Crystal structures of taxane-tubulin complexes: Implications for the mechanism of microtubule stabilization by Taxol

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Abstract

Paclitaxel (Taxol®) is a first-line chemotherapeutic drug that promotes the curved-to-straight conformational transition of tubulin, an activation step that is necessary for microtubule formation. Crystallization of Taxol bound to tubulin has been long elusive. We found that baccatin III, the core structure of paclitaxel which lacks the C13 side chain, readily co-crystallizes with curved tubulin. Tailor-made taxanes with alternative side chains also co-crystallized, allowing us to investigate their binding modes. Interestingly, these Taxol derived compounds lost their microtubule stabilizing activity and cytotoxicity but kept their full microtubule binding affinity, and all induced lattice expansion upon binding. Additional nuclear magnetic resonance studies propose that Taxol binds to a small fraction of straight tubulin present in solution. Our results suggest a mode of action of Taxol, where the core structure is responsible for the interacting energy while the bulky hydrophobic C13 side chain enables binding selectively to straight tubulin and promotes stabilization.

50 **Introduction**

51 Taxanes are the main choice among other chemotherapeutic agents for the
52 treatment of several solid tumors such as ovarian, lung and breast cancer, as well as
53 advanced Kaposi's sarcoma(*1*). The three active taxanes in clinical use, paclitaxel
54 (Taxol®; Ptx), docetaxel (Taxotere®; Dcx) and cabazitaxel (Jevtana®), are part of a big
55 family of chemically diverse compounds that bind to the taxane site of the αβ-tubulin
56 heterodimer(*2*) **(Fig. 1A)**. This family of compounds also includes other non-taxane
57 molecules and a wide range of analogs derived thereof. Because taxane-site targeting
58 compounds promote tubulin assembly into microtubules (MTs)(*3*), they are called MT-
59 stabilizing agents (MSAs). From the thermodynamic point of view, taxane-site MSAs
60 contribute to MT assembly by their preferred binding to them(*4*). Tubulin displays two
61 known conformations, related to its assembly state: a straight conformation (s-tubulin)
62 present in MTs and zinc-induced sheets(*5, 6*), and a curved conformation (c-tubulin)
63 observed in unassembled tubulin, which is non-related to the nucleotide bound to the
64 regulatory site of β-tubulin (GTP or GDP)(*7, 8*). The curved-to-straight conformational
65 transition is required for MT-assembly. Current hypotheses(*9-11*) establish that the
66 activation of tubulin for assembly could be accomplished by different pathways and each
67 MSA could favor a specific one, depending on its structure and its interaction pattern with
68 unassembled, c-tubulin. Structural studies of taxane-site agents(*10, 12*) bound to MTs or
69 unassembled tubulin have shed light on the binding mode to s- and c-tubulin tubulin,
70 respectively. Nevertheless, their underlying mechanisms of action are still a matter of
71 debate. On one hand, zampanolide and epothilone induce the complete restructuring of
72 the βM-loop in unassembled, c-tubulin **(Fig. 1B)**, which is a key secondary structural
73 element required for the stabilization of lateral contacts between protofilaments (PF)
74 within the MT(*12*). These compounds mainly connect the base of the βM-loop with both
75 the N-terminal domain helix βH6 and the central helix βH7 (**Fig. 1B**), similarly to
76 dictyostatins and discodermolides(*9, 13*). However, neither dictyostatins nor
77 discodermolides fold the βM-loop into a helix, which suggests a different mechanism.
78 The interaction of Ptx with MTs affects the longitudinal tubulin interface along
79 protofiliaments (PFs), increasing the axial repeat of tubulin dimers (i.e., causing a lattice
80 expansion)(*10*). The stabilizing effect promoted in assembled, s-tubulin involves subtle
81 movements in the N-terminal domain helix βH1, the central helix βH7 and the
82 intermediate domain loop βS9-βS10 (**Fig. 1B**)(*10*). However, the molecular details of

how Ptx favors these movements and promotes MT assembly and stabilization are unknown yet.

Ptx is one of the drugs in the World Health Organization's List of Essential Medicines, but the appearance of side effects (e.g., neurotoxicity) compromises chemotherapy cycles during cancer treatment. Understanding the underlying mechanism of MT stabilization by taxanes is highly required to lay the groundwork for future and safer drugs. To date, all efforts to obtain high-resolution crystal structures and hence, detailed information on the Ptx, Dcx or cabazitaxel interacting network within the taxane-site have been unssucessful(*7, 8, 14*). Here, we have taken a multidisciplinary approach to unveil how Ptx recognizes the taxane site and stabilizes MTs. From the Ptx core structure (baccatin III) to specifically tailored Ptx derivatives with alternative engineered C13 tails, we have combined macromolecular crystallographic high-resolution structures with biochemical, cell biology, nuclear magnetic resonance (NMR) and X-ray fiber diffraction studies to explain how clinically used taxanes induce MT assembly from c-tubulin and how they stabilize MTs.

Results

The bulky group at ring A position of the C13 side chain precludes binding to curved conformation and is essential for activity.

To determine the high-resolution structure of a taxane bound to tubulin we performed both soaking and co-crystallization experiments using the previously described crystal system consisting of a protein complex composed of two αβ-tubulin heterodimers, the stathmin-like protein RB3 and the tubulin tyrosine ligase (termed T_2R-TTL)(*12, 14*) as well as with the DARPin D1-tubulin crystal system (TD1)(*8*). Upon failure with a first series of tested taxanes comprising Ptx, Dcx, the more soluble 3'N-aminopaclitaxel (N-AB-PT, **Fig. 1C**)(*15*) and the engineered high affinity taxanes Chitax 40(*16*) and Chitax 68(*17*) (**Fig. 1C**), we approached the challenge from a different angle. We started with baccatin III, a precursor in the biosynthesis of Ptx that contains both the C2-benzoyloxy ring C and the C10 acetate ester, but lacks the C13 side chain with both the 3'-N-benzamido phenyl ring A and the 3'-phenyl ring B moieties(*18*).

Baccatin III (**Fig. 1C**) is reported to be biologically inactive(*19-21*) despite of having μM affinity for MTs(*22*) (ref. 22 describes a weak *in cell* activity as well). It is

116 known that taxanes bind with high affinity to assembled s-tubulin(*23*) while having
117 undetectable affinity for unassembled c-tubulin(*4*). We found that baccatin III shows a
118 detectable affinity ($Kb_{25°C}$ 3.0±0.5x10^3 M^{-1}) to unassembled c-tubulin (**Fig. 3C)**, in the
119 same range as the ones previously measured for other compounds able to bind and co-
120 crystallize with tubulin, such as epothilone A 0.8±0.3×10^4 M^{-1} (*24*) and discodermolide
121 2.0±0.7×10^4 M^{-1} (*25*). Therefore, we hypothesized that the presence of the C13 side chain
122 of the taxanes aforementioned might preclude the binding to the c-tubulin form present
123 in both the T_2R-TTL and the TD1 complexes. Subsequently, we obtained a T_2R-TTL-
124 baccatin III complex structure at 1.9Å resolution (**Fig. 2A, Fig. S1A & D, Table S1**)
125 (PDB ID 7A7I) and found that baccatin III binds to the taxane site with its C2-benzoyloxy
126 ring C stacked between the side chains of βH229 and βL275 in the leucine rich pocket
127 formed by the side chains of βC213, βL217, βL219, βD226, βH229, βL230 and βL275.
128 Its carbonyl oxygen forms a weak hydrogen bond to the main chain amide of βR278 (**Fig.
129 2A**). The C10 acetate is exposed to the solvent and together with the C12 methyl is in
130 Van der Waals distance to βG370 of the βS9-βS10 loop. Furthermore, the oxetane oxygen
131 and the C13 hydroxyl form hydrogen bonds to the main chain amide of βT276 and to
132 βH229 NE2, respectively. The C4 acetate is buried in the hydrophobic pocket formed by
133 βL230, βA233, βF272, βP274, βL275, βM302, βL371 and the aliphatic portion of the
134 βR369 side chain. Strikingly, we only found well-defined electron density for the amino-
135 terminal section of the βM-loop up to βR278, while the remaining portion of the βM-
136 loop was disordered. This suggests that baccatin III does not promote the stabilization of
137 this region into a helical conformation, in contrast to what has been previously observed
138 for both the taxane-site binders zampanolide and epothilone A(*12*).

139 Aiming to understand the implication of Ptx's C13 ring A moiety (or its equivalent
140 tert-butyl in Dcx) on tubulin activation and to understand why it precludes binding to c-
141 tubulin, we took a synthetic effort to obtain new taxane ligands with modified side chains
142 able to crystallize in complex with c-tubulin. We produced a series of modified taxanes
143 bearing smaller groups at the ring A position of Ptx: acrylamide (**2a**), haloacetamide (**2b,
144 2c**) and isocyanate (**2d**) (**Fig 1C**). Since we measured binding affinity of **2a** to
145 unassembled tubulin dimers ($Kb_{25°C}$ 0.8±0.3x10^3 M^{-1}) (**Fig. 3C),** but not of N-AB-PT(*15*),
146 Chitax 40(*16*) or Chitax 68(*17*), we concluded that the new modification applied to the
147 Ptx structure increases the binding of the compounds for the unassembled c-tubulin. In
148 fact, we unequivocally found difference electron densities at the taxane sites of T_2R-TTL

149 crystals soaked with **2a** (**Fig S1B, E**) (PDB ID 7A7F) and **2b** (**Fig S1C, F**) (PDB ID
150 7A7J). The corresponding structures were refined to 1.95 Å and 2.35 Å resolution,
151 respectively. Although these densities displayed continuity between the 3'-N moieties of
152 these ligands and the side chain of βH229, covalent adduct formation was discarded both
153 biochemically **(see extended data)** and crystallographically with the help of anomalous
154 data collected from a T_2R-TTL-**2b** crystal at the bromine peak wavelength (0.91501 Å,
155 **Fig S1G**).

156 The structure showed that compound **2a** forms comparable interactions with both
157 the C2-benzoyloxy ring C and the oxetane moieties as described for baccatin III (**Fig.**
158 **2B,C**). However, the core ring is tilted towards helix βH6 and strand βS7 by 18.5˚ (angle
159 between the two C1-C9 axis**;** $rmsd_{bacIII-2a}$ of 0.794 Å for 39 core atoms), adopting a pose
160 that is closer to that observed for Ptx bound to s-tubulin (PDB ID 6WVR; $rmsd_{2a-Ptx}$ of
161 0.845 Å for 56 core atoms **Fig. 4B;** $rmsd_{bacIII-Ptx}$ of 1.048 Å for 42 core atoms **Fig. 4B**).
162 Similar to Ptx bound to s-tubulin, the C39 carbonyl of the C13-3'-N-acrylamide moiety
163 of **2a** forms an equivalent hydrogen bond to the NE2 of βH229 (**Fig. 2B, 4B**). The
164 acetamide moiety is exposed to the solvent and forms water-mediated hydrogen bonds to
165 the side chains of βE22 and βR369, a space that is otherwise occupied by water molecules
166 in the baccatin III structure. In the context of Ptx-bound MTs the 3'-N-benzamido phenyl
167 ring A moiety of Ptx occupies this space and the βD26 side chain replaces the βR369 side
168 chain, which adopts a flipped-out conformation (PDB ID 6WVR, **Fig. 2B, Fig. 4B**). The
169 absence of the C10 acetate in **2a** compared to baccatin III has no impact on the
170 orientations and conformations of both the βS9-βS10 and the βM-loops, which are
171 closely similar in both structures (**Fig. 2C**). In both structures, we found the βT5-loops in
172 the "out" conformation.

173 Interestingly, we found that the replacement of ring A by an acrylamide moiety
174 has a deep impact in the compound's activity showing a four orders of magnitude lower
175 cytotoxicity than Ptx in KB and HeLa tumoral cells (**Table 1**). Biochemically **2a** and **2b**
176 behaved as weaker assembly inducers compared to Ptx (PEDTA buffer, **Fig. 3AB**).
177 Because both the tubulin assembly induction power and the cytotoxicity of MT stabilizing
178 agents is strongly linked to the binding affinity for MTs(*13, 23, 26*) we expected a
179 strongly compromised ability to bind MTs for **2a** and **2b**. Surprisingly, that was not the
180 case and the binding constants of **2a** ($0.46 \pm 0.06 \times 10^7$ M^{-1}) and **2b** ($0.32 \pm 0.03 \times 10^7$ M^{-1})
181 to MTs were similar to the one observed for Ptx ($1.43 \pm 0.17 \times 10^7$ M^{-1} (*23*)). Furthermore,

182 also baccatin III (inactive compound) displayed a significant binding affinity for
183 assembled MTs $1.5x10^5M^{-1}$ (*22*), which is even higher than that of other taxanes with
184 detectable activity(*16*). Therefore, we concluded that the increase of binding affinity for
185 the curved conformation (and the subsequently reduction of the preferential binding for
186 the straight one) could be linked to decreased MT stabilizing activity and cytotoxicity.

187

188 *Ptx binds to the straight conformation of unassembled tubulin in solution.*

189 To obtain information about the transient Ptx/unassembled tubulin interactions in
190 solution we approached TR-NOESY experiments. Surprisingly, despite both the low
191 solubility of Ptx and the reported very low binding constant of Ptx to unassembled,
192 c-tubulin ($< 10^3$ M^{-1})(*4*), we found weak but clear signals in the spectra (**Fig. 5C**). In the
193 presence of MTs, the spectra displayed strong signals (**Fig. 5D**), as expected(*25*). As a
194 control we acquired the NOESY spectrum of Ptx in the absence of tubulin, no peaks were
195 detected (not shown) indicating that all the signals observed in the presence of tubulin are
196 TR-NOESY ones. Strikingly, the correlation between both spectra was almost perfect
197 (the observed displacement in the position of the cross peaks is due to the different
198 temperature at which the spectra were recorded 298 K for unassembled tubulin vs 310 K
199 for MTs), suggesting that the conformation of Ptx was similar when bound to
200 unassembled, c-tubulin (Kb $< 10^3$ M^{-1}) or assembled s-tubulin (Kb 10^7 M^{-1})(*23*). Instead,
201 previous studies with epothilone and discodermolide revealed differences on the bound
202 conformation depending on the tubulin assembly state(*24, 25*). The quantitative analysis
203 of the TR-NOESY spectra (Table S2), indicates that the conformation adopted by Ptx in
204 solution (**Fig. 5A**) is nearly coincident with this determined by cryo-EM. Considering
205 that Ptx binding to unassembled, c-tubulin is not detected either by biochemical methods
206 nor by macromolecular crystallography, we assumed that the equal conformation of Ptx
207 bound to assembled and unassembled tubulin could be attributed to the observation of
208 Ptx bound to a small fraction of s-tubulin (**see discussion and extended discussion**).

209 To further characterize the ligand binding epitope and to probe the pharmacophore
210 region of Ptx bound to tubulin, we acquired STD-NMR spectra of tubulin preparations
211 obtained under unassembled and assembled conditions. Similar to what has been
212 described for Dcx(*25*), we found STD signals under both studied conditions (assembled
213 and unassembled tubulin with very similar profiles **(Fig. S3)**, denoting a similar binding
214 mode of Ptx under both conditions. However, experiments performed with **2a** under

215 unassembled and assembled tubulin conditions **(Fig. S4)** showed subtle differences in the
216 C13 side chain section corresponding to the acrylamide moiety that replaces the aromatic
217 ring A in Ptx, thereby suggesting two distinct interaction patterns of this moiety
218 depending on the tubulin conformation. Additionally, similar STD profiles of baccatin III
219 bound to unassembled and assembled tubulin **(Fig. S5)** were found, as expected, because
220 this compound lacks the C13 side chain responsible for the differential interaction for c-
221 and s-tubulin. Together, these results support that the weak signals of Ptx bound to
222 unassembled tubulin arise from the compound bound to the small proportion of
223 unassembled s-tubulin in equilibrium with c-tubulin.

224

225 *C13 side chain promoted perturbations in unassembled c-tubulin, strengthen on straight*
226 *MTs.*

227 To understand the contribution of the C13 side chain to the observed differences
228 in biochemical and biological activity and in the NMR-experiments, we further
229 investigated the perturbations induced by binding of baccatin III and **2a** to the
230 unassembled c-tubulin β chains. To this aim, we first superimposed the structures of apo
231 (PDB ID 4I55), baccatin III and **2a** bound states onto the N-terminal β-sheets of β-tubulin
232 (residues 3-9, 63-66, 132-138, 163-169 and 198-202; $rmsd_{BacIII}$ 0.08 Å of 29 C_α; $rmsd_{\mathbf{2a}}$
233 0.10 Å of 29 C_α) and calculated the rmsd-differences between both the apo and the taxane
234 bound states using the rmsdByRes function in PyMOL (**Fig. 6B, D**). The rmsd-
235 differences were also plotted and mapped onto the corresponding structures to highlight
236 the major regions of perturbation (**Fig. 6A, C**). Comparable perturbations were observed
237 for backbone and all atoms in the helix βH2', the βT5-loop, the βM-loop basis, the helix
238 βH9, and both the helix βH11-βH11' and βH12 regions of both the ligands, while the
239 rmsd-values for both the βS2'-βS2" and the βS9-βS10 loops were higher for **2a**. To
240 investigate the effect of the observed perturbations on the relative domain arrangements
241 in β-tubulin of the individual complexes, we further superimposed the β-tubulin chains
242 onto their central helices βH7. For baccatin III, a mild relative twist between the N-
243 terminal and the intermediate domains was observed (**Fig. S2**; **Suppl. Movie M1, M2**),
244 while binding of **2a** rather caused both the N-terminal and intermediate domains of the
245 β-tubulin molecule to slightly move apart (**Fig. S2; Suppl. Movie M3, M4**).

246 Since these subtle movements were observed in the c-tubulin conformations in the
247 context of a crystal lattice, we sought to compare them to the perturbations that occur
248 upon Ptx binding in the context of MTs. To this aim, we performed the same type of
249 analysis by superimposing the N-terminal β-sheets of β-tubulin from the cryo-EM
250 reconstruction of Ptx-bound GDP-MTs (PDB ID 6WVR)(*27*) onto the corresponding
251 domains of the undecorated apo GDP-MT structure (PDB ID 6DPV; rmsd 0.304 Å 30
252 C_{α})(*28*). The rmsd-analysis of Ptx bound MTs revealed comparable but more pronounced
253 perturbations to the ones observed for **2a** for the βS2'-S2", the βH3, the βS9-βS10 and
254 the βH11-βH12 regions, with a more prominent effect on the βM-loop (**Fig. 4C, D**).
255 These observations suggested that perturbations induced by the C13 side chain may have
256 an impact on stabilizing the MT-lattice, since they occur at positions that contribute to
257 lateral contacts in MTs, which is in agreement with the results published by Debs and
258 co-workers(*27*), where Ptx-bound MTs displayed higher curvature and distinct βM-loop
259 movements.

260

261 *C13 side chain is not required for lattice expansion but affects lateral contacts in MTs.*

262 The main structural effect of Ptx on the MT lattice is a longitudinal expansion that
263 have been related to the stabilization effect of this compound(*29, 30*). We have previously
264 validated X-ray fiber diffraction of shear-flow aligned MTs as an accurate technique to
265 discern main MT lattice structural features(*31, 32*) and, we used it to elucidate how our
266 Ptx derivatives with reduced activities affect the structure of MTs.

267 The meridional diffraction patterns are related to axial helical repetitions on the
268 MT lattice and show a layer line at 4 nm (**Fig. 7**) and, when the lattice is expanded, a
269 second weaker layer line at 8 nm due to the length differences between α- and
270 β- tubulin(*31*). Additionally, the position of the 1 nm layer line (4th harmonic of the 4 nm
271 layer line) also moves towards the center of the image when lattice expansion occurs.
272 Both, Ptx (**Fig. 7A**) and Dcx (**Fig. 7B**) induced MTs expansion, as expected(*29*). And this
273 expansion occurred in either case, when the addition of the drug was previous to assembly
274 into MT using GTP-bound or GDP-bound tubulin (named "pre-" in **Table S3**) or when
275 added directly to assembled MTs (named "post-" in **Table S3**). This was in clear
276 opposition to previous observations in which expansion was only observed in
277 pre-addition experiments(*10*). Interestingly, **2a** (**Fig. 7C**), **2b** (**Fig. 7D**) and baccatin III

278 (**Fig. 7E**) showed similar MT expanded lattices indicating that the lattice expansion is not
279 related with taxane's MT stabilizing activity. Note that the diffraction patterns of MTs
280 stabilized with **2a** or **2b** showed a diffuse 1 nm layer line showing variations on the
281 monomer rise, which is in clear contrast to all other analyzed taxanes that displayed a
282 sharp band (i.e., a fixed monomer rise). Furthermore, we found that the effect of these
283 compounds on the lateral interactions (equatorial diffraction patterns) differed and,
284 thereby variations in the C13 side chain might modulate the lateral interactions between
285 PFs (**Table S3**).

286

287

288 **Discussion**

289 Previous studies of MT stabilization by Ptx controversially discussed the
290 interaction of the drug with the βM-loop and lattice effects, through lateral versus
291 longitudinal contacts(*10, 29, 33*) and communication between β-tubulin domains through
292 core helix βH7(*34*). Here, we undertook a unifying approach to discern which parts of
293 the Ptx molecule are involved in protein recognition, in inducing conformational changes
294 on tubulin and MT and, in its final stabilization activity. We have studied and compared
295 the structural effects as well as the biochemical and cellular activity of the Ptx core region,
296 baccatin III, and of two engineered Ptx derivatives, **2a** and **2b,** with modifications at the
297 C13 side tail affecting ring A. We have gained insight into our understanding of the
298 molecular mechanism of action of Ptx finding that: (i) the taxane core (baccatin III) is
299 responsible for most of the interacting energy of Ptx but it is only marginally involved in
300 the stabilization activity, (ii) the bulky hydrophobic group at 3'-N of the C13 side tail
301 distinguishes the s- from the c-tubulin conformation and promotes stabilization, and, (iii)
302 the lattice expansion is unrelated to the MT stabilization mechanism of Ptx.

303 The high-resolution crystallographic data show that the taxane core region reduces
304 the flexibility of the M-loop by inducing a partial structuring of its N-terminal section.
305 Further changes occur in the presence of a C13 side chain (**2a** and **2b**), tilting the
306 positioning of the Ptx core region by 18.5º within the pocket, and inducing a subtle
307 reorientation of the tubulin domains. Therefore, tubulin conformational changes related
308 to the binding of taxanes are initiated when bound to c-tubulin (in solution or at the tip of
309 MTs(*35*)) and strengthen upon tubulin straightening through the formation of polymer
310 contacts. Despite we have not observed a complete folding of the βM-loop upon baccatin

311 III, **2a** and **2b** binding, perturbations were detected in the c-tubulin structures, which were
312 comparable but weaker to the ones observed upon Ptx-binding to assembled MTs.
313 Moreover, fiber diffraction studies showed differences on the lateral arrangement of
314 shear-flow aligned MTs bound to these compounds. This suggests that Ptx and Ptx-
315 derived compounds indeed lock the lateral contacts to promote MT lattice stability
316 through interactions with the N-terminal section of the βM-loop, which is in agreement
317 with both the observations reported by Manka and Moores(*33*) and by Debs and co-
318 workers(*27*). Interestingly, fiber diffraction studies also showed the same MT-lattice
319 expansion for these analogs as the one observed in the presence of the active compounds
320 Ptx and Dcx. This structural feature is not related to the stabilization power since all
321 taxanes and Ptx-derived compounds similarly induced MT axial expansion but their
322 stabilization power ranged from highly stabilized MTs (Ptx and Dcx) to lower (**2a**) or
323 none or very weak (bacattin III). Therefore, the axial effect of these taxanes and hence,
324 the longitudinal contacts might not be the main reason of the stabilizing activity and this
325 activity may rely more on the effect of these compounds on the lateral interactions.

326 Importantly, the Ptx structure is optimized for its activity and changes introduced
327 at the ring A of the C13 side chain severely hamper the MT stabilizing activity. These
328 changes did not affect the binding to s-tubulin in MTs since the binding modes of **2a** and
329 **2b** were very similar to the ones described for Ptx-bound to MTs(*10, 29, 33*) (**Fig. S6**),
330 but significantly enhanced the interaction with unassembled c-tubulin. The curved-to-
331 straight conformational change in tubulin induces subtle modifications at the taxane-site
332 that were also previously detected by changes of the STD profiles of other taxane site
333 binders (discodermolide and epothilones)(*24, 25*). In contrast, no changes in the STD
334 profiles were detected for Ptx samples. Moreover, TR-NOESY determined, Ptx-bound
335 conformations in unassembled tubulin and MTs were identical (**Figs 5; S3; S4; S5**)
336 (detailed information about the conformational studies are given in supporting
337 information, including the quantitative analysis of the NOE intensities by taking into
338 account the full matrix relaxation treatment implemented in MSpin software(*36*)). We
339 hypothesize that very likely we are detecting the binding of Ptx to a small fraction of
340 unassembled, s-tubulin. This binding event would shift the equilibrium towards the
341 straight conformation due to the higher affinity **(Fig. 8, extended discussion)** and hence,
342 Ptx-bound unassembled s-tubulin would easily nucleate and would be more prone to
343 assembly because the energy needed for the curved-to-straight transition is provided by

344 Ptx binding **(extended discussion)**. Furthermore, these nucleated, straight oligomers
345 (previously observed in(*37*)) would be less prone to disassembly. In the case of assembled
346 MTs, the effect of the preferential binding implies that drug-bound subunits would be
347 restricted to bend (bending would require release of the tightly bound drug), therefore
348 preserving the stabilizing contacts. Concordantly, compounds with higher differential
349 affinity between c- vs s-tubulin conformations (Ptx and Dcx) are better assembly inducers
350 and more cytotoxic than those with lower difference of affinities (Ptx derivatives of this
351 work) (**Fig. 3** and **Table 1**), supporting a link between preferential binding for the s-
352 tubulin conformation and the stabilizing activity.

353 We have seen that several secondary structural elements are perturbed upon Ptx
354 derivative binding. Moreover, we observed a major structural difference in proximity of
355 the position occupied by the 3'-N-benzamido phenyl ring A moiety of Ptx in MTs: In the
356 c-tubulin-**2a** structure**,** the βR369 side chain occupies the same space as the βD26 side
357 chain in the context of the s-tubulin conformation in MTs. These changes could explain
358 the observed differences of binding affinity for the unassembled and the assembled
359 tubulin structures. The C13 side chain is involved in the interaction with helix βH1, which
360 is flanked by the βH1-βS2 loop (the lock in the key-lock lateral interplay with the βM-
361 loop). Upon transition to the assembled s-tubulin state, this space is narrowed down by
362 the side chains of βD26, βK19, βE22 and βH229 to form a favorable environment for the
363 interaction with the ring A moiety, which may lock the Ptx-bound tubulin in the straight
364 conformation (**Fig. 9B**). In the absence of the C13 side chain (baccatin III) or in the
365 presence of the less bulky moieties in ring A position (**2a** and **2b**), ligand binding is likely
366 less affected by the conformational transition from curved to straight, since much looser
367 interactions can still be established to the charged residue side chains of the βS9-βS10
368 loop and the helix H1 through water molecules (**Fig. 2C, Fig. 9A**). However, they may
369 not be strong enough to lock the tubulin in the straight conformation. These observations
370 suggest that modifications of the C13 side chain greatly affect the differential binding
371 affinity of Ptx derived taxanes for unassembled c- vs. assembled s-tubulin in MTs.

372 In summary, our results support the essential role of the ring A in C13 side chain
373 of Ptx in the mechanisms of MT stabilization. The presence of the ring A in the side chain
374 precludes binding to c-tubulin and contributes to the consolidation of lateral contacts over
375 the axial ones.
376

377 **Materials and Methods**

378 *Proteins and ligands*

379 Purified calf brain tubulin and chemicals were obtained as previously described(*4,*
380 *38*). Paclitaxel (Taxol®) (Ptx) was from Alfa Aesar Chemical, Docetaxel (Taxotere®)
381 (Dcx) was kindly provided by Rhône Poulenc Rorer, Aventis (Schiltigheim, France),
382 baccatin III was from Sigma, Flutax-2, Chitax 40, 3'N-aminopaclitaxel (N-AB-PT) and
383 Chitax-68 were synthesized as described(*15-17, 39*). All compounds were diluted in
384 99.8% D6-DMSO (Merck) to a final concentration of 20 mM and stored at -20 ºC. Their
385 solubility in aqueous media was determined as described in(*40*), Flutax-2 was found
386 soluble, while a 100 μM solubility was found for docetaxel and a 50 μM for both
387 paclitaxel and Chitax40.

388

389 *Biochemistry and cell biology*

390 HeLa S3 and HeLa βIII(*41*) cells were cultured in Dulbecco Modified Eagle
391 Medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine (2 mM),
392 gentamycin (40 μg/mL), penicillin (100 IU/mL), and streptomycin (100 μg/mL). The
393 medium for HeLa βIII cells was additionally supplemented with geneticin (0.5 mg/mL).
394 KB-3-1 (HeLa cells) derived from a cervical carcinoma and its multidrug resistant
395 counterpart KB-V1 (*42*) were grown in DMEM, 10% FCS, 1 mM piruvate and penicillin
396 (50 IU/mL) streptomycin (50 mg/mL), the latter cell line with 1 mg/mL of vinblastine.
397 Compounds were screened for toxicity against HeLa cells (KB-3-1, KB-V1, HeLa S3 and
398 HeLa βIII) using MTT assay following previously described protocols with minor
399 modifications(*43*). Briefly, cells were seeded in 96-well plates using regular DMEM
400 medium and incubated for 24 h. Then, cells were treated with vehicle or compound
401 diluted in the DMEM medium without serum for 72 h. The vehicle and compound
402 solution was changed once every 24 h. MTT solution (5 mg/ml) was added into each well
403 and further incubated for another 2 h. Finally, DMSO was added to stop the MTT reaction
404 and then, optical density was measured at 570 nm using a Molecular Devices SpectraMax
405 M5 (Molecular Devices, USA). The statistical significance of differences in IC_{50} values
406 were evaluated using the t-test option implemented in the Sigma Plot 13 software package
407 (version 13, Systat Software, Inc., San Jose, CA, USA).

408 Polymerization of 20 μM Tubulin in PEDTA buffer (10 mM sodium phosphate
409 (NaPi), 1 mM EDTA, 1 mM GTP, pH 6.7) plus 4 or 7 mM $MgCl_2$, was monitored in the
410 presence of 25 μM taxanes by turbidity at 350 nm employing a Thermo Appliskan plate
411 reader (Thermo Fisher, Waltham, MA, USA). The binding constants of the compounds
412 to the taxane binding site of stabilized cross-linked microtubules (MTs) were measured
413 by Flutax-2 displacement as previously described(*23*).

414 The binding constants of both **2a** and baccatin III to unassembled dimeric tubulin
415 were measured by centrifugation. Increasing amounts of dimeric tubulin (up to 150 μM)
416 prepared in NaPi-GTP buffer (10 mM sodium phosphate, 0.1 mM GTP, pH 7.0) were
417 incubated with a fixed concentration (50 μM) of either baccatin III or **2a**, incubated for
418 30 min at 25 ºC and centrifuged at 100000 rpm in a TLA-100.2 rotor for 2h at 25 ºC.
419 Then, samples were divided into upper (100 μL) and lower (100 μL) parts and 100 μL of
420 NaPi were added to both of them. Afterwards, 10 μM of either docetaxel or paclitaxel
421 were added as internal standard and samples were subjected three times to an organic
422 extraction using dichloromethane (v:v). Dichloromethane was removed by evaporation
423 and samples were resuspended in methanol 70%. Finally, ligand content was analyzed
424 using an HPLC system (Agilent 1100 Series) and samples were separated using a Zorbax
425 Eclipse XDB-C18 column (Methanol 70% isocratic condition; 20 minutes runs). Tubulin
426 content was determined by BCA for each sample. Ligand concentration in the upper 100
427 μL was considered as free concentration, while this in the lower 100 μL was considered
428 as the sum of bound and free concentrations. Binding constants of tubulin for the ligand
429 were calculated assuming a single binding site per tubulin dimer using SIGMAPLOT
430 14.5 Sigmastat Software Inc.

431 Binding constant of **2a** to cross-linked assembled MTs was measured as
432 previously described(*16*).

433 Reactivity of **2a** with tubulin and MTs was studied using organic solvent
434 extraction and HPLC. Reaction of the compound with MTs was studied by incubating 25
435 μM of tubulin in 10 mM NaPi, 1mM EDTA, 0.1 mM GTP and 7 mM $MgCl_2$ buffer pH
436 6.7, in the presence of 27.5 μM of **2a** (polymerizing conditions). Duplicated samples were
437 collected from the reaction tube at 0', 30', 1h, 2h and 4h and were centrifuged at 50000
438 rpm in a TLA100.2 rotor for 20'. Subsequently, supernatant was collected and a similar
439 volume of buffer was used to dissolve the pellet. Paclitaxel at 10 μM was added to all the

440 samples as internal standard. Duplicated samples were subjected to organic solvent
441 extraction either with 3 times of dichloromethane (v:v) or with the milder solvent ethyl
442 acetate, and the organic solvent was evaporated afterwards. Residues left were
443 resuspended in 70% methanol/Water and developed isocratically using an Agilent
444 1100 HPLC system connected to a reverse phase column Zorbax Eclipse XDB-C18
445 (mobile phase 70% methanol) with detection at 254 and 273 nm to quantify **2a** using the
446 internal standard.

447 Reaction of **2a** with dimeric tubulin was also studied. 20 μM tubulin in 10 mM
448 NaPi, 1 mM EDTA, 0.1 mM GTP buffer pH 7.0 (non-polymerizing conditions) was
449 incubated for 2 hours with 22.5 μM of **2a** at 4°C and 37°C, after incubation 10 μM of
450 paclitaxel was added to all the samples as internal standard. Samples were further
451 developed and analyzed as described above. As control, a sample of **2a** in the absence of
452 tubulin was processed and analyzed in the same way.

453

454 *NMR sample preparation and experiments*

455 Samples of ligand bound to non-polymerized α/β–tubulin -heterodimers were
456 prepared in 3 mm NMR tubes using a 300 μM concentration of baccatin III or 150 μM of
457 paclitaxel and 10 μM of tubulin in D_2O (10 mM NaPi, 0.1 mM GTP pD 7.3) as described
458 in(*25*). Samples of ligands bound to MTs were prepared in 3 mm NMR tubes using a 150
459 μM of paclitaxel and 20 μM of tubulin in D_2O (10 mM KPi, 0.1 mM GMPCPP, 6 mM
460 $MgCl_2$, pD 7.0) as described in(*25*). An 8% DMSO was added to the buffer to solubilize
461 the ligand. NMR spectra were then recorded at 298 K (dimeric tubulin samples) or 310
462 K (polymeric tubulin samples) in D_2O on a Bruker AVANCE 500 MHz or on a 700 MHz
463 spectrometer equipped with a triple-channel cryoprobe as described in(*25*). Additionally,
464 the oligomerization state of the tubulin samples for the NMR experiments was analyzed
465 by sedimentation velocity in a Beckman Optima XL-I analytical ultracentrifuge as
466 described(*25*).

467

468 *Crystallization, Data Collection and Structure Determination*

469 Crystals of T_2R-TTL were generated as described(*12, 14*). Suitable T_2R-TTL
470 crystals were soaked for 8h in reservoir solutions (2-4% PEG 4K, 2-10% glycerol, 30
471 mM $MgCl_2$, 30 mM $CaCl_2$, 0.1 M MES/Imidazole pH 6.7) containing either 10 mM

472 baccatin III, 5 mM **2a** or **2b.** Ssubsequently, crystals were flash cooled in liquid nitrogen
473 following a brief transfer into cryo solutions containing the reservoir supplemented with
474 16% and 20% glycerol. All data were collected at beamline X06DA at the Swiss Light
475 Source (Paul Scherrer Institut, Villigen, Switzerland). Images were indexed and
476 processed using XDS(*44*). Structure solution using the difference Fourier method and
477 refinement were performed using PHENIX(*45*). Model building was carried out
478 iteratively using the Coot software(*46*). Data collection and refinement statistics for all
479 three T_2R-TTL-complexes are given in Supplemental Table S1. Molecular graphics and
480 analyses were performed with PyMol (The PyMOL Molecular Graphics System, Version
481 2.3.2, Schrödinger, LLC). To compare the structures of both baccatin III and **2a**
482 complexes in the curved tubulin (c-tubulin) conformation to the straight tubulin (s-
483 tubulin) in paclitaxel stabilized MT (PDB ID 5SYF), all structures were superimposed
484 onto the taxane-site of **2a** (residues 208-219+225-237+318-320+359-376+272-276+287-
485 296; $rmsd_{BacIII}$ 0.171 Å (48 C_α atoms), $rmsd_{5SYF}$ 0.868 Å (52 C_α atoms)).

486

487 *MT shear-flow alignment and X-ray fiber diffraction experiments*

488 X-ray fiber diffraction data were collected in BL11-NDC-SWEET beamline of
489 ALBA Synchrotron at a λ=0.827 nm as described in(*32*). Radial structural parameters
490 (MT diameter and average inter-PT distances) and dimer/monomer length (from the 4th
491 harmonic of the first layer-line signals) were determined as described in(*32*).

492

493

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495

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J.F.D., A.C. J. E-G., S.K., Y.M. and S.L.; Visualization: A.E.P. and M.A.O. Writing—original draft: J.F.D. Writing—review & editing: A.E.P., W-S.F., M.O.S., J.M.A. and K-H.A.

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652 **Figures and Tables**

653

654 **Table 1.** IC50[1] values (μM) of tested compounds in Ptx sensitive KB (HeLa), HeLa S3
655 and in Ptx resistant KBV (HeLa) P-gp overexpressing and HeLa βIII-tubulin
656 overexpressing cells.

657

Compound	KB μM	KBV μM	HeLa S3 μM	HeLa βIII μM
2a	0.6±0.1	>20	11.5±0.8	11.8±0.3
2b	10±3	>20	17.7±0.6	16.6±0.3
2c	13±1	>20	12.9±0.8	>20
2d	>20	>20	>20	>20
Ptx[2]	0.0019 ± 0.0001	9± 4	0.0011 ± 0.0004	0.034 ± 0.009

658

659 [1]IC50 values of growth inhibition in a panel of HeLa (KB and Hela S3) cervical
660 carcinoma cells expressing P-glycoprotein (KBV) or the βIII isotype of tubulin (Hela
661 βIII), compared to the isogenic parental lines. Values are presented as the mean±SE of
662 three independent measurements[2]. Data from reference(*47*).

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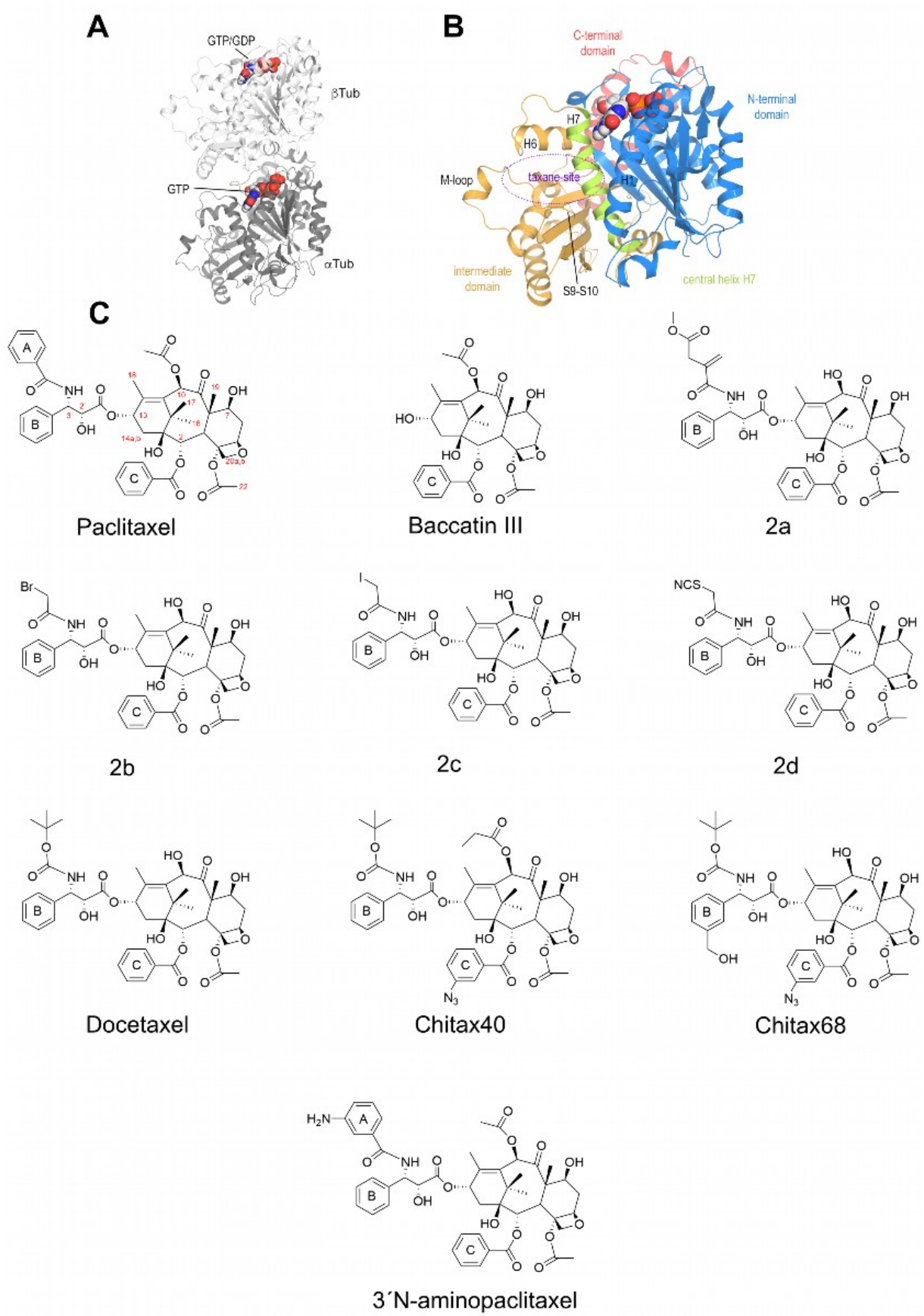


Figure 1. (**A, B**) Structural features of tubulin. (**C**) Structures of taxanes used in this study.

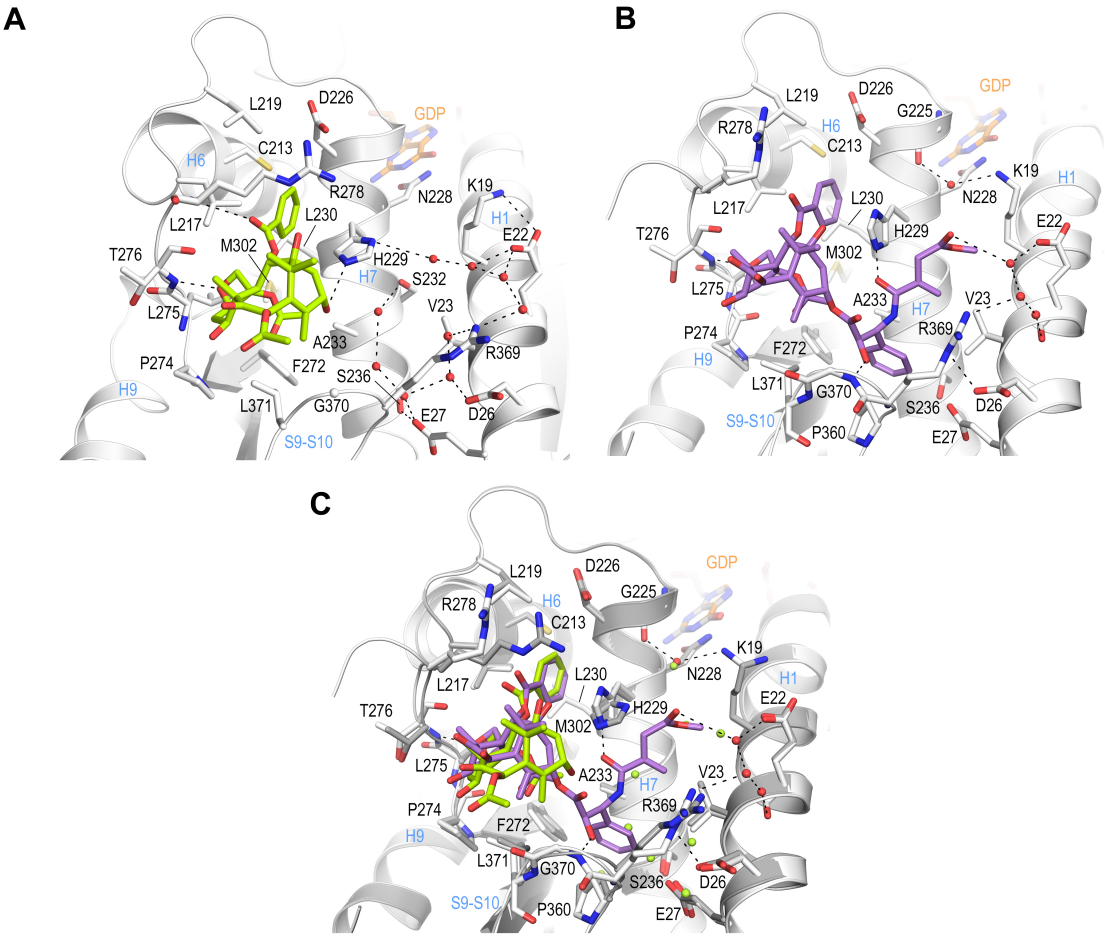
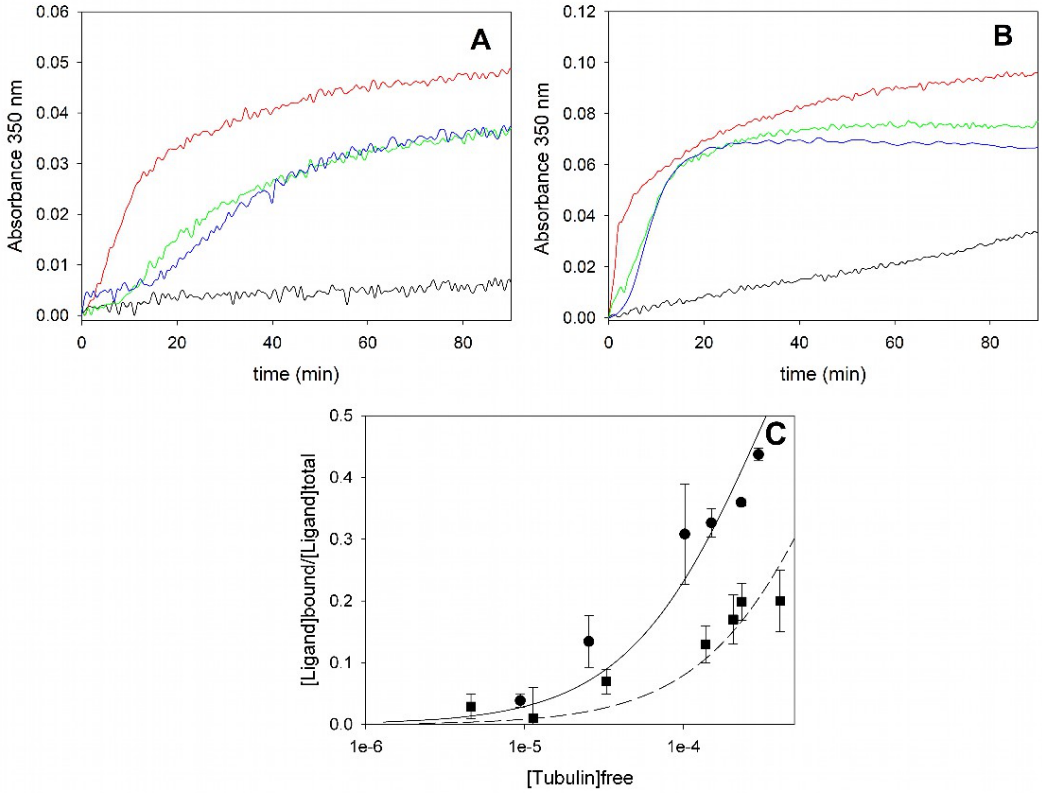


Figure 2. Crystal structure of T_2R-TTL-baccatin III (PDB ID 7A7I) and T_2R-TTL-2a (PDB ID 7A7F) complexes. (**A**) Close-up view of the interaction network observed between baccatin III (lemon) and tubulin (light gray). Interacting residues of tubulin are shown in stick representation and are labeled. 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 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.



679

680 **Figure 3. Biochemical characterization of the tubulin-2a and 2b interaction.** Time-
681 course assembly of 25 μM tubulin in 10 mM phosphate buffer, 1 mM EDTA, 1 mM GTP,
682 pH 6.7, with 4 **(A)** or 7 mM **(B)** $MgCl_2$ in the presence of 27.5 μM Ptx (red), **2a** (green),
683 **2b** (blue) or vehicle (DMSO, black). (**C**) Isotherm of binding of 50 μM baccatin III
684 (circles and solid line) or **2a** (squares and dashed line) to tubulin. Error bars are standard
685 errors of three independent measurements. Lines are the fitting to a single site model.

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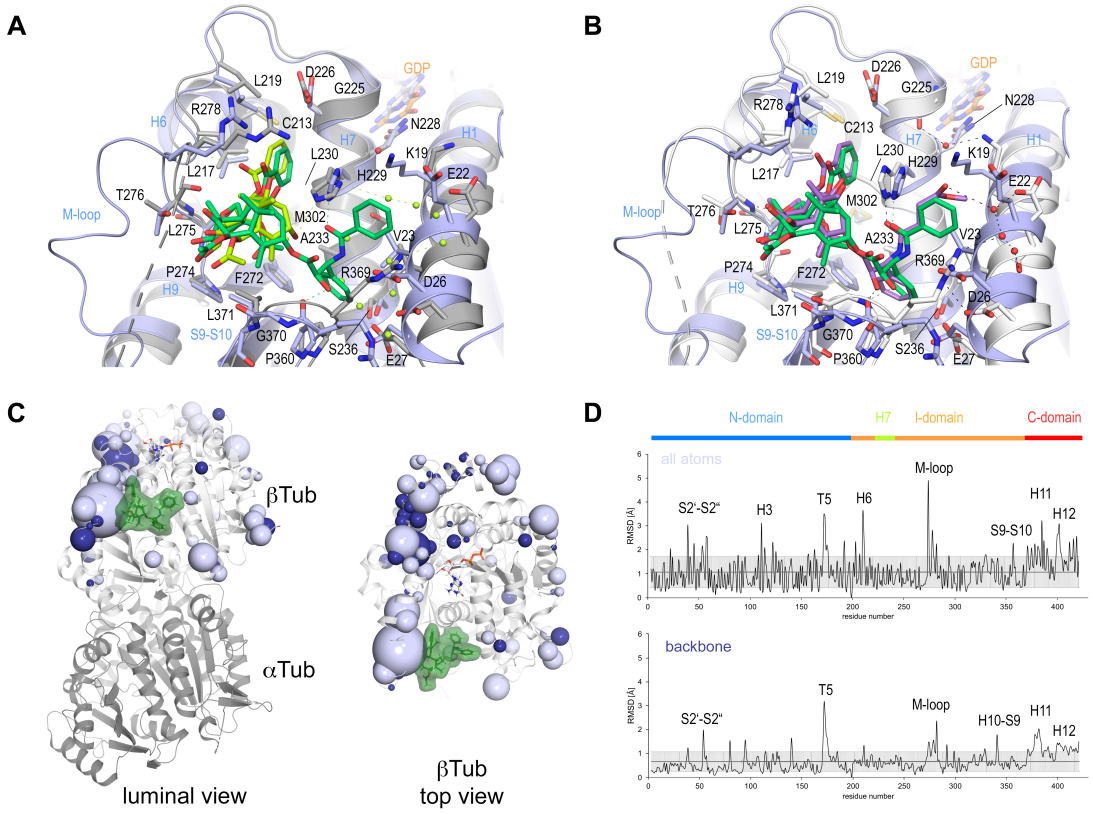

Figure 4. Comparison of taxane binding to unassembled c- vs. assembled, s- tubulin. (**A**) Close-up view of the superimposed baccatin III bound (ligand in lemon; protein in grey ribbon and sticks) to unassembled, c-tubulin and Ptx bound to assembled, s-tubulin as found in a MT (PDB ID 6WVR; ligand in 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 unassembled tubulin (ligand in violet; protein in grey ribbon and sticks) and Ptx bound to assembled tubulin (PDB ID 6WVR; ligand in dark green; protein in slate ribbon and sticks) structures (rmsd 0.826 Å (52 C_α atoms) using the same settings as in (**A**). (**C**) Perturbations on β-tubulin induced by Ptx upon binding to assembled tubulin in MTs (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 Ptx-bound, assembled MTs are represented as light (all atom rmsd) and 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 all atoms (top) and backbone (bottom) positions between the Ptx bound (PDB ID 6WVR) and the apo (PDB ID 6DPV) assembled tubulin state in MTs. The grey error bar represents the average rmsd ± standard deviation. The top bar is colored according to the following domain assignment: N-domain (marine blue), I-domain (orange), central helix βH7 (lemon) and 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 30 C_α).

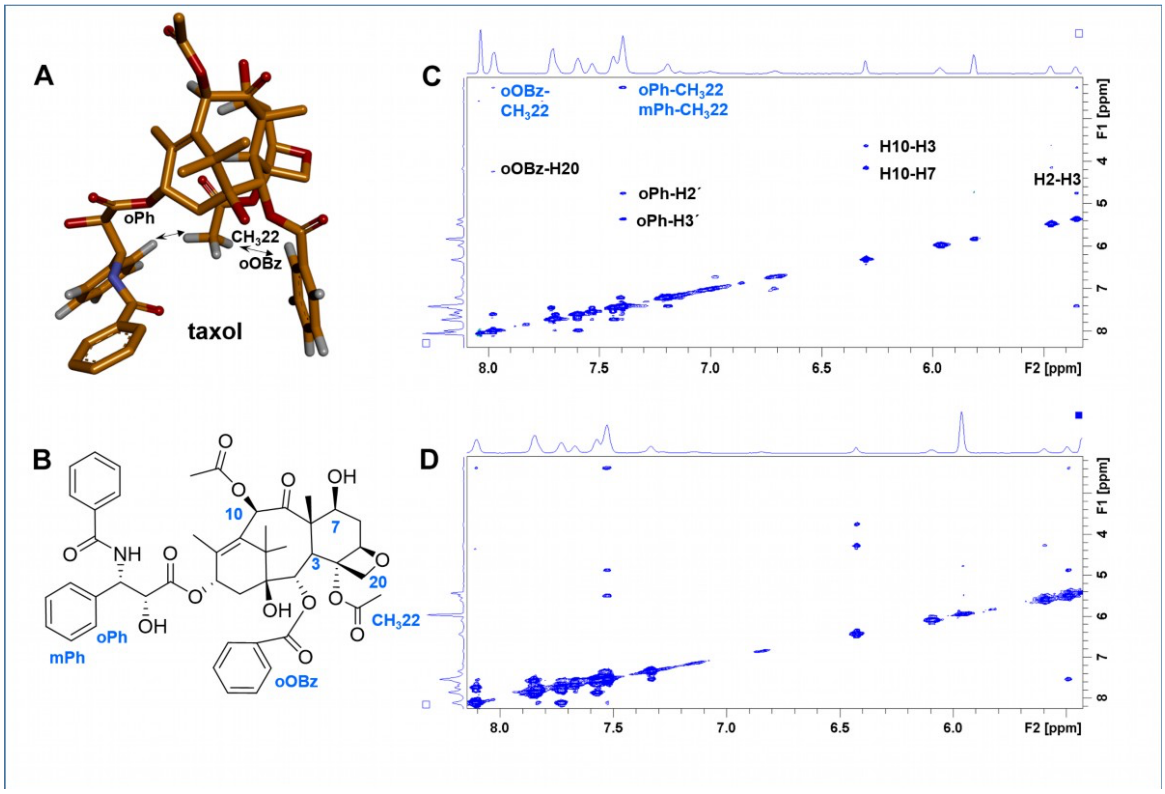

Figure 5. NMR-TR-NOESY characterization of the Ptx-tubulin interaction. (**A**) Bioactive structure of Ptx as determined by TR-NOESY. (**B**) Chemical structure of Ptx, indicating the protons involved in the TR-NOESY detected interactions. (**C**) TR-NOESY spectra (298 K) of Ptx bound to tubulin dimers. (**D**) TR-NOESY spectra (310 K) of Ptx bound to MTs.

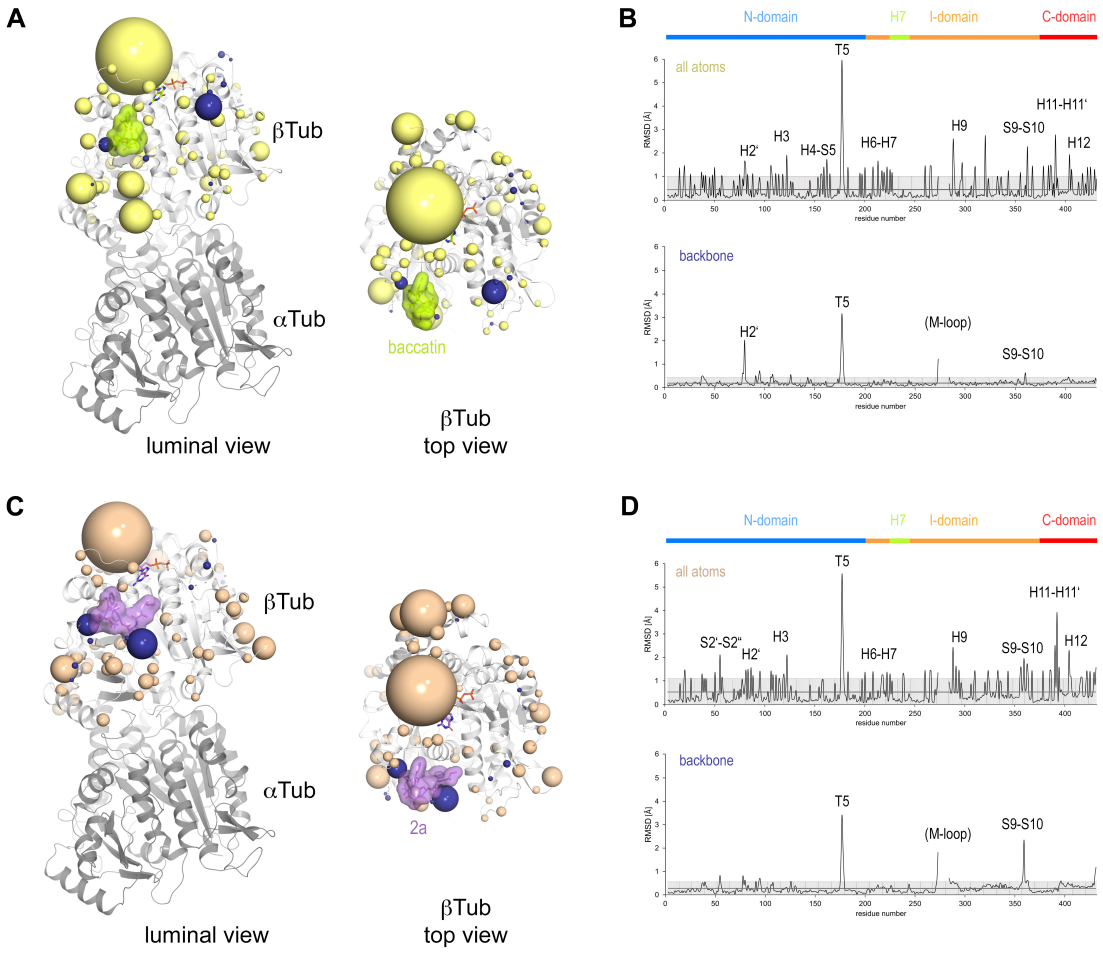

Figure 6. Perturbations induced by taxane binding to unassembled, c-tubulin. (**A**) Perturbations on all residue (yellow) and backbone atoms (dark blue) of the β-tubulin chain induced by baccatin III upon binding to unassembled tubulin. The tubulin chains are in ribbon representation and are colored in dark (α-tubulin) and light (β-tubulin) grey, respectively. The rmsd-values of the superimposed unbound and baccatin III bound, unassembled tubulin are represented as pale yellow (all atom rmsd) and 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 all atoms (top) and backbone (bottom) positions between the baccatin bound and the apo (PDB ID 4I55) unassembled tubulin state. The grey error bar represents the average rmsd ± standard deviation. The top bar is colored according to the following domain assignment: N-domain (marine blue), I-domain (orange), central helix H7 (lemon), 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 29 C_α). (**C**) Perturbations on all residue (wheat) and backbone atoms (dark blue) of the β-tubulin chain induced by **2a** upon binding to unassembled tubulin. (**D**) rmsd plots of the all atoms (top) and backbone (bottom) positions between the **2a** bound and the apo (PDB ID 4I55) unassembled tubulin state (rmsd 0.10 Å of 29 C_α). The same display settings as in (**B**) are applied.

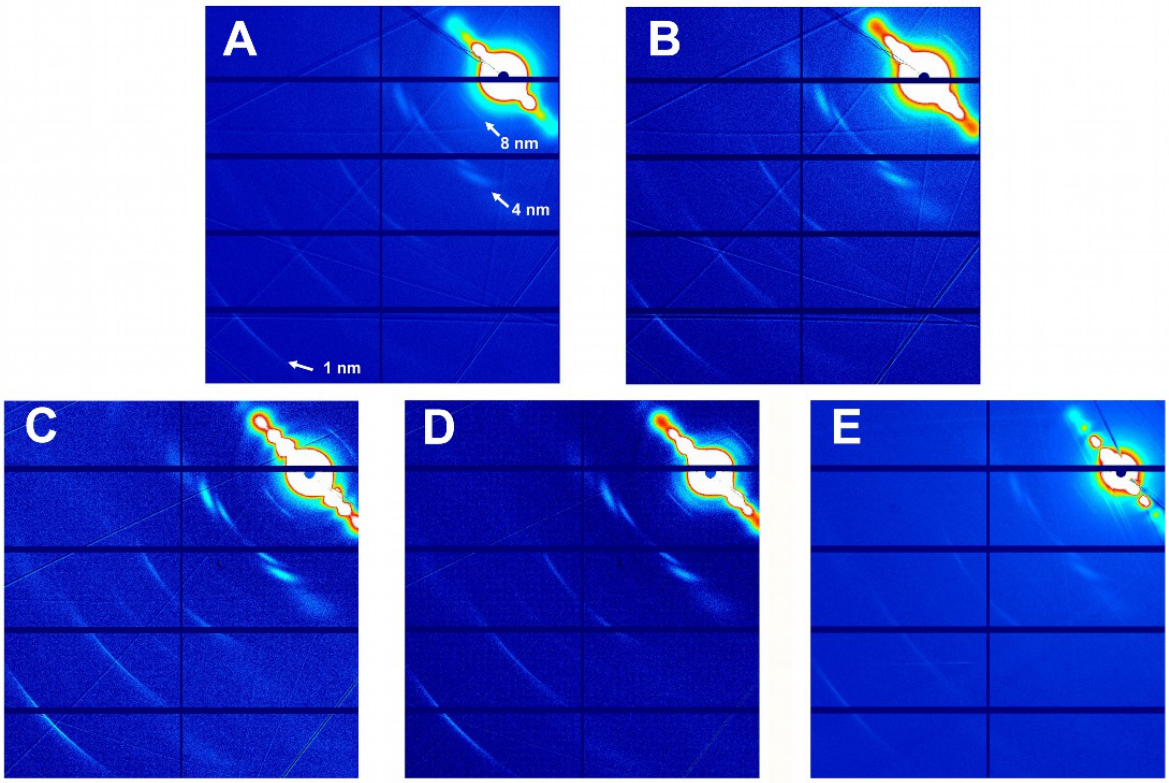

Figure 7. Fiber diffraction patterns of MTs assembled from (**A**) GTP-tubulin and Ptx, (**B**) GTP-tubulin and Dtx, (**C**) GTP-tubulin and **2a**, (**D**) GTP-tubulin and **2b**, and (**E**) GTP-tubulin and baccatin III.

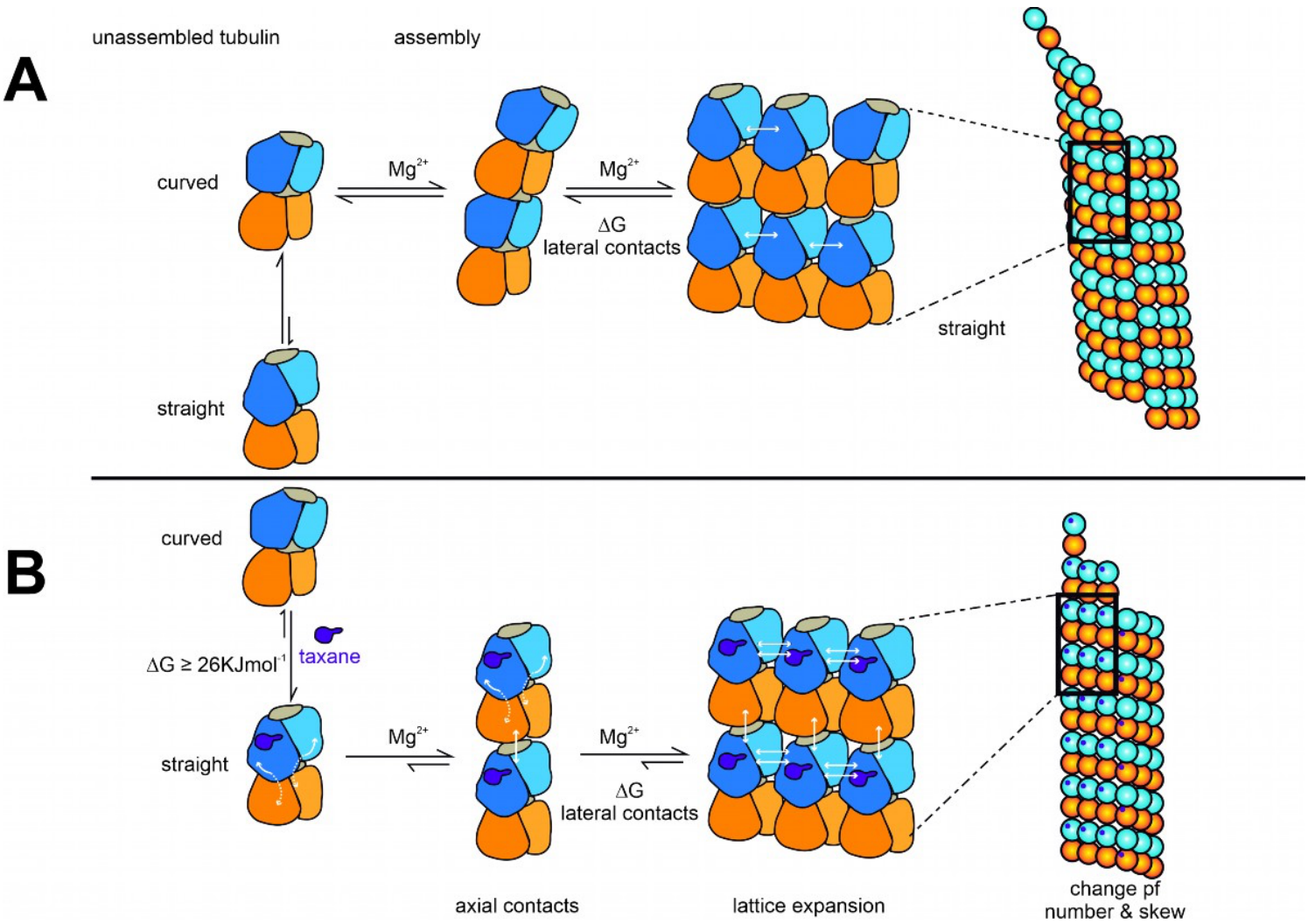

Figure 8. Schematic representation of the mechanism of taxane-induced MT assembly. **(A)** MT assembly scheme with a classical bidimensional nucleus for cooperative polymerization. **(B)** Taxane-induced MT assembly scheme with an activated, s-tubulin for cooperative polymerization. In both panels, α-Tubulin is in orange (Nt-domain light orange and, intermediate domain dark orange), β-tubulin is in blue (Nt-domain light blue and, intermediate domain dark blue), while the nucleotide binding site in tubulin is in gray. Taxane molecules in (B) are depicted in purple. White arrows show direct and allosteric protein-protein interactions.

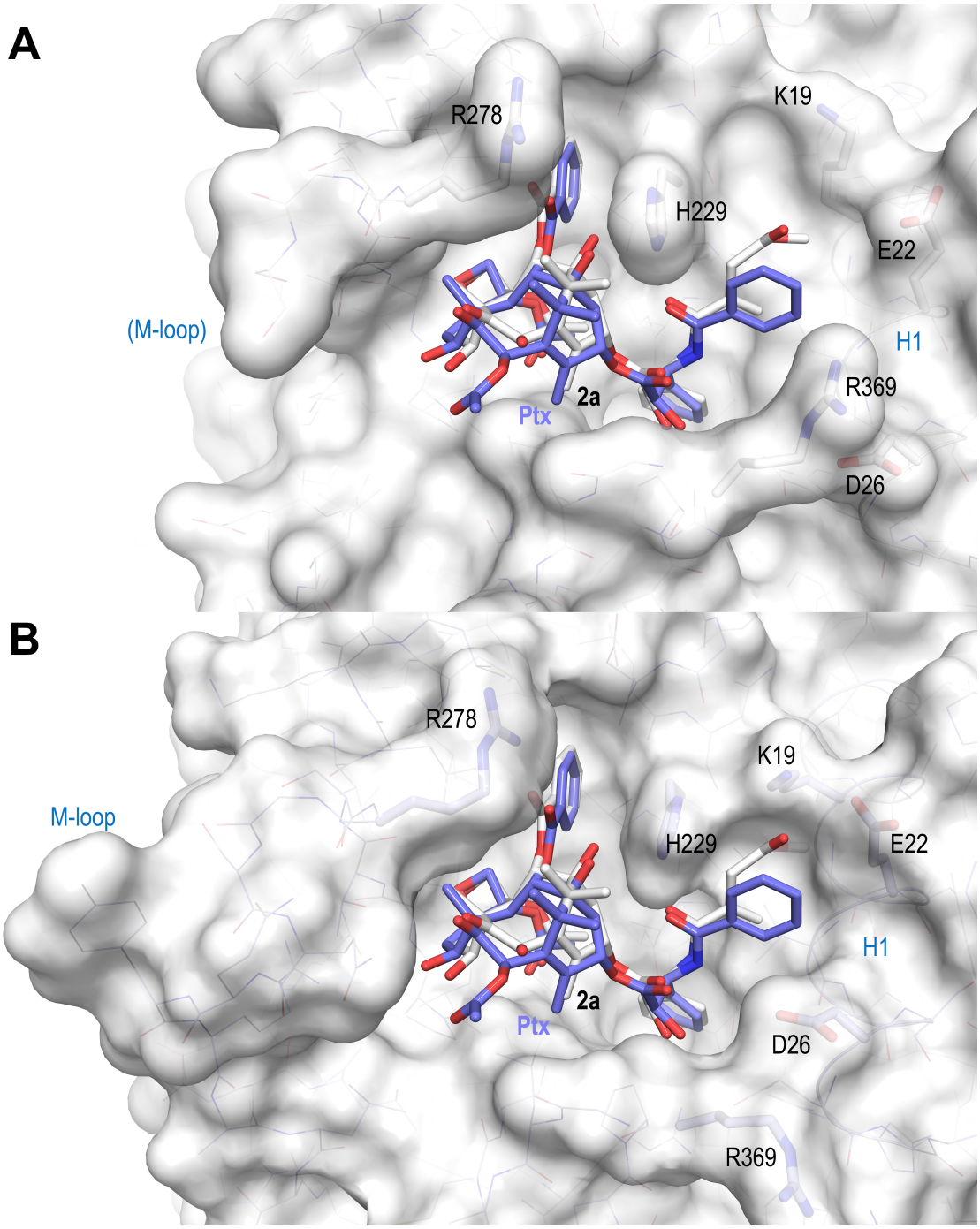

Figure 9. Surface representations of liganded taxane sites in both (A) the c- and (B) s-tubulin conformational state. The structures of **2a** (white) and Ptx (slate) bound to MTs (PDB ID 6WVR) were superimposed onto their central helices βH7. The side chains of the βM-loop residue βR278 and of residues surrounding the C13 side chains of the ligands are in stick representation and are labeled. Helix βH1 is highlighted in ribbon representation