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Griseofulvin Efficiently Induces Apoptosis in *In Vitro* Treatment of Lymphoma and Multiple Myeloma

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Abstract. *Background/Aim: Recent innovations in the development of systemic and targeted therapies have improved survival and quality of life in multiple myeloma (MM) patients. However, in most cases, this hematological malignancy of monoclonal B-lymphocytes remains incurable. Exaggerated Wnt/β-catenin signaling has been demonstrated in lymphoma and MM, therefore targeting related signaling molecules might represent a promising therapy approach. Griseofulvin, a widely used antifungal drug, is chemically related to other known Wnt-inhibitors and we recently demonstrated its potent in vivo efficacy in a murine myeloma model. Materials and Methods: The anti-tumor apoptotic effect of griseofulvin at doses ranging from 0.1-200 μM was investigated on a total of ten human and two murine myeloma/lymphoma cell lines, as determined by 3'3-dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. Results: Griseofulvin significantly induced apoptosis in all investigated myeloma and lymphoma cell lines in a dose-dependent manner, while healthy control cells were less sensitive. Conclusion: Given the known safety profile and apoptosis induction at low effective doses, our data warrant further in vitro and in vivo studies utilizing griseofulvin as a potential therapy agent for MM and lymphoma.*

Multiple myeloma (MM) represents a hematological neoplasm characterized by monoclonal malignant secretory plasma cells in the bone marrow and is commonly accompanied by monoclonal protein in peripheral blood and/or urine (1, 2). Innovative therapy strategies, including immunomodulatory drugs (IMiDs) like bortezomib, lenalidomide and thalidomide improve both treatment outcome and patient survival. Also cell-based therapies have been proven to be feasible and effective in initial clinical trials (3, 4). However, despite recent innovations, sustainable treatment strategies are still indispensable since the majority of patients might eventually experience relapse of disease.

The activation of the Wnt pathway, usually restricted to embryonic development, represents a tumor-specific signaling pathway and has been shown to induce and maintain oncogenic effects, particularly in the oncogenesis and promotion of lymphoma and MM (5-14). Hence, a specific inhibition of Wnt signaling suppresses tumor progression and, thereby, renders Wnt signaling molecules an interesting therapeutic target for MM (14, 15).

Our recent studies confirmed the *in vitro* and *in vivo* efficacy of several agents by targeting Wnt/beta-catenin signaling molecules, especially in hematopoietic types of cancer (16-30). Griseofulvin, as the investigated drug, is chemically related to other known Wnt inhibitors and has already shown anticarcinogenic properties *in vivo* (31). Here, we demonstrated *in vitro* treatment efficacy and selective induction of apoptosis by griseofulvin in a broad range of myeloma and lymphoma cells.

Materials and Methods

Cell lines and culture conditions. Cell lines were obtained from DSMZ (Braunschweig, Germany) or ATCC (LGC Standards, Wesel, Germany) and incubated at 37˚C with 5% CO_2 at 90 % humidity.

The human myeloma cell lines KMS 18, OPM-2, RPMI-8226 and U-266 (all obtained from DMSZ) were cultured in RPMI-1640-medium (PAA, Pasching, Austria), supplemented with 5% heat-inactivated fetal calf serum (FCS; Invitrogen, Darmstadt, Germany) and 1% penicillin-streptomycin (Seromed, Jülich, Germany). Human lymphoma cell lines Raji, SU-DHL-4, Oci Ly 8 Lam 53 and primary chronic lymphocytic leukemia (CLL) cells were cultured

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under identical conditions as human myeloma cell lines. MPC-11 is a murine plasmocytoma cell line and RAW 264.7 is a leukemia monocyte macrophage cell line. Cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FCS and 1% penicillin/streptomycin. RAW 264.7 cells were harvested by using 0.05% trypsin-EDTA solution (Invitrogen).

The human colon fibroblast cell line CCD-18Co was obtained from ATCC (LGC Standards) and cultured in ATCC-formulated Eagle's minimum essential medium (LGC Standards) supplemented with 15% of heat-inactivated FCS and 1% penicillin-streptomycin. Cells were harvested by 0.05% trypsin-EDTA solution (Invitrogen), centrifuged at 1,200 × *g* for 7 min and re-suspended in 1 ml media to define the cell count. Media were renewed at least every 3 days.

Human samples. Peripheral blood lymphocytes (PBLs) were isolated from blood samples of healthy volunteers using Ficoll density gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Blood from buffy coats was diluted 1:2 with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) (both from PAA) and used for a Ficoll gradient (Lymphoprep). The leukocyte layer was transferred to new tubes after centrifugation at 800 × *g* for 30 minutes. Cells were washed three times with PBS/1%BSA and re-suspended in RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 2.5% HEPES buffer solution (PAA).

Drugs and chemical reagents. Griseofulvin was purchased from Sigma-Aldrich (Steinheim, Germany) and tested at concentrations ranging from 0.1 to 200 μM for 72 h. For CCD-18Co cells, concentrations up to 400 μM were tested.

3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining. Reduced mitochondrial transmembrane potential is known to occur late in the apoptotic process. We used DiOC6 staining and flow cytometry to assess the mitochondrial transmembrane potential. Therefore, 1×10^5 cells were plated in 3 ml medium in 6-well plates. Griseofulvin was dissolved in dimethyl sulfoxide (DMSO) (Invitrogen) and added to the medium at different concentrations for three days. Staining with DiOC6 for detecting viable cells and with PI, which binds to DNA in necrotic cells, was used for the apoptosis assay, measured by a fluorescence-activated cell sorter (FACS) (BD FACSCanto II; Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The medium containing drug-treated cells was transferred from each well into a glass tube. Then, cells were centrifuged at 800 × *g* for 7 min, washed with phosphate buffered saline (PBS, pH 7.4) (Roti-Stock 10x, purchased from CarlRoth, Karlsruhe, Germany) and stained after repeated centrifugation by adding 500 μl staining solution (RPMI-1640, 0.5% bovine serum albumin (BSA), 80 nM DiOC6) for 15 min at 37˚C. After another washing step with PBS/1% BSA, cells were re-suspended in 500 μl PBS/1% BSA. FACS analysis was performed immediately after the addition of 5 μl PI solution (100 μg/ ml) with a BD FACSCanto II (Becton Dickinson Biosciences) flow cytometer. Approximately 10,000 counts were made for each sample. In this assay, viable cells show high fluorescence intensity for DiOC6 and a low fluorescence for PI. Necrotic cells fluoresce in an opposite manner, with high intensity for PI and a low intensity for DiOC6. Early apoptotic cells show low fluorescence for both DiOC6 and PI. Cells with high fluorescence intensity for both DiOC6 and PI correspond either to late apoptotic cells as apoptotic bodies or debris.

Table I. *Half-maximal inhibitory concentration (IC_{50}) of griseofulvin for human lymphoma, human and murine multiple myeloma, murine leukemia and control cell lines. CCD-18Co cells and peripheral blood lymphocytes (PBLs) served as controls. A total of 1×10^5 cells were cultured under different concentrations of griseofulvin for 72 h. Cell viability and apoptosis was measured by 3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. CCD-18Co cells were investigated by the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium-bromide (MTT) assay. Results represent the mean of data from three independent experiments each.*

	IC_{50} (μM)
Cell line	Griseofulvin
KMS 18	9 μM
OPM-2	45 μM
RPMI-8226	26 μM
U-266	18 μM
MPC-11	44 μM
Primary CLL cells	80 μM
Raji	33 μM
RAW 264,7	28 μM
Oci Ly 8 Lam 53	30 μM
SU DHL 4	22 μM
CCD-18Co	>400 μM
PBL	180 μM

Cell viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT). The efficacy of griseofulvin in CCD-18Co cells was determined by cell viability in MTT assay. Viable cells convert the yellow MTT (Sigma Aldrich) into purple formazan when taken-up into mitochondria. Previously, cells were plated at 1×10^4 well/ 100 μl in 96-well plates and left to adhere overnight in the incubator. Twenty-four h later media were removed and renewed containing various concentrations of griseofulvin. After 69 h, 1 μl MTT (5 mg/ml) was added to each well and incubated for another 3-h period. Then, 80 μl of the media were removed and 50 μl of acidified isopropanol was added for cell lysis. After shaking for 10 min, the amount of formazan was measured at 565 nM. The measured amount of formazan in treated cells was compared to untreated cells.

Statistical analysis. Values are given as mean±standard deviation (SD). At least three separate and independent experiments were performed with each cell line. Paired, two-tailed Student's *t*-test was used for statistical analysis. A *p*-value less than 0.05 was considered significant.

Results

Titration of griseofulvin. The mean 50% inhibitory concentration (IC_{50}) after 72 h was calculated following titration. Griseofulvin concentrations leading to a significant decrease in viability of all tested myeloma and lymphoma cells were, therefore, determined. PBLs and CCD-18Co colonic fibroblasts served as healthy controls. All, except CCD-18Co, cells were investigated by DiOC6 and PI staining

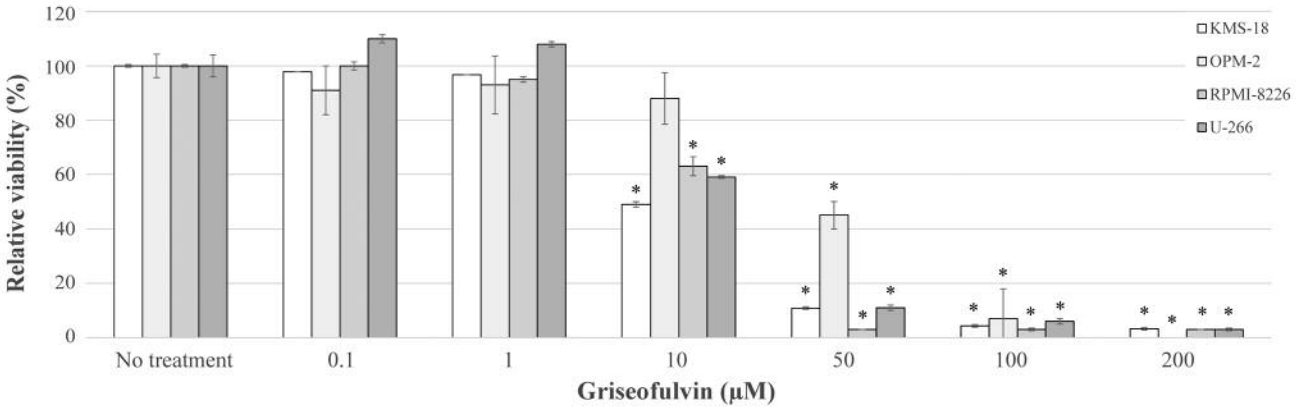

Figure 1. *Effect of griseofulvin on viability of KMS-18, OPM-2, RPMI-8226 and U-266 human myeloma cells. Cells were cultured with griseofulvin for 72 h. Cell viability and apoptosis was measured by 3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. Results represent data from three independent experiments. Data are shown as the mean±SD. *p<0.05 compared to untreated cells.*

in flow cytometry. CCD-18Co cells were investigated by MTT. IC_{50} values of griseofulvin employed after 72 h of incubation are given in Table I.

Effect of griseofulvin on viability of human myeloma cells. The viability of all investigated myeloma cells decreased in a concentration-dependent manner following the addition of griseofulvin. Low concentrations, starting from 10 μM, were required for a significant apoptosis induction in most human myeloma cells except OPM-2 cells, which tolerated doses up to 50 μM without a significant decline in viability. Results are shown in Figure 1. Figure 2 (Panel A and B) shows the corresponding flow cytometry results.

Effect of griseofulvin on viability of human lymphoma cells. Exposure to griseofulvin also significantly decreased lymphoma cell viability in all tested cell lines. The IC_{50} of Raji, SU-DHL-4 and Oci Ly 8 Lam 53 was attained after treatment with 33 μM, 22 μM and 30 μM, respectively; a significant induction of apoptosis was registered, comparably with myeloma cells, at a griseofulvin concentration of 10 μM. Primary CLL cells were least susceptible to the toxicity of griseofulvin with an IC_{50} of 80 μM. Figure 3 presents the respective results. Figure 2 (Panel C) presents the corresponding flow cytometry results.

Effect of griseofulvin on viability of murine cells. The effects of griseofulvin treatment in human myeloma and lymphoma cells were also reproducible in murine myeloma and leukemia monocyte macrophage cells. Required griseofulvin concentrations for a significant decrease of viability in RAW 264.7 cells were comparable to those for human myeloma and lymphoma cells and slightly higher in MPC-11 cells with an IC_{50} of 28 μM and 41 μM, respectively. Results are given in Figure 4.

Effect of griseofulvin on viability of healthy controls. We chose CCD-18Co colon fibroblasts and PBLs in order to analyze the toxicity of griseofulvin towards healthy stroma cells and lymphocytes, respectively. CCD18-Co cells and PBLs tolerated higher concentrations of griseofulvin compared to myeloma and lymphoma cell lines tested. Results are shown in Figure 5.

Discussion

MM represents a systemic malignant neoplasm caused by degenerated plasma cells, mainly due to frequent gene mutations and/or chromosomal translocations (30). Today's therapy schedules are built upon a primary initiated high-dose chemotherapy followed by facultative hematopoietic stem cell transplantation (32-35). Recent therapy innovations enriched our therapeutic repertoire and led to both increased patient survival and improved quality of life. However, up to now, most MM patients remain incurable when solely treated with chemotherapy (3, 36, 37).

Meanwhile, targeting tumor-specific signaling pathways promoting tumor differentiation and proliferation represents an established therapeutic approach in cancer research and treatment. As a promising example of abrogated signaling pathways, targeting canonical Wnt signaling might, thus, play a pivotal role for treatment of MM (10-14). Development and propagation of MM cells is, *inter alia*, dependent on the bone marrow microenvironment. Bone marrow stromal cells were shown to provide Wnt ligands encouraging an exaggerated proliferation of MM cells (37-39). The inhibition of Wnt/β-catenin signaling, in return, retards MM growth as evidenced by numerous *in vitro* and *in vivo* studies (40).

Our laboratory recently revealed several drugs as potent inducers of apoptosis in lymphoma and myeloma cells *in vitro* and partially proved *in vivo* efficacy in subsequent animal

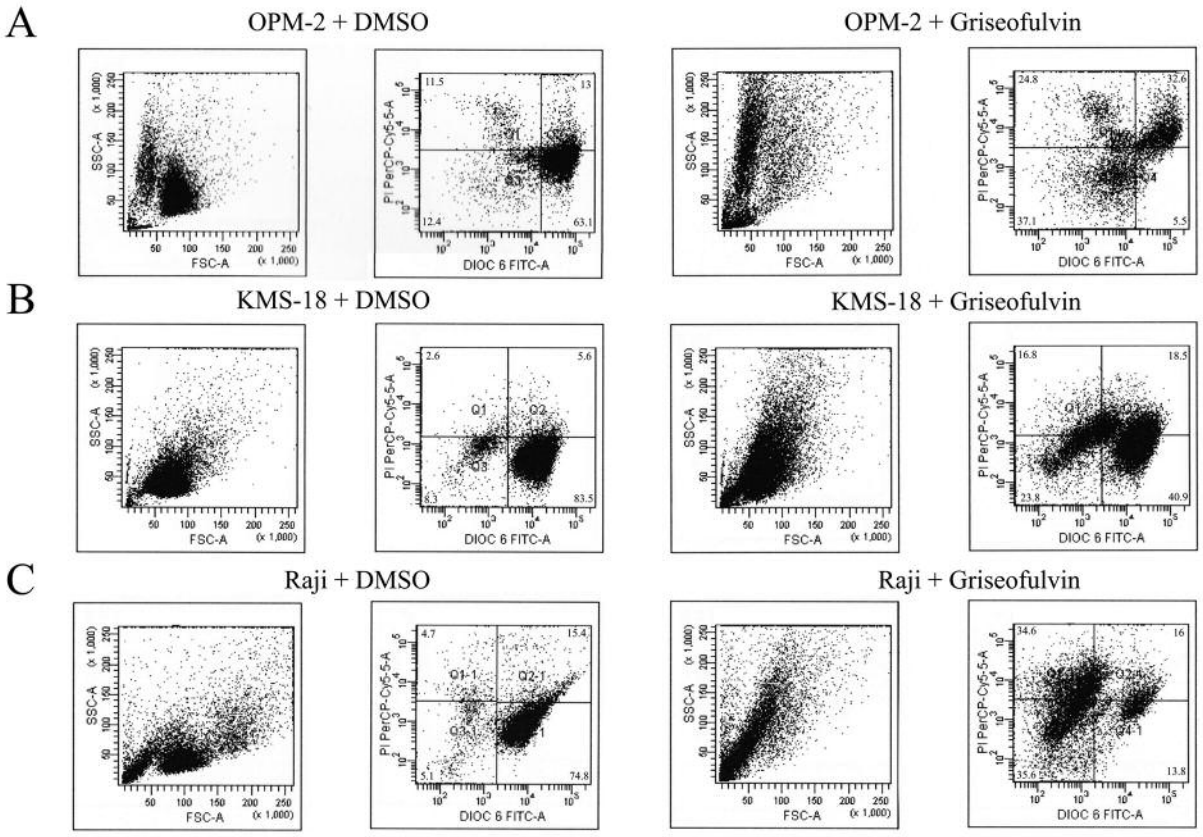

Figure 2. *Exemplary results generated by flow cytometry. Within the quarters, the relative number of cells is given in percentages. (A) OPM-2 cells before and after treatment with 100 μM griseofulvin. (B) KMS-18 cells before and after treatment with 10 μM griseofulvin. (C) Raji cells before and after treatment with 50 μM griseofulvin. Cells were treated with griseofulvin at different concentrations. Flow cytometry was performed seventy-two hours after incubation.*

studies. Four of these drugs were already shown to inhibit the Wnt pathway through targeting either β-catenin itself or its downstream factors (16-26, 40, 41). Owing to their chemical relationship to well-documented Wnt-inhibitors, such an inhibitory potential is also conceivable for the remaining agents.

Griseofulvin is an orally active antifungal drug, first isolated from *Penicillium griseofulvum* in 1939, used for the treatment of several dermatophytoses (39). Its mechanism of action was attributed to a selective inhibition of microtubule depolymerization and induction of abnormal cell mitosis by blocking at the G_2/M phase. These antiproliferative and antimitotic effects are merely weak in mammalian cells following the intake of sufficient fungicidal doses (42-44).

Griseofulvin-promoted abnormal microtubule stabilization was shown to induce a cascade of events leading to apoptosis. In HT 29 colorectal adenocarcinoma cells, low doses of 10 μM induced apoptosis, whereas higher doses of >20 μM initiated significant G_2/M arrest. Higher doses (>20 μM) caused an increase of cell death and G_2/M mitotic arrest. Griseofulvin-induced G_2/M arrest was not solely attributed to the induction of abnormal mitotic spindle formation but also to the elevation of cyclin B1/cdc2 kinase activity and the down-regulation of myelin transcription factor-1 (myt-1) protein expression. Additionally, caspase 3 activation and B-cell lymphoma 2 (Bcl-2) hyperphosphorylation were supposed to be the mechanisms of griseofulvin-induced apoptosis (44). Another study demonstrated that very high concentrations of griseofulvin (>100 μM) were required to inhibit microtubule polymerization in HeLa cervical cancer cells, albeit much lower drug concentrations (1-20 μM) effectively suppressed the dynamic instability of microtubules; the authors concluded that the primary mechanism of action by which griseofulvin inhibits mitosis in human cells is by suppressing spindle microtubules dynamics similar to other antimitotic drugs as vinca alkaloids and taxanes (44). Besides that, treatment of adrenocortical cancer cells with a griseofulvin concentration of 40 μM for 24 h resulted in a significant induction of apoptosis, as shown by caspase 3/7 cleavage (46).

Interestingly, in HL-60 leukemia cells, the activation of the nuclear factor-kappa B (NF-κB) pathway, as well as the activation of c-Jun N-terminal kinases (JNKs), significantly promoted the phosphorylation of Bcl-2 resulting in G_2/M

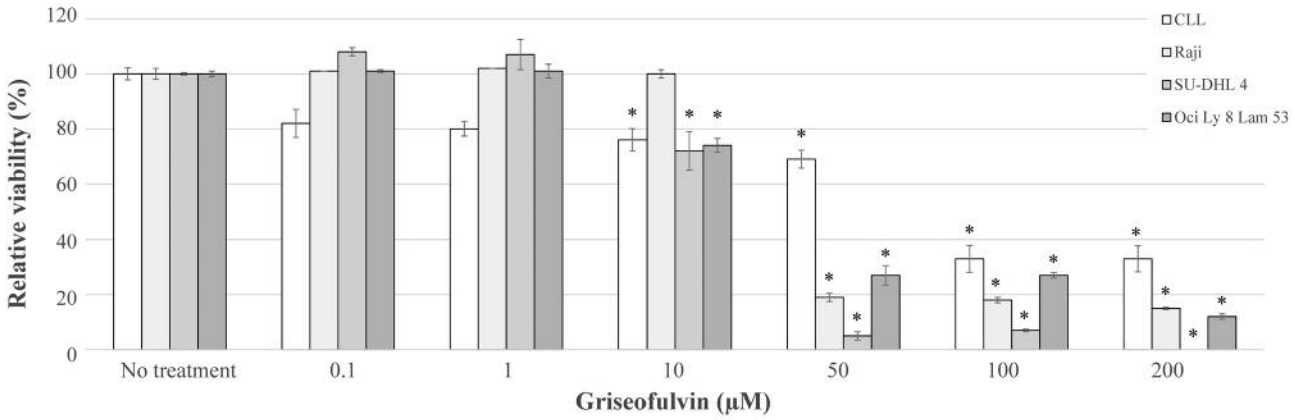

Figure 3. *Effect of griseofulvin on viability of primary CLL, Raji, SU DHL 4 and Oci Ly 8 Lam 53 human lymphoma cells. Cells were cultured with griseofulvin for 72 h. Cell viability and apoptosis was measured by 3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. Results represent data from three separate experiments each. Data are shown as mean±SD. *p<0.05 compared to untreated cells.*

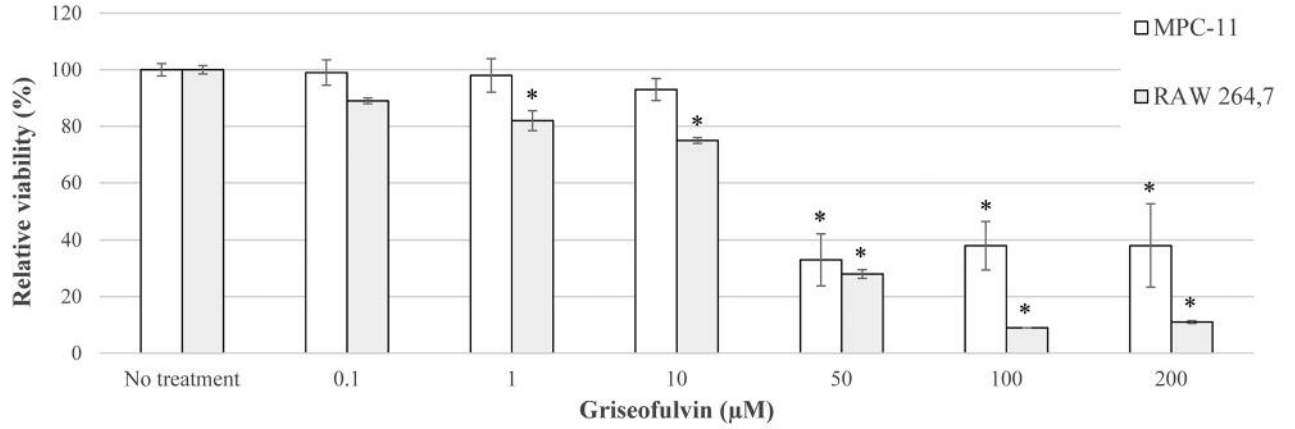

Figure 4. *Effect of griseofulvin on viability of MPC-11 and RAW 264.7 murine myeloma and leukemia cells, respectively. Cells were cultured with griseofulvin for 72 h. Cell viability and apoptosis was measured by 3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. Results represent data from three separate experiments each. Data are shown as mean±SD. *p<0.05 compared to untreated cells.*

cell-cycle arrest and induction of apoptosis (47); this is of major importance since the majority of the above mentioned signaling pathways and molecules also interfere with the Wnt pathway, particularly within the scope of hematological neoplasms. Despite those initial results, the effect of griseofulvin on both MM and lymphoma was solely addressed *in vivo* by a recent study of our workgroup using a murine myeloma cell model in which we could demonstrate prolonged survival and reduced tumor growth in myeloma-bearing mice (31), whilst *in vitro* data were lacking.

Our presented *in vitro* data indicate that griseofulvin affects the growth of multiple myeloma and lymphoma since it significantly reduced the viability of all tested myeloma and lymphoma cell lines by apoptosis induction due to reduced mitochondrial membrane potentials. Thereby, both human and murine cells were equally affected in a dose-dependent manner. Doses of approximately 10 µM significantly decreased cell viability in most myeloma and lymphoma cell lines tested. Interestingly, CCD-18Co colonic fibroblasts and PBLs, serving as healthy controls, tolerated higher drug concentrations. These data and the given safety profile of griseofulvin, as a commonly used anti-fungicidal drug, emphasize its favorable tolerability.

Due to its influence on Wnt-associated signaling molecules in leukemia and other malignancies, griseofulvin might also interfere with signaling molecules embedded in the Wnt and associated signaling pathways in lymphoma and multiple myeloma. Griseofulvin demonstrated a significant cytotoxic potential towards both MM and lymphoma cells by apoptosis induction and slightly decreased the viability of healthy controls. Hence, advanced studies investigating its potential as novel therapeutic drug for MM and lymphoma are warranted.

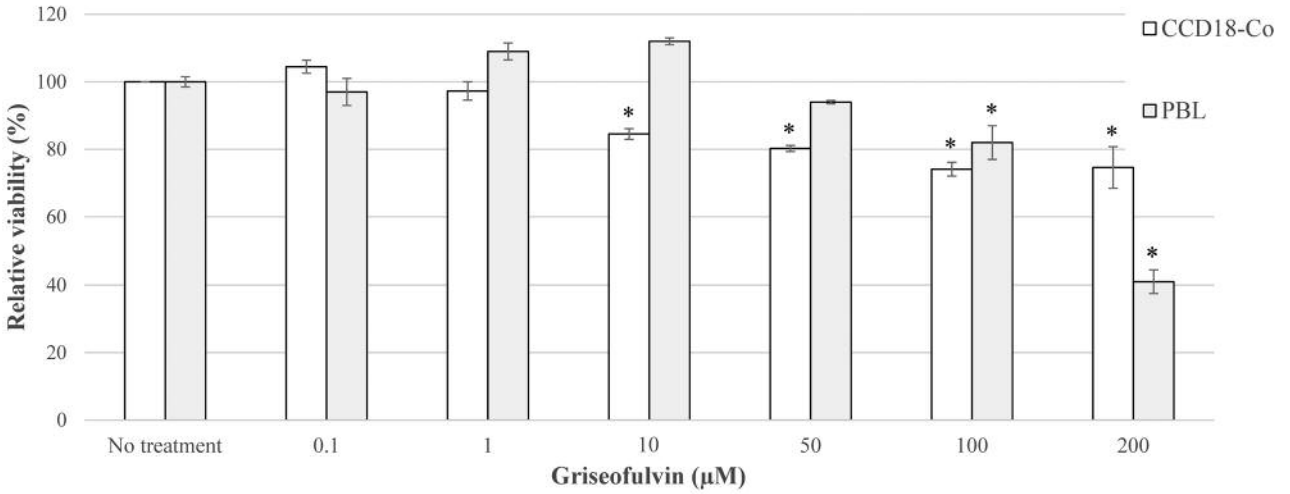

Figure 5. *Effect of griseofulvin on viability of CCD-18Co cells and peripheral blood lymphocytes (PBLs) that served as healthy controls. Cells were cultured with griseofulvin for 72 h. For CCD-18Co cells, viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. For PBLs, viability and apoptosis was measured by 3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining in flow cytometry. Results represent data from three separate experiments each. Data are shown as mean±SD. *p<0.05 compared to untreated cells.*

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