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In vivo efficacy of griseofulvin against multiple myeloma

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ABSTRACT

We recently confirmed that ciclopirox olamine inhibits Wnt/beta catenin signalling in myeloma. Griseofulvin (GF) has similar chemical features as compared to ciclopirox olamine. In this study the anti-tumor effect of GF was investigated. GF demonstrated a major apoptotic activity in various human and murine myeloma and lymphoma cell lines as well as in human primary cells. In vivo, tumor growth as well as overall survival were significantly reduced in mice treated with GF as compared to untreated mice. In conclusion, our results reveal a significant selective induction of apoptosis by GF and suggest a significant in vivo effect against myeloma.

1. Introduction

Major progress has been achieved in the treatment of multiple myeloma by the introduction of novel agents like thalidomide, lenalidomide and bortezomib. Nevertheless, multiple myeloma remains an incurable disease. In newly diagnosed patients, the combination of lenalidomide and dexamethasone has a response rate of 91%.

Several groups have shown that the Wnt/beta catenin pathway plays an important role in the regulation of cell proliferation, differentiation and apoptosis [1–3]. Aberrant activation of the Wnt signaling pathway has major oncogenic effects [4–7]. In the canonical Wnt pathway, the secreted Wnt proteins bind to a receptor complex, consisting of a member of the Frizzled (Fzd) family and the low-density lipoprotein-receptor-related proteins (LRP) 5 or LRP6. Subsequently, the cytoplasmic adaptor protein disheveled (Dvl) is phosphorylated and inhibits glycogen synthase kinase (GSK)-3β activity through its association with axin. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it interacts with T cell (TCF) and lymphoid-enhancing (LEF) factors to activate transcription of Wnt target genes [4,5,8]. In addition, it has been demonstrated that the Wnt pathway is activated in lymphoma. Thus, the Wnt/beta catenin signaling molecules are attractive candidates for development of targeted approaches in lymphoma treatment.

In a more recent work, our group confirmed that the antifungal agent ciclopirox olamine (CIC) inhibits Wnt/beta catenin signaling [9]. Ciclopirox olamine (CIC) is a synthetic antifungal agent used topically for the treatment of yeast infections in humans and is degraded by glucoronidation [10]. It serves as a chelator of polyvalent metal cations (e.g. Fe^{3+} and Al^{3+}) resulting in the inhibition of metal depending enzymes, occurring in the metabolism of the cell. Furthermore, it blocks the cell cycle near the G1/S phase boundary [11].

Griseofulvin (GF), an ethanolamine salt of the hydroxamic acid derivative piroctone, is a pyridone derivate as is CIC [12]. It is used as an antifungal drug.

In this study, we investigated the effect of griseofulvin (GF) on multiple myeloma and lymphoma cells in vitro and in vivo in a murine myeloma model.

2. Methods

2.1. Cell lines and culture conditions

The lymphoma cell lines LAM-53, SU-DHL-4, Daudi and Raji, as well as the myeloma cell lines OPM-2, RPMI-8226 and U-266 (all obtained from DSMZ, Collection of Microorganisms and Cell Culture, Braunschweig, Germany) were cultured in RPMI-1640 Medium consisting of 10% heat-inactivated fetal calf serum (FCS, Invit-

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rogen, Karlsruhe, Germany), 2.5% 1 M HEPES, and 1x penicillin/streptomycin (all from PAA Laboratories GmbH, Cölbe, Germany). Cells were cultured at a density of 3.3×10^5 cells ml^{-1} and incubated at 37 °C with 5% CO_2 and 95% humidity.

MPC11 (DMSZ, Braunschweig, Germany) is a murine plasmocytoma cell line derived from the Balb/c strain expressing IgG2b. Cells were cultured in RPMI 1640 medium (PAA Laboratories GmbH, Austria) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine (both from PAA, Cölbe, Germany), 100 U/ml penicillin/100 U/ml streptomycin (both from Seromed, Jülich, Germany) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Human samples

Peripheral blood mononuclear cells (PBMC) and PBL were isolated from blood samples of healthy volunteers by Ficoll density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). In addition, bone marrow samples from patients with myeloma were obtained. Ethics approval had been obtained according to the guidelines of our institution.

2.3. Drugs and chemical reagents

In our experiments the following drugs were used: Thalidomide (Grünenthal Pharma GmbH, Aachen, Germany), lenalidomide was obtained from Celgene (Munich, Germany), and griseofulvin (PO) from Spinnrad, Bonn, Germany. All drugs were tested at various concentrations for 24–72 h. In addition, in this study griseofulvin (GF, Spinnrad, Bonn, Germany) was applied orally.

2.4. $DiOC_6$ and PI-staining

1×10^5 cells were cultured in 3 ml medium in 6-well plates. GF was dissolved in DMSO, and added in optimized concentrations between 10 μM (CIC) and 30 μM (EA) alone or in combination with the therapeutic agents at various concentrations for 3 days. The apoptosis assay was performed with 3′,3-dihexyloxacarbocyanine iodide ($DiOC_6$) detecting mitochondrial membrane potential in viable cells, and propidium iodide (PI) which binds to DNA in necrotic cells, measured by a fluorescence-activated cell sorter (FACS).

2.5. Fluorescence-activated cell analysis

For FACS analysis, 500 μl staining solution containing 80 nM $DiOC_6$ in FACS buffer, consisting of deficient RPMI-medium with 0.5% bovine serum albumin (BSA), was mixed with equal volumes of the cell sample in a glass tube and incubated at 37 °C for 15 min. After a washing step with PBS/BSA 1% the cells were re-suspended in 500 μl PBS/BSA 1%. After addition of 5 μl PI-solution (100 μg/ml) the cells were analyzed by FACS. Using this assay, viable cells reveal high fluorescence intensity for $DiOC_6$ and a low expression for PI. In contrary, apoptotic cells show a low expression for $DiOC_6$ and also a low expression for PI (2). Finally, necrotic cells show a low expression for $DiOC_6$ and a high expression for PI.

A mean IC_{50} value in myeloma cells was determined using the mean of the IC_{50} results determined in OPM-2, U266 and RPMI 8226 cells.

2.6. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood of healthy donors by Ficoll-Hypaque density gradient centrifugation. Blood from buffy coats was mixed 1:2 with PBS/1% BSA (both PAA, Cölbe, Germany) and used for a ficoll gradient (LymphoPrep, PAA, Cölbe, Germany). After the centrifugation at 800×*g* for 30 min, the leukocyte layer was removed and transferred to new tubes. Subsequently, these cells were washed 3 times with PBS/1% BSA and re-suspended in fresh medium, consisting of RPMI medium (PAA, Cölbe, Germany) with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany), 2.5% hepes buffer solution (PAA, Cölbe, Germany), and 1% penicillin/streptomycin (PAA, Cölbe, Germany).

2.7. Bone marrow samples

Bone marrow samples from patients with myeloma were taken after informed consent, single cell suspensions generated and cells ficolled. Cells were incubated with or without GF for 3 days and measured for viability.

2.8. Western blot

We analyzed the effect of GF on the Wnt/beta catenin pathway by Western blot. Western blot was performed as described recently [13].

2.9. Animal studies

All animal experiments were done at least in duplicate with groups of six BALB/c mice (Charles River, Sulzfeld, Germany). 5×10^5 MCP11 murine myeloma cells were injected per mouse subcutaneously into Balb/c mice. Mice were treated orally with 450 μg/day PO. Overall survival and tumor growth were measured. Tumor volume was calculated as follows: volume = length × $width^2$ × 0.52. Animals were killed when tumor volume reached 2000 mm^3.

Table 1
The effect of griseofulvin, thalidomide,and lenalidomide on myeloma (OPM-2, U266, RPMI 8226 and KMS18) and lymphoma (LAM53, SU-DHL-4 and Raji) cell lines was assayed. PBMC and PBL derived from healthy individuals were used as controls. 1×10^5 cells were cultured with each compound using various concentrations for 3 days. Then cell viability was measured by $DIOC_6$ staining by flow cytometry and IC 50 rates were determined. Results represent data from 2 to 4 separate experiments each.

Cell line	IC_{50} of griseofulvin	IC_{50} of thalidomide	IC_{50} of lenalidomide
OPM-2	ND	53 μM	13 μM
U266	18 μM	300 μM	34 μM
RPMI 8226	26 μM	>400 μM	2 μM
Raji	33 μM	>400 μM	>1000 μM
OCI- Ly8 LAM 53	30 μM	>400 μM	>1000 μM
SU-DHL 4	22 μM	>400 μM	>1000 μM
MPC 11	41 μM	ND	ND
PBMC	180 μM	>400 μM	>400 μM
PBL	80 μM	>400 μM	>200 μM

2.10. Statistical analysis

For statistical analysis, the numbers of the results comprising the relative viability are expressed as the mean ± standard error of the mean (SEM). Different sample sizes (n) were chosen for different cell lines. Student's t-test was used for statistical analysis. A p value below 0.05 was considered significant. Statistical survival analyses were performed with the software GraphPad InStat, Version 3.0.0 (GraphPad Software, San Diego, CA), applied the Mann-Whitney test (non-paired, non-parametric).

3. Results

3.1. Effect of GF in vitro on viability of various cell lines

GF significantly decreased the viability of all myeloma cell lines in vitro (Table 1 and Figs. 1–4). Even small dosages of griseofulvin were toxic, so the IC_{50} could be reached with less than 50 μM of griseofulvin for the human myeloma cell lines U-266 (18 μM), RPMI-8226 (26 μM) and murine MPC-11 (41 μM).

Similar results were obtained for human lymphoma cell lines Raji (33 μM), OCI-Ly-LAM-53 (30 μM) and SU-DHL 4 (22 μM) (Table 1).

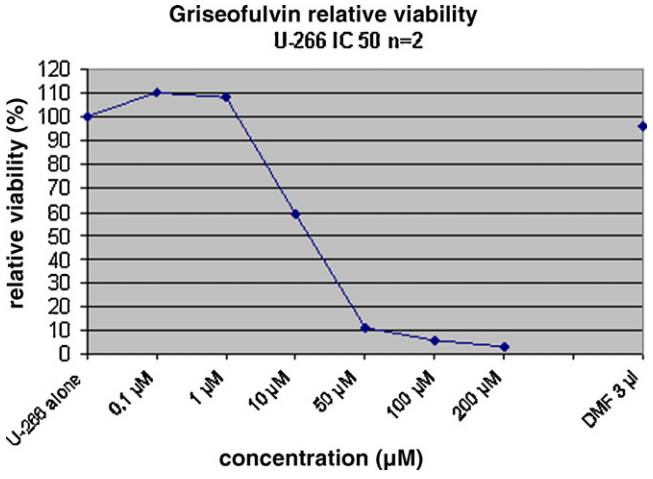

Fig. 1. Flow cytometric analysis of griseofulvin in U266 cells. 1×10^5 cells were cultured with various concentrations of griseofulvin for 3 days. Then cell viability was measured by $DIOC_6$ staining in flow cytometry. Results represent data from two separate experiments. Data are shown as mean ± SD.