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A Novel Polyacetylene Significantly Inhibits Angiogenesis and Promotes Apoptosis in Human Endothelial Cells through Activation of the CDK Inhibitors and Caspase-7

Abstract

A novel bioactive polyacetylene compound, 1,2-dihydroxy-5(*E*)-tridecene-7,9,11-triyne (compound **1**), was identified from the *Bidens pilosa* extract using an *ex vivo* primary human umbilical vein endothelium cell (HUVEC) bioassay-guided fractionation protocol. Our results demonstrate that compound **1** (at 2.5 μg/mL) possessed significant anti-angiogenic effects, as manifested by an inhibition of HUVEC proliferation, migration, and the formation of tube-like structures in collagen gel. Moreover, compound **1** induced HUVECs to undergo cell death in a concentration- and time-dependent manner. The mechanisms underlying these pharmacological effects include reduced expression of cell cycle mediators such as CDK4, cyclins D1 and A, retinoblastoma (Rb) and vascular endothelial growth factor receptor 1 (VEGFR-1), and promotion of caspase-mediated activation of CDK inhibitors p21(Cip1) and p27(Kip). Moreover, apoptotic induction in HUVECs mediated by compound **1** was found to be in part through overexpression of FasL protein, down-regulation of anti-apoptotic Bcl-2, and activation of caspase-7 and poly(ADP-ribose) polymerase. This study demonstrates the potent anti-angiogenic and apoptotic activities of compound **1**, suggesting that phytocompounds such as polyacetylenes deserve more attention regarding their potential as candidates for anti-angiogenic therapeutics.

Key words
Bidens pilosa · Compositae · polyacetylenes · HUVEC · angiogenesis · apoptosis · CDK inhibitors

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Introduction

Tumor growth and metastasis depend upon angiogenesis – the formation of new blood vessels from pre-existing endothelium [1]. Angiogenesis is a complex process of endothelial cell proliferation and migration, and capillary tube formation [2]. Angiogenesis is an essential process for tumor cell expansion and colonization of distant organs; therefore, the inhibition of tumor angiogenesis offers great promise for the treatment of cancer [3]. Endothelial cells are one important type of cells involved in angiogenesis and acquire all the information required for angiogenic processes *in vitro*. During the past few years, researchers

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and clinical scientists have spent considerable efforts in the search for novel agents for use as anti-angiogenic therapeutics against cancer using *in vitro* or *ex vivo* endothelial cell bioassay systems. A systematic search for useful bioactivities or therapeutic effects (e.g., for cancer prevention) from medicinal plants is now considered to be a rational approach in nutraceutical and drug research.

Bidens pilosa L., an annual Compositae herb mainly found in tropical and subtropical regions, is a commonly used folk medicine and herbal tea constituent. Plant extracts from *B. pilosa* exhibit a number of bioactivities such as prevention of autoimmune diabetes in non-obese diabetic mice [4], anti-tumor effects in a leukemia cell line [5], anti-microbial [6], and anti-hyperglycemic [7] effects. We were the first group to show chemopreventive and anti-inflammatory effects of specific phytocompounds isolated from *B. pilosa* [8]. In addition, a bioactive fraction and two derived polyacetylenes from *B. pilosa* extract were observed to function as anti-angiogenic agents [9].

In the present study, angiogenic inhibition-guided bioassays coupled with liquid chromatographic fractionation has allowed us to identify a novel polyacetylene, structurally elucidated as 1,2-dihydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**), which exhibited significant anti-angiogenic activity. The anti-angiogenic effect of compound **1** was illustrated by its inhibitory effect on the ability of HUVECs to divide and migrate in response to angiogenic factors and to form tubes in collagen gels. Compound **1** could also induce cell death in primary HUVECs. The possible molecular mechanism underlying anti-angiogenesis or apoptosis mediated by the novel compound **1** in HUVECs was characterized.

Materials and Methods

Materials

Human bFGF and VEGF-A were purchased from Pepro Tech (Rocky Hill, NJ, USA) and EGM2 medium was from BioWittaker (Walkersville, MD, USA). Endothelial Cell Growth Supplement and heparin sulfate were from Life Technology (Gaithersburg, MD, USA). BSA, gelatin, phorbol myristate acetate and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Renaissance Chemiluminescence Reagent Plus was from NEN Life Science Products (Boston, MA, USA). Antibodies against p21(Cip1), p57(Kip2), cyclin A, cyclin E, β-actin and poly(ADP-ribose) polymerase (Santa Cruz Biotechnology; Santa Cruz, CA, USA), cyclin D1 and α-tubulin (Neo Markers; Fremont, CA, USA), p27(Kip1), caspase 7 and retinoblastoma protein (Rb) (BD PharMingen; San Diego, CA, USA), and p53 (Oncogene Research Products; Boston, MA, USA) were used. Geimsa stain was from Merck (Darmstadt, Germany) and zVAD was from Calbiochem Biochemicals (San Diego, CA, USA). All chemicals and solvents used were of reagent or HPLC grade.

Compound isolation and structural elucidation

B. pilosa Linn. var. *radiata* (Compositae), as authenticated by Dr. Sheng-Yang Wang, National Chung-Hsing University, was collected and a voucher specimen (No. 11519) was deposited at the Herbarium of Research Center for Biodiversity, Academia Sinica, Taiwan. Fresh whole plants (2.5 kg) was extracted with 25 L of 70% ethanol at room temperature. Total ethanolic extract (~130.0 g) was partitioned with ethyl acetate and *n*-butanol yielding EA, BuOH and water fractions. The EA fraction (18 g) was chromatographed over silica gel (347 g) with an MeOH/CH_2Cl_2 solvent gradient to yield 8 subfractions (Ea – Eh). The Ee fraction (3.5 g), having the most significant anti-angiogenic activity, was further fractionated using an RP-18 column (Phenomenex Luna 3 μ C18, 150 × 4.6 mm) with an acetonitrile/H_2O solvent gradient. 1,2-Dihydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**), 1,2-dihydroxytrideca-5,7,9,11-tetrayne (**2**), 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (**3**) [9], and ethyl caffeate (**4**) [10] were isolated from the Ee fraction. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DMX-500 spectrometer. EI-MS were obtained on a JEOL JMS-HX 300 mass spectrometer.

*1,2-Dihydroxy-5(*E*)-tridecene-7,9,11-triyne* (**1**): Amorphous colorless solid; IR: ν_{max} = 3363, 2937, 2223, 1045, 956, 867 cm^{-1}; $[\alpha]_D^{25}$: 28.6 (CH_3OH, *c* 1.2); ^{1}H- and ^{13}C-NMR data: see Table **1S** in the Supporting Information; EI-MS: *m/z* (rel intensity) = 202 $[M]^+$ (10), 184 $[M-H_2O]^+$ (17), 171 $[M-CH_2OH]^+$ (13), 152 (30), 139 (70), 127 (100), 115 (51), 101 (42); HR-EI-MS: *m/z* = 202.0996 (calcd. for $C_{13}H_{14}O_2$: 202.0994).

Cell lines

Primary HUVECs were obtained from fresh human umbilical veins as described with a slight modification [11]. Isolated HUVECs were seeded on gelatinized dishes and maintained in EGM2. To avoid genetic variation resulting from different individuals, HUVECs from 5 or more different donors were pooled. HUVECs at < 6 passages were used for experiments. Human lung carcinoma cell line A549 was maintained as described by ATCC (Manassas, VA, USA, HACAT keratinocyte line was maintained as previously described [12].

Cell proliferation assay

HUVECs (5,000 cells/well) were cultured in starvation medium containing 1% FBS and 0.1% BSA in M199 and treated for 48 h with 1 ng/mL bFGF together with vehicle (0.1% DMSO) or compound **1**. A549 and HACAT cells at the same seeding density were starved overnight with serum-free medium containing 0.1% BSA and then treated for 48 h with compound **1** or vehicle. Cell proliferation assays were performed using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay kit (Promega; Madison, WI, USA). All assays were performed in quadruplicate, and 2 to 3 independent experiments were conducted.

Crystal violet staining

HUVECs were treated with vehicle or compound **1** for 48 h in quadruplicate under the same conditions as for the proliferation assay. Cells were washed and fixed for 15 min with methanol and stained for 10 min with 0.2% crystal violet. After extensive washes, crystal dye from stained cells was eluted with 2% SDS. The amount of dye in each treatment was quantified with an ELISA reader at OD_{595}.

Tube formation assay

Tube formation by endothelial cells seeded in collagen gel was performed as previously described [13]. The rat tail collagen was isolated as described [14]. HUVECs (3×10^5 cells/well) was mixed with medium containing 5.7 × M199, 1 mM HEPES, 0.125%

$NaHCO_3$, 1 mM NaOH, 1.5 mM $CaCl_2$ and 0.2% rat tail tendon collagen on ice and then added to a 6-well plate. After formation of a collagen gel at 37 °C, M199 medium supplemented with 1% FBS, 0.5 mg/mL ECGS, 20 U/mL heparin, 0.08 mM PMA, 0.05 mg/mL ascorbic acid, 40 ng/mL VEGF and 40 ng/mL bFGF, with or without phytocompound, was added to each well and incubated at 37 °C for 6 days with drug replenishment every 2 days.

Cell migration assay

HUVECs (2.2×10^4 cells/well) in M199 medium with 0.1% BSA were plated in quadruplicate at the bottom of a modified Boyden chamber (Neuro Probe, Inc.; Gaithersburg, MD, USA). The procedure for detecting the effect of phytocompound on HUVEC migration followed a published method [9]. Either bFGF (1 ng/mL) or VEGF-A (5 ng/mL) was used to attract HUVEC migration. The number of cells per 9 high power fields (HPF) at 400× magnification was counted. This experiment was independently repeated two to three times.

Western blotting

Serum-deprived HUVECs were treated with 0.1% DMSO or compound **1** at 2.5 or 10 μg/mL in the presence of bFGF, then lysed in boiled lysis buffer containing 1% SDS and 10 mM Tris-HCl (pH 7.4). Protein content was measured by Bradford protein assay. Proteins were resolved by SDS-PAGE and subjected to immunoblotting using Renaissance Chemiluminescence Reagent Plus. α-Tubulin, β-actin and glyceraldehydes 3-phosphate dehydrogenase (GA3PDH) were used as loading controls.

Statistical analysis

Statistical significance was determined by one-way ANOVA with Fischer's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Supporting information

NMR spectral data for compound **1** are available as Supporting Information.

Results and Discussion

The metabolite profile of the bioactive fraction (Ee) obtained from *B. pilosa* extracts was analyzed using HPLC (Fig. **1A**). One new polyacetylene, 1,2-dihydroxy-5(*E*)-tridecene-7,9,11-triyne

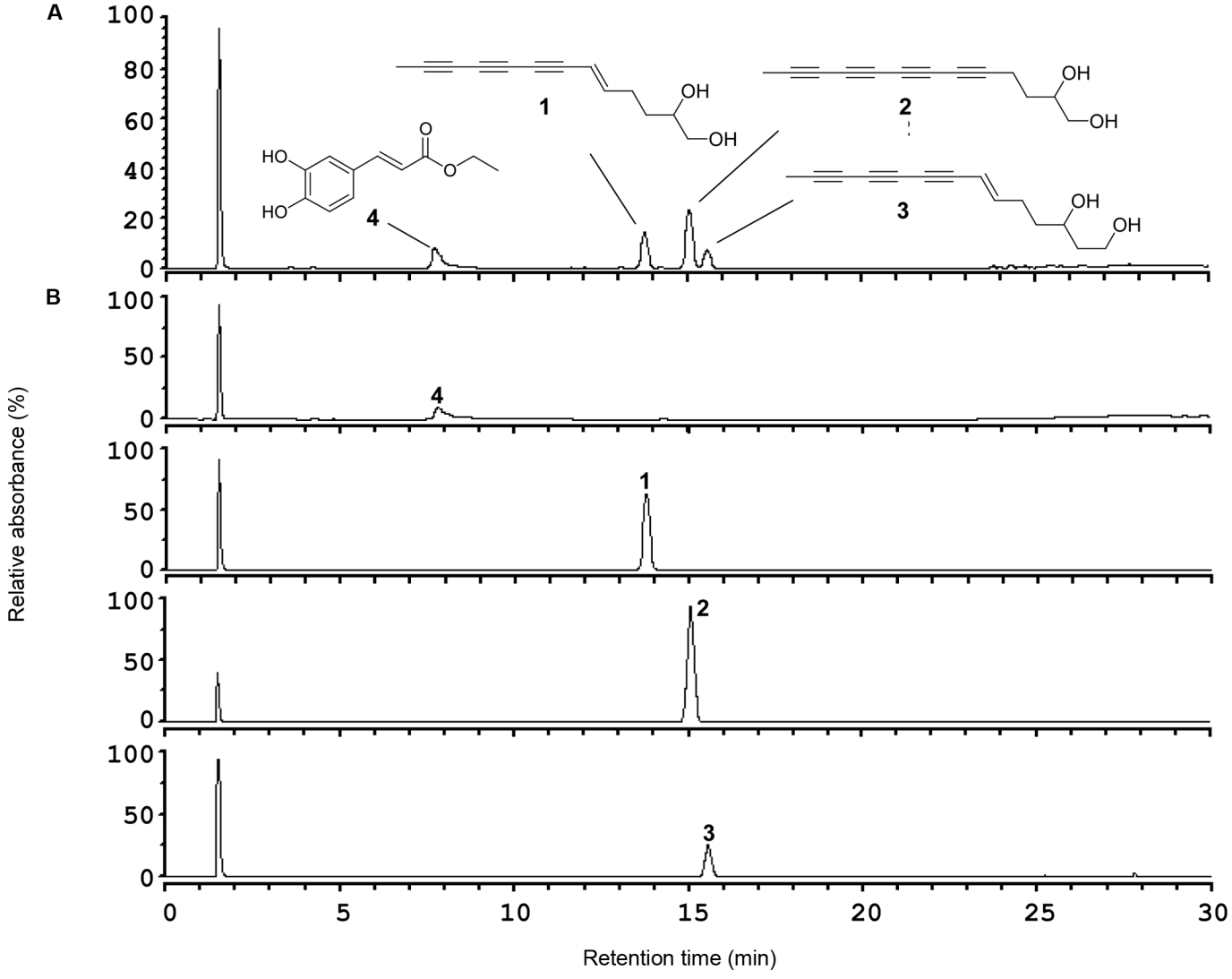

Fig. **1** HPLC traces of the *B. pilosa* bioactive fraction. **A** HPLC profile and the chemical structures of the major constituent compounds of the Ee fraction at 230 nm. **B** Pure compounds **1 – 4** were eluted using the same column conditions as in **A**.

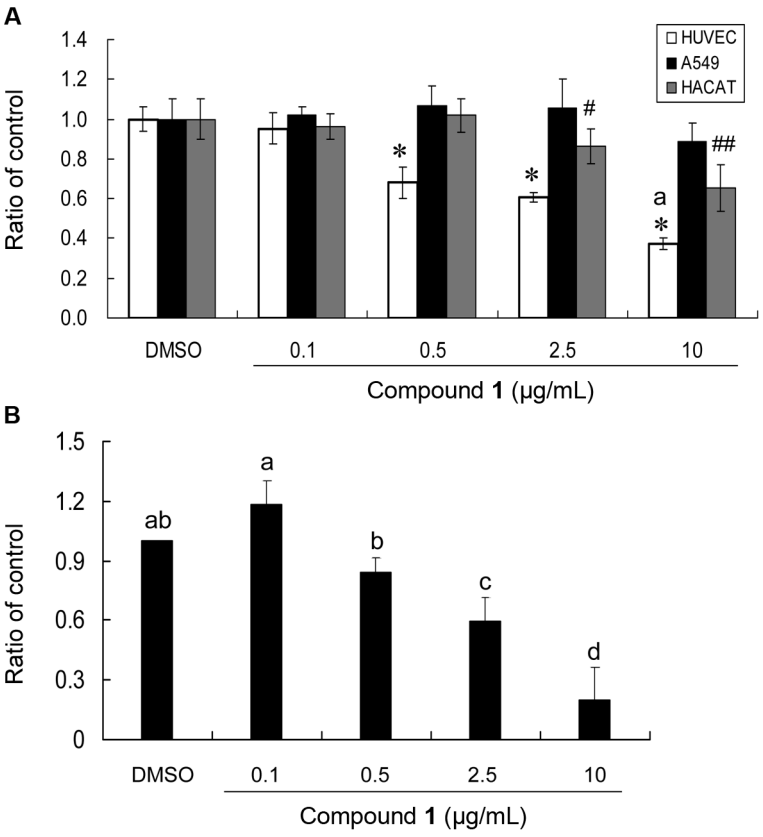

Fig. 2 Concentration-effect of compound **1** on HUVEC, A549 and HACAT proliferation (**A**), and HUVEC viability (**B**). HUVECs, A549, and HACAT cells were treated for 48 h with vehicle or compound **1** in the presence of bFGF. Data are expressed as mean ratio of the vehicle control (mean ± SD, n = 8). * P <0.0001, # P <0.05, ## P <0.0001. a P < 0.0005 between 10 μg/mL and 0.5 or 2.5 μg/mL compound **1** treatments. Bars with different letters in (**B**) are significant differences between treatments (P < 0.05).

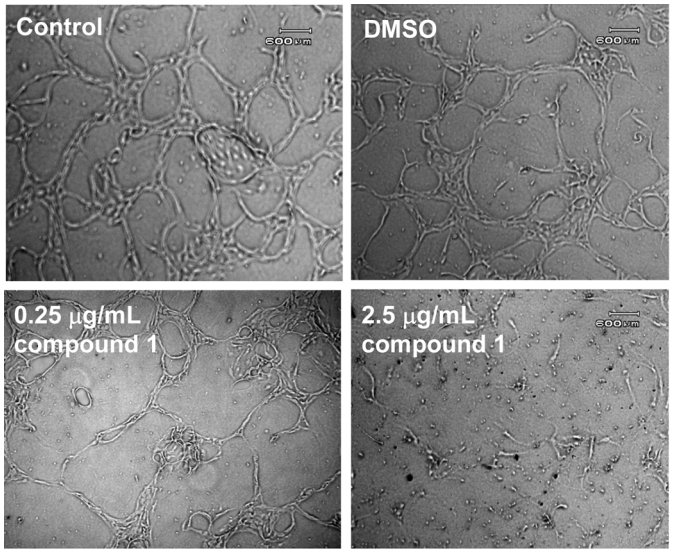

Fig. 3 Inhibition of tube formation by compound **1** in HUVECs. Cells seeded in collagen gels were treated for 6 days without (control) or with 0.1% DMSO (vehicle) or compound **1** in tube-forming medium. The tube-like structures in each well were recorded under a light microscope (200× magnification).

(**1**), along with three known compounds (**2**–**4**) isolated from this fraction were used as standards to trace the retention times in the Ee fraction (Fig. **1B**). Compound **1** had high-resolution mass spectroscopy (HR-EI-MS) and ^{13}C-NMR data consistent with the molecular formula $C_{13}H_{14}O_2$, which indicated a hydrogen deficiency index of 7. The IR spectra showed hydroxy groups (3363 cm^{-1}), acetylenic groups (2223 cm^{-1}), and a conjugated double bond (956 cm^{-1}). The ^{1}H-NMR spectrum of compound **1** (Table **1S**, Supporting Information) exhibited signals for a single methyl group [δ_H = 1.98 (s, H_3-13)], 4 methylene protons [δ_H = 1.46 and 1.61 (each 1H, m, H_2-3), and 2.24, 2.32 (each 1 H, m, H_2-4)], 2 oxymethene protons [δ_H = 3.42 (1H, dd, J = 11.6, 6.0 Hz, H_a-1) and 3.46 (1H, dd, J = 11.6, 4.8 Hz, H_b-1)], 1 oxymethine proton [δ_H = 3.56 (1H, m, H-2)], and 2 olefinic protons [δ_H = 5.61 (1H, d, J = 16.0 Hz, H-6) and 6.43 (1H, dt, J = 16.0, 7.2 Hz)], which correspond to a double bond with the *E* configuration adjacent to the polyene moiety. ^{13}C-NMR and DEPT spectra of the compound indicated 1 CH_3, 3 CH_2, 3 CH, and 6 C, including 2 carbons to which oxygen was attached [δ_C = 67.2 (C-1) and 72.3 (C-2)]. 1,3-Dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (**3**) [15] had similar ^{1}H- and ^{13}C-NMR data as compound **1** on the polyene moiety. From the molecular formula and the above evidence, this phytocompound was considered to be an ene-triyne with a terminal methyl group and a vicinal diol on the other terminus. This structure was confirmed by COSY correlations for H_2-1/H-2; H-2/H_2-3; H_2-3/H_2-4; H_2-4/H-5; and H-5/H-6. Therefore, this new compound was elucidated as 1,2-dihydroxy-5(*E*)-tridecene-7,9,11-triyne.

To examine whether the anti-angiogenic effect of compound **1** was in part mediated by the specific inhibition of cell proliferation of endothelial cells, a key step of angiogenesis, we treated HUVECs, lung carcinoma A549 cells and HACAT keratinocytes with compound **1** at concentrations ranging from 0.1 to 10 μg/mL. Compound **1** significantly and concentration-dependently attenuated bFGF-induced growth of HUVECs at 0.5 – 2.5 μg/mL, with an IC_{50} = ca. 5.7 μg/mL (Fig. **2A**). At 10 μg/mL, only 38% of the HUVECs were viable, however, 65% of the HACAT cells and

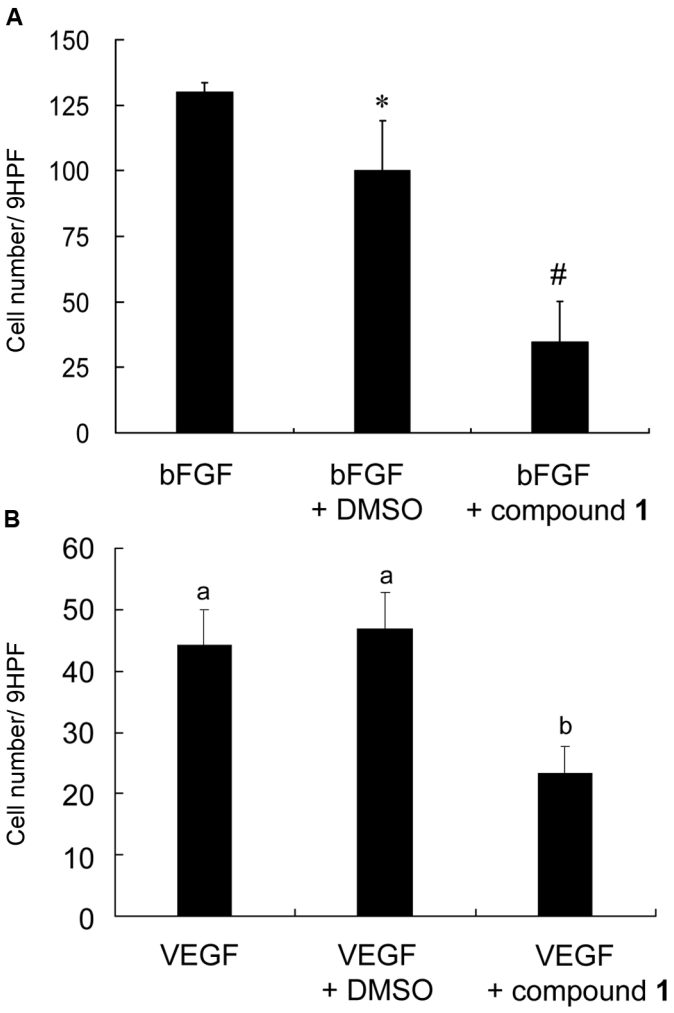

Fig. **4** Migration of HUVECs mediated by compound **1.** HUVECs were treated with 0.1% DMSO or compound **1** at 2.5 μg/mL in the presence of bFGF (**A**) or VEGF (**B**). The total cell number in 9 high power fields (HPF) (400× magnification) on the upper side of the porous membrane after 6-h migration was used to represent the migratory cells responding to stimulation. Data are expressed as mean ± SD (n = 8–12). * $P < 0.05$, # $P < 0.0005$. Bars with different letters indicate significant differences between treatments ($P < 0.0001$).

89% of the A549 cells remained viable. We further performed crystal violet staining to examine the remaining living cells in compound **1**-treated HUVECs. Fig. **2B** shows that compound **1** could induce significant cell death at 2.5 and 10 μg/mL ($P < 0.05$), with no detectable effect at 0.1–0.5 μg/mL. These results indicate that compound **1** at low doses (0.5–2.5 μg/mL) selectively attenuated the proliferation of HUVECs, but not of A549 cells and HACAT keratinocytes, at least in part, via induction of cell death.

Endothelial cells form tube-like structures when seeded in collagen gels. The effect of compound **1** on tube formation of HUVECs in tube-promoting medium supplemented without (control) or with vehicle (0.1% DMSO) or compound **1** at 0.25 and 2.5 μg/mL was assayed for 6 days. As shown in Fig. **3**, DMSO had no effect on the tube-like structures as compared with the control cells. In contrast, compound **1**, at 2.5 μg/mL, completely attenuated the formation of tube-like structures. We also investigated the effect of compound **1** on HUVEC migration toward bFGF or VEGF-A. Fig. **4** shows that compound **1** (2.5 μg/mL) significantly attenuated the ability of HUVECs to migrate toward bFGF (**A**) or VEGF-A (**B**). A DMSO effect was observed on HUVEC migration toward bFGF but not VEGF-A, suggesting a possible batch variation of HUVECs. Together, our results indicate that compound **1** manifested an anti-angiogenic effect *in vitro* by inhibiting endothelial cell tube formation in collagen gels and migration.

Since VEGF-A-mediated signaling via VEGF receptors plays a critical role in angiogenesis, we next examined if compound **1** had any effect on the expression of VEGF-A, VEGFR1 and VEGFR2. Immunoblotting shows that compound **1** reduced the expression of VEGFR-1 but not of VEGFR2 (Fig. **5A**) while having no effect on the level of VEGF-A (data not shown) in conditioned medium. This finding is consistent with a recent report that wogonin inhibits interleukin-6-induced angiogenesis partly via down-regulation of VEGFR1 but not VEGFR2 protein [16].

Progression of the cell cycle through G1 and entry into the S phase is regulated by cyclins D1-, E- and A-associated kinases (CDKs), Rb, and CDKs inhibitors (CKIs) [17]. D-type cyclins are synthesized in early G1 and behave as positive regulators of CDK4 and CDK6 kinases, which elicit phosphorylation of Rb [18]. CyclinE/CDK2 complexes appear later in the G1 phase and probably regulate the G1/S transition [19]. We used immunoblotting to examine whether treating HUVECs for 24 h with 2.5 or 10 μg/mL compound **1** affected the expression of CDK4 (a cyclin D1-associated kinase), CDK2 (a cyclin E- and A-associated kinase), Rb, and cyclins D1, E and A proteins. Fig. **5B** shows that the level of Rb and CDK4 proteins but not that of CDK2 was suppressed in HUVECs treated with 10 μg/mL compound **1,** as compared with vehicle-treated cells. These results indicate that compound **1** may play a role in the early G1 phase of the cell cycle, resulting in a blockade of HUVEC growth. Fig. **5C** shows that the expression of cyclin D1 and A but not that of cyclin E was inhibited by compound **1**. Decreased expression of CDK4, Rb, cyclins D1 and A has been observed in apoptotic endothelial cells following serum withdrawal [20]. The data in our study therefore indicate that compound **1** at concentrations as high as 10 μg/mL might induce apoptosis. Consistent with this notion, compound **1** at high (10 μg/mL) but not at low concentration (2.5 μg/mL) reduced the expression of anti-apoptotic Bcl-2 and increased cytosolic cytochrome c (Fig. **5D**) while having no effect on proapoptotic Bax (data not shown). We also observed that apoptotic DNA (sub-G1 peak) increased dose- and time-dependently with compound **1** treatment in HUVECs, with more than 30% apoptotic cells appearing after 48–56 h of treatment (data not shown).

CKIs, namely p21(Cip), p27(Kip1) and p57(Kip), play a critical role in the negative control of cyclin/CDK complexes and cell growth [21], [22]. Compound **1** was observed to significantly reduce the level of p21(Cip1) and p27(Kip1) but not of p57(Kip2) in HUVECs, as compared to vehicle control (Fig. **6A**). The specific cleavage forms of p21(Cip1) and p27(Kip1) were also detected (Fig. **6A**), indicating a possible involvement of caspase(s) in this anti-proliferation process. Cleavage of p21(Cip1) and p27(Kip1) into smaller fragments by caspase-3 or caspase-3-like enzymes has been observed in apoptotic endothelial cells induced by growth factor deprivation [20]. To determine whether caspases

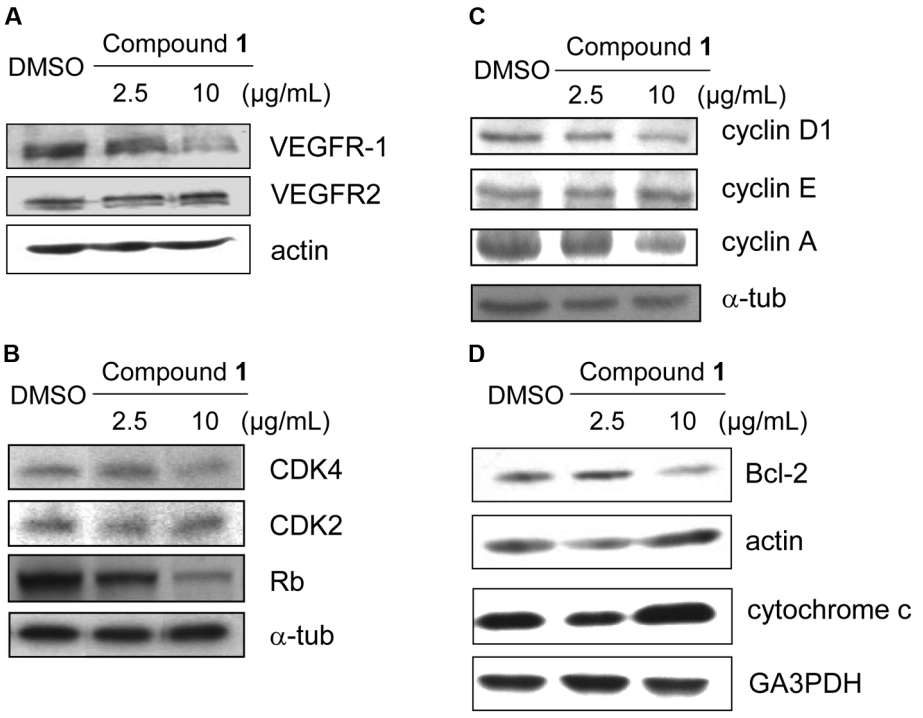

Fig. **5** Deregulation of VEGFR-1, CDK4, Rb, cyclin D1, cyclin A, and Bcl-2 in compound **1**-treated HUVECs. HUVECs were treated for 24 h with or without compound **1** in the presence of bFGF. Protein lysates were subjected to SDS-PAGE followed by immunoblotting.

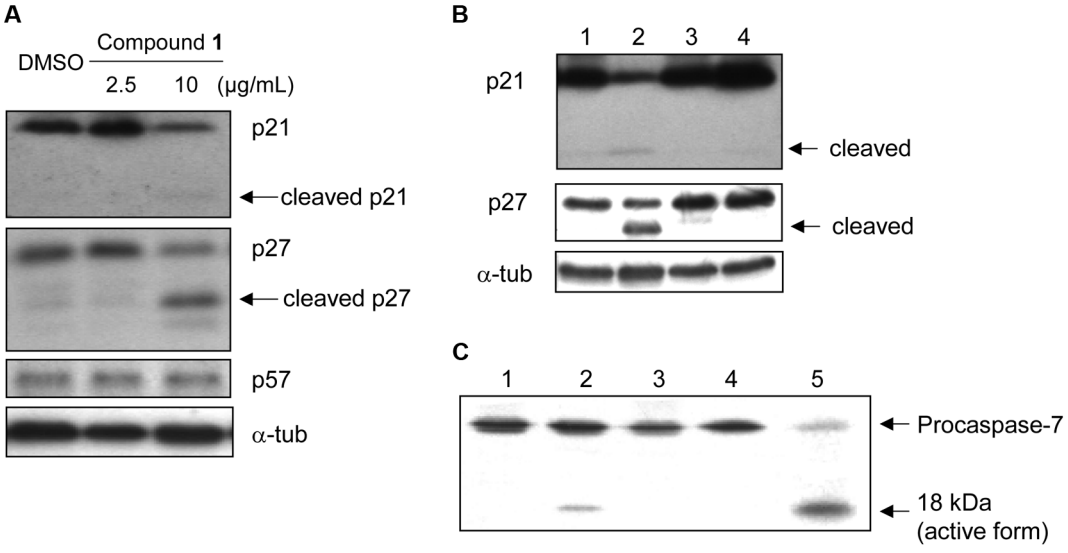

Fig. **6** Caspase-mediated cleavage of p21(Cip1) and p27(Kip1) in compound **1**-treated HUVECs. HUVECs were treated for 24 h with or without compound **1** in the presence of bFGF. Protein lysates were subjected to SDS-PAGE followed by immunoblotting. **A** The arrow indicates the cleaved forms of p21 (Cip1) or p27 (Kip1). Lanes 1–4 in **B** and **C** were DMSO, compound **1** (10 μg/mL), 50 μM zVAD alone, and compound **1** plus zVAD, respectively. Lane 5 in **C** represents HUVECs treated with compound **1** (10 μg/mL) only for 48 h.

were involved in the proteolytic cleavage of p21(Cip1) and p27(Kip1), we treated proliferating HUVECs with 10 μg/mL compound **1** in the presence or absence of the caspase inhibitor I (zVAD). Fig. **6B** shows that zVAD indeed abolished the specific cleavage of p21(Cip1) and p27(Kip1) to smaller fragments in compound **1**-treated HUVECs. However, no detectable activation of caspase-3 was observed in compound **1**-treated cells (data not shown). Nevertheless, we observed a time-dependent activation of procaspase 7 accompanied with the appearance of active 18-kDa caspase 7 (Fig. **6C**). These data suggest that proteolytic activation of caspase-7 may be responsible for the compound **1**-mediated cleavage of p21(Cip1) and p27(Kip1) in HUVECs.

We examined the caspase-mediated cleavage of PARP, a hallmark of apoptosis, in compound **1**-treated HUVECs. Fig. **7A** demonstrates that PARP was time-dependently activated in HUVECs treated with compound **1**. We then analyzed whether compound **1**-induced apoptosis in HUVECs was triggered by the overexpression of FasL, a known death ligand involved in mediating mammalian cell apoptosis. Flow cytometry and immunoblotting analyses showed that indeed the FasL expression increased upon the treatment of HUVECs with compound **1** (Fig. **7B**), suggesting that the induction of apoptosis in HUVECs by compound **1** is partly through the up-regulation of FasL protein expression.

In summary, we have identified here a novel polyacetylene from *B. pilosa* which possesses specific, however, selective and differential effects against angiogenesis and promotion of apoptosis in HUVECs. These activities are sufficiently novel for the compound to be developed as an anti-angiogenic agent for cancer therapy. The effect of compound **1** on tumor angiogenesis *in vivo* is of high interest and worthy of further investigation.

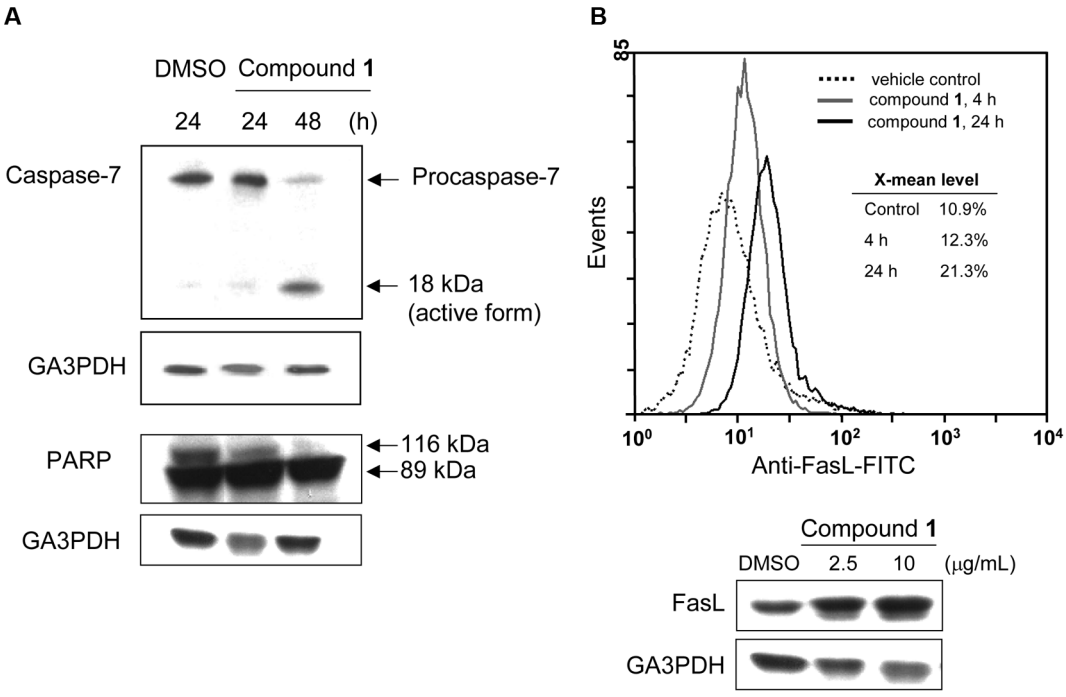

Fig. **7** Activation of procaspase 7 and PARP in compound **1**-treated HUVECs. **A** HUVECs were treated with vehicle or 10 μg/mL of compound **1** in the presence of bFGF. Protein lysates were subjected to immunoblotting against specific antibodies PARP (116 and 89 kDa), procaspase 7 and its cleaved form (18 kDa). **B** FasL expression in compound **1**-treated HUVECs. FasL expression was quantified using immunoblotting or flow cytometry, by staining cells with mouse anti-human FasL IgG (clone NOK-1), followed by FITC-conjugated rat anti-mouse IgG. Fluorescent intensities (mean X values) obtained from vehicle control cells and HUVECs treated with compound **1** for 4 h or 24 h were analyzed using a Coulter EPICS XL flow cytometer.

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