JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Antiangiogenic Activity of Hypoxylonol C

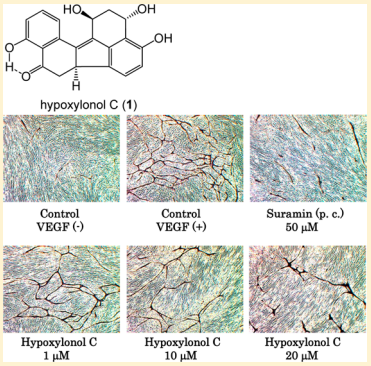
Miyuki Fukai,[†] Toshihiro Suzuki,[‡] Ikuko Nagasawa,[†] Kaoru Kinoshita,[†] Kunio Takahashi,[†] and Kiyotaka Koyama*[,†]

[†]Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University, Noshio 2-522-1 Kiyose-shi, Tokyo 204-8588, Japan

[‡]Department of Analytical Biochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan

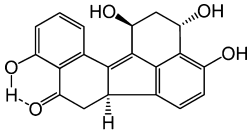
Supporting Information

ABSTRACT: Hypoxylonol C (**1**), isolated from the inedible mushroom *Hypoxylon truncatum*, exhibited inhibitory activities against the migration and tube formation of HUVECs. A cDNA microarray analysis was performed to investigate the target of hypoxylonol C (**1**) in HUVECs, and it was found that the genes related to cell cycle and adhesion were down-regulated. The down-regulation of mRNA levels of cell cycle and adhesion genes was confirmed by real-time RT-PCR. Cell cycle arrest and suppression of adhesion molecule expression might be plausible mechanisms of actions for the antiangiogenic activity of hypoxylonol C (**1**).



Tumor angiogenesis, defined as the formation of new capillaries from existing vessels toward a tumor, plays an important role in the growth of tumor cells. This phenomenon involves several steps such as proliferation, migration, and tube formation of vascular endothelial cells. Tumors can grow at an accelerated pace by receiving nutrients and oxygen and by removal of waste products; moreover they can metastasize to other organs through new vessels. Therefore, tumor angiogenesis inhibitors represent a promising strategy for the treatment of cancer.

We have previously reported a benzo[*j*]fluoranthene derivative, hypoxylonol C (**1**), as a major component of the mushroom *Hypoxylon truncatum* (Schweinitz:Fries) J. H. Miller (Xylariaceae). This compound showed antiproliferative activity against human umbilical vein endothelial cells (HUVECs).[1,2] In this paper, we report the antiangiogenic activity and propose the targeted genes of HUVECs affected by hypoxylonol C (**1**).



hypoxylonol C (**1**)

Hypoxylonol C (**1**) was found to inhibit the first step of angiogenesis, the proliferation of vascular endothelial cells with an IC_{50} value of 21 μM.[2] We examined the effect of hypoxylonol C (**1**) against the migration of HUVECs. HUVECs were seeded in an inner chamber with hypoxylonol C (**1**), and vascular endothelial growth factor (VEGF) was added in an outer chamber. Induced by VEGF, HUVECs migrated to the reverse side of the membrane through the pores. Inhibition of migration was determined by counting the number of migrated cells. The number of migrated cells was significantly decreased at the concentration of 1, 10, and 20 μM of hypoxylonol C (**1**). This compound inhibited VEGF-induced migration of HUVECs in a dose-dependent manner (Figure 1). Next, the inhibitory effect against tube formation was tested. HUVECs and normal human dermal fibroblasts (NHDFs) were cocultured with 20 ng/mL VEGF and four concentrations of hypoxylonol C (**1**) for 11 days. After staining of formed tubes, the network patterns were photographed, and the number of branching points and the length of tubes were quantified. HUVECs stimulated with VEGF formed networks, while cells without VEGF did not. The number of branching points was significantly decreased at the concentration of 1, 10, and 20 μM of hypoxylonol C (**1**). Also, the length of formed tubes was significantly decreased with the presence of hypoxylonol C (**1**). Hypoxylonol C (**1**) inhibited VEGF-induced tube formation of HUVECs in a dose-dependent manner (Figure 2).

Microarray analysis is a useful method to understand the molecular mechanism by which compounds affect cell behavior.[3,4] We investigated the gene expression profile of HUVECs treated with VEGF and hypoxylonol C (**1**) using cDNA microarray analysis to understand the mechanism of antiangiogenic activity of hypoxylonol C (**1**).

As a result, 1489 and 861 genes were up- and down-regulated, respectively (Supporting Information). Pathway analysis using DAVID (http://david.abcc.ncifcrf.gov/

Received: September 10, 2013
Published: March 4, 2014

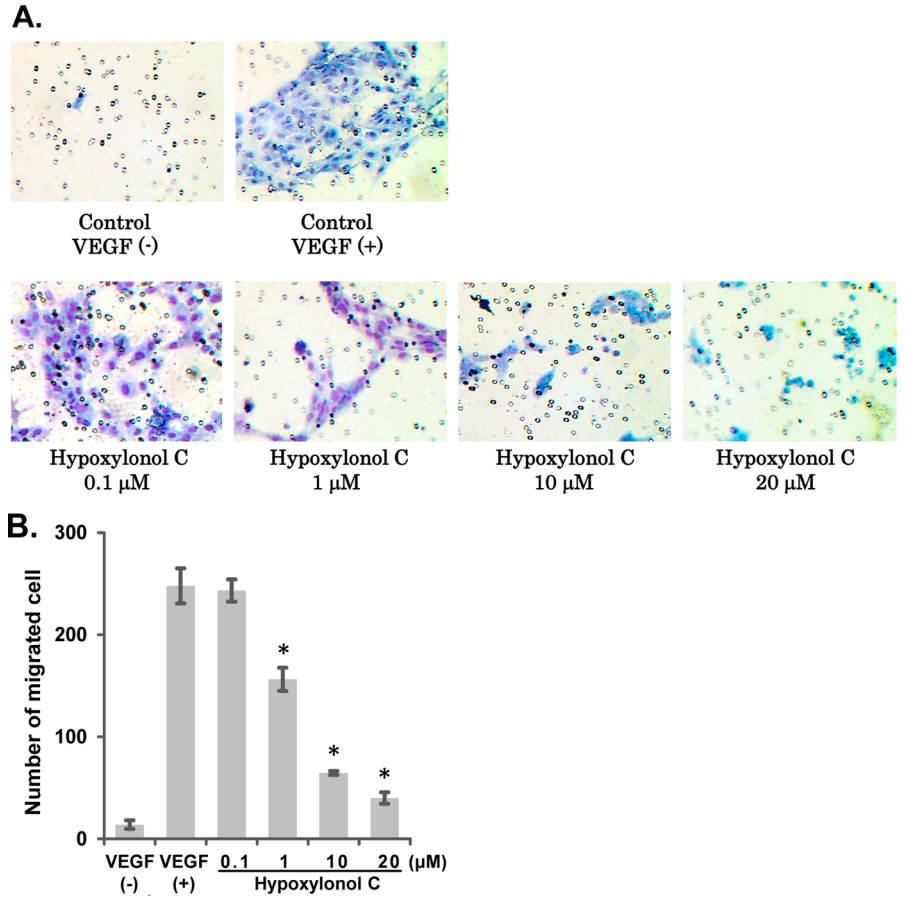


Figure 1. Inhibitory effect of hypoxylonol C (**1**) on the migration of HUVECs. HUVECs (5×10^4 cells/well) with 0.1, 1, 10, and 20 μM hypoxylonol C (**1**) were seeded in the inner chamber. The outer chamber (24-well plate) of VEGF (−) as control was filled with the same medium. The outer chambers of VEGF (+) as control and 1, 10, and 20 μM hypoxylonol C (**1**) were filled with a medium containing 20 ng/mL VEGF. After 22 h of incubation at 37 °C in 5% CO_2, the nonmigrated cells on the upper surface of the membrane were removed. The migrated cells were then fixed and stained. The migrated cells were photographed under a microscope (A) and counted manually. Bars indicate means ± SE in triplicate tests. *$p < 0.05$ compared to VEGF (+) control (B).

summary.jsp) and KEGG (http://www.genome.jp/kegg/pathway.html) suggested that several cell cycle- and adhesion-related genes were down-regulated. To confirm the results of the microarray analysis, we performed real-time reverse transcription-polymerase chain reaction (real-time RT-PCR); the relative mRNA levels of cyclins A_2 and B_1, cyclin-dependent kinase (CDK) 1, integrins α_3 and β_1, and vascular endothelial (VE)-cadherin were clearly down-regulated (Figure 3).

The cell cycle is controlled by phosphorylation of CDKs by forming complexes with cyclins, molecules that are synthesized and degraded during each cell cycle. The expression of cyclin A is required for the S phase transition and the control of DNA replication. CDK1−cyclin B complex regulates several events during the G2 to M transition and progression through mitosis.[5] TR-644, a combretastatin A-4 analogue, caused G2/M phase arrest of HUVECs accompanied by a decreased expression of cyclin B_1 and CDK1.[6] Delphinidin, an anthocyanin compound, inhibits endothelial cell proliferation and migration by inducing a down-regulation of cyclin A expression.[7]

Integrins are a family of heterodimeric transmembrane adhesion receptors, composed of α- and β-subunits, which bind to the extracellular matrix. Specifically, this receptor modulates the functional connection between focal adhesion and the actin skeleton that is required to drive cell migration.[8,9] VE-cadherin is an endothelial-specific cell−cell adhesion protein of the adherens junction complex. This adhesion protein is indispensable for maturation, extension, and remodeling of blood vessels that are characteristic of angiogenesis.[9,10] Genistein, a major isoflavone found in soybeans, is known as an angiogenesis inhibitor, which causes the down-regulation of VE-cadherin and integrin levels in HUVECs.[4] It has been suggested that hypoxylonol C (**1**) showed the antiangiogenic effect *in vitro* by down-regulating the level of cell cycle- and adhesion-related molecules in a manner similar to genistein. Six antiangiogenic inhibitors, bevacizumab, sorafenib, sunitinib, pazopanib, axitinib, and regorafenib, are currently used clinically in Japan. Bevacizumab is an antibody drug that binds the ligand of KDR (VEGFR-2) and inhibits the binding of VEGF and KDR. The remainder are tyrosine kinase inhibitors that inhibit KDR and its intracellular signaling related to vascular endothelial cell survival, proliferation, and migration. In contrast, cilengitide, a cyclic pentapeptide, is the first antiangiogenesis inhibitor candidate targeting the adhesion molecules, integrins. This drug induces apoptosis of endothelial cells via the inhibition of the interaction between integrins and their ligands.[11] Adhesion molecule inhibitors are expected to become a new class of anticancer agents.

In conclusion, it is suggested that hypoxylonol C (**1**) has a dual effect against HUVECs: the arrest of cell cycle at the G2/

dx.doi.org/10.1021/np400687y | *J. Nat. Prod.* 2014, 77, 1065−1068

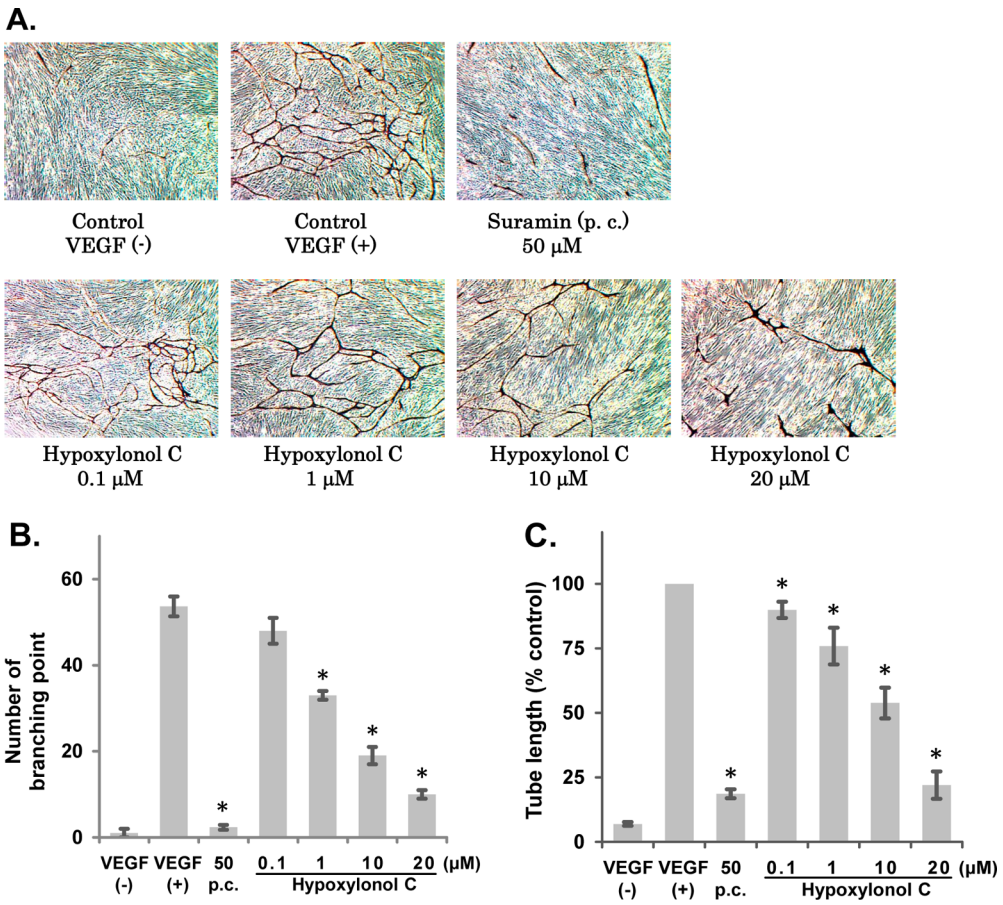


Figure 2. Inhibitory effect of **1** on the tube formation of HUVECs. HUVECs and NHDFs were cocultured on the plate. The wells of VEGF (−) as control were filled with the medium without VEGF. Those of VEGF (+) as control, suramin as positive control, and 0.1, 1, 10, and 20 μM hypoxylonol C (**1**) were filled with a medium containing 10 ng/mL VEGF. After incubation for 11 days, cells were fixed and stained with anti-human CD31 antibody. After drying overnight, the wells were photographed under a microscope (A). The number of branching points was counted (B). The tube length measurements were presented as percentage of the VEGF (+) control (C). Bars indicate means ± SE in triplicate tests. *$p < 0.05$ compared to VEGF (+) control.

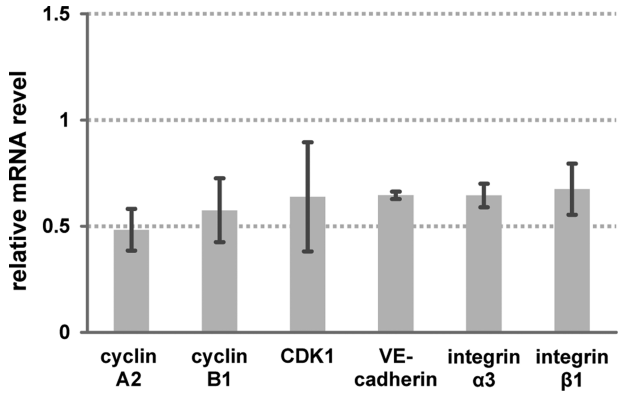


Figure 3. Effects of hypoxylonol C (**1**) on the cell cycle- and adhesion-related gene expression levels in HUVECs. Total RNAs were isolated from HUVECs cultured with 1 nM VEGF and 20 μM hypoxylonol C (**1**) or 0.1% DMSO for 24 h at 37 °C in 5% CO_2. Reverse transcription and real-time PCR were performed as described in the Experimental Section. Relative mRNA expression levels of cells treated with hypoxylonol C (**1**) compared to those of cells treated with DMSO were calculated by the $2^{-\Delta\Delta CT}$ method. 18s rRNA was used as a control. Bars indicate means ± SE in triplicate tests.

M phases by down-regulation of cell cycle-related gene expressions and the inhibition of angiogenesis of vascular endothelial cells by suppressing the expression of adhesion molecules.

EXPERIMENTAL SECTION

General Experimental Procedures. Hypoxylonol C (**1**; purity = 95.9%) was isolated from the $CHCl_3$ extract of the fruiting bodies of *Hypoxylon truncatum*, as described previously.[2] Microscopic photographs were taken by CKX31 (Olympus, Tokyo, Japan) and WRAYCAM G900 (Wraymer, Inc., Osaka, Japan) microscopes. A microarray slide was hybridized using a Takara Hybridization chamber TX711 (Takara Bio, Inc., Shiga, Japan). The fluorescence images of the microarray slide were scanned and detected with a 3D-Gene Scanner 3000 (Toray, Tokyo, Japan). Real-time PCR was performed by using a 7300 system (Life Technologies, CA, USA). TaqMan gene expression assays CDK1 (Hs00938777_m1), CDK2 (Hs01548894_m1), CDK4 (Hs01565683_g1), CDK6 (Hs01026371_m1), cyclin A_2 (Hs00996788_m1), cyclin B_1 (Hs00259126_m1), cyclin D_1 (Hs00765553_m1), cyclin E_2 (Hs00180319_m1), integrin α_3 (Hs01076873_m1), integrin β_1 (Hs00559595_m1), VE-cadherin (Hs00901463_m1), and 18s rRNA (Hs03928985_g1) were purchased from Life Technologies.

Cell Culture. HUVECs were purchased from Lonza Walkersville, Inc., MD, USA, and cultured using EGM-2 (Lonza Walkersville, Inc.) at 37 °C in 5% CO_2.

Migration Assay. Inhibition of cell migration was determined using a PET membrane filter from the inner chamber of an 8 μm Falcon cell culture insert (Becton, Dickinson and Company, NJ, USA).

dx.doi.org/10.1021/np400687y | *J. Nat. Prod.* 2014, 77, 1065−1068

HUVECs (5×10^4 cells/well) suspended in EBM-2 medium containing 0.2% FBS with various concentrations of **1** dissolved in DMSO were seeded in the inner chamber. Next, the inner chamber was placed into the outer chamber (24-well plate), which was filled with the same medium containing 20 ng/mL VEGF. After 22 h incubation at 37 °C in 5% CO_2, the nonmigrated cells on the upper surface of the membrane were removed by wiping with cotton swabs. The cells were then fixed and stained using the Diff-Quik system (Sysmex, Hyogo, Japan). The cells, which migrated through the pores to the reverse side of the membrane, were photographed under a microscope and counted manually using six different microscopic fields (200×).

Tube Formation Assay. The tube formation assay was performed using an angiogenesis kit (Kurabo, Tokyo, Japan). HUVECs and NHDFs were cocultured in 24-well plates and incubated with optimized medium. After 3 h of incubation at 37 °C in 5% CO_2, the medium was replaced with fresh medium containing 10 ng/mL VEGF and different concentrations of **1**, and the plates were incubated for 11 days at 37 °C in 5% CO_2 (medium was replaced on days 4, 7, and 9). After 11 days, the formed tubes were fixed with 70% EtOH and stained with mouse anti-human CD31, goat anti-mouse IgG alkaline phosphatase conjugate, and BCIP/NBT. Tube network patterns were identified under a microscope (200×) and photographed. Both the number of branching points and the length of formed tube were quantified as described previously.[12] Suramin, which is an antagonist against growth factors, well known as an antiangiogenic agent, was used as a positive control.[13]

mRNA Preparation and cDNA Microarray Analysis. HUVECs cultured in collagen-coated 6φ dishes (3.5×10^4 cells/cm^2) were starved in 1% FBS-EBM-2 medium for 24 h at 37 °C in 5% CO_2. After starvation, the cells were exposed to 1 nM VEGF and 20 μM **1** or DMSO and incubated for 24 h at 37 °C in 5% CO_2. The cells were homogenized by using Qiazol lysis reagent and QIAshredder (QIAGEN, Venlo, Netherlands). Total cellular RNAs were isolated by using miRNeasy Mini (Qiagen) and amplified using the Ambion Amino Allyl aRNA kit (Life Technologies, CA, USA). Amplified RNAs were labeled with Amersham Cy5 or Cy3 mono-reactive dye (GE Healthcare UK Ltd., Buckinghamshire, England). A 3D-Gene Human Oligo chip 25k (TORAY), which was loaded with 24460 human genes, was used. The slide was hybridized with Cy5- and Cy3-labeled cDNA for 16 h at 37 °C and scanned to obtain fluorescence images. The intensities of Cy5 and Cy3 of each spot were detected, quantified, and normalized by adjusting the median of Cy3/Cy5 to 1. Cy3/Cy5 values of $\geq$1.5 and $\leq$0.67 were considered to be up- and down-regulated on each gene, respectively. The pathway analysis was performed by DAVID 6.7 (National Institute of Allergy and Infectious Diseases (NIAID, NIH), MD, USA).

Real-Time RT-PCR. Total cellular RNAs were isolated from HUVECs exposed with 1 nM VEGF and 20 μM **1** or DMSO for 24 h as described above. Reverse transcription was performed with SuperScript VILO MasterMix according to the manufacturer's instructions. Real-time PCR was performed with TapMan Universal PCR Master Mix II and TaqMan gene expression assays (Life Technologies). 18s rRNA was used as a control. Changes in expression level were calculated as relative mRNA level by the $2^{-\Delta\Delta CT}$ method.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effects of hypoxylonol C on the cell cycle- and adhesion-related gene expression levels in HUVECs and down-regulated genes in HUVECs treated with hypoxylonol C. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-42-495-8913. Fax: +81-42-495-8912. E-mail: kiyotaka@my-pharm.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was partially supported by the Japan Society for the Promotion of Science (JSPS) AA Scientific Platform Program (2010−2012), by a grant from the High-Tech Research Center Project, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (S0801043), and by a Sasakawa Scientific Research Grant from the Japan Science Society (24-324).

■ REFERENCES

(1) Koyama, K.; Kuramochi, D.; Kinoshita, K.; Takahashi, K. *J. Nat. Prod.* **2002**, *65*, 1489−1490.
(2) Fukai, M.; Tsukada, M.; Miki, K.; Suzuki, T.; Sugita, T.; Kinoshita, K.; Takahashi, K.; Shiro, M.; Koyama, K. *J. Nat. Prod.* **2012**, *75*, 22−25.
(3) Kim, J.; Shim, J.; Lee, S.; Kim, K.; Rha, S.; Chung, H.; Kwon, H. *Jpn. J. Cancer Res.* **2002**, *93*, 1378−1385.
(4) Piao, M.; Mori, D.; Satoh, T.; Sugita, Y.; Tokunaga, O. *Endothelium* **2006**, *13*, 249−266.
(5) Malumbres, M.; Barbacid, M. *Trends Biochem. Sci.* **2005**, *30*, 630−641.
(6) Lum, H.; Malik, A. B. *Am. J. Physiol.* **1994**, *267*, L223−L241.
(7) Lamalice, L.; Boeuf, F. L.; Huot, J. *Circ. Res.* **2007**, *100*, 782−794.
(8) Harris, E. S.; Nelson, W. J. *Curr. Opin. Cell Biol.* **2010**, *22*, 651−658.
(9) Favot, L.; Martin, S.; Keravis, T.; Andriantsitohaina, R.; Lugnier, C. *Cardiovasc. Res.* **2003**, *59*, 479−487.
(10) Porcù, E.; Viola, G.; Bortolozzi, R.; Persano, L.; Mitola, S.; Ronca, R.; Presta, M.; Romagnoli, R.; Baraldi, P. G.; Basso, G. *Angiogenesis* **2013**, *16*, 647−662.
(11) Mas-Moruno, C.; Rechenmacher, F.; Kessler, H. *Anticancer Agents Med. Chem.* **2010**, *10*, 753−768.
(12) Liu, Y.; Veena, C. K.; Morgan, J. B.; Kaleem, A. M.; Jekabsons, M. B.; Nagle, D. G.; Zhou, Y. *J. Biol. Chem.* **2009**, *284*, 5859−5868.
(13) Bishop, E. T.; Bell, G. T.; Bloor, S.; Broom, I. J.; Hendry, N. F. K.; Wheatley, D. N. *Angiogenesis* **1999**, *3*, 335−344.