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T-Taxol and the Electron Crystallographic Density in β-Tubulin

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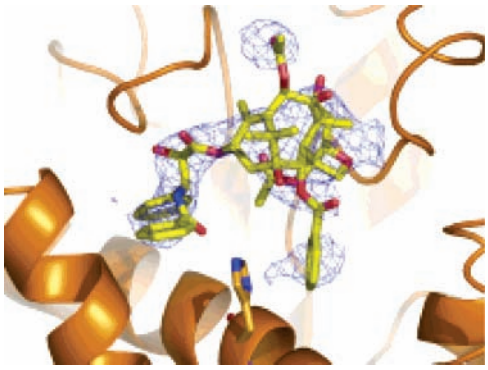
ABSTRACT



T-Taxol has been proposed as the bioactive conformation on β-tubulin and subsequently utilized in the design of a series of highly active bridged taxane analogues. A modified T-form with a reversed C-13 side chain orientation has recently been proposed as an equally plausible bioactive shape. A comparison of the two spatial alternatives within the tubulin binding site electron crystallographic density suggests strongly that T-Taxol is the bioactive conformation.

The diterpenoid paclitaxel (Taxol, PTX), first reported by Wall in 1971 in an NIH natural product screening program,[1] was recognized as a microtubule stabilizing agent only 10 years later by Horwitz and colleagues.[2] The compound became a drug of major clinical importance in the 1990s. Currently, it is used for the treatment of breast and ovarian cancers, AIDS-related Kaposi's sarcoma, and a wide variety of other cancers.[3] The compound's importance as an anticancer drug has motivated a generation of investigation into its chemistry and mechanism of action. Chemists have modified virtually every position on the baccatin core rings and on the multiple side chains.[4−6] More recent work has led to the development of several analogues of paclitaxel that are in clinical trial as second-generation taxanes.[7]

The design of simpler taxanes,[8] as well as analogues that are less toxic and capable of escaping resistance,[9] would be hastened if three-dimensional structural details concerning the binding of paclitaxel on β-tubulin could be routinely and unambiguously applied by practitioners in the field. We believe such guidance is currently available.[10] However, reservations continue to be expressed. In the present inves-

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tigation, we focus on a recent study by the Ojima−Horwitz group.[11]

The first proposals for the bioactive conformation of PTX were based on analyses in solution that derived single conformations from averaged ^{1}H NMR data. The so-called "polar"[12] and "nonpolar"[13] conformers, each involving hydrophobic collapse between one of the C-3′ phenyls and the C-2 phenyl, were both conceived to play this role. Subsequently, a similar analysis was inspiration for a "common pharmacophore" hypothesis of ligand binding on tubulin.[14] A more recent combined REDOR−NMR and fluorescence spectroscopy study likewise settled on the phenyl-clustered conformer as the bound form of PTX.[15] These propositions stimulated the syntheses of numerous bridged PTX analogues attempting to mimic the proposed bioactive conformations, only two of which match PTX in its ability to stabilize microtubules.[11,16] None of the compounds are competitive with paclitaxel as cytotoxins.[17]

A breakthrough took the form of a 3.7 Å resolution structure of the $\alpha\beta$-tubulin dimer in sheets stabilized by Zn^{2+} ions and PTX.[18] At this level of resolution, the conformation of the drug could not be determined. However, subsequent refinement at 3.5 Å[10b] and an approach characterized by coupling PTX-NMR and X-ray analysis, molecular modeling and electron crystallography (EC) defined the bioactive conformer as T-Taxol.[10a] A critical feature of the latter is the lack of clustering of distant phenyl rings in order to allow His227 to reside between the C-3′ benzamido and C-2 benzoyl phenyl groups.

Although doubts have been raised that the tubulin sheet model housing the T-Taxol conformation is representative of genuine microtubules,[11,19] several observations reinforce the proposition that the taxane binding site is shared between them. First, the T-Taxol tubulin model explains the capacity of epothilones A and B to assemble and stabilize purified yeast microtubules (*Saccharomyces cerevisiae*) by contrast with PTX's inability to do so.[20] The same model accurately predicted the mutation of five yeast tubulin binding site residues that restored paclitaxel's tubulin assembly activity.[21] Second, it is completely consistent with all known acquired tubulin mutations in resistant human cell lines in response to persistent taxane drug exposure.[9b] Third and most compelling, unlike all other previous bridging strategies, the design of bridged taxanes based on the T-Taxol conformation has achieved *both* tubulin polymerization capacities *and* cytotoxicities in several cell lines superior to PTX.[22] Since the T-Taxol geometry is predictive of activity in this context, while both types of experiments involve genuine microtubules, it implies that the microtubule and tubulin sheet binding sites are sufficiently similar to serve as mutual mimics.

Despite the observations outlined above, the T-Taxol form has recently been criticized[11] for its inability to comply with two intramolecular distances obtained by solid-state REDOR NMR experiments, measurements that initially suggested the collapsed polar form of PTX as the bound conformation.[15] Specifically, $d1$ and $d2$ in a derivative of PTX fluorinated at the *para*-position of the C-2 benzoyl phenyl (2-FB-PT, Figure 1) were reported to be 9.8 ± 0.5 and 10.3 ± 0.5 Å,

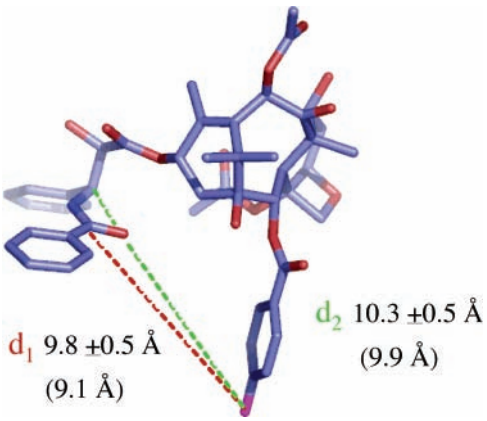

Figure 1. A 3D representation of T-Taxol illustrating the solid-state REDOR−NMR determined $^{13}C\cdots F$ distances[15] and those derived from the computationally refined tubulin/T-Taxol complex[10] (in parentheses).

respectively. According to Geney, Ojima, and co-workers,[11] T-Taxol sustains values of 8.1 and 9.3 Å, respectively, well outside the reported REDOR errors. As a result, these authors proposed a modified T-Taxol conformation ("REDOR−Taxol") as the tubulin bound form (see below). Unfortu-

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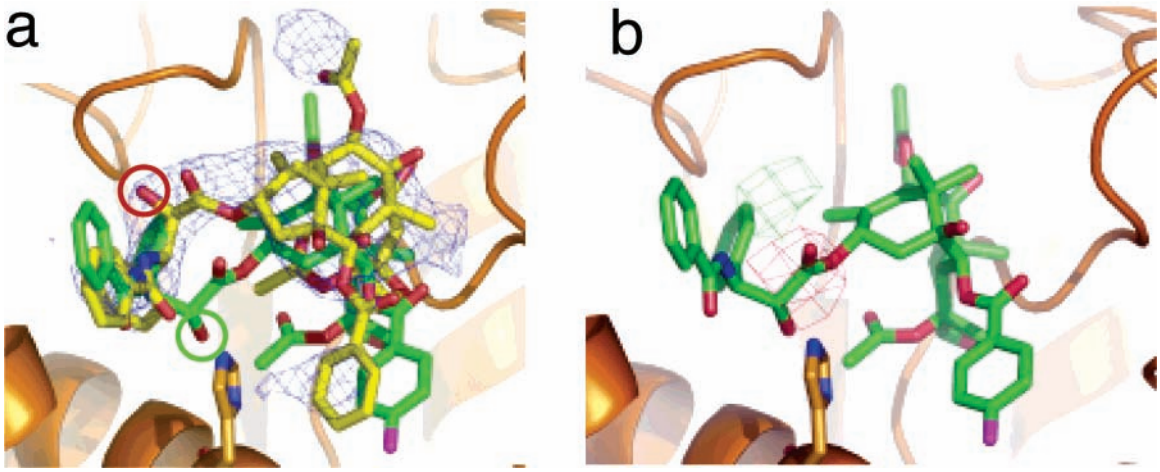

Figure 2. The structures of T-Taxol (cpk) and the New York Taxol model (PTX-NY, green) superimposed in the β-tubulin binding site. The latter structure incorporates a fluorine atom at the *para*-position of the C-2 benzoyl phenyl (i.e., 2-FB-PT). (a) The $2F_{obs}-F_{calc}$ omit map is shown as a blue 3D contour. (b) Difference maps ($F_{obs}-F_{calc}$) for the PTX-NY structure. Green corresponds to unfilled density, red to incorrectly filled density.

nately, the internal interatomic distances were taken from the refined electron crystallographic structure of Taxol−tubulin[10b] and not from the model used to derive T-Taxol.[10a] The latter sustains *d*1 and *d*2 values of 9.1 and 9.9 Å, respectively, the lower value falling outside the conservative ±0.5 Å error bar by 0.2 Å. Interestingly, the model selected by the Geney−Ojima group for further work was recorded as having *d*1/*d*2 = 10.0/9.4 Å, the second of which is also outside the reported 0.5 Å error boundary by 0.4 Å. Thus, both the Emory and New York ligand models are reasonable REDOR−Taxols. We show below that the diminutive differences between long cross-molecule distances are far less important for defining the bound conformation of the PTX ligand than the conformation of the C-13 side chain. We also demonstrate that only T-Taxol is compatible with the protein−ligand complex determined by electron crystallography.

The *xyz* coordinates of the Geney−Ojima et al. PTX binding model[11] have not been made available. However, the essential difference between the New York (PTX-NY) and the Emory models resides in the conformation of the C-13 side chain from C-1′ to C-3′. The former model can be reconstructed by adopting the dihedral angles reported for this chain of atoms[11] (Figure 2a, green conformer). In our original report, the C-2′ OH group experiences a hydrogen bond with the backbone NH of Gly370 on the loop that spans β-sheet strands B9 and B10.[10a] The corresponding OH group for T-Taxol is shown at the upper left in Figure 2a, circled in red. The new proposal involves a conformational reorientation that directs the same group toward the hydrophobic basin but within hydrogen bonding distance of His227 on Helix 7. The corresponding OH is circled in green in Figure 2a.

A necessity for the structure of molecule bound to a protein as determined by either X-ray crystallography or electron crystallography is that the ligand model fit the corresponding density as derived from experimental amplitudes and phases. Figure 2a portrays the $2F_{obs}-F_{calc}$ density map[23] as a blue contour grid as derived from the original measurements of Nogales et al.[10b,18] The 3.7 Å resolution occasions missing density around C-2 and C-10. However, strong density along the T-Taxol C-13 side chain from C-1′ to C-3′ (yellow) demonstrates that this conformer matches the available experimental data perfectly. Manual superposition of T-Taxol and New York models within β-tubulin according to the visual recipe recently reported[24] illustrates that the baccatin cores and the three terminal phenyl groups occupy similar regions of space (Figure 2a). However, while the PTX-NY conformation is a reasonable mimic of the T-Taxol binding rotamer at its extremities, the conformationally inverted C-2′ center in the modified T-Taxol (green) falls well outside the EC density associated with C-13 to C-3′.

Another means of evaluating the fit of a protein−ligand model is to generate $F_{obs}-F_{calc}$ difference Fourier maps.[23] Figure 2b displays versions of these maps for the PTX-NY conformer as positioned in Figure 2a. To focus specifically on the C-13 side chain, the maps have been truncated to this region alone. Where model atoms lie outside $2F_{obs}-F_{calc}$ contours, the $F_{obs}-F_{calc}$ map portrays them within negative (i.e., red) contours. Positive (i.e., green) contours highlight the correct locations for the same atoms. In other words, the green grid represents density derived from experiment, but unfilled by the ligand model. The red density corresponds to a region of the binding pocket that has been incorrectly filled by the taxane conformation. Taken together, the maps clearly distinguish the two conformations, indicate that the C-13 side chain of the PTX-NY structure has been

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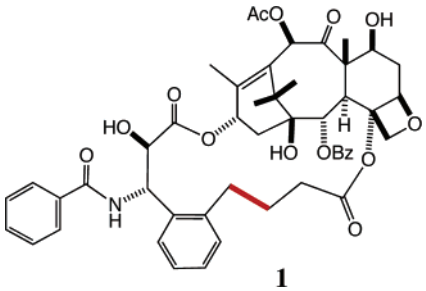
improperly positioned in the binding site, and affirm that T-Taxol is the conformer that best fits the EC density.



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To ensure that these results have not been compromised by the manual docking guided by Ojima's recent report,[24] we performed low-temperature molecular dynamics on the New York conformation for 2-FB-PT in the tubulin binding site (see Supporting Information for details). Throughout the simulations, the C2′−OH···N−His227 distance was constrained to a 2.5−3.5 Å window with the His227 side chain reorienting itself toward the C-2′ OH group. Once the MD simulations provided a stable model (see Supporting Information), the constraints were removed and full optimization of the tubulin−PTX-NY complex was performed with the MMFF94 force field holding the protein backbone fixed. The resulting *d*1 and *d*2 distances were measured at 11.0 and 10.4 Å, respectively. The corresponding $2F_{obs}-F_{calc}$ and $F_{obs}-F_{calc}$ density maps were constructed analogous to those in Figure 2. They are virtually identical in their representation that the C-13 side chain is most likely misplaced.

One other issue is pertinent. The T-Taxol conformer has been used to design highly active taxane analogues with short bridges as a result of H···H distances measured at 2.5−2.9 Å between the C-4 acetate methyl group and the *ortho*-position of the C-3′ phenyl. For example, compound **1**, incorporating a two-carbon tether between these centers, is 2−13-fold more cytotoxic than PTX and twice as effective in the tubulin aggregation assay.[22] We wondered if the New York proposal would be predictive of this taxane modification. The corresponding H···H separations in the MD/optimized structure described in the previous paragraph fall from 3.5 to 5.3 Å. While these quantities might possibly provide insight for bridging between the two centers in question, inspection of the New York conformer suggests that the location of C-2′ OH group would interfere with such a bridge. To test this idea, we inserted the two-carbon spacer shown for **1** into the conformer depicted in Figure 2b and optimized its geometry with MMFFs. As expected, the structure (**1**-NY) experienced conformational reorganization around the C-13 side chain to avoid steric clash. Superposition of the New York conformation (PTX-NY, green) and the corresponding tethered, optimized variant depicted in Figure 3 (magenta) shows that both the C-2′ OH's (red arrow) and the C-3′ phenyl groups experience serious displacement. See the Supporting Information for the parallel experiment in β-tubulin.

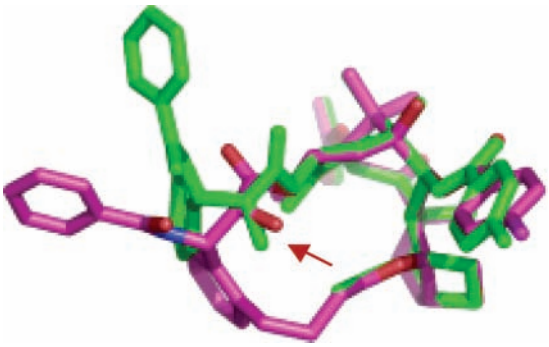

Figure 3. Superposition of PTX-NY shown in Figure 2b (green) and the same conformer enhanced with a two-carbon bridge as shown in **1** followed by MMFF94 optimization (magenta). The arrow marks the location of the two C-2′ OH groups.

In summary, the T-Taxol binding conformation matches the electron crystallographic density and is predictive of highly active bridged taxanes such as **1**. While the recently proposed C-13 variant (Figure 2B) meets neither requirement, it does have the power to suggest reasonably active alternative bridged structures.[11] However, since T-Taxol is likewise predictive of such analogues,[25] we conclude that the C-13 side chain in the New York PTX conformation is most likely misplaced, and that T-Taxol is the taxane binding form on β-tubulin most consistent with the electron crystallographic density.

Acknowledgment. We are grateful to Dennis Liotta (Emory University) for encouragement and support, and Jim Nettles (Emory University) for advice on constructing and displaying the density maps. Dedicated to Professor Iwao Ojima on his 60th birthday.

Supporting Information Available: Computational details and procedures for map generation. This material is available free of charge via the Internet at http://pubs.acs.org.

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