Ring Polymers of Tubulin Induced by Binding of Natural Antimitotic Peptides

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Summary: A number of natural products with potent antimitotic activity are peptides and depsipeptides that bind to tubulin, provoke depolymerization of microtubules, and induce formation of single layer rings of tubulin dimers. These peptides are all hydrophobic and small relative to tubulin (3-5 amino acid residues), yet induce rings polymers with properties that can differ significantly in size and self-association. In addition, these compounds exhibit potent cytotoxicity that varies by several hundred fold from one compound to another. Cryptophycin induces unusually homogeneous rings, composed of eight tubulin dimers, that are stable to dilution at least to nanomolar tubulin concentrations.

Keywords: biopolymers; cryptophycin; microtubules; ring polymers; supramolecular structures

Introduction

Microtubules (MT) are the largest of the three polymer fiber systems that together comprise the cytoskeleton of eukaryotic cells. MT are dynamic noncovalent polymers composed of a structural subunit, the heterodimer tubulin, and a large number of associated proteins which regulate assembly, promote directed movement along the MT surface, and form signaling complexes on MT. Assemblies of MT provide the cell with polarity (such as inside – outside directionality), and regulate directed intracellular movement. Notably, arrays of MT comprise the mitotic spindle, the molecular machine responsible for accurate separation of the two sets of chromosomes at cell division.

This highly complex and dynamic structure is very sensitive to alteration or disruption of MT dynamics or function. A large number of small molecules are known that are capable of doing this, and are collectively known as mitotic poisons. The vast majority of these exert their effect by binding to and altering tubulin, and many of these are, or are derived

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from, natural products. The large number of these found in nature suggests that MT and mitosis are particularly vulnerable targets to which organisms have evolved chemical attacks, presumably as a defense from predators or as a way to achieve advantage over competitors.

Antimicrotubule Agents

Antimitotic agents targeting MT fall into several classes, based on their non-overlapping binding sites on the tubulin dimer. The classic mitotic poison colchicine defines one of the major binding sites on tubulin, and many natural, semisynthetic, and synthetic compounds are known that bind to this site. A second site is defined by the binding of vinblastine and related vinca alkaloids. Many natural compounds unrelated to vinblastine nonetheless have binding sites that appear to at least overlap with that of vinblastine. These compounds tend to be larger in molecular mass than those binding to the colchicine site. A third site is defined by the binding of taxol, and a small but growing group of compounds are known whose binding is at the taxol site. An additional "site" is the cysteine sulfhydryl groups exposed on the tubulin dimer.

Table 1. Properties of molecules binding to the three major drug binding sites on tubulin. These are grouped by their effect on MT, and then by their binding site. Colchicine site agents tend to be smaller molecules than vinca site binders.

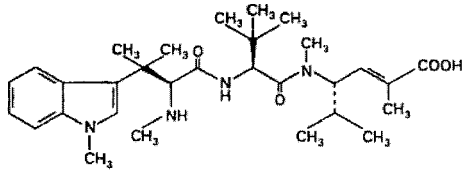
MT effect	Depolymerizing		Hyperstablizing
Binding site on:	Tubulin dimer		Tubulin polymer (MT)
Binding site:	Colchicine	Vinca	Taxol
Examples:	Colchicine Podophyllotoxin Combretastatin Nocodazole	Vinblastine Vincristine Maytansine Rhizoxin	Taxol Epothilone Discodermolide
Average MW, Da	~ 370	~750	~650
Example (MW), Da	Colchicine (400)	Vinblastine (800)	Taxol (850)

Binding at all of these except for the taxol site results in inhibition of MT polymerization, while occupation of the taxol site results in hyperstabilization of the MT and inhibition of depolymerization. Both polymerization and depolymerization are required for the normal dynamic functioning of MT and indeed all of these agents have been shown to alter dynamics at lower concentrations than those required for effects on total polymer mass[1]. The properties of agents binding to these binding sites are summarized in Table 1, with a few examples of molecules in each class.

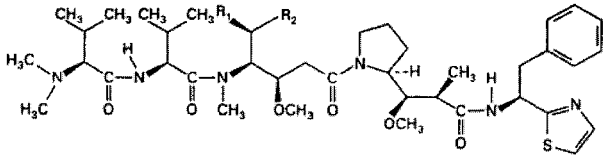
Vinca Domain Peptide Agents

In addition to vinca site agents such as those listed in Table 1, a subclass of compounds is composed of peptides and depsipeptides. These inhibit the binding of vinblastine to tubulin, as required for a vinca site agent, but their inhibition is not competitive, leading to the suggestion that their binding site(s) are overlapping but non-identical with that for vinblastine. This has given rise to the name "vinca domain" for the collection of binding sites overlapping with that for vinblastine[2].

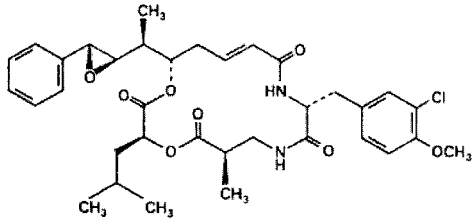
Three interesting examples of the peptide agents binding in the vinca domain are hemiasterlin, cryptophycin 1, and dolastatin 10 [3]. These are, respectively, a tripeptide, a cyclic four residue depsipeptide, and a linear pentapeptide, and in all three the amino acid residues are highly modified. All three are hydrophobic small peptides, and all are mutually competitive inhibitors of binding. Their structures are given in Figure 1. Like all vinca agents, these strongly destabilize MT and disfavor polymerization. Unlike many other destabilizing agents, these compounds do not simply prevent tubulin from polymerizing into MT, the tubulin remaining as dimers. Rather, these compounds induce tubulin to form alternative oligomeric structures[4]. Vinblastine also does this, inducing the formation of linear spirals of indefinite length. These compounds also induce formation of oligomers, but these are closed oligomers, specifically rings of defined size.



Hemiasterlin



Dolastatin 10: $R_1 = CH_3$; $R_2 = CH_3CH_2$
Isomer 2: $R_1 = CH_3CH_2$; $R_2 = CH_3$



Cryptophycin 1

Fig. 1. Structures of the three peptide antimitotic agents.

Structure and Properties of the Peptide-Induced Rings

Interestingly, the rings formed by the three peptides are similar but distinct. The properties of the rings were revealed by electron microscopy (yielding the ring diameter, and, by STEM analysis, the mass), a combination of sedimentation velocity (to yield sedimentation coefficients) and dynamic light scattering (to yield diffusion coefficients), the latter combination providing the ring mass by the Svedberg equation[5,6]. The results are presented in Table 2.

The rings are all formed by a single linear chain of dimers, unlike other known tubulin rings, e.g. the tubulin GDP-Mg rings which are double unequal nested rings[7], or the Rev-tubulin rings[8], which are double equal stacked rings. While the peptide rings are all single filament rings, their diameter, and hence curvature, are not the same.

Table 2. Properties of peptide-induced tubulin ring polymers.[5,6]

Drug	Diameter EM, nm	Sedimentation Coeffifient (*s*)	Diffusioin Coefficient (cm^2sec^{-1})x10^7	Ring Mass kDa	Number of Tubulin Dimers
Cryptophycin	27 ± 1	16	1.8	820	8
Hemiasterlin, Dolastatin	42 ± 2	20	1.3	1400	14

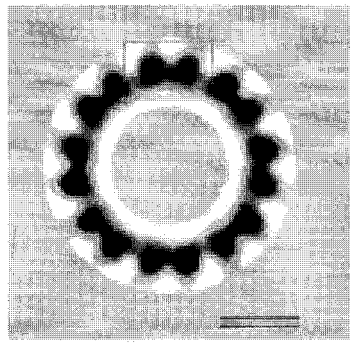


Fig. 2. The cryptophycin-tubulin ring, reconstructed from cryoelectron microscopy[5]. An individual tubulin dimer is outlined in the box; the β-subunit is on the left. The bar = 10 nm.

The cryptophycin rings are the smallest tubulin rings reported[5,9] , composed of only 8 dimers. We find the same size rings with cryptophycin-1, the natural product, or cryptophycin-52, an analog in clinical trials. The rings are also very homogeneous, with both compounds. This uniformity has facilitated structural analysis of these rings by cryoelectron microscopy and image reconstruction[5]. The resulting image, Figure 2, shows the 8-dimer ring of uniform polarity (no reversals of apparent handedness), with a clearly larger inter-dimer spacing than intra-dimer spacing. There are two points of curvature in each dimer: a larger angle (32°) at the inter-dimer contact and a smaller angle (13°) at the intra-dimer contact. Additionally, the normally smooth surface on the outside of the MT is now on the interior of the ring, and the scalloped morphology normally on the interior of the MT is now evident on the outside of the ring. Clearly cryptophycin has had the effect of causing a normal MT protofilament to curl into a ring, with the dimer surface normally

exposed on the outside of the MT now confined in the center of the ring. Protofilaments need not pre-exist for ring formation; addition of cryptophycin (or dolastain or hemiasterlin) to tubulin dimers results in the same ring structures as if added to MT. Dolastatin and hemiasterlin rings are the same diameter and are both larger than cryptophycin rings, composed mostly of 14 dimers, and are more heterogeneous in size. While these two ring types have the same morphology, they differ in that the dolastatin rings self-associate readily, causing significant increases in solution turbidity due to formation of structures composed of stacked rings that grow large enough to settle under gravity[3,10]. Hemiasterlin rings, like cryptophycin rings, do not self-associate under the same conditions, and the solutions remain water-clear.

The rings also differ in their stability to dilution. Fluorescence correlation spectroscopy (FCS) allows the diffusion coefficient of the rings to be estimated at a very wide range of concentrations. This analysis showed that cryptophycin rings exhibit no measurable depolymerization even when diluted to 1 nM total tubulin. Dolastatin rings depolymerized measurably below 10 nM, while hemiasterlin rings were significantly depolymerized at 100 nM [11]. This is the same order as the cytotoxicity of these compounds.

A comparison of the cytotoxicities of these compounds in presented in Table 3. This table also compares these compounds with vinblastine and taxol, demonstrating the significantly increased potency of these compounds (as much as 500-fold compared to taxol).

Table 3. Cytotoxicity of peptides on different cell lines. The IC_{50} (in nM) for a 4-day incubation wih drug is shown for ovarian (1A9), prostate (PC3), and breast (MCF7) carcinoma, and for lymphoma (Ca46) cell lines.[11]

Drug	1A9	PC3	MCF7	Ca46
Cryptophycin	0.01	0.016	0.01	0.012
Dolastatin	0.05	0.08	0.04	0.06
Hemiasterlin	0.24	0.6	0.3	0.2
Vinblastine	1.1	1.9	1	1.1
Taxol	5	8	6	6

These results suggest that the molecular properties of the rings may reflect parameters of the peptide-tubulin interaction that are significant for cell toxicity, whether or not the rings actually form inside treated cells. Additionally, it highlights the ability of these small molecules to induce formation of nanoscopic structures of high reproducibility. Finally,

the modular synthesis of these compounds[2] offers the possibility of "tuning" the interaction with tubulin by alteration of the structure of the peptides.

It is interesting to consider several unresolved points. Why do the peptides induce rings of differing curvature? Why do the rings differ in stability to dilution? Why do dolastatin rings aggregate but the others do not?

Computational docking studies of cryptophycin 52[9] and cryptophycin 1, dolastatin 10, and hemiasterlin[12] have indicated that the binding site for these compounds is on ß-tubulin adjacent to the exchangeable GTP site, in the inter-dimer interface. The site is somewhat to the "side" of the dimer and hence is involved in lateral interactions with the neighboring protofilament in a MT[9]. Nonetheless, occupancy of this site requires an upward displacement of helix H10 of the α-subunit of the next dimer[12], and this displacement may be the origin of the curvature observed at the inter-dimer contact[5]. It will be interesting to see if the displacement required by docking of cryptophycin is more than that required for hemiasterlin and dolastatin, as would be necessary to produce smaller rings.

The rings differ in stability to dilution. One explanation of this would be that this stability simply reflects the tightness of binding. The binding site is located at the contact site between neighboring dimers and is predicted to feature contacts between the peptide and both sides of the inter-dimer junction[9,12]. Therefore an increased binding affinity would plausibly result in tighter association of neighboring dimers and hence a more stable ring polymer. However, the binding affinities deduced from computational docking, as well as available experimental values, indicate the wrong trend for this explanation[12]. Hemiasterlin binding affinity is more than 10 fold higher than that for cryptophycin, yet the cryptophycin rings are far more stable than those formed with hemiasterlin. Hence, the explanation for stability differences must be more subtle.

The rings differ in association behavior, and the reason is not clear. Dolastatin rings aggregate readily while the other rings do not. At room temperature and pH 7, dolastatin rings associate to form structures that generate significant turbidity. These structures become large enough that they settle out of solution under 1 g. Under similar conditions, the cryptophycin and hemiasterlin rings do not aggregate, as indicated by the absence of turbidity, and by unchanged behavior in FCS. It seems remarkable that these rings are so different, since the peptides are all small hydrophobic peptides that bind to the same site on tubulin, the tubulin is the same in all cases, and the buffer is also the same. The rings

that are produced have the same morphology (i.e. the dimer surface exposed on the outside of the MT is on the inside of the ring), and are even the same diameter in the case of hemiasterlin and dolastatin. A related puzzle is that addition of substoichiometric amounts of cryptophycin or hemiasterlin to a mixture of dolastatin and tubulin poisons the self-association of the resulting rings[3]. In the case of cryptophycin addition, this might be rationalized based on the smaller size of the cryptophycin rings: perhaps the association requires rings of similar size. But this would fail to account for the behavior of the hemiasterlin – dolastatin rings, which are identical in size. Thus there must be some subtle difference in conformation of the rings. Since small angle neutron scattering reveals that the dolastatin rings stack like coins to form these structures[10], the differences presumable are to be found in the surfaces of the rings that contact in these stacks. These dimer surfaces are those that are involved in lateral contacts in MT. Further research will be required to reveal the nature of these subtle changes as well as the details that account for the size and stability differences.

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