

# Histone deacetylase inhibitor valproic acid inhibits proliferation and induces apoptosis in KM3 cells via downregulating VEGF receptor

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## Abstract

The expression of vascular endothelial growth factor receptor 1 (VEGFR-1) in human multiple myeloma KM3 cells in vitro, effects of valproic acid (VPA), as a histone deacetylase inhibitor, on cell proliferation and apoptosis and the underlying molecular mechanism were investigated. The effects of VPA on the growth of KM3 cells were studied by MTT assay. The apoptosis rate was determined with flow cytometry. The mRNA level of VEGFR was determined by RT-PCR; and immunocytochemistry was used to detect the protein level of ac-H4 and VEGFR. VPA inhibited proliferation of KM3 cells in a time- and dose-dependent manner. Treatment with VPA (4, 2, 1 and 0.5 mmol/L) for 48h, the apoptosis rates of KM3 cells were (13.27±3.54)%, (22.13±1.20)%, (24.41±2.23)% and (40.62±4.28)% respectively. The expression of VEGFR-1 in KM3 cells were decreased in VPA-treated group by the immunocytochemistry and RT-PCR, whereas the acetylated histone H4(ac-H4) accumulated. It suggested VPA could decrease the expression of VEGFR-1 in KM3 cells, and it might play an important role in regulating the proliferation and apoptosis of multiple myeloma cell line KM3 cells. These results provide the framework for clinical trials.

Despite recent advances in treatment, multiple myeloma remains incurable in the majority of patients. Histone deacetylase (HDAC) inhibitors form a new class of anticancer drugs and several of these drugs are currently being investigated in clinical trials [1,2]. Valproic acid (VPA) is a shortchain fatty acid with a long history of clinical use as an anti-convulsant [3]. However, VPA also inhibits HDAC and induces apoptosis in selected solid tumors [4,5] as well as in hematologic neoplasms [6]. Vascular endothelial growth factor (VEGF) is an important signaling protein involved in both vasculogenesis

and angiogenesis [7,8]. The aim of this study was to define the effects of VPA on multiple myeloma cells and the underlying mechanism on VEGF and VEGF receptors.

## MATERIALS AND METHODS

### *Drugs and reagents*

VPA, MTT was purchased from the Sigma (USA). VPA was prepared in sterile phosphate-buffered saline immediately before use. Anti-vascular endothelial growth factor receptor 1(VEGFR1)

antibody and streptavidin peroxidase (SP) reagent kits were purchased from Boster company (China). anti-ac-H4 antibody were purchased from Santa Cruz (USA). The apoptosis kit get from Jingmei Company (China). RT-PCR kit and associated reagents were purchased from Takara biotechnology company (Japan).

#### Cell culture

Human multiple myeloma cells lines KM3 were kindly provided by professor Houjian of Shanghai Changzheng hospital. It was cultured in RPMI-1640 (Gibco) culture medium containing 10% fetal calf serum and 2 mmol/L L-glutamine at 37°C in a 5% CO<sub>2</sub> incubator. Cell grouping: VPA-untreated group, VPA-treated groups (0.5, 1.0, 2.0, 4.0 mmol/L). Pass the generation 2 or 3 days and use it at logarithmic phase.

#### Cell proliferation assays

The MTT-test was used for cell viability studies as recommended. VPA was added to cells in different concentrations (0.5, 1.0, 2.0, 4.0 mmol/L) and cells were incubated for 24, 48, 72 hours respectively.

The absorbance (A) was detected in the microplate reader at 490 nm wavelength using a 96-well multiscanner autoreader (Biotech Instruments, USA). Data were obtained in three independent experimental sets. The cell proliferation inhibition rate was calculated according to the following formula: Cell proliferation inhibition rate (%)=[1-(A of the experimental samples/A of the control)]×100%.

#### Assessment of apoptosis

The extent of apoptosis was evaluated by annexinV staining. KM3 Cell line were incubated in the presence of VPA (0.5, 1.0, 2.0, 4.0 mmol/L) for 48 hours and stained with annexinV-fluorescein isothiocyanate and propidium iodide. Samples were analyzed by flow cytometry. Similar data were obtained in at least three independent experimental sets. And the data were analyzed using WinMDI2. 9 software.

#### Reverse-transcriptase polymerase chain reaction analyses of VEGF-receptors

Total RNA was prepared with Trizol Reagent (Gibco Life Technologies) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by ultraviolet spectrophotometry and RNA was stored at -70°C until analyzed.

For RT of extracted RNA to Complementary DNA (cDNA), 1.0 µg of total RNA template was incubated for 60 minutes at 37°C in a 20 µl reaction volume containing 1.5×first-strand buffer (50 mmol/L Tris/HCl, 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>) 4 µl, 10 mmol/l dNTPs 2 µl, 0.5 mmol/L of oligod T<sub>18</sub> 1 µl, ribonuclease inhibitor RNasin 20U and AMV reverse transcriptase 240 U. The reaction was stopped by incubating at 95°C for 5 minutes and the samples were placed on ice or stored in -20°C for further analysis.

Subsequently, PCR reactions were performed in a thermal cycler (Gene Amp PCR System PTC-200, MJ Research, USA ): 3 µl RT product were amplified in a volume of 25 µl containing 1×PCR buffer (10 mmol/L Tris-HCl, pH 9.0, 50 mmol/L KCl), 1.0 mmol/L MgCl<sub>2</sub>, 200 µM each deoxynucleotide, 0.6 pmol/µl each primer, and 0.06 U/µl Taq DNA polymerase. The following primers were used: sense primer 5'-CAA GTG GCC AGA GGC ATG GAG TT-3' (corresponding to nucleotides 13262 to 13284) and antisense primer 5'-GAT GTA GTC TTT ACC ATC CTG TTG-3' (corresponding to nucleotides 13736 to 13759) for Fms-like tyrosine kinase-1 (Flt-1); 5'-CAA CAA AGT CGG GAG AGG AG-3'(sense) and 5'-ATG ACG ATG GAC AAG TAG CC-3' (antisense) for kinase insert domain-containing receptor (KDR). The thermal cycle profile consisted of denaturing at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes. The samples were amplified for 32 cycles. The integrity of messenger RNA of all samples was confirmed by amplification of β-actin. PCR products were separated on a 1% agarose gel and photographed. It was analyzed with a computerized scanner with image analyzing software (Kodak, USA).

#### Immunohistochemistry for VEGFR and (ac-H4)

VPA (4mmol/l, 48h) treated cells were collected and plated onto a glass slide, dried for one hour, and fixed with 2% paraformaldehyde for 1h at room temperature. And then the procedure was carried out as described in SABC method. Briefly speaking, sildes was washed by PBS and was blocked by 10% serum from the species from which the secondary antibody was taken for 20 minutes at room temperature in a humidified chamber. Wash with PBS and then incubate it with goat anti-flt-1 antibody and rabbit anti-AC-H4 antibody overnight respectively. At the same time Routine negative controls were performed by application of PBS instead of the primary antibody. Next day the cells were treated with biotinylated secondary antibody and peroxidase-labeled streptavidin followed by diaminobenzidine (DAB), and the cells were viewed by an Olmpus microscope and their individual A valules were recorded and analysis by PPIAS 1000 Image Analysis System.

#### Statistical analysis

The data are presented as means ± SD using SPSS13.0. Comparative data were analyzed by multivariate analysis and Student t-test for paired data with significance defined as p<0.05.

## RESULTS

#### VPA inhibits proliferation of multiple myeloma cell lines KM3 cell

KM3 cell were sensitive to VPA in a dose- and time-dependent manner (p<0.05). After 48 hours of incubation, the IC<sub>50</sub> was observed to be approximately 4 mM for KM3. As shown in Figure 1, cell proliferation at a

**Table 1.** The antiproliferation effect of VPA(mmol/L) in the KM3 cells (x±s, n=12). The results is expressed by the cell proliferation inhibition ratio.

GROUPS	VPA 0.5 mmol/L	VPA 1.0 mmol/L	VPA 2.0 mmol/L	VPA 4.0 mmol/L
24h	8.72%±3.58%	17.13%±4.21%	24.15%±2.98%	32.90%±1.25%
48h	20.42%±4.86%	34.63%±5.72%	41.92%±5.86%	54.16%±5.08%
72h	22.77%±2.54%	44.34%±6.45%	56.14%±1.33%	83.26%±2.28%

concentration of 4 mM VPA was reduced to 70%, 50% and 10% in KM3 cells after 24, 48, 72 hours. (Table 1)

VPA shows a strong induction of apoptosis in KM3 cell lines.

After 48 hours of treatment with VPA (0.5, 1.0, 2.0, 4.0 mmol/L), the apoptosis rate approximately 10%, 20%, 25% and 40% of KM3 respectively (Figures 2), VPA can significantly induce apoptosis of KM3 in a dose depended manner (p<0.05), Table 2.

VPA leads to decreased expression of VEGFR-1 by KM3 cells.

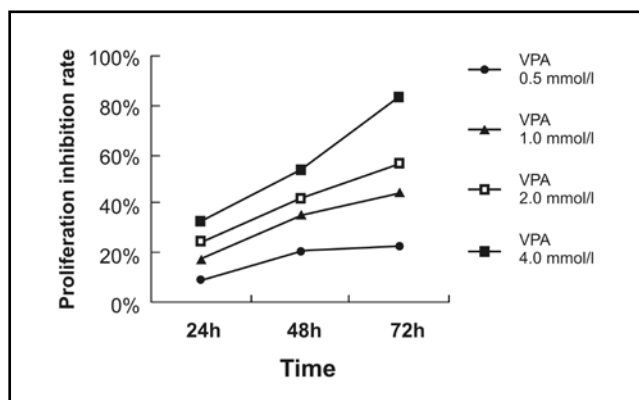
We examined VEGFR production of KM3 by RT-PCR. Only VEGFR-1 (flt-1) was detected in KM3 cell. Treatment with 2mM VPA for 48 hours led to a significant (p<0.05) decrease of VEGFR-1 expression by 50% in KM3 cells in comparison to the production by untreated cells (Figure 3).

VPA leads to acetylation of histone H4 and down-regulates VEGFR-1 in KM3 cells.

The result revealed that the expression of flt-1 and an-H4 was positive in KM3 cell and flt-1 was significantly decreased whereas ac-H4 was significantly (p<0.05) increased after the cells was treated by 4 mmol/L VPA for 48h compared with the untreated cells (Figure 4). photos were analyzed using a HPIAS 1000 Image Analysis System and A value of VEGFR-1 and ac-H4 were 0.3129±0.0231, 0.1215±0.0210 for untreated cells and 0.0982±0.0231, 0.4231±0.0170 for treated cells respectively (p<0.05).

**DISCUSSION**

Nucleosomes are the fundamental unit of chromatin structure, and it provides the higher-order packaging and compaction of the DNA about 10,000 fold. The nucleosome core particle consists of a highly conserved basic proteins, histone around which 146bp of DNA are wrapped. Over the past decade, extensive genetic, biochemical, and cytological studies have revealed that in addition to their structural role, the histone proteins are also involved in regulation of gene expression [9]. Post-translational modifications of histone tails, such as acetylation, phosphorylation and methylation has emerged as common denominators in regulating several biological functions. Acetylation is probably the best understood of these modification reactions. The enzymes involved in this process are Histone acetyl transferases (HATs) and Histone deacetylases (HDACs). Hyperacetylation leads to over expression of a particular gene whereas hypoacetylation leads to its repression. Thus HAT and HDAC activity control the level of acetylation and in turn, can regulate the gene expression and its biological functions [10]. Any alterations in the enzyme activity leads to aberrant acetylation. Aberrant acetylation has been linked to cellular transformation and development of cancer, suggesting that both HATs and HDACs play an important role in carcinogenesis [11]. Histone deacetylase inhibitors are small molecules and restore the acetylation to normal level, induce cell cycle arrest, differentiation, and apoptosis, suggesting their promising anticancer activity [12,13,14]. Results of clinical trials with several of these

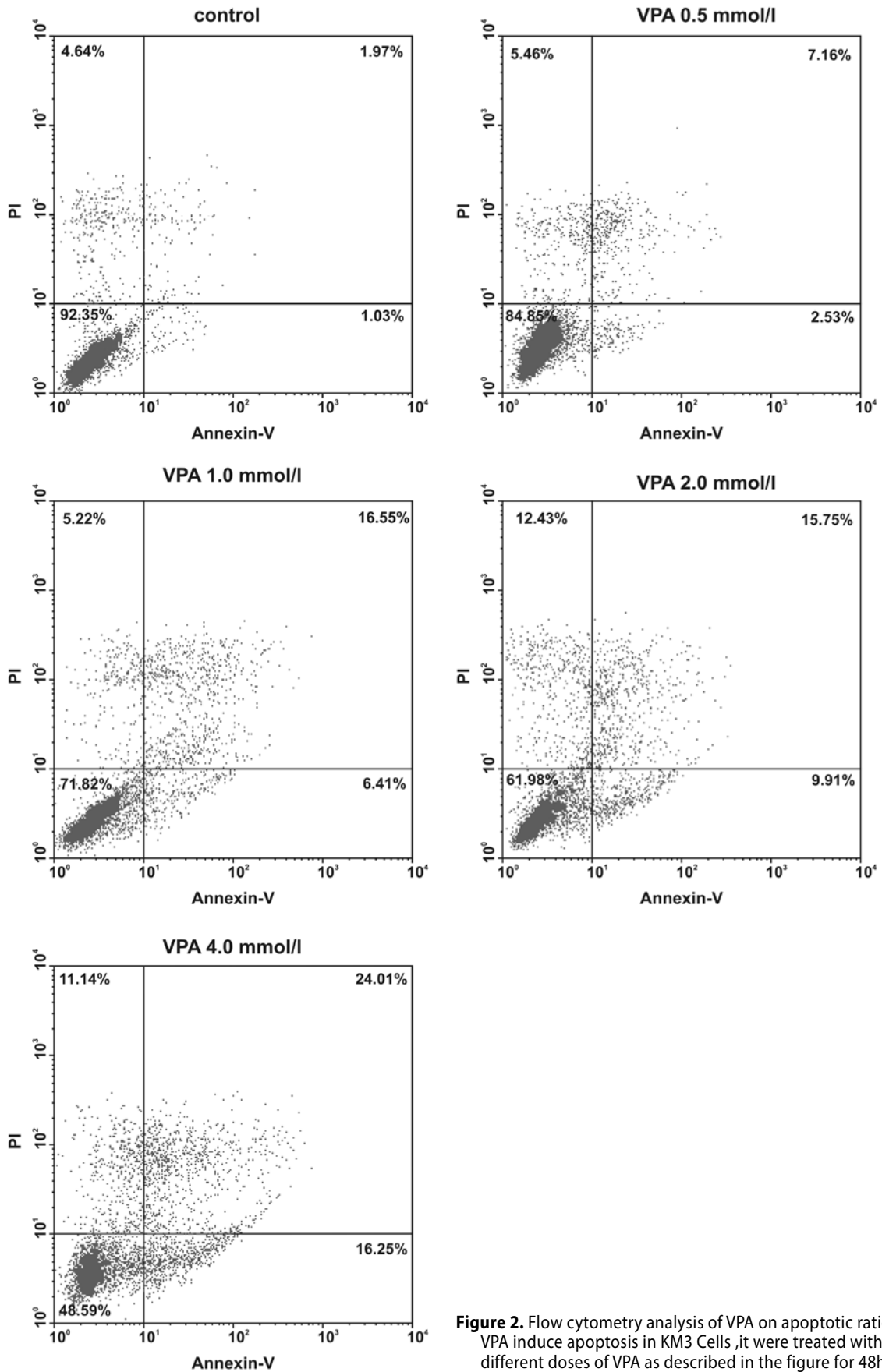


**Figure 1.** VPA (mmol/L) inhibits growth of KM3 cell. cell viability was assessed by MTT assay.

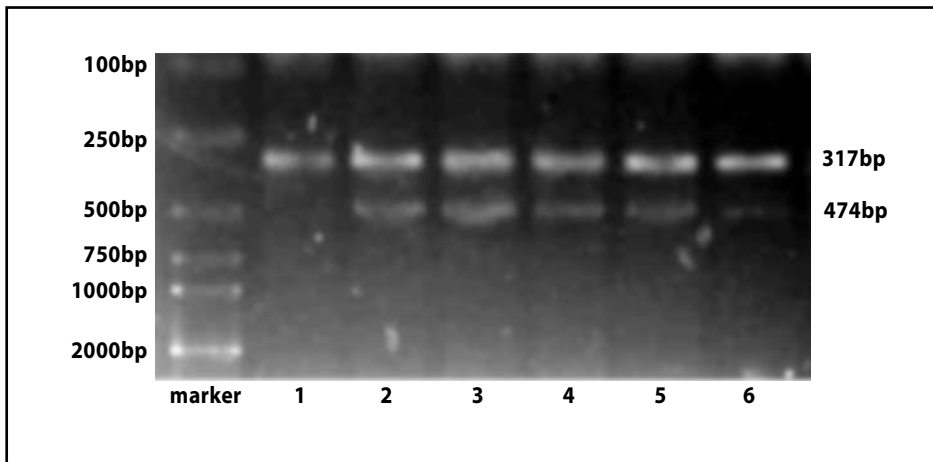
**Table 2.** The effect of VPA (different doses) on the apoptosis in KM3 cells(x±s, n=3).

GROUPS (48h)	The rate of apoptosis
control	3.96%±1.88%
VPA 0.5 mmol/l	13.27%±3.54%*
VPA 1.0 mmol/l	22.31%±1.20%*
VPA 2.0 mmol/l	24.41%±2.23%*
VPA 4.0 mmol/l	40.62%±4.28%*#

\*p<0.05, compared with control. #p<0.05, compared with other groups



**Figure 2.** Flow cytometry analysis of VPA on apoptotic ratio. VPA induce apoptosis in KM3 Cells ,it were treated with different doses of VPA as described in the figure for 48h.



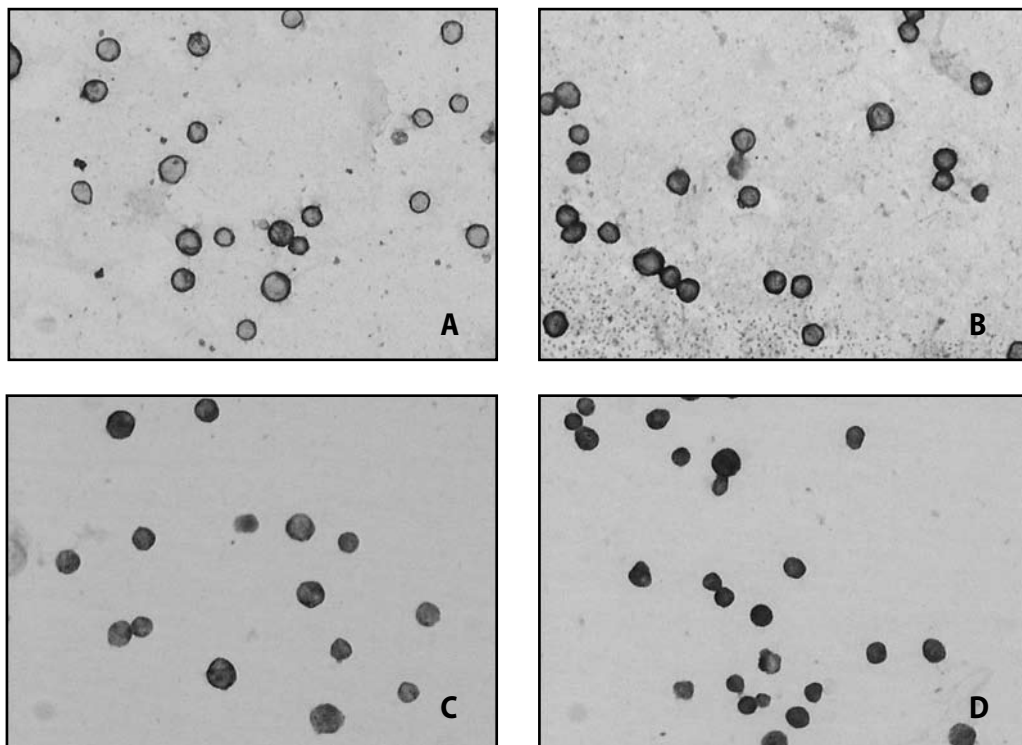
**Figure 3.** VPA downregulates the mRNA level of vesicular endothelial growth factor receptor 1(VEGFR-1/flt-1). 1 is the lane for KDR ,2-6 were incubated with VPA (0, 0.5, 1.0, 2.0, 4.0 mmol/L) for 48h respectively.  $\beta$ -actin is 317bp; flt-1 is 474bp.

agents have indicated that they are well tolerated at doses that have anti-tumor activity [1,2,15,16].

It has been reported that selected HDAC inhibitors are effective against multiple myeloma [17, 18]. However, no HDAC inhibitor is so far available for broad clinical use. The anticonvulsant VPA has HDAC inhibiting activity [3]. It induces apoptosis in some solid tumors[4,5] and in selected hematologic disorders [6]. Clinical trials with VPA in acute myeloid leukemia and myelodysplastic syndromes have been initiated [19]. Few reports so far has investigated the antitumor activity of VPA in

multiple myeloma. We evaluated the effects of VPA on human myeloma cell lines KM3 Cell.

We found an increase of acetylated histone H4 in myeloma cell lines KM3 cell after treatment with VPA. VPA dose- and time-dependently inhibited proliferation after 24h, 48h, 72h of treatment. Flow cytometric apoptosis analysis revealed a marked increase of apoptosis rate after VPA treatment. It was consistent with its effect of inhibiting proliferation. The mechanism underlying this maybe VPA increase the acetylation level



**Figure 4.** The expression of VEGFR-1 and ac-H4 in VPA(4 mM, for 48h) treated cells by immunochemistry ( $\times 200$ ). **A:** flt-1 in control group. **B:** flt-1 in VPA treated group. **C:** ac-H4 in control group. **D:** ac-H4 in VPA treated group.

of histone and the chromosome structure more open, so that nucleic acid enzyme have an access to DNA.

In the bone marrow microenvironment of MM, VEGF is essential for tumor growth and survival [20,21], and Myeloma cells are known to produce VEGF and VEGF receptors [22]. Binding of VEGF<sub>165</sub> to MM cells triggers VEGFR-1 tyrosine phosphorylation. Subsequently the downstream signaling pathways are activated: (1) a PI3-kinase/protein kinase Cα(PKCα)-dependent cascade mediating MM cell migration on fibronectin, (2) a MEK-extracellular signal-regulated protein kinase (ERK) pathway mediating MM cell proliferation [23]. It has been reported that NVP-LAQ824 significantly inhibited VEGF production and thus has an anti-angiogenic effect in many tumor cell lines [24]. In the present study we investigated the influence of sub-apoptotic dose of VPA on the expression of VEGFR-1, a key factor of the autocrine loop of VEGF, and found mRNA and protein levels of the VEGFR-1 were decreased markedly after VPA treatment. These findings suggest that VPA could have an anti-tumor effect in the bone marrow microenvironment in the patients of multiple myeloma by disturbing the autocrine loop of VEGF.

In this study we showed that VPA acts as an HDAC inhibitor in multiple myeloma cell line KM3 cells, potently inhibits tumor growth and markedly induces apoptosis. In addition to its direct antitumor effect, VPA reduces VEGFR-1 production in KM3 cells, and this may partly cause the proliferation inhibition effect of VPA. These data provide the framework for clinical trials with valproic acid in multiple myeloma.

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