

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

Salam Nasreddine

Fatima Salameh

Doctoral School of Science and Technology,
Research Platform for Environmental Science (PRASE),
Faculty of Sciences, Lebanese University, Lebanon

Khodor Haidar Hassan

Department of Physical Therapy, Faculty of Public Health-Section I,
Lebanese University, Hadath, Lebanon

Ahmad Daher

Mohamad Nasser

Hassan Rammal

Akram Hijazi

Doctoral School of Science and Technology,
Research Platform for Environmental Science (PRASE),
Faculty of Sciences, Lebanese University, Lebanon

Alissar Cheaib

Department of Biology, Faculty of Sciences-Section IV,
Lebanese University, Bekaa, Lebanon

Ola Hadadeh

Department of Biology, Faculty of Sciences,
Lebanese University, Hadath, Lebanon

Hussein Fayyad-Kazan

Mohamad Mortada

Bassam Badran

Doctoral School of Science and Technology,
Research Platform for Environmental Science (PRASE),
Faculty of Sciences, Lebanese University, Lebanon

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 metastasis 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 world 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 toxic 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].

HDI 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 investigated 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% CO₂.

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% CO₂ 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% CO₂ 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% CO₂ 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% CO₂ 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 0.7×10^6 per 25 cm² 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 μ g of total RNA using kit iScriptTM (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 \pm 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 1 and 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].

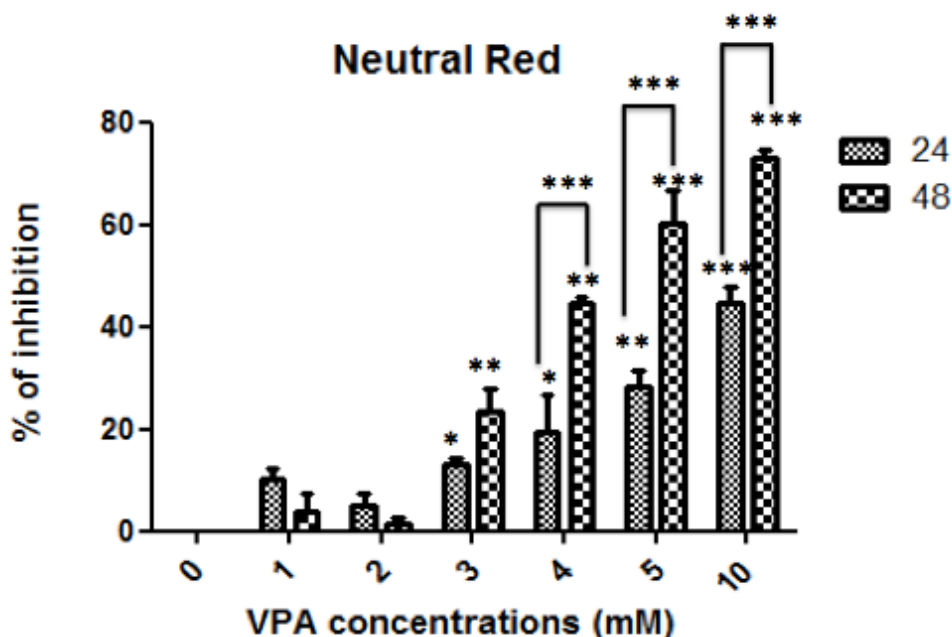


Figure 1: Valproic acid (VPA) affect the survival of the SKOV3 cells.

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 = $\frac{OD(\text{optical density}) \text{ of non-treated cells} - OD \text{ of treated cells}}{OD \text{ of non-treated cells}} \times 100$. Results represent % inhibition of cell survival of SKOV3 cells compared to control. Experiments were done in triplicates and results represent the mean \pm SEM of three independent

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

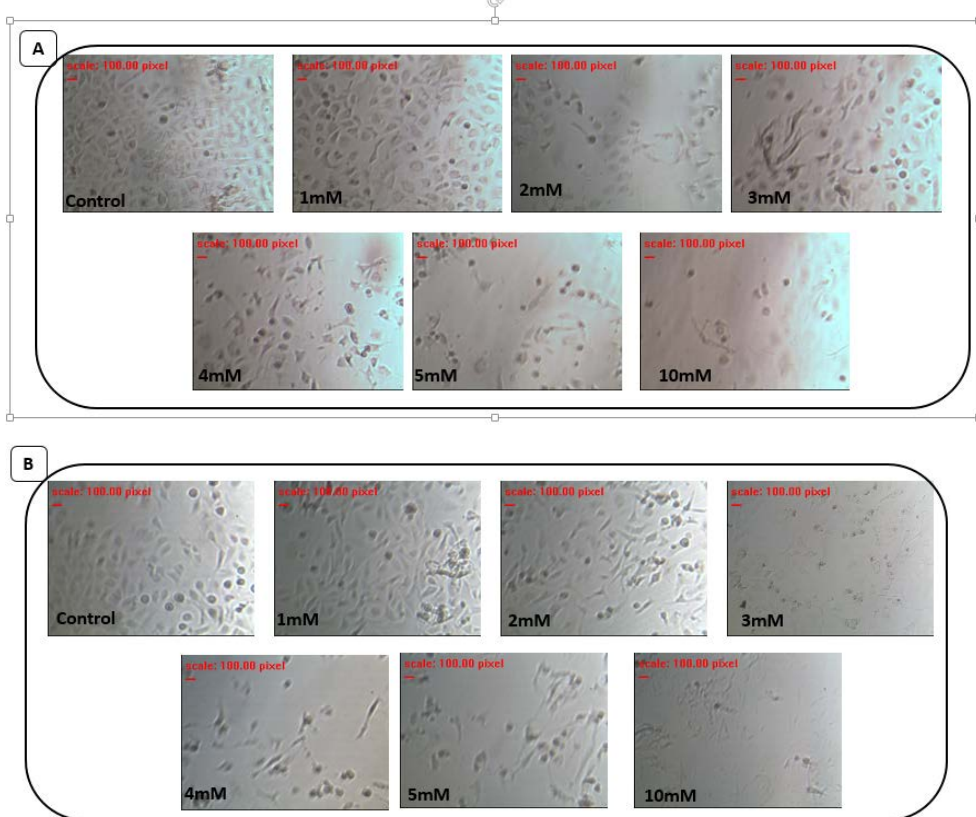


Figure 2: Microscopic view of SKOV-3 cells after 24 (A) and 48 hours (B) incubation with increasing concentrations (1, 2, 3, 4, 5 and 10 mM) of VPA. The results presented are from one experiment representative of three carried out, and were photographed with a microscope ($\times 40$).

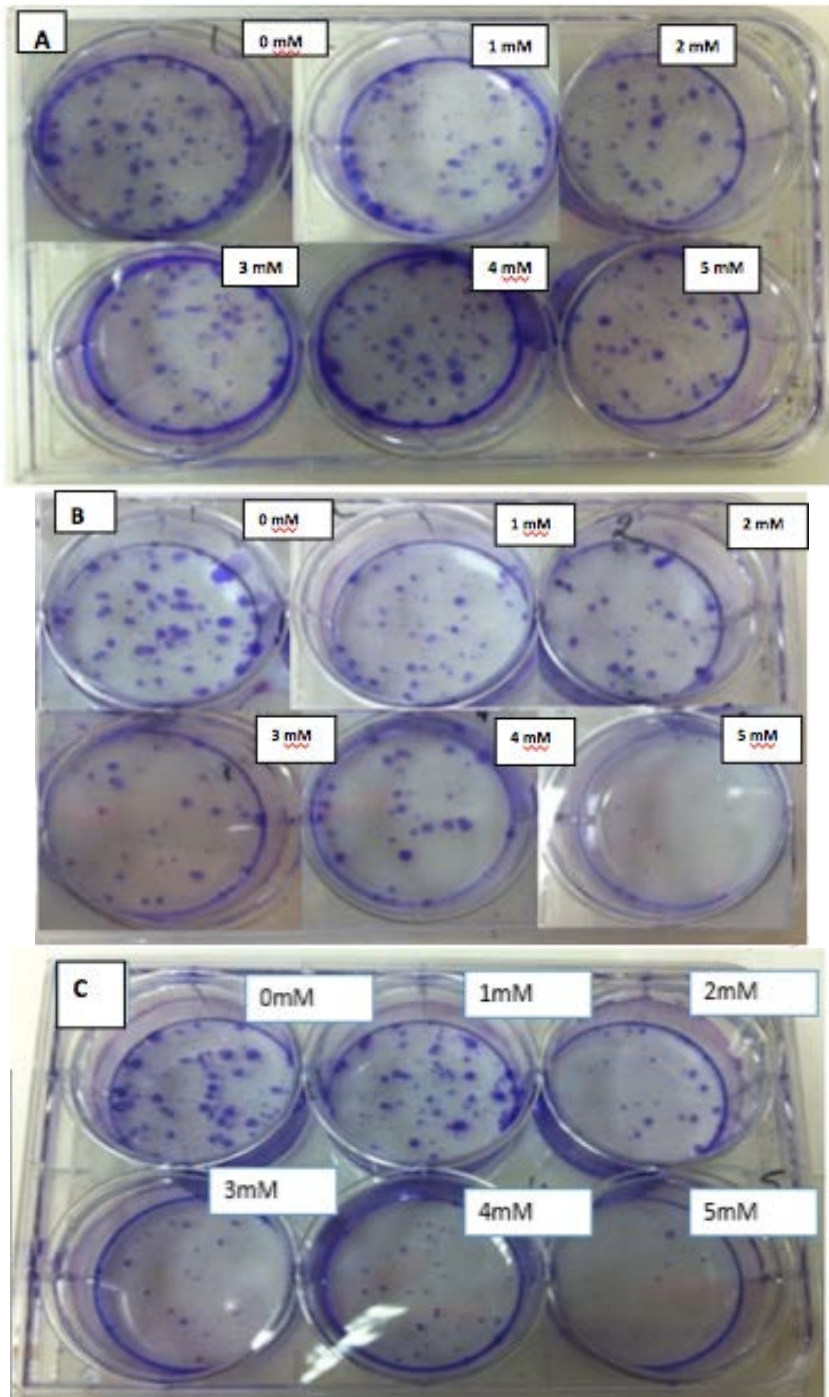


Figure 3: Cell colonies (SKOV3) formed after VPA treatment. SKOV-3 was treated with 0 (control), 1, 2, 3, 4 or 5 mM of VPA for 12 (A), 24 (B) or 48 h (C). After 10 days, colonies were stained with crystal violet. The colony formation are counted manually with naked eye.

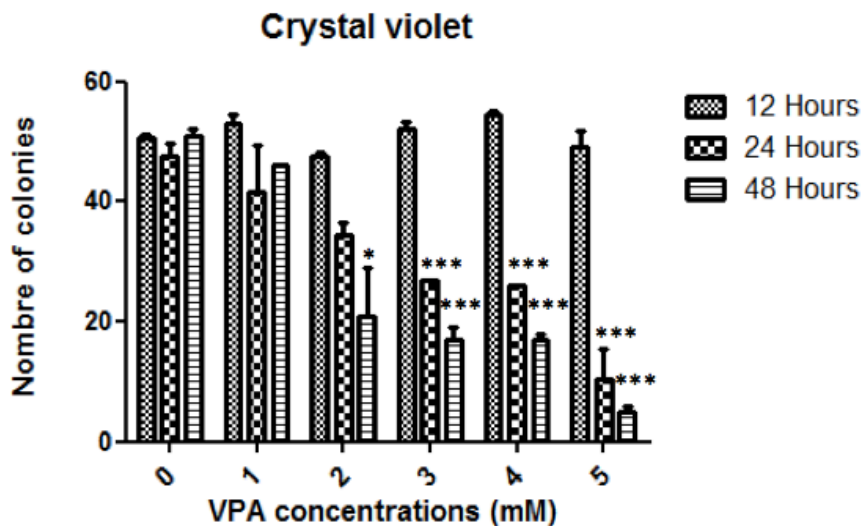


Figure 4: Treatment with valproic acid (VPA) affects the clonogenic survival of the SKOV3 cells. SKOV3 cells were incubated with different concentrations of VPA (1, 2, 3.4 and 5 mM) for 12, 24 and 48 hours. Clonogenic survival was made by counting cell colonies. The colony formation on the 6-well plates 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).

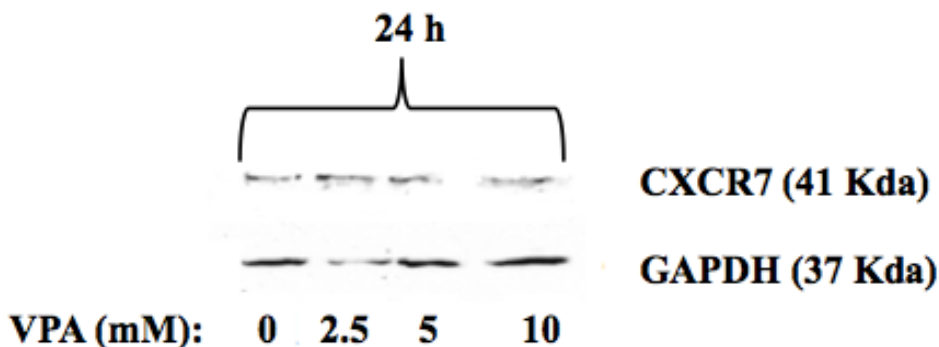


Figure 5: Effect of VPA on the protein expression of CXCR7. Whole cell lysate of SKOV-3 was prepared after 24 hours of exposure to VPA (2.5, 5 and 10 mM). The proteins were extracted in RIPA as described in the Materials and methods. 100 µg of each extraction were loaded into separate lanes of a 10% 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.

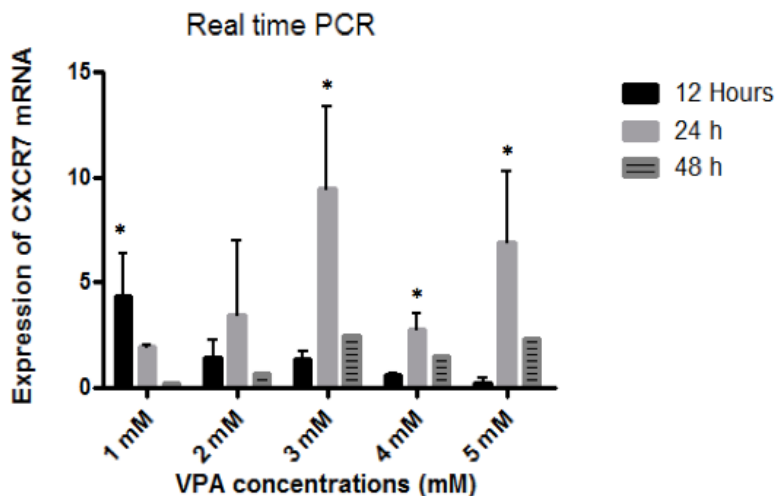


Figure 6: Effect of VPA on the expression of CXCR7 mRNA. CXCR7 mRNA levels were quantified by real-time PCR and are expressed as CXCR7 content normalized to that of EF-1 alpha. The cells were treated or not with 1, 2, 3, 4 and 5 mM VPA for 12, 24, 48 hours. Error bars represent standard error of the 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 *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 HDACi 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 potential and novel attractive agent for treatment of epithelial ovarian cancer.

Acknowledgement

This work was supported by Central Administration of the Lebanese University. We thank Dr. Karl BALABANIAN (UMR 996, Clamart, France) for providing us with the EOC cell lines.

Abreviations

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).

References:

- Permuth-Wey, J., & Sellers, T. (2008). Epidemiology of ovarian cancer. *Methods in Molecular Biology*, 413-37.
- Jemal, A., Siegel, R. et al. (2008). Cancer Statistics, 2008. *CA: A Cancer Journal for Clinicians*, 71-96.
- Juarez, J., Bendall, L. et al. (2004). Chemokines and their Receptors as Therapeutic Targets: The Role of the SDF-1 / CXCR4 Axis. *Current Pharmaceutical Design*, 1245-1259.
- Burns, J., Summers, B. et al. (2006). A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine*, 2201-2213.
- Balabanian, K., Lagane, B. et al. (2005). The Chemokine SDF-1/CXCL12 Binds to and Signals through the Orphan Receptor RDC1 in T Lymphocytes. *Journal of Biological Chemistry*, 35760-35766.
- Scotton, C., Wilson, J. et al. (2002). Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Research*, 5930-5938.
- Kryczek, I., Wei, S. et al. (2006). Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *AJP: Cell Physiology*, C987-C995.
- Tsutsumi, H., Tanaka, T. et al. (2007). Therapeutic potential of the chemokine receptor CXCR4 antagonists as multifunctional agents. *Biopolymers*, 279-289.
- Infantino, S., Moepps, B. et al. (2006). Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. *Journal of Immunology*, 2197-207.
- Furuya, M., Suyama, T. et al. (2007). Up-regulation of CXC chemokines and their receptors: Implications for proinflammatory microenvironments of ovarian carcinomas and endometriosis. *Human Pathology*, 1676-1687.

- Broxmeyer, H., & Orschell, C. (2005). Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *Journal of Experimental Medicine*, 1307-1318.
- Zhang, T., Somasundaram, R. et al. (2005). CXC chemokine ligand 12 (stromal cell-derived factor 1 alpha) and CXCR4-dependent migration of CTLs toward melanoma cells in organotypic culture. *Journal of Immunology*, 5856-5863.
- Glaser, K., Staver, M. et al. (2013). Gene Expression Profiling of Multiple Histone Deacetylase (HDAC) Inhibitors: Defining a Common Gene Set Produced by HDAC Inhibition in T24 and MDA Carcinoma Cell Lines. *Molecular Cancer Therapeutics*, 151-63.
- Shan, Z., Feng-Nian, R. et al. (2012). Effects of Valproic Acid on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer in Vitro and in Vivo. *Asian Pacific Journal of Cancer Prevention*, 3977-3982.
- Minucci, S., & Pelicci, P. (2006). Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nature Reviews Cancer*, 38-51.
- Marquez-Curtis, L., Qiu, Y. et al. (2014). Migration, proliferation, and differentiation of cord blood mesenchymal stromal cells treated with histone deacetylase inhibitor valproic Acid. *Stem Cells International*.
- Borenfreund, E., & Puerner, J. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Letters*, 119-124.
- Malek, S., Lee, G. et al. (2011). Phytochemical and Cytotoxic Investigations of Curcuma mangga Rhizomes. *Molecules*, 4539-4548.
- Cho, J., Cho, C. et al. (2012). Crude aqueous extracts of *Pluchea indica* (L.) Less. inhibit proliferation and migration of cancer cells through induction of p53-dependent cell death. *BMC Complementary and Alternative Medicine*, 265-265.
- Sá, N., Cavalcante, T. et al. (2011). Antimicrobial and antibiofilm action of Casbane Diterpene from *Croton nepetaefolius* against oral bacteria. *Archives of Oral Biology*, 550-555.
- Barber, P., Vojnovic, B., Kelly, J., Mayes, C., Boulton, P., Woodcock, M., & Joiner, M. (2001). Automated counting of mammalian cell colonies. *Physics in Medicine and Biology*, 63-76.
- Kwiecińska, P., Taubøll, E. et al. (2012). Comparison of the effects of valproic acid and levetiracetam on apoptosis in the human ovarian cancer cell line OVCAR-3. *Pharmacol Rep*, 603-14.
- Hasanzadeh-Moghadam, M., Edalatmanesh Mohamad, A. et al. (2014). Comparative Analysis of Expression of Chemokine Receptors CXCR4, CXCR6, CCR1 and CX3CR in Human Adipose-Driven Mesenchymal Stem Cell with Valproic Acid. *International Journal of Pediatric*, 93-93.

- Xia, Q., Sung, J. et al. (2006). Chronic Administration of Valproic Acid Inhibits Prostate Cancer Cell Growth In vitro and In vivo. *CANCER RESEARCH*, 7237-7244.
- Qian, X., Ara, G. et al.(2008). Activity of the histone deacetylase inhibitor belinostat (PXD101) in preclinical models of prostate cancer. *International Journal of Cancer*, 1400-1410.
- Carducci, M., Nelson, J. et al. (1996). Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clinical Cancer Research*, 379–387-379–387.
- Chen, Y., Tsai, T. et al. (2011). Combined valproic acid and celecoxib treatment induced synergistic cytotoxicity and apoptosis in neuroblastoma cells. *Anticancer Research*, 2231-9.
- Gui, C., Ngo, L. et al. (2004). Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proceedings of the National Academy of Sciences*, 1241-1246.
- Vallo, S., Xi, W. et al. (2011). HDAC inhibition delays cell cycle progression of human bladder cancer cells in vitro. *Anti-Cancer Drugs*, 1002-9.
- Rodriguez-Menendez, V., Gilardini, A. et al. (2008). Valproate protective effects on cisplatin-induced peripheral neuropathy: An in vitro and in vivo study. *Anticancer Research*, 335-42.
- Chou, C., Wu, M. et al. (2011). HDAC Inhibition Decreases the Expression of EGFR in Colorectal Cancer Cells. *PLoS ONE*, E18087-E18087.
- Wedel, S., Hudak, L. et al. (2011). Impact of combined HDAC and mTOR inhibition on adhesion, migration and invasion of prostate cancer cells. *Clinical & Experimental Metastasis*, 479-491.
- Leiva, M., Moretti, S. et al. (2012). Valproic acid induces differentiation and transient tumor regression, but spares leukemia-initiating activity in mouse models of APL. *Leukemia*, 1630-7.
- Dive, C., & Wyllie, A. (1993). Apoptosis and cancer chemotherapy. *Cancer Chemotherapy*, 21-56.
- Kim, M., Blake, M. et al. (2003). Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. *Cancer Research*, 7291-300.
- Glaser, K. (2007). HDAC inhibitors: Clinical update and mechanismbased potential. *Biochemical Pharmacology*, 659-71.
- Tumber, A., Collins, L. et al. (2007). The histone deacetylase inhibitor PXD101 synergises with 5-fluorouracil to inhibit colon cancer cell growth in vitro and in vivo. *Cancer Chemotherapy and Pharmacology*, 275-283.
- Lin, C., Lai, H. et al. (2008). Valproic acid resensitizes cisplatin-resistant ovarian cancer cells. *Cancer Science*, 1218-1226.