The molecular recognition of epothilones by microtubules and tubulin dimers revealed by biochemical and NMR approaches

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Abstract

The binding of epothilones to dimeric tubulin and to microtubules has been studied by means of biochemical and NMR techniques. We have determined the binding constants of epothilone A (EpoA) and B (EpoB) to dimeric tubulin, which are four orders of magnitude lower than those for microtubules, and we have elucidated the conformation and binding epitopes of EpoA and EpoB when bound to tubulin dimers and microtubules in solution.

The determined conformation of epothilones when bound to dimeric tubulin is similar to that found by X-ray crystallographic techniques for the binding of EpoA to the Tubulin/RB3/TTL complex; it is markedly different from that reported for EpoA bound to zinc-induced sheets obtained by electron crystallography. Likewise, only the X-ray structure of EpoA bound to the Tubulin/RB3/TTL complex at the luminal site, but not the electron crystallography structure, is compatible with the results obtained by STD on the binding epitope of EpoA bound to dimeric tubulin, thus confirming that the allosteric change (structuring of the M-loop) is the biochemical mechanism of induction of tubulin assembly by epothilones. TR-NOESY signals of EpoA bound to microtubules have been obtained supporting the interaction with a transient binding site with a fast exchange rate (pore site), consistent with the notion that epothilones access the luminal site through the pore site, as has also been observed for taxanes. Finally, the differences in the tubulin binding affinities of a series of epothilone analogs has been quantitatively explained using the newly determined binding pose and the COMBINE methodology.

Introduction.

Among the non-taxane microtubule-stabilizing agents (MSA) targeting the paclitaxel-binding site, the epothilones (Figure 1) are those that have attained the greatest relevance for anticancer drug development. Their superior solubilities and higher microtubule binding affinities as compared with paclitaxel, in combination with their lack of susceptibility to drug resistance related to the overexpression of P-gp and other membrane-bound efflux pumps, have made them important candidates for drug development against tumors with the MDR phenotype. As the first culmination of epothilone-based drug development efforts, Ixempra[®] (the lactam analog of epothilone B, ixabepilone) entered the market for the treatment of advanced breast cancer in 2007 (1).

Figure 1.- Binding of EpoA (filled squares) and EpoB (filled circles) to tubulin in D₂O 10 mM sodium phosphate, 0.1 mM GTP, pH* 7.0 at 25°C, data are the average of 3 independent measurements. Inset Chemical structure of epothilones employed in this study.

Paclitaxel and its functional biomimetics (epothilones, discodermolide, dictyostatin, cyclostreptin, zampanolide, etc.) (2) are efficient microtubule binders, through interactions with one or both of the previously described taxane-related microtubule binding sites, which are located either at the lumen or at the pore type I(3-7). There is a strict 1:1 stoichiometry with the $\alpha\beta$ -tubulin heterodimer in assembled microtubules, which indicates that binding to both sites is mutually exclusive (2,8-9).

Electron crystallographic data have indicated that taxanes and epothilones bind to the luminal site (3-4) in Zn^{2+} -assembled tubulin sheets. X-ray crystallographic data confirmed the same binding site for epothilones in unassembled tubulin, although in a conformation and orientation (7) different from the one suggested by electron crystallography (4).

Kinetic and biochemical evidence have indicated that at least one taxane, 7-hexaflutax, exclusively binds to the pore site in assembled tubulin (10), while the binding process for other fluorescent taxanes (i.e., Flutax-1 and Flutax-2) to microtubules involves at least two distinct steps. The first step, which represents the actual molecular recognition event, can be assigned to ligand binding to the pore site, while the second one involves an internalization process that leads to ligand transfer to the luminal binding site. Fittingly, cyclostreptin and derivatives are able to covalently label assembled tubulin at both the luminal and the pore site (5,11). In contrast, zampanolide only labels the luminal site, both in dimeric tubulin and in microtubules (12). Finally, detailed NMR studies have shown that at least a fraction of the pore sites in microtubules are occupied by docetaxel or discodermolide (6). Up to now, no experimental data were available to distinguish if

epothilone-type microtubule binders first recognize the pore site or if they bind to the luminal site directly.

While structural evidence for the weak binding of microtubule-stabilizing agents to dimeric tubulin is lacking, such an event has to be inferred in order to explain the observed nucleation of microtubules under experimental conditions that do not permit spontaneous tubulin assembly (13). Some years ago, initial NMR evidence was obtained for the binding of epothilone A (EpoA) and also discodermolide to tubulin in an inhomogeneous non-assembled state (14-15). Direct evidence of MSA binding to dimeric tubulin was subsequently obtained for cyclostreptin (5, 11), and zampanolide (12); these compounds covalently label dimeric tubulin only at the pore or at the luminal site, respectively. The interactions of docetaxel and discodermolide with dimeric tubulin have been characterized using biochemical and NMR methods. It was demonstrated that both compounds indeed bind to dimeric tubulin, although with affinities in the range of 10^4 M^{-1} at 25°C, which are several orders of magnitude lower than those for binding to microtubules (6). Most recently, EpoA was shown to bind to tubulin at the luminal site, but with tubulin engaged in a ternary complex with RB3 and tubulin-tyrosine ligase (7). Thus, the binding mode of epothilones to free soluble tubulin dimers and the strength of the interaction have remained unknown.

From a conformational perspective, the detailed characterization of the tubulin-bound structure of epothilones has been a topic of continuous interest since 1996. The solid state conformation of EpoA (as deduced by either X-ray crystallography or solid state NMR) is known (16-17), and so are its conformations in organic solvents (18). In non-polar solvents, the most populated conformer is very similar to the X-ray structure obtained from crystals that had been grown in dichloromethane (16).

However, the true, tubulin-bound, bioactive conformation of the epothilones has remained a controversial issue during these years. The first tubulin-bound conformation of EpoA was determined by solution state NMR experiments using a non-polymerized soluble form of $\alpha\beta$ -tubulin (14). However, subsequent studies of the tubulin/EpoA complex by electron crystallography of tubulin sheets (4) suggested a markedly different binding mode. In support of the NMR-derived conformation, the latter has been found to be significantly populated for the free ligand in water, while this was not the case for the electron crystallography-derived structure (19). Strikingly, the recent X-ray structure of the above mentioned tubulin/RB3/tubulin-tyrosine ligase/EpoA complex (7) shows an epothilone conformation similar to the NMR structure described by Carlomagno and coworkers (14), although the orientation of the ligand in the binding site is different from both the ones proposed either by Carlomagno (20) or Nettles (4). While this would still need to be proven experimentally, it is at least conceivable that these discrepancies might reflect a dependence of the epothilone binding-mode on the actual tubulin polymerization state being studied. Additionally, the presence of different protein binding sites or crystallographic constraints could affect the experimental observations.

In this context, we now report on the fine structural details of the binding of epothilones to unassembled dimeric tubulin and to stabilized assembled microtubules. NMR techniques have been employed to elucidate the binding epitopes of EpoA and EpoB, while the binding constants for the different complexes were determined using direct biochemical methods. 3D models of the resulting bioactive structures bound to both tubulin states have been derived from the experimental data and are compared to the previously reported structures. Finally, the structural model of tubulin-bound epothilones was

employed in a quantitative structure-affinity study of the epothilone chemotype by means of the Comparative Binding Energy (COMBINE) analysis method (21). Taken together, our results indicate that the binding of epothilones to microtubules involves an initial interaction of the ligand with the pore site and a posterior translocation to the luminal site, in a manner analogous to that already described for other MSA. At the structural level, our data show that the tubulin-bound conformation of epothilones and their orientation in the binding site on free tubulin dimers in solution are identical with those observed in the structure of the recently reported trimeric complex (7).

Results and discussion

The binding of epothilones to dimeric tubulin.

MSA induce microtubule assembly under conditions where tubulin is unable to assemble by itself (GDP-bound). Thus, it is likely that they bind (although weakly) to unassembled tubulin. In previous work, the binding of docetaxel and discodermolide to dimeric tubulin has been detected using centrifugation and HPLC techniques (6).

In the absence of Mg^{2+} , tubulin remains dimeric even at high concentrations and in the presence of Epothilone, as is shown by analytical ultracentrifugation controls (Figure S1C and Table S1). Preparative centrifugation assays indicated that EpoA and EpoB (Figure 1) co-sediment with dimeric tubulin. Although tubulin concentration is not constant among the lower part of the centrifuge tube, this method measures a true chemical equilibrium as long as the protein oligomerization state is not dependent on the concentration (which is the case) and assuming fast ligand binding, is formally equivalent to an equilibrium dialysis (22). The binding constant of epothilones to dimeric tubulin could be then estimated assuming a 1:1 stoichiometry and was found to be $0.8 \pm 0.3 \times 10^4$ for

EpoA and $2.1 \pm 0.5 \times 10^4$ for EpoB at 25°C. These values are consistent with the differences between these two compounds in their affinities for microtubules(9), but they are several orders of magnitude lower (K_a 26°C $7.5 \pm 1 \times 10^7$ M⁻¹ EpoA and $150 \pm 15 \times 10^7$ M⁻¹ EpoB). The values are similar to the values determined for the binding of discodermolide to dimeric tubulin (K_a $2.0 \pm 0.7 \times 10^4$ M⁻¹), but they are much higher than those of paclitaxel and docetaxel; the latter has much lower affinity for unassembled tubulin ($K_a < 10^3$ M⁻¹) (6,13).

Saturation Transfer Difference analysis of epothilones bound to dimeric tubulin and to microtubules.

STD-NMR experiments are widely employed to detect ligand binding to protein receptors and to characterize the ligand binding epitope (23-24) under particular kinetic conditions. The binding of epothilones to dimeric tubulin in 10 mM NaPi, 0.1 mM GTP pH* 7.0 (pH* is the apparent pH measured by a pH electrode in a D₂O solution) was monitored by STD (Figure 2A, 2B). Competition experiments with another MSA (discodermolide) were also performed to assess the specificity of binding. The addition of a molar excess of discodermolide removed the characteristic STD peaks of the epothilones (Figure 2E), thus indicating that epothilones and discodermolide bind to the same tubulin site.

The STD signals obtained for EpoA or EpoB bound to dimeric tubulin were very similar, although some differences were also detected. For both compounds, the signals corresponding to H13, H15, H17, H19, and Me21 (and H12 of EpoA) showed STD intensities above average (Figure 2F, solid lines). Analysis of this data allowed the determination of the key ligand epitope involved in the interaction with dimeric tubulin. The binding process between epothilones and microtubules (25) displays fast kinetics, thus

Figure 2.- Off resonance (lower line) and STD (upper line) ^1H NMR spectra (500 MHz) and STD spectrum of (A) EpoA bound to dimeric tubulin (B) EpoA bound to microtubules, (C) EpoB bound to dimeric tubulin (D) EpoB bound to microtubules (E) Decrease of STD signal of EpoA bound to tubulin dimers vs discodermolide concentration. (peak 6.51 ppm, H17). (F) Saturation transfer difference of EpoA (black symbols and lines) and EpoB (red symbols and lines) protons in the states bound to dimer (circles and solid lines) and microtubules (squares and dashed lines).

suggesting that these molecules should enter the luminal site through transient interactions with the external pore. However, no STD or TR-NOESY signals for epothilones bound to assembled microtubules have been obtained until now. Strikingly, this omission contrasts with the observation of such signals for other paclitaxel biomimetics, such as discodermolide, dictyostatin, or docetaxel (6,26). STD/TR-NOESY signals arise from the compound in fast exchange between the solvent and the binding site (27-28), thus fast dissociation kinetics is required to observe these signals. Since similar kinetic parameters have been deduced for the formation and dissociation of epothilone- and paclitaxel-microtubule complexes, the fact that STD/TR-NOESY signals have remained elusive for epothilone/microtubule complexes could indicate that either epothilones are not bound to the pore site in assembled microtubules to any significant extent or that they might directly interact with the high affinity (and therefore slow-exchanging) luminal site.

As expected, it was not possible to obtain STD signals or TR-NOESY cross peaks for epothilones bound to microtubules under the experimental pH* 6.7 and buffer conditions previously employed for other MSAs (6). However, TR-NOESY spectra of good quality were obtained when the pH* was lowered to 6.5. This detail is consistent with the pH-dependence of the kinetics observed for Flutax-1 binding to assembled microtubules (29).

The observed STD (Figures 2C, 2D) intensities with microtubule-bound epothilones were much more homogeneous than those obtained for the complexes with dimeric tubulin (Figure 2F, dashed lines). Only the signals corresponding to the side chain protons (as well as H13 of EpoB) were above average. This may suggest that MSA binding to the tubulin polymer results in different contacts between the ligand and the receptor in an assembled

state. The data suggest that the entire macrocycle is involved in the binding of epothilones to microtubules.

The comparison of the STD data of EpoA/EpoB bound to unassembled tubulin or microtubules (Figure 2F) reveals an increase in the average STD value (%) from 7.5 (EpoA) and 13.1 (EpoB) to 35.6 (EpoA) and 24.2 (EpoB) for the microtubule-bound ligands. A similar increase of the average STD value has been observed for docetaxel or discodermolide binding (6). This observation is not unexpected and probably reflects a slower rotational relaxation due to the larger receptor size in the case of the microtubule complexes.

The conformation of epothilones bound to dimeric tubulin and to microtubules

TR-NOESY experiments were employed to assess the conformational features of EpoA and EpoB bound to dimeric tubulin and to microtubules. Strong negative TR-NOESY cross peaks were observed for both EpoA and EpoB in the presence of dimeric tubulin and microtubules (Figures 3A, 3B, 3C and 3D), thus indicating binding of both molecules.

Analysis of the NMR data permitted the determination of the conformation of both epothilones when bound to dimeric tubulin and to microtubules. The structures obtained were similar in both cases (Figure 4) and they also resembled the tubulin-bound conformation previously described for EpoA bound to non-polymerized tubulin(14) (the first conformation of EpoA determined in an aqueous buffer(14, 30)) except for the O1C1C2C3 dihedral angle (however it should be considered that this angle is difficult to define by using proton NMR due to the ester group involved in it); this conformation is

Figure 3.- TR-NOESY spectra (mixing time: 300 ms, except panel A in which the mixing time is 200 ms) of the different ligands in the presence of dimeric tubulin and microtubules (D₂O, 298 K) (Panels, A, EpoA, and C EpoB,) and microtubules (D₂O, 310K) (Panels B, Epothilone A and D, Epothilone B).

highly populated when the free ligand is in aqueous solution, as the same authors also reported later(19), indicating that the bioactive structure is preorganized in solution. This finding is in strong disagreement with the tubulin-bound conformation of EpoA bound to zinc-stabilized tubulin sheets, as obtained by electron crystallography(4), especially regarding the preferred conformation of the macrolactone ring (Figure 4D).

The comparison of the dihedral angles is given in the supporting information (Table S2). The bound conformation obtained for EpoB (Figure 4) was derived from the analysis of the key NOEs encoding relevant conformational information (key NOEs are depicted in Figure 3A). In particular, the existence of a weak H19-H17 versus a strong Me27-H19 NOE clearly indicated that the syn-periplanar disposition of the C16-C17-C18-C19 torsion of the side chain is substantially more populated than the anti-periplanar arrangement,

Figure 4.- Bioactive conformations of Epothilone bound to tubulin.

which is populated (>80%) in the presence of both dimeric or polymerized tubulin (Figure 4). This observation is also in agreement with previous reports on the conformation of EpoA bound to tubulin aggregates (14) and with the electron crystallography-derived model of the tubulin-bound structure of EpoA (4). The observed NOEs (strong H3-H6) were also in agreement with a gauche disposition around the C2-C3-C4-C5 torsion in the bound state to both dimeric tubulin as well as microtubules. This geometry, which has also been postulated by Carlomagno and co-workers (14), markedly differs from the major conformer observed in organic solvents. Regarding the orientation of the epoxide ring, the presence of a strong H13-H15 NOE for both epothilones bound to either dimeric tubulin or to microtubules can only be explained if the oxygen atom of the epoxide in the macrolactone is directed outwards, as has been reported for EpoA bound to non-polymerized tubulin (14) and corresponds to the preferred conformation in organic solvents (31). This result is in sharp contrast with the epoxide orientation for the tubulin-bound EpoA conformation as suggested by electron crystallography, which has the oxygen atom of the epoxide ring directed towards the center of the macrocycle. Additional differences regarding the relative orientation of the thiazole side chain with respect to the macrolactone ring were also found between the epothilone conformations deduced herein relative to the electron crystallography-derived geometry. The presence of a strong NOE correlation between protons H15-H17 supports one specific orientation, again this is similar to that deduced for EpoA bound to non-polymerized tubulin (14).

Figure 5.- (A) Solutions found for the docking of the microtubule bound form of EpoB (purple) compared with this previously found(6) for discodermolide (green) into the pore type I of microtubules, the four tubulin heterodimers forming the pore are labeled 1 (grey), 2 (blue) 3 (green) and 4 (orange). Comparison between experimental and calculated (from(7) structure) STD data (CORCEMA-ST) of EpoA (B) and EpoB (C) bound to dimers. Comparison between experimental and theoretical (model of figure 5A) STD data (CORCEMA-ST) for EpoA (D) and EpoB (E) in the presence of microtubules. Experimental STD effects (Solid line and circles) calculated ones (dashed line and squares).

NMR-guided modelling of the bioactive conformations.

The STD and TR-NOESY data were then employed to derive a detailed 3D model of the tubulin bound conformations of the epothilones. For the case of the α/β -tubulin heterodimer, the data are fully compatible with the the X-ray derived coordinates of the recently reported structure of Prota et al. (7) (EpoA in complex with unassembled tubulin). When these coordinates were used in the CORCEMA-ST calculations, EpoA and EpoB STD values back-calculated from this structure were found to be in excellent agreement with our experimental data for unassembled tubulin, NRMSD value 11.6% and 16.3% Figure 5B and 5C. Therefore, the binding mode detected in solution is in agreement with the X-ray determined structure.

This is not the case when the STD signals from epothilones bound to microtubules were employed, which indicated that the signals were not arising from the compound bound to the luminal site. In contrast, when EpoB was modeled into the pore site, employing the CORCEMA-ST method as described (6), the best solution displayed a good fit with an NMRS value of 19.5% (Figures 5A and 5E), comparable with the results obtained for discodermolide and docetaxel (6). The interaction involves EpoB binding to the bottom part of the pore, between the two β subunits of adjacent protofilaments. This interaction model resembles the one proposed for discodermolide and has also been suggested as an alternative pose for docetaxel (6) in the area indicated by Freedman et al. (32). The best solution for EpoA is identical with the one found for EpoB, which displays a slightly higher NMRS value, 21.5% (Figure 5D).

In this binding pose for EpoB, the ketone carbonyl group at position 5 is engaged in a hydrogen bond with the hydroxyl group of β_{left} (the β subunit of the left protofilament)

Thr220, the hydroxyl group at position 3 forms two hydrogen bonds with β_{left} Lys218 and β_{left} Phe214 and the thiazole ring establishes a hydrogen bond with β_{left} Lys218 and a cation π interaction with β_{right} Lys124.

It is worth noting that β_{left} Thr220, β_{left} Lys218 and β_{left} Phe214 are also key residues in the binding model for the interaction of discodermolide with the pore site of microtubules described in our previous work. According to our models, the hydroxyl group at position 3 of epothilones is involved in the same hydrogen bonds as the hydroxyl group at position eleven of discodermolide.

COMBINE analysis of the epothilone-tubulin interaction

Given the fact that the binding pose employed in our previous COMBINE analysis (21) has proven to be incompatible with the recent experimental data on the EpoA-tubulin complex (PDB entry 4I50) solved at 2.3 Å resolution, the latter has been employed to apply the COMBINE methodology to rationalize the experimental binding free energies (ΔG) of a set of 22 structurally different epothilones (Table S3) and provide information on the relative importance of the amino acids that constitute the binding site (9,33).

In its complex with β -tubulin, the O1, OH3, OH7, and N20 groups of EpoA are hydrogen bonded to the main-chain NH of Thr276 (as is the oxetane oxygen of paclitaxel), the side-chain amide nitrogen of Gln281, the side-chain carboxylate oxygen of Asp226, and the side-chain hydroxyl group of Thr276, respectively (7). In addition, inspection of the electron density using the program COOT (Crystallographic Object-Oriented Toolkit)(34) revealed an unreported water molecule that is likely to mediate hydrogen-bonding interactions between the carbonyl oxygen at position 5 of EpoA and both the main chain

NH of Arg278 and the main chain CO of Leu217, thus contributing to the stabilization of the M-loop in a helical conformation (Figure S2).

Epothilones bind to the luminal site in unassembled dimeric tubulin.

From a biochemical perspective, the major aim of this work was to clarify those issues still pending for a complete structural characterization of the interaction of tubulin with epothilones. In particular, whether if epothilones do bind to dimeric tubulin in solution and, if so, to determine the corresponding binding parameters. Although the binding of epothilones to different unassembled forms of tubulin (7,14,20) has been reported, no direct evidence for the binding of these compounds to tubulin dimers, which are the functional units that assemble to form microtubules, had been obtained previously. Our results establish, for the first time, that epothilones do bind to unassembled tubulin, although with low affinity ($K_a \sim 10^4 \text{ M}^{-1}$). This low affinity binding results from the opposing contributions of the intrinsically higher affinity of the ligand binding to an assembled binding site in its final state (i.e. in the state that is present in microtubules) and the energetic cost associated with the structural changes of the protein required to adopt the active assembling conformation and its integration in the microtubule structure. The energetic cost may be identified with the structuring of the β -tubulin M-loop upon EpoA binding described by Protá et al. (7) making it adopt the ordered structure found in the assembled states of the protein, (3) and thus the protein becomes more prone to assembly and the energy needed for an activated dimeric tubulin to assemble into microtubules is different for each ligand (2). Since the M-loop is already structured in assembled microtubules, it is logical to assign (at least partially since other contributions such as the

energy needed for the curve to straight transition may be considered) the differences (22.6 kJmol⁻¹ EpoA, 27.7 kJmol⁻¹ EpoB and 32 kJmol⁻¹ discodermolide) in free energy of binding between microtubules (K_a 26°C 7.5±1x10⁷ M⁻¹ EpoA, 150±15x10⁷ M⁻¹ EpoB, 872±82 x10⁷ M⁻¹ discodermolide) and unassembled tubulin (K_a 25°C 0.8±0.3x10⁴ M⁻¹ EpoA, 2.1±0.5x10⁴ M⁻¹ EpoB and 2.0±0.7x10⁴ M⁻¹ discodermolide) to the energy needed to structure the loop.

For the first time, we have also observed TR-NOESY signals for epothilones bound to microtubules. Since the detection of TR-NOESY signals depends on the existence of fast dissociation kinetics on the relaxation time-scale, these signals must reflect interactions of the ligands with a binding site that permits fast exchange between the free and bound states. As this is not the case for the luminal site of the microtubules either for taxanes (29) or epothilones (25), the TR-NOESY signals can be safely assigned to the pore site, as for docetaxel and discodermolide (6). This finding is indicative of the existence of pre-dissociation interactions at the pore site similar to those proposed for other MSA, such as docetaxel and discodermolide (6), which in turn suggests that a similar general mechanism of microtubule binding may be operative for all taxol site MSA. For dimeric tubulin, the molecular recognition process is established through the interactions with the luminal site as described by Prota et al. (7), while access to the microtubule luminal site proceeds through a temporary interaction with the pore site (25).

For microtubule-bound epothilones the observation of STD and TR-NOESY signals is dependent on the pH of the buffer. Since the pore site does not feature any titratable groups in the pH* range close to 6.5, the fact that STD and TR-NOESY signals are observed at lower pH* may be attributed to the protonation of His229 in the luminal site, which is close to the more hydrophobic southern part of the epothilone molecule.

Protonation of this residue should weaken the strength of the van der Waals interactions of the ligand with the luminal site, thereby increasing the percentage the ligand molecules in the fast dissociating pore site and allowing the observation of signals from this site.

Bioactive conformation of epothilones bound to dimers and microtubules.

The combined NMR/modelling approach described above has permitted elucidation of the conformation of EpoA and EpoB bound to the tubulin α/β -heterodimer and to microtubules. The EpoA and EpoB bioactive conformations as well as their binding epitopes for dimeric tubulin and microtubules have also been obtained. The conformations of EpoA and EpoB bound to dimeric tubulin and to microtubules are very similar and they closely resemble the conformation previously described by Carlomagno et al. for EpoA bound to non-assembled tubulin (14). This conformation is also dominant in the ensemble of conformations of EpoA free in water (19).

However, our structure strongly deviates from the conformation that has been suggested for EpoA bound to zinc-stabilized tubulin sheets based on electron crystallography and modeling (PDB entry 1TVK) (4), especially with regard to the conformation of the macrolactone ring. In fact, this latter conformation has not been detected in solution, either in the free state or in the bound state to dimeric tubulin, microtubules, or oligomeric tubulin aggregates.

COMBINE QSAR analysis of the epothilone-tubulin interaction

With the binding mode of epothilones for the luminal binding site of microtubules established, it became feasible to rationalize some of the structure-affinity relationships that we have reported previously for a series of epothilone analogs (9, 33). For example, the structure readily explains the moderately small losses of affinity in **EPKT2** and **EPKT3** brought about by the lack of OH3 or the relatively large gains in binding free energy observed for **EP3** and **EP19** due to the presence of a thiomethyl group on the thiazole ring, with the latter presumably occupying an adjacent pocket. Nonetheless, in order to assess the effect of these and other structural changes (replacement of the epoxide moiety with a cyclopropane or cyclobutane ring, inversion of configuration at C12 and C15, etc.) simultaneously and quantitatively, a chemometric tool is needed that analyzes both the affinity data and the structural features of all the compounds. When the gCOMBINE program(35) automatically calculated all the van der Waals (AMBER force field) and electrostatic interaction energies between each ligand and every protein residue, and then subjected these computed values (X variables) to multivariate statistical analysis using Partial Least Squares (a.k.a. Projection to Latent Structures, PLS), a good correlation was found with the experimental binding free energies (Y variable), which span a relatively narrow range of 20 kJ·mol⁻¹ (Figure 6A).

Figure 6.- (A) Correlation between predicted and calculated binding free energies in the COMBINE model with 3 latent variables ($r^2 = 0.92$, $q^2 = 0.82$). Some representative epothilones have been labelled for reference. Molecular surface of β -tubulin color-coded according to the normalized PLS pseudocoefficients (ramping from blue, most positive, to red, most negative) of the van der Waals (B) and electrostatic (C) interactions selected by the 3-PC COMBINE model derived for the 22 epothilones studied (displayed as sticks). Note the extra water molecule that is proposed to bridge good hydrogen-bonding interactions between the carbonyl oxygen at position 5 of the epothilones and both the main-chain NH of Arg278 and the main-chain CO of Leu217.

Importantly, the origin of the differences in ΔG values was mapped onto protein residues that line the binding site and their distinct role in modulating the binding affinities was quantified by means of signed PLS pseudocoefficients (Figure 6B and C and Table S4), which are used by the COMBINE methodology to properly weigh the calculated interaction energies (36-37). Since energy values in the refined complexes are usually negative (i.e. attractive), a positive coefficient will make the calculated binding free energy more negative, and vice versa. Thus, the importance of the direct hydrogen bonds established between the OH3 group of the epothilones and the side-chain amide nitrogen of Gln281 is reflected in the large positive coefficient assigned to the electrostatic term involving this residue. The fact that the lack of this hydroxyl is more deleterious for **EPKT2** than for **EPKT3** can be rationalized on the basis of a better electrostatic interaction between the amide and the π electrons in the double bond. Other hydrogen-bonding interactions are also modulated to different extents, as is the electrostatic interaction between the heteroaromatic moiety in the substituent attached to C15 and the side-chains of both Arg284 and Glu290, which make up the floor of the groove where the different substituents attached to the macrocycle are located. The van der Waals term, on the other hand, is heavily dominated by His229, Phe272, Pro274, Gln281 and Leu230, but increased ligand interactions with other residues, particularly Ala233 and Leu275, leads to a loss of binding affinity, which most likely reflects the existence of a steric clash(37).

The QSAR results obtained using the revised binding pose for EpoA represent an improvement over previous attempts that could only relate calculated binding energies to experimental binding enthalpies for some epothilones (21) and nicely rationalize the experimental findings that the binding of epothilones to mammalian tubulin is severely

decreased in the presence of T276I and R284Q mutations to the extent that they cause drug resistance (38). They are also in accord with the fact that Lys19 and His229 stand out among the five amino acid changes in yeast tubulin that drastically increase the cytotoxicity of EpoB (39).

Methods

Proteins and ligands

Calf brain tubulin (W-tubulin) was purified as described (40) or purchased (T238) from Cytoskeleton for the analytical ultracentrifugation control experiments. Docetaxel (Taxotere) was kindly provided by Sanofi-Aventis. Epothilones A and B were gifts from the Novartis Institute for Biomedical Research. Discodermolide was synthesized as described (41). All compounds were diluted in 99.8% D6-DMSO (Merck) to a final concentration of 20 mM and stored at -20 °C.

Binding of microtubule stabilizing agents to dimeric tubulin.

The binding of microtubule-stabilizing agents to dimeric tubulin was determined by centrifugation. Samples containing 50 μM of the desired compound in D_2O containing 10 mM sodium phosphate, 0.1 mM GTP pH* 7.0 (or the same buffer in H_2O) were incubated with increasing concentrations of tubulin up to 130 μM at 25°C. The samples were centrifuged at 100.000 rpm in a TLA 100 rotor in a Beckman Optima TLX ultracentrifuge for 120 minutes. The upper- and lower-most 100 μl were carefully collected, and the concentration of tubulin was determined spectrophotometrically, using an extinction coefficient of $107000 \text{ M}^{-1} \text{ cm}^{-1}$ at 275 nm in 10 mM phosphate buffer 1% SDS (42),

employing a Thermo Evolution 300 LC spectrophotometer. The top half was depleted of protein, which concentrated in the bottom half 10 μ M docetaxel was added as internal standard to 100 μ l of both parts and extracted three times with an excess volume of dichloromethane, dried in vacuum and, dissolved again in 25 μ l of 60% (v/v) methanol. The samples were then analyzed in an Agilent 1100 HPLC, employing a Zorbax Eclipse XDB-C18 column, using a gradient from 60 to 70% of methanol in water at 1 ml/min in 15 minutes.

Preparation of NMR samples.

The protocol for preparation of the samples with dimeric tubulin, and their analysis by analytical ultracentrifugation was as described (6). For the experiments carried out with dimeric tubulin and in order to further exclude any aggregation effect due to MSA binding, NMR samples of 13 μ M tubulin equilibrated by gel chromatography in 10 mM NaPi, 0.1 mM GTP, pH* 7.0, incubated with 0.5 mM EpoA, were analyzed by sedimentation velocity. These samples contained 90-94% of $\alpha\beta$ -tubulin dimers with $s_{20,w} = 5.8$ S(43) and a few percent of 9S and 12S oligomers, irrespective of epothilone addition (see Supp. Figure S1A and Table S1), thus indicating that the epothilone-tubulin interactions observed by NMR experiments involve only tubulin dimers.

In contrast, equilibration of tubulin by two-day dialysis in 2.5 mM calcium/sodium phosphate-D₂O buffer exactly as in previous studies (14-15,44-45) resulted in extensive tubulin aggregation. The protein partially precipitated and the fraction remaining in solution consisted of large aggregates and only a 17-20% of a 6.4 S component. This ~6S peak was somewhat broader than 5.8 S dimeric tubulin. Addition of 0.5 mM EpoA somewhat

modified the aggregation pattern but did not stabilize the ~6S component (Figure S1). Control experiments (not shown) indicate that the aggregation is due to the method previously employed to prepare the protein sample for the NMR experiment, since T238-tubulin (Cytoskeleton) behaves similarly to W-tubulin when processed through gel filtration chromatography.

For the ligands bound to microtubules, 20 μ M of tubulin in 10 mM KPi, 0.1 mM GMPCPP, 6 mM MgCl₂ pH* 6.5, was prepared as described (6) except for the pH* of the buffers employed to equilibrate it (pH* 6.8 instead of 7.0) the final pH* of the sample was then 6.5 instead of 6.7. After equilibration 300 μ M of the desired compound was added to the sample, which was incubated at 37°C for 30 minutes prior to the measurement, conditions in which the sample is assembled into microtubules with the ligand bound to the paclitaxel site.

NMR Experiments

NMR spectra were recorded at 298 K (dimeric tubulin samples) or 310 K (polymeric tubulin samples) in D₂O on a Bruker AVANCE 500 MHz spectrometer as described (6). STD and TR-NOE experiments were performed at least twice for each sample as described (6). All NMR spectra shown are representative experiments.

Conformational search and docking of ligands.

The expected STD effects for the ligands bound to non-polymerized tubulin α/β -heterodimers and microtubules were calculated using the CORCEMA-ST program (46). For these full relaxation matrix calculations, the overall rotational motion correlation time

τ_c for the free state was always set to 0.75 ns (values between 0.25 and 1 ns were tested), since NOESY cross peaks for the free molecule were basically zero at room temperature and 500 MHz. τ_c for the bound state was set to 60 ns for non-polymerized α/β -heterodimers (τ_c estimated with HYDROPRO (47)) and 100 ns for microtubules (optimized in the CORCEMA-ST calculation). An order parameter $S^2=0.85$ was employed to account for the fast rotation of the methyl groups, as implemented in CORCEMA-ST.

In order to fit the experimental STD effects and TR-NOE intensities, off-rate constants between 5-100000 s^{-1} were tested. Optimal agreement was achieved using $k_{off}=100 s^{-1}$ for microtubules. For the non-polymerized tubulin α/β -heterodimers, the best fit was achieved using a $k_{off}=8 s^{-1}$ for EpoA and $10 s^{-1}$ for EpoB

Conformational search calculations were performed using the MacroModel/Batchmin (48) package (version 9.6) and the OPLS2005 all-atom force field as previously described (6).

Docking of the ligands was performed using the AutoDock 4.0 program (49) employing the additional CORCEMA-ST (50) based scoring function as already illustrated (6).

COMBINE Analysis.

The structures of β -tubulin in complex with EpoA (PDB entry 4I50) and the different epothilone analogs studied were constructed as described before (21) but employing the crystallographic binding pose of the ligand.

Affinity maps for methyl, hydroxyl, keto and amino functional groups were calculated with the cGRILL program, an improved version of our CGRID code (51) that

relies on interaction energy calculations for selected probes on 3D grids, as pioneered by Goodford in his well-known GRID program (52) The cubic grid for cGRILL calculations was defined as the space delimited by the axis-parallel box containing the co-crystallized EpoA ligand, augmented by 5 Å in each axis direction, and the resulting energy maps (Supp. Info.) were of assistance to orientate some of the substituents (9). All the complexes were refined by energy minimization using the steepest descent algorithm until the root-mean-square of the potential energy gradient was below $0.1 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-1}$. The set of refined ligand-receptor complexes (including one water molecule, as explained below) was then used as input to the gCOMBINE program (35), which automatically calculated all the van der Waals (AMBER force field) and electrostatic interaction energies (dielectric constant = 4.0 Debye) between each ligand and every protein residue. The data matrix containing the computed energy components (X variables) was then subjected to multivariate statistical analysis using partial least squares (PLS) in order to find a correlation with the experimental binding free energies (Y variable). The leave-one-out method was used for cross-validation purposes. Comparable results were obtained when random groups of 3 elements per group were excluded from the training set each time and the procedure was repeated 5 times, as described (35), or when Goodford's images method was employed to compute the electrostatic interactions. The molecular graphics program PyMOL (v. 0.99rc6, DeLano Scientific) was employed for molecular editing, visualization and representation.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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