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Increased *In Vivo* Efficacy of Lenalidomide by Addition of Piroctone Olamine

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Abstract. *Background: It was recently confirmed that the antifungal agent ciclopirox olamine inhibits Wnt/beta catenin signaling in myeloma. Piroctone olamine (PO) has very similar chemical features to ciclopirox olamine. Materials and Methods: This study investigated the antitumor effect of PO in vitro and in vivo in a murine myeloma model. Results: PO 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, was significantly reduced in mice treated with PO as compared to untreated mice. Interestingly, concerning tumor growth and survival of the animals, a significant additive effect was seen by the combination of lenalidomide plus PO as compared to single application. Conclusion: These results reveal a significant selective induction of apoptosis by PO and suggest a significant in vivo effect against myeloma.*

Major progress has been achieved in the treatment of multiple myeloma by the introduction of novel agents such as 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% (1).

Several groups have shown that the Wnt/beta catenin pathway plays an important role in the regulation of cell proliferation, differentiation and apoptosis (2-4). Aberrant activation of the Wnt signaling pathway has major oncogenic effects (5-8). 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. Un-phosphorylated 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 (5, 6, 9). In addition, it has been demonstrated that the Wnt pathway is activated in lymphoma (10). Thus, the Wnt/beta catenin signaling molecules are attractive candidates for the development of targeted approaches in lymphoma treatment.

It was recently confirmed that the antifungal agent ciclopirox olamine (CIC) inhibits Wnt/beta catenin signaling (10). CIC is a synthetic antifungal agent used topically for the treatment of yeast infections in humans and is degraded by glucoronidation (11). It serves as a chelator of polyvalent metal cations (*e.g.* Fe^{3+} and Al^{3+}) resulting in the inhibition of metal-dependent enzymes, in the metabolism of the cell. Furthermore, it blocks the cell cycle near the G_1/S phase boundary (12).

Piroctone olamine (PO), an ethanolamine salt of the hydroxamic acid derivative piroctone, is a pyridone derivate as is CIC. PO was first synthesized by Schwarzkopf–Henkel, Düsseldorf, Germany in 1979. It is a component of many cosmetic products such as anti-dandruff shampoo and is known to have bactericidal effects on gram-positive and gram-negative bacteria as well as fungicidial effects. The agent penetrates the cell membrane and forms complexes with iron (III) ions inhibiting energy metabolism in mitochondria. In addition, it has been described as a new active collagenase inhibitor (13).

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This study investigated the effect of PO on multiple myeloma and lymphoma cells *in vitro* and *in vivo* in a murine myeloma model.

Materials and Methods

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

MPC11 (DMSZ) 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 humidified 5% CO_2 atmosphere.

Human samples. Peripheral blood mononuclear cells (PBMCs) and peripheral blood lymphocytes (PBLs) were isolated from blood samples of two anonymous healthy volunteers by Ficoll density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). In addition, bone marrow samples from two patients with myeloma were obtained. Ethics approval had been obtained according to the guidelines of the host institution and all samples were taken after signed informed consent.

Drugs and chemical reagents. The following drugs were used in this study: thalidomide (Grünenthal Pharma GmbH, Aachen, Germany), lenalidomide (Celgene, Munich, Germany), and PO (Spinnrad, Bonn, Germany). All drugs were tested at various concentrations for 24-72 h. In addition, PO was applied orally.

$DiOC_6$ and propidium iodide (PI)-staining. A total of 1×10^5 cells were cultured in 3 ml medium in 6-well plates. The compounds EA and CIC were 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 three days. The apoptosis assay was performed with 3'3-dihexyloxacarbocyanine iodide ($DiOC_6$) detecting mitochondrial membrane potential in viable cells, and PI, which binds to DNA in necrotic cells, measured by a fluorescence-activated cell sorter (FACS).

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 intensity for PI. On the contrary, apoptotic cells show a low intensity for $DiOC_6$ and also a low intensity for PI (2). Finally, necrotic cells show a low intensity for $DiOC_6$ and a high intensity for PI.

Table I. *The effect of PO on myeloma (OPM-2, U-266, RPMI-8226 and KMS18) and lymphoma (LAM-53, SU-DHL-4 and Raji) cell lines was assayed.*

Cell line	IC_{50} (μm)		
	Thalidomide	Lenalidomide	Piroctone olamine
OPM-2	53	13	0.7
U-266	300	34	0.6
RPMI-8226	>400	2	0.6
KMS18	ND	ND	0.6
Raji	>400	>1000	0.6
Oci Ly8 LAM-53	>400	>1000	0.6
SU-DHL-4	>400	>1000	0.7
MPC-11	ND	ND	0.8
PBL	>400	>200	18

PBLs derived from healthy individuals were used as controls. 1×10^5 cells were cultured with each compound using various concentrations for three days. Then cell viability was measured by $DIOC_6$ staining by flow cytometry and IC_{50} values were determined. Results represent data from 2 to 4 separate experiments each. ND: Not done.

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.

Isolation of 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 three times with PBS/1%BSA and re-suspended in fresh medium, consisting of RPMI medium (PAA, Cölbe, Germany) with 10% FCS (Invitrogen), 2.5% HEPES buffer solution, and 1% penicillin/streptomycin (both PAA, Cölbe, Germany).

Bone marrow samples. Single-cell suspensions were generated from patient bone marrow samples and the cells were ficolled. Cells were incubated with or without CIC for three days and measured for viability.

Western blot. The effect of PO on the Wnt/beta catenin pathway was analyzed by Western blot. Western blot was performed as described recently (14).

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

Statistical analysis. For statistical analysis, results comprising the relative viability were expressed as the mean±standard error of the mean (SEM). Different sample sizes (n) were chosen for different

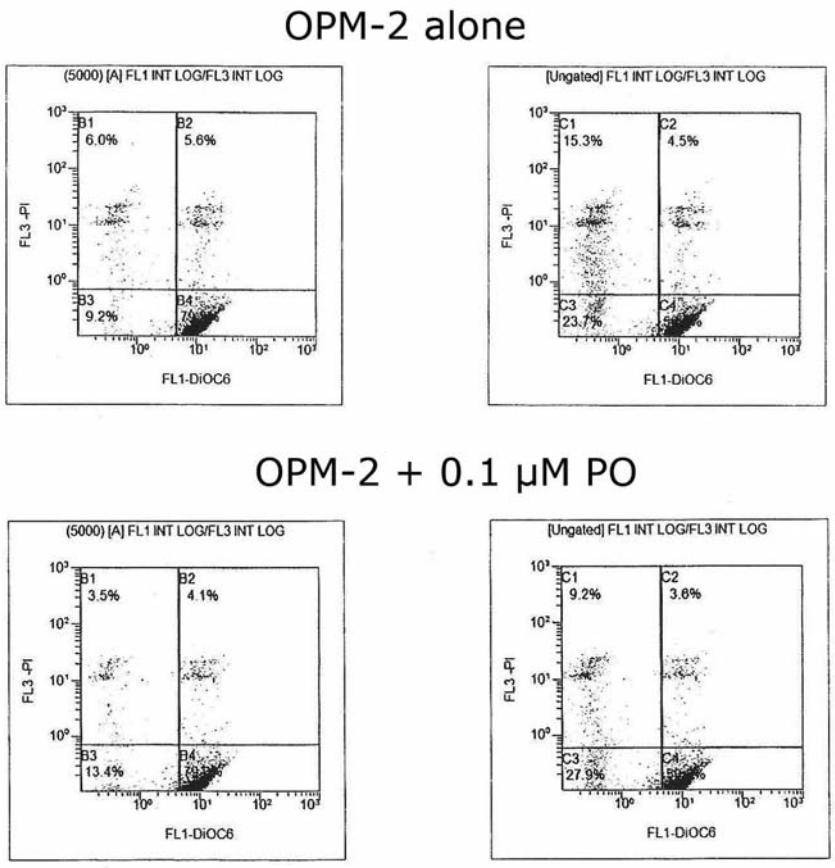

Figure 1. *Flow cytometric analysis of PO in human OPM-2 myeloma cells. 1×10^5 cells were cultured with or without 0.1 µM PO for three days. Cell viability was measured by DIOC$_6$ staining in flow cytometry. A representative experiment is shown.*

cell lines. Student's *t*-test was used for statistical analysis. A *p*-value <0.05 was considered significant. Statistical survival analyses were performed with the software GraphPad InStat, Version 3.0.0 (GraphPad Software, San Diego, CA, USA). The Mann-Whitney test (non-paired, non-parametric) was also applied.

Results

Effect of PO in vitro on viability of various cell lines. PO significantly decreased the viability of all myeloma cell lines *in vitro* (Table I, Figures 1 and 2). Even small dosages of PO were toxic, so the IC_{50} was mostly achieved with less than 1 µM of PO for the human myeloma cell lines OPM-2 (0.7 µM), U-266 (0.6 µM), RPMI-8226 (0.6 µM), KMS18 (0.6 µM) and murine MPC-11 (0.8 µM).

Similar results were obtained for human lymphoma cell lines Raji (0.6 µM), Oci-Ly-Lam-53 (0.6 µM) and SU-DHL-4 (0.7 µM) (Table I). In contrast, in PBLs (18 µM), which were used as control cells, PO was less efficient (Table I).

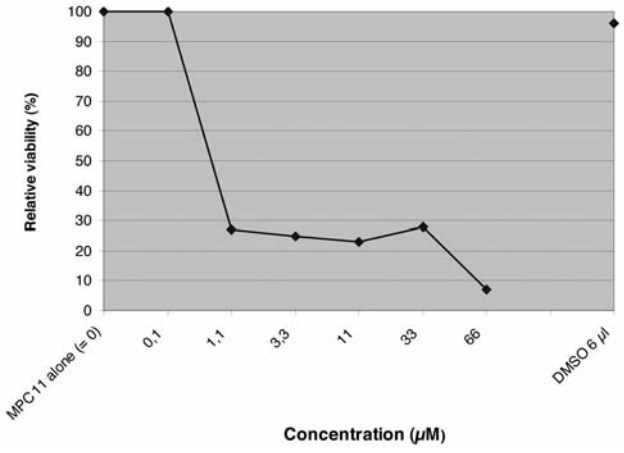

Figure 2. *Flow cytometric analysis of PO in murine MPC11 myeloma cells. 1×10^5 cells were cultured with various concentrations of PO for three days. Then cell viability was measured by DIOC$_6$ staining in flow cytometry. Results represent data from two separate experiments. Data are shown as means.*

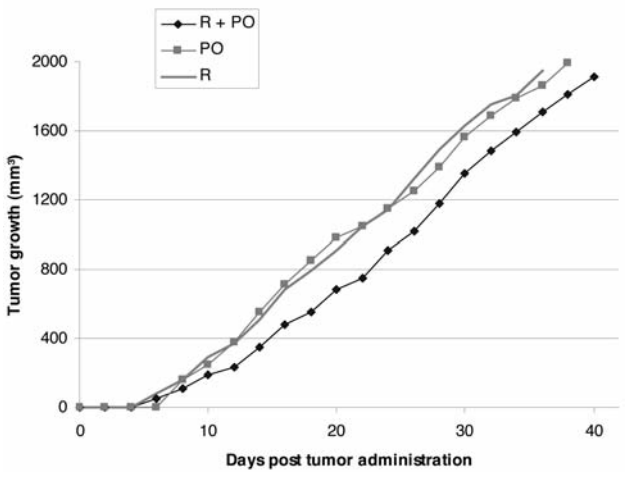

Figure 3. *In vivo effect of PO in MPC11-bearing mice. PO was administered orally by gavage at a concentration of 450 μg/day/mouse. Tumor growth in mice is presented. Six animals were used in each group. The observation time was 60 days.*

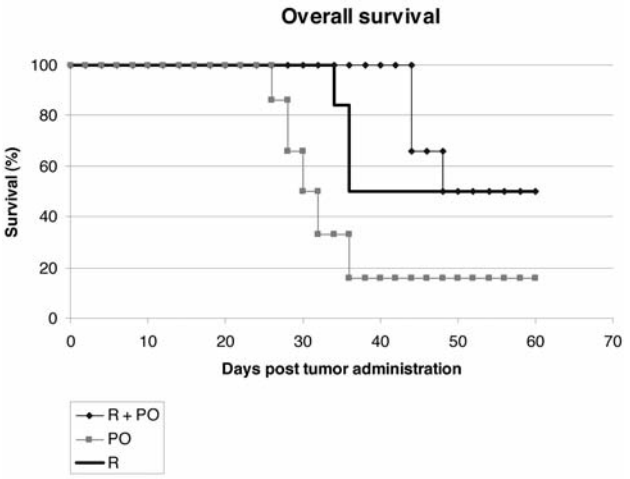

Figure 4. *In vivo effect of PO in MPC11-bearing mice. PO was administered orally by gavage at a concentration of 450 μg/day/mouse. Overall survival of mice is presented. Six animals were used in each group. The observation time was 60 days.*

Cytotoxic activity was shown to be *via* induction of apoptosis (Figure 1).

Effect of DMSO on viability of cell lines. In contrast to PBMCs and lymphoma cell lines, the myeloma cell line OPM-2 was highly sensitive to DMSO and showed a decrease of relative viability to 66.8±2.3%.

Effect of thalidomide, lenalidomide and PO on viability of myeloma cell lines. All three compounds significantly ($p<0.05$) decreased the viability of myeloma cell lines *in vitro* (Table I, Figure 2). A mean IC_{50} value in myeloma cells was determined: lenalidomide (16.3 μM) and PO (0.6 μM) were more effective than thalidomide (>251 μM; Table I).

In contrast, the effect on normal PBLs was marginal for PO and lenalidomide. Here, no effect was found for thalidomide (Table I).

Effect of thalidomide, lenalidomide and PO on viability of lymphoma cell lines. Only PO significantly ($p<0.05$) decreased the viability of lymphoma cell lines *in vitro* (Table I). In contrast, lenalidomide and thalidomide showed no effect (Table I).

Effect of PO on the Wnt/beta catenin pathway in myeloma cells. The effect of EA on β-catenin expression in myeloma cells was tested. MPC11 cells were found to be ß-catenin-positive (data not shown).

Effect of PO on viability of myeloma primary cells. Preliminary data suggest that PO inhibits the growth of primary myeloma cells derived from bone marrow from patients with multiple myeloma (data not shown).

Effect of PO on myeloma cells in vivo. All animals in the control group developed a tumor larger than 2000 mm^3 and therefore had to be sacrificed by day 22 (Figure 3).

PO was administered by gavage with a daily dosage of 450 μg per animal in the treatment group. After the MPC11 wild-type cells were administered subcutaneously into the back of the animals on day eight, solid tumor nodules formed after a few days in five out of six animals. The overall survival time was significantly ($p<0.05$) longer as compared to the control group (Figure 4).

With respect to the toxicity of PO administration, no side-effects were observed at the applied dose.

In addition, the combination of lenalidomide plus PO was tested *in vivo*. Interestingly, concerning tumor growth and survival of the animals, a significant ($p<0.05$) additive effect was seen with the combination as compared to the single application (Figure 4).

Discussion

The Wnt signaling pathway has been shown to play a critical role in the early phases of B lymphocyte development. Multiple myeloma cells, but not cells from healthy donors and patients with monoclonal gammopathy of undetermined significance or

other plasma cell dyscrasias involving bone marrow express the Wnt-signaling antagonist DKK1 (15). It has been reported that secretion of DKK1 by MM cells likely contributes to the formation of osteolytic lesions in this disease by inhibiting Wnt signaling: the latter being essential for osteoblast differentiation and survival. Changes of DKK1 expression in MM cells can be traced through disturbances in the JNK signaling cascade which is differentially modulated by oxidative stress and interactions between MM cells with osteoclasts, *in vitro*. Despite its role as a tumor suppressor and mediator of apoptosis in other cell types, including osteoblasts, the data indicate that *DKK1*, a stress-responsive gene in MM, does not mediate apoptotic signaling, is not activated by TP53, and its forced overexpression does not inhibit cell growth or sensitize MM cells towards apoptosis, following treatment with either thalidomide or lenalidomide. Therefore, specific strategies may be beneficial to modulate persistent activation of the JNK pathway in preventing disease progression and treating myeloma associated bone disease by inhibiting DKK1 expression (15).

Many hypotheses have been proposed to explain the molecular mechanism of thalidomide's teratogenicity, in particular regarding limb defects. Most *in vivo* experimental evidence has been provided for a model suggesting the generation of oxidative stress by thalidomide with subsequent down-regulation of both Wnt and Akt survival pathways. In addition, transcription factors Tbx5 and Sall4 are involved in thalidomide-induced molecular pathology (16).

Since thalidomide down-regulates the Wnt pathway and is used for treatment of myeloma patients, testing the combination of thalidomide and Wnt inhibitors is of particular interest. A synergistic effect of the combination of thalidomide, PO, CIC and EA has recently been demonstrated in myeloma cells. In the lymphoma cell lines SU-DHL4 and LAM-53 the Wnt inhibitors CIC and EA induce apoptosis through down-regulation of beta-catenin, an important molecule within the Wnt pathway.

This study showed that the daily oral administration of PO leads to apoptosis of myeloma and lymphoma cells *in vitro* and *in vivo*. Combined with previous results, these data suggest that PO, like EA and CIC, may inhibit Wnt/beta-catenin signaling in myeloma cell lines and thus significantly prolong the overall survival time *in vivo* without deteriorating the general condition of the animals. These results might lead to new treatment options in patients with multiple myeloma and should be followed-up in further studies for the benefit of such patients.

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