

EFFECT OF VALPROIC ACID IN COMPARISON WITH VORINOSTAT ON CELL GROWTH INHIBITION AND APOPTOSIS INDUCTION IN THE HUMAN COLON CANCER SW48 CELLS *IN VITRO*

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Aim: Acetylation levels of histones are the result of the balance between histone acetyltransferases and histone deacetylases activities, which plays an important role in chromatin remodeling and regulation of gene expression. Histone deacetylases inhibitors such as valproic acid, vorinostat have attracted interest because of their ability to induce differentiation and apoptosis of cancer cells. The current study was designed to assess the effect of valproic acid in comparison to and in combination with vorinostat on cell growth inhibition and apoptosis induction in the human colon cancer SW48 cells. **Materials and Methods:** The colon cancer SW48 cells were seeded and treated with various doses of valproic acid and vorinostat and MTT assay and flow cytometric assay were done to determine cell viability and cell apoptosis, respectively. **Results:** All concentrations of both agents reduced viability significantly in a dose- and time-dependent fashion ($p < 0.004$). Both compounds, either single or combined agents, induced apoptosis significantly, whereas the ratio of the apoptotic cells treated with combined agents was more significant than the single. **Conclusion:** Our findings suggest that valproic acid and vorinostat can significantly inhibit cell growth and induce apoptosis in colon cancer SW48 cells. **Key Words:** valproic acid, vorinostat, apoptosis, colon cancer.

Colorectal cancer is currently the third most common cancer in men and the second in women and also the fourth cause of cancer death worldwide. The incidence rate of the cancer is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 [1]. Genetic and epigenetic alterations are the most causes of human cancer. Fortunately, epigenetic changes are reversible by drugs targeting the epigenome, including histone modifications and DNA methylation [2].

Epigenetic modifications such as posttranslational modification of nucleosomal histones play an important role in gene expression in the eukaryotic cells. These modifications have a significant role in tumorigenesis and cancer induction. DNA hypermethylation and histone deacetylation are the most epigenetic alternations, which lead to tumor suppressor genes silencing and cancer induction. Acetylation of histones is one of the most important mechanisms of regulation of the gene expression [3]. Acetylation of histones regulated by two opposing groups of enzymes, including histone acetyltransferases (HATs) and histone deacetylases (HDACs) is associated with increased transcription. Acetylation levels of histones are the result of the balance between HATs and HDACs activities, which plays an important role in chromatin remodeling and regulation of gene expression. HATs add acetyl groups to amino acid of histone proteins, whereas HDACs have opposite activity and remove the acetyl groups. Histone hypoacetylation induced

by HDACs activity is associated with gene silencing. These enzymes account for as critical regulators of cell growth, differentiation, and apoptotic programs [4–6]. HDACs have been classified into four classes (comprising > 18 isoenzymes) including class I (HDACs 1, 2, 3 and 8), class II (HDACs 4, 5, 6, 7, 9 and 10), class III (sir2 family) and class IV (HDAC11) [7–11]. The class I HDACs (1, 2, 3 and 8) and the class II HDAC (4) are expressed in the normal colon cell. Class I HDACs play a role in promoting colonic cell proliferation. It has been demonstrated that Knockdown of these enzymes reduces growth of colon cancer cells (HCT116, HT29 and SW480). Several studies have demonstrated increased expression of the class I HDACs (HDACs 1, 2, 3 and 8) in colon tumor cells [12–18]. HDAC inhibitors (HDACIs) have attracted interest because of their ability to induce differentiation and apoptosis of cancer cells.

It has been reported that several HDACIs such as valproic acid (VPA), vorinostat (suberoylanilide hydroxamic acid, SAHA), trichostatin A (TSA) and sodium butyrate can induce differentiation, cell cycle arrest and apoptosis in colon cancer cell lines *in vitro* [19–24]. Up to date there was no data on the anti-proliferative and apoptotic effect of VPA and vorinostat on colon cancer cell line. Therefore, we examined the effect of VPA in comparison to and in combination with vorinostat on viability and apoptosis of colon cancer SW48 cell line in the current study.

MATERIALS AND METHODS

Cell line and culturing. Human colon carcinoma SW48 cell line were obtained from the National Cell Bank of Iran-Pasteur Institute and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% antibiotics,

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Abbreviations used: HATs – histone acetyltransferases; HDACIs – histone deacetylases inhibitors; HDACs – histone deacetylases; TSA – trichostatin A; VPA – valproic acid.

including penicillin G sodium (Sigma, USA), streptomycin sulfate and amphotericin B (Sigma, USA) at 37 °C in 5% CO₂ to promote attachment. The cells were cultured and exposed to drugs after they reached > 80% confluence and routinely observation was done for the presence of Mycoplasma. Vorinostat (Sigma, USA) was dissolved in DMSO as a 10 mmol/l stock solution and diluted in order to different concentrations preparation. VPA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in the culture medium to prepare a stock solution, which was further diluted with culture medium to yield various concentrations of VPA. Other materials, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin-EDTA, FBS, RPMI-1640, Annexin-V-(FITC) and propidium iodide (PI, Becton-Dickinson, San Diego, CA, USA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany).

Cell viability assay. The effect of VPA and vorinostat individually and in combination on the cell viability was measured by MTT assay. First, the cells were seeded into 96-well plates (4 · 10⁵ cells per well) and

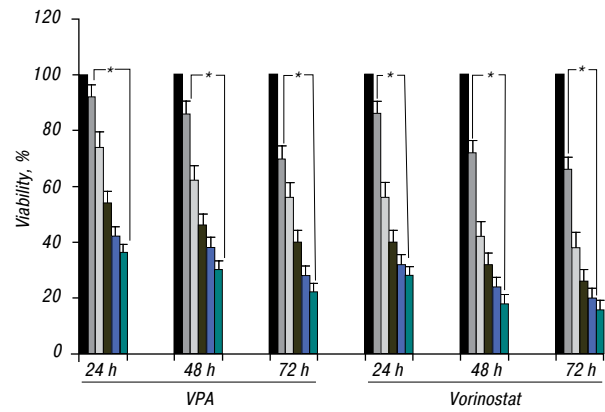


Fig. 1. Effect of VPA (0.5, 1, 5, 10 and 25 μM) and vorinostat (0.5, 1, 5, 10 and 25 μM) on the SW48 cell viability. The effects were determined by the MTT assay. Data are presented as mean ± SD from at least triplicate wells and 3 independent experiments. Asterisks (*) indicate significant differences between treated cells and the control group. The first column of each group belongs to control group

allowed to adhere for 24 h and then culture medium was replaced with medium containing different doses of VPA (0.5, 1, 5, 10 and 25 μM) and vorinostat (0.5, 1, 5, 10 and 25 μM) except control groups for different time periods, control groups were incubated with

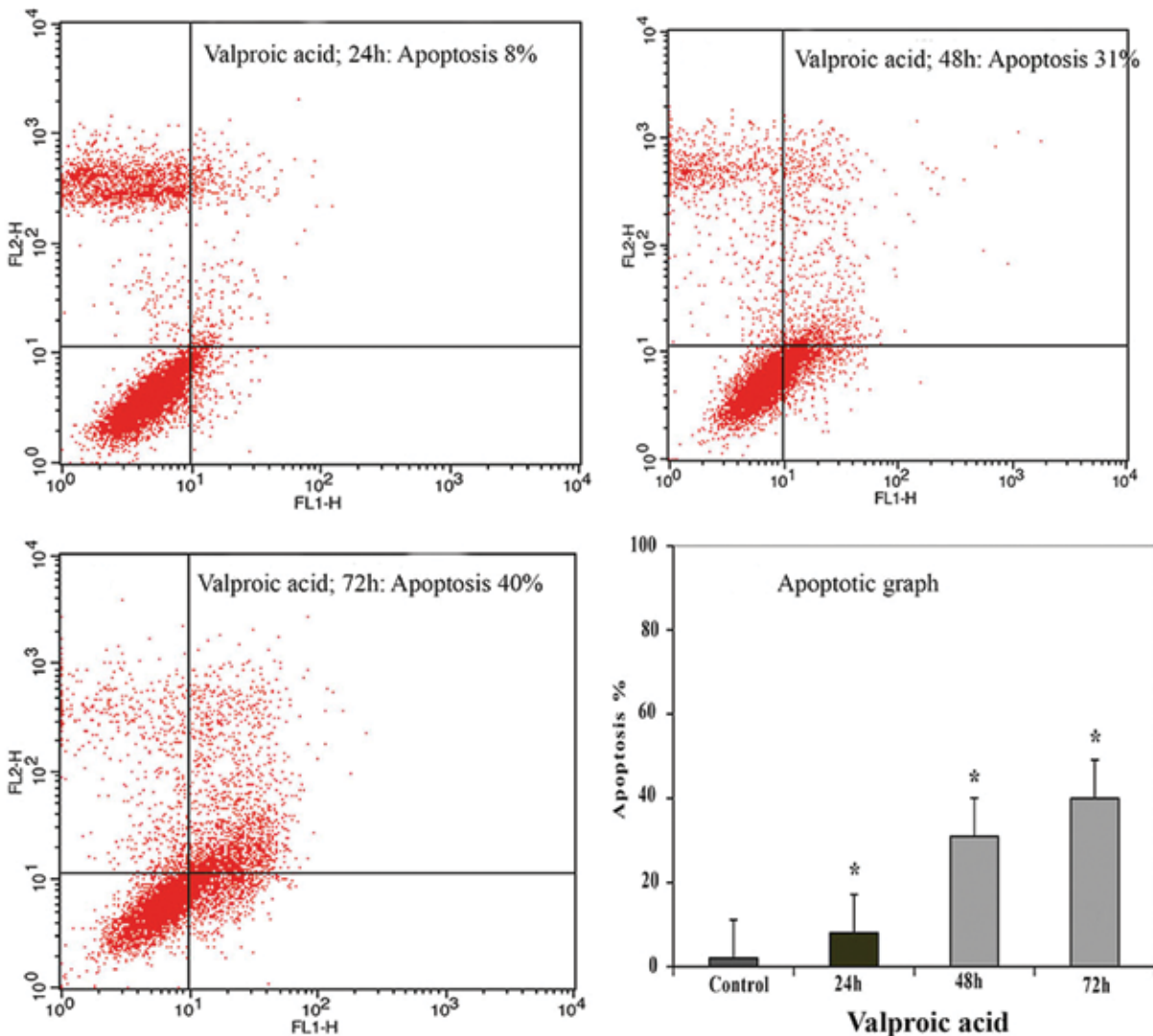


Fig. 2. Apoptotic effects of VPA on SW48 cells. Significant apoptosis was shown at different time periods (24, 48, and 72 h) in a dose dependent manner (**p* < 0.001)

DMSO only. After incubation times (24, 48 and 72 h), the cells were washed twice with FBS, a medium containing MTT was added and maintained for 4 h and finally the formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm. All experiments were repeated three times.

Cell apoptosis assay. For detection of cell apoptosis, the cells were cultured in 24-well plates at a density of $4 \cdot 10^5$ cells/well and incubated overnight before exposure to medium containing drugs. After cell adhesion, the cells treated with VPA (5 μ M) and vorinostat (1 μ M), based on IC_{50} values, as alone and combined for 24, 48, and 72 h. After treatment times, all the adherent cells were harvested by trypsinization, washed with PBS and resuspended in Binding buffer ($1 \times$). Annexin-V-(FITC) and propidium iodide were used for staining according to the protocol. Finally, the apoptotic cells were counted by FACScan™ flow cytometer (Becton Dickinson, Heidelberg, Germany).

Statistical analysis. The data were obtained from three tests and are shown as means \pm standard deviations. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA) and

Turkey test. A significant difference was considered as $p < 0.05$.

RESULTS

Result of cell viability assay. The effects of the VPA and vorinostat on the colon cancer SW48 cell viability were assessed by MTT assay after treatment with various doses of the compounds (as mentioned above). As can be seen in Fig. 1, the effective doses of the VPA and vorinostat that inhibited 50% cell growth were 5 μ M and 1 μ M, respectively. All concentrations of both compounds reduced viability significantly in a dose-dependent fashion ($p < 0.004$).

Result of flow cytometric assay. The SW48 cells were treated with VPA (5 μ M) and vorinostat (1 μ M) at different times (24, 48 and 72 h) as mentioned in Materials and Methods. By flow cytometry, we assessed the ability of VPA and vorinostat, alone and combined, to induce apoptosis in colon cancer SW48 cell line. As shown in Fig. 2, VPA induced significant apoptosis at all experimental time points ($p < 0.001$). Apoptotic effect of vorinostat was significant as shown in Fig. 3 ($p < 0.001$). The fraction of the

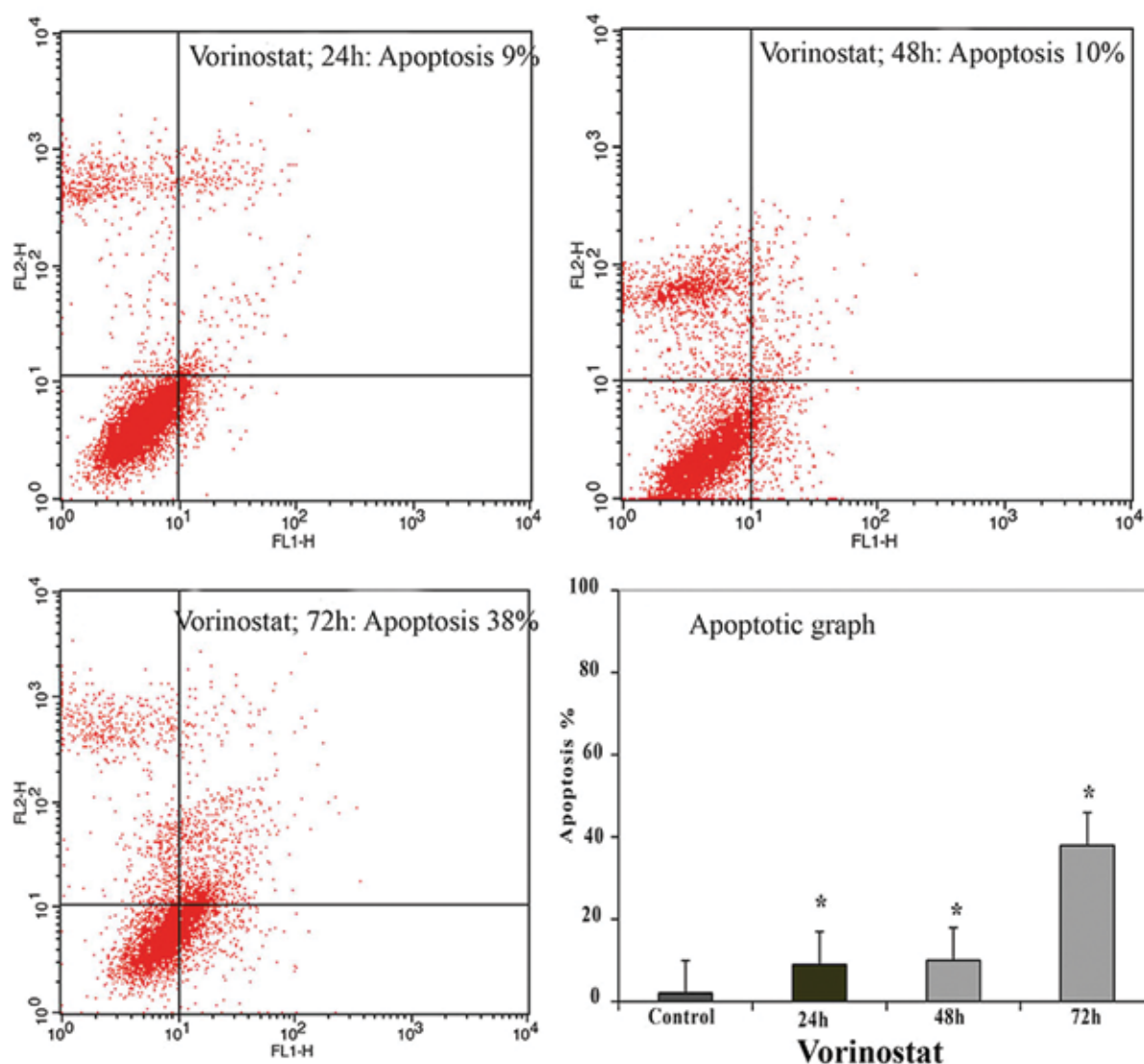


Fig. 3. Apoptotic effects of vorinostat on SW48 cells. Significant apoptosis was shown at different time periods (24, 48, and 72 h) in a dose dependent manner (* $p < 0.001$)

apoptotic cells with combined agents was superior to that with the single agents. The percentage of apoptotic cells is indicated in the Table. Relative analysis between VPA and vorinostat treatment groups at different times indicated that VPA induced apoptosis more significantly than vorinostat. Maximal apoptosis was seen in the group, which received VPA in combination with vorinostat for 72 h ($p < 0.001$) (Fig. 4).

Table. Percentage of apoptosis

<i>p</i> -value	Apoptosis, %	Duration, h	Dose, μ M	Drug
< 0.001	8	24	5	VPA
< 0.001	31	48	5	VPA
< 0.001	40	72	5	VPA
< 0.001	9	24	1	Vorinostat
< 0.001	10	48	1	Vorinostat
< 0.001	38	72	1	Vorinostat
< 0.001	46	24	5/1	VPA/vorinostat
< 0.001	51	48	5/1	VPA/vorinostat
< 0.001	56	72	5/1	VPA/vorinostat

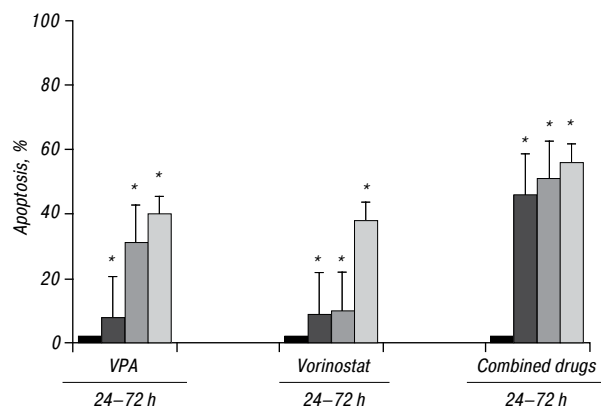


Fig. 4. Apoptotic effect of VPA and vorinostat on SW48 cells. The cells were treated with VPA (5 μ M), vorinostat (1 μ M) and VPA/vorinostat (5/1 μ M) for 24, 48 and 72 h. Data are presented as mean \pm standard error of the mean from at least three different experiments. Asterisks (*) indicate significant differences between treated cells and the control group. * $p < 0.01$ as compared to the control group

DISCUSSION

Cancer can be induced by genetic and epigenetic alterations, epigenetic changes such as histone deacetylation, DNA methylation and non-coding RNA deregulation. Histones deacetylation influence gene transcription including down regulation of the several tumor suppressor genes [25, 26]. HDACs have potent and specific anticancer activities and have emerged as a potential strategy to reverse silenced genes associated with cancer [27]. Apoptotic and antiproliferative effects of HDACs such as butyrate, TSA, vorinostat, benzamides (MS-275) and VPA on colon cancer have been reported by several researchers [28–30]. We, for the first time, show that VPA and vorinostat (individually and in combination) can inhibit viability and induce apoptosis in colon cancer SW48 cell line. Similar to our data, it has been reported that vorinostat has apoptotic and antiproliferative effects on colon cancer HCT116 and HT29 cell lines [31]. There are several reports that indicate inhibitory and apoptotic effects of VPA on the other cancers such as Panc1 and PaCa44 pancreatic cancer cells [32], human melanoma G-361 cell [33], ovarian cancer OVCAR-3 cell [34],

gastric cancer cell [35], and AML1/ETO-positive leukemic cells [36]. As mentioned above, vorinostat exerted significant antiproliferative and apoptotic effect. This effect has been reported by several previous works on 320 HSR colon cancer [37], HCT116 and SW480 colon cancer [38], prostate cancer LNCaP, PC-3 and TSU-Pr1 [39], human ovarian cancer SK-OV-3, OVCAR-3, TOV-21G, OV-90, and TOV-112D [40], breast cancer SKBr-3, MCF-7, and MDA-MB-468 [41]. In the present study, the combination of VPA and vorinostat gave a significant increase in the apoptotic cells and also VPA had a stronger apoptotic effect than vorinostat [32]. HDACs cause acetylated histones to reactivate tumor suppressor genes and induce apoptosis in cancer cells.

These compounds act by different mechanisms and pathways, including activation of the extrinsic and/or intrinsic apoptotic pathways, growth arrest, mitotic cell death and reactive oxygen species (ROS)-induced cell death [42]. Regulatory mechanisms of action of VPA include HDACs, AKT, GSK3 α and β , the phosphoinositol pathway, the ERK pathway, the tricarboxylic acid cycle, the OXPHOS system, and GABA [43]. VPA can sensitize cells to TRAIL/Apo2L-mediated apoptosis by increasing expression of DR4 and DR5 and modulates expression of p21^{WAF1}/CDKN1A [44], a CDK associated with cell cycle arrest in G₁/S phase, and induce apoptosis by an extrinsic pathway involving engagement of the caspase-8-dependent cascade [45–48]. Several studies have demonstrated that vorinostat can inhibit classes I, II and IV, but not the NAD-dependent class III enzymes. It can induce histones H3 and H4 acetylation associated with the proximal promoter of the *CDKN1A* gene, decrease HDAC1 and Myc, and ROS accumulation in transformed cultured cells [49–51]. Vorinostat decreases the expression of Bcl-2, Bcl-x_L and XIAP and enhances the proapoptotic protein expression such as Bax and Bak in breast cancer [52]. Taken together, our finding indicated that VPA and vorinostat could induce apoptosis in colon cancer SW48 cell line if used separately or in combination.

CONCLUSION

Collectively, our report suggests an important role of VPA and vorinostat on apoptosis induction and cell growth inhibition of colon cancer SW48 cell line. The function of these compounds in inducing apoptosis is very important since it may provide a new preventive and therapeutic strategy for cancer treatment.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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