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Differences in Paclitaxel and Docetaxel Interactions with Tubulin Detected by Mutagenesis of Yeast Tubulin

Robert D. Winefield^a, Ruth A. Entwistle^a, Travis B. Foland^a, Gerald H. Lushington^b[Director], and Richard H. Himes^{a,*}[Professor]

^aDepartment of Molecular Biosciences University of Kansas Lawrence, Kansas 66045-7534

^bMolecular Graphics & Modeling Laboratory University of Kansas Lawrence, Kansas 66045-7582

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Paclitaxel and a semi-synthetic analogue docetaxel (Scheme) are two taxane anti-tumor agents that are used against a number of cancers.[1] The taxanes bind to the β -subunit of the dimeric protein α,β -tubulin in microtubules in a 1:1 molar ratio, resulting in a decrease in the dynamic nature of microtubules leading to mitotic arrest and apoptotic cell death.[2] The taxanes also promote the assembly of tubulin into microtubules.[3] Docetaxel is two to three times as effective as paclitaxel in promoting the assembly of mammalian brain tubulin *in vitro* and has a binding constant that is greater than that of paclitaxel by the same factor.[4] We have been using site-directed mutagenesis of *Saccharomyces cerevisiae* β -tubulin to examine the taxane binding site in tubulin. Although wild-type *S. cerevisiae* tubulin does not bind taxanes, we were able to instill taxane binding by making five mutations in β -tubulin. [5] The rationale for choosing the five sites to mutate was based on the electron crystal structure of the mammalian brain tubulin-paclitaxel complex.[6] This structure indicated that the amino acid side chains, K19, V23, D26, H227 and F270, are important in taxane binding. In *S. cerevisiae* β -tubulin these sites are occupied by different amino acids, A19, T23, G26, N227, and Y270. When we exchanged the five residues for those that occur in brain β -tubulin, yeast tubulin was able to bind paclitaxel.[5] We are currently in the process of determining the relative importance of each residue to taxane binding by systematically reversing our original mutations. As a screen to measure the effects of the changes, we are using a cell-based assay in which we examine effects of the mutations on cell proliferation. To be able to use a cell-based assay we introduced the mutated β -tubulin gene into a yeast strain that has diminished multidrug transport activity[7] to produce a strain that is sensitive to paclitaxel (AD1-8-tax).[8] In the course of these studies we found interesting differences in the sensitivity of the various strains to paclitaxel and docetaxel.

The effect of the two taxanes on the growth of the strains is presented in Table 1. The letters B and Y refer to the five residues found in brain and *S. cerevisiae* tubulin, respectively. The data show that 25 μ M of either taxane completely inhibited growth of the strain with brain β -tubulin residues at the five positions (BBBBB), but had no effect on the strain with yeast residues (YYYYY). When positions 23, 26, and 270 were changed back to the residues in yeast β -tubulin, the strains (BYBBB, BBYBB, BBBBY) were insensitive to both taxanes, indicating the importance of these residues to taxane binding. However, substituting a yeast residue at position 19 (YBBBB) or 227 (BBBYB) did not affect the sensitivity to paclitaxel,

i.e., the paclitaxel ID₅₀ values were essentially unchanged. On the other hand, the sensitivity of these two strains to docetaxel decreased. The K19A and H227N mutations caused the ID₅₀ for docetaxel to increase approximately 5 to 6-fold.

To further examine the effect of the H227N mutation, tubulin was purified from this strain (BBBYB) as well as from AD1-8-tax (BBBBB) and used in *in vitro* assembly and binding experiments. Data presented in Fig. 1 demonstrate that assembly was dependent on the taxanes and that, in the case of tubulin from strain BBBBB (Fig. 1A), the EC₅₀ values for paclitaxel and docetaxel were 1.1 μ M and 0.36 μ M, respectively. However, with tubulin from the H227N (BBBYB) strain, the values were 2.2 μ M for paclitaxel and 3.0 μ M for docetaxel (Fig. 1B). Thus, the H227N mutation increased the ED₅₀ for docetaxel 8.3-fold but had a much less effect on the ED₅₀ of paclitaxel (2-fold increase). The formation of microtubules in these experiments was verified by electron microscopy (data not shown). The effect of the H227N mutation on the tubulin-docetaxel interaction was also seen in a competition-binding assay. Preformed microtubules were incubated with 5 μ M ³H-paclitaxel and a varying concentration of docetaxel. The data in Fig. 2 show that the H227N mutation decreased the interaction of docetaxel with yeast tubulin by a factor of 3 to 4, i.e., a 3 to 4 higher concentration of docetaxel was required to decrease paclitaxel binding by 50%.

To help explain the consequences of having Asn instead of His at residue 227 on paclitaxel and docetaxel activity, we modeled the T-taxol conformation of bound paclitaxel[6] into the binding sites of the two proteins. The T-taxol conformation appears to be the most widely accepted conformation for tubulin-bound paclitaxel[11–14]. We assumed that the basic conformers for paclitaxel and docetaxel would be largely conserved relative to the T-taxol structure reported in the 1JFF crystal structure for both proteins, and thus constructed preliminary models for each of the four relevant complexes; paclitaxel-BBBBB, docetaxel-BBBBB, paclitaxel-BBBYB, and docetaxel-BBBYB, by simply editing the original crystal structure accordingly and effecting local receptor relaxation in the vicinity of the ligand.

From these computations, we derived a purely enthalpic estimate that docetaxel bound more strongly than paclitaxel to the BBBBB tubulin by 0.43 kcal/mol and that paclitaxel bound more strongly than docetaxel to the BBYB tubulin by 1.94 kcal/mol. The main source of the enthalpy difference in the BBBBB case is van der Waals interactions, which are derived from stronger interactions between the bulkier docetaxel *t*-butyl group at the 3'-*N* position with H227 and V23 than are achieved by the longer, thinner phenyl at this position on paclitaxel. In the case of the BBYB tubulin, our calculations suggest that docetaxel may lose a significant portion of its van der Waals advantage relative to paclitaxel by virtue of the His to Asn mutation (Asn is a poorer lipophile). Paclitaxel, however, might gain a binding advantage to BBYB tubulin by virtue of stronger H-bonding between its benzamide carbonyl and the side chain N227 NH donor site (2.22 Å) than is possible for the carbamate carbonyl of docetaxel (2.48 Å) (Fig. 3). The combined effects of the decreased van der Waals interaction of docetaxel and the increased H-bonding of paclitaxel to BBYB tubulin might thus result in stronger binding by paclitaxel.

In comparing the predicted binding conformers for paclitaxel and docetaxel with BBBBB and YBBBB our calculations suggest that the hydrogen atoms on the C₈ of the K19 side chain approach within 4.0 Å of the *t*-butyl group of docetaxel (a favorable distance from the perspective of van der Waals interactions) but that no K19 atom comes within 6.1 Å of any atom on the corresponding paclitaxel benzamide ring. Mutation of the lysine to a smaller alanine might thus remove a favorable lipophilic contact from docetaxel, but suggests very minimal effect on paclitaxel binding.

In summary, we have shown that the relative effects of paclitaxel and docetaxel on tubulin can be affected differently by mutations in β -tubulin. The yeast tubulin mutagenesis approach that we have developed is a useful tool in the development of new taxane and other microtubule anti-mitotic agents.

Experimental Section

Tubulin mutations and purification

Mutations were introduced into the single β -tubulin gene (TUB2) of the *S. cerevisiae* strain AD 12345678 (AD1-8)[7] using procedures described previously.[8] Thus, wild-type β -tubulin is not produced in the mutant strains. β -Tubulin in these strains also carries a His₆ tag at the C-terminus. His₆-tagged tubulin was purified using a previously developed immobilized metal chelating chromatography procedure.[5]

Yeast proliferation assay

The cell proliferation assays were conducted in triplicate in 96-well plates containing 2,000 cells/well in YPD-media (2% glucose, 1% yeast extract, and 2% peptone) supplemented with pen/strep (100 U/mL penicillin and 100 μ g/mL streptomycin) and varying concentrations of paclitaxel or docetaxel. After incubation at 30 °C for 24–27 h in a humidified chamber, the optical densities were determined at 620 nm using a plate reader.

Assembly assay

Freshly cycled tubulin (250 pmol) in 30 mM PEM (30 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, 0.5 mM GTP, pH 6.9) was assembled in a 50 μ L volume in the presence of varying concentrations of each taxane for 30 min at 30 °C. Microtubules were collected by sedimentation at 100,000 $\times$ g for 5 min in a Beckman TL-100 ultracentrifuge. The Bradford assay[10] was used to determine the protein concentration in the supernatant and in the pellet suspended in 0.1 M NaOH. Data were fitted with the sigmoidal dose response non-linear curve fitting equation found in Prism 4.03 (Graphpad Software Inc., La Jolla, CA).

Competition-binding assay

Freshly cycled tubulin was assembled (250 pmol) in 100 mM PEM (100 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, 0.5 mM GTP, pH 6.9) in a 50 μ L volume at 30 °C for 30 min. The preformed microtubules were then incubated in the presence of a mixture of 5 μ M ³H-paclitaxel (420 μ Ci/ μ mol) and a varying concentration of unlabelled docetaxel. After 30 min, microtubules were collected by centrifugation and suspended in 0.1 M NaOH. Protein was determined by the Bradford assay[10] and ³H-paclitaxel by scintillation counting. In the absence of docetaxel, incorporation of ³H-paclitaxel was 0.65 mol/mol tubulin.

Molecular modeling

All of the simulations were performed in SYBYL with the Tripos force field[15] and Gasteiger-Marsili electrostatics[16] within a non-bonding threshold of 8.0 Å. Initial structures for the ligand-tubulin complexes were constructed from the tubulin crystal structure (PDB ID 1JFF).[17] Docetaxel complexes were created by hand-editing the native paclitaxel structure. Models for mutants were created by in silico mutation within SYBYL Biopolymer toolkit. All other energetic and convergence parameters were left at default values. Interaction enthalpies were determined as the difference between the energy of the complex and the sum of the energies of the isolated ligand and receptor. To allow for relaxation effects specific to each complex, the ligand plus all residues within 8.0 Å of the ligand were permitted to relax for 100 molecular mechanics steps, with the remainder of the

tubulin structure held fixed. The position of each ligand was further optimized by allowing it to relax to molecular mechanics convergence with the entire receptor held fixed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effect of the H227N mutation on the assembly of yeast tubulin. The conditions of the experiment are described in the Experimental Section. A, strain BBBBB. B, strain BBYB. Error bars refer to standard error of the mean.

Figure 2. Effect of the H227N mutation on the displacement of ^3H -paclitaxel by docetaxel. The conditions of the experiment are described in the Experimental Section.

Figure 3.

A molecular model showing the H227 and N227 residues in the tubulin-paclitaxel and tubulin-docetaxel complexes. The position of paclitaxel and residues interacting with it are shown (black) superimposed over docetaxel and residues interacting with it (green). Helices (purple), β -strands (yellow), and loops (light blue), are shown along with oxygen (red), nitrogen (dark blue), and hydrogen atoms (light blue spheres). Helices and β -strands are labeled sequentially according to the β -tubulin sequence along with the C-3' benzamido (a), the C-3' phenyl (b), and the C2-benzoyl phenyl groups (c) belonging to paclitaxel and docetaxel. In the BBBYB complexes formation of an H-bond between the N227 side chain and the carbonyl oxygen of the paclitaxel benzamido and the docetaxel carbamate groups is shown.

Scheme.
Structures of paclitaxel and docetaxel.

Table 1

Growth inhibition by paclitaxel and docetaxel

Strain	Paclitaxel, percent ^a	Paclitaxel, ID ₅₀ , μM ^b	Docetaxel, percent ^a	Docetaxel, ID ₅₀ , μM ^b
BBBBB	97	6.3 ± 0.3	100	4.5 ± 0.1
YYYYY	0	----	0	----
YBBBB	92	4.6 ± 0.4	67	22 ± 1.0
BYBBB	0	----	0	----
BBYBB	0	----	0	----
BBBYB	100	4.3 ± 0.2	37	26 ± 2.6
BBBBY	0	----	0	----

^a Inhibition at a concentration of 25 μM.^b Standard deviations are shown.