Flunarizine Exhibits *In Vitro* Efficacy Against Lymphoma and Multiple Myeloma Cells

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Abstract. *Background/Aim: Novel agents such as lenalidomide and bortezomib have significantly improved today's therapy of multiple myeloma. Despite recent innovations, new therapeutic options are needed. The Wingless-related integration site (WNT) pathway is aberrantly activated in lymphoma and myeloma and therefore renders WNT signaling molecules attractive for the development of targeted therapies. Flunarizine was used in this study as it has chemical features similar to those of other known WNT inhibitors and already proven proapoptotic properties in leukemia cells. Materials and Methods: The antitumor apoptotic effect of flunarizine at doses ranging from 0.1-200 μM was investigated on three human lymphoma cell lines, one murine and four human myeloma cell lines by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining in flow cytometry. Results: Flunarizine induced significant apoptotic activity in all tested myeloma and lymphoma cell lines in a dose-dependent manner. Conclusion: Our results reveal a significant selective induction of apoptosis by flunarizine and suggest an in vivo effect against lymphoma and myeloma.*

Multiple myeloma (MM) is a hematological neoplasia of post-germinal center B-lymphocytes, with an accumulation of malignant plasma cells in the bone marrow, and mostly occurs with monoclonal protein being present in either peripheral blood or urine. A delayed diagnosis due to nonspecific clinical symptoms, particularly during the onset of the disease, is often seen in patients suffering from MM (1). Bortezomib, lenalidomide and thalidomide significantly improved treatment outcome and patient survival over the past decade, but most patients might still experience disease relapse, emphasizing the need for innovative treatment lines.

Wingless-related integration site (WNT)/β-catenin signaling represents an interesting target in cancer therapy as it is involved in apoptosis induction, differentiation and regulation of cell proliferation. Thus, aberrant activation of the WNT signaling pathway contributes to oncogenic effects (2-6). A pivotal role is played by β-catenin as a downstream effector in the canonical WNT signaling pathway. When WNT ligands are lacking, the cytosolic fraction of β-catenin forms a destruction complex comprising axin, adenomatous polyposis coli, casein kinase and glycogen synthase kinase-3β (GSK3β). This destruction complex is responsible for the phosphorylation of cytosolic β-catenin, which is subsequently ubiquitinated by cellular β-transducin repeat-containing proteins and thereafter degraded by the proteasome (7). Binding of secreted WNT ligands to Frizzled receptors and the co-receptor low density lipoprotein receptor-related protein 5, or 6, induces increased phosphorylation of the cytoplasmatic adaptor protein Disheveled that inhibits GSK3β activity and thereby causes accumulation of stable β-catenin. Non-phosphorylated β-catenin translocates into the nucleus to interact with lymphoid enhancer-binding factor and T-cell factor, which induces the transcription of WNT target genes such as *cMYC* and *cyclin D1* (5, 6). As the inhibition of the WNT signaling pathway results in suppression of MM progression, influencing WNT signaling might represent a promising therapeutic approach (8-13).

In our previous studies we confirmed that ethacrynic acid (EA), ciclopirox olamine (CIC), piceatannol and piroctone olamine (PO) inhibit the WNT/β-catenin pathway and might be effective in the therapy of various types of cancers, especially hematopoietic types (14-24).

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Key Words: Flunarizine, multiple myeloma, lymphoma, cancer therapy, WNT, pathway.

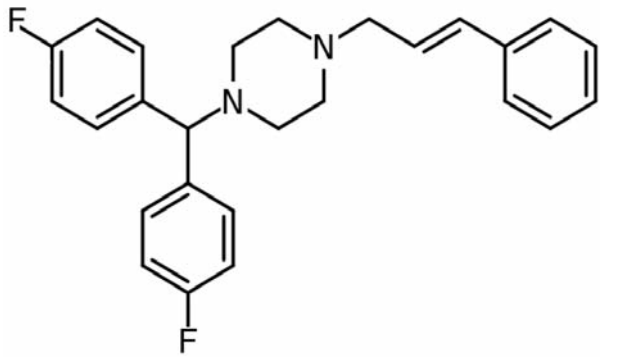

Figure 1. *Structural formula of flunarizine.*

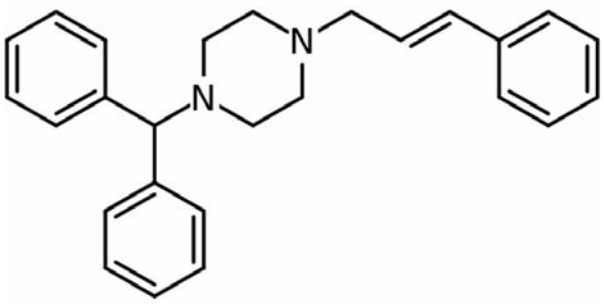

Figure 2. *Structural formula of cinnarizine.*

Recently, we showed a chemical analog of flunarizine {1-[*bis*(4-fluorophenyl)methyl]-4-[(2*E*)-3-phenylprop-2-en-1-yl]piperazine}, cinnarizine, to be an effective inducer of apoptosis in hematological malignancies (25). Structural formulae of flunarizine and cinnarizine are shown in Figures 1 and 2, respectively. Flunarizine is also distantly related to CIC and PO. Thus, here we investigated its cytotoxic potential on myeloma and lymphoma cells.

Materials and Methods

Cell lines and culture conditions. Cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) or the American Type Culture Collection (ATCC-LGC Standards, Wesel, Germany) and incubated at 37˚C with 5% CO_2 and at 90% humidity.

The human myeloma cell lines KMS 18, OPM-2, RPMI-8226 and U-266 (all 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). The human lymphoma cell lines Oci Ly 8 Lam 53, Raji and SU DHL 4 were cultured under identical conditions as human myeloma cell lines. MPC-11 (ATCC) is a murine plasmocytoma cell line. Cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FCS and 1% penicillin/streptomycin. The human colon fibroblast cell line CCD-18Co was obtained from the 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 using 0.05% trypsin-EDTA solution (Invitrogen), centrifuged at 1,200× *g* for 7 min and suspended in 1 ml medium to determine the cell count. The medium was renewed every three days.

Drugs and chemical reagents. Flunarizine was used in this study. Flunarizine was purchased from Sigma-Aldrich (Steinheim, Germany) and tested at different concentrations for 72 h.

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. Flunarizine was dissolved in dimethyl sulfoxide (DMSO) (Invitrogen) and added to the medium at an optimized concentration 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).

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 10×, 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 (BD Biosciences, Heidelberg, Germany) 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. Apoptotic cells show low fluorescence for both DiOC6 and PI. Cells with high fluorescence intensity for both DiOC6 and PI correspond either to debris or apoptotic bodies.

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

Results

Titration of flunarizine. We determined the optimal concentrations of flunarizine which led to a significant decrease in viability of myeloma and lymphoma cells. As controls CCD-18Co colon fibroblasts were investigated by FACS analysis. The mean 50% inhibitory concentration (IC_{50}) after 72 h was detected by titration. IC_{50} values of flunarizine employed after 72 h of incubation are given in Table I.

Table I. *Inhibitory concentration (IC_{50}) of flunarizine for human and murine lymphoma, multiple myeloma and control cell lines. CCD18-Co cells served as controls. A total of 1×10^5 cells were cultured with different concentrations of flunarizine for three days. Cell viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining. Results represent the mean of data from three independent experiments each.*

Cell line	Flunarizine IC_{50} (μM)
KMS 18	36 μM
OPM 2	29 μM
RPMI 8226	39 μM
U 266	35 μM
MPC 11	38 μM
Oci Ly 8 Lam 53	37 μM
Raji	25 μM
SU DHL 4	55 μM
CCD18-Co	76 μM

Effect of flunarizine on viability of human and murine myeloma cells. The viability of all tested human myeloma cells was affected by flunarizine. Administered concentrations of flunarizine starting from 50 μM significantly reduced the viability of myeloma cells in a concentration-dependent manner. Concentrations necessary to induce apoptosis of murine MPC-11 myeloma cells were comparable to those needed for human myeloma cells. Flunarizine at 38 μM triggered apoptosis of approximately 50% of MPC-11 cells. All results are shown in Figure 3. Figure 5 summarizes the significant decrease in viability after exposure to 50 μM flunarizine. Figure 6 A shows the corresponding flow cytometry results.

Effect of flunarizine on viability of human lymphoma cells. Exposure to flunarizine also strongly reduced lymphoma cell viability and triggered a significant selective induction of apoptosis in all tested cell lines. The IC_{50} values for Oci Ly 8 Lam 53 and Raji lymphoma cells were 35 μM and 25 μM, respectively. SU DHL 4 lymphoma cells were least susceptible to the toxicity of flunarizine; at least 55 μM flunarizine was required to reduce their viability to a level of 50% (Figure 4). Figure 5 summarizes the significant decrease in viability of cell lines after exposure to 50 μM flunarizine. Figure 6 B presents the respective flow cytometry results.

Effect of flunarizine on viability of healthy controls. We chose CCD-18Co colon fibroblasts in order to analyze the toxicity of flunarizine towards healthy cells. CCD18-Co cells tolerated high doses of flunarizine as concentrations of more than 50 μM were required for a significant induction of apoptosis. Results are shown in Figures 3-5.

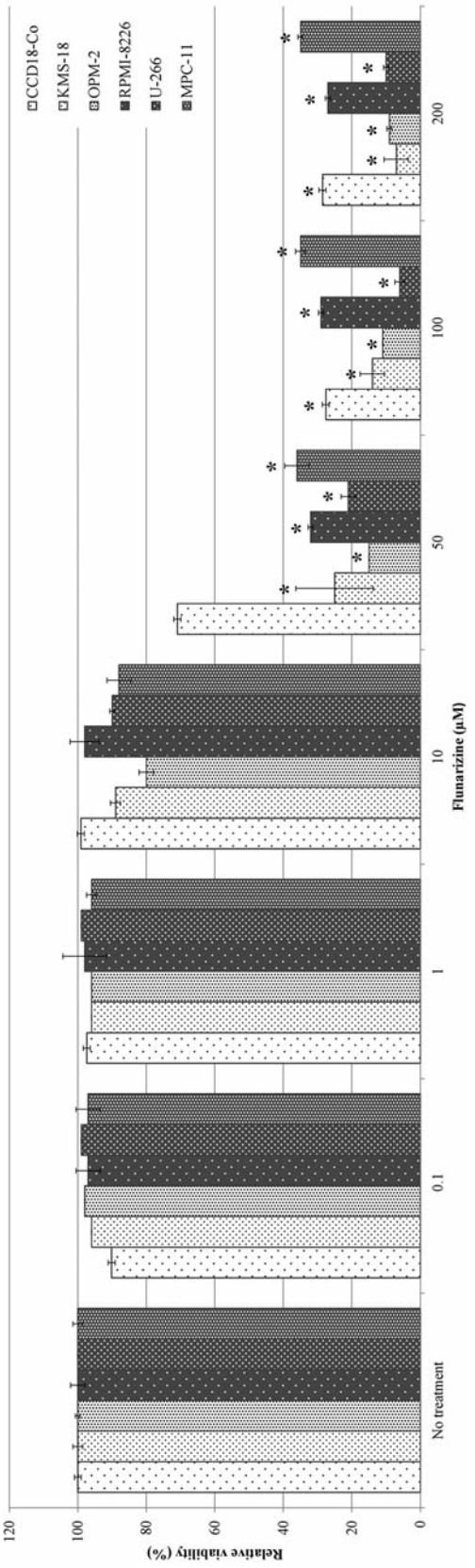

Figure 3. *Effect of flunarizine on viability of KMS-18, OPM-2, RPMI-8226, U-266 and MPC-11 human and murine myeloma cells and CCD-18Co colonic fibroblasts. CCD-18Co cells were used as healthy controls. Cells were cultured with flunarizine for three days. Viability was measured by 3' 3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Data are shown as the mean±SD of experiments performed in triplicate. *$p<0.05$ compared to untreated cells.*

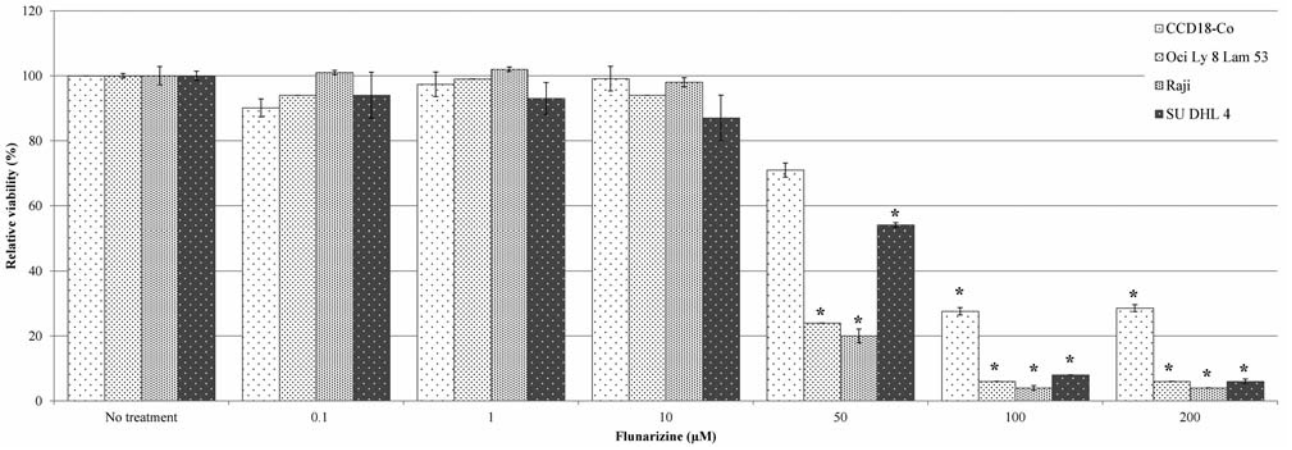

Figure 4. *Effect of flunarizine on viability of Oci Ly 8 Lam 53, Raji and SU DHL 4 human lymphoma cells and CCD-18Co colon fibroblasts. CCD-18Co cells were used as healthy controls. Cells were cultured with flunarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide-staining using flow cytometry. Results represent data from three independent experiments. Data are shown as the mean±SD. *p<0.05 compared to untreated cells.*

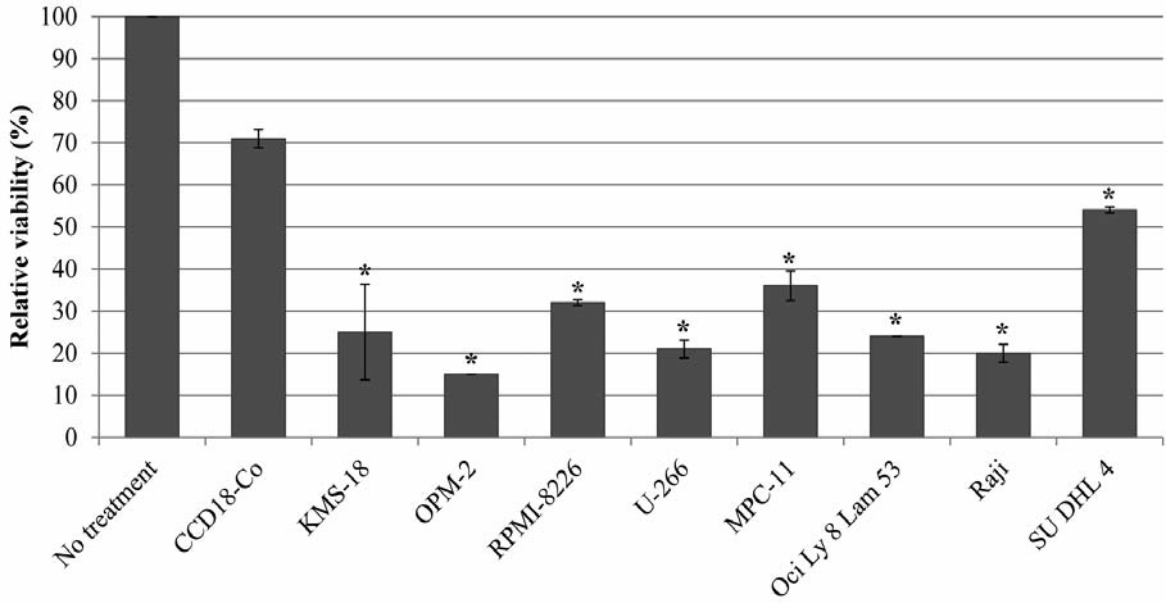

Figure 5. *Flunarizine at 50 μM significantly reduced the viability of all tested human and murine lymphoma and myeloma cell lines. However, the viability of CCD18-Co cells which served as controls was not significantly reduced. Cells were cultured with flunarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Results represent data from three independent experiments. Data are shown as the mean±SD. *p<0.05 compared to untreated cells.*

Discussion

MM represents a malignant neoplasm of plasma cells caused by frequent gene mutations with or without chromosomal translocations (26). In light of the latest research, therapy is based on high-dose chemotherapy, following hematopoietic stem cell transplantation (27-29). Despite several therapy innovations, the introduction of new pharmaceutical agents is still warranted as MM currently remains incurable with current chemotherapeutic treatment approaches (1).

Targeting the WNT signaling pathway might become a promising treatment approach since WNT signaling represents a perfect example of abrogated signaling pathways in MM (8-13). Recent studies confirmed that WNT ligands

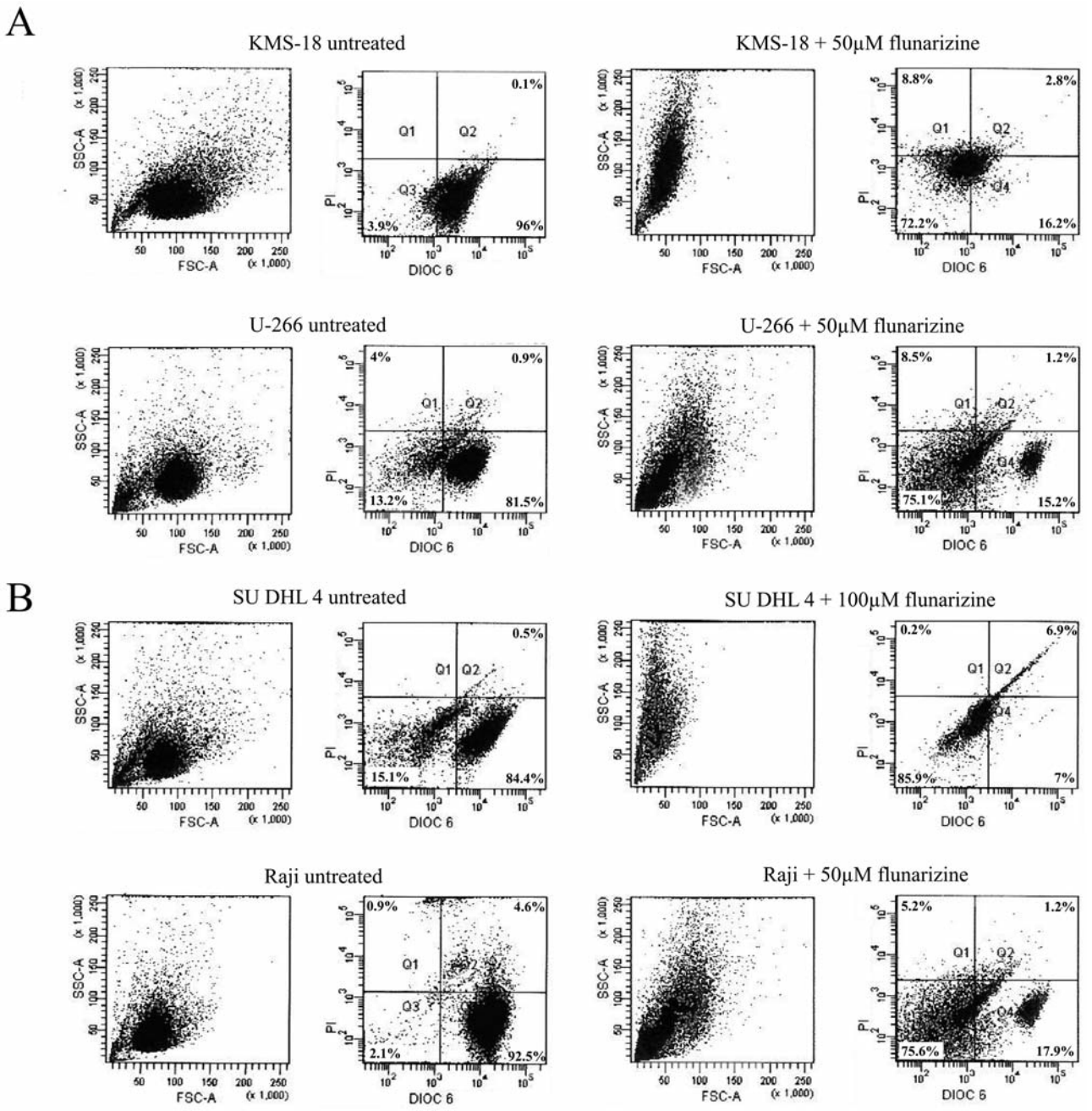

Figure 6. *Exemplary results generated by flow cytometry. Within the quarters the relative number of cells is given in percentages. KMS-18, U-266, SU DHL 4 and Raji cells were treated with flunarizine at different concentrations. Seventy-two hours after incubation, flow cytometry was performed. (A) Myeloma cells before and after treatment with flunarizine. (B) Lymphoma cells before and after treatment with flunarizine.*

induce enhanced proliferation of MM cells, and inhibition of WNT/β-catenin signaling suppresses MM growth (30-33). Hence, current MM therapy might be improved by the implementation of innovative WNT inhibitors.

We recently demonstrated that treatment with PIC, EA, CIC and PO significantly reduced proliferation of lymphoma and myeloma cells *in vitro* by influencing WNT signaling through targeting either β-catenin itself or its downstream factors. Subsequent *in vivo* studies revealed the potential of EA, CIC and PO as they significantly reduced tumor growth and prolonged overall survival duration in myeloma-bearing mice (14-24, 34). More recently, we proved cinnarizine to be

effective in the treatment of hematological malignancies (25). These promising effects on both cancer cell survival and WNT signaling made it tempting to determine whether flunarizine, which is an analog of cinnarizine and distantly related to CIC and PO, exhibits any cytotoxic effects towards MM and lymphoma cells.

Flunarizine is a selective calcium entry blocker with calmodulin-binding properties and histamine H1-blocking activity. It was first synthesized by Janssen Pharmaceutica in 1967 as a derivative of piperazine. Flunarizine is commonly used in the prophylaxis of migraine, occlusive peripheral vascular disease, vertigo of central and peripheral origin, and as an adjuvant in the therapy of epilepsy. In contrast to its chemical analog cinnarizine, it has a long plasma half-life and is administered only once a day. Animal studies revealed that high single doses of flunarizine are well-tolerated in adult rats, mice, guinea pigs, and dogs of both sexes as there is a wide safety margin. Daily oral doses of up to 20 mg/kg for three months did not produce any significant toxicity (35).

Previous *in vitro* studies already showed that the efficacy of chemotherapy was significantly enhanced when flunarizine was combined with common chemotherapeutic agents. Interestingly, this effect was also observed in drug-resistant neoplasms as flunarizine enhanced the activity of melphalan against drug-resistant rhabdomyosarcoma, positively modulated doxorubicin-resistance in multidrug-resistant human colonic adenocarcinoma cells, and slightly enhanced the sensitivity of multidrug-resistant primary human renal cell carcinomas towards treatment with vinblastine (36-38). Additionally, flunarizine treatment inhibited the *in vitro* migration of melanoma cells, and the growth rate and survival fraction of B16 murine melanoma cells (39, 40). Combined treatment with vincristine enhanced intracellular levels of vincristine in melanoma-bearing mice and significantly prolonged the median survival of the animals as compared with controls, which were solely treated with vincristine (41). Dose-dependent growth inhibition of prostate cancer cells was also observed (42). It was recently confirmed that flunarizine induces caspase-10-dependent apoptosis of Jurkat T-leukemia cells, but not of breast or colon carcinoma cells. Flunarizine treatment also resulted in production of reactive oxygen species, dissipation of mitochondrial transmembrane potential, release of cytochrome *c* from mitochondria, and caspase-9 activation. Moreover, no significant toxicity towards healthy cells was observed (43).

These underlying apoptotic mechanisms of flunarizine emphasize its anti-carcinogenic potential, particularly for hematological malignancies. Our results corroborate these findings for lymphoma and MM as our data indicate that flunarizine significantly influences the proliferation of cells of hematological malignancies by selective induction of apoptosis. We observed that flunarizine treatment induced apoptosis in all tested myeloma and lymphoma cell lines. Human and murine cells were equally affected and comparable flunarizine concentrations were sufficient for apoptosis induction. Doses of approximately 35 μM reduced cell viability by 50% in most myeloma and lymphoma cell lines. However, SU DHL 4 lymphoma cells tolerated higher doses of flunarizine. Interestingly, CCD-18Co colonic fibroblasts, which served as healthy controls, tolerated higher doses of flunarizine as doses lower than 100 μM did not significantly influence viability, thus suggesting a favorable tolerability of flunarizine concerning healthy tissues.

Taken together, flunarizine exhibits selective toxicity towards lymphoma and MM cells and might also interfere with WNT signaling or other associated pathways due to its chemical relationship to other known WNT inhibitors. Hence, flunarizine might represent a sustainable anticancer drug and further *in vitro* and *in vivo* experiments are warranted.

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Leonard Christopher Schmeel and Frederic Carsten Schmeel have contributed equally to this work as joint first Authors.

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