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Dissecting Paclitaxel–Microtubule Association: Quantitative Assessment of the 2′-OH Group

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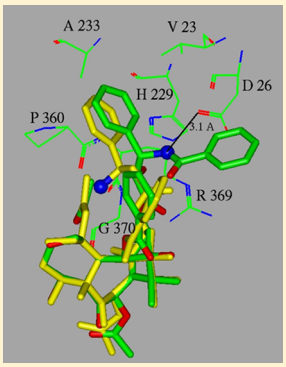
ABSTRACT: Paclitaxel (PTX) is a microtubule-stabilizing agent that is widely used in cancer chemotherapy. This structurally complex natural product acts by binding to β-tubulin in assembled microtubules. The 2′-hydroxyl group in the flexible side chain of PTX is an absolute requirement for activity, but its precise role in the drug–receptor interaction has not been specifically investigated. The contribution of the 2′-OH group to the affinity and tubulin-assembly efficacy of PTX has been evaluated through quantitative analysis of PTX derivatives possessing side chain deletions: 2′-deoxy-PTX, *N*-debenzoyl-2′-deoxy-PTX, and baccatin III. The affinity of 2′-deoxy-PTX for stabilized microtubules was more than 100-fold lower than that of PTX and only ∼3-fold greater than the microtubule affinity of baccatin III. No microtubule binding activity was detected for the analogue *N*-debenzoyl-2′-deoxy-PTX. The tubulin-assembly efficacy of each ligand was consistent with the microtubule binding affinity, as was the trend in cytotoxicities. Molecular dynamics simulations revealed that the 2′-OH group of PTX can form a persistent hydrogen bond with D26 within the microtubule binding site. The absence of this interaction between 2′-deoxy-PTX and the receptor can account for the difference in binding free energy. Computational analyses also provide a possible explanation for why *N*-debenzoyl-2′-deoxy-PTX is inactive, in spite of the fact that it is essentially a substituted baccatin III. We propose that the hydrogen bonding interaction between the 2′-OH group and D26 is the most important stabilizing interaction that PTX forms with tubulin in the region of the C-13 side chain. We further hypothesize that the substituents at the 3′-position function to orient the 2′-OH group for a productive hydrogen bonding interaction with the protein.



The diterpenoids paclitaxel (PTX, Taxol) and docetaxel (TXT, Taxotere) are highly effective drugs for the treatment of a variety of cancers. PTX, TXT, and related taxanes act by interfering with cellular microtubule dynamics through binding to β-tubulin.[1] PTX was the first substance found to bind to microtubules and stabilize them against disassembly.[2] The clinical success of PTX and TXT prompted many studies of structure–activity relationships for this class of compounds and searches for other molecular entities that display PTX-like activity. One of these classes of molecules, the epothilones, seems particularly promising. For example, the epothilone derivative ixabepilone (Ixempra) is approved by the U.S. Food and Drug Administration for the treatment of breast cancer. Still, the taxane class of molecules continues to be explored for new anticancer agents, with a half-dozen PTX-related molecules in various stages of clinical trials.[3]

The first crystallographic structure of tubulin was a breakthrough not only in the understanding of the protein but also in the molecular mechanism of PTX's interaction with microtubules.[4] Binding to a site facing the microtubule lumen, PTX locks tubulin into its active, "straight" conformation, strengthening contacts between protofilaments and tubulin dimers in the microtubule lattice. The precise binding mode of PTX could not be ascertained from the original data, however. The rigid core of the molecule was visualized, but the binding site conformation of the flexible C-13 side chain could not be clearly delineated. The C-13 side chain of PTX is critical for its antitumor activity, and therefore, understanding the interactions between this portion of the molecule and the protein is of utmost importance in understanding precisely how this drug works on a molecular level. Subsequent molecular modeling combined with the electron crystallography density data led to the proposal of the "T-Taxol" model of the microtubule-bound ligand.[5] Experimental data that support this conformation for the tubulin-bound ligand have been presented,[6−8] but this conformation is not universally accepted.[9−11]

Many of the so-called "second-generation" taxanes differ from PTX and TXT in the C-13 side chain. (See Figure 1 for the structure and numbering system of PTX.) If the correct absolute configuration of the C-3′ position is maintained, the substituents on the C-3′ amide and the C-3′ atom can be replaced with a variety of hydrophobic groups, yielding highly active taxanes.[11] The hydroxyl substituent at the C-2′ position,

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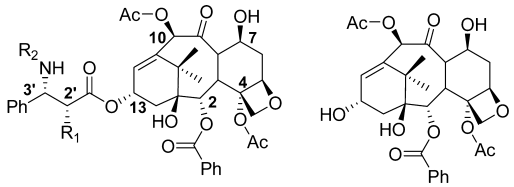

PTX	R_1: OH	R_2: Bz
2'-deoxy-PTX	R_1: H	R_2: Bz
N-debenzoyl-2'-deoxy-PTX	R_1: H	R_2: H

Figure 1. Structure of PTX and deletion analogues.

however, is absolutely essential. Acetylation completely abolishes binding of PTX to microtubules.[12] Replacing the hydroxyl group with fluorine or thiol decreases the cytotoxicity of the PTX by 2 orders of magnitude.[13,14] It has long been assumed that the 2′-OH group participates in an important hydrogen bonding interaction between the ligand and the protein. To date, though, the precise role of the critical 2′-hydroxyl group in the association and activity of PTX has not been directly addressed.

In this study, we have quantified the role of the 2′-OH group in the affinity and efficacy of PTX for tubulin. We have synthesized two "deletion" PTX derivatives: 2′-deoxy-PTX, which lacks the 2′-OH group, and *N*-debenzoyl-2′-deoxy-PTX, which lacks both the 2′-OH group and the 3′-benzoyl group. Their affinities for GMPcPP-stabilized microtubules, their efficacies as promoters of pure tubulin assembly, and their cytoxicities have been measured and compared with those of the parent molecule, PTX, and the paclitaxel derivative devoid of the side chain, baccatin III. We find that the 2′-OH group accounts for 80% of the binding free energy of the entire side chain. Therefore, the 2′-OH group is energetically and functionally the most important component of the side chain; the lack of the hydroxyl group renders 2′-deoxy-PTX more like baccatin III than PTX in activity. Somewhat surprisingly, we could detect no microtubule binding or cytotoxicity for the double-deletion derivative *N*-debenzoyl-2′-deoxy-PTX at concentrations up to its maximal solubility. In this case, the presence of the partial side chain at C-13 is more deleterious to microtubule binding than its complete absence.

Computational analyses were performed to understand the behavior of these molecules. Molecular dynamics simulations were performed for PTX and *N*-debenzoyl-2′-deoxy-PTX within the binding site in the structures of Protein Data Bank (PDB) entry 1JFF[15] as well as T-Taxol–tubulin.[5] These results confirm the expected presence of a hydrogen bonding interaction between the 2′-OH group and the protein, but our model identifies D26 as the primary hydrogen bond acceptor in the binding site, which has not heretofore been emphasized in this role. The modeling results also provide an explanation for how a PTX derivative with a side chain can be much less active than baccatin III, the derivative that has no side chain at all.

MATERIALS AND METHODS

All chemicals used were analytical grade and purchased from Sigma Chemicals unless mentioned otherwise. The compounds 2′-deoxy-PTX and *N*-debenzoyl-2′-deoxy-PTX were synthesized on the basis of the methods of Walker et al.[16] and Long et al.[17] N-AB-PT and GMPcPP were previously synthesized.[18] Stock solutions of all taxanes were made in DMSO. All references to percent DMSO concentration are volume to volume. The concentrations of *N*-debenzoyl-2′-deoxy-PTX, 2′-deoxy-PTX, and PTX were determined in DMSO using an $\varepsilon_{273,DMSO}$ of 1.7×10^3 M^{-1} cm^{-1},[19] and the concentrations of baccatin III and N-AB-PT were determined using an $\varepsilon_{276,DMSO}$ of 1.19×10^3 M^{-1} cm^{-1} for baccatin III[20] and an $\varepsilon_{320,DMSO}$ of 2.08×10^3 M^{-1} cm^{-1} for N-AB-PT.[18]

Tubulin Purification and Protein Determination. Tubulin was prepared by two cycles of temperature-dependent assembly and disassembly followed by ion exchange chromatography.[21] The purified tubulin was drop-frozen in liquid nitrogen. Prior to use, the frozen pellets were gently thawed and then desalted into PME buffer [100 mM PIPES, 1 mM $MgSO_4$, and 2 mM EGTA (pH 6.90) at 25 °C] by the method of Penefsky.[22] The concentration of tubulin was determined spectrophotometrically on a Hewlett-Packard model 8453 diode array spectrophotometer using an extinction coefficient of 1.23 M^{-1} cm^{-1} at 278 nm.

Promotion of Tubulin Assembly. Tubulin assembly was monitored by the apparent absorption at 350 nm using a Hewlett-Packard model 8453 absorption spectrophotometer with a multicell holder that was maintained at 37 °C with a circulating water bath. Tubulin in PME buffer containing 0.1 mM GTP was equilibrated at 37 °C in the spectrophotometer, and a baseline was recorded. Assembly was initiated by adding the taxane in DMSO. The progress of the reaction was monitored by the increase in the apparent absorption at 350 nm as a function of time until a steady state was reached. The extent of microtubule assembly was determined as the difference between the apparent absorption at steady state and that at the baseline. The concentration of DMSO was maintained at ≤4% depending upon the requirement of the experiments.

Preparation of GDP-Tubulin and GMPcPP-Tubulin. GDP-tubulin and GMPcPP-tubulin, in which the E-site nucleotide is fully replaced by GDP and GMPcPP, respectively, were prepared as described previously.[23,24] Unbound GDP and excess GMPcPP were not removed from the system.

Affinity of Ligands for GMPcPP-Stabilized Microtubules. The affinity of taxanes for GMPcPP-stabilized microtubules was assessed by competition with a fluorescent derivative of PTX, N-AB-PT, as described previously[7] with the following modifications. The concentrations of GMPcPP microtubules and N-AB-PT were 1 μM each instead of 5 μM, and the concentration of DMSO in the experiments was 15%[25] instead of 4% because of the low solubility of the compounds. The fluorescence emission spectrum of each sample was collected on a Jobin Yvon FluoroMax3 spectrofluorometer (excitation wavelength of 320 nm) in a cell holder maintained at 37 °C. A 2 mm × 10 mm quartz cuvette was used for the measurements and was oriented such that the excitation beam passed through the short path. Appropriate background spectra were subtracted before data analysis. EC_{50} values of ligands for GMPcPP-stabilized microtubules were determined by a plot of F/F_0 versus the log of the taxane concentration, where F_0 is the fluorescence emission intensity at 413 nm of N-AB-PT bound to GMPcPP-stabilized microtubules in the absence of nonfluorescent analogues and F is the fluorescence emission intensity at 413 nm of N-AB-PT in the presence of a given concentration of the nonfluorescent ligand. An inhibition constant (K_i) for each ligand was determined using a one-site competition relation: $K_i = EC_{50}/(1 + [\text{N-AB-PT}]/K_{PTX})$, where K_{PTX} is the dissociation constant for N-AB-PT with GMPcPP-

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polymerized tubulin (15 nM[23]) and [N-AB-PT] is 1 μM. The inhibition constant reflects the relative dissociation constant (K_d) or association constant ($1/K_d$) for the nonfluorescent ligand binding to microtubules.

Critical Concentration Determination. Critical concentrations (C_{crit}) of tubulin assembly in the presence of PTX, 2′-deoxy-PTX, or baccatin III were determined by assembly experiments in PME buffer containing 2% DMSO performed at 37 °C. Various concentrations of tubulin in PME buffer (containing 2% DMSO) were polymerized with the ligands, and the extent of assembly was measured. Tubulin:ligand concentration ratios of 1:0.5, 1:1, and 1:1.3 were used for PTX, 2′-deoxy-PTX, and baccatin III, respectively. Critical concentrations were obtained as the *x*-intercepts of plots of the apparent absorption at 350 nm at steady state (ΔA_{350}) versus tubulin concentration.

Determination of Cytotoxicity. PC3 cells were grown in HAMS F-12K medium supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cytotoxicity assay was performed on the basis of the method of Skehan et al.[26] IC_{50} values were obtained from plots of the absorbance of SRB at 570 nm versus taxane concentration.

Computational Simulations of PTX and *N*-Debenzoyl-2′-deoxy-PTX. Computational simulations involving PTX were performed on structures of PDB entry 1JFF[15] and T-Taxol−tubulin. The structure of T-Taxol−tubulin was created using coordinates from the Supporting Information of ref 27. The structure of tubulin-bound *N*-debenzoyl-2′-deoxy-PTX was created from 1JFF by removing the 2′-OH group and *N*-benzoyl group of PTX.

Computational simulations for all the structures described above were performed using AMBER 9, a suite of programs for molecular dynamics simulations of proteins and nucleic acids, developed by The Scripps Research Institute (TSRI) [The Scripps Research Institute, The AMBER Molecular Dynamics Package (http://amber.scripps.edu)]. Visualization and manipulation of the structures were performed using either DS Visualizer or Insight II Viewer (Accelrys, San Diego, CA).

The AMBER force field (2003 version) was used for the protein and water, and the force field parameters for the ligands (PTX, GTP, and GDP) were obtained from GAFF (general AMBER force field). The partial charges for the ligands (PTX, GTP, and GDP) were calculated using the electrostatic potential (ESP) fit method in the DMol3 module of Materials Studio version 4.1 from Accelrys.[28] Each system was neutralized by adding 31 Na^+ ions and was immersed in a TIP3P box of water[29] extending 15 Å from the surface of the protein, and periodic boundary conditions were applied.

All the structures mentioned at the beginning of this section were equilibrated for 120 ps at 300 K with a 1 g/cm^3 water density and minimized. Additionally, for the two PTX structures, equilibration was followed by a long-term (10 ns) dynamics session, and the time evolutions of the distances between the 2′-OH group from side chain oxygens of D26 as well as the backbone amides of G370 and R369 were recorded for each structure.

RESULTS

Tubulin-Assembly Activity of PTX Analogues. Initial assessment of the in vitro activity of the taxanes was performed by measuring tubulin assembly in the presence of PTX and the other molecules (Figure 2). Like PTX, 2′-deoxy-PTX induces tubulin to assemble into normal microtubules [confirmed by electron microscopy (data not shown)]. Baccatin III is weakly active, but *N*-debenzoyl-2′-deoxy-PTX does not promote tubulin assembly under these conditions.

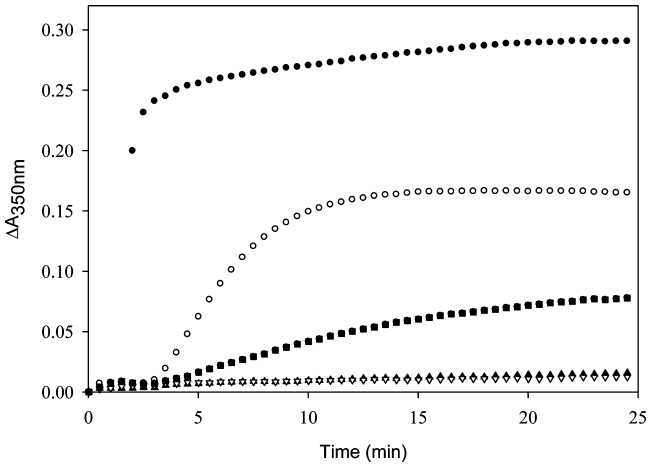

Figure 2. Effect of taxanes on tubulin assembly. Tubulin (5 μM) was treated with 15 μM PTX (●), 2′-deoxy-PTX (○), *N*-debenzoyl-2′-deoxy-PTX (▲), baccatin III (■), or no drug (▽). Experiments were performed in PME buffer containing 0.1 mM GTP and 4% DMSO at 37 °C as described in Materials and Methods. Assembly was monitored by the increase in the apparent absorption at 350 nm.

Relative Binding Affinity for Stabilized Microtubules. Polymerization assays do not provide a direct assessment of a ligand's affinity for tubulin because of the linkage between tubulin binding and microtubule assembly.[30] Association constants for assembly-promoting ligands binding to microtubules can be determined under conditions in which the concentration of polymerized tubulin is essentially constant, such as with cross-linked microtubules[31] or with GMPcPP-stabilized microtubules.[32] Most quantitative evaluations of equilibrium binding parameters for PTX site ligands have been performed by measuring displacement of a fluorescent PTX bound to stabilized microtubules.[33] In this investigation, the apparent equilibrium constants for the PTX derivatives were assessed using GMPcPP-stabilized microtubules and the fluorescent PTX analogue *N*-debenzoyl-*N*-(*m*-aminobenzoyl)-paclitaxel (N-AB-PT) as the reporter ligand. The competition curves are shown in Figure 3. Curves for the ligands other than PTX are truncated at the maximal solubility of each ligand under the experimental conditions (15% DMSO in PME buffer). It is seen that removal of the 2′-OH group shifts the binding curve to the right of PTX, closer to that of baccatin III than that of PTX. No inhibition of binding of N-AB-PT to microtubules was observed with *N*-debenzoyl-2′-deoxy-PTX at its highest soluble concentration, 45 μM. The apparent association constants for the binding of taxanes to GMPcPP-stabilized microtubules at 37 °C, calculated as described in Materials and Methods, are listed in Table 1. Note that the apparent association constants for binding of PTX and baccatin III to microtubules under these conditions are in good agreement with literature values,[31,34] confirming that the high concentration of DMSO in this assay does not adversely affect the results.[25] Binding free energies were calculated from the apparent association constants. These values are listed in Table 1.

Tubulin-Assembly Efficacy of Taxanes. The efficacies of PTX, 2′-deoxy-PTX, and baccatin III were measured in terms

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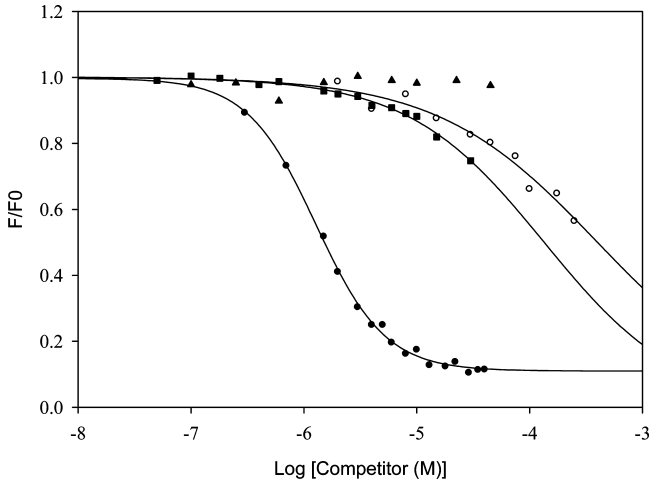

Figure 3. Competition binding curves for binding of taxanes to GMPcPP microtubules. PTX (●), 2′-deoxy-PTX (■), *N*-debenzoyl-2′-deoxy-PTX (▲), or baccatin III (○) in DMSO was added to solutions of GMPcPP-stabilized microtubules (1 μM) containing N-AB-PT (1 μM). The samples were incubated at 37 °C for 30 min, and the emission intensity of N-AB-PT was recorded for each sample as described in Materials and Methods. The concentration of DMSO was maintained at 15%.

Table 1. Equilibrium Binding Parameters for Binding of Taxanes to GMPcPP-Stabilized Microtubules[a]

taxane	$K_{a,app}$ ($\times 10^6$ M^{-1})[b]	$\Delta G°$ (kcal/mol)[c]
PTX	53 ± 2	−11 ± 0.02
2′-deoxy-PTX	0.50 ± 0.01	−8.1 ± 0.01
baccatin III	0.17 ± 0.03	−7.4 ± 0.09
N-debenzoyl-2′-deoxy-PTX	–	–

[a]All measurements were performed at 37 °C as described in Materials and Methods. [b]Apparent equilibrium constant for binding of taxane to GMPcPP-stabilized microtubules. [c]Apparent standard free energy change associated with binding of ligands to GMPcPP-stabilized microtubules.

of their effect on the critical concentration (C_{crit}) of tubulin assembly (Figure 4). C_{crit} of tubulin assembly is the minimal concentration of tubulin required for microtubule formation under a particular set of experimental conditions.[35] The inverse of C_{crit} is a good approximation of the apparent elongation constant (K_p) for tubulin assembly. The apparent elongation constant is a measure of the affinity of the growing microtubule end for an unassembled tubulin dimer.

Critical concentrations were determined by measuring microtubule assembly caused by the ligands at varying concentrations of protein in the presence of 2% DMSO (Figure 4). Because there was no detectable binding of 2′-deoxy-3′-debenzoyl-PTX to tubulin in the competition assay, this ligand was excluded from the experiments. Ideally, critical concentration measurements should be performed under conditions in which the PTX binding site is saturated with ligand. Unfortunately, 2′-deoxy-PTX is insufficiently soluble for this protocol. Instead, critical concentrations were measured at ligand:tubulin concentration ratios that provide the same fractional occupation of the PTX binding site based on the apparent equilibrium constants determined here for binding of taxane to GMPcPP-stabilized microtubules. Tubulin:taxane concentration ratios used were 1:0.5 for PTX, 1:1 for 2′-deoxy-PTX, and 1:1.3 for baccatin III. The critical concentration of tubulin in the absence of a promoter was also measured. Critical concentrations and the corresponding elongation constants and free energies of elongation are listed in Table 2. Under these experimental conditions, PTX lowers the critical concentration of tubulin by a factor of 9, which corresponds to a difference in free energy of elongation of 1.3 kcal/mol. Approximately half of this free energy difference is lost by removal of the 2′-OH group.

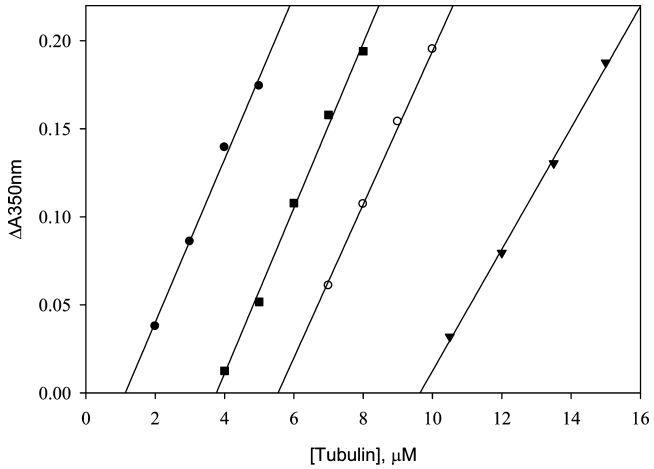

Figure 4. Critical concentration of tubulin assembly in the presence of taxanes. PTX (●), 2′-deoxy-PTX (■), baccatin III (○), and 4% DMSO (▼) were used to assemble varying concentrations of tubulin at 37 °C as described in Materials and Methods. Taxane:tubulin ratios were maintained at 1:0.5, 1:1, and 1:1.3 for PTX, 2′-deoxy-PTX, and baccatin III, respectively. The extent of assembly was measured by the increase in the apparent absorption at 350 nm (ΔA_{350}). The critical concentration was obtained from the *x*-intercept of each plot.

Table 2. Effects of Taxanes on the Critical Concentration of Tubulin Assembly

agent	C_{crit} (μM)[a]	K_p ($\times 10^5$ M^{-1})[b]	$\Delta G°_{app}$ (kcal/mol)[c]
PTX	1.1 ± 0.1	9.1 ± 0.1	−8.4 ± 0.06
2′-deoxy-PTX	3.7 ± 0.2	2.7 ± 0.1	−7.7 ± 0.03
baccatin III	5.6 ± 0.2	1.8 ± 0.05	−7.4 ± 0.02
2% DMSO	9.6 ± 0.5	1.0 ± 0.05	−7.1 ± 0.03

[a]Critical concentration of tubulin assembly at 37 °C. [b]Apparent equilibrium constant for microtubule growth at 37 °C ($K_p = 1/C_{crit}$). [c]Apparent standard free energy change at steady state.

Cytotoxicity of Taxanes. Cytotoxicities of taxanes in PC3 cells are listed in Table 3. As expected, 2′-deoxy-PTX is much less cytotoxic than PTX. A comparison of the relative cytotoxicities of the four taxanes is revealing. PTX is 350-fold more cytotoxic than 2′-deoxy-PTX, but 2′-deoxy-PTX is only ~6-fold more cytotoxic than baccatin III. Again, removal of just

Table 3. Cytotoxicities of Taxanes for PC3 Cells[a]

taxane	IC_{50} (nM)	log(IC_{50})
PTX	1.41 ± 0.32	−8.8
2′-deoxy-PTX	526 ± 82	−6.3
baccatin III	~3000	~−5.5
N-debenzoyl-2′-deoxy-PTX	>10000[b]	–

[a]Cytotoxicities of ligands were measured as described in Materials and Methods. [b]No cytotoxicity observed at 10 μM, the highest concentration tested.

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the 2′-OH group is almost as deleterious to activity as removal of the entire side chain, and removal of both the 2′-OH and 3′-benzoyl groups renders the molecule inactive.

Computational Analysis of the 2′-OH Group and *N*-Benzoyl Group of PTX. Possible binding site interactions between PTX and tubulin were probed by molecular dynamics simulations. Two different initial structures were studied: PDB entry 1JFF and T-Taxol–tubulin (coordinates from the Supporting Information of ref 27). The structures were minimized as described in Materials and Methods, and amino acid residues within 6 Å of the 2′-oxygen and 3′-nitrogen of PTX were identified (Figure 5A,B). Except for the orientation

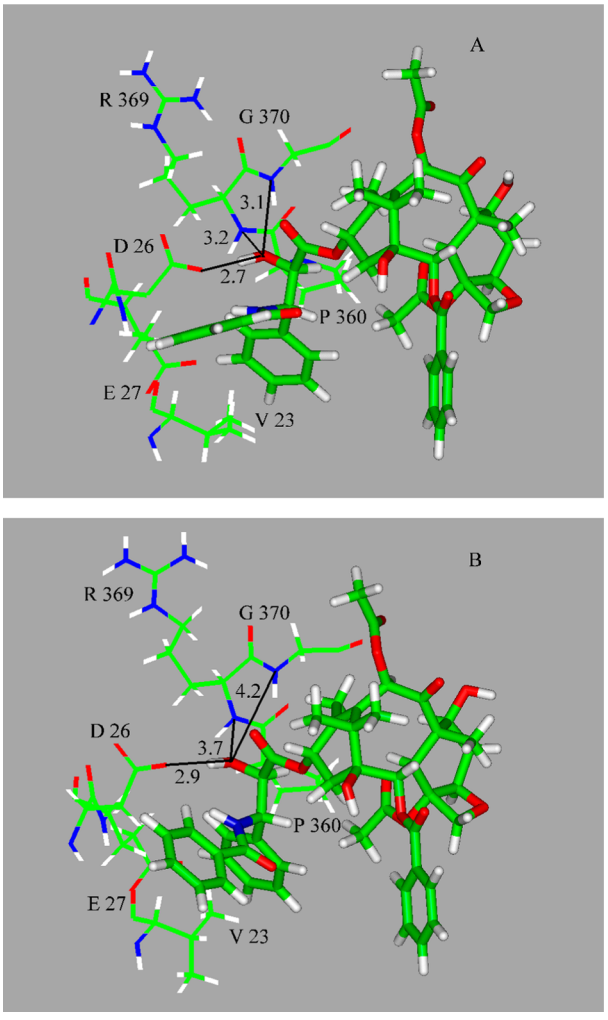

Figure 5. Illustration of PTX bound to tubulin in minimized structures from initial coordinates of PDB entry 1JFF (A) or T-Taxol (B). Residues within 6 Å of the 2′-OH group are labeled. Distances between the oxygen of the 2′-OH group and potential hydrogen bond acceptors (carboxylic acid of D26 and backbone amides of G370 and R369) are shown as solid lines.

of the 3′-benzamide, the two minimized structures are very similar. The 2′-OH group is within hydrogen bonding distance of three possible donors: backbone nitrogens of R369 and G370, previously noted by Snyder et al.,[5] and a side chain oxygen of D26, which has not been explicitly implicated in binding site models of PTX. To evaluate the candidates participating in hydrogen bonding interaction with the 2′-OH group, the two structures were subjected to long-term dynamics (10 ns, described in Materials and Methods), and the time evolutions of the distances of the 2′-OH group from the side chain of D26 and the backbone amides of G370 and R369 were recorded (Figure 6). In the 1JFF structure, the distances

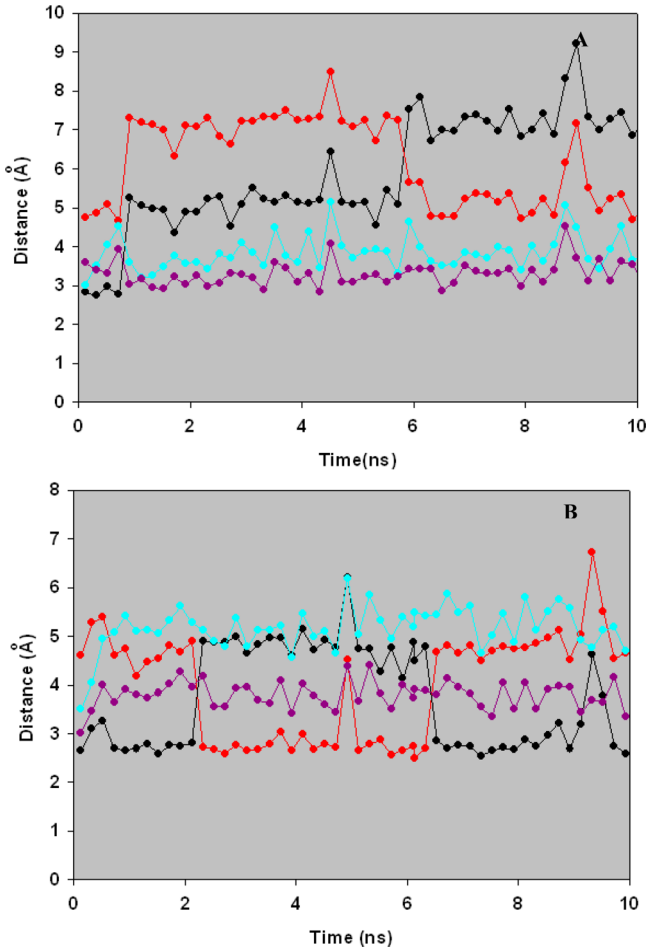

Figure 6. Time evolution of the distances between the 2′-OH group of PTX and selected atoms in the protein during dynamics. The structures from Figure 5 were subjected to a 10 ns dynamic run using AMBER 9 at 300 K as described in Materials and Methods. Distances between the oxygen of the 2′-OH group and the carboxylate oxygens of D26 (red and black) and the nitrogen atoms of the backbone amides of G370 (purple) and R369 (cyan) are shown: (A) long-term dynamics of PDB entry 1JFF and (B) long-term dynamics of the T-Taxol–tubulin structure.

between the peptide bond nitrogens and the 2′-oxygen are essentially constant over the period, while the carboxylate oxygens are quickly displaced outside of typical hydrogen bonding distance. In contrast, in the T-Taxol structure, the shortest distance over the dynamics period is between the 2′-oxygen and one of the carboxylate oxygens of D26. The carboxylate oxygens exchange positions with one another twice during the simulation. The amide nitrogen of G370 remains within ~4 Å of the 2′-OH group, while the amide nitrogen of R349 moves to a position more than 5 Å from the 2′-OH group.

The binding site within 6 Å of the 3′-nitrogen of PTX in 1JFF consisted of R369, G370, P360, D26, H229, A233, and V23. The side chain of D26 was found to be 3.1 Å from this nitrogen. In the complex of *N*-debenzoyl-2′-deoxy-PTX equilibrated with tubulin, the unsubstituted 3′-amino group

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flipped away from the side chain of D26 (Figure 7). It should be noted that the pK_a of benzylamine is 9.3, rendering the 3′-amine in *N*-debenzoyl-2′-deoxy-PTX positively charged in aqueous solutions at neutral pH. It is therefore somewhat surprising that the positively charged amine is repulsed from the carboxylate-containing binding site rather than attracted to it. It may be that the overall hydrophobicity of the binding overwhelms any advantage that would be attained from a salt bridge between the ammonium ion and the anionic side chain. To test this hypothesis, simulations were also performed with a neutral 3′-amine on the ligand. In this case, no flipping of the amino group was observed.

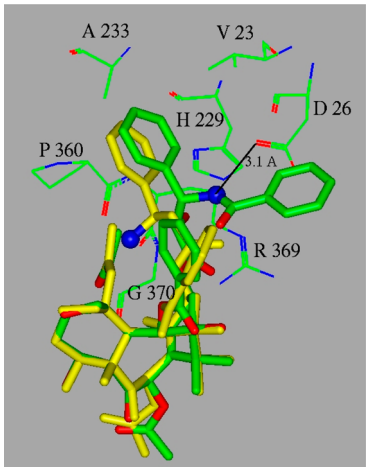

Figure 7. Schematic diagram of the β-subunit showing the microtubule binding site within 6 Å of the 3′-nitrogen of PTX in PDB entry 1JFF. The 3′-nitrogen of PTX (green) and that of *N*-debenzoyl-2′-deoxy-PTX (yellow) are shown as blue spheres. *N*-Debenzoyl-2′-deoxy-PTX was created from PTX in 1JFF by removing the 2′-OH group and the *N*-benzoyl group. The resulting structure was equilibrated, minimized, and superimposed with respect to the baccatin core on PTX in the equilibrated and minimized 1JFF structure. The side chain of D26 was found 3.1 Å (shown by the black line) from the 3′-nitrogen of PTX.

DISCUSSION

Multiple structure−activity studies have demonstrated the importance of the 2′-OH group of PTX for its microtubule assembly and anticancer activity. It was recognized more than 25 years ago that acylation of the 2′-OH group results in the loss of PTX's tubulin activity.[36] Replacement of the 2′-OH group with other functional groups yields inactive molecules, even if the proper stereochemistry is retained. All of the prior investigations into the role of this substitutent in the PTX−tubulin interaction relied on tubulin polymerization or cytotoxicity assays for activity data, which are valuable but do not specifically assess binding site interactions. In this work, the role of the 2′-OH group in the energetics of the PTX−tubulin complex has been directly examined through quantitative analysis of truncated derivatives of PTX.

The affinity of 2′-deoxy-PTX for GMPcPP-stabilized microtubules is 2 orders of magnitude lower than the affinity of PTX for microtubules. This corresponds to a difference in the standard free energy of binding between the two ligands of ∼3 kcal/mol, which is reasonable for removal of a hydrogen bonding interaction between the ligand and the receptor. Removal of the rest of the side chain has a relatively minor effect on complex formation: the affinity of 2′-deoxy-PTX for GMPcPP-stabilized microtubules is only ∼3-fold higher than the affinity of baccatin III for microtubules, and the standard free energies of binding for the two ligands differ by ∼0.7 kcal/mol. Thus, 80% of the contribution of the entire C-13 side chain to the stability of the PTX−tubulin complex can be attributed to the 2′-OH group.

This analysis does not consider the change in ligand conformational entropy that should occur when the PTX side chain is immobilized in the binding site, which will affect binding of PTX and 2′-deoxy-PTX but not baccatin III to microtubules. We believe that the conformational entropy differences between the molecules are not large enough to alter our conclusions. Buey et al. determined thermodynamic parameters for binding of PTX to cross-linked microtubules by measuring the apparent equilibrium constant as a function of temperature.[37] At 37 °C, the association is entropically unfavored by approximately −2 ± 1 kcal/mol (ΔS°_{app} = −29.3 ± 13.1 J mol^{-1} K^{-1}). Because there are a number of species that can undergo entropic changes upon binding of the ligand to microtubules, including protein moieties and water molecules, the contribution of the side chain cannot be extracted from the overall value, but in view of the fact that the unfavorable entropy change is a relatively small contribution to the overall free energy of binding for the entire PTX molecule (ΔH°_{app} = −12.3 ± 1.0 kcal/mol, and ΔG°_{app} = −10.0 ± 0.05 kcal/mol at 37 °C), the differences in ligand conformational entropies should also be minor contributors to the overall binding energetics of the ligands.

The relative differences in binding free energies for the ligands are also reflected in their efficacies. The effect of taxane binding on tubulin assembly is assessed by the ligand's effect on tubulin critical concentration, which can be converted into an apparent standard free energy of elongation. Of the 1 kcal/mol difference between the elongation free energies of PTX- and baccatin III-induced tubulin assembly, 0.7 kcal/mol can be attributed to the 2′-OH group. These numbers must be interpreted cautiously, however, because the PTX binding site is only partially occupied under the experimental conditions. Nevertheless, these data show the same trend as the binding site affinities: removal of just the 2′-OH group produces a molecule with efficacy that is more similar to that of baccatin III than to that of PTX as a promoter of tubulin assembly. Cellular effects of the three ligands parallel the in vitro tubulin results. Baccatin III is ∼3.3 log units less active than PTX, and 2′-deoxy-PTX is 2.6 log units less active than PTX. Again, ∼80% of the difference in the activities of baccatin III and PTX can be attributed to the 2′-OH group.

Coupling these results with fact that changing the substituents on the 3′-amide and 3′-carbon produces only minor alterations in the affinity and activity of taxanes,[38,39] we hypothesize that the primary role of the rest of the side chain is to properly position the 2′-OH group to donate a hydrogen bond to the receptor site.

Energy minimization revealed three candidates for the hydrogen bond acceptor in the PTX binding site: the amide nitrogens of R369 and G370 and the carboxylate of D26. Interestingly, molecular dynamics simulations identified different hydrogen bond acceptors with the two initial structures. One interpretation of these results is that the hydrogen bonds can fluctuate between the various acceptors, which seem to be the backbone amide nitrogens in PDB entry 1JFF and the carboxylate oxygens in the T-Taxol structure. If these results are considered in light of biochemical data, a different picture

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emerges. Himes and co-workers have performed a number of elegant experiments using site-directed mutagenesis on yeast tubulin. Tubulin from *Saccharomyces cerevisiae* is insensitive to PTX, and no binding to microtubules formed from the wild-type protein has been observed.[40] Five residues in the PTX binding site are different in mammalian and yeast tubulin: K19, V23, D26, H227. and F270 in mammalian β-tubulin are replaced with A19, T23, G26, N227, and Y270, respectively, in yeast. When these five residues in yeast are mutated to the mammalian amino acids, the modified protein binds PTX.[41] A subsequent study of mutants with four of the five yeast amino acids changed to their mammalian counterparts indicates that K19 or H227 can be substituted with A or N, respectively, without a loss of PTX binding. Mutants that possess the yeast amino acid at V23, D26, or F270, however, are unable to bind PTX. Entwistle et al. proposed that the F270Y mutation places a hydrophilic group in a hydrophobic pocket, close to the p-carbon on the C-3′ phenyl ring, such that both steric and electronic factors of the substitution are responsible for the lack of observed binding of PTX to this mutant.[42] The V23T mutation replaces a methyl group with a hydroxyl, which would provide a less favorable environment for the other aromatic ring on the side chain (3′-*N*-benzoyl). We propose that the deleterious effect of the D26G mutation is due to the loss of the important hydrogen bonding interaction with the 2′-OH group rather than to loss of van der Waals interaction between the side chain methylene and the aromatic ring of the 3′-*N*-benzoyl substituent.

Other biological data support the importance of D26 in the PTX binding site on tubulin. A PTX-resistant KB-3-1 line that had an D26E mutation in β-tubulin also had microtubules that are less stable than those of the unexposed cells.[43] It is not known, however, if the mutated tubulin had an altered affinity for PTX.

Gueritte-Voegelein et al.[44] found that that the removal of the *N*-benzoyl group causes a 94% loss of tubulin-assembly activity of PTX. We find here that the removal of the *N*-benzoyl group causes a complete loss of tubulin-assembly activity as well as the cytotoxicity and the microtubule affinity of 2′-deoxy-PTX. This could be attributed to the loss of hydrophobic interactions as suggested by Bode et al.[40] between the *N*-benzoyl group and methylene groups of V23, K19, and D26.[5] However, computational simulations with PTX in PDB entry 1JFF and *N*-debenzoyl-2′-deoxy-PTX created from PTX in 1JFF reveal that the removal of the *N*-benzoyl group causes flipping of the unsubstituted 3′-amino group to the exterior of the receptor site. Flipping was also observed upon simulations of PTX in the T-Taxol−tubulin structure and *N*-debenzoyl-2′-deoxy-PTX created from PTX in the T-Taxol−tubulin structure (data not shown). We therefore propose that the removal of the *N*-benzoyl group not only removes the anchoring interactions between this group and the protein but also renders the conformation of the C-13 side chain essentially destructive to the overall activity of the PTX derivative.

SUMMARY AND CONCLUSIONS

The quantitative contribution of 2′-OH group as well as the function of the *N*-benzoyl group in the association of PTX with microtubules has been studied. Removal of this group decreases the affinity, efficacy, and cytotoxicity of the taxane almost as much as deletion of the entire C-13 side chain of PTX. The importance of the 2′-OH group in the C-13 side chain is attributed to its ability to form a hydrogen bond with residue D26 on β-tubulin. It can therefore be said that this hydrogen bonding interaction is the most important interaction that PTX forms with protein in the region of the C-13 side chain. We propose that the hydrophobic substituents on the C-3′ atom and amide nitrogen function primarily to hold the side chain in a conformation conducive to the formation of a productive hydrogen bonding interaction between the 2′-OH group and the protein.

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ABBREVIATIONS

DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; G-50, Sephadex G-50; GDP, guanosine 5′-diphosphate; GMPcPP, guanylyl-(α,β)-methylene-diphosphonate; GTP, guanosine 5′-triphosphate; $MgSO_4$, magnesium sulfate; N-AB-PT, 3′-*N*-*m*-aminobenzamido-3′-*N*-debenzamidopaclitaxel; PTX, paclitaxel or Taxol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PME, 100 mM PIPES, 1 mM $MgSO_4$, and 2 mM EGTA (pH 6.90) at 25 °C.

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