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Identification of resveratrol derivative 3,3′,4,4′,5,5′-hexamethoxy-trans-stilbene as a novel pro-angiogenic small-molecule compound

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ABSTRACT

The potential to promote neovascularization in ischemic tissues using exogenous agents is an attractive avenue for therapeutics. To identify novel pro-angiogenic small-molecule compound, we screened a series of resveratrol methylated derivatives and identified 3,3′,4,4′, 5,5′-hexamethoxy-trans-stilbene (3,3′,4,4′,5,5′-HMS) potently promotes proliferation, migration, invasion and tube formation of human umbilical vein VECs (HUVECs) *in vitro*. Furthermore, 3,3′,4,4′,5,5′-HMS accelerates neo-vessels sprouting of rat aortic rings *ex vivo*, and neovascularization of chick chorioallantoic membrane (CAM) and mouse matrigel plugs *in vivo*. Microarray analyses show that the level of early growth response 1 (EGR-1), an inducible pro-angiogenic gene regulatory factor, was upregulated. The upregulation of EGR-1 was confirmed by semiquantitative RT-PCR, quantitative real-time PCR and western blotting analyses. In addition, the levels of several pro-angiogenic factors including transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), nitric oxide (NO), and the activity of endothelial NO synthase (eNOS) were elevated in 3,3′,4,4′,5,5′-HMS-treated HUVECs. Inhibition of NO synthase by L-NAME blocked the pro-angiogenic effects of 3,3′,4,4′,5,5′-HMS. Our research shows that 3,3′,4,4′,5,5′-HMS dramatically promoted angiogenesis *in vitro, ex vivo* and *in vivo,* which might represent a novel potential agent for the development of therapeutic drugs to treat ischemic diseases.

1. Introduction

Approaches for promoting neovessel development in ischemic tissue include administration of pro-angiogenic growth factors, either as recombinant proteins or as gene therapy, and more recently, to transplantation of stem or progenitor cells (Cooke and Losordo, 2015). Protein delivery is the most straightforward strategy but is very difficult to target and maintain pro-angiogenic activity at a desired location. Moreover, most recombinant proteins are quickly eliminated by the mononuclear phagocyte system with only a small fraction reaching the specific site of interest (Chu and Wang, 2012). Although gene therapy can lead to high level of protein efficiency providing a sustained source of pro-angiogenic growth factors, its disadvantages include relatively low transfection rates, risk of uptake of vectors and gene expression at distant sites with unwanted effects in non-target tissues as well as the inability to withdraw treatment rapidly (Chu and Wang, 2012). Compared with single pro-angiogenic growth factor therapies, cell therapies have emerged as a promising avenue for therapeutic angiogenesis. However, it is a great challenge to collect enough cells on the order of millions per patient. Moreover, the viability of these cells is usually very low *in vivo*, and even worse, most cells fail to integrate with the host tissue and die soon after delivery (Chu and Wang, 2012). The use of small-molecule compounds for therapeutic angiogenesis would obviate many disadvantages associated with the pro-angiogenic growth factors delivery or cell therapy (Murphy et al., 2006). Furthermore, most small-molecule compounds have the advantages of extended *in vivo* stability, low cost, and scalable production, so they are particularly appealing for potential regenerative medicine applications (Segar et al., 2013).

Natural products (NPs) are believed to be an important source of new clinical drugs, including structural modification of natural small-molecule compounds or synthesis of new compounds designed following a natural compound as a model (Chen et al., 2015; Cragg and Newman, 2013). Resveratrol (*trans*-3,4′,5-trihydroxystilbene; RVT), a phytoalexin found in many types of foods, such as the skin of grapes, peanuts, mulberries, red wine, *Polygonum cuspidatum* Sieb et Zucc and so on (Zhang et al., 2015b), is one such NP and has been shown to have a regulatory effect on angiogenesis. Unfortunately, RVT exhibits opposite effects on angiogenesis, either pro- or anti-angiogenic effects, depending on

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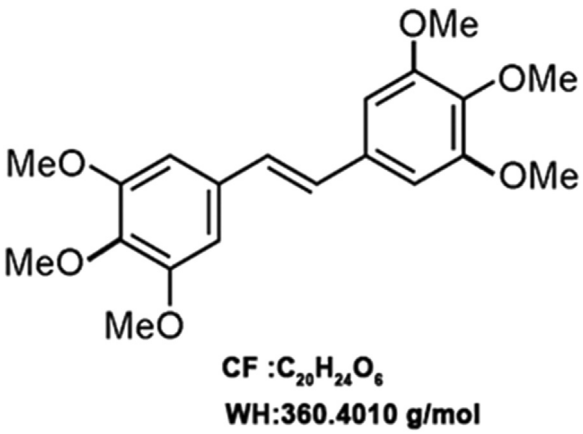

Fig. 1. The chemical structure of 3,3′,4,4′,5,5′-hexamethoxy-trans-stilbene (3,3′,4,4′,5,5′-HMS). 3,4,5,3′,4′,5′-HMS has the molecular formula $C_{20}H_{24}O_6$ and a molecular weight of 360.4010 g/mol.

the situation, applied dosage and different cell type (Wang et al., 2010).

Modification of RVT has predominantly been concerned with the introduction of additional hydroxy moieties into the trihydroxystilbene framework and various degrees of methylation of the phenol groups (Gosslau et al., 2005). An increasing number of studies have demonstrated that many RVT methylated derivatives showed higher bioavailability and biological activity than RVT (Kapetanovic et al., 2011; Sale et al., 2005; Walle, 2011). To discover novel small-molecule compound promoting angiogenesis, we synthesized a series of RVT methylated derivatives. After screening, we found that 3,3′,4,4′,5,5′-hexamethoxy-trans-stilbene (3,3′,4,4′,5,5′-HMS, Fig. 1) potently promoted proliferation of human umbilical vein VECs (HUVECs) *in vitro*. In the present study, we aimed to identify the pro-angiogenic effect of 3,3′,4,4′,5,5′-HMS both *in vitro* and *in vivo* and determine the corresponding mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

3,3′,4,4′,5,5′-HMS was synthesized in our laboratory (purity was established as at least 99% by HPLC analysis) and dissolved in dimethyl sulfoxide (DMSO) to form a 0.1 mol/L stock solution, which was stored at −20 °C in small aliquots until needed and then diluted to various concentrations as needed. Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA, USA). MCDB 131 medium, protease inhibitor cocktail, nitr-L-arginine methyl ester (L-NAME), the primary antibodies anti-early growth response-1 (Egr-1), anti-β-actin, anti-von Willebrand factor (vWF), anti-CD31 and HRP-conjugated secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibody anti-transforming growth factor β1 (TGF-β1) was purchase from Abcam (Cambridge, MA, USA). The primary antibody anti-vascular endothelial growth factor (VEGF) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primary antibodies anti-endothelial NO synthase (eNOS) and anti-phosphorylated (p)-eNOS were purchased from ABclonal (Cambridge, MA, USA). Vascular endothelial growth factor (VEGF), VEGF and transforming growth factor β1 (TGF-β1) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). DMSO, 3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyl-tetrazolium (MTT), Tween 20, Tris, sodium dodecylsulfate (SDS), glycerol, β-mercaptoethanol, bromophenol blue, Coomassie brilliant blue, bovine serum albumin (BSA) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Shanghai Sangon Biotech (Shanghai, China). Matrigel was purchased from BD Biosciences (San Diego, CA, USA). Nitric oxide (NO) and eNOS detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). TRIzol reagent was acquired from Invitrogen (Carlsbad, CA, USA). PrimeScriptTM RT reagent kit and SYBRH PremixEx TaqTM were purchased from TaKaRa (Kyoto, Japan). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA).

2.2. Animals

8-week-old Sprague-Dawley rats were purchased from Laboratory Animal Center of Zhengzhou University, China. 8-week-old C57BL/6 mice were obtained from Department of Laboratory Animal Science, Peking University Health Science Center (PUHSC), China. The experimental animals were housed under standard conditions of humidity, room temperature and dark-light cycles, and fed certified laboratory feed (Labofeed H, ISO 22000). Animals underwent deep anesthesia with isoflurane prior to any procedures to minimize suffering. When the experiments were terminated, the animals were killed in a CO_2 chamber.

2.3. Cell culture

HUVECs were obtained in our laboratory as previously described (Jaffe et al., 1973). Cells (1–8 passage) were cultured in MCDB 131 medium, supplemented with 15% FBS and 20 ng/ml VEGF in a humidified incubator at 37 °C with 5% CO_2. The identity of HUVECs was confirmed by their cobblestone morphology and strong positive immunoreactivity to vWF.

2.4. Exposure of HUVECs to 3,3′,4,4′,5,5′-HMS

HUVECs were plated in 24-well cell culture plates. When cells reached 80% confluency, they were treated with DMSO (80 μM) as negative control groups, VEGF (20 ng/ml) as positive controls or various concentrations (0, 5, 10, 20, 40, or 80 μM) of 3,3′,4,4′,5,5′-HMS in basal MCDB 131 medium without FBS and VEGF as test groups. The morphological changes of HUVECs were observed and images were taken under a phase contrast microscope (Nikon, Japan).

2.5. Cell proliferation assay

Cell proliferation was quantified by counting the cell number directly and a MTT assay. After treatment as mentioned above, the HUVECs in each group were resuspended in MCDB 131 culture medium following digestion. A drop of the cell suspension of each sample was placed on a hemacytometer (QiuJing, Shanghai, China) and the number of cells were counted under a phase-contrast microscopy (Nikon, Japan). For MTT measurements, the HUVECs were seeded in 96-well cell plates and cultured in completed MCDB 131 medium for 24 h. The cells were then treated as described above for 20 or 44 h. Twenty μl of MTT solution (0.5 mg/ml) was added to each well and incubated at 37 °C for another 4 h to allow the formation of blue formazan crystals. Residual MTT and crystals dissolved by incubation with DMSO (100 μl each well) were carefully removed. Plates were shaken for 10 min, and the absorbance was measured at 450 nm using an Epoch™ spectrophotometer (BioTek Instruments, Inc. Vermont, USA).