GF-15, a novel inhibitor of centrosomal clustering, suppresses tumor cell growth in vitro and in vivo

Marc S. Raab1,2,3, Iris Breitkreutz3,4, Simon Anderhub5, Mads H. Rønnest6,7, Blanka Leber5, Thomas O. Larsen7, Ludmila Weiz2,5, Gleb Konotop2, Patrick J. Hayden3, Klaus Podar3,4, Johannes Fruehaut8, Felix Nissen9, Walter Mier9, Uwe Haberkorn9, Anthony D. Ho1, Hartmut Goldschmidt1,4, Kenneth C. Anderson3, Mads H. Clausen6, and Alwin Krämer1,5

1Dept. of Internal Medicine V, University of Heidelberg, Heidelberg, Germany; 2Max-Eder Group Experimental Therapies for Hematologic Malignancies, German Cancer Research Center (DKFZ), Heidelberg, Germany; 3Dept. of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; 4National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany; 5Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center and Dept. of Internal Medicine V, University of Heidelberg, Heidelberg, Germany; 6Center for Nanomedicine and Theranostics & Dept. of Chemistry, Technical University of Denmark, Kgs. Lyngby, Denmark; 7Center for Microbial Biotechnology, Department of Systems Biology, Kgs. Lyngby, Denmark; 8Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; 9Dept. of Nuclear Medicine, University Hospital Heidelberg, Heidelberg, Germany;

The authors disclose no potential conflict of interest.

Short title: Centrosomal cluster inhibition

Abstract word count: 199
Text word count: 4404
Figure count: 6
Suppl. figure count: 5
Reference count: 41

Correspondence: Alwin Krämer, Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center and Dept. of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany, Phone: +49-6221-42-1440; Fax: +49-6221-42-1444, Email: a.kraemer@dkfz.de
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 (IC\textsubscript{50}) for proliferation and survival were in the range of 1-5 \textmu M 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 demonstrate the \textit{in vitro} and \textit{in vivo} anti-tumor efficacy of a prototype small molecule inhibitor of centrosomal clustering and strongly support the further evaluation of this new class of molecules.
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 behaviour 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 appear to have mechanisms that suppress multipolar division, the best studied being clustering of supernumerary centrosomes into two 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 non-transformed 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 (IC\textsubscript{50}) 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\textsuperscript{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 \textit{in vitro} and \textit{in vivo}. 
Materials and methods

Materials

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'(2'-benzyloxy-6'-methylcyclohex-2'-en-4'-one) (2'-benzyloxy-2'-demethoxygriseofulvin; GF-15) was synthesized following the procedures described recently. Caspase 8, caspase 9, γ-tubulin, BubR1 and PARP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The centrin antibody was kindly provided by J. Salisbury (Rochester).

Cell culture

All human MM cell lines (RPMI-8226, OPM-2, NCI-H929, OPM-1, KMS-12BM, KMS-12PE, KMS-11, KMS-18, U-266, MM1.S, LR5, Dox40, MM1.R, PAT1) and primary patient MM cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA). 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), THLE3 (liver cells), peripheral blood mononuclear cells (PBMC), and primary bone marrow stromal cells (BMSC) served as non-malignant controls. All solid tumor cell lines and non-malignant controls were grown in DMEM medium supplemented with 10% heat-inactivated FBS (Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA). All cell lines are regularly authenticated by fingerprinting before back-up freezing and are kept less than four 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 (IRB) of the University of Heidelberg, MM patient cells (96% CD38+ CD45RA-) were obtained as described.

Cell lysis and immunoblotting

Cell lysis and Western blot analysis were performed as described previously.
Evaluation of cell viability
Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma Chemical, St Louis, MO) colorimetric assay, as previously described\textsuperscript{23}. Briefly, cells were plated in 96-well microtitre plates at a density of 2-3 x 10\textsuperscript{4} cells per well, and each plate was incubated for 24 and/or 48 h, with MTT added to each well for at least 4 h. The absorbance of each well was measured at 570/630 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). Each condition was analyzed in at least three replicates, and the results are presented as the mean ± standard deviation of replicates of a representative experiment that was repeated at least three times.

DNA synthesis and cell proliferation assay
Cell proliferation was assessed by measuring [$^{3}$H]-thymidine uptake, as described in prior studies\textsuperscript{22}.

Measurement of caspase 3/7 activation
Caspase 3/7 activation was analyzed using the Apoptosis Detection Kit from Promega (Mannheim, Germany) according to the recommendations of the manufacturer.

Flow cytometry
Cell cycle analysis by flow cytometry was performed as previously described\textsuperscript{13}.

Immunofluorescence
Immunofluorescence staining was performed as described\textsuperscript{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 GF15 on tubulin polymerization was assessed using the tubulin polymerization assay kit (Cytoskeleton, Denver, CO) 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 $3 \times 10^6$ OPM2 or HT29 cells in 100 mL RPMI 1640 medium, together with 100 mL matrigel (Becton Dickinson Biosciences, Bedford, MA). When a tumor was measurable, mice were assigned to a GF-15 treatment group or the control group. GF-15 was dissolved in 100% DMSO and given daily five times a week by i.p. 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 = $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$). 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 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**

$^{[125\text{I}/131\text{I}]-(2\text{S},6'\text{R})-(7\text{-Chloro-4,6-dimethoxy-benzofuran-3-one})-2\text{-spiro-1'-(2'-(4-iodobenzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) was prepared by thallation-iodination with 125I-iodide or 131I-iodide (Perkin Elmer, Dreieich, Germany) of (2\text{S},6'\text{R})-(7\text{-chloro-4,6-dimethoxy-benzofuran-3-one})-2\text{-spiro-1'-(6'-methyl-2'-(4-trimethylsilylbenzyloxy)cyclohex-2'-ene-4'-one))$, which was in turn synthesized from $p$-trimethylsilylbenzyl alcohol\textsuperscript{25} using a known method\textsuperscript{21}. The radiolabeled analogue was compared to a sample of \textit{(2S,6'R)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'- (4-iodobenzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one)} prepared from $p$-iodobenzyl alcohol\textsuperscript{22} and found to be identical by HPLC-MS analysis.

**Scintigraphic in vivo imaging**

For imaging studies, 200 µL of a solution of the $^{125\text{I}}$-labeled GF-15 analogue (5 MBq/mouse) was injected into the tail vein of six week old female NMRI mice. Scintigraphic images were taken using a gamma camera (Biospace, France). The accumulation of the radioactive tracer was monitored by static planar images at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after injection.

**Biodistribution studies**
131I-labeled GF-15 analogue (1 MBq/mouse) was injected via the tail vein of six 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 γ-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 125I-labeled GF-15 analogue in human serum at 37 °C. Aliquots were taken at several points in time, and the degradation was stopped by precipitation of the serum proteins with acetonitrile. After incubation for 30 min at 0 °C and a further centrifugation step the clear supernatant was analyzed by reverse-phase HPLC on a Chromolith Performance RP-18e 100 × 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, Ferguson, MO) 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 > 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’s t test or a one-way ANOVA with Dunnett’s Multiple Comparison Test for interkinetochore distances. Overall survival in animal studies was measured using the Kaplan-Meier method (* P>0.01; ** 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 mitosis when compared to griseofulvin itself. Initial testing for the ability to inhibit centrosomal clustering was performed in SCC114 cells, an oral squamous cell carcinoma line showing pronounced centrosome amplification. GF-15 (2'-benzyloxy-2'-demethoxygriseofulvin) is significantly more potent with regard to the induction of spindle multipolarity than griseofulvin (Figure 1A). The EC$_{50}$ value of GF-15 for multipolar spindle induction was 900 nM, corresponding to a 27-fold increase in activity compared to griseofulvin. Importantly, SCC114 cells that became resistant to GF-15 after long-term culture with increasing doses (0.2 – 1 μM) of the compound over a period of ten weeks, showed significantly less centrosome amplification and formed fewer multipolar spindles upon treatment with therapeutic doses (5 μM) of the drug (Figure 1B, Figure S1).

To test for the contribution of centrosome declustering to total multipolar mitosis induction after treatment with GF-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 h (Figure 1C). Intriguingly, at the highest analyzable concentration (1.5 μM), GF-15 induced centrosome declustering — as defined by the detection of two centrioles at each spindle pole of multipolar mitoses (Figure 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 two spindle poles despite cells were 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\textsuperscript{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 \( \mu \text{M} \) GF-15 for 24 h (Figure 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 during metaphase and BubR1 should remain at affected kinetochores. As shown in Figure 1F, kinetochores of multipolar metaphases in GF-15 treated cells stained positive for BubR1 similarly to cells exposed to low-dose taxol, used as a positive control. In contrast, BubR1 was absent from bipolar metaphases in vehicle treated control cells. Moreover, no MAD2 signal as a marker for disturbed spindle attachment could be detected at kinetochores of GF-15 induced multipolar metaphases, in contrast to kinetochores of cells treated with nocodazole as a positive control (data not shown). These findings demonstrate that GF-15 reduces spindle tension and thereby activates the spindle assembly checkpoint, but does not disturb microtubule attachment to kinetochores. This corroborates earlier findings showing that reduced spindle tension after siRNA-mediated depletion of kinetochore and spindle components leads to the formation of multipolar spindles\textsuperscript{19,20}.

**Effects of griseofulvin and GF-15 on tubulin polymerization in vitro**

To exclude the hypothesis that loss of spindle tension upon GF-15 exposure is merely a consequence of tubulin depolymerization, the effects of griseofulvin and GF-15 on the polymerization of purified porcine brain tubulin into microtubules *in vitro* were analyzed using a fluorescence assay\textsuperscript{25}. Corroborating earlier data, inhibition of brain tubulin polymerization required very high concentrations of griseofulvin\textsuperscript{26,31}. Similarly, the GF-15 concentrations needed for inhibition of tubulin polymerization (25 \( \mu \text{M} \)) were about 25-fold above those required for induction of spindle multipolarity (Figure 1G).
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\textsuperscript{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\textsuperscript{28} (Figure 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\textsuperscript{14,29}. Treatment of HeLa-PLK4 cells with increasing concentrations of GF-15 for 24 h, starting 48 h 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 (Figure 2A,B; Figure S3). In addition, compared to uninduced cells, GF-15 preferentially decreased the viability of HeLa cells after PLK4-induced centriole overduplication (Figure 2C).

GF-15 is active against a broad spectrum of cancer cell lines \textit{in vitro}

Next, we examined the effect of GF-15 on the growth of several different cancer cell lines. GF-15 exhibits potent cytotoxicity in a concentration-dependent manner against a broad spectrum of tumor cell types including colon, cervix, glioblastoma, pancreas, leukemia, and myeloma-derived cell lines (Figure 3A). As compared to solid tumor cell lines, multiple myeloma and leukemia cell lines were particularly susceptible to the cytotoxic and anti-proliferative effect of GF-15 with IC\textsubscript{50} values ranging from 1 - 2.5 $\mu$M. In contrast, GF-15 did not induce significant cytotoxicity in non-malignant control cell lines (Figure 3A) or peripheral blood mononuclear cells from healthy volunteers even after stimulation with phytohemagglutinin (PHA), providing an overall selectivity index of 10- to 30-fold when compared to IC\textsubscript{50} values of cancer cell lines (Figure 3B). These data suggest that GF-15 exhibits both potent and selective cytotoxicity against malignant cells.

GF-15 is rapidly eliminated \textit{in vivo}

Derived from its parental molecule griseofulvin, GF-15 has been modified at the 2'-position (Figure 1A). We therefore sought to analyze the \textit{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 $^{125}\text{I}$-labeled GF-15-analogue. HPLC analysis showed only slow degradation of this molecule in human serum with a half-life of 48 h at 37 °C. The cleavage products resulting from degradation are presumably 4-iodobenzyl alcohol and griseofulvinic 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 h after injection (Figure 3C,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 (i.p.) application.

**GF-15 exhibits in vivo anti-tumor 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 (BNX) 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 (ten mice per cell line) or 100 mg/kg (ten mice per cell line) i.p. five days per week for two weeks was started when tumors became palpable. Ten mice per cell line served as a control cohort and received i.p. 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 i.p. (Figures 4A, 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 $P<0.001$; figure 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 favourable as body weight was not affected by treatment with GF-15 compared with untreated controls (Figure 4C). Importantly, examination of histological 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 20mg/kg, $P<0.001$ for 100mg/kg; figure 4D,E). In addition, GF-15 significantly increased the mitotic index in treated tumors versus controls (Figure S5).
Induction of spindle multipolarity, mitotic arrest and apoptosis by GF-15

As already depicted in Figure 3A, myeloma cell lines are among those tumor types with particular susceptibility to GF-15. Since myeloma cells are known to harbor supernumerary centrosomes\textsuperscript{3-5}, we used multiple myeloma cells as a model system for further in-depth analyses. Similar to OPM2, RPMI-8226, NCI-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 (Figure 5A,B). Moreover, primary myeloma cells freshly isolated from the bone marrow of three (out of ten) heavily pre-treated patients with relapsed myeloma showed marked cytotoxic effects upon treatment with GF-15. Lack of efficacy in the remaining seven myeloma samples was most likely due to lack of proliferation, a common phenomenon of primary myeloma cells in tissue culture. This was in stark contrast to their corresponding proliferating bone marrow stromal cells (BMSC), which displayed virtually no cytotoxicity when exposed to GF-15 (Figure 5C). Likewise, after 24 h of treatment with 3 μM GF-15, no significant induction of spindle multipolarity could be detected in mitotic primary BMSCs, whereas >80% of mitoses were multipolar in NCI H929, OPM2, and RPMI 8226 cells (Figure 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 G\textsubscript{2}/M cell cycle arrest within 12 h of treatment followed by an increase of the sub-G\textsubscript{1} population compared to mock-treated cells (Figure 5E, upper panel). Indicating the induction of apoptosis, the increase of the sub-G\textsubscript{1} population was concentration-dependent (Figure 5E, lower panel), analogous to the effect of griseofulvin in SCC114 cells\textsuperscript{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 (Figure 5F). Cleavage fragments of these proteins became detectable at 0.5 μM and strongly increased at 3 μM of GF-15. Importantly, exposure of primary BMSCs to 3 μM of GF-15 for 24 h did not induce activation of effector caspases 3 and 7 compared to OPM2 cells (Figure 5G).

GF-15 inhibits myeloma cell growth triggered by bone marrow stromal cells

In addition to the effects mediated by growth factors and cytokines within the myeloma bone marrow 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 (Figure 6A,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 Figures 5C and 5D, GF-15 did not impact on BMSCs, as determined by MTT assays and spindle polarity analysis.

Evaluation of combinations of GF-15 with other anti-myeloma agents

Clinical experience in the therapeutic management of multiple myeloma supports the notion that drug combinations can induce higher response rates when compared with single-agent treatment23,30. 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). While GF-15 together with bortezomib resulted in additive effects according to isobologram analysis (e.g., CI=0.98 for 1.5 µM GF-15 with 1.5 nM bortezomib), the combination with melphalan led to a marked abrogation of GF-15 induced cytotoxicity (CI=1.4 for 3 µM GF-15 with 5 µM melphalan, Figure 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\(^{26,33,35}\). 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 \textit{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 two 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 \(\mu\text{M}\)), while induction of spindle multipolarity by griseofulvin occurred at an \(\text{EC}_{50}\) of 25 \(\mu\text{M}\) in SCC114 cells. Similarly, GF-15 inhibits tubulin polymerization only at concentrations of or above 25 \(\mu\text{M}\), whereas the \(\text{EC}_{50}\) value for multipolar spindle induction was 900 nM 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\cite{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\cite{35,39}. Interestingly, interference with microtubule dynamics has been described to cause loss of spindle tension across kinetochores\cite{40,41}. 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$_{50}$ value for multipolar spindle induction \textit{in vitro} was 900 nM in SCC114 cells, corresponding to a 27-fold increased activity compared to griseofulvin itself. Also, whereas griseofulvin inhibits cell proliferation only weakly with half-maximal inhibition occurring at 25 $\mu$M\cite{13}, GF-15 led to inhibition of tumor cell growth \textit{in vitro} at IC$_{50}$ values of 1 to 3 $\mu$M, with almost no impact on the viability of non-malignant control cell lines. Similarly, while 3 $\mu$M GF-15 effectively decreased the viability of primary patient myeloma cells, the drug induced neither spindle multipolarity nor cell death in primary bone marrow stromal cells or primary peripheral blood mononuclear cells even after mitogenic stimulation. \textit{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 prolonged survival in mice treated with either 20 or 100 mg/kg GF-15 i.p. daily for five days a week. Since GF-15 is only
poorly soluble in water and its biological half-life is short with little drug left 1 h after administration, it is expected that optimization of medicinal chemistry and application schedules will lead to a further improvement of the drug’s 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\textsuperscript{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.
Acknowledgement

We thank Ingrid Hoffmann, PhD, German Cancer Research Center, for providing the conditional HeLa-PLK4 cell line and J. Salisbury (Rochester) for the centrin antibody. We also thank Anja Baumann for excellent technical assistance. This work was supported by a grant of the Max-Eder-Program, Deutsche Krebshilfe (M.S.R.); the Hopp-Foundation (H.G.); the DFG (A.K.); a Deutsche Krebshilfe grant (M.S.R. and A.K.); the Tumorzentrum Heidelberg/Mannheim (A.K.); the Danish Research Council (ref. 274-07-0561) (M.H.R, T.O.L. and M.H.C); the Danish Cancer Society and Karen Krieger Fonden (M.H.C.); the National Institutes of Health grants RO CA50947, PO-1 CA78378, and P50 CA100707 (K.C.A.). K.C.A. is an ACS Clinical Research Professor.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Authorship

M.S.R. and I.B. contributed equally to this work. Contribution: M.S.R. designed, performed, and analyzed research and wrote the manuscript; I.B., S.A. and M.H.C. designed, performed, and analyzed research; M.H.R., B.L., T.O.L., L.W., G.K., P.J.H., K.P., J.F., F.N. performed and analyzed research; W.M., U.H., A.D.H., H.G., K.C.A. analyzed data and provided expert advice; A.K. designed the project, analyzed research and wrote the manuscript. All authors approved the final manuscript.
References


15. Silkworth WT, Nardi IK, Scholl LM, Cimini D. Multipolar spindle pole coalescence is a major source of kinetochore mis-attachment and chromosome mis-segregation in cancer cells. PLOS One 2009;4(8):e6564


Figure legends

Figure 1. GF-15 is a potent and specific inducer of spindle multipolarity. (A) Chemical structures of griseofulvin and GF-15. SCC114 cells stably expressing α-tubulin were treated with increasing concentrations of griseofulvin or GF-15 for 24 h, EC₅₀ 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 γ-tubulin/centrin co-immunostaining. (**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. 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‴″) according to their regular versus amplified centriole content. Cells were treated with GF-15 or vehicle only for 24 h, spindle poles were counted by γ-tubulin staining, centrioles by centrin staining. Inserts show enlargements of centrin signals at each spindle pole. All scale bars represent 5 µm (E) GF-15 reduces spindle tension in PC-3 cells exposed to GF-15 (1 µM; 24 h). Interkinetochore distances from multipolar and bipolar spindles (table) are given as mean and SD. Cells were stained for CREST (green), HEC1 (red), γ-tubulin (purple), and DNA (DAPI; blue) (right panel). Sister kinetochores were identified by spatial correlation of HEC1 and CREST as a marker of the interkinetochore space. Distances between corresponding HEC1 signals were measured. (F) GF-15 triggers the spindle assembly checkpoint. PC-3 cells were exposed to DMSO control, taxol (10nM) as a positive control and GF-15
(0.75 and 1.5 µM, respectively) for 24h and stained for α-tubulin, BubR1, and DAPI. BubR1 staining of kinetochores indicates an active spindle assembly checkpoint. (G) The effects of increasing concentrations of griseofulvin (GF, left) or GF-15 (right), respectively, on tubulin polymerization were measured by relative fluorescence intensity.

All quantitative data shown are the mean +/- SD of three independent experiments.

**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 (B) α-tubulin (red), centrin (green), and DNA (blue). 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 to 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 Figure 3. Scale bar represents 5 µm. (C) Viable cells were assessed by MTT cleavage during the last 4 h of 48 h cultures of HeLa-PLK4 cells with and without doxycycline (+/-Dox, 48h) with increasing concentrations of GF-15. All quantitative data shown are the mean +/- SD of three independent experiments performed in triplicate.

**Figure 3. GF-15 selectively inhibits growth of tumor cells in vitro and in vivo.**

(A) IC_{50} values of cell lines of indicated origins. Unless otherwise indicated, viable
cells were measured by MTT cleavage during the last 4 h of 48 h cultures. Data shown are the mean +/- SD of experiments performed in triplicate. (B) Peripheral blood mononuclear cells (PBMCs) of three healthy donors stimulated with phytohemagglutinin (PHA) do not show significant toxicity upon treatment with indicated concentrations of GF-15. PBMCs were incubated with PHA 24h before exposure to GF-15 for 48h. Column colors represent corresponding PBMC samples with or without PHA, respectively. Viable cells are expressed as x-fold of respective control. (C) Whole-body scintigraphic images of NMRI mice at indicated times after intravenous injection of a $^{125}$I-labeled GF-15 analogue. (D) Biodistribution of a $^{131}$I-labeled GF-15 analogue at different times after intravenous administration to NMRI mice ($n = 12$). Data are expressed as mean %ID/g +/- SD of each time point. ID: injected dose; p.i.: post injection. All quantitative data shown are the mean +/- SD of three independent experiments.

**Figure 4. GF-15 decreases tumor growth, prolongs survival and induces mitotic aberrations in a multiple myeloma xenograft mouse model.** (A) Beige-nude Xid mice were subcutaneously inoculated in the right flank with $3 \times 10^6$ OPM2 cells. Treatment by intraperitoneal injection (vehicle alone or indicated concentrations of GF-15) was started when tumors were measurable. Arrows indicate treatment stop. Tumor burden was measured every alternating day using an electronic caliper. Tumor volume is presented as means +/- SE. (B) Survival was evaluated using Kaplan-Meier curves and log-rank analysis. (C) Body weight was evaluated three times per week. Data shown are the mean +/- SD. (* $P<0.01$; ** $P<0.001$) (D) In H&E-stained tumor sections ($n = 3$ per cohort) at least 200 mitotic cells were analyzed for mitotic aberrations. (E) Dose-dependent effects of GF-15 on mitotic figures. Paraffin-
embedded sections of tumor tissue, explanted 24 h after last treatment, were H&E-stained and analyzed by light microscopy. Representative microscopic images are shown.

**Figure 5. GF-15 specifically induces growth inhibition, spindle multipolarity, cell cycle arrest, and apoptosis in multiple myeloma cells.** (A) Dose-related effects of GF-15 on cell survival (48 h) and (B) proliferation (24 h) on indicated multiple myeloma (MM) cell lines. Cell proliferation was assessed by uptake of [³H]-thymidine during the last 8 h of 24 h cultures. (C) GF-15 differentially inhibits cell survival of primary cells from MM patients compared to patient bone marrow stromal cells (BMSCs). (D) GF-15 selectively induces multipolar mitotic spindles in MM cells (NCI H929, OPM2, RPMI 8226; left to right) compared to primary BMSCs from three MM patients. At least 200 mitotic cells were counted for spindle polarity after staining for γ-tubulin, Eg5, and DAPI. (E) Synchronized OPM2 cells arrest in G₂/M phase upon treatment with GF-15 followed by an increase of the sub-G₁ population (upper panel). The increase of the sub-G₁ population is dose-dependent (lower panel). OPM2 cells were exposed for indicated times to indicated concentrations of GF-15 and subsequently stained with propidium iodide after ethanol fixation. (F) GF-15 triggers apoptotic cell death in MM cells. OPM2 cells were exposed to indicated concentrations of GF-15 for 24 h, followed either by immunoblot analysis of lysates with indicated antibodies or (G) ELISA-based assessment of activation of effector caspases 3 and 7. Identical treatment of BMSCs showed no significant induction of caspase 3/7 activation compared to OPM2 cells. Results are expressed as x-fold of control. CF: cleaved form of respective protein

All quantitative data shown are the mean +/- SD of three independent experiments performed in triplicate.
Figure 6. GF-15 abrogates the growth advantage of tumor cells conferred by adhesion to primary bone marrow stromal cells. (A-B) Indicated MM cell lines were cultured with or without primary bone marrow stromal cells (BMSCs). GF-15 was added at indicated concentrations and proliferation was measured by [³H]-thymidine uptake during the last 8 h of 24 h cultures. (C) Co-treatment of MM cells with melphalan partially abrogates the growth inhibitory effects of GF-15. OPM2 cells were treated with indicated concentrations of GF-15 with or without 5 or 10 μM. All quantitative data shown are the mean +/- SD of three independent experiments performed in triplicate.
Figure 1

A. Griseofulvin

Griseofulvin

EC<sub>50</sub> = 25 µM

B. Sensitive vs. Resistant

Supernumerary Centrosomes (%)

C. Mitotic cells (%)

D. γ-tubulin, centrin, DAPI, merge

Regular bipolar

Amplified clustered

E. Interkinetochore distance

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (µm)</th>
<th>SD (µm)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1.70</td>
<td>0.32</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Bipolar</td>
<td>1.52</td>
<td>0.32</td>
<td>0.01</td>
</tr>
<tr>
<td>GF-15</td>
<td>1.54</td>
<td>0.34</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Multi-polar</td>
<td>1.35</td>
<td>0.32</td>
<td>&gt; 0.01</td>
</tr>
</tbody>
</table>

F. α-tubulin, BubR1, DAPI, merge

DMSO

GF-15

G. Relative fluorescence units

GF

GF-15
Figure 2

A) Bar graph showing the percentage of anaphases with amplified centrosomes. The x-axis represents different concentrations of DMSO (0, 0.5, 0.75, 1, 1.25 μM), and the y-axis shows the percentage of anaphases with amplified centrosomes. The graph compares BP clustered and MP declustered conditions.

B) Images showing immunofluorescence staining of α-tubulin, centrin, DAPI, and a merge of all channels for different samples treated with DMSO and GF-15.

C) Line graph depicting the viable cell counts (% of control) for cells treated with and without Dox. The x-axis represents concentrations of GF-15 (0, 0.25, 0.5, 0.75, 1, 1.25 μM), and the y-axis shows the viable cell counts as a percentage of control.
Figure 3

A

Myeloma
Myeloma res.
Leukemia
Solid tumors
Non-malignant controls

0 10 20 30 40 50μM

B

PHA

PBMCs

Viable cells (x-fold)

0 0.5 1 1.5 2 2.5

0 5 10 30 50 μM

C

D

% ID / g

Blood Heart Lung Spleen Liver Kidney Muscle Intestine Brain Skin

10 min p.i.
30 min p.i.
1 h p.i.
4 h p.i.
8 h p.i.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2012 American Association for Cancer Research.
Figure 5

A: Graph showing the percentage of viable cells in control and treated samples over time. The x-axis represents the treatment time in hours (0h, 6h, 12h, 24h), and the y-axis represents the viability percentage.

B: Graph showing the percentage of proliferation in different cell lines. The x-axis represents the treatment time in hours (0h, 6h, 12h, 24h), and the y-axis represents the proliferation percentage.

C: Bar graph comparing the viability of primary MM cells and BMSCs treated with GF-15 at different concentrations. The x-axis represents the cell types, and the y-axis represents the viability percentage.

D: Bar graph comparing the spindle multipolarity in BMSCs and MM cell lines treated with GF-15 at 3 µM. The x-axis represents the cell type, and the y-axis represents the spindle multipolarity percentage.

E: Flow cytometry histograms showing the cell cycle distribution of cells treated with GF-15 and Mock for different times.

F: Western blot analysis showing the expression levels of various proteins (α-tubulin, Caspase-8, Caspase-9, Caspase-3, PARP, GAPDH) in BMSCs and OPM 2 treated with GF-15 at different concentrations (0, 0.5, 1, 3, 5 µM) after 24 hours.

G: Bar graph showing the fold change in Caspase 3/7 expression in BMSCs and OPM 2 treated with GF-15 at 3 µM compared to control.
Figure 6

(A) Graph showing \( ^{3}H(dT) \) uptake/cpm for BMSC, OPM2, and BMSC/OPM2 cells at different concentrations of GF-15 (0, 1, 3, 5 \( \mu M \)).

(B) Graph showing \( ^{3}H(dT) \) uptake/cpm for BMSC, RPMI 8226, and BMSC/RPMI 8226 cells at different concentrations of GF-15 (0, 1, 3, 5 \( \mu M \)).

(C) Graph showing viable cells (% of control) for control, 5 \( \mu M \), and 10 \( \mu M \) Mel at different concentrations of GF-15 (0, 1.5, 3 \( \mu M \)).
GF-15, a novel inhibitor of centrosomal clustering, suppresses tumor cell growth in vitro and in vivo

Marc S Raab, Iris Breitkreutz, Simon Anderhub, et al.

Cancer Res  Published OnlineFirst August 31, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-2026

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/08/31/0008-5472.CAN-12-2026.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.