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The Binding Mode of Side Chain- and C3-Modified Epothilones to Tubulin

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The tubulin-binding mode of C3- and C15-modified analogues of epothilone A (Epo A) was determined by NMR spectroscopy and computational methods and compared with the existing structural models of tubulin-bound natural Epo A. Only minor differences were observed in the conformation of the macrocycle between Epo A and the C3-modified analogues investigated. In particular, 3-deoxy- (compound **2**) and 3-deoxy-2,3-didehydro-Epo A (**3**) were found to adopt similar conformations in the tubulin-binding cleft as Epo A, thus indicating that the 3-OH group is not essential for epothilones to assume their bioactive conformation. None of the available models of the tubulin–epothilone complex is able to fully recapitulate the differences in tubulin-polymerizing activity and microtubule-binding affinity between C20-modified epothilones **6** (C20-propyl), **7** (C20-butyl), and **8** (C20-hydroxypropyl). Based on the results of transferred NOE experiments in the presence of tubulin, the isomeric C15 quinoline-based Epo B analogues **4** and **5** show very similar orientations of the side chain, irrespective of the position of the nitrogen atom in the quinoline ring. The quinoline side chain stacks on the imidazole moiety of β-His227 with equal efficiency in both cases, thus suggesting that the aromatic side chain moiety in epothilones contributes to tubulin binding through strong van der Waals interactions with the protein rather than hydrogen bonding involving the heteroaromatic nitrogen atom. These conclusions are in line with existing tubulin polymerization and microtubule-binding data for **4**, **5**, and Epo B.

Introduction

Epothilones are 16-membered bacterial macrolides that block cell proliferation through the alteration of the dynamic equilibrium between tubulin heterodimers and microtubules (MTs).[1] Similar to the established clinical antitumor agent paclitaxel (Taxol®), epothilones induce tubulin polymerization in vitro and stabilize MTs even under normally destabilizing conditions.[2,3] At the cellular level, interference with MT functionality leads to mitotic arrest in the G_2–M phase of the cell cycle and induction of apoptosis, thus making epothilones powerful lead structures for anticancer drug discovery. As demonstrated by competition experiments with radioactive paclitaxel[2,3] or a fluorescently labeled paclitaxel derivative,[4] epothilones bind to the paclitaxel binding site of MTs with moderate to high affinity. Apparent K_i values of 1.4 μM[2] (0.6 μM)[3] and 0.71 μM[2] (0.4 μM)[3] at 37 °C have been reported for epothilone A (Epo A, **1a**; Figure 1) and epothilone B (Epo B, **1b**; Figure 1), respectively; affinity constants for binding to stabilized MTs have been determined at 2.93×10^7 and 6.08×10^8 M^{-1} for Epo A and B, respectively.[4] However, in contrast to paclitaxel, epothilones are also active in vitro against multidrug-resistant human cancer cells,[3,5] and they inhibit the growth of paclitaxel-resistant tumors in vivo.[5] Furthermore, they exhibit superior physicochemical properties over paclitaxel, such as higher water solubility.[6]

Since the elucidation of their relative and absolute configuration in 1996[6] a large number of total synthesis routes[7] have been developed for natural epothilones, and hundreds of synthetic analogues and semisynthetic derivatives were prepared

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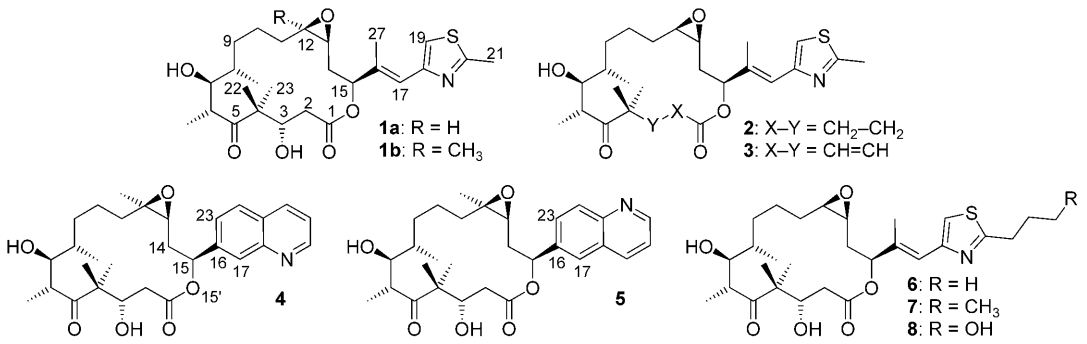

Figure 1. Structures and atom numbering of Epo A (**1a**), Epo B (**1b**), 3-deoxy-Epo A (**2**), 3-deoxy-2,3-didehydro-Epo A (**3**), quinoline-based Epo B analogues **4** and **5**, and 20-propyl-Epo A (**6**), 20-butyl-Epo A (**7**), and 20-hydroxypropyl-Epo A (**8**).

for SAR studies.[8] The most advanced of these compounds recently received regulatory approval in the US (ixabepilone, Ixempra®),[9,10] while several others are currently in advanced clinical trials.[11]

Ever since the first report on the tubulin-polymerizing activity of epothilones[2] there has been an intense debate over the molecular basis of their interactions with the tubulin/MT system.[12] Based on the observation of overlapping binding sites between paclitaxel and epothilones,[2–4] several pharmacophore models were developed,[13–16] each of which can reproduce some aspect of the epothilone SAR. At the same time, experimental studies have indicated a high level of structural similarity for epothilones between the crystalline (single-crystal X-ray structure),[6] polycrystalline,[17] and solution state in organic solvents.[6,18–20] The conformational preferences of Epo A (**1a**) in aqueous solution partially differ from those in organic solvents;[21] importantly, they were found to include the tubulin-bound conformation that was derived from solution NMR experiments with non-polymerized tubulin,[22] which is significantly populated by the free ligand in an aqueous environment. On the other hand, a distinctly different structure has been suggested for Epo A (**1a**) when bound to Zn^{2+}-stabilized polymeric tubulin sheets on the basis of electron crystallographic (EC) studies at intermediate resolution (2.9–4.2 Å).[23] These latter studies have also raised doubts about the presence of a common pharmacophore between paclitaxel and epothilones. While this has called into question the various pharmacophore models that had been developed prior to the disclosure of the EC-based structure of the Epo A–tubulin complex,[12] the hypothesis of a common pharmacophore between paclitaxel and epothilones has been re-strengthened recently by an extensive computational study.[24]

SAR studies with epothilones have so far largely focused on the effects of structural modifications on tubulin polymerization, MT binding, in vitro cellular activity, and in vivo antitumor activity, with comparably little attention paid to possible changes in the tubulin-bound conformation that might be associated with these structural alterations. One notable exception is the work performed by Taylor and co-workers,[18,20] who carefully investigated the activity and conformational properties of a series of variously methylated epothilone analogues. However, this work did not include the direct observation or computational modeling of these analogues as part of a tubulin–epothilone complex. In the work reported herein, we investigated the tubulin-bound conformation of a series of epothilone analogues by means of solution NMR spectroscopy, under conditions that were previously used to determine the bioactive conformation of Epo A (**1a**), that is, by transferred NOE measurements of mixtures of soluble tubulin oligomers and an excess of weakly bound ligand.[22] This approach enables a direct comparison of the tubulin-bound structures of the structural analogues with that of the unmodified natural product. Following refinement by density functional theory (DFT) calculations, the structures were also docked into the tubulin binding cleft starting from the previously described orientation of bound Epo A (**1a**) as derived by the INPHARMA method.[21a]

As changes in the protein-bound conformation of bioactive ligands would be expected to be associated with changes in their biological activity, the compounds investigated span a range of tubulin-polymerizing and/or antiproliferative activities. This includes analogues with activity similar to that of the corresponding natural epothilone, but also compounds with significantly decreased cellular potency. Specifically, NMR techniques were employed to investigate 3-deoxy-Epo A derivatives **2** and **3** and the quinoline-based Epo B analogues **4** and **5** (Figure 1). In addition, computational studies were performed for the C20-propyl, -butyl, and -hydroxypropyl analogues **6**, **7**, and **8**, respectively (Figure 1).

We previously determined the conformational properties of analogues **2** and **3** free in aqueous solution, which we found to be very similar to those of Epo A (**1a**) in both cases.[22] Overall, the conformation of **1a**, **2**, and **3** in water closely resembled the NMR-derived structure of tubulin-bound Epo A (**1a**);[21] in particular, a strong preference was observed for an antiperiplanar C1–C2–C3–C4 torsion, even in the case of **2**. These findings suggested that **2** and **3** would also show similar *tubulin-bound* conformations as Epo A (**1a**), but this hypothesis needed to be confirmed experimentally.

Quinoline-based Epo B analogues **4** and **5** belong to a class of side-chain-modified epothilone variants that have generally been found to retain the *tubulin-polymerizing* potency of the corresponding parent natural product, independent of the position of the nitrogen atom in the heterocycle.[25] Thus, both **4** and **5** are potent inducers of tubulin polymerization and they

both show similar growth inhibitory activity against human cancer cell lines as Epo B.[25,26] This is in contrast with the corresponding monocyclic pyridine-based Epo B analogues (for which the pyridine ring simply replaces the thiazole moiety in Epo B), where the 4-pyridyl derivative is clearly less active than the 2-pyridyl analogue (the nitrogen atom of which occupies the same position as the nitrogen in the thiazole ring of natural Epo B).[27] Contrary to these earlier observations on tubulin polymerization induction, however, more recent findings have shown that the position of the nitrogen atom in the quinoline side chain of **4** and **5** does affect the *MT binding affinity* of these analogues, with **4** binding to stabilized, cross-linked MTs with ~10-fold higher affinity than **5**. To understand how the interactions of the quinoline side chain with β-tubulin might differ between **4** and **5**, we determined the conformational preferences of the C15 side chain of these analogues in the tubulin-bound state, employing transferred NOEs;[28] in addition, the experimental approach was complemented by ab initio calculations.

SAR studies on C21-modified analogues of epothilones have pointed to a clear size limitation for the substituent at the 2-position of the thiazole ring, with bulkier groups leading to significantly decreased biological activity.[8] For example, C20-*tert*-butyl-Epo B has been reported to be a significantly less potent tubulin-polymerizing and antiproliferative agent than Epo B (with a C20-methyl substituent).[8b] However, in general, the conclusions about a negative correlation between the size of the C20 substituent and the biological activity of the corresponding epothilone analogues are derived from data scattered throughout the literature, and no systematic and directed study on this question has been performed. Using computational methods we have now interrogated the existing structural models of tubulin-bound epothilones for their ability to rationalize the apparent inverse relationship between the size of the C20 substituent and tubulin-polymerizing as well as cellular activity. To provide a sound experimental basis for these studies we prepared Epo A (**1 a**) analogues **6**–**8**, which incorporate a C20-propyl, -butyl, and -hydroxypropyl substituent, respectively, and we determined their effects on tubulin polymerization, their binding constants for stabilized microtubules, and their in vitro growth inhibitory activity. Quite surprisingly, none of these analogues has been investigated previously and neither have the corresponding Epo B derivatives; in contrast, C20-ethyl-Epo B (Epo B_{10}) has been shown to be virtually equipotent with Epo B.[29]

Results and Discussion

C3-modified epothilones

In a previous study, only small differences had been observed between **2**, **3**, and Epo A (**1 a**) in their EC_{50} values for the induction of tubulin polymerization, thus suggesting that all three compounds can promote the assembly of soluble tubulin into MT-like polymers with similar efficiency.[22] These findings have been reconfirmed in this study, even if the EC_{50} value for **2** is somewhat higher than previously reported (Table 1).[22] In contrast, more distinct activity differences are present between **2**, **3**, and Epo A (**1 a**) at the level of cancer cell growth inhibition, with analogue **2** being clearly less active than **3** and, in particular, Epo A (**1 a**) (Table 2). While the diminished cellular potency of **2** (relative to **3** and Epo A) is in line with the trend shown by the tubulin polymerization data, the magnitude of the differences is still striking (e.g., the ratio of IC_{50} values for **2** and Epo A against the MCF-7 breast cancer cell line is ~20, versus a less than twofold difference in EC_{50} values for tubulin polymerization). Similar discrepancies between tubulin-polymerizing capacity and antiproliferative activity have also been observed in other studies and may be caused by the modulation of cellular activity by parameters unrelated to ligand–target interactions, such as cellular uptake or intracellular distribution of ligands. At the same time, it should be noted that EC_{50} values for tubulin polymerization induction are most valuable for the unequivocal identification of compounds with poor tubulin assembly properties, while they are less suited for the high-resolution quantitative differentiation between potent assembly inducers (at least under the conditions employed in our experiments). To address this issue, we also determined the MT binding constants of compounds **2** and **3** using methodology that was developed previously in one of our laboratories and involves the displacement of the fluorescent paclitaxel derivative Flutax-2 from stabilized MTs.[30]

Table 1. Interactions of compounds **1**–**8** with tubulin/microtubules.

Compd	EC_{50} [μM][a]	K_b MTs [10^7 M^{-1}][b]	ΔG^0_{app} MT [kJ mol^{-1}][c]	ΔH^0_{app} MT [kJ mol^{-1}][d]	ΔS^0_{app} MT [J mol^{-1} K^{-1}][e]
1 a[f]	3.9 ± 0.6	3.63 ± 0.5	−44.5 ± 0.3	−65 ± 2	−68 ± 9
1 b[f]	3.0 ± 0.3	75.0 ± 7.4	−52.6 ± 0.5	−70 ± 7	−60 ± 23
2	7.2 ± 1.2	0.581 ± 0.051	−39.9 ± 0.2	−39 ± 10	5 ± 31
3	4.3 ± 0.4	1.7 ± 0.5	−42.6 ± 0.6	−66 ± 25	−69 ± 80
4[g]	3.2 ± 0.4	91.8 ± 13.2	−52.8 ± 0.3	ND[h]	ND[h]
5[g]	4.3 ± 0.8	6.92 ± 0.22	−46.2 ± 0.3	ND[h]	ND[h]
6	5.0 ± 0.3	2.2 ± 0.5	−43.3 ± 0.5	−42 ± 19	7 ± 61
7	8.1 ± 0.9	1.2 ± 0.2	−41.7 ± 0.4	−10 ± 3	103 ± 11
8	9.2 ± 2.1	0.245 ± 0.014	−37.7 ± 0.1	−21 ± 4	56 ± 15

[a] Concentration required to induce 50% of maximal tubulin polymerization at 25 °C (10 μM porcine brain α/β-tubulin). EC_{50} values of 5.6, 4.8, and 4.6 μM were reported in ref. [22] for **2**, **3**, and Epo A (**1 a**), respectively. In our hands, these differences are within the normal variability of the experiments, especially when different batches of tubulin are involved. For all three compounds the SDs for the EC_{50} values determined in the current study and those reported in ref. [22] overlap. For compound **5** the EC_{50} value obtained for Epo A (**1 a**) in the corresponding (parallel) control experiment was 4.4 ± 0.5 μM. [b] Association constant with glutaraldehyde-stabilized MTs at 35 °C, as determined by the displacement of Flutax-2.[30] [c] Binding free energy for the association with glutaraldehyde-stabilized MTs. [d] Change in enthalpy for the association with stabilized MTs. [e] Entropy change for the association with stabilized MTs; errors are SEM for all parameters. [f] Thermodynamic data for MT binding from ref. [4]. [g] Data from ref. [26]. [h] Not determined.

As illustrated by the data summarized in Table 1, the unsaturated epothilone analogue **3**, in which a torsion angle of 180° about the C2–C3 bond (as suggested for the NMR-derived bioactive conformation of Epo A) is enforced by an *E* double bond, binds to MTs with almost the same affinity as Epo A (**1 a**) ($\Delta\Delta G^0_{app} \approx 2$ kJ mol^{-1}). In addition, both compounds show virtually identical ΔH^0_{app} and ΔS^0_{app} values (Table 1), which clearly suggests that a 3-OH group is not an essential requirement for the high-affinity binding of epothilone-type macrolides to MTs. Binding affinity is decreased for analogue **2**, which binds to stabilized microtubules with an affinity constant (K_b) of 5.81×10^6 M^{-1}, compared with 36.3×10^6 M^{-1} for Epo A (**1 a**); thus, at 35 °C 3-deoxy-Epo A (**2**) binds to cross-linked microtubules with about sixfold lower affinity than Epo A (**1 a**), corresponding to a difference in apparent binding free energy of ~5 kJ mol^{-1} (Table 1). Interestingly, the ΔS^0_{app} value for the binding of **2** to stabilized MTs is significantly more positive than for Epo A (**1 a**), while the enthalpy change associated with the transition from the free to the bound state is clearly less favorable for **2**. No straightforward explanation is possible for these changes in thermodynamic parameters, which represent the combined changes associated with each individual systems component, including protein, ligand, and solvent. Given the very similar conformations of **1 a**, **2**, and **3** both free in solution and in the tubulin-bound state (see below), it seems unlikely that changes in ligand conformation per se strongly contribute to the differences in behavior between **1 a** (or **3**) and **2**.

As discussed above in the Introduction, previous conformational studies on analogues **2** and **3** free in aqueous solution in both cases had revealed similar torsion angle distributions as for natural Epo A (**1 a**) across the entire structure.[22] Using transferred-NOE-restrained simulated annealing calculations[31] followed by DFT refinement, we have now also determined the conformations of **2** and **3** in the tubulin-bound state (with the DFT-refined structures representing the closest energy minima to the NMR-derived conformations).

The tubulin-bound structures of **2** and **3** are almost superimposable with the NMR-based tubulin-bound structure of Epo A (**1 a**) (figure S1), in close analogy to our previous observations for the free solution state;[22] in addition, docking of the bound structures of **2** and **3** to tubulin indicated that both compounds are able to engage in similar contacts with the protein as are found for Epo A (**1 a**) (Figure 2 and figures S2 and S3). Specifically, this includes: a) an electrostatic interaction between the C7-OH group of the epothilone macrocycle and Arg282 of β-tubulin, b) solvent shielding of the hydrophobic C15–C18 region due to close (hydrophobic) contacts with Arg276 of the M loop of tubulin, c) a face-to-face orientation of the thiazole moiety and the imidazole ring of His227 (π stacking), and d) hydrophobic contacts between the C8–C12 part of the macrolide ring and the floor of the binding cleft. Hence, the removal of the 3-OH group in Epo A (**1 a**) does not lead to changes in the bioactive conformation, independent of the absence (compound **2**) or presence (compound **3**) of a conformational constraint about the C2–C3 bond. Collectively, our data indicate that **1 a**, **2**, and **3** all assume a single bioactive conformation, for which the 3-OH group (in the case of Epo A) is predicted not to engage in direct interactions with tubulin. At the same time, notable differences are observed in MT binding affinity between analogue **2** and Epo A (**1 a**). While these differences are of limited magnitude and may be rationalized without having to invoke specific interactions of the 3-OH group with tubulin, our data do not definitively exclude alternative tubulin-binding modes for epothilones, especially in light of the limited accuracy of the tubulin structure used in our previous INPHARMA studies.[21a]

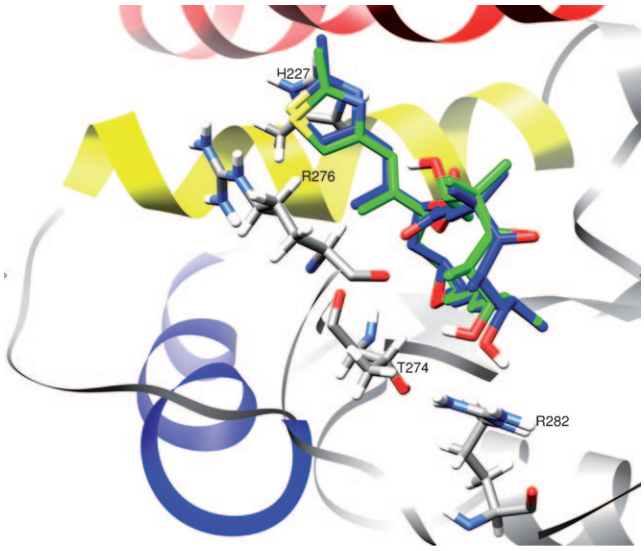

Figure 2. The structures of *E*-3-deoxy-2,3-didehydro-Epo A (**3**, blue) and Epo A (**1 a**, green) docked into the paclitaxel binding site of β-tubulin. The binding site is represented by ribbons (H1 helix: red, H6 helix: blue, H7 helix: yellow). Amino acids discussed in the text are labeled. A face-to-face orientation of the thiazole ring of **3**/**1 a** and the imidazole side chain of β-His227, as well as the close proximity of the C7-OH group of the macrolide ring to Arg282 are clearly discernible. Hydrophobic contacts occur between the C15–C18 region of the macrocycle and the aliphatic portion of the Arg276 side chain and between the C8–C12 part of the ligands and the hydrophobic floor of the binding cleft. The docked structure of **2** and the comparison of the tubulin binding modes of **1 a** and **2** are shown in the Supporting Information (figure S2).

C15-modified epothilones

Quinoline-based Epo B analogues **4** and **5** were previously found to exhibit highly potent antiproliferative activity similar to that of the parent compound Epo B (**1 b**)[25,26] (Table 2). The cellular activity of these analogues is independent of the position of the nitrogen atom in the quinoline ring system, in contrast with the effects observed for the corresponding isomeric pyridyl epothilones, for which Epo B-like activity is observed only if the nitrogen atom is positioned *ortho* to the vinyl linker between the heterocycle and the macrolide ring.[27] In addition, **4** and **5** were also concluded to exhibit similar tubulin-polymerizing activity, based on the corresponding EC_{50} values (Table 1).[25,26] This put into question the notion of specific hydrogen bonding between the heterocyclic nitrogen atom and β-His227, as has been proposed for the EC-derived structural model of the tubulin–Epo A complex (with the thiazole nitro-

Table 2. Antiproliferative activity of Epo A/B (**1a**/**1b**) and of epothilone analogues **2**–**8**.

Compd	IC_{50} [nM][a]		
	MCF-7 (breast)	PC-3M (prostate)	HCT-116 (colon)
1a	2.9 ± 0.3	6.4 ± 1.5	2.8 ± 0.4
1b	0.33 ± 0.01	ND[c]	0.34 ± 0.03
2	58.4 ± 6.8	79.0 ± 21.3	84.4 ± 11.6
3	8.7 ± 2.4	24.8 ± 4.1	16.2 ± 1.8
4[b]	0.59 ± 0.08	ND[c]	0.22 ± 0.04
5[b]	0.74 ± 0.14	ND[c]	0.57 ± 0.07
6	3.8 ± 1.0	4.9 ± 0.6	7.9 ± 0.8
7	381 ± 70	409 ± 93	278 ± 41
8	435 ± 107	523 ± 52	397 ± 21

[a] Cells were exposed to compounds for 72 h. Data represent the mean ± SD of at least three experiments; see the Experimental Section for details. [b] Data from ref. [26]. [c] Not determined.

gen atom acting as a hydrogen bond acceptor).[23] On the other hand, more recent studies in our laboratories have revealed a >10-fold difference between **4** and **5** in their binding affinity for stabilized microtubules ($K_b = 91.8 \times 10^7\ M^{-1}$ for **4** and $6.92 \times 10^7\ M^{-1}$ for **5**; Table 1),[26] thus indicating that the binding of these analogues to preformed stabilized MTs does in fact depend on nitrogen positioning in the heterocycle.

To gain a better understanding of the role of the quinoline side chain of epothilone analogues **4** and **5** in tubulin binding, we determined the side chain conformations of these ligands in the tubulin-bound state, employing DFT calculations and transferred NOEs.[28] First, rotational barriers around the C15–C16 bond were calculated at the B3LYP (Onsager)/6-31G* level. The rotation around the C15–C16 bond in **4** and **5** has a twofold symmetry with two nearly degenerate minima and a theoretical rotational barrier of ~5 kcal mol^{-1} (Figure 3).

Next, transferred NOEs in the presence of tubulin were used to determine the orientation of the side chain in the tubulin–**4** and tubulin–**5** complexes. For both analogues **4** and **5** the NOEs between the C14 methylene protons (H141 and H142) and H23 at a mixing time of 40 ms were found to be larger than those between H141/H142 and H17, suggesting a dihedral angle O15′–C15–C16–C23 of ~240°. The opposite trend is observed for the NOEs between H15 and the side chain protons H17 and H23. Although a quantitative assessment of the NOE data would have to take into account spin-diffusion effects, the comparison of specific NOE intensities between **4** and **5** clearly allows discrimination between the two energetically favored conformations of Figure 3. Thus, both **4** and **5** bind to β-tubulin with the same orientation of the quinoline side chain relative to the macrolactone ring, that is, with torsion angles O15′–C15–C16–C23 of 240° and the nitrogen atoms pointing in two different directions (Figure 4; note that C23 in **4** is located *para* to the pyridine ring nitrogen atom, whereas it is *meta* to the nitrogen in analogue **5**). The side chain binding mode of analogues **4** and **5** in both cases is characterized by the π–π stacking of the aromatic quinoline ring system on the imidazole side chain of His227; the same type of interaction has been suggested for the NMR-derived

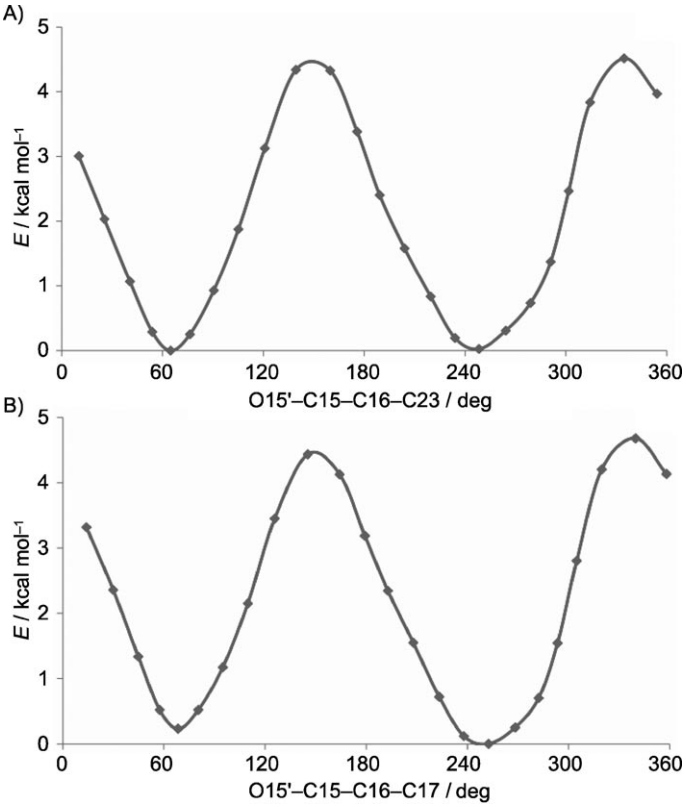

Figure 3. Computed B3LYP (Onsager, water)/6-31G* potential curves for rotation around the C15–C16 bond: A) **4**, B) **5**. The torsion angles were scanned in 15° increments, optimizing the geometry in each step.

tubulin-bound structure of Epo A (**1a**), in which the thiazole ring is located above the imidazole moiety of His227 without any evidence for hydrogen bonding between the two heteroaromatic ring systems. Likewise, no hydrogen bond can be discerned between the quinoline nitrogen and the imidazole side chain of His227 in the tubulin-bound model derived here for analogue **4**. The lower MT binding affinity of **5** (relative to **4**; Table 1) may then be a consequence of unfavorable interactions between the quinoline nitrogen in this analogue (which is now in contact with protein side chains) and the protein, or the less favorable solvation of the solvent-exposed edge of the quinoline side chain, which no longer presents a hydrophilic heteroatom, or both.

Docking studies with **4** and **5** were based on the model of the tubulin-bound structure of Epo A (**1a**), and it might be argued that the structures derived here for the complexes between **4** or **5** and tubulin (as depicted in Figure 4) are thus biased in favor of side chain stacking with His227 rather than hydrogen bonding (in the case of **4**). As discussed in the Introduction, the EC-derived model of tubulin-bound Epo A (**1a**) suggests a "side-by-side" arrangement of the thiazole ring and the imidazole moiety of His227, which are connected through a crucial hydrogen bond.[23] In principle, the same arrangement could be invoked for quinoline-based analogues **4** and **5**, and the decrease in MT binding for **5** could then simply reflect a loss of a crucial hydrogen bond with the protein. Alternatively,

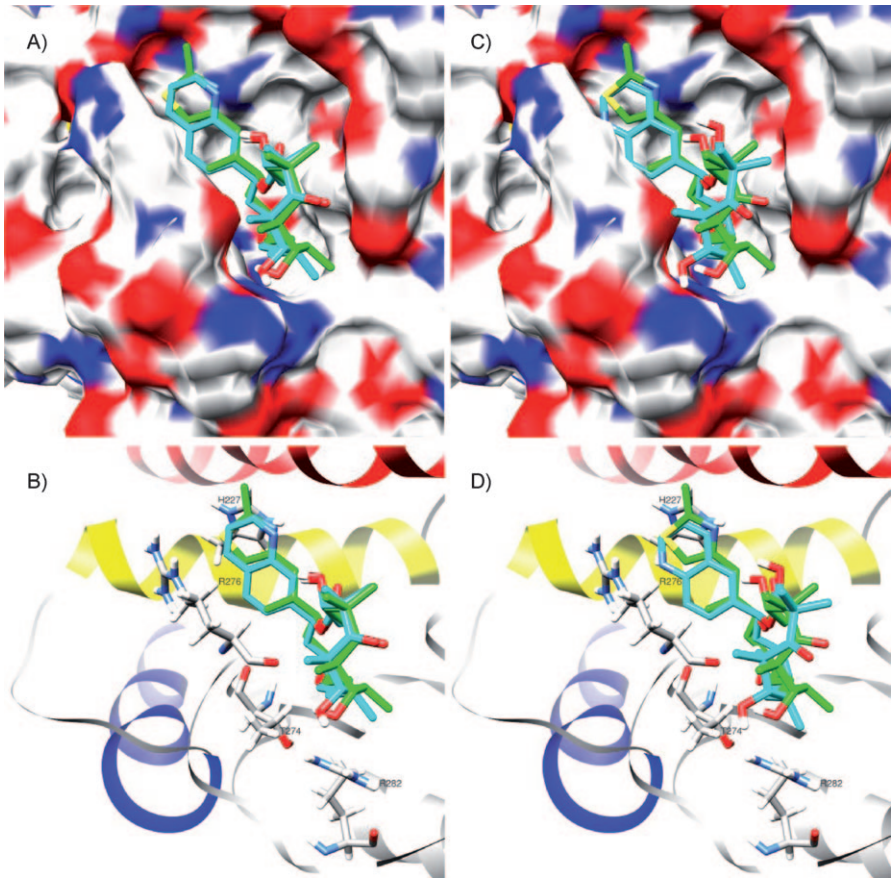

Figure 4. Superimposed structures of tubulin-bound Epo A (**1a**, green) and analogues **4** (A,B) or **5** (C,D) in the tubulin binding cleft (compounds **4** and **5** are shown in cyan). The bound conformations of **4** and **5** were derived by conserving the conformation of the macrocycle in the Epo A (**1a**) structure and adjustment of the quinoline orientation according to the transferred NOE values. The docked structures of the tubulin–**4** and tubulin–**5** complexes were generated by superimposing the macrolide rings of **4** and **5** with that of Epo A followed by energy minimization and water refinement protocols, during which the quinoline side chains of **4** and **5** were left free to reorient.

the hydrogen bond may be maintained by a ~180° rotation about the O15′–C15–C16–C23 bond in **5** and simultaneous repositioning of the entire molecule in the binding pocket. Although these alternative structural hypotheses may not seem implausible a priori, it is difficult to see how they could explain the fact that **5** binds to cross-linked MTs with even higher affinity than Epo A (**1a**; Table 1). In contrast, in the model presented in Figure 4, the stacking interaction between the side chain heterocycle and the imidazole moiety of His227, which is an essential feature of the NMR-derived model of tubulin-bound Epo A (**1a**), is maintained—or even enhanced, due to the more extended aromatic system—without the need for an unfavorable change in the overall positioning of the ligand in the binding pocket. However, as indicated above for the models of the bioactive conformations of analogues **2** and **3**, alternative tubulin-binding modes for **4** and **5** cannot be ruled out with certainty.

C21-modified epothilone

Being a prominent part of the epothilone pharmacophore, and as discussed in the Introduction, the C15-thiazolylalkenyl side chain of epothilones has been a frequent subject of structure–activity studies.[8] Modifications of this moiety have included the replacement of the thiazole ring by a number of other aromatic groups as well as the incorporation of a variety of C20 substituents of various sizes and polarity as a replacement of the natural methyl group. These studies have shown that small substituents at C20 (either polar or nonpolar) can lead to compounds with excellent in vitro and in vivo antitumor activity (e.g., CH_2OH, CH_2NH_2, or SCH_3),[8] while bulky hydrophobic groups tend to result in decreased activity,[8] although this question has not been investigated in a systematic fashion. In addition, little information is available on the biochemical and cellular effects of polar C20 substituents of larger size, especially those terminating in a polar group.

Inspection of the tubulin-related data presented in Table 1 for C20-propyl- (**6**), C20-butyl- (**7**), and C20-hydroxypropyl-Epo A (**8**) indicate that the tubulin-polymerizing activity of analogue **6** is similar to that of Epo A (**1a**); in comparison, both **7** and **8** are less potent inducers of tubulin polymerization. Relative to analogue **6**, EC_{50} values for the induction of tubulin polymerization by **7** and **8** are increased by a factor of ~2, which is similar to the ratio of EC_{50} values for **2** and **1a**. As illustrated by the latter pair of compounds, however, such small numerical differences in tubulin-polymerizing activity can be associated with significantly more pronounced differences in antiproliferative potency, and similar observations have also been reported in other studies. The rank order of MT binding affinities observed for **6**, **7**, and **8** qualitatively resembles the potency order for the induction of tubulin polymerization, with a C20-propyl substituent giving the highest and a C20-hydroxypropyl substituent the lowest binding constants (Table 1). However, while the difference in MT binding constants between **6** and **7** is less than twofold (K_b: 2.2×10^7 and $1.2\times10^7\ M^{-1}$, respectively; Table 1), the affinity of C20-hydroxypropyl derivative **8** for cross-linked MTs is clearly decreased (approximate ninefold difference in K_b values between **6** and **8**; Table 1). Much larger dif-

ferences between **6** and **7** or **8** are apparent at the cellular level, with the antiproliferative activity of **6** being similar to that of Epo A (**1a**), while both **7** and **8** are at least 40-fold (and up to >100-fold) less active than **6** (Table 2). Thus, the cellular activity of differently C20-substituted epothilone analogues appears to be governed by stringent steric requirements, with a very steep decline in activity occurring at the level of a (linear) four-(heavy) atom substituent. Most notably, the decrease in cellular potency by far exceeds the decrease in either tubulin-polymerizing activity or MT binding affinity that is associated with the increase in the length of the C20 substituent; as for **2** and **1a**, these findings indicate that relative differences in tubulin-polymerizing activity do not reliably predict the cellular potency of epothilone analogues. Given the multitude of parameters that affect cellular potency, apart from direct ligand–protein interactions, this observation may not be too surprising, even without having to invoke one or more additional targets for epothilones for which no real evidence has been reported. On the other hand, it is important to remember that the process of microtubule assembly triggered by microtubule-stabilizing agents is still poorly understood at the molecular level. For example, it is unclear whether polymerization induction occurs through ligand binding to isolated tubulin heterodimers or to pre-existing tubulin oligomers, and it is unknown whether the binding site for epothilones on soluble tubulin heterodimers is identical with the binding site in a microtubular assembly. With respect to the relationship between tubulin-polymerizing activity and cellular potency, one might also speculate that this may involve nonlinear effects, such that a disproportionate increase in antiproliferative activity could occur upon exceeding a particular threshold for tubulin-polymerizing activity.

Notwithstanding these uncertainties, the consistently low performance of the C20-hydroxypropyl derivative **8**, which shows the highest EC_{50} value for tubulin polymerization, the lowest K_b value for MT binding, and the highest IC_{50} values for cell growth inhibition (of the three C20-modified analogues **6**, **7**, and **8**) led us to investigate whether the decreased capacity of this compound to interact with the tubulin/MT system would also be reflected in changes in its tubulin-bound conformation relative to Epo A (**1a**). Based on the results of transferred NOE measurements under the same conditions that had been employed for the study of **1a**/**2**–**5**, analogue **8** was indeed found to interact with soluble tubulin. The experimental data are compatible with a conformation of the macrolide ring in tubulin-bound **8** that closely resembles that of Epo A (**1a**) (data not shown), but unfortunately, the conformation of the C20-hydroxypropyl moiety is not well defined by the NMR restraints due to the overlap of the NMR resonances of the geminal protons of the methylene groups.

In the absence of experimental data on the conformation of the C20 substituent in **8**, compounds **7** and **8** were docked into the epothilone binding pocket of β-tubulin, based on the superposition of the macrolide ring of **7** and **8** with that of tubulin-bound Epo A (**1a**) in the NMR-derived model.[21a] According to the docking studies, C20-modified analogues **7** and **8** can be well accommodated in the tubulin binding pocket, with a 4-(heavy) atom substituent at C20 (as in **7** and **8**) either in an extended conformation or in a conformation where the hydroxypropyl (or butyl) side chain is folded toward the nitrogen atom of the thiazole ring. The results of the modeling studies based on the NMR-derived model of the tubulin–Epo A complex[21a] do not provide a direct explanation for the differences between **1a**, **6**, **7**, and **8** in their tubulin-polymerizing activity and MT binding affinity. At the same time, however, they are also not compatible with the EC-derived model of the tubulin-bound conformation of epothilones,[23] as in this model a two-carbon substituent at C20 should provide for the most favorable contacts with tubulin, while *both* three- (**6**) and four-carbon (**7** and **8**) substituents would appear to be suboptimal.[32]

Conclusions

We have investigated the interactions of a series of epothilone analogues with soluble tubulin/tubulin oligomers using biochemical, computational, and NMR methods, attempting to recapitulate the biochemical effects caused by modifications of the epothilone structure by the conformational properties of the corresponding ligand–tubulin complexes.

The tubulin-bound structures of C3-modified epothilone derivatives **2** and **3** clearly demonstrate that neither the simple removal of the 3-OH group nor the concomitant conformational restriction of the C2–C3 bond to a *trans* geometry significantly alter the overall conformation of the macrolide ring in the tubulin-bound state, as compared with the NMR-derived bioactive conformation of Epo A (**1a**).[21] This observation is in excellent agreement with the conformational preferences of these analogues free in aqueous solution[22] and it is in line with their tubulin-polymerizing activity.[22]

Quinoline-based Epo B analogues **4** and **5** bind to tubulin with very similar side chain geometries. As a consequence, the heterocyclic nitrogen atom is buried by protein side chains for **5**, whereas it occupies a solvent-exposed position in the case of **4**. This latter difference may well explain the superior MT binding affinity of **4** over that of **5**. At the same time, the side chain in both cases stacks efficiently with the imidazole moiety of His227, an observation that is in excellent agreement with the fact that even analogue **5** binds to MTs with at least similar affinity as that of Epo A (**1a**).

Relative to Epo A (**1a**), C20-butyl- (**7**) and C20-hydroxypropyl-Epo A (**8**) exhibit decreased tubulin-polymerizing and significantly diminished antiproliferative activity. In contrast, C20-propyl-Epo A (**6**) is essentially equipotent with Epo A (**1a**). We are unable to explain these differences in biochemical properties on the basis of either of the experimental structural models available for the Epo A–tubulin complex. While the EC-derived model predicts decreased tubulin binding even for the C20-propyl derivative **6**, the NMR model can accommodate three- and four-carbon C20 substituents equally well (and thus would not predict any significant differences between **6**, **7**, and **8**). At the same time, the relative binding affinities of **6**, **7**, and **8** for stabilized MTs differ substantially from the relative cellular activities of the three compounds. These findings un-

derscore the multi-causality of cellular effects of chemical probes and thus the problems associated with the interpretation of cellular data in terms of a single ligand–receptor interaction, even for structurally closely related compounds. In this context it is interesting to note that MT binding of compound **6** as the most potent C20-modified analogue is clearly enthalpy driven, whereas the entropic contribution dominates for **7** and **8** (Table 1). These findings are in line with previous observations on a different set of epothilone analogues, which suggest that compounds with a more favorable entropic contribution to MT binding tend to be less effective inhibitors of cell proliferation than those with more favorable binding enthalpies.[4] However, the molecular basis for this empirical correlation has not been elucidated.

In summary, our findings highlight the complex nature of the interactions of low-molecular-weight ligands with the tubulin/MT system, making it difficult, if not perhaps impossible, to establish firm correlations between effects that occur at different levels of the system, such as (soluble) tubulin-bound ligand structures, tubulin polymerization, binding to preformed MTs, and the translation of these parameters into cellular activity. However, with regard to the specific question of the bioactive conformation of epothilones, it is notable that the tubulin-bound conformations of all analogues investigated in this study converged toward the same family of closely related structures, and are thus similar to the NMR-derived structure of tubulin-bound Epo A (**1 a**).[21]

Experimental Section

Sample preparation. Epothilone A (**1 a**) was a generous gift of Novartis Pharma AG, Basel (Switzerland). The syntheses of compounds **2** and **3**[22] as well as **4** and **5**[26] have been published. Epothilone analogues **6**–**8** were prepared from Epo A (**1 a**) by semisynthesis. Experimental details for the preparation these compounds will be published elsewhere. Analytical data for **6**–**8** are included in the Supporting Information.

For sample preparation 53 μL of tubulin solution (bovine brain tubulin, 66 μM), purchased from Cytoskeleton Inc. (Denver, CO, USA), were diluted to 350 μL and dialyzed twice against 1.5 L aqueous calcium phosphate buffer (1.5 mM, adjusted to pH 7.0 with NaOH, 12 h) and then for 12 h against 15 mL D_2O buffer (1.5 mM calcium phosphate) at 4 °C. Samples were prepared by dissolving the epothilone derivatives in [D_6]DMSO and diluting 14 μL aliquots with a D_2O solution of non-polymerized tubulin to a final concentration of 500 μM epothilone derivative and 10 μM tubulin. [D_6]DMSO was used as co-solvent to increase the solubility of epothilones to a final fraction of 5% *v/v* [D_6]DMSO.

NMR experiments were carried out on Bruker 600, 700, 800 and 900 MHz spectrometers. Resonance assignment was performed using HSQC,[33] HMBC,[34] E.COSY,[35] TOCSY,[36] and NOESY[37] experiments. NOESY buildups were measured with 0.25 or 0.5 mM solutions of epothilone derivatives at mixing times of 40, 80, 120, 160, 200, and 400 ms at 298 K without water suppression. The NOE buildups were analyzed with Felix (Felix NMR Inc., CA, USA).

Structure calculations were performed using restrained simulated annealing from a single starting template as implemented in XPLOR-NIH 2.13.[38] NOE intensities were used in the full relaxation matrix approach. An extensive description of the protocol applied is given in the Supporting Information.

DFT calculations. The structures obtained in the NOE-restrained simulated annealing computations were refined at the DFT level using the Hybrid B3LYP function and the 6-31G* basis set.[39,40] Solvent effects were included in the geometry optimization using the Onsager method,[41,42] with sphere radii of 6.6 Å and a water relative dielectric constant of 78.39. All computations were done with the Gaussian 03 software package.[43] To obtain torsional profiles of the C15–C16 bond rotation, relaxed scans in 15° increments were performed at the same level for **4** and **5**.

Docking calculations. The HADDOCK protocol[44] as implemented in the CNS program[45] was used to generate models for the complexes of epothilone derivatives **2** and **3** with β-tubulin. Initial structures were constructed by placing the bound structures (employing UCSF Chimera[46]), resulting from the DFT refinement of the NOE-restrained simulated annealing calculations mentioned above, at the position of Epo A (**1 a**) in the previously published Epo A–β-tubulin model.[21a] This model is based on the structure of *tubulin* in the Epo A–tubulin complex as determined by Nettles et al. (PDB ID: 1TVK).[23] The complex was hydrated with a water layer of ~8 Å, followed by energy minimization and water refinement. Full flexibility of the protein was allowed throughout, whilst the conformations of the epothilone derivatives were kept rigid throughout the docking calculations. Methyl and hydroxy groups were allowed to rotate. Initial structures of compounds **7** and **8** were built by attachment of a butyl or hydroxypropyl substituent, respectively, to C20 of Epo A (**1 a**) in the Epo A–β-tubulin model derived from solution NMR[21a] after removal of the C21 methyl group. For compounds **4** and **5**, the bound conformation was derived retaining the DFT-refined Epo A (**1 a**) macrolide conformation, while the orientation of the side chain was derived from transferred NOE data. During the docking protocol of **4** and **5**, the quinoline ring was allowed to freely rotate around the C15–C16 bond. Docking models and molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).[46]

Tubulin polymerization. EC_{50} values for the induction of tubulin polymerization (i.e., the concentration required to induce 50% of the maximum α/β-tubulin polymerization achievable) were determined with 10 μM porcine brain tubulin. Tubulin polymerization was assessed through turbidity measurements at λ 340 nm (A_{340}).[47] For a given compound concentration, an equilibrium state between soluble and polymerized tubulin is indicated by a stable plateau in A_{340}. Maximum tubulin polymerization is reached when a further increase in compound concentration no longer results in an increase of the plateau value for A_{340}. Similar maximum values for A_{340} were observed for all six compounds investigated in this study. EC_{50} values in Table 1 represent the mean ± SD of three independent experiments.

Microtubule binding. Binding constants of epothilone analogues **2**, **3**, **6**–**8** for glutaraldehyde-stabilized microtubules (MTs) were determined at 35 °C by the method previously described by Buey et al.,[30] which is based on the displacement of Flutax-2. Binding constants were calculated using Equigra v. 5.[48] Thermodynamic parameters (apparent ΔG^0, ΔH^0, and ΔS^0) were calculated as described.[29]

Cellular activity. IC_{50} values for human cancer cell growth inhibition were determined by quantifying the protein content of fixed cells by methylene blue staining after a 72 h exposure period of

cells to compounds. For further experimental details, see ref. [27]. IC_{50} values in Table 1 represent the mean ±SD of three independent experiments.

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