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Effects of 5-aza-2ˈ-deoxycytidine and Valproic Acid on Epigenetic-modifying DNMT1 Gene Expression, Apoptosis Induction and Cell Viability in Hepatocellular Carcinoma WCH-17 cell line

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Abstract
Background: DNA molecule of the eukaryotic cells is found in the form of a nucleoprotein complex named chromatin. Two epigenetic modifications are critical for transcriptional control of genes, including acetylation and DNA methylation. Hypermethylation of tumor suppressor genes is catalyzed by various DNA methyltransferase enzymes (DNMTs), including DNMT1, DNMT2, and DNMT3. The most well characterized DNA demetilating and histone deacetylase inhibitor drugs are 5-aza-2ˈ-deoxycytidine (5-Aza-CdR) and valproic acid (VPA), respectively. The purpose of the current study was to analyze the effects of 5-Aza-CdR and VPA on cell growth, apoptosis, and DNMT1 gene expression in the WCH-17 hepatocellular carcinoma (HCC) cell line.
Materials and Methods: In this descriptive analytical study, MTT assay, flow cytometry assay, and Quantitative Real-Time RT-PCRwere done to evaluate proliferative and apoptotic effects and also gene expression.
Results: Both compounds inhibited the cell growth and induced apoptosis significantly in a dose and time depended fashion. Additionally, 5-Aza-CdR down-regulated DNMT1 gene expression. The relative expression of DNMT1 was 0.40 and 0.20 ($P < 0.001$) at different times, respectively. The percentage of VPA- treated apoptotic cells were reduced by about 28 and 34 % ($P<0.001$) and that of 5-Aza-CdR-treated were reduced by about 34 and 44 % ($P<0.001$) after treatment time periods.
Conclusion: In the current study, it was observed that 5-Aza-CdR and VPA could significantly inhibit the growth of WCH-17 cell and played a significant role in apoptosis. It was also found that 5-Aza-CdR could decrease DNMT1 gene expression.
Keywords: Apoptosis, 5-aza-2ˈ-deoxycytidine, DNA methyltransferase 1, Hepatocellular carcinoma, Valproic Acid

Introduction

In the eukaryotic cells, DNA molecule is presented in the form of chromatin, the basic unit of chromatin is the nucleosome (1). The architecture and organization of nucleosome is critical for gene regulation (2). In fact, the genomic cell is divided into euchromatin and hetero-chromatin by packaging DNA into chromatin. Two epigenetic modifications are critical for transcriptional control of genes, including acetylation of the amino-terminal tails of histones (histone H3 and H4) and DNA methylation. These modifications are done by histone acetyltransferases (HATs) and DNA methyltransfrases (DNMTs) respectively. DNA methylation leads to gene inactivity and transcriptional silencing (3). All eukaryotes use histone acetylation to control transcription (4). Cancer is a disease that results from genetic and epigenetic changes (5, 6). DNA methylation, as an epigenetic process, affects chromatin organization and gene expression. Hypermethylation of CpG islands of the gene promoter is associated with transcriptional silencing of the involved genes (5). Aberrant promoter DNA hypermethylation of the tumor suppressor genes leads to cancer (7, 8). Hypermethylation of tumor suppressor genes has been reported in certain

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malignancies such as breast, cervical, liver, gallbladder, ovarian, kidney, pancreas, colorectal, and prostate cancers (9). Various studies have shown that an overall increase in the enzymatic DNA methyltransferase activity occurs in cancers versus normal tissues (10).Hypermethylation of tumor suppressor genes is catalyzed by a group of DNA methyltransferase enzymes. In eukaryotic cells, there are three groups of DNMTs, including DNMT1, DNMT2, and DNMT3 (11, 12). Besides, modification of histones regulated by a balance between HATs and HDACs activities is a general function of the transcriptional machinery. Therefore, histone acetylation is closely related to transcriptional activation and is a reversible process (13-15). HATs add an acetyl group to N-terminal lysine residues in histones, resulting in increased accessibility of regulatory proteins to DNA. Accordingly, activity of histone acetyltransferases leads to acetylation of the core histones, facilitating transcription. Histone deacetylation is performed by histone deacetylases (HDACs). It has been demonstrated that HDACs has a significant role in tumoroigenesis. In fact, recruitment of these enzymes causes decreased histone acetylation through compaction of chromatin, resulting in transcriptional repression (16-18). The most well characterized DNA metilating drugs is 5-aza-2'-deoxycytidine (5-Aza-CdR) which inhibits DNA cytosine methylation and reactivates silenced genes (19, 20). HDAC inhibitors (HDACIs) can inhibit cell proliferation, induce cell apoptosis, and enhance cell differentiation in different cancers (21). Histone deacetylase inhibitor valproic acid (VPA) is a short-chain fatty acid class of HDACIs which inhibits HDACs (22), resulting apoptosis induction. However, there have been no reports on the effects of 5-Aza-CdR alone or in combination with VPA on WCH-17 hepatocellular carcinoma (HCC) cell line. Therefore, the aim of the present study was to investigate the effects of these compounds on cell proliferation, cell apoptosis, and DNMT1 gene expression of hepatocellular carcinoma WCH-17 cell line.

Materials and Methods

This is a descriptive analytical study. Hepatocellular carcinoma WCH-17 cells were provided from the National Cell Bank of Iran-Pasteur Institute, maintained in Dulbecco's modified Eagle's medium (DMEM) (St. Louis, MO, USA), and supplemented with 10% fetal bovine serum (FBS) and antibiotic (penicillin, streptomycin and amphotericin B). Trypsin-EDTA, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), FBS, DMEM, streptomycin, and penicillin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck Co. (Darmstadt, Germany). VPA (2-propyl-pentanoic acid) and 5-Aza-CdR (pH 6.8) were provided from Sigma (St. Louis, MO) and dissolved in serum-free medium and PBS;respectively, to make a stock solution. The stock solution was further diluted with culture medium to obtained final concentrations of VPA and 5-Aza-CdR.

Cell Culture and Cell Proliferation Assay

WCH-17 cells were cultured with DMEM (pH 7.2–7.4) supplemented with 1% sodium pyruvate (sigma), 1.5 g/L sodium bicarbonate, 10% FBS, and 1% antibiotics at 37°C in 5% CO2 to promote attachment. After suitableconfluencyt, 5 × 105 cells were cultured into 96-well plates at a density of 5 × 105 live cells per well, allowed to adhere for 24 h and subsequently treated with medium containing different doses of 5-Aza-CdR (1, 2.5, 5, 7.5 and 10 μM) and VPA (1, 2.5, 5, 7.5 and 10 μM) after 24 h to determine IC50 value. After one and two days, the effect of 5-Aza-CdR and VPA was assessed by MTT assay according to standard protocols. In this regard, the cells were washed twice with PBS, a fresh

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medium containing 0.5 mg/mLMTT was added, and finally after 4 h incubation, the formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 570 nm. All experiments were repeated three times, with at least three measurements (triplicates).

Cell Cycle Analysis and Apoptosis

The assessment of apoptosis was carried out using flow cytometry, WCH-17 cells were plated (at a density of 5 × 105 cells/well) in 24-well treated with 5-Aza-2′-deoxycytidine (2 μM) and VPA (3 μM), a concentration within the range employed previously (23) for 24 and 48 h. In combined treatment group, the cells were treated with 5-Aza-CdR for 24 h and then exposed to VPA for further 24 h prior harvesting. After treatment, all the adherent cells were harvested by trypsinization, washed with cold phosphate-buffered saline (PBS), and resuspended in Binding buffer (1x). In order to staning, annexin-V-(FITC) and propidium iodide (PI) were used according to the manufacturer's protocol. Finally, the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Quantitative real-time RT-PCR was done to quantitatively estimate the expression of DNMT1 gene in 5-Aza-CdR (2 μM)-treated WCH-17 cells at different times. WCH-17 Cells were plated at a density of 5 × 105 cells/well in six-well plates; after 24 h, they were treated with 5-Aza-CdR (2μM) for 24h and 48 h. After treatment times, total RNA was isolated by RNeasy mini kit and treated by RNase free DNase to eliminate the genomic DNA. The concentration of RNA was determined using a Biophotometer (Eppendorf). Total RNA (100 ng) was reverse transcribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Real-time RT-PCR was performed similar to our previous work [24].DNMT1 primers were used as referenced article (24) which their sequences are shown in Table I.GAPDH was used as a reference gene for internal control.

TableI. Primer sequences used in the study

Genes	Primer sequences
DNMT1	
Forward:	5'-TAC CTG GAC GAC CCT GAC CTC-3'
Revers:	5'-CGT TGG CAT CAA AGA TGG ACA-3'

Statistical Analysis

The effects of 5-Aza-CdR and valproic acid on cell proliferation and apoptosis were analyzed with one-way ANOVA. The relative quantification (comparative method) was calculated using the ΔΔct method. All samples were normalized to the Δct value of a reference gene to obtain a Δct value (Δct target - Δct reference). The results were expressed as means ± standard deviation. Differences were consideredsignificant at $p < 0.05$.

Results

Result of MTT Assay

The effects of the 5-Aza-CdR and VPA on the WCH-17 cells viability were assessed after treatment with various concentrations of agents (as mentioned previously) by MTT assay. As shown in Figure 1, 5-Aza-CdR and VPA significantly inhibited cell cell viability in a dose and time dependent manner ($P < 0.001$) in all three experimental versus control groups. 5-Aza-CdR inhibited growth in all treated groups by 80% to 28% at 24 h and 74% to 18% at 48 h ($P < 0.001$) and also VPA inhibited growth in all treated groups by 70% to 20% at 24 h and 66% and 16 % at 48 h ($P < 0.001$). Reduction of cell viability by 50% (half maximalinhibitory concentration, IC50) required 2 μm 5-Aza-CdR and 3μM VPA. For consistency of the result, each experiment was repeated three times. The percentage of cell viability with various dose of drugs at

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different time periods (24 and 48 h) are shown in Figure 1 (P<0.001).

VPA Induction of Apoptosis

To investigate the apoptotic effects of 5-Aza-CdR (2 µM) and VPA (3 µM) on the WCH-17 cells, cells were treated with these compounds (alone and combined) at a different time periods (24 and 48 h) and flow cytometry was performed to observe the apoptotic cells. As shown in Figure 2, flow cytometry revealed that 5-Aza-CdR and VPA (alone and combined) induced significant apoptosis versus control group. The percentage of VPA- treated apoptotic cells were reduced by about 28% and 34 % (P<0.001) and that of 5-Aza-CdR-treated 34% and 44 % (P<0.001) after treatment times (24 h and 48 h), respectively. Maximal apoptotic cell (72 %, P<0.001) was observed in the group which received 5-Aza-CdR for 24 h and then VPA for 24 h and minimal apoptotic cell was observed in the group which received VPA alone for 24 h.

Result of Quantitative Real-Time RT-PCR

To assess the effect of 5-Aza-CdR on DNMT1 gene expression, the effect of 5-Aza-CdR was determined by using quantitative real-time RT-PCR analysis at different time periods (24 and 48 h). Using quantitative RT-PCR, 5-Aza-CdR was shown to decrease DNMT1 gene expression significantly at different times. The relative expression of DNMT1 was 0.40 and 0.20 (P < 0.001) after 24h and 48h, respectively. In conclusion, 5-Aza-CdR decreased DNMT1 expression significantly as shown in Figure 3. Data were presented as means ± S.E.M.

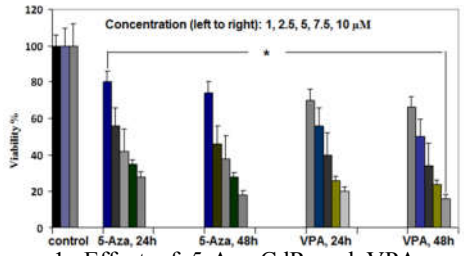

Figure 1. Effect of 5-Aza-CdR and VPA on the WCH-17 cells viability. The viable cells were determined by the MTT assay and calculated in comparison to the control groups. (*p < 0.001). Data are presented as mean ± SD from at least triplicate wells and 3 independent experiments.

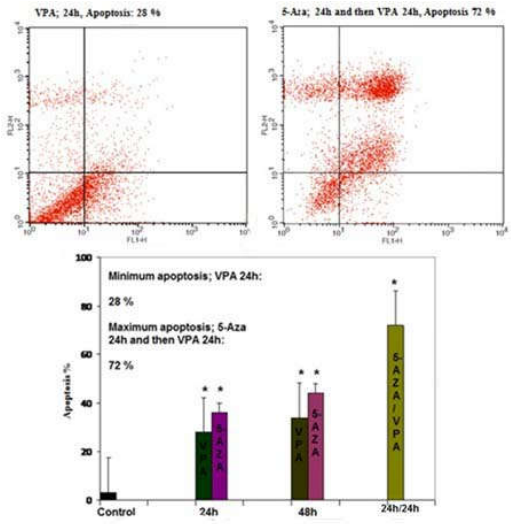

Figure 2. 5-Aza-CdR and VPA (alone and combined) promoted apoptosis in WCH-17 cells. Both agents induced apoptosis in WCH-17 cellssignificantly. Combined drug induced more apoptotic cells in WCH-17 cells than that of each drug alone (*p<0.001 compared with control group).

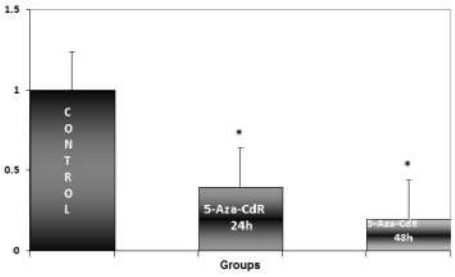

Figure3. Relative expression level of DNMT1.

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Discussion

The use of chromatin-modifying drugs such as DNA methylating agents and histone deacetylase inhibitors to restore chromatin structure is an attractive therapeutic approach for cancer treatment. The involvement of DNA hypermethylation on the regulation of gene expression has been reported. The role of DNA methylation in tumorigenesis and 5-Aza-CdR as a DNA demethylating agent has been well recognized (25). HDAC inhibitors can influence gene expression and reactivation of silenced genes during cancer treatment, resulting in cancer cell growth inhibition and apoptosis induction (26). In fact, inhibitory and apoptotic effect of HDAC inhibitors have been observed in a variety of solid and hematological cancers. Several studies have demonstrated that VPA can induce histone acetylation, growth arrest, and cell differentiation in several cancers (27).Given that DNA methylating agents and HDAC inhibitors affect cell viability, apoptosis and silenced gene expression, the effects of 5-Aza-CdR, and VPA on viability, apoptosis, and DNMT1 gene expression were assessed in this study. It was found that these compounds (alone and combined) inhibited proliferation, induced apoptosis, and also inhibited DNMT1 gene expression.Indeed, each drug alone had the capacity to induce apoptosis and inhibit cell growth, and the combination of them had additive effect. In agreement with the current report, it was shown that 5-Aza-CdR down-regulated DNMT1 mRNA levels, resulting in global demethylation and reactivation of a silenced hemimethylated p16INK4a gene in colon cancer cells HCT-116 (28). Other studies showed that 5-Aza-CdR induced 15-LOX-1 expression and cell apoptosis in Caco-2 colon cancer cells in a time and dose dependent manner (29). Additionally, the effect of 5-Aza-CdR on other methylated genes was indicated in previous studies (30). VPA has been increasingly used for a variety of cancer treatment idividually or in combination with other compounds.VPA induces apoptosis in prostate cancer cells by TMPRSS2-ERG down-regulation (31). Various studies reported that VPA induced differentiation of leukemic and apoptosis of glioma cells and also inhibited angiogenesis (32, 33). VPA inhibits Akt1 and Akt2 gene expression in HeLacervicals cancer cells resulting in apoptosis induction apoptosis (34). In vitro experiment showed that this drug inhibited growth of epithelial ovarian cancer SKOV3 cells (35). Furthermore, VPA induces G1 phase cell cycle arrest by increasing expression of the cyclin-dependent kinase (cdk) inhibitor p21WAF1/CIP1 (36).This study suggested that 5-Aza-CdR combined with VPA induced cell cycle arrest and inhibited cell growth in WCH-17 cells. Inconsistent with our results, it has been shown that the combination of 5-Aza-CdR and VPA could induce apoptosis and reactivate tumor suppressor gene. An in vitro study indicated that the 5-Aza-CdR combined with VPA inhibited cell growth, induced apoptosis, and reactivated p57KIP2 and p21CIP1 in human leukemias (37). In addition, it was indicated that the combination of 5-Aza-CdR and VPA could restore RARb2 expression and histone H3 acetylation to the silenced RARb2-RARE in MCF-7 cells (38). Althoughthe combination of 5-Aza-CdR and VPA showed an inhibiting, apoptotic and down-regulating effect in the current work, it is necessary to confirm the efficacy of 5-Aza-CdR combined with VPA on methylation, acetylation, and also enzymatic process involved in apoptosis and cell growth inhibition.

Conclusion

In the current study, it was observed that 5-Aza-CdR and VPA (alone and combined) could inhibit the growth and induce apoptosis of HCC in vitro, suggesting that they could be used in the treatment of HCC.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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