Synthesis and Anti-Angiogenic Activity of Cortistatin Analogs

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Analogs of cortistatins, a series of anti-angiogenic compounds isolated from the Indonesian marine sponge Cortisium simplex, were synthesized from estrone by using the Suzuki-Miyaura coupling reaction as the key step. The estrone-isoquinoline hybridized compound showed selective inhibitory activity against the proliferation and VEGF-induced migration of HUVEC.

Key words: Cortisium simplex; cortistatin A; synthesis; HUVEC; angiogenesis

Cortistatins A-L are a series of unprecedented isoquinolynyl steroids isolated from the Indonesian marine sponge, Cortisium simplex, by Kobayashi et al.1–3 Among them, cortistatins A (1a) and J (1j) showed the strongest inhibitory activity against the proliferation of human umbilical vein endothelial cells (HUVEC), but were almost inactive against KB3-1, Neuro2A, K562 and NHDF cells. Thus, 1a could be a promising selective anti-angiogenic agent. The synthesis of 1a from the steroid, prednisone, has been achieved by Baran’s group,4 and several synthetic studies have been reported.5,6 The structure-activity relationship studies of cortistatins7 have revealed that a) introduction of an oxygen function on the D-ring diminishes the activity, b) the isoquinoline ring is crucial, and c) the dihydroxy group on the A-ring is removable. Taking these observations into account, we designed a simpler compound, namely, estrone-isoquinoline hybrid A (EI-hybrid A, 2). Although the diene system on the BC ring has been reported as likely to be important,7,8 2 could be easily prepared from commercially available estrone, and its bioassay will provide useful information to identify further structure-activity relationships, espe-
Results and Discussion

Synthesis of EI-hybrid A (2)

For the coupling reaction of the steroidal ring and the isoquinoline, estrone (3) was converted to corresponding stannane 5\textsuperscript{a} and borate 6\textsuperscript{a} via enol triflate 4\textsuperscript{a,8,9}. Bromoisouquinoline (7) was prepared according to Gensler’s procedure as a 2:1 mixture of 7/5-isomers.\textsuperscript{10}

The Stille coupling reaction between 5 and 7 did not give any coupling product, so the Suzuki-Miyaura coupling reaction using borate 6\textsuperscript{a} was then examined. Among the palladium catalysts [Pd(PPh\textsubscript{3})\textsubscript{4}, Pd(PhCN)\textsubscript{2}Cl\textsubscript{2}, etc.], additives (PPh\textsubscript{3} and AsPh\textsubscript{3}), bases (KF, CsF, NaOH, Ag\textsubscript{2}O, K\textsubscript{2}CO\textsubscript{3}, Cs\textsubscript{2}CO\textsubscript{3}, etc.) and solvents (DMF, THF, and H\textsubscript{2}O) tested, the combination of Pd(dppf)Cl\textsubscript{2}, Na\textsubscript{2}CO\textsubscript{3} and THF-H\textsubscript{2}O afforded 8 in a 14\% yield. When this reaction was carried out under microwave irradiation in DMF-H\textsubscript{2}O, the yield was doubled, and most of starting 6 was recovered. Unexpectedly, the undesired 5'-isomer was preferentially formed. A trial of the Stille coupling reaction between

triflate 4 and 7-(trimethylstannyl)isoquinoline, as Baran has reported using the corresponding vinyl iodide instead of 4\textsuperscript{a,15} resulted in the decomposition of both compounds.

Hydrogenation of the 16,17-double bond of 8 proceeded stereoselectively on 10\% Pd/C for 5 h to give 9. A prolonged reaction time resulted in decomposition of the isoquinoline ring. Finally, deprotection of the TBS group using TBAF afforded EI-hybrid A (2) as a 1:3 mixture of 7/5'-isomers. 8 was also treated with TBAF to give Δ\textsuperscript{16}-EI-hybrid A (10). Since these 7/5'-isomers were inseparable, 2 and 10 were subjected to the bioassay in preliminary studies.

Biological activities of the synthetic analogs

The synthetic compounds, EI-hybrid A (2) and Δ\textsuperscript{16}-EI-hybrid A (10), were tested by several bioassays. Estrone (3) and 7/5-bromoisoquinoline (7) were also included for reference.

EI-Hybrid A (2) selectively inhibited endothelial cell proliferation

To investigate the biological activity of EI-hybrid A (2), we conducted a cell growth inhibition assay for several cell lines, HeLa, SH-SY5Y, NIH3T3, and HUVEC. Cell proliferation was measured by using the WST-8 reagent. 2 selectively inhibited endothelial cell (HUVEC) proliferation (the IC\textsubscript{50} value of 2 was 9.1 μM), but not the proliferation of tumor cells (HeLa and SH-SY5Y) and fibroblast cells (NIH3T3) (Table 1). In contrast, estrone (3), bromoisouquinoline (7), and Δ\textsuperscript{16}-EI-hybrid A (10) did not have any inhibitory effect on each of these cell lines.

EI-Hybrid A (2) inhibited VEGF-induced migration of HUVEC

An important step in the development of pathological angiogenesis is thought to involve the signaling pathway

\textsuperscript{*} 3-Methoxy-16-(tributylstannyl)estr-1,3,5,16-tetraene has already been reported: see lit.\textsuperscript{9}
of VEGF, a key regulator of malignant tumor growth.\textsuperscript{11)} Therefore, we next examined the inhibitory effect of 2 on the VEGF-induced migration of HUVEC for an anti-angiogenic effect. In the migration assay, a VEGF treatment significantly induced the migration of HUVEC for 12 h. The induction of migration by VEGF was inhibited by 2 in the range of 3 to 10\(\mu\)M without significant cell toxicity (Fig. 1). These results suggest that EI-hybrid A had an anti-angiogenic effect on HUVEC.

**EI-Hybrid A (2) did not inhibit the VEGF signaling pathway of HUVEC**

It is known that ERK is involved in both the cell growth and migration of endothelial cells.\textsuperscript{12)} To characterize the effect of 2 on the angiogenesis signaling pathway of HUVEC, we investigated the VEGF-induced phosphorylation of ERK. In a western blotting analysis, ERK was not phosphorylated after 18 h of serum starvation, but was significantly phosphorylated after VEGF addition (Fig. 2). 2 did not inhibit VEGF-induced ERK phosphorylation. These results suggest that 2 inhibited a downstream factor of ERK or another pathway concerned with the cells.

Not only was the proliferation of HUVEC cells inhibited by 2 about 10 times more strongly than other cells were, but the migration of HUVEC cells induced by VEGF was also inhibited at the level of 3\(\mu\)M. Furthermore, 2 did not show inhibitory activity against VEGF-induced ERK phosphorylation. Hence, we hypothesize that its site of action was the downstream factor of ERK, or another pathway for cell migration. Further studies are necessary to clarify the molecular target of 2 (and cortistatins) by using labeled probe compounds or an exhaustive evaluation from a cDNA microarray and proteome analysis. The inactivity of \(\Delta^{16}\)-EI-hybrid A (10) indicates that the hydrophobicity of the D-ring as well as the stereochemical angle of isoquinoline-steroid bond of cortistatins were crucial for these activities.

**Conclusions**

Analogs of cortistatins, a series of anti-angiogenic compounds isolated from the Indonesian marine sponge *Cortisium simplex*, were synthesized from estrone. The estrone-isoquinoline hybrid (EI-hybrid A, 2) is a candidate for a new anti-angiogenic agent due to its inhibitory activities against the proliferation and migration of HUVEC cells, although the experiments have been carried out only for endothelial cells and the active compound seemed to be the minor 7’-isomer contained in 2. More selective synthetic studies of 2 and further analogs, including natural cortistatins, for biological investigation are in progress.
Experimental

$^1$H-NMR spectra: Varian Inova 500 (500 MHz in CDCl$_3$). Mass spectra: Jeol JMS-700. Microwave reactor: CEM Discover (2455 MHz, 300 W). Column chromatography: Merck silica gel 60 (63–212 μm) and Kanto silica gel 60N (spherical, neutral, 100–210 μm).

Synthesis of the compounds.

(8R,9S,13S,14S)-3-(tet-Butyldimethylsilyloxy)-17-(trimethylstannyl)estra-1,3,5,16-tetraene (5). A mixture of $^4$D$_1$ (150 mg, 0.290 mmol), LiCl (86.5 mg, 2.04 mmol), Pd(PPh)$_3$$_4$ (33.8 mg, 29.2 μmol), and (Me$_3$Si)$_2$O (50 μL, 79 μg, 0.24 μmol) in THF (15 mL) was stirred at 60°C for 2 h. A 1.0 M potassium-phosphate buffer was added to the mixture which was then extracted with EtOAc. The extract was dried (MgSO$_4$) and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (hexane/EtOAc = 10:1) to give 5 (91.0 mg, 0.171 mmol, 59.0%) as a white solid. $^1$H-NMR δ: 0.161 [9 H; s, d, J = 53.1 (Me$_3$119Sn) Hz; d, J = 52.7 (Me$_3$171Sn) Hz], 0.19 (6 H, s, Me$_3$Si), 0.75 (3 H, s, 18-H), 0.98 (9 H, s, t-Bu), 1.38–1.66 (5 H, m), 1.91 (1 H, m), 2.08 (1 H, ddd, J = 14.6, 11.2, 10.0 Hz), 2.25 (2 H, m), 2.32 (1 H, m), 2.76–2.91 (2 H, m), 5.91 [1 H; dd, J = 2.9, 1.5 (16-HCCSn) Hz; pseudo d, J = 40 (16-HCC$_{199}$$^{179}$Sn) Hz], 6.56 (1 H, d, J = 2.4 Hz, 4-H), 6.61 (1 H, dd, J = 8.3, 2.4 Hz, 2-H), 7.11 (1 H, d, J = 8.3 Hz, 1-H). HR-EL-MS: $m/z$ [M–Me]$^+$: calcd. for C$_{27}$H$_{43}$OSi$_{120}$Sn, 517.1949; found, 517.1949; M$^{**}$: calcd. for C$_{27}$H$_{43}$OSi$_{120}$Sn, 532.2183; found, 532.2188.

(8R,9S,13S,14S)-3-(tet-Butyldimethylsilyloxy)-17-(isoquinolin-5'-/7'-yl)estra-1,3,5,16-tetraene (6, 5'-/7'-isomers = 3:1). A 10-mL (φ10 × 90 mm) glass vessel charged with $^6$D$_1$ (100 mg, 0.202 mmol), 7'/5-bromoisoquinoline$_{10}$ (7, 34 mg, 0.168 mmol), PdCl$_2$(dpff) (20 mg, 24 μmol) and Na$_2$CO$_3$ (36.0 mg, 0.338 mmol) in DMF-H$_2$O (5:1, 4.8 mL) was stirred under microwave irradiation at 100°C for 1 h. After being cooled to 20°C, the mixture was extracted with EtOAc. The extract was dried (MgSO$_4$) and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (hexane/EtOAc = 10:1) to give 6 (61.2 mg, 0.124 mmol) and 8 [31.4 mg, 63.2 μmol, 31.3% (89.2% brsm)] as a brown solid. $^1$H-NMR δ: 0.18 (6 H, s, Me$_3$Si), 0.979 (9 H, s, t-Bu), 0.985 (3 H, s, CMe), 1.5–2.6 (10 H, m), 2.8–3.0 (3 H, m), 5.84 (0.75 H, m, 16-H), 6.15 (0.25 H, m, 16-H), 6.57–6.65 (2 H, m), 7.09 (0.75 H, d, J = 8.8 Hz), 7.14 (0.25 H, d, J = 8.3 Hz), 7.53 (0.75 H, dd, J = 6.8, 1.0 Hz, 4'-H), 7.59 (0.75 H, t, J = 7.6 Hz, 7'-H), 7.62 (0.25 H, d, J = 5.4 Hz, 4'-H), 7.76 (0.25 H, d, J = 8.8 Hz, 5'/6'-H), 7.79 (0.25 H, dd, J = 8.8, 1.5 Hz, 5'/6'-H), 7.89 (0.75 H, d, J = 6.3 Hz, 6'/8'-H), 7.90 (0.75 H, d, J = 7.8 Hz, 6'/8'-H), 7.95 (0.25 H, s, 1'H), 8.49 (0.25 H, d, J = 4.9 Hz, 3'-H), 8.50 (0.75 H, d, J = 4.9 Hz, 3'-H), 9.23 (0.25 H, s, 1'H), 9.24 (0.75 H, s, 1'H). HR-FAB-MS: $m/z$ [M + H]$^+$: calcd. for C$_{33}$H$_{42}$NO$_3$, 496.3036; found, 496.3040.

(8R,9S,13S,14S,17R)-3-(tet-Butyldimethylsilyloxy)-17-(isoquinolin-5'/7'-yl)estra-1,3,5,16-tetraene (9, 5'/7'-isomers = 3:1). A suspension of $^8$ (80.0 mg, 0.161 mmol) and 10% Pd/C (40 mg) in EtOAc (3 mL) was stirred at room temperature under hydrogen for 5 h. The reaction mixture was filtered through a Celite pad and concentrated in vacuo to give 9 (60.2 mg, 0.121 mmol, 75.1%) as a white solid. $^1$H-NMR δ: 0.18 (6 H, s, Me$_3$Si), 0.54 (0.75 H, s, 18-H), 0.62 (2.25 H, s, 18-H), 0.97 (9 H, s, t-Bu), 1.15–1.75 (6 H, m), 1.9–2.1 (3 H, m), 2.15 (1 H, m), 2.18–2.35 (3 H, m), 2.84 (2 H, m), 3.74 (1 H, t, J = 9.6 Hz), 6.56 (1 H, s, 4-H), 6.58 (1 H, pseudo d, J = 7.8 Hz, 2-H), 7.07 (0.75 H, d, J = 7.8 Hz), 7.11 (0.25 H, d, J = 8.8 Hz), 7.56–7.64 (1.25 H, m), 7.75 (0.75 H, d, J = 6.8 Hz), 7.81 (0.25 H, s, 8'-H), 7.85 (0.75 H, d, J = 8.3 Hz), 7.89 (0.25 H, m, J = 5.9 Hz), 8.47 (0.25 H, d, J = 5.4 Hz, 3'-H), 8.52 (0.75 H, d, J = 5.9 Hz, 3'-H), 9.23 (0.25 H, s, 1'-H), 9.24 (0.75 H, s, 1'-H). HR-FAB-MS: $m/z$ [M + H]$^+$: calcd. for C$_{33}$H$_{44}$NO$_3$, 498.3192; found, 498.3192.
(0.25 H, m), 8.04 (0.75 H, d, J = 5.9 H, 4'-H), 8.48 (0.25 H, d, J = 5.8 Hz, 3'-H), 8.53 (0.75 H, d, J = 5.9 Hz, 3'-H), 9.23 (0.25 H, s, 1'-H), 9.24 (0.75 H, s, 1'-H). HR-FAB-MS: m/z [M+H]+: calcd. for C_{27}H_{39}NO, 384.2327; found, 384.2329.

Bioassays.

Cells and reagents. HeLa, SH-SYSY, and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS; JRH Bioscience, Lenexa, KS, USA), 50 units/ml of penicillin, and 50 µg/ml of streptomycin (Sigma). Human umbilical vein endothelial cells (HUVEC) were cultured in HuMedia-EG2 (Kurabo, Osaka, Japan) containing 2% FCS in 5% CO₂ at 37 °C. Recombinant human VEGF was obtained from R&D Systems (Minneapolis, MN, USA). The anti-p44/42 MAP kinase (ERK1/2) antibody (#9102) and anti-phospho-p44/42 MAPK (T202/Y204) antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). SU5614 was obtained from Merck Biosciences.

Cell growth inhibition assay. Cell survival was determined by a WST-8 assay kit (Kishida Kagaku, Osaka, Japan). Briefly, cells (5 × 10³ cells/well) in 96-well plates were incubated overnight. The cells were then treated with various concentrations (0, 0.001, 0.01, 0.1, 1, 10, 100 µM) of EI-hybrid A (2), estrone (3), 7/5-bromoisoquinoline (7), and Δ^{15}-EI-hybrid A (10). After 48 h of incubation, the WST-8 reagents were added to the culture. After 2 h of incubation, the absorbance at 450 nm was measured with a multi-detection microplate reader (Dainippon Pharmaceutical, Osaka, Japan). The absorbance was correlated with the number of living cells. The number of living cells (% control) was calculated by the following formula: (number of total cells – number of dead cells)/number of total cells × 100.

Migration assay. The migration assay was performed in a 24-well modified Chemotaxicell chamber (Kurabo, Osaka, Japan). The chamber was placed in a 24-well plate containing 500 µl of HuMedia-EG2 with 0.1% FCS. HUVEC (1 × 10⁵ cells/well) were incubated in the upper chamber for 30 min and treated with or without a chemical compound for 1 h. After VEGF (3.1 ng/ml) stimulation, the cells were allowed to migrate from the upper to the lower chamber for 12 h in 5% CO₂ at 37 °C. The non-migratory cells were removed from the upper chamber by wiping the upper surface with a swab. The migrated cells were fixed for 30 min with methanol and stained with hematoxylin. After washing with H₂O, the number of migrated cells was counted in four different fields under a microscope. Cell viability was measured with trypan blue assay. HUVEC were treated with or without a chemical compound for 12 h. After washing with phosphate buffered saline (PBS), the cells were stained with trypan blue. The cell viability (% of control) was calculated by the following formula: (number of total cells – number of dead cells)/number of total cells × 100.

Western blot analysis. HUVEC (2 × 10⁴ cells/well) were starved with an M199 medium for 18 h and then treated with various concentrations of 2, 3, 7, or 10 for 2 h. After VEGF (3.1 ng/ml) stimulation for 5 min, the cells were lysed with a lysis buffer (20 mM HEPES at pH 7.5, 5 mM EDTA, 150 mM NaCl, 1.5 mM MgCl₂, 5 mM Na₃PO₄, 50 mM NaF, 10% glycerol, 0.2% Triton X-100, 2 mM Na₂VO₃, and 1 mM PMSF, a protease inhibitor mixture (complete; Roche Diagnostics). The cell lysate was separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with a 5% non-fat dry milk buffer and probed with specific antibodies. The proteins were visualized with enhanced chemiluminescence substrate detection reagents (Pierce, Rockford, IL, USA).

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