The Effects of 5-Aza-2’-Deoxycytidine and Valproic Acid on Apoptosis Induction and Cell Growth Inhibition in Colon Cancer HT 29 Cell Line

Abstract

Background: Epigenetic changes, including DNA methylation and histone modification, alter gene expression without the nucleotide template alterations and are associated with all stages of tumor formation and progression. Previously, we investigated the effects of DNA demethylating agents and histone deacetylase inhibitors on hepatocellular carcinoma and colon cancers. The current study aimed to investigate the effects of 5-aza-2’-deoxycytidine (5-AZA-CdR, decitabine) and valproic acid (VPA), individually as well as combined on apoptosis induction and cell growth inhibition in colon cancer HT 29 cell line. Methods: The effect of the compounds on the cell viability was measured by MTT assay. To determine cell apoptosis, the cells were treated with 5-aza-CdR and VPA. Propidium iodide was used for staining and the cells were analyzed using flow cytometry. Results: Both agents decreased cell viability in a time and dose-dependent manner significantly (P < 0.002). The results of flow cytometry demonstrated that 5-aza-CdR and VPA induced apoptosis significantly as opposed to control groups. Maximal percentage of apoptotic cells was obtained after 48 h with combined treatment. Conclusions: Our findings suggest that 5-aza-CdR and VPA can significantly inhibit cell growth and induce apoptosis in colon cancer HT 29 cell line.

Keywords: Apoptosis, colonic neoplasms, decitabine, valproic acid

Introduction

Colon cancer is the fourth leading cause of cancer deaths in the world. Cellular pathway disruptions that control cell proliferation and cell death play an important role in tumorigenesis, initiation, and progression of cancer. Epigenetic changes including DNA methylation and histone modification, alter gene expression without the nucleotide template alterations and are associated with all stages of tumor formation and progression. One of the best-known epigenetic changes that inactivate tumor suppressor genes is promoter CpG island hypermethylation mediated by DNA methyltransferases (DNMTs). Of all epigenetic changes, DNA hypermethylation, which inactivates transcription of tumor suppressor genes (TSGs) leading to gene silencing, has been reported in many cancers including colorectal, prostate, pancreatic, bladder, ovarian, and breast cancers. In mammalian cells, the DNMTs are divided into two major families, according to the structure and functions, including maintenance methyltransferase (DNMT1) and de novo methyltransferases (DNMT3a, DNMT3b, and DNMT3L). Furthermore, DNMT2 displays a weak DNA methyltransferase activity. The high expression level of DNMT1 seen in the S-phase of the cell cycle makes it a target for DNMT inhibitors (DNMTIs) in cancer treatment. Other chromatin modifications associated with silenced TSGs are histone modifications such as acetylation and deacetylation. The histone acetylation levels are the result of the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) and histone acetyl-transferase (HAT) activities. HDACs remove the acetyl groups resulting in chromatin compaction. Mammalian HDACs can be subdivided into different groups according to their homology with yeast HDACs including class I (HDACs 1, 2, 3, and 8) and class II which are further subdivided into two subclasses: subclass IIa (HDAC 4, 7, and 9) and IIb (HDAC 6 and 10).

In vitro studies have revealed that DNA demethylating agents are involved in the reactivation of the silenced genes such as demethylation reactivation of the silenced genes such as...
Previously, we investigated the effects of DNA DNMT inhibitor compounds such as curcumin, genistein, EGCG, resveratrol, equol, and parthenolide act through DNMTs inhibition. DNMT inhibitor 5-Aza-2′-deoxycytidine (5-aza-CdR) has strong potential to inhibit DNMT resulting in a progressive loss of methylation and reactivation of gene expression.

It is a prodrug that requires activation through phosphorylation by deoxycytidine kinase, which catalyzes the phosphorylation of deoxycytidine to form deoxycytidine monophosphate. The nucleotide analog, 5-aza-CdR, is incorporated into DNA and produces an irreversible inactivation of DNA methyltransferase. This compound is an S-phase specific agent that reactivates silent tumor-suppressor genes (TSGs) by DNA demethylation resulting in cell differentiation, cell growth inhibition, and cell apoptosis. HDACIs can be divided into four groups structurally including hydroxamic acids, small molecular weight carboxylates, cyclic peptides, and benzamides. There are more than 15 HDACIs some of which include SAHA (vorinostat), phenyl-butyrate, valproic acid (VPA), trapoxin A, and trichostatin A.

In cells cultured with HDACIs, these compounds can upregulate the expression of both death receptors and their ligands in transformed cells. These agents upregulated proapoptotic proteins of the Bcl-2 family such as Bax, Bak, Bim, Bmf, and Bik and decrease Bcl-X	extsubscript{L}, Bcl-w, and Mcl-1. The low concentration of HDACIs induces G\textsubscript{1} arrest while high concentrations induce both G\textsubscript{1} and G\textsubscript{2}/M arrests associated with induction of p21, which inhibits CDKs regulating G\textsubscript{i}/S transition (CDK2), and G\textsubscript{i} progression (CDK4/6).

VPA, an 8-carbon branched-chained fatty acid has been reported as a well-known anticancer agent belonging to the small molecular weight carboxylates. This compound causes hyperacetylation of the N-terminal tails of histones H3 and H4 in vivo and in vitro and inhibits HDAC activity, it binds to the catalytic center thereby blocking substrate access. Previously, we investigated the effects of DNA demethylating agents and histone deacetylase inhibitors on hepatocellular carcinoma and colon cancers which encouraged us to design this study. The current study aimed to investigate the effects of 5-aza-CdR and VPA on apoptosis induction and cell growth inhibition in colon cancer HT 29 cell line.

**Methods**

Colon cancer HT 29 cells were purchased from the National Cell Bank of Iran, Pasteur Institute and were maintained in Dulbecco’s modified Eagle’s medium (DMEM). Culture media contains 100 mL/L fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37°C in a humidified atmosphere of 5% carbon dioxide (CO2). In addition, 5-aza-CdR and VPA were provided from Sigma (St. Louis, MO, USA) and dissolved in culture medium and phosphate-buffered saline (PBS), respectively at a final concentration of 100 mM to prepare a suitable stock solution. All of the other test concentrations were prepared by diluting the stock solution. DMEM, FBS, and 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany).

### Cell viability assay

The effect of 5-aza-CdR and VPA on the cell viability was measured by MTT assay. The cells were seeded into the 96-well plates at 5 x 10\textsuperscript{4} cells/well and allowed to adhere overnight. After 24 h, the culture medium was replaced with medium containing different concentrations of VPA (0.5, 1, 2.5, 5, and 10 µM) and 5-aza-CdR (0.5, 1, 2.5, 5, and 10 µM), except control groups which were treated with a drug-free medium for different periods. After treatment times (24 and 48 h), the viable cells were determined by an MTT assay, as described elsewhere.

### Apoptosis assay

The colon cancer HT 29 cell, 5 x 10\textsuperscript{4} cells, were treated with 5-aza-CdR at a concentration of 2 µM and VPA at a concentration of 5 µM and the cells were harvested by trypsin solution at the end of the treatment (after 24 and 48 h). Besides, one group was treated with 5-aza-CdR at a concentration of 2µM for 24 h followed by VPA at a concentration of 5 µM for another 24 h. After treatment times, the cells were washed with cold phosphate-buffered saline (PBS) and resuspended in binding buffer (1x). Finally, Annexin-V-FITC and propidium iodide (PI, Becton-Dickinson, San Diego, CA) were used for staining according to the manufacturer’s instructions and the cells were analyzed using flow cytometry.

### Statistical analysis

The data were obtained from three tests and are shown as means ± standard deviations. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA) and Turkey test. A significant difference was considered as P < 0.05.

### Results

#### Result of cell viability assay

The colon cancer HT 29 cell were cultured in the 96-well plates and treated with VPA (0.5, 1, 2.5, 5, and 10 µM) and 5-aa-CdR (0.5, 1, 2.5, 5, and 10 µM) for 24 and 48 h and evaluated by MTT assay to determine cell viability. The results are shown in Figure 1, comparing the percentage of cell viability in treated cells with untreated control.
groups. As shown in Figure 1, both compounds decreased cell viability in a time and dose-dependent manner significantly \((P < 0.002)\). The IC50 values of 5-aza-CdR and VPA were 2 \(\mu\)M and 5 \(\mu\)M, respectively.

**Result of apoptosis assay**

To determine the apoptotic effect of the compounds, the propidium iodide (PI) and Annexin-V/FITC staining were done. The results demonstrated that 5-aza-CdR at a concentration of 2 \(\mu\)M and VPA at a concentration of 5 \(\mu\)M induced apoptosis significantly as opposed to control groups. A similar result was observed in the group treated with 5-aza-CdR at a concentration of 2 \(\mu\)M for 24 h followed by VPA at a concentration of 5 \(\mu\)M for another 24 h. The percentage of apoptotic cells in 5-aza-CdR-treated groups was 47 and 82% and in VPA-treated groups were 85 and 94%, respectively \((P < 0.001)\). The percentage of apoptotic cells in the group which treated with 5-aza-CdR at a concentration of 2 \(\mu\)M for 24 h and followed by VPA at a concentration of 5 \(\mu\)M for another 24 h were 96% \((P < 0.001)\). Maximal percentage of apoptotic cells was obtained after 48 h with combined treatment [Figures 2 and 3].

**Discussion**

Colon cancer is a leading cause of cancer deaths worldwide. One of the basic causes of the colon cancer initiation and progression is the accumulation of a variety of epigenetic changes such as aberrant DNA methylation and histone deacetylation in colon epithelial cells. Epigenetic assessment of colon cancer has indicated aberrantly methylated and histone deacetylated genes that alter miRNA expression. \[21\] DNA hypermethylation-mediated epigenetic silencing of TSGs has emphasized the development and application of anticancer drugs which work by inhibition of DNA methylation and restore silenced genes by reprogramming the genes involved in cancer initiation and progression. DNMTIs modulate the cellular epigenome by reversing the hypermethylation, resulting in renewed transcription of silenced TSGs. \[22\]

HDACIs are natural or synthetic agents that have broad functions in cancer treatment. These compounds can change the balance between HATs and HDACs, resulting in the accumulation of acetylated histones/nonhistone proteins, which reactivates silenced TSGs. \[23\] Previously, we reported the effect of DNA demethylating agents such as genistein and histone deacetylase inhibitors such as VPA on cancers. \[13-17\] The result of the current study indicated that 5-aza-CdR and VPA (individually and combined) can inhibit cell growth and induce significant apoptosis in colon cancer cells. One of the major mechanisms of DNA demethylating agents and histone deacetylase inhibitors is silenced TSGs reactivation as we reported estrogen receptor alpha (ER\(\alpha\)) reactivation and DNMTs suppression previously. It has been reported that 5-aza-CR down-regulates DNMT1, DNMT3a, and DNMT3b gene expression, arrests the cell cycle at the G2/M phase and induces apoptosis by a joined activation of p53 protein and its downstream effectors p21WAF1 and GADD45 in the colon cancer cell line HCT-116. \[24\] Other studies have demonstrated that the 5-aza-CdR treatment affected the cell cycle and caused accumulation of cells in the G0/G1 phase by up-regulating the silenced tumor suppressor Ras association domain family 1A (RASSF1A) gene in Caco-2 cells. \[25\] Furthermore, it has been shown that treatment with 5-aza-CdR up-regulates endothelial PAS domain-containing protein 1 (EPAS1) expression in

![Figure 1: Effect of VPA (0.5, 1, 2.5, 5, and 10 \(\mu\)M) and 5-aza-CdR (0.5, 1, 2.5, 5, and 10 \(\mu\)M) on colon cancer HT 29 cell viability determined by the MTT assay. Data are presented as mean ± SD from at least triplicate wells and three independent experiments. Both compounds with all concentrations indicated a significant inhibitory effect. The first column of each group belongs to the control group](image1)

![Figure 2: Apoptotic effects of VPA and 5-aza-CdR. Both compounds induced significant apoptosis in colon cancer HT 29 cells. The apoptotic cell percentage in the group treated with 5-aza-CdR (24 h) and then VPA (24 h) were more significant than that of each drug alone. \((P < 0.001\) compared with control group)](image2)
In all, our finding indicated that 5-aza-CdR and VPA blocks cell growth, and reactivation of p57KIP2 and p21CIP1 in leukemic cell lines HL-60 and MOLT4. [27] Based on our result, combined treatment indicated a more significant apoptotic effect than that of a single agent alone. In constant with our finding, it has been reported that VPA inhibits cell growth and induces apoptosis in the HT-29 and LoVo colon cancer cell lines. It modulates the expression of PPARγ, a possible heterodimer of RXRs. VPA exerts its effect by modulation of the pro- and antiapoptotic factors, BAX and BCL2, respectively, it enhances the expression of BAX and down-regulates BCL2 expression in the HT-29 and LoVo colon cancer cell lines. [27] VPA blocks cell growth, possibly through the synthesis of p21WAF1/CIP1 and p27KIP1. It can exert by two main pathways resulting in programmed cell death including death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Activation of both caspase-8 and caspase-9 has been demonstrated in VPA-treated colorectal cells. [28] Other investigators have shown that DNA methyltransferase/histone deacetylase inhibitor combinations reverse the aberrant epigenetic gene silencing which leads to apoptotic induction in myeloid neoplasms. [29] Furthermore, histone deacetylase inhibitor trichostatin A can significantly upregulate DNA methylase inhibitor 5-aza-CdR-induced MAGE-A1, -A2, -A3, and -A12 genes expression in breast cancer (MDA-MB-231, GI101, and MTSV1.7) and colon cancer (WiDr) cell lines. [31] In all, our finding indicated that 5-aza-CdR and VPA could induce apoptosis in colon cancer HT 29 cell line, if used separately or in combination. 

Conclusions

In conclusion, our finding shows that 5-aza-CdR and VPA can inhibit in vitro colon cancer HT 29 cells and induce their apoptosis. Our results provide experimental support and a new therapeutic strategy into novel therapies for colon cancer. Meanwhile, the current study is limited to in vitro experiments, hence further evaluations will be required for a better understanding of the effects of these compounds on colon cancer.

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Conflicts of interest

There are no conflicts of interest.

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