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EFFECT OF VALPROIC ACID AND ZEBULARINE ON *SOCS-1* AND *SOCS-3* GENE EXPRESSION IN COLON CARCINOMA SW 48 CELL LINE

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***Background*: Two epigenetic modifications such as histone acetylation and DNA methylation have been known as critical players of gene regulation. Hypermethylation and deacetylation of suppressors of cytokine signaling family *SOCS-1* and *SOCS-3* have been shown in many solid cancers. Previously, we evaluated the effect of 5-aza-2′-deoxycytidine and valproic acid on hepatocellular carcinoma and colon cancer cells. *Aim:* The present study was designed to assess the effect of valproic acid in comparison to zebularine on *SOCS-1* and *SOCS-3* gene expression, cell growth inhibition and apoptosis induction in colon carcinoma SW48 cell line. *Materials and Methods*: SW48 cells were treated with valproic acid or zebularine for 24 h and 48 h. The effect of the compounds on cell viability, *SOCS-1* and *SOCS-3* gene expression, and apoptosis induction was evaluated. Reverse transcription polymerase chain reaction analysis and flow cytometry were applied. *Results*: Both agents inhibited cell growth in a time- and dose-dependent fashion. The apoptotic effect was observed in cells treated with valproic acid (7.5 μM) but not zebularine (75 μM). The valproic acid but not zebularine upregulated *SOCS-1* and *SOCS-3* gene expression. *Conclusion*: Epigenetic modulation can reactivate silenced tumor suppressor genes *SOCS-1* and *SOCS-3* through histone acetylation resulting in apoptosis induction.**
***Key Words*: valproic acid, zebularine, *SOCS-1, SOCS-3*, colon carcinoma.**

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The nucleosomes and histone proteins are the fundamental building blocks of eukaryotic chromatin. The modification of histones plays an important role in the regulation of gene expression. Various histone post-translational modifications not only regulate chromatin structure but also recruit remodeling enzymes. Histone modifications can affect DNA replication, repair, and recombination. There are at least eight distinct types of histone modifications, one of which is histone acetylation regulated by the opposing activity of two families of enzymes, including histone deacetylases and histone acetyl transferases [1].

Two epigenetic modifications have been known as critical players of regulation, including histone acetylation which appears to be used by all eukaryotes and DNA methylation which has been implicated as a critical factor of control for enhancing transcriptional silencing [2]. DNA methylation is catalyzed by DNA methyl transferase enzymes (DNMTs) including DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L existing in mammalian cells [3]. Cancer is driven by the accumulation of epigenetic changes such as histone deacetylation and DNA methylation.

Hypermethylation and deacetylation of tumor suppressor genes (TSGs) such as suppressors of cytokine signaling (SOCS) family have been reported in several cancers [4]. This family consists of eight members, including SOCS-1 to SOCS-7 and cytokine-inducible SH2 protein [5]. The aberrant methylation of *SOCS-1* has been reported in colorectal cancer [6, 7], human hepatocellular carcinoma (HCC) [8], and pancreatic cancer [9]. Farther, the aberrant methylation of SOCS-3 has been demonstrated in HCC, the squamous cell carcinoma of the head and neck, and colon cancer [10-13]. In addition to hypermethylation, deacetylation of *SOCS-1* and *SOCS-3* in colorectal cancer [14], and *SOCS-1* in human cervical carcinoma cell lines CaSki, HeLa, and SiHa has been demonstrated [4]. DNMTs activity has an important role in tumorigenesis, and the activity of these enzymes can be inhibited by DNMT inhibitors including 5-azacytidine, 5-aza-2′-deoxycytidine (5-AZA-CdR), and zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-1] [15]. In T24 bladder carcinoma cells, zebularine can reactivate *p16* gene by remethylation of its 5'-region [16]. DNMT1 inhibition by zebularine results in growth inhibition and apoptosis induction in HCC HepG2 and pancreatic cancer cells [17]. Furthermore, DNA demethylation in colon cancer HT-29 cells by 5-AZA-CdR, as a DNMT inhibitor, recovers the constitutive expression of *SOCS-1* [18]. 5-AZA-CdR can also reactivate *SOCS-3, SOCS-5*, and *SOCS-7* in colon cancer HT-29 cells [19].

Histone deacetylase inhibitors (HDACIs) represent an emerging class of therapeutic agents with potential anticancer activity. Valproic acid (VPA) is a well-known HDACI promising as a therapeutic compound for cancer treatment. VPA induces growth arrest in various cancer cell lines, e.g. colon cancer HT-29 cells and pancreatic cancer cells [20] affecting the expression of the genes involved in cell growth inhibition and

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Abbreviations used: 5-AZA-CdR – 5-aza-2′-deoxycytidine; DNMT – DNA methyl transferase; HCC – human hepatocellular carcinoma; HDACI – histone deacetylase inhibitor; MTT – 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide; PCR – polymerase chain reaction; PI – propidium iodide; SOCS – suppressors of cytokine signaling; TSG – tumor suppressor gene; VPA – valproic acid.

apoptosis induction [21]. It has been shown that HDACI trichostatin A up-regulates *SOCS-1* gene expression in human cervical carcinoma cell lines CaSki, HeLa, and SiHa but not ME-180 cells [4]. Other studies have indicated that HDACI sodium butyrate increases *SOCS-1* and *SOCS-3* expression by triggering the promoter-associated histone acetylation of these genes in K562 and HEL cell lines originated from human myeloid leukemia [22].

Previously, we evaluated the effect of 5-AZA-CdR and VPA on HCC and colon cancer cells [23–25]. The present study was designed to assess the VPA effect on *SOCS-1* and *SOCS-3* gene expression, cell growth inhibition and apoptosis induction in colon carcinoma SW48 cell line in comparison with zebularine.

MATERIALS AND METHODS

Cells and reagents. Human colon carcinoma SW48 cell line was purchased from the National Cell Bank of Iran-Pasteur Institute and maintained in Dulbecco's modified Eagle's medium containing 100 mL/L fetal bovine serum and antibiotics (50 µg/ml streptomycin and 50 U/ml penicillin) at 37 °C in a humidified atmosphere of 5% CO_2. VPA and zebularine (Sigma, USA) were dissolved in distilled water as a stock solution. By diluting these solutions, all working solutions were provided. Antibiotics, 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Annexin-V (FITC), propidium iodide (PI), trypsin-EDTA, dimethyl sulfoxide, Dulbecco's modified Eagle's medium, and phosphate-buffered saline were purchased from Sigma (USA). Real-time polymerase chain reaction (PCR) kits (qPCR MasterMix Plus for SYBR Green I dNTP) and total RNA extraction kit (TRIZOL reagent) were obtained from Applied Biosystems Inc. (USA).

Cell viability. SW48 cells were seeded into 96-well plates (5×10^5 cells per well). After 24 h, the cells were treated with VPA (1, 2.5, 5, 7.5, and 10 µM) or zebularine (10, 25, 50, 75, and 100 µM) for 24 h and 48 h. Following the treatment, the effect of the compounds on cell viability was evaluated by MTT assay according to standard protocols. Finally, the optical density was detected by a microplate reader at a wavelength of 570 nM. Each experiment was repeated in triplicates.

Apoptosis assay. The cells were detached from the surface of the plates by trypsinization and the single-cell suspension was prepared. The cells were washed with cold phosphate-buffered saline and resuspended in annexin binding buffer (0.01 M HEPES pH 7.4; 0.14 M NaCl, 2.5 mM $CaCl_2$) and stained with annexin V-FITC and PI staining solution in the dark at room temperature for 15 min according to the manufacturer's protocol. After incubation, the samples were analyzed by flow cytometry in FACScan flow cytometer (Becton Dickinson, Germany).

Quantitative reverse-transcription PCR analysis. Total RNA was isolated from colon carcinoma SW48 cells treated with VPA (7.5 µM) or zebularine (75 µM) using Trizol reagent (Invitrogen, USA). cDNA was synthesized from total RNA with Superscript III reverse transcriptase (Invitrogen, USA). The expression of mRNAs was measured by quantitative real-time PCR using StepOnePlus (Applied Biosystem, USA) instrument using SYBER green PCR kit (TaKaRa Bio). The amplification reactions were performed as mentioned previously [26]. The primer sequences for *SOCS-1* and *SOCS-3* genes are shown in Table 1. *GAPDH* was used as an endogenous control. Data were analyzed using the comparative Ct (ΔΔCt) method.

This study was approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1397.100 of January 16 2019.

RESULTS

Cell viability. To determine cell growth inhibitory effect of VPA and zebularine on SW48 cells, the cells were treated with VPA (1, 2.5, 5, 7.5, and 10 µM) and zebularine (10, 25, 50, 75, and 100 µM) for 24 h and 48 h and then the viability was determined using MTT assay. As shown in Fig. 1, both compounds decreased the count of the viable SW48 cells significantly in a time- and dose-dependent fashion ($p < 0.035$). IC_{50} values

Table 1. Primer sequences of *SOCS-1* and *SOCS-3* used in the present study

Primer name	Primer sequences (5' to 3')	Reference
SOCS-1 forward	AGAC CCCTTCTCACCTCTTG	[27]
SOCS-1 reverse	CTGCACAGCA GAAAATAAAGC	
SOCS-3 forward	TCCCCCCAGAAGAGCCTATTAC	[27]
SOCS-3 reverse	TCCG ACAGAGATGCTGAAGAGTG	

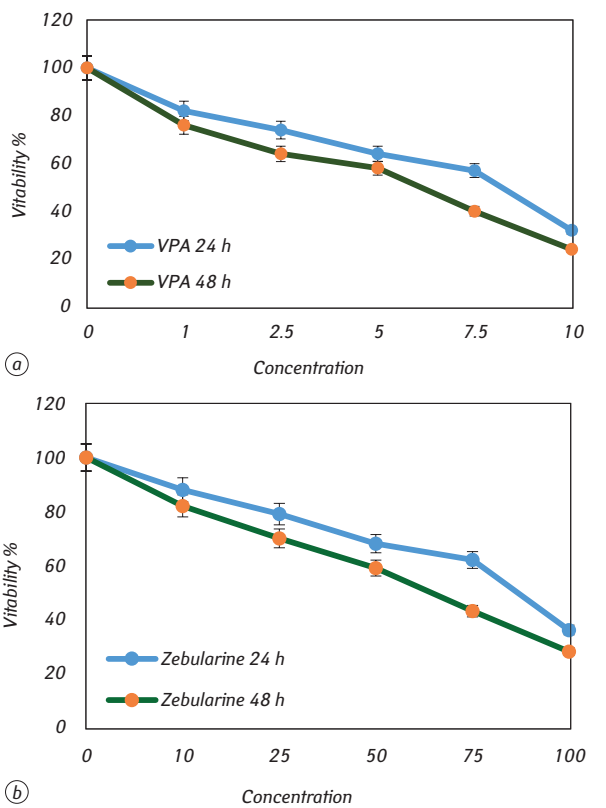

Fig. 1. The effect of VPA (*a*) and zebularine (*b*) on the viability of SW48 cells. The cells were treated with and without different concentrations of VPA and zebularine for 24 h and 48 h and subsequently, the cell viability was evaluated by MTT assay. Each experiment was conducted in triplicate. Mean values from the three experiments ± standard error of mean are shown

were calculated approximately as 7.5 μM for VPA and 75 μM for zebularine.

Apoptosis induction. The cells were treated with VPA or zebularine for 24 h and 48 h and stained with Annexin V in combination with PI. As depicted in Fig. 2, significant differences were observed between cells treated with VPA for 24 h and 48 h in comparison with controls. No significant differences were observed between the cells treated with zebularine in comparison with controls as shown in Fig. 3. The percentage of the apoptotic cells is given in Table 2. Three independent experiments were performed for each concentration.

SOCS-1 and SOCS-3 expression. The effect of VPA (7.5 μM) and zebularine (75 μM) on *SOCS-1* and *SOCS-3* genes expression was evaluated by quantitative real-time reverse transcription PCR analysis. The treatment with VPA (7.5 μM) for 24 h and 48 h up-regulated *SOCS-1* and *SOCS-3* expression significantly. In contrast, zebularine did not change *SOCS-1* and *SOCS-3* expression levels (Table 3).

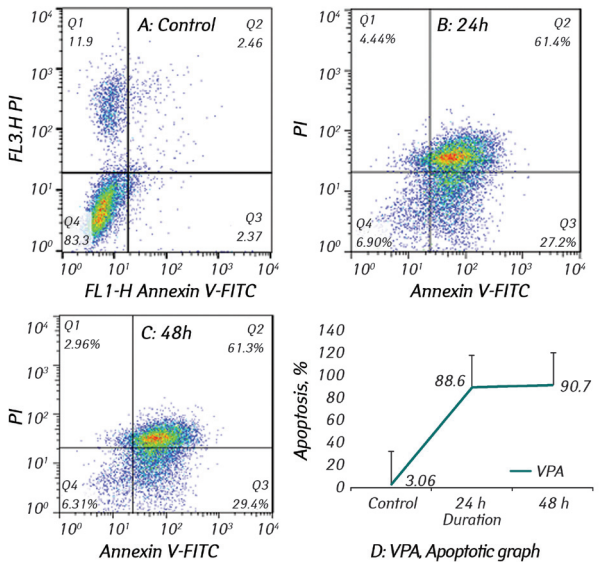

Fig. 2. The apoptosis-inducing effect of VPA on SW48 cells was investigated by flow cytometric analysis using Annexin V and. Result of flow cytometry indicated that VPA induced significant apoptosis in this cell line

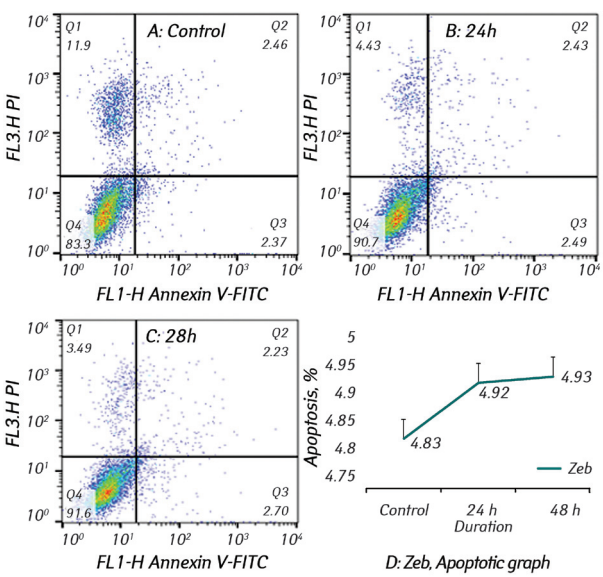

Fig. 3. The apoptosis-inducing effect of zebularine on SW48 cells was investigated by flow cytometric analysis using Annexin V and PI. Result of flow cytometry indicated that zebularine had no significant apoptotic effect on this cell line

DISCUSSION

Hypermethylation of CpG islands and histone deacetylation represent the epigenetic events that are not accompanied by the changes in DNA sequences. They play a major causal role in cancer and represent an alternative mechanism to inactivate TSGs. The presence of epigenetic alterations in tumorigenesis is now widely accepted [28].The possibility to restore silenced TSGs has generated considerable interest in the development of DNMTs inhibitors. Additionally, HDACIs have been known as a potential strategy to reactivate silenced TSGs associated with cancer. In the present study, we observed that VPA inhibited cell growth, induced apoptosis, and reactivated *SOCS-1* and *SOCS-3* genes expression significantly.

SOCS comprises several members including SOCS-1, SOCS-2, and SOCS-3, which are encoded by genes located in 16p13.13, 12q, 17q25.3, respectively. They are stimulated by cytokines and act as the negative regulators of cytokine signaling. The SOCS proteins affect the functional state of the cells via three basic mechanisms. First, they bind to phosphotyrosines on the receptors and block the recruitment of signal transducers, such as STATs, to the receptor. Second, they can bind directly to janus kinases or their receptors that leads to the inhibition of janus kinase activity. Third, these proteins interact with the cullin 2 and elongin BC complex, facilitating the ubiquitination of janus kinases and, presumably, the receptors [29]. In colon cancer, SOCS-1 has been demonstrated to bind to directly janus kinase 2 inhibiting its catalytic activity while SOCS-3 binds to glycoprotein 130 (gp130)-related receptors [30]. It has been reported that overexpression of SOCS-3 inhibits janus kinase/signal transducer and activator of transcription 3 signaling and induces apoptosis

Table 2. The percentage of apoptotic cells treated with VPA and zebularine

Drug	Dose (μM)	Duration (h)	Apoptosis (%)	*p* (as compared with untreated cells)
Untreated cells	–		4.8	
VPA	7.5	24	88.6	0.001
VPA	7.5	48	90.7	0.001
Zebularine	75	24	4.9	0.993
Zebularine	75	48	4.9	0.992

Table 3. The relative expression levels of *SOCS-1* and *SOCS-3*

Gene	Drug	Dose (μM)	Duration (h)	Relative expression level	*p*
SOCS-1	VPA	7.5 μM	24	2.6	0.001
SOCS-1	VPA	7.5 μM	48	2.9	0.001
SOCS-3	VPA	7.5 μM	24	2.4	0.001
SOCS-3	VPA	7.5 μM	48	2.8	0.001
SOCS-1	Zebularine	75 μM	24	1.1	0.874
SOCS-1	Zebularine	75 μM	48	1.2	0.807
SOCS-3	Zebularine	75 μM	24	1.15	0.603
SOCS-3	Zebularine	75 μM	48	1.25	0.570

in colon cancer cells [31]. Several studies have shown that SOCS-3 overexpression reduces colon cancer SW480 cells, Caco2, and hepatocellular carcinoma by inhibition of interleukin 6/signal transducer and activator of transcription 3 and tumor necrosis factor α/nuclear factor-kappa B pathways [32].

Accumulating evidence has demonstrated that HDACI sodium butyrate upregulates *SOCS-1* and *SOCS-3* resulting in janus kinase 2/signal transducer and activator of transcription signaling inhibition [22]. Similarly, another HDACI, trichostatin A increases *SOCS-1* and *SOCS-3* expression leading to the inhibition of janus kinase 2/signal transducer and activator of transcription 3 signaling in colon cancer cell [14]. It has been indicated that SOCS-1 and SOCS-3 are the most effective in inhibiting interleukin 6-mediated signaling pathways [33]. Meanwhile, HDACIs modify the acetylation state of a large number of cellular proteins involved in tumorigenic processes.

In the current study, zebularine had no significant effect on *SOCS-1* and *SOCS-3* genes reactivation and apoptosis induction. Meanwhile, we could not find any study to report the significant effect of zebularine (75 μM) on *SOCS-1* and *SOCS-3* gene expression in colon cancer. Other researchers have demonstrated that DNMT inhibitor 5-AZA-CdR increases *SOCS-1* expression in colon cancer [7]. However, the treatment of breast cancer cells with the 5-AZA-CdR inhibits cell growth by activating *SOCS-1* [34]. In the ovarian PEO1 cell line, treatment with 5-AZA-CdR increases *SOCS-2* expression [26]. Finally, it has been reported that zebularine inhibits breast cancer MDA-MB-231 and MCF-7 cell growth and induces apoptosis in a dose and time-dependent manner, with an IC_{50} of 150 μM in 96 h [35]. In human head and neck cancer SCC-9 and SCC-25 cells, zebularine can increase significant apoptotic-related proteins at a concentration of 350 μM in 96 h [36].

Zebularine can induce apoptosis in colon cancer cells by other molecular mechanisms as we reported previously. Our previous work indicated that it can induce apoptosis by inhibition of DNMT1 3a, and 3b, resulting in reactivation of *p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2* gene expression, cell growth inhibition and apoptosis induction in colon cancer LS180 cell line [37]. *In vitro* studies have shown that zebularine is involved in the reactivation of the silenced genes, such as cyclin-dependent kinase inhibitor p16 in colon cancer cell line resulting in apoptosis induction [38]. Several studies have indicated that zebularine-induced cell death is dependent on *p53* up-regulation and *GRP78* and *p62* gene down-regulation in colorectal cancer [39]. In the current study, we did not investigate various molecular mechanisms of the apoptotic effect of zebularine such as reactivation of cyclin-dependent kinase inhibitors. Taken together, the effects of zebularine in SW48 cells could be further investigated at higher concentrations and longer treatment periods. The evaluation of other pathways possibly affected by zebularine is also worthwhile.

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CONFLICT OF INTREST

The authors report no conflict of interest.

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