Original Article
Suberoylanilide hydroxamic acid inhibits proliferation and promotes apoptosis of mouse myeloma Sp2/0 cells through activating p53/caspase-3 signaling

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Abstract: Multiple myeloma (MM) is considered as one of the largest contributors to cancer-related deaths in the world. Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, is used extensively for treating cancers. However, the effect of SAHA and its underlying mechanisms in MM remain elusive. In this study, the viability of mouse myeloma Sp2/0 cells was assessed by CCK-8 assay. Proliferation of mouse myeloma Sp2/0 cells was evaluated by 5-ethynyl-20-deoxyuridine and colony formation assay. Apoptosis was determined by flow cytometry using Annexin V-APC/7-AAD apoptosis kit. Western blot was carried out to detect protein levels of PCNA, Bax, p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3 and cleaved PARP. SAHA decreased the viability of mouse myeloma Sp2/0 cells in a dose- and a time-dependent manner. Furthermore, SAHA repressed proliferation of mouse myeloma Sp2/0 cells involving a decreased number of EdU positive cells and a reduced colony-forming ability. Furthermore, SAHA promoted apoptosis of mouse myeloma Sp2/0 cells, as evidenced by the decreased level of PCNA and the increased level of Bax. Moreover, SAHA activated the p53/Caspase-3 signaling pathway, accompanying with upregulation of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3 and PARP protein. Taken together, these findings suggest that SAHA inhibits proliferation and promotes apoptosis of mouse myeloma Sp2/0 cells through activating the p53/Caspase-3 signaling pathway.

Keywords: Suberoylanilide hydroxamic acid, proliferation, apoptosis, myeloma

Introduction

Multiple myeloma (MM), which is also referred to as plasma cell myeloma, is the second most common hematological malignancy, accounting for low remission rate and high relapse rate [1]. Globally, MM is estimated to result in 101100 deaths in 2015 [2]. MM is hallmarked by clonal expansion of malignant plasma cells in the bone marrow [3]. Clinically, MM can manifest as bone pain, bleeding, renal impairment, amyloidosis, heterogeneous anemia, immune dysfunction, hypercalcaemia, and infections [4]. Currently, the aim of treatment of MM is to control clinical symptoms, improve quality of life and prolong lifespan [5]. Despite substantial advances in clinical treatment over the past decade, MM is still considered to be an incurable disease [6]. Therefore, expanding our knowledge of the physiological mechanism of MM is crucial to capture MM.

Mounting studies have documented that epigenetic modifications, such as histone acetylation, play an important role in the occurrence and development of human cancers [7]. Histone acetylation can affect the dynamics of chromatin folding during gene transcription [8]. In the organism, histone acetyltransferase (HAT) and histone deacetylase (HDAC) play antagonistically important roles in the modification of histone acetylation and deacetylation [9]. HAT transfers an acetyl group from acetyl-CoA to form ε-N-acetyl lysine amino on histone and ultimately contributes to increased gene expression [10]. Conversely, HDAC removes acetyl groups from anε-N-acetyl lysine amino on histone and leads to inhibition of gene expression [10]. Recently, a growing body of evidence suggests that an imbalance between HAT and HDAC is tightly associated with the occurrence and development of human cancers [11, 12]. Therefore, targeting

HDAC has become a novel target for cancer therapy.

By inhibition of histone deacetylase, histone deacetylase inhibitor (HDACi) leads to accumulation of acetylated nucleosomal histones and exerts the anti-tumor efficiency through repressing the proliferation and promoting the apoptosis of cancer cells [13]. Suberoylanilide hydroxamic acid (SAHA), otherwise known as vorinostat, is the first histone deacetylase inhibitor granted by the US Food and Drug Administration for the treatment of cutaneous t-cell lymphoma. SAHA can induce cell differentiation and block cell cycle through inhibiting class I HDACs, class II HDACs and class IV HDACs and is used extensively as a novel antineoplastic target therapy drug to treat cancers [14, 15]. Previously, SAHA has been documented to inhibit cell growth in several cancers [16, 17]. However, the effect of SAHA and its underlying molecular mechanism in MM is still not well characterized.

In our study, we explored the effect of SAHA on proliferation and apoptosis in mouse myeloma Sp2/0 cells and its underlying molecular mechanism was further investigated. Our findings conclude that SAHA represents a promising antineoplastic drug for the treatment of MM.

Materials and methods

Cell culture

Mouse myeloma Sp2/0 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and were incubated in the RPMI-1640 medium (Solarbio, Beijing, China) supplemented with 10% calf serum (Solarbio), 100 U/ml penicillin (Solarbio), and 100 g/ml streptomycin (Solarbio) at 37°C in humidified 5% CO_2 and 95% air. Mouse myeloma Sp2/0 cells were treated with different concentration (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA for 24 or exposed to 2 µM SAHA for different duration (0, 12, 24, 36, 48 and 72 hours).

Cell counting kit-8 (CCK-8) assay

The viability of mouse myeloma Sp2/0 cells was evaluated by using CCK-8 (Solarbio). In brief, mouse myeloma Sp2/0 cells were seeded in 96-well plates and incubated with increasing concentration (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA in a 5% CO_2 incubator at 37°C. After 48

hours of incubation, CCK-8 solution was added into each well, and plates were detected employing a microplate reader set at 450 nm.

5-ethynyl-20-deoxyuridine (EdU) assay

Proliferation rates of mouse myeloma Sp2/0 cells were determined with EdU (Beyotime, Shanghai, China). In brief, mouse myeloma Sp2/0 cells were treated with increasing concentration (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA for 48 hours. Mouse myeloma Sp2/0 cells were cultured in the RPMI-1640 medium contained 10 µM EDU for 2 hours, followed by fixation with 4% paraformaldehyde for 15 minutes. Subsequently, mouse myeloma Sp2/0 cells were counterstained with Hoechst 33342 and visualized under a fluorescence microscopy. Pictures were photographed and analyzed by mage J software.

Colony formation assay

The colony-forming ability of mouse myeloma Sp2/0 cells was determined by colony formation assay. Mouse myeloma Sp2/0 cells were seeded in 6-well plates and incubated with indicated concentration of SAHA. After 14 days of incubation, mouse myeloma Sp2/0 cells were fixed with methanol and stained with 0.1% crystal violet. Colonies greater than 2 mm in diameter were counted.

Flow cytometry

Apoptosis was determined by flow cytometry using Annexin V-APC/7-AAD apoptosis kit (KeyGEN BioTECH, Nanjing, China). Mouse myeloma Sp2/0 cells were incubated with increasing concentration of SAHA for 48 hours. Mouse myeloma Sp2/0 cells were washed and resuspended in cold Annexin-binding buffer. Then, cells were probed with Annexin V-APC for 10 minutes, followed by incubation with 7-AAD for 5 minutes at room temperature in dark. Cells were resuspended in PBS and analyzed by flow cytometry within 4 hours.

Western blot assay

Following incubation with the indicated concentrations of SAHA, mouse myeloma Sp2/0 cells were harvested and lysed in RIPA buffer at 4°C. The protein concentration was evaluated using BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved
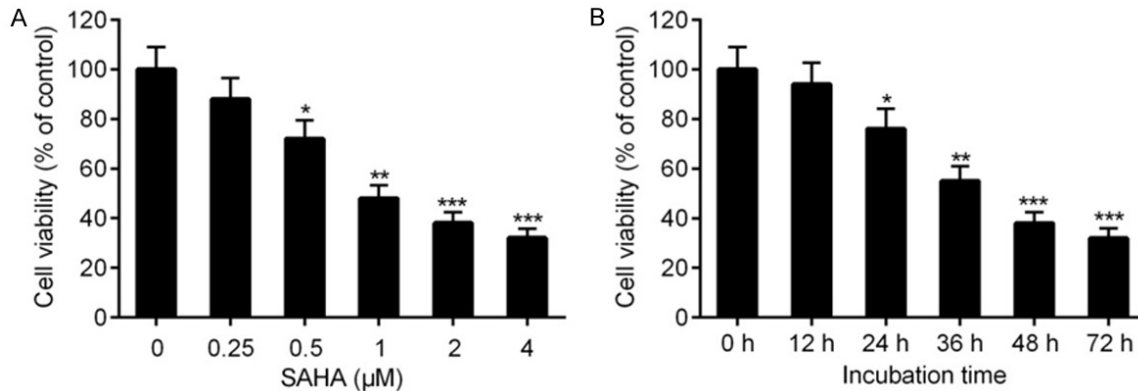
Figure 1. Cytotoxic effects of SAHA on mouse myeloma Sp2/0 cells. A. Mouse myeloma Sp2/0 cells were treated with increasing concentrations (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA for 48 hours. SAHA strikingly decreased the viability of mouse myeloma Sp2/0 cells, in a dose-dependent manner. B. Mouse myeloma Sp2/0 cells were exposed to 2 µM SAHA for different duration (0, 12, 24, 36, 48 and 72 hours). SAHA markedly decreased the viability of mouse myeloma Sp2/0 cells, in a time-dependent manner. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

in 10% SDS-PAGE and electrotransferred onto PVDF membranes. After blocking with 5% skim milk, membranes were immunoblotted with primary antibodies against proliferating cell nuclear antigen (PCNA; Cell Signaling Technology, Danvers, MA, USA), Bax (Cell Signaling Technology), p53 (Novus, Littleton, Colorado, USA), cleaved caspase-8 (Novus), cleaved caspase-9 (Novus), cleaved caspase-3 (Novus) and cleaved Poly (ADP-ribose) polymerase (PARP; Novus) at 4°C overnight. After washing in PBS, membranes were incubated for 2 hours at room temperature with horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). Bands were developed using ECL (Pierce), and analyzed by Image J software.

Statistical analysis

Values are expressed as mean ± SD for a series of experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey post hoc test using SPSS 20.0 statistical analysis software. *P* value of 0.05 or less was considered statistically significant.

Results

The cytotoxic effects of SAHA on mouse myeloma Sp2/0 cells

To address the cytotoxic effects of SAHA, CCK-8 assay was performed in mouse myeloma Sp2/0 cells treated with indicated concentrations (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA for 48 hours or exposed to 2 µM SAHA for different duration

(0, 12, 24, 36, 48 and 72 hours). As displayed in **Figure 1A** and **1B**, SAHA treatment strikingly decreased the viability of mouse myeloma Sp2/0 cells, in a dose-and a time-dependent manner. Moreover, there was no obvious difference on the viability of mouse myeloma Sp2/0 cells exposed to 2 µM SAHA at 48 or 72 hours. Thus, our findings suggested that SAHA treatment induced a dose- and a time-dependent cytotoxic effect in mouse myeloma Sp2/0 cells.

SAHA inhibits the proliferation of mouse myeloma Sp2/0 cells

To explore the cytotoxic effects of SAHA on the proliferation of mouse myeloma Sp2/0 cells, mouse myeloma Sp2/0 cells were incubated with indicated concentration (0, 0.25, 0.5, 1, 2 and 4 µM) of SAHA for 48 hours and the proliferation of mouse myeloma Sp2/0 cells were assessed by EdU assay and colony formation assay. As a result, SAHA strikingly decreased the number of EdU positive cells, in a dose-dependent manner (**Figure 2A**). As detected by colony formation assay, SAHA administration led to a dose-dependent reduction in the colony-forming ability of mouse myeloma Sp2/0 cells (**Figure 2B**). Together, these data indicated that SAHA inhibited the proliferation of mouse myeloma Sp2/0 cells in a dose-dependent manner.

SAHA promotes the apoptosis of mouse myeloma Sp2/0 cells

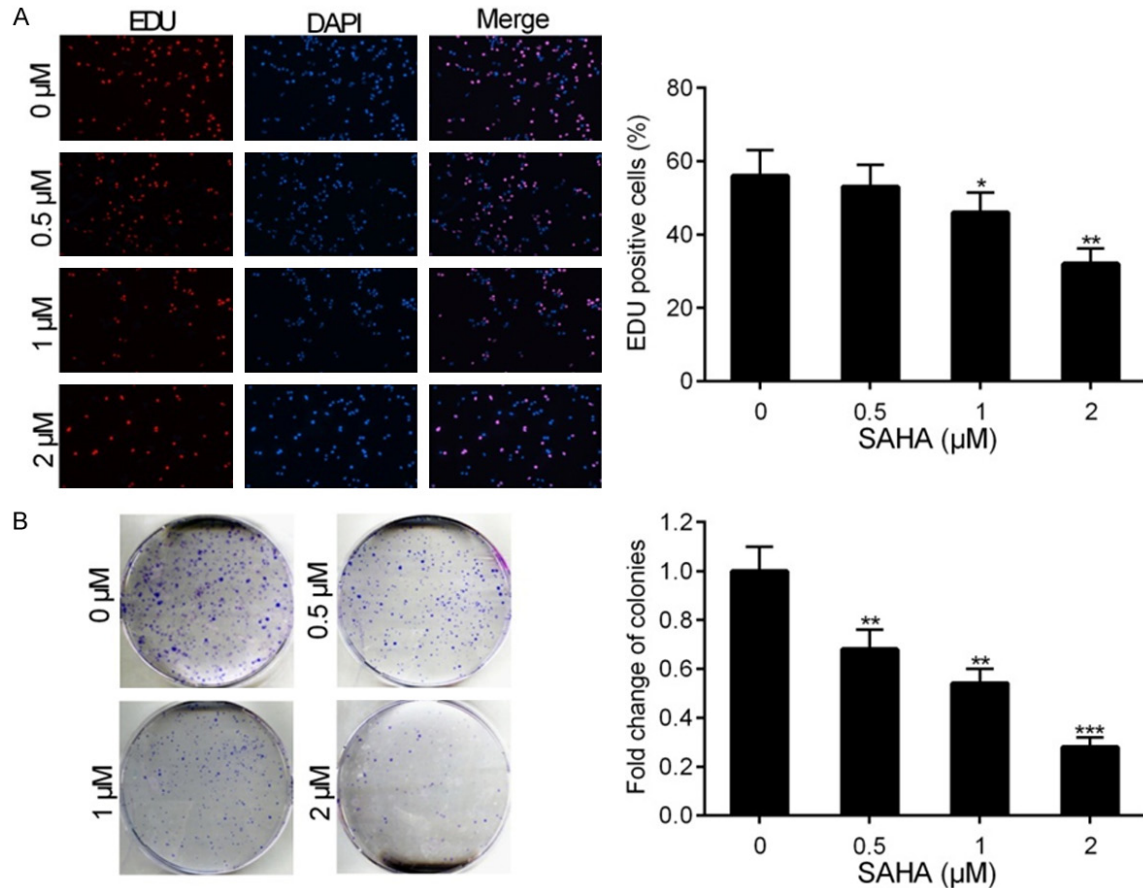We investigated whether the cytotoxic effects of SAHA was contributable to the apoptosis of

Figure 2. SAHA inhibits the proliferation of mouse myeloma Sp2/0 cells. Mouse myeloma Sp2/0 cells were incubated with increasing concentration (0, 0.25, 0.5, 1, 2 and 4 μM) of SAHA for 48 hours. A. SAHA treatment led to a dose-dependent reduction in the number of EdU positive cells. B. At 14 days after incubation, the colony-forming ability of mouse myeloma Sp2/0 cells was determined by colony formation assay. SAHA remarkably reduced the colony-forming ability of mouse myeloma Sp2/0 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mouse myeloma Sp2/0 cells using flow cytometry. After 48 hour treatment of increasing concentration (0, 0.25, 0.5, 1, 2 and 4 μM) of SAHA, SAHA treatment led to a dose-dependent elevation of apoptosis in mouse myeloma Sp2/0 cells (**Figure 3A**). To further inquiry the mechanism by which SAHA promote apoptosis of mouse myeloma Sp2/0 cells, the levels of PCNA and Bax were measured by Western blot. SAHA treatment decreased the protein level of PCNA in a dose-dependent manner (**Figure 3B**). In contrast, SAHA treatment increased the protein level of Bax in a dose-dependent manner (**Figure 3C**). Therefore, these findings uncovered that SAHA promoted apoptosis of mouse myeloma Sp2/0 cells.

SAHA activates p53/caspase-3 signaling pathway in mouse myeloma Sp2/0 cells

To validate whether the cytotoxic effects of SAHA were mediated by the p53/Caspase-3

signaling pathway, the protein levels of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved PARP were detected by using Western blot. After 48 hours of treatment of increasing concentration (0, 0.25, 0.5, 1, 2 and 4 μM) of SAHA, the protein levels of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved PARP were conspicuously increased in mouse myeloma Sp2/0 cells treated with indicated concentration of SAHA (**Figure 4A-E**). Collectively, SAHA activated p53/Caspase-3 signaling pathway in mouse myeloma Sp2/0 cells.

Discussion

MM is a malignant tumor of hematopoietic system, which take account for 1% of human malignant tumors and 10% of hematologic malignancies [18]. MM often occurs in old people, which morbidity increases with age [19]. MM is closely related with increased monoclo-
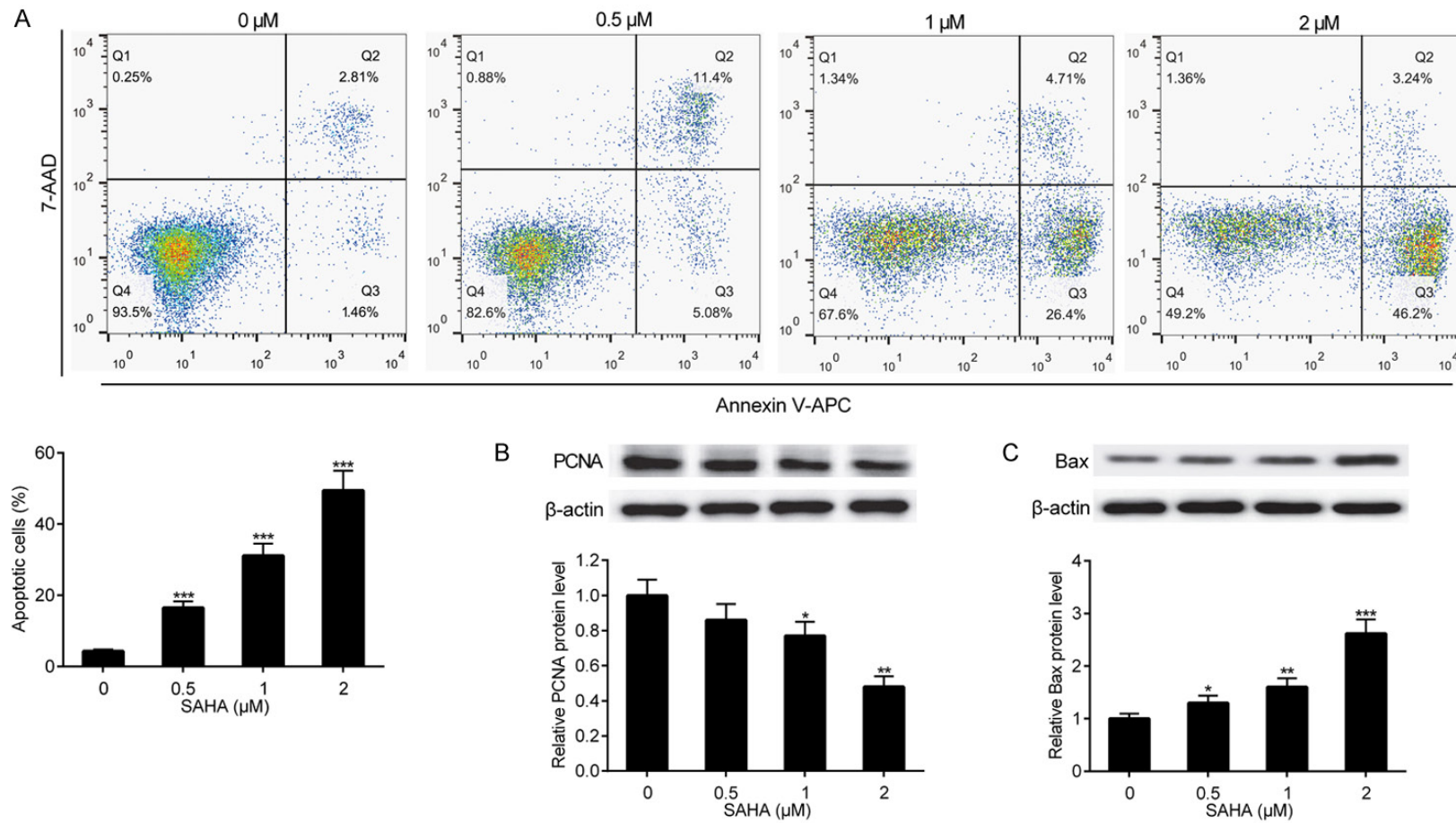
Figure 3. SAHA promotes the apoptosis of mouse myeloma Sp2/0 cells. A-C: After 48 hour treatment of increasing concentration (0, 0.25, 0.5, 1, 2 and 4 μM) of SAHA, the apoptosis was determined by flow cytometry and the protein levels of PCNA and Bax were measured by Western blot. SAHA promoted the apoptosis of mouse myeloma Sp2/0 cells, as evidenced by the decreased level of PCNA and the increased level of Bax. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
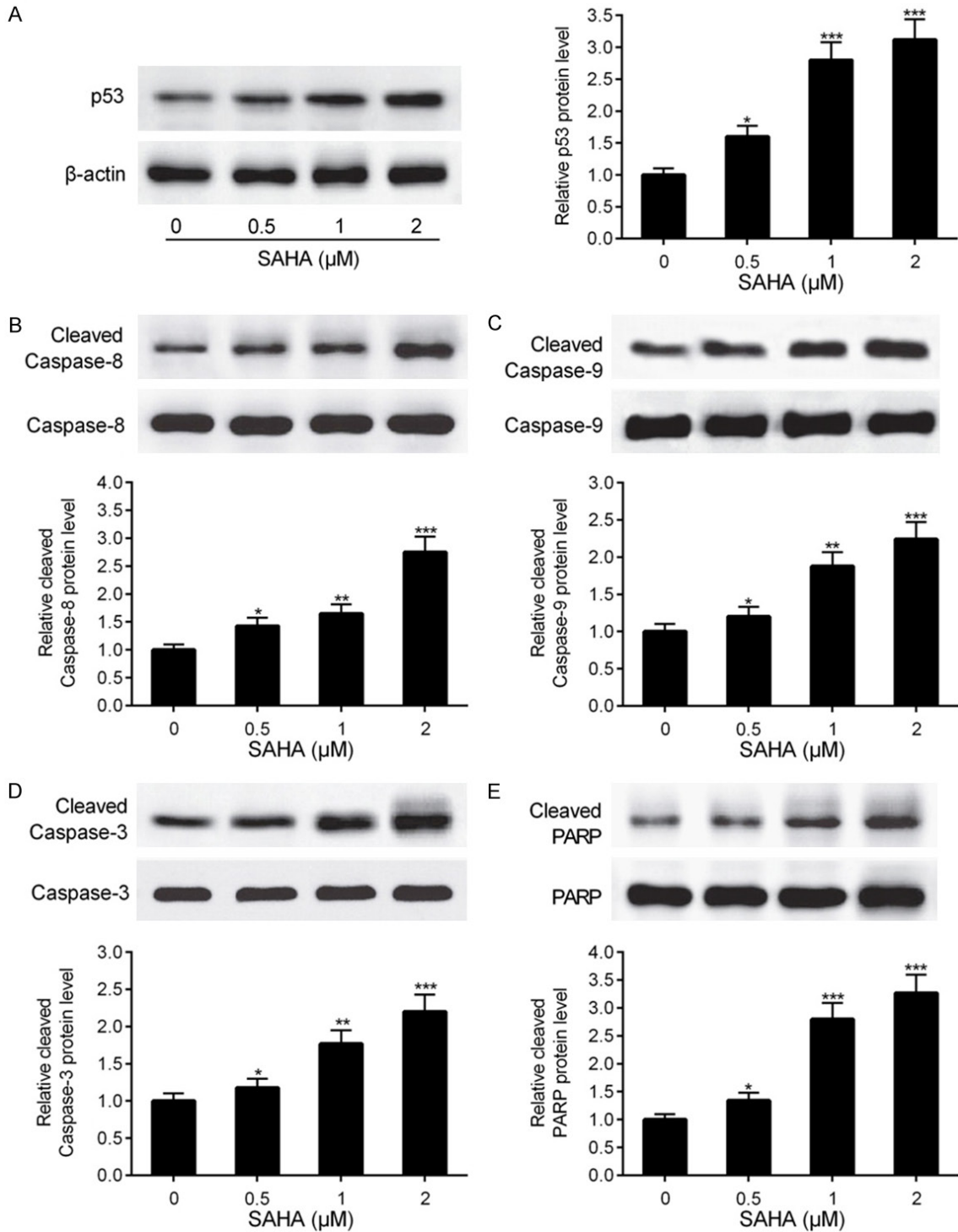
Figure 4. SAHA activates p53/Caspase-3 signaling pathway in mouse myeloma Sp2/0 cells. A-E. After 48 hour treatment of increasing concentration (0, 0.25, 0.5, 1, 2 and 4 μM) of SAHA, the protein levels of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved PARP were detected by using Western blot. SAHA administration increased the protein levels of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved PARP, in a dose-dependent manner. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

nal immunoglobulin levels. Malignant plasma cells invade the skeletal system and cause areas of extensive bone destruction [20]. Currently, drug therapy is one of the main treat-

ments for MM and increased the 5-year survival from 32.6% to 46.6% [21]. Many drugs are used for MM treatment, such as bortezomib, thalidomide, ixazomib and melphalan. Nevertheless, most of them have proven to have harmful side effects [22]. As an example, large dosages of melphalan obviously improve the therapeutic effect, but it can cause permanent damage of hematopoietic stem cells [23]. Additionally, combination treatment of thalidomide and lenalidomide markedly increased the incidence of deep vein thrombosis in MM patients [24]. Furthermore, ixazomib treatment may lead to thrombocytopenia, peripheral neuropathy and gastrointestinal adverse reactions [25]. Thus, searching for the new effective drugs for the treatment of MM remains a priority.

SAHA has been the focus of increasing attention due to its anti-tumor effect in numerous cancers. Recently, several lines of evidence have shown that SAHA plays a crucial role in the treatment of human cancers. For instance, Lee et al. found that SAHA repressed the proliferation of SK-Mel-5 cells through upregulation of activin A and activation of the Smad signaling pathway [26]. Peela et al. suggested that SAHA decreased microtubule polarization and suppressed cell migration and proliferation of SUM159 breast cancer cells [27]. You et al. demonstrated that SAHA increased ROS levels and induced GSH depletion and thioredoxin1-mediated apoptosis in lung cancer cells. Moreover, SAHA downregulated Trx1 level via upregulation of miR-129-5p through activating ASK-JNK/p38 kinase pathway [28]. Grabarska et al. uncovered that SAHA repressed cell proliferation, induced hyperacetylation of histone H3 K9, K14, and K23 and triggered apoptosis in RK33 and RK45 cells. Furthermore, SAHA blocked cell cycle through upregulating *CDKN1A* and downregulating *CCND1* [29]. Kunnimalaiyaan et al. indicated that SAHA administration reduced cell viability and colony forming ability of hepatocellular carcinoma cells through inducing apoptosis and blocking cell cycle. In addition, SAHA repressed Notch, AKT, and Raf-1 pathways but not the STAT3 pathway in hepatocellular carcinoma cell lines [30]. Hsu et al. demonstrated that SAHA inhibited cell proliferation, triggered apoptosis, and repressed the stemness properties of glioma stem-like cells. Additionally, SAHA at a low con-

centration resulted in cell cycle arrest via upregulating p21, and induction of premature senescence via activation of the p38/p53 pathway [31]. Arhoma et al. stated that SAHA inhibited MM cells growth and enhanced tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis through activation of caspase-8 and -9 [32]. In our study, SAHA reduced the viability of mouse myeloma Sp2/0 cells in a concentration- and time-dependent manner. Furthermore, SAHA repressed the proliferation of mouse myeloma Sp2/0 cells involving the decreased number of EdU positive cells and the reduced colony-forming ability.

Apoptosis is an active form of cell death in an attempt to in order to maintain homeostasis, which is regulated by polygene. Experimental evidence in the last two decades indicates that apoptosis is characteristically impaired in cancer cells and induction of apoptosis in cancer cells is one of the strategies of the treatment of cancers [33]. Previous studies have demonstrated that the p53/Capsase-3 signaling pathway is implicated in the development of human cancers [34]. PARP is a multifunctional nuclear protein which involved in a variety of cellular processes such as DNA repair and apoptosis [35]. p53, a tumor suppressor, induces DNA repair and plays a vital role in cell cycle arrest and cell apoptosis. In pathological conditions like cancers, activated PARP can induce apoptosis through depletion of ATP and production of PAR, which in turn stimulate mitochondria to release apoptosis-inducing factor and ultimately cause cell death [36]. Furthermore, activated PARP leads to the accumulation of p53, which interacts with the members of the Bcl-2 family and results in the translocation of Bax from the cytosol to the mitochondria, the increase of mitochondria membrane permeability and release of cytochrome *c*. Then, cytochrome *c* binds to apoptotic protein-activating factor-1 and procaspase-9 to form an "apoptosome", which leads to activation of caspase-9 and caspase-3 and finally results in cell apoptosis [37]. Recruitment of p53 can lead to an activation of Fas. Activated Fas causes caspase-8 activation, which cleaves full-length BID and leads to the release of cytochrome *c*, the formation of apoptosome and cascade reaction of caspase and ultimately results in cell apoptosis [38]. Our results indicate that SAHA promoted the apoptosis of mouse myeloma Sp2/0 cells

and the SAHA-mediated apoptosis was related with the p53/Caspase-3 signaling pathway. Indeed, SAHA increased the protein levels of p53, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved PARP, in a dose-dependent manner.

In summary, we found that SAHA inhibited proliferation and triggered apoptosis of mouse myeloma Sp2/0 cells in a concentration- and a time-dependent manner. Our results demonstrate that the cytotoxic effect of SAHA on mouse myeloma Sp2/0 cells might be mediated through p53/Caspase-3 signaling. Furthermore, our study indicates that SAHA is a promising antineoplastic drug for the treatment of MM and provides experimental evidence for clinical application of SAHA.

Disclosure of conflict of interest

None.

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