Abstract. Background/Aim: Recent investigations have shown that the Wnt signaling pathway is constitutively activated in multiple myeloma (MM), thereby promoting an exaggerated cell proliferation. Thus, influencing the Wnt pathway might represent a promising target in myeloma treatment. Materials and Methods: The present study investigated whether a combination of ethacrynic acid (EA) and ciclopirox olamine (CIC) with piceatannol (PIC) would influence the Wnt pathway and viability of human and murine myeloma cell lines by using DiOC₆ and propidium iodide (PI) staining, flow cytometry and immunoblotting. Results: The combination of EA with PIC as well as the combination of CIC with PIC had a significant additive effect on the vitality of myeloma cells compared to single-agent application, while healthy cells remained mainly unaffected. Additionally, EA and CIC altered the expression of β-catenin itself and its downstream factors. Conclusion: A combination of Wnt inhibitors could lead to novel treatment options for MM patients.

Multiple myeloma (MM) represents a hematological neoplasia of post-germinal center B-lymphocytes that is characterized by accumulation of malignant secretory plasma cells in the bone marrow. Owing to its heterogeneous and unspecific clinical symptoms that may vary, among others, from anemia-related fatigue, bone pain to immunodeficiency (1), diagnosis can be delayed, especially during the onset of the disease. In the US approximately 22,000 cases are expected to be newly-diagnosed in 2013 and more than 10,000 cases of MM-related deaths are estimated (2), thus MM remains the second most common hematopoietic malignancy.

MM is primarily diagnosed in elderly patients with a median age at diagnosis of 69 (3). By establishing new therapeutic regimens that include novel drug agents such as bortezomib, lenalidomide and thalidomide, outcome and patient survival could significantly be improved over the last decade. Despite this major progress in the treatment of MM most patients relapse and it eventually remains an incurable disease. This is the reason why new biologically-based treatment strategies are urgently needed.

The Wnt/beta-catenin signaling pathway appears to be a promising target in cancer therapy as it has been shown to be involved in apoptosis induction, differentiation and regulation of cell proliferation. An aberrant activation of Wnt signaling pathway has major oncogenic effects (4-8). β-catenin, as a downstream effector in the canonical Wnt signaling pathway, plays a key role. Provided there is no stimulation by Wnt-ligands, cytosolic β-Catenin usually forms a destruction complex consisting of axin, adenomatous polyposis coli (APC), casein kinase (CK) and glycogen synthase kinase-3 β, GSK-3 β), phosphorylating β-catenin. Phosphorylated β-catenin is ubiquitinated by cellular β-transducin repeat-containing proteins (β-TrCP) and afterwards degraded by the proteasome (9). In the canonical pathway, binding of secreted Wnt-ligands to Frizzled receptors and the co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 leads to an increased phosphorylation of the cytoplasmatic adaptor protein disheveled (Dvl), that inhibits glycogen synthase kinase (GSK-3 β) activity and thereby causes stabilization and accumulation of β-catenin. Hence un-phosphorylated β-catenin is ubiquitinated by cellular β-transducin repeat-containing proteins (β-TrCP) and afterwards degraded by the proteasome (9). In the canonical pathway, binding of secreted Wnt-ligands to Frizzled receptors and the co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 leads to an increased phosphorylation of the cytoplasmatic adaptor protein disheveled (Dvl), that inhibits glycogen synthase kinase (GSK-3 β) activity and thereby causes stabilization and accumulation of β-catenin. Hence un-phosphorylated β-catenin is able to translocate into the nucleus, where an interaction with lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) induces the transcription of Wnt target genes like c-myc and cyclin-D1 (7, 8). An enhanced activation of Wnt pathway with an overexpression of β-catenin has been shown in MM, although no known
mutations of Wnt signaling and β-catenin themselves have been reported (10-14). As inhibition of Wnt signaling pathway results in suppressed progression of MM, influencing Wnt signaling could be an interesting therapeutic approach (14, 15).

In addition, we recently confirmed that piceatannol (PIC 3,3',4,5'-tetrahydroxystilbene), a naturally-occurring polyphenol and an analog of resveratrol (trans-3,5,4'-trihydroxystilbene), inhibits the Wnt/beta-catenin signaling pathway and selectively induces cell death in myeloma cell lines (F. Carsten Schmeel and L. Christopher Schmeel, submitted). Furthermore PIC sensitizes MM cells to chemotherapeutic drug-mediated apoptosis (16). The commonly used diuretic agent ethacrynic acid (EA) and the anti-fungal agent ciclopirox olamine (CIC), both inhibitors of the Wnt signaling cascade, have already been shown to be effective agents in MM therapy (17, 18).

In the present study, we tested the effect of PIC in combination with either EA or CIC to determine whether they have an additive effect on cytotoxicity of myeloma cells. We demonstrated that both combinations exhibit a therapeutically-synergistic effect, with no apparent increase in toxicity compared to either drug used as single-agent.

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% CO2 at 90% humidity. The human myeloma cell lines OPM-2, RPMI-8226 and U-266 (DMSZ, 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). MPC-11 (ATCC, LGC Standards GmbH, Wesel, Germany) 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 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 rpm for 7 min and resuspended in 1 mL media to define the cell count. Medium was renewed every 3 days.

Drugs and chemical reagents. The following drugs were used in this study: EA, CIC and PIC. All agents were purchased from Sigma-Aldrich (Steinheim, Germany). The drugs were tested at different concentrations with an incubation time of 72 h.

In our flow cytometric experiments PIC was used at a concentration range from 10-60 μM on all cell lines, EA was used for MPC-11, OPM-2, RPMI-8226 and U-266 cells at a concentration of 27.5, 30, 6 and 35 μM, respectively, and CIC was used at 1, 2, 1 and 1.5 μM, respectively. On CCD18Co cells, EA and CIC were used at a concentration of 35 and 2 μM, respectively.

For immunoblotting, EA was used for MPC-11, OPM-2, RPMI-8226 and U-266 cells at a concentration of 40, 30, 5, and 45 μM, respectively, CIC was used at a concentration of 1.5, 2.5, 1.25 and 2 μM, respectively.

DiOC6 and propidium iodide (PI)-staining. Reduced mitochondrial transmembrane potential is known to occur late in the apoptotic process. We assessed mitochondrial transmembrane potential by using DiOC6 staining and flow cytometry. 1×105 cells were plated in 3 mL medium in 6-well plates. EA, CIC or PIC was dissolved in Dimethyl sulfoxide (DMSO) (Invitrogen) and added to the medium at an optimized concentration alone or in combination with the other drugs in different concentrations for three days. Staining with 3’3-dihexyloxacarbocyanine iodide (DiOC6) for detecting mitochondrial membrane potential in viable cells as well as 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 phosphate buffered saline (PBS, pH 7.4) (Roth-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 resuspended 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. Approximately 10,000 counts were made for each sample.

Viable cells showed high fluorescence intensity for DiOC6 and a low expression for PI. Necrotic cells expressed the opposite effect, high fluorescence intensity for PI and a low intensity for DiOC6. Apoptotic cells showed low expression for both, DiOC6 and PI. Cells that showed high fluorescence intensity for both, DiOC6 and PI, may correspond either to debris or apoptotic bodies.

Immunoblotting. Cells were treated with the indicated amounts of EA or CIC for 72 h. More than 1×106 cells were collected by centrifugation at 800 × g for 7 min. Then cells were washed twice with ice-cold PBS, transferred in a centrifuge tube and resuspended in 1 mL lysis buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, all purchased from ThermoScientific (Bonn, Germany). The suspension was placed on
ice for 45 min, interrupted by occasional vortexing and afterwards centrifuged at 13,000 x g for 20 min at 4˚C. The supernatants were stored at –80˚C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Munich, Germany). Samples containing 80 μg of protein were boiled in SDS sample buffer (Roti-Load 1, Carl Roth GmBH&Co. KG, Karlsruhe, Germany) and then loaded on 10% SDS/PAGE, thereafter blotted onto PVDF membrane (Immobilon-P, EMD Millipore Corporation, Billerica, MA, USA). Membranes containing transferred proteins were blocked by incubation in blocking solution (Roti-Block, Carl Roth, Karlsruhe, Germany) for 30 min, followed by immunoblotting using anti-β-catenin antibody (1:500), anti-TCF-4 antibody (1:1000), anti-LEF-1 antibody (1:1000), anti-c-myc antibody (1:1000), and anti-β-actin antibody (1:1000) (all antibodies were from Santa Cruz Biotechnology, CA, USA). Appropriate horseradish peroxidase-conjugated anti-IgG was used as secondary antibody (Santa Cruz Biotechnology, CA, USA). The membranes were developed using a chemiluminescence system (Luminata forte, EMD Millipore Corporation, Billerica, MA, USA), stripped with Re-blot Plus (EMD Millipore Corporation, Billerica, MA, USA) and re-probed to analyze given membranes with different antibodies.

Figure 1. Effect of PIC in combination with EA on viability of MPC-11 (A), OPM-2 (B), RPMI-8226 (C) and U-266 (D) myeloma cells. Cells were cultured with different compounds for three days. Viability was measured by DiOC6 and PI staining and flow cytometry. Results represent data of MPC-11 n=5, OPM-2 n=3, RPMI-8226 n=4 and U-266 n=3 separate experiments, respectively. Data are shown as mean±SD.

Figure 2. Effect of PIC in combination with either EA or CIC on viability of human CCD-18Co colon fibroblasts serving as controls. After incubation with 60 μM of PIC and either 35μM EA or 2 μM CIC for 72 h, cell viability was measured by DiOC6 and PI staining and flow cytometry. Results represent data from three separate experiments for CCD-18Co colon fibroblasts (n=3). Data are shown as mean±SD.
Statistical analysis. Values are presented as mean ± standard deviation (SD). Different sample sizes (n) were chosen for different cell lines. Student’s t-test was used for statistical analysis. A p-value of less than 0.05 was considered significant.

Results

Effect of PIC in combination with EA or CIC on viability of myeloma cell lines. Our recent studies have shown, that PIC exhibits selective toxicity on MPC-11, OPM-2, RPMI-8226 and U-266 myeloma cells and leads to a significant decrease in viability with a mean 50% inhibitory concentration (IC$_{50}$) after 72 h in the 24-33 μM range (F. Carsten Schmeel and L. Christopher Schmeel, submitted). However, achieved effects were dependent on the respective cell line and administered concentration. Therefore we determined the optimal concentration by titration of PIC in combination with EA and CIC and investigated whether the combination of these Wnt inhibitors would produce additional cytotoxicity. IC$_{50}$ values
of the either single-agent are presented in Table I. As a control, human CCD-18Co colon fibroblasts were investigated by FACS analysis.

**Effect of PIC in combination with EA on viability of human and murine myeloma cells and CCD-18Co colon fibroblasts serving as control cell line.** Both PIC and EA used as single-agent therapy showed a strong cytotoxic effect. Figure 1 reveals, however, that the combination of PIC plus EA led to a significantly higher decrease in viability in MPC-11, OPM-2, RPMI-8226 and U-266 myeloma cell lines than compared to either single-agent application, suggesting a synergistic effect for this combination. Interestingly, toxic effects on CCD-18Co control cells were similar to those obtained with EA alone (Figure 2). Figure 3A shows flow-cytometric results.

**Effect of PIC in combination with CIC on viability of human and murine myeloma cells and CCD-18Co colon fibroblasts serving as control cell line.** Used as single-agents, both PIC and CIC showed a toxic effect on the MPC-11, OPM-2, RPMI-8226 and U-266 myeloma cells, but induced a significantly higher decrease in viability of MPC-11, RPMI-8226 and U-266 cell lines when used in combination, suggesting a synergistic effect for this combination. However, the combination of PIC and CIC did not lead to a significantly higher decrease in viability of OPM-2 cells. Figure 4 illustrates the relevant results. Furthermore the combination of PIC and CIC showed a slightly higher cytotoxicity than compared to either single-agent application, resulting in a higher decrease in viability of the control cell line CCD-18Co colon fibroblasts. In Figure 2 the toxic effects of the respective agents on the CCD-18Co control cell line are shown. Figure 3B shows flow-cytometric results.

**Effect of EA on the Wnt/ beta-catenin pathway in human and murine myeloma cells.** We investigated the effect of EA on the Wnt/ beta-catenin pathway and therefore included several proteins in our study that are well-known as essential components of the Wnt-pathway (Figure 5). After exposure to EA, β-catenin expression was down-regulated in MPC-11,
RPMI-8226 and U-266 cells, in contrast to OPM-2 cells, where β-catenin expression was slightly increased. The human OPM-2, RPMI-8226 and U-266 myeloma cell lines were found to be LEF-1-negative. Murine MPC-11 cells, however, expressed LEF-1 which was down-regulated after EA therapy. The expression of TCF-4 decreased in OPM-2 and MPC-11 cells in comparison to RPMI-8226 and U-266 cells where the expression was mostly unaffected by EA. The presence of EA impaired the levels of c-myc in MPC-11 cells and slightly in OPM-2 cells while RPMI-8226 and U266 cells providing merely weak signals were mostly unaffected.

Figure 5. Western blot analysis of EA and CIC effects on the Wnt/beta-catenin pathway in human and murine myeloma cells by. Analysis was performed under untreated conditions and under treatment with either EA or CIC for 72 h. After cells were lysed western blot analysis was performed by using 80 μg of whole-cell lysates for β-catenin, C-myc, TCF-4 and LEF-1. Alpha-actin immunoblotting served as the loading control. Membranes were stripped and re-probed.

Effect of CIC on the Wnt/beta-catenin pathway in human and murine myeloma cells. When exposed to CIC, β-catenin expression decreased in MPC-11 and RPMI-8226 cells and slightly increased in OPM-2 and U266 cells. In murine MPC-11 myeloma cells, LEF-1 level was slightly down-regulated when CIC was added. TCF-4 expression was decreased by CIC in MPC-11 and OPM-2 cell lines, increased in RPMI-8226 and unaffected in U266 cells. The c-myc level was impaired by CIC in MPC-11 and slightly in OPM-2 cells, whereas RPMI-8226 and U266 cells were unaffected. Figure 5 shows the relevant results generated by western blot experiments.

Discussion

The canonical Wnt signaling pathway represents a perfect example of abrogated signaling pathways in MM (10-14). Development and proliferation of MM cells depends on the bone marrow microenvironment, wherein bone marrow stromal cells release Wnt ligands which enhance Wnt signaling in MM, consequently leading to an increased proliferation activity of MM cells (19-21).

Previous investigations of our group revealed both EA and CIC to be efficient inducers of apoptosis in lymphoma and myeloma cells. Besides, both drugs rendered the tested cell lines more sensitive to other agents and influenced the expression of β-catenin in at least one of our tested cell lines (17, 18, 22, 23). More recently we observed that PIC, also potently induces apoptosis in MM cells by influencing the Wnt signaling pathway through targeting either β-catenin itself or its downstream factors (F. Carsten Schmeel and L. Christopher Schmeel, submitted). Therefore, we tested EA, as well as CIC in combination with PIC.

We chose PIC since it produced significant anti-tumor effects in prior investigations. It was observed that PIC inhibits serine/threonine kinases, in particular Syk-kinases which are involved in many intracellular signaling processes (24). PIC demonstrated its capacity to abrogate PI3K/Akt/mTOR signaling, to diminish nuclear factor kappa-B (NF-κB) transcriptional activity and DNA binding of NF-κB as well as PIC inhibits IL-6/STAT3 signaling (25-27). In several malignancies an arrest of cancer cells in the S phase is seen by PIC treatment (28). Based on our results we corroborate the findings of Alas and Bonavida (16) describing that PIC can sensitize MM cells to a battery of chemotherapeutic drugs, particularly with regard to EA and CIC.

CIC is a synthetic anti-fungal agent, which is used to treat mycoses. Apart from its ordinary topical use in dermatology, CIC recently showed diverse anti-carcinogenic properties (18, 29-33). However, understanding the exact mechanisms of CIC’s antitumor effects is still in its beginning. Recent studies attributed these effects to iron-chelation and thus to inhibition of iron-dependent enzymes like ribonucleotide reductase (30). It was shown that CIC has the ability to slow down cell-cycle progression from G1/G0 to S phase in tumor cells through reduced levels of cyclines and cyclin-dependent kinases (CDKs) and up-regulation of CDK inhibitor p21.
Apoptosis is induced by the down-regulation of anti-apoptotic proteins (Bcl-xL and survivin) and increased levels of pro-apoptotic Bcl-2, subsequently leading to apoptosis (31). Recent investigations, including our prior studies, observed a down-regulation of Wnt signaling in colon and breast cancer cells as well as in lymphoma cells upon treatment with iron chelators such as CIC (18, 32, 33).

Analog to these findings in other cancer cells, the present study also indicates an influence of CIC on the Wnt signaling pathway in MM by abrogating the levels of β-catenin as a key protein within the Wnt pathway, thus leading to an altered expression of TCF-4 and c-myc, both downstream β-catenin factors. Additionally, murine MPC-11 cells showed decreased levels of LEF-1, a protein acting as transcription factor in association with TCF-4 and β-catenin.

As stated in several prior studies EA, a loop diuretic agent which was once commonly used, revealed a variety of interesting properties besides the inhibition of the Na⁺-K⁺-2Cl⁻ kidney symport. EA has been shown to be cytotoxic towards different cancer cells, including MM cells, and additionally led to an increase of other drugs’ cytotoxicity (17, 22, 34). The inhibition of glutathione-S-transferase (GST), causing increased cellular oxidative stress through higher glutathione (GSH) levels, was supposed to be the biological effect by which EA induces apoptosis. Yet another study did not confirm this finding, demonstrating an independence between GSH levels and EA-related induction of apoptosis (35). N-acetyl-L-cystein (NAC) could protect cancer cells from apoptosis upon treatment with EA while GSH levels remained increased (35). Apart from the inhibition of GST, recent studies reported EA to be an inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen activated protein kinase (MAPK) signaling (35, 36). In a previous study of our group, we showed that EA influences the Wnt signaling pathway in chronic lymphocytic leukemia cells by interaction with the LEF-1 protein, subsequently inducing a de-stabilization of the LEF-1/β-catenin complex. The expression of the Wnt target genes cyclin-D1, LEF-1 and fibronectin was also suppressed (23).

Regarding MM, our experiments are in accordance with this report of Lu D et al., demonstrating that EA affects the expression of crucial proteins associated to the Wnt pathway. In detail, the expression of β-catenin was altered in all cell lines tested, hence affecting TCF-4 and c-myc levels in most of the tested cell lines. In the case of murine MPC-11 cells, exposition to EA also impaired the expression of LEF-1.

Most interestingly, we could demonstrate a significant and synergistic apoptotic effect of either EA or CIC in combination with PIC in all tested myeloma cell lines. This might result from combined Wnt-antagonism. Although multiple effects due to an interaction of Wnt-antagonism and other associated signaling pathways seem imaginable, we focused solely on the effects of Wnt signaling in our experiments. To what extent the action mechanisms of the three substances synergistically complement each other and where an interaction exactly takes place with regard to the Wnt pathway should be subject of further investigations. In the case of PIC and EA, however, additional synergistic effects may be observed due to common inhibition of NF-κB and MAPK signaling.

In conclusion, our results reveal a synergistic effect of the combination of EA as well as CIC with PIC which leads to an increased cytotoxicity in myeloma cells. Combining Wnt inhibitors might, therefore, lead to improved treatment options for MM patients. Through the present study, we report on several details, thus confirming the notion that both EA and CIC inhibit the canonical Wnt pathway through targeting either β-catenin itself or its downstream factors.

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