Original Article

Investigation of the Effect of 5-Aza-2'-Deoxycytidine in Comparison to and in Combination with Trichostatin A on *p16INK4a*, *p14ARF*, *p15INK4b* Gene Expression, Cell Growth Inhibition and Apoptosis Induction in Colon Cancer Caco-2 Cell Line

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Abstract

Background: The cell cycle is divided into four phases, G1, G2, S, and M phase. The mammalian cell cycle is controlled and governed by the kinase complexes including cyclin and the cyclin-dependent kinase (CDK), cyclin-CDK complexes. The activity of the complexes is regulated by cyclin-dependent kinase inhibitors (CDKIs), the INK4, and the CDK interacting protein/kinase inhibitory protein (CIP/KIP) families. Promoter hypermethylation and histone deacetylation of CDKIs have been reported in several cancers. These changes can be reversed by DNA demethylating agents, such as decitabine, 5-Aza-2′-deoxycytidine (5-Aza-CdR), and histone deacetylase inhibitors (HDACIs), such as trichostatin A. Previously, we reported the effect of 5-Aza-CdR and trichostatin A (TSA) on hepatocellular carcinoma (HCC). The present study aimed to investigate the effect of 5-Aza-CdR in comparison to and in combination with trichostatin A on *p16INK4a*, *p14ARF*, *p15INK4b* genes expression, cell growth inhibition and apoptosis induction in colon cancer Caco-2 cell line. **Methods:** The Caco-2 cells were cultured and treated with 5-Aza-CdR and TSA (alone and combined). The cell viability, apoptosis, and relative gene expression were determined by MTT assay, flow cytometry, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), respectively. **Results:** Both compounds inhibited cell growth, induced apoptosis, and up-regulated the *p16INK4a, p14ARF, p15INK4b* gene significantly. The TSA had a more significant effect in comparison to 5-Aza-CdR. Furthermore, maximal apoptosis and up-regulation were observed with combined treatment. **Conclusions:** our finding indicated that 5-Aza-CdR and TSA can epigenetically re-activate the *p16INK4a, p14ARF, p15INK4b* gene resulting in cell growth inhibition and apoptosis induction in colon cancer.

Keywords: *Colonic neoplasms, cyclin-dependent kinase inhibitors, decitabine, trichostatin A*

Introduction

The most important decision that every mammalian live-cell must make is whether to continue cell division or to exit the cell cycle and reach a quiescent state. The cell cycle is divided into four phases, G1, G2, S, and M phase. The mammalian cell cycle is controlled and governed by the kinase complexes including cyclin, as a regulatory subunit, and the cyclin-dependent kinase (CDK), as a catalytic subunit together named cyclin-CDK complexes. The kinases are very essential for the accuracy of DNA replication and chromosome segregation. They are activated by phosphorylation/dephosphorylation events and binding to regulatory subunits, cyclins. The activity of complexes, cyclin-CDK complexes, is regulated at several levels including phosphorylation of CDK subunit, the availability of the cyclin subunit, inhibitory molecules named CDK inhibitors (CKIs). In mammals, these inhibitors are divided into two groups, the INK4 and the CDK interacting protein/kinase inhibitory protein (CIP/KIP) families.[1] The INK4 family inhibits CDK4/6 and comprises *p16INK4a, p14ARF, p15INK4b*. The CIP/KIP family includes *p21CIP1, p27KIP1, and p57KIP2*.[2,3] Promoter hypermethylation of INK4[4] and CIP/KIP family,[5] as CKIs, have been reported in several cancers. INK4 family (*p14ARF, p15INK4b, and p16INK4a* genes) located on chromosome 9p21 is hypermethylated

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in various cancers.[6] It has been reported that overexpression of DNA methyltransferase (DNMTs) results in promoter hypermethylation of the INK4 family leads to cancer induction in several tissues such as liver and lung.[7-9] The role of methylation in cancer induction and tumorigenesis was inferred from epigenetic studies of normal and neoplastic tissues. In proliferating cells, DNA hypermethylation is critically dependent on the continued expression of DNMTs.[10] The enzymes involved in DNA methylation are DNMTs, which catalyze the transfer of a methyl group from S-adenosyl-methionine to cytosine residues to form 5-methylcytosine, a modified base found at CpG sites of the cellular genome. This alteration can suppress the expression of tumor suppressor genes (TSGs) such as the INK4 family. Overexpression of TSGs plays a significant role in the induction and development of many cancers comprising gastric cancer,[11] ovarian cancer,[12] and colorectal cancers.[13-15] DNA demethylating agent decitabine, 5-Aza-2′-deoxycytidine (5-Aza-CdR), can revert hypermethylation of INK4 and CIP/KIP genes in colorectal cancer.[16-18] Histone deacetylases (HDACs) regulate biological processes by deacetylation of histones and non-histone proteins. The overexpression of HDACs has been demonstrated in numerous cancer types.[19] The histone deacetylase inhibitors (HDACIs) reactivate the INK4 and CIP/CIP gene families causing G1 phase arrest. It has been reported that HDACI trichostatin A (TSA) treatment induces the expression of INK4 and CIP/CIP genes in various solid and hematologic cancers.[20-22] [Previously, we reported the effect of 5-Aza-CdR and TSA on hepatocellular carcinoma (HCC).[23,24]

The present study aimed to investigate the effect of 5-Aza-CdR in comparison to and in combination with TSA on *p16INK4a, p14ARF, p15INK4b* genes expression, cell growth inhibition and apoptosis induction in colon cancer Caco-2 cell line.

Methods

Materials

The human colon cancer Caco-2 cell line was provided from the National Cell Bank of Iran-Pasteur Institute and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum 10% and antibiotics in a humidified atmosphere of 5% CO2 in air at 37°C.

5-Aza-CdR and TSA were purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) to make a master stock solution.

Further concentration was obtained by diluting the provided solution. Other compounds including, antibiotics, DMSO, 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), phosphate-buffered saline (PBS), DMEM, trypsin-EDTA, Annexin-V-(FITC), propidium iodide (PI), were purchased from Sigma. Total RNA extraction kit (TRIZOL reagent) and real-time polymerase chain reaction (RT-PCR) kits (qPCR MasterMix Plus for SYBR Green I dNTP) were obtained from Applied Biosystems Inc. (Foster, CA, USA).

Cell culture and cell viability

This work was approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1398.023. To perform the present study, the Caco-2 cells were cultured in DMEM supplemented with 10% FBS, sodium pyruvate, antibiotics, and sodium bicarbonate at 37°C in 5% CO_2 overnight and then seeded into 96-well plates (5×10^5 cells per well).

After 24 h of culture, the growth medium was removed and experimental medium containing various doses of 5-Aza-CdR (0, 1, 2.5, 5, 10, and 20 μm) and TSA (0, 0.5, 1, 2.5, 5, and 10 μm) was added. The control groups received DMSO only, at a concentration of 0.05%. The cells were treated with 5-Aza-CdR and TSA for 24 and 48 h. Subsequently, the cell viability was evaluated by MTT assay according to standard protocols, a quantitative colorimetric assay based on the living cell's ability, to determine cell growth in the treated and untreated control groups. This technique is based on the activity of cellular enzymes which reduces the tetrazolium salt MTT resulting in a dark-blue formazan product. The product is dissolvable in DMSO through which the number of viable cells can be indicated. Therefore, the MTT solution was added to each well for 4 h at 37°C, the MTT solution was removed, DMSO was added and shaken 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

For apoptosis determination, the Caco-2 cells were cultured at a density of 4×10^5 cells/well and incubated overnight without treatment. Subsequently, the Caco-2 cells were treated with 5-Aza-CdR (5 μm) and TSA (2.5 μm), as alone and combined, for 24 and 48 h and then the culture medium was removed and the cells were harvested by trypsinization, washed with cold PBS, and resuspended in Binding buffer (1×).

To determine apoptotic cells, Annexin-V-(FITC) and PI were used according to the protocol and the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time quantitative reverse transcription-polymerase

Chain reaction (qRT-PCR)

To determine the relative expression level of p16INK4a, p14ARF, p15INK4b gene, qRT-PCR was done. The

Caco-2 cells were treated with 5-Aza-CdR (5 μm) and TSA (2.5 μm), as alone and combined, for different periods (24 and 48 h), based on IC5o values, and total RNA from the untreated and treated Caco-2 cells was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer protocol and treated by RNase-free DNase (Qiagen) to eliminate the genomic DNA.

The concentration of RNA was determined using a BioPhotometer (Biowave II Germany). Total RNA (100 ng) was reverse-transcribed to complementary DNA (cDNA) by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, K1622 for 100 reactions), according to the protocol.

Real-time RT-PCR was performed by the Maxima SYBR Green RoxqPCR master mix kit (Fermentas). The primer sequences were obtained from the published article in which their sequences are indicated in Table 1. GAPDH was used as an endogenous control. Data were analyzed using the comparative Ct (ΔΔct) method.

Results

Result of cell viability by the MTT assay

The viability of Caco-2 cells treated with various concentrations of 5-Aza-CdR and TSA was measured by MTT assay. As shown in Figure 1, both compounds induced significant cell growth inhibition with all experimental concentrations as a dose- and time-dependent manner ($P < 0.001$). IC50 values were obtained with approximately 5 and 2.5 μm for 5-Aza-CdR and TSA, respectively.

Result of cell apoptosis assay

To determine whether 5-Aza-CdR (5 μm) and TSA (2.5 μm) could induce apoptosis, the Caco-2 cells were stained using annexin-V-(FITC) and PI. As demonstrated in Figures 2 and 3, significant differences were observed by comparing the amounts of the treated cells to the untreated cells. Both compounds induced apoptosis in a time-dependent manner ($P < 0.001$). Furthermore, combined treatment h alone ad a more significant effect in comparison to each agent ($P < 0.001$), Figure 4. Besides, TSA had a more significant effect in comparison to 5-Aza-CdR ($P < 0.001$). Besides maximal apoptosis was seen in the combined treatment groups after 48 h of treatment ($P < 0.001$), Figure 5.

Table 1: The primer sequences of *p16INK4a, p14ARF, p15INK4b* genes

Primer	Primer sequences (5′ to 3′)	Reference
$p14^{ARF}$		[25]
Forward	GTGGGTTTTAGTTTGTAGTT	
Reverse	AAACCTTTCCTACCTAATCT	
p15INK4b		[26]
Forward	AAGCTGAGCCCAGGT CTCCTA	
Reverse	CCACCGTTGGCCGTAAACT	
p16INK4a		[27]
Forward	CCCGCTTTCGTAGTTTTCAT	
Reverse	TTATTTGAGCTTTGGTTCTG	

Result of determination of genes expression

The result of RT-PCR analysis demonstrated that treatment with 5-Aza-CdR (5 μm) and TSA (2.5 μm), as alone and combined, at 24 and 48 h up-regulated p16INK4a, p14ARF, p15INK4b significantly ($P < 0.001$), Table 2 and Figures 6, 7. Finally, TSA had a more significant effect on gene expression in comparison to 5-Aza-CdR ($P < 0.001$). Besides maximal up-regulation was seen in the combined treatment groups after 24 and 48 h of treatment as indicated in Figure 8.

Discussion

Epigenetic alterations such as histone acetylation and DNA methylation act to regulate gene expression in normal mammalian proliferating cells. DNA hypermethylation plays a major role in tumorigenesis through transcriptional silencing of TSGs. Other modifications include histone deacetylation affect local compaction of chromatin structure leads to TSGs silencing.[28]

Recently, it has become clear that the silencing of cancer-related TSGs INK4 is not exclusively a result of mutations or deletions, but it can also be because of histone deacetylation and DNA hypermethylation. These changes, deacetylation, and hypermethylation have been reported in several cancers.[29-32] Histone acetylation and DNA methylation of the TSGs such as p16 and p21 can be modified with TSA and 5-Aza-CdR in colorectal cancer.[33-35]

In the current study, we reported that 5-Aza-CdR and TSA can inhibit Caco-2 cell growth and induce apoptosis. Besides, we decided to evaluate the molecular

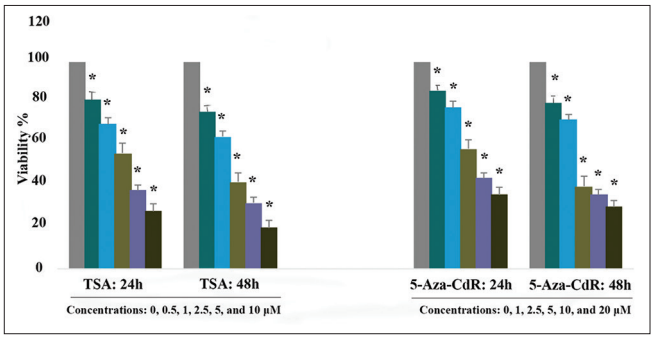

Figure 1: *In-vitro* effects of the various concentrations of 5-Aza-CdR and TSA on colon cancer Caco-2 cell viability (24 and 48 h). The first column of each group belongs to the control group. Values are means of three experiments in triplicate. Asterisks (*) demonstrate significant differences between treated and untreated control groups

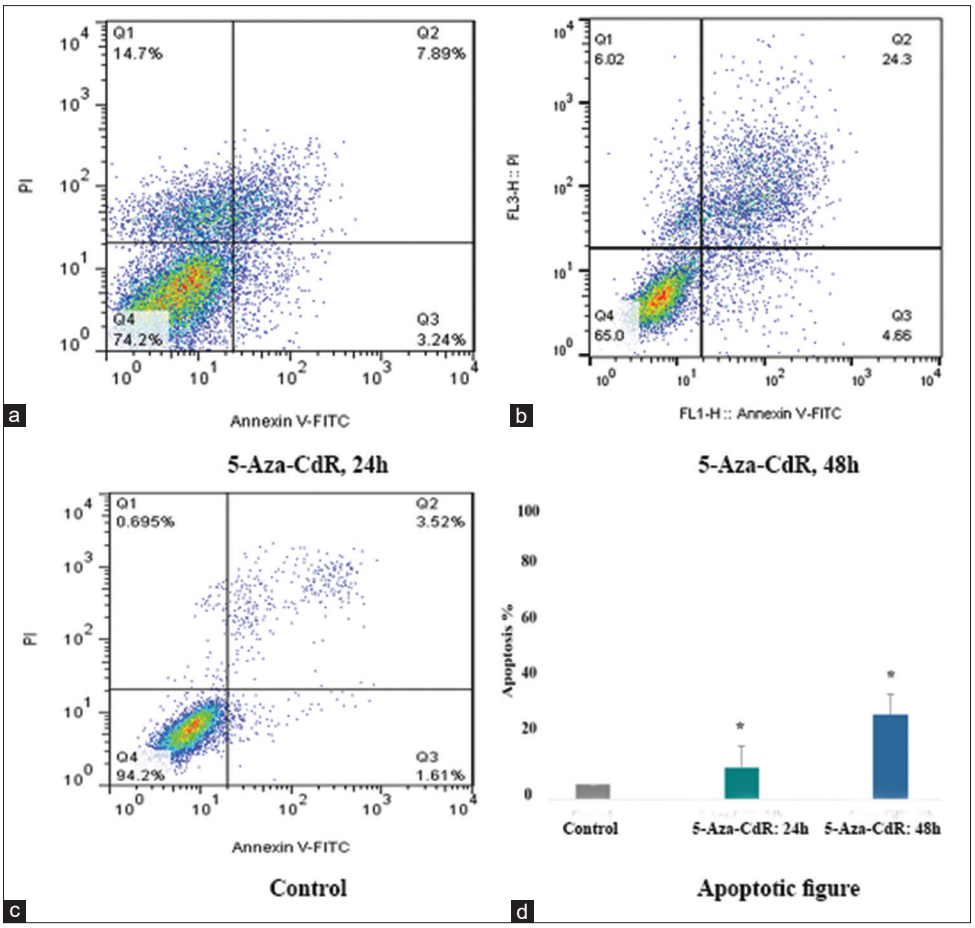

Figure 2: The apoptotic effect of 5-Aza-CdR (5 μm) on Caco-2 cell versus control groups (24 and 48 h) evaluated by flow cytometric analysis. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. Asterisks (*) demonstrate significant differences between treated and untreated control groups. A: 5-Aza-CdR (5 μm) treated cells at 24h, B: 5-Aza-CdR (5 μm) treated cells at 48h, C: Control group, D: Apoptotic graph

Table 2: The relative expression level of *p16INK4a, p14ARF, p15INK4b* genes

Cell line	Gene	Drug	Dose (μm)	Duration (h)	Expression	*P*
Caco-2	p14ARF	5-Aza-CdR	5 μm	24	2.6	0.001
Caco-2	p14ARF	5-Aza-CdR	5 μm	48	2.9	0.001
Caco-2	p15INK4b	5-Aza-CdR	5 μm	24	2.4	0.001
Caco-2	p15INK4b	5-Aza-CdR	5 μm	48	3	0.001
Caco-2	p16INK4a	5-Aza-CdR	5 μm	24	2.5	0.001
Caco-2	p16INK4a	5-Aza-CdR	5 μm	48	3.1	0.001
Caco-2	p14ARF	TSA	2.5 μm	24	2.9	0.001
Caco-2	p14ARF	TSA	2.5 μm	48	3.3	0.001
Caco-2	p15INK4b	TSA	2.5 μm	24	2.7	0.001
Caco-2	p15INK4b	TSA	2.5 μm	48	3	0.001
Caco-2	p16INK4a	TSA	2.5 μm	24	2.8	0.001
Caco-2	p16INK4a	TSA	2.5 μm	48	3.1	0.001
Caco-2	p14ARF	Combined	5 + 2.5 μm	24	3.2	0.001
Caco-2	p14ARF	Combined	5 + 2.5 μm	48	3.8	0.001
Caco-2	p15INK4b	Combined	5 + 2.5 μm	24	3.3	0.001
Caco-2	p15INK4b	Combined	5 + 2.5 μm	48	3.6	0.001
Caco-2	p16INK4a	Combined	5 + 2.5 μm	24	2.9	0.001
Caco-2	p16INK4a	Combined	5 + 2.5 μm	48	3.7	0.001

mechanisms of this effect. In the case of the pathway, we assessed the effect of compounds (alone and combined) on *p16INK4a, p14ARF, p15INK4b* genes expression and found that both agents up-regulated

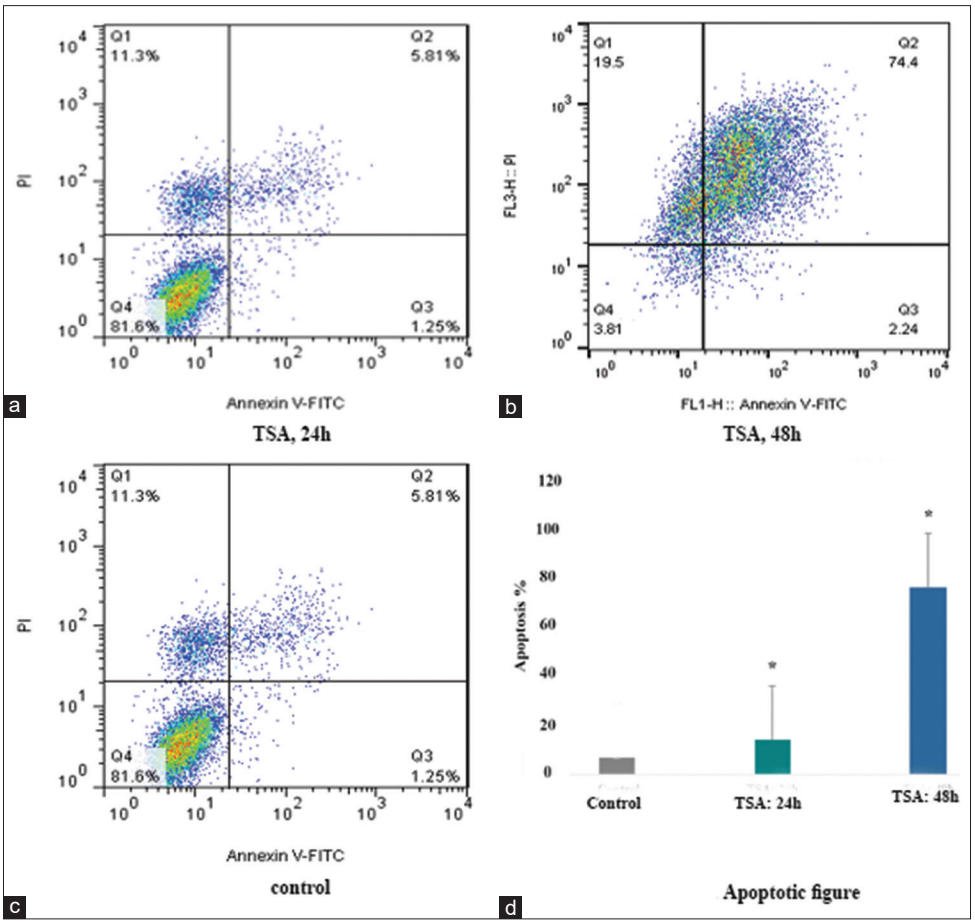

Figure 3: The apoptotic effect of TSA (2.5 μm) on Caco-2 cell versus control groups (24 and 48 h) evaluated by flow cytometric analysis. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. Asterisks (*) demonstrate significant differences between treated and untreated control groups. A: TSA (2.5 μm) treated cells at 24 h, B: TSA (2.5 μm) treated cells at 48h, C: Control group, D: Apoptotic graph

the genes significantly. Furthermore, TSA indicated a more significant effect in comparison to 5-Aza-CdR. Additionally, maximum apoptosis and gene expression were seen with combined treatment.

Similar pathways have been reported by other researchers. It has been shown that Aza-CdR can restore p14ARF in colon cancer RKO cell,[36] p16 in colon cancer HCT116, SW480, L174 T, Co115 cells,[37] and p15 in RKO human colorectal cell.[38] The same molecular mechanism has been indicated in other cancers including human gastric cancer cells,[39,40] human lung cancer cells,[41] and ovarian cancer.[42] Inconsistent with our result, it has been demonstrated that histone deacetylase inhibitor TSA activates the *p15INK4b* gene in human colorectal carcinoma cell line HCT116.[22] It should be noted that the INK4 family is suppressed by DNMTs overexpression. Aberrant DNA methylation is catalyzed by DNMTs. Thus, abnormal variations of these enzymes can contribute to tumorigenesis. The DNMTs are known to date include DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. In colorectal cancer, the over-activity of DNMT3a and DNMT3b leads to cancer induction.[43] Therefore, methylated inactivation of the INK4 pathway is one of the mechanisms of cancer induction in colon cancer. It could be restored by DNMTIs such as 5-Aza-CdR.

Furthermore, the HDAC family has emerged as an important regulator of colorectal cell transformation and maturation. They play a major role in cancer induction and development. These enzymes remove the acetyl groups resulting in the compacted chromatin and cell cycle regulator gene silencing.[44] The overexpression of HDACs represses transcription of INK4 and CIP/KIP families such as *p15INK4b* and *p21WAF1/CIP1*, which in turn, induces cancer induction.[45] As a result, HDACIs can be a suitable choice for silenced TSGs reactivation.

In summary, 5-Aza-CdR and TSA can reactivate INK4 genes by which induce apoptosis in colon cancer. They may play this role by inhibition of the activity of DNMTs and HDACs. We didn't evaluate the effect of the mentioned compounds on the DNMTs and HDACs. Therefore, this assessment is recommended.

Conclusions

In conclusion, our finding indicated that DNA

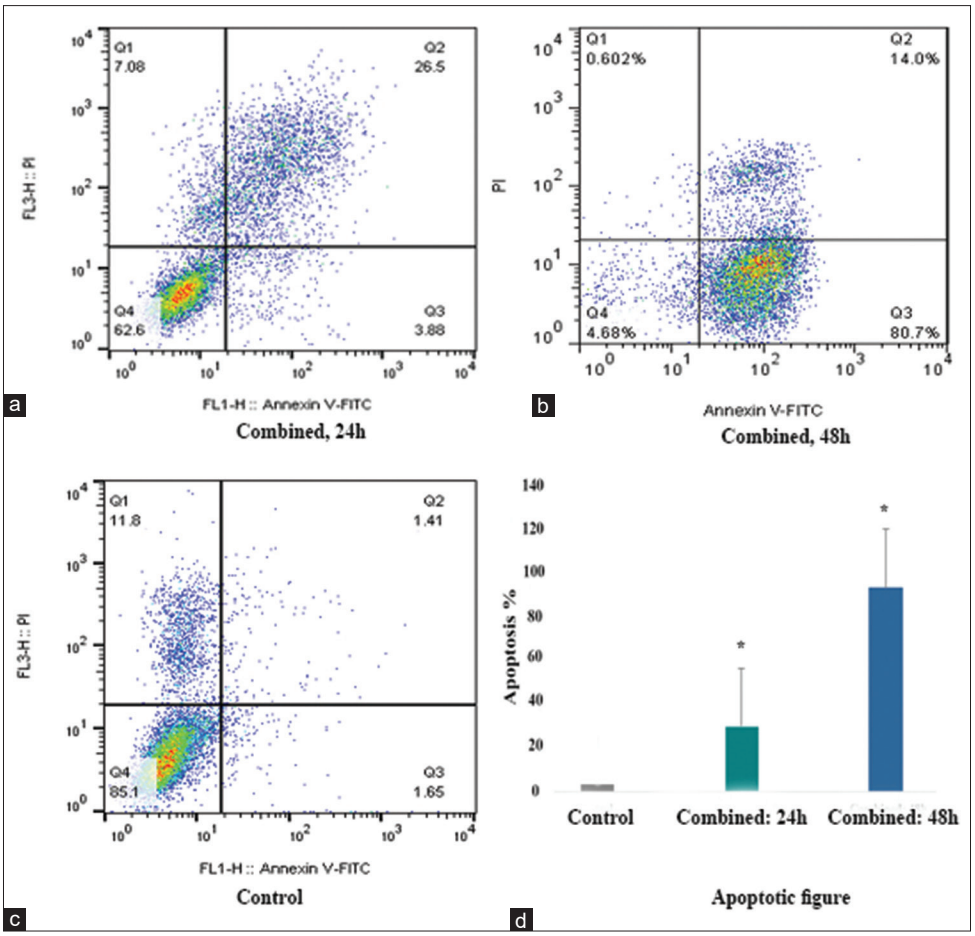

Figure 4: The apoptotic effect of 5-Aza-CdR (5 µm) in combination with TSA (2.5 µm) on Caco-2 cell versus control groups (24 and 48 h) investigated by flow cytometric analysis. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. Asterisks (*) demonstrate significant differences between treated and untreated control groups. A: Combined, 5-Aza-CdR (5 µm) in combination with TSA (2.5 µm), treated cells at 24h, B: Combined, 5-Aza-CdR (5 µm) in combination with TSA (2.5 µm), treated cells at 24h, C: Control group, D: Apoptotic graph

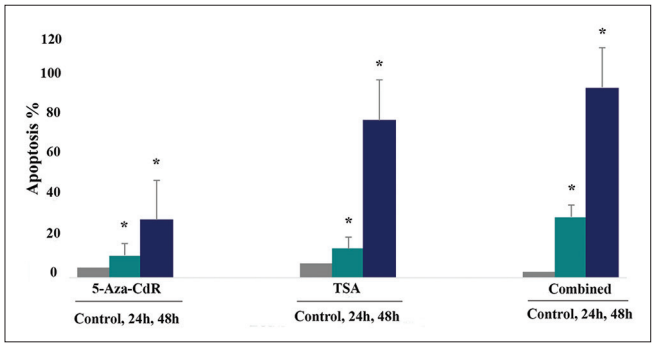

Figure 5: The comparative apoptotic effects of 5-Aza-CdR (5 µm) and TSA (2.5 µm), as alone and combined, on Caco-2 cell at 24 and 48 h. As shown above, the first column of each group belongs to the control group and the others belong to treated cells at 24 and 48 h. Asterisks (*) indicate significant differences between the treated and untreated control groups

demethylating agent 5-Aza-CdR and histone deacetylase inhibitor TSA can epigenetically re-activate the *p16INK4a, p14ARF, p15INK4b* gene resulting in cell growth inhibition and apoptosis induction in colon cancer. Thus, this result suggests a dependence of the *p16INK4a, p14ARF, p15INK4b* gene silencing through hypermethylation and histone deacetylation by a mechanism that involves the up-regulation of DNA methyltransferases and histone deacetylases. Therefore, the evaluation of the effect of 5-Aza-CdR and TSA on these enzymes is recommended.

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Conflicts of interest

There are no conflicts of interest.

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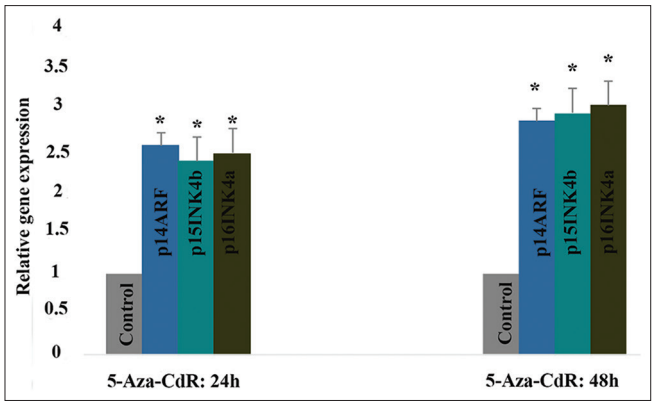

Figure 6: The relative expression level of *p16INK4a, p14ARF, p15INK4b* genes in the Caco-2 cell line treated with 5-Aza-CdR (5 μm) versus untreated control groups (24 and 48 h). The first column of each group belongs to the untreated control group. Asterisks (*) indicate significant differences between the treated and untreated groups

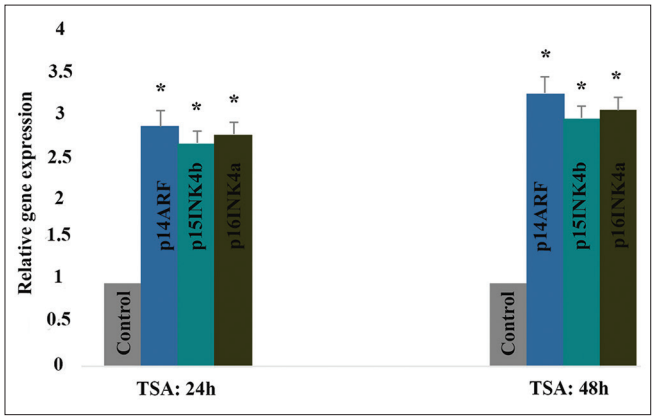

Figure 7: The relative expression level of *p16INK4a, p14ARF, p15INK4b* genes in the Caco-2 cell line treated with TSA (2.5 μm) versus untreated control groups (24 and 48 h). The first column of each group belongs to the untreated control group. Asterisks (*) indicate significant differences between the treated and untreated groups

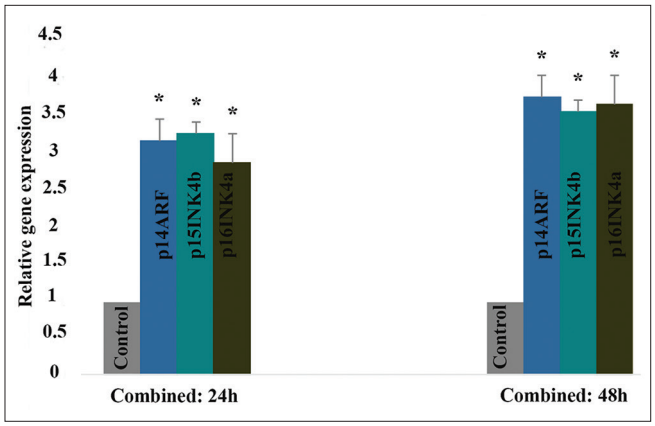

Figure 8: The relative expression level of *p16INK4a, p14ARF, p15INK4b* genes in the Caco-2 cell line treated with 5-Aza-CdR (5 μm) in combination with TSA (2.5 μm) versus untreated control groups (24 and 48 h). The first column of each group belongs to the untreated control group. Asterisks (*) indicate significant differences between the treated and untreated groups

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