Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication

Ying-Chun Wang, Xu Yang, Lan-Hua Xing, Wei-Zong Kong

Ying-Chun Wang, Xu Yang, Lan-Hua Xing, Wei-Zong Kong, Department of Gastroenterology, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, Liaoning Province, China

Author contributions: Wang YC designed research and analyzed data; Yang X performed research, analyzed data and wrote the paper; Xing LH performed research; Kong WZ performed research.

Correspondence to: Ying-Chun Wang, MD, Professor, Department of Gastroenterology, Affiliated Zhongshan Hospital of Dalian University, No. 6 Jiefang Street, Zhongshan District, Dalian 116001, Liaoning Province, China. wych_1648@126.com

Telephone: +86-411-62893717 Fax: +86-411-62893555

Received: May 20, 2013 Revised: July 4, 2013

Accepted: July 12, 2013

Abstract

AIM: To investigate the effects of suberoylanilide hydroxamic acid (SAHA) on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and hepatitis B virus (HBV) replication.

METHODS: HepG2.2.15 cells were treated with different concentrations of SAHA. Cell morphology was examined by confocal laser scanning microscopy, and cell proliferation was determined using a MTT colorimetric assay. Flow cytometry was used to detect apoptosis and determine cell cycle phase, while hepatitis B surface antigen and hepatitis B e antigen content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure hepatitis B virus (HBV) DNA in cell lysate.

RESULTS: Cell proliferation rates were significantly reduced by the addition of SAHA. The inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment with SAHA, the early cell apoptotic rate increased from 3.25% to 21.02% (P = 0.049). After 48 h of treatment, hepatitis B surface antigen and hepatitis B e antigen content increased from 12.33 ± 0.62 to 25.42 ± 2.67 (P = 0.020) and 28.92 ± 1.24 to 50.48 ± 1.85 (P = 0.026), respectively. Furthermore, HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 (P = 0.029).

CONCLUSION: SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Key words: Human hepatocellular carcinoma; HepG2.2.15 cells; Suberoylanilide hydroxamic acid; Hepatitis B virus

Core tip: HepG2.2.15 cells were treated with different concentrations of suberoylanilide hydroxamic acid (SAHA). Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure hepatitis B virus (HBV) DNA in cell lysate. Results found that, the inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment, the early cell apoptotic rate increased. After 48 h of treatment, HBsAg and HBeAg content both increased. Furthermore, HBV DNA content increased. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.
INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. The worldwide incidence of HCC ranks fifth out of all malignant tumors, and the number of patients with HCC in China accounts for more than half of total cases in the world[1]. Etiological factors of HCC vary for different countries and areas. Histone deacetylase inhibitors (HDACIs) are a series of new anti-cancer drugs with a wide scope of application. In recent years, HDACIs have generated considerable interest due to their high efficiency to inhibit a variety of solid tumors with low toxicity[2-6]. In the current study, the effects of suberoylanilide hydroxamic acid (SAHA), a potent HDAC-1, on proliferation and apoptosis of human HCC cells HepG2.2.15 and hepatitis B virus (HBV) replication were investigated. The study objective was to characterize a potentially new treatment option for HCC.

MATERIALS AND METHODS

Cell culture and treatment

HepG2.2.15 cells (obtained from the Cell Center of the Chinese Academy of Medical Sciences; prepared by transfection of HepG2 cells with HBV genome) were maintained in DMEM (HyClone Laboratories, Inc., New England, United States) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin and 380 mg/L G418 in a thermostatic and sealed incubator (37 ℃, 5% CO₂). About 100 mmol/L SAHA (Sigma-Aldrich Corp, Missouri, United States) in dimethylsulfoxide (DMSO) was prepared and stored at -20 ℃ until further use. HepG2.2.15 cells were divided into a control group and several treatment groups to receive different concentrations of SAHA. The adherent cells were washed down with 0.25% trypsin, followed by passage.

Inhibition of cell proliferation

An MTT colorimetric assay was used to monitor inhibition of cell proliferation by the addition of different concentrations of SAHA to cell culture medium. For three 96-well plates, 100 μL HepG2.2.15 cells (1 × 10⁴ cells/mL) was added to each well and incubated for 12 h at 37 ℃ in 5% CO₂. Once cells were adhered to the wells, SAHA was added to a final concentration of 2.5, 5, 7.5 or 10 μmol/L. Wells without SAHA were used as negative controls. After the addition of SAHA, a culture plate was incubated for 24, 48 or 72 h. Cell morphology was examined by confocal laser scanning microscopy (CLSM). Then, 20 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h, 150 μL of DMSO was added, followed by mixing for 10 min. Lastly, absorbance (A) at 490 nm was measured using a microplate reader. The inhibition rate of cell proliferation was calculated as follows: Cell proliferation inhibition rate (%) = (1 - A_SAHAGroup / A_Negative control group) × 100%.

Detection of apoptosis and determination of cell cycle phase

Control group and SAHA groups (2.5 and 5 μmol/L) were cultured for 24 and 48 h respectively. The single cell suspension was then prepared. After centrifugation at 2000 g for 5 min, the cell pellet was resuspended in 0.5 mL PBS (final concentration, 1 × 10⁵ cells/mL). For detection of apoptosis, binding buffer (500 μL) and 5 μL annexin V-fluorescein isothiocyanate (Annexin V-FITC) were added to the cell resuspension, followed by 5 μL propidium iodide (PI) (Nanjing KGI Biological Technology Co., Ltd., Nanjing, China). After incubation for 5-15 min (room temperature, avoiding light), samples were subjected to flow cytometry (FCM). For determination of cell cycle phase, 5 mL of obtained cell resuspension was infused into 70% cold ethanol, followed by fixation at 4 ℃ overnight. During the next day, the cell solution was centrifuged at 800 r/min for 15 min, followed by two phosphate buffer saline (PBS) washes and resuspension in 0.4 mL PBS. RNaseA was added to a final concentration of 50 μg/mL, followed by digestion for 30 min in a 37 ℃ water bath. Lastly, PI was added to a final concentration of 65 μg/mL, followed by incubation for 30 min. After filtration through a nylon mesh, FCM was conducted.

Determination of hepatitis B surface antigen and hepatitis B e antigen content

HepG2.2.15 cells (2.5 × 10³ cells/mL) were plated onto a 6-well plate. In triplicate, 1 μL SAHA (7.5 μmol/L) or an equivalent volume of DMSO was added to an individual well. After incubation for 72 h, cells were centrifuged at 800 r/min for 5 min. The supernatant was collected, and the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen content were quantitated using quantitative chemiluminescence detection kits in i4000s R automatic chemiluminescence immunoassay analyser (R.D. Abbott Company, Inc., California, United States).

Determination of HBV DNA content

A 6-well cell culture plate was prepared as previously described above. HBV negative and positive controls were prepared as follows: 100 μL of cell supernatant was mixed with an equal amount of DNA extraction liquid (shaking for 15 s), followed by centrifugation at 12000 g for 10 min to remove supernatant. Then, 20 μL of DNA extraction liquid was added to the sediment, followed by incubation for 10 min in a 100 ℃ water bath.

HBV-polymerase chain reaction (PCR) reaction liquid (35.6 μL) and Taq enzyme (0.4 μL) were mixed in a 0.2 mL Eppendorf tube. Two μL of treated sample supernatant was then added to each tube and centrifuged at 8000 g for several seconds. Quantitative fluorescent PCR was performed under the following conditions: 95 ℃ for 3 min; 94 ℃ for 15 s (40 cycles); 60 ℃ for 30 s (40 cycles).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis
was performed using SPSS 16.0 statistical software. Single factor analysis of variance and t tests were conducted for comparison among multiple groups. P < 0.05 was considered as statistically significant.

RESULTS

Effect of SAHA on cell morphology
CLSM showed that proliferation of untreated HepG2.2.15 cells was rapid, and the cells were compactly arranged. The adjacent cells fused into pieces, with clear edge. The cytoplasm was small, with a large nucleus. In SAHA-treated groups, cell proliferation rate was significantly slower. There were sparse adherent cells, with blurred configuration. The cytoplasm increased, presenting morphological changes similar to normal cells (Figure 1). 

Effect of SAHA on cell proliferation
Multiple concentrations of SAHA could inhibit proliferation of HepG2.2.15 cells, and the inhibitory rate increased with increasing concentrations of SAHA (P < 0.05). With each SAHA concentration, the inhibition rate gradually increased with prolonged treatment time (P < 0.05). Taken together, the inhibitory effect of SAHA on cell proliferation was time- and dose-dependent (Table 1).

Effect of SAHA on cell apoptosis and cell cycle
After 24 h of treatment with 2.5 μmol/L SAHA, early apoptosis rate of HepG2.2.15 cells increased from 3.25% to 16.28% (P = 0.032), and the middle-late apoptosis rate increased from 1.08% to 5.16% (P = 0.035). In the 5 μmol/L SAHA group, early and middle-late apoptosis rate increased from 3.25% to 21.02% (P = 0.041) and 1.08% to 10.70% (P = 0.045), respectively (Table 2 and Figure 2). After 24 h of treatment with 2.5 and 5 μmol/L SAHA, the proportion of G0/G1 phase cells increased from 50.3% to 65.3% and 66.3%, respectively, and the proportion of S phase cells decreased from 34.9% to 20.6%, respectively. Most cells were arrested in the G0/G1 phase (Table 3).

HBsAg and HBeAg content and HBV DNA content
Positive expression of HBsAg and HBeAg in the SAHA group and control group, respectively, was observed. After 48 h of treatment with SAHA, HBsAg and HBeAg content increased from 12.33 ± 0.62 to 25.42 ± 2.67 (P = 0.020) and 28.92 ± 1.24 to 50.48 ± 1.85 (P = 0.026), respectively, and HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 (P = 0.029).

DISCUSSION
Abnormality of any step of epigenetics can affect gene
expression or function, leading to the occurrence of
disease, such as cancer. As a main epigenetic pattern,
histone acetylation is closely related with tumor occur-
rence. HDACIs are often used to alter histone acetylation
for treatment of cancer.[9]. SAHA is a broad-spectrum
HDACI, and was approved for treating T-cell lymphoma
in 2006 (in phase I and II clinical trial). It has obvious
inhibitory effects on histone deacetylase, and can inhibit
the growth of HCC cells by arresting cell cycle progression
and inducing cell differentiation and apoptosis. HDACIs
have been shown to exhibit a broad-spectrum inhibitory activity on blood and solid tumors.[10-11].

Unrestricted division and proliferation is an important
feature of tumor cells. Detection of an inhibitory effect
on tumor cell proliferation is a basic index for screening
of anti-tumor drugs. In this study, CLSM and a MTT
colorimetric assay were used to show that cell prolifera-
tion rates was significantly decreased by treatment with
SAHA. Specifically, the number of cells was reduced, and
adherent cells became sparse. Time- and dose-dependen-
cies of SAHA inhibition on cell proliferation were evi-
dent. The cytoplasm increases, presenting morphological changes similar to normal cells, which is consistent with results from a previous study.[2].

Apoptosis is programmed cell death. The process of
apoptosis and the clearance of apoptotic cells is one of
the most important factors for maintaining liver health.
In this study, after 24 h of treatment with 5 μmol/L
SAHA, early apoptosis rate and middle-late apopto-
sis rate of HepG2.2.15 cells increased from 3.25% to
21.02% (P = 0.029) and 1.08% to 10.70% (P = 0.045),
respectively, indicating that SAHA may interfere with
the balance between apoptosis and anti-apoptosis, induce
the expressions of pro-apoptotic genes (Bmf, Bim, TRAIL,
and DR3)[13], and activate the expression of transcription
factor E2F1. Furthermore, SAHA can induce the ex-
pression of apoptosis signal-regulating kinase 1 (ASK1),
which promotes apoptosis of tumor cells through the
death receptor and intracellular apoptotic pathways.[14,15].
In addition, SAHA can activate the expression of pro-
apoptotic proteins, including Bax and Bak, and inhibit ex-
pression of anti-apoptosis proteins, including Bcl-2 and
Bcl-xL, thus inducing apoptosis of tumor cells.[16]. In the
extracellular apoptotic pathway, activated caspase-8 can
cleave BID to truncated BID (tBid), as well as cause\nCyt C release and Bax expression, leading to activation of cas-
pase-9 and caspase-3. Caspase-3 can promote activation
and cleavage of PARP to subsequently activate the intra-
cellular apoptotic pathway.[17]. SAHA has been shown to
induce transcription of CDK inhibitor p21/waf1 in T24
bladder cancer cells, reducing proliferation and increasing
apoptosis.[14].

Abnormality of cell cycle regulation is one of intrinsic
factors for tumor occurrence. HDACIs can arrest tumor
 cell cycle, inhibiting growth. Results of this study show
that, after 24 h of treatment with 5 μmol/L SAHA, the
proportion of G0/G1 phase cells increases from 50.3%
to 65.3%, and the proportion of S phase cells decreases
from 34.9% to 20.6%. Most cells were arrested in the
G0/G1 phase and induced to undergo apoptosis. This
may be related to increased expression of CDK inhibitor
p21/waf1, which is induced by SAHA treatment. Nearly
all HDACIs can induce expression of p21/waf1 to inhibit
the activities of cyclin and CDK, resulting in cell
cycle arrest and inhibition of differentiation. In addition,
SAHA has been shown to influence expression of p27.
After SAHA treatment, the degree of histone acetylation
is elevated, stabilizing the activity of p53 (an important
intracellular tumor suppressor protein) and leading to
cell cycle arrest.[18-20]. The Ras-Raf-MEK-ERK pathway is
closely related with tumor cell proliferation. ERK can be
activated by various growth factors, leading to interaction
with transcription factors (mitogen, c-Jun, c-fos, c-Myc,
cERK1) and nuclear proteins to promote the transcrip-
tion and expression of a variety of oncogenes and genes
related to cell cycle regulation, thus promoting cell prolif-
eration and inhibiting apoptosis.[21-22].

HBV is a risk factor for development of HCC. An
epidemiological survey demonstrated that the carrying
rate of HBsAg in China is 7.18%. HBV can be actively
replicated in patients with HCC, causing further liver damage.\(^\text{24-27}\) HepG2.2.15 cells can continuously excrete intact HBV Dane particles into culture media. Upon treatment with SAHA, HBsAg and HBeAg content were 2.06 and 1.75 times greater than the control group, respectively, and HBV DNA content was 1.83 times greater than the control group. Taken together, SAHA stimulated replication of HBV. Histone acetylation is involved in regulation of gene transcription. After treatment with SAHA, the level of histone acetylation in HBV DNA is increased, and chromosome structure became incompact. This facilitates the combination of transcription factor with HBV DNA polymerase, thus stimulating HBV replication. However, this mechanism needs further validation. SAHA is an effective drug for HBV-negative HCC patients, but should be cautiously used in HBV-positive HCC patients in combination with anti-HBV drugs.

**COMMENTS**

**Background**

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Its occurrence is related to the multiple-step development process of different genetic alterations. At present, there is no effective treatment method. Suberoylanilide hydroxamic acid (SAHA) is a newly discovered anti-tumor drug which has broad application prospect. It exhibits inhibitory effect of tumor growth, which is been further confirmed in clinical trials.

**Research frontiers**

Histone deacetylase inhibitors (HDACis) are a class of new anticancer drugs emerging in recent years, which has attracted widespread attention. Previous clinical trials find that, SAHA has broad-spectrum anti-hematological and solid tumor activities, with good tolerance. However, the effect of SAHA on hepatitis B virus (HBV) replication has not been reported.

**Innovations and breakthroughs**

SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

**Applications**

SAHA has been applied in previous clinical trials. Results show that, it has broad-spectrum anti-hematological and solid tumor activities. SAHA can inhibit HepG2.2.15 cell proliferation, deduce the differentiation, and promote the apoptosis. At the same time, it can stimulate the replication of HBV. Therefore, SAHA should be cautiously used for treatment of HCC, and be combined with anti-HBV drugs if necessary. It can be used in the treatment of HBV-negative HCC patients.

**Peer review**

HCC is one of the most common malignant tumors, and HDACis are a series of new anticancer drugs with wide scope of application. In this manuscript, the authors investigated the effects of suberoylanilide hydroxamic acid, a potent HDACi on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and HBV replication. The manuscript is very well written.

**REFERENCES**

1. Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol* 2001; 2: 533-543 [PMID: 11905707 DOI: 10.1016/S1470-2247(01)00486-7]

2. Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell* 2003; 4: 13-18 [PMID: 12892709 DOI: 10.1016/S1535-6108(03)00165-X]

3. Kelly WK, O’Connor OA, Marks PA. Histone deacetylase inhibitors: from target to clinical trials. *Expert Opin Investig Drugs* 2002; 11: 1695-1713 [PMID: 12457432 DOI: 10.1517/13
Wang YC et al. Effects of SAHA on HBV replication

Inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. *Mol Cancer Ther* 2002; 1: 937-941 [PMID: 12481415]

19 Hirata H, Hinoda Y, Nakajima K, Kawamoto K, Kikuno N, Ueno K, Yamamura S, Zaman MS, Khatri G, Chen Y, Saini S, Majid S, Deng G, Ishii N, Daiya R. Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. *Int J Cancer* 2011; 128: 1793-1803 [PMID: 20549706 DOI: 10.1002/ijc.25507]

15 Belinsky SA, Grimes MJ, Picchi MA, Mitchell HD, Stidley CA, Tesfaigzi Y, Channell MM, Liu Y, Casero RA, Baylin SB, Reed MD, Tellez CS, March TH. Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer Res* 2011; 71: 454-462 [PMID: 21224363 DOI: 10.1158/0008-5472.CAN-10-3184]

22 Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* 2010; 1802: 396-405 [PMID: 20079433 DOI: 10.1016/j.bbadis.2009.12.009]

23 Xu Q, Lu R, Zhu ZF, Ly JQ, Wang LJ, Zhang W, Hu JW, Meng J, Lin G, Yao Z. Effects of tyroservatide on histone acetylation in lung carcinoma cells. *Int J Cancer* 2011; 128: 460-472 [PMID: 20309941 DOI: 10.1002/ijc.25346]

24 Chuang C, Lin SH, Huang F, Pan J, Josic D, Yu-Lee LY. Acetylation of RNA processing proteins and cell cycle proteins in mitosis. *J Proteome Res* 2010; 9: 4554-4564 [PMID: 20812760 DOI: 10.1021/pr100281h]

24 Wands JR. Prevention of hepatocellular carcinoma. *N Engl J Med* 2004; 351: 1567-1570 [PMID: 15470221 DOI: 10.1056/NEJMe048237]

25 Zhuang H. Chronic hepatitis B virus infection, its treatment and prevention. *Zhonghua Ganzangbing Zazhi* 2005; 13: 324-325 [PMID: 15918962]

26 Tian L, He S, Li X, Hu WY, Peng PL, Wang F, Gao CY, Ren H, Tang KF. Long-fragment RNA inhibits hepatitis B virus gene replication and expression in HepG2.2.15 cells. *Zhonghua Ganzangbing Zazhi* 2011; 19: 44-47 [PMID: 21272458 DOI: 10.3760/cma.j.issn.1007-3418.2011.01.012]

27 Han YF, Zhao J, Ma LY, Yin JH, Chang WJ, Zhang HW, Cao GW. Factors predicting occurrence and prognosis of hepatitis-B-virus-related hepatocellular carcinoma. *World J Gastroenterol* 2011; 17: 4258-4270 [PMID: 22090781 DOI: 10.3748/wjg.v17.i38.4258]

P- Reviewers Balachandar V, Germani G  S- Editor Wang JL  L- Editor A  E- Editor Li JY
