Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles
Implications for the role of microtubule dynamics in mitosis

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Summary
Inhibition of mitosis by many drugs that bind to tubulin has been attributed to depolymerization of microtubules. However, we found previously that low concentrations of vinblastine and vincristine blocked mitosis in HeLa cells with little or no depolymerization of spindle microtubules, and spindles appeared morphologically normal or nearly normal. In the present study, we characterized the effects of vinblastine, podophyllotoxin and nocodazole over broad concentration ranges on mitotic spindle organization in HeLa cells. These three drugs are known to affect the dynamics of microtubule polymerization in vitro and to depolymerize microtubules in cells. We wanted to probe further whether mitotic inhibition by these drugs is brought about by a more subtle effect on the microtubules than net microtubule depolymerization. We compared the effects of vinblastine, podophyllotoxin and nocodazole on the organization of spindle microtubules, chromosomes and centrosomes, and on the total mass of microtubules. Spindle organization was examined by immunofluorescence microscopy, and microtubule polymer mass was assayed on isolated cytoskeletons by a quantitative enzyme-linked immunoadsorbence assay for tubulin. As the drug concentration was increased, the organization of microtubules changed in the same way with all three drugs. The changes were associated with mitotic arrest, but were not necessarily accompanied by net microtubule depolymerization. With podophyllotoxin, mitotic arrest was accompanied by microtubule depolymerization. In contrast, with vinblastine and nocodazole, mitotic arrest occurred in the presence of a full complement of spindle microtubules. All three drugs induced a nearly identical rearrangement of spindle microtubules, an increasingly aberrant organization of metaphase chromosomes, and fragmentation of centrosomes. The data suggest that these anti-mitotic drugs block mitosis primarily by inhibiting the dynamics of spindle microtubules rather than by simply depolymerizing the microtubules.

Key words: mitosis, microtubule dynamics, vinblastine, podophyllotoxin, nocodazole.

Introduction
Inhibition of mitosis by several anti-mitotic drugs including vinblastine, podophyllotoxin and nocodazole, that can depolymerize microtubules in vivo and in vitro, has been considered to occur by a mechanism involving depolymerization of microtubules (e.g. see Malawista et al. 1968; Wilson and Bryan, 1974; DeBrabander et al. 1976; Hoebeke et al. 1976; Zieve et al. 1980; reviewed by Dustin, 1984). However, we found that inhibition of mitosis in HeLa cells by low concentrations of vincristine and vinblastine occurs with little or no depolymerization of spindle microtubules (measured by enzyme-linked immunoadsorbence assay (ELISA) of tubulin in isolated cytoskeletons; Jordan et al. 1991). By immunofluorescence microscopy with an antibody to tubulin, blocked metaphase spindles appear morphologically normal or exhibit only slight abnormalities in microtubule organization. We also found that vinblastine significantly inhibits the exchange of tubulin at the ends of in vitro reassembled microtubules without exerting significant effects on the total mass of microtubule polymer (Jordan and Wilson, 1990). These results indicated that vincristine and vinblastine inhibit mitosis, not by inducing microtubule depolymerization, but by stabilizing the dynamics of the spindle microtubules. We therefore wanted to determine whether other microtubule-depolymerizing drugs inhibit mitosis by depolymerizing spindle microtubules or by a more subtle action on the microtubules.

In the present study, we characterized further the effects of vinblastine, and we characterized the effects of podophyllotoxin and nocodazole on mitotic accumulation, the mass of cellular microtubules, and the organization of the spindle microtubules, chromosomes and centrosomes in HeLa cells. We found that while each drug blocked mitosis with very different effects on microtubule polymer levels, all three drugs induced a
nearly identical concentration-dependent series of rearrangements of spindle microtubules, centrosomes and chromosomes. The series was characterized by a drug-concentration-dependent: (1) increase in the length and number of astral microtubules; (2) decrease in the length of the central spindle; (3) increase in the number of chromosomes that were found near the spindle poles rather than in the metaphase plate; and (4) fragmentation of centrosomal material. With low concentrations of vinblastine and nocodazole, these changes occurred in the absence of any net depolymerization of microtubules.

The observations reported in the present study, together with data on the effects of vinblastine, podophyllotoxin and nocodazole on the dynamics of microtubules in vitro (see Discussion), suggest that these three antimitotic drugs, and perhaps others as well, inhibit mitosis primarily by inhibiting microtubule dynamics. The data support the idea that the dynamic behavior of microtubules is crucial to the progress of mitosis and the cell cycle.

Materials and methods

Cell culture

HeLa S3 cells were provided by Dr. Jeannette Bulinski (Columbia University, New York, NY) or American Type Culture Collection (Rockville, MD). Cells were grown in monolayers at 37°C without antibiotics in 5% CO2 as previously described (Jordan et al. 1991). Subcultures of cells for assay of polymer mass were performed at a density of 1.5 × 10^6 cells/ml in 15 ml. Approximately 20 h later fresh medium plus or minus drug was added. Cells were harvested for assay of polymerized and soluble tubulin 18-20 h after drug addition. Subcultures of cells for immunofluorescence microscopy and for assays of proliferation were plated at a density of 4 × 10^4/2 ml in 35 mm dishes containing no. 1 glass coverslips freshly coated with polylysine (50 μg/ml, 2 h, 37°C, followed by a rinse with water and a rinse with medium). Approximately 40 h later, fresh medium containing drug (or no drug) was added. At this time, cells were scraped from some coverslips and counted by hemocytometer to determine cell number at the time of drug addition. Cell viability was determined by exclusion of trypan blue. At 18-20 h after drug addition samples of control and drug-treated cells were counted to determine the increase in cell number. Two to four independent experiments were performed with each drug to determine the concentration dependence for inhibition of cell division. Simultaneously, parallel coverslips of cells were fixed and processed for immunofluorescence microscopy. Vinblastine was a gift from Eli Lilly and Co., Indianapolis, IN. Podophyllotoxin was obtained from Aldrich (Milwaukee, WI) and nocodazole from Janssen Pharmaceutical (Beerse, Belgium).

Immunofluorescence microscopy and determination of concentration dependence for metaphase arrest and spindle reorganization

Cells grown on coverslips were prepared for immunofluorescence microscopy as described previously (Jordan et al. 1991). Coverslips were incubated with mouse monoclonal antibody (E7, IgG; a gift from Dr. Michael Klymkowsky, University of Colorado, Boulder, CO; Chu and Klymkowsky, 1989) that is specific for β-tubulin in HeLa cell extracts (data not shown), centrosomes were detected using antisierum 5051, a human autoimmune anti-centrosomal antisierum (a gift from Dr. S. Doxsey, University of California, San Francisco, CA; Calarco-Gillam et al. 1983), and chromosomes were stained with DAPI (4,6-diamino-2-phenylindole; Sigma Chemical, St. Louis, MO). Second antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-human IgG (Cappel, West Chester, PA). The percentage of cells arrested in metaphase was counted on preparations double-stained with DAPI and for anti-tubulin immunofluorescence; at least 400 cells were counted at each drug concentration tested; two or three independent experiments were performed for each drug. At drug concentrations that were just sufficient to induce metaphase arrest, metaphase was clearly distinguishable from other stages of mitosis by the characteristic compact metaphase plate of chromosomes. However, at high drug concentrations associated with the formation of types III and IV mitotic spindles (see Results for description of types), cells with highly condensed masses of chromatin were arbitrarily called "metaphase" as has been the convention in the literature. Sufficient numbers of mitotic cells were counted to acquire a minimum of 50 anaphase and/or metaphase cells to determine the anaphase/metaphase ratio. Between 50 and several hundred metaphases were scored for each drug concentration to determine the frequencies of normal and types I-IV spindles.

The distance between the two poles was measured on metaphase spindles that had been triply stained with DAPI and with anti-centrosomal and anti-tubulin antibodies. Measurement was done directly on the coverslip preparation using a 40× Olympus oil immersion objective and an eyepiece reticle. Only spindles that had both centrosomes in the plane of focus were measured. A minimum of 22 spindles were measured for each drug concentration. Photo-micrographs were obtained using a Zeiss Photomicroscope III equipped with an epi-fluorescence condenser and a 40× Olympus UVFL oil immersion objective of numerical aperture 1.3 as described previously (Jordan et al. 1991).

Quantitation of tubulin in microtubules

Cells were released from flasks by gentle scraping, collected by centrifugation, and resuspended for counting and for collection of stabilized microtubules in cytoskeletons as described in detail previously (Thrower et al. 1991). Tubulin in microtubules was determined using an enzyme-linked immunosorbence assay (ELISA) (Thrower et al. 1991) using a monoclonal antibody to beta tubulin (1-1.1, IgM, kappa class; Ball et al. 1986). The tubulin standard was three-times-cycled microtubule-associated protein (MAP)-depleted bovine brain tubulin prepared as described by Farrell et al. (1987). Between 2 and 6 independent determinations of microtubule polymer mass were made for each drug concentration.

Results

We incubated HeLa cells for a duration of one cell cycle with a range of concentrations of vinblastine, podophyllotoxin and nocodazole. Cells were then fixed and processed for fluorescence microscopy to determine the arrangement of chromatin or chromosomes using DAPI, the microtubules using anti-tubulin immunofluorescence, and the centrosomes using 5051, a human
scleroderma autoimmune antiserum. The percentage of cells in mitosis or in a mitotic-like stage after drug treatment was determined from the stained preparations. In parallel, we isolated stabilized cytoskeletons and determined the total mass of tubulin in the form of microtubules that remained after drug treatment as compared with control cells.

Effects of vinblastine on mitosis
Mitotic arrest and microtubule polymer mass
Incubation with vinblastine induced cells to accumulate at a stage resembling mitotic metaphase in a concentration-dependent manner (Fig. 1A, circles). A total of 50% of the cells accumulated in metaphase (Kmet) at 0.8 nM drug (Table 1), and a peak of maximal accumulation occurred at 6 nM vinblastine (Fig. 1A). Vinblastine inhibited cell division concomitant with metaphase arrest; half-maximal inhibition of cell division (Kdiv) occurred at 0.45 nM vinblastine (Table 1).

The ratio of the number of cells in anaphase to the number of cells in metaphase at each vinblastine concentration was determined to ascertain whether accumulation of cells in metaphase by vinblastine was due to slowing of mitosis relative to the entire cell cycle, or whether it was due to a block of mitosis at metaphase. An increase in the number of cells in metaphase and a proportional increase in the number of cells in anaphase would indicate that mitosis was slowed, whereas an increase in the number of cells in metaphase and a decrease in the number of cells in anaphase would indicate a metaphase block. Vinblastine induced a decrease in the ratio of cells in anaphase to cells in metaphase in a concentration-dependent manner, with 50% decrease in the ratio (Kanap/met) occurring at 0.4 nM drug (Table 1). At 1.6 nM vinblastine, the anaphase/metaphase ratio was zero (data not shown). Thus, vinblastine blocked cells at metaphase of mitosis.

Vinblastine affected the total mass of cellular microtubule polymer in a complex fashion with respect to drug concentration and with respect to metaphase accumulation (Fig. 1A, squares). Metaphase accumulation occurred without any reduction in total microtubule polymer mass at vinblastine concentrations between 0.1 and 6 nM; maximal metaphase accumulation occurred in the concentration range in which microtubule polymer mass was unaltered. Microtubule polymer mass was reduced by 50% (Kdep) at 11 nM vinblastine, 14 times the concentration required for 50% accumulation of cells at metaphase (Kdep/Kmet, Table 1). Microtubules were completely depolymerized at 100 nM vinblastine. Vinblastine at concentrations greater than 1 μM induced formation of vinblastine-tubulin paracrystals, resulting in increased polymer in isolated cytoskeletons (described further below).

Table 1. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic block, the mass of microtubule polymer and cell division

<table>
<thead>
<tr>
<th>Drug</th>
<th>Kmet</th>
<th>Kmet/Kdep (nM)</th>
<th>Kdep</th>
<th>Kdiv</th>
<th>Kdep/Kmet</th>
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<tr>
<td>Vinblastine</td>
<td>0.80</td>
<td>0.40</td>
<td>11</td>
<td>0.45</td>
<td>14</td>
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<tr>
<td>Podophyllotoxin</td>
<td>30</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>0.5</td>
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<tr>
<td>Nocodazole</td>
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<td>12</td>
<td>600</td>
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Kmet, drug concentration that induced accumulation of 50% of cells in metaphase after incubation for 18-20 h. From the data of Figs 1A, 4A and 6A. Kmet/Kdep, drug concentration that induced a 50% decrease of microtubule polymer mass as determined by quantitation of tubulin in cytoskeletons isolated from cells 18-20 h after drug addition. From the data of Figs 1A, 4A and 6A. Kdiv, drug concentration that induced inhibition of cell division by 50% after treatment of exponentially growing cells for 18-20 h. The values were determined from plots of the percentage inhibition of increase in number of cells after incubation for 18-20 h in the presence of drug vs the drug concentration (data not shown). For example, the value for vinblastine was derived from Fig. 1 of Jordan et al. (1991).

Fig. 1. Metaphase arrest and microtubule depolymerization and spindle length after incubating HeLa cells with increasing concentrations of vinblastine. (A) Percentage of cells in metaphase (circles) and percentage decrease in mass of polymerized microtubules compared with control cells (squares). At concentrations of 10 μM and 100 μM vinblastine, the polymer mass increased as a result of formation of vinblastine-tubulin paracrystals. The relatively large standard errors of polymer mass measurements at 10 and 100 μM vinblastine are probably due to the small number of assays carried out at these concentrations and to cell death, which was prevalent in this vinblastine concentration range (data not shown). (B) Percentage decrease of interpolar distance of normal, type 1 and type II spindles.

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[Graph showing the percentage of cells in metaphase and the percentage decrease in polymer mass as a function of vinblastine concentration, with error bars indicating standard deviation.

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Fig. 2. Microtubule, chromosome, and centrosome arrangement of two normal (control) cells in metaphase (the cell on the left and the cell on the right). Other cells (more dimly stained) are in interphase. (A) Indirect immunofluorescence using anti-tubulin monoclonal antibody followed by a fluorescein isothiocyanate-conjugated second antibody on HeLa cells fixed and stained as described in Materials and methods, (a) DAPI stain of metaphase plates of chromosomes (arrows) in the same cells, (a') Indirect immunofluorescence of centrosomes (arrows) using 5051, a human scleroderma autoimmune antisemimm, followed by a rhodamine-conjugated second antibody, in the same cells. Bar, 10 μm.

In this and other micrographs, staining of the same cell or group of cells using different antibodies and fluorochromes is designated by one alphabet character, as A, a or a'.

Effects of vinblastine on spindle organization

Metaphase spindles of control cells contained primarily kinetochore and interpolar microtubules; the few astral microtubules that were present were typically very short and were often barely detectable (Fig. 2A). Condensed chromosomes (Fig. 2A, arrows) were in a compact metaphase plate located midway between the two spindle poles. A single compact mass of centrosomal protein was located at each pole (Fig. 2A, arrows). The poles were separated by a distance of 7.9 ± 0.4 μm.

After incubation with low concentrations of vinblastine (0.4-1.6 nM), between 12% and 69% of the cells were blocked in metaphase (Fig. 1A). Yet, by immunofluorescence microscopy, many of the metaphase spindles were morphologically indistinguishable from metaphase spindles of control cells. (The proportion of all metaphase spindles that appeared normal was 69% after incubation with 0.4 nM vinblastine; the proportion that appeared normal diminished to 9% of all metaphase spindles with 1.6 nM vinblastine.) However, some spindles were clearly abnormal. The abnormal spindles could be described in terms of four types (I-IV), characterized by increasing degrees of disorganization (Jordan et al. 1991). Type I spindles were nearly normal-looking bipolar spindles (Fig. 3A, arrows), except that one or a few chromosomes were near one or both spindle poles instead of being aligned with the majority of the chromosomes at the metaphase plate (Fig. 3a, arrows). Also, the astral microtubules of type I spindles were typically more numerous and somewhat longer than those of normal spindles (Fig. 3A, arrows).

Type II abnormal spindles were bipolar (Fig. 3C,c',c") but the microtubules and chromosomes exhibited more extensive rearrangement than those of type I spindles. More chromosomes were located at one or both poles in type II spindles than in type I spindles (Fig. 3c, arrows). Astral microtubules were prominent and often were very long (Fig. 3C). The average distance between...
Fig. 3
the two poles in type I and type II spindles was 26% shorter than the distance between poles of normal spindles (Jordan et al. 1991). Thus in both type I and type II spindles the astral microtubules were longer and the kinetochore microtubules were shorter than normal. The distinction between type I and II spindles was subjective and was made primarily to emphasize the qualitative continuum of changes that occurred with increasing drug concentration. Type III spindles (Fig. 3C,c,c',D,d,d') were essentially monopolar and consisted of a ball of condensed chromatin (Fig. 3c,d), one or more star-shaped aggregates of microtubules (Fig. 3C,D), and one or more masses of centrosomal material (described further below) (Fig. 3c',d').

Between 0.2 nM and 1.6 nM vinblastine, the arrested cell population contained a mixture of normal spindles and abnormal types I, II and III spindles. For example, 12% of the cells contained metaphase spindles at 0.4 nM vinblastine; of those spindles 69% were normal, 16% were types I and II, and 15% were type III. As the drug concentration was increased from 0.2 nM to 1.6 nM vinblastine, the frequency of normal and types I and II spindles diminished and the frequency of type III spindles increased. For example, 48% of the cells contained metaphase spindles at 0.8 nM vinblastine; of those spindles 16% were normal, 12% were types I and II, and 72% were type III. Above 1.6 nM vinblastine, as for example at 3 nM vinblastine, 82% of the cells contained metaphase spindles, but there were no normal-looking spindles; all spindles were types I, II or III. At 6 nM vinblastine, 86% of the cells contained metaphase spindles, but there were no bipolar spindles and all spindles were type III (Fig. 3D,d,d').

Type IV mitotic figures (Fig. 3E,e,F) had no microtubules and contained nondescript aggregates of condensed chromosomes. Type IV mitotic figures occurred only upon incubation with vinblastine at concentrations of 50 nM and higher; at these concentrations no microtubule polymer was detected in isolated cytoskeletal isolates (e.g. 100 nM vinblastine; Fig. 1A, squares).

Small punctate aggregates of tubulin were observed by immunofluorescence microscopy at vinblastine concentrations between 6 nM and 1000 nM. The aggregates were present in very small numbers at vinblastine concentrations of 6 nM (Fig. 3D); their number increased after incubation of cells with vinblastine at concentrations that resulted in loss of measurable microtubule polymer. At 100 nM and 1000 nM vinblastine they were the only tubulin-containing structures visible by immunofluorescence (Fig. 3E,F). The aggregates were located at the cell periphery in cells displaying type III spindles (Fig. 3D) and were scattered throughout the cytoplasm in cells displaying type IV mitotic figures (Fig. 3E,F).

The centrosomal material of cells incubated with very low concentrations of vinblastine (0.4 nM or less) formed a single compact mass in interphase cells or two compact masses in mitotic cells, one at each spindle pole (not shown). Between 0.8 and 13 nM vinblastine, centrosomal material in mitotic cells displaying normal and types I, II and III spindles was fragmented into multiple pieces that were located at or near the points of convergence of microtubule arrays. The fragmentation of centrosomal material induced by 0.8 nM vinblastine is shown in three cells in Fig. 3B. The location of fragmented centrosomal material at the foci of microtubule arrays is shown in Fig. 3C,c,c',D,d,d'. Above 50 nM vinblastine, mitotic centrosomes were compact (data not shown); this occurred concomitant with total microtubule depolymerization (Fig. 1A). In contrast to the effects of vinblastine on centrosomal material in mitotic cells, the centrosomal material of interphase cells was not fragmented at any vinblastine concentration examined (data not shown).

With increasing vinblastine concentration between 0.1 nM and 6 nM, the distance between the spindle poles decreased. Central spindles were half as long as central spindles in control cells after incubation with 3.6 nM vinblastine (Fig. 1B).

**Viblastine-tubulin paracrystal formation**

Tubulin paracrystals formed only at high vinblastine concentrations (> 1 μM), concomitant with an increase in the mass of polymeric tubulin in cytoskeletal isolates (Fig. 1A, squares). At 10 μM vinblastine, cells contained several long, large, polygonal tubulin paracrystals (Fig. 3G). The chromatin was either interphase-like (diffuse with an apparent nuclear membrane) or mitotic (condensed and aggregated with no apparent nuclear membrane) (not shown). At a vinblastine concentration of 100 μM, cells contained large numbers of small, globular paracrystals (Fig. 3H), and the chromatin often appeared extremely condensed (not shown).

**Effects of podophyllotoxin on mitosis**

The effects of podophyllotoxin on mitotic arrest and spindle organization appeared similar to the effects of vinblastine in some ways, but there also were significant differences in the mode of action of the two drugs.

**Mitotic arrest and microtubule polymer mass**

Like vinblastine, podophyllotoxin induced cells to accumulate in mitotic metaphase (Fig. 4A, circles). A total of 50% of the cells accumulated in metaphase ($K_{met}$) at approximately 30 nM podophyllotoxin (Table 1) and a peak of maximal metaphase accumulation occurred at a podophyllotoxin concentration of 100 nM (Fig. 4A). Incubation of cells with podophyllotoxin, like incubation with vinblastine, decreased the ratio of cells in anaphase to cells in metaphase in a concentration-dependent manner, with 50% decrease in the ratio occurring at 5 nM podophyllotoxin ($K_{ana/met}$, Table 1). The anaphase/metaphase ratio was zero at 33 nM podophyllotoxin. Half-maximal inhibition of cell division ($K_{div}$) occurred at 20 nM podophyllotoxin (Table 1).

In contrast to the action of low concentrations of vinblastine, metaphase accumulation by low concentrations of podophyllotoxin was associated with a high degree of microtubule depolymerization (Fig. 4A,
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Fig. 4. Podophyllotoxin concentration dependence of metaphase arrest and microtubule depolymerization (A) and of spindle and cell reorganization (B,C) in HeLa cells after treatment with podophyllotoxin at the stated concentrations for the duration of one cell cycle. (A) Accumulation of cells in metaphase was concomitant with depolymerization of microtubules. Percentage of cells in metaphase (open circles) and percentage decrease in mass of polymerized microtubules (filled squares). (B) Percentage of metaphases that were types I or II (open triangles) and types III and IV (filled triangles). The percentage of metaphase spindles that appeared normal equals 100% minus the percentages indicated by the filled and open triangles. (C) Percentage decrease of interpolar distance measured on bipolar spindles (normal, type I and type II).

squares; Table 1, $K_{dep}/K_{mec}=0.5$). For example, 50% depolymerization of microtubules occurred at 15 nM podophyllotoxin while only 8% of the cells were arrested in metaphase (Table 1, $K_{dep}$; Fig. 4A, circles).

Spindle organization
Between 3.3 nM and 18 nM podophyllotoxin, metaphase spindles were frequently types I and II, exhibiting chromosomes located near the spindle poles and more numerous, frequently longer, astral microtubules than control spindles (Fig. 5A,a,b,C,c). The reorganization of spindle microtubules, chromosomes and centrosomes induced by podophyllotoxin was qualitatively similar to that induced by incubation with vinblastine (see Fig. 3A,a,C,c). Type III spindles were prevalent at 18 and 32 nM podophyllotoxin. Small star-shaped aggregates of microtubules were induced by podophyllotoxin (Fig. 5D,E), similar to but smaller than the star-shaped aggregates induced by vinblastine (Fig. 3C,D). Fig. 5b shows a particularly clear example of the arrangement of polar chromosomes in a type I spindle. Polar chromosomes were frequently distributed unequally at the two poles after incubation with vinblastine, podophyllotoxin or nocodazole (see below), suggesting that segregation of chromatids had not occurred.

By contrast with vinblastine, podophyllotoxin induced significant distortions of spindle morphology without inducing accumulation of cells at metaphase or inhibiting cell division. For example, only 2.2% of the cells contained metaphase spindles at 6 nM podophyllotoxin, but 22% of the spindles were types I and II (Fig. 4B, open triangles). In addition, there was no net microtubule depolymerization at this concentration (Fig. 4A), and no inhibition of cell division. Even at 10 nM podophyllotoxin, only 5% of the cells were in metaphase (Fig. 4A, open circles), and there was no detectable inhibition of cell proliferation (data not shown). However, spindle organization was significantly affected (Fig. 4B, 5C,c). Spindles were 23% shorter than control spindles (Fig. 4C) and were often abnormal (26% of the spindles were types I, II or III) (Fig. 4B). In addition, the anaphase/metaphase ratio was reduced by 60% as compared with controls.

The observation that 10 nM podophyllotoxin induced a low frequency of cells in anaphase relative to cells in metaphase and induced significant distortions of spindle morphology without inhibiting cell division was initially puzzling. A plausible explanation, however, is that the rate of progression through metaphase was slowed by low concentrations of podophyllotoxin, but metaphase was not blocked, and anaphase and cytokinesis occurred normally. These data suggest that the mechanisms involved in proper construction of the metaphase spindle are more sensitive to podophyllotoxin than the mechanisms responsible for progression from metaphase to anaphase. The data also indicate that abnormal spindles do not necessarily result in metaphase blockage, but that such spindles can proceed through metaphase and the cells can divide.

The extent of spindle damage and the effects of the damage on mitotic accumulation increased dramatically between 18 nM and 33 nM podophyllotoxin (Figs 4A, 5D,d,E). At 18 nM podophyllotoxin, only 8% of the cells were in metaphase (Fig. 4A, open circles). Of cells in metaphase, 32% had spindles with normal organization, 23% of the spindles were types I or II, and 45% were types III or IV (Fig. 4B). Bipolar spindles present at this concentration were 63% shorter than control spindles (Fig. 4C, open squares), and the mass of microtubules was reduced by 52% as compared with
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Fig. 5. Microtubule, chromosome and centrosome arrangement in HeLa cells after 18-20 h incubation in podophyllotoxin at the specified concentrations. (A,a) 3 nM; (B,b) 18 nM; (C,c) 10 nM; (D,d) 18 nM; (E) 33 nM; (F) 37 nM; (G,g) 110 nM podophyllotoxin. 
(A,B,C,D,E,F,G) Anti-tubulin immunofluorescence; (a,b,c,g) DAPI staining of chromosomes; (d) anti-centrosomal immunofluorescence. (A,a) Type I abnormal spindle induced by 3 nM podophyllotoxin; arrows point to tufts of long astral microtubules in (A) and chromosomes located near the spindle poles in a; (B,b) type I abnormal spindle induced by 18 nM podophyllotoxin; arrows in b point to chromosomes located near one pole, there were none at the opposite pole; astral microtubules are not prominent in this particular spindle but there are a few at the arrow in B. (C,c) Two type II spindles induced by 10 nM podophyllotoxin; arrows point to long astral microtubules in C and to large numbers of chromosomes located near the spindle poles in c. (D,d) Three small spindles induced by incubation with 18 nM podophyllotoxin. All three spindles contain star-shaped aggregates of microtubules. The upper two spindles contain fragmented centrosomal material. The chromosomes of the lower two spindles were condensed primarily into one spherical mass characteristic of type III spindles. The chromosomes of the upper spindle retained a slightly increased density where one might expect the metaphase plate, characteristic of a type II spindle (chromosomes not shown). (E) Star-shaped aggregates of microtubules in type III spindles (asterisks) and punctate aggregates of tubulin in type IV spindles (arrows) induced by 33 nM podophyllotoxin. (F) Microtubules of interphase cells induced by incubation in 37 nM podophyllotoxin. (G) Diffuse tubulin stain in both mitotic (labelled m) and interphase (labelled i) cells induced by incubation with 110 nM podophyllotoxin; and (g) parallel DAPI staining of the same cells showing either masses of condensed mitotic chromatin or chromosomes (m) or diffuse interphase chromatin (i). Bars, 10 μm. Small bar in a shows magnification of A,a and C-g; large bar in b shows magnification of B and b.

Control cells (Fig. 4A, squares). After incubation with 33 nM podophyllotoxin, 65% of the cells were in metaphase, and all of the mitotic figures were types III and IV (Fig. 4B, closed triangles). Mitotic cells lacked microtubules altogether (Fig. 5E, arrows), or contained very small star-shaped aggregates of short microtubules (Fig. 5E, asterisks). Short microtubules remained in some interphase cells (Fig. 5F). Concomitantly, the microtubule mass measured in isolated cytoskeletons was reduced by 80% as compared with controls (Fig. 4A, squares). At podophyllotoxin concentrations greater than or equal to 100 nM, no microtubules were present in interphase or mitotic cells (Fig. 4A, closed squares; Fig. 5G), and all mitotic figures were type IV (Fig. 5G,g).

The centrosomal material was fragmented to some degree at 10 nM podophyllotoxin and it was markedly fragmented at 18 nM podophyllotoxin (Fig. 5d). However, the centrosomal material was compact (data not shown) at high podophyllotoxin concentrations (≥ 100 nM), which induced total microtubule depolymerization (Fig. 4A and Fig. 5G).

Effects of nocodazole on mitosis

The effects of very low concentrations of nocodazole on mitotic arrest and spindle organization were similar to the effects of vinblastine in several significant ways.

Mitotic arrest and microtubule polymer mass

Like incubation of cells with vinblastine and podophyllotoxin, incubation of cells with nocodazole induced metaphase accumulation. A total of 50% of the cells had accumulated in metaphase ($K_{met}$) at 54 nM nocodazole (Fig. 6A, circles; Table 1), and maximal accumulation occurred at 100 nM nocodazole. Unlike vinblastine and podophyllotoxin, the percentage ac-
cumulation did not diminish at high concentrations of nocodazole (Fig. 6A; compare with Figs 1A, 4A). Like vinblastine and podophyllotoxin, incubation with nocodazole resulted in a concentration-dependent decrease in the ratio of cells in anaphase to cells in metaphase, with 50% decrease in the ratio \( K_{an/m} \) occurring at 12 nM nocodazole (Table 1), and a ratio of zero at 100 nM nocodazole. Half-maximal inhibition of cell division \( K_{div} \) occurred at 22 nM nocodazole (Table 1).

At concentrations of nocodazole (54 nM) that induced 50% accumulation of cells in mitosis, there was little or no detectable microtubule depolymerization (Table 1, Fig. 6A). Some microtubule depolymerization was associated with high levels of metaphase arrest, but the degree of depolymerization was slight compared with depolymerization associated with metaphase arrest induced by podophyllotoxin (Fig. 4A). For example, the microtubule polymer level was reduced by only 30% at 100 nM nocodazole, a concentration that induced maximal mitotic accumulation. By comparison, maximal metaphase accumulation with podophyllotoxin was accompanied by 100% loss of polymer (Fig. 4A). An 11-fold higher concentration of nocodazole was required to depolymerize half of the microtubule polymer than was required to induce 50% accumulation of cells at metaphase \( K_{dep}/K_{met} \) (Table 1).

**Spindle organization**

Between 3 nM and 100 nM nocodazole, metaphase spindles were frequently types I and II, exhibiting pole-associated chromosomes and lengthened, more numerous astral microtubules than control spindles (Fig. 7A,a,a',B,b,b'). At 33 nM and higher nocodazole concentrations, spindles were frequently type III, consisting of star-shaped aggregates of microtubules, and type IV (Fig. 7B,b,b',C,c,c'). The reorganization of spindle microtubules, chromosomes and centrosomes induced by nocodazole was qualitatively similar to that induced by incubation with vinblastine and podophyllotoxin (see Figs 3 and 5).

The organization of a typical type I spindle is clearly visible in Fig. 7A,a,a'. It is evident that the chromosomes that are not included in the metaphase plate are located at the ends of tufts of astral microtubules rather than strictly at the centrosomes. Chromosomes with a polar location were often located at the ends of astral microtubules after incubation of cells with all three drugs, but the relationship is easier to visualize in the microscope than in micrographs. Types I and II spindles occurred with relatively high frequency with nocodazole as compared with the other drugs. A maximum of 39% of all spindles were types I and II with nocodazole (Fig. 6B, open triangles); whereas the maximum induced by podophyllotoxin was 23% (Fig. 4B) and the maximum induced by vinblastine was 17% (Jordan et al. 1991). However, as with vinblastine and podophyllotoxin, some cells arrested in metaphase had normal spindles. For example, after incubation with 33 nM nocodazole, 22% of cells were arrested in metaphase.
nocodazole induced significant microtubule depolymerization. By immunofluorescence microscopy, it was seen that high concentrations of nocodazole (≥ 1 μM) induced nearly complete depolymerization of microtubules. Punctate aggregates of tubulin were scattered throughout the cytoplasm of the cells, and only a few interphase cells contained short microtubule fragments (Fig. 7D). However, some microtubule polymer was present in isolated cytoskeletons (20-40% of controls; Fig. 6A). The presence of measurable cytoskeletal tubulin polymer but the nearly complete absence of stained microtubule polymer in the corresponding microscopic preparations in this concentration range was the only inconsistency noted between the biochemical measurement of polymer mass and impressions of microtubule polymer levels obtained by microscopy. The measurable cytoskeletal tubulin polymer after incubation with nocodazole at concentrations ≥ 1 μM is probably attributable to the tubulin aggregates and microtubule fragments present at these concentrations.

**Discussion**

Motility of cilia and flagella occurs by the interaction of the motor protein dynein with an array of stable microtubules (reviewed by Warner, 1979). Such stable microtubules clearly function as passive supports whose assembly dynamics are not important in motility. Studies from many laboratories have also documented the probable importance of microtubule-associated motor proteins in mitosis (e.g. see Pfarr et al. 1990; Steuer et al. 1990; Hyman and Mitchison, 1991). However, mitotic spindle microtubules are highly dynamic, and it is reasonable to think that the dynamics of the microtubules may be important in one or more aspects of mitosis (e.g. see Saxton et al. 1984; Salmon et al. 1984; Mitchison, 1989).

Many drugs that inhibit mitosis are thought to act by destroying spindle microtubules, and the actions of these drugs at high concentrations appear to result from depletion of microtubule polymer (Malawista et al. 1968; Wilson and Bryan, 1974; De Brabander et al. 1976; Sluder, 1979; Zieve et al. 1980). However, we found that at low concentrations, vinblastine, podophyllotoxin, nocodazole, colchicine and taxol inhibit the exchange of tubulin at microtubule ends in vitro without exerting significant effects on the total mass of microtubule polymer (Jordan and Farrell, 1983; Wilson et al. 1985; Wilson and Farrell, 1986; Jordan and Wilson, 1990; R. Toso, M. A. Jordan and L. Wilson, unpublished results). Podophyllotoxin has been shown to inhibit dynamic instability in microtubule suspensions using radionuclide exchange methodology and electron microscopy (Schilstra et al. 1989). Thus, each of the drugs can inhibit tubulin exchange at microtubule ends in vitro without significantly altering the mass of assembled microtubules. In other words, at low drug concentrations, the association and dissociation rate constants for tubulin addition to one or both microtubule ends are suppressed in ways that stabilize microtubule dynamics but do not alter the equilibrium between microtubule polymer and soluble tubulin. It is likely that the powerful capacity of vinblastine and nocodazole to inhibit microtubule dynamics and stabilize microtubule ends in vitro and a similar alteration in vivo is responsible for their ability to block mitosis. With podophyllotoxin, inhibition of microtubule dynamics in concert with net microtubule depolymerization appear to be responsible for mitotic block.

**Effects of vinblastine, podophyllotoxin and nocodazole on mitosis**

**Effects on spindle organization**

Metaphase accumulation by vinblastine, podophyllo-
toxin and nocodazole was associated with concentration-dependent rearrangements in the organization of spindle microtubules and chromosomes that were similar for all three drugs. At the lowest effective concentrations of all the drugs, many blocked spindles were indistinguishable from normal (control) metaphase spindles (Figs 4B, 6B). The mildest perturbations of spindle organization in spindles that showed abnormalities were increasing numbers and lengths of astral microtubules, decreasing lengths of the central spindles, the presence of one or a few chromosomes located near the spindle poles, and fragmentation of centrosomal material (Figs 1B, 3, 4C, 5, 6C, 7) (types I and II).

Types I and II abnormal spindles appeared to result from a redistribution of microtubule polymer from the central spindle to the asters. The distance between the poles decreased, and the number and length of astral microtubules increased. Thus, the interpolar and kinetochore microtubules must have shortened, while the astral microtubules got longer. Such length changes could occur by a common action of the three drugs on a mechanism that controls the dynamics of tubulin addition and loss at the ends of the microtubules. For example, prevention of catastrophic depolymerization of astral microtubules could result in increased numbers and increased lengths of astral microtubules.

One of the most sensitive effects of the three drugs was on congression of chromosomes to form the metaphase plate. At the lowest effective concentrations of the three drugs, most chromosomes congressed normally, while in some cells one or a few chromosomes appeared unable to congress. With increasing drug concentrations, increasing numbers of chromosomes were found at or near the spindle poles. Thus, the correct attachment of microtubules to both kinetochores of a chromatid pair must be highly sensitive to the drugs. During prometaphase, the astral microtubules repeatedly grow and shorten, apparently probing the cytoplasm until kinetochore attachment is achieved (Rieder et al. 1990; Hayden et al. 1990). One can envision that interfering with growing and shortening of the astral microtubules in prometaphase might result in the inability of the microtubules from one pole to reach the kinetochore of a chromosome located near the opposite pole. Alternatively, the attachment of the microtubule to the kinetochore may be blocked by the presence of the drug at the end of the microtubule.

Low concentrations of vinblastine, podophyllotoxin and nocodazole produced high proportions (16-39%) of spindles that were bipolar but contained imperfectly congressed chromosomes (types I and II). This observation raises the question of whether a cell that eventually overcomes mitotic block (either during incubation with drug or after drug removal) will undergo accurate chromosome segregation or not. Little information appears to exist concerning induction of chromosome nondisjunction by these three drugs. However, other drugs (Colcemid, diethylstilbestrol) that affect microtubule polymerization dynamics have been found to cause aneuploidy (Kato and Yosida, 1970; Sharp and Parry, 1985; Wheeler et al., 1986). The prevalence of abnormal bipolar spindles (types I and II) observed with vinblastine, podophyllotoxin, nocodazole (Figs 4B, 6B), other vinca alkaloids (Jordan et al. 1991), and colchicine and taxol (M. A. Jordan, D. Thrower and L. Wilson, unpublished results), suggests that any drug that affects microtubule dynamics may have the capacity to induce aneuploidy. Nicklas (1985) proposed, in words that presage the observations reported here, that "a little explored but perhaps very important source of aneuploidy stems from the delicate balance that must be struck between a spindle that is too stable for reorientation to occur and one that is so unstable that reorientation continues indefinitely".

At higher levels of metaphase accumulation with all three drugs, the proportions of normal and type I spindles decreased and types II and III became more prevalent. Type III spindles were not bipolar. There was no distinguishable central plate of chromosomes; rather the chromosomes were arranged in a ball enclosing one or more star-shaped aggregates of microtubules. In type III spindles there was no separation of centrosomal material into two distinct masses. Rather the centrosome consisted of multiple fragments that were generally located at the foci of the microtubule array(s). The mechanism by which type III monopolar spindles form may involve inhibition of spindle pole separation during prophase. By whatever mechanism they form, type III spindles appear to represent an extreme in drug concentration-dependent redistribution of microtubule polymer from the central spindle to the asters.

Sufficiently high concentrations of all three drugs caused microtubule depolymerization as determined by ELISA (discussed below). The loss of microtubule polymer in spindles was also clearly evident by immunofluorescence microscopy (e.g. Figs 3F, 5G). At sufficiently high concentrations of all three drugs, no microtubules were visible. Depolymerization resulted in the appearance of a type IV abnormal mitotic figure consisting of a ball of condensed chromosomes and no microtubules; centrosomes were compact and located apparently randomly. Types III and IV spindles are typical of the c-mitotic figures described by Levan (1938) and Ostergren (1944) in their early studies on colchicine.

Effects on the mass of microtubule polymer

All three drugs induced accumulation of cells in a metaphase-like stage of mitosis. However, there was no clear relationship between the extent of mitotic accumulation and microtubule depolymerization. For example, upon incubation of cells with concentrations of vinblastine or nocodazole that induced 50% accumulation of cells in metaphase, there was little or no detectable microtubule depolymerization (Figs 1A, 6A). Thus, vinblastine and nocodazole appear to block mitotic spindle function without changing the amount of microtubule polymer. In contrast, metaphase accumulation with podophyllotoxin was accompanied by depolymerization of microtubules. For example, at the

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podophyllotoxin concentration that induced 50% accumulation of cells at metaphase (30 nM), the drug induced an 85% loss of microtubule polymer (Fig. 4A).

A possible error in the foregoing analysis may arise by comparing the microtubule polymer mass in cultures consisting predominantly of mitotic cells after incubation with drugs with the microtubule mass in control cultures that consist predominantly of interphase cells. It has not been previously determined whether mitotic cells have an inherently greater mass of microtubules than interphase cells. In the present study, we compared the microtubule content of interphase cells with the microtubule content of a population of cells that was enriched in mitotic stages but that had not been incubated with any drug that induced metaphase block. We measured isolated cytoskeletons from HeLa cells that were synchronized in mitosis using a double thymidine block (Rao and Engelberg, 1966) followed by mitotic shake-off (Robbins and Scharf, 1966). The mean mitotic index of the synchronized populations at the time of cytoskeletal isolation was 45%, whereas the mitotic index of the control populations was 2-3%. Tubulin in polymer form comprised 0.79 (± 0.24)% of the total protein in the synchronized mitotic populations (n=5) compared with 1.03 (± 0.04)% for control populations (n=117). In addition, Y. Zhai and G.G. Borisy (University of Wisconsin, Madison, WI) measured the mass of microtubule polymer in individual LLC-PK cells after microinjection of fluorescent tubulin and found that, at 37°C, mitotic cells contained 90% ± 5% of the microtubule polymer mass of interphase cells (personal communication). Thus, the mass of microtubule polymer does not appear to increase significantly, if at all, during mitosis.

In addition, two other lines of evidence suggest that any mitosis-associated increase in microtubule polymer mass must be small or nonexistent. First, Bulinski et al. (1980) found that the total tubulin concentration in synchronized HeLa cells in mitosis was the same as the total tubulin concentration in a mixed cell population. We found, after incubation with vinca alkaloids, podophyllotoxin and nocodazole over the range of concentrations used in this study, that the soluble (unpolymerized) tubulin concentration remained constant as the concentration of tubulin in polymer form was altered (Jordan et al. 1991, and unpublished data). In other words, the soluble tubulin concentration remained constant whether the cells were predominantly in mitosis or whether they were predominantly in interphase. Thus, mitotic cells do not have a significantly higher fraction of tubulin in polymer form than interphase cells, and from the results of Bulinski et al. (1980) we know that mitotic cells do not have more total tubulin than interphase cells.

Second, incubation of cells with the drug taxol enhances microtubule polymerization (Schiff et al. 1979; Schiff and Horwitz, 1980). However, in a separate study, we found that taxol at low concentrations can induce approximately 33% mitotic accumulation in HeLa cells without increasing the microtubule polymer mass as determined by ELISA of tubulin in isolated cytoskeletons (M. A. Jordan, D. Thrower and L. Wilson, unpublished experiments). If mitotic cells contained more microtubule polymer than interphase cells, one would expect to measure higher polymer levels in such a population of cells blocked in mitosis by taxol compared with control cells that were predominantly in interphase.

Another possible error in the analysis of the relationship between mitotic accumulation and microtubule polymer mass with vinblastine, podophyllotoxin and nocodazole could arise if vinblastine or nocodazole induced the formation of a non-microtubule tubulin polymer. However, only trace amounts of small aggregates of tubulin were observed after incubation of cells with 6.4 nM vinblastine (Fig. 3D) or 100 nM nocodazole (data not shown). These concentrations were higher than the concentrations of vinblastine and nocodazole that induced mitotic accumulation (Figs IA, 6A). In addition, no paracrystals or macrotubules (Bensch and Malawista, 1969) were found after incubation of HeLa cells with 2 nM or 10 nM vinblastine, as determined by electron microscopy (K. Wendell, L. Wilson and M. A. Jordan, unpublished data). After incubation with 10 nM vinblastine the mean diameter of microtubules was 24.9 nm. Some close alignment of microtubules was observed by electron microscopy; this may account for the thick appearance of the microtubule stain by immunofluorescence microscopy (Fig. 3D).

**Effects on the distance between spindle poles**

The concentration-dependent decrease in pole-to-pole distance that occurred with vinblastine, podophyllotoxin and nocodazole (Figs 1B, 4C, 6C) indicates that all three drugs diminish the forces holding the two poles apart. Suppression of dynamic instability or treadmilling of kinetochore microtubules (Mitchison et al. 1986; Hamaguchi et al. 1987; Mitchison, 1989) or steric hindrance of kinetochore/microtubule attachment might lead to a disruption of the balance of forces in the spindle and a resultant shortening of the spindle (for a discussion of these forces, see Nicklas, 1988). It is conceivable that the poleward forces inherent in treadmilling or fluxing microtubules that are continually adding subunits at their (+) ends (kinetochore and/or interpolar microtubules) are instrumental in maintaining centrosomal separation. In support of the idea that treadmilling of kinetochore microtubules is responsible for pole-to-pole separation, Sawin and Mitchison (1991a,b) induced formation of asymmetric half-spindles in vitro composed of treadmilling or fluxing microtubules that extended between a centrosome and a mass of chromatin. Thus, interactions among the components of a single half-spindle are sufficient to maintain chromosome/centrosome separation and, therefore, in a whole spindle, centrosome/centrosome separation. Perhaps vinblastine, podophyllotoxin and nocodazole reduce the pole-to-pole distance by inhibiting the treadmilling dynamics that may be responsible for maintenance of the separation between the poles.
Effects on centrosomal organization

The mitotic centrosome appears to play a significant role in mitosis. One component (p34\textsuperscript{cdk 2} protein kinase) of the mitotic control factor MPF is localized in centrosomes of mitotic HeLa cells (Bailly et al. 1989). Centrosomes nucleate microtubules (Kuriyama and Borisy, 1981), and a cyclical phosphorylation-and-dephosphorylation of centrosomal proteins parallels the increase in microtubule nucleating activity of centrosomes early in mitosis and the subsequent decrease in nucleating activity at anaphase (Robbins et al. 1968; Kuriyama and Borisy, 1981; Vandré and Borisy, 1989). Using high concentrations of several antimitotic drugs Kung et al. (1990) and Alfa et al. (1990) obtained evidence suggesting that intact microtubules play an essential role in the activation and/or inactivation of mitotic control proteins.

Sellitto and Kuriyama (1988) found that pericentriolar material was dispersed after treatment of CHO cells with the antimitotic drug colcemid, but not after treatment with high concentrations of nocodazole (>330 nM). In agreement with their results, we found that centrosomes of HeLa cells were compact after incubation with nocodazole at concentrations greater than 330 nM (Fig. 7c'). However, we found that, at lower concentrations of nocodazole, as well as with vinblastine and podophyllotoxin, the organization of the centrosomal material was altered in ways that were similar for all three drugs. Specifically, centrosomal material was fragmented by concentrations of the three drugs that affected the organization of spindle microtubules but did not totally destroy the microtubules. Fragmented centrosomal aggregates were always located at the foci of microtubule arrays (Figs 3C,c',D,d', 5D,d, 7A,a',B,b'). Curiously, at drug concentrations that induced complete microtubule depolymerization, the centrosomal material of mitotic cells was compact, and the centrosomes of interphase cells were compact at all concentrations of the three drugs. These results support the idea that organization of the mitotic spindle microtubules by centrosomes is not simply a one-way street, but that intact microtubules can alter the organization of the centrosome. In addition, they suggest that microtubule dynamics may also exert an important role in regulating the structure, and perhaps the function, of mitotic centrosomes.

Cell cycle

We have shown that vinblastine, podophyllotoxin and nocodazole can arrest the cell cycle at metaphase in cells having apparently correctly assembled spindles. As the drug concentration is increased, the organization of arrested metaphase spindles is altered in qualitatively the same way with all three drugs. With vinblastine and nocodazole, metaphase arrest occurs in the presence of a normal complement of microtubule polymer. The metaphase/anaphase transition appears to be selectively sensitive to drug effects on microtubule-dependent functions. Microtubule-dependent processes required for progression of cells through interphase into mitosis were not inhibited by drug concentrations that induced blockage of mitosis at the metaphase/anaphase transition. Our results support the idea that not simply microtubules, but dynamic microtubules, are in some way crucial to mitosis. Spindle microtubules are known to be considerably more dynamic than microtubules in interphase cells (Saxton et al. 1984; Salmon et al. 1984). Murray and Kirschner (1989) postulated that a correctly assembled spindle may be required for the degradation of cyclin and the exit from mitosis. It seems reasonable to think that the increased dynamics of spindle microtubules may be critical to the correct assembly of the spindle at a level that is not detectable by light microscopy, or they may be critical for signalling the transition from metaphase to anaphase.

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