RESEARCH ARTICLE

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The Effect of 5-aza,2'-deoxyCytidine (5 AZA CdR or Decitabine) on Extrinsic, Intrinsic, and *JAK/STAT* Pathways in Neuroblastoma and Glioblastoma Cells Lines

Masumeh Sanaei, Fraidoon Kavoosi*

Abstract

Epigenetic changes such as histone deacetylation and DNA methylation play to regulate gene expression. DNA methylation plays a major role in cancer induction via transcriptional silencing of critical regulators such as tumor suppressor genes (TSGs). One approach to inhibit TSGs inactivation is to use chemical compounds, DNA methyltransferase inhibitors (DNMTIs). Previously, we investigated the effect of 5-aza-2'-deoxycytidine (5 AZA CdR or decitabine) on colon cancer and hepatocellular carcinoma cell lines. The present study aimed to investigate the effect of 5 AZA CdR on extrinsic (*DR4, DR5, FAS, FAS-L*, and *TRAIL* genes), intrinsic [pro- (Bax, Bak, and Bim) and anti- (Bcl-2, Bcl-xL, and Mcl-1) apoptotic genes], and *JAK/STAT* (*SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A,* and *STAT5B* genes) pathways in neuroblastoma (*IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3*, and *UKF-NB-4*) and glioblastoma (*SF-767, SF-763, A-172, U-87 MG*, and *U-251 MG)* cell lines. **Materials and Methods:** The neuroblastoma and glioblastoma cells were cultured and treated with 5 AZA CdR. To determine cell viability, cell apoptosis, and the relative gene expression level, MTT assay, flow cytometry assay, and qRT-PCR were done respectively. **Results:** 5 AZA CdR changed the expression level of the genes of the extrinsic, intrinsic, and JAK/STAT pathways by which induced cell apoptosis and inhibited cell growth in neuroblastoma and glioblastoma cell lines. **Conclusion:** 5 AZA CdR can play its role through extrinsic, intrinsic, and JAK/STAT pathways to induce cell apoptosis.

Keywords: 5 AZA CdR- tumor suppressor gene- neoplasms

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Introduction

Epigenetic changes such as histone deacetylation and DNA methylation play to regulate gene expression. In fact, epigenetic changes are susceptible to change and are excellent candidates to explain how certain factors may increase the risk of tumorigenesis and cancer induction. However, DNA methylation plays a major role in cancer via transcriptional silencing of critical regulators such as tumor suppressor genes (TSGs). Basically, tumorigenesis is directed by changes in two different groups of genes: TSGs that inhibit cell growth and oncogenes that promote this process. Meanwhile, chromatin modifications, such as DNA methylation, affect local chromatin structure without any changes in DNA sequences. The major step in tumorigenesis is gene inactivation by hypermethylation of CpG islands located in the promoter region. In mammals, DNA methylation occurs at the C5 position of cytosine, mostly within CpG dinucleotides (Grønbaek et al., 2007). Specific enzymes such as DNA methyltransferases (DNMTs) play a major role in DNA methylation and cause reduced expression of TSGs, resulting in cancer induction and progression. In mammals, DNA methylation is regulated by DNA methyltransferases (DNMTs), including *DNMT1, DNMT3A*, and DNMT3B (Zhang et al., 2017) The DNMTs are often overexpressed in various human cancers. DNMTs are important epigenetic targets for cancer treatment since DNA methylation is a reversible process. These enzymes are promising targets for the treatment of various types of cancers (Zhang et al., 2020; Yu et al., 2019). One approach to inhibit TSGs inactivation is to use chemical compounds, DNA methyltransferase inhibitors (DNMTIs), to reverse DNA hypermethylation. These compounds include 5-aza-2'-deoxycytidine (5 AZA CdR or decitabine), 5-aza-cytidine (5-aza-C), 5-fluoro-2'-deoxycytidine (FdCyd), 2-H pyrimidinone-1-β-D(2'-deoxyriboside) (zebularine), and pseudoisocytidine (Gowher et al., 2004). Previously, we investigated the effect of 5 AZA CdR on colon cancer (Sanaei et al., 2021; Sanaei et al., 2020; Sanaei et al., 2020) and hepatocellular carcinoma cell lines (Sanaei et al., 2020; Sanaei et al., 2020). This compound plays its role through multiple mechanisms comprising extrinsic, intrinsic, and *JAK/STAT* pathways. Recent experimental works have indicated that

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5 AZA CdR induces apoptosis by re-activation of extrinsic pathway (FAS-ligand up-regulation) in neoplastic cells (Karlic et al., 2011). Further, this agent induces apoptosis via the intrinsic (mitochondrial) pathway (Mcl-1 cleavage; Bax, Puma, and Noxa up-regulation) (Kiziltepe et al., 2007). It has been reported that 5 AZA CdR suppresses cancer cell growth through JAK/STAT pathway, regulation of downstream targets of JAK2/STAT3/STAT5 signaling including Bcl-2, $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$ (Sanaei et al., 2021). The present study aimed to investigate the effect of 5 AZA CdR on extrinsic (*DR4, DR5, FAS, FAS-L*, and *TRAIL* genes), intrinsic [pro- (*Bax, Bak*, and *Bim*) and anti- (*Bcl-2, Bcl-xL,* and *Mcl-1*) apoptotic genes], and *JAK/STAT* (*SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A, and STAT5B* genes) pathways in neuroblastoma (*IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3, and UKF-NB-4*) and glioblastoma (*SF-767, SF-763, A-172, U-87 MG*, and *U-251 MG*) cell lines.

Materials and Methods

Materials

Human neuroblastoma (*IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3*, and *UKF-NB-4*) and glioblastoma (*SF-767, SF-763, A-172, U-87 MG*, and *U-251 MG*) cell lines were purchased from the National Cell Bank of Iran-Pasteur Institute. 5 AZA CdR and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma (St. Louis, MO, USA). The 5 AZA CdR was dissolved in dimethyl sulfoxide (DMSO) to make a work-stock solution. Further concentrations of 5 AZA CdR were obtained by diluting the provided stock solution. Other necessary materials and kits were purchased as provided for our previous works (Sanaei. et al., 2020; Sanaei et al., 2018). The cells were maintained in DMEM supplemented with fetal bovine serum of 10% and antibiotics in a humidified atmosphere of 5% CO_2 in air at 37°C. This work was approved by the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1399.078.

Cell culture and cell viability

All cell lines were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO_2 for 24 h. Subsequently, all cell lines were seeded into 96-well plates (3×10^5 cells per well). After 24h, the culture medium was replaced with an experimental medium containing various concentrations of 5 AZA CdR. The human neuroblastoma and the glioblastoma cell lines were treated with 5 AZA CdR (0, 1, 2.5, 5, 7.5, 10, 15, and 20 µM) for 24h, the control cells were treated with the same amount of solvents, DMSO. After 24 h of treatment, all treated and untreated cells were investigated by MTT assay according to Standard protocols to determine cell viability, the MTT solution was added to each well for 4 h at 37°C and then it was changed by DMSO for 10 min to dissolve all of the crystals. Finally, the optical density was detected by a microplate reader at a wavelength of 570 nM. Each experiment was repeated three times (triplicates).

Cell apoptosis assay

To determine cell apoptosis, all cell lines were cultured at a density of 3×10^5 cells/well and incubated overnight. Then All of the cell lines were treated with 5 AZA CdR, based on IC_{50} values indicated in table 1, for 24h, the control cells were treated with the same amount of solvent, DMSO. Subsequently, the cells were harvested by trypsinization, washed with cold PBS, and resuspended in a Binding buffer (1x). Finally, Annexin-V-(FITC) and propidium iodide (PI) were used according to the protocol to determine the apoptotic cells by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was done to determine the relative expression level of the extrinsic (*DR4, DR5, FAS*, *FAS-L*, and *TRAIL*), intrinsic [pro- (Bax, Bak, and Bim) and anti- (*Bcl-2, Bcl-xL*, and *Mcl-1*)], and JAK/STAT (*SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A,* and *STAT5B* genes) genes expression. The cell lines were cultured at a density of 3×10^5 cells/well and treated with 5 AZA CdR, based on IC_{50} values indicated in table 1, for 24 h, except control groups which were treated with DMSO only. Then qRT-PCR was done as our previous works (Kavoosi et al., 2018; Sanaei et al., 2019). The primer sequences are addressed and shown in table 2.

Statistical analysis

The database was set up with the SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA) and Graph Pad Prism 8.0 for data analysis. Results are expressed as mean ± standard deviation (SD) for n=3 independent experiments. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA). A significant difference was considered as $P < 0.05$.

Results

Result of cell viability by the MTT assay

The cell viability of the neuroblastoma and glioblastoma cell lines treated with 5 AZA CdR (0, 1, 2.5, 5, 7.5, 10, 15, and 20 µM) for 24h was investigated by MTT assay thereby the activities of cellular enzymes produced a dark-blue formazan crystal by the tetrazolium salt MTT reduction. To determine the viable neuroblastoma and glioblastoma cells, the crystals were dissolvable in DMSO. As indicated in Figures 1 and 2, 5 AZA CdR induced significant cell growth inhibition in all treated groups in a dose-dependent manner.

Result of cell apoptosis assay

To determine cell apoptosis, the neuroblastoma and glioblastoma cells were treated with 5 AZA CdR for 24h. Subsequently, the cells were stained using annexin-V-(FITC) and PI to determine apoptotic cells. As indicated in figures 3-5, this compound induced cell apoptosis significantly ($P<0.0001$). Based on statistical analysis, a significant difference was seen between treated and untreated cell groups. Maximal and minimal apoptosis was seen in SK-N-AS and U-87 MG cells treated with 5 AZA CdR (24 h) respectively.

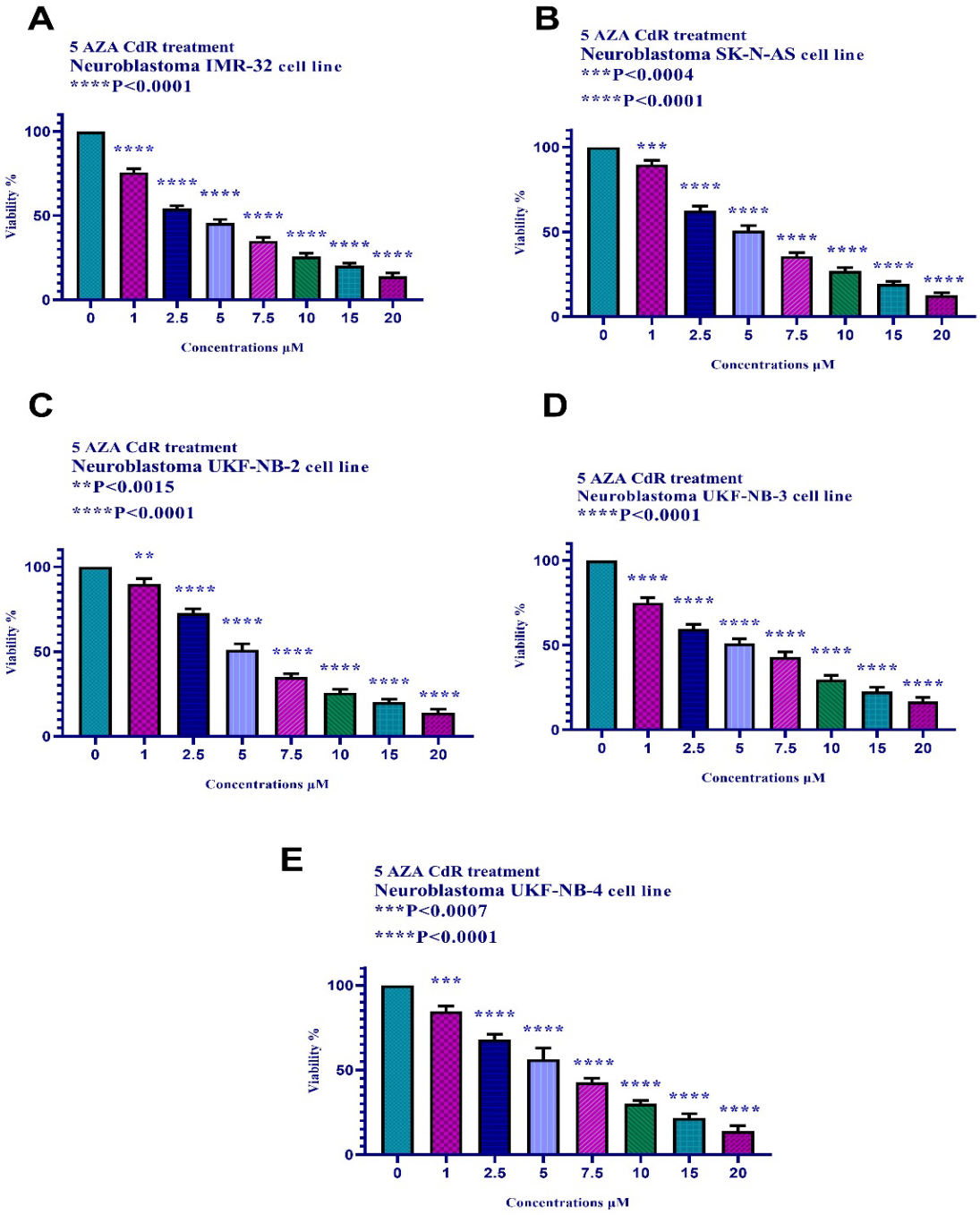

Figure 1. The Effect of 5-AZA-CdR on the Viability of Neuroblastoma Cell Lines. The cells were treated without and with different doses of 5-AZA-CdR for 24 and the cell viability was evaluated by MTT assay. Each experiment was achieved in triplicate. Mean values from the three experiments ± standard error of mean are indicated. Asterisks indicate significant differences between treated and untreated cells. **P <0.0015, ***P <0.0007, ***P <0.0004, and ****P< 0.0001.

Result of determination of genes expression

Result of determination of genes expression in hepatocellular carcinoma SK-Hep 1

Neuroblastoma

5 AZA CdR and extrinsic pathway

To determine the expression level of the *DR4, DR5, FAS, FAS-L*, and *TRAIL* genes, neuroblastoma cell lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in table 2, was evaluated by quantitative real-time RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of *DR4, DR5, FAS, FAS-L,* and *TRAIL* genes significantly, Figure 6. *P< 0.0001.

5 AZA CdR and intrinsic pathway

To determine the expression level of the *Bax, Bak, Bim, Bcl-2, Bcl-xL*, and *Mcl-1* genes, neuroblastoma cell lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in Table 2. The relative expression level was

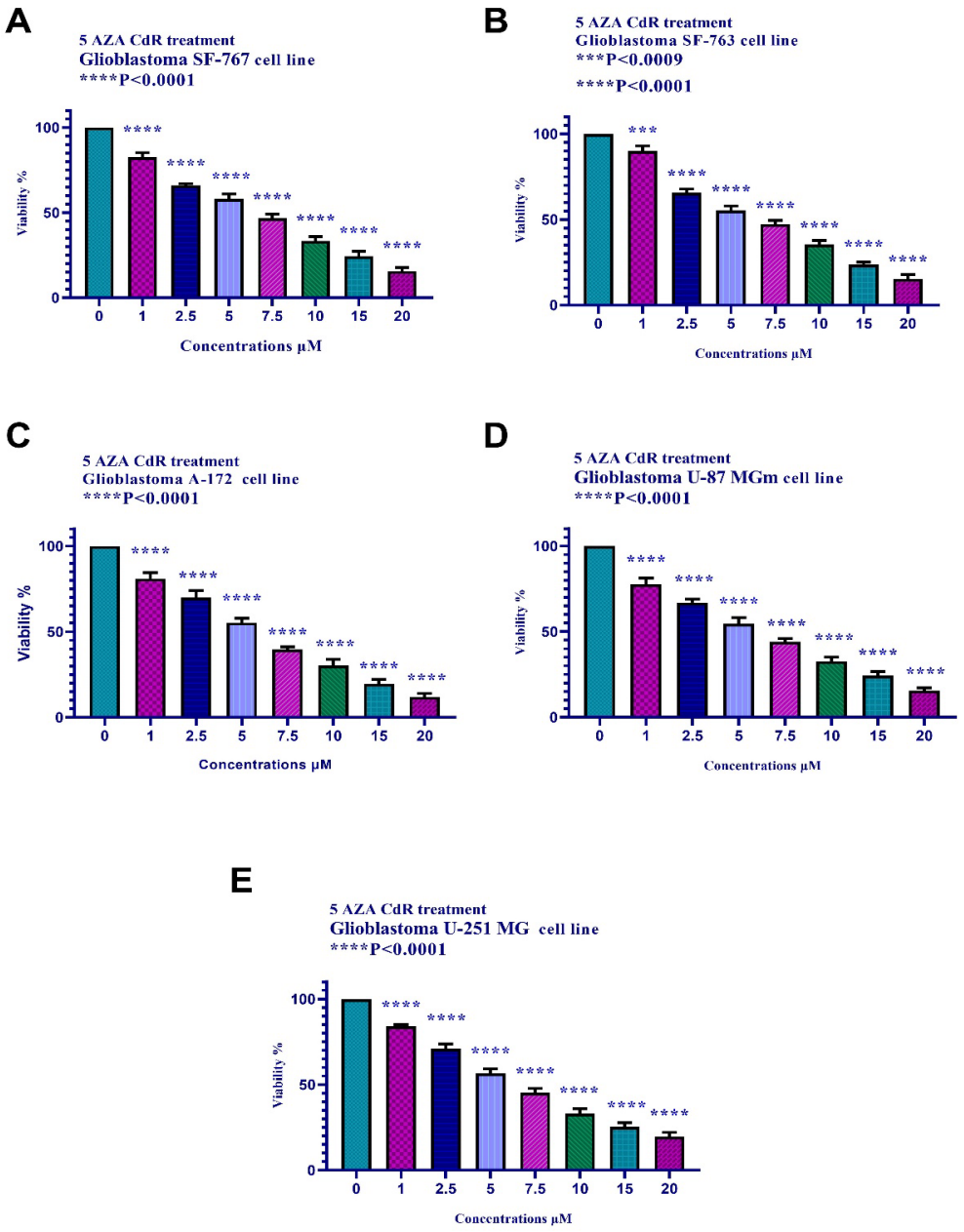

Figure 2. The Effect of 5 AZA CdR on the Viability of Glioblastoma Cell Lines. The cells were treated without and with different doses of 5 AZA CdR for 24 and the cell viability was evaluated by MTT assay. Each experiment was achieved in triplicate. Mean values from the three experiments ± standard error of mean are indicated. Asterisks indicate significant differences between treated and untreated cells. ***P <0.0009, and ****P< 0.0001.

Table 1. IC_{50} Values

Cell line	Drug	Duration/Hour	IC_{50}	$LogIC_{50}$	R squared
Neuroblastoma IMR-32	5-AZA-CdR	24	2.178	0.3381	0.9358
Neuroblastoma SK-N-AS	5-AZA-CdR	24	3.154	0.4989	0.982
Neuroblastoma UKF-NB-2	5-AZA-CdR	24	4.003	0.6024	0.9871
Neuroblastoma UKF-NB-3	5-AZA-CdR	24	4.15	0.618	0.9218
Neuroblastoma UKF-NB-4	5-AZA-CdR	24	5.259	0.7209	0.9723
Glioblastoma SF-767	5-AZA-CdR	24	6.929 5-AZA-CdR	0.8407	0.9561
Glioblastoma SF-763	5-AZA-CdR	24	5.306	0.7248	0.971
Glioblastoma A-172	5-AZA-CdR	24	6.812	0.8333	0.9655
Glioblastoma U-87 MG	5-AZA-CdR	24	6.37	0.8041	0.9458
Glioblastoma U-251 MG	5-AZA-CdR	24	5.609	0.7489	0.9698

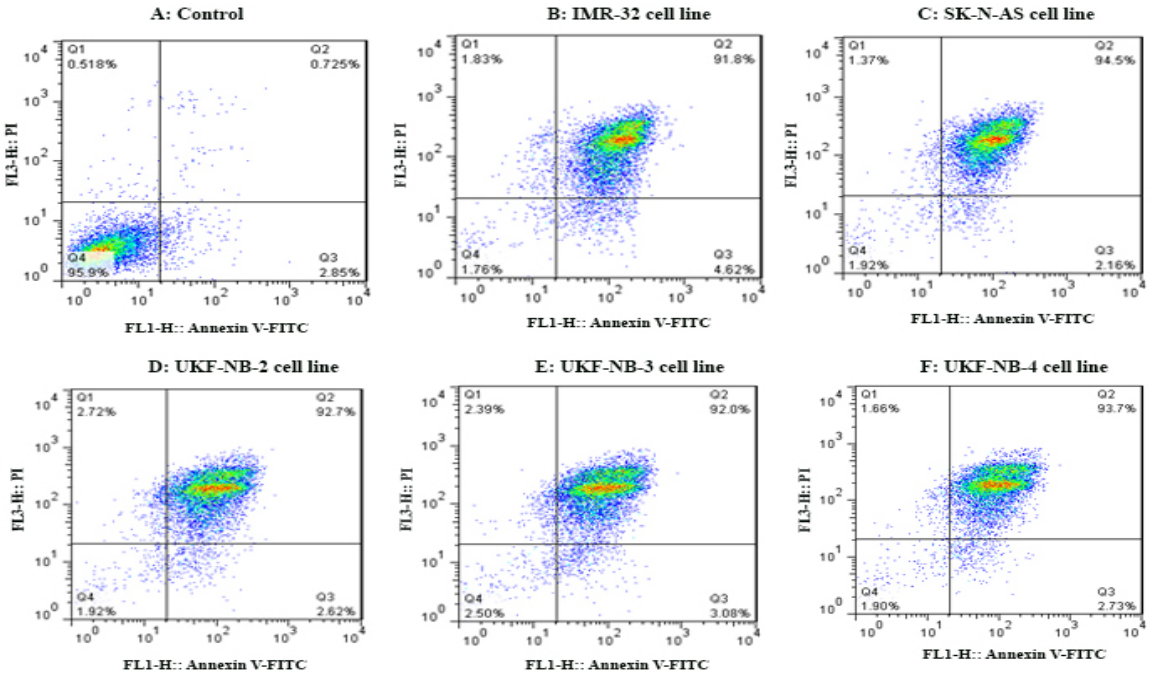

Figure 3. The Apoptosis-Inducing Effect of 5-AZA-CdR was Investigated by Flow Cytometric Analysis of Neuroblastoma Cells Stained with Annexin V and PI. The result indicated that 5-AZA-CdR induced cell apoptosis after 24 h of treatment significantly. P<0.0001.

evaluated by quantitative real-time RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of Bax, Bak, and Bim genes and down-regulated the expression level of *Bcl-2, Bcl-xL*, and *Mcl-1* genes significantly as indicated in Figure 7 *P< 0.0001.

5 AZA CdR and JAK/STAT pathway

To determine the expression level of the *SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A,* and *STAT5B* genes, neuroblastoma cell lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in Table 2. The relative expression level was evaluated by quantitative real-time

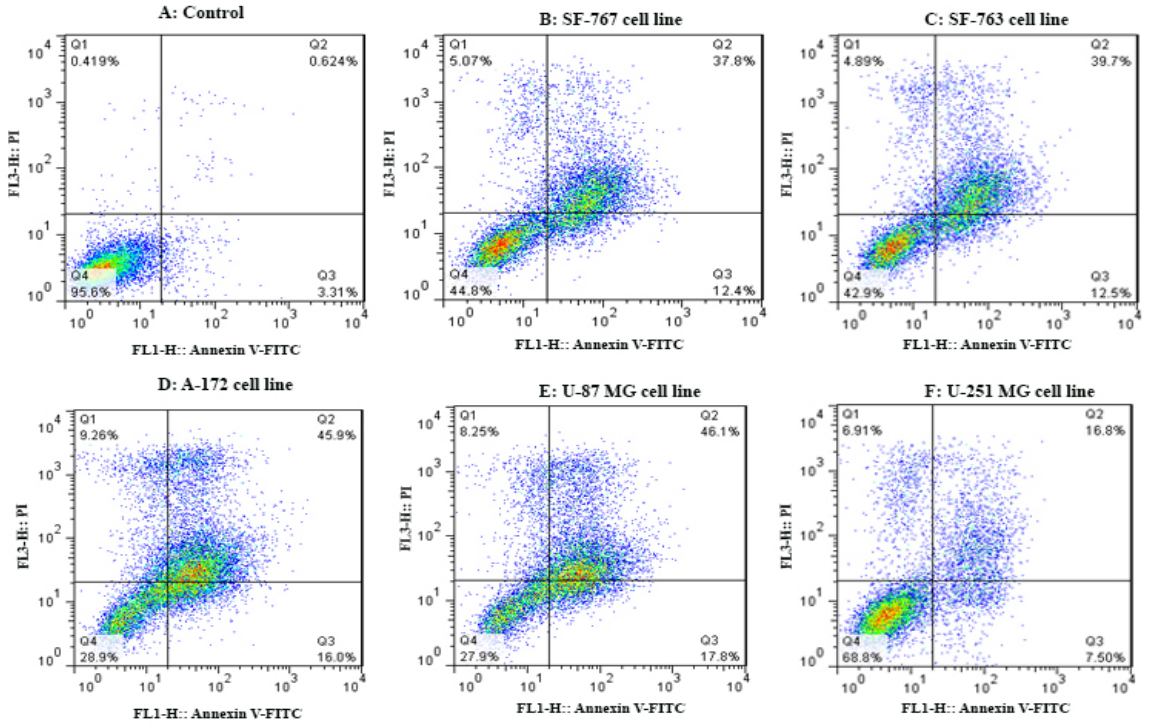

Figure 4. The Apoptosis-Inducing Effect of 5-AZA-CdR was Investigated by Flow Cytometric Analysis of Glioblastoma Cells Stained with Annexin V and PI. The result indicated that 5-AZA-CdR induced cell apoptosis after 24 h of treatment significantly. P<0.0001.

Table 2. The Primer Sequences of *DR4, DR5, FAS, FAS-L, TRAIL, Bax, Bak, Bim, Bcl-xL, Mcl-1, SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* Genes were Used in the Current Study.

Primer	Primer sequences (5' to 3')	Product length	Reference
DR4		299 bp	Nakamoto et al., 2006
Forward	CAGAACATCCTGGAGCCTGTAAC		
Reverse	ATGTCCATTGCCTGATTCTTTGTG		
DR5		389 bp	Nakamoto et al., 2006
Forward	TGCAGCCGTAGTCTTGATTG		
Reverse	GCACCAAG TCTGCAAAGTCA		
FAS			Tao et al., 2012
Forward	TTCTGCCATAAGCCCTGTCC	103 bp	
Reverse	TGTACTCCTTCCCTTCTTGG		
FAS-L			Tao et al., 2012
Forward	GCCTGTGTCTCCTTGTGATG	222 bp	
Reverse	TGGACTTGCCTGTTAAATGGG		
TRAIL		103 bp	Inoue et al., 213
Forward	GAAGCAACACATTGTCTTCTCCAA		
Reverse	TTGCTCAGGAATGAATGCCC		
Bax		77 bp	Cao et al., 2002
Forward	AGTAACATGGAGCTGCAGAGGAT		
Reverse	GCTGCCACTCGGAAAAAGAC		
Bak		82 bp	Ierano et al, 2013
Forward	CCTGCCCTCTGCTTCTGA		
Reverse	CTGCTGATGGCGGTAAAAA		
Bim		101 bp	Zhang et al., 2017
Forward	ATTACCAAGCAGCCGAAGAC		
Reverse	TCCGCAAAGAACCTGTCAAT		
Bcl-2		147 bp	Xu et al., 2012
Forward	TGGCCAGGGTCAGAGTTAAA		
Reverse	TGGCCTCTCTTGCGGAGTA		
Bcl-xL		62 bp	Zhang et al., 2008
Forward	TCCTTGTCTACGCTTTCCACG		
Reverse	GGTCGCATTGTGGCCTTT		
Mcl-1		198 bp	Wang et al., 2014
Forward	AAAGCCTGTCTGCCAAAT		
Reverse	CCTATAAACCCACCACTC		
SOCS1		119 bp	Masood et al., 2013
Forward	TTTTTCGCCCTTAGCGTGA		
Reverse	AGCAGCTCGAAGAGGCAGTC		
SOCS3		109 bp	Leon et al., 2009
Forward	GGCCACTCTTCAGCATCTC		
Reverse	ATCGTACTGGTCCAGGAACTC		
JAK1			Chen et al., 2019
Forward	CCACTACCGGATGAGGTTCTA	213	
Reverse	GGGTCTCGAATAGGAGCCAG		
JAK2			Xiong et al., 2009
Forward	GATGAGAATAGCCAAAGAAAACG	160	
Reverse	TTGCTGAATAAATCTGCGAAAT		
STAT3			Xiong et al., 2009
Forward	GCTTTTGTCAGCGATGGAGT	174	
Reverse	ATTTGTTGACGGGTCTGAAGTT		
STAT5A			Xiong et al., 2009
Forward	AATGAGAACACCCGCAACG	101	
Reverse	TTCCTGAAGTGGGCACTGAG		

Table 2. Continued

Primer	Primer sequences (5' to 3')	Product length	Reference
STAT5B			Xiong et al., 2009
Forward	ACTGCTAAAGCTGTTGATGGATAC	174	
Reverse	TGAGTCAGGGTTCTGTGGGTA		
GAPDH			Zhao et al., 2016
Forward	TGTGGGCATCAATGGATTTGG	116	
Reverse	ACACCATGTATTCCGGGTCAAT		

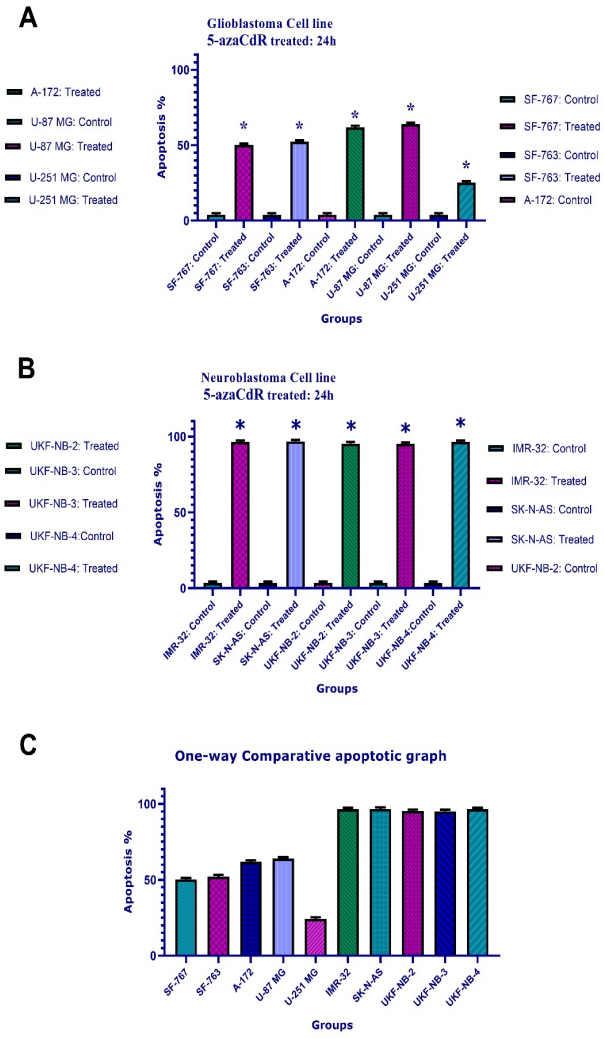

Figure 5. Part A. The apoptotic effect of 5-AZA-CdR on glioblastoma cell lines versus control groups after 24h of treatment. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. The results of the statistical analysis indicate significant differences between treated and untreated cells. *P< 0.0001. Part B. The apoptotic effect of 5-AZA-CdR on neuroblastoma cell lines versus control groups after 24h of treatment. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. The results of the statistical analysis indicate significant differences between treated and untreated cells. *P< 0.0001. Part C. Comparative analysis of the effect of 5-AZA-CdR on neuroblastoma and glioblastoma cell lines. Maximal and minimal apoptosis was seen in SK-N-AS and U-87 MG cells treated with 5-AZA-CdR (24 h) respectively.

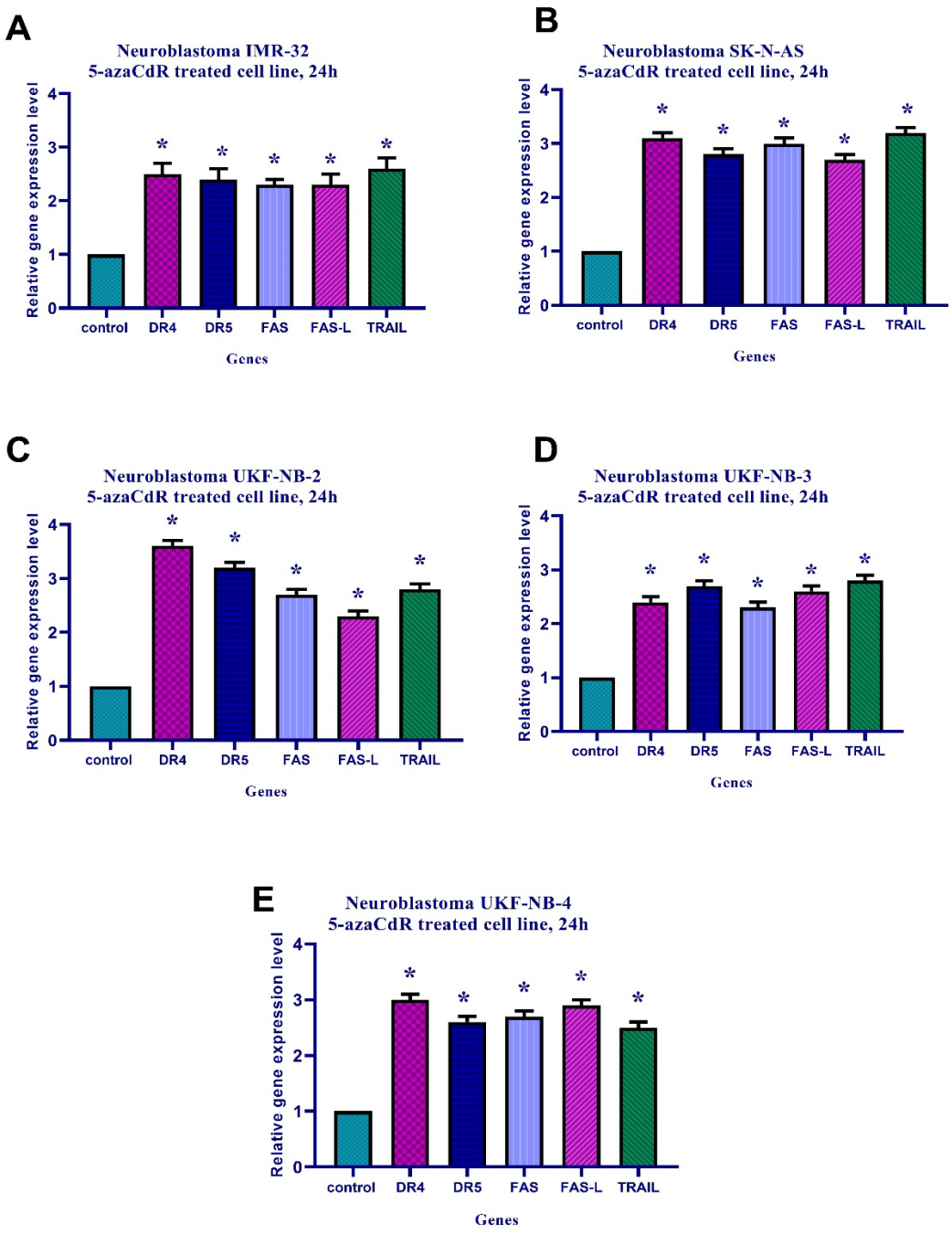

Figure 6. The Relative Expression Level of *DR4, DR5, FAS, FAS-L,* and *TRAIL* Genes Expression in Neuroblastoma Cell Lines Treated with 5-AZA-CdR after 24h of Treatment. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound up-regulated the expression of *DR4, DR5, FAS, FAS-L*, and *TRAIL* genes significantly. *P< 0.0001.

RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of *SOCS1* and *SOCS3* genes and down-regulated the expression level of *JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes significantly, Figure 8 *P< 0.0001.

Glioblastoma

5 AZA CdR and extrinsic pathway

To determine the expression level of the *DR4, DR5, FAS, FAS-L*, and *TRAIL* genes, glioblastoma cell lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in Table 2, was evaluated by quantitative real-time RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of *DR4, DR5, FAS, FAS-L,* and *TRAIL* genes significantly, Figure 9. *P< 0.0001.

5 AZA CdR and intrinsic pathway

To determine the expression level of the *Bax, Bak, Bim, Bcl-2, Bcl-xL*, and *Mcl-1* genes, glioblastoma cell

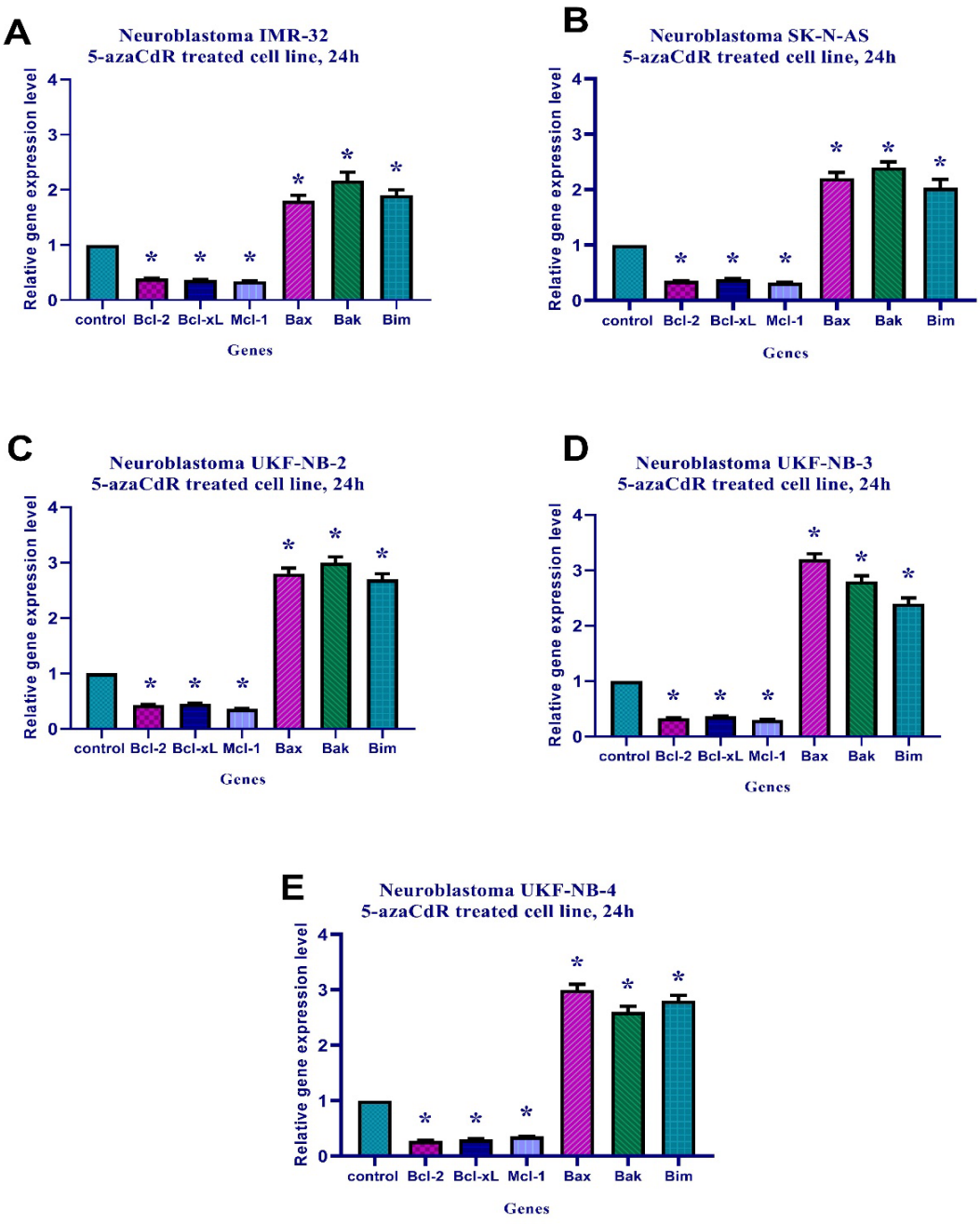

Figure 7. The Relative Expression Level of *Bax, Bak, Bim, Bcl-2, Bcl-xL*, and *Mcl-1* Genes Expression in Neuroblastoma Cell Lines Treated with 5-AZA-CdR at 24h. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound upregulated the expression of *Bax, Bak,* and *Bim* and downregulated the expression of *Bcl-2, Bcl-xL,* and *Mcl-1* genes significantly. *P< 0.0001

lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in Table 2. The relative expression level was evaluated by quantitative real-time RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of *Bax, Bak*, and *Bim* genes and down-regulated the expression level of *Bcl-2, Bcl-xL*, and *Mcl-1* genes significantly as indicated in figure 10. *P< 0.0001.

5 AZA CdR and JAK/STAT pathway

To determine the expression level of the *SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes, glioblastoma cell lines were treated with 5 AZA CdR, based on IC_{50} values demonstrated in Table 2. The relative expression level was evaluated by quantitative real-time RT-PCR analysis. The result of the quantitative real-time RT-PCR indicated that treatment with 5 AZA CdR upregulated the expression level of *SOCS1* and *SOCS3* genes and down-regulated the expression level of *JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes significantly, Figure 11 *P< 0.0001.

Discussion

It has been reported that DNMTIs induce

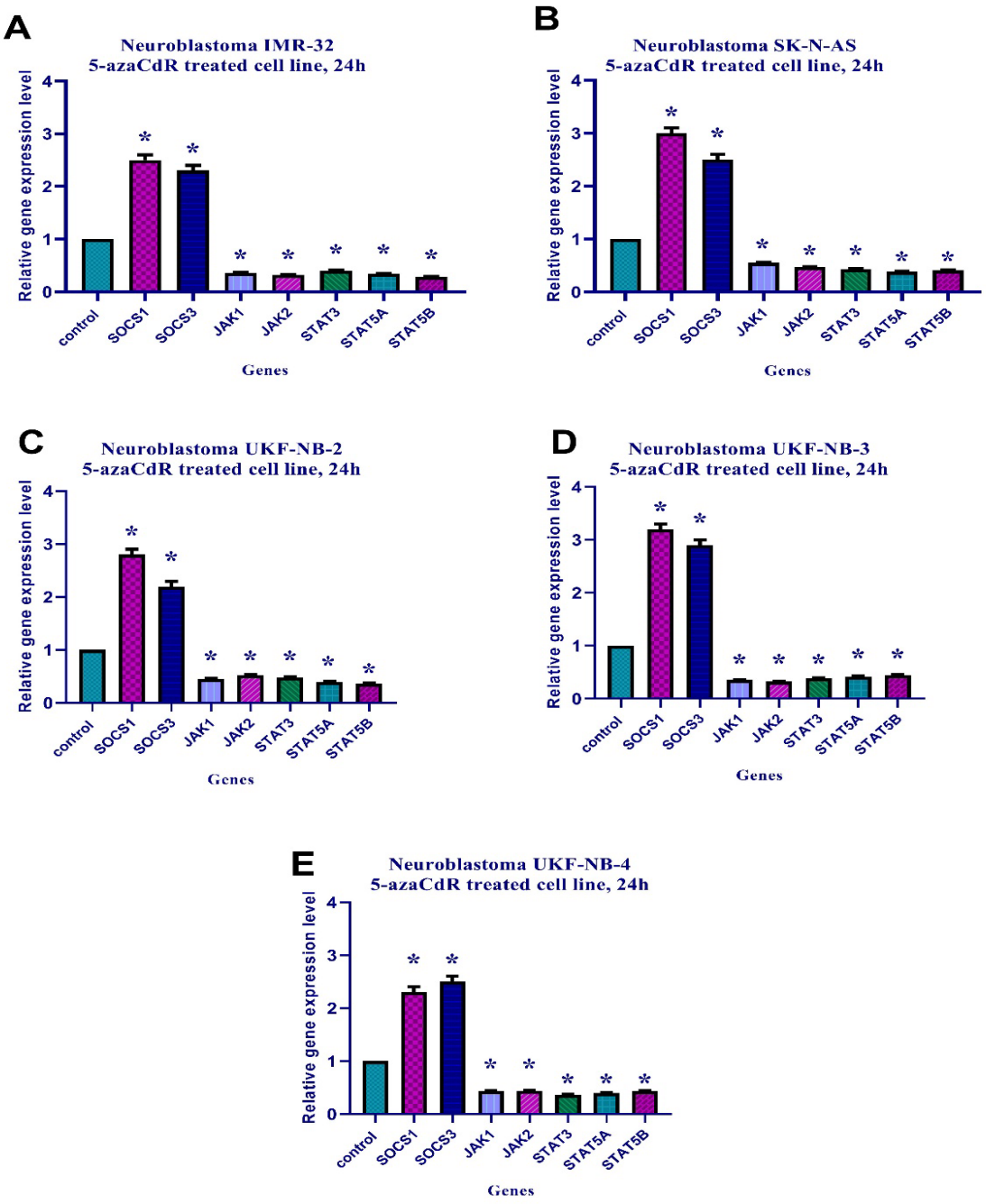

Figure 8. The Relative Expression Level of *SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* Expression in Neuroblastoma Cell Lines Treated with 5-AZA-CdR at 24h. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound up-regulated the expression of *SOCS1* and *SOCS3* and downregulated *JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes expression significantly. *P< 0.0001.

apoptosis throw extrinsic (Zhao et al., 2013), intrinsic (Ruiz-Magaña et al., 2012) and *JAK/STAT* (Calvisi et al., 2006) apoptotic pathways. In the present study, we indicated that 5 AZA CdR induced apoptosis via extrinsic (up-regulation of DR4, DR5, FAS, *FAS-L*, and *TRAIL* genes), intrinsic (up-regulation of Bax, Bak, and Bim, and down-regulation of *Bcl-2, Bcl-xL,* and *Mcl-1* genes), and JAK/STAT (up-regulation of *SOCS1* and *SOCS3* and down-regulation of *JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes) pathways in neuroblastoma (*IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3*, and *UKF-NB-4)* and glioblastoma (*SF-767, SF-763, A-172, U-87 MG,* and *U-251 MG*) cell lines. Similarly, in vitro studies have shown that 5 AZA CdR can induce apoptosis via an extrinsic pathway (re-expression of FAS and DR4 gene) in colon cancer and AML cell lines (Torres et al., 201; Zhang et al., 2018). Further, it up-regulates DR4 mRNA in choriocarcinoma (BeWo, JEG 3, JAR) and HTR-8/SVneo trophoblast cell lines (Wu et al., 2016). As we reported in this work, it has been shown that 5 AZA CdR and MS-275 play their roles by intrinsic pathway (up-regulation of p21waf1 and down-regulation of Mcl-1) in MV4-11 TP53 R248W cell line (Nishioka et al., 2011). Besides, 5 AZA CdR inhibits cell growth and induces

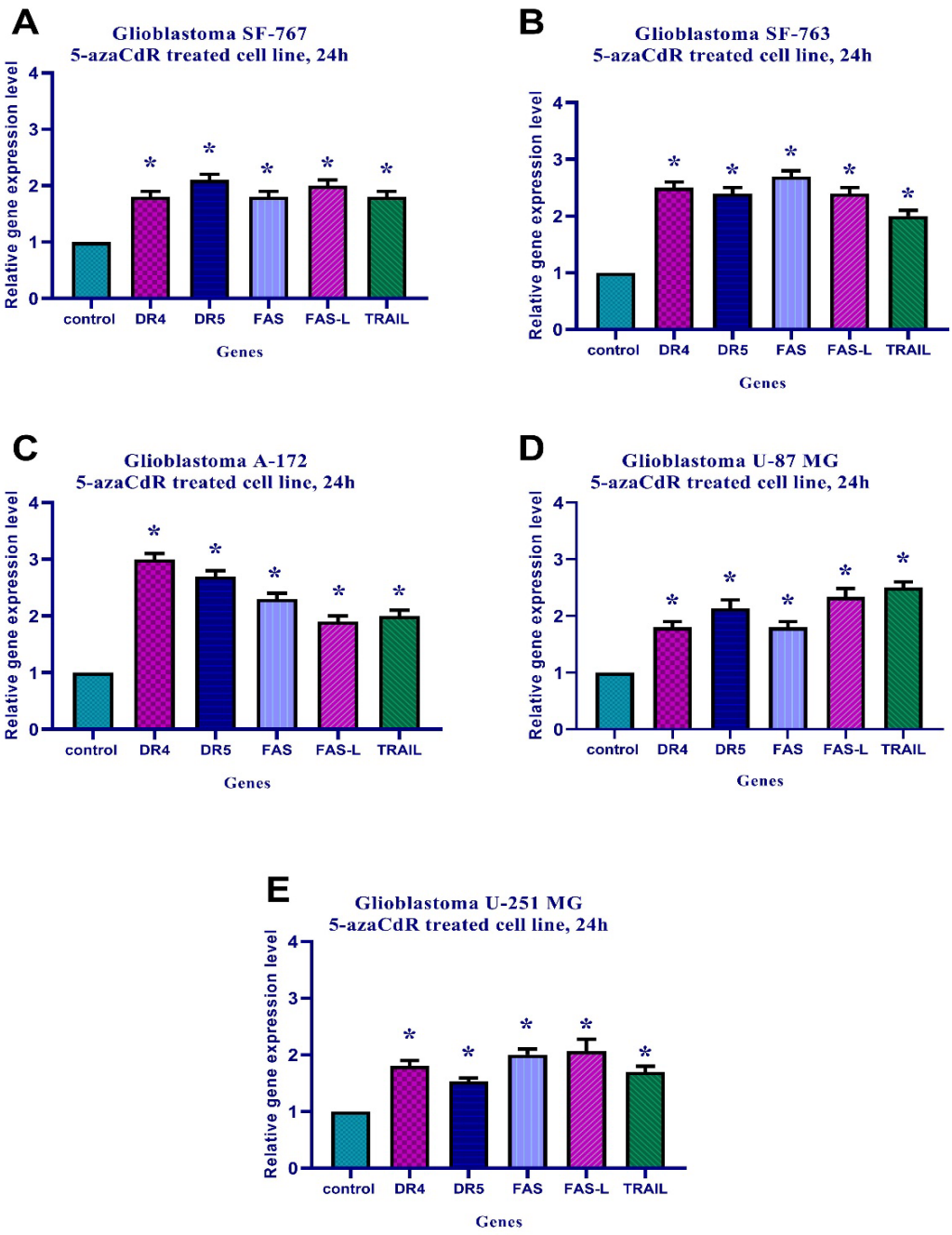

Figure 9. The Relative Expression Level of *DR4, DR5, FAS, FAS-L*, and *TRAIL* Genes Expression in Glioblastoma Cell Lines Treated with 5-AZA-CdR after 24h of Treatment. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound up-regulated the expression of *DR4, DR5, FAS, FAS-L*, and *TRAIL* genes significantly. *P< 0.0001.

apoptosis by MCL-1 down-regulation in the AML cell line (Tsao et al., 2012). Other researchers have shown that this compound up-regulates the Bax gene and leads to apoptosis induction in the human pancreatic cancer cell line (PANC-1) (Dastjerdi et al., 2018). 5 AZA CdR-induced apoptosis in the human leukemia cell lines U937 and HL60 is correlated with the downregulation of anti-apoptotic Bcl-2, cIAP-1, XIAP, and cIAP-2 protein levels, the cleavage of Bid proteins, the activation of caspases resulting in cell apoptosis (Shin et al., 2012). Another apoptotic molecular mechanism of 5 AZA CdR includes *JAK/STAT* pathway. This agent reactivates the suppressor of the cytokine signaling-1 (*SOCS-1*) gene and affected the Janus kinase/signal transducers and activators of the transcription (*JAK/STAT*) pathway in pancreatic cancer cells (Fukushima et al., 2003). In multiple myeloma, this compound plays its role via STAT3 down-regulation (Chim et al., 2004). Further, in hepatocellular carcinoma and endometrial cell lines, it up-regulates *SOCS-3* expression (Niwa et al., 2005; Chen et al., 2015). Meanwhile, it induces apoptosis through both *JAK/STAT* (*SOCS3* re-expression) and intrinsic (down-regulation of *BCL2* and *BCL-XL*) apoptosis pathways in mantle cell lymphoma (MCL) (Molavi et al., 2013). It should be noted that the

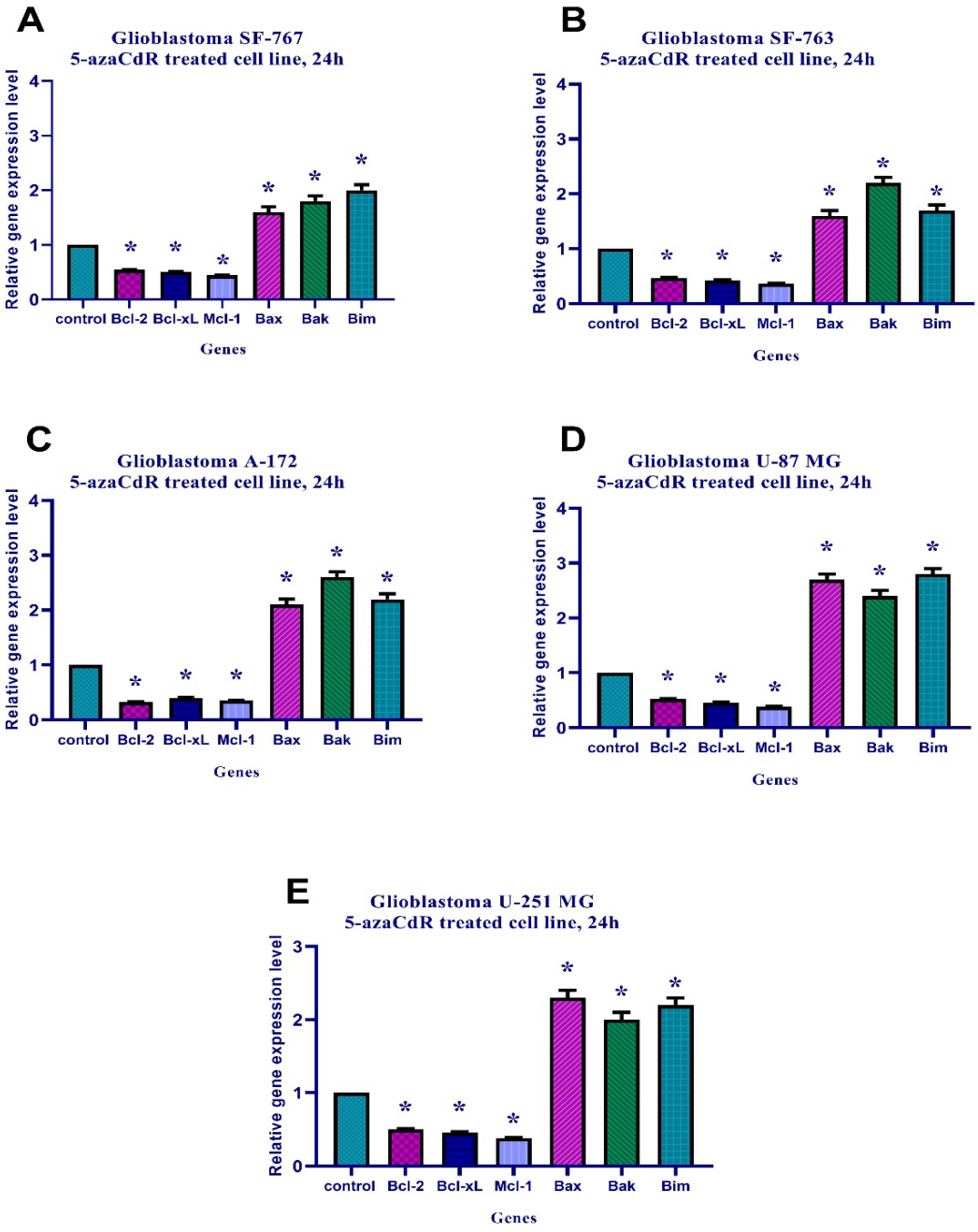

Figure 10. The Relative Expression Level of *Bax, Bak, Bim, Bcl-2, Bcl-xL*, and *Mcl-1* Genes Expression in Glioblastoma Cell Lines Treated with 5-AZA-CdR at 24h. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound upregulated the expression of *Bax, Bak, and Bim* and downregulated the expression of *Bcl-2, Bcl-xL*, and *Mcl-1* genes significantly. *P< 0.0001.

mentioned pathways are not the only pathway of 5 AZA CdR. Previously, we reported that 5 AZA CdR can induce apoptosis by DNMT 1 inhibition, and CIP/KIP family (p21, p27, and p57) genes up-regulation in colon cancer SW480 cell line (Sanaei et al., 2019). Besides, recent studies have demonstrated that 5 AZA CdR reactivates the expression of *BRCA1*, and *p53* and increases p15, p16, and *BRCA2* genes in Breast cancer stem cells (BCSCs) (Phan et al., 2016). Furthermore, treatment with 5 AZA CdR induces upregulation of *p16, FHIT, CRBP1, WWOX*, and *DLC-1* in the gastric cancer cell line (He et al., 2015). Finally, 5 AZA CdR induces apoptosis by various molecular mechanisms, we investigated only three ways comprising extrinsic, intrinsic, and JAK/STAT apoptotic pathways in neuroblastoma (*IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3,* and *UKF-NB-4*) and glioblastoma (*SF-767, SF-763, A-172, U-87 MG*, and *U-251 MG*) cell lines. Therefore, the investigation of other mechanisms is recommended.

In conclusion, our results indicated that 5 AZA CdR can induce its apoptotic effect through several molecular mechanisms including intrinsic, extrinsic, and *JAK/STAT* pathways in neuroblastoma and glioblastoma cell lines. Besides, this compound changes the relative expression

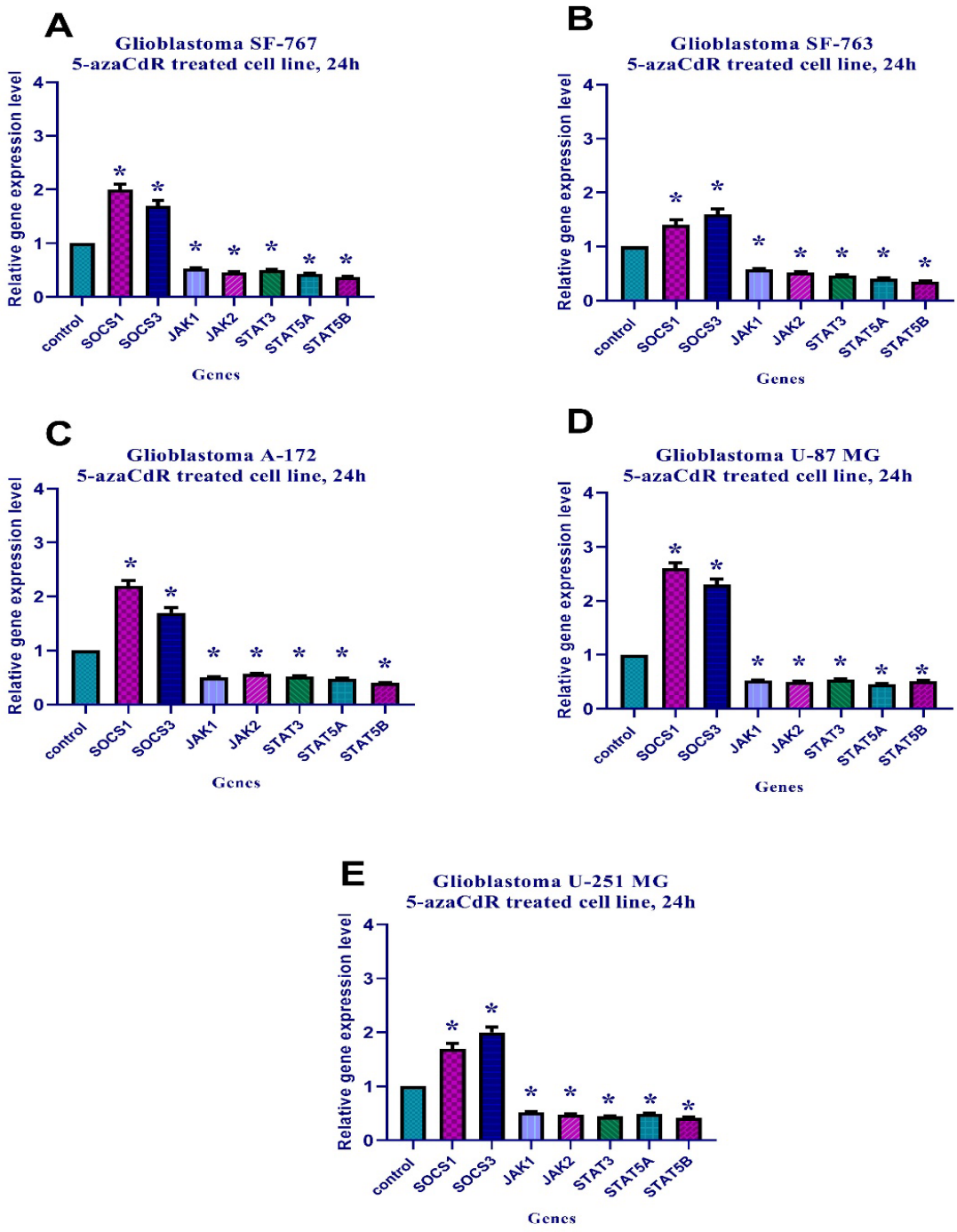

Figure 11. The Relative Expression Level of *SOCS1, SOCS3, JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* Expression in Glioblastoma Cell Lines Treated with 5-AZA-CdR at 24h. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that this compound up-regulated the expression of *SOCS1* and *SOCS3* and downregulated *JAK1, JAK2, STAT3, STAT5A*, and *STAT5B* genes expression significantly. *P< 0.0001.

level of the pro-and anti-apoptotic genes by which inhibits cell growth inhibition and induces apoptosis induction. Our findings will form the basis for further studies on the effects of 5 AZA CdR in neuroblastoma and glioblastoma.

Author Contribution Statement

All authors generated the ideas and contributed to the writing of the manuscript, acquisition of data, analysis, interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis, and technical or material support. All authors approved the final revision..

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Conflict Of Interest

The authors report no conflict of interest.

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