

## Effect of 5-aza-2'-deoxycytidine on Estrogen Receptor Alpha/Beta and DNA Methyltransferase 1 Genes Expression, Apoptosis Induction, and Cell Growth Prevention of the Colon Cancer HT 29 Cell Line

### Abstract

**Background:** Cellular activity such as gene expression is regulated by epigenetic mechanisms and modifications. In mammals, DNA methylation is an essential component of the epigenetic machinery of the cells. DNA hypermethylation of the several tumor suppressor genes (TSGs) is associated with transcriptional gene silencing resulting in colon tumorigenesis. Overexpression of DNA methyltransferase 1 (DNMT1) in colon cancer has been reported in several studies. The methylation of estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ) have been demonstrated in various cancers. Previously, we indicated that genistein can reactivate ER $\alpha$  in hepatocellular carcinoma (HCC). The present study was designed to investigate the effect of 5-aza-2'-deoxycytidine (5-aza-CdR) on ER $\alpha$ /ER $\beta$  and DNMT1 gene expression, apoptosis induction, and cell viability inhibition of the colon carcinoma HT 29 cell line. **Methods** The effect of 5-Aza-CdR on the colon carcinoma HT 29 cell viability was measured by MTT assay. To determine the apoptotic cells, the cells were assessed using the Annexin V-FITC/PI detection kit. The expression of ER $\alpha$ , ER $\beta$ , and DNMT1 genes was determined using real-time quantitative RT-PCR. **Results:** The results indicated that 5-Aza-CdR can inhibit cell growth significantly versus control groups, induce significant apoptosis, down-regulate DNMT1, and up-regulate ER $\alpha$  and ER $\beta$  genes expression at different time periods. The percentage of apoptotic cells was 85.83% and 86.84% after 24 and 48 h, respectively ( $P < 0.01$ ). The IC<sub>50</sub> value for 5-Aza-CdR was obtained at 2.5  $\mu$ M. **Conclusions:** 5-Aza-CdR can up-regulate ER $\alpha$  and ER $\beta$  genes expression through DNMT1 down-regulation resulting in apoptosis induction and cell growth prevention.

**Keywords:** 5-aza-2'-deoxycytidine, colon cancer, epigenetic

### Introduction

The genetic information in human cells is stored and encoded which contains basic information controlling and directing gene transcription and expression. Cellular activity such as gene expression is regulated by epigenetic mechanisms and modifications. These modifications and alterations modify gene expression patterns lead to controlling different biological processes including genomic imprinting, cell proliferation, cell differentiation, cell survival, pre-mRNA processing, and X chromosome inactivation.<sup>[1]</sup> Epigenetic mechanisms involved inappropriate gene expression include post-translational modifications of histone proteins, DNA hydroxymethylation, DNA methylation, nucleosome remodeling, and regulation by non-coding RNAs.<sup>[2,3]</sup> Dysregulation of epigenetic modification such as aberrant DNA methylation profiles

and histone modifications attribute as a pathogenic mechanism of cancers.<sup>[4]</sup> In mammals, DNA methylation is an essential component of the epigenetic machinery of the cells. This methylation consists of covalent attachment of a methyl group to the 5-position of cytosine residues in 5'-CG-3' dinucleotides catalyzed by DNA methyltransferases (DNMTs), the predominant epigenetic modification in mammals. The mammalian DNMT family classified into DNMT1, DNMT3a, DNMT3b, and DNMT3L. DNMT1 is involved in the maintenance of methylation and the most abundant DNMTs. DNMT3 acts as a de novo methyltransferase and consists of two subgroups including DNMT3a and DNMT3b. DNMT2 can methylate RNA instead of DNA.<sup>[5]</sup> Colon cancer arises through the accumulation of multiple genetic and epigenetic alterations including DNA methylation and covalent

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### Masumeh Sanaei, Fraidoon Kavooosi

Research Center for Non-Communicable Diseases, Jahrom University of Medical Sciences, Jahrom, Fars Province, Iran

### Address for correspondence:

Dr. Faridoon Kavooosi, Research Center for Non-Communicable Diseases, Jahrom University of Medical Sciences, Jahrom, Fars Province, Postal Code: 74148-46199, Iran. E-mail: [kavooosifraidoon@gmail.com](mailto:kavooosifraidoon@gmail.com)

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histone modifications. DNA hypermethylation of promoter CpG islands, regions of DNA with a high G + C content, of the several tumor suppressor genes (TSGs) is associated with transcriptional gene silencing resulting in colon tumorigenesis.<sup>[6]</sup> Overexpression of DNMT1 in colon cancer has been reported in several studies.<sup>[7]</sup> Several researchers have reported that the methylation of estrogen receptor alpha (ER $\alpha$ ) plays an important role in colon cancer.<sup>[8]</sup> ER $\alpha$  is encoded by the estrogen receptor 1 (ESR1) gene and modulates gene expression by interactions with promoter response elements.<sup>[9]</sup> In addition to ER $\alpha$ , estrogen receptor beta (ER $\beta$ ) is the predominant estrogen receptor in the malignant colonic epithelium. ER $\beta$  expression is reduced during colonic tumorigenesis. ER $\beta$  and ER $\alpha$  are encoded by two separate genes including ESR1 and ESR2 located on different chromosomes.<sup>[10]</sup> The methylation of ER has been demonstrated in prostate cancer LNCaP, PC3, ND1, DUPro, and DU145 cell lines,<sup>[11,12]</sup> breast cancer MDA-MB-231 cell line,<sup>[13]</sup> and endometriosis.<sup>[14]</sup> Several works have demonstrated that 5-aza-2'-deoxycytidine (5-aza-CdR), a potent and non-selective DNA methylation inhibitor, can affect genomic methylation and reactivate several silenced genes such as CDO1, HSPC105, MAGEA3, and RNF113B in colorectal cancer HT29, SW480, SW48, and HCT116 cell lines.<sup>[15,16]</sup> Similarly, it has been reported that 5-aza-CdR activate ER with decreased DNMT1 expression and activity, partial demethylation of the ER CpG island, in breast cancer cell lines MDA-MB-231 and MDA-MB-435.<sup>[17]</sup>

Previously, we indicated that DNA demethylating agent genistein (GE) can reactivate ER $\alpha$  in hepatocellular carcinoma (HCC) Hep G2 cell line.<sup>[18]</sup> The present study was designed to investigate the effect of 5-aza-CdR on estrogen receptor alpha/beta and DNMT1 gene expression, apoptosis induction, and cell growth inhibition of the colon carcinoma HT 29 cell line.

## Methods

### Materials

The colon carcinoma HT 29 cell line was purchased from the National Cell Bank of Iran Pasteur Institute. 5-aza-CdR, 3 (4,5-dimethyl-2-thiazolyl) 2, 5-diphenyl -2H-tetrazolium bromide (MTT), and RPMI-1640 were supplied by Sigma-Aldrich (Sigma-Aldrich, Louis, MI, USA). The Annexin V and propidium iodide (PI) apoptosis kit were purchased from Life Technologies. Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany). Total RNA extraction kit (TRIZOL reagent) and real-time polymerase chain reaction (PCR) kits (qPCR Master Mix Plus for SYBR Green I dNTP) were obtained from Applied Biosystems Inc. (Foster, CA, USA).

### Cell Culture

The colon carcinoma HT 29 cells were maintained in RPMI-1640 containing 100 ml/l fetal bovine serum with

100,000 U/l penicillin and 100 mg/l streptomycin and grown in a humidified incubator at 37°C containing 5% CO<sub>2</sub>. 5-Aza-CdR was dissolved at a concentration of 100  $\mu$ M in DMSO to provide a stock solution, and all of the other test concentrations were provided by dilution of this stock solution. The final DMSO concentration did not exceed 0.1%, and all control groups were administered 0.1% DMSO concentration.

### Cell Viability Assay

The effect of 5-Aza-CdR on the colon carcinoma HT 29 cell viability was measured by MTT assay. The HT 29 cells were cultured with the culture medium. After suitable confluency,  $5 \times 10^5$  cells per each well were transferred into 96-well plates and allowed to adhere overnight. After 24 h of cell seeding, the cells were treated with medium containing different doses of 5-Aza-CdR (1, 2.5, 5, 10 and 20  $\mu$ M) except control groups for 24 and 48 h; the control groups were received an equal volume of solvent, DMSO. After exposure to the various concentrations of the compounds for the mentioned time, the cells were trypsinized, and the viable cell population was determined using MTT.

### Flow Cytometric Analysis of Apoptosis

To determine the apoptotic cells, treated and untreated cells were assessed using the Annexin V-FITC/PI detection kit. In this regard, the HT 29 cells were cultured in 24-well plates at a density of  $5 \times 10^5$  cells/well and incubated and treated with 5-Aza-CdR (2.5  $\mu$ M) for 24 and 48 h, according to IC50 values. After treatment times, all the adherent cells were harvested with trypsin-EDTA, washed with cold fetal bovine serum, and resuspended in binding buffer (1 $\times$ ). Annexin-V-FITC and PI were used for staining according to the protocol. Finally, the apoptotic cells were counted by FACScan™ flow cytometer (Becton Dickinson, Heidelberg, Germany).

### Determination of Gene Expression by Quantitative Real-time RT-PCR

To determine ER $\alpha$ , ER $\beta$ , and DNMT1 gene expression, the cells were treated with 5-Aza-CdR (2.5) for 24 and 48 h. After treatment with 5-Aza-CdR, total RNA of the control and treated cells was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and then pretreated with RNase-free DNase (Qiagen) to remove the genomic DNA prior to cDNA synthesis. The RNA concentration was determined using a Biophotometer (Eppendorf, Hamburg, Germany). Total RNA (100 ng) was reverse transcribed to cDNA using the RevertAid™ First Strand cDNA synthesis kit (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Real-time RT-PCR was performed with the Maxima SYBR Green ROX qPCR Master Mix Kit (Fermentas) as described

previously.<sup>[11]</sup> ER $\alpha$ , ER $\beta$ , DNMT1, and GAPDH primer sequences were obtained from our previous article,<sup>[11,18-20]</sup> which their sequences are shown in Table 1. GAPDH was used as an endogenous control.

### Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. The data were obtained from three separate tests and are indicated as means  $\pm$  standard deviations.

Statistical comparisons between treated and untreated groups were achieved with ANOVA (one-way ANOVA) and Tukey's test. A significant difference was considered  $P < 0.05$ .

## Results

### In vitro effects of 5-Aza-CdR on the LCL-PI 11cells growth

The colon carcinoma HT 29 cells were treated with 5-Aza-CdR (1, 2.5, 5, 10, and 20  $\mu$ M) vs. control groups for 24 and 48 h; control groups received an equal volume of solvent and evaluated by MTT assay to determine cell viability. The results indicated that 5-Aza-CdR can inhibit cell growth significantly versus control groups. As shown in Figure 1, all concentrations of 5-Aza-CdR inhibited cell growth significantly ( $P < 0.001$ ). IC50 values for 5-Aza-CdR were obtained at 2.5  $\mu$ M.

### Effects of 5-Aza-CdR on the LCL-PI 11cells apoptosis

The apoptotic cells were obtained using a flow cytometry assay. The HT 29 cells were treated with 5-Aza-CdR at a concentration of 2.5  $\mu$ M for 24 and 48 h except for control groups. As indicated in Figure 2, 5-Aza-CdR induced significant apoptosis at deferent time periods.

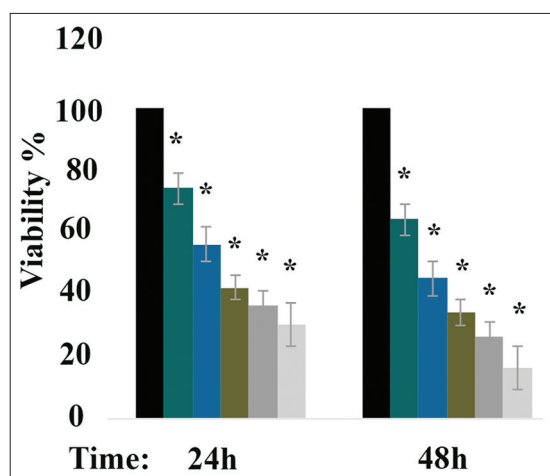


Figure 1: The effect of 5-Aza-CdR on HT 29 cell proliferation. The cells were seeded and treated with 5-Aza-CdR (1, 2.5, 5, 10, and 20  $\mu$ M) for 24 and 48 h, and the cell viability was determined by the MTT assay. The first column of each group represents control group. Data are presented as mean  $\pm$  standard error of the mean from at least three different experiments. Significant differences between treated and control group are indicated by asterisks (\*)

The percentage of apoptotic cells was 85.83% and 86.84% after 24 and 48 h, respectively ( $P < 0.01$ ).

### Effects of 5-Aza-CdR on DNMT1, ER $\alpha$ , and ER $\beta$ genes expression

To determine the effects of 5-Aza-CdR on DNMT1, ER $\alpha$ , and ER $\beta$  genes expression, real-time RT-PCR was carried out. The results demonstrated that 5-Aza-CdR significantly down-regulated DNMT1 and up-regulated ER $\alpha$  and ER $\beta$  genes expression at different time periods (24 and 48 h) as shown in Table 2 and Figure 3. As indicated in the table, 5-Aza-CdR had a more significant effect on DNMT1 and ER $\beta$  genes expression in comparison to ER $\alpha$ .

## Discussion

DNA methylation plays an important role in the regulation of expression of tumor TSGs, genomic stability, and genomic stability. Aberrant methylation of CpG islands of the promoter region is associated with at least half of all genes involves in cancers, these islands are normally methylation-free.<sup>[21]</sup> This process is catalyzed by three DNMTs include DNMT1, DNMT3a, and DNMT3b. Thus, DNA methylation patterns can be considered as biomarkers for cancer diagnosis, prognosis, prevention, and treatment of cancers. Several works have reported that colon cancer is strongly associated with aberrant DNA methylation profiles.<sup>[22]</sup> ER $\alpha$  and ER $\beta$  are encoded by two separate genes including ESR1 and ESR2 located on different chromosomes and have different expression patterns in different tissues.<sup>[23]</sup> Hypermethylation of the ER $\alpha$  gene has been reported in most human colon cancers.<sup>[24]</sup> ER $\beta$  is the predominant estrogen receptor in the malignant colonic epithelium.<sup>[25]</sup> A selective loss of ER- $\beta$  protein expression

Table 1: ER $\alpha$ , ER $\beta$ , and DNMT1 primer sequences

Primer name	Primer sequence (5'→3')
ER $\alpha$ Forward	AGACATGAGAGCTGCCAAC
ER $\alpha$ Reverse	GCCAGGCACATTCTAGAAGG
ER $\beta$ Forward	AGAGTCCCTGGTGTGAAGCAAG
ER $\beta$ Reverse	GACAGCGCAGAAGTGAGCATC
DNMT1 Forward	GAG GAAGCTGCTAAGGAC TAG TTC
DNMT1 Reverse	ACT CCA CAA TTT GAT CAC TAA ATC
GAPDH Forward	TCCCATCACCATCTTCCA
GAPDH Reverse	CATCACGCCACAGTTTCC

Table 2: The relative expression level of DNMT1, ER $\alpha$ , and ER $\beta$  genes

Gene	Drug	Dose ( $\mu$ M)	Duration (h)	Expression	P
DNMT1	5-Aza-CdR	2.5	24	0.5	0.067
DNMT1	5-Aza-CdR	2.5	48	0.3	0.017
ER $\alpha$	5-Aza-CdR	2.5	24	1.3	0.003
ER $\alpha$	5-Aza-CdR	2.5	48	2.6	0.001
ER $\beta$	5-Aza-CdR	2.5	24	2.4	0.338
ER $\beta$	5-Aza-CdR	2.5	48	3.2	0.001

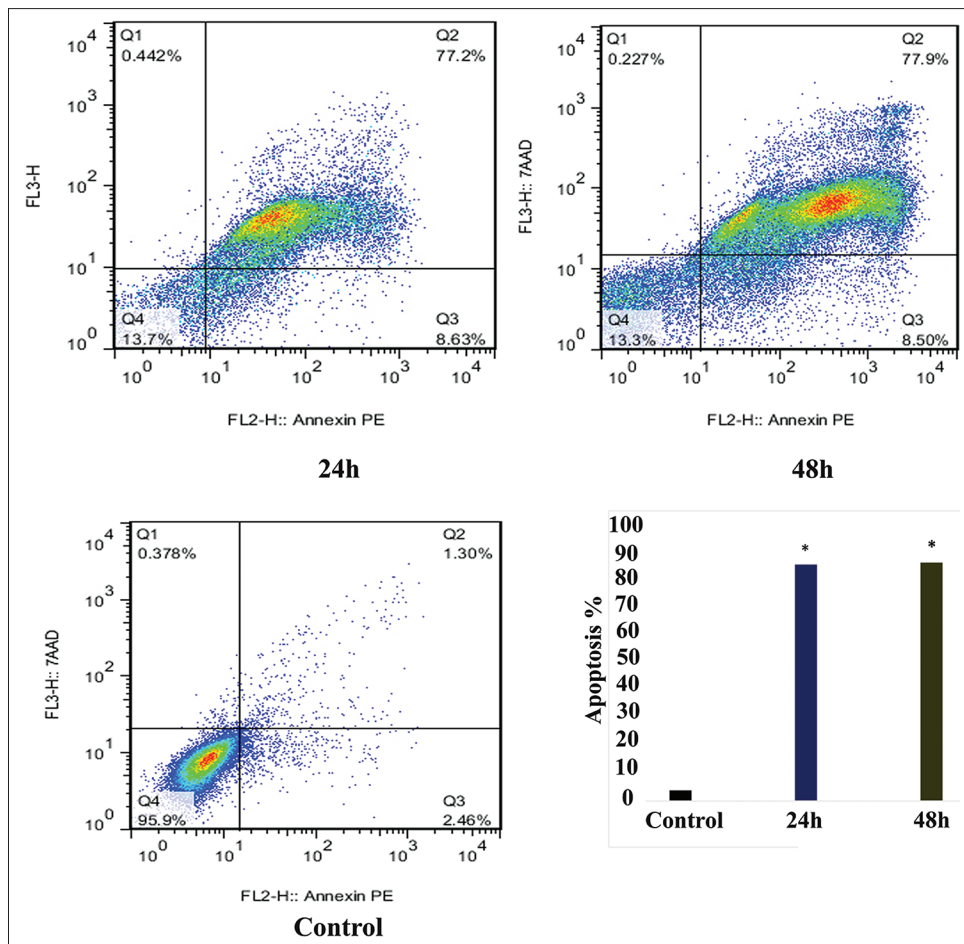


Figure 2: The apoptosis-inducing effect of 5-Aza-CdR (2.5  $\mu$ M) investigated by flow cytometric analysis of HT 29 cell. As shown above, the significant apoptotic effect of 5-Aza-CdR was seen after 24 and 48 h of treatment

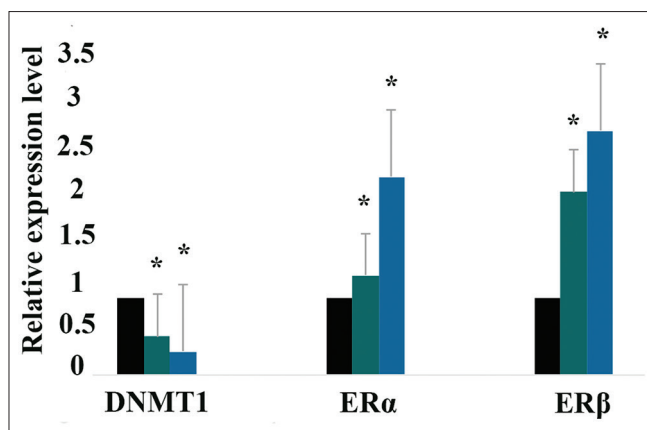


Figure 3: Relative expression of DNMT1, ER $\alpha$ , and ER $\beta$  in response to 5-Aza-CdR (2.5  $\mu$ M). Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that 5-Aza-CdR decreased DNMT1 and increased ER $\alpha$  and ER $\beta$  expression significantly. In each group of columns, the first column belongs to untreated cells, control groups; the second column belongs to treated cells in 24 h; and third column belongs to treated cells in 48 h. The results were obtained from three independent experiments and were expressed as mean  $\pm$  standard error of the mean. Asterisks (\*) indicate significant differences between treated and the control groups

because of hypermethylation has been reported in malignant colon tissue.<sup>[26]</sup> Significant demethylation has been reported

in colon cancer SW48, SW480, and HCT116 cells after treatment with 5-Aza-CdR.<sup>[15]</sup> Previously, we reported that DNA demethylating agent GE reactivated ER $\alpha$  in HCC HepG 2 cells.<sup>[18]</sup> In the present study, we observed that 5-Aza-CdR inhibited colon cancer HT 29 cell growth and induced apoptosis. This result encouraged us to evaluate the mechanism of the agent. Therefore, we evaluated the effect of 5-Aza-CdR on DNMT1, ER $\alpha$ , and ER $\beta$  genes expression. The results indicated that 5-Aza-CdR down-regulated DNMT1 and up-regulated ER $\alpha$  and ER $\beta$  genes expression. In line with our result, it has been reported that 5-Aza-CdR inhibits the growth of breast cancer significantly by inhibiting the expression of DNMTs.<sup>[27]</sup> Similarly, it has demonstrated that 5-Aza-CdR reactivates ER $\alpha$  through DNMT1 down-regulation in breast cancer MDA-MB-435 and MDA-MB-231 cell lines.<sup>[17]</sup> In ovarian cancer SKOV3 cell line, 5-Aza-CdR inhibits ovarian cancer cell growth by ER $\beta$  promoter demethylation.<sup>[28]</sup> Reactivation of ESR1 after 5-Aza-CdR treatment in HCC HepG2, HuH2, HLE, HLF, and SK-Hep1 cells has been shown by other researchers.<sup>[29]</sup> Epigenetic regulation and reactivation of ER $\beta$  by 5-Aza-CdR has been reported in prostate cancer too.<sup>[30]</sup> Restoration of TBX5 expression is

another mechanisms of 5-Aza-CdR in colon cancer DLD-1, HT-29, LOVO, SW480, SW620, CaCO<sub>2</sub>, and CL14 cells.<sup>[31]</sup> In summary, DNMT1 inhibition is one of the mechanisms of 5-Aza-CdR which reactivates ER $\alpha$  and ER $\beta$  expression resulting in cell growth inhibition and apoptosis induction. In this study, we did not evaluate the effect of 5-Aza-CdR on histone deacetylase and stilation status of ER $\alpha$  and ER $\beta$  genes. Therefore, this evaluation is recommended.

## Conclusions

5-Aza-CdR can up-regulate ER $\alpha$  and ER $\beta$  genes expression through DNMT1 down-regulation resulting in apoptosis induction and cell growth inhibition in colon cancer HT 29 cell line.

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## Conflicts of interest

There are no conflicts of interest.

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