

RESEARCH ARTICLE

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# In Vitro Effect of the Histone Deacetylase Inhibitor Valproic Acid on Viability and Apoptosis of the PLC/PRF5 Human Hepatocellular Carcinoma Cell Line

Masumeh Sanaei<sup>1</sup>, Fraidoon Kavooosi<sup>1\*</sup>, Abazar Roustazadeh<sup>1</sup>, Hossein Shahsavani<sup>2</sup>

## Abstract

The nucleosome is the fundamental building block of eukaryotic chromatin formed by DNA and histone proteins. Chromatin modifications such as acetylation, methylation, and phosphorylation are necessary for protection, replication, and gene transcription. Histone deacetylases (HDACs) are a group of enzymes that remove acetyl groups to re-establish positive charges on histones and aberrant deacetylation may lead to tumorigenesis in different tissues. Histone deacetylase inhibitors (HDACIs) are a class of chemotherapeutic agent that can reactivate gene expression and inhibit the growth of tumor cells by histone deacetylase inhibition. HDACI valproic acid (VPA) has shown potent anticancer effects in vitro and in vivo. Previously, we reported that VAP can inhibit the growth and induce apoptosis of human colon carcinoma HT 29 and hepatocellular carcinoma HepG 2 cells. The aim of the present study was to access the effect of VPA on proliferation and apoptosis of the human hepatocellular carcinoma (HCC) PLC/PRF5 cell line. **Materials and Methods:** PLC/PRF5 cells were treated with VPA and then MTT and flow cytometry assays were used to determine the effects on viability and apoptosis, respectively. **Results:** VPA inhibited cell growth and induced apoptosis in PLC/PRF5 cells significantly. **Discussion:** Our results clearly demonstrated that VPA has inhibitory and apoptotic effects. **Conclusion:** VPA can significantly inhibit the growth of HCC cells and play a significant role in apoptosis induction.

**Keywords:** Valproic acid- proliferation- apoptosis- hepatocellular carcinoma

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## Introduction

Nucleosome is the fundamental building block of eukaryotic chromatin formed by DNA and histone proteins (Strahl et al., 2003). Each nucleosome, basic unit of chromatin, is composed of an octamer of core histone proteins around which two super-helical turns of DNA are wrapped. Chromatin is a higher-order nucleoprotein complex by which the cell protects genetic information (Ito, 2003; Peterson et al., 2004). Chromatin modifications such as acetylation, methylation, and phosphorylation are necessary for chromatin protection, replication, DNA repair and gene transcription (Kouzarides, 2007). The correlation between histone acetylation and gene transcription has been reported. Acetylation of histone proteins occurs at specific lysine residues, all of which occur in the amino-terminal domains of the core histones, is closely linked to transcriptional activation (Choudhary et al., 2009; Berger, 2002; Heintzman et al., 2009). Addition and removal of acetate groups is catalyzed by specific enzymes (Zhang et al., 2005). Histone

acetyltransferases (HATs) are a group of enzymes that transfers the acetyl moiety to the internal lysine residues and histone deacetylases (HDACs) remove the acetyl groups by which re-establish the positive charge in the histones (Shahbazian et al., 2007). The balance between acetylation and deacetylation of the lysine of histone proteins plays an important role in the regulation of gene expression (Shahbazian et al., 2007; Iizuka et al., 2003). Aberrant deacetylation of histone proteins leads to tumorigenesis in different tissues (Luo et al., 2000) and deacetylation of lysine is a common event in human cancer (Fraga et al., 2005). Histone deacetylase inhibitors (HDACIs) are a class of chemotherapeutic agent that can reactivate gene expression and inhibit the growth of tumor cells and also induce apoptosis in a wide range of tumor-derived cell lines by histone deacetylase inhibition (Marks et al., 2001; Johnstone, 2002). HDACI valproic acid (VPA) has shown potent anticancer effects in vitro and in vivo (Duenas-Gonzalez et al., 2008). Previously, we reported that VAP can inhibit the growth and induce apoptosis of human colon carcinoma

<sup>1</sup>Research Center for Non-Communicable Diseases, <sup>2</sup>Student of Research Committee, Jahrom University of Medical Sciences, Jahrom, Iran. \*For Correspondence: Kavooosifraidoon@gmail.com

HT 29 (Sanaei et al., 2016) and hepatocellular carcinoma (HCC) HepG 2 cells significantly (Sanaei et al., 2017). To establish that VPA can inhibit viability and induce apoptosis in HCC PLC/PRF5 cell line, the present study was designed to investigate whether proliferation and apoptosis are altered by this compound.

## Materials and Methods

### Chemical agents

Valproic acid (2-propyl-pentanoic acid) was obtained from Sigma (St. Louis, MO) and dissolved in serum-free medium to make a stock solution which was further diluted with culture medium to yield different concentrations. Fetal bovine serum (FBS), Trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), phosphate-buffered saline (PBS), penicillin and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany).

### Cell line

Human hepatocellular carcinoma cell line (PLC/PRF5) was obtained from the National Cell Bank of Iran-Pasteur Institute and maintained as monolayers in DMEM (St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37 °C (5% CO<sub>2</sub>/air atmosphere).

### Cell viability assay

Hepatocellular carcinoma, PLC/PRF5, cells were cultured into 96-well plates at a density of  $4 \times 10^5$  cells per well. Following a 24 h culture period, the cells were treated with medium alone or with medium containing various concentrations of VPA (0, 1, 5, 10 and 20  $\mu$ M) for 24, 48 and 72 h, except control groups which treated with drug-free medium. After treatment time (24, 48 and 72 h), treated viable cells were determined by MTT assay according to the manufacturer's instructions, treated and untreated (control groups) cells were washed twice with PBS and then a fresh medium containing MTT (0.5 mg/mL) was added and finally, after 4-hour of incubation the culture media were replaced with 100  $\mu$ l of DMSO and the dye absorbance was measured spectrophotometrically at 570 nm. All experiments were repeated three times.

### Detection of cell apoptosis

The PLC/PRF5 cells,  $5 \times 10^5$  cells, were treated with 5  $\mu$ M VPA for 24, 48 and 72 h and then all the adherent cells were harvested by trypsin solution to produce a single cell suspension, which washed with cold phosphate-buffered saline (PBS) and re-suspended in Binding buffer (1x). An annexin-V-(FITC) and propidium iodide (PI, Becton-Dickinson, San Diego, CA) were used for staining according to the manufacturer's instructions. Finally the apoptotic cells were analyzed using flow cytometry.

### Statistical analysis

The database was set up with the SPSS 16.0 software

package for analysis. The data were acquired from three tests and are shown as means  $\pm$  standard deviations.

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

## Results

### Cell growth inhibition of PLC/PRF5 by VPA

To assess the effect of VPA on the growth of PLC/PRF5 cell line, the cells were exposed to various doses of the agent, as mentioned above, for 24, 48 and 72 h and then MTT assay was done. As can be seen in Figure 1, VPA had a strong growth inhibitory effect at all concentrations in a dose and time dependent manner. The effective dose of VPA that inhibited 50% growth of the PLC/PRF5 cells at 24 h was 5  $\mu$ M. The percentage of inhibition in 5  $\mu$ M VPA treated cells were approximately 52% as compared to the control group.

### Apoptotic induction of PLC/PRF5 by VPA

The results of flow cytometry assay demonstrated that VPA with concentration of 5  $\mu$ M induced apoptosis in PLC/PRF5 cells significantly versus control groups. The Percentage of apoptotic cells in VPA-treated groups were 15, 36 and 60 % after 24, 48 and 72 h respectively ( $P < 0.001$ ). Maximal Percentage of apoptotic cells were obtained after 72 h Figure. 2 and 3.

## Discussion

Histone proteins comprise the protein backbone of the chromatin structure. Histone acetyltransferases acetylate the lysine residues of histones by which diminish their ability to bind to DNA. Besides, acetylated histone inhibits higher-order structures of chromatin provided accessibility for transcription factors. HDACs compact chromatin structure by removal of acetyl groups from histone proteins, thereby prevent the transcription of genes involved in tumorigenesis (Marmorstein et al., 2007; Glozak et al., 2007; Shogren-Knaak et al., 2006). Histone deacetylase inhibitors can induce growth arrest, differentiation and apoptosis of tumor cells in vitro and

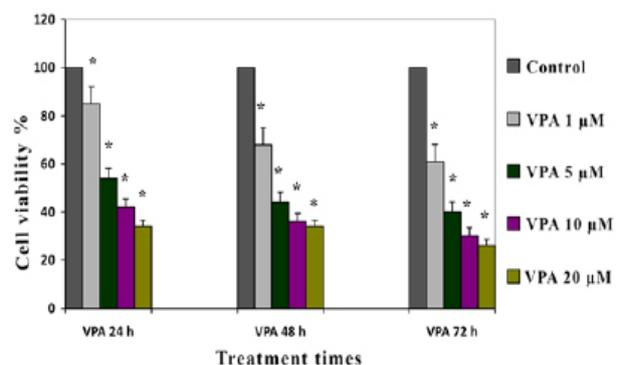


Figure 1. In Vitro Effect of VPA on PLC/PRF5 Cell Growth Assessed by MTT Assay. Values are means of three experiments in triplicate. Asterisks (\*) indicate significant differences between VPA treated groups and the control groups. \* $P < 0.001$  as compared to the control group.

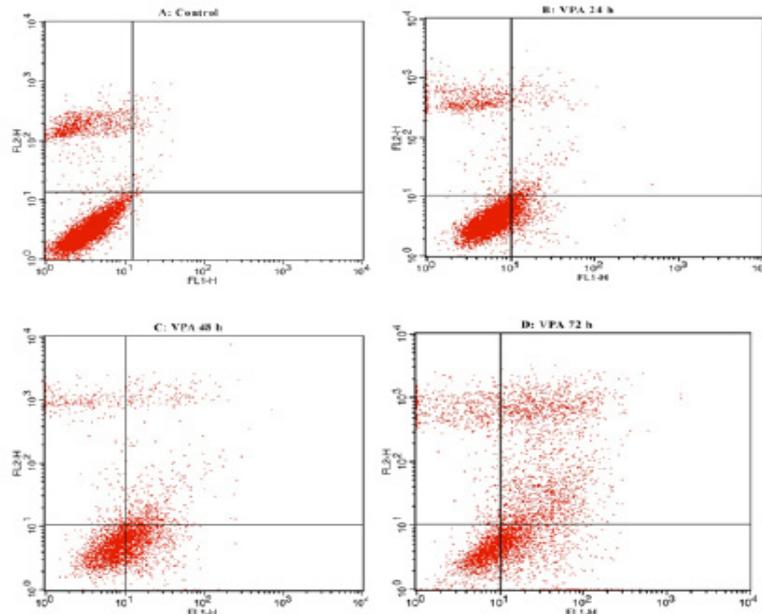


Figure 2. VPA-induced Apoptosis in PLC/PRF5 Cells after 24, 48 and 72 h of Treatment Tested by Flow Cytometric Analysis. VPA Significantly Induced Apoptosis in this Cell Line.

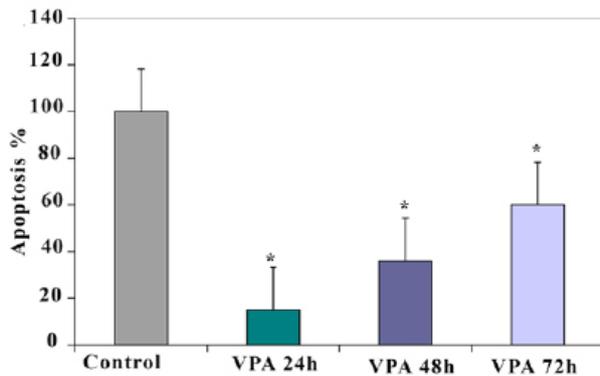


Figure 3. Apoptotic Effect of VPA on PLC/PRF5 Cells. Data are Means of Three Experiments in Triplicate. Asterisks (\*) indicate significant differences between VPA treated and the control groups. \*P < 0.001 as compared to the control group.

in vivo (Marks et al., 2000). HDAC inhibitors can be divided into several structural classes, including cyclic peptides, hydroxamates, benzamides and aliphatic acids. The aliphatic acids include phenylbutyrate, butyrate and valproic acid (Bolden et al., 2006; Dokmanovic et al., 2005; Miller et al., 2003; Rasheed et al., 2007; Xu et al., 2007).

In the present report, we studied the effect of VPA on viability and apoptosis of HCC PLC/PRF5 cells. The result demonstrated that VPA inhibited the growth and induced apoptosis of PLC/PRF5 cells in a dose and time dependent fashion. This result is consistent with previous studies that indicated VPA exerts an apoptotic and inhibitory effect on HT 29 (Sanaei et al., 2016) and HepG 2 (Sanaei et al., 2017) cell lines.

In agreement with our results, dose- and time-dependent growth inhibition and apoptosis induction of VPA on HeLa cervical cancer cells has been reported. VPA acts through various pathways. It can inhibit the activities of cytosol

and nuclear histone deacetylase (Han et al., 2013) and also activate Notch-1 signaling in human GI and pulmonary carcinoid cancer cells (Greenblatt et al., 2007). It has been shown that VPA induces apoptosis by activation of caspases-3, -8, and -9, cytochrome c release from mitochondria, DNA fragmentation and phosphatidylserine externalization (Kawagoe et al., 2002). VPA, however, activates Wnt-dependent gene expression through inhibition of HDAC following which increases the expression of  $\beta$ -catenin and de-represses Tcf/Lef (Phiel et al., 2001). Furthermore, it has been reported that VPA can act by the induction of histone (H3 and H4) hyperacetylation, restoration of p16/CDK4 pathway, activation of p21 and suppression of CMYC oncogene (Li et al., 2005). In the present study, we have not assessed apoptotic signaling pathway and molecular mechanism, suggesting that it should be evaluated in HCC PLC/PRF5 and other HCC cell lines.

In conclusion, VPA inhibits cell growth and induces apoptosis of HCC PLC/PRF5 cells in vitro, suggesting that it may be a potential agent for HCC treatment.

#### Conflict of interest

The authors report no conflict of interest.

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