Suppression of Microtubule Dynamics by Epothilone B Is Associated with Mitotic Arrest

Kathy Kamath and Mary Ann Jordan

Department of Molecular Cellular and Developmental Biology and the Neuroscience Research Institute, University of California, Santa Barbara, California, 93106

ABSTRACT

The epothilones are a group of novel microtubule-targeted, antimitotic compounds that have a paclitaxel-like, assembly enhancing effect on tubulin in vitro as well as in cultured cells. We hypothesize that epothilones induce mitotic arrest by suppressing microtubule dynamics. To test this hypothesis, we used MCF7 cells stably transfected with GFP-alpha-tubulin to analyze microtubule dynamics at three concentrations of epothilone B, one that induced no mitotic arrest (0.2 nM, 20 h), one that induced one-third maximal mitotic arrest (IC50, 2 nM, 20 h), and one that induced half-maximal mitotic arrest (IC33, 3.5 nM, 20 h). We found that epothilone B suppressed microtubule dynamics in a concentration-dependent manner coincident with mitotic block. At 0.2 nM epothilone B, dynamics were not significantly altered. At 2 nM epothilone B (IC50), the mean growth and shortening rates were decreased by 38 and 27%, respectively. Dynamicity was decreased by 47%. At the IC33, 80% of the cells had nearly complete stabilization of microtubule dynamics, and no anaphase or telophase figures were observed. Comparison of the effects of epothilone B on microtubule dynamics with those of paclitaxel indicated that both drugs alter the same microtubule dynamic parameters to a similar extent. At the IC50 for mitotic arrest, dynamicity was reduced by 54% by paclitaxel compared with 62% for epothilone B. In 65% of the cells treated with paclitaxel, the microtubules were completely stabilized. Thus, the effects of epothilone B on microtubule dynamics are remarkably similar to those of paclitaxel, suggesting that both drugs induce mitotic block by a similar mechanism.

INTRODUCTION

Microtubules are the cellular target for a growing class of naturally derived antitumor compounds. Although several of these compounds, such as paclitaxel, are clinically effective, their success has been tempered by serious side effects, including neurotoxicity and acquired resistance (1, 2). These shortcomings have driven efforts to identify other naturally occurring compounds with similar properties, which might overcome these obstacles.

The epothilones are novel microtubule-targeted compounds produced by the myxobacterium Sorangium cellulosum (3, 4). First identified in a screen for secondary metabolites with antifungal activity (3), epothilones were subsequently shown to have a paclitaxel-like effect on tubulin in vitro (4), as well as in cultured cells (5). Like paclitaxel, epothilones can induce the polymerization of microtubules in the absence of GTP or microtubule-associated proteins and stabilize preformed microtubules against conditions favoring depolymerization, including dilution, cold temperatures or Ca2+ (4, 6). Competition studies with [3H]paclitaxel indicate that epothilones share the same or an overlapping binding site on tubulin (4, 6). In cultured cells, both compounds cause mitotic arrest and aberrant spindle formation at nanomolar concentrations without significant bundling of interphase microtubules (4). Epothilones also suppress growth in human tumor xenografts (5). Several features of epothilones have made them attractive candidates for antitumor drug development. Unlike paclitaxel, they are amenable to total synthesis and chemical manipulation (7). Epothilones are more water soluble than paclitaxel (8), eliminating the need for delivery in Cremaphor, a vehicle responsible for some of the side effects of paclitaxel treatment (1). Finally, epothilones are effective against paclitaxel-resistant cells and tumors that overexpress P-glycoprotein, a membrane efflux pump that greatly decreases intracellular paclitaxel concentrations (4, 5).

Both in vitro and in cells, microtubules are dynamic polymers that grow and shorten by the addition and loss of tubulin subunits at the microtubule ends (9, 10). Although at relatively high concentrations, microtubule-targeted compounds can be subdivided into two classes based on their ability to either induce polymerization of tubulin or induce microtubule disassembly, one of the most potent activities of these compounds appears to be suppression of microtubule dynamics (11). Microtubule dynamics are critical, particularly during mitosis, when microtubules are responsible for the capture and alignment of chromosomes on the metaphase plate and subsequent separation to the two daughter cells at anaphase. Although the mechanism of mitotic arrest by microtubule-targeted antimitotic agents is poorly understood, it is believed to be a result of suppression of microtubule dynamics, which prevents chromosome alignment at the metaphase plate, resulting in a sustained block at or before the metaphase-anaphase transition. Cells ultimately die by apoptosis (12, 13), which can occur directly after mitotic arrest or after an aberrant exit from mitosis into multinucleate interphase (14).

We wanted to determine whether epothilone B affects microtubule dynamics and if so whether it affects dynamics similarly to paclitaxel at concentrations that induce mitotic arrest. Because the dynamics of most spindle microtubules cannot be imaged in cells, we have analyzed microtubule dynamics in living tumor cells during interphase that were incubated with epothilone B over a range of concentrations and compared its effects with those of paclitaxel. We found that epothilone B induced an almost complete stabilization of microtubule dynamics at a concentration that induced one-half of the maximal mitotic arrest at 20 h. In addition, we found that epothilone B suppressed microtubule dynamics qualitatively and quantitatively similarly to paclitaxel.

MATERIALS AND METHODS

Cell Culture. MCF7 breast carcinoma cells (American Type Culture Collection, Manassas, VA) or MCF7 cells stably transfected with GFP-alpha-tubulin (Clontech, Palo Alto, CA) were cultured in DMEM supplemented with 10% fetal bovine serum, nonessential amino acids, and 0.1% penicillin/streptomycin (Sigma, St. Louis, MO) at 37°C in 5% CO2. Transfection of MCF7 cells was carried out with Superfect transfection reagent (Qiagen, Germantown, MD). Cells stably expressing GFP-alpha-tubulin were selected in G418 (0.8 mg/ml; Biowhittaker, Walkersville, MD). Individual colonies were screened for optimal GFP expression by fluorescence microscopy and then maintained in media supplemented with 0.4 mg/ml G418.

Stock Drug Solutions. Synthetic epothilone B (Calbiochem, San Diego, CA) and paclitaxel (Sigma) were dissolved in DMSO at stock concentrations of 100 μM and 10 mM, respectively, and stored at –80°C. Drugs were diluted to 10 μM in ethanol before a final dilution in media for each experiment.

The abbreviation used is: GFP, green fluorescent protein.

References

1 Supported by NIH Grant CA57291 (M. A. J.).

2 To whom requests for reprints should be addressed, at Department of Molecular Cellular and Developmental Biology and the Neuroscience Research Institute, University of California, Santa Barbara, CA 93106.

3 The abbreviation used is: GFP, green fluorescent protein.
To prevent photobleaching and reduce photodamage, Oxyrase (30 nM for epothilone B and 2 nM for paclitaxel) was incubated in the absence or presence of drug at a range of concentrations (0.3–30 nM for epothilone B and 2–72 nM for paclitaxel) for 20 h. Media were collected and cells were rinsed with versene (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and 0.5 mM EDTA), detached by trypsinization, and added back to the media to ensure that floating and poorly attached mitotic cells were included in the analysis. Cells were fixed with 10% formalin for 30 min, permeabilized in ice-cold methanol for 10 min, and stained with 4,6-diamidino-phenylindole to visualize nuclei.

Mitotic indices were determined by microscopy. Results are the mean and SE of seven experiments in which each of which 500 cells were counted for each concentration. The IC50 and IC90 are the drug concentrations that experimentally induced one-third and one-half of the maximal mitotic accumulation at 20 h, respectively. Mitotic arrest profiles for the drug solvents, DMSO and ethanol, were also examined; they did not alter mitotic arrest or microtubule dynamics under the conditions used.

Preparation of Cells for Analysis of Microtubule Dynamics. Cells were prepared as above, except that to promote cell spreading, cells were seeded onto glass coverslips that had been pretreated with poly-L-lysine (0.4 mg/ml; Sigma) for 2 h, followed by laminin and fibronectin (Life Technologies, Inc., Carlsbad, CA) at 10 and 20 μg/ml, respectively, for an additional 2 h at 37°C. In addition, cells were incubated with drug for 6 h, an incubation duration that allows attainment of an equilibrium drug concentration in cells (15). Cells had begun to accumulate in mitosis at that time, but there were still sufficient flat cells in interphase available for analysis of microtubule dynamics. At later time points, many interphase cells became somewhat rounded, some reverted to an interphase-like state apparently after abnormal mitotic exit (14), and some were apoptotic (data not shown). Before analysis, coverslips were transferred to recording media (culture media lacking phenol red and sodium bicarbonate, buffered with 25 mM HEPES and supplemented with 3.5 grams/liter glucose). To prevent photobleaching and reduce photodamage, Oxyrase (30 μL/mL; Oxyrase, Inc., Mansfield, OH) was added to the recording media immediately before sealing cells in a double coverslip chamber.

Time-Lapse Microscopy and Image Acquisition. Microtubules were observed using a Nikon Eclipse E800 fluorescence microscope with a plan apochromat 1.4 N.A. ×100 objective lens. The stage was enclosed in a Plexiglas box and maintained at 36 ± 1°C by a forced air heating system. Thirty images of each cell were acquired at 4-s intervals using a Hamamatsu Orca II (Middlesex, NJ) digital camera driven by Metamorph software (Universal Imaging, Media, PA) using 600 ms exposure time, a gain of three, and 2 × 2 binning to enhance brightness.

Analysis of Microtubule Dynamics. The positions of the plus ends of microtubules over time were tracked using the Metamorph Track Points program (Universal Imaging, Media, PA), exported to Microsoft Excel and analyzed using Real Time Measurement software (16). The lengths of individual microtubules were graphed as a function of time. Individual growth and shortening rates were determined by linear regression. Changes of ≥0.5 μm between two points were called growth or shortening events. Changes in length of <0.5 μm were considered to be periods of attenuated dynamics or pause. Greater than 50 microtubules were analyzed for each condition except for 3.5 nM epothilone B, in which only 34 microtubules were analyzed because of the lack of dynamic microtubules at this concentration. Results are the mean and SE of at least three independent experiments.

The time-based catastrophe frequency for each microtubule was calculated by dividing the number of catastrophes per microtubule by the time spent in growth or attenuation. The time-based rescue frequency per microtubule was calculated by dividing the total number of rescues per microtubule by the time spent shortening. The distance-based catastrophe and rescue frequencies were calculated by dividing the total number of rescues per microtubule by the total life span of the microtubule. Microtubules that were visible for ≥1 min were included in the frequency analysis.

RESULTS

Mitotic Arrest by Epothilone B. In the absence of epothilone B, 2.5% of MCF7 cells were in mitosis, as determined by counting of fixed and stained cells (see “Materials and Methods”). After the addition of epothilone B, as cells entered M phase of the cell cycle, many of them were unable to progress through mitosis, and they began to accumulate in mitosis. As shown in Fig. 1, after 20-h incubation with epothilone B at a range of concentrations, the percentage of cells in mitosis ranged from 3.7% at 0.3 nM to a maximum of 35–45% at 10 nM epothilone B. Many of the cells that were not blocked in mitosis were in an abnormal multinucleate interphase and may have undergone an abortive mitosis and reverted to interphase without successful cytokinesis (12, 14). To examine the relationship between mitotic block and drug effects on microtubule dynamics, we measured the effects of epothilone B on microtubule dynamic instability at one drug concentration that induced no mitotic arrest (0.2 nM) and at two concentrations that blocked mitosis to some degree but not completely (2 nM, the concentration that induced 33% of the maximal arrest at 20 h [IC50], and 3.5 nM, the concentration that induced 50% of maximal arrest at 20 h [IC90]).

Suppression of Microtubule Dynamics in Living Cells by Epothilone B. The effects of epothilone B on microtubule dynamic instability were determined in MCF7 cells that had been stably transfected with GFP-α-tubulin (“Materials and Methods”). At the flat lamellar edge of interphase cells, GFP-labeled microtubules are readily visible and can be followed for several minutes by time-lapse fluorescence microscopy. Fig. 2 shows several frames of time-lapse sequences of an untreated control cell (top panel) and of a cell incubated with 3.5 nM epothilone B (bottom panel). It was qualitatively clear that 3.5 nM epothilone B stabilized microtubule dynamics. Microtubules in control cells (Fig. 2A) shortened approximately twice as fast as they grew and underwent relatively long changes in length during both growth and shortening events. Most microtubules grew and/or shortened during the course of each control time-lapse sequence. Importantly, in epothilone B-treated cells, nearly all microtubules were completely stabilized, as shown in Fig. 2B. The few microtubules that were dynamic (Table 1) grew and shortened more slowly and for shorter lengths than in control cells.

The position of each microtubule end with time was determined and graphed to generate a “life history plot” from which parameters of microtubule dynamics were determined. As shown in Table 1 and Fig. 3, at 0.2 nM epothilone B, dynamics were not significantly altered. However, at higher drug concentrations (which induced mitotic arrest), epothilone B suppressed microtubule dynamics in a concentration-dependent manner. Parameters were determined from the dy-
dynamic microtubules in the presence of 2 and 3.5 nM epothilone B, e.g., at 2 nM epothilone B, the mean growth rate was decreased from 13.3 ± 4.5 μm/min in control cells to 8.3 ± 3.0 μm/min, a decrease of 38%. The mean shortening rate was similarly decreased by 27%, from 26.3 ± 10.1 μm/min to 19.3 ± 8.4 μm/min. The mean durations of individual growth and shortening events were unchanged compared with controls. Thus, as a result of the slower rates, the mean length a microtubule grew was reduced from 4.2 ± 3.5 μm to 2.3 ± 1.8 μm, a decrease of 45%, and the mean length shortened decreased from 6.1 ± 4.6 μm to 2.9 ± 2.1 μm, a reduction of 52%. Finally, dynamicity, which represents the total tubulin exchange/min decreased by 47% at 2 nM epothilone B but was virtually unchanged at 0.2 nM (~3%).

At 3.5 nM epothilone B, microtubules were almost completely stabilized, and most cells had few if any dynamic microtubules. Of 24 cells examined, only 8 had any microtubules that grew or shortened in a 2-min time period, whereas all 21 control cells had an abundance of dynamic microtubules (~70–90% were dynamic). Because the analysis at 3.5 nM epothilone B included only dynamic microtubules, the reported values (Table 1) significantly underestimate the stabilizing effect of epothilone B on microtubules.

The frequencies of catastrophe and rescue appear to be important parameters in determining microtubule function. The catastrophe frequency is the frequency with which the microtubules switch from either pause or growth to shortening. The rescue frequency is the frequency with which the microtubules switch from shortening to either growth or pause. Both the time- and length-based rescue frequencies increased in a concentration-dependent manner (Table 2). Rescue frequency per minute increased by 53 and 62%, and rescue frequency per micron shortened by 104 and 165% at 2 nM and 3.5 nM, respectively. The increase in rescue frequency is attributable, in part, to the method of analysis. In the presence of epothilone B, microtubule ends remain within the visible thin peripheral portion of the cell where the microtubule network is less dense, and thus, the rescue events can be seen. Microtubules in control cells shorten for longer distances, bringing their ends deeper into the thicker regions of the cell where individual microtubules are no longer distinguishable, and thus, many of the shortening events appear unrescued. The length-based catastrophe frequency also increased at the higher concentrations of epothilone B (by 51 and 78% at 2 and 3.5 nM epothilone B, respectively).

Although in general, epothilone B strongly stabilized microtubules, the extreme ends exhibited frequent minute changes in length, i.e., the ends “chattered.” Sometimes these events were >0.5 μm in length and thus were counted as growth or shortening events, yet other times they fell below this cutoff and were not included in the frequency analysis (Fig. 4). This “chattering” effect also contributed to the increases observed in both the catastrophe and rescue frequencies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>0.2 nM Epo no mitotic arrest</th>
<th>% change</th>
<th>2 nM Epo IC₅₀</th>
<th>% change</th>
<th>3.5 nM Epo IC₅₀</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (μm/min)</td>
<td>13.3 ± 4.5</td>
<td>12.0 ± 4.0</td>
<td>−10</td>
<td>8.3 ± 3.0⁹</td>
<td>−38</td>
<td>7.5 ± 3.0</td>
<td>−44</td>
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<tr>
<td>Growth duration (min)</td>
<td>0.32 ± 0.24</td>
<td>0.29 ± 0.22</td>
<td>−9</td>
<td>0.30 ± 0.23</td>
<td>−6</td>
<td>0.28 ± 0.27</td>
<td>−12</td>
</tr>
<tr>
<td>Growth length (μm)</td>
<td>4.2 ± 3.5</td>
<td>3.3 ± 2.7</td>
<td>−22</td>
<td>2.3 ± 1.8</td>
<td>−45</td>
<td>1.7 ± 1.4</td>
<td>−59</td>
</tr>
<tr>
<td>Shortening rate (μm/min)</td>
<td>26.3 ± 10.1</td>
<td>24.4 ± 10.9</td>
<td>−7</td>
<td>19.3 ± 8.4</td>
<td>−27</td>
<td>14.9 ± 6.4</td>
<td>−44</td>
</tr>
<tr>
<td>Shortening duration (min)</td>
<td>0.21 ± 0.11</td>
<td>0.19 ± 0.11</td>
<td>−10</td>
<td>0.15 ± 0.07</td>
<td>−29</td>
<td>0.15 ± 0.11</td>
<td>−29</td>
</tr>
<tr>
<td>Shortening length (μm)</td>
<td>6.1 ± 4.6</td>
<td>5.1 ± 4.6</td>
<td>−16</td>
<td>2.9 ± 2.1</td>
<td>−52</td>
<td>2.1 ± 1.4</td>
<td>−67</td>
</tr>
<tr>
<td>Pause duration (min)</td>
<td>0.27 ± 0.23</td>
<td>0.23 ± 0.18</td>
<td>−15</td>
<td>0.24 ± 0.2</td>
<td>−11</td>
<td>0.30 ± 0.27</td>
<td>+11</td>
</tr>
<tr>
<td>Dynamicity (μm/min)</td>
<td>10.4 ± 4.7</td>
<td>10.1 ± 4.4</td>
<td>−3</td>
<td>6.3 ± 2.8</td>
<td>−47</td>
<td>3.9 ± 1.6</td>
<td>−62</td>
</tr>
<tr>
<td>% time spent:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growing</td>
<td>39.5</td>
<td>39.9</td>
<td></td>
<td>38.7</td>
<td></td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>Shortening</td>
<td>19.4</td>
<td>22.3</td>
<td></td>
<td>15.9</td>
<td></td>
<td>12.1</td>
<td></td>
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<tr>
<td>Paused</td>
<td>41.1</td>
<td>37.8</td>
<td></td>
<td>45.4</td>
<td></td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>Number of microtubules</td>
<td>97</td>
<td>51</td>
<td></td>
<td>79</td>
<td></td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Number of cells</td>
<td>19</td>
<td>10</td>
<td></td>
<td>19</td>
<td></td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

⁹ Values in bold type differ significantly from control at ≥99% confidence level by Student’s t test. Values are mean and SD.

Underestates the real effects on the percentage of time spent paused (see text).
The suppressive effects of epothilone B on dynamic instability were also reflected in the total time that microtubules spent in growing, shortening, and paused states, particularly at 3.5 nM epothilone B. The percentages of time spent growing and shortening decreased by 8 and 7.3%, respectively, leading to an increase of 15.3% in the percentage of time spent in a paused state. Although the increased time spent in a paused state accurately reflects the behavior of the dynamic microtubules, because there were few dynamic microtubules at 3.5 nM epothilone B, it is an underestimate of the percentage of time that the total population of microtubules were in a state of attenuated dynamics at this concentration.

Can Cells Complete Anaphase at 3.5 nM Epothilone B with Significantly Impaired Dynamics? We determined the ratio of the number of postmetaphase (anaphase and telophase) cells to the number that were in metaphase or prometaphase over the concentration range that induced mitotic block (Table 3). Interestingly, the ratio decreased from 0.18 in controls (n = 114) to 0.03 (n = 288) at 1 nM epothilone B and 0 (n = 457) at 3 nM epothilone B, indicating that at epothilone B concentrations ≥3.0 nM, cells were unable to progress from metaphase to anaphase.

Epothilone B Suppresses Microtubule Dynamics Similarly to Paclitaxel. We compared the effects of epothilone B with those of paclitaxel at the IC₅₀ for mitotic block. MCF7 cells incubated with a range of paclitaxel concentrations (2–72 nM) for 20 h reached a maximum of ~30% mitotic arrest at 24 nM compared with the 40% mitotic arrest seen with epothilone B. The IC₅₀ for mitotic arrest was 7.5 nM.

At the IC₅₀ for mitotic arrest by paclitaxel, 35% (11 of 30) of cells possessed dynamic microtubules (compared with 20% at the IC₅₀ with epothilone B). Of those cells with microtubules undergoing some dynamic instability, the number of stable microtubules was greatly increased over controls. As was also the case with epothilone B, no postmetaphase cells were observed at ≥7.5 nM paclitaxel.

At the IC₅₀ for mitotic arrest, epothilone B and paclitaxel qualitatively and quantitatively suppressed microtubule dynamics similarly (Table 4 and Fig. 4, B and C), e.g., at 3.5 nM epothilone B and 7.5 nM paclitaxel.

Table 2. Epothilone B increases the catastrophe and rescue frequencies

<table>
<thead>
<tr>
<th>Epothilone B concentration</th>
<th>Catastrophe frequency/min</th>
<th>% change</th>
<th>Rescue frequency/min</th>
<th>% change</th>
<th>Catastrophe frequency/μm</th>
<th>% change</th>
<th>Rescue frequency/μm</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.1 ± 0.92</td>
<td>0.29</td>
<td>4.7 ± 2.8</td>
<td>16</td>
<td>0.37 ± 0.45</td>
<td>11</td>
<td>0.23 ± 0.21</td>
<td>26</td>
</tr>
<tr>
<td>0.2 nM Epo no mitotic arrest</td>
<td>1.5 ± 0.94</td>
<td>+29</td>
<td>5.5 ± 2.4</td>
<td>+16</td>
<td>0.33 ± 0.23</td>
<td>−11</td>
<td>0.29 ± 0.21</td>
<td>+26</td>
</tr>
<tr>
<td>2 nM Epo IC₅₀</td>
<td>1.4 ± 0.79</td>
<td>+20</td>
<td>7.2 ± 3.0**</td>
<td>+53</td>
<td>0.56 ± 0.54**</td>
<td>+51</td>
<td>0.66 ± 0.60**</td>
<td>+78</td>
</tr>
<tr>
<td>3.5 nM Epo IC₅₀</td>
<td>1.2 ± 0.61</td>
<td>+9</td>
<td>7.6 ± 3.7**</td>
<td>+62</td>
<td>0.47 ± 0.31**</td>
<td>+104</td>
<td>0.61 ± 0.43**</td>
<td>+165</td>
</tr>
</tbody>
</table>

* and ** values differ significantly from control at ≥95% and ≥99% confidence levels, respectively, by Student’s t test. Values are the mean ± SD.

Fig. 3. Concentration dependence for suppression of microtubule growth and shortening rates (A), lengths (B), and dynamicity (C) by epothilone B. In A and B, growth and shortening are denoted by and , respectively. Results are the means and SEs of at least three independent experiments.

Fig. 4. Life history plots of microtubules from control cells (A) or cells incubated with 3.5 nM epothilone B (B) or 7.5 nM paclitaxel (C) (the IC₅₀ for mitotic accumulation). Microtubules are more stable in the presence of either drug as compared with controls. Although microtubules were highly stabilized by epothilone B and paclitaxel, microtubule ends often “chattered” or appeared to be undergoing an increased number of minute transitions (catastrophes and rescues) compared with untreated cells (arrows in B).
paclitaxel, the mean growth rates were reduced by 44 and 41% and the mean shortening rates by 44 and 57%, respectively. Mean growth lengths were reduced by 59 and 42%, and the mean shortening lengths were reduced by 67 and 63% for epothilone B and paclitaxel, respectively. The length-based catastrophe frequency as well as both length- and time-based rescue frequencies were increased for both drugs. The net effect of these changes was a reduction in overall dynamicity of 62% for 3.5 nM epothilone B and 54% for 7.5 nM paclitaxel.

**DISCUSSION**

By quantitative time-lapse fluorescence microscopy of living human tumor cells stably expressing GFP-α-tubulin, we found that epothilone B suppressed microtubule dynamics in a concentration-dependent manner coincident with concentrations that induced mitotic block. At the IC$_{50}$ for mitotic arrest, most microtubules were completely stabilized and nondynamic. At the IC$_{33}$ and IC$_{50}$ for mitotic arrest, the dynamic microtubules grew and shortened more slowly and for shorter lengths on average than microtubules from control cells. In addition, the percentage of time that microtubules spent paused increased. The overall dynamicity was consequently decreased by 47 and 62% at the IC$_{33}$ and IC$_{50}$, respectively. The length-based rescue frequency also significantly increased, consistent with the decrease in the average length shortened.

Surprisingly, the length-based catastrophe frequency also increased in a concentration-dependent manner. The increase is consistent with the microtubule “chatter” observed, especially at the highest epothilone B concentration (3.5 nM). Despite the very strong overall stabilization, the extreme ends of microtubules underwent small changes in length. Microtubules from paclitaxel-treated cells also exhibited chattering at their ends. One model to explain this is that in the presence of either drug, microtubules continue to grow and shorten, but each event becomes rapidly stabilized as a depolymerizing microtubule encounters an epothilone B or paclitaxel molecule bound to the microtubule or as a drug molecule binds to a growing microtubule, resulting in an increased number of small growing and shortening events per unit length compared with control cells. Because epothilone B and paclitaxel bind along the sides of the microtubule lattice and not preferentially to the ends (17), the extreme ends of the microtubules may remain free to grow and shorten for short distances until they encounter a bound drug molecule. Support for this model comes from two earlier studies with paclitaxel. Wilson et al. (1985) found that when in vitro paclitaxel-stabilized microtubules were pulsed with radiolabeled GTP, they showed an initial burst of incorporation of tubulin-GTP, suggesting that the paclitaxel may not have prevented dynamic instability at the extreme ends (18). Derry et al. (1995) found that transition frequencies were unaffected even at concentrations of paclitaxel that markedly stabilized dynamics in vitro, again suggesting that the extreme ends were free to undergo some dynamic instability (19).

It is surprising that at 3.5 nM epothilone B, the IC$_{50}$ for mitotic block, most microtubules were completely stable. Even though the percentage of cells in mitosis continued to increase at concentrations $>3.5$ nM epothilone B, the postmetaphase:metaphase ratio indicated that no cells were able to progress into anaphase. Thus, mitosis was completely arrested. The stability of the microtubules at the IC$_{50}$ supports the conclusion that mitotic arrest is a result of impaired microtubule dynamics. However, we note that measurement of microtubule dynamics in interphase cells as was performed in this study may conceivably underestimate the dynamics of mitotic microtubules (discussed further below).

Why does the percentage of cells in mitosis increase at $>3.5$ nM epothilone B? It is conceivable that mitotic accumulation is the result of two separate phenomena. Between 0.3 and 3 nM epothilone B, mitotic accumulation may result from a slowing of mitosis, although cells may ultimately pass through to anaphase. Subsequent to mitotic arrest, cells can undergo an abnormal mitotic exit in which anaphase does not occur, but the chromosomes decondense and revert to an interphase-like state, resulting in a multinucleated cell (12, 14). At drug concentrations of $>3.5$ nM, no cells were able to progress into anaphase. The increase in the percentage of mitotic cells at higher drug concentrations ($>3.5$ nM epothilone B) may result from an increased duration of the mitotic state before undergoing an abnormal mitotic exit.

**How Does Suppression of Microtubule Dynamics Induce Mitotic Arrest?** Microtubule dynamics are cell cycle dependent reflecting their distinct roles in quiescent and dividing cells. During interphase, microtubules appear to play a predominantly structural role and are dynamic primarily near the cell periphery. Microtubule growth and shortening occur over relatively short distances. In mitosis, however, microtubules are responsible for chromosome capture and alignment at the metaphase plate and separation of the chromosomes at anaphase. New microtubules appear to be constantly generated from the spindle poles and grow outward over relatively large distances to search for and attach to chromosomes via their kinetochores (20). If no chromosome is encountered, a growing microtubule eventually transitions to shortening (depolymerization). Once a chromosome is attached to microtubules emanating from both spindle poles, it becomes aligned at the metaphase plate. Consistent with these roles, Rusan et al. (21) found that microtubule growth and shortening rates did not change significantly between interphase and mitosis; however, the lengths and durations of growth and shortening events increased 2-fold. By significantly reducing the length a microtubule can grow or shorten before being stabilized, epothilone B and paclitaxel appear to slow chromosome alignment at low concentrations and prevent alignment completely at higher concentrations.

**Epothilone B Affects Microtubule Dynamics Nearly Identically to Paclitaxel.** Epothilone B shares the same or an overlapping binding site with paclitaxel and has been reported to have the same effects as paclitaxel.
as paclitaxel on purified microtubules in vitro and on mitotic spindle structure and function in cultured cells (4, 5). We reasoned that if a change in microtubule dynamics were responsible for these effects, then microtubule dynamics might be suppressed similarly at concentrations of both drugs that induce a similar degree of arrest. We found that epothilone B and paclitaxel alter the same microtubule dynamic parameters and to a similar extent. At the IC50 for mitotic arrest, dynamicity was reduced by 54% by paclitaxel compared with 62% for epothilone B. In addition, no anaphase or telophase figures were observed at the IC50 for either drug, and in 65% of the cells treated with paclitaxel, the microtubules were completely stabilized compared with 80% for epothilone B. Thus, the effects of epothilone B on microtubule dynamics are remarkably similar to those of paclitaxel, suggesting that both drugs work by the same mechanism to induce mitotic block. Because epothilone B is chemically malleable, overcomes P-glycoprotein-mediated paclitaxel resistance, and is more water soluble than paclitaxel, it has been an attractive compound for drug development (4, 5, 8). Phase I and II clinical trials are under way (22–24).

How Do Cells Enter and Exit Mitosis Without Dynamic Microtubules? The observation that microtubule dynamics are almost completely stabilized in the presence of paclitaxel and epothilone B raises the question of how the microtubule network can completely disassemble at the G2–M transition, assemble a mitotic spindle, and subsequently reestablish an interphase microtubule array all with an apparent lack of microtubule turnover. There are several possibilities. We measured microtubules in interphase; it is conceivable that microtubule dynamics are suppressed less efficiently during mitosis by these compounds than they are during interphase. Several stabilizing microtubule-associated proteins are inactivated by phosphorylation at the onset of mitosis (25), which could potentially destabilize microtubules enough so that mitosis could proceed, albeit at a much slower rate. In addition, microtubule destabilizers, such as the microtubule-severing protein katanin, the catastrophe-promoting factor statmin/op18, or an as yet unidentified protein that is transiently activated at the transitions into or out of mitosis (25), could disassemble drug-stabilized microtubules. A protein that sets a precedent for this kind of activity is the microtubule motor protein MCAK. MCAK is a catastrophe-promoting protein that can depolymerize paclitaxel-stabilized microtubules in vitro (26). Although MCAK is not likely to be involved in the disassembly process itself (dominant negative constructs have no effect on mitotic entry: Ref. 27), it confirms the existence of microtubule destabilizers that can operate even in the presence of stabilizing drugs. To fully answer this question, it will be important to determine which, if any, of these proteins can act on drug-stabilized microtubules. In addition to providing insight into the mitotic arrest induced by microtubule-stabilizing agents, it may also reveal novel proteins that contribute to drug resistance.

ACKNOWLEDGMENTS

We thank Drs. Clare Waterman-Storer and Pat Wadsworth for invaluable help in assembling the necessary equipment and learning the techniques of measuring microtubule dynamics. We thank Janis Bunker for transfection of cells with GFP-tubulin.

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### Suppression of Microtubule Dynamics by Epothilone B Is Associated with Mitotic Arrest

Kathy Kamath and Mary Ann Jordan


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