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ORIGINAL ARTICLE

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A quantitative evaluation of the effects of inhibitors of tubulin assembly on polymerization induced by discodermolide, epothilone B, and paclitaxel

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Abstract *Purpose*: To determine whether inhibitors of microtubule assembly inhibit polymerization induced by discodermolide and epothilone B, as well as paclitaxel, and to quantitatively measure such effects. *Methods*: Inhibition was quantitated by measuring polymer formation either by turbidimetry or by centrifugation, and the amount of inhibitor required to inhibit 50% relative to an appropriate control reaction was determined. *Results*: The inhibitory drugs evaluated were four colchicine site agents (combretastatin A-4, podophyllotoxin, nocodazole, and *N*-acetylcolchinol-*O*-methyl ether), maytansine, which competitively inhibits the binding of *Catharanthus* alkaloids to tubulin, halichondrin B and phomopsin A, which noncompetitively inhibit the binding of *Catharanthus* alkaloids to tubulin, and the depsipeptide dolastatin 15. While relative inhibitory effects were highly variable, a few broad generalizations can be made. First, assembly reactions that were either enhanced or dependent upon all three stimulatory drugs were subject to inhibition by all inhibitors. Second, the more readily the tubulin assembled, the greater the concentration of inhibitor required to inhibit polymerization. Drug IC_{50} values were generally lowest with no stimulatory drug and highest when discodermolide was present; IC_{50} values were higher as reaction temperature increased; and IC_{50} values were higher as the tubulin concentration increased. Third, inhibition of assembly by inhibitors of *Catharanthus* alkaloid binding to tubulin changed much less as a function of changes in reaction conditions than inhibition by inhibitors of colchicine binding. *Conclusions*: Since there was no apparent quantitative predictability of combined drug interactions with tubulin, any combination of interest must be studied in detail.

Keywords Discodermolide · Epothilone B · Paclitaxel · Tubulin assembly

Abbreviations *MAPs* Microtubule-associated proteins · *Mes* 4-Morpholineethanesulfonate

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Introduction

Paclitaxel, together with structurally related taxoids, was for many years a unique antimitotic agent [22]. Low concentrations of all drugs, including paclitaxel, that interact with tubulin suppress microtubule dynamics, a term that encompasses the multiple reactions involved in the rapid assembly and disassembly of microtubules, particularly those forming the spindle [11]. Nevertheless, paclitaxel has distinct cellular and biochemical properties [19] when compared with most drugs that interact with tubulin. Cells treated with paclitaxel display bundled microtubules at interphase or, at mitosis, bizarre, malformed "spindles," whereas in cells treated with most antimitotic compounds the microtubules disappear. At the biochemical level paclitaxel enhances assembly of tubulin into hyperstable microtubules, while most drugs inhibit tubulin polymerization.

Since 1995, additional natural products have been described that act by a similar mechanism of action. These compounds include the epothilones [4], discodermolide [24], and eleutherobin [17], all of which

competitively inhibit the binding of [^{3}H]paclitaxel to microtubules. Such an inhibitory pattern implies the drugs bind at the same or, perhaps, an overlapping site on tubulin as does paclitaxel [8, 13, 14]. Epothilone B and discodermolide are especially interesting, since they bind to tubulin more avidly than does paclitaxel. This increased activity is also manifested by their being more active, and at lower concentrations, than paclitaxel in inducing tubulin assembly [13, 14].

It has long been recognized that the effects of inhibitors of tubulin assembly dominate assembly induction by the taxoids [15, 23]. Inhibition occurs in taxoid-induced assembly, as well as in drug-independent tubulin polymerization, but there has been little quantitative evaluation of mixed drug effects. Moreover, there is negligible published data about the effects of inhibitors of assembly on the reactions induced by the recently discovered agents. We therefore examined quantitatively eight inhibitors of assembly in combination with paclitaxel and in combination with the more potent enhancers of assembly, epothilone B and discodermolide.

Materials and methods

Materials

Tubulin freed of MAPs and heat-treated MAPs [7] were prepared from bovine brain and discodermolide was synthesized [21] as before. For experiments for determination of the critical concentration of tubulin without MAPs and GTP but with 10 μM paclitaxel or discodermolide, the tubulin was freed of unbound nucleotide by gel filtration chromatography [6] and dialyzed against 0.1 *M* Mes (pH 6.9 with NaOH in 1 *M* stock solution). Epothilone B was provided by Merck Research Laboratories. Dolastatin 15 and combretastatin A-4 were gifts from Dr. G.A. Pettit (Arizona State University), and *N*-acetylcolchinol-*O*-methyl ether was a gift from Dr. A. Brossi (National Institute of Diabetes and Digestive and Kidney Diseases). Paclitaxel, maytansine, and halichondrin B were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. Phomopsin A was from Calbiochem, and nocodazole and podophyllotoxin were from Aldrich.

Methods

Tubulin polymerization was evaluated either turbidimetrically or by centrifugation. In the turbidimetric studies, assembly was followed at 350 nm in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. Reaction mixtures contained 1.0 mg/ml tubulin, 0.5 mg/ml heat-treated MAPs, 0.1 *M* Mes (pH 6.9), 0.1 m*M* GTP, varying amounts of inhibitory drug, stimulatory drug, if present, at 10 μM, and 4% dimethyl sulfoxide. The stimulatory drug was added to the other components in cuvettes held at 0°C after baselines were established. Unless the reaction was followed at 0°C, the temperature was jumped to 30°C. The assembly reactions were followed for 30 min, at which point the turbidity readings were taken for calculation of IC_{50} values.

When mixed drug effects were examined in the cold, centrifugation was used for the assay to process larger numbers of samples in individual experiments. Reaction mixtures (0.1 ml) were prepared as described above, with the final addition of either epothilone B or discodermolide. The reaction mixtures were left on ice for 15 min and transferred to an ice-cold Beckman TLA 100 rotor and centrifuged at 30,000 rpm for 10 min in an Optima TLX miniultracentrifuge prechilled to 2°C. An aliquot was removed from the top of the supernatant, and its protein content determined by the Lowry method. The amount of protein in the supernatant of control samples (neither inhibitory nor stimulatory drug) averaged 91% of that in the uncentrifuged reaction mixture. With 10 μM discodermolide, the supernatant protein averaged 35% of the total protein; and with 10 μM epothilone B, the supernatant protein averaged 48% of the total. For this assay, the IC_{50} value was defined as the concentration of inhibitory drug that reduced supernatant protein attributable to the presence of discodermolide or epothilone B in the reaction mixture by 50%:

$$\begin{aligned}\%\ \text{inhibition} = 100 - [&(\text{no drug supernatant protein} \\ &- \text{inhibitory drug mixed} \\ &\text{with stimulatory drug supernatant protein})/ \\ &(\text{no drug supernatant protein} \\ &- \text{supernatant protein with discodermolide or} \\ &\text{epothilone B only})] \times 100\end{aligned}$$

Drug-induced disassembly of preformed polymer was determined by the same centrifugal technique, except that rotor and centrifuge were at 30°C. Polymer was formed under the reaction conditions described above (first paragraph Methods section), except that 1% dimethyl sulfoxide was present during the assembly phase (30 min at 30°C). Aliquots from polymer-containing reaction mixtures were added to inhibitory drugs at varying concentrations (final dimethyl sulfoxide concentration, 4%), and the incubation was continued for another 30 min at 30°C. Following centrifugation, the protein concentrations of aliquots removed from the top of each supernatant were determined, and disassembly was measured in terms of the increase in protein concentration. The drug concentrations that caused 50% disassembly of polymer (EC_{50} values) were determined. All data were corrected for the amount of protein removed from the supernatant by centrifugation of reaction mixtures containing neither GTP nor drug ($8.3 \pm 2\%$ in this series of experiments). The mathematical calculations were essentially as described above for the 0°C assembly inhibition experiments.

The critical concentration for polymerization of tubulin without MAPs and without GTP in the presence of 10 μM paclitaxel or discodermolide at 37°C was determined by centrifugation. Reaction mixtures (0.1 ml) contained varying concentrations of tubulin, 0.1 *M* Mes (pH 6.9), 4% dimethyl sulfoxide, and drugs as appropriate. After 30 min at 37°C the reaction mixtures were centrifuged for 15 min at 14,000 rpm at room temperature (about 22°C) in an Eppendorf 5417C centrifuge. An aliquot was removed from the supernatant, and its protein content determined by the Lowry method. IC_{50} values for drugs were determined by the same method at tubulin concentrations as indicated. The mathematical calculation of IC_{50} values was as described above for the 0°C experiments.

Results and discussion

Inhibitory drugs examined

Our major criterion was that the selected drugs cause no turbidity development. We chose podophyllotoxin [27], combretastatin A-4 [16], nocodazole [9], and *N*-acetylcolchinol-*O*-methyl ether [10, 12] (four drugs that inhibit colchicine binding to tubulin), and maytansine [18], halichondrin B [2], phomopsin A [1, 25], and dolastatin 15 [3] (four drugs that probably bind in the vinca domain of tubulin, although the binding site of dolastatin 15 is uncertain). Dolastatin 15 does not inhibit vinblastine binding to tubulin [3], although both

vinblastine and vincristine do inhibit dolastatin 15 binding to tubulin [5].

Inhibitory effects on MAP-dependent assembly at 30°C

We initially examined the effects of each of the eight inhibitors on microtubule assembly, followed turbidimetrically, in a reconstituted system containing purified tubulin, heat-treated MAPs, and GTP (Table 1). Even when no IC_{50} value was determined, partial inhibition of assembly was always observed. This was also true in the centrifugal studies presented below.

It is apparent from the data presented in Table 1 that, while all the inhibitory drugs inhibited assembly enhanced by paclitaxel, epothilone B, or discodermolide, quantitative aspects of the inhibitory effects varied with the inhibitor studied. Overall, there was a major difference between the drugs that inhibited the binding of *Catharanthus* alkaloids to tubulin and those that inhibited colchicine binding, with dolastatin 15 more closely resembling the latter group. The *Catharanthus* alkaloid inhibitors, especially maytansine, were much less affected in their ability to inhibit microtubule assembly by the presence of a stimulatory drug in the reaction mixture than were the colchicine inhibitors. As two examples, the IC_{50} values obtained for halichondrin B rose from 0.8 μM without stimulatory drug to 2.7 μM with paclitaxel to 3.3 μM with epothilone B to 4.7 μM with discodermolide; for podophyllotoxin, equivalent values were 2.3, 13, 31, and 280 μM, respectively. The distinct behavior of dolastatin 15, as compared with the drugs that are unambiguous *Catharanthus* alkaloid inhibitors, probably derives from its relatively weak interaction with tubulin. Recent work has shown that dolastatin 15 has a high apparent K_d value (about 30 μM) for its binding to tubulin and that its binding is inhibited by most vinca domain drugs [5]. It is also likely that this low affinity for tubulin accounts for the inability of dolastatin 15 to inhibit vinblastine binding to tubulin [3].

In addition, in most cases, the more potent the stimulatory drug, the greater the concentration of inhibitory drug required to inhibit assembly by 50%. The order of inhibitory IC_{50} values was generally no drug < paclitaxel < epothilone B < discodermolide. For the exceptions, maytansine and phomopsin A, the IC_{50} values obtained in the presence of epothilone B were slightly lower than the values obtained in the presence of paclitaxel.

Inhibitory effects on MAP-dependent assembly at 0°C

When we began these studies, we thought the IC_{50} values of the inhibitory drugs would increase as assembly became more facile. This expectation was partially fulfilled by the observation that IC_{50} values tended to increase with stimulatory drug potency, but we had not expected such wide quantitative variability between different inhibitory agents of apparent similar potency in the absence of a stimulatory drug (compare maytansine with nocodazole, or phomopsin A with combretastatin A-4). We decided to extend our analysis to drug-enhanced assembly at 0°C, although there was significant microtubule assembly under our reaction conditions at this temperature only with epothilone B and discodermolide (Fig. 1; cf. references 14 and 24). In this case we anticipated that, assembly being less facile at 0°C, inhibitory drug IC_{50} values would be lower at 0°C than they were at 30°C, and that IC_{50} values obtained in the presence of discodermolide would be higher than those obtained in the presence of epothilone B.

The actual findings were only partially in agreement with these predictions and are summarized in Table 2. As at 30°C, the IC_{50} values obtained in the presence of discodermolide were higher than those obtained in the presence of epothilone B, but, as at 30°C, the differences with the *Catharanthus* alkaloid inhibitors was minimal. Moreover, with these inhibitors, the IC_{50} values obtained at 0°C were almost identical to those obtained at 30°C in the presence of either discodermolide or

Table 1 Inhibition of microtubule assembly with MAPs and GTP at 30°C by inhibitory drugs with or without paclitaxel, epothilone B, or discodermolide. Values are the means ± SD of at least three experiments, except for those in which no IC_{50} value could be obtained; the latter experiments were performed twice

Inhibitory drug added	No stimulatory drug IC_{50} (μM)	Paclitaxel		Epothilone B		Discodermolide	
		IC_{50} (μM)	Relative to no stimulatory drug	IC_{50} (μM)	Relative to no stimulatory drug	IC_{50} (μM)	Relative to no stimulatory drug
Maytansine	2.7 ± 0.3	3.3 ± 0.1	1.2	2.9 ± 0.1	1.1	4.7 ± 2	1.7
Halichondrin B	0.80 ± 0.1	2.7 ± 0.1	3.4	3.3 ± 0.3	4.1	4.7 ± 0.3	5.9
Phomopsin A	0.62 ± 0.1	7.4 ± 2	12	7.2 ± 0.9	12	11 ± 1	18
Dolastatin 15	5.4 ± 0.5	370 ± 35	69	> 600	> 111	> 650	> 120
Nocodazole	3.2 ± 0.2	39 ± 5	12	100 ± 5	31	> 150	> 47
Combretastatin A-4	0.65 ± 0.1	2.5 ± 0.6	3.8	32 ± 4	49	300[a]	460
Podophyllotoxin	2.3 ± 0.2	13 ± 2	5.7	31 ± 5	13	280 ± 40	120
N-acetylcolchinol-*O*-methyl ether	3.3 ± 0.3	23 ± 3	7.0	120 ± 20	36	> 300	> 91

[a]The same value was obtained in all experiments

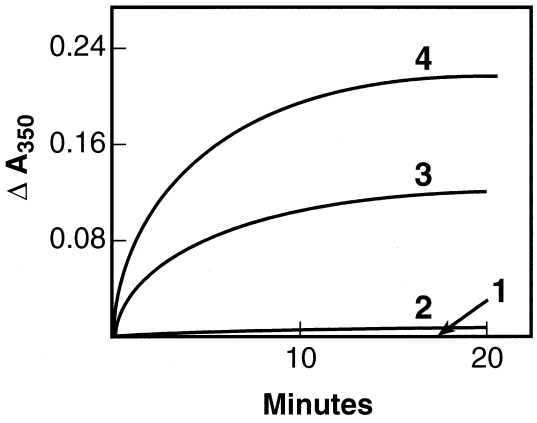

Fig. 1 Stimulation of MAP-dependent microtubule assembly at 0°C by discodermolide or epothilone B but not by paclitaxel. Reaction mixtures were prepared as described in detail in the text. Stimulatory drugs were added at time zero, with the reaction mixtures continuously at 0°C, as follows: *curve 1* none, *curve 2* 10 μ*M* paclitaxel, *curve 3* 10 μ*M* epothilone B, *curve 4* 10 μ*M* discodermolide

Table 2 Inhibition of microtubule assembly with MAPs and GTP at 0°C by inhibitory drugs with epothilone B or discodermolide. SD, standard deviation. Values are the means ± SD of at least two determinations; if no SD is shown, the same value was obtained twice

Inhibitory drug added	IC_{50} (μ*M*)	
	Epothilone B	Discodermolide
Maytansine	3.6 ± 0.6	4.1 ± 0.6
Halichondrin B	1.8 ± 0.1	2.4
Phomopsin A	7.0 ± 0.2	10 ± 3
Nocodazole	6.4 ± 0.2	25 ± 0.8
Combretastatin A-4	2.9 ± 0.2	8.5 ± 1
Podophyllotoxin	28 ± 2	100
N-acetylcolchinol-*O*-methyl ether	31 ± 11	100

epothilone B. With the colchicine site drugs the IC_{50} values obtained in the presence of discodermolide were all substantially lower at 0°C than the analogous values obtained at 30°C, but with epothilone B two different patterns were observed. Nocodazole, combretastatin A-4, and *N*-acetylcolchinol-*O*-methyl ether had IC_{50} values at 0°C that were significantly lower than the 30°C values, but the values obtained for podophyllotoxin were nearly identical at the two temperatures.

Effects of assembly inhibitors on preformed microtubules

In the studies summarized in Table 1, the stimulatory drug was added last because of the extensive assembly that occurs at 0°C with epothilone B and discodermolide (Fig. 1). We also examined the effects of inhibitors on preformed polymers, which were incubated for 30 min at 30°C following addition of the inhibitors (Table 3). The amount of inhibitor causing 50% disassembly (EC_{50} value) was determined. Inhibitor concentrations up to 300 μ*M* were used, but we noted precipitated nocodazole in the pellet at 150 μ*M*. In addition, we found that aggregates induced by phomopsin A [25] were pelleted when the peptide was present at 15–20 μ*M*. This prevented evaluation of the ability of phomopsin A to induce disassembly of drug-induced microtubules (the control EC_{50} value was 8.1 μ*M*).

Without a stimulatory drug, all the inhibitors caused extensive polymer disassembly, but disassembly EC_{50} values were higher than the IC_{50} values (Table 1) when drugs were added prior to polymerization. The smallest difference in values, about 2-fold, was observed with maytansine, and the largest, almost 90-fold, with combretastatin A-4.

With drug-induced polymers, in general disassembly became more feeble as the stimulatory drug became more potent. Specific disassembly effects were as unpredictable as the inhibitory effects described above. For example, while maytansine was equally effective in causing 50% disassembly of paclitaxel polymer and control polymer at low micromolar concentrations, even 300 μ*M* maytansine did not cause 50% disassembly of either the epothilone B or the discodermolide polymer (30% and 11% disassembly, respectively). While halichondrin B was fourfold more potent than combretastatin A-4 in causing control polymer disassembly

Table 3 Drug-induced disassembly of microtubules formed with MAPs and GTP at 30°C with or without paclitaxel, epothilone B, or discodermolide. Values are the means ± SD of two experiments

Inhibitory drug added	No stimulatory drug	Paclitaxel		Epothilone B		Discodermolide	
	EC_{50} (μ*M*)	EC_{50} (μ*M*)	Relative to no stimulatory drug	EC_{50} (μ*M*)	Relative to no stimulatory drug	EC_{50} (μ*M*)	Relative to no stimulatory drug
Maytansine	6.2 ± 0.3	5.4 ± 0.1	0.9	> 300	> 48	> 300	> 48
Halichondrin B	14 ± 2	32 ± 0.2	2.3	> 300	> 48	> 300	> 48
Dolastatin 15	77 ± 6	> 300	> 3.9	> 300	> 3.9	> 300	> 3.9
Nocodazole	23 ± 6	> 150[a]	> 6.5	> 150[a]	> 6.5	> 150[a]	> 6.5
Combretastatin A-4	56 ± 7	110 ± 1	2.0	220 ± 20	3.9	> 300	> 5.4
Podophyllotoxin	99 ± 20	> 300	> 3.0	> 300	> 3.0	> 300	> 3.0
N-acetylcolchinol-*O*-methyl ether	27 ± 10	> 300	> 11	> 300	> 11	> 300	> 11

[a]Maximum solubility based on apparent drug precipitate in pellets

(EC_{50} values of 14 and 56 μ*M*, respectively), both compounds showed about a twofold reduction in their ability to induce disassembly of the paclitaxel polymer (EC_{50} values of 32 and 110 μ*M*, respectively). Only combretastatin A-4 was able to induce 50% disassembly of the epothilone B polymer (EC_{50} value 220 μ*M*) of all inhibitors examined, and none caused 50% disassembly of the discodermolide-induced polymer. At 300 μ*M*, halichondrin B caused 30% disassembly of the epothilone B polymer and 12% disassembly of the discodermolide polymer, and combretastatin A-4 caused 31% disassembly of the discodermolide polymer.

The four remaining inhibitors were unable to cause 50% disassembly of any of the drug-stabilized polymers. Their disassembly effects at 300 μ*M* (saturating drug in the case of nocodazole) on polymers induced by paclitaxel, epothilone B, and discodermolide, respectively, were as follows: podophyllotoxin, 44%, 30%, and 13%; nocodazole, 43%, 25%, and 8.1%; *N*-acetylcolchinol-*O*-methyl ether, 38%, 18%, and 9.4%; and dolastatin 15, 6.6%, 1.5%, and 4.1%. Even in this group of compounds, no predictions for disassembly of the drug-stabilized polymer could be derived from EC_{50} values for disassembly of control polymer. Thus, despite nearly identical disassembly effects on control microtubules for podophyllotoxin and dolastatin 15 (EC_{50} values of 80 and 77 μ*M*, respectively), podophyllotoxin partially disassembles the paclitaxel and epothilone B polymers, while dolastatin 15 is almost totally inactive.

Critical concentration studies

One of the favored models for tubulin assembly is the nucleation-condensation hypothesis, originally formulated by Wegner and Engel [26] to describe the kinetics of actin assembly. This model envisages a minimal concentration of monomer (i.e., $\alpha\beta$-tubulin dimer in microtubule assembly) required for assembly to occur. This required minimum concentration was termed the "critical concentration." In previous studies the tubulin critical concentration under all reaction conditions examined was lower with epothilone B than with paclitaxel [14] and lower still with discodermolide [13]. We wondered whether the apparent differences in IC_{50} values with the colchicine site drugs in the presence of the stimulatory drugs (Tables 1 and 2) had resulted at least in part from these differences in critical concentrations.

Previously the highest tubulin critical concentrations were obtained when both MAPs and GTP were omitted from reaction mixtures, and we reasoned that this reaction condition would give the most reliable data. To obtain an unambiguous answer, we limited the comparison to paclitaxel and discodermolide. As noted above, these two agents had previously been shown to have the largest differences in critical concentrations, with epothilone B yielding intermediate values. Moreover, paclitaxel and discodermolide yielded the largest difference in IC_{50} values obtained with the colchicine site drugs.

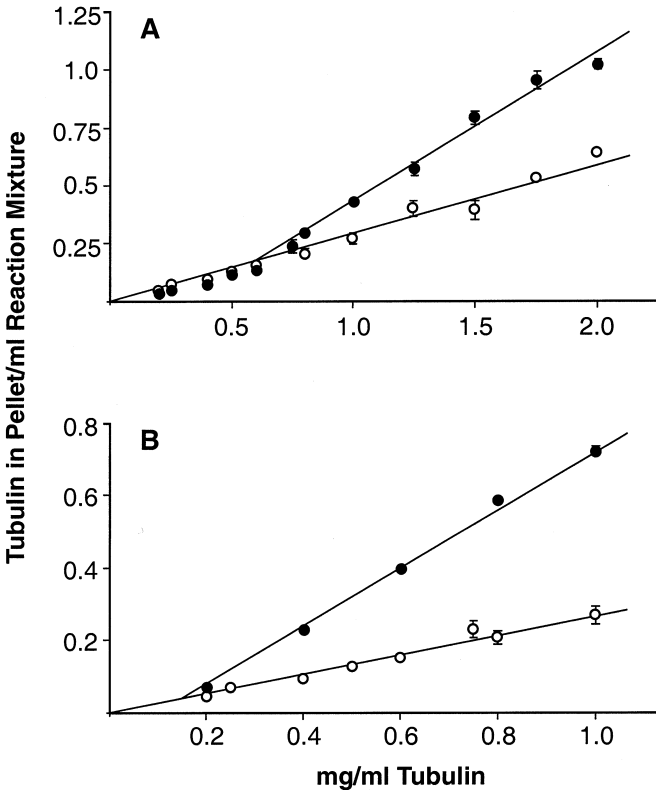

Fig. 2A, B Determination of the critical concentrations of tubulin in the presence of 10 μ*M* paclitaxel (**A**) or 10 μ*M* discodermolide (**B**). Reaction mixtures contained tubulin at the indicated concentrations, 4% dimethyl sulfoxide, 0.1 *M* Mes (pH 6.9), and either no drug (○) or, as indicated, 10 μ*M* paclitaxel or 10 μ*M* discodermolide (●). Values obtained in all experiments were averaged for preparation of the figure, with standard errors shown, unless the error bar is smaller than the symbol

Figure 2 summarizes the experiments in which the tubulin critical concentrations were determined in the presence of 10 μ*M* paclitaxel (Fig. 2A) or 10 μ*M* discodermolide (Fig. 2B). Reaction mixtures were incubated at 37°C for 30 min prior to centrifugation. Although electron microscopy demonstrated that no polymer formation occurred in the absence of drug, there nevertheless was a significant amount of tubulin that was removed by centrifugation in the control samples (about 20–25% as compared with about 8–10% at 0°C and 30°C, see Methods section), and this was a linear function of the original protein concentration. We took the critical concentration to be the tubulin concentration where the curve derived from the values obtained in the presence of the stimulatory drug intersects the control curve. These values were 0.15 mg/ml with discodermolide and 0.59 mg/ml with paclitaxel. The data shown in Fig. 2 are the averages from all experiments. (Two experiments were performed with discodermolide, both yielding critical concentrations of 0.15 mg/ml, and three with paclitaxel, yielding critical concentrations of 0.51, 0.54, and 0.72 mg/ml.)

Table 4 Inhibition of tubulin polymerization in the absence of MAPs and GTP with paclitaxel or discodermolide at equivalent multiples of the tubulin critical concentration. Values are means ± SD of at least two determinations; if no SD is shown, the same value was obtained twice

Inhibitory drug added	IC_{50} (μ*M*)		
	Paclitaxel (tubulin, 1.77 mg/ml)	Discodermolide (tubulin, 0.45 mg/ml)	Discodermolide (tubulin, 1.77 mg/ml)
Maytansine	3.5 ± 0.5	1.1 ± 0.3	7.4 ± 0.08
Nocodazole	6.8 ± 0.4	4.2 ± 0.4	> 150
Combretastatin A-4	2.5 ± 0.2	1.2 ± 0.09	10 ± 0.07
Podophyllotoxin	3.9	2.1 ± 0.05	18 ± 0.2
N-acetylcolchinol-*O*-methyl ether	2.5 ± 0.3	1.8 ± 0.5	12 ± 0.2

We examined the inhibitory effects of the colchicine site drugs and maytansine on assembly of tubulin alone dependent on either 10 μ*M* paclitaxel or discodermolide at three times the critical concentrations for tubulin, 1.77 mg/ml and 0.45 mg/ml, respectively. Note that, unlike the experiments described above where stimulatory drug and tubulin were stoichiometric at 10 μ*M*, in the paclitaxel system there was almost twice as much tubulin as stimulatory drug, while in the discodermolide system it was the stimulatory drug that was in twofold molar excess over tubulin. We also examined inhibitory effects with 10 μ*M* discodermolide with tubulin at 1.77 mg/ml. The results obtained are shown in Table 4. It is clear that tubulin concentration, probably as a manifestation of different critical concentrations with different stimulatory drugs under different reaction conditions, played a significant role in the inhibitory effects observed with different drugs. Again, however, no pattern common to all inhibitory drugs was observed.

At three times the presumptive critical concentrations, the inhibitory drugs were all more inhibitory with discodermolide than with paclitaxel. However, there seemed to be some quantitative variability, ranging from over a threefold difference in the IC_{50} values obtained for maytansine to a 50% difference in the values obtained for *N*-acetylcolchinol-*O*-methyl ether and nocodazole. When the tubulin concentration was raised in the discodermolide system, the IC_{50} values obtained with all drugs except nocodazole rose about seven- to eightfold, while with nocodazole only partial inhibition occurred even at the highest concentration examined.

Conclusions

All inhibitors of microtubule assembly that were examined inhibited tubulin polymerization induced by paclitaxel, epothilone B, and discodermolide. Quantitative aspects of these inhibitory effects could not be completely generalized, although in many cases, particularly with colchicine site drugs, higher concentrations of inhibitor were required as the stimulatory drug became more potent or assembly was facilitated by higher temperatures, MAPs, and GTP. There was only a relatively minor effect of changes in reaction conditions on the potency of vinca domain drugs as inhibitors of assembly. It is tempting to speculate that these differences between the colchicine and *Catharanthus* inhibitors derive from their distinct binding locations on β-tubulin. Much evidence suggests that the colchicine site lies at the interface of the tubulin subunits. In contrast, vinca domain drugs probably bind near the exchangeable nucleotide site, which the electron crystallographic model of tubulin shows is at the opposite end of β-tubulin [20]. Thus, vinca domain drugs probably bind at the interface between two different αβ-heterodimers in a protofilament. This could explain the greater sensitivity of paclitaxel/epothilone B/discodermolide-induced assembly to vinca domain drugs as compared with colchicine site drugs. In any case, there was no apparent quantitative predictability of combined drug interactions with tubulin, so that any combination of interest must be studied in some detail. Similar unpredictability may occur if drug combinations are examined for effects on dynamic instability. Lower concentrations of both inhibitory and stimulatory agents often have marked effects on dynamic parameters such as growing and shortening rates, and it would be interesting to know if the differences observed here between the colchicine and vinca classes of drugs in their effects with stimulatory agents also apply to microtubule dynamics.

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