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COMMUNICATIONS TO THE EDITOR

Anti-angiogenic Activities of Novel Isocoumarins, AGI-7 and Sescandelin

Sir:

Angiogenesis is a fundamental process by which new blood vessels are formed and is composed of several steps including proteolytic degradation of basement membrane in the vessels, directed migration and proliferation of endothelial cells to provide cells for the new vessels. In adults, angiogenesis occurs under several physiological conditions including maturation of the corpus luteum, inflammation, wound healing and delayed hypersensitivity reactions, however, uncontrolled angiogenesis can contribute to a number of pathological processes such as rheumatoid arthritis, diabetic retinopathy and tumor growth and metastasis.[1] Thus anti-angiogenic therapy was postulated to be an attractive approach for the treatment of such diseases, especially cancer.[2]

Several angiogenic inhibitors have been developed for targeting angiogenic factors or their receptors, extracellular matrix compounds, and vascular endothelial cell proliferation. Among them, thalidomide and TNP-470 are known to inhibit the growth of endothelial cells specifically and are in clinical trials.[3,4]

In screening of anti-angiogenic substances inhibiting differentiation of endothelial cells to the capillary-like structure on Matrigel, we isolated a new compound 6,8-dihydroxy-4-acetylisocoumarin, which named AGI-7 (**1**), along with sescandelin (**2**) from an unidentified fungal strain by bioassay-guided fractionation and isolation. In this communication, we describe the isolation, identification, and anti-angiogenic activity of compounds **1** and **2**.

The producing organism, an unidentified fungal strain Y70832, was isolated from a soil sample collected at a field in Kongju, Korea. The seed culture was incubated in a medium consisting of 2.0% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.05% magnesium sulfate, and 0.1% KH_2PO_4 and incubated for 3 days at 27°C and 150 rpm on a rotary shaker. For the production of isocoumarin compounds, 300 ml of seed culture was transferred into a jar fermentor containing 6 liters of the same medium. The inhibitory activity of culture broth in the tube formation assay reached a maximum at 6 days. After the fermentation, an equal volume of acetone was added to the culture broth and then the mixture was filtered. The filtrate was concentrated *in vacuo* to a small volume and then the residue was extracted with EtOAc. The EtOAc extract (3.8 g) was chromatographed on a silica gel (Kieslgel 60, Merck) eluted with a CH_2Cl_2 - MeOH step gradient system (CH_2Cl_2, CH_2Cl_2 - MeOH, 20 : 1, 10 : 1, MeOH, each 1 liter) to obtain 4 fractions. The active fraction, which was eluted with the solvent ratio at 20 : 1, was concentrated *in vacuo*. The residue (2.2 g) was rechromatographed on RP-18 (70~230 mesh, YMC Co.) eluted with CH_3CN - H_2O (1 : 1). The active fractions were combined, concentrated *in vacuo* and subjected to Sephadex LH20 column chromatography with MeOH. The active fractions were concentrated *in vacuo* to give a pale greyish powder (320 mg). Compounds **1** and **2** were finally purified with preparative HPLC (J'sphere ODS-H80, 20×150 mm, eluent: linear gradient from 15 to 70% CH_3CN in H_2O, flow rate: 5 ml/minute, 220 nm) to afford 17 mg of **1** and 162 mg of **2**, respectively.

Compound **2** gave positive responses to the iodine, sulfuric acid, and ferric chloride tests. The molecular formula of **2** was determined as $C_{11}H_{10}O_5$ by HRFAB-MS (*m/z* $[M+H]^+$; calcd. 223.0607, found 223.0603). The UV, IR, ^1H, and ^{13}C NMR spectra of **2** were comparable those of the 6,8-dihydroxyisocoumarin compound, sescandelin reported previously.[5] Compound **1** also gave positive reaction to iodine, sulfuric acid, and ferric chloride tests. The molecular formula of **1** was determined as $C_{11}H_8O_5$ by HRFAB-MS (*m/z* $[M+H]^+$; calcd. 221.0450, found 221.0448). The UV spectrum of **1** was very similar to that of **2** except for a strong absorption at 263 nm, indicating that another UV chromophore was present in 6,8-dihydroxyisocoumarin skeleton. The IR spectrum exhibited the presence of a hydroxyl group (3440 cm^{-1}), and two carbonyl groups (1685 cm^{-1} and 1640 cm^{-1}). The ^1H NMR spectrum of **1** in DMSO-d_6 revealed two aromatic protons with *meta* coupling at δ 7.59 (1H, d, J=2.4 Hz) and 6.43 (1H, d, J=2.4 Hz), and an olefinic proton at δ 8.57 (1H, s). In addition, one methyl group and two hydroxyl protons appeared at δ 2.50 (3H, s), 11.01 (H, s), and 11.03 (1H, br s), respectively. These spectral data were closely related to those of **2**, except for the presence of the signal at δ 2.50 assignable to an acetyl group instead of two characteristic signals from the H-9 and -10 of **2**. The ^{13}C NMR and DEPT spectra showed the presence of 11 carbons comprised of one methyl carbon, three non-substituted aromatic methine

carbons, seven quarternary carbons including two carbonyl groups as shown in Table 1. Among the difference in carbon signals between **1** and **2**, the signal for the hydroxy methine carbon (C-9) of **2** has been replaced by the carbonyl signal at δ 196.3. This result indicated that the side chain of **1** would be an acetyl group instead of a hydroxyethyl group of **2**. This conclusion is in agreement with not only two mass unit difference of **1** from **2** in HRFAB-MS but also the down field shifts of H-3 and -5 signals of **1** due to the carbonyl group effect. The position of the acetyl group was further confirmed by HMBC spectra. In the HMBC spectra of **1**, the olefinic proton at δ 8.57 was correlated with C-1 (δ 163.5), -4 (116.7), -4a (135.0), and -9 (196.3), and the methyl protons at δ 2.50 were correlated with C-9 (δ 196.3). On the basis of these spectroscopic analyses, the structure of **1** was determined to be 6,8-dihydroxy-4-acetylisocoumarin and named AGI-7 (Fig. 1).

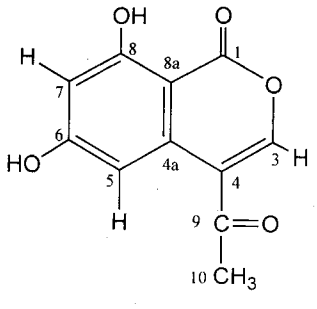
Various natural and synthetic isocoumarins have been found, among them some representative 4-substituted-3-nonsubstituted natural isocoumarins are such as oosponol, its reduction product oospoglycol,[6] 4-acetyl-6,8-dihydroxy-5-methylisocoumarin,[7] and sescandelin.[5] It has been reported that these isocoumarin compounds possess antibiotic activities against plant cells, bacteria and plant-pathogenic fungi.[5,8] Another isocoumarin, cytogenin has been isolated from the culture filtrate of *Streptoverticillium eurocidicum* using cytotoxicity and antitumor activity assays.[9] Recently, an isocoumarin derivative, NM-3, was synthesized from cytogenin and both NM-3 and cytogenin showed anti-angiogenic effects in the mouse dorsal air sac assay system.[10] Moreover, it has been demonstrated that NM-3 is selectively cytotoxic to human umbilical vein endothelial cells (HUVECs) and is a useful anti-angiogenic agent for combination studies with ionizing radiation in cancer therapy.[11] Sescandelin (**2**) was originally isolated from the culture filtrate of *Sesquicillium candelabrum* IFO 30556 as a root-promoting substance.[5] The detailed biological activities of sescandelin other than root-promoting activity have not been reported yet. However, we found that sescandelin and its reduction product, AGI-7 are capable of suppressing angiogenesis.

To determine the inhibitory effect of the compounds **1** and **2** on the angiogenesis *in vitro*, HUVECs were used as a model on the basis that they migrate and differentiate into capillary tubes on Matrigel. The cultured HUVECs were

Table 1. ^{1}H and ^{13}C NMR data of AGI-7 in DMSO-d_6.

Position	δ_C (70 MHz)	δ_H (300 MHz)
1	163.5	-
3	155.1	8.57 (1H, s)
4	116.7	-
4a	135.0	-
5	103.9	7.59 (1H, d, J=2.4Hz)
6	166.2	-
7	102.6	6.43 (1H, d, J=2.4Hz)
8	163.1	-
8a	98.3	-
9	196.3	-
10	28.4	2.50 (3H, s)
6-OH	-	11.03 (1H, br s)
8-OH	-	11.01 (1H, s)

Fig. 1. Chemical structures of AGI-7 (**1**) and sescandelin (**2**).



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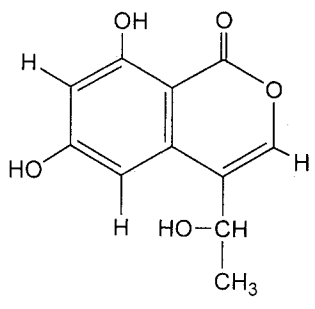

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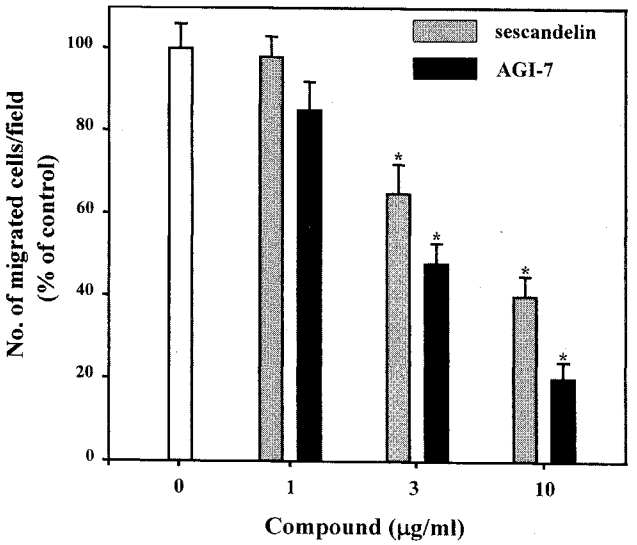
plated on Matrigel coated 96-well plates with an M199 medium supplemented with 20% FBS, 2 ng/ml bFGF and 100 μg/ml heparin at a density of 2×10^4 cells/well. The two compounds were independently added at various concentrations into the wells and the plate was incubated for 18~48 hours in a CO_2 incubator. The formation of capillary-like tube was monitored under a microscope during the incubation. The cells in the well containing vehicle (DMSO) became to be more robust and longer hollow tube networks as the incubation proceeded, but the tubes in the wells containing compound **1** or **2** were broken and shortened. AGI-7 (**1**) completely inhibited tube formation at a concentration of 1.0 μg/ml and was still effective at a concentration as low as 0.2 μg/ml. Sescandelin (**2**) also completely inhibited the differentiation of HUVECs into capillary tubes at a concentration of 10 μg/ml and was effective at a concentration as low as 4 μg/ml(data not shown). Thus, AGI-7 exhibited more potent inhibitory activity on the tube formation of HUVECs than sescandelin.

Next, we investigated the effect of these compounds on the migration of HUVECs.[12] Both compounds dose-dependently inhibited the migration of HUVECs as assessed by the transwell-migration assay (Fig. 2). Consistent with the tube formation assay, compound **1** showed more potent inhibitory activity on the migration of HUVECs. In addition, the effects of compounds **1** and **2** on the proliferation of HUVECs were investigated by MTT assay. As a control, we also measured the effect of these compounds on the proliferation of other cancer cell lines such as HeLa, and HT-1080 cells. Compounds **1** and **2** did

Fig. 2. Effect of AGI-7 (**1**) and sescandelin (**2**) on the migration of HUVECs.



Migration of HUVEC was measured in a modified Boyden chamber (24-multiwell). After 4 hours at 37°C in 5% CO_2, non-migratory cells on the upper membrane surface were removed with a cotton swab and the cells which traversed and spread on the lower surface of the filter were fixed and stained with eosin and hematoxylin. The number of migratory cells per membrane was enumerated using a microscope with a ×20 objective. Each data point is the average number of cells in four random fields. Each determination represents the average±S.D. of two individual wells. The asterisks indicate statistical significance determined by Students's t test ($p<0.01$).

Table 2. Anti-angiogenic activity of AGI-7 (**1**) and sescandelin (**2**) in CAM assay.

Compound	Concentration (μg/egg)	Total eggs showing anti-angiogenesis	Total eggs Tested	% Inhibition
Vehicle	-	3	20	15.0
Retinoic acid	1	15	19	78.9
Sescandelin	1	6	22	27.3
	5	12	18	66.7
AGI-7	1	10	20	50.0
	5	15	18	83.3

Samples were applied to the CAM surface of 4.5-day chick embryo. Two days later, about 1 to 2 ml of 10% fat emulsion was injected into the CAM and the result was observed under a microscope. As a positive control, 1 μg/egg of retinoic acid was used and a negative control was vehicle-treated coverslip. When the CAM showed avascular zone to similar degree of retinoic acid-treated CAM, which had few vessels compared with control, the response was scored as positive and calculated by the percentage of positive eggs among the total numbers of eggs tested.

not significantly inhibit the proliferation of HUVECs or that of HeLa and HT-1080 cells at concentrations that inhibited the tube formation and migration of HUVECs (data not shown).

In order to confirm the anti-angiogenic activity of these compounds, the chorioallantoic membrane (CAM) assay was carried out as described previously.[13] As shown in Table 2, both compounds significantly inhibited the developmental neovascularization of chick embryos in a dose dependent manner. Sescandelin inhibited angiogenesis by 27.3% at a concentration of 1 μg/egg and by 66.7% at a concentration of 5 μg/egg. On the other hand, AGI-7 inhibited angiogenesis by 50.0% at a concentration of 1 μg/egg and by 83.3% at a concentration of 5 μg/egg, indicating that the anti-angiogenic activity of AGI-7 is better than that of sescandelin.

Both migration and differentiation of endothelial cells into tubular structure are critical and essential events in the new blood vessel formation. Compound **1** and **2** significantly inhibited the migration and tube formation of HUVECs without significant cytotoxicity. Therefore, it is possible that anti-angiogenic activities of AGI-7 and sescandelin may be partially explained by the inhibition of migration and of tube formation of endothelial cells among several angiogenesis steps, however, more detailed evaluation on anti-angiogenic mechanisms of these compounds remains to be investigated.

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