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Anticancer Effects of Valproic Acid on Esophageal Stem-like Carcinoma Cells*

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Abstract Valproic acid (VPA) is a histone deacetylase inhibitor that has been an object of interest to clinicians for its promising potency in cancer therapy, as it induces apoptosis and differentiation, and enhances of chemotherapy sensitivity. Esophageal squamous cell carcinoma (ESCC) is a malignant disease with growing incidence and low survival rate. Due to limited information on VPA activity in ESCC cells, we aimed to determine effects of VPA on chemotherapy responsiveness and expression of malignant markers in ESCC stem-like cells. Upon coadministration of non-toxic VPA + cisplatin (DDP), paclitaxel and 5-fluorouracil, viability of KYSE30 cells was assessed, and induced apoptosis was evaluated by DAPI staining, DNA laddering and flow cytometry. In addition, real time RT-PCR was performed to study changes in the expression of *P21*, *CD44* and *BMI-1* upon treatments. MTT test demonstrated that VPA significantly ($P < 0.05$) increased toxicity of DDP, which was confirmed by DNA laddering, flow cytometry analysis and significant ($P < 0.05$) overexpression of *P21*. Moreover, real time RT-PCR results indicated significant ($P < 0.05$) down regulation of *CD44* and *BMI-1* after VPA administration. Present attempt provided evidence, for the first time, that VPA not only improved responsiveness of esophageal stem-like cancer cells to DDP, also negatively regulated cancer stem cells markers in these cells.

Key words valproic acid, esophageal squamous cell carcinoma, synergic activity
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Esophageal cancer is the eighth common malignancy and fourth cause of cancer related death worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant form of esophageal cancer with variable geographical distribution; highest-risk areas include North of Iran and China with 90% of ESCC cases [2]. Despite advances in therapeutic modalities, ESCC incidence and mortality are increasing due to the late diagnosis of the disease, and therapy resistance of neoplastic cells [3]. Cancer stem cells (CSCs) are malignant cells with stemness properties that seem to be responsible for recurrence and metastasis of human cancers. In case of ESCC, for instance, recent reports indicated that eradication or attenuation of quiescent CSCs, rather than proliferative non-CSCs, could lead to more promising and long-lasting therapeutic outcomes[4].

Valproic acid (VPA, 2-propylpentanoic acid) is a saturated short-chain, branched fatty acid that has been used as an anticonvulsant and mood stabilizer for more than four decades[5]. This small molecule shows histone deacetylase (HDAC) inhibitory effects, and induces differentiation and apoptosis in a broad spectrum of human malignant cells[6–11]. In addition, VPA sensitizes cancer cells to ionizing radiation [12–14], and acts synergistically with other anticancer drugs [7, 10, 15–17]. Accordingly, the multimodal efficacy of VPA, in single use or with other therapeutic agents, could help us to achieve more success in cancer treatment.

Although pharmacokinetics of VPA has been well explained in many cancers, application of VPA in clinical practice for gastrointestinal cancers, including esophageal cancer, still needs to be documented. Therefore, we aimed to study VPA effects on chemotherapy responsiveness of ESCC stem-like cells, as well as expression of CSC markers, for the first time. In this regard, KYSE30 cells were treated with non-toxic VPA alone or in combination with cisplatin

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(DDP), paclitaxel (PTX) and 5-fluorouracil (5-FU). Then, cell viability, induced apoptosis and expression of *P21* (apoptosis mediator), *CD44* and *BMI-1* (CSC markers) were evaluated.

1 Materials and methods

1.1 Cell culture and treatment

In present study, KYSE30 cells, obtained from Pasteur Institute (Tehran, Iran), were cultured in Roswell park memorial institute (RPMI) 1640/Ham's F12 medium (Biowest) supplemented with 10 % fetal bovine serum (FBS, Biowest), and incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air. To determine the half maximal inhibitory concentration (IC_{50}) of VPA, cells were seeded at a density of 5000 cell/well in 96-well tissue culture plates (Falcon Becton-Dickinson), and incubated with increasing concentrations of VPA(Sigma, 5, 10, 20 and 40 mmol/L) for three consecutive days.

1.2 Cell viability assay

To study viability of KYSE30 cells treated with VPA alone or in combination with cisplatin (DDP, Mylan), paclitaxel (PTX, Actavis) and 5-fluorouracil (5-FU, Ebewe Pharma), the thiazolyl blue (MTT) assay was used. To do so, 24, 48 and 72 h after treatments, 20 μl of MTT dye (Atocel, 5 g/L) was added to each well, and plates were incubated for 4 h at 37℃. Then, media was replaced by dimethyl sulfoxide (DMSO, 150 μl/well) and optic densities of wells were measured at 570 nm by ELISA plate reader (Awareness). All tests were performed in triplicates, and cells were checked for morphological alterations by an invert microscope (Olympus). To note, percentages of increased cytotoxicity in combinatorial treatments were calculated by dividing the mean absorbance of treated (VPA + drug) cells to the mean absorbance of control cells (only treated with relevant anticancer drug).

1.3 Demonstration of apoptotic morphology

To further evaluate effects of VPA on cytotoxicity of DDP, KYSE30 cells were stained with 4 min, 6-diamidino-2 phenylindole(DAPI). In this regard, 72 h after administration of 5 mmol/L VPA, 1 mg/L DDP or 5 mmol/L VPA + 1 mg/L DDP, cells were fixed in 4% paraformaldehyde (Sigma), incubated with 0.1% Triton X-100 containing 2 mg/L DAPI (Merck) at 37℃ for 10 min. After washing with phosphate buffered saline (PBS), cells were examined under fluorescent microscope (Olympus), while chromatin condensation was the criteria used to demonstrate apoptosis.

1.4 DNA laddering

To detect DNA fragmentation, DMSO-sodium dodecyl sulphate (SDS)-Tris-EDTA (TE) method was used[18]. Briefly, treated cells were washed with PBS, and 100 μl of DMSO was added directly to the cell pellet and mixed well. Then, 100 μl of TE buffer (Merck, pH 7.4) with 2% SDS (Merck) was added followed by vortexing. The resulting solution was centrifuged at 12 000 r/min at 4℃ for 5 min, and 35 μl of the supernatant was loaded on 1.5% agarose gel (Fermentas). Finally, electrophoresis was conducted for 50 min in an electric field of 80 V.

1.5 Apoptosis detection

In current study, apoptosis was assessed flow cytometrically by FITC annexin V apoptosis detection kit with propidium iodide (BioLegend) according to the manufacturer's protocol. Briefly, treated cells were collected, resuspended in staining buffer, and stained with FITC-annexin V and propidium iodide for 15 min at room temperature in the dark. Then, binding buffer was added, and cells were analyzed by flow cytometry (BD FACSCalibur) using appropriate filters.

1.6 Real time reverse transcription-polymerase chain reaction (RT-PCR)

The total cellular RNA was extracted from treated and control KYSE30 cells using RNX-plus (Cinnagen) according to the manufacturer's instruction. As soon as RNAs were treated with RNase-free DNase Ⅰ (Thermo Scientific), cDNAs were synthesized using oligo-dT, dNTPs, RNase inhibitor, and M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. Then, fidelity of amplified cDNAs was confirmed by PCR using GAPDH primers (forward: GACCACTTTGTCAAGCTCATTTCC and reverse: GTGAGGGTCTCTCTCTTCCTCTTGT), followed by agarose gel electrophoresis. Real time RT-PCR was performed in iQ5 real-time PCR detection system (Bio-Rad) using SYBR green master mix (Pars Toos) and specific primers for P21 (forward: GGAAGACCATGTGGACCTGT and reverse: GGCGTTTGGAGTGGTAGAAA), CD44 (forward: CGGACACCATGGACAAGTTT and reverse: GAAAGCCTTGCAGAGGTCAG) and BMI-1 (forward: CTGCAGCTCGCTTCAAGATG and reverse: CACACACATCAGGTGGGGAT). To note, GAPDH transcripts were used as internal control, and fold change expression was calculated as $2^{(-\Delta\Delta CT)}$. For *P21* primers, PCR cycling

conditions were 95℃ for 4 min, [95℃ for 15 s, 55℃ for 15 s, 72℃ for 15 s] (50 cycles), while for CD44 and BMI-1 primers, thermal conditions were as 95℃ for 4 min, [95℃ for 30 s, 59℃ for 30 s, 72℃ for 30 s] (40 cycles).

1.7 Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparison test using SPSS software. Values were expressed as mean± SEM, and $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 VPA enhanced toxicity of DDP

To study effects of VPA on KYSE30 stem-like cancer cells, we first calculated IC_{50} values of VPA that were 35, 25 and 18 mmol/L after 24, 48 and 72 h, respectively (data not shown). Then, synergic activity of VPA with DDP, PTX and 5-FU was assessed by combinatorial treatments. In this regard, cells were cultured in the presence of 5 mmol/L VPA + DDP (1 and 2 mg/L), PTX (1 and 2 mg/L), and 5-FU (2.5 and 5 mg/L), all in concentrations lower that their IC_{50} [19], for three consecutive days. Results of MTT assay indicated that non-toxic VPA significantly ($P < 0.05$) enhanced toxicity of DDP (1 mg/L), specifically 72 h after combinatorial treatment (Figure 1). In case of PTX and 5-FU, however, increased cytotoxicity was not considerable.

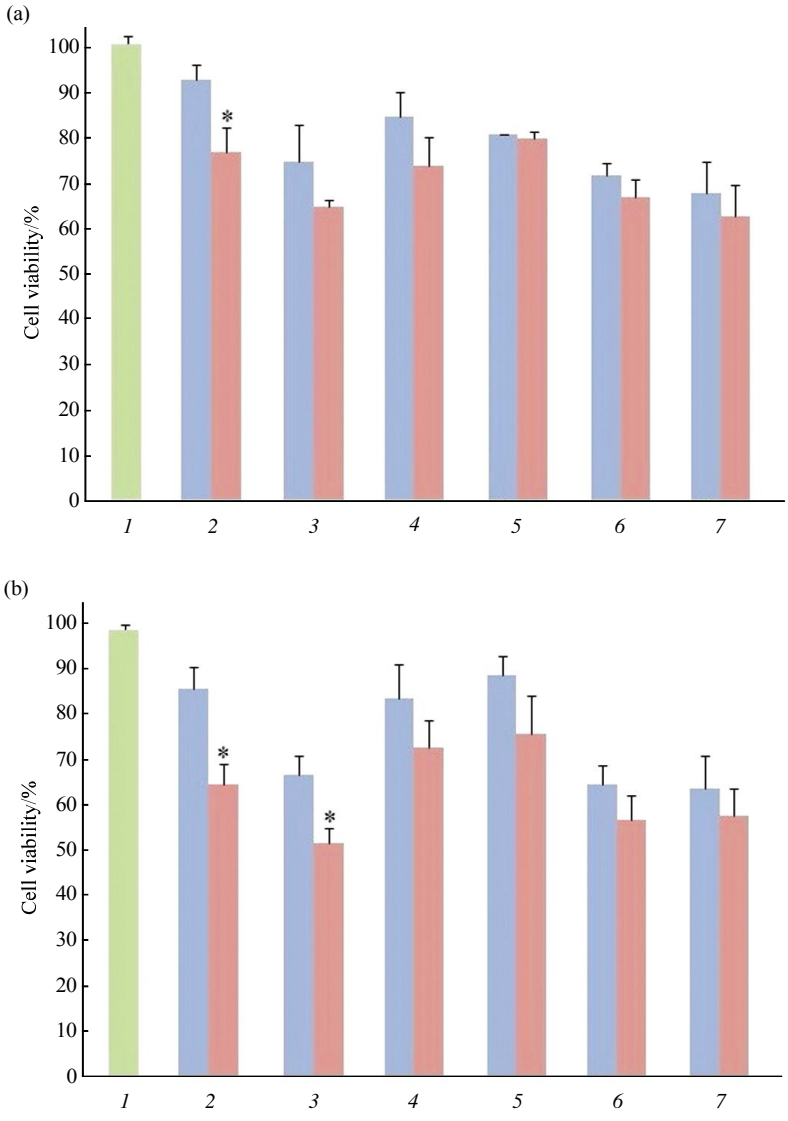

Fig. 1 Synergic activity of VPA enhanced toxicity of DDP in KYSE30 cells

Time-based dose response analysis of KYSE30 cells to VPA alone or in combination with DDP (1 and 2 mg/L), PTX (1 and 2 mg/L), and 5-FU (2.5 and 5 mg/L) for 48 (a) and 72 (b) h. * Indicates significant ($P < 0.05$) difference with 1 mg/L DDP alone. □ : –VPA(5 mmol/L); ■ : +VPA(5 mmol/L); *1*: VPA(5 mmol/L); *2*: DDP(1 mg/L); *3*: DDP(2 mg/L); *4*: PTX(1 mg/L); *5*: PTX(2 mg/L); *6*: 5-FU(2.5 mg/L); *7*: 5-FU(5 mg/L).

2.2 Synergic effects of VPA increased apoptosis induced by DDP

As presented in Figure 2, morphological observations revealed that in comparison with KYSE30 cells treated with 5 mmol/L VPA or 1 mg/L DDP for 72 h, prominent cytoplasmic granulations were more obvious after coadministration of 5 mmol/L VPA+1 mg/L DDP. Moreover, demonstrating apoptotic morphology by DAPI staining revealed that 24% of cells treated with 5 mmol/L VPA + 1 mg/L DDP presented nuclear fragmentation (Figure 2f), which was higher than in cells treated with 5 mmol/L VPA (1.5%, Figure 2d) or 1 mg/L DDP (18%, Figure 2e). To further analyze combinatorial effects of VPA + DDP on DNA structure, DNA laddering was applied on cells treated with 5 mmol/L VPA, 1 mg/L DDP, or 5 mmol/L VPA + 1 mg/L DDP for 72 h. As shown in Figure 2g, only in cells with combinatorial treatment DNA fragmentation was observed, which supports results obtained from MTT assay and DAPI staining. Figure 2h shows effects of VPA and DDP, alone and in combination, on the percentage of alive and necrotic cells, as well as early and late apoptotic cells. As presented, almost the same percentage (27.8% and 29.8%) of cells became early and late apoptotic upon 5 mmol/L VPA + 1 mg/L DDP treatment, which was unlike cells treated VPA and DDP alone.

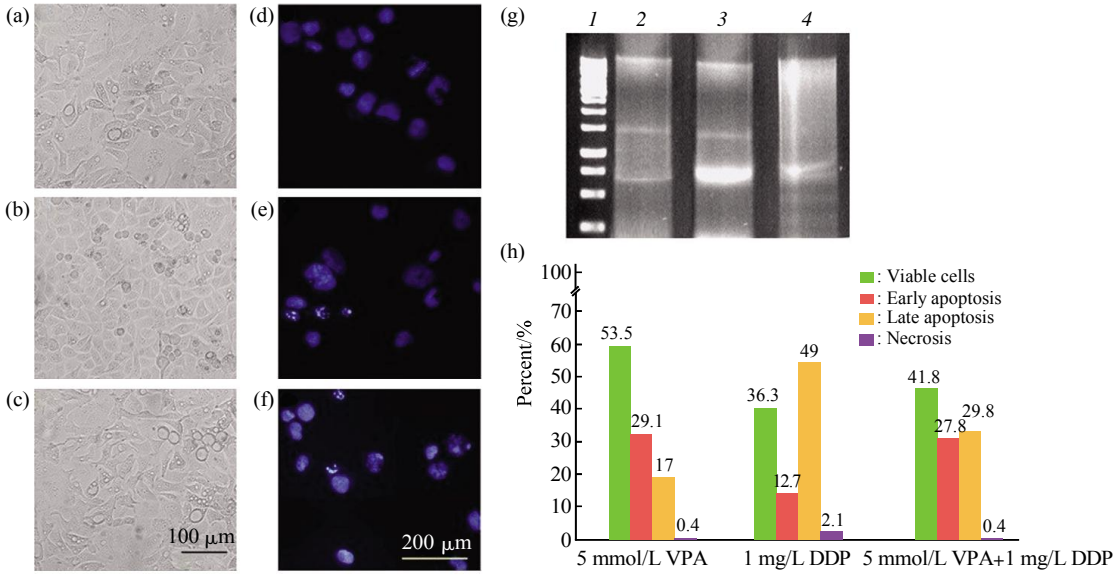

Fig. 2 VPA increased apoptosis induced by DDP in terms of cytoplasmic granulation, nuclear and DNA fragmentations and emergence of apoptotic cells

Phase contrast photomicrographs of KYSE30 cells treated with 5 mmol/L VPA (a), 1 mg/L DDP (b), and 5 mmol/L VPA + 1 mg/L DDP (c) indicates cytoplasmic granulations after combinatorial treatment. DAPI staining (d～f) revealed nuclear fragmentation upon administration of 1 mg/L DDP (e) and 5 mmol/L VPA + 1 mg/L DDP (f), but not 5 mmol/L VPA (d). Application of DMSO-SDS-TE method for detection of DNA fragmentation revealed DNA ladders of KYSE30 cells treated with 5 mmol/L VPA + 1 mg/L DDP (g). *1*: Ladder; *2*: 5 mmol/L VPA; *3*: 5 mg/L DDP; *4*: 5 mmol/L VPA+ 1 mg/L DDP. Differentiating alive KYSE30 cells from necrotic and apoptotic cells by flow cytometry analysis revealed almost the same percentage of early and late apoptotic cells in 5 mmol/L VPA + 1 mg/L DDP treatment, unlike cells treated VPA or DDP alone (h).

2.3 Coadministration of VPA and DDP enhanced the expression of P21

To further elucidate the mechanism of VPA synergic activity, the expression of *P21*, a gene involved in apoptosis induced by VPA and DDP, was studied. Figure 3a shows relative fold changes in the expression of *P21* in each treatment over untreated KYSE30 cells. As presented, coadministration of 5 mmol/L VPA and 1 mg/L DDP significantly($P < 0.05$) enhanced *P21* expression in KYSE30 cells, in comparison with cells only treated with DDP. This confirms results of MTT assay, DNA laddering and flow cytometry.

2.4 VPA downregulated the expression of CD44 and BMI-1

To have a better understanding of VPA anticancer potential, we also investigated effects of VPA, as a single agent, on the expression of *CD44* and *BMI-1*, CSC markers highly expressed it KYSE30 cells. As shown in Figure 3b, 72 h after administration of VPA (2.5 and 5 mmol/L), significant($P < 0.05$) downregulation was detected in the expression of both markers.

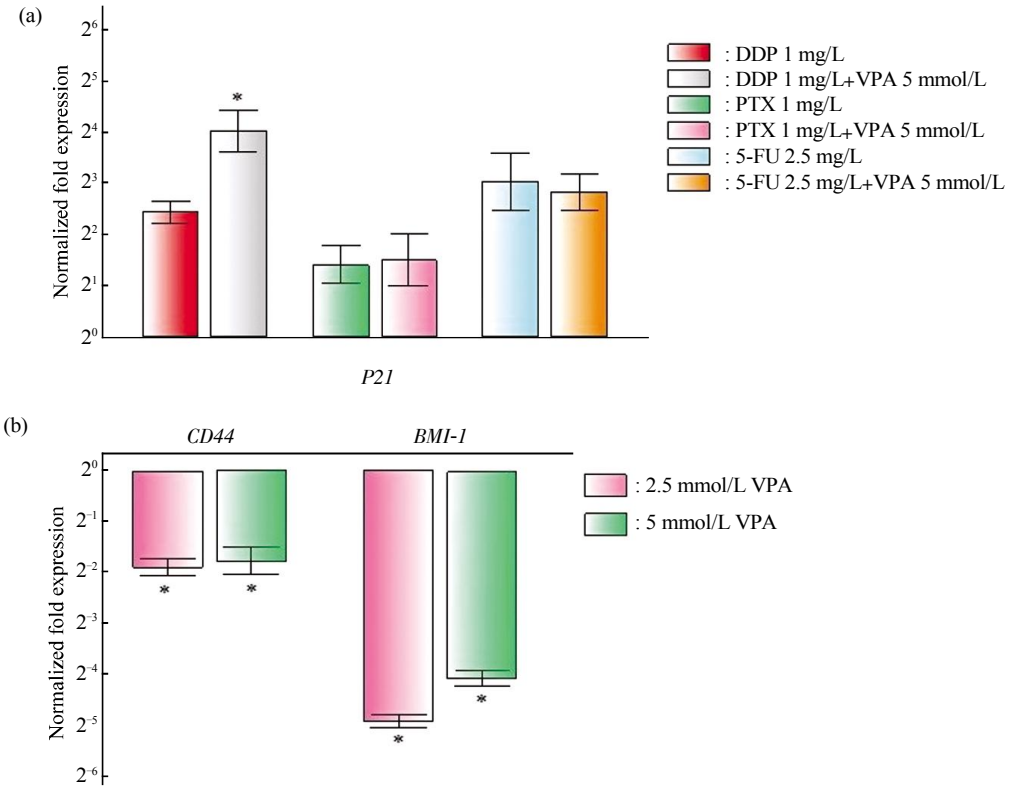

Fig. 3 VPA induced changes in the expression of P21, CD44 and BMI-1

Real time RT-PCR analysis of *P21* expression after drug treatments, alone or in combination with 5 mmol/L VPA (a). Changes induced by VPA (2.5 and 5 mmol/L) in the expression of *CD44* and *BMI-1*, after 72 h (b). To note, normalized values were plotted as relative fold change over untreated control KYSE30 cells. * Indicates significant ($P < 0.05$) difference with 1 mg/L DDP in (a) and with untreated KYSE30 cells in (b).

3 Discussion

Despite advances in cancer therapy, the incidence of esophageal carcinoma, which is among the top 10 life-threatening cancers worldwide, is increasing. High-risk areas for ESCC, the most common type of esophageal cancer, are Caspian littoral region in Iran and North-Central China [2]. Unfortunately, late and incorrect diagnoses, and developing resistance to chemoradiotherpy are main reasons for high mortality of ESCC[3]. In this regard, designing novel therapeutic approaches that affect esophageal CSCs would help clinicians to reduce the risk of tumor relapse in ESCC patients [4]. In present study, we examined whether VPA, a HDAC inhibitor (HDACI) with wide range of biological activities, could improve sensitivity of esophageal stem-like cancer cells to anticancer drugs and downregulate CSC markers, for the first time.

There is a great interest in using VPA rather than other class-selective HDACIs in cancer studies, since VPA has long biological half-life, shows pharmacological activities at nontoxic therapeutic concentrations, and is easily administrated [5]. More importantly, utilization of VPA is not limited to single-agent cancer therapy, since it enhances sensitivity of malignant cells to radiotherapy [12–14] and improves efficacy of conventional therapeutic agents by its synergic activity [7, 10, 15–17]. In current attempt,

results of MTT assay indicated that non-toxic VPA significantly ($P < 0.05$) increased responsiveness of KYSE30 cells to DDP, which is routinely used for ESCC treatment [20]. This was in line with morphological observations, nuclear alterations, DNA fragmentation and early and late apoptosis detected by flow cytometry. Previous reports indicated that VPA enhanced efficacy of anticancer drugs ellipticin [15], nanotaxol[7], retinoic acid[8], methotrexate[16], mitomycin C, adriamycin and DDP[10]. In addition, a recent report indicated that in head and neck squamous cell carcinoma (HNSCC) CSCs, VPA potentiated cytotoxicity of DDP by suppressing its specific transporters, ABCC2 and ABCC6[21]. This explains and confirms current results, although we provided new evidence for synergic effects of VPA on ESCC stem-like cells.

To better evaluate effects of VPA in combinatorial treatments, we examined the expression of *P21*, a gene involved in apoptosis induced by anticancer agents. Comparing the expression of *P21* between cells treated with DDP, PTX and 5-FU alone and in combination with VPA only indicated significant ($P < 0.05$) changes after coadministration of DDP and VPA. Increased expression of *P21* has been previously reported in KYSE30 cells upon DDP treatment [22]. Nevertheless, we indicated improved efficacy of DDP (in a P21-dependent manner) upon combinatorial use with VPA that might be, to some extent, due to increased accumulation of this drug inside the cells.

Beside its synergic activity, VPA induces differentiation in many types of cancer cells. For instance, VPA treatment of thyroid cancer cells, as well as HNSCC and neural CSCs reduced stemness (OCT4, SOX2, NANOG and hTERT) and CSC (CD44 and CD133) markers, while increased lineage differentiation molecules [11, 21, 23]. Since CD44 and BMI-1 are important markers for esophageal cancer diagnosis[4], and KYSE30 cell line is a suitable model for studying $CD44^+$ and $BMI\text{-}1^+$ ESCC stem-like cells[24], effects of non-toxic VPA was also investigated on the expression of both molecules in present study. Results of real time RT-PCR indicated significant ($P < 0.05$) downregulation of *CD44* and *BMI-1* upon administration of 2.5 and 5 mmol/L VPA in KYSE30 cells, which is in line with previous reports on other types of CSCs[11, 21, 23]. Furthermore, since downregulation of *CD44* and *BMI-1* has been linked to enhanced responsiveness of ovarian, glioblastoma, osteosarcoma, nasopharyngeal and lung cancer cells to DDP [25−30], negative regulatory effects of VPA on *CD44* and *BMI-1* might also result in its synergic effects.

In conclusion, present results indicated great pharmaceutical value of VPA in ESCC treatment, since it enhanced chemosensitivity of esophageal stem-like cancer cells, and reduced expression of CSC markers. Accordingly, effects of VPA, alone or in combination with other therapeutic modalities, make this small molecule a good candidate for future *in vivo* and clinical studies on esophageal carcinoma.

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