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Bioorganic & Medicinal Chemistry 15 (2007) 6758–6762
Bioorganic & Medicinal Chemistry

Structure–activity relationship and biological property of cortistatins, anti-angiogenic spongean steroidal alkaloids

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Received 17 July 2007; revised 31 July 2007; accepted 1 August 2007
Available online 21 August 2007

Abstract—Previously, bioassay-guided separation led us to isolate eleven novel steroidal alkaloids named cortistatins from the marine sponge *Corticium simplex*. These cortistatins were classified into three types based on the chemical structure of the side chain part, that is, isoquinoline, *N*-methyl piperidine or 3-methylpyridine units. From the structure–activity relationship study, the isoquinoline unit in the side chain was found to be crucial for the anti-angiogenic activity of cortistatins. Cortistatin A (**1**) showed cytostatic growth-inhibitory activity against human umbilical vein endothelial cells (HUVECs). Cortistatin A (**1**) also inhibited VEGF-induced migration of HUVECs and bFGF-induced tubular formation. Although cortistatin A (**1**) showed no effect on VEGF-induced phosphorylation of ERK1/2 and p38, which are one of the signaling pathways for migration and tubular formation, the phosphorylation of the unidentified 110 kDa protein in HUVECs was inhibited by the treatment with cortistatin A.

1. Introduction

Angiogenesis is an essential process in normal physiology, while uncontrolled angiogenesis is pathological and often associated with several diseases such as atherosclerosis, arthritis, diabetic retinopathy, and cancer.[1] Tumor angiogenesis involves several steps including degradation of extracellular matrix by metalloproteinases, proliferation, migration, and tubular formation of endothelial cells. Each of these steps is tightly regulated by angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and so on.[2] It has been shown that growth of solid tumor requires a corresponding increase of vascularization, and each step in angiogenesis has potential as a target for development of anti-tumor agents.[3]

In the course of our search for anti-angiogenic substances[4,5] from marine organisms, we isolated eleven steroidal alkaloids named cortistatins from the marine sponge *Corticium simplex* on the basis of bioassay-guided separation and presented their structure elucidation and anti-proliferative activity against HUVECs briefly.[6–8] In this paper, the detailed structure–activity relationship and biological property of cortistatins are presented.

2. Result and discussion

2.1. Structure–activity relationship

Each cortistatin has the same characteristic seven-membered B ring system, and these cortistatins are classified into three types by the difference of the side chain structure (Fig. 1). Cortistatins A (**1**)–D (**4**) and cortistatins J (**10**), K (**8**), and L (**11**) having isoquinoline unit in the side chain part showed potent anti-proliferative activity against HUVECs (IC_{50} = 1.8 nM–1.1 μM), in which the selective index in comparison with those of normal human dermal fibroblast (NHDF) and several cancer cell lines was up to 9500-fold (Table 1). Among cortistatins A (**1**)–D (**4**), cortistatin A (**1**) having most simple functionality showed the strongest growth inhibitory activity against HUVECs, and cortistatins A (**1**) and C (**3**)

Keywords: Cortistatin; Marine sponge; Anti-angiogenic effect; HUVECs; Tubular formation; Migration.
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doi:10.1016/j.bmc.2007.08.017

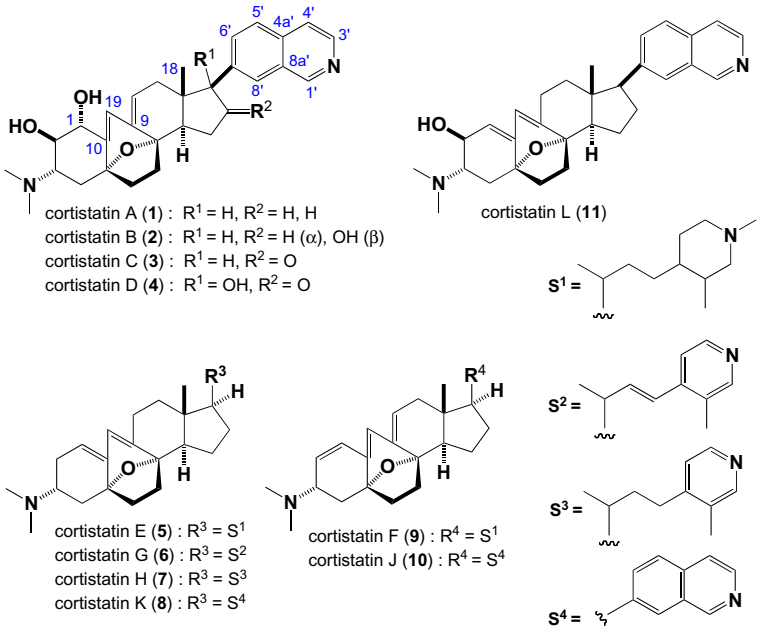

Figure 1. Structures of cortistatins.

Table 1. Growth inhibition of cortistatins against HUVECs and various type of cell lines

Cell line	A (**1**)		B (**2**)		C (**3**)		D (**4**)		E (**5**)	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
HUVEC	0.0018	1	1.1	1	0.019	1	0.15	1	0.45	1
KB3-1	7.0	3900	120	110	150	7900	55	460	2.5	6
Neuro2A	6.0	3300	160	150	180	9500	>300	n.d.	1.9	4
K562	7.0	3900	200	180	>300	n.d.	>300	n.d.	2.8	6
NHDF	6.0	3300	>300	n.d.	>300	n.d.	>300	n.d.	1.9	4

G (**6**)		H (**7**)		K (**8**)		F (**9**)		J (**10**)		L (**11**)	
IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
0.80	1	0.35	1	0.04	1	1.9	1	0.008	1	0.023	1
8.9	11	2.3	7	10.2	250	10.8	6	9.1	1100	14	610
4.0	5	2.2	6	3.0	80	4.0	2	3.3	410	2.8	120
3.8	5	2.7	8	3.9	100	4.0	2	3.3	410	4.3	190
2.9	4	2.7	8	2.5	60	4.1	2	2.4	300	2.4	100

showed extremely high selective index against HUVECs. The hydroxyl group at 16- or 17-positions in cortistatins B (**2**) and D (**4**) significantly decreased inhibitory activity against HUVECs. On the other hand, cortistatins E (**5**), F (**9**), G (**6**), and H (**7**) having *N*-methyl piperidine or 3-methylpyridine units in the side chain part showed only weak anti-proliferative activity (IC_{50} = 0.35–1.9 μM) against HUVECs and no selectivity (less than 11-fold) between HUVECs and other cell lines. Among cortistatins A (**1**), J (**10**), K (**8**), and L (**11**), cortistatin J (**10**) having triene system showed stronger activity with high selectivity than cortistatin K (**8**) having 1(10),9(19)-diene system and cortistatin L (**11**) having 2β-hydroxyl-1(10),9(19)-diene system. These data indicated that the position of diene system is more important for the selectivity against HUVECs than the presence of the hydroxyl group in the ring A. Although additional investigation is needed to elucidate the relationship between the functional groups on the ring A and the anti-proliferative activity, these data suggested that the isoquinoline unit in the side chain part was essential for the anti-angiogenic activity of cortistatins and the characteristic functionality in the A and B rings might be important for the selective growth-inhibitory activity against HUVECs.

2.2. Cytostatic effect of cortistatin A (1) against HUVECs

Based on the cell morphology of the cortistatin A-treated HUVECs, the cell proliferation seemed to be prevented without any cytotoxic effect (data not shown). Therefore, to confirm whether cortistatin A (**1**) is able to inhibit cell proliferation cytostatically or not, the

effect of the exposing time with **1** was examined by WST-8 colorimetric assay. As shown in Figure 2, the control HUVECs grew in time-dependent manner, and the OD_{450} value reached 1.2 after 96 h incubation. However, proliferation of HUVECs was prevented with 100 nM concentration of **1**, and the OD_{450} showed constant values (0.5) from 48 h to 96 h. On the other hand, when HUVECs were incubated for 24 h in the presence of **1** and then washed to remove **1**, HUVECs were able to re-start proliferation after additional 24 h incubation, and then the OD_{450} value was increased in time-dependent fashion. These data indicated that compound **1** inhibited cell proliferation of HUVECs due to cytostatic effect. Cortistatin A (**1**) might bind reversibly with target molecule.

2.3. Effect of cortistatin A (1) on migration and tubular formation of HUVECs

Next, we examined inhibitory effect of cortistatin A (**1**) on the migration of HUVECs using chemotactic chamber method. As shown in Figure 3, the 165 cells/area of HUVECs were migrated to the reverse side of the membrane filter coated with fibronectin by the stimulation of VEGF (20 ng/ml). When HUVECs were pre-incubated with **1** (0.2–2.0 μM) for 12 h, the number of migrated cells was decreased in dose-dependent manner, and 2.0 μM of **1** prevented the migration of cells from 165 cells/area to 80 cells/area. Next, the inhibitory effect of **1** on bFGF-induced tubular formation was evaluated by Matrigel tubular formation assay (Fig. 4). When HUVECs were plated on the Matrigel in the presence of bFGF, the cells aligned with high motility and cell–cell communication and formed a tight tubular network within 10 h (Fig. 4a). However, the tubular network was prevented by the 12 h pre-treatment with 2.0–200 nM concentration of **1** (Fig. 4b–d). These results indicated

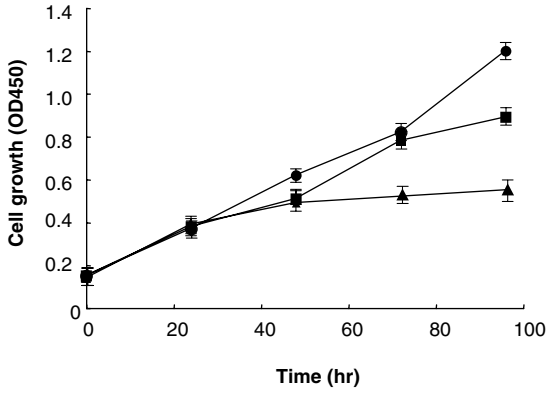

Figure 2. Effect of the exposing time of cortistatin A (**1**) on the growth of HUVECs. The HUVECs in the 96-well plates (1.0×10^3 cells/well/100 μl) were incubated for 0 h, 24 h, 48 h, 72 h, and 96 h in the presence (▲) or absence (●) of 100 nM concentration of **1**. To confirm the effect of the exposing time of **1**, the compound **1** was removed after 24 h exposure, and then the cells were incubated for additional indicated time (■). The cell proliferation was detected by WST-8 colorimetric reagent. Each point represents means ± SD the value of OD_{450} from three independent wells.

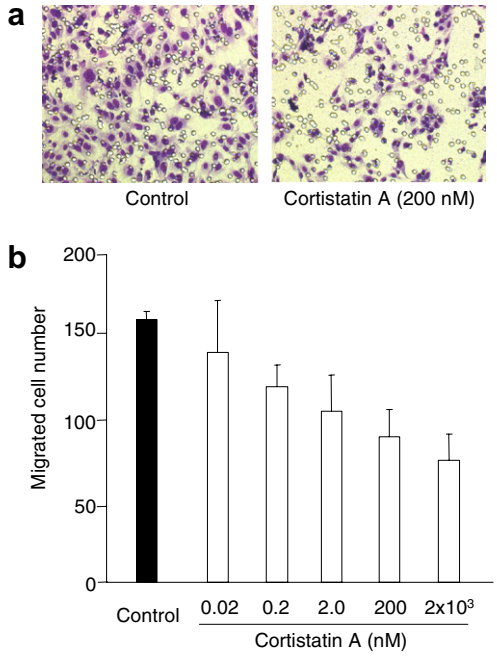

Figure 3. Effect of coristatin A (**1**) on the migration of HUVECs induced by VEGF. HUVECs (1.5×10^5 cells) were treated with the indicated concentrations of **1** and stimulated with VEGF (20 ng/ml). After 6 h, the migrated cells to the reverse side of the membrane filter were counted in the six different microscopic fields. The data are presented as means ± SD (b). The representative figure of the migrated HUVECs is shown (a).

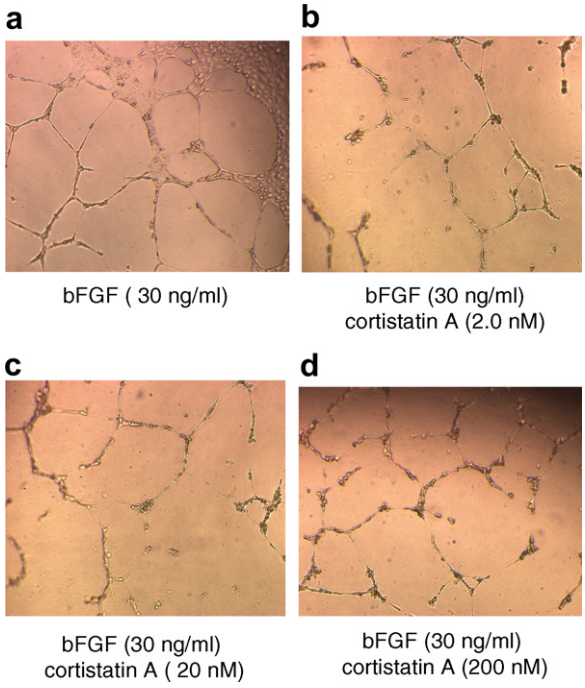

Figure 4. Effect of cortistatin A (**1**) on the tubular formation of HUVECs induced by bFGF. HUVECs (1.0×10^4 cells) were pre-treated with the indicated concentrations of **1** for 12 h. Then, the cells were suspended in HuMedia-EB2 medium supplemented with bFGF (30 ng/ml) and 0.2% FBS, and inoculated onto the solidified Matrigel in 96-well plates. After 10 h, the images were captured under a microscope.

that cortistatin A (**1**) inhibited migration and tubular formation of HUVECs.

2.4. Effect of cortistatin A (1) on protein phosphorylation in HUVECs

Proliferation and migration of HUVECs requires activation of ERK1/2 MAP kinase and p38 MAP kinase pathway induced by the growth factor such as VEGF and bFGF.[9] To analyze action mechanism of cortistatin A (**1**), the effect of **1** on the phosphorylation of ERK1/2 and p38 was examined by using Western blotting method (Fig. 5a and b). Although HUVECs were prevented 50% of cell proliferation by the treatment with 0.01–1.0 μM concentration of **1**, the levels of the phosphorylated ERK1/2 and phosphorylated p38 stimulated by VEGF were not affected by the treatment with these concentrations of **1**. These results suggested that cortistatin A (**1**) inhibited proliferation and migration of HUVECs without inhibition of the phosphorylation of ERK1/2 and p38. Furthermore, to examine the effect of **1** on protein phosphorylation, the levels of the phosphorylated tyrosine in HUVECs were compared with those of the cortistatin A-treated HUVECs by using anti-phosphor-tyrosine antibody. As shown in Figure 6, the 110 kDa protein in HUVECs was phosphorylated by VEGF stimulation, whereas the phosphorylation of this protein in HUVECs was not observed in the absence of VEGF. Then, the phosphorylation of this protein by VEGF-stimulation was reduced remarkably by the treatment with 0.01–1.0 μM concentration of **1**. Gerber et al. previously reported that PI3 kinase/AKT pathway was involved in growth signal transduction induced by angiogenic factor, and this pathway also plays an important role for regulation of actin cytoskeleton.[10,11] Further studies are needed to confirm the relationship between this 110 kDa protein and PI3 kinase/AKT pathway. Interestingly, in our preliminary proteome analysis, cytoskeleton-related tropomyosin was identified as one of the expressed proteins in HUVECs by the cortistatin A-treatment. Further analysis is currently under way.

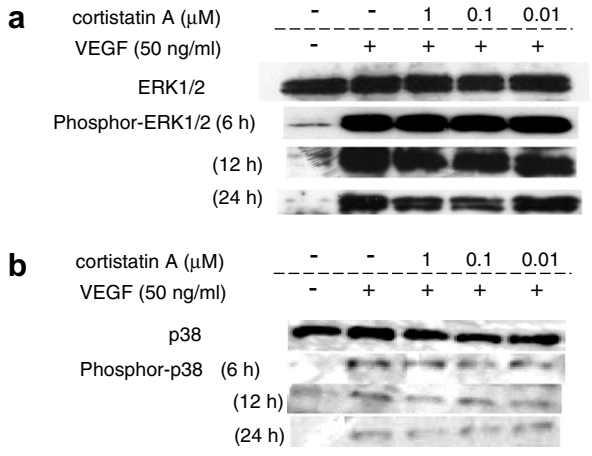

Figure 5. Effect of cortistatin A (**1**) on the phosphorylation of ERK1/2 and p38. HUVECs were pre-incubated with the indicated concentrations of **1** for 6 h, 12 h or 24 h, and then the cells were stimulated with or without VEGF (50 ng/ml) for 15 min. The cell lysate (10 μg/protein) was resolved by SDS–PAGE and detected by each monoclonal antibody.

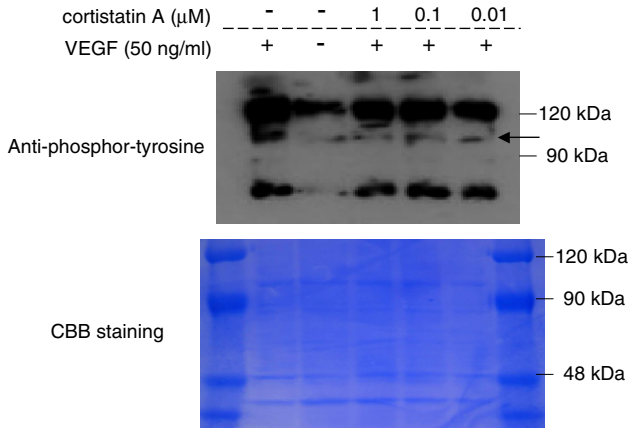

Figure 6. Effect of cortistatin A (**1**) on the level of the phosphorylated tyrosine. HUVECs were pre-incubated with the indicated concentrations of **1** for 12 h, and then the cells were stimulated with or without VEGF (50 ng/ml) for 15 min. The cell lysate (10 μg protein) was separated by SDS–PAGE and detected by monoclonal anti-phosphor-tyrosine antibody.

3. Experimental

3.1. General experimental procedures

Dulbecco's Modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). WST-8 colorimetric reagent was from Nacalai Tesque, Inc. (Kyoto, Japan). The Chemotaxicell chamber (8 μm) was obtained from Kurabo Inc. (Osaka, Japan). Human recombinant $VEGF_{165}$, human recombinant bFGF were from Pepro Tech EC Ltd (London, UK). A monoclonal Anti-p44/42 MAP kinase antibody, Anti-phosphor-p44/42 MAP kinase antibody, Anti-p38 MAP kinase, and Anti-phosphor-p38 MAP kinase were from Cell Signaling (Danvers, MA). A monoclonal Anti-phosphor-tyrosine antibody was obtained from Upstate (Lake Placid, NY). The other reagents were purchased from Sigma Chemical Co., Ltd (St. Louis, MO) or Nacalai Tesque, Inc. (Kyoto, Japan). Cortistatins were isolated from the marine sponge *C. simplex*, which was collected in 2001 at west end of Flores Island, Indonesia.[6–8]

3.2. Assay for anti-proliferative activity against HUVECs and several cell lines

HUVECs (5×10^5 cells/vial) and NHDF (5×10^5 cells/vial) were purchased from Kurabo Inc. and grown in HuMedia-EG2 medium with growth supplements (Kurabo Inc.) and Medium 106S with growth supplements (Kurabo Inc.), respectively.

Human KB epidermoid carcinoma cells (KB3-1) were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS) and kanamycin (50 μg/ml). Human chronic myelogenous

leukemia cells (K562) and murine neuroblastoma cells (Neuro2A) were cultured in RPMI 1640 medium or in Dulbecco's Modified Eagle's medium (DMEM), respectively, supplemented with 10% FBS and kanamycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.

Each suspension of HUVECs or other cell lines in the culture medium was plated into each well of 96-well plates (2×10^3 cells/well/100 μl). After 24 h, test compounds were added, and then the plates were incubated for an additional 72 h in a humidified atmosphere of 5% CO_2 at 37 °C. The cell proliferation was detected by WST-8 colorimetric reagent. The IC_{50} value was determined by linear interpolation from the growth inhibition curve. We assessed selectivity of the anti-proliferative activity (Selective Index, SI) from the differences of IC_{50} values against HUVECs and other cell lines. In the case for confirming cytostatic effect of **1** against HUVECs, the cells (1.0×10^3 cells/well/100 μl) were plated into 96-well plates. After 24 h, **1** was added and incubated for additional 24 h. Then, the cells were washed three times with pre-warmed growth medium.

3.3. VEGF-induced migration assay

The polycarbonate filter of the inner chamber (Chemotaxicell chamber, 8 μm) was soaked in fibronectin solution (1.33 μg/ml) for 1 h at 37 °C and dried in vacuo. HUVECs were treated with different concentrations of **1** in serum and growth factor starved medium for 12 h. Then HUVECs (1.5×10^4 cells) were suspended in HuMedia-EB2 medium containing 0.2% FBS and seeded in the inner chamber. The inner chamber was placed into the outer chamber (24-well plate), which was filled with the same medium containing VEGF (20 ng/ml). After 6 h incubation at 37 °C, the non-migrated cells on the upper surface of the filter were removed by wiping with cotton swabs, and the cells were fixed with 70% EtOH and stained with Giemsa. The cells, which migrated through the filter to the reverse side, were counted manually at six different areas under a microscope (200×).

3.4. bFGF-induced Matrigel tubular formation assay

HUVECs were treated with the different concentrations of **1** in serum and growth factor starved medium for 12 h. 96-Well plates were coated with 50 μl of Matrigel (11.55 mg/ml) and incubated at 37 °C for 1 h to promote gelation. The HUVECs were trypsinized, washed with HEPES buffer, counted, and resuspended in HuMedia-EB2 medium supplemented with bFGF (30 ng/ml) and 0.2% FBS. Then, the HUVECs (1×10^4 cells/well) were seeded onto the solidified Matrigel in 96-well plates. The plates were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 10 h, tubular network patterns were captured through an inverted phase contrast microscope and photographed.

3.5. Immunoblotting assay

HUVECs were cultured in petri dish with serum and growth factors (bFGF and EGF) starved HuMedia-EG2 medium for 24 h. The confluent cells were then pre-incubated with the indicated concentrations of **1** for 6 h, 12 h or 24 h and stimulated with VEGF (50 ng/ml) for 15 min at 37 °C. The cells were rinsed with ice-cold PBS and lysed in lysis buffer (50 mM Tris–HCl (pH 8.0), 137 mM NaCl, 200 mM EDTA, 1% Triton X-100, and 10% glycerol containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail-DMSO solution, and 1 mM sodium vanadate). The cell lysate was subjected to SDS–PAGE and transferred onto PVDF membranes (Amersham, UK). The membrane was incubated with the blocking solution and probed with the primary antibodies (anti-phosphor-tyrosine antibody, anti-p44/42 MAP kinase antibody, anti-phosphor-p44/42 MAP kinase antibody, anti-p38 antibody, and anti-phosphor-p38 antibody). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, UK).

Acknowledgment

This study was financially supported by Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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