Effect of 5-aza-2'-deoxycytidine on p16INK4a, p14ARF, p15INK4b Genes Expression, Cell Viability, and Apoptosis in PLC/PRF5 and MIA Paca-2 Cell Lines

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Abstract
Background: Mammalian cell division is regulated by a complex includes cyclin-dependent kinases (Cdks) and cyclins. Cdk/cyclin complex. The activity of the complex is regulated by Cdk inhibitors (CKIs) compressing cyclin molecules. These kinases p(Cip/Kip) family, p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2) (1). In fact, Cdks are like the engine, cyclins are considered to be the gears, and Cdk inhibitors serve as brakes to halt cell cycle under abnormal conditions (2). The genomic region of 9p21 houses two members of the INK4 family, including p15INK4b and p16INK4a, and an

Materials and Methods: In this laboratory trial, both cell lines were treated with 5-aza-CdR (0, 1, 2.5, 5, 10, 15, and 20 μM) to determine cell viability and then with 3 μM to obtain cell apoptosis and relative gene expression. The cell viability, apoptosis, and genes expression were investigated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay, flow cytometry, and Real-Time quantitative reverse-transcription polymerase chain reaction (qRT-PCR), respectively.

Results: 5-aza-CdR indicated significant inhibitory effect with all used concentrations (P = 0.003). The apoptotic effect of 5-aza-CdR on PLC/PRF5 cells in comparison to pancreatic cancer MIA Paca-2 cells was more significant (P= 0.001). Real-time quantitative PCR analysis revealed that treatment with 5-aza-CdR (3 μM) for 24 and 48h up-regulated p16INK4a, p14ARF, p15INK4b genes expression significantly (P=0.040).

Conclusion: Reactivation of p16INK4a, p14ARF, p15INK4b genes by 5-aza-CdR can induce apoptosis and inhibit cell viability in HCC, PLC/PRF5, and pancreatic cancer, MIA Paca-2 cell lines.

Keywords: Apoptosis, 5-aza-2'-deoxycytidine-5'-monophosphate, Gene expression, Viability

Introduction
The living cells are required to divide to produce new cells, daughter cells. This process is regulated by two classes of molecules compressing cyclin-dependent kinases (Cdks) and cyclins, the regulatory subunits of Cdks. These kinases play an important role during cell cycle using different cell-cycle stage-specific cyclins. Under normal conditions, the functions of Cdk/cyclin complexes are regulated by Cdk inhibitors (CKIs) which include inhibitors of CDK4 (INK4) family (p16INK4a, p15INK4b, p18INK4c, p19INK4d) and two families of Cdk inhibitors (CKIs), including the inhibitors of CDK4 (INK4) family (p16INK4a, p15INK4b, p18INK4c, p19INK4d) and the CDK-interacting protein/kinase inhibitory protein (Cip/Kip) family (p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2) (1). In fact, Cdks are like the engine, cyclins are considered to be the gears, and Cdk inhibitors serve as brakes to halt cell cycle under abnormal conditions (2).
unrelated gene p14ARF (3). The methylation of p16INK4a, p15INK4b, and p14ARF has been reported in various cancers such as colon, breast, bladder, pancreatic, and liver cancers (4-6). DNA hypermethylation is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b. Overexpression of these genes has been reported in human hepatocellular carcinoma (HCC) (7) and pancreatic cancer (8). Finally, DNA hypermethylation is associated with chromatin compaction, resulting in tumor suppressor genes (TSGs) silencing and tumorigenesis. DNA demethylating agents can reactivate hypermethylated TSGs by demethylation of these silenced genes. There are four FDA-approved epigenetic drugs, including two histone deacetylase inhibitors (HDACIs), vorinostat and valproic acid, and two DNMT inhibitors, decitabine, 5-aza-2’-deoxycytidine (5-aza-CdR), and decitabine. Success in epigenetic therapy with compounds such as suberoylanilide hydroxamic acid (SAHA) and 5-aza-CdR has been achieved in both solid and hematological cancers. DNA demethylating agent 5-aza-CdR can reactivate silenced genes by DNA demethylation. Several studies have indicated that this agent can induce apoptosis in numerous cancer cell lines (9-11). One of the mechanisms by which 5-aza-CdR affect tumor cells is silenced TSGs reactivation. It has been reported that DNA demethylating agent, 5-aza-CdR, can reactivate silenced p16INK4a gene in HCC HepG2 cell line, resulting in cell cycle arrested in G1 and apoptosis induction (12). There are similar reports about the effect of this agent on the reactivation of p16INK4a in bladder cancer (13), and p14ARF in esophageal cancer (14). Other researchers have shown that DNA demethylating agent zebularine reactivates p15INK4B gene expression in acute myeloid leukemia (AML) (15). Previously, the effect of 5-aza-CdR on the DNMT1 gene expression and cell apoptosis in HCC WCH-17 cell line has been reported (16). Besides, the effect of DNA demethylating agent genistein (GE) on DNMT1, DNMT3a, and DNMT3b genes inhibition in hepatocellular carcinoma Hep G2 cell line has been evaluated (17). The current study was assigned to investigate the effect of 5-aza-CdR on the expression of p16INK4a, p14ARF, p15INK4b genes, cell viability, and apoptosis in human hepatocellular carcinoma, PLC/PRF5, and pancreatic cancer, MIA Paca-2, cell lines. This work was a laboratory trial study.

Materials and Methods
This laboratory trial was done with the human HCC, PLC/PRF5, and pancreatic cancer, MIA Paca-2, cell lines. The cell lines were obtained from the National Cell Bank of Iran-Pasteur Institute and maintained as a monolayer in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 mL/L fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) at 37º C in a humidified atmosphere. 5-aza-CdR was provided from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, provided from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, provided from Sigma at a final concentration of 100 μM to provide a working stock solution. By diluting this solution, all other working solutions were provided. Annexin-V-(FITC), propidium iodide (PI), dimethyl sulfoxide (DMSO), Trypsin-EDTA, DMEM, 3-[4, 5-dimethyl-2-thiazoly]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Phosphate-buffered saline (PBS), and antibiotics 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 a lab trial study approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1397.100.
Cell culture and cell viability
Both cell lines were cultured with DMEM supplemented with sodium bicarbonate, antibiotics, sodium pyruvate, 10% FBS, at 37°C in 5% CO2 overnight and subsequently plated into 96-well plates, 5 x 10^3 cells per well. Next day, each cell line was treated with 5-aza-CdR (0, 1, 2.5, 5, 10, 15, and 20μM) for different periods, 24h and 48h. Following treatment, the effect of 5-aza-CdR was evaluated by MTT assay according to the manufacturer's protocols. In this regard, the plated cells were incubated with MTT for 4 h and then the medium was changed with100 μl DMSO per well. Subsequently, the plates were placed for 5 min at room temperature, so that the formazan crystals were dissolved in DMSO and the absorbance was measured by a microplate reader at a wavelength of 570 nM. Each experiment was repeated three times (triplicates).

Cell apoptosis analysis
To assess the cell apoptosis by flow cytometry, both cell lines were seeded in 24-well plate, at a density of 5 x 10^5 per well, and treated with 5-aza-CdR (3 μM) according to the half-maximal inhibitory concentration (IC50), for 24 and 48 h except the control groups which incubated with DMSO only. After treatment with 5-aza-CdR, all the treated and untreated cells, control groups, were collected by trypsinization, washed with cold PBS, and re-suspended in Binding buffer (1x). To determine the apoptotic cells, the cells were stained with annexin-V-(FITC) and PI according to the manufacturer's protocol, the samples were subjected to flow cytometry, and the apoptotic cells were investigated by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis
The qRT-PCR was done to determine the expression of p16INK4a, p14ARF, p15INK4b genes. Therefore, the HCC PLC/PRF5 and pancreatic cancer MIA Paca-2 cells were treated with 5-aza-CdR (3 μM) according to IC50 value, and total cell RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and treated by RNase-free DNase (Qiagen). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Target mRNA expression was measured by qRT-PCR with QuantiTect SYBR Green RT-PCR Kit (Qiagen). Real-time RT-PCR reactions for cDNA amplification were performed as mentioned previously (17). All used primer sequences are indicated in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Data were analyzed using the comparative Ct (ΔΔct) method.

Results
Cell viability by the MTT assay
The viability of HCC, PLC/PRF5, and pancreatic cancer, MIA Paca-2, cells treated with 5-aza-CdR (0, 1, 5, 10, 15, and 20μM) was determined by MTT assay. As indicated in Figure 1, the inhibitory effect of 5-aza-CdR on both cell lines was dependent on the concentration and incubation time. 5-aza-CdR inhibited cell growth significantly with all used concentrations (P< 0.003). IC50 values were obtained with approximately 3 μM of 5-aza-CdR.
Both cell lines were treated with and without different doses of 5-aza-CdR for different periods, 24 and 48 h, and the viability was assessed by MTT assay. Each experiment was done in triplicate. Mean values from the three experiments ± standard error of mean are demonstrated. Each group of columns represents 5-aza treated cells at a concentration of 0 (control) 1, 2.5, 5, 10, 15, and 20 μM, respectively. Asterisks (*) indicate significant differences between treated and untreated cells.
Cell apoptosis assay
The apoptotic cells of treated and control groups were investigated by flow cytometry. The PLC/PRF5 and MIA Paca-2 cells were treated with 5-aza-CdR. After 24 and 48 h of treatment, the cells were stained using annexin-V-(FITC) and PI. As depicted in Figure 2 and Figure 3, significant differences were observed between the numbers of apoptotic cells in all treated groups compared to control groups. More apoptotic effect was observed in PLC/PRF5 cells in comparison to MIA Paca-2 cells. Besides, 5-aza-CdR induced apoptosis in PLC/PRF5 cells as a time-dependent manner (Table II and Figure 4).

As shown above, 5-aza-CdR indicated a time-dependent apoptotic effect. As depicted in Figure 2, significant differences were observed between the numbers of apoptotic cells in all treated groups compared to control groups. Asterisks (*) indicate significant differences between treated cells and untreated control groups.

As depicted in Figure 3, significant differences were observed between the numbers of apoptotic cells in all treated groups compared to control groups. Asterisks (*) indicate significant differences between treated and untreated control groups.

Effect of 5-aza-CdR on genes expression
The effect of 5-aza-CdR (3 μM) on the expression of p16INK4a, p14ARF, p15INK4b genes was evaluated by quantitative Real-Time RT-PCR analysis. Real-time quantitative PCR analysis revealed that 5-aza-CdR (3 μM) up-regulated p16INK4a, p14ARF, p15INK4b genes expression in PLC/PRF5 and MIA Paca-2 cells significantly. The 5-aza-CdR had a more significant effect on the genes expression in PLC/PRF5 cell in comparison to MIA Paca-2 cell (Table III and Figure 5).

As shown above, 5-aza-CdR up-regulated p16INK4a, p14ARF, p15INK4b significantly. Asterisks (*) indicate significant differences between treated and untreated (control) groups. Data are presented as means ± standard error of the mean.
Figure 2. The apoptosis-inducing effect of 5-aza-CdR on HCC PLC/PRF5 cells investigated by flow cytometric analysis.

Figure 3. The apoptosis-inducing effect of 5-aza-CdR on pancreatic cancer MIA Paca-2 cells investigated by flow cytometric analysis.
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Figure 4. Comparative analysis of the apoptotic effect of 5-aza-CdR on PLC/PRF5 and MIA Paca-2 cells treated with 5-aza-CdR for 24 and 48h. As shown above, 5-aza-CdR had a stronger apoptotic effect on HCC PLC/PRF5 cells.

Figure 5. The relative expression level of p16INK4a, p14ARF, p15INK4b treated with 5-aza-CdR in HCC PLC/PRF5 cells and pancreatic cancer MIA Paca-2 cells

Table I: The primer sequences of p16INK4a, p14ARF, and p15INK4b genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5′ to 3′)</th>
<th>Reference</th>
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<tr>
<td>p14ARF Forward</td>
<td>GTGGGTTTTTAGTTGTAGTT</td>
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<tr>
<td>p14ARF Reverse</td>
<td>AAACCTTTCTACCTAACT</td>
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</tr>
<tr>
<td>p15INK4b Forward</td>
<td>AAGCTGAGCCAGGTCTCCTA</td>
<td>19</td>
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<tr>
<td>p15INK4b Reverse</td>
<td>CCACCCTTTGCCGTAATACT</td>
<td></td>
</tr>
<tr>
<td>p16INK4a Forward</td>
<td>CCCGCTTTCTAGTTTTTCAT</td>
<td>20</td>
</tr>
<tr>
<td>p16INK4a Reverse</td>
<td>TTATTGTAGCTTTTGGTCTG</td>
<td></td>
</tr>
</tbody>
</table>
Table II: The percentage of apoptotic cells treated with 5-Aza-CdR (3 μM) at 24 and 48h

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell line</th>
<th>Dose (μM)</th>
<th>Duration (h)</th>
<th>Apoptosis (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Aza-CdR</td>
<td>PLC/PRF5</td>
<td>3</td>
<td>24</td>
<td>88.5</td>
<td>0.001</td>
</tr>
<tr>
<td>5-Aza-CdR</td>
<td>PLC/PRF5</td>
<td>3</td>
<td>48</td>
<td>95.6</td>
<td>0.001</td>
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<tr>
<td>5-Aza-CdR</td>
<td>MIA Paca-2</td>
<td>3</td>
<td>24</td>
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<tr>
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<td>MIA Paca-2</td>
<td>3</td>
<td>48</td>
<td>19.49</td>
<td>0.001</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell line</th>
<th>Drug</th>
<th>Dose (μM)</th>
<th>Duration (h)</th>
<th>Expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p14ARF</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>24</td>
<td>2.4</td>
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<tr>
<td>p15INK4b</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>24</td>
<td>2.2</td>
<td>0.005</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>24</td>
<td>2.6</td>
<td>0.002</td>
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<tr>
<td>p14ARF</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>3.1</td>
<td>0.005</td>
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<tr>
<td>p15INK4b</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>3</td>
<td>0.002</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>PLC/PRF5</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>3.4</td>
<td>0.001</td>
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<tr>
<td>p14ARF</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>24</td>
<td>2</td>
<td>0.019</td>
</tr>
<tr>
<td>p15INK4b</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>2.1</td>
<td>0.001</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>2</td>
<td>0.001</td>
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<tr>
<td>p14ARF</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>2.7</td>
<td>0.001</td>
</tr>
<tr>
<td>p15INK4b</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>2.5</td>
<td>0.001</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>MIA Paca-2</td>
<td>5-Aza-CdR</td>
<td>3 μM</td>
<td>48</td>
<td>2.8</td>
<td>0.040</td>
</tr>
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</table>

Discussion

One of the main epigenetic causes of cancer is aberrant methylation of the TSGs that induces gene silenced and loss of the function of the genes (21). This change compacts chromatin structure, resulting in the gene inactivation. CIP/KIP (p21, p27, and p57) and INK4 (p15, p16, p18, and p19) are two families of cyclin-dependent kinase inhibitors targeted by methylation in various cancers. Many recent studies have indicated that various types of cyclin-dependent kinase are frequently inactivated in HCC and pancreatic cancers (22, 23). The ability of 5-Aza-CdR to restore the genes involved in the cell cycle has been widely indicated (24). The results of the current study indicated a positive correlation between DNA demethylating agent 5-Aza-CdR and TSGs reactivation. The present study reported that 5-Aza-CdR reactivated p16INK4a, p14ARF, p15INK4b genes, inhibited cell growth and induced apoptosis in PLC/PRF5 and MIA Paca-2 cell lines significantly. Furthermore, 5-Aza-CdR indicated a more strong effect on PLC/PRF5 cells than MIA Paca-2 cells. According to the present result, one of the molecular mechanisms by which 5-Aza-CdR affected PLC/PRF5 and MIA Paca-2 cells was re-activation of TSGs, p16INK4a, p14ARF, and p15INK4b. Similarly, another researcher has reported that 5-aza-CdR affects HepG2 cell line by p16INK4a re-activation (12). This compound effect on the reactivation of cyclin-dependent kinase inhibitors is shown in other cancers. For example, it can reactivate p16INK4a and p15INK4b genes in neuroendocrine gastroenteropancreatic (GEP) tumors (25), p21WAF1/CIP1 gene in prostate cancer cell lines DU145, 1542 NP PC3, and LNCaP (26), p16INK4a and p53 in pancreatic cancer cell lines AsPC-1 and SW1990 (27), and p27 in gastric cancer (28). Additionally, zebularine is a member of the DNA methyltransferase family that acts as a DNMT inhibitor. It has been reported that this agent can reactivate p16INK4a, p21, and p27 genes in CFPAC-1 pancreatic cancer, PC3 prostate cancer, T24 bladder cancer, CALU-1 lung cancer cells, HT-29 colon cancer, and HCT15, SW48 (29). DNA methyltransferase inhibitors demethylate hypermethylated TSGs through inhibition of DNA methyltransferases activity. Previously, it
has been demonstrated that GE inhibited DNMT1, DNMT3a, and DNMT3b genes activity in HCC (17). In agreement with this report, 5-aza-CdR reverses DNA methylation of CDKN2A, RASSF1A, HTLF, RUNX3, and AKAP12B genes by DNMT1 and DNMT3b inhibition has been indicated in gastric cancer (30). In human endometrial cancer cell lines, 5-aza-CdR induces cell cycle arrest, cell growth inhibition, and cell apoptosis by inhibition of DNMT3B activity (31). Several researchers have reported that 5-aza-CdR restores caspases expression in many human cancers, including medulloblastoma, neuroblastoma, Ewing tumors, melanoma or small cell lung carcinoma (32). Taken together, these results indicated that reactivation of cyclin- dependent kinase inhibitors was one of the several mechanisms by which 5-aza-CdR fulfilled its role.

**Conclusion**

The findings of this study revealed that reactivation of p16INK4a, p14ARF, p15INK4b genes by 5-aza-CdR could inhibit cell viability and induce apoptosis in hepatocellular carcinoma PLC/PRF5 and pancreatic cancer, MIA Paca-2 cell lines.

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**Conflict of interest**

The authors declare no conflict of interest.

**References**


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