The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma

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The aim of this study was to evaluate the effects of valproic acid (VPA), as a histone deacetylase inhibitor, on myeloma cell lines and on sorted human bone marrow multiple myeloma cells. VPA induced accumulation of acetylated histones, potently inhibited proliferation in a dose-dependent manner and induced apoptosis in all myeloma cell lines tested as well as in sorted primary multiple myeloma cells. Cell cycle analysis indicated an arrest in G0/1 phase in response to VPA. Accumulation of p21 and reduced levels of cyclin D1 were detected. The production of vascular endothelial growth factor was significantly inhibited by VPA. These results provide the framework for clinical trials.

Key words: multiple myeloma, HDAC inhibitor, valproic acid, vascular endothelial growth factor.

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Despite recent advances in treatment, multiple myeloma remains incurable in the majority of patients.[1-3] Histone deacetylase (HDAC) inhibitors form a new class of anticancer drugs[4] and several of these drugs are currently being investigated in clinical trials.[5] Valproic acid (VPA) is a short-chain fatty acid with a long history of clinical use as an anticonvulsant.[6] However, VPA also inhibits HDAC and induces apoptosis in selected solid tumors[7,8] as well as in hematologic neoplasias.[9] The aim of this study was to define the effects of VPA on multiple myeloma cells.

Design and Methods

Cells and reagents. Human multiple myeloma cells lines OPM-2, NCI-H929 and LP-1 were cultured as recommended. Myeloma cells from bone marrow aspirates from patients with multiple myeloma were sorted immunomagnetically using MACS mouse anti-human CD138 beads as previously described.[10] Valproic acid was prepared in sterile phosphate-buffered saline immediately before use. The investigations were approved by the ethics committee of the University Hospital Charité in Berlin, Germany, in accordance with the Declaration of Helsinki and informed consent to the use of their biological material was obtained from all patients.

Cell proliferation assays. The MTT-test was used for cell viability studies. In dose-response studies, VPA was added to cells in different concentrations and cells were incubated for 48 hours. Data were obtained in three independent experimental sets.

Assessment of apoptosis. The extent of apoptosis was evaluated by annexin V staining. Cell lines or immunomagnetically sorted bone marrow multiple myeloma cells from patients were incubated in the presence of the HDAC inhibitor and stained with annexin V–fluorescein isothiocyanate and propidium iodide. Samples were analyzed by flow cytometry. Similar data were obtained in at least two independent experimental sets.

Cell cycle analysis. In order to evaluate the effects of VPA on cell cycle distribution of multiple myeloma cells, cell lines were exposed to VPA or solvent. After DNA staining, cells were analyzed on a FACS flow cytometer using ModFit software.

Western blot analysis. Protein of lysates from sorted bone marrow multiple myeloma cells and from cell lines were separated by gradient SDS–PAGE and blotted on PVDF membranes. Membranes were incubated with antibodies against acetylated histone H3, p21 and p27. Binding of the peroxidase-conjugated secondary antibody was detected by chemiluminescence. As an internal loading control, anti-β-actin antibodies were used.

Measurement of vascular endothelial growth factor (VEGF) in cell culture supernatants. Cells were cultured and incubated with VPA. After incubation, cell culture supernatants were collected for VEGF analysis and cell pellets were lysed and protein content determined. VEGF production was measured using a commercially available VEGF-enzyme-linked immunosorbent assay (R&D). The test was performed in two independent sets of experiments. Results of VEGF measurements were normalized to protein content and set in relation to control concentrations.

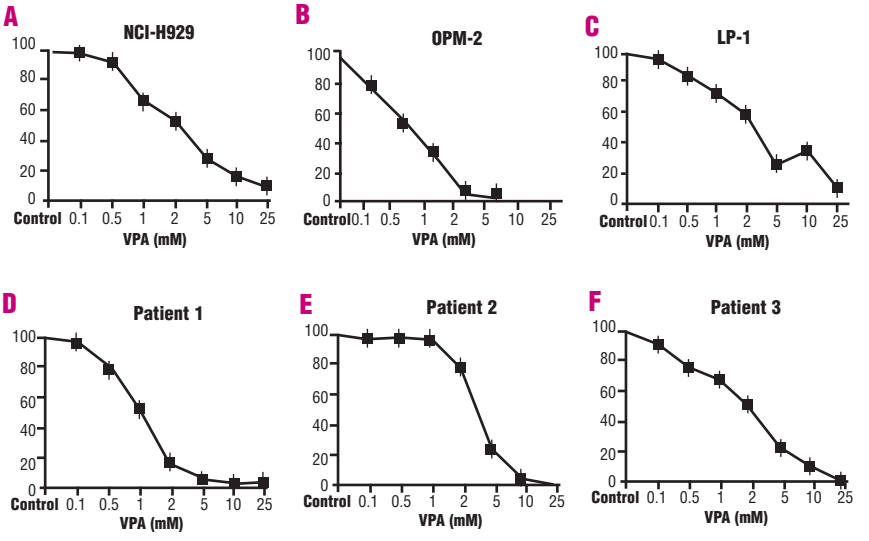


Figure 1. Valproic acid inhibits growth of multiple myeloma cell lines and sorted bone marrow myeloma cells. (A-C) The multiple myeloma cell lines NCI-H929 (A), OPM-2 (B) and LP-1 (C) were incubated with varying concentrations of valproic acid for 48 hours. Cell viability was assessed by the MTT assay. Results are presented as percentage of viable control cells treated with solvent alone. Values represent the means±SD for three separate experiments performed in sextuplets. (D-F) Myeloma cells were sorted from bone marrow aspirates from patients with multiple myeloma and incubated for 48 h with varying concentrations of valproic acid and cell viability was measured by the MTT assay.

Proteasome activity assay. Cells were washed with phosphate-buffered saline and lysed in lysis buffer. After estimation of protein content, lysates were incubated in duplicate with substrate buffer containing 100 μM of a fluorogenic substrate (Suc-LLVY-AMC). Fluorescence was measured and normalized to protein content.

Statistical analysis

The data are presented as means ± SD. When applicable, statistical analysis was performed using the Mann-Whitney-U test. All data were confirmed in at least two independent sets of experiments. IC_{50}-values were calculated using the median-effect plot method.

Results and Discussion

VPA inhibits proliferation of multiple myeloma cell lines. Human myeloma cell lines were exposed to various concentrations of VPA for 48 hours and their viability was subsequently measured by the MTT assay. All cell lines tested were sensitive to VPA in a dose-dependent manner. After 48 hours of incubation, the IC_{50} was observed to be approximately 0.5 mM for OPM-2, 2 mM for NCI-H929 and 2.5 mM for LP-1. As shown in Figure 1, cell proliferation at a concentration of 2 mM VPA was reduced to 10% in OPM-2, 50% in NCI-H929 and 60% in LP-1 cells after 48 hours.

VPA inhibits proliferation of primary multiple myeloma cells. The influence of VPA on sorted bone marrow multiple myeloma cells from patients was also investigated using the MTT assay. Freshly sorted cells were incubated with VPA for 48 hours. Multiple myeloma cells of three different patients were responsive to treatment with VPA (Figure 1) with interindividual differences. Calculated IC_{50}-values were approximately 0.8 mM, 1.3 mM and 3.2 mM VPA. After 48 hours of incubation with VPA, relative cell viability at 2 mM was 20% for patient #1, 80% for patient #2 and 50% for patient #3.

VPA induces apoptosis in multiple myeloma cell lines and in bone marrow myeloma cells from patients. VPA showed a strong induction of specific apoptosis in all cell lines tested. After 48 hours of treatment with 1 mM VPA, approximately 50%, 50% and 30% of OPM-2, NCI-H929 and LP-1 cell lines, respectively underwent specific apoptosis, (Figures 2 A-C), whereas 72 hours of incubation with 5 mM VPA resulted in apoptosis of about >85% of all cell lines tested.

Immunomagnetically sorted bone marrow multiple myeloma cells from two patients showed specific apoptosis rates of 32% and 19% after 48 hours of incubation with 1 mM VPA (Figure 2 D-E).

VPA leads to G0/G1 cell cycle arrest in myeloma cell lines. To investigate cell cycle modifications in response to VPA, myeloma cell lines were subjected to cell cycle analysis. Cells were exposed to VPA for 48 and 72 hours. Flow cytometric evaluation showed a significant decrease of cells in S phase and a G0/G1 cell cycle arrest after 72 hours treatment with VPA (*data not shown*).

VPA leads to accumulation of acetylated histones and influences $p21^{WAF1}$ and $p27^{KIP1}$ protein levels in myeloma cell lines and in bone marrow myeloma cells. OPM-2 and NCI-H929 were exposed to 5 mM VPA for 0, 4, 8 or 24 hours. Bone marrow myeloma cells isolated from multiple myeloma patients were incubated with either 5 mM VPA or solvent over a period of 24 hours, lysed and subjected to western blotting. Cells treated with VPA showed an increased acetylation of histones H3. p21WAF1 protein levels were increased in the cell lines. Levels of p27KIP1 remained constant in NCI-H929 and OPM-2. Equal loading of lanes was confirmed by incubation with an anti-β-actin-antibody (Figure 3).

Activity of the 20S-proteasome is not altered by treatment with VPA. In contrast to results of a recent study,[11] in our cell lines treatment with VPA had no measurable effect on 20S-proteasome activity, even at concentrations that definitely induced apoptosis (*data not shown*).

VPA leads to decreased expression of VEGF by myeloma cells. We examined the influence of VPA on VEGF production in cell culture supernatants of the cell lines

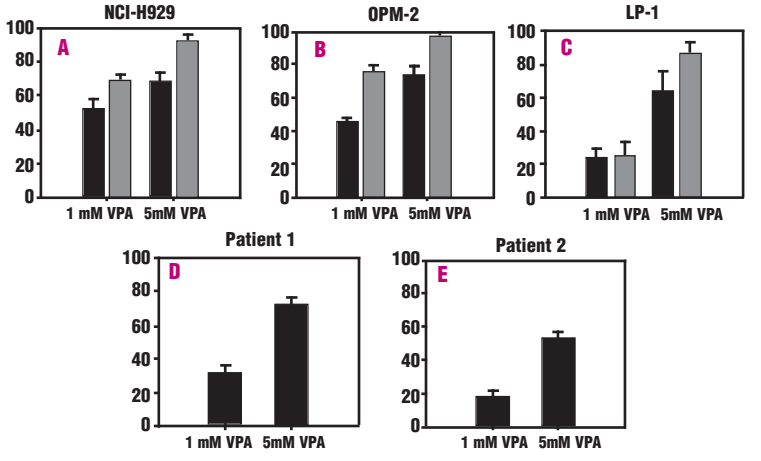
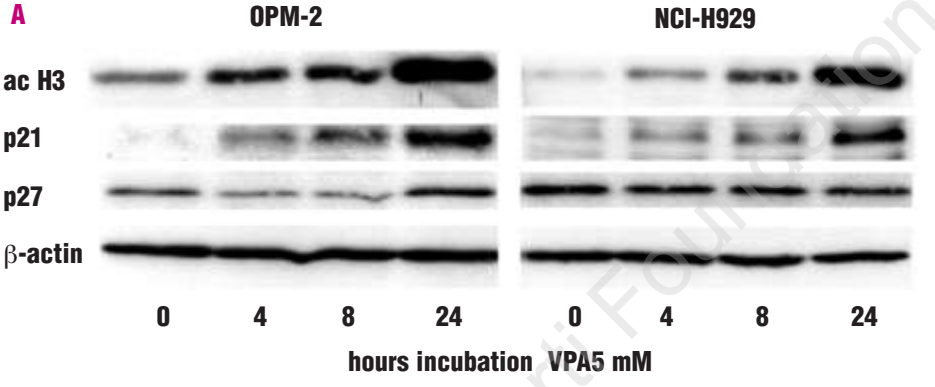


Figure 2. Valproic acid induces apoptosis in human myeloma cell lines and immunomagnetically sorted bone marrow multiple myeloma cells from patients. (A-C) The cell lines NCI-H929 (A), OPM-2 (B) and LP-1 (C) and sorted primary myeloma cells from two patients (D,E) were treated with 1 mM and 5 mM VPA for 48 h, cell lines were additionally incubated for 72 h. Cells were stained with anti-annexin V-FITC antibody to detect early apoptosis and propidium iodide to detect late apoptosis and analyzed by flow cytometry. Specific apoptosis was calculated as described in the Design and Methods section. Dark bars represent specific apoptosis after 48 h and bright bars after 72 h of incubation with valproic acid. Results are means ± SD from two independent sets of experiments. All data were significant at the $p<0.05$ level.



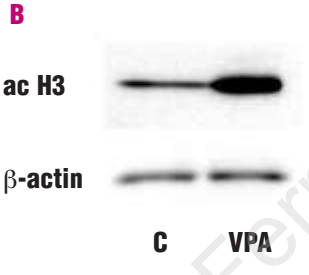


Figure 3. Valproic acid leads to acetylation of histones and upregulates p21. (A) Multiple myeloma cell lines OPM-2 and NCI-H929 were incubated for 0, 4, 8 or 24 h with VPA 5 mM. At the end of these periods, the cells were lysed and subjected to western blot analysis. Each lane was loaded with 50 μg of protein; blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading and quality of protein transfer. Two independent studies yielded equivalent results. (B) Primary sorted multiple myeloma cells from a patient were incubated for 24 h with VPA 5 mM, lysed and subjected to western blot analysis. Each lane was loaded with 25 μg of protein. After incubation with anti-acetylated histone H3 antibodies, the membrane was stripped and reprobed with anti-β-actin antibodies to ensure equal loading and quality of protein transfer.

OPM-2 and NCI-H929. Treatment with 0.1 mM VPA for 48 hours led to a significant ($p<0.05$) decrease of VEGF concentration by 20% in OPM-2 cells in comparison to the production by untreated cells; incubation with 0.5 mM VPA reduced VEGF production by 39% ($p<0.05$). The VEGF concentration in cell culture supernatants of NCI-H929 decreased to 58% ($p<0.05$) when cells were cultured for 72 hours with 1 mM VPA in comparison to untreated controls.

It has been reported that selected HDAC inhibitors are effective against multiple myeloma.[14,15] However, no HDAC inhibitor is so far available for broad clinical use. The anticonvulsant VPA has HDAC inhibiting activity.[6] It induces apoptosis in some solid tumors[7-8] and in selected hematologic disorders.[9] Clinical trials with VPA in acute myeloid leukemia and myelodysplastic syndromes have been initiated.[16] No report so far has investigated the antitumor activity of VPA in multiple myeloma. We evaluated the effects of VPA on human myeloma cell lines as well as on multiple myeloma cells isolated from bone marrow aspirates.

We found an increase of acetylated histone H3 in myeloma cell lines and in sorted primary myeloma cells after treatment with VPA. In all of the cell lines tested, as well as in primary multiple myeloma cells, VPA dose-dependently inhibited proliferation after 48 hours of treatment. Protein levels of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$ increased markedly after VPA treatment, whereas $p27^{KIP1}$ levels remained stable. These findings are consistent with other reports[17] and support the theory of increased $p21^{WAF1}$-expression as a possible mechanism by which VPA inhibits tumor growth. Flow cytometric cell cycle analysis revealed a marked increase of cells in G0/G1-phase after VPA treatment. In order to investigate whether decreased proliferation was accompanied by an increased rate of apoptosis, we performed annexin V flow cytometry on bone marrow multiple myeloma cells and myeloma cell lines. Results

showed a markedly increased rate of apoptosis after 48 hours of treatment with VPA. Recently, it was suggested that VPA may also have proteasome-inhibiting activity.[11] However, in our experiments proteasome activity was not altered by incubation with VPA.

Angiogenesis is essential for tumor growth and survival.[12] Myeloma cell lines are known to produce VEGF.[18–20] In the present study we investigated the influence of sub-apoptotic doses of VPA on the secretion of VEGF, a potent stimulator of angiogenesis. VPA significantly inhibited VEGF production in myeloma cells. These data suggest that VPA could have an anti-angiogenic effect in the bone marrow microenvironment in patients with multiple myeloma.

In this study we showed that VPA acts as an HDAC inhibitor in multiple myeloma cells, induces G1 cell cycle arrest, potently inhibits tumor growth and markedly induces apoptosis. In addition to its direct antitumor effect, VPA reduces VEGF production in myeloma cells. These data provide the framework for clinical trials with valproic acid in multiple myeloma.

MK performed most experiments, MK, UH, OS contributed to the conception, analysis and interpretation of data, drafting the article and final approval of the version to be published. IZ, JS, CJ, CF contributed to the analysis and interpretation of data. P-MK contributed to proteasome related work. The authors reported no potential conflicts of interest.

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