Anti-angiogenic Activity of Terpestacin, a Bicyclo Sesterterpene from *Embellisia chlamydospora*

HYE JIN JUNG[a], HYANG BURM LEE[b], CHANG JIN KIM[b], JUNG-RAE RHO[c], JONGHEON SHIN[d] and HO JEONG KWON[a,*]

[a] Department of Bioscience and Biotechnology, Institute of Bioscience, Sejong University, Seoul 143-747, Korea
[b] Korea Research Institute of Bioscience & Biotechnology, Taejon, Korea
[c] Marine Natural Products Laboratory, Korea Ocean Research and Development Institute, Ansan 425-600, Korea
[d] Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 110-460, Korea

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Angiogenesis, the process of new blood vessel formation using endothelial cells, is a crucial process for solid tumor growth and metastasis[1,2]. Accordingly, an efficient inhibition of angiogenesis could be important in the treatment of human cancers[3,4]. Although several chemicals and peptides are being developed as anti-angiogenic agents[5~11], anti-angiogenic chemicals with novel structures can be valuable tools in a chemical genetics approach to study angiogenesis as well as for the development of new anti-angiogenic therapeutic drugs. Based on this idea, we undertook a phenotypic screen for small molecules with anti-angiogenic activity amongst microbial metabolites. As a result, we found the known compound, terpestacin, in a culture extract of *Embellisia chlamydospora* KF208 that exhibited anti-angiogenic activities both *in vitro* and *in vivo*.

Terpestacin (Fig. 1), a bicyclo 5~15-fused sesterterpene, was originally isolated from *Arthrinium* sp. as a syncytium formation inhibitor (IC_{50}=0.46 mg/ml) in HIV infection[12]. In addition, this compound was isolated from *Bipolaris cynodontis* as a phytotoxin[13]. However, there has been no report on the anti-angiogenic activity of the compound and its production by *Embellisia chlamydospora*. In this study, we demonstrate a novel biological activity of terpestacin: inhibition of angiogenesis.

Terpestacin was obtained from the culture extract of *Embellisia chlamydospora* KF208 as the procedure described by OKA *et al.*[12]. Basic fibroblast growth factor (bFGF) was purchased from Upstate Biotechnology (Lake Placid, NY), cell culture media from Life Technology (Grand Island, NY), Matrigel from Collaborative Biomedical Products (Bedford, MA), and Transwell plates from Corning Costar (Cambridge, MA). Early passage (4~8 passages) bovine aortic endothelial cells (BAECs) were kindly provided by Dr. JO at the NIH of Korea. BAECs were grown in MEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

Fig. 1. Chemical structure of terpestacin ($C_{25}H_{38}O_4$).

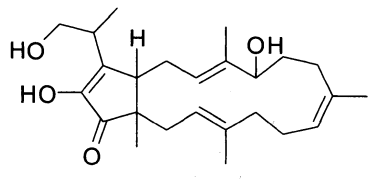
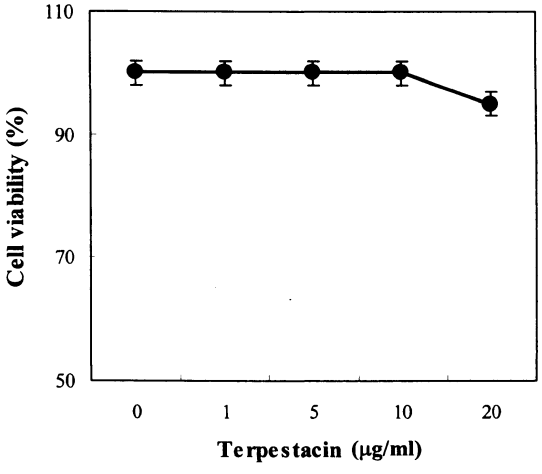


Fig. 2. Effect of terpestacin on viability of BAECs.



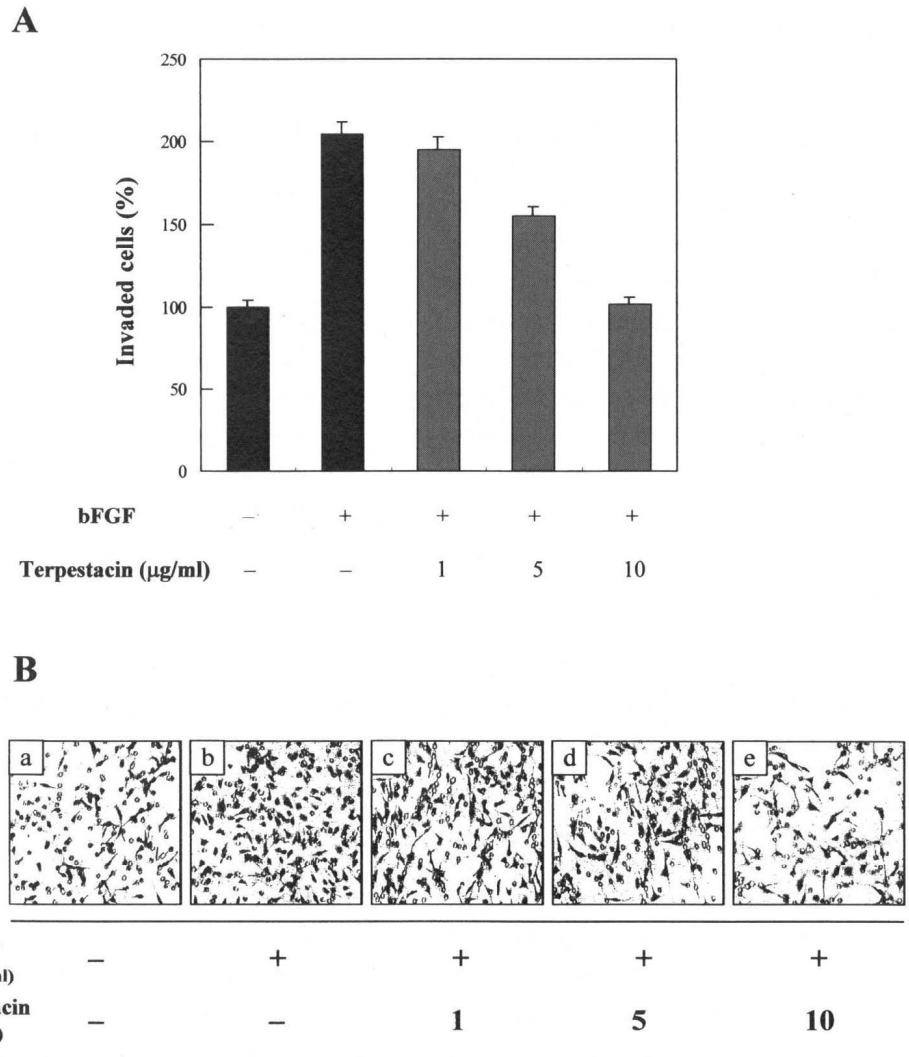
BAECs were treated with various concentrations of terpestacin (0~20 μg/ml) and incubated for 72 hours. The cell viability was measured by trypan blue assay. The experiment was repeated twice independently.

* Corresponding author: kwonhj@sejong.ac.kr

First, to determine the optimum dose of terpestacin with no cytotoxicity for angiogenesis assays, endothelial cell viability assay was performed using the trypan blue exclusion method. The BAECs were seeded at a density of 1.5×10^4 cells per well in 24-well culture plates. Terpestacin (0~20 μg/ml) was added to each well and incubated for up to 72 hours. After 72 hours, cells were stained with trypan blue and then counted by hemacytometer. The viability of BAECs was more than 95% even at 20 μg/ml of terpestacin treatment for 72 hours (Fig. 2). Therefore, angiogenesis assays were performed in a concentration range of 1 to 20 μg/ml of terpestacin in the absence of cytotoxicity.

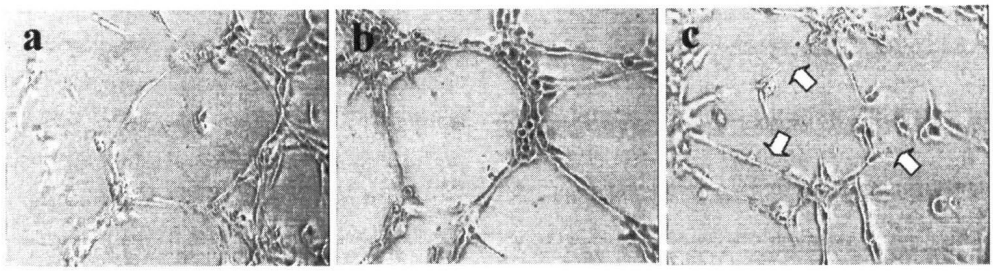
To explore the anti-angiogenic activity of terpestacin, an *in vitro* endothelial cell invasion assay was conducted as described previously by KWON *et al.*[9]. Since endothelial cell invasion is a crucial step for the spreading and migration of cells, the inhibition of this step has been considered as an important property for anti-angiogenic agents[14,15]. Thus, the invasiveness of endothelial cells was performed *in vitro* using a transwell chamber system with 8.0-μm-pore-polycarbonate filter inserts. The lower side of the filter was coated with 10 μl gelatin (1 mg/ml), whereas

Fig. 3. Effect of terpestacin on the invasion of BAECs.



BAECs were seeded at a density of 1×10^5 cells/well and treated with and without bFGF (30 ng/ml). The cells were then treated with the agent. (A) Inhibitory activity of terpestacin on endothelial cell invasion. Serum-starved BAECs left in serum-free medium (Control) or treated with bFGF in the presence or absence of terpestacin was used for invasion assay. The experiment was repeated twice independently. (B) Microscopic observation of invaded cells (×100 magnification): (a) control; (b) bFGF alone; (c) bFGF+terpestacin (1 μg/ml); (d) bFGF+terpestacin (5 μg/ml); (e) bFGF+terpestacin (10 μg/ml).

Fig. 4. Effect of terpestacin on the tube formation of BAECs.



(a) BAECs were seeded on Matrigel-coated wells at a density of 1×10^5 cells/well without bFGF and the agent. (b) The BAECs were stimulated with bFGF (30 ng/ml) without the agent. (c) The BAECs were stimulated with bFGF and treated with 10 μg/ml terpestacin. Photographs were taken 8 hours after the agent treatment. Arrow indicates dramatic inhibition of the tube formation. The experiment was repeated twice independently.

the upper side was coated with 10 μl of the Matrigel. BAECs (1×10^5 cells) were placed in the upper part of the filter. The chamber was then incubated at 37°C for 18 hours. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting the total number of cells in the lower side of the filter using optical microscopy at ×100 magnification. As shown in Fig. 3, bFGF effectively induced cell invasion through the filter compared to that of the control. However, terpestacin (1~10 μg/ml) dose-dependently inhibited bFGF-induced invasion of BAECs.

Next, the inhibitory effect of terpestacin on capillary tube formation, another key phenotype for endothelial cell differentiation of angiogenesis[14], was examined. Thus, 150 μl Matrigel (10 mg/ml) was placed in a 48-well culture plate and polymerized for 2 hours at 37°C. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Subsequently, the morphological changes of the cells and tube formation were observed under a microscope and photographed at ×100 magnification using a JVC digital camera (VICTOR, Japan). In the absence of bFGF, cultured BAECs on the Matrigel normally formed incomplete and narrow tube-like structures (Fig. 4A) but the capillary network formation was stimulated by the treatment of bFGF resulting in elongated and robust tube-like structures (Fig. 4B). As shown in Fig. 4C, terpestacin (10 μg/ml) efficiently inhibited the tube formation induced by bFGF. However, trypan blue staining of formed tubes showed no stained cells, implying that the inhibition of tube formation by terpestacin was not merely due to the cytotoxic effect of the compound (data not shown). Interestingly, the inhibitory concentration in these *in vitro* angiogenesis assays was much less than that of syncytium formation (0.46 mg/ml), suggesting that the mode of action of terpestacin in these biological activities appears to be different from each other.

Table 1. Effect of terpestacin on *in vivo* angiogenesis of CAM.

Drug	Inhibited egg / live egg	Inhibition ratio (%)
Empty	2/21	10
Retinoic acid (1 μg/egg)	17/20	85
Terpestacin		
(10 μg/egg)	7/15	47
(100 μg/egg)	9/14	64
(200 μg/egg)	18/21	86

The anti-angiogenic activity of terpestacin was also validated *in vivo* using chorioallantoic membrane (CAM) from growing chick embryo. Fertilized chick eggs were kept in a humidified incubator at 37°C for 3 days. About 2 ml of egg albumin was then removed with a hypodermic needle allowing the CAM and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed and the shell membrane peeled away. At the stage of a 4.5-day old chick embryo, terpestacin (10~200 μg/egg)-loaded thermanox cover slips were air-dried and applied to the CAM surface. Two days later, 2 ml

of 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope. Since retinoic acid (RA) is known as an anti-angiogenic compound, RA was used as a positive control for anti-angiogenic responses[11]. When the CAM treated with sample showing an avascular zone to a similar degree of RA-treated CAM that had very few vessels compared to empty cover slip, the response was scored as positive, and calculated based on the percentage of positive eggs to the total number of eggs tested. As a result, the inhibition of the angiogenesis of retinoic acid, the positive control, was 85% (n=20), and that of empty cover slips was 10% (n=21). Terpestacin dose-dependently inhibited the neovascularization of chorioallantoic membrane from chick embryo without showing any rupture or toxicity of preexisting vessels (Table 1).

All results shown herein strongly demonstrate that terpestacin inhibits angiogenesis without affecting endothelial cell viability both *in vitro* and *in vivo*, and suggest that the compound appears to have a unique mode of action different from its known biological activities. Accordingly, it can be developed as an anti-angiogenic agent for chemical genetics study of angiogenesis. Interestingly, the bicyclic ring structure of terpestacin is similar to that of radicicol, an inhibitor of molecular chaperone heat shock protein (HSP) 90, which exhibited the morphological reversion activity of oncogene-transformed fibroblasts[16] and a potent anti-angiogenic activity[17]. However, the mode of actions of these two compounds may be differ since morphological change of the cells by terpestacin treatment is different from that of radicicol (data not shown). Notably, terpestacin inhibits extracellular signal-regulated kinase (ERK) activity in the cells (data not shown). The activation of the mitogen-activated protein kinase (MAPK)/ERK signaling pathway has been highlighted as a fundamental pathway that modulates a number of cellular process including cell proliferation, migration, and invasion[18]. Therefore, the mode of anti-angiogenic activity of terpestacin appears to be related to the regulatory mechanism of ERK pathway. Detailed studies on the effect of terpestacin in ERK pathway as well as the identification of cellular target of the compound will help to define the mechanism of the compound.

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