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HIGHLIGHT

Marine natural products that interfere with multiple cytoskeletal protein interactions

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Various marine natural products that target cytoskeletal proteins have been discovered. A few of these compounds have recently been shown to induce or inhibit the protein–protein interactions. Lobophorolide, an actin filament-disrupting macrolide, binds to actin with a unique 2:2 stoichiometry in which two lobophorolide molecules cooperate to stabilize an actin dimer. Adociasulfates, merotriterpenoid derivatives, inhibit microtubule-stimulated ATPase activity of a motor protein kinesin by blocking both the binding of microtubules and the processive motion of kinesin along microtubules. The antitumor macrolide aplyronine A synergistically binds to tubulin in association with actin, and prevents spindle formation and mitosis. In this highlight, we address recent chemical biology studies on these mechanistically-attractive marine natural products. These findings may be useful for the design and development of new pharmacological tools and therapeutic agents.

1 Introduction

The modulation of protein–protein interactions (PPI) with small molecules has become an important issue in the fields of basic research and drug discovery.[1] Protein–protein interactions can either be stabilized or disrupted to achieve significant physiological effects. For example, the immunosuppressive macrolides FK506 and rapamycin, approved as Prograf® and Rapamune®, respectively, are well-known PPI inducers that are used in organ transplantation.[2] While they bind to the same cellular target, the immunophilin FKBP12, their second targets are different enzymes (the phosphatase calcineurin and the kinase FRAP, respectively) to regulate a variety of cellular signaling pathways. Due to the high diversity of protein complex interfaces, PPI inhibitors are also expected to show higher specificity compared to inhibitors that target conserved active sites of enzymes.[3] Therefore, the manipulation of PPI is a promising approach for therapeutic intervention. The discovery and design of PPI inhibitors and stabilizers have recently been reviewed.[4]

The cytoskeleton of eukaryotes has three major components: microfilaments (actin filaments), microtubules, and intermediate filaments. These higher-order structures form a network of fibers through the association of monomeric proteins (actin, tubulin, keratin, vimentin, etc.), and thus the stabilization or inhibition of the PPIs of such cytoskeletal proteins directly leads to the regulation of cytoskeletal dynamics. Microtubules consist of polymerized α- and β-tubulin heterodimers, and play important roles in cell proliferation, trafficking, signaling, and migration.[5] Several tubulin-targeting natural products, which interfere with microtubule dynamics, have been discovered and are widely used in cancer chemotherapy.[6,7] As the representative antitumor drugs, taxols and epothilones increase the formation of microtubules by stabilizing the interaction between tubulin α/β-heterodimers. On the other hand, the vinca alkaloids vincristine and vinblastine destabilize microtubule formation by disrupting tubulin oligomerization. With regard to marine natural products, new non-taxane microtubule dynamics inhibitor, eribulin mesylate (Halaven®), binds to the plus end of microtubules and blocks the growth phase of microtubule dynamics, leading to irreversible mitotic arrest and cell death by apoptosis.[8,9] Eribulin is a macrocyclic ketone derivative of halichondrin B, and is the most structurally complex, non-peptidic drug. Another promising compound is dolastatin 10, a peptide obtained from the Indian Ocean sea hare *Dolabella* sp., which has been shown to have unprecedented potency in antineoplastic and tubulin assembly systems.[10] Brentuximab vedotin (Adcetris®), the mAb-targeted dolastatin 10 analog, has been approved for Hodgkin's lymphoma and systemic anaplastic large cell lymphoma.[11]

As mentioned above, several structurally and functionally diverse secondary metabolites that target cytoskeletal proteins have been discovered in the marine environment. A few of these compounds have recently been shown to regulate PPIs in hitherto unprecedented ways. In this highlight, we focus on chemical biology studies of several marine natural products that interfere with "multiple" cytoskeletal protein–protein interactions.

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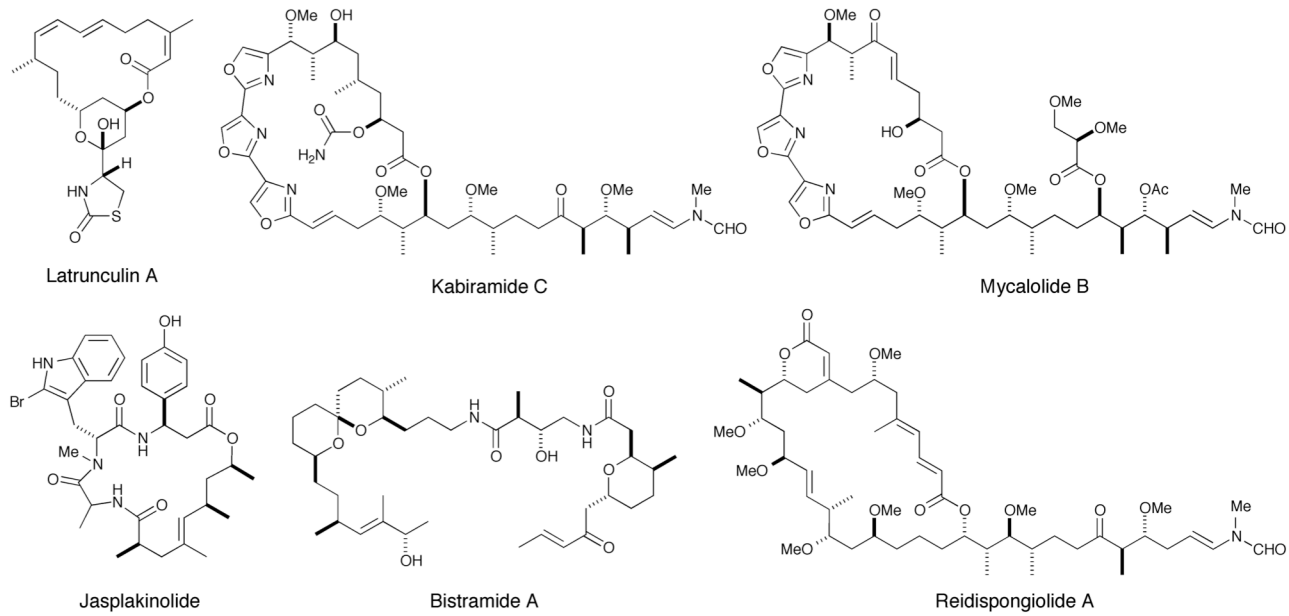

Fig. 1 Structures of actin-targeting marine natural products.

2 Lobophorolide and related actin-binding dimeric/monomeric macrolides

Actin is the most abundant protein in the eukaryotic cytoskeleton and is essential for the regulation of various cellular functions, such as muscle contraction, cell division, and the migration of tumor cells.[12] The dynamics of actin assembly (polymerization/depolymerization) are regulated by numerous actin-binding proteins. For example, thymosin β4 and profilin bind to monomeric actin and physically inhibit its addition to growing filaments, while CapZ binds to filament ends and inhibits both monomer addition and dissociation.

Along with endogenous actin-binding proteins, various small agents that target actin have been discovered, and some show potent cytotoxicity (Fig. 1).[13–15] In the 1980s, the Kashman group identified latrunculins and investigated their biological properties: latrunculins were the first actin-binding substances to be isolated from marine origin.[16] To date, various actin-depolymerizing agents have been found in marine invertebrates. For example, ulapualides,[17] mycalolides,[18] kabiramides,[19] sphinxolides/reidispongiolides,[20] and bistramides,[21,22] show potent cytotoxicity by disrupting actin polymerization dynamics. In addition, various actin-polymerization-stimulating or -blocking molecules have been shown to induce apoptosis at sub-μM concentrations; examples include jasplakinolide,[23] latrunculin A,[24] and mycalolide B.[25] Thus, these agents are not only useful for investigating actin dynamics in cells, but also may be of therapeutic value as antitumor compounds. A great deal of attention has been paid to the synthesis and function of actin-targeting compounds and their use as effective molecular probes in the field of chemical biology.

Masaki Kita received his Ph.D. in 2005 from Nagoya University under the direction of Professor Daisuke Uemura. He became Assistant Professor at Nagoya University in 2001, and moved to University of Tsukuba in 2007, and was promoted to Associate Professor in 2011. He was honored with Young Scientist's Research Award in Natural Products Chemistry in 2006, the Incentive Award in Symposium on Chemistry of Natural Products in 2010, and the Chemical Society of Japan Award for Young Chemists in 2011. His research is directed at the diverse natural products that regulate biologically and physiologically intriguing phenomena on the basis of chemical biology.

Hideo Kigoshi completed his doctoral work in 1989 under the direction of Professor Kiyoyuki Yamada at Nagoya University. He was a postdoctoral fellow with Professor Elias J. Corey (Harvard University) in 1990–1991. He became Assistant Professor at Nagoya University in 1984 and was promoted to Associate Professor in 1994. He moved to University of Tsukuba in 2000 as Professor of Chemistry. His research interests lie in the field of chemistry and chemical biology of the bioactive natural products. He received the Chemical Society of Japan Award for Young Chemists in 1993.

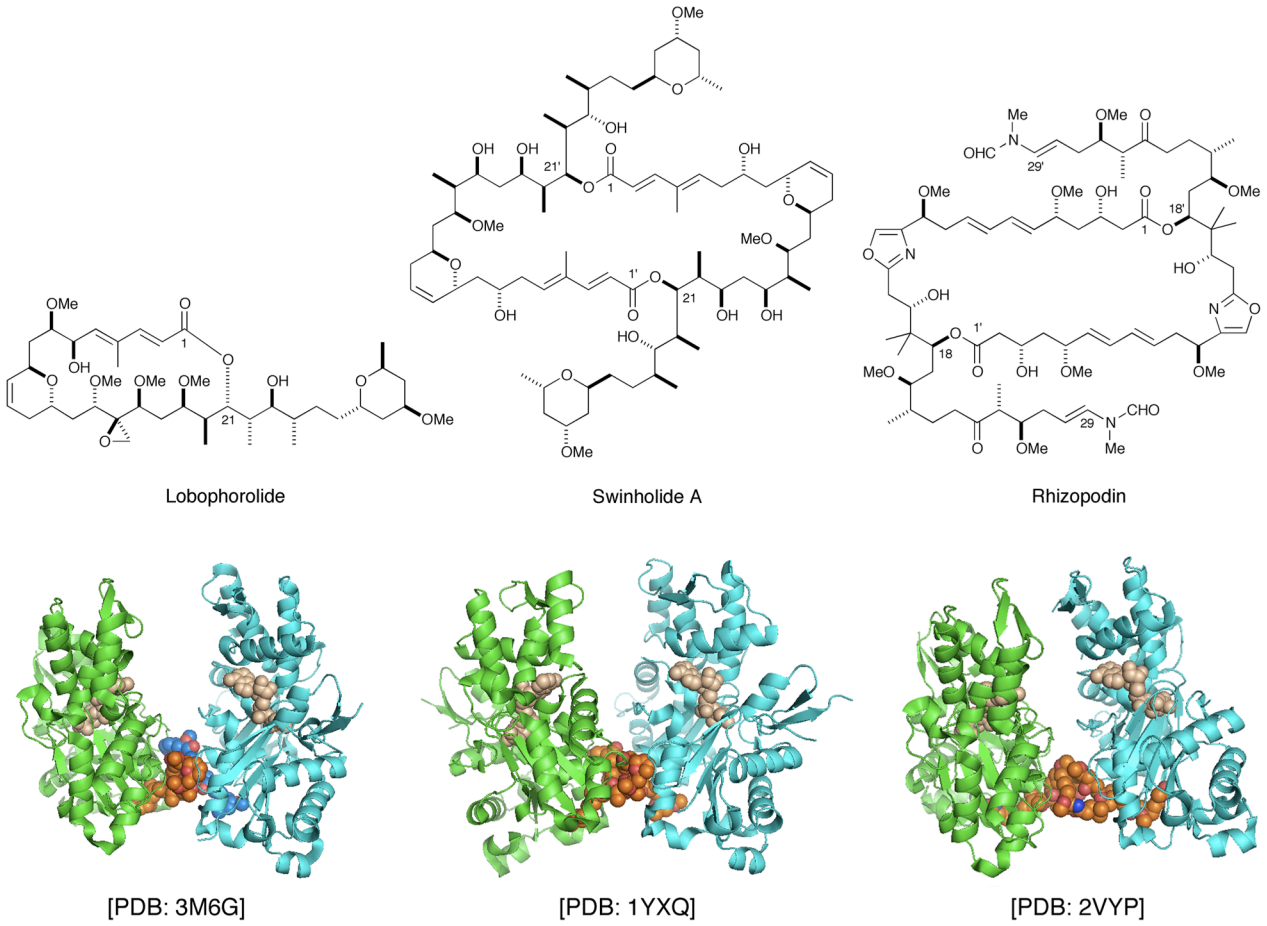

Fig. 2 Structures of lobophorolide, swinholide A, and rhizopodin (top) and their actin complexes (bottom). Two lobopholide molecules are shown in orange and blue CPK models, and their bound actin subunits are shown in green and cyan cartoon representations. Similarly, dimeric lactones (swinholide A and rhizopodin) and ATP molecules in the actin complexes are shown in orange and wheat CPK models, respectively.

Several actin-binding macrolides have similar structural features: a highly variable 24- to 26-membered macrocyclic ring; a linear aliphatic side-chain (tail) part; and an *N*-methyl enamide moiety. These compounds bind to the barbed end of actin to form a 1:1 complex, which inhibits further longitudinal interactions between adjacent actin filament subunits. As a result, sequestration of globular actin (G-actin) and severing of filamentous actin (F-actin) occurs. In 2003, an X-ray analysis of the actin–kabiramide C complex revealed that this macrolide intercalates into the hydrophobic cleft between subdomains (SD) 1 and 3 of actin by using its tail part.[26] Since then, 1:1 complexes of actin with other agents, such as aplyronine A,[27] sphinxolide B,[28] reidispongiolides A and C,[28] swinholide A,[29] and bistramide A,[30] have been reported, and these show similar contacts between actin and macrolides.[15] These results suggest that their tail parts are important for their high affinity toward actin as well as their cytotoxic functionality.

Swinholide A is a 44-membered, C_2-symmetric, dimeric lactone from the marine sponge *Theonella swinhoei* (Fig. 2).[31] Due to the presence of two tetrahydropyran (THP) ring-terminated side chains, swinholide forms an actin-macrolide complex with 2:1 stoichiometry in which each side chain binds to the SD 1 and 3 hydrophobic clefts of two different actin molecules.[29,32] A 40-membered bis-lactone congener of swinholide A, named bistheonellide A (or misakinolide A), was identified from the same sponge species.[33] Both swinholide A and bistheonellide A intervene between two actin molecules, form a tertiary complex with each of its side chains bound to G-actin, and inhibit polymerization by sequestering G-actin from incorporation into F-actin.[34]

Along with swinholides, a 38-membered bis-lactone, rhizopodin, isolated from the myxobacterium *Myxococcus stipitatus*, is a bivalent inhibitor that forms ternary complexes with actin.[35] Rhizopodin bears two oxazole rings and side chains that terminate in *N*-methyl enamide groups. Its revised structure and absolute ~~chemistry~~ configuration were established by NMR and molecular modeling studies [36,37] as well as the X-ray crystallographic analysis of an actin–rhizopodin complex.[38] A C_2-symmetric rhizopodin dilactone bears two enamide side chains, each of which binds to a single G-actin molecule, resulting in a ternary 2:1 complex. The binding position of rhizopodin in actin and the conformation of its enamide part are similar to those of related actin-depolymerizing monomeric macrolides, such as kabiramide C, reidispongiolide A, and sphinxolide B.

On the other hand, in 2003, a cytotoxic and antifungal marine macrolide, lobophorolide, was isolated from the brown alga *Lobophora variegate*, with sub-μM activity against several

pathogenic and saprophytic marine fungi.[39] While the natural concentration of lobophorolide is low (ca. 1.2×10^{-4}% of plant dry mass), this molecule is considered to account for most of the antifungal activity of *L. variegate.* Lobophorolide bears a 22-membered macrolactone ring with a THP ring-terminated side chain. Interestingly, its polyketide backbone is half of swinholide A. The structural and biological similarity between lobophorolide and swinholide suggested that one lobophorolide molecule must bind to the barbed end of G-actin, as with other actin-depolymerizing macrolides.

In 2010, however, the structure of lobophorolide bound to actin was determined at 2.0 Å resolution by X-ray analysis; lobophorolide binds to actin with a unique 2:2 stoichiometry in which two lobophorolide molecules cooperate to stabilize an actin dimer.[40] Each macrocyclic ring part forms a dimerization interface with actin molecules, and the two actin molecules themselves do not directly interact with each other in the 2:2 complex. While the twist angles of actin subunits are slightly different, the quaternary actin–lobophorolide complex is remarkably similar to the 2:1 actin–swinholide A and actin–rhizopodin complexes. Allingham et al. proposed that structural congeners of lobophorolide, such as scytophycins and tolytoxin, might also interact with actin with similar 2:2 stoichiometry systems.[40] This finding highlights that some actin-binding monomeric lactones can sequester the actin dimer from the actin network, which potently inhibits the further nuclearization and polymerization of actin to regulate microfilament dynamics.

3 Adociasulfates: Motor protein ATPase modulators

Eukaryotic cells depend on actin and microtubule-mediated events that are regulated by motor proteins. The cytoskeletal motor protein myosin moves along microfilaments by interacting with actin, while dynein and kinesin are representative microtubule motor proteins. Members of the kinesin superfamily of motor proteins are vital to several eukaryotic cellular processes, such as vesicle transport and mitosis.[41,42] The general kinesin structure includes two heavy chains with motor heads (ca. 320 amino acids) that hydrolyze ATP and step along microtubules. Kinesin also has a coiled-coil region to facilitate dimerization and a tail domain that attaches to cellular cargo for transport.

Inhibitors of kinesin have been expected to be valuable as probes for exploring cell physiology and as potential therapeutics. In 1998, a sulfated triterpenoid hydroquinone compound, adociasulfate-2 (AS-2), isolated from the marine sponge *Haliclona* (also known as *Adocia*) sp., was shown to be a unique kinesin inhibitor by competing with microtubules for binding (Fig. 3).[43] AS-2 binds to kinesin and interferes with microtubule binding with minor effects on nucleotide interactions.[44] Adociasulfates specifically inhibit members of the kinesin superfamily and H^+-ATPase proton pump enzymes, where their activity has been linked to the presence of at least one sulfate group.[45] In addition, AS-2 has little effect on microtubule polymerization. Preliminary *in vivo* experiments demonstrated that AS-2 had no effect on HeLa cell proliferation, while direct injection into early (syncytium stage) *Drosophila* embryos revealed that 65 μM of AS-2 caused striking defects in microtubule and chromosome organization followed by the arrest of nuclear division.[43] These results indicate how difficult it is for AS-2 to permeate membranes due to the presence of two charged sulfate moieties.

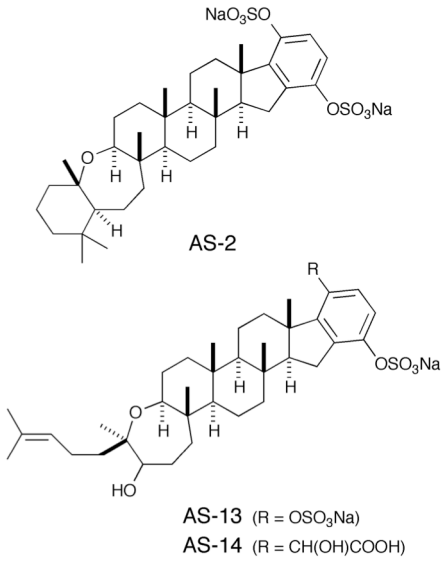

Fig. 3 Structures of adociasulfates.

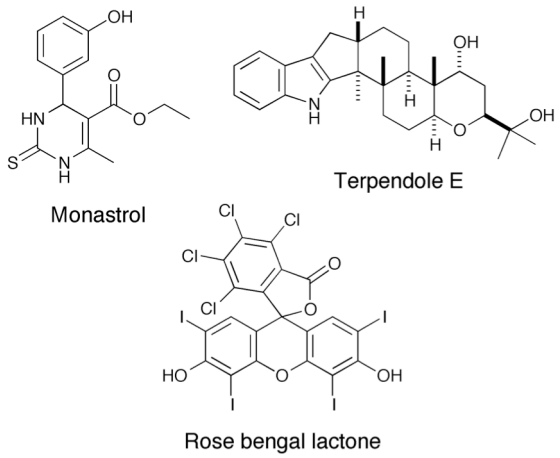

Fig. 4 Structures of kinesin inhibitors.

Since the discovery of AS-2, several small kinesin inhibitors have been identified. For example, monastrol [46] and terpendole E [47] have been shown to inhibit the ATPase activity of the mitotic kinesin Eg5 (also known as KIF11) allosterically, thus allowing ATP binding but preventing ADP release (Fig. 4). Meanwhile, adociasulfates are kinesin inhibitors with mechanisms of action that involve competition for binding to microtubules. Like AS-2, rose bengal lactone specifically inhibits the microtubule-stimulated ATPase activity of human kinesin.[48] This dye is competitive with microtubules, but not with ATP.

Monastrol and AS-2 have been used as chemical probes to clarify new structural conformations and to gain additional mechanistic insights into kinesin motor proteins. The crystal structure of the ADP–Eg5–monastrol ternary complex revealed that monastrol binds in an allosteric binding pocket and inhibits ADP release, leading to large conformational rearrangements of native Eg5.[49] Meanwhile, Brier and co-workers reported that formation of Eg5/AS-2 complex increased the solvent-

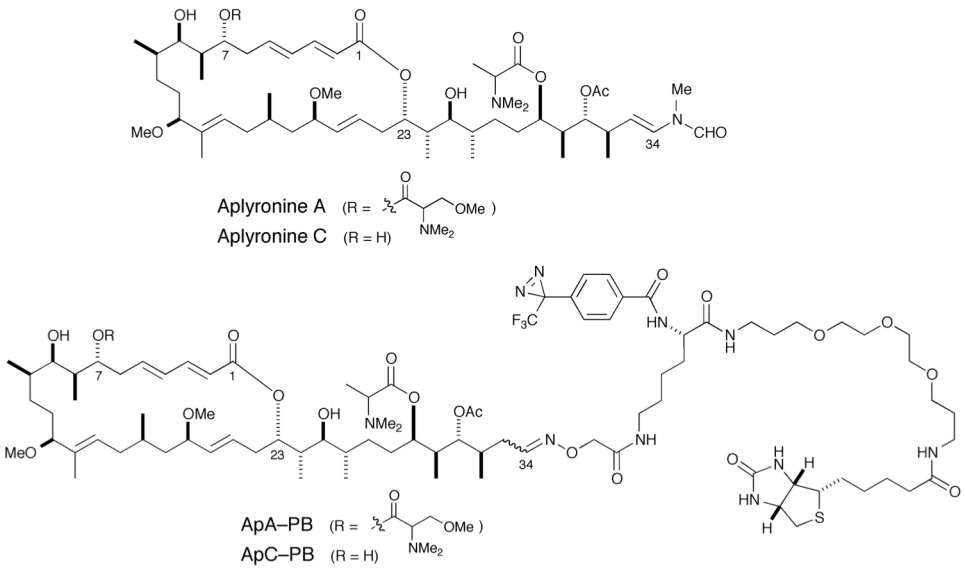

Fig. 5 Structures of aplyronins and their photoaffinity biotin probes.

accessible region located at the microtubule interface of Eg5, by measuring hydrogen/deuterium exchange with mass spectrometry.[44] These results indicated that AS-2 binds to the putative microtubule-binding site, which is the region opposite that for monastrol and known Eg5 inhibitors.

In addition, two adociasulfate derivatives, AS-13 and AS-14, were recently isolated from the sponge *Cladocroce aculeata*.[50] Through the use of single-molecule biophysical measurements (*in vitro* motility assay using kinesin heavy chain isoform 5A and binding microtubules), both adociasulfates were shown to inhibit the microtubule-stimulated ATPase activity of kinesin by blocking both the binding of microtubules and the processive motion of kinesin along microtubules. Kinesin was still inhibited when the 5′-sulfate in AS-13 was replaced by a glycolic acid moiety in AS-14. The negative charge of α-hydroxyacid may be responsible for the bioactivity of 5′-sulfate derivatives. Finally, adociasulfates might be valuable tools for studying the mechanism and function of motor proteins in the kinesin superfamily, and their use might lead to additional mechanistic insights into the regulation of microtubule dynamics.

4 Aplyronine A: a chemical probe approach and mechanism of action

Sea hares (family Aplysiidae) are a rich source of bioactive substances. Over the past 40 years, the genera *Aplysia* and *Dolabella* have afforded various bioactive secondary metabolites, including antitumor compounds.[51,52] Aplyronine A was originally isolated from the sea hare *A. kurodai* guided by a cytotoxicity against human carcinoma HeLa S3 cell line (Fig. 5).[53,54] Aplyronine A was revealed to be an inseparable mixture of four diastereomers with respect to two amino acid esters (ca. 1.1:1 and 3:1 for the *N*,*N*,*O*-trimethylserine and *N*,*N*-dimethylalanine ester moieties, respectively): this was confirmed by the asymmetric synthesis of aplyronine A as a diastereomeric mixture of amino acid esters with the same ratios as in the natural compound.[55] Aplyronines B and C, two minor congeners of aplyronine A, were also synthesized by the same group in 1996.[56] Recently, Paterson and co-workers accomplished a highly stereo-controlled total synthesis of aplyronine C.[57]

Aplyronines A–C exhibit cytotoxicity against HeLa S3 cells with the IC_{50} values of 0.48, 3.1, and 21 μg/mL, respectively.[58–60] Aplyronine A is much more cytotoxic than aplyronine C, which lacks the C7 trimethylserine ester moiety. Aplyronine A exhibits potent antitumor activity *in vivo* against several tumor cell lines, including P388 leukemia, Lewis lung carcinoma and Ehrlich carcinoma. Aplyronine A forms a 1:1 complex with G-actin, and inhibits the polymerization of G-actin to F-actin.[61] For aplyronine A to reveal strong cytotoxicity, the combination of the macrolide ring part and the side-chain part was found to be essential, since both the macrolactone analog (C1–C23) and the side-chain moiety (C21–C34) alone showed extremely weak cytotoxicity.[62,63] Along with a synthetic approach, interaction between the side-chain part of aplyronine A and actin has also been demonstrated by photo-affinity labeling experiments.[64] Thus, the side-chain part of aplyronine A is essential for both its cytotoxicity and actin-depolymerizing activity. On the other hand, its actin-depolymerizing activity is not influenced by the C7 trimethylserine ester, the conjugated diene and two hydroxyl groups.

As mentioned above, aplyronine A binds to a hydrophobic cleft between SD 1 and 3 of actin by intercalating its side-chain part.[27] Meanwhile, its positively-charged C7 trimethylserine moiety protrudes toward the bulk solvent region of the 1:1 complex. It was recently shown that aplyronine A significantly reduced cellular viability and induced DNA fragmentation at 1 nM in HeLa S3 and human leukemia HL60 cell lines.[25] In addition, treatment with aplyronine A at 1 nM led to potent caspase 3 activation in HeLa S3 cells, while treatment with aplyronine C did not, even at 100 nM.[65] These significant differences in apoptosis-related activities were consistent with their cytotoxicity, which highlighted the importance of the C7 trimethylserine moiety for the potent activities of aplyronine A. Overall, these various findings suggested that aplyronine A first binds to actin to form an actin–aplyronine A complex, which then binds to another biomolecule to exhibit potent cytotoxicity.

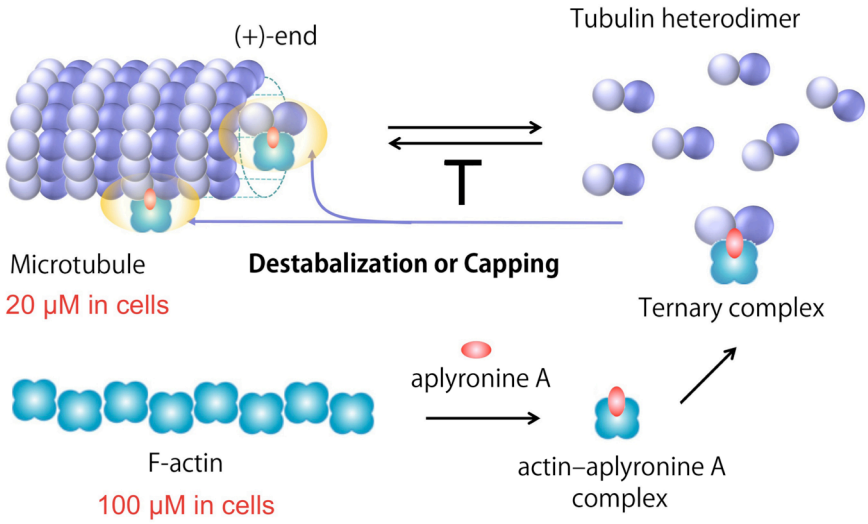

Fig. 6 Proposed inhibition mechanism of microtubule assembly by an actin–aplyronine A complex.

To identify the hypothetical secondary target molecules of aplyronine A and to further investigate its mode of action, chemical probes were prepared. Based on the finding that the C34 enamide moiety of aplyronine A can be replaced with hydrogen bond acceptors (i.e., imines and hydrazones) without a significant loss of activity,[65,66] acidic hydrolysis of the *N*-methyl enamide moiety of natural aplyronine A followed by condensation with an alkoxyamine linker afforded a photoaffinity biotin derivative (ApA–PB) and its aplyronine C analog (ApC–PB).[67] ApA–PB showed potent cytotoxicity against HeLa S3 cells (IC_{50} 1.2 nM) and inhibited actin polymerization, as with aplyronine A. In contrast, ApC–PB was ~260-fold less cytotoxic than ApA–PB, and we expected that ApC–PB could serve as a useful negative probe.

With the use of aplyronine probes, *in situ* photoreaction with ApA–PB in HeLa S3 cells and subsequent affinity purification using NeutrAvidin agarose were carried out. Notably, actin and several proteins (52–55 and 58 kDa) were detected as ApA-target proteins.[68] Blotting analysis with streptavidin-HRP conjugate and peptide mass fingerprinting revealed that β-tubulin was covalently bound to ApA–PB. In contrast, only actin was photolabeled to the ApC–PB control. Together, these results suggest that aplyronine A interacts with both actin and tubulin.

While actin and aplyronine A alone each had little effect on tubulin polymerization *in vitro*, their 1:1 complex delayed the nucleation and growth phases, and reduced the final polymer mass of tubulin.[68] Gel permeation HPLC analysis revealed the formation of a 1:1:1 complex of actin–aplyronine A–tubulin heterodimer (145 kDa). The binding constant of the actin–aplyronine A complex to tubulin heterodimer was estimated to be 3.0×10^6 M^{-1} on the basis of a Scatchard plot analysis. In contrast, the actin–aplyronine C complex did not attenuate microtubule growth or interact with tubulin. Immunostaining experiments showed that HeLa S3 cells treated with 100 pM aplyronine A had irregular, multipolar spindle structures with unaligned chromosomes. The same treatment inhibited cell-cycle progression in M-phase. In contrast, treatment with aplyronine C had no detectable effects on spindle formation, and inhibited cell-cycle progression in M-phase only at 100 nM. Therefore, the significant differences in cytotoxicity, mitosis inhibition, and apoptogenic effects of these two compounds are likely to be due to the tubulin-binding properties of aplyronine A.

How does aplyronine A inhibit spindle microtubule dynamics at the lowest effective concentration? Due to the high concentration of intracellular actin (ca. 100 μM) and tubulin (ca. 20 μM) and the high affinity of actin–aplyronine A for tubulin heterodimer, aplyronine A might first bind to cellular actin and then interact with liberated tubulin heterodimer in cytoplasm to form a ternary complex (Fig. 6). It has been shown that treatment with substoichiometric concentrations of vinblastine, colchicine, or other antimitotic agents does not depolymerize spindle microtubules, but rather blocks mitosis.[69] Similarly, low concentrations of paclitaxel also block mitosis by kinetically stabilizing spindle microtubules without changing the mass of polymerized microtubules.[70,71] It is possible that a few molecules of the actin–aplyronine A–tubulin ternary complex would bind at the microtubule plus end or copolymerize into the microtubule lattice to affect cytoskeleton dynamics.

Microtubule–actin interactions underlie many fundamental cellular processes, such as cell motility, neuronal pathfinding, cell division, and cortical flow.[72] A variety of proteins mediate microtubule–actin interactions and regulate their dynamics. Our recent study proposed that aplyronine A, the first microtubule inhibitor that also binds to actin and affects microfilament dynamics, modulates the coordination between microtubules and actin. Actin is one of the most abundant cytoplasmic proteins, and thus it is possible that a variety of actin-targeting agents interact with multiple cellular targets via PPIs. Our findings regarding aplyronine A should provide further insights into the molecular mechanisms of structurally diverse natural products that regulate cytoskeletal dynamics.

5 Outlook

We have focused on several marine natural products that target cytoskeletal proteins in unique fashions. Lobophorolide is the first example that stabilizes and sequesters an actin dimer with 2:2 stoichiometry systems, which is particularly noteworthy. To the best of our knowledge, adociasulfates and aplyronine A are the first examples to interfere with the microtubule/kinesin or

actin/tubulin interactions. The complexity and diversity of natural products might lead to multiple interactions with as-yet-unidentified intracellular biomolecules, such as motor proteins and signaling molecules. The use of PPI-stabilizing or -inhibiting scaffolds could have important implications for simplified mimetic design, and also provides new insight into ways to design molecules that tether proteins using different binding surfaces.

Due to the scarcity of available marine natural sources, there have been few exhaustive biological and physiological evaluations or *in vivo* preclinical trials that have examined antitumor effects. Thus, further precise and practical chemical syntheses of cytoskeletal protein-targeting compounds and the development of their derivatives are important, and should offer better perspectives for the design and development of new pharmacological tools and therapeutic agents.

6 Acknowledgements

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