Drug Binding Modes

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Structural Basis of the Activity of the Microtubule-Stabilizing Agent Epothilone A Studied by NMR Spectroscopy in Solution**

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Tubulin exerts a fundamental role in cell division.[1] The protein exists in a dynamic equilibrium between a heterodimeric form constituted by α- and β-tubulin subunits and a polymeric form (microtubules (MTs)). Several classes of natural products that bind to tubulin and interfere with the polymerization equilibrium possess cytotoxic activity. Paclitaxel (PTX), which inhibits cancer-cell growth through the stabilization of cellular MTs,[2] has become one of the most important clinical anticancer drugs for the treatment of a wide range of solid human cancers. However, taxanes such as PTX are associated with severe side effects,[3] and are ineffective in colon and prostate cancer. In the search for tubulin-binding drugs with an improved pharmacological profile, epothilones appear to be extraordinarily promising, as they show a low propensity to induce multidrug resistance,[4,5] are better deliverable to cells owing to their higher solubility, and possess cytotoxic activity on cell lines resistant to PTX.[6]

The demonstration that epothilones bind to the same tubulin binding site as PTX[6] has provided the basis for the development of common pharmacophore models.[7–10] However, neither the molecular-modeling data nor the enormous amount of structure–activity relationship (SAR) data available for epothilones[11] have succeeded in providing a coherent picture of the binding mode of the drugs to tubulin. The structure of epothilone A (EpoA) bound to nonpolymerized tubulin in solution determined by NMR spectroscopy[12,13] confirmed the importance of the thiazole side chain for binding, but left open the question of the orientation of EpoA in the tubulin binding pocket. This question is addressed herein, where we derive the binding mode of EpoA to nonpolymerized tubulin in solution.

The complex of EpoA with tubulin polymerized in zinc-stabilized sheets has been recently studied by electron crystallography (EC).[14] The tubulin-bound conformation of EpoA reported in the study is in strong disagreement with the tubulin-bound conformation of EpoA determined in solution by NMR spectroscopy, in particular, with respect to the conformation of the macrolide ring.[12] Additionally, the EC-derived model of the EpoA–tubulin complex contradicts some of the available SAR data. In this context, it becomes relevant to derive the binding mode of EpoA to nonpolymerized tubulin, as this event can be considered the first of a complicated series of intermolecular interactions leading to the epothilone-induced formation of MTs. The high flexibility of the M loop, the S9–S10 loop, and the H6–H7 loop forming the binding pocket could be responsible for different binding modes of EpoA to tubulin depending on the polymerization state.

The binding of EpoA to nonpolymerized tubulin cannot be detected in presence of Mg^{2+} ions, as under these conditions the binding and MT-assembly reactions are linked.[15] In the present and previous studies,[12] we prevented the epothilone-driven assembly of tubulin into MTs by operating in a buffer that contained Ca^{2+} instead of Mg^{2+} ions. Under these conditions, where the MT assembly is disfavored, and only a fraction of the tubulin polymerizes in the form of MT sheets (Figure S1 in the Supporting Information), we can detect the weak binding of EpoA to nonpolymerized tubulin. This weak binding escapes detection by biochemical methods, owing to the large dissociation rate constant ($k_{off} \gg 100$ Hz, as measured by relaxation–dispersion experiments), but can be easily detected by NMR spectroscopy.

The binding of EpoA to nonpolymerized tubulin can be considered functionally relevant for the following reasons: a) EpoA binds to tubulin in its completely nonpolymerized form (Figure S1A in the Supporting Information) and induces the formation of elongated polymers (Figure S2B in the

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Supporting Information), confirming that the interaction of EpoA with nonpolymerized tubulin is the first and required step for polymer assembly. b) We have observed protein-mediated interligand nuclear Overhauser effects (NOEs) among all the ligands that are known to bind to the taxane-binding pocket (epothilones, taxanes, discodermolide, and baccatins),[16,17] but not between ligands binding to different sites (for example, EpoA and vinblastine; data not shown). These results confirm that MT-stabilizing agents bind to the same binding pocket in nonpolymerized tubulin as they do in the MTs; thus, the binding is specific. c) Although the weaker affinity of EpoA for nonpolymerized tubulin with respect to assembled MTs indicates that the structural details of the protein are different in the two forms, it is unlikely that the protein–ligand interactions are completely dissimilar for polymerized and nonpolymerized tubulin. Such a hypothesis would imply a large conformational rearrangement of the epothilone-binding pocket, which cannot be reconciled with the observation that EpoA induces tubulin polymerization even under conditions that disfavor polymerization, such as at low temperature, in the absence of guanosine 5′-triphosphate (GTP), or in the presence of Ca^{2+} ions.

The binding mode of EpoA to tubulin in solution (Figure 1 a,b) was derived using a combination of the interligand NOE for pharmacophore mapping (INPHARMA) methodology[16] and molecular modeling (see the Supporting Information). The novel INPHARMA methodology relies on the observation of intermolecularly transferred NOEs[18] for two ligands binding competitively and consecutively to the same binding site of a common target protein with an equilibrium dissociation constant (K_d) in the micromolar range. These NOEs are exclusively mediated by spin-diffusion through protons of the protein and are, therefore, dependent on the specific interactions of each of the two ligands with the protein. An accurate and quantitative evaluation of such effects leads to the determination of the binding modes of the two ligands.

Our experimental system consisted of a mixture of tubulin, EpoA, and baccatin III (BacIII; Figure 1 c), in a concentration ratio of 1:50:50. We chose BacIII as the second ligand because it shares a large portion of the chemical structure of PTX, but lacks the side chain at C13 that is

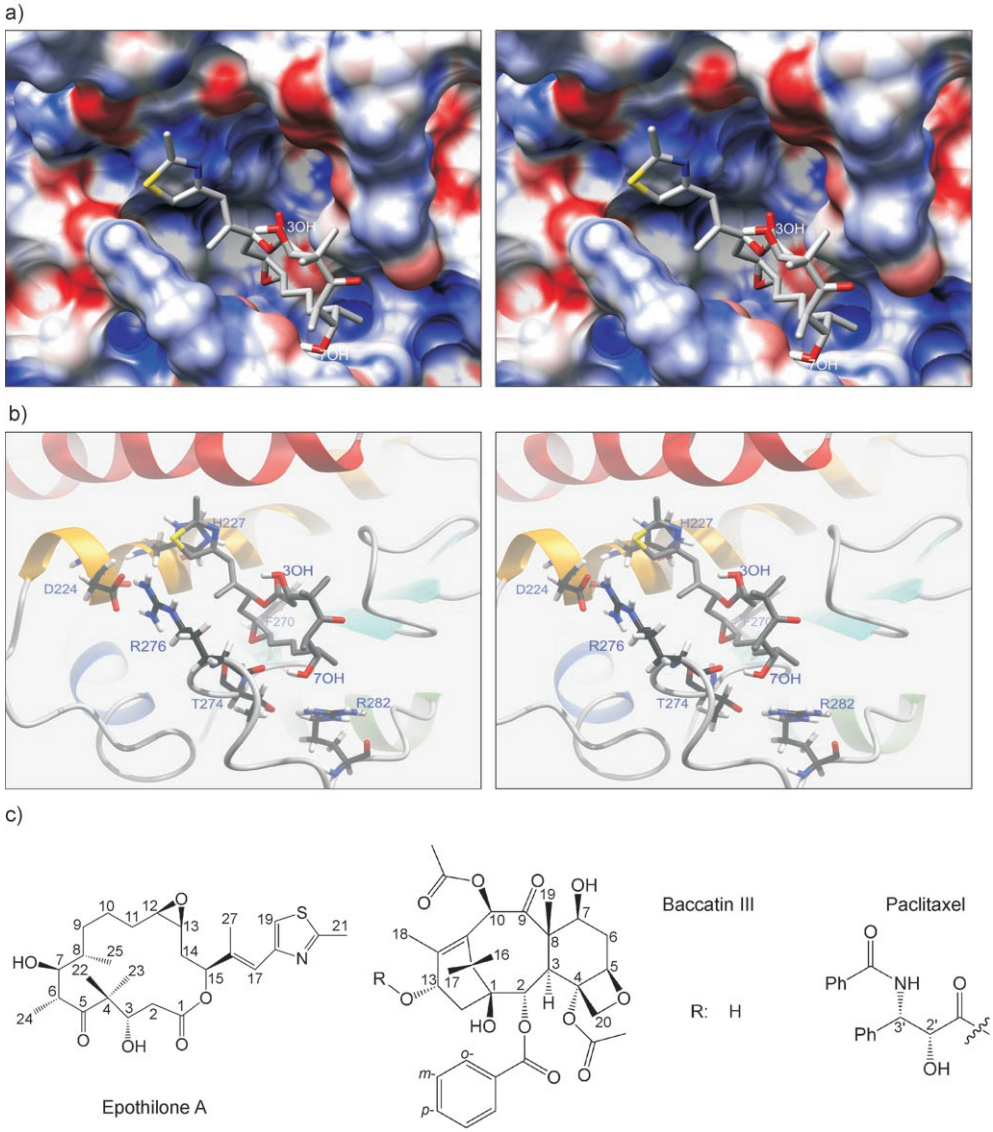

Figure 1. a,b) Stereoviews of the model of the EpoA–tubulin complex that best reproduces the experimental interligand NOEs. EpoA is represented by rods (C gray, H white, N blue, O red, S yellow). In (a), the solvent-accessible surface of tubulin is colored by the electrostatic potential (red: negative, blue: positive). In (b), the binding site on tubulin is represented by rods and ribbons (red: H1 helix, yellow: H7 helix, blue: H6 helix). The thiazole ring of EpoA interacts with the aromatic ring of the H227 residue of tubulin, while the 7OH group of EpoA is situated close to the T274 and R282 residues. The C8–C12 hydrophobic stretch of EpoA is directed towards the hydrophobic floor at the lower right side of the binding site. The aliphatic side chain of the R276 residue entertains hydrophobic contacts with the EpoA side chain, while the guanidinium group of R276 interacts face-to-edge with the thiazole ring. A schematic summary of all the hydrophobic and electrostatic contacts between tubulin and EpoA is given in Figure S3 in the Supporting Information. C) Chemical structures of EpoA (half-maximum inhibitory concentration (IC50) = 3.10 nM), BacIII, and PTX (IC50 = 1.30 nM).[15]

responsible for the very poor solubility of PTX. Although relatively ineffective in promoting tubulin polymerization, BacIII binds to tubulin in the same binding pocket as EpoA and is, therefore, a suitable competitor for EpoA.[16] Additionally, BacIII can be turned into a promoter of MT assembly that competes with PTX for the same binding site by the introduction of an azido group at the *meta* position on the phenyl ring of the benzoyloxy group at C2,[9] which confirms that BacIII binds to tubulin in a specific manner. Herein, 5000 binding modes of each ligand (EpoA and BacIII) to tubulin were generated by molecular modeling and were rated on the basis of the interligand NOEs. Note that we do not assume that the binding modes of BacIII and PTX to tubulin are the same, as the very poor biological activity of BacIII could indicate that this ligand binds to tubulin in a different pose than PTX.

We also measured intermolecular NOEs between EpoA and DCX in the presence of tubulin. We could observe intermolecular NOEs between several regions of the two compounds, confirming that EpoA binds in the same pocket as DCX and PTX. However, owing to the low concentration of DCX (0.05 mM), the interligand NOEs were too weak to be quantitatively interpreted.

The structure of the EpoA–tubulin complex that uniquely satisfies the experimental intermolecularly transferred NOEs is shown in Figure 1 a,b. It represents the NMR-determined binding mode of EpoA to soluble tubulin. We can exclude that the EC-derived binding mode of EpoA to tubulin[14] is present in solution, as this model provides a very poor agreement between calculated and experimental intermolecular NOE data with all BacIII–tubulin binding modes.

The NMR-derived EpoA–tubulin complex offers a good explanation of the SAR data available for epothilone analogues and tubulin mutants. Most importantly, the NMR-derived binding mode of EpoA to tubulin partially resembles the EC-derived binding mode of PTX to polymerized tubulin (Figure 2).[19] In contrast, no features of the pharmacophore of PTX are in common with the EC-derived structure of EpoA with polymerized tubulin.

The position of the thiazole ring in the EpoA–tubulin complex is close to that of the phenyl ring of the benzamido group at C3′ of PTX in the PTX–tubulin complex (Figure 2). The thiazole ring of EpoA and the imidazole ring of the H227 residue are stacked in a face-to-face orientation, though they are tilted at an angle of approximately 30° with respect to one another (Figure 1 b). The two proton-bearing nitrogen atoms of the imidazole ring are situated above the sulfur and nitrogen atoms of the thiazole ring, respectively, allowing a favorable overlap between the delocalized π system of the imidazole ring and the π orbitals at the sulfur and nitrogen atoms of the thiazole ring, which contain a high electron density. The nitrogen atom of the thiazole ring is not involved in any hydrogen bonds, although potentially a water-mediated hydrogen bond could be formed with the side chain of the D24 residue (not shown in Figure 1). In contrast, in the EC-derived model of the EpoA–tubulin complex,[14] the two aromatic rings are situated edge-to-edge, an orientation seldom observed in protein cores,[20] and the nitrogen atom of the thiazole ring accepts a hydrogen bond from the histidine side chain.

SAR data for epothilone analogues with the thiazole ring substituted by different isomeric pyridine moieties revealed the relevance of the nitrogen-atom position in cell assays.[21] A decrease of the cellular potency by approximately one order of magnitude for pyridine-based analogues with the nitrogen atom in positions other than *ortho* to the vinyl linker was interpreted as an indication that the thiazole nitrogen atom is involved in contacts with tubulin side chains. However, the much less pronounced impact of the nitrogen atom observed in tubulin-polymerization assays[22,23] suggests that the relevance of the position of the thiazole nitrogen atom in cellular assays is related to factors other than direct interaction with tubulin. This data does not support the presence of the hydrogen bond between the thiazole ring and the H227 residue proposed in the EC-derived structure of the EpoA–tubulin complex. Instead, the SAR data is in agreement with our model, which predicts a more modest influence of the nitrogen atom on the electronic distribution in the thiazole π orbitals and, therefore, on its face-to-face interaction with the H227 residue.

The R276 residue in the M loop of tubulin runs along the C15–C18 hydrophobic stretch of the EpoA side chain, terminating in a face-to-edge interaction between the guanidinium group and the thiazole ring; a salt bridge is formed between the positively charged arginine residue and the negatively charged D224 residue of the H7 helix (Figure 1 a,b).

Inversion of the stereochemical configuration at C3 is detrimental for the biological activity of epothilones, whereas

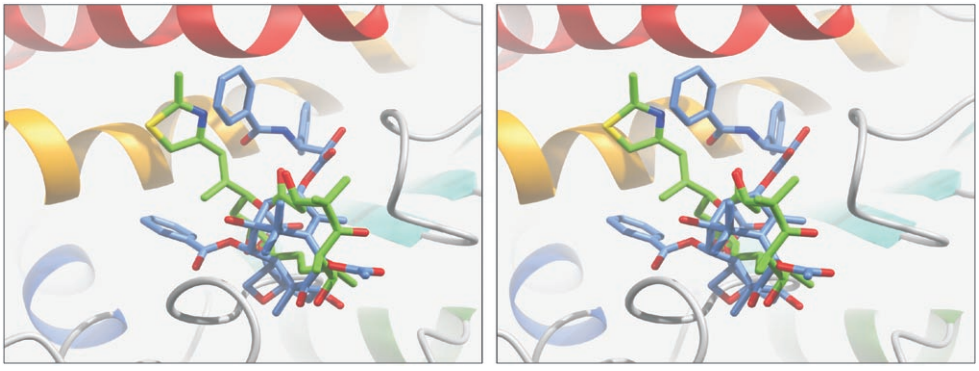

Figure 2. Stereoview of the superposition of the NMR-derived EpoA (rods; C green, N blue, O red, S yellow) binding mode and the EC-derived PTX (rods; C light blue, N blue, O red) binding mode to β-tubulin (ribbons; red: H1 helix, yellow: H7 helix, blue: H6 helix).[19] The thiazole ring of EpoA roughly overlaps with the phenyl ring of the benzamido group at C3′ of PTX. Additionally, the 3OH group of EpoA is close to the 1′CO and 2′OH groups of PTX.

(*E*)-3-deoxy-2,3-didehydroepothilones, which lack the hydroxyl group at C3, retain most of the tubulin-polymerization activity.[24] These data underline the importance of preserving a *trans* configuration of the C1-C2-C3-C4 chain in the bioactive conformation. In our conformation of EpoA, a steric clash of the 3OH group with the H6 atom would destabilize the *trans* configuration in epothilones with inverted stereochemistry at C3. In contrast, in the EC-derived structure of the EpoA–tubulin complex, the C1-C2-C3-C4 torsion angle is close to 90°.

In our NMR-derived model of the EpoA–tubulin complex, the 3OH group is not involved in close contacts with the protein. In the EC-derived model, the presence of a critical hydrogen bond between the 3OH group and the T274 residue is not reconcilable with the high activity of (*E*)-3-deoxy-2,3-didehydroepothilones, which lack the hydroxyl group, or of 3-deoxyepothilones, which lack both the hydroxyl group and the structural constrainment of the additional double bond.[25] Additionally, we observe that the hydrophilic carbonyl and hydroxyl groups at C1′ and C2′ of PTX, respectively, are placed in a similar position to the 3OH group of EpoA, without any evident contacts with tubulin (Figure 2).[19]

The positively charged side chain of the R282 residue entertains extensive electrostatic contacts with the polar 7OH group of EpoA (Figure 3). This interaction locks the side chain of the R282 residue in an optimal position for forming a hydrogen bond with the side chain of the T274 residue, thereby, stabilizing a specific conformation of the tubulin M loop, which might be relevant for microtubule stabilization. The involvement of the T274 and R282 residues in this network of electrostatic interactions provides a rationale for the loss of activity of epothilones in cell lines containing either the T274I or the R282Q mutation (40-fold and 57-fold activity loss, respectively).[10] This pattern of electrostatic interactions is not present in the PTX–tubulin complex, in agreement with the higher tolerance of PTX to the T274I and R282Q mutations.[10]

The epoxide ring of EpoA is situated at the entrance of the hydrophobic pocket hosting the F270 residue; however, EpoA does not enter this pocket, as opposed to PTX, which locates the phenyl ring of its benzoyloxy group at C2 close to F270. In agreement with this observation, epothilones retain activity in cell lines exhibiting the F270V mutation, whereas PTX undergoes a 27-fold decrease in activity.[26]

Most importantly, our structure accounts for the higher potency of EpoB (IC50 = 0.3 nM), which bears a methyl group at C12, with respect to EpoA. Small apolar substituents at C12 increase the activity of epothilones, as they are placed in the hydrophobic pocket hosting the F270 and L369 residues, which is left empty by EpoA. In contrast, the activity difference between EpoB and EpoA is not well explained by the EC-derived structure of the EpoA–tubulin complex, in which a methyl group at C12 of EpoA would be projected towards the protein surface.

Recent work by the Knossow and Nogales laboratories[27,28] suggests that microtubule stabilization occurs through a straightening of the curvature at the interface of the α- and β-tubulin subunits in protofilaments. In particular, a translation of the H7 helix, which drags along the H6–H7 loop and the H6 helix, is the major player in determining the curvature at the interface between the heterodimers.[27] We propose that EpoA induces tubulin polymerization by stabilizing the straight form of the protofilaments, even in the absence of GTP, and that it does so by locking the relative positions of the H7 helix and the M loop. In our structure, EpoA acts as a linker between these two structural elements by mediating a contact between the R276 residue in the M loop and the D224 residue in the H7 helix, and by directly interacting with the H227 residue in the H7 helix and the R282 and T274 residues in the M loop .

In conclusion, the NMR-derived model of the EpoA–tubulin complex provides a rationale for the SAR data available for the drug and establishes a relationship between the functional mechanism of epothilones and their binding mode to tubulin. The differences between the structures of the EpoA–tubulin complex in solution and in zinc-stabilized sheets, and the incompatibility of the EC-derived structure with the NMR spectroscopic data underline the importance of accessing structural data for the EpoA–tubulin complex in different polymerization states. We expect our results to be helpful for the development of improved therapeutic molecules based on the common pharmacophore of EpoA and PTX.

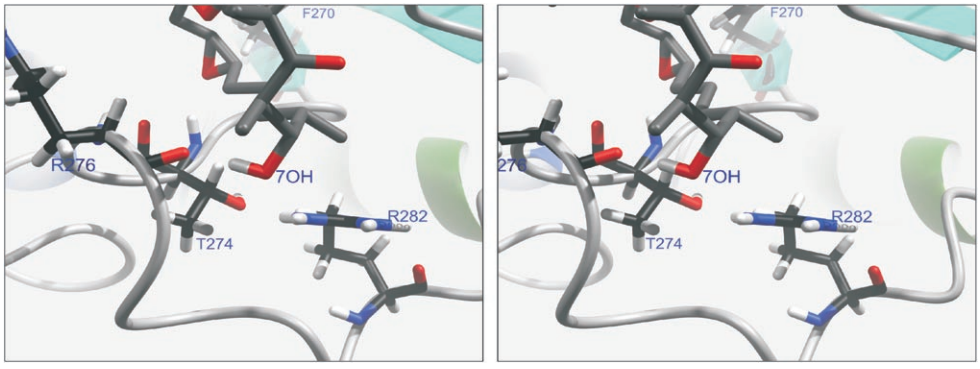

Figure 3. Stereoview of the interactions of the 7OH group of EpoA with the R282 and T274 residues of tubulin (rods; C gray, H white, N blue, O red, S yellow). Electrostatic interactions between the guanidinium group of R282 and the 7OH group of EpoA define the conformation of the M loop of tubulin, which favors the formation of a hydrogen bond between R282 and T274.

Experimental Section

NMR spectroscopy: The sample consisted of a mixture of EpoA (0.6 mM), BacIII (0.6 mM), and tubulin (12 μM; Cytoskeleton Inc., Denver (USA)) in D_2O containing phosphate (3 mM), calcium (1.5 mM), and sodium (ca. 0.7 mM) ions at pH 7.0. Inter-ligand NOEs were measured in NOESY spectra acquired at 900 MHz and 298 K with mixing times of 20, 40, 70, 100, and 200 ms.

Computational analysis: As the experimental system consists of two ligands binding competitively and mutually exclusively

to tubulin, we described the system with a *pair* of structural models, one for the EpoA–tubulin complex and one for the BacIII–tubulin complex. Our approach to obtain a structural model of the EpoA–tubulin complex consisted of three steps: first, we generated 5000 models for each of the two ligands complexed with tubulin and combined them pairwise; second, we calculated the theoretical intermolecular NOEs between EpoA and BacIII for each of the 25000000 pairs; third, we rejected or retained pairs of structures depending on how well the calculated intermolecular NOEs reproduced the experimental ones. After the application of selection criteria to evaluate the agreement between the calculated and experimental NOEs in each pair of models (see the Supporting Information), one family of model pairs was uniquely identified. The binding mode of BacIII to tubulin in this family is discussed in the Supporting Information (Figure S2).

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