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Characterization of the Colchicine Binding Site on Avian Tubulin Isotype β VI†

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Abstract

Tubulin, the basic component of microtubules, is present in most eukaryotic cells as multiple gene products, called isotypes. The major tubulin isotypes are highly conserved in terms of structure and drug binding capabilities. The tubulin isotype β VI, however, is significantly divergent from the other isotypes in sequence, assembly properties and function. It is the major β -tubulin isotype of hematopoietic tissue and forms the microtubules of platelet marginal bands. The interaction of the major tubulin isotypes β I, β II, β III and β IV with antimicrotubule drugs has been widely studied, but little is known about the drug binding properties of tubulin isotype β VI. In this investigation, we characterized the activity of various colchicine-site ligands with tubulin isolated from *Gallus gallus* erythrocytes (CeTb), which is ~95% β VI. Colchicine binding is thought to be a universal property of higher eukaryotic tubulin; however, we were unable to detect colchicine binding to CeTb under any experimental conditions. Podophyllotoxin and nocodazole, other colchicine-site ligands with divergent structures, were able to inhibit paclitaxel-induced CeTb assembly. Surprisingly, the colchicine isomer allocolchicine also inhibited CeTb assembly and displayed measurable, moderate affinity for CeTb ($K_a = 0.18 \times 10^5 \text{ M}^{-1}$ vs. $5.0 \times 10^5 \text{ M}^{-1}$ for bovine brain tubulin). Since allocolchicine and colchicine differ in their C ring structures, the two C-ring colchicine analogues were also tested for CeTb binding. Kinetic experiments indicate that thiocolchicine and chlorocolchicine bind to CeTb, but very slowly and with low affinity. Molecular modeling of CeTb identified five divergent amino acid residues within 6 Å of the colchicine binding site compared to β I, β II, and β IV; three of these amino acids are also altered in β III-tubulin. Interestingly, the altered amino acids are in the vicinity of the A ring region of the colchicine binding site rather than the C ring region. We propose that the amino acid differences in the binding site constrict the A ring binding domain in CeTb, which interferes with the positioning of the trimethoxyphenyl A ring and prevents C ring binding site interactions from efficiently occurring. Allocolchicine is able to accommodate the altered binding mode because of its smaller ring size and more flexible C ring substituents. The sequence of the colchicine binding domain of CeTb β VI-isotype is almost identical to that of its human hematopoietic counterpart. Thus, through analysis of the interactions of ligands with CeTb, it may be possible to discover colchicine site ligands that specifically target tubulin in human hematopoietic cells.

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SUPPORTING INFORMATION AVAILABLE

The inhibition of paclitaxel induced CeTb assembly by allocolchicine, an illustration of three dimensional alignment of tubulin-bound colchicine and podophyllotoxin, and sequence alignment of chicken erythrocyte β VI and human β I tubulin. This material is available free of charge via the internet at <http://pubs.acs.org>.

Microtubules are the target for a wide variety of substances whose utility ranges from herbicides and fungicides to anticancer drugs (1–3). The vast majority of these molecules act by binding to the protein tubulin, an α,β -heterodimer that forms the core of the microtubule. Tubulin is a highly conserved and ubiquitous protein, typically containing only 2–8% amino acid sequence divergence and found in every eukaryotic cell. Organisms possess multiple genes for each tubulin subunit, which are referred to as isotypes (4). Each tubulin isotype is also subject to posttranslational modifications, so the microheterogeneity of tubulin within a single cell can be enormous (5). Although this is true for both α and β tubulin, much more is known about β -isotypes because they contain the binding sites of clinically relevant drugs (6). The mammalian and avian β -isotypes have been classified into seven categories (5). Some of these isotypes, such as β I and β IVb, are distributed widely among different tissues (7). Others seem to be restricted to specific organs and cells. Particular interest has been paid to the β III-isotype, which is normally found in specific cells in the brain, testis and colon. Overexpression of this isotype in several types of cancer cells has been correlated with paclitaxel resistance and poor prognosis (7–11).

Although tubulin exists in multiple isotypes, its drug binding properties with respect to the antimitotic agent colchicine are almost universally conserved among higher order eukaryotes (12). The affinity of the protein for colchicine decreases in lower order eukaryotes, but even tubulin from yeast such as *Saccharomyces cerevisiae* weakly binds colchicine (13). Tubulin from bovine brain (BbTb), a common source of purified tubulin, is composed of β -tubulin isotypes β I, β II, β III and β IV in the relative amounts of 3, 58, 25 and 13%, respectively (4). Studies with mammalian brain tubulin have established that colchicine binding to tubulin is slow, highly temperature dependent and effectively irreversible. The binding is accompanied by conformational changes in the protein and unique alterations in optical spectra of colchicine (14). Intraspecies differences in colchicine binding properties of tubulin were first detected by kinetic studies. Biphasic curves corresponding to parallel reactions were observed for the association of colchicine with BbTb (15). Ludueña and coworkers demonstrated that the minor phase of the association kinetics reaction is due to colchicine binding to the β III-isotype of bovine brain tubulin (16). Subsequent drug binding measurements performed on purified isotypes demonstrated that all of the β -tubulin isotypes investigated have a slightly different affinity for colchicine and other colchicine site ligands. The differences in affinity, however, are relatively small for most ligands, normally varying from a factor of 2 to a factor of 10 difference in association constants (17–21).

The major β -tubulin isotype in hematopoietic tissue, β VI, resists the observation that the primary sequence is conserved within higher order eukaryotes (22). Referred to as β 1-tubulin when derived from mouse or human, it forms the marginal band in platelets, and its sequence divergence is thought to reflect its specialized structural role in these cells. Chick β VI tubulin can coassemble *in vivo* into microtubules in mammalian cells (23), but β 1-tubulin knock-out mice have thrombocytopenia and defective marginal bands (24). Of the seven β -tubulin categories, the β VI-isotype has the most diverse primary sequence. For example, avian β VI exhibits overall 17% divergence in its amino acid sequence compared with other avian β -tubulins. It is more hydrophobic, containing seven fewer net negative charges, and assembles *in vitro* more efficiently but with lower nucleation rate than brain tubulin (22,25). Additionally, it assembles into microtubules that are longer than those composed of brain tubulin (26). The conspicuous divergence of the β VI from all other β -isotypes prompts the study of its interaction with colchicine site drugs that bind well to the brain tubulin. Chicken erythrocytes were chosen as the tubulin source, since avian erythrocyte tubulin is ~95% β VI and the assembly properties of the pure protein have been well studied (27,28).

In this work, we investigate the effect of avian β VI sequence divergence on the colchicine binding site and drug binding properties thereof as compared to mammalian brain tubulin. The ligands explored were colchicine, allicolchicine, podophyllotoxin, chlorocolchicine, thiocolchicine, and nocodazole, all of which are known to bind BbTb at the colchicine site. The results of these studies are discussed in light of sequence differences within the colchicine binding site of β -tubulin isotypes.

MATERIALS AND METHODS

Chemicals used were analytical grade and purchased from Sigma-Aldrich. All experiments with tubulin were performed in PME buffer (100 mM Pipes, 1 mM MgSO_4 , 2 mM EGTA, pH 6.90 at 25°C) with freshly added 0.1 mM GTP. Spectrograde solvents were used in absorption and fluorescence spectroscopy. Allicolchicine, thiocolchicine and chlorocolchicine were synthesized in our lab (29). All colchicinoids and allicolchicine were pure by thin-layer chromatography and their structures were confirmed by proton NMR spectroscopy and mass spectrometry.

Stock solutions of paclitaxel, podophyllotoxin, nocodazole, allicolchicine chlorocolchicine and thiocolchicine were made in DMSO. Colchicine stock solutions were made in water or in buffer. Ligand concentrations were determined spectrophotometrically using the following extinction coefficients: paclitaxel ($\epsilon_{273 \text{ DMSO}} = 0.17 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), colchicine ($\epsilon_{353 \text{ water}} = 1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (30), allicolchicine ($\epsilon_{295 \text{ DMSO}} = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), nocodazole ($\epsilon_{324 \text{ DMSO}} = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), podophyllotoxin ($\epsilon_{294 \text{ DMSO}} = 0.45 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (31), chlorocolchicine ($\epsilon_{353 \text{ DMSO}} = 0.97 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and thiocolchicine ($\epsilon_{380 \text{ DMSO}} = 1.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (29).

Tubulin Purification

BbTb was prepared by two cycles of temperature dependent assembly-disassembly as described by Williams and Lee (32). The protein solution from the end of the second cycle was subjected to ion exchange chromatography on phosphocellulose resin to yield purified tubulin.

Chicken erythrocyte tubulin (CeTb) was isolated from whole blood purchased from Pel-Freez Biologicals (Rogers, AR) using a procedure adapted from Sackett (33). Briefly, the erythrocytes were pelleted at $2500 \times g$ for 10 min at 4°C and washed with 0.9% saline. The pellet was resuspended in 0.6 volume of PMCG (100 mM PIPES, 1 mM MgCl_2 , 2 mM CaCl_2 , 0.1 mM GTP). The suspension was sonicated at 4°C to disrupt the cells and centrifuged at $10,000 \times g$ at the same temperature for 30 min. The supernatant was incubated with DE52 chromatography resin previously equilibrated with PME. The resin was washed with PME to remove hemoglobin and centrifuged at 1,250 rpm in an IEC Centra-7R refrigerated centrifuge at 4°C for 1 min to pellet the resin. This was followed 3 washings and centrifugations with PMCG buffer supplemented with 0.25 M glutamate buffer. Finally tubulin was eluted using 0.75 M glutamate in PMCG buffer. The CeTb was pure by SDS-PAGE.

The MAP-free BbTb and CeTb were drop frozen in liquid nitrogen. Prior to use, the frozen pellets were gently thawed and then desalted into PME buffer by the method of Penefsky (34). The concentration of BbTb or CeTb was determined spectrophotometrically using an extinction coefficient of $1.23 (\text{mg/mL})^{-1} \text{ cm}^{-1}$ at 278 nm in PME buffer (35).

IC₅₀ Determination

The IC₅₀ of the assembly inhibiting agent is defined as the total concentration of inhibitor required to reduce the extent of tubulin polymerization by 50% of its value in the absence of added inhibitor. The procedure was as follows: BbTb or CeTb (5 μM) in PME was incubated

with varying concentrations of colchicine, podophyllotoxin or nocodazole at 25°C for 90, 25 and 40 min, respectively, in the presence of 0.1 mM GTP. The incubated protein samples were equilibrated to 37°C in the sample cells of a Hewlett-Packard 8453 UV-Vis spectrophotometer with a thermostatted multicell holder and a baseline was recorded. Paclitaxel in DMSO was added to a concentration of 5 μ M to initiate polymerization. The final concentration of DMSO was kept at 2% (v/v). The extent of polymerization was assessed as the plateau value of apparent absorption at 400 nm for colchicine and 350 nm for other ligands. The IC₅₀ values were calculated from nonlinear regression fits of sigmoidal plots of percent polymerization vs. ligand concentration using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA).

Fluorescence Spectroscopy

All fluorescence measurements were performed in a Jobin Yvon Fluorolog 3 spectrofluorometer in a thermostated cell. A 2 × 10 mm fluorescence cell was oriented such that the excitation beam passed through shorter pathlength and the temperature was maintained at 25°C using a circulating water bath.

Measurement of Association Kinetics

Colchicine—The kinetics of colchicine binding to tubulin were monitored by increase in colchicine fluorescence or decrease in intrinsic protein fluorescence as a function of time. Experiments monitoring fluorescence enhancement of colchicine in the presence of CeTb or BbTb were carried out as follows: A solution of colchicine (150 μ M) in PME containing 0.1 mM GTP was equilibrated in fluorescence cell in the spectrofluorometer. Tubulin was added to the final concentration of 1 μ M. The sample was rapidly mixed and the shutters were opened to monitor the fluorescence. The excitation and emission wavelengths used were 340 nm and 440 nm, respectively. The kinetics of intrinsic fluorescence quenching of tubulin in the presence of colchicine were measured as described above except that colchicine was added to a pre-equilibrated tubulin solution and the excitation and emission wavelengths used were 295 nm and 335 nm, respectively.

Allocolchicine—The association of allocolchicine with tubulin as a function of time was observed by monitoring the increase in allocolchicine fluorescence that accompanies tubulin binding (36). All measurements of allocolchicine association kinetics were performed under the same conditions as that for colchicine fluorescence enhancement kinetics except that 10% DMSO was included in the samples to ensure the solubility of ligand. The excitation and emission wavelengths used were 330 nm and 400 nm, respectively.

Thiocolchicine and Chlorocolchicine

The kinetics of thiocolchicine or chlorocolchicine binding to tubulin were assessed by recording the decrease in tubulin fluorescence as function of time, excitation and emission wavelengths of 295 nm and 335 nm, respectively. The experiments with CeTb were performed in the same manner as for colchicine except that DMSO (10 % v/v) was included in the samples to promote the solubility of ligands. Kinetics of ligand binding to BbTb were measured using 20 μ M ligand because the chlorocolchicine reaction occurred too quickly at the higher concentration to accurately measure on the spectrofluorometer. The apparent second order rate constants for the colchicinoids binding to BbTb were comparable with those measured previously (30,37).

Kinetic Data Analysis

The data of increasing ligand fluorescence or decreasing protein fluorescence as a function of time were fit to double exponential for BbTb:

$$F_t = Ae^{-\alpha t} + Be^{-\beta t} + C$$

where F_t is the fluorescence at time t , α and β are the observed pseudo-first-order rate constants of the fast and slow phases, respectively, A and B are the amplitudes of these two phases, and C is an integration constant. When the data were from increasing fluorescence intensity, F_t was the observed fluorescence intensity at each time interval after subtraction of the initial fluorescence value. For data of decreasing fluorescence intensity, F_t was defined as the absolute value of the fluorescence at time zero minus the fluorescence value at each time interval. The parameters A , B , α , and β were obtained by nonlinear curve fitting software from SigmaPlot 10.0. The apparent second order rate constant for the fast phase for each ligand ($k_{on,app}$) was calculated as $k_{on,app} = \alpha/c$ where ' c ' is the total concentration of the ligand.

The fluorescence data as a function of time for ligand binding to CeTb were fit to a single exponential

$$F_t = Ae^{-\alpha t} + C$$

where F_t is the fluorescence at time t , defined in the same manner as for the BbTb experiments, α is the observed pseudo-first-order rate constant, A is the amplitude, and C is an integration constant. The data were fit using SigmaPlot and the apparent second order rate constants for each ligand were calculated as described above.

Association of Alcolchicine with Tubulin: Equilibrium Measurements

The association constant of alcolchicine for BbTb or CeTb at 25°C was determined by fluorescence titration. Samples of BbTb or CeTb (1 μ M) in PME and 0.1 mM GTP were incubated with varying concentrations of alcolchicine at 25°C for 90 min. The samples were excited at 315 nm and the emission intensity at 400 nm was measured. The final DMSO concentration was maintained at 10%. Blank spectra were taken of the buffer without fluorophore and subtracted to correct for background fluorescence.

The emission intensity of alcolchicine was corrected for inner filter effects as described by Lakowicz (38) using the equation:

$$F_{corr} = F_{obs} \text{ antilog } [(A_{ex} + A_{em})/2]$$

where F_{obs} is the observed emission intensity of alcolchicine, F_{corr} is the emission intensity corrected for inner filter effect, A_{ex} and A_{em} are the absorbances of alcolchicine at the excitation (315 nm) and emission (400 nm) wavelengths respectively. A_{ex} was divided by 5 before applying to the equation, to account for the shorter path of the excitation beam.

The corrected data were then analyzed according to the following equation (36):

$$\alpha/[allo]_{free} = -K\alpha + nK$$

where α = fraction of tubulin bound, or $[complex]/To$, where To is the total tubulin concentration, $[allo]_{free}$ is the concentration of the unbound ligand (calculated as $[allo]_{free} = [allo]_0 - \alpha To$, where $[allo]_0$ is the total alcolchicine concentration), n (number of binding sites) = 1, and K is the association constant.

The fraction of tubulin bound (α) was determined by:

$$\alpha = F/F_{\max}$$

where $F_{\max}$ is fluorescence intensity at saturating concentration of allocolchicine and F is the fluorescence measured at any allocolchicine concentration.

Inhibition of Allocolchicine Binding to Tubulin by Podophyllotoxin

Podophyllotoxin inhibits allocolchicine binding to tubulin by blocking the binding site (39). To confirm the binding of podophyllotoxin as well as allocolchicine to CeTb at the same discrete binding site, we compared the binding of allocolchicine to CeTb in the presence and absence of podophyllotoxin. A solution of CeTb (1 μ M) in PME buffer containing 0.1 mM GTP was incubated with 150 μ M of podophyllotoxin in the fluorescence cell at 25°C for 25 min. Allocolchicine was added to the final concentration of 150 μ M and the kinetics of binding were followed as described above. The DMSO concentration was limited to 10%. For a reasonable comparison, similar experiment was performed with BbTb except that the concentration of podophyllotoxin and allocolchicine were 50 μ M each.

Sequence Alignment and Structural Superimposition of Chicken Erythrocyte Tubulin on Mammalian Brain Tubulin (1SA0)

The sequence and structure of BbTb bound to colchicine was downloaded from the RSCB Protein Data Bank (1SA0). The sequences of chicken tubulin isotype β VI and human isotype β 1 were downloaded from the NCBI data base (accession numbers P09207 and NP_110400, respectively). Linear sequence alignment was performed using Clustal W2 (European Bioinformatics Institute). The 1SA0 coordinates of the $\alpha\beta$ dimer including GTP, GDP, and colchicine (SH group of the CN2 ligand was replaced by H) were used as starting coordinates for minimization of the mammalian brain tubulin dimer. For the minimization of the avian β VI dimer (α 1, β VI), substitutions were made in the 1SA0 β -chain using the sequence of the chicken β VI-isotype and α -chain was kept the same as in 1SA0 structure. The AMBER 9 suite of programs (Scripps Research Institute) was used to perform the minimizations. To neutralize the systems, 33 and 31 Na^+ ions were added to the mammalian brain and avian β VI dimer structures, respectively. Both structures were immersed in TIP3P boxes of water extending 15 Å from the surface of the dimers. The minimizations were performed using periodic boundary conditions. The minimized chicken erythrocyte tubulin structure was superimposed on the minimized mammalian brain tubulin structure using the DS Visualizer by Accerlys (San Diego, CA). Residues belonging to the β -chains within 6Å from any atoms of the colchicine were identified in both the proteins. Both three dimensional structures were aligned and the extended colchicine binding domains, encompassing the S4, S5, S6, S8 and S9 sheets, H7 and H8 helices and the T7 loop as described by Ravelli et al. (40) were searched for divergent residues.

We considered only the β subunits of the proteins for our study because the α subunits do not differ significantly across the isotypes (4). Additionally, we aligned the α 1 of chicken tubulin with the α subunit of 1SA0 and did not observe difference in α -tubulin sequence close to the colchicine binding site.

RESULTS

Linear Alignment and Structure Superimposition

Linear sequence alignment of avian tubulin β VI chain with the β chain of 1SA0 reveals 67 divergent residues across the entire length of avian β VI (Figure 2), the highest proportion of which is in the hypervariable C-terminus. A three dimensional structure for avian β VI-tubulin

was constructed from the 1SA0 crystal structure as described in *Materials and Methods*. Three dimensional structure superimposition of the two β -tubulin chains shows that a total of 36 residues fall within 6 Å of colchicine. This region is comprised of residues 198, 200, 235–240, 245–246, 248–257, 311–317, 345, 347–352, 374 and 376. Of these 36 residues, 5 substitutions are observed in the β VI-isotype: Y200-F, C239-S, A315-C, V316-I and T351-V (Figure 3). The divergent residues are located in helices H7-H8 and sheets S8-S9 (Figures 2 and 3). The alignment shows 2 further mutations in the extended colchicine binding domain: T136-I in S4 and A231-L in H7 (Figure 2).

The colchicine binding region defined here differs slightly from that assigned by Huzil et al., which contains 29 amino acids (41). The only significant difference between the two models is that Y200-F is not included in their binding site sequence.

Effect of Colchicine on CeTb Polymerization

The effect of colchicine on the ability of CeTb to assemble in the presence of paclitaxel was investigated and compared to that of BbTb under identical conditions. CeTb has a lower critical concentration than BbTb and has a greater tendency to form oligomers, which slow the rate of assembly (26,27). In order to minimize the amount of oligomers in solution, low tubulin concentrations were employed. CeTb assembled in the presence of paclitaxel formed normal microtubules, which was confirmed by electron microscopy (data not shown). CeTb and BbTb assembly was monitored by apparent light scattering as described under *Experimental Procedures*. Colchicine decreased both the rate and extent of BbTb assembly, as expected (data not shown). Colchicine did not, however, affect either the rate or extent of CeTb polymerization at any of the concentrations tested. No inhibition of CeTb polymerization was observed at colchicine concentrations up to 140 μ M (Figure 4A). A well defined dose-response curve (Figure 4b) yields an IC_{50} of 2.7 ± 0.03 μ M for BbTb. (Table 1).

Kinetics of Colchicine Binding to CeTb

Two possibilities were considered for the absence of an inhibitory effect of colchicine on CeTb assembly: either the ligand does not bind well to the colchicine site, or binding occurs but the resulting complex does not inhibit CeTb assembly. Colchicine fluorescence emission intensity is enhanced and tubulin fluorescence is quenched upon complex formation (12). Therefore, both signals were probed to detect colchicine binding to CeTb. The results were compared to those from colchicine binding to BbTb under identical conditions. Figure 5A shows that there is negligible enhancement in the fluorescence of colchicine upon incubation with CeTb relative to that with BbTb. Tubulin-bound fluorescence of colchicine is due in part to a highly rigid environment for the ligand after binding (42), which may be different for CeTb. Many colchicine-site ligands quench BbTb fluorescence, and this signal may also be used to monitor ligand binding (12). Colchicine does not, however, significantly quench the intrinsic fluorescence of CeTb (Figure 5B). Both of these results indicate a lack of colchicine binding to CeTb.

Effect of Podophyllotoxin on Paclitaxel-Induced Tubulin Assembly

Unassembled CeTb may contain a substantial amount of oligomers, particularly at high protein concentrations (25). Colchicine binds to soluble tubulin but not to microtubules (43). Therefore, one explanation for the lack of colchicine binding activity in CeTb is that the aggregation state of the protein in CeTb does not allow access to the colchicine binding site. Schönbrunn et al. showed that podophyllotoxin is able to dissolve CeTb ring aggregates, and they concluded that podophyllotoxin is probably able to bind to tubulin aggregates (44). The effect of podophyllotoxin on the assembly of CeTb was therefore assessed. Figure 6A shows that podophyllotoxin causes a concentration dependent decrease in CeTb assembly, and the data fit a normal dose-response curve (Figure 6B). The IC_{50} for podophyllotoxin inhibition of

CeTb assembly is about 15-fold greater than that determined for BbTb under the same experimental conditions (Table 1).

Effect of Nocodazole on Paclitaxel-Induced Tubulin Assembly

Nocodazole is an antimicrotubule agent without apparent structural similarity to colchicine that is a competitive inhibitor of colchicine binding to tubulin (45). Like colchicine, the affinity of nocodazole for tubulin varies with the β -tubulin isotype, and each drug binds with lowest affinity to $\alpha\beta$ III-tubulin (18,21). It was therefore of interest to determine whether the similarity between nocodazole and colchicine binding would extend to the predominant α I/ β VI-isotype in CeTb. The effect of nocodazole on CeTb and BbTb assembly was assessed (Figure 7). Nocodazole is less potent than podophyllotoxin as an inhibitor of CeTb assembly (Table 1), but the difference in the effect of drug on CeTb and BbTb assembly is smaller for nocodazole than for podophyllotoxin (~11-fold vs. ~15-fold).

Effect of Alcolchicine on CeTb Polymerization

Alcolchicine is a structural isomer of colchicine whose interaction with BbTb has been thoroughly documented (36,46). Overall the characteristics of the associations of the two molecules with BbTb are very similar, but the mechanistic steps to form the final complexes are significantly different. The effect of alcolchicine on the assembly of CeTb was therefore examined. The IC_{50} of CeTb assembly inhibition by alcolchicine could not be quantified because of limited solubility of ligand under the experimental conditions (5 μ M CeTb and 2% DMSO). However, when the tubulin concentration was dropped to 2 μ M, inhibition of tubulin assembly by alcolchicine was observed at soluble concentrations (Supplementary Material, Figure S1). It was found that 20 μ M alcolchicine causes complete inhibition of 2 μ M CeTb assembly in the presence of 2 μ M paclitaxel. The IC_{50} value for alcolchicine inhibition of 2 μ M CeTb assembly is about 10-fold greater than the IC_{50} for BbTb under the same conditions (Table 1).

Association of Alcolchicine with CeTb

The association of alcolchicine with CeTb was studied in terms of the effect of CeTb on alcolchicine fluorescence, and the data were compared with those of alcolchicine binding to BbTb. Like colchicine, the fluorescence of alcolchicine is dramatically enhanced upon binding to bovine brain tubulin. Unlike colchicine, the fluorescent properties of the tubulin bound species can be emulated by solvent (36). The effect of CeTb and BbTb on the fluorescence of alcolchicine was compared (Figure 8). Both sources of tubulin enhance the fluorescence of alcolchicine. The emission intensity of alcolchicine in the presence of CeTb is about 5-fold less than that of alcolchicine-BbTb under identical experimental conditions. The lower intensity of alcolchicine-CeTb could be due to a number of factors: a difference in the association constants of alcolchicine for the two tubulins, differences in the quantum yields of the complexes, presence of inactive forms of CeTb in the solution, or a combination of these. Note that the emission maximum of alcolchicine with CeTb is blue shifted 5 nm from that of alcolchicine-BbTb, which indicates that the environment experienced by alcolchicine with CeTb is more hydrophobic than its environment in the colchicine binding site of BbTb (36). This observation is in agreement with the amino acid substitutions in the β IV-isotype binding site, which are considerably more hydrophobic than that of β I, β II, and β IV-isotypes (grand average of hydrophobicity for YCAVT and FSCIV = 1.30 and 2.64, respectively).

The difference in fluorescence intensity of bound alcolchicine over that of free ligand allows the ligand emission at 400 nm to be used to monitor its association with tubulin. The association kinetics of alcolchicine with CeTb and BbTb were studied under pseudo-first-order conditions by monitoring the increase in ligand emission intensity as function of time. As seen

in Figure 9, allocolchicine binding to BbTb is much faster than allocolchicine binding to CeTb. Under identical conditions, the binding with BbTb attains a plateau within 600 sec whereas that with CeTb attains a plateau in 1300 sec. The association of allocolchicine with CeTb is also monophasic, whereas the association with BbTb is biphasic, as expected. At 25°C the association rate constant for CeTb is $14.5 \pm 0.09 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). For BbTb, the association rate constant for the fast phase of allocolchicine binding is $343 \pm 7 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2), which is in accordance with previous experimental results (36).

Enhancement of allocolchicine fluorescence upon tubulin binding was also used to assess the affinity of allocolchicine for CeTb. Emission intensity was recorded as a function of allocolchicine concentration with a fixed concentration of tubulin at 25°C. The fluorescence enhancement plateaus at 150 μM for 1 μM of CeTb (Figure 10). A similar experiment with BbTb shows that that fluorescence enhancement of plateaus at about 20 μM (Figure 10). The fluorescence titrations of allocolchicine binding to CeTb and BbTb were fit to a single site isotherm, which yielded association constants of $0.18 \times 10^5 \text{ M}^{-1}$ and $5.0 \times 10^5 \text{ M}^{-1}$ for CeTb and BbTb, respectively.

Inhibition of Allocolchicine Binding to CeTb by Podophyllotoxin

Comparing the characteristics of allocolchicine binding to CeTb to those of allocolchicine binding to BbTb assumes that the ligand is occupying the colchicine binding site on both proteins. Since podophyllotoxin is a competitive inhibitor of colchicine binding to BbTb (47), the effect of podophyllotoxin on allocolchicine binding to CeTb was assessed. Figure 11 shows the kinetics of allocolchicine association with CeTb or BbTb in the presence and absence of podophyllotoxin. Preincubation of tubulin with podophyllotoxin causes complete inhibition of allocolchicine binding to CeTb.

Kinetics of Chlorocolchicine and Thiocolchicine Binding to BbTb and CeTb

The difference between the CeTb-inactive molecule colchicine and the CeTb-active molecule allocolchicine is the structure of the C ring. We wished to explore whether a less dramatic alteration in the C ring region of a colchicinoid could produce a molecule with CeTb activity. Thiocolchicine (Figure 1) differs from colchicine by one atom in the C ring substituent; this alteration slightly increases the rate constant and equilibrium constant for BbTb binding (30). Chlorocolchicine (10-demethoxy-10-chlorocolchicine) is slightly more potent than colchicine as an inhibitor of BbTb assembly (29). The rates of the three colchicinoids binding to tubulin at a single concentration and temperature were measured under pseudo-first order conditions (20 μM colchicinoid, 1 μM BbTb). Figure 12A shows the kinetic traces of the ligands binding to BbTb. The biphasic curves for ligands binding to BbTb were fit to the appropriate equation (see *Materials and Methods*), and the second order rate constants of the fast phase were calculated (Table 2). The kinetic experiments were repeated with CeTb (Figure 12B), except that the ligand concentration was increased to 150 μM . Colchicine caused very little quenching of CeTb fluorescence over the nearly 2 hour period of the experiment, but the data for the two other colchicinoids could be fit to a single exponential decay and rate constants were calculated (Table 2). Under these experimental conditions, the binding rates for the colchicinoids are in the order of chlorocolchicine > thiocolchicine > colchicine for both sources of tubulin.

DISCUSSION

Colchicine binding to mammalian tubulin is both unique and ubiquitous. The specificity of colchicine binding to tubulin over all other cellular proteins was demonstrated when tubulin was first identified as the primary protein of the microtubule as well as the colchicine binding protein in the cell. All mammalian tubulin examined to date is able to bind colchicine (12). The idea that there might be some heterogeneity in mammalian tubulins toward colchicine was

confirmed by Ludueña's group, who developed antibodies to different β -tubulin isotypes. The second kinetic phase for colchicine binding to brain tubulin was shown to be due to the β III-isotype, which makes up about 25% of neural β -tubulin (48). Equilibrium binding data show that molecules that bind to the colchicine site on tubulin also bind with different affinities to various β -isotypes (18,21,49). A common theme to the colchicine site binding ligands is that the slowest, weakest binding occurs with the β III-isotype. This is the most diverse isotype in tubulins isolated from mammalian brain and is the only one that shows heterogeneity at the colchicine binding site. The colchicine binding site of β III-isotype, defined as a region within 6 Å of colchicine, differs from β I, β II, and β IV-isotypes at 3 positions: C239-S, A315-T, and T351-V. Biochemical studies have identified C239 as an important amino acid in the binding site (50), and Joe et al. propose that this difference could be a key feature in the lower affinity of β III-isotype for colchicine (51). Huzil et al. postulate that replacement of the smaller amino acids A315 and T351 with T and V, respectively, may cause disruption in the interactions with adjacent helices 9 and 10, resulting in an overall narrowing of the colchicine binding site and thereby reducing the affinity for colchicine (41).

Our modeling of the β VI-isotype identifies 5 substitutions in the colchicine binding site: Y200-F, C239-S, A315-C, V316-I and T351-V, which are located in helices H7-H8, sheets S8-S9 and loop T7. The three variant positions in β III-isotype are also different in β VI-isotype, although the amino acid at position 315 is C in β VI-isotype and T in β III-isotype. Within the colchicine binding site, the differences between β III-isotype and β VI-isotype are very subtle: loss of a hydroxyl (Y-F), addition of a methyl (L-I), and substitution of an oxygen with a sulfur atom (T-C). Yet these changes are sufficient to render colchicine binding to β VI-isotype undetectable. The apparent lack of colchicine binding to CeTb therefore may be due in part to a further narrowing of the colchicine binding site relative to that of β III-isotype by the additional methyl group at position 316 and the larger sulfur atom at position 315.

The A ring of colchicine has been described as an "anchor" for the placement of the C ring of the molecule within the binding site (52). The effect of a colchicine site ligand on tubulin conformation is dictated by interactions between the C ring domain of the binding site on tubulin and the inhibitor. Podophyllotoxin and colchicine exemplify this statement. Both ligands produce the same end result – inhibition of tubulin assembly – by binding to the same region of the protein. However, the path by which the inhibitory complex is formed and the nature of the final complex is very different for the two ligands. Colchicine binds to tubulin in a two step process that is revealed by evaluation of the concentration dependence of the apparent second order rate constant (15). A tubulin conformational change occurs in the slow unimolecular second step of the process, and this conformational change can be detected by multiple techniques. In contrast, podophyllotoxin binds to tubulin in a single bimolecular reaction (47), and the conformation of the podophyllotoxin-tubulin complex cannot be differentiated from that of unliganded tubulin by direct means (e.g., spectroscopic changes, tubulin GTPase activity) (14).

Although they share a common structural feature, podophyllotoxin and colchicine do not occupy the binding site on tubulin in the same way. In fact, the trimethoxyphenyl rings of colchicine and podophyllotoxin bound to tubulin are almost perpendicular to each other in the crystal structures (see Figure S2 in Supplementary Material). Colchicine derivatives, though, would be expected to have very similar orientations in the binding site owing to the high rigidity of the fused tricyclic scaffold. The constricted nature of the A ring domain of the CeTb binding site may not allow the molecule to penetrate as deeply into the protein, and thus stabilizing interactions between the tropone ring and the protein may not be accessible to the ligand in the narrower β VI-tubulin binding site. Allocolchicine may be able to make some productive contacts with the protein because of the relatively flexible methyl ester and therefore possess some CeTb binding activity. The C-10 substituent of thiocolchicine is larger than the methoxy

group of colchicine, but the C-10 heteroatoms of both thio- and chlorocolchicine are more polarizable, which may account for the weak CeTb binding observed for these two ligands.

The data presented here may have bearing on clinical applications of colchicine site drugs. It has previously been suggested that hematopoietic mammalian $\beta 1^*$ -tubulin is the equivalent of avian βVI , but it was also observed that the two proteins contained many differences in amino acid sequence (53). When the sequences for human $\beta 1$ (NP_110400), and avian βVI (P09207) are aligned, we find that, although there is significant sequence divergence, the colchicine binding site and the colchicine binding domain as defined in this manuscript contain only 1 non-conservative mutation (Figure S3 in Supplementary Material). In mammals, $\beta 1$ -tubulin expression occurs in mature megakaryocytes and blood platelets, up to 90% of the total β -tubulin content in these cells being the $\beta 1$ -isoform (54). Transgenic mice lacking $\beta 1$ -tubulin have fewer platelets than wild type mice (24). These platelets also have reduced microtubule content and are structurally and functionally defective. It has long been known that one of the side effects of many microtubule-active drugs is myelosuppression, and it is possible that the hematological toxicity of these drugs may be the result of their effect on microtubules of hematopoietic cells (55,56). Although there are differences in the overall sequences of the two β -tubulins (16% nonidentical), only two of the 36 amino acids surrounding the colchicine binding site on βVI -tubulin is different in human $\beta 1$ -tubulin: Val236 and Glu198 in chicken βVI -tubulin are Ile and Ala in human $\beta 1$ -tubulin, respectively. It stands to reason that human $\beta 1$ may exhibit the same divergent ligand binding behavior seen in avian βVI . Indeed, it has been recently shown cells containing human $\beta 1$ are insensitive to the colchicine binding site ligand 2-methoxyestradiol (57). Thus, measurements of ligand binding to CeTb may provide a way to predict hematological toxicity of potential new colchicine site drugs and a screen for potential molecules for targeted chemotherapy of hematopoietic cell disorders.

CONCLUSION

In this manuscript we have provided evidence that avian βVI tubulin possess unique drug binding characteristics with respect to the colchicine site that are remarkably different from any other higher order eukaryotic tubulin studied to date. We could not find any evidence of colchicine binding up to the concentrations tested in any of our assays. Whether CeTb will bind colchicine at higher concentrations is currently under investigation in our lab, but regardless, it is at least the first report of a higher eukaryotic tubulin that exhibits this behavior.

Interestingly, although the colchicine site in avian βVI does not appear to bind colchicine, it maintains an ability to bind to other colchicine site ligands, which also inhibit microtubule polymerization. This suggests that the binding site on βVI has not lost function, but is simply different than the site on other tubulins with regard to shape, electronics, or both. With this in mind, it is possible that small molecules can be found which will preferentially bind to the avian βVI colchicine over other tubulins. This could translate into a targeting mechanism for hematopoietic tissue *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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*Not the same as class I β , which is also called βI in some nomenclature systems. See reference ⁵ for a discussion of tubulin isotype nomenclature.

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Abbreviations

ATP	Adenosine 5'-triphosphate
BbTb	bovine brain tubulin
CeTb	chicken erythrocyte tubulin
DEAE	diethylaminoethyl cellulose
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
G-50	Sephadex G-50
GTP	guanosine 5'-triphosphate
MAPs	microtubule associated proteins
MgSO ₄	magnesium sulfate
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PME	0.1 M PIPES, 1 mM MgSO ₄ , and 2 mM EGTA, pH 6.90 at 25°C.

R = OCH₃: Colchicine
SCH₃: Thiocolchicine
Cl: Chlorocolchicine

Podophyllotoxin

Nocodazole

Allocolchicine

FIGURE 1.

Structures of colchicine, chlorocolchicine, thiocolchicine, podophyllotoxin, nocodazole and allocolchicine.

1SA0	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGSYHGSDSLQLERINVYYNEAAGNKYV	60
TUBB6_CHICK	MREIVHLQIGQCGNQIGAKFWEVISDEHGIDIAGNYCGNASLQLERINVYFNEAYSHKYV	60
	* * * * *	
1SA0	PRAILVDLEPGTMDSVRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV	120
TUBB6_CHICK	PRSILVDLEPGTMDSVRSSKIGPLFRPDNFIHGNSGAGNNWAKGHYTEGAELIENVMDVV	120
	* * * * *	
1SA0	RKESESCDCLQGFQLTHSLGGGTGSGMGTLISKIREEYPDRIMNTFSVVPSPKVSDTVV	180
TUBB6_CHICK	RNECESCDCLQGFQLIHSLLGGGTGSGMGTLINKIREEYPDRIMNTFSVVPSPKVSDTVV	180
	* * * *	
1SA0	EPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCL	240
TUBB6_CHICK	EPYNAILSIIHQLIENTDETFCIDNEALYDICFRTLKLTNPITYGDLNHLVSLTMSGVTTSL	240
	* * * *	
1SA0	RFPGQLNADLRKLAVNMVFFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM	300
TUBB6_CHICK	RFPGQLNADLRKLAVNMVFFPRLHFFMPGFAPLTARGSQQYRALSVPPELTQQMFDAKNMM	300
	* *	
1SA0	AACDPRHGRYLTVAAVFRGRMSMKEVDEQMLNVQKNSSYFVEWIPNNVKTAVCDIPPRG	360
TUBB6_CHICK	AACDPRRGRYLTVACIFRGRMSTREVDEQLLSVQTKNSSYFVEWIPNNVKVAVCDIPPRG	360
	* ** ** *	
1SA0	LKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS	420
TUBB6_CHICK	LKMAATFIGNNTAIQELFIRVSEQFSAMFRRKAFLHWYTGEGMDEMEFSEAEGNTNDLVS	420
	* * * * *	
1SA0	EYQQYQDATAD-EQGEFEEEEGEDEA	445
TUBB6_CHICK	EYQQYQDATADVVEEYEEAEASPEKET	446
	* ** * * *	

FIGURE 2.

Sequence alignment of β subunit of 1SA0 and *Gallus gallus* isotype β VI. Residues colored in red are those which were within 6Å of the binding site. Residues highlighted in cyan are those in the colchicine binding domain. Mutations are marked with a star underneath the alignment.

FIGURE 3.

Schematic diagram of differences in mammalian brain β tubulin (from 1SA0 structure) and isotype β VI of chicken in the colchicine binding site. The 1SA0-derived coordinates of α/β heterodimer including GTP, GDP and colchicine (SH group was replaced by H) were used as starting coordinates for minimization procedure for the mammalian brain tubulin dimer. The chicken erythrocyte tubulin dimer was created from 1SA0 and the two dimers were minimized and superimposed as described under *Materials and Methods*. The residues containing any atom within 6Å of bound colchicine were selected. Only the divergent residues within this cut-off are shown in small ball-and-stick representation in blue (chicken isotype β VI) and brown (mammalian brain β -tubulin). The helices, β -sheets and loops for mammalian brain β -tubulin

and chicken isotype β VI are shown as purple and yellow ribbons respectively. The colchicine is shown in large ball-and-stick representation with C, O and N colored green, red and dark blue, respectively. Only the mammalian structure colchicine is shown as it differs only slightly from the colchicine in the chicken β VI structure. Five substitutions; Y200-F, C239-S, A315-C, V316-I and T351-V, were observed in chicken isotype β VI relative to mammalian brain β -tubulin within the 6 Å cut-off.

FIGURE 4.

(A) Effect of colchicine on paclitaxel induced polymerization of CeTb. CeTb (5 μM) in PME buffer and 0.1 mM GTP was incubated with increasing concentrations of colchicine for 90 min at 25° C. The samples were then treated with paclitaxel (5 μM) at 37° C and the polymerization was monitored in terms of apparent absorption at 400 nm. The colchicine concentrations were 0 μM ($\blacktriangle$), 80 μM ($\bullet$), 100 μM ($\circ$), 120 μM ($\blacksquare$) and 140 μM ($\square$). (B) Plot for IC_{50} of colchicine for BbTb (3) and Cetb (3). The IC_{50} of BbTb was 2.7 μM and that for CeTb was >140 μM .

FIGURE 5.

(**A**) Effect of BbTb (---) and CeTb (.....) on fluorescence of colchicine. Tubulin (1 μ M) was added to colchicine (150 μ M) in PME buffer in presence of 0.1 mM GTP at 25°C and enhancement in colchicine fluorescence at 440 nm was monitored as function of time. (**B**) Effect of colchicine on intrinsic fluorescence of BbTb (---) and CeTb (.....). Colchicine (150 μ M) was added to tubulin (1 μ M) in PME buffer in presence of 0.1 mM GTP at 25°C and the quenching of intrinsic protein fluorescence at 335 nm was monitored as function of time.

FIGURE 6.

(A) Effect of podophyllotoxin on paclitaxel induced polymerization of CeTb. CeTb (5 μM) in PME buffer and 0.1 mM GTP was incubated with increasing concentrations of podophyllotoxin for 25 min at 25° C. The samples were then treated with paclitaxel (5 μM) at 37 °C and the polymerization was monitored in terms of apparent absorption at 400 nm. The podophyllotoxin concentrations were 0 μM ($\blacktriangle$), 5 μM ($\bullet$), 10 μM ($\circ$), 15 μM ($\blacksquare$), 20 μM ($\square$), 30 μM ($\star$), 40 μM ($\star$), and 60 μM ($\blacktriangledown$). (B) Plot for IC_{50} of podophyllotoxin for BbTb ($\bullet$) and Cetb ($\circ$). The IC_{50} of BbTb was 1.6 μM and that for CeTb 25 μM .

FIGURE 7.

Plot for IC_{50} of nocodazole for BbTb (●) and Cetb (○). Tubulin (5 μ M) in PME buffer and 0.1 mM GTP was incubated with increasing concentrations of nocodazole for 40 min at 25° C. The samples were then treated with paclitaxel (5 μ M) at 37°C and the polymerization was monitored in terms of apparent absorption at 350 nm. The IC_{50} of nocodazole for BbTb was 7.7 μ M and that for CeTb was 87 μ M.

FIGURE 8.

Emission spectra of allocolchicine in the presence of BbTb (—) and CeTb (.....). Allocolchicine (150 μ M) was incubated with tubulin (1 μ M) at 25°C for 90 min and the ligand emission spectra were obtained by exciting the samples at 315 nm.

FIGURE 9.

Enhancement of allocolchicine fluorescence upon binding to BbTb (●) and CeTb (▲) at 25°C as a function of ligand concentration. The excitation wavelength was 315 nm. Spectra were collected as described under *Materials and Methods*.

FIGURE 10.

Enhancement of allocolchicine fluorescence upon binding of BbTb (---) and CeTb (.....) as function of time. Tubulin 1 μM was added to an allocolchicine solution (150 μM) at 25° C. The excitation and emission wavelengths were 330 and 400 nm, respectively. The kinetics of binding were evaluated as described under *Materials and Methods*.

FIGURE 11.

Competition of allicolchicine binding to tubulin by podophyllotoxin. The kinetics of allicolchicine binding to CeTb (A) and BbTb (B) in the absence (.....) and presence (- - -) of podophyllotoxin were measured. CeTb 1 μ M was incubated with 0 μ M and 150 μ M podophyllotoxin for 25 min at 25° C. At the end of incubation, allicolchicine (150 μ M) was added to the solution and binding was studied in terms of increase in allicolchicine fluorescence as a function of time. For experiment with BbTb, 50 μ M of each ligand was used. DMSO was limited to 10%. The excitation and emission wavelengths were 330 and 400 nm, respectively.

FIGURE 12.

Quenching of intrinsic fluorescence of BbTb (**A**) and CeTb (**B**) by colchicine (—), thiocolchicine (.....) and chlorocolchicine (- - -), as a function of time. Ligands were added to tubulin solutions to yield a final concentration of 1 μ M of tubulin and 20 μ M ligands in A and 1 μ M of tubulin and 150 μ M ligands in B at 25°C. The excitation and emission wavelengths were 295 and 335 nm, respectively.

Table 1

Inhibition of Taxol-Induced Assembly of BbTb or CeTb by Colchicine-Site Ligands

Ligand	[Tubulin], μM	IC ₅₀ , μM	
		BbTb	CeTb
Colchicine	5	2.7 ± 0.03	$\gg 140$
Podophyllotoxin	5	1.6 ± 0.02	25 ± 1
Nocodazole	5	7.7 ± 0.2	87 ± 2
Allocolchicine	5	2.2 ± 0.09	ND ^a
Allocolchicine	2	0.5	5

^aNo inhibition of assembly observed at the solubility limit under these experimental conditions (40 μM).

Table 2

Association Rate Constants of the Binding of Colchicine, Alcolcolchicine, Thiocolchicine and Chlorocolchicine to BbTb and CeTb.

Drug	BbTb	CeTb
	$k_f, M^{-1} s^{-1a}$	$k, M^{-1} s^{-1b}$
Colchicine	45.3 ± 0.7	----
Alcolcolchicine	343 ± 7	14.5 ± 0.09
Thiocolchicine	129 ± 5	2.20 ± 0.03
Chlorocolchicine	367 ± 7	11.7 ± 0.4

^a k_f is the apparent second order rate constant of the fast phase of biphasic binding,

^b k is the second order rate constant for monophasic binding. All measurements were performed at 25°C as described under *Materials and Methods*.