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Effect of valproic acid on extrinsic and intrinsic apoptotic pathways, cell viability and apoptosis in hepatocellular carcinoma PLC/PRF5 cell line

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ABSTRACT

Inhibitors of histone deacetylase enzymes induce various molecular and extracellular effects that lead to their anticancer role. In this study, the result of valproic acid on the expression of genes of extrinsic pathway and intrinsic pathway of apoptosis, cell viability, and apoptosis in liver cancer PLC/PRF5 cell line was designed. For this purpose, PLC/PRF5 liver cancer cells were cultured, and after the overlapping of the cells reached about 80%, the cells were collected with trypsin and after washing, they were cultured on a plate with a concentration of 3 x 10^5. After 24 hours, the culture medium was treated with a medium containing valproic acid (the control group received only DMSO). 24, 48, and 72 hours after treatment to determine the cell viability, apoptotic cells, gene expression, MTT, flow cytometry, and Real-time techniques. The results showed that valproic acid significantly inhibited cell growth, induced apoptosis, and decreased the expression of Bcl-2 and Bcl-xL genes. Also, it increased the expression of DR4, DR5, FAS, FAS-L, TRAIL, BAX, BAK, and APAF1 genes. In general, valproic acid seems to play its apoptotic role through intrinsic and extrinsic pathways in liver cancer.

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Introduction

Histone acetylation and deacetylation play an essential role in transcription and gene expression (1). The acetylation status of histone and non-histone proteins is determined by histone acetyl-transferases (HATs) and histone deacetylases (HDACs), respectively (2). Histone acetyltransferase enzymes add acetyl groups to lysine, and histone deacetylase enzymes do the opposite. Histone acetylation causes the chromatin structure to open, and as a result, the transcription of genes is activated (3, 4).

Inhibitors of histone deacetylase enzymes induce various molecular and extracellular effects that lead to their anticancer role. These compounds can initiate various biological responses that lead to inhibiting the cell growth of cancer cells (5, 6). These drugs can cause apoptosis in cancer cells in two different ways, including internal or intrinsic/mitochondrial or receptor death. These drugs selectively change gene expression by changing the structure of DNA and by changing the proteins involved in transcription (7). As mentioned, drugs play their role by inhibiting histone deacetylase enzymes by increasing the expression of the genes of the internal pathway of cell apoptosis (APAF1, BAX, and BAK) and the external pathway of apoptosis (FAS-L, and TNF-α, TRAIL, DR5, FAS) and decreasing the expression Anti-apoptotic genes (7, 8).

There are natural and artificial histone deacetylase inhibitor compounds that target different groups of histone deacetylase enzymes. Several groups of these compounds have been identified, including hydroxamic organic acids, Benzamides such as MS-275, short-chain fatty acids such as valproic acid (VPA), and Cyclic tetrapeptides (9, 10). It has been reported that histone deacetylase inhibitory compounds (VPA, SAHA, and TSA) can induce apoptosis by modulating apoptosis regulators such as 1-Bcl2, BcIXL, Mcl BcLXs, Apaf-1, NOXA, TRAIL-R1, TRAIL-R2, caspase-8. In addition, TSA induces apoptosis in colon cancer cell line HT116 cells HT29 by increasing the expression of pro-apoptotic proteins such as Bax and decreasing the expression of anti-apoptotic proteins such as Bc1-2 and Bcl-xL (10). Laboratory research has shown that TSA stops pancreatic cancer cells in the G1-M phase by reducing the expression of Bc1-2, Mcl-1, and Bcl-XL genes. Several studies have shown that most hepatocellular liver cancers (carcinoma, HCCs) express the death receptor gene (Fas) and its ligand (FasL) (11, 12).

Studies have shown that histone deacetylase inhibitor drugs inhibit cell growth and cause apoptosis in HeG2 and SMMC-772 liver cancer cells by activating the intracellular (mitochondrial) pathway (3, 12, 13). Research has shown valproic acid induces apoptosis in HepG2 liver cancer cells by activating intracellular and extracellular apoptosis pathways. SAHA drug causes apoptosis in HepG2, HepG3B, and SK-Hep1 liver cancer cells by increasing the expression of DR5 and TRAIL and activating caspase-8, which are components of the extracellular apoptosis pathway (14).

The effect of drugs that inhibit histone deacetylase enzymes is dose-dependent, time-dependent, and cell-dependent, and the result is also reported. These compounds on some liver cancer cell lines cannot be generalized to other lines, and there is no report about the effect of the valproic

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acid drug on the intracellular and extracellular apoptosis pathway in the PLC/PRF5 cell line (15, 16). Therefore, in this research, we investigated the effect of the valproic acid drug on the expression of genes and cell viability and apoptosis rate of external pathways, including DR4, DR5, FAS, FAS-L, TRAIL, and internal pathways of BAX, BAK APAF1, Bcl-2, and Bcl-xL, in liver cancer of PLC/PRF5 cell line.

Materials and Methods

PLC/PRF 5 liver cancer cells were purchased from Charles River Cell Bank (Virginia, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) solution enriched with fetal bovine serum, FBS, and containing antibiotics, including penicillin. We kept g sodium, streptomycin (streptomycin sulfate), and amphotericin B at 37 degrees Celsius. We purchased valproic acid medicine and other necessary materials such as trypsin, trypsin-EDTA culture medium, DMEM, Annexin-V, propidium iodide (PI), and gene expression kit, including Total RNA extraction kit (TRIZOL reagent) and Real-time polymerase chain reaction kits from Sigma.

Determination of cell viability by MTT assay

First, liver cancer cells were cultured, and after the overlap of the cells reached about 80%, the cells were collected with trypsin, and after washing, they were cultured in 96-well plates with a concentration of 3x105. After 24 hours, the culture medium was replaced with the medium containing the drug valproic acid with different concentrations (0.1, 5, 10, and 20 µM). The cells were treated with this drug (the control group received only DMSO). Also, 24, 48, and 72 hours after treatment, the cells of the experimental and control groups were washed with PBS, and then MTT solution was added for 4 hours. After 4 hours, the colored crystals created were dissolved with DMSO, and the amount of color produced was checked with a wavelength of 570. For more certainty, each experiment was repeated three times.

Determination of apoptotic cells by flow cytometry assay

To determine the amount of cell apoptosis, liver cancer cells were cultured in 24 plates with a concentration of 3x105. After 24 hours, the culture solution was replaced with a solution containing valproic acid with a concentration of 5 µM (except for the control group that received DMSO). After 24, 48, and 72 hours of treatment, all cells were collected using trypsin. They were washed with PBS solution and stained with annexin and propidium iodide, and apoptotic cells were counted with a FACScanTM flow cytometer.

Determination of gene expression by real-time quantitative reverse transcription polymerase chain reaction (Real-time RT-PCR)

To determine the gene expression level, liver cancer cells were cultured in 24 plates with a concentration of 3 x 105. After 24 hours, the culture solution was replaced with a solution containing the drug valproic acid with a concentration of 5 µM (except for the control group that received DMSO). After 24, 48, and 72 hours of treatment, all RNA cells were extracted by RNeasy mini kit according to the relevant protocol and to remove genomic DNA. The obtained RNA was treated with an RNase-free DNase kit. RNA concentration was determined using a BioPhotometer.

Using the RevertAid™ First Strand cDNA Synthesis Kit, cDNA was made from the resulting RNA, and finally, Real-time RT-PCR was performed using Cybergreen. The GAPDH gene was used as a control. The sequence of the primers used is specified in Table 1.

Table1. Sequence of studied primers and related references.

Gene Name		Primer Sequence (5'-3')	Product Length	Reference
BAK	Forward	GCCCAGGACACAGAGGAGGTTTTC	528	(17)
	Reverse	AAACTGGCCCAACAGAACCACACC		
FAS-L	Forward	GGCCCATTTAACAGGCAAGT	73	(18)
	Reverse	CAGGACAATTCCATAGGTGTCTTC		
FAS	Forward	AGGACATGGCTTAGAAGTGGAAATA	80	(18)
	Reverse	TACAAAAAAAGTTTGGTTTACATCTGC		
DR5	Forward	CTCGAGGTCCTGCTGTTGGTGAGT	71	(19)
	Reverse	GAGCTCGGGAATTTACACCAAGTGGAG		
DR4	Forward	CTCGAGAAGTTTGTCGTCGTCGGGGT	70	(19)
	Reverse	GAG-CTCCCGTTCTTCCTCCGACTC		
TRAIL	Forward	CCAGAGGAAGAAGCAACACATTG	96	(20)
	Reverse	GCCCACTCCTTGATGATTCCC		
APAF1	Forward	AAAAGGGGATAGAACCAGAGGTGG	68	(21)
	Reverse	TGCGGCACCTCAAGTCTTC		
Bcl-2	Forward	TGTTGTTCAAACGGGATTCA	136	(22)
	Reverse	GGCTGGGCACATTTACTGTT		
Bcl-xL	Forward	GTTCCCTTTCCTTCCATCC	123	(23)
	Reverse	TAGCCAGTCCAGAGGTGAG		
GAPDH	Forward	GAGTCAACGGATTTGGTCGT	131	(22)
	Reverse	GACAAGCTTCCCGTTCTCAG		

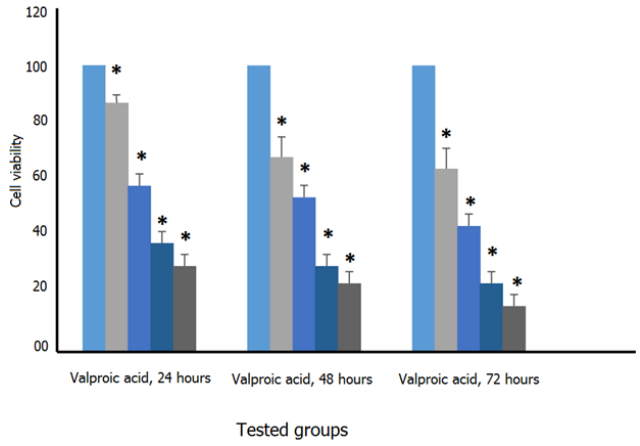

Figure 1. The result of determining the living cells in liver cancer PLC/PRF5 cell line that were treated with valproic acid drug with different concentrations (0.1, 5, 10, and 20 µM). The asterisk (*) indicates a significant difference between the drug-treated and control groups (P<0.001). In each group, from left to right, the first bar corresponds to the control group, and the other bars correspond to the groups treated with valproic acid with concentrations of 0.1, 5, 10, and 20 µM.

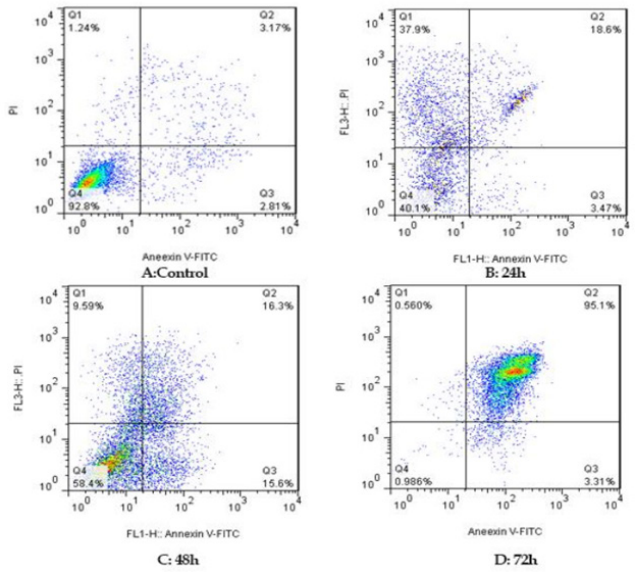

Figure 2. The result of determining apoptotic cells in the liver cancer of PLC/PRF5 cell line was treated with valproic acid drug with a 5µM. The asterisk indicates a significant difference between the drug-treated and control groups (p<0.001).

Results

The result of determining the number of living cells

Liver cancer cells were treated with valproic acid drug with different concentrations (10, 5, 1.0, and 20 µM) for 24, 48, and 72 hours and then the number of living cells was determined by the MTT technique. As shown in Figure 1, valproic acid with all concentrations used was able to significantly inhibit cell growth in a dose- and time-dependent manner (P<0.001). The adequate amount of this compound, which could inhibit the growth of 50% of cells, was 5 µM.

The result of the determination of cell apoptosis

To determine apoptotic cells, liver cancer cells were treated with valproic acid (5 µM) for 48, 24, and 72 hours. Then, the number of apoptotic cells was determined using annexin and propidium iodide staining (Figure 2). As shown in Figure 3, this combination significantly induced apoptosis time-dependent (P<0.001). The percentage of apoptotic cells (after 24, 48 and 72 hours) were 22.07, 31.9 and 98.32, respectively (p<0.001). The maximum amount of apoptosis was observed after 72 hours.

The result of determining gene expression

The result of determining gene expression showed that valproic acid (5µM) could significantly decrease the expression of Bcl-2 and Bcl-xL genes, and increase the expression of TRAIL, DR4, DR5, FAS, FAS-L, BAX, BAK and APAF1 (Figures 4 and 5). The expression levels of these genes are specified in Tables 2 and 3.

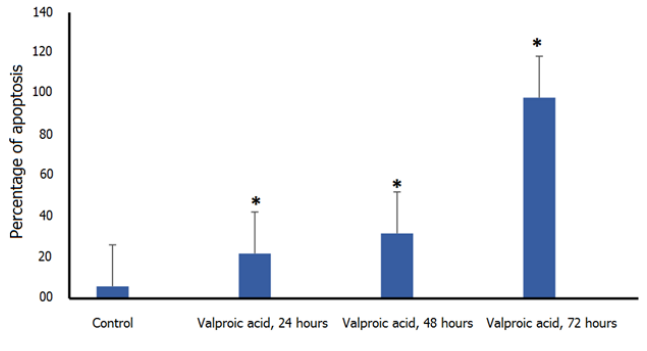

Figure 3. Apoptotic effect of the valproic acid drug with a concentration of 5µM after 24, 48, and 72 hours of treatment; the asterisk (*) indicates a significant difference between the experimental and control groups (p<0.001).

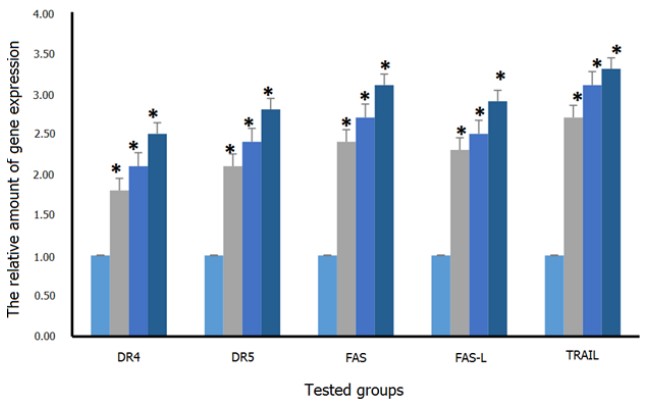

Figure 4. The result of determining the expression of extrinsic pathway genes (DR4, DR5, FAS, FAS-L, TRAIL) in PLC/PRF5 cell line liver cancer treated with valproic acid. The asterisk (*) indicates a significant difference between the drug-treated and control groups. In each group, from left to right, the first bar corresponds to the control group, and the other bars correspond to the groups treated with valproic acid for 48, 24, and 72 hours, respectively.

Discussion

Cancer is the uncontrolled proliferation of cells that, due to various genetic and epigenetic reasons, cell division goes out of control, and the division process becomes cancerous (23-25). Among the epigenetic factors that cause cancer is the silencing of tumor suppressor genes, TSGs. Among the factors that cause the silencing of these genes

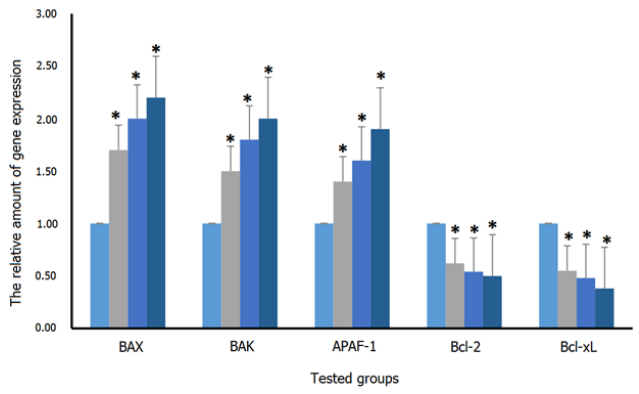

Figure 5. The result of determining the expression of BAX, BAK, APAF1, Bcl-2, and Bcl-xL internal pathway genes in PLC/PRF5 cell line liver cancer treated with valproic acid. The asterisk (*) indicates a significant difference between the drug-treated and control groups. In each group, from left to right, the first bar corresponds to the control group, and the other columns correspond to the groups treated with valproic acid for 24, 48, and 72 hours.

are promoter region methylation and histone deacetylation. Histone deacetylase enzymes play a pivotal role in gene expression through the modification of chromatin structure (26, 27). The activity of these enzymes causes the removal of the acetyl group from the histone lysine and, as a result, the condensation of DNA, which in turn reduces the access of transcription factors to DNA and causes gene silencing. This process induces cancer in cancer-suppressing genes (27). Therefore, inhibitors of histone deacetylase enzymes can be the target of cancer treatment. By inhibiting the activity of histone deacetylase enzymes, these compounds cause the re-expression of cancer-suppressing genes and induce apoptosis (23).

It has been reported that histone deacetylase enzymes can cause apoptosis of cancer cells through two external (cytoplasmic) and internal (mitochondrial) pathways. Some of the regulators of these two pathways are Bcl-2, BclXL, Mcl-1, BclXs, Apaf-1, NOXA, TRAIL-R1, TRAIL-R2, and caspase-8 genes (28, 29). The present research showed that valproic acid could induce apopto-

Table2. The effect of valproic acid (5 µM) on the expression of extrinsic pathway genes of cell apoptosis in PLC/PRF5 cell line.

Gene	Duration (h)	Relative gene expression level	P-value
DR4	24	1.8	0.001
DR4	48	2.1	0.001
DR4	72	2.5	0.001
DR5	24	2.1	0.001
DR5	48	2.4	0.001
DR5	72	2.8	0.001
FAS	24	2.4	0.001
FAS	48	2.7	0.001
FAS	72	3.1	0.001
FAS-L	24	2.3	0.001
FAS-L	48	2.5	0.001
FAS-L	72	2.9	0.001
TRAIL	24	2.7	0.001
TRAIL	48	3.1	0.001
TRAIL	72	3.3	0.001

Table3. The effect of valproic acid (5 µM) on the expression of internal pathway genes of cell apoptosis in PLC/PRF5 cell line.

Gene	Duration (h)	Relative gene expression level	P-value
BAX	24	1.7	0.001
BAX	48	2	0.001
BAX	72	2.2	0.001
BAK	24	1.5	0.001
BAK	48	1.8	0.001
BAK	72	2	0.001
APAF1	24	1.4	0.005
APAF1	48	1.6	0.001
APAF1	72	1.9	0.001
Bcl-2	24	0.62	0.001
Bcl-2	48	0.54	0.001
Bcl-2	72	0.5	0.001
Bcl-xL	24	0.55	0.001
Bcl-xL	48	0.48	0.001
Bcl-xL	72	0.38	0.001

sis and inhibit cell growth in PLC/PRF5 type liver cancer cells from both external and internal pathways. In the external path, this drug increased the expression of internal genes in DR4, DR5, FAS, FAS-L, and TRAIL pathways, increased the expression of BAX, BAK, and APAF1 genes, and decreased the expression of Bcl-2 and Bcl-xL genes.

The activation of these two pathways inhibited cell growth and apoptosis in liver cancer cells. Similar to this result, other researchers have also reported (29). Laboratory studies have shown that VPA causes apoptosis in BHT101 and N-PA thyroid cancer cells through the internal pathway (7). It has been reported that this compound induces apoptosis in head and neck cancer cells (HNSCC) by activating both paths. In liver cancer, it has been reported that this compound induces apoptosis by increasing the expression of DR4 and DR5 and decreasing the expression of Bcl2 and Bcl-xl. This means that this combination uses both internal and external routes (30).

Similar molecular pathways have been reported for trichostatin-A drug (from other histone deacetylase inhibitor family drugs) (31). This drug causes apoptosis in blood cancer cells by activating internal and external pathways. Other studies have reported that trichostatin-A and sodium butyrate cause apoptosis in glioma cancer cells by increasing the expression of Bad and decreasing the expression of BCL-2 and BCL-XL. Sodium butyrate drug causes apoptosis in pancreatic cancer cells by reducing the expression of Bcl-2 and Bcl-Xl (18).

In HT-29 colon cancer cells, this compound causes apoptosis by activating the internal pathway (32). All the cases reported by other researchers mentioned above confirm the report of this work (29, 31). In addition to the above routes, different routes have also been mentioned for valproic acid. Previously, it was reported that this combination causes the re-expression of kinase-dependent cyclin inhibitors such as P27, P21, and P27 in colon cancer (33). It has also been reported that trichostatin-A induces apoptosis in the HCT116 colon cancer cell line by increasing the expression of P15 and P21 (34). In general, according to the results, it can be concluded that valproic acid is a good candidate for the cancer treatment process. In this research, we did not investigate the effect of the valproic acid drug on the number of proteins of the internal and external pathways of apoptosis. Therefore, protein analysis of these two pathways is suggested.

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