Baccatin III Induces Assembly of Purified Tubulin into Long Microtubules†

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Received December 12, 2000; Revised Manuscript Received March 16, 2001

ABSTRACT: Baccatin III is widely considered to be an inactive derivative of Taxol. We have reexamined its effect on in vitro assembly of tubulin under a variety of conditions. We found baccatin III to be active in all circumstances in which Taxol is active: it assembled GTP−tubulin, GDP−tubulin, and microtubule protein into normal microtubules and stabilized these polymers against cold-induced disassembly. The effect of baccatin III on in vitro microtubule assembly was quantitatively assessed through determination of critical concentrations, which can be used to obtain the apparent equilibrium constants for the addition of tubulin subunits to growing microtubules. The apparent equilibrium constants for the growth reaction for baccatin III-induced GTP−tubulin and GDP−tubulin assembly measured at 37 °C were 4.2−4.6-fold less than those measured for Taxol-induced GTP−tubulin and GDP−tubulin assembly. These data indicate that the entire Taxol side chain contributes only about −1 kcal/mol to the apparent standard free energy of microtubule growth at 37 °C regardless of the nature of the E site nucleotide. These data also support the idea that the majority of the interactions between Taxol and tubulin that affect this equilibrium occur between the baccatin portion of the molecule and the binding site. We have also observed a structural difference in microtubules formed using baccatin III and Taxol. Baccatin III-induced microtubules were routinely much longer than those assembled by Taxol, even when very high concentrations of baccatin III were employed. One interpretation of these data is that baccatin III and Taxol differ in their abilities to nucleate GTP−tubulin. This difference in activity may have bearing on the large disparity in cytotoxicity of the two molecules.

Taxol is a natural product that has shown great utility in cancer chemotherapy. Currently a front-line agent in the treatment of breast and ovarian cancers, it has shown efficacy in single agent and combination chemotherapy of a variety of neoplasms (*1*−*3*). The therapeutic effect of Taxol (**1**, Figure 1) and related molecules such as Taxotere (**2**, Figure 1) is believed to be the result of the drugs' interactions with cellular microtubules. The effect of Taxol on microtubules in vitro is well-known: Taxol will induce tubulin, the major component of the microtubule, to assemble into microtubules under normally prohibitive conditions and will stabilize these polymers against disassembly (*4*, *5*). It binds to a single site on the β-subunit of the tubulin heterodimer (*6*). Taxol binding to tubulin is normally observed only under conditions in which tubulin assembly occurs, indicating that Taxol binding to tubulin is tightly linked to protein self-association (*5*, *7*). Very low concentrations of Taxol that have little observable effect on polymer mass can nevertheless inhibit cellular mitosis. At these drug concentrations, the dynamic behavior of microtubules is suppressed. It is now believed that the effect of Taxol and other antimitotic drugs on microtubule dynamics rather than their effects on the bulk properties of microtubules is sufficient to inhibit cell proliferation (*8*, *9*).

Taxol was initially available only by purification of *Taxus brevifolia* bark extracts due it its very complicated structure.

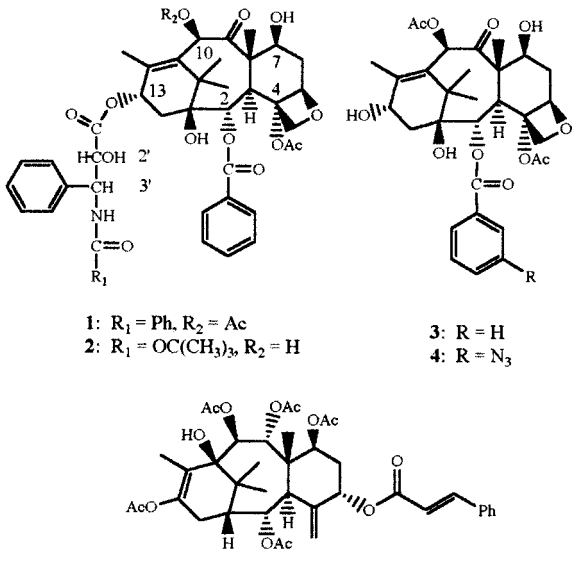

FIGURE 1: Structures of Taxol (**1**), Taxotere (**2**), baccatin III (**3**), 2-*m*-azidobaccatin III (**4**), and taxuspine D (**5**).

It is presently manufactured by semisynthesis: the ring system of the molecule, 10-deacetylbaccatin III, is isolated from the needles of the yew, and a synthetic side chain is attached to the baccatin core (*10*). One goal of research in this field is to discover or design molecules that possess the beneficial activities of Taxol but not its structural intricacy. Understanding the interaction of Taxol with its receptor on a molecular level is important information for development of these new drugs.

† This work was supported by NIH Grant CA 69571.

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10.1021/bi002816i CCC: $20.00 © 2001 American Chemical Society
Published on Web 05/17/2001

Toward this end, extensive structure−activity studies have been performed (*10*−*15*). One of the early observations in Taxol structure−activity relationship investigations was that removal of the C-13 side chain completely abolished its antimitotic and antimicrotubule activity (*16*). Thus, a tremendous amount of effort has been expended to understand how the side chain interacts with the receptor site. However, more recent reports on studies performed with baccatin III (**3**, Figure 1) have led to a confusing picture of the role of the side chain. Baccatin III is known to stabilize microtubules against cold-induced disassembly, but the extent of stabilization depends on the source of the protein. Baccatin III is almost equipotent with Taxol in stabilizing microtubules from *Physarum* but is 60-fold less active than Taxol when mammalian brain microtubules are examined (*17*). It is inactive as a promoter of MTP assembly in MES/glycerol buffer and is not cytotoxic in J744.2 cells at concentrations up to 10 μM (*16*). In a later report, baccatin III was found to be cytotoxic in a variety of cell lines, although at concentrations 300−4000 times greater than required for Taxol. In this same study, high concentrations of baccatin III inhibited tubulin assembly into microtubules (*18*). Most recently, debenzoyl-2-(*m*-azidobenzoyl)baccatin III [2-*m*-azidobaccatin III (**4**, Figure 1)] has been shown to be highly active as a promoter of in vitro microtubule assembly and is 25- to 45-fold less cytotoxic than Taxol (I_{50} = 68 and 300 nM for 2-*m*-azidobaccatin III in A549 and SKOV3 cells, respectively). Baccatin III itself does not induce MTP assembly in MES/glycerol buffer at drug concentrations up to 10 μM (1 mg/mL MTP) and is not cytotoxic at much higher concentrations (1 μM) in both cell lines (*19*). In contrast, baccatin III causes mitotic arrest in Bcap37 and KB cells but is about 100-fold less potent than Taxol in these systems (*20*). And finally, recent natural product isolations from the Japanese yew (*Taxus cuspidate* Seib. et Zucc.) have yielded taxane-related diterpenoids that lack a structure equivalent to the C-13 side chain of Taxol. Some of these, such as taxuspine D (**5**, Figure 1), are nearly as active as Taxol as inhibitors of calcium-induced microtubule depolymerization (*21*−*23*). The most active compounds in these extracts tend to lack the oxitane D ring of Taxol and have a cinnamoyl acetate functionality at C-5.

We have undertaken a systematic study of baccatin III's effect on in vitro microtubule assembly in an attempt to understand how this ligand interacts with microtubules and perhaps to clarify some of the conflicting reports.[1] We found that baccatin III can indeed mimic Taxol's effects on the bulk properties of microtubules. Measurement of critical concentrations for baccatin III-induced tubulin assembly enabled us to quantitatively assess the effect of this ligand on apparent equilibrium constants for the microtubule growth reaction. The significance of these findings for the mechanism of action of Taxol is discussed.

EXPERIMENTAL PROCEDURES

Materials. PIPES,[2] EGTA, GTP (type II S), GDP (sodium salt type I), and phosphoenolpyruvate were obtained from Sigma. Pyruvate kinase (rabbit muscle) was obtained from Boehringer Mannheim. The buffers used were as follows: PME buffer, 100 mM PIPES, 1 mM $MgSO_4$, and 2 mM EGTA, pH 6.9; PMEG buffer, PME buffer containing 0.1 mM GTP. Baccatin III was obtained from the Natural Products Division of the National Cancer Institute. Paclitaxel was a gift of the late Matthew Suffness of the National Cancer Institute.

Purity of Baccatin III and Taxol. To ensure that the data were due to baccatin III activity and not to contaminating Taxol, the purity of the baccatin III was evaluated by HPLC. Chromatography was performed on a Supelco Discovery C-8 column (12.5 cm × 4.5 mm, 5 μm particle size). A linear gradient of acetonitrile/water was employed (30:70 to 60:40 over 15 min, flow rate 1 mL/min). The retention times of baccatin III and of Taxol were 8.3 and 14.4 min, respectively (absorbance detector at 227 nm). No Taxol was detected in the baccatin III sample, which corresponds to a minimum purity of 99.5% for baccatin III.

Tubulin Purification and Protein Determination. Microtubule protein (MTP) and tubulin were prepared by two cycles of assembly/disassembly as described by Williams and Lee (*24*). The protein solution from the end of the second cycle was either directly frozen in liquid nitrogen as MTP or subjected to phosphocellulose chromatography to yield purified tubulin prior to drop freezing in liquid nitrogen. Prior to use the frozen pellets were gently thawed, centrifuged at 1000*g* for 10 min at 4 °C, and then desalted into the desired buffer on Sephadex G-50 columns (*25*). The concentrations of tubulin were determined spectrophotometrically [ϵ_{278nm} = 1.23 $(mg/mL)^{-1}$ cm^{-1} PMEG buffer (*26*)]. The concentrations of baccatin III and Taxol were also determined spectrophotometrically. The extinction coefficient for baccatin III was determined in our laboratory (ϵ_{276nm} = 1.19×10^3 M^{-1} cm^{-1} in DMSO); the extinction coefficient for Taxol (ϵ_{273nm} = 1.7×10^3 M^{-1} cm^{-1} in ethanol) was obtained from the literature (*5*).

Preparation of Tubulin−*GDP.* Tubulin−GDP, in which the exchangeable site (E site) GTP was completely replaced by GDP, was prepared by a modified procedure from Seckler et al. (*27*). Briefly, tubulin (40 μM) in PME buffer was incubated with excess GDP (5 mM) on ice for 30 min. Unbound GTP and GDP were removed by rapid gel filtration on Sephadex G-50 columns equilibrated with PME buffer. Tubulin−GDP was evaluated for nucleotide content using HPLC. Nucleotides were extracted from tubulin by the method of Seckler et al. (*27*). HPLC analysis of the extracts, performed as described previously (*28*), showed a 1:1 ratio of GTP:GDP.

Assembly and Disassembly of GTP−*Tubulin, GDP*−*Tubulin, and MTP.* (*A*) *Assembly Assays.* Tubulin polymerization was monitored by apparent light scattering using a Hewlett-Packard 8453 absorption spectrophotometer with a thermostated multicell holder that was maintained at 37 °C with a circulating water bath. The basic experimental protocol was as follows: tubulin or MTP in PME buffer was equilibrated in the sample cell, and a baseline was recorded.

[1] A portion of this work was presented at the 39th Annual Meeting of the American Society for Cell Biology, December 1999.

[2] Abbreviations: MTP, microtubule protein; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PME buffer, 100 mM PIPES, 1 mM $MgSO_4$, and 2 mM EGTA, pH 6.9; PMEG buffer, PME buffer containing 0.1 mM GTP, pH 6.9.

Assembly was initiated by addition of baccatin III or Taxol in DMSO. The final concentration of DMSO was 4% or less (v/v), which does not affect the rate or extent of tubulin assembly under these conditions (unpublished results). The apparent absorption at 350 nm was recorded at 30 s intervals until a steady state was reached. The extent of assembly was determined from the difference in the initial and plateau absorption values. When polymerizations of GTP−tubulin were performed, 1 mM GTP and a GTP regenerating system (3 mM phosphoenolpyruvate + 0.02 mg/mL pyruvate kinase) were included in the cuvette. When polymerizations of GDP−tubulin were performed, 1 mM GDP was included in the cuvette. MTP polymerizations were performed in PME buffer with no added nucleotide.

Since we observed different microtubule length distributions for baccatin III and Taxol (vide infra), the wavelength dependence of the plateau turbidity was checked for microtubules formed by each drug with GTP−tubulin and GDP−tubulin. All plots of $\log(\Delta A)$ vs $\log(\lambda)$ were linear, and the slopes of the plots were −2.7 to −3.0, confirming that turbidity could be used to quantify the mass of polymerized protein (*29*).

The extent of assembly induced by baccatin III was also evaluated by a sedimentation experiment. Samples of 10 μM GTP−tubulin containing varying concentrations of baccatin III were incubated in microcentrifuge tubes for 30 min at 37 °C. Assembled tubulin was pelleted by centrifugation at 34 000 rpm for 45 min at 37 °C in a Ti 42.2 type rotor on a Beckman L-60 ultracentrifuge. The supernatant was carefully removed, and the amount of unassembled tubulin was determined by the method of Bradford (*30*). The concentration of polymerized tubulin was determined by subtracting the concentration of unassembled tubulin from the initial concentration of tubulin.

(B) Disassembly Assays. The disassembly of tubulin microtubules was evaluated by determining initial rates of cold-induced disassembly as described by Lataste et al. (*17*). Briefly, tubulin (2 mg/mL) in PME buffer containing 1 mM GTP was assembled in the spectrometer at 37 °C in the presence of baccatin III, Taxol, or no drug. After steady state was reached, the temperature of the cell holder was decreased by switching to a second bath held at 4 °C. Disassembly was monitored by the decrease in apparent absorption at 350 nm. The initial rate of disassembly was calculated from the data as described by Lataste et al. (*17*). The rate of disassembly of the control without drug (V_0) was compared to the rate of disassembly with varying concentrations of baccatin III or Taxol (V). The I_{50} for disassembly was the concentration of the drug at which V/V_0 equals 0.5.

I_{50} Determination. The I_{50} for drug-induced assembly is the concentration of drug at which the extent of assembly is 50% of the maximum value. This parameter is often determined from a plot of percent assembly vs drug concentration (*31*, *32*). A linear relationship between the two parameters is obtained at intermediate points in the curve (i.e., at about 25% to 75% activity). If, however, the entire activity range is evaluated, the assembly vs [drug] plot will show the typical nonlinear dose−response curve. In this work, we collected data over the entire range of the dose−response curve. This enabled us to calculate the I_{50} values from nonlinear regression fits of rectangular hyperbolas (response vs drug concentration). The equation used was

$$A_{350\text{nm}} = A_{\text{max}}[\text{drug}]/(I_{50} + [\text{drug}])$$

Fitting was done using the nonlinear regression software within SigmaPlot 2000 (Jandel Scientific).

Critical Concentrations for Tubulin in the Presence of Baccatin III or Taxol. The critical concentrations of tubulin in the presence of baccatin III or Taxol were determined from assembly experiments performed at 37 °C. The drug concentration was kept constant, and the extent of assembly was measured at different tubulin concentrations. Critical concentrations were calculated from the *x*-intercepts of plots of apparent $A_{350\text{nm}}$ vs tubulin concentration. Critical concentrations of the protein in the absence of drug were measured as a control.

Electron Microscopy. Tubulin polymers formed under different experimental conditions were examined by electron microscopy. Single drops of the assembled tubulin (microtubules) solutions were put on 200 mesh hydrophilic carbon-coated grids. Great care was taken to avoid shearing the microtubules. After 1 min, excess solution was removed with filter paper, and the grid was rinsed with 1 mM ammonium acetate. The grids were negatively stained with 1% (w/v) aqueous uranyl acetate. Excess stain was taken off by filter paper after 1 min. Electron micrographs were obtained using Hitachi 7000 TEM operated at 100 kV.

Measurement of Microtubule Lengths. The lengths of 500 Taxol-induced microtubules and 400 baccatin III-induced microtubules were measured from digitized photographs of electron micrographs using SigmaScan Pro (Version 4.0, Jandel Scientific). Length distribution analyses were performed with SigmaPlot 2000 (Jandel Scientific).

RESULTS

Taxol induces purified tubulin to assemble into microtubules in a concentration-dependent manner. The extent of polymerization plateaus at a 1:1 molar ratio of Taxol:tubulin (*4*, *5*). Baccatin III also induced purified tubulin to polymerize in a concentration-dependent manner (Figure 2). The difference in polymerization activity of the two molecules was surprisingly small: half-maximal assembly of 10 μM GTP−tubulin was reached with 0.48 μM Taxol and with ∼4.0 μM baccatin III (Table 1). The relationship between drug concentration and extent of assembly was more complex in the case of baccatin III than for Taxol. This difference is more clearly illustrated by curve fitting. The polymerization data could be routinely fitted to a simple rectangular hyperbola in most instances (Figure 2A). In the case of baccatin III-induced assembly of GTP−tubulin, however, a better fit was obtained when the data were treated as the sum of two rectangular hyperbolas (Figure 2B). The I_{50} values calculated by the two methods were similar although not identical (Table 1).

The biphasic nature of the dose−response curve did not appear to be due to a difference in the polymers produced by the two drugs. The extent of baccatin III-induced microtubule assembly was also determined by separating the polymerized protein from unassembled tubulin by centrifugation (data not shown). Comparable results were obtained using both methods, indicating that the data from apparent light scattering were not affected by polymer morphology.

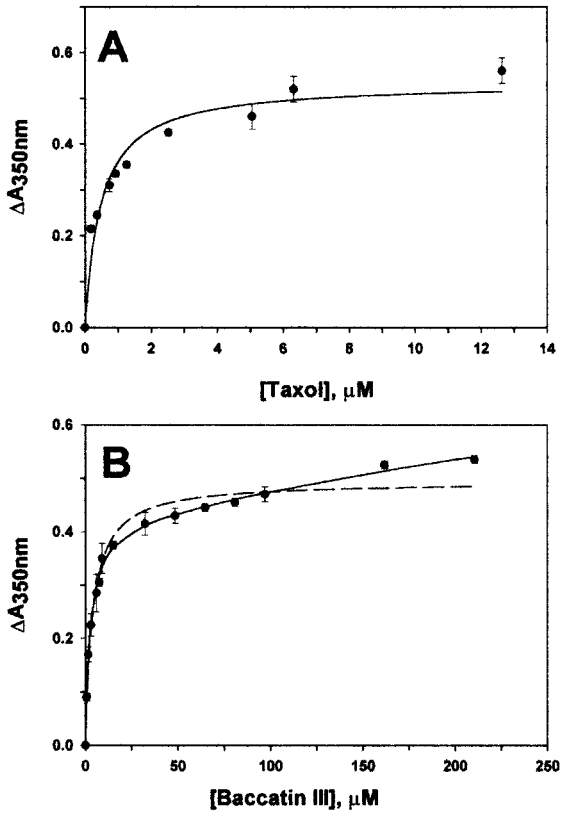

FIGURE 2: Effect of Taxol or baccatin III on the assembly of GTP−tubulin. Tubulin (10 μM) was treated with varying concentrations of Taxol (panel A) or baccatin III (panel B) in PME buffer at 37 °C as described under Experimental Procedures. GTP (1 mM) and a GTP regenerating system were included in all samples. Assembly was monitored by apparent absorption at 350 nm. Panel A: Taxol data. The solid line is a fit of the assembly data to a single rectangular hyperbola. Panel B: Baccatin III data. The solid line is a fit of the assembly data to a double rectangular hyperbola; the dashed line is a fit of the data to a single rectangular hyperbola.

Table 1: Effect of Taxol and Baccatin III on the Assembly and Disassembly of Tubulin and Microtubule Protein

	I_{50} (μM)[a] for	
protein	Taxol	baccatin II
GTP−tubulin[b]	0.48 ± 0.09[c]	4.0 ± 0.49;[c] 2.6 ± 0.27[d]
GDP−tubulin	4.0 ± 0.28[c]	>400
MTP	1.1 ± 0.23[c]	27 ± 4.0[c]
GTP−tubulin disassembly[e]	1.9 ± 0.8	18 ± 3.0

[a] I_{50} is the concentration of drug required to obtain 50% of the maximum extent of assembly measured by ΔA_{350nm}. The concentration of protein was 10 μM in all experiments unless otherwise noted. [b] Contains the GTP regenerating system. [c] I_{50} and standard deviation from fitting the data to a single rectangular hyperbola. [d] I_{50} and standard deviation from fitting the data to a double rectangular hyperbola. [e] Cold-induced disassembly, determined as described under Experimental Procedures.

Dose−response curves obtained in other I_{50} determinations were a single hyperbola (data not shown).

Baccatin III was also able to stabilize microtubules against depolymerization by cold. The initial rate of depolymerization was measured as a function of drug concentration (Figure 3). The disassembly rate decreased with increasing concentrations of Taxol or baccatin III. The I_{50} for inhibition of depolymerization was 1.9 μM for Taxol and 18 μM for baccatin III (Table 1).

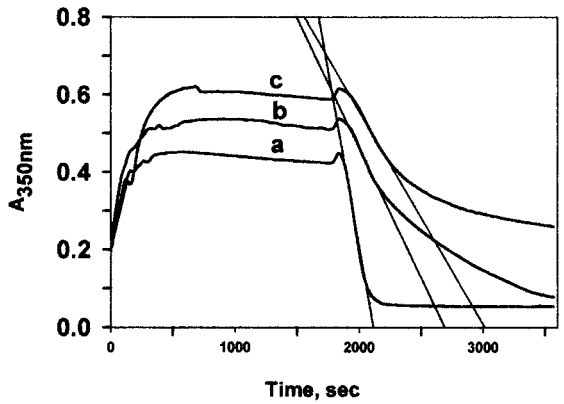

FIGURE 3: Effect of Taxol or baccatin III on the cold-induced disassembly of GTP−tubulin. Tubulin (10 μM) in PMEG buffer was polymerized with varying concentrations of Taxol or baccatin III. After steady state was reached, the temperature of the cuvette was decreased to 4 °C. The initial rate of disassembly of the polymers was from the initial slopes (straight lines) determined as described by Lastate et al. (*17*). Curve a is the control without drug; curve b contains 50 μM baccatin III; curve c contains 5 μM Taxol.

We noted that tubulin assembled by baccatin III in the absence of exogenous GTP tended to reach a plateau and then disassemble, a behavior that has been reported for Taxol (*33*, *34*). Adding GTP to these samples increased the duration and extent of the plateau in a concentration-dependent manner (data not shown). It was therefore possible that the interaction of baccatin III with microtubules might be influenced by the nucleotide content of the protein. We attempted to assemble GDP−tubulin with Taxol and baccatin III. The I_{50} for Taxol-induced assembly of 10 μM GDP−tubulin was 4 μM, about 10-fold greater than the I_{50} for GTP−tubulin under the same conditions. Baccatin III, however, was only weakly active as an inducer of GDP−tubulin assembly under these conditions, even at very high concentrations of baccatin III.

MAPs are known to affect the manner in which Taxol interacts with polymerized tubulin (*35*−*37*). The effect of baccatin III on the polymerization of microtubule protein was therefore examined. MTP was induced to assemble in the absence of exogenous GTP by addition of Taxol or baccatin III. Baccatin III was again less potent than Taxol in this assay (Table 1).

I_{50} values are useful for comparing activities of antimicrotubule ligands but do not provide quantitative information pertinent to the molecular mechanisms of the drugs. The reciprocal of the critical concentration provides a good estimate of the apparent binding equilibrium constant for a tubulin dimer to the polymer, which will in turn give an estimate of the standard free energy change for the growth reaction (*29*, *38*). Critical concentrations were measured for baccatin III-induced assembly of GTP−tubulin, GDP−tubulin, and MTP. Critical concentrations for Taxol-induced assembly were measured under the same conditions so that direct comparisons between the two ligands could be made. Assembly of tubulin in the presence of Taxol plateaus at a molar ratio of 1:1 Taxol:tubulin (*5*, *39*, and Figure 2A). The extent of assembly of GTP−tubulin by baccatin III reached a maximum at a molar ratio of about 10:1 (Figure 2B). The critical concentrations were therefore determined at Taxol and baccatin III concentrations of 10 and 100 μM, respectively. No change in the critical concentration was observed

Table 2: Critical Concentrations and Apparent Growth Equilibrium Parameters for Tubulin Assembly Induced by Taxol and Baccatin III

	C_{crit} (μM)[a]	app K_p ($M^{-1} \times 10^{-6}$)	$-\Delta G°_{app}$ (kcal/mol)[c]
GTP−tubulin/Taxol	0.37 ± 0.07[d]	2.7 ± 0.51	9.1 ± 0.12
GTP−tubulin/baccatin III	1.4 ± 0.15	0.69 ± 0.07	8.2 ± 0.06
GTP−tubulin	18 ± 2.0	0.055 ± 0.006	6.7 ± 0.07
GDP−tubulin/Taxol	1.4 ± 0.45	0.74 ± 0.25	8.4 ± 0.21
GDP−tubulin/baccatin III	9.6 ± 0.2	0.10 ± 0.002	7.1 ± 0.01
GDP−tubulin	>30[e]		
MTP/Taxol	0.66 ± 0.11	1.5 ± 0.25	8.8 ± 0.10
MTP/baccatin III	2.0 ± 0.25	0.49 ± 0.06	8.1 ± 0.08
MTP	>30		

[a] Critical concentration, 37 °C. [b] Apparent equilibrium growth constant, 37 °C. $K_p = 1/C_{crit}$. [c] Apparent standard free energy change for the growth reaction, 37 °C, calculated from $\Delta G°_{app} = -RT \ln(K_p)$. [d] Mean ± standard deviation. [e] No assembly was observed at protein concentrations ≤30 μM.

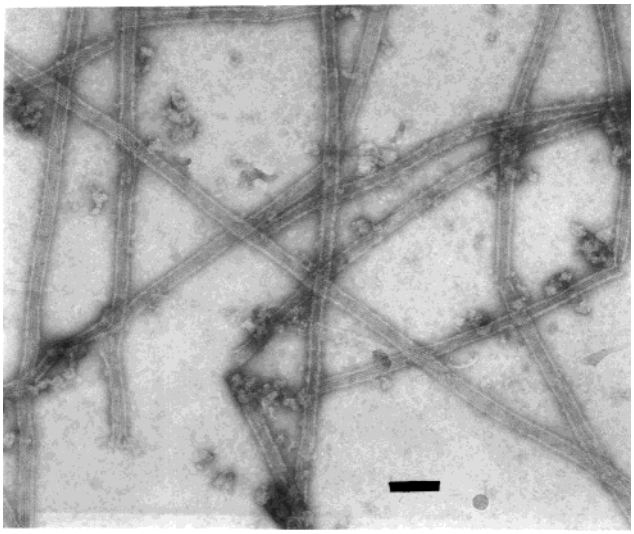

FIGURE 4: Electron micrograph of microtubules formed with baccatin III. Polymers formed by Taxol- or baccatin III-induced assembly of tubulin were examined by electron microscopy. Normal microtubules were observed when GTP−tubulin, GDP−tubulin, and MTP were assembled with either drug. Illustrated here are microtubules formed from MTP by baccatin III. A solution of MTP (1 mg/mL) containing 300 μM baccatin III in PME buffer was incubated at 37 °C for 30 min prior to analysis. Bar = 0.1 μm.

when the concentration of baccatin III was increased to 250 μM.

The critical concentrations for tubulin assembly in the presence of baccatin III or Taxol are summarized in Table 2. Like Taxol, baccatin III induced GTP−tubulin, GDP−tubulin, and MTP to assemble into microtubules. Substitution of GDP for GTP into the E site of tubulin resulted in a 6.0−6.6-fold increase in the critical concentrations of both ligands. Baccatin III was 4.0−4.5-fold less active than Taxol regardless of the nucleotide content of tubulin and 3-fold less active when MAPs were present.

The polymers formed by baccatin III and Taxol under the various experimental conditions were examined by electron microscopy. Normal microtubules were observed under all conditions (Figure 4). One notable difference between the two drugs was the length of the microtubules. Taxol microtubules were markedly shorter than microtubules assembled by baccatin III (Figure 5).

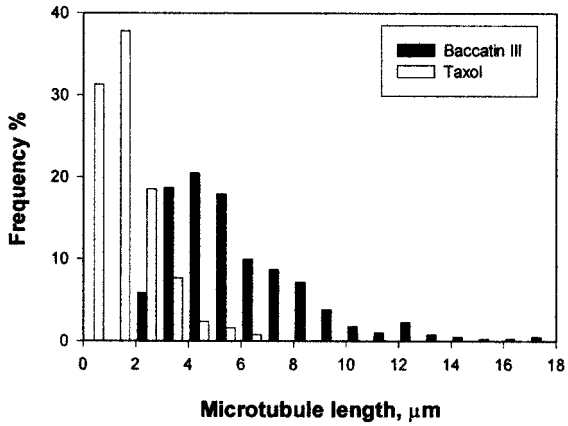

FIGURE 5: Length distribution of microtubules formed by Taxol and baccatin III. Microtubules formed from GTP−tubulin, GDP−tubulin, and MTP in the presence of baccatin III were routinely longer than microtubules formed with Taxol. Illustrated here is a distribution of microtubule lengths resulting from treatment of MTP with 10 μM Taxol (dark bars) or 300 μM baccatin III (light bars). In this illustration, the mean length of Taxol-induced microtubules was 2.0 ± 1.0 μm, and the mean length of baccatin III-induced microtubules was 6.5 ± 2.6 μm.

DISCUSSION

Baccatin III is widely assumed to be an inactive derivative of Taxol. Labeling the compound "inactive" is an oversimplification of its biological activity and is, in fact, inconsistent with early reports concerning this molecule (*17*). In this work, we have evaluated the effect of baccatin III on in vitro assembly of tubulin under a variety of conditions. We have also subjected Taxol to these same experiments in order to have a direct comparison between the two drugs. Like Taxol, baccatin III induced pure tubulin and microtubule protein to assemble into normal microtubules regardless of the nucleotide content of the protein and stabilized the polymers against cold-induced disassembly. The difference in the two ligands was the degree of their activity. Under our standard assay conditions, the I_{50} for baccatin III-induced tubulin assembly was only 5−8-fold greater than the I_{50} for Taxol-induced assembly *provided that sufficient GTP was present.* When the solution became depleted of GTP, depolymerization of tubulin occurred. When the E site was completely occupied by GDP rather than GTP, no significant activity for baccatin III was observed. Inclusion of MAPs, in the form of MTP, seemed to counteract partially the absence of exogeneous GTP. This latter result concurs with previous reports for Taxol (*36*).

Thus, if the I_{50} values are used as the criterion for in vitro activity, three different conclusions could be reached. Either baccatin III is nearly as potent as Taxol (GTP−tubulin results), completely inactive (GDP−tubulin results), or moderately active (MTP and disassembly results).

I_{50} values are dependent on the protein concentration in the assay. What is required to sort out the conflicting conclusions suggested by the I_{50} results is an activity measurement that is a direct assessment of the ligand−receptor interaction. The critical concentration, the concentration of protein below which no polymerization can occur, is a property the system that is independent of protein concentration. In nucleated polymerization processes such as microtubule assembly, the reciprocal of the critical concentration is a very close approximation to the equilibrium

constant for polymer growth (K_p). The K_p can then be converted to an apparent standard free energy of binding. Therefore, the effect of the drug on the affinity of the microtubule end for free tubulin can be quantitatively assessed through determination of critical concentrations (*29*, *38*).

In the case of microtubule assembly in the presence of Taxol and related compounds, polymerization and ligand binding are linked processes. Thus, the apparent K_p is a function of both processes. Evaluation of the thermodynamic parameters for each equilibrium process is beyond the scope of this report. It is noted that, in the presence of excess Taxol, the apparent K_p is a good approximation of the elongation equilibrium constant for the drug-liganded microtubule (*5*, *39*, *41*). The concentrations of Taxol used in the critical concentration determinations are large enough to be saturating (*5*, *39*, *40*). The critical concentration for baccatin III-induced assembly of GTP−tubulin was unchanged when the ligand concentration was increased from 100 to 250 μM, indicating that the K_p derived from these data represents the apparent elongation equilibrium constant.

Critical concentrations for drug-induced microtubule assembly and their corresponding elongation equilibrium constants and standard free energy changes are summarized in Table 2. It is seen that the difference in the observed free energy change for Taxol-induced assembly of GTP−tubulin vs baccatin III-induced assembly of GTP−tubulin is about −1 kcal/mol. When the nucleotide in the E site was GDP rather than GTP, the difference in the observed free energy change was unchanged within experimental error. These data indicate that the entire Taxol side chain contributes only about −1 kcal/mol to the apparent standard free energy change for elongation at 37 °C regardless of the nature of the nucleotide. This is a remarkably small difference. Diaz and Andreu have measured the apparent thermodynamic parameters of Taxol- and Taxotere-induced tubulin assembly under a variety of solution conditions (*5*, *39*). They found that the structural difference in the side chains of the two ligands (see Figure 1) translated into a difference in the apparent standard free energy change of microtubule growth of 0.4 kcal/mol. Taken together, these data show that although the side chain of the taxoid affects the apparent equilibrium constant for polymer growth, the majority of the ligand−receptor interactions that affect this equilibrium occur between the baccatin portion of the molecule and the binding site. This conclusion supports the hypothesis that the baccatin portion of Taxol is the primary pharmacophore for the receptor site (*19*).

It is unclear how the absence of the Taxol side chain results in a different affinity of tubulin for the microtubule ends. It may be that the side chain is responsible for a difference in protein conformation that subtly affects the interaction between the microtubule and tubulin. We observed that microtubules formed with baccatin III were significantly longer than those formed with Taxol (Figure 5). This observation seems to indicate that the two drugs differently affect polymer nucleation. A conformational difference that affects nucleation could conceivably affect polymer propagation as well.

This work has a bearing on the other assays that show no activity for baccatin III or show less activity than reported here.[3] Baccatin III exhibited almost no activity in assembling GDP−tubulin under our "normal" assay conditions (10 μM tubulin in PME buffer) but was very active when GTP was included in the buffer. The critical concentration of GDP−tubulin under these solution conditions was approximately the same as the concentration of tubulin in the solution. Therefore, very little assembly would be observed no matter how much baccatin III was added to the solution.

We have insufficient information to understand why baccatin III cytotoxicity is detected in some instances (*18*, *20*) and not in others (*16*, *19*). It may be that the difference in cytotoxicity is due to nontubulin factors. For example, compounds that are potent in in vitro assembly assays can be much less active in cytotoxicity assays (*42*). Intriguingly, it has been reported that taxuspines that are potent inhibitors of microtubule polymerization (Figure 1) are less cytotoxic than Taxol derivatives with equal in vitro potency (*23*). Alternatively, it may be that the ability of Taxol and related substances to nucleate tubulin polymers is the physiologically relevant activity of these drugs.

[3] Pengsuparp et al. (*18*) found that relatively high concentrations of baccatin III would actually inhibit the assembly of MTP and the binding of colchicine to tubulin. We have seen no activity of this sort in our studies.

ACKNOWLEDGMENT

We thank Phoung Doan for technical assistance early in the project and Mr. Henry Eichelberger and Dr. Barbara Poliks for assistance in obtaining the electron micrographs.

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BI002816I