Original Article

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Comparative Analysis of the Effects of Valproic Acid and Tamoxifen on Proliferation, and Apoptosis of Human Hepatocellular Carcinoma WCH 17 CellLine

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Abstract

Background: Histone deacetylation of tumor suppressor genes such as estrogen receptor alpha (ERα) can induce cancer, which is reversible by epi-drugs such as valproic acid (VPA). The previous result indicated that tamoxifen (TAM) induced apoptosis in hepatocellular carcinoma (HCC). This study was designed to assess the apoptotic and antiproliferative effects of VPA and TAM and also the effect of VPA on ERα gene expression in HCC.

Material and Methods: The cells were treated with various doses of VPA and TAM and the MTT assay, Real-Time RT-PCR, and flow cytometry assay were done to determine viability, ERα gene expression, and apoptosis.

Results: Both agents inhibited viability and induced apoptosis. ERα gene expression was increased by VPA, which in turn increased the apoptotic effect of TAM. The half-maximum inhibitory concentration (IC50) value for VPA and TAM was 5 and 20 μM respectively. VPA inhibited cell growth by 88 % to 38 % at 24 h ($P < 0.001$) and 76 % to 28 % at 48 h ($P < 0.002$) and also TAM inhibited by 92 % to 40 % at 24 h ($P < 0.006$) and 84 % to 32 % at 48 h ($P < 0.001$). The percentage of VPA- treated apoptotic cells were reduced by about 35 and 43 % ($P <0.001$) and that of TAM-treated 32 and 38 % ($P <0.001$) after 24 and 48 h, respectively.

Conclusion: VPA and TAM can significantly inhibit viability and induce apoptosis and also VPA play a significant role in ERα reactivation.

Keywords: Hepatocellular Carcinoma, Tamoxifen,Valproic acid

Introduction

Hepatocellular carcinoma (HCC), primary liver cancer, accounts for the fifth most common cancer in men and the seventh one in women worldwide. The disease is the third most common cause of cancer related death. The main recognized risk factors of HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (1). Genetic and epigenetic alterations can lead to the genomic unstability and transformation of normal hepatocyte to liver cancer. Alterations in tumor suppressor genes, epigenetic changes, such as estrogen receptor alpha (ERα) that change gene expression and cell function, appear to promote liver tumorigenesis (2). Changes in the chromatin status of tumor suppressor genes include acetylation, methylation, phosphorylation, and sumoylation (3). The levels of histone acetylation play a crucial role in gene expression (4). Histone acetylation is a reversible modification controlled by the antagonistic actions of two types of enzymes, histone acetylases (HATs) which catalyze the transfer of acetyl groups from acetyl CoA to the ε-amino group of the lysine residue and histone deacetylases (HDACs) which promote the removal of the acetyl group from the acetylated residue (3, 6). Together, these two groups determine the acetylation status of histones. HDAC inhibitors (HDACIs) interact with ERα, belong to a large superfamily of nuclear receptors that modulate the expression of genes, results in suppression of its transcriptional activity (6). Finally, histone deacetylation is not only involved in

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tumorigenesis but also in metastasis and tumor invasion (7). Fortunately, epigenetic alterations are reversible by epi-drugs which can reverse epigenetic alternation and re-activate gene expression(8-10).HDACIs can induce differentiation of malignant cells (11). To date, more than 15 HDACIs have been known, the common mechanism of action of these compounds is to bind a critical Zn2+ ion required for catalytic function of the HDAC enzyme (12, 13).During the last decade, a number of HDACIs have been recognized that inhibit the growth of cancer cell and induce apoptosis and differentiation (14). Several experimental and clinical studies have demonstrated that HDACIs such as valproic acid (VAP), Trichostatin A, and phenyl butyrate can inhibit cancer cell growth, enhance cell differentiation, and induce apoptosis (15, 16). Tamoxifen (TAM) has been used for treating breast cancers for many years, this compound changes steroid binding domain by which prevents the gene expression and affects growth of cancer cells (2). Apoptotic and antiproliferative effects of VPA have been reported in many cancers such as gastric cancer BGC823 cell, breast cancer cell, SK-HEP-1 hepatoblasma cells, hepatocellular carcinoma HepG2 cell and human cholangiocarcinoma cells (17).One of the mechanisms by which TAM induces apoptosis is ER inhibition. Besides, non-ER-mediated mechanisms of this compound have been reported, including modulation of signaling proteins such as protein kinase C (PKC), transforming growth factor-β (TGFβ), calmodulin, and the protooncogene c-myc. Furthermore, mitochondrial permeability transition (MPT), oxidative stress, ceramide generation as well as changes in cell membrane fluidity may play an important role in TAM-induced apoptosis (18). It has recently reported that VPA in combination with TAM inhibit proliferation of MCF-7 breast cancer cells better than with either agent alone. Besides, VPA cooperates with TAM in a variety of ERα positive cell lines and also is more effective when combined with other antiestrogens (19). However, few studies have evaluated the effect of VPA on cell viability and ERα gene expression and also the effect of VPA in combination with TAM on apoptosis induction of HCC (WCH 17) cell line. Therefore, the present study was planned and performed to evaluate the effects of the compounds on WCH17 cell line.

Materials and Methods

Human hepatocellular carcinoma WCH-17 cells were purchased from the National Cell Bank of Iran-Pasteur Institute and maintained in Dulbecco's modified Eagle's medium (DMEM) (St. Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotic (penicillin, streptomycin and amphotericin B). Total RNA extraction Kit (TRIZOL reagent), Real-time PCR kits (qPCRMasterMix Plus for SYBR Green I dNTP), FBS (fetal bovine serum), and MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma (Sigma, St. Louis, MO). DMSO (dimethyl sulfoxide) was obtained from Merck Co. (Darmstadt, Germany). Valproic acid (2-propyl-pentanoic acid) and tamoxifen were purchased from Sigma (St. Louis, MO) and dissolved in serum-free medium and DMSO respectively to make a stock solution, DMSO was present at 0.01–0.3% in the medium based on the half-maximum inhibitory concentration(IC50) index. The stock solution was further diluted with cell culture medium to yield final VPA and tamoxifen concentrations. All other chemicals were obtained from the best available sources.

Cell Culture and Cell Viability Assay

WCH-17 cells were cultured with DMEM (pH 7.2–7.4) supplemented with 1% sodium pyruvate (sigma), 1.5 g/L sodium bicarbonate, 10% fetal bovine serum and 1% antibiotics, including 1% penicillin/streptomycin and 25 ug/ml

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amphotericin B (sigma) at 37°C in 5% CO2 to promote attachment. When cells became > 80% confluent, the cells were seeded into 96-well plates at a density of 5 × 10^5 live cells per well, incubated for 24 hours under standard conditions to allow cell attachment and subsequently treated with medium containing different doses of VPA (1, 2.5, 5, 7.5 and 10 μM) and TAM (1, 5, 10, 20, 40) to determine cell viability. After 24 and 48 h of the treatment, the effect of VPA and TAM was measured by the methylthiazolyldiphenyl-tetrazolium bromide rapid colorimetric assay according to standard protocols, the cells were washed twice with phosphate-buffered salin (PBS), a fresh medium containing MTT (0.5 mg/mL) was added and incubated at 37°C for 4 hours and then the formazan crystals were dissolved in acidic isopropanol and finally the absorbance was measured at 570 nm. All experiments were repeated three times, with at least three measurements (triplicates).

Cell Apoptosis Assay

The assessment of apoptosis was carried out by flow cytometry assay. Briefly, WCH-17 Cells were seeded at a density of 5 × 10^5 cells/well in 24-well cluster plates treated with VPA (5 μM) without and with TAM (20 μM)for 24 and 48 h. In combined treatment groups, the cells were treated with VPA for 24 h and then exposed to TAM for further 24 h prior to harvesting. After treatment times, all the adherent cells were harvested by trypsinization, washed with cold PBS, and resuspended in Binding buffer (1x). 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 counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The WCH-17 cells were treated with VPA (5 μM). After 24 and 48 h, Quantitative real-time RT-PCR was done to determine the ERα gene expression. In this regard, 5 × 10^5 cells/well were cultured in six-well plates and then treated with VPA after 24 h of culture. After 24 and 48 h of treatment, total RNA was isolated by RNeasy mini kit (Qiagen) according to the manufacturer's protocol and then treated by RNase free DNase (Qiagen) to eliminate the genomic DNA. Determination of RNA concentration was done by using a Biophotometer (Eppendorf). By using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas), Total RNA (100 ng) was reversetranscribed to cDNA according to protocol. Real-time RT-PCR was performed by the Maxima SYBR Green RoxqPCR master mix kit (Fermentas). The ERα primers selection was done through references, article (20) as shown in TableI. Real-time PCR reactions were performed using the Steponeplus (Applied Biosystem). Thermal cycling conditions for ERα were an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95˚C for 15 seconds, annealing at 60˚c for 20 seconds and extension at 72˚c for 20 seconds. Data analysis was done by comparative Ct (ΔΔct) method, the ERα gene expression level was calculated via determination of the ratio between the amount of ERα gene and that of endogenous control. To determine melting temperature of specific amplification products and primer dimmers, Melting curve was used. These experiments were carried out at least three times. GAPDH was used as a reference gene for internal control.

Results

Result of Determination of IC50 Value by MTT Assay

The effects of VPA and TAM on the WCH-17 cells proliferation were assessed by MTT assay after exposure to various concentrations of the drugs at defferent

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time periods. As shown in Figure 1, VPA and TAM reduced the number of viable cells in all three experimental versus control groups in a time- and dose-dependent manner significantly. VPA inhibited cell growth in all treated groups by 88 % to 38 % at 24 h ($P < 0.001$) and 76 % to 28 % at 48 h ($P < 0.002$) and also TAM inhibited cell growth in all treated groups by 92 % to 40 % at 24 h ($P < 0.006$) and 84 % and 32 % at 48 h ($P < 0.001$). Reduction of cell viability by 50% (IC50) required 5 µm VPA and 20 µM TAM. Each experiment was repeated three times for consistency of the results.

VPA Induction of Apoptosis

To determine the apoptotic effects of VPA (5 µM) and TAM (20 µM) on the WCH-17 cells, the cells were treated with the drugs at a different time periods (as mentioned in the methods) and flow cytometry was performed to observe the apoptotic cells. Result of flow cytometry revealed that VPA and TAM (alone and combined) induced significant apoptosis versus control group as shown in Figure2. The percentage of VPA- treated apoptotic cells were reduced by about 35 and 43 % ($P<0.001$) and that of TAM-treated 32 and 38 % ($P<0.001$) after 24 h and 48 h, respectively. Maximal apoptotic cell (64 %, $P<0.001$) was observed in the group which treated with VPA (24 h) and then TAM (24 h) and minimal apoptotic cell (32 %, $P < 0.001$) was observed in the group which received TAM alone for 24 h (Figures2 and 3).

Result of Determination of Gene Expression by Quantitative Real-Time RT-PCR

The effect of VPA (5 µM) on ERα gene expression was evaluated by quantitative real-time RT-PCR analysis at different time periods (24 and 48 h). Using quantitative RT-PCR, VPA was shown to increase ERα gene expression at different time periods significantly. The relative expression of ERα gene was 2.2 and 2.9 ($P < 0.001$) in different time periods respectively as depicted in Figure 4. Data are presented as means ± S.E.M (Standard error of mean).

TableI: Real time polymerase chain reaction primers used in the study

Genes	Primer sequences
ERα	
Forward:	5'-AGA CAT GAG AGC TGC CAA CC-3'
Reverse:	5'-GCC AGG CAC ATT CTA GAA GG-3'

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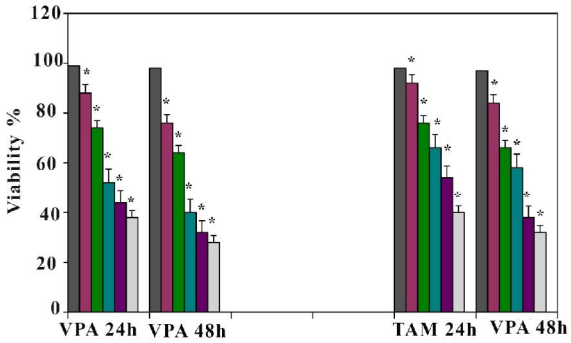

Figure 1. Effect of VPA and TAM on the WCH-17 cell viability determined by the MTT assay and calculated as a ratio, comparing treated to the control group. Data are presented as mean ± SD from at least triplicate wells and 3 independent experiments. Asterisks (*) indicate significant differences between treated cells and the control group. Drug concentrations in each group from left to right are 1, 2.5, 5, 7.5 and 10 µM for VPA (24 and 48 h) and for TAM 1, 5, 10, 20 and 40 µM (24 and 48 h). The first column of each group belongs to control group.

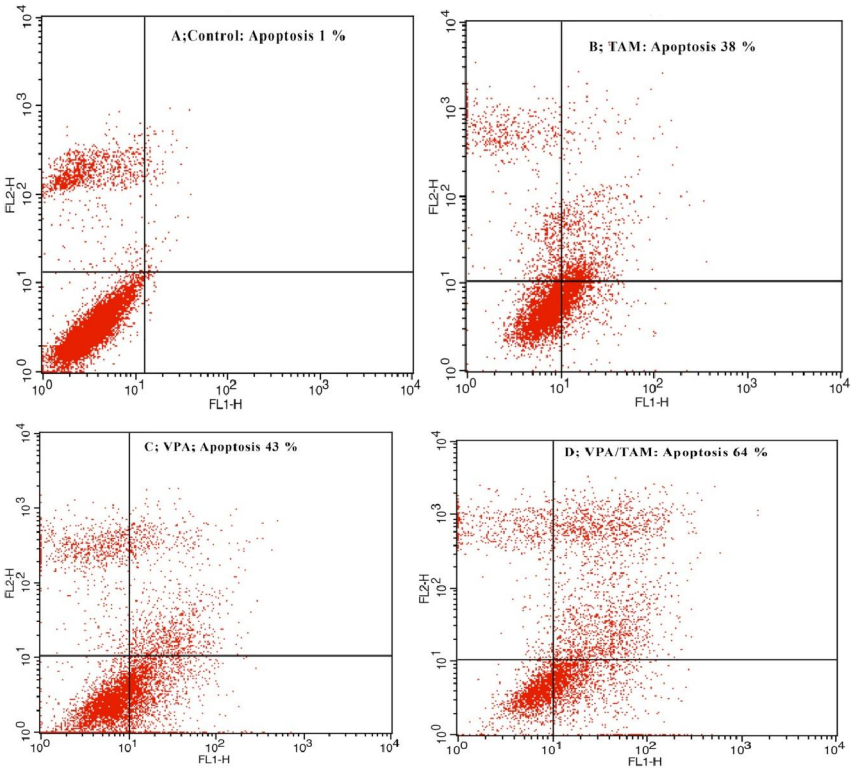

Figure 2. Apoptotic effects of VPA and TAM. VPA and TAM induced significant apoptosis in WCH-17 cells. The apoptotic cell percentage in the group treated with VPA (24 h) and then TAM (24 h) were more significant than that of each drug alone. (*p<0.001 compared with control group). A: control, B: VPA 48 h, C: TAM 48 h, D: VPA 24 h and then TAM 24 h.

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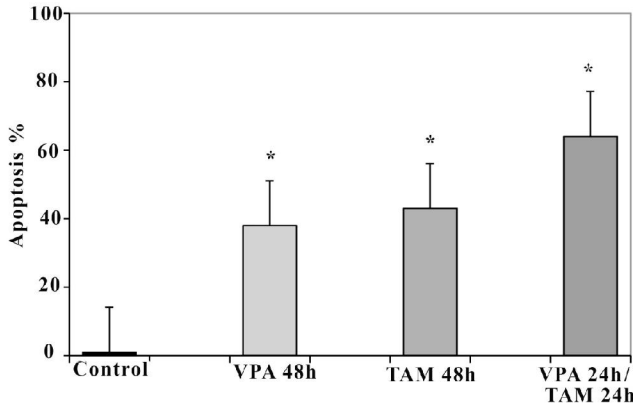

Figure 3. Apoptotic effects of VPA and TAM (alone and combined) on WCH 17 cell. Combined drug treatment induced apoptosis more significant than each drug alone ($P < 0.001$). Asterisks (*) indicate significant differences between treated cells and the control group.

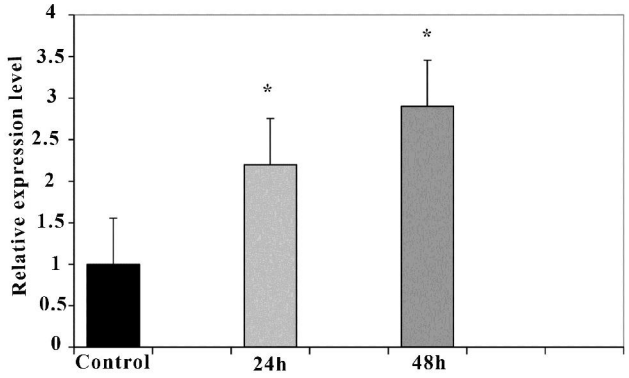

Figure 4. Relative expression level of ERα. As shown above, VPA increased ERα gene expression significantly (P, 0.001). Data are presented as mean ± standard error of the mean from at least three different experiments. Asterisks (*) indicate significant differences between treated cells and the control group.

Discussion

Epigenetic mechanisms such as histone modifications are essential for normal gene expression patterns in mammals. Histone deacetylation, which is done by histone deacetylase activity enzymes, in tumor suppressor genes result in the heritable silencing of genes and tumorigenesis. Fortunately, histone deacetylationisa reversible process and deacetylated histone can be reacetylated by histone deacetylase inhibition resulting gene expression (21).HDACIs are potent inducers components that induce apoptosis by HDAC inhibition. These compounds can act alone, but synergize with other targeted anticancer drugs are more effective in cancer therapy. In the present study, VPA restored the ERα gene expression and induced cell growth inhibition and

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apoptosis in WCH 17 cell line. Apoptotic effect of VPA was increased when the cells were treated with VPA for 24 h and then TAM was added for 24 h. Furthermore, VPA and TAM inhibited cell proliferation with a dose and time dependent manner. In the previous studies, VPA induced apoptosis in hepatocellular carcinoma HepG 2 (22) and colon carcinoma HT 29 cell lines (23). Consistent with these findings, the antiproliferative effect of VPA in human hepatocellular cancer cells (HUH7) and ovarian cancer SKOV3 cells in a dose and time dependent manner have been reported (24, 25). Other regulatory mechanism of VPA includes histone deacetylases, phosphoinositol and ERK pathway, AKt, GSK3 α and β, the OXPHOS system, and the tricarboxylic acid cycle (26). By increasing expression of DR4 and DR5, it can modulate expression of p21WAF1/CDKN1AAnd sensitize cells to TRAIL/Apo2L-mediated apoptosis (27). In the ERG-positive prostate cancer cells, it induces apoptosis by upregulation of P21/Waf1/CIP1 and repression of TMPRSS2-ERG expression (28). Besides, it acts through down-regulation of TMPRSS2-ERG in prostate cancer (29) and inhibition of Akt1 and Akt2 genes HeLa cervical cancer cells (30-32). Other studies have reported that valproic acid and butyrate induce apoptosis in HeLa cervical cancer cells by inhibition of gene expression of Akt1 and Akt2, which is mediated through the caspase-dependent pathways (16). Increasing of pro-apoptotic proteins such as TRAIL and p21 is one of the molecular mechanisms by which the HDACIs induce apoptosis (33, 34). In the current study, the result showed that tamoxifen inhibits proliferation and induces apoptosis with a time and dose dependent manner. Previous findings indicated that TAM induces apoptosis in hepatocellular carcinoma PLC/PRF5 and HepG 2 cell line (20, 35). Similar to these findings, it has been reported that TAM induces apoptosis in ER positive breast cancer (36). One of the mechanism of apoptotic induction of TAM is caspase-3-like proteases activity induction and c-Jun NH2-terminal kinase (JNK) 1 activation which has been reported in MDA-MB-231 and BT-20 cell line (37). In addition to estrogen receptor (ER) inhibition, which is the primary mechanism of action of TAM, it acts by modulation of signaling proteins such as protein kinase C (PKC), calmodulin, transforming growth factor-β (TGFβ), and the protooncogene c-myc in breast cancer cell (18). In the present experiment, apoptotic effect of VPA was increased when VPA treatment followed by TAM treatment.The similar effect of combined treatment with VPA and TAM has been demonstrated in Ishikawa adenocarcinoma cells (19). Other works have indicated that TAM induces apoptosis in ER positive MCF7 breast cancer cell reactivated by trichostatin A, Tamoxifen mainly binds to ER modulating the ER-mediated transcriptional cascade (38).

Conclusion

In conclusion, VPA induced apoptosis and augmented the apoptotic effect of TAM by reactivation of the ERα gene, suggesting that it could be used in the treatment of HCC alone or in combination with TAM.

Acknowledgments

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Conflict of interest

The authors report no conflict of interest.

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