Experimed 2024; 14(3): 154-160

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1500633

Evaluation of the Molecular Effects of the Anticancer Adjuvant Valproic Acid on HEK293T Cells

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Cite this article as: Olgun Celebioglu HB, Ekici Y, Yilmaz A, Tuncer FN. Evaluation of the molecular effects of the anticancer adjuvant valproic acid on HEK293T cells. Experimed 2024; 14(3): 154-160.

ABSTRACT

Objective: Valproic acid (VPA) can induce apoptosis while inhibiting proliferation in various cancer cells. This study adopted a novel strategy for investigating the molecular effects of VPA on non-cancerous cells. Building upon our previous work, which examined the effects of VPA in PANC-1 cells, we now turn our attention to HEK293T cells to determine the effect of VPA in non-cancerous cells.

Materials and Methods: HEK293T cells were treated with 2.5 and 5-mM VPA. Flow cytometry analyses were performed on the 24, 48, 72, and 96 h. Assays for apoptosis and proliferation were conducted using annexin V/ propidium iodide (PI) staining and carboxyfluorescein-succinimidyl ester (CFSE) dilution, respectively.

Results: No statistical significance was detected between the two different doses of VPA-treated cells and the controls at any time point. While early apoptosis values remained stable until 72 h in all groups, an increase was monitored in cells subjected to VPA for 96 h.

Conclusion: Preliminary results indicated no proliferative effect of VPA treatment. However, it may induce apoptosis in long-term incubations. Nevertheless, additional doses of VPA at increased concentrations should be administered to explore cytotoxic levels and their impact on proliferation and apoptosis.

Keywords: HEK293T, VPA, apoptosis, proliferation

INTRODUCTION

Multicellular organisms maintain a delicate balance between cell proliferation, differentiation, and death, which is crucial for sustainable tissue homeostasis (1). Dysregulation of the cell cycle machinery leads to malignant phenotypes through uncontrolled cell proliferation and suppressed cell death (2). Hence, malfunctions, mutations, or inactivations in these systems frequently lead to cancer development.

Additionally, epigenetic mechanisms have essential roles in maintaining normal growth and development through modulating gene expression machinery, particularly in the methylation of DNA, modifications of histones, and expression of miRNA, together with the regulation of the cell cycle and apoptosis (3). Several enzymes are responsible for these epigenetic alterations, such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs), and DNA methyltransferases (DNMTs) (4). Cancer is an aggressive malignancy caused by abnormal and uncontrolled cell division, in which gene expression changes, impaired apoptosis, and proliferation dynamics are increased along with cell cycle disruptions (5). DNMT and HDAC inhibitors are widely under investigation for their potential use as epigenetic drugs with significant attention (3).

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Submitted: 13.06.2024 **Revision Requested:** 12.10.2024 **Last Revision Received:** 21.10.2024 **Accepted:** 25.10.2024 **Published Online:** 29.11.2024

Hence, DNMT inhibitors are promising agents for epigenetic drug development. Many genes are mutated or abnormally activated during the development of cancer, and impaired HDAC levels were reported to repress tumor suppressor genes (TSGs) along with the induction of tumorigenesis (4, 6). Eventually, several HDAC inhibitors have been used in the field of cancer treatment with increasing attention. Target-specific inhibitors without substantial side effects are required for effective anticancer treatment (3).

Fatty acid valproic acid (2-propylvaleric acid, VPA) has been frequently used for treating several neuropathies, such as migraine, bipolar disorder, and epilepsy, for decades (7, 8). This anticonvulsant drug functions as an HDAC inhibitor that alters gene expression in many crucial cellular processes (9). VPA inhibition on HDACs influences cell survival by enhancing cellular growth and differentiation, while inhibiting apoptosis and inflammation (10). Hence, the effects of VPA on HDAC inhibition have become widely preferred in cancer therapies because of elevated expression of HDACs in tumor cells and its cost-effective features (11). Combined with conventional treatment methods, molecular-targeted therapies have gained enormous attention as anticancer strategies. Consequently, this synergistic therapeutic approach has been shown to suppress tumor proliferation and metastasis in numerous cancer types (6). Although numerous experimental studies have investigated the effects and benefits of non-toxic VPA on cancer cells, little is known about the molecular response of VPA on healthy human cells compared with cancer cells. With this aim in mind, we used the VPA-treated HEK293T cell line as a model for healthy cells to observe the effects of VPA treatment on non-cancerous cells.

MATERIALS AND METHODS

Cell Culture

The human embryonic epithelial kidney (HEK293T) cell line was collected from the American Type Culture Collection (ATCC®CRL-3216™; Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Medium with high glucose (DMEM, Cat. No: 2LM-D1110, Biosera, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Cat. No:1027, GIBCO, Thermo Fisher Scientific, USA), and 0.5% streptomycin (Sigma Chemicals, USA) at 37ºC under 5% CO_2. Cell detaching was conducted using 0.25% Trypsin–EDTA (Cat. No: LM-T1720, Biosera, France). Optimized cell densities and IC_{50} values of VPA concentrations for flow cytometry measurements were obtained from a recently published study by Ekici et al. (12) as $1x10^5$ cells/well on 24-well plates treated with 0, 2.5, or 5 mM of VPA. This study aimed to assess the impact of various VPA doses on two distinct cell concentrations to determine the optimal IC_{50} value of the agent. Two different cell quantities ($1x10^5$ and $5x10^5$ cells/well) were cultured in 24-well plates. Consequently, VPA concentrations of 0.25, 0.5, 1, 2.5, and 5 mM/mL were administered. The apoptotic and proliferative dynamics of the cells were evaluated using flow cytometry over the subsequent 4 days. In conclusion, no discrepancies were observed in cell quantities, and the effective IC_{50} was determined to be 2.5 mM/mL (12). In addition, cells were treated with 1 mL of DMEM containing different concentrations of VPA (P4543, Sigma Chemicals, USA) of 0,

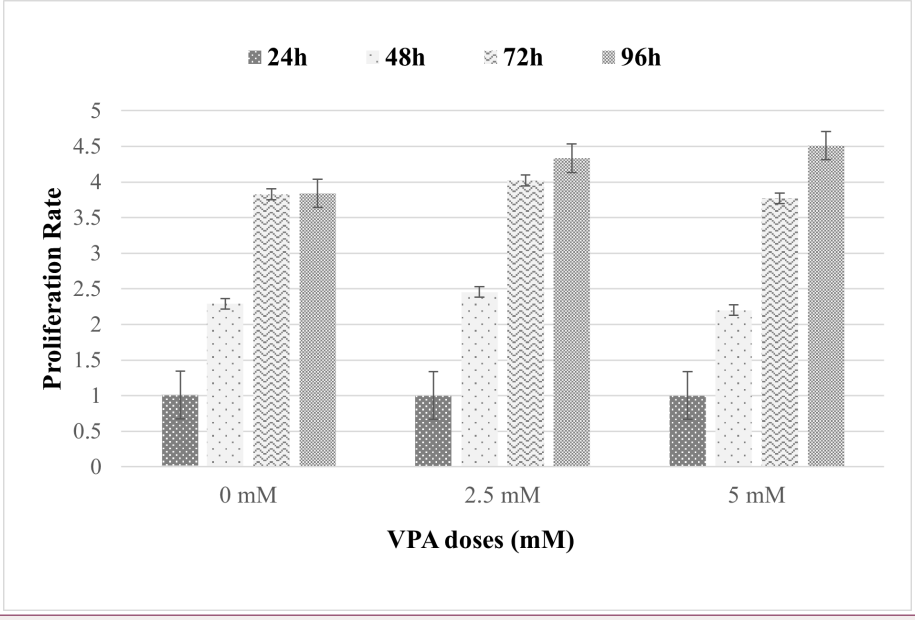


Figure 1. Cell proliferation results.
The average measurements from duplicate experiments are displayed for cells treated with 2.5 or 5 mM of VPA and the control (0 mM) groups at 24, 48, 72, or 96 h. The calculated results indicate the variance in the number of cell doublings, which were normalized to the number of untreated control cells (0 mM VPA). When VPA-treated cells (2.5 and 5 mM) were compared with untreated cells, Tukey's multiple comparison tests did not reveal any significance for both concentrations.

Table 1. The calculations of cell proliferation ascertained by flow cytometry measurements

Time (h)	VPA concentrations		
	0 mM	2.5 mM	5 mM
24	1	1	1
48	2.29 ± 0.08	2.45 ± 0.08	2.20 ± 0.05
72	3.82 ± 0.04	4.02 ± 0.09	3.77 ± 0.03
96	3.84 ± 0.30	4.33 ± 0.25	4.51 ± 0.34

The average data from two independent proliferation experiments are presented as time (hours) versus VPA concentration (millimolar, mM). The calculated results indicate the variance in the number of cell doublings, which were normalized to the number of untreated control cells (0 mM VPA). The variance in cell doublings between VPA-induced and control cells was determined using the *2n=Fcontrol/FVPA; n=ln(Fcontrol/FVPA)/ln2* formula, wherein F represents the detected CFSE fluorescence intensity and n is the deviation in cell division numbers (14, 15). Values are expressed as percentage (means ± SD).

2.5, and 5 mM to perform proliferation and apoptosis experiments. Subsequently, the proliferative and apoptotic activities of cells were measured at the 24, 48, 72, and 96 hours. The untreated cell group was utilized as a negative control for each VPA dose and duration. Carboxyfluorescein-succinimidyl ester (CFSE, 5 µM final concentration) and annexin V/ propidium iodide (PI) stains were applied to all groups of cells prior to flow cytometry measurements. Moreover, $1x10^5$ unstained cells/well were cultured in the absence of VPA , which were used as negative controls. Flow cytometric analysis was performed using a NovoCyte flow cytometry (Agilent, USA).

Cell Proliferation

Proliferation measurements followed subsequent staining of cells with CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Cell culturing, monitoring, and calculation protocols were used, as described by Ekici et al. (12). All experiments were performed in duplicates.

Cell Apoptosis

"Dead Cell Apoptosis Kit with Annexin V-fluorescein isothiocyanate (FITC) and PI, for flow cytometry (Thermo Fisher Scientific, USA)" kit was used, which allows the determination of early and late apoptosis and the differentiation of apoptosis from necrosis (13). Apoptosis measurement protocols were followed, as described by Ekici et al. (12). All experiments were performed in duplicates following the manufacturer's instructions.

Statistical Analyses

All data obtained from proliferation and apoptosis assays were statistically analyzed using two-way analysis of variance (ANOVA), followed by post hoc Tukey multiple comparisons. The Student's t-test was used to analyze the variability in the percentage of apoptosis between various time points. When $p \leq 0.05$, the data was deemed significant and presented as means ± standard deviation (SD). All statistical calculations were performed using GraphPad Prism Software.

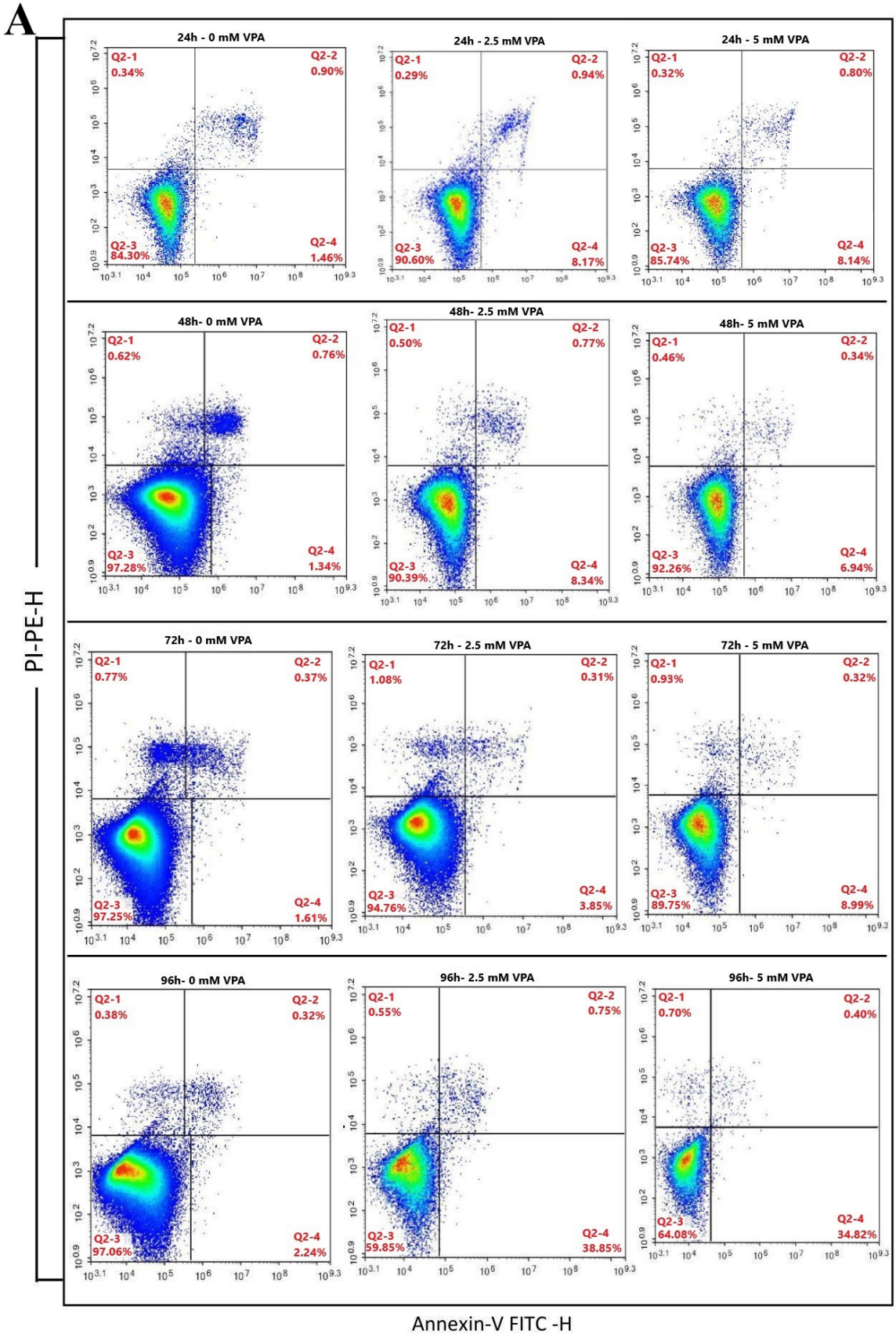
RESULTS

Cell Proliferation Results

The study evaluated the proliferative effects of different VPA doses on cells compared with the untreated control group. When the response of cells was considered in terms of increasing doses of VPA over the same period, the reactions of cells did not differ significantly. When two different VPA concentrations (2.5 and 5 mM) were compared with untreated cells, Tukey's multiple comparison tests did not reveal any significance for both concentrations ($p=0.4184$) (Figure 1). The difference in cell doublings between VPA-induced cells and control cells was determined by utilizing the *2n=Fcontrol/FVPA; n=ln(Fcontrol/FVPA)/ln2* formula, wherein F represents the detected CFSE mean fluorescence intensity and n is the number of cell divisions (14, 15). Table 1 presents the proliferation findings of cells from flow cytometry analyses.

Cell Apoptosis Results

In the scope of this study, the effects of different VPA doses at various time intervals on cell apoptosis and necrosis were evaluated. According to the dot plots of the log data obtained from flow cytometry measurements, the percentages of live, early apoptotic, late apoptotic, and necrotic cells at all time intervals are presented in Table 2. Following these results, the early apoptotic features of all groups remained stable for up to 72 h; an increase was observed at the 96th in VPA-treated


A
24h - 0 mM VPA
Q2-1 0.34%
Q2-2 0.90%
Q2-3 84.30%
Q2-4 1.46%
24h - 2.5 mM VPA
Q2-1 0.29%
Q2-2 0.94%
Q2-3 90.60%
Q2-4 8.17%
24h - 5 mM VPA
Q2-1 0.32%
Q2-2 0.80%
Q2-3 85.74%
Q2-4 8.14%
48h- 0 mM VPA
Q2-1 0.62%
Q2-2 0.76%
Q2-3 97.28%
Q2-4 1.34%
48h- 2.5 mM VPA
Q2-1 0.50%
Q2-2 0.77%
Q2-3 90.39%
Q2-4 8.34%
48h- 5 mM VPA
Q2-1 0.46%
Q2-2 0.34%
Q2-3 92.26%
Q2-4 6.94%
72h - 0 mM VPA
Q2-1 0.77%
Q2-2 0.37%
Q2-3 97.25%
Q2-4 1.61%
72h - 2.5 mM VPA
Q2-1 1.08%
Q2-2 0.31%
Q2-3 94.76%
Q2-4 3.85%
72h - 5 mM VPA
Q2-1 0.93%
Q2-2 0.32%
Q2-3 89.75%
Q2-4 8.99%
96h- 0 mM VPA
Q2-1 0.38%
Q2-2 0.32%
Q2-3 97.06%
Q2-4 2.24%
96h- 2.5 mM VPA
Q2-1 0.55%
Q2-2 0.75%
Q2-3 59.85%
Q2-4 38.85%
96h- 5 mM VPA
Q2-1 0.70%
Q2-2 0.40%
Q2-3 64.08%
Q2-4 34.82%
PI-PE-H
Annexin-V FITC -H

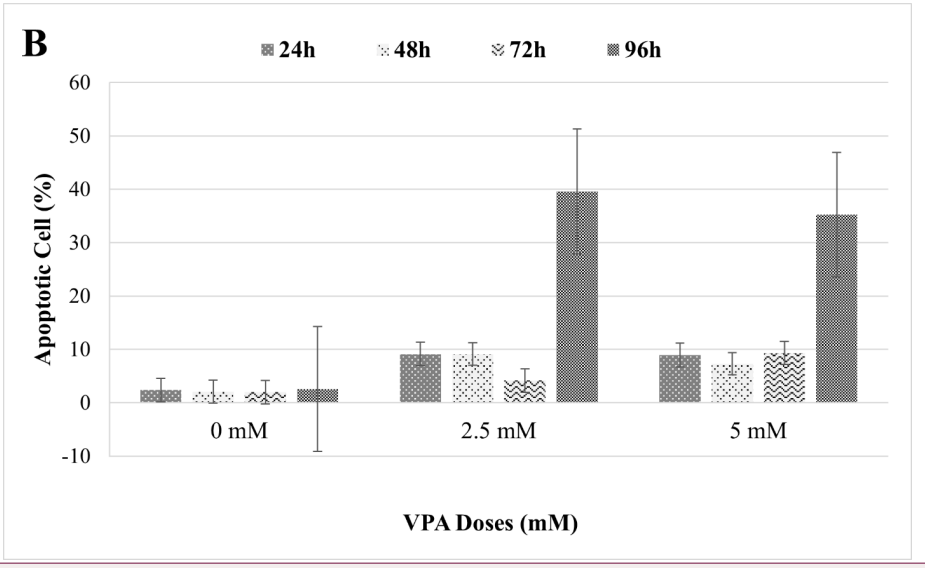


Figure 2. Cell apoptosis results.
The measurements are illustrated from a single experiment for the cells subjected to 2.5 mM and 5 mM VPA and the control (0 mM) groups at the 24, 48, 72, and 96h. A) Representative results of flow cytometric dot plots of cells for Annexin V-PI counterstain. Untreated control cells (on the left panels) and VPA-treated cells (2.5 mM VPA in the middle and 5 mM VPA on the right panels) are exhibited. In the lower left area (Q2-3), live cells are displayed, whereas the lower right (Q2-4) denotes early apoptosis. The upper right (Q2-3) shows late apoptosis, and the upper left (Q2-1) shows necrotic/dead cells. Cells were gated using untreated cells for subsequent analyses. The scatter plot illustrates the apoptosis percentages of cells treated with 2.5 mM and 5 mM VPA and the control groups across three independent experiments. B) The total (early and late) apoptotic cell percentages were compared between the untreated and VPA-treated groups. Over time, the percentage of apoptosis in cells treated with VPA exhibited a significant increase compared to the control groups. The experiments were conducted in independent duplicates. The error bars represent the +/– standard error of the means of replicates.

cells ($p<0.0001$) (Figure 2). VPA treatment seems to cause a significant increase in the early apoptotic cell numbers compared with the control cells.

DISCUSSION

Cancer is a leading cause of death worldwide (9). Clinical studies have demonstrated that the combination of targeted molecular agents with conventional chemotherapy effectively enhances the inhibition of tumor growth and metastasis in patients with cancer. Hence, VPA is a noteworthy adjuvant drug due to its well-documented adverse effects, which are dose-dependent and reversible in severe toxicity (6, 7).

The utilization of VPA in clinical settings for various malignancies is advancing; however, the specific effects of these HDAC inhibitors in normal non-cancerous cells and the molecular responses elicited by them are limitedly understood (16). Stapnes et al. investigated four different HDAC inhibitors, including VPA on $CD34^+$ acute myeloid leukemia (AML) cells, normal bone marrow $CD34^+$ cells, and acute lymphoblastic leukemia (ALL) blasts, in terms of proliferation and viability (17). Their results revealed that normal $CD34^+$ hematopoietic cells differed from cancerous cells, as they showed growth enhancement at the highest VPA concentrations with no antiproliferative effects. It can be implied that this difference might be caused by the drug's effects on specific cell populations associated with leukemia but not normal cells (17). In accordance with these findings, our study revealed parallel results; when examining the impact of varying doses of VPA over time, cell proliferation demonstrated a significant linear increase for up to three days following administration. VPA administration did not affect the proliferation kinetics of HEK293T cells, even at varying concentrations. The results indicate that there were consistent outcomes between the experimental and control groups for a period of up to three days. We previously demonstrated that VPA inhibits the proliferation of pancreatic ductal carcinoma, a highly fatal type of cancer. These findings suggest that VPA operates through different mechanisms in healthy cells compared with cancer cells, potentially offering insights into targeted therapeutic strategies (12).

The primary objective of cancer treatment is to prevent cancer cells from replicating by destroying DNA signals or inflammation, both of which lead to apoptosis. However, while achieving this, minimizing damage to healthy cells is as essential as destroying existing cancer cells. Our findings showed that the early apoptotic features remained stable for up to 72 h in the 2.5 and 5 mM VPA and control groups. However, an increase was observed in VPA-treated cells at the 96^{th} hour ($p<0.001$). A study on epilepsy indicated that a

Table 2. Representative data of flow cytometry analysis of cells stained with Annexin V-FITC and propidium iodide

Time (h)	VPA concentration (mM)	Live Cells (%)	Early Apoptotic Cells (%)	Late Apoptotic Cells (%)	Necrotic Cells (%)
24	0 mM	84.30	1.46	0.90	0.34
	2.5 mM	90.60	8.17	0.94	0.29
	5 mM	85.74	8.14	0.80	0.32
48	0 mM	97.28	1.34	0.76	0.62
	2.5 mM	90.39	8.34	0.77	0.50
	5 mM	92.26	6.94	0.34	0.46
72	0 mM	97.25	1.61	0.37	0.77
	2.5 mM	94.76	3.85	0.31	1.08
	5 mM	89.75	8.99	0.32	0.93
96	0 mM	97.06	2.24	0.32	0.38
	2.5 mM	59.85	38.85	0.75	0.55
	5 mM	64.08	34.82	0.40	0.70

Percentage data from a single experiment for cells subjected to 2.5 mM and 5 mM VPA and control (0 mM) groups at the 24, 48, 72, and 96. The Dead Cell Apoptosis Kit with Annexin V-FITC and PI was used for flow cytometry analysis. Detailed data represent percentage means of live, early apoptotic, late apoptotic, and necrotic cell numbers at all time intervals, obtained from dot plots of cells for Annexin V-PI counterstain, as presented in Figure 2.

clinical dose of 1 mM VPA is considered safe as an antiepileptic medication, whereas 2.7 mM VPA results in low toxicity with benign side effects. Nevertheless, a dosage of 5.1 mM can lead to severe and life-threatening effects (18). When the results of this study are combined with our own findings, VPA increases cell damage over time at doses exceeding 2.5 mM. In our previous study, the IC_{50} value of VPA in PANC-1 cells was 2.5 mM. At this dose, we observed the onset of early apoptosis at 48 h (12). These results suggest that further research is needed at concentrations between 1 and 2.5 mM to determine the effective dose. This approach may help prevent toxic effects that cause physiological deterioration in healthy cells.

Acknowledgments: The findings of this study were presented as an e-poster at the Medical Biology and Genetics Society Conference 2021.

Ethics Committee Approval: Only cell culture material was used in this study and no living material that would require ethics committee approval was used.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study – H.B.O.C., F.N.T.; Data Acquisition – H.B.O.C., Y.E., A.Y., F.N.T.; Data Analysis/Interpretation - H.B.O.C., Y.E., F.N.T.; Drafting Manuscript – H.B.O.C., Y.E., F.N.T.; Critical Revision of Manuscript – H.B.O.C., Y.E., F.N.T.; Final Approval and Accountability – H.B.O.C., Y.E., F.N.T.

Conflict of Interest: The authors declare no conflict of interest.

Financial Disclosure: This study is granted by the Scientific Research Projects Coordination Unit of Istanbul University (TDP-2019-32763).

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