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Basic Study

Suberoylanilide hydroxamic acid upregulates reticulophagy receptor expression and promotes cell death in hepatocellular carcinoma cells

Jia-Yao Li, Tian Tian, Bing Han, Ting Yang, Yi-Xin Guo, Jia-Yu Wu, Yu-Si Chen, Qin Yang, Ru-Jia Xie

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Hepatocellular carcinoma (HCC) is a common clinical condition with a poor prognosis and few effective treatment options. Potent anticancer agents for treating HCC must be identified. Epigenetics plays an essential role in HCC tumorigenesis. Suberoylanilide hydroxamic acid (SAHA), the most common histone deacetylase inhibitor agent, triggers many forms of cell death in HCC. However, the underlying mechanism of action remains unclear. Family with sequence similarity 134 member B (FAM134B)-induced reticulophagy, a selective autophagic pathway, participates in the decision of cell fate and exhibits anti-cancer activity. This study focused on the relationship between FAM134B-induced reticulophagy and SAHA-mediated cell death.

AIM

To elucidate potential roles and underlying molecular mechanisms of reticulophagy in SAHA-induced HCC cell death.

METHODS

The viability, apoptosis, cell cycle, migration, and invasion of SAHA-treated Huh7 and MHCC97L cells were measured. Proteins related to the reticulophagy

pathway, mitochondria-endoplasmic reticulum (ER) contact sites, intrinsic mitochondrial apoptosis, and histone acetylation were quantified using western blotting. ER and lysosome colocalization, and mitochondrial Ca²⁺ levels were characterized *via* confocal microscopy. The level of cell death was evaluated through Hoechst 33342 staining and propidium iodide colocalization. Chromatin immunoprecipitation was used to verify histone H4 lysine-16 acetylation in the *FAM134B* promoter region.

RESULTS

After SAHA treatment, the proliferation of Huh7 and MHCC97L cells was significantly inhibited, and the migration and invasion abilities were greatly blocked *in vitro*. This promoted apoptosis and caused G1 phase cells to increase in a concentration-dependent manner. Following treatment with SAHA, ER-phagy was activated, thereby triggering autophagy-mediated cell death of HCC cells *in vitro*. Western blotting and chromatin immunoprecipitation assays confirmed that SAHA regulated *FAM134B* expression by enhancing the histone H4 lysine-16 acetylation in the *FAM134B* promoter region. Further, SAHA disturbed the Ca²⁺ homeostasis and upregulated the level of autocrine motility factor receptor and proteins related to mitochondria-endoplasmic reticulum contact sites in HCC cells. Additionally, SAHA decreased the mitochondrial membrane potential levels, thereby accelerating the activation of the reticulophagy-mediated mitochondrial apoptosis pathway and promoting HCC cell death *in vitro*.

CONCLUSION

SAHA stimulates *FAM134B*-mediated ER-phagy to synergistically enhance the mitochondrial apoptotic pathway, thereby enhancing HCC cell death.

Key Words: Suberoylanilide hydroxamic acid; Histone H4 lysine-16; Reticulophagy; Apoptosis; Autophagic cell death; Hepatocellular carcinoma

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Core Tip: Family with sequence similarity 134 member B (*FAM134B*) is considered to be a tumor suppressor protein that can play a pivotal role in inhibiting hepatocellular carcinoma (HCC) cells. In addition, *FAM134B* acts as a putative reticulophagy receptor in the regulation of the reticulophagy process. Furthermore, suberoylanilide hydroxamic acid (SAHA) upregulates *FAM134B* expression in HCC cells and promotes apoptosis and autophagy-mediated cell death. Thus, *FAM134B*-mediated reticulophagy synergizes with SAHA to induce HCC cell death. Our findings offer novel insights into the mechanism underlying SAHA-induced HCC cell death.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the most well-known and prevalent primary liver cancer in China. The mortality rates with HCC have consistently increased annually[1]. Immune checkpoint therapies have recently emerged as noteworthy treatments for HCC[2-4]. A considerable proportion of patients, approximately 70% with advanced HCC, fail to derive benefits from immunotherapy[5]. Thus, pursuing more potent anticancer medications to combat HCC must persist. Owing to its high degree of malignancy, poor prognosis, and relatively limited range of treatment strategies, it is necessary to seek more powerful anticancer agents for treating HCC. In the past decade, accumulating evidence has validated the role that epigenetics plays in HCC tumorigenesis[6,7]. Epigenetic regulation changes the transcriptional activity of key genes without altering DNA sequences[8,9].

Epigenetic regulation occurs primarily through DNA methylation, post-translational histone modification, chromatin remodeling, and non-coding RNA-mediated gene silencing[10]. Current therapies targeting epigenetic modifications to cancer mainly include DNA methyltransferases and histone deacetylases (HDACs) as well as microRNAs (miRNAs). Due to the widespread existence of DNA methylation variation in HCC, a variety of corresponding regulators of DNA methyltransferase have been developed[11]. At the same time, miRNAs such as miRNA-148a are used in anti-HCC therapy by combining with oncolytic viruses.

Histone acetylation modification, induced by HDACs and histone acetyltransferases, is a prominent mode of epigenetic regulation[12]. The histone acetylation/deacetylation balance is dynamically regulated to maintain global chromatin structure[13]. Therefore, any dysregulation may contribute to altered gene expression, leading to pathological conditions, such as HCC. Suberoylanilide hydroxamic acid (SAHA) represents the most typical HDAC inhibitor (HDACi)

and was the first of its kind to be approved for human treatment. So far, SAHA has been found to induce the differentiation of malignant tumor cells and accelerate apoptosis *in vitro* and *in vivo*[14]. According to our previous results, SAHA may act as a potential initiator of endoplasmic reticulum (ER) stress-associated apoptosis in HepG2 hepatoma cells by activating ER stress-related apoptotic pathways[15]. However, it is still unknown whether SAHA utilizes a new mechanism to induce HCC cell death through some different therapeutic targets.

Acetylation of histone H4 lysine 16 (H4K16ac) is important for gene initiation[16]. Recently, researchers have found that H4K16ac is closely associated with autophagy induction and significantly correlated with autophagy regulation. Moreover, deacetylase inhibitors can promote the upregulation of H4K16ac and lead to autophagic death of cancer cells [17]. However, the regulatory mechanism underlying the induction of H4K16ac-mediated reticulophagy is unclear.

Family with sequence similarity 134 member B (*FAM134B*) has been proposed as a cancer suppressor gene[18,19]. Numerous researchers have demonstrated that in colorectal carcinoma, the presence of *FAM134B* limits the overgrowth and suppresses the proliferation of cancer cells[20,21]. In addition, *FAM134B* acts as a putative reticulophagy receptor in regulating ER turnover and maintaining calcium homeostasis by remodeling ER[22,23]. Recent findings have also identified that *FAM134B*-mediated ER-phagy may regulate ER-mitochondrion interaction[24]. As the largest cellular organelle, the ER can interact with mitochondria through multiple contact sites, termed mitochondria-ER contact sites (MERCs).

Many ER-related and mitochondria-related proteins have been discovered at MERCs, including the inositol 1, 4, 5-trisphosphate receptor type 1 (IP3R1)/glucose-regulated protein 75 (GRP75)/voltage-dependent anion channel 1 (VDAC1) complex, which is a central component of MERCs that contributes to calcium exchange regulation[25,26]. Recent research has suggested that Ca²⁺ deregulation between ER and mitochondria by MERCs led to mitochondrial calcium overload, thereby activating the mitochondria-associated apoptotic pathway[27]. In the present study, we verified that SAHA treatment augmented *FAM134B* expression and facilitated Huh7 and MHCC97L HCC cell apoptosis; however, the regulatory mechanisms underlying this effect remain unknown. In our study, we elucidated potential roles and underlying molecular mechanisms of reticulophagy in SAHA-induced HCC cell death. Our findings may offer new perspectives for clinical trials of HCC.

MATERIALS AND METHODS

Cell culture and treatments

The human Huh7 and MHCC97L cell lines were derived from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultivated at 37 °C in a 5% CO₂-supplemented atmosphere and maintained in high-glucose Dulbecco's modified Eagle's medium (ESscience, Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, United States) and 1% penicillin-streptomycin (BioInd, Beit-Haemek, Israel). SAHA (Abcam, Cambridge, United Kingdom) was dissolved in dimethyl sulfoxide as a 5 mmol/L stock solution and then diluted with the complete medium to achieve ultimate concentrations of 0-24 μmol/L. Both cell lines were exposed to SAHA or vehicle treated with 0.1% dimethyl sulfoxide.

Cell counting kit-8 assay

Cell counting kit-8 (CCK-8) assay was applied to evaluate the anti-proliferative effects of SAHA. Briefly, MHCC97L and Huh7 cells (5 × 10³) were plated onto 96-well plates and given 24 h to adhere before being treated with SAHA. The cells were then exposed to various doses of SAHA (0, 0.5, 1, 3, 6, 9, 12, 18, and 24 μmol/L) for 48 h. Subsequently, 10 μL CCK-8 reagent (Solarbio, Beijing, China) was added to each well and incubated for 4 h. Absorbance at 450 nm was recorded using a spectrophotometer (BioRad, Hercules, United States). The IC₅₀ of SAHA was calculated using GraphPad Prism software (v9.0.0, GraphPad Software, La Jolla, CA, United States).

Flow cytometry

The data regarding the apoptosis and cell cycle of HCC cells treated with 0, 1, 3, 6, and 12 μmol/L SAHA were obtained *via* standard flow cytometry (NovoCyte, Agilent, Santa Clara, CA, United States). The apoptosis assay was carried out using a cellular apoptosis detection kit (KeyGEN BioTECH, Nanjing, China) following the manufacturer's protocol. Briefly, Huh7 and MHCC97L cells were plated onto 6-well plates at 1 × 10⁵ cells per well before adding SAHA solution. Then, each group of cells was harvested, rinsed with chilled phosphate buffered saline (PBS), and loaded with binding buffer. The cells were labelled with Annexin V-FITC/propidium iodide (PI) solution, and the stained cells were calculated using the flow cytometer. Similarly, cell cycle analysis was conducted using the flow cytometer following the manufacturer's instructions. Each group of cells was collected and washed thrice with chilled PBS. The cells were fixed with 70% ethanol and stained with PI/RNase I solution (KeyGEN BioTECH), and the percentages of cells in the G1, S, and G2 stages were measured using the flow cytometer. The inbuilt software NovoExpress® 1.4.1 was used for statistical analysis.

Wound healing assay

The wound healing assay was monitored to evaluate the effect of SAHA on HCC cell migration. Briefly, Huh7 and MHCC97L cells (3 × 10⁴) were planted onto 6-well plates, grown until they formed an optically confluent monolayer, and then wounded with a sterile 200 μL micropipette tip. Upon treatment with 0 μmol/L or 3 μmol/L SAHA for 48 h, HCC cells were photographed using a microscope (× 40).

Transwell assay

The cell invasion and migration assay *in vitro* was carried out using 24 transwell plates divided into upper and lower chambers using sterile polycarbonate with 8 μm pore size (Corning Life Science, Corning, NY, United States). The sterile polycarbonate covered 200 μL Matrigel (BD Biosciences, San Jose, CA, United States) in the cell invasion assay but not in the cell migration assay. Upon treatment with 0 $\mu\text{mol/L}$ or 3 $\mu\text{mol/L}$ SAHA for 48 h, cells that passed through the pore were stained with crystal violet, photographed, and quantified.

Western blot analysis

Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA for 48 h. Then, the cells were rinsed with pre-chilled PBS, lysed in 100 μL RIPA lysis buffer with protease inhibitor (Solarbio), and collected with cell scrapers. Protein samples were boiled for 5 min, and total protein extracts were subjected to standard sodium-dodecyl sulfate gel electrophoresis and subsequently removed to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, United States). The membranes were blocked with rapid blocking solution and stained overnight with the corresponding primary antibodies, including FAM134B (Proteintech; Wuhan, China 1:1500), CCPG1 (Proteintech; 1:1500), LC3 (Abcam; 1:2000), ATG12 (Cell Signaling Technology; Danvers, MA, United States 1:1500), H4 (Proteintech; 1:2000), total acH4 (Proteintech; 1:2000), H3K27ac (Cell Signaling Technology; 1:6000), H4K5ac (Cell Signaling Technology; 1:6000), H4K12ac (Cell Signaling Technology; 1:6000), H4K16ac (Abcam; 1:10000), GRP75 (Abcam; 1:2000), VDAC1 (Abcam; 1:2000), IP3R (Abcam; 1:1000), autocrine motility factor receptor (AMFR) (Proteintech; 1:500), cyt c (Cell Signaling Technology; 1:1500), cleaved caspase-3 (Cell Signaling Technology; 1:1500), Bax (Cell Signaling Technology; 1:1500), Bcl-2 (Cell Signaling Technology; 1:1500), and β -actin (Abcam; 1:1000), followed by incubation with the corresponding secondary antibodies (1:8000). Polyvinylidene fluoride membranes carrying proteins were treated with enhanced chemiluminescence reagent (Solarbio), and ImageLab software was used to observe the protein bands.

Live imaging of the ER and lysosomes

ER-trackers and Lyso-trackers (Beyotime; Nanjing, China), the specific organelle dyes, were applied to stain and locate ER and lysosomes, respectively. Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA for 48 h. Then, the cells were coincubated with the two tracking dyes at 37 $^{\circ}\text{C}$ for 45 min and rinsed thrice with PBS. The stained cells were viewed at $\times 200$ magnification under a confocal microscope (Olympus, Tokyo, Japan) and immediately imaged.

Hoechst 33342/PI double chromatin staining assay

Huh7 and MHCC97L cells (3×10^4) were seeded in confocal laser Petri dishes. The cells were pretreated with 0.5 nmol/L bafilomycin A1 (MedChemexpress, NJ, United States) for 12 h and then exposed with 12 $\mu\text{mol/L}$ SAHA for 48 h. Subsequently, Hoechst 33342/PI double fluorescent chromatin staining assay was conducted using a Viastain™ Hoechst 33342/PI viability kit (Beyotime). Cells were stained with Hoechst 33342 and PI for 30 min at 25 $^{\circ}\text{C}$ in the dark. The labelled cells were observed with a confocal microscope under $\times 400$ magnification.

Mitochondrial calcium labeling

Rhod-2 AM Red (Abcam), a specific Ca^{2+} indicator, was applied to detect the level of mitochondrial Ca^{2+} . Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA for 48 h and rinsed thrice with Hank's balanced salt solution. The treated cells were labelled with a mixture of Mito-Tracker Green (Beyotime) and Rhod-2 AM Red (Beyotime) and incubated for 50 min. The labelled cells were observed with a confocal microscope under $\times 400$ magnification.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay of Huh7 cells was conducted as described previously[28]. Huh7 cells treated with 0 $\mu\text{mol/L}$ or 6 $\mu\text{mol/L}$ SAHA were subjected to ChIP assay using a ChIP Kit (Thermo Fisher Scientific, Waltham, MA, United States). In brief, the Huh7 cells were cross-linked with 1% formaldehyde and lysed with sodium-dodecyl sulfate lysis buffer. The lysate was sonicated and centrifuged (9000 \times g/min) to harvest chromatin fragments (200-1000 bp). Immunoprecipitation was conducted with the following antibodies: anti-H4K16ac (1:50); rabbit IgG (1:50); and anti-RNA polymerase II (1:50). A no-antibody sample was used as input control. Input DNA and ChIP DNA were detected *via* quantitative polymerase chain reaction.

JC-1 fluorescence mitochondrial imaging

The JC-1 fluorescence mitochondrial imaging technique was applied to examine the mitochondrial membrane potential of HCC cells treated with 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA. Briefly, Huh7 and MHCC97L cells (1×10^5) were plated onto 6-well plates before adding SAHA solution and incubated with JC-1 fluorescence solution in an incubator for 20 min. Analysis was performed using a flow cytometer.

Statistical analysis

Data were processed and analyzed, and experimental graphs were prepared using GraphPad Prism software. All experiments were conducted in biological triplicate. All data are shown as means \pm standard deviation. One-way analysis of variance was conducted for multigroup comparisons. *P* values less than 0.05 were considered to indicate significance.

RESULTS

SAHA suppressed the overgrowth of HCC cells by inducing G1 phase arrest and apoptosis *in vitro*

In our previous study, we confirmed that SAHA suppressed the overgrowth of HepG2 cells and mediated apoptosis by promoting the ER stress-associated apoptotic pathway[15]. However, this was not demonstrated in Huh7 and MHCC97L cells. As presented in **Figure 1A**, HCC cell growth was notably suppressed in SAHA-treated cells; the semi-lethal dose was 12 $\mu\text{mol/L}$. Apoptosis in Huh7 and MHCC97L cells was determined using flow cytometry with different SAHA concentrations. Consistent with previous studies, SAHA induced apoptosis in Huh7 and MHCC97L cells (**Figures 1B and C**). The percentage of specific cell populations that were early apoptotic and late apoptotic throughout the apoptotic stage is shown in **Figure 1C**. In addition, cell cycle assay results showed that an increasing number of cells were blocked in the G1 phase (**Figures 1D-F**).

SAHA suppressed the migration and invasion of HCC cells

Both Huh7 and MHCC97L cells were tested under various treatment conditions (0 $\mu\text{mol/L}$ or 3 $\mu\text{mol/L}$ SAHA) to assess their ability to cross a membrane from low to high nutrient solution *in vitro*. Serum was used as an inducer, mimicking the process of tumor cell invasion into adjacent tissues *in vivo*. Cells invading through the semiporous membrane into the underlying medium containing a high serum concentration were photographed under an optical microscope (**Figures 2A and B**) and quantitatively analyzed (**Figures 2C and D**). The model cells were compared with the control sample, which was assumed to represent 100% invasion without any treatment. Huh7 and MHCC97L cells showed 78% and 68% lower migration and 44% and 30% lower invasion following treatment with 3 $\mu\text{mol/L}$ SAHA, respectively. Further wound-healing tests were used to verify how cancer cells interact and move. The results showed a gradual decrease in cell migration distance after SAHA treatment (**Figure 2E**). Overall, SAHA resulted in reduced cell invasion and migration in HCC cells.

SAHA enhanced the level of ER-phagy and augmented autophagy-mediated cell death in HCC cells

The effect of SAHA on FAM134B-mediated ER-phagy was assessed using western blotting. In HCC cells, SAHA increases the expression of proteins linked to the reticulophagy-related signaling pathway. We found increases in the expression of FAM134B, CCPG1, and autophagy-related protein Atg12 and the LC3-II/LC3-I ratio (**Figures 3A-C**). In the process of reticulophagy, ER fragments were delivered to lysosomes for final degradation. To detect the final state of ER autophagic lysosome formation, we used organelle markers that could trace the ER and lysosomes to detect the colocalization of both. We found that colocalization coverage of the ER and lysosomes increased in Huh7 and MHCC97L cells under SAHA treatment (**Figure 3D**). The above results suggested that SAHA could enhance the level of ER-phagy in HCC cells.

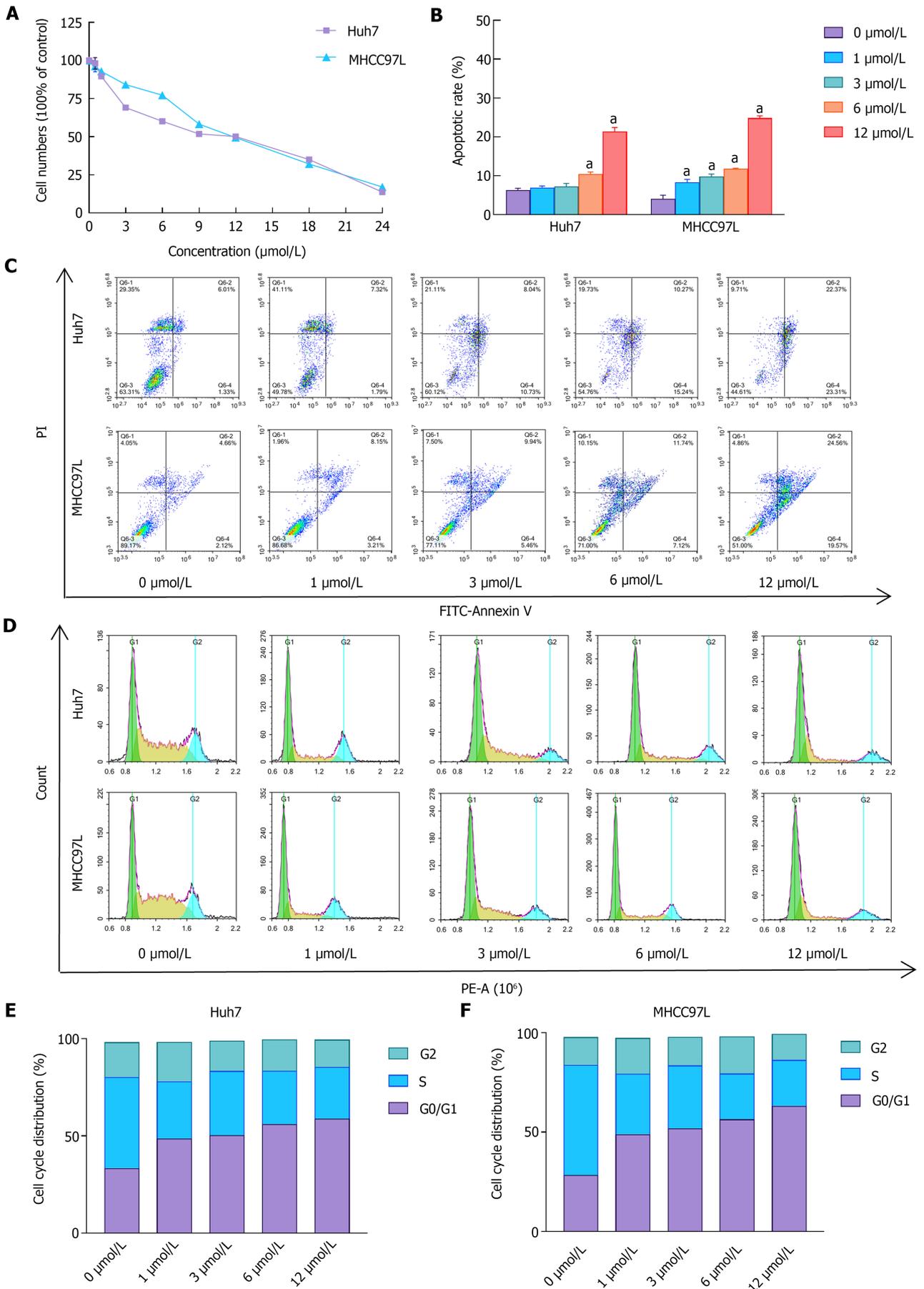
Previous research has reported that appropriate autophagy is a protective response under cellular stress conditions, but uncontrolled autophagy leads to autophagy-mediated cell death[29]. To further verify whether autophagic death mode exists in HCC cells under SAHA treatment, Huh7 and MHCC97L cells were pretreated with BafA1, a specific inhibitor of the late phase of autophagy that restrains autophagosomal fusion with lysosomes, 12 h before SAHA treatment. Nuclear double staining with Hoechst 33342 and PI was conducted to observe the level of cell death in the treated cells. The results showed that HCC cells treated with 12 $\mu\text{mol/L}$ SAHA for 48 h after pretreatment with BafA1 exhibited significantly lower cell death rates compared with cells treated with 12 $\mu\text{mol/L}$ SAHA alone, indicating that autophagy-mediated cell death was involved in SAHA-induced HCC cell death (**Figure 3E**).

SAHA upregulated the level of histone acetylation modification and enhanced H4K16ac in the promoter region of FAM134B in HCC cells

After SAHA treatment, the levels of H3K27ac, total H4ac, H4K5ac, H4K12ac, and H4K16ac in Huh7 and MHCC97L cells were determined using western blotting. We found that various doses of SAHA could upregulate the levels of these proteins (**Figures 4A-C**). Histone hyperacetylation results in gene transcription activation, and recent findings have shown that H4K16ac is linked to the regulation of autophagy-related genes; therefore, we further focused on the regulatory role of H4K16ac in gene expression. We conducted ChIP assays to confirm whether the regulation of *FAM134B* transcription by SAHA was mediated by H4K16ac upregulation. Our results showed that H4K16ac in the *FAM134B* promoter region was significantly increased in Huh7 cells (**Figure 4D**).

SAHA disturbed Ca^{2+} homeostasis and upregulated the expression of AMFR and MERCS-related proteins in HCC cells

After SAHA treatment, the level of cytosolic Ca^{2+} in Huh7 and MHCC97L cells was determined using Rhod-2 AM Red staining, and mitochondrial Ca^{2+} was colocalized with Mito-Tracker Green and Rhod-2 AM Red. The results showed that SAHA markedly elevated cytosolic and mitochondrial Ca^{2+} levels (**Figures 5A and B**). Classical papers reported that increased cytosolic Ca^{2+} could increase the expression of AMFR, which targets the outer mitochondrial membrane (OMM) for ubiquitination and degradation[30-32]. We evaluated the protein level of AMFR in Huh7 and MHCC97L cells and found that SAHA could considerably increase AMFR protein levels. The IP3R-GRP75-VDAC1 complex is one of the most significant components of MERCS, which regulates ER-mitochondrial calcium flux. To determine the altered expression of MERCS in response to SAHA, the levels of IP3R, GRP75, and VDAC1 were examined using protein blots. Similar to our hypothesis, SAHA administration raised the protein levels of the above three proteins relative to the control group (**Figures 5C-E**). These findings showed that SAHA contributed to calcium buildup in the mitochondria by enhancing the interaction between the ER and mitochondria.



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Figure 1 Impact of suberoylanilide hydroxamic acid treatment on cell viability, cell cycle dispersion, and cellular apoptosis in Huh7 and

MHCC97L cells. A: Huh7 and MHCC97L cells were treated with 0, 0.5, 1, 3, 6, 9, 12, 18, and 24 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid (SAHA) for 48 h. The proliferation of Huh7 and MHCC97L cells upon SAHA treatment was monitored using a cell counting kit-8 assay; B-F: Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA for 48 h. The proportions of apoptotic cells and the cell cycle dispersion were detected using flow cytometer. Experiments were repeated thrice. Representative results from three independent replicate assays are shown. Data are exhibited as mean \pm standard deviation. ^a $P < 0.05$ vs 0 $\mu\text{mol/L}$ group ($n = 3$). PI: Propidium iodide.

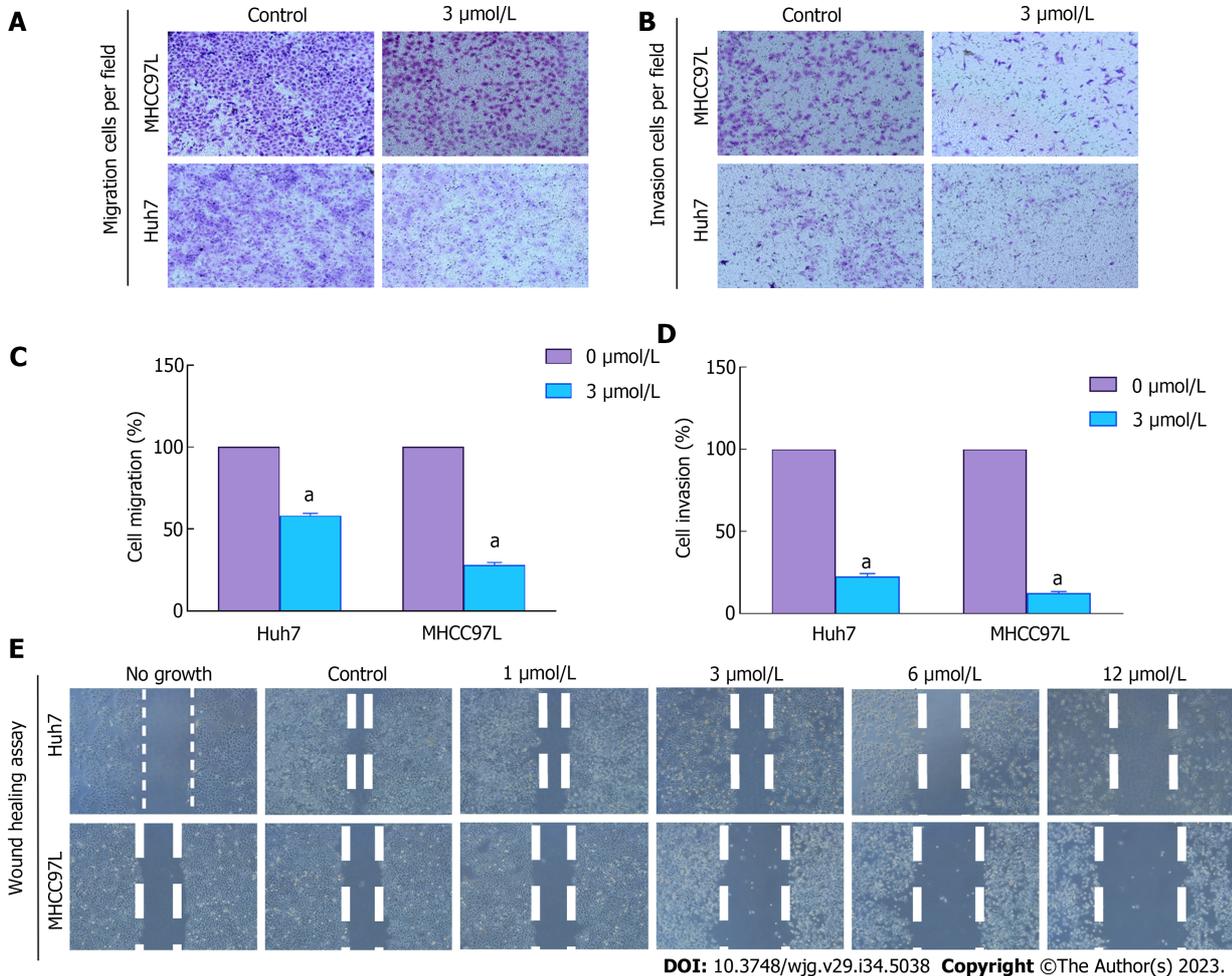


Figure 2 Impact of suberoylanilide hydroxamic acid treatment on the migration and invasion of Huh7 and MHCC97L cells. A and B: During experimental verification, the mobile ability of hepatocellular carcinoma cells could not be observed in the 6 $\mu\text{mol/L}$ and 12 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid (SAHA) groups and even the cell growth was directly inhibited. Therefore, we selected 3 $\mu\text{mol/L}$ SAHA to treat Huh7 and MHCC97L cells in a transwell assay. Both cell lines were vehicle-treated with 0.1% dimethyl sulfoxide or exposed to 3 $\mu\text{mol/L}$ SAHA for 48 h. The migration and invasion behavior of cells was evaluated by calculating the cells stained using crystal violet. Representative pictures of invaded hepatocellular carcinoma cells from three independent replicate assays are exhibited; C and D: Statistical graphs; E: In the cell scratch experiment, the space in the middle was artificially divided into regions to observe the motility of Huh7 and MHCC97L cells upon treatment with various doses of SAHA (0, 1, 3, 6, and 12 $\mu\text{mol/L}$) for 48 h. All cells were observed under an inverted microscope with a 40-fold objective lens. Data are exhibited as mean \pm standard deviation. ^a $P < 0.05$ vs control group ($n = 3$).

SAHA treatment activated the mitochondrial apoptotic pathway by decreasing mitochondrial membrane potential

After SAHA treatment, mitochondrial membrane potential was monitored *via* JC-1 staining. We found that SAHA treatment reduced mitochondrial membrane potential (Figures 6A and B). To investigate the particular SAHA-induced apoptotic processes, the levels of cyt c, caspase-3, and proteins related to the Bcl-2 family were detected using western blotting. The experimental results revealed that SAHA upregulated the expression of cyt c, cleaved caspase-3, and Bax and downregulated the expression of Bcl-2 (Figures 6C-E). These results clearly indicated that the mitochondrial membrane potential was at a lower level, and the mitochondrial apoptotic pathway was activated under the action of SAHA.

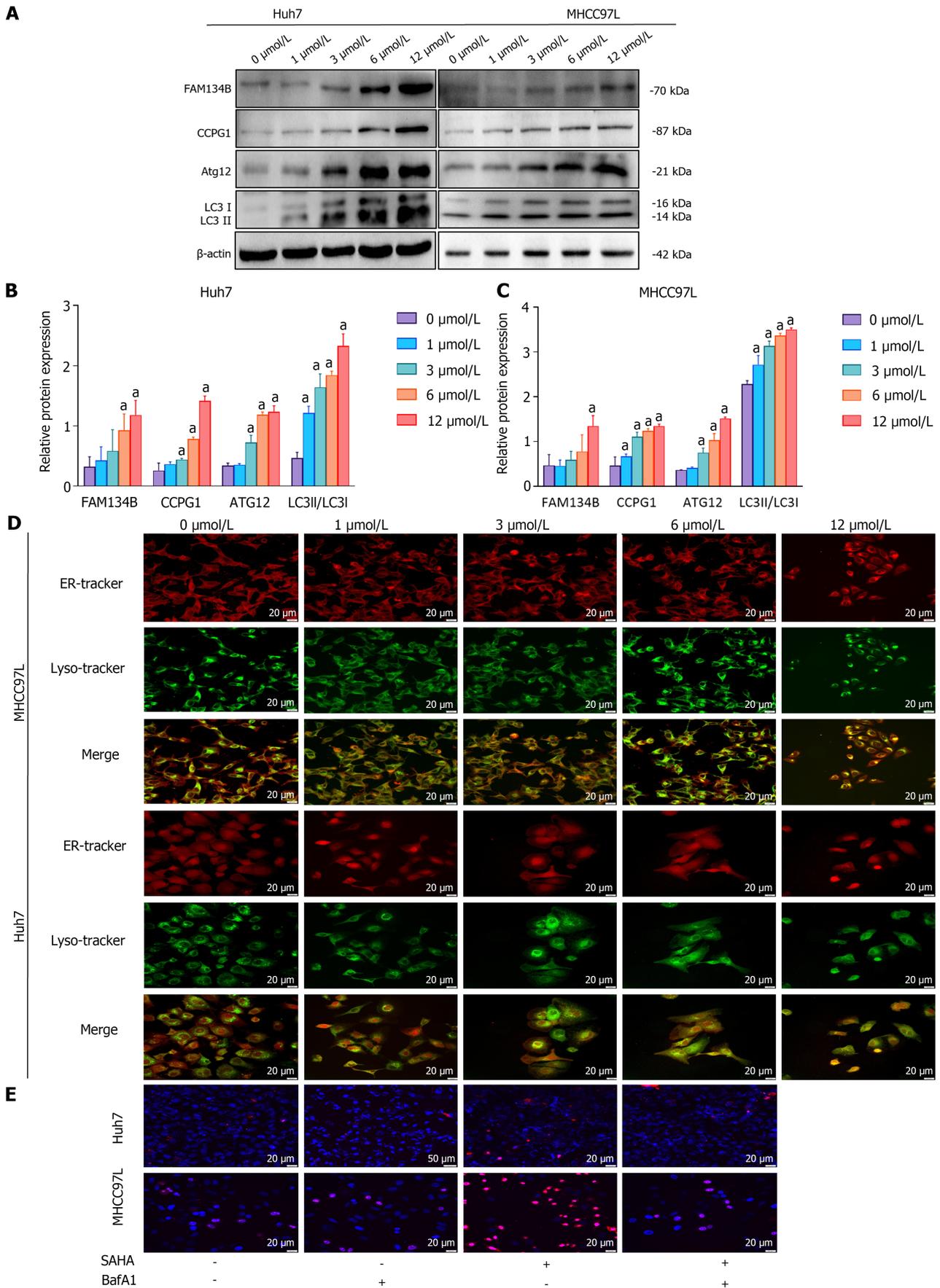
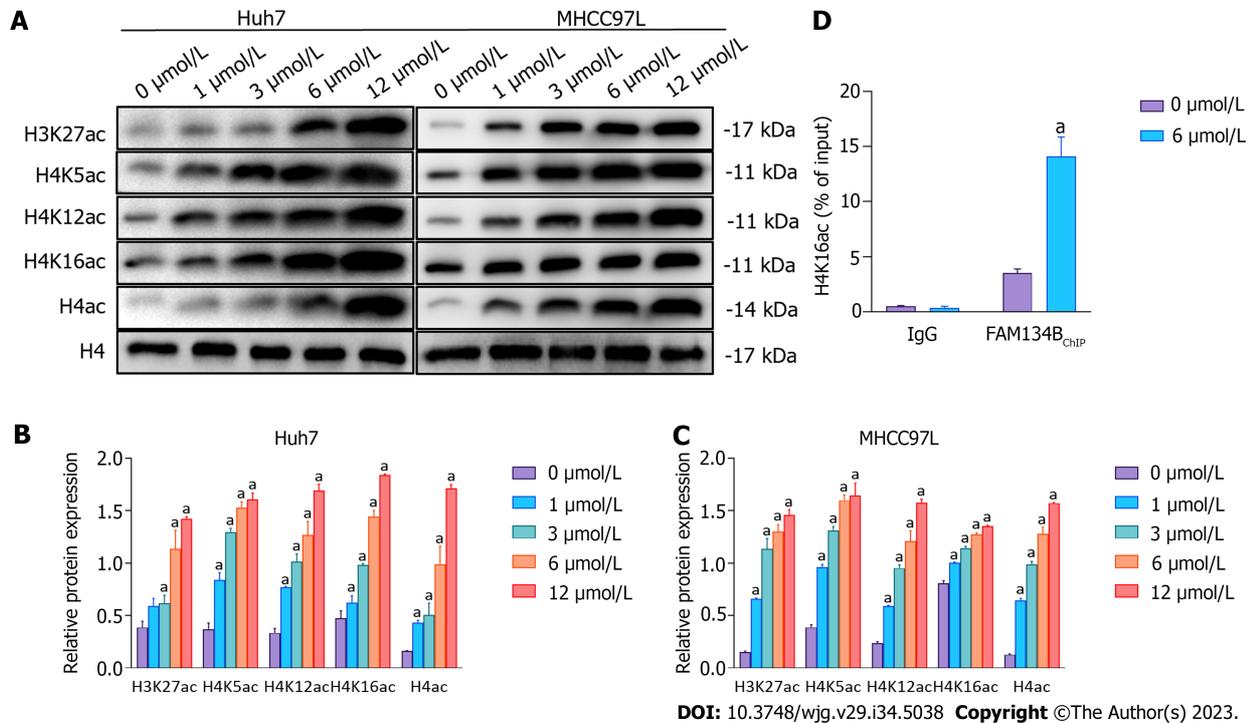


Figure 3 Impact of suberoylanilide hydroxamic acid treatment on the level of endoplasmic reticulum-phagy and autophagy-mediated cell death in Huh7 and MHCC97L cells. A-C: Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid for 48 h.

Proteins related to the reticulophagy pathway were detected using western blotting. Relative protein expression levels were normalized to β -actin; D: The colocalization coverage of the endoplasmic reticulum and lysosomes were observed using a confocal microscope ($\times 400$). Representative pictures from three independent replicate assays are exhibited; E: Huh7 and MHCC97L cells were pretreated with 0.5 nmol/L BafA1 for 12 h, followed by 12 μ mol/L suberoylanilide hydroxamic acid treatment for 48 h. Hoechst 33342/propidium iodide (PI) double staining localization assay was utilized to observe nuclear coagulation. After Hoechst 33342/PI double staining, the death rate of hepatocellular carcinoma cells was quantified based on the ratio of pink cells/blue cells. Experiments were repeated thrice. Representative pictures from the three independent replicate assays are shown. Data are exhibited as mean \pm standard deviation. ^a $P < 0.05$ vs 0 μ mol/L group. SAHA: Suberoylanilide hydroxamic acid; ER: Endoplasmic reticulum; FAM134B: Family with sequence similarity 134 member B.



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Figure 4 Impact of suberoylanilide hydroxamic acid treatment on the levels of histone H4 lysine 16 acetylation in the promoter region of family with sequence similarity 134 member B in Huh7 and MHCC97L cells. A-C: Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 μ mol/L suberoylanilide hydroxamic acid for 48 h. Relative protein levels of histone H3 lysine 27 acetylation (H3K27ac), total H4ac, H4K5ac, H4K12ac, and H4K16ac were normalized to total histone H4. Representative western blot images from three independent replicate assays are exhibited; D: Huh7 cells were exposed to 0 μ mol/L or 6 μ mol/L suberoylanilide hydroxamic acid for 48 h. Quantification of the acetylated histone H4K16-related promoter region of family with sequence similarity 134 member B in Huh7 cells was measured using chromatin immunoprecipitation assay and quantitative polymerase chain reaction. Data are exhibited as mean \pm standard deviation. ^a $P < 0.05$ vs 0 μ mol/L group ($n = 3$). FAM134B: Family with sequence similarity 134 member B; ChIP: Chromatin immunoprecipitation.

DISCUSSION

SAHA, also known as vorinostat, is an HDACi that has shown tumor-suppressive properties in clinical trials[33]. Findings have revealed that SAHA could suppress cell proliferation and accelerate apoptosis in multiple malignant tumor cells. Scholars have demonstrated that SAHA increased the expression of death receptor 5 in liver cancer cells, which led to the initiation of the death receptor-mediated apoptosis pathway[34]. Furthermore, SAHA contributed to the initiation of the mitochondria-related apoptosis pathway by enhancing the protein level of Bim and Bax[35]. Our previous study found that SAHA could initiate the ER stress-related apoptotic pathway to foster apoptosis in liver cancer cells by upregulating the protein level of CHOP, a transcription factor that accelerates proapoptotic gene transcription[15]. In the present study, we found that SAHA suppressed the proliferation and induced cell cycle arrest in Huh7 and MHCC97L cells. Moreover, SAHA initiated Huh7 and MHCC97L cell apoptosis.

We also indicated that SAHA could augment the expression of *FAM134B*, a putative cancer suppressor gene. Studies have uncovered the pivotal role of *FAM134B* in multiple malignancies. Lee *et al*[20] reported that *FAM134B* may exert anticancer effects by influencing mitochondrial function and inducing cell cycle arrest in colon cancer. *FAM134B* also acts as a cancer suppressor in breast carcinoma. Upregulating *FAM134B* expression was significantly correlated with a higher survival in patients with breast carcinoma[36]. Zhong[37] confirmed that *FAM134B* was decreased in liver cancer, and its decreased expression was correlated with malignant liver cancer. In contrast, upregulation of *FAM134B* inhibited HepG2 cell proliferation and enhanced cell apoptosis. *FAM134B* was proposed as the first discovered mammalian receptor of reticulophagy[38,39], a type of selective phagocytosis. As an ER-anchored protein, *FAM134B* mediates the sequestration of ER fragments into phagophore membranes through its LC3-interacting region, which binds the autophagy modifier protein LC3[40-42]. In the present study, we detected the proteins related to ER-phagy in Huh7 and MHCC97L cells

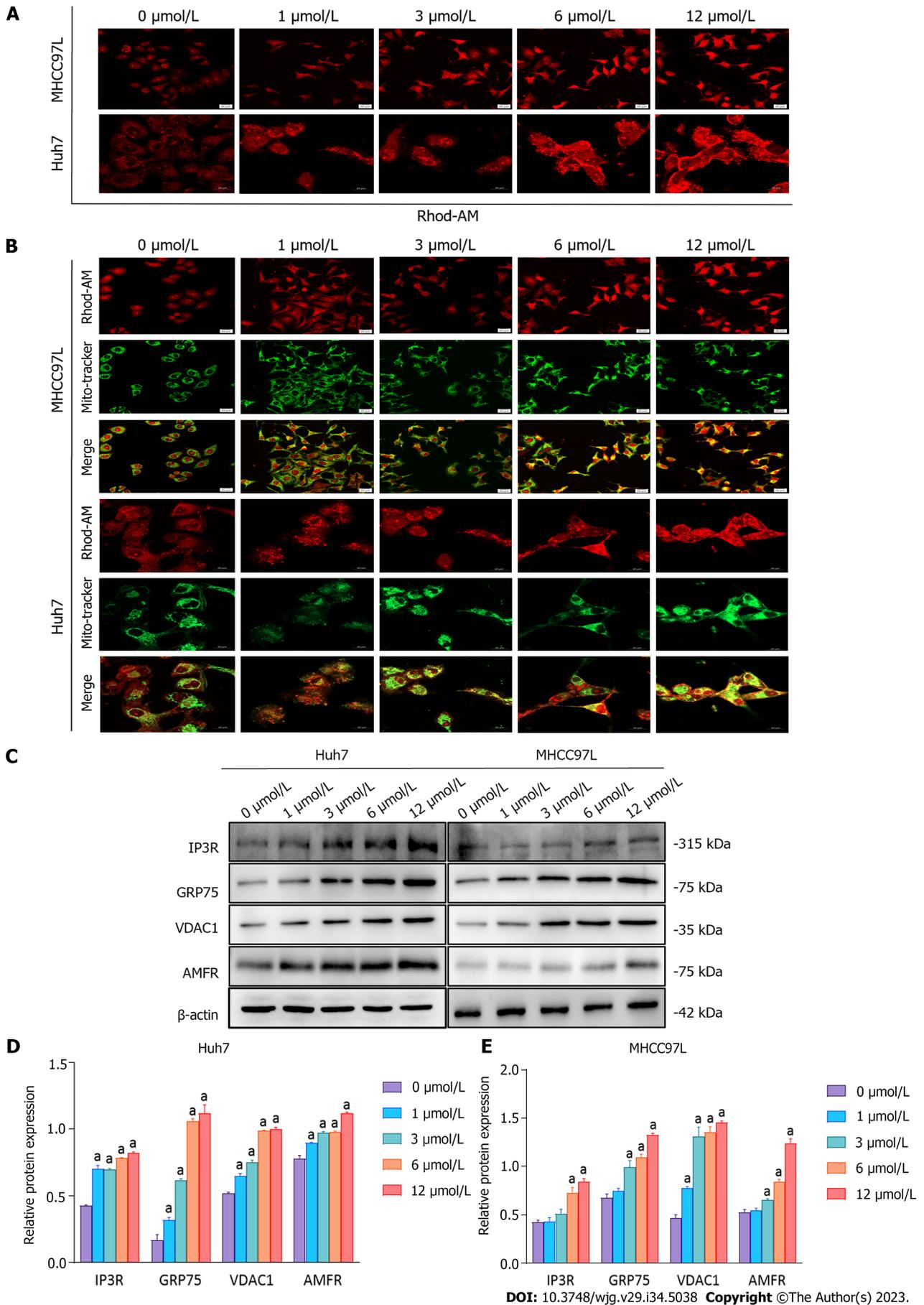


Figure 5 Impact of suberoylanilide hydroxamic acid treatment on Ca²⁺ homeostasis and the expression of autocrine motility factor

receptor and proteins related to mitochondria-endoplasmic reticulum contact sites in Huh7 and MHCC97L cells. A: Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid (SAHA) for 48 h and stained with Rhod-2 AM. The cells were observed under a confocal microscope ($\times 400$ magnification); B: Huh7 and MHCC97L cells were exposed to 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ SAHA for 48 h and simultaneously stained with Mito-Tracker Green and Rhod-2 AM Red. The cells were observed under a confocal microscope ($\times 400$ magnification). The scale is 100 μm ; C-E: Representative western blot images showed the expression of inositol 1, 4, 5-trisphosphate receptor type 1, glucose-regulated protein 75, voltage-dependent anion channel 1, and autocrine motility factor receptor in Huh7 and MHCC97L cells treated with SAHA for 48 h. Relative protein expression levels were normalized to β -actin. Data are exhibited as mean \pm standard deviation. $^aP < 0.05$ vs 0 $\mu\text{mol/L}$ group ($n = 3$). AMFR: Autocrine motility factor receptor; IP3R1: Inositol 1, 4, 5-trisphosphate receptor type 1; GRP75: Glucose-regulated protein 75; VDAC1: Voltage-dependent anion channel 1.

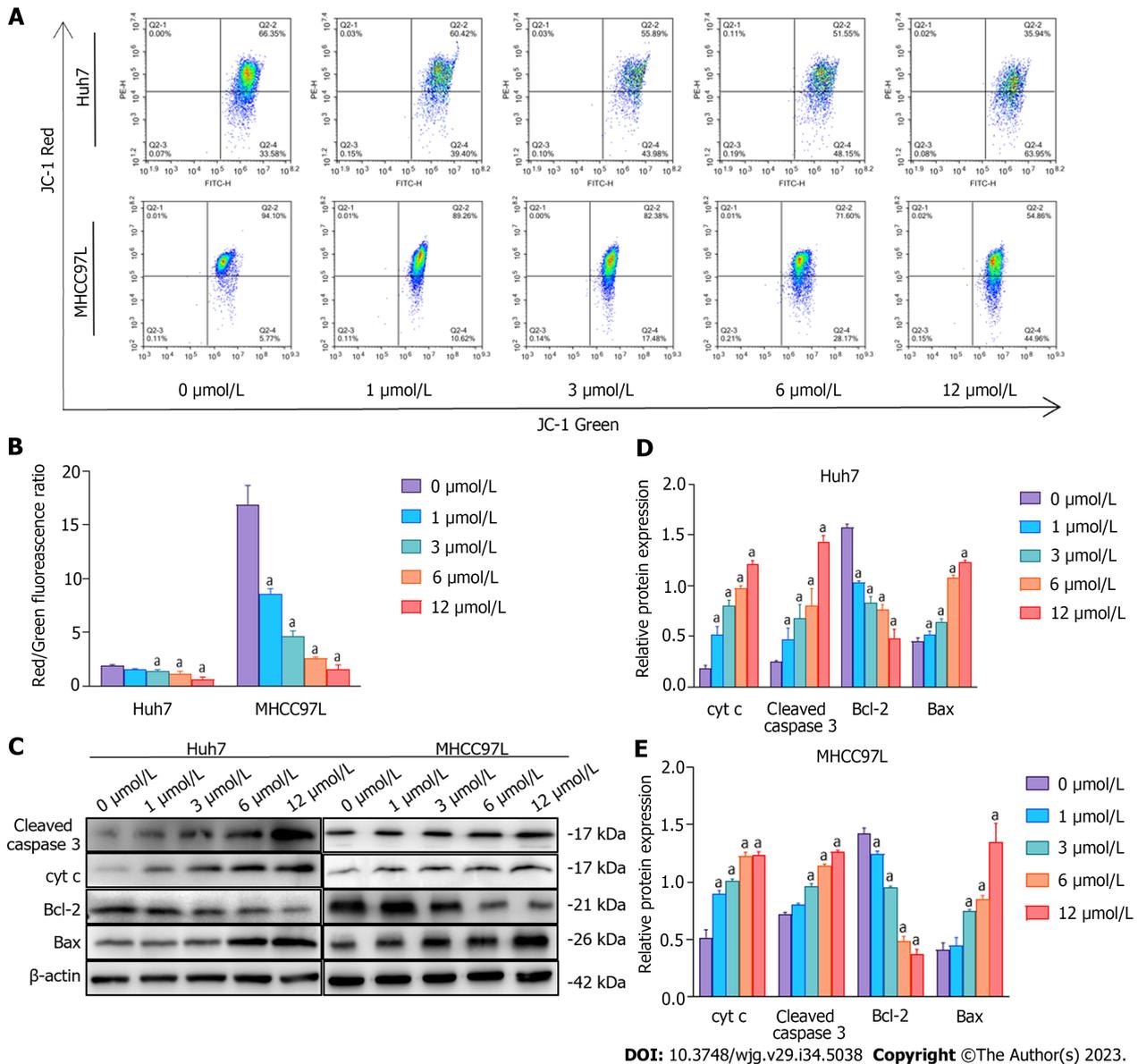


Figure 6 The impact of suberoylanilide hydroxamic acid treatment on mitochondrial membrane potential and the expression of proteins related to the mitochondrial apoptotic pathway in Huh7 and MHCC97L cells. A and B: After being treated with 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid for 48 h, the mitochondrial membrane potential in Huh7 and MHCC97L cells was analyzed using flow cytometry with bivariable JC-1 dye (mitochondrial membrane potential probe). Representative pictures from three independent replicate assays are exhibited; C-E: Representative western blot images showed the related protein expression in Huh7 and MHCC97L cells treated with 0, 1, 3, 6, and 12 $\mu\text{mol/L}$ suberoylanilide hydroxamic acid for 48 h. Relative protein expression levels were normalized to β -actin. Data are exhibited as mean \pm standard deviation. $^aP < 0.05$ vs 0 $\mu\text{mol/L}$ group ($n = 3$).

treated with various doses of SAHA. We found that the expression levels of FAM134B, Atg12, and LC3II/LC3I were considerably upregulated. Moreover, SAHA augmented the colocalization of ER and lysosomes in Huh7 and MHCC97L cells. The above results indicated that SAHA treatment enhanced the level of ER-phagy.

ER-phagy is a protective response under cellular stress conditions, but uncontrolled autophagy leads to the depletion of organelles and key proteins, which in turn lead to caspase-independent cell death, also known as autophagy-mediated

cell death[43]. Recent findings have revealed that SAHA could induce autophagy-mediated cell death in malignant tumor cells[44,45]. In fact, a decrease in cell viability detected using CCK-8 was not the same as apoptosis induction, as revealed in this study. We found that 12 $\mu\text{mol/L}$ SAHA resulted in markedly reduced Huh7 and MHCC97L cell proliferation levels (Figure 1), with proliferation being inhibited by approximately 50%; however, the rate of early apoptosis was only about 25%. This phenomenon reveals that SAHA may also elicit other types of cell death to suppress the proliferation of Huh7 and MHCC97L cells, such as autophagy-mediated cell death.

To confirm this hypothesis, we investigated cells with or without pretreatment with BafA1, a specific inhibitor of the late phase of autophagy that restrains autophagosomal fusion with lysosomes. Notably, BafA1-mediated autophagy inhibition reduced SAHA-induced cell death. These results confirmed that SAHA could enhance autophagy-mediated cell death, potentially by promoting FAM134B-mediated ER-phagy. However, the specific mechanism by which SAHA regulated FAM134B expression in Huh7 and MHCC97L cells remains elusive. As an HDACi, the essential role of SAHA is the enhancement of histone acetylation. In previous research, we revealed that SAHA markedly augmented the acetylated histones H4K5 and H4K12 in HepG2 cells[15]. In the present study, SAHA was shown to augment H4K16ac. In addition, Füllgrabe *et al*[17] reported that H4K16ac was linked to altered gene expression, including the modulation of autophagy-related genes; however, it was unclear whether H4K16ac regulated *FAM134B* transcription and its associated ER-phagy. We used ChIP to detect H4K16ac in the *FAM134B* promoter region. Our findings revealed that FAM134B promoter H4K16ac was considerably elevated in Huh7 cells exposed to SAHA, which enhanced the *FAM134B* transcription.

As a key organelle in eukaryotic cells, the ER contributes to protein synthesis and maintenance of calcium homeostasis [46,47]; hence, ER dysfunction results in the agglomeration of protein aggregates and disturbance of calcium homeostasis in the ER. Moreover, the ER contributes to organelle communication[27,48]. For example, the ER can interact with mitochondria through MERCS, as the short distance (15-20 nm) between the ER and OMM enables ER-anchored proteins to interact with OMM proteins[49]. The IP3R1-GRP75-VDAC1 complex is a central component of MERCS, which is involved in regulating calcium flow[50]. Emerging evidence has indicated that the imbalance of Ca^{2+} between the ER and mitochondria by MERCS leads to calcium overload in the mitochondria, which activates the mitochondria-associated apoptotic pathway[51].

Furthermore, under ER stress conditions, increased cytosolic Ca^{2+} would elevate the level of ER E3 ligase AMFR, which targets the OMM for ubiquitination and degradation[32]. High AMFR levels accelerate OMM degradation, which brings the inner mitochondrial membrane closer to the ER, thus promoting interplay between the ER and mitochondria[30]. In the present study, we revealed that SAHA upregulated the expression of MERCS-related proteins, including IP3R1, GRP75, and VDAC1, thereby enhancing the exchange of Ca^{2+} from the ER to the mitochondria, along with mitochondrial Ca^{2+} overload. Additionally, SAHA augmented cytosolic Ca^{2+} and increased AMFR expression, thus decreasing mitochondrial membrane potential and elevating mitochondrial membrane permeability, resulting in the release of proapoptotic proteins. As expected, we found that SAHA upregulated the expression level of mitochondria-dependent apoptotic proteins, including cytochrome c, cleaved caspase-3, and Bax but downregulated the expression of Bcl-2. The above results revealed that SAHA treatment enhanced the interplay between the ER and mitochondria and promoted Ca^{2+} transmission from the ER to the mitochondria, thereby activating the mitochondria-related apoptotic pathway.

This study had a few limitations. Despite the presence of numerous reticulophagy receptors, we focused on only FAM134B in this manuscript. For comprehensive knowledge, our team will verify multiple receptors in subsequent laboratory studies. Knocking down the gene encoding FAM134B could verify if it is a key gene in the pathway leading to HCC death. In this experiment, H4K16ac, a highly relevant acetylation site, was selected for the study. However, SAHA acts as a broad-spectrum deacetylase inhibitor influencing numerous acetylation sites, which need to be further verified in subsequent experiments. Results of this study indicated that SAHA can inhibit the proliferation of liver cancer cells *in vitro*; however, *in vivo* analyses could confirm the consistency of its effectiveness. Basic medical research serves the clinic, and the clinical verification of various aspects is more persuasive. Our study provided basic information that aids ongoing and prospective *in vivo* experiment.

CONCLUSION

The HDACi SAHA initiated apoptosis and autophagy-mediated cell death in Huh7 and MHCC97L cells to exert antitumor activity in HCC. Our results underscore a crucial link between the induction of ER-phagy and H4K16ac-linked *FAM134B* gene expression, which facilitates FAM134B-mediated ER-phagy. Moreover, we presented evidence that suggested that SAHA induced the mitochondria-associated apoptotic pathway in Huh7 and MHCC97L cells by enhancing the interplay between the ER and mitochondria and promoting Ca^{2+} exchange. In summary, SAHA promoted FAM134B-mediated ER-phagy, which acted synergistically with the mitochondrial apoptotic pathway to promote HCC cell death.

ARTICLE HIGHLIGHTS

Research background

Suberoylanilide hydroxamic acid (SAHA) has been demonstrated to trigger multiple forms of cell death in hepatocellular carcinoma (HCC). Family with sequence similarity 134 member B (*FAM134B*), a reticulophagy receptor, has been

recognized as a cancer suppressor protein in multiple tumors, including HCC. However, few researchers have focused on the relationship between reticulophagy and SAHA-induced HCC cell death.

Research motivation

Reticulophagy is involved in a variety of human cancer pathologies. However, its specific function in the modulation of SAHA-initiated HCC cell death remains unproven.

Research objectives

To validate the potential regulatory mechanisms of the FAM134B-mediated reticulophagy in SAHA-induced HCC cell death.

Research methods

The proliferation, apoptosis, and cell cycle of SAHA-treated Huh7 and MHCC97L cells were quantified using cell counting kit-8 and flow cytometry. The migration and invasion of Huh7 and MHCC97L cells were measured using the transwell assay. Proteins related to the reticulophagy pathway, mitochondria-endoplasmic reticulum contact sites, intrinsic mitochondrial apoptosis, and histone H4K16 acetylation were detected using western blotting. ER and lysosome co-localization, and mitochondrial Ca²⁺ levels were observed *via* confocal microscopy. Autophagy-mediated cell death was validated through Hoechst33342 staining and propidium iodide colocalization. The enrichment of histone H4 lysine 16 acetylation in the *FAM134B* promoter region was determined using chromatin immunoprecipitation.

Research results

SAHA treatment augmented the expression of proteins related to the reticulophagy pathway and enhanced the level of reticulophagy in HCC cells. Chromatin immunoprecipitation experiments confirmed that SAHA regulated FAM134B expression by increasing the histone H4 lysine 16 acetylation in the *FAM134B* promoter region. SAHA interfered with Ca²⁺ homeostasis in HCC cells and upregulated the expression of autocrine motility factor receptor-related and mitochondria-endoplasmic reticulum contact sites-related proteins. Furthermore, SAHA reduced mitochondrial membrane potential and aggravated the activation of the reticulophagy-mediated mitochondrial apoptosis pathway and HCC cell death.

Research conclusions

SAHA stimulated excessive reticulophagy and induced autophagy-mediated cell death, which acted synergistically with the mitochondria-dependent apoptotic pathway to facilitate HCC cell death.

Research perspectives

FAM134B-induced reticulophagy may further provide a novel avenue for more effective interventions in HCC treatment. Our results confirmed that reticulophagy participates in SAHA-induced apoptosis and autophagy-mediated cell death in HCC cells, where SAHA-induced regulation of FAM134B expression *via* histone H4 lysine 16 is the key to HCC cell death.

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FOOTNOTES

Author contributions: Xie RJ and Yang Q contributed to the experimental conception and design; Li JY, Tian T, and Han B conducted the experiments; Yang T and Guo YX collected and assembled the experimental data; Li JY, Chen YS, and Wu JY contributed to data analysis and interpretation; Li JY and Xie RJ wrote the article; All authors approved the final manuscript. Li JY, Tian T, and Han B contributed equally to this work.

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