Original Article

Effect of Zebularine on Apoptotic Pathways in Hepatocellular Carcinoma Cell Lines

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Abstract

Background: The alteration of DNA cytosine methylation is one of the most common epigenetic changes that can play a significant role in human cancers. The enzymes involved in DNA methylation of promoter regions of the genes are DNA methyltransferases (DNMTs). The therapeutic activities and apoptotic effects of DNA methyltransferase inhibitors (DNMTIs) have been reported in various cancers. This study was assigned to assess the effect of zebularine on intrinsic and extrinsic pathways, DNAT 1, 3a, and 3b, p21, and p53, viability, and apoptosis in hepatocellular carcinoma (HCC) cell lines. **Methods:** Hepatocellular carcinoma cell lines (HCCLM3, MHCC97H, and MHCC97L) were purchased from the National Cell Bank of Iran, Pasteur Institute, treated with zebularine, and the MTT assay was performed. Then, flow cytometry assay and real-time RT-PCR analysis were performed with zebularine. Statistical comparisons between groups were made using GraphPad Prism software version 8.0. A significant difference was considered as $P < 0.05$. **Results:** Zebularine up-regulated DR4, DR5, FAS, FAS-L, TRAIL, Bax, Bak, Bim, p21WAF/CIP1 (p21), and p53 and down-regulated DNMTs (DNAT 1, 3a, and 3b), Bcl-2, Bcl-xL, and Mcl-1, significantly resulting in apoptosis induction in HCC cell lines. Maximal and minimal apoptosis was seen in HCCLM3 and MHCC97L cell lines, respectively. **Conclusions:** Our findings indicated that DNMTI zebularine can induce apoptosis and inhibit cell growth through both pathways (extrinsic and intrinsic) in HCC cell lines HCCLM3, MHCC97H, and MHCC97L.

Keywords: *Apoptosis, extrinsic, intrinsic, pathway, zebularine*

Introduction

The alteration of DNA cytosine methylation is one of the most common epigenetic changes that can play a significant role in human cancers. The enzymes involved in this process are DNA methyltransferases (DNMTs). These enzymes catalyze the transfer of a methyl group to cytosine residues, resulting in chromatin compaction and gene silencing. In various types of cancers, aberrant methylation has been reported for many cancer-related genes and tumor suppressor genes (TSGs), which lead to silencing of genes expression.[1] In eukaryotes, DNMTs are grouped into one family and divided into five members including DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L.[2] Overexpression of DNMTs has been reported in various human cancers.[3] Re-activation of silenced TSGs can lead to suppression of tumor growth. The compounds that can reverse DNA methylation, DNA demethylating agents (DNA methyltransferase inhibitors, DNMTIs), include nucleoside and non-nucleoside inhibitors of DNMTs. The pharmacological inhibition of DNMTs provides a novel opportunity for cancer therapy.[4] The therapeutic activities and apoptotic effects of DNMTIs, such as cytosine nucleoside analogs, 5-aza-2ʾ-deoxycytidine (decitabine, DAC), 5-azacytidine (azacitidine, 5AC), and pyrimidine-2-one ribofuranoside (zebularine, Zeb), have been reported in various cancers.[5] It should be noted that apoptosis is triggered by two major mechanisms: the extrinsic pathway, the binding of death ligands to death receptors, or the intrinsic "mitochondrial" pathway. *In vitro* studies have indicated that DNMTIs induce apoptosis through the activation of both pathways.[6,7] It has been shown that decitabine and zebularine induce apoptosis and cell cycle arrest through the intrinsic apoptotic pathway, BAX, and BAK activation.[7] Experimental studies have shown that zebularine induces apoptosis through the activation of intrinsic and

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extrinsic apoptosis pathways, leading to the up-regulation of $p21^{WAF/CIP1}$ (p21) in human bladder cancer cells.[8] Other researchers have reported that DNMTI 5-Aza-CdR induces p53 and p21 expression in prostatic cancer cells.[9] Finally, DNMTIs can induce apoptosis through the activation of both extrinsic and intrinsic pathways. The intrinsic pathway includes positive (apoptotic) regulators, such as p53, Noxa, Bid, and Bax, and negative regulators (anti-apoptotic), e.g., Bcl-2, Bcl-X_L, Mcl-1, and NF-$_K$B.[10] The extrinsic apoptotic pathway is triggered by the binding of death ligands to death receptors (DRs) such as TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, and TRAIL-R4/DcR2.[11] This study was designed to assess the effect of zebularine on mitochondrial/intrinsic and cytoplasmic/extrinsic pathways, DNAT 1, 3a, and 3b, p21 and p53, viability, and apoptosis in hepatocellular carcinoma (HCC) cell lines HCCLM3, MHCC97H, and MHCC97L. The intrinsic pathway includes pro-apoptotic (Bax, Bak, and Bim) and anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) genes, and the extrinsic pathway comprises DR4, DR5, FAS, FAS-L, and TRAIL genes.

Methods

Materials

HCC cell lines (HCCLM3, MHCC97H, and MHCC97L) were obtained from the National Cell Bank of Iran-Pasteur Institute. The zebularine and DMEM were provided from Sigma (St. Louis, MO, USA). To make a work stock solution, zebularine was dissolved in DMSO. By diluting the stock solution, further experimental concentrations were provided. Other kits and necessary materials were obtained as provided for our previous works.[12-14] The HCC cells (HCCLM3, MHCC97H, and MHCC97L) were maintained in DMEM supplemented with fetal bovine serum 10% and antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin). This is a laboratory trial study and 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

HCC cell lines (HCCLM3, MHCC97H, and MHCC97L) were cultured in DMEM supplemented with 10% FBS and antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO_2 for 24 h. Subsequently, all the HCC cell lines were seeded into 96-well plates (3×10^5 cells per well). After 1 day, the culture medium was removed and a culture medium containing various doses of zebularine (0, 10, 25, 50, 75, and 100 μM) was added, except control groups which were treated with DMSO at a concentration of 0.05%. After 24 and 48 h of treatment with zebularine, all treated and untreated HCC cells were evaluated by MTT assay to obtain cell viability, and the MTT solution (5 mg/mL) was added to each well and allowed incubation for 4 h at 37°C. To dissolve all the crystals, the solution was replaced by DMSO for 10 min. Subsequently, the absorbance spectrum was determined by a microplate reader at a wavelength of 570 nM.

Cell apoptosis assay

To determine HCC (HCCLM3, MHCC97H, and MHCC97L) cell apoptosis, the cells were cultured at a density of 3×10^5 cells per well and incubated overnight and then were treated with zebularine, based on IC50 values [Table 1], for 24 and 48 h. After treatment time, the cells were prepared for flow cytometry by trypsinization, washing twice with cold PBS, and then stained with Annexin V-FITC and propidium iodide (PI). The apoptotic cells were determined by FACScan flow cytometry.

Real-time quantitative reverse transcription polymerase *Chain reaction (qRT-PCR)*

The qRT-PCR was performed to determine the relative of the Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 qRT-PCR. To investigate the gene expression, the HCC cells were treated with zebularine, based on IC50 indicated in Table 1, for 24 and 48 h, except control groups that were treated with DMSO only. Then, qRT-PCR was performed as our previous works.[12] The primer sequences used in the current study are shown in Table 2.[13-24]

Statistical analysis

Data from three independent experiments were analyzed with one-way analysis of variance (ANOVA) with Tukey's post hoc test that was used to compare the groups using GraphPad Prism software version 8.0. A significant difference is expressed as $P < 0.05$.

Results

Cell viability

The cell viability of the HCCLM3, MHCC97H, and MHCC97L cells treated with zebularine at a concentration of 0, 10, 25, 50, 75, and 100 μM was investigated by MTT assay to determine cell viability and IC50 values. To determine the HCC viable cells, the crystals were dissolvable in DMSO. As shown in Figure 1, zebularine induced significant cell growth inhibition in a dose-dependent manner ($P < 0.0001$). IC50 values were calculated by GraphPad Prism 8. They are shown in Table 1.

Table 1: IC50 values

Cell line	Duration/Hour	IC50 Value	LogIC50	R-squared
HCCLM3	24	56.75	1.754	0.9824
HCCLM3	48	36.78	1.566	0.9844
MHCC97H	24	59.72	1.766	0.9949
MHCC97H	48	43.14	1.635	0.9982
MHCC97L	24	64.93	1.812	0.9721
MHCC97L	48	56.19	1.750	0.9903

Table 2: The primer sequences of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, p53, and GAPDH

Primer	Primer sequences (5' to 3')	Product length	Reference
Bax		77 bp	13
Forward	AGTAACATGGAGCTGCAGAGGAT		
Reverse	GCTGCCACTCGGAAAAAGAC		
Bak		360 bp	14
Forward	ACGCTATGACTCAGAGTTCC		
Reverse	CTTCGTACCACAAACTGGCC		
Bim		101 bp	15
Forward	ATTACCAAGCAGCCGAAGAC		
Reverse	TCCGCAAAGAACCTGTCAAT		
Bcl-2		147 bp	16
Forward	TGGCCAGGGTCAGAGTTAAA		
Reverse	TGGCCTCTCTTGCGGAGTA		
Bcl-xL		62 bp	17
Forward	TCCTTGTCTACGCTTTCCACG		
Reverse	GGTCGCATTGTGGCCTTT		
Mcl-1		198 bp	18
Forward	AAAGCCTGTCTGCCAAAT		
Reverse	CCTATAAACCCACCACTC		
DR4		299 bp	19
Forward	CAGAACATCCTGGAGCCTGTAAC		
Reverse	ATGTCCATTGCCTGATTCTTTGTG		
DR5		389 bp	19
Forward	TGCAGCCGTAGTCTTGATTG		
Reverse	GCACCAAGTCTGCAAAGTCA		
FAS		336 bp	20
Forward	ATGCTGGGCATCTGGACCCT		
Reverse	GCCATGTCCTTCATCACACAA		
FAS-L		345 bp	20
Forward	CAAGTCCAATCAAGGTCCATGCC		
Reverse	CAGAGAGAGCTCAGATACGTTGAC		
TRAIL		68 bp	21
Forward	GCTCTGGGCCGCAAAAT		
Reverse	TGCAAGTTGCTCAGGAATGAA		
DNMT1		206 bp	22
Forward	GAGGAAGCTGCTAAGGACTAGTTC		
Reverse	ACTCCACAATTTGATCACTAAATC		
DNMT3a		370 bp	22
Forward	GGAGGCTGAGAAGAAAGCCAAGGT		
Reverse	TTTGCCGTCTCCGAACCACATGAC		
DNMT3b		195 bp	22
Forward	TACACAGACGTGTCCAACATGGGC		
Reverse	GGATGCCTTCAGGAATCACACCTC		
P21		103 bp	23
Forward	GCTCAGGGGAGCAGGCTGAAG		
Reverse	CGGCGTTTGGAGTGGTAGAAATCTGT		
P53		118 bp	23
Forward	TCAACAAGATGTTTTGCCAACTG		

Contd...

Table 2: Contd...

Primer	Primer sequences (5' to 3')	Product length	Reference
Reverse	ATGTGCTGTGACTGCTTGTAGATG		
GAPDH		100 bp	24
Forward	GTCAGCCGCATCTTCTTTTG		
Reverse	GCGCCCAATACGACCAAATC		

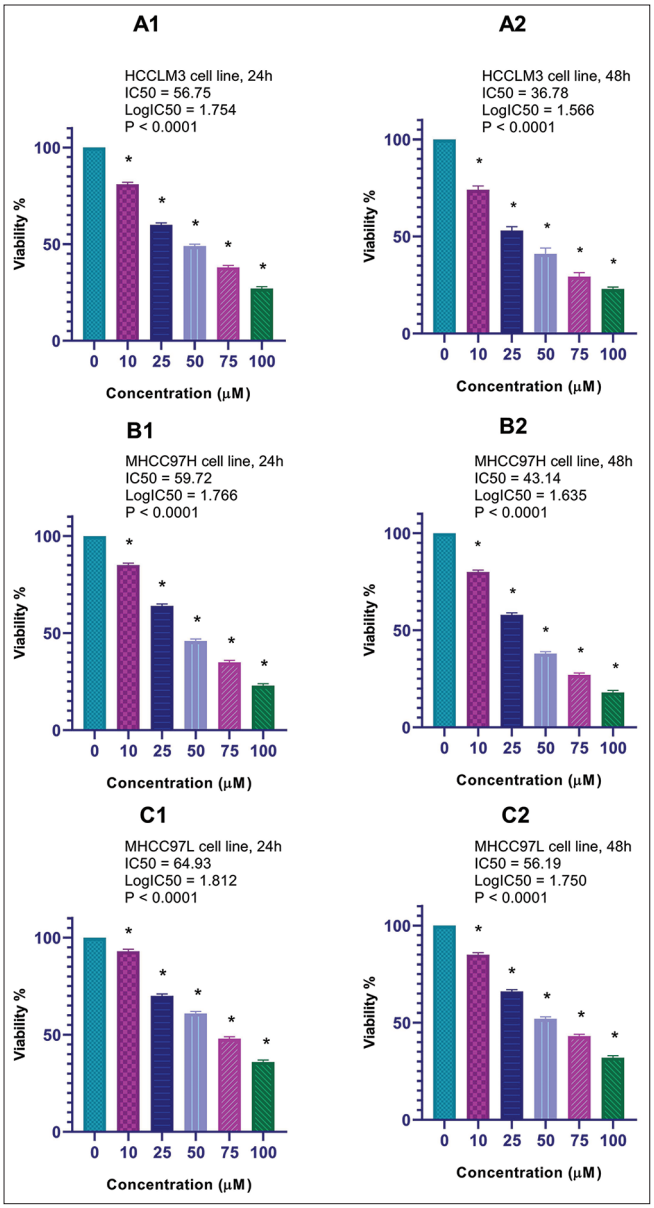

Figure 1: The effect of zebularine (0, 10, 25, 50, 75, and 100 μM) on the viability of HCC cell lines, HCCLM3, MHCC97H, and MHCC97L. The HCC cell lines were treated without and with different doses of zebularine for 24 and 48 h, and the cell viability was investigated by MTT assay. Asterisks (*) indicate significant differences between treated and untreated cells

Cell apoptosis

To determine cell apoptosis, the HCC (HCCLM3, MHCC97H, and MHCC97L) cells were treated with zebularine, based on IC50 values, for 24 and 48 h.

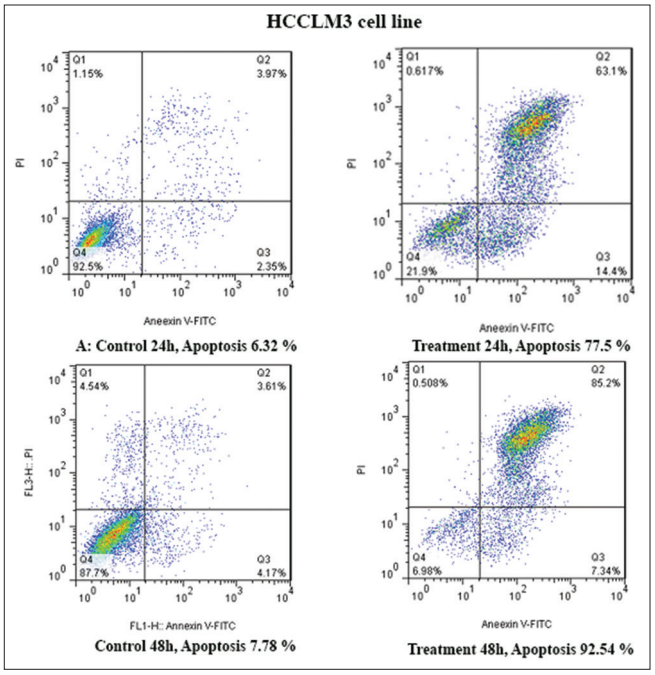

Figure 2: The apoptosis-inducing effect of zebularine was investigated by flow cytometric analysis of HCCLM3 cells. The result indicated that zebularine induced cell apoptosis after 24 and 48 h of treatment significantly

Subsequently, the cells were stained using Annexin V-FITC and PI to obtain apoptotic cells in both early and late apoptosis stages. As indicated in Figures 2–5, this compound induced cell apoptosis in all treated groups significantly ($P < 0.001$). The percentages of zebularine-treated apoptotic cells were reduced by about 77.5 and 92.54% ($P < 0.0001$) in HCCLM3 cell line, 38.5 and 59.52% ($P < 0.0001$) in MHCC97H cell line, and 19.76 and 46.87% ($P < 0.0001$) in MHCC97L cell line after 24 h and 48 h, respectively. Maximal and minimal apoptosis was seen in HCCLM3 and MHCC97L cell lines, respectively.

Result of determination of genes expression

HCCLM3 cell line

The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in HCCLM3 cells treated with zebularine for 24 and 48 h was assessed by quantitative real-time RT-PCR analysis. The results demonstrated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and

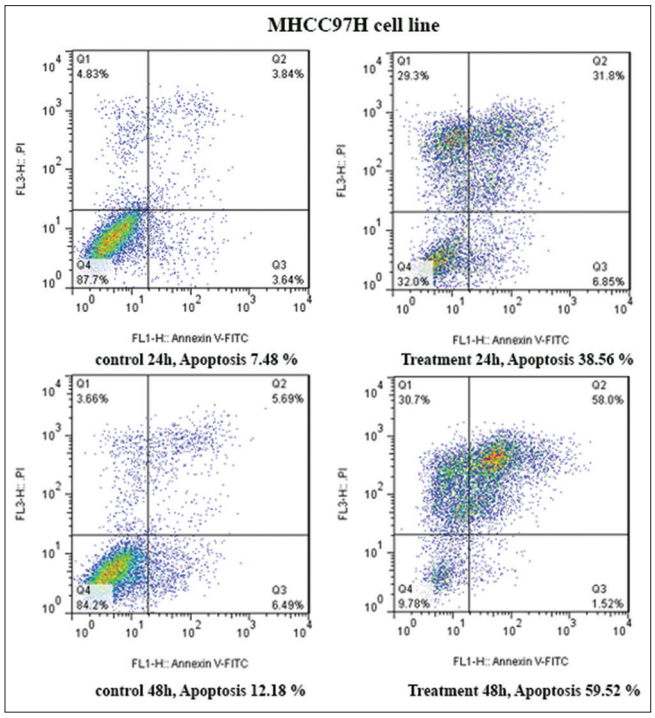

Figure 3: The apoptosis-inducing effect of zebularine was investigated by flow cytometric analysis of MHCC97H cells. The result indicated that zebularine induced cell apoptosis after 24 and 48 h of treatment significantly

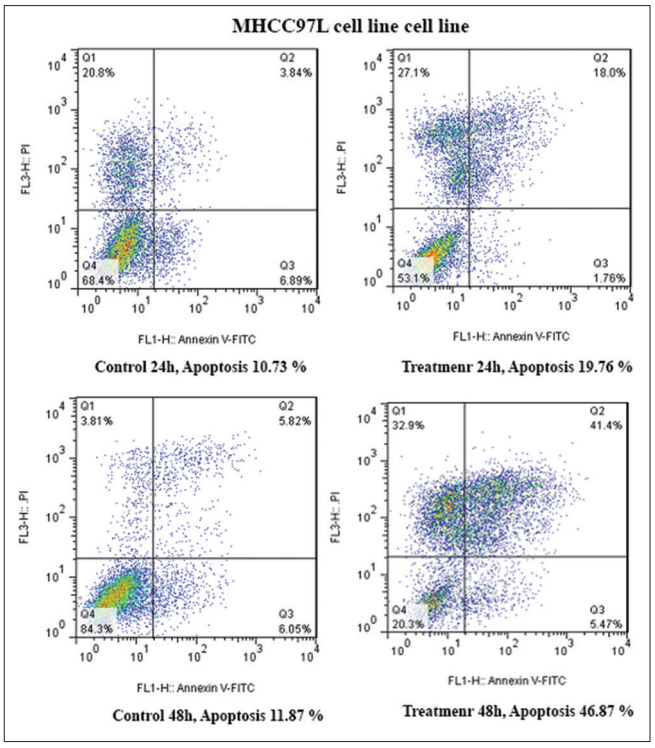

Figure 4: The apoptosis-inducing effect of zebularine was investigated by flow cytometric analysis of MHCC97L cells stained with Annexin V and propidium iodide. The result indicated that zebularine induced cell apoptosis after 24 and 48 h of treatment significantly

TRAIL, p21, and p53 and down-regulated the expression of Bcl-2, Bcl-xL, Mcl-1, DNA methyltransferase 1, 3a, and 3b significantly, Figure 6.

MHCC97H cell line

The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in MHCC97H cells treated with zebularine for 24 and 48 h was assessed by quantitative real-time RT-PCR analysis. The results indicated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of Mcl-1, DNA methyltransferase 1, 3a, and 3b significantly. It could not down-regulate Bcl-2 and Bcl-xL significantly after 24 and 48 h of treatment [Figure 7].

MHCC97L cell line

The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in MHCC97L cells treated with zebularine for 24 and 48 h was assessed by quantitative real-time RT-PCR analysis. The results indicated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of DNA methyltransferase 1, 3a, and 3b significantly. It could not down-regulate Mcl-1, Bcl-2, and Bcl-xL significantly after 24 and 48 h of treatment [Figure 8].

Discussion

Epigenetic modifications (e.g., DNA methylation of the promoter region of the genes) act to regulate gene transcription and expression. Indeed, DNA hypermethylation of the promoter region plays an important role in cancer induction through transcriptional silencing of TSGs. The DNMTIs can induce functional re-expression and up-regulation of aberrantly silenced genes, leading to cell growth inhibition and apoptosis induction.[25] These compounds can induce apoptosis through both extrinsic and intrinsic molecular mechanisms.[26,27] In the present study, we indicated that zebularine up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of Bcl-2, Bcl-xL, Mcl-1, DNA methyltransferase 1, 3a, and 3b, significantly resulting in cell growth inhibition and apoptosis induction in HCC cell lines. Similar to our findings, it has been shown that zebularine up-regulated $p21^{WAF/CIP1}$ and p53 and down-regulated the anti-apoptotic protein Bcl-2 level in HCC HepG2 cell line.[28] *In vitro* studies have demonstrated that zebularine induces changes in apoptotic proteins by DNMTs inhibition and alteration in caspase-3, Bcl2, Bax, and PARP cleavage in breast cancer.[29] Our previous work demonstrated that zebularine decreased DNMTs (DNMT1, 3a, and 3b) and increased p21, p27, and p57 genes expression significantly in LS 180 cell line.[30] Further, it

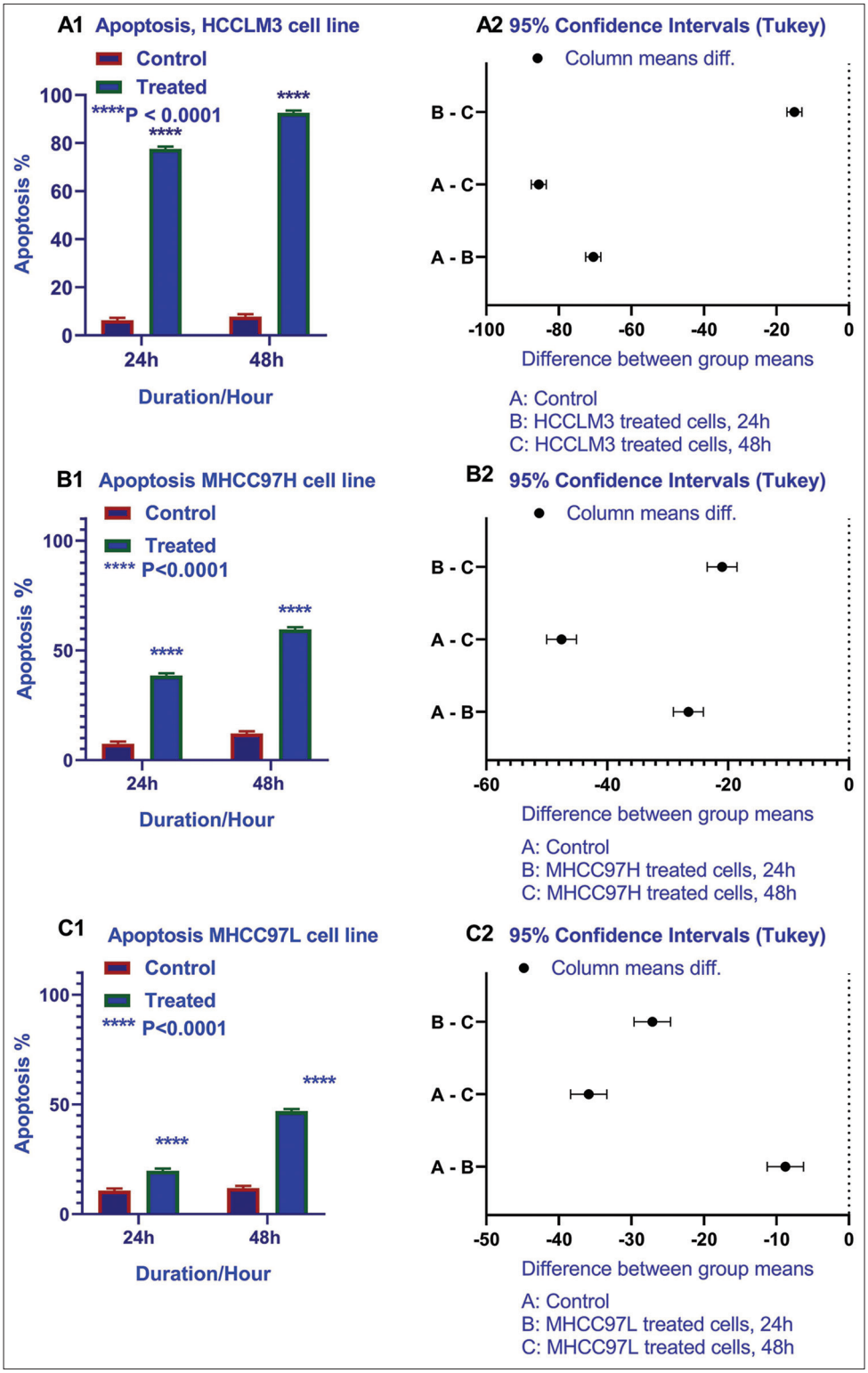

Figure 5: The apoptotic effect of zebularine on HCCLM3, MHCC97H, and MHCC97L cells versus control groups at different periods (24 and 48 h). The results of the statistical analysis indicate significant differences between treated and untreated cells

has been reported that DNA methyltransferase inhibitor azacitidine enhances caspase-3- and caspase-8-dependent apoptosis by re-expression of DR4 in ovarian cancer.[31] Another DNA demethylating agent decitabine has been shown to induce cell apoptosis by down-regulation of anti-apoptotic protein levels (such as XIAP, Bcl-2, cIAP-1, and cIAP-2), the activation of caspases, the cleavage of Bid proteins, and the collapse of mitochondrial membrane potential (MMP) in human leukemia cell lines (U937 and HL60).[32] Furthermore, this compound down-regulates the

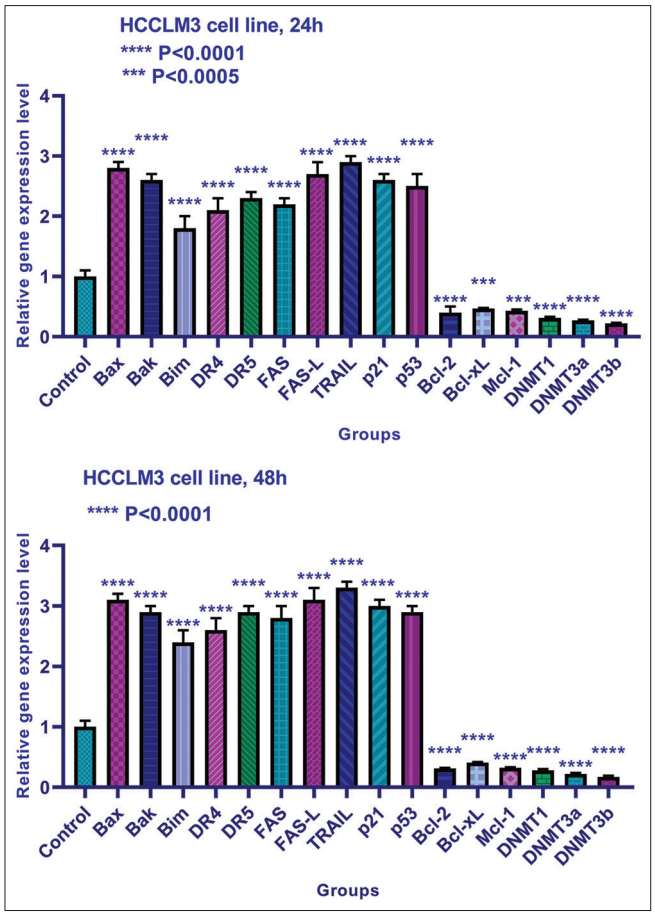

Figure 6: The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in HCCLM3 cells treated with zebularine for 24 and 48 h. The results indicated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of Bcl-2, Bcl-xL, Mcl-1, DNA methyltransferase 1, 3a, and 3b significantly. Asterisks (*) indicate significant differences between treated cells and the control group

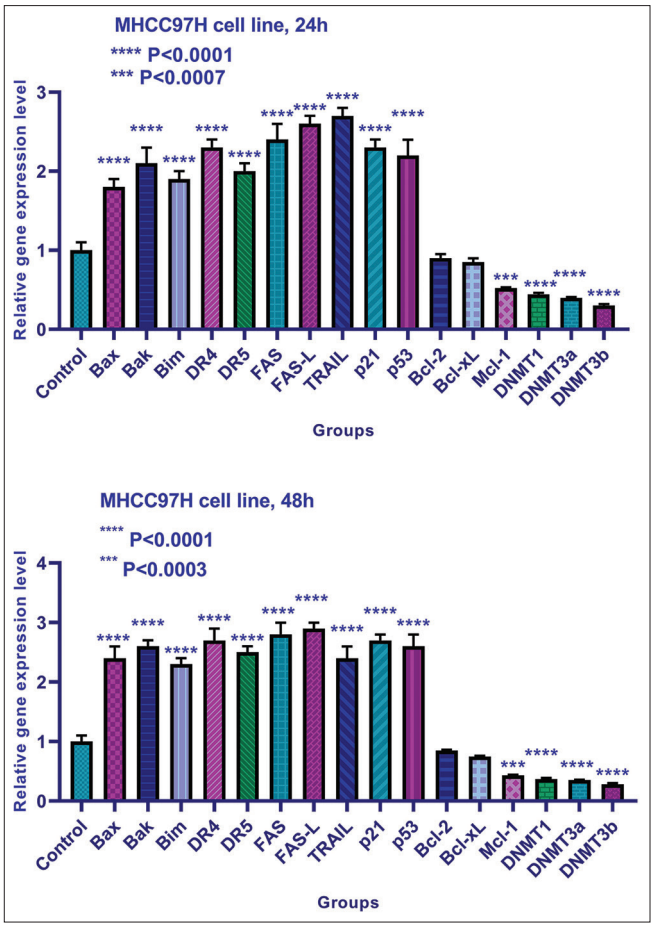

Figure 7: The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in MHCC97H cells treated with zebularine for 24 and 48 h. The results demonstrated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of Mcl-1, DNA methyltransferase 1, 3a, and 3b significantly. It could not down-regulate Bcl-2 and Bcl-xL significantly after 24 and 48 h of treatment. Asterisks (*) indicate significant differences between treated cells and the control group

expression of anti-apoptotic Bcl-2 protein, up-regulates the protein expression levels of pro-apoptotic proteins Bax, p53, and cytochrome c, and increases the cleavage of procaspases 8 and 9 in colorectal cancer Caco-2 cell line.[33] In the current study, zebularine had no significant effect on the expression of Bcl-2 and Bcl-xL in the MHCC97H cell line and also on the expression of Bcl-2, Bcl-xL, and Mcl-1 in MHCC97L cell line after 24 and 48 h of treatment. An apoptotic effect of zebularine at a concentration of 100 μM has been reported in human lens epithelial cells[34] and also at a concentration of 350 μM in head and neck cancer cells.[35] Therefore, zebularine may alter the expression of these three genes with a high concentration in MHCC97H and MHCC97L cell lines. The evaluation of the high concentrations of zebularine on these cell lines and genes is recommended.

Conclusions

In conclusion, our findings indicated that DNMTI zebularine can induce cell apoptosis and inhibit cell growth via both extrinsic and intrinsic apoptotic pathways in HCC HCCLM3, MHCC97H, and MHCC97L cell lines.

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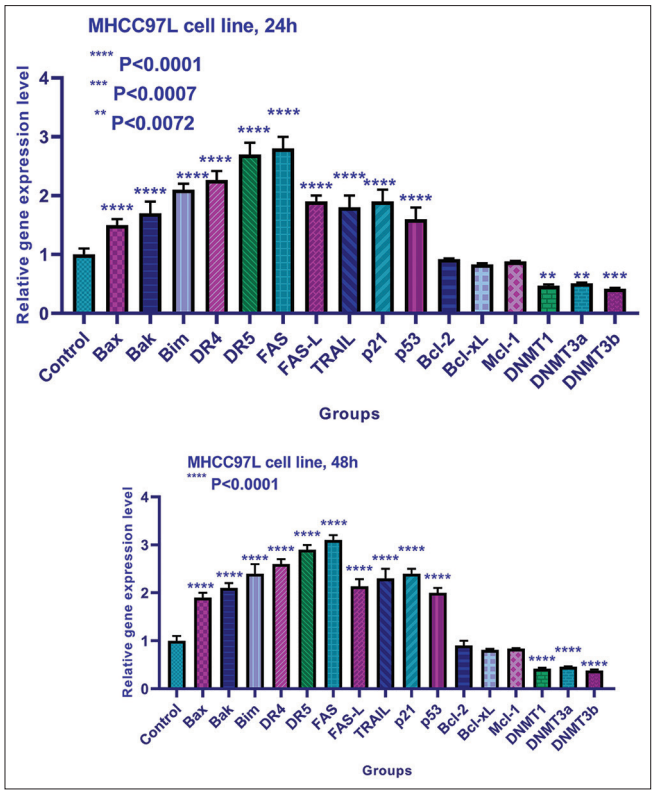

Figure 8: The relative expression level of Bcl-2, Bcl-xL, Mcl-1, Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, TRAIL, DNA methyltransferase 1, 3a, and 3b, p21, and p53 in MHCC97L cells treated with zebularine for 24 and 48 h. The results demonstrated that this compound up-regulated the expression of Bax, Bak, Bim, DR4, DR5, FAS, FAS-L, and TRAIL, p21, and p53 and down-regulated the expression of DNA methyltransferase 1, 3a, and 3b significantly. It could not down-regulate Mcl-1, Bcl-2, and Bcl-xL significantly after 24 and 48 h of treatment. Asterisks (*) indicate significant differences between treated cells and the control group

Conflicts of interest

There are no conflicts of interest.

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References

1. Momparler RL, Bovenzi V. DNA methylation and cancer. J Cell Physiol 2000;183:145-54.
2. Margot JB, Ehrenhofer-Murray AE, Leonhardt H. Interactions within the mammalian DNA methyltransferase family. BMC Mol Biol 2003;4:1-9.
3. Haggarty P, Hoad G, Harris SE, Starr JM, Fox HC, Deary IJ, *et al*. Human intelligence and polymorphisms in the DNA methyltransferase genes involved in epigenetic marking. PLoS One 2010;5:11329-35.
4. Brown R, Strathdee G. Epigenetic cancer therapies: DNA methyltransferase inhibitors. Expert Opin Investig Drugs 2002;11:747-54.
5. Flotho C, Claus R, Batz C, Schneider M, Sandrock I, Ihde S, *et al*. The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. Leukemia 2009;23:1019-28.
6. Matthews GM, Lefebure M, Doyle MA, Shortt J, Ellul J, Chesi M, *et al*. Preclinical screening of histone deacetylase inhibitors combined with ABT-737, rhTRAIL/MD5-1 or 5-azacytidine using syngeneic Vk MYC multiple myeloma. Cell Death Dis 2013;4:798-806.
7. Ruiz-Magaña MJ, Rodríguez-Vargas JM, Morales JC, Saldivia MA, Schulze-Osthoff K, Ruiz-Ruiz C. The DNA methyltransferase inhibitors zebularine and decitabine induce mitochondria-mediated apoptosis and DNA damage in p53 mutant leukemic T cells. Int J Cancer 2012;130:1195-207.
8. Walia YK, Sharma V. Role of HDACs and DNMTs in cancer therapy: A review. Asian J Adv Basic Sci 2013;1:62-78.
9. Pulukuri SMK, Rao JS. Activation of p53/p21Waf1/Cip1 pathway by 5-aza-2'-deoxycytidine inhibits cell proliferation, induces pro-apoptotic genes and mitogen-activated protein kinases in human prostate cancer cells. Int J Oncol 2005;26:863-71.
10. Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: Molecular mechanisms. J Pathol JPTLAS 2003;199:275-88.
11. Yagita H, Takeda K, Hayakawa Y, Smyth MJ, Okumura K. TRAIL and its receptors as targets for cancer therapy. Cancer Sci 2004;95:777-83.
12. Sanaei M, Kavoosi F, Salehi H. Genistein and trichostatin a induction of estrogen receptor alpha gene expression, apoptosis and cell growth inhibition in hepatocellular carcinoma HepG 2 cells. Asian Pac J Cancer Prev 2017;18:3445-51.
13. 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;8:869-76.
14. Huang Y-W, Wang L-S, Chang H-L, Ye W, Dowd MK, Wan PJ, *et al*. Molecular mechanisms of (-)-gossypol-induced apoptosis in human prostate cancer cells. Anticancer Res 2006;26:1925-33.
15. 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;8:24119-26.
16. 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;7:33262-70.
17. 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;14:3069-76.
18. 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;13:98-104.
19. 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;29:625-35.
20. Puppo F, Contini P, Ghio M, Brenci S, Scudeletti M, Filaci G, *et al*. Soluble human MHC class I molecules induce soluble fas ligand secretion and trigger apoptosis in activated CD8 + Fas (CD95)+ T lymphocytes. Int Immunol 2000;12:195-203.
21. Herbeuval J-P, Boasso A, Grivel J-C, Hardy AW, Anderson SA, Dolan MJ, *et al*. TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its *in vitro* production by antigen-presenting cells. Blood 2005;105:2458-64.
22. Sanaei M, Kavoosi F, Roustazadeh A, Golestan F. Effect of genistein in comparison with trichostatin a on reactivation of DNMTs genes in hepatocellular carcinoma. JCTH 2018;6:141-8.
23. Pirouzpanah MB, Sabzichi M, Pirouzpanah S, Chavoshi H, Samadi N. Silibilin-induces apoptosis in breast cancer cells by modulating p53, p21, Bak and Bcl-XL pathways. Asian Pac J

Cancer Prev 2015;16:2087-92.
24. Wiklund ED, Bramsen JB, Hulf T, Dyrskjøt L, Ramanathan R, Hansen TB, *et al*. Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. IJC 2011;128:1327-34.
25. Gray SG, Baird A-M, O'Kelly F, Nikolaidis G, Almgren M, Meunier A, *et al*. Gemcitabine reactivates epigenetically silenced genes and functions as a DNA methyltransferase inhibitor. Int J Mol Med 2012;30:1505-11.
26. Soncini M, Santoro F, Gutierrez A, Frigè G, Romanenghi M, Botrugno OA, *et al*. The DNA demethylating agent decitabine activates the TRAIL pathway and induces apoptosis in acute myeloid leukemia. Biochim Biophys Acta Mol Basis Dis 2013;1832:114-20.
27. Jin S, Cojocari D, Purkal JJ, Popovic R, Talaty NN, Xiao Y, *et al*. 5-Azacitidine induces NOXA to prime AML cells for venetoclax-mediated apoptosis. Clin Cancer Res 2020;26:3371-83.
28. Nakamura K, Aizawa K, Nakabayashi K, Kato N, Yamauchi J, Hata K, *et al*. DNA methyltransferase inhibitor zebularine inhibits human hepatic carcinoma cells proliferation and induces apoptosis. PloS One 2013;8:54036-42.
29. Billam M, Sobolewski MD, Davidson NE. Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. Breast Cancer Res Treat 2010;120:581-92.
30. Sanaei M, Kavoosi F. Investigation of the effect of zebularine in comparison to and in combination with trichostatin A on p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2, DNA methyltransferases and histone deacetylases in colon cancer LS 180 cell line. Asian Pac J Cancer Prev 2020;21:1819-28.
31. Li Y, Hu W, Shen D-Y, Kavanagh JJ, Fu S. Azacitidine enhances sensitivity of platinum-resistant ovarian cancer cells to carboplatin through induction of apoptosis. AJOG 2009;200:177-86.
32. Shin DY, Park Y-S, Yang K, Kim G-Y, Kim W-J, Han MH, *et al*. Decitabine, a DNA methyltransferase inhibitor, induces apoptosis in human leukemia cells through intracellular reactive oxygen species generation. Int J Oncol 2012;41:910-8.
33. Najem SA, Khawaja G, Hodroj MH, Rizk S. Synergistic effect of epigenetic inhibitors decitabine and suberoylanilide hydroxamic acid on colorectal cancer in vitro. Curr Mol Pharmacol 2019;12:281-300.
34. Zhou P, Lu Y, Sun X-H. Effects of a novel DNA methyltransferase inhibitor Zebularine on human lens epithelial cells. Mol Vis 2012;18:22-9.
35. Napso T, Fares F. Zebularine induces prolonged apoptosis effects via the caspase-3/PARP pathway in head and neck cancer cells. Int J Oncol 2014;44:1971-9.