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Pseudolarix acid B inhibits angiogenesis by antagonizing the vascular endothelial growth factor-mediated anti-apoptotic effect

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Abstract

Angiogenesis is controlled by a number of growth factors, including vascular endothelial growth factor (VEGF). In this study, pseudolarix acid B, isolated from the traditional Chinese medicinal plant *Pseudolarix kaempferi* and originally identified as an early pregnancy-terminating agent, was evaluated for its potential as an angiogenesis inhibitor, using in vitro and in vivo models. After exposure to pseudolarix acid B 0.625–5 μM for 72 h, the proliferation of human umbilical vein endothelial cells was significantly inhibited. Pseudolarix acid B 0.313–2.5 μM for 24 h potently blocked the VEGF-induced tube formation of human umbilical vein endothelial cells in a dose-dependent manner. Matrigel plug assays disclosed that pseudolarix acid B reduced angiogenesis induced by VEGF in vivo. In addition, pseudolarix acid B antagonized VEGF-mediated anti-apoptotic effects on serum-deprived human umbilical vein endothelial cells and increased apoptosis of endothelial cells induced by VEGF in Matrigel plug assays. Moreover, pseudolarix acid B significantly inhibited VEGF-induced tyrosine phosphorylation of kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1), in correlation with a marked decrease in the phosphorylation of Akt and extracellular signal-regulated kinases (ERK). These findings collectively suggest that pseudolarix acid B possesses anti-angiogenic activity. One of the main anti-angiogenesis mechanisms of pseudolarix acid B may involve antagonism of the VEGF-mediated anti-apoptosis effect via inhibition of KDR/flk-1, ERK1/2, and Akt phosphorylation in endothelial cells.

Keywords: Pseudolarix acid B; Angiogenesis; Umbilical vein endothelial cell; Apoptosis; VEGF; KDR; (Human)

1. Introduction

Angiogenesis, the formation of new capillaries from pre-existing vessels, plays a key role in several physiological events, including embryonic development, follicular growth, and wound healing. Numerous pathological conditions, including diabetic retinopathy, atherosclerosis, various inflammatory diseases, tumor growth and metastasis, are characterized by undesirable neovascularization. (Folkman, 1995). Angiogenesis is tightly modulated through a balance of positive and negative regulatory factors. To date, several growth factors have been identified as possible mediators of angiogenesis. Among these, vascular endothelial growth factor (VEGF) is the most important positive angiogenic factor (Battegay, 1995; Veikkola et al., 2000).

VEGF is a homodimeric 32- to 42-kDa heparin-binding glycoprotein, which exerts multiple biological functions via two high affinity tyrosine-kinase receptors, specifically,

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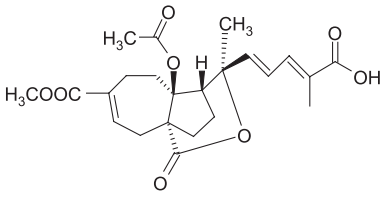

Fig. 1. Chemical structure of pseudolarix acid B.

vascular endothelial cell growth factor receptor-1 (VEGF-1 receptor; fms-like tyrosine kinase-1, flt-1) and VEGF-2 receptor (fetal liver kinase-1/kinase insert domain-containing receptor, flk-1/KDR). In addition to stimulating endothelial cell proliferation, sprouting, migration, and morphogenesis, VEGF functions as an important anti-apoptotic factor for endothelial cells during angiogenesis (Neufeld et al., 1999; Harmey and Bouchier, 2002). This anti-apoptotic function of VEGF is possibly crucial for its pro-angiogenic function. Of the two receptors of VEGF, KDR is predominantly involved in this anti-apoptotic effect. Following activation by VEGF, KDR transduces the anti-apoptotic signal via phosphatidylinositol 3-kinase (PI3-K), which further activates Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) pathways (Zachary, 2001; Zachary and Gliki, 2001).

The dependence of numerous pathological conditions on angiogenesis has led to the proposal that these diseases may be controlled by the use of anti-angiogenic agents (Pepper, 1997). Investigations of anti-angiogenic agents have been conducted in preclinical and clinical trials, and multiple agents have been developed, such as fragments of naturally occurring proteins, neutralizing antibodies to angiogenic factors, kinase inhibitors, and antibiotic derivatives (Gourley and Williamson, 2000; Ellis et al., 2001). In addition to these inhibitors, many anti-angiogenic compounds (e.g., genistein, curcumin, and quercetin) have been derived from plants (Marwick, 2001; Tosetti et al., 2002; Tan et al., 2003). Our group has an ongoing interest in identifying novel anti-angiogenic inhibitors from small molecular compounds extracted from Chinese natural plants. In this study, we assess the in vitro and in vivo anti-angiogenic activity of pseudolarix acid B (Fig. 1), a natural compound from a traditional Chinese medicine. To further elucidate the anti-angiogenic mechanism, we established a human umbilical vein endothelial cells apoptosis model induced by serum deprivation and investigated the influence of pseudolarix acid B on the anti-apoptotic effect mediated by VEGF.

2. Materials and methods

2.1. Materials

Type-I collagenases, the antibody to vWF (von Willebrand factor), endothelial cell growth supplement (ECGS), epidermal growth factor (EGF), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), 4,6-diamino-2-phenylindole (DAPI) and propidium iodide were purchased from Sigma (St. Louis, MO, USA). M199 medium and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). The 3D collagen cell culture system was obtained from Chemicon Int. (Temecula, CA, USA). VEGF was purchased from R&D Systems (Minneapolis, MN, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit was obtained from Promega (Madison, WI, USA). Matrigel and primary monoclonal antibody against CD31 were obtained from BD Biosciences, (San Jose, CA, USA). Primary antibodies for phosphorylated KDR, Akt, ERK and total protein kinase of KDR, Akt, ERK were obtained from New England Biolabs (Beverly, MA, USA). Secondary antibodies were obtained from Calbiochem. (San Diego, CA, USA). The enhanced chemiluminescence detection system used was purchased from Pierce Biotech. (Rockford, IL, USA).

2.2. Extraction and isolation of compounds

Dried root bark (10 kg) of *Pseudolarix kaempferi* was ground and percolated with 95% ethanol. After filtration and removal of the solvent, the ethanol extract was suspended in 2 l of 5% $NaHCO_3$ solution and immediately extracted with ethyl acetate to obtain a neutral ethyl acetate-soluble fraction. The aqueous solution was treated with 5% HCl solution to about pH 6 and extracted with ethyl acetate again to obtain an acidic ethyl acetate-soluble fraction. The acidic fraction was subjected to silica gel column chromatography using a gradient solvent system of petroleum–ethyl acetate (4:1 to 2:1) followed by chloroform–methanol (10:1 to 0:1). Seven major fractions (1–7) were collected. Fraction 3, which contained one major compound, was recrystallized from petroleum ether–actone (4:1) to yield pseudolarix acid B, which was extensively recrystallized in 95% ethanol to afford pseudolarix acid B with a purity of 99.3%, checked by high-performance liquid chromatography (HPLC).

Pseudolarix acid B was dissolved in dimethyl sulfoxide (DMSO). The concentration of DMSO was maintained below 0.1% (v/v) in pseudolarix acid B-treated groups. DMSO [0.1% (v/v)] was used as a vehicle control throughout the study.

2.3. Cell culture

Human umbilical vein endothelial cells were isolated from human umbilical cord veins by digestion with 0.1% type-I collagenases at 37 °C for 15 min, and their identity was confirmed by immunofluorescence staining for the vWF. Human umbilical vein endothelial cells were cultured in M199 medium supplemented with 20% heat-inactivated FBS, 30 μg/ml ECGS, 10 ng/ml EGF, 100 units/ml penicillin and 100 μg/ml streptomycin in a 37 °C incubator

with a humidified atmosphere containing 5% CO_2. Cells at passages 3–7 were used in experiments (Jaffe et al., 1973).

For serum starvation, human umbilical vein endothelial cells were cultured in complete medium, as described above, until 90–100% confluence, washed twice with phosphate buffered saline (PBS) and cultured in serum-free medium (M199 medium containing 0.1% bovine serum albumin without ECGS or EGF) for the indicated time periods.

2.4. Measurement of human umbilical vein endothelial cells growth inhibition

Inhibition of human umbilical vein endothelial cells growth by pseudolarix acid B was measured using the MTT assay. Briefly, human umbilical vein endothelial cells were seeded into 96-well microculture plates at a density of 8×10^3 cells/well and allowed to attach for 24 h before the addition of pseudolarix acid B. Cells were exposed to 0.625, 1.25, 2.5, and 5 μM pseudolarix acid B for 72 h. Each concentration of pseudolarix acid B was tested in triplicate. Next, 20 μl of 5 mg/ml MTT was added to each well and plates were incubated for 4 h at 37 °C. 'Triplex solution (10% sodium dodecyl sulfate (SDS)–5% isobutanol–0.012 M HCl)' was added and the plates were incubated for 12–20 h at 37 °C. Optical density (OD) was measured on a plate reader (VERSAmax, Molecular Devices, USA) at a wavelength of 570 nm. Media and DMSO control wells, in which pseudolarix acid B was absent, were included in all the experiments. The rate of inhibition of cell proliferation was calculated using the following formula: Growth inhibition (%)=$(OD_{control}-OD_{treated})/OD_{control}\times100$. Data were obtained from three independent experiments.

2.5. Tube formation assay

The tube formation assay was conducted using a 3D collagen cell culture system. Briefly, 96-well plates were coated with a mixture of collagen, 5×M199 medium, and neutralizing buffer according to the manufacturer's instructions. After the mixture had solidified at 37 °C, human umbilical vein endothelial cells (1.5×10^4 cells) suspended in 2% FBS medium without or with VEGF (50 ng/ml), containing either vehicle or various concentrations of pseudolarix acid B, were seeded on the collagen. Following incubation for 24 h, capillary networks were photographed using a phase-contrast microscope (Olympus, IX×70, Japan), and the number of tubes was quantified from five random fields (Ashton et al., 1999).

2.6. Matrigel plug assay

The effect of pseudolarix acid B on angiogenesis in vivo was examined using a Matrigel plug assay. In brief, chilled Matrigel (0.4 ml) containing 20 units/ml heparin and 150 ng/ml VEGF with or without various doses of pseudolarix acid B was injected subcutaneously into 7- to 8-week-old female C57BL/6J mice. After 7 days, Matrigel plugs were recovered and fixed in 10% neutral-buffered formalin solution. The plugs were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

2.7. Determination of apoptosis

Three methods, including DAPI staining, TUNEL staining, and flow cytometric analysis, were used to detect cell apoptosis. Subconfluent human umbilical vein endothelial cells seeded on gelatin-coated glass coverslips were incubated in 10% serum or serum-free medium for 24 h. The latter medium was supplemented with vehicle, 2.5 μM pseudolarix acid B, 50 ng/ml VEGF alone, or 50 ng/ml VEGF plus pseudolarix acid B (1.25 or 2.5 μM). After treatment for another 24 h, cells were fixed in 4% paraformaldehyde for DAPI and TUNEL staining. Stained cells on coverslips were observed and photographed under a fluorescent microscope (Olympus, Japan). For flow cytometric analysis, subconfluent human umbilical vein endothelial cells in six-well plates were treated as described above. Following treatment, cells were collected and washed with ice-cold PBS. DNA was stained by incubation with 50 μg/ml propidium iodide solution for 60 min. The DNA content was measured by flow cytometry (Becton Dickinson Labware, Franklin Lakes, NJ), and the percentage of subdiploid cells was quantified.

2.8. Immunofluorescent double staining for CD31 and TUNEL examined by confocal microscopy

The Matrigel plug assay was performed as described above. Matrigel plugs recovered from mice were embedded in optimum cutting temperature compound (Miles, IN) and rapidly frozen in liquid nitrogen. Frozen tissue sections (10 μm) were fixed with cold acetone for 5 min and incubated overnight at 4 °C with primary monoclonal antibody against CD31. Sections were incubated for 1 h at room temperature with secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC). TUNEL staining was performed as specified above. Computer-assisted image analysis of fluorescence was performed using a confocal microscopy scanning laser microscope (Leica TCS, Germany).

2.9. Western blot analysis

Human umbilical vein endothelial cells cultured in serum-free medium for 18–20 h were incubated in fresh serum-free medium in the absence or presence of various concentrations of pseudolarix acid B for 2 h, followed by incubation with 50 ng/ml VEGF for 15 min. Cell lysates were prepared in lysis buffer (2 mM sodium orthovanadate, 50 mM NaF, 20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM sodium pyrophosphate, 10% glycerol, 0.2%

Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice for 30 min. Samples were cleared by centrifugation for 15 min at 15,000 rpm at 4 °C. Equal amounts of protein were separated on a SDS polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies for phosphorylated KDR, Akt, ERK and total protein kinase of KDR, Akt, ERK, followed by incubation for 1 h with secondary antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system.

2.10. Data analysis

Results are expressed as $\bar{X}\pm$S.E., and statistical significance was assessed using Student's *t*-test.

3. Results

3.1. Inhibitory effect of pseudolarix acid B on the growth of human umbilical vein endothelial cells

Pseudolarix acid B 0.625–5 μM significantly inhibited the proliferation of human umbilical vein endothelial cells for 72 h in a concentration-dependent manner with an IC_{50} value of approximately 1.58 μM (Fig. 2). Pseudolarix acid B had no effect on the growth of human umbilical vein endothelial cells at 0.313 or 0.625 μM, and exhibited a slight growth inhibition at 1.25, 2.5, and 5.0 μM of, respectively, 19.3%, 22.6%, and 26.3% after an exposure period of 24 h. Therefore, the conditions of pseudolarix acid B treatment for investigating the anti-angiogenic effect on human umbilical vein endothelial cells were chosen to be a concentration less than 2.5 μM and for an exposure of 24 h.

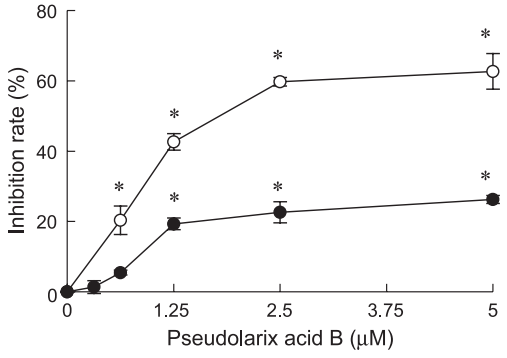

Fig. 2. Effect of pseudolarix acid B on growth inhibition of human umbilical vein endothelial cells. Endothelial cells seeded in 96-well plates were exposed to pseudolarix acid B for 24 h (●) or 72 h (○), and growth inhibition was examined in MTT assays. Values are expressed as means±S.E. from three independent experiments. *P<0.001 vs. control, n=3.

3.2. Anti-angiogenic effect of pseudolarix acid B in vitro

After a 24-h incubation on collagen, human umbilical vein endothelial cells incubated with 2% FBS failed to form tubular networks. When the cells were incubated with 2% FBS plus VEGF, they migrated and organized into extensive and enclosed tubular networks, resembling capillaries. The tube formation triggered by VEGF was reversed by pseudolarix acid B 0.313–2.5 μM in a dose-dependent manner, resulting in the formation of incomplete and sparse tubular networks (Fig. 3). When compared to the growth inhibition effect of pseudolarix acid B at 24 h, the inhibitory effects of pseudolarix acid B on tube formation was significantly higher than that on growth inhibition at corresponding concentrations. Pseudolarix acid B at non-cytotoxic concentrations, 0.313 and 0.625 μM, still inhibited tube formation by 46.9% and 77.3%. This showed that the inhibition by pseudolarix acid B on endothelial cell tube formation was not induced by its cytotoxic action.

3.3. Anti-angiogenic effect of pseudolarix acid B in vivo

We further assessed the in vivo anti-angiogenic effect of pseudolarix acid B using Matrigel plug assays. Seven days after subcutaneous injection of Matrigel into mice, plugs containing VEGF exhibited intense neovascularization, whereas those supplemented with 8 mM or 16 mM pseudolarix acid B exhibited less vessel formation. These results demonstrate that pseudolarix acid B suppresses angiogenesis in vivo. Matrigel plugs supplemented with heparin alone did not display neovessel formation (Fig. 4).

3.4. Suppression of the VEGF-mediated anti-apoptotic effect on human umbilical vein endothelial cells by pseudolarix acid B

To characterize the anti-angiogenic mechanism of action of pseudolarix acid B, we investigated whether the compound inhibits VEGF-mediated anti-apoptosis of human umbilical vein endothelial cells cultured in serum-free medium. Serum deprivation for 24 h resulted in large numbers of apoptotic human umbilical vein endothelial cells (28.4%) compared to the number of apoptotic cells under 10% serum culture conditions (8.4%), as determined by flow cytometry. Addition of 50 ng/ml VEGF inhibited the apoptosis of human umbilical vein endothelial cells induced by serum deprivation (apoptotic rate of 15.5%). Notably, human umbilical vein endothelial cells incubated with VEGF and pseudolarix acid B (1.25 and 2.5 μM) displayed significantly more apoptosis (28.5% and 30.3% respectively) than did cells incubated with VEGF alone. Treatment with pseudolarix acid B (2.5 μM) in the absence of VEGF slightly enhanced the apoptotic rate of human umbilical vein endothelial cells (38.3%), compared to that of cells grown under conditions of serum deprivation. However, no

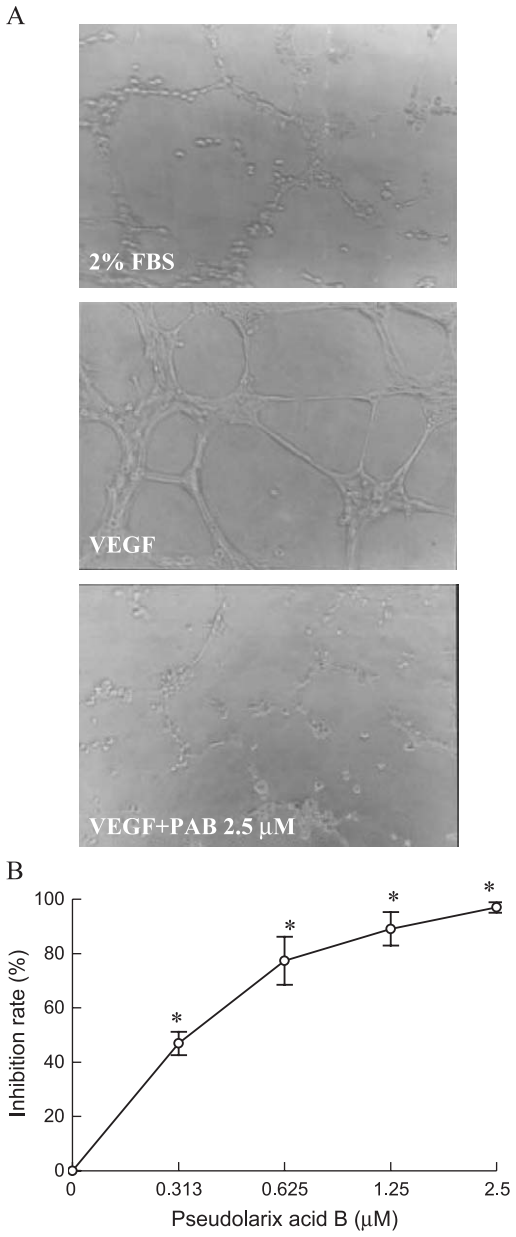

Fig. 3. Effect of pseudolarix acid B on VEGF-induced human umbilical vein endothelial cells tube formation. Various concentrations of pseudolarix acid B supplemented with 50 ng/ml VEGF were added to collagen. After incubation for 24 h, capillary networks were photographed and quantified. (A) Representative photographs from three independent experiments. Magnification: ×100. (B) Rate of inhibition of human umbilical vein endothelial cells tube formation by pseudolarix acid B. The number of tube structures was measured and expressed as a percentage of control. Results are expressed as means±S.E., *P<0.001 vs. control, n=5.

significant differences (P>0.05) were observed in the rates of apoptosis between the two groups (Fig. 5A). Quantitative results were supported by DAPI staining, which revealed morphological changes characteristic of apoptosis, including nuclear condensation and apoptotic bodies (Fig. 5B), and by the TUNEL assay, which showed biochemical alterations typical of apoptotic fragmentation of DNA (Fig. 5C). The data indicated that VEGF prevents human umbilical vein endothelial cells from undergoing apoptosis induced by serum starvation and that pseudolarix acid B antagonizes the VEGF-mediated anti-apoptotic effects on human umbilical vein endothelial cells cultured in serum-free medium.

3.5. Antagonism of VEGF-mediated anti-apoptosis of endothelial cells in vivo by pseudolarix acid B

To further confirm that pseudolarix acid B could suppresses the VEGF-mediated anti-apoptotic effect on endothelial cells, the apoptotic endothelial cells present in the neovasculature induced by VEGF in an in vivo Matrigel plug model were identified by immunofluorescent double staining with an endothelial cell marker, CD31, and the apoptosis marker, TUNEL. Immunofluorescent staining

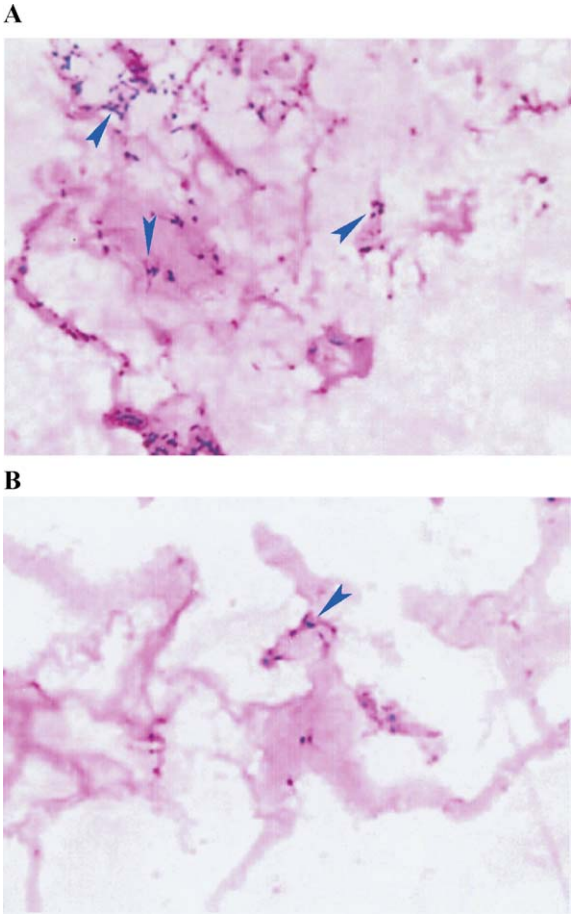

Fig. 4. Angiogenesis inhibition by pseudolarix acid B in the Matrigel plug assay. The chilled Matrigel (0.4 ml) containing 20 units/ml heparin and 150 ng/ml VEGF, with or without various doses of pseudolarix acid B, was injected subcutaneously in C57BL/6J mice. After 7 days, mice were killed and the excised Matrigel plugs were tested for hematoxylin and eosin staining. (A) Control Matrigel plugs containing heparin and VEGF. (B) Matrigel plugs containing heparin, VEGF and pseudolarix acid B (16 mM) (×200). Each photograph represents data obtained from at least five mice. Similar results were obtained from three separate experiments. Arrows indicate neo-angiogenesis.

revealed a significant number of CD31-positive endothelial cells forming neovasculature structures in the VEGF group and few TUNEL-positive cells. Treatment with pseudolarix acid B (8 or 16 mM) resulted in an increased number of apoptotic endothelial cells (Fig. 6). From these findings, we conclude that pseudolarix acid B significantly inhibits angiogenesis in vivo by suppressing the VEGF-mediated anti-apoptotic effect on endothelial cells.

3.6. Inhibition of VEGF-dependent phosphorylation of KDR, Akt, and ERK by pseudolarix acid B

To determine the potential involvement of protein kinase pathways in the pseudolarix acid B-induced suppression of the VEGF-mediated anti-apoptotic effect, we analyzed the phosphorylation status of KDR, Akt, and ERK. As illustrated in Fig. 7, we observed basal

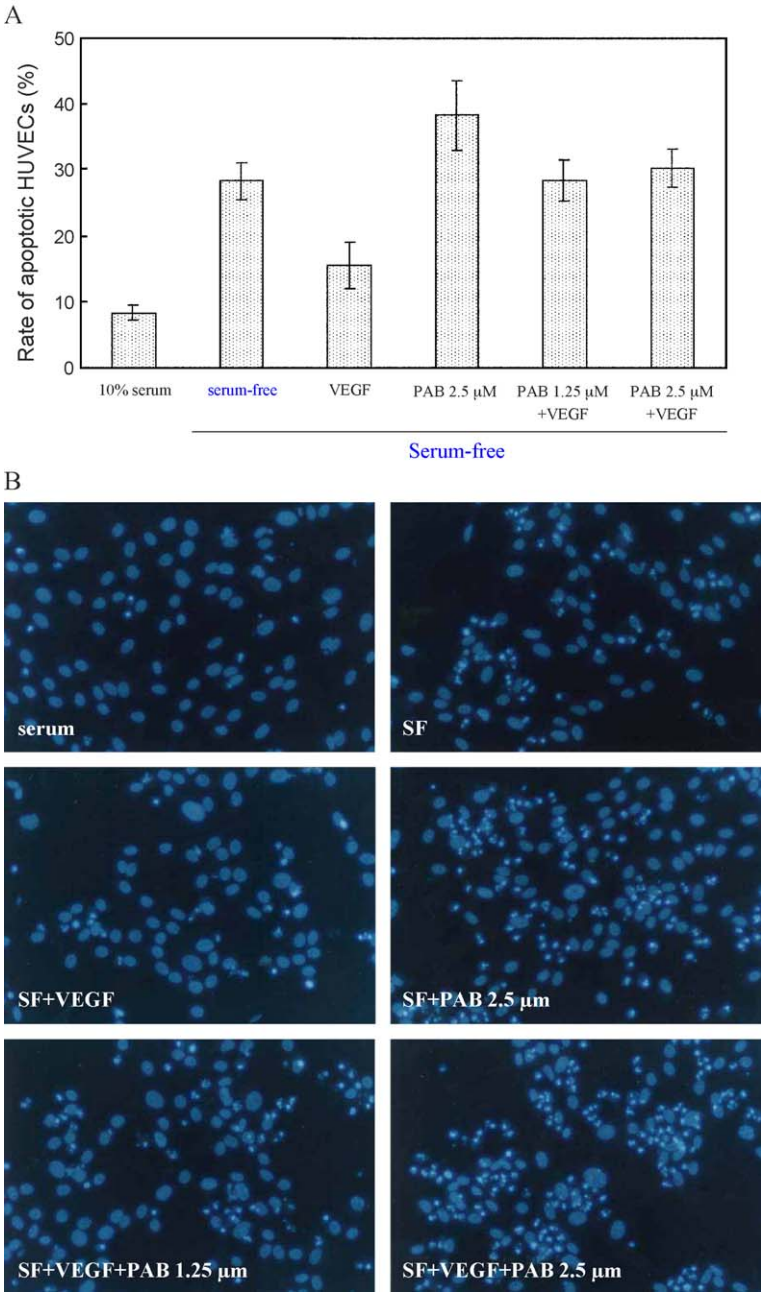

Fig. 5. Antagonism by pseudolarix acid B of VEGF-mediated anti-apoptotic effects in vitro. Subconfluent human umbilical vein endothelial cells were incubated in either 10% serum or serum-free medium containing 2.5 μM pseudolarix acid B, 50 ng/ml VEGF, or 50 ng/ml VEGF in the presence of pseudolarix acid B (1.25 or 2.5 μM) for 24 h. (A) Quantification of apoptotic human umbilical vein endothelial cells by flow cytometric analysis of propidium iodide staining. Data are expressed as means±S.E. from a triplicate assay. 10% serum vs. serum-free, $P<0.05$; serum-free vs. serum-free+VEGF, $P<0.05$; serum-free vs. serum-free+PAB (2.5 μM), $P>0.05$; serum-free+VEGF vs. serum-free+VEGF+PAB (1.25 μM), $P<0.05$; serum-free+VEGF vs. serum-free+VEGF+PAB (2.5 μM), $P<0.05$. Representative photographs of apoptotic human umbilical vein endothelial cells stained with DAPI (B) and detected by TUNEL assays (C) from three independent experiments (×100). *SF: serum-free; PAB: pseudolarix acid B.

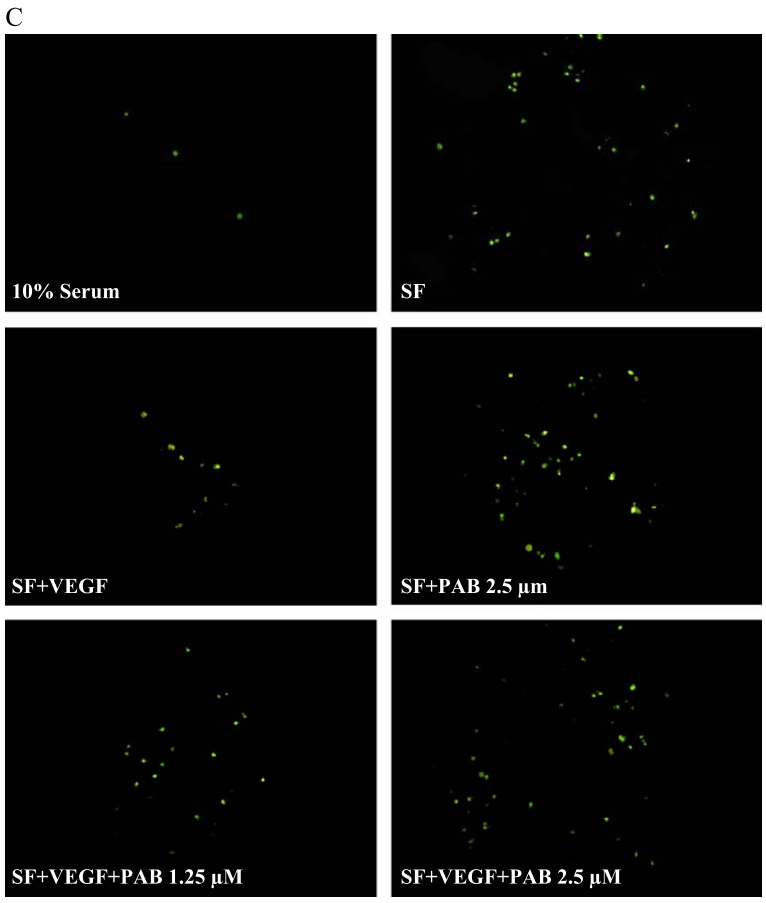

Fig. 5 (*continued*).

phosphorylation of KDR, Akt and ERK in human umbilical vein endothelial cells incubated in serum-free medium. Following stimulation by VEGF, higher levels of phosphorylation of KDR, Akt, and ERK were observed. Pretreatment with pseudolarix acid B (1.25 or 2.5 μM) inhibited the phosphorylation of KDR induced by VEGF, in correlation with a marked decrease in the efficiency of VEGF in inducing pERK and pAKT activation. In addition, incubation of human umbilical vein endothelial cells with pseudolarix acid B in the absence of VEGF resulted in a weak decrease in the basal phosphorylation of KDR, Akt, and ERK, but there was no statistical difference when compared to those of human umbilical vein endothelial cells incubated under serum-free conditions. Treatment with pseudolarix acid B caused no change in the level of total protein of KDR, Akt, and ERK (Fig. 7). These observations suggest that reduction in the phosphorylation levels of KDR, Akt, and ERK by pseudolarix acid

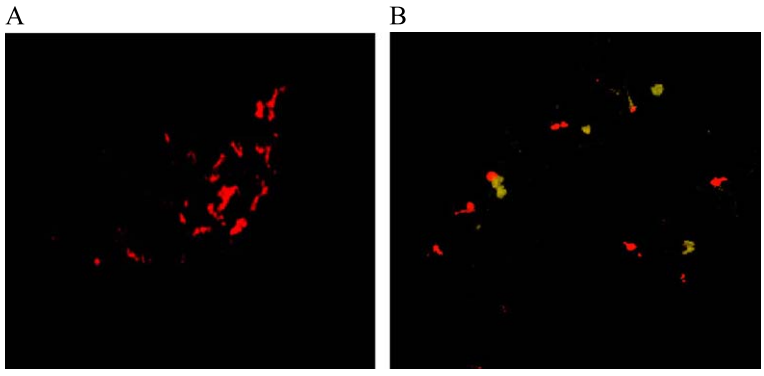

Fig. 6. Antagonism by pseudolarix acid B of the VEGF-mediated anti-apoptotic effect in vivo. Apoptotic endothelial cells forming new vessels induced by VEGF in a Matrigel plug as examined by immunofluorescent double staining for CD31 (labeled with TRITC; red) and TUNEL (labeled with FITC; green). Representative photographs of control (A) and 16 mM pseudolarix acid B (B) from a triplicate assay (×400). Cells stained red represent non-apoptotic endothelial cells; whereas those stained both red and green (yellow appearance) signify apoptotic endothelial cells. n=5.

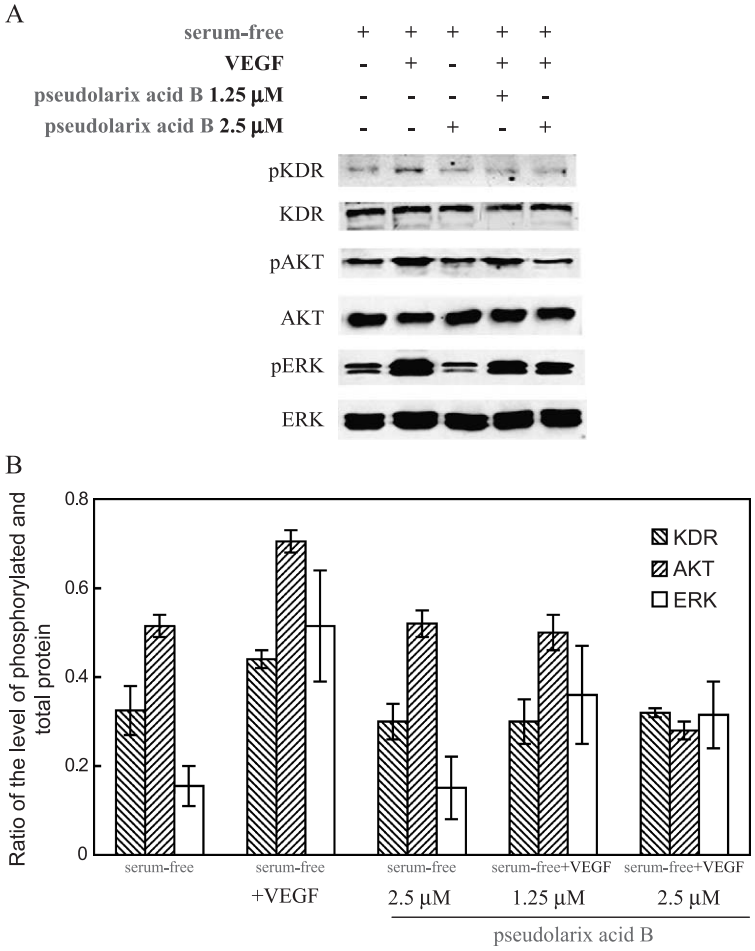

Fig. 7. Inhibition of VEGF-dependent phosphorylation of KDR, Akt, and ERK. Subconfluent human umbilical vein endothelial cells were incubated in serum-free medium for 18–20 h. Next, human umbilical vein endothelial cells were incubated in fresh serum-free medium in the absence or presence of various concentrations of pseudolarix acid B for 2 h, followed by treatment with 50 ng/ml VEGF for 15 min. Western blot assays were conducted as specified in Materials and methods. (A) Representative photographs from three separate experiments. (B) Densitometric analysis disclosed the relative ratio of phosphorylated KDR/total KDR, phosphorylated Akt/total Akt, and phosphorylated ERK/total ERK. Data are presented as means±S.E. of the ratios from three independent experiments. serum-free vs. serum-free+VEGF, $P<0.05$; serum-free vs. serum-free+pseudolarix acid B (2.5 μM), $P>0.05$; serum-free+VEGF vs. serum-free+VEGF+pseudolarix acid B (1.25 μM), $P<0.05$; serum-free+VEGF vs. serum-free+VEGF+pseudolarix acid B (2.5 μM), $P<0.05$.

B may be involved in the antagonism of the VEGF-mediated anti-apoptotic effect.

4. Discussion

P. kaempferi is a plant indigenous to the east of China. Its root bark, known as 'Tu-jin-Pi', is used as a traditional Chinese medicine for the treatment of skin disease caused by microbial infection. Pseudolarix acid B is one of the most important natural diterpenoid compounds isolated from the root bark of *P. kaempferi* Gord. The compound possesses anti-fungal, anti-fertility, and cytotoxic activities (Zhou et al., 1983; Pan et al., 1990). Among these functions, the anti-fertility effect of pseudolarix acid B is well established, and it is recognized as an early pregnancy-terminating agent in China. Treatment with 5–40 mg/kg pseudolarix acid B for 7–15 days after mating terminated early pregnancies in treated rats, rabbits, and dogs and caused a decrease in endo- and myometrial blood flow and necrosis of the endothelium (Wang et al., 1982; Wang et al., 1988; Wang et al., 1991; Zhang et al., 1990). However, the mechanism of the anti-fertility effect is currently unclear. It is well known that pregnancies are strictly angiogenesis-dependent. We propose that the anti-angiogenic effect of pseudolarix acid B may account for its early pregnancy-terminating activity. The decrease in endo- and myometrial blood flow and necrosis during treatment may be due to inhibition of angiogenesis and ischemia. Consistent with this propose, pseudolarix acid B potently inhibited the proliferation, invasion, migration, adhesion and tube formation of human microvascular endothelial cells as well as the neo-vascularization of chick embryo (data will be published).

The angiogenesis process can be separated into the following main steps: degradation of the basement membrane of existing blood vessels, migration and proliferation of endothelial cells, and organization of

endothelial cells into capillary tubes (Klagsbrun and Moses, 1999). This process is critically dependent on the preservation of endothelial cell viability. Considerable attention has been focused on VEGF as a survival factor for endothelial cells in angiogenesis. VEGF prevents the apoptosis of endothelial cells induced by serum starvation (Gerber et al., 1998). In vivo studies established that VEGF potently inhibited apoptosis of the endothelial cells of newly formed retinal vessels in neonatal rats exposed to hypoxia (Alon et al., 1995). Jain et al. (1998) demonstrated that VEGF protects endothelial cells of newly formed immature tumor blood vessels from apoptosis. Bruns et al. (2000) reported that therapies targeting KDR not only inhibited angiogenesis but also led to apoptosis of endothelial cells in liver metastases from colon carcinoma. These studies collectively confirm that VEGF is an important anti-apoptotic factor for endothelial cells in newly formed vessels. There is increasing evidence that the induction of endothelial cell apoptosis is an important anti-angiogenic mechanism (Brooks et al., 1994). In the present study, we demonstrated that pseudolarix acid B significantly inhibited the proliferation of human umbilical vein endothelial cells. VEGF could induce the tube formation of human umbilical vein endothelial cells, which could clearly be inhibited by pseudolarix acid B at concentrations that hardly affected cell proliferation. In addition, pseudolarix acid B abrogated the anti-apoptotic effect mediated by VEGF. The results suggest that suppression of the VEGF-mediated anti-apoptotic effect by pseudolarix acid B may contribute to its anti-angiogenic mechanism. This theory was further confirmed by the finding that pseudolarix acid B induced apoptosis of the endothelial cells that formed new vessels in Matrigel plug assays. The dual cytotoxic and anti-angiogenetic activities of pseudolarix acid B are consistent with those of some other conventional anti-cancer agents (Miller et al., 2001; Schirner, 2000), such as cyclophosphamide (Albertsson et al., 2003), camptothecin (Clements et al., 1999), docetaxel (Grant et al., 2003), and 10-hydroxycamptothecin (Xiao et al., 2001). Thus this type of anti-tumor substance might have potential as a new approach to treating malignant tumors.

The biological effects of VEGF are mainly mediated via two surface receptors mainly expressed on endothelial cells, specifically, VEGF-1 (Flt-1) and VEGF-2 (KDR or Flk-1). KDR strongly induces kinase activity in response to VEGF. VEGF binding stimulates KDR dimerization and autophosphorylation, and generates numerous binding sites for molecules with Src-Homology 2 (SH2) domains of appropriate specificity, leading to successive activation of several protein kinase cascades. Two prominent signaling pathways may play a central role in the VEGF-mediated anti-apoptotic effect on endothelial cells. One is the PI3K/Akt pathway. Once activated, Akt inhibits apoptosis in a number of ways, including phosphorylation and inactivation of the proapoptotic Bcl-2 homologue, Bad, and the apoptosis-initiating enzyme caspase-9 (Gerber et al., 1998; Liu et al., 2000). The classic MAPK/ERK pathway is another key component in the transduction of signals for survival. This pathway consists of a linear cascade of protein kinases, specifically, Raf, MEK, and MAPK/ERK (Liu et al., 2002). Data from the current study clearly demonstrate that pseudolarix acid B inhibits the phosphorylation of KDR induced by VEGF, but does not affect the total protein level of KDR. Furthermore, pseudolarix acid B decreased the phosphorylation of Akt and ERK, the downstream signal molecules of KDR, but did not affect the total protein content of the respective protein kinases. These results verify that pseudolarix acid B inhibits KDR autophosphorylation activated by VEGF in human umbilical vein endothelial cells, and subsequently influences the functions of two main downstream signaling pathways, PI3K/Akt and MAPK/ERK, and then inhibits the VEGF-mediated anti-apoptotic effect. Pseudolarix acid B additionally induced a weak decrease in the basal level of phosphorylation of KDR, Akt, and ERK in the absence of exogenous VEGF. These changes are consistent with the finding that pseudolarix acid B induces apoptosis only slightly in human umbilical vein endothelial cells cultured in the absence of exogenous VEGF. These data further corroborate the suggestion that the antagonism by pseudolarix acid B of the VEGF-mediated anti-apoptotic effect is mediated via inhibition of the phosphorylation and activation of KDR upon VEGF binding, and successive dephosphorylation of its downstream signaling molecules, Akt and ERK. To date, there are no reports on any compounds that suppress the VEGF-elicited anti-apoptosis effect via synchronous inhibition of phosphorylation of KDR as well as Akt and ERK, which are crucial proteins of KDR downstream signaling pathways. Thus, pseudolarix acid B may be a novel potent angiogenesis inhibitor that possesses this particular mechanism.

In conclusion, pseudolarix acid B displays potent anti-angiogenic properties. These properties may be attributed to antagonism of the VEGF-mediated anti-apoptosis effect via the dephosphorylation and inactivation of KDR/flk-1, followed by down-regulation of the PI3K/Akt and MAPK/ERK signaling pathways. The compound may be an effective agent against angiogenesis-related diseases, such as solid tumors, and rheumatoid arthritis. However, the solubility of pseudolarix acid B is poor. We are now synthesizing a series of derivatives of pseudolarix acid B in order to find a good solubility compound which also possesses potent anti-angiogenic effect.

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References

Albertsson, P., Lennenaes, B., Norrby, K., 2003. Chemotherapy and antiangiogenesis: drug-specific effects on microvessel sprouting. APMIS 111, 995–1003.

Ashton, A.W., Yokota, R., John, G., Zhaoi, S., Suadicanii, S.O., 1999. Inhibition of endothelial cell migration, intercellular communication, and vascular tube formation by thromboxane A2. J. Biol. Chem. 274, 35562–35570.

Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J., Keshet, E., 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat. Med. 1, 1024–1028.

Battegay, E.J., 1995. Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. J. Mol. Med. 73, 333–346.

Brooks, P.C., Montgomery, A.M., Rosenfeld, M., Reisfeld, R.A., Hu, T., Klier, G., Cheresh, D.A., 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79, 1157–1164.

Bruns, C.J., Liu, W., Davis, D.W., Shaheen, R.M., McConkey, D.J., Wilson, M.R., Bucana, C.D., Hicklin, D.J., Ellis, L.M., 2000. Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer 89, 488–499.

Clements, M.K., Jones, C.B., Cumming, M., Daoud, S.S., 1999. Antiangiogenic potential of camptothecin and topotecan. Cancer Chemother. Pharmacol. 44, 411–416.

Ellis, L.M., Liu, W., Fan, F., Reinmuth, N., Shaheen, R.M., Jung, Y.D., Ahmad, S., 2001. Role of angiogenesis inhibitors in cancer treatment. Oncology (Huntingt.) 15, 39–46.

Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1, 27–31.

Gerber, H.P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V., Ferrara, N., 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. 273, 30336–30343.

Gourley, M., Williamson, J.S., 2000. Angiogenesis: new targets for the development of anticancer chemotherapies. Curr. Pharm. Des. 6, 417–439.

Grant, D.S., Williams, T.L., Zahaczewsky, M., Dicker, A.P., 2003. Comparison of antiangiogenic activities using paxlitaxel and docetaxel. Int. J. Cancer 104, 121–129.

Harmey, J.H., Bouchier, D., 2002. Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. BioEssays 24, 280–283.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745–2756.

Jain, R.K., Safabakhsh, N., Sckell, A., Chen, Y., Jiang, P., Benjamin, L., Yuan, F., Keshet, E., 1998. Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. Proc. Natl. Acad. Sci. U. S. A. 95, 10820–10825.

Klagsbrun, M., Moses, M.A., 1999. Molecular angiogenesis. Chem. Biol. 6, 217–224.

Liu, W., Ahmad, S.A., Reinmuth, N., Shaheen, R.M., Jung, Y.D., Fan, F., Ellis, L.M., 2000. Endothelial cell survival and apoptosis in the tumor vasculature. Apoptosis 5, 323–328.

Liu, W., Reinmuth, N., Stoeltzing, O., Parikh, A.A., Fan, F., Ahmad, S.A., Jung, Y.D., Ellis, L.M., 2002. Anti-angiogenic therapy targeting factors that enhance endothelial cell survival. Semin. Oncol. 3, 96–103.

Marwick, C., 2001. Natural compounds show anti-angiogenic activity. J. Natl. Cancer Inst. 93, 1685.

Miller, K.D., Sweeney, C.J., Sledge, G.W., 2001. Redefining the target: chemotherapeutics as antiangiogenics. J. Clin. Oncol. 19, 1195–1206.

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 1, 9–22.

Pan, D.J., Li, Z.L., Hu, C.Q., Chen, K., Chang, J.J., Lee, K.H., 1990. The cytotoxic principles of *Pseudolarix kaempferi*: pseudolaric acid-A and -B and related derivatives. Planta Med. 56, 383–385.

Pepper, M.S., 1997. Manipulating angiogenesis: from basic science to the bedside. Arterioscler. Thromb. Vasc. Biol. 17, 605–619.

Schirner, M., 2000. Anti-angiogenic chemotherapeutic agents. Cancer Metastasis Rev. 19, 67–73.

Tan, W., Lin, L., Li, M., Zhang, Y.X., Tong, Y., Xiao, D., Ding, J., 2003. Quercetin, a dietary-derived flavonoid, possesses anti-angiogenic potential. Eur. J. Pharmacol. 459, 255–262.

Tosetti, F., Ferrari, N., Flora, S., Albini, A., 2002. Angioprevention: angiogenesis is a common and key target for cancer chemopreventive agents. FASEB J. 16, 2–14.

Veikkola, T., Karkkainen, M., Claesson, L., 2000. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res. 60, 203–212.

Wang, W.C., Lu, R.F., Zhao, S.X., Zhu, Y.Z., 1982. Antifertility effect of pseudolarix acid B. Acta Pharm. Sin. 3, 188–192.

Wang, W.C., Lu, R.F., Zhao, S.X., Gu, Z.P., 1988. Comparison of early pregnancy-terminating effect and toxicity between pseudolarix acids A and B. Acta Pharm. Sin. 9, 445–448.

Wang, W.C., Gu, Z.P., Koo, A., Chen, W.S., 1991. Effects of pseudolaric acid B on blood flows of endometrium and myometrium in pregnant rats. Acta Pharm. Sin. 12, 423–425.

Xiao, D., Tan, W.F., Li, M.H., Ding, J., 2001. Antiangiogenic potential of 10-hydroxycamptothecin. Life Sci. 69, 1619–1628.

Zachary, I., 2001a. Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. Am. J. Physiol., Cell Physiol. 280, 1375–1386.

Zachary, I., Gliki, G., 2001b. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc. Res. 49, 568–581.

Zhang, Y.L., Lu, R.Z., Yan, E.L., 1990. Inhibition of ova fertilizability by pseudolarix acid B in hamster. Acta Pharm. Sin. 9, 60–62.

Zhou, B.N., Ying, B.P., Song, G.Q., Chen, Z.X., Han, J., Yan, Y.F., 1983. Pseudolarix acids from *Pseudolarix kaempferi*. Planta Med. 47, 35–37.