Identification of Griseofulvin as an Inhibitor of Centrosomal Clustering in a Phenotype-Based Screen

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Abstract
A major drawback of cancer chemotherapy is the lack of tumor-specific targets which would allow for the selective eradication of malignant cells without affecting healthy tissues. In contrast with normal cells, most tumor cells contain multiple centrosomes, associated with the formation of multipolar mitotic spindles and chromosome segregation defects. Many tumor cells regain mitotic stability after clonal selection by the coalescence of multiple centrosomes into two functional spindle poles. To overcome the limitations of current cancer treatments, we have developed a cell-based screening strategy to identify small molecules that inhibit centrosomal clustering and thus force tumor cells with supernumerary centrosomes to undergo multipolar mitoses, and subsequently, apoptosis. Using a chemotaxonomic selection of fungi from a large culture collection, a relatively small but diverse natural product extract library was generated. Screening of this compound library led to the identification of griseofulvin, which induced multipolar spindles by inhibition of centrosome coalescence, mitotic arrest, and subsequent cell death in tumor cell lines but not in diploid fibroblasts and keratinocytes with a normal centrosome content. The inhibition of centrosome clustering by griseofulvin was not restricted to mitotic cells but did occur during interphase as well. Whereas the formation of multipolar spindles was dynein-independent, depolymerization of interphase microtubules seemed to be mechanistically involved in centrosomal declustering. In summary, by taking advantage of the tumor-specific phenotype of centrosomal clustering, we have developed a screening strategy that might lead to the identification of drugs which selectively target tumor cells and spare healthy tissues. [Cancer Res 2007;67(13):6342–50]

Introduction
Centrosomes are small cytoplasmic organelles which consist of a pair of centrioles embedded in pericentriolar material and act as microtubule organizing centers (1). During mitosis, centrosomes function as spindle poles, directing the formation of bipolar spindles, a process essential for accurate chromosome segregation (2, 3). Centrosomes duplicate precisely once per cell cycle to assure spindle bipolarity, with each daughter cell receiving one centrosome upon cytokinesis.

Centrosome amplification has been frequently observed in both solid tumors and hematologic malignancies, and is linked to tumorigenesis and aneuploidy (4–8). The extent of centrosomal aberrations is correlated with the degree of chromosomal instability and clinical aggressiveness of the malignant neoplasias (9–12). In mitosis, supernumerary centrosomes can lead to the formation of multipolar spindles which are responsible for chromosome malsegregation with subsequent aneuploidy and which can be found in many tumor types (5). Multipolar spindles, however, are antagonistic to cell viability. Most progeny derived from a defective mitosis will undergo apoptosis, but few daughter cells, receiving the appropriate chromosome complement and gene dosage, will survive and contribute, via clonal selection, to a population of aneuploid tumor cells. The survivors, however, must overcome the condition of supernumerary centrosomes in order to divide efficiently. To regain secondary karyotype stability, many tumor cells have developed a mechanism termed centrosomal clustering that prevents the formation of multipolar spindles by coalescence of multiple centrosomes into two functional spindle poles (13–15).

Centrosome positioning in the center of interphase cells is accomplished by pulling forces applied to microtubules by dynein, which serves to keep the centrosome away from the cell margin, and microtubule pushing by actomyosin-driven forces directed toward the cell center (16). Several pieces of evidence suggest that the minus end–directed microtubule motor protein dynein is involved in microtubule minus end–bundling for the establishment of bipolar spindles (17–19). A current model suggests that the function of dynein to tether spindle pole microtubules into bundles requires NuMA, which might use the motor activity of dynein to become localized to centrosomes (20). At the spindle poles, it forms a matrix to hold microtubule minus ends together. Analogous, in cells with multiple centrosomes, centrosomal clustering seems to be mediated by dynein (14). Recent data show that only cells with spindle-associated dynein were capable of coalescing multiple centrosomes into two spindle poles. Spindle multipolarity, on the other hand, was found to follow the overexpression of NuMA which interferes with the spindle localization of dynein (14). With the exception of the involvement of dynein and NuMA, the molecular mechanisms responsible for clustering of multiple centrosomes into two spindle poles of tumor cells are unknown.

The only known small molecules that specifically affect the mitotic machinery target either tubulin (21) or the plus end–directed motor protein Eg5, a mitotic kinesin required for spindle bipolarity (22). Whereas Vinca alkaloids and taxanes disrupt
spindle function by inhibiting or increasing microtubule polymerization, inhibition of Eg5 activity by monastrol leads to impaired microtubule-dependent centrosome separation and formation of monopolar spindles. Both Vinca alkaloids and taxanes are used as anticancer drugs and Eg5 is currently evaluated as a potential target for antineoplastic drug development (23). However, neither microtubule poisoning nor Eg5 inhibition selectively affects tumor cells, explaining the side effects and dose limitations of antimitotic drugs in clinical use.

Supernumerary centrosomes almost exclusively occur in a wide variety of neoplastic disorders but not in nontransformed cells. Therefore, inhibition of centrosomal clustering with consequential induction of multipolar spindles and subsequent cell death would specifically target tumor cells with no effect on normal cells with a regular centrosome content. To identify cell-permeable small molecules that inhibit centrosomal clustering in cells with supernumery centrosomes, we developed a cell-based screening strategy founded on the visualization of microtubules and chromatin.

Natural products have proved to be rich sources of novel anticancer lead compounds during the past 20 years (24). Therefore, we decided to screen a fungal extract library for compounds inhibiting centrosomal clustering. The fungal extracts were selected based on a chemotaxonomic screening approach (25), in order to increase the chemodiversity to be tested. An initial screening effort using extracts from different *Penicillium* species led to the identification of griseofulvin as an inhibitor of centrosome coalescence in several different tumor cell lines.

### Materials and Methods

**Cell culture.** All cell lines were cultured in DMEM (PAA Laboratories) supplemented with 10% FCS (PAA Laboratories). UPCECS114 (S114) cells stably expressing green fluorescent protein (GFP)-α-tubulin were generated by transfection (Fugene 6). (Roche Diagnostics) of the transgene in pEGFP-C1 (Clontech) and maintained under selective pressure by the addition of geniticin (Invitrogen). Primary normal human epidermal keratinocytes (NHEK; PromoCell) were cultured in Keratinocyte Growth Medium 2 (PromoCell). When indicated, griseofulvin (Sigma) was added to the culture medium. Griseofulvin was dissolved in DMSO (Sigma). In all experiments, the final DMSO concentration was 0.1%.

**Preparation of fungal extracts.** For fungal strains, preparation of fungal extracts, fractionation of fungal extracts, isolation of pure compounds, identification of griseofulvin by high-performance liquid chromatography–diode array detection–mass spectrometry (HPLC-DAD-MS), and synthesis of griseofulvin derivatives, full experimental details can be found in the supplementary information accompanying this article.

**Antibodies.** The following antibodies were used: mouse monoclonal antibodies to Eg5 (Transduction Laboratories), α-tubulin (DM1A), γ-tubulin (GTU-88; Sigma), β-tubulin (A1), ε-tubulin (H280), PARP (F-2; Santa Cruz), dynein light intermediate chain (Chemicon International), and NuMA (Calbiochem); rabbit polyclonal antibodies to γ-tubulin, centrin (Sigma), pericentrin (Covance), actin (I-19; Santa Cruz), and phospho-S10-histone H3 (Upstate Biotechnology). A mouse monoclonal antibody to centrin and a rabbit polyclonal antibody to α-Tubulin were kindly provided by J.L. Salisbury and E.A. Nigg, respectively.

**Immunofluorescence.** Immunofluorescence staining was done as described (26). The following fluorochrome-conjugated secondary antibodies were used: anti-rabbit Alexa 488 (Molecular Probes; Invitrogen) and anti-mouse Cy3 (Jackson ImmunoResearch Laboratories). Immunostained cells were examined using a Zeiss Axiowert 200 M fluorescence microscope. Images were processed with Photoshop software (Adobe).

**Time-lapse video microscopy.** For live-cell imaging, GFP-α-tubulin expressing S114 cells were grown in CO2-independent Leibovitz medium (Life Technologies, Invitrogen) on plastic dishes (μ-dishes, Ibidi). Live-cell imaging was carried out using a Nikon TE2000-U inverted microscope equipped with differential interference contrast optics and an Orca AG camera (Hamamatsu), driven by NIS-Element AR software (Nikon). Individual GFP-α-tubulin expressing cells containing bipolar or multipolar spindles were detected by immunofluorescence and followed using differential interference contrast imaging.

**Flow cytometry.** Cell cycle analysis by flow cytometry including the quantification of cells in mitosis by phospho-S10-histone H3 staining was done as previously described (27).

**Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolium) assay.** The cytotoxicity assay was done as previously described (28).

**Isolation and analysis of human centrosomes.** Centrosomes from S114 cells were isolated as previously described (26, 29).

**Measurement of Annexin V–positive cells.** Phosphatidylserine externalization was analyzed using the Apoptosis Detection Kit I from Becton Dickinson according to the recommendations of the manufacturer.

### Results

**Screening procedure for the identification of small molecules that inhibit centrosomal clustering.** To identify small molecules that inhibit centrosome coalescence and thereby induce multipolar mitoses, we first generated S114 cells that stably express GFP-α-tubulin (Supplementary Fig, S1A). S114 is an oral squamous cell carcinoma cell line showing pronounced centrosomal clustering (14). Despite the presence of supernumerary centrosomes in 64.5% of S114 cells as detected by immunostaining against centrin and γ-tubulin, only 3.6% of the cells in mitosis harbored multipolar spindles (Supplementary Fig, S1B and C).

Because 13.6% of exponentially growing, unmanipulated S114 cells were in mitosis, sufficient mitotic cells for the evaluation of the spindle polarity status were available. GFP-α-tubulin expressing S114 cells were grown in 96-well plates to near-confluence, treated with *Penicillium* extracts for 7 h, fixed, and examined by fluorescence microscopy. Three hundred mitotic cells per well were analyzed with the percentage of mitotic cells with multipolar spindles being the read-out. Nine 4-fold dilutions of each extract, covering a final concentration range from micromolar to nanomolar were analyzed. On each plate, three wells were treated with DMSO only to generate a control population. Experiments were done twice in parallel to provide a replicate data set. Extracts producing a significant increase in the percentage of multipolar mitoses were fractionated by HPLC into 24 fractions each. Subsequently, all fractions were reanalyzed by the screening procedure described above. In positive fractions, the detection of compounds eluting from the HPLC column was done by UV detection and subsequent mass spectrometry. Major compounds in positive fractions were further purified for retesting using semi-preparative HPLC.

**Selection of fungal cultures for the natural product extract library.** To generate a natural product compound library representing a chemodiversity as large as possible, only one or few fungal strains of the same species should be included. This is because strains of the same species usually produce very similar metabolite profiles of up to hundreds of natural products, and thereby represent the same chemotype (25). For this study, it was therefore decided to include just two strains of each of the species that previously had been analyzed and chemotyped by HPLC-DAD-MS profiling (data not shown).

**Identification of griseofulvin from a *Penicillium* extract library.** Using the screening procedure described here, three extracts from the fungal extract library were found to induce a
significant increase in the percentage of multipolar mitoses (Supplementary Fig. S2). Two extracts originated from Penicillium berlincense (IBT 18288 and 19440), strongly indicating that the observed activity was due to the same compound(s) in the two extracts. The third extract originated from Penicillium faroense (IBT 22543). Therefore, extracts of IBT 18288 and IBT 22543 were further separated by semipreparative HPLC, and single fractions from a total of 24 fractions each were shown to account for the induction of spindle multipolarity (data not shown). Analysis by HPLC-DAD-MS of the positive fractions, as well as a further purified fraction (Supplementary Fig. S3), identified griseofulvin as the active compound.

Induction of multipolar mitoses by griseofulvin. Griseofulvin (Sigma) induced multipolar mitoses in a concentration-dependent fashion in four human cancer cell lines, but not in normal fibroblasts or NHEK (Fig. 1A). After 24 h of treatment with 100 μmol/L of griseofulvin, >86% of mitoses were multipolar in SCC114, HeLa, U2OS, and MCF7 cells. In contrast, 87% and 82% of mitotic fibroblasts and NHEK harbored bipolar spindles, respectively. At 20 μmol/L of griseofulvin, multipolar spindles ranged from 20% in MCF7 cells to 53% in SCC114 cells, whereas in BJ fibroblasts and NHEK, only 3% and 10% of metaphases were multipolar. Coimmunostaining of griseofulvin-treated SCC114 cells with antibodies to Eg5 and phospho-S10-histone H3 revealed that virtually all multipolar cells were indeed mitotic (Fig. 1B). To prove that each spindle pole in cells with multipolar spindles contains complete centrosomes and not only acentriolar centrosome-related bodies (30), SCC114 cells stably expressing GFP-α-tubulin were treated with griseofulvin and immunostained with an antibody to centrin as a centriolar marker. Almost all spindle poles in cells with multipolar spindles contained two centrin signals, clearly indicating that each spindle pole consists of a complete centrosome (Fig. 1C).

Induction of multipolar mitoses by griseofulvin. To examine the effect of multipolar mitosis induction on cell cycle progression, griseofulvin-treated cells were stained with propidium iodide and subsequently analyzed by flow cytometry. Treatment with griseofulvin induced a concentration-dependent G2-M cell cycle arrest in all cell lines examined (Fig. 2A; top; Supplementary Table S1). However, whereas 91.2 ± 3.9% and 65.6 ± 7.6% of SCC114 and HeLa cells were arrested in G2-M phase, respectively, only 19.1 ± 3.8% of BJ fibroblasts were in G2-M phase at 24 h after treatment with 20 μmol/L of griseofulvin. To discriminate between cell cycle arrest in G2 and M phases of the cell cycle, cells were immunostained with an antibody to phospho-S10-histone H3 as mitosis marker (27, 31). Two-variable flow cytometry analysis revealed that treatment with 35 μmol/L of griseofulvin induced an increase in the percentage of mitotic cells and a corresponding gradual decrease in the percentage of cells in G1, S, and G2 phases of the cell cycle (Fig. 2A, bottom). Whereas the percentage of SCC114 and HeLa cells in mitosis increased from 15.0 ± 8.9% and 4.0% prior to griseofulvin treatment to 67.3 ± 8.3% and 35.6% at 24 h, respectively, only 5.5% of BJ fibroblasts were in mitosis 24 h after 35 μmol/L of griseofulvin as compared with 4.1% prior to treatment. Importantly, the ability of griseofulvin to induce a G2-M arrest closely paralleled its capacity to induce multipolar spindles (Supplementary Fig. S4).

Inhibition of cell proliferation by griseofulvin. Next, we determined the ability of griseofulvin to inhibit cell proliferation in cancer cell lines and normal BJ fibroblasts. Again, the effect of griseofulvin on proliferation closely paralleled its ability to induce multipolar spindles and mitotic cell cycle arrest as shown in Figs. 1A and 2A. Griseofulvin inhibited cell growth in a concentration-dependent manner, with half-maximal inhibition occurring at 35 μmol/L in SCC114 and 75 μmol/L in HeLa cells (Fig. 2B). In BJ fibroblasts, even the highest griseofulvin concentration used (100 μmol/L) led to only 38 ± 1% growth inhibition. Thus, concentrations needed for the induction of both multipolar spindles and mitotic cell cycle arrest by griseofulvin correlate well with its ability to inhibit proliferation in SCC114. Normal fibroblasts, on the other hand, were less sensitive to griseofulvin-induced cytotoxicity, paralleling our findings with multipolar spindle induction and mitotic cell cycle arrest.

Induction of apoptosis by griseofulvin. Griseofulvin seemed to induce apoptosis in SCC114 as indicated by the detection of a sub-G1 peak by flow cytometry after 48 h of treatment (data not shown). Cell lysates were prepared from SCC114 cells treated with 35 μmol/L of griseofulvin and were evaluated for the induction of...
apoptosis by detection of the p85 fragment of PARP, a downstream target of activated caspase 3. PARP cleavage was detected at 24 h and strongly increased at 48 h (Fig. 3A). In addition, phosphatidyserine externalization was measured by Annexin V staining as another variable of apoptosis. In contrast to mock-treated SCC114 cells, 70.95 ± 0.39% of the cells treated with 35 μmol/L of griseofulvin stained positive for Annexin V after 48 h of incubation (Fig. 3B). To show that cells treated with griseofulvin do indeed die because of multipolar spindle formation, we examined the effect of griseofulvin on the fate of bipolar versus multipolar live SCC114 cells. Continuous time-lapse video microscopy of SCC114 cells treated with 35 μmol/L of griseofulvin revealed that 12 out of 15 cells (80%) containing multipolar spindles died after prolonged mitosis by apoptotic blebbing (Fig. 3C, top). In contrast, only one out of five (20%) SCC114 cells with a bipolar spindle examined eventually exited from mitosis despite the continuous presence of griseofulvin (Fig. 3C, bottom). This difference was statistically significant (t test; P = 0.009).

**Griseofulvin inhibits centrosome coalescence in interphase cells.** Like SCC114 cells, N115 mouse neuroblastoma cells contain large numbers of centrioles, and yet undergo mostly bipolar divisions (5, 13, 15). In these cells, multiple centrioles aggregate to single, unusually large “compound” centrosomes during interphase (Fig. 4A). To examine whether the inhibition of centrosomal clustering is mitosis-specific or also occurs in interphase cells, both mock- and griseofulvin-treated interphase N115 cells were immunostained for γ-tubulin. Analogous to mitotic cells, griseofulvin led to a concentration-dependent inhibition of centrosome coalescence in interphase cells (Fig. 4A). After 24 h of treatment with 50 μmol/L of griseofulvin, 68 ± 1.5% of N115 cells lacked clustering of centrioles. Instead, centrosomes were found to be dispersed all over the cytoplasm (Fig. 4B).

To evaluate whether the inhibition of centrosomal clustering in interphase cells was caused by the destruction of the microtubule network, both mock- and griseofulvin-treated interphase N115 cells were immunostained for α-tubulin. Griseofulvin inhibited the orderly formation of interphase microtubules in a concentration-dependent manner. Whereas a less severe phenotype with dispersion of multiple centrosomes within a single perinuclear α-tubulin meshwork predominated at lower griseofulvin concentrations (Fig. 4C and D), higher concentrations resulted in widely separated centrosomes leading to multiple microtubule nucleation sites with short and convoluted microtubules. Furthermore, a nuclear enlargement of cells treated with griseofulvin was observed even though the DNA content, as analyzed by flow cytometry, was not increased as compared with untreated control cells (Fig. 4A; data not shown).

**Griseofulvin depletes neither cytoplasmic dynein nor NuMA from the mitotic spindle.** Recently, it was proposed that overcoming centrosomal clustering involved the dissociation of cytoplasmic dynein from the mitotic spindle (14). In addition, overexpression of NuMA has been described to induce spindle multipolarity via delocalization of dynein from the spindle (14). To determine the localization of dynein and NuMA, both mock- and griseofulvin-treated SCC114 cells were immunostained for α-tubulin and NuMA (Fig. 5A and B). To examine whether the inhibition of centrosomal clustering is mitosis-specific or also occurs in interphase cells, both mock- and griseofulvin-treated interphase N115 cells were immunostained for α-tubulin. Griseofulvin inhibited the orderly formation of interphase microtubules in a concentration-dependent manner. Whereas a less severe phenotype with dispersion of multiple centrosomes within a single perinuclear α-tubulin meshwork predominated at lower griseofulvin concentrations (Fig. 4C and D), higher concentrations resulted in widely separated centrosomes leading to multiple microtubule nucleation sites with short and convoluted microtubules. Furthermore, a nuclear enlargement of cells treated with griseofulvin was observed even though the DNA content, as analyzed by flow cytometry, was not increased as compared with untreated control cells (Fig. 4A; data not shown).

**Griseofulvin does not affect major centrosome constituents.** To evaluate whether the inhibition of centrosomal clustering by griseofulvin was caused by interference with core centrosomal proteins, centrosomal extracts were prepared from both mock- and griseofulvin-treated SCC114 cells (26, 29). The levels and mobilities of centrin, c-Nap1, β-tubulin, and ε-tubulin remained unchanged.
when assayed by immunoblotting in centrosomal extract preparations after 24 h of incubation with 35 μmol/L of griseofulvin (Fig. 5C). Pericentrin and γ-tubulin levels increased slightly after griseofulvin treatment.

2'-substituted derivatives of griseofulvin have an enhanced capacity for multipolar mitosis induction. Recently, it has been described that 2'-substituted derivatives of griseofulvin display a stronger inhibitory activity on microtubule formation as compared with griseofulvin itself (32). To gain insight into the mechanism of multipolar spindle formation by griseofulvin, we synthesized griseofulvin derivatives with different 2'-substitutions (Fig. 6A). Whereas side chain elongation led to increased multipolar spindle formation with 2'-demethoxy-2'-proproxygriseofulvin (compound 5) being the most active compound and approximately 10 times more potent than griseofulvin itself, reduction of the 2'-substituent to a keto group completely abolished the activity (Fig. 6B).

Discussion

The overall goal of the study presented here has been to identify small molecules that induce the formation of multipolar spindles by the inhibition of centrosomal clustering in cells with supernumerary centrosomes. For that, we have developed a cell-based screening strategy employing squamous cell carcinoma cells which harbor extra copies of centrosomes and nevertheless divide in a strictly bipolar manner as a model system (14). Bipolar spindle formation in tumor cells is accomplished by the coalescence of multiple centrosomes into two spindle poles (13, 14). Because multipolar spindles do not alert the spindle checkpoint controlling the exit from mitosis (33) but aberrant cell divisions provoke apoptotic cell death (34, 35), we have speculated that interference with mechanisms leading to centrosomal clustering will force tumor cells with supernumerary centrosomes to undergo multipolar mitoses and consequently apoptosis.

Using a fungal extract library, this approach led to the identification of griseofulvin from three independent *Penicillium* extracts. Griseofulvin induced multipolar spindles by the inhibition of centrosome coalescence, mitotic arrest, and subsequent cell death in several tumor cell lines but not in normal fibroblasts. The mechanism of action seems to be the disruption of the interphase microtubule network by inhibition of microtubule polymerization, resulting in the dispersion of supernumerary centrosomes throughout the cytoplasm.

Griseofulvin, an orally active, nontoxic antifungal drug derived from several species of *Penicillium*, has been used for many years for the treatment of tinea capitis and other dermatophyte infections (36, 37). Early studies showed that griseofulvin inhibits mitosis in sensitive fungi in a manner resembling the actions of colchicine (21). The antiproliferative and antimitotic effects of griseofulvin in mammalian cells are weak, with inhibition requiring micromolar concentrations (38, 39). The question of whether inhibition of mitosis by griseofulvin involves microtubule depolymerization or some other action on microtubules in human cells has remained unsettled. Although griseofulvin is able to bind to
mammalian brain tubulin and is able to inhibit the polymerization of microtubules in vitro, conflicting results regarding its ability to interfere with the cytoplasmic microtubule network in vivo have been published (32, 38–41). Depending on the cell lines used, the griseofulvin concentrations necessary to disrupt microtubules ranged between 10 and 100 μmol/L. Comparable concentrations of griseofulvin led to mitotic arrest in several cell lines.

We have found that the griseofulvin concentrations necessary for the induction of multipolar spindles are similar to those required for the inhibition of mitosis and cell proliferation, suggesting that multipolar spindles lead to aberrant cell divisions and subsequent cell death. As a direct proof of this concept, we were able to show that griseofulvin-induced cytotoxicity was limited to cells with multipolar spindles; however, cells with bipolar spindles, despite experiencing a prolonged mitosis, eventually divide and survive in the presence of griseofulvin.

Multipolar mitosis induction by griseofulvin might be related to an effect seen with low concentrations of microtubule-stabilizing antimitotic drugs like paclitaxel, epothilone B, and discodermolide (42). Analogous to our results with griseofulvin, the drug concentrations required for growth inhibition with paclitaxel and discodermolide were close to the concentrations necessary for the induction of multipolar mitoses. In contrast to microtubule-stabilizing agents, microtubule-destabilizing drugs like vinblastine, colchicine, and nocodazole have been found to be unable to induce multipolar mitoses (42). Although griseofulvin formally belongs to the group of microtubule-destabilizing agents, we have shown here that it leads to a prominent induction of multipolar spindles.

Griseofulvin induces multipolar mitoses by inhibition of centrosomal clustering in tumor cells with supernumerary centrosomes, then no such effect should be seen in healthy cells with a normal centrosome content. In keeping with this prediction, griseofulvin did not induce multipolar cell divisions in normal fibroblasts and keratinocytes. Importantly, as compared with the tumor cell lines examined, the griseofulvin concentrations required to kill fibroblasts were considerably higher. Nevertheless, similar concentrations of the drug induced a G2-M phase arrest in both tumor cell lines and fibroblasts. These data support the recent

Figure 4. Inhibition of centrosomal clustering in interphase N115 cells by griseofulvin. A, exponentially growing N115 cells treated with 50 μmol/L of griseofulvin for 24 h captured in interphase were immunostained with anti-γ-tubulin. Mock-treated cells were used as a control. Arrows, aggregates of multiple centrioles. B, concentration-dependent inhibition of centrosome coalescence in interphase N115 cells after incubation with the indicated concentrations of griseofulvin for 24 h. C, disruption of the interphase microtubule network by griseofulvin. Exponentially growing N115 cells treated with 50 μmol/L of griseofulvin for 24 h captured in interphase were immunostained with antibodies to α-tubulin and γ-tubulin. At lower griseofulvin concentrations, a less severe phenotype with multiple centrosomes dispersed within a single perinuclear α-tubulin meshwork predominated (middle). At higher concentrations, only short and convoluted microtubules surrounding widely separated, cytoplasmically dispersed centrosomes were found (bottom). Mock-treated cells were used as a control (top). D, concentration-dependent disruption of the interphase microtubule network in N115 cells after incubation with the indicated concentrations of griseofulvin for 24 h.
suggestion that the mechanism underlying aberrant mitosis may not be the same as that responsible for mitotic blockage, and that the former determines the sensitivity of cells to griseofulvin and paclitaxel-like drugs (42).

Supernumerary centrosomes in tumor cells are usually found in close proximity to each other during interphase and become separated to form multipolar spindles only at the onset of mitosis (13). The mechanisms that hold multiple interphase centrosomes together but allow for the formation of multipolar spindles in late G2 phase are largely unknown but might be analogous to those controlling centrosome positioning in normal cells with two centrosomes (16). Drugs that interfere with centrosome coalescence might exert their action either during interphase or specifically in cells approaching mitosis. Interestingly, inhibition of centrosome clustering by griseofulvin was not restricted to mitotic cells but did occur during interphase as well. As microtubules are critically involved in the positioning of centrosomes at the center of interphase cells and griseofulvin binds to tubulin in vitro, we reasoned that the declustering of multiple centrosomes was caused by the destruction of the interphase microtubule network. In keeping with this assumption, we found that griseofulvin led to a concentration-dependent disruption of interphase microtubules, with dispersion of centrosomes within a single perinuclear α-tubulin mass at lower concentrations and completely disorganized, short, and convoluted microtubules surrounding widely separated centrosomes at higher concentrations. Importantly, the griseofulvin concentrations necessary for the disruption of interphase microtubules were similar to those needed for loss of interphase centrosomal clustering on one hand and the induction of multipolar spindles on the other hand. These findings suggest that in tumor cells with multiple centrosomes, induction of multipolar mitoses by griseofulvin was brought about by the suppression of centrosome coalescence via disruption of the interphase microtubule network. In further support of this hypothesis, we found that 2'-demethoxy-2'-propoxygriseofulvin, a griseofulvin analogue which has recently been reported to display a stronger inhibitory activity on microtubule formation as compared with griseofulvin itself (32), was also significantly more active with regard to the induction of multipolar spindle formation.

Although binding of griseofulvin to both the α- and β-subunits of mammalian brain tubulin has been described (43), its mechanism of interaction with tubulin is still poorly understood. Interactions of microtubule-targeted drugs with centrosome-specific forms of tubulin like γ-, δ-, and ε-tubulin have long been debated (21, 42). Because paclitaxel induces multipolar mitoses similar to griseofulvin and has been reported to preferentially bind to centrosomes (44), interference with centrosomal tubulins may be an additional mechanism responsible for the inhibition of centrosomal clustering.
Overexpression of NuMA has been described to perturb the ability of tumor cells to coalesce supernumerary centrosomes into a bipolar spindle (14). This effect seems to be mediated by delocalization of the motor protein dynein from the mitotic spindle as a prerequisite of multipolar spindle formation (14). In contrast to these findings, the induction of multipolar spindles by griseofulvin was not associated with the depletion of dynein or NuMA from the mitotic spindle or with changes in the expression levels of these proteins. Also, the inhibition of dynein activity by orthovanadate did not diminish the capacity of griseofulvin to induce multipolar spindles. Together with our observation that inhibition of centrosomal clustering by griseofulvin was not restricted to mitotic cells but could be observed in interphase cells as well, these data argue for a mechanism of multipolar spindle formation that is independent from spindle-associated dynein.

In view of its ability to stabilize microtubule dynamics and to inhibit microtubule polymerization in human cells, it is remarkable that griseofulvin is well-tolerated when given to humans for the treatment of fungal infections. However, in vitro griseofulvin induces numerous chromosomal aberrations in all the systems analyzed (36). In mice and rats, prolonged treatment with griseofulvin provoked the development of multiple hepatomas (36). Of note, in adult rats and mice, ~90% of hepatocytes were polyploid (46). In these animals, mononuclear 4N hepatocytes arise from binuclear hepatocytes via clustering pairs of centrosomes at opposing spindle poles and subsequent progression through a bipolar mitosis (46). From this, aneuploidy induction and formation of hepatocarcinomas would be predicted based on the ability of griseofulvin to prevent centrosomal clustering and to induce multipolar cell divisions. Apart from considerably shorter treatment times, another reason for the lack of hepatocarcinoma induction by griseofulvin in man might be the comparatively low number of polyploid cells (20–30%) in the adult human liver (46).

In summary, by taking advantage of the tumor-specific phenotype of centrosomal clustering and a chemotaxonomic approach for maximization of chemodiversity to be tested, we have developed a combined screening strategy that might lead to the identification of new drugs which selectively target tumor cells and spare healthy tissues. The identification of griseofulvin from three independent Penicillium extracts proves the effectiveness of the cell-based screening strategy described here. Together with a recent report indicating that griseofulvin, either alone or in combination with nocodazole, inhibits tumor formation in nude mice (47), and in view of its lack of significant toxicity in humans, the data presented here supports the notion that griseofulvin might be useful for the treatment of cancer.

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**Figure 6.** 1,2-substituted derivatives of griseofulvin have an enhanced capacity for multipolar mitosis induction. A, structures of a series of novel 1,2-substituted griseofulvin analogues. 1, Griseofulvin; 2, (2S,6R)-(7-chloro-4,6-dimethoxybenzofuran-3-on)-2-spiro-1-(6-methyl-cyclohexane-2',4'-dione) (griseofulvic acid); 3, (2S,6R)-(7-chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1-(2'-ethoxy-6'-methyl-cyclohex-3-en-4'-one); 4, (2S,6R)-(7-chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1-(2'-isoproxy-6'-methyl-cyclohex-2'en-4'-one); 5, (2S,6R)-(7-chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1-(6'-methyl-2'-isoproxy-cyclohex-2'en-4'-one) (2'-demethoxy-2'-isoproxy-griseofulvin). B, concentration-dependent induction of multipolar mitotic spindles in SCC114 cells by 1,2-substituted derivatives of griseofulvin. Cells were incubated with the indicated concentrations for 24 h.

in cells with supernumerary centrosomes. However, we did not find a significant impairment of centrosome assembly by griseofulvin. The levels and mobilities of major centrosomal constituents including centrin, c-Nap1, 6-tubulin, and α-tubulin, representing components of centrioles, pericentriolar matrix, and centriolar linker structures remained completely unchanged when assayed by immunoblotting in centrosomal extract preparations. Pericentrin and γ-tubulin levels slightly increased after griseofulvin treatment, fitting to the mitotic recruitment of these proteins to each individual spindle pole (45). Therefore, although not completely excluded, interference of griseofulvin with centrosome assembly seems unlikely.

Recently, the microtubule-associated proteins NuMA and dynein have been implicated as a critical part of the centrosome-coalescing machinery in cells with amplified centrosomes (14).
Identification of Griseofulvin as an Inhibitor of Centrosomal Clustering in a Phenotype-Based Screen

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