Original Paper

A Novel Natural Product-Derived Compound, Vestaine A₁, Exerts both Pro-Angiogenic and Anti-Permeability Activity via a Different Pathway from VEGF

Yoko Ishimoto^a Yuki Hirota-Takahata^b Emi Kurosawa^b Jun Chiba^c Yuko Iwadate^b
Yoshiko Onozawa^a Toru Hasegawa^d Akihiro Tamura^d Masahiro Tanaka^b
Hideki Kobayashi^a

^aFrontier Research Laboratories IV, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, ^bNatural Product Research Group, Discovery Science and Technology Department, Drug Discovery and Biomedical Technology Unit, Daiichi Sankyo RD Novare Co., Ltd., Tokyo, ^cMedicinal Chemistry Research Laboratories VI, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, ^dResearch Group II, Organic Synthesis Department, Drug Discovery and Biomedical Technology Unit, Daiichi Sankyo RD Novare Co., Ltd., Tokyo, Japan

Key Words

Angiogenesis • Permeability • Vessel maturation • Vessel stabilization • Natural product • Co-culture

Abstract

Background/Aims: Vascular endothelial growth factor (VEGF) is a key molecule in the regulation of both angiogenesis and vascular permeability. However, it is known that overproduction of VEGF induces abnormal blood vessel formation and these vessels cause several disease pathologies, such as diabetic retinopathy. The purpose of this study was to find novel vasoactive compounds which have different properties from VEGF. **Methods/Results:** We screened a natural product library using a co-culture angiogenic assay of endothelial cells and fibroblasts. By focusing on morphological changes of endothelial cells, we isolated the novel compounds vestaine A₁ and vestaine B₁ from the cultured broth of an actinomycete strain, *Streptomyces* sp. SANK 63697. Vestaine A₁ enhanced tube formation of endothelial cells in Matrigel and suppressed cell death induced by serum deprivation. Vestaine A₁ activated both MEK1/2 and PI-3 kinase pathways independently of the VEGF pathway in a dose- and time-dependent fashion. Finally, vestaine A₁ potently suppressed VEGF-induced vascular permeability both *in vitro* and *in vivo*. **Conclusion:** Vestaine A₁ has the potential to exhibit both pro-angiogenic and anti-permeability properties, and would therefore be useful for therapeutic treatment for abnormal vascular permeability-related diseases.

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Introduction

Blood vessels are extended throughout the body, deliver nutrients and oxygen into all tissues, and take away waste products. To maintain vascular integrity, our body constantly produces new vessels through angiogenesis, and then enhances stabilization of such newly formed vessels through vessel maturation. In adults, this process is tightly regulated by the coordinated function among pro-angiogenic factors, anti-angiogenic factors, extracellular matrix production, degradation, and so on [1].

Angiogenesis is a process critical to the formation of new vessels and maintenance of vessel integrity. However, it is known that excess angiogenesis induces fragile vessel formation and these vessels are problematic in many clinical settings, causing complications such as diabetic retinopathy and cerebral hemorrhage. In the retinas of diabetic patients, the decrease in blood flow and thrombosis formation induces a hypoxic condition, and continuous hypoxia induces abrupt new vessel formation. Such abruptly formed vessels are structurally fragile, leaky, poor in oxygen delivery, and prone to cause hemorrhage. These properties cause further dysfunction in diabetic retinopathy, leading to blindness [2]. At the acute phase of stroke, fragile new blood vessels are also induced by ischemia at the distal site of the thrombus in the brain. Thrombolytic drugs such as recombinant thrombin are useful for treating the condition. However, adaptation of thrombolytic therapy is limited because reperfusion by thrombolysis sometimes causes hemorrhaging from the fragile vessels [3, 4]. Additionally, leaky vessels are also observed in tumors. The highly hypoxic condition in solid tumors induces formation of fragile vessels. These structurally abnormal vessels are leaky and poor in oxygen delivery, resulting in a decrease in anti-tumor drug delivery, and are one of the causes of resistance to radiation therapy [5]. Therefore, a treatment to eliminate the fragile vessels by suppressing excess angiogenesis or enhancing vessel maturation would be useful for treating these clinical conditions.

Vascular endothelial growth factor (VEGF) is a key molecule of angiogenesis and also plays a critical role in abnormal vessel formation. In several clinical settings, overproduction of VEGF has been observed in lesions where abnormal vessel formation causes problems [6-9]. Recently, anti-VEGF antibodies have been approved to treat eye diseases such as age-related macular degeneration and cancer, and show steady efficacy [1, 10, 11]. Therefore, medicines which accomplish abnormal vessel elimination through mechanisms different from anti-VEGF therapy would be useful to provide other options for patients showing resistance or insufficient response to the anti-VEGF therapy.

In this study, we focused on identifying novel vasoactive compounds with a mechanism of action different from VEGF's action. We screened a natural product library using a co-culture assay of endothelial cells with fibroblasts and found two novel compounds, vestaine A₁ and vestaine B₁. We also confirmed that vestaine A₁ possesses both pro-angiogenic and anti-vascular permeability activity, which suggests the possibility that vestaine A₁ is useful for therapeutic treatment.

Materials and Methods

Reagents

HuMedia-EG2 and VasculLife EnGS Comp Kit were purchased from KURABO and fetal bovine serum (FBS) from Hyclone. Medium-199 (M199), collagen-I solution, and Alamar blue were purchased from Thermo Fisher Scientific Inc. CELLBANKER-1 was purchased from Takara. Hexadimethrine bromide, anti- β -tubulin antibody, and Evans Blue were purchased from Sigma. Cignal™ Lenti Positive Control (GFP) and RNeasy mini kit were purchased from Qiagen. Growth factor reduced Matrigel was purchased from Corning. Recombinant human VEGF165 and recombinant human FGF-basic (bFGF) were purchased from Peprotech. PD98059 and VEGF receptor 2 (VEGFR2) inhibitor were purchased from Calbiochem. Complete EDTA-free was purchased from Roche and phosphatase inhibitor cocktail from Nacalai Tesque, Inc. LY294002, anti-phospho-Akt (Ser473), anti-Akt, phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-VEGFR2 (Tyr1175), and anti-VEGFR2 antibodies were purchased from Cell Signaling Technology, Inc. ECL prime

Western Blotting Detection Reagents were purchased from GE Healthcare Life Sciences. Power SYBR Green PCR Master Mix and Taqman Gene Expression Master Mix were purchased from Applied Biosystems. Vestaine A₁ (natural-**1**) and vestaine B₁ were isolated from the extract of an actinomycete strain, *Streptomyces* sp. SANK 63697. All other chemicals used were of reagent grade.

Synthesis for vestaine A₁ (synthetic-1)

All starting materials and synthesis reagents were obtained commercially. Flash column chromatography was performed with Biotage SNAP Cartridge HP-Sil, and Silicycle SilicaSep. The ¹H NMR spectra were recorded on a JEOL GSX 500 -NMR spectrometer, and chemical shifts are given in ppm (δ) from tetramethylsilane as an internal standard. The spectral splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. The mass spectra were recorded on a JEOL LC mate spectrometer (ESI).

14-Methylpentadec-1-en-3-ol (4)

To a cooled 10-Bromo-1-decanol (**1**) (14 g, 50 mmol) at -10°C was added a solution of 0.1 mol/L lithium tetrachloro cuprate (II) in THF (50 ml, 5.0 mmol) and 1 mol/L isobutyl magnesium bromide solution in THF (150 ml, 150 mmol) were added under an atmosphere of nitrogen. After being stirred at that temperature for 2 h, the reaction was quenched by addition of sat. NH₄Cl solution and extracted with EtOAc. The extract was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure to afford **2** as a colorless oil.

A mixture of **2** (50 mmol) and dimethyl sulfoxide (DMSO) (17.8 ml, 250 mmol) in dichloromethane (100 ml) was cooled to 0°C under an atmosphere of nitrogen. To the reaction mixture, triethylamine (27.9 ml, 200 mmol) and Py•SO₃ (19.9 g, 125 mmol) were added at that temperature and stirred for 2 h. After being stirred at room temperature for 30 min., the reaction mixture was concentrated. The resulting residue was diluted with water and extracted with hexane. The extract was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure to afford **3** as a colorless oil.

A solution of **3** (50 mmol) in THF (150 ml) was cooled to 0°C under atmosphere of nitrogen. To the reaction mixture, 1 mol/L vinyl magnesium bromide solution in THF (65 ml, 65 mmol) was added at that temperature. After being stirred at that temperature for 30 min, the reaction was quenched by the addition of sat. NH₄Cl solution and extracted with EtOAc. The extract was washed with brine, and dried over Na₂SO₄. After removing the solvent, the resulting mixture was purified by flash column chromatography on silica gel with hexane/EtOAc (4:1, v/v) as eluent to give **4** (10.3 g, 86% 3 steps) as a colorless oil.

¹H NMR (CDCl₃) δ: 0.86 (6H, d, *J* = 6.9 Hz), 1.13-1.55 (22H, m), 4.07-4.12 (1H, m), 5.09-5.11 (1H, m), 5.20-5.21 (1H, m), 5.83-5.90 (1H, m).

14-Methylpentadec-1-en-3-one (5)

A mixture of 14-methylpentadec-1-en-3-ol (**4**) (6.0 g, 25 mmol) and MnO₂ (75 g) in chloroform (150 ml) was stirred at 50°C for 5 h. To a reaction mixture, MnO₂ (45 g) was added and stirred for 1 day. The mixture was filtered through a celite pad and the resulting filtrate was concentrated. The residue was diluted with chloroform (150 ml). To a reaction mixture, MnO₂ (90 g) was added and stirred for 3 days at room temperature. After being filtered through a celite pad, the resulting filtrate was concentrated to afford **5** (2.5 g, 42%) as a colorless oil.

¹H NMR (CDCl₃) δ: 0.86 (6H, d, *J* = 6.9 Hz), 1.13-1.35 (16H, m), 1.47-1.64 (3H, m), 2.58 (2H, t, *J* = 7.6 Hz), 5.82 (1H, d, *J* = 10.7 Hz), 6.21 (1H, d, *J* = 17.6 Hz), 6.35 (1H, dd, *J* = 17.6, 10.7 Hz).

1,2-Dibromo-14-methylpentadecan-3-one (6)

To a cooled solution of 14-methylpentadec-1-en-3-one (**5**) (2.5 g, 10.5 mmol) in dichloromethane (100 ml), a solution of bromine (0.60 ml, 11.5 mmol) in dichloromethane (5 ml) was added at 0°C. The reaction was quenched by addition of sat. Na₂SO₄ solution and extracted with dichloromethane. The extract was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure to give **6** (3.5 g, 84%) as a colorless oil.

¹H NMR (CDCl₃) δ: 0.86 (6H, d, *J* = 6.9 Hz), 1.12-1.18 (2H, m), 1.20-1.38 (14H, m), 1.47-1.54 (1H, m), 1.61-1.68 (2H, m), 2.55-2.62 (1H, m), 2.73-2.79 (1H, m), 3.63 (1H, dd, *J* = 9.9, 3.8 Hz), 3.96 (1H, t, *J* = 10.3 Hz), 4.50-4.54 (1H, m).

Methyl N-acetyl-S-(2-bromo-14-methyl-3-oxopentadecyl)-L-cysteine (8)

To a solution of 1,2-dibromo-14-methylpentadecan-3-one (**6**) (3.5 g, 8.79 mmol) in THF (100 ml), TEA (2.7 ml, 19.3 mmol) was added at 0°C under atmosphere of nitrogen. After being stirred for 15 min at that temperature, *N*-acetyl-L-cysteine (1.6 g, 9.67 mmol) was added to the reaction mixture and stirred for 1 h. The reaction mixture was warmed up to room temperature and stirred for 1 h. The reaction was quenched by addition of 1 N HCl (30 ml) at 0°C and extracted with ethyl acetate. The extract was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure to give **7** as a colorless oil.

To a solution of **7** (8.79 mmol) in THF/MeOH (100/30 ml), 2 mol/L trimethylsilyldiazomethane in ether solution (8.0 ml, 16 mmol) was added and stirred for 1 h. After removing the solvent, the residue was purified by flash column chromatography in silica gel with hexane/EtOAc (From 100/0 to 0/100, v/v) as eluent to give **8** (1.5 g, 35%, 2 steps) as a colorless oil.

¹H NMR (CDCl₃) δ: 0.86 (6H, d, *J* = 6.9 Hz), 1.10-1.35 (16H, m), 1.47-1.68 (3H, m), 2.05-2.07 (3H, m), 2.50-3.28 (5H, m), 3.45-3.49 and 4.34-4.40 (total 1H, each m, amide isomer), 3.65-3.80 (4H, m), 4.79-4.90 (1H, m), 6.19-6.35 (1H, m).

(2R)-Acetylamino-3-[[14-methyl-3-oxo-2-(trimethylammonio)pentadecyl]sulfanyl]propanoate (Vestaine A₁ [synthetic-1])

To a solution of methyl *N*-acetyl-S-(2-bromo-14-methyl-3-oxopentadecyl)-L-cysteine (**8**) (1.5 g, 3.03 mmol) in THF (30 ml), 2 mol/L dimethylamine in THF solution (15 ml, 30 mmol) was added at 0°C under an atmosphere of nitrogen. The reaction mixture was stirred for 1 day with gradual warming up to room temperature. The reaction was quenched by water and extracted with ethyl acetate. The extract was washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure to give **9** as a colorless oil.

A mixture of **9** (3.03 mmol) and MeI (0.94 ml, 15.3 mmol) in CH₃CN (14 ml) was irradiated by microwave reactor at 90°C for 1.5 h. The reaction mixture was concentrated to afford **10** as a colorless oil.

A mixture of **10** and 3 N HCl (20 ml) was heated 80°C for 3 h with stirring. After concentrated the reaction mixture, CH₃CN was added to the residue and dissolved. After removing the unsolved material by filtration, the mother liquid was concentrated and purified by reverse phase HPLC (Nacalai cosmosil 15C₁₈-MS-II, CH₃CN/water = From 1/99 to 90/10, v/v) as eluent to give vestaine A₁ (synthetic-1) (27 mg, 2%, 3 steps) as a colorless amorphous solid.

¹H NMR and MS (ESI) indicated The ¹H NMR spectrum of synthetic vestaine A₁ (**11**) accorded with that of the natural product.

Cell culture

Human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts were obtained from KURABO. HUVECs were cultured in collagen-I coated culture dishes in HuMedia-EG2. Cells were used between passage 3 and 7. Fibroblasts were cultured in culture dishes in HuMedia-EG2 and used between passage 3 and 10. All cells were incubated at 37°C under a humidified atmosphere of 5% CO₂ in air.

Establishment of EGFP-labeled HUVEC (GFP-HUVEC)

HUVECs were seeded at 5 × 10⁵ cells per dish in collagen-I coated 10 cm dishes with 10 ml of VasculLife EnGS Comp Kit. After 1 day, the medium was replaced with ice-cold HuMedia-EG2, and infected with 250 μl of Signal-Lenti Positive Control (GFP) supplemented with 4 μg/ml hexadimethrine bromide. After the second passage, the cells were cultured using HuMedia-EG2. The infected cells were passaged four times, and stored using CELLBANKER-1 at -80°C.

A co-culture assay of endothelial cells with fibroblasts: VS-G assay

Fibroblasts were seeded in 96-well black clear bottom plates (Corning). GFP-HUVECs were seeded at 7.5 × 10³ cells onto the confluent fibroblasts' feeder layers in HuMedia-EG2. In the case of co-treatment with vestaine A₁ and VEGF, the number of GFP-HUVECs was 3.5 × 10³ cells. After adhesion of GFP-HUVECs to the fibroblasts' layers, cells were washed by assay medium (M199, 0.5% FBS), cultured broths or test compounds in assay medium were added, and incubated at 37°C for 3 days. Cell images were captured using an In Cell Analyzer 6000 (GE) or ImageXpress ULTRA (Molecular Devices). The value of area covered by GFP-HUVECs was analyzed using IN Cell Investigator 1.6 or MetaXpress and its value was used as the *in vitro* activity. The wells treated with 0.25% DMSO in assay medium were used as a negative control (0% activity), and the wells treated with 50 ng/ml of VEGF were used as a positive control (100% activity). *In vitro* activity

was calculated as below:

In vitro activity (%) = $100 \times [(test\ compound) - (negative\ control)] / [(positive\ control) - (negative\ control)]$.

For graphs, *in vitro* activity was shown using the number of maximum area value of treatment with vestaine A₁ as a base of 100 and half maximal effective concentration (EC₅₀) was calculated using SAS System Release 9.2 (SAS institute Institute Inc.).

Matrigel tube formation assay

Growth factor reduced Matrigel was added to cold 24-well plates, and incubated at 37°C for more than 30 min. After test compounds were added, HUVECs were seeded at 1×10^5 cells onto the Matrigel, and incubated for 37°C. After 24 h, images of the cells were captured under microscopy.

Measurement of cell survival activity

Confluent HUVECs were treated with test compounds in serum deprived assay medium (M199, 0.5% FBS) at 37°C for 24 h. The medium was replaced with 100 µl of fresh assay medium containing Alamar blue reagent, and incubated at 37°C for 3 h. The fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength. The wells treated with 0.25% DMSO in assay medium were used as a negative control (0% survival), and the wells cultured in growth medium (HuMedia-EG2) were used as a positive control (100% survival). Survival activity was calculated as below:

Cell survival activity (%) = $100 \times [(test\ compound) - (negative\ control)] / [(positive\ control) - (negative\ control)]$.

Western blotting

HUVECs were seeded into collagen-I coated plates, and grown to confluence. HUVECs were starved in M199 containing 0.5% FBS for 3 h. After pre-treatment with an inhibitor for 15 min, the cells were treated with vestaine A₁ for various amounts of time. They were lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete EDTA-free, and phosphatase inhibitor cocktail) on ice for 15 min and centrifuged at 15,000 rpm for 15 min. The supernatants were collected as samples, mixed with NuPAGE® LDS Sample Buffer (Thermo Fisher Scientific Inc), and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to PVDF membrane (Millipore), blocked with BlockACE (DS Pharmabiomedical) in phosphate-buffered saline, pH 7.4, and 0.05% Tween 20 for 1 h at room temperature, and incubated overnight at 4°C with primary antibody (1:1000). The membranes were incubated with diluted horseradish peroxidase-linked secondary antibodies (1:5000) for 1 h at room temperature, and then antibodies were detected using ECL prime Western Blotting Detection Reagents.

In vitro endothelial permeability assay

Endothelial barrier function was evaluated by measuring transendothelial electric resistance (TER) using an xCELLigence Real Time Cell Analyzer (RTCA) DP instrument (ACEA Bioscience) and by referencing the manufacturer's instructions and the previous paper [12]. HUVECs were seeded into the collagen-I coated wells of E-plate VIEW 16 (16 electrodes) and incubated for 24 h. After washing the electrodes with new medium, HUVECs were treated with VEGF (100 ng/ml) for 3 h. Test compounds were added to the E-plate VIEW 16 and the impedance termed "cell index (CI)" was continuously measured in the following hours. The wells cultured in growth medium (vehicle -) were used as vehicle control, and the wells treated with VEGF (VEGF -) were used as VEGF only. For analysis, impedance values were normalized to each time point average impedance value of vehicle control as baseline normalized impedance (termed "baseline normalized CI") according to the manufacturer's instructions. The mathematical formula was shown below:

% of vehicle of baseline normalized CI = $100 \times [(test\ compound) - (VEGF\ only)] / [(vehicle\ control) - (VEGF\ only)]$.

In vivo vascular leakage assay

All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. Six to seven week old C57/BL6 mice were injected intraperitoneally with saline or test compounds. After 4 h, 100 µl of 1.5% Evans Blue was injected into their jugular veins. As soon as the mice were anesthetized with isoflurane inhalation, VEGF (60 ng per ear) was administrated intradermally into ears and circulated for 30 min. The mice were euthanized

by CO₂ inhalation, and their ears were removed. The Evans Blue was extracted in DMSO and its amount was measured at 650 nm with a SpectraMax M5 (Molecular Devices).

Statistical analysis

Each value is expressed as mean ± SEM. Dunnett's multiple comparison test was used to evaluate results from data in Fig. 3B and Fig. 5B. The difference between vehicle- and VEGF-control was compared using a Student's *t*-test. For data in Fig. 5A, multiple pairwise comparisons were made using a Student's *t*-test with Bonferroni corrections. Differences were considered significant if *p* < 0.05.

Results

Vestaine A₁ and vestaine B₁ as novel vasoactive compounds

To obtain novel vasoactive compounds which possess different properties from VEGF, we conducted a screening in a well-established co-culture assay of endothelial cells-fibroblasts (VS-G assay). In the VS-G assay, GFP-labeled human umbilical vein endothelial cells (GFP-HUVECs) were seeded onto feeder fibroblasts, treated with test samples for 3 days, and morphological changes of GFP-HUVECs were analyzed by image analyzer. VEGF treatment readily induces a tube-like structure change of endothelial cells, reflecting strong angiogenic activity of VEGF (Fig. 1A). In the screening for the compounds which induced different morphological changes of endothelial cells from VEGF, we tested about 13,000 culture extracts of the microorganisms. As a result, we found the unique island-shaped morphological change in the extract of an actinomycete strain, *Streptomyces* sp. SANK 63697 (Fig. 1A). From the broth of the SANK 63697, we isolated two active compounds, vestaine A₁ and vestaine B₁. Physico-chemical analysis revealed that vestaine A₁ and vestaine B₁ are novel compounds which consist of *N*-acetylcystein and alkyl chain moieties (Fig. 1B). Details of the fermentation, isolation, physico-chemical properties, and structural elucidations of vestaines were reported independently [13]. The difference between vestaine A₁ and vestaine B₁ is the length of their alkyl chains and the activity of vestaine A₁ and B₁ in VS-G assay was approximately the same potent. The purified vestaine A₁ dose-dependently induced island-shaped morphology in the VS-G assay (Fig. 1C). Based on the identified structure information of natural vestaine A₁ (natural-1), the total synthesis of vestaine A₁ (synthetic-1) was conducted (Fig. 2). We confirmed that synthesized vestaine A₁ (synthetic-1) showed equal potency as purified vestaine A₁ (natural-1) in the VS-G assay (Fig. 1C). Hereafter, we used the synthetic-1 as a representative active compound from SANK 63697 for following biological assays. In the VS-G assay, the effect of VEGF is saturated at 50 ng/ml, so we hypothesized that no additive effect was observed by co-treatment with the compound which acts VEGF pathway and 50 ng/ml of VEGF. In fact, teleocidin B₃, which activates general pathways as a tumor promoter and induced a tube-like morphology in the VS-G assay (Fig. 1A), showed no additive effect by co-treatment with VEGF (Fig. 1D). Co-treatment with vestaine A₁ and VEGF showed synergistic effects in the VS-G assay (Fig. 1D and 1E). These results supported that vestaine A₁ affected endothelial cells via different mechanisms from the VEGF pathway.

The existence of trimethylamine in vestaine A₁ raised the possibility that vestaine A₁ is prone to be degraded around the site and the consequent metabolites exert its function in the VS-G assay. To examine this possibility, the possible metabolite of vestaine A₁, compound-b and compound-c, were synthesized and analyzed in the VS-G assay (Table 1). Neither compound-b nor compound-c showed the activity. On the other hand, compound-a, which conserved most of the chemical structure of vestaine A₁, maintained the activity (Table 1), suggesting that all of the components of vestaine A₁ are related to its activity and vestaine A₁ itself is the active body. Collectively these data indicate that vestaines are new potent vasoactive compounds derived from natural product.

Vestaine A₁ induced endothelial tube formation and promoted cell survival

Then the biological effects of vestaines on endothelial cells were further analyzed. Since the VS-G assay contained both endothelial cells and fibroblasts, we analyzed direct effects

Fig. 1. *Streptomyces* sp. SANK 63697 derived active compounds. (A) Morphologies of GFP-HUVECs in VS-G assay treated with 0.25% DMSO, VEGF (50 ng/ml), broth of *Streptomyces* sp. SANK 63697, or teleocidin B₃ (220 nM). Magnification of the representative morphology was achieved by a 10-fold lens. (B) Structures of vestaine A₁ and vestaine B₁. (C) The activity of purified product from the broth of *Streptomyces* sp. SANK 63697 (natural-1) and chemically synthesized compound (synthetic-1) in VS-G assay. Natural-1 and synthetic-1 were evaluated in the same plate. The value of natural-1 is the black line and that of synthetic-1 is the dotted line. (D) Morphologies of GFP-HUVECs in VS-G assay treated at 220 nM of teleocidin B₃ or at various concentrations of vestaine A₁ alone, or with 50 ng/ml of VEGF. Magnification of the representative morphology was achieved by a 10-fold lens. (E) HUVECs were treated at various concentrations of vestaine A₁ alone or with 50 ng/ml of VEGF. The value of treatment with vestaine A₁ is the black line and that of the co-treatment with vestaine A₁ and VEGF is the dotted line. *In vitro* activity was shown using the number of maximum area value of treatment with vestaine A₁ as a base of 100. Data is representative of three independent experiments. Each value is represented as the mean ± range (n = 2 wells per group).

of vestaine A₁ on endothelial cells using a Matrigel tube formation assay and survival assay. In the Matrigel tube formation assay, vestaine A₁ strongly induced tight tube-like structure

Fig. 2. Scheme of vestaine A₁ synthesis.

Fig. 3. Vestaine A₁ induced endothelial tube formation and promoted cell survival. (A) HUVECs were treated with 1 μ M of vestaine A₁ and cultured on Matrigel for 24 h. Morphologies were observed by optical microscope. Magnification of the representative morphology was achieved by a 4-fold lens. (B) Serum starved HUVECs were treated with 0.25% DMSO, VEGF (50 ng/ml), bFGF (25 ng/ml), or vestaine A₁ (10, 100, or 1000 nM) for 24 h. Survival activity was measured by Alamar blue. Data is representative of three independent experiments. Each value is represented as the mean $\pm$ SEM (n = 4 wells per group). Statistically significant differences were determined by a Dunnett's multiple comparison test vs. VEGF (*: p < 0.05).

formation (Fig. 3A). In the cell survival assay, vestaine A₁ dose-dependently suppressed serum-deprivation induced cell death at the equivalent level as VEGF and bFGF, two major survival factors of endothelial cells (Fig. 3B). These results indicate that vestaine A₁ directly affects endothelial cells and has the potential to induce tube formation and promote endothelial survival, two features of angiogenesis.

Table 1. *In vitro* activity in VS-G assay of vestaine A₁ derivatives. Vestaine A₁ derivatives were evaluated by VS-G assay. EC₅₀ was calculated using the number of maximum area value of vestaine A₁ as a base of 100 and shown the value in two or three independent study

Compound	Structure	EC ₅₀ in VS-G assay
Vestaine A ₁		60-403 nM
Compound-a		248-475 nM
Compound-b		not active (EC ₅₀ > 12.5 μM)
Compound-c		not active (EC ₅₀ > 12.5 μM)

Fig. 4. Vestaine A₁ induced both proliferation and survival signals in endothelial cells. (A) HUVECs were treated with vestaine A₁ at various concentrations for 5 min and the level of the phosphorylated ERK or Akt were determined using Western blotting. (B) HUVECs were treated with 1 μM of vestaine A₁ for various amounts of time. (C) After pre-treatment of inhibitors for 15 min, vestaine A₁ was treated at 1 μM for 5 min. The treatment concentrations are below: PD98059 (PD, 5 μM), LY294002 (LY, 5 μM), and VEGFR2 inhibitor (R2 inh., 0.5 μM). (D) HUVECs were treated with 1 μM of vestaine A₁ or 50 ng/ml of VEGF for 5 min. Data is representative of three independent experiments.

Vestaine A₁ induced both ERK and Akt signals in endothelial cells via a VEGFR2-independent pathway

Then, we analyzed signaling pathways activated by vestaine A₁ in endothelial cells. It is well-known that many vasoactive factors such as VEGF, bFGF, and angiopoietin-1 exert their various vascular activities through the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) in endothelial cells [14, 15]. ERK is known to be activated in the stage of proliferation and angiogenesis and Akt is mainly required for maintenance and quiescence of endothelial cells [16]. To evaluate the effect of vestaine A₁ on ERK and Akt pathways, HUVECs were treated with different doses of vestaine A₁ for different intervals and the level of the phosphorylated ERK or Akt was determined using Western blotting. Vestaine A₁ dose-dependently induced both ERK and Akt phosphorylation (Fig. 4A). The effective concentration was shown to be similar to that in the VS-G assay. The maximum activation was observed at 10 min after treatment (Fig. 4B), which indicates that vestaine A₁ induces ERK and Akt phosphorylation within a relatively short period of time. ERK and Akt phosphorylation was inhibited by MEK1/2 inhibitor (PD98059) or PI3-K inhibitor (LY294002), respectively (Fig. 4C), suggesting that vestaine A₁ activates ERK via a MEK1/2-

Fig. 5. Vestaine A₁ suppressed VEGF-induced vascular leakage. (A) After the pre-treatment with 100 ng/ml of VEGF for 3 h, HUVECs were incubated with 1 μ M of vestaine A₁ and the impedance was continuously measured in the following hours using a xCELLigence real-time cell analyzer DP system. Impedance values were normalized to each time point average impedance value of vehicle as baseline normalized impedance (termed “baseline normalized Cell Index (CI)”) according to the mathematical formula in Materials and Methods. The graph shows the average baseline normalized CI of 3 h after the treatment of vestaine A₁. Statistically significant differences were determined by a Student's *t*-test with Bonferroni corrections as follow: **p* < 0.05 vs. vehicle control, #*p* < 0.05 vs. VEGF control. Data is representative of three independent experiments. Each value is represented as the mean $\pm$ SEM (n = 4 wells per group). (B) After the intraperitoneal injection of vestaine A₁ (0.1 or 1 mg/kg) for 4 h, vascular leakage was induced by the injection of VEGF (60 ng per ear) into the ears of mice and the amount of Evans Blue was determined as an indicator of vessel permeability. The difference between vehicle and VEGF control was compared using a Student's *t*-test (**p* < 0.05). Statistically significant differences were determined by a Dunnett's multiple comparison test vs. VEGF control (ˆ*p* < 0.05). Each value is represented as the mean $\pm$ SEM (n = 8 per group).

dependent pathway and Akt via a PI3-kinase-dependent pathway. A VEGFR2 inhibitor didn't affect the phosphorylation status of ERK and Akt induced by vestaine A₁ (Fig. 4C). Furthermore, VEGF strongly induced VEGFR2 and ERK phosphorylation but weakly induced Akt phosphorylation, whereas vestaine A₁ didn't induce VEGFR2 phosphorylation and strongly induced both ERK and Akt phosphorylation (Fig. 4D). These results indicate that the strengths and patterns of ERK and Akt pathway activation induced by vestaine A₁ are different from that of VEGF, further supporting the possibility that vestaine A₁ could exert a different vasoactive activity from VEGF.

Vestaine A₁ suppressed VEGF-induced vascular leakage

To further elucidate the difference between vestaine A₁ and VEGF action on endothelial cells, we focused on the effects on vascular permeability regulation. VEGF has been known as not only a pro-angiogenic factor but also as a potent stimulator which increases vascular permeability [17, 18]. First, we investigated the effect of vestaine A₁ on barrier function by measuring the impedance using a xCELLigence real-time cell analyzer DP system. Single treatment with vestaine A₁ elevated HUVEC impedance (Fig. 5A). VEGF strikingly decreased the HUVEC impedance, and addition of vestaine A₁ completely recovered the impedance decrease (Fig. 5A). These results suggest that vestaine A₁ enhances endothelial barrier function *in vitro* and also can antagonize the increase of vascular permeability induced by VEGF.

Next, we tested the effect of vestaine A₁ on vascular permeability *in vivo*. Vascular permeability was assessed by measuring Evans Blue dye leakage from ear vessels in mice. VEGF strongly induced leakage of Evans Blue dye, and pretreatment of vestaine A₁ clearly

suppressed vascular permeability induced by VEGF (Fig. 5B). These results suggest that vestaine A₁ also has a suppressive effect on vascular permeability *in vivo*.

Discussion

Angiogenesis is a process critical to maintaining blood vessel integrity, but excess angiogenesis results in the formation of fragile vessels which are largely dysfunctional and cause various problems in several clinical settings. VEGF is a key angiogenic factor and permeability mediator. Excess angiogenesis and the resulting permeability increase triggered by VEGF overproduction is reported as one of the causes of several disease pathologies, such as retinopathy and cerebral hemorrhage. In this context, a treatment which supports angiogenesis, but can antagonize the undesirable effects of VEGF would be useful for treating diseases caused by fragile blood vessels. In the present study, we screened natural products and identified vestaine A₁ and vestaine B₁ as vasoactive compounds. Vestaine A₁ showed the intriguing properties of having both pro-angiogenic and anti-permeability activity.

Vestaine A₁ showed several properties as a pro-angiogenic factor, such as tube formation enhancement on Matrigel (Fig. 3A), promotion of cell survival upon serum deprivation (Fig. 3B), and ERK and Akt activation (Fig. 4A, 4B, 4C, and 4D). However, these effects are supposed to be caused by the signaling pathways different from VEGF. In the VS-G assay, vestaine A₁ promoted an island-shaped morphological change of endothelial cells, whereas VEGF induced a tube-like morphology (Fig. 2A). Recent papers have demonstrated that the difference in the pattern of morphological change in the co-culture assay reflects the difference in the modes of function of vasoactive compounds [19-21]. In agreement with the above papers, although teleocidin B₃ which induced a tube-like morphology in the VS-G assay had no additive effect by co-treatment with VEGF, co-treatment with vestaine A₁ and VEGF showed synergistic effects in the VS-G assay (Fig. 1D and 1E). Furthermore, vestaine A₁ induced ERK and Akt phosphorylation without affecting the VEGF pathway (Fig. 4C). These findings strongly suggest that vestaine A₁ works on endothelial cells via a different pathway from VEGF and has different biological effects on endothelial cells from VEGF. Actually we confirmed that in permeability control vestaine A₁ functioned to antagonize VEGF (Fig. 5A and 5B). The determination of mechanism of action of vestaine A₁, especially identification of target molecules, would provide new insight into the understanding of angiogenesis regulation. Further examination is needed to specify the mechanism.

Vestaine A₁ dramatically enhanced barrier function of endothelial cells both *in vitro* (Fig. 5A) and *in vivo* (Fig. 5B), indicating that vestaine A₁ would work not only to induce angiogenesis but also to enhance vessel maturation. Vascular permeability regulation is critical to maintaining proper transportation of nutrition or chemicals in tissues and to regulate inflammation. VEGF is a well-known inducer of vascular permeability. Excess VEGF induces fragile and leaky vessels with low adhesion molecule expression and poor coverage by mural cells, and causes various problems in disease pathologies through disruption of permeability regulation [17, 18]. Vestaine A₁ suppresses VEGF-induced vascular permeability (Fig. 5A and 5B), suggesting that vestaine A₁ could normalize the abnormal vessels induced by excess VEGF and enhance vascular maturation. Sphingosine 1-phosphate (S1P), the lipid mediator derived from sphingolipid, is a well-known vascular maturation factor. S1P plays a role in promoting angiogenesis and enhancing vascular maturation via its receptor S1PRs, especially via S1P₁ [22]. Using *in vitro* and *in vivo* models, it has been reported that S1P and S1P₁ agonists promote endothelial survival and angiogenesis by activating both the ERK and Akt pathways [23, 24] and reverse endothelial permeability induced by growth factors, LPS, and ischemia-induced injury [25-28]. The similarities between vestaine A₁ and S1P₁ agonists in their biological functions and activated pathways further support that vestaine A₁ would function as both a pro-angiogenic and vessel maturation factor. Although we didn't detect any S1P₁ agonistic activity of vestaine A₁ (unpublished data), it would be intriguing to evaluate crosstalk among the vestaine A₁ pathway and S1P₁ signaling.

As discussed above, the determination of a target molecule of vestaine A₁ is critical to understanding the mechanism of action. So far we do not have many clues to find the target. One interesting structural property of vestaines is betaine structure. Betaine is a neutral chemical compound both with the positively charged part and with the negatively charged part in the same molecule. Trimethylglycine is a typical biological compound having a betaine structure, and it has been known that betaine is involved in methylation reactions and detoxification of homocysteine. Homocysteine is harmful for vessels since it inhibits angiogenesis by preventing proliferation and migration of endothelial cells [29, 30]. Trimethylglycine is known to scavenge the harmful effect following homocysteine using methyl groups to afford methionine [31, 32]. Thus, we expected that the betaine portion of vestaine A₁ might work in the same manner as that of trimethylglycine and exert its effect on endothelial cells. However, trimethylglycine, homocysteine, and *N*-acetyl-L-cysteine methyl ester showed no effect either alone or in combination with vestaine A₁ in the VS-G assay (unpublished data), which suggests that vestaine A₁ provides a protective effect to endothelial cells through a different mechanism from trimethylglycine. Additionally, to elucidate which is an active body, vestaine A₁ or its metabolites, we evaluated the derivatives of vestaine A₁. Table 1 showed that vestaine A₁ itself is an active body and all of the components of vestaine A₁ is necessary for its activity. Further examination would be needed to reveal the molecular pathway of vestaine A₁.

One of the physiologically important effects of vestaine A₁ is suppression of vascular permeability. There are many diseases associated with elevation in vascular permeability. For example, in the lesions of patients suffering from retinopathy, brain hemorrhage, and cancer, bleeding and hypoxia induced by immature and fragile vessels cause disease progression and resistance to therapy. Additionally, in acute respiratory distress syndrome/acute lung injury (ARDS/ALI), the leakage from pulmonary vessels induced by inflammatory cytokines impairs lung functions [33]. Recently, it has been reported that inhibiting agents for vascular permeability are effective for the treatment in animal models. Peng et al. have indicated that S1P and SEW-2871, a selective S1P₁ agonist, decreased pulmonary endothelial leakage in murine and canine LPS models, which are generally used as an ARDS/ALI model [26, 22, 34-37, 25]. These findings suggest that enhancement of vascular integrity may be effective for suppression of vascular leakage. Thus, it would be meaningful to explore the possibility to utilize vestaine A₁ for therapeutic treatment as an inhibitor of vascular permeability in a pathological condition. Further investigation of vestaine A₁ in a disease model relating to increased permeability would be important.

In conclusion, the present study revealed that vestaine A₁ is a unique novel compound with both pro-angiogenic and anti-vascular permeability activity. Although the mechanism of vestaine A₁ action remains to be elucidated, we speculate that identification of unknown target molecules and the pathway of vestaine A₁ for regulating endothelial functions may provide useful insight into vascular biology and also lead to a new therapy for abnormal vascular permeability-related diseases.

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Disclosure Statement

None.

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