In Vitro Efficacy of Cinnarizine Against Lymphoma and Multiple Myeloma

LEONARD CHRISTOPHER SCHMEEL[1*], FREDERIC CARSTEN SCHMEEL[1,2], YOUNG KIM[1], SABINE BLAUM-FEDER[1], TOMOYUKI ENDO[3] and INGO G.H. SCHMIDT-WOLF[1]

[1]*Department of Internal Medicine III, Center for Integrated Oncology (CIO), University Hospital Bonn, Bonn, Germany;*
[2]*Department of Radiology, University Hospital Bonn, Bonn, Germany;*
[3]*Department of Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan*

Abstract. *Background/Aim: Multiple myeloma, a well-known but still incurable disease, is a hematological malignancy of B-lymphocytes. While standard chemotherapy regimens have been used for years, novel agents, such as lenalidomide and bortezomib, have become an essential part of today's therapies. Nevertheless, new therapeutical strategies are required in the future. Aberrant activation of wingless-related integration site (WNT)/β-catenin signaling promotes the development of several types of cancer. Recently, it has been demonstrated that the WNT pathway is also activated in lymphoma and myeloma. Thus, the WNT signaling molecules are attractive candidates for the development of targeted therapies. To this extent, we recently confirmed that the diuretic agent ethacrynic acid (EA) and the antifungal agent ciclopirox olamine (CIC) inhibit WNT signaling. Cinnarizine has similar chemical features to those of CIC. Materials and Methods: Thus, in this study the antitumor effect of cinnarizine on myeloma and lymphoma cells was investigated by DiOC6 and propidium iodide (PI)-staining in flow cytometry. Results: Cinnarizine triggered a significant apoptotic activity in all tested myeloma and lymphoma cell lines in a concentration-dependent manner. Interestingly, healthy cells were mainly unaffected. Conclusion: These results reveal a significant selective induction of apoptosis by cinnarizine that might result from an inhibition of WNT signaling and suggest an in vivo efficacy against lymphoma and myeloma.*

Multiple myeloma (MM) is a hematological neoplasia of post-germinal center B-lymphocytes characterized by accumulation of malignant secretory plasma cells in the bone marrow and mostly occurs with monoclonal protein in either peripheral blood or urine. Due to heterogeneous and unspecific clinical symptoms, diagnosis can be delayed, particularly during the onset of the disease (1). MM is primarily diagnosed in elderly patients, with a median age at diagnosis of 69 years (2). New therapeutic regimens, including bortezomib, lenalidomide and thalidomide, significantly improved treatment outcome and patient survival over the past decade. But despite this major progress in the treatment of MM, most patients experience disease relapse, underlining the need for new treatment strategies.

The wingless-related integration site (WNT)/β-catenin signaling pathway represents an interesting target in cancer therapy as it has been shown to be involved in apoptosis induction, differentiation and regulation of cell proliferation. Aberrant activation of WNT signaling pathway has major oncogenic effects (3-7). β-catenin as a downstream effector in the canonical WNT signaling pathway plays a key role. Without stimulation by WNT ligands, cytosolic β-catenin forms a destruction complex consisting of axin, adenomatous polyposis coli, casein kinase and glycogen synthase kinase-3β, phosphorylating β-catenin. Phosphorylated β-catenin is ubiquitinated by cellular β-transducin repeat-containing proteins and afterwards degraded by the proteasome (8). In the canonical pathway, binding of secreted WNT ligands to FRIZZLED receptors and the coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 leads to an increased phosphorylation of the cytoplasmatic adaptor protein DISHEVELED, which inhibits glycogen synthase kinase 3 β activity and thereby causes stabilization and accumulation of β-catenin. Hence unphosphorylated β-catenin is able to translocate into the nucleus, where an interaction with lymphoid enhancer-binding factor and T-cell factor induces the transcription of WNT target genes such as c-MYC and cyclin D1 (6, 7). Enhanced WNT signaling and an overexpression of β-catenin has been shown in MM (9-

*These Authors contributed equally to this work.

Correspondence to: Professor Dr. med. Ingo G.H. Schmidt-Wolf, University Hospital Bonn, Center for Integrated Oncology (CIO), Sigmund-Freud-Straße 25, 53105 Bonn, Germany. Tel: +49 22828717050, Fax: +49 2282879080059, e-mail: Ingo.Schmidt-Wolf@ukb.uni-bonn.de

Key Words: Cinnarizine, multiple myeloma, lymphoma, cancer therapy, WNT.

13). As the inhibition of WNT signaling pathway results in suppressed progression of MM (13, 14), influencing WNT signaling could be a valuable therapeutic approach (13, 14).

In our previous studies, we confirmed that ethacrynic acid (EA), ciclopirox olamine (CIC) and piroctone olamine (PO) inhibit the WNT/β-catenin pathway and might be effective in the therapy of various types of cancer, especially hematopoietic (15-24). More recently, we discovered piceatannol (PIC) to be another biological WNT inhibitor, corroborating an efficacy in apoptosis induction in cancer cells due to alterations in WNT-mediated protein expression (25).

With respect to its chemical features, cinnarizine is distantly related to CIC and PO. For this reason, we investigated the cytotoxic effect of cinnarizine on myeloma and lymphoma cells. Here we demonstrate for the first time that cinnarizine exhibits selective toxicity and triggers apoptosis induction in 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 and at 90% humidity.

The human myeloma cell lines KMS 18, OPM-2, RPMI-8226 and U-266 (all obtained from DMSZ (Braunschweig, Germany) 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 and RAW 264,7 (ATCC, LGC Standards GmbH, Wesel, Germany) are murine cell lines. MPC-11 is a murine plasmocytoma cell line and RAW 264,7 is a leukaemia 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 the ATCC (LGC Standards, Wesel, Germany) and cultured in ATCC-formulated Eagle's minimum essential medium (LGC Standards, Wesel, Germany) supplemented with 15% heat-inactivated FCS and 1% penicillin/streptomycin. Cells were harvested by using 0.05% trypsin-EDTA solution (Invitrogen), centrifuged at 241.5 × *g* for 7 minutes and resuspended in 1 ml medium to define the cell count. The medium was renewed every three 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 resuspended in RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 2.5% HEPES buffer solution (PAA).

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

3'3-Dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) staining. Reduced mitochondrial transmembrane potential occurs late in the apoptotic process. We used DiOC6 staining and flow cytometry to assess the mitochondrial transmembrane potential. A total of 1×10^5 cells were plated in 3 ml medium in 6-well plates. Cinnarizine was dissolved in dimethyl sulfoxide (DMSO) (Invitrogen) and added to the medium at an optimized concentration for three days. Staining with DiOC6 for detection of mitochondrial membrane potential in viable cells and with propidium iodide, 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 PBS (pH 7.4) (Roti-Stock 10×; CarlRoth, Karlsruhe, Germany) and stained after repeated centrifugation by adding 500 μl staining solution (RPMI-1640, 0.5% 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 flow cytometer (BD Biosciences, Heidelberg, Germany). Approximately 10,000 counts were made for each sample.

In this assay, viable cells show high fluorescence intensity for DiOC6 and a low intensity for PI. Necrotic cells express the opposite effect, high fluorescence intensity for PI and a low intensity for DiOC6. Apoptotic cells exhibit low florescence for both DiOC6 and PI. Cells that showed high fluorescence intensity for both DiOC6 and PI may correspond either to debris or apoptotic bodies.

Statistical analysis. Values are given as the mean±standard deviation (SD). At least three separate 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 cinnarizine. As a first step, we determined the optimal concentrations of cinnarizine which led to a significant decrease in viability of all tested myeloma and lymphoma cells. As controls, human PBLs and CCD-18Co colon fibroblasts were investigated by FACS analysis. The mean 50% inhibitory concentration (IC_{50}) after 72 hours was detected by titration. The IC_{50} values of cinnarizine after 72 hours of incubation are shown in Table I.

Effect of cinnarizine on viability of human myeloma cells. The viability of all tested human myeloma cells was affected by cinnarizine. Administered concentrations of cinnarizine starting from 20 μM significantly reduced the viability of myeloma cells in a concentration-dependent manner. Maximum efficacy was observed with concentrations higher than 30 μM. KMS-18 cells, however,

required higher doses for significant apoptosis induction. Results are shown in Figure 1.

Effect of cinnarizine on viability of human lymphoma cells. Exposure to cinnarizine also strongly reduced lymphoma cell viability and triggered a significant selective induction of apoptosis in all tested cell lines. The IC_{50} for Oci Ly 8 Lam 53 and Raji cells was attained after treatment with approximately equal concentrations of 37 μM and 36 μM, respectively. SU DHL 4 lymphoma cells were least susceptible to the toxicity of cinnarizine. At least 180 μM cinnarizine were required to reduce their viability to a level of 50%. Figure 2 presents the respective results.

Effect of cinnarizine on viability of murine myeloma cells and murine macrophages. The effects of cinnarizine treatment on human myeloma and lymphoma cells were reproducible in murine myeloma cells as well as in murine macrophages and are shown in Figure 3. Concentrations necessary to induce apoptosis in MPC-11 myeloma cells were comparable to those in human myeloma cells. Cinnarizine at 42 μM triggered apoptosis in approximately 50% of MPC-11 cells. Additionally, RAW 264,7 monocytes were investigated to determine if cinnarizine exhibits any toxicity towards myeloid cells. A significant reduction of the viability of RAW 264,7 cells occurred at concentrations exceeding 100 μM.

Effect of cinnarizine on viability of healthy controls. We chose two heterogeneous control cell lines in order to analyze the toxicity of cinnarizine towards different tissue types. PBLs derived from healthy donors and CCD18-Co colon fibroblasts were exposed to cinnarizine. Both cell types, PBLs and colon fibroblasts, showed a low sensitivity to cinnarizine, since treatment with high concentrations of cinnarizine did not result in a significant reduction of viability of PBLs or CD18-Co cells, even after exposure to concentrations up to 400 μM. The results are summarized in Figure 4.

Table I. *The half maximal inhibitory concentration (IC_{50}) of cinnarizine for human and murine lymphoma, multiple myeloma and control cell lines. Peripheral blood lymphocytes derived from healthy volunteers and CCD-18Co cells were used as controls. A total of 1×10^5 cells were cultured with different concentrations of cinnarizine for three days. Cell viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining. Results represent data from three experiments each.*

Cell line	IC_{50} (μM) Cinnarizine
KMS 18	76
OPM 2	49
RPMI 8226	36
U 266	32
MPC 11	42
RAW 264,7	102
Oci Ly 8 Lam 53	37
Raji	36
SU DHL 4	180
PBL	>400
CCD18co	>400

Discussion

MM represents a malignant neoplasm of plasma cells caused by frequent gene mutations, with or without chromosomal translocations (26). Currently, treatment is characterized by a primarily initiated high-dose therapy with chemotherapeutics, optionally followed by hematopoietic stem cell transplantation (26-29). In the last decades, numerous innovations have been achieved in the development of innovative therapeutic agents and their transition into clinical practice (30-31). But despite these treatment innovations, MM currently remains incurable in patients treated solely with chemotherapy (3), emphasizing the need for new therapeutic agents.

In this context, targeting the canonical WNT pathway might be an interesting approach as WNT signaling represents an excellent example of abrogated signaling pathways in MM (9-13). Development and proliferation of MM cells is, among others, dependent on the bone marrow microenvironment, wherein bone marrow stromal cells enhance WNT signaling by the release of WNT ligands, consequently leading to an enhanced proliferation activity of MM cells (31-33). Hence, it follows that inhibition of WNT/β-catenin signaling suppresses MM growth (34) and thus the WNT signaling pathway has emerged as an attractive therapeutic target for MM.

Recently, our workgroup revealed four drugs, PIC, EA, CIC and PO to be efficient inducers of apoptosis of lymphoma and myeloma cells *in vitro*. The latter three have already proven their efficacy in *in vivo* studies. They significantly reduced tumor growth and prolonged overall survival in myeloma-bearing mice. All four drugs rendered the tested cell lines more sensitive to other agents and influenced the WNT pathway through targeting either β-catenin itself or its downstream factors (15-25, 34). These promising effects on both cancer cell survival and WNT signaling encouraged us to determine whether cinnarizine, which is distantly related to CIC and PO regarding chemical properties, displays any cytotoxicity towards MM and lymphoma cells.

Cinnarizine [1-(diphenylmethyl)-4-(3-phenylprop-2-en-1-yl)piperazine] is a selective antagonist of T-type voltage-operated calcium ion channels and features antihistaminic, antiserotoninergic and antidopaminergic properties. It was

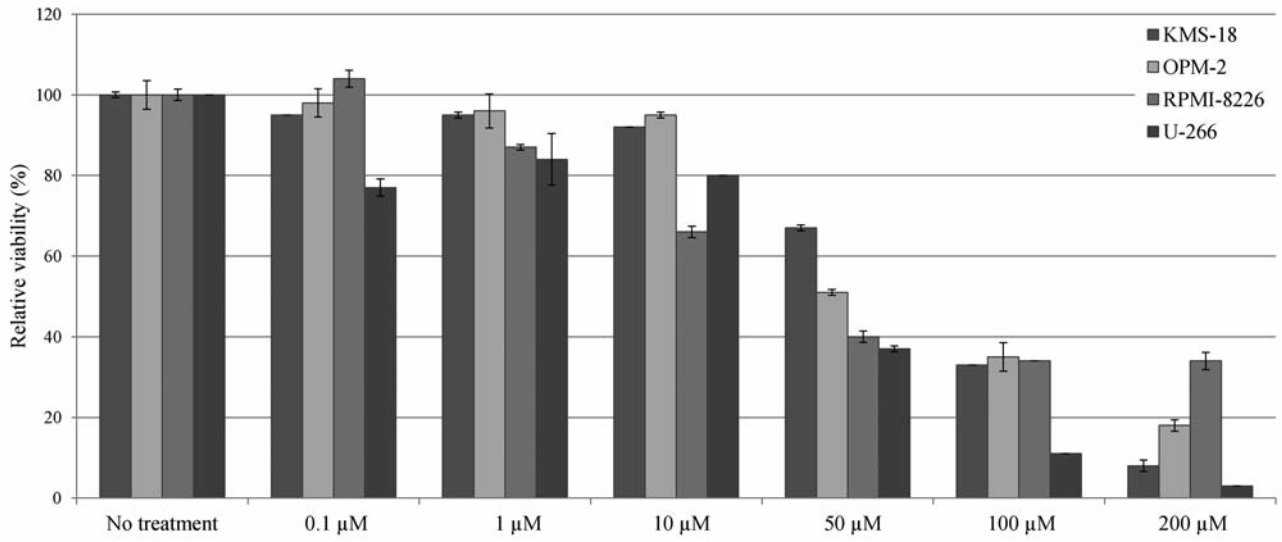

Figure 1. *Effect of cinnarizine on viability of KMS-18, OPM-2, RPMI-8226 and U-266 human myeloma cells. Cells were cultured with cinnarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Results represent data from three separate experiments each. Data are shown as the mean±SD.*

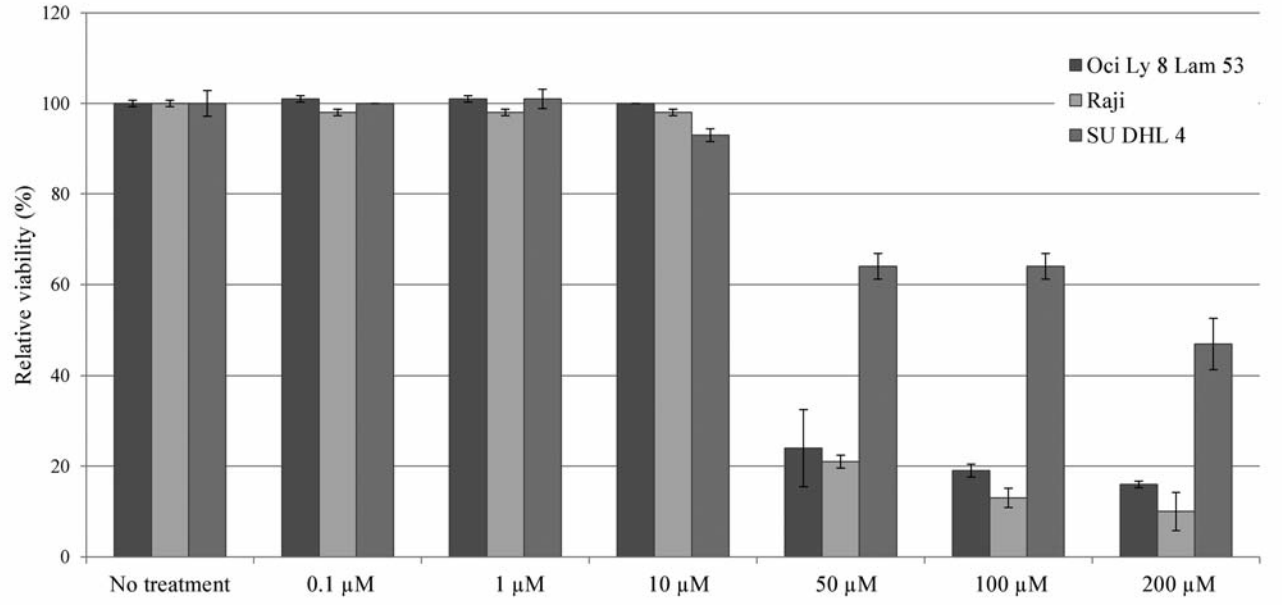

Figure 2. *Effect of cinnarizine on viability of Oci Ly 8 Lam 53, Raji and SU DHL 4 human lymphoma cells. Cells were cultured with cinnarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Results represent data from three separate experiments each. Data are shown as the mean±SD.*

first synthesized by Janssen Pharmaceutica in 1955 as a derivative of piperazine. Cinnarizine is commonly used to treat nausea and vomiting associated with motion sickness, vertigo and Ménière's disease (35). A less frequent therapeutic indication is seasickness. The signal transmission between the vestibular apparatus of the inner ear and the vomiting center of the hypothalamus is affected due to a decreased activity of the vestibular hair cells (36). Interestingly, cinnarizine had positive effects in the treatment of chemotherapy-related side-effects, such as

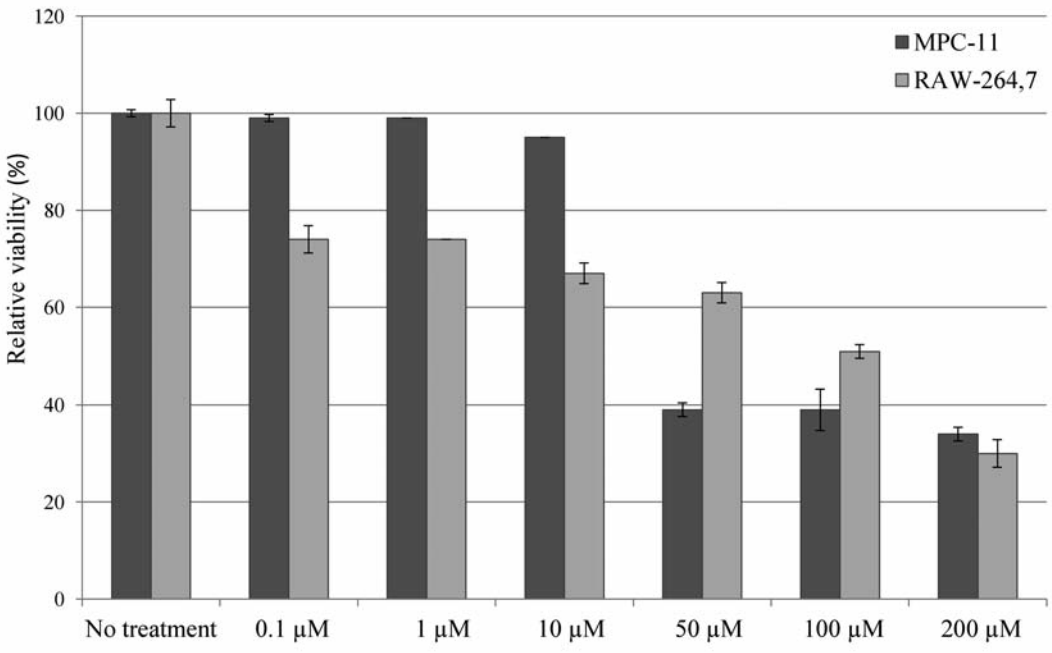

Figure 3. Effect of cinnarizine on viability of MPC-11 and RAW 264,7 murine myeloma cells and macrophages. Cells were cultured with cinnarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Results represent data from three separate experiments each. Data are shown as mean±SD.

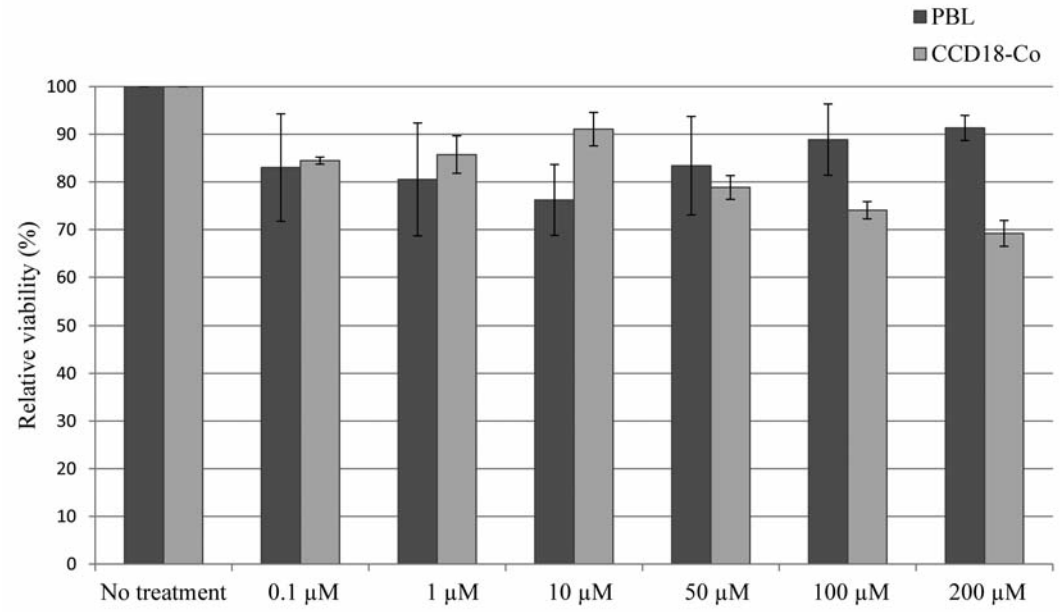

Figure 4. Effect of cinnarizine on viability of PBLs and CCD18-Co cells which served as controls. Cells were cultured with cinnarizine for three days. Viability was measured by 3'3-Dihexyloxacarbocyanine iodide and propidium iodide staining using flow cytometry. Results represent data from three separate experiments each. Data are shown as the mean±SD.

dizziness, loss of appetite, nausea and vomiting (37). In a retrospective study of 47 patients, cinnarizine was also found to be a valuable treatment option for idiopathic urticarial vasculitis (38). Apart from that, cinnarizine-induced calcium channel blockage-mediated vasorelaxation, predominantly occurring in brain vessels and possibly improving brain oxygen supply, might explain potential nootropic effects (39).

We found only a few studies investigating any other possible qualities of cinnarizine beyond those described above. Especially its utilization in oncology and its potential influence on neoplasia was not addressed so far. However, our data indicate that cinnarizine might have an impact on the proliferation of hematological malignancies by selective induction of apoptosis. We showed that cinnarizine triggered apoptosis in all tested myeloma and lymphoma cell lines. Human and murine cells were equally affected and comparable cinnarizine concentrations were sufficient for apoptosis induction. Doses of approximately 35 μM reduced cell viability by 50% in most myeloma and lymphoma cell lines. However, KMS-18 and OPM-2 myeloma cells, as well as SU DHL 4 lymphoma cells, were less sensitive to cinnarizine. Murine myeloid cells (RAW 264,7 murine macrophages) also tolerated higher concentrations as 102 μM reduced viability by 50%. Most interestingly, healthy controls were mainly unaffected by cinnarizine, since even doses of 400 μM did not significantly decrease cell viability, thus suggesting a favorable tolerability of healthy tissues.

Owing to its chemical relationship to other known WNT inhibitors, cinnarizine might also have the potential to interfere with signaling molecules embedded in the WNT or associated signaling pathways.

But despite these valuable results it is questionable whether such high concentrations are tolerated by patients as these doses might possibly contribute to adverse reactions, particularly in the light of cinnarizine's narrow therapeutic margin. However, cinnarizine revealed its selective cytotoxicity towards MM and lymphoma cells and hardly altered the viability of healthy cells. Hence, it seems to be a sustainable anticarcinogenic agent and further *in vitro* and *in vivo* experiments should be considered to provide deeper insight into the molecular mechanism and to determine possible *in vivo* efficacy.

Acknowledgements

L. C. Schmeel and F. C. Schmeel contributed equally to this work as joint first Authors.

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Received September 23, 2014
Revised October 20, 2014
Accepted October 27, 2014