and migration. Our results indicate that PE exerts an anti-angiogenic activity associated with inhibition of VEGFR2 signaling, and an anti-tumor activity associated with decreased proliferation of tumor cells and increased apoptosis of both endothelial cells and tumor cells.

ABBREVIATIONS

ATCC, American type culture collection; CAM, chorioallantoic membrane; DMSO, dimethylsulfoxide; EGF, epithelial growth factor; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinases; H-22, hepatoma 22; HMEC, human microvascular endothelial cell; HUVEC, human umbilical vein endothelial cells; IC₅₀, 50% inhibitory concentration; KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase; MMP, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density; PBS, phosphate buffer saline; S-180, aSarcoma 180; SD, standard deviation; SRB, sulforhodamine B; TUNEL, terminal deoxynucleotidyl transferase mediated nick end labeling; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

INTRODUCTION

Angiogenesis is formation of new vessels from preexisting capillaries. Among the known angiogenic growth factor and cytokines, VEGF and its corresponding receptors are indispensable in regulating multiple facets of the angiogenic processes. As angiogenesis plays a central role in tumor growth, progression, invasion and metastasis,¹ inhibition of this process provides a potential strategy for cancer treatment.² Researchers are currently seeking to develop new angiogenesis inhibitors³ from sources such as cleaved proteins, monoclonal antibodies, synthesized small molecules and natural products.⁴ However, recent studies have suggested that sole blockage of angiogenesis may not be sufficient to fully suppress malignancies,^{5,6} indicating that dual treatments may be more effective. For example, the VEGF inhibitor Avastin was shown to extend the lives of colon cancer patients when given intravenously in combination with standard chemotherapy drugs (irinotecan, 5-FU and leucovorin), but was not effective when used as a single treatment

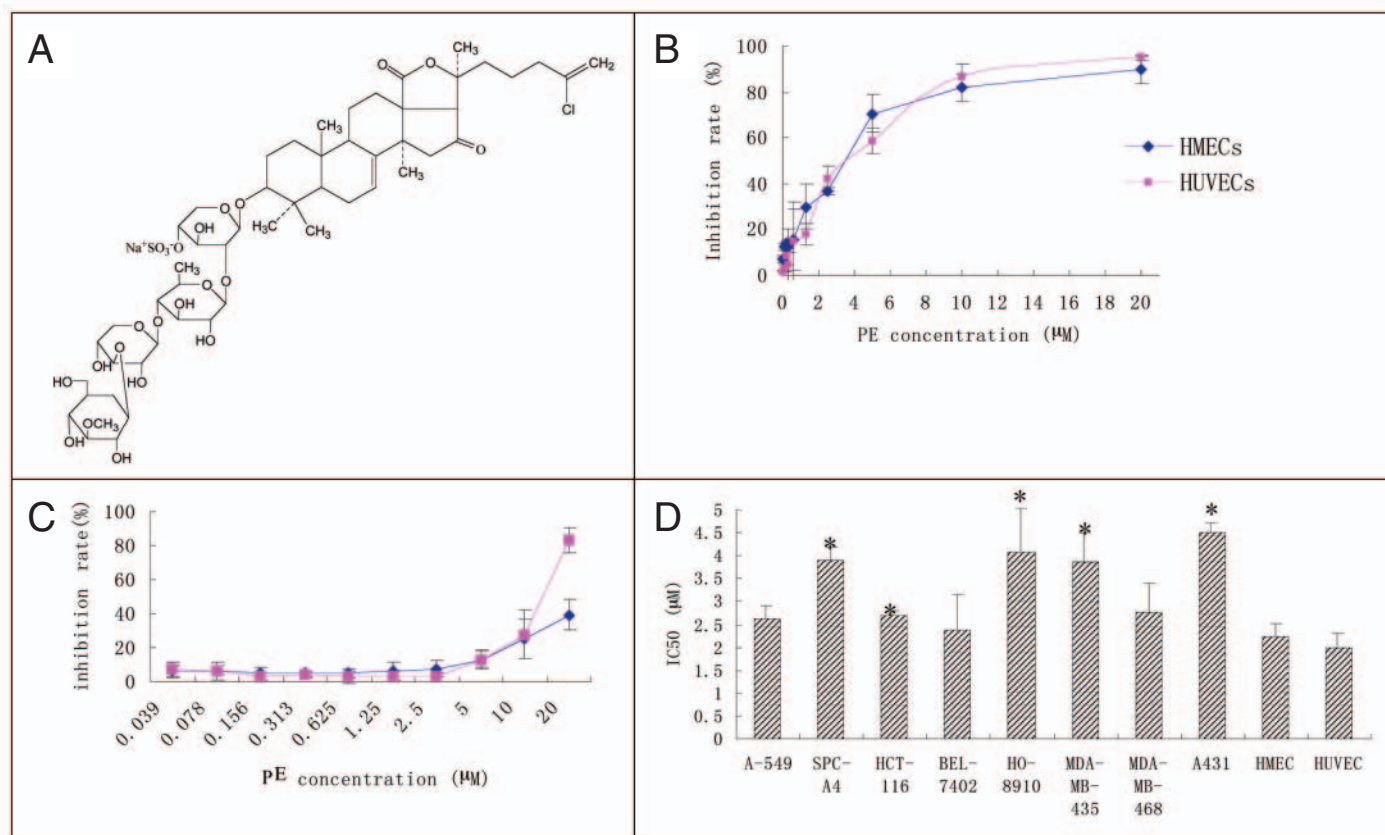


Figure 1. Inhibitory effect of PE on endothelial and tumor cell proliferation. (A) The chemical structure of PE. (B) HMECs and HUVECs were treated with PE for 72 h and the effect on cell growth was measured using the SRB method. (C) HMECs were treated with PE for 8 or 24 h and measured using the SRB method. (D) IC₅₀ values for the effect of PE on endothelial and tumor cells. *P < 0.01 vs. PE treated HMECs. Each value is the mean ± SD of three independent experiments.

drug.⁷ Thus, it would seem valuable to seek molecules that simultaneously confer both anti-angiogenic and anti-tumoral activities.

Marine-derived natural products contain a variety of chemotherapeutic compounds that have been shown to prevent the development of malignancies,⁸ and several marine-derived molecules are currently in or entering clinical trials in cancer therapy,⁹ promising that marine natural products act as a rich source for cancer therapy. Notably, we discovered that serials marine-derived chemotherapeutic agents exert anti-angiogenic properties. Of them, PE, a novel compound isolated from the sea cucumber *pentacta quadrangularis*, drew our special interests, due to its both anti-angiogenesis and anti-tumor activity upon our screening in several in vitro models.

Here, we examined the ability of PE to suppress angiogenesis and tumor development in vitro and in vivo, and examined possible mechanisms of action. Our results indicate that PE has strong anti-angiogenic activity, which may account in part for its anti-tumor activities. The potent anti-angiogenic activity of PE at least partly due to inhibition of VEGF receptor 2 signaling, and its anti-tumoral effects was associated with decreased tumor cell proliferation and increased tumor cell apoptosis. Thus, PE may be viewed as a possible candidate molecule for cancer therapeutics.

MATERIALS AND METHODS

Materials. Soluble KDR/Flk-1 was purchased from Calbiochem. Anti-pERK, Anti-pKDR and Anti-pAKT were obtained from Cell Signaling Technology. The anti-CD-31, anti-VEGF, anti-pFAK and

anti-paxillin antibodies were obtained from Santa Cruz Biotechnology. The Prolong anti-fade kit and the fluorescent Alexa Fluor 546-conjugated secondary antibody were purchased from Molecular Probes. The cell culture mediums (M199 and MCDB131), endothelial cell growth supplement (ECGS), VEGF165, MTT, Heparin, 5-FU, Suramin, and all other reagents were purchased from Sigma.

Isolation and purification of PE. PE was isolated from the sea cucumber *Pentacta quadrangularis*. Specimens were collected near Guangdong Province, China, and identified by Prof. J. R. Fang of the Fujian Institute of Oceanic Research (China). Air-dried body walls (5 kg, dried weight) of *P. quadrangularis* were cut into pieces and extracted twice with refluxing ethanol. The combined extracts were evaporated in vacuo and further partitioned between water and chloroform. The water layer was extracted with n-butanol and the organic layer was evaporated in vacuo to yield n-butanol extracts. The n-butanol extracts were concentrated, and the extracted residue was dissolved in water. Samples were desalted with a DA101 resin column (60 x 30 cm), with the inorganic salts and polar impurities eluted with water, and the crude glycoside fraction (8.1 g) subsequently eluted with 80% ethanol. The latter fraction was separated by flash chromatography on silica gel (6/40 cm; CHCl₃/MeOH/H₂O 7.0:3.0:0.2, 20 ml/min) to yield a crude PE-containing fraction. This fraction was further separated by HPLC (Zorbax 300 SB-C18, 9.4 mm x 25 cm, 55% MeOH/H₂O, flow rate 1.5 ml/min) to yield pure PE. The PE was dissolved in DMSO and diluted to the desired concentrations before use, with the final DMSO concentration maintained <0.05% in the various treatment groups. The chemical structure of PE was determined using ¹H and ¹³C NMR spectra, ESI-MS and the IR spectrum, as previously described (Fig. 1A).

Cell lines and cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase

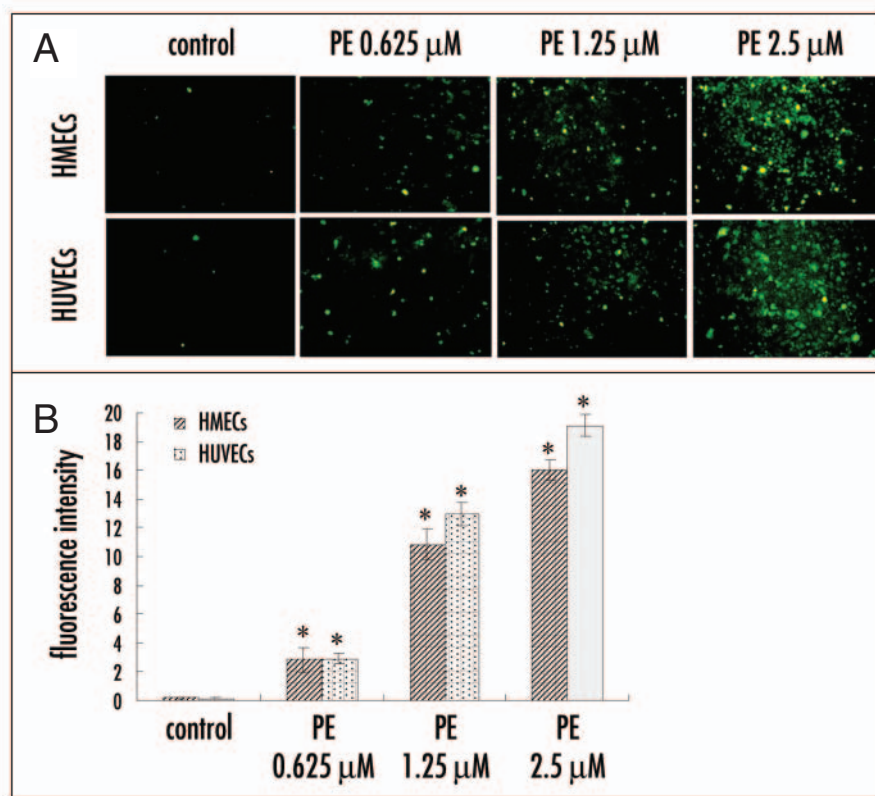


Figure 2. Effect of PE on endothelial cell apoptosis. (A) HMECs and HUVECs were treated with PE for 36 h and TUNEL staining was used to examine apoptosis. (B) Fluorescence intensity of TUNEL staining in HMECs and HUVECs undergoing PE treatment. Results are expressed as the mean \pm SD of three separate experiments. * $p < 0.01$ vs. control.

treatment as described previously.¹⁰ Cells from passages 2–6 were grown in Medium 199 containing 100 unit/ml penicillin, 10 ng/ml EGF, 3 μ g/ml endothelial cell growth supplement (ECGS), and 20% fetal bovine serum (FBS). Human dermal microvascular endothelial cells (HMECs) were obtained from the ATCC, and cultured in MCDB-131 containing 15% FBS, 1 ng/ml EGF and 1 mg/ml hydrocortisone. Gastric adenocarcinoma cell line MKN-28 was obtained from the Japanese Foundation for Cancer Research (JFCR) and cultured in 1640 culture medium containing 10% FBS. Colon adenocarcinoma cell line HCT-116, and breast adenocarcinoma cell lines MDA-MB-468 and MCF-7 were obtained from the ATCC and were cultured in 5A, L-15 and DMEM media, respectively, containing 10% FBS. Hepatocellular carcinoma cell line BEL-7402, lung adenocarcinoma cell line SPC-A4 and ovarian epitheloid carcinoma cell line HO-8910 were obtained from the Shanghai Institute of Biochemistry and Cell Biology (SIBCB), and cultured in 1640 culture medium containing 10% FBS. All cells were maintained at 37°C under a humidified 95% and 5% (v/v) mixture of air and CO₂.

Cell proliferation assay. Endothelial cells were seeded into 96-well plates at a density of 8×10^3 cells/well. After the cells were incubated overnight, the medium in each well was replaced by fresh MCDB131 medium containing different concentrations of PE or 0.01% DMSO (v/v). After incubation for 72 h, the medium was removed from each well and the relative number of cells was determined in triplicate wells using the SRB assay, as described previously.¹¹

Endothelial cell differentiation assay. Matrigel was thawed at 4°C, and 60 μ l aliquots were quickly added to each well of a 96-well plate and allowed to solidify for 30 min at 37°C. Endothelial cells (2.5×10^4 cells/well) were seeded onto the Matrigel and cultured in MCDB131 medium containing different concentrations of PE or 0.01% DMSO (v/v) for 12 h. For analysis

of tube formation, the enclosed networks from five randomly chosen fields were counted and photographed under a microscope (IX70, Olympus, Japan). The total length of the tube structure in each photograph were measured using the Adobe Photoshop software,¹² and inhibition of tube formation was calculated as $[1 - (\text{tube length}_{\text{treated}}/\text{tube length}_{\text{control}})] \times 100\%$.

Endothelial cell migration assay. Briefly, the chemotactic motilities of endothelial cells were assayed using Transwell Boyden Chambers (Costar, MA, USA) with 6.5-mm diameter polycarbonate filters (8 μ m pore size).¹³ The lower surface of each filter was coated with 10 μ g of gelatin, and 600 μ l M199 medium containing 1% FBS and 10 ng/ml VEGF was placed in each lower well. Endothelial cells were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS, and 100 μ l of the cell suspension was loaded into each upper well, along with various concentrations of PE or 0.01% DMSO (v/v). The chambers were then incubated at 37°C for 8 h, whereupon cells were fixed and stained with crystal violet. Nonmigrating cells on the upper surface of the filter were removed with a cotton swab, and chemotaxis was quantified by counting the cells that had migrated to the lower side of the filter, as visualized under a microscope (IX70, Olympus, Japan). Ten random fields were counted for each assay. The inhibition of migration was calculated as $[1 - (\text{migrated cells}_{\text{treated}}/\text{migrated cells}_{\text{control}})] \times 100\%$.

Chicken embryo chorioallantoic membrane (CAM) assay. The CAM angiogenesis assay was performed as described previously, with some modifications.¹⁴ Briefly, fertilized chicken eggs were incubated in a humidified egg incubator (Lyon, CA, USA) for eight days, and then a small hole was punched into the broad side of the egg and a window was carefully created in the eggshell. Various amounts of PE or 0.01% DMSO (v/v) were air-dried onto sterile glass coverslips of approximately 1 mm². The coverslips were placed onto well-vascularized sites of the CAM of the developing chick embryos, and the eggs were returned to the humidified egg incubator. Forty-eight hours later, the sites were evaluated and recorded with stereomicroscopic photography (MS5, Leica, Switzerland). Angiogenesis was quantified by counting the number of blood vessel branch points in each photo. A positive anti-angiogenic effect was scored when the microvessels were obviously reduced under the coverslips. At least ten viable embryos were tested for each treatment.

Cell adhesion assay. The efficiency of cell adhesion was determined by measuring the number of cells that adhered to a given substrate.¹⁵ Fibronectin was diluted in sterile water (10 mg/ml), aliquotted to 96-well plates 100 μ l per well, and incubated overnight at 4°C. The samples were blocked with 1% BSA at 37°C for 1 h, and then 0.5×10^5 cells in 100 μ l of prewarmed serum-free medium were seeded into each well and allowed to adhere at 37°C for 1 h with various concentrations of PE or 0.01% DMSO (v/v). Nonadherent cells were rinsed off with PBS and the remaining cells were fixed with 4% paraformaldehyde for 10 min, then stained with 0.5% crystal violet in 4% paraformaldehyde for 5 min and rinsed with water. Cells were solubilized by the addition of 100 μ l of 1% SDS and quantified in a microtiter plate reader at 590 nm with a multi-well spectrophotometer (VERSAmax, Molecular Devices, CA, USA).

TUNEL assay. Cellular apoptosis was determined using the TUNEL assay.¹⁶ Endothelial cells were grown to 75% confluence on coverslips and incubated for 36 h with various amounts of PE or 0.01% DMSO (v/v). The TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end-labeling) assay was performed with the In Situ Cell Death Detection Kit (Roche Diagnostics, Barcelona, Spain), according to the manufacturer's instructions. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized

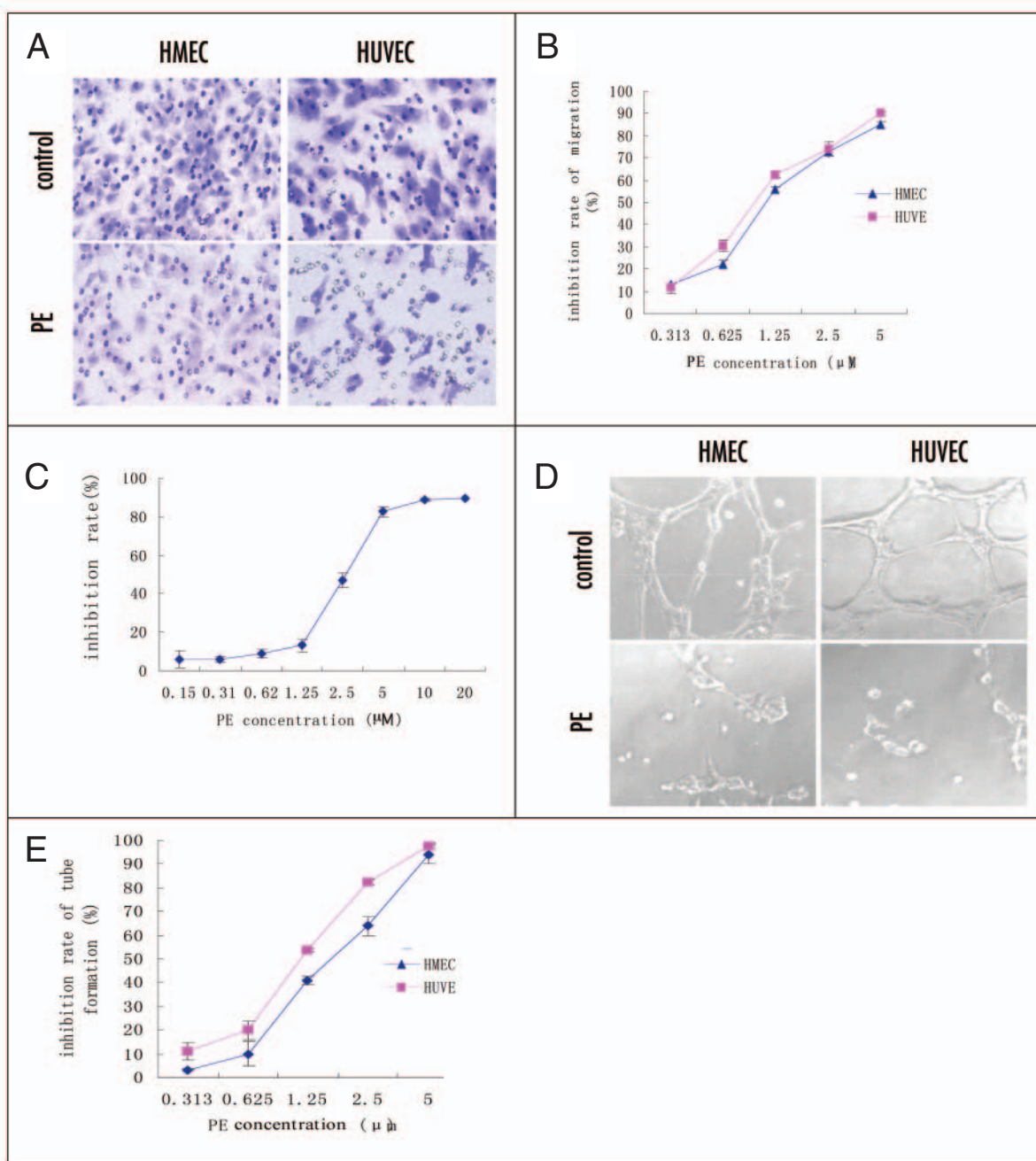


Figure 3. Effect of PE on endothelial cell function. (A) Endothelial cells seeded in Transwell Boyden Chambers were incubated for 8 h with or without 1.25 μM PE. Migrated cells on the lower surface of the filter were stained with crystal violet and counted manually from five random fields. (B) Overall rate of PE-induced inhibition of endothelial migration. (C) Effect of PE on endothelial cell adhesion. HMECs were plated on fibronectin-coated wells with or without the indicated concentration of PE for 1 h. The relative number of attached cells was assessed by staining with crystal violet, and the absorbance value was determined at 590 nm. (D) Endothelial cells seeded in Matrigel-coated 96-well plates were incubated for 24 h with or without 1.25 μM PE. The enclosed networks of tubes were photographed from five randomly chosen microscopic fields. (E) Overall rate of PE-induced inhibition of tube formation. Results are expressed as mean \pm SD of three separate experiments.

with 0.1% Triton X-100 and 0.1% sodium citrate (freshly prepared), and labeled with fluorescein-12-dUTP. Apoptosis was detected by fluorescence microscopy (Olympus, BX51, Japan) and quantified by the Image-Pro Plus 5.0 image analysis software (Media Cybernetics Inc, Maryland).

Western blotting analysis. Confluent HMECs were incubated for 24 h in MCDB131 containing 1% FBS, incubated for 1 h in MCDB131 without FBS in the presence PE or DMSO control, and then stimulated by the addition of VEGF (50 ng/ml) for 10 min. After stimulation, cells were lysed in lysis buffer (20 mM Tris/HCl, pH 8.0, 2 mM EDTA, 137 mM NaCl, 1 mM

Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100). Lysates were clarified by centrifugation at 15,000 $\times g$ for 10 min, resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature and then probed with primary antibody overnight at 4°C. Immunoreactive bands were visualized by incubation with horseradish peroxidase-conjugated second antibodies and application of an enhanced chemiluminescent (ECL) system (Amersham Biosciences). Each experiment was repeated at least 3 times, with representative blots presented.

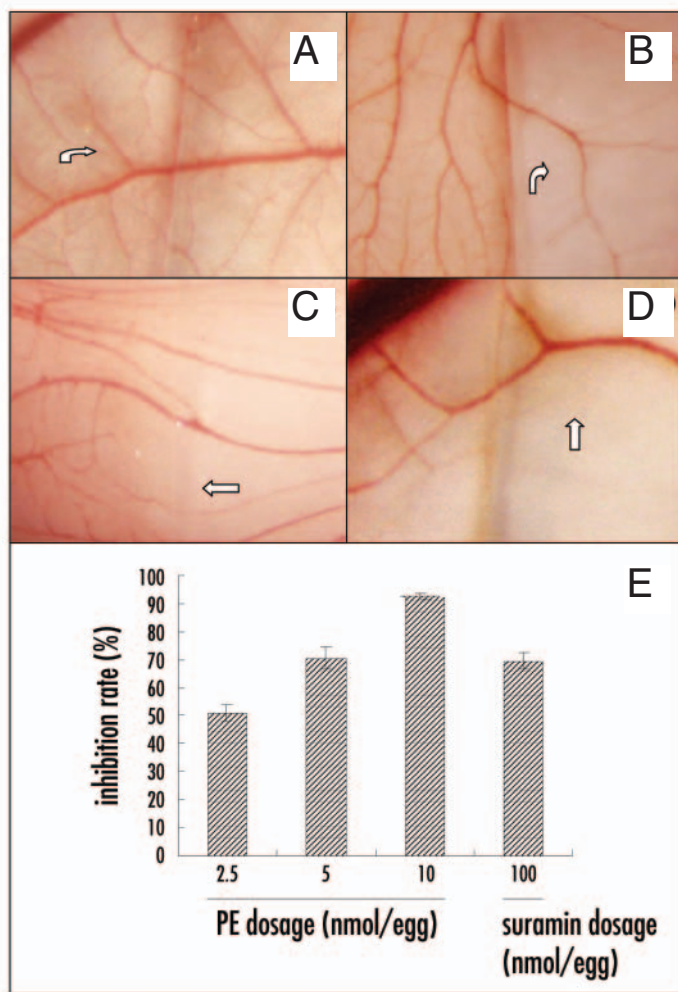


Figure 4. Effect of PE on CAM. Fertilized eggs were incubated continuously for 8 days, and then a window was opened to expose the CAM and PE was added to a final concentration of A) 0 nM/egg (solvent control), B) 2.5 nM/egg, C) 5 nM/egg or D) 10 nM/egg. The eggs were incubated for another 48 h, and then the treated CAMs were harvested and photographed. E) Overall inhibition rate of PE on CAM angiogenesis, assessed by quantification of the blood vessel branch points in each photograph. Each value is the mean \pm SD from ten eggs.

In vivo tumor growth inhibition assay. Female KM mice (6–8 weeks of age) were used to study inhibition of tumor growth in vivo. The use of lab animals was in accordance with guidelines of the Experimental Animal Association of China, certificate number: SCXK (Shanghai; 2003-0003). Sarcoma 180 cells or hepatoma 22 cells (2.5×10^6) were subcutaneously implanted into the axilla of mice on day 0, and mice were randomly grouped (8 mice per group) on day 1. Normal saline and dilutions of PE (1, 2 and 3 mg/kg, dissolved in normal saline) were delivered intravenously to nonanaesthetized mice once daily for seven days. Animals were sacrificed 24 h after the last administration, and mice and tumor weights were measured. The tumor growth inhibition rates were calculated as follows:

$$\text{Tumor growth inhibition rate} = \frac{\text{Tumor weight of control} - \text{Tumor weight of drug-treated}}{\text{Tumor weight of control}} \times 100\%$$

Immunofluorescence. Paraffin-embedded tumor tissue sections (5 μ m-thick) putted on slides were dewaxed by heating at 60°C, washed in xylene and rehydrated through a graded series of ethanol and double distilled water

washes. The slides were washed with PBS and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate (freshly prepared). The samples were blocked with PBS/5% BSA for 1 h, incubated with the anti-CD31 polyclonal antibody for 1 h at room temperature, washed three times in PBS, and then incubated for 1 h at room temperature with a secondary antibody conjugated to fluorescent Alexa Fluor 546 (Molecular Probes). Samples were finally washed three times in PBS and mounted with coverslips using Prolong anti-fade medium (Molecular Probes). Immunofluorescent staining was observed and photographed using a fluorescence microscope. For double staining, TUNEL detection was performed as described above, followed by anti-CD-31 detection. All fluorescence photographs were taken under a fluorescence microscope (Olympus, BX51, Japan) and analyzed with the Image-Pro Plus image analysis software (Media Cybernetics Inc, Maryland). Fluorescence intensities were gathered from three random fields per slice (from three random slices per tumor) and presented as the mean \pm SD.

Data analysis. All results were expressed as mean \pm SD, and statistical significance was assessed by Student's t-test.

RESULTS

PE inhibits proliferation of HMECs, HUVECs and tumor cell lines. As angiogenesis involves local proliferation of endothelial cells, we initially investigated the effects of PE on proliferation of HMECs and HUVECs. Treatment with PE inhibited the growth of cultured HMECs and HUVECs in a concentration-dependent manner (Fig. 1B), with IC₅₀ values of $2.22 \pm 0.31 \mu\text{M}$ and $1.98 \pm 0.32 \mu\text{M}$, respectively. Treatment of HMECs with 5 μM of PE for 8 or 24 h did not have any detectable effect on HMEC proliferation (Fig. 1C), so this concentration of PE was used for the HMEC migration and tube formation assays, to ensure that the detected abilities of PE to inhibit endothelial cell functions were specific effects rather than the results of general cytotoxicity. In addition, PE treatment inhibited the proliferation of all tested tumor cell lines, displaying slightly higher IC₅₀ values ranging from 2.4 to 4.1 μM (Fig. 1D).

PE induces apoptosis of endothelial cells. Anti-proliferation and subsequent anti-angiogenesis have been correlated with several underlying mechanisms,¹⁷ including induction of apoptosis.¹⁸ Accordingly, we used TUNEL staining to investigate whether PE treatment induced endothelial cell apoptosis. HMECs and HUVECs exposed to 0.625, 1.25, and 2.5 μM PE for 24 h showed a slight induction of apoptosis (data not shown), while treatment for 36 h resulted in dose-dependent induction of apoptosis (Fig. 2).

PE hinders endothelial cell migration. As endothelial cell migration is a prerequisite for angiogenesis, we explored the effect of PE on directional cell motility using a Transwell Boyden Chamber assay. As shown in Figure 3A, incubation of control HMECs or HUVECs in the chamber for 8 h resulted in large-scale migration of endothelial cells to the lower side of the filter. In contrast, treatment with PE (0.313–5 μM) dose-dependently inhibited HMEC and HUVEC migration, yielding IC₅₀ values of $1.36 \pm 0.12 \mu\text{M}$ and $1.09 \pm 0.01 \mu\text{M}$, respectively.

PE suppresses endothelial cell adhesion. Adhesion of endothelial cells to the extracellular matrix enables the cells to respond to growth factors by migrating, proliferating and forming new blood vessels. Therefore, we investigated whether PE affects adhesion of endothelial cells to fibronectin, a common component of the extracellular matrix. Control HMECs attached efficiently to fibronectin after plating for 1 h, whereas HMECs treated with 0.1–20 μM PE showed a dose-dependent decrease in cell adhesion (Fig. 3C), with an IC₅₀ value of $2.84 \pm 0.18 \mu\text{M}$.

PE disrupts the capillary tube formation of endothelial cells. As organization of endothelial cells into a network of tubes is a late event during angiogenesis, we used a Matrigel-induced tube formation assay to determine whether PE treatment inhibits endothelial cell differentiation. Matrigel-cultured control HMECs migrated and organized into capillary-like enclosed tubular networks, whereas those treated with various concentrations of PE for 8 h showed dose-dependent inhibition of tube formation (Fig. 3D). Furthermore, PE treatment disrupted tube structure, leading to the development of incomplete tube morphologies. Similar results were obtained in

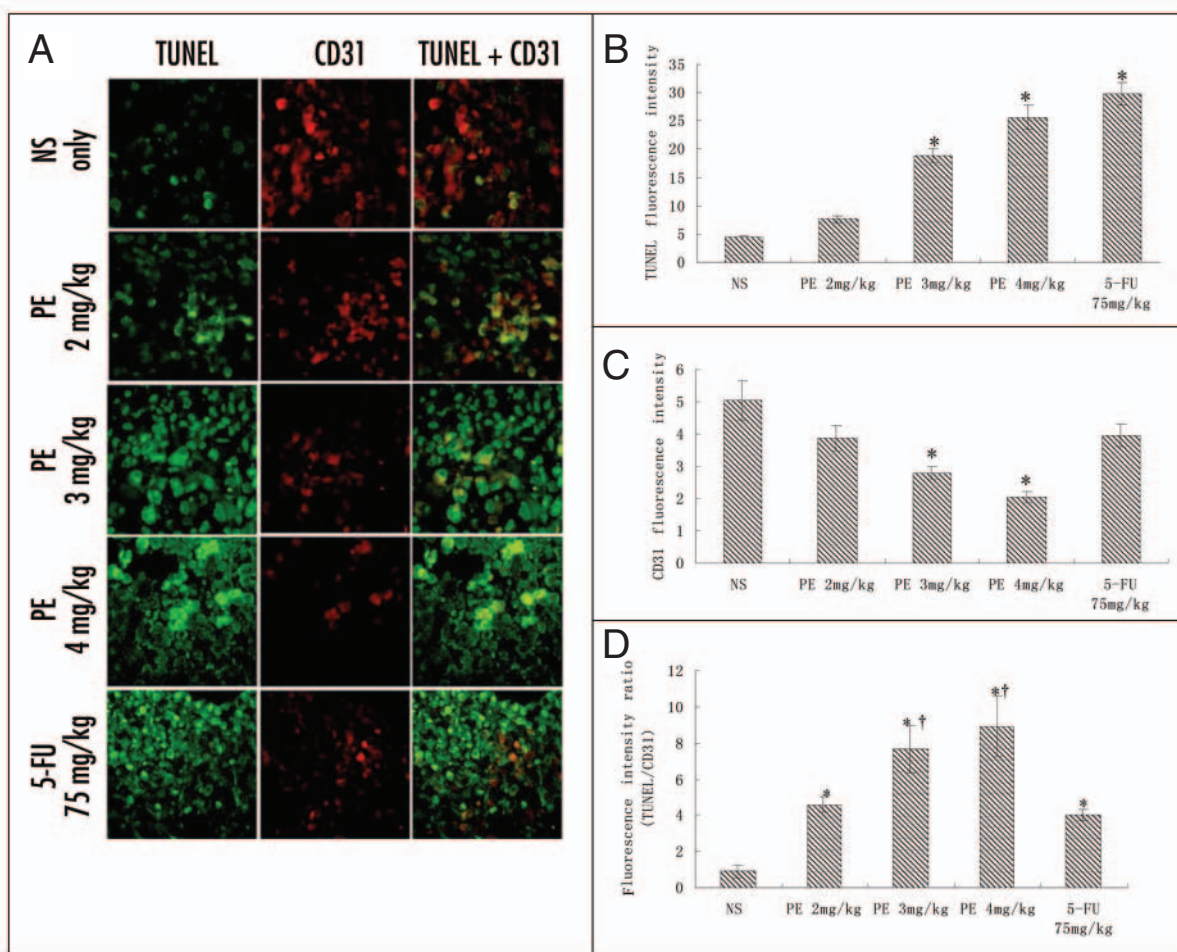


Figure 5. Immunofluorescent evaluation of tumor tissues. (A) Apoptosis detection in tumor tissues. Sequential staining for TUNEL and CD31 was performed in mouse sarcoma 180 tumor tissue sections taken from the normal saline (NS)-, PE- and 5-FU-treated groups. (B) Effect of PE and 5-FU on apoptosis of tumor cells. The data were gathered from three random fields per slice (from three random slices per tumor) and are presented as apoptotic fluorescence intensity (mean \pm SD). * $p < 0.01$ vs. control. (C) Effect of PE and 5-FU on tumor blood vessels. The data were gathered from three random fields per slice (from three random slices per tumor) and are presented as endothelial cell fluorescence intensity (mean \pm SD). * $p < 0.01$ vs. control. (D) Effect of PE and 5-FU on apoptosis of tumor-associated endothelial cells. The data were gathered from three random fields per slice (from three random slices per tumor) and are expressed as the mean \pm SD of the fluorescence intensity ratio (TUNEL/CD31). * $p < 0.01$ vs. control, $p < 0.01$ vs. 5-FU group.

HUVECs. PE at concentrations of 0.313, 0.625, 1.25, 2.5 and 5 μ M reduced tube formation by 3.28, 9.95, 41.03, 63.84, and 94.18% in HMECs and by 11.10, 19.94, 53.66, 82.38 and 97.77% in HUVECs, compared to control cells. The IC_{50} values were 1.52 ± 0.05 μ M in HMECs and 1.18 ± 0.02 μ M in HUVECs.

PE reduces neovascularization of the CAM. The CAM of the chicken embryo provides a unique model for evaluating new blood vessel formation and the response of new vessels to potentially anti-angiogenic agents. Using this model, we examined the potential anti-angiogenic activities of PE in vivo. In control eggs, blood vessels formed densely branching vascular networks (Fig. 4A). Treatment with PE caused a dramatic, dose-dependent inhibition of blood vessel numbers and branching patterns, with PE concentrations of 2.5, 5 and 10 nM/egg yielding inhibition rates of 51, 70.6 and 92.6%, respectively (Fig. 4B). Notably, PE at 10 nM/egg suppressed new blood vessel development not only in the treatment area, but also in the surrounding area. Moreover, 5 nM/egg PE had a similar anti-angiogenic effect to that of 100 nM/egg of suramin, a well-known angiogenesis inhibitor⁴ (69% inhibition).

PE counteracts tumor angiogenesis and induces apoptosis of endothelial cells in vivo. To further explore the anti-angiogenic and anti-tumor effects of PE in vivo, we next examined the effect of PE in two mouse models,

those harboring tumors induced by implantation of sarcoma 180 cells, and those harboring tumors induced by hepatoma 22 cells. Administration of 2, 3 and 4 mg/kg PE for 7 consecutive days following implantation of sarcoma 180 cells hindered tumor growth by 28.2, 55.6 and 60.7%, respectively (Table 1), while administration of 1, 2 and 3 mg/kg PE for seven consecutive days following implantation of hepatoma 22 cells suppressed tumor growth by 20.6, 46.1 and 59.4%, respectively (Table 2). These results indicate that PE has anti-tumoral activity in vivo.

Tumor specimens from PE-treated and control mice implanted with sarcoma 180 model cells were subjected to immunofluorescent staining with anti-CD31 for detection of endothelial cells and TUNEL staining for visualization of apoptosis. Anti-CD31 immunofluorescent staining revealed that PE dramatically decreased tumor microvessel density in a concentration-dependent fashion (Fig. 5A), while TUNEL staining revealed that treatment with 4 mg/kg PE induced a 9.8-fold increase in apoptotic tumor endothelial cells (Fig. 5D) and a 5.6-fold increase in apoptotic tumor cells (Fig. 5B). Collectively, these in vitro and in vivo results indicate that PE counteracts tumor angiogenesis and induces apoptosis of tumor endothelial cells. In contrast, while 5-FU (a representative chemotherapeutic agent) inhibited tumor growth and induced tumor cell apoptosis, this drug exerted almost no effect on tumor angiogenesis or endothelial cell apoptosis.

Table 1 Effect of PE on the in vivo growth of sarcoma 180 tumors

Tumor	Treatment group	Dosage (mg kg ⁻¹ d ⁻¹) × d	Mice/number (Initial/End)	Body weight (g) (Initial/End)	Tumor weight (g)	Inhibition rate (%)	p value
Sarcoma 180	Normal Saline	—	16/16	21.5/31.5	1.17 ± 0.41	—	—
	PE	2 × 7	8/8	21.6/26.8	0.84 ± 0.42	28.2	>0.05
	PE	3 × 7	8/8	21.3/20.9	0.52 ± 0.35	55.6	<0.01
	PE	4 × 7	8/8	21.3/20.4	0.46 ± 0.27	60.7	<0.01
	5-FU	75 × 2	8/8	21.5/24.3	0.19 ± 0.11	83.8	<0.01

Sarcoma 180 cells were implanted in mouse models, and the indicated drugs were administered once daily for seven days. On day eight after implantation of cells, mouse tumor tissues were harvested and weighed and the tumor growth inhibition rates were calculated. The data were expressed as mean ± SD from three independent experiments.

PE inactivates VEGF-induced tyrosine phosphorylation of VEGFR2 and arrests downstream signaling in HMECs. Angiogenesis and subsequent cancer growth and progression are often modulated by vascular endothelial growth factor (VEGF), a key mediator of angiogenesis that is produced by tumor cells. VEGF stimulates endothelial cell growth via binding and activation of the VEGF receptor 2 (KDR/Flk-1), which triggers downstream signaling pathways. Accordingly, we examined the effect of PE on VEGF₁₆₅-induced cellular KDR phosphorylation using Western blot analysis. When HMECs were stimulated with 50 ng/ml VEGF₁₆₅ for 5 min, KDR was strongly tyrosine phosphorylated. In contrast, preincubation of HMECs with 1.25–5 μM PE for 1 h prior to VEGF₁₆₅ stimulation reduced KDR tyrosine phosphorylation in a dose-dependent manner (Fig. 6). To examine signaling downstream of KDR, we further visualized the levels of activated FAK (downstream of KDR), paxillin (which associates with FAK and plays an important role in cell adhesion and migration,¹⁹), Akt (which regulates cell survival) and ERK (which regulates mitogenicity²⁰) in PE-treated cells. We found that VEGF₁₆₅ treatment stimulated the phosphorylation of these proteins in control cells, but that this phosphorylation was dose-dependently inhibited by 1 hr pretreatment of cells with 1.25–5 μM PE. These data suggest that PE can arrest multiple VEGF-associated signaling events in endothelial cells.

DISCUSSION

Once focused solely on tumor-selective cytotoxicity, cancer therapy research has branched out into considerations of anti-angiogenesis drugs arising from both synthetic and natural backgrounds. Here, we show that PE, a novel marine-derived compound, has both anti-angiogenic and anti-tumoral activities in vitro and in vivo. We found that noncytotoxic concentrations of PE are capable of inhibiting the main steps involved in angiogenesis, including endothelial cell proliferation, migration, adhesion and tube formation. In addition, these same concentrations of PE had a marked anti-angiogenic effect on our in vivo CAM model. Notably, our CAM assay revealed that

treatment with 5 nM/egg PE inhibited small vessel development comparably to the effect of 100 nM/egg of suramine, a well-known angiogenesis inhibitor.⁴ Further in vivo investigations in sarcoma 180 mouse tumor models demonstrated that PE treatment reduced blood vessel density in sarcoma tissues and dramatically increased endothelial cell apoptosis in tumors. And finally, PE exhibited significant anti-tumor activities in vivo, characterized by inhibition of sarcoma and hepatoma growth. Thus, our results show that PE is capable of conferring both anti-angiogenic and anti-tumoral activities in vitro and in vivo. In contrast, while 5-FU significantly inhibited tumor growth and induced tumor cell apoptosis, this well-known chemotherapeutic drug did not affect endothelial cell apoptosis or angiogenesis, indicating that these two drugs likely represent different modes of action.

Induction of apoptosis in tumor cells and tumor-associated endothelial cells is critical to anti-tumoral and anti-angiogenic activities,¹⁸ making induction of apoptosis a favorable strategy for anti-cancer therapeutics. Immunofluorescent analysis showed that PE treatment reduced the volumes of mouse sarcoma 180-induced tumors by triggering apoptosis of both tumor cells and tumor-associated endothelial cells. Endothelial cells were targeted more highly than tumor cells, indicating PE inhibited tumor growth mainly by inducing endothelial apoptosis rather than by inducing tumor cells apoptosis. This result consisted with other laboratory evidences that cytotoxic chemotherapy and anti-angiogenic therapy are each dependent on endothelial cell apoptosis.¹⁸ During cytotoxic chemotherapy, apoptosis of endothelial cells in the vascular bed of tumors precedes apoptosis of tumor cells.^{18,21} Administration of an angiogenesis inhibitor can increase tumor cell apoptosis and inhibit tumor growth by inhibiting endothelial proliferation and migration and/or by inducing endothelial apoptosis.¹⁸

Table 2 Effect of PE on the in vivo growth of hepatoma 22 tumors

Tumor group	Treatment (mg kg ⁻¹ d ⁻¹) × d	Dosage number (Initial/End)	Mice/number (Initial/End)	Body weight (g)	Tumor weight (%)	Inhibition rate (%)	p value
Hepatoma 22	Normal Saline	—	20/20	21.5/30.4	2.01 ± 0.43	—	—
	PE	1 × 7	10/10	21.5/29.3	1.31 ± 0.56	34.8	<0.01
	PE	2 × 7	10/10	21.5/25.1	1.45 ± 0.45	27.9	>0.05
	PE	3 × 7	10/10	21.6/23.5	0.96 ± 0.40	52.2	<0.01
	5-FU	75 × 2	10/10	21.7/24.4	0.37 ± 0.26	81.6	<0.01

Hepatoma 22 cells were implanted in mouse models, and the indicated drugs were administered once daily for seven days. On day eight after implantation of cells, mouse tumor tissues were harvested and weighed and the tumor growth inhibition rates were calculated. The data were expressed as mean ± SD from three independent experiments.

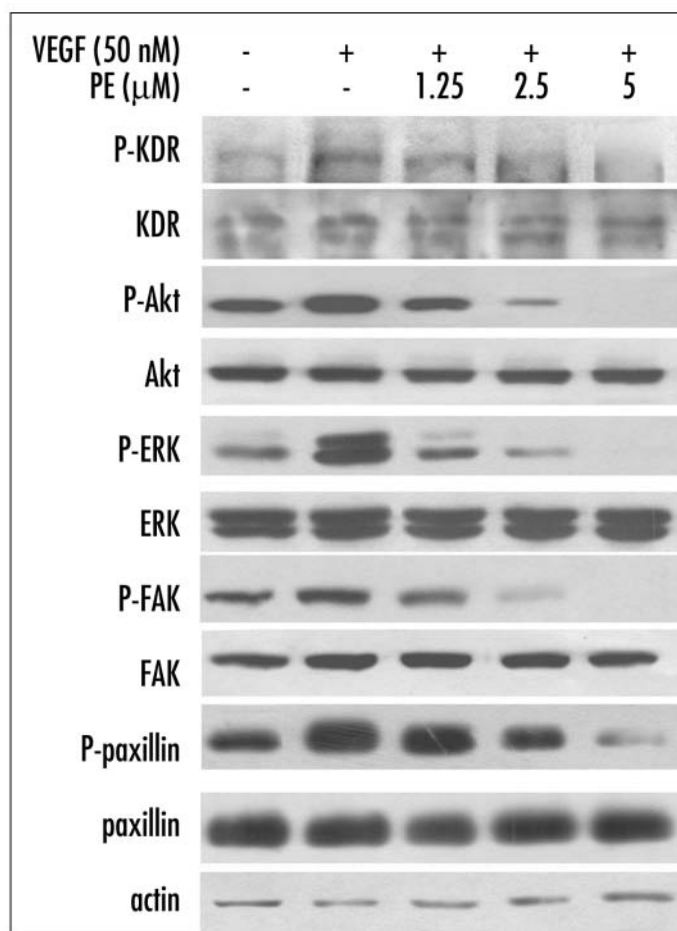


Figure 6. Immunoblot detection of tyrosine phosphorylation. The indicated cell lines were incubated with various concentrations of PE for 1 h at 37°C and stimulated for 5 min with the indicated growth factors. The cells were lysed and the resultant proteins were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with anti-phosphotyrosine antibody, stripped and reincubated with antibodies targeting the indicated signaling molecules.

The angiogenesis of endothelial cells is mediated by a number of important mitogenic factors, including VEGF, which binds to its receptor (VEGF Receptor 2/KDR) to trigger proliferation and migration of endothelial cells.^{22,23,24} Inhibition of VEGF-triggered cascades generally leads to profound anti-angiogenesis and subsequent anti-tumor activity.^{25,26} Here, we show that PE inhibits VEGF¹⁶⁵-induced KDR phosphorylation in HMECs, and dramatically hinders downstream KDR signaling by decreasing the activation (phosphorylation) of FAK, paxillin, Akt and ERK, which are required for the mitogenic activities of VEGF in endothelial cells. Since Akt inhibition is central to initiation of the apoptotic cascade, the apoptosis-induced action of PE on endothelial cells probably benefits its inhibition on Akt activation. Similarly, since FAK and ERK phosphorylation is involved in VEGF-induced endothelial cell migration and cell adhesion, PE-induced decreases in FAK and ERK activation may account for the observed effect of PE on endothelial cell migration and adhesion. Although future work will be required to fully elucidate the involved pathways, these results seem to indicate that PE exerts its anti-angiogenic effects (at least in part) by blocking VEGF-induced KDR activation and downstream signaling.

Recent studies have indicated that in cancer therapy, anti-angiogenic agents should be used in synergistic combinations with traditional cytotoxic or other molecular-targeting agents capable of providing anti-tumoral activities. Thus, it would seem beneficial to utilize single agents capable of acting against both angiogenesis and tumor growth. It is demonstrated that some (not all) conventional anti-cancer drugs possess dual cytotoxic and anti-angiogenic effects, including camptothecin, docetaxel and cyclophosphamide.^{27,28} On the other hand, many cytotoxic anti-tumor drugs have not the anti-angiogenic activities, even in the cytotoxic doses, such as carboplatin, mitomycin and etoposide. Here, we show that the marine-derived compound, PE, has both anti-tumoral and anti-angiogenic activities, suggesting that it may fill the need for a dual-acting agent. Future work will be required to identify the structure-activity relationship and structural optimization of PE. However, the present study provides strong evidence that this marine-derived compound may prove to be an attractive candidate for further preclinical testing as a new anti-neoplastic agent.

Reference

- Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; 1:27-31.
- Matter A. Tumor angiogenesis as a therapeutic target. *Drug Discov Today* 2001; 6:1005-24.
- Eskens FA. Angiogenesis inhibitors in clinical development; where are we now and where are we going? *Br J Cancer* 2004; 90:1-7.
- Deplanque G, Harris AL. Anti-angiogenic agents: Clinical trial design and therapies in development. *Eur J Cancer* 2000; 36:1713-24.
- Cattley RC, Radinsky RR. Cancer therapeutics: Understanding the mechanism of action. *Toxicol Pathol* 2004; 32:116-21.
- Rehman S, Jayson GC. Molecular imaging of antiangiogenic agents. *Oncologist* 2005; 10:92-103.
- Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 2003; 21:60-5.
- Haefner B. Drugs from the deep: Marine natural products as drug candidates. *Drug Discov Today* 2003; 8:536-44.
- Newman DJ, Cragg GM. Advanced preclinical and clinical trials of natural products and related compounds from marine sources. *Curr Med Chem* 2004; 11:1693-713.
- Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* 1988; 107:1589-98.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; 82:1107-12.
- Soeda S, Kozako T, Iwata K, Shimeno H. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: Its possible mechanism of action. *Biochim Biophys Acta* 2000; 1497:127-34.
- Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 1997; 272:15442-51.
- Tan DC, Kini RM, Jois SD, Lim DK, Xin L, Ge R. A small peptide derived from Flt-1 (VEGFR-1) functions as an angiogenic inhibitor. *FEBS Lett* 2001; 494:150-6.
- Orecchia A, Lacial PM, Schietroma C, Morea V, Zambruno G, Failla CM. Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the alpha 5 beta 1 integrin. *J Cell Sci* 2003; 116:3479-89.
- Gu J, Fujibayashi A, Yamada KM, Sekiguchi K. Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways. *J Biol Chem* 2002; 277:19922-8.
- Gupta MK, Qin RY. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* 2003; 9:1144-55.
- Folkman J. Angiogenesis and apoptosis. *Semin Cancer Biol* 2003; 13:159-67.
- Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor-protein kinase signaling. *Annu Rev Cell Dev Biol* 2003; 19:173-206.
- Zwick E, Bange J, Ullrich A. Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol Med* 2002; 8:17-23.
- Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000; 60:1878-86.
- Takahashi A, Sasaki H, Kim SJ, Tobisu K, Kakizoe T, Tsukamoto T, et al. Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 1994; 54:4233-7.

23. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; 55:3964-8.
24. Zachary I, Glick G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res* 2001; 49:568-81.
25. Madhusudan S, Harris AL. Drug inhibition of angiogenesis. *Curr Opin Pharmacol* 2002; 2:403-14.
26. Sun L, McMahon G. Inhibition of tumor angiogenesis by synthetic receptor tyrosine kinase inhibitors. *Drug Discov Today* 2000; 5:344-53.
27. Sweeney CJ, Miller KD, Sissons SE, Nozaki S, Heilman DK, Shen J, Sledge Jr GW. The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. *Cancer Res* 2001; 61:3369-72.
28. Albertsson P, Lennernas B, Norrby K. Chemotherapy and antiangiogenesis: Drug-specific effects on microvessel sprouting. *APMIS* 2003; 111:995-1003.