Kinetic suppression of microtubule dynamic instability by griseofulvin: Implications for its possible use in the treatment of cancer

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The antifungal drug griseofulvin inhibits mitosis strongly in fungal cells and weakly in mammalian cells by affecting mitotic spindle microtubule (MT) function. Griseofulvin also blocks cell-cycle progression at G_2/M and induces apoptosis in human tumor cell lines. Despite extensive study, the mechanism by which the drug inhibits mitosis in human cells remains unclear. Here, we analyzed the ability of griseofulvin to inhibit cell proliferation and mitosis and to affect MT polymerization and organization in HeLa cells together with its ability to affect MT polymerization and dynamic instability *in vitro*. Griseofulvin inhibited cell-cycle progression at prometaphase/anaphase of mitosis in parallel with its ability to inhibit cell proliferation. At its mitotic IC_{50} of 20 μM, spindles in blocked cells displayed nearly normal quantities of MTs and MT organization similar to spindles blocked by more powerful MT-targeted drugs. Similar to previously published data, we found that very high concentrations of griseofulvin (>100 μM) were required to inhibit MT polymerization *in vitro*. However, much lower drug concentrations (1–20 μM) strongly suppressed the dynamic instability behavior of the MTs. We suggest that the primary mechanism by which griseofulvin inhibits mitosis in human cells is by suppressing spindle MT dynamics in a manner qualitatively similar to that of much more powerful antimitotic drugs, including the vinca alkaloids and the taxanes. In view of griseofulvin's lack of significant toxicity in humans, we further suggest that it could be useful as an adjuvant in combination with more powerful drugs for the treatment of cancer.

cancer chemotherapy | mitosis

Griseofulvin, an orally active nontoxic antifungal antimitotic drug derived from several species of *Penicillium* (1), has been used for many years for the treatment of *Tinea capitis* (ringworm) and other dermatophyte infections (2). Its mechanism of action has been thought to involve the selective inhibition of fungal cell mitosis in association with its accumulation in the keratin layers of the epidermis (1). Early studies demonstrated that griseofulvin inhibits mitosis in sensitive fungi in a manner resembling the actions of colchicine and other antimitotic drugs that act in mammalian cells by disrupting spindle microtubule (MT) function (3), although its precise mechanism of action in sensitive fungi remains unclear. The antiproliferative and antimitotic effects of griseofulvin in mammalian cells are very weak, with inhibition requiring high micromolar concentrations (4, 5).

The question of whether inhibition of mitosis by griseofulvin involves MT depolymerization or some other action on MTs in human cells has remained unsettled. Specifically, griseofulvin clearly inhibits MT polymerization coincident with inhibition of mitosis in echinoderm eggs (6), and inhibition of mitosis at high griseofulvin concentrations in 3T3 cells occurs together with depolymerization of the spindle MTs (5). In contrast, inhibition of mitosis by griseofulvin in HeLa cells occurred in the absence of significant spindle MT depolymerization (4), which was interpreted as indicating that mitotic inhibition was caused by the impairment of the organization or function of the spindle MTs rather than their depolymerization. Griseofulvin is able to bind weakly to mammalian-brain tubulin and to inhibit the polymerization of MTs *in vitro*; however, inhibition of brain tubulin polymerization requires very high concentrations of griseofulvin (5, 7).

MTs exhibit two forms of nonequilibrium dynamics, treadmilling and dynamic instability (3). Most of the earlier work on griseofulvin was carried out before the realization that mitotic spindle MTs are highly dynamic, and that the rapid dynamics of the MTs, not just their presence, are critical for proper spindle function (3, 8–13). Further, most antimitotic MT-targeted drugs that act on animal cells suppress MT dynamics at concentrations that often are far below the concentrations required to inhibit (e.g., the vinca alkaloids) (9) or increase (e.g., paclitaxel) (10, 11) MT polymerization. Further, several antimitotic drugs that weakly inhibit MT polymerization in cells and *in vitro*, including noscapine (12), estramustine (13), and benomyl (14), suppress MT dynamic instability and treadmilling remarkably strongly in the absence of appreciable inhibition of polymerization.

In pursuing the earlier results of Grisham *et al.* (4), we reasoned that, like the aforementioned drugs, griseofulvin might inhibit proliferation and mitosis in human cancer cells primarily by suppressing MT dynamics. Thus, we analyzed the ability of griseofulvin to inhibit the polymerization of brain tubulin into MTs and its ability to modulate MT dynamic instability *in vitro* together with its ability to inhibit cell proliferation and mitosis and to affect MT organization in HeLa cells. We found that griseofulvin inhibited mitosis in parallel with its ability to inhibit cell proliferation, and that at its IC_{50} for inhibition of mitosis (20 μM), the organization of the MTs in the blocked spindles appeared nearly normal. In addition, griseofulvin bound weakly to tubulin, and inhibition of MT polymerization *in vitro* required griseofulvin concentrations >100 μM. In contrast, griseofulvin suppressed the dynamic instability of the MTs remarkably strongly (IC_{50} for overall dynamicity, <1 μM). Our data strongly support the hypothesis that inhibition of mitosis in human cancer cells is due to the suppression of spindle MT dynamics. A recent report indicates that griseofulvin either alone or in combination with nocodazole inhibits tumor production in athymic mice (15). Taking these observations together with the data described in this work, we suggest that griseofulvin might be useful in combination with more powerful MT-targeted drugs or with agents acting on novel targets for the treatment of cancer.

Materials and Methods

Reagents. Griseofulvin, GTP, EGTA, colchicine, piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes), and Mes were purchased

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Abbreviations: MT, microtubule; MAP, MT-associated protein; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid).

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from Sigma. Phosphocellulose (P11) was obtained from Whatman. All other chemicals used were of analytical grade.

Purification of Goat and Bovine Brain Tubulins. Goat brain MT protein, tubulin plus MT-associated proteins (MAPs), was isolated as described in refs. 14 and 16. Bovine MT protein was obtained as described in ref. 17. Goat and bovine tubulin were separated from the MAPs by using phosphocellulose chromatography and stored at −80°C. The tubulin concentration was determined by the Bradford method using BSA as the standard (18).

The Effects of Griseofulvin on Glutamate-Induced MT Polymerization *in Vitro*. Goat brain tubulin (11 μM) was polymerized for 30 min at 37°C in 25 mM Pipes buffer, pH 6.8, containing 1 M sodium glutamate, 1 mM GTP, and 5 mM $MgCl_2$ in the absence or presence of griseofulvin. The polymerized MTs were sedimented by centrifugation (52,000 × *g* for 40 min), and the MT polymer mass was determined. Samples for transmission electron microscopy were prepared as described in ref. 14, and MT structures were observed with a Tecnai $G^2$12 electron microscope (FEI, The Netherlands) at ×43,000 magnification.

Effects of Griseofulvin on Seeded MT Assembly. MT nucleating seeds were constructed by polymerizing goat brain tubulin (3.5 mg/ml) in the presence of 6 M glycerol and 1 mM GTP at 37°C for 45 min and shearing the MTs into small fragments by repeated passage through a 25-gauge needle. For measurement of polymer level, desired griseofulvin concentrations were first incubated with 10 μM tubulin at 37°C for 10 min. The MT seeds were then added (a 1:5 ratio of tubulin in the suspension of seeds to soluble tubulin; final tubulin concentration of 12 μM). Polymerization was started by adding 1 mM GTP and incubating at 37°C for 45 min. The MT mass was determined after sedimenting the MTs at 50,000 × *g* (32°C) for 45 min.

Griseofulvin Binding to Tubulin. Goat brain tubulin (1 μM) was incubated with griseofulvin (0–100 μM) for 30 min at 37°C in 25 mM Pipes buffer, pH 6.8. The fluorescence intensity at 335 nm was measured by using a 0.3-cm-pathlength cuvette and 295 nm as the excitation wavelength. Fluorescence intensities were corrected for the inner filter effect as described in refs. 13 and 19 and were used to determine the dissociation constant (K_d) for griseofulvin binding to tubulin (13, 19).

Effects of Griseofulvin on the Aggregation of Tubulin. Tubulin (5 μM) was mixed with different concentrations (5–100 μM) of griseofulvin in 25 mM Pipes buffer, pH 6.8, and the ligand-induced aggregation of tubulin dimers was monitored by 90° light scattering at 400 nm by using a spectrofluorometer (FP-6500, Jasco, Tokyo). We also used a Zeta Plus analyzer (Brookhaven Instruments, Holtsville, NY) to analyze the size of tubulin aggregates.

Cell Culture, Cell Proliferation, Immunofluorescence Microscopy, and Mitotic Index Assays. HeLa cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air as described in ref. 14. Griseofulvin was dissolved in 100% DMSO; the final DMSO concentration in all experiments was 0.1%. The effects of griseofulvin on cell proliferation were determined in 24-well tissue culture plates by counting the cells with a hemocytometer (Bright-Line, Hauser Scientific, Horsham, PA). The cells were seeded at 5×10^4 cells per ml, and after 24 h, the medium was replaced with fresh medium containing either DMSO vehicle (control) or griseofulvin (0–120 μM), and incubation was continued for an additional 40 h. Both the attached and unattached cells were harvested and combined after trypsinization (0.025% trypsin, 10 min) and counted to determine the percent inhibition of proliferation.

Immunofluorescence microscopy was performed as described in ref. 14. The cells were seeded on poly(L-lysine)-coated coverslips at a density of 1×10^5 cells per ml contained in 24-well tissue culture plates. After 40 h of drug treatment, cells were fixed in 3.7% formaldehyde for 30 min at 37°C and then treated with cold 100% methanol (−20°C) for 20 min. After the nonspecific sites had been blocked with 2% BSA/PBS, the cells were incubated with mouse monoclonal anti-α-tubulin antibody (Sigma) at 1:300 dilution for 2 h at 37°C. Coverslips were then rinsed with BSA/PBS and incubated with anti-mouse IgG antibody labeled with Alexa Fluor 568 (Molecular Probes) at 1:100 dilution for 1 h at 37°C. Coverslips were then rinsed with PBS and incubated with DAPI (1 μg/ml) for 20 s. The MTs and DNA were observed with an Eclipse TE-2000 U microscope (Nikon). The images were analyzed by using IMAGEPRO PLUS software (Media Cybernetics, Silver Spring, MD).

Mitotic indices were determined by Wright–Giemsa staining (14). Briefly, the cells were plated at a density of 5×10^4 cells per ml in 24-well plates 24 h before the addition of griseofulvin. The cells were then treated with different concentrations of griseofulvin and incubated for an additional 20 or 40 h. Both the attached and unattached cells were harvested and combined after trypsinization (0.025% trypsin, 10 min), washed with PBS, and then treated with 0.5× PBS for 10 min on ice. The cells were fixed with methanol/acetic acid solution (3:1, vol/vol). Finally, the cell suspensions were spread onto cold slides, air-dried, and stained with a 10% Giemsa solution.

Analysis of MT Dynamic Instability. Purified tubulin (12 μM) was polymerized for 30 min at the ends of sea urchin (*Strongylocentrotus purpuratus*) axonemal seeds at 37°C in the presence or absence of the desired concentrations of griseofulvin in 87 mM Pipes/36 mM Mes/1.4 mM $MgCl_2$/1 mM EGTA, pH 6.8. The dynamic instability at the plus-ends of individual MTs was recorded (8). The ends were designated as either plus or minus on the basis of the growth rate, the number of MTs that grew at opposite ends of the seeds, and the relative lengths of the MTs. MT length changes with time were analyzed 30 min after initiation of polymerization when the MTs had reached steady state (8). Data points were collected at 3- to 5-s intervals. A MT was considered to be in a growth phase if it increased in length by >0.2 μm at a rate >0.3 μm/min. MTs showing length changes ≤0.2 μm over the duration of six data points were considered to be in an attenuated state. Twenty to 30 MTs were analyzed for each experimental condition.

Results

Inhibition of HeLa Cell Proliferation and Mitosis by Griseofulvin. We first determined the relationship between the ability of griseofulvin to inhibit mitosis in HeLa cells and its ability to inhibit cell proliferation. Griseofulvin inhibited cell proliferation weakly and in a concentration-dependent fashion, with half-maximal inhibition occurring at 25 ± 4 μM (Fig. 1). The effects of griseofulvin on mitosis closely paralleled its ability to inhibit proliferation as shown in Fig. 1. At 20 h of drug treatment, 49% of the cells were blocked at mitosis at 20 μM griseofulvin, and 58% were blocked at 30 μM griseofulvin (Fig. 1). At 40 h of drug treatment, ≈35% and ≈43% of the cells were arrested in mitosis by 20 and 40 μM griseofulvin, respectively (data not shown). Thus, the block of mitosis in HeLa cells by griseofulvin correlates well with its ability to inhibit proliferation of the cells.

The organization of the MTs and chromosomes in the blocked cell spindles was analyzed by immunofluorescence microscopy using antibodies against tubulin and DAPI staining of the chromosomes. In control cells, the bipolar metaphase spindles and the tight distribution of the chromosomes were similar to those described in refs. 20 and 21 (Fig. 2). At 20 μM griseofulvin (40 h of drug treatment), which blocked proliferation and mitosis

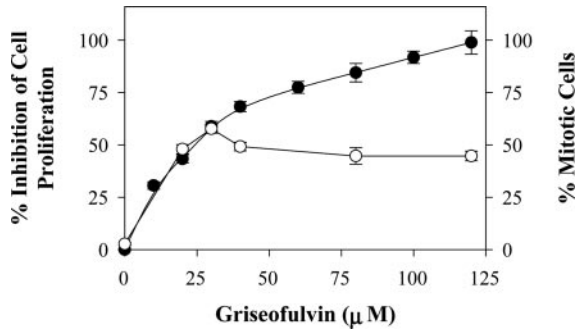

Fig. 1. Inhibition of proliferation (●) and mitotic progression (○) in HeLa cells by griseofulvin. HeLa cells were treated with various concentrations of griseofulvin (0–120 μM) for 40 h, and the percent inhibition of proliferation was determined by counting the cells. The mitotic index was determined by using the Wright–Giemsa staining method after incubating the cells with various concentrations of griseofulvin (0–120 μM) for 20 h. In the absence of griseofulvin, 2.8 ± 0.5% of the DMSO-treated cells were found to be in mitosis.

by ≈45% and ≈35%, respectively, most of the spindles were bipolar and appeared nearly normal, with a few chromosomes not aligned properly on the metaphase plate (Fig. 2). At 40 μM griseofulvin, most of the spindles were bipolar, but the MTs were substantially disrupted (Fig. 2). At 120 μM griseofulvin, a concentration that inhibited proliferation by close to 100%, no spindle MTs were present in the blocked cells and the chromosomes were arranged in nondescript ball-shaped clusters (Fig. 2).

The effects of griseofulvin on the organization of MTs in interphase cells at a given griseofulvin concentration were less pronounced than its effects on the spindle MTs. At 40 h of incubation with 20 and 40 μM griseofulvin, which inhibited proliferation by ≈45% and >70%, respectively, the interphase MTs were nearly intact. However, at 40 μM griseofulvin, 85–90% of the interphase cells were multinuclear and significantly larger than control cells (Fig. 3). At 120 μM griseofulvin, which completely inhibited proliferation, ≈50% of the cells remained in interphase and had substantial numbers of MTs, although the

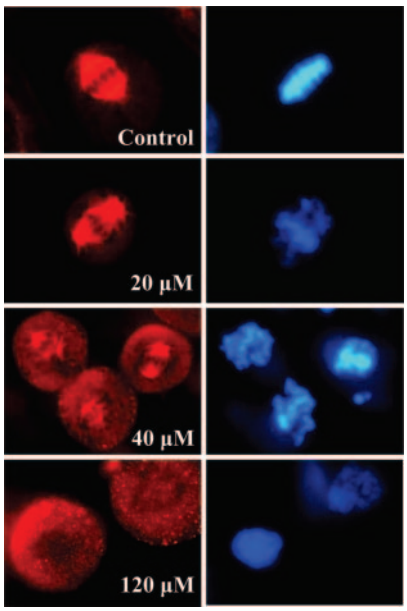

Fig. 2. Effects of griseofulvin on spindle MTs and chromosome organization. HeLa cells were incubated with the indicated concentrations of griseofulvin for 40 h. Spindle MTs (red) and chromosomes (blue) were analyzed as described in *Materials and Methods*.

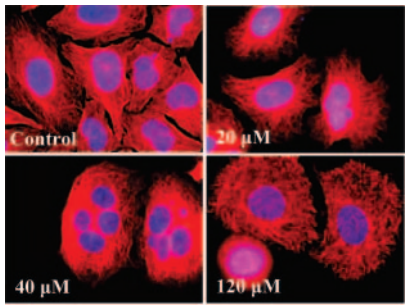

Fig. 3. Effects of 20, 40, and 120 μM griseofulvin on interphase MTs in HeLa cells. HeLa cells were incubated with the indicated concentrations of griseofulvin for 40 h.

density of the MTs was decreased, compared with the density in control cells. Interestingly, most of the interphase cells were mononuclear, suggesting that they were unable to progress in the cell cycle.

Effects of Griseofulvin on Tubulin Polymerization into MTs *in Vitro* and the Binding of Griseofulvin to Tubulin. The effects of griseofulvin on the polymerization of purified goat brain tubulin into MTs *in vitro* were analyzed with polymer mass sedimentation assays. As shown in Fig. 4*A*, a griseofulvin concentration as high as 100 μM had no effect on glutamate-induced polymerization. Identical results were obtained when polymerization was carried out with purified tubulin in the absence of glutamate by using glycerol seeds to nucleate polymerization (data not shown). We analyzed the structure of the MTs by transmission electron microscopy when the MTs were polymerized beginning with pure tubulin in the presence of 0.8 M glutamate and with a MAP-rich MT protein preparation in the absence of glutamate. We did not detect any alteration of structure at 5, 20, or 50 μM griseofulvin under any polymerization conditions (data not shown). Similarly, 100 μM griseofulvin did not change MT structure in the presence of MAPs. These data are consistent with previous work showing that 60 μM griseofulvin does not change MT structure (4). However, we did find that 100 μM griseofulvin altered MT structure in the presence of 0.8 M glutamate (data not shown).

To determine the affinity constant for the binding of griseofulvin to tubulin, we took advantage of the fact that the binding of griseofulvin to tubulin reduces tubulin's intrinsic tryptophan fluorescence (22). Analysis of the reduction in tubulin fluorescence as a function of griseofulvin concentration yielded a very weak dissociation constant of 300 ± 12 μM (Fig. 4 *B* and *C*). This relatively weak binding constant is in the same range but is 2.4-fold weaker than that reported by Chaudhuri and Luduena (22). A difference of this magnitude could simply be due to the buffer conditions, subtle differences in the posttranslational status of the purified tubulin, or various experimental conditions used in two studies. We also examined the effect of griseofulvin on the secondary structure of tubulin by far-UV circular dichroism spectroscopy and found that a concentration as high as 100 μM did not affect tubulin's secondary structure (data not shown). Further, no aggregation of tubulin dimers was detected in the absence and presence of different griseofulvin concentrations (5–100 μM) by 90° light scattering (data not shown). However, under similar conditions, vinblastine (30 μM) was found to induce aggregation of tubulin dimers strongly (data not shown). The dynamic light-scattering experiment also showed that up to 50 μM griseofulvin did not induce detectable aggregation of tubulin dimers. However, 100 μM griseofulvin induced tubulin aggregation weakly (data not shown).

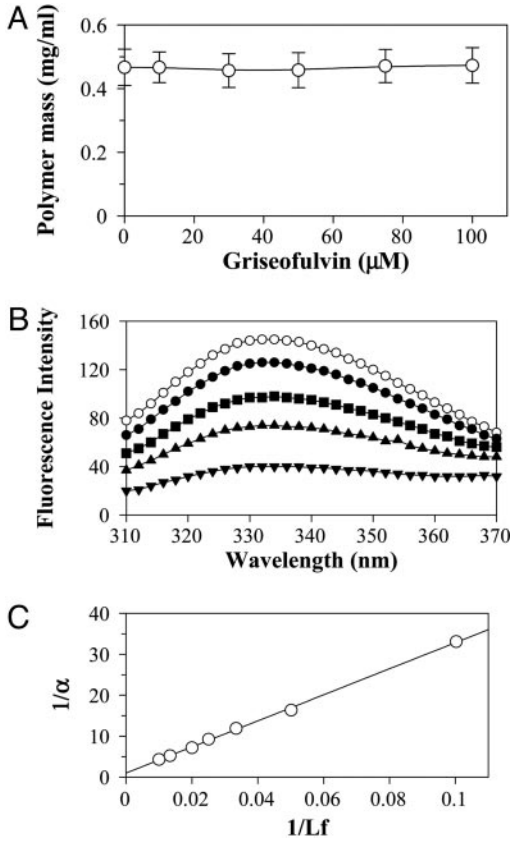

Fig. 4. Effects of griseofulvin on tubulin polymerization and its binding to tubulin. (*A*) Effects of griseofulvin on the MT polymer mass. Tubulin (11 μM) in Pipes buffer, pH 6.8, containing 1 mM GTP and 1 M sodium glutamate was polymerized into MTs in the absence or presence of different concentrations of griseofulvin for 30 min at 37°C. Polymers were sedimented, and the polymer mass was determined as described in *Materials and Methods*. (*B*) Binding of griseofulvin to tubulin. Binding to tubulin was determined by monitoring the tubulin's intrinsic tryptophan fluorescence. Tubulin (1 μM) was incubated in 25 mM Pipes buffer with various concentrations of griseofulvin from 0 (○), 10 (●), 30 (■), 50 (▲), and 100 (▼) μM, and emission spectra were recorded after 30 min of incubation at 37°C. The fluorescence intensity at 335 nm was used to calculate the K_d for griseofulvin. (*C*) Double-reciprocal plot for the binding of griseofulvin to tubulin. α, fraction of binding sites occupied by griseofulvin; Lf, free griseofulvin concentration.

Suppression of MT Dynamic Instability at Steady State *in Vitro* by Griseofulvin. A number of MT-targeted antimitotic drugs, including vinblastine (9) and paclitaxel (10), suppress MT dynamic instability at much lower concentrations than those required to change the MT polymer mass (3). Thus, we wanted to determine whether griseofulvin possessed similar capability. Griseofulvin did indeed suppress dynamic instability at concentrations far below those required to inhibit polymerization. Life-history traces of individual steady-state bovine brain MTs in the absence or presence of 20 μM griseofulvin are shown in Fig. 5. Control MTs displayed typical growing and shortening dynamics (Fig. 5*A*), whereas the dynamics of MTs treated with 20 μM griseofulvin were strongly reduced (Fig. 5*B*). The actions of griseofulvin on the individual dynamic instability parameters were determined quantitatively (Table 1). Specifically, griseofulvin relatively strongly suppressed the rate of growth and shortening. For example, 5 μM griseofulvin reduced the growth rate by 50% and the shortening rate by 70%. In addition, 5 μM griseofulvin also reduced the mean length shortened per shortening event by 40% but did not reduce the mean length of growth. At or near steady state, both *in vitro* and *in vivo*, MTs spend a fraction of

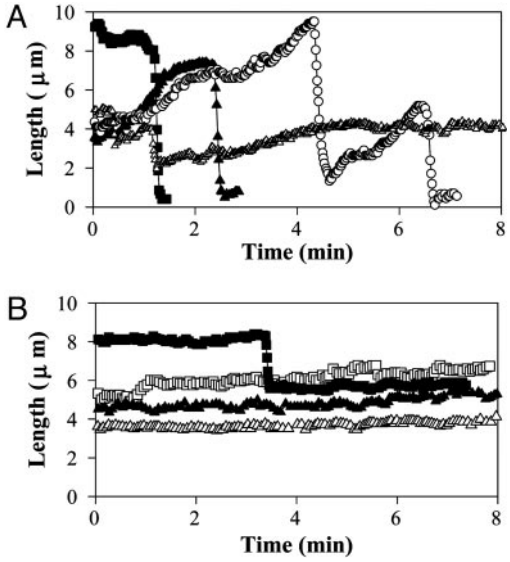

Fig. 5. Length changes of individual MTs at their plus ends at steady state in the absence (*A*) or presence (*B*) of 20 μM griseofulvin (see *Materials and Methods*).

time in an attenuated or paused state, neither growing nor shortening detectably. As shown in Table 1, griseofulvin strongly reduced the fraction of time the MTs spent growing and shortening and increased the percentage of time that the MTs spent in the attenuated state. Specifically, at the highest griseofulvin concentration examined (20 μM), griseofulvin increased the fraction of time the MTs spent in the attenuated state by 8-fold, from ≈6% of the time to 47% of the time.

The transition frequencies among the growing, shortening, and attenuated phases, which may reflect the gain and loss of the stabilizing tubulin–GTP or tubulin–GDP–P_i cap at MT ends, are considered to be important in the regulation of MT dynamics in cells. Griseofulvin strongly suppressed both the rescue and catastrophe frequencies (Table 1). For example, 5 μM griseofulvin reduced the catastrophe frequency by 60% and the rescue frequency by 30%. These data, together with the results indicating that griseofulvin inhibits the rate of shortening, indicate that griseofulvin acts directly at the MT plus ends. Dynamicity is a measure of the overall visually detectable growth and shortening at a MT end per unit time. As shown in Table 1, griseofulvin strongly suppressed the dynamicity in a concentration-dependent manner, with 20 μM griseofulvin suppressing this parameter by ≈10-fold.

Discussion

Suppression of MT Dynamic Instability and the Inhibition of Mitosis in HeLa Cells by Griseofulvin. We have found that griseofulvin, an oral antifungal drug targeted to tubulin and/or MTs, inhibits mitosis in HeLa cells at the metaphase/anaphase transition in parallel with its ability to inhibit cell proliferation. The results strongly support the idea that inhibition of proliferation in these cells by griseofulvin is due to the inhibition of mitosis. Similar to the results of previous studies (4, 5, 7), inhibition was weak, with half-maximal inhibition of proliferation and mitosis occurring at a griseofulvin concentration of ≈25–30 μM. Examination of the mitotic spindles and interphase MT organization in blocked cells at the IC_{50} revealed that the spindles were bipolar with nearly normal MT organization, with several chromosomes located at or near the poles. However, the interphase MT array in the cells at griseofulvin's IC_{50} was not detectably altered. Such a prometaphase/metaphase block with little change in spindle MT orga-

Table 1. Effect of griseofulvin on the dynamics of individual microtubules

Parameter	Griseofulvin concentration, μM				
	0	0.5	1	5	20
Rate, μm/min					
Growing	1 ± 0.10	0.73 ± 0.11	0.84 ± 0.20	0.5 ± 0.07	0.44 ± 0.01
Shortening	19.9 ± 0.22	15.6 ± 1.3	12.4 ± 0.22	6 ± 0.24	5.1 ± 0.24
Length change, μm					
Growing	1.2 ± 1.1	1.4 ± 1.3	1.64 ± 1.6	1.4 ± 1.3	1.4 ± 1.3
Shortening	4.5 ± 4.3	4.9 ± 1.1	4.1 ± 3.9	2.4 ± 2.2	1.8 ± 1.6
Percent time in phase					
Growing	78.8	59.7	64.4	39.4	48.5
Shortening	15.1	7.7	5.8	7.4	4.1
Attenuation	6.1	32.6	29.8	53.2	47.4
Frequency, no./min					
Catastrophe	0.49 ± 0.10	0.2 ± 0.04	0.08 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
Rescue	1.9 ± 0.48	1.3 ± 0.42	1.1 ± 0.27	0.68 ± 0.20	1.1 ± 0.36
Dynamicity, μm/min	2.6	1.2	0.85	0.35	0.28

Data are given as mean ± SEM.

nization and in the absence of detectable changes in MT organization in interphase cells is similar to that induced by relatively low concentrations of much more powerful antimitotic drugs, including vinblastine, paclitaxel, and cryptophycin 52 (3). Although the potency of the more powerful drugs is much higher than that of griseofulvin, these drugs, like griseofulvin, inhibit or stimulate MT polymerization at high drug concentrations but at their lowest effective concentrations, they suppress MT dynamic instability without affecting the polymer mass (9, 11).

In keeping with the results described in ref. 22, we found that griseofulvin bound weakly to soluble tubulin (K_d, ≈300 μM). But surprisingly, when the ability of griseofulvin to inhibit the polymerization of purified MAP-free tubulin into MTs was analyzed at concentrations as high as 100 μM, when ≈30% of the tubulin would have been drug-bound, griseofulvin did not affect the polymer mass, indicating that the binding of griseofulvin to tubulin does not inactivate the tubulin by sequestering it.

Griseofulvin suppressed the steady-state dynamic instability behavior of the MTs surprisingly strongly relative to its ability to inhibit polymerization. Specifically, at 20 μM griseofulvin, the IC_{50} for inhibition of proliferation and mitosis in HeLa cells, the MT-shortening rate was reduced by ≈75%; the percent time that the MTs remained in an attenuated state, neither growing nor shortening detectably, was increased by ≈8- to 9-fold; and the overall dynamicity was reduced by nearly 90%. These data strongly support the idea that inhibition of proliferation and mitosis in HeLa cells by griseofulvin is brought about by the suppression of mitotic spindle MT dynamics.

The Mechanism of Action of Griseofulvin. Although griseofulvin has been shown to inhibit mitosis at metaphase by acting either on MT polymerization or MT organization in a large number of cell types, including fungal cells, echinoderm embryos, and mouse and human cultured cells, its mechanism of action has remained unclear. For example, Grisham *et al.* (4) found that when mitosis was inhibited in HeLa cells by 20 μM griseofulvin, the spindle MTs were present, appeared normal in structure, and were normally attached to the spindle poles and the kinetochores, as revealed by electron microscopy. Similar observations were made in HeLa cells blocked at moderate griseofulvin concentrations (10–20 μM), whereas high griseofulvin concentrations (≥50 μM) did destroy the MTs (5). These results can now be understood in light of the results described here, indicating that *in vitro* griseofulvin suppresses MT dynamic instability at concentrations that do not affect the MT polymer mass. We suggest that the suppression of spindle MT dynamics may be the major mechanism by which griseofulvin inhibits mitosis in HeLa cells and perhaps in other human cells. Such a mechanism is consistent with the actions of more powerful antimitotic drugs, including vinblastine and paclitaxel, which also suppress MT dynamics at concentrations that are well below those necessary to affect MT polymer mass (9, 10). The dynamic instability behavior of individual MTs cannot be analyzed in HeLa cells, because the cells are not sufficiently flat for such an analysis. However, it will be worthwhile to carry out such an analysis in a favorable human tumor cell line, such as MCF7 cells.

The reported effects of griseofulvin on the polymerization of mammalian-brain MTs *in vitro* are somewhat more difficult to rationalize. Some of the differences may be due in part to the fact that inhibition of MT polymerization by griseofulvin requires much higher griseofulvin concentrations when polymerization is carried out in the absence, rather than in the presence, of MAPs (7). In the present study, griseofulvin did not affect the polymerization of MAP-free tubulin into MTs at a concentration as high as 100 μM (Fig. 5), and consistent with these results, Sloboda *et al.* (23) found that inhibition of polymerization required extremely high griseofulvin concentrations (e.g., 500–800 μM). Thus, a sufficiently high griseofulvin concentration can inhibit the polymerization of brain tubulin into MTs *in vitro*. In contrast, several groups have found that griseofulvin inhibits the polymerization of tubulin plus MAPs into MTs *in vitro* in the same concentration range as used here. Specifically, Roobol *et al.* (24) found that griseofulvin concentrations between 20 and 200 μM inhibited polymerization when MAPs were present. Roobol *et al.* (24) and Weber *et al.* (7) reported that griseofulvin could induce the aggregation of MT protein (tubulin plus MAPs) at cold temperatures. Related to these results, Roobol *et al.* (25) found that griseofulvin can bind efficiently to sheep-brain MAPs *in vitro* at griseofulvin concentrations that inhibit MT polymerization. Such data suggest that the ability of griseofulvin to inhibit MT polymerization when MAPs are present may be related to an ability of griseofulvin to affect MAP–tubulin interactions.

How Might Griseofulvin Suppress MT Dynamic Instability? Griseofulvin strongly suppressed the steady-state dynamic instability of MAP-free bovine-brain MTs *in vitro* at concentrations well below those required to reduce the polymer mass. Significant suppression of the shortening rate and the catastrophe frequency at the plus ends occurred at griseofulvin concentrations as low as 0.5 μM, when, based on the affinity of griseofulvin for tubulin, very little soluble tubulin could have been bound to the drug (<1

mol of griseofulvin bound per 100 mol of tubulin). These data indicate that griseofulvin must suppress MT dynamics at its lowest effective concentrations by an end-poisoning mechanism. The only way that the drug could inhibit plus-end shortening at steady state is by acting directly at the plus end.

It is noteworthy that griseofulvin does not inhibit the polymerization of MTs to steady state, yet once steady state is achieved, it strongly inhibits the rate and extent of MT shortening. Perhaps somewhat like the action of colchicine–tubulin complex at MT ends (26, 27), griseofulvin–tubulin complexes become incorporated at the MT ends, thus forming a copolymer. Such a mechanism also may occur with benomyl (14). The incorporation of the griseofulvin–tubulin complexes, although not able to inhibit tubulin addition, strongly reduces tubulin loss by increasing the strength of the interactions between tubulin subunits in the lattice. Perhaps griseofulvin increases the stability at the MT end by modifying the size or chemical nature of the stabilizing cap (28).

The Possible Use of Griseofulvin for the Treatment of Cancer. In view of its ability to stabilize MT dynamics and to inhibit mitosis in human cells, it is rather remarkable that griseofulvin is so well tolerated when given to humans for the treatment of fungal infections. The drug can cause toxicities in animals that would be predicted based on its mechanism of action (29, 30), but such adverse reactions do not appear to occur to an appreciable extent in humans (2). One major reason for the relative safety of griseofulvin at the clinically useful doses may be the fact that it accumulates to high levels in the keratin layers of the skin, where it acts on the growth of dermatophytes. Another important factor appears to be that the drug's effects on mitosis in sensitive fungal cells occur at concentrations that are substantially below those required to inhibit mitosis in human cells (31, 32). Brian (31) found that the inhibitory concentrations of griseofulvin against various dermatophytes *in vitro* range from 0.4 to 1.7 μM, well below the concentration range required to inhibit mitosis in HeLa cells.

Of considerable interest is a report by Ho *et al.* (15) that in athymic mice carrying human tumor xenografts griseofulvin synergistically inhibits tumor growth in combination with nocodazole. These authors suggested that griseofulvin might be valuable in combination with other drugs for the treatment of cancer. Our data support this idea. It is attractive to consider the use of nontoxic concentrations of griseofulvin for the treatment of cancer in combination with other anticancer drugs and even other MT-targeted drugs. A mild suppression of MT dynamics by griseofulvin in tumor cells, combined with the effects of more powerful drugs working through other mechanisms, might provide a therapeutic advantage for treatment of certain tumors.

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