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Anti-angiogenic effects of Shiraiachrome A, a compound isolated from a Chinese folk medicine used to treat rheumatoid arthritis

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Abstract

The Chinese folk medicine *Shiraia bambusicola* has long been utilized in the treatment of rheumatoid arthritis, a disease in which angiogenesis plays an important role. We report here the isolation of the compound Shiraiachrome A from *S. bambusicola* and the demonstration of its anti-angiogenic properties. We found that Shiraiachrome A significantly inhibited the proliferation, migration, and tube formation of human microvascular endothelial cells (HMEC) in a dose-dependent manner, with average IC_{50} values of 2.1 ± 0.36, 1.97 ± 0.44, and 1.65 ± 0.59 μM, respectively. In addition, Shiraiachrome A inhibited the formation of new microvessels in a rat aorta culture model as well as in the chick embryo chorioallantoic membrane (CAM) assay. Investigation of the mechanism of action of Shiraiachrome A demonstrated that this compound suppressed the autophosphorylation of four receptor tyrosine kinases (RTKs), with IC_{50} values ranging from 2.2 to 4.3 μM. These results suggest that Shiraiachrome A inhibits angiogenesis by blocking growth factor-stimulated autophosphorylation of RTKs. These findings also indicate that Shiraiachrome A may be a potent therapeutic agent for angiogenesis-related diseases such as cancer, rheumatoid arthritis, and diabetic retinopathy.

Keywords: Shiraiachrome A; Angiogenesis inhibitor; Arthritis; Receptor tyrosine kinase

1. Introduction

Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature (Folkman, 1995), is a complex process that occurs in response to an increase in tissue mass and the concomitant requirement for increased delivery of oxygen and nutrients. This process has been shown to occur during wound healing and tumorigenesis as well as during the disease processes that take place in rheumatoid arthritis and several skin diseases (Reynolds and Redmer, 1998; Benn et al., 1996; Folkman, 1995; Yamamoto et al., 1997). Angiogenesis is usually triggered by increased levels of pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (Hanahan and Folkman, 1996), which in turn induce the phosphorylation of their respective receptors on the surface of endothelial cells, and finally results in the formation of a new blood vessel (Risau, 1995). Disruptions in the signal transduction pathways of these pro-angiogenic factors, however, may result in the blockage of angiogenesis (Shawver et al., 1997).

The importance of angiogenesis in the pathogenesis of various diseases suggests that inhibition of angiogenesis, or

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anti-angiogenesis, may be a promising approach in their treatment (Deplanque and Harris, 2000; Matter, 2001). China has a long history of using natural medicines to treat various diseases, including those related to angiogenesis. Thus, it is reasonable to hypothesize that natural medicines used to treat angiogenesis-related diseases may contain compounds that have anti-angiogenic properties. Based on those concerning, our strategy is to explore potential anti-angiogenic compounds from traditional Chinese medicine used to treat angiogenesis-related diseases. By the help of ancient experience of administration, the approach is called bioassay-guided separation. Briefly, traditional Chinese medicine used to treat angiogenesis-related diseases was subjected to 95% ethanol to give crude extracts. The crude extracts were separated into four fractions by using solvent partition method. The bioactive fractions were subjected to further bioassay-guided separation using various chromatographic methods to give the desired active components. In this report, we show that the traditional Chinese medicine *S. bambusicola*, which is used to treat rheumatoid arthritis, contains the active component Shiraiachrome A. We further show that Shiraiachrome A has significant anti-angiogenic properties, including the inhibition of autophosphorylation of multiple receptor tyrosine kinases (RTKs).

2. Materials and methods

2.1. Extraction and purification of Shiraiachrome A

Powdered *S. bambusicola* was refluxed with 95% ethanol three times. Following evaporation of the ethanol in vacuo, the aqueous residue was extracted with petroleum ether and then with chloroform. The chloroform fraction was subjected to column chromatography over silica gel using a gradient of petroleum ether and acetone (5:1 → 1:1) as eluent. Shiraiachrome A was isolated from the 3:1 petroleum ether/acetone fraction and further purified by filtration through a Sephadex LH-20 column, using ethanol as eluent. The Shiraiachrome A isolated by this procedure was over 98% pure, as determined by high-performance liquid chromatography (HPLC). The structure of Shiraiachrome A is shown in Fig. 1. Shiraiachrome A was dissolved in dimethyl sulfoxide (DMSO) and diluted to desired concentrations before use. The concentration of DMSO was kept below 0.1% in treated groups. DMSO 0.1% was used as a vehicle control throughout the study.

2.2. Other reagents

Suramin, an angiogenesis inhibitor under phase II clinical development, as well as VEGF and PDGF-BB homodimer (PDGF-BB) were purchased from Sigma (St. Louis, MO). PD153035 [4-(3-bromoanilino)-6,7-dimethoxyquinazoline], a specific inhibitor of EGF receptor tyrosine kinase, was from Calbiochem (Darmstadt, Germany). The fetal liver

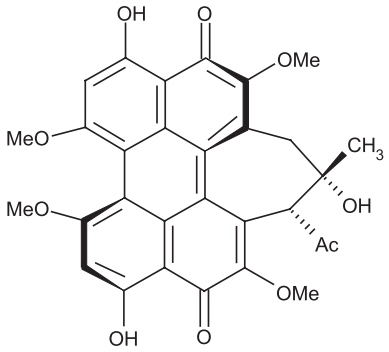

Fig. 1. Chemical structure of Shiraiachrome A.

kinase-1 (Flk-1) inhibitor SU5416 (3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-iodolin-2-one) was the kind gift of Dr. Fajun Nan (National Center for Drug Screening, Shanghai, P.R. China), and bFGF was kindly provided by Yisheng Pharmaceutical Factory (Zhuhai, P.R. China). EGF was purchased from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-phosphotyrosine antibody (PY99) and rabbit polyclonal antibodies against Flk-1, FGF_1 receptor, $PDGF_\beta$ receptor, and EGF receptor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Matrigel was obtained from Becton Dickinson Labware (Bedford, MA).

2.3. Cell lines and cell culture

NIH-3T3 cells highly expressing full-length Flk-1 protein were the kind gift of Dr. Axel Ullrich (Max-Planck-Institute für Biochemie, Martinsried, Germany) and were cultured in Dulbecco's modified eagle medium (DMEM, Gibco-BRL, Grand Island, NY) containing 200 μg/ml G418 (Sigma). NIH-3T3 cells, human microvascular endothelial cells (HMEC), and the human breast cancer cell line MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA). NIH-3T3 cells and HMEC cells were cultured in DMEM (Gibco-BRL). MDA-MB-468 cells were cultured in RPMI 1640 (Gibco-BRL). All cell culture media contained 20% heat-inactivated fetal bovine serum, 100-units/ml penicillin, 100-μg/ml streptomycin, and 2 mM L-glutamine. Cells were maintained at 37 °C in an incubator containing a humidified 5% CO_2/95% air atmosphere.

2.4. HMEC proliferation assay

The effect of Shiraiachrome A on the growth of HMEC was measured using the Sulforhodamine B (SRB; Sigma) method. Briefly, cells were plated in 96-well plates (5×10^3 cells/90 μl/well) in DMEM containing 20% heat-inactivated fetal bovine serum and cultured at 37 °C for 24 h. Ten microliters of each serial dilution of Shiraiachrome A in medium containing 0.1% DMSO was added to each well, and the plates were incubated at 37 °C for 72 h. The cells were fixed by gentle addition of 100-μl cold (4 °C) 10% trichloroacetic acid to each well, followed by incubation at 4

°C for 1 h. The plates were washed five times with deionized water, allowed to air-dry, and stained by addition of 100-μl SRB solution [0.4% SRB (w/v) in 1% acetic acid (v/v)] to each well for 15 min. The plates were washed five times with 1% acetic acid to remove unbound dye and allowed to air-dry. The bound dye in each well was dissolved in 10 mM Tris base (pH 10.5), and the optical density at 515 nm was measured with a multiwell spectrophotometer (VERSAmax, Molecular Devices, USA). The inhibition of proliferation was calculated as $[1-(A_{515\ \text{treated}}/A_{515\ \text{control}})]\times 100\%$. The result was also expressed as IC_{50} (i.e., the drug concentration that reduced the absorbance observed in untreated cells by 50%), which was calculated by the Logit method. The mean IC_{50} was determined from the results of three independent tests.

2.5. HMEC migration assay

Migration of HMEC was determined in a transwell Boyden chamber (Costar, MA, USA) containing a polycarbonate filter with a pore size of 8 μm (Ashton et al., 1999) coated with 0.2% gelatin. In the standard assay, 0.1-ml cell suspension (2×10^5 cells/ml) containing Shiraiachrome A or 0.1% DMSO (v/v) was added to the upper compartment of each well. The lower compartment contained 0.6-ml DMEM supplemented with the same concentration of Shiraiachrome A or DMSO. After incubation for 24 h at 37 °C, the filter was removed and fixed with ethanol. Cells remaining on the upper surface of the filter (nonmigrated) were removed by gentle scraping. Cells on the lower surface of the filter (migrated) were stained with eosin and counted manually in five random fields. The inhibition of migration was calculated as $[1-(\text{migrated cells}_{\text{treated}}/\text{migrated cells}_{\text{control}})]\times 100\%$.

2.6. HMEC tube formation assay

The tube formation assay was performed to determine the effect of Shiraiachrome A on angiogenesis in vitro. Briefly, a 96-well plate coated with 0.1-ml matrigel per well was allowed to solidify at 37 °C for 1 h. Each well was seeded with 1×10^4 HMEC and cultured in DMEM containing various concentrations of Shiraiachrome A or 0.1% DMSO (v/v) for 24 h. The enclosed networks of tubes were photographed from five randomly chosen fields under a microscope (Olympus, IX70, Japan). The total length of the tube structures in each photograph was measured using Adobe Photoshop software (Soeda et al., 2000). Inhibition of tube formation was calculated as $[1-(\text{tube length}_{\text{treated}}/\text{tube length}_{\text{control}})]\times 100\%$.

2.7. Rat aorta cultures

The thoracic aorta of Sprague–Dawley rats was dissected into 1-mm long rings, rinsed eight times with culture medium, embedded in matrigel, and incubated in serum-free endothelial cell basal medium containing 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Nicosia and Ottinetti, 1990; Carlini et al., 1995). Serial dilutions of Shiraiachrome A were added on day 2, and the cells were cultured for another 4 days, replacing the supernatant every 24 h. The microvessel growth was photographed on day 6. To confirm the presence of endothelial cells in the microvessels, the rat aorta sections were immunohistochemically stained with rabbit polyclonal antibodies against the endothelial cell-specific markers factor VIII (1:100) and CD31 (1:200) (Santa Cruz, CA).

2.8. Chicken chorioallantoic membrane assay

Groups of 10 fertilized chicken eggs were transferred to an egg incubator (Lyon, CA), maintained at 37 °C and 50% humidity, and grown for 9 days. The chicken chorioallantoic membrane (CAM) was separated from the shell membrane by drilling two small holes, one at the broad end of the egg where the air sac is located and the second at a position 90° from the first. The cell membrane was carefully pushed down on the second hole to detach the CAM from the shell. Gentle suction was applied at the hole located at the broad end of the egg to create a false air sac directly over the CAM. A 1-cm^2 window was removed from the eggshell immediately over the second hole. Filter paper disks saturated with compounds or 0.1% DMSO (v/v) were placed on the areas between preexisting vessels, and the embryos were further incubated for 48 h. The neovascular zones under the disks were photographed at 10× magnification under a stereomicroscope (Leica, MS5, Switzerland). Angiogenesis was quantified by counting the number of blood vessel branch points on the photo.

2.9. Cellular RTK autophosphorylation assay

NIH 3T3 cells overexpressing Flk-1 were plated onto 96-well plates (3×10^4 cells/well) in DMEM plus 10% fetal bovine serum and cultured overnight. The medium was removed, and the cells were cultured in DMEM for 24 h at 37 °C. Serial dilutions of compounds were added, and the cells were incubated for 1 h. Phosphorylation was stimulated by the addition of 250 ng/ml human recombinant VEGF. After 5 min at 37 °C, the cells were washed with phosphate-buffered saline (PBS) and lysed with HNTG [20 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, 10% glycerol] containing 5 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, and 5 mM EDTA. Lysates were transferred to 96-well plates precoated with polyclonal anti-Flk-1 antibody and incubated for 2 h. Tyrosine phosphorylation was detected by sequential incubation with PY99 (1:500), biotinylated anti-mouse antibody (Burlingame, CA) (1:1000), and horseradish–streptavidin–peroxidase (Burlingame) (1:500), each for 1 h at 37 °C. After several washes with PBS containing 0.2% Tween-20, the wells were stained with 2,2′ -azino-bis(3-

ethylbenzthiazoline)-6-sulfonic acid (ABTS) (Roche, Basel, Switzerland). The reaction was stopped by adding an equal volume of 2% (w/v) sodium dodecyl sulfate (SDS), and the amount of color development was read in the multiwell spectrophotometer at 405 nm (Zaman et al., 1999).

Enzyme-linked immunosorbent assays (ELISAs) for $PDGF_{\beta}$ receptor, FGF_1 receptor, and EGF receptor were performed according to standard procedures. Protein phosphotyrosine was detected as described for the Flk-1 assay.

2.10. Immunoblotting

NIH-3T3 cells overexpressing Flk-1 were plated onto 6-well plates, grown to confluence, and starved overnight. Dilutions of compound were added and incubated for 1 h at 37 °C. Flk-1 autophosphorylation was stimulated by the addition of 100 ng/ml VEGF for 5 min. The cells were lysed with HNTG, and the lysates were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated sequentially with mouse anti-phosphotyrosine antibody and rabbit anti-Flk-1 antibody, and immune complexes were detected following incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Calbiochem). Immunoblotting for the other tyrosine kinase receptors, $PDGF_{\beta}$ receptor, FGF_1 receptor, and EGF receptor, was performed according to similar protocols.

2.11. Data analysis

All results are expressed as mean ± S.D. Statistical significance was assessed by Student's *t*-test.

3. Results

3.1. Effects of Shiraiachrome A on growth of HMEC

The effects of Shiraiachrome A on the growth of HMEC were determined at both 24 and 72 h. At concentrations of 0.313 and 0.625 μM, Shiraiachrome A did not inhibit the proliferation of HMEC at either time point. When treated with 1.25, 2.5, and 5 μM Shiraiachrome A for 72 h, however, the proliferation of these cells was inhibited in a dose-dependent manner and yielded inhibition rates of 13.9%, 50.1%, and 96.6%, respectively (Fig. 2). At 24 h, 2.5 and 5.0 μM Shiraiachrome A also showed slight inhibition of cell growth with inhibition rates of 9.8% and 16.2% (Fig. 2).

3.2. Effects of Shiraiachrome A on migration of HMEC

Endothelial cell migration, which occurs through the process of chemotaxis, is indispensable for angiogenesis. The incubation for 24 h resulted in a large number of HMEC cells migrated to the lower side of the filter in control group (Fig. 3A). When we tested the effects of Shiraiachrome A on the migration of HMEC, we observed significant, dose-dependent inhibition at all Shiraiachrome A concentrations above 0.625 μM (Fig. 3). The inhibition rates were 19.6%, 26.5%, 58.6%, and 79.4% for 0.625, 1.25, 2.5, and 5 μM Shiraiachrome A, respectively (Fig. 3B and C). Since Shiraiachrome A had no obvious effect on the growth of HMEC (Fig. 2), it is likely that its inhibition of cell migration is specific and not due to any cytotoxic effect.

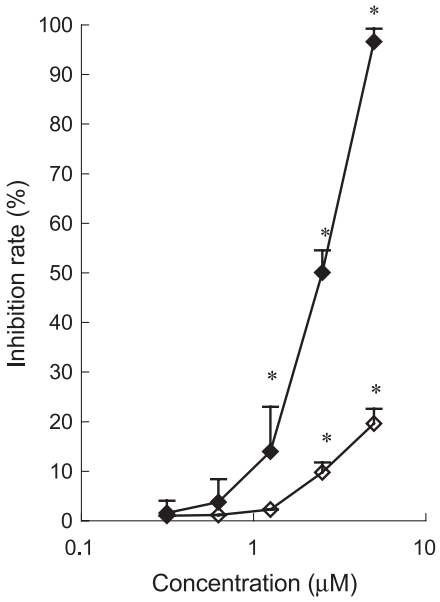

Fig. 2. Inhibitory effect of Shiraiachrome A on HMEC proliferation. HMEC seeded in 96-well plates were treated with Shiraiachrome A for 24 h (◇) or 72 h (♦), and the effect on cell growth measured using the SRB method. Each value is the mean ± S.D. of three independent experiments. *$P < 0.001$ vs. control.

3.3. Effects of Shiraiachrome A on tube destruction

In the later stages of angiogenesis, endothelial cells have to rearrange themselves into a tube in order to form a new small blood vessel. The endothelial cell tube formation assay utilized here is an in vitro model of this process. HMEC incubated on matrigel for 24 h differentiate into an extensive and enclosed network of tubes (Fig. 4A). When exposed to Shiraiachrome A at concentrations above 0.625 μM, however, the tube structure was severely disrupted, resulting in an incomplete and sparse tube network (Fig. 4B). Shiraiachrome A at concentrations of 0.625, 1.25, 2.5, and 5 μM reduced tube formation by 13.2%, 32.9%, 69.0%, and 88.3%, respectively, compared with the control group (Fig. 4C).

3.4. Effects of Shiraiachrome A on microvessel outgrowth in rat aorta

When a section of rat aorta is embedded in matrigel and cultured under certain circumstances, microvessels can be

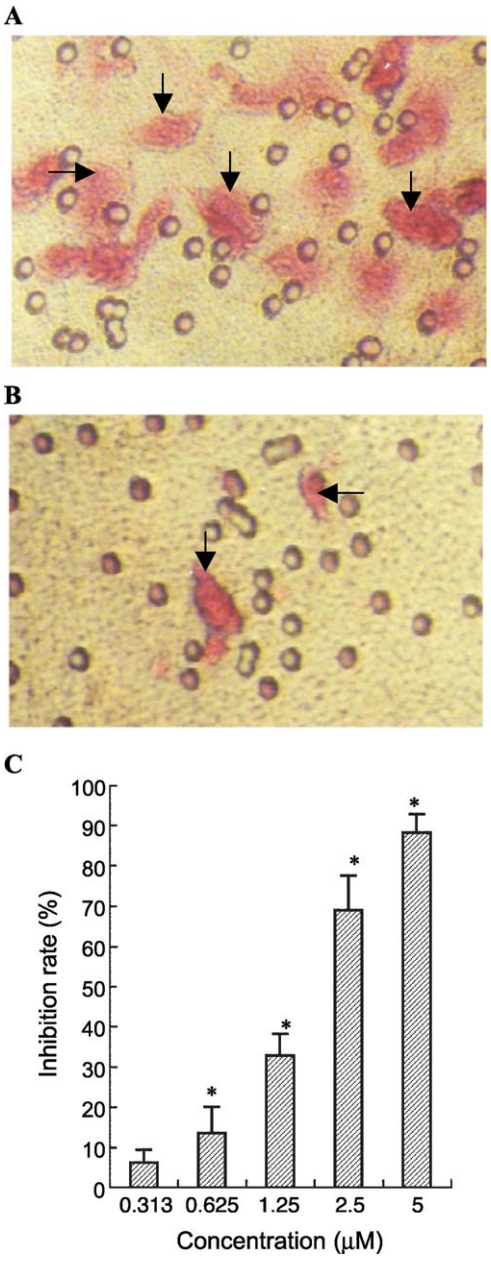

Fig. 3. Effect of Shiraiachrome A on HMEC migration. HMEC seeded in transwell Boyden Chambers were incubated for 24 h with (A) medium alone or (B) 2.5 μM Shiraiachrome A. Migrated cells (arrows indicating) on the lower surface of the filter were stained with eosin and counted manually from five random fields. Representative microscopic fields are shown. (C) Overall inhibition of migration by Shiraiachrome A. Results are expressed as the mean ± S.D. of three separate experiments. *$P<0.001$ vs. control.

observed growing out of the section. This phenomenon is the result of colligation from endothelial cell proliferation, migration, and tube formation, thus closely approximating in vivo angiogenesis. We observed the initial formation of microvessels on day 2 or 3, followed by an increase in their number and length (Fig. 5A). When incubated with 10 and 20 μM Shiraiachrome A, we observed regression of the microvessels, while lower concentrations of this compound (2.5 or 5 μM) retarded their growth (Fig. 5B). We observed staining of the sprout with antibodies to factor VIII and CD31 (not shown); the positive staining for both antibodies confirmed that these sprouts were microvessels.

3.5. Anti-angiogenic activity of Shiraiachrome A in the CAM assay

The ability of Shiraiachrome A to inhibit vascular development was compared to that of suramin, a known anti-angiogenic compound. In the absence of Shiraiachrome A, the area of the CAM below the disks showed no

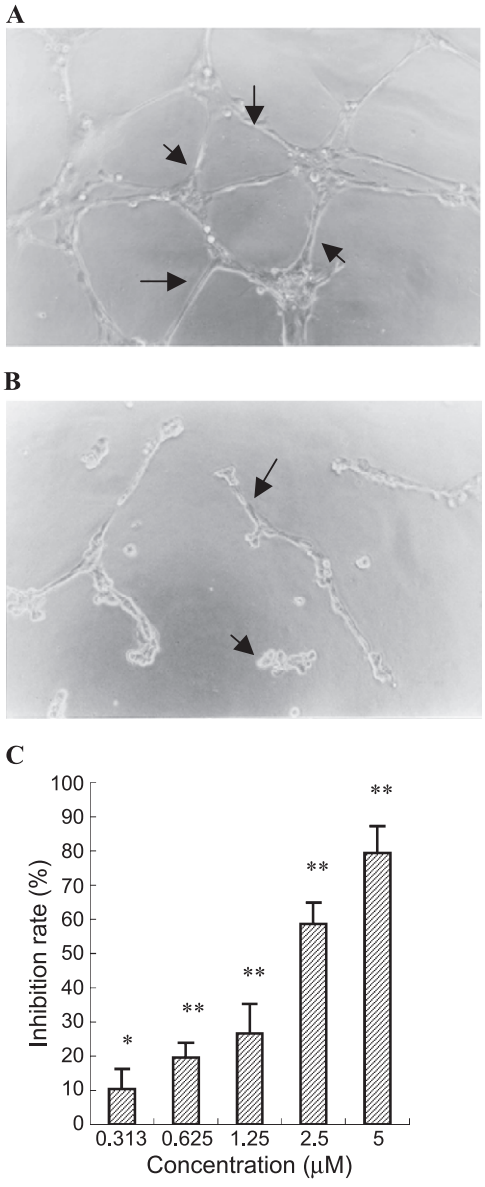

Fig. 4. Effect of Shiraiachrome A on HMEC tube formation. HMEC seeded in matrigel-coated 96-well plates were incubated for 24 h with (A) medium alone or (B) 2.5μM Shiraiachrome A. The enclosed networks of tubes (arrows indicating) were photographed from five randomly chosen fields under a microscope. (C) Overall inhibition rate of tube formation by Shiraiachrome A. Results are expressed as mean ± S.D. of three separate experiments. *$P<0.05$ vs. control, **$P<0.001$ vs. control.

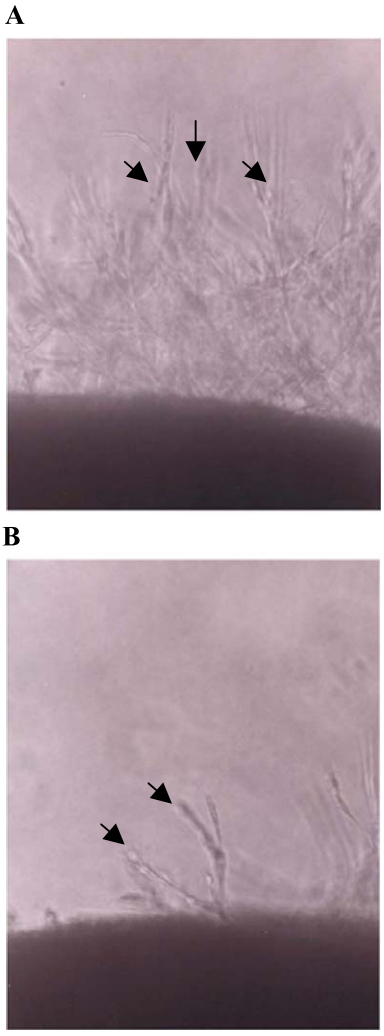

Fig. 5. Effect of Shiraiachrome A on microvessel outgrowths arising from rat aorta sections. Sections were embedded in matrigel and cultured in base endothelial culture medium for 24 h. Shiraiachrome A was added on day 2, and microvessel (arrows indicating) growth was photographed on day 6. (A) Shiraiachrome A, 0 μM; (B) Shiraiachrome A, 5 μM.

alteration in vascular density, and a normal branching pattern of blood vessels was present (Fig. 6A). After 48 h of treatment with Shiraiachrome A, the branching pattern of the blood vessels below the disks was dramatically decreased (Fig. 6B) and in a dose-dependent manner. Although suramin is a potent inhibitor of angiogenesis now undergoing phase II clinical development, 10 nmol Shiraiachrome A/egg had a comparative effect compared with 125-nmol suramin/egg (Fig. 6C).

3.6. Inhibition of cellular RTK autophosphorylation

The effect of Shiraiachrome A on autophosphorylation of various growth factor receptors was assayed in several cell lines (Fig. 7). To ensure that these cell lines were suitable for this assay, a pretest was performed to assess tyrosine phosphorylation induced by stimulus with the corresponding growth factor. As shown in Fig. 7, each cell line was sufficiently stimulated to study the effect of Shiraiachrome A on RTK autophosphorylation. We selected 250 ng/ml VEGF to stimulate autophosphorylation of Flk-1 and PDGF-BB 125 ng/ml for $PDGF_{\beta}$ receptor, bFGF 125 ng/ml for FGF_1 receptor, and EGF 62.5 ng/ml for EGF receptor, respectively. When we tested the

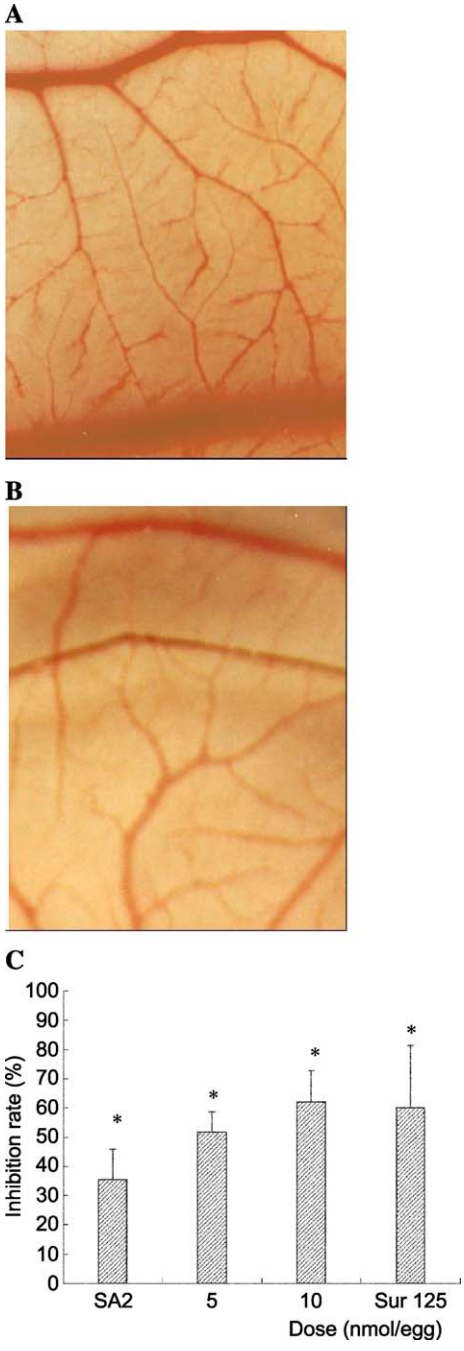

Fig. 6. Effect of Shiraiachrome A on CAM. CAM were treated with Shiraiachrome A for 48 h, harvested and photographed. (A) Shiraiachrome A, 0 nmol/egg; (B) Shiraiachrome A, 5 nmol/egg. (C) Angiogenesis was quantified by counting the number of blood vessel branch points in each photograph (SA for Shiraiachrome A, Sur for suramin). Each value is the mean ± S.D. of 10 eggs. *$P < 0.001$ vs. control.

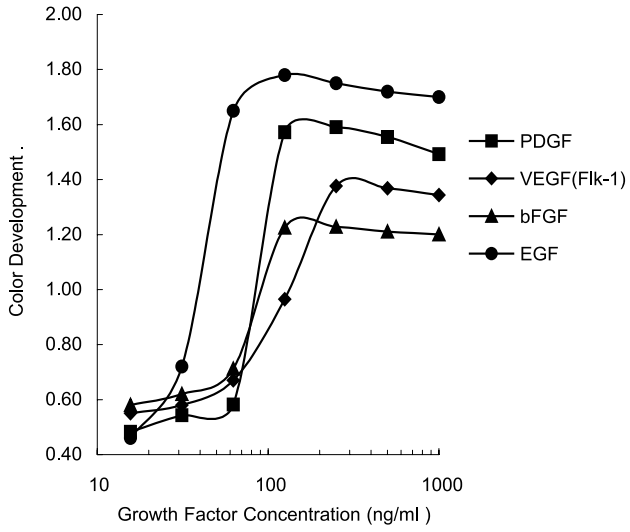

Fig. 7. Effect of Shiraiachrome A on tyrosine phosphorylation. (A) Flk-1 NIH-3T3, NIH-3T3, and MDA-MB-468 cells were seeded onto 96-well plates, grown to confluence, and starved overnight. Flk-1 NIH-3T3 cells were incubated for 5 min with VEGF (♦); NIH 3T3 cells were incubated for 5 min with bFGF (▲) or PDGF-BB (■); and MDA-MB-468 cells were incubated for 5 min with EGF (●). Cells were lysed and tyrosine phosphorylation was quantified by ELISA.

effects of Shiraiachrome A, we found that this compound possessed a broadly inhibitory, dose-dependent effect on autophosphorylation of all four RTKs tested. As shown in Table 1, Shiraiachrome A yielded IC_{50}s of 3.4 ± 1.1, 2.2 ± 1.0, 2.7 ± 1.6, and 4.3 ± 2.3 μM for Flk-1, FGF_1 receptor, $PDGF_\beta$ receptor, and EGF receptor, respectively. As positive controls, we utilized two compounds with known specific effects on RTKs: PD153035, a potent specific EGF receptor inhibitor, inhibited autophosphorylation only of EGF receptor, with an IC_{50} of 2.6 ± 0.9 μM, but had no effect on the other RTKs; and SU5416, a highly selective inhibitor of Flk-1, inhibited autophosphorylation of Flk-1, with an IC_{50} of 2.9 ± 0.8 μM, but did not alter that of the other RTKs.

To confirm that the decreased signal in the ELISAs was due to the inhibition of tyrosine phosphorylation rather than to decreased phosphorylation of associated proteins or loss of RTK proteins, the phosphorylation state of the RTKs was also determined by immunoblotting. We found that treatment with the corresponding growth factor increased RTK

Table 1
Effect of Shiraiachrome A on cell-based RTK autophosphorylation

Compound	Flk-1	FGF_1 receptor	$PDGF_\beta$ receptor	EGF receptor
Shiraiachrome A	3.4 ± 1.1	2.2 ± 1.0	2.7 ± 1.6	4.3 ± 2.3
PD153035	>50	>50	>50	2.6 ± 0.9
SU5416	2.9 ± 0.8	>50	>50	>50

Cells were treated with various concentrations of Shiraiachrome A for 1 h and stimulated for 5 min with their respective growth factors, as described in the legend to Fig. 7. Autophosphorylated proteins were detected as described in Materials and methods. IC_{50} values (in μM) are the mean ± S.D. of three experiments.

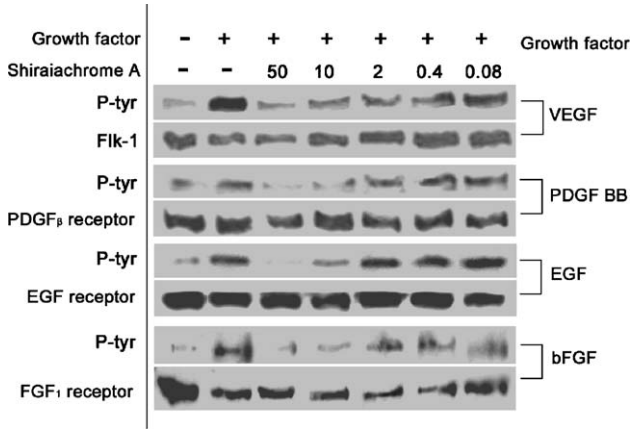

Fig. 8. Immunoblot detection of tyrosine phosphorylation. Cell lines were incubated with various concentrations of Shiraiachrome A for 1 h at 37 °C and stimulated for 5 min with the respective growth factor, as described in the legend to Fig. 7. The cells were lysed and the resultant proteins were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated with anti-phosphotyrosine antibody, stripped, and reincubated with antibodies targeting the respective receptors.

phosphorylation, while 50 and 10 μM Shiraiachrome A significantly decreased the phosphotyrosine signals of all four RTKs (Fig. 8). When the blots were stripped and reblotted with antibodies against corresponding RTKs to verify that the loss of phosphotyrosine was not due to the loss of RTK total proteins, we found that Shiraiachrome A specifically inhibited autophosphorylation of the four RTKs but had no effect on the phosphorylation of associated proteins (Fig. 8).

4. Discussion

We show here the isolation of Shiraiachrome A, an anti-angiogenic compound present in the traditional Chinese medicine *S. bambusicola*. This traditional Chinese medicine is actually a fungus that grows on bamboo. It is found mainly in several provinces in southern China, including Sichuan, Anhui, Jiangsu, and Zhejiang, and it has been found to be effective in treating rheumatoid arthritis (Chen and Lai, 1998; Hu, 1994; Lin et al., 2002).

Rheumatoid arthritis is an angiogenesis-related disease, in which the expansion of the synovial pannus results in local hypoxia, thus driving the need for compensatory neovascularization to increase the supply of nutrients and oxygen to the joint (Blake et al., 1989; Stevens et al., 1991). The vascular endothelium, which contains circulating mediators of inflammation, controls the trafficking of cells and molecules from the bloodstream toward the synovial tissue in rheumatoid arthritis. Angiogenesis allows the pannus to develop and grow, thereby promoting cartilage and bone destruction and joint remodeling (Walsh, 1999).

Angiogenesis inhibitors have been demonstrated to be effective in animal models of arthritis (Peacock et al., 1992;

Oliver et al., 1994, 1995). More convincingly, some anti-rheumatic drugs currently in clinical use or in trials, such as indomethacin, methotrexate, and corticosteroids, possess anti-angiogenic potential (Jones et al., 1999; Billington, 1991; Colville-Nash et al., 1993). Thus, it is possible that, throughout history, people have been unconsciously using angiogenesis inhibitors or herbs containing anti-angiogenic elements to treat angiogenesis-related diseases. Our isolation of the anti-angiogenic compound Shiraiachrome A in a traditional Chinese medicine used to treat rheumatoid arthritis provides strong support for this hypothesis.

Angiogenesis includes various steps, such as the degradation of basement membrane and endothelial cell migration, proliferation, rearrangement, as well as other morphological changes, which finally forms a new blood vessel (Risau, 1995). We have demonstrated the dose-dependent anti-angiogenic properties of Shiraiachrome A using a series of in vitro models that include the most salient features of angiogenesis, including endothelial cell proliferation, migration, and tube formation. The multistage inhibition of angiogenesis by Shiraiachrome A was further confirmed using ex vivo rat aorta culture and CAM models. In the latter assay, Shiraiachrome A demonstrated greater anti-angiogenic activity than the control drug suramin, thus providing further evidence that the natural compound we isolated possesses potent anti-angiogenic properties.

Angiogenesis is regulated by the balance between pro- and anti-angiogenic stimuli (Hanahan and Folkman, 1996). One of the growth factors that promote angiogenesis (Folkman and Klagsbrun, 1987; Senger et al., 1993) is VEGF, whose receptors, VEGFR-1 and VEGFR-2 (Flk-1/kinase insert domain receptor, KDR), are preferentially expressed in endothelial cells. The biological activity of VEGF occurs primarily through its binding to VEGFR-2, and this interaction has been shown to be mitogenic for endothelial cells as well as being required for the maturation of these cells (Veikkola and Alitalo, 1999; Ferrara, 1999). Moreover, using various molecular validation approaches, VEGFR-2 has been shown to be necessary for angiogenesis. Other growth factor signaling pathways have also been found to promote angiogenesis, including FGF–FGF receptor, PDGF–PDGF receptor, and EGF–EGF receptor (Laird et al., 2000). FGF is the first identified angiogenesis factor and promotes angiogenesis strongly. PDGF and EGF play much-complicated roles in angiogenesis and can promote angiogenesis both directly and indirectly. We have shown here that Shiraiachrome A inhibits autophosphorylation of all four of these RTKs, with IC_{50}s ranging from 2.2 to 4.3 μM, suggesting that the anti-angiogenic effects of Shiraiachrome A are due, at least in part, to its inhibition of RTK.

To develop a RTK inhibitor, a specific one is preferable in principle to avoid side effects. Several RTK inhibitors are currently in clinical trials. There is evidence, however, that specific RTK inhibitors may not be effective in suppressing angiogenesis both in vitro and in vivo. For example, while the highly selective Flk-1 inhibitor SU5416 has been shown to inhibit VEGF-driven mitogenesis of HUVEC, with an IC_{50} of 0.04 ± 0.02 μM, it has little effect on FGF-dependent mitogenesis of these same cells ($IC_{50} = 50$ μM) (Fong et al., 1999). Furthermore, SU5416 has been found to only partially block EGF-induced neovascularization of mouse cornea, in contrast to ZD1839, a selective inhibitor of EGF receptor, which almost completely inhibits the activity of EGF in this model (Hirata et al., 2002). Thus, it is likely that a series of growth factors is involved in angiogenesis, each playing its own role but having overlapping pathways; thus, block one RTK may not be able to block angiogenesis process effectively (Hanahan and Folkman, 1996). Since simultaneous inhibition of several growth factors may be more effective than inhibition of each individually, a wide spectrum RTK inhibitor such as Shiraiachrome A may be an effective inhibitor of angiogenesis. Of course, there is the possibility that a wide spectrum kinase inhibitor may cause severe side effects and should be noted in future research and development.

In conclusion, Shiraiachrome A is a potent angiogenesis inhibitor. This compound may be an effective therapeutic agent in the treatment of diseases such as cancer and rheumatoid arthritis that require new blood vessel formation. What more, the discovery of Shiraiachrome A also provided us some new thought in developing angiogenesis inhibitors. Further investigations of Shiraiachrome A on angiogenesis-related disease models are underway in our laboratory.

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