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Epigenetic modulation of gene expression of human leukemia cell lines – induction of cell death and senescence

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Histone deacetylase inhibitors (HDACi) are emerging new class of anticancer agents that act by inhibiting cell growth, inducing cell cycle arrest and apoptosis of various cancer cells. However, in some conditions, apoptosis can be blocked and non apoptotic cell death and irreversible growth arrest, namely senescence, can be activated as potential tumor-suppressor mechanism. Here we evaluated the dosage effects of HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) in a series of human leukaemia cell lines. We investigated, what concentration of SAHA and VPA can optimally induce apoptosis, growth inhibition or stress-induced premature senescence. We have found that SAHA inhibited proliferation and induced apoptosis in concentration 1000x lower than VPA. The senescence phenotype was preferentially induced by lower dosage of HDACi and required longer incubation time (5 days) while apoptosis was induced by higher dosage and appeared already after 24h. The optimal doses for the induction of cell death are 2,5-5 μM of SAHA and 2,5-5 mM of VPA. These doses of HDACi induce both apoptosis and senescence of studied leukemia cell lines.

Keywords: senescence, apoptosis, histone deacetylase inhibitors (HDACi) suberoylanilide hydroxamic acid (SAHA), valproic acid (VPA)

Recently, the use of histone deacetylase inhibitors (HDACi) as anti-cancer agents has received great attention. Histone deacetylase enzymes (HDACs) are the class of proteins that catalyze removal of acetyl groups from lysine residues of histone or non-histone proteins that regulate the expression of genes and proteins essential for cell survival [1-3]. The acetylation status of histones can alter the chromatin structure, thereby modulating the gene expression. This process is controled by the relative activities of histone acetyltransferases (HATs) and HDACs [4]. Disturbance of the HAT-HDAC dynamics appers to result in the development of cancer. The aberrant recruitment of HDAC is closely associated with leukemogenesis through silencing the expression of genes involved in the hematopoietic cell differentiation [5]. Number of a small-molecule HDAC inhibitors (HDACi) have been developed as a new promising class of anticancer drugs [2, 6-8]. Natural and synthetic inhibitors have been characterized. Despite the fact that these inhibitors are structurally different, they share the capacity to induce cell differentiation [9], apoptosis [10], growth inhibition [11] and cell cycle arrest in a variety of malignant cells [12]. Several HDAC inhibitors are currently in clinical trials as anti-cancer agents, because they show an inhibitory activity against cancer at well-tolerated doses [13]. Some of them, however, have a short half-life and exert a significant toxic side effects in vivo [14]. Suberoylanilide hydroxamic acid (SAHA, vorinostat) belongs to the hydroxamic acid-based hybrid polar compounds and is a prototypic compound of HDACi [6]. SAHA increases the intracellular acetylation of histones H2B, H3 and H4 and also increases the expression of p21 and Bax while decreasing the expression of STAT6 and levels of phospho-STAT6. All these actions ultimately lead to the activation of apoptosis and other forms of cell death [15]. Valproic acid (2-propylpentanoic acid, VPA) is a member of branched short chain fatty acids and has been widely used in the management of various types of epilepsy for decades. VPA affects the growth and differentiation of some cell types. It induces differentiation of neuroblastoma cells, inhibits their growth [16] and induces apoptosis in selected solid tumors as well as in hematological neoplasias[17,18].

Induction of cell death or cell growth arrest upon the treatment with anticancer drugs correlates with the tumor response. Recently the most extensively studied mechanisms of cancer treatment is the induction of cell death by apoptosis, necrosis,or autophagy, or induction of senescence. Numerous studies have shown that in cells where apoptosis is blocked, non-apoptotic cell death or irreversible cell growth arrest namely senescence, can be activated as potential tumor-suppressor

mechanism [19,20]. Senescence is the physiological program of the irreversible terminal growth arrest occurring in both normal and immortalized cells in response to telomeric alterations, and also to sublethal stress and inappropriate oncogenic signaling. The term "stress-induced premature senescence" is used for senescence resulting from the stress or DNA damage induced by the effect of chemoterapeutic drugs on cancer cells. Senescent cells develop the characteristic phenotype, including the enlarged, flattened morphology, prominent nucleus, senescence-associated heterochromatin foci (SAHF), and also the senescence-associated β-galactosidase (SAβ-gal) activity [21,22,23]. When senescence is induced in cancer cells, the upregulation of inflammatory cytokines triggers an innate immune response, that targets the tumor cells [24]. Cellular senescence prevents some cells from initiating cancer, but can paradoxically promote cancer development in neighbouring cells [22].

Here we examine the drug dosage of SAHA or VPA, which can optimally induce apoptosis, growth inhibition or stress-induced premature senescence in leukemic cell lines JURL-MK1, MOLM-7, HEL, HL-60. We have found that SAHA inhibited proliferation and induced apoptosis in concentration 1000x lower than VPA. Both SAHA and VPA can induce senescence and apoptosis at low drug concentrations, however by longer treatment (5 days) rather senescence than apoptosis (2 days) is induced. The use of lower treatment concentrations can limit the undesired side effects.

Material and methods:

Chemicals. Suberoylanilide hydroxamic acid (SAHA) was purchased from Alexis Corporation (San Diego, CA, USA) and was dissolved in DMSO as stock solution. The maximum concentration of DMSO in the experiments was less than 0,1%. Valproic acid sodium salt was purchased from Alexis Corporation (San Diego, CA, USA) and was dissolved in water. The APO-BrdU™ TUNEL assay kit was purchased from Invitrogen (Carlsbad, California, USA. The senescence β-Galaktosidase Staining Kit was purchased from Cell Signaling Technology, Denver, USA.

Cultivation of cell lines. Myeloid-megakaryocytic (MOLM-7, JURL-MK1), myeloid-promyelocytic (HL-60), erytroid-megakaryocytic cell lines (HEL) were maintained in suspension culture in medium RPMI 1640 containing 10% fetal bovine serum (FBS), 150 mg/l L-glutamine, 50 000 U/ml penicillin and 50 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. They were diluted to a density of 2 to 4 x 10^5 cells per ml three times a week. Cells were harvested in the exponential growth phase. Viability was assessed by Trypan blue exclusion test.

Detection of cell viability and growth inhibition. Cell lines were seeded and allowed to reach the exponential growth for 24 hours. Cells were plated at the initial density of 2,5 x 10^5 cells/ml. SAHA was used in 0,5 to 10 μM concentrations. Valproic acid was used in 0,5 to 10 mM concentrations to inhibit proliferation and induce cell death. After 24, 48 and 72 hours of drug treatment, cells were harvested and viable cell were counted by Trypan blue dye exclusion method (at least 200 cells were counted in each assay). Cell viability was calculated as the percentage of values of untreated control cells.

Growth inhibitory effect of HDACi was assayed by measuring the uptake (incorporation) of ^{3}H-thymidine. Shortly the treated cells as well as the appropriate controls in triplicates were pulsed with 24 kBq (6-^{3}H)-thymidine (Institute for Reasearch, Development and Aplication of Radioisotopes, Prague, Czech Republic) of the specific activity 980 (Gbq/mmol) for 4 h and the cells were collected using Scatron cell harvester. The incorporated radioactivity into the newly synthesized DNA was measured on the beta scintillation counter. The mean values of triplicate experiments expressed in counts per minute (cpm) were calculated. The inhibition of proliferation was expressed as the percentage of cpm of cells that were treated in relation to untreated cells, cpm (untreated), according to the formula:

Inhibition of proliferation [%] = [cpm (untreated) – cpm (treated)] / cpm (untreated)] * 100%

Cell cycle analysis: MOLM-7, JURL-MK1, HL-60 cells (10^6 cells) treated by SAHA (concentrations 10 μM, 5 μM, 2,5 μM, 1 μM, 0,5 μM), valproic acid (concentrations 10 mM, 5 mM, 2,5 mM, 1mM, 0,5 mM) were collected by centrifugation after 24, 48 and 72 hours, suspended in 4.5ml of ice-cold 70% ethanol, incubated for 30min at 10°C and kept for 5-7 days at -20°C. The samples were then washed once in PBS and incubated for 30min at room temperature in 1ml of the modified Vindelovs propidium iodide buffer (10 mM Tris, pH 8, 1 mM NaCl, 0,1% Triton X-100, 20 μg/ml PI and 10K units of ribonuclease A). The fluorescence excited at 488nm was then measured using Coulter Epics XL flow cytometer. The histograms were analysed using the FLOWJo (Flow Cytometry Analysis Software) software.

Assessment of apoptosis by TUNEL assay. The TUNEL assay was performed employing the APO-BrDU TUNEL Assay Kit (Invitrogen, California, USA) following the standard manufacturer's protocol. Briefly, MOLM-7, JURL-MK1, HL-60 cells treated by SAHA and VPA as indicated above. After drug treatment, cells were washed in PBS, adjusted to a density of (1-2) x 10^6 cells/ml and fixed with 1% paraformaldehyde solution in PBS for 15 min. Subsequently, the cells were pelleted and permeabilized with the ice-cold 70% ethanol for 18 hours at -20 °C. To label DNA strand breaks, 1-2 x 10^6/ml cells were incubated with 50 μl of TUNEL reaction mixture for 60 min at 37°C. The extent of DNA labeling with Alexa Fluor 488 dye-labeled anti-BrdU antibody was determined by flow cytometry.

Detection of senescent cells. MOLM-7, HL-60 and JURL-MK1 cells were seeded in 12-well plates in culture medium containing, SAHA (5 μM, 2,5 μM, 1 μM) or VPA (5 mM, 2,5 mM, 1 mM) and the cells were incubated for 24h, 48h, 72h or 120 h. SA-β-gal staining was performed using a senescence staining kit (Cell signaling). In brief, cells were fixed for 10 min in fixative solution (2% formaldehyde, 0,2% glutaraldehyde), washed and incubated at 37°C with X-gal (1mg/ml), dissolved in solution containing 40 mM citric acid pH 6,5; 5 mM potas-

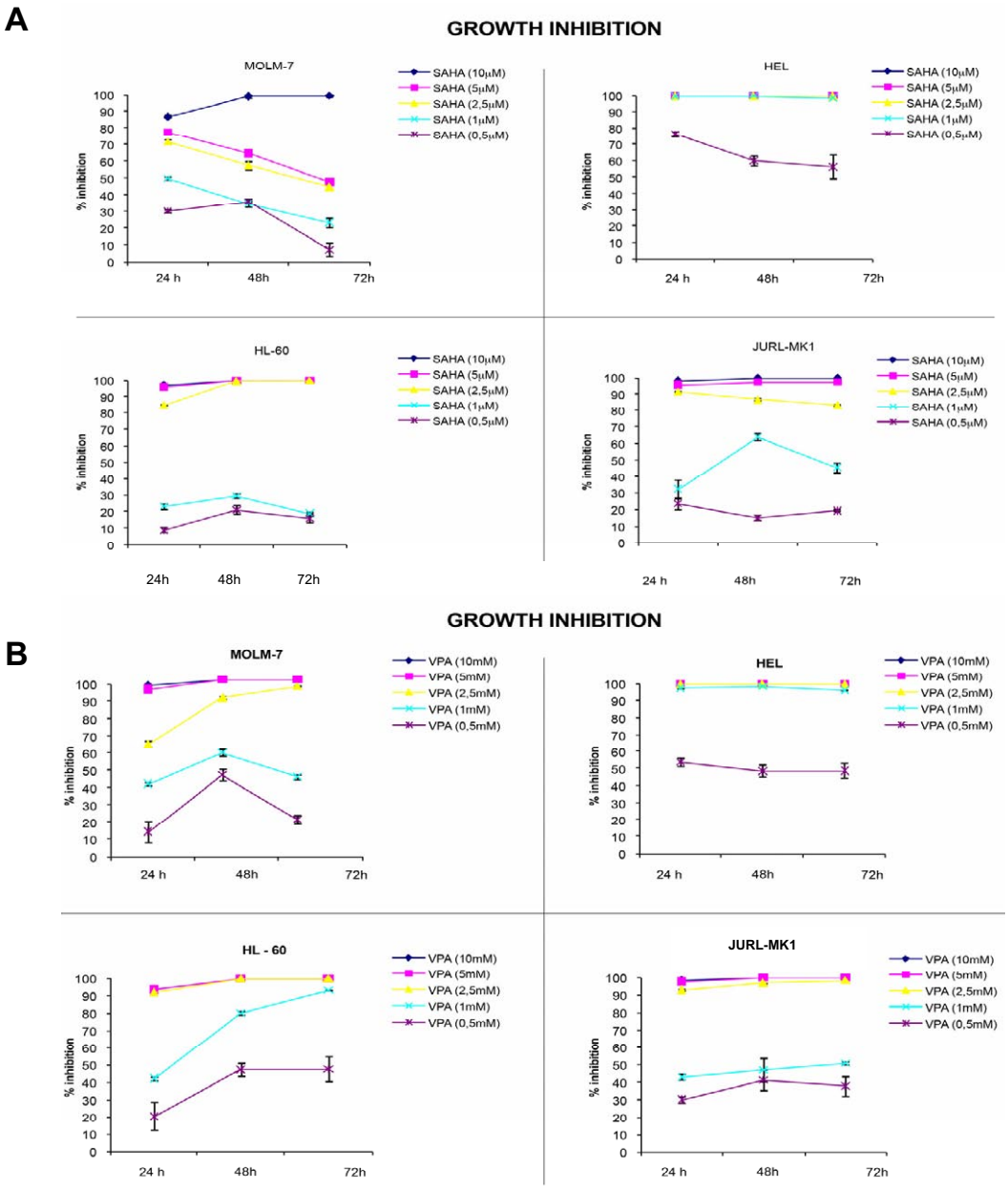

Figure 1. Effect of SAHA and VPA on the proliferation of MOLM-7, HEL, HL-60 and JURL-MK1 leukemic cell lines. Cells were treated with various concentrations (0,5-10 μM) of SAHA (A) and (0,5-10 mM) of VPA (B) for up to 72h.The cell aliquots in triplicates were assayed on proliferation with ^{3}H-thymidine for 4 h. The cells were harvested and the ^{3}H-thymidine radioactivity was measured in counts/min (cpm). Results are expressed as percentage of untreated control cells and represent mean and standard deviation (SD) of three independent experiments.

sium ferrokyanide, 5 mM potassium ferrikyanide, 150 mM NaCl, and 2 mM $MgCl_2$. After 24 h incubation, photographs were taken under the phase microscope.

Results

Both SAHA and VPA inhibited proliferation of leukemic cell lines*:* We evaluated the growth-inhibitory effect of HDACi at concentrations 0,5-10 mM of VPA and/or 0,5-10 mM of SAHA on the variety of leukemic cell lines. Myeloid-megakaryocytic (MOLM-7, JURL-MK1), myeloid-promyelocytic (HL-60) and erytroid-megakaryocytic (HEL) cell lines were treated by SAHA and by VPA for 72h. Both SAHA and VPA inhibited the proliferation of leukemic cell lines in the dose-dependent manner. As shown in Fig.1.A,B the strongest growth-inhibitory effect exhibited concentrations 2,5-10 μM SAHA and

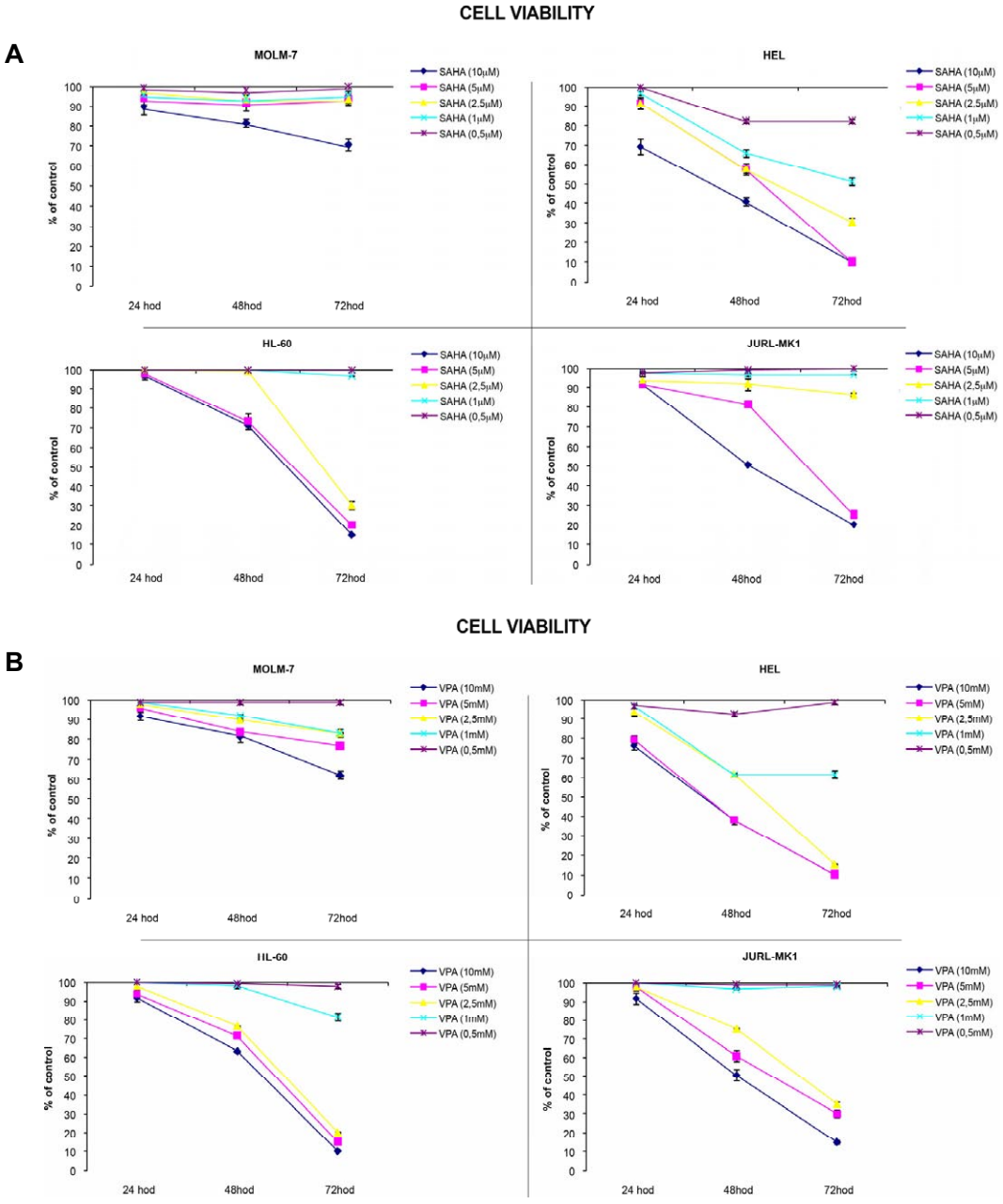

Figure 2. Viability of MOLM-7, HEL, HL-60 and JURL leukemic cell lines after treatment with SAHA (0,5-10 μM) (A) and VPA (0,5-10 mM) (B) for up to 72h. Viability was asessed by Trypan blue exclusion test. Results are expressed as percentage of untreated control cells and represent mean and standard deviation (SD) of three independent experiments.

2,5-10 mM VPA on JURL-MK1, HL-60 and HEL cells during the 72h treatment. In contrast, the antiproliferative effect of SAHA in concentrations 0,5-5 μM and VPA in concetrations 0,5-2,5 mM on MOLM-7 was weaker and decreased after the prolonged incubation. The most sensitive leukemic cell line to the treatment by HDACi was the erytroleukemia cell line HEL. The 50% inhibitory concentration (IC50) of HDACi on HEL cells was found to be 0,3 μM SAHA or 0,3 mM VPA. (Tab.1).

The number of Trypan blue-positive cells gradually increased with the increasing concentration of HDACi during the treatment period. The effect of HDACi in the lowest concentrations (0,5-1 mM VPA or 0,5-1 μM SAHA) on the viability of JURL-MK1, HL-60 and MOLM-7 cells was negligible (Fig. 2.A,B). The most pronounced cytotoxin effect was observed in HEL cells for both SAHA and VPA. On the other hand, MOLM-7 cells were the most resistent.

Table 1. The growth inhibitory effects of SAHA and VPA on HL-60, HEL, MOLM-7 and JURL-MK1 leukemic cell lines. Cells were treated with various concentrations (0,5-10 μM) of SAHA and (0,5-10 mM) of VPA for 24, 48 and 72 hours. Each value indicates the mean IC50. Results are expressed as the mean and standard deviation of three independent experiments.

Cell line name	IC50					
	24h		48h		72h	
	SAHA (μM)	VPA (mM)	SAHA (μM)	VPA (mM)	SAHA (μM)	VPA (mM)
HL-60	1,5 ± 0,03	1,1 ± 0,3	1,1 ± 0,05	0,4 ± 0,02	1,3 ± 0,1	0,3 ± 0,04
HEL	0,3 ± 0,01	0,4 ± 0,02	0,2 ± 0,02	0,3 ± 0,03	0,3 ± 0,05	0,3 ± 0,04
MOLM-7	1,2 ± 0,2	1,5 ± 0,04	0,9 ± 0,08	0,6 ± 0,03	3,8 ± 0,2	1,0 ± 0,1
JURL-MK1	1,2 ± 0,2	1,0 ± 0,05	1,7 ± 0,1	0,7 ± 0,1	1,1 ± 0,05	0,7 ± 0,05

DNA fragmentation and tunel assay: DNA fragmentation is considered to be the hallmark of the culminating apoptotic process. The cellular fraction having reached this stage of ordered auto-destruction can be determined by the TUNEL method based on the enzymatic fluorescence labeling of DNA strand breaks. Our data have shown that SAHA in concentrations 1 μM and lower, or VPA in concentrations 1 mM and lower, did not induce apoptosis of JURL-MK1 cells during the 72 hours period of treatment. Higher doses of HDACi (2,5-10 μM) SAHA or (2,5-10 mM) VPA caused an increase of TUNEL-positive cells within the 72h (Fig 3.A). In HL-60 cells, the highest level of DNA fragmentation was achieved 72h after the addition of 10 μM SAHA or 10 mM VPA and it reached 58% cells in culture. The lower concentrations of HDACi caused an increase of the TUNEL-positive HL-60 cells to 20%-35% for SAHA and 25%-45% for VPA (Fig.3.B). In MOLM-7 cells, concentrations 0,5–10 μM SAHA or 2,5-10 mM VPA induced apoptosis. The highest level of DNA fragmentation was achieved 72h after the addition of 10 mM VPA and it reached 70% of all cells (Fig. 3.C).

Cell cycle analysis: The fraction of cells in the individual cell cycle phases were assessed during the incubation with SAHA or VPA. The treatment of JURL-MK1 cells with 2,5–10 μM SAHA resulted in the accumulation of cells in sub-G1 phase of the cell cycle. The concentration of 0,5 μM SAHA did not influence the cell cycle of JURL-MK1 cells, there was the same distribution as in the controls (Fig.4). VPA in the concentration (5 mM) induced G1/G0 cell cycle arrest after 48h of treatment and SAHA (1 μM) induced G1/G0 cell cycle arrest after 48h and 72h of treatment of JURL-MK1 cells. During the period of treatment (up to 72h), the highest concentration of VPA (10 mM) showed a growing accumulation of cells in the subG1 phase. The lower concentrations of VPA (0,5-1 mM) induced the same distribution as in controls (Fig.4). In HL-60 and MOLM-

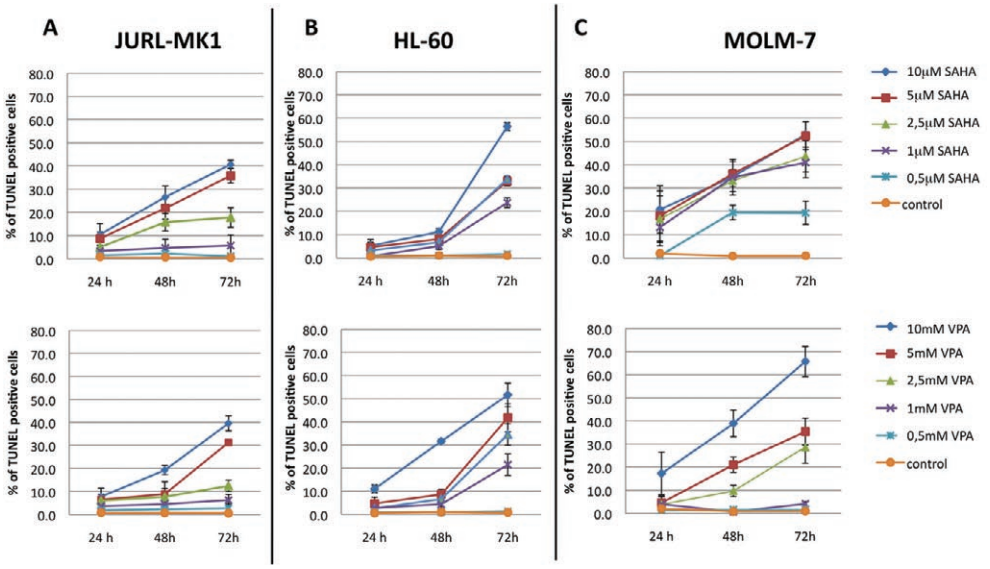

Figure 3. Flow cytometry analysis of JURL-MK1 (A), HL-60 (B), MOLM-7 (C) cells induced to apoptosis by HDACi in concentrations (0,5-10 μM for SAHA and 0,5-10 mM for VPA). Fractions of apoptic cells were determined by TUNEL assay after 24h, 48h and 72h. Data are reported as mean±SD of three independent experiments.

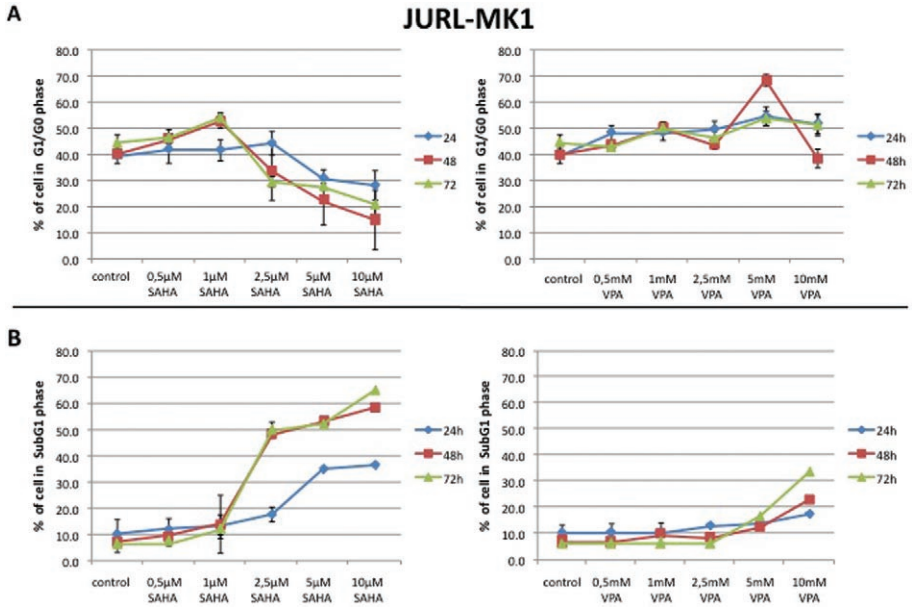

Figure 4. Cell cycle analysis of JURL-MK1 by flow cytometry. The JURL-MK1 were treated with HDACi in concentrations (0,5-10 μM for SAHA and 0,5-10 mM for VPA) for 24, 48 and 72 hours (A) Data indicated the percentage of cells in G1/G0 phase. (B) Data indicated the percentage of cells in subG1 phase. Data are reported as mean±SD of three independent experiments.

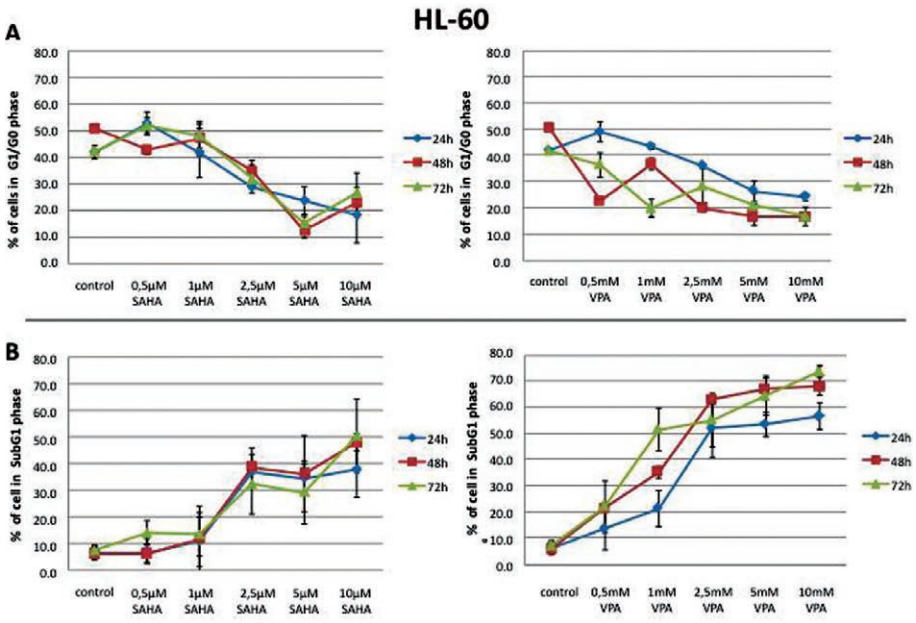

Figure 5. Cell cycle analysis of HL-60 by flow cytometry. The HL-60 were treated with HDACi in concentrations (0,5-10 μM for SAHA and 0,5-10 mM for VPA) for 24, 48 and 72 hours (A) Data indicated the percentage of cells in G1/G0 phase. (B) Data indicated the percentage of cells in subG1 phase. Data are reported as mean±SD of three independent experiments.

7 cells, no cell cycle arrest in G1/G0 phase was detected in the presence of SAHA or VPA. However, an increasing number of cells accumulated in the sub-G1 region of the cell cycle during the treatment with increasing concentrations of SAHA and VPA was noted, as is shown in the grafs on Fig. 5,6.

Induction of senescence. HDAC inhibitors can induce the growth arrest and apoptosis in many cell types, and they have been recently reported to induce a senescence-like state in normal human fibroblasts [25,26]. Therefore, we attempted to determine whether the HDAC inhibitors SAHA and VPA

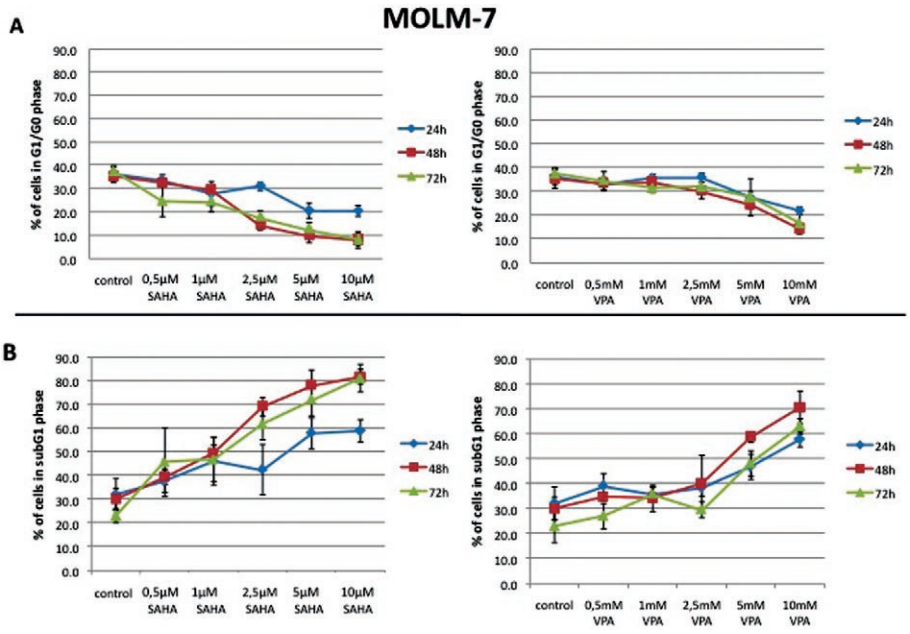

Figure 6. Cell cycle analysis of MOLM-7 by flow cytometry. The MOLM-7 were treated with HDACi in concentrations (0,5-10 μM for SAHA and 0,5-10 mM for VPA) for 24, 48 and 72 hours (A) Data indicated the percentage of cells in G1/G0 phase. (B) Data indicated the percentage of cells in subG1 phase. Data are reported as mean±SD of three independent experiments.

can induce the cellular senescence of MOLM-7, HL-60 and JURL-MK1 leukemic cell lines. As biomarker for the cellular senescence we used the senescence-associated-β-galaktosidase (SA-β-gal) aktivity [27]. MOLM-7, HL-60, JURL- MK1 cells were cultured for 24h, 48h, 72h, 96h and 120h in 1; 2,5; 5 mM VPA and 1; 2,5; 5 μM SAHA containing medium. The JURL-MK1, HL-60 and MOLM-7 cells cultured for 24h, 48h, 72h, 96h in the presence of SAHA or VPA did not express SA-β-gal activity. However, after 120h (5 days) JURL-MK1, HL-60 and MOLM-7 cells treated by HDAC inhibitors acquired the perinuclear staining for SA-β-gal activity and morphology associated with senescent cells (Fig.7). Untreated MOLM-7, HL-60 and JURL-MK1 cells had insignificant SA-β-gal acitivity.

Discussion

Epigenetic mechanisms may play an important role in the initiation of tumors by dysregulation of the expression of specific genes [28,29]. Evidence suggests, however, that histone deacetylase inhibitors (HDACi) can reactivate the gene expression and inhibit the growth and survival of tumor cells [30]. Chromatin remodeling by HDACi can induce a DNA damage-like response by the activation of DNA damage kinase ATM [31] and by the formation of DNA strand breaks [32]. The initiation of senescence or apoptosis can be the cellular answer to the DNA damage induced by HDACi [32,33].

Senescent cells appear typically flattened, enlarged, with the increasing cytoplasmatic granularity and display an enhanced activity of senescence-associated β-galactosidase (SA-β-gal) when assessed in acidic pH [27,34]. Senescent cells remain viable, metabolically active, possess the typical transcriptional profile which distinguishes them from quiescent cells [35]. Such cells can be easily eliminated by the innate immune system, particulary, by natural killer (NK) cells [36].

Apoptosis is characterized by cellular blebbing, exposure of phosphatidylserine at the cell surface, shrinkage of the cell core, DNA condensation and formation of apoptotic bodies, which can be rapidly phagocytized and digested by macrophages or neighbouring cells [37].

In the present study, we evaluated the effects of dosage of HDAC inhibitors SAHA and VPA in series of leukaemia cell lines. We investigated what concentration of SAHA or VPA can optimally induce the cell cycle arrest and what concentration of either compound is able to induce apoptosis or senescence. The mechanism which decides whether cells are driven to senescence or to apoptosis is not known. It has been shown by others that during the apoptotic treatment of tumors the resistance to the particular agent can occur. Some senescent tumor cells can however produce cytokines that may paradoxically initiate the tumor growth of other cells. Nevertheless such senescent cells may also initiate the immmune response to cancer cells. From this point of view it is important to select the anticancer agent which will be able to induce both apoptosis and senescence of cancer initiating cells.

Inhibitory effect of HDACi on cell proliferation has been described in both normal and transformed cells [38]. In our study the growth inhibition was observed in all leukemic cell

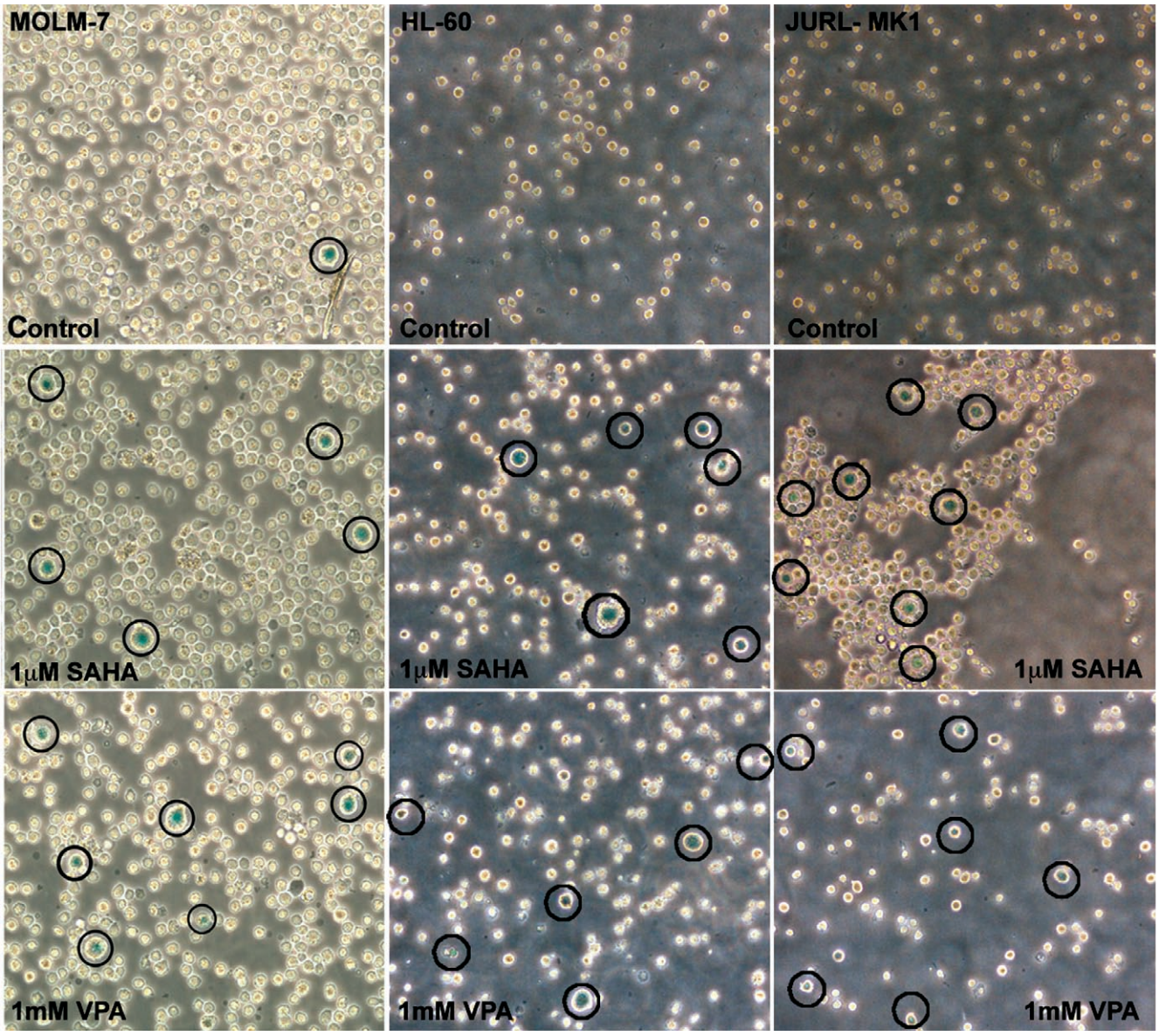

Figure 7. Effects of HDACi on cell senescence-like growth arrest in MOLM-7, HL-60 and JURL-MK1 cells. After 5 days, cells were fixed, analysed for SA-β-gal staining (a senescence marker) using a phase-contrast microscope and photographed, after 24h, at x 200 magnification. The level of SA-β-gal staining was also determined in untreated MOLM-7, HL-60 and JURL-MK1 cells propagated in parallel (controls).

lines tested and was dose-dependent. The most inhibitory concentration range on the proliferation of leukemic cell lines was 2,5-10 μM SAHA and 2,5-10 mM VPA. We also revealed IC50 at a range of 0,2-3,8 μM of SAHA and 0,3-1,6 mM of VPA during the 72h incubation.

Several HDAC inhibitors have been reported to induce apoptosis in many tumor cell types. In our experiments, the induction of apoptosis of individual cell types by SAHA and/or VPA was variable. MOLM-7 cells undergo extensive and fast concentration dependent apoptosis. These results were confirmed by the cell cycle analysis (Fig. 6), where the cell cycle arrest in G1/G0 phase was not detected. During the treatment, however, the increasing number of cells accumulated in the sub-G1 region of the cell cycle was detected. In contrast to MOLM-7 cells, JURL-MK1 cell line undergoes slower apoptosis in a lower number of cells, as is shown on the Tunel-positive cell fraction (Fig. 3.A). Probably, they undergo other forms of cell death (necrosis or autophagy) as is indicated by the higher fraction of Trypan-blue positive cells during the treatment (Fig. 2). At higher concentration range of SAHA and the highest concentration of VPA JURL-MK1 cells triggered apoptosis as judged from the high cell number accumulated in

the sub-G1 phase. However, at lower concentration of SAHA the JURL-MK1 cells uderwent G1/G0 arrest

We also tested the activity of the senescence-associated β-galactosidase (SA-β-Gal) that distinguished the senescent cells from other living cells. The senescence phenotype can be induced by doxorubicin, aphidicolin, cisplatine, ionizing radiation, cytarabine, etoposide, depending on the cellular context and concentration [39]. Tumor cell senescence is also induced by differentiating agents including sodium butyrate and retinoids [40]. Our results indicate, that after 24h, 48h, 72h, 96h HDAC inhibitors did not cause senescence-like arrest process of JURL-MK1, HL-60 and MOLM-7 cells. However, after 5 days of treatment SAHA at concentrations 1 and 2,5 μM and VPA at 1 and 2,5 mM induced senescence-like phenotype of JURL-MK1, HL-60 and MOLM-7 cells as shown by the staining of SA-β-Gal (Fig. 7). In contrast, the higher doses of HDACi induced more apoptosis than senescence, and this observation is in accordance with the finding of Schwarze et al [38].

These findings indicate that the mechanism of SAHA/VPA action is complex and the resulting effect depends on the individual cell context. In conclusion our data demonstrate that the effect of SAHA is stronger than VPA even in the 1000x lower concentration of SAHA used in the induction of apoptosis and inhibition of proliferation. Nevertheless, the effect of VPA action is moderate and it induces predominantly the proliferation arrest, but after the prolonged treatment apoptosis was induced. HDAC inhibitors induces more strongly and quickly apoptosis than senescence. Longer incubation time (5 days) and lower concentration of HDACi is needed for induction of senescence while apoptosis appeared already after 24h by higher doses of HDACi (mainly by SAHA). The ideal induction doses are 2,5-5 μM SAHA and 2,5-5 mM VPA. Both agents induce apoptosis and senescence.

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