RESEARCH ARTICLE

Effects of Valproic Acid on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer in Vitro and in Vivo

Zhao Shan[1]*, Rong Feng-Nian[2], Geng Jie[3], Zhou Ting[2]

Abstract

Inhibitors of histone deacetylase activity are emerging as a potentially important new class of anticancer agents. In this study, we assessed the anticancer effects of valproic acid (VPA) on ovarian cancer in vitro and in vivo. Cultured SKOV3 cells were treated by VPA with different concentrations and time, then the effects on cell growth, cell cycle, apoptosis, and related events were investigated. A human ovarian cancer model transplanted subcutaneously in nude mice was established, and the efficacy of VPA used alone and in combination with diammine dichloroplatinum (DDP) to inhibit the growth of tumors was also assessed. Proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. The cell cycle distribution changed one treatment with VPA, with decrease in the number of S-phase cells and increase in G1-phase. VPA could significantly inhibit the growth of the epithelial ovarian cancer SKOV3 cells in vivo without toxic side effects. Treatment with VPA combined with DDP demonstrated enhanced anticancer effects. The result of flow cytometry (FCM) indicated that after VPA in vitro and in vivo, the expression of E-cadherin was increased whereas vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) were decreased. This study suggests that VPA could be a novel attractive agent for treatment of ovarian cancer.

Keywords: Valproic acid - ovarian cancer - apoptosis - DDP

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Introduction

Ovarian cancer has the highest mortality rate among gynecologic malignancies, with a 5-year survival rate of 39% in 1990 (Boring et al., 1992). Most neoplastic ovarian tumors produce few symptom until the disease is widely disseminated throughout the abdominal cavity, for this reason, the chemotherapeutic treatment is a attractive therapeutic strategy to improve the efficacy of surgery, reduce the chance of recurrence. But many patients present drug resistance gradually and these common agents maybe associated with some potentially life-threatening toxic side effects. So finding some new agents which has better anticancer effect without toxicities is an area of intensive investigation.

It has been well established that the occurrence and development of cancers involve a substantial change in functions of both oncogenes and tumor suppressor genes. One of the most important mechanisms in chromatin remodeling is the posttranslational modification of the N-terminal tails of histones by acetylation, which are key steps in the regulation of gene expression, consequently affecting proper cell function, differentiation and proliferation. More than thirty years ago, Vincent Allfrey and collaborators reported a strong correlation between increased levels of histone acetylation and gene over expression (Allfrey et al., 1964). The genesis of cancer, malignant dissemination of tumor cells are, at least in part, based on the up-regulation of histone deacetylases (HDACs). So HDACIs are emerging as promising clinical therapeutics for cancer (Bellarosa et al., 2012; Feng et al., 2012; Francisco et al., 2012).

Some HDACIs are of limited therapeutic use, due to poor bioavailability in vivo and toxic side effects at high doses (Marks et al., 2001). The short chain fatty acid valproic acid (VPA), one of the HDACIs, has a good tolerability and safety profile. It emerged in 1997 as an antineoplastic agent as well, when findings indicated that the substance inhibited proliferation and induced differentiation of primitive neuroectodermal tumor cells in vitro and in vivo (Cinatl Jr et al., 1997) The anti cancer characteristics of VPA has meanwhile been investigated in preclinical models of a variety of solid and hematologic tumors. Currently, the drug is in phase II trials (Daud et al., 2009). While these agents show promise, their mechanism(s) of action and selective toxicity against tumor cells have not yet been adequately defined. The current study was designed to define the biological therapeutic effects of VPA in the treatment of ovarian cancer in vitro and in vivo.

*[1]Department of Obstetrics and Gynecology, the First Affiliated Hospital of Guangxi Medical University, Nanning, [2]Department of Obstetrics and Gynecology, Shandong Qianfoshan Hospital, Jinan, [3]Department of Reproduction, The 174 Hospital of the PLA, Xiamen, China *For correspondence: zhaoshan81641@yahoo.com.cn*

Materials and Methods

The human ovarian cancer cell line SKOV3 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). It was routine maintained in RPMI 1640 containing 100 mL/L fetal bovine serum (FBS), 100U/mL penicillin, 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 50 mL/L CO_2. VPA was obtained from Sigma and dissolved in phosphate-buffered saline (PBS) at concentration of 1, 2, 3, 4,5mmol/L, PBS was added to culture medias a negative control.

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipenyl (MTT) dye reduction assay. Cells were seeded at the density of 5×10^4 per well in 96-well plates in RPMI-1640 containing 100μl /L FBS and incubated as described above. After 24 hours, fresh medium was added; containing VPA at concentrations of 0, 1, 2, 3, 4, 5 mmol/L, and every concentration has 10 plates. The plates were allowed to stand 1-3 days at 37°C, 5% CO_2. We observed the cells morphous under light microscope every day. After the termination of the culture, MTT (0.5 mg/mL) was added for an additional 4 hours. Absorbance at a wavelength of 492 nm was determined for each well using a microplate ELISA reader. So we can get optical density (OD) of every well. Inhibition ratio of different concentrations was calculated.

The percentage of G_1, S, and G_2-M phase of the cell cycle was determined by a modified Vindelov propodiumiodide DNA staining procedure. Briefly, cells cultured after 48 h and 72 h were isolated from the culture dishes with trypsin, washed once in fresh media, and resuspended at a concentration of 1 x 106 cells/ml. The cell suspension (200 μl) was then incubated with 500 μl of a propidium iodide solution [0.006 g of PI, 70 units of RNase, 0.058 g of NaCl, 0.121 g of Trisma base, and 0.1 ml of NP40; volume to 100 ml (pH 8.0)] overnight at 4°C. The following day, the stained cell nuclei were run on a Coulter Epix XL flow cytometer. The ungated histogram was evaluated using the Phoenix flow DNA modeling system to determine the percentage of cells in G_1, S, and G_2-M phase. On the left side of G_1 summit is $SubG_1$, it represented the cell apoptotic index.

Cells cultured after 48 h were subsequently dissociated into a single-cell suspension, and resuspended at a concentration of 1 x 10^6 cells/ml. Cells were initially incubated with rat-monoclonal anti-VEGF, anti-E-cadherin and anti-MMP-9 antibody (1:100, Zymed, USA) respectively at room temperature for 30 min, followed bylabeling with anti-rat fluorescein-isothiocynate (FITC)-conjugated second antibody (1:100, Center Laboratories QiLu Hospital). That was also performed at room temperature for 30 min. Flow cytometry and cell sorting were completed on a Coulter Epix XL flow cytometer. Facility using Expo 32 ADC Analysis Program. Fluorescence index (FI) was used to demonstrate the protein lever of VEGF, E-cadherin and MMP-9 (Morkve et al., 1991).

Twenty four 6-week-old weighing 19-21 g immunodeficient beige/nude/xid nu/nu female mice were purchased from Chinese Academy of Medical Sciences, and maintained under pathogen-free conditions with irradiated chow. The experimental procedures on the use and care of animals had been approved by the Ethics Committee of Shandong University. In our experiment, 5 x 10^6 SKOV3 cells in 0.2 mL PBS was subcutaneously injected into the trunk of 24 mice, leading to the formation of a tumor per animal. Human ovarian cancer model transplanted subcutaneously in nude mice was established, and randomly divided into 4 groups (6 mice per group): (1) control group, received PBS 0.2ml only; (2) experimental group, including: VPA group, DDP group and VPA+DDP group. Treatment was initiated intraperitoneally when established tumors of 0.2 cm-0.3 cm diameter were detected; VPA group was treated with VPA in doses of 500 mg/kg/day in 0.2 mL for 30 days. DDP group was treated with DDP in doses of 3mg/kg every 6 days, for 5 times. VPA+DDP group was treated VPA and DDP. Tumors were measured every week with vernier calipers. Tumor volume was calculated using the following formula: volume x length x width x height x 0.5236 respectively. Draw the growth curve of different drugs. At the end of the experiment, animals were sacrificed after which careful resection was performed and tumor weights were measured, and calculated the Tumor growth inhibition (TGI). TGI (%) = (1 - Vt/Vc) × 100%, where Vt is the mean tumor volume of the arsenic-treated group, and Vc the mean tumor volume of the control group. Tumor, brain, liver, spleen, and kidney specimens were fixed and stained for histologic analysis.

Tumors and normal organs were detected apoptosis by flow cytometry, the protein levers of VEGF, E-cadherin and MMP-9 were also assaied by FCM.

All results were presented as mean ±standard error. Date was analyzed by a computer software SPSS10.0, statistical significance was investigated by F test and $P<0.05$ was considered statistically significant.

Results

We initially examined whether assertion of the VPA to SKOV3 cells cultures produced any effect and cellular morphology. Figure 1 is a photomicrograph composite of SKOV3 cells incubated in the presence and absence of VPA (4 mmol/L 48 h). Under light microscope, cells in the control group grew bloomy and were clustered. The shapes were long, shuttle and polygon. The cell shapes were plump. When the concentration of VPA was less than 2 mmol/L, the growth of cells in the experimental group were not inhibited evidently. As the concentration of VPA rise to 3 mmol/L or more, the growth of the cells was inhibited and the normal cells were dropped off. The residual attached cells were scattered or rickled. The shape of the residual attached cells was round or ellipse. The cytoplast was crimpled. Photomicrographs in the Figure 1 B showed the SKOV3 cells treated with VPA (4 mmol/L, 48 h). As is apparent from the figure, within 48h, VPA had a dramatic effect on the morphology of cells, and the cells density was reduced.

To test whether the reduced cell density after VPA treatment is the result of changes in proliferation, we used MTT to determine the optical density, and then calculated

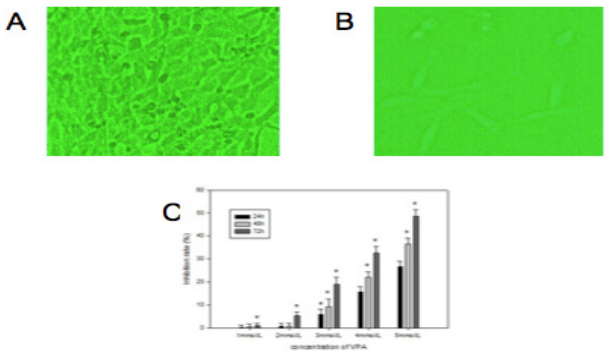

Figure 1. Inhibition Rate of VPA on SKOV3 Cells Growth. Under light microscope, cells in the control group grew bloomly and were clustered. The shapes were long, shuttle and polygon. The cell shapes were plump (as in A). As the concentration of VPA rise to 3 mmol/L or more, the growth of the cells was inhibited and the normal cells were dropped off. The residual attached cells were scattered or rickled. The shape of the residual attached cells were round or ellipse (as in B). We used MTT to determine the optical density after VPA treatment, and then calculated the inhibition ratio. It shows VPA producing a significant reduction of proliferation in cells. When the concentration of VPA was between 3 to 5 mmol/L, the growth of SKOV3 cells was inhibited evidently in vitro (as in C). The proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. *: p<0.05 for the difference in each concentrations and each times. Error bars represent standard deviations

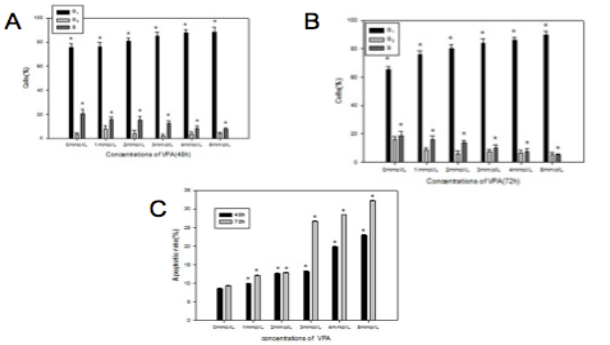

Figure 2. Cell Cycle Analysis of SKOV3 Cells by Flow Cytometry. Cells cultured with different concentrations of VPA after 48 h and 72 h were isolated from the culture dishes with trypsin. The ungated histogram was evaluated using the Phoenix flow DNA modeling system to determine the percentage of cells in G_1, S, and G_2 phase. After treated with VPA for 48 h, S-phase cells were decreased and of G_1-phase cells were increased, but the changes of the G_2-phase and M-phase were not significant (as in A). After 72 h, this effects were more significant (as in B). Apoptotic rate was increased in a dose and time dependent fashion and reached differences statistical significance in each group.*: p<0.05 for the difference in each concentrations and each times. Error bars represent standard deviations

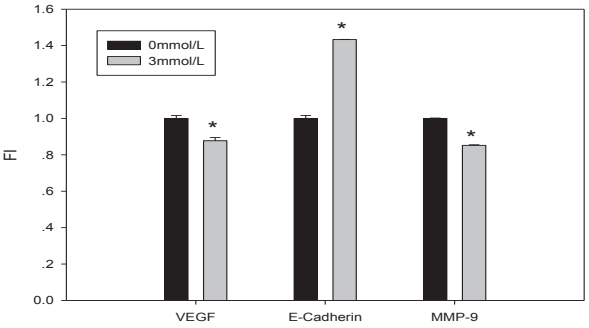

Figure 3. The VEGF, E-cadherin and MMP-9 Protein Level of SKOV3 Cells Treated with VPA of Different Concentrations for 48h by FCM. After treated with VPA at concentration of 3mmoL/L for 48 hours ,the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, there was a significant difference among each group(P<0.01)

the inhibition ratio. Figure 2C shows VPA producing a significant reduction of proliferation in cells. When the concentration of VPA was between 3 to 5 mmol/L, the growth of SKOV3 cells was inhibited evidently in vitro. After using VPA for 72 h, the cell inhibition rate was 18.92%, 32.70%, 48.65% respectively. The proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion.

We used FCM to determine whether the reduction in the number of cells was the result of increased apoptosis and cell cycle arrest. The cell cycle distribution changed while treated with VPA for 24 h, decrease of S-phase cells and increase of G_1-phase cells, but the changes of the G_2-phase and M-phase were not significant as in Figure 2. The percentage of $SubG_1$ was increased in a dose and time dependent fashion and reached differences statistical significance in each group (P<0.05). The $SubG_1$ respected the apoptosis rate, as showed in Figure 2C, Apoptotic rate of SKOV3 cells treated with VPA of different concentrations for different times, the differences has statistical significance in each group (P<0.05).

In our study, we tested expression of genes associates with angiogenesis and metastasis, to examine whether VPA can inhibite neoplasms metastasis and angiogenesis. We used FCM to detect VEGF, E-cadherin and MMP-9 protein levers. After treated with VPA at concentration of 3mmoL/L for 48 hours ,the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased,as showed in Figure 3, there was a significant difference among each group (P<0.01).

Finally we detected the anticancer effect of VPA and sensitization in combine with DDP on the growth of human ovarian cancer transplanted subcutaneously in nude mice. Under light, in the control group cancer cells growth were active, nucleus were hyperchromasia, with a lot of cellular atyoia and karyokine. But in the tumors of experimental groups, there was zone of necrosis, a number of characteristic apoptotic cells were found, the karyoplasmic ratio was smaller, we also found inflammatory cell infiltrate in tumors of experimental groups. However, in the control groups there were few. In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. The inhibiting rate of VPA group, DDP group and VPA+DDP group were as follows: 40.7%, 45.3%, 58%, as showed in Figure 4. They were significantly higher than control group (P<0.01).The apoptotic rate of experimental groups were as follows: (27.05±1.63)%, (35.93±3.89)%, (42.59±2.55)%, which were significantly higher than control group (16.73±2.82)% (P<0.01). The cell cycle distribution changed in experimental group, the S-phase was decrease. The result (Figure 5) of FCM indicated

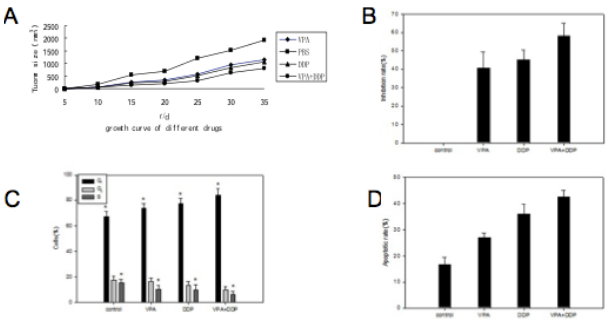

Figure 4. Inhibition Effects After Treatment with Different Drugs in Nude Mice. Human ovarian cancer model transplanted subcutaneously in nude mice was established, and randomly divided into 4 groups. Treatment was initiated intraperitoneally when established tumors of 0.2 cm - 0.3 cm diameter were detected, At the end of the experiment, tumor weights were measured, draw the growth curve of different drugs (as in A). In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. After used with VPA combined DDP, the tumors were the smallest, there was a significant difference among each group (P<0.01). The inhibiting rate of VPA group, DDP group and VPA+DDP group were as follows: 40.7%, 45.3%, 58% (as in B), they were significantly higher than control group (P<0.01). The cell cycle distribution changed in experimental group, the S-phase was decrease (as in C).The apoptotic rate of experimental groups were as follows: (27.05±1.63)%, (35.93±3.89)%, (42.59±2.55)% (as in D), which were significantly higher than control group (16.73±2.82)% (P<0.01)

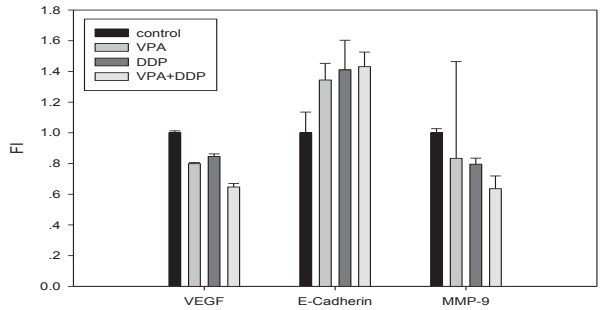

Figure 5. The VEGF, E-cadherin and MMP-9 Protein Level of Human Ovarian Cancer Transplanted Subcutaneously in Nude Mice. The result of FCM indicated that after using VPA, the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, there was a significant difference among each group (P<0.05)

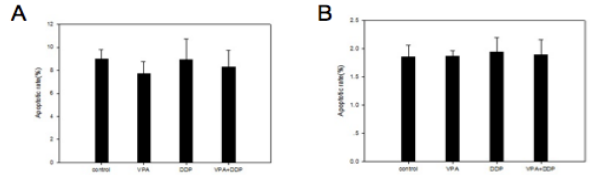

Figure 6. Apoptotic Rates of Liver and Kidney. There were no toxic reactions during the course. The apoptotic rates of the normal organs tissues (A is liver, B is kidney) in nude mice were very low (P>0.05)

that after using VPA, the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, there was a significant difference among each group (P<0.05). There were no toxic reactions during the course. The apoptotic rates of the normal organs tissues in nude mice were very low (P>0.05), as showed in Figure 6. Treated with VPA combined with DDP can enhance anticancer effects.

Discussion

Ovarian cancer turned to be the biggest risk of women's health. Chemotherapy as one of the assisted therapeutic measure once encouraged both patients and gynecologists in the battle against ovarian cancer now facing great challenge due to its serious side effect. With the development of apoptosis and oncology, we began to define neoplasm in a brand new angle.

Valproic acid, a short chain fatty acid, has been used extensively in the clinic for the treatment of a variety of seizure disorders as well as for the treatment of manic depressive bipolar illness. The advantages of VPA include low cost, favorable safety profile, and oral dosing. Recently it has been identified that VPA is an effective inhibitor of HDAC activity at physiologically relevant concentrations. It is also becoming evident that the efficacy of VPA in vivo is in part due to inhibition of cancer cell migration, invasion and angiogenesis (Chou et al., 2011; Wedel et al., 2011; Leiva et al., 2012). This gave us the inspiration to see whether VPA could have the similar effects in ovarian cancer.

In our in vitro experiment, the proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. Our study showed VPA can inhibit the proliferation of cancer cells.

The reasons for this effect is completely obscure but might be related to the specific subset of genetic defects affecting regulation of the cell cycle or apoptosis in cancer cells. Researches have showed HDACIs have cytostatic activity characterized by a G_1 phase cell cycle arrest that is associated with the increased expression of the cyclin-dependent kinase (cdk) inhibitor p21WAF1/CIP1 (Chen et al., 2011). HDACIs result in diminished proliferation due to cell cycle blocks at the G_1 and G_2-M checkpoints, consistent with an association between HDAC activity and cell cycle control genes (Ogryzko et al., 1996). Cell cycle arrest maybe the main mechanism of inhibition effects by VPA (Vallo et al., 2011).

Then we use FCM to determine this mechanism. The cell cycle distribution changed while treated with VPA, decreasing of S-phase cells and increasing of G_1-phase cells, but the changes of the G_2-phase and M-phase were not significant. Diminished proliferation of ovarian cancer cells is the result of a reduction in the S-phase due to cell cycle blocks at the G_1 checkpoint. The percentage of $SubG_1$ was increased in a dose and time dependent fashion and reached differences statistical significance in each group (P<0.05). We have shown that treatment with VPA dramatically and significantly increase the number of apoptotic cells.

The growth and persistence of solid tumors and their dissemination are dependent on angiogenesis and metastasis. It has been demonstrated that long-term application of VPA is necessary to delay tumor cell growth and block metastatic processes (Blaheta et al., 2007;

Osuka et al., 2012). Based on our in vitro and in vivo data, we found that VEGF and MMP-9 were reduced but the expression of E-cadherin was increased after treated with VPA. It demonstrated that VPA can interrupt tumor angiogenesis and metastasis by down regulate the VEGF protein and up-regulate E-cadherin protein and MMP-9 protein.

Primary cytoreductive surgery followed by platinum based chemotherapy (e.g., DDP) is the standard of care for treatment of ovarian cancer. This treatment is associated with high response rates, but the disease is characterized by recurrence and the subsequent development of resistance to chemotherapy. Resistance to chemotherapy has been associated with decreased susceptibility to apoptosis, introducing the possibility that cell death determinants may influence the outcome of treatment (Dive et al., 1993).

The findings have indicated that combinations of a HDACIs and a chemotherapeutic agent could either induce either additive or synergistic cytotoxic effects. Furthermore, HDACIs increase the efficiency of several anticancer drugs that target the DNA (Kim et al., 2003; Hede et al., 2006; Glaser, 2007; Tumber et al., 2007). The study of Lin CT results clearly show that VPA not only exhibits synergistic cytotoxicity with cisplatin in all of the ovarian carcinoma cells tested, but also can resensitize the cells that have acquired resistance to cisplatin. Consistent with the increased cytotoxicity, cotreatment with VPA was shown to upregulate the cisplatin-mediated DNA damage (Lin et al., 2008). VPA is an antiepileptic drug with hypothetically putative neuroprotective effect. Which has protective effects on cisplatin-induced peripheral neuropathy: an in vitro and in vivo study (Rodriguez-Menendez et al., 2008). In our vivo investigation, we assay the anticancer effect of DDP combining with VPA. We found in the group of DDP+VPA, the tumors were the smallest, and the apoptosis rate was higher than the other groups, the strong antiproliferative effect of DDP+VPA on human ovarian cancer model may be caused, in part, by the induction of apoptosis, the prominent cell cycle arrest of malignant cells is likely to account for this effect. We also have shown that treatment with these two agents significantly increases the expression of E-cadherin, reduces the expressions of VEGF and MMP-9. Suggesting again of dramatically tumor suppressor function in response to the inhibition of combination drugs. All of these data demonstrated VPA can enhance the anticancer effect of DDP, revese DDP-resistance.

VPA is a commonly used and effective antiepileptic drug for some patients with epilepsy. The side effects of VPA include gastrointestinal disturbances, elevation of hepatic enzymes, hyperammonemia, neurological disturbances, alopecia, weight gain, pancreatitis and thrombocytopenia (Dreifuss et al., 1988). VPA therapy may cause adverse effects other than renal injuries with hepatotoxicity being well known and developing in 15–30% of patients receiving VPA therapy (Anderson, 2002). In this study, no body weight loss or over toxic effects in the treated animals, which could be related to the drug treatment, was observed. The apoptotic rates of the normal organs tissues in nude mice were very low. Therefore, VPA seemed well tolerated and safe. Still, this conclusion is made with caution since side effects in humans may develop during protracted treatment.

In conclusion, VPA can significantly inhibit the growth of the epithelial ovarian cancer SKOV3 cells in vitro and in vivo; this antitumor activity was not accompanied by any major side effects. The key mechanism of cell growth inhibition by VPA in tumor growth may relate to the induction of cell cycle arrest and apoptosis. HDACIs show selective cytotoxicity against tumor cells with little toxicity for normal cells.

The basis for the selective toxicity of HDACIs remains unclear but it could be related to the significant HDAC over-expression observed in cancer cells. Treated with VPA combined with DDP can enhance anticancer effects. VPA can decrease the expression of VEGF and MMP-9 protein, meanwhile, it can increase the expression of E-cadherin protein. So VPA may inhibit the growth of ovarian cancer by inhibiting the neoplasms angiogenesis and metastasis. This study suggests that VPA could be a perspective and novel attractive agent for treatment of ovarian cancer either as a single agent or complementary and in combination with other drugs. More clinical studies should be designed to find a safe does with the best effects and the lowest toxic reaction.

Acknowledgements

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