

Received 2020-03-31
Revised 2020-07-13
Accepted 2020-07-13

Effect of 5-aza-2'-deoxycytidine on *p27Kip1*, *p21Cip1/Waf1/Sdi1*, *p57Kip2*, and *DNA methyltransferase 1* Genes Expression, Cell Growth Inhibition and Apoptosis Induction in Colon Cancer SW 480 and SW 948 Cell Lines

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Abstract

Background: Dysregulation of the cell cycle has been reported in various cancers. Inactivation of the cyclin-dependent kinases inhibitors (CDKIs), CIP/KIP family, such as *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* genes because of hypermethylation has been shown in several cancers. Treatment with DNA demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) has been indicated that affect genomic methylation and resulting in silenced genes reactivation in colon cancer. Previously, we evaluated the effect of 5-Aza-CdR on *DNA methyltransferase 1 (DNMT1)* gene expression in hepatocellular carcinoma (HCC) which encouraged us to design the current study. The present study aimed to evaluate the effect of 5-Aza-CdR on *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2*, and *DNMT1* genes expression, cell growth inhibition and apoptosis induction in colon cancer SW 480 and SW 948 cell lines. **Materials and Methods:** The effect of 5-aza-CdR on the SW 480 and SW 948 cells growth, apoptosis induction and genes expression were assessed by MTT assay, flow cytometry, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis respectively. **Results:** 5-aza-CdR inhibited cell growth as time- and dose-dependent manner significantly ($P < 0.001$). The agent reactivated *p15INK4*, *p16INK4*, *p18INK4*, and *p19INK4* genes expression and induced apoptosis at a concentration of 5 μ M significantly. Besides, 5-aza-CdR had a more significant effect on the SW 480 cell line in comparison to SW 948 cell line. **Conclusion:** 5-Aza-CdR plays a key role in the up-regulation of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* and down-regulation of *DNMT1* genes resulting in cell growth inhibition and apoptosis induction. [GMJ.2020;9:e1899] DOI: [10.31661/gmj.v9i0.1899](https://doi.org/10.31661/gmj.v9i0.1899)

Keywords: 5-aza-CdR; *Cip/Kip* Genes; *DNMT1*; Colon Cancer

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Introduction

Cancer is considered to be a disease of the cell cycle deregulation, the most frequent alterations during cancer induction and tumor development which underlies the aberrant cell proliferation. Cell cycle progression is a highly ordered, tightly-regulated process that involves several checkpoints that evaluate extracellular cell growth signals, cell size, and DNA integrity [1]. The cell cycle progression is driven by the cyclin-dependent kinase (CDK) family, the key regulators of cell-cycle transitions, and their regulatory partners, the cyclins. The level of cyclins fluctuates throughout the cell cycle. In mammalian cells, G1 phase progression is driven by cyclin *D-CDK4*, cyclin *D-CDK6*, and cyclin *E-CDK2*. Cyclin *A-CDK2* involve in S phase initiation and cyclin *B-CDK1* regulates G2 phase progression [2]. Both the cyclin D and E and their associated kinases are necessary for entry into and progression through the G1 phase of the cell cycle. The *CDKs* family are the major targets for deregulation in cancer and the misregulated *CDKs* lead to unscheduled cell proliferation as well as chromosomal and genomic instability [3]. Inhibition of cyclin-dependent kinases activity may be effective anti-cancer therapeutics. These kinases can be negatively regulated by two groups of cyclin-dependent kinases inhibitors (CDKIs) including INK4 and Cip/Kip families. INK4 proteins, specific for the *Cdk4* subfamily, include *p16INK4a*, *p15INK4b*, *p18INK4c*, and *p19INK4d* and interact with the monomeric *CDKs*. The Cip/Kip family includes *p21Cip1*, *p27Kip1*, and *p57Kip2* which contact both the CDK and cyclin subunits and can inhibit CDK-cyclin heterodimers [4]. DNA methylation can affect chromatin structure, which, in turn, can alter tumor suppressor genes (TSGs) expression. These changes have been involved in tumorigenesis. Hypermethylation of CDKIs has been reported in various cancers such as colon cancer [5]. DNA methylation is brought by a family of enzymes known as the DNA methyltransferases (DNMTs) including DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3a, DNMT3b with its isoforms, and DNMT3L [6]. Treatment with DNA demethylating agent 5-aza-2'-de-

oxycytidine (5-Aza-CdR) has been indicated that affect genomic methylation and resulting in silenced genes reactivation in colon cancer [7, 8]. In addition to colon cancer, it has been reported that 5-aza-CdR increases *p53/p21Waf1/Cip1* expression in lung cancer and prostate cancer [9, 10], *p27kip1* in esophageal squamous cell carcinoma [11], and *p57KIP2* in lung and breast cancers [12]. Previously, we evaluated the effect of 5-Aza-CdR on *DNMT1* gene expression in hepatocellular carcinoma (HCC) which encouraged us to design the current study [13]. The present study aimed to evaluate the effect of 5-Aza-CdR on *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2*, and *DNA methyltransferase 1* genes expression, cell growth inhibition and cell apoptosis induction in colon cancer SW 480 and SW 948 cell lines.

Materials and Methods

Human colon cancer SW 480 and SW 948 cell lines were purchased from the National Cell Bank of Iran-Pasteur Institute and cultured 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 was provided from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma) at a final concentration of 100 μM to obtain a stock solution. All other working solutions were provided by diluting the stock solution. Antibiotics, DMSO, trypsin-EDTA, 3-[4, 5-dimethyl-2-thiazoly]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Annexin-V-(FITC), propidium iodide (PI), DMEM, and Phosphate-buffered saline (PBS) were purchased from Sigma. Total RNA extraction kit (TRIZOL reagent) and real-time polymerase chain reaction (PCR) kits (qPCR MasterMix Plus for SYBR Green I dNTP) were obtained from Applied Biosystems Inc. (Foster, CA, USA). This work was approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1397.138.

Cell Growth and Viability

The effect of 5-aza-CdR on the in vitro growth of colon cancer SW 480 and SW 948 cell

lines was determined by MTT assay. Briefly, the cells were seeded at the density of 5×10^5 cells per well onto a 96-well plate for 24h. The cells were subsequently treated with 5-aza-CdR (0.5, 1, 2.5, 5, and 10 μ M) for 24h and 48h. After treatment times, 20 μ l of MTT (0.5%) in PBS was added to each well and the incubation was continued for 4 h at 37°C and then the culture medium was replaced with DMSO (200 μ l) and finally, the optical density was detected by a microplate reader at a wavelength of 570 nm. Each sample was performed in triplicate.

Flow Cytometry Analysis

The percentage of colon cancer SW 480 and SW 948 apoptotic cells was evaluated by staining the cells with annexin V-FITC, a sensitive probe for identifying cells undergoing apoptosis in an early stage which precedes the loss of membrane integrity, and PI, allowing to distinguish annexin V-single positive cells undergoing the late apoptosis according to the manufacturer's protocol. Before flow cytometry analysis, the cells were plated at a density of 5×10^5 per well in 24-well plate and treated with 5-aza-CdR (1 μ M) for different periods (24 and 48 h) except for the control groups, these groups were incubated with DMSO only. Subsequently, all adherent and floating cells were collected by trypsinization and washed with cold PBS and resuspended in binding buffer for 10 min at room temperature in the dark. Finally, the cells were incubated with annexin-V-(FITC) and PI according to the manufacturer's protocol and

the double-stained cells were subsequently analyzed by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). Three independent experiments were performed for each concentration.

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

Total RNA was extracted from 5-aza-CdR (1 μ M) treated colon cancer cell, SW 480 and SW 948, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated by RNase-free DNase (Qiagen). Subsequently, One microgram RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) to generate the single-stranded cDNAs. The expression of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2*, and *DNMT1* genes were quantified using the quantitative SYBR Green PCR kit (TaKaRa Bio, Japan) according to the manufacturer's protocol and measured by quantitative real-time PCR using StepOnePlus (Applied Biosystem, USA) instrument. Primers sequences of the mentioned genes are indicated in Table-1. GAPDH was used as an endogenous control. Data were analyzed using the comparative Ct ($\Delta\Delta$ ct) method. The thermocycling condition was as described previously [14].

Results

Result of Cell Viability by the MTT Assay

To examine the inhibitory effect of 5-aza-CdR on colon cancer SW 480 and SW 948 cells, we

Table 1. The Primer Sequences of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2*, and *DNMT1* Genes.

Primer name		Primer sequences (5' to 3')	References
<i>DNMT1</i>	Forward	GAG GAA GCT GCT AAG GAC TAG TTC	[15]
	Reverse	ACT CCA CAA TTT GAT CAC TAA ATC	
<i>P21</i>	Forward	AGG CGC CAT GTC AGA ACC GGC TGG	[16]
	Reverse	GGA AGG TAG AGC TTG GGC AGG C	
<i>P 27</i>	Forward	ATG TCA AAC GTG CGA GTG TCT AAC	[16]
	Reverse	TTA CGT TTG ACG TCT TCT GAG GCC A	
<i>P 57</i>	Forward	GCGGCGATCAAGAAGCTGTC	[17]
	Reverse	CCGTTTGCTGCTACATGAAC	
<i>GAPDH</i>	Forward	TCCCATCACCATCTTCCA	[17]
	Reverse	CATCACGCCACAGTTTCC	

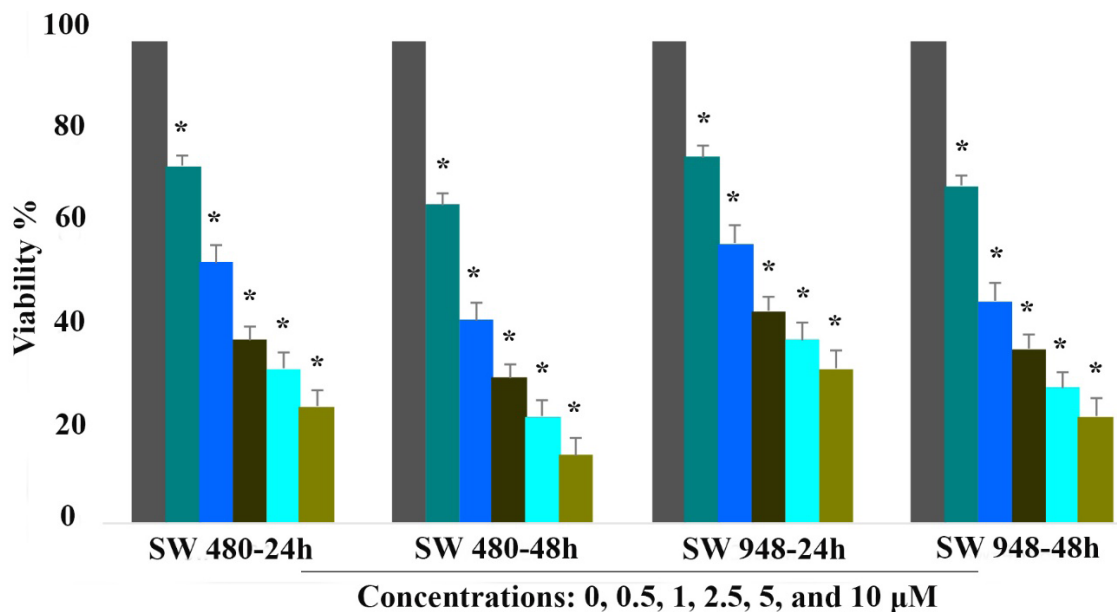


Figure 1. The effect of 5-aza-CdR on colon cancer SW 480 and SW 948 cells viability. The cells were with different concentrations of 5-aza-CdR (0.5, 1, 2.5, 5, and 10 μM) for 24 and 48 h and then the cell viability was evaluated by MTT assay. Each experiment was done in triplicate Asterisks (*) indicate significant differences between treated and untreated cells.

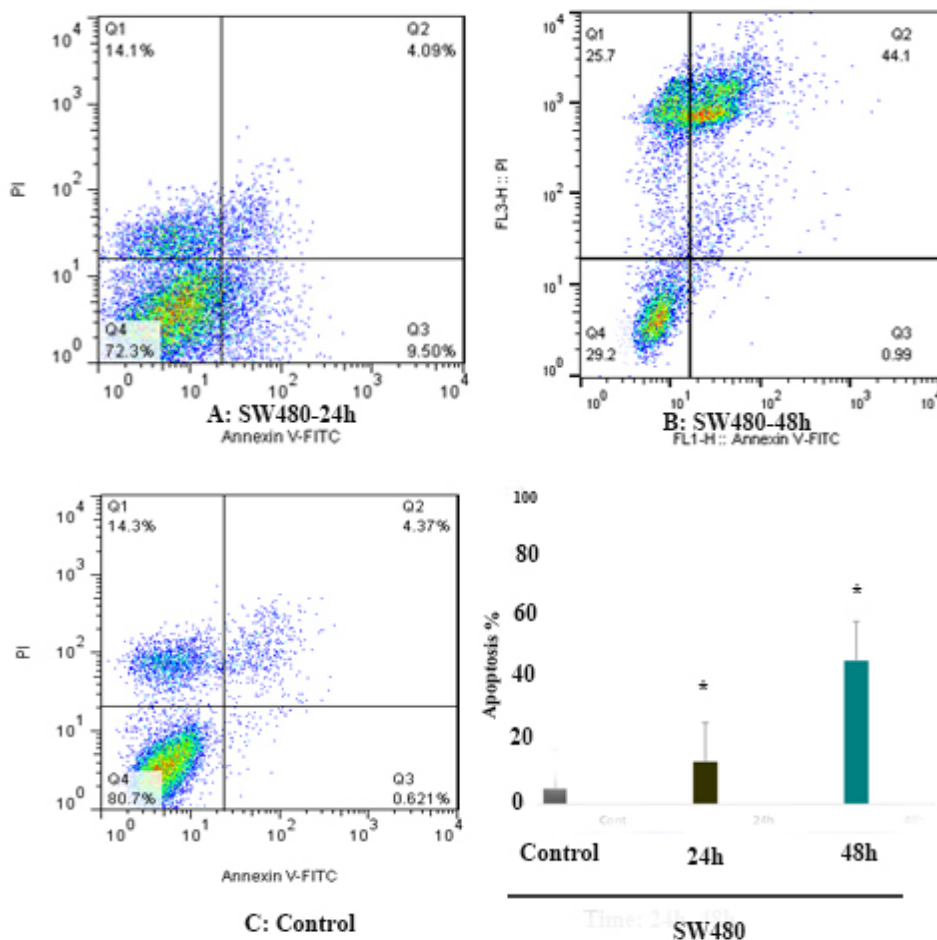


Figure 2. The apoptosis-inducing effect of 5-aza-CdR on colon cancer SW 480 cells investigated by flow cytometry assay. As shown above, 5-aza-CdR induced apoptosis as a time-dependent manner. Asterisks (*) indicate significant differences between treated and untreated cells.

treated the cells with various concentrations of the agent (0.5, 1, 2.5, 5, and 10 μM) for 24 and 48h. The viability of SW 480 and SW 948 cells was determined by MTT assay. As shown in Figure-1, 5-aza-CdR inhibited cell growth significantly in a time- and dose-dependent manner ($P < 0.001$). The IC₅₀ value was obtained with approximately 1 μM of 5-aza-CdR.

Result of Cell Apoptosis Assay

Cell apoptosis was measured by Annexin V and PI staining. To determine the effect of 5-aza-CdR (1 μM) on SW 480 and SW 948 cells apoptosis, the cells were stained using annexin-V-(FITC) to detect the apoptotic cells in an early stage and PI to detect annexin V-single positive cells from the cells subjected to necrotic processes. After treatment times (24 and 48 h), the treated and untreated cells were collected by trypsinization and labeled with annexin V and PI. Representative graphs are indicated in Figures-2 and 3. As shown, significant differences were observed between the numbers of apoptotic cells in SW 480

treated groups compared to untreated control groups, Figure-2. In SW 948 cell line, the significant apoptotic effect was observed after 48 h of treatment as indicated in Figure-3. As indicated in Figure-4, 5-aza-CdR had a more significant apoptotic effect on the SW 480 cell line in comparison to SW 948 cell line. The percentage of the apoptotic cells is shown in Table-2.

Result of Determination of Genes Expression

The effect of 5-aza-CdR (1 μM) on *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2*, and *DNMT1* genes expression was evaluated by quantitative real-time RT-PCR analysis. The result of RT-PCR analysis revealed that treatment with 5-aza-CdR for 24 and 48 h up-regulated *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* and down-regulated *DNMT1* genes significantly in SW 480 cells (Figure-5). No significant effect was observed in SW 948 after 24h of treatment but treatment with 5-aza-CdR for 48h induced significant up-regulation of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* and down-regulation of *DNMT1* genes (Fig-

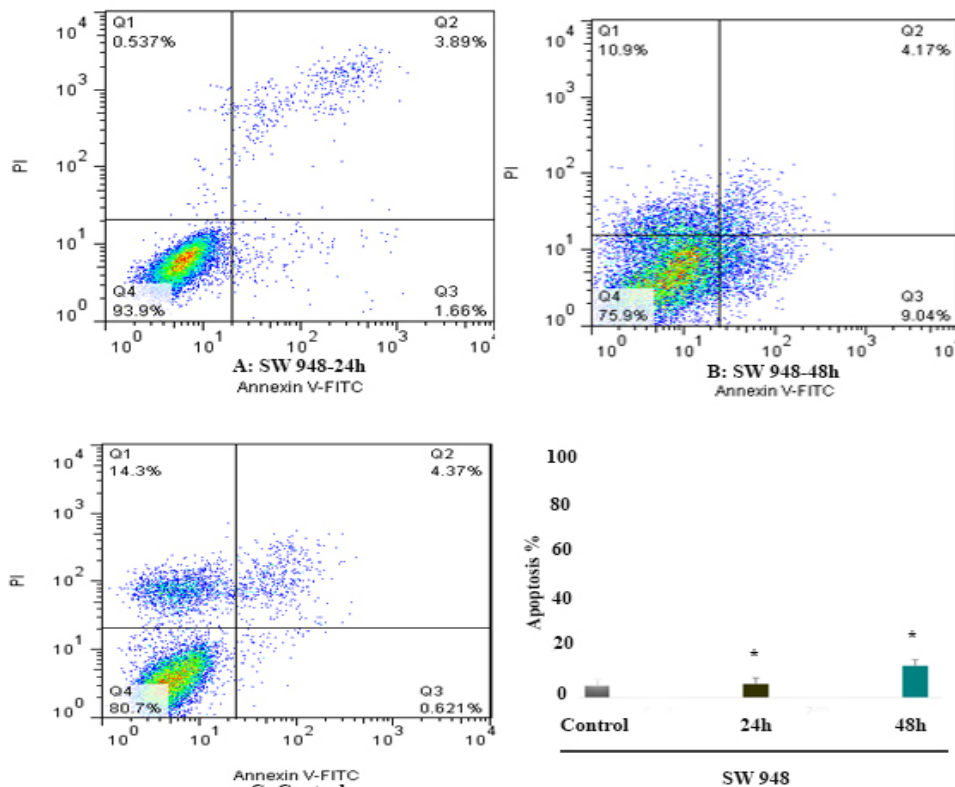


Figure 3. The apoptosis-inducing effect of 5-aza-CdR on colon cancer SW 948 cells investigated by flow cytometry assay. As shown above, 5-aza-CdR induced apoptosis as a time-dependent manner. Asterisks (*) indicate significant differences between treated and untreated cells.

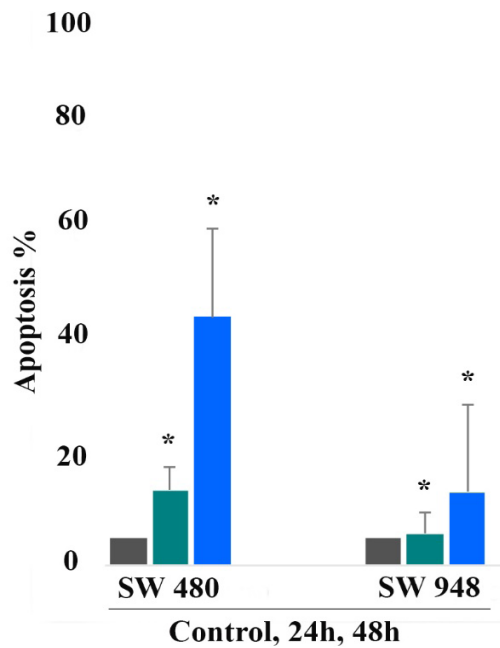


Figure 4. Comparative analysis of the apoptotic effect of 5-aza-CdR on SW 480 and SW 948 cell lines (24 and 48h). As shown, a more significant effect was observed in SW 480 in comparison to SW 948 cell line.

ure-6). The relative expression of all of the genes has been shown in Table-3.

Discussion

Dysregulation of the cell cycle has been reported in various cancers. Inactivation of the CIP/KIP family such as *p21*, *p27*, and *p57* genes because of hypermethylation has been shown in several cancers including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) [18, 19], and colorectal cancers [20]. DNA demethylating agents such as 5-aza-CdR can reactive hypermethylated cyclin-dependent kinase inhibitors in hepatocellular cancer Hep G2 [21], human ovarian cancer SKOV3 cells [22], pancreatic cancer [23], and colon cancer [24]. In this study, we indicated that 5-aza-CdR inhibits cell growth and induces apoptosis in SW 480 and SW 948 cell lines as a time- and dose-dependent manner. To evaluate the mechanisms involved

Table 2. The Percentage of Apoptotic Cells Treated with 5-Aza-CdR at Different Time Periods.

Cell line	Drug	Dose (μM)	Duration (h)	Apoptosis (%)	P-value
SW 480	5-Aza-CdR	5	24	13.59	0.001
SW 480	5-Aza-CdR	5	48	45.09	0.001
SW 948	5-Aza-CdR	5	24	5.55	0.690
SW 948	5-Aza-CdR	5	48	13.21	0.001

Table 3. Relative Expression Level of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, *p57Kip2* and *DNMT1* Genes.

Cell line	Gene	Drug	Dose (μM)	Duration (h)	Expression	P-value
Sw 480	<i>p21Cip1/Waf1/Sdi1</i>	5-Aza-CdR	5 μM	24	2.6	0.001
Sw 480	<i>p21Cip1/Waf1/Sdi1</i>	5-Aza-CdR	5 μM	48	2.9	0.001
Sw 480	<i>p27Kip1</i>	5-Aza-CdR	5 μM	24	2.5	0.001
Sw 480	<i>p27Kip1</i>	5-Aza-CdR	5 μM	48	3	0.001
Sw 480	<i>p57Kip2</i>	5-Aza-CdR	5 μM	24	2.8	0.001
Sw 480	<i>p57Kip2</i>	5-Aza-CdR	5 μM	48	3.3	0.001
Sw 480	<i>DNMT1</i>	5-Aza-CdR	5 μM	24	0.7	0.028
Sw 480	<i>DNMT1</i>	5-Aza-CdR	5 μM	48	0.4	0.001
SW 948	<i>p21Cip1/Waf1/Sdi1</i>	5-Aza-CdR	5 μM	24	0.85	0.70
SW 948	<i>p21Cip1/Waf1/Sdi1</i>	5-Aza-CdR	5 μM	48	1.8	0.001
SW 948	<i>p27Kip1</i>	5-Aza-CdR	5 μM	24	0.8	0.47
SW 948	<i>p27Kip1</i>	5-Aza-CdR	5 μM	48	2	0.001
SW 948	<i>p57Kip2</i>	5-Aza-CdR	5 μM	24	0.95	0.99
SW 948	<i>p57Kip2</i>	5-Aza-CdR	5 μM	48	1.9	0.001
SW 948	<i>DNMT1</i>	5-Aza-CdR	5 μM	24	0.9	0.90
SW 948	<i>DNMT1</i>	5-Aza-CdR	5 μM	48	0.6	0.001

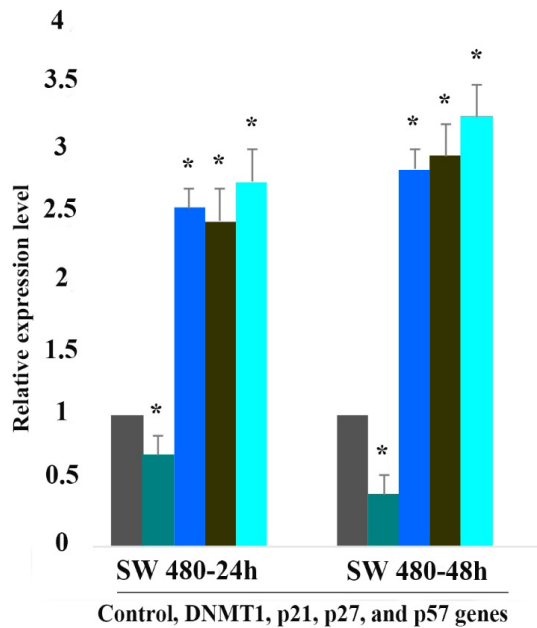


Figure 5. The relative expression level of p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2 and DNMT1 genes treated with 5-aza-CdR in SW 480 cells. Significant up-regulation of p21Cip1/Waf1/Sdi1, p27Kip1, and p57Kip2 and down-regulation of DNMT1 genes was observed after treatment with the compound at different times (24 and 48h). Asterisks (*) indicate significant differences between treated and control groups. Data are presented as the means \pm standard error of the mean.

in cell apoptosis following treatment with 5-aza-CdR, we investigated the genes expression and found that this agent plays its role through the up-regulation of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* and down-regulation of DNMT1 genes expression. Besides, we demonstrated that 5-aza-CdR had a more significant effect on the SW 480 cell line in comparison to SW 948 cell line. Similar pathways have been reported in other cancers. In HCC SMMC-7721 cell lines, 5-aza-CdR can reactivate *p15*, *p16*, *p21* genes by DNA demethylation [25]. In prostate cancer PC3, LN-CaP and DU145 cell lines, the demethylating agent 5-Aza-CdR can up-regulate *p21WAF1/CIP1* mRNA expression [26]. The demethylation of *p27kip1* cells treated with 5-Aza-CdR has been demonstrated in the gastric cancer cell line too [27]. Other researchers have been shown that 5-Aza-CdR can restore methylated *p57KIP2* in lung and breast cancer cell lines [28]. It has been established that deregulation in the function of CDK inhibitors can result in tumorigenesis processes. There are two families of CDK-inhibitors: INK4 and CIP/KIP class. These two families differ in the particular cyclin families that they interact with. The

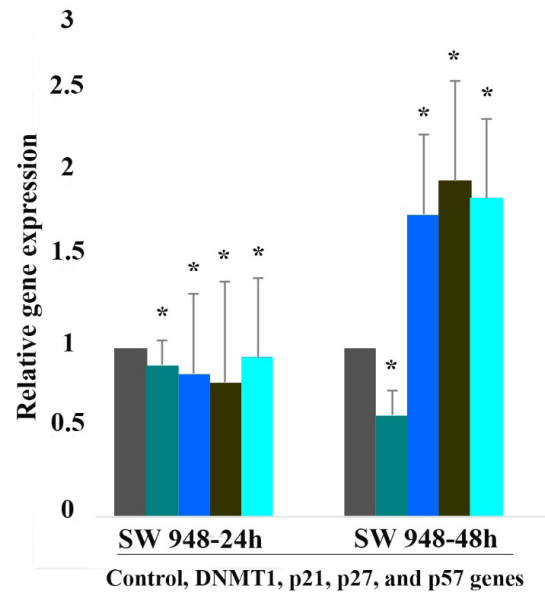


Figure 6. The relative expression level of p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2 and DNMT1 genes treated with 5-aza-CdR in SW 948 cells. Significant up-regulation of p21Cip1/Waf1/Sdi1, p27Kip1, and p57Kip2 and down-regulation of DNMT1 genes was observed after treatment with the compound for 48h. No significant effect was observed after 24h of treatment. Asterisks (*) indicate significant differences between treated and control groups. Data are presented as the means \pm standard error of the mean.

kinase inhibitor family, CIP/KIP, is composed of three proteins that interact with other cyclin families. From a mechanistic standpoint, CDK-inhibitors can be used as an anti-cancer drug by blocking CDK's [29]. As we reported in the current article, others have indicated a decreased expression of DNMT1 has been reported following treatment with 5-Aza-CdR in colorectal cancer HCT 116, HT-29, MIP101, and RKO cell lines [30]. Furthermore, it has been indicated that 5-Aza-CdR demethylates the promoter sequence of *TIMP-3* and *p16* in HCT116 colon cancer [31]. Additionally, 5-AzadC induce the 15-lipoxygenase-1 (15-LOX-1) expression in human colon cancer cells which increases 13-S-hydroxyoctadecadienoic acid levels, cell growth inhibition, and apoptosis induction in these cells [32]. All reports mentioned above are inconsistent with our results. In addition to Cyp/Kip pathway and DNMT1 inhibition which reported by our teamwork, other molecular mechanisms have been reported for 5-Aza-CdR. It induces apoptosis by up-regulation of human and mouse *TNFR1* (TNFRSF1) genes [33]. Another study has been demonstrated that this agent restores the expression of *TBX5* in colon

cancer cell lines SW620, HT-29, SW620 and CaCO2 [34]. In gastrointestinal cancers, it can induce apoptosis by re-activation of silenced death-associated protein kinase expression [35]. Finally, the up-regulation of Cip/Kip genes and down-regulation of DNMT1 gene is not the only molecular pathways of apoptosis in colon cancer. The following of other mechanisms is recommended.

Conclusion

In summary, we have demonstrated that 5-Aza-CdR plays a key role in re-activation of *p21Cip1/Waf1/Sdi1*, *p27Kip1*, and *p57Kip2* and down-regulation of DNMT1 genes resulting in cell growth inhibition and apoptosis

induction. This pathway may be an effective molecular target for colon cancer treatment through the re-activation of Cip/Kip and inhibition of *DNMT1* genes.

Acknowledgment

This article was supported by the adjutancy of research of Jahrom University of Medical Sciences, Iran. The article has been extracted from Ms. Sedighe Nasiri's thesis.

Conflict of Interest

The authors report no conflict of interest.

References

1. Park M-T, Lee S-J. Cell cycle and cancer. *J. Biochem. Mol. Biol.* 2003; 36(1):60-5.
2. Williams GH, Stoeber K. The cell cycle and cancer. *The Journal of pathology.* 2012; 226(2):352-64.
3. Collins I, Garrett MD. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. *Curr Opin Pharm.* 2005; 5(4):366-73.
4. Malumbres M. Cyclin-dependent kinases. *Genome Biol.* 2014; 15(6):122.
5. Lu R, Wang X, Chen Z-F, Sun D-F, Tian X-Q, Fang J-Y. Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells. *J Biol Chem.* 2007; 282(16):12249-59.
6. Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol.* 2004; 22(22):4632-42.
7. Roulois D, Yau HL, Singhanian R, Wang Y, Danesh A, Shen SY, et al. DNA-demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. *Cell* 2015; 162(5):961-73.
8. Mossman D, Kim K-T, Scott RJ. Demethylation by 5-aza-2'-deoxycytidine in colorectal cancer cells targets genomic DNA whilst promoter CpG island methylation persists. *BMC Cancer.* 2010; 10(1):366
9. Zhu W-G, Hileman T, Ke Y, Wang P, Lu S, Duan W, et al. 5-aza-2'-deoxycytidine activates the p53/p21Waf1/Cip1 pathway to inhibit cell proliferation. *J Biol Chem.* 2004; 279(15):15161-6.
10. Bott S, Arya M, Kirby R, Williamson M. p21 WAF1/CIP1 gene is inactivated in metastatic prostatic cancer cell lines by promoter methylation. *Prostate Cancer Prostatic Dis.* 2005; 8(4):321.
11. Ling Y, Zhang C, Xu Y, Zhu J, Zhu C, Lu M, et al. Promoter methylation-associated silencing of p27kip1 gene with metastasis in esophageal squamous cell carcinoma. *Mol Med Report.* 2014; 9(3):1075-9
12. Kobatake T, Yano M, Toyooka S, Tsukuda K, Dote H, Kikuchi T, et al. Aberrant methylation of p57KIP2 gene in lung and breast cancers and malignant mesotheliomas. *Oncol Rep.* 2004; 12(5):1087-92.
13. Sanaei M, Kavvoosi F. Effects of 5-aza-2'-deoxycytidine and Valproic Acid on Epigenetic-modifying DNMT1 Gene Expression, Apoptosis Induction and Cell Viability in Hepatocellular Carcinoma WCH-17 cell line. *Iranian Journal of Pediatric Hematology & Oncology.* 2019; 9(2).
14. Sanaei M, Kavvoosi F, Roustazadeh A, Golestan F. Effect of Genistein on DNMT1 Gene Expression and Cell Proliferation of Hepatocellular Carcinoma HepG2 Cell Line. *Global Journal of Medicine Researches and Studies.* 2017; 4(1).
15. Sanaei M, Kavvoosi F, Roustazadeh A, Golestan F. Effect of genistein in comparison with trichostatin a on reactivation of DNMTs genes in hepatocellular carcinoma. *Journal of*

- clinical and translational hepatology. 2018; 6(2):141.
16. Gao F-H, Hu X-H, Li W, Liu H, Zhang Y-J, Guo Z-Y, et al. Oridonin induces apoptosis and senescence in colorectal cancer cells by increasing histone hyperacetylation and regulation of p16, p21, p27 and c-myc. *BMC Cancer*. 2010; 10(1):610.
 17. Oya M, Schulz W. Decreased expression of p57 KIP2 mRNA in human bladder cancer. *Br. J. Cancer*. 2000; 83(5):626.
 18. Chim C, Wong A, Kwong Y. Epigenetic inactivation of the CIP/KIP cell-cycle control pathway in acute leukemias. *Am J Hematol*. 2005; 80(4):282-7.
 19. Brakensiek K, Langer F, Kreipe H, Lehmann U. Absence of p21CIP1, p27KIP1 and p57KIP2 methylation in MDS and AML. *Leukemia Res*. 2005; 29(11):1357-60.
 20. Xu X-L, Yu J, Zhang H-Y, Sun M-H, Gu J, Du X, et al. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World journal of gastroenterology: WJG*. 2004; 10(23):3441.
 21. Peng Y-T, Wu W-R, Chen L-R, Kuo K-K, Tsai C-H, Huang Y-T, et al. Upregulation of cyclin-dependent kinase inhibitors CDKN1B and CDKN1C in hepatocellular carcinoma-derived cells via goniothalamin-mediated protein stabilization and epigenetic modifications. *Toxicology reports*. 2015; 2:322-32.
 22. Zhao Y, Li Q, Wu X, Chen P. Upregulation of p27Kip1 by demethylation sensitizes cisplatin-resistant human ovarian cancer SKOV3 cells. *Mol Med Report*. 2016; 14(2):1659-66.
 23. Lee K-H, Lotterman C, Karikari C, Omura N, Feldmann G, Habbe N, et al. Epigenetic silencing of MicroRNA miR-107 regulates cyclin-dependent kinase 6 expression in pancreatic cancer. *Pancreatol*. 2009; 9(3):293-301.
 24. Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, Hartig R, et al. RETRACTION: 5-aza-Cytidine Is a Potent Inhibitor of DNA Methyltransferase 3a and Induces Apoptosis in HCT-116 Colon Cancer Cells via Gadd45-and p53-Dependent Mechanisms. *J Pharmacol Exp Ther*. 2005; 312(2):525-36.
 25. Tao S-F, Zhang C-S, Guo X-L, Xu Y, Zhang S-S, Song J-R, et al. Anti-tumor effect of 5-aza-2'-deoxycytidine by inhibiting telomerase activity in hepatocellular carcinoma cells. *World journal of gastroenterology: WJG*. 2012; 18(19):2334.
 26. Bott S, Arya M, Kirby R, Williamson M. p21 WAF1/CIP1 gene is inactivated in metastatic prostatic cancer cell lines by promoter methylation. *Prostate Cancer Prostatic Dis*. 2005;8(4):321.
 27. WANG H-I, ZHANG J, LI Y, WANG X-q. Experimental studies of 5-Aza-CdR on p27kip1 gene's abnormal methylation in gastric cancer cell line [J]. *Practical Pharmacy and Clinical Remedies*. 2011; 2.
 28. Kobatake T, Yano M, Toyooka S, Tsukuda K, Dote H, Kikuchi T, et al. Aberrant methylation of p57KIP2 gene in lung and breast cancers and malignant mesotheliomas. *Oncol Rep*. 2004;12(5):1087-92.
 29. Sandal T. Molecular aspects of the mammalian cell cycle and cancer. *The oncologist*. 2002;7(1):73-81.
 30. Cheetham S, Tang M, Mesak F, Kennecke H, Owen D, Tai I. SPARC promoter hypermethylation in colorectal cancers can be reversed by 5-Aza-2' deoxycytidine to increase SPARC expression and improve therapy response. *Br J Cancer*. 2008;98(11):1810.
 31. Wang L-S, Kuo C-T, Cho S-J, Seguin C, Siddiqui J, Stoner K, et al. Black raspberry-derived anthocyanins demethylate tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells. *Nutr Cancer*. 2013;65(1):118-25.
 32. Hsi LC, Xi X, Wu Y, Lippman SM. The methyltransferase inhibitor 5-aza-2-deoxycytidine induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells. *Mol Cancer Ther*. 2005;4(11):1740-6.
 33. Kaminski R, Kozar K, Niderla J, Grzela T, Wilczynski G, Skierski JS, et al. Demethylating agent 5-aza-2'-deoxycytidine enhances expression of TNFR1 and promotes TNF-mediated apoptosis in vitro and in vivo. *Oncol Rep*. 2004;12(3):509-16.
 34. Yu J, Ma X, Cheung K, Li X, Tian L, Wang S, et al. Epigenetic inactivation of T-box transcription factor 5, a novel tumor suppressor gene, is associated with colon cancer. *Oncogene*. 2010; 29(49):6464.
 35. Satoh A, Toyota M, Itoh F, Kikuchi T, Obata T, Sasaki Y, et al. DNA methylation and histone deacetylation associated with silencing DAP kinase gene expression in colorectal and gastric cancers. *Br J Cancer*. 2002; 86(11):1817.