

The Interaction of Microtubules with Stabilizers Characterized at Biochemical and Structural Levels

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Abstract Since the discovery of paclitaxel and its peculiar mechanism of cytotoxicity, which has made it and its analogues widely used antitumour drugs, great effort has been made to understand the way they produce their effect in microtubules and to find other products that share this effect without the undesired side effects of low solubility and development of multidrug resistance by tumour cells. This chapter reviews the actual knowledge about the biochemical and structural mechanisms of microtubule stabilization by microtubule stabilizing agents, and illustrates the way paclitaxel and its biomimetics induce microtubule assembly, the thermodynamics of their binding, the way they reach their binding site and the conformation they have when bound.

Keywords Antitumour drugs, Bioconformer, Microtubule stabilizing agents, Paclitaxel

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Abbreviations

MSA	Microtubule stabilizing agent
Cr	critical concentration
GMPCPP	Guanosine-5'-[(α,β)-methylene]triphosphate
PEDTA	10mM phosphate, 1mM EDTA pH6.7 buffer
RMSD	Root mean square deviation, NOE, Nuclear Overhauser Enhancement
REDOR	Rotational Echo Double Resonance; tr-NOE, transferred Nuclear Overhauser Enhancement
NOESY/STD	Nuclear Overhauser Enhancement spectroscopy/saturation transfer difference

1 Biochemical Background on Microtubule Stabilizing Agents. What is a Microtubule Stabilizer?

1.1 Microtubules and Drugs

Small molecules modulating microtubule assembly have played major roles as tools in microtubule research, in a manner closely related to their chemotherapeutic interest [1]. Tubulin was first purified in the last century as the colchicine-binding protein proposed to be the subunit of cellular microtubules [2]. More recently, a colchicine derivative was employed to help crystallization and determine the structure of tubulin by X-ray diffraction [3]. The colchicine, vinblastine [4] and paclitaxel [5] sites are main drug binding sites of tubulin, to which many other substances bind. The discovery of microtubule stabilization by paclitaxel [6] prompted its clinical development [7] and a burst of research on new MSAs, as well as the generalized use of paclitaxel or docetaxel as convenient reagents to assemble (see Fig. 1), stabilize or detect microtubules in the laboratory. One example is the development

of the active fluorescent paclitaxel derivatives Flutax-1 and Flutax-2 [8] employed to study cellular microtubules [9] (see Fig. 2) and as probes of the paclitaxel binding site [10, 11]. The electron crystallography determination of the structure of tubulin was made on two-dimensional tubulin crystals assembled with zinc and paclitaxel, providing the first atomic view of tubulin and bound paclitaxel [5, 12]. In this structure, tubulin forms protofilaments which associate in a different fashion with respect to a microtubule.

1.2 Function and Evolution

It was speculated that the paclitaxel binding site of tubulin might bind an unknown endogenous regulator of microtubule assembly [6], but such a substance has not been convincingly documented so far. Therefore, we do not know

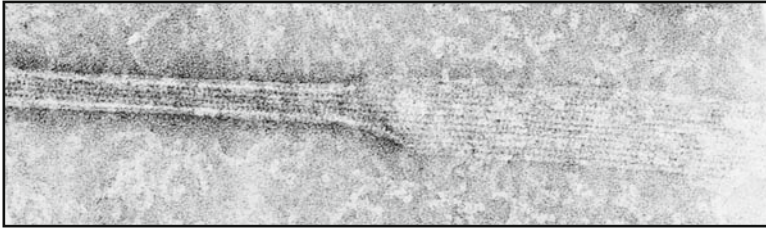


Fig. 1 Electron micrograph of a negatively stained microtubule assembled from purified tubulin and docetaxel. The *left side* shows the lateral projection of the protofilaments forming a microtubule cylinder ~24nm in diameter which has opened into a sheet on the *right side*. There are also tubulin oligomers in the image

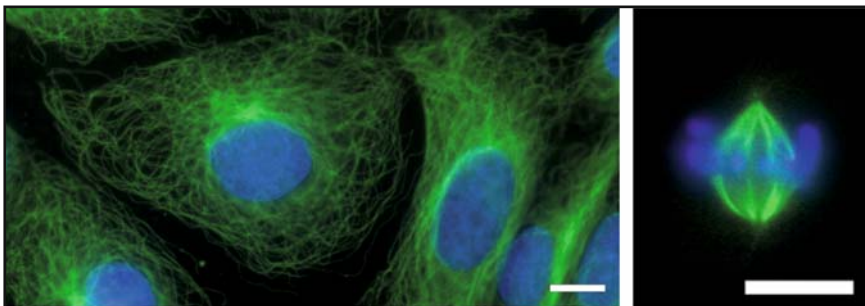


Fig. 2 *Left*: cytoplasmic microtubules in interphase kidney epithelial cells imaged with the fluorescent paclitaxel derivative Flutax-2 (*green*) and nuclear DNA stained with Hoescht 33342 (*blue*). *Right*: mitotic spindle from a dividing metaphase cell with similarly imaged microtubules and chromosomes. *Bars* indicate 10 μ m (micrographs courtesy of Isabel Barasoain)

whether the paclitaxel binding cavity has any physiological functions. The zones of microtubule associated proteins and motor proteins do not, in most cases, overlap with the paclitaxel or other drug binding sites. Tubulin promiscuously binds a variety of exogenous small molecules, particularly from natural sources, some of which are plant poisons known since antiquity, which suggests that the production of microtubule poisons is an effective defensive mechanism against predators. Interestingly, a simpler relative of tubulin, bacterial cell division protein FtsZ [13], forms tubulin-like protofilaments which do not associate into microtubules [14] and does not bind most of these substances (JMA, unpublished), suggesting that the binding of antimetabolic drugs by tubulin appeared with its ability to associate laterally into microtubules, or that plants and sponges have not developed a defensive mechanism targeting bacterial FtsZ.

1.3 What an MSA is and How it Works

A microtubule stabilizer or destabilizer molecule is, in a general thermodynamic definition, a molecule able to change the ratio between assembled and dimeric tubulin; to do so, it has to bind to microtubules differently from unassembled tubulin. A ligand which preferentially binds to the dimeric protein will inhibit polymer formation, whereas a ligand which binds more to the polymers than to the dimeric protein will stabilize the polymers, due to thermodynamic linkage [15]. This definition implies neither knowledge of the site of binding nor of the changes in the structural dynamics of ligand and protein upon binding. Since from a structural point of view a microtubule modulator may modify, either directly or allosterically, the polymerization interfaces of the tubulin molecule, one may obviously wish to know the mechanism by which such a ligand modifies microtubule assembly, which requires kinetic and structural investigation. Structural modifications may in principle range from local side-chain rearrangements to significant domain movements, but translate in a few kilojoules per mol tubulin of microtubule stabilization. GTP is a natural cofactor that binds at the axial association interfaces between tubulin subunits [5, 16]. Several microtubule inhibitors are in a broad sense interfacial ligands [17] which bind like wedges at or near association interfaces between tubulin molecules [4] and therefore perturb their polymerization geometry to different extents. MSAs subtly modify microtubule structure. Paclitaxel, by binding near the lateral association interface between protofilaments, reduces the average number of protofilaments in microtubules made of purified tubulin from 13 to 12 [18], whereas its side-chain analogue docetaxel does not [19], and the fluorescent analogues Flutax-1 and Flutax-2 carrying a bulky fluorescent moiety increase the average number of protofilaments from 13 to 14 [10]. Finally, any substance that stabilizes cellular microtubules may be considered an MSA irrespective of its molecular target or mechanism.

1.4 Chemical Diversity of MSAs in Short

MSAs with different chemical structures have been discovered in different natural sources. Taxanes come from plants, epothilones and cyclostreptin are of microbial origin, whereas discodermolide, dictyostatin, eleutherobin, laulimalide and peloruside were discovered in sea organisms (for a classification and MSA structures see [20]). We do not know of MSAs from a purely synthetic chemistry not related to natural products.

1.5 Three MSA Binding Sites in Microtubules

Many MSAs, including epothilones, discodermolide, dictyostatin, eleutherobin, sarcodictyin and a steroid derivative reversibly compete with taxanes to bind to the β -tubulin subunit in microtubules whereas cyclostreptin irreversibly inhibits taxane binding [20, 21]. Cyclostreptin binds covalently to a microtubule pore and to the luminal taxoid binding site, indicating the entry pathway of taxanes into microtubules (Fig. 3) [21]. Binding to one site appears to exclude binding to the other, apparently because both sites use residues from the β -tubulin loop between helices H6 and H7 [22]. This opens the possibility that some MSAs may stop at the pore and not reach the luminal site, as seems to be the case, at least partially, for fluorescent paclitaxel derivatives Flutax-2 and Hexaflutax [23]. On the other hand, laulimalide [24] and peloruside [25] share a binding site biochemically distinct from the paclitaxel site. Peloruside virtually docks into a zone of α -tubulin equivalent to that occupied by paclitaxel in β -tubulin [26, 27], yet its binding site has not been experimentally located. We still do not know whether any more binding sites for stabilizing ligands may exist in microtubules.

1.6 Mechanisms and Cellular Consequences of Microtubule Stabilization

Microtubule stabilizing agents at relatively high concentrations induce assembly of all tubulin available into microtubules, typically with accumulation of microtubule bundles in cells. The paclitaxel ligation of most tubulin molecules in a microtubule has interesting structural consequences, modifying microtubule flexural rigidity [28] and the curvature of dissociated protofilaments [29]. However, MSAs are active at lower concentrations at which they bind substoichiometrically to only a fraction of tubulin molecules in a microtubule. Under these substoichiometric conditions they suppress microtubule dynamic instability, a property in common with microtubule inhibitors, resulting in mitotic block or impairment, which eventually triggers tumour cell death [1] or senescence [30]. In addition, microtubule drugs can target tumour vasculature [1].

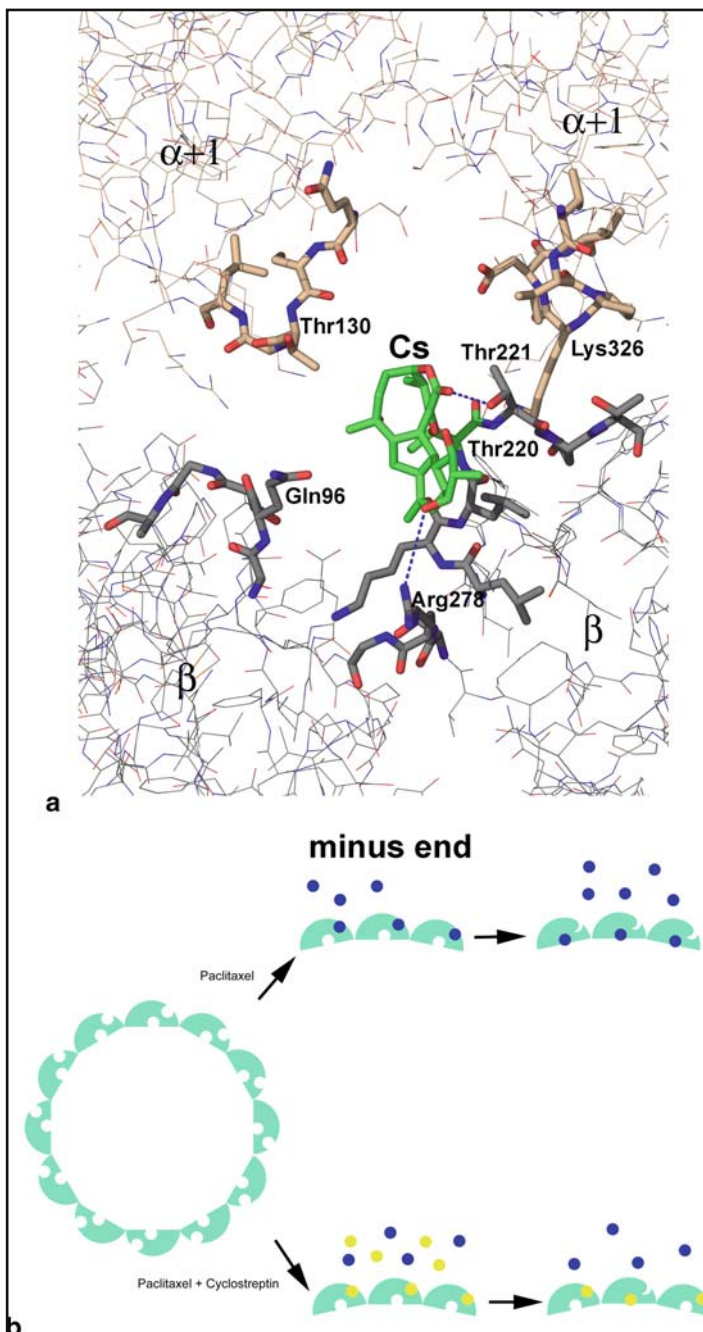


Fig. 3 **a** Model for the binding of cyclostreptin at the proposed initial MSA binding site at pore type I. Taken from [21]. **b** Scheme of the route of paclitaxel to its luminal site in the microtubules. Paclitaxel binds to the external site at the pores of the microtubules, being later transported to its luminal site while the external site gets blocked. In the presence of cyclostreptin the external site gets irreversibly blocked; thus, paclitaxel cannot reach the luminal site

2 Thermodynamics and Kinetic Mechanisms of the Interaction of Stabilizing Agents with Microtubules. How do they Bind?

2.1 *Thermodynamics of MSA Induced Microtubule Assembly. How do they Induce Assembly?*

The main ability of microtubule stabilizing agents and the basis of their mechanism of action is their capacity to induce microtubule assembly. Although different models [31, 32], based on the structure of the paclitaxel binding site [5], have been proposed to explain the paclitaxel mechanism of action on the basis of a structural effect on the tubulin molecule, it is unlikely, given the wide structural diversity of paclitaxel binding site ligands and the fact that there are at least three different binding sites (the internal and external paclitaxel sites and laulimalide one) that all of them produce the same structural effect at the atomic level on the microtubules.

On the other hand, from a thermodynamical point of view, a common mechanism for assembly induction can be proposed. All microtubule stabilizing agents bind tightly to the assembled form, while they do not bind with a measurable affinity to the dimeric tubulin [33], indicating that the taxane binding pocket is either not formed or not-completely formed in non-microtubular tubulin.

Considering the difference in affinities for the assembled and dimeric tubulin, it is straightforward to deduce that the compounds should displace the assembly equilibrium towards the polymerized form, as they fill the binding sites in microtubules.

However, preferential binding does not explain how microtubule assembly starts. Highly active microtubule stabilizing agents induce tubulin assembly in solution conditions in which no microtubules exist, and thus in which there is no equilibrium to be displaced [20, 33], implying that microtubule stabilizing agents have to bind unassembled tubulin (in dimeric or oligomeric form) to make it assemble into microtubules.

Microtubule assembly in the absence of ligands follows a non-covalent nucleated condensation polymerization, characterized by cooperative behaviour and by the presence of a critical concentration C_r , below which no significant formation of large polymers takes place [33] (Fig. 4). It can be demonstrated [34] that the apparent equilibrium constant for the growth reaction, i.e. the addition of a protomer to the polymer, is in good approximation, equal to the reciprocal critical concentration $K_{el_app} = C_r^{-1}$, which renders the apparent standard free energy change of elongation amenable to simple measurement (as long as nucleotide hydrolysis is disregarded).

For ligand-induced assembly an apparent critical concentration, dependent on the ligand concentration, is observed [33]. Paclitaxel- and epothilone-induced assembly of GDP-tubulin have been extensively characterized. Under these conditions, in which tubulin is unable to assemble in the absence of ligand, assembly and binding are linked processes, there being no assembled unligated tubulin and showing an apparent critical concentration that saturates with ligand concentration (Fig. 5 of [35] and Fig. 2 of [36]), the latter not being compatible with a simple mechanism in which MSA stabilize microtubules binding to the empty sites in them.

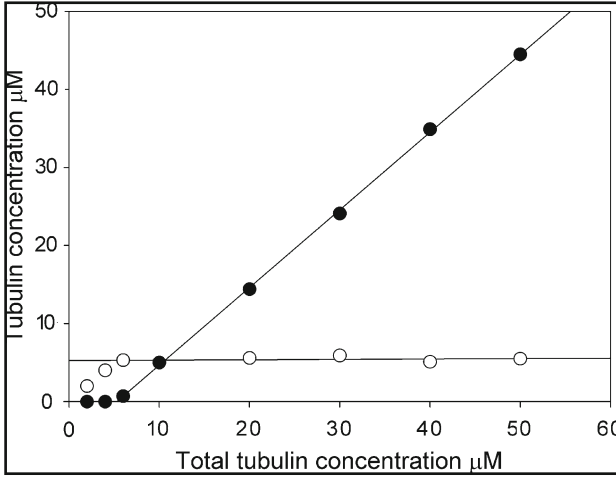
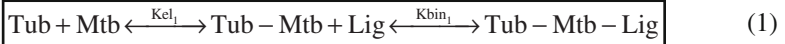


Fig. 4 Polymerization of GTP-tubulin in 3.4 M glycerol, 10mM sodium phosphate, 1mM EGTA, 1mM GTP, pH 6.7 buffer at 37°C measured by sedimentation. *Solid circles*, pelleted tubulin; *hollow circles*, tubulin in the supernatant. The critical concentration determined is 5.3 μM ; under this total concentration no tubulin is pelleted while over this total concentration all tubulin in excess is pelleted

In principle, ligand induced tubulin assembly may proceed in two different ways [33] (Fig. 5), the most intuitive being the ligand-facilitated pathway (stabilization by binding to the empty sites, in which elongation precedes binding, Eq.1, Fig. 5a), i.e. the binding of a tubulin dimer creates a new high affinity binding site for the ligand, whose binding displaces the equilibrium towards the assembled form.



Although this mechanism cannot explain microtubule induced assembly in conditions in which tubulin is unable to assemble in absence of ligands, the ligand facilitated pathway is supported by the fact that paclitaxel does not bind tightly to unassembled tubulin (at least under non-assembly conditions) [33].

However, there is a strong argument against the ligand facilitated pathway: if the reaction proceeds via the ligand-facilitated pathway, the apparent elongation constant K_{el_app} (the equilibrium constant of addition of a dimer to the end of the microtubule), which reflects the power of assembly induction, should depend linearly on the free concentration of ligand and its binding constant to the empty site (Eq. 2).

$$\boxed{K_{el_app} = \frac{[\text{Tub} - \text{Mtb} - \text{Lig}] + [\text{Tub} - \text{Mtb}]}{[\text{Tub}][\text{Mtb}]} = K_{el_1} (1 + [\text{Ligand}]K_{b_1})} \quad (2)$$

This means that given two different ligands, at the same concentration, their power of assembly induction should depend linearly on the binding constant of the ligand,

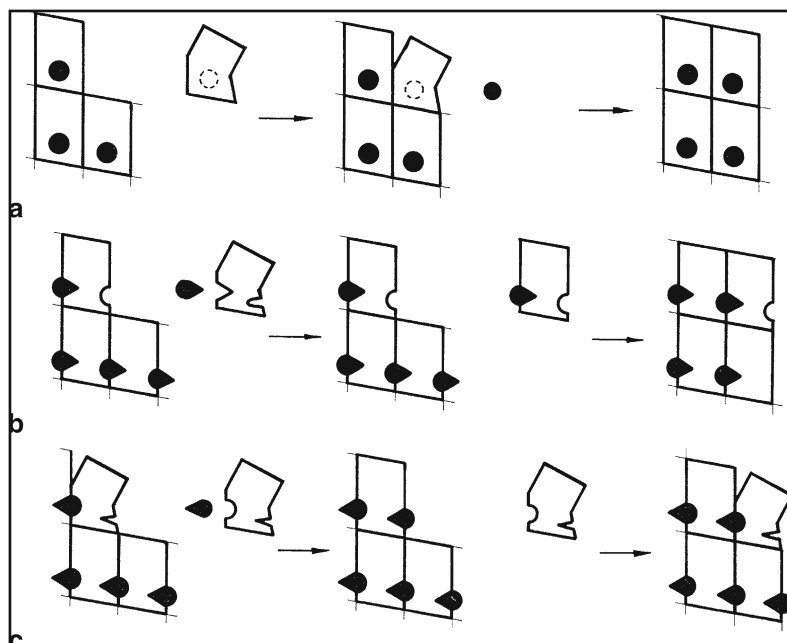
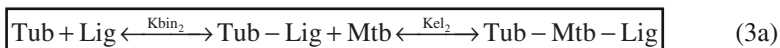


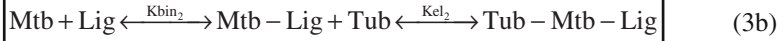
Fig. 5 Models of ligand induced tubulin assembly. The ligand induced addition of a tubulin molecule to the microtubule lattice is represented. **a** Ligand facilitated pathway, a ligand molecule binds to an empty site at the end of a microtubule thus stabilizing the microtubule end. **b** Ligand mediated pathway 1. The ligand binds to unassembled tubulin and the ligated dimer has a higher affinity for the microtubules. **c** Ligand mediated pathway 2. The binding of a ligand to a non completed site at the end of the microtubule increases the affinity for the binding of the next dimer. Adapted from [33]

independently of the specific effect that the ligand causes on tubulin. Equation(2) predicts a continuous increase of Kel_{app} observed at overstoichiometric concentrations of microtubule stabilizing agents.

Apparently this is not the case. Although, in general, better binders are better assembly inductors, there are compounds that significantly deviate from the best regression line (see Fig. 4a of [36], and Fig. 4a [20]), while a saturating behaviour of critical concentration Cr (inverse of apparent elongation constant Kel_{app}) with ligand concentration is observed (Fig. 2 of [36] and Fig. 5 of [35]).

This saturating behaviour observed is explained with both possible ligand-mediated pathways (binding precedes elongation), which are thermodynamically equivalent: (1) the ligand binds to unassembled tubulin and the ligated dimer has a higher affinity for the microtubules, so decreasing the critical concentration (3a, Fig. 5b); (2) the binding of a ligand to a non completed site at the end of the microtubule increases the affinity for the binding of the next dimer (Eq. 3b, Fig. 5c).





Mass action law predicts that if the ligand-mediated pathway were the only one that worked, the apparent elongation constant should saturate with the ligand concentration (Eq. 4), notwithstanding which of the pathways the reaction followed (in case path 3b is followed) the term $([\text{Tub}-\text{Lig}]+[\text{Tub}]) [\text{Mtb}]$ should be replaced by $([\text{Mtb}-\text{Lig}] + [\text{Mtb}]) [\text{Tub}]$.

$$\boxed{K_{\text{el}}^{\text{app}} = \frac{[\text{Tub} - \text{Mtb} - \text{Lig}]}{([\text{Tub} - \text{Lig}] + [\text{Tub}])[\text{Mtb}]} = \frac{K_{\text{el}_2} K_{\text{bin}_2} [\text{Ligand}]}{(1 + K_{\text{bin}_2} [\text{Ligand}])}} \quad (4)$$

So the elongation constant measured will depend on the elongation constant of the ligated dimer, which will depend on the specific effect that the ligand employed causes upon binding to a tubulin molecule.

However, since microtubule stabilizing agents will bind tightly to unoccupied sites, it is reasonable to assume that both mechanisms should work; however, in this case the apparent critical concentration will saturate following Eq. (5) (as in Eq. 4); in the case where path 3b is followed the term $([\text{Tub}-\text{Lig}]+[\text{Tub}])[\text{Mtb}]$ should be replaced by $([\text{Mtb}-\text{Lig}] + [\text{Mtb}]) [\text{Tub}]$:

$$\boxed{K_{\text{el}}^{\text{app}} = \frac{[\text{Mtb} - \text{Tub}] + [\text{Mtb} - \text{Tub} - \text{Lig}]}{([\text{Tub}] + [\text{Tub} - \text{Lig}])[\text{Mtb}]} = K_{\text{el}_2} K_{\text{bin}_2} \frac{[\text{Ligand}] + \frac{1}{K_{\text{bin}_1}}}{(1 + K_{\text{bin}_2} [\text{Ligand}])}} \quad (5)$$

In practice, since at the paclitaxel or epothilone concentrations necessary to induce assembly, the concentrations of free ligand are of the order of 10^{-6} M, and $1/K_{\text{bin}_1}$ for the strong assembly inducers is of the order of 10^{-7} to 10^{-9} M, the term $1/K_{\text{bin}_1}$ can be neglected and so Eq. (5) becomes equivalent to Eq. (4).

Following Eqs. (4) or (5), it can be deduced that the measured $K_{\text{el}}^{\text{app}}$ in saturation conditions (the experimental conditions) corresponds to the K_{el} of the ligated tubulin (or K_{el} of unligated tubulin to the ligated microtubule end), i.e. K_{el_2} .

Although the ligand mediated pathways easily explain both the experimental results of assembly induction in conditions in which tubulin cannot assemble in the absence of the ligands and the saturation behaviour of $K_{\text{el}}^{\text{app}}$, it seems contradictory with the absence of observed binding of ligands to dimeric tubulin. Nevertheless, evidence of ligand binding to dimeric tubulin and to an alternative binding site in microtubules was obtained, supporting the hypothesis that the ligand mediated pathway is involved in the assembly induction by the microtubule stabilizing agents. Cyclostreptin, a covalent binding microtubule stabilizing agent of the paclitaxel site [37], binds to the tubulin dimer [21] although with a slow kinetic rate in non-assembly conditions, and epothilone binding to tubulin to a mixed state of dimers and non-microtubule assemblies have been detected by NMR [38].

Thus, the most plausible hypothesis is that microtubule stabilizing agents induce microtubule assembly via a mechanism involving binding to dimeric tubulin. As described in Eq.(4), although microtubule stabilizing agents bind with high affinity to the

empty sites in the microtubules, the equilibrium of the assembly process (K_{app}) is determined by the change in the assembly properties of the dimer after binding to the microtubule stabilizing agent (K_{el_2}), and not by the binding affinity to the site itself (K_{bin_2}).

2.2 Thermodynamics of MSA Binding to Microtubules. How to Make Them Sticky?

Microtubule stabilizing agents work because they bind microtubules. Improving this property is important for their action, since the better they bind to tubulin, the better cytotoxic agents they are [20, 36, 39]. In addition to this, high affinity or covalent microtubule stabilizing agents get trapped inside the cell due to the chemical potential of their interaction with tubulin, thus escaping the detoxification mechanism by membrane pumps overexpression, used by multidrug resistant cells [21, 39].

Early studies of the interaction between microtubule stabilizing agents with their binding sites were greatly hampered by the thermodynamics of ligand induced assembly [6, 40]. Since microtubule stabilizing agents significantly perturb the assembly state of the protein, assembly and binding are linked processes and thus it is difficult to separate the contributions from both assembly and binding. It is thus, important to find conditions in which the assembly is not significantly perturbed due to the presence of the ligand.

Our first attempt to characterize the binding of paclitaxel and docetaxel to microtubules [35] was done at high concentrations of tubulin. Since in the conditions of the assay most of the tubulin gets assembled, the perturbation in the amount of assembled polymer is minimal, and a rigorous comparison between the binding affinity and the power of assembly induction of paclitaxel and docetaxel can be carried out. The study shows a single common binding site for paclitaxel and docetaxel for which docetaxel has double the affinity than paclitaxel (Fig. 6). However, it was not possible to measure directly the binding constant of paclitaxel and docetaxel. In order to do so, it would be necessary to have empty assembled binding sites. Although empty sites can be assembled in the absence of ligand at high concentrations of tubulin, the high affinity observed for paclitaxel and docetaxel made it impossible to find conditions in which the reaction is not completely displaced towards the bound state.

The problem was overcome with the use of mildly fixed microtubules [10] in which the paclitaxel binding site is unaltered, while protected from cold and dilution depolymerisation and a paclitaxel molecule bound to a fluorescent probe (either fluorescein or difluorofluorescein) [8]. The fluorescent derivatives of paclitaxel were then tested to check that they compete with taxoids for the same site, with the same 1:1 stoichiometry producing the same cellular effects as paclitaxel and docetaxel, thus it can be assumed that they bind for the same site [9]. Using these stabilized microtubules, it is possible to determine precisely the binding constant of the fluorescent taxoid which was found to be of the order of 10^8 M^{-1} at 25°C .

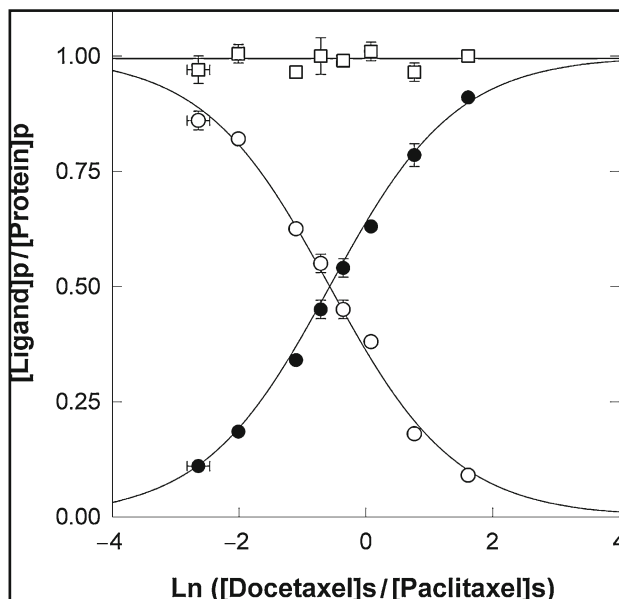


Fig. 6 Competition between ^3H -paclitaxel and ^{14}C -docetaxel for binding to microtubules. $11.3\ \mu\text{M}$ tubulin was assembled at 37°C in PEDTA, 1mM GDP, 1mM GTP, 8mM MgCl_2 , $\text{pH}6.7$ by the addition of paclitaxel and docetaxel at a total concentration of $20\ \mu\text{M}$, at different molar ratios of paclitaxel to docetaxel. The total concentration of microtubules was $11.0 \pm 0.10\ \mu\text{M}$; the concentration of tubulin in supernatants (not polymerized tubulin) varied between ca. 0.4 (in paclitaxel excess) and $0.2\ \mu\text{M}$ (in docetaxel excess). *Open circles*, ^3H -paclitaxel bound per polymerized tubulin dimer; *solid circles*, ^{14}C -docetaxel bound; *squares*, total ligand (paclitaxel plus docetaxel) bound per polymerized tubulin dimer. The *solid lines* are the best fit to the data, employing a simple competition model of the two ligands for the same site, taken from [35]

Having a fluorescent probe with a known binding site affinity, it was possible to design competition tests for the evaluation of the binding affinity of paclitaxel, docetaxel and baccatin III to microtubules [11, 22] that can be used for fast and precise evaluation of a large series of compounds thus allowing precise studies of structure-affinity relationship [36] and to classify the microtubule stabilizing agents according to their binding sites [20, 24, 25].

The test has been used to measure the binding affinity of a set of 19 epothilones [36]. The study showed that it is possible to optimize the binding affinity of complex molecules by studying the effect of single changes in the substituents of the core of the molecule and combining the most favourable substitutions to obtain a 500-fold increase in the binding affinity of the epothilone molecule from Epothilone A (compound 1) to *cis*-CP-*tmt*-Epo B (compound 19) (Fig. 7).

Moreover, the study demonstrates that the cytotoxicity of the compound on tumour cells is related to the binding affinity, being affinity of the compounds a variable to maximize in order to obtain more cytotoxic compounds.

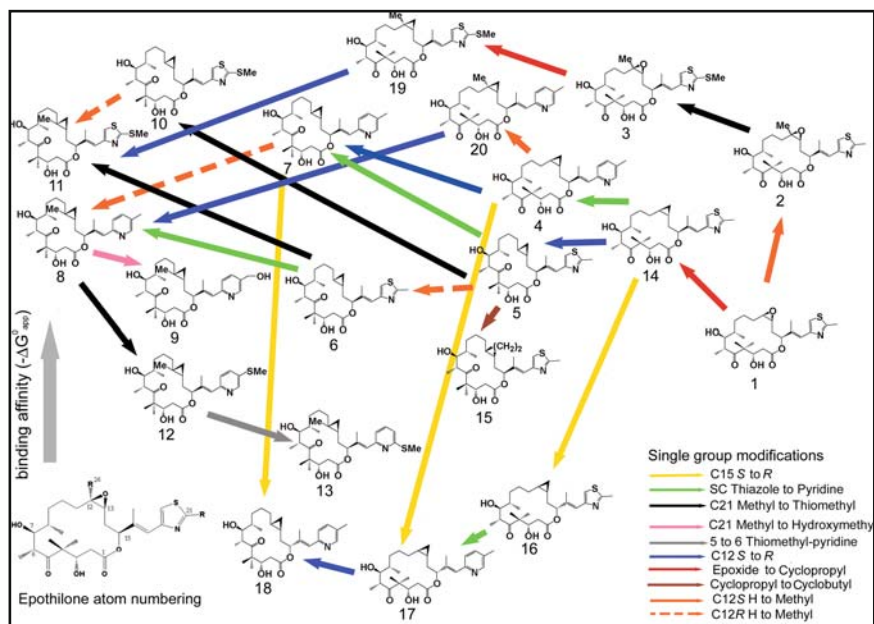


Fig. 7 Scheme of the structures of epothilone analogues studied in [36], the chemical differences between them, and the effect of these modifications in the free energy of binding to their site in microtubules at 35°C. Taken from [36]

The latter was generalized [20] for all paclitaxel binding site ligands known thus showing that the higher the affinity of the paclitaxel binding site the more cytotoxic the ligand.

From the equilibrium studies of microtubule stabilizing agents binding to the taxane site we learned the following: Microtubule stabilizing agents of the paclitaxel site bind microtubules with a well defined 1:1 stoichiometry. Binding affinity for a given fixed conformation of the molecule core can be modulated by selecting the side chain substituents with the highest contributions for the free energy of binding and combining them into a single molecule. The affinity of a compound is predictive of its cytotoxicity; thus it is possible to design more cytotoxic taxanes by studying the effect of each substitution on the binding affinity to select those which provide larger free energy changes to the binding.

2.3 Kinetics of MSA Binding to Microtubules. How Do They Reach Their Site in the Microtubules?

The kinetics of binding of ligands to a binding site provides information about the way the ligands reach the site. They may bind fast, indicating an easily accessible site, or they may bind slowly indicating an occluded site. They may bind in a single

step, indicating simple binding to a site or in successive steps, indicating a processing after early binding to a site.

One of the first effects of taxanes in microtubules observed was the fact that the structure of the paclitaxel-induced microtubules is different from that of the microtubules assembled in its absence [18]. Moreover, paclitaxel is able to change the structure of preformed docetaxel-induced assembled microtubules within a time range of the order of tens of seconds, indicating a fast exchange of taxanes in the site [41].

The modification of the microtubule structure should come from the perturbation of the interprotofilament contacts, which allows the accommodation of extra protofilaments in the microtubule lattice. The experimental fact that taxane binding modifies the interprotofilament contacts rapidly lead to the conclusion that the taxane binding site in microtubules was located in the interprotofilament space [18, 19].

The first structural location of the taxane binding site [42] placed it in the interprotofilament space, thus supporting the biochemical results. However, this changed when the first high resolution 3D structure of the paclitaxel-tubulin complex was solved by electron-crystallography of a two-dimensional zinc-induced tubulin polymer [5]. The fitting of this structure into a three-dimensional reconstruction of microtubules from cryoelectron microscopy allowed a pseudo atomic resolution model of microtubules [43] in which the paclitaxel binding site was placed inside the lumen of the microtubules hidden from the outer solvent.

The kinetics of taxane binding to microtubule were subsequently determined; taxanes and epothilones [10, 22, 23, 44] bind to microtubules extremely fast (Fig. 8, Table 1).

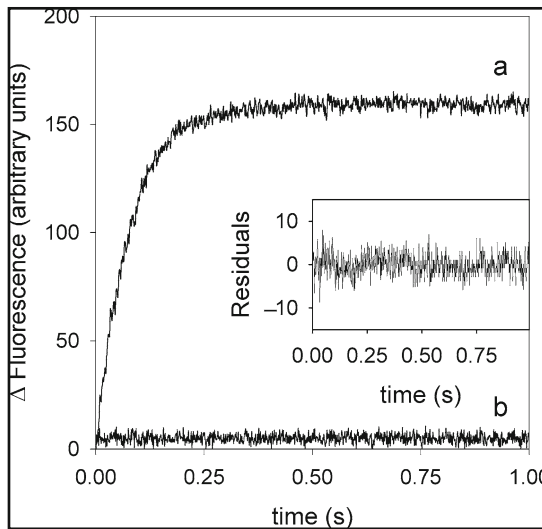


Fig. 8 Kinetics of binding of Flutax1 to microtubules at 35°C. In the stopped-flow device a 1 μM solution of Flutax1 was mixed with 25 μM pure tubulin assembled into microtubules (concentration of sites 20 μM) (final concentrations of 500 nM Flutax and 10 μM sites) in the absence (a) and presence (b) of 50 μM docetaxel. Curve a is fitted to an exponential decay. *Inset*: residues between the experimental and theoretical curves. Taken from [10]

Table 1 Kinetic rates of taxane site ligands binding to microtubules (37°C)

	$k_{+1} \times 10^5 \text{M}^{-1} \text{s}^{-1}$	$k_{-1} \text{s}^{-1}$	$k_{+2} \text{s}^{-1}$	$k_{-2} \text{s}^{-1}$
Flutax-1 ^a	6.10 ± 0.22	0.99 ± 0.27	0.0243 ± 0.0007	0.026 ± 0.0012
Flutax-2 ^a	13.83 ± 0.18	1.63 ± 0.18	2.7 ± 0.8	0.022 ± 0.0012
Paclitaxel ^b	3.63 ± 0.08	ND	ND	0.091 ± 0.0061
Epothilone A ^c	3.3 ± 0.03	ND	ND	0.138 ± 0.37 (slow) 0.463 ± 0.32 (fast)

k_{+1} and k_{-1} – kinetic on and off rate constants of the first fast bimolecular step

k_{+2} and k_{-2} – kinetic on and off rate constants of the second slow monomolecular step

^aData from [10]

^bData from [22]

^cData from [44]

The binding of taxanes has been well characterized [10, 22] and shows a series of consecutive reactions involving a first fast bimolecular step (k_{+1} and k_{-1}), a second slow monomolecular step (k_{+2} , k_{-2}) and a third step which is the structural change involving the change in the number of microtubule protofilaments. It can be proved numerically that the first bimolecular fast step of binding is diffusion controlled, thus indicating that taxanes can not directly bind to the luminal site [22].

Since the luminal site of microtubules was well supported by the structural data and also by the fact that mutations in the luminal site confer resistance to taxanes [45], an alternative mechanism with binding to an initial exposed binding site located in pore type I of the microtubule wall and later transportation of the ligand to the luminal site was proposed in [22] (Fig. 9).

The existence of the external site was further confirmed with a fluorescein-tagged taxane, Hexaflutax, specially tailored with a spacer between the taxane moiety and the fluorescein tag, long enough to allow binding to an external site while keeping the fluorescent tag exposed to an antibody but short enough to avoid antibody binding if the taxane moiety is bound to the luminal site. It was shown that monoclonal and polyclonal antibodies bind to Hexaflutax bound to microtubules at a kinetic rate consistent with a diffusion controlled bimolecular reaction between two objects of the size of a microtubule and an antibody, forming a stable ternary complex, thus indicating that at least a significant part of the taxane is bound to an external site [23].

The route of taxanes was finally unveiled with the use of a covalent ligand of the taxane binding site [21]. Cyclostreptin, a bacterial natural product [46, 47] with weak, but irreversible tubulin assembly activity and strong apparent binding affinity for the paclitaxel site, covalently labels both a residue placed in the luminal site of paclitaxel Asn228 and a residue previously proposed to be in the external binding site Thr220 (Fig. 3a).

From these results a structural binding pathway consistent with that observed kinetically was proposed [21]. Paclitaxel binds fast to a site located in the surface of the microtubules, into the pore type I. Then it has to be transferred to the second luminal, final location. The transfer involves probably the switch of some of the elements of the first site, since only one molecule of paclitaxel can bind to each molecule of β -tubulin (Fig. 3b).

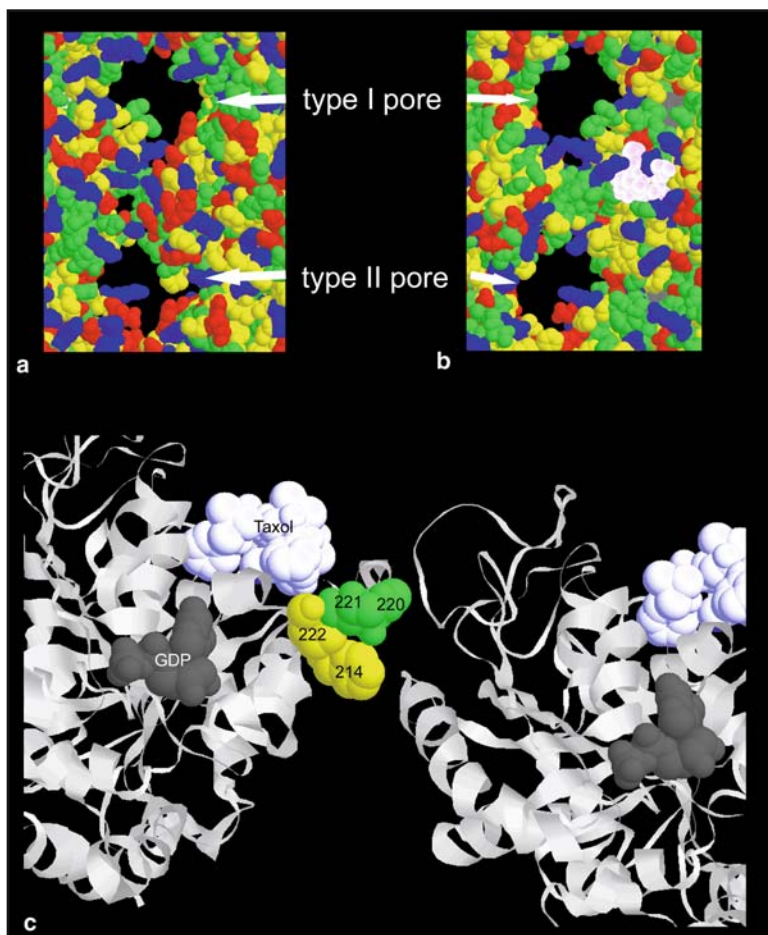


Fig. 9 Insight of the outer (a) and (b) inner surface of a high resolution microtubule model, showing two different types of pores, I and II (see text). *Green beads*, polar residues; *yellow beads*, hydrophobic residues; *red beads*, acid residues; *blue beads*, basic residues; *white beads*, paclitaxel bound at its site; *grey beads*, nucleotide. Detail of a pore type I viewed from above (c). Ribbon representation of two neighbour β -tubulin subunits as seen from the plus end of the microtubule, paclitaxel, GDP and the four residues forming a putative taxoid binding site are shown in Van der Waals representation. Taken from [22]

The kinetic information can be used as well to deduce if a compound is bound homogeneously or not. The kinetics of flutax and paclitaxel dissociation from β -tubulin are monophasic, which indicates a single rate limiting step, consistent with most of the compound bound to the same site. However, dissociation of Epothilone A from the binding site shows biphasic behaviour, which would be consistent with the compound distributed between the external and the luminal site, with a temperature-dependant equilibrium (Table 2) which favours the outer site at higher temperatures.

Table 2 Kinetic rates of epothilone A dissociation^a

	25°C	30°C	35°C	37°C	40°C
$k_{\text{fast}} \text{ s}^{-1}$	ND	ND	0.445	0.463	0.635
A_{fast}	ND	ND	0.25	0.35	0.38
$k_{\text{slow}} \text{ s}^{-1}$	0.020	0.052	0.126	0.138	0.199
A_{slow}	1	1	0.74	0.65	0.62

k_{fast} – apparent kinetic rate constant of the fast phase

A_{fast} – amplitude of the fast phase

k_{slow} – apparent kinetic rate constant of the slow phase

A_{slow} – amplitude of the slow phase

^aDetermined for this work as described in [44]

From the kinetics of ligand binding to microtubules we can obtain valuable information about the way paclitaxel microtubule stabilizing agents reach their binding site. They bind to an external binding site located in the pores of the microtubule wall from which they are totally or partially relocated to an inner luminal site.

3 The Bioactive Conformations of MSA. What Do They Look Like When Bound?

Many attempts have been made over the last years to deduce the actual pharmacophore/s for the recognition of MSA by microtubules. For newly designed MSA to serve as effective anticancer drugs, it seems reasonable that they should be able to achieve a conformation compatible with the binding pocket of the target protein. On this basis, many scientists have focused on determining the bioactive conformation of the different MSAs.

3.1 Paclitaxel and Related Molecules. The Single Conformer Hypothesis

The study of the tubulin-bound conformation of paclitaxel has resulted in a number of protein-ligand models, partially or fully based on the electron diffraction structure of $\alpha\beta$ -tubulin in paclitaxel-stabilized Zn^{2+} -induced sheets [5, 12]. Obviously, the nature of the paclitaxel binding site and the paclitaxel conformation in the binding site have key implications for the design of new MSA. A deep knowledge of the bioactive conformation would also help to explain how compounds as structurally diverse as the epothilones [48], discodermolide [49], and eleutherobin [50] have very similar mechanisms of action.

After the first photoaffinity labelling studies, which were the first methods used to define the paclitaxel binding site on tubulin, and indeed allowed identification of different amino acids of β -tubulin as putative parts of the binding site [51], the initial efforts to correlate activity (or binding) with the solid state, solution or modelled conformations of MSA in their free states led to the first pharmacophore proposals

[52]. At first, the existence of only a very limited set of conformations for these molecules was assumed [53], while more recently, it was considered that most of these molecules are intrinsically flexible and may assume a variety of shapes [54].

Previous investigations on this topic up to the beginning of 2002 have already been reviewed [55] and further expanded more recently [56], gathering the knowledge of the conformation of these molecules under different conditions. Up to 2001, also corresponding to the availability of the $\alpha\beta$ -tubulin coordinates from the electron crystallography structure [5, 12], the attempts to codify the bound conformation of MSA were derived from ligand conformations acquired in the free state by either X-ray crystallography or NMR studies. An assumed or modelled bioactive form was then employed as a template for superimposition by other ligands to try to deduce the pharmacophore [57].

After the availability of the 3.7Å resolution electron crystallographic structure of paclitaxel bound to $\alpha\beta$ -tubulin [5], further refined at 3.5Å resolution [12], different research groups tried to probe the binding conformation of MSA at the key β -subunit. Nevertheless, although paclitaxel indeed stabilized the actual microtubule sheets examined by electron crystallography, the first coordinates of tubulin deposited (1TUB) contained the single crystal coordinates for docetaxel [58] instead of the actual ligand, due to problems to characterize fully either the binding mode or the conformation of bound paclitaxel. On this basis, only qualitative statements could first be made concerning the details of ligand binding. Nonetheless, the location of the drug was consistent with the former photoaffinity labelling results and showed that paclitaxel occupied a hydrophobic cleft on β -tubulin. Furthermore, the ligand density suggested that one of the phenyl rings at the C-3' terminus was near the top of helix H1, while the C-2 benzoyl moiety was close to H5 and to the H5–H6 loop. In the refined structure (1JFF) [12], the taxane ring was better defined, but the densities for both the 2-phenyl side-chain and the *N'*-phenyl group still remained low, suggesting certain mobility of these groups. Nevertheless, according to the authors, the refined paclitaxel structure adopted a geometry very similar to that determined independently by energy-based refinement [59], except for the key torsional rotations of the side-chain phenyl rings. We will return to this point below.

Before these investigations, most of the models of the paclitaxel-tubulin interaction had been directly extrapolated from the conformations of paclitaxel found either in polar [60] or non-polar media [61] and from the single crystal X-ray structure of docetaxel (Fig. 10a) [58].

3.2 Paclitaxel and Related Molecules. Flexibility

Indeed, before 2000, single conformers had been basically considered, although it was possible that the multiple torsional degrees of freedom of these molecules would produce a complex multidimensional energy surface, and therefore a variety of putative 3D geometries capable of interacting with the tubulin binding site [62, 63]. Numerous research teams pursued different indirect approaches to the pharmacophore

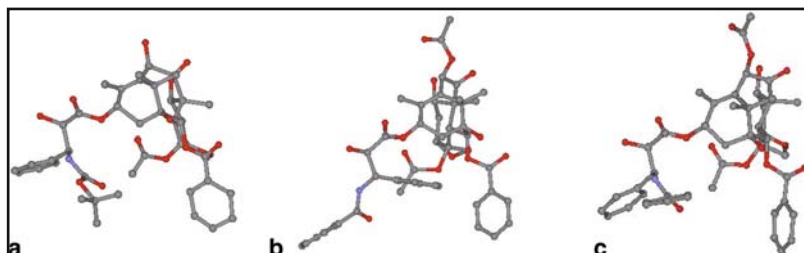


Fig. 10 a X-ray structure of docetaxel [58]. b Polar conformer of paclitaxel. c T-Taxol conformer

determination. The first and earliest attempts relied on studies of the structure–activity relationship of the taxane skeleton [52–54, 64] which included quantitative calculations [53]. These studies demonstrated that the presence and the spatial disposition of the three flexible side chains at C-4, C-2 and C-13 defined the conformations proposed or determined in both solution, in the solid state and at the β -tubulin binding site, since the baccatin core (A–D rings) was conformationally well-defined [65].

A more complete structure-based approach used (bio-)physical methods, in particular X-ray, NMR spectroscopy and/or molecular modelling, to study the structure and conformations of paclitaxel in its uncomplexed state, trying to extrapolate these findings to the bound form. Single crystal X-ray analysis in the solid state defined different conformers for different analogues [66], while NMR analysis in polar and non-polar media indeed identified others [67–73]. In all cases, these reports assumed a major single conformation, although later Snyder’s group, through deconvolution of the NMR data obtained for paclitaxel in CDCl_3 [62] and $\text{D}_2\text{O}/\text{DMSO-d}_6$ [100] solutions, identified many (9–10) conformations in these solvents, none of them showing a population above 30%. Nevertheless, the structural details from NMR were interpreted in terms of the key features of the dominant conformations: the NOE data in polar solvents showed that, to some extent, the C-3’ phenyl group is hydrophobically collapsed with the C-2 benzoyl phenyl (the “polar” conformer, Fig. 10b), while in non-polar solvents the data evidenced an analogous phenyl–phenyl association between the C-3’ benzamido phenyl and the one at the terminus of the C-2 side chain (the “nonpolar” conformer) [52–54, 57, 74, 75]. As previously reviewed [55], both the polar and the nonpolar conformations were proposed as the bioactive forms. It was also speculated that an eclipsed conformer related to the polar form could be the one recognized by the tubulin binding site [75].

3.3 *Synthetic Analogues. Quest for the Actual Bioactive Geometry*

These interpretations prompted many organic chemists to prepare synthetic analogues, bridging different regions of the paclitaxel molecule, aimed at locking or at least favouring the putative bioactive conformation of paclitaxel on tubulin

(see below for further discussion) [56, 76]. Wide discussion and controversy among different groups has been established on the bioactive three-dimensional form of paclitaxel. In a first REDOR NMR study, F-¹³C distances between the fluorine of a 2-(*p*-fluorobenzoyl) paclitaxel and both the C-3' amide carbonyl and C-3' methine carbons were estimated [77], permitting a serious attempt to determine key aspects of the ligand conformation at the β -tubulin binding site [78]. Complementary measurements of fluorescence-resonance energy transfer suggested a model for the paclitaxel binding mode. In a second study, a distance of 6.5 Å was determined between the fluorine atoms of 2-(*p*-fluorobenzoyl)-3'-(*p*-fluorophenyl)-10-acetyl-docetaxel [79]. Recent studies using a variety of fluorinated and deuterated analogues, also using REDOR [80] have led the authors of this investigation to conclude that the bound form is in agreement with the so-called T-Taxol geometry (see below) [59, 81], and not with other REDOR-based geometry proposed by another research group, dubbing New York (NY-Taxol) [78].

3.4 The T-Taxol Conformer

Computational studies have been of paramount importance in order to integrate the results of the experimental studies indicated above with the electron crystallographic data known. According to Snyder and co-workers, a satisfactory and experimentally verifiable model of the tubulin-binding site and of the conformation of paclitaxel has been obtained by computational methods on the first electron crystallographic model. In this context, a new paclitaxel conformer, T-Taxol (Fig. 10c), was proposed [59, 81, 82].

Further refinement of the electron crystallographic structure of tubulin-paclitaxel at 3.5 Å resolution delivered a similar result. Nevertheless, the T-Taxol model has not been completely accepted as the actual bioactive conformation [78]. It is evident that the low 3.5–3.7 Å resolution of the complex limits the precision of the resulting model. In addition, the Zn²⁺-stabilized tubulin preparation employed in the electron crystallographic study involves antiparallel protofilaments organized in sheets, which strongly differ from genuine microtubules. Consequently, concern has been expressed that the sheets may not be representative of cellular microtubules and that sheet-bound paclitaxel geometry may differ from its bioactive form in microtubules.

In this context, using the different models as templates, different synthetic organic chemistry groups have prepared cyclic, conformationally-constrained analogues based on the T-Taxol structure, leading to molecules with potencies similar or even greater than the lead structure [56, 76, 78, 83]. Different research groups have introduced constraints into the paclitaxel molecule involving the C-4, C-2 and C-13 side chains. Interestingly, it has been reported that forming a tether between the C-4 acetate methyl and the *ortho*- center of the C-3' phenyl, in agreement with the proposed T-Taxol conformation, permits the access to compounds with much better activities (3-, 30- or 50-fold) than the activity of paclitaxel, even when tested

against both paclitaxel- and epothilone-resistant cell lines. It has been proposed that the origin of the bioactivities is related to the degree of rigidification introduced by the C-4–C-3' bridging moieties. While for paclitaxel, the T-form was estimated to be present in only 2–5% of the variety of conformations [59, 83], the key constrained compounds appeared with 76 and 86% contributions from T-Taxol-like geometries. Nevertheless, the authors have also warned that the significant activity increases measured for these substances could not be attributed completely to conformational biasing, although it seems to be a dominating factor. Indeed, they proposed that reinforcing the T-Taxol conformation is a necessary but not sufficient condition to elicit high levels of drug potency [83]. Especially, in addition to the appropriate molecular conformation, the ligand must also show a tubulin-compatible molecular volume.

3.5 Epothilones. Different Geometries are Found Under Different Experimental Conditions

As for all MSAs, the investigation of the complex of epothilones [57] with tubulin has also been attempted. The solid state (deduced by either X-ray or solid state NMR) structure of free epothilone B is known, as well as its conformations in organic solvents [84–86]. However, its actual bioactive conformation is also a matter of debate.

For the free state, the major conformation of the macrocycle in solution is similar to that found in the crystal, although there was no preferred conformation of the thiazole side chain, which showed a substantial freedom of the side chain around the C17–C18 bond.

In contrast, approach to the structure determination by solid-state NMR [86], has proposed a more defined geometry of both the macrocycle and the side chain. In fact, the 10 solid-state NMR structures derived were very well defined, while the average RMSD of the heavy atom to the X-ray structure was only 0.75 Å. When comparing assignments of the solid-state NMR with the previously reported solution-state NMR data [84], a qualitative correlation was found between the chemical-shift variations observed and the possibility of intermolecular hydrogen-bonding [86].

The first study of the bioactive conformation of epothilones was conducted through solution-state NMR for epothilone A, which lacks the methyl group at C12, bound to non-microtubular $\alpha\beta$ -tubulin [38, 87]. Almost immediately, the tubulin/epothilone A complex was studied through electron-crystallography for Zn²⁺-stabilized $\alpha\beta$ -tubulin layers [88]. The conformation of the tubulin-bound epothilone was strikingly different in the two studies, suggesting the need for further investigation. As this discrepancy may reflect the dependence of the epothilone binding mode on the tubulin polymerization state, further studies are still expected.

From the viewpoint of NMR, the existence of specific and transient binding of epothilone A to non-microtubular tubulin enabled the structural analysis of the active conformation [38, 87] of the epothilones by using trNOESY experiments, in

particular, using the interligand NOE for pharmacophore mapping (INPHARMA) methodology [89] and molecular modeling.

For the NMR structural investigation of the epothilone-tubulin complex in solution, microtubule assembly was prevented. Evident conformational differences were observed between the X-ray crystal structure and the tubulin-bound NMR conformation of epothilone A. However, the authors rationalized many structure-activity relationship data available on the tubulin-polymerization activity of several epothilone derivatives [90]. According to these authors, the electron crystallography-derived model [88] of the epothilone A-tubulin complex did not support some of the structure activity relationship data. Nevertheless, as also stated in [89], the high flexibility of the M loop, the S9–S10 loop, and the H6–H7 loop forming the binding pocket could be responsible for different binding modes of epothilone A to tubulin, depending on the polymerization state.

Although, according to the NMR analysis in non-polar solvents [84], the most populated conformer is indeed very similar to the X-ray conformer, evidences of other conformers are also clearly observed in the NMR data in solution for the free state. The comparison of the torsion angles of epothilone A in the tubulin-bound and in the free conformation revealed two major changes: the first occurred in the O1–C6 region, while the second affected the orientation of the thiazole ring respect to the C16–C17 double bond. Neither the C6–C7 dihedral angle, nor the C10–C15 region exhibited significant conformational changes upon binding to tubulin. The most significant difference between the free and tubulin-bound conformations of epothilone occurred in the side chain with the thiazole ring. In the geometry of the bound conformation, the nitrogen atom of the thiazole ring becomes more accessible for potential formation of hydrogen bonds with the protein, which may more than offset the deduced high-energy epothilone side-chain conformation. The design of epothilone analogues should now use the knowledge about the conformation of epothilone in its tubulin-bound state.

3.6 *Discodermolide and Dictyostatin. The Free and Bound Conformers*

An elegant recent work by Carlomagno et al. [91] has reported a non-microtubular-tubulin-bound conformation of discodermolide. Since the conditions in which the discodermolide conformation was determined tubulin is not assembled into microtubules, it might be possible that the tubulin-bound conformation observed therein [91] is not identical to the conformation bound to microtubules. Nevertheless, under these experimental conditions, the tubulin-bound conformation of discodermolide in solution from NMR tr-NOE data is a compact globular shape. With the help of both protein-mediated interligand NOE signals between discodermolide and Epothilone [91] and the SAR data available for the two drugs, a common pharmacophore model was proposed. Similarities and differences in the pharmacophore of the two drugs may provide a rationale for the analogous but not fully equivalent

biological activities of the two natural products. Also, the similarity between the tubulin-bound conformation of discodermolide and its X-ray structure [92] provides a rationale for the powerful biological activity of the marine natural product dictyostatin, a 22-membered macrocyclic lactone which is structurally and biogenetically related to discodermolide. Not surprisingly, the tubulin-bound conformation of discodermolide derived therein [91] was closely related to the solution structure of dictyostatin [93], which supports a common mechanism for the MT-stabilizing activity of the two compounds.

A variety of NMR data, including trNOESY/STD, line broadening analysis, and STD-based NMR competition experiments, assisted by molecular mechanics calculations, have also been recently employed to deduce the microtubule-bound conformation of the previously mentioned two MSA, discodermolide and dictyostatin [94]. Since it is well known that tubulin in plain phosphate buffer does not assemble into microtubules, and it has been described that the microtubule taxoid binding site does not exist in dimeric tubulin, at least with an affinity higher than millimolar, we rather preferred to search for biochemical conditions in which stable microtubules were assembled from native tubulin with GMPCPP [27]. The NMR data obtained indicated that microtubules recognize discodermolide through a conformational selection process, in which the half-chair conformer (and not the predominant in water solution, skew boat form) of the lactone moiety is bound by the receptor. There are minor changes in the rest of the molecular skeleton between the major conformer in water solution and that bound to assembled microtubules. Indeed, despite the many torsional degrees of freedom of discodermolide, intramolecular interactions within the molecule and hydration strongly affect its conformational features, which indeed only shows conformational mobility around a fairly narrow part of the molecule. This evidence contrasts strongly with the observations on other solvents, in which discodermolide shows different degrees of flexibility. Therefore, while in acetonitrile [95] or dimethyl sulfoxide [96], the different torsional degrees of freedom of discodermolide show different flexibilities, the orientation of the C5–C24 chain is highly pre-organized in water solution [94] with respect to that bound by tubulin, probably to minimize entropic penalties. There are some slight changes in the deduced microtubules-bound conformation with respect to that described by Carlomagno et al. using non-assembled tubulin (Fig. 11). The structure provided by Carlomagno et al. [91] shows a chair for the six-membered ring and its orientation is different from the rest of the chain. In any case, the presentation of the molecule is remarkably similar, despite the change in the mode of preparation of tubulin in the two cases. In particular, van der Waals contacts and torsional constraints strongly bias its conformational behaviour. Yet this feature serves to modulate the presentation of polar and non-polar surfaces to interact with the binding site of tubulin. A model of the binding mode of discodermolide to tubulin has also been proposed, involving the β -tubulin monomer by using docking simulations [94]. This model involves the taxane binding site of tubulin and comprises both polar and non polar interactions between discodermolide and the receptor.

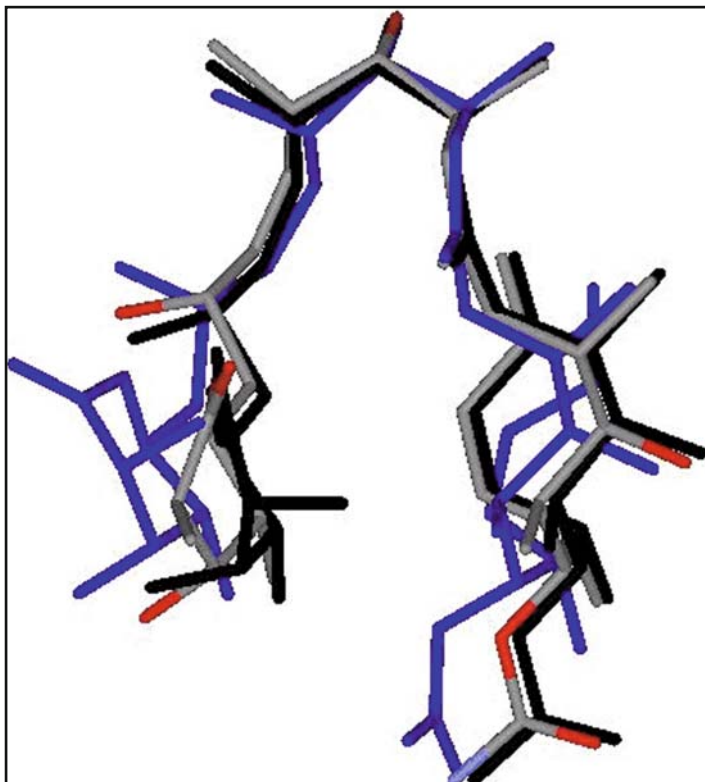


Fig. 11 Superimposition of the X-ray structure of discodermolide (*black*) [92], bound to non-assembled tubulin (*blue*) [91] and bound to microtubules [94]. Minor adjustments are observed

The microtubule-bound conformation of an analogous MSA, dictyostatin, has also been derived by using the same combined protocol of NMR spectroscopy and molecular docking [94]. The bound geometry deduced (Fig. 12) presents some key conformational differences against the major one existing in solution [97] around torsion angles at the C-7 region, and additionally displays mobility (even when bound) along the lateral C22–C26 chain. In any case, the bound conformer of dictyostatin resembles that of discodermolide and provides very similar contacts with the receptor. Competition experiments with a paclitaxel binding site ligand (Baccatin III [11]) have indicated that both molecules compete with the taxane-binding site, providing further support to previously described biochemical data.

3.7 *Peluroside A*

Peluroside A bound to microtubules has also been investigated. Two low energy conformations of Peluroside A have been found in water solution [27], which

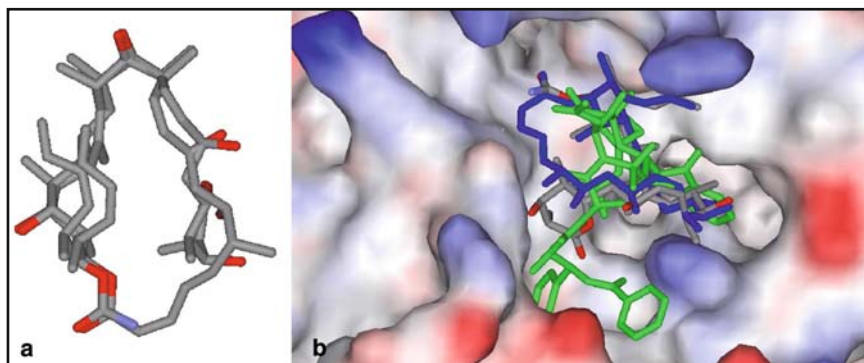


Fig. 12 **a** Superimposition of the bound conformers of discodermolide and dictyostatin bound to assembled microtubules [94]. **b** AUTODOCK solution of discodermolide and dictyostatin (*blue*) bound to tubulin (1JFF), also compared with bound paclitaxel (*green*)

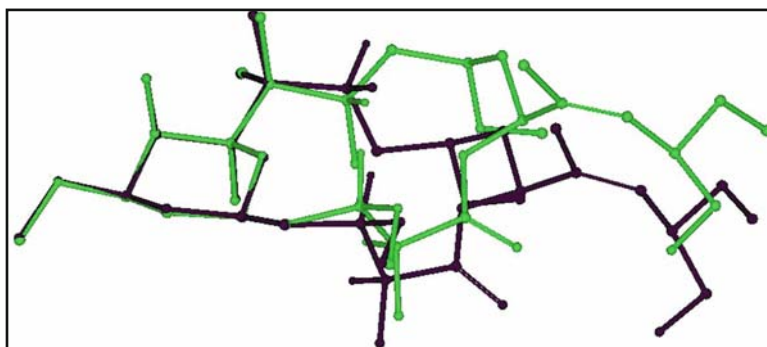


Fig. 13 Superimposition of the two major conformers of Peloruside A coexisting in water solution [27]

mainly differ in the relative orientation of the C10–C15 region (Fig. 13). One of these geometries (A) is in agreement with that postulated for Peloruside A in chloroform solution [98, 99]. From the viewpoint of energy, and using MM3*, the new conformer (B) is more stable than A. The major energy component that stabilizes B is solvation energy. Nevertheless, according to molecular dynamics simulations, both conformers are flexible along the backbone, with somehow higher variations for the lateral chain. Nevertheless, despite the large size of the macrocyclic ring, intramolecular interactions (van der Waals contacts and torsional constraints) within the Peloruside A ring strongly affect the conformational features of this molecule, which indeed only shows conformational mobility around a fairly narrow part of the molecule. Yet, this existing conformational freedom, in the presence of a given solvent, serves to modulate the presentation of polar and nonpolar surfaces to interact with the binding site.

Peluroside A was the first microtubule stabilizing agent whose conformation has been determined bound to microtubules (those of Paclitaxel and Epothilone were determined in non-microtubular tubulin [5, 12, 38, 91]). In the bound state, the NMR data, assisted by molecular mechanics calculations and docking experiments, indicated that only one (that present in water, B) of the two major conformations existing in water solution is bound to microtubules (α -tubulin). A model of the binding mode to tubulin has also been proposed [27], involving the α -tubulin monomer, in contrast with paclitaxel, which binds to the β -monomer.

It can be expected that the near future will provide tubulin-ligand structures with sufficient accuracy to define precisely the conformation and binding mode for these compounds and thereby validate or reject the current set of models. For the time being, the methodologies schematically reviewed here may provide additional data for understanding the action of MSAs and hopefully to design new potent analogues.

Note added in proof: Recently, taxane binding to microtubules has been optimized by affinity dissection and incremental construction of high affinity analogues of paclitaxel which are not affected by multidrug resistance in P-glycoprotein overexpressing cells [101]. The conformational preferences of epothilone A and C3-modified derivatives in aqueous solution have been found to include the previously proposed bioactive conformation bound to non-microtubular tubulin [102]. A different site of peloruside binding in beta-tubulin has been proposed from hydrogen-deuterium exchange and data-directed molecular docking [103]

References

1. Jordan MA, Wilson L (2004) *Nat Rev Cancer* 4:253
2. Weisenberg RC, Borisy GG, Taylor EW (1968) *Biochemistry* 7:4466
3. Ravelli RB, Gigant B, Curmi PA, Jourdain I, Lachkar S, Sobel A, Knossow M (2004) *Nature* 428:198
4. Gigant B, Wang C, Ravelli RB, Roussi F, Steinmetz MO, Curmi PA, Sobel A, Knossow M (2005) *Nature* 435:519
5. Nogales E, Wolf SG, Downing KH (1998) *Nature* 391:199
6. Schiff PB, Fant J, Horwitz SB (1979) *Nature* 277:665
7. Wall ME, Wani MC (1995) *Cancer Res* 55:753
8. Souto AA, Acuña AU, Andreu JM, Barasoain I, Abal M, Amat-Guerri F (1996) *Angew Chem Int Ed Engl* 34:2710
9. Evangelio JA, Abal M, Barasoain I, Souto AA, Lillo MP, Acuña AU, Amat-Guerri F, Andreu JM (1998) *Cell Motil Cytoskeleton* 39:73
10. Díaz JF, Strobe R, Engelborghs Y, Souto AA, Andreu JM (2000) *J Biol Chem* 275:26265
11. Andreu JM, Barasoain I (2001) *Biochemistry* 40:11975
12. Lowe J, Li H, Downing KH, Nogales E (2001) *J Mol Biol* 313:1045
13. Lowe J, Amos LA (1998) *Nature* 391:203
14. Huecas S, Llorca O, Boskovic J, Martín-Benito J, Valpuesta JM, Andreu JM (2008) Energetics and geometry of FtsZ polymers: nucleated self-assembly of single protofilaments. *Biophys J* 94, 1798
15. Wyman J, Gill SJ (1990) *Binding and linkage*. University Science Books, Mill Valley, CA

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16. Menéndez M, Rivas G, Díaz JF, Andreu JM (1998) *J Biol Chem* 273:167
17. Pommier Y, Cherfils J (2005) *Trends Pharmacol Sci* 26:138
18. Andreu JM, Bordas J, Díaz JF, García de Ancos J, Gil R, Medrano FJ, Nogales E, Pantos E, Towns-Andrews E (1992) *J Mol Biol* 226:169
19. Andreu JM, Díaz JF, Gil R, de Pereda JM, García de Lacoba M, Peyrot V, Briand C, Towns-Andrews E, Bordas J (1994) *J Biol Chem* 269:31785
20. Buey RM, Barasoain I, Jackson E, Meyer A, Giannakakou P, Paterson I, Mooberry S, Andreu JM, Díaz JF (2005) *Chem Biol* 12:1269
21. Buey RM, Calvo E, Barasoain I, Pineda O, Edler MC, Matesanz R, Cerezo G, Vanderwal CD, Day BW, Sorensen EJ, Lopez JA, Andreu JM, Hamel E, Díaz JF (2007) *Nat Chem Biol* 3:117
22. Díaz JF, Barasoain I, Andreu JM (2003) *J Biol Chem* 278:8407
23. Díaz JF, Barasoain I, Souto AA, Amat-Guerri F, Andreu JM (2005) *J Biol Chem* 280:3928
24. Pryor DE, O'Brate A, Bilcer G, Diaz JF, Wang Y, Kabaki M, Jung MK, Andreu JM, Ghosh AK, Giannakakou P, Hamel E (2002) *Biochemistry* 41:9109
25. Gaitanos TN, Buey RM, Díaz JF, Northcote PT, Teesdale-Spittle P, Andreu JM, Miller JH (2004) *Cancer Res* 64:5063
26. Pineda O, Farras J, Maccari L, Manetti F, Botta M, Vilarrasa J (2004) *Bioorg Med Chem Lett* 14:4825
27. Jimenez-Barbero J, Canales A, Northcote PT, Buey RM, Andreu JM, Diaz JF (2006) *J Am Chem Soc* 128:8757
28. Dye RB, Fink SP, Williams RC (1993) *J Biol Chem* 268:6847
29. Elie-Caille C, Severin F, Helenius J, Howard J, Muller DJ, Hyman AA (2007) *Current Biology* 17:1765
30. Klein LE, Freeze BS, Smith AB, Horwitz SB (2005) *Cell Cycle* 4:501
31. Amos LA, Lowe J (1999) *Chem Biol* 6:R65
32. Li H, DeRosier DJ, Nicholson WV, Nogales E, Downing KH (2002) *Structure (Camb)* 10:1317
33. Díaz JF, Menéndez M, Andreu JM (1993) *Biochemistry* 32:10067
34. Oosawa F, Asakura S (1975) *Thermodynamics of the polymerization of protein*, Academic, London
35. Díaz JF, Andreu JM (1993) *Biochemistry* 32:2747
36. Buey RM, Díaz JF, Andreu JM, O'Brate A, Giannakakou P, Nicolaou KC, Sasmal PK, Ritzén A, Namoto K (2004) *Chem Biol* 11:225
37. Edler MC, Buey RM, Gussio R, Marcus AI, Vanderwal CD, Sorensen EJ, Díaz JF, Giannakakou P, Hamel E (2005) *Biochemistry* 44:11525
38. Carlomagno T, Blommers MJ, Meiler J, Jahnke W, Schupp T, Petersen F, Schinzer D, Altmann KH, Griesinger C (2003) *Angew Chem Int Ed Engl* 42:2511
39. Yang C, Barasoain I, Li X, Matesanz R, Liu R, Sharom FJ, Díaz JF, Fang W (2007) *Chem Med Chem* 2:691
40. Schiff PB, Horwitz SB (1981) *Biochemistry* 20:3247
41. Díaz JF, Valpuesta JM, Chacón P, Diakun G, Andreu JM (1998) *J Biol Chem* 273:33803
42. Nogales E, Wolf SG, Khan IA, Luduena RF, Downing KH (1995) *Nature* 375:424
43. Nogales E, Whittaker M, Milligan RA, Downing KH (1999) *Cell* 96:79
44. Díaz JF, Buey RM (2007) In: Zhou J (ed) *Methods in molecular medicine*, vol 137. Humana, Totowa, NJ p 245
45. Giannakakou P, Sackett DL, Kang YK, Zhan Z, Buters JT, Fojo T, Poruchynsky MS (1997) *J Biol Chem* 272:17118
46. Sato B, Muramatsu H, Miyauchi M, Hori Y, Takase S, Hino M, Hashimoto S, Terano H (2000) *J Antibiot (Tokyo)* 53:123
47. Sato B, Nakajima H, Hori Y, Hino M, Hashimoto S, Terano H (2000) *J Antibiot (Tokyo)* 53:204
48. Nicolaou KC, Roschangar F, Vourloumis D (1998) *Angew Chem Int Ed Engl* 37:2015
49. Kalesse M (2000) *Chembiochem* 1:171
50. Lindel T, Jensen PR, Fenical W, Long BH, Casazza AM, Carboni J, Fairchild CR (1997) *J Am Chem Soc* 119:8744
51. Rao S, He LF, Chakravarty S, Ojima I, Orr GA, Horwitz SB (1999) *J Biol Chem* 274:37990
52. Czaplinski KHA, Grunewald GL (1994) *Bioorg Med Chem Lett* 4:2211

53. Morita H, Gonda A, Wei L, Takeya K, Itokawa H (1997) *Bioorg Med Chem Lett* 7:2387
54. Wang M, Xia X, Kim Y, Hwang D, Jansen JM, Botta M, Liotta DC, Snyder JP (1999) *Org Lett* 1:43
55. Jiménez-Barbero J, Amat-Guerri F, Snyder JP (2002) *Curr Med Chem Anti-Canc Agents* 2:91
56. Kingston DGI, Bane S, Snyder JP (2005) *Cell Cycle* 4:279
57. Giannakakou P, Gussio R, Nogales E, Downing KH, Zaharevitz D, Bollbuck B, Poy G, Sackett D, Nicolaou KC, Fojo T (2000) *Proc Natl Acad Sci USA* 97:2904
58. Gueritte-Voegelein F, Guenard D, Mangatal L, Potier P, Guilhem J, Cesario M, Pascard C (1990) *Acta Cryst C* 46:781
59. Snyder JP, Nettles JH, Cornett B, Downing KH, Nogales E (2001) *Proc Natl Acad Sci USA* 98:5312
60. Vandervelde DG, Georg GI, Grunewald GL, Gunn GW, Mitscher LA (1993) *J Am Chem Soc* 115:11650
61. Williams HJ, Scott AI, Dieden RA, Swindell CS, Chirlian LE, Francl MM, Heerding JM, Krauss NE (1994) *Can J Chem* 72:252
62. Snyder JP, Nevins N, Cicero DO, Jasen J (2000) *J Am Chem Soc* 122:724
63. Johnson SA, Alcaraz AA, Snyder JP (2005) *Org Lett* 7:5549
64. Zhu QQ, Guo ZR, Huang N, Wang MM, Chu FM (1997) *J Med Chem* 40:4319
65. Moyna G, Williams HJ, Scott AI, Ringel I, Gorodetsky R, Swindell CS (1997) *J Med Chem* 40:3305
66. Mastropaolo D, Camerman A, Luo YG, Brayer GD, Camerman N (1995) *Proc Natl Acad Sci USA* 92:6920
67. Gómez Paloma L, Guy RK, Wrasidlo W, Nicolaou KC (1994) *Chem Biol* 1:107
68. Dubois J, Guenard D, Gueritte-Voegelein F, Guedira N, Potier P, Gillet B, Beloeil JC (1993) *Tetrahedron* 49:6533
69. Cachau RE, Gussio R, Beutler JA, Chmurny GN, Hilton BD, Muschik GM, Erickson JW (1994) *Int J Supercomput Appl High Perform Comput* 8:24
70. Boge TC, Himes RH, Vandervelde DG, Georg GI (1994) *J Med Chem* 37:3337
71. Ojima I, Kuduk SD, Chakravarty S, Ourevitch M, Begue JP (1997) *J Am Chem Soc* 119:5519
72. Georg GI, Harriman GCB, Hepperle M, Clowers JS, VanderVelde DG, Himes RH (1996) *J Org Chem* 61:2664
73. Jiménez-Barbero J, Souto AA, Abal M, Barasoain I, Evangelio JA, Acuña AU, Andreu JM, Amat-Guerri F (1998) *Bioorg Med Chem* 6:1857
74. Ojima I, Chakravarty S, Inoue T, Lin S, He L, Horwitz SB, Kuduk SD, Danishefsky SJ (1999) *Proc Natl Acad Sci USA* 96:4256
75. Magnani M, Ortuso F, Soro S, Alcaro S, Tramontano A, Botta M (2006) *Febs J* 273:3301
76. Shanker N, Kingston DGI, Ganesh T, Yang C, Alcaraz AA, Geballe MT, Banerjee A, McGee D, Snyder JP, Bane S (2007) *Biochemistry* 46:11514
77. Li YK, Poliks B, Cegelski L, Poliks M, Gryczynski Z, Piszczek G, Jagtap PG, Studelska DR, Kingston DGI, Schaefer J, Bane S (2000) *Biochemistry* 39:281
78. Geney R, Sun L, Pera P, Bernacki RJ, Xia SJ, Horwitz SB, Simmerling CL, Ojima I (2005) *Chem Biol* 12:339
79. Ojima I, Inoue T, Chakravarty S (1999) *J Fluorine Chem* 97:3
80. Paik Y, Yang C, Metaferia B, Tang SB, Bane S, Ravindra R, Shanker N, Alcaraz AA, Johnson SA, Schaefer J, O'Connor RD, Cegelski L, Snyder JP, Kingston DGI (2007) *J Am Chem Soc* 129:361
81. Alcaraz AA, Mehta AK, Johnson SA, Snyder JP (2006) *J Med Chem* 49:2478
82. Ganesh T, Guza RC, Bane S, Ravindra R, Shanker N, Lakdawala AS, Snyder JP, Kingston DGI (2004) *Proc Natl Acad Sci USA* 101:10006
83. Ganesh T, Yang C, Norris A, Glass T, Bane S, Ravindra R, Banerjee A, Metaferia B, Thomas SL, Giannakakou P, Alcaraz AA, Lakdawala AS, Snyder JP, Kingston DGI (2007) *J Med Chem* 50:713
84. Höfle G, Bedorf N, Steinmetz H, Schomburg D, Gerth K, Reichenbach H (1996) *Angew Chem Int Ed Engl* 35:1567

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85. Taylor RE, Zajicek J (1999) *J Org Chem* 64:7224
86. Lange A, Schupp T, Petersen F, Carlomagno T, Baldus M (2007) *Chemmedchem* 2:522
87. Carlomagno T, Sanchez VM, Blommers MJ, Griesinger C (2003) *Angew Chem Int Ed Engl* 42:2515
88. Nettles JH, Li H, Cornett B, Krahn JM, Snyder JP, Downing KH (2004) *Science* 305:866
89. Reese M, Sanchez-Pedregal VM, Kubicek K, Meiler J, Blommers MJJ, Griesinger C, Carlomagno T (2007) *Angew Chem Int Ed Engl* 46:1864
90. Nicolaou KC, Scarpelli R, Bollbuck B, Werschkun B, Pereira MMA, Wartmann M, Altmann KH, Zaharevitz D, Gussio R, Giannakakou P (2000) *Chem Biol* 7:593
91. Sanchez-Pedregal VM, Kubicek K, Meiler J, Lyothier I, Paterson I, Carlomagno T (2006) *Angew Chem Int Ed Engl* 45:7388
92. Gunasekera SP, Gunasekera M, Longley RE, Schulte GK (1990) *J Org Chem* 55:4912
93. Paterson I, Britton R, Delgado O, Meyer A, Poullennec KG (2004) *Angew Chem Int Ed Engl* 43:4629
94. Canales A, Matesanz R, Gardner NM, Andreu JM, Paterson I, Díaz JF, Jiménez-Barbero J (2008) The bound conformation of microtubule-stabilizing agents (II): NMR insights on the bioactive 3D structure of discodermolide and dictyostatin. *Chemistry* (DOI: 10.1002/chem.200800039)
95. Smith AB III, LaMarche MJ, Falcone-Hindley M (2001) *Org Lett* 3:695
96. Monteagudo E, Cicero DO, Cornett B, Myles DC, Snyder JP (2001) *J Am Chem Soc* 123:6929
97. Paterson I, Britton R, Delgado O, Wright AE (2004) *Chem Commun (Camb)*:632
98. West LM, Northcote PT, Battershill CN (2000) *J Org Chem* 65:445
99. Liao XB, Wu YS, De Brabander JK (2003) *Angew Chem Int Ed Engl* 42:1648
100. Snyder JP, Nevins N, Jiménez-Barbero J, Cicero D, Jansen JM. Unpublished
101. Matesanz R, Barasoain I, Yang CG, Wang L, Li X, de Inés C, Coderch C, Gago F, Barbero JJ, Andreu JM, Fang WS, Díaz JF. (2008) *Chem Biol*.15:573
102. Erdélyi M, Pfeiffer B, Hauenstein K, Fohrer J, Gertsch J, Altmann KH, Carlomagno T. (2008) *J Med Chem.* 51:1469
103. Huzil JT, Chik JK, Slyszyk GW, Freedman H, Tuszynski J, Taylor RE, Sackett DL, Schriemer DC. (2008) *J Mol Biol.* 378:1016