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R-(−)-β-O-methylsynephrine, a natural product, inhibits VEGF-induced angiogenesis *in vitro* and *in vivo*

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ABSTRACT

R-(−)-β-O-methylsynephrine (OMe-Syn) is an active compound isolated from a plant of the Rutaceae family. We conducted cell proliferation assays on various cell lines and found that OMe-Syn more strongly inhibited the growth of human umbilical vein endothelial cells (HUVECs) than that of other normal and cancer cell lines tested. In angiogenesis assays, it inhibited vascular endothelial growth factor (VEGF)-induced invasion and tube formation of HUVECs with no toxicity. The anti-angiogenic activity of OMe-Syn was also validated *in vivo* using the chorioallantonic membrane (CAM) assay in growing chick embryos. Expression of the growth factors VEGF, hepatocyte growth factor, and basic fibroblast growth factor was suppressed by OMe-Syn in a dose-dependent manner. Taken together, our results indicate that this compound could be a novel basis for a small molecule targeting angiogenesis.

1. Introduction

Angiogenesis is defined as new blood vessel formation from existing parent vessels through the generation of endothelial cells [1]. This process is regulated by several angiogenic factors leading to induction of persistent angiogenesis by tumors [2]. Pathological angiogenesis can lead to cancer and various inflammatory and ischemic diseases. Therefore, controlling the balance between pro- and anti-angiogenic factors has been one of the key strategies of therapies against cancer and other diseases [3].

Vascular endothelial growth factor (VEGF) ligands are glycoproteins that contribute to vessel development during angiogenesis [4]. These usually exist as homodimers, except for VEGF-A, VEGF-B, and placental growth factor (PlGF), which can form heterodimeric molecules [5]. Vascular endothelial growth factor receptors (VEGFRs) are members of the receptor tyrosine kinase (RTK) superfamily. Binding of VEGFs to their receptor leads to formation of homo- or hetero-dimers of the receptors. These activated VEGFRs induce autophosphorylation of tyrosine residues and trigger the signal transduction cascades of angiogenesis [4,6].

Natural products have played a significant role in the development of new bioactive small molecules and have provided leads for drug development. Several natural compounds show activity as anti-viral, anti-bacterial, and anti-cancer agents [7]. Among these, paclitaxel (Taxol®), a microtubule stabilizer of plant origin, is a complex diterpene that was isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. It is one of the most important anti-cancer drugs, and its analog is used for the production of docetaxel (Taxotere®), which is also in clinical use for cancer treatment [8].

As part of the identification of bioactive natural products that can also be used as tools to probe cellular mechanism of disease, we have built a small natural product fragment library, which contains compounds with MW <250, clogP ⩽4, hydrogen bond donor ⩽4, hydrogen bond acceptor ⩽5, percentage polar surface <45, rotational bond ⩽6. The physicochemical properties reflect indirectly that these compounds show both a reasonable aqueous solubility and a membrane permeation profile [9,10]. Our effort to identify small molecules targeting angiogenesis has led to the identification of *R*-(−)-β-O-methylsynephrine (OMe-Syn), which exhibits potent growth inhibition on human umbilical vein endothelial cells (HUVECs). OMe-Syn is a natural compound isolated from a plant of the Rutaceae family and has a unique chemical structure that is distinct from known angiogenesis inhibitors. To investigate its anti-angiogenic activity, we conducted *in vitro* and *in vivo* angiogenesis assays and found that the compound effectively inhibited VEGF-induced angiogenesis both *in vitro* and *in vivo*. Accordingly, this study is the first report of the anti-angiogenic activities of OMe-Syn and provides both a novel basis for a small molecule probe to decipher the exciting biology of angiogenesis and a new candidate for drug development targeting angiogenesis-related disorders.

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2. Materials and methods

2.1. Reagents

R-(−)-O-methylsynephrine (OMe-Syn) was purified from a plant of the Rutacea family by our group. Endothelial growth medium-2 (EGM-2) was purchased from Lonza (Walkersville, MD). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF) and Matrigel were obtained from KOMA Biotech. (Seoul, Korea) and BD Bioscience (Bedford, MA), respectively. Transwell chamber systems were from Corning Costar (Corning, NY) and the Multiple Simultaneous Tag (MUSTag) cytokine assay kit was from Synthera Technologies (Tokyo, Japan).

2.2. Isolation and structure elucidation of OMe-Syn

The isolation has been previously reported in general [11]. Ground *Clausena emarginata* (100 g), was extracted with methanol. The extract was passed through a strong cation exchange flash column Dowex 50WX8 (20 g) and the alkaloid fraction eluted with methanol–10% ammonia. The fraction was dried and chromatographed on a preparative C18 column (Betasil C18, 21.2 × 150 mm, 5 μm; Thermo Electro Corporation). A separation was performed at a flow rate of 10 mL/min, starting with 100% water (1% TFA) gradient to 60% acetonitrile (1% TFA)–40% water (1% TFA) in 50 min, then gradient to 100% acetonitrile (1% TFA) in the next 10 min. β-O-methylsynephrine (600 mg) was eluted at 5 min. Structure of the compound was elucidated by ^{1}H NMR and 2D NMR (gCOSY, gHMBC, gHSQC). Stereochemistry of the compound was confirmed as R based on the optical value $[\alpha]_D$ −26.3 (c = 0.01, methanol). *R*-(−)-O-methylsynephrine was previously isolated from *Coryphantha* species [12].

2.3. Cell culture and proliferation assay

HUVECs were grown for 7–11 passages in EGM-2 medium supplemented with 10% FBS. HEK293 (human embryonic kidney) and HeLa (human cervical carcinoma) cells were grown in DMEM with 10% FBS and 1% antibiotics. HT1080 (human fibrosarcoma) cells were grown in MEM containing 10% FBS and 1% antibiotics. All cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, and cytotoxicity was analyzed using the Trypan blue exclusion assay.

2.4. Capillary tube formation assay

Matrigel (10 mg/mL) was used to coat a 48-well plate and allowed to polymerize for 1 h at 37 °C. HUVECs (6×10^4 cells) were seeded on the surface of the Matrigel, and then test compounds were added for 4–16 h at 37 °C in the presence or absence of VEGF (50 ng/mL). Morphological changes in the cells and formation of tubular structures were observed under a microscope (IX71, Olympus) and photographed at 100 × magnification (DP70, Olympus).

2.5. Chemoinvasion assay

To examine the invasiveness of HUVECs *in vitro*, we used a Transwell chamber system with 8.0-μm pore polycarbonate filter inserts. Briefly, the lower side of the filter was coated with gelatin (10 μL, 1 mg/mL) and the upper side was coated with Matrigel (10 μL, 3 mg/mL). Test compounds were added to the lower chamber in the presence of VEGF (50 ng/mL) and HUVECs (7×10^5 cells) were placed in the upper chamber of the filter. The chamber was incubated at 37 °C for 18 h, and then the cells were fixed with 70% methanol and stained with hematoxylin and eosin. The invasiveness of cells was measured by counting the number of whole cells in the lower side of the filter using a microscope at 100 × magnification, and cells were photographed at 100 × magnification.

2.6. Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously [13]. Fertilized chicken eggs were kept in a humidified incubator at 37 °C for 3 days. About 2–3 mL of egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. On day 5, a 2.5-cm diameter window was made with a razor and tweezers, and a compound-loaded thermanox coverslip (NUNC, Rochester, NY) was applied to the CAM surface. After further incubation for 2 days, 2–3 mL of Intralipose (Greencross Co, Korea) was injected beneath the CAM and the membrane was observed under a microscope. Retinoic acid (RA) was used as a positive control.

2.7. Multiple simultaneous tag (MUSTag) cytokine assay

To measure expression levels of VEGF, hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF), the MUSTag cytokine assay, which was designed to simultaneously detect multiple cytokines, was used as described previously [14]. Briefly, HUVECs were seeded into a 48-well plate (1×10^5 cells/mL) and starved overnight in media containing 0.5% FBS. Cells were pretreated with the test compound for 1 h and induced with VEGF (10 ng/mL) for 5 h. Cell culture supernatant was added to the ELISA plate, and the antigens allowed to bind to the bottom of the wells, which contained immobilized capture antibodies. Next, MUSTag antibody was applied and oligonucleotide was removed from MUSTag with *Eco*RI. The released DNA was measured by quantitative real-time PCR.

2.8. Statistical analysis

Results are expressed as the mean ± standard error (SE). Student's *t*-test was used to determine the statistical significance between control and test groups. A *p*-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. R-(−)-β-O-methylsynephrine (OMe-Syn) is a new and potent growth inhibitor of HUVECs

OMe-Syn (Fig. 1A) is a natural product that was isolated from a plant of the Rutaceae family. To explore whether this compound affects angiogenesis, we first examined the effect of OMe-Syn on the growth of various cell lines. Interestingly, OMe-Syn showed stronger growth inhibition of human umbilical vein endothelial cells (HUVECs) than of other normal and cancer cell lines (Fig. 1B). Next, cell viability was examined using a Trypan blue exclusion assay. As shown in Fig. 1C, OMe-Syn exhibited no cytotoxicity on HUVECs at concentrations up to 50 μM for 3 days. Accordingly, the following studies were performed using a concentration range of 10–20 μM.

3.2. OMe-Syn showed anti-angiogenic activities in vitro and in vivo

To investigate the anti-angiogenic activity of OMe-Syn, we conducted a number of angiogenesis assays *in vitro* and *in vivo*. First,

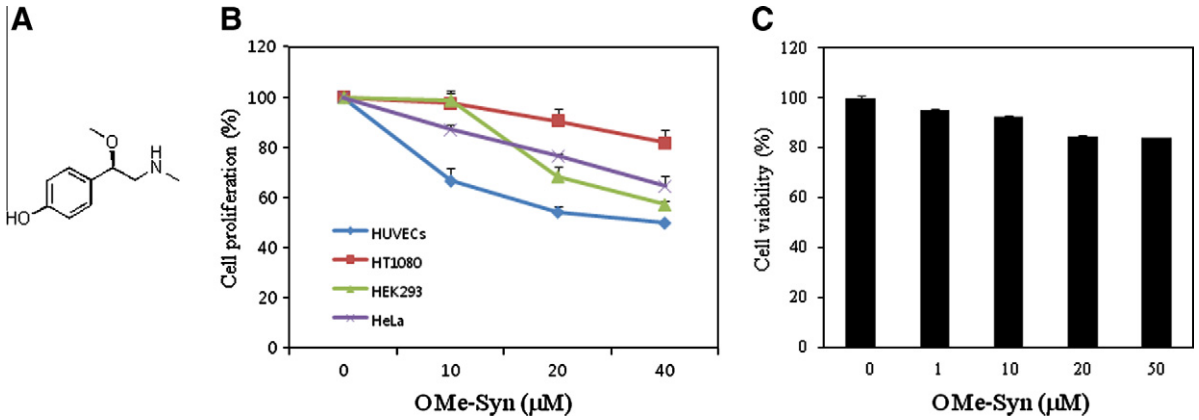

Fig. 1. Chemical structure and specificity of OMe-Syn. (A) Chemical structure of OMe-Syn. (B) The effect of OMe-Syn on cell proliferation. Cells were treated with OMe-Syn (0–40 μM) for 3 days and cell growth was measured using the MTT colorimetric assay. (C) Cell viability was examined using a Trypan blue exclusion assay. OMe-Syn exhibited no toxicity on HUVECs at concentrations up to 50 μM for 3 days.

we performed a tube formation assay using HUVECs. In the presence of VEGF, cultured cells on the Matrigel formed a massive network of tubes compared with non-treated cells. However, OMe-Syn inhibited VEGF-induced tube formation in a dose-dependent manner without showing any toxicity (Fig. 2A). To further characterize the *in vitro* anti-angiogenic activity of this compound, we tested the inhibitory activity of the compound on invasiveness of HUVECs. While VEGF cells efficiently induced invasion of HUVECs, OMe-Syn suppressed this VEGF-induced invasion in a dose-dependent manner (Fig. 2B). These data indicate that OMe-Syn effectively inhibited VEGF-induced angiogenesis *in vitro*.

Next, the effect of OMe-Syn on blood vessel formation *in vivo* was analyzed using the chick embryo chorioallantoic membrane (CAM) assay in growing chick embryos (Fig. 3). After treatment with the compound for 2 days, the CAM was observed under a microscope. OMe-Syn inhibited capillary formation (67%, $n = 14$) during CAM development in a dose-dependent manner without showing any rupture or toxicity to blood vessels. The solvent control (EtOH) exhibited 16.5% inhibition ($n = 18$). Retinoic acid (RA) was used as a positive control for anti-angiogenic response.

3.3. OMe-Syn inhibited expression of pro-angiogenic factors VEGF, HGF, and bFGF under VEGF-induced conditions

To further confirm these findings, a multiple simultaneous tag (MUSTag) cytokine assay was performed. This assay enables simultaneous detection of three key pro-angiogenic factors (VEGF, HGF, and bFGF) with high sensitivity. To investigate the effect of OMe-Syn on VEGF-induced expression of growth factors, we conducted this assay using conditioned media of HUVECs treated with the compound. The expression levels of VEGF, HGF, and bFGF in HUVECs were suppressed by OMe-Syn in a dose-dependent manner. Suberoylanilide hydroxamic acid (SAHA), a known histone deacetylase (HDAC) inhibitor, was used as a positive control (Fig. 4).

In this study, we provide the first evidence of anti-angiogenic activities of OMe-Syn. The compound inhibited VEGF-induced angiogenesis in a dose-dependent manner both *in vitro* and *in vivo*. In addition, expression of the pro-angiogenic factors VEGF, HGF, and bFGF in HUVECs was suppressed by OMe-Syn in a dose-dependent manner. OMe-Syn possesses lead-like physicochemical

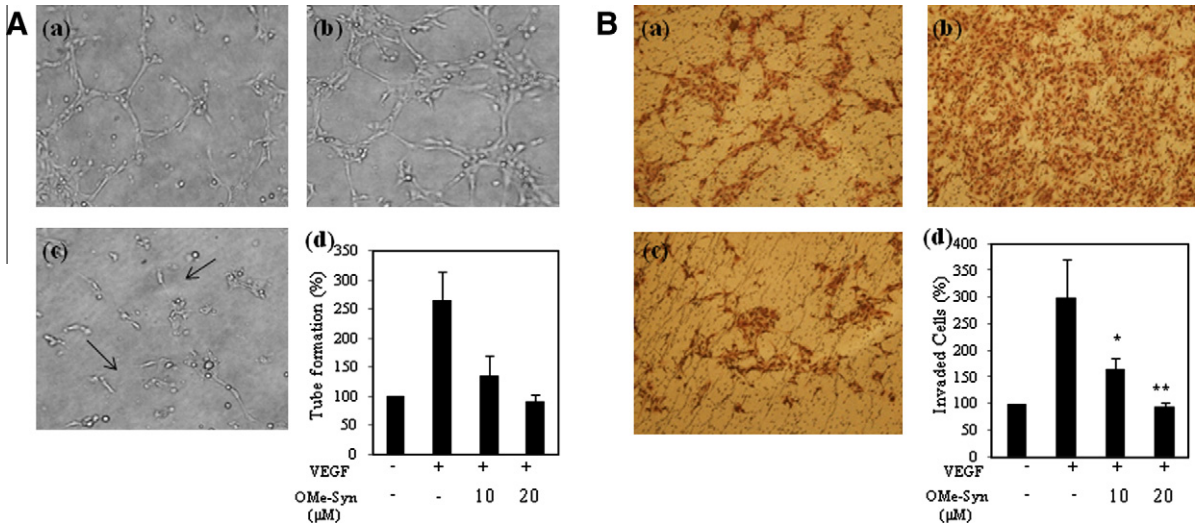

Fig. 2. Anti-angiogenic activities of OMe-Syn *in vitro*. (A) Tube formation assay: (a) non-treated cells, (b) VEGF (50 ng/mL) alone, (c) co-treatment with VEGF (50 ng/mL) and OMe-Syn (20 μM) and (d) quantitative analysis of the tube formation assay. Treatment of HUVECs with OMe-Syn resulted in dose-dependent inhibition of tube formation induced by VEGF. Arrows indicate broken tubes following treatment with OMe-Syn. (B) Effect of OMe-Syn on VEGF-induced invasion of HUVECs: (a) non-treated cells, (b) VEGF (50 ng/mL) alone, (c) OMe-Syn (20 μM) and VEGF (50 ng/mL) and (d) quantitative analysis of invaded cells. OMe-Syn inhibited VEGF-induced invasion of HUVECs in a dose-dependent manner. $^{*}p < 0.005$ versus VEGF control; $^{**}p < 0.001$ versus VEGF control. Each value represents mean ± SE from three independent experiments.

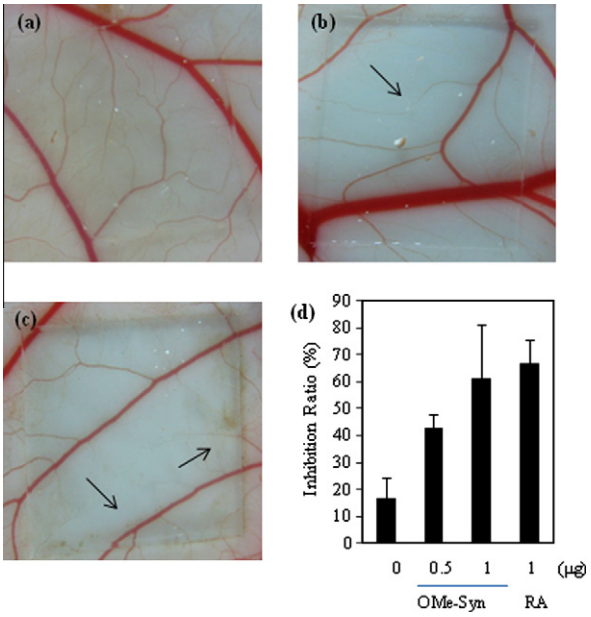

Fig. 3. *In vivo* anti-angiogenic activity of OMe-Syn: (a) EtOH control, (b) RA (1 μg/egg), and (c) OMe-Syn (1 μg/egg) were applied to the CAM and the membrane was observed. Arrows indicate inhibition of neovascularization of CAM by the compound. (d) The inhibition ratio was quantified as the percentage of eggs showing inhibition relative to the total number of eggs tested.

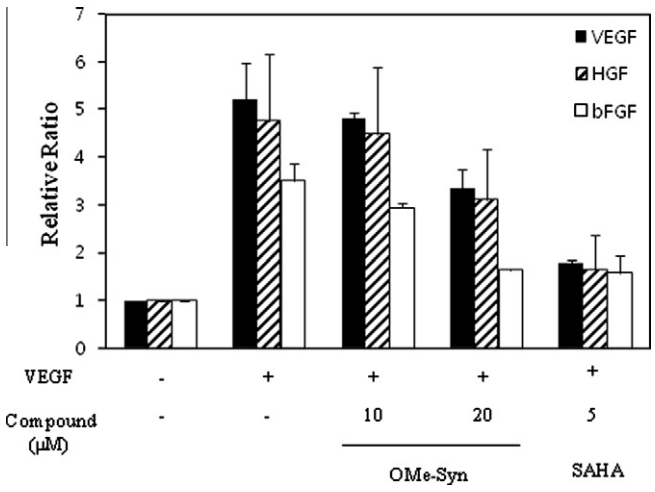

Fig. 4. Effect of OMe-Syn on VEGF-induced growth factor expression. The MUSTag cytokine assay was conducted as described in section 2. OMe-Syn inhibited VEGF-induced expression of growth factors VEGF, HGF, and bFGF in a dose-dependent manner. Suberoylanilide hydroxamic acid (SAHA) was used as a positive control. Results shown are representative of three independent experiments.

properties, conferring good solubility. Moreover, its structure is unique and simple: attractive properties for chemical synthesis. Therefore, this compound could be a novel basis for small molecules targeting angiogenesis and its derivatives could have therapeutic significance in the treatment of angiogenesis-related diseases. Further study to identify the targets of OMe-Syn will elucidate the detailed mechanism of angiogenesis inhibition and provide new insights into the key players of angiogenesis.

Acknowledgments

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