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Epothilone and Paclitaxel: Unexpected Differences in Promoting the Assembly and Stabilization of Yeast Microtubules[†]

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ABSTRACT: Paclitaxel (Taxol) and the epothilones are antimitotic agents that promote the assembly of mammalian tubulin and stabilization of microtubules. The epothilones competitively inhibit the binding of paclitaxel to mammalian brain tubulin, suggesting that the two types of compounds share a common binding site in tubulin, despite the lack of structural similarities. It is known that paclitaxel does not stabilize microtubules formed in vitro from *Saccharomyces cerevisiae* tubulin; thus, it would be expected that the epothilones would not affect yeast microtubules. However, we found that epothilone A and B do stimulate the formation of microtubules from purified yeast tubulin. In addition, epothilone B severely dampens the dynamics of yeast microtubules in vitro in a manner similar to the effect of paclitaxel on mammalian microtubules. We used current models describing paclitaxel and epothilone binding to mammalian β-tubulin to explain why paclitaxel apparently fails to bind to yeast tubulin. We propose that three amino acid substitutions in the N-terminal region and at position 227 in yeast β-tubulin weaken the interaction of the 3′-benzamido group of paclitaxel with the protein. These results also indicate that mutagenesis of yeast tubulin could help define the sites of interaction with paclitaxel and the epothilones.

Antimitotic agents that are directed toward mammalian tubulin and microtubules can be divided into two classes on the basis of their effects on the assembly of tubulin into microtubules. Compounds such as colchicine and the Vinca alkaloids bind to tubulin, inhibit the assembly reaction, and at high concentrations, depolymerize microtubules. Paclitaxel, the epothilones, and several others belong to a second class of compounds that promotes the assembly reaction by binding to polymerized tubulin and stabilizes the microtubule polymer. Compounds from both classes of antimitotic agents have been found to dampen the dynamic nature of microtubules (*1*). In fact, inhibition of microtubule dynamics appears to be the mechanism by which antimitotic agents inhibit the proliferation of tumor cells (*1*).

Paclitaxel was the first natural product shown to promote tubulin assembly and stabilize microtubules (*2*) and has been successfully employed in the clinical treatment of various forms of cancer. Subsequently, the epothilones were isolated from the myxobacterium *Sorangium cellulosum* (*3*, *4*) and were shown to have a similar mechanism of action (*4*, *5*). The epothilones are of particular interest as potential antitumor agents because they are effective against some multidrug resistant cell lines that are resistant to paclitaxel

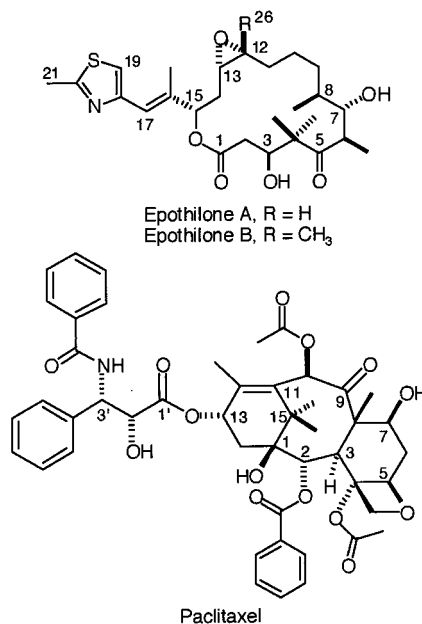Epothilone A, R = H
Epothilone B, R = CH₃

Paclitaxel

FIGURE 1: Structures of paclitaxel and epothilones A and B.

and they are more water-soluble than paclitaxel. In studies with mammalian brain tubulin, it was found that the epothilones competitively inhibit the binding of paclitaxel to polymerized tubulin (*4*, *5*). This finding suggests that the two compounds share a common binding site or overlapping sites, despite the lack of structural similarities (Figure 1). However, as we show in this paper, the epothilones, but not paclitaxel, promote the assembly yeast microtubules in vitro.

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EXPERIMENTAL PROCEDURES

Materials. Cryptophycin A was provided as a gift from Merck Research Laboratories, West Point, PA. Colchicine, podophyllotoxin, and vinblastine were purchased from Sigma Chemical Co., St. Louis, MO. Paclitaxel was purchased from Hauser Chemical Research Inc., Boulder, CO. Epothilone A and B were isolated from *S. cellulosum* using a modification of published procedures (*3, 4*). Stock solutions of the antimitotic compounds were prepared in DMSO.[1]

Purification of Bovine and Yeast Tubulin. Yeast tubulin was purified from the haploid strains MGY1 (*6*) and BGY1. Both strains have a His$_6$ tag just before the stop codon at the carboxy terminus of β-tubulin. BGY1 lacks the minor α-tubulin gene *TUB3* and was constructed from CDY1 (a gift from C. A. Dougherty, Johns Hopkins University, Baltimore, MD). Yeast tubulin was purified to apparent homogeneity, as judged by a single band on SDS−PAGE gels, using a His$_6$-based affinity purification procedure.[2] Microtubule protein was prepared from bovine brain by the method of Tiwari and Suprenant (*7*), and MAP-free brain tubulin was purified by the method of Algaier and Himes (*8*).

Tubulin Assembly In Vitro. To test the effects of assembly inhibitors on yeast tubulin assembly, reactions were performed at a tubulin concentration of 5 μM in PEM (100 mM Pipes, 1 mM EGTA, and 1 mM MgSO$_4$ (pH 6.9)) and 1 mM GTP at 30 °C. The inhibitors (colchicine, vinblastine, podophyllotoxin, or cryptophycin A) were present at a concentration of 10 mM. Both the control and experimental samples also contained 4% DMSO. The final reaction volume was 40−45 μL. Polymerized tubulin was pelleted for 10 min at 100 000g, and the pellets were suspended in 0.1 M NaOH. The protein concentration of the pellets and supernatants were measured using the Bradford method (*9*).

The effects of the assembly promoters epothilone A and B and paclitaxel on the assembly of bovine and yeast tubulin were examined in 50-PEM (50 mM Pipes, 1 mM EGTA, and 1 mM MgSO$_4$ (pH 6.9)), supplemented with 50 μM GTP and at a tubulin concentration of 5 μM. Control and experimental samples also contained 4% DMSO. After reaching steady-state, samples were fixed in 0.25% glutaraldehyde and negatively stained for electron microscopy.

Microtubule Dynamics. A nucleated assembly assay with *Chlamydomonas* axonemes (*10*) was used to monitor microtubule dynamics by video-enhanced differential interference contrast light microscopy using a modified protocol of Walker et al. (*11*). Yeast tubulin (1.8 μM) in filter-sterilized PEM and 50 μM GTP, containing either 4% DMSO or 10 μM epothilone B and 4% DMSO, were incubated in the perfusion chamber on the microscope slide for 30 min at 30 °C to achieve steady-state. The microtubules were recorded for no longer than 90 min after steady-state was reached. For each experimental condition, 12−14 microtubules were analyzed using the RTM software kindly provided by E. D. Salmon, University of North Carolina, Chapel Hill, NC.

Length measurements were taken every 30−45 s over the lifetime of the microtubule or, in the case of shortening events, as often as possible (2−3 s^{-1}). A growth phase was defined as lasting >5.5 min with a time averaged growth rate of >4.8 mm/h, and shortening rates were >2.9 mm/h. Catastrophe events are defined as the transition from growth or attenuation to shortening. Rescue events are defined as the transition from shortening to growth or attenuation. Catastrophe frequency is defined as the total number of shortening events divided by the total time. Standard deviations were calculated as the frequency of catastrophe divided by the square root of the number of events observed. The parameter dynamicity refers to the rate of tubulin exchange per microtubule end and was calculated according to Toso et al. (*12*).

RESULTS

Effects of Antimitotic Agents on Yeast Tubulin Assembly. We examined the effects of several antimitotic agents on the assembly reaction of purified *S. cerevisiae* tubulin. Confirming a previous report (*13*), we found that colchicine present at a 2-fold molar excess over tubulin was ineffective as an inhibitor of yeast tubulin assembly. We also found that vinblastine, podophyllotoxin, and cryptophycin A, also tested at 2-fold molar excesses over tubulin, had no effect on yeast tubulin assembly. At this molar ratio, the antimitotic compounds would have inhibited bovine brain tubulin assembly.

To test the possible stimulatory effects of paclitaxel and the epothilones, conditions were sought that did not support assembly in the absence of these compounds. This was particularly important for yeast tubulin because of its low critical protein concentration for microtubule assembly as compared to brain tubulin (*14*). For example, common tubulin assembly conditions (100 mM Pipes buffer and 1 mM GTP) do not support the assembly of 10 μM bovine brain tubulin in the absence of stimulatory agents, such as DMSO, paclitaxel, or the epothilones. In contrast, yeast tubulin assembles robustly under these conditions. Thus, assembly conditions were modified to prevent the self-assembly of yeast tubulin in the absence of the epothilones. The conditions that were eventually used include 5 μM tubulin, 50 mM Pipes, and 50 μM GTP (although we found that the epothilones stimulate bovine and yeast tubulin assembly in the absence of GTP).

Paclitaxel, present at a 5-fold molar excess over yeast tubulin, did not stimulate the formation of microtubules. The absence of activity by paclitaxel is consistent with an earlier report that this compound does not stabilize yeast microtubules in vitro (*15*). We were surprised then when we found that epothilone A and B stimulated the assembly of yeast tubulin. Under the conditions used, the formation of yeast microtubules was dependent on the presence of epothilone A or B and was complete within 15 min. Data comparing the epothilone B concentration dependence of the assembly of brain and yeast tubulins are presented in Figure 2. A small degree of aggregation of yeast tubulin occurred in the absence of epothilone B; however, microtubules were not observed using electron microscopy. On the other hand, bona fide microtubules were observed in the presence of epothilone B (Figure 3). The EC$_{50}$ values were 1.2 ± 0.2 μM and 1.3 ± 0.4 μM for brain and yeast tubulin, respectively. Paclitaxel

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[1] Abbreviations: Pipes, 1,4-piperazinediethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.
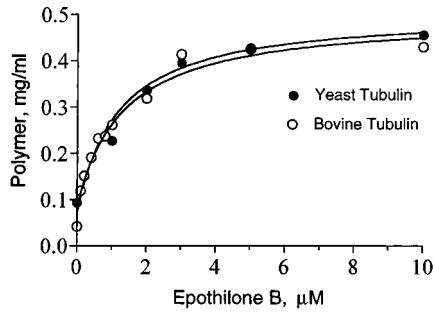
[2] Manuscript in preparation.

FIGURE 2: Assembly of yeast and bovine brain tubulin in the presence of epothilone B. The reactions were performed using yeast or bovine brain tubulin at a concentration of 5 μM in a buffer containing 50 mM Pipes, 1 mM $MgSO_4$, 1 mM EGTA (pH 6.9), and 50 μM GTP. Incubation was for 30 min at 30 °C.
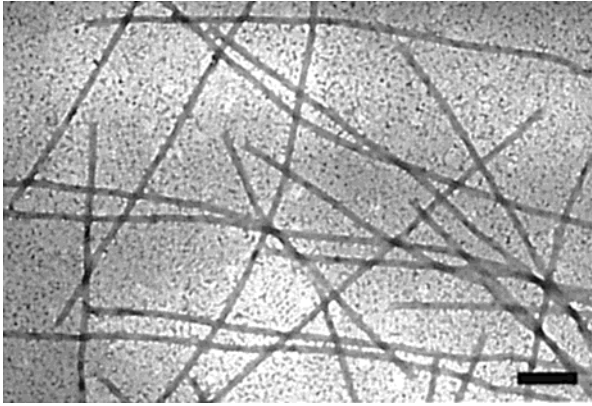

FIGURE 3: Electron micrograph of negatively stained yeast microtubules formed in the presence of 10 μM epothilone B. The experimental conditions were as described in Figure 2 (bar = 0.2 μm).

or baccatin III (paclitaxel lacking the C-13 side chain) at 25 μM did not inhibit the stimulatory effect of 5 μM epothilone B. This result eliminates the possibility that paclitaxel or baccatin III binds to a site in yeast tubulin that is shared with the epothilones but without promoting microtubule formation.

Effect of Epothilone B on Microtubule Dynamics In Vitro. In addition to stimulating the assembly of mammalian tubulin, paclitaxel also dampens microtubule dynamics (*16*). The term microtubule dynamics describes the characteristic behavior of microtubules at polymer mass steady state, in which individual microtubules continue to grow while others remain attenuated or undergo sudden depolymerization (a catastrophe) (*17*). Depolymerization can continue to completion or until a rescue event occurs when the microtubule switches to growth or becomes attenuated. Such dynamics are observed in vitro and in vivo and are essential to microtubule function.

To determine the effects of epothilone B on yeast microtubule dynamics, we measured steady-state dynamics at the plus ends of microtubules grown from *Chlamydomonas* axonemes in vitro using video-enhanced differential interference contrast microscopy. Samples contained 4% DMSO because the epothilone B stock solution was prepared in DMSO. The results are presented in Table 1 and Figure 4. As shown by the representative lifetime history plots in Figure 4B, epothilone B clearly dampened microtubule dynamics. Microtubules in the absence of epothilone B spent

Table 1: Effects of Epothilone B on the Dynamics of Yeast Microtubules In Vitro

	4% DMSO[a]	4% DMSO with epothilone B (10 μM)[b]
time spent in		
growth phase, %	87.0	16.3
shrinkage phase, %	0.2	0.0
attenuated phase, %	12.8	83.7
total time, min	265	317
growth rate, μm/h	10.2 ± 1.8 (12)[c]	6.0 ± 1.2 (5)
shrinkage rate, μm/min	65.3 ± 8.0 (6)[d]	NA[e]
catastrophe frequency, min^{-1}	0.019 ± 0.008 (5)	<0.003[e]
rescue frequency, min^{-1}	<0.004[f]	<0.003[f]
dynamicity, dimers/s	7.9	0.9

[a] Total number of microtubules analyzed was 14. [b] Total number of microtubules analyzed was 12. [c] Results are reported as the mean ± one standard deviation. Number of events in parentheses. [d] Shrinkage events exceed catastrophes because of brief pauses during disassembly. [e] No catastrophe events were observed in 317 min. [f] No rescue events were observed for either of the experimental conditions.
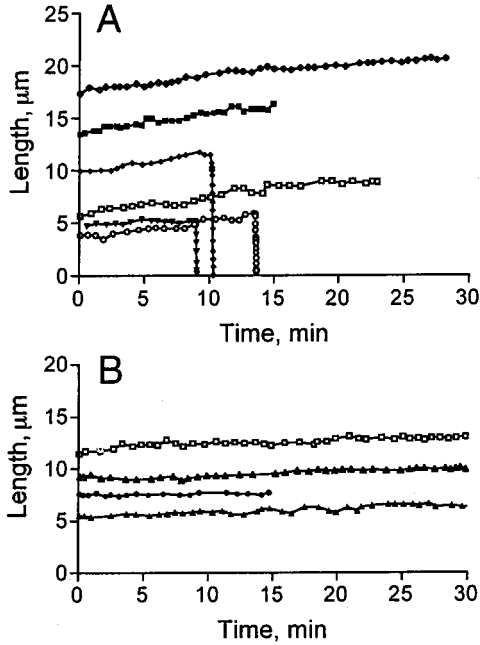

FIGURE 4: Yeast microtubule lifetime history plots in the absence and presence of epothilone B. Microtubules were assembled from yeast tubulin using *Chlamydomonas* axonemes as described in Experimental Procedures in the presence of (A) 4% DMSO or (B) 4% DMSO and 10 μM epothilone B. Individual microtubules are represented by different symbols.

most of their time in a slow growth phase, except for periods of rapid and complete disassembly. This behavior of yeast microtubules in vitro has been observed in previous studies (*14*). When epothilone B was present, the microtubules were much less dynamic (Figure 4B). The most pronounced effects of epothilone B were on the frequency of catastrophes and on the proportion of time microtubules spent in the growth, shrinkage, and attenuated phases (Table 1). The catastrophe frequency of 0.019 min^{-1} in the absence of epothilone B is lower than that obtained in past studies (*14, 18*), undoubtedly because of the presence of DMSO, a known microtubule stabilizer. When epothilone B was present, no catastrophes were observed. No rescue events were observed in the presence or absence of epothilone B. The absence of rescue

events with yeast microtubules in vitro has been observed previously (*14*, *18*). The microtubules in DMSO spent 87% of the time in the growth phase (Table 1). Epothilone B lowered this value dramatically to 16% while the percent time microtubules were attenuated rose from 13% to 84%. Thus, the overall dynamicity, or tubulin dimer turnover, was decreased by epothilone B by a factor of 9.

Although epothilone B binds to yeast microtubules and dampens microtubule dynamics in vitro almost completely, it did not inhibit the proliferation of *S. cerevisiae* at a concentration of 150 μM and it did not change the DNA content distribution of cells as detected by FACS analysis. This finding is consistent with earlier reports, which found that the zygomycete *Mucor heimalis* and some plant fungi were the only organisms whose growth were inhibited when the epothilones were tested against a variety of yeast and fungi (*3*). Obvious explanations for the absence of an effect on yeast proliferation are that the epothilones cannot penetrate the yeast cell wall or that they undergo metabolic transformation.

DISCUSSION

Our findings demonstrate that the epothilones, but not paclitaxel, promote the assembly of yeast tubulin. To explain this apparent disparity in binding affinity, we used current models of paclitaxel and epothilone binding to mammalian microtubules. Several laboratories have used theoretical and molecular modeling approaches to identify a common pharmacophore for paclitaxel and epothilones and to determine the bound conformation of the compounds (*19*−*22*). To predict the binding sites on β-tubulin, the structures were docked onto the 3.7 Å electron crystal structure of the zinc-induced tubulin polymer (*22*) that also shows the electron density of bound paclitaxel (*23*). These modeling studies provide a rationale for why the epothilones, but not paclitaxel, bind to yeast microtubules. In one model, the bound form of paclitaxel assumes a "T" conformation in which the C-2 phenyl ring is nearly equidistant from the 3′-phenyl and 3′-benzamidophenyl rings (*22*). REDOR NMR studies of tubulin polymers formed using an analogue of paclitaxel, containing ^{13}C, ^{15}N, and ^{19}F, are consistent with this structure as well as a "hydrophobically collapsed" structure (*20*). Experimental evidence from photoaffinity labeling (*24*−*26*) and structure/activity relationship studies have been used to support the predictions of sites in tubulin that interact with the drugs. In addition, results from studies of β-tubulin mutant mammalian cell lines that have decreased sensitivity to paclitaxel and epothilones, or to paclitaxel but not to epothilones (*21*, *27*, *28*), add support to some of the predictions. Data from these different experimental approaches indicate that residues in the 1−31, 217−231, 270−289, and 358−372[3] regions of β-tubulin interact with or form hydrophobic sites for paclitaxel. A model for epothilone binding proposes that residues in the 217−231 and 270−282 regions make contact with this antimitotic agent (*21*). Pig brain β-tubulin contains 445 amino acids, and *S. cerevisiae* β-tubulin contains 457. While there is diversity

between these two sequences at the C-terminus, there is 76% identity through residue 441. Most of the residues in the four regions that have been implicated in paclitaxel binding are identical in brain and yeast β-tubulin. However, there are a few key differences, specifically at positions 19, 23, 26, 227, 231, and 270. In the model describing the binding of the T conformer of paclitaxel to brain tubulin, the isopropyl group of V23 juxtaposes the C-3′-benzamidophenyl ring (*22*). In yeast tubulin, a threonine residue is at this position. The increase in polarity caused by this substitution could certainly affect binding of paclitaxel. In addition, the methylene groups of K19, E22, and D26 make short contacts with the C-3′-benzamidophenyl ring (*22*). In yeast tubulin, K19 is replaced by alanine, and D26 is replaced by glycine. The two changes would significantly reduce the number of methylene groups. K19, V23, and D26 are found in the 1−31 peptide, which was identified as a peptide that cross-linked to a photoaffinity analogue of paclitaxel (*24*). These residues have not been implicated in epothilone binding according to proposed models (*21*). Therefore, these changes would not be predicted to disrupt epothilone binding to yeast tubulin.

Position 227 in β-tubulin is another site that may contribute to the difference in paclitaxel binding to yeast and brain tubulin. Histidine occupies this position in brain β-tubulin. In the T binding model described previously, this residue lies between the C-2 phenyl and the C-13 side chain and is stacked between the C-2 and C-3′-benzamidophenyl rings. In yeast tubulin, an asparagine residue occupies the 227 position. This change would eliminate the π electron interactions between the imidazole and the C-2 and benzamidophenyl rings. Two other residues in brain β-tubulin, A231 and F270, are part of a hydrophobic basin that sits below the C-4 acetate (*22*). In yeast β-tubulin, a serine and tyrosine residue occupy positions 231 and 270, respectively. Although these residues are more polar than the corresponding residues in brain tubulin, it is difficult to predict how these changes would affect paclitaxel binding.

We suggest that weak binding of the 3′-benzamido group of paclitaxel is a major contributing factor for the lack of activity of the antimitotic agent in promoting yeast tubulin assembly. Analogues of paclitaxel lacking the benzamido group have been tested for mammalian brain tubulin assembly activity and were found to have about 6% of the activity of paclitaxel (*29*, *30*). This result indicates that the benzamido group significantly contributes to the binding affinity of paclitaxel. A recent study found that the baccatin III ring provides about 75% of the binding energy (*31*); however, we found that baccatin III, which lacks the C-13 side chain, was ineffective in promoting yeast tubulin assembly. This result is similar to what has been reported previously for brain tubulin (*32*).

Our results suggest that yeast tubulin is a valuable tool in the attempt to determine the binding sites of taxol-like compounds in tubulin. Mutagenesis experiments are planned to test our hypotheses.

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[3] Regions 217−231, 270−289, and 358−372 would be 219−233, 272−289, and 360−374, respectively, using the sequence aligned to α-tubulin (*23*).

REFERENCES

1. Wilson, L., and Jordan, M. A. (1995) *Chem. Biol. 2*, 569−573.
2. Schiff, P. B., Fant, J., and Horwitz, S. B. (1979) *Nature 277*, 665−667.
3. Gerth, K., Bedorf, N., Höfle, G., Irschik, H., and Reichenbach, H. (1996) *J. Antibiot. 49*, 560−563.
4. Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E., and Woods, C. M. (1995) *Cancer Res. 55*, 2325−2333.
5. Kowalski, R. J., Giannakakou, P., and Hamel, E. (1997) *J. Biol. Chem. 272*, 2534−2541.
6. Gupta, M. L., Jr., Bode, C. J., Dougherty, C. A., Marquez, R. T., and Himes, R. H. (2001) *Cell Motil. Cytoskeleton 49*, 67−77.
7. Tiwara, S. C., and Suprenant, K. A. (1993) *Anal. Biochem. 215*, 96−103.
8. Algaier, J., and Himes, R. H. (1988) *Biochim. Biophys. Acta 954*, 235−243.
9. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
10. Whitman, G. B. (1986) *Methods Cell Biol. 134*, 280−290.
11. Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. F., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988) *J. Cell Biol. 107*, 1437−1448.
12. Toso, R. J., Jordan, M. A., Farrell, K. W., Matsumoto, B., and Wilson, L. (1993) *Biochemistry 32*, 1285−1293.
13. Kilmartin, J. V. (1981) *Biochemistry 20*, 3629−3633.
14. Davis, A., Sage, C. R., Wilson, L., and Farrell, K. W. (1993) *Biochemistry 32*, 8823−8835.
15. Barnes, G., Louie, K. A., and Botstein, D. (1992) *Mol. Biol. Cell 3*, 29−47.
16. Derry, W. B., Wilson, L., and Jordan, M. A. (1995) *Biochemistry 34*, 2203−2211.
17. Mitchison, T., and Kirschner, M. (1984) *Nature 312*, 237−242.
18. Sage, C. R., Davis, A. S., Dougherty, C. A., Sullivan, K., and Farrell, K. W. (1995) *Cell Motil. Cytoskeleton 30*, 285−300.
19. Ojima, I., Chakravarty, S., Inoue, T., Lin, S., He, L., Horwitz, S. B., Kuduk, S. D., and Danishefsky, S. J. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 4256−4261.
20. Li, Y., Poliks, B., Cegelski, L., Poliks, M., Grycynski, Z., Piszczek, G., Jagtap, P. G., Studelska, D. R., Kingston, D. G. I., Schaefer, J., and Bane, S. (2000) *Biochemistry 39*, 281−291.
21. Giannakakou, P., Gussio, R., Nogales, E., Downing, K. H., Zaharevitz, D., Bollbuck, B., Poy, G., Sackett, D., Nicolaou, K. C., and Fojo, T. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 2904−2909.
22. Snyder, J. P., Nettles, J. H., Cornett, B., Downing, K. H., and Nogales, E. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 5312−5316.
23. Nogales, E., Wolf, S. H., and Downing, K. H. (1998) *Nature 391*, 199−203.
24. Rao, S., Krauss, N. E., Heerding, J. M., Swindell, C. S., Ringel, I., Orr, G. A., and Horwitz, S. B. (1994) *J. Biol. Chem. 269*, 3132−3134.
25. Rao, S., Orr, G. A., Chaudhary, A. G., Kingston, D. G. I., and Horwitz, S. B. (1995) *J. Biol. Chem. 270*, 20235−20238.
26. Rao, S., He, L., Chakravarty, S., Ojima, I., Orr, G. A., and Horwitz, S. B. (1999) *J. Biol. Chem. 274*, 37990−37994.
27. Giannakakou, P., Sackett, D. L., Kang, Y.-K., Zhan, Z., Buters, J. T. M., Fojo, T., and Poruchynsky, M. S. (1997) *J. Biol. Chem. 272*, 17118−17125.
28. Gonzalez-Garay, M. L., Chang, L., Blade, K., Menick, D. R., and Cabral, F. (1999) *J. Biol. Chem. 274*, 23875−23882.
29. Guéritte-Voegelein, F., Guénard, D., Lavelle, F., Le Goff, M.-T., Mangatal, L., and Potier, P. (1991) *J. Med. Chem. 34*, 992−998.
30. Swindell, C. S., Krauss, N. E., Horwitz, S. B., and Ringel, I. (1991) *J. Med. Chem. 34*, 1176−1184.
31. Andreu, J. M., and Barasoain, I. (2001) *Biochemistry 40*, 11975−11984.
32. Lataste, H., Senilh, V., Wright, M., Guénard, D., and Potier, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4090−4094.