Research in Pharmaceutical Sciences, August 2022; 17(4): 392-409
Received: 09-12-2021
Peer Reviewed: 17-01-2022
Revised: 05-02-2022
Accepted: 16-05-2022
Published: 14-07-2022

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

The effect of valproic acid on intrinsic, extrinsic, and JAK/STAT pathways in neuroblastoma and glioblastoma cell lines

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Abstract

Background and purpose: Epigenetics has been defined as the study of mitotically heritable alterations in gene expression that are not caused by changes in DNA sequence. Epigenetic-mediated silencing of a gene includes genomic imprinting, histone deacetylation, DNA methylation, and RNA-associated silencing. Cell growth and cell proliferation are inhibited by some histone deacetylase and histone inhibitors. This study was designed to investigate the effect of valproic acid (VPA) on extrinsic, intrinsic, and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways in neuroblastoma and glioblastoma cell lines.
Experimental approach: The neuroblastoma and glioblastoma cells were cultured and treated with VPA. MTT assay was done to determine cell viability. Besides, a flow cytometry assay was performed to determine apoptotic cells and finally, the relative gene expression level was evaluated by qRT-PCR.
Findings / Results: VPA changed the expression level of the genes of the extrinsic, intrinsic, and JAK/STAT pathways which induced cell apoptosis and inhibited cell growth in the neuroblastoma and glioblastoma cells. In the neuroblastoma cell lines, VPA upregulated the expression level of FAS, FAS-L, DR4, DR5, and TRAIL genes significantly. Additionally, it significantly up-regulated the expression level of Bak, Bax, and Bim genes and down-regulated the expression level of Bcl-xL, Bcl-2, and Mcl-1 genes in both neuroblastoma and glioblastoma cell lines.
Conclusion and implications: VPA induced cell apoptosis through extrinsic, intrinsic, and JAK/STAT pathways.

Keywords: Apoptosis; Gene Expression; Neoplasms; Valproic acid.

INTRODUCTION

The term epigenetics describes the study of the genetic material alterations and refers to the alterations in gene expression without any changes in DNA structure. Epigenetic-mediated silencing of the gene includes genomic imprinting, histone deacetylation, DNA methylation, and RNA-associated silencing (1). Chromatin alterations (*e.g.* DNA cytosine methylation and histone deacetylation) have been associated with all stages of cancer induction and progression. These two processes are important epigenetic molecular mechanisms that have significant roles in gene regulation and gene expression (2).

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Numerous *in vitro* studies have demonstrated that tumor suppressor genes (TSGs) have been indicated to be silenced in human cancers. The silencing of TSGs is accompanied by a change in DNA hypermethylation and histone deacetylation. These processes are directed by changes in two groups of genes including oncogenes that promote cell survival and cell growth and TSGs that inhibit cell growth. As mentioned, epigenetic alterations include heritable changes in gene expression and changes in the DNA sequences (3).

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Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.350240

Human cancer cells indicate DNA methylation status which is established by the activity of DNA methyltransferase enzymes (DNMTs). In mammals, DNA methylation is achieved by DNMTs (DNMT1, DNMT3A, and DNMT3B) (4). Enzymatic histone acetylation and deacetylation represent major S-adenosyl-L-methionine to DNA cytosine bases (4). These enzymatic processes represent major molecular mechanisms controlling gene expression. The balance between histone acetylation and histone deacetylation plays a major role in the transcription and regulation of gene transcription. Histone acetylation and deacetylation are induced by histone acetyltransferases and histone deacetylases (HDACs) activities, respectively. HDACs comprise a family of 18 enzymes classified I-IV. In mammals, these enzymes are classified into HDACs I-III (5). The potential reversibility of epigenetic alterations offers valuable opportunities for novel anticancer drugs that can reactivate epigenetically silenced TSGs. DNMTs and HDACs inhibition have been approved for the treatment of various cancers (6).

DNMT inhibitors (DNMTIs) represent promising new compounds for cancer treatment. There are several compounds such as 5-aza,2'-deoxycytidine (5-azaCdR), and zebularine that have been characterized as DNMTIs. The most broadly-used DNMTIs, 5-azaCdR which is clinically named decitabine, has been indicated to be capable of transcriptionally activating TSGs with unmethylated promoters, also leading to increasing histone acetylation (7).

In addition to DNMTIs, some HDAC inhibitors (HDACIs) cause tumor cell growth inhibition. These compounds represent a broad family of chemical agents that includes four main structural groups comprising short-chain fatty acids (*e.g.* butyrate and valproic acid, VPA), hydroxamates (such as trichostatin A and benzamides (*e.g.* MS-275/SNDX-275), and cyclic tetrapeptides (8). Previously, we investigated the effect of VPA on human hepatocellular carcinoma and colon cancer cell lines (9).

HDACIs play their apoptotic roles through various molecular mechanisms. Recently, *in vitro* studies have shown that HDACIs exert their apoptotic role through various molecular mechanisms. They induce apoptosis *via* mitochondrial (intrinsic pathway) and death receptor (extrinsic pathway) (10). HDACIs activates the extrinsic apoptotic pathway which is initiated by the binding of death receptors, including tumor necrosis factor receptor-1 (TNFR-1), Fas (Apo-1 or CD95), TNF-related apoptosis-inducing ligand (TRAIL or Apo2-L) receptors (DR-4 and -5), DR-3 (Apo3) and DR-6 to their ligands, such as TNF, FAS ligand (FAS-L), TRAIL, and TL1A (Apo3L), resulting in activation of caspases. These compounds can upregulate the expression of both death receptors and their ligands of transformed cells *in vitro* and *in vivo*. In addition to the extrinsic pathway, these agents activate the intrinsic apoptotic pathway which is mediated by mitochondria, with the release of mitochondrial intermembrane proteins, such as cytochrome c, and the consequent activation of caspases. It is regulated, in part, by pro and anti-apoptotic proteins of the Bcl-2 family. HDACIs up-regulate pro-apoptotic proteins of the Bcl-2 families, such as Bim, Bmf, Bak, Bax, and Bik (11).

Furthermore, DNMTIs can induce cell apoptosis through both intrinsic and extrinsic apoptotic pathways (12). Other researchers have shown that HDACIs can play their roles through the JAK/STAT pathway, up-regulation of the suppressors of cytokine signaling (SOCS) family (*e.g.* SOCS1 and SOCS3), and inhibition of JAK/STAT signaling (13). A similar pathway has been reported for DNMTIs (14).

Our previous work indicated that trichostatin A and zebularine can up-regulate SOCS-1 and SOCS-3 gene expression in colon carcinoma SW48 cell lines (15). All HDACIs have been reported to activate either an extrinsic or intrinsic pathway or both of these cell death pathways in many cancer models. Additionally, it has been reported that VPA plays its apoptotic role in a tissue, time, and dose-dependent manner. Therefore, VPA can indicate different effects depending on the cell line, time, and concentration (16).

Regarding the aforementioned manner of VPA, we decided to examine its effect on neuroblastoma and glioblastoma cell lines. It could be the novelty of the present study. The present study was designed to investigate the effect of VPA on extrinsic (FAS, FAS-L, DR4, DR5, and TRAIL genes), intrinsic (pro-apoptotic genes: Bak, Bax, and Bim, and anti-apoptotic genes: Bcl-2, Bcl-xL, and

Mcl-1), and JAK/STAT (SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B genes) pathways in neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cell lines.

MATERIALS AND METHODS

Human neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cell lines were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, I.R. Iran. VPA and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). To make a work-stock solution the VPA was dissolved in sterile water. By diluting the stock solution, further experimental concentrations were provided. This study was approved by the Ethics Committee of the Jahrom University of Medical Science with a code number of IR.JUMS.REC.1399.078.

Cell culture and cell viability

Human neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cells were cultured in DMEM supplemented with fetal bovine serum 10% (Nichirei Biosciences, Inc., Tokyo, Japan) and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) at 37 °C in 5% CO_2 for 24 h. Subsequently, all of the neuroblastoma and glioblastoma cell lines were seeded into 96-well plates (3×10^5 cells per well). After one day, the culture medium was removed and a culture medium containing various concentrations of VPA was added. The human neuroblastoma and glioblastoma cells were treated with VPA (0, 0.5, 1, 2.5, 5, 7.5, 10, and 15 μM) for 24 h, the control cells were treated with the same amount of solvent, sterile water (concentration zero of VPA). After 24 h of treatment with VPA, all treated and untreated neuroblastoma and glioblastoma cells were evaluated by MTT assay to obtain cell viability, and the MTT solution (5 mg/mL) was added to each well and allowed to incubate for 4 h at 37 ℃. To dissolve all of the crystals, the solution was replaced by DMSO for 10 min. Subsequently, the absorbance spectrum was determined by a microplate reader (Bio-Rad Hercules, CA, USA) at a wavelength of 570 nM.

Cell apoptosis assay

To determine cell apoptosis, the neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cells were cultured at a density of 3×10^5 cells/well and incubated overnight. Then, all of the cell lines were treated with VPA (IMR-32: 2.697 μM, UKF-NB-2: 2.560 μM, SK-N-AS: 2.391 μM, UKF-NB-3: 3.341 μM, UKF-NB-4: 3.703 μM, SF-763: 6.809 μM, SF-767: 5.385 μM, A-172: 6.269 μM, U-87 MG: 5.999 μM, and U-251 MG: 6.165 μM), based on the half-maximal inhibitory concentration (IC_{50}) values (Table 1) for 24 h, the control groups were treated with the same amount of solvent, sterile water. The neuroblastoma and glioblastoma cells were prepared for flow cytometry by trypsinization, washing twice with cold phosphate-buffered saline (PBS), and then stained with annexin V-fluorescein isothiocyanate (FITC; eBioscience Inc., San Diego, CA, USA) and propidium iodide (PI; eBioscience Inc., San Diego, CA, USA). The apoptotic cells were determined by FACScan flow cytometry.

Table 1. The IC_{50} values of valproic acid in neuroblastoma and glioblastoma cell lines after 24 h.

Cell line	IC_{50} (μM)	Log (IC_{50})	R squared
Neuroblastoma IMR-32	2.697	-0.09449 to 0.9620	0.8557
Neuroblastoma SK-N-AS	2.391	-0.1631 to 0.9238	0.8405
Neuroblastoma UKF-NB-2	2.560	0.01641 to 0.7996	0.9090
Neuroblastoma UKF-NB-3	3.341	0.02896 to 1.075	0.8505
Neuroblastoma UKF-NB-4	3.703	0.1668 to 0.9980	0.9046
Glioblastoma SF-767	5.385	0.3505 to 1.168	0.9142
Glioblastoma SF-763	6.809	0.5523 to 1.152	0.9471
Glioblastoma A-172	6.269	0.4999 to 1.130	0.9435
Glioblastoma U-87 MG	5.999	0.3959 to 1.228	0.9077
Glioblastoma U-251 MG	6.165	0.3523 to 1.321	0.8866

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was done to determine the relative expression of apoptotic extrinsic genes (FAS, FAS-L, DR4, DR5, and TRAIL), intrinsic genes (pro-apoptotic genes including Bak, Bax, and Bim and anti-apoptotic genes including Bcl-2, Bcl-xL, and Mcl-1), and JAK/STAT genes (SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B). The neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cells were cultured at a density of 3×10^5 cells/well and treated with VPA (IMR-32: 2.697 µM, UKF-NB-2: 2.560 µM, SK-N-AS: 2.391 µM, UKF-NB-3: 3.341 µM, UKF-NB-4: 3.703 µM, SF-763: 6.809 µM, SF-767: 5.385 µM, A-172: 6.269 µM, U-87 MG: 5.999 µM, and U-251 MG: 6.165 µM), based on IC_{50} values (Table 1), for 24 h, except the control groups which were treated with sterile water only. qRT-PCR was performed to estimate the expression of target genes using qRT-PCR arrays according to the manufacturer's protocol. We extracted total RNA from each cell line by RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and then was treated it with RNase-free DNase (Qiagen, Hilden, Germany) to eliminate the genomic DNA. The RNA concentration was determined using a BioPhotometer (Biowave II, Germany). Total RNA (100 ng) was reverse-transcribed to complementary DNA (cDNA) using the RevertAid™ first-strand cDNA synthesis kit (Fermentas, K1622 for 100 reactions, USA) according to the manufacturer's instructions. Next, qRT-PCR was performed by the Maxima™ SYBR Green/ROX qPCR master mix (2 × 1.25 mL, K0221). GAPDH was used as an internal control. RT-PCR reactions were performed using the Steponeplus (BD FACs Calibur StepOne plus v2.2, Applied Biosystems, USA). Thermal cycling conditions are followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s. Cycle threshold (Ct) values were transformed to the gene copy number of the template cDNA using the comparative Ct method (also known as the $2^{-\Delta\Delta Ct}$ method). The primer sequences (17-30) used in this study are demonstrated in Table 2

Statistical analysis

Data, indicated as mean ± SD, from three independent experiments were analyzed with a one-way analysis of variance (ANOVA) followed by Turkey using Graphpad Prism Software version 8.0. $P < 0.05$ was considered significant.

Table 2. The primer sequences of the genes used in the current study.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Product length	Reference
DR4	CAGAACATCCTGGAGCCTGTAAC	ATGTCCATTGCCTGATTCTTTGTG	299 bp	17
DR5	TGCAGCCGTAGTCTTGATTG	GCACCAAG TCTGCAAAGTCA	389 bp	17
FAS	TTCTGCCATAAGCCCTGTCC	TGTACTCCTTCCCTTCTTGG	103 bp	18
FAS-L	GCCTGTGTCTCCTTGTGATG	TGGACTTGCCTGTTAAATGGG	222 bp	18
TRAIL	GAAGCAACACATTGTCTTCTCCAA	TTGCTCAGGAATGAATGCCC	103 bp	19
Bax	AGTAACATGGAGCTGCAGAGGAT	GCTGCCACTCGGAAAAAGAC	77 bp	20
Bak	CCTGCCCTCTGCTTCTGA	CTGCTGATGGCGGTAAAAA	82 bp	21
Bim	ATTACCAAGCAGCCGAAGAC	TCCGCAAAGAACCTGTCAAT	101 bp	22
Bcl-2	TGGCCAGGGTCAGAGTTAAA	TGGCCTCTCTTGCGGAGTA	147 bp	23
Bcl-xL	TCCTTGTCTACGCTTTCCACG	GGTCGCATTGTGGCCTTT	62 bp	24
Mcl-1	AAAGCCTGTCTGCCAAAT	CCTATAAACCCACCACTC	198 bp	25
SOCS1	TTTTTCGCCCTTAGCGTGA	AGCAGCTCGAAGAGGCAGTC	119 bp	26
SOCS3	GGCCACTCTTCAGCATCTC	ATCGTACTGGTCCAGGAACTC	109 bp	27
JAK1	CCACTACCGGATGAGGTTCTA	GGGTCTCGAATAGGAGCCAG	213 bp	28
JAK2	GATGAGAATAGCCAAAGAAAACG	TTGCTGAATAAATCTGCGAAAT	160 bp	29
STAT3	GCTTTTGTCAGCGATGGAGT	ATTTGTTGACGGGTCTGAAGTT	174 bp	29
STAT5A	AATGAGAACACCCGCAACG	TTCCTGAAGTGGGCACTGAG	101 bp	29
STAT5B	ACTGCTAAAGCTGTTGATGGATAC	TGAGTCAGGGTTCTGTGGGTA	174 bp	29
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT	116 bp	30

RESULTS

Cell viability

The cell viability of the neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cells treated with VPA (0, 0.5, 1, 2.5, 5, 7.5, 10, and 15 μM) for 24 h was investigated by MTT assay to determine cell viability and IC_{50} values (Table 1). Figures 1 and 2 show that VPA induced a significant cell growth inhibition in all treated groups in a concentration-dependent manner. This experiment was repeated three times for each group. The IC_{50} values of VPA for each cell line were obtained as follows; IMR-32: 2.697 μM, UKF-NB-2: 2.560 μM, SK-N-AS: 2.391 μM, UKF-NB-3: 3.341 μM, UKF-NB-4: 3.703 μM, SF-763: 6.809 μM, SF-767: 5.385 μM, A-172: 6.269 μM, U-87 MG: 5.999 μM, and U-251 MG: 6.165 μM.

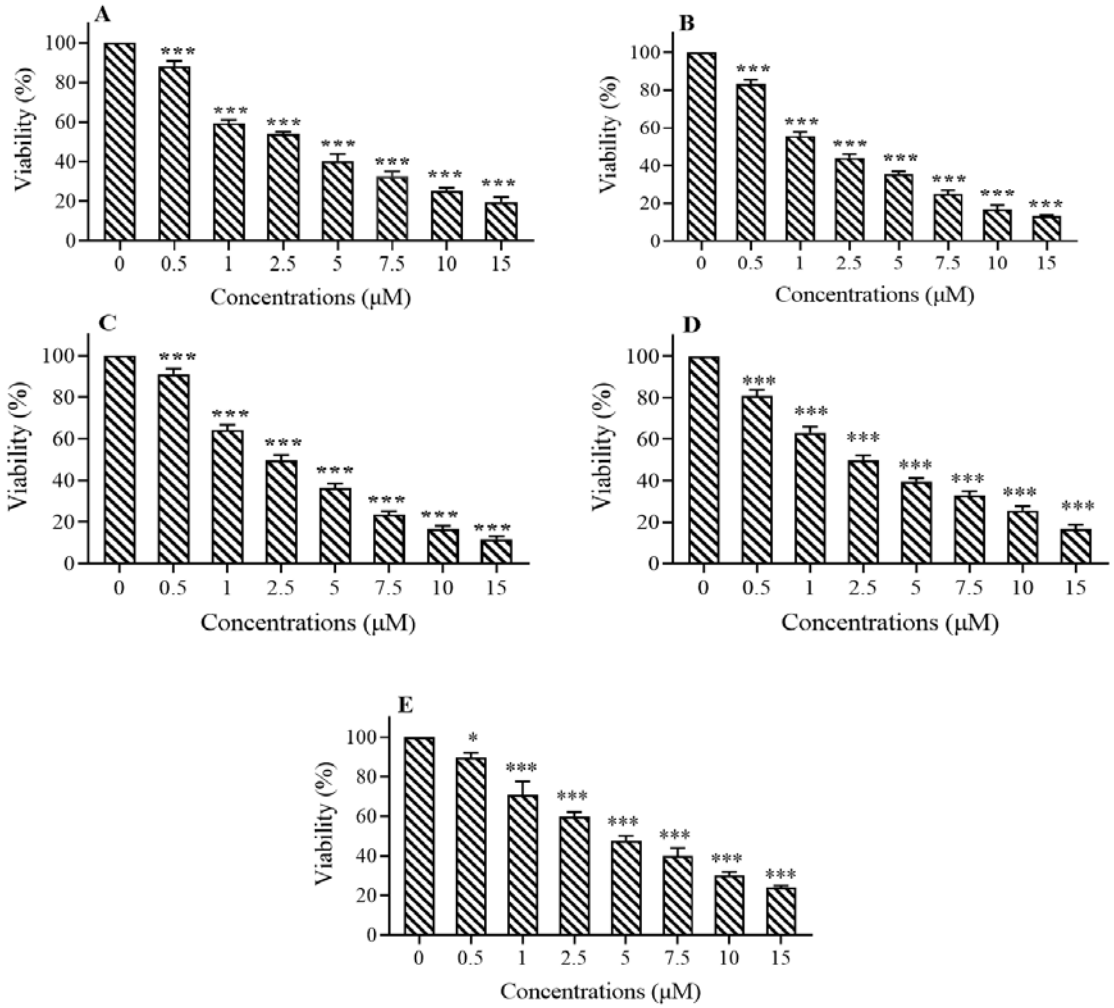

Fig. 1. The effect of VPA on the viability of neuroblastoma cell lines; (A) IMR-32, (B) SK-N-AS, (C) UKF-NB-2, (D) UKF-NB-3, and (E) UKF-NB-4. The cells were treated with different concentrations of VPA for 24 h and then the cell viability was investigated by MTT assay. **P* < 0.05 and ****P* < 0.001 indicate significant differences compared to the control group (untreated cells). VPA, Valproic acid.

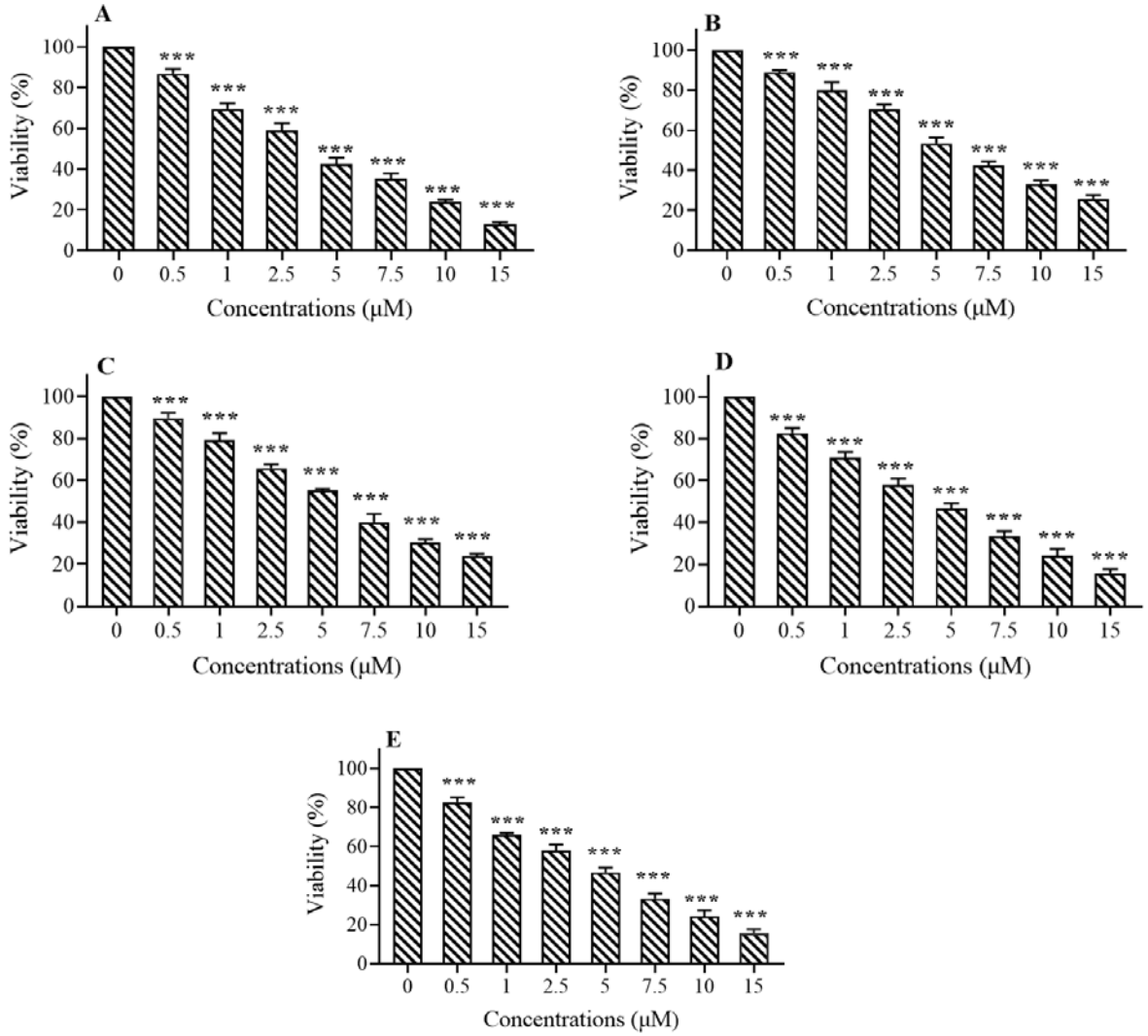

Fig. 2. The effect of VPA on the viability of glioblastoma cell lines; (A) (A) SF-767, (B) SF-763, (C) A-172, (D) U-87 MG, and (E) U-251 MG). The cells were treated with different concentrations of VPA for 24 h and then the cell viability was investigated by MTT assay. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells). VPA, Valproic acid.

Cell apoptosis

As indicated in Figs. 3-5, VPA significantly induced apoptosis in all cell lines compared to their respective control groups. Percentage of apoptotic cells were obtained as follows; IMR-32: 96.44%, UKF-NB-2: 94.6%, SK-N-AS: 93.9%, UKF-NB-3: 96.83%, UKF-NB-4: 96.99%, SF-763: 85.4%, SF-767: 86.2%, A-172: 91.9%, U-87 MG: 88.97%, and U-251 MG: 87.94%. Maximal and minimal cell apoptosis percentages were seen in UKF-NB-4 and SF-763 cells treated with VPA, respectively.

Gene expression in neuroblastoma cells

The effect of VPA on apoptotic extrinsic pathway

The expression level of the FAS, FAS-L, DR4, DR5, and TRAIL genes was investigated in neuroblastoma cells (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) which were treated with VPA at IC_{50} values (Table 2). The result demonstrated that treatment with VPA significantly up-regulated the expression level of FAS, FAS-L, DR4, DR5, and TRAIL genes in comparison with the control group (Fig. 6).

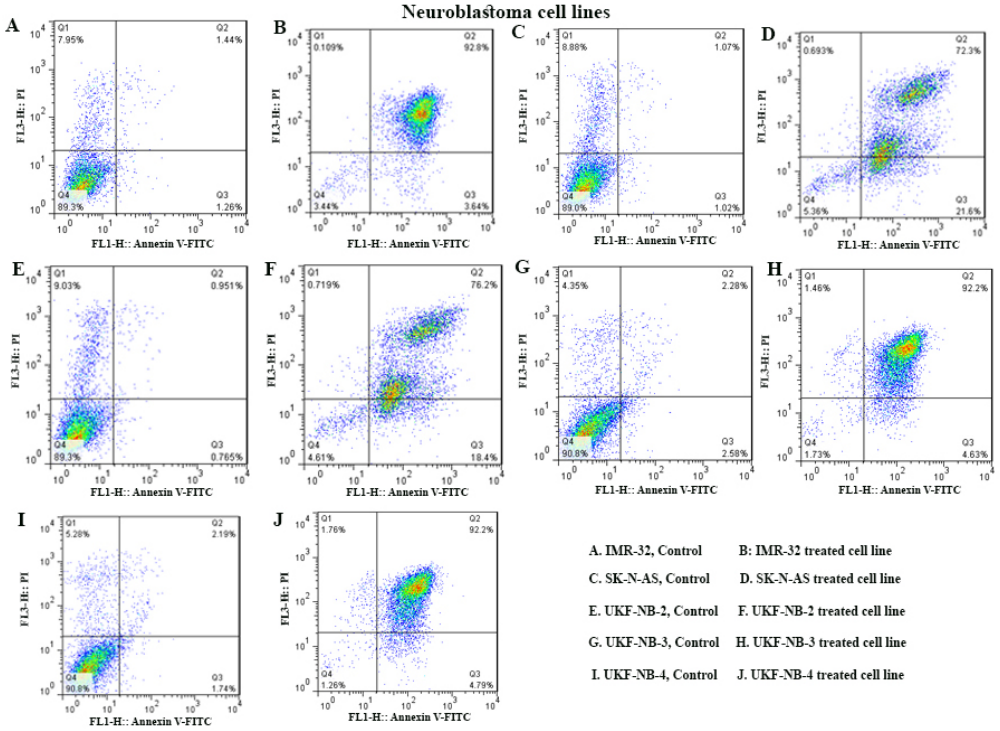

Fig. 3. Evaluation of the apoptosis-inducing effect of VPA on neuroblastoma cells through flow cytometry plots using annexin V-FITC/ propidium iodide (PI) staining. The cells treated with VPA at IC_{50}s; IMR-32: 2.697 µM, UKF-NB-2: 2.560 µM, SK-N-AS: 2.391 µM, UKF-NB-3: 3.341 µM, UKF-NB-4: 3.703 µM. VPA, Valproic acid.

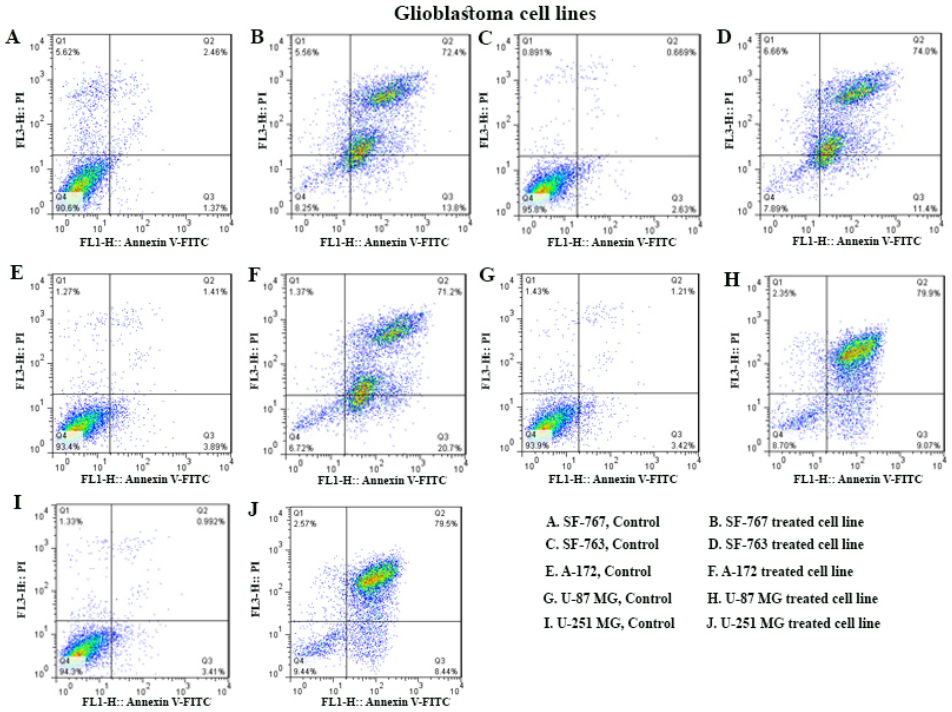

Fig. 4. Evaluation of the apoptosis-inducing effect of VPA on glioblastoma cells through flow cytometry plots using annexin V-FITC/ propidium iodide (PI) staining. The cells treated with VPA at IC_{50}s; SF-767: 5.385 µM, SF-763: 6.809 µM, A-172: 6.269 µM, U-87 MG: 5.999 µM, and U-251 MG: 6.165 µM. VPA, Valproic acid.

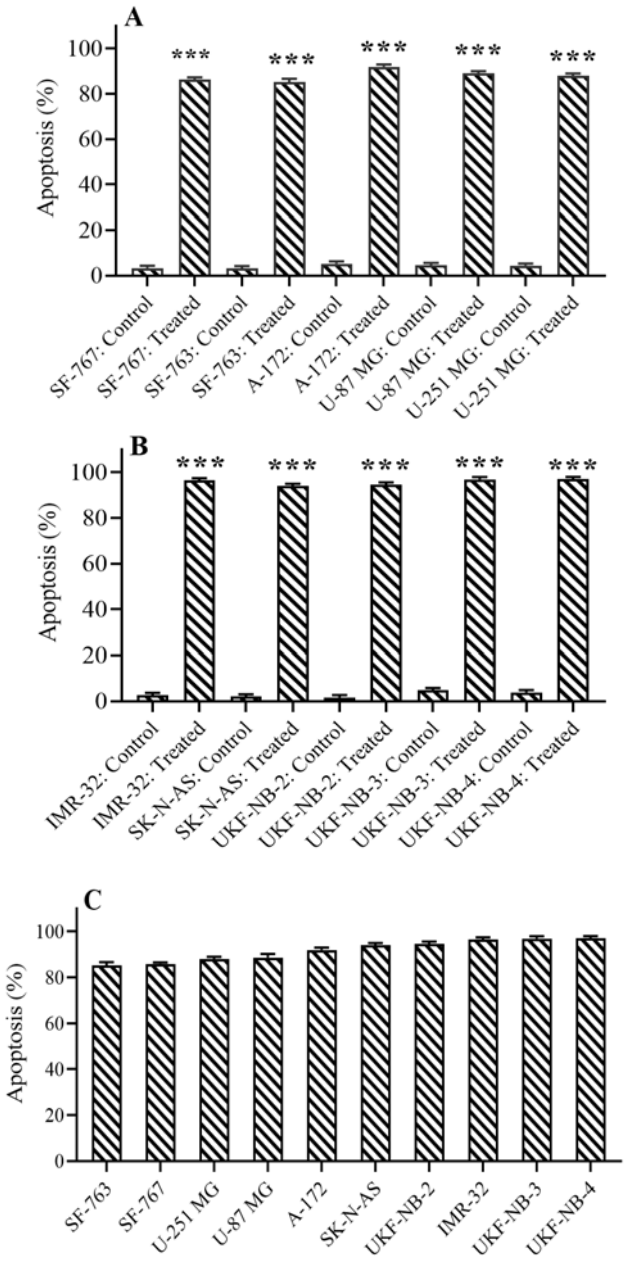

Fig. 5. The apoptotic effect of VPA on glioblastoma and neuroblastoma cells. (A) Glioblastoma cell lines treated with VPA at IC_{50}s for 24 h; SF-763: 6.809 µM, SF-767: 5.385 µM, A-172: 6.269 µM, U-87 MG: 5.999 µM, and U-251 MG: 6.165 µM versus their respective control groups; (B) neuroblastoma cell lines treated with VPA at IC_{50}s for 24 h; IMR-32: 2.697 µM, UKF-NB-2: 2.560 µM, SK-N-AS: 2.391 µM, UKF-NB-3: 3.341 µM, UKF-NB-4: 3.703 µM versus their respective control groups; (C) comparative analysis of the effect of VPA on neuroblastoma and glioblastoma cells. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells). VPA, Valproic acid.

The effect of VPA on apoptotic intrinsic pathway

The expression level of the Bak, Bax, Bim, Bcl-xL, Bcl-2 and Mcl-1 genes were determined in neuroblastoma cells (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) which were treated with VPA at IC_{50} values (Table 2). The results of the qRT-PCR indicated that treatment with VPA leads to up-regulating the expression level of Bak, Bax, and Bim genes and down-regulating the expression level of Bcl-xL, Bcl-2, and Mcl-1 genes significantly (Fig. 7).

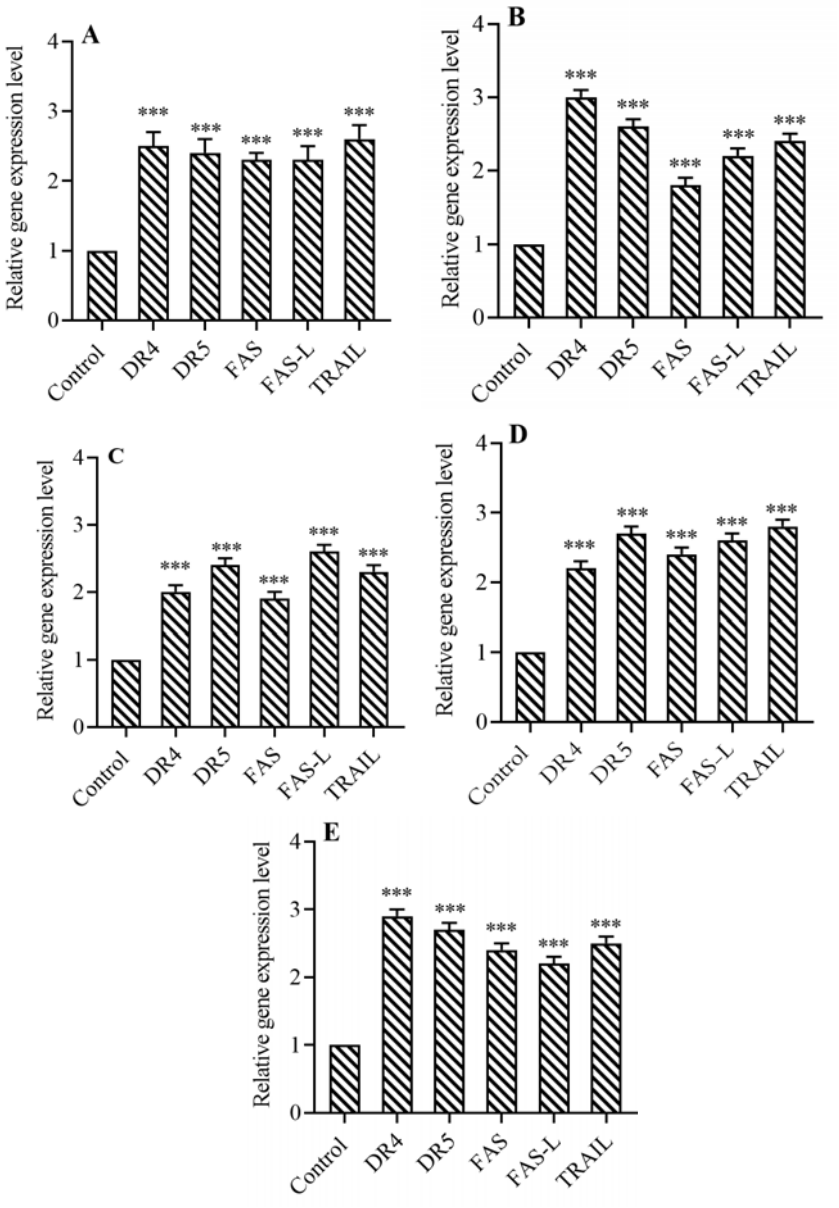

Fig. 6. The relative expression level of DR4, DR5, FAS, FAS-L, and TRAIL genes, involved in the extrinsic apoptotic pathway, in neuroblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) IMR-32: 2.697 μM, (B) SK-N-AS: 2.391 μM, (C) UKF-NB-2: 2.560 μM, (D) UKF-NB-3: 3.341 μM, and (E) UKF-NB-4: 3.703 μM. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

The effect of VPA on apoptotic JAK/STAT pathway

To determine the expression level of the SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B genes, neuroblastoma cells (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) were treated with VPA at IC_{50} values (Table 2). The results of qRT-PCR demonstrated that treatment with VPA up-regulates the expression level of SOCS1 and SOCS3 genes and down-regulates the expression level of JAK1, JAK2, STAT3, STAT5A, and STAT5B genes significantly (Fig. 8).

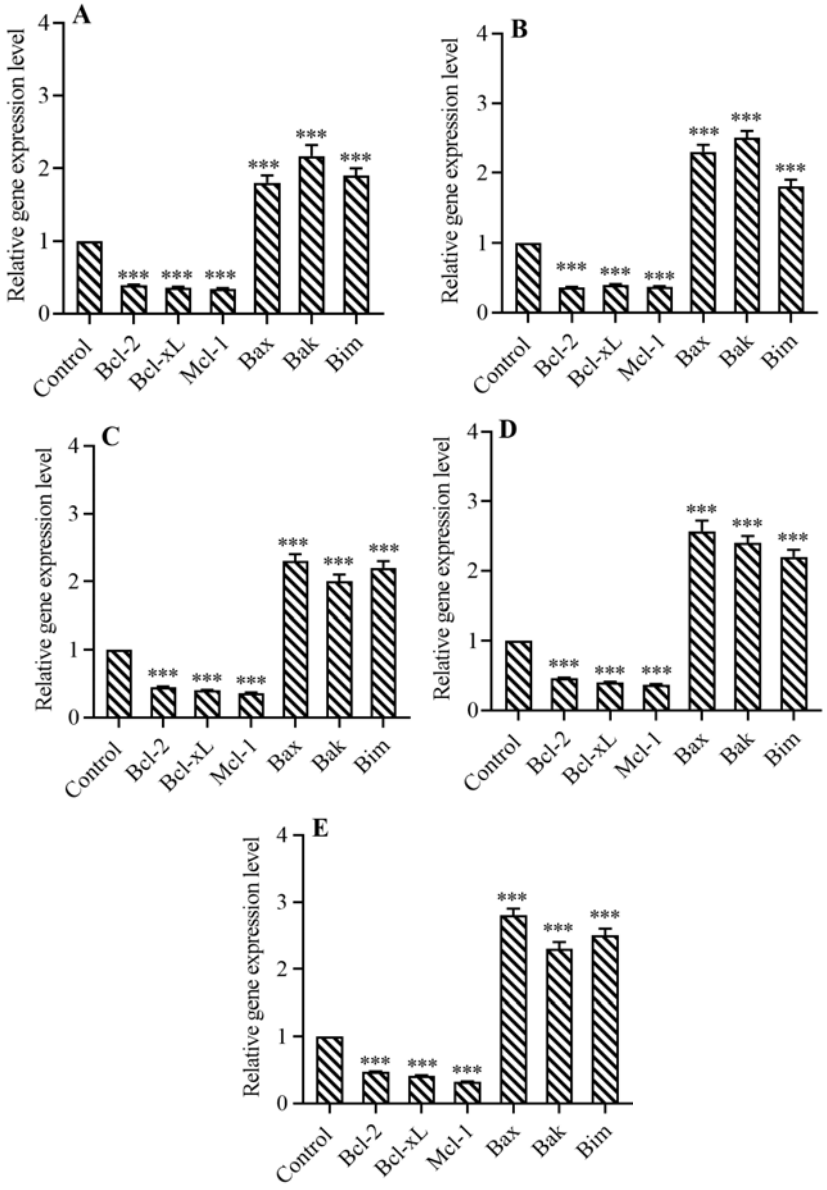

Fig. 7. The relative expression level of Bcl-2, Bcl-xL, Mcl-1 Bax, Bak, and Bim genes, involved in the extrinsic apoptotic pathway, in neuroblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) IMR-32: 2.697 µM, (B) SK-N-AS: 2.391 µM, (C) UKF-NB-2: 2.560 µM, (D) UKF-NB-3: 3.341 µM, and (E) UKF-NB-4: 3.703 µM. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

Genes expression in glioblastoma cells

The effect of VPA on apoptotic extrinsic pathway

To determine the expression level of the FAS, FAS-L, DR4, DR5, and TRAIL genes, glioblastoma cells (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) were treated with VPA IC_{50} values (Table 2). The results of qRT-PCR demonstrated that treatment with VPA led to up-regulating the expression of FAS, FAS-L, DR4, DR5, and TRAIL genes in A-172, U-87 MG, and U-251 MG cell lines significantly. Besides, it up-regulated FAS, FAS-L, and TRAIL gene expression in SF-767 and SF-763 cell lines significantly, whereas it could not induce a significant up-regulation of DR4 and DR5 genes in these cell lines (Fig. 9). Therefore, we decided to evaluate the

expression of these genes with high concentrations and more duration of VPA. In this regard, these two cell lines (SF-767 and SF-763) were treated with VPA at 7.5 μM and 10 μM for 24 h to evaluate FAS, FAS-L, DR4, DR5, and TRAIL genes expression. Additionally, we treated these two cell lines with the previous concentrations of VPA (SF-767 cell line treated with 5.3 and SF-763 cell line with 6.8 μM) for 48 h and 72 h to evaluate FAS, FAS-L, DR4, DR5, and TRAIL genes expression. Both methods indicated that VPA up-regulated DR4 and DR5 gene expressions after 48 and 72 h, significantly. In addition to these genes, it induced a strong up-regulation of the expression of other genes (FAS, FAS-L, and TRAIL) (Figs. 10-11).

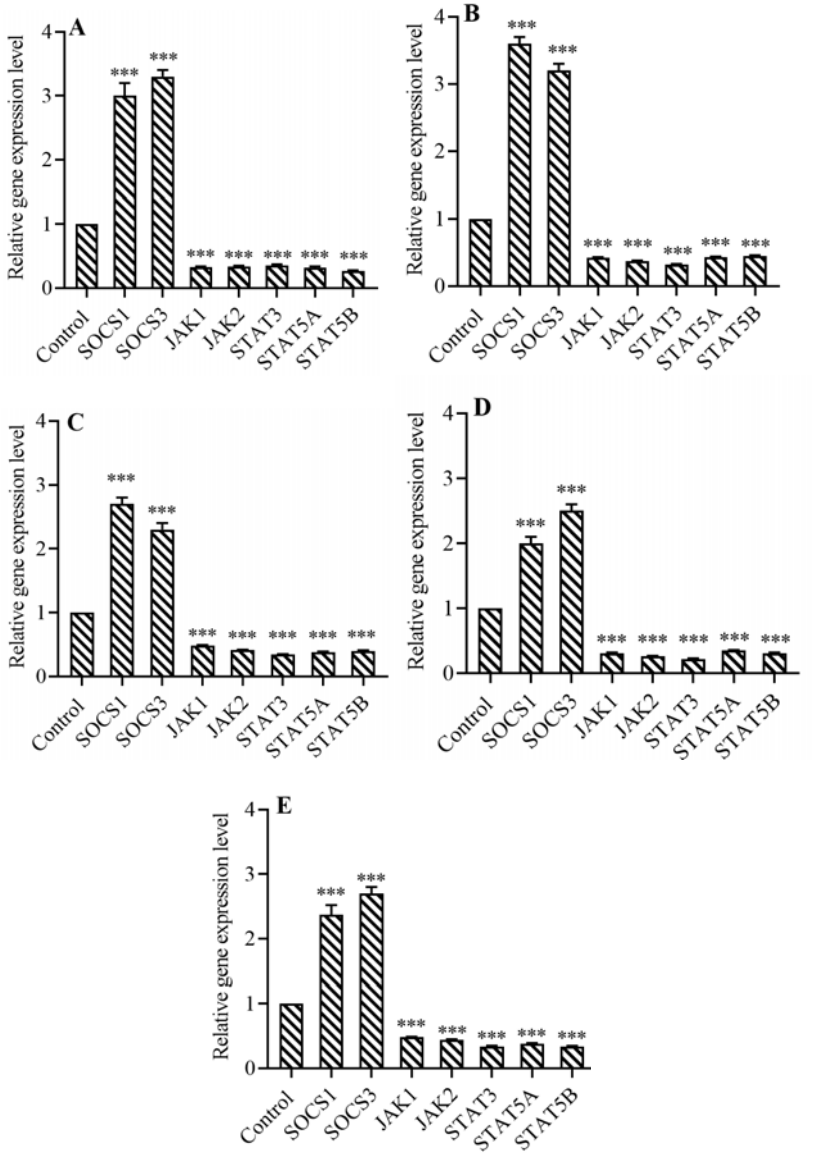

Fig. 8. The relative expression level of SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B genes, involved in the extrinsic apoptotic pathway, in neuroblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) IMR-32: 2.697 μM, (B) SK-N-AS: 2.391 μM, (C) UKF-NB-2: 2.560 μM, (D) UKF-NB-3: 3.341 μM, and (E) UKF-NB-4: 3.703 μM. ***P < 0.001 indicates significant differences compared to the control group (untreated cells).

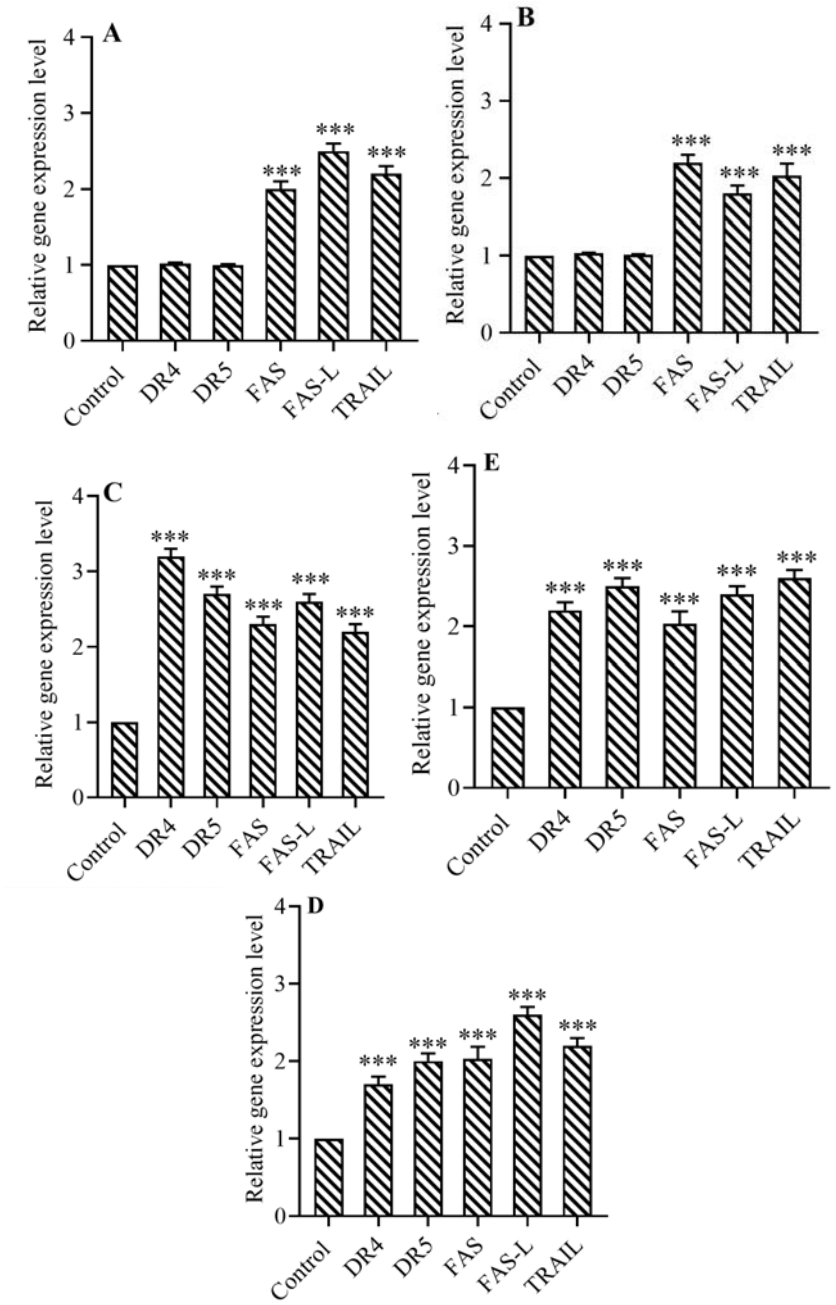

Fig. 9. The relative expression level of DR4, DR5, FAS, FAS-L, and TRAIL genes, involved in the extrinsic apoptotic pathway, in glioblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) SF-767: 5.385 μM, (B) SF-763: 6.809 μM, (C) A-172: 6.269 μM, (D) U-87 MG: 5.999 μM, and (E) U-251 MG: 6.165 μM). ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

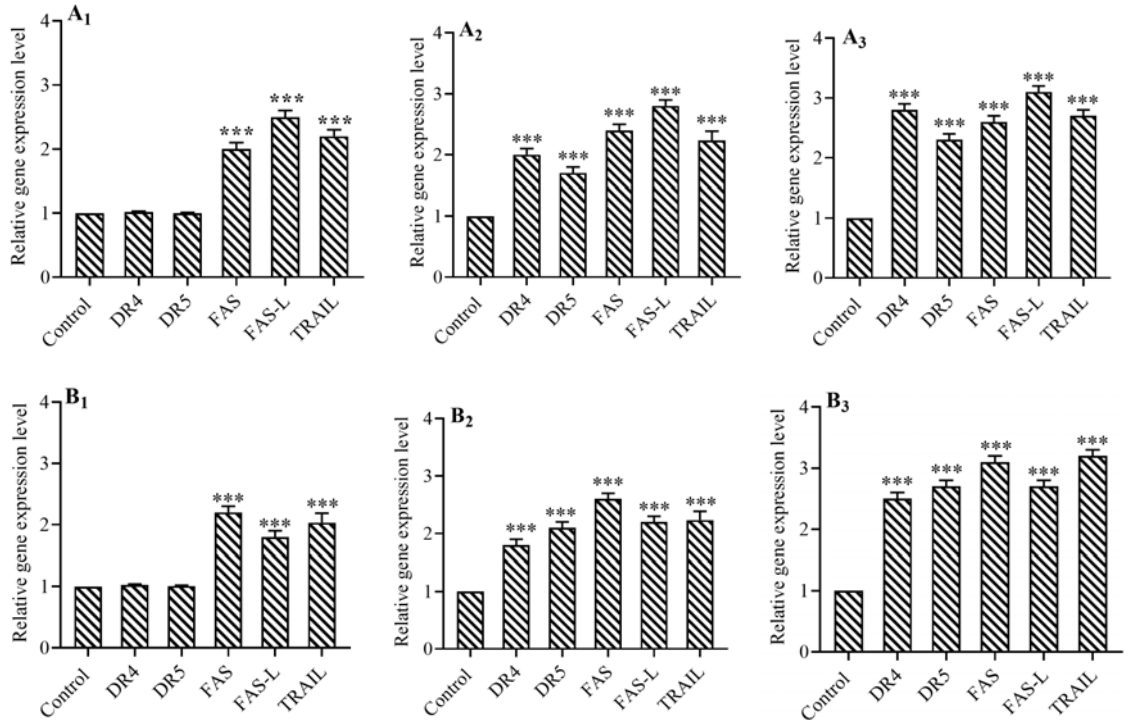

Fig. 10. The relative expression level of DR4, DR5, FAS, FAS-L, and TRAIL genes, involved in the extrinsic apoptotic pathway, in glioblastoma (A) SF-767 and (B) SF-763 cell lines treated for 24 h with valproic acid at IC_{50}s (A_1 and B_1) 6.809 Mm, (A_2 and B_2) 7.5 Mm, and (A_3 and B_3) 10 Mm. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

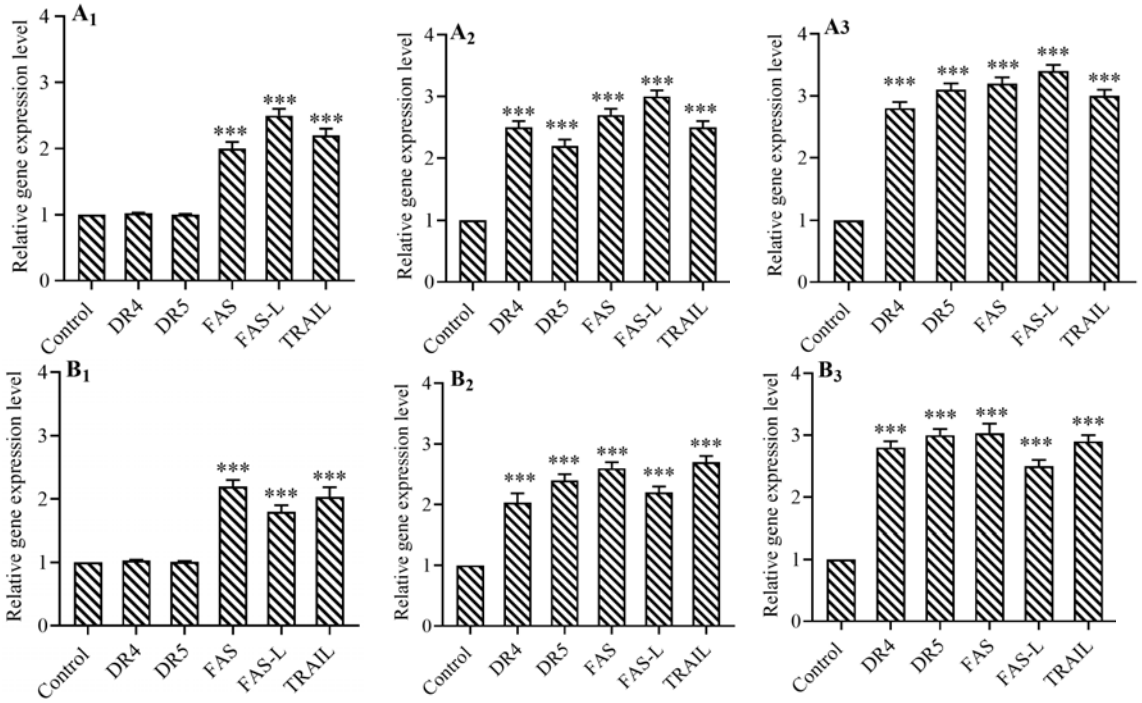

Fig. 11. The relative expression level of DR4, DR5, FAS, FAS-L, and TRAIL genes in the glioblastoma cell line. (A^1-A^3) SF-767 cells were treated with valproic acid at 5.385 μM for 24, 48, and 72 h; and (B^1-B^3) SF-763 cells treated with valproic acid at 6.809 μM for 24, 48, and 72 h. ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

The effect of VPA on apoptotic intrinsic pathway

To determine the expression level of the Bak, Bax, Bim, Bcl-xL, Bcl-2, and Mcl-1 genes, glioblastoma cells (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) were treated with VPA at IC_{50} values (Table 2). The results of qRT-PCR indicated that treatment with VPA upregulated the expression level of Bax, Bak, and Bim genes and down-regulated the expression level of Bcl-xL, Bcl-2, and Mcl-1 genes significantly (Fig. 12).

The effect of VPA on apoptotic JAK/STAT pathway

The expression level of the SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B genes was determined in glioblastoma cells (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) which were treated with VPA at IC_{50} values (Table 2). The relative expression level was evaluated by analysis. The results of qRT-PCR indicated that treatment with VPA up-regulated the expression of the SOCS1 and SOCS3 and down-regulated the expression of JAK1, JAK2, STAT3, STAT5A, and STAT5B genes significantly (Fig. 13).

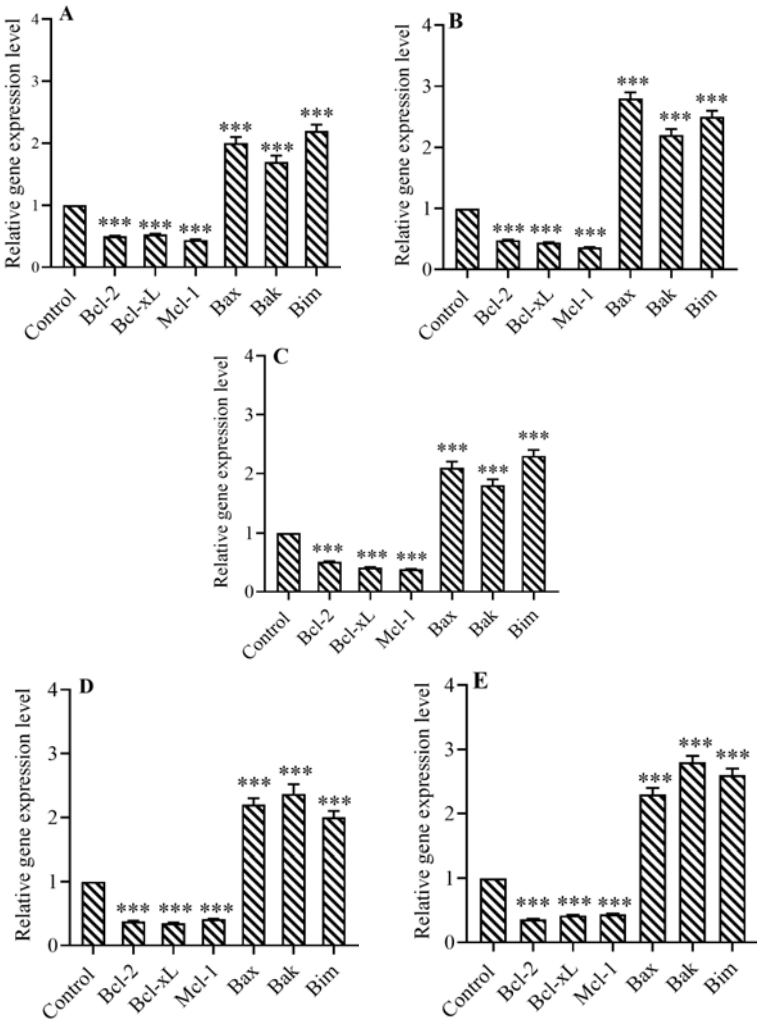

Fig. 12. The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, and Bim genes, involved in the extrinsic apoptotic pathway, in glioblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) SF-767: 5.385 µM, (B) SF-763: 6.809 µM, (C) A-172: 6.269 µM, (D) U-87 MG: 5.999 µM, and (E) U-251 MG: 6.165 µM). ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

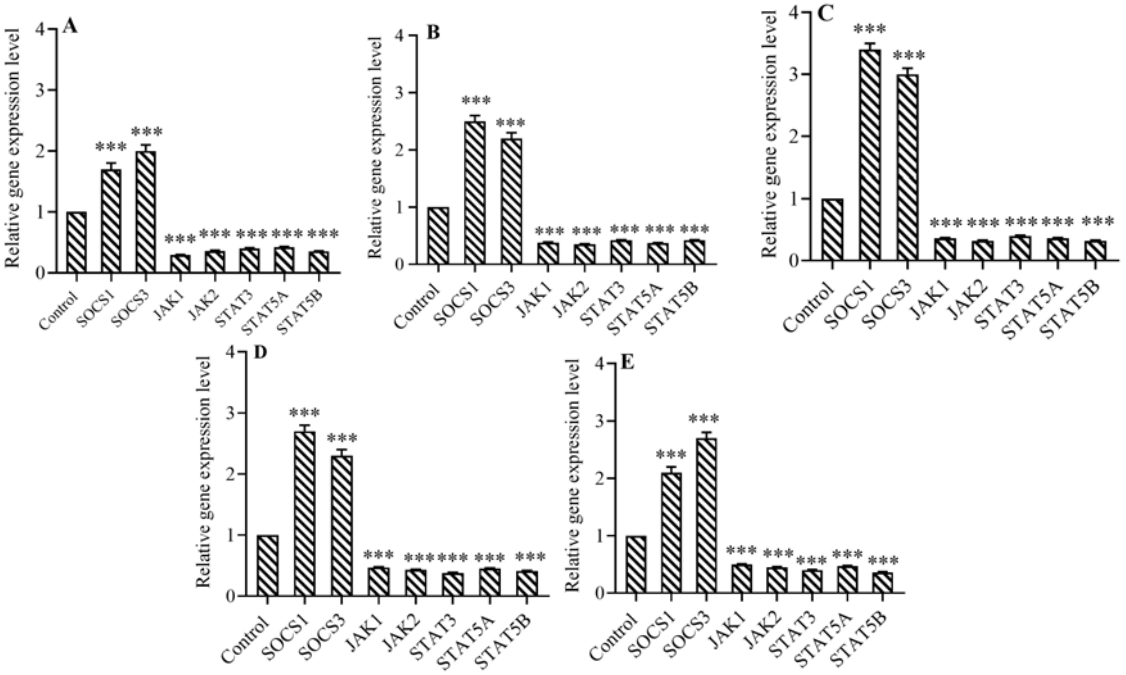

Fig. 13. The relative expression level of SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B genes, involved in the extrinsic apoptotic pathway, in glioblastoma cell lines treated for 24 h with valproic acid at IC_{50}s; (A) SF-767: 5.385 μM, (B) SF-763: 6.809 μM, (C) A-172: 6.269 μM, (D) U-87 MG: 5.999 μM, and (E) U-251 MG: 6.165 μM). ***$P < 0.001$ indicates significant differences compared to the control group (untreated cells).

DISCUSSION

Recently, *in vitro* studies have indicated that the effect of VPA on cell proliferation and differentiation of various cancer cells, *e.g.* neuroblastoma cells, is linked with its effect on HDACs activities. Several experimental works have shown that VPA plays its role through the reactivation of silenced TSGs (31).

It has been reported that VPA can induce apoptosis through intrinsic, extrinsic (32), and JAK/STAT (33) pathways. The result of the current study indicated that VPA can induce apoptosis *via* three molecular mechanisms including intrinsic, extrinsic, and JAK/STAT pathways. We demonstrated that VPA induced apoptosis by these mechanisms in the neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cell lines. The results indicated that VPA played its role through extrinsic, intrinsic, and JAK/STAT pathways, it up-regulated FAS, FAS-L, DR4, DR5, TRAIL, Bak, Bax, Bim, SOCS1, and SOCS3 and down-regulated Bcl-xL, Bcl-2, Mcl-1, JAK1, JAK2, STAT3, STAT5A, and STAT5B gene expression resulting in cell growth inhibition and apoptosis induction in the all of the neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) cell lines.

Similar to our report, Shankar *et al.* have shown that HDACIs such as suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydroxamide, MS-275, and trichostatin A induce apoptosis through both extrinsic and intrinsic pathways; up-regulation of DR4, DR5, Bim, Bak, Bax, PUMA, and Noxa, and down-regulation of Bcl-XL, Bcl-2, Mcl-1, and cFLIP, the release of mitochondrial proteins to the cytosol, and activation of caspases (34).

Several studies have reported that cell death pathways involve the intrinsic and extrinsic pathways, autophagic cell death, and mitotic catastrophe/cell death. The cell response to HDACIs depends on the nature of HDACIs, concentration and duration of exposure, and the cell context. The extrinsic apoptotic pathway is initiated by the binding of DRs, including Fas, TNFR-1, DR-3, DR-4, DR-5, and DR-6, to their ligands, such as FAS-L, TRAIL, TNF, and TL1A (Apo3L), leading to activation of

caspases (8 and 10). These compounds can up-regulate the expression of both death receptors and their ligands (35).

Similarly, Nakata *et al*. reported that HDACIs such as trichostatin A, SAHA, and sodium butyrate up-regulates death receptor expression (extrinsic pathway activation) in various cancer cell lines (36). Additionally, it has been reported that VPA induces apoptosis *via* the extrinsic (death receptor) apoptotic pathway (37). Besides, in a study by Catalano *et al*. it has been shown that VPA induces apoptosis by activating the intrinsic pathway, caspases 3 and 9 activations (38). Further, HDACIs SAHA, sodium butyrate, and trichostatin A strongly reduce neuroblastoma cell viability and induce cell cycle arrest in the G2/M phase by the activation of the BimEL and Bid and inactivation of the Bcl-xL, XIAP, and RIP (39). As we reported in this article VPA induced apoptosis and inhibited cell growth through extrinsic, intrinsic, and JAK/STAT pathways in glioblastoma (SF-767, SF-763, A-172, U-87 MG, and U-251 MG) cell lines.

Similar to our report, other researchers have shown that VPA increases the mitochondrial release of cytochrome c and apoptosis-inducing factor in human glioblastoma cells, intrinsic apoptotic activation (40). Furthermore, this compound decreases mRNA and protein levels of Bcl-2, whereas it increases Bax in the C6 glioma cell line (41).

The results from molecular studies have demonstrated that cell apoptosis occurs *via* activation of the extrinsic and intrinsic apoptosis pathways in glioblastoma C6 and T98G cells treated with HDACI, SAHA (42).

In addition, VPA could not upregulate the expression of DR4 and DR5 in glioblastoma cell lines (SF-763 and SF-767), it up-regulated FAS, FAS-L, and TRAIL genes expression in SF-767 and SF-763 cell lines significantly, whereas it could not induce significant up-regulation of DR4 and DR5 genes in these cell lines. Significant up-regulation of the expression of DR4 and DR5 genes were observed with increased concentration of VPA and duration of treatment in SF-767 and SF-763 cell line. Finally, VPA can induce apoptosis through several molecular mechanisms such as extrinsic, intrinsic, and JAK/STAT pathways in a time- and concentration-dependent manner. Inconsistent with our result, we could not find any study to report the significant effect of VPA on the extrinsic pathway in glioblastoma. Therefore, an investigation of high concentrations of VPA on other cell lines of glioblastoma is recommended.

CONCLUSION

Our findings indicated that VPA can induce its apoptotic effect through several pathways including extrinsic, intrinsic, and JAK/STAT pathways in neuroblastoma (IMR-32, UKF-NB-2, SK-N-AS, UKF-NB-3, and UKF-NB-4) and glioblastoma (SF-763, SF-767, A-172, U-87 MG, and U-251 MG) cell lines. Additionally, VPA changed the relative expression level of the genes involved in the extrinsic pathway in a concentration- and time-dependent manner. Our findings will form the basis of further research on the effects of VPA in neuroblastoma and glioblastoma cells. Because histone deacetylation is a potentially reversible change, the epigenetic histone modification by HDACIs such as VPA represents new opportunities for cancer management by reactivation of TSGs silencing including the genes associated with extrinsic, intrinsic, and JAK/STAT pathways. This efficacy of VPA in neuroblastoma and glioblastoma cancer cells suggests a role for VPA in the treatment of these cancers.

Acknowledgments

The present work was financially supported by the Adjutancy of Research of Jahrom University of Medical Sciences, Iran through Grant No. IR.JUMS.REC.1399.078.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

All authors contributed to the conception and design of the study, critical revision, acquisition of data, analysis and interpretation of data, manuscript writing, and revised the article. All authors approved the final revision of the article.

REFERENCES

1. Sanaei M, Kavoosi F. Effect of vorinostat on INK4 family and HDACs 1, 2, and 3 in pancreatic cancer and hepatocellular carcinoma. Res Pharm Sci. 2021;16(3):260-268. DOI: 10.4103/1735-5362.314824.
2. Liu X, Luo M, Wu K. Epigenetic interplay of histone modifications and DNA methylation mediated by HDA6. Plant Signal Behav. 2012;7(6):633-635. DOI: 10.4161/psb.19994.
3. Grønbaek K, Hother C, Jones PA. Epigenetic changes in cancer. Apmis. 2007;115(10):1039-1059. DOI: 10.1111/j.1600-0463.2007.apm_636.xml.x.
4. Naghitorabi M, Mohammadi Asl J, Mir Mohammad Sadeghi H, Rabbani M, Jafarian-Dehkordi A, Javanmard HS. Quantitative evaluation of DNMT3B promoter methylation in breast cancer patients using differential high resolution melting analysis. Res Pharm Sci. 2013;8(3):167-175. PMID: 24019826.
5. Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: what are the cancer relevant targets? Cancer Lett. 2009;277(1):8-21. DOI: 10.1016/J.CANLET.2008.08.016.
6. Ganesan A, Nolan L, Crabb SJ, Packham G. Epigenetic therapy: histone acetylation, DNA methylation and anti-cancer drug discovery. Curr Cancer Drug Targets. 2009;9(8):963-981. DOI: 10.2174/156800909790192428.
7. Laird PW. Cancer epigenetics. Hum Mol Genet. 2005;14(Suppl1):R65-R76. DOI:10.1093/hmg/ddi113.
8. Walkinshaw DR, Yang XJ. Histone deacetylase inhibitors as novel anticancer therapeutics. Curr Oncol. 2008;15(5):237-243. PMID: 19008999.
9. Kavoosi F, Sanaei M. Comparative analysis of the effects of valproic acid and tamoxifen on proliferation, and apoptosis of human hepatocellular carcinoma WCH 17 celllin. Iran J Ped Hematol Oncol. 2018;8(1):12-20.
10. Sanaei M, Kavoosi F. Histone deacetylases and histone deacetylase inhibitors: molecular mechanisms of action in various cancers. Adv Biomed Res. 2019;8:63-72. DOI: 10.4103/abr.abr_142_19.
11. Xu SW, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene. 2007;26(37):5541-5552. DOI: 10.1038/sj.onc.1210620.
12. Wang H, Cai S, Ernstberger A, Bailey BJ, Wang MZ, Cai W, *et al*. Temozolomide-mediated DNA methylation in human myeloid precursor cells: differential involvement of intrinsic and extrinsic apoptotic pathways. Clin Cancer Res. 2013;19(10):2699-2709. DOI: 10.1158/1078-0432.CCR-12-2671.
13. Xiong H, Du W, Zhang YJ, Hong J, Su W-Y, Tang J-T, *et al*. Trichostatin A, a histone deacetylase inhibitor, suppresses JAK2/STAT3 signaling via inducing the promoter-associated histone acetylation of SOCS1 and SOCS3 in human colorectal cancer cells. Mol carcinog. 2012;51(2):174-184. DOI: 10.1002/mc.20777.
14. Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, *et al*. DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development. 2005;132(15):3345-3356. DOI: 10.1242/dev.01912.
15. Sanaei M, Kavoosi F, Behjoo H. Effect of valproic acid and zebularine on SOCS-1 and SOCS-3 gene expression in colon carcinoma SW48 cell line. Exp Oncol. 2020;42(3):183-187. DOI: 10.32471/exp-oncology.2312-8852.vol-42-no-3.15113.
16. Kortenhorst MSQ, Isharwal S, Van Diest PJ, Chowdhury WH, Marlow C, Carducci MA, *et al*. Valproic acid causes dose-and time-dependent changes in nuclear structure in prostate cancer cells *in vitro* and *in vivo*. Mol Cancer Ther. 2009;8(4):802-808. DOI: 10.1158/1535-7163.MCT-08-1076.
17. Nakamoto N, Higuchi H, Kanamori H, Kurita S, Tada S, Takaishi H, *et al*. Cyclooxygenase-2 inhibitor and interferon-β synergistically induce apoptosis in human hepatoma cells *in vitro* and *in vivo*. Int J Oncol. 2006;29(3): 625-635. PMID: 16865278.
18. Tao J, Qiu B, Zhang D, Wang Y. Expression levels of Fas/Fas-L mRNA in human brain glioma stem cells. Mol Med Rep. 2012; 5(5):1202-1206. DOI: 10.3892/mmr.2012.791.
19. Inoue T, Anai S, Onishi S, Miyake M, Tanaka N, Hirayama A, *et al*. Inhibition of COX-2 expression by topical diclofenac enhanced radiation sensitivity *via* enhancement of TRAIL in human prostate adenocarcinoma xenograft model. BMC urol. 2013; 13:1-9. DOI: 10.1186/1471-2490-13-1.
20. Cao XX, Mohuiddin I, Chada S, Mhashilkar AM, Ozvaran MK, McConkey DJ, *et al.* Adenoviral transfer of mda-7 leads to BAX up-regulation and apoptosis in mesothelioma cells, and is abrogated by over-expression of BCL-XL. Mol Med. 2002; 8(12):869-876. DOI: 10.1007/BF03402093.
21. Ierano C, Chakraborty A, Nicolae A, Bahr J, Zhan Z, Pittaluga S, *et al*. Loss of the proteins Bak and Bax prevents apoptosis mediated by histone deacetylase inhibitors. Cell Cycle. 2013;12(17):2829-2838. DOI: 10.4161/cc.25914.
22. Zhang X-Q, Dong J-J, Cai T, Shen X, Zhou X-J, Liao L. High glucose induces apoptosis *via* upregulation of Bim expression in proximal tubule epithelial cells. Oncotarget 2017;8(15): 24119-24129. DOI: 10.18632/oncotarget.15491.
23. Xu Y, Liu L, Qiu X, Liu Z, Li H, Li Z, *et al*. CCL21/CCR7 prevents apoptosis *via* the ERK pathway in human non-small cell lung cancer cells. PLoS One. 2012;7(3): e33262-1-9. DOI: 10.1371/journal.pone.0033262.

24. Zhang Y-L, Pang L-Q, Wu Y, Wang X-Y, Wang C-Q, Fan Y. Significance of Bcl-xL in human colon carcinoma. World J gastroenterol:. 2008;14(19):3069-3073.
    DOI: 10.3748/wjg.14.3069.
25. Wang B, Ni Z, Dai X, Qin L, Li X, Xu L, *et al*. The Bcl-2/xL inhibitor ABT-263 increases the stability of Mcl-1 mRNA and protein in hepatocellular carcinoma cells. Mol Cancer. 2014;13:98-109.
    DOI: 10.1186/1476-4598-13-98.
26. Masood KI, Rottenberg ME, Salahuddin N, Irfan M, Rao N, Carow B, *et al*. Expression of *M. tuberculosis*-induced suppressor of cytokine signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of *tuberculosis*. BMC Infect Dis. 2013; 13:13-21.
    DOI: 10.1186/1471-2334-13-13.
27. Leon AJ, Gomez E, Garrote JA, Bernardo D, Barrera A, Marcos JL, *et al*. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. Mediators inflam. 2009;2009:580450-1-10.DOI: 10.1155/2009/580450.
28. Chen B, Lai J, Dai D, Chen R, Li X, Liao N. JAK1 as a prognostic marker and its correlation with immune infiltrates in breast cancer. Aging (Albany NY). 2019;11(23):11124-11135.
    DOI: 10.18632/aging.102514.
29. Xiong H, Chen Z-F, Liang Q-C, Du W, Chen H-M, Su W-Y, *et al*. Inhibition of DNA methyltransferase induces G2 cell cycle arrest and apoptosis in human colorectal cancer cells *via* inhibition of JAK2/STAT3/STAT5 signalling. J Cell Mol Med. 2009;13(98):3668-3679.
    DOI: 10.1111/j.1582-4934.2009.00661.x.
30. Zhao S, Guo J, Zhao Y, Fei C, Zheng Q, Li X, *et al*. Chidamide, a novel histone deacetylase inhibitor, inhibits the viability of MDS and AML cells by suppressing JAK2/STAT3 signaling. Am J Transl Res. 2016;8(7): 3169-3178.
    PMID: 27508038.
31. Sanaei M, Kavoosi F, Safarzadeh M. Effect of valproic acid on SOCS-1, SOCS-2, SOCS-3, SOCS-5, SOCS6, and SOCS-7 genes expression and cell growth inhibition in colon carcinoma. Gastroenterol Hepatol Bed Bench. 2022;15(1): 39-44)..
32. Arvidsson Y, Johanson V, Pfragner R, Wängberg B, Nilsson O. Cytotoxic effects of valproic acid on neuroendocrine tumour cells. Neuroendocrinology. 2016;103(5):578-591.
    DOI: 10.1159/000441849.
33. Santos J, Hubert T, Milthorpe BK. Valproic acid promotes early neural differentiation in adult mesenchymal stem cells through protein signalling pathways. Cells. 2020;9(3):619-644.
    DOI: 10.3390/cells9030619.
34. Shankar S, Singh TR, Fandy TE, Luetrakul T, Ross DD, Srivastava RK. Interactive effects of histone deacetylase inhibitors and TRAIL on apoptosis in human leukemia cells: involvement of both death receptor and mitochondrial pathways. Int J Mol Med. 2005;16(6):1125-1138.
    PMID: 16273296.
35. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene. 2007;26(37):5541-5552.
    DOI: 10.1038/sj.onc.1210620.
36. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene. 2004;23(37):6261-6271.
    DOI: 10.1038/sj.onc.1207830.
37. Lagneaux L, Gillet N, Stamatopoulos B, Delforge A, Dejeneffe M, Massy M, *et al*. Valproic acid induces apoptosis in chronic lymphocytic leukemia cells through activation of the death receptor pathway and potentiates TRAIL response. Exp Hematol. 2007;35(10):1527-1537.
    DOI: 10.1016/j.exphem.2007.06.014.
38. Catalano MG, Fortunati N, Pugliese M, Costantino L, Poli R, Bosco O, *et al*. Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. J Clin Endocrinol Metab. 2005;90(3):1383-1389.
    DOI: 10.1210/jc.2004-1355.
39. Mühlethaler-Mottet A, Meier R, Flahaut M, Bourloud KB, Nardou K, Joseph J-M, *et al*. Complex molecular mechanisms cooperate to mediate histone deacetylase inhibitors anti-tumour activity in neuroblastoma cells. Mol Cancer. 2008;7:55-66.
    DOI: 10.1186/1476-4598-7-55.
40. Choudhury SR, Karmakar S, Banik NL, Ray SK. Valproic acid induced differentiation and potentiated efficacy of taxol and nanotaxol for controlling growth of human glioblastoma LN18 and T98G cells. Neurochem Res. 2011;36(12):2292-2305.
    DOI: 10.1007/s11064-011-0554-7.
41. Zhou Y, Xu Y, Wang H, Niu J, Hou H, Jiang Y. Histone deacetylase inhibitor, valproic acid, radiosensitizes the C6 glioma cell line *in vitro*. Oncol Lett. 2014;7(1): 203-208.
    DOI: 10.3892/ol.2013.1666.
42. Khathayer F, Taylor MA, Ray SK. Synergism of 4HPR and SAHA increases anti-tumor actions in glioblastoma cells. Apoptosis. 2020;25(3-4):217-232.
    DOI: 10.1007/s10495-020-01590-9.