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Cochlioquinone A1, a New Anti-Angiogenic Agent from *Bipolaris zeicola*

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Abstract—Cochlioquinone A1 (CoA1) was newly isolated from the culture extract of *Bipolaris zeicola* as a potent anti-angiogenic agent. CoA1 inhibited in vitro angiogenesis of bovine aortic endothelial cells (BAECs) such as bFGF-induced tube formation and invasion at the concentration (1 μg/mL) without cytotoxicity. Notably, CoA1 exhibited more potent inhibition activity for the growth of BAECs than that of normal and cancer cell lines investigated in this study. These results demonstrate that CoA1 is a new anti-angiogenic agent and can be developed as a new therapeutic agent for angiogenesis-related diseases.

Introduction

Angiogenesis, the process of new blood vessels formation, is essential for a number of normal physiological processes such as embryonic development, wound healing, and tissue or organ regeneration.[1,2] However, persistent unregulated angiogenesis drives angiogenic diseases such as rheumatoid arthritis, diabetic retinopathy, solid tumor, hemangioma, and psoriasis.[3–5] The angiogenic process is consisted of multi-steps such as stimulation of endothelial cells growth by tumour cytokine, degradation of extracellular matrix proteins by metalloproteinases, migration of endothelial cells mediated by cell membrane adhesion molecules called integrins, endothelial cell proliferation, and tube formation.[6,7] Accordingly, inhibition of each of these processes is considered as a promising strategy for the treatment of cancer and other human diseases related with angiogenesis.[3,5]

Several angiogenesis inhibitors from natural products and chemical synthesis have been developed for this purpose. These include angiostatin, endostatin, and canstatin as peptide inhibitors, and thalidomide, TNP470, radicicol, and FK228 as low molecular weight compounds from microbial metabolites, and marimastat and sesquicillin as metalloproteinases inhibitors.[8–15] Some of these compounds are currently undergoing clinical phase trials, yet new 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.

Through our continuing efforts in screening new anti-angiogenic agents from natural products, cochlioquinone A1 (CoA1) was newly isolated from a fungal strain, *Bipolaris zeicola,* as a potent anti-angiogenic agent. Herein, we report the isolation, structure determination, and anti-angiogenic activity of CoA1.

Results and Discussion

Isolation and structure determination of CoA1

Phenotypic cell based screen for small molecules with anti-angiogenic activity was conducted with 2000 microbial culture extracts. Tube formation assay was applied for monitoring the phenotypic activity of the

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libraries as described in the Experimental. As a result, culture extract from fungus *B. zeicola* showed notable activity on the assay. Thereafter, the active compound was purified from the culture extract of *B. zeicola* as described in the Experimental.

The active compound was identified as a new derivative of cochlioquinone A (CoA) on the basis of HRFAB-MS, ^{1}H and ^{13}C NMR, ^{1}H–^{13}C and ^{1}H–^{1}H COSY, HMBC, and rotating frame NOE (ROESY) analyses (Fig. 1). CoA has been previously reported as a nematocidal agent and diacylglycerol kinase inhibitor from *Helminthosporium sativum* and *Drechslera sacchari*, respectively.[16,17] However, there has been no report on this new derivative of CoA and its activity related with angiogenesis. Notably, a new cochlioquinone derivative named as cochlioquinone A1 (CoA1) contains an acetone moiety added to one of the quinone carbonyl groups commonly found in cochlioquinone analogues. The exact mass calculated for CoA1, $C_{33}H_{50}O_9$, 590.7561, observed $(M+H)^+$ 591.3529 and the chemical composition was supported by the observed ^{1}H and ^{13}C NMR spectral data. In the ^{13}C NMR spectrum, the chemical shifts of 31 signals out of 33 signals were in good agreement with those for C-1 – C-5 and C-32 – C-33 of CoA, and/or C-6 – C-31 of cochlioquinol which was previously reported,[18] suggesting it is an analogue of a new cochlioquinone derivative. The spectral data were similar to those of CoA except that the signals at C-7 were replaced by acetomethyl group ($–CH_2–CO–CH_3$) instead of carbonyl group (C=O). The NMR spectral data of the compound indicated the presence of $–CH_2–CO–CH_3$ moiety [δ H 2.49 (1H, d, $J=15.18$, Hz), 2.82 (1H, d, $J=15.24$, Hz), 2.28 (3H, s) ppm, δ C 46.48 (CH_2), 209.57 (CO) and 33.20 (CH_3) ppm] at C-29 to C-31. ^{1}H–^{13}C COSY and HMBC experiments also verified the chemical structure of CoA1.

Anti-angiogenic activity of CoA1

We first investigated the effect of CoA1 on the growth of various cell lines including bovine aortic endothelial cells (BAECs) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. As shown in Figure 2, CoA1 inhibited the proliferation of each cell lines with a different growth inhibitory spectrum. Notably, CoA1 potently and selectively inhibits the growth of BAECs than that of normal (CHANG) or cancer cell (HT29, HeLa, HT1080, B16) lines. IC_{50} values of each cell line are shown in Table 1. These data demonstrate that CoA1 may exhibit the anti-angiogenic activity by specific inhibition of endothelial cell growth. The viability of endothelial cells was not affected up to 3 μg/mL of CoA1 treatment, implying that the growth inhibitory activity of CoA1 shown Figure 2 is not due to mere cytotoxicity of the compound. It is also noteworthy that the growth of highly metastatic melanoma cells, B16/BL6, is moderately inhibited by CoA1, suggesting that CoA1 can also inhibit the growth of highly proliferative metastatic cells.

We, next, investigated the effect of CoA1 on angiogenic phenotypes of endothelial cells such as cell invasion and tube formation using in vitro assays.[19,20] Basic fibroblast growth factor (bFGF) was used as a chemoattractant or an angiogenic factor.[7] 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. As shown in Figure 3A and B, bFGF effectively induced cell invasion through the filter compared to that of the control. However, CoA1 (1, 3 μg/mL) dose-dependently inhibited bFGF-induced invasion of BAECs. Next, the inhibitory effect of purified CoA1 on capillary tube formation, another key phenotype for endothelial cell differentiation of angiogenesis was examined. In the absence of bFGF, cultured BAECs on the Matrigel normally formed incomplete and narrow tube-like structures but the capillary network formation that was stimulated by the treatment of bFGF resulting in elongated and robust tube-like structures. As shown in Figure 3C, CoA1 efficiently inhibited the tube formation induced by bFGF. Trypan blue staining was performed in parallel with in vitro angiogenesis assays

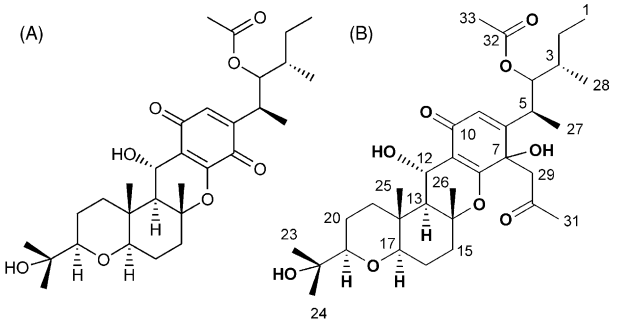

Figure 1. The structure of CoA (A) and CoA1 (B).

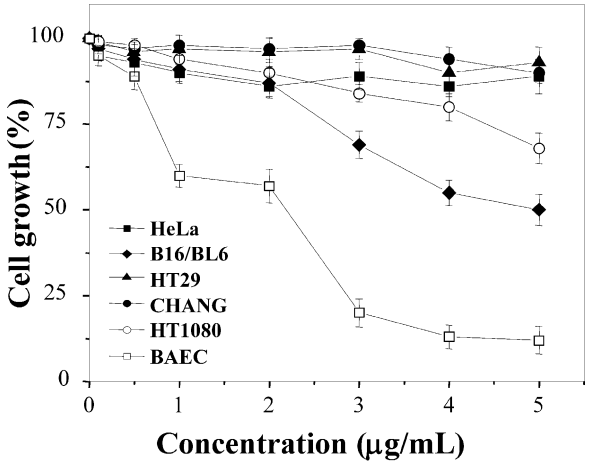

Figure 2. Effect of CoA1 on the growth of various cell lines. Cell growth was measured using MTT colorimetric assay. Data represent mean±SE from three independent experiments.

Table 1. IC_{50} values of CoA1 on various cell lines

Cell lines	HeLa	B16/BL6	HT29	CHANG	HT1080	BAEC
IC_{50} (μg/mL)	15	5	20	15	10	1

and cytotoxicity was not shown at concentrations used in this study (data not shown).

Together, these results demonstrate that CoA1 potently inhibits angiogenesis without affecting endothelial cell viability, and suggest that the compound can be developed as a novel anti-angiogenic agent.

How CoA1 inhibits angiogenesis has not been fully defined yet. Previous reports demonstrated that epicochlioquinone A, a stereoisomer of cochlioquinone A, is an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT) that may possibly be involved in angiogenesis through the regulation of cholesterol esterification/ cholesteryl ester accumulation.[21–24] Consistent with the results, we found that CoA significantly inhibited bFGF-induced invasion and tube formation of BAECs, but the potential of the activity was relatively weaker than that of CoA1 (data not shown). This result also suggests that the increased hydrophobicity of CoA1 by replacement of carbonyl group (C=O) of CoA at C-7 position to acetomethyl group (CH_2–CO–CH_3) may contribute the anti-angiogenic activity of CoA1. We also investigated the effect of CoA1 on histone deacetylase (HDAC) that plays a key role in angiogenesis.[25] CoA1 did not inhibit the enzyme activity both in vitro and in vivo, suggesting the molecular target of CoA1 is not HDAC (data not shown). Detailed studies on the effect of CoA1 on the endothelial cell growth signal such as VEGF or HIF1α-mediated signal or target identification using affinity matrix based purification or phage display biopanning will help to decipher the mode of action of CoA1.

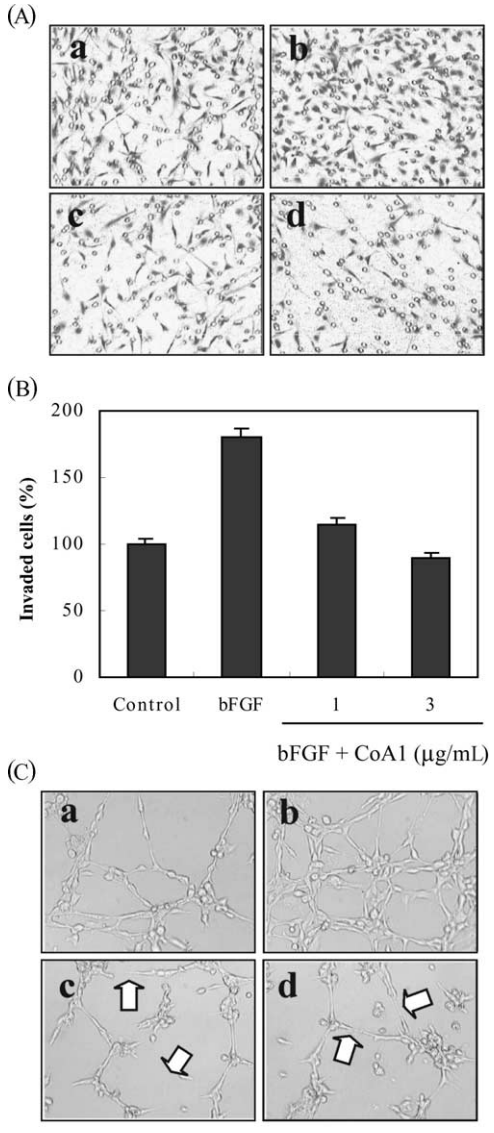

Figure 3. Effect of CoA1 on angiogenic phenotypes of endothelial cells. (A) Microscopic observation of invaded cells (×100 magnification). (a) Control; (b) bFGF alone; (c) bFGF + CoA1 (1 μg/mL); (d) bFGF + CoA1 (3 μg/mL). (B) Inhibitory activity of CoA1 on endothelial cell invasion. Serum-starved BAECs left in serum-free medium (Control) or treated with bFGF in the presence or absence of CoA1 were used for invasion assay. (C) Effect of CoA1 on tube forming ability of BAECs. (a) Control; (b) bFGF alone; (c) bFGF + CoA1 (1 μg/mL); (d) bFGF + CoA1 (3 μg/mL). Arrows indicate the inhibition of tube formation by CoA1.

Experimental

General procedures

The HRFAB-MS spectrum was obtained using a Jeol JMSHX 110 mass spectrometer. The UV spectra were recorded on a Simadzu UV-3000. The ^{1}H- and ^{13}C NMR spectra were determined on Brucker AC 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$, using the tetramethylsilane (TMS) as an internal standard. High performance liquid chromatography (HPLC) separations were performed on Shimadzu (Kyoto, Japan), UV–vis detector, using a Cosmosil octadecyl silica (ODS) column.

Isolation

B. zeicola isolated from maize grain was cultured on potato sucrose agar plates (1000 plates, 90 mm in diameter) in the dark at 26 °C for 2 weeks. The agar plates were then soaked in 100% acetone, and the acetone extract was filtered and concentrated in vacuo below 40 °C. The concentrated residue was extracted with *n*-hexane, and aqueous layer was re-extracted with ethyl acetate. The solvent was removed in vacuo to obtain the crude preparation. This material was chromatographed on a stepwise silica gel column using a solvent gradient system from chloroform, chloroform / methanol (9:1→1:1), methanol (v/v). Fraction (F2) exhibiting the activity out of eight fractions, as evidenced by bioassay, was further purified by Sep-pak C18 cartridges, and then reverse-phase HPLC on a Cosmosil C_{18} column (20×250 mm i.d) and solvent system of 80% aqueous methanol (flow rate, 4 mL/min) to afford CoA1 (24.2 mg) in pure form.

CoA1. A dark yellow gum: $[\alpha]_D^{24}$ +278.5 (*c* 0.85, EtOH); IR V_{max} (KBr) cm^{-1}: 3425, 2983, 2950, 1710, 1668, 1570. UV λ_{max} (EtOH) nm (ε): 255 (9550), 327 (6760); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.07 (1H, s, H-11), 5.35 (1H, bs, 7-OH), 5.09 (1H, dd, J = 10.8, 2.22 Hz, H-4), 4.59 (1H, bs, 12-OH), 4.18 (1H, d, J = 10.44 Hz, H-12),

3.23 (2H, dd, H-21), 3.13 (1H, dd, $J=11.76$, 3.72 Hz, H-17), 3.03 (1H, m, H-5), 2.82 (1H, d, $J=15.24$ Hz, H-29a), 2.49 (1H, d, $J=15.18$ Hz, H-29b), 2.49 (1H, H-19), 2.28 (3H, s, H-31), 2.05 (1H, H-15a), 1.97 (1H, H-15b), 1.94 (3H, s, H-33), 1.79 (1H, m, H-3), 1.74 (1H, m, H-16a), 1.64 (1H, m, H-20a), 1.59 (1H, d, $J=10.38$ Hz, H-13), 1.53 (1H, m, H-16b), 1.41 (1H, m, H-20b), 1.27 (3H, s, H-26), 1.172 (3H, s, H-24), 1.166 (3H, s, H-23), 1.14 (3H, d, $J=6.72$ Hz, H-27), 0.99 (3H, s, H-25), 0.94 (3H, t, $J=6.84$ Hz, H-1), 0.92 (3H, d, $J=7.44$ Hz, H-28). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 209.57 (C-30), 187.16 (C-10), 169.66 (C-32), 168.40 (C-8), 160.90 (C-6), 124.61 (C-11), 110.62 (C-9), 85.13 (C-21), 83.91 (C-17), 82.60 (C-14), 79.68 (C-4), 71.85 (C-22), 71.82 (C-7), 62.94 (C-12), 52.37 (C-13), 46.48 (C-29), 38.39 (C-19), 37.49 (C-15), 36.61 (C-18), 35.86 (C-3), 35.85 (C-5), 33.20 (C-31), 25.92 (C-24), 25.87 (C-16), 25.10 (C-2), 23.66 (C-23), 21.51 (C-20), 21.34 (C-26), 20.80 (C-33), 19.75 (C-27), 16.47 (C-28), 12.49 (C-25), 11.84 (C-1).

Cell culture and growth assay

Early passages (4–8 passages) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. Jo at KNIH. BAECs were grown in MEM supplemented with 10% fetal bovine serum (FBS, Life Technology, Grand Island, NY, USA). CHANG (immortalized hepatocyte derived from normal human liver), HeLa (cervical carcinoma), and HT1080 (fibrosarcoma) cells were maintained in DMEM, and HT29 (colon carcinoma) and B16/BL6 (murine melanoma) cells in RPMI1640 containing 10% FBS. All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Cell growth assay was carried out using MTT colorimetric assay. Cells were inoculated at a density of 5×10^3 cells per well in 96-well culture plates and incubated for 24 h for stabilization. Various concentrations of CoA1 were added to each well and incubated for 3 days and performed MTT assay. 50 μL of MTT (2 mg/mL stock solution, Sigma, St. Louis, MO, USA) was added and the plate was incubated for an additional 4 h. After removal of medium, 100 μL of DMSO was added. The plate was read at 540 nm by universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Chemoinvasion assay

The invasiveness of BAECs was examined in vitro using a Transwell chamber system with 8.0 μm pore-sized polycarbonate filter inserts (Corning Costar, Cambridge, MA). Briefly, the lower side of the filter was coated with 10 μL gelatin (1 mg/mL) and the upper side was coated with 10 μL Matrigel (3 mg/mL). 6 μL BSA (100 mg/mL), 2 μL bFGF (10 μg/mL, Upstate Biotechnology, Lake Placid, NY, USA), and CoA1 were added in the lower wells placed in 600 μL MEM. BAECs (1×10^5 cells) were placed in the upper part of the filter and the chamber was then incubated at 37 °C for 18 h. The cells were fixed with 70% methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting the total number of invaded cells in the lower side of the filter using an optical microscope at a ×100 magnification.

Tube formation assay

Matrigel (150 μL, 10 mg/mL, Collaborative Biomedical Products, Bedford, MA, USA) was coated in a 48-well culture plate and polymerized for 2 h at 37 °C. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/mL). Then, CoA1 was added and incubated for 6–18 h. The morphological changes of cells were observed under microscope and photographed at ×100 magnification using JVC digital camera (Victor, Yokohama, Japan). Cytotoxicity of tube-forming endo-thelial cells was evaluated by trypan blue staining.

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