GF-15, a Novel Inhibitor of Centrosomal Clustering, Suppresses Tumor Cell Growth In Vitro and In Vivo

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Abstract

In contrast to normal cells, malignant cells are frequently aneuploid and contain multiple centrosomes. To allow for bipolar mitotic division, supernumerary centrosomes are clustered into two functional spindle poles in many cancer cells. Recently, we have shown that griseofulvin forces tumor cells with supernumerary centrosomes to undergo multipolar mitoses resulting in apoptotic cell death. Here, we describe the characterization of the novel small molecule GF-15, a derivative of griseofulvin, as a potent inhibitor of centrosomal clustering in malignant cells. At concentrations where GF-15 had no significant impact on tubulin polymerization, spindle tension was markedly reduced in mitotic cells upon exposure to GF-15. Moreover, isogenic cells with conditional centrosome amplification were more sensitive to GF-15 than parental controls. In a wide array of tumor cell lines, mean inhibitory concentrations (IC50) for proliferation and survival were in the range of 1 to 5 μmol/L and were associated with apoptotic cell death. Importantly, treatment of mouse xenograft models of human colon cancer and multiple myeloma resulted in tumor growth inhibition and significantly prolonged survival. These results show the in vitro and in vivo antitumor efficacy of a prototype small molecule inhibitor of centrosomal clustering and strongly support the further evaluation of this new class of molecules. Cancer Res; 72(20); 1–12. ©2012 AACR.

Introduction

Centrosomes are small cytoplasmic organelles, which consist of a pair of centrioles embedded in pericentriolar material and act as microtubule-organizing centers. During mitosis, centrosomes function as spindle poles, directing the formation of bipolar spindles, a process essential for accurate chromosomal segregation (1, 2). Centrosomes duplicate precisely once per cell cycle to assure spindle bipolarity, with each daughter cell receiving one centrosome upon cytokinesis. Centrosome amplification is frequent in both solid tumors and hematologic malignancies, and is linked to tumorigenesis and aneuploidy (3–10). The extent of centrosomal aberrations correlates with the degree of chromosomal instability and malignant behavior in tumor cell lines, mouse tumor models, and human tumors (6, 9–12).

In mitosis, supernumerary centrosomes can lead to the formation of multipolar spindles, which is a hallmark of many tumor types (8). Multipolar cell division, however, is antagonistic to cell viability (13, 14). Most progeny derived from a multipolar mitosis will undergo apoptosis. To circumvent this problem, many cancer cells seem to have mechanisms that suppress multipolar division, the best studied being clustering of supernumerary centrosomes into 2 spindle poles enabling bipolar division (8, 13–20). Bipolar spindle formation via centrosomal clustering is associated with an increased frequency of lagging chromosomes during anaphase, thereby explaining the link between supernumerary centrosomes and chromosomal instability (14, 15).

The mechanisms of centrosomal clustering in tumor cells are incompletely understood. Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest the involvement of the spindle assembly checkpoint and spindle tension as controlled by the cortical actin cytoskeleton, cell adhesion molecules as well as centrosome and kinetochore components in this process (19, 20).

Supernumerary centrosomes are almost exclusively found in a wide variety of neoplastic disorders but rarely 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 regular centrosome content (8, 17). Using a phenotype-based screening strategy, we have recently found that griseofulvin induces spindle multipolarity, mitotic arrest, and subsequent cell death in multiple tumor cell lines but not in diploid fibroblasts and keratinocytes with normal centrosome content (13). Chemical optimization of griseofulvin led to the development of compounds with significantly increased activity and mean inhibitory concentrations (IC50) of proliferation and survival in the lower micromolar range when applied to the human squamous cell carcinoma cell line SCC114, which had been used for the initial screening (21).

Here, we present evidence that the exposure of tumor cells to the griseofulvin derivative GF-15 leads to reduced spindle tension, spindle multipolarity, and the inhibition of centrosomal clustering, culminating in the induction of apoptosis in vitro and in vivo.

Materials and Methods

Materials

Materials and Methods

\((2S,6R)-(\text{7-Chloro}-4,6\text{-dimethoxy-benzofuran-3-on})-2\text{-spiro}
\((2\text{-benzoyloxy-6\text{-methylcyclohex}-2\text{-en-4-one}) \text{-(2\text{-benzyloxy-2\text{-demethoxygriseofulvin}})}\) GF-15 was synthesized following the procedures described recently (21), Caspase-8, caspase-9, γ-tubulin, BubR1, and PARP antibodies were purchased from Santa Cruz Biotechnology. The centrin antibody was kindly provided by J. Salisbury (Rochester).

Cell culture

All human multiple myeloma (MM) cell lines (RPMI-8226, OPM-2, NCI-H929, OPM-1, KMS-12BM, KMS-12PE, KMS-11, KMS-18, U-266, MM1S, L85, Dox80, MM1.R) and primary patient MM cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Harlan), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro). Leukemia lines used were HEL, MOLM14, and KU812 and cultured as described above. Solid tumor cell lines comprised HeLa (cervical carcinoma), HT29, HCT116, SW480 (colorectal carcinoma), PANC1, PACA1 (pancreatic carcinoma), and LN229 (glioblastoma). HS4, KM105, KM104 (bone marrow stromal cells, BMSC), THLE3 (liver cells), peripheral blood mononuclear cells (PBMC), and primary BMSCs served as nonmalignant controls. All solid tumor cell lines and nonmalignant controls were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated FBS (Harlan), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro). All cell lines are regularly authenticated by fingerprinting before backup freezing and are kept less than 4 months in culture.

Isolation of patient tumor cells

Patients provided informed consent in accordance with the Declaration of Helsinki. Following approval by the Institutional Review Board of the University of Heidelberg, MM patient cells (96% CD38+ CD45RA-) were obtained as described (22).

Cell lysis and immunoblotting

Cell lysis and Western blot analysis were conducted as described previously (22).

Evaluation of cell viability

Cell viability was examined using the MTT (Sigma Chemical) colorimetric assay, as previously described (23). Briefly, cells were plated in 96-well microtitre plates at a density of 2–3 × 104 cells per well, and each plate was incubated for 24 and/or 48 hours, with MTT added to each well for at least 4 hours. The absorbance of each well was measured at 570/630 nm using a spectrophotometer (Molecular Devices). Each condition was analyzed in at least 3 replicates, and the results are presented as the mean ± SD of replicates of a representative experiment that was repeated at least 3 times.

DNA synthesis and cell proliferation assay

Cell proliferation was assessed by measuring [3H]-thymidine uptake, as described in prior studies (22).

Measurement of caspase-3/7 activation

Caspase-3/7 activation was analyzed using the Apoptosis Detection Kit from Promega according to the recommendations of the manufacturer.

Flow cytometry

Cell-cycle analysis by flow cytometry was conducted as previously described (13).

Immunofluorescence

Immunofluorescence staining was conducted as described (24). The following fluorochrome-conjugated secondary antibodies were used: anti-rabbit Alexa 488 (Molecular Probes) and anti-mouse Cy3 (Jackson ImmunoResearch Laboratories). Immunostained cells were examined using a Zeiss Axiovert 200 M fluorescence microscope. Images were processed with Photoshop software (Adobe).

Tubulin polymerization assay

The effect of GF-15 on tubulin polymerization was assessed using the tubulin polymerization assay kit (Cytoskeleton) according to the manufacturer’s recommendation.

Xenograft mouse models

To determine the in vivo activity of GF-15, beige-nude Xid mice were inoculated s.c. in the right flank with 3 × 106 OPM2 or HT29 cells in 100 mL RPMI 1640 medium, together with 100 mL matrigel (Becton Dickinson Biosciences). When a tumor was measurable, mice were assigned to a GF-15 treatment group or the control group. GF-15 was dissolved in 100% dimethyl sulfoxide (DMSO) and given daily 5 times a week by intraperitoneal injection for indicated periods. The control group received the carrier alone at the same schedule and route of administration. Tumor burden was measured every alternate day using a calliper [calculated volume = 4π/3 × (width/2)2 × (length/2)]. Animals were sacrificed when their tumors reached 2 cm in diameter, became exulcerated, or when the mice were moribund. Survival was evaluated from the first...
EC_{50} = 25 \mu mol/L

Griseofulvin

EC_{50} = 0.9 \mu mol/L

GF-15

A

Sensitivity and resistance to Griseofulvin and GF-15.

B

Histology images showing normal bipolar, regular bipolar, declustered, and aberrant mitotic cells.

C

Graph showing relative fluorescence units vs. time for Control, 25, 50, 75, and 100 \mu mol/L.

D

Supernumerary centrosomes (%)

E

Interkinetochore distance

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F

\(\alpha\)-tubulin, BubR1, DAPI, and Merge images for DMSO, Taxol, GF-15.

G

Relative fluorescence units vs. time for Control, 25, 50, 75, and 100 \mu mol/L.
day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

**Preparation of the radiolabeled GF-15 analogue**

\(1^{25}\text{I}^{131}\text{I}^{2-}(2,5'-6')\text{R}^{7-}(7-\text{Chloro}-4,6-\text{dimethoxy-benzofuran-3-one})-2\text{-spiro-1'-}(2'-6'-\text{methyl-cyclohex-2'-ene-4'-one})\) was prepared by thallation-iodination with \(1^{25}\text{I}^{131}\text{I}^{2-}\)iodide or \(1^{31}\text{I}^{131}\text{I}^{2-}\)iodide (Perkin Elmer) of \((2,5'-6')\text{R}^{7-}(7-\text{chboro}-4,6-\text{dimethoxy-benzofuran-3-one})-2\text{-spiro-1'-}(6'-\text{methyl-2'-}(4-\text{trimethylsilylbenzoxyl})-cyclohex-2'-ene-4'-one)\), which was in turn synthesized from \(p\)-trimethylsilylbenzyl alcohol (25) using a known method (21). The radiolabeled analogue was compared with a sample of \((2,5'-6')\text{R}^{7-}(7-\text{chboro}-4,6-\text{dimethoxy-benzofuran-3-one})-2\text{-spiro-1'-}(2'-4(\text{iodobenzoxyl})-6'-\text{methyl-cyclohex-2'-ene-4'-one})\) prepared from \(p\)-iodobenzyl alcohol (22) and found to be identical by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis.

**Scintigraphic in vivo imaging**

For imaging studies, 200 \(\mu\text{L}\) of a solution of the \(1^{25}\text{I}^{131}\text{I}^{2-}\)labeled GF-15 analogue (5 MBq/mouse) was injected into the tail vein of 6-week-old female NMRI mice. Scintigraphic images were taken using a gamma camera (Biospace). The accumulation of the radioactive tracer was monitored by static planar images at 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours after injection.

**Biodistribution studies**

\(1^{31}\text{I}^{131}\text{I}^{2-}\)labeled GF-15 analogue (1 MBq/mouse) was injected via the tail vein of 6-week-old female NMRI mice. At the time points specified, the animals were sacrificed, weighed, and dissected. Organs or tissues were blotted dry and weighed. The radioactivity was measured in a \(\gamma\)-counter along with a sample of the injection solution to calculate the percentage of injected dose per gram of tissue (% ID/g).

**Stability experiments**

The serum stability was determined by incubation of the \(1^{25}\text{I}^{131}\text{I}^{2-}\)labeled GF-15 analogue in human serum at 37°C. All cohorts were taken at several points in time, and the degradation was stopped by precipitation of the serum proteins with acetonitrile. After incubation for 30 minutes at 0°C and a further centrifugation step the clear supernatant was analyzed by reverse-phase HPLC on a Chromolith Performance RP-18e 100 \(\times 4.6\) mm column using water and acetonitrile containing 0.1% trifluoroacetic acid as the eluent.

**Isobologram analysis**

For combination studies, data from MTT assays were converted into values representing the fraction of growth affected (FA) in drug-treated versus untreated cells and analyzed using CalcuSyn software program (Biosoft) based on the Chou-Talalay method. A combination index (CI) smaller than 0.9 indicates synergism, whereas 0.9 to 1.1 indicates additive and more than 1.1 antagonistic effects.

**Statistical analysis**

The statistical significance of differences observed in GF-15-treated versus control cell cultures and mice was determined using an unpaired Student \(t\) test or a 1-way ANOVA with Dunnett’s Multiple Comparison Test for interkinetochore distances. Overall survival in animal studies was measured using the Kaplan–Meier method (\(\ast\), \(P > 0.01\); \(\ast\ast\), \(P > 0.001\)).

**Results**

**GF-15 leads to multipolar mitosis induction in the upper nanomolar range**

Recently, we have shown that 2'-modified derivatives of griseofulvin are more potent inducers of multipolar mitoses when compared with griseofulvin itself (21). Initial testing for the ability to inhibit centrosomal clustering was conducted in SCC114 cells, an oral squamous cell carcinoma line showing pronounced centrosome amplification (13, 18). GF-15 (2'-benzoxyl-2'-demethoxygriseofulvin) is significantly more potent with regard to the induction of spindle multipolarity than griseofulvin (Fig. 1A). The \(EC_{50}\) value of GF-15 for multipolar spindle induction was 900 nmol/L, corresponding to a 27-fold increase in activity compared with griseofulvin. Importantly, SCC114 cells that became resistant to GF-15 after long-term culture with increasing doses (0.2–1 \(\mu\text{mol/L}\)) of the compound over a period of 10 weeks, showed significantly less centrosome amplification and formed fewer multipolar spindles upon treatment with therapeutic doses (5 \(\mu\text{mol/L}\)) of the drug (Fig. 1B, Supplementary Fig. S1).

To test for the contribution of centrosome declustering to total multipolar mitosis induction after treatment with GF-15 is a potent and specific inducer of spindle multipolarity. A, chemical structures of griseofulvin and GF-15. SCC114 cells stably expressing \(\alpha\)-tubulin were treated with increasing concentrations of griseofulvin or GF-15 for 24 hours. \(EC_{50}\) of spindle multipolarity was assessed by immunofluorescence microscopy. B, SCC114 cells resistant to GF-15 after long-term culture with increasing concentrations of GF-15 display significantly fewer cells with supernumerary centrosomes than wild-type SCC114. Centrosomes were counted in interphase cells by \(\gamma\)-tubulin/centrin communostaining. \(\ast\ast\), \(P < 0.001\), C, in PC-3 prostate cancer cells, GF-15 induces centrosomal declustering (Declustered) in cells with amplified centrosomes and spindle multipolarity by other means (aberrant) in cells with regular centrosome content in a concentration-dependent manner. In the dashed line depicts the overall percentage of PC-3 cells with centrosome amplification. D, spindle phenotypes of PC-3 cells upon treatment with vehicle only (D', D'') or GF-15 (D''', D'''').

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15, the PC-3 prostate carcinoma cell line, which harbors supernumerary centrosomes in 28 ± 4% of the cells, was treated with increasing concentrations of the drug for 24 hours (Fig. 1C). Intriguingly, at the highest analyzable concentration (1.5 μmol/L), GF-15 induced centrosome declustering—as defined by the detection of 2 centrioles at each spindle pole of multipolar mitoses (Fig. 1D)—in 27 ± 4% of the cells, thereby closely matching the total percentage of cells with supernumerary centrosomes. At the lowest concentration tested (0.375 μM), multipolarity induction was mostly due to inhibition of centrosomal clustering. With increasing doses of GF-15, the contribution of multipolarity induction by other means gradually increased. This phenomenon was arbitrarily termed "aberrant," defined by the presence of centrioles at only 2 spindle poles despite cells undergoing multipolar mitoses. From these results, it may be concluded that the mechanisms responsible for holding supernumerary centrosomes together might be similar to the forces that bundle microtubules into a bipolar spindle array in cells with a regular centrosome content. However, differential sensitivities of both mechanisms may provide a therapeutic window to preferentially target centrosome clustering at lower dose levels.

Recently, we and others have shown that spindle tension is required for centrosomal clustering (19, 20). To directly measure spindle tension, we determined interkinetochore distances in mitotic PC-3 cells with either bipolar or multipolar spindles after treatment with 1 μmol/L GF-15 for 24 hours (Fig. 1E). Tension across sister kinetochores was substantially reduced by GF-15, as indicated by shorter interkinetochore distances in multipolar metaphase cells. To a lesser but still significant extent, interkinetochore distances were also reduced in metaphase cells that remained bipolar despite treatment with GF-15. If spindle tension is disturbed, the spindle assembly checkpoint cannot get sufficiently satisfied (19, 29). Treatment of HeLa–PLK4 cells with increasing concentrations of GF-15 for 24 hours, starting 48 hours after induction of PLK4 expression by addition of doxycycline, led to a dose-dependent, marked increase in the frequency of multipolar anaphases with declustered centrosomes (Fig. 2A and B; Supplementary Fig. S3). In addition, compared with uninduced cells, GF-15 preferentially decreased the viability of HeLa cells after PLK4-induced centriole overduplication (Fig. 2C).

**GF-15 causes multipolar anaphases and cell death in cells with supernumerary centrosomes**

Cells with supernumerary centrosomes only rarely undergo multipolar divisions. Instead they pass through a transient multipolar intermediate state followed by centrosome clustering and bipolar anaphase (14, 15). To ascertain that GF-15 indeed induces multipolar cell divisions and subsequent cell death in cells with supernumerary centrosomes, HeLa cells were induced to contain extra centrioles by conditional overexpression of PLK4 (28; Supplementary Fig. S2). In these cells, in the second cell cycle after centriole overduplication, supernumerary centrioles disengage before duplication and multipolar intermediates are common in the following mitosis (14, 29). Treatment of HeLa–PLK4 cells with increasing concentrations of GF-15 for 24 hours, starting 48 hours after induction of PLK4 expression by addition of doxycycline, led to a dose-dependent, marked increase in the frequency of multipolar anaphases with declustered centrosomes (Fig. 2A and B; Supplementary Fig. S3). In addition, compared with uninduced cells, GF-15 preferentially decreased the viability of HeLa cells after PLK4-induced centriole overduplication (Fig. 2C).

**GF-15 is active against a broad spectrum of cancer cell lines in vitro**

Next, we examined the effect of GF-15 on the growth of several different cancer cell lines. GF-15 exhibits potent cytoxicity in a concentration-dependent manner against a broad spectrum of tumor cell types including colon, cervix, glioblastoma, pancreas, leukemia, and myeloma-derived cell lines (Fig. 3A). As compared with solid tumor cell lines, MM and leukemia cell lines were particularly susceptible to the cytotoxic and antiproliferative effect of GF-15 with IC₅₀ values ranging from 1 to 2.5 μmol/L. In contrast, GF-15 did not induce significant cytotoxicity in nonmalignant control cell lines (Fig. 3A) or PBMCs from healthy volunteers even after stimulation with phytohemagglutinin (PHA), providing an overall selectivity index of 10- to 30-fold when compared with IC₅₀ values of cancer cell lines (Fig. 3B). These data suggest that GF-15 exhibits both potent and selective cytotoxicity against malignant cells.

**GF-15 is rapidly eliminated in vivo**

Derived from its parental molecule griseofulvin, GF-15 has been modified at the 2’-position (Fig. 1A). We therefore sought to analyze the in vivo stability and pharmacokinetics of this new compound. By introducing a p-iodobenzyl group in the 2’-position of the griseofulvin molecule, we generated a 125I-labeled GF-15-analogue. HPLC analysis showed only slow degradation of this molecule in human serum with a half-life...
of 48 hours at 37°C. The cleavage products resulting from degradation are presumably 4-iodobenzyl alcohol and griseofulvic acid, consistent with the analogue undergoing hydrolysis (data not shown). After i.v. application of trace amounts of this analogue, rapid renal clearance was observed within the first 6 hours after injection (Fig. 3C and D). In light of the clearance data and the poor solubility of GF-15 at higher concentrations, we went on to investigate its in vivo efficacy after intraperitoneal application.

GF-15 exhibits in vivo antitumor activity in xenograft mouse models

In view of the potent and selective in vitro activity of GF-15 against different cancer cell lines, we next examined the in vivo effect of GF-15 on human tumor growth in immunodeficient mice. Two cohorts, each consisting of 30 immunodeficient beige-nude-Xid mice were inoculated with either 3 \times 10^6 OPM2 myeloma or HT29 colon cancer cells s.c. in the right flank. Treatment with a daily dose of 20 mg/kg (10 mice per cell line) or 100 mg/kg (10 mice per cell line) intraperitoneally 5 days per week for 2 weeks was started when tumors became palpable. Ten mice per cell line served as a control cohort and received intraperitoneal injections of the vehicle alone. GF-15 treatment decreased tumor growth in all cohorts of treated mice with a greater effect in the group that received 100 mg/kg intraperitoneally (Figs. 4A, Supplementary Fig. S4). Kaplan–Meier and log-rank analysis revealed a significant prolongation of survival for the GF-15-treated mice inoculated with OPM2 myeloma cells compared with the vehicle-treated controls (log-rank \( P < 0.001 \); Fig. 4B). For mice inoculated with HT29 colon cancer cells survival analysis was not feasible because tumors in the control group rapidly became exulcerated and therefore had to be sacrificed according to institutional regulations. The toxicity profile of GF-15 seems to be quite favorable as body weight was not affected by treatment with GF-15 compared with untreated controls (Fig. 4C). Importantly, examination of histologic tumor sections revealed a dose-dependent, significant increase of aberrant and multipolar mitoses in the GF-15-treated mice compared with controls (\( P < 0.01 \) for 20 mg/kg, \( P < 0.001 \) for 100 mg/kg; Fig. 4D and E). In addition, GF-15 significantly increased the mitotic index in treated tumors versus controls (Supplementary Fig. S5).

Induction of spindle multipolarity, mitotic arrest and apoptosis by GF-15

As already depicted in Fig. 3A, myeloma cell lines are among those tumor types with particular susceptibility to GF-15. Because myeloma cells are known to harbor supernumerary centrosomes (3–5), we used MM cells as a model system for further in-depth analyses. Similar to OPM2, RPMI-8226, NCI-

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**Figure 2.** Centriole amplification sensitizes cells to treatment with GF-15. A, dose-dependent declustering of amplified centrosomes and induction of multipolar (MP) versus clustered bipolar (BP) cell divisions by GF-15 in HeLa cells conditionally expressing PLK4, stained for α-tubulin (red), centrin (green), and DNA (blue); B, At least 100 anaphases of cells with amplified centrioles were counted for each concentration. The first panel from the top shows a regular bipolar cell division without centriole amplification compared with the second panel with amplified centrioles clustered into bipolar spindle poles. The third panel displays partial declustering of amplified centrioles resulting in tripolar cell division, whereas the fourth panel represents aberrant multipolarity in a cell without amplified centrioles (second centriole at lower pole out of focus). See also Supplementary Fig. S3. Scale bar, 5 μm. C, viable cells were assessed by MTT cleavage during the last 4 hours of 48-hour cultures of HeLa-PLK4 cells with and without doxycycline (±Dox, 48 hours) with increasing concentrations of GF-15. All quantitative data shown are the mean ± SD of 3 independent experiments conducted in triplicate.
H929, OPM1, and KMS12BM myeloma cells, myeloma cell lines resistant to doxorubicin (Dox40), melphalan (LR5), and dexamethasone (MM1.R) are susceptible to GF-15 as well (Fig. 5A and B). Moreover, primary myeloma cells freshly isolated from the bone marrow of 3 (out of 10) heavily pretreated patients with relapsed myeloma showed marked cytotoxic effects upon treatment with GF-15. Lack of efficacy in the remaining 7 myeloma samples was most likely due to lack of proliferation, a
This was in stark contrast to their corresponding proliferating BMSCs, which displayed virtually no cytotoxicity when exposed to GF-15 (Fig. 5C). Likewise, after 24 hours of treatment with 3 µmol/L GF-15, no significant induction of spindle multipolarity could be detected in mitotic primary BMSCs, whereas more than 80% of mitoses were multipolar in NCI H929, OPM2, and RPMI 8226 cells (Fig. 5D). To examine the effect of multipolar mitosis induction on cell-cycle progression, starvation-synchronized OPM2 cells were exposed to GF-15, stained with propidium iodide, and subsequently analyzed by flow cytometry. GF-15 induced a pronounced G2–M cell-cycle arrest within 12 hours of treatment followed by an increase of the sub-G1 population compared with mock-treated cells (Fig. 5E, top panel). Indicating the induction of apoptosis, the increase of the sub-G1 population was concentration-dependent (Fig. 5E, bottom panel), analogous to the effect of griseofulvin in SCC114 cells (13). To further verify apoptotic cell death triggered by GF-15, protein profiling in GF-15-treated MM cells showed dose-dependent cleavage of caspase-8, caspase-9, caspase-3, and PARP (Fig. 5F). Cleavage fragments of these proteins became detectable at 0.5 µmol/L and strongly increased at 3 µmol/L of GF-15. Importantly, exposure of primary BMSCs to 3 µmol/L of GF-15 for 24 hours did not induce activation of effector caspases-3 and -7 compared with OPM2 cells (Fig. 5G).

GF-15 inhibits myeloma cell growth triggered by BMSCs

In addition to the effects mediated by growth factors and cytokines within the myeloma bone marrow
Figure 5. GF-15 specifically induces growth inhibition, spindle multipolarity, cell-cycle arrest, and apoptosis in MM cells. A and B, dose-related effects of GF-15 on cell survival (48 hours; A) and proliferation (24 hours; B) on indicated MM cell lines. Cell proliferation was assessed by uptake of [3H]-thymidine during the last 8 hours of 24-hour cultures. C, GF-15 differentially inhibits cell survival of primary cells from MM patients compared with patient BMSCs. D, GF-15 selectively induces multipolar mitotic spindles in MM cells (NCI H929, OPM2, RPMI 8226; left to right) compared with primary BMSCs from 3 MM patients. At least
microenvironment, direct myeloma–stroma contact also triggers tumor cell growth and mediates drug resistance. We therefore evaluated the effect of GF-15 on myeloma cell proliferation induced by the stimulatory effect of BMSCs (Fig. 6A and B). Binding of OPM2 or RPMI-8226 cells to primary BMSCs triggered increased myeloma cell proliferation, which was completely abrogated by GF-15. Importantly, as also shown in Figs. 5C and D, GF-15 did not impact on BMSCs, as determined by MTT assays and spindle polarity analysis.

**Evaluation of combinations of GF-15 with other antimyeloma agents**

Clinical experience in the therapeutic management of MM supports the notion that drug combinations can induce higher response rates when compared with single-agent treatment (23, 30). We therefore evaluated the effects of combinations of GF-15 with other established anti-MM drugs on the viability of MM cells. Specifically, GF-15 was combined with conventional agents (melphalan) as well as with more recently developed compounds (bortezomib). Although GF-15 together with bortezomib resulted in additive effects according to isobologram analysis (e.g., CI = 0.98 for 1.5 µmol/L GF-15 with 1.5 nmol/L bortezomib), the combination with melphalan led to a marked abrogation of GF-15 induced cytotoxicity (CI = 1.4 for 3 µmol/L GF-15 with 5 µmol/L melphalan, Fig. 6C). This is consistent with an S-phase arrest induced by the DNA-damaging drug melphalan, thereby preventing entry into mitosis of melphalan-exposed cells and underlines the specificity of GF-15 for cells in G2–M phase of the cell cycle.

**Discussion**

We show here that GF-15, a derivative of griseofulvin, leads to multipolar cell division, loss of spindle tension, centrosomal declustering, and subsequent tumor-specific cell death both in tissue culture and in xenograft mouse models.

Griseofulvin has been used for many years for the treatment of dermatophyte infections (31). Mechanistically, it inhibits mitosis in sensitive fungi (32) and mammalian cells (33, 34) but whether mitotic arrest is a consequence of microtubule depolymerization or some other action on microtubules in both fungi and human cells is still unclear (27, 28, 33, 35). Interestingly, in a recent comparison of analogues in fungal and mammalian cells we found that GF-15 was less active against dermatophytes than griseofulvin (36). Although griseofulvin has been reported to bind to mammalian brain tubulin and to inhibit microtubule polymerization in vitro, it does so only at concentrations significantly higher than those needed for spindle multipolarity induction in cancer cells (13, 35). Also, whether griseofulvin binds to tubulin directly or to microtubule-associated proteins remains unclear (27, 35, 37, 38). It was reported more than 30 years ago that griseofulvin treatment induces spindle multipolarity with each mitotic center containing 2 centrioles in HeLa cells in the absence of significant spindle microtubule depolymerization (33). In accordance with these findings, our data show that griseofulvin significantly inhibits polymerization of purified tubulin only at the highest concentration tested (100 µmol/L), although induction
of spindle multipolarity by griseofulvin occurred at an EC\textsubscript{50} of 25 \textmu M in SCC114 cells. Similarly, GF-15 inhibits tubulin polymerization only at concentrations of or above 25 \textmu M, whereas the EC\textsubscript{50} value for multipolar spindle induction was 900 \textmu M in SCC114 cells for this compound. These findings suggest that induction of spindle multipolarity by griseofulvin and its analog GF-15 is not sufficiently explained by their inhibitory action on tubulin polymerization.

It has recently been shown that cells with supernumerary centrosomes pass through a transient multipolar spindle intermediate state before centrosome clustering and subsequent bipolar anaphase occur (14, 15). At low concentrations GF-15 leads to multipolar metaphases with centrioles at each pole in cells with extra centrioles. At higher concentrations spindle multipolarity with acentrosomal spindle pole formation is induced. This is consistent with the concept that clustering extra centrosomes in cancer cells might be mechanistically related to focusing microtubules into a bipolar spindle array in normal cells. Analysis of GF-15-treated HeLa cells conditionally overexpressing PLK4 revealed that spindle multipolarity is carried on into anaphase/telophase with centrioles at each pole in a substantial proportion of cells with PLK4-induced centriole amplification. Moreover, treatment with GF-15 increased the death rates of HeLa cells after induction of PLK4 expression, suggesting that GF-15 preferentially kills cells with amplified centrioles.

Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest that only an intact spindle assembly checkpoint allows for sufficient time for centrosomal clustering to occur and that spindle tension is necessary for clustering of supernumerary centrosomes into a bipolar mitotic spindle array (19, 20). Determination of spindle tension in mitotic cells revealed that tension across sister kinetochores was substantially reduced by GF-15 in both multipolar metaphase cells and metaphase cells that remained bipolar despite treatment with GF-15. In addition to microtubule depolymerization or stabilization at higher concentrations, most microtubule-interacting drugs, including griseofulvin, have been proposed to exert their effects by suppressing microtubule dynamics (35, 38). Interestingly, interference with microtubule dynamics has been described to cause loss of spindle tension across kinetochores (39, 40). Therefore, it is conceivable that GF-15 inhibits microtubule dynamic instability with subsequent loss of spindle tension, centrosome declustering in cells with supernumerary centrosomes, multipolar cell division, and ultimately cell death.

For GF-15, the EC\textsubscript{50} value for multipolar spindle induction in vitro was 900 \textmu M in SCC114 cells, corresponding to a 27-fold increased activity compared with griseofulvin itself. Also, whereas griseofulvin inhibits cell proliferation only weakly with half-maximal inhibition occurring at 25 \textmu M (13), GF-15 led to inhibition of tumor cell growth in vitro at IC\textsubscript{50} values of 1 to 3 \textmu M, with almost no impact on the viability of nonmalignant control cells. Similarly, although 3 \textmu M GF-15 effectively decreased the viability of primary patient myeloma cells, the drug induced neither spindle multipolarity nor cell death in primary BMSCs or primary PBMCs even after mitogenic stimulation. In vivo, GF-15 was well tolerated and effective in mouse myeloma and colon cancer xenograft models, as evidenced by significant inhibition of tumor growth, the induction of spindle multipolarity in tumor xenograft cells and protracted survival in mice treated with either 20 or 100 mg/kg GF-15 intraperitoneally daily for 5 days a week. Because GF-15 is only poorly soluble in water and its biologic half-life is short with little drug left 1 hour after administration, it is expected that optimization of medicinal chemistry and application schedules will lead to a further improvement of the drugs potency and of this novel therapeutic strategy in general.

In summary, we have shown that GF-15 potently inhibits tumor cell growth in vitro and in vivo. From a mechanistic point of view our data reveal that GF-15 reduces spindle tension, possibly via inhibition of microtubule dynamic instability, leading to spindle multipolarity in cells with supernumerary centrosomes, similar to what has been described for the siRNA-mediated depletion of several spindle and kinetochore components (19, 20). These observations, coupled with GF-15’s lack of major toxicity in a preclinical mouse model and the lack of significant toxicity of griseofulvin in humans, provide the framework for further clinical development of GF-15 in particular and centrosomal cluster inhibitors in general, directed at improving patient outcome.

Disclosure of Potential Conflicts of Interest

K.C. Anderson is an ACS Clinical Research Professor. No potential conflicts of interest were disclosed by the other authors.

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