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VALPROIC ACID INDUCES APOPTOSIS AND INCREASES CXCR7 EXPRESSION IN EPITHELIAL OVARIAN CANCER CELL LINE SKOV-3.

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Abstract

Background: The chemokine receptor, CXCR7 is described to play a

biologically relevant role in tumor growth and spread. Recently, it was reported that CXCR7 overexpression is associated with an *unfavorable prognosis* and metastatis of epithelial ovarian cancer (EOC). Aware that, several reports indicated that Histone deacetylases (HDACs) regulate the expression and activity of many proteins involved in both cancer initiation and progression, the aim of this work, was to study the effect of the HDAC inhibitor valproic acid (VPA) on the *expression* of *CXCR7* as well as its impact on survival function in the epithelial ovarian cell line (SKOV-3).

Methods: cells were cultured with varying concentrations of VPA (1, 2, 3, 4, 5 and 10 mM) for different durations (0, 12 h, 24 h and 48 h). Cell survival was assessed by Neutral red assay and by colony counting which being stained with crystal violet. CXCR7 expression was determined at mRNA level using *quantitative real-time* PCR (qRT-PCR) or at the protein level using western blotting.

Results: VPA reduces cell survival of SKOV-3 cancer cells. The inhibition effect of VPA was dose and time-dependent. Exposure to VPA at concentrations above 2 mM at 24 h resulted in an increase expression of CXCR7 at both the mRNA and protein levels.

Conclusion: These observations provide, for the first time, a better insight into the epigenetic mechanisms involved in regulating CXCR7 expression in EOC and will open new avenues for evaluating drugs that specifically stimulate the apoptosis of EOC with minimal unwanted side effects.

Keywords: Valproic acid, chemokine receptors, epigenetic regulation, apoptosis, epithelial ovarian cancer cell line SKOV-3.

Introduction

Epithelial ovarian cancer (EOC) is the sixth most commonly diagnosed cancer among women in the word and the second most common gynecological cancer [1]. EOC is the fifth leading cause of death among women in developed countries due to the continued inability to detect early stage (I) disease [2]. Since, most women with EOC have disseminated disease (i.e. expansion in the abdominal cavity) at the time of diagnosis, chemotherapy is needed to reduce the chance of recurrence. However, many patients gradually develop resistance to the drugs associated with their toxic effects. Hence, identifying some new agents that have better anticancer effect without being toxics is highly appreciated.

Accumulating evidences suggest that chemokines can affect tumour growth either directly, by acting as autocrine and/or paracrine growth factors or indirectly by promoting or inhibiting angiogenesis and inducing tumour metastasis. The best-characterized chemokine in this regard is CXCL12/SDF-1 which acts as an autocrine factor and proangiogenic inducer

of several cancers [3]. For many years it was thought that CXCR4 is the sole receptor for this chemokine. However, in 2005, a second chemokine receptor, CXCR7, was identified, which binds to CXCL12 with high affinity [4,5]. CXCR7 also binds to a second chemokine, CXCL11/I-TAC, one of the ligands for CXCR3 [4]. The chemokine CXCL12 and its receptors, CXCR4 and CXCR7, have been implicated in cancer progression and metastasis [6-9]. Recent studies show an increase in the expression of CXCL12 and CXCR4/CXCR7 in different malignancies, including epithelial ovarian cancer and ovarian cancer cell lines [10-12]. Several studies have also reported that treating cells with histone deacetylase inhibitors (HDI) treatment like valproic acid (VPA) results in an up-regulated expression of many genes [13].

HDIs are potential anticancer agents because of their abilities to alter gene expression, induce apoptosis and growth arrest of tumor cells and stimulate differentiation [12]. It has been demonstrated that VPA could significantly inhibit the growth of various cancer cells including the epithelial ovarian cancer cells, *in vivo*, without toxic side effects [14, 15]. In this study we invetigated the effect of VPA on the viability of EOC cells derived from the EOC cell line, SKOV-3. Aware that only little is known, so far, about the mechanisms involved in regulating CXCR7 expression. However, a recent study reported that the histone deacetylase inhibitor (HDI) valproic acid (VPA) increases CXCR7 and CXCR4 expression in Mesenchymal stromal cells derived from cord blood [16]. For this reason, we investigated whether VPA enhances the expression of CXCR7 in the EOC cell line, SKOV-3.

Materials and Methods

Cells and Cell Culture

Human epithelial ovarian carcinoma cell line SKOV3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 medium (Sigma Chemical Company) supplemented with 0.1mg/ ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS). SKOV-3 was maintained at 37 ° C, under an atmosphere containing 5% CO_2.

Neutral Red Analysis

Cell viability was performed using Neutral Red assay based on the initial protocol as described earlier [17, 18]. Neutral Red, a chromogenic dye, is an indicator of lysosomal activity. Live cells demonstrate a chromogenic change with Neutral Red that is detected spectrophotometrically. Briefly, cells were detached from the tissue culture flask with 2 ml of trypsin solution. The cell pellet was obtained by

centrifugation at 1.000 rpm for 5 minutes. The density of the viable cells was counted by the trypan blue exclusion in a haemocytometer. Cells were then plated in 96-well microtiter plate, at a concentration of 8×10^3 cells/well and incubated in a humidified 37°C, 5% CO_2 incubator that allows the cells to adhere. After 24 h, the cells were treated with six different concentrations of Valproic acid (VPA): 1, 2, 3, 4, 5 and 10 mM each being tested in three replicates. VPA were purchased from Sigma-Aldrich. The plates were incubated for 24 and 48 h at 37°C in a 5% CO_2 incubator. The untreated cells were regarded as a negative control, whilst cells incubated only with ethanol (0.5%, v/v) were used as a vehicle control. No effect due to the ethanol was observed. At 24 and 48 h, the old medium was replaced with 100 μl of fresh medium containing 40 μg/ml neutral red and incubated for 3 h. This is to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. Then, the media was discarded and cells were washed twice with 100 μl of 1X PBS. The intracellular accumulation of neutral red dye was extracted in 200 μl of a 50% ethanol-1% acetic acid lysing solution.

The optical density (OD) of the eluted dye was read at 490 nm using a microplate reader. The experiments were conducted in triplicates. The percentage of inhibition of each of the test samples was calculated according to the following formula using the OD values obtained: **Percentage of inhibition (%) = (OD control − OD sample)/OD control × 100.**

Focus formation assay- Staining of colonies

One hundred cells/well were seeded in 6-well plates and incubated at 37°C, 5% CO_2 for 24 hours. Following a 24-hour incubation, various concentrations of VPA were added for different durations (0, 12 h, 24 h and 48 h). After 10 days, the content of each well was removed and the colonies were washed twice with 500 μL of sterilized water, to remove cells weakly adhered. The attached colonies was quantified using crystal violet staining at 5mg/ml in 25% methanol [19-21]. Briefly, 500 μL of a solution sodium acetate/formalin 2% were distributed in each well, in order to fix the adhered cells, and left for 15 min. After this time, the solution sodium acetate/formalin 2% was removed and 500 μL of crystal violet were added to each well. After approximately 20-30 min, the stain was removed by washing the wells three times under cold water and the plates were left at room temperature to dry before colony counting. The colony formation are counted manually with naked eye.

Total RNA extraction

2×10^5 cells/well were seeded in 6-well plates and incubated at 37°C, 5% CO_2 for 24 hours. Following a 24-hour incubation, various concentrations of

VPA (1, 2, 3, 4 and 5 mM) were added for different durations (0, 12 h, 24 h and 48 h). Cell line was homogenized using *TriPure* reagent (Roche, USA) following the manufacturer's instructions. After complete dissociation of nucleoprotein complexes, phase separation was achieved with chloroform and centrifugation. The precipitated RNA from the aqueous phase was washed with 75% ethanol. The RNA was dried and dissolved in RNase-free water. The amount and quality of the extracted RNA were assessed by spectrophotometry using NanoDrop.

Western blot analysis

SKOV-3 cells were seeded at 0.7 x 10^6 per 25 cm^2 plate overnight. The next day VPA was added to cells at various concentrations (2.5, 5, 10 mM) for 24 h. Total cell lysate was prepared using the lysis buffer RIPA (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 50 mM Tris, pH 8, 20 mM, NaF, 2 mM EGTA, 0.5% levamisole, 1 mM NaVO4) (Roche). Protein concentration was determined using Bradford method (Bio-Rad). The protein complexes were separated on 10% gradient SDS-PAGE gel. Membrane was incubated with CXCR7 (Sigma) and GAPDH (Sigma) primary antibodies. Proteins were detected using ECL detection kit (Bio-Rad) according to the manufacturer's protocol. The immune complexes were visualized with the use of the ECL Plus kit (Amersham) according to the manufacturer's protocol. The autoradiographs obtained were scanned and the band intensity quantified utilizing TotalLab software (Nonlinear Dynamics, Newcastle on Tyne, UK). All bands were normalized with respect to GAPDH.

Quantitative RT-PCR studies

Total RNA was isolated from SKOV-3 cells treated with 1, 2, 3, 4 and 5 mM VPA for different durations (0, 12 h, 24 h and 48 h). First strand cDNA was synthesized with 1 μg of total RNA using kit iScript^{TM} (Bio-Rad). We amplified the resulting cDNA by real-time PCR on a Bio- Rad Mini Opticon real-time PCR system, with the IQ SYBR green supermix kit (Bio-Rad). The data were analyzed with Opticon Monitor 3 software (Bio-Rad). EF-1 alpha was used to normalize samples. The primers used were as follows: CXCR7 (forward: 55-'TGCATCTCTTCGACTACTCAGA- 3', reverse: 5' GGCATGTTGGGACACATCAC-3') and EF-1 alpha (forward: 5'- CTGAACCATCCAGGCCAAAT- 3', reverse: 5'- GCCGTGTGGCAATCCAAT- 3').

Statistical analysis

All results were presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5

(GraphPad Software Inc., CA, USA). Two-way ANOVA was used to calculate *P* values (p). Groups that are significantly different from control are indicated in the figures as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

VPA reduces cell survival of SKOV-3 cancer cells

Previous studies suggest that VPA is effective on EOC cell lines [14]; therefore we examined its effect on SKOV-3 cells [14, 22]. First we tested whether VPA could have any effect on SKOV-3 cell growth.

Upon using the Neutral Red Cytotoxicity/ Viability Assay to measure the viability of the SKOV-3 cells, an evaluation of the antiproliferative activity of various concentrations of VPA (1, 2, 3, 4, 5 and 10 mM) was performed after 24 and 48 hours of treating this cancerous cell line. The neutral red assay shows that the VPA has no significant antiproliferative effect after 24 h and 48 h of treatment at a concentration of 1 or 2 mM. The percentage of survival inhibition was increased by 13-fold after 24 h and by 23-fold after 48 h of treatment with 3 mM VPA (Figures 1and 2). The percentage of survival inhibition reached 19, 28 and 44-fold increase, at the concentration of 4, 5 and 10 mM, respectively after 24 h of VPA treatment (Figure 1 and 2A). VPA also significantly increased the percentage of survival inhibition after 48 h by 45-fold and 60-fold at the concentration of 4 and 5 mM of VPA, respectively (Figure 1 and 2B). The highest antiproliferative activity ($p<0.001$) on the SKOV-3 cell line was 73-fold increase at 10 mM VPA after 48 h (Figure 1 and 2B). The percentage of survival inhibition was compared to untreated cells. The antiproliferative activity was further confirmed by another technique based on colony counting which being stained with crystal violet.

SKOV-3 cells did not show significant effects in colony formation after 12 h of VPA treatment with 1, 2, 3, 4 and 5 mM (Figures 3A and 4). When treated with 3, 4 and 5 mM VPA at 24 h, SKOV-3 cells showed significant decrease in colony formation ($p<0.001$) by 73-fold, 74-fold and 89-fold, respectively (Figures 3B and 4). When treated with 2, 3 and 4 mM VPA at 48 h, SKOV-3 cells showed significant decrease in colony formation by 79-fold, 82-fold and 83-fold, respectively (Figures 3C and 4). The highest inhibition activity of SKOV-3 colony formation ($p<0.001$) is 95-fold with 5 mM VPA at 48 h (Figure 3C and 4). The inhibition of colony formation was compared to untreated cells. Taken together these data suggest that this drug inhibits cell viability of SKOV-3 in a dose- and time- dependent manner (Figures 1-4).

VPA Increases the Gene and Protein Expression of CXCR7 in SKOV-3

Next, we tested the effect of VPA on CXCR7 mRNA/protein

expression. Western blot assay revealed that CXCR7 protein was induced with 2.5 mM TSA at 24 h (Figure 5). The induction of CXCR7 mRNA was further confirmed with quantitative RT-PCR. The result of quantitative RT-PCR shows that the VPA has no significant effect in CXCR7 mRNA expression after 24 and 48 h of treatment at 2, 3, 4 and 5 mM (Figure 6). Likewise, 1 mM VPA has no significant effect in CXCR7 mRNA expression after 48 h of treatment (Figure 6). Four-fold induction of CXCR7 mRNA at 1mM VPA was observed after 12 h treatment (Figure 6). We observed nine-fold induction of CXCR7 mRNA at 3 mM VPA, two-fold increase induction at 4 mM VPA and seven-fold increase induction at 5 mM after 48 h treatment (Figure 6), suggesting that VPA activates CXCR7 transcription. These results are consistent with the induction of CXCR7 by VPA in blood mesenchymal stromal cells [16, 23].

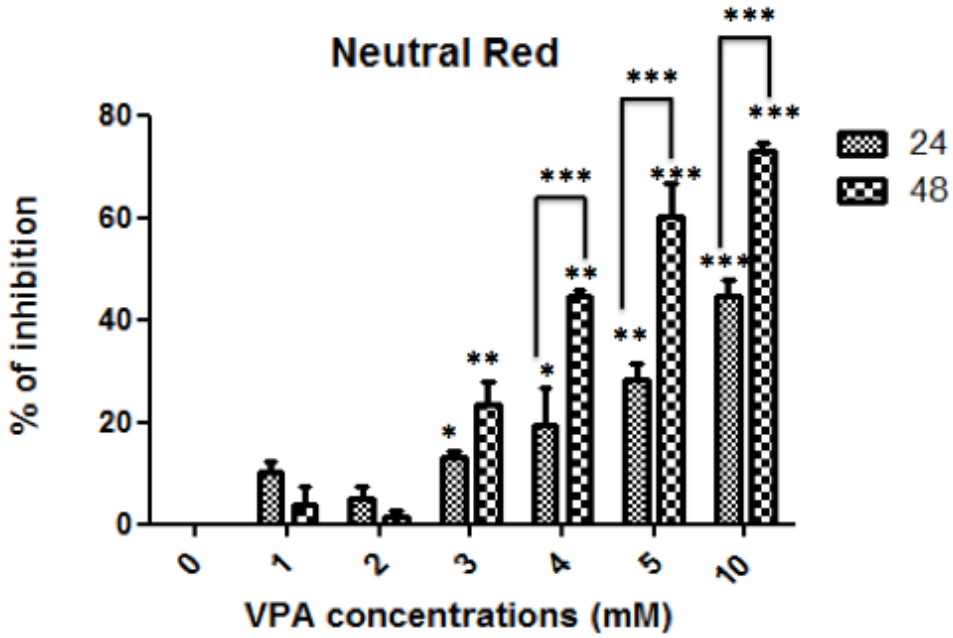

Figure 1: Valproic acid (VPA) affect the survival of the SKOV3 cells.

The SKOV3 cells were incubated with different concentrations of VPA (1, 2, 3.4, 5 and 10 mM) for 24 and 48 hours. Cell viability was estimated by the neutral red (NR) test. The harvested cells are exposed to the dye (NR). The cells are lysed and the optical density (OD) is measured at 490 nm. % Inhibition = OD (optical density) of non-treated cells - OD of treated cells / OD of non-treated cells × 100. Results represent % inhibtion of cell survival of SKOV3 cells compared to control. Experiments were done in triplicates and results represent the mean ± SEM of three independent

experiments. * p <0.05; ** P <0.01; *** P <0.001 (Two-way ANOVA).

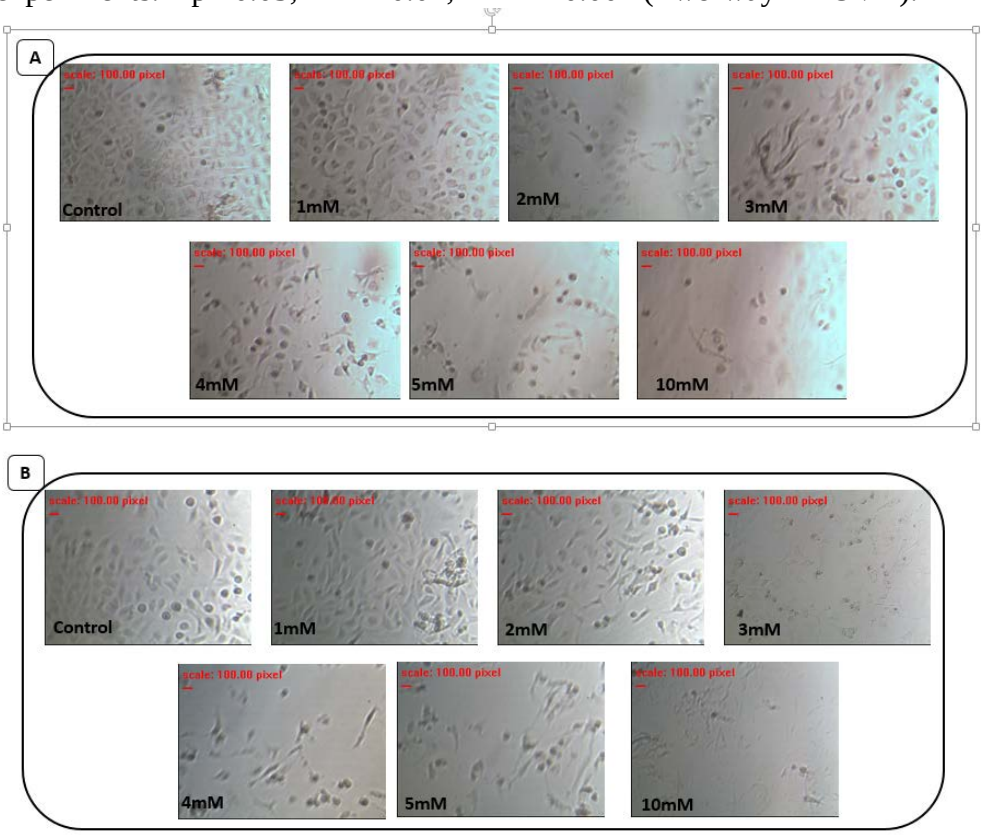

Figure 2: Microscopic view of SKOV-3 cells after 24 (A) and 48 hours (B) incubation with increasing concentrations (1, 2, 3, 4, 5 and 10 mM) of VPA. The results presented are from one experiment representative of three carried out, and were photographed with a microscope (× 40).

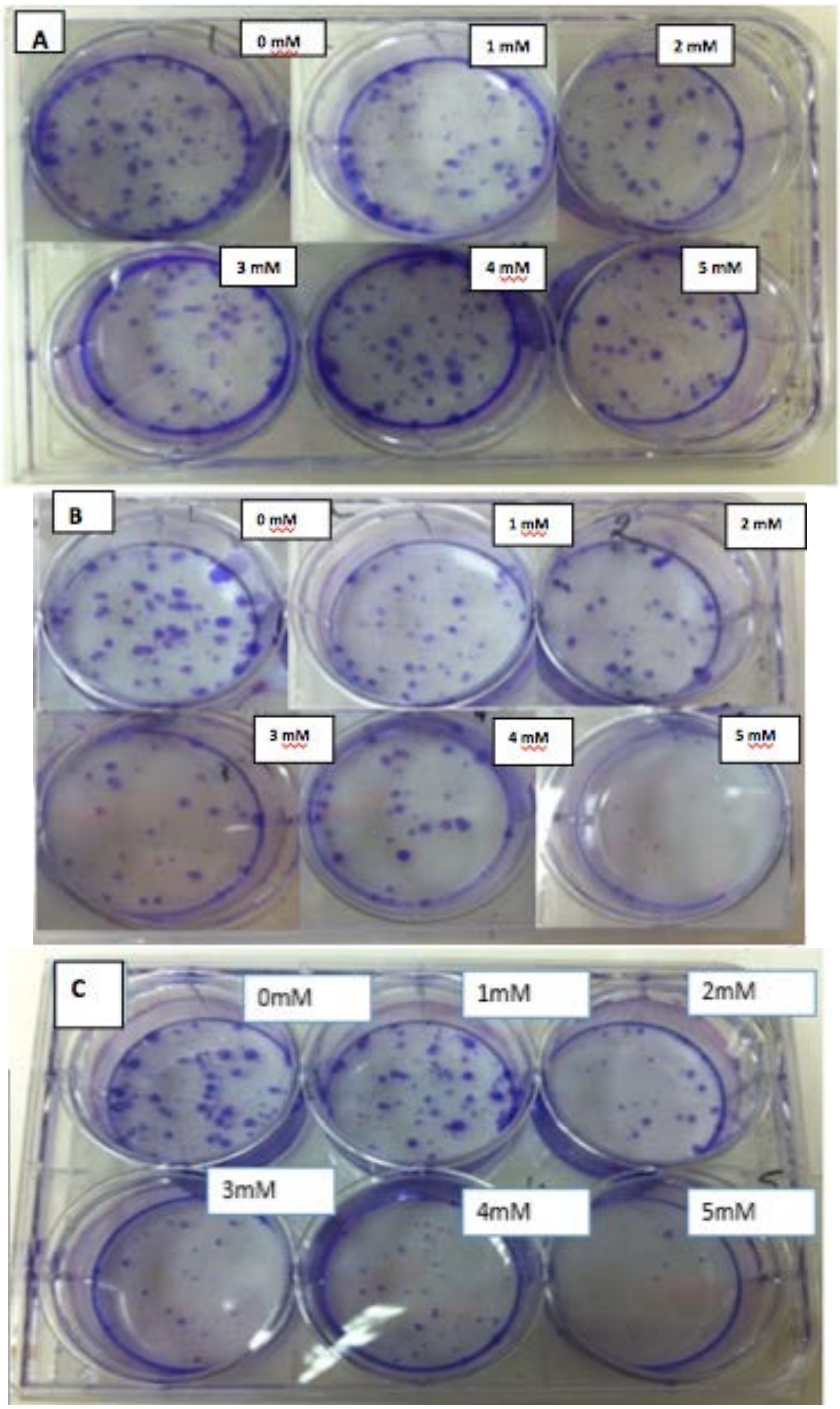

Figure 3: Cell colonies (SKOV3) formed after VPA treatment. SKOV-3 was treated with 0 (control), 1, 2, 3, 4 or 5 mM of VPA for 12 (A), 24 (B) or 48 h (C). After 10 days, colonies were stained with crystal violet. The colony formation are counted manually with naked eye.

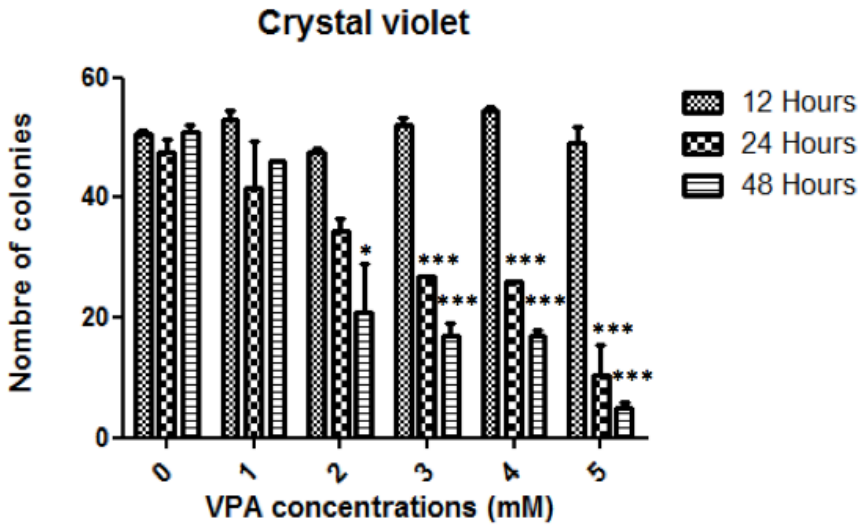

Figure 4: Treatment with valproic acid (VPA) affects the clonogenic survival of the SKOV3 cells. SKOV3 cells were incubated with different concentrations of VPA (1, 2, 3.4 and 5 mM) for 12, 24 and 48 hours. Clonogenic survival was made by counting cell colonies. The colony formation on the 6-well plates seeded with 100 cells after 10 days, are counted manually with naked eye after staining with crystal violet. Results represent the number of clones of SKOV3 cells compared to control. Error bars represent standard error of the mean (SEM). * p <0.05; ** P <0.01; *** P <0.001 (Two-way ANOVA).

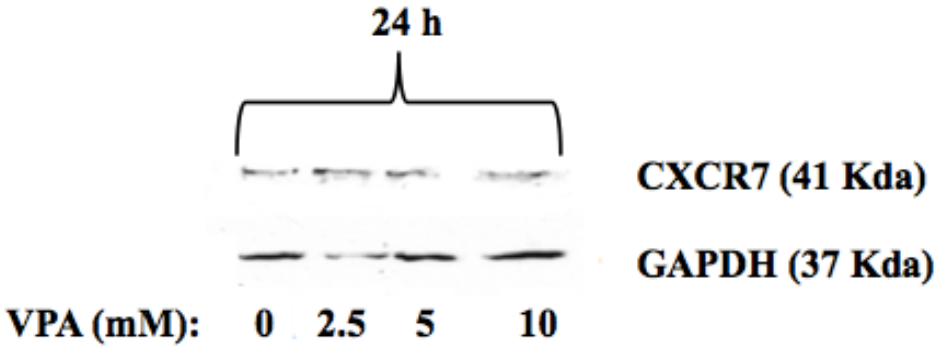

Figure 5: Effect of VPA on the protein expression of CXCR7. Whole cell lysate of SKOV-3 was prepared after 24 hours of exposure to VPA (2.5, 5 and 10 mM). The proteins were extracted in RIPA as described in the Materials and methods. 100 µg of each extraction were loaded into separate lanes of a 10% SDS-polyacrylamide gel electrophoresis and after transfer to nitrocellulose membranes were probed with antibodies against CXCR7. GAPDH was used as a control to ensure equal loading.

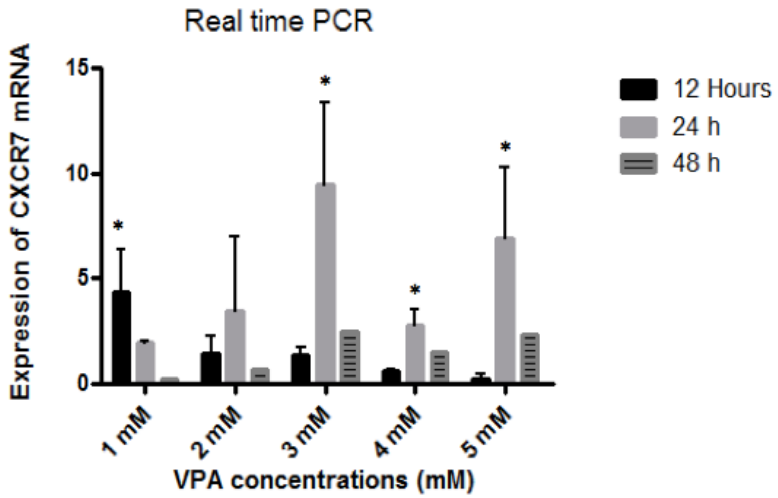

Figure 6: Effect of VPA on the expression of CXCR7 mRNA. CXCR7 mRNA levels were quantified by real-time PCR and are expressed as CXCR7 content normalized to that of EF-1 alpha. The cells were treated or not with 1, 2, 3, 4 and 5 mM VPA for 12, 24, 48 hours. Error bars represent standard error of the mean (SEM). * $p < 0.05$ (Two-way ANOVA).

Discussion

Ovarian cancer is the fifth most deadly cancer in women [2]. Chemotherapy is effective against advanced ovarian cancer, but this treatment must be adjusted because of its serious side effects. Therefore, identifying new treatments with better anticancer effect without toxicities are highly needed. With the development of agents with epigenetic effects involved in transcriptional regulation, cell growth, apoptosis, differentiation and proliferation, researchers started to seriously study these agents as a potential new cancer treatment [12].

Deregulated activity of histone deacetylases (HDAC) is usually observed in many cancer types. Treatment with HDAC inhibitors (HDACIs) can suppress cell proliferation and differentiation and induce apoptosis of ovarian cancer cells suggesting that HDACs are required to maintain cancer cell survival [24-26]. However, the role of HDAC inhibitors in regulating gene transcription that support tumor properties is poorly understood. Some studies show that the anti-proliferative activity of HDACIs is a result of cell cycle arrest in G1 that is associated with the increased expression of the cyclin-dependent kinase (cdk) inhibitor $p21^{WAF1/CIP1}$ [27-28]. Among different HDACIs, valproic acid (VPA) is most commonly studied for its effect on cell growth arrest [29].

Valproic acid has been widely used in the treatment of seizure disorders and for the treatment of bipolar depressive and epileptic illness [30]. A new study shows that VPA, even at low concentration, serve as an

effective inhibitor of HDACs. VPA can interrupt tumor angiogenesis and metastasis of EOC by down regulating the VEGF protein and up-regulating E-cadherin protein and MMP-9 protein [14]. In *vivo*, the role of VPA becomes very important especially in the inhibition of cancer cell migration, angiogenesis and invasion [31-33]. A recent study shows that VPA can significantly inhibit the growth of the epithelial ovarian cancer cells in *vivo*; this antitumor activity was not accompanied by any major side effects [14]. Such observation enabled us to check whether VPA could have similar effects on the survival of EOC cell lines, SKOV-3.

The standard treatment for ovarian cancer is surgery followed by platinum based chemotherapy (e.g., DDP or cisplatin). This treatment is associated with high response rates. However, the disease is characterized by recurrence and the subsequent development of resistance to chemotherapy [34]. Some studies showed that HDACIs increase the efficiency of several anticancer drugs that target the DNA [35-37]. Interestingly, one report [38] indicated that VPA exhibits synergistic cytotoxicity with cisplatin and can also resensitize the cells that have acquired resistance to cisplatin in all of the ovarian carcinoma cells tested. The cotreatment with VPA was shown to upregulate the cisplatin-mediated DNA damage [38].

In this study we found that the viability of SKOV3 cells was significantly inhibited by VPA in a dose and time dependent. Our study shows that the VPA has an antiproliferative effect on cancer cells. Because so little is known about the anticancer mechanisms triggered by VPA, future studies are necessary to better understand the mechanism of cell death induction by VPA.

Chemokines are important in the pathogenesis of several tumors. Early studies demonstrated an evident correlation between the expression of chemokine receptors and the tumor growth, angiogenesis, and metastasis in various human malignant tumors. The chemokine SDF-1 and its receptors, CXCR4 and CXCR7, are implicated in cancer progression, angiogenesis and metastasis [6-9]. Recent studies reported an increase in the expression of CXCL12 and CXCR4/CXCR7 in different malignancies, including epithelial ovarian cancer and ovarian cancer cell lines [10-12]. Several studies have also reported an up-regulated expression of many genes after treating cells with a histone deacetylase inhibitor (HDI) like VPA [13]. However, how these HDAC inhibitors affect the transcriptional network that sustain tumor properties is still poorly understood. A recent study reported that valproic acid (VPA) increases CXCR7 and CXCR4 expression in Mesenchymal stromal cells derived from cord blood [16]. For this reason, we decided to study the effect of valproic acid on CXCR7 gene expression in the EOC cell line, SKOV-3. Our results revealed that the expression levels of CXCR7 protein /mRNA were significantly enhanced in the presence of 2.5 to 3 mM

VPA for 24 h. This study suggests that VPA could be a potentail and novel attractive agent for treatment of epithelial ovarian cancer.

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Abrevations

Epithelial ovarian cancer (EOC), histone deacetylases (HDACs), valproic acid (VPA), histone deacetylase inhibitor (HDI), fetal bovine serum (FBS), optical density (OD), standard error of the mean (SEM), cyclin-dependent kinase (cdk).

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