Structural insight into the stabilization of microtubules by taxanes

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Abstract

Paclitaxel (Taxol®) is a taxane and a first-line chemotherapeutic drug that stabilizes microtubules. While the interaction of paclitaxel with microtubules is well described, the current lack of high-resolution structural information on a tubulin-taxane complex precludes a comprehensive description of the binding determinants that affect the drug's mechanism of action. Here, we solved the crystal structure of the core baccatin III moiety of paclitaxel lacking the C13 side chain in complex with tubulin at 1.9 Å resolution. Based on this information, we engineered two tailor-made taxanes with modified C13 side chains, solved their crystal structures in complex with tubulin, and analyzed their effects along with those of paclitaxel, docetaxel, and baccatin III on the microtubule lattice by X-ray fiber diffraction. We then compared high-resolution structures of ligand-bound tubulin and microtubule complexes with apo forms and used molecular dynamics simulations to understand the consequences of taxane binding to tubulin as well as to simplified protofilament and microtubule-lattice models. Our combined approach sheds light on three mechanistic questions. Firstly, taxanes bind better to microtubules as compared to unassembled tubulin due to a dual structural mechanism: Tubulin assembly is linked to a conformational reorganization of the βM loop, which otherwise occludes ligand access to the taxane site, while the bulky C13 side chains preferentially recognize the microtubule-assembled over the unassembled conformational state of tubulin. Second, the occupancy of the taxane site by a ligand has no influence on the straightness of tubulin protofilaments. Finally, binding of the taxane core to the taxane site displaces the S9-S10 loop of β-tubulin resulting in microtubule expansion. Our results provide detailed new insights into the mechanism of microtubule-stabilization by taxanes.

49 **Introduction**

50 The taxane paclitaxel is a drug included in the World Health Organization's List
51 of Essential Medicines (*1*). Taxanes, either alone or in combination with other
52 chemotherapeutic agents, are important drugs for the treatment of several solid tumors,
53 such as ovarian, lung, and breast cancer, as well as advanced Kaposi's sarcoma (*2-5*). The
54 three taxanes in clinical use, paclitaxel (Taxol®), docetaxel (Taxotere®), and cabazitaxel
55 (Jevtana®), are part of a large family of chemically diverse compounds that bind to the
56 so-called "taxane site" of the αβ-tubulin heterodimer (*6, 7*) (Fig. 1A,B), the building
57 block of microtubules. However, the appearance of peripheral sensory neuropathy and
58 other side effects caused by taxanes compromises treatment efficacy in the long term (*8*).
59 Thus, understanding the underlying mechanism of microtubule stabilization by this class
60 of antitubulin agents is an important requirement for future and safer drug development
61 efforts.

62 Because taxane-site ligands stabilize microtubules and suppress their dynamics,
63 they are collectively called microtubule-stabilizing agents. Several structures of
64 microtubules in complex with taxane-site agents have been recently analyzed and solved
65 by cryo-electron microscopy to resolutions ranging between ~3 and ~5 Å. For paclitaxel,
66 it was initially suggested that the drug acts on longitudinal tubulin contacts along
67 protofilaments in microtubules by allosterically expanding the microtubule lattice in the
68 direction of its long filament axis (*9-11*), a notion that is also consistent with X-ray fiber
69 diffraction data (*12*). However, more recent studies suggest that paclitaxel enhances
70 lattice flexibility and acts on lateral tubulin contacts between protofilaments in
71 microtubules through interactions with the M-loop of the β-tubulin subunit (βM loop)
72 (*13-15*).

73 Besides directly acting on microtubules, taxane-site ligands also have the capacity
74 to bind to unassembled tubulin dimers and promote their assembly into microtubules (*16-*
75 *20*). Several structures of non-taxane agents bound to the taxane-site of tubulin have been
76 solved to resolutions ranging from 2.4 to 1.7 Å by X-ray crystallography (*21-24*). These
77 data suggested that one mode of action of some taxane-site ligands such as zampanolide
78 or epothilone A on unassembled tubulin is to stabilize lateral tubulin contacts between
79 protofilaments within microtubules by structuring and stabilizing the β–tubulin M–loop
80 into a short α-helix (*21*). In contrast, the absence of a helical structure for this segment in
81 the presence of the taxane-site ligands dictyostatin and discodermolide (*22, 23*) suggests

82 a different, still poorly understood mechanism of microtubule stabilization for these two
83 classes of non-taxane agents.

84 In the case of taxanes, one hypothesis is that they preferentially bind to a specific
85 conformation of tubulin. It is well established that tubulin displays two prominent
86 conformations that are related to its assembly state (reviewed in (*25*)): a "straight"
87 conformation present in assembled microtubules (denoted "straight tubulin" hereafter)
88 and a "curved" conformation observed in unassembled tubulin (denoted "curved tubulin"
89 hereafter). The "curved-to-straight" conformational transition is required for the
90 formation of lateral tubulin contacts between protofilaments in the main shaft of
91 microtubules. Some data suggest that the activation mechanism of taxanes facilitates the
92 curved-to-straight conformational transition by preferentially binding to the straight
93 conformation of tubulin (*26-28*).

94 Structural information of a taxane in complex with unassembled tubulin is
95 currently unavailable. With the aim of providing insight into the mechanism of action of
96 this important class of anticancer drugs and into the tubulin-taxane interaction, we solved
97 the high-resolution structures of three different taxanes bound to curved tubulin by X-ray
98 crystallography. We further analyzed the effects of different taxanes on the microtubule
99 lattice by X-ray fiber diffraction. These studies were complemented with molecular
100 dynamics (MD) simulations that shed light on issues that were not amenable to
101 experimental verification. Taken together, our results suggest that the main reason for the
102 differential affinity of taxane-site ligands for assembled tubulin and unassembled tubulin
103 arises from two terms. Firstly, the stabilization of the β M loop in an "out" conformation
104 compatible with the formation of specific lateral contacts in microtubules and secondly,
105 the selectivity of the bulky C13 side chain for the assembled, straight conformational state
106 of tubulin. Finally, we found that the occupancy of the taxane site results in a
107 displacement of the S9-S10 loop of β-tubulin that accounts for the observed microtubule
108 expansion with no influence, however, on the straightness of tubulin protofilaments.
109
110 **Results**
111 *High-resolution crystal structure of a tubulin-taxane complex*

112 To determine the high-resolution structure of a taxane bound to curved tubulin,
113 we performed both soaking and co-crystallization experiments using the previously
114 described protein complexes termed T_2R-TTL and TD1. The former complex is

115 composed of two αβ-tubulin heterodimers bound head-to-tail, the stathmin-like protein
116 RB3, and the tubulin tyrosine ligase (*21, 29*); the latter complex contains one αβ-tubulin
117 heterodimer and the DARPin D1 (*30*). We did not succeed in procuring any valuable
118 structural information from these two crystal ensembles using a first series of taxanes
119 comprising paclitaxel, docetaxel, the more soluble 3'-*N*-*m*-aminobenzamido-3'-*N*-
120 debenzamidopaclitaxel (N-AB-PT)(*31*), and the engineered, high-affinity taxanes Chitax
121 40(*32*) and Chitax 68(*33*). We thus decided to approach the issue from a different angle
122 and started off with baccatin III, a precursor in the biosynthesis of paclitaxel that contains
123 both the C2-benzoyloxy ring C and the C10 acetate ester, but lacks the C13 side chain
124 with both the 3'-N-benzamido phenyl ring A and the 3'-phenyl ring B moieties (*34*) (**Fig.**
125 **1C**). Notably, baccatin III is largely biologically inactive despite displaying micromolar
126 affinity for microtubules (*35-38*).

127 We found that baccatin III shows detectable affinity (K_b 25 ºC 3.0±0.5x10^3 M^{-1})
128 to unassembled tubulin, which is in the same range as for other compounds that have been
129 co-crystallized with tubulin, such as epothilone A 0.8±0.3×10^4 M^{-1} (*39*) and
130 discodermolide 2.0±0.7×10^4 M^{-1} (*40*). Therefore, we hypothesized that the presence of
131 the C13 side chain of the aforementioned taxanes might preclude the binding to the curved
132 tubulin form present in both the T_2R-TTL and the TD1 complexes. Subsequently, we
133 succeeded in obtaining a T_2R-TTL-baccatin III complex structure that was solved at 1.9
134 Å resolution (**Fig. Sup. 1A,D**; **Table 1**). We found that the ligand binds to the taxane site
135 of curved tubulin with its C2-benzoyloxy ring C stacked between the side chains of
136 βH229 and βL275 in the leucine-rich β-tubulin pocket lined by the side chains of βC213,
137 βL217, βL219, βD226, βH229, βL230 and βL275 (**Fig. 2A**, **3A**). Its carbonyl oxygen
138 forms a weak hydrogen bond to the main chain amide of βR278. The C10 acetate is
139 exposed to the solvent and, together with the C12 methyl, is within van der Waals distance
140 to βG370 of the βS9-βS10 loop. Furthermore, the oxetane oxygen and the C13 hydroxyl
141 accept hydrogen bonds from the main chain amide nitrogen of βT276 and the βH229
142 imidazole NE2, respectively. The C4 acetate is buried in the hydrophobic pocket made
143 up by βL230, βA233, βF272, βP274, βL275, βM302, βL371, and the aliphatic portion of
144 the βR369 side chain.

145

146

147 *Generation of paclitaxel analogs that bind to tubulin crystals*

148 Aiming to understand the implication on tubulin activation of paclitaxel's bulky
149 and hydrophobic C13 ring A moiety (or its equivalent *tert*-butyl in docetaxel) and to
150 elucidate the reason why it apparently precludes binding to T_2R-TTL and TD1 crystals
151 (see above), we devoted a synthetic effort to obtaining new taxane ligands with modified
152 C13 side chains. We produced a series of modified taxanes bearing smaller groups than
153 paclitaxel at the 3'-N position, namely, acrylamide **2a**, haloacetamides **2b** and **2c**, and
154 isothiocyanate **2d** (**Fig. 1C**). We could measure binding of **2a** to unassembled tubulin
155 dimers ($K_{b25°C}$ 0.8±0.3x10^3 M^{-1}), but not of N-AB-PT(*31*), Chitax 40(*32*) or Chitax
156 68(*33*), thus indicating that the modification of the paclitaxel structure increased the
157 binding affinity for unassembled tubulin. In fact (**Fig. Sup. 1B, C, E, F**), we found
158 unequivocal difference electron densities at the taxane site of β-tubulin in T_2R-TTL
159 crystals soaked with **2a** and **2b** and refined the corresponding structures to 1.95 and 2.35
160 Å resolution, respectively (**Table 1**).

161 Interestingly, the electron densities of compounds **2a** and **2b** displayed a
162 continuity between the 3'-N-attached moieties of both ligands and the side chain of
163 βH229 of β-tubulin, suggesting the possible formation of a covalent adduct. For further
164 validation, we collected additional X-ray diffraction data on T_2R-TTL crystals soaked
165 with the haloacetamide derivative **2b** at the bromine peak wavelength of 0.91501 Å. After
166 rigid body and restrained refinement, we detected two clear anomalous difference peaks
167 in electron densities at the taxane sites of the two tubulin dimers in the T_2R-TTL crystals
168 soaked with **2b** (**Fig. Supp. 1F, G**), which did not support covalent bond formation.
169 Furthermore, refinement cycles performed in parallel with **2a** modeled in both the
170 covalent and the non-covalent form, resulted in clear electron density for the non-covalent
171 model, while red difference peaks for the covalent form were always present after
172 refinement (not shown). Accordingly, we interpreted the continuous electron density
173 observed in the T_2R-TTL-**2a** structure as a strong hydrogen bond between the βH229
174 NE2 and the C39 carbonyl of the ligand side chain rather than a covalent bond (**Fig. 2B**).

175 The T_2R-TTL-**2a** complex structure revealed that **2a** engages in comparable
176 interactions to curved tubulin by means of both its C2-benzoyloxy ring C and its oxetane
177 moieties, as found for baccatin III (**Fig. 2B, C**). However, the core ring system of **2a** is
178 tilted towards helix βH6 and strand βS7 by ~20˚ (angle between the two C1-C9 axis;
179 $rmsd_{bacIII-2a}$ of 0.794 Å for 39 core atoms), thereby adopting a pose that is closer to that

180 observed for paclitaxel bound to straight tubulin in microtubules (PDB ID 6WVR; $rmsd_{2a-paclitaxel}$ of 0.845 Å for 56 core atoms; $rmsd_{bacIII-paclitaxel}$ of 1.048 Å for 42 core atoms; **Fig. 3B**).

183 Similar to paclitaxel bound to straight tubulin, the C39 carbonyl of the C13-3'-N-acrylamide moiety of **2a** forms a hydrogen bond to the βH229 NE2 in curved tubulin (**Figs 2B** and **3B**). The terminal ester moiety of 2a is exposed to the solvent and it forms water-mediated hydrogen bonds to the side chains of βE22 and βR369 of β-tubulin; it lodges within a space that is otherwise occupied by crystallographic water molecules in the curved tubulin-baccatin III structure. In the context of paclitaxel-bound microtubules (straight tubulin), the same space is occupied by the 3'-N-benzamido phenyl ring A of paclitaxel and the side chain of βD26 replaces that of βR369 and adopts a flipped-out conformation (**Figs 2B** and **3B**). The absence of the C10 acetate in **2a** relative to baccatin III has little impact on the conformation of the secondary structural elements shaping the taxane site (**Fig. 2C**).

194 Together, these structural data provide for the first time a high-resolution structural description of the interaction of taxanes harboring a C13 side chain with unassembled, curved tubulin. They indicate that the main interaction energy of this class of antitubulin agents is mediated by their common baccatin III core moieties. They further reveal that the taxane pose in both curved and straight tubulin is very similar. Overall, our results suggest that the tubulin-**2a** structure is an excellent model to study the interaction of paclitaxel with curved tubulin at high resolution and that X-ray crystallography is a valuable method to analyze the molecular mechanism of action of taxane site microtubule-stabilizing agents.

203

204 *Conformational changes upon taxane binding to curved and straight tubulin*

205 Next, we investigated the conformational changes induced by binding of baccatin III and **2a** to curved tubulin. To this end, we first superimposed the crystal structures of apo tubulin (PDB ID 4I55), tubulin-baccatin III, and tubulin-**2a** onto the N-terminal β-sheets of β-tubulin (residues 3-9, 63-66, 132-138, 163-169, and 198-202; $rmsd_{BacIII}$ 0.08 Å of 29 C_α; $rmsd_{\mathbf{2a}}$ 0.10 Å of 29 C_α), and calculated the root-mean-square deviations (rmsd) between the apo and the two ligand-bound states. These rmsd values were also

211 plotted and mapped onto the corresponding structures to highlight the major regions of
212 conformational change.

213 As shown in **Fig. 4**, significant and comparable conformational changes were
214 observed for backbone atoms of the βT5 loop and the N-terminal segment of the βM loop
215 in both the tubulin-baccatin III and tubulin-**2a** complex structures. Interestingly, the βT5
216 loop that is prominently involved in establishing longitudinal tubulin contacts along
217 protofilaments is oriented in the active "out" conformation in both structures (*41*). This
218 observation indicates an allosteric crosstalk between the taxane site and the βT5 loop
219 possibly via the central helix βH7 and the guanosine nucleotide bound to β-tubulin. In
220 the case of the βM loop, we only found well-defined electron densities for its N-terminal
221 section up to residue βR278, while the remaining portion of the loop appeared disordered
222 in both complex structures. This partial βM loop structuring has been observed previously
223 in tubulin complexes with the taxane-site ligands dictyostatin and discodermolide ((*22,*
224 *23*); note that the taxane-site ligands zampanolide and epothilone A promote the
225 structuring of the βM loop into a helical conformation (*21*)). A direct effect of taxanes on
226 the βM loop is consistent with the notion that paclitaxel stabilizes this secondary
227 structural element in two discrete conformations giving rise to heterogeneous lateral
228 microtubule-lattice contacts (*14*). We also found significant conformational changes in
229 the βS9-βS10 loop, which were more prominent in tubulin-**2a** than in tubulin-baccatin
230 III. This finding can be explained by the presence of a C13 side chain in **2a** that needs
231 more room for accommodation inside the taxane site compared to baccatin III, which
232 lacks a C13 side chain. Finally, we observed a conformational change of the H2' helix in
233 the tubulin-baccatin III structure, which was absent in tubulin-**2a**.

234 To investigate the effect of the observed conformational changes on the relative
235 domain arrangements in β-tubulin of the individual complexes, we further superimposed
236 the β-tubulin chains of apo tubulin (PDB ID 4I55), tubulin-baccatin III and tubulin-**2a**
237 onto their central βH7 helices (residues 224-243). For tubulin-baccatin III, a subtle
238 relative twist between the N-terminal and the intermediate domains was observed (**Fig.**
239 **4**; **Fig. Sup. 2**; **Movies M1 and M2**), while binding of **2a** rather caused both the N-
240 terminal and intermediate domains of β-tubulin to move slightly apart (**Fig. 4**; **Fig. Sup.**
241 **2**; **Movies M3 and M4**). Thus, taxane binding to tubulin causes global, but subtle
242 conformational rearrangements.

243 We next wondered whether similar conformational changes are also observed in
244 straight tubulin in the context of a microtubule upon paclitaxel binding. To this end, we
245 performed the same type of analysis by superimposing the N-terminal β-sheets of β-
246 tubulin from the cryo-EM reconstruction of paclitaxel-bound GDP-microtubules (PDB
247 ID 6WVR) onto the corresponding domains of the undecorated apo GDP-microtubule
248 structure (PDB ID 6DPV; rmsd 0.304 Å 30 C_α). The rmsd analysis revealed similar
249 significant conformational changes of both the βT5 and the βM-loops as observed for the
250 taxanes bound to curved tubulin, however, no prominent perturbations of the βS9-βS10
251 loop could be detected. In addition, we found significant conformational changes of the
252 βS2'-βS2'' loop (the interacting part of the βM loop with the neighboring protofilament)
253 and the C-terminal βH11-βH12 helix region (**Fig. 3CD**), which were not detected in the
254 curved tubulin structures.

255 Together, these results suggest that taxane binding in the context of the
256 microtubule should have an effect on the lateral contact established by the βM loop. An
257 effect that cannot be detectable in curved tubulin as this contact does not exist in the
258 crystal. Moreover, we observe an activation effect on the T5-loop, but do not see any
259 direct structural evidence for that, therefore it should be exerted via a crosstalk through
260 the nucleotide.

261

262 *Effects of taxanes on microtubule lattice parameters*

263 We have previously validated X-ray fiber diffraction of shear-flow aligned
264 microtubules as an accurate technique to determine microtubule lattice parameters (*12,*
265 *42*). In such diffraction patterns, the meridional 4-nm layer line is related to the axial
266 helical rise of tubulin monomers in the microtubule lattice. When the lattice is expanded
267 in the direction of the helix axis, a second, weaker 8-nm layer line emerges due to the
268 length difference between the α- and β-tubulin subunits (*42*) and the position of the 1-nm
269 layer line corresponding to the fourth harmonic of the 4-nm layer line moves towards the
270 center of the image.

271 We used this method to analyze the effect of different conditions on the
272 microtubule lattice (**Fig. 5, Table 2**). We first analyzed microtubules that were assembled
273 in the presence of either GTP (producing GDP-microtubules) or the slowly hydrolyzable
274 GTP analogue GMPCPP (producing GMPCPP-microtubules) and found that the tubulin

275 dimer rise increased by 0.24 nm (from 8.12±0.02 nm to 8.36±0.02 nm, respectively) in
276 the presence of GMPCPP, a distance that is consistent with that found in previous studies.
277 Concomitantly, the microtubule radius increased from 11.42±0.1 nm for GDP-
278 microtubules to 11.63±0.1 nm for GMPCPP-microtubules, which translates into an
279 increase of the average protofilament number (av. PF nr.) from 12.9 to 13.3, respectively.
280 An increase in both tubulin dimer rise and number of protofilaments for GMPCPP-
281 microtubules compared to GDP-microtubules has been reported previously ((*9, 11, 43,*
282 *44*)).

283 As shown in **Fig. 5** and **Table 2**, and when compared to GDP-bound microtubules, both
284 paclitaxel-bound microtubules and docetaxel-bound microtubules displayed a similar
285 lattice expansion of 0.24 nm as seen for GMPCPP-bound microtubules. Interestingly,
286 while paclitaxel-bound microtubules show a reduced microtubule radius of 10.97±0.1 nm
287 (av. PF nr., 12.21), docetaxel-bound microtubules displayed a radius of 11.53±0.1 nm
288 (av. PF nr., 12.9), which is similar to the value obtained for GDP-microtubules. In the
289 case of paclitaxel, this expansion occurred either when the drug was added before the
290 polymerization reaction was started with GTP- or GDP-tubulin, or when it was added to
291 preformed microtubules, in concordance with rapid structural transitions of microtubules
292 observed upon taxane addition(*45*). Interestingly, microtubules with bound **2a**, **2b** or
293 baccatin III showed similar lattice expansion as those bound to paclitaxel or docetaxel.
294 Note that the diffraction patterns of microtubules stabilized with **2a** or **2b** showed a
295 diffuse 1-nm layer line that reflects variations in the tubulin monomer (and consequently
296 dimer) rise, in clear contrast to those bound by paclitaxel and docetaxel, which displayed
297 a sharp band, i.e., a robust monomer rise.

298 Taken together, these results suggest that taxanes with or without a C13 side chain
299 have the capacity to expand the microtubule lattice and thus affect longitudinal tubulin
300 contacts along protofilaments. They further indicate that the nature of the C13 side chain
301 can affect the radius of a microtubule and thus lateral tubulin contacts between
302 protofilaments. We note, however, that microtubules assembled in the presence of
303 baccatin III, which lacks a C13 side chain, display the same radius as paclitaxel-bound
304 microtubules. Thus, the presence of a C13 side chain *per se* does not seem to modulate
305 lateral tubulin contacts between protofilaments.

306

307

308 *Molecular dynamics simulation analysis of taxane binding to tubulin, protofilament, and*
309 *microtubule lattice models*

310 Although recent advances in the structural biology of tubulin and microtubules
311 have shed light on the mode of action of taxane-site microtubule stabilizers (*11, 13-15*),
312 there are three main mechanistic questions that remain unsolved. First, previous studies
313 have shown that paclitaxel, docetaxel, epothilone A and discodermolide bind to
314 microtubules with much higher affinities compared to unassembled tubulin (*19, 46, 47*),
315 and that covalent binders like zampanolide react slowly with unassembled tubulin
316 compared to microtubules (*48*). Furthermore, in the present work we have seen that
317 baccatin III and the engineered compounds **2a** and **2b** exhibit sufficient affinity to bind
318 to curved tubulin in T_2R-TTL crystals, while paclitaxel and docetaxel do not. Similarly,
319 the molecular basis accounting for the fact that the affinity for tubulin is two orders of
320 magnitude lower for baccatin III compared to the affinity for microtubules ($3x10^3$ M^{-1} vs
321 $1.5x10^5$ M^{-1} (*38*)) remains also unknown. A second mechanistic question that is
322 unanswered is whether taxanes that bind better to straight tubulin and promote tubulin
323 assembly (i.e., they lower the critical concentration for tubulin assembly (*19, 47*)) are able
324 to induce tubulin straightening upon binding to unassembled tubulin (*27*) or whether they
325 simply provide the necessary free energy by specifically recognizing the microtubule-
326 assembled tubulin form. Finally, we presently lack a molecular rationale explaining the
327 characteristic microtubule-lattice expansion observed upon interaction of taxanes with
328 microtubules (*10-15*).

329 Although the high-resolution crystallographic structures discussed above provide
330 detailed information of the taxane binding site for the ligands, no large differences were
331 observed between apo and taxane-bound tubulin structures, indicating that additional
332 ligand effects may be related to the dynamic behavior of the protein. For these reasons
333 and to gain further insight into the underlying mechanism of taxane-induced microtubule
334 stabilization, we next used molecular dynamics (MD) simulations to study the behavior
335 of different tubulin assemblies in solution. To this end, we built three types of fully
336 solvated molecular models representing the different oligomerization states of tubulin: (i)
337 the αβ-tubulin heterodimer; (ii) a short protofilament consisting of three longitudinally
338 concatenated tubulin dimers ((αβ-tubulin)$_3$); and (iii) a minimalist representation of a
339 microtubule lattice (*49*) made up of two laterally associated protofilament fragments ((α_1-
340 β_1-α_2)/($\alpha_{1'}$-$\beta_{1'}$-$\alpha_{2'}$)). All models were created in their apo- and taxane-bound forms.

341 Baccatin III, **2a**, and paclitaxel were chosen as representative taxane ligands for our fully
342 atomistic simulations.

343 In concordance with the structural results, the MD simulations of the αβ-tubulin
344 heterodimer pointed to the βM loop as the most likely structural element responsible for
345 the selective recognition of the microtubule-assembled tubulin form by taxanes. All
346 taxane-site ligands, including paclitaxel, docetaxel (*19*), discodermolide (*40*), epothilone
347 A (*39*), and **2a** show a loss of affinity of at least four orders of magnitude when binding
348 to unassembled tubulin relative to binding to microtubules. Even baccatin III, which lacks
349 the side chain altogether, has an affinity for the unassembled state that is still two orders
350 of magnitude lower compared to the microtubule-assembled state ($3x10^3$ M^{-1} vs $1.5x10^5$
351 M^{-1}), a finding that is not explained by our crystallographic data. In our simulations of
352 the tubulin dimer model, we found that the βM loop is the most flexible region (**Fig. S3**
353 top), in good accord with the fact that no density is usually observed for this β-tubulin
354 element in most crystallographic structures. During the course of the MD simulations,
355 this loop was not structured as an α-helix in any of the models studied; instead, it was
356 found to assume a relatively stable, extended hairpin conformation that interacted with
357 and blocked access to the taxane site. Even when the βM loop was initially modeled as
358 an α-helix (as present in all microtubule structures (*11, 13-15*)), this secondary structure
359 element was rapidly lost during the simulated trajectory (**Fig. S3** bottom) regardless of
360 whether or not baccatin III, **2a**, or paclitaxel was bound at the taxane site (**Fig. 6AB**). One
361 likely reason for this behavior is that the bound taxanes do not establish any long-lasting
362 hydrogen-bonding interactions with the amino acids making up this loop (βL275-βL286)
363 so as to stabilize it into an α-helix, as epothilone A and zampanolide do (*21*). Therefore,
364 β-hairpin conformation of the βM loop may compete efficiently with the binding of
365 ligands to the taxane-site (**Movie M5**). As a consequence, the free energy of ligand
366 binding to tubulin dimers with a βM loop partially occluding the taxane site would be
367 expected to be much lower (in the 10^3—10^4 M^{-1} range) than the free energy of binding to
368 microtubules, as is indeed the case (*19, 39, 40*). When considering paclitaxel, entry into
369 the taxane site is further hampered by the fact that this bulky and highly hydrophobic
370 molecule can adopt alternatively collapsed conformations in solution that are different
371 from its bioactive, tubulin-bound T-shape conformation (*50, 51*). The alternative
372 paclitaxel conformations that are inexistent in the case of baccatin III or **2a** further reduce
373 the apparent binding affinity below the solubility limit of the ligand (**Fig. 6C**). These

374 considerations might explain why we failed to obtain crystal structures of tubulin-
375 paclitaxel and tubulin-docetaxel complexes. Conversely, we think that the laxer binding
376 of the less bulky baccatin III and **2a** molecules compared to paclitaxel (**Fig. S4**) may
377 explain the success in obtaining co-crystal structures with tubulin.

378 The intermolecular hydrogen bond involving the oxetane O5 and the backbone
379 NH of βT276 is a common feature to all three tubulin-taxane complexes, both in crystals
380 (baccatin III and **2a**) and throughout the simulated MD trajectories (all three ligands).
381 Paclitaxel and **2a** establish two other long-lived hydrogen bonds during our simulations,
382 namely O4':(NE2)βH229 and O2':(O=C)βR369, which may involve ─depending on
383 context─ a βR369-βG370 backbone rearrangement. In turn, the hydroxyl group at C13
384 of baccatin III alternates between acting as a direct or water-mediated hydrogen bond
385 donor or an acceptor to/from (NE2)βH229 and (O=C)βR369, respectively. In the case of
386 **2a** (and **2b**), on the other hand, it seems that the smaller and more flexible substituents at
387 the C3' position – relative to those present in paclitaxel and docetaxel– allow an
388 adaptation of the βR369-βG370 backbone in the crystal lattice that does not appear to be
389 feasible for the pharmacologically used taxanes.

390 It has been reported previously that paclitaxel is able to prevent the straight-to-
391 curved conformational transition in GDP-bound microtubules (*27*). However, our
392 simulations indicate that protofilaments are curved both in the absence and in the presence
393 of paclitaxel (**Fig. 6D**), which suggests little or no direct influence of taxanes on the
394 straight-to-curved conformational transition of tubulin. On the other hand, and similar to
395 unassembled tubulin, although in our simulations of the taxane-bound protofilament the
396 occupancy of the taxane site by the ligand constrains the available conformational space
397 of the βM loop compared to that of the apo form, the loop still fails to adopt a well-defined
398 secondary structure in the absence of additional stabilizing interactions with a
399 neighboring protofilament.

400 Finally, we used a minimalist model of a solvated microtubule lattice in which we
401 could study and compare two taxane-binding sites (β_1 and $\beta_{1'}$), namely, an interfacial one
402 that is highly preorganized for the binding of taxanes due to the stabilization of the
403 βM loop into an α-helix by lateral lattice contacts (site 1), and another one that is fully
404 exposed to the solvent (site 2). We found that the solvent-exposed paclitaxel-bound
405 βM loop is not permanently structured as an α-helix, as expected, and that the major

406 ligand interactions at site 2 are essentially the same as in the paclitaxel-bound αβ-tubulin
407 heterodimer and the protofilament model (**Fig. S4**). On the other hand, in site 1 dispersion
408 forces, additional H-bonds, the hydrophobic effect, and decreased ligand entropy confer
409 to the studied compounds (paclitaxel, baccatin III, and **2a**) higher binding free energies
410 and longer residence times (i.e., lower k_{off} values) relative to the tubulin dimer and the
411 exposed taxane site 2 (**Fig. S5**). The three H-bond-mediated anchoring points, namely
412 O5:(NH)βT276, O4':(NE2)βH229, and O2':(O=C)βR369, are the same as those
413 observed in the microtubule-paclitaxel complex structure (*13*). The hydrogen bond
414 between the amide carbonyl O4' and the imidazole N^{ε} of βHis229 is maintained in all the
415 **2a** and paclitaxel complexes studied even though this interaction fluctuates substantially,
416 as does the stacking of βHis229 on the benzoyl phenyl ring. However, the most important
417 interaction that is strengthened laterally when a taxane is bound is that involving βTyr283,
418 whose position in the βM loop is fixated by segment ^{85}QIFR^{88} of loop βT3 from the
419 neighboring β-tubulin subunit, as seen previously for other taxane-site ligands like, *e.g.*,
420 zampanolide and taccalonolide AJ (*49*) (**Fig. S5**).

421 Importantly, our simulations consistently reproduce the axial lattice expansion
422 observed upon paclitaxel binding (*11-13*). We found that the expansion mainly originates
423 from displacement of the βS9-βS10 loop caused by the Φ/Ψ backbone rearrangement in
424 the βR369-βG370 stretch. Because the βS9-βS10 loop acts as a lid covering and stapling
425 the bound taxanes in their final location, this motion propagates toward the attached α-
426 tubulin subunit so that the distance between the α_1- and β_2-tubulin subunits of two
427 longitudinally aligned, consecutive tubulin dimers increases (**Fig. 6E**), a feature that
428 could not be detected in the taxane-bound crystal structures of curved tubulin.

429 Taken together, these analyses suggest that (i) taxanes bind better to the
430 microtubule-assembled over the unassembled state of tubulin due to the preorganization
431 of the βM loop that otherwise is stabilized in conformations that are incompatible with
432 high-affinity taxane binding; (ii) the bulky C13 side chains preferentially recognize the
433 assembled over the unassembled state of tubulin; (iii) the occupancy of the taxane site
434 has no influence on the straightness of tubulin protofilaments; and (iv) the displacement
435 of the βS9-βS10 loop of β-tubulin by the bound taxane results in microtubule expansion.

436

437

438 **Discussion**

439 Previous studies on taxanes left us with several important open issues related to
440 their molecular mechanism of microtubule stabilization. Why do they preferentially bind
441 to the microtubule-assembled over the unassembled tubulin state? Are they involved in
442 structuring of the βM loop, a molecular process that is required for microtubule assembly?
443 Why do they distort/affect the microtubule lattice? Here, we used a combination of ligand
444 engineering, structural biology, and computational approaches to gain insight into these
445 pending questions.

446 Firstly, we used a rational synthetic approach to dissect which parts of the
447 paclitaxel molecule are involved in particular aspects of tubulin recognition and
448 microtubule stabilization. Our results reveal that paclitaxel's baccatin III core is
449 responsible for filling most of the taxane site and for the key O5:NH(βT276) hydrogen-
450 bonding interaction that is established between taxanes and β-tubulin. However, this
451 interaction has only a marginal effect on the drug's microtubule-stabilizing effect (*35-*
452 *37*), which requires the C13 side chain to increase the selectivity of the drugs for
453 microtubules over unassembled tubulin. On the other hand, we found that ring A of
454 paclitaxel precludes binding of the drug to the T_2R-TTL and TD1 crystals, while two
455 taxanes with a modified, smaller C13 side chain (**2a** and **2b**) can bind due to the reduced
456 size of their 3'-acylamino substituents and increased flexibility relative to paclitaxel.
457 These smaller substituents allow the newly synthesized taxane derivatives to bind to
458 curved, unassembled tubulin –while keeping their binding poses very similar to that
459 described for paclitaxel when bound to straight tubulin in microtubules– by allowing
460 adaptation of the whole ligand to a rearranged βArg369-βGly370 backbone in the crystal
461 lattice.

462 Regarding the selective recognition of microtubules by taxanes, we found that it
463 arises from two different terms. The first one is the differential interaction of the bulky
464 C13 side chains with straight and curved tubulin. Our structural analysis reveals that a
465 major structural difference is the environment of the position occupied by the 3'-*N*-
466 benzamido phenyl ring A moiety of paclitaxel in microtubules: in the T_2R-TTL-**2a**
467 structure, the βR369 side chain occupies the same space as the βD26 side chain does in
468 the context of the assembled tubulin conformation in microtubules. The C13 side chain
469 is involved in the interaction with helix βH1 that is flanked by the βH1-βS2. Upon

470 transition to the microtubule-assembled, straight tubulin state, this space is narrowed
471 down by the side chains of βD26, βK19, βE22, and βH229 to form a favorable
472 environment for the interaction with ring A, which may lock the paclitaxel-bound tubulin
473 in the straight conformation (**Fig. 7B**). In the absence of the C13 side chain (baccatin III)
474 or in the presence of less bulky and "articulated" moieties at the ring A position (**2a** and
475 **2b**; their substituents at the N3 position have a rotatable bond in the middle), ligand
476 binding is likely to be less affected by the curved-to-straight conformational transition,
477 since much looser interactions can still be established with the charged residue side chains
478 of the βS9-βS10 loop and helix H1 through water molecules (**Fig. 2C**, **Fig. 7A**). A second
479 term that accounts for selectivity is occupancy of the taxane site by the βM loop in the
480 absence of lateral contacts, which would be a general mechanism that accounts for the
481 loss of at least four orders of magnitude in affinity when binding to unassembled tubulin
482 relative to microtubules for all taxane-site ligands, including paclitaxel, docetaxel (*19*),
483 discodermolide (*40*), epothilones (*39*), and **2a** (this paper). Our MD simulations of the
484 drug-free tubulin dimer shed new light on why taxanes and other taxane-site ligands bind
485 tubulin dimers with affinities much lower than those reported for microtubules. Whereas
486 in assembled microtubules the βM loop is structured as an α-helix and the preorganized
487 taxane-site is empty and ready to accommodate a ligand, in the unassembled tubulin
488 dimer –as well as in the models of isolated protofilaments and the solvent-exposed site
489 of the minimalist microtubule representation– this same loop displays a large
490 conformational heterogeneity and can adopt a hairpin conformation that allows it to
491 interact with the taxane site and thus to inhibit ligand binding (**Fig. S5**). Moreover, when
492 the tubulin dimer with the βM loop in an α-helical conformation was simulated in
493 complex with baccatin III, **2a**, and paclitaxel, an evolution was systematically observed
494 consisting of βM loop disordering similar to that likely responsible for the lack of electron
495 density in the crystallographic apo structures. The MD analysis indicates that the
496 conformational freedom of the βM loop in unassembled tubulin allows it to occupy the
497 taxane-binding pocket in such a way as to preclude (or compete with) ligand binding. On
498 the other hand, the free energy contribution of taxane-site ligands for microtubule
499 assembly arises from the preferential recognition of the taxane-site conformation present
500 in microtubules (*52*).

501 Our results point to the βM loop as an essential structural element for the mode of
502 action of paclitaxel and other clinically used taxanes. Our high-resolution structural

503 analysis of baccatin III in complex with tubulin suggests that even this simplified taxane
504 is able to reduce the flexibility of the βM loop by inducing a partial structuring of its N-
505 terminal region. Further changes occur in the presence of a small C13 side chain, as in **2a**
506 and **2b**, compared to paclitaxel, such as tilting the position of their baccatin III core region
507 by ~20º within the binding pocket and inducing a subtle reorientation of tubulin domains
508 with respect to one another. Despite the fact that we did not observe a complete
509 structuring of the βM loop upon baccatin III, **2a**, or **2b** binding in their respective crystal
510 structures or during MD simulations of free dimers and protofilaments, conformational
511 changes were detected in this β-tubulin region that are in consonance with those observed
512 upon paclitaxel binding to microtubules. Furthermore, our X-ray fiber diffraction studies
513 indicate differences in lateral contacts of shear-flow aligned microtubules bound to
514 baccatin III, **2a**, or **2b**. This observation suggests that paclitaxel and the novel taxanes
515 reported here indeed affect lateral contacts so as to promote microtubule stability through
516 interactions with the N-terminal section of the βM loop, in good agreement with
517 observations reported previously (*14, 15*).

518 Finally, we found that binding of taxanes to assembled microtubules results in a
519 displacement of the βS9-βS10 loop, which promotes a lattice expansion. The description
520 of the effect of paclitaxel on microtubule lattice parameters has been controversial. Initial
521 analyses suggested that paclitaxel induces lattice expansion (*10, 11*); however,
522 subsequent studies reported only a minor effect (*13-15*). Our present results reinforce the
523 view that lattice expansion is indeed a general consequence of taxane binding and does
524 not require the presence of a C13 side chain. Since baccatin III is essentially biologically
525 inert (*35-38*), our data further indicate that lattice expansion is not an important factor
526 contributing to the mechanism of microtubule stabilization by paclitaxel. Our MD
527 analyses offer a plausible explanation for the taxane-induced longitudinal expansion of
528 microtubules. Although in the complexes with **2a** and **2b** –but not in that with baccatin
529 III–, the crystal structures show that (NH)βG370 hydrogen bonds to the taxane side chain,
530 the simulated complexes indicate that in solution it is the (O=C)βR369 that consistently
531 acts as the hydrogen bond acceptor for the O2' hydroxyl of taxanes. In our view, these
532 findings point to the βS9-βS10 loop as a major structural element that changes on taxane
533 binding, and this change is transmitted to the following α-tubulin subunits on both sides,
534 hence the stretching or longitudinal expansion of the concatenated tubulin dimers. The
535 fact that we observed the Φ/Ψ backbone rearrangement in the βR369-βG370 stretch upon

536 cooling down the tubulin-paclitaxel complex obtained after the targeted MD procedure
537 (**Movie M7**) points to expansion of the cavity and consolidation of the hydrogen-bonding
538 network as the main factors responsible for this conformational change.

539 In conclusion, our combined experimental and computational approach allowed
540 us to describe the tubulin-taxane interaction in atomic detail and assess the structural
541 determinants for binding. Our structural analyses further suggest a mode of action of
542 paclitaxel by means of which its core moiety provides the main tubulin-interaction
543 network whilst its C13 side chain enables selective recognition of the prestructured βM
544 loop of the microtubule-assembled tubulin state. Such differential recognition is expected
545 to promote microtubule formation and stabilization. On the other hand, the longitudinal
546 expansion of the microtubule lattices arises from the accommodation of the taxane core
547 within the site, a process that is, however, not related to the microtubule stabilization
548 mechanism of taxanes.
549

550 **Materials and Methods**

551 *Proteins and ligands*

552 Purified calf brain tubulin and chemicals were obtained as previously
553 described(*19, 53*). Paclitaxel (Taxol®) was from Alfa Aesar Chemical, docetaxel
554 (Taxotere®) was kindly provided by Rhône Poulenc Rorer, Aventis (Schiltigheim,
555 France), baccatin III was from Sigma, Flutax-2, Chitax 40, 3'N-aminopaclitaxel (N-AB-
556 PT) and Chitax-68 were synthesized as described(*31-33, 46*). All compounds were diluted
557 in 99.8% DMSO-D6 (Merck) to a final concentration of 20 mM and stored at -20 ºC.
558 Their solubility in aqueous media was determined as described in(*54*), Flutax-2 was found
559 soluble, while a 100 μM solubility was found for docetaxel and a 50 μM for both
560 paclitaxel and Chitax40.

561

562 *Synthesis of taxoids **2a-2d**.*
563

564 **General Experimental Procedures.** ^{1}H and ^{13}C NMR spectra were recorded on Varian
565 400, 500 MHz spectrometers or a Bruker AVANCE III 600 MHz NMR spectrometer.
566 Mass spectra (ESI) was measured on JEOL Accu TOF CS (JMS T100CS). Reagents were
567 purchased from J&K and Alfa Aesar Chemical companies. All anhydrous solvents were
568 purified and dried according to standard procedures, unless otherwise indicated.

569 Reactions were monitored by TLC (silica gel, GF254) with UV light and
570 H_2SO_4-anisaldehyde spray visualization. The purity of the final compounds was analyzed
571 by HPLC.
572
573 **7, 10-*O*-di(triethylsilyl)-10-deacetylbaccatin III (4)**
574 To a stirred solution of **3** (1.82 g, 3.3 mmol) in anhydrous tetrahydrofuran (THF) (36 mL)
575 under argon, 4-dimethylaminopyridine (DMAP) (400 mg, 3.3 mmol), triethylamine
576 (TEA) (8.3 mL, 69.4 mmol) and (chlorotriethylsilane) TESCl (4.5 mL, 26.4 mmol) was
577 added dropwise. After the reaction mixture was stirred at room temperature (RT) for 5.5
578 h, the solution of anhydrous LiBr (291 mg, 3.3 mmol) in anhydrous THF (1.8 mL) was
579 added, the reaction mixture was refluxed at 65-70 °C for 7 h. Once cooled down, the
580 mixture was diluted with ethyl acetate (200 mL). The mixture was washed with saturated
581 aqueous $NaHCO_3$ solution (200 mL) and brine (200 mL), and dried over anhydrous
582 Na_2SO_4. The organic layer was evaporated under reduced pressure. Purification of the
583 crude product by silica gel chromatography (acetone: petroleum ether=1:7) gave 84%
584 yield of product **4** (2.13 g) as a light yellow oil: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.55-0.71
585 p.p.m. (m, 12H), 0.94-1.02 (m, 18H), 1.04 (s, 3H), 1.18 (s, 3H), 1.65 (s, 3H), 1.85-1.91
586 (m, 1H), 2.01 (s, 3H), 2.22-2.28 (m, 5H), 2.49-2.57 (m, 1H), 3.91 (d, J = 6.8 Hz, 1H),
587 4.14 (d, J = 8.0 Hz, 1H), 4.27 (d, J = 8.4 Hz, 1H), 4.42 (dd, J = 10.4 Hz, 6.8 Hz, 1H),
588 4.81 (t, J = 8.0 Hz, 1H), 4.93 (d, J = 8.0 Hz, 1H), 5.21 (s, 1H), 5.61 (d, J = 7.2 Hz, 1H),
589 7.45 (t, J = 7.6 Hz, 2H), 7.57 (t, J = 7.2 Hz, 1H), 8.09 (d, J = 7.2 Hz, 2H). The ^{1}H NMR
590 data are identical to those for 7, 10-*O*-di(triethylsilyl)-10-deacetylbaccatin III in(*55*).
591
592 **7,10-*O*-di(triethylsilyl)-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*N*-(de-*tert*-**
593 **butoxycarbonyl)- 3'-*N*-(benzyloxycarbonyl)docetaxel (6)**
594 A stirred solution of **4** (2.12 g, 2.74 mmol) in anhydrous THF (35.7 mL) under argon was
595 cooled to -45 °C and lithium bis(trimethylsilyl)amide (LHMDS) (0.9 M in
596 methylcyclohexane, 4.6 mL, 4.11 mmol) was added dropwise. The reaction mixture was
597 stirred for 20 min at -45 °C and then, the solution of **5**(*56*) (1.352 g, 3.288 mmol) in
598 anhydrous THF (9 mL) was added and the reaction mixture was stirred for 100 min at the
599 same temperature. Afterwards, the mixture was quenched with saturated aqueous NH_4Cl
600 solution (10 mL) and extracted with ethyl acetate (200 mL*2). The organic layer was
601 washed with saturated aqueous NH_4Cl solution (100 mL) and brine (100 mL), dried over
602 anhydrous Na_2SO_4. Solvent was removed under reduced pressure. Purification of the
603 crude product by silica gel chromatography (acetone: petroleum ether=1:10~1:7) gave

604 79% yield of product **6** (2.57 g) as a light yellow oil: ^{1}H-NMR (400 MHz, $CDCl_3$): δ -
605 0.31 p.p.m. (s, 3H), -0.08 (s, 3H), 0.56-0.72 (m, 12H), 0.75 (s, 9H), 0.95-1.03 (m, 18H),
606 1.20 (s, 3H), 1.22 (s, 3H), 1.69 (s, 3H), 1.84 (s, 3H), 1.88-1.96 (m, 2H), 2.33-2.39 (m,
607 1H), 2.49-2.54 (m, 4H), 3.86 (d, J = 6.8 Hz, 1H), 4.21, 4.29 (ABq, J = 8.4 Hz, each 1H),
608 4.41 (dd, J = 10.4 Hz, 6.4 Hz, 1H), 4.55 (s, 1H), 4.93 (d, J = 8.4 Hz, 1H), 4.97, 5.02 (ABq,
609 J = 12.4 Hz, each 1H), 5.16 (s, 1H), 5.37 (d, J = 8.8 Hz, 1H), 5.67 (d, J = 7.2 Hz, 1H),
610 5.72 (d, J = 9.6 Hz, 1H), 6.25 (t, J = 8.4 Hz, 1H), 7.20-7.32 (m, 8H), 7.38 (t, J = 7.2 Hz,
611 2H), 7.48 (t, J = 7.6 Hz, 2H), 7.57 (t, J = 7.6 Hz, 1H), 8.13 (d, J = 7.2 Hz, 2H); ^{13}C-NMR
612 (150 MHz, $CDCl_3$): δ -6.0, -5.4, 5.3, 5.9, 6.9, 10.5, 13.7, 14.1, 14.2, 18.1, 20.9, 21.0, 22.7,
613 23.1, 25.4, 26.5, 29.3, 29.6, 29.7, 31.9, 35.6, 37.3, 43.2, 46.6, 57.2, 58.3, 60.3, 66.8, 71.4,
614 72.6, 75.2, 75.3, 75.5, 76.7, 78.9, 81.2, 84.0, 126.4, 127.7, 127.8, 128.0, 128.4, 128.6,
615 129.5, 130.2, 133.4, 134.2, 136.3, 137.7, 138.6, 155.7, 167.0, 170.1, 171.1, 171.2, 205.2;
616 ESIMS *m/z* 1184.6 $[M + H]^+$.
617
618 **7,10-*O*-di(triethylsilyl)-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*N*-(de-*tert*-**
619 **butoxycarbonyl)docetaxel (7)**
620 To a stirred solution of **6** (2.54 g, 2.14 mmol) in methanol (50 mL), 10% Pd/C (250 mg)
621 was added under H_2 and the reaction mixture was stirred at room temperature for 20 h.
622 The mixture was diluted with methanol (50 mL), filtered and washed with methanol. The
623 organic layer was evaporated under reduced pressure. Purification of the crude product
624 by silica gel chromatography (acetone: petroleum ether=1:8) gave 64% yield of product
625 **7** (1.44 g) as a colorless oil with 18% yield of **6** (0.46 g) recovery: ^{1}H-NMR (500 MHz,
626 DMSO-d_6): δ -0.05 p.p.m. (s, 3H), -0.04 (s, 3H), 0.52-0.62 (m, 12H), 0.84 (s, 9H), 0.90-
627 0.95 (m, 18H), 1.05 (s, 6H), 1.52 (s, 3H), 1.65-1.70 (m, 4H), 1.79-1.84 (m, 1H), 2.02-
628 2.07 (m, 2H), 2.33 (s, 3H), 3.68 (d, J = 7.0 Hz, 1H), 4.02-4.05 (m, 2H), 4.14, 4.30 (ABq,
629 J = 6.0 Hz, each 1H), 4.32 (dd, J = 10.5 Hz, 6.5 Hz, 1H), 4.60 (s, 1H), 4.93 (d, J = 9.5
630 Hz, 1H), 5.06 (s, 1H), 5.44 (d, J = 7.0 Hz, 1H), 5.89 (t, J = 9.0 Hz, 1H), 7.20-7.22 (m,
631 1H), 7.35-7.36 (m, 5H), 7.60 (t, J = 7.5 Hz, 2H), 7.70 (t, J = 7.5 Hz, 1H), 7.98 (d, J = 7.0
632 Hz, 2H); ^{13}C-NMR (125 MHz, DMSO-d_6): δ -5.4, -5.3, 4.8, 5.4, 6.7, 6.8, 10.1, 13.7, 17.9,
633 20.7, 22.6, 25.5, 26.3, 34.9, 36.8, 42.9, 45.9, 57.7, 58.9, 70.4, 72.4, 74.5, 75.0, 75.5, 76.6,
634 78.0, 80.0, 83.0, 124.2, 127.3, 128.0, 128.6, 129.5, 130.0, 133.4, 134.2, 137.1, 141.6,
635 165.2, 169.8, 172.1, 204.7; ESIMS *m/z* 1050.5 $[M + H]^+$.
636
637

638 **3'-*N*-(de-*tert*-butoxycarbonyl)-3'-*N*-(4-methoxy-2-methylene-4-**
639 **oxobutanoyl)docetaxel (2a)**
640 To a stirred solution of **7** (43.6 mg, 0.042 mmol) in anhydrous dichloromethane (DCM)
641 (0.34 mL) under Argon, *N,N'*-Dicyclohexylcarbodiimide (DCC) (17.1 mg, 0.083 mmol),
642 DMAP (2.5 mg, 0.020 mmol) and the solution of itaconic acid monomethyl ester(*57*) (9.2
643 mg, 0.064 mmol) in DCM (0.15 mL) was added in ice bath. Then, the mixture was stirred
644 for 2 h at room temperature. The mixture was diluted with ethyl acetate (30 mL), filtered
645 by celite and washed with ethyl acetate (30 mL). The organic layer was evaporated under
646 reduced pressure. Purification of the crude product by silica gel chromatography
647 (acetone: hexane=1:9) gave crude product. Subsequently, to a stirred solution of the crude
648 product in acetonitrile (1.7 mL), pyridine (1.0 mL, 12.1 mmol) and HF (0.52 mL, 12.1
649 mmol) was added and the reaction was stirred at RT for 24 h. Following that, the mixture
650 was diluted with ethyl acetate (50 mL), washed with brine (20 mL), extracted with ethyl
651 acetate (20 mL), and dried over anhydrous Na_2SO_4. The organic layer was evaporated
652 under reduced pressure. Purification of the crude product by silica gel chromatography
653 (acetone: petroleum ether=1:2) gave 32% yield (for two steps) of compound **2a** (11.0 mg)
654 as a white solid: ^{1}H-NMR (600 MHz, CD_3COCD_3): δ 1.11 p.p.m. (s, 3H), 1.20 (s, 3H),
655 1.69 (s, 3H), 1.80-1.84 (m, 1H), 1.88 (d, J = 1.2 Hz, 3H), 2.16-2.20 (m, 1H), 2.30-2.34
656 (m, 1H), 2.39-2.45 (m, 4H), 3.34 (s, 2H), 3.61 (s, 3H), 3.89 (d, J = 6.6 Hz, 1H), 4.13, 4.18
657 (ABq, J = 8.4 Hz, each 1H), 4.25 (dd, J = 11.4 Hz, 6.6 Hz, 1H), 4.69 (d, J = 4.2 Hz, 1H),
658 4.95 (dd, J = 9.6 Hz, 1.8 Hz, 1H), 5.24 (s, 1H), 5.53 (d, J = 4.2 Hz, 1H), 5.65 (d, J = 7.2
659 Hz, 1H), 5.73 (d, J = 1.2 Hz, 1H), 6.15-6.18 (m, 2H), 7.27 (t, J = 7.2 Hz, 1H), 7.38 (t, J
660 = 7.8 Hz, 2H), 7.46 (d, J = 7.2 Hz, 2H), 7.54 (t, J = 8.4 Hz, 2H), 7.64 (t, J = 7.2 Hz, 1H),
661 8.09 (dd, J = 8.4 Hz, 1.2 Hz, 2H); ^{13}C-NMR (150 MHz, CD_3COCD_3): δ 9.1, 13.1, 20.2,
662 21.7, 25.9, 35.4, 36.1, 38.4, 42.9, 46.1, 51.1, 54.8, 57.2, 70.9, 71.0, 73.2, 73.8, 74.7, 75.6,
663 77.1, 80.5, 83.9, 126.8, 127.0, 127.5, 127.9, 128.2, 129.6, 129.9, 132.8, 134.6, 136.1,
664 137.3, 138.9, 165.5, 166.4, 169.3, 170.0, 172.4, 210.1; HRMS (*m/z*): $[M+Na]^+$ calcd for
665 $C_{44}H_{51}NaNO_{15}$, 856.3259; found, 856.3157.
666
667 **3'-*N*-(de-*tert*-butoxycarbonyl)-3'-*N*-(2-bromoacetyl)docetaxel (2b)**
668 To a stirred solution of **7** (90 mg, 0.086 mmol) in anhydrous DCM (0.9 mL) under Argon,
669 DCC (53.2 mg, 0.26 mmol), DMAP (10.5 mg, 0.086 mmol) and the solution of
670 bromoacetic acid (35.9 mg, 0.26 mmol) in DCM (0.1 mL) was added in ice bath. Then,
671 the mixture was stirred for 2 h at room temperature. The mixture was diluted with ethyl
672 acetate (30 mL), filtered by celite and washed with ethyl acetate (30 mL). The organic

673 layer was evaporated under reduced pressure. Purification of the crude product by silica
674 gel chromatography (acetone: hexane=1:9) gave crude product (71 mg). Then, a stirred
675 solution of the crude product (54 mg) was solved in 5% HCl/methanol (0.41 mL) in ice
676 bath, and the reaction was stirred in ice bath for 1 h and at room temperature for 12 h.
677 Afterwards, the mixture was diluted with ethyl acetate (50 mL), washed with brine (20
678 mL), extracted with ethyl acetate (20 mL) and dried over anhydrous Na_2SO_4. The organic
679 layer was evaporated under reduced pressure. Purification of the crude product by silica
680 gel chromatography (acetone: petroleum ether=1:1.5) gave 40% yield (for two steps) of
681 compound **2b** (28.5 mg) as a white solid: ^{1}H-NMR (600 MHz, CD_3COCD_3): δ 1.11 p.p.m.
682 (s, 3H), 1.18 (s, 3H), 1.69 (s, 3H), 1.80-1.84 (m, 1H), 1.88 (d, J = 1.2 Hz, 3H), 2.16-2.20
683 (m, 1H), 2.29-2.33 (m, 1H), 2.38 (s, 3H), 2.40-2.45 (m, 1H), 3.89 (d, J = 7.2 Hz, 1H),
684 3.95, 4.00 (ABq, J = 12.0 Hz, each 1H), 4.13, 4.18 (ABq, J = 8.4 Hz, each 1H), 4.23 (dd,
685 J = 11.4 Hz, 6.6 Hz, 1H), 4.70 (d, J = 4.2 Hz, 1H), 4.95 (dd, J = 9.6 Hz, 1.8 Hz, 1H), 5.23
686 (s, 1H), 5.49 (d, J = 4.2 Hz, 1H), 5.65 (d, J = 7.2 Hz, 1H), 6.16 (t, J = 8.4 Hz, 1H), 7.28
687 (t, J = 7.2 Hz, 1H), 7.39 (t, J = 7.2 Hz, 2H), 7.47 (d, J = 7.2 Hz, 2H), 7.56 (t, J = 7.8 Hz,
688 2H), 7.65 (t, J = 7.2 Hz, 1H), 8.09 (d, J = 8.4 Hz, 2H); ^{13}C-NMR (150 MHz, CD_3COCD_3):
689 δ 9.1, 13.1, 20.2, 21.7, 25.8, 35.4, 36.0, 42.8, 46.0, 55.3, 57.2, 59.4, 70.9, 73.0, 73.7, 74.7,
690 75.6, 77.1, 80.5, 83.8, 126.8, 127.2, 128.0, 128.1, 129.5, 129.9, 132.8, 136.1, 137.2,
691 138.3, 165.4, 165.9, 170.0, 172.1, 210.0; HRMS (*m/z*): $[M+Na]^+$ calcd for
692 $C_{40}H_{46}NaBrNO_{13}$, 850.2153; found, 850.2037.
693
694 **3'-*N*-(de-*tert*-butoxycarbonyl)-3'-*N*-(2-iodoacetyl)docetaxel (2c)**
695 Taxoid **2c** was synthesized with iodoacetic acid following the similar procedure for **2b**.
696 Yield of 48% (for two steps), 28.5 mg, white solid: ^{1}H-NMR (600 MHz, CD_3COCD_3): δ
697 1.12 p.p.m. (s, 3H), 1.19 (s, 3H), 1.71 (s, 3H), 1.80-1.85 (m, 1H), 1.90 (d, J = 1.2 Hz,
698 3H), 2.22-2.26 (m, 1H), 2.34-2.38 (m, 1H), 2.40 (s, 3H), 2.41-2.46 (m, 1H), 3.82, 3.87
699 (ABq, J = 9.6 Hz, each 1H), 3.91 (d, J = 7.2 Hz, 1H), 4.14, 4.19 (ABq, J = 8.4 Hz, each
700 1H), 4.27 (dd, J = 10.8 Hz, 6.6 Hz, 1H), 4.71 (d, J = 3.6 Hz, 1H), 4.95 (dd, J = 9.6 Hz,
701 2.4 Hz, 1H), 5.23 (s, 1H), 5.51 (d, J = 3.6 Hz, 1H), 5.66 (d, J = 7.2 Hz, 1H), 6.20 (t, J =
702 9.0 Hz, 1H), 7.29 (t, J = 7.2 Hz, 1H), 7.39 (t, J = 7.2 Hz, 2H), 7.48 (d, J = 7.2 Hz, 2H),
703 7.56 (t, J = 7.8 Hz, 2H), 7.65 (t, J = 7.8 Hz, 1H), 8.11 (d, J = 9.0 Hz, 1.8 Hz, 2H); ^{13}C-
704 NMR (150 MHz, CD_3COCD_3): δ 9.3, 13.3, 20.4, 21.9, 26.1, 35.6, 36.3, 43.0, 46.3, 55.3,
705 57.4, 59.6, 71.1, 71.2, 73.2, 74.0, 74.9, 75.8, 77.4, 80.7, 84.0, 127.0, 127.3, 128.2, 128.3,
706 129.8, 130.1, 133.0, 136.3, 137.4, 138.7, 165.7, 167.7, 170.2, 172.4, 210.2; ESIMS *m/z*
707 876.2 $[M + H]^+$, 898.2 $[M + Na]^+$.

3'-*N*-(de-*tert*-butoxycarbonyl)-3'-*N*-(2-azidoacetyl)docetaxel (2e)

Taxoid **2e** was synthesized with azidoacetic acid(*58*) following the similar procedure for **2a**. Yield of 83% (for two steps), 25.0 mg, colorless oil: ^{1}H-NMR (500 MHz, CD_3COCD_3): δ 1.12 p.p.m. (s, 3H), 1.18 (s, 3H), 1.69 (s, 3H), 1.79-1.85 (m, 1H), 1.87 (s, 3H), 2.12-2.17 (m, 1H), 2.27-2.32 (m, 1H), 2.36 (s, 3H), 2.40-2.46 (m, 1H), 3.89 (d, J = 7.0 Hz, 1H), 4.01 (s, 2H), 4.13, 4.17 (ABq, J = 8.0 Hz, each 1H), 4.25 (dd, J = 11.0 Hz, 6.5 Hz, 1H), 4.67 (d, J = 4.5 Hz, 1H), 4.95 (d, J = 8.0 Hz, 1H), 5.23 (s, 1H), 5.50 (d, J = 4.5 Hz, 1H), 5.65 (d, J = 7.5 Hz, 1H), 6.16 (t, J = 9.0 Hz, 1H), 7.28 (t, J = 7.5 Hz, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.47 (d, J = 7.5 Hz, 2H), 7.56 (t, J = 7.5 Hz, 2H), 7.65 (t, J = 7.0 Hz, 1H), 8.09 (d, J = 7.5 Hz, 2H); ^{13}C-NMR (125 MHz, CD_3COCD_3): δ 9.3, 13.3, 20.4, 22.0, 26.0, 35.7, 36.3, 43.0, 46.3, 51.3, 55.4, 57.4, 71.0, 71.2, 73.5, 74.0, 74.9, 75.8, 77.4, 80.7, 84.1, 127.1, 127.5, 128.3, 128.4, 129.8, 130.1, 133.0, 136.4, 137.4, 138.8, 165.6, 167.2, 170.2, 172.4, 210.3; ESIMS *m/z* 813.3 $[M + Na]^+$.

3'-*N*-(de-*tert*-butoxycarbonyl)-3'-*N*-(2-isothiocyanatoacetyl)docetaxel (2d)

To a stirred solution of **2e** (16.4 mg, 0.021 mmol) in anhydrous THF (0.32 mL) under Argon, Ph_3P (8.5 mg, 0.032 mmol) and CS_2 (12.6 μL, 0.21 mmol) were added and the mixture was stirred for 50 h at room temperature. The mixture was evaporated under reduced pressure. Purification of the crude product by silica gel chromatography (acetone: hexane=1:9) gave 83% yield of compound **2d** (28.5 mg) as a white solid: ^{1}H-NMR (600 MHz, CD_3COCD_3): δ 1.08 p.p.m. (s, 3H), 1.10 (s, 3H), 1.67 (s, 3H), 1.76-1.84 (m, 5H), 2.06-2.09 (m, 1H), 2.36 (s, 3H), 2.40-2.45 (m, 1H), 3.85 (d, J = 7.2 Hz, 1H), 4.11, 4.15 (ABq, J = 7.8 Hz, each 1H), 4.18-4.25 (m, 3H), 4.94 (dd, J = 9.6 Hz, 1.8 Hz, 1H), 5.20 (s, 1H), 5.59 (d, J = 7.2 Hz, 1H), 5.65 (d, J = 6.6 Hz, 1H), 6.02 (t, J = 9.0 Hz, 1H), 6.17 (d, J = 10.2 Hz, 1H), 7.25 (t, J = 7.2 Hz, 1H), 7.38 (t, J = 7.8 Hz, 2H), 7.54 (d, J = 7.8 Hz, 2H), 7.61 (t, J = 7.8 Hz, 2H), 7.69 (t, J = 7.8 Hz, 1H), 8.04 (dd, J = 8.4 Hz, 1.2 Hz, 2H); ^{13}C-NMR (150 MHz, CD_3COCD_3): δ 9.0, 13.0, 19.9, 21.7, 25.6, 35.2, 35.9, 42.6, 46.0, 47.4, 57.1, 59.4, 69.7, 69.8, 70.8, 73.6, 74.5, 75.5, 77.0, 80.3, 83.8, 128.0, 128.1, 128.5, 129.4, 129.8, 132.9, 135.5, 136.1, 136.9, 165.3, 169.9, 171.7, 173.3, 209.8; HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{41}H_{46}NaN_2O_{13}S$, 829.2721; found, 829.2619.

Crystallization, Data Collection and Structure Determination

Crystals of T_2R-TTL were generated as described (*21, 29*). Suitable T_2R-TTL crystals were soaked for 8h in reservoir solutions (2-4% PEG 4K, 2-10% glycerol, 30

742 mM $MgCl_2$, 30 mM $CaCl_2$, 0.1 M MES/Imidazole pH 6.7) containing either 10 mM
743 baccatin III, 5 mM **2a** or **2b.** Subsequently, crystals were flash cooled in liquid nitrogen
744 following a brief transfer into cryo solutions containing the reservoir supplemented with
745 16% and 20% glycerol. All data were collected at beamline X06DA at the Swiss Light
746 Source (Paul Scherrer Institut, Villigen PSI, Switzerland). Images were indexed and
747 processed using XDS(*59*). Structure solution using the difference Fourier method and
748 refinement were performed using PHENIX(*60*). Model building was carried out
749 iteratively using the Coot software(*61*). Data collection and refinement statistics for all
750 three T_2R-TTL-complexes are given in Table 1. Molecular graphics and analyses were
751 performed with PyMol (The PyMOL Molecular Graphics System, Version 2.3.2,
752 Schrödinger, LLC). To compare the structures of both baccatin III and **2a** complexes in
753 the curved tubulin conformation to the straight tubulin in paclitaxel stabilized microtubule
754 (PDB ID 6WVR), all structures were superimposed onto the taxane-site of **2a** (residues
755 208-219+225-237+318-320+359-376+272-276+287-296; $rmsd_{BacIII}$ 0.171 Å (48 C_α
756 atoms), $rmsd_{5SYF}$ 0.868 Å (52 C_α atoms)).

757

758 *Biochemistry*

759 The binding constants of both **2a** and baccatin III to unassembled dimeric tubulin
760 were measured by centrifugation. Increasing amounts of dimeric tubulin (up to 150 μM)
761 prepared in NaPi-GTP buffer (10 mM sodium phosphate, 0.1 mM GTP, pH 7.0) were
762 incubated with a fixed concentration (50 μM) of either baccatin III or **2a**, incubated for
763 30 min at 25 ºC and centrifuged at 100000 rpm in a TLA-100.2 rotor for 2h at 25 ºC.
764 Then, samples were divided into upper (100 μL) and lower (100 μL) parts and 100 μL of
765 NaPi were added to both of them. Afterwards, 10 μM of either docetaxel or paclitaxel
766 were added as internal standard and samples were subjected three times to an organic
767 extraction using dichloromethane (v:v). Dichloromethane was removed by evaporation
768 and samples were resuspended in methanol 70%. Finally, ligand content was analyzed
769 using an HPLC system (Agilent 1100 Series) and samples were separated using a Zorbax
770 Eclipse XDB-C18 column (Methanol 70% isocratic condition; 20 minutes runs). Tubulin
771 content was determined by BCA for each sample. Ligand concentration in the upper 100
772 μL was considered as free concentration, while this in the lower 100 μL was considered
773 as the sum of bound and free concentrations. Binding constants of tubulin for the ligand

774 were calculated assuming a single binding site per tubulin dimer using SIGMAPLOT
775 14.5 Sigmastat Software Inc.

776

777 *Microtubule shear-flow alignment and X-ray fiber diffraction experiments*

778 X-ray fiber diffraction data were collected in BL11-NDC-SWEET beamline of
779 ALBA Synchrotron at a λ=0.827 nm as described in(*12*). Radial structural parameters
780 (microtubule diameter and average inter-PT distances) and dimer/monomer length (from
781 the 4th harmonic of the first layer-line signals) were determined as described in(*12*).

782

783 *Molecular Modeling*

784 ***In silico model building and molecular dynamics simulations.***

785 Our reduced representation of a straight microtubule (MT) for simulation purposes
786 consisted of the α_1:β_1:α_2 subunits from one protofilament (PF) together with the closely
787 interacting $\alpha_{1'}$:$\beta_{1'}$:$\alpha_{2'}$ subunits from a neighboring PF, as found in the cryo-EM
788 reconstruction of an undecorated MT in complex with zampanolide (PDB code 5SYG,
789 3.5 Å resolution)(*13*). Likewise, α_1:β_1 made up the isolated dimer, and three concatenated
790 α:β dimers provided the starting straight PF. Missing residues 39-48 in the four α subunits
791 were added, and the partially hydrated Ca^{2+} coordinated by Asp39, Thr41, Gly44, and
792 Glu55 was replaced by Mg^{2+}. Computation of the protonation state of titratable groups at
793 pH 6.8 and addition of hydrogen atoms to each protein ensemble were carried out using
794 the H++ 3.0 Web server (*62*). Nonetheless, in agreement with previous work from our
795 group, the side chain carboxylic group of βGlu200 in the colchicine-binding site was
796 considered to be protonated (*63*) and a disulfide bond was created to link the side chains
797 of βCys241 and βCys356 (*49*). The four guanosine-triphosphate (GTP) and two
798 guanosine-diphosphate (GDP) molecules in the nucleotide-binding sites of α and
799 β tubulin, respectively, were kept, together with their coordinated Mg^{2+} ions and
800 hydrating water molecules. For consistency with the Protein Data Bank, residue
801 numbering and secondary structure assignment herein follow the α-tubulin-based
802 definitions given by Löwe *et al*. (*64*).

803 The initial molecular models of the taxane complexes were built by best-fit
804 superposition of β-tubulin in their respective crystallographic complexes, as reported here
805 for baccatin and **2a** –and previously for paclitaxel– (*51*), onto the MT, PF or α:β dimer

806 structure described above. *Ab initio* geometry optimization of baccatin, paclitaxel and **2a**,
807 followed by derivation of atom-centered RESP charges, (*65*) was achieved using a 6-
808 31G* basis set, the Density Functional Tight-Binding (DFTB) method, and the IEF-SCRF
809 continuum solvent model (*66*), as implemented in program Gaussian 09 (Revision
810 D.01)(*67*). The *gaff* (*68*) and *ff14SB* (*69*) AMBER force fields were used for ligand and
811 protein atoms, respectively. The molecular graphics program PyMOL (v. 1.8,
812 Schrödinger LLC) was employed for structure visualization, molecular editing and figure
813 preparation.
814 All the ligand:tubulin complexes and their respective apo forms were solvated into
815 a cubic box of TIP3P water molecules –with a minimal distance of the protein to the
816 borders of 12 Å– and neutralized by addition of a sufficient number of Na^+ ions. These
817 ensembles were simulated under periodic boundary conditions and electrostatic
818 interactions were computed using the particle mesh Ewald method (*70*) with a grid
819 spacing of 1 Å. The cutoff distance for the non-bonded interactions was 9 Å and the
820 SHAKE algorithm (*71*) was applied to all bonds involving hydrogens so that an
821 integration step of 2.0 fs could be used. All hydrogens and water molecules were first
822 reoriented in the electric field of the solute and then all protein residues, ligands,
823 counterions and waters were relaxed by performing 5 000 steps of steepest descent
824 followed by 50 000 steps of conjugate gradient energy minimization. The resulting
825 geometry-optimized coordinate sets were used as input for the molecular dynamics (MD)
826 simulations at a constant pressure of 1 atm and 300 K using the *pmemd.cuda_SPFP*
827 engine (*72*) as implemented in AMBER 18 for GeForce Nvidia GTX 980 graphics
828 processing units. Ligands, water molecules and counterions were first relaxed around the
829 positionally restrained protein (1 kcal mol^{-1} $Å^{-2}$ on Cα atoms) during a heating and
830 equilibration period lasting 0.5 ns. For the remaining simulation time (from 250 to 1200
831 ns depending on the system) the macromolecular ensembles were allowed to evolve and
832 coordinates were collected every 0.1 ns for further analysis by means of the *cpptraj*
833 module in AMBER (*73*). Positional restraints were used only in the case of the apo and
834 ligand-bound MT, in which case a weak harmonic restraint (0.5 mol^{-1} $Å^{-2}$) on all
835 Cα atoms (except for those in amino acids 276-374 of both β subunits that make up
836 the βM loops and a large part of the α:β interfaces) was employed to preserve the overall
837 architecture observed in the cryo-EM structure. Snapshots taken every 5 ns were cooled
838 down from 300 to 273 K over a 1-ns period using a simulated annealing procedure (*74*);

839 the geometries of these "frozen" complexes were then optimized by carrying out an
840 energy minimization until the root-mean-square of the Cartesian elements of the gradient
841 was less than 0.01 kcal·mol^{-1}·Å^{-1}. The resulting ensembles of low-energy and
842 geometrically optimized representative structures, which are expected to be closer to the
843 global energy minima (*49*), were used to calculate the residue-based, solvent-corrected
844 interaction energies.
845
846 ***Geometry and Binding Energy Analysis.***
847 Both the trajectory snapshots and the sets of representative optimized coordinates
848 for each complex studied were analyzed in geometrical terms with the aid of the *cpptraj*
849 routines (*73*) from the AmberTools18 suite. Estimations of the solvent-corrected binding
850 energies were provided by our in-house MM-ISMSA software (*75*), which makes use of
851 a sigmoidal, distance-dependent dielectric function (*76*), and also provides a per-residue
852 decomposition into van der Waals, coulombic, apolar, and desolvation contributions.
853
854 ***Steered MD simulations.***
855 The macromolecular assemblies composed of an α:β dimer in complex with either
856 baccatin or paclitaxel, as obtained after 5 ns of MD equilibration at 300 K, were
857 additionally subjected to a targeted MD dynamics (tMD) procedure by means of which
858 the trajectories were biased so as to force ligand exit firstly and then re-entry into the
859 binding site. The tMD approach was followed essentially as described (*77*) and made use
860 of the parallel implementation of the AMBER *sander.MPI* code running on 4 CPUs,
861 which allows the solvent molecules to move freely and follow the dynamics of ligand and
862 protein. A restraint was defined in terms of a mass-weighted root-mean-square (rms)
863 superposition to the final reference structure (target) that is applied in the force field as
864 an extra energy term of the form $E = 0.5\, k_r\, N\, (rmsd - trmsd)^2$, where k_r is the force
865 constant, N is the number of atoms, and *trmsd* is the target rms deviation. A negative
866 value of the force constant (–0.5 kcal mol^{-1} Å^{-2} over 0.5 ns using only the ligand's oxygen
867 atoms in the rms definition) was employed to force the ligand coordinates away from the
868 initial docking location whereas a positive one was used to find a low-energy path leading
869 from the unbound ligand obtained from the previous procedure back to the initial target
870 structure.
871 Whereas the same value of 0.5 kcal mol^{-1} Å^{-2} over 0.5 ns proved sufficient to bring
872 baccatin back to its binding pocket, it was considerably more cumbersome to achieve the

same goal in the case of paclitaxel, in which case it was imperative to apply additional conformational restraints to fixate both the T-shape of the ligand and an α-helical βM loop for reasons discussed in the text.

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908 Coordinates and structure factors have been deposited at the Protein Data Bank
909 (www.rcsb.org) under accession numbers PDB: 8BDE (T_2R-TTL-**BacIII**), 8BDF (T_2R-
910 TTL-**2a**) and 8BDG ((T_2R-TTL-**2b**).
911
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1128 **Figures and Tables**

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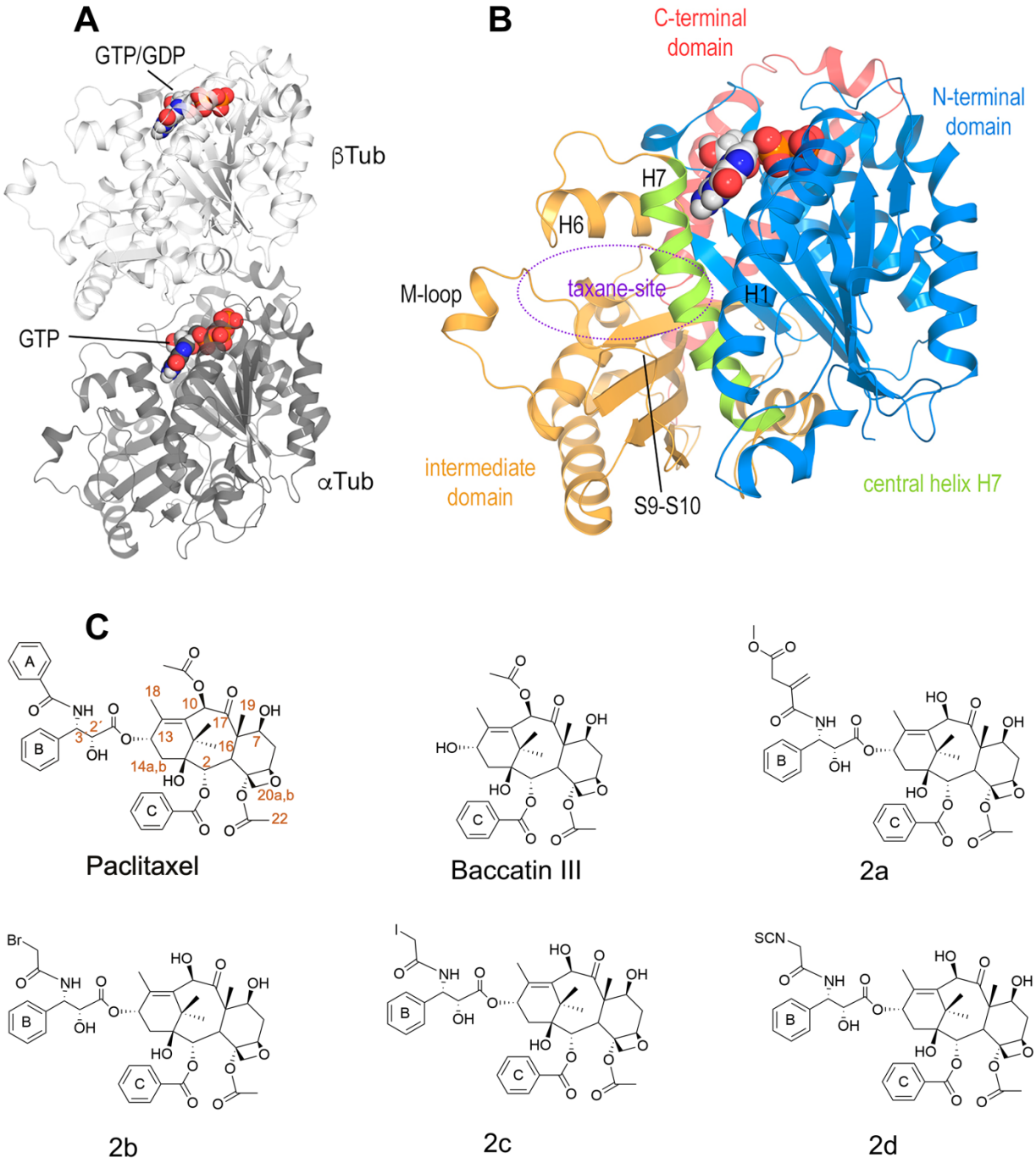

1130

1131 **Figure 1.** (**A**) Tubulin heterodimer (α-tubulin in gray and β-tubulin in white) in ribbon
1132 representation, where nucleotide binding sites have been highlighted in sphere
1133 representation **(B)** Structural features of the tubulin β-subunit. (**C**) Structures of taxanes
1134 used in this study.

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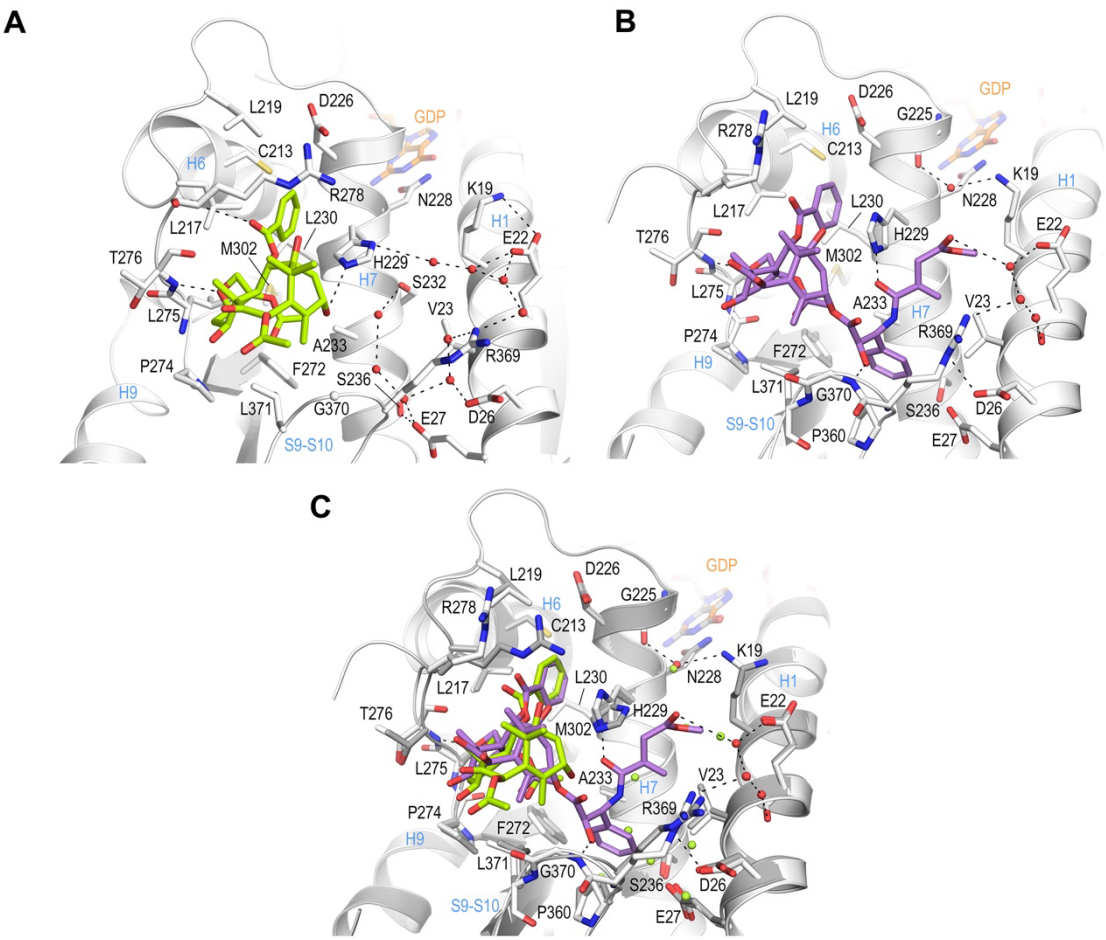


Figure 2. Crystal structure of T_2R-TTL-baccatin III and T_2R-TTL-2a complexes. (**A**) Close-up view of the interaction network observed between baccatin III (lemon) and β-tubulin (light gray). Interacting residues of tubulin are shown in stick representation and are labeled. Oxygen and nitrogen atoms are colored red and blue, respectively; carbon atoms are in lemon (baccatin III) or light gray (tubulin). Hydrogen bonds are depicted as black dashed lines. Secondary structural elements of tubulin are labeled in blue. (**B**) Close-up view of the interaction of **2a** (violet) with β-tubulin in the same view and representation as in (**A**). (**C**) The same close-up view as in (**A**) and (**B**) with the superimposed baccatin III (lemon) and **2a** (violet) complex structures. Water molecules belonging to the baccatin III structure are represented as lemon spheres.

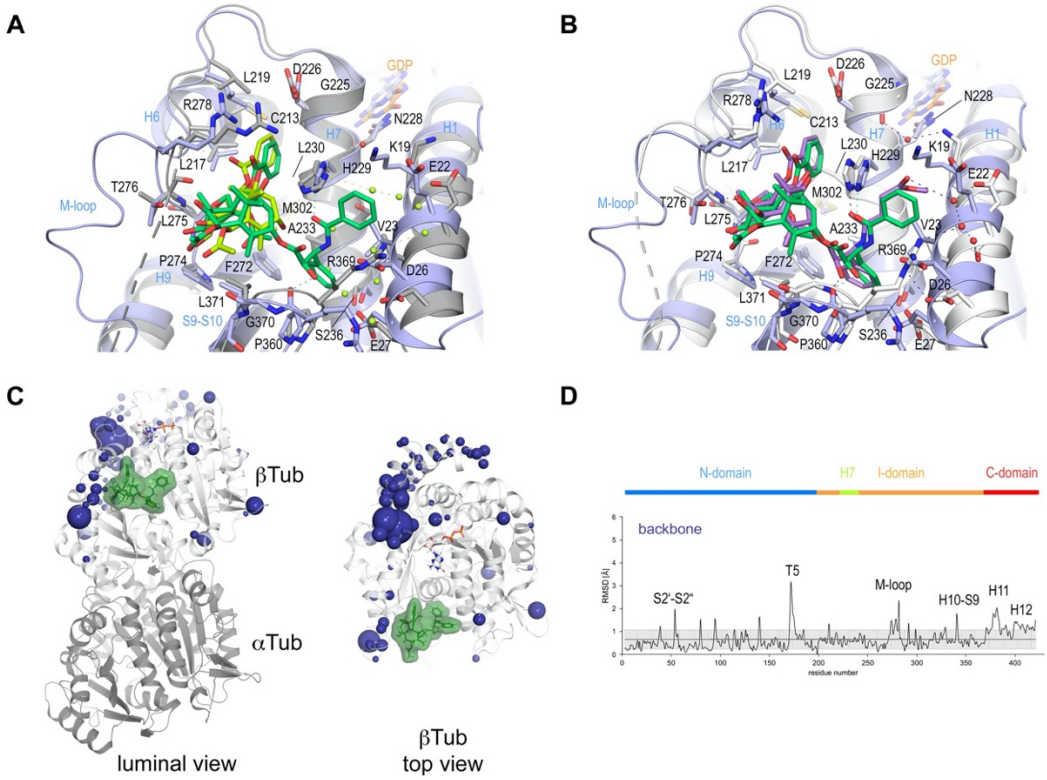


Figure 3. Comparison of taxane binding to unassembled curved versus assembled straight tubulin. (**A**) Close-up view of the superimposed baccatin III bound (ligand in lemon; protein in grey ribbon and sticks) to curved tubulin and paclitaxel bound to straight tubulin as found in a microtubule (PDB ID 6WVR; ligand in dark green; protein in slate ribbon and sticks) structures. Interacting residues of tubulin are shown in stick representation and are labeled. Oxygen and nitrogen atoms are colored red and blue, respectively. Hydrogen bonds are depicted as black dashed lines. Secondary structural elements of tubulin are labeled in blue. Water molecules belonging to the baccatin III structure are represented as lemon spheres. The structures were superimposed onto their taxane-sites (residues 208–219 + 225–237 + 272–276 + 286–296 + 318–320 + 359–376; rmsd 0.894 Å (52 C_α atoms). (**B**) Close-up view of superimposed **2a** bound to curved tubulin (ligand in violet; protein in grey ribbon and sticks) and paclitaxel bound to straight tubulin (PDB ID 6WVR; ligand in dark green; protein in slate ribbon and sticks) structures (rmsd 0.826 Å over 52 C_α atoms) using the same settings as in (**A**). (**C**) Conformational changes on β-tubulin induced by paclitaxel upon binding to straight tubulin in microtubules (PDB ID 6WVR). The α-tubulin and β-tubulin chains are in ribbon representation and are colored in dark and light grey, respectively. The rmsd differences between unbound and paclitaxel-bound straight tubulin are represented as dark (backbone rmsd) blue spheres. Only the rmsd-differences above a threshold of average ± standard deviation are displayed. The sphere-radii correspond to the average-subtracted rmsd-values displayed in panel (**D**). (**D**) Rmsd plots of backbone positions between the paclitaxel bound (PDB ID 6WVR) and the apo (PDB ID 6DPV) straight tubulin in microtubules. The grey error bar represents the average rmsd ± standard deviation. The top bar is colored according to the following domain assignment: N-terminal domain (N-domain., marine blue), intermediate domain (I-domain, orange), central helix βH7 (lemon) and C-terminal domain (C-domain, red). The β-tubulin chains of the corresponding structures were superimposed onto their β-tubulin N-terminal β-sheets (rmsd 0.304 Å over 30 C_α).

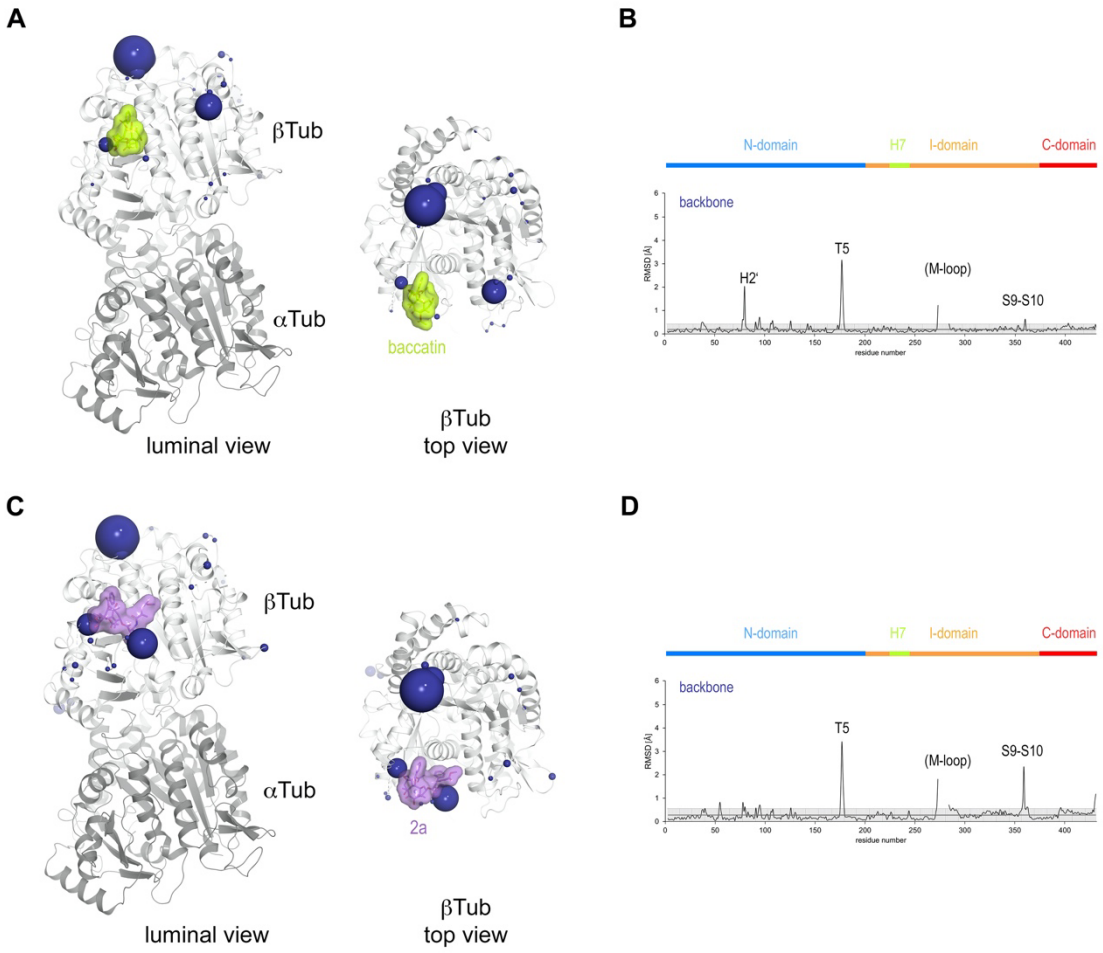


Figure 4. Conformational changes induced by taxane binding to unassembled, curved tubulin. (**A**) Conformational changes on the backbone atoms (dark blue) of the β-tubulin chain induced by baccatin III upon binding to curved tubulin. The tubulin chains are in ribbon representation and are colored in dark (α-tubulin) and light (β-tubulin) grey, respectively. The rmsd-values of the superimposed unbound and baccatin III bound curved tubulin are represented as dark blue (backbone rmsd) spheres, respectively. Only the rmsd-values above a threshold of average + standard deviation are displayed. The sphere-radii correspond to the average-subtracted rmsd-values displayed in panel (**B**). (**B**) Rmsd plots of the backbone (bottom) positions between the baccatin bound and the apo (PDB ID 4I55) curved tubulin state. The grey error bar represents the average rmsd ± standard deviation. The top bar is colored according to the following domain assignment: N-terminal domain (N-domain, marine blue), intermediate domain (I-domain, orange), central helix H7 (lemon), C-terminal domain (C-domain, red). The β-tubulin chains of the corresponding structures were superimposed onto their β-tubulin N-terminal β-sheet (rmsd 0.08 Å over 29 C_α). (**C**) Conformational changes on the backbone atoms (dark blue) of the β-tubulin chain induced by **2a** upon binding to curved tubulin. (**D**) Rmsd plots of the backbone (bottom) positions between the **2a** bound and the apo (PDB ID 4I55) curved tubulin state (rmsd 0.10 Å over 29 C_α). The same display settings as in (**B**) are applied.

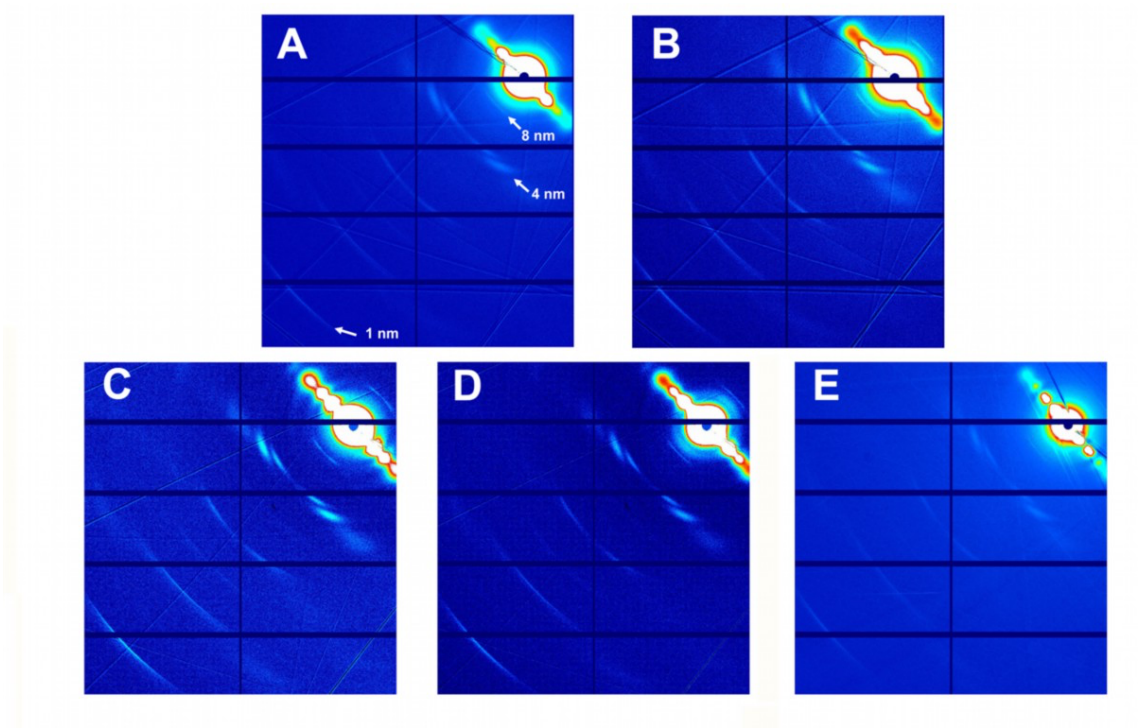


Figure 5.- Fiber diffraction patterns of microtubules. Microtubules assembled from GTP-tubulin and paclitaxel (**A**), GTP-tubulin and docetaxel (**B**), GTP-tubulin and **2a** (**C**), GTP-tubulin and **2b** (**D**), and GTP-tubulin and baccatin III (**E**) are shown.

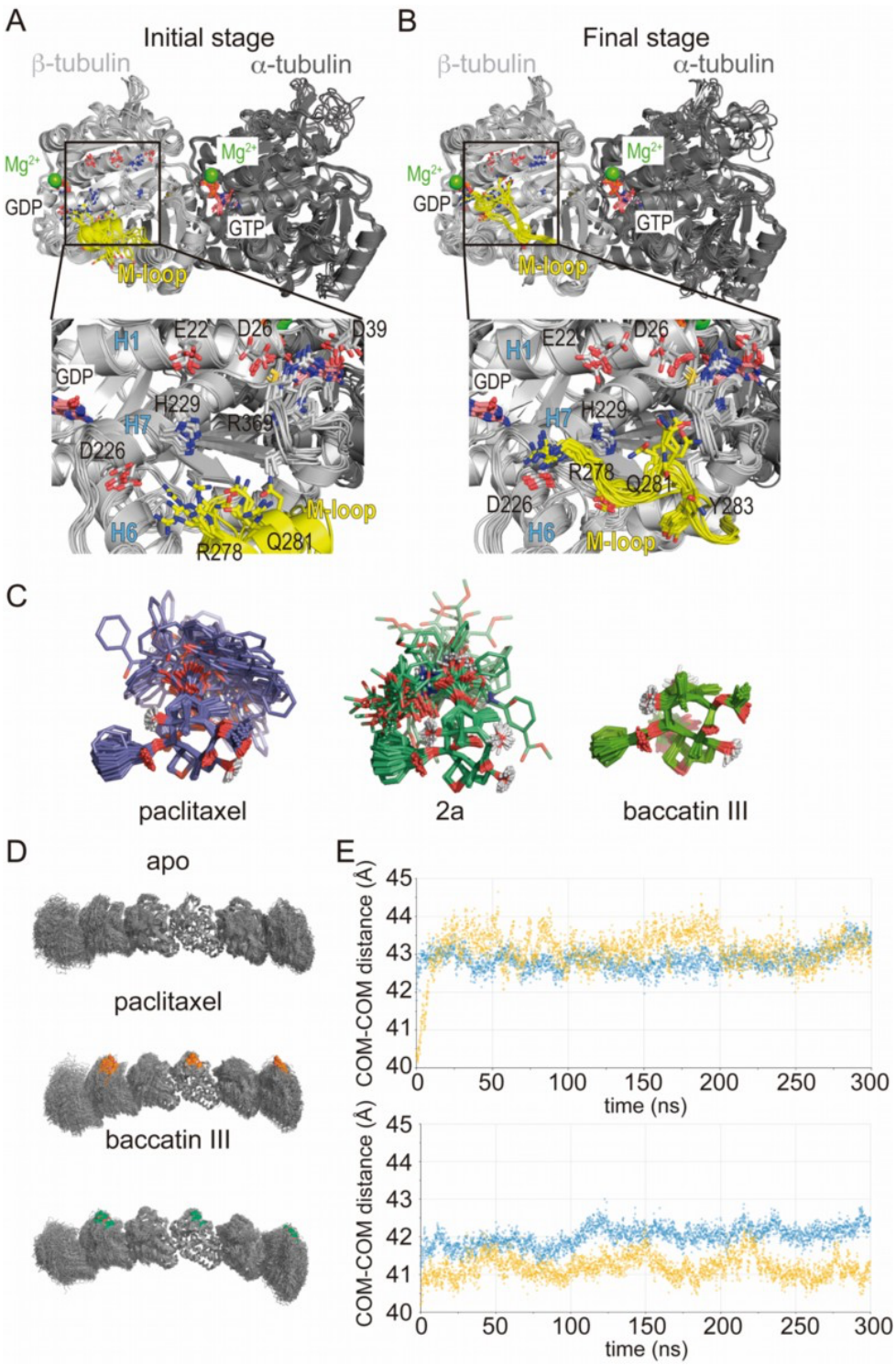


Figure 6.- **Molecular dynamics simulation of tubulin-taxane complexes. (A,B)** MD simulation of the free αβ-tubulin dimer. **(A)** Initial stage of the simulation, starting from a βM loop (residues β275-β286; yellow) organized as a α-helix akin to what is observed in a microtubule and **(B)** after 100 ns of a MD simulation. **(C)** Overlaid snapshots taken every 5 ns over the course of a 250-ns MD simulation of paclitaxel (left), **2a** (middle), or baccatin III (right). **(D)** Snapshots of the protofilament model bound to paclitaxel or baccatin III and apo form. **(E)** Time evolution of the intermonomer distances (measured between the respective centers of mass; α2-β2 in blue and β2-α3 in yellow) in the simulated apo and liganded protofilaments.

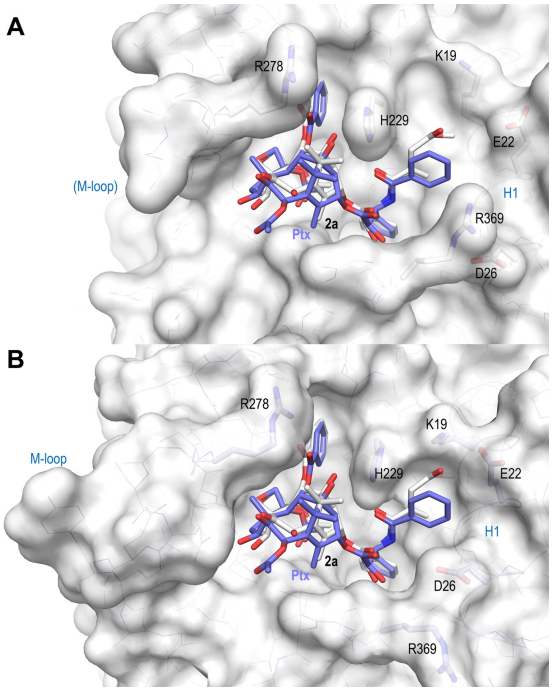


Figure 7. Surface representations of liganded taxane sites in both the curved and straight tubulin conformational states. (A) Curved tubulin; (B) straight tubulin. The structures of **2a** (white) and paclitaxel (slate) bound to microtubules (PDB ID 6WVR) were superimposed onto their central helices βH7. The side chains of the βM loop residue βR278 and of residues surrounding the C13 side chains of the ligands are in stick representation and are labeled. Helix βH1 is highlighted in ribbon representation.

1221 **Table 1 X-ray data collection and refinement statistics**
1222

	T_2R-TTL-BacIII	T_2R-TTL-**2a**	T_2R-TTL-**2b**
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions			
a, *b*, *c* (Å)	104.1, 157.2, 179.2	104.8, 157.9, 179.1	105.3, 158.6, 179.2
Resolution (Å)	49.2-1.9 (1.95-1.90)	49.3-1.95 (2.00-1.95)	49.4-2.35 (2.41-2.35)
R_{merge}*(%)*	10.7 (491.9)	13.3 (516.6)	17.4 (403.5)
R_{meas} *(%)*	11.1 (513.1)	13.6 (526.1)	17.7 (410.8)
R_{pim} *(%)*	3.3 (147.5)	2.9 (102.9)	2.6 (57.7)
I / σI	16.5 (0.5)	20.1 (0.7)	20.1 (0.9)
CC half	100 (17.8)	100 (31.4)	99.9 (46.6)
Completeness (%)	100 (99.8)	100 (100)	100 (100)
Redundancy	13.5 (12.4)	27.3 (27.8)	28.5 (28.3)
Refinement			
Resolution (Å)	49.2-1.9	49.3-1.95	49.4-2.35
No. unique reflections	229654	215774	125168
R_{work} / R_{free}	19.2 / 21.8	18.9 / 21.6	18.3 / 21.4
No. atoms			
Protein	17555	17389	17227
Ligand	42	120	
Water	861	883	166
Average *B*-factors (Å^2)			
Protein	59.0	62.9	76.1
Ligand (chain B / D)	n.a. / 109.2	111.4 / 102.8	146.6 / 144.9
Water	56.2	60.3	59.4
Wilson *B*-factor	41.7	43.1	56.9
R.m.s. deviations			
Bond lengths (Å)	0.003	0.003	0.002
Bond angles (°)	0.642	0.655	0.550
Ramachandran statistics			
Favored regions (%)	98.1	98.1	98.0
Allowed regions (%)	1.8	1.8	2.0
Outliers (%)	0.1	0.1	0

1223 *For each structure, data were collected from a single crystal. *Values in parentheses are
1224 for highest-resolution shell.
1225

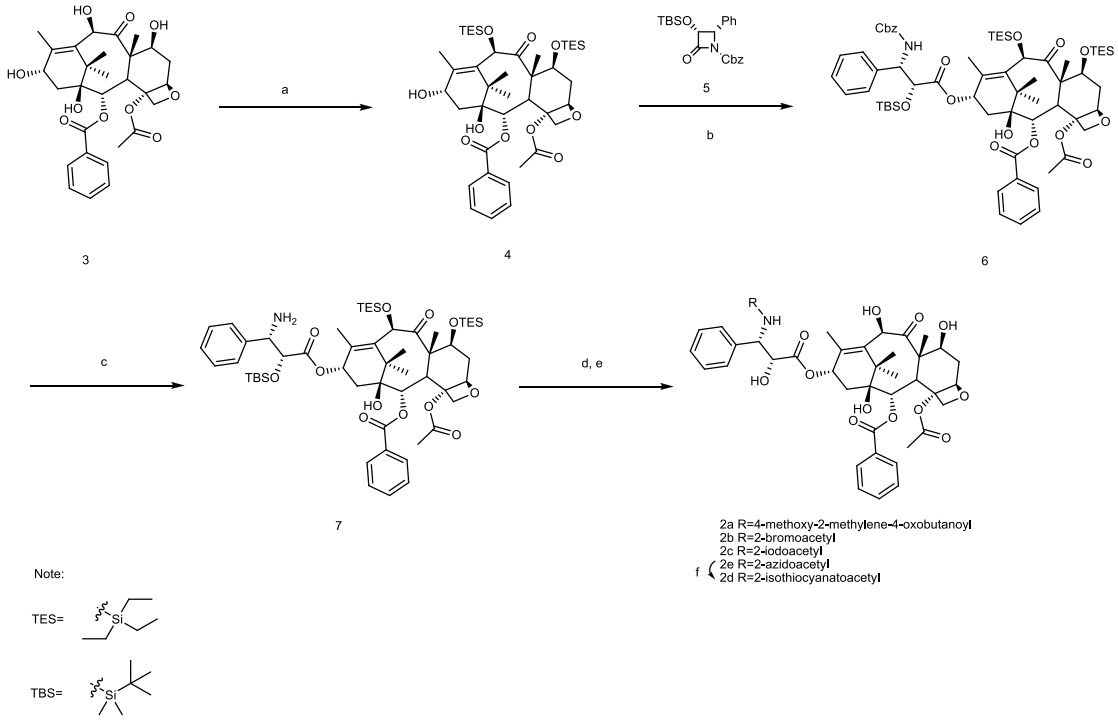
1226 **Table 2. Structural parameters of microtubules assembled in the presence of different nucleotides and drugs*.**

	paclitaxel-Pre microtubules	paclitaxel- Post microtubules	paclitaxel-GDP Tubulin	GDP-microtubules	GMPCPP-microtubules	Docetaxel-microtubules	Baccatin III -microtubules	2a-microtubules	2b-microtubules
microtubule radius (nm)	10.97±0.10	11.04±0.51	10.98±0.47	11.42±0.10	11.63±0.10	11.53±0.10	11.06±0.35	11.27±0.57	11.60±0.36
Avg. PF number	12.21±0.10	12.28±0.71	12.23±0.65	12.91±0.10	13.29±0.10	12.90±0.10	12.29±0.39	12.63±0.72	12.99±0.40
Inter-PF distances (nm)	5.58±0.01	5.59±0.33	5.57±0.29	5.50±0.03	5.45±0.03	5.57±0.01	5.61±0.18	5.55±0.31	5.56±0.17
Avg. monomer length (nm)	4.18±0.01	4.18±0.01	4.18±0.01	4.06±0.01	4.18±0.010	4.18±0.01	4.16±0.03	4.15±0.03	4.13±0.03
1 nm band peak position (nm^{-1})	6.02±0.01	6.02±0.01	6.02±0.01	6.19±0.01	6.02±0.01	6.02±0.01	6.04±0.5	6.06±0.05	6.08±0.05
8 nm band	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes

1227

1228 *Errors are SE of three independent

1229



Scheme 1. Reagents and conditions: (a) TESCl, TEA, DMAP, LiBr, THF, r.t. to 70 °C, 84%; (b) LHMDS, THF, -45 °C, 79%; (c) 10% Pd/C, H_2, MeOH, 64%; (d) DCC, DMAP, acid, DCM, 0 °C to r.t.; (e) HF, Py, CH_3CN or 5% HCl/MeOH, 32% for **2a**, 40% for **2b**, 48% for **2c** and 83% for **2e** for two steps (d, e); (f) PPh_3, CS_2, THF, 83%.

Movie legends

Movie M1. Conformational transition from apo to baccatin III bound, unassembled tubulin state. Top view on β-tubulin (onto the "plus end" in the context of a microtubule).

Movie M2. Conformational transition from apo to baccatin III bound, unassembled tubulin state. Luminal view on β-tubulin (view from the lumen in the context of a microtubule).

Movie M3. Conformational transition from apo to **2a** bound, unassembled tubulin state. Top view on β-tubulin (onto the "plus end" in the context of a microtubule).

Movie M4. Conformational transition from apo to **2a** bound, unassembled tubulin state. Luminal view on β-tubulin (view from the lumen in the context of a microtubule).

Movie M5. MD movie of the apo tubulin dimer showing the occupancy of the taxane site by the βM loop. 1 μs simulation, 1 snapshot every 5 ns, βM loop in yellow.

Movie M6. Simulation of ligand exit and entry using targeted MD for baccatin III unbinding from and binding to αβ-tubulin.

Movie M7. Simulation of ligand exit and entry using targeted MD for paclitaxel unbinding from and binding to αβ-tubulin.

Supplemental Figures

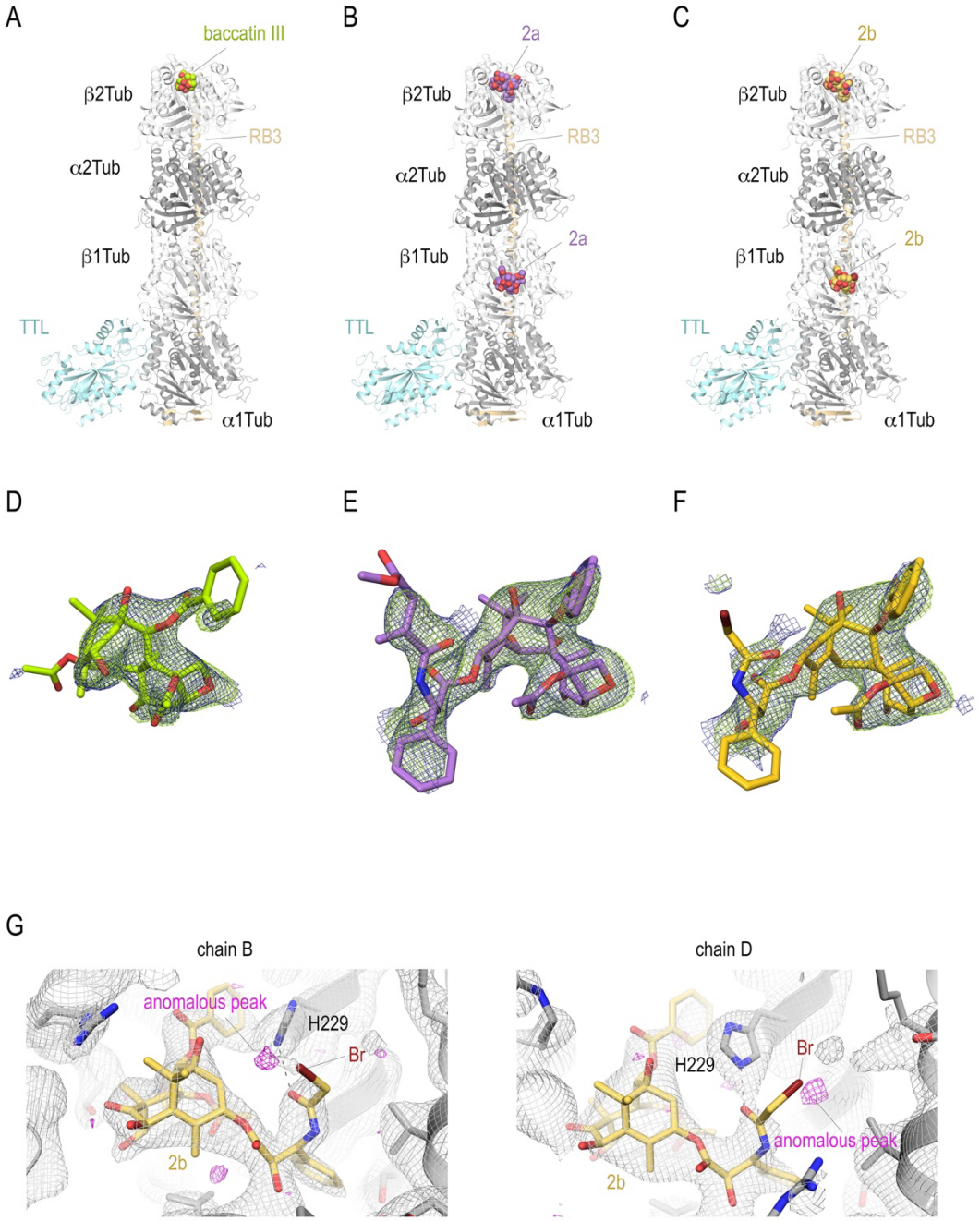


Figure S1. T_2R-TTL structures in complex with baccatin III, 2a, and 2b. Overall view of the T_2R-TTL-baccatin III **(A),** the T_2R-TTL-**2a** and **(B)**, and the T_2R-TTL-**2b** crystal structures. The α- and β-tubulin chains are colored in dark and light grey, respectively. The TTL chains (cyan) and the RB3 (yellow-orange) are shown in ribbon representation. The tubulin-bound ligands are displayed as spheres and are colored following the same color scheme as in the main figures. (**D-F**) Electron-density maps highlighting the bound baccatin III, **2a,** and **2b.** The SigmaA-weighted 2mFo - DFc (dark blue mesh) and mFo - DFc (light green mesh) omit maps are contoured at +1.0σ and +3.0σ, respectively. The map calculations excluded the atoms of the corresponding ligands. (**G**) Anomalous density peaks detected in both the binding sites in chains B and D of T_2R-TTL for the bromine moiety of compound **2b**.

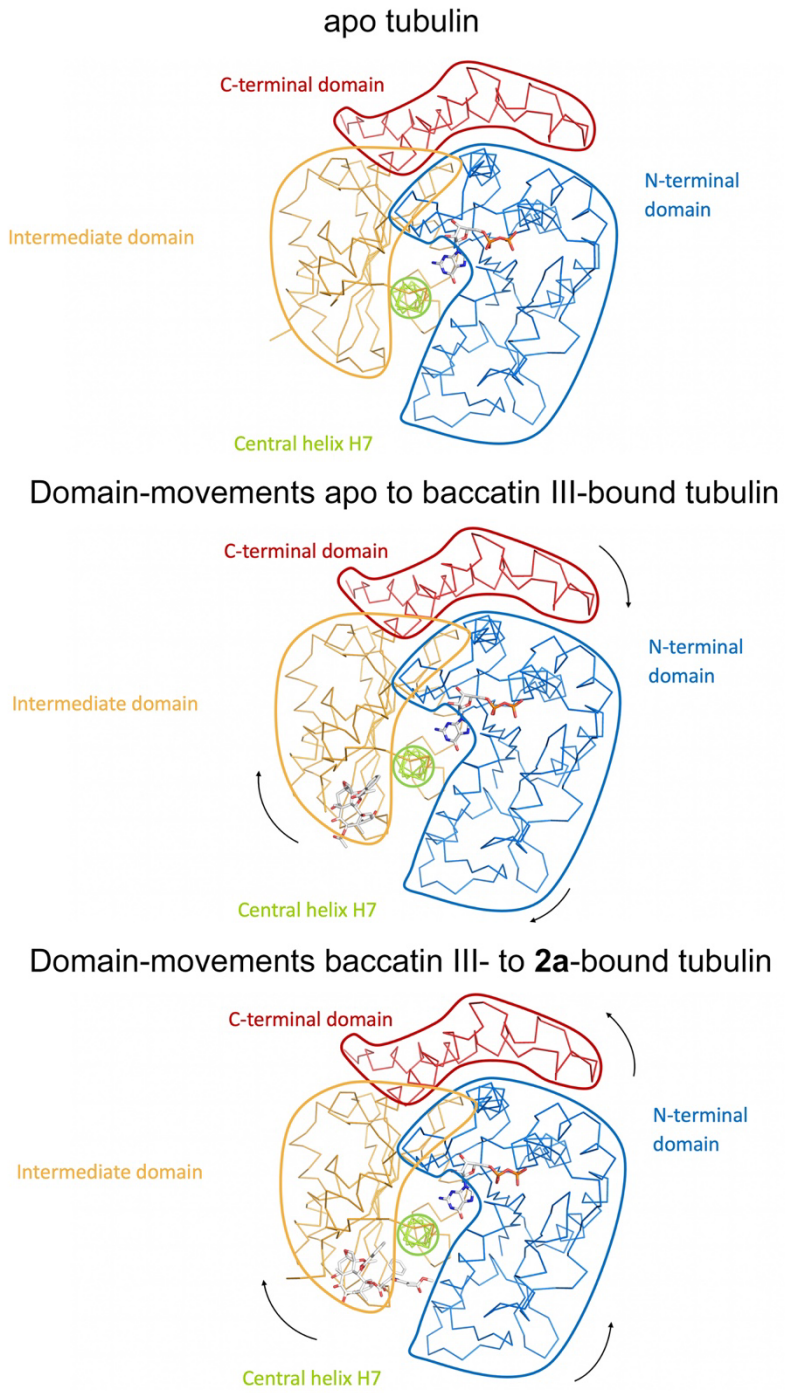


Figure S2. Schematic representation of subtle domain movements observed from apo to baccatin III- to 2a-bound curved tubulin. The three structures were superimposed onto their central helices βH7 to highlight better the subtle domain movements relative to each other. The individual domains are colored according to their domain assignment and their borders are contoured using the same color scheme: N-terminal domain (N-domain, marine blue), intermediate domain (I-domain, orange), central helix βH7 (lemon), C-terminal domain (C-domain, red). The directions of the individual movements are highlighted with black arrows.

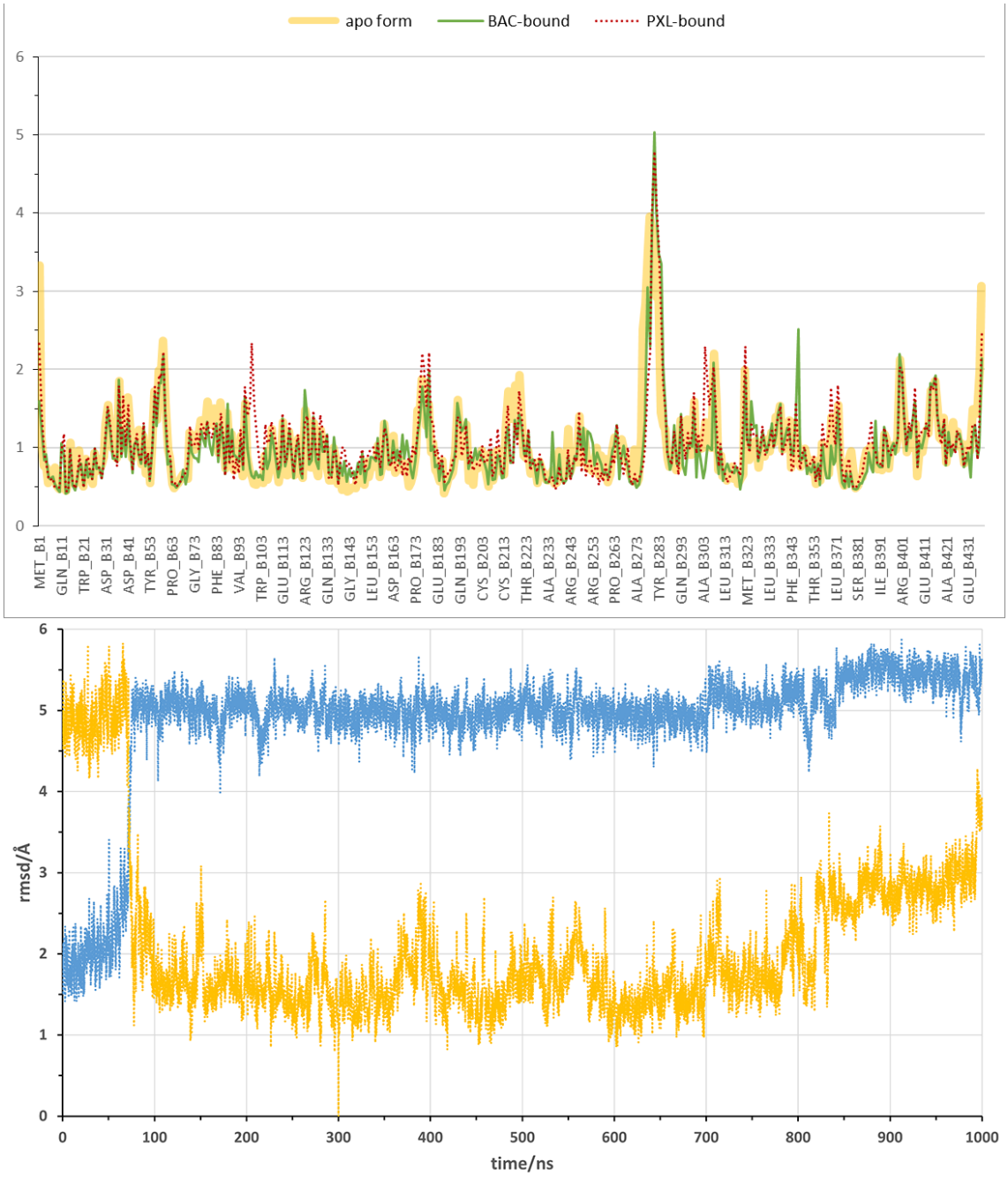


Figure S3. Flexibility of β subunit and βM loop during the αβ-tubulin dimer MD simulation. (Top) Mass-weighted positional fluctuations (or root-mean-square fluctuations, Å) by residue for atoms in the β subunit of the αβ-tubulin dimer over the course of 0.6 μs of MD simulation, in the apo form (yellow line) and in complex with baccatin III (green line) or paclitaxel (red dotted line). **(Bottom)** Evolution of the conformation of the βM loop in the 1.0 μs simulation of the αβ-tubulin dimer free in solution. The Cα root-mean-square deviation is measured with respect to either the initial α-helical structure (blue line) or the extended hairpin conformation that was stabilized at 300 ns (orange line).

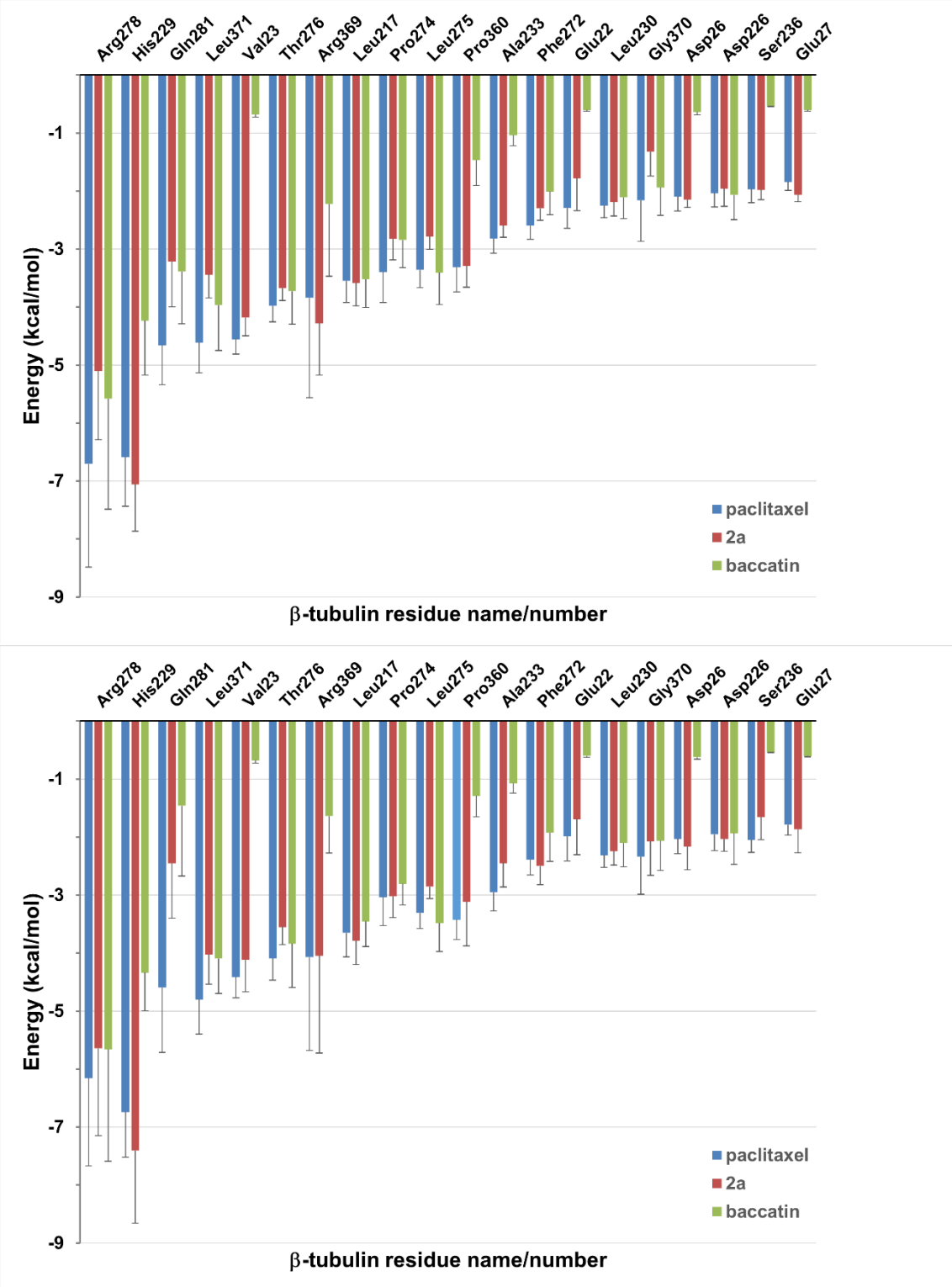


Figure S4.- Solvent-corrected interaction energies between individual β1-tubulin residues and ligands throughout the MD simulations of the minimalist representation of a microtubule. (**A**) The interfacial site 1 between neighboring protofilamentes. (**B**) The solvent-exposed site 2. These per-residue energies, which together represent a "binding fingerprint", were calculated by means of the program MM-ISMSA (*75*) using 120 complex structures from the MD simulations after equilibration (5-600 ns), cooling down to 273 K and energy minimization. A cut-off of 1.5 kcal mol^{-1} was used in the plot for enhanced clarity. Bars are standard errors.

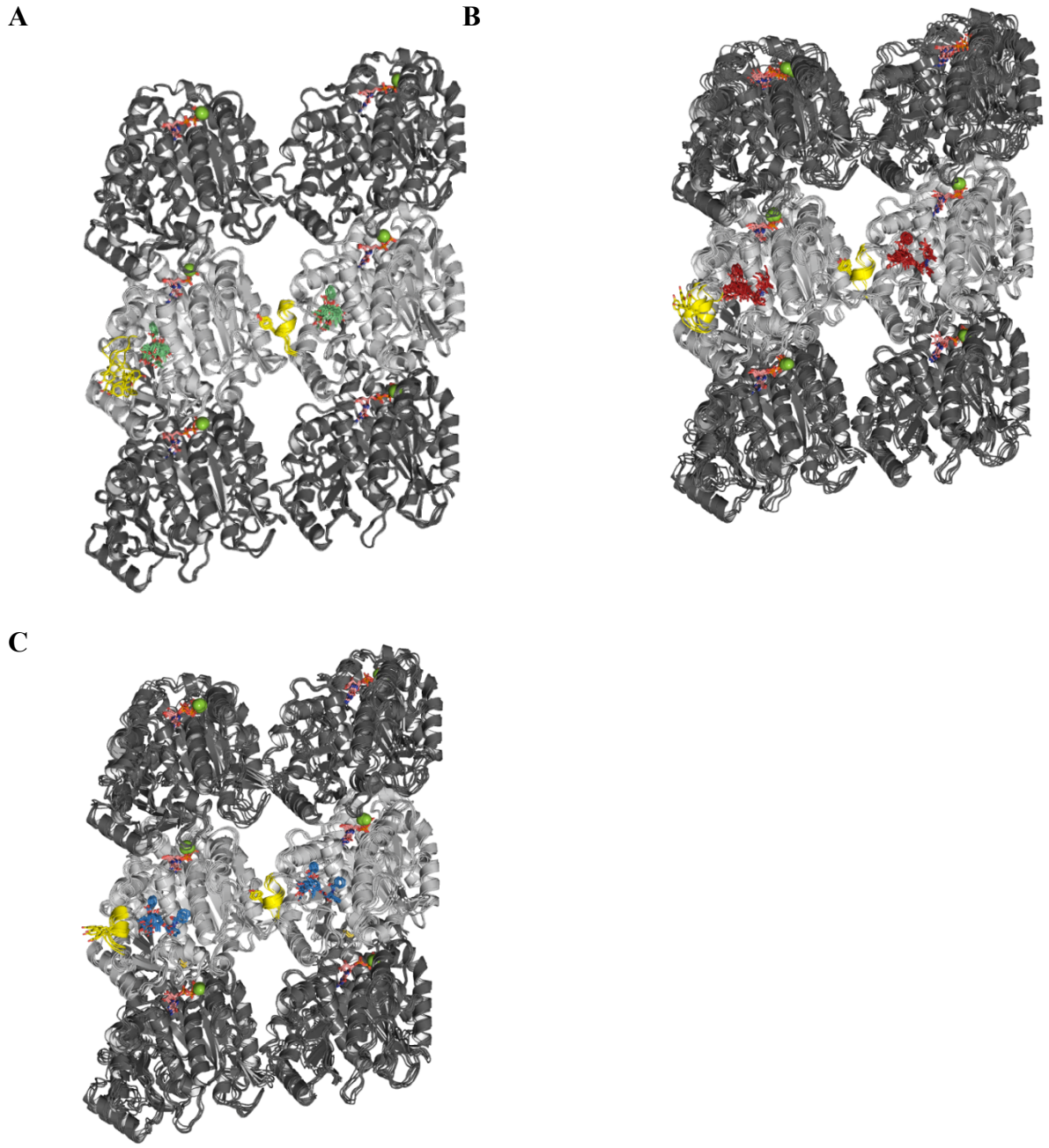


Figure S5.- MD simulations of minimalist representations of a microtubule ((α$_1$-β$_1$-α$_2$)/(α$_{1'}$-β$_{1'}$-α$_{2'}$)) in complex with baccatin III (green, A), 2a (red, B), or paclitaxel (blue, C). α- (dark grey) and β-tubulin (light grey) are displayed as ribbons, with the βM loop colored in yellow and the side chain of Tyr283 as sticks. GDP and GTP are shown as sticks, with C atoms colored in salmon. Mg^{2+} ions are displayed as green spheres. Each set of five overlaid structures represents a conformational ensemble made up of snapshots spaced by 5 ns taken from the equilibrated part of the trajectory and then cooled down to 273 K and energy minimized. Site 1 (at the top of each figure) is located at the interface between two neighboring protofilaments whereas site 2 (at the bottom of each figure) is devoid of any lateral contacts but exposed to the bulk solvent instead.