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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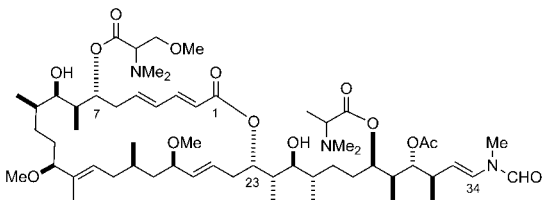
THESIS SUMMARY

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)

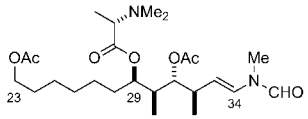


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Figure 1. Structure of ApA and analog **4**

In Chapter 3, 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 the 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. We also conducted multistep ultracentrifugation strategy to get short-MT fragments that bound to actin–ApA complex (**Figure 2**). A broken short-fragment with some proteins in the outer surface was observed by the negative staining-EM, but the condition was not enough homogenous due to the presence of aggregated complex.

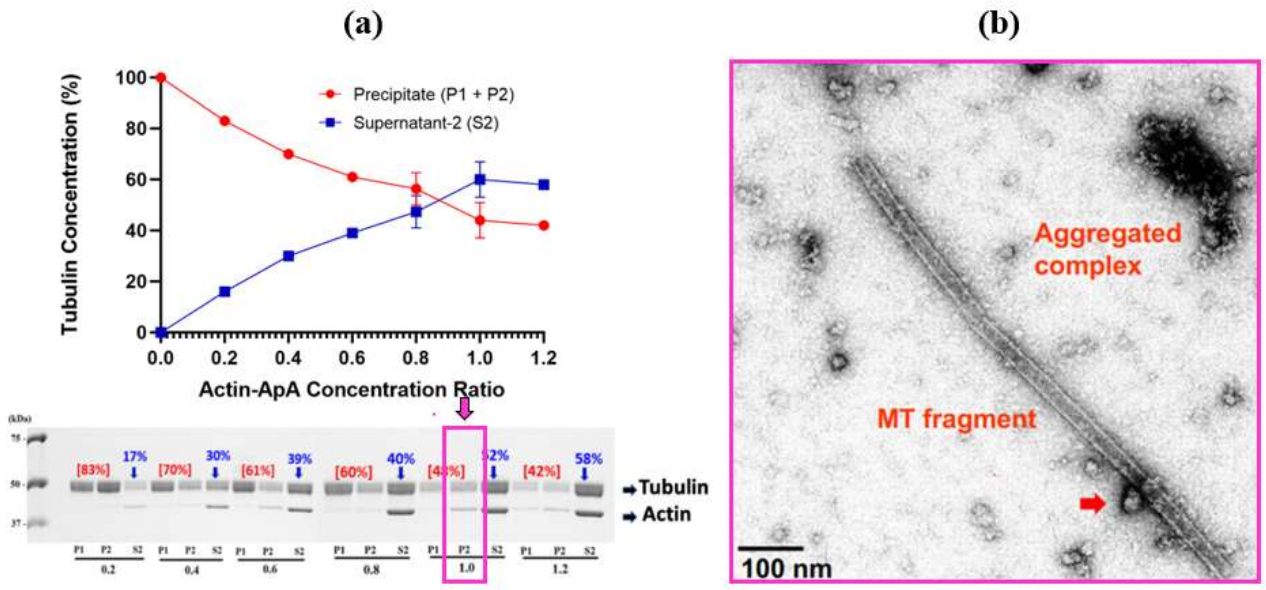

Figure 2. Multiultracentrifugation strategy to get MT fragments bound to Actin–ApA complex. (a) Actin–ApA complex worked as dose-dependent manner in breaking p-MT. (b) MT fragment mixed with actin–ApA complex in P2 fraction.

The second strategy was to directly mix actin–ApA complex with tubulin α,β-heterodimer without polymerization. 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 their single particles were not observed in the current study, we successfully showed that actin–ApA complex induced MT disassembly *in vitro*.

In Chapter 4, 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 heterodimer in the top four docking models, which are important for MT assembly and interaction with various cellular proteins. Molecular dynamics simulation revealed that only two HTCs (HTC-1 and HTC-3) were enough stable during 5 ns simulation. Meanwhile, the ligand movement dynamics showed that HTC-3 was the most plausible model, where the helix 12 of α-tubulin tightly interacted with the actin–ApA complex. The C7 *N*,*N*,*O*-trimethylserine (TMSer) ester of ApA, an essential group for its potent cytotoxicity and PPI-inducing effect. To understand how the C7 *S/R* TMSer ester affects PPI, comparative study was conducted between *S*-ester and *R*-ester. It was found that *S*-ester had a better properties compared with *R*-ester from the viewpoints of both the HTC stability and ligand movement dynamics. This simulation gave an important insight that the *S*-TMSer ester could be mainly responsible for the PPI stabilization between actin and tubulin. The simulation of *S*-TMSer ester also clearly explained that this moiety induced α-tubulin outward movement and interacted with D396, R422, and E429 on the helix-12 of α-tubulin to bend the angle by around 20° (**Figure 3**).

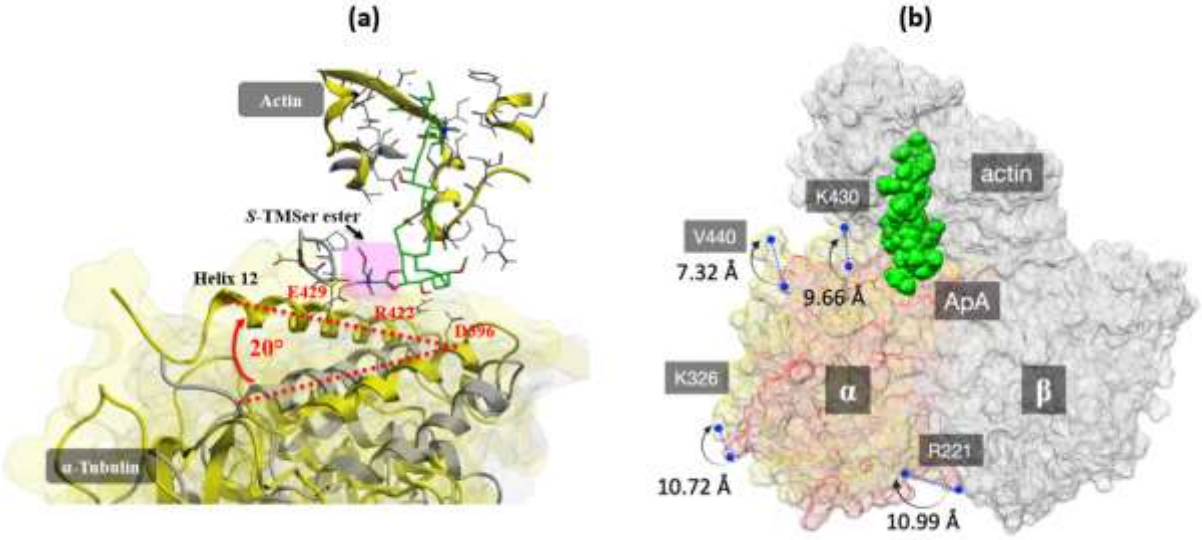

Figure 3. Interaction between the TMSer ester of ApA (green) and the helix-12 residues of α-tubulin in HTC-3 obtained by molecular modeling. (a) Movement of helix-12 of α-tubulin induced by PPI with the actin–ApA complex. (b) The α-tubulin models before (red) and after (yellow) MD simulation with the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

These findings clearly explain how the actin–ApA–tubulin HTC inhibits MT dynamics. The actin–ApA complex could interact with the tubulin α,β-heterodimer in a 1:1 ratio to form HTC, in which the conformation of α,β-tubulin has a bent, nonlinear structure on the actin–ApA–tubulin HTC. Firstly, the formed HTC could bind to the plus (+) end of MT to inhibit tubulin assembly. Secondly, the actin–ApA complex would bind to the middle part of MT to form the internal HTC with the induced outward movement of α,β-tubulin, which might cause rapid MT disassembly.

In Chapter 5, structurally-simplified C29–C34 side-chain analogs of ApA were 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 other synthetic analogs **2**–**5**, 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. Molecular docking study with actin showed that the **4** had an enough low binding free energy (ΔG –8.60 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, pull-down experiments were carried out using cell lysate to demonstrate the binding selectivity or specify the target of analog **4**. These results showed that **4** was noncovalently but highly specifically bound to actin, and that both **4** and **4**-**bio** had enough potency as actin-affinity tags. This result was strongly supported by Western blot (WB) analysis using an anti-actin antibody. Our study proved that the actin-binding side-chain analogs of ApA might serve as versatile actin-affinity tags (**Figure 4**).

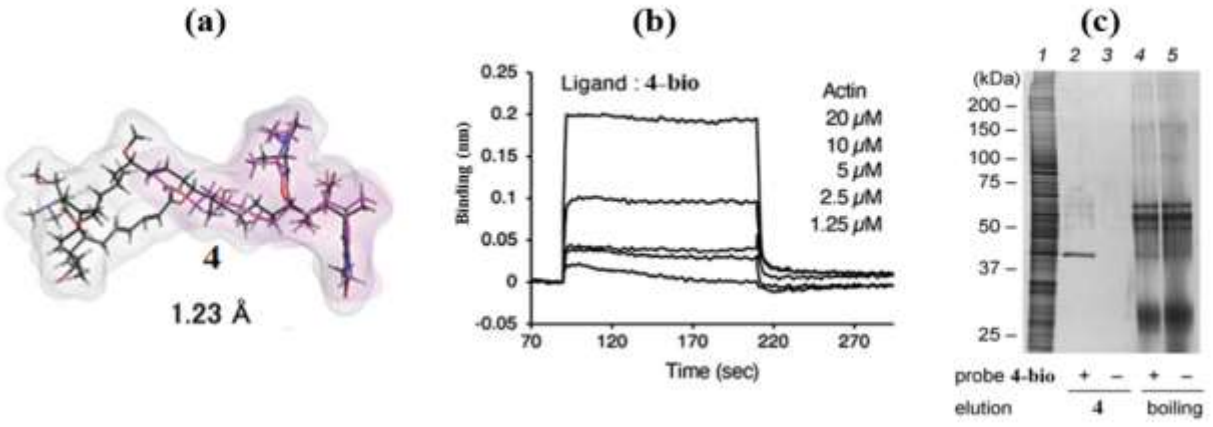

Figure 4. Simplified side-chain analog **4** as a potential candidate as actin-affinity tag. (a) Binding mode comparison between **4** (magenta) with native ApA (grey). (b) Binding kinetics of **4** as a moderate actin binder. (c) Pull-down experiments using cell lysate and **4**.

In conclusion, this study demonstrated that MT dynamics was affected by the actin–ApA complex *in vitro* and explained the molecular mechanism of HTC *in silico.* Further purification of HTC using gel-permeation HPLC and structural determination using cryo-EM are promising for understanding the mode of action of ApA as a potent PPI inducer. We also have successfully simulated the interactions between the actin–ApA complex and the tubulin α,β-heterodimer and obtained reliable HTC models that can explain the mechanism of the inhibitory effect of ApA on MT dynamics. In addition, structurally simplified side-chain analog of ApA was developed, which would accelerate the modes of action studies on cytoskeletal dynamics, as well as the development of PPI-based anticancer agents and other types of drug leads.