Recent Progress in Study on the Biologically-Active Natural Products

Search for Anti-angiogenic Substances from Natural Sources

Naoyuki Kotoku, Masayoshi Arai, and Motomasa Kobayashi*

Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan.
Received September 30, 2015

As angiogenesis is critical for tumor growth and metastasis, potent and selective anti-angiogenic agents with novel modes of action are highly needed for anti-cancer drug discovery. In this review, our studies focusing on the search for anti-angiogenic substances from natural sources, such as bastadins, globostellatic acid X methyl esters and cortistatins from marine sponges, and pyrpyropenes from marine-derived fungus, together with senegasaponins from medicinal plant, are summarized.

Key words anti-angiogenesis; marine organism; natural product; antitumor substance

1. Introduction

Angiogenesis, the formation of new blood capillaries from preexisting blood vessels, is highly regulated in a stable physiological condition. By contrast, several diseases including cancer, rheumatoid arthritis, diabetic retinopathy, and various inflammatory diseases, are often associated with uncontrolled angiogenesis. In particular, a growing tumor needs an extensive network of capillaries to provide nutrients and oxygen. In addition, these new blood vessels provide a way for tumor cells to enter into the circulation and to metastasize to another organ. Therefore, substances that inhibit angiogenesis have been expected to be promising therapeutic agents for the treatment of cancer.1

Tumor angiogenesis involves several processes including digestion of the extracellular matrix by metalloproteinases, endothelial proliferation, migration, and tube formation. Each of these processes is tightly controlled by angiogenic factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). Today, several anti-angiogenic agents targeting the VEGF signal transduction pathway, such as bevacizumab (a neutralizing antibody to VEGF), sorafenib, and sunitinib (small molecule inhibitors of VEGF receptor tyrosine kinases), have been approved for cancer chemotherapy, and many other inhibitors are in clinical trial.2 However, new lead compounds having novel modes of action are still needed because of the narrow adaptation range, problems of side effects, drug resistance, and so on, in existing therapies.3,4

Based on the background described above, the search for anti-angiogenic substances from natural sources, such as phytochemicals or marine natural products, are being conducted worldwide.5,6 We have focused on the evidence that endothelial cells are involved in all processes of angiogenesis. Thus, selective anti-proliferative substances against endothelial cells are promising as angiogenic inhibitors with various modes of action, targeting not only VEGF but also other unknown molecules/pathways.7,8 In the course of our study on bioactive substances from marine organisms, we focused on the search for novel anti-angiogenic substances by cell-based assay using human endothelial cells. In this paper, our studies concerning the search for selective anti-proliferative substances against human umbilical vein endothelial cells (HUVECs) from natural sources, together with mechanistic/synthetic studies of the active compounds, are reviewed.

2. Anti-angiogenic Substances from Natural Sources

2.1. Bastadins from the Marine Sponge Ianthella basta
Bastadins are cyclic or acyclic tetramers of brominated-tyrosine derivatives possessing oxime moieties as a structural feature.9–23 More than twenty bastadins have been identified so far from marine sponges of Ianthella sp., Psammaphyllis sp., and Dendrilla sp., and they have been known to exhibit some interesting biological activities, such as antibacterial23 and cytoxic26–13 activities, enzyme inhibitory activities against inositol-5’-phosphate dehydrogenase,24 topoisomerase II and dihydrofolate reductase,21 selective inhibitory activity toward δ-opioid receptors,13 and interaction with the intracellular ryanodine receptor-1 (RyR-1) calcium channel complex.15,16

Based on a growth inhibitory assay using HUVECs and KB3-1 cells, we have identified bastadins as selective anti-proliferative substances from the Indonesian marine sponge of Ianthella basta (Fig. 1). Bastadin 6 (I), a major constituent, exhibited selective growth inhibitory activity against HUVECs (IC50: 0.052 µM) through the induction of apoptosis, whereas it showed weak growth inhibition against fibroblasts (3Y1) and tumor cells (KB3-1, K562, Neuro2A), with selectivity index of 17–106-fold. Bastadin 6 (I) also inhibited in vitro VEGF-induced migration and bFGF-induced tube formation of HUVECs. Moreover, compound 1 was found to be effective in vivo. Thus, intraperitoneally administered compound 1 almost completely blocked VEGF and bFGF-induced neovascularization in an in vivo mouse corneal assay, and showed anti-tumor effects against nude mice subcutaneously inoculated with human epidermoid carcinoma A431 cells.22

Further examination of the sponge extract provided seven more bastadins (2–8, Fig. 1) as active constituents. Structure–activity relationship (SAR) study revealed that bastadins having a bastarane skeleton (2–5) exhibited similarly potent anti-proliferative activity against HUVECs as that of bastadin 6 (I). On the other hand, isobastarane-type congeners (bastadins 13 (6) and 19 (7)) showed moderate activity, and bastadin...
3 (8), having an acyclic structure, showed only weak activity, with lower selectivity. These results indicated that the bastarane skeleton is an important structural feature for exhibiting potent and selective anti-angiogenic effect of bastadins.23)

To analyze the cause of these differences, a conformational search of bastadins using molecular mechanics calculations was analyzed. We found that bastarane-type bastadins (such as 1 or 3) have moderately rigid conformation, whereas bastadins with an isobastarane skeleton (6 or 7) have relatively flexible conformation. This implies that the conformational properties might be related to differences in bioactivity between bastarane and isobastarane. A similar modeling study was performed by Crews, in which several distinct conformations were proposed to exist in isobastarane-type compounds, whereas the bastarane macrocycle maintained a similar overall shape.24)

For a sustainable supply of the compound, and from the viewpoint of medicinal chemistry, we developed a practical synthetic method for the preparation of bastadin 6 (I) (Fig. 2). The key reaction in our synthetic route is the novel oxidative coupling of the \( o_o \)-dibromophenol derivative (9 and 11), mediated by Ce(NH\(_4\))\(_2\)(NO\(_3\))\(_6\), followed by condensation and subsequent macrocycle formation of the resulting two segments, 10 and 12, to give bastadin 6 (I) in 23% overall yield.25)

Utilizing the above synthetic method, the preparation of some analogue compounds of bastadin 6 (1) and their SAR study was also executed (Fig. 2). This revealed that an oxime moiety was essential for the potent anti-proliferative activity of 1 against HUVECs, because protection or deletion of the oxime moiety caused almost a complete loss of the growth inhibitory activity. A reduction in the number of bromine atoms also resulted in diminished activity and selectivity. Similar results were obtained by the substitution of two bromine atoms in neighboring phenolic hydroxyl groups to an amine or guanidine group.26)

2.2. Globostellatic Acid X Methyl Esters from the Marine Sponge Rhabdastrella globostellata From the Indonesian marine sponge Rhabdastrella globostellata, novel isomalabaricane-type triterpenes with characteristic conjugated polyene side chains, named globostellatic acid X methyl esters (13–16, Fig. 3), were isolated as HUVEC-selective growth inhibitory substances.27) Isolation of similar isomalabaricane-type triterpenes from marine sponges of Rhabdastrella sp., Jaspiris sp., Stelletta sp. and Geodia sp. have been reported mainly as cytotoxic compounds.28) Compounds 13–16 showed selective anti-proliferative activity against HUVECs, with IC\(_{50}\) values of 0.06–0.4 \( \mu \)M. Among them, compounds 15 and 16, having 13E-geometry, showed higher HUVEC-selectivity (80 to 250-fold) in comparison with several cancer cell lines. Compound 16 inhibited VEGF-induced migration and bFGF-induced tubular formation of HUVECs. Mechanistic studies revealed that compound 16 inhibited the growth of HUVECs through inducing apoptosis, whereas it did not affect the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 of HUVECs.

We also isolated some congeners having an oxidized side chain (17–20, Fig. 3), and clarified that those compounds exhibited weaker anti-proliferative activity toward HUVECs, as well as lower selectivity. Furthermore, compound 21, having a saturated side chain, and obtained from compound 16 by hydrogenation, completely lost its anti-proliferative activity (Fig. 4). These results indicated that the unfunctionalized 13E-penta-ene side chain might be the crucial moiety for exhibiting potent and selective anti-proliferative activity against HUVECs. In addition, the deacetylation product (compound 22) resulted in diminished activity and selectivity.

We also synthesized some structurally simplified analogues of 16, based on the SAR described above. It revealed that the model compound 23, having a BC-ring structure and conjugated penta-ene side chain, showed moderate anti-proliferative activity against HUVECs (IC\(_{50}\): 2.6 \( \mu \)M), with 6.5-fold selectivity over KB 3-1 cells (IC\(_{50}\): 17 \( \mu \)M)29) (Fig. 4).
2.3. Cortistatins from the Marine Sponge Corticium simplex

A family of alkaloids named cortistatins A (24) to D (27), with a highly oxidized and rearranged steroidal skeleton, was identified from the Indonesian marine sponge Corticium simplex as HUVEC-selective anti-proliferative substances (30) (Fig. 5). Detailed NMR analyses revealed the stereostructure of cortistatin A (24), consisting of a 9(10–19)-abeo-androstane skeleton with an isoquinoline appendage, and X-ray crystallographic and circular dichroism (CD) analyses (31) confirmed the absolute stereostructure of 24. We found that cortistatin A (24), a major constituent, showed cytostatic anti-proliferative activity against HUVECs at the concentration range of 100 pM to 1 µM, while remarkably high selectivity was observed over some other cell lines, including tumor cells and normal fibroblast cells, with selective index of more than 3000-fold. Cortistatin A (24), at 2 nM concentration, also inhibited the migration and tubular formation of HUVECs induced by VEGF or bFGF, respectively. Therefore, cortistatins might have considerable potential as novel anti-angiogenic drug leads.

In order to elucidate the pharmacophore of cortistatins, we further examined the extract of the same marine sponge, which resulted in the isolation of seven more derivatives named cortistatins E (28) to L (34) (Fig. 5). Cortistatins E (28), F (29), G (30), and H (31) were clarified to possess N-methyl piperidine or 3-methylpyridine units in the side chain. These four congeners showed only weak anti-proliferative activity (IC50: 0.35–1.9 µM) against HUVECs with no selectivity between HUVECs and other cell lines. (32) On the other hand,
cortistatins J (32), K (33), and L (34), having an isoquinoline unit, were found to show selective anti-proliferative activity against HUVECs at 8–40 nM, respectively, in which the selective index were 60–9500-fold in comparison with those of some other cell lines. These data suggested that the isoquinoline unit in the side chain was essential for the anti-proliferative activity of cortistatins against HUVECs. Among these, cortistatin J (32), having a triene system, showed stronger activity with higher selectivity than either cortistatin K (33), having 1(10),9(19)-diene system, or cortistatin L (34), having a 2β-hydroxyl-1(10),9(19)-diene system. These data indicated that the position of the diene system is more important for the selectivity against HUVECs than the presence of the hydroxyl group(s) in the ring A, although additional investigation is needed to elucidate the relationship between the functional groups on ring A and the anti-proliferative activity.33) Previously, several abeo-9(10–19)-type steroidal alkaloids were isolated from the marine sponge Corticium sp.34) Oxidation at C-19 and subsequent rearrangement might occur to give the abeo-9(10–19)-type skeleton, supported by the isolation of 19-acetoxylated alkaloid plakinamine I.35) Furthermore, stigmastane-type steroidal alkaloids having an N-methyl piperidine unit, together with an N-methyl pyrrolidine unit in the side chain, have also been isolated.36,37)

Proliferation and migration of HUVECs requires the activation of ERK1/2 mitogen-activated protein kinase (MAPK) and p38 MAPK pathways induced by a growth factor, such as VEGF and bFGF. Thus, the effect of cortistatin A (24) on the phosphorylation of ERK1/2 and p38 was examined using the Western blotting method. Although the proliferation of HUVECs was prevented 50% by treatment with 0.01–1.0 µM concentration of 24, the phosphorylation levels of ERK1/2 and p38 stimulated by VEGF were not affected by the treatment of 24. These results suggest that cortistatin A (24) inhibits the proliferation and migration of HUVECs without inhibiting the phosphorylation of ERK1/2 and p38.38)

Nicolau and colleagues reported that some kinases, such as Rho-associated, coiled-coil containing protein kinase (ROCK), cyclin-dependent kinase 8 (CDK8), and cyclin-dependent kinase 11 (CDK11), may be targets of cortistatin A (24).39) On the other hand, other studies have suggested that CDK8 and 11 were not related to the anti-angiogenic activity of 24.40,41) Target identification studies of cortistatin A (24) by our group are ongoing.

The unique structure and characteristic biological properties of cortistatin A (24) have attracted synthetic chemists from all over the world, and a number of synthetic reports, including six total syntheses, have appeared.42–50) We also reported the synthetic study of 24, in which the 7-endo-selective intramolecular Heck reaction and oxy-Michael reaction were used for elaborating the B-ring core structure of 24.51–53) On the other hand, only small quantities of the final compound could be obtained in most cases because the many reaction steps were needed for the total synthesis. In addition, there has been no

![Fig. 4. Chemical Derivatization Products (21, 22) of 13E,17E-Globostellatic Acid X Methyl Ester and Synthetic Model Compound (23)](image)

![Fig. 5. Chemical Structures of Cortistatins (24–34)](image)
report on the in vivo anti-tumor effect of cortistatins. Therefore, we decided to engage in the synthetic study of structurally simplified and in vivo-active analogues of cortistatins.\textsuperscript{54–56}) Considering the above SAR of naturally occurring congeners and the 3-D structure of 24, analogue compounds having a similar CD-ring and a simplified AB-ring were designed and synthesized. Among those prepared, compound 35 (Fig. 6), in which the isoquinoline moiety was appended to the planar tetracyclic core structure, showed potent anti-proliferative activity against HUVECs (IC\textsubscript{50}: 0.035 \(\mu\)M), together with high selectivity (100–300-fold) over some other cell lines. Furthermore, the analogue 35 also showed in vivo anti-angiogenic activity and significant anti-tumor effect by oral administration.\textsuperscript{57,58})

2.4. Pyripyropenes from a Marine-Derived Fungus of Aspergillus sp. Pyripyropenes, structurally classified as meroterpenoids, were originally isolated from the culture broth of Aspergillus fumigatus as potent and selective inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT), on the guidance of an enzyme assay using rat liver microsomes.\textsuperscript{59,60}) ACAT is now considered a therapeutic target of atherosclerosis and hyperlipidemia, and extensive SAR studies of the synthetic derivatives of pyripyropenes have been conducted.\textsuperscript{61–63}) We have re-discovered pyripyropenes A (36), B (37), and D (38) as selective anti-proliferative agents against HUVECs from a marine-derived fungus of Aspergillus sp. GF5 (Fig. 7). Compounds 36, 37, and 38 exhibited anti-proliferative activity against HUVECs with IC\textsubscript{50} values of 1.8, 0.1, and 1.8 \(\mu\)M, respectively, whereas no effect was observed against KB3-1 cells up to 100 \(\mu\)M concentration.\textsuperscript{64}) Pyripyropene A (36) inhibited the VEGF-induced migration and tubular formation of HUVECs in a dose-dependent manner up to 10 \(\mu\)M concentration, while no inhibition against the VEGF-induced auto-phosphoryration of ERK1/2, p38, or Akt was observed with 10 \(\mu\)M treatment of 36. These results indicate that pyripyropene A (36) inhibits the proliferation and migration of HUVECs with-

Fig. 6. Superimposed View of Cortistatin A (24) and Synthetic Analogue (35)

Fig. 7. Chemical Structures of Pyripyropenes (36–38)

Fig. 8. Chemical Structures of Senegasaponins (39–43) and Their Degradation Products (44, 45)
out inhibition of these signal transduction pathways.

2.5. Senegasaponins from Senega Radix Senega Radix, the root of *Polygala senega*, has been used clinically as an expectorant. Several saponin constituents from *Polygala senega* have been found to exhibit inhibitory activity of ethanol absorption, hypoglycemic activity, and immunological adjuvant activity, while the HUVEC-selective growth inhibitory activity of senegasaponins has not yet been reported. In our screening study from medicinal plant resources, we isolated triterpenoidal saponins [senegin II (39), senegin III (40), senegin IV (41), senegasaponin a (42), and senegasaponin b (43)] as selective anti-proliferative substances against HUVECs. These saponins exhibited anti-proliferative activity against HUVECs with IC$_{50}$ values of 5.9, 1.1, 6.2, 0.92, and 0.59 µM, respectively. The selective index was 7–100-fold in comparison with those for several cancer cell lines. Subsequent chemical modification study revealed that desacetyl mixture of senegasaponins (44) and tenuifolin (45) exhibited no anti-proliferative activity against either HUVECs or tumor cells, indicating that the 28-O-glycoside moiety and methoxycinnamoyl group were essential for HUVEC-selective growth inhibition. Senegin III (40) inhibited VEGF-induced *in vitro* tubular formation of HUVECs and bFGF-induced *in vivo* neovascularization in the mouse Matrigel plug assay. Moreover, intraperitoneally administered senegen III (40) (2.5 mg/kg dose) suppressed tumor growth in the ddY mice subcutaneously (s.c.)-inoculated with murine sarcoma S180 cells, without side effects such as weight loss or diarrhea. The combined effect with cisplatin was also observed.

Analysis of the action mechanism of senegin III (40) disclosed that 40 (0.1–10 µM) showed no inhibitory effect against the VEGF-stimulated auto-phosphorylation of ERK1/2, p38, or Akt, suggesting that the target of 40 would not be related to the VEGF pathway. Next, we examined the effect of senegin III (40) on the induction of pigment epithelium-derived factor (PEDF), which was the reported target molecule of ginsenoside Rb1, a major active constituent of ginseng saponin. Each group exhibited its own characteristic pharmacophore. This evidence indicates that our screening system has potential to explore promising anti-angiogenic substances with various modes of action, targeting not only VEGF but also unknown molecules/pathways. Further mechanical study is now under way.

**Acknowledgments** This study was financially supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Uehara Memorial Foundation, the Tokyo Biochemical Research Foundation, and Adaptable and Seamless Technology Transfer Program through Target-Driven R&D (A-STEP, Exploratory Research) from the Japan Science and Technology Agency.

**Conflict of Interest** The authors declare no conflict of interest.

**References**

1. Carmeliet P., Jain R. K., *Nature* (London), 407, 249–257 (2000).
2. Musumeci F., Radi M., Bruullo C., Schenone S., *J. Med. Chem.*, 55, 10797–10822 (2012).
3. Carmeliet P., Jain R. K., *Nature* (London), 473, 298–307 (2011).
4. Sennino B., McDonald D. M., *Nat. Rev. Cancer*, 12, 699–709 (2012).
5. Eggert U. S., *Nat. Chem. Biol.*, 9, 206–209 (2013).
6. Swinney D. C., Anthony J., *Nat. Rev. Drug Discov.*, 10, 507–519 (2011).
7. Kadioglu O., Geo E. J., Efferth T., *Med. Aromat. Plants*, 2, 134 (2013).
8. Wang Y.-Q., Miao Z.-H., *Mar. Drugs*, 11, 903–933 (2013).
9. Kazlauskas R., Lindgard R. O., Murphy P. T., Wells R. J., Blount J. F., *Aust. J. Chem.*, 34, 765–786 (1981).
10. Pordesimo E. O., Schmitz F. J., *J. Org. Chem.*, 55, 4704–4709 (1990).
11. Miao S., Andersen R. J., Allen T. M., *J. Nat. Prod.*, 53, 1441–1446 (1990).
12. Carney J. R., Scheuier P. J., Kelly-Borges M., *J. Nat. Prod.*, 56, 153–157 (1993).
13. Reddy A. V., Kavinder K., Narasimhulu M., Sridevi A., Satyana- rayana N., Konadip A. K., Venkateswarlu Y., *Bioorg. Med. Chem. Lett.*, 14, 4452–4457 (2006).
14. Jauparas M., Rali T., Laney M., Schatzman R. C., Diaz M. C., Schmitz F. J., Pordesimo E. O., Crews P., *Tetrahedron*, 50, 7367–7374 (1994).
15. Carroll A. R., Kaiser S. M., Davis R. A., Moni R. W., Hooper J. N., Quinn R. J., *J. Nat. Prod.*, 73, 1173–1176 (2010).
16. Mack M. M., Molinski T. F., Buck E. D., Pessah I. N., *J. Biol. Chem.*, 269, 23236–23249 (1994).
17. Franklin M. A., Penn S. G., Lebrilla C. B., Lam T. H., Pessah I. N., *Molinski T. F., J. Nat. Prod.*, 59, 1121–1127 (1996).
18. Butler M. S., Lim T. K., Capon R. J., Hammond L. S., *Aust. J. Chem.*, 44, 287–296 (1991).
19. Dexter A. F., Garson M. J., Hemling M. E., *Aust. J. Chem.*, 56, 782–786 (1993).
20. Park S.-K., Jurek J., Carney J. R., Scheuier P. J., *J. Nat. Prod.*, 57, 407–410 (1994).
21. Coll J. C., Kearns P. S., Rideout J. A., Sankar V., *J. Nat. Prod.*, 65, 753–756 (2002).
22. Aoki S., Cho S., Ono M., Kuwano T., Nakao S., Kuwano M., Nakagawa S., Gao J.-Q., Mayumi T., Shibuya M., Kobayashi M., *Anticancer Drugs*, 17, 269–278 (2006).
23. Aoki S., Cho S., Hiramatsu A., Kotoku N., Kobayashi M., *J. Nat. Med.*, 60, 231–235 (2006).
24. Inman W. D., Crews P., *J. Nat. Prod.*, 74, 402–410 (2011).
25. Kotoku N., Tsujita H., Hiramatsu A., Mori C., Koizumi N., Kobayashi M., *Tetrahedron*, 61, 7211–7218 (2005).
