報告番号	※　　第　　号

主　論　文　の　要　旨

論文題目　Mode of Action Studies on Antitumor Macrolide Aplyronine A and Development of Actin-affinity Tags
（抗腫瘍性マクロリド・アプリロニン A の作用機序に関する研究およびアクチン親和性タグの開発）

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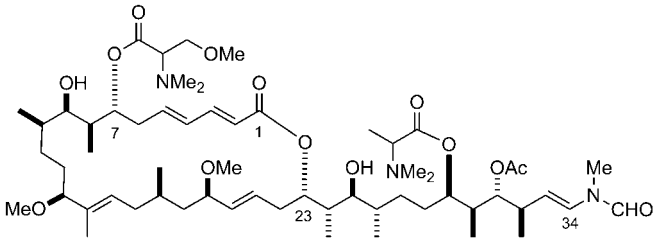
論　文　内　容　の　要　旨

Protein-protein interactions (PPIs) have received considerable attention and shown tremendous potential as intervention targets for various diseases, including cancer, neurodegenerative diseases, and infectious diseases. Several macrolides and macrolactams, such as the antitumor agent rapamycin and the immunosuppressant FK-506, have been shown to induce PPIs to exert specific and potent biological activities. Aplyronine (ApA), a 24-membered antitumor macrolide isolated from the sea hare *Aplysia kurodai*, also promotes a unique PPI between the two major cytoskeletal proteins, actin and tubulin (Figure 1). The formation of a 1:1:1 heterotrimeric complex (HTC) of actin–ApA–tubulin α,β-heterodimer was established by gel-permeation HPLC, surface plasmon resonance analysis, and photoaffinity labeling experiments. However, the detailed binding mode of HTC and the inhibitory mechanism of ApA on microtubule (MT) dynamics have been unclear. To establish the binding mode of the actin–ApA complex on the tubulin α/β-heterodimer, cryo-electron microscopy analysis and molecular dynamics simulation studies were performed.



Aplyronine A (ApA)

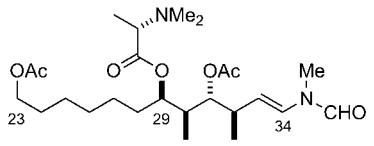


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Figure 1. Structures of ApA and analog **4**.

Firstly, to clarify the binding position of actin–ApA complex on tubulin, direct observation was performed using electron microscopes (EM) based on two strategies. The first one was for the actin–ApA complex to interact with paclitaxel-stabilized MTs. Negative staining-EM analysis showed that MTs were almost completely disrupted by the treatment with actin–ApA complex only for 5 min, and actin–ApA complex worked dose-dependently on MT disruption. In cryo-EM studies, no interaction between actin–ApA complex and the outer surface of MT was observed, but some defects and protofilaments of MTs treated with actin–ApA complex were observed. The second strategy was to mix actin–ApA complex directly with tubulin α,β-heterodimer without polymerization. As a result, the tubulin α,β-heterodimer particles were annotated with a size of 10 nm in length. While the HTC observed by negative staining-EM was non-homogeneous, and the single particles of HTC were not observed in the current study, we successfully showed that actin–ApA complex disrupted tubulin assembly *in vitro*.

To accelerate the mode of action studies of ApA, blind protein–protein docking and molecular dynamics simulation between actin–ApA complex and tubulin α,β-heterodimer was examined. These results showed that actin–ApA complex interacted with the C-terminal domain (CTD) of tubulin in the top four docking models, which are important for MT assembly and interaction with various cellular proteins. On the most plausible HTC model (HTC-3), the helix 12 of α-tubulin tightly bound to the actin–ApA complex (Figure 2). The C7 *N*,*N*,*O*-trimethylserine (TMSer) ester of ApA, an essential group for its potent cytotoxicity and PPI-inducing effect, interacted with D396, R422, and E429 on the helix-12 of α-tubulin to bend the angle by around 20°. These results suggested that the conformation of α,β-tubulin has a bended, nonlinear structure on the actin–ApA–tubulin HTC, which might potently inhibit tubulin heterodimer assembly and/or cause MT disassembly.

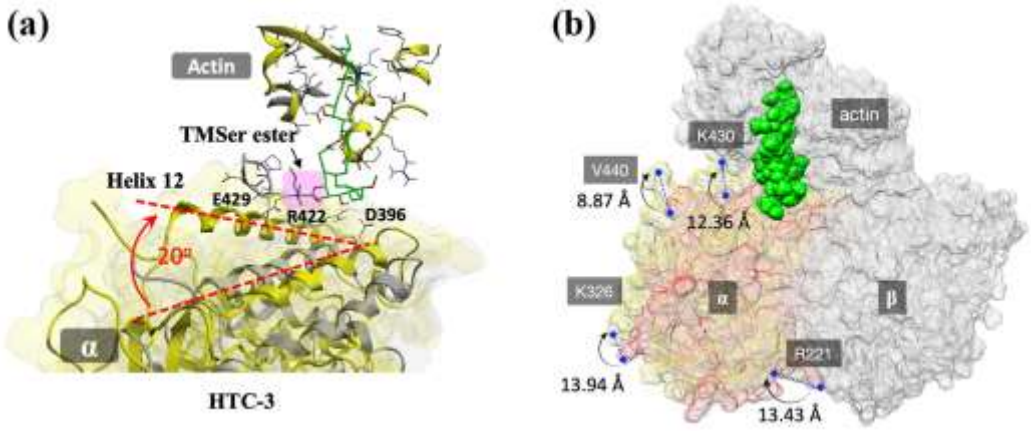

Figure 2. Interaction between the TMSer ester of ApA (green) and the helix-12 residues of α-tubulin in HTC-3 obtained by molecular modeling. (a) Movement of helix-12 of α-tubulin induced by PPI with the actin–ApA complex. (b) The α-tubulin models before (red) and after (yellow) MD simulation with the distances of the Cα atoms on selected residues.

Furthermore, structurally-simplified C29–C34 side-chain analogs of ApA have been developed based on the structure-activity relationship studies of aplyronines. Actin-depolymerizing assay showed that analog **4** had the most potent activity (16%) compared to ApA, which possessed the C23 acyloxy group, the C29 *N*,*N*-dimethyl-L-alanine (DMAla) ester and the C34 *N*-methyl enamide moiety (Figure 1). To analyze the specific interactions of ApA side-chain analogs, their biotin derivatives were prepared. Binding kinetics analysis showed that active analog **4-bio** was a moderate actin binder with K_D 10.1 μM (Figure 3). Molecular docking study with actin showed that **4** had an enough low binding free energy (ΔG –8.6 kcal/mol) and had a similar binding mode with the native ApA (RMSD = 1.23 Å). These results suggested that presence of the C29 DMAla ester was highly important for the strong binding to actin. Finally, the actin in HCT116 cell lysate were specifically affinity-purified by the above biotin analogs. Our study demonstrated that the actin-binding side-chain analogs of ApA might serve as versatile actin affinity tags.

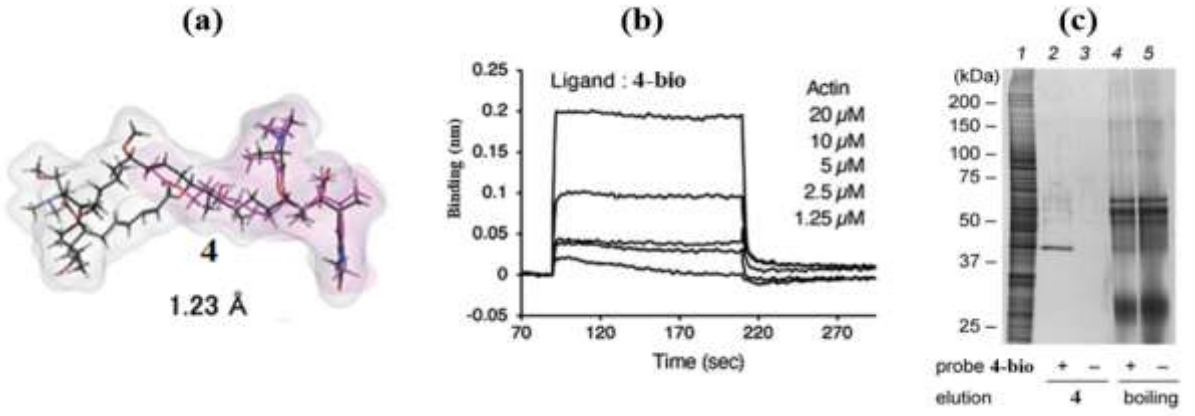

Figure 3. Simplified side-chain analog **4** as a potential candidate as actin-affinity tag. (a) Binding mode comparison between **4** (magenta) with native ApA (grey). (b) Binding kinetics of **4** as a moderate actin binder. (c) Pull-down experiments using cell lysate and **4**.

In summary, negative staining-EM and cryo-EM analysis revealed that actin-ApA complex rapidly disrupted the paclitaxel-stabilized MT. Computational study proposed a reasonable molecular mechanism for the MT dynamics inhibitory effect of ApA by changing the binding mode of actin–ApA–tubulin HTC and the conformation of α,β-tubulin heterodimer. These findings have the potential to contribute to the design and development of future PPI-based drug leads. Furthermore, we developed structurally simplified side-chain analogs of ApA that selectively bound to actin. The use of actin-affinity tags will accelerate the study of the modes of action related to cytoskeletal dynamics, as well as the development of PPI-based anticancer agents and other kinds of drug leads.