Triptolide induces cell-cycle arrest and apoptosis of human multiple myeloma cells in vitro via altering expression of histone demethylase LSD1 and JMJD2B

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Aim: To elucidate the relationship between triptolide-induced changes in histone methylation and its antitumor effect on human multiple myeloma (MM) cells in vitro.

Methods: Human multiple myeloma cell line RPMI8226 was used. Apoptosis was evaluated using Annexin-V-FITC/PI-labeled flow cytometry, Hoechst 33258 staining, and transmission electron microscopy. Flow cytometry was used to detect the cell cycle distribution of the apoptotic cells. The presence of the LSD1, JMJD2B, H3K4me2, H3K9me2, and H3K36me2 proteins was verified by Western blot analysis. Semi-quantitative real-time PCR was performed to examine the expression of LSD1 and JMJD2B.

Results: Triptolide (10–160 nmol/L) suppressed the proliferation of MM cells in a dose- and time-dependent manner with an IC50 value of 99.2±9.0 nmol/L at 24 h. Triptolide (50 nmol/L) induced G0/G1 cell cycle arrest in MM cells. The agent (50–150 nmol/L) induced apoptosis of MM cells in a dose-dependent manner. The same concentrations of triptolide suppressed the expression of dimethylated H3K4, dimethylated H3K9 and dimethylated H3K36 by altering the expression of histone demethylase LSD1 and JMJD2B without affecting the expression of histone demethylase LSD1.

Conclusion: Triptolide potently inhibits the growth of MM cells via regulating the expression of histone demethylase LSD1 and JMJD2B, which lead to abnormal histone methylation.

Keywords: triptolide; multiple myeloma; apoptosis; cell cycle arrest; LSD1; JMJD2B
expressed in triptolide-treated MM cells, which was the first evidence that triptolide induced epigenetic changes by regulating histone lysine methylation[^15].

LSD1 was the first histone lysine demethylase to be discovered and is highly specific for di- and mono-methylated H3K4 and H3K9; LSD1 has been identified as a component of transcriptional repressor complexes comprising transcriptional corepressor protein (CoREST) and HDAC1/2. ChIP-DAL analyses revealed that LSD1 targets the promoters of an array of genes that are involved in several important cellular processes, including cellular growth, proliferation, apoptosis and fate specification. JMJ2B, a member of the JMJ2 family, which mainly consists of oncogenes, demethylates di- and trimethylated H3K9 and H3K36[^16]. Compared with studies examining DNA methylation, studies on histone methylation, histone demethylases and their mechanisms of action in MM are scarce.

In this study, we aimed to investigate changes in the expression of the histone demethylases LSD1 and JMJ2B induced by triptolide in the MM cell line RPMI8226. We analyzed H3K4 dimethylation, H3K9 and H3K36 dimethylation states and examined the connections between apoptosis, histone methylation changes and demethylase regulation by triptolide to determine whether triptolide exerts its anti-myeloma effects through the regulation of histone demethylases in MM cells.

### Materials and methods

#### Reagents

Triptolide (empirical formula: C_{20}H_{24}O_{6}, molecular weight: 360.40, purity: >98%; Figure 1) was purchased from Sigma-Aldrich (St Louis, MO, USA). Triptolide was dissolved in dimethylsulfoxide (DMSO), stored at -20 °C, and thawed before use. RPMI-1640 medium was purchased from Gibco (Gaithersburg, MD, USA). Propidium iodide (PI), DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). TRIzol for RNA extraction was purchased from Invitrogen (Carlsbad, CA, USA), and the cDNA synthesis kit was obtained from Toyobo Biologics. SYBR Green PCR master mix was purchased from Applied Biosystems (Foster City, CA, USA).

#### Cell lines and culture

The human MM cell line RPMI8226 was purchased from Biomart Co Ltd (Shanghai, China); peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors (after informed consent had been obtained) by Ficoll-Hypaque density gradient centrifugation. The two kinds of cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 IU/mL, 100 µg/mL, respectively), 2 mmol/L glutamine, and 10 mmol/L HEPES buffer at 37°C in a humidified 5% CO₂ atmosphere. During the expansion phase, RPMI8226 cells were maintained at a concentration of 0.5–1×10⁶ cells/mL in culture medium. The appropriate number of cells for use in the experiments was determined. RPMI8226 cells were treated with 50, 100, or 150 nmol/L triptolide for 48 h, except where otherwise specified.

#### Cell proliferation assay

The anti-proliferative effect of triptolide in RPMI8226 cells was determined by MTT assay. RPMI8226 cells (5×10⁴ per well) were exposed to various concentrations of triptolide (0–160 nmol/L) for 24, 48, and 72 h in 96-well plates. The experiments were performed in triplicate. After incubation with triptolide, 20 µL MTT solution (5 mg/mL in PBS) was added to each well, the plates were incubated for 4–6 h, the supernatants were discarded and 150 µL of DMSO was added. Once the blue crystals were dissolved, the optical density (OD) was measured at 570 nm using a 96-well multisensor autoreader (Biotec Instruments, NY, USA). The inhibition of cell proliferation was determined using the following formula: 

\[
\text{inhibition of cell proliferation (\%)} = \left(1 - \frac{\text{OD of the experimental samples}}{\text{OD of the control}}\right) \times 100\% \quad (n=3, \text{mean±SD})
\]

The tripotlide concentration that caused 50% inhibition of cell proliferation was defined as half of the maximal inhibitory concentration (IC₅₀).

#### Cell cycle distribution analysis

RPMI8226 cells (1×10⁶ cells/well) were treated with different concentrations of triptolide (0 or 50 nmol/L) for 48 h and were harvested. After being washed, the cells were fixed with 75% ice-cold ethanol and were maintained overnight at 4 °C. The cells were collected and resuspended in PBS containing 40 µg/mL PI, 0.1 mg/mL RNase, and 5% Triton X-100 and were incubated at 37 °C for 30 min. The cells were evaluated by flow cytometry (BD, San Diego, CA, USA). Data was collected from at least 10000 cells for each sample. The distribution of cells across the cell-cycle phases was analyzed using CELLQuest.

#### Annexin-V/PI staining assay

An immunofluorescent flow cytometry assay was used to evaluate the ability of triptolide to induce apoptosis in RPMI8226 cells. The cells were seeded in 6-well plates (1×10⁶ cells/well) and treated with varying concentrations of triptolide. PBMCs from 12 healthy donors were treated with 150 nmol/L triptolide for 48 h. The cells were harvested, washed with cold PBS, and were resuspended in 100 µL binding buffer. Subsequently, phosphatidyl serine on the surface of apoptotic cells

![Image](Chemical structure of triptolide.)
was detected using Annexin V/FITC and a PI apoptosis detection kit according to the manufacturer’s instructions (Bender Med Systems Inc, Burlingame, CA, USA). The number of apoptotic cells was evaluated by flow cytometry (BD, San Diego, CA, USA). At least 10000 cells were analyzed.

**Hoechst 33258 staining assay**

Nuclear fragmentation was visualized by staining apoptotic nuclei with Hoechst 33258. Cells treated with varying concentrations of triptolide were collected and washed and were fixed with a mixture of acetic acid:ethanol (1:3) for 10 min before deposition onto polylysine-coated cover slips. The samples were subsequently permeabilized with 0.25% Triton X-100 for 5 min and stained with 1 µg/mL Hoechst 33258 for 30 min at 37°C. The slides were mounted with glycerol-PBS, and the images were visualized and captured using a FV500 confocal microscope (Olympus, Tokyo, Japan).

**Morphological observation by transmission electron microscopy**

The ultrastructural appearance of the apoptotic cells was examined by transmission electron microscopy. After incubation with 150 nmol/L triptolide for 48 h, the cells were harvested and pre-fixed with 2% glutaraldehyde, post-fixed with 1% osmic acid, dehydrated in graded ethanol, embedded in resin, and cut into sections on an ultramicrotome. The ultra-thin sections were doubly stained with uranyl acetate and lead citrate and were examined by transmission electron microscopy using a Hitachi H-7500 instrument (Hitachi, Tokyo, Japan).

**Western blot analysis**

Western blot analysis was used to evaluate the levels of LSD1, JMJD2B, and histones (dimethylated H3K4, dimethylated H3K9, and dimethylated H3K36) present in RPMI8226 cells exposed to triptolide.

Cells were treated with varying concentrations of triptolide and collected. The sample lysates (25 µg of protein per lane) were prepared, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gels. The proteins were electro-transferred onto polyvinylidene difluoride membranes. The membranes were subsequently incubated with diluted primary antibodies (1:500–1:1000, Table 1) for 2 h. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, USA) for 1 h at 25°C. The immune complexes were visualized using the enhanced chemiluminescence reagent Super Signal Substrate (Pierce Biotechnology, Rockford, IL, USA). The band intensity was measured and quantified with a chemiluminescence detection system (Chemidoc XRS, Bio-Rad, USA). The protein levels were normalized relative to β-actin, and each group was subsequently normalized to the control (100%).

**Real-time PCR analysis**

Total RNA was isolated from each group using TRIzol according to the manufacturer’s instructions. For each sample, 3 µg total RNA was reverse transcribed into cDNA. Real-time quantitative PCR was performed with SYBR Green PCR master mix in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

All mRNA expression levels are expressed as a ratio relative to the control in each experiment, and β-actin was used as internal reference. The following primers for human genes were used: LSD1 forward, 5’-GTGCTCTGGTTGCGGTGCT-3’, reverse, 5’-CCCCGAAGAAGAGTCGTG-3’; JMJD2B forward, 5’-GGAACGGATGAGAAGGTG-3’, reverse, 5’-GGTTAGTGACCCGCGTGCGG-3’; β-actin forward, 5’-GCCGACTTCTCTTCCAAATGTC-3’, reverse, 5’-GCAGGCAGTGAGATCATATT-3’.

**Immunofluorescence staining**

An immunofluorescence staining assay was performed to evaluate the effects of triptolide on LSD1 expression in MM cells. The cells were fixed with 4% paraformaldehyde for 10 min and were permeabilized with 0.25% Triton X-100 for 5 min. The cells were subsequently washed twice with PBS and blocked in 3% bovine serum albumin. The rabbit monoclonal anti-LSD1 antibody was diluted 1:100 and incubated with 1×106 cells in a total volume of 100 µL for 1 h; non-immunoreactive IgG was used as a negative control. After being washed, the cells were incubated for 1 h with a TRITC-labeled goat anti-rabbit secondary antibody (Pierce, Rockford, USA), diluted in PBS and were stained with Hoechst 33258 (10 µg/mL) for 10 min. Images were visualized and captured using a FV500 confocal microscope (Olympus, Tokyo, Japan). The LSD1 levels were estimated as the mean fluorescence intensity after subtracting the fluorescence of the negative control cells.

**Table 1. Information about the primary antibodies used for Western blot and immunofluorescence staining.**

| Antibody       | Company        | Catalog | Source                      | Molecular weight |
|----------------|----------------|---------|-----------------------------|------------------|
| Anti-H3K4me2   | Upstate        | #07-436 | Rabbit polyclonal antibody  | 17 kDa            |
| Anti-H3K9me2   | Upstate        | #07-212 | Rabbit polyclonal antibody  | 17 kDa            |
| Anti-H3K36me2  | Cell Signaling | #2901S  | Rabbit polyclonal antibody  | 17 kDa            |
| Anti-JMJD2B    | Cell Signaling | #2139   | Rabbit monoclonal antibody  | 150 kDa           |
| Anti-β-actin   | Santa Cruz     | sc-47778| Mouse monoclonal antibody   | 43 kDa            |
anti-JMJD2B antibody cannot be used for immunofluorescence staining.

**Statistical analysis**

The data are expressed as the mean±SD of three independent experiments. Also, *t*-tests were used to evaluate the differences between treated samples and controls. A *P* value <0.05 was considered to be significant.

**Results**

**Effects of triptolide on the proliferation of RPMI8226 cells**

The inhibitory effect of triptolide on MM cell proliferation was examined in RPMI8226 cells. Figure 2 shows that triptolide had a dose- and time-dependent anti-proliferative effect on RPMI8226 cells at 0–160 nmol/L after exposure for 24, 48, and 72 h. The number of viable cells decreased as the concentration of triptolide increased. The IC_{50} value at 24 h was 99.2±9.0 nmol/L. As the exposure time increased, the IC_{50} values gradually decreased. The IC_{50} values after 48 and 72 h of treatment were 61.6±5.8 nmol/L and 23.3±2.6 nmol/L, respectively.

**Triptolide induced RPMI8226 cell-cycle arrest**

To investigate whether triptolide was able to inhibit DNA synthesis, the effect of triptolide on cell-cycle progression in RPMI8226 cells was measured by flow cytometry. After incubation with triptolide at 50 nmol/L for 48 h, RPMI8226 cells were analyzed for alterations in their cell-cycle distribution (Figure 3); the proportion of G_{0}/G_{1} phase cells significantly increased following the treatment with triptolide, with the G_{2} phase cells also increasing slightly. Meanwhile, the fraction of cells in S phase decreased accordingly. This result indicates that triptolide-induced apoptosis is cell cycle-dependent (Table 2).

**Triptolide induced apoptosis in RPMI8226 cells and PBMCs**

Apoptosis induction by triptolide was observed in RPMI8226 cells using various methods. In Figure 4, the degree of apoptosis, as measured by flow cytometry, is quantitatively expressed as the percentage of cells that were Annexin V-positive in the presence of triptolide. For the triptolide concentrations in the range 50–150 nmol/L, the total percentage of apoptotic cells, designated as Annexin V-positive and PI-negative cells, gradually increased from 26.0%±0.6% to 40.3%±0.9%, which was statistically different from the control (2.9%±0.3%).

In addition, we observed the effects of triptolide on apoptosis in RPMI8226 cells by visualizing the Hoechst 33258 staining of live cell nuclei using laser scanning confocal microscopy. Nuclei in the control cells were regular in shape. However, the cells became shrunken and dark after treatment with 50 nmol/L triptolide, and the nuclei showed chromatin condensation and marginalization or nuclear beading. The number of apoptotic cells gradually increased as the triptolide concentration increased, and at high concentrations (100 or 150 nmol/L),
the cells showed morphological features typical of apoptotic cells (Figure 5).

To confirm further the induction of apoptosis by triptolide and to visualize better the ultrastructural changes occurring in MM apoptotic cells, we also used electron microscopy. Apoptotic bodies were observed in triptolide-treated cells; these bodies are spherical protuberances containing fragmented and segregated chromatin clumps separating from the cell surface (Figure 6). Conversely, the untreated RPMI8226 cells had intact plasma membranes and ordered chromatin folding (Figure 6).

The experiments to detect apoptosis by flow cytometry were also performed on PBMCs isolated from 12 healthy donors after the cells had been treated with 150 nmol/L triptolide for 48 h. The amount of apoptosis induced by triptolide increased in 8 of the 12 donors (75%), but only 2 donors (17%) showed an increase of more than 30%, and 5 donors (41.7%) showed an increase of more than 20% (Table 3).

Triptolide decreased dimethylated H3K4 expression

H3K4 methylation is generally associated with active transcription\(^{16}\). RPMI8226 cells were treated with triptolide concentrations of 50, 100, or 150 nmol/L for 48 h. The levels of dimethylated H3K4 were measured by Western blot analysis. As shown in Figure 7B, the expression of dimethylated H3K4 decreased in a dose-dependent manner, and the rates of inhibition by 50, 100, and 150 nmol/L of triptolide were 17.5%, 53.6%, and 70.3%, respectively (\(P<0.05\) vs untreated control). We also found that the global trimethylated H3K4 level decreased in a dose-dependent manner after treatment with...
increasing triptolide concentrations in another MM cell line, U266[17].

Table 3. Apoptosis of PBMC from 12 healthy persons treated with triptolide (150 nmol/L, 48 h).

| Gender | Age (year) | Control (%) | T-treated (%) |
|--------|------------|-------------|---------------|
|        | Early apoptosis | Late apoptosis | Early apoptosis | Late apoptosis |
| 1 M    | 26         | 3.9         | 3.2           | 3.5          | 3.6    |
| 2 M    | 53         | 7.1         | 1.3           | 38.3         | 10.1   |
| 3 F    | 48         | 5.1         | 2.9           | 30.8         | 15.6   |
| 4 M    | 40         | 22.3        | 3.5           | 45.8         | 6.5    |
| 5 M    | 56         | 53.3        | 3.1           | 54.7         | 3.6    |
| 6 M    | 55         | 30.9        | 5.9           | 36.6         | 10.2   |
| 7 M    | 58         | 3.6         | 12.2          | 14.4         | 26.4   |
| 8 F    | 61         | 8.9         | 16.2          | 19.3         | 33.5   |
| 9 F    | 24         | 10.9        | 4.5           | 19.1         | 3.6    |
| 10 M   | 31         | 21.8        | 3.9           | 36.1         | 2.9    |
| 11 M   | 26         | 8.9         | 2.3           | 7.8          | 3.5    |
| 12 M   | 27         | 7.9         | 3.9           | 3.9          | 3.5    |

**Triptolide decreased dimethylated H3K9 expression**

To date, H3K9 methylation has been examined primarily at repressed promoter regions and is thought to act as a docking site for the chromodomain of HP1. H3K9 methylation and HP1 were also found to be associated with the transcribed regions of all active genes examined[13]. RPMI8226 cells were treated with triptolide concentrations of 50, 100, or 150 nmol/L for 48 h. After triptolide treatment, the levels of dimethylated H3K9 were measured by Western blot analysis. The global levels of dimethylated H3K9 significantly decreased after exposure to triptolide. The rates of inhibition by 50, 100, and 150 nmol/L triptolide were 34.7%, 29.4%, and 39.6%, respectively; (P<0.05 vs untreated control) (Figure 7B). We also found that the expression of trimethylated H3K9 decreased in RPMI8226 cells, and monomethylated H3K9 was elevated in U266 cells in a dose-dependent manner following treatment with increasing concentrations of triptolide[15, 18].

**Triptolide decreased dimethylated H3K36 expression**

H3K36 methylation has primarily been characterized as an active mark that facilitates transcriptional elongation[19]. RPMI8226 cells were treated with triptolide concentrations of...
50, 100, or 150 nmol/L for 48 h. Western blot analysis with an antibody against H3K36me2 was used to measured histone H3K36 dimethylation. Triptolide treatment for 48 h led to a significant reduction in the level of H3K36me2 in a dose-dependent manner. The rates of inhibition by 50, 100, and 150 nmol/L triptolide for H3K36me2 were 13.9%, 25.2%, and 35.8%, respectively (Figure 7B). In addition, H3K36 trimethylation was also found to be reduced by triptolide in U266 cells in a dose-dependent manner[17].

**Triptolide altered the expression of the histone demethylase LSD1 in RPMI8226 cells**

LSD1 is a flavin-dependent monoamine oxidase that demethylates both di- and mono-methylated H3K4 and H3K9, which is a post-translational modification that is associated with gene activation[20–22]. To investigate the potential mechanisms by which triptolide downregulates dimethylated H3K4 and dimethylated H3K9, RPMI8226 cells were treated with triptolide (50, 100, or 150 nmol/L), and LSD1 protein levels were analyzed using an antibody against human LSD1. As shown in Figure 7A, triptolide-treated cells showed significantly higher levels of LSD1 compared with the control (P<0.05 vs untreated control), which could be responsible for the decrease in dimethylated H3K4 and dimethylated H3K9. Consistent with the Western-blot results, immunofluorescence analysis showed that there was a significant increase in LSD1 in cells exposed to triptolide (shown in Figure 8).

We extracted total RNA from triptolide-treated RPMI8226 cells and analyzed LSD1 mRNA by real-time PCR to investigate the effects of triptolide on the transcriptional expression of LSD1. As shown in Figure 9A, the levels of LSD1 mRNA in RPMI8226 cells were increased slightly after exposure to triptolide for 48 h compared with the control, but this change was not statistically significant. Therefore, triptolide could not regulate the activity of LSD1 at the transcriptional level (P>0.05).

**Triptolide decreased the expression of the histone demethylase JMJD2B in RPMI8226 cells**

Unlike LSD1, JMJD2B actively mediates the removal of chromosomal tri- and dimethylated H3K36 and H3K9 well before the replication of chromatin[23]. We examined whether JMJD2B played a role in the triptolide-mediated induction of H3K36 and H3K9 dimethylation. RPMI8226 cells were treated with triptolide (50, 100, or 150 nmol/L), and JMJD2B protein levels were analyzed using an antibody against human JMJD2B. As shown in Figure 7A, JMJD2B levels declined as the concentration of triptolide increased (P<0.05 vs untreated control). The rates of inhibition by 50, 100, and 150 nmol/L triptolide at 48 h were 5.5%, 16.8%, and 66.5%, respectively, compared with the control (P<0.05 vs untreated control) (Figure 7B).

To determine whether triptolide affected the transcriptional expression of the JMJD2B gene, we extracted total RNA from triptolide-treated RPMI8226 cells and amplified JMJD2B by semiquantitative real-time PCR. As shown in Figure 9B, triptolide inhibited the mRNA expression of JMJD2B in a dose-dependent manner (P<0.05). A low concentration of triptolide
(50 nmol/L) inhibited the expression of JMJD2B mRNA by 18%. However, the difference in inhibition rates between the 100 and 150 nmol/L triptolide treatments, which were 53% and 56%, respectively, was not significant (P>0.05). This result indicates that triptolide regulates JMJD2B activity at the transcriptional level.

Discussion

MM is a plasma cell neoplasm characterized by the accumulation of terminally differentiated monoclonal plasma cells in the bone marrow and, usually, the presence of monoclonal immunoglobulin in the blood and/or urine[24]. MM accounts for approximately 1% of malignant diseases and is the second most commonly diagnosed hematological malignancy[25]. The outcome for patients with MM is highly variable. The median overall survival time is 3–4 years[25]. Despite major improvements in treatments including high dose chemotherapy and autologous bone marrow transplantation, the disease remains incurable. Myelomas with no response to initial chemotherapy, incomplete response rates and the eventual emergence of drug resistance remain major obstacles, indicating an urgent need for novel therapeutic agents.

Cancer has traditionally been viewed as primarily a genetic disorder. However, it has become apparent that, in addition to genetic mutations, epigenetic abnormalities also play important roles in the pathogenesis of tumors[26]. Epigenetic control mechanisms involve changes in gene expression patterns, due to the modification of DNA and/or histones[15]. Modifications to histone N-terminal tails affect chromatin structure, gene transcription, and DNA replication and repair.

Highly specific histone modifications are enriched in target tumor genes and work together with other components to form a complex that interacts with the target genes to induce or inhibit DNA methylation, thereby causing transcriptional repression or activation[27, 28]. The N-terminal tails of histones undergo several types of post-translational modifications, including methylation, acetylation, phosphorylation and ubiquitination. Unlike other histone modifications, such as acetylation, phosphorylation and ubiquitination, histone methylation is important because of its highly specific effects on gene regulation[14]. These modifications remain stable between methylation and demethylation events, which are dynamically regulated by histone methyltransferases and demethylases. It has been demonstrated that abnormalities affecting histone methylation-modifying enzymes lead to aberrant histone modifications, which may occur at gene promoters and induce the improper expression or repression of individual genes that play important roles in tumorigenesis. Recently, the overexpression, amplification and mutation of several histone demethylases have been linked to cell proliferation, cell apoptosis, gene expression, genome stability, and the genesis of cancer.

Triptolide has multiple pharmacological activities, including anti-inflammatory, immunosuppressive, male anti-fertility and anticancer effects[29]. Research into its mechanisms of action has revealed that it potently inhibits monocyte activation, activates caspases and other proapoptotic signaling cas-
LSD1) could increase p53-dependent germ cell apoptosis and plays a unique role in meiotic DNA double-strand break (DSB) repair[44]. In addition, knockdown of LSD1 delays the initial induction of p53 in response to DNA damage, which leads to reduced activation of p21 and MDM2, resulting in a decrease in p53-mediated apoptosis. Likewise, in p53-deficient cells, LSD1 can regulate DNA-damage-induced cell death by promoting E2F1 accumulation during DNA damage and the activation of its proapoptotic target gene p73, a major mediator of p53-independent apoptosis[45]. In contrast, Huang et al found that knockdown of LSD1 increases the transcriptional activation of p21 and MDM2 by p53[46, 47]. However, the LSD1 complex has been shown to be required for the repressive activity of an oncogenic I kappa B protein, and a functional link has been established between the E3 ligase TBLR1 and NF-kappaB[48].

In our study, triptolide promoted the expression of LSD1 at the translational level in a dose-dependent manner. LSD1 could exert its proapoptotic effects by directly demethylating non-histone proteins, such as p53 and E2F1 in multiple myeloma cells. LSD1 can also induce apoptosis through its H3K4 demethylase activity. H3K4me2 is highly enriched at transcriptionally competent or active gene promoters. Accord-
ing non-histone proteins, such as p53 and E2F1 in multiple

Our experiments demonstrated that histone H3K9 and H3K36 demethylation were dose-dependently reduced by triptolide, as were JMJD2B mRNA and protein levels. These results suggest that the downregulation of H3K9me2 was partly due to the decrease in JMJD2B in RPMI8226 cells because the levels of H3K9me2 formed by the demethylation of H3K9me3 were significantly reduced. Triptolide may exert its proapoptotic effects partly by directly downregulating JMJD2B in multiple myeloma cells.

In addition, JMJD2B could demethylate trimethylated H3K36 to dimethylated H3K36. H3K36me2/me3 are enriched mostly in the transcribed region of active genes and are associated with the later stages of transcription in actively transcribed regions[49]. However, a direct association between H3K36me2 and proapoptotic genes has not been proven; further investigation will be required to clarify this relationship.

In our study, the downregulation of JMJD2B may be partially responsible for the reduction in H3K9me2 and H3K36me2 in triptolide-treated cells. LSD1 and JMJD2B are assumed to be two targets for triptolide as an anti-MM drug.

In summary, our experiments showed that triptolide inhibited proliferation and induced apoptosis and G0/G1 cell cycle arrest in the MM cell line RPMI8226 in a dose-dependent manner. Moreover, this study provided the first evidence that triptolide could restore epigenetic changes by regulating the histone demethylases LSD1 and JMJD2B, thereby offering a novel view of the anti-MM mechanism of triptolide.

Abbreviations
MM, multiple myeloma; BM, bone marrow; PI, propidium iodide; DMSO, dimethylsulfoxide; MTTR, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PBMC, peripheral blood mononuclear cells; SDS, sodium dodecylsulfate; TBS, Tris-buffered saline; H3K4me2, dimethyl-lysine 4 on histone H3; H3K9me2, dimethyl-lysine 9 on histone H3; H3K36me2, dimethyl-lysine 36 on histone H3; ECL, enhanced chemiluminescence; BSA, bovine serum albumin; LSD1, lysine-specific demethylase 1; JMJD2B, the second member of the Jumonji domain 2 family of proteins. PBS, phosphate-buffered saline.

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Author contribution
Yan CHEN, Ling-lan ZENG, and Chun ZHANG designed the research; Lu WEN, Rui LI, Fei ZHAO, and Yuan LIU performed the experiments; Lu WEN analyzed the data and wrote the paper.
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