Modulation of microtubule interprotofilament interactions by modified taxanes.

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Abstract.

Microtubules assembled with paclitaxel and docetaxel differ in the number of protofilaments, reflecting modification of the lateral association between αβ-tubulin molecules in the microtubule wall. These modifications of microtubule structure, through a non-yet characterized mechanism, are most likely related to the changes in tubulin-tubulin interactions responsible for microtubule stabilization by these antitumor compounds. We have used a set of modified taxanes to study the structural mechanism of microtubule stabilization by these ligands.

Using Small Angle X-ray Scattering, we have determined how modifications in the shape and size of the taxane substituents result in changes in the interprotofilament angles and in their number. The observed effects have been explained using NMR-aided docking and molecular dynamic simulations of taxane binding at the microtubule pore and luminal sites. Modeling results indicate that modification of the size of substituents at positions C7 and C10 of the taxane core influence the conformation of three key elements in microtubule lateral interactions (M-loop, S3 beta strand and H3 helix) that modulate the contacts between adjacent protofilaments. Whereas, modifications of the substituents at position C2 slightly rearrange the ligand in the binding site, modifying the interaction of the C7 substituent with the M-loop.
Introduction.

Fully assembled microtubules are long hollow cylinders of approximately 25 nm diameter composed of laterally associated tubulin protofilaments. The number of protofilaments per microtubule, can range from 10 to 18, although the number is usually 13 in vivo. In contrast, this number is variable in vitro and depends on the experimental conditions. It should be stressed that the actual protofilament number is heterogeneous and follows a distribution. In fact, the number of protofilaments is given as an average figure (1).

Taxanes are a class of anti-tumor drugs in clinical use (2), which exert their cytotoxic action through the microtubule cytoskeleton. Paclitaxel (Figure 1) and its chemical relatives, such as docetaxel, belong to this group of drugs (3). Taxanes prevent microtubule disassembly by activation of GDP-bound tubulin (4-5). They were the first available class of compounds with microtubule-stabilizing agent (MSA) activity.

Several models have been proposed (see (6) for a review) to explain the structural mechanisms of microtubule assembly, the most simplistic one proposes the one by one addition of tubulin dimers to the growing end of a nucleus (7). In our case, MSA-induced microtubule assembly, the structural pathway of MSA-induced microtubule assembly has been studied using Small Angle X-ray Scattering (SAXS), (8). Under the biochemical conditions of this study, and in the presence of MSA, the first structural step of the assembly involves oligomerization of tubulin dimers in the presence of Mg$^{2+}$ ions, in a head to tail way, to form linear oligomers, called protofilaments. These linear oligomers laterally associate to form the nucleus, (this process is mediated by the MSA) which grows until the number of protofilaments are sufficient to display cylindrical morphology, with the correct interprotofilament angle (Figure 1). This so-called ‘Interprotofilament angle’ is defined as the average angle between protofilaments in the cylindrical cross section (see inset in Figure 2A). The nucleus then elongates by incorporating new tubulin subunits to the growing end of the cylinder. This model is compatible with previous observations of intermediate sheet structures (9) and with the observed fast growth of existing microtubules from elongation of sheet-like structures at the growing ends (10). However, we should point out that although the MSA-induced microtubule assembly, which we are studying in this paper, can be considered as model system of the physiological GTP-induced microtubule assembly, the exact mechanism of GTP-induced tubulin assembly both in vivo and in vitro is controversial (11-12) and thus microtubule formation may proceed through a different structural pathway.

In earlier studies on the interaction of taxanes with microtubules we found that the structure of paclitaxel-induced microtubules is different from the structure of those assembled in the absence of this drug (13). In the presence of paclitaxel, microtubules assembled with an average of 12.1 protofilaments (13), which is rather different to the average number of protofilaments found in vitro (13.4). Strikingly, when assembled in the presence of docetaxel, the microtubules have an identical average protofilament number to that found in the absence of drugs (14). The most straightforward explanation for this
observation would be that these compounds can alter the way in which protofilaments interact laterally. In addition, the exchange of paclitaxel for docetaxel, or the addition of Flutax-2 (a chemically modified fluorescent analogue) to microtubules preassembled in the absence of drugs resulted in rapid changes (within a time scale of less than 1 minute) in the number of protofilaments (8,15-16). Therefore, the mechanism of MSA-induced assembly should modify the interprotofilament angle and subsequently the average number of microtubule protofilaments.

Although high resolution electron diffraction located the main paclitaxel binding site on the internal surface of microtubules (17), rather remote from the interprotofilament space, our studies (18-21) have provided evidence of the existence another binding site for MSAs. This site is on the vicinity of residue T220 of β-tubulin (at the outer surface of the pore in the microtubule wall). On the other hand, it has been described that binding of paclitaxel to the luminal site of microtubules facilitates the interaction between the S7-H9 loop (M-loop) of β-tubulin and H1-S2 loop of the adjacent subunit (17). This enhanced M-loop H1-S2-loop interaction has been proposed as one of the key reasons for the microtubule-stabilizing activity of paclitaxel (22). In both cases, (binding in the outer surface of the pore or at the luminal site), MSA-tubulin interactions take place close to the interprotofilament region, so it is not unreasonable to imagine that they should modify the corresponding angle and thus, the microtubule structure.

Herein, we have employed SAXS to determine the average microtubule diameter and the number of protofilaments. By comparing SAXS data of microtubules assembled in the presence of a series of different modified taxanes (Figure 1) it has been possible to shed light on the mechanisms involved in the modification of the microtubule structure by these ligands. On this basis, the goal of this work has been to obtain insights, at molecular and atomic resolution, into the mechanisms of MSA-induced microtubule assembly by understanding how ligands with different chemical functional groups may modify the interactions between protofilaments. These events are probably related to those which modulate tubulin-tubulin interactions and induce microtubule assembly. Therefore, taxane derivatives with different sizes and shapes have been selected from a library of active compounds (23). In particular, a number of molecules have been selected for our study according to different observations; since the first differences in the observed number of protofilaments involved paclitaxel and docetaxel, which mainly differ in the substitutions at the C13 lateral chain (at the “eastern” part of the molecule), these two molecules and related analogues have been initially selected for our study. However, between these two key molecules there is also one smaller modification at position 10 (in the “north” part of the taxane core), which was also chosen for further exploration. On the other hand, we also decided to explore the consequences of the chemical differences between paclitaxel, Flutax-1 and Flutax-2, which involve the C7 lateral chain (at the “north-east” part of the taxane core). Additionally, the effect of modifications at the C2 side chain (in the “southern” part) has also been evaluated, since they have a significant influence on the binding of taxanes to microtubules (23).
Materials and Methods

Ligands and protein.

Purified calf brain tubulin and chemicals were purified, synthesized and used as described (5,16,18,23-24). Baccatin III was from Sigma-Aldrich (St. Louis, USA). All compounds were dissolved at 50 mM concentration in D_6-DMSO.

X-ray scattering measurements.

Tubulin was equilibrated in 10 mM sodium phosphate, 1 mM EDTA, 0.1 mM GTP, buffer pH 7.0 through a Sephadex G-25 medium column (25x9 mm), and the protein was centrifuged for 20 min at 90.000xg in a TLA120.2 rotor in a Optima TLX centrifuge (Beckman) to remove aggregates. The tubulin concentration was then determined spectrophotometrically as described (25). MgCl_2 (7 mM) and up to 1 mM GTP were added to the sample (final pH 6.7) and the desired ligand or DMSO (vehicle) in a 10% stoichiometric excess over the protein concentration was added, and the samples were incubated for 20 minutes at 37ºC and kept at 25ºC before recording the scattering patterns.

SAXS data collection was performed either at BM26B station (DUBBLE) of the ESRF in Grenoble (France) or at a Bruker NANOSTAR system (see Supplementary information for details).

The low angle X-ray scattering pattern of microtubules in solution can be described as the Fourier transform of a hollow cylinder. To a first approximation, the intensity is given by the zero'th order Bessel function \( J_0(qR) \), \( q \) being the scattering angle and \( R \), the cylinder radius (26). The position of the first scattering maximum is therefore a sensitive measure for the radius of the microtubule, via the relation \( J_0(1.22/2R) \) since in a solution scattering pattern these are not distorted due to overlap between the higher order diffraction maxima of the helical lattice (13-14).

The differences in diameter between microtubules assembled in the presence of different taxanes can be interpreted, to a good approximation, as changes in the average number of protofilaments making up the cylinder wall.

NMR sample preparation and experiments

The samples of the ligands bound to microtubules were prepared, measured at 310 K in D_2O on Bruker AVANCE 500 MHz or 700 MHz spectrometers and analyzed as described (21). Off-rate constants between 1-200000 s\(^{-1}\) were tested in order to fit the experimental STD effects and TR-NOE intensities. Optimal agreement was achieved for \( k_{off} = 90 \) s\(^{-1}\) for cephalomanine, Chitax-1, Chitax-4 and Chitax-17 and for 110 s\(^{-1}\) for Flutax-2.

Docking and molecular dynamics calculations.

Docking of the ligands was performed as described (21) (see
Supplementary information for details).

Autodock poses with the best fitting between experimental and calculated STD values were refined by using molecular dynamic simulations (MD) performed with AMBER 9 (27), (500 ps equilibration time, 2 ns acquisition time). For each compound 100 structures were saved along the last ns of the MD trajectory. The predicted STD values of each structure were obtained with CORCEMA-ST and the average STD values of these 100 structures were considered for comparison against the experimental data. Finally, the Normalized Root-Mean-Square Deviation (NRMSD) between these average STD values and the experimental STD were calculated for each proton of the ligand.

The volume of the Solvent Excluded Surface (V-SES) by the side chains of the taxanes was calculated using the MSMS procedure (28) to calculate the excluded surface and the Chimera program (29) to calculate the volume under the excluded surface.

Results

SAXS determination of the sizes of the ligand induced microtubules

Tubulin assembly was induced in the presence of the different ligands and the corresponding SAXS profiles were recorded for each microtubule population. Figure 2 shows the comparison of the profiles for selected molecules differing in a single position of the taxane side chain. From these profiles, the positions of the first order Bessel function maxima $J_{01}$ were determined and appropriate controls were performed to assure that the position of $J_n$ peak (proportional to the distance between the center of the protofilaments) remained constant in all cases. From these data the average microtubule diameters and average protofilament number were calculated (Table I).

Except for the case of Chitax-14 in which the valleys are remarkably less pronounced, indicating a larger proportion of open microtubular sheets (8), the width of the peaks and the ratio between the maximum of the $J_{01}$ peak and the minimum between $J_{01}$ and $J_{02}$ is very similar for all the compounds studied. This indicates that the distribution of protofilament numbers and the proportion of open microtubular sheets should be similar for all ligand induced microtubules and also similar to these previously described by electron microscopy (1,8,13-14).

Effect of the modification of the ligand size, at selected positions, in the microtubule structure.

The effects of single point changes of the taxane molecule, on the interprotofilament contacts can be then calculated from the changes in the number of protofilaments (Table II). When the SAXS patterns of paclitaxel-induced microtubules (Figure 2A, green line) are compared with patterns of
microtubules assembled in the presence of Chitax-21 and cephalomannine (paclitaxel equivalents with specific modifications only at the C13 side chain) the differences in the position of the J01 maxima fell within the experimental error (Table II) (Figure 2A, black and red lines, respectively). The same result was obtained for the rest of the taxanes with single point modifications at C13 side chain (Table II). Only the removal of the C13 side chain (from paclitaxel to baccatin III) resulted in a large decrease of 1.7 units in the average number of protofilaments.

The result indicates that the change in microtubule structure observed between paclitaxel-induced and docetaxel-induced microtubules (13-14) was actually not due to the modification at the side chain. However the results point towards a strong influence of the volume of the side chain in the interaction with the binding site. In fact the size of the two side chains involved (phenyl for tert-butyl) is nearly the same as it is shown by the calculation of the change in the volume of the Solvent Excluded Surface (V-SES) by the side chain (Table II) (-11 Å3). Exchange of these two side chains for a different one with a similar size (butylen) (phenyl for butylen +15 Å3) does not result in an appreciable change in the protofilament number while removing of the side chain which results in a volume change of -256 Å3 results in a large decrease of the average number of protofilaments.

It is therefore evident that the difference observed between the structure of the microtubules induced by paclitaxel and those induced by docetaxel should arise from the difference of the group at position 10 (acetyl group vs. a free OH moiety, for paclitaxel and docetaxel respectively), and corresponds to a volume decrease of 42 Å3.

An increase of volume of the position 10 resulted in a small but consistent decrease in the number of protofilaments of the induced microtubules. The introduction of acetyl (cephalomannine, red line, Figure 2B) or propionyl (+53 Å3) groups (Chitax-18, black line, Figure 2B) at position 10 resulted in a decrease of the microtubule diameter as compared with Chitax-17 (green line, Figure 2B) (Table II). When an acetyl group was introduced as a single point modification at position 10, the average decrease in the number of protofilaments was 0.7. When a propionyl group was introduced, the decrease corresponded to 1.0 protofilaments.

An opposite effect is seen for the other group in the north face of the molecule, C7 also pointing towards the interprotofilament space in both binding sites. The introduction of bulky groups instead of the free hydroxyl, such as fluorescein (+366 Å3) and difluorofluorescein (+375 Å3) (Flutax-1 and Flutax-2), at this position resulted in a large increase in the microtubule diameter (16). However, the relatively large size of these groups may result in additional interactions. Therefore a further experiment comparison was also performed for analogues bearing a smaller propionyl side chain (+53 Å3). Thus, the effect observed could be compared to that monitored for the equivalent change at position 10.

The introduction of a propionyl side chain at C7 (Chitax-17->Chitax-1) resulted in an increase in the diameter of the microtubules (Figure 2C, green
line for Chitax-17 and black line for Chitax-1), equivalent to 1.4 extra protofilaments. Accordingly, this variation was similar to that observed for the introduction of the fluorescein moieties mentioned above, with 1.6 extra protofilaments (see Table II). Interestingly, the introduction of a bulkier group, as in 7-hexaflutax (+453 Å³), resulted in the largest change in diameter observed, corresponding to 2.1 protofilaments, as also shown in Table II.

Since the “south face” of the molecule and, more precisely, the benzyl moiety at C2 position of the taxane ring strongly influences the interactions of these compounds with the luminal site (23) and, very probably, also with the pore site, (20) we have studied the modifications at C2 that highly modulate the binding affinity. In both cases, the C2-modified molecules with groups at the meta position of the ring, which increase the binding affinity, amplified the microtubule diameter (Table II). An average increase of 0.7 units was observed for compounds bearing an azide substituent, which added extra 35 Å³ (-N₃, Chitax-14, black line in Figure 2D) and an average increase of 0.8 units for the methoxy-containing analogues, which added 24 Å³ (-OCH₃, Chitax-13, green line in Figure 2D).

**NMR and molecular modeling.**

Three-dimensional models of the ligands bound to the two alternative binding sites (the luminal and the pore site) were obtained in order to understand the effects, at the structural level, of ligand binding to microtubules. A combined NMR and modeling approach was employed when NMR experiments could be performed. Unfortunately, taxane solubility problems precluded the general use of the combined procedure. However, in the case of docetaxel (21),(23), paclitaxel, chitax-42 and flutax-2 (this work), NMR-based information could be obtained regarding the bioactive conformations and the binding epitopes of the ligands, which was employed as a guide for the modeling procedures.

STD experiments detect magnetization transfer from the protein to a bound ligand. Only bound ligands show STD signals and, as in any NOE-type experiment, the STD effect observed depends on the distance between the protons of the protein and those of the ligand, thus providing a useful tool to detect the ligand epitope and to structurally probe the binding site. This information is of paramount importance to improve the docking models. However, there are kinetic requirements for these experiments to be successful. Indeed, STD and TR-NOESY (Transferred NOE effect) experiments, (which permits deducing the bioactive conformation of the ligand) require a fast off-rate in the relaxation time scale. Therefore, as previously discussed, the characteristic slow dissociation of the ligands from the luminal site in the microtubules precludes the observation of TR-NOESY and STD signals from the ligand when bound to this site (23). As a matter of fact, the calculated STD-profiles of the taxanes bound to the luminal site cannot reproduce those experimentally determined (21). Thus, the NMR-based information for these systems can only be employed to model the pore-bound poses.

Three model compounds were selected for the construction of the
model “average” STD profile of taxanes. Paclitaxel and docetaxel were selected as lead compounds in the series, while Chitax-42 (a low affinity compound with a modified linker at C2) was chosen to determine if all active taxanes bind to microtubules in the same way. Flutax-2 (16) was also measured as an example of those analogues bearing bulky probes at C7. STD and TR-NOESY data were acquired for paclitaxel, Chitax-42 and Flutax-2 (experimental data are shown in Figure 3A, a higher resolution version of the TR-NOESY spectra is available as supplemental information (Figure S1)). Docetaxel data was taken from our previous studies (21,23). Regarding the taxane part of the molecules, the molecular conformation deduced from TR-NOESY spectra was found to be identical to that previously described for docetaxel (21,23).

Flutax-2 was modeled in the pore site (Figure 3B), employing its experimentally measured STD profile (Figure 3C), as described in Materials and Methods. The normalized root mean square deviation factor (NRMSD) of the best model was 19.38%, in between those obtained for the previously published models of docetaxel 9.9% and discodermolide 22.4 % bound to microtubules (21). Given the bulky group at C7 which may be involved in additional interactions with the binding site, STD effects of Flutax-2 protons were not employed to build the model “averaged” STD profile for the common protons of paclitaxel, which was later use to model the non-fluorescent ligands.

Four selected ligands (cephalominine, Chitax-1, Chitax-4 and Chitax-17, which differ in single points at C7 (Chitax-17 and cephalomannine), C10 (Chitax-17 and Chitax-1) and C2 (Chitax-1 and Chitax-4)), were modeled in the pore site employing the model “average” STD profile of taxanes. These ligands were chosen because of their possible influence on the microtubule interprotofilament contacts described above. As in the case of Flutax-2, the compounds were first docked into the pore site, and produced different poses. Following this procedure, the geometries of the docking poses were employed to calculate the expected STD profiles (30). The poses were then classified by comparing their expected STD values to those of the model “average” taxane (Figure 3D), and their geometries were compared to those experimentally determined for docetaxel (23), and to those estimated for the T-Taxol conformation (31). Based on the conservation of the TR-NOESY profile, only those models in which the structure of the ligand was compatible with either of these geometries were further considered (Table S1). NRMSD between the STD estimations was considered to quantify the similarities, as described in the experimental section. The NRMSD values obtained (Cephalomannine 18.93%, Chitax-1 18.26%, Chitax-17 16.23 %, Chitax-4 18.79%) and the models were in between those determined for the best structures previously obtained for docetaxel and discodermolide (21) (Figures 4A-C). The ligands were found to bind between the tubulin β-subunit, close to the luminal site (β1, following the Magnani nomenclature (32)), and the α-subunit of the next dimer in the protofilament (α2, see Figure 5A of (21), cyan structure), as previously described for docetaxel (21). However, although the location of the binding site was similar to that described by Magnani (but rather different to that found by Freedman et al. (33), close to subunits β1 and β4), the binding pose of the
ligands was rather different to that described by Magnani (32).

Alternatively, the four ligands were modeled in the luminal binding site (1JFF model) (17) by direct docking of the geometries constructed from the TR-NOESY-determined docetaxel conformation (23), as previously described (34) (Figures 4E-H).

The geometries of the “best” docking poses were considered as initial structures for MD simulations. In this way, the influence of the different ligands on the interprotofilament contacts were evaluated. As hypothesized, the different chemical moieties at the peripheral substitutions made distinct and specific contacts with determined protein regions. Local perturbation of the conformations of the H3 and S3 elements of the β-tubulin subunit at the right side of the pore (orange subunit) was observed when the ligand was bound to the pore site (Figures 4A-C). Alternatively, perturbations in the topology of the β-tubulin M-loop, which contains the luminal site, were detected when the ligand was bound to this site (Figures 4E-H). These changes on the protein conformation are likely to result in variations of the interprotofilament contacts.

Figure S2 shows the effect of the cephalomannine, Chitax-17, Chitax-1, and Chitax-4 series in the M-loop. They induced 12.6, 13.4, 14.8, and 15.2 protofilament-containing microtubules, respectively. It could be deduced that these molecules, especially cephalomannine and Chitax-17, induced the closing of the M-loop towards the luminal site, thus giving rise to the formation of microtubules with a low number of protofilaments.

Discussion

In order to better understand the mechanism of MSA-induced microtubule assembly and why interprotofilament contacts and subsequently the microtubule structure are altered as compared with GTP-induced assembly, different taxanes with groups of different sizes at selected positions were employed. We then observed how the modification in the taxane shape is reflected in the microtubule structure, assuming that it is unlikely that the general features of the interaction of the ligand with the binding sites would be altered by these minor modifications. NMR-directed models of the formed complexes have been constructed in order to understand, at high resolution, how the different taxanes modify microtubule structure, and cause stabilization. In this way we have constructed a data-directed model of the interaction of taxanes with both the pore and the luminal site of microtubules, and of the conformational effects that binding to these sites produce in contacting tubulin subunits. Thus, the SAXS data, and the observed modifications in the number of protofilaments, can be discussed in terms of the NMR-directed docking models obtained.
How does ligand binding to the pore and luminal sites affect microtubule structure

In order to properly understand the effects of the changes in the ligand side chains on the interprotofilament structure, we have carefully examined the models of the complexes of the ligands with both binding sites. Our initial working hypothesis was that the interaction of the different taxane ligands with the pore binding site (19,35), located between the protofilaments, should be the one most likely involved in the modifications observed in the interprotofilament angle and thus the average number of protofilaments out of which the microtubule cylindrical structure is composed. In agreement with this hypothesis, the only compound which is known to bind exclusively to the microtubule pore, 7-hexaflutax (18,20), is the ligand that produces the largest effect on the microtubule structure.

Given the transient nature of the interaction of all taxanes except Hexaflutax with the pore site, it is only possible to determine the ratio of compound bound to each of the sites for the fluorescent compounds Flutax-1 (10% outer site) and Flutax-2 (1% outer site) (16). However, for docetaxel, STD and TR-NOESY NMR analysis of the ligand-receptor systems also indicates that a significant percentage of non-fluorescent taxanes would have to be bound to the pore site (23).

However, the strong difference between the ratios of binding of these three compounds Flutax-1, Flutax-2 and Hexaflutax to both sites as compared with the similarity of sizes of the groups (366, 375 and 453 Å³) and the microtubules induced by these drugs (14.6, 14.6 and 15.1 pf) suggests that either the effect is exerted through both the luminal and the pore sites or that only a small proportion of the ligand is enough to produce the effect on microtubule structure. Thus, in order to gain information about the specific effect, both models of the interaction with the pore and with the luminal site have to be analyzed.

The results indicate that the main effect of ligands at both the pore site and the luminal site is due to the modification of the size of groups at positions C7 and C10. The analysis of the 3D models of the ligands derived from NMR and docking simulations with O-substituents at the "North face" of the molecule (taxane positions C7 and C10) reveals a well defined interaction mode with the pore site. The groups at these positions strongly interact with the secondary H3 and S3 structure elements of the β-tubulin subunit at the adjacent protofilament modifying their molecular conformations (Figure 4D and Figure S2A), while the positions which are relevant to the binding, C2 and C13, are oriented towards the center of the protofilament (Figure 4). When no substituents are present at the hydroxyl groups at C7 and C10, the groups are exposed to the solvent making favorable interactions with the water molecules, and in turn does not interact with the protein (accordingly, compounds with no substituents at the hydroxyl groups at C7 and C10, such as docetaxel, Chitax-15, and Chitax-17, have little influence on the microtubule diameter). Fittingly, when a hydrophobic group is present at this location, the protein rearranges its conformation to protect the non polar areas from the solvent. When the substitution takes place at C7, which is close to
the surface of the pore, the H3 helix changes its topology, resulting in a more compact conformation the bigger the side chain is. This in turn, results in protofilament-protofilament interactions with larger interprotofilament angles. Alternatively, for the C10-substituted analogues, the modified position is closer to the inner surface. In this case, the tubulin S3 strand rearranges to close the interprotofilament contacts in the inner part of the protein. This process involves a reduction in the interprotofilament angle, and thus thinner microtubules, with less protofilaments, are formed. The effect on the pore structure can be clearly seen in Figure 4. In the presence of cephalomannine (Figure 4A), which contains an acetyl group at position 10 and a hydroxyl at position 7, the H3 helix is not well structured (magenta) and is located far from the ligand. In contrast, when the C10 acetyl is removed and a propionyl group is introduced at C7 (Chitax-1, Figure 4B), the helix becomes stable and interacts with the ligand. A similar effect can be observed with Flutax-2 (Figure 4C).

The modeling indicates that when the ligands are bound to the luminal site, the influence of the ligands is exerted through changes in the orientation of the tubulin M-loop (see Figures 4D-G and Figure S2B). A similar effect has been observed by Mitra and Sept (36) in a large scale molecular simulation, employing the T-taxol conformation (37). This indicates that, as previously proposed, the effect on this loop is one of the reasons for the microtubule stabilizing effect. Substitutions at taxane positions C7 and C10 produce different orientations of this secondary tubulin structure element. There are different structures of the M-loop (Figure S2B) when an acyl substituent is at position 10, as in cephalomannine (yellow loop), or when a large substituent is located at position 7, as in Chitax-1 (green loop). Interestingly, for non-substituted hydroxyl groups at C7 and C10 (as in Chitax-17), the M-loop adopts the intermediate orientation (white loop) between those described above. Looking at the structural basis for the observed variations, the presence of the acyl substituent at position 10 induces changes in the orientation of the M-loop, which shifts inwards due to contacts between the acyl moiety with Arg278 and Thr276. These interactions result in the existence of closer contacts between protofilaments in the luminal part of the microtubule wall and therefore in smaller interprotofilament angles. Microtubules assembled in the presence of Chitax-17 resulted in a microtubule cylinder with an average of 13.4 protofilaments, while cephalomannine-microtubules only contained 12.6. In contrast, the presence of substituents at taxane position 7 induced the outward shifting of the M-loop, due to contacts between the acyl group and Gln282. This alternative shift results in the assembly of microtubules with larger interprotofilament angles. In fact, Chitax-17 microtubules have 13.4 protofilaments, while Chitax-1 microtubules have 14.8.

The models of the ligands docked both into the pore and into the luminal binding site (Figure 4) indicate that the C13 side chain is not interacting with the loops responsible for the interprotofilament contacts. Thus, the large change observed in its absence should be the result of a different pose of baccatin III in the sites, further away from the S3 and H3 loops in the pore site and from the M-loop in the luminal site.
The increase in volume of the meta group of the benzyl moiety at taxane position C2 produced alternative variations at other locations in the luminal binding pocket. As a key example, incorporation of an azide moiety at the C2 aromatic ring resulted in a closer interaction of the ligand with His229, which has been proposed as the main reason for the observed increase in affinity (23). This intermolecular interaction slightly modified the presentation of the ligand and forced a large movement of the M-loop towards the interprotofilament space (Figure S2B, blue loop). This motion produced the concomitant change in the number of protofilaments, which increases from 14.8 for Chitax-1 to 15.2, for Chitax-4. This effect can also be easily observed by inspecting Figure 4. In the presence of just a hydroxyl group (as for Chitax-17, Figure 4H), the M-loop packs closer to the ligand than in the presence of an acetyl group at position 7 (Chitax-1, Figure 4F). This effect is reinforced with the addition of the N3 moiety to the aromatic ring at C2 (as for Chitax-4, figure 4G).

Conclusions

The mechanisms of ligand induced microtubule stabilization have been explored with a set of taxane analogs. These ligands modify the structure of these polymers by altering the interprotofilament contacts as detected by Small Angle X-Ray scattering. Modeling protocols, assisted by NMR experiments, have been used for different protein complexes holding ligands bound at the pore site or at the luminal site. The modeled 3D structures have been employed to analyze the microtubule structural changes detected, providing plausible explanations on the structural influence of these compounds to modulate microtubule assembly.

Although it is not strictly possible to isolate the individual effects arising from ligand binding to the luminal or to the pore site, simulations indicate that the binding process strongly influences the interactions at three tubulin regions, with different well-defined secondary-structure elements. These regions, the S7-H9 loop (M-loop), helix H3, and the S3 strand (Figure 4D) have been described as key elements for interprotofilament interactions (17) and could be selectively targeted by employing the adequately designed analogues.

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References


Table I: Structural data of the ligand induced microtubules

<table>
<thead>
<tr>
<th>Compound</th>
<th>$J_{01}$ (nm$^{-1}$)$^a$</th>
<th>Mean helical radius (nm)$^b$</th>
<th>Average protofilament number$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baccatin III</td>
<td>0.0595±0.0006</td>
<td>10.3±0.2</td>
<td>11.3±0.3</td>
</tr>
<tr>
<td>Cephalomannine</td>
<td>0.0535±0.0002</td>
<td>11.4±0.1</td>
<td>12.6±0.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0518±0.0004</td>
<td>11.8±0.1</td>
<td>13.0±0.1</td>
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<tr>
<td>Docetaxel</td>
<td>0.0480±0.0005</td>
<td>12.7±0.2</td>
<td>14.0±0.2</td>
</tr>
<tr>
<td>Chitax-1</td>
<td>0.0455±0.0002</td>
<td>13.4±0.1</td>
<td>14.8±0.1</td>
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<tr>
<td>Chitax-4</td>
<td>0.0443±0.0008</td>
<td>13.8±0.3</td>
<td>15.2±0.3</td>
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<tr>
<td>Chitax-5</td>
<td>0.0435±0.0005</td>
<td>14.0±0.1</td>
<td>15.5±0.1</td>
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<td>Chitax-11</td>
<td>0.0498±0.0003</td>
<td>12.2±0.1</td>
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<td>Chitax-12</td>
<td>0.0501±0.0003</td>
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<td>Chitax-13</td>
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<td>13.0±0.1</td>
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<td>Chitax-14</td>
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<td>Chitax-15</td>
<td>0.0504±0.0003</td>
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<td>Chitax-17</td>
<td>0.0501±0.0003</td>
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<td>Chitax-18</td>
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<td>12.4±0.1</td>
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<td>Chitax-19</td>
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<td>Chitax-20</td>
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<td>13.4±0.1</td>
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<td>Chitax-21</td>
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<td>Chitax-40</td>
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<td>Flutax-1</td>
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<td>Flutax-2</td>
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<td>Hexaflutax</td>
<td>0.0446±0.0003</td>
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<td>15.1±0.1</td>
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</tbody>
</table>

$^a$ $J_{01}$: position of the $J_{01}$ maxima in the scattering profiles obtained from three independent measurements. Fitting of the peaks was done using the curve-fitting software tool TableCurve2D 5.01 (Systat Software Inc. Richmond, Ca). The error is the error of the best fit.

$^b$ Radii is the helical radii estimated from the positions of the first subsidiary maximum of the $J_0$ Bessel function $J_{01}=(1.22/2R)$ (26).

$^c$ Calculated from the mean helical radius and the microtubule interprotofilament spacing (5.7 nm) (14).
Table II: Changes in protofilament numbers due to single point modifications.

<table>
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<tr>
<th>Modification type</th>
<th>Modification</th>
<th>Compounds</th>
<th>Change in pf number</th>
<th>Average change(^a)</th>
<th>(\Delta)V-SES (Å(^3))(^b)</th>
</tr>
</thead>
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<tr>
<td>C2</td>
<td>Benzoyl to 3-N(_3) benzoyl</td>
<td>P-&gt;12</td>
<td>+0.4</td>
<td>+0.7±0.2</td>
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<td></td>
<td></td>
<td>C-&gt;14</td>
<td>+1.0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>18-&gt;20</td>
<td>+1.0</td>
<td></td>
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<td>1-&gt;4</td>
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<td></td>
<td>Benzoyl to 3-OCH(_3) benzoyl</td>
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<td>+0.8±0.3</td>
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<td>18-&gt;19</td>
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<td>C7</td>
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<td>+1.6</td>
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<td>-OH to diFluoro-Fluorescein</td>
<td>P-&gt;Flutax-2</td>
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<td>+1.6</td>
<td>+375</td>
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<td>-OH to (CH2)6-Fluorescein</td>
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<td>+453</td>
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<td>C13</td>
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<tr>
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<td>PTX-&gt;DXL</td>
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<td>CPH-&gt;DXL</td>
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<td>PTX-&gt;None</td>
<td>P-&gt;Bill</td>
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<td>-1.7</td>
<td>-256</td>
</tr>
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</table>

\(^a\) Errors are the standard error where applicable
\(^b\) V-SES Volume of Solvent Excluded Surface
Figure legends.

Figure 1: Upper panel: **Structural pathway of MSA-induced microtubule assembly**, adapted from (8). Lower panel: **Chemical structures of the compounds employed in the study**.

Figure 2: **Effect of single modifications in the different side chains on the SAXS profiles of microtubules assembled in the presence of different taxanes.** A. Effect of changes in C13 side chain. SAXS profiles of microtubules assembled in the presence of Chitax-21 (black line), Cephalomannine (red line) or paclitaxel (green line) Inset: Schematic drawing of a microtubule indicating the interprotofilament angle. B. Effect of changes in C10 side chain. SAXS profiles of microtubules assembled in the presence of Chitax-18 (black line), Cephalomannine (red line) or Chitax-17 (green line) C. Effect of changes in C7 side chain. SAXS profiles of microtubules assembled in the presence of Chitax-1 (black line), Cephalomannine (red line) or Chitax-17 (green line). D. Effect of changes in C2 side chain. SAXS profiles of microtubules assembled in the presence of Chitax-13 (black line), Cephalomannine (red line) or Chitax-14 (green line).

Figure 3.- **NMR directed modeling of the ligands bound to the pore site.** A.- Off-resonance NMR experiment (500 MHz) (lower line) and STD spectra (upper line) of Flutax-2 bound to microtubules, Inset: TR-NOESY spectra (mixing time: 200 ms) of Flutax-2 in the presence of microtubules (D2O, 310 K) (a large high resolution version of this file is available as supplementary information). B.-Best model of Flutax-2 docked into the pore site. Protein residues considered in CORCEMA-ST calculations are shown in sticks. C.- Experimental STD profile of Flutax-2 bound to microtubules (black line and circles). Calculated STD profile of the best model of Flutax-2 docked into the pore site (red line and squares). D.- Average STD profile of the common protons of paclitaxel, docetaxel and Chitax-42 (black line and circles). Calculated STD profile of cephalomannine docked into the pore site (red line and squares). Calculated STD profile of Chitax-1 docked into the pore site (green line and squares). Calculated STD profile of Chitax-17 docked into the pore site (blue line and squares). Calculated STD profile of Chitax-4 docked into the pore site (pink line and squares). X-axis values of Figures 3C and 3D are placed over the symbols and represent the common protons of taxane core and/or the fluorescein moiety of Flutax-2. The order of the points have been selected so points representing protons close in the graph are as well close in the taxane molecule (protons not observable by STD keep its space but are not plotted), spacing and connectivity of the points are arbitrary for presentation purposes.

Figure 4.- **Models of ligands bound to microtubules** Panels A-C Ligands bound to pore site: (A) Cephalomannine, (B) Chitax-1, (C) Flutax-2, (Magenta, H3 Helix of β3-tubulin subunit, White, S3 of β3-tubulin subunit) Panel D Scheme of the interaction of ligand at both binding sites with the structural elements responsible for the interprotofilament interaction. The scheme shows the position of H3, S3 and the M-loop in the interprotofilament interface with respect with the two possible binding sites for the taxanes, and the substituents of the ligand that interact with them altering the interprotofilament interactions.
contacts. A ligand bound to the pore site will interact with H3 through the substituent at position C7, increasing the interprotofilament angle and with S3 through the substituent at position C10 decreasing the angle. A ligand bound to the luminal site will interact with the M-loop through the substituent at position C10 producing the same effect as in the pore site. Microtubule is seen from the plus end. Panels E-H ligands bound to luminal site (E) Cephalomannine. (F) Chitax-1. (G) Chitax-4. (H) Chitax-17. Magenta H3 Helix of β3-tubulin subunit, White S3 of β3-tubulin subunit. Yellow M-loop of β1-tubulin subunit.
Figure 1
Figure 2
Figure 3
Figure 4