Original Article
Effect of histone deacetylase inhibitor SAHA combined with sorafenib on HepG2 cells

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Abstract: This research aimed to investigate the effect of histone deacetylase inhibitor suberoylamilide hydroxamic acid (SAHA) combined with sorafenib (SRFN) on the proliferation, apoptosis, migration, and invasion of cultured HepG2 cells. After treatment with SAHA, SRFN, or SAHA combined with SRFN, the proliferation, cycle/apoptosis, and migration/invasion related protein expression of HepG2 cells was investigated using MTT, flow cytometry, scratch wound, and Western blot assays. Within the concentration range investigated, SAHA or SRFN inhibited the proliferation of HepG2 cells in a concentration and time-dependent manner, and SAHA combined with SRFN enhanced the anti-proliferative effect. After treatment with SAHA, SRFN or SAHA combined with SRFN, the G0/G1 phase of cell cycle in HepG2 cells increased by 87.16, 68.81 or 109.17%, respectively, compared with control group. The apoptotic rate of HepG2 cells in SAHA or SRFN group (19.72 or 12.01%) was significantly ($P < 0.05$) higher than that of control group (4.67%), but significantly ($P < 0.05$) lower than that of the SAHA combined with SRFN group (31.00%). SAHA, SRFN, or SAHA combined with SRFN significantly ($P < 0.05$) up-regulated the expression of p21 and E-cadherin proteins and down-regulated the expression of Cyclin D1, Bcl-2, MMP-2, and vimentin proteins without effect on the expression of Bax and MMP-9 proteins. SAHA or SRFN inhibited the proliferation, migration and invasion of HepG2 cells and promoted their apoptosis by up-regulating the expression of p21 and E-cadherin proteins and down-regulating the expression of Cyclin D1, Bcl-2, MMP-2, and vimentin proteins. There was a synergistic or additive effect for these above effects between SAHA and SRFN.

Keywords: Histone deacetylase inhibitor SAHA, sorafenib, hepatoma, drug combination

Introduction

The mortality rate of hepatocellular carcinoma (HCC), one of the most common malignant tumors, is very high in China. Annual new cases of HCC in China occupy up to 45% around the world, and now the incidence of hepatoma in China is the highest in the world [1]. The first cause of mortality among malignant tumors in rural is HCC, which is the second mortality cause in urban areas. Surgical treatments are the first-line therapeutic protocols for HCC at present, but 70% of patients diagnosed with HCC have lost the opportunity of surgical treatments because HCC is characterized by high degree of deterioration, concealment of pathogenesis, and low diagnostic rate in early stage [2]. Therefore, it is urgent to investigate the non-operative therapies for HCC. Histone deacetylase inhibitor (HDACI) suberoylamilide

hydroxamic acid (SAHA) and sorafenib (SRFN) are clinically commonly used drugs for HCC, and they are mainly used to treat some HCC patients who cannot be treated with surgical treatments [3, 4].

Histone deacetylase (HDAC) plays an important role in structural modification and regulation of gene expression of chromosome. The present investigation shows that malignant cell transformation can significantly enhance HDAC activity, which can result in disequilibrium of gene expression. Hence, HDAC is a potential anti-tumor target [5], and its inhibitor has been developed for treating cancer. A number of clinical trials have already confirmed the clinical therapeutic effect of SRFN, a kind of molecular-targeted drug with multiple targets, on middle and advanced stage primary HCC, and it is widely used to treat advanced stage and non-
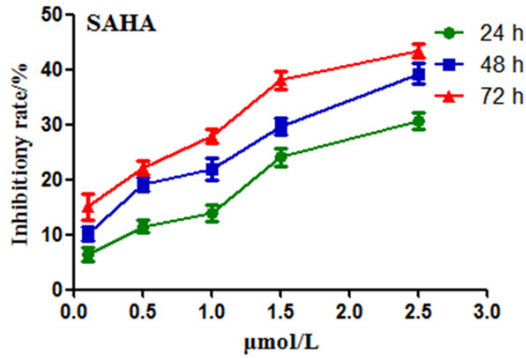
Figure 1. Inhibitory effect of SAHA on proliferation of HepG2 cells.
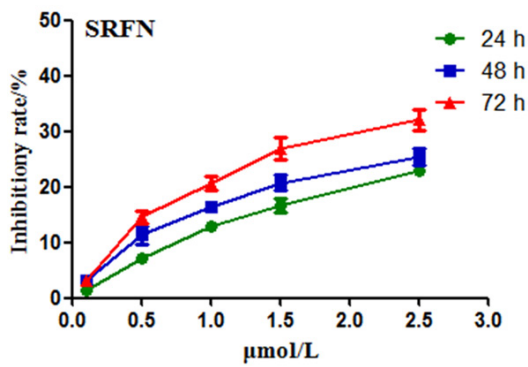
Figure 2. Inhibitory effect of SRFN on proliferation of HepG2 cells.

operative HCC [6, 7]. Although HDACI or SRFN can be used alone to treat HCC, recent investigation indicates that the combined use of SAHA and SRFN can enhance therapeutic effect, reduce toxic side effects, and achieve the effect of comprehensive treatment on HCC [8]. However, the basis of reasonable drug combination is to deeply understand the mechanism of used drugs. In this work, the effects of SAHA, SRFN and SAHA combined with SRFN on growth, proliferation, cell cycle, and related proteins expression of cultured HepG2 cells were investigated to find potential synergy mechanism between SAHA and SRFN.

Materials and methods

Experimental materials

HepG2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Pancreatic enzymes, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, United States). MTT cell proliferation and cytotoxicity assay kit, cell total protein extraction kit, and the BCA protein assay kit were purchased from Beyotime (Shanghai, China). Primary antibody for p21, Cyclin D1, Bax, Bcl-2, MMP-2, vimentin, and E-cadherin proteins and second antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). This study was approved by the Ethics Committee of the Affiliated Hospital of Beihua University.

Cell culture and grouping

HepG2 cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were divided into 4 groups: control, SAHA (treatment with 1.5 µmol/L SAHA), SRFN (treatment with 1.5 µmol/L SRFN) and SAHA combined with SRFN (treatment with 1.5 µmol/L SAHA and 1.5 µmol/L SRFN) groups.

MTT assay

MTT assay was conducted to evaluate the effect of SAHA, SRFN, or SAHA combined with SRFN on proliferation of HepG2 cells. Briefly, HepG2 cells were seeded onto a 96-well plate at 5×10^4 cells/well. HepG2 cells were treated with SAHA or SRFN at different concentrations of 0.1, 0.5, 1, 1.5, or 2.5 µmol/L for 24, 48, or 72 h. Cells treated with 0 µmol/L of SAHA or SRFN for 24, 48, or 72 h were regarded as the control group. After treatment with MTT kit based on a standard protocol, the optical density (OD) of each well was measured at 570 nm. Each group set up 4 wells, and tests were repeated three times. The average OD value was regarded as final OD value. The inhibitory rate of SAHA or SRFN against proliferation of HepG2 cells was determined using the following equation: Inhibitory rate (%) = (OD control - OD treatment)/OD control × 100.

Flow cytometry assay

Flow cytometry assay was performed to investigate the effect of SAHA, SRFN, or SAHA combined with SRFN on cycle and apoptosis of HepG2 cells. After treatment with above drugs for 72 h, cells were digested with pancreatic enzymes, washed with PBS twice, and then centrifuged at 2000 rpm for min to obtain cells.

Table 1. Inhibitory effect of SAHA combined with SRFN on proliferation of HepG2 cells

		SAHA Concentration (µmol/L)					
		0	0.1	0.5	1.0	1.5	2.5
SRFN concentration (µmol/L)	0	---	10.09±2.01	19.17±2.15	21.84±1.88	29.75±2.89	39.23±2.53
	0.1	3.07±0.46	11.38±1.94	20.97±1.59	23.64±2.36	30.28±1.13	41.44±2.18
	0.5	11.37±1.24	16.68±3.34	23.67±3.64	26.79±3.45	38.02±2.94	49.96±3.87
	1.0	16.47±1.91	23.41±2.89	29.41±1.18	35.93±3.69	45.01±3.02	50.03±3.21
	1.5	20.76±1.86	26.85±3.03	35.46±2.85	43.48±3.25	50.79±2.46	53.51±3.84
	2.5	25.41±2.67	30.69±3.74	36.72±3.02	44.09±3.94	49.83±4.03	55.64±4.12

Cells (1-5 × 10^4) were resuspended in 500 µL of binding buffer, and then 5 µL of Annexin V-FITC and 5 µL of Propidium Iodide were added into the above binding buffer. Cells were incubated for 15 min in the dark at room temperature. Cells were analyzed by flow cytometry with 488 nm of excitation wavelength and 530 nm of emission wavelength. The apoptotic rate and cycle of HepG2 cells were analyzed by FCSExpress 3.0 software.

Scratch wound assay

Scratch wound assay was used to investigate the effect of SAHA, SRFN, or SAHA combined with SRFN on migration of HepG2 cells. HepG2 cells were seeded onto 6-well plates at 5 × 10^5 cells/well. After treatment with above drugs for 24 h, 10 µL of pipet tip was vertically used to create a scratch of the cell monolayer. The wounded monolayer was rinsed with PBS three times to remove the scratched cells and then cultured for 24 h in serum-free DMEM in a humidified atmosphere containing 5% CO_2 at 37°C. Migration of the monolayer cells into the wounded area was observed by photographing.

Transwell assay

Transwell assay was used to investigate the effect of SAHA, SRFN, or SAHA combined with SRFN on invasion of HepG2 cells. Matrigel: FBS-free DMEM (1:3) was added into the top chamber of Transwells at 60 µL/chamber, and then Transwells was placed for 2 h at 37°C. Logarithmic growth phase cells were cultured for 24 h in serum-free DMEM and then cells were made into cell suspension at a density of 1 × 10^5 cells/mL. The 200 µL of cell suspension were added into the top chamber with Matrigel-coated membrane. The inserts were placed into the bottom chamber wells of a

24-well plate containing 500 µL of DMEM with 10% FBS for 24 h of incubation. Cells were fixed in 4% formaldehyde for 15 min, followed by staining in 0.25% of crystal violet for 25 min. Inserts were washed with sterile water before air drying. After air drying in sterile ultraclean workbench, the membranes were photographed, and cell counts were determined.

Western blot assay

After treatment with SAHA, SRFN, or SAHA combined with SRFN, the total proteins of HepG2 cells were extracted with cell total protein extraction kit. Protein concentration was determined using BCA protein assay kit. Then total proteins (100 µg) were separated using SDS-PAGE and transferred to a PVDF membrane. Following blocking, the membrane was orderly incubated with primary antibody and second antibody. After coloration, the brightness of protein band was analyzed by Image-J software.

Statistical analysis

All data are represented as mean ± standard deviation (SD). The differences among different groups were analyzed by independent-samples *t* test in SPSS 19.0. Differences were considered to be statistically significant at *P* < 0.05.

Results

Effect of SAHA, SRFN, or SAHA combined with SRFN on proliferation of HepG2 cells

As shown in **Figures 1** and **2**, the results of MTT assay showed that SAHA or SRFN inhibited the proliferation of HepG2 cells in concentration/time-dependent manner. Meanwhile, SAHA combined with SRFN enhanced the anti-proliferative effect (**Table 1**).
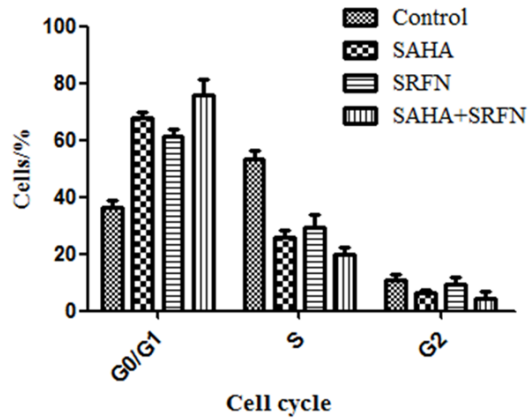
Figure 3. Inhibitory effect of SAHA, SRFN, or SAHA combined with SRFN on cycle of HepG2 cells.
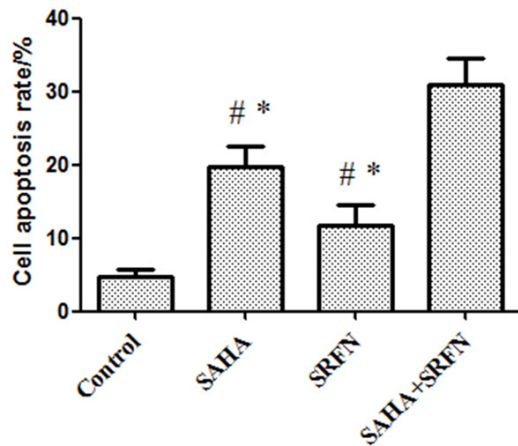
Figure 4. Inductive effect of SAHA, SRFN, or SAHA combined with SRFN on apoptosis of HepG2 cells. [#]*P* < 0.05, compared with the control group. [*]*P* < 0.05, compared with the SAHA combined with SRFN group.

Effect of SAHA, SRFN, or SAHA combined with SRFN on cycle and apoptosis of HepG2 cells

As shown in **Figure 3**, SAHA or SRFN induced the G0/G1 phase arrest of HepG2 cells. The G0/G1 phase of the cell cycle in SAHA or SRFN group increased by 87.16 or 68.81%, respectively, compared with the control group. The G0/G1 phase of the cell cycle in SAHA combined with SRFN group increased by 11.76% relative to SAHA group and 23.91% relative to SRFN group. As shown in **Figure 4**, the apoptotic rate of HepG2 cells in the SAHA or SRFN group (19.72 or 12.01%) was significantly ($P <$ 0.05) higher than that of the control group (4.67%), but significantly ($P <$ 0.05) lower than

that of the SAHA combined with SRFN group (31.00%).

Effect of SAHA, SRFN, or SAHA combined with SRFN on migration of HepG2 cells

HepG2 cells were treated with SAHA, SRFN, and combined treatment, separately. The migration ability was 56.0%, 47.0%, or 3.5% respectively compared with the control group (**Figure 5**).

Effect of SAHA, SRFN, or SAHA combined with SRFN on invasion of HepG2 cells

HepG2 cells were treated with SAHA, SRFN, and combined treatment, separately. The invasion ability was 69.0%, 47.8%, or 15.0% respectively compared with the control group (**Figure 6**).
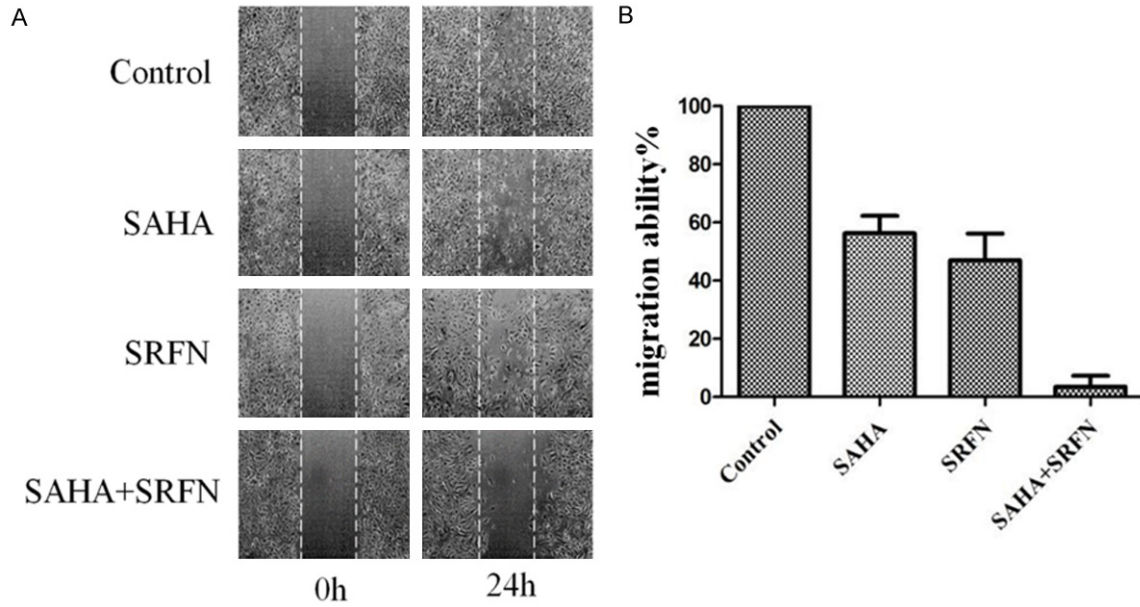
Effect of SAHA, SRFN, or SAHA combined with SRFN on related proteins expression of HepG2 cells

The proliferation, apoptosis, migration or invasion-related proteins expression in HepG2 cells were investigated by Western blot assay. SAHA or SRFN significantly ($P <$ 0.05) up-regulated the expression of p21 and E-cadherin proteins relative to the control group. The expression of p21 and E-cadherin proteins in SAHA combined with SRFN group were significantly ($P <$ 0.05) higher than those of the SAHA or SRFN group. SAHA or SRFN significantly ($P <$ 0.05) down-regulated the expression of Cyclin D1, Bcl-2, MMP-2 and vimentin proteins without effect on the expression of Bax and MMP-9 proteins. The expression of Cyclin D1, Bcl-2, MMP-2 and vimentin proteins in SAHA combined with SRFN group were significantly ($P <$ 0.05) lower than those of the SAHA or SRFN group (**Figure 7**).

Discussion

HDACI are novel anti-tumor drugs characterized by good therapeutic effects and no obvious toxic side effects [9]. HDACI are a hotspot of anti-tumor drug development at present and show good prospects for application in treating tumors. HDACI exerts anti-tumor biological effect through inhibiting HDAC activity, regulating histone acetylation status, promoting transcription, and expression of anti-tumor transcription factors, and regulating related signal-

Figure 5. Effect of SAHA, SRFN, or SAHA combined with SRFN on the migration ability of HepG2 cells. A. Scratch wound assay of different treatments at 0 h and 24 h. B. The relative invasion% of SAHA, SRFN, or SAHA combined with SRFN compared with the control.
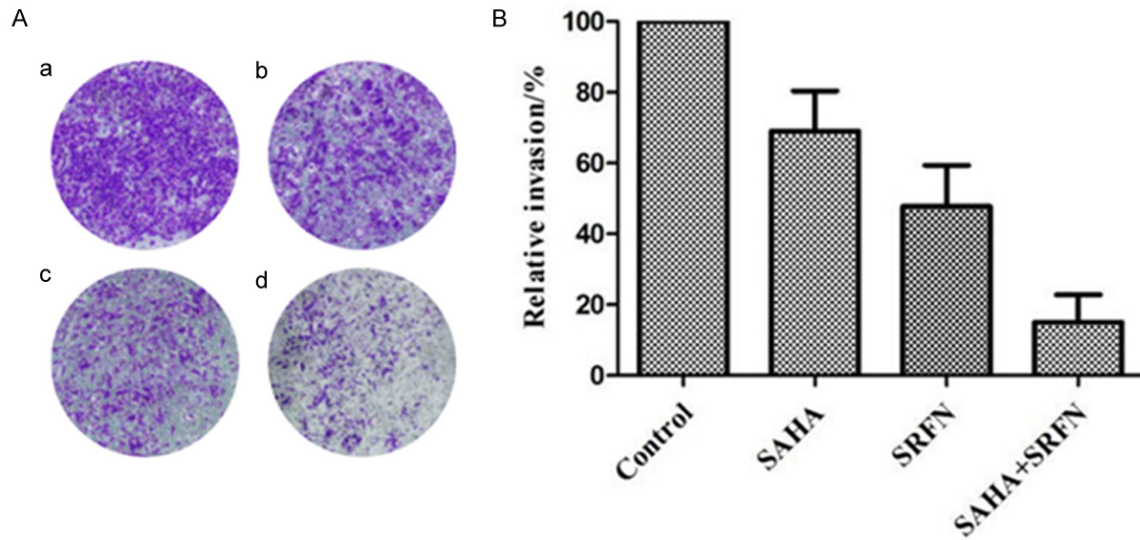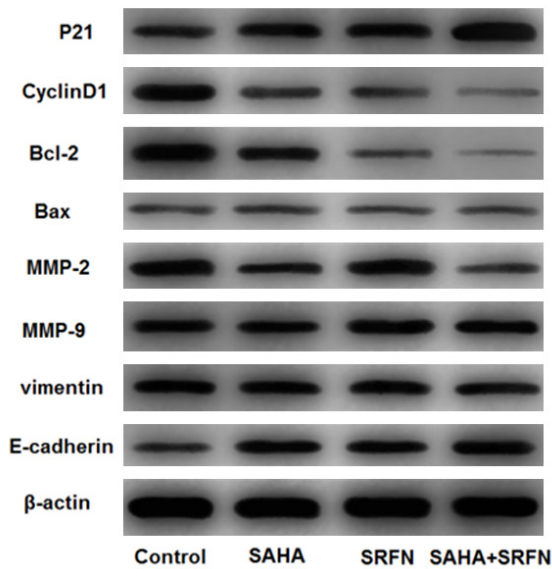

Figure 6. Effect of SAHA, SRFN, or SAHA combined with SRFN on the invasion of HepG2 cells. A. (a-d) Transwell results of HepG2 treated with control reagent, SAHA, SRFN, and SAHA combined with SRFN. B. Relative invasion of HepG2 cells by different treatments.

ing pathways [10]. HDACI affects the biological behavior characteristics of tumor cells or tumor from multiple aspects such as growth, differentiation, and apoptosis of cells, formation, growth, metastasis and invasion of tumor, activating immune system, and eliminating free radicals. The anti-tumor mechanisms of HDACI are mainly related to arresting cell cycle, inducing apoptosis, regulating transcription and expression of related anti-tumor genes, and inhibiting tumor metastasis and invasion [10]. SAHA, one of the most representative HDACI, was developed based on the interrelations among abnormal histone acetylation status,

Figure 7. Comparison among proliferation, apoptosis, migration, or invasion-related proteins expression in SAHA, SRFN, or SAHA combined with SRFN groups. #P < 0.05, compared with the control group. *P < 0.05, compared with the SAHA combined with SRFN group.
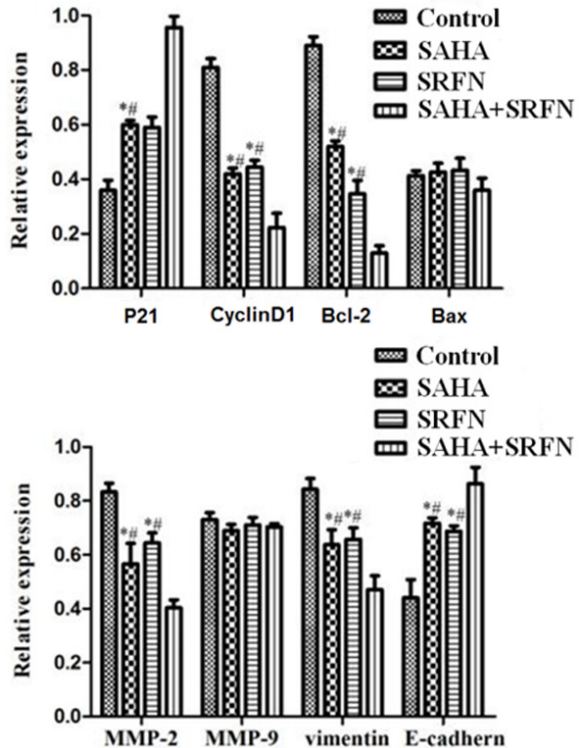
nucleic acid, chromatin, and proliferation and differentiation of tumor cells. SAHA, the second generation ketones hydroxy acids HDACI, can exert high anti-tumor biological effect under micromole level [11]. The investigation shows that SAHA can achieve the goal of treating cancer by regulating genes and increase the sensibility of tumor cells for other chemotherapy [8]. Although the targets of SAHA and SRFN are different, proliferation, differentiation, invasion, and migration of tumor cells are regulated by deoxyribonucleic acid, indicating that their combined use maybe have a synergistic anti-tumor effect.

This work found that SAHA combined with SRFN showed higher inhibitory effect on proliferation, migration and invasion of HepG2 cells and inductive effect on apoptosis of HepG2 cells than SAHA and SRFN. The investigation of Cha TL et al. [12] indicated that although the action targets of different anti-tumor drugs are different, they all show anti-tumor activity by inducing apoptosis of tumor cell and changing internal environment of tumor tissue. There is an important link between cell cycle arrest and apoptosis of cells. This work found that SAHA or SRFN induced the G0/G1 phase arrest of HepG2 cells. The G0/G1 phase of the cell cycle

in SAHA combined with SRFN group was increased by 11.76% relative to SAHA group and 23.91% relative to SRFN group. Moreover, the apoptotic rate of HepG2 cells in SAHA combined with SRFN group was significantly higher than that of SAHA or SRFN group, indicating that there was a synergistic or additive effect for inducing apoptosis of HepG2 cells between SAHA and SRFN.

Meanwhile, further investigations were performed to explore the mechanism of the synergistic or additive effect between SAHA and SRFN on apoptosis of HepG2 cells. This work found that the expression of p21 protein in SAHA combined with SRFN group were significantly higher than that of the SAHA or SRFN group, and expression of Cyclin D1 and Bcl-2 proteins in SAHA combined with SRFN group was significantly lower than those of the SAHA or SRFN group. *p21* a cell differentiation-related gene can inhibit proliferation and differentiation of cells by inducing the G1/G0 phase arrest of cells. If drugs up-regulate expression of *p21* in tumor cells, the ratio of the G1/G0 phase of tumor cells is increased. Then the proliferation of tumor cells is inhibited and death of tumor cells happen. *Cyclin D1* an important cell G1/S phase change-related gene shows

high expression in many tumor cells, and inhibiting its high expression can induce the G1/S phase change arrest of cells, which can induce the G1/G0 phase arrest of cells [13]. Bcl-2 can inhibit apoptosis of cell by regulating many factors. So, SAHA combined with SRFN induced apoptosis of HepG2 cells by inducing the G1/G0 phase arrest of cells related to the up-regulation of *p21* and the down-regulation of *Cyclin D1*, and the apoptosis of HepG2 cells can be further promoted by down-regulating Bcl-2.

Moreover, this work found that SAHA combined with SRFN showed higher inhibitory effect on invasion and migration of HepG2 cells than SAHA or SRFN. Abilities of invasion and migration of tumor cells play an important role in promoting disease progression of primary tumor, and reducing the abilities of invasion and migration of tumor cells is an important aspect of anti-tumor drugs to inhibit disease progression of tumor.

Furthermore, epithelial-mesenchymal transition (EMT) of tumor cells can increase their ability of invasion and migration, leading to greater tumorigenicity [14]. MMP-2, MMP-9, vimentin, and E-cadherin are some important iconic proteins for EMT [15, 16]. MMP-2 and MMP-9 are two important enzyme MMPs family, and the existing investigation indicates that MMPs are the most important proteolytic enzymes in EMT process. The expression of MMP-2 and MMP-9 in lung carcinoma cell are significantly up-regulated in EMT process of lung carcinoma cell, related to abilities of invasion and migration of lung carcinoma cell [17]. Yang et al. [18] also found that the expression of MMP-2 is positively related to ability of migration of hepatoma cells.

However, vimentin is regarded as a marker of interstitial performance in EMT process. Jacob U *et al.* [15] found that vimentin can promote the migration and transfer of breast cancer cells. E-cadherin shows opposite functions in EMT process of tumor cells relative to MMPs and vimentin proteins. E-cadherin plays an important role in mutual adsorption among cells or basal membrane materials. E-cadherin an epithelial marker in EMT process can inhibit migration of tumor cells, and the up-regulation of E-cadherin protein expression indicates the increase of abilities of migration and invasion of tumor cells [16]. This work found that SAHA combined with SRFN significantly down-regulated the expression of MMP-2 and vimentin proteins and up-regulated the expression of E-cadherin protein relative to SAHA and SRFN, indicating the molecular mechanisms of drug combination between SAHA and SRFN against migration and invasion of HepG2 cells.

SAHA or SRFN inhibited the proliferation, migration, and invasion of HepG2 cells and promoted their apoptosis by up-regulating the expression of p21 and E-cadherin proteins and down-regulating expression of Cyclin D1, Bcl-2, MMP-2, and vimentin proteins. Finally, there was a synergistic or additive effect for these above effects between SAHA and SRFN.

Acknowledgements

Disclosure of conflict of interest

None.

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