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Inhibition of Microtubule Assembly by a Complex of Actin and Antitumor Macrolide Aplyronine A

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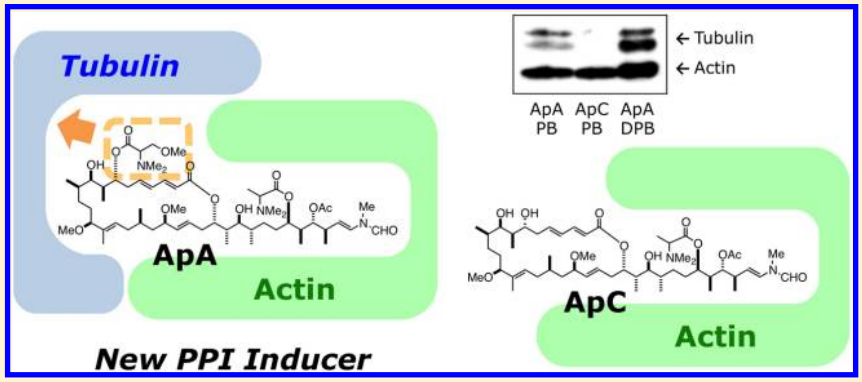
Supporting Information



ABSTRACT: Aplyronine A (ApA) is a marine natural product that shows potent antitumor activity. While both ApA and ApC, a derivative of ApA that lacks a trimethylserine ester moiety, inhibit actin polymerization in vitro to the same extent, only ApA shows potent cytotoxicity. Therefore, the molecular targets and mechanisms of action of ApA in cells have remained unclear. We report that ApA inhibits tubulin polymerization in a hitherto unprecedented way. ApA forms a 1:1:1 heterotrimeric complex with actin and tubulin, in association with actin synergistically binding to tubulin, and inhibits tubulin polymerization. Tubulin-targeting agents have been widely used in cancer chemotherapy, but there are no previous descriptions of microtubule inhibitors that also bind to actin and affect microtubule assembly. ApA inhibits spindle formation and mitosis in HeLa S3 cells at 100 pM, a much lower concentration than is needed for the disassembly of the actin cytoskeleton. The results of the present study indicate that ApA represents a rare type of natural product, which binds to two different cytoplasmic proteins to exert highly potent biological activities.

INTRODUCTION

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. The dynamics of actin assembly (polymerization/depolymerization) are regulated by numerous actin-binding proteins.[1] For example, thymosin β4 and profilin bind to monomeric actin and physically inhibit its addition to growing filaments. CapZ binds to filament ends and inhibits both monomer addition and dissociation, while the Arp2/3 complex nucleates actin assembly and stabilizes actin filaments (F-actin) by cross-linking.

Along with these endogenous proteins, various small agents that target actin have also been discovered and some show potent cytotoxicity.[2] For example, ulapualides,[3] mycalolides,[4] kabiramides,[5] sphinxolides/reidispongiolides,[6] swinholides,[7] and bistramides[8] are all actin-depolymerizing agents of marine origin that have recently been characterized. These actin-targeting agents typically disrupt actin cytoskeleton dynamics at concentrations below 100 nM without the breakdown of stress fibers, which induces potent cytotoxicity. In addition, various actin-polymerization-stimulating or -blocking molecules have been shown to induce apoptosis; examples include cytochalasin D (100 nM against T cells),[9] jasplakinolide (100 nM against HL-60 cells[10] and T cells[11]), latrunculin A (1−10 μM against MKN45 or NUGC-4 cells),[12] and mycalolide B (100 nM against HL-60 cells).[13] Thus, these agents are not only useful for investigating actin dynamics in cells but also may be of therapeutic value as antitumor compounds.

Among the actin-targeting natural products, the apoptogenic marine macrolide aplyronine A (ApA, **1**) depolymerizes F-actin and shows antitumor activities in mouse xenograft models (e.g., T/C 545%, 0.08 mg kg^{-1}, against P388 leukemia).[14] Previous

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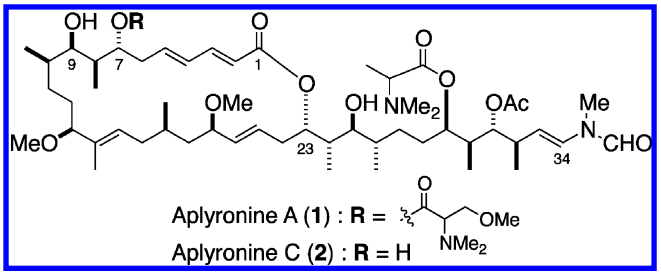
studies showed that ApA inhibits polymerization of actin by forming a 1:1 complex with the globular monomeric molecule (K_d = 100 nM).[15] X-ray analysis of the actin−ApA complex revealed that ApA intercalates into the hydrophobic cleft between subdomains (SD) 1 and 3 of actin by using its side chain.[16] Complexes of actin with other agents, such as kabiramide C,[17] sphinxolide B,[18] reidispongiolides A and C,[18] swinholide A,[19] and bistramide A,[20] have also been reported. The contacts between actin and each macrolide are similar to those observed in the actin−ApA complex.



Although the interaction with actin is mediated by the C24−C34 side chain of ApA, results of structure−activity studies indicated that both the macrolactone part (C1−C23) and the C24−C34 side chain are necessary for cytotoxicity at pM concentrations.[21] For example, aplyronine C (ApC, **2**), which lacks the C7 trimethylserine ester moiety, exhibits 1000-fold less cytotoxicity, but as much actin-depolymerizing activity, as ApA. The binding affinity of ApA to actin is comparable to those of ApC and mycalolide B. However, ApA induces caspase-dependent apoptosis in HeLa S3 cells at 1 nM, while ApC and mycalolide B do not, even at 100 nM.[22] Therefore, the potent cytotoxicity and apoptogenic effect of ApA was not entirely accounted for by its F-actin-severing properties, and its molecular targets and mechanisms of action remained unclear.

By using fluorescence microscopy observations and photoaffinity-tag experiments, we now show that ApA synergistically binds to tubulin in association with actin, inhibits tubulin polymerization, and prevents spindle formation and mitosis in tumor cells. Tubulin-targeting natural products and their synthetic derivatives have been widely used in cancer chemotherapy.[23] To our knowledge, however, there are no previous descriptions of microtubule inhibitors that also bind to actin and affect microfilament dynamics.[24] Our studies of ApA provide further insights into the molecular mechanisms of structurally diverse natural products that regulate cytoskeletal dynamics.

RESULTS

Low Concentrations of ApA, but Not ApC, Induced Cell Death without Actin Destabilization. As previously described, ApA is approximately 1000-fold more cytotoxic than ApC on the basis of the MTT assay (Figure S1, Supporting Information).[14,21,22] We used fluorescence microscopy to examine the relationships between the cytotoxicity and actin-destabilizing properties of aplyronines. Both 100 nM ApA and ApC caused rapid disassembly of the actin cytoskeleton in HeLa S3, a human cervical carcinoma cell line (Figure 1). These properties on HeLa cells were similar to those of typical actin-targeting agents (e.g., 100−1000 nM latrunculin A, cytochalasin D, and mycalolide B).[25] Meanwhile, treatment with 100-fold more dilute samples (1 nM) of ApA or ApC induced no detectable alteration of the actin cytoskeleton. These results suggest that the potent cytotoxicity of ApA may not be due solely to its F-actin severing properties.

ApA Binds to Tubulin by Interaction with Actin in Cells and in Vitro. The C7 ester group of ApA, a rare modification among natural products, protrudes toward the bulk solvent region of the actin−ApA complex.[16] Due to the basicity and hydrophilic nature of the trialkylamine structure, we hypothesized that the C7 trimethylserine ester group might be involved in interactions with biomolecules other than actin, as with various posttranslational modifications such as the

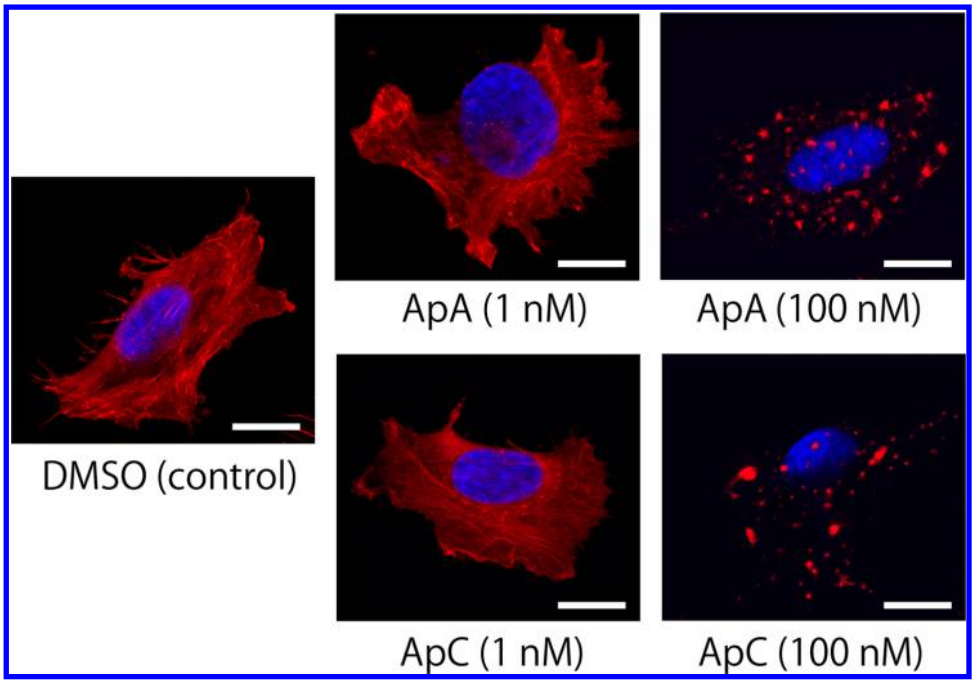


Figure 1. Confocal fluorescence images of HeLa S3 cells treated with aplyronines for 2 h. Cells were stained with rhodamine−phalloidin (red, F-actin) and DAPI (blue, nucleus). Scale bars: 20 μm.

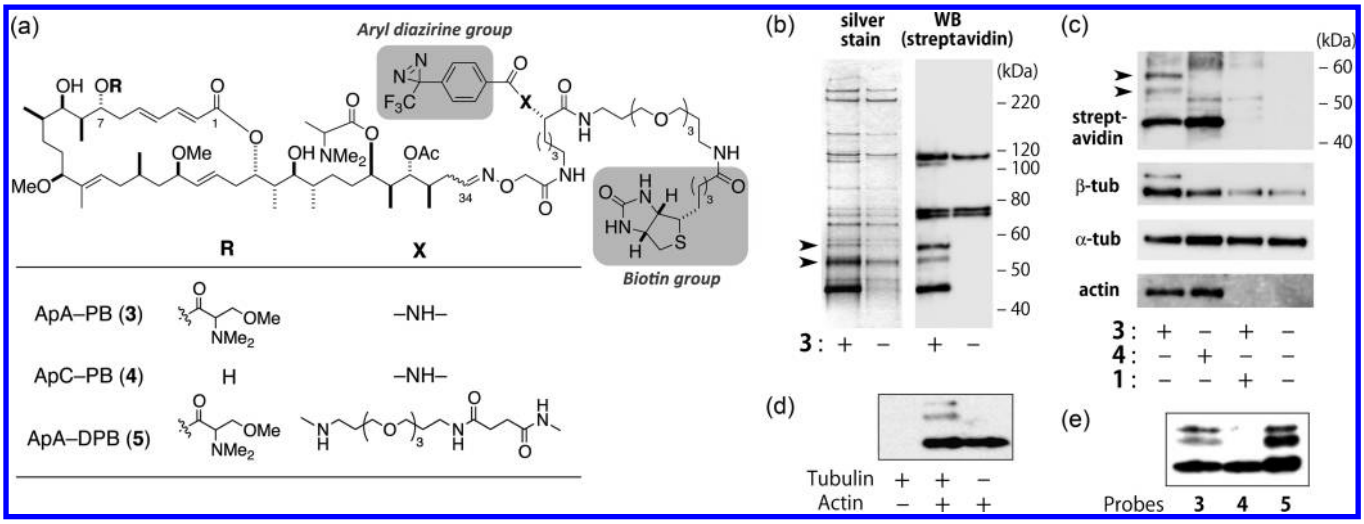


Figure 2. Actin–ApA complex binding to tubulin in situ and in vitro. (a) Structures of aplyronine photoaffinity derivatives. (b, c) In situ photolabeling experiments. In (b), HeLa S3 cells were treated with or without ApA–PB and then irradiated with UV (365 nm) for 15 min. Labeled proteins were affinity-purified with NeutrAvidin agarose, subjected to 10% SDS-PAGE, and detected with silver stain or HRP-conjugated streptavidin. The arrowheads show the proteins that were identified as α/β-tubulin. Several nonspecific biotinylated proteins (72–74 and 110–120 kDa) were also detected. In (c), HeLa S3 cells were treated with ApA–PB or ApC–PB (2.5 μM) in the absence or presence of excess ApA (25 μM). Labeled proteins were detected by immunoblotting analysis. The arrowheads show the protein bands (55 and 58 kDa) that covalently bond to ApA–PB. (d, e), In vitro binding experiments on photoaffinity derivatives (0.67 μM) to purified actin (from rabbit muscle, 0.67 μM) and/or tubulin (from porcine brain, 0.33 μM for the heterodimer) at 4 °C. After photoreaction, the reaction mixtures were lyophilized and directly analyzed by SDS-PAGE. Labeled proteins were detected with HRP-conjugated streptavidin. In (d), tubulin and/or actin was photolabeled with ApA–PB. In (e), tubulin and actin were photolabeled with the aplyronine derivatives **3**–**5**.

phosphorylation of signaling molecules and the sulfation of peptidoglycans.

To identify additional target proteins of ApA, we prepared two photoaffinity biotin derivatives, ApA–PB (**3**) and ApC–PB (**4**) (Figure 2a).[26] ApA–PB showed potent cytotoxicity against HeLa S3 cells (IC_{50} = 1.2 nM), whereas ApC–PB was ~260-fold less cytotoxic than ApA–PB. We previously reported that modifications to the side chain of ApA, such as C34 alcohol or C25 acetate derivatives, decreased its affinity for actin.[21] Apart from the hydrophobic binding of the side chain moiety (C24–C33), the terminal *N*-methyl enamide part of ApA is located in a hydrophilic environment and interacts with water molecules inside actin. It has been shown that this moiety can be replaced by oximes or hydrazones without a significant loss of activity.[22,27] In fact, ApA–PB inhibited actin polymerization as with ApA, as confirmed by an F-actin sedimentation assay.[26] ApA–PB and ApC–PB showed comparable reactivities toward actin in cell lysate,[26] suggesting that they have similar affinities for actin.

We performed photolabeling experiments with living tumor cells instead of cell lysate. Actin (43–45 kDa) and several proteins (52–55, and 58 kDa) were detected, following in situ photoreaction with ApA–PB in the HeLa S3 cells cooled on ice and subsequent affinity purification using NeutrAvidin agarose and silver staining (Figure 2b). Blotting analysis with streptavidin–HRP conjugate revealed biotinylated bands at 45, 55, and 58 kDa, consistent with the silver staining results. Mass analysis of tryptic peptide fragments established that the silver-stained bands at 52–55 and 58 kDa included α- and β-tubulin (Figure S2, Supporting Information). Since ApA–PB most effectively photolabeled actin, considerable amounts of nonlabeled α/β-tubulins might be eluted as adducts of actin–ApA–PB conjugate, which were observed as a major silver-stained band (52–55 kDa). Anti-β-tubulin antibody identified the 58 kDa band as β-tubulin that was covalently bound to ApA–PB (Figure 2c). In contrast, only actin (45 kDa) was photolabeled to the ApC–PB control. Together, this suggests that ApA interacts with both actin and tubulin.

To demonstrate the specificity of ApA–PB binding to tubulin, we performed a competition experiment (Figure 2c). Excess ApA inhibited the covalent binding of ApA–PB to both actin and β-tubulin, and the total amount of β-tubulin was reduced to control level. The presence of photoaffinity derivatives had no significant effect on the amount of α-tubulin detected. Our results suggest that ApA binds selectively to actin and β-tubulin in cells.

Through the use of ApC–PB, the amounts of affinity-purified α- and β-tubulin were slightly higher than those in the control (Figure 2c and Figure S3a (Supporting Information)). While actin was predominantly photolabeled, several proteins (i.e., 51 and 60–65 kDa) were also labeled, as with the control (ApA–PB + excess ApA). It is possible that a tubulin heterodimer was purified as the adduct of these unspecific proteins, without direct interaction with the actin–ApC–PB conjugate.

In vitro photolabeling experiments with purified proteins were carried out to investigate specific interactions among ApA, actin, and tubulin. ApA–PB formed a covalent bond with actin but not with tubulin alone (Figure 2d). In the presence of actin, however, tubulin was photolabeled with ApA–PB and detected as two biotin-labeled protein bands (55 and 58 kDa), as in the in situ experiments. In contrast, ApC–PB did not bind to tubulin, even in the presence of actin (Figure 2e).

The density ratios of two tubulin bands (55 and 58 kDa) in Figure 2d,e were less reproducible (Figure S3b, Supporting Information). The sizes of the original α- and β-tubulin were 52–55 kDa, and we were unable to distinguish whether the lower biotinylated tubulin bands corresponded to α-tubulin only or an α/β-tubulin mixture. Photolabeling of tubulin by ApA–PB might occur at several places following the random

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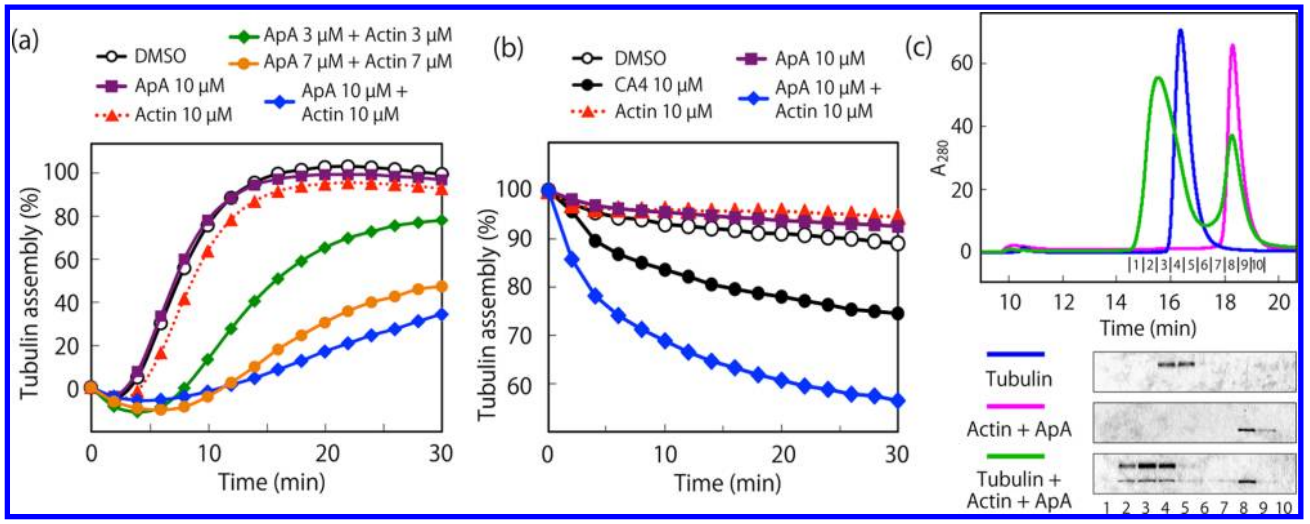

Figure 3. ApA and actin synergistically inhibit tubulin polymerization, enhance microtubule depolymerization, and form a ternary complex with the tubulin heterodimer in vitro. (a) Effects of ApA and actin on tubulin polymerization. Tubulin (2 mg/mL, 20 μM for the heterodimer) was polymerized with 20% glycerol at 37 °C, as monitored by an increase in DAPI fluorescence ($\lambda_{ex}/\lambda_{em}$ 360/450 nm). (b) Synergistic effect of ApA and actin on microtubule depolymerization. Various agents were added to polymerized tubulin (2 mg/mL) with paclitaxel (10 μM), and their abilities to attenuate turbidity were monitored at 350 nm. (c) Gel permeation HPLC analysis of the binding of actin–ApA complex to tubulin: buffer, 50 mM PIPES·K (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$; column, TSKgel SuperSW3000 (ϕ 4.6 × 300 mm); flow rate, 0.2 mL/min; temperature, 8 °C; detection, UV 280 nm. Binding was monitored by the appearance of a faster mobility peak (~15.5 min, corresponding to a 1:1:1 complex of actin–ApA–tubulin heterodimer, green trace) at 18.3 min, excess actin + ApA. Blue trace: tubulin heterodimer (100 kDa). Pink trace: actin–ApA complex (45 kDa). Eluted proteins (fractions 1–10) were analyzed by SDS-PAGE under reduceing conditions and detected with CBB stain (bottom). Due to the high concentration of Mg^{2+} ion, ligand-free actin was polymerized (see also Figure S6 (Supporting Information)) and was not detected as a single peak under these tubulin-stabilized buffer conditions.

movement of a flexible diazirine moiety on the ternary complex. It is possible that labeled β-tubulin was detected as split bands.

Although stoichiometric amounts of tubulin were used, the amount of tubulin photolabeled by ApA–PB in Figure 2d,e was far less than that of actin. The C24–C34 side chain of ApA binds to the hydrophobic cleft of SD 1 and 3 of actin,[16,28] and the aryldiazirine group in ApA–PB is believed to be located inside of actin. Therefore, we prepared an ApA double-PEG-linked photoaffinity biotin derivative (ApA–DPB, **5**) to enhance the flexibility of the photoreacting group and allow it to reach toward the C7 trimethylserine ester moiety (Figures 2a and Figure S4 (Supporting Information)). ApA–DPB showed potent cytotoxicity against HeLa S3 cells (IC_{50} 0.54 nM), similar to the case for ApA–PB and other ApA derivatives.[26,27] As predicted, treatment with ApA–DPB increased the amounts of both photolabeled tubulins and actin, in comparison to treatment with ApA–PB (Figure 2e and Figure S3a (Supporting Information)).

Due to the present poor availability of ApC, ApC–DPB has not yet been prepared. To clarify the specificity of the photoreactions of DPB derivatives toward tubulin, we hope to perform labeling studies with ApC–DPB in future studies.

ApA and Actin Synergistically Inhibit Microtubule Polymerization. We next examined whether the actin–ApA complex affects microtubule assembly. While actin and ApA alone had little effect on in vitro tubulin polymerization, their 1:1 complex delayed nucleation and growth phases and reduced the final polymer mass of tubulin in a dose-dependent manner (Figure 3a). In contrast, the actin–ApC complex failed to attenuate microtubule growth (Figure S5, Supporting Information). Actin and ApA synergistically depolymerized microtubules stabilized by paclitaxel (Figure 3b), and this effect surpassed that of combretastatin A-4 (CA4), a microtubule inhibitor that binds to the colchicine site of β-tubulin. Ultracentrifugation experiments similarly showed that actin and ApA synergistically depolymerized microtubules stabilized with paclitaxel (Figure S6, Supporting Information).

When the actin–ApA complex and tubulin were coanalyzed by gel permeation HPLC, a mobility peak appeared that was faster (green) than both the α/β-tubulin heterodimer (blue) and actin–ApA complex peaks (pink), and the last two peaks were reduced (Figure 3c, top). The retention times and SDS-PAGE analysis revealed that the faster mobility peak corresponded to the 1:1:1 complex of actin–ApA–tubulin heterodimer (145 kDa) (Figure 3c, bottom, and Figure S7 (Supporting Information)). A densitometric analysis of the CBB-stained bands showed that the quantitative ratios of tubulin/actin in lanes 2 and 3 were 1.6:1 and 2.7:1, respectively (Figure S8 (Supporting Information)). These values were consistent with the calculated value for 1:1 ratios of 100 kDa tubulin heterodimer/43 kDa actin. While we have no evidence regarding the stoichiometry of ApA in the ternary complex, it is likely that the complex binds to a tubulin heterodimer on the basis of the structure of the actin–ApA complex. This ternary complex was stable in solution for at least 1 h, despite being in equilibrium with the actin–ApA complex and the liberated tubulin heterodimer. The ternary complex peaks increased depending on the amount of actin, and 49% actin contributed to the formation of a ternary complex when an equivalent amount of actin was added (Figure S9a (Supporting Information)). The binding constant of the actin–ApA complex to tubulin heterodimer was estimated to be 3.0×10^6 M^{-1} on the basis of a Scatchard plot analysis (Figure S9b (Supporting Information)). In contrast, the actin–ApC complex did not interact with tubulin, and these compounds were separately eluted at their original retention times (Figure S5 (Supporting Information)).

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ApA Inhibits Spindle Formation and Mitosis in Tumor Cells. Immunostaining experiments showed that HeLa S3 cells treated with 100 pM ApA had irregular, multipolar spindle structures with unaligned chromosomes (Figure 4b). The same

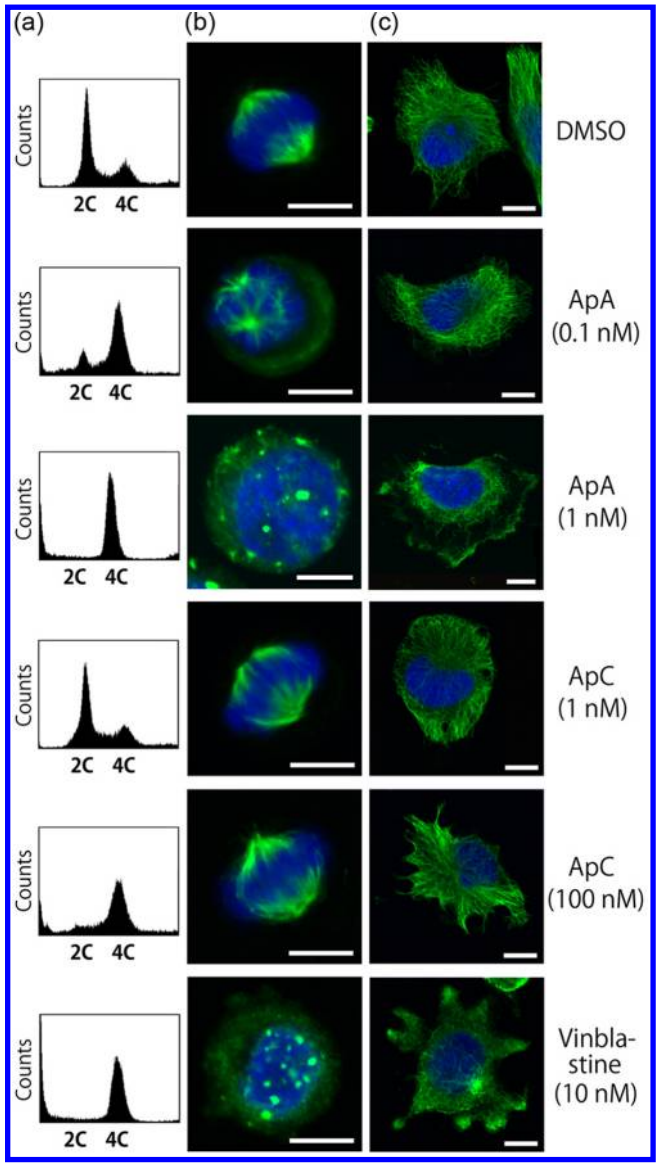

Figure 4. ApA inhibits spindle formation and mitosis at a concentration that is much less than that which causes actin depolymerization. (a) Cell cycle analysis. HeLa S3 cells were treated with various agents for 18 h followed by PI staining. (b, c) Confocal fluorescence images of HeLa S3 cells treated with various agents for 6 h. Cells were immunostained with anti-α-tubulin (green) and costained with DAPI (blue). (b) Metaphase cells. (c) Interphase cells. Scale bars: 10 μm.

treatment inhibited cell-cycle progression in M-phase (Figure 4a). Irregular spindles were observed in 36%, 52%, and 91% of synchronized mitotic cells by treatment with 0.1, 1, and 10 nM ApA, respectively, but in only 13% of control cells (Figure S10 (Supporting Information)). Treatment with 1 nM ApA partially disrupted microtubule structures in interphase cells (Figure 4c) and induced apoptosis,[22] comparable to effects of treatment with 10 nM vinblastine. In contrast, treatment with ApC had no detectable effects on spindle formation and inhibited cell-cycle progression in M-phase only at 100 nM, the same concentration that caused complete disassembly of the actin cytoskeleton (Figure 1). The observed inhibitory effects of ApA on spindle formation and mitosis might be essential for its antitumor activity, in accordance with caspase-dependent apoptosis.[29,13]

DISCUSSION

We previously reported that ApA does not interact with known molecular targets of antitumor agents, such as DNA, microtubules, and cell cycle regulating proteins.[30] Our present results show that ApA targets tubulin in association with actin, thereby inhibiting microtubule assembly at a very low concentration. To the best of our knowledge, there have been no previous reports that small actin-binding agents disturb microtubule assembly. Previous pull-down experiments using biotinylated derivatives of aplyronines revealed that Arp2 and Arp3 (actin-related proteins) were affinity-purified as binding proteins along with actin from HeLa S3 cell lysate.[27] However, Arp2 and Arp3 did not covalently bind to ApA–PB or ApC–PB.[26] It is noted that no tubulin in the cell lysate was photolabeled or affinity-purified with ApA–PB. In contrast, in situ photolabeling experiments established that the cellular targets of ApA are both actin and tubulin, consistent with those of in vitro and in vivo effects on cytoskeleton filaments. These critical differences between in vitro and in situ photolabeling experiments might be due to the instability of microtubules under the lysis conditions we used.

Due to the high concentration of intracellular actin and the high affinity of aplyronines for actin,[15] they might be trapped within cells as an actin complex. It is possible to imagine that the intracellular concentrations of aplyronines could be higher than the extracellular concentrations, which might enhance their cytotoxic effects. However, we previously showed that fluorescent derivatives of ApA and ApC are similarly incorporated into HeLa S3 cells and are not easily excluded from the cytoplasm.[22] These results suggest that ApA and ApC have similar cellular permeabilities and accumulation levels. Since these two compounds have almost the same effects on actin assembly in vitro, the significant differences in cytotoxicity, mitosis inhibition, and apoptogenic effects[22] of ApA and ApC are likely to be due to the tubulin-binding properties of ApA.

Through the use of in situ photolabeling experiments, we demonstrated that ApA binds selectively to actin and β-tubulin in cells. In contrast to β-tubulin, the amount of affinity-purified α-tubulin with ApA–PB was similar to that of the control (Figure 2c). This result suggests that the tight association of the subunits of the tubulin heterodimer need to be dissociated, which is incompatible with gel permeation HPLC analysis. A possible explanation is that ApA–PB covalently bound to β-tubulin might destabilize the association of tubulin subunits. Alternatively, it is possible that α-tubulin is more labile than β-isomer under the cell lysis or affinity-purification conditions we used, which might enhance its dissociation from the resin.

How is the stable ternary complex of actin–ApA–tubulin formed? To answer this question, we compared the structures of the actin–latrunculin A[31] and actin–kabiramide C[17] complexes with that of the actin–ApA complex (Figure S11 (Supporting Information)). The structures of actin in the three complexes highly overlapped, and the RMSD values of the main chain atoms were 0.88 and 0.96 Å, respectively. Thus, the actin bound to ApA does not have a unique conformation. As described above, the side chain of ApA, which has the same structure as that of ApC, binds to actin, but the C7

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trimethylserine ester does not. Presumably, the positively charged C7 ester group that protrudes from the actin−ApA complex might generate a unique tubulin-binding site on the actin−ApA complex, driving the interaction with tubulin α/β-heterodimer (probably on the β-isomer). However, it is also possible that the actin−ApA complex undergoes a structural rearrangement upon binding to tubulin.

Due to the unstable nature of diluted tubulin, an in vitro tubulin polymerization assay and gel-permeation HPLC analysis could not be performed at a sub-μM range. In cell-based assays, however, ApA potently inhibited spindle formation and mitosis at 100 pM, while actin filament disassembly occurred only at 100 nM. Within cells, ApA might first bind to actin and then interact with liberated tubulin heterodimer to form a ternary complex. A few molecules of the ternary complex would then bind at the microtubule plus end or copolymerize into the microtubule lattice, as in treatment with substoichiometric concentrations of vinblastine, colchicine, or other antimitotic agents.[23] At low concentrations, vinblastine does not depolymerize spindle microtubules but blocks mitosis, and cells die by apoptosis.[32] Similarly, low concentrations of paclitaxel block mitosis by kinetically stabilizing spindle microtubules without changing the mass of polymerized microtubules.[33] It is possible that the actin−ApA complex might inhibit spindle microtubule dynamics at the lowest effective concentration by forming a ternary complex.

Microtubule−actin interactions underlie many fundamental cellular processes, such as cell motility, neuronal pathfinding, cell division, and cortical flow.[34] A variety of proteins mediate microtubule−actin interactions and regulate their dynamics. Among those, mammalian diaphanous-related (mDia) formin proteins not only nucleate and assemble linear actin filaments but also directly bind to microtubules and regulate their stabilization.[35a] A member of the mDia family, mDia3, associates with the kinetochore and contributes to chromosome alignment in the M-phase.[35b] Another F-actin-associated protein, drebrin, binds to EB3 to coordinate the F-actin−microtubule interactions responsible for neuritogenesis.[36] It is possible that ApA modulates the coordination between the microtubules and actin and affects cytoskeleton dynamics by mimicking such microtubule-targeting, actin-binding proteins.

Only a few natural products have been shown to simultaneously interact with more than one biomacromolecule, such as FK506, an immunosuppressive macrolide, which targets FKBP and calcineurin.[37] Thus, ApA represents a relatively rare type of compound, which binds to two different cytoplasmic proteins and forms a ternary complex. Rapamycin, a macrolide structurally related to FK506, also binds to FKBP with high affinity, but the FKBP−rapamycin complex targets mTOR to inhibit the activation of lymphocytes in a different manner.[38] Actin is an abundant cytoplasmic protein similar to FKBP. Likewise, it is possible that actin-targeting agents interact with multiple cellular targets via protein−protein interactions. The results of these studies have potential in the design and development of newly classified pharmacological tools and therapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

Text, tables, and figures giving all experimental procedures, characterization data for all compounds, ^{1}H and ^{13}C NMR spectra, and details of biological evaluations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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