RESEARCH ARTICLE

Effects of Sodium Valproate on the Growth of Human Ovarian Cancer Cell Line HO8910

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Abstract

To explore a possible new treatment for human ovarian cancer, we studied the effects of sodium valproate on the growth of the HO8910 human cell line. HO8910 cells were cultured in vitro and treated with different concentrations of sodium valproate. Cell proliferation, cell cycling, and apoptosis were measured by flow cytometry, cell morphology under a microscope, and expression levels of WWOX and P27 by Western blotting and RT-PCR. Tumor xenografts were established to determine *in vivo* effects of sodium valproate. Our results showed that cell proliferation was decreased with increasing concentration of sodium valproate, with features of cytoplasmic retraction and floating cells. Moreover, cell cycle analysis revealed a higher apoptosis rate and G_0/G_1 phase in the sodium valproate experimental group than in the control group. In addition, protein expression levels of WWOX and P27 were elevated. Importantly, sodium valproate decreased *in vivo* xenograft tumor burden and up-regulated WWOX and P27 expression in nude mice. In conclusion, sodium valproate might play a role in inhibition and control of ovarian cancer cell line HO8910 by inhibiting cell proliferation, interfering with the cell cycle and promoting apoptosis, so that it may be effective in the clinical treatment of ovarian cancer.

Keywords: Ovarian cancer - apoptosis - WWOX - P27 - sodium valproate

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Introduction

Sodium valproate, as a member of the short chain fatty acids family, is a broad-spectrum anti-epileptic drug that has been extensively used in clinical practices. It is also a histone deacetylase inhibitor, which suppresses the activity of histone deacetylating (Tryfon et al., 2009). Histone deacetylase inhibitors are promising new class of anti-cancerous agents that affect proliferation, differentiation, and apop¬tosis in both solid and hematologic tumors (Blaheta et al., 2002). In the nucleus, increased levels of histone acety¬lation lead to relaxation of the chromatin structure, allowing access of transcription factors and increased transcription, while decreased levels of acetylation are associated with repressed tran¬scription. Therefore, inhibition of the function of histone acetylation affects the regulation of gene expression, cell growth, differentiation, and apoptosis (Duenas-Gonzalez et al., 2008).

Sodium valproate has been shown to reduce cancer proliferation to some extent by inducing cancer cell apoptosis, differentiation and cycle arrest (Davies, 2002; Michaelis et al., 2007; Shi et al., 2007; Dutertre et al., 2010; Li et al., 2012). Therefore, we hypothesized that sodium valproate is a remarkable drug and could be used as a new treatment for tumors. In this present study, we selected human ovarian cancer cell line HO8910 as an experimental cell model and the effects of sodium valproate has been investigated on its proliferation, cell cycle, and apoptosis.

Materials and Methods

Cell Culture

HO8910 ovarian cancer cell line, obtained from the Gynecology Department Laboratory of our hospital, Xuzhou, China, were cultured in RPMI 1640 (Hangzhou Sijiqing Biology Engineering Materials Co., Ltd. China) medium supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biology Engineering Materials Co., Ltd. China), penicillin and streptomycin, in a humidified atmosphere containing 5% CO_2 at 37°C.

Experimental grouping

The HO8910 cells were randomly divided into a control group and experiment groups. The experiment groups were cultured in the medium with 1.0, 2.0, 3.0 and 4.0 mmol/L concentrations of sodium valproate (Sigma aldrich, USA), while normal culture medium had been used in the control group.

Methylthiazol tetrazolium (MTT) Cell Proliferation Assay

MTT cell proliferation assay kit (Beyotime, China) was used for this assay. Briefly, HO8910 cells were trypsinized

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and plated in triplicate into 96-well plates (200µl each well) and then incubated for 24h to allow cell attachment. In the experiment groups, cells were treated with 1.0, 2.0, 3.0, and 4.0mmol/L concentrations of sodium valproate, while normal culture medium was used in the control group. After 48h, the MTT assay was performed by using 20µl of serum-free medium containing MTT (0.5 g/l) and incubating at 37C for 4 h. Then, 150µL DMSO was added to each well, and all the plates were shaken at room temperature for 10min. Optical density (OD) was measured at 490nm using a spectrophotometer microplate reader, and the cellular proliferation graphs were plotted.

Cellular morphological changes observed under microscope

HO8910 cells were trypsinized, added into 24-wells plates (1ml each well), and then incubated for 24h in the serum-free medium. In the experiment groups, cells were treated with 1.0, 2.0, 3.0, 4.0 mmol/L concentrations of sodium valproate, while normal culture medium was used in the control group. After 48h, cells were examined under microscope for cell growth and morphology.

Flow Cytometric analysis for Cell Cycle and apoptosis

Cell cycle assay: This assay was performed by cell cycle detection kit (Beijing Biosea Biotechnology Co., Ltd., China). Briefly, HO8910 cells were plated into 6-wells plates (3×10^4/ml, 2ml each well) and then incubated for 24h in serum-free medium. The cells were treated with normal culture medium for control groups and different concentrations of sodium valproate (1.0, 2.0, 3.0, and 4.0mmol/L respectively) for experimental groups. After 48h treatment, the cells were harvested, fixed with 70% ethanol, and then adjusted to the concentration of 1×10^6/ml. According to the manufacturer's instruction, the cells were incubated in 0.5ml propidium iodide with RnaseA for 30 min at 4°C in the dark. Results were analyzed by Flow Cytometry (6 color FACSCalibur, Promega, CellQuest was analysis software).

Cell apoptosis assay: This assay was performed by Annexin V-FITC/PI Apoptosis Detection Kit purchased from Beijing Biosea Biotechnology Co., Ltd., China. In short, HO8910 cells were plated into 6-wells plates (6×10^4 cells/well) and then incubated for 24h in the serum-free medium. In the experiment groups, cells were treated with above mentioned concentrations of sodium valproate; normal culture medium was used in the control group. After 48h, the percentage of apoptotic cells was measured according to manufacturer's instructions.

Western blot analysis

HO8910 cells (3×10^4/ml) were trypsinized and harvested as the concentration of 2×10^6 in 100ml medium. In the experiment groups, cells were treated with above mentioned concentrations of sodium valproate, while the cells in the control group were treated with normal culture medium. The cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C for 24h. After cell attachment, the mediums were replaced with the fresh RPMI1640 medium, in which different doses of sodium valproate were added for the experiment and control groups. After 48h, the cells were harvested and protein concentration was determined by a BCA protein assay kit (Sigma aldrich, USA) 30µg proteins were subjected to SDS-PAGE and electro-blotted on a PVDF membrane. The membranes were blocked with 5% fat-free milk solution at room temperature for 4h. After that, the anti-WWOX and anti-P27 antibodies at 1:250 ratios (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) were incubated for 12h at 4°C. The secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) conjugated with horseradish peroxidase with dilution ratio 1:1000 was incubated for 3h incubation at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) kit (Xuzhou College Biochemistry Laboratory, China). The same nitrocellulose membrane was stripped and incubated with β-actin monoclonal antibody (Xuzhou College Biochemistry Laboratory, China) at a 1:2000 dilution for 2 h, which acted as a control for loading and blotting.

Xenograft Studies

20 Female BALB/c nude mice (Shanghai Experiment Animal Center, Chinese Academy of Medical Sciences, China), 4-6weeks old, were used. Trypsinized HO8910 cells suspension (2×10^6/ml) was subcutaneously injected per mouse at right forelimb. After one week, the existence of tumor had been confirmed at subcutaneous tissue of the nude mice. Then after, mice were equally divided into two groups: the experiment group and the control group. They were maintained at 25-27°C , 45%-50% humidity, fresh air, and pathogen-free conditions. In the control group, the mice were allowed to free access for food and water, while, in the experimental group, the water was substituted by 0.4% sodium valproate solution. The nude mice were observed daily and the death date had been recorded. After the mouse death, it was anatomized immediately and then the tumor sizes were measured. The tissues from the control and experimental groups were collected. The expressions of WWOX and P27 were detected by western blot as described earlier.

RT-PCR

Total RNA was extracted using TRIpure Reagent Total RNA Extraction kit purchased from Shanghai Jierui, China. RT-PCR was performed using TIANscript RT Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. To detect the expression of WWOX, forward primers (5'-CACGCATTTTAGAAGAATGG-3') and reverse primers (5'-GACAGCAGCACAGTACACG-3') were designed. For P27, forward primer 5'-ATCCACTCTCTGCCCGGTGTTTTT-3' and reverse primer 5'-ATGGCAGACCCAATGGAGCCCA-3' were used. WWOX and P27 expression levels were normalized with GAPDH expression levels. GAPDH forward primers (5'-TCCTGTGGCATCCACGAAACT-3') and reverse primers (5'-GAAGCATTTGCGGTGGACGAT-3') were used. All primers were synthesized by Shanghai Bioengineering, China. RT-PCR for all genes was performed for 30 cycles. The condition for WWOX

PCR: 30s at 94°C (denaturing temperature), 45s at 55°C (annealing temperature), and 45s at 65°C (extension temperature). For P27 PCR: 30s at 94°C (denaturing temperature), 30s at 58°C (annealing temperature), and 40s at 65°C (extension temperature). For GAPDH, 30s at 94°C (denaturing temperature), 30s at 62°C (annealing temperature), 1 min at 72°C (extension temperature). The product visualization was performed after the electrophoresis of PCR products (WWOX 598bp, P27 444bp and GAPDH 315bp).

Statistical analysis

Statistical and graphic analyses were performed with the SPSS 13.0 software (Chicago, USA). All results were expressed in mean ± SD. Comparisons between two groups were made using Student's t test; multiple-group analyses were made by one-way ANOVA test and two groups comparisons in multiple-groups were made by q test. P <0.05 was considered significantly different.

Results

The effect of sodium valproate on the proliferation of HO8910 cells

MTT cell proliferation assay results showed that there was a significant decrease (P <0.05) in cell proliferation (ODs) in the experimental groups than the control group (p=0.032). Moreover, a gradual decrease in cell proliferation was also observed with the gradual increase of sodium valproate concentration within the experimental groups. Importantly, these differences were significant (P <0.05) at each level among different experimental groups (Figure 1). These results suggested that ovarian cancer HO8910 cells treated with sodium valproate had a profound dose-dependent inhibition of growth.

The effect of sodium valproate on the morphology of HO8910 cells

After 48h incubation of cells with sodium valproate, the morphology of HO8910 cells was observed under microscope. Cells cultured in control media grew well, attached in clumps and sheets with the increased diopter and the smooth margins. The numerous cells at fissiparous stage had been observed (Figure 2A). In the experimental group of 1mmol/L sodium valproate, few apoptotic cells at early stage were observed. Moreover, cells turned to small and spherical with concentrated cytoplasm with decreased diopter and coarse margins (Figure 2B). Cells treated with increasing concentrations of sodium valproate showed markedly morphological changes. More apoptotic cells were found and the cells became more spherical with aggravated concentration of cytoplasm and with an increase in the floating cells (Figure 2CDE).

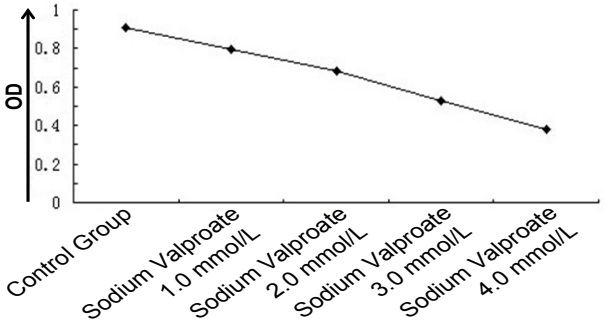

Figure 1. The Effect of Sodium Valproate on the Proliferation of HO8910 Cells. Gradual significant decrease in proliferation of HO8910 cells with gradual increase of sodium valproate concentration (P <0.05). OD: optical density

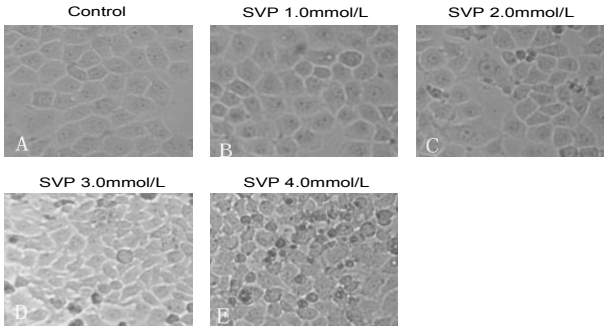

Figure 2. The Effect of Sodium Valproate on the Morphology of HO8910 Cells. Marked morphological changes in cells observed under microscope with increasing concentrations of sodium valproate (SVP). Cells became more spherical with aggravated concentration of cytoplasm and with increased floating cells

Table 1. The Effect of Sodium Valproate on the HO8910 Cellular Cycle (%mean ± SD)

Groups	Go/G1	S	G2/M
Control group	50.2±0.82	39.36±0.78	10.44±0.96
sodium valproate 1.0mmol/L	Δ55.78±0.79*	34.84±0.67	9.38±1.58
sodium valproate 2.0mmol/L	Δ59.64±0.64*	29.02±1.32	11.34±1.23
sodium valproate 3.0mmol/L	Δ70.32±1.02*	19.39±0.83	10.29±0.68
sodium valproate 4.0mmol/L	Δ76.34±0.96*	15.31±0.92	8.35±0.94

Compared with Control group * P < 0.05; Different experimental groups ΔP < 0.05

The effect of sodium valproate on the cell cycle of HO8910 cells

Flow cytometric analysis of cell cycle assay indicated that a significant higher number of cells in the experimental groups with various doses (1.0, 2.0, 3.0 and 4.0mmol/L respectively) of sodium valproate were blocked at G_0/G_1 phase compared with the control group (P <0.05). In addition, with the increased dose of sodium valproate, the percentages of cells that blocked at S phase obviously decreased (Table 1).

The effect of sodium valproate on the apoptosis rate of HO8910 cells

Flow cytometric analysis for the percentage apoptosis rates of the experimental groups with 1.0, 2.0, 3.0 and 4.0 mmol/L doses of sodium valproate were 10.39±1.1, 29.31±1.19, 38.7±1.23 and 46.96±1.37. Our results indicated that all apoptotic rates in the experimental groups from low to high doses of sodium valproate were statistically and gradually higher (P <0.05) as compared to the control group (4.4±1.16) (Figure 3). Thus, sodium valproate induces apoptosis in HO8910 cells in dose-

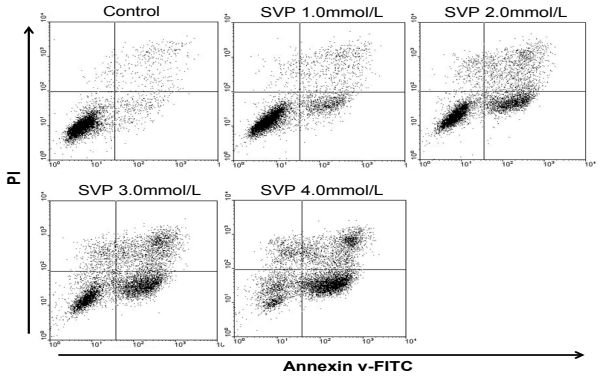

Figure 3. The Effect of Sodium Valproate on the Apoptosis Rate of HO8910 Cells. Representative dot plots of Annexin V/PI analysis in control and experimental groups after treating with different concentrations of sodium valproate (SVP). The lower-right phase is the early apoptotic cells. PI: propidium iodide

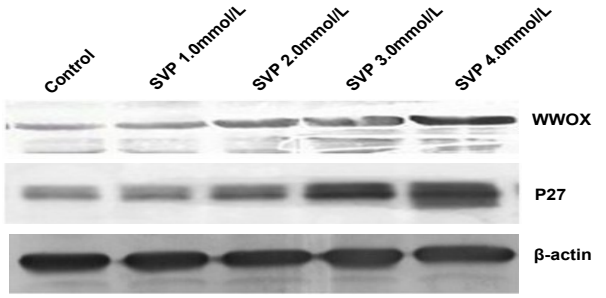

Figure 4. The Effect of Sodium Valproate on Protein Expression of WWOX and P27. Western blot analysis depicts sodium valproate dose-dependent up-regulation of WWOX and P21 protein expression. β-actin was used as the loading control

dependent manner.

The effect of sodium valproate on the expression levels of WWOX and P27

Western blots studies showed that the expression levels of WWOX in the experimental groups was significantly and gradually increased (P <0.05) compared with control group. The increase of the protein was in a dose-dependent manner. Similarly, the expression levels of P27 was also significantly and gradually increased (P <0.05) with the increase concentrations of sodium valproate (Figure 4). In addition, correlation analysis indicated that sodium valproate doses had a positive correlation with the expression levels of P27 (p=0.038, r=0.801). These results suggested that sodium valproate induce expression of tumor suppressor genes in a dose-dependent manner.

The effect of sodium valproate on tumor burden induced by HO9810 cells in vivo

Tumors were detected after one week in all the nude mice injected with the HO8910 cells. The average tumor size of the experimental group (fed on sodium valproate solution) was significantly lesser (1264.59±24.83 vs. 2155.62±73.38mm3, P <0.05) than control group. The average survival time was significantly higher in the experimental group (47.12±0.73 vs. 34.58±0.46 days, P <0.05) compared to the control group. These results indicate that sodium valproate reduce tumor burden and

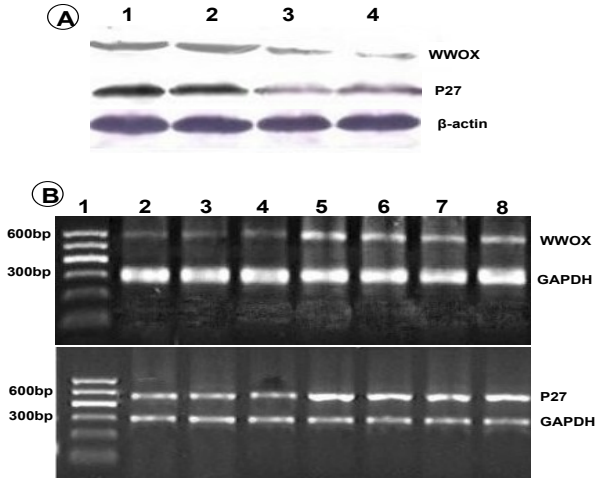

Figure 5. Proteins and mRNA Expression of WWOX and P27 in Xenograft Tumors in Nude Mice. A. Western blot analysis of WWOX and P21 protein expression in the tumors of representatives experimental (1, 2) and control (3, 4) groups. β-actin was used as a control. B. RT-PCR analysis of WWOX and P27 mRNA expression in the tumors of representatives control and experimental groups. G3PDH was used as a housekeeping gene (as a control). 1: Maker; 2, 3, 4: Control group; 5, 6, 7, 8: Experimental group

increase survival time.

Tumoral proteins and mRNA expression of WWOX and P27 in nude mice

Western blot results showed that the expression levels of WWOX and P27 proteins were significantly higher in the experimental group (fed on sodium valproate solution) than in the control group (0.46±0.065 & 0.39±0.018 vs. 0.15±0.025 & 0.20±0.067, P <0.05 respectively) (Figure 5A). Similarly, RT-PCR results indicated that the expression levels of WWOX and P27 mRNA were also significantly higher in the experimental group than in the control group (0.305±0.015 & 0.926±0.034 vs. 0.193±0.056 & 0.702±0.047, P <0.05 respectively) (Figure 5B). These results demonstrated that sodium valproate induce *in vivo* expression of tumor suppressor genes (WWOX and P27) both at the levels of mRNA and protein.

Discussion

There are several kinds of ovarian cancers, however, epithelial ovarian carcinomas account for 85 to 90 percent of all cancers of the ovaries. It is a leading gynecologic cancer with high mortality rate and poses a great threat to female health for its failure in early diagnosis and treatment (Jemal et al., 2011). Therefore, the search for effective and successful therapies for this cancer is mandatory.

Sodium valproate, sodium salt of valproic acid, is a histone deacetylase inhibitor. Acetylation of histones is regulated by the opposing activities of histone acetyltransferase or histone deacetylase. Chromatin structure and gene expression and regulation are controlled under the dynamic balance of histone acetylation and deacetylation. It is becoming a hot topic that histone deacetylase inhibitors (HDIs) are promising anticancer agents. It is generally believed that HDIs

induce acetylation of histones, modify the expression of oncogenes and tumor suppressor genes, rescue normal cell growth and differentiation, and thus elicit anti-cancer effect (Balakin et al., 2007). Sodium valproate is a traditional anti-epilepsy drug with few side effects and excellent tolerability for long-term use. Therefore, sodium valproate as a strong and safe HDI which has a selective cytotoxic effect on cancer cells but no serious influence on normal cells.

Recent evidences suggested that sodium valproate participates in multiple processes to treat cancer i.e. blocking tumor cells signaling, down-regulating the proliferation of tumor cell and the production of vascular growth factor, and inducing cell cycle arrest (Venugopal et al., 2011). Some researches revealed up-regulation of Bak protein expression, degradation of cyclin D1 and an increase in cyclin-dependent kinases p21 and p27 after sodium valproate treatment. In addition, sodium valproate also increased the levels of full-length Notch-1 and active Notch-1 intracellular domain, which intern inhibit proliferation and induced apoptosis of neoplasm (Greenblatt et al., 2007; Fortunati et al., 2008; Platta et al., 2008). Sami et al. in cervical cancer found that sodium valproate can increase the expression of histone H3 acetylation and up-regulate p21 expression (Sami et al., 2008). Moreover, long-term administration of sodium valproate had a cytostatic effect resulting in a significant reduction of tumor growth. Another study revealed that sodium valproate suppress in vitro proliferation of endometrial cancer cells in nude mice and increase the proportion of cells at G_0/G_1 phases of the cell cycle by possible increase of p21, p27, and E-cadherin (Takai et al., 2004).

To further evaluate whether sodium valproate can inhibit the proliferation of ovarian cancer cells, we investigated the effect of different doses of sodium valproate on HO8910 cells in vitro, and in BALB/c nude mice *in vivo*. Our result demonstrated that sodium valproate inhibit the growth of HO8910 cells in a dose-dependent manner. Moreover, flow cytometric and microscopic examination data indicated that sodium valproate not only has cytostatic effect and block the cell cycle at G_0/G_1 or S phase, but also promoted the apoptosis of HO8910 cells. In addition, the effect of sodium valproate has an obvious association with its concentrations. Based upon these facts, we extend our study on the possible molecular mechanisms of sodium valproate on HO8910 cells. Western blot analysis indicated that WWOX and P27 expression levels were increased in HO8910 cells treated with various doses of sodium valproate. These result suggested that sodium valproate up-regulate WWOX and P27 expression levels and then inhibit growth and induce apoptosis in HO8910 cells. Our *in vivo* experiments on HO8910 cell induced tumor in nude mice also revealed that sodium valproate treated mice showed decrease tumor burden, and up-regulate tumor suppressors WWOX and P27, thus confirming the inhibitive effect of sodium valproate on HO8910 cells.

In summary, we showed that sodium valproate inhibits HO8910 cells growth in a dose-dependent manner. We also present evidences that sodium valproate up-regulate tumor suppressor genes WWOX and P27 in vitro as well as *in vivo* in these cancerous cells. Taken together, sodium valproate has a potential role in inhibition and control of ovarian cancer cell line HO8910, and could be the basis of future ovarian cancer treatments.

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The author(s) declare that they have no competing interests.

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