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ORIGINAL ARTICLE

Effects and mechanism of suberoylanilide hydroxamic acid on the proliferation and apoptosis of human hepatoma cell line Bel-7402

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Summary

Purpose: *To investigate the effects and mechanism of suberoylanilide hydroxamic acid (SAHA) on the proliferation and apoptosis of human hepatoma cell line Bel-7402.*

Methods: *SAHA treatment and control groups were designed in this study. To observe the morphological characteristics and the inhibition of cell proliferation, we conducted confocal microscopy and methyl thiazolyl tetrazolium (MTT) assay, respectively. Changes in cell apoptosis and cell cycle were then determined by flow cytometry. Real-time polymerase chain reaction (RT-PCR) was also conducted to detect the mRNA expressions of p53, bcl-2 and bax genes. Caspase-3 protein activity was determined by spectrophotometry.*

Results: *Cell proliferation in the SAHA treatment group could be inhibited in a time- and dose-dependent manner. FCM analysis showed that the early apoptosis rate in the SAHA treatment group increased significantly. Furthermore, cell cycle was arrested at the S phase. RT-PCR assay confirmed that SAHA could upregulate the mRNA expressions of p53 and bax genes. By comparison, SAHA could downregulate the mRNA expression of bcl-2. SAHA induced apoptosis by activating the caspase-3 pathway.*

Conclusion: *SAHA inhibited cell proliferation and promoted human hepatoma Bel-7402 cell apoptosis by affecting caspase-3 protein activity and mRNA expressions of p53, bcl-2 and bax genes.*

Key words: *apoptosis, bax, bcl-2, hepatocellular carcinoma, p53, SAHA*

Introduction

The occurrence and development of hepatocellular carcinoma (HCC) involve a complex of various signal transduction pathways disorders, which are closely related to epigenetics. Among these pathways, histone acetylation has been the focus of many studies. In this process, histone acetyltransferase and histone deacetylase (HDAC) are important enzymes whose dysfunction results in tumorigenesis [1,2]. To eradicate tumor cells, researchers investigated SAHA. SAHA is a new class of broad-spectrum HDAC inhibitors that has entered phase I and II clinical trials, showing a broad spectrum of activities against haematological malignancies and solid tumors with better tolerance compared to trichostatin A (TSA) [3-7]. However, the mechanism of SAHA-induced apoptosis in human hepatoma cells remains unclear. The present study aimed to investigate the effect and mechanism of SAHA on human hepatoma Bel-7402 cell line proliferation and apoptosis.

Methods

Cell culture

Adherent cell culture method was used. Bel-7402 cells (Nanjing KGI Biological Technology Development Co., Ltd., China) were cultured in Dulbecco's modified Eagle medium (Sigma, California, USA) with 100 ml/L foetal bovine serum (Sigma, California, USA) and 10 ml/L penicillin-streptomycin antibiotics. The following

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Table 1. Effects of SAHA with different concentrations on Bel-7402 cell proliferation activity (A490 value) (mean±SD)

Group	*12 hrs*	*24 hrs*	*48 hrs*	*72 hrs*
Control	1.108±0.257	1.108±0.257	1.108±0.257	1.108±0.257
2.5μmol/L	0.493±0.150	0.948±0.109	1.012±0.043	0.645±0.072
5.0μmol/L	0.583±0.029	0.918±0.066*	0.794±0.030**	0.553±0.043**
7.5μmol/L	0.745±0.053**	0.865±0.043*	0.565±0.055* *	0.431±0.024**

* $p<0.05$, ** $p<0.01$, compared with the control group
SD: standard deviation

conditions were used: 37 °C; saturated humidity; and 5% CO_2. A control group and SAHA treatment groups with different concentrations and culturing times were established.

Laser confocal microscopy

SAHA (Sigma, USA) with concentrations of 5.0 and 7.5 μmol/L were used to treat Bel-7402 cells. A negative control group was created. The experimental groups were treated for 24 and 48 hrs, and then a laser confocal microscope was used to observe any morphological changes.

Methyl thiazolyl tetrazolium (MTT) assay

Cells in the logarithmic growth phase were selected. The cell density was adjusted to 1×10^4/mL and the cells were then inoculated in a 96-well culture plate (100 μL per well). The cells were routinely cultured for 12 hrs and afterwards SAHA was added to treat the cells at 0, 2.5, 5.0 and 7.5 μmol/L concentrations (3 wells per set). After further cell culture for 12, 24, 48 and 72 hrs, 20 μL of 5 g/L MTT solution was added to each well and the cells were cultured for another 4 hrs. The culture medium was aspirated and discarded after centrifugation; 200 μL of DMSO were then added. Low-speed oscillation was performed for 10 min to allow the blue crystalline particles to dissolve sufficiently. A microplate reader (Leidu Shenzhen Electronics Ltd., China; λ=490 nm) was used to measure the absorbance (A) of each well. The control was then zeroed. Tumor cell growth inhibition rate (%) was estimated as follows: (1–A of SAHA treatment group/A of the control group) × 100.

Flow cytometry

According to the MTT results, 5.0 and 7.5 μmol/L of the effective and less toxic 24 and 48 hrs SAHA treatment groups were selected to continue the experiment. A negative control group was created. Bel-7402 cells were treated with SAHA and routinely cultured; afterwards, single-cell suspension was prepared using 0.25% trypsin. The cells were collected and washed in PBS twice; 500 μL of PBS solution were added to prepare the cell suspension. The solution was transferred into 5 mL of 70% ethanol with a syringe, sealed and fixed overnight at 4 °C. RNase A was added to a final concentration of approximately 50 μg/mL and incubated for 30 min at 37 °C. Propidium iodide (PI) was also added to a final concentration of 65 μg/mL. The solution was then placed in an ice bath and dark-stained for 30 min. The cells were mixed, filtered using a 300-mesh nylon, and tested by flow cytometry (BD Biosciences, New Jersey, USA).

AnnexinV/Pl double staining

The negative control group and the Bel-7402 cells treated with SAHA at different concentrations and for different times were digested and collected using non-EDTA trypsin. The solution was centrifuged and the supernatant was discarded and washed twice with PBS. The cells were resuspended, 5 μL of annexin V-fluorescein isothiocyanate (Annexin V-FITC) were added and mixed, and also 5 μL PI were added and mixed. The solution was incubated at room temperature, protected from light for 15 min and assessed by flow cytometry.

RT-PCR

After 5.0 and 7.5 μmol/L SAHA to treat the Bel-7402 cells for 24 and 48 hrs, the total RNA of the cells in each group was extracted using RNAiso Plus reagent Takara Biotechnology Co, Dalian, China) according to the manufacturer's instructions. Afterwards, 2 μL of total RNA were placed in a 200 μL tube and the appropriate reagents were added in accordance with the reverse transcription kit instructions. A total of 20 μL of the solution was prepared and mixed. The RT conditions for the machine were as follows: incubated for 60 min at 42°C; heated for 5 min at 70 °C to terminate the reaction; and saved at −20 °C for subsequent use. cDNA was then reverse-transcribed. The gene products in each group were amplified by PCR (Fermentas Company, Canada). GAPDH was used as an internal control. The upstream primer was 5′-AGG TCG GAG TCA ACG GAT TTG-3′ and the downstream primer was 5′-GTG ATG GCA TGG ACT GTG GT 3′. The product length was 532 bp. The p53 upstream primer was 5′-CTC CTC AGC ATC TTA TCC GAG T-3′ and the downstream primer was 5′-GCT GTT CCG TCC CAG TAG ATT A-3′. The product length was 239 bp. The Bcl-2 upstream primer was 5′-ATG TGT GTG GAG AGC GTC AAC-3′ and the reverse primer was 5′-AGA GAC AGC CAG GAG AAA TCA AAC-3′. The product length was 182 bp. The Bax upstream primer was 5′-AAG CTG AGC GAG TGT CTC AAG -3′ and the downstream primer was 5'-CAA AGT AGA AAA GGG CGA CAA C-3'. The product length was 178 bp. The amplifi-

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7.5μmol/L	0.745±0.053**	0.865±0.043*	0.565±0.055**	0.431±0.024**

* p<0.05, ** p<0.01, compared with the control group
SD: standard deviation

cation parameters were as follows: 95°C for 3 min, 95°C for 30 s; 54 °C for 30 s; 72 °C for 11 s; and 35 cycles. The PCR amplification product (20 μL) was added to 2% agarose gel to perform electrophoresis. Quantitative images were analyzed by ethidium bromide staining. The results were statistically analyzed after computer image gray scan and digital conversion. The relative expression of the target gene was expressed by the ratio of the optical density (OD) of the target gene band and the OD of the GAPDH band.

Caspase-3 protein activity detection

SAHA (5.0 and 7.5 μmol/L) was used to treat cells for 24 and 48 hrs, respectively. All of the adherent and suspended cells were collected. Caspase-3 was detected using caspase-3 spectrophotometric ion kit (Nanjing KGI Biotechnology Development Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Absorbance was determined using a microplate reader (λ=450 nm). The degree of caspase-3 protein activation was determined by calculating the multiples of the OD for the treated and control groups.

Statistics

One-way ANOVA method was used to analyse all values of data, which were expressed as mean±standard deviation. A p-value <0.05 was considered statistically significant.

SPSS 17.0 software (SPSS Inc., Chicago, IL) was used in statistical analyses.

Results

Morphological changes

Confocal microscopy revealed that the cells in the control group were spindle-shaped and the cell bodies were large, highly proliferative and closely adherent. Fewer cells were detected after SAHA treatment. These cells showed smaller cell bodies and exhibited shrinkage. An increased number of floating cells and decreased adhesion ability were observed.

Detection of inhibition rate for cell proliferation by MTT assay

Bel-7402 cells were treated with >5.0 μmol/L SAHA for 24 hrs. The results showed that the cell activity (OD) was significantly lower and decreased significantly as concentration and culturing time increased. Hence, cell proliferation inhibition increased as concentration and culturing time increased. The p-values of the groups treated for 24 and 48 hrs were 0.037 and 0.009, respectively, for the group treated with 5.0 μmol/L SAHA, and 0.018 and 0.000, respectively, for the group treated with 7.5 μmol/L. The differences were statistically significant (Table 1). To calculate the rate at which SAHA inhibited the growth of Bel-7402 cells, the rates of inhibition of the Bel-7402 cells treated with 5.0 mol/L and 7.5 mol/L SAHA for 48 hrs (28.6% and 49.9%, respectively) were compared with those treated for 24 hrs (24.6% and 30.3%, respectively). The inhibition rate was significantly increased. The experiments showed that SAHA could inhibit the proliferation of human hepatoma Bel-7402 cells in a time- and dose-dependent manner.

Detection of cell cycle and apoptosis using flow cytometry

After treatment with different concentrations of SAHA (5.0 and 7.5 μmol/L) for 24 hrs, the S phase cells significantly increased in number from 24.320±0.170% to 32.060±0.160% and 33.983±0.204% (p=0.000), respectively. For the cells treated for 48 hrs, the S phase cells significantly increased in number from 24.332±1.180% to 32.251±0.533% and 33.610±0.081% (p=0.000), respectively. The differences were statistically significant. The cell cycle was arrested in the S phase (Table 2). The AnnexinV/Pl double staining results showed that the early apoptosis rate (p=0.030, p=0.000) and the middle and late apoptosis rates (p=0.000) in the groups treated with 5.0 and 7.5 μmol/L SAHA for 24 hrs increased significantly compared with those in the control group. The differences were statistically significant (Figure 1; Table 3).

Detection of mRNA expressions for apoptosis-related genes (p53, bcl-2, and bax) using RT-PCR

After treatment with SAHA at different con-

Table 2. Effects of SAHA with different concentrations on Bel-7402 cell proliferation activity (A490 value) (mean±SD)

Time	*Cell cycle*		*SAHA (µmol/L)*	
		Control	*5.0*	*7.5*
24 hrs	G0/G1	54.627±0.93	55.450±0.580	53.360±0.880
	S	24.320±0.170	32.060±0.160*	33.983±0.204*
	G2/M	21.043±1.100	11.300±2.057*	12.560±1.230*
48 hrs	G0/G1	54.660±0.700	55.360±0.480	53.480±0.640
	S	24.332±1.180	32.251±0.533*	33.610±0.081*
	G2/M	21.010±0.480	10.030±0.560*	14.440±0.800*

* p<0.01, compared with the control group
SD: standard deviation

Table 3. Effects of SAHA with different concentrations on Bel-7402 cells apoptosis (mean±SD)

Group	*24 hrs*		*48 hrs*	
	Early stage	*Middle and late stage*	*Early stage*	*Middle and late stage*
Control	0.192±0.040	0.130±0.030	0.192±0.040	0.130±0.030
5.0µmol/L	1.657±0.592*	3.240±0.240**	14.470±2.260**	13.940±1.050**
7.5µmol/L	8.930±0.940**	6.960±0.710**	26.250±0.550**	24.270±0.590**

* p<0.05, ** p<0.01 compared with the control group
SD: standard deviation

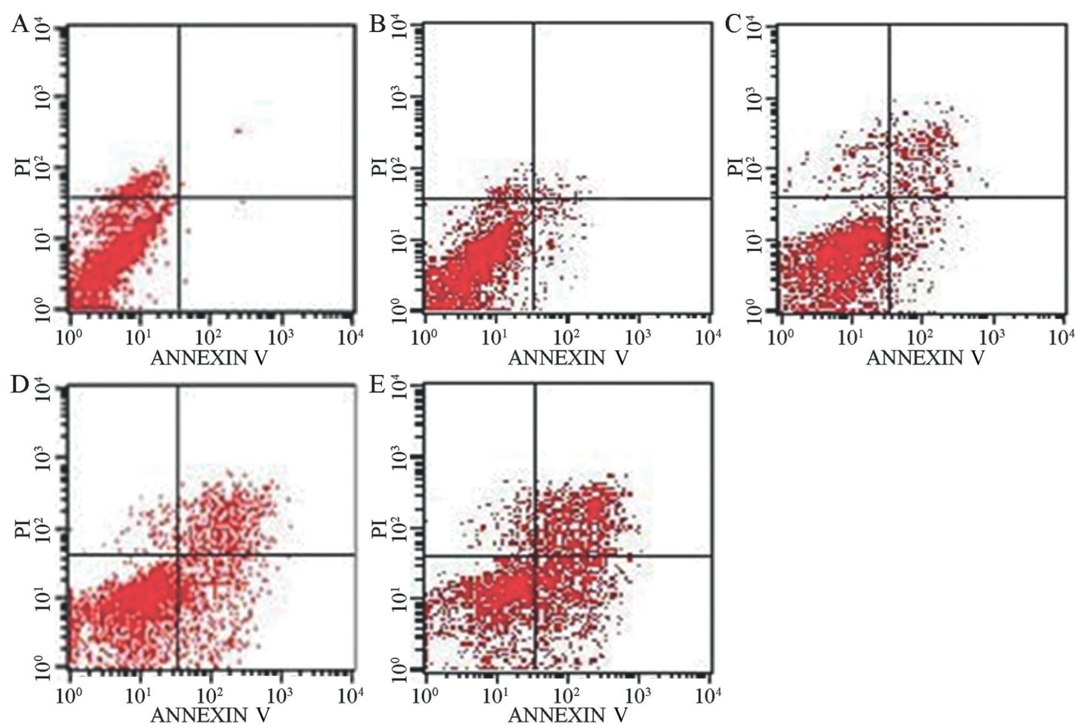

Figure 1. Flow cytometry showing the effects of SAHA with different concentrations on Bel-7402 cell apoptosis. **A:** Control group; **B:** 5.0 µmol/L 24 hrs; **C:** 7.5 µmol/L 24 hrs; **D:** 5.0 µmol/L 48 hrs; **E:** 7.5 µmol/L 48 hrs. The cell apoptosis increased with the increase of dose and time

centrations (5.0 and 7.5 µmol/L) for 24 hrs, the p53 mRNA expression level increased from 1.026±0.046 to 1.569±0.228 and 1.738±0.172, respectively; the differences were statistically significant (p=0.008, p=0.002). The bax mRNA expression level increased from 0.284±0.002 to 0.735±0.022 and 0.843±0.054, respectively; the differences were statistically significant (p=0.000). The bcl-2 mRNA expression level increased from 0.715±0.067 to 0.548±0.009 and 0.436±0.045; the differences were statistically significant (p=0.002, p=0.000). After treatment for 48 hrs, the p53 mRNA expression level increased to 2.813±0.287 and 3.266±0.154, respective-

ly; the differences were statistically significant (p=0.000). Bax mRNA expression level increased to 1.420±0.063 and 1.660±0.190, respectively; the differences were statistically significant (p=0.000). bcl-2 mRNA expression level decreased to 0.367±0.007 and 0.242±0.001, respectively; the differences were statistically significant (p=0.000; Figure 2).

Effect of SAHA on caspase-3 protein activity of Bel-7402 cells

After treatment with SAHA at different concentrations (5.0 and 7.5 μmol/L) for 24 hrs, caspase-3 protein activity increased from 0.415±0.068 to 0.825±0.022 and 1.066±0.206, respectively; the differences were statistically significant (p=0.009; p=0.001). After treatment for 48 hrs, caspase-3 protein activity increased from 0.415±0.068 to 1.433±0.229 and 2.032±0.014, respectively; the differences were statistically significant (p=0.000).

Discussion

As a hydroxylamine HDAC inhibitor, SAHA highly induces histone acetylation by inhibiting HDAC activity; as a result, transcription factors bind to the DNA and stimulate specific gene expression to inhibit tumor cell proliferation, arrest the cell cycle and induce apoptosis, thereby blocking tumor cell growth [8-10].

SAHA was experimentally observed to inhibit cell proliferation, reduce the number of cells, reduce the size of the cell body, increase the number of floating cells and decrease adhesion. The observed inhibition increased with SAHA concentration and culturing time. In the present study, SAHA decreased Bel-7402 cell activity ($p<0.05$ or $p<0.01$) and inhibited cell proliferation in a time- and dose-dependent manner. After the cells were treated with different concentrations of SAHA (5.0 and 7.5 μmol/L) for 24 hrs, the early apoptotic rate increased from 0.192±0.040% to 1.657±0.592% ($p<0.05$) and 8.930±0.940% ($p<0.01$), respectively. After the cells were treated for 48 hrs, the early apoptotic rate increased to 14.470±2.260% and 26.250±0.550% ($p<0.01$), respectively. The differences were statistically significant, suggesting that SAHA may inhibit HDAC1 expression and histone deacetylation; SAHA may also stimulate the p21 tumor suppressor gene [11]. Previous studies have shown that various drugs can induce cell apoptosis of SMMC-7721 cells in the Bel-7402 human hepatoma cell line or breast cancer MCF-7 cell line by arresting the cell cycle in the S phase; this condition inhibits cell growth [12-15]. The experimental results also showed that the number of S phase cells increased significantly ($p<0.01$) after the Bel-7402 cells were treated with different concentrations of SAHA (5.0 and 7.5 μmol/L) for 24 and 48 hrs, thereby inhibiting the growth of human hepatoma Bel-7402 cell line and inducing apoptosis. This result is consistent with that in a previous study [16], where the DNA replication fork collides with chemotherapy drug complexes and initiates cell-specific cytotoxicity of the S phase, thereby arresting the cell cycle in the S phase.

However, the major process of inducing apoptosis involves the activation of a caspase protein cascade; this activation is currently the only mechanism that stimulates this cascade [17]. Once this cascade is activated, caspase protein activates the downstream caspase proteins, leading to apoptosis [12,18]. In this experiment, caspase-3 protein activity increased significantly ($p<0.01$) compared with the control group after the Bel-7402 cells were treated with different concentrations of SAHA (5.0 and 7.5 μmol/L) for 24 and 48 hrs. Proteins in the Bcl-2 family, which are classi-

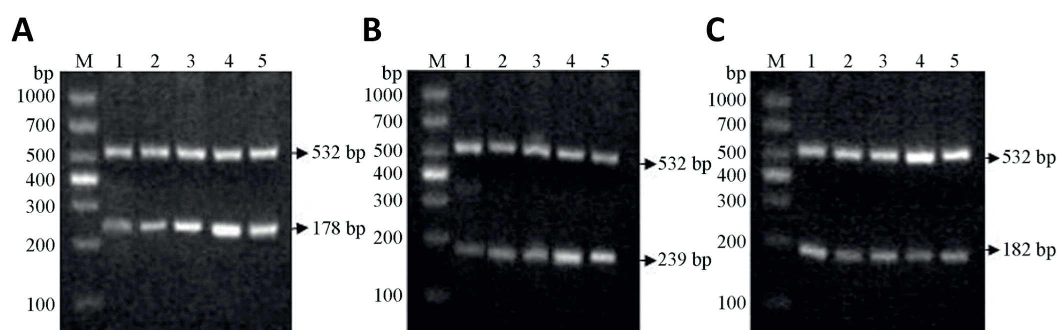

Figure 2. Effects of SAHA on mRNA expression of Bel-7402 cell apoptosis-related genes. **A:** p53 gene. **B:** Bax gene. **C:** Bcl-2 gene. **1:** Control group; **2:** 5.0 μmol/L 24 hrs; **3:** 7.5 μmol/L 24 hrs; **4:** 5.0 μmol/L 48 hrs; **5:** 7.5 μmol/L 48 hrs. The expression levels of p53 and Bax increased, but the expression level of Bcl-2 decreased with the increase of dose and time.

fied into anti-apoptotic (e.g., bcl-2 and bcl-XL) and pro-apoptotic (e.g., bax and bak) proteins, exhibit a major function in the mitochondrial pathway of apoptosis. The ratio of bax to bcl-2 also influences the regulation of the apoptosis program. A large number of studies focused on the functions of genes, such as bcl-2 and cyclinD1 in adrenal tumors, bladder cancer and other malignancies. This result demonstrated that bcl-2 and cyclinD1 exhibit high expression in these tumors. Bcl-2 binds to 1.4.5 inositol triphosphate (IP3) receptor to hinder IP3 cell signal transduction pathways and the release of endoplasmic reticulum Ca^{2+}, thereby inhibiting apoptosis [19-21].

p53 is a common precipitating factor of apoptosis, which is considered as the executioner of apoptosis [22]. In the current experiment, the mRNA expression of p53 was significantly higher ($p<0.01$) in the SAHA treatment group than in the control group. Thus, SAHA may increase the mRNA expression of bax ($p<0.01$) and decrease the mRNA expression of bcl-2 ($p<0.01$) possibly by activating p53 gene expression; as a result, caspase-3-dependent mitochondrial apoptotic pathway is activated ($p<0.05$) and Bel-7402 cell apoptosis is induced [23-26].

Further studies should be conducted to determine the clinical efficacy of SAHA on patients with HCC. Further studies should also be performed to investigate whether or not this efficacy is associated with clinicopathological parameters and whether or not the patients exhibit adverse reactions. Other aspects of the treatment in question should also be analysed.

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