Drug Binding

DOI: 10.1002/anie.201001946

Interaction of Epothilone B (Patupilone) with Microtubules as Detected by Two-Dimensional Solid-State NMR Spectroscopy**

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Significant progress has been made in the use of solid-state NMR (ssNMR) spectroscopy for the study of heterogeneous biomolecular systems that are not amenable to conventional techniques in structural biology. For example, ssNMR spectroscopy has been used to structurally characterize amyloid fibrils and small-molecule binding to membrane-embedded proteins (see, for example, Refs. [1,2]). Ligand binding is also of utmost importance in other cellular compartments. For example, a variety of pharmacological compounds have been developed that trigger apoptosis by accelerating the polymerization of αβ-tubulin into microtubules (MTs).[3] MTs exist in a dynamic equilibrium with the nonpolymerized form, tubulin, a heterodimeric protein consisting of one α-tubulin subunit and one β-tubulin subunit.[4] The dynamic behavior of MTs plays a crucial role in cell division; MTs are thus important targets for anticancer-drug design.[5] Tubulin-binding agents, such as Paclitaxel (PTX), are amongst the most widely used chemotherapeutic drugs in cancer therapy. Their efficacy against a variety of human cancers has been successfully demonstrated,[3] and taxanes or related compounds appear promising according to the results of their clinical trials.[6] However, taxanes, such as PTX, are associated with numerous side effects, and are ineffective against several types of cancer.[7] A new class of anticancer compounds, 16-membered-ring macrocyclic lactones known as epothilones, were discovered by Gerth, Höfle, and co-workers from the myxobacterium *Sorangium cellulosum*.[8,9] Epothilones are reported to be more water-soluble than PTX, and to retain cytotoxicity independent of multidrug resistance.[8,10]

Previously, it was demonstrated that PTX and epothilones share a common binding pocket on the β-tubulin surface, and a common pharmacophore for various tubulin-binding agents was hypothesized.[11] However, various efforts, such as molecular modeling and the collation of structure–activity-relationship (SAR) data, have not produced a coherent picture of the binding mode of drugs to tubulin.[12] Electron crystallography (EC)[13] and solution-state NMR spectroscopy[14,15] were used to gain an understanding of the mode of interaction of epothilones with αβ-tubulin on a structural level. In the EC approach, a complex of epothilone A (epoA) with αβ-tubulin polymerized in zinc-stabilized sheets was studied at a resolution of 2.9 Å. The results suggested that ligands with different chemical structures exploit the tubulin-binding pocket in a unique and independent manner.[13] Solution-state NMR spectroscopy of epoA interacting with nonpolymerized αβ-tubulin suggested a common pharmacophore for Paclitaxel and epothilone. Both the model derived by NMR spectroscopy and the EC structure were discussed with respect to existing SAR data.[15]

Herein, we show the utility of ssNMR spectroscopy for the direct inference of information about the binding of the drug, in this case epothilone B (patupilone; Figure 3c), to the biologically relevant polymerized form of tubulin: microtubules. Previously, we studied the structure of free patupilone in the microcrystalline state.[16] Patupilone, which differs from epoA through the presence of a methyl group at C12, is a more potent microtubule stabilizer than epoA and PTX.[11] Our results showed that the structure of patupilone in its crystalline form is identical to the structure of patupilone as an amorphous powder; however, there are significant differences between the ssNMR spectrum and the NMR spectrum recorded in dimethyl sulfoxide (DMSO).[16] Since the 3D structure determined by ssNMR spectroscopy[16] largely agrees with previously reported structures, such differences would be consistent with a general ability of patupilone to be engaged in intermolecular interactions in the solid state through crystal contacts.

The MT/patupilone complexes investigated by ssNMR spectroscopy were well-ordered intact tubules, as seen by

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[**] This research was funded by the Volkswagen Foundation.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201001946.

electron microscopy (Figure 1a). We estimate that the resulting complex contained labeled patupilone in a 1:10[4] ligand/protein ratio (w/w), with a total amount of approximately 0.05 mg of patupilone. Thus, approximately 30 αβ-tubulin heterodimers were present in the complex per patupilone molecule. Under such conditions, the use of double-quantum-filtering (DQF) techniques facilitates the unambiguous detection of ligand signals.[2,17]

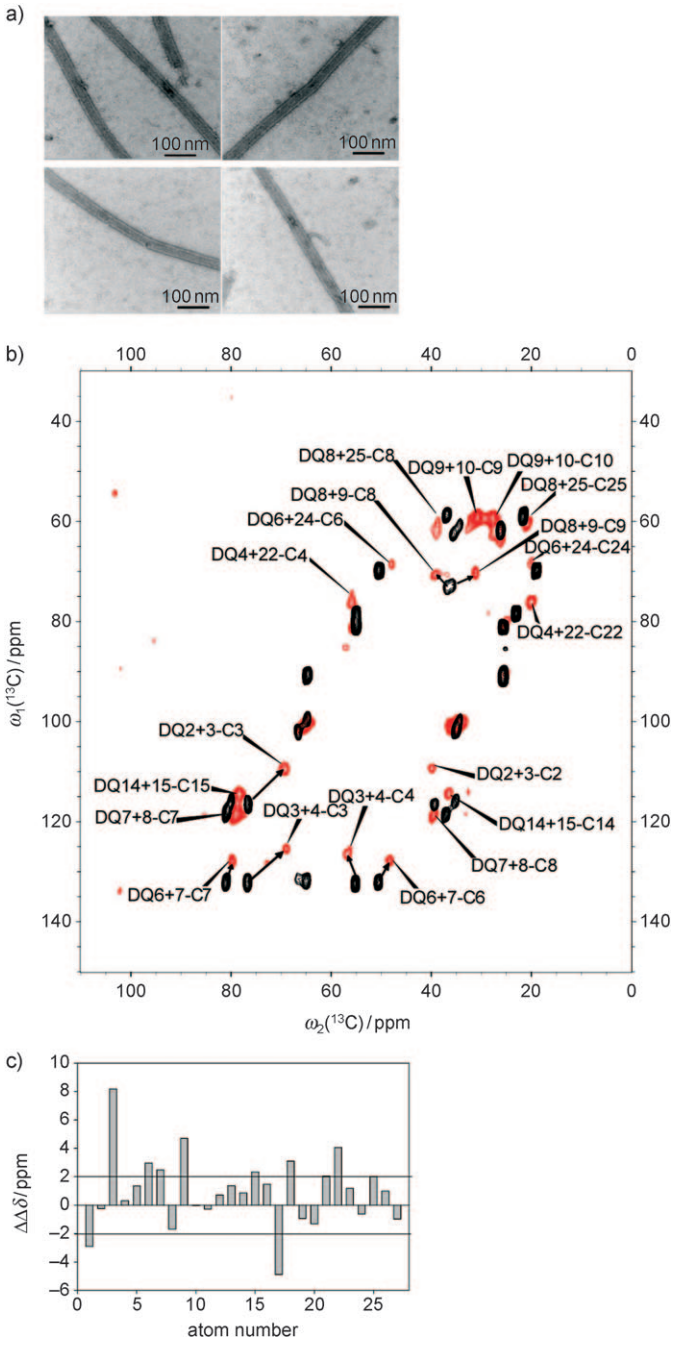

Figure 1. a) TEM image of microtubules complexed with patupilone. b) Aliphatic region of 2D ^{13}C–^{13}C (2Q,1Q) NMR correlation spectra for free patupilone (black) and patupilone in a complex with MT (red). Cross-correlations for different atoms and strong signal shifts are indicated. c) Chemical-shift deviation of each carbon atom in patupilone upon binding to MT. The horizontal lines indicate approximately four times the (one-quantum) ^{13}C line width. This value is relevant for evaluation of the significance of chemical-shift differences.

In Figure 1b, we compare data obtained for free (black) and complexed (red) patupilone. In both cases, high-resolution ssNMR spectra were obtained, and a single set of ssNMR resonances was apparent. The observation of a single set of ssNMR resonances suggests that patupilone—MT interactions are characterized by strong binding, in line with recent ssNMR studies of protein binding to MTs,[18] the results of which suggested that patupilone–MT interactions are characterized by strong binding. Indeed, fluorescence-based cellular assays showed very strong binding of patupilone to microtubules, with a K_d value of (36.04 ± 1.58) nM; for comparison, PTX has a K_d value of 100 nM.[19]

Cross-correlations originating from directly dipolar coupled ^{13}C resonances of patupilone in the complex were readily identified (red) by comparison with the spectrum of free patupilone (black) on the basis of ssNMR assignments reported earlier[16] and confirmed by a sequential walk through the 2D spectrum. Figure 1c shows a plot of chemical-shift deviations for patupilone in the complex with tubulin for each ^{13}C position. For several resonances, we detected perturbations that considerably exceeded the ^{13}C line width. Four carbon positions, namely, C3, C9, C17, and C22, exhibited a chemical-shift perturbation larger than 3 ppm, and an additional six positions (C1, C5, C6, C8, C15, and C18) showed a perturbation of more than 2 ppm.

On the basis of previous EC and solution-state NMR spectroscopic results, we subsequently conducted a structural analysis of the observed chemical-shift changes. Figure 2 shows the binding mode of epoA with αβ-tubulin as determined by EC (Figure 2a)[13] and NMR spectroscopy (Figure 2b).[15] Atomic positions for which ssNMR chemical-shift perturbations were observed that were larger than 3 ppm or in the range 2–3 ppm are colored red and orange, respectively.

The chemical-shift changes observed between the microcrystalline and the MT-bound form of patupilone advocate a unique tight interaction between the drug and tubulin. Such chemical-shift changes may result from direct changes in the conformation of epothilone or may be due to alterations in the interaction network. For example, C3 showed the largest chemical-shift change of more than 7 ppm upon binding. In the crystal, the OH group at this position forms a hydrogen bond with the epoxide at C12, C13.[9] This interaction is manifested in a change in the chemical shift of 4 ppm relative to that observed for patupilone dissolved in DMSO.

In the EC model of the MT-bound form, the 3-OH group and the side-chain OH group of T274 of tubulin form a hydrogen bond with high affinity (Figure 3a). In the structure determined by NMR spectroscopy, the OH group faces the solvent, but a conformational change with respect to the solid-state structure upon binding to tubulin rotates the C–OH bond parallel to the carbonyl C1–O double bond (Figure 3b). This conformational change, as well as the difference in the chemical nature of an epoxide oxygen atom and the oxygen atom of a hydroxy group, could account for the change in the chemical shift of C3. The observed chemical shift is thus equally well explained by the EC or NMR structure. From

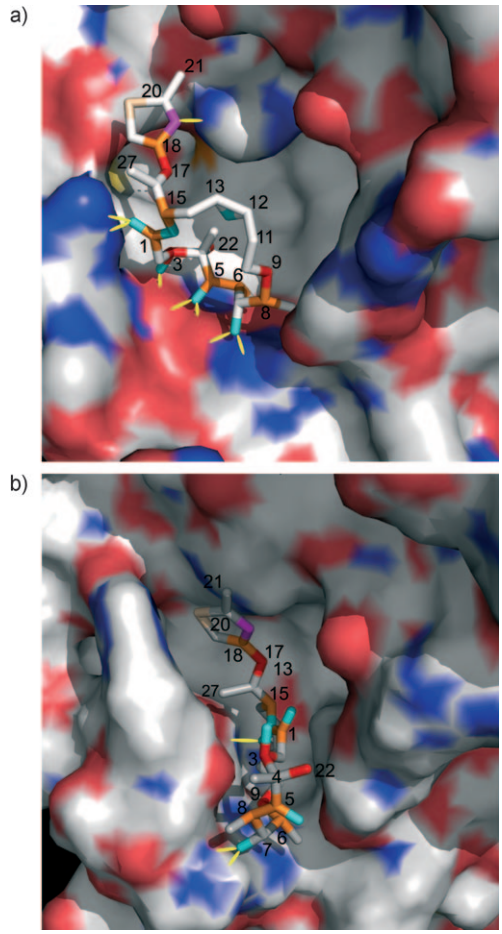

Figure 2. Views of the epoA–tubulin complex showing the different orientations of epoA in the tubulin pocket for the structures derived from a) EC and b) solution-state NMR spectroscopy (see also the Supporting Information). For atoms colored red and orange, an ssNMR chemical-shift deviation of more than 3 ppm and more than 2 ppm was observed, respectively; magenta: N, yellow: S, cyan: O. The charge surface of tubulin is shown as positive (blue), negative (red), and hydrophobic (gray). Yellow lines show polar contacts between epoA and tubulin.

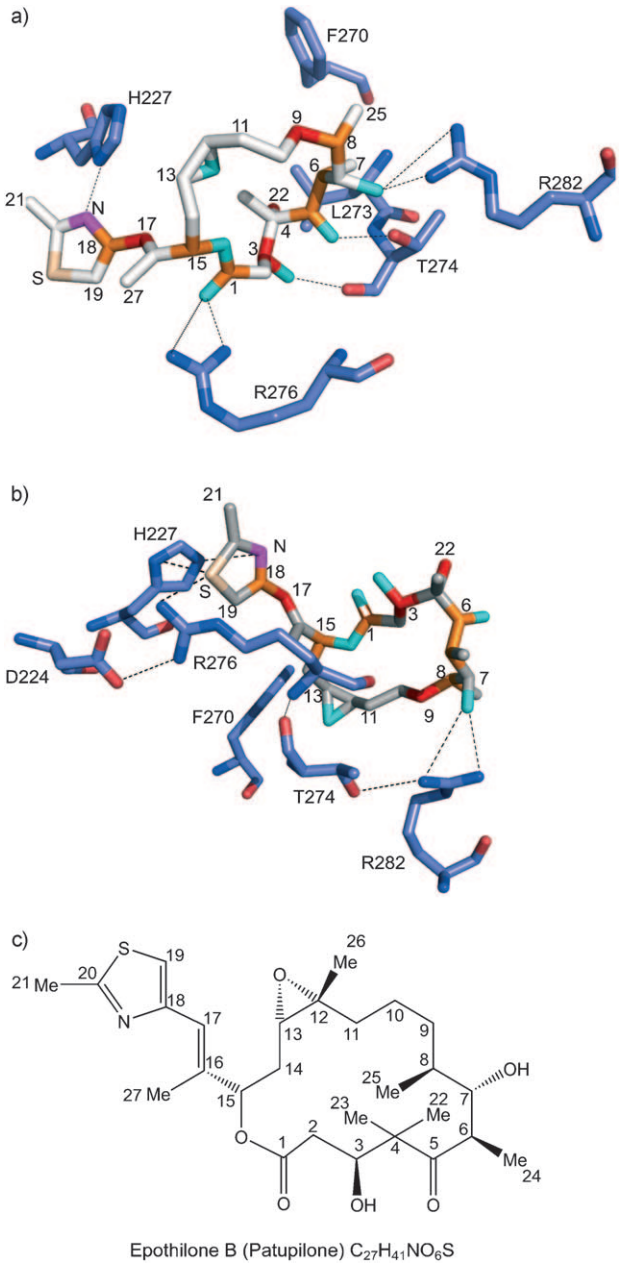

Figure 3. Interactions between epoA and tubulin. a) Dotted lines indicate hydrogen bonds between OH groups of epoA and various amino acids of tubulin in the EC-derived model. b) Dotted lines indicate interactions between epoA and various amino acids of tubulin in the model derived by NMR spectroscopy. For atoms of epoA colored red and orange, an ssNMR chemical-shift deviation of more than 3 ppm and more than 2 ppm was observed, respectively; magenta: N, yellow: S, cyan: O. c) Chemical structure of patupilone.

SAR data, the importance of the C3 hydroxy group is still controversial. The replacement of C3–OH with a cyano group, which would be a hydrogen-bond acceptor in the EC model, reduces the polymerization activity of patupilone.[20] On the other hand, (*E*)-2,3-dehydroepothilones, which lack the C3 hydroxy group, are equally efficient in polymerization acceleration and even in cancer-cell models, and retain the bound conformation; these observations call the importance of this group into question.[21,22]

We also observed large chemical-shift changes for atoms C17 and C18 of patupilone, which are near the nitrogen atom of the thiazole ring. Both findings can be readily explained by hydrogen-bonding interactions, as postulated in the EC study. According to EC, this moiety forms a hydrogen bond with H227 (Figure 3a). However, this hydrogen bond is controversial in terms of SAR data. Although drug-induced mutations in cancer cells hinted at a hydrogen bond,[22] the unchanged effect of epothilone derivatives with altered nitrogen-atom positions in the benzothiazole or in the quinoline ring upon tubulin polymerization appears to be incompatible with a hydrogen bond to the nitrogen atom.[23,24] However, the model derived from NMR spectroscopy proposes a direct interaction between the guanidinium side chain of R276 and the thiazole ring of patupilone. Such an interaction could also explain the chemical-shift changes observed for atoms C17 and C18. Thus, the large chemical-

shift changes observed for these resonances are again in agreement with both models.

Finally, C10–C12 exhibit very similar chemical shifts in the free crystal and in the MT-bound form. These atoms are in a hydrophobic environment in the free crystal. According to the EC model, they are exposed to water in the tubulin complex and thus are expected to exhibit chemical-shift changes. By contrast, according to the NMR model, they do not change environment from the free crystal to the MT-bound form, as the hydrophobic side chain of R276 is in close proximity to these atoms. This model would be more in line with our experimental observations.

The chemical-shift information presented herein cannot unambiguously resolve the apparent differences between earlier models derived by EC and solution-state NMR spectroscopy of the epoA–MT complex. However, we have identified atomic positions in the drug that undergo clear changes in their chemical shift upon MT binding. Such information is useful for further pharmacological optimization and provides the basis for refinement of the binding mode of patupilone, for example, through the comparison of ssNMR chemical-shift values with data computed from first principles (see, for example, Ref. [25]).

Experimental Section

Sample preparation: ^{13}C-labeled patupilone was obtained from the myxobacterium *Sorangium cellulose* So ce90 as described previously.[9] A polycrystalline sample was prepared by dissolving [^{13}C]patupilone (3 mg) and unlabeled patupilone (18 mg)in methanol/water (3:1).[16] Tubulin purified by phosphocellulose chromatography (PC-tubulin), including a MAP-depleting step (MAP = microtubule-associate protein), was prepared as described previously.[26] Tubulin (40 mg mL^{-1}, 1 mL) and guanosine triphosphate (1 mM) were incubated at 37 °C for 5 min to initiate microtubule polymerization. Isotope-labeled patupilone (5.4 mg) dissolved in DMSO (50 μL) was then added to a final concentration of 10 mM, and polymerization was continued for 20 min. Microtubules-containing labeled patupilone were obtained at a concentration of approximately 400 μM.

ssNMR spectroscopy: All NMR spectroscopic experiments were performed on a wide-bore 600 MHz (^{1}H resonance frequency) spectrometer with a 4 mm double-channel (^{1}H, ^{13}C) probe head. Experiments on polycrystalline patupilone were conducted at approximately 5 °C (effective temperature), whereas experiments involving the patupilone–MT complex were performed at approximately −35 °C; that is, with a frozen solution. The magic-angle-spinning rate was 7 kHz; two-pulse phase modulation[27] at a 105 kHz radio-frequency amplitude was used for proton decoupling during free-evolution and detection. Ramped radio-frequency proton pulses for 600 μs were used for cross-polarization. For the spectrum of MT-bound patupilone, 175 t_1 experiments with a maximum t_1 value of 1.785 ms were carried out with 1664 scans per slice. POST-C7[28] was used in DQF experiments during DQ excitation and reconversion periods. ^{13}C resonances were calibrated by using adamantane as an external reference and setting its upfield resonance to 31.47 ppm.

Received: April 1, 2010
Revised: June 14, 2010
Published online: August 31, 2010

Keywords: antitumor agents · drug binding · microtubules · NMR spectroscopy · structure elucidation

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