Differential Effects of Paclitaxel (Taxol) Analogs Modified at Positions C-2, C-7, and C-3′ on Tubulin Polymerization and Polymer Stabilization: Identification of a Hyperactive Paclitaxel Derivative†

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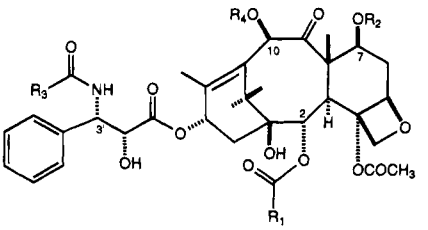
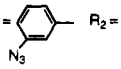
ABSTRACT: Our finding that an analog of paclitaxel (Taxol) modified at position C-2 (2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel) was substantially more active than paclitaxel in promoting tubulin assembly [Chaudhary et al. (1994) *J. Am. Chem. Soc. 116,* 4097−4098] led us to perform an analysis of the modulating effects of microtubule-associated proteins, GTP, and temperature on assembly and polymer stability. The analog always showed superior activity to paclitaxel in inducing polymerization where it fails to occur without drug, probably indicating a greater ability than paclitaxel to "hypernucleate" assembly. In contrast, much smaller differences in effects on polymer stability were observed. The analysis was extended to a large series of derivatives modified at positions C-2, C-7, C-10, and C-3′, including docetaxel, a clinically important analog of paclitaxel. While analog stabilization of polymer was frequently observed, neither qualitative nor quantitative analysis of this property reliably predicted whether a compound would have enhanced hypernucleation activity relative to that of paclitaxel. Stabilization was often observed at substoichiometric analog concentrations, while even superstoichiometric concentrations of most compounds failed to induce extensive tubulin polymerization at low temperatures or in the absence of microtubule-associated proteins or GTP. Docetaxel was intermediate in activity between paclitaxel and 2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel in promoting assembly reactions. We conclude that the hypernucleation of tubulin assembly and polymer stabilization observed with paclitaxel represent two distinct properties of the drug. Our findings suggest that paclitaxel, docetaxel, and 2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel are able to interact with progressively smaller assemblages of tubulin at low temperatures or in the absence of microtubule-associated proteins or GTP.

Antimitotic agents, by interfering with the microtubule system, inhibit cell growth and have potential roles in the treatment of neoplastic diseases. Most compounds in this class inhibit microtubule formation and may cause disassembly of existing microtubules. Exceptions are the taxoids paclitaxel and docetaxel [Taxol (**1**) and Taxotere (**2**); structures in Figure 1], which have great promise in cancer treatment (for reviews, see Kingston, 1991; Rowinsky & Donehower, 1992; Kingston et al., 1993; Nicolaou et al., 1994). Paclitaxel not only enhances microtubule assembly and stabilizes microtubules to disassembly induced by cold, calcium, and dilution (Schiff et al., 1979), but it also obviates many of the normal requirements for tubulin polymerization. Paclitaxel permits assembly reactions to occur at low temperatures and in the absence of microtubule-associated proteins (MAPs)[1] and GTP (Hamel et al., 1981; Kumar, 1981; Schiff & Horwitz, 1981; Thompson et al., 1981).



1 Paclitaxel: $R_1 = R_3 = Ph$, $R_2 = H$, $R_4 = Ac$

2 Docetaxel: $R_1 = Ph$, $R_2 = R_4 = H$, $R_3 = t\text{-BuO}$

3 R_1 =  $R_2 = H$, $R_3 = Ph$, $R_4 = Ac$

4 $R_1 = PhOCH_2$, $R_2 = H$, $R_3 = Ph$, $R_4 = Ac$

5 $R_1 = R_3 = Ph$, $R_2 = PhCO$, $R_4 = Ac$

6 $R_1 = Ph$, $R_2 = H$, $R_3 = PhOCH_2$, $R_4 = Ac$

FIGURE 1: Structural formulas of paclitaxel and paclitaxel analogs. Nomenclature for compounds **3**−**6** as follows: compound **3**, 2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel; compound **4**, 2-debenzoyl-2-(phenoxyacetyl)paclitaxel; compound **5**, 7-benzoylpaclitaxel; compound **6**, *N*-debenzoyl-*N*-(phenoxyacetyl)paclitaxel.

We, as well as others, have been interested in using photoaffinity labeling as an approach to localizing the paclitaxel binding site on tubulin (Georg et al., 1992; Rao et al., 1992; Combeau et al., 1994; Swindell et al., 1994). We successively prepared analogs derivatized at position C-7

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[1] Abbreviations: MAPs, microtubule-associated proteins; Mes, 4-morpholineethanesulfonate.

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(Rimoldi et al., 1993), C-3′, and C-2 (Chaudhary et al., 1994), but thus far none was satisfactory for further study in terms of stoichiometry, specificity, and photodependence for covalent bond formation. Because we wished to avoid photoderivatized MAPs, we examined analog effects first in a glutamate−GTP polymerization system in which assembly occurs without MAPs, and subsequently in a Mes−GTP system in which assembly requires MAPs unless paclitaxel is present. With the C-7-modified analogs stable polymers were formed in glutamate−GTP, but failed to form in Mes−GTP (Rimoldi et al., 1993). Even in glutamate, assembly without drug or with the C-7 analogs required a higher reaction temperature than polymerization with paclitaxel. These properties were observed whether or not the C-7 substituent was potentially photoreactive. All C-3′-modified agents we examined were similar in their properties to the C-7-modified analogs, inducing formation of stable polymer in glutamate−GTP at 37 °C, but not in the cold or in Mes−GTP. In contrast to our experience, docetaxel, with a major C-3′ modification, is superior to paclitaxel in its interactions with tubulin (Guéritte-Voegelein et al., 1991; Ringel & Horwitz, 1991; Díaz & Andreu, 1993; Georg et al., 1994).

Our initial C-2-modified derivatives had reduced activity relative to paclitaxel. However, when compound **3** (structure in Figure 1), with a *m*-azido group in the benzoyl residue, was added to tubulin in glutamate−GTP on ice (without exposure to light), the reaction mixture rapidly became turbid. Markedly enhanced assembly also occurred in Mes-GTP (Chaudhary et al., 1994). Other analogs with *meta* (as well as *para* and *ortho*) substituents were prepared. Although none was as potent as compound **3**, compounds with nonreactive *meta* groups (methoxy and chloro) also were much more active than paclitaxel (Chaudhary et al., 1994).

These initial findings caused us to wonder whether the specific effects of MAPs and GTP would be altered in reactions modulated by paclitaxel analogs that differed in activity from the parent compound. We were especially interested in whether enhanced activity with compound **3** and docetaxel would be independent of reaction conditions. We also wished to determine whether differences were quantitative, simply reflecting relative drug affinities for tubulin, or whether there was a qualitative difference in drug−tubulin interactions as a function of drug structure. The compounds selcted for presentation here are shown in Figure 1.

MATERIALS AND METHODS

Materials. Electrophoretically homogeneous bovine brain tubulin and heat-treated MAPs (Hamel & Lin, 1984) and compounds **3**, **4** (Chaudhary et al., 1994), and **5** (Rimoldi et al., 1993) were prepared as described previously. GTP was repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephacel. Paclitaxel (**1**) was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute.

The tubulin used here underwent gel filtration on Sephadex G-50 (superfine) in 1.0 M monosodium glutamate (pH 6.6 with HCl) to remove unbound nucleotide and contains about 1 molar equivalent each of nonexchangeable GTP and exchangeable GDP. The bound GDP is tightly retained by tubulin in glutamate (Hamel et al., 1984). After gel filtration the tubulin was precipitated with additional monosodium glutamate (final concentration, 2 M) and the protein harvested by centrifugation (0 °C, 30 min, 35 000 rpm, Beckman Ti45 rotor). The pellet was made into a thick slurry with water, and this was dialyzed for 4 h with one buffer change against 1.0 M monosodium glutamate, redissolving the tubulin. The tubulin, concentration 54 mg/mL, was stored in liquid nitrogen. Since glutamate can replace MAPs as an inducer of tubulin polymerization (Hamel & Lin, 1984), for experiments in which the critical tubulin concentration was determined, the tubulin was dialyzed for 4 h against 0.1 M Mes (pH 6.9 with NaOH) with one buffer change. The protein (50 mg/mL) was stored in liquid nitrogen.

Methods. The synthesis of docetaxel and compound **6** will be detailed elsewhere. In brief, 10-deacetylbaccatin III was protected as its 7,10-bis((trichloroethoxy)carbonyl) derivative and esterified at position C-13 with (4*S*,5*R*)-*N*-(*tert*-butoxycarbonyl)-2,2-dimethyl-4-phenyl-5-oxazolidinecarboxylic acid (Commerçon et al., 1992). Removal of the oxazolidine protecting group with formic acid followed by acylation with di-*tert*-butyl dicarbonate yielded a derivative of docetaxel protected at positions 7 and 10. This was converted to docetaxel with zinc in acetic acid. Compound **6** was prepared by a similar pathway from 7-(triethylsilyl)baccatin III. Acylation with Commerçon's acid (Commerçon et al., 1992) was followed by deprotection with formic acid and *N*-acylation with phenoxyacetic acid/dicyclohexyl carbodiimide/(dimethylamino)pyridine.

Tubulin assembly was followed turbidimetrically at 350 nm in Gilford Model 250 recording spectrophotometers equipped with electronic temperature controllers. With these devices temperature rises at 0.5 °C/s and falls at 0.1 °C/s. Depolymerization IC_{50} values (Lataste et al., 1984) were obtained from reaction mixtures containing 1.0 M monosodium glutamate, 1.0 mM $MgCl_2$, 1.0 mg/mL tubulin, 0.4 mM GTP, and varying drug concentrations (dimethyl sulfoxide, 4% v/v). Polymerization was initiated by a 0 to 37 °C temperature jump. After 20 min the temperature was reset to 0 °C, and the maximum depolymerization rates were determined. The concentration of drug required to reduce the depolymerization rate by 50% was determined by interpolation between experimental values.

Otherwise, all polymerization reactions were performed in 0.1 M Mes, taken from a 1.0 M stock solution adjusted to pH 6.9 with NaOH. Other components were as indicated, with the standard concentrations being 1.0 mg/mL tubulin, 0.5 mg/mL heat-treated MAPs, 100 μM GTP, and 4% (v/v) dimethyl sulfoxide. We used the suboptimal pH of 6.9 and omitted exogenous Mg^{2+} in these studies to be able to reliably study assembly reactions with compound **3**, which can be complete within a few minutes even at 0 °C.

For negative stain electron microscopy, a 5−10 μL droplet was placed on a carbon-coated formavar-treated 200-mesh copper grid and within 10 s washed off with several drops of 0.5% (w/v) uranyl acetate. Grids were subsequently examined in a Zeiss Model 10CA electron microscope. Reactions were followed turbidimetrically with sequential incubation for 15 min each at 0, 10, and 37 °C, and 30 min at 0 °C. Aliquots were removed and placed on grids whenever there was a ΔA_{350} value over 0.1 unit. In no case was a major morphological difference observed in the electron microscope between specimens taken at different temperatures.

Table 1: IC_{50} Values of Paclitaxel and Paclitaxel Analogs for the Rate of Depolymerization of Tubulin Polymers Formed in 1 M Glutamate with GTP

compd	IC_{50} (μM) ± SE	compd	IC_{50} (μM) ± SE
paclitaxel (**1**)	0.42 ± 0.03	**4**	2.0 ± 0.1
docetaxel (**2**)	0.28 ± 0.02	**5**	0.65 ± 0.01
3	0.31 ± 0.01	**6**	0.78 ± 0.06

[a] The maximum depolymerization rate was defined as the greatest interval drop in turbidity following reduction of reaction temperature. The sample changer had 4 cuvettes, dwell time was 5 s per position, and the interval between readings was 26 s. At least three independent determinations were performed with each compound.

RESULTS

The Compounds Examined and Their Effects on Stabilization of Tubulin Polymers Formed in Glutamate–GTP. Our initial observations with compound **3**, that it had enhanced activity as compared with paclitaxel, were reiterated under every reaction condition examined (see below). Several C-2-modified analogs with reduced ability to stimulate assembly were carefully studied, and our results with compound **4** will be described here. All C-7- and C-3′-modified analogs that we synthesized and studied in detail were able to stabilize tubulin polymer, usually at low concentrations, but they invariably had reduced activity in enhancing assembly. Reactions with compounds **5** (C-7-modified) and **6** (C-3′-modified) are presented here. Docetaxel was synthesized in order to determine whether our reaction systems were in accord with those of others (Guéritte-Voegelein et al., 1991; Ringel & Horwitz, 1991; Díaz & Andreu, 1993; Georg et al., 1994), and we, too, found docetaxel more active than paclitaxel (see below). Docetaxel differs structurally from paclitaxel at position C-10 as well as C-3′ (see Figure 1). We therefore studied 10-deacetylpaclitaxel and 10-acetyldocetaxel under all reaction conditions and found the C-10 modification had no effect (data not presented).

Table 1 summarizes results with paclitaxel, docetaxel, and compounds **3**–**6** as inhibitors of cold-induced depolymerization (Lataste et al., 1984). Polymer was formed from 10 μM tubulin, with assembly induced with glutamate and GTP, and very low drug concentrations inhibited the disassembly rate by 50%. Docetaxel and compound **3** were slightly more active (IC_{50} values, 0.3 μM) than paclitaxel (IC_{50} value, 0.4 μM), and compounds **5** and **6** slightly less active (IC_{50} values of 0.7 and 0.8 μM). Compound **4** was least active, with an IC_{50} value of 2 μM.

Polymerization Studies. In the tubulin polymerization studies that follow (Figures 2–5), turbidity tracings are labeled with the compound numbers shown in Figure 1 to indicate the agent used in the reaction, and negative data are not presented. Only in the "complete system" containing both GTP and MAPs (Figure 2) was there a reaction without drug (indicated by "0"). Drugs were added to otherwise complete reaction mixtures at 0 °C, and turbidity changes were monitored successively at 0, 10, and 37 °C, followed by evaluation of polymer stability at 10 and 0 °C.

Activity with MAPs and GTP. Figure 2A contrasts reactions without drug and with paclitaxel (**1**) to those with compounds **3** and **4**, with drugs at 10 μM. Without drug, assembly occurred only at 37 °C, and the microtubules depolymerized at 10 °C. With paclitaxel, minimal assembly occurred at 0 °C, with 55–60% of the total polymerization

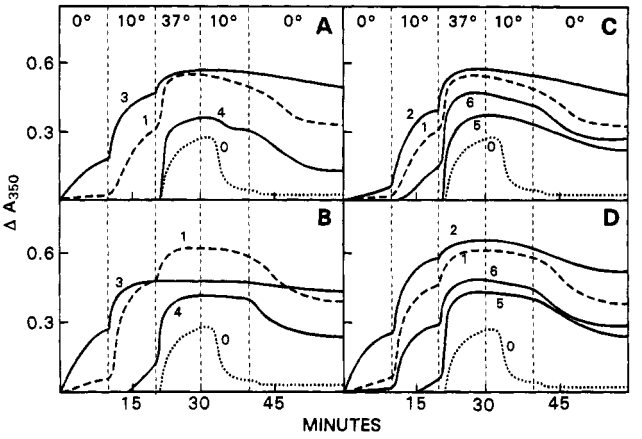

FIGURE 2: Polymerization with both MAPs and GTP. Reaction mixtures contained 0.1 M Mes (pH 6.9), 1.0 mg/mL (10 μM) tubulin, 0.5 mg/mL heat-treated MAPs, 100 μM GTP, 4% (v/v) dimethyl sulfoxide, and drugs at 10 (A, C) or 40 (B, D) μM. At the times indicated by the vertical dashed lines, the temperature controller was set at the temperature to the right of the line. Curves 0 (dotted lines), the reaction without drug; curves 1 (dashed lines), the reactions with paclitaxel. The numbers on the curves correspond to the drug numbers as indicated in Figure 1. A and B: C-2-modified analogs. C and D: C-7- and C-3′-modified analogs.

reaction occurring at 10 °C. The paclitaxel polymer was essentially stable at 10 °C, but an overall 40% drop in turbidity occurred following incubation at 0 °C. With compound **3**, turbidity began to rise at 0 °C, with another large increase occurring at 10 °C. The polymer formed was almost completely cold stable. With compound **4**, there was no polymer formation in the cold, but a brisk reaction occurred at 37 °C. Slight loss of turbidity occurred at 10 °C, with an overall 70% drop following the 0 °C incubation.

One explanation for drug differences in polymerization and depolymerization properties is different affinities for tubulin or tubulin oligomer/polymer. An approach to evaluating this possibility might be competitive ligand binding assays, but our efforts to develop an assay using radiolabeled paclitaxel have been unsuccessful. Therefore, we attempted to gain insight into relative affinities by asking whether higher analog concentrations would make less active agents like compound **4** behave more like paclitaxel and paclitaxel like compound **3**. Figure 2B summarizes experiments with drugs at 40 μM, and relative activities observed with 10 μM drug were little changed.

In Figure 2C docetaxel (**2**) and compounds **5** and **6** are compared to paclitaxel (**1**) at 10 μM and to the control. The reaction with docetaxel was moderately more vigorous than that with paclitaxel, and the polymer was more cold stable. The reactions with compounds **5** and **6** were less vigorous than the paclitaxel reaction. The polymers formed with these agents were comparable in stability to the paclitaxel polymer. With drugs at 40 μM (Figure 2D), the chief difference from 10 μM was an enhanced reaction with docetaxel at 0 °C.

Activity with MAPs but not GTP. Without GTP, assembly occurred at 37 °C with paclitaxel (**1**) at either 10 (Figure 3A) or 40 μM (Figure 3B). Omitting nucleotide almost eliminated assembly at 10 °C. Figure 3A presents the effects of the analogs at 10 μM. With compound **3** the chief effect of eliminating GTP was some reduction in polymerization at 0 °C. With docetaxel (**2**) assembly was slightly more vigorous than with paclitaxel, but polymerization at lower temperatures was much weaker than with GTP. Compounds

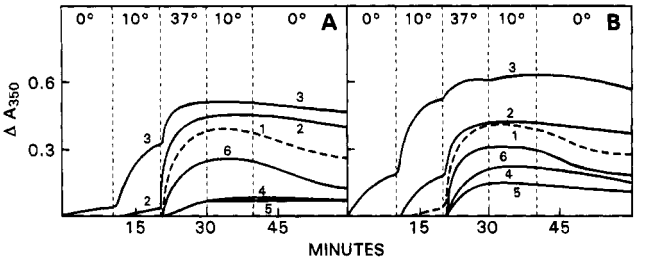

FIGURE 3: Polymerization with MAPs but not GTP. Reaction mixtures contained 0.1 M Mes, 1.0 mg/mL tubulin, 0.5 mg/mL heat-treated MAPs, 4% dimethyl sulfoxide, and drugs at 10 (A) or 40 (B) μM. Temperature as indicated. Curves 1 (dashed lines), the reactions with paclitaxel. The numbers on the curves correspond to the drug numbers as indicated in Figure 1.

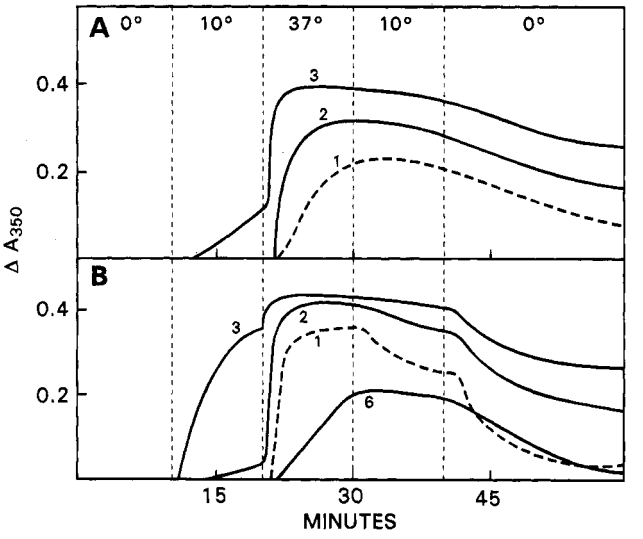

FIGURE 4: Polymerization with GTP but not MAPs. Reaction mixtures contained 0.1 M Mes, 1.0 mg/mL tubulin, 100 μM GTP, 4% dimethyl sulfoxide, and drugs at 10 (A) or 40 (B) μM. Temperature as indicated. Curves 1 (dashed lines), the reactions with paclitaxel. The numbers on the curves correspond to the drug numbers as indicated in Figure 1.

4 and **5** had feeble activity at 37 °C, and compound **6** was moderately less active than paclitaxel. With 40 μM drug (Figure 3B) there was little difference in relative activities. Polymer stability to cold was little changed by excluding GTP from the reaction.

Activity with GTP but Not MAPs. Without MAPs, polymerization with 10 μM drug only occurred with paclitaxel (**1**), docetaxel (**2**), and compound **3** (Figure 4A). The most active agent was compound **3**, which still supported some assembly at lower temperatures. With drugs at 40 μM (Figure 4B), polymerization reactions were enhanced, and sluggish assembly occurred with compound **6**. The assembly reaction with 40 μM paclitaxel differed little from that with 10 μM docetaxel, and that with 40 μM docetaxel resembled that with 10 μM compound **3**. With 40 μM compound **3**, assembly was almost complete at 10 °C. The enhanced reactions observed with 40 μM as opposed to 10 μM drug could indicate reduced affinity of tubulin for all active compounds under this reaction condition. The polymers formed with the higher drug concentration were generally more cold labile than those formed with 10 μM drug. This could be due to smaller average size of polymer, but at least with paclitaxel and compound **3** (see below), this reaction condition yielded the greatest amount of polymer of aberrant morphology.

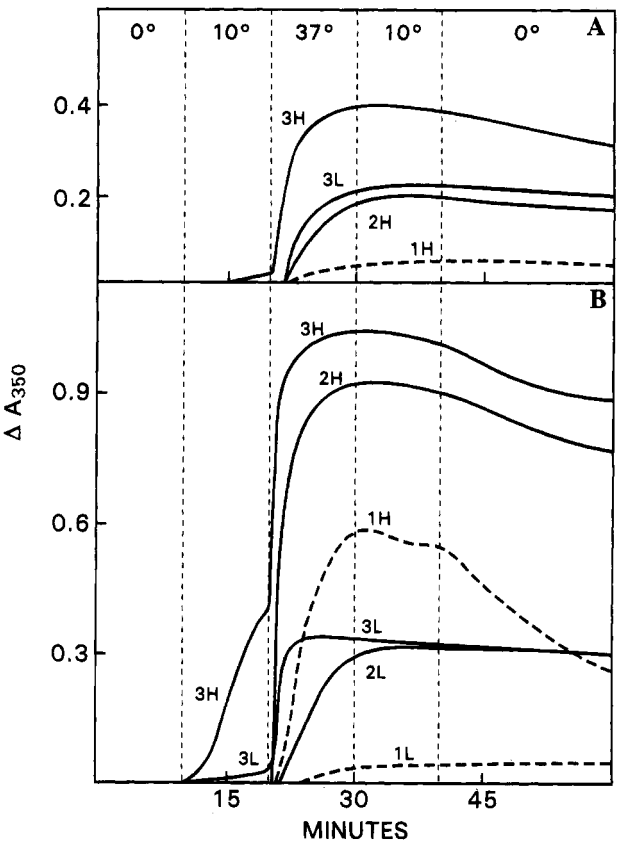

FIGURE 5: Polymerization with neither MAPs nor GTP. Reaction mixtures contained 0.1 M Mes, 1.0 mg/mL (A) or 2.0 mg/mL (B) tubulin, and 4% dimethyl sulfoxide. Temperature as indicated. Dashed curves, reactions with paclitaxel. The numbers on the curves correspond to the drug numbers as indicated in Figure 1, with "L" indicating the drug concentration was 10 μM, and "H" indicating the drug concentration was 40 μM.

Activity with neither GTP nor MAPs. Without MAPs or GTP with 10 μM drug, assembly occurred only with compound **3** at 37 °C (Figure 5A). With 40 μM compound **3** more extensive formation of the cold-stable polymer occurred. With 40 μM drug, a slight reaction occurred with paclitaxel (**1**) and a moderate reaction with docetaxel (**2**). As with GTP only, the reaction with 40 μM docetaxel did not differ greatly from that with 10 μM compound **3**, an effect that could be attributed to relative affinities of the drugs for tubulin.

Tubulin concentration affected the results obtained. At 2.0 mg/mL (Figure 5B) and 10 μM drug, there was a minimal reaction with paclitaxel (**1**) and a moderate reaction with docetaxel (**2**) at 37 °C. When drug concentration was increased to 40 μM, there was substantial assembly at 37 °C with paclitaxel, producing a polymer that partially depolymerized at 0 °C. With 40 μM docetaxel, rapid and extensive assembly occurred at 37 °C. With 10 μM compound **3**, there was a slight reaction at 10 °C and a rapid rise in turbidity at 37 °C. With the agent at 40 μM, the 10 °C reaction was much more extensive, and there was further rapid and extensive polymerization at 37 °C.

Critical Concentrations of Tubulin. As a potentially more quantitative approach to relative drug affinities for tubulin, we determined critical tubulin concentrations with paclitaxel and the five analogs (Table 2). The highest concentration of tubulin examined was 8.0 mg/mL, and the ratio of MAPs to tubulin was held constant when MAPs were included in

Table 2: Critical Concentrations of Tubulin (mg/mL) with Paclitaxel and Analogs under Different Reaction Conditions[a]

	neither GTP nor MAPs		GTP only		MAPs only		both GTP and MAPs	
drug	10 °C	37 °C	10 °C	37 °C	10 °C	37 °C	10 °C	37 °C
none	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0	0.3
paclitaxel (10 μM)	>8.0	2.8	>8.0	0.6	>8.0	0.5	0.3	<0.1
paclitaxel (40 μM)	>8.0	1.2	2.5	0.3	0.4	0.2	0.1	<0.1
docetaxel (10 μM)	>8.0	1.2	2.6	0.3	0.5	0.3	0.1	<0.1
docetaxel (40 μM)	4.3	0.6	1.5	<0.1	0.2	<0.1	<0.1	<0.1
compound **3** (10 μM)	1.8	0.4	0.4	<0.1	0.2	<0.1	<0.1	<0.1
compound **3** (40 μM)	0.9	0.2	0.2	<0.1	0.1	<0.1	<0.1	<0.1
compound **4** (10 μM)	>8.0	>8.0	>8.0	>8.0	>8.0	0.7	>8.0	0.2
compound **4** (40 μM)	>8.0	>8.0	>8.0	2.8	>8.0	0.3	1.2	0.2
compound **5** (10 μM)	>8.0	>8.0	>8.0	1.8	>8.0	0.3	>8.0	0.2
compound **5** (40 μM)	>8.0	>8.0	>8.0	2.1	>8.0	0.3	0.7	0.1
compound **6** (10 μM)	>8.0	>8.0	>8.0	1.6	>8.0	0.3	0.6	0.2
compound **6** (40 μM)	>8.0	4.1	>8.0	1.4	>8.0	0.3	0.3	<0.1

[a] Critical concentrations were measured in reaction mixtures containing 0.1 M Mes (pH 6.9), drug as indicated (final dimethyl sulfoxide at 4%), and, if present, 100 μM GTP and MAPs at half the concentration of the tubulin (in mg/mL). Drug was the final addition to the reaction mixture. Reactions were followed for 10 min at 0 °C, 15 min at 10 °C, and 20 min at 37 °C. The final turbidity readings (as ΔA_{350} units) at the indicated temperatures were used in determination of the critical concentrations, with turbidity plotted against tubulin concentration. The critical concentration was taken as the intercept on the concentration axis. Generally, the 10 and 37 °C studies required use of different concentration ranges. Typically, in the drug-free controls, there was no increase in turbidity at 10 °C, and turbidity values at this temperature were uncorrected for determination of critical concentration. At 37 °C, however, there was always a rise in turbidity in the absence of drug. Without both GTP and MAPs, this reaction was not cold reversible and ceased when the temperature was reduced. This reaction was a linear function of tubulin concentration, and an appropriate correction was made to values obtained with drug (i.e., the turbidity change with drug was only attributed to the drug if it exceeded the change without drug). All values are averages from two independent experiments, with differences between duplicate determinations no greater than 0.1 mg/mL (for some of the lower critical concentrations) or 15%. In addition, there were initial experiments that established the concentration ranges required for detailed studies.

the reaction. In the controls without drug, no reaction was observed at 10 °C, and at 37 °C assembly only occurred with both GTP and MAPs.

These data reiterate the above findings, and they suggest that differential affinity of taxoids for tubulin plays a role in the differing polymerization patterns observed.[2] In terms of relative activity of the different reaction systems, a clear order has emerged: MAPs/GTP > MAPs only > GTP only > no MAPs/no GTP. In general, a 4-fold increase in drug concentration (from 10 to 40 μM) reduced the critical concentration by half at both 10 and 37 °C. There are two types of exception to this generalization. First, in a few cases where the critical concentration at 10 μM drug was over 8.0 mg/mL, the critical concentration with 40 μM drug was low (paclitaxel, MAPs only, 10 °C; compounds **4** and **5**, MAPs/GTP, 10 °C). Second, in a few cases there was little difference in the critical concentrations with the two drug concentrations (at 37 °C with compounds **5** and **6** in the GTP-only and the MAPs-only reaction conditions, and with compound **4** at 37 °C with MAPs/GTP).

With the more active compounds (paclitaxel, docetaxel, compound **3**) in the more restrictive reaction conditions (GTP only; no MAPs/no GTP), the critical concentrations at 40 μM/10 °C were always higher than with 10 μM/37 °C, while with MAPs the critical concentrations at 40 μM/10 °C and 10 μM/37 °C were similar. With the less active compounds the critical concentrations at 40 μM/10 °C were higher than those at 10 μM/37 °C under all reaction conditions. This may indicate either a differential enhancement of activity with the weaker agents at the higher temperature or perhaps requirement for formation of higher order structures prior to binding (see Discussion).

Morphological Studies. Since compound **3** is more potent than paclitaxel in inducing polymerization under all conditions examined, it was important to determine whether there were any morphological differences between polymer formed with the two agents. These studies were performed with tubulin at 1.0 mg/mL and 10 μM drug. If significant turbidity developed or persisted in a series of temperature steps (0, 10, 37, 0 °C), we removed aliquots from reaction mixtures followed turbidimetrically for evaluation by electron microscopy. We observed no significant difference in any sample as a function of temperature, and Figures 6 and 7 present examples of polymer observed at 37 °C (each reaction condition observed in two independent experiments).

Figure 6 compares polymer formed with paclitaxel and compound **3** in the presence of MAPs, both with and without GTP. In all cases the predominant polymer had the typical morphological appearance of microtubules. With both compounds with GTP only, a mixed polymer of microtubules and open sheets was observed, with sheets predominating. Often the microtubules would emerge from open sheets, as if the closure step was delayed or inhibited. Higher power views are presented in Figure 7A for compound **3** and Figure 7B for paclitaxel. With compound **3** in the absence of both MAPs and GTP, microtubules were again frequently observed and seemed to be the predominant polymer (Figure 7C). (Paclitaxel was inactive under this condition unless the tubulin concentration was higher.)

DISCUSSION

Polymer Stability and "Hypernucleation". With a variety of paclitaxel analogs, cold stabilization of polymer does not correlate well with activity under "restrictive reaction conditions" (low temperature; no MAPs; no GTP) where tubulin normally does not polymerize. Warmer temperatures, MAPs, and GTP all enhance nucleation reactions. The reduced requirements for these elements represent manifestations of paclitaxel's ability to "hypernucleate" microtubules, as do a lower critical concentration for tubulin and increased number of short microtubules (Schiff et al., 1979).

[2] Alternatively, it may be argued that critical concentration data primarily provide information about altered nucleation properties rather than about relative drug affinities for tubulin (see Discussion).

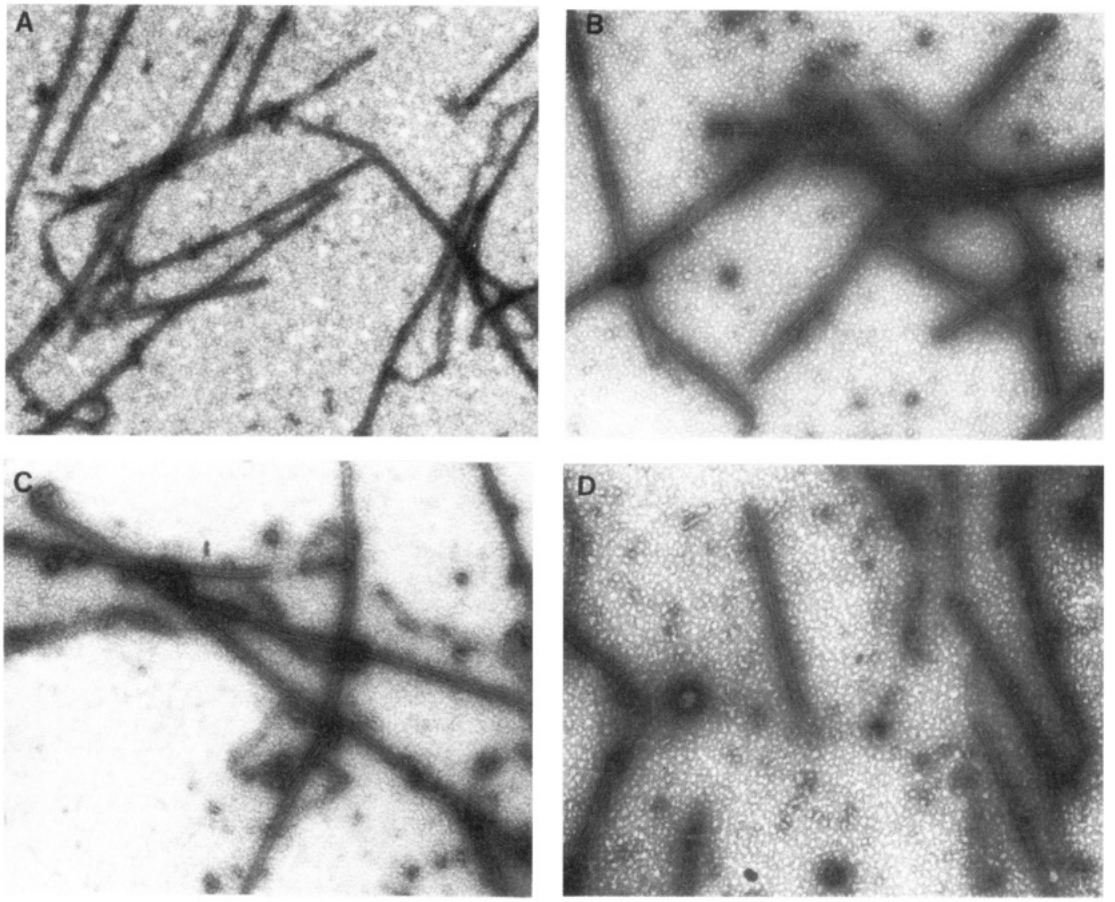

FIGURE 6: Polymer formed with paclitaxel (A, C) or compound **3** (B, D) with both MAPs and GTP (A, B) or with MAPs only (C, D). Magnification 36000×. Reaction mixtures contained, as indicated, 0.1 M Mes, 1.0 mg/mL tubulin, 100 μM GTP, 0.5 mg/mL heat-treated MAPs, 4% dimethyl sulfoxide, and paclitaxel or compound **3** at 10 μM. Images prepared from samples taken at 37 °C.

The dissociation of the stabilization and hypernucleation properties of paclitaxel is most striking in compound **5** but also occurs with compound **6**. With a temperature jump from 0 to 37 °C under conditions where nucleation occurs without drug, differences from the paclitaxel control were minimal. Polymer formed with either analog was as cold stable as that formed with paclitaxel. When cold stabilization was evaluated as a function of drug concentration, there were minimal differences between paclitaxel and the analogs (IC_{50} values of 0.4, 0.7, and 0.8 μM for paclitaxel, **5**, and **6**). The decreased activity of these analogs relative to paclitaxel only became apparent under restrictive reaction conditions or when critical tubulin concentrations were measured.

Conversely, the markedly enhanced hypernucleation activity of compound **3** could not be predicted from the cold stability of the polymer formed in its presence. In the concentration study of stabilization, the IC_{50} values for **3** and paclitaxel were nearly equivalent. Enhanced activity of **3** was only detected through use of restrictive reaction conditions.

The restrictive reactions were affected in tandem with all analogs. Those with limited ability to support polymerization at low temperatures were also deficient when either MAPs or GTP was not included in the reaction mixture and had higher critical concentrations for tubulin than that of paclitaxel. Compound **3** and, to a lesser extent, docetaxel were more active than paclitaxel under all restrictive conditions and had lower tubulin critical concentrations.

Implications for the Mechanism of Interaction of Taxoids with Tubulin. Studies with radiolabeled paclitaxel and 7-acetylpaclitaxel established that these agents bind in stoichiometric amounts much more readily to microtubules than to unpolymerized tubulin (Parness & Horwitz, 1981; Takoudju et al., 1988). This implies that binding to polymer must be an important mechanism in its cytotoxic action, for both interphase and mitotic cells possess complex microtubule arrays (cf. Manfredi et al., 1982).

Nonetheless, in cell-free systems, paclitaxel induces extensive microtubule assembly under conditions where it otherwise does not occur (Hamel et al., 1981; Kumar, 1981; Schiff & Horwitz, 1981; Thompson et al., 1981). Compound **3** and docetaxel were even more potent than paclitaxel, and lower critical concentrations were obtained with both analogs. These agents induce assembly under conditions where there is no evidence for the existence of microtubules, and this implies that they bind to small assemblages of protein.

Is the potential binding site as small as a single α-β heterodimer? This seems unlikely from our critical concentration studies, for the very existence of a critical concentration demonstrates that the rate and extent of the assembly reaction is a sigmoidal function of tubulin concentration. This in turn is most consistent with tubulin−tubulin interactions being a prerequisite for drug-induced polymerization. With **3**, docetaxel, and paclitaxel, the critical concentration of tubulin varied with drug concentration and temperature, as well as the presence of MAPs and/or GTP in the reaction mixture. With these three agents, the critical concentration was reduced when the drug concentration or reaction temperature was increased or when GTP or MAPs were added, with the addition of MAPs having the greatest impact.

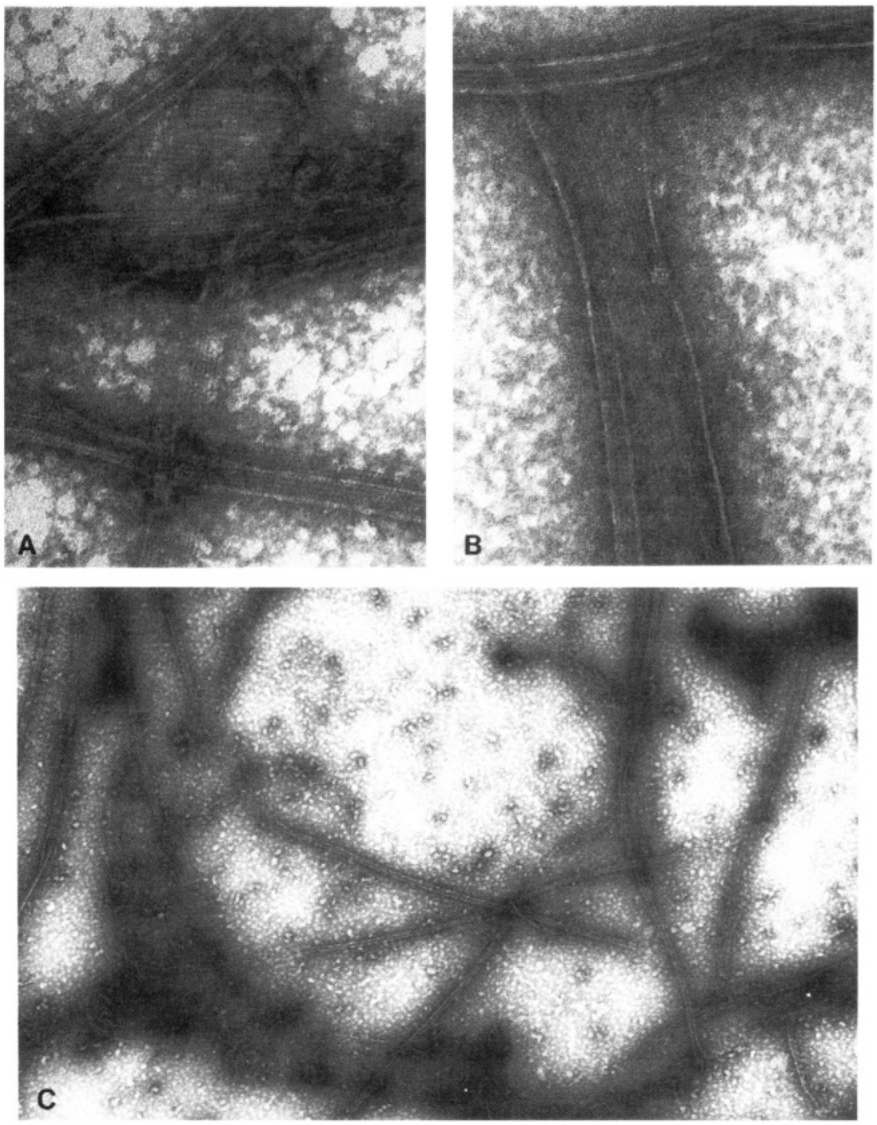

FIGURE 7: Polymer formed with compound **3** (A) or paclitaxel (B) with GTP only or with compound **3** with neither GTP nor MAPs (C). A and B: Magnification 180000×. C: magnification 36000×. Reaction mixtures contained, as indicated, 0.1 M Mes, 1.0 mg/mL tubulin, 100 μM GTP, 4% dimethyl sulfoxide, and paclitaxel or compound **3** at 10 μM. Images prepared from samples taken at 37 °C.

These are all factors, as is an increased tubulin concentration, known to enhance the poorly understood nucleation phase of microtubule assembly. The progressively increasing activity of paclitaxel, docetaxel, and compound **3** in inducing microtubule assembly could indicate their increasing activity as stabilizing agents for transient nucleation centers and progressive reduction in the minimal size of such centers. The high activity of compound **3** may indicate that a taxoid could convert a single tubulin heterodimer into an active nucleation center as a result of a conformational change in the protein. Such an agent would presumably yield a critical concentration of zero under all reaction conditions. The poor activity of compound **5** (bulky substituent at C-7) as an inducer of nucleation combined with its strong activity in stabilizing polymer implies a significant change in the conformation of the paclitaxel binding site when stabilized nucleation centers are converted to microtubules.

Structure−Activity Correlations. We examined analogs with a variety of modifications at several positions in the paclitaxel molecule. Different modifications at either position C-3′ or C-2 can yield compounds that are either more active or less active than paclitaxel. Examples of more active analogs are docetaxel (at C-3′), in agreement with previous reports (Guéritte-Voegelein et al., 1991; Ringel & Horwitz, 1991; Díaz & Andreu, 1993; Georg et al., 1994), and **3** (at C-2); of less active analogs, **6** (at C-3′) (cf. Guéritte-Voegelein et al., 1991) and **4** (at C-2) (cf. Chen et al., 1993d, 1994). Such seemingly disparate findings are readily understood in terms of the probable conformation of active taxoids in aqueous solution (Dubois et al., 1993; Vander Velde et al., 1993; Williams et al., 1993). In contrast to the crystal structure of docetaxel (Guéritte-Voegelein et al., 1990), in which the C-13 and C-2 substituents are well separated, in more polar solvents the two hydrophobic substituents move close together. They thus form a "side chain complex" that may also impose conformational constraints on the taxoid skeleton. Besides the azido group in compound **3**, we found that several *meta* substituents in the C-2 benzoyl group enhance activity relative to paclitaxel, while *para* substituents all lead to reduced activity (Chaudhary et al., 1994). We postulate that the former analogs have an enhanced interaction between the substituted C-2 benzoyl group and the C-13 side chain, while the latter have a reduced interaction.

Lataste et al. (1984) showed that the C-13 side chain was required for an effective interaction of taxoids with porcine

tubulin, since baccatin III, lacking only the C-13 substituent, was 50-fold less active than paclitaxel. Tubulin obtained from the amoeba *Physarum polycephalum* interacted equally well with baccatin III and paclitaxel. Assuming active site conservation, the primary interaction between taxoids and tubulin probably occurs via the taxoid skeleton rather than through the side chain complex. Our findings with bulky C-7 substituents (cf. Georg et al., 1992; Rimoldi et al., 1993) support this interpretation. Other studies (Lataste et al., 1984; Mellado et al., 1984; Ringel & Horwitz, 1987; Chen et al., 1993b,c) have described paclitaxel analogs with C-7 modifications as equivalent in activity to paclitaxel, but the derivatives were generally nonbulky at C-7. Moreover, only with 7-acetylpaclitaxel was a restrictive reaction condition examined (Mellado et al., 1984). We find that compounds with bulky C-7 substituents have limited ability to enhance assembly reactions but do stabilize polymer to cold. If the primary interaction of tubulin with paclitaxel occurs through the taxoid skeleton, the reduced activity of C-7-substituted analogs indicates that this region of the paclitaxel molecule comes into close contact with the protein during nucleation and that contacts between drug and protein change in the microtubule.

The 10-acetyl group does not affect the activity of paclitaxel or docetaxel in the reaction conditions examined, in agreement with previous work (McLaughlin et al., 1981; Parness et al., 1982; Lataste et al., 1984; Guéritte-Voegelein et al., 1991; Chaudhary & Kingston, 1993; Chen et al., 1993a; Georg et al., 1994). Thus, the C-10 region is an attractive site for photoactive, fluorescent, or spin labels.

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