RESEARCH ARTICLE

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Effect of Valproic Acid on the Class I Histone Deacetylase 1, 2 and 3, Tumor Suppressor Genes *p21WAF1/CIP1* and *p53*, and Intrinsic Mitochondrial Apoptotic Pathway, Pro- (*Bax*, *Bak*, and *Bim*) and anti- (*Bcl-2, Bcl-xL,* and Mcl-1) Apoptotic Genes Expression, Cell Viability, and Apoptosis Induction in Hepatocellular Carcinoma HepG2 Cell Line

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Abstract

Backgrounds: Hepatocellular carcinoma (HCC), Primary liver cancer, is the fifth most common cancer in men. Histone deacetylation causes chromatin condensation resulting in gene silencing and tumorigenesis. These enzymes have become a novel target for the treatment of cancer. Histone deacetylase inhibitors (HDACIs) can reactivate tumor suppressor genes (*TSGs*) by inhibition of histone deacetylases (HDACs) activity leads to apoptosis induction in cancer cells. Further, these compounds can induce apoptosis through the intrinsic/mitochondrial pathway. Previously, we reported the effect of valproic acid (VPA) and trichostatin A (TSA) on TSGs $p21^{WAF1/CIP1}$ (p21), $p27^{Kip1}$ (p27), and $p57^{Kip2}$ (P57) and also HDAC1 in colon cancer. The present study was designed to investigate the effect of VPA on the class I histone deacetylase (HDAC) 1, 2 and 3, TSGs p21and p53, and intrinsic mitochondrial pathway, pro- (Bax, Bak, and Bim) and anti- (Bcl-2, Bcl-xL, and Mcl-1) apoptotic genes, viability, and apoptosis in HCC HepG2 cell line. **Materials and Methods:** The HepG2 cells were cultured and treated with VPA. To determine viability, apoptosis, and the relative expression level of the mentioned genes, MTT assay, cell apoptosis assay, and qRT-PCR were done respectively. **Results:** VPA downregulated class I histone deacetylase (HDAC) 1, 2, and 3, Bcl-2, Bcl-xL, and Mcl-1 and upregulated p21, p53, Bax, Bak, and Bim resulting in apoptosis induction. **Conclusion:** VPA can induce apoptosis via activation of the intrinsic mitochondrial apoptotic pathway and also epigenetic reactivation of p21 and p53 through inhibition of class I HDAC 1, 2 and 3, activity.

Keywords: Extrinsic- intrinsic- pathway- apoptosis

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Introduction

Hepatocellular carcinoma (HCC), Primary liver cancer, is the fifth most common cancer in men, the seventh one in women and the second leading cause of cancer mortality worldwide. It is a common malignancy in developing countries and its incidence is on the rise in the developing world. The epidemiology of the cancer is unique since its risk factors, compressing hepatitis C and B, have been established (Tang et al., 2018; Bosetti et al., 2014). There are several universal aberrations common to all cancers especially HCC, one of which being the epigenetic silencing of the promoter region of tumor suppressor genes (TSGs) (Kazanets et al., 2016). DNA methylation and histone deacetylation of the TSGs are the most common types of epigenetic alterations that induce cancer and tumorigenesis. These changes can lead to the silencing of TSGs resulting in cancer induction (Ho et al., 2013). The DNA methyltransferases (DNMTs) are responsible for aberrant DNA methylation patterns leading to gene silencing. These enzymes include DNMT1, DNMT1b, DNMT2, DNMT3a, and DNMT3b (Liu et al., 2003). Additionally, histone acetylation and deacetylation play a key role in the regulation of gene expression, histone acetyltransferases (HATs) catalyze the acetylation of histones and relaxes chromatin structure to increase the accessibility of transcription factors to the promoter regions of the target genes. Just the opposite, histone deacetylases (HDACs) remove the acetyl groups from histones and repress gene transcription (Glozak

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et al., 2007). Histone deacetylation causes chromatin condensation, while decondensation is caused by histone acetylation. According to phylogenetic analyses and sequence homology, the HDAC family consists of at least 18 members divided into two families and four classes in eukaryotic cells. These two families are classical and sirtuin families. HDACs are categorized into class I (HDACs 1, 2, 3, and 8), class IIa (HDACs 4, 5, 7, and 9), class IIb (HDACs 6 and 10), class III (SIRTs), and class IV (HDAC11) (Zhang et al., 2007). These enzymes have become a novel target for the treatment of cancer. Inhibition of HDACs activity by histone deacetylase inhibitors leads to the acetylation of histones and nonhistone proteins resulting in reactivation of TSGs and apoptosis induction. HDACIs can be divided into five classes of compounds: (I) hydroxamic acids (hydroxamates, e.g. trichostatin A, TSA); (II) short-chain fatty (aliphatic) acids (such as butyrate, phenylbutyrate, and valproic acid, VPA); (III) benzamides; (IV) cyclic tetrapeptides; and (V) sirtuin inhibitors (Dokmanovic et al., 2007; Eckschlager et al., 2017). Previously, we reported the effect of histone deacetylase inhibitors VPA and TSA on epigenetic reactivation of tumor suppressor genes *p21, p27*, and *p57* via down-regulation of HDAC1 in colon cancer SW480 cell line (Sanaei et al., 2019). In addition to this epigenetic pathway, other researchers have indicated that histone deacetylase inhibitors can induce apoptosis through the intrinsic/mitochondrial pathway in various cancers. In vitro studies have demonstrated that HDACIs activate the intrinsic pathway via the upregulation of several pro-apoptotic BH3-only *Bcl-2* family genes compressing *Bid, Bim*, and *Bmf* (Matthews et al., 2012). It has been shown that sodium butyrate (NaBt) treatment results in a marked down-regulation of Bcl-xL expression, mitochondrial membrane depolarization, cytochrome c release from mitochondria, activation of caspase-9 and -3 and apoptosis induction in pancreatic cancer (Natoni et al., 2005).

Various anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, etc.) and pro-, (Bax, Bak, Bim, Bid, etc.) proteins regulate intrinsic/mitochondrial pathway. HDACIs including TSA, FK228, SAHA, and LBH589, decrease the expression of anti-apoptotic Bcl-2, Bcl-xL, and XIAP, and enhance the expression of proapoptotic proteins Bax and Bak. Meanwhile, it has been reported that HDACIs activate either an extrinsic or intrinsic pathway or both of these cell death pathways in various cancers (Rikiishi et al., 2011). With the results of our previous work and other reports, the present study was designed to investigate the effect of VPA on the class I histone deacetylase (HDAC) 1, 2 and 3, TSGs p21 and p53 and intrinsic mitochondrial apoptotic pathway, pro- (*Bax, Bak*, and *Bim*) and anti- (*Bcl-2, Bcl-xL*, and *Mcl-1*) apoptotic gene expression, cell viability, and apoptosis induction in hepatocellular carcinoma HepG2 cell line.

Materials and Methods

Materials

The human hepatocellular carcinoma HepG2 cell line was kindly provided from the National Cell Bank of Iran-Pasteur Institute and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and fetal bovine serum 10% in a humidified atmosphere of 5% CO_2 in air at 37°C. All reagents and materials including VPA, various kits, and instruments used in cell culture, MTT assay, flow cytometry assay, RNA extraction, reverse transcription, and Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) were provided as obtained previously (Kavoosi et al., 2018; Kavoosi et al., 2018; Sanaei et al., 2019). This is a lab-trial work approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC. 1394.131.

Cell culture and cell viability

The HepG2 cell viability was measured using 3 (4,5 dimethyl 2 thiazolyl) 2, 5 diphenyl 2H tetrazolium bromide (MTT) assay. The cells were cultured in 96-well plates at a density of 5 × 105 cells per well and treated with VPA (1, 5, 10, 25, and 50 µM) for 24, 48, and 72 h. The control groups received the solvent of the drug, serum-free Medium. After incubation for different periods, the cells were exposed to MTT, MTT (0.5 mg/mL) was added to each well for 4 h. Finally, the blue MTT formazan, the MTT metabolite, was dissolved in DMSO (200 mL) and the optical density was detected by a microplate reader at a wavelength of 570 nM. Each experiment was repeated three times (triplicates).

Cell apoptosis assay

To determine the apoptotic HepG2 cells, the cells were treated with cultured and seeded at a density of 5×10^5 cells/well and treated with VPA, based on IC50 values, at a concentration of 5.32, 4.27, and 3.03 µM for 24, 48, and 72 h respectively. Following treatment, the HepG2 cells were harvested by trypsin and washed twice with PBS and then resuspended in Binding buffer (1x). Finally, the cells were stained with annexin V-FITC (5 µl) and propidium iodide (5 µl) in the dark at room temperature for 15 min and counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was performed to determine the relative expression level of class I HDAC 1, 2, and 3, TSGs p21, p53, pro- (Bax, Bak, and Bim) and antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1) genes expression. The HepG2 cells were cultured at a density of 5×10^5 cells/well and treated with VPA, based on IC_{50} values, at a concentration of 5.32, 4.27, and 3.03 µM for 24, 48, and 72 h respectively. After 24, 48, and 72 h of incubation, the total RNA was harvested using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer protocol and treated by RNase-free DNase (Qiagen). The RNA was transcribed to complementary DNA (cDNA). QRT-PCR was done as described previously (Sanaei et al., 2018). The program for the PCR was as we reported previously (Sanaei et al., 2018). The primer sequences of the genes are indicated in Table 1. GAPDH was used as an endogenous control. Data were analyzed using the comparative Ct (ΔΔct) method.

Results

Result of cell viability by the MTT assay

The viability of HCC HepG2 cells treated with VPA (1, 5, 10, 25, and 50 µM) for various periods (24, 48, and 72 h) was measured by MTT assay, the activity of cellular enzymes produced a dark-blue formazan which is dissolvable in DMSO to determine the number of viable cells. As shown in Figure 1, TSA induced significant cell growth inhibition with a dose- and time-dependent manner (P< 0.001). The IC_{50} value was obtained with approximately 5.32, 4.27, and 3.03 µM for 24, 48, and 72 h respectively.

Result of cell apoptosis assay

To determine HepG2 cell apoptosis, the cells were treated with VPA, based on IC_{50} values, for 24, 48, and 72 h and stained using annexin-V-(FITC) and PI to

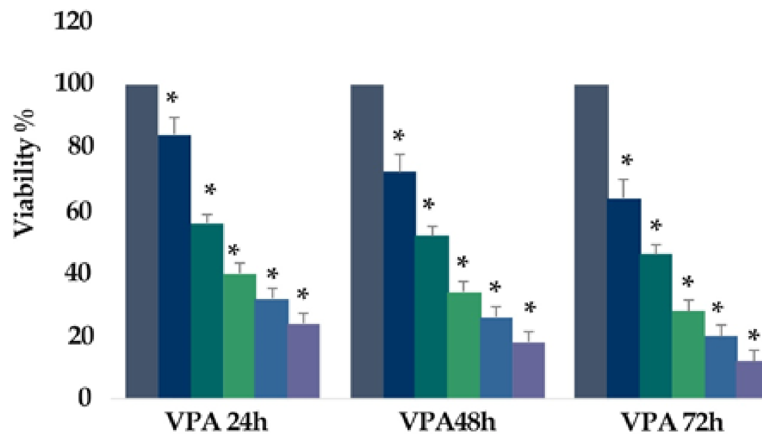

Figure 1. In Vitro Effects of VPA (1, 5, 10, 25, and 50 µM) on HepG2 Cells Viability Determined by MTT Assay at 24, 48, and 72 h. As indicated above, the first column of each group belongs to the control group. Values are means of three experiments in triplicate. Asterisks (*) demonstrate significant differences between treated and untreated control groups. A significant difference was considered as P < 0.05.

Table 1. The Primer Sequences of Class I HDAC 1, 2, and 3, p21, p53, pro- (Bax, Bak, and Bim) and Antiapoptotic (*Bcl-2, Bcl-xL,* and *Mcl-1*) Genes

Primer name	Primer sequences (5' to 3')	References
p21 Forward	ATTAGCAGCGGAACAAGG AGTCAGACAT	Huang et al., (2005)
p21 Reverse	CTGTGAAAGACACAGAACAGTACAGG GT	Huang et al., (2005)
P53 Forward	CACTAAGCGAGCACTG	Huang et al., (2005)
P53 Reverse	GGAGGTAGACTGACCC	Huang et al., (2005)
GAPDH Forward	AGCAATGCCTCCTGCACCACCAAC	Huang et al., (2005)
GAPDH Reverse	CCGGAGGGGCCA TCCACAGTCT	Huang et al., (2005)
HDAC1 Forward	AACTGGGGACCTACGG	Huang et al., (2005)
HDAC1 Reverse	ACTTGGCGTGT CCTT	Huang et al., (2005)
HDAC2 Forward	GTTG CTCGATGTTGGAC	Huang et al., (2005)
HDAC2 Reverse	CCAGGTGCATGAGGTA	Huang et al., (2005)
HDAC3 Forward	CCCTGCGGGATGGCATTGATGA	Liu et al., (2015)
HDAC3 Reverse	AGCCCAGAGAGTCAGCTCCACA	Liu et al., (2015)
Bim Forward	TGC GCC CGG AGA TAC	Liu et al., (2005)
Bim Reverse	CCT CCT TGT GTA AGT TTC GTT GAA C	Liu et al., (2005)
Bak Forward	GCCCAGGACACAGAGGAGGTTTTC	Hasan et al., (2006)
Bak Reverse	AAACTGGCCCAACAGAACCACACC	Hasan et al., (2006)
Bax Forward	CCA GCT CTG AGC AGA TCA TG	Spampanato et al., (2012)
Bax Reverse	CCA GCT CTG AGC AGA TCA TG	Spampanato et al., (2012)
Bcl-2 Forward	GAC TTC GCC GAG ATG TCC AG	Spampanato et al., (2012)
Bcl-2 Reverse	CAG GTG CCG GTT CAG GTA CT	Spampanato et al., (2012)
Bcl-xL Forward	GTTCCCTTTCCTTCCATCC	Sharifi et al., (2015)
Bcl-xL Reverse	TAGCCAGTCCAGAGGTGAG	Sharifi et al., (2015)
Mcl-1 Forward	AGGCTGGGATGGGTTTGTG	Zhang et al., (2012)
Mcl-1 Reverse	CACATTCCTGATGCCACCTTCT	Zhang et al., (2012)

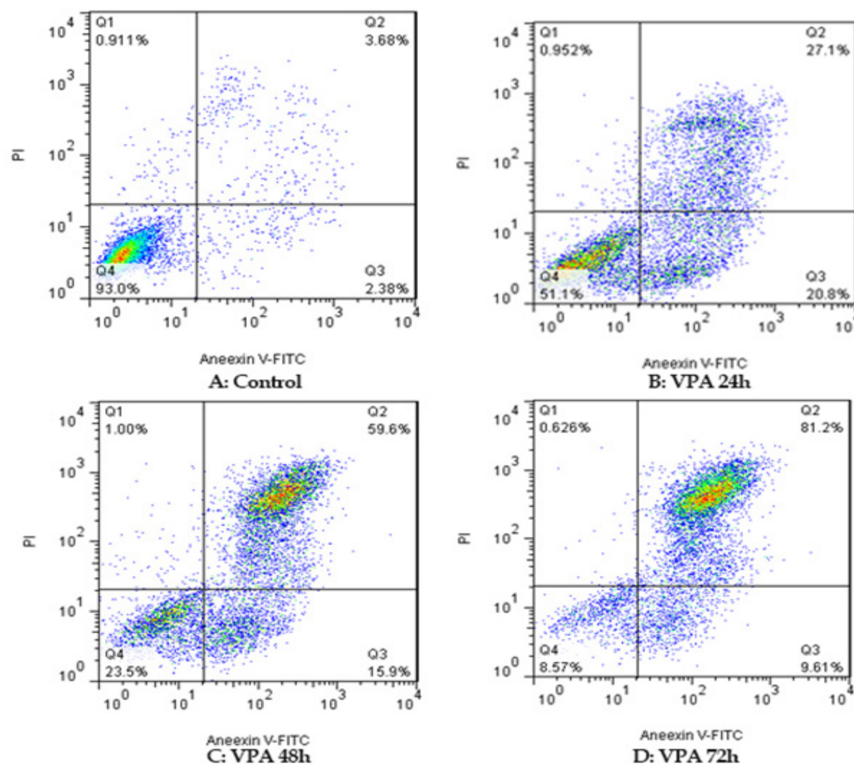

Figure 2. The Apoptotic Effect of VPA on HepG2 Cells versus Control Groups at Different Periods (24, 48, and 72 h). The cells were treated with VPA for different periods and then the apoptotic effect was evaluated by flow cytometric analysis. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean.

Table 2. Percentage of Apoptotic Cells in the HepG2 Cell Groups Treated with VPA at Different Periods.

Drug	Dose/ µM	Duration/ h	Apoptosis %	P-value
VPA	5.32	24	47.9	P < 0.001
VPA	4.27	48	75.5	P < 0.001
VPA	3.03	72	90.63	P < 0.001

distinguish apoptotic cells in the early and late apoptosis stage. As indicated in Figure 2, VPA induced apoptosis significantly. It induced apoptosis in a time-dependent manner, Fgures 3. The percentage of HepG2 apoptotic cells is shown in Table 2.

Result of determination of genes expression

The effect of VPA (5.32, 4.27, and 3.03 µM) on class I HDAC 1, 2 and 3, TSGs p21 and p53, pro- (*Bax, Bak,* and *Bim*) and anti- (*Bcl-2, Bcl-xL*, and *Mcl-1*) apoptotic genes expression was assessed by quantitative real-time RT-PCR analysis. The results indicated that treatment with this agent (24, 48, and 72 h) up-regulated p21, *p53*, pro- (*Bax, Bak*, and *Bim*) apoptotic genes and down-regulated *HDAC 1, 2* and *3*, *Bcl-2, Bcl-xL*, and *Mcl-1* genes expression significantly, Figures 4 and 5.

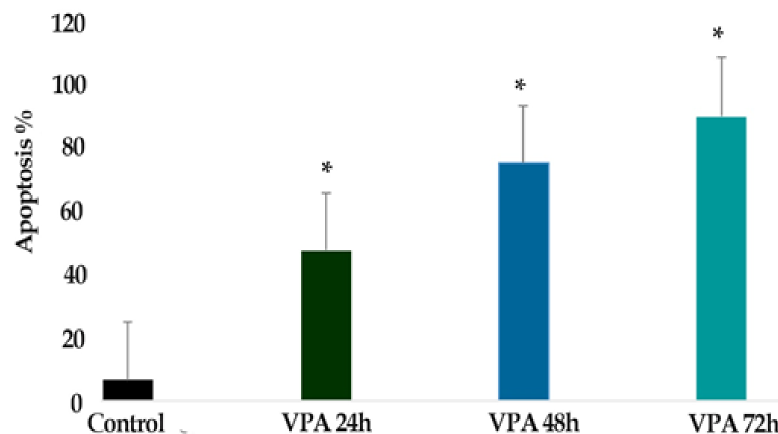

Figure 3. The Apoptotic Effect of VPA after 24, 48, and 72 h of Treatment. Asterisks (*) indicate significant differences between the treated and untreated control groups. As demonstrated above VPA had a time-dependent manner. Asterisks (*) demonstrate significant differences between treated and untreated control groups. A significant difference was considered as P < 0.05.

Table 3. The Effect of VPA on Class I HDAC 1, 2, and 3, p21, and p53 Genes Expression in the HCC HepG2 Cell Line

Cell line	Gene	Duration (h)	Expression	P-value
HepG2	*HDAC1*	24	0.54	0.001
HepG2	*HDAC1*	48	0.46	0.001
HepG2	*HDAC1*	72	0.3	0.001
HepG2	*HDAC2*	24	0.51	0.001
HepG2	*HDAC2*	48	0.42	0.001
HepG2	*HDAC2*	72	0.31	0.001
HepG2	*HDAC3*	24	0.47	0.001
HepG2	*HDAC3*	48	0.4	0.001
HepG2	*HDAC3*	72	0.26	0.001
HepG2	*P21*	24	2.1	0.001
HepG2	*P21*	48	2.7	0.001
HepG2	*P21*	72	3.3	0.001
HepG2	*P53*	24	2.4	0.001
HepG2	*P53*	48	2.8	0.001
HepG2	*P53*	72	3.5	0.001

Table 4. The Effect of VPA on the Pro- (Bax, Bak, and Bim) and Anti- (Bcl-2, Bcl-xL, and Mcl-1) Apoptotic Gene Expression in HCC HepG2 Cell Line

Cell line	Gene	Duration (h)	Expression	P-value
HepG2	*Bcl-2*	24	0.52	0.001
HepG2	*Bcl-2*	48	0.41	0.001
HepG2	*Bcl-2*	72	0.34	0.001
HepG2	*Bcl-xL*	24	0.47	0.001
HepG2	*Bcl-xL*	48	0.38	0.001
HepG2	*Bcl-xL*	72	0.26	0.001
HepG2	*Mcl-1*	24	0.44	0.001
HepG2	*Mcl-1*	48	0.36	0.001
HepG2	*Mcl-1*	72	0.28	0.001
HepG2	*Bax*	24	2.2	0.001
HepG2	*Bax*	48	2.8	0.001
HepG2	*Bax*	72	3.4	0.001
HepG2	*Bak*	24	2.4	0.001
HepG2	*Bak*	48	2.9	0.001
HepG2	*Bak*	72	3.2	0.001
HepG2	*Bim*	24	2.5	0.001
HepG2	*Bim*	48	2.8	0.001
HepG2	*Bim*	72	3.1	0.001

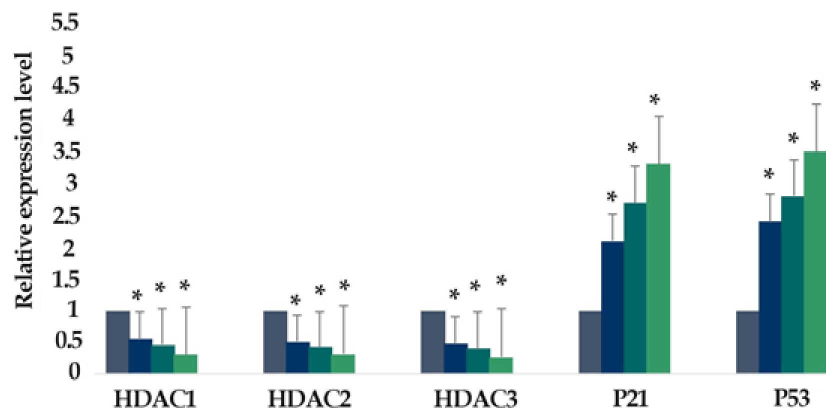

Figure 4. The Relative Expression Level of Class *I HDAC 1, 2*, and *3, p21, and p53* genes in the HepG2 Cell Line Treated with VPA versus Untreated Control Groups at Different Periods (24, 48, and 72 h). The first column of each group belongs to the control group and the others belong to the treated cells with VPA at 24, 48, and 72 h. Asterisks (*) indicate significant differences between the treated and untreated groups. A significant difference was considered as P < 0.05

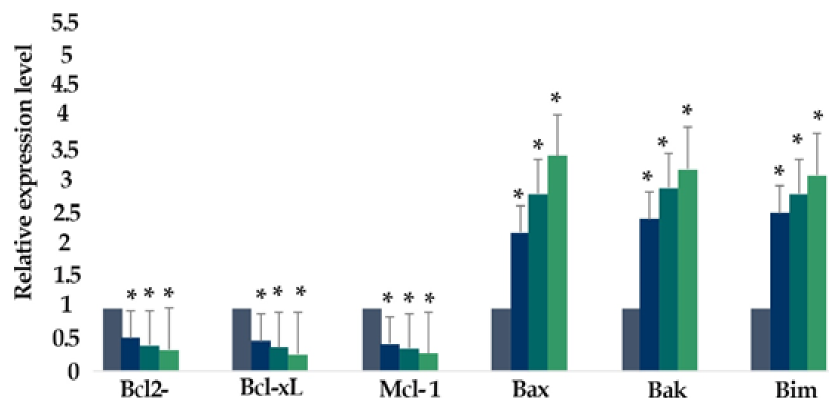

Figure 5. The Relative Expression Level of *Bcl-2, Bcl-xL, Mcl-1, Bax, Bak*, and *Bim* Genes in the HepG2 Cell Line Treated with VPA versus Untreated Control Groups at Different Periods (24, 48, and 72 h). The first column of each group belongs to the control group and the others belong to the treated cells with VPA at 24, 48, and 72 h. Asterisks (*) indicate significant differences between the treated and untreated groups. A significant difference was considered as P < 0.05.

The relative expression level of the genes is indicated in Tables 3 and 4.

Discussion

HDACIs are a relatively new class of anti-cancer drugs that play important roles in epigenetic and non-epigenetic regulation, cell cycle arrest, and apoptosis in cancer cells. Epigenetic alterations caused by imbalances between HDACs and HATs can affect global transcriptional profiles. In fact, TSGs, such as p53, is suppressed in many cancers by aberrant epigenetic changes. HDAC-mediated deacetylation changes the transcriptional activity of transcriptional factors such as p53, E2F, c-Myc, NF-kB, HIF-1α, smad7, ER, and AR. HDACs upregulate the intrinsic and extrinsic apoptosis mechanism via the induction of the proapoptotic genes (e.g. *Bmf* and *Bim*), and TRAIL and DR5 respectively (Kim et al., 2011). It has been reported that the apoptosis mechanism of HDACIs involve activation of both the extrinsic (receptor-mediated) and the intrinsic (mitochondrial-mediated) pathways. Overall, all HDACIs have been reported to activate either an intrinsic, or extrinsic pathway or both of these cell death mechanisms in various cancers (Rosato et al., 2003). In the current study, we indicated that VPA can inhibit HepG2 cell growth and induce apoptosis by both p53-dependent and -independent mechanisms. We first demonstrated that VPA can up-regulate p21 and p53 by down-regulation of HDAC 1, 2, and 3. This result encouraged us to evaluate the p53-independent mechanism of VPA. This evaluation demonstrated that VPA can play through an intrinsic mitochondrial apoptotic pathway, up-regulation of *Bax, Bak*, and *Bim* and down-regulation of *Bcl-2, Bcl-xL*, and *Mcl-1* genes expression.

In line with our findings, it has been reported that histone deacetylase inhibitor TSA induces G2/M cell cycle arrest and Bax-dependent apoptosis in HCT116 and HT29 colorectal cancer cells by both p53-dependent and -independent pathways (Meng et al., 2012). Similar to our result, in vitro study has shown that histone deacetylase inhibitors VPA and TSA induce apoptosis by up-regulation of p21/Waf1/CIP1 and p53 and inhibition of HDAC1 activity in prostate cancer cells (Fortson et al., 2011). It inhibits both classes I and II HDACs with resultant hyperacetylation of histone H3 and H4. Altered expression of TSGs such as the cyclin dependent kinase inhibitor p21 has been reported in the cells exposed to VPA treatment (Li et al., 2005). In HCC, it induces down-regulation of Notch signaling via suppressing the expression of Notch1 with an increase of p21 and p63 (Sun et al., 2015). It has been indicated that VPA can downregulate cyclins A and D1, HDAC1, and up-regulate the expression of P21 in hepatocellular carcinoma HepG2 cell line (Yang et al., 2015). Furthermore, histone deacetylase inhibitor TSA increases expression of cyclin A, p2l, bax and (pro)-caspase 3, while downregulates bcl-2 in the hepatoma cells HepG2, Hepa1–6, MH1C1, and Hep1B (Herold et al., 2002). As we reported in this study, the intrinsic (mitochondrial-mediated) apoptotic pathway of histone deacetylase inhibitors have been reported by other researchers. It has been shown that HDACIs up-regulate Bax, Bak, and Bim and down-regulate Bcl-xL, Mcl-1, and XIAP in melanoma (Gillespie et al., 2006).

Another study has shown that these compounds induce apoptosis primarily through inactivation of anti-apoptotic Bcl-2 family members by increases in Bim and Noxa in CLL/lymphoma (Inoue et al., 2007). Similarly, it has been demonstrated that VPA, TSA, and SAHA modulate the regulators of apoptosis, including TRAIL-R1, TRAIL-R2, caspase 8, Bcl-2, Bcl-XL, Mcl-1, Apaf-1, NOXA, and survivin in human melanoma cell lines (WM266, WM115, A375, SK-Mel28) (Facchetti et al., 2004). However, one of the pathways being targeted for cancer therapy is the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family of proteins including Bcl-w, Bcl-XL, Bfl1/A-1, Bcl-2, Mcl-1, and Bcl-B (Kang et al., 2009). Finally, VPA can induce apoptosis by the intrinsic apoptotic pathway and reactivation of TSGs.

In conclusion, we indicated that HDAC inhibitor VPA induces apoptosis of hepatocellular carcinoma HepG2 cells via activation of the intrinsic mitochondrial apoptotic pathway, up-regulation of pro- (*Bax, Bak*, and *Bim*) apoptotic and down-regulation of anti- (*Bcl-2, Bcl-xL*, and *Mcl-1*) apoptotic genes and also epigenetic reactivation of *p21* and *p53* through inhibition of class I histone deacetylase (HDAC) 1, 2 and 3 activities.

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Conflict of interest
The authors report no conflict of interest.

References

Bosetti C, Turati F, La Vecchia C (2007). Hepatocellular carcinoma epidemiology. *Best Pract Res Clin Gastroenterol*, **28**, 753-70.

Dokmanovic M, Clarke C, Marks PA (2007). Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res*, **5**, 981-9.

Eckschlager T, Plch J, Stiborova M, et al (2017). Histone deacetylase inhibitors as anticancer drugs. *Int J Mol Sci*, **18**, 1414.

Facchetti F, Previdi S, Ballarini M, et al (2004). Modulation of pro-and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. *Apoptosis*, **9**, 573-82.

Fortson WS, Kayarthodi S, Fujimura Y, et al (2011). Histone deacetylase inhibitors, valproic acid and trichostatin-A induce apoptosis and affect acetylation status of p53 in ERG-positive prostate cancer cells. *Int J Oncol*, **39**, 111-9.

Gillespie S, Borrow J, Zhang XD, et al (2006). Bim plays a crucial role in synergistic induction of apoptosis by the histone deacetylase inhibitor SBHA and TRAIL in melanoma cells. *Apoptosis*, **11**, 2251-65.

Glozak M, Seto E (2007). Histone deacetylases and cancer. *Oncogene*, **26**, 5420–32

Hasan Z, Ashraf M, Tayyebi A, et al (2006). leprae inhibits apoptosis in THP-1 cells by downregulation of Bad and Bak and upregulation of Mcl-1 gene expression. *BMC*

Microbiol, **6**, 78-86.
Herold C, Ganslmayer M, Ocker M, et al (2002). The histone-deacetylase inhibitor Trichostatin A blocks proliferation and triggers apoptotic programs in hepatoma cells. *J Hepatol*, **36**, 233-40.
Ho AS, Turcan S, Chan TA (2013). Epigenetic therapy: use of agents targeting deacetylation and methylation in cancer management. *Onco Targets Ther*, **6**, 223-9.
Huang B, Laban M, Leung CH, et al (2005). Inhibition of histone deacetylase 2 increases apoptosis and p21 Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ*, **12**, 395-404.
Inoue S, Riley J, Gant TW, et al (2007). Apoptosis induced by histone deacetylase inhibitors in leukemic cells is mediated by Bim and Noxa. *Leukemia*, **21**, 1773-82.
Kang MH, Reynolds CP (2009). Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res*, **15**, 1126-32.
Kazanets A, Shorstova T, Hilmi K, et al (2016). Epigenetic silencing of tumor suppressor genes: Paradigms, puzzles, and potential. *Biochim Biophys Acta Rev Cancer*, **1865**, 275-88.
Kim H-J, Bae S-C (2011). Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *Am J Transl Res*, **3**, 166-271.
Li X-N, Shu Q, Su JM-F, et al (2005). Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. *Mol Cancer Ther*, **4**, 1912-22.
Liu K, Wang YF, Cantemir C, et al (2003). Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. *Mol Cell Biol*, **23**, 2709-19.
Liu X, Wang JH, Li S, et al (2015). Histone deacetylase 3 expression correlates with vasculogenic mimicry through the phosphoinositide3-kinase/ERK–MMP–laminin5γ2 signaling pathway. *Cancer Sci*, **106**, 857-66.
Matthews GM, Newbold A, Johnstone RW (2012). Intrinsic and extrinsic apoptotic pathway signaling as determinants of histone deacetylase inhibitor antitumor activity. *Adv Cancer Res*, **5**, 165-97.
Meng J, Zhang H-H, Zhou C-X, et al (2012). The histone deacetylase inhibitor trichostatin A induces cell cycle arrest and apoptosis in colorectal cancer cells via p53-dependent and-independent pathways. *Oncol Rep*, **28**, 384-8.
Natoni F, Diolordi L, Santoni C, et al (2005). Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways. *Biochim Biophys Acta Rev Cancer*, **1745**, 318-29.
Rikiishi H (2011). Autophagic and apoptotic effects of HDAC inhibitors on cancer cells.Biochim *Biophys Acta Mol Cell Res*, **5**, 1-9.
Rosato RR, Almenara JA, Dai Y, et al (2003). Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells. *Mol Cancer Ther*, **2**, 1273-84.
Sanaei M, Kavoosi F (2019). Effects of 5-aza-2ˊ-deoxycytidine and Valproic Acid on Epigenetic-modifying DNMT1 gene expression, apoptosis induction and cell viability in hepatocellular carcinoma WCH-17 cell line. *Iran J Ped Hematol Oncol*, **9**, 83-90
Sanaei M, Kavoosi F (2019). Effect of 5-aza-2'-deoxycytidine in comparison to valproic acid and trichostatin A on histone deacetylase 1, DNA methyltransferase 1, and CIP/KIP family (p21, p27, and p57) genes expression, cell growth inhibition, and apoptosis induction in colon cancer SW480 cell line. *Adv Biomed Res*, **8**, 52-60.
Sanaei M, Kavoosi F (2018). Comparative analysis of the effects of valproic acid and tamoxifen on proliferation, and apoptosis of human hepatocellular carcinoma WCH 17 celllin. *Iran J Ped Hematol Oncol*, **8**, 12-20.
Sanaei M, Kavoosi F (2018). Effect of curcumin and trichostatin a on the expression of DNA methyltransfrase 1 in hepatocellular carcinoma cell line hepa 1-6. *Iran J Ped Hematol Oncol*, **8**, 193-201.
Sanaei M, Kavoosi F, Roustazadeh A, et al (2018). Effect of genistein in comparison with trichostatin a on reactivation of DNMTs genes in hepatocellular carcinoma. *J Clin Transl Hepatol*, **6**, 141-145.
Sharifi S, Barar J, Hejazi MS, et al (2015). Doxorubicin changes Bax/Bcl-xL ratio, caspase-8 and 9 in breast cancer cells. *Adv Pharm Bull*, **5**, 351-8.
Spampanato C, De Maria S, Sarnataro M, et al (2012). Simvastatin inhibits cancer cell growth by inducing apoptosis correlated to activation of Bax and down-regulation of BCL-2 gene expression. *Int J Oncol*, **40**, 935-941.
Sun G, Mackey LV, Coy DH, et al (2015). The histone deacetylase inhibitor vaproic acid induces cell growth arrest in hepatocellular carcinoma cells via suppressing notch signaling. *J Cancer*, **6**, 996-1002.
Tang A, Hallouch O, Chernyak V, et al (2018). Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. *Abdom Radiol*, **43**, 13-25.
Tan T-T, Degenhardt K, Nelson DA, et al (2005). Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell*, **7**, 227-38.
Yang W, Zhao X, Pei F, et al (2015). Activation of the intrinsic apoptosis pathway contributes to the induction of apoptosis in hepatocellular carcinoma cells by valproic acid. *Oncol Lett*, **9**, 881-6.
Zhang J, He J, Xia J, et al (2012). Delayed apoptosis by neutrophils from COPD patients is associated with altered bak, bcl-xl, and mcl-1 mRNA expression. *Diagn Pathol*, **7**, 65.
Zhang X, Yuan Z, Zhang Y, et al (2007). HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Mol Cell*, **27**, 197-213.

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