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Activities of the Microtubule-stabilizing Agents Epothilones A and B with Purified Tubulin and in Cells Resistant to Paclitaxel (Taxol®)*

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Epothilones A and B, natural products with minimal structural analogy to taxoids, have effects similar to those of paclitaxel (Taxol®) in cultured cells and on microtubule protein, but differ from paclitaxel in retaining activity in multidrug-resistant cells. We examined interactions of the epothilones with purified tubulin and additional cell lines, including a paclitaxel-resistant ovarian carcinoma line with an altered β-tubulin. The epothilones, like paclitaxel, induced tubulin to form microtubules at low temperatures and without GTP and/or microtubule-associated proteins. The epothilones are competitive inhibitors of the binding of [^{3}H]paclitaxel to tubulin polymers. The apparent K_i values for epothilones A and B were 1.4 and 0.7 μM by Hanes analysis and 0.6 and 0.4 μM by Dixon analysis. In the paclitaxel-sensitive human cell lines we examined, epothilone B had greater antiproliferative activity than epothilone A or paclitaxel, while epothilone A was usually less active than paclitaxel. A multidrug-resistant colon carcinoma line and the paclitaxel-resistant ovarian line retained sensitivity to the epothilones. With *Potorous tridactylis* kidney epithelial (PtK_2) cells examined by indirect immunofluorescence, microtubule bundles appeared more rapidly following epothilone B treatment, and there were different proportions of various mitotic aberrations following treatment with different drugs.

Many facets of cellular growth depend on microtubules. They provide support for organelles and membranes, resist compressive forces, provide tracks for intracellular vesicular transport and sorting, and form spindle fibers for chromosomal separation and segregation (1). Composed of the heterodimeric protein tubulin, microtubules are dynamic structures that undergo periods of slow growth and rapid shortening both *in vitro* and in cells (2–4). Cellular functions of microtubules probably depend on this phenomenon. Jordan *et al.* (5) have shown that significant alterations of dynamic instability occur with a variety of antimitotic drugs that interact with tubulin. An increasing number of such compounds, displaying great structural diversity, have become available, and the most potent of these agents were initially isolated from natural sources (6). Typically, such compounds inhibit tubulin polymerization, and all lead to mitotic arrest. Ptx[1] (Taxol®) and docetaxel (Taxotere®) (structures in Fig. 1), however, stabilize microtubules (7, 8). These complex diterpenes are currently being used to treat a variety of solid tumors (9, 10).

Recently, three non-taxoid compounds have also been shown to stabilize microtubules. The macrolides EpA and EpB, from the bacterium *Sorangium cellulosum* (11), and the polyhydroxylated alkatetraene lactone Dcd (structures in Fig. 1), from the marine sponge *Discodermia dissoluta* (12), share with Ptx the ability to arrest cells in mitosis, cause formation of bundles of intracellular microtubules in non-mitotic cells, and induce the formation of hyperstable tubulin polymers.

Because the initial studies with the epothilones were performed with microtubule protein (11), we decided it was important to examine the interactions of these new drugs with purified tubulin and characterize the effects of MAPs and GTP on these interactions. We compared interactions between tubulin and Ptx with those between tubulin and the epothilones under a variety of conditions. Of particular interest to us was the nature of the binding site of the epothilones on microtubules because of the lack of structural similarity between the taxoids, Dcd, and the epothilones. Despite this dissimilarity, we observed a competitive pattern of inhibition by EpA and EpB on the binding of [^{3}H]Ptx to polymer formed with purified tubulin. EpB was the most effective compound in inducing assembly of tubulin polymers, while the potency of EpA was similar to that of Ptx.

We also report initial studies on the antiproliferative effects of EpA and EpB in cultured cells. With *Potorous tridactylis* kidney epithelial (PtK_2) cells, we confirmed the cellular reorganization of microtubules described by Bollag *et al.* (11). We found that EpB was more potent than Ptx and EpA, which tended to be less potent than Ptx, in inhibiting the growth of five human tumor cell lines. The epothilones retained their potency in cell lines resistant to Ptx.

EXPERIMENTAL PROCEDURES

Materials—EpA and EpB were isolated from *S. cellulosum* (11). The compounds were generously provided by Dr. Michael Goetz of Merck Research Laboratories. Ptx and [^{3}H]Ptx (19–23 mCi/mmol) were obtained from the Drug Synthesis and Chemistry Branch, NCI (Rockville, MD). Drugs were dissolved in dimethyl sulfoxide, and control samples contained equivalent amounts of solvent. Heat-treated MAPs and electrophoretically homogeneous tubulin were prepared from bovine brain (13). Tubulin was freed from unbound nucleotide by gel-filtration chromatography, reconcentrated, and dialyzed against 0.1 M Mes (pH 6.9) as described elsewhere (8). The tubulin was centrifuged to remove aggre-

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[1] The abbreviations used are: Ptx, paclitaxel; EpA, epothilone A; EpB, epothilone B; Dcd, discodermolide; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAP, microtubule-associated protein; Mes, 4-morpholineethanesulfonic acid; MEM, minimal essential medium; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; MDR, multidrug-resistant; $[T]_{cr}$, tubulin critical concentration; PtK_2 cell, *P. tridactylis* kidney epithelial cell.

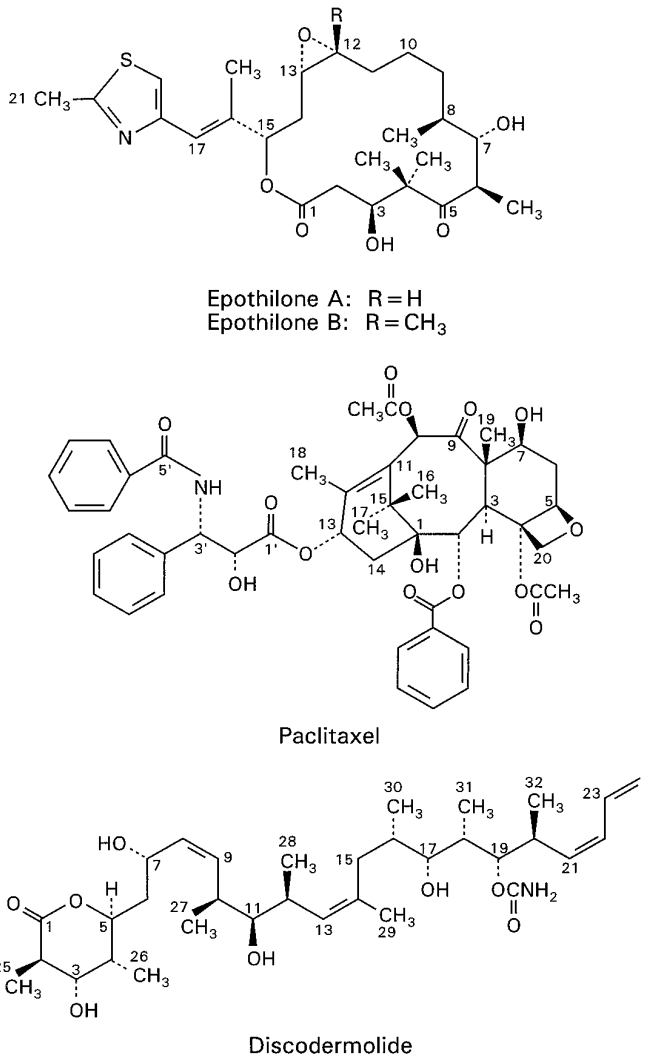

FIG. 1. **Structures of EpA and EpB, Ptx, and Dcd.** The configurations of the chiral centers of the epothilones are those reported by Höfle *et al.* (35).

gated protein and stored in liquid nitrogen in 0.2-ml aliquots. Human Burkitt lymphoma CA46 cells were a gift from Dr. P. O'Connor, NCI (Bethesda, MD); *P. tridactylis* kidney epithelial (PtK_2) cells were from the American Type Culture Collection. The human ovarian carcinoma line 1A9 (14) was used to generate a Ptx-resistant line, 1A9 (PTX22), by incubating the cells with increasing concentrations of Ptx in the presence of verapamil.[2] The human colon carcinoma line SW620 and its subline SW620AD-300, which overexpresses P-glycoprotein, were described elsewhere (15). Culture media and FCS were from Life Technologies, Inc., Permanox and glass slides from Nunc, murine monoclonal anti-β-tubulin antibody and GTP from Sigma, and Texas Red-conjugated goat-anti-mouse IgG and ProLong antifade fluorescent mounting media from Molecular Probes. GTP and ddGTP (from Pharmacia) were repurified by anion exchange chromatography on DEAE-cellulose.

Inhibition of Burkitt Lymphoma Cell Growth—Cells were grown in 5-ml suspension cultures at 37°C in a humidified 5% CO_2 atmosphere in RPMI medium supplemented to 2 mM glutamine, 12% FCS, 10 μg/ml gentamicin sulfate, 1% (v/v) dimethyl sulfoxide, and varying drug concentrations. The initial inoculum was 10^5 cells/ml. For determination of IC_{50} values, cells were grown for 72 h and counted in a Coulter counter. The IC_{50} drug concentration inhibited increase in cell number by 50%.

Inhibition of Carcinoma Cell Growth—Cells were continually incubated in RPMI medium containing 10% FCS, 12 μg/ml gentamicin sulfate, and 2 mM glutamine. The medium for the Ptx-resistant ovarian line also contained 5 μg/ml verapamil (to inhibit expression of P-glycoprotein) and 15 ng/ml Ptx. Prior to drug treatment, the Ptx-resistant ovarian cells were removed from Ptx-containing media for 5–7 days. Drug effects were determined in 96-well microtiter plates, with cells fixed and stained for protein (16).

Indirect Immunofluorescence—Confluent monolayers of PtK_2 cells, grown on 25-cm^2 plates, were suspended by incubation with 7 ml/plate of 0.05% trypsin and 0.53 mM EDTA in Hank's balanced salt solution. Suspended cells were diluted 1:40 with MEM containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10% FCS, 2 mM glutamine, and 10 μg/ml gentamicin sulfate and allowed to attach and grow for 24 h at 37°C in a humid 5% CO_2 atmosphere on sterile, eight-well Permanox or glass cell-culture slides (300 μl/well, 10^3 cells/well). Medium was removed and attached cells were incubated in supplemented MEM containing 10 μM drug at 37°C. After various incubation times, medium was removed, and cells were rinsed with PBS at 37°C. Attached cells were fixed in −20°C methanol for 10 min, rinsed three times with PBS, and incubated in PBS containing 0.1% Triton X-100 and 10% FCS for 30 min at room temperature to block nonspecific antibody binding sites. The blocking buffer was removed, and the cells were incubated for 1 h at 37°C with antibody to β-tubulin (200 μl/well, 1:200 dilution in blocking buffer). Cells were washed three times with PBS and incubated for 45 min with Texas Red-conjugated goat anti-mouse IgG (150 μl/well, 1:50 dilution in PBS). Following removal of the secondary antibody by two washes with PBS, diaminopropidium iodide (2 μg/ml) dissolved in PBS was added to stain DNA. Slides were wet-mounted in ProLong according to the manufacturer's procedures. Cells were examined with a Nikon Optiphot-2 epifluorescence microscope and photographs taken with Kodak T-MAX 100 film.

Tubulin Assembly—Polymerization was followed turbidimetrically at 350 nm in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. Base-line levels were established with the reaction mixtures, containing all components except drug, held at 0°C. Drug was added and mixed into the reaction mixture, and the reaction was followed as indicated at 0°C. Temperature settings were changed as indicated, rising in the reaction mixtures at 0.5°C/s and falling at 0.1°C/s.

Inhibition of [^{3}H]Ptx Binding to Polymer—For all binding studies, tubulin polymer was preformed in the absence of drugs for 30 min at 37°C in reaction mixtures containing 2 μM tubulin, 20 μM ddGTP, and 0.75 M monosodium glutamate (2 M stock solution adjusted to pH 6.6 with HCl). This condition induced nearly complete assembly of tubulin into polymer (data not presented; *cf.* Refs. 17–19). Mixtures of EpA or EpB with [^{3}H]Ptx in varying concentrations were added to preformed polymer and incubated for 30 min at 37°C. Bound [^{3}H]Ptx was separated from free [^{3}H]Ptx by centrifugation of the reaction mixtures at 14,000 rpm in an Eppendorf microcentrifuge for 20 min at room temperature. Greater than 90% of added protein sedimented under these conditions even in the absence of drug. Protein and radiolabel in both supernatants and pellets (dissolved in 0.1 M NaOH overnight and neutralized with 0.1 M HCl) were quantitated by the Lowry procedure and liquid scintillation counting. Data from at least three independent experiments were combined for data analysis.

Electron Microscopy—During polymerization reactions 10 μl aliquots were removed at various times and placed on 200-mesh carbon-coated, Formvar-treated, copper grids. Samples were stained with several drops of 0.5% uranyl acetate and examined in a Zeiss model 10CA electron microscope.

RESULTS

Effects of EpA and EpB on Cultured Cells—Table I summarizes inhibitory effects of EpA, EpB, and Ptx on the growth of five human tumor cell lines. In all cases EpB was more potent than Ptx or EpA. Burkitt cells treated with EpA or EpB accumulated in mitotic arrest (data not presented; *cf.* Ref. 11). We confirmed the observation of Bollag *et al.* (11) that the epothilones were not P-glycoprotein substrates. The MDR colon carcinoma line SW620AD-300, with over a 1000-fold increased resistance to Ptx relative to its parental line, had sensitivity to EpA and EpB similar to that of the parental line.

The ovarian carcinoma cells were evaluated because of the characterization of the biochemical properties of tubulins derived from the parental line and from a Ptx-resistant line[2] that strongly indicate that resistance was caused by an altered β-tubulin. It is therefore notable that the parental and Ptx-

[2] P. Giannakakou, D. L. Sackett, Y.-K. Kang, Z. Zhang, J. Regis, T. Fojo, and M. Poruchynsky, manuscript in preparation.

TABLE I
Inhibitory effects of Ptx, EpA, and EpB on growth of human cancer cells

Drug	IC_{50} (relative resistance)[a]				
	Burkitt lymphoma[b]	Colon carcinoma[c]		Ovarian carcinoma[d]	
		Parental	Ptx-resistant	Parental	Ptx-resistant
			nM		
Ptx	6.4	0.2	250 (1250)	2	43 (22)
EpA	9.6	2	3 (1.5)	2	3 (1.5)
EpB	1.8	0.1	0.3 (3)	0.06	0.1 (1.7)

[a] The drug concentration that inhibits cell growth by 50%. Cell number was measured with the Burkitt cells, cell protein with the carcinoma cells. Relative resistance was obtained by dividing the IC_{50} value of the resistant line by the IC_{50} value of the parental line.
[b] Cell line CA46.
[c] The parental line is SW620, and the Ptx-resistant line is SW620AD-300, which overexpresses P-glycoprotein, resulting in multidrug resistance (15).
[d] The parental line, designated 1A9, was derived as a subclone of line A2780 (14). The resistant line was designated 1A9(PTX22), and it was derived by growing the parental cells in Ptx and verapamil. It expresses a modified β-tubulin and will be described in detail elsewhere.

resistant lines had nearly equal sensitivity to both EpA and EpB while retaining significant resistance to several potent taxoids we have examined, including docetaxel and 2-debenzoyl-2-*meta*-azidobenzoylpaclitaxel.[3]

PtK_2 cells were used to evaluate the effects of EpA, EpB, and Ptx on intracellular microtubules. IC_{50} values, determined by counting both adherent and detached cells, were 40, 44, and 120 nM for EpA, EpB, and Ptx, respectively. We confirmed the findings of Bollag *et al.* (11) that EpA and EpB, like Ptx, enhance intracellular microtubule assembly.

Fig. 2 illustrates the effects of these drugs on cellular microtubules at 2, 6, and 24 h. *Panels A–C* show control cells; *D–F*, cells treated with Ptx; *G–I*, with EpB; and *J–L*, with EpA. All three drugs caused microtubules to become more bundled and to develop multiple regions of nucleation (*arrows* in Fig. 2, *E*, *G*, and *K*) in interphase cells (those with nuclei). Bundling of microtubules at 2 h was more extensive following EpB treatment than treatment with the other drugs. After 24 h (Fig. 2, *F*, *I*, and *L*), microtubules were found almost exclusively in large bundles. Each drug examined produced similar cellular changes overall, but a few morphological differences were noted, especially in mitotic cells.

A variety of mitotic aberrations were observed as early as 2 h and were maximal at 6 h (Table II). With 10 μM drug, normal spindles were never observed, and microtubule organization in mitotic cells (those with condensed chromosomes and no apparent nuclear membrane) was distinct from that in interphase cells. This suggests that, despite the high stability of microtubules formed in biochemical systems with these drugs (see below), substantial microtubule turnover occurs in cells (*cf.* Refs. 5 and 20).

Most mitotic cells could be placed in one of three categories (Table II). Each subtype was observed following treatment with all three drugs, but relative proportions varied. Ptx produced more punctate-stained spindle aberrations (Fig. 2, *D* and *E*, *arrowheads*), probably representing multiple microtubule organizing centers or spindle poles. Short filaments resembling astral microtubules were attached to the multiple spindle poles, and these are listed as "aster spindles" in Table II. Astral microtubules appeared to be longer in epothilone-treated cells than in Ptx-treated cells. With EpB most mitotic cells had complex arrays of relatively long microtubules that formed a spiral pattern. Such "spiral spindles" had only one apparent or two poorly separated microtubule organizing centers (Fig. 2*H*, *arrowhead*). The third distinct type of mitotic cell was most abundant following treatment with EpA. These "spike spindles" (not shown) had short clusters of thick microtubules originating from one or two microtubule organizing centers, and they may represent a variant of the aster spindles resulting when the microtubule organizing center remained intact. We do not know if these aberrant spindles represent any sort of progression, although the spikes were observed only at the later time points, and with both EpA and Ptx asters were observed before either spirals or spikes. Finally, additional mitotic cells had patterns of microtubule organization that did not fit any of these categories (Fig. 2*J*, *arrowhead*).

Effects of EpA and EpB on the Polymerization of Purified Tubulin with and without MAPs and/or GTP—Ptx and other taxoids induce tubulin assembly under conditions where the reaction usually does not occur, and taxoid-induced polymers are more stable than normal microtubules. Thus, with these drugs assembly can occur at low temperatures and in the absence of MAPs and/or exogenous GTP, and the polymers formed are resistant to disassembly by calcium, dilution, or low temperatures (7, 8, 21–24).

In their studies Bollag *et al.* (11) showed that EpA and EpB induced the assembly of microtubule protein in the absence of GTP at 37°C, with substoichiometric (relative to tubulin) concentrations of the scarcer EpB inducing more extensive assembly than an equivalent concentration of EpA or Ptx, which had nearly identical activities. They also showed that microtubules induced by the epothilones were resistant to cold- or calcium-induced disassembly. However, no studies were performed with purified tubulin and MAPs to define their relative importance in the interactions of polymer with the epothilones, nor was assembly at low temperatures evaluated.

To address these issues we performed the experiments presented in Fig. 3, examining assembly reactions induced by the epothilones and Ptx. Assembly was followed at a series of progressively higher temperatures, and polymer stability was evaluated by a final incubation at 0°C. *Panel A* presents reactions with both MAPs and GTP, *panel B* with MAPs only, and *panel C* with GTP only. Equimolar concentrations (10 μM) of tubulin and drug were used. EpB always caused the most vigorous polymerization of tubulin, while the relative activities observed with EpA and Ptx depended on the reaction condition.

In the presence of GTP and MAPs (Fig. 3*A*), the only condition that supported microtubule assembly without drug, EpB and to a lesser extent EpA, induced tubulin polymerization at 0°C, whereas assembly with Ptx required a higher temperature. Assembly was near its maximum with all compounds after 10 min at 25°C, with the highest turbidity reading obtained with EpB. When the reaction temperature was reduced to 0°C, there was a slight decrease in turbidity with EpA and Ptx but not with EpB. Tubulin polymer formed without drug disassembled to near base-line level at 0°C, and, without drug, assembly required a 25°C incubation. Electron micrographs of aliquots taken from reaction mixtures after 1 h at 0°C with all three drugs showed that microtubules remained intact (data

[3] P. Giannakakou, P. G. I. Kingston, and E. Hamel, unpublished observations.

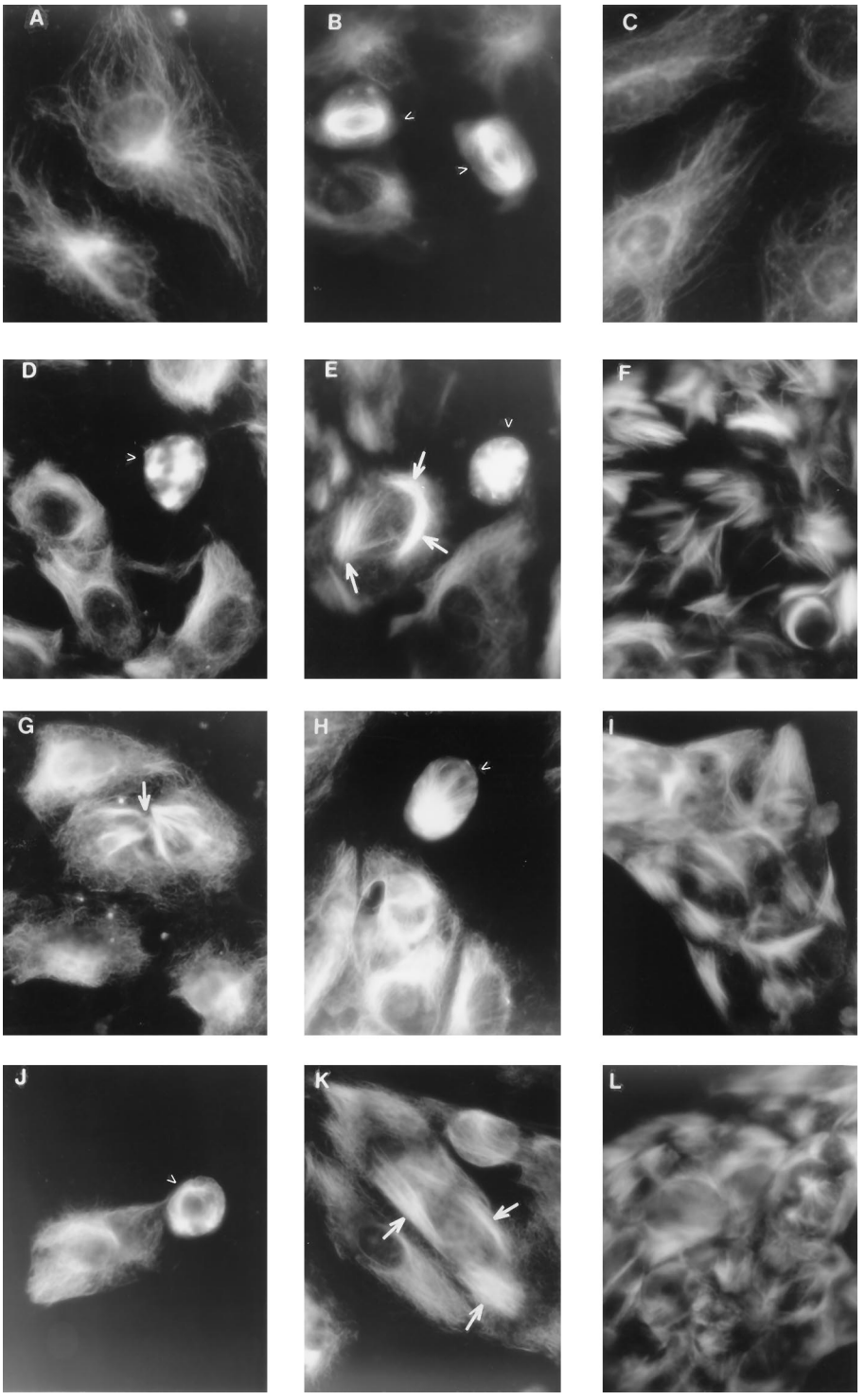

FIG. 2. **Effects of Ptx, EpB, and EpA on the microtubules of PtK$_2$ cells.** *A–C*, no drug; *D–F*, Ptx; *G–I*, EpB; *J–L*, EpA. Cells were grown for 2 h (*A*, *D*, *G*, *J*), 6 h (*B*, *E*, *H*, *K*), or 24 h (*C*, *F*, *I*, *L*) at 37°C prior to fixation and staining. Magnification, ×346. In *panels E*, *G*, and *K*, *arrows* indicate nonmitotic cells with multiple nucleation centers. In *panels B*, *D*, *E*, *H*, and *J, arrowheads* indicate mitotic cells.

not shown). Addition of 5 mM $CaCl_2$ at 0°C resulted in nearly complete disassembly of Ptx and EpA polymers, while the EpB polymer remained intact (data not shown). This total stability of the EpB-induced polymer is thus identical to that observed with Dcd (12).

When reaction mixtures contained tubulin and MAPs but no GTP (Fig. 3*B*), more vigorous assembly occurred with EpA or EpB than with Ptx. The epothilones induced assembly at 10°C, and maximum turbidity readings occurred at 25°C. With Ptx, no assembly occurred until the temperature reached 25°C, and a 37°C incubation was required for maximum assembly. When the temperature was returned to 0°C, little disassembly occurred with any of the drug-induced polymers.

A different pattern was obtained when GTP, but not MAPs, was added (Fig. 3*C*). While each drug nucleated polymer growth at 25°C, under this reaction condition greater apparent activity (see below) was observed with Ptx than with EpA. EpB induced near maximum turbidity at 25°C, whereas Ptx and EpA caused modest reactions. At 37°C, little additional assembly occurred with EpB, but further polymerization was observed with Ptx and EpA. The turbidity reading with Ptx was nearly twice that which occurred with EpA. When the reaction temperature was returned to 0°C, the epothilone-induced polymer was somewhat more resistant to disassembly than the Ptx-induced polymer.

In the absence of both GTP and MAPs, none of the drugs

TABLE II
Mitotic index and aberrations induced by Ptx, EpA, and EpB in PtK_2 cells
Cells were cultured, stained, and examined as described in detail in the text.

Drug	Incubation	Mitotic index[a]	Aster spindles[b]	Spiral spindles[b]	Spike spindles[b]
	h		%	%	%
Ptx	2	11	47	0	0
	6	23	67	2	0
	12	17	31	41	21
EpA	2	12	63	0	0
	6	18	25	13	28
	12	17	35	9	47
EpB	2	10	5	45	0
	6	15	14	67	11
	12	13	13	55	28

[a] The percentage of examined cells judged to be mitotic by the presence of condensed chromosomes and no apparent nuclear membrane. An average of 219 cells was counted from each specimen (range, 153–308). The mitotic index of untreated cells was 1.5.
[b] These percentages refer to the mitotic population only.

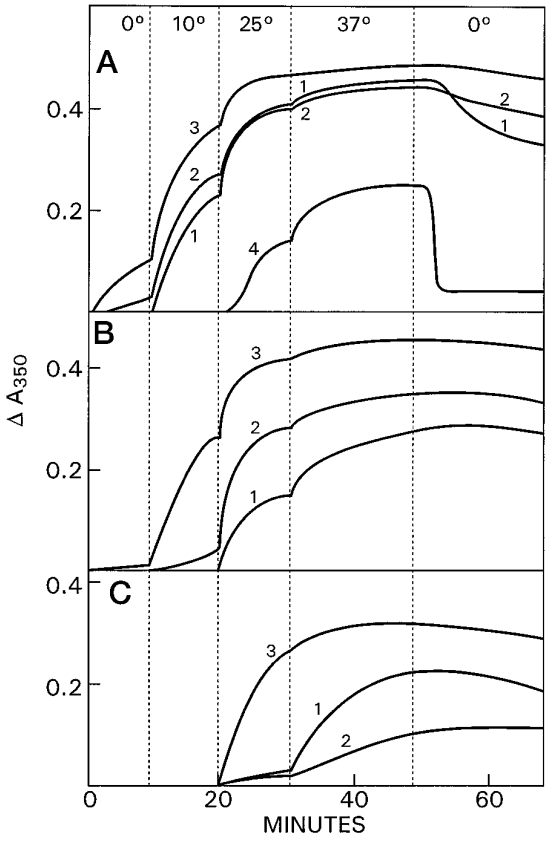

FIG. 3. **Tubulin polymerization reactions induced by Ptx (*curves 1*), EpA (*curves 2*), and EpB (*curves 3*) in the presence of both MAPs and GTP (*panel A*), MAPs only (*panel B*), and GTP only (*panel C*).** Reaction mixtures (0.25 ml) contained 1.0 mg/ml (10 μM) tubulin, 10 μM drug, 4% dimethyl sulfoxide, 0.1 M Mes (pH 6.9), and, if present, 100 μM GTP and heat-treated MAPs at 0.5 mg/ml. Temperature settings were changed at the times indicated by the *vertical dashed lines* to the left of each temperature. *Curve 4* in *panel A* represents a reaction mixture containing 4% dimethyl sulfoxide but no drug. No reaction occurred without drug under the conditions shown in *panels B* and *C*.

stimulated significant tubulin polymerization at 10 μM drug + 10 μM tubulin. Reactions did occur at higher concentrations of tubulin or drug.

The ability of the taxoids, Dcd, and the epothilones to induce assembly at low temperatures and without MAPs and/or GTP are probably manifestations of the ability of these agents to cause hypernucleation of tubulin polymerization. Perhaps the best quantitative measure of this property is the $[T]_{cr}$. We recently reported on $[T]_{cr}$ values obtained with a series of taxoids that included compounds both more and less active than Ptx (8). The quantitative results were in excellent agreement with the qualitative effects of these agents on assembly at low temperatures and in the absence of MAPs or GTP. We therefore compared $[T]_{cr}$ values of the 37°C reactions induced by the epothilones with those induced by Ptx at 10 μM drug (Table III).

With MAPs and GTP, the $[T]_{cr}$ with all three drugs was less than 1 μM, as compared with 3 μM without drug. Differences between drugs were readily detected under the other reaction conditions. Generally the values obtained were in accord with the qualitative results shown in Fig. 3. With MAPs only, Ptx and EpA yielded $[T]_{cr}$ values of 1.4–1.7 μM and EpB, 0.8 μM. With GTP only, the $[T]_{cr}$ with EpB was 2 μM, less than half those obtained with EpA and Ptx (4.3–4.5 μM). With neither MAPs nor GTP, the $[T]_{cr}$ values were still higher, 7.4 μM with EpB, 22 μM with Ptx, and 19 μM with EpA. In summary, EpA and Ptx have similar $[T]_{cr}$ values under all reaction conditions, while the $[T]_{cr}$ values with EpB are about 50% lower.

Another parameter of nucleation is microtubule lengths. These were quantitated on samples taken from reaction mixtures containing equimolar (10 μM) tubulin and drug (Table IV) under conditions where polymerization occurred. With MAPs and GTP, all three drugs yielded polymer with a shorter average length than that formed without drug, consistent with enhanced nucleation. The EpB polymers were shorter and the EpA polymers longer than the Ptx polymers. With MAPs but not GTP, microtubules were longer than those formed in the complete system with all three drugs. The EpA polymers were slightly longer than the Ptx polymers, and the EpB microtubules were about half as long as those formed with the other drugs. With GTP but not MAPs, average microtubule length further increased, but the relative lengths induced with the three drugs remained about the same.

Polymer Morphology—In the current studies, all samples taken for electron microscopic evaluation displayed primarily the morphology of microtubules, with occasional, short areas of open microtubules (ribbons). There were no obvious differences between the Ptx-induced polymers and those formed with the epothilones, other than the length differences summarized above. Predominant microtubule morphology was observed under all reaction conditions (MAPs + GTP, MAPs only, GTP only, no MAPs/no GTP) and whether or not calcium had been added to the reaction. Moreover, microtubules persisted for a least 1 h at 0°C following incubation at higher temperatures.

Competitive Binding of Epothilones and Ptx to Microtubules—Despite the similarity of the effects of the epothilones and Ptx on assembly reactions, the disparate structures of these compounds caused us to undertake studies to gain infor-

TABLE III
$[T]_{cr}$ values for assembly with Ptx, EpA, and EpB

Reaction mixtures contained 10 μM drug, 4% dimethyl sulfoxide, 0.1 M Mes (pH 6.9), and, if present, 100 μM GTP and MAPs at half the concentration (in mg/ml) of tubulin. Drug was the final addition to the reaction mixture. Reaction mixtures were incubated as shown in Fig. 3. $[T]_{cr}$ values were determined from final turbidity readings at 350 nm, with turbidity plotted against the tubulin concentration. The $[T]_{cr}$ was taken as the intercept on the concentration axis. Data from at least two independent experiments were pooled to derive the values shown in the table, with analysis by linear regression using the software program "Origin" from Microcal Software Inc., Northampton, MA.

Drug	$[T]_{cr}$ ± S.E.			
	MAPs and GTP[a]	MAPs only	GTP only	No MAPs or GTP
		μM		
Ptx	0.24 ± 0.3	1.7 ± 0.4	4.3 ± 0.4	22 ± 1
EpA	0.28 ± 0.4	1.4 ± 0.4	4.5 ± 0.8	19 ± 2
EpB	0.19 ± 0.3	0.80 ± 0.3	2.0 ± 0.3	7.4 ± 1

[a] Without drug, the $[T]_{cr}$ under this reaction condition was 3.0 ± 0.05 μM.

TABLE IV
Microtubule lengths after assembly with Ptx, EpA, and EpB

Electron micrographs were taken on samples from reaction mixtures containing 1.0 mg/ml (10 μM) tubulin, 10 μM drug, 4% dimethyl sulfoxide, 0.1 M Mes (pH 6.9), and, if present, 100 μM GTP and heat-treated MAPs at 0.5 mg/ml. Drug was the final addition to the reaction mixture. Reaction mixtures were incubated as shown in Fig. 3. After 20 min at 37 °C, samples were applied to grids, which were used to prepare the micrographs used in the analyses presented in the table. Measurements were made on photographs at magnification ×4800. A minimum of 100 microtubules was measured for each reaction condition.

Drug	Average polymer length (μm) ± S.D. (relative to lengths with Ptx)		
	MAPs and GTP	MAPs only	GTP only
None	4.3 ± 2 (2.3)		
Ptx	1.9 ± 1 (1.0)	2.6 ± 1 (1.0)	6.0 ± 3 (1.0)
EpA	2.8 ± 2 (1.5)	3.0 ± 2 (1.2)	6.6 ± 5 (1.1)
EpB	1.1 ± 0.6 (0.58)	1.4 ± 0.7 (0.54)	3.5 ± 2 (0.58)

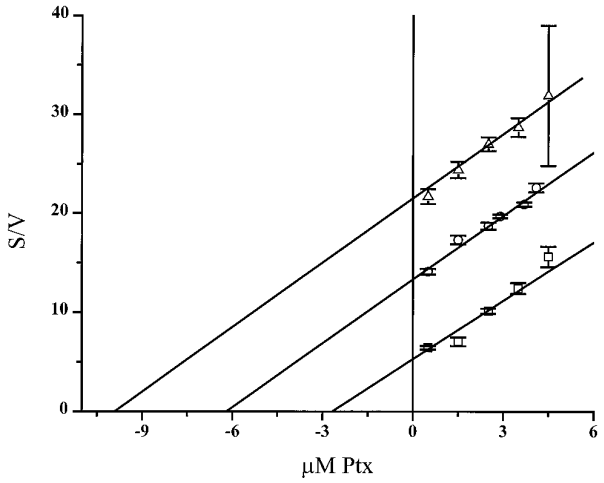

FIG. 4. **Competitive inhibition of [^{3}H]Ptx binding to tubulin polymer by EpB, as demonstrated by Hanes analysis.** The reaction mixtures contained the components described in the text, together with the indicated concentrations of [^{3}H]Ptx and EpB at 0 (□), 1.0 (○), or 2.0 (△) μM. See "Experimental Procedures" for further details. The ordinate units are μM Ptx × pmol of Ptx bound/μg of tubulin.

mation about their binding site(s). With purified tubulin, we confirmed the findings of Bollag *et al.* (11) with microtubule protein that both EpA and EpB displaced [^{3}H]Ptx from preformed polymers, but we found EpB distinctly more potent than EpA. In detailed kinetic experiments we demonstrated that both EpA and EpB are competitive inhibitors of the binding of [^{3}H]Ptx to polymer. (A similar finding, as a control experiment, was made with docetaxel, generously provided by Dr. D. G. I. Kingston.)

Fig. 4 presents our binding data with EpB in the Hanes format, in which a competitive inhibitor generates a family of parallel curves at different inhibitor concentrations. Analysis of these data and similar, but less extensive, data with EpA yielded apparent K_i values of 1.4 μM for EpA and 0.71 μM for EpB from the abscissa intercepts (25). (The value obtained for docetaxel was 1.2 μM.) Dixon analysis of the same data yielded slightly different apparent K_i values, 0.6 and 0.4 μM for EpA and EpB, respectively.

DISCUSSION

EpA and EpB are non-taxoid natural products of bacterial origin that Bollag *et al.* (11) found paralleled Ptx in their effects on cultured cells and in promoting the assembly of microtubule protein. They further found that EpA and EpB displaced [^{3}H]Ptx from microtubules, implying that the target for these agents is tubulin itself. In the cellular studies, Ptx was more effective than the epothilones in inhibiting the growth of two cell lines, and EpB was 2.5 times more effective than Ptx in one line. Most importantly, both EpA and EpB remained cytotoxic for the MDR line KBV-1, which was highly resistant to Ptx. In the biochemical studies, EpB was more active than EpA in inducing microtubule assembly but less active than EpA in displacing [^{3}H]Ptx from polymer.

A reisolation of the epothilones allowed us to further evaluate these compounds. First, we wanted to confirm that the target of the epothilones was in fact tubulin by studying interactions of EpA and EpB with purified tubulin and to evaluate specific effects of MAPs, GTP, and reduced temperature in assembly reactions induced by these agents. Second, we wanted to determine whether or not the epothilones bound to tubulin in polymer in the same or a different site than Ptx by kinetic criteria. Third, we wanted to determine the relative effects of the epothilones and Ptx on the growth of additional cell lines, in particular lines resistant to Ptx. Fourth, we wanted to determine whether subtle differences in microtubule rearrangement could be detected in cells treated with EpA and/or EpB in comparison with those treated with Ptx.

We unequivocally showed that tubulin is the target of the epothilones. These agents, like Ptx, other taxoids, and Dcd will induce purified tubulin to form microtubules both with and without GTP in the reaction mixture. MAPs do not appear to form part of the binding site for EpA and EpB, and the quantitative effects of MAPs (Tables III and IV) are equivalent for Ptx and the epothilones.

Under all conditions studied, EpB was more active than both EpA and Ptx, suggesting that the methyl group at C-12 enhances a hydrophobic interaction between drug and tubulin. More extensive assembly occurred at lower temperatures with EpB, the $[T]_{cr}$ was half that observed with the other drugs, and shorter microtubules were formed with EpB. EpB is thus a more potent inducer of microtubule nucleation reactions than either Ptx or EpA.

The comparison of effects of EpA and Ptx is more complex. With MAPs as a component of the reaction mixture, assembly was slightly more extensive with EpA than with Ptx, with

assembly occurring somewhat more readily at lower temperatures. Without MAPs but with GTP, the Ptx reaction was more extensive than the EpA reaction. Although we could detect no significant difference in the $[T]_{cr}$ between EpA and Ptx, EpA microtubules were longer than Ptx microtubules. This may indicate that there are subtle differences in the relative effects of Ptx and EpA on nucleation and elongation reactions.

For our second goal, to obtain kinetic evidence for the binding of Ptx and the epothilones at the same or different sites on tubulin polymers, we devised a reaction condition with tubulin as the only protein component in which virtually all of the tubulin was first driven into microtubules. We exploited the finding that ddGTP is more potent than virtually any other nucleotide in inducing tubulin polymerization (17–19). Initial displacement studies (constant [^{3}H]Ptx, varying epothilone concentrations) demonstrated that EpB was more potent than EpA, in contrast to the results of Bollag *et al.* (11). When both substrate ([^{3}H]Ptx) and inhibitor (epothilone) concentrations were varied and analyzed, we obtained classic competitive inhibition patterns with EpA and EpB, as well as with the docetaxel control. Generally, this is interpreted as indicating binding at the same site, although binding in overlapping sites or allosteric phenomena are probably not excluded. The apparent K_i values derived from these experiments indicated that EpB bound 1.5–2 times as tightly as EpA, relative values comparable to those obtained for the $[T]_{cr}$ value and microtubule lengths (the EpB values about half the EpA values). It is possible that the difference between the relative activities of EpA and EpB in inhibiting [^{3}H]Ptx binding that we found *versus* those observed by Bollag *et al.* (11) with microtubule protein may indicate a modulating effect of MAPs in the latter system.

The relationship of the Ptx site on polymer to other drug binding sites on tubulin is uncertain. Binding of radiolabeled taxoids has only been observed with polymer, and many inhibitors of assembly inhibit both taxoid-driven assembly and binding of taxoid to tubulin (23, 26–28). Recent observations suggest that Ptx and the colchicine tropolone ring interact with the same region of β-tubulin (29–31) (*cf.* Ref. 32). The Ptx-like activity of Dcd and the epothilones may strengthen this conclusion. A computer analysis of compounds of unknown activity for structural features shared with colchicine site drugs was the first indication that Dcd was a tubulin active agent (33). In the epothilones, a prominent structural feature is the thiazole moiety in the side chain at C-15. This is reminiscent of the thiazoline ring of curacin A, a structurally unique competitive inhibitor of colchicine binding (34). Ongoing structure-activity studies with curacin A analogs have demonstrated that activity is retained when the thiazoline ring is oxidized to a thiazole ring and largely lost when the thiazoline ring is disrupted.[4] These observations may indicate that the thiazoline ring of curacin A interacts at the same site on tubulin as the tropolone ring of colchicine and that the thiazole ring of the epothilones is essential for their activity as inducers of tubulin assembly.

For our third goal, we studied several human cancer cell lines: 1) the Burkitt lymphoma CA46 line because of the high mitotic index it yields with anti-tubulin agents; 2) the colon carcinoma line SW620, for comparison with a line derived from it, SW620AD-300, that is highly resistant to Ptx on the basis of overexpression of P-glycoprotein; 3) the ovarian carcinoma line 1A9, for comparison with a line derived from it, 1A9(PTX22), that has moderate resistance to Ptx based on expression of a modified β-tubulin. The Burkitt cells were 3-fold more sensitive to EpB than to Ptx, while EpA was about 50% less active than Ptx. The parental colon cells were 2-fold more sensitive to EpB than to Ptx, but EpA was 10-fold less active. Importantly, however, we found that the MDR colon line, like the MDR line studied by Bollag *et al.* (11), remained sensitive to both epothilones. Thus, the Ptx-resistant cells were over 80-fold more sensitive to EpA and over 800-fold more sensitive to EpB than to Ptx. The parental ovarian line was equally sensitive to Ptx and EpA, but over 30-fold more sensitive to EpB. Again the derived Ptx-resistant line retained nearly complete sensitivity to both epothilones, so that it was 14-fold more sensitive to EpA than to Ptx, and over 400-fold more sensitive to EpB. In conjunction with the immunofluorescence studies, we found that PtK_2 cells were about 3-fold more sensitive to EpA and EpB than to Ptx. Höfle *et al.* (35) reported that among the cell lines they had examined, EpB was usually twice as active as EpA.

The epothilones deserve a thorough evaluation as potential anticancer drugs. The scarcer EpB is usually more active against cells in culture relative to Ptx and EpA, concordant with its greater activity in promoting tubulin assembly and in inhibiting the binding of [^{3}H]Ptx to polymers prepared from purified tubulin. The epothilones can be obtained in bulk from fermentation cultures (11, 36), are active in MDR cell lines resistant to Ptx (Ref. 11; Table I), are active in a cell line with Ptx resistance based on an alteration in β-tubulin (Table I), and are 30-fold more soluble than Ptx in aqueous solution (35), thus permitting easier administration to patients and avoiding side effects of Ptx administration attributed to the vehicle (9).

Finally, we evaluated over time the effects of Ptx, EpA, and EpB on the microtubule network of PtK_2 cells treated with 10 μM drug. At this high concentration, used to minimize effects of differential cytotoxicity, there were no clear-cut differences between the compounds. Massive microtubule bundles were apparent at 2 h only with EpB, probably because of its tighter binding to tubulin polymers. In mitotic cells (those with condensed chromosomes), three types of microtubule arrays were observed with all drugs. The first were multiple nucleation centers that had short microtubules attached that resembled short astral fibers. Cells with these structures were more abundant following Ptx or EpA treatment, but the attached microtubules were longer in epothilone-treated cells. The second tended to have a single nucleation center, perhaps derived from an undivided centrosome, and the microtubules were long and arranged in a spiral pattern. Cells with these structures were most abundant following treatment with EpB. The third tended to have one or two nucleation centers with multiple short microtubules originating at these centers. Cells with these spike-like spindle aberrations were most abundant in EpA-treated cells. There is no ready explanation for these mitotic differences from our biochemical studies. They may derive from different effects of the epothilones *versus* Ptx on microtubule dynamics (8, 20). Such effects may be more critical in mitotic as opposed to interphase cells.

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