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Structural Basis of Swinholide A Binding to Actin

Brief Communication

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Summary

Marine toxins targeting the actin cytoskeleton represent a new and promising class of anti-cancer compounds. Here we present a 2.0 Å resolution structure of swinholide A, a marine macrolide, bound to two actin molecules. The structure demonstrates that the actin dimer in the complex does not represent a physiologically relevant entity, for the two actin molecules do not interact with each other. The swinholide A actin binding site is the same as that targeted by toxins of the trisoxazole family and numerous actin binding proteins, highlighting the importance of this site in actin polymerization. The observed structure reveals the mechanism of action of swinholide A and provides a structural framework about which to design new agents directed at the cytoskeleton.

Introduction

There is considerable interest in finding and designing small molecule inhibitors of the actin cytoskeleton with the hope that they might serve as a new class of therapeutic agents [1–3], analogously to the tremendously successful microtubule-directed anti-cancer drugs [4]. One such compound with potent cytoskeletal effects is the marine macrolide toxin, swinholide A [5–8], that, based on its antitumor activity, has been included in the National Cancer Institute's Molecular Targets Development Program (http://home.ncifcrf.gov/mtdp/name_sor.html). The molecular target of swinholide A is actin, a ubiquitous and abundant protein crucial for cell motility and cytokinesis in all eukaryotes.

Actin is characterized by a dynamic interplay between its filamentous F-actin and globular G-actin forms. The relationship between these two forms is highly regulated and influenced by many cellular processes and components. Therefore, any alteration in this state, such as increased filament lifetime by capping or decreased filament length by severing, has a profound consequence on all cytoskeletal-dependent processes, even though comparatively few actin molecules may be directly affected. Therefore, agents that influence the balance between the filamentous and globular forms offer great opportunities for controlling numerous cellular functions.

Swinholide A is one of the better-characterized membrane permeable and specific inhibitors of actin filaments network and is actively used in the cell biological studies [9, 10]. It is a symmetric macrolide and has been shown to bind two actins [7, 8]. Cytotoxicity of swinholide A is proposed to result from its actin filament severing and actin monomer sequestering activities, although the precise mechanism of swinholide A interaction with actin is unknown. To resolve this question and to provide a framework for designing new pharmacological compounds, we have crystallized and determined the structure of the actin-swinholide A complex by X-ray diffraction by taking advantage of the fact that actin complexed with swinholide A does not polymerize.

Results and Discussion

Overview of the Structure

Crystals of actin-swinholide A grew in the presence of 50 mM $MgCl_2$ and belong to the space group $P2_1$ with the two actins bound to a single swinholide A in the asymmetric unit cell (Table 1). The structure was solved by molecular replacement where one of the actin molecules is highly ordered and the other shows well-defined thermal movement. The crystal packing consists of the two sheets, each formed exclusively by one of the crystallographically independent actin molecules (monomer A or B). The general topology of crystal contacts within each of these sheets is similar and closely resembles packing found in another actin crystal belonging to space group $P2_1$ space group [11], PDB code 1QZ6. However, the two sheets of actin molecules are not identical in the crystal of the actin-swinholide complex. Each actin in layer "A" forms 3318 $Å^2$ contact area with the neighbor protein molecules, while each actin in layer "B" has only 1946 $Å^2$ contact area with its neighbors. This provides the physical explanation for the high degree of order in one sheet (A) and greater degree of mobility in the other (B).

The overall structure of the complex is shown in Figure 1A. It exhibits the inherent 2-fold symmetry of swinholide A (Figure 2B). Actin is found in a typical "closed" G-actin conformation quite similar to all other available structures of actin complexed with various actin binding proteins or small molecules (RMS deviation 0.5–1.0 Å).

The electron density for swinholide A is unequivocal and in full agreement with the stereochemical assignments made based on the crystal structure of its free brominated diketone derivative [12]. The conformation of swinholide A in the complex is quite different from one observed for the free molecule—the macrolide ring

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Table 1. Summary of Crystallographic Statistics

Diffraction Data	
Space group	$P2_1$
Unit cell (Å °)	A = 68.0 b = 76.8 c = 98.4 β = 101.2
Resolution (Å)	50-2.0
Reflections, total/unique	224403/66151
Average I/σ[a]	24.8 (3.6)
Completeness [a] (%)	99.4 (95.7)
R_{merge}[a] (%)	5.5 (30.1)
Refinement and Model Statistics	
Number of atoms, actin/toxin/MgATP/solvent	5603/98/64/291
R_{work}/R_{free}[a] (%)	18.4/21.9 (22.4/25.9)
Average B factor (Å^2)	26.2
Ramachandran plot, favored/allowed (%)	93.2/6.8
Rmsd bonds/Rmsd angles	0.015 Å/1.65°

[a] Data in parentheses represent the highest resolution shell.

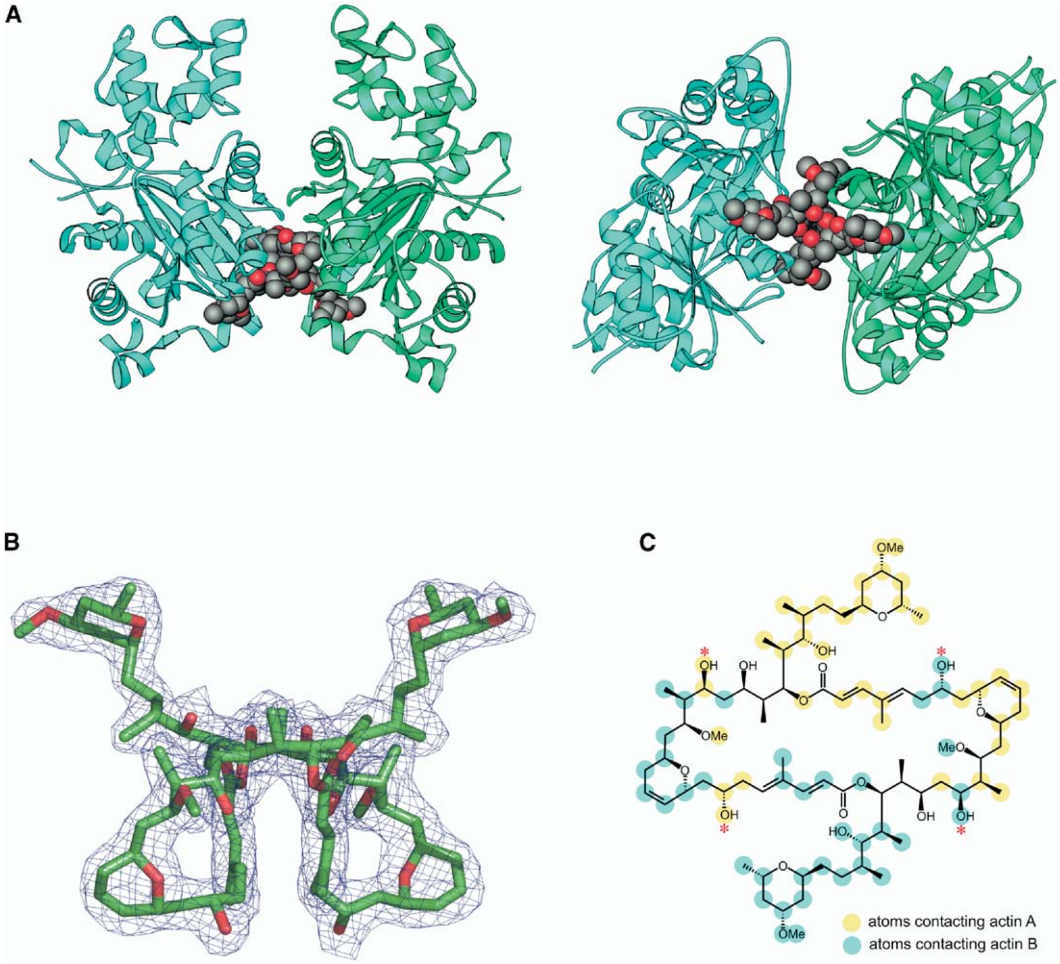

Figure 1. Structure, Electron Density, and Ligand Contacts for the Actin-Swinholide A Complex

(A) Shows a side and bottom view of the complex where Swinholide A is drawn in a space filling representation and actin is depicted in a ribbon representation. The actin molecules are colored in cyan and green, respectively.

(B) Shows an omit electron density map for swinholide A contoured at 3σ calculated with coefficients of the form ($F_o - F_c$).

(C) Maps the interaction of each actin molecule on a chemical structure of swinholide A. Atoms highlighted with colored circles make direct contact with one of the actin molecules. Yellow circles indicate contacts with molecule A whereas blue circles indicate interactions with molecule B. The hydroxyl groups that form hydrogen bonds with actin are labeled with asterisks.

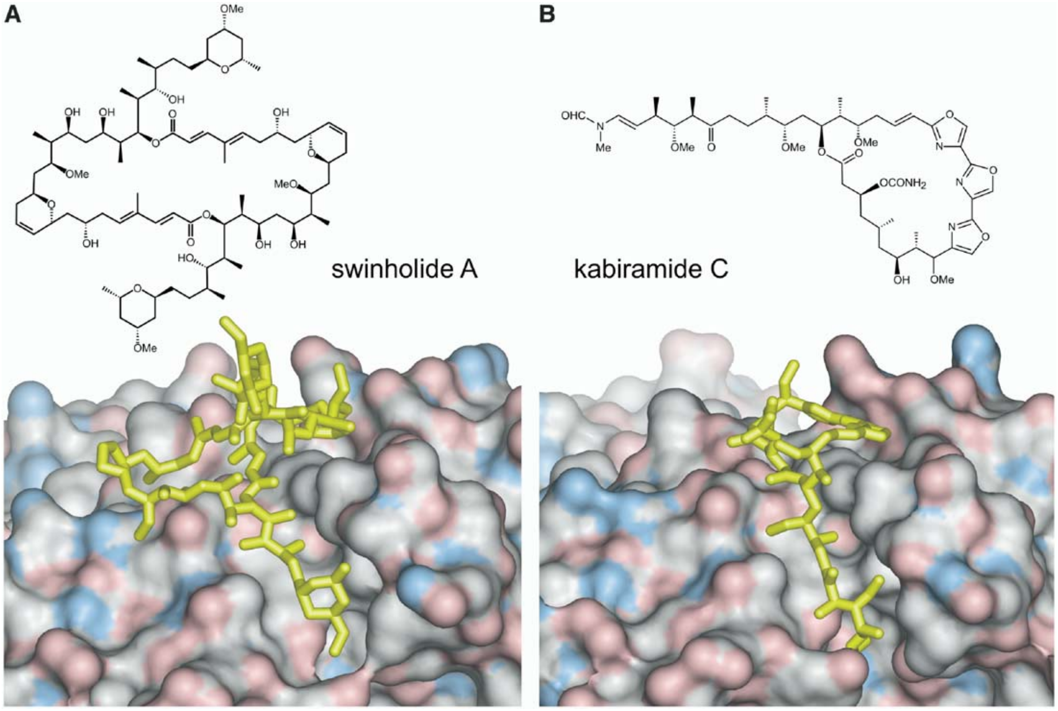

Figure 2. Swinholide A and Kabiramide C Occupy the Same Binding Site on Actin

(A and B) Shown are the chemical structures of the toxins and their complexes with actin. Actin is shown as a molecular surface and toxins are depicted in a ball-and-sticks representation. It clearly reveals that the side chains of the macrolides lie in the hydrophobic groove that lies between subdomains 1 and 3 of actin. The overall conformation of actin in both complexes are the same, but small differences in the groups that constitute the binding site are clearly evident.

adopts a figure eight-like conformation that enables both of its side chains to swing away from the ring to interact with two actin molecules. Contacts between the toxin and protein are extensive and distributed approximately equally between the macrolide ring and side chain moieties of swinholide A. Upon binding of swinholide to two actins, a total of 2330 Å^2 of molecular surface area is buried where 36% of the swinholide A molecular surface area becomes inaccessible. Interestingly, the complete interaction of a single actin molecule with swinholide A cannot be described in terms of a simple division of the 2-fold symmetric macrolide. Instead, atoms from both halves of the toxin make contact with a single actin molecule (Figure 1C).

The solved structure unequivocally shows that a previous conclusion that swinholide A stabilizes the disulfide-linked “lower” actin dimer [7] was in error. The latter consists of an anti-parallel arrangement of actin monomers that is stabilized by S-S crosslinking between two Cys374 residues [13, 14]. In contrast, the two actins in the complex seen here, for all intents and purposes, do not interact with each other, with the exception of a salt bridge between opposing Asp25 and Lys328 residues. Additionally, no disulfide bonds are observed anywhere in the crystal. Thus, it is clear that the actin dimer bound to swinholide A represents a nonphysiological entity that is incompatible neither with the generally accepted helical model of F-actin [15], nor with the lower dimer, the putative intermediate in the F-actin nucleation [14].

Actin Binding Site and Comparison to Other Toxins

As might be expected based on the chemical composition of the toxin, the interaction between swinholide A and actin is mostly of hydrophobic nature. The actin binding site can be divided into two parts (Figure 2A). A hydrophobic patch on the surface of the protein (residues Ala144, Gly146, Ile341-Leu349), interacts with the macrolide ring, and the hydrophobic cleft between actin subdomains 1 and 3, into which the swinholide A side chain is inserted (residues Gly168, Tyr143, Thr148, Tyr169, Leu346, Ile345, Leu349, Thr351, Met355). Additionally, hydrogen bonds between carbonyl oxygens of Ser145 and Gly146 and hydroxyl groups of swinholide A (O2 and O5, respectively) contribute to the observed conformation of the bound toxin. As proposed earlier [11], it is very likely that the hydrophobic cleft between actin subdomains 1 and 3 is crucial for the actin monomers interaction with each other in the polymeric F-actin structure. This provides an attractive explanation as to why swinholide A inhibits polymerization and severs actin filaments.

The overall actin binding site for swinholide A is nearly identical to the binding site for members of the trisoxasole family of marine toxins identified previously [11]. This is remarkable given that there is no obvious structural similarity between these families of macrolides (Figure 2). There are several implications of this unexpected finding. First, swinholide A is expected to compete with other toxins—those of the trisoxazole family (over 30 members so far) and a number of macrolides that share essentially the same aliphatic side chain, the presumed major determinant of their binding to actin in this location—reidispongiolides, sphinxolides, aplyronins, scytophycins, and tolytoxins (reviewed in [1]). Additionally, as the same binding site on actin is targeted by numerous actin binding proteins, our finding identifies swinholide A and its structural homologs such as misakinolide A [16] as new class of small molecule biomimetics of proteins that regulate actin dynamics in the cell [11, 17]. Second, analogously to kabiramide C, swinholide A is expected to stabilize the closed actin conformation and inhibit nucleotide exchange, as indeed has been observed [8]. Finally, following the line of argument provided for the trisoxazole-containing toxins [11, 17] it predicts that swinholide A, alone or in the complex with actin, should be capable of capping the "barbed" end (exposed subdomains 1 and 3) of actin filaments.

Swinholide A filament severing activity implies that the toxin is capable of intercalating between the neighbor actin protomers in the filament. Again, by analogy to the trisoxazole macrolides, this process is likely to proceed in two steps—first the macrolytic ring anchors the molecule on a protein surface followed by insertion of the tail in the hydrophobic cavity [17]. In this regard, it is notable that misakinolide A, with its four carbons shorter ring but identical side chain, does not sever polymeric actin [16]. This is probably because the macrolide moiety of misakinolide A, being shorter and thus less flexible, cannot adopt a figure eight-like conformation that appears to be a prerequisite for productive binding to actin. The acceptance of ligands of very different structure within the same binding region on actin strongly suggests that other chemical frameworks that provide similar binding surfaces may exist or can be designed.

Significance

The structure and mechanism of action of small molecule inhibitors of the actin cytoskeleton are of great interest because of their potential to become a new class of anti-cancer agents. Here we report the structure of one of such compounds, swinholide A, bound to actin, which shows that its binding site on G-actin overlaps substantially with that targeted by trisoxazole-containing macrolides and highlights the importance of the targeted hydrophobic surface on actin. Thus, a second group of toxins is shown to employ the same mechanism of action as seen before. The acceptance of ligands of very different structure within the same binding region on actin strongly suggests that other chemical frameworks that provide similar binding determinants can be designed. This study opens the way for attempts at rational design of swinholide A analogs with improved pharmacological characteristics.

Experimental Procedures

Crystals of actin-swinholide A complex were grown at 4°C by small-scale batch method [18]. 5 μl of 10 mg/ml actin-swinholide A complex was mixed with 5 μl of precipitant solution containing 13%–15% dimethyl polyethylene glycol 5000, 100 mM HEPPS, 100 mM $MgCl_2$, 1.0 mM TCEP, and 1 mM NaN_3 (pH 8.5). The solution was spun to remove any precipitate and drops were immediately streak-seeded with microcrystals from hanging drop crystallization. For cryopreservation, the crystals were first transferred into precipitant solution, then, in three equal steps of increasing solute concentrations, into freezing solution (25% dimethyl polyethylene glycol 5000, 18% ethylene glycol, 100 mM HEPPS, and 175 mM $MgCl_2$ [pH 8.5]), and frozen in a nitrogen stream at 100 K. Diffraction data were collected to 2.0 Å resolution as 370 frames of 0.5° oscillations with R-AXIS IV image plate detector utilizing Cu Kα radiation generated by a Rigaku RU300 operated at 50 kV and 95 mA and focused with Osmic Blue mirrors. The data were processed with HKL2000 [19]. The structure was solved using Molrep [20] with two copies of actin in asymmetric unit cell (starting model PDB code 1J6Z). The refinement was performed with Refmac [21] utilizing TLS refinement [22] which resulted in a dramatic improvement of the model fit to the experimental electron density followed by restrained individual B factors refinement. Geometrical restraints used for swinholide A were the same as those observed in the X-ray structure of its diketone derivative [12]. Ligand-protein contacts were analyzed with the LPC software [23], http://pdb.weizmann.ac.il:8500/osc-bin/lpccsu/. Molecular surface buried at the interface between molecules was calculated with CNS version 1.1 [24]. Figures were prepared with Pymol (http://www.pymol.org).

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Accession Numbers

Coordinates and structure factors have been deposited in the Protein Data Bank under ID code 1YXQ.