Combination of PPP and LBH589 in multiple myeloma

The HDAC inhibitor LBH589 enhances the anti-myeloma effects of the IGF-1 RTK inhibitor picropodophyllin

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Statement of translational relevance

Multiple myeloma (MM) remains an incurable cancer. Picropodophyllin (PPP) is an IGF-1RTK inhibitor with promising anti-MM effects. Here, we describe the ability of HDAC inhibitor LBH589 to potentiate the effects of PPP in human cell lines and murine cells from the 5T33MM model. Our results show that combinatorial treatment synergistically decreases cell survival compared to single treatment. We saw increased cell death and stronger cleavage of caspase-8 after combinatorial treatment. Cell cycle analysis revealed G2/M arrest and subsequent down-regulation of cyclin B1, -E and –D2. Using Affymetrix array we observed an alteration on genes regulating apoptosis and cell adhesion. Finally, using the 5T33MM model we saw an significantly increased survival of myeloma inoculated mice compared to control groups. These results provide a better understanding of the molecular mechanisms underlying the anti-MM effects of PPP and LBH589 and supports the rationale for the clinical evaluation of the combination in MM therapy.
Abstract

Purpose: We have previously demonstrated the use of the IGF-1RTK inhibitor picropodophyllin (PPP) as an attractive strategy to combat multiple myeloma (MM) in vitro and in vivo. After a combinatorial drug screening the HDAC inhibitor LBH589 was shown to act in synergy with PPP reducing survival of MM cells. In the current study we tried to elucidate the molecular mechanisms underlying this combinatorial effect.

Experimental design: The in vitro anti-MM effects of PPP and LBH589 alone and in combination were evaluated by studying apoptosis, cell cycle distribution and downstream transcriptome using both human MM cell lines and cells from the murine 5T33MM model. In vivo the effect on survival of 5T33MM inoculated mice was evaluated.

Results: In the human MM cell line RPMI8226 treatment with PPP and LBH589 in combination resulted in a 5-fold increase of apoptosis and an additive effect on the cleavage of the active forms of caspase-8 was observed as compared to the single drug treatments. Cell cycle analysis revealed an accumulation of cells in the G2/M phase and subsequent down-regulation of cell cycle regulating proteins. These data were also confirmed in the 5T33MM cells in vitro. Also, the transcriptome was analyzed by Affymetrix arrays showing gene expression alterations mainly in categories of genes regulating apoptosis and cell adhesion. Combined treatment in vivo resulted in a significantly prolonged survival of 5T33MM inoculated mice.

Conclusions: The results indicate an improved MM treatment opportunity in using a combination of PPP and LBH589.
Introduction

Multiple myeloma (MM) is characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM), overproduction of monoclonal immunoglobulins and bone lesions (1, 2). MM is the second most prevalent hematological cancer that despite emerging novel therapies remains fatal. Importantly, there is a large interclonal variation between MM patients with only a few identified common chromosomal translocations to the IgH locus (3, 4) and anomalous gene expression reflecting activated extrinsic or intrinsic signaling pathways (5). However, these genetic abnormalities have only in a few cases been proven useful as prognostic markers or therapeutic targets (4, 6). Rather, the tumor microenvironment i.e. the tumor-host interactions and soluble growth factors expressed and produced by bone marrow stromal cells (7) are essential for MM tumor development and now constitute the focus for the development of emerging MM therapies (8). The bidirectional interactions between the MM cells and the BM stromal cells contribute to the survival and proliferation of the malignant cells via the activation of signaling pathways including the Ras/Raf/MAPK, the JAK/STAT3 and the PI-3K/Akt/ cascade (9).

Many MM growth factors have been identified, among which the insulin-like growth factor type 1 (IGF-1) is considered to play a major role in the tumor growth (10), supporting the survival and proliferation of both IL-6 dependent (11) and independent MM cells (12). Besides the anti-apoptotic and the proliferative effects, IGF-1 also contributes to VEGF-secretion which stimulates the angiogenic process and contributes to the homing of MM cells (13) as well as their resistance to cytotoxic treatment (14-16). All these features make IGF-1 an attractive target for therapeutic intervention in MM. Moreover, the fact that IGF-1 receptor (IGF-1R) signaling is not an absolute requirement for maintenance of normal adult cell homeostasis, has encouraged the development of IGF-1R inhibitors for clinical use in MM (17, 18).

Picropodophyllin (PPP), a member of the cycloolignan family, has been shown to be a potent inhibitor of the IGF-1R tyrosine kinase (IGF-1RTK) activity (19). We have previously demonstrated strong anti-tumor effects of the IGF-1RTK inhibitor PPP in vitro and in vivo using the 5T33MM and 5T2MM...
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murine models, as well as in a panel of human MM cell lines (16, 20). PPP inhibited the in vitro proliferation of MM cells and reduced the secretion of VEGF. Furthermore, PPP dramatically reduced tumor burden, angiogenesis and bone disease in vivo and significantly increased the survival rate of 5T2MM diseased mice when used in a therapeutical setting (20, 21). However, no selective drug is likely to be sufficient for single use in a therapeutic setting. PPP constitutes no exception to this notion and despite these encouraging data, the PPP treated mice eventually relapsed and showed signs of morbidity.

To overcome drug resistance and relapse in vivo we studied the possibility of treating MM with PPP in drug combination. By using an integrated SAIGAN Core System for high throughput screening a broad spectrum of substances were analyzed for synergistic effects with PPP. From this high throughput screening, HDAC inhibitors (HDACi) were shown to act in synergy with PPP and were therefore selected for further studies. HDACi represent a class of agents with anti-tumor activity against several hematological and solid cancers as they may act to reactivate tumor suppressor genes, inhibit cell cycle progression and induce apoptosis (22).

LBH589, the cinnamic acid hydroxamate, is an HDACi acting at nanomolar concentrations and inhibiting MM growth with promising results. LBH589 has previously been shown to induce apoptosis, enhance drug cytotoxicity (23, 24) and induces cell cycle arrest of MM cells (25). Using primary MM cells from patients and two MM cell lines, we previously demonstrated (26) that LBH589 may reactivate gene expression silenced by histone modifications as marked by the Polycomb repressive complex (PcG) in MM. The PcG has previously been found important for preserving self-renewal capacity in embryonic stem cells. Indeed, gene reactivation by LBH589 of selected genes was confirmed in the 5T33MM murine model. In parallel, LBH589 treatment resulted in a reduction of tumor load in vivo and increased overall survival in treated mice.

In the current study we demonstrate that the combinatorial use of PPP and LBH589 more efficiently inhibits cell proliferation by down-regulating the cell cycle proteins, cdk6 and cyclin D2, in the 5T33MM cells, and cyclin E, cyclin B1 and cyclin D2 in RPMI8226 cells. Moreover, treatment with
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both drugs also down-regulates the anti-apoptotic proteins Mcl-1 and Bcl-2 and enhances the apoptotic effect in RPMI8226 cells.

The anti-tumor effects of PPP in combination with LBH589 in vivo were studied using the 5T33MM murine model, in a fully syngeneic bone marrow microenvironment and showed a significant increase in overall survival as compared to the single-drug treatments.
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Material and methods

Animals

C57BL/KaLwRij mice, purchased from Harlan CPB (Horst, The Netherlands) were used at 6 to 10 weeks of age. Animals had free access to food and water and were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, VUB. (License no. LA1230281)

Cells

The 5T33MM cells originated spontaneously in elderly C57BL/KaLwRij mice and have since been propagated *in vivo*, by intravenous transfer of the diseased marrow in young syngeneic mice. This model closely represents the human disease with respect to clinical (i.e., selective localization in the BM, serum M component, and angiogenesis) and molecular (i.e., adhesion and chemokine profile) characteristics (27-29). When showing clear signs of morbidity, mice were sacrificed, and isolation of the MM cells was performed as previously described (30, 31). MM cells obtained from the diseased mice were 95% pure. These cells only survive for a short period *in vitro*.

The human MM cell lines RPMI8226 (32), Karpas707 (33), LP-1 (34) and OPM-2 (35) were maintained in RPMI1640 (Flow, Irvine, United Kingdom) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA), glutamine (2 mM), and antibiotics (penicillin 100 U/mL and streptomycin 50 g/mL) at 37°C in a humidified 5% CO2 in-air atmosphere.

Reagents

For the in vitro experiments LBH589 (Panobinostat, Novartis Pharmaceuticals Inc.) and Picropodophyllin (PPP) (19) were dissolved in dimethyl sulfoxide or in ethanol (DMSO/Ethanol; final concentration < 0.01 %) and aliquots were stored at -20°C (LBH589) and 4°C or -20°C (PPP). For the in vivo experiments LBH589 was dissolved in a saline solution and PPP was mixed into the food.
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Screening of drugs in combination with PPP

RPMI8226 and Karpas707 cells were seeded in drug-prepared 384 well plates at a density of 10 000 cells/well. The fluorometric microculture cytotoxicity assay (FMCA) was used for measurement of the cytotoxic effect and is based on hydrolysis of fluorescein diacetate (FDA). An integrated SAIGAN Core System containing Orca robot, CO2 incubator, dispenser, washer, delidding station, plate hotels, barcode reader, liquid handler (Biomek 2000) and a multipurpose reader (Fluostar Optima) enabled high through-put readout of cell survival after drug treatment. The combinatorial study was designed as suggested in the CalcuSun manual (BioSoft, Cambridge UK). 9 concentrations were tested and all combinations were investigated both with simultaneous and sequential addition of the drugs. The data were analyzed using the median-effect method of Chou and Talalay (36) using the CalcuSyn software version 2 (BioSoft, Cambridge, UK). The substances used in combination with PPP were etopside, mTOR inhibitor rapamycin, 5-FU, Vincristine, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA), dexamethasone, bortezomib (velcade/PS-341), 17-AAG/geldanamycin, As203 and the p38 inhibitor SB203580.

Viability assays

Purified 5T33MM cells were cultured during 24h in RPMI1640 medium with 10% serum as described above using different concentrations of LBH589 and/or PPP. Viability was measured using CellTiter-Glo Luminiscent Viability Assay (Promega, Madison, WI, USA) according to manufacturer’s instructions, determining the number of viable cells in culture based on quantitation of adenosine 5’ triphosphate (ATP).

To assess the viability of the human MM cell lines we performed Resazurin assay using AlamarBlue (Sigma) quantifying cell proliferation and cytotoxicity in relation to the presence of metabolically active cells (37). RPMI8226, Karpas707, LP-1 and OPM-2 were incubated in round-bottomed (RPMI8226, LP-1 and OPM-2) or flat-bottomed (Karpas707) 96-well plates with different concentrations of LBH589, PPP or in combination of both. After the indicated times, 10% AlamarBlue
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was added to the wells, followed by incubation for 1-3h at 37° C in a humidified 5% CO2 in-air atmosphere. Wallac Victor Multilabel Counter (Wallac, Yurku, Finland) was used for analysis of the fluorescence. Mean was calculated from triplicate wells and subtracted from mean of blank wells resulting in ΔFluorescence. The relative number of viable cells was expressed as percentage of untreated cells and calculated as 100 x ΔFluorescence (treated cells) / ΔFluorescence (untreated cells).

Analysis of cell cycle phase distribution and apoptosis

RPMI8226 cells were cultured in 6-well plates for 24h in the presence of reagents, LBH589, PPP or in a combination of both. Analysis of cell cycle distribution was performed according to Vindelöf (38), where propidium iodide (PI)-stained nuclei were analyzed by flow cytometry (FACScan, Becton Dickinson, San José, CA), and modfit LT 3.1 Analysis Software (Verity Software House, Topsham, ME) was used to calculate the distribution of the cell cycle phases. Briefly, approximately 10^6 cells were washed in cold PBS, lysed in NP-40 buffer and treated with trypsin (0.03 mg/ml) for 10 minutes at room temperature. Trypsin inhibitor (0.5 mg/mL) and ribonuclease A (0.1 mg/mL) were added followed by 10 minutes incubation in room temperature. Finally, the cells were exposed to PI (0.42 mg/mL) for 15 minutes at 4° C. The stained nuclei were analyzed as previously described.

Apoptosis was quantified by staining with Annexin V (AV)-fluorescein isothiocyanate (FITC) and PI using TACSTM Annexin V-FITC Apoptosis Kit (R&D Systems). The samples were treated as instructed by manufacturer followed by analysis using flow cytometry (FACScan) showing apoptotic cells as Annexin V-positive/PI-negative cells and the necrotic cells as Annexin V-positive /PI-positive cells.

Western blot analysis

RPMI8226 and 5T3MM cells were incubated with different concentrations LBH589 and/or PPP. After different time points, cell pellets were harvested, lysed and protein extracts were blotted as previously described (26, 39). Primary antibodies were used against CDK2, CDK4, cyclin D2, cyclin D1, Bcl-xL, Bcl-2, actin, cyclin B, cyclin E, IGF1-Rβ, Mcl-1, CDK1, CDK6 (Santa Cruz Biotechnology, Santa
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Cruz, CA), actin, Caspase-3, Caspase-8 and Caspase-9 (Cell Signaling Technology) and survivin (R&D Systems). Immunodetection of actin was used as loading control.

**Gene array**

RPMI8226 cells were cultured in 6-well plates for 6 and 24h in the presence of LBH589, PPP or in combination of both. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Expression study was performed using whole Human Genome U133 Plus 2.0 Affymetrix oligo arrays (Affymetrix, Santa Clara, CA).

**Survival experiment**

Preliminary experiments demonstrated that the suboptimal in vivo dose of PPP is 1.5 mg a day while that of LBH589 is 2.5 mg/kg (data not shown). At day 0 C57BL/KalwRij mice were inoculated with 5x10⁵ 5T33MM cells purified as described above. Mice were assigned to different treatment groups receiving 1.5 mg PPP/5 g food/day/mouse (n=8), 2.5 mg/kg LBH589 (n=8; daily i.p. injection) or a combination of both (n=8). The vehicle group (n=8) received normal food and a 0.9% NaCl solution (daily i.p. injection). As control an untreated, disease-free group (n=8) was used. Each mouse was sacrificed when showing signs of morbidity (paralysis of the hind legs).

**Statistical analysis**

For the in vitro data the Student t-test or the 2-way ANOVA and for the in vivo survival analysis the Kaplan-Meier analysis method was used. A p-value <0.05 was considered significant. For microarray analysis 2-way ANOVA was used to find the differentially regulated genes and to do the Hierarchical clustering using software GeneSpring GX v11 (Agilent Technologies, Santa Clara, CA). We used the Chou and Talalay method (36) to calculate combination index (CI). CI >1, =1 or <1 indicate antagonism, additive effect or synergy, respectively. For the calculations of the CI-values a computer software was used (ComboSyn, Inc. 599 Mill Run, Paramus, NJ, 07653, USA).
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Results

Screening of drugs acting in synergy with PPP

Our previous studies have shown that treatment with PPP in vivo reduced tumor load and prolonged the survival of the 5TMM diseased mice. However, eventually the myeloma tumor reappears (21). For the purpose of finding suitable partners acting in synergy or addition to the PPP, a panel of selected substances including conventional drugs used in MM high-dose chemotherapy, as well as classes of new anti-tumor agents recently added to the potential therapeutic armamentarium against MM were analyzed. The drugs used in the combinatorial assay were etoposide, mTOR inhibitor rapamycin, 5-FU, Vincristine, SAHA, dexamethasone, bortezomib (Velcade/PS-341), 17-AAG/ geldanamycin, As2O3 and the p38 inhibitor SB203580. The survival analysis of the schedule dependency of PPP showed that of these selected substances, the p38 inhibitor SB203580, Bortezomib (Velcade/ PS-341) and the histone deacetylase inhibitor (SAHA) will sensitize the cells to IGF-1RTK inhibitor PPP (data not shown). HDACi were subjected to further studies.

The effects of PPP and/or LBH589 on cell survival in mouse and human myeloma cells in vitro

For further analysis on the use of HDACi in combination with PPP, LBH589 was selected due to its potency as a novel developed hydroxamic-acid derived HDAC inhibitor at present in clinical trials for MM.

We first examined the effects of both drugs on cell viability using four human MM cell lines: RPMI8226, Karpas707, LP-1 and OPM-2. Cells were exposed to increasing concentrations of PPP and/or LBH589. After 72h the relative number of viable cells was determined by using the resazurin assay. All cell lines responded dose-dependently to LBH589 and PPP as single drugs. The IC50 values for LBH589 where at low nanomolar range for all cell lines (16.0 nM for the RPMI8226 cells; 11.0 nM for the Karpas707 cells; 7.42 nM for the LP-1 cells and 2.8 nM for the OPM-2 cells. Data not shown). When treated with PPP all the human cell lines showed similar IC50 values (0.33 μM for the RPMI8226 cells; 0.30 μM for the Karpas707 cells; 0.31μM for the LP-1 cells and 0.24 μM for the OPM-2. Data not shown). Simultaneous treatment of these cell lines with different concentrations of
PPP and LBH589 resulted in a significant decrease in cell survival when compared to the single drugs (p < 0.05), with the only exception of the low combination in the RPMI8226 cells. When we combined both drugs at intermediate and high concentrations, synergistic effects were observed for all the cell lines (CI < 0.90). For the Karpas707 cells synergy was also observed at low combination treatment (Figure 1A-D). We then examined the effects of both drugs, alone and in combination, on the survival of 5T33MM murine cells. After 24h treatment the IC50 values were 8.22 nM for LBH589 and 1.58 µM for PPP (Data not shown). The survival of 5T33MM cells was significant lower when treated with both drugs as compared to the single treatments (p < 0.05), and synergy was demonstrated for all the combinations with CI values lower than 0.90 (Figure 1E).

**The effects of PPP and/or LBH589 on cell cycle distribution and cell cycle proteins**

Drugs effect on cell cycle proteins was analyzed by Western blotting. After 24h in RPMI8226, a more pronounced down-regulation of cyclin E, cyclin B1 and cyclin D2 could be detected in the combinatorial design compared to single dose treatment. Cyclin-dependent kinase 1 (cdk1) also got slightly down-regulated in the combinatorial design but not in the single drug treatment. The expression of cyclin-dependent kinase 4 (cdk4) and 6 (cdk6) remained unchanged (Figure 2A).

Cell cycle distribution demonstrated that exposure of RPMI8226 cells to PPP increased the fraction of cells in G2/M phase from 14 to 32% after 24h, with a corresponding decrease of fraction of cells in G1/G0 and S phases. Treatment with LBH589 for 24h increased the number of cells in G1/G0 phase from 42 to 69%, with a corresponding decrease of fraction of cells in S and G2/M phases. For the combinatorial design the amount of cells increased in the G2/M, from 14 to 32% after 24h (Figure 2C).

Western blot analysis of 5T33MM cells after 24h demonstrated that the expression of most analyzed cell cycle proteins remained unchanged compared to the single treatments (data not shown). Cyclin-dependent kinase 6 (cdk6) expression was reduced by PPP while completely inhibited in the combinatorial design. An increased down-regulation of cyclin D2 could also be detected in the combinatorial design compared to the single treatments (Figure 2B).
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The effect of PPP and/or LBH589 on apoptosis

Both drugs, individually, caused an increase in the relative number of apoptotic and late apoptotic/necrotic cells compared to the control in RPMI8226 cells, but to lesser extent as compared to the combinatorial design. After 48h, a 5-fold increase of apoptotic and late apoptotic/necrotic cells was detected in the combinatorial design compared to the untreated (p<0.001). A significant increase of apoptotic and late apoptotic/necrotic cells were found in the combinatorial design compared to LBH589 p<0.01 for the 24h time point, p<0.001 for the 48 and 72h time points and PPP p<0.001 for all three time points (Figure 3A).

A clear cleavage of caspase -9, -8 and -3 caspases could first be detected after 24h treatment of RPMI8226 cells (Figure 3B, D and F). LBH589 induced stronger cleavage of the active forms (p37/35 and p17) of caspase 9 than PPP. The same could be detected in cleavage of caspase 3. Treatment with both drugs induced a stronger cleavage of the active forms (p43/41 and p18) of caspase 8 compared to the single drug treatment. Similar results were observed in the 5T33MM cells (Figure 3C, E and G).

We then evaluated whether the expression of the apoptosis related molecules Mcl-1, Bcl-xL, Bcl-2, Bim and survivin were affected in the cells after treatment with the drugs. The protein levels of these molecules were analyzed by Western blotting. For the human cell line RPMI8226 a clear down-regulation of the expression of the anti-apoptotic protein Mcl-1, could be detected after 24h of treatment with PPP and in the combinatorial design (Figure 4A). Bcl-2 was reduced when using both drugs after 24h in the RPMI8226 (Figure 4A) cells and the 5T33MM cells (Figure 4B). No regulation of survivin or Bim expression could, however be detected in neither RPMI8226 nor 5T33MM cells (data not shown). In the 5T33MM cells we also observed a down-regulation of BCL-xL in the combination treatment (Figure 4B). Treatment with the drugs also induced a clear down-regulation of IGF1-Rβ in the combinatorial design in both the RPMI8226 and the 5T33MM cells (Figure 4C and D).
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**Gene Array data**

The drug combination has more differential enhanced levels of expression, seen for several genes at 24h of drug combination than the single drugs alone (Supplementary table 1). Microarray was validated using Real time PCR (data not shown). Functional analysis of the regulated genes was done using the software DAVID and several important categories were found, out of which 6.7% were apoptotic genes, 6.3% were cell adhesion, 4.5% were hematopoiesis, and 2.2% DNA packaging (Figure 5).

**Survival of 5T33MM mice using a combination of LBH589 and PPP**

Using the 5TMM murine models our groups have previously demonstrated the anti-myeloma effect of both compounds (16, 20, 26). LBH589 and PPP were able to reduce tumor load and increase the survival rate when used at optimal concentrations. Here we examined the effect of the combinatorial treatment using sub-optimal concentrations of both compounds. *In vivo* treatment of 5T33MM inoculated mice with a combination of LBH589 (2.5 mg/kg/day) and PPP (1.5 mg/day) resulted in a prolonged survival when compared to the vehicle group (p<0.0001), the LBH589 treated group (p<0.05) and the PPP treated group (p<0.0001) (Figure 6).
Discussion

New treatment approaches are necessary to overcome MM disease. Not only has the expression of IGF-1R been associated with progressive disease and drug resistance (40), the use of small synthetic molecules and neutralizing antibodies targeting the IGF-1R signal have been proven useful in tumor models of MM in vitro and in vivo as demonstrated by us and others (17, 20, 21, 41). The most salient result speaking in favor of the IGF1-1R as a candidate for novel MM therapy is the modest effects of the IGF-1 on the growth of normal non-malignant cells. Accumulating evidence during the last decade suggests an important role for IGF-1 in migration, survival and expansion of MM cells in the BM microenvironment and in VEGF induced angiogenesis (7, 10). Recently, shedding some light on the control of apoptosis mediated by microenvironmental factors, we showed that IGF-1 may silence gene expression of pro-apoptosis genes i.e. Bim by epigenetic mechanisms (39).

As a result of MM acquiring additional genetic lesions during its progression in vivo compensatory pathways of survival will eventually emerge as a result of drug exposure (42). Drugs targeting the IGF-1R present no exception (40, 43) and approaches targeting the IGF-1R are therefore unlikely to be curative as a solo therapy in MM. In line with this notion, remaining MM cells after treatment with the RTK inhibitor PPP in the in vivo 5T2MM model, although initially reducing tumor load, eventually lead to relapse and mortality (20).

In this paper we report the synergistic cytotoxicity in MM cells using the selective IGF-1R inhibitor PPP in combination with the HDACi LBH589, panobinostat. LBH589 was selected from a large drug screen aiming at finding candidate drugs enhancing the effect of PPP. The drug screen in this study included conventional drugs used in MM high-dose chemotherapy, immunomodulatory analogs, the proteasome inhibitor bortezomib (Velcade/PS-341), and drugs selectively targeting parallel pathways of myeloma survival, i.e. mTOR and p38 inhibitors and were also subjected to schedule dependency with PPP.

HDACi as single agents are in clinical trials for treatment of MM patients (44). However, several publications have pointed to the potential use of using HDACi in combination with conventional
chemotherapeutic drugs. Recently, LBH589 was shown to have synergistic anti-myeloma activity in combination with dexamethasone, bortezomib or melphalan (23, 25).

The synergistic effect of LBH589 and PPP could be demonstrated in the human cell lines RPMI8226, Karpas707, LP-1 and OPM-2 and also in the 5T33MM murine cells. Simultaneous treatment with both drugs showed statistically significant synergistic inhibition of cell survival with CI values lower than 0.90. Using the RPMI8226 human MM cell line for in-depth studies, simultaneous treatment with LBH589 and PPP resulted in an increase of caspase -9, -8 and -3 activity and apoptotic and late apoptotic/necrotic cells as compared to controls. The combination of LBH589 and PPP could be monitored as an accumulation of cells in the G2/M phase, and subsequent down-regulation of cell cycle regulated proteins. The effect of both compounds on the expression of cyclin B1, -E and –D2 was additive, as demonstrated by Western blot. Gene expression analysis reveals that affected genes belong to important categories like apoptosis, hematopoiesis, cell adhesion and DNA packaging. However, it must be pointed out that the drugs used in the present combination are at suboptimal concentrations and are not likely to correspond to previous results when using the drugs in solo.

Experiments using CD138+ primary cells from four MM patients showed that one out of four patients responded to suboptimal concentrations of PPP with a reduction of viability with >50%. A combinatorial effect was observed in the patient responding to PPP (data not shown).

The BM microenvironment plays an important role in the expansion and survival of the MM cells (45). The 5T33MM model offers a unique possibility to study the benefits of in vivo drug combinations in a therapeutic setting since the tumor grows in a syngeneic BM microenvironment (28). Using this model we could demonstrate that the combination of PPP and LBH589 is able to prolong the survival rate of 5T33MM diseased mice suggesting that this therapy is able to evade the favorable impact of the tumor microenvironment on tumor progression and expansion.

The importance of targeting drug resistant tumor cells is also well recognized. However, the definition of this population with stem cell and tumor initiating properties is a matter of constant debate, most recently extensively discussed at the 52nd annual meeting of ASH. The two current opposing views on
the definition of the tumor stem cell is either the existence of MM progenitors resembling normal memory B cells that display properties of normal stem cells (46), or that the bulk of MM cells has an inborn potential of tumor initiating capacity or stemness, expressing a gene profile preferentially allowing for proliferation over differentiation (26). The view favoring the presence of a few circulating tumor stem cells would presume all darwinistic selection of the tumor to occur in a small population of non-proliferating tumor cells, while the latter would allow for genetic alterations leading to clonal variation and acquired drug resistance during disease progression. The recently published hypothesis by us of the potential of tumor stemness lying within the epigenetically silenced gene profile of MM is an attractive idea due to the fact that it may be reverted by pharmacological intervention. In our recent paper (26) the pan-HDACi LBH589 was used to activate the defined gene signature underexpressed in MM as compared to normal BM plasma cells. Although LBH589 was shown to act as a reactivator of gene expression silenced by Polycomb-mediated mechanisms (26), drugs directly targeting components of the silencing complex would be preferred. Further studies are certainly needed to define the tumor initiating population in MM in order to design selective treatment combinations for successful targeting.

In conclusion, we show here that the combination of LBH589 with PPP, a potent inhibitor of the IGF-1R signaling, more efficiently inhibits cell proliferation, down-regulates anti-apoptotic proteins and enhances the apoptotic effect compared to single drug treatment. The combination also significantly prolongs the survival in the 5T33MM \textit{in vivo} murine model compared to the single drugs, suggesting that PPP in combination with LBH589 should be a candidate for improved treatment of MM patients.

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References


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Combination of PPP and LBH589 in multiple myeloma


Figure legends

Figure 1. Combined treatment of PPP and LBH589 significantly reduces growth in MM cell lines and 5T33MM cells compared to single dose treatment. (A) and (B): RPMI 8226 and Karpas707 were incubated for 72 hours with PPP and/or LBH589 (low 0.1 µM PPP / 5 nM LBH589; intermediate 0.2 µM PPP / 10 nM LBH589; high 0.375 µM PPP / 20 nM LBH589) followed by Resazurin assay. At least 3 experiments were performed in triplicates, data is presented as mean percentage of control ±SD. (C) and (D): LP-1 and OPM-2 were incubated for 72 hours with PPP and /or LBH589 (low 0.2 µM PPP / 5 nM LBH589; intermediate 0.4 µM PPP / 5 nM LBH589; high 0.4 µM PPP / 10 nM LBH589) followed by Resazurin assay. At least two experiments were performed, data is presented as mean percentage of control ±SD. (E): 5T33MM cells were incubated for 24 hours with PPP and/or LBH589 (low 0.5 µM PPP / 2.5 nM LBH589; intermediate 1.0 µM PPP / 5.0 nM; high 1.5 µM PPP / 7.5 nM LBH589) followed by cellTiter-Glo Luminiscent Viability assay. Asterisks (* P < 0.05, ** P < 0.01, *** P < 0.001) mark statically significantly differences between groups. CI = combination index.

Figure 2. Combined treatment of PPP and LBH589 down-regulates cell cycle proteins more efficiently than single dose treatment. (A) RPMI 8226 cells were incubated with PPP and/or LBH589 (0.375 µM / 20 nM) for 24 hours and (B) 5T33MM cells for 24h. Western blot was performed using the indicated antibodies. Actin was used as loading control. (C) Cell cycle distribution after treatment with PPP and/or LBH589 (0.375µM / 20 nM) for 24 hours followed by analysis using Vindelöv method.

Figure 3. Combined treatment of PPP and LBH589 increases the amount of apoptotic cells compared to single drug treatment and activate caspases. RPMI 8226 cells were incubated with PPP and/or LBH589 (0.375µM / 20 nM) and 5T33MM cells were incubated with PPP and/or LBH589

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(1.5 µM / 7.5 nM) (A) RPMI 8226 cells were treated with PPP and/or LBH589 (0.375 µM / 20 nM) for 24, 48 and 72 hours followed by AV/PI staining and flow cytometry analysis. At least 3 independent experiments were performed. Data is presented as mean percentage apoptotic and late apoptotic/necrotic cells ±SD. (B) RPMI 8226 and (C) 5T33MM cells, caspase 9. (D) RPMI 8226 and (E) 5T33MM cells, caspase 8. (F) RPMI 8226 and (G) 5T33MM cells, caspase 3.

**Figure 4. The effects of PPP and LBH589 on apoptotic proteins.** RPMI 8226 cells were incubated with PPP and/or LBH589 (0.375µM / 20 nM) for 24 hours and 5T33MM PPP and/or LBH589 (1.5 µM / 7.5 nM) for 24h (A) and (C) RPMI 8226 cells and (B) and (D) 5T33MM cells. Expression of anti-apoptotic proteins were analyzed by western blotting using specific antibodies. Actin was used as a loading control.

**Figure 5. Gene Array data.** Pie chart depicting the percentage differentially regulated genes present in the different functional categories.

**Figure 6. Combined treatment of PPP and LBH589 increases survival compared to single dose treatment in the 5T33MM mice model.** Survival was studied using Kaplan-Meier analysis method. At day 0 C57BL/KalwRij mice were injected with 0.5 x 10⁶ 5T33MM cells. At day 0 the mice were either assigned to a PPP group receiving 1.5 mg/kg, a LBH589 group receiving 2.5 mg/kg, a combo group receiving 1.5 mg/kg PPP and 2.5 mg/kg LBH589, a vehicle group receiving 0.9 % NaCl solution or to an untreated, disease-free group as a control group (naïve mice). The treatment continued until each animal showed signs of morbidity.
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Figure A shows the percentage of Annexin V/PI^+ cells relative to control at 24h, 48h, and 72h. The findings are statistically significant as indicated by the asterisks.

Figure B and C depict Western blot analysis for Caspase 9 at 24h, showing the effects of LBH589 and PPP treatments.

Figure D and E display Western blot analysis for Caspase 8 and 3 respectively, including the p57, p47, p37/35, p17, p43/41, p18, p19/17, and actin bands.

Figure F and G illustrate the effects of LBH589 and PPP on Caspase 3 at 24h, with corresponding Western blot images for p19/17 and actin.
DNA packaging: 2%
Haematopoiesis: 5%
Adhesion molecules: 6%
Apoptosis: 7%
Others: 80%
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The HDAC inhibitor LBH589 enhances the anti-myeloma effects of the IGF-1 RTK inhibitor picropodophyllin

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