Conformation Analysis

DOI: 10.1002/anie.200906828

The Tubulin-Bound Structure of the Antimitotic Drug Tubulysin

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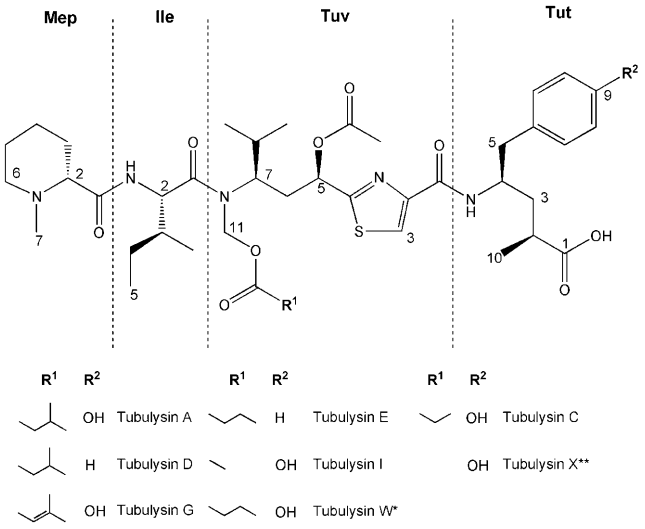
Tubulysin is a highly cytotoxic peptide isolated from the myxobacterial species *Archangium gephyra* and *Angiococcus disciformis*.[1] It consists of *N*-methylpipecolic acid (Mep), L-isoleucine (Ile), and the two unusual and novel amino acids tubuvaline (Tuv) and tubutyrosine (Tut)[2] (Scheme 1). Tubulysin displays extremely potent cytotoxic activity in mammalian cells, including multidrug-resistant cell lines, with IC_{50} values in the lower nanomolar range.[1,3] The cytotoxic activity of tubulysin is connected with its ability to interfere with microtubule (MT) dynamics and to inhibit tubulin polymerization both in vivo and in vitro.[2] With respect to its peptidic nature and biological activity, tubulysin is closely related to dolastatin-10, an anticancer agent derived from the marine mollusc *Dolabella auricularia* (see Scheme S1 in the Supporting Information). Other tubulin ligands, such as vinblastine and colchicines, share the MT-destabilizing activity of tubulysin while showing a very different chemical structure (see Scheme S1 in the Supporting Information).



Scheme 1. Chemical structure of natural tubulysins; Mep, *N*-methylpipecolic acid; Ile, isoleucine; Tuv, tubuvaline; Tut, tubutyrosine (* = deacetyl; ** = *N*,*O*-deacetyl).

The binding sites of vinblastine and colchicines to tubulin have been elucidated by X-ray crystallography[4,5] and provided the basis to propose a mechanism for the cytotoxic activity of these two MT-destabilizing agents. Tubulysin A (TBS, Scheme 1) strongly inhibits the binding of vinblastine to tubulin; however, the experiments suggest a noncompetitive pattern of inhibition,[2] as previously observed for other antimitotic peptides, such as dolastatin-10 and phomopsin A.[6a–c] Instead, antimitotic polyketides, such as rhizoxin[6a] and disorazol,[2] have been shown to inhibit binding of vinblastine to tubulin in a competitive manner. This led to the proposal of two binding sites for MT-destabilizing agents on tubulin, a *Vinca* site where the *Vinca* alkaloids bind and a peptide site for phomopsin and TBS binding, both sites situated in a region of the β-tubulin called the *Vinca* domain.

In addition to the many classes of MT-destabilizing agents, other groups of compounds, such as the epothilones and paclitaxel, function as MT-polymerizing enhancers. In vitro studies showed that induction of polymerization of tubulin by these compounds is strongly inhibited in the presence of TBS,[2] and that TBS is able to dissolve MTs formed in the presence of paclitaxel or epothilone.[2]

Knowledge of the structure of the tubulin–TBS complex is essential to understand the mechanisms of action of TBS and the interplay between this MT-destabilizing agent and other tubulin ligands. Direct structural information on the interaction of natural products with tubulin includes complexes of tubulin with either taxol or epothilone A (EpoA) as the polymerizing agents,[7–9] and with vinblastine and colchicines as the depolymerizing agents[4,5] (see Figure S1 in the Supporting Information). However, in the case of TBS structural knowledge is limited to the unbound conformation of TBS in a methanol/water mixture, determined by X-ray diffraction, or in dimethyl sulfoxide (DMSO), studied by NMR spectroscopy.[3]

Herein, we describe the bioactive tubulin-bound conformation of TBS, as determined by NMR structural analysis in aqueous solution using transferred NOE (tr-NOE) data. We find that the bioactive conformation of TBS dramatically differs from that in the solid state determined by X-ray crystallography. The conformational differences between the unbound and tubulin-bound forms presented here allow the first rationalization of the biological data available on the tubulin depolymerization activity of tubulysins.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200906828.

There are only weak cross-peaks observed in the NOESY spectra of tubulin-free samples of TBS, as expected for a small molecule tumbling rapidly in solution. In contrast, the NOESY spectra of a 500 μM solution of TBS in the presence of 10 μM tubulin show intense cross-peaks (tr-NOE), thus indicating that TBS binds to soluble tubulin. In the presence of tubulysin and under the conditions described in the Experimental Section, tubulin is either dimeric or forms rings (see Figure S2 in the Supporting Information).

A total of 197 nonredundant tr-NOE peaks were identified in the NOESY spectrum of a 500 μM aqueous solution of TBS in the presence of 10 μM tubulin, and were used as restraints in the structure calculation (see Figure S3 in the Supporting Information). The full relaxation matrix approach was used to calculate the structure of tubulin-bound TBS. This method minimizes the difference between the computed and the experimental 2D NOE intensities while accounting for spin-diffusion effects. The structure calculation converged to a unique family comprising the 12 lowest-energy conformers (Figure 1; heavy atoms, root mean square deviation 0.33 Å).

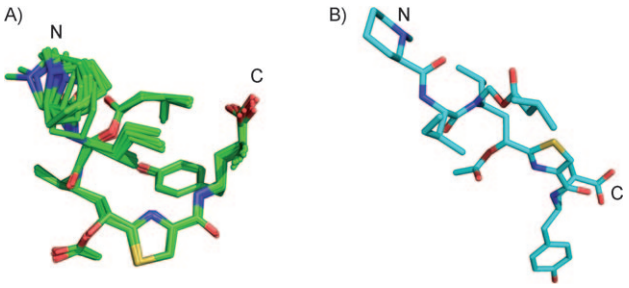

Figure 1. Conformation of A) tubulin-bound (overlap of the best twelve NMR structures) and B) free TBS. Color code: blue N, red O, yellow S, green/cyan C.

The tubulin-bound conformation of TBS (Figure 1 A, green) is compared to the free (unbound) conformation of TBS determined by X-ray crystallography (Figure 1 B, cyan).[3] The compact structure of tubulin-bound TBS largely differs from the extended unbound conformation. In the bound state, the nitrogen atom of the thiazole ring of Tuv and the aromatic ring of the Tut form a basal platform at the bottom of the molecule upon which the *O*-acyl *N*,*O*-acetal side chain of Tuv packs to form a hydrophobic core. The Ile and the methyl group at the C-terminal end of the Tut also contribute to this core. The *O*-acetylmethyl group closes the hydrophobic pocket at the bottom. The remaining backbone towards the N-terminal *N*-pipecolic acid protrudes from this core region, as does the *O*-acetyl group, both of which are located on the same side of the basal platform. Comparison of the dihedral angles of the tubulin-bound and free conformations of TBS revealed several major differences, which are described in detail in the Supporting Information (Table S1).

The piperidine ring of the *N*-pipecolic acid shows only sparse interresidual tr-NOE data, whereas the expected number of intraresidual signals from the methylene groups is present in the NOESY spectra. The NOE data are not consistent with a single conformation of the ring but indicate a mixture of twisted boat and chair conformations in the ratio 60:40 (Figure 1). Consistently with this conformational flexibility, the piperidine ring points away from the rest of the molecule and is likely not to be part of the pharmacophore. In contrast, in the tubulin-unbound structure the piperidine ring adopts a well-defined chair conformation.

Figure 2 shows a summary of the structure–activity relationship (SAR) data[10,11] mapped on the three-dimensional structure of tubulin-bound tubulysin. The tertiary

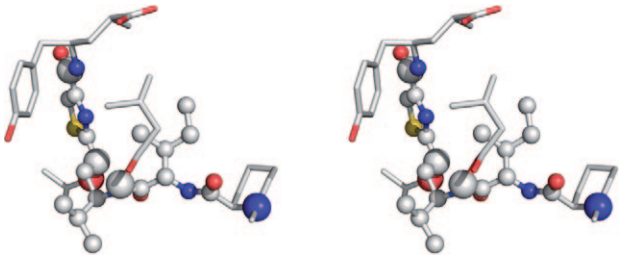

Figure 2. Stereo view of the tubulin-bound three-dimensional structure of TBS mapping of the SAR data available from the literature. The radius of the spheres, which represent C (gray), N (blue), S (yellow), and O atoms (red), is proportional to the relevance of the site for cellular activity. No sphere indicates that the corresponding atoms can be eliminated without affecting the bioactivity; middle-size spheres represent the sites where modifications were not tested, while the large spheres represent the sites where modification of stereochemistry or suppression of the functionality negatively impacts activity.

amine group of the Mep residue at the N terminus is essential for activity, but very simple low-molecular-weight substituents are acceptable at this site. Neither the tyrosylethyl nor the γ-carboxy groups of the C-terminal Tut residue are relevant for cytotoxicity. Similarly, both labile Tuv C5-acetyl and *O*-acyl *N*,*O*-acetal groups can be eliminated without affecting cellular activity. On the other hand, a change in the stereochemistry at the Tuv C5 atom negatively impacts the ability of TBS to destabilize MTs.

The tubulin-bound structure of TBS allows rationalization of these SAR data. The piperidine ring of the N-terminal Mep, the tyrosylethyl and γ-carboxy groups of the C-terminal Tut, and the *O*-acyl *N*,*O*-acetal group are all situated on the same side of the molecule and are all dispensable for biological activity. On the other hand, the hydrophobic skeleton of the molecule, which assumes a T-shaped form described by the thiazole ring, the valine side chain of Tuv, and the Ile side chain, seems to be essential, as indicated by the relevance of the stereochemistry at the Tuv C5 atom. On the opposite side of the T-shaped core, the hydrophilic tail of the Mep residue is likely to be involved in a hydrogen bond with tubulin side chains, as indicated by the relevance of the tertiary amine group at the N terminus of TBS.

Tubulysin has been shown to inhibit the binding of vinblastine to tubulin in a noncompetitive manner.[2] Thus, the location of the tubulin-binding site of TBS remains unknown. To address this question, we applied the INPHARMA method[12,13] to two samples: 1) TBS (500 μM), EpoA (500 μM), and tubulin (10 μM); and 2) TBS (500 μM), vinblastine (500 μM), and tubulin (7 μM). In INPHARMA,

protein-mediated interligand NOEs can be observed between two ligands binding weakly and competitively to the same binding pocket of a macromolecular receptor. In agreement with the biological data, which suggest a noncompetitive inhibition of the tubulin binding of vinblastine by TBS, we did not observe any protein-mediated interligand NOEs between TBS and vinblastine. However, we cannot exclude that the failure in the observation of the interligand NOEs may result from the slow dissociation constant, k_{off}, of vinblastine rather than the presence of two different binding sites for the two drugs.

Surprisingly, we observed interligand NOEs between EpoA and TBS, which suggests that the two drugs share a common binding site on tubulin (see Table S2 and Figure S4 in the Supporting Information). Tubulin-mediated interligand NOEs are observed homogeneously for almost all protons of the two drugs, thus suggesting that they do not bind simultaneously to neighboring pockets. Furthermore, the piperidine ring of TBS does not show any tubulin-mediated interligand NOEs, which confirms that this ring is not in contact with tubulin. The existence of tubulin-mediated interligand NOEs between EpoA and TBS could imply that the taxane binding pocket, to date identified as an exclusive binding site for MT-stabilizing agents, accommodates MT-destabilizing agents as well. This in turn poses the fundamental question about which structural features make a tubulin ligand either a MT-stabilizing or a MT-destabilizing agent.

Another possible explanation for the observation of interligand NOEs between EpoA and TBS would be the competitive binding of the two drugs to a pocket different from the taxane one. Recent work by Diaz et al.[14] has identified a second binding site for MT-stabilizing agents, the cyclostreptin binding site, which is adjacent to the taxane binding site on the other side of the M-loop and includes residues F214, T220, T221, and P222 of the H6–H7 loop. This binding site was suggested to be partially present in dimeric tubulin as well, and is proposed to represent the entry gate for MT-stabilizing agents to the lumenal taxane binding pocket in MTs. Thus, it is conceivable that EpoA and TBS share the cyclostreptin binding pocket in nonpolymerized tubulin.

Recently, the binding mode to tubulin of soblidotin, a dolastatin-10 analogue lacking the thiazole ring, has been determined by X-ray crystallography using αβ-tubulin in complex with the RB3 stathmin-like domain.[15] An overlap of the tubulin-bound conformation of soblidotin and tubulysin is shown in Figure S5 in the Supporting Information. There is a remarkable overlap between the pharmacophore of the two drugs, both in the overall shape at the C termini of the peptides and in the position of both the aromatic and polar groups. In this study, the binding site of soblidotin to tubulin partially overlaps with that of vinblastine, thus contradicting previous reports on the noncompetitive nature of the inhibition of vinblastine binding by dolastatin-10.[6] Notwithstanding this contradiction, we note that the dolastatin-10 binding site identified in the crystallographic study[15] marginally overlaps with the cyclostreptin binding site,[14] especially in the involvement of the H6–H7 loop. Assuming that dolastatin-10 and tubulysin share a common binding site on tubulin, the results from our INPHARMA NOE experiments might indicate that EpoA and tubulysin both bind to soluble tubulin at a site close to the cyclostreptin binding pocket.

Further investigations are in progress to confirm or disprove the competitive binding of tubulysin and EpoA, either to the taxane or to the cyclostreptin binding pockets of tubulin.

Experimental Section

Tubulin preparation: Bovine brain tubulin was purchased from Cytoskeleton Inc. (Denver, CO, USA; Product No. T238) and was prepared for the NMR measurements as described previously.[16]

Sample preparation: TBS stock solution was prepared by dissolving TBS in [D_6]DMSO (25 μL), and then mixed with tubulin solution to a final volume of 280 μL. The final sample contained 5% [D_6]DMSO, 10 μM tubulin, and 500 μM TBS. The presence of DMSO increased the solubility of TBS. Tubulin-free NMR samples of TBS were prepared by diluting the [D_6]DMSO stock solution of TBS in the NMR buffer.

NMR spectroscopy: NMR experiments were performed on a Bruker 900 MHz spectrometer. Resonances of TBS were assigned from COSY, TOCSY, HSQC, and HMBC spectra. A series of NOESY experiments was recorded at 25 °C with mixing times of 40, 70, 100, and 150 ms on tubulin–TBS and tubulin-free TBS samples. NMR data were processed with NMRPipe[17] and analyzed with FELIX (Accelrys Software Inc., CA, USA); 221 cross-peaks were identified in the NOESY spectra. Build-up curves were extracted for each cross-peak and 197 nonredundant NOEs were used as restraints in the structure calculation.

Structure calculation: 120 structures were calculated with XPLOR-NIH 2.13[18] using restrained simulated annealing (SA) from a single extended starting template. NOEs were used in the full relaxation matrix approach. From the initial rates of NOE build-up curves of those proton pairs the distance of which was independent of the conformation, a value of approximately 9 ns was estimated for the effective correlation time ($\tau_{c,eff}$), which correlated well with the result of the XPLOR-NIH grid search routine. The SA protocol comprised one high-temperature phase (2000 K), two cooling phases (from 2000 to 1000 K in steps of 50 K and from 1000 to 100 K), and a final 200 steps of energy minimization. The same weight was applied to all experimental peak intensities. Since stereospecific assignment of the methylene groups was missing, the in-house XPLOR function SWAP was used for optimizing the energy. Protons in methyl groups were averaged as $\langle r^{-3}\rangle^{-1/3}$. The quality of the fit of the structures to the experimental NOE data was determined with the generalized R-factor R^n, with $n = 1/6$. To test the consistency of the experimental data, the NOE information from 10 and 15% of the data was completely omitted from the experimental restraint list. Calculations run with the reduced sets of NOE data converged consistently to the same result. All our tests proved that the tubulin-bound conformation of TBS was exclusively determined by the experimental NMR data.

Structures have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) under accession number BMRB-20121.

Interligand tr-NOEs: Protein-mediated interligand NOEs between EpoA and TBS were observed in a NOESY spectrum acquired at 900 MHz by a sample containing tubulin (10 μM), EpoA (500 μM), and TBS (500 μM) in a D_2O/[D_6]DMSO (95:5) solution. The mixing times of the NOESY spectra were 450, 275, 200, 150, and 100 ms.

Received: December 3, 2009
Revised: February 19, 2010
Published online: May 21, 2010

Keywords: conformation analysis · cytotoxicity · microtubules · NMR spectroscopy · tubulysin

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