Carbendazim Inhibits Cancer Cell Proliferation by Suppressing Microtubule Dynamics

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ABSTRACT

Carbendazim (methyl 2-benzimidazolecarbamate) is widely used as a systemic fungicide in human food production and appears to act on fungal tubulin. However, it also inhibits proliferation of human cancer cells, including drug- and multidrug-resistant and p53-deficient cell lines. Because of its promising preclinical antitumor activity, it has undergone phase I clinical trials and is under further clinical development. Although it weakly inhibits polymerization of brain microtubules and induces G₂/M arrest in tumor cells, its mechanism of action in human cells has not been fully elucidated. We examined its mechanism of action in MCF7 human breast cancer cells and found that it inhibits proliferation (IC₅₀, 10 μ M) and half-maximally arrests mitosis at a similar concentration (8 μ M), in concert with suppression of microtubule dynamic instability without appreciable microtubule depolymerization. It induces

mitotic spindle abnormalities and reduces the metaphase inter-centromere distance of sister chromatids, indicating reduction of tension on kinetochores, thus leading to metaphase arrest. With microtubules assembled *in vitro* from pure tubulin, carbendazim also suppresses dynamic instability, reducing the dynamicity by 50% at 10 μ M, with only minimal (21%) reduction of polymer mass. Carbendazim binds to mammalian tubulin (K_d , 42.8 $\pm$ 4.0 μ M). Unlike some benzimidazoles that bind to the colchicine site in tubulin, carbendazim neither competes with colchicine nor competes with vinblastine for binding to brain tubulin. Thus, carbendazim binds to an as yet unidentified site in tubulin and inhibits tumor cell proliferation by suppressing the growing and shortening phases of microtubule dynamic instability, thus inducing mitotic arrest.

Carbendazim (methyl 2-benzimidazolecarbamate) (Fig. 1, inset) is a systemic fungicide that is used globally in production of cereals, oils, fruits, vegetables, and ornamentals, thus leading to varying levels of carbendazim on human food products. Carbendazim also inhibits proliferation of mammalian tumor cells. Specifically, it exhibits broad antiproliferative activity against both drug- and multidrug-resistant and p53-deficient cell lines including murine melanoma, human breast, ovarian, lung, leukemia, and colon carcinoma cell lines, arresting the cells at the G₂/M phase of the cell cycle and inducing apoptosis (Hammond et al., 2001). It is also active *in vivo* against human pancreatic, lung, prostate, colon, and breast tumor xenografts (Hao et al., 2002). Because of its promising preclinical antitumor activity, it has undergone phase I clinical trials and is under further clinical development for treatment of cancer. However, its mechanism of action has not been clearly elucidated.

Tubulin seems to be the primary target in fungal cells for carbendazim (Davidse, 1986). In addition, carbendazim weakly inhibits polymerization of mammalian tubulin into microtubules (Friedman and Platzter, 1978; Lacey and Watson, 1985). Microtubules are essential components of the cytoskeleton whose dynamic behaviors are critical for cell cycle progress. They display two types of dynamic behavior, treadmilling and dynamic instability. Treadmilling is the net addition of tubulin at one microtubule end and the net loss of tubulin at the other end (Panda et al., 1999). The second dynamic behavior, dynamic instability, is the stochastic switching between growing and shortening phases at microtubule ends (Mitchison and Kirschner, 1984). The rapid dynamics of mitotic spindle microtubules are important for the proper and time-sensitive attachment of chromosomes to the spindle, congression of the chromosomes to the metaphase plate, and induction of tension on kinetochores, all of which are essential for passage through the metaphase/anaphase spindle checkpoint. Suppression of spindle microtubule dynamics by drugs such as vinca alkaloids and taxanes leads to arrest of cell cycle at mitosis and often to cell death (Jordan and Wilson, 1998).

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ABBREVIATIONS: ANS, 8-anilino-naphthalene-1-sulfonic acid; DMSO, dimethyl sulfoxide; PIPES, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; CENP-B-GFP, centromere-binding protein B-GFP; DAPI, 4,6-diamidino-2-phenylindole; MAP, microtubule-associated protein.

Fig. 1. Inhibition of MCF7/GFP cell proliferation (●) and induction of mitotic index (bars) by carbendazim. Cells were seeded in six-well plates for 24 h, followed by incubation with 0.5 to 50 μ M carbendazim for 48 h. Live cells were counted by hemacytometer after staining with trypan blue for estimating cell proliferation. Mitotic cells were determined after fixation and staining with DAPI and phosphohistone H3 antibody. Results are mean and S.E.M. of three experiments. Inset, structure of carbendazim.

Microtubule-targeted drugs can bind to several domains in tubulin, including the vinca domain, the colchicine site, and the taxane site (Hamel, 1996). However, the location of the benzimidazole binding site in tubulin is somewhat controversial. Competition studies have indicated that some benzimidazoles, other than carbendazim, bind to mammalian tubulin at the colchicine binding site (Friedman and Platzer, 1978), and colchicine competitively inhibits the binding of carbendazim to fungal tubulin (Davidse and Flach, 1977). However, benomyl, the parent compound of carbendazim, seems to bind a novel site in mammalian tubulin (Gupta et al., 2004). In addition, tubulin mutations that confer benzimidazole sensitivity have suggested a novel binding site for benzimidazoles (Downing, 2000).

In the present study, we found that carbendazim inhibited proliferation of MCF7 breast cancer cells in concert with mitotic arrest without appreciably depolymerizing microtubules but with strong suppression of microtubule dynamic instability. Mitotic arrest was associated with distinct spindle abnormalities and with reduction of tension on chromosome kinetochores. A direct action on microtubules was confirmed by observations of similar suppression of dynamic instability with purified reassembled mammalian microtubules, also in the absence of significant microtubule depolymerization. We also found that, like benomyl, carbendazim does not bind at the colchicine or vinblastine binding sites of mammalian tubulin. These results indicate that the primary antitumor mechanism of action of carbendazim is suppression of spindle microtubule dynamics, leading to mitotic arrest and ultimately to cell death.

Materials and Methods

Materials. Carbendazim (Pestanal), 8-anilinoanthracene-1-sulfonic acid (ANS), vinblastine sulfate, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Bio-gel P-10 gel was from Bio-Rad (Hercules, CA), fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA), fibronectin was from Invitrogen (Carlsbad, CA), [3 H]vinblastine sulfate was from Moravak Biochemicals (Brea, CA), [methoxy- 3 H]colchicine from PerkinElmer Life and Analytical Sciences (Waltham, MA), and Ready Protein liquid scintillation cocktail was from Beckman

Coulter (Fullerton, CA). Carbendazim (8 mM) was dissolved in DMSO, stored at 25°C, and all assays were performed with appropriate DMSO vehicle controls. By examining the increase in the fluorescence (excitation and emission wavelengths were 281 and 307 nm, respectively) of carbendazim solutions after centrifugation (clinical centrifuge, 3000 rpm), we determined the solubility limit of carbendazim to be 70 to 80 μ M under the conditions of our experiments (50 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 1% DMSO, 25°C, data not shown).

Cell Culture. Human MCF7 breast cancer cells stably transfected with green fluorescent protein- α -tubulin (Clontech, Mountain View, CA) (MCF7/GFP cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.1% penicillin/streptomycin, and nonessential amino acids in a humidified incubator (37°C, 5% CO_2) (Jordan et al., 2005). Doubling time was approximately 29 h. For measuring sister kinetochore separation, human osteosarcoma (U-2 OS) cells transfected with centromere-binding protein B-GFP (CENP-B-GFP) (Okounneva et al., 2003) were maintained similarly.

Proliferation and Mitotic Arrest. MCF7/GFP cells seeded at 3×10^4 cells/ml in six-well plates were allowed to adhere (24 h), after which the medium was replaced with medium containing carbendazim. Cells were incubated with a range of carbendazim concentrations (0.5–50 μ M) for 24 h, rinsed with Versene (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , 0.5 mM EDTA, pH 7.2), and detached with trypsin in Versene. Both adherent and floating cells were collected. For determination of cell proliferation, cells were stained with trypan blue and counted by hemacytometer. IC_{50} was determined as the concentration at which $100 \times (T - T_0)/(C - T_0) = 50$, where T = final cell number, C = final control cell number, and T_0 = cell number at time 0. Results are mean and S.E.M. of three independent experiments.

For determination of mitotic index, cells on coverslips were fixed in 10% formalin for 20 min (25°C) followed by methanol (4°C) for 10 min. Cells were immunostained with phosphohistone H3 antibody (Alexa Fluor 488 conjugate) (Cell Signaling Technology Inc., Danvers, MA) and mounted with Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (for staining chromosomes and chromatin) (Invitrogen). Cells were examined using an inverted fluorescence Eclipse E800 microscope (Nikon, Melville, NY). Results are mean and S.E.M. of three experiments in which 500 cells were counted for each drug concentration.

Immunofluorescence Microscopy. MCF7/GFP cells were grown on poly-L-lysine-coated coverslips (to enhance cell attachment). After 24 h, cells were incubated with carbendazim for 48 h and fixed as described above. Cells were incubated with 10% normal goat serum and then with a mouse anti- α -tubulin monoclonal antibody (Sigma-Aldrich) overnight at 4°C. After washing, cells were stained with fluorescein isothiocyanate-conjugated goat antimouse IgG antibody (Sigma-Aldrich) (1 h, 25°C) and DAPI. Stained cells were mounted and examined using an inverted fluorescence Eclipse E800 microscope, fitted with 60 $\times$ and 100 $\times$ (numerical aperture 1.4 for both) objectives and an Orca II CCD (Hamamatsu Corporation, Bridgewater, NJ) camera supported by Metamorph (version 4.6) imaging software (Molecular Devices, Sunnyvale, CA).

Analysis of Microtubule Dynamic Instability in Living Interphase Cells. The dynamic instability behavior of individual microtubules was studied in the thin peripheral area of living interphase cells as described previously (Kamath et al., 2006). MCF7/GFP cells were grown for 48 h on glass coverslips. After incubation with carbendazim (4 h, 5–30 μ M) or appropriate DMSO-containing controls, cells were transferred to recording medium. Images were recorded after 15 min for a maximum of 2 h after sealing coverslips in a double-coverslipped chamber ($36 \pm 1^\circ C$). The position of the plus ends of microtubules over time was tracked and graphed as microtubule length versus time (life history plots) in ImageJ software (National Institutes of Health, Bethesda, MD), and the parameters of microtubule dynamic instability were determined. Changes of

>0.5 μm between two points were considered growth or shortening events. Changes in length of <0.5 μm were considered as periods of attenuation or pause. The transition frequency at which microtubules switched from phase of growth or attenuation to shortening (catastrophe) and shortening to growth or attenuation (rescue) were determined as described previously (Kamath et al., 2006). Dynamicity is the total rate of measurable tubulin exchange at microtubule ends (attributable to growing and shortening) (Kamath et al., 2006). Results are from 30 to 70 individual microtubules from 8 to 15 cells for each condition.

Measurement of Sister Kinetochore Separation. U-2 OS cells transfected with a CENP-B-GFP (Okouneva et al., 2003) were grown on coverslips for 24 h, followed by incubation with carbendazim (5–30 μM , 24 h), then fixed and examined as described above. Images were acquired as stacks of 1 μm slices through the spindle. Measurement was done using ImageJ (National Institutes of Health), and data are mean and S.E.M. of between 102 and 227 pairs for each condition.

Purification and Polymerization of Tubulin. Microtubule protein consisting of approximately 70% tubulin and approximately 30% microtubule-associated proteins (MAPs) was isolated from bovine brain by three cycles of polymerization and depolymerization. MAP-free tubulin (>99%) was purified from the microtubule protein by phosphocellulose chromatography, drop-frozen in liquid nitrogen, and stored at -70°C (Panda et al., 1999). Protein was determined by Bradford assay (Bradford, 1976) using bovine serum albumin as the standard.

Tubulin (23 μM) was mixed with a range of carbendazim concentrations (10–50 μM) in 100 mM PIPES, 1 mM MgCl_2 , 1 mM EGTA, and 1 mM GTP. Polymerization was initiated by adding microtubule-nucleating seeds (Jordan et al., 2005) to the tubulin solution (1:10 dilution, seeds/tubulin) and was monitored at 30°C for 60 min by turbidimetry at 350 nm using a Beckman DU 640 Spectrophotometer.

Determination of Microtubule Dynamic Instability Parameters in Vitro. The dynamic instability behavior at plus ends of individual purified steady-state microtubules was studied by video-enhanced differential contrast microscopy as described previously (Kamath et al., 2006). In brief, tubulin (14 μM) was mixed with sea urchin axonemal seeds and polymerized to steady state (30 min, 30°C) in 87 mM PIPES, 36 mM MES, 1.4 mM MgCl_2 , 1 mM EDTA, pH 6.8, and 2 mM GTP, in the absence or presence of carbendazim (5–30 μM) (<0.5% DMSO). Microtubules were recorded for a maximum of 45 min after reaching steady state. The positions of microtubule plus ends were tracked and analyzed as described previously (Kamath et al., 2006). With purified microtubules, we considered a microtubule to be growing if its length increased >0.3 μm at a rate >0.3 $\mu\text{m}/\text{min}$. Shortening events were identified by a length change >1 μm at a rate of >2 $\mu\text{m}/\text{min}$. Microtubules that changed <0.3 $\mu\text{m}/\text{min}$ over a duration of four data points were considered to be in an attenuated or paused state, neither growing nor shortening detectably. Thirty microtubules were analyzed for each experimental condition.

Carbendazim Binding Affinity for Tubulin. Tubulin (2 μM) was incubated with carbendazim (10–80 μM) (1% DMSO) in 25 mM PIPES, 3 mM MgSO_4 , and 1 mM EGTA at pH 6.8, 25°C for 30 min. The fluorescent hydrophobic probe, ANS (40 μM), was added, and incubation continued for an additional 15 min (Bhattacharyya and Wolff, 1975; Gupta et al., 2004). Fluorescence measurements were performed using a PerkinElmer LS 50B Fluorescence Spectrometer and a 0.3-cm path length cuvette. The excitation and emission wavelengths were 400 and 470 nm, respectively. Spectra were taken by multiple scans, and buffer blank values were subtracted from all measurements. Absorbance measurements for inner filter effects were performed in a Beckman DU 640 Spectrophotometer using a 1-cm path length cuvette. Corrections for the inner filter effects were made as described previously (Gupta et al., 2004). The fraction of the binding sites (X) occupied by carbendazim was determined from the

equation $X = (F - F_o)/F_{\text{max}}$, where F_o is the fluorescence intensity of tubulin-ANS in the absence of carbendazim, F is the corrected fluorescence intensity of tubulin-ANS in the presence of carbendazim, and F_{max} was calculated from the plot of $1/(F - F_o)$ versus $1/[\text{carbendazim}]$. The dissociation constant (K_d) was determined using the relationship, $1/X = 1 + K_d/L_f$, where L_f is the free carbendazim concentration, and $L_f = C - X[Y]$, where C is total concentration of carbendazim, and $[Y]$ is the molar concentration of ligand-binding sites assuming a single binding site per tubulin dimer. Results are mean and S.E.M. for four experiments.

Assay for Colchicine Binding Competition. Tubulin was incubated with carbendazim under two different experimental conditions. Bovine brain tubulin (9 μM) was incubated simultaneously with [methoxy- ^3H]colchicine (20 μM , final specific activity, 7.8 mCi/mmol) and carbendazim (10–80 μM) (1.6% DMSO, final concentration) for 16 h, or the tubulin was first incubated with carbendazim (10–80 μM) for 2 h followed by the addition of [^3H]colchicine (5 μM) for 1 h. Binding reactions were carried out in 50 mM PIPES, 1 mM MgCl_2 , and 1 mM EGTA, containing 2.5 μM vinblastine sulfate to stabilize colchicine binding activity. The tubulin-colchicine complex was separated from free colchicine by gel filtration (Pfeffer et al., 1976) on $1.5 \times 18\text{-cm}$ columns of Bio-Gel P10. Radioactivity was determined by scintillation counting.

Assay for Vinblastine Binding Competition. Bovine brain tubulin (5 μM) was preincubated with carbendazim (10–40 μM) (0.8% DMSO, final concentration) for 30 min, followed by incubation with [^3H]vinblastine sulfate (5 μM , final specific activity, 21 mCi/mmol) for 30 min. Binding reactions were carried out in 50 mM PIPES, 1 mM MgCl_2 , and 1 mM EGTA at 24°C . Bound vinblastine was separated by use of an MPS micropartition device with YMT membrane (Centrifree; Millipore, Billerica, MA) centrifuged at 4000 rpm for 5 min at 24°C (Jordan et al., 1998). The concentration of unbound vinblastine was determined by measuring radioactivity in the centrifugate, and bound vinblastine was determined by subtracting unbound vinblastine from total vinblastine. Background radioactivity measured in the absence of tubulin was subtracted from the experimental values. Results are mean and S.E.M. for two experiments.

Results

Carbendazim Arrested Mitosis in MCF7/GFP Cells in Concert with Inhibition of Proliferation. MCF7/GFP cells were incubated in the presence or absence of carbendazim (0.5–50 μM , 24 h) and the increase in live cell number was determined (*Materials and Methods*). In addition, to determine specifically the percentage of cells arrested in mitosis, we collected both floating and attached cells, fixed and stained them with histone H3 antibody and DAPI, and counted mitotic and interphase cells in a large fields of cells (*Materials and Methods*). As shown in Fig. 1, carbendazim inhibited cell proliferation and arrested mitosis in concentration-dependent manners. The IC_{50} for proliferation was 10 μM , and half-maximal mitotic accumulation occurred at a similar concentration, 8 μM . Inhibition of cell proliferation was also determined using a sulforhodamine B assay and yielded similar results (IC_{50} , 8 μM , data not shown). In addition to an increase in mitotic cells, carbendazim also induced multinucleation of interphase cells, most probably as a downstream consequence of arrested mitosis. Between 70 and 75% of the cells were in either a mitotic or a multinucleated interphase stage after incubation with 20 to 50 μM carbendazim (data not shown). Increases in multinucleated cells and induction of apoptosis (Hammond et al., 2001) probably account for the increased inhibition of proliferation observed at 20 to 50 μM carbendazim (Fig. 1).

Effects of Carbendazim on Cell Morphology, Microtubule Organization, and Polymer Mass. The effects of carbendazim on microtubule organization in interphase and mitotic cells are shown in Fig. 2, A to J. Control cells in interphase had normal fine filamentous arrays of microtubules (Fig. 2A), and mitotic cells had well organized bipolar spindles with compact metaphase plates of condensed chromosomes (Fig. 2B). At low concentrations of carbendazim (below the IC_{50} of 8 μ M), most spindles were normal, but some were abnormal bipolar spindles with uncongressed chromosomes and longer, more prominent astral microtubules (Fig. 2, C and D). Some bipolar spindles were asymmetric, with more microtubules and chromosomes on one side of the spindle (Fig. 2C). At higher concentrations of carbendazim ($2 \times IC_{50}$, 15 μ M), interphase microtubules were less well spread, were pulled back from the cell periphery, and appeared thicker and slightly depolymerized (Fig. 2E). Mostly tripolar spindles and some uncongressed chromosomes occurred in mitotic cells (Fig. 2F). Thirty μ M carbendazim further reduced microtubule polymer mass in interphase cells (Fig. 2G). More than 60% of cells were mitotic, and all spindles were multipolar, with approximately five poles (Fig. 2H). At a concentration of 50 μ M, carbendazim nearly completely depolymerized the microtubule network in interphase cells (Fig. 2I) and induced highly multipolar spindles with many small clusters of chromosomes in mitotic cells (Fig. 2J).

Carbendazim Suppressed Microtubule Dynamic Instability in Living Cells at Concentrations That Arrest Mitosis. The effects of carbendazim on dynamic instability were determined in living interphase MCF7 cells expressing GFP-tubulin as a marker to visualize the microtubules. After incubation of cells with carbendazim for 4 h to allow establishment of an equilibrium intracellular drug concentration, fluorescence time-lapse images of GFP-labeled microtubules in the flat peripheral regions of living cells were captured, and microtubule length changes were measured and analyzed (*Materials and Methods*). Control microtubules alternated between phases of growing and shortening and an attenuated or paused state, neither growing nor shortening detectably. Control microtubules grew at a rate of 9.5 ± 0.4 μ m/min and rapidly shortened at 17.8 ± 1.1 μ m/min (Table 1). At a concentration below the mitotic IC_{50} , carbendazim (5 μ M) predominantly and significantly reduced the growth and shortening lengths [from 1.9 and 3.4 μ m, respectively, in controls to 1.4 and 2.3 μ m, respectively ($p < 0.01$ and $p < 0.001$, respectively)]. Other dynamic instability parameters were strongly suppressed with increasing concentration of the drug. At 15 μ M carbendazim ($2 \times IC_{50}$), the growth rate was reduced by 15%, whereas the shortening rate was decreased by 38%, and starting at this concentration, the dynamicity of the microtubules was significantly reduced (by 41%, $p < 0.001$). At the highest concentration of carbendazim studied (30 μ M), several of the parameters were strongly suppressed. The growth rates and shortening rates were reduced by 26 and 58%, respectively, and the growth and shortening lengths decreased by more than 50%. These reductions in rates and lengths resulted in an overall 66% reduction in microtubule dynamicity (Table 1; Fig. 3). The results indicate that, in cells, suppression of microtubule dynamic instability by carbendazim occurs in concert with mitotic arrest and inhibition of proliferation.

Carbendazim Reduced Tension across Sister Kinetochores at Metaphase of Mitosis. The distance separating sister centromeres in a metaphase spindle is a measure of the tension on kinetochores induced by dynamic microtubules and their associated proteins (Waters et al., 1996; McIntosh et al., 2002; Okouneva et al., 2003). Kinetochore tension is required for passage through the metaphase/anaphase cell cycle checkpoint (Nicklas, 1997). We used U-2 OS cells stably expressing the fluorescent centromere binding protein CENP-B-GFP, a model system we have used previously (Okouneva et al., 2003), to measure the effects of carbendazim on the intercentromere distance. The IC_{50} for U-2 OS cell proliferation was 20 μ M. Carbendazim decreased the intercentromere distance in a concentration-dependent manner from 1.1 ± 0.2 μ m in control metaphase cells to 1.0 ± 0.2 μ m at 5 μ M, to 0.8 ± 0.2 μ m at 15 μ M, and to 0.7 ± 0.1 μ m at 30 μ M carbendazim (a 37% reduction) ($p < 0.001$), indicating that it reduced tension on kinetochores and supporting the conclusion that suppression of the dynamics of mitotic spindle microtubules by carbendazim results in mitotic arrest.

Inhibition of Tubulin Polymerization into Microtubules by Carbendazim. Because carbendazim seemed to act in mammalian cancer cells principally by suppressing microtubule dynamic instability, we also examined its effects on microtubules assembled from purified mammalian tubulin. Using turbidimetry to measure microtubule polymerization, we found that carbendazim did not inhibit assembly of MAP-containing bovine brain tubulin at concentrations as high as 50 μ M (data not shown). However, when MAP-depleted bovine brain tubulin was used, the drug modestly inhibited microtubule polymerization by 21% at 10 μ M, by 22% at 30 μ M, and by 35% at 50 μ M (60 min), with an IC_{50} that was estimated to be approximately 70 μ M (data not shown).

The Suppression of Dynamic Instability by Carbendazim in Vitro Qualitatively and Quantitatively Resembles Its Effects on Microtubule Dynamic Instability in Cells. The effects of carbendazim on dynamic instability at plus ends of microtubules assembled to steady state with purified tubulin (14 μ M) in vitro were measured at concentrations below the IC_{50} for inhibiting microtubule polymerization (*Materials and Methods*). Life history plots of typical changes in microtubule lengths in controls are shown in Fig. 4A, and the effects of 30 μ M carbendazim on the length changes are shown in Fig. 4B. Carbendazim suppressed microtubule dynamic instability in vitro in a similar manner and at similar concentrations as in cells (Table 2; Fig. 4C). A 27% decrease in shortening rate occurred in vitro at the lowest concentration of carbendazim (5 μ M). At 10 μ M, the growth rate and length and the catastrophe frequency were also significantly decreased by 32, 26, and 50%, respectively. In addition, at this concentration, dynamicity was inhibited by 50%. Suppression of the same parameters increased at 30 μ M carbendazim, and there was a pronounced 78% suppression of the dynamicity. Carbendazim also increased the percentage of time that the microtubules spent in an attenuated state while decreasing the percentage of time spent growing and shortening (Fig. 4C). Although no significant reduction in the catastrophe frequency was observed in cells, with reassembled microtubules in vitro, carbendazim reduced the catastrophe frequency by 50% both at 10 and 30 μ M. Thus, all parameters of dynamic instability, except for the catastrophe frequency and the shortening length, were suppressed in a very

Fig. 2. Effects of carbendazim on microtubule spindle organization in MCF7/GFP cells. Cells were fixed and stained with anti- α -tubulin to stain microtubules and DAPI to stain chromosomes and chromatin (*Materials and Methods*). Control interphase cell with normal dense network of microtubules (A), control mitotic cell with well defined compact metaphase plate (B), 5 μ M carbendazim induced asymmetric mitosis (C) and uncongressed chromosomes in mitosis (D), 15 μ M carbendazim induced depolymerization of microtubules in interphase cells (E) and tripolar spindles in mitotic cells (F), 30 μ M carbendazim depolymerized many microtubules in interphase cells (G) and induced multipolar spindles (H), increased depolymerization of microtubule network at 50 μ M carbendazim in interphase cells (I), and highly multipolar spindle (J).

similar fashion in living cells as with purified microtubules. These results indicate that the effects of carbendazim on dynamic instability in cells mechanistically reproduce its effects with highly purified microtubules.

Carbendazim Binds with Moderate Affinity to Mammalian Tubulin. ANS, a hydrophobic probe that strongly fluoresces when adsorbed to certain proteins (Brand and Gohlke, 1972), was used to determine the dissociation constant for carbendazim-tubulin binding. Carbendazim concentration-dependently increased the fluorescence of the tubulin-ANS complex (Fig. 5A). The dissociation constant (K_d) was calculated by linear regression of the binding data assuming a single binding site per tubulin dimer and was found to be 42.8 ± 4.0 μ M (Fig. 5A), indicating that carbendazim has moderate affinity for bovine brain tubulin.

Carbendazim Does Not Competitively Inhibit Colchicine Binding to Tubulin. To determine whether carbendazim competed with colchicine for its binding site on tubulin, we studied the effect of carbendazim on the binding of [3 H]colchicine to tubulin. Colchicine binding is a slow, temperature-dependent process, and although it is non-covalent, it is poorly reversible (Wilson and Bryan, 1974). [3 H]Colchicine (20 μ M) and a range of concentrations of carbendazim were incubated for 16 h with tubulin, and bound colchicine was determined by gel filtration (*Materials and Methods*). Carbendazim had no effect on the stoichiometry of colchicine binding to tubulin (Fig. 5B). The stoichiometry was approximately 0.6 to 0.7, equivalent to that reported earlier (Weisenberg et al., 1968; Wilson and Bryan, 1974). In a second experimental design, to favor or allow enhanced binding of carbendazim to tubulin, we preincubated carbendazim with tubulin before colchicine addition and reduced the [3 H]colchicine concentration (5 μ M), which reduced the stoichiometry of colchicine binding to tubulin to approximately 0.1 to 0.2. However, as the carbendazim concentration was increased, there was no reduction in the binding of [3 H]colchicine to tubulin but rather a very slight increase (Fig. 5B), again indicating the absence of competition between the two drugs for tubulin binding.

Carbendazim Does Not Competitively Inhibit Vinblastine Binding to Tubulin. We used centrifugal ultrafiltration to determine whether carbendazim competes with vinblastine for binding to tubulin. A range of concentrations of carbendazim were preincubated with tubulin, followed by [3 H]vinblastine addition. The unbound vinblastine was separated by centrifugal filtration, and the bound vinblastine was determined as the difference between total and unbound vinblastine. The stoichiometry of vinblastine/tubulin was approximately 0.7. Carbendazim did not inhibit the binding of [3 H]vinblastine to tubulin (Fig. 5C), indicating that it does not compete for the vinblastine site.

Discussion

Carbendazim's Antiproliferative Mechanism in MCF7/GFP Breast Cancer Cells Is Suppression of Microtubule Dynamics. Carbendazim inhibited MCF7 cell proliferation with an IC_{50} of 10 μ M and induced half-maximal mitotic arrest at a similar concentration (8 μ M), indicating that mitotic arrest is its primary antiproliferative action. At concentrations less than the IC_{50} values, carbendazim induced formation of abnormal bipolar and asymmetric spindles with uncongressed chro-

TABLE 1

Suppression of microtubule dynamic instability by carbendazim in living MCF7/GFP cells

Values are expressed as mean $\pm$ S.E.M. Results are from 30 to 70 individual microtubules from 8 to 15 cells for each condition.

	Control	Carbendazim, 5 μ M	Change	Carbendazim, 15 μ M	Change	Carbendazim, 30 μ M	Change
			%		%		%
Growth rate (μ m/min)	9.5 $\pm$ 0.4	9.0 $\pm$ 0.4	-5	8.1 $\pm$ 0.3**	-15	7.0 $\pm$ 0.6***	-26
Growth length (μ m)	1.9 $\pm$ 0.1	1.4 $\pm$ 0.1**	-26	1.6 $\pm$ 0.1	-16	0.8 $\pm$ 0.1***	-58
Shortening rate (μ m/min)	17.8 $\pm$ 1.1	15.8 $\pm$ 1.0	-11	11.1 $\pm$ 0.7***	-38	7.4 $\pm$ 0.7***	-58
Shortening length (μ m)	3.4 $\pm$ 0.3	2.3 $\pm$ 0.2***	-32	1.9 $\pm$ 0.1***	-44	1.0 $\pm$ 0.1***	-71
Catastrophe frequency (per min)	2.5 $\pm$ 0.2	3.1 $\pm$ 0.3	+24	2.3 $\pm$ 0.2	-8	2.2 $\pm$ 0.3	-12
Rescue frequency (per min)	7.3 $\pm$ 0.9	8.3 $\pm$ 0.6	+14%	5.9 $\pm$ 0.5	-19	7.2 $\pm$ 1.0	-1
Dynamicity (μ m/min)	9.5 $\pm$ 0.8	8.9 $\pm$ 0.9	-6	5.6 $\pm$ 0.5***	-41	3.2 $\pm$ 0.6***	-66
Percentage of total time growing	30.5	29.9		28.2		18.7	
Percentage of time shortening	27.2	25.9		25.8		23.2	
Percentage of total time attenuated	42.3	44.2		46.0		58.1	

* Values differ significantly from controls, $p < 0.05$, as determined by Student's t test.** Values differ significantly from controls, $p < 0.01$, as determined by Student's t test.*** Values differ significantly from controls, $p < 0.001$, as determined by Student's t test.

Fig. 3. Concentration-dependent suppression of dynamic instability parameters in living MCF7/GFP cells by carbendazim. Effect of increasing concentrations of carbendazim on the growth rate ($\diamond$), growth length ($\square$), shortening rate ($\triangle$), shortening length ($\blacktriangledown$), and dynamicity ($\bullet$). Data are from Table 1.

mosomes and multinucleate interphase cells. Similar to reports in human granulosa cells (Can and Albertini, 1997), higher carbendazim concentrations induced multipolar and abnormal metaphase spindles and multinucleated interphase cells. Carbendazim suppressed microtubule dynamic instability during interphase, reduced tension across kinetochore pairs during metaphase, and it arrested mitotic progression in a manner indicating that mitotic block is because of suppression of dynamics. Thus, carbendazim joins the microtubule-targeted drugs, including taxanes and vinca alkaloids, that suppress microtubule dynamics in concert with mitotic arrest (Kamath et al., 2006; Jordan and Kamath, 2007). Similar to these drugs, low concentrations of carbendazim induced no substantial microtubule depolymerization in association with mitotic arrest.

Near the mitotic IC_{50} (8 μ M), the most prominent actions of carbendazim on interphase microtubule dynamics were to suppress microtubule growth and shortening rates and lengths, resulting in strong suppression of overall dynamicity. At 15 μ M carbendazim, growth and shortening rates and dynamicity were decreased by 15, 38, and 41%, respectively, whereas at even lower concentrations (5 μ M), the growth and shortening lengths were significantly decreased. Among the drugs that suppress microtubule dynamics in concert with mitotic arrest, carbendazim's actions most closely resemble those of vinblastine. Both drugs inhibit shortening and growth, whereas several other microtubule-depolymerizing drugs, including vinflunine, eribulin, and 2-methoxyestra-

Fig. 4. Concentration-dependent suppression of in vitro dynamic instability of microtubule plus ends by carbendazim. Life history traces of length changes of individual control microtubules (A) and microtubules assembled in the presence of 30 μ M carbendazim (B) show carbendazim's suppressive effect on dynamic instability. C, effect of increasing concentrations of carbendazim on the growth rate ($\diamond$), growth length ($\square$), shortening rate ($\triangle$), and dynamicity ($\bullet$) of reassembled bovine brain microtubules in vitro. Data are from Table 2.

TABLE 2

Suppression of microtubule dynamic instability by carbendazim at plus ends of microtubules assembled to steady state in vitro with purified bovine brain tubulin

Values are expressed as mean $\pm$ S.E.M. Results are from 30 individual microtubules for 10 min for each condition.

	Control	Carbendazim, 5 μ M	Change	Carbendazim, 10 μ M	Change	Carbendazim, 30 μ M	Change
			%		%		%
Growth rate (μ m/min)	3.4 $\pm$ 0.3	3.2 $\pm$ 0.3	-6	2.3 $\pm$ 0.3*	-32	1.5 $\pm$ 0.1***	-56
Growth length (μ m)	1.9 $\pm$ 0.1	1.6 $\pm$ 0.1	-16	1.4 $\pm$ 0.1***	-26	0.9 $\pm$ 0.1***	-53
Shortening rate (μ m/min)	11.0 $\pm$ 0.8	8.0 $\pm$ 0.6***	-27	7.7 $\pm$ 0.8***	-30	4.6 $\pm$ 0.4***	-58
Shortening length (μ m)	1.8 $\pm$ 0.1	2.0 $\pm$ 0.1	+11	1.6 $\pm$ 0.1	-11	1.4 $\pm$ 0.2	-22
Catastrophe frequency (per min)	0.4 $\pm$ 0.1	0.3 $\pm$ 0.0	-25	0.2 $\pm$ 0.0*	-50	0.2 $\pm$ 0.0***	-50
Rescue frequency (per min)	5.6 $\pm$ 0.9	4.6 $\pm$ 1.5	-18	7.1 $\pm$ 1.7	-27	7.7 $\pm$ 1.8	+38
Dynamicity (μ m/min)	1.8 $\pm$ 0.3	1.2 $\pm$ 0.2	-33	0.9 $\pm$ 0.1**	-50	0.4 $\pm$ 0.1***	-78
Percentage of total time growing	40.3	31.6		29.6		21.6	
Percentage of total time shortening	7.3	7.3		5.7		4.1	
Percentage of total time attenuated	52.4	61.0		64.7		74.3	

* Values differ significantly from controls, $p < 0.05$, as determined by Student's t test.

** Values differ significantly from controls, $p < 0.01$, as determined by Student's t test.

*** Values differ significantly from controls, $p < 0.001$, as determined by Student's t test.

diol, do not inhibit shortening (Jordan and Kamath, 2007). Vinblastine potently regulates dynamic instability by binding to microtubule ends (Jordan and Wilson, 2008), and suppression of the shortening rate and extent by carbendazim suggests that it also acts at microtubule ends. If carbendazim acts primarily by binding to microtubule ends, the high concentrations required may be explained by its relatively low tubulin binding affinity.

Loss of Intercentromere Tension Indicates Suppression of Mitotic Spindle Microtubule Dynamics. Sister kinetochores/centromeres are linked by elastic heterochromatin (Shelby et al., 1996) and oscillate between stretching apart and relaxing together under the influence of the attached dynamic microtubules (Skibbens et al., 1993). The tension generated by microtubules and/or motors is necessary for passage through the cell cycle checkpoint (Nicklas, 1997; McIntosh et al., 2002). The intercentromere distance is an indicator of this tension. Inhibition of spindle microtubule dynamics and reduction in spindle tension by vinca alkaloids, eribulin, and paclitaxel (Taxol) lead to mitotic arrest (Kelling et al., 2003; Okounova et al., 2003, 2008). Likewise, carbendazim concentrations that arrested mitosis also reduced the intercentromere distance by 27% at 15 μ M, indicating that suppression of spindle microtubule dynamics contributed to mitotic arrest.

Effects of Carbendazim on Microtubules in Vitro. With purified MAP-depleted mammalian tubulin, carbendazim reduced the microtubule mass in a concentration-dependent manner, with an IC_{50} of approximately 70 μ M. Consistent with previous results (Winder et al., 2001), we found no effect on polymerization of MAP-rich tubulin at ≤ 50 μ M carbendazim, although the IC_{50} of approximately 90 μ M determined by Friedman and Platzer (1978) indicates that carbendazim weakly inhibits MAP-rich tubulin assembly.

Carbendazim strongly suppressed dynamic instability of purified mammalian microtubules in manners and at concentrations very similar to its cellular actions, without significant microtubule depolymerization. The carbendazim concentrations that suppressed dynamic instability (5–30 μ M) were lower than the IC_{50} for inhibition of polymerization (70 μ M). Even at a low concentration of 5 μ M carbendazim, the shortening rate was inhibited significantly, whereas the growth rate and length were reduced at ≥ 10 μ M. Carbendazim did not affect the transition frequencies in cells but decreased the catastrophe frequency in vitro. One possible

explanation for the difference is that cells contain microtubule regulatory proteins that can induce catastrophe (Jordan and Wilson, 2008), possibly overriding the catastrophe-reducing effects of carbendazim. Dynamicity was reduced by approximately 48% at a carbendazim concentration approximately equimolar with tubulin (10 and 14 μ M, respectively), whereas in contrast, several potent drugs such as vinca alkaloids and taxanes reduce dynamicity at concentrations substoichiometric to tubulin (Jordan and Wilson, 2008).

Suppression of microtubule dynamics by carbendazim was similar to that induced by the parent compound benomyl, which also inhibits proliferation and mitosis in mammalian cells. Benomyl binds more tightly to tubulin than carbendazim (K_d of approximately 12 and 43 μ M, respectively), and the two drugs have similar IC_{50} s for inhibition of tubulin polymerization (approximately 70 μ M) (Gupta et al., 2004). Both drugs reduced growth and shortening rates in vitro. In contrast to carbendazim, benomyl did not alter the catastrophe frequency in vitro (Gupta et al., 2004), although because catastrophes are rare events, the total measurement time may have obscured detection of a benomyl-induced change in catastrophe frequency. On the basis of the high percentage (85%) of benomyl bound to tubulin when polymerization was inhibited by 50%, it was suggested that benomyl may copolymerize with tubulin into microtubules (Gupta et al., 2004). Because the affinity of carbendazim for tubulin is one fourth of benomyl's affinity, less tubulin will be bound by carbendazim at the low micromolar concentrations that suppressed dynamicity. Carbendazim's ability to suppress both growth and shortening events and the catastrophe frequency indicates strongly that carbendazim binds to microtubule ends. In addition, carbendazim may conceivably copolymerize into microtubules that could contribute to suppression of the shortening rate. However, given that vinblastine suppresses shortening without copolymerizing into microtubules and given that the proportion of tubulin bound by carbendazim at concentrations that suppress dynamic instability is relatively small compared with that of benomyl at similar concentrations, we cannot discern whether carbendazim copolymerizes into microtubules.

A difference between carbendazim and other microtubule-targeted drugs is that carbendazim suppresses microtubule dynamicity in cells at similar media concentrations as with purified microtubules. For several other drugs, media drug

Fig. 5. Determination of binding affinity and binding site for carbendazim in tubulin. **A**, effects of carbendazim on tubulin-bound ANS fluorescence. Tubulin ($2 \mu\text{M}$) was incubated with carbendazim for 30 min, followed by incubation with ANS ($40 \mu\text{M}$) for 15 min (*Materials and Methods*). Inset, double-reciprocal plot of binding of carbendazim to tubulin. X and L_f represent the fraction of the binding sites and the free carbendazim concentration, respectively. The dissociation constant (K_d) was determined using the relationship, $1/X = 1 + K_d/L_f$. Results are expressed as mean and S.E.M. for four experiments. **B**, inhibition of [^3H]colchicine binding by carbendazim. Tubulin ($9 \mu\text{M}$) was incubated with [^3H]colchicine ($20 \mu\text{M}$) and increasing concentrations of carbendazim simultaneously for 16 h (●) or preincubated with a range of concentrations of carbendazim for 2 h followed by incubation with $5 \mu\text{M}$ colchicine for 1 h (□). The tubulin-colchicine complex was separated from free colchicine by gel filtration, and radioactivity of bound colchicine was determined. Data for preincubation experiment are mean and S.E.M. of two independent experiments; S.E.M. values are smaller than the dimensions of the symbols. **C**, inhibition of [^3H]vinblastine binding by carbendazim. Tubulin ($5 \mu\text{M}$) was incubated with increasing concentrations of carbendazim for 30 min, followed by incubation with [^3H]vinblastine ($5 \mu\text{M}$) for 30 min. The unbound vinblastine was separated by centrifugal filtration, and radioactivity was determined. The concentration of bound vinblastine was calculated from the difference between total and unbound vinblastine. Results are expressed as mean and S.E.M. for two experiments.

concentrations that induce half-maximal mitotic arrest and suppress dynamicity in cells are much lower than the concentrations required to suppress dynamics in vitro because of intracellular drug accumulation. For example, 2-methoxyestradiol is concentrated approximately 10-fold in MCF7 cells (Ka-

math et al., 2006), vinblastine is concentrated 31- to 63-fold in HeLa cells, and paclitaxel accumulates several 100-fold (Jordan and Kamath, 2007). Our results suggest that carbendazim does not accumulate intracellularly, or its actions may be inhibited by cellular MAPs. Alternatively, because of its low aqueous solubility, it might be sequestered in a cellular compartment and be unavailable to bind to microtubules.

Carbendazim Binds to Tubulin. Carbendazim concentration-dependently increased the fluorescence of tubulin-bound ANS, giving a K_d of $42.8 \pm 4.0 \mu\text{M}$, a lower binding affinity than benomyl (K_d , $11.9 \pm 1.2 \mu\text{M}$; Gupta et al., 2004), and 40- to 400-fold lower affinity than colchicine (K_d , $0.3\text{--}0.91 \mu\text{M}$; Luduena, 1979). The affinity of carbendazim for tubulin is less than that of benomyl, most probably because it lacks the position 1 side chain of benomyl. Although the affinity of carbendazim for tubulin is relatively weak, it is similar to that of other investigatory cancer drugs, such as the noscapine analog EM015 (K_d , $40 \pm 8 \mu\text{M}$) (Aneja et al., 2006).

Carbendazim Does Not Compete with Vinblastine or Colchicine for Binding to Mammalian Tubulin. It is interesting that although the same parameters of microtubule dynamics are suppressed by carbendazim and vinblastine, carbendazim does not compete with [^3H]vinblastine for binding to tubulin. Some benzimidazole derivatives have been reported to bind to the colchicine site, whereas others bind to a specific benzimidazole binding site in the core of β -tubulin (Friedman and Platzer, 1978; Downing, 2000; Richards et al., 2000; Gupta et al., 2004). Like benomyl (Gupta et al., 2004), carbendazim did not compete with colchicine for binding to brain tubulin. Benzimidazole derivatives, such as mebendazole, oxbendazole, and fenbendazole, competitive inhibitors of colchicine (Friedman and Platzer, 1978; Friedman and Platzer, 1980), have large moieties in the 5-position that are lacking in benomyl and carbendazim. The moieties at the 5-position may account for their greater potency in inhibiting polymerization and their competitive inhibition of colchicine binding. Thus, carbendazim and benomyl may bind to a novel site near the colchicine site, but because of their less complex structures, they do not inhibit colchicine binding.

In summary, carbendazim binds to purified mammalian tubulin and suppresses dynamic instability of purified mammalian microtubules. In cells, it suppresses microtubule dynamics in a similar manner and at similar concentrations in association with mitotic arrest and inhibition of proliferation. It induces abnormal mitotic spindles with unattached kinetochores and decreases tension at sister kinetochores, both leading to arrest at the metaphase/anaphase spindle checkpoint and indicating that suppression of mitotic spindle microtubule dynamics is the mechanism of mitotic arrest. Because of its broad spectrum of action, carbendazim is potentially an efficacious addition to cancer chemotherapy. An open question is whether long-term human exposure to carbendazim used as a fungicide may have predominantly positive tumor-preventive effects or potential negative effects associated with induction of mitotic abnormalities.

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References

- Aneja R, Lopus M, Zhou J, Vangapandu SN, Ghaleb A, Yao J, Nettles JH, Zhou B, Gupta M, Panda D, Chandra R, and Joshi HC (2006) Rational design of the microtubule-targeting anti-breast cancer drug EM015. *Cancer Res* **66**:3782–3791.
- Bhattacharyya B and Wolff J (1975) The interaction of 1-anilino-8-naphthalene sulfonate with tubulin: a site independent of the colchicine-binding site. *Arch Biochem Biophys* **167**:264–269.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Brand L and Gohlke JR (1972) Fluorescence probes for structure. *Annu Rev Biochem* **41**:843–868.
- Can A and Albertini DF (1997) M-phase specific centrosome-microtubule alterations induced by the fungicide MBC in human granulosa cells. *Mutat Res* **373**:139–151.
- Davidse LC (1986) Benzimidazole fungicides: mechanism of action and biological impact. *Annu Rev Phytopathol* **24**:43–65.
- Davidse LC and Flach W (1977). Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. *J Cell Biol* **72**:174–193.
- Downing KH (2000) Structural basis for the action of drugs that affect microtubule dynamics. *Expert Opin Ther Targets* **4**:219–237.
- Friedman PA and Platzer EG (1978) Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochim Biophys Acta* **544**:605–614.
- Friedman PA and Platzer EG (1980) Interaction of anthelmintic benzimidazoles with *Ascaris suum* embryonic tubulin. *Biochim Biophys Acta* **630**:271–278.
- Gupta K, Bishop J, Peck A, Brown J, Wilson L, and Panda D (2004) Antimitotic antifungal compound benomyl inhibits brain microtubule polymerization and dynamics and cancer cell proliferation at mitosis, by binding to a novel site in tubulin. *Biochemistry* **43**:6645–6655.
- Hamel E (1996) Antimitotic natural products and their interactions with tubulin. *Med Res Rev* **16**:207–231.
- Hammond LA, Davidson K, Lawrence R, Camden JB, Von Hoff DD, Weitman S, and Izbicke E (2001) Exploring the mechanisms of action of FB642 at the cellular level. *J Cancer Res Clin Oncol* **127**:301–313.
- Hao D, Rizzo JD, Stringer S, Moore RV, Marty J, Dexter DL, Mangold GL, Camden JB, Von Hoff DD, and Weitman SD (2002) Preclinical antitumor activity and pharmacokinetics of methyl-2-benzimidazolecarbamate (FB642). *Invest New Drugs* **20**:261–270.
- Jordan MA and Kamath K (2007) How do microtubule-targeted drugs work? An overview. *Curr Cancer Drug Targets* **7**:730–742.
- Jordan MA and Wilson L (1998) Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr Opin Cell Biol* **10**:123–130.
- Jordan MA and Wilson L (2008) Microtubule dynamics: Mechanisms and regulation by microtubule-associated proteins and drugs *in vitro* and in cells, in *The Role of Microtubules in Cell Biology, Neurobiology, and Oncology* (Fojo T ed) pp 47–81, Humana Press, Totowa, NJ.
- Jordan MA, Kamath K, Manna T, Okouneva T, Miller HP, Davis C, Littlefield BA, and Wilson L (2005) The primary antimitotic mechanism of action of the synthetic halichondrin E7389 is suppression of microtubule growth. *Mol Cancer Ther* **4**:1086–1095.
- Jordan MA, Walker D, de Arruda M, Barlozzari T, and Panda D (1998) Suppression of microtubule dynamics by binding of cemadotin to tubulin: possible mechanism for its antitumor action. *Biochemistry* **37**:17571–17578.
- Kamath K, Okouneva T, Larson G, Panda D, Wilson L, and Jordan MA (2006) 2-Methoxyestradiol suppresses microtubule dynamics and arrests mitosis without depolymerizing microtubules. *Mol Cancer Ther* **5**:2225–2233.
- Kelling J, Sullivan K, Wilson L, and Jordan MA (2003) Suppression of centromere dynamics by Taxol in living osteosarcoma cells. *Cancer Res* **63**:2794–2801.
- Lacey E and Watson TR (1985). Structure-activity relationships of benzimidazole carbamates as inhibitors of mammalian tubulin, *in vitro*. *Biochem Pharmacol* **34**:1073–1077.
- Ludueno RF (1979) Biochemistry of tubulin, in *Microtubules* (Robert K and Hyams J eds) pp 94–95, Academic Press, New York.
- McIntosh JR, Grishchuk EL, and West RR (2002) Chromosome-microtubule interactions during mitosis. *Annu Rev Cell Dev Biol* **18**:193–219.
- Mitchison T and Kirschner M (1984). Dynamic instability of microtubule growth. *Nature* **312**:237–242.
- Nicklas RB (1997) How cells get the right chromosomes. *Science* **275**:632–637.
- Okouneva T, Azarenko O, Wilson L, Littlefield BA, and Jordan MA (2008) Inhibition of centromere dynamics by eribulin (E7389) during mitotic metaphase. *Mol Cancer Ther* **7**:2003–2011.
- Okouneva T, Hill BT, Wilson L, and Jordan MA (2003) The effects of vinflunine, vinorelbine, and vinblastine on centromere dynamics. *Mol Cancer Ther* **2**:427–436.
- Panda D, Miller HP, and Wilson L (1999) Rapid treadmilling of brain microtubules free of microtubule-associated proteins in vitro and its suppression by tau. *Proc Natl Acad Sci U S A* **96**:12459–12464.
- Pfeffer TA, Asnes CF, and Wilson L (1976) Properties of tubulin in unfertilized sea urchin eggs: quantitation and characterization by the colchicine-binding reaction. *J Cell Biol* **69**:599–607.
- Richards KL, Anders KR, Nogales E, Schwartz K, Downing KH, and Botstein D (2000) Structure-function relationships in yeast tubulins. *Mol Biol Cell* **11**:1887–1903.
- Shelby RD, Hahn KM, and Sullivan KF (1996) Dynamic elastic behavior of alpha-satellite DNA domains visualized *in situ* in living human cells. *J Cell Biol* **135**:545–557.
- Skibbens RV, Skeen VP, and Salmon ED (1993) Directional instability of kinetochore motility during chromosome congression and segregation in mitotic newt lung cells: a push-pull mechanism. *J Cell Biol* **122**:859–875.
- Waters JC, Skibbens RV, and Salmon ED (1996) Oscillating mitotic newt lung cell kinetochores are, on average, under tension and rarely push. *J Cell Sci* **109**:2823–2831.
- Weisenberg RC, Borisy GG, and Taylor EW (1968) The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry* **7**:4466–4479.
- Wilson L and Bryan J (1974) Biochemical and pharmacological properties of microtubules. *Adv Cell Mol Biol* **3**:21–72.
- Winder BS, Strandgaard CS, and Miller MG (2001) The role of GTP binding and microtubule-associated proteins in the inhibition of microtubule assembly by carbenazim. *Toxicol Sci* **59**:138–146.

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