

## Effect of Histone Deacetylase Inhibitors Trichostatin A and Valproic Acid on Etoposide-induced Apoptosis in Leukemia Cells

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**Abstract.** *Background: Histone deacetylase inhibitors (HDACi) have been extensively studied as potential candidates for treatment of various malignancies, including leukemia, since they not only induce growth inhibition, cell cycle arrest and apoptosis of cancer cells, but can also increase the sensitivity of cancer cells to chemotherapeutic drugs. The aim of this study was to investigate the effect of two HDACi, trichostatin A (TSA) and valproic acid (VPA), on etoposide-induced apoptosis in human leukemia cell lines. Materials and Methods: Viability, apoptosis rate, caspase activity, mitochondrial membrane potential and expression of BCL2 mRNA were assessed in HL60 and U937 cell lines treated with 250 nM TSA or 1.25 mM VPA alone or followed by 5 μM etoposide. Results: Preincubation of HL60 cells with TSA or VPA significantly potentiated etoposide-induced cytotoxicity and apoptosis, which was associated with activation of caspases and loss of mitochondrial membrane potential. Similar effects were not observed in U937 cells. Expression of BCL2 mRNA was strongly down-regulated after treatment of cells with HDACi alone but did not show additive effect with etoposide. Conclusion: Combination of HDACi with etoposide can have a synergistic effect on increased apoptosis in leukemia cells but this effect depends on the cancer cell type and other factors such as the concentration of drugs and the administration schedule.*

Histone deacetylase inhibitors (HDACi) inhibit deacetylation of both histones and nonhistone cellular proteins, inducing histone acetylation and subsequent chromatin relaxation, which facilitates transcription of target genes, especially

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those involved in cell proliferation, differentiation and apoptosis (1). Various HDACi have been described to cause numerous effects in malignant cell lines including cell death, induction of differentiation, cell-cycle arrest, enhanced production of reactive oxygen species, altered cell migration, and apoptosis (2-5). HDACi are therefore emerging as a promising new class of anticancer agents with low toxicity toward normal nonmalignant cells (6, 7).

Trichostatin A (TSA), the most common HDACi, is an antifungal antibiotic derived from *Streptomyces hygroscopicus* that inhibits mammalian histone deacetylase classes I and II and effectively induces apoptosis in various cancer cell types including leukemias (8-10). Valproic acid (VPA), a class I selective HDACi already known for years as an antiepileptic drug, has been also shown to exert antileukemic activity (11-14).

HDACi are also being investigated in combination with chemotherapeutic drugs in the treatment of malignant diseases. Previous studies showed that TSA increased sensitivity of gastric cancer cell lines to chemotherapeutic drugs, including 5-fluorouracil, paclitaxel and irinotecan (15). It was also reported that TSA potentiated the antitumor effects of gemcitabine and cisplatin in human bladder cancer cells (16, 17). Preclinical and clinical studies have shown antileukemic effects when VPA was used in combination with other chemotherapeutic agents including idarubicin (18), 5-aza-2'-deoxycytidine (19), gemtuzumab ozogamicin (20) and cytarabine (21).

Etoposide is a widely used anticancer drug (22). It has been shown that both etoposide and HDACi activities are mediated by their interaction with topoisomerase II, which leads to DNA strand breaks, inhibition of DNA replication, and apoptotic cell death (23-26). Interestingly, in combination treatment with HDACi and etoposide, both agonist and antagonist effects on cytotoxicity have been reported in different leukemia cell lines (26-30).

In order to clarify this issue, we investigated the effect of two HDACi, TSA and VPA, combined with etoposide, on apoptosis in HL60 and U937 human leukemia cell lines.

## Materials and Methods

**Drugs.** TSA, VPA and etoposide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of drugs were aliquoted and kept frozen at  $-20^{\circ}\text{C}$ .

**Cell culture.** Cultures of HL60 (ATTC CCL 240) and U937 (ATCC CRL 1593) cells (Manassas, VA, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50  $\mu\text{g}/\text{ml}$  gentamycin (all from Invitrogen, Grand Island, NY, USA). Cells were cultured at  $37^{\circ}\text{C}$  in a humidified (5%  $\text{CO}_2$ ) atmosphere and were used for experiments in the phase of exponential growth. They were seeded at  $0.1 \times 10^6/\text{ml}$  in 25  $\text{cm}^2$  culture flasks (NUNC, Thermo Scientific, Roskilde, Denmark).

**Cell proliferation assay.** Cell growth was assessed by AlamarBlue assay (Biosource, Camarillo, CA, USA), according to the manufacturer's instructions. Briefly, the cells were seeded into 96-well plate at a density  $2 \times 10^4$  cells per well (100  $\mu\text{l}$ ) in culture medium alone or with drugs and incubated for a specified amount of time. After the incubation period, AlamarBlue was added to the wells at a final concentration of 10% and the plates were incubated for 4 hours at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . The intensity of fluorescence emitted due to reduction of Alamar Blue was measured with Microplate Fluorescence Reader FL600 (Bio-Tek Instruments, Inc., VT, USA) (excitation: 530 nm, emission: 590 nm). Cell viability was expressed as a percentage of the control viability (100%).

**HDAC activity assay.** HDAC activity was measured by using a fluorescence HDAC Cell-Based Activity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. By using a cell-permeable HDAC substrate, the activity of various protein lysine-specific deacetylases, including HDAC1-containing complexes, can be measured in whole intact cells. Briefly, cells were seeded in a 96-well clear-bottom black plate at a density of  $5 \times 10^4$  cells/well. The HDAC reaction was initiated by adding 10  $\mu\text{l}$  Boc-Lys(AC)-AMC substrate to each well, and the incubation was carried out at  $37^{\circ}\text{C}$  for 2 hours. The lysis/developer solution was then added, and the mixture was incubated for 15 minutes at  $37^{\circ}\text{C}$ . Fluorescence of samples was measured using microplate fluorescence reader, with excitation at 360 nm and emission at 460 nm. HDAC activity was expressed as a percentage of that measured in control cells (100%).

**Identification and quantification of apoptotic cells with Hoechst 33342.** Nuclear morphology of control and treated cells was studied by fluorescence microscopy after staining cell nuclei with Hoechst 33342 (Sigma), as described previously (31), with some modifications. Briefly, cytospin slides were washed in PBS (pH 7.4), fixed in formaldehyde (4% solution in PBS) for 15 minutes at room temperature and then incubated with Hoechst 33342 (5  $\mu\text{g}/\text{ml}$  in distilled water) for 10 minutes at room temperature. They were then washed in PBS, mounted in glycerin/PBS solution (1:2) and coverslipped. Cells (at least 200 per slide) were examined under a fluorescence microscope (Olympus BX50) equipped with MNU2 filter and the percentage of apoptotic cell nuclei was calculated in each sample.

**Caspase activity assay.** Caspase activity was measured using Apo-ONE Homogeneous caspase-3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Cells were seeded

in a 384-well plate at  $2 \times 10^4$  cells/20  $\mu\text{l}$  per well in culture medium, and 20  $\mu\text{l}$  of caspase-3/7 reagent was added to each well. The reaction was carried out for 2 hours at room temperature in the dark, and the measurement of fluorescence was then performed in microplate fluorescence reader with excitation wavelength of 485 nm and emission wavelength 530 nm. Caspase activity was expressed as relative fluorescence intensity (control cells: 100%).

**Detection of changes in mitochondrial membrane potential  $\Delta\psi\text{m}$ .** The mitochondrial membrane potential was determined using a fluorescence Mitochondrial Permeability Transition Detection kit MitoPT™ JC-1 (AbD Serotec, Kidlington, Oxford, UK) according to the manufacturer's instructions. JC-1 dye exhibits potential-dependent accumulation in the mitochondria indicated by a fluorescence emission shift from green ( $\sim 529$  nm) to red ( $\sim 590$  nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Briefly, cells were resuspended at a density of  $1 \times 10^6$  cells in 1 ml of MitoPT™ JC-1 solution and were incubated for 15 minutes in the dark at  $37^{\circ}\text{C}$  in an atmosphere containing 5%  $\text{CO}_2$ . After incubation, cells were resuspended in the assay buffer and seeded in a 96-well clear bottom black plate at a density of  $1 \times 10^5$  cells/100  $\mu\text{l}$  per well. Fluorescence was monitored in a microplate fluorescence reader by measuring both the monomer (527 nm emission; green) and J-aggregate (590 nm emission; red) forms of JC-1 following 488 nm excitation.  $\Delta\psi\text{m}$  was calculated as J-aggregate to monomer fluorescence intensity ratio and in treated cells was expressed as a percentage of the one in control cells (100%).

**RNA isolation.** In order to assess the expression of *BCL2* mRNA, total RNA was extracted from cultured cells using TRIzol reagent, according to the protocol provided by the manufacturer (Invitrogen). The concentration of total RNA was assessed by measuring the absorbance at 260 nm, and RNA integrity was determined by 1.5% agarose gel electrophoresis. The isolated RNA samples were stored at  $-80^{\circ}\text{C}$  until RT-PCR analysis.

**Reverse transcription of RNA.** Total RNA from the cell samples was reverse-transcribed using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Life Science Europe, Bremen, Germany) according to the manufacturer's instructions. For reverse transcription (RT), 3  $\mu\text{g}$  of total RNA were mixed with 1  $\mu\text{l}$  Oligo d(T) primer (0.5  $\mu\text{g}/\mu\text{l}$ ) and water pretreated with diethylpyrocarbonate ( $\text{H}_2\text{O}$ -DEPC), and preincubated for 5 min at  $70^{\circ}\text{C}$ . After preincubation, other components were added to the mixture: 4  $\mu\text{l}$  of 5 $\times$  concentrated RT buffer (250 mM Tris-HCl, 250 mM KCl, 20 mM  $\text{MgCl}_2$ , 50 mM DTT, pH 8.3 at  $25^{\circ}\text{C}$ ), 1  $\mu\text{l}$  RNase inhibitor (20 U/ $\mu\text{l}$ ), and 2  $\mu\text{l}$  deoxyribonucleotide triphosphates (dNTPs, 10 mM). Following incubation at  $37^{\circ}\text{C}$  for 5 min, 1  $\mu\text{l}$  RevertAid M-MuLV Reverse Transcriptase (200 U/ $\mu\text{l}$ ) in a total volume of 20  $\mu\text{l}$  was added. The mixture was first incubated for 60 minutes at  $42^{\circ}\text{C}$ , then for the final 10 minutes at  $70^{\circ}\text{C}$ , and subsequently stored at  $20^{\circ}\text{C}$ .

**Polymerase chain reaction (PCR).** The PCR reactions were run in total volumes of 25  $\mu\text{l}$  containing: 2  $\mu\text{l}$  of synthesized cDNA, 0.2  $\mu\text{M}$  of each primer, 0.04 U/ $\mu\text{l}$  DNA polymerase in 10 mM buffer Tris-HCl pH 8.8 (supplemented with 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.1% Triton X-100) (Finnzymes, Espoo, Finland), 0.2 mM dNTPs (Roche, Mannheim, Germany) and  $\text{H}_2\text{O}$ -DEPC. For the *BCL2*

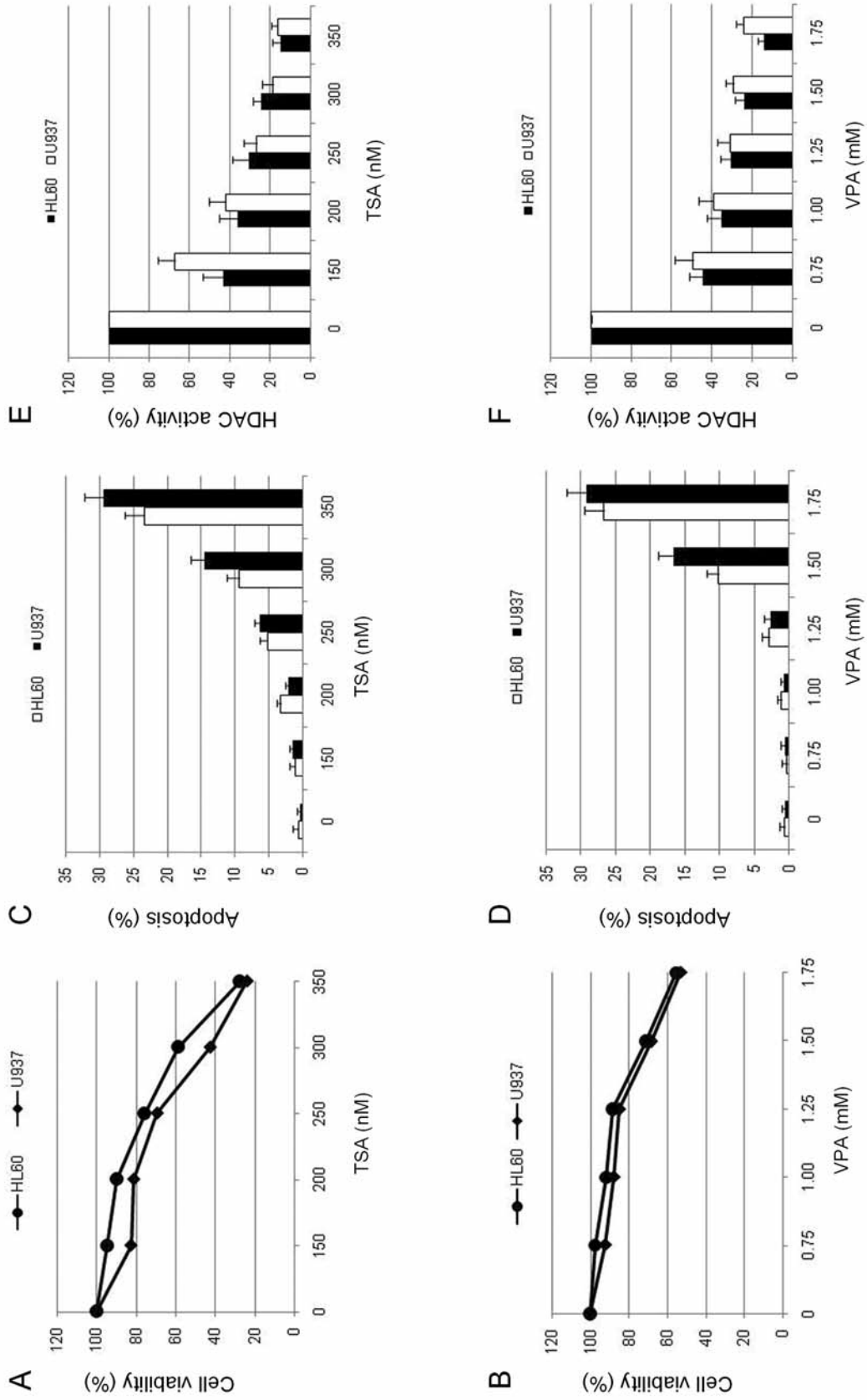


Figure 1. Effect of trichostatin A (TSA) and valproic acid (VPA) on cell viability (A, B), apoptosis (C, D) and histone deacetylase (HDAC) activity (E, F) in HL60 and U937 cells. TSA and VPA decreased cell viability, increased apoptosis and lowered HDAC activity in a dose-dependent manner.

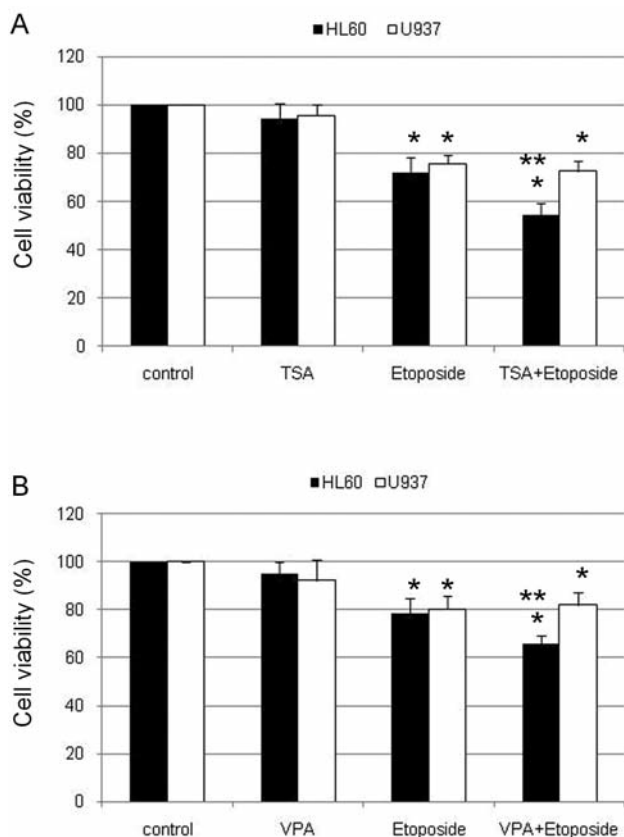


Figure 2. Effect of etoposide on the viability of HL60 and U937 cells pretreated with TSA (A) or VPA (B). Each value is the mean $\pm$ SD of 5 measurements. Significant difference of cell viability from the corresponding group of \*untreated (control) cells ( $p < 0.05$ ) and from \*\*etoposide-treated cells ( $p < 0.03$ ).

gene, after an initial 5 minute denaturation at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s for 28 cycles, with the final incubation at 72°C for 8 minutes (32). For the  $\beta$ -actin gene, after an initial 5 minute denaturation at 94°C, amplification was performed under the following conditions: 94°C for 30s, 54°C for 30 s, and 72°C for 2 minutes for 28 cycles, with the final incubation at 72°C for 8 minutes (33).  $\beta$ -Actin was used as an internal standard to normalize all samples for potential variations in mRNA content. The reaction products were analyzed by electrophoresis on 2.5% agarose gel (Sigma Chemical Co.), stained with ethidium bromide, directly visualized under UV light and photographed. The PCR primer sets were as follows: 5'-GAC TTC GCC GAG ATG TCC AG-3' and 5'-TCA CTT GTG GCT CAG ATA GG-3' for BCL2 (product size: 390 bp); 5'-CTG TCT GGC GGC ACC ACC AT -3' and 5'-GCA ACT AAG TCA TAG TCC GC -3' for  $\beta$ -actin (product size: ~300 bp).

**Statistical analysis.** The results were expressed as means $\pm$ SD of five independent experiments. Statistical analysis was performed by use of two-tailed Student's *t*-test for unpaired data. *p*-values  $< 0.05$  were considered statistically significant.

## Results

**Effects of TSA and VPA on cell viability, apoptosis and HDAC activity.** HL60 and U937 cells were exposed to increasing concentrations of HDAC inhibitors: TSA (150 to 350 nM) or VPA (0.75 to 1.75 mM) for 24 hours. The cell viability was inhibited by TSA and VPA in a dose-dependent manner (Figure 1 A and B). No significant apoptosis (increase up to ~5%) was induced by lower concentrations of either HDACi. Concentrations higher than 250 nM of TSA and 1.25 mM of VPA caused  $>10\%$  increase in apoptosis in both cell lines (Figure 1 C and D).

After TSA or VPA treatment the HDAC activity was significantly reduced in both HL60 and U937 cells in a dose-dependent manner (Figure 1 E and F).

Concentrations of 250 nM TSA and 1.25 mM VPA were chosen for further studies, being the highest concentrations having a relatively low toxic effect on the cells (~80% viability and 5% increase in apoptosis). At these concentrations of the drugs, HDAC activity was reduced to approximately 30% in both cell lines (Figure 1 E and F).

**Effect of etoposide after pretreatment with TSA or VPA. Cell viability:** HL60 and U937 cells were treated with 250 nM TSA or 1.25 mM VPA for 24 hours before subsequent treatment with 5  $\mu$ M etoposide for 24 hours. The dose of etoposide was selected to cause approximately 20% reduction in cell viability. TSA and VPA alone had no significant effect on cell viability in either cell line. Preincubation with TSA and with VPA increased the sensitivity of HL60 cells to etoposide, whereas in U937 cells, this effect was not observed (Figure 2).

**Apoptosis:** Apoptosis was assessed using Hoechst 33342-stained cytospin preparations observed under fluorescence microscopy (Figure 3 A-D).

Treatment with TSA or VPA alone did not induce a significant increase in apoptosis of HL60 and U937 cells. Etoposide-induced apoptosis was significantly potentiated after pretreatment with TSA and VPA in HL60 cells but not in U937 cells (Figure 3 E and F).

**Caspase activity:** TSA and VPA did not significantly alter caspase-3/7 activity in HL60 and U937 cells. Treatment with 5  $\mu$ M etoposide induced a significant increase in caspase activity in both cell lines compared to untreated cells. The combination of TSA or VPA and etoposide significantly increased the caspase activity compared to the treatment with etoposide alone in HL60 cells, but not in U937 cells (Figure 4).

**Mitochondrial membrane potential ( $\Delta\psi_m$ ):** Treatment with TSA or VPA alone caused a slight decrease in  $\Delta\psi_m$ , while etoposide alone more strongly reduced  $\Delta\psi_m$  in HL60 and

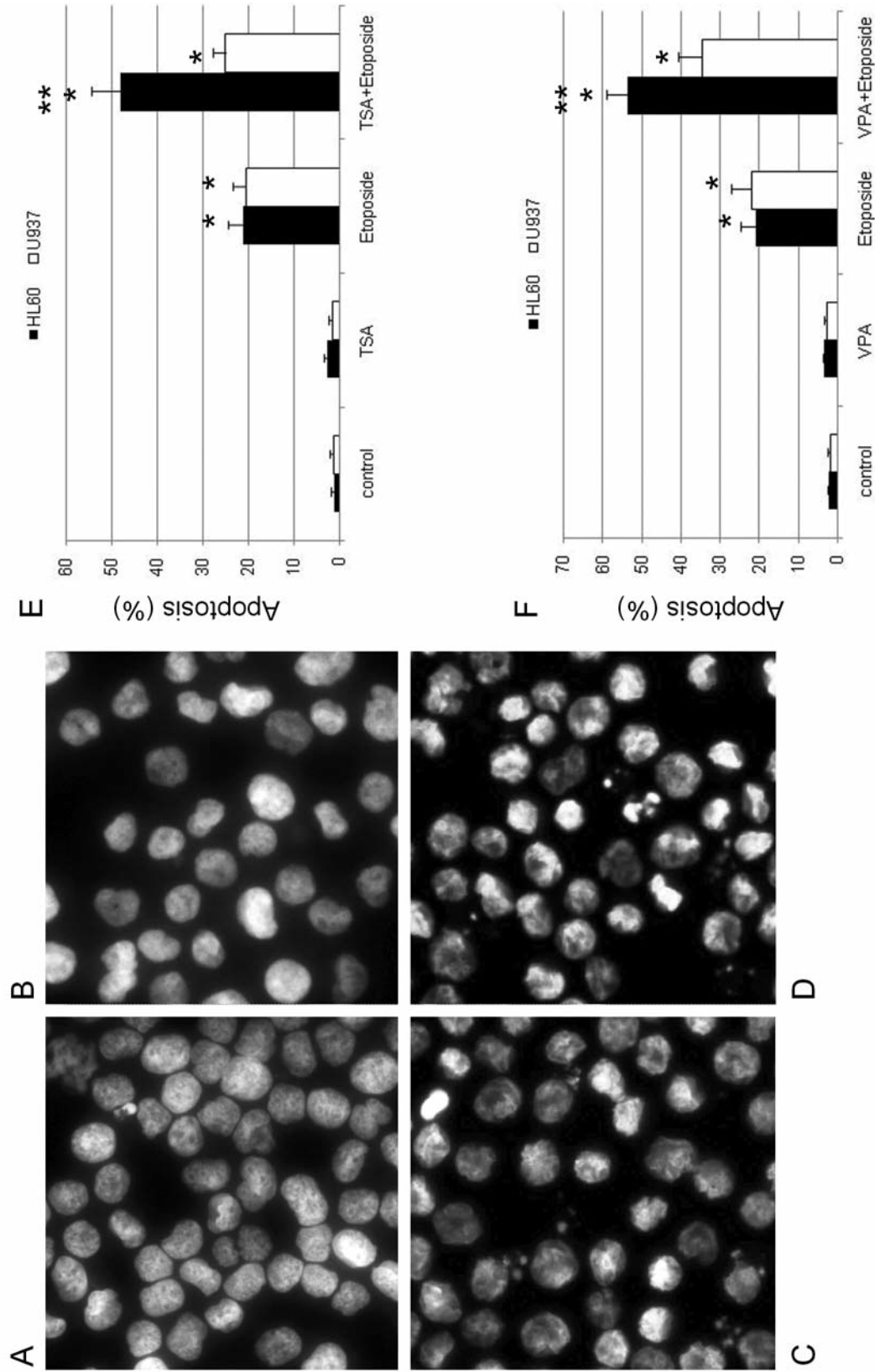


Figure 3. Effect of etoposide on apoptosis of pretreated HL60 and U937 cells. Representative micrographs of Hoechst 33342-stained HL60 cells: untreated control (A), TSA-treated cells (B), etoposide-treated cells (C), TSA/etoposide-treated cells (D) (magnification  $\times 200$ ). Quantification of apoptosis in HL60 and U937 cells treated with TSA (E) and VPA (F). Each value is the mean $\pm$ SD of 5 measurements. Significant difference in apoptosis from the corresponding group of \*untreated (control) cells ( $p < 0.05$ ) and from \*\*etoposide-treated cells ( $p < 0.04$ ).

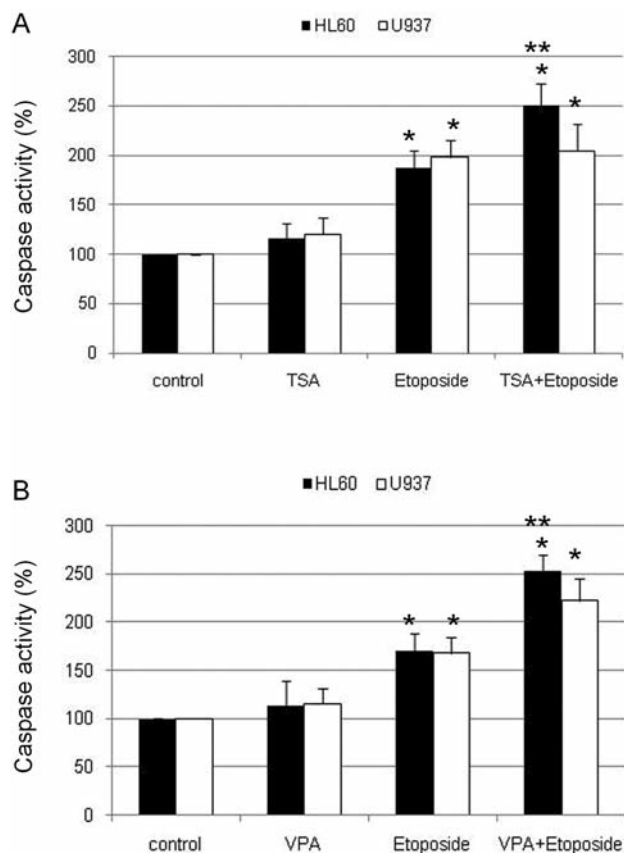


Figure 4. Effect of etoposide on caspase activity of HL60 and U937 cells pretreated with TSA (A) and VPA (B). Each value is the mean±SD of 5 measurements. Significant difference of caspase activity from the corresponding group of \*untreated (control) cells ( $p<0.05$ ) and from \*\*etoposide-treated cells ( $p<0.05$ ).

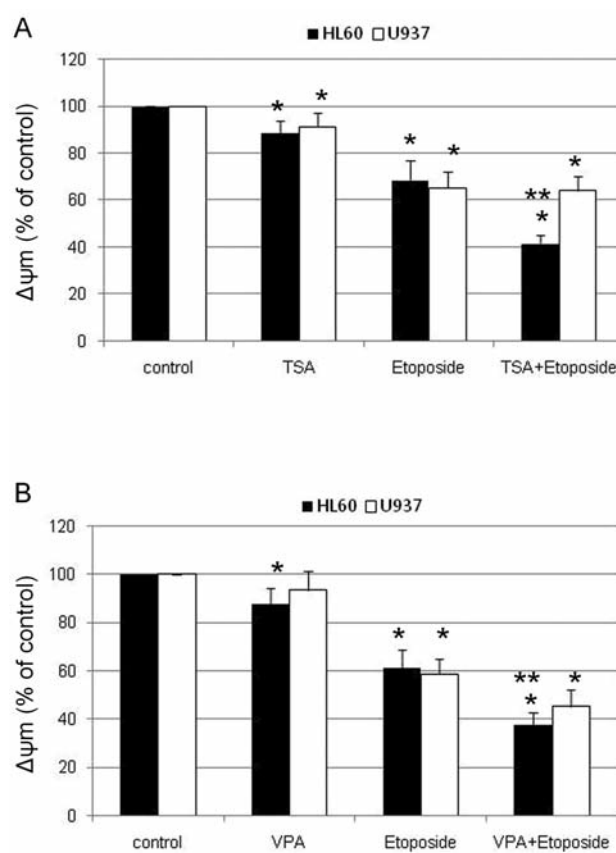


Figure 5. Effect of etoposide on the change in mitochondrial membrane potential ( $\Delta\psi_m$ ) of HL60 and U937 cells pretreated with TSA (A) and VPA (B). Each value is the mean±SD of 5 measurements. Significant difference of  $\Delta\psi_m$  from the corresponding group of \*untreated (control) cells ( $p<0.05$ ) and from \*\*etoposide-treated cells ( $p<0.04$ ).

U937 cells compared to untreated cells. The combined treatment of HL-60 cells with TSA or VPA and etoposide resulted in a significant reduction of  $\Delta\psi_m$  compared to the treatment with etoposide alone, but such an effect was not observed in U937 cells (Figure 5).

**BCL2 mRNA expression:** As compared with untreated cells, TSA and VPA as single agents reduced *BCL2* mRNA level in both cell lines. Expression of *BCL2* mRNA also decreased after treatment with TSA or VPA and etoposide in HL60 cells. In U937 cells, inhibition of *BCL2* mRNA expression following treatment with TSA in combination with etoposide was weaker as compared with that observed after VPA and etoposide treatment. However, no significant differences were observed in either cell line between *BCL2* mRNA levels after treatment with a single HADCi and after combined treatment with HADCi and etoposide (Figure 6).

## Discussion

Apart from assessing the net apoptosis rate, we investigated some apoptosis-related parameters: activity of caspases, mitochondrial membrane potential and expression of *BCL2* mRNA. Apoptosis is usually deregulated in cancer cells because of increased expression of antiapoptotic proteins and/or decreased expression of proapoptotic proteins (34). Mitochondria are important regulators of extrinsic as well as intrinsic apoptosis pathways, and loss of mitochondrial membrane potential, accompanied by opening of permeability transition pores, are early events in the apoptotic cascade. Mitochondrial function is controlled by several factors including pro- and antiapoptotic members of the *BCL2* family (35).

In the present study, preincubation of HL60 promyelocytic leukemia cells with 250 nM of TSA or 1.25 mM of VPA for 24 hours significantly potentiated etoposide-induced

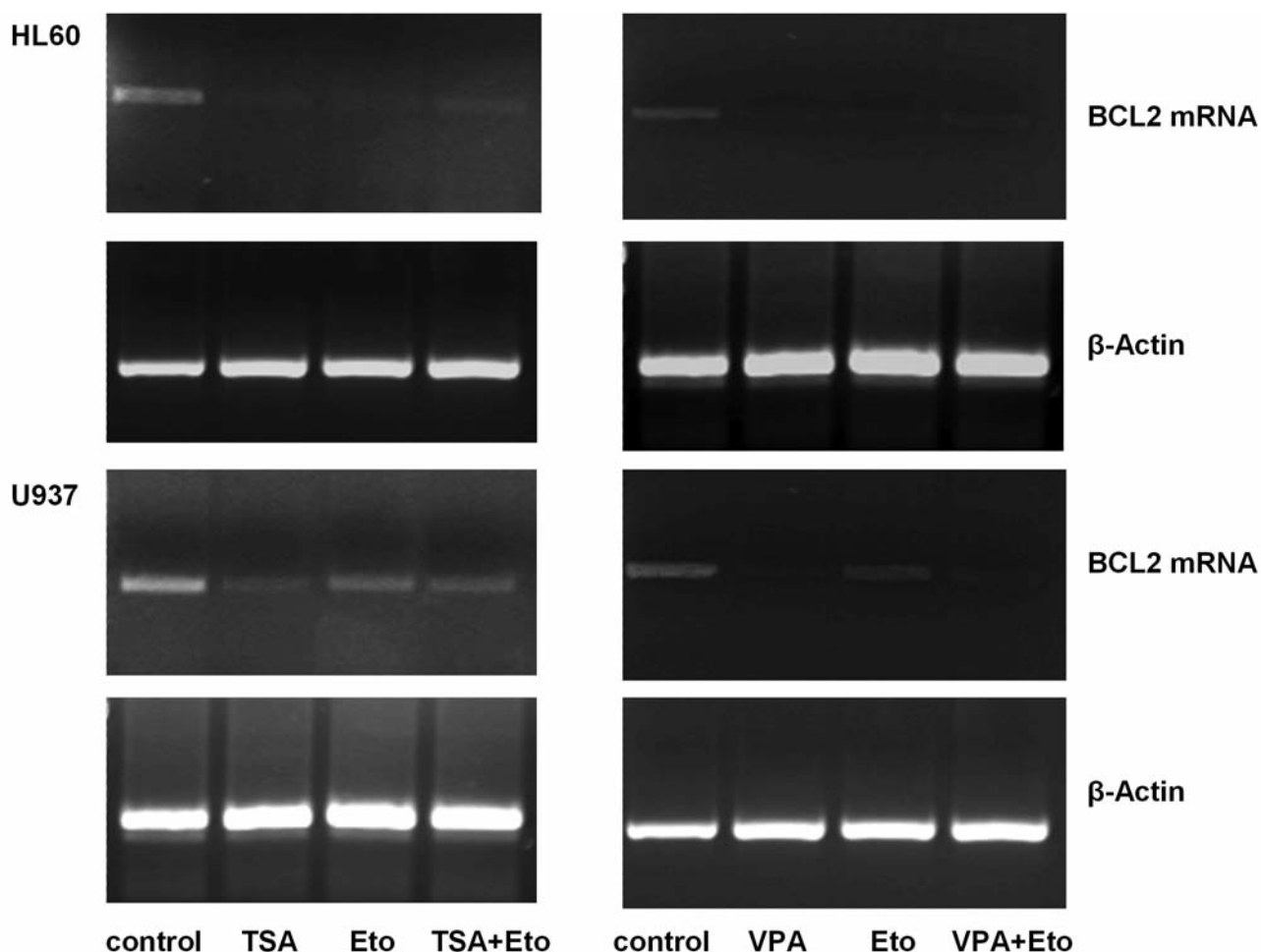


Figure 6. Effect of etoposide (Eto) on *BCL2* mRNA expression in HL60 and U937 cells pretreated with TSA and VPA in reverse transcription-PCR analysis.  $\beta$ -Actin expression levels were used as controls for RNA.

apoptosis, which was associated with activation of caspases and loss of mitochondrial membrane potential. Accordingly, cell viability significantly decreased after combined treatment with HDACi and etoposide.

Such an effect was not observed in U937 monocytic leukemia cells after pretreatment with HDACi. As compared to cells treated by etoposide alone, no significant changes in apoptosis rate, caspase activity or mitochondrial membrane potential were detected. This absence of a synergistic effect of HDACi and etoposide could be at least partially related to the weaker inhibition of *BCL2* mRNA expression in U937 cells, observed especially after TSA and TSA with etoposide treatments.

The enhanced caspase activity and decrease in mitochondrial membrane potential mostly corresponded with an increase in the net apoptosis rate after HDACi and etoposide treatment. However, expression of *BCL2* mRNA was strongly down-regulated after treatment of cells with

HDACi alone and did not show any additive effect with etoposide. It has been shown that antileukemia activities of TSA and VPA can be explained by down-regulation of *BCL2* (15). This suggests that other pro- or antiapoptotic factors are also influenced by the combination treatment. As recently demonstrated in lymphoma cells treated with HDACi and etoposide or cisplatin, activation of caspases and up-regulation of BES1-interacting Myc-like protein 3 (BIM3) and *BCL2*-associated X protein (BAX) are not correlated with *BCL2* inhibition (36).

Our findings showing different response to HDACi combined with etoposide in two different leukemia cell lines correspond with results reported by other authors. On one hand, HDACi were found to enhance etoposide-triggered apoptosis in tumor cells (26, 36-41). On the other hand, inhibition of apoptosis was also observed after such treatment (27, 28, 42). Hauswald *et al.* (28) demonstrated that HDACi, including TSA, may induce expression of

multidrug resistance-associated genes in leukemia cells and that combinations of HDACi and anticancer drugs can lead to reduced cytotoxic effects by influencing multidrug resistance-associated transport of drugs across the cell membrane.

It should be noted that cell response to HDACi combined with anticancer drugs can depend upon a variety of factors. Leukemia cell lines display different phenotypes and bear diverse chromosomal abnormalities (43). This variability can in part explain some of the apparently conflicting results. Another reason may relate to the differences in drug concentrations and the exposure conditions used. Cytotoxicity in combined treatment can be schedule-specific: synergistic effect on lung tumor cells was observed when HDACi and a DNA-damaging drug were applied simultaneously, while sequential treatment (HDACi followed by etoposide or cisplatin) caused an antagonistic effect (42).

In summary, combination of HDACi with anticancer agents may induce increased apoptosis in cancer cells but this effect depends on multiple factors, such as cancer cell type, concentration of drugs within the range showing less additive toxicity for normal cells, and administration schedule. The influence of all these factors should be taken into consideration in preclinical trials assessing the efficiency of combined treatment with HDACi and other anticancer drugs.

### Conflict of Interest

None declared.

### Acknowledgements

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