

Andrographolide inhibits multiple myeloma cells by inhibiting the TLR4/NF- κ B signaling pathway

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Abstract. Andrographolide is an active component from the extract of *Andrographis paniculata* [(Burm.f) Nees], a medicinal plant from the Acanthaceae family. Pharmacological studies have revealed that andrographolide possesses anti-bacterial, anti-inflammatory, anti-viral, immune regulatory and hepatoprotective properties, and is efficacious in the treatment of cardiovascular diseases, while exhibiting low toxicity and low cost. The present study aimed to determine the inhibitory effects of andrographolide on the growth of multiple myeloma (MM) cells and its possible impact on the Toll-like receptor (TLR)4/nuclear factor (NF)- κ B signaling pathway. Cell proliferation was detected using an MTT assay, cellular apoptosis was measured using flow cytometry, and caspase-9/3 activation were assessed using colorimetric assay kits. Furthermore, TLR4 and NF- κ B protein expression was determined by western blot analysis. The results revealed that andrographolide reduced the proliferation, while increasing cellular apoptosis and caspase-9/3 activation of MM cells, in addition to downregulating the expression of TLR4 and NF- κ B protein. Of note, TLR4- or NF- κ B-targeting small-interfering (si)RNA enhanced the andrographolide-induced inhibition of cell proliferation and induction of apoptosis of MM cells. The results of the present study therefore suggested that andrographolide inhibited multiple myeloma cells via the TLR4/NF- κ B signaling pathway.

Introduction

Multiple myeloma (MM) is a malignant blood cancer type with the characteristic of plasma-cell clonal proliferation, which accounts for ~10% of all hematological malignancies and has a yearly increasing incidence rate (1). In recent years,

with the application of novel chemotherapeutic drugs and improvements in treatment methods, as well as progress in the development and optimization of supportive treatments, 50-70% of patients receive effective chemotherapy; however, multiple cycles of chemotherapeutic treatments cause drug resistance, leading to refractory MM (2).

Studies on *Drosophila (D.) melanogaster* have led to the discovery of the Toll gene, which mainly determines the developmental direction of the front and lateral body axes in *D. melanogaster* as well as the non-specific immune response. Toll genes encode Toll-like receptors (TLR); the first TLR identified on the human cell surface displaying homology with *D. melanogaster* TLRs was TLR4 (3). Studies have shown that signaling pathways induced by TLRs, including TLR4 and TLR9, are important in tumor formation, and that the upregulation of TLRs may be closely associated with the development of cancer types, including gastric and colon cancer (4).

Nuclear factor (NF)- κ B is a key nuclear transcription factor which, under normal conditions, exists in the inactive forms of homologous or heterodimers in the cytoplasm of almost all types of cells, and which is associated with multiple cellular activities, including the activation of immune cells, development of T- and B-lymphocytes, stress response and cell apoptosis (5). Recent studies have shown that NF- κ B is closely associated with the occurrence of hematopoietic malignancies, including leukemia, lymphoma and MM (6).

Andrographolide (Fig. 1) is a diterpene lactone compound extracted from *Andrographis paniculata* [(Burm.f) Nees], a medicinal plant from the Acanthaceae family, and is one of the major active components of the traditional Chinese medicine Andrographis with a content of up to 1.8% in the leaves (7). In China, Andrographis is being mass-produced as a raw material for the isolation of andrographolide used as an anti-inflammatory drug in formulations including Kalii Dehydrographolidi Succinas and Andrographis injection (8). Pharmacological studies have shown that andrographolide has anti-inflammatory, anti-bacterial, anti-viral, anti-tumor, immunoregulatory and hepato- and gallbladder-protective effects, as well as beneficial effects on cardiovascular diseases, with characteristics of low toxicity and low cost (9-12). However, to date, the potential of andrographolide to be used in the treatment of human MM has not been studied. The present study provided experimental evidence for the anti-cancer efficacy of andrographolide on MM cells; in

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addition, the mechanism of action and potential regulatory molecules involved, including TLR4 and NF- κ B, were assessed.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was provided by Invitrogen (Thermo Fisher Scientific). Annexin V/propidium iodide (PI) was purchased from eBioscience (San Diego, CA, USA). Caspase-9/3 activation ELISA colorimetric assay kits were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The bicinchoninic acid (BCA) Protein Assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Cells and cell culture. The OPM1 human myeloma cell line was purchased from Shanghai Cell Bank (Shanghai, China) and cultured in complete DMEM with 10% heat-inactivated FBS, 100 U/ml penicillin and streptomycin (100 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability assay. OPM1 cells were seeded into 96-well plates at 1x10⁴/well and allowed to attach overnight, following which they were treated with 1.0, 5.0 or 10.0 μ M andrographolide (Sigma-Aldrich; purity, >98%) for 24, 48 or 72 h according to the procedure of a previous study (13). Subsequently, 20 μ l MTT (5 mg/ml) was added to each well and plates were cultured for an additional 4 h, followed by aspiration of the media, addition of 150 μ l dimethylsulfoxide (Invitrogen; Thermo Fisher Scientific) to each well and agitation for 20 min. The absorbance values were determined at 550 nm using an automatic microplate reader (Wallac Victor 1420; PerkinElmer, Inc., Waltham, MA, USA).

Flow cytometric analysis. OPM1 cells were inoculated into six-well plates at 2x10⁶/well and treated with 1.0, 5.0 and 10.0 μ M andrographolide for 24 h. Each well was washed twice with phosphate-buffered saline (PBS) and following trypsinization (Beyotime Institute of Biotechnology), cells were suspended in 1 ml binding buffer. Annexin V (5 μ l) was added and cells were incubated for 15 min in the dark. Subsequently, 5 μ l PI was added and cells were incubated for 30 min in the dark on ice. The apoptotic rate of OPM1 cells was then assessed by flow cytometry (FACSCalibur; BD Biosciences) with 1x10⁶ events recorded.

Caspase-9/3 activation. OPM1 cells were seeded into 96-well plates at 1x10⁴/well and treated with 1.0, 5.0 or 10.0 μ M andrographolide for 24 h. Caspase-9/3 activation in OPM1 cells was determined using ELISA colorimetric assay kits. Caspase-9 inhibitor LEHD-pNA and caspase-3 inhibitor Ac-DEVD-pNA were added to each well, and caspase-9/3 activation-associated fluorescence was detected at the wavelength of 405 nm using an automatic microplate reader (Wallac Victor 1420).

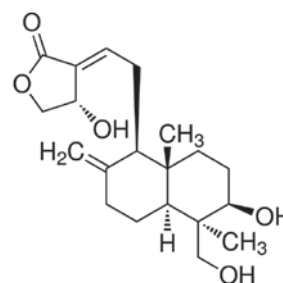


Figure 1. Chemical structure of andrographolide.

Western blot analysis. OPM1 cells were inoculated into six-well plates at 2x10⁶/well and treated with 1.0, 5.0 or 10.0 μ M andrographolide for 24 h. Each well was washed twice with PBS and incubated with ice-cold lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice. The protein contents were determined using the BCA Protein Assay kit. Following loading of 10 μ g protein per lane, total protein was fractionated by 10% SDS-PAGE and transfer onto a polyvinylidene difluoride membrane at 4°C over 2 h. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 prior to incubation with anti-TLR4 (cat. no. sc-293072; 1:1,000; Santa Cruz Biotechnology, Inc.); anti-NF- κ B (cat. no. sc-56735; 1:1,000; Santa Cruz Biotechnology, Inc.) and β -actin (cat. no. AC106; 1:1,000; Beyotime Institute of Biotechnology, Inc.) overnight at 4°C with agitation. After extensive washing, membranes were incubated with secondary antibody (1:3,000; Tiangen, Beijing, China) for enhanced chemiluminescence (ECL) detection using Pierce ECL Western Blotting substrate (cat. no. 32109; Thermo Fisher Scientific).

Transfection of TLR4 small interfering (si)RNA and NF- κ B siRNA. TLR4 siRNA and NF- κ B siRNA were chemically synthesized by BeastBio Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: TLR4 5'-GATCCCGACTTACAGTTTCTACGTTTCAAGAGAACGTAGAACTGTAAGTCGTTA-3' and 5'-AGCTTAACGACTTACAGTTTCTACGTTTCTTGAACGTAGAACTGTAAGTCGG-3'; and NF- κ B: 5'-CCCCTTCCAAGTTCCCTATA-3' and 5'-GGACATATGAGACCTTCAA-3'. OPM1 cells were seeded into six-well plates at 2x10⁶/well. 100 pmol TLR4 siRNA or NF- κ B siRNA were transfected into OPM1 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. All quantitative values were obtained from experiments performed at least three times. Values are expressed as the mean \pm standard deviation. Statistical significance was analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Andrographolide inhibits the proliferation of MM cells. To investigate whether andrographolide inhibited the proliferation

of MM cells, OPM1 cells were treated with andrographolide (1, 5 or 10 μ M) for 24, 48 or 72 h and subjected to the MTT assay. As shown in Fig. 2, andrographolide significantly inhibited the proliferation of MM cells *in vitro* in a dose- and time-dependent manner (Fig. 2).

Andrographolide induces apoptosis of MM cells. To detect whether andrographolide induced apoptosis of MM cells, OPM1 cells were treated with andrographolide (1, 5 or 10 μ M) for 24 h and subjected to Annexin V/PI double staining followed by flow-cytometric evaluation. As shown in Fig. 3, andrographolide significantly induced apoptosis of MM cells *in vitro* in a dose-dependent manner.

Andrographolide induces caspase-9/3 activation of MM cells. To evaluate whether andrographolide induced caspase-9/3 activation in MM cells, OPM1 cells were treated with andrographolide (1, 5 or 10 μ M) for 24 h and subjected to a colorimetric ELISA assay. As shown in Fig. 4A and B, andrographolide effectively increased caspase-9/3 activation in MM cells *in vitro* in a dose-dependent manner (Fig. 4A and B).

Andrographolide inhibits TLR4 protein expression in MM cells. To further investigate the potential regulatory mechanisms of the effects exerted by andrographolide, the TLR4 protein expression of MM cells was determined using western blots analysis. As shown in Fig. 5A and B, andrographolide effectively reduced the levels of TLR4 protein expression in OPM1 cells in a dose-dependent manner (Fig. 5A and B).

Andrographolide inhibits NF- κ B protein expression in MM cells. To further elucidate the potential regulatory mechanism of andrographolide on the growth of MM cells, NF- κ B protein expression in MM cells was detected using western blot analysis. As shown in Fig. 6A and B, andrographolide effectively reduced the level of NF- κ B protein expression in OPM1 cells in a dose-dependent manner (Fig. 6A and B).

TLR4 siRNA enhances andrographolide-mediated inhibition of cell proliferation and induction of apoptosis of MM cells. To further confirm whether the TLR4/NF- κ B signaling pathway was the functional target of andrographolide, TLR4 siRNA was transfected into MM cells. As shown in Fig. 7A and B, TLR4 siRNA inhibited the TLR4 protein expression in OPM1 cells. Of note, TLR4 siRNA efficiently enhanced the andrographolide-mediated inhibition of the cell proliferation and induction of apoptosis of MM cells (Fig. 7C and D). These results indicated that downregulation of TLR4 expression significantly enhanced the anti-cancer effects of andrographolide on MM cells.

TLR4 siRNA regulates NF- κ B protein expression in MM cells. To further confirm whether the TLR4/NF- κ B signaling pathway was the functional target of andrographolide, the NF- κ B protein expression of MM cells was detected using western blot analysis. As shown in Fig. 8A and B, TLR4 siRNA significantly inhibited the expression of NF- κ B protein in MM cells. The results indicated that downregulation of TLR4 expression significantly enhanced the inhibitory

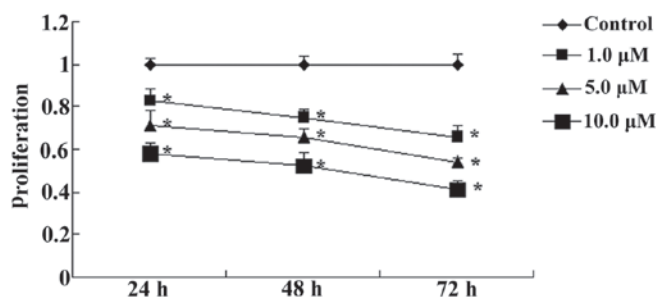


Figure 2. Andrographolide inhibits the proliferation of multiple myeloma cells. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. control.

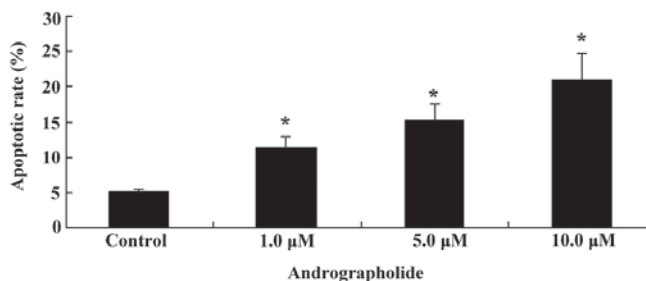


Figure 3. Andrographolide induces apoptosis of multiple myeloma cells. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. control.

effects of andrographolide on NF- κ B expression in the MM cells.

NF- κ B siRNA enhances andrographolide-mediated reduction of cell proliferation and induction of apoptosis of MM cells. To further assess whether the TLR4/NF- κ B signaling pathway is a functional target of andrographolide, NF- κ B siRNA was transfected into MM cells. As shown in Fig. 9A and B, NF- κ B siRNA inhibited the protein expression of NF- κ B in MM cells. Of note, NF- κ B siRNA markedly enhanced andrographolide-mediated inhibition of cell proliferation and induction of apoptosis of MM cells (Fig. 9C and D). These results indicated that downregulation of NF- κ B expression significantly enhanced the anti-cancer effects of andrographolide on MM cells.

Discussion

MM is a neoplasm of the blood, which is common in the elderly; its major cause is the proliferation of malignant plasma cells in the blood. With the increasing mean age of the Chinese population, the incidence of MM has been increasing (14). Autologous hematopoietic stem-cell transplantation can improve the number of normal blood plasma cells in cancer patients to prolong the survival time to a certain extent; however, as the age of patients with MM is generally high, stem-cell transplantation is not suitable for most of the patients (15). In the present study, andrographolide restrained the proliferation of MM cells in a dose- and time-dependent manner. Furthermore, andrographolide induced cellular apoptosis and caspase-9/3 activation in MM cells in a dose-dependent manner. Yang *et al* (16) reported that andrographolide induced apoptosis in glioma cells through the extracellular signal-regulated

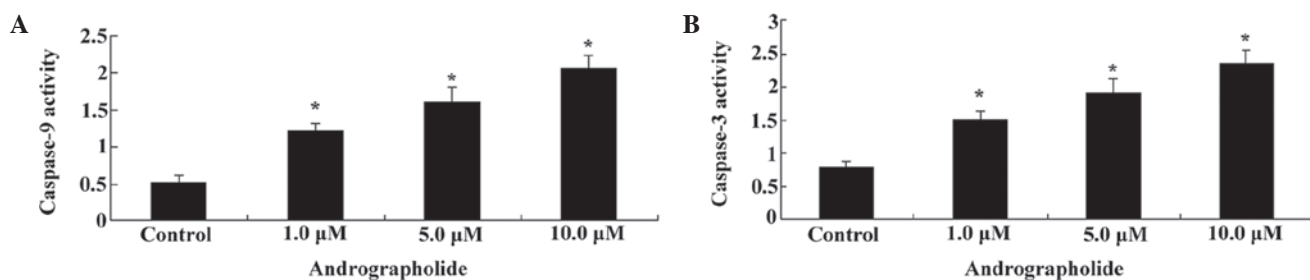


Figure 4. Andrographolide activates caspase-9/3 in multiple myeloma cells. (A) Caspase-9 and (B) caspase-3 expression levels. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. control.

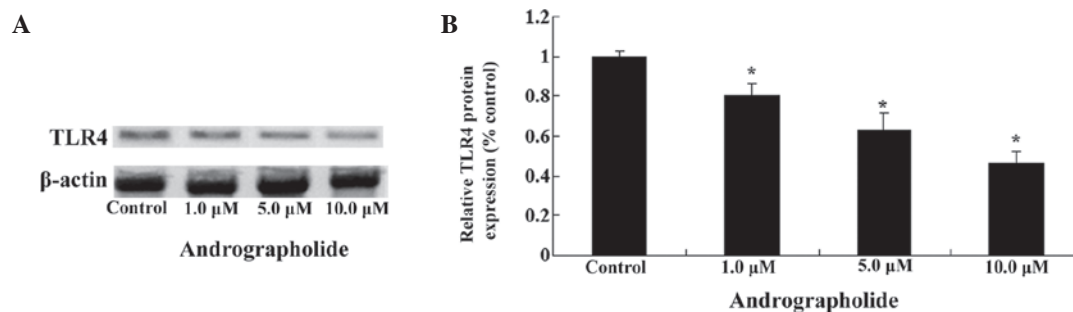


Figure 5. Andrographolide inhibits TLR4 protein expression in multiple myeloma cells. (A) Representative western blot showing TLR4 protein expression. (B) TLR4 expression was quantified by densitometric analysis with normalization to β -actin. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. control. TLR, Toll-like receptor.

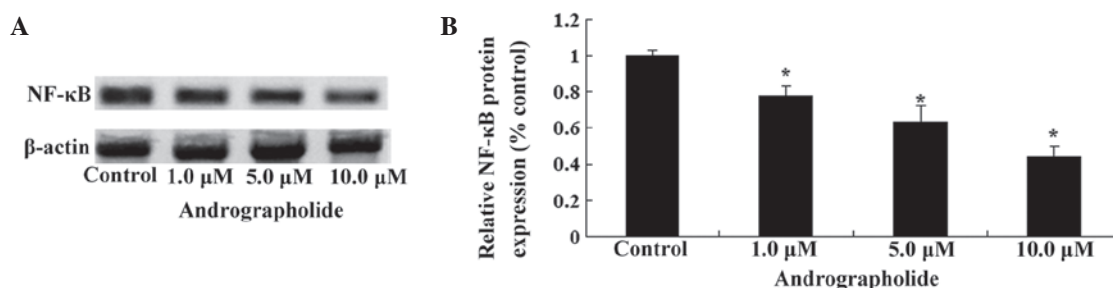


Figure 6. Andrographolide inhibits NF- κ B protein expression in multiple myeloma cells. (A) Representative western blot showing NF- κ B protein expression. (B) NF- κ B expression was quantified by densitometric analysis with normalization to β -actin. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. control. NF- κ B, nuclear factor κ B.

kinase/p53/caspase 7/poly(adenosine triphosphatase ribose) polymerase signaling pathway. Furthermore, andrographolide was shown to inhibit tumor angiogenesis through downregulation of vascular endothelial growth factor (VEGF)/VEGF receptor 2/mitogen-activated protein kinase pathway (17).

In humans, 11 types of TLRs have been identified, among which TLR4 was the first TLR found in mammals (3). Lipopolysaccharide (LPS) is the exogenous ligand of TLR4 and an *in vivo* study has shown that LPS stimulates the growth and metastasis of tumor cells (18). TLR4 is expressed in a variety of murine tumor-cell lines, and LPS-activated TLR4 signaling is conducive to tumor cells escaping from the microenvironment of immune surveillance; in addition, following siRNA-mediated TLR4 silencing, the inhibition of tumor cell growth was enhanced, which thus prolonged the survival time of mouse models with tumors (19). TLR4 is expressed on the surface of human ovarian cancer epithelial cells and can induce proliferation as well as enhance the

production of cell cytokines following activation by LPS; therefore, it can be speculated that tumor cells regulate the tumor microenvironment via TLR4 and influence the activity of immune cells (20-22). The exogenous ligand of TLR4, LPS, promotes the proliferation of MM cells. Studies have revealed that andrographolide inhibits the growth of melanoma (23) and insulinoma (24) through inhibition of the TLR4/NF- κ B signaling pathway. The present study demonstrated that andrographolide suppressed the protein expression levels of TLR4 in a dose-dependent manner. Furthermore, TLR4 siRNA enhanced andrographolide-mediated inhibition of cell proliferation and induction of apoptosis, while restraining the protein expression of NF- κ B in MM cells.

NF- κ B is a protein which can specifically combine with κ B sites in a variety of gene promoters or enhancers to promote transcription (25). It regulates the expression of numerous genes, including cytokines, adhesion molecules, chemokines, immune factors, oxidative stress-associated

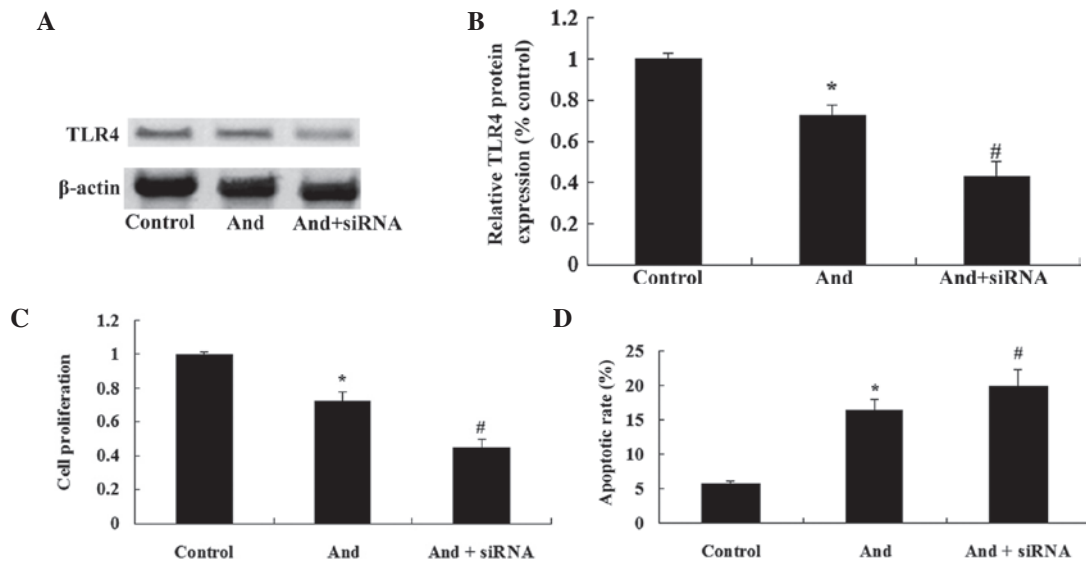


Figure 7. TLR4 siRNA regulates andrographolide-induced the cell proliferation and apoptosis of MM cells. (A) Representative western blot and (B) quantified expression levels obtained by densitometric analysis with normalization to β -actin confirming that TLR4 siRNA regulates TLR4 protein expression. (C) The reduction of cell proliferation and (D) the enhancement of apoptosis of MM cells by andrographolide were enhanced by TLR4 siRNA. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ compared with control group, # $P < 0.01$ compared with $5.0 \mu\text{M}$ andrographolide-treated group. MM, multiple myeloma; And, andrographolide; TLR, Toll-like receptor; siRNA small interfering RNA.

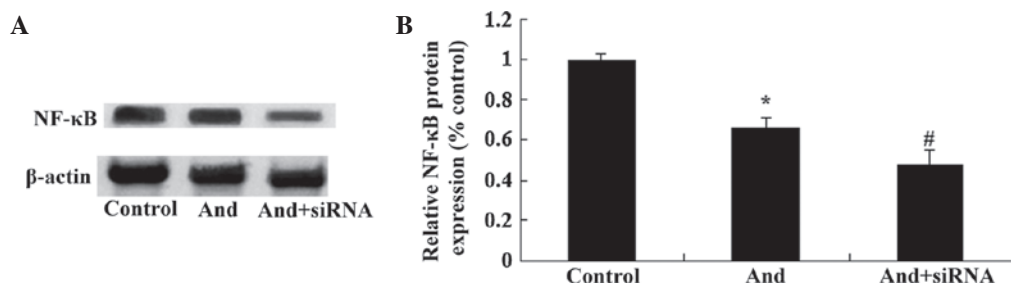


Figure 8. TLR4 siRNA regulates NF- κ B protein expression of MM cells. (A) Representative western blots and (B) quantified expression levels obtained by densitometric analysis with normalization to β -actin confirming that TLR4 siRNA regulates NF- κ B protein expression in MM cells. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ compared with control group, # $P < 0.01$ compared with $5.0 \mu\text{M}$ andrographolide-treated group. MM, multiple myeloma; And, andrographolide; TLR, Toll-like receptor; siRNA small interfering RNA; NF- κ B, nuclear factor kappa B.

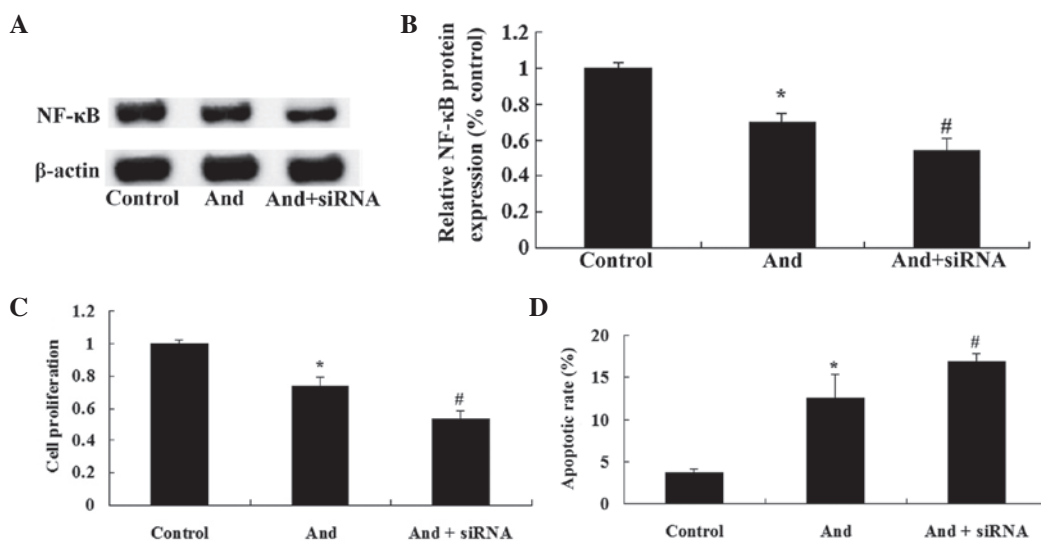


Figure 9. NF- κ B siRNA regulates andrographolide-mediated reduction of cell proliferation and enhancement of apoptosis of MM cells. (A) Representative western blots and (B) quantified expression levels obtained by densitometric analysis with normalization to β -actin confirming that NF- κ B siRNA regulates NF- κ B protein expression. (C) The reduction of cell proliferation and (D) the enhancement of apoptosis of MM cells by andrographolide were enhanced by NF- κ B siRNA. Values are expressed as the mean \pm standard deviation. * $P < 0.01$ compared with control group, # $P < 0.01$ compared with $5.0 \mu\text{M}$ andrographolide-treated group. MM, multiple myeloma; And, andrographolide; siRNA small interfering RNA; NF- κ B, nuclear factor kappa B.

enzymes and transcription factors, and therefore has a variety of biological functions, including the participation in inflammatory immune responses, the regulation of cell apoptosis, self-transcription, cell cycle regulation, tumorigenesis and drug resistance (26,27). As a transcription factor with a designated DNA-binding sequence, NF- κ B has an important role in solid tumor-cell proliferation and transformation as well as tumor development; furthermore, it is closely associated with neoplasms of the blood system (28). Luo *et al* (13) showed that andrographolide inhibited the activation of NF- κ B as well as matrix metalloproteinase-9 activity in H3255 lung cancer cells. Furthermore, Wang *et al* (29) demonstrated that andrographolide inhibited the proliferation of oral squamous cell carcinogenesis through inhibition of NF- κ B inactivation. In the present study, andrographolide decreased the protein expression NF- κ B in MM cells. Of note, NF- κ B siRNA significantly enhanced andrographolide-mediated inhibition of cell proliferation and induction of apoptosis of MM cells.

In conclusion, to the best of our knowledge, the present study was the first to show that andrographolide suppressed the proliferation and promoted apoptosis and caspase-9/3 activation in MM cells. The underlying mechanism may involve the suppression of the TLR4/NF- κ B signaling pathway. It is thus suggested that andrographolide may a promising candidate anti-cancer drug for the clinical treatment of MM through the TLR4/NF- κ B signaling pathway.

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