Previously, we evaluated the effect of trichostatin A (TSA) on the expression of DNA methyltransferase 1 (DNMT1) in Hepatocellular Carcinoma (HCC). Fragile histidine triad (FHIT) and WW domain-containing oxidoreductase (WWOX) are two of the most common down-regulated genes in many cancers located on chromosome 3p14.2 and 16q23.3–24.1 respectively. The aim of the current study was to assess the effect of TSA on these genes expression, cell growth, and apoptosis in HCC WCH 17 cell. The cells were seeded and treated with TSA at different times. Then, MTT assay, flow cytometry, and qRT-PCR were achieved to determine viability, apoptosis and gene expression respectively.

Cell growth was significantly inhibited, 92 to 36% after 24 h, 86 to 28% after 48 h, and 78 to 24% after 72 h. The results of flow cytometry confirmed that TSA increased apoptosis compared to the control group, the apoptosis percentage increased to 12%, 16%, and 18% in comparison to control groups (2%). Significant up-regulation of the genes was observed in all treated groups. We concluded that re-expression of silenced WWOX and FHIT genes could be achieved by TSA resulting in cell growth inhibition and apoptosis induction in WCH 17 cell.

Keywords: Trichostatin A. FHIT. WWOX. Apoptosis. Cancer.
(HDACIs) can restore silenced gene transcription by inducing acetylation of histones, transcription factors and other proteins regulating transcription. These compounds can be structurally grouped into at least four classes including cyclic peptides (e.g. tyrocidine A), hydroximates (e.g. trichostatin A: TSA), aliphatic acids (e.g. valproic acid) and benzamides (e.g. MS-275) (Xu et al., 2007; Kim et al., 2011). It has been reported that TSA can alter the levels of acetylated histones associated with re-expression of specific cancer-related genes such as insulin-like growth factor binding protein 2 (IGFBP2), integrin, basigin (BSG), quiescin Q6 (QSCN6), superoxide dismutase 3 (SOD3), nerve growth factor receptor (NGFR), and p53-induced protein (PIG11) in HCC (Chiba et al., 2004). WW domain-containing oxidoreductase (WWOX, which is located at 16q23.3-24.1) has been reported as a tumor suppressor gene. Several in vivo and in vitro studies have indicated significant down-regulation of WWOX in many cancers and also indicated that up-regulation of WWOX induces apoptosis and inhibits cell growth (Lin et al., 2015). Fragile histidine triad (FHIT) is one of the most common down-regulated genes in many cancers that is located on 3p14.2, a region of high fragility (FRA3B), loss of FHIT protein is one of the earliest landmarks of cancer. The loss of this protein has been reported in several cancers such as breast, lung, stomach, esophagus, pancreas, skin, kidney, cervix, and other epithelial tissues. Similarly, WWOX down-regulation has been demonstrated in a wide variety of human cancers (Karras et al., 2016). Up-regulation of FHIT and WWOX after treatment with TSA has been reported in gastric cancer (He et al., 2015). In HCC HepG2 cells, TSA can inhibit cell growth and induce apoptosis through up-regulation of FHIT gene (Li et al., 2011). Previously, we evaluated the effect of TSA on the expression of DNA methyltransferase 1 (DNMT1) in hepatocellular carcinoma cell line Hepa 1-6 (Sanaei, 2018). With regard to the other results and our previous report, we designed to assess the effect of TSA on FHIT and WWOX expression, cell growth inhibition, and apoptosis induction in hepatocellular carcinoma WCH 17 cell line.

**MATERIAL AND METHODS**

Hepatocellular carcinoma WCH 17 cell line was provided from the National Cell Bank of Iran, Pasteur Institute and were maintained in Dulbecco’s modified Eagle’s medium (DMEM).

Culture media contains fetal bovine serum (FBS), 100 mL/L, and antibiotics including penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in a humidified atmosphere of 5% CO₂. TSA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) in order to prepare a stock solution and then all necessary drug concentrations were provided by diluting the stock solution. All of the other compounds, including fetal bovine serum (FBS), 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Real-time PCR kits (qPCR Master Mix Plus for SYBR Green I dNTP), Total RNA Extraction Kit (TRIZOL reagent), trypsin-EDTA, Annexin-V-(FITC) and propidium iodide were obtained from Sigma (PI, Becton-Dickinson, San Diego, CA). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany).

**Cell growth and viability assay**

The effects of TSA on the WCH 17 cell viability were measured using MTT assay. First, the cells were seeded and cultured at a density of 5 × 10⁵ cells per well into 96-well plates. After 24 h, the culture medium was replaced with a medium containing different doses of TSA (0.5, 1, 2.5, 5, 10, and 25 μM), the control groups were treated with DMSO only, DMSO was present at 0.01-0.3% in the medium based on IC50 index. After treatment times (24, 48, and 72 h), the cells were washed twice with FBS, maintained in a medium containing MTT for 4 h, then the culture media were replaced with 200 μL of DMSO and finally, the optical density was measured at 570 nm with a microplate reader.

**Cell apoptosis assay**

To determine the apoptotic effect, the WCH 17 cells were seeded in 24-well plates at a density of 4 × 10⁵ cells/well and incubated overnight before treating with TSA. After 24 h, the cells treated with TSA (5 μM) at different time periods (24, 48, and 72 h), based on IC50 values. After treatment times, the culture medium was discarded and all the treated or untreated cells were harvested by trypsinization, washed with cold PBS, and then the cells were re-suspended in 0.5 mL of a binding buffer (1x). Finally, Annexin-V-(FITC) and propidium iodide (PI) were added to stain the cells according to the protocol and the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).
Real-Time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

To determine whether TSA could reactivate the FHIT and WWOX genes expression, qRT-PCR was performed. In this regard, the cells were treated with TSA (5 μM, based on IC50 values) for different time periods (24, 48 and 72 h) and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the protocol and treated by Rnase-free DNase (Qiagen) to eliminate the genomic DNA before cDNA synthesis. The RNA concentration and purity were determined using a BioPhotometer (Biowave II Germany). By using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, K1622 for 100 reactions), total RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) using oligo-dT primers and Superscript II Reverse Transcriptase according to the protocol. Real-time RT-PCR reactions for cDNA amplification were performed as previous work (Sanaei et al., 2018) and data were analyzed using the comparative Ct (ΔΔct) method. GAPDH was used as an endogenous control. The primers sequences are shown in table I.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWOX forward</td>
<td>GAGTTCCTGAGCGAGTGGAC located on exon 1</td>
<td>14</td>
</tr>
<tr>
<td>WWOX reverse</td>
<td>CCCAGGAATTCCTGCTT located on exon 9</td>
<td>Park et al., 2004</td>
</tr>
<tr>
<td>FHIT forward</td>
<td>AGCTCACATTCTAGTCAGCCT</td>
<td>Yuan et al., 2000</td>
</tr>
<tr>
<td>FHIT reverse</td>
<td>GCCAATTCCCAGATG</td>
<td>Yuan et al., 2000</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>AGCAAGAGCACAAGAGGAAG</td>
<td>Midorikawa et al., 2009</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>GTCTACATGGCAACTGTTGAG</td>
<td>Midorikawa et al., 2009</td>
</tr>
</tbody>
</table>

Statistical analysis

The database was set up with the SPSS (version 16.0) for analysis. The data were acquired from three tests and were shown as means ± standard deviations. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA) and Turkey tests. A significant difference was considered as P < 0.05.

RESULTS

Effect of TSA on viability of WCH 17 cell

In order to assess the inhibitory effects of TSA on the hepatocellular carcinoma WCH 17 cell using MTT assay, the cells were treated with TSA (0.5, 1, 2.5, 5, 10, and 25 μM) for different time periods, as mentioned above. Under different concentrations of TSA, cell growth was significantly inhibited, 92 to 36% after 24 h (P<0.003), 86 to 28% after 48 h (P<0.001), and 78 to 24% after 72 h (P<0.001) of treatment as shown in figure 1. TSA efficiently inhibited the cell viability in a time- and concentration-dependent manner compared with DMSO-treated groups. The result of MTT assay demonstrated that TSA inhibited cell growth with an IC50 of ~5 μM.
FIGURE 1 – Effect of TSA on WCH 17 cells viability determined by MTT assay. Data are presented as mean ± standard deviation from triplicate wells and three independent experiments. Asterisks (*) indicate significant differences between treated cells and the control group. The first column of each group belongs to the control group.

TSA induces apoptosis in WCH 17 cells

The apoptosis-inducing analysis of TSA on WCH 17 cells was carried out by flow cytometry using Annexin V-FITC and PI staining. After incubation of WCH 17 cells with a single of TSA, flow cytometric analysis was performed to determine whether TSA can induce apoptosis in WCH 17 cells. As shown in Figure 2, the result of flow cytometry confirmed that TSA treatment (5 μM) induced significant apoptosis in WCH 17 cells, the apoptosis percentage increased to 12% (p< 0.001), 16% (p<0.001), and 18% (p<0.001) after 24, 48 and 72 h respectively. Maximal apoptosis was seen in after 72 h of treatment (Figure 3).

FIGURE 2 – The apoptotic effects of TSA with a concentration of 5 μM. The compound induced significant apoptosis in WCH 17 cells at different time periods (24, 48 and 72 h). In the panels, the lower left quadrant shows viable cells, which exclude PI and are negative for FITC-Annexin V binding (annexin V–negative/PI-negative). Apoptotic cells staining with annexin V-FITC, but not PI (annexin V–positive/PI-negative), appear in the lower right (LR) quadrant of data plots. Late apoptotic/necrotic cells appear in the upper right (UR) quadrant, staining with both PI and annexin V-FITC, cells positive for FITC-Annexin V binding and for PI uptake and finally necrotic cells are presented in the left upper quadrant (annexin V–negative/PI-positive).
Effects of trichostatin A on FHIT and WWOX genes expression, cell growth inhibition and apoptosis induction in hepatocellular carcinoma

WCH 17 cell line

FIGURE 3 – Apoptotic effect of TSA after different time periods.

Effect of TSA treatment on the expression of FHIT and WWOX gene expression

To determine relative expression levels of FHIT and WWOX genes in WCH 17 cells treated with TSA (5 μM) for different time periods (24, 48, and 72 h), we performed quantitative real-time PCR using WCH 17 cell line. We observed that FHIT and WWOX genes were significantly up-regulated in WCH 17 cell line. The maximum increase was observed with 5 μM TSA after 72 h as shown in Figure 4. The relative expression of FHIT gene was increased to 1.7, 2.1, and 2.6 (p<0.001) and the relative expression of WWOX gene was increased to 2.3, 3.9, and 4.4 (p<0.001) at different times respectively. The relative expression of WWOX gene was more significant than that of the FHIT gene. Besides, TSA increased FHIT and WWOX genes expression in a time-dependent manner significantly.

FIGURE 4 – Relative expression levels of FHIT and WWOX genes. The cells were treated with TSA (5 μM) for different time periods (24, 48, and 72 h) and Quantitative real-time RT-PCR was done to determine the FHIT and WWOX genes expression. The first column of each group belongs control group and other columns belong various period times (24, 48, and 72 h) respectively. Data are presented as the mean ± standard error of the mean from three different experiments. Asterisks (*) indicate significant differences between treated cells and the control group. The first column of each group belongs to the control groups.
DISCUSSION

Carcinogenesis is a multistep process mediated by several factors such as epigenetic modifications, these modifications have been reported in almost all cancers types. These changes occur in key oncogenes, tumor suppressor genes, resulting in cancer induction and progression. The most commonly epigenetic changes include histone histone lysine methylation and demethylation, DNA methylation, histone lysine acetylation and deacetylation (Shanmugam et al., 2018). WWOX is a candidate tumor suppressor gene which suppresses the transactivation function of several transcription factors implied in neoplasia by sequestering them in the cytoplasm. It participates in a number of cellular processes such as cell growth, differentiation, apoptosis, and tumor suppression (Del Mare et al., 2009). Fragile FHIT gene is another tumor suppressor gene that its product has established its role in protecting against tumorigenesis and tumor development. The loss of FHIT protein expression has been demonstrated in many cancers, FHIT overexpression can induce apoptosis in various cancers (Okumura et al., 2009). The result of the current study indicated that TSA can inhibit cell growth and induce apoptosis in WCH 17 cells significantly. In order to find the molecular mechanism of TSA, we evaluated the expression of WWOX and FHIT genes in this cell line. The result demonstrated that this compound up-regulated WWOX and FHIT genes expression significantly. Inconsistent with our report, overexpression of WWOX gene has been demonstrated in breast cancer BT-474, MDA MB-231, HCC1937 cell lines after treatment with 5-aza-2'-deoxycytidine and TSA resulting in cell growth inhibition and apoptosis induction suppresses cancer cell growth and induces apoptosis in vitro, suggesting that reactivation of the WWOX signal pathway is one of the molecular mechanisms of apoptotic induction in breast cancer (Iliopoulos et al., 2007).

Similarly, up-regulation of the WWOX gene after TSA treatment has been reported in gastric cancer GC cell line (Maeda et al., 2010). In addition, treatment with TSA and 5-aza-2'-deoxycytidine leads to significant up-regulation of WWOX mRNA expression in human prostate LNCaP, DU145, PWR-1E, and PC-3 cell lines and transgenic adenocarcinoma mouse prostate (TRAMP) tissues (Qin et al., 2006). In ovarian cancer cell line HO8910, cell cycle analysis has revealed cell cycle arrest in G0/G1 phase and elevated protein expression level of WWOX after treatment with valproic acid, a histone deacetylase inhibitor (Yan et al., 2012). As mentioned above, we indicated that TSA re-activated FHIT gene expression significantly. Similar to our report, it has been demonstrated that TSA increases the expression of FHIT mRNA and protein by which inhibits the proliferation of breast cancer cell MCF-7 (Liu et al., 2012). Other researchers have reported that TSA increases FHIT mRNA and FHIT protein expression in the human hepatocellular carcinoma HepG2 cell line resulting in inhibition of the enzymatic activity of histone deacetylases and cell proliferation (Wang et al., 2010). In lung cancer H1299 cells, TSA can restore FHIT expression significantly (Cantar et al., 2007). A novel molecular pathway for FHIT-mediated apoptosis signaling in human lung cancer is the inactivation of the PI3K-Akt-survivin pathway. Akt is known as a member of the PI3K family which is activated in response to the generation of phosphatidylinositides and activated Akt in turn signals to a variety of key downstream molecules, including caspase-9, Bad, mTOR and GSK-3, the sum of which is to promote cell survival and to suppress cell death (Semba et al., 2006). In summary, TSA can inhibit cell growth and induce apoptosis by re-activation of WWOX and FHIT genes in HCC WCH 17 cell line.

CONCLUSION

We concluded that re-expression of silenced WWOX and FHIT genes could be achieved by histone deacetylase inhibitor trichostatin A resulting in cell growth inhibition and apoptosis induction in HCC WCH 17 cell line.

ACKNOWLEDGMENT

This article was supported by adjutancy of research of Jahrom University of Medical Sciences, Iran. The article has been extracted from Ms. Hossein Karami’s thesis.

CONFLICT OF INTEREST

The authors report no conflict of interest.

REFERENCES

Effects of trichostatin A on FHIT and WWOX genes expression, cell growth inhibition and apoptosis induction in hepatocellular carcinoma
WCH 17 cell line


Received for publication on 14th January 2019
Accepted for publication on 18th April 2019