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Histone deacetylase inhibitor LMK-235-mediated HO-1 expression induces apoptosis in multiple myeloma cells via the JNK/AP-1 signaling pathway

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ABSTRACT

Aims: Histone deacetylase inhibitors (HDACis) are promising anticancer drugs that open new areas of epigenetic drug discovery. Multiple myeloma (MM) is a malignant tumor of the blood system that is difficult to cure and often relapses. Here, we investigated the in vitro effects of a novel HDACi, LMK-235, on MM cells, and explored the underlying mechanisms.

Main methods: Real-time PCR and western blot were used to measure the expression of HDAC4 and HO-1 in MM cells treated with LMK-235. si-RNA was used to transfect MM cells. Hemin or ZnPP was combined to regulate heme oxygenase-1 (HO-1), and a pathway inhibitor was added to measure changes in the JNK/AP-1 signaling pathway. Apoptosis and proliferation were assessed by flow cytometry and CCK-8 assay, respectively.

Key findings: We found that LMK-235, a selective inhibitor of class IIA HDAC4/5, induced apoptosis of MM cells by downregulating HO-1 that is closely related to HDAC4. LMK-235 increased phosphorylation of JNK and c -Jun in MM cells. Downregulation of HO-1 expression in combination with LMK-235 expression further activated phosphorylation of JNK and c-Jun and induced apoptosis in MM cells. When the JNK inhibitor SP600125 was used in combination, the apoptosis phenomenon was reversed. However, when HO-1 was upregulated, LMK-235-mediated phosphorylation of JNK and c-Jun was inhibited, and apoptosis of MM cells began to decrease.

Significance: These data suggest that LMK-235 has potent anti-myeloma activity through regulation of HO-1-induced apoptosis via the JNK/AP-1 pathway. This provides a new concept for the treatment of multiple myeloma.

1. Introduction

Multiple myeloma (MM) is an invasive hematological malignancy characterized by clonal proliferation of abnormal plasma cells in the bone marrow [1,2]. New cases of multiple myeloma diagnosed each year account for about 13% of global blood cancer and 1% of all cancers [3]. Despite the fact that agents such as bortezomib, thalidomide, lenalidomide and autologous stem cell transplantation have significantly contributed to the improvement of survival outcomes in MM patients [4,5], MM remains an incurable disease, with a high likelihood of relapse. Therefore, it is essential to identify alterative targets and additional therapeutic strategies for MM.

In recent years, epigenetic modification has emerged as a method for treating various diseases. Acetylation is one such method. It occurs as a post-translational modification of the ε-amino group of lysine residues of a cellular protein; histones are the most studied substrate [6]. Histone deacetylase inhibitors (HDACis) increase histone acetylation, induce apoptosis and differentiation and inhibit tumor cell proliferation [7,8]. HDAC inhibitors have been shown to exhibit potent anticancer activity in preclinical and clinical studies, and have become novel drugs for the treatment of hematological malignancies. Among them, suberoylanilide hydroxamic acid SAHA (known also as vorinostat) and cyclic depsipeptide romidepsin have been approved by the FDA for the treatment of relapsed and drug-resistant T-cell lymphoma (CTCL) [9]. Pabitastat (HDACi, LBH589) has been used to treat multiple myeloma (MM) [10].

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Most HDAC inhibitors have been considered 'pan-inhibitors' that inhibit all enzymes with comparable potency; however, in other cases, more specific inhibitors may be superior to treatment and may cause fewer adverse effects [11]. Class IIA HDACs have unique functions that are different from those other HDACs; they contain an N-terminal domain that allows interaction with tissue-specific transcription factors and recruitment to their target genes. They play a vital role in organogenesis and cell differentiation [12]. Studies have shown that the inhibition of HDAC4 expression by miR-125a-5p has anti-tumor effects in breast cancer cells [13]. As a specific HDAC inhibitor, LMK-235 showed preferential activity against class IIA HDAC4/5 [11,14]. Our previous studies confirmed that LMK-235 caused apoptosis in diffuse large B-cell lymphoma via the NF-kB pathway [15]; however, the precise mechanisms for LMK-235 anti-MM action remain elusive. Here, we studied the effect of LMK-235 on MM cell apoptosis and explored its mechanism of action, providing new concepts for the treatment of MM.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the process of heme catabolism. It catalyzes conversion of hemoglobin into biliverdin, iron and carbon monoxide [16]. It is constitutively expressed in various tumor cells [17] and effectively protects cells with anti-apoptotic properties; HO-1 also promotes proliferation and induces drug resistance [18,19]. The relationship between overexpression of HO-1 and apoptosis and proliferation has been studied recently [20,21]. Our previous studies also confirmed that HO-1 plays an important role in hematological diseases, including acute myeloid leukemia, chronic myeloid leukemia and diffuse large B-cell lymphoma [22–24]. Activator protein-1 (AP-1) is the transcription factor that plays central roles in the expression of many genes involved in immune and inflammatory responses [25]. AP-1 controls many cellular processes, including differentiation, proliferation, and apoptosis [26]. In this study, we hypothesized that HO-1 was involved in LMK-235-mediated MM apoptosis and may be a potential therapeutic target.

We first evaluated the activity of LMK-235 on MM cells and found that LMK-235 may be associated with HO-1 by affecting HDAC4 activity. We further regulated the expression of HO-1 and verified its activation of JNK/AP1 signaling. It is involved in the mechanism of action of LMK-235 in MM cells and mediated apoptosis of MM cells. This may provide a new strategy for the treatment of MM.

2. Materials and methods

2.1. Reagents and antibodies

In this research, the reagents were employed as follows: specific selective HDACi LMK-235 (MedChemExpress, USA); fetal bovine serum (Gibco BRL); RPMi-1640 medium (gibco BRL); dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO,USA); annexin V-fluorescein isothiocyanate (fiTC)/propidium liodide (Pi) apoptosis detection kit (BD Biosciences, San Jose, CA, USA); The bought HO-1 inhibitor ZnPPIX was from Cayman Chemical (Ann Arbor, MI,USA). Antibodies specific for HDAC4, HDAC5, HDAC6, β-actin, Acetylated α-tubulin, p-JNK, p-c-Jun, Bcl-2, Bad, Bax and HO-1 were purchased from MDL biotech (Beijing, China). Secondary antibodies (Li-Cor Corp., Lincoln, NE, USA); and TRIzol reagent (Life Technologies).

2.2. Patient samples

Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors to obtain mononuclear cells. CD138 + plasma cells from patients with MM and healthy donors were purified using CD138 (syndecan-1) microbeads with a Miltenyi Magnetic Cell Sorting System (Miltenyi Biotec, Auburn, California, USA).Institutional Review Board approval was obtained from Guizhou Medical University Hospital (Guiyang, Guizhou, China). The study was carried out in accordance with the modified Helsinki Declaration and the protocol was approved by our ethical review boards before study initiation. Informed consent was obtained from all patients and healthy volunteers.

2.3. Cell lines and cell culture

The bought Human MM cell line RPMI8226 and U266 cells were from Cobioer Bioscience (Nanjing, China). The cryopreserved MM cell lines were in Guizhou Hematopoietic Stem Cell Transplantation Center Laboratory (Guiyang, China). MM cell lines were kept in RPMI 1640 medium (Sigma-Aldrich), in which 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen, Carlsbad, CA, USA) were added. All cells were incubated in an incubator at 37 °C, keeping 95% humidity and 5% CO_2. All of the tests mentioned above were carried out employing logarithmic growth cells (3–6 × 10^5 cells/ml).

2.4. Cell viability assay

Cells were inoculated into 96-well plates with a density of 5000/well. The cells were then cultured overnight and processed through diverse concentrations (0, 1, 2, 3, 4and 5 μmol/L) of LMK-235, which lasted 24 and 48 h, respectively. The inhibition of LMK-235 was measured employing the cell counting kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Haimen, China) and duplicated three times. The survival rate (SR) was calculated from the equation listed below: SR (%) = (A Treatment / A Control) × 100%. The GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) was required to measure the concentration which can make 50% cytotoxicity (IC50).

2.5. Apoptosis assay

On the basis of the supplier's illustrations, the measurement of apoptosis was required using double staining of annexin-V–FITC and propidium iodide (PI) (7Sea Biotech, Shanghai, China). The MM cells were processed through fresh drug preparations and culture medium, then used PBS as washing buffer, and suspended in a 100 μl combination buffer again containing 5 μl of annexin-V (BD Pharmingen, San Diego, California, USA). After adding 7 μl of PI, the cells were studied using flow cytometry, and data were processed by FACSCalibur flow cytometry (BD Biosciences). The experimental data showed the average values of three independent experiments.

2.6. siRNA transfection

Antagonism of HDACs (si-HDAC4 and si-HDAC6) by siRNA inhibited the expression of the endogenous HDACs. The interference siRNA (si-NC) was employed as a negative contrast. The siRNA sequence was originally developed by TranSheep Bio-Tech Co., Ltd. (Shanghai, China) and finally synthesized for application.

The target sequences of these include:

Si-HDAC4, 5′-GCGUCUUAUUGAACUUAUUTT-3′(sense), 5′-AAUAA GUUCAAUAAGACGCTT-3′(anti sense).

Si-HDAC6, 5′-CCAUUGCCUACGAGUUUAATT-3′(sense), 5′-UUAAA CUCGUAGGCAAUGGTT-3′(anti sense).

With each transfection concentration of siRNA was 50 nM. On the basis of the supplier's illustrations, cells were electroporated employing transfection buffer and Soly-fecter (TranSheep Bio-Tech Co., Ltd., Shanghai, China). After 48 h of transfection, the collected cells were used for quantitative real-time polymerase chain reaction or reagent treatment. The transfected cells were cultured for 48 h, Western blot was then performed.

2.7. Quantitative real-time PCR assay

On the basis of the supplier's illustrations, total RNAs were picked up from cell lines and primary mononuclear cell samples employing Trizol reagent (Invitrogen, Carlsbad, CA, USA). PCR was performed

employing the SYBR Green PCR Master Mix (TianGen Biotech, Beijing, China) and the PRISM 7500 real-time PCR detection system (ABI, USA). The levels of gene expression relative to that of β-actin gene transcript were analyzed.

The sequences of primers were as follows:
HDAC4-F 5′-GCCCACACACTCCTCTAC-3′;
HDAC4-R 5′-AAGCCTGACGAACACTGA-3′;
HDAC6-F 5′-GCACGCTGTCTCATCCTACCT-3′;
HDAC6-R 5′-CCCGAGTTTTCATCTTTTCTGTG-3′;
HDAC1-F 5′-CTACTACGACGGGGATGTT-3′;
HDAC1-R 5′-CTTTGTGAGGGCGATAGA-3′;
HO-1-F 5′-ACCCATGACACCAAGGACCAGA-3′;
HO-1-R 5′-GTGTAAGGACCCATCGGAGAAGC-3′;
β-actin-F 5′-GAGACCTTCAACACCCCAGC-3′;
β-actin-R 5′-ATGTCACGCACGATTTCCC-3′.

The cDNA specimens were blended with a total volume of 20 ml of primer and SYBR Master Mix. The thermal cycling conditions employed in the protocol were 94 °C for 1 min, followed by 94 °C for 10s and 60 °C for 15 s and cycled 40 times.

2.8. Western blot analysis

Firstly, cells from different groups were gathered. After washing twice in ice-cold PBS, the cells were dissolved using ultrasound in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton ´100, 2% SDS, and 1% sodium deoxycholate) comprising 1 mM phenylmethanesulfonyl fluoride (Solarbio Science & Technology, Beijing, China). The lysate was shifted to EP tubes and held the action of Centrifugal effect at 12,000*g* for 10 min at 4 °C. Then the supernatant was gathered and blended with loading buffer. The resulting solution was boiled for 10 min and then aliquots were stored at −80 °C as far as they were used. The same amounts of protein (50–100 mg) were put in and followed by the separation of 10% SDS-PAGE, and shifted to a PVDF membrane (Millipore Corporation, Milford, MA, USA), then under the condition of 4 °C in 5% skim milk overnight closed in Tris buffer, with the correlative antibodies imprinting the membrane for 2 h. After the washing process, it was an hour's incubation at room temperature with secondary antibodies (HRP-conjugated goat anti-rabbit or anti-mouse; Beyotime, Shanghai; China). On the basis of the supplier's illustrations, the protein bands were detected on the membrane by strengthening chemiluminescence (7sea Biotech, Shanghai, China). Quantity one 4.6.2 of the image processing software was employed in order to make the quantization of the strength of protein bands for each treatment three times at lowest.

2.9. Statistical analysis

Statistical analysis of data values was performed using GraphPad Prism 6.0 software (Graphpad Software, Inc., USA). All data values are indicated as average ± standard deviation. Variance and *t*-test were employed for analyzing statistically. When P value was < 0.05, it was regarded as statistical significance.

3. Results

3.1. Levels of HDAC4 and HDAC5 in MM patients and MM cell lines

We first examined the expression of HDAC4 and HDAC5 in bone marrow biopsies of newly diagnosed MM patients and healthy donors by real-time PCR and Western blot to investigate the association of class IIA HDAC with MM biology. Patient information is listed in Table 1. Thirty newly diagnosed MM patients were collected at the Hematopoietic Stem Cell Laboratory of the Affiliated Hospital of Guizhou Medical University from 2017 to 2019. We measured the expression of HDAC4 mRNA and protein in MM patients and normal donors. Real-time PCR and western blot showed that HDAC4 mRNA and protein expressions were significantly higher in MM patients than in healthy donors, and HDAC4 expressions were higher in the high-risk group than in the low-risk MM group at different ISS stages ($^{*}P < 0.05$,$^{**}P < 0.01$), suggesting tumor selectivity. Although mRNA expression levels of HDAC5 were higher than those of healthy donors, the difference in mRNA and protein expression of HDAC5 was not significant at various ISS stages (Fig. 1A, B). Furthermore, HDAC4 expression was higher in MM cells than HDAC5 (Fig. 1A). Subsequently, we examined the expression of HDAC4 protein in MM cell lines and CD138+ primary cells of MM patients. The HDAC4 protein was overexpressed in all tested MM cells and only slightly expressed in peripheral blood mononuclear cells from normal subjects (Fig. 1C). This suggests that HDAC4 is a prognostic indicator.

Table 1
Patient characteristics.

Patient	Age (years)	Sex	Serum β2M (mg/l)	Serum albumin (g/l)	ISS stage
1	61	Male	1.55	41.53	I
2	76	Male	2.18	37.60	I
3	58	Female	1.49	41.33	I
4	62	Male	3.17	40.10	I
5	45	Female	2.51	38.65	I
6	47	Female	2.04	36.11	I
7	51	Female	1.14	47.13	I
8	43	Male	3.01	36.92	I
9	60	Male	4.20	47.73	II
10	59	Female	4.06	48.20	II
11	72	Male	3.83	32.78	II
12	61	Male	5.01	40.20	II
13	62	Female	3.95	39.64	II
14	71	Male	5.38	40.24	II
15	57	Female	4.77	46.91	II
16	72	Male	5.42	40.63	II
17	55	Male	5.40	41.90	II
18	51	Female	3.79	32.82	II
19	60	Female	4.67	46.91	II
20	44	Male	6.68	26.40	III
21	66	Female	9.82	31.49	III
22	58	Female	11.67	20.70	III
23	58	Female	14.86	33.49	III
24	77	Female	6.97	39.64	III
25	62	Male	7.81	34.50	III
26	74	Male	10.05	32.19	III
27	46	Female	5.68	28.52	III
28	70	Male	7.83	34.06	III
29	68	Female	6.38	29.57	III
30	70	Male	10.65	35.31	III

All patients were newly diagnosed and had not received hematology-related treatment.

3.2. LMK-235 induced apoptosis of MM cells

To investigate the apoptotic and growth inhibitory properties of the specific HDAC4/5 inhibitor LMK-235 on MM cells, we first tested the survival of MM cell lines (RPMI8226, U266) after LMK-235 treatment by the CCK8 method. As shown in Fig. 2A, LMK-235 significantly inhibited the survival of the MM cell line (RPMI8226, U266) in a dose-dependent manner. We then compared cell viability of RPMI8226 cells treated with the same concentration of LMK-235 (3 μmol/L) for 24 and 48 h. We found that the survival rate of RPMI8226 cells after 48 h of LMK-235 treatment was higher than that after 24 h of treatment. U266 cells showed similar results.

We examined the apoptotic rate of two multiple myeloma cell lines, RPMI8226 and U266, after treatment with LMK-235, by annexin-V-PI staining to further validate the results above (Fig. 2B). We treated cells with various concentrations of LMK-235 (1, 2, 3, 4, 5 μmol/L) for 24 h. Both MM cell lines (RPMI8226, U266) were significantly more prone to apoptosis. When we treated with 5 μmol/L LMK-235 for 24 h, the

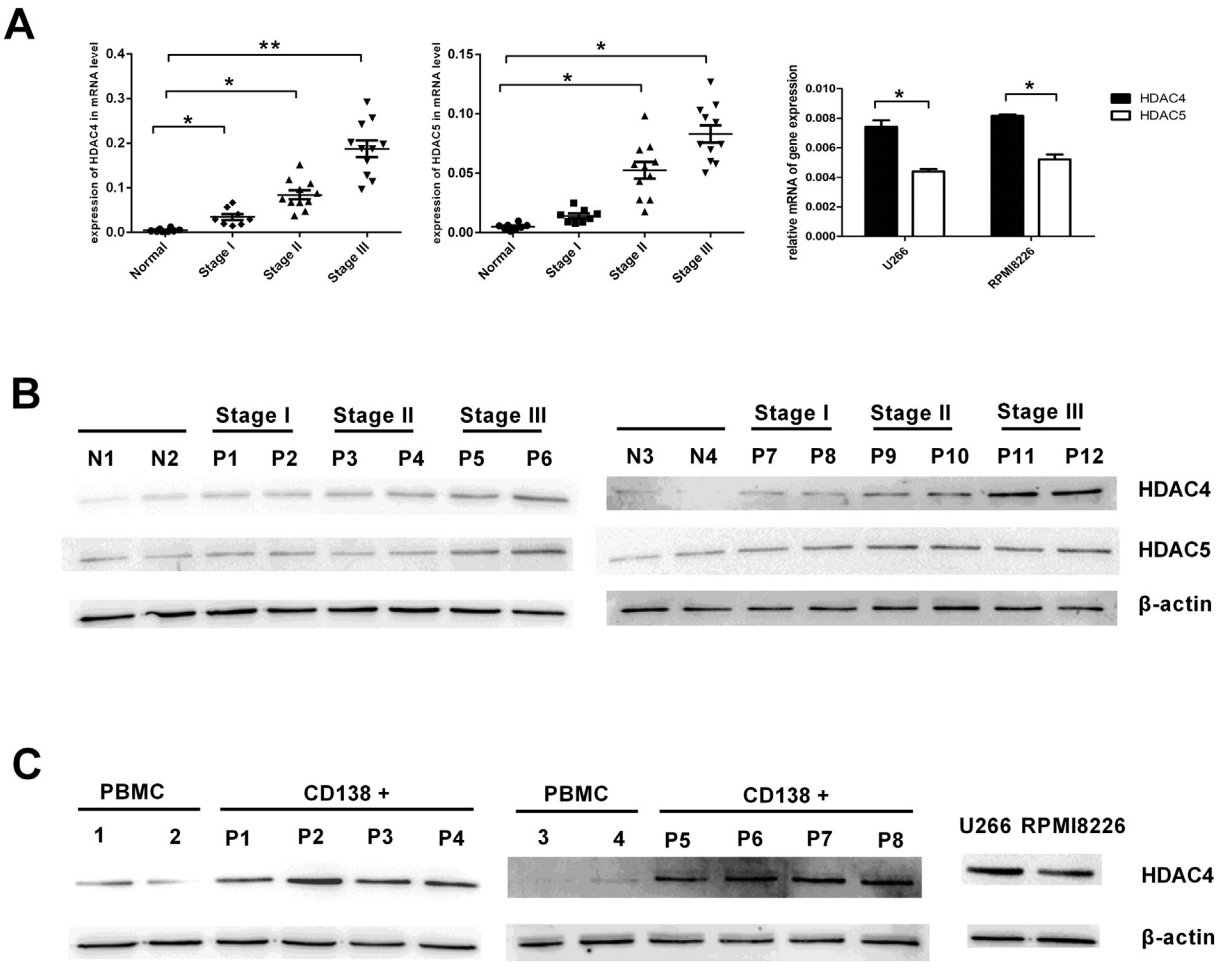


Fig. 1. Levels of HDAC4 and HDAC5 in MM patients and MM cell lines. (A) Detection of HDAC4 and HDAC5 mRNA expression levels in CD138 + cells from stage I to stage III MM patients and healthy donors by real-time PCR, and expression of HDAC4 and HDAC5 mRNA in RPMI8226 and U266 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (B) Detection of HDAC4 and HDAC5 protein expression in MM patients from stage I to stage III by Western blot. Four healthy donors were selected as negative controls. The characteristics of MM patients are shown in Table 1. (C) Cell lysates were extracted from MM cell lines and peripheral blood mononuclear cells (PBMC) were isolated from four normal donors and eight randomly selected MM patients. Protein levels of HDAC4 were analyzed by Western blot. β-Actin was used as a protein loading control. All experiments were repeated three times.

apoptotic rate of U266 was 62.29%, while the apoptosis rate of RPMI8226 cells significantly increased to 68.60%. Simultaneously, we measured the apoptosis rate of the two strains of MM cell line (RPMI8226, U266) treated with the same concentration of LMK-235 for 24 h and 48 h. The apoptosis rate of RPMI8226 cells after 48 h of LMK-235 treatment was significantly higher than the rate after 24 h of treatment, and difference in apoptosis of cells was most significant when the concentration of LMK-235 was 3 μmol/L. U266 cells showed similar results. This is consistent with prior cell viability. To further investigate whether LMK-235 promotes apoptosis in MM cells, we measured apoptosis rates in RPMI8226 and U266 cells treated with the same dose of LMK-235 (3 μmol/L) for 6, 12, 18, 24 and 48 h, respectively (Fig. 2C).We found that LMK-235 did not affect apoptosis in MM cells at 12 h and before; however, apoptosis began to change at 18 h after drug treatment, and the apoptosis rate began to increase significantly after 24 h. The maximum effect was achieved in 48 h. LMK-235 mediated apoptosis in DLBCL cells in a time-dependent manner. These results suggest that the specific HDAC inhibitor LMK-235 plays an important role in the growth inhibition of MM cells and can induce apoptosis of MM cells.

3.3. The down-regulation of HO-1 expression in MM cells by LMK-235 was closely related to HDAC4

Our team previously demonstrated that HO-1 was highly expressed in MM cells, and that HO-1 was associated with HDAC [27]. Therefore, we predicted that the MM cell effect induced by LMK-235 was related to expression of HO-1. To validate our hypothesis, we treated MM cells with various concentrations of LMK-235 (1, 2, 3, 4 and 5 μmol/L) for 24 h and analyzed the expression of related genes. The mRNA expression levels of HDAC4 and HO-1 in RPMI8226 cells decreased in a dose-dependent manner (Fig. 3A). Although LMK-235 blocked other HDACs at μM doses, including HDAC6 and HDAC1, our data show that LMK-235 at a concentration of 1–5 μmol/L in MM cells had no significant effect on HDAC6 and HDAC1 expression. (Fig. 3B). We also analyzed the expression of HO-1 and HDAC4 at the protein level in MM cells after 24 h of LMK-235 treatment by western blot. As expected, expression levels of HO-1 and HDAC4 were downregulated in RPMI8226 cells. The protein expression level of acetylated histone H3 increased as the concentration of LMK-235 increased (Fig. 3C). At the same time, the protein expression levels of HDAC6 and acetylated α-tubulin did not show significant differences (Fig. 3E). These results suggest that LMK-235 regulates the expression of HDAC4 and HO-1 mRNA and protein in MM cells.

We treated MM cells at various time points with 3 μmol/L LMK-235

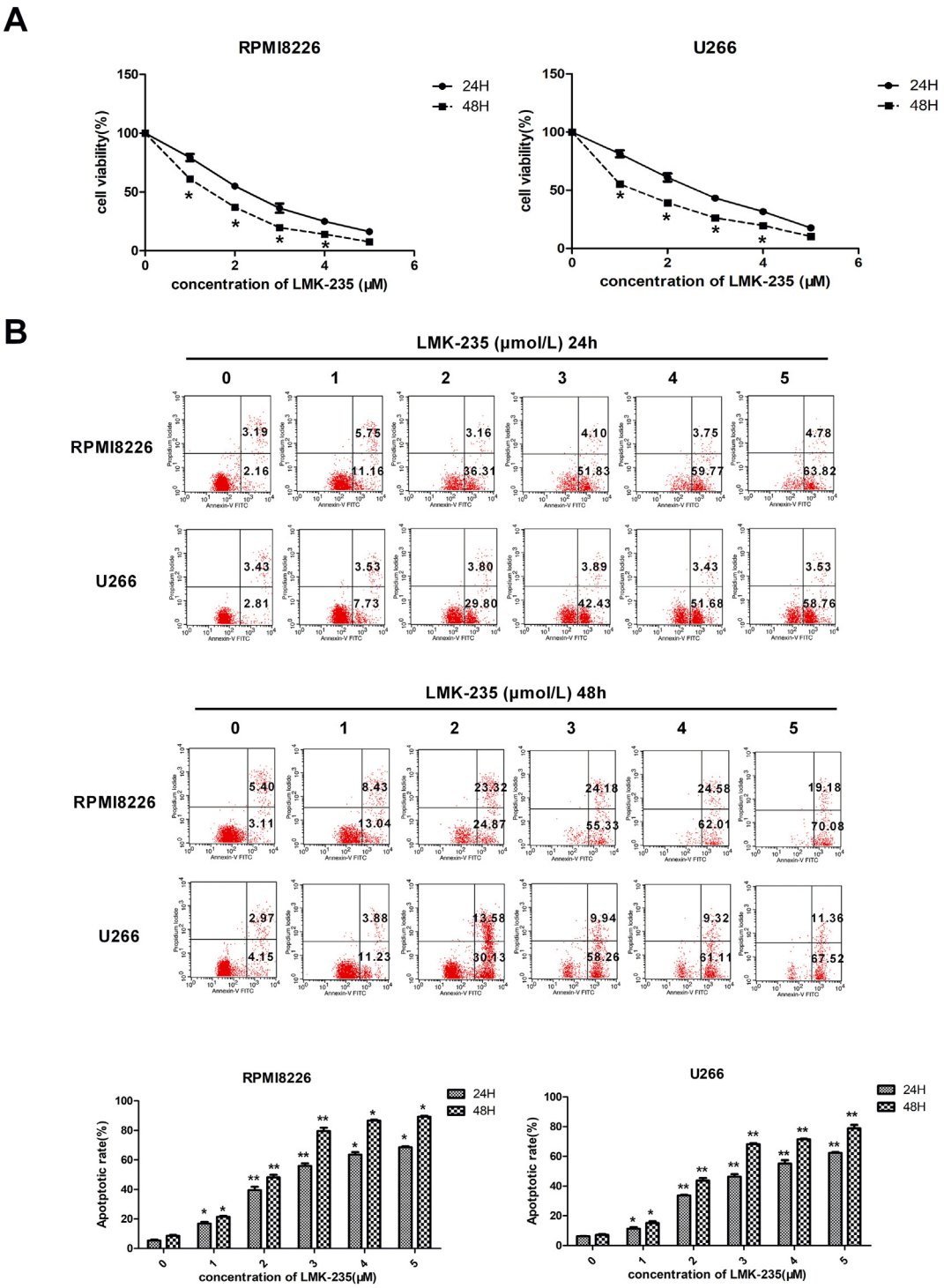


Fig. 2. LMK-235 induced apoptosis of MM cells. (A) RPMI8226 and U266 cell lines were cultured in the absence or presence of LMK-235 at various concentrations. After culture for 24 and 48 h, cell viability was measured by CCK8 assay. *$P < 0.05$, **$P < 0.01$ vs. control. (B) After treatment with LMK-235 for 24 h and 48 h, apoptosis was determined by flow cytometry. (C) The MM cell lines RPMI8226 and U266 cells were plated in triplicate and treated with 3 μmol/L LMK-235 for 6 h, 12 h, 18 h, 24 h and 48 h, respectively. Flow cytometry was used to determine apoptosis.*$P < 0.05$, **$P < 0.01$ vs. control group. All experiments were repeated three times.

in subsequent experiments. Protein level expression of HDAC4 and HO-1 was analyzed. We found that the expression of HDAC4 and HO-1 did not change at 6 and 12 h after treatment, and the expression was downregulated at 18 h of treatment. After treatment for 24 h, the expression levels of both proteins were downregulated. When treated for 48 h, expression levels of HDAC4 and HO-1 were significantly inhibited. A correlation between inhibition of expression of HDAC4 and HO-1 and apoptosis was demonstrated (Fig. 3D). To determine if LMK-235 alone had any effect on the cells, we treated MM cells with various concentrations of LMK-235 for 12 h (Fig. 3F), demonstrating that the tested concentrations of LMK-235 did not affect apoptosis. We also performed protein level analysis of HDAC4 and HO-1. We found that protein expression of HDAC4 and HO-1 did not change for 12 h when LMK-235 did not cause apoptosis. These data further demonstrate that HDAC4

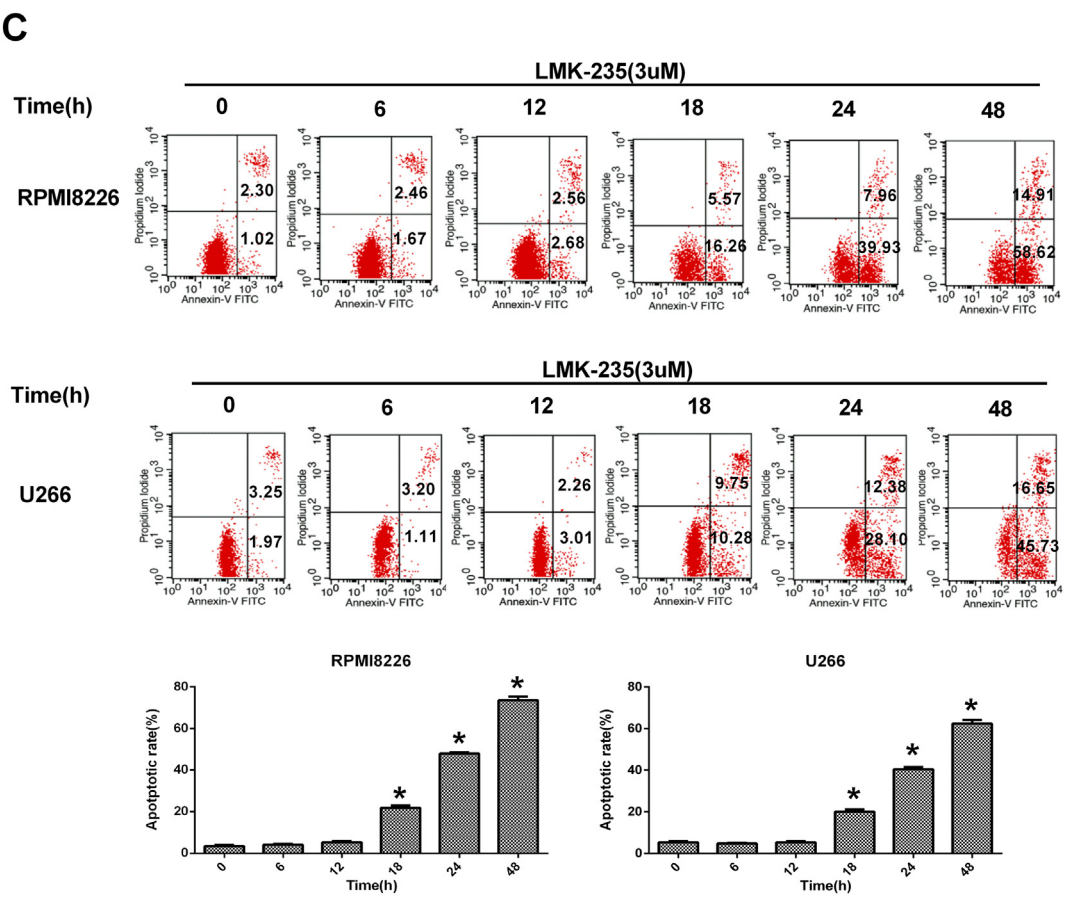


Fig. 2. (*continued*)

and HO-1 are involved in LMK-235-mediated MM cell apoptosis.

To explore the mechanism by which LMK-235 regulates HO-1, we next verified whether LMK-235 acts as an inhibitor of HDAC4/HDAC5 activity and regulates HO-1 expression by affecting HDAC4. We found that LMK-235 induced down-regulation of HDAC4 expression in RPMI8226 cells. First, we treated RPMI8226 cells with siRNA for HDAC4 (si-HDAC4), or used an empty siRNA (si-NC) as a negative control to exclude the side effects of transfection or the incorporation of RNA into cells. RPMI8226 cells were treated with si-HDAC4 and NC siRNA for 48 h, and real-time PCR verified successful transfection (Fig. 3G). We also examined mRNA expression levels of HO-1 after silencing the HDAC4 gene and found that expression of HO-1 was inhibited. Subsequently, we analyzed protein levels of HO-1 in MM cells RPMI8226 after transfection with si-HDAC4, and Western blot showed that HDAC4 silencing inhibited the expression of HO-1 protein (Fig. 3G). In addition, our data also suggested that HO-1 mRNA and protein expression levels were not significantly inhibited after silencing the HDAC6 gene in RPMI8226 cells (Fig. 3H). This suggests that down-regulation of HO-1 expression by LMK-235 in MM cells was mainly associated with HDAC4, but not with HDAC6.

3.4. Up-regulation of HO-1 expression inhibited apoptosis of MM cells with low expression of HDAC4

HO-1 is an anti-apoptotic molecule that may be a potential target for disease treatment. Our previous studies showed that the HDAC inhibitor LMK-235 regulated HO-1 expression and was associated with HDAC4. Therefore, we next investigated whether HO-1 was involved in LMK-235-mediated apoptosis of MM cells. We first stimulated the MM cell line with Hemin (5 μM) or ZnPP (0.1 μM) and analyzed expression of HO-1. As shown in Fig. 4A, real-time PCR showed that 5 μM Hemin up-regulated HO-1 expression, however, 0 .1μM ZnPP down-regulated HO-1 expression.

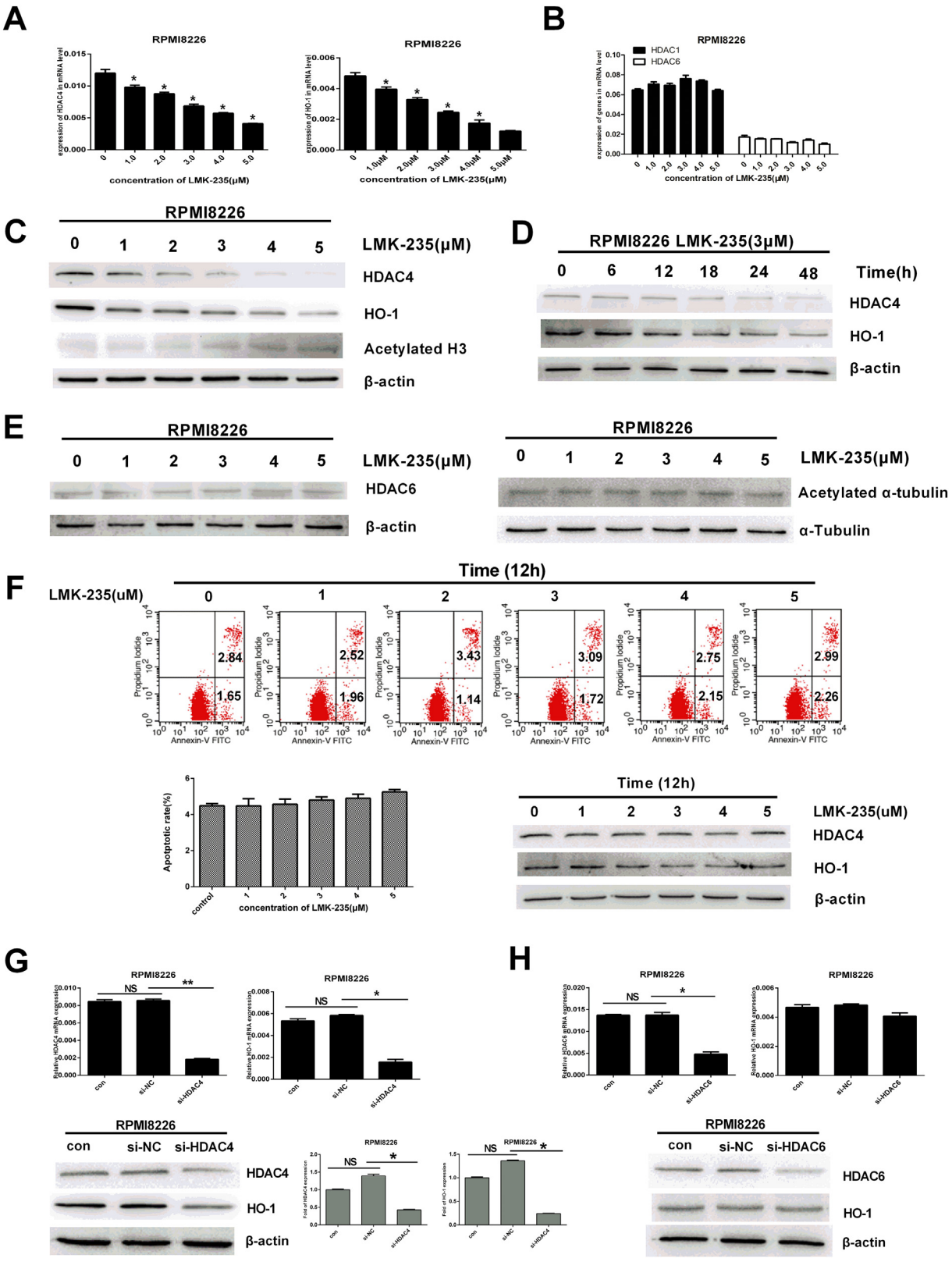
Silencing HDAC4 has been shown to inhibit HO-1 expression. In the next experiment, we used si-HDAC4 transfection combined with 5 μM Hemin to treat RPMI8226 cells. Western blot analysis (Fig. 4B) showed that down-regulation of HO-1 caused by silencing HDAC4 was weakened after the combined use of Hemin.

We then examined the change in apoptotic rate after treatment of MM cells with si-HDAC4 or Hemin (Fig. 4C). We found that the apoptosis rate of RPMI8226 and U266 cells increased significantly after transfection with si-HDAC4, and the apoptosis of RPMI8226 cells was slightly higher than that of U266 cells, reaching 40.67%. We found that these changes were observed after combined Hemin treatment. The apoptosis rate of RPMI8226 cells treated with si-HDAC4 and Hemin was 26.54%. Similarly, apoptosis of U266 cells was also significantly reduced. Therefore, we conclude that Hemin inhibits apoptosis in MM cells, and upregulation of HO-1 expression attenuates apoptosis in MM cells with low expression of HDAC4.

3.5. LMK-235 activated the JNK pathway and increased AP-1 activity

To elucidate how LMK-235 affects apoptosis through some pathway in MM, we incubated U266 and RPMI8226 cells with 1, 3 and 5 μm/l LMK-235 for 24 h and then tested protein expression in particular pathways by Western blot. We found that LMK-235 increased the protein level of phosphorylated JNK in a dose-dependent manner as the concentration increased, while there was no significant change in total JNK levels, suggesting that LMK-235 exposure activates the JNK pathway. At the same time, we detected a significant increase in p-c-Jun levels and increased AP-1 activity by Western blot analysis (Fig. 5A).

Since LMK-235 inhibited cell growth and caused apoptosis, we



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incubated RPMI8226 and U266 cells with 1, 3 and 5 μm/l LMK-235 for 24 h to observe changes in related apoptotic proteins (Fig. 5B). Expression of the anti-apoptotic protein Bcl-2 was significantly reduced by LMK-235 treatment in a dose-dependent manner. Simultaneously, the expression of the pro-apoptotic proteins Bax and Bad were increased.

For further verification, we treated MM cells at various time points with 3 μmol/L LMK-235. Protein level expression of p-JNK, p-c-Jun and BCL-2 family were analyzed (Fig. 5C). We found that the expression of p-JNK, p-c-Jun did not change at 6 and 12 h after treatment, and slightly up-regulated at 18 h of treatment. After 24 h of treatment,

Fig. 3. The down-regulation of HO-1 expression in MM cells by LMK-235 was closely related to HDAC4. (A) RPMI8226 cells were cultured in the absence or presence of LMK-235 at different concentrations for 24 h, HDAC4 and HO-1 mRNA expressions were analyzed by real-time PCR. *$P < 0.05$, **$P < 0.01$ vs. control. (B) RPMI8226 cells were cultured in the absence or presence of LMK-235 at different concentrations for 24 h, HDAC1 and HDAC6 mRNA expressions were analyzed by real-time PCR. (C) RPMI8226 cell lines were cultured for 24 h in the absence or presence of LMK-235 at different concentrations. Then the protein levels of HO-1, HDAC4 and AC-H3 were analyzed by Western blot. (D) The protein level expression of HDAC4 and HO-1 was analyzedat at different time points with 3 μmol/L LMK-235 by Western blot. β-Actin was used as loading control. (E) The expression levels of HDAC6 and acetylated α-tubulin in RPMI8226 cells were analyzed by Western blot. (F) After 12 h of treatment with LMK-235, protein level analysis was performed on HDAC4 and HO-1. (G) After RPMI8226 cells were transfected with control siRNA or HDAC4 siRNA for 48 h, HDAC4 and HO-1 mRNA expressions were examined by real-time PCR. *$P < 0.05$, **$P < 0.01$ vs. control. After treatment with si-HDAC4 or NC siRNA (si-NC) for 48 h, the expression levels of HDAC4 and HO-1 protein in RPMI8226 cells were analyzed by Western blot. *$P < 0.05$, **$P < 0.01$ vs. control. (H) The expression levels of HDAC6 and HO-1 in RPMI8226 cells were analyzed by real-time PCR and Western blot. All experiments were repeated three times.

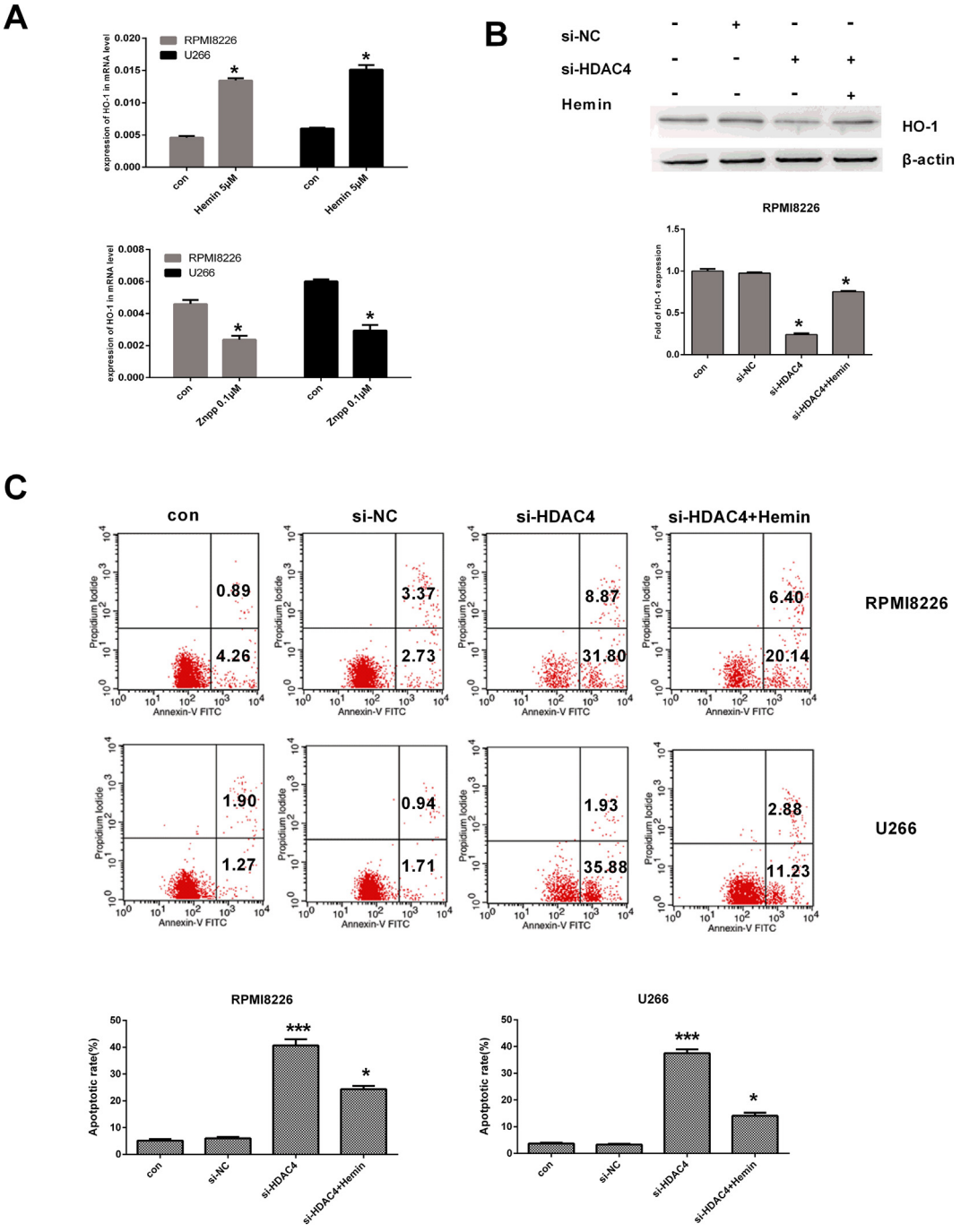

Fig. 4. Up-regulation of HO-1 expression inhibited apoptosis of MM cells with low expression of HDAC4. (A) HO-1 mRNA expressions in RPMI8226 and U266 cells were examined by real-time PCR after regulated by 5 μmol Hemin or 0 .1μmol ZnPP. *$P < 0.05$, **$P < 0.01$ vs. control. (B) Protein levels of HO-1 in RPMI8226 cells which were induced with si-NC or si-HDAC4 for 48 h or combined with 5 μM Hemin for 24 h. (C) Cell apoptosis rates were tested by flow cytometry. All experiments were performed three times. Data are represented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the control group.

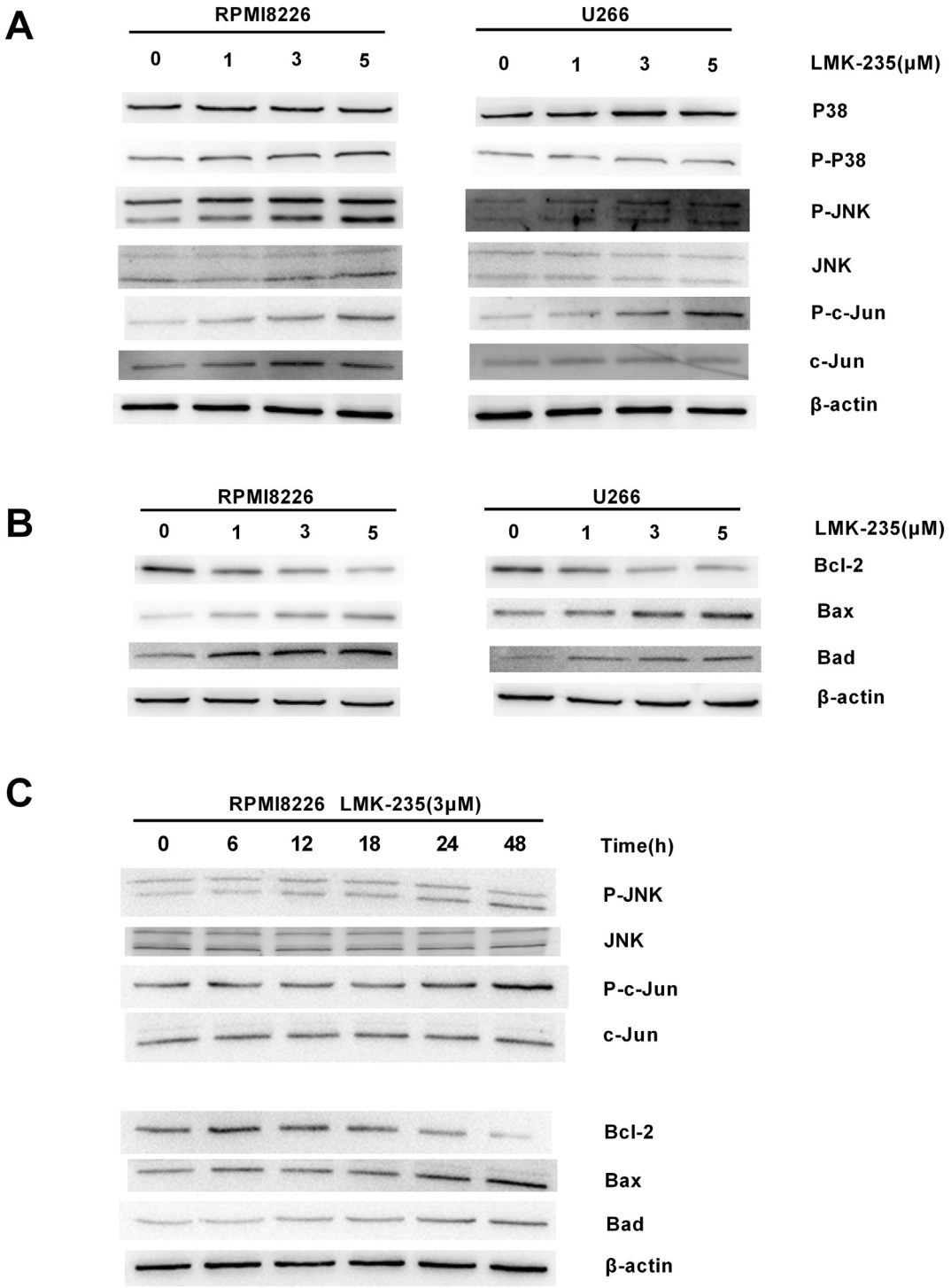

Fig. 5. LMK-235 activated the JNK pathway and increased AP-1 activity. (A) Western blot analysis showed that phosphorylation of JNK (p-JNK) was activated after LMK-235 treatment in MM cells, but total JNK was not affected. (B) Western blot tested the protein levels of Bcl-2, Bax and Bad in MM cells treated with different concentrations of LMK-235. (C)The expression of p-JNK, p-c-Jun and BCL-2 family proteins in MM cells treated with 3 μmol/L LMK-235 at different time was detected by Western blot. All experiments were repeated three times.

phosphorylation of JNK and c-Jun was significantly activated. When treated for 48 h, the expression levels of p-JNK and p-c-Jun were further increased. At the same time, the protein level of the BCL-2 family also showed a meaningful trend. It was confirmed that LMK-235 regulates apoptosis of MM cells by activating the JNK/AP-1 pathway and regulating the expression of apoptosis-related proteins.

3.6. LMK-235 mediated HO-1 expression and induced MM cell apoptosis by activating the JNK/AP-1 pathway

Previous studies confirmed that LMK-235 activates the JNK/AP1 pathway. Therefore, in order to verify the role of HO-1 in this pathway, we first incubated RPMI8226 cells with 3 μm/l LMK-235 for 24 h (Fig. 6A). Consistent with previous results, MM cells began to undergo apoptosis. Subsequently, we combined LMK-235 with 5 μm/l Hemin to

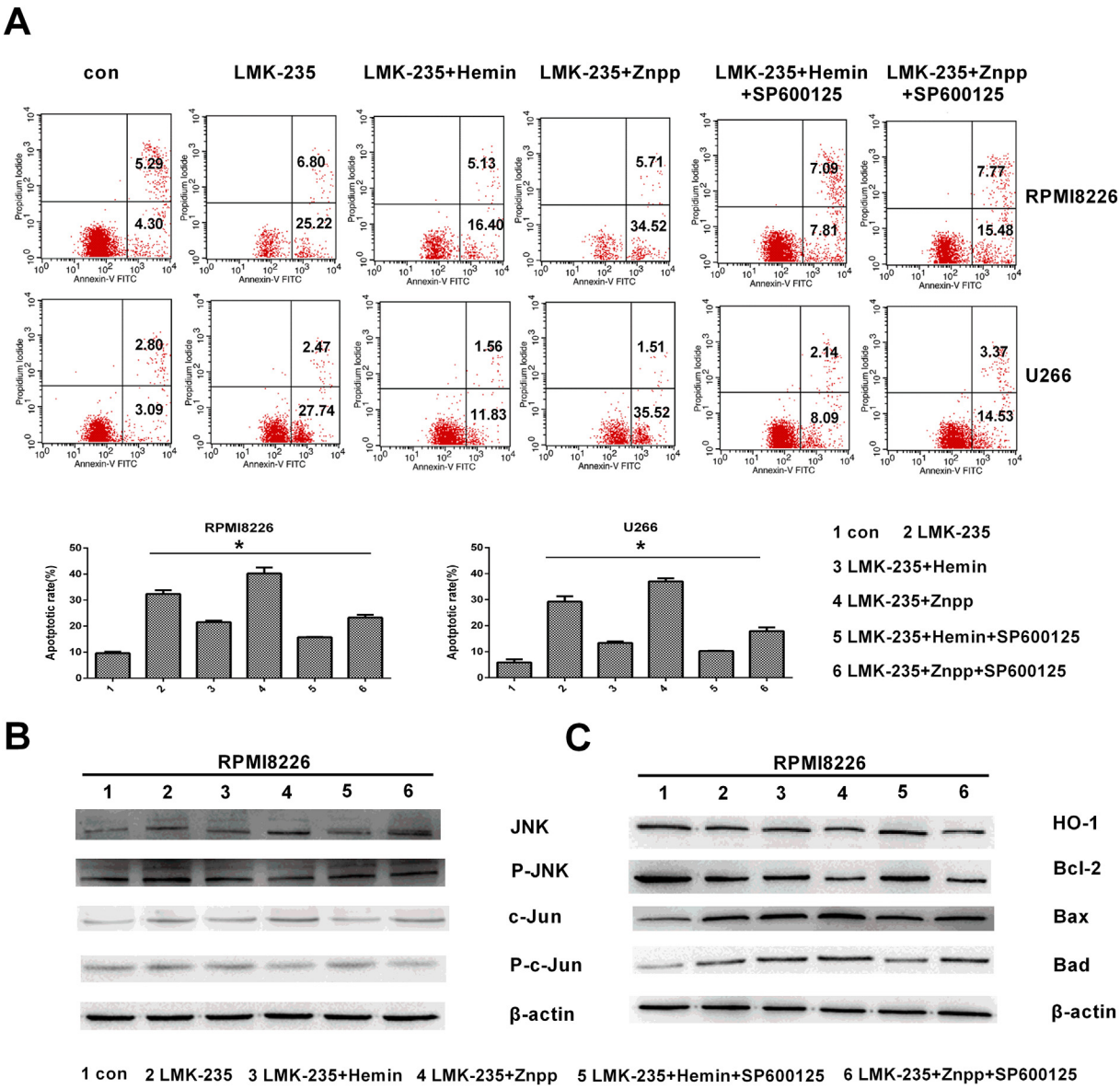

Fig. 6. LMK-235 mediated HO-1 expression and induced MM cell apoptosis by activating the JNK/AP-1 pathway.(A) Cells were incubated with 3 μM LMK-235 for 24 h after treatment with or without 5 μM Hemin and 0 .1μM ZnPP for 24 h, respectively, in different cells after treated with 10 μM SP600125 or not. Apoptosis rates of MM cells were tested by flow cytometry. $*P < 0.05$, $**P < 0.01$, vs. control. (B) The protein levels of JNK, P-JNK, c-Jun and P-c-Jun were then evaluated by Western blot. (C) The protein levels of HO-1, Bcl-2, Bax, Bad were then evaluated by Western blot. All experiments were repeated three times.

up-regulate HO-1 or 0.1 μm/l ZnPP to down-regulate HO-1, and then observed changes in apoptotic rates of the two MM cell lines. We found that the apoptosis rate of RPMI8226 cells decreased from 32.02% to 21.53% after Hemin combined treatment. Upregulation of HO-1 inhibited MM cell apoptosis induced by LMK-235. However, after ZnPP, the apoptotic rate increased to 40.23%, and the down-regulation of HO-1 promoted MM cell apoptosis induced by LMK-235. On this basis, we added the JNK pathway pharmacological inhibitor SP600125 to inhibit the JNK pathway after HO-1 regulation in MM cells. At this time, the apoptosis rate of RPMI8226 cells was further suppressed in the above results. U266 cells showed similar results.

We performed a more in-depth validation of RPMI8226 cells by Western blot (Fig. 6B). After treating the cells in the manner described above, we measured phosphorylation levels of JNK and c-Jun and the total levels of JNK and c-Jun. We found that LMK-235 activated the phosphorylation of JNK and c-Jun. LMK-235 combined with Hemin enhanced p-JNK and p-c-Jun to increase AP-1 activity, however, Binding of LMK-235 to ZnPP inhibited phosphorylation of JNK. After the addition of SP600125, the JNK pathway was further downregulated in both cases. At the same time, phosphorylation of c-Jun was inhibited using SP600125. We conclude from these results that LMK-235 regulates the JNK/AP1 pathway through HO-1 and induces apoptosis of MM cells by activating this pathway.

We also examined the expression of HO-1 and apoptosis-related proteins in MM cells as described above (Fig. 6C). LMK-235 combined with Hemin or ZnPP regulated HO-1 expression, however, SP600125 did not affect HO-1 expression, suggesting that HO-1 is a factor located upstream of the JNK pathway. The JNK pathway is regulated. Western blot also showed that down-regulation of HO-1 reduced the expression of anti-apoptotic Bcl-2 after LMK-235 combined with Hemin or ZnPP. up-regulation of HO-1increased expression of Bcl-2, and SP600125 further increased Bcl-2 expression. By contrast, after LMK-235 combined with Hemin or ZnPP treatment, down-regulation of HO-1 enhanced the expression of Bax and Bad, up-regulation of HO-1 attenuated the expression of Bax and Bad, and SP600125 further attenuated Bax and Bad expression. This is consistent with previous apoptotic

results. Finally, we conclude that LMK-235 activates the JNK/AP1 pathway by regulating HO-1, further affecting the expression of apoptosis-related proteins, and induces apoptosis in MM cells.

4. Discussion

Multiple myeloma (MM) remains an incurable malignant tumor. It is a genetically-heterogeneous disease characterized by conversion of B cells into malignant cells [28]. Therefore, the identification of novel therapeutic approaches and potential therapeutic targets is critical [29]. The acetylation of the ε-amino group of lysine residues in histones induces changes in chromatin structure that are primarily involved in the activation of DNA transcription and replication; this is one of the most significant epigenetic mechanisms affecting human cancer development [30]. In recent years, histone deacetylase inhibitors have shown effective anticancer activity in preclinical and clinical studies, and have become new drugs for the treatment of hematological malignancies [9,10,31]. These agents have been studied extensively for to their ability to reverse neoplastic phenotypes and to exert tumoricidal activities.

The function of class IIA HDACs in MM is essentially unknown. In our study, we explored the mechanism of action of the novel HDAC4/5 specific inhibitor LMK-235 on MM apoptosis and confirmed this effect. The regulation of HO-1 is related. First, we found that LMK-235 affected the growth activity of MM cell lines and induced MM cell apoptosis in a dose- and time-dependent manner within a specific range. We also found that LMK-235 downregulates HO-1 expression in this dose range and is associated with inhibition of HDAC4. Studies have shown that increasing the level of acetylated α-tubulin by HDAC6 deletion can be a potential target for therapeutic strategies in a variety of neurodegenerative diseases [32]. Although LMK-235 blocked other HDACs at μM doses, including HDAC6 and HDAC1, our data show that LMK-235 had no significant effect on HDAC6 and HDAC1 expression in MM cells. By α-tubulin and histone acetylation assays, we observed that LMK-235 did not increase the levels of acetylated α-tubulin in MM cells. It was further confirmed that the main factor for LMK-235 to regulate HO-1 is HDAC4. Expression levels of HO-1 were related to proliferation, drug resistance, and apoptosis of various cell types, including MM [33,34]. Therefore, we hypothesized that HO-1 played an important role in LMK-235-mediated apoptosis of MM cells. To confirm this, we next examined changes in several HO-1-related pathways in MM cells treated with LMK-235. We found that LMK-235 down-regulated the expression of phosphorylated JNK and c-Jun and activated the JNK/AP-1 signaling pathway. Next, we determined whether regulation of HO-1 affected the cellular effects of LMK-235 on MM. We combined LMK-235 with hemin to up-regulate HO-1 and found that MM cell apoptosis induced by LMK-235 was inhibited, and that the phosphorylated JNK levels were attenuated. However, combination with ZnPP down-regulated HO-1, and the apoptotic rate increased, promoting apoptosis of MM cells induced by LMK-235, and the expression of P-JNK and P-c-Jun was further promoted. There were no significant changes in the overall levels of JNK and c-Jun. Therefore, we added the JNK inhibitor SP600125. This time, LMK-235 induced MM cell apoptosis in the above case was further reversed, and activation of AP-1 by LMK-235 was also inhibited. SP600125 did not affect the expression of HO-1. We also found that the expression of apoptosis-related proteins changed correspondingly after the above treatment. Thus, we conclude that LMK-235 induces HO-1 expression to modulate apoptosis-related proteins and promote apoptosis in MM cells via the JNK/AP-1 pathway.

Although some HDACs have been established as effective targets for anti-tumor drugs, the role of class IIA HDACs in the development of cancer and their potential as drug targets remains unclear. In this paper, we demonstrated that LMK-235, a selective inhibitor of HDAC4/5, mediated low expression of HO-1 affected MM cell apoptosis via the JNK/AP-1 pathway. This suggested that LMK-235 has an effect on the proliferation and apoptosis of MM. We studied the apoptosis effect of LMK-235 on MM and its mechanism only in a certain concentration range, and confirmed its anti-tumor activity against MM cells; however, the drug concentration of MM cells is larger or smaller, its impact requires further research. In conclusion, we demonstrate that LMK-235 promotes significant apoptosis in MM cells and explores its underlying mechanisms. This provides a new foundation for future clinical research and treatment concepts for MM.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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