

Triptolide induces apoptosis through the calcium/calmodulin-dependent protein kinase kinase β /AMP-activated protein kinase signaling pathway in non-small cell lung cancer cells

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Abstract. Triptolide, a triterpene extracted from the Chinese herb *Tripterygium wilfordii*, has been reported to exert multiple bioactivities, including immunosuppressive, anti-inflammatory and anticancer effects. Although the anticancer effect of triptolide has attracted significant attention, the specific anticancer mechanism in non-small-cell lung cancer (NSCLC) remains unclear. The present study aimed to investigate the anticancer effect of triptolide in the H1395 NSCLC cell line and to determine its mechanism of action. The results revealed that triptolide significantly inhibited the cell viability of NSCLC cells in a dose-dependent manner, which was suggested to be through inducing apoptosis. In addition, triptolide was revealed to activate the calcium (Ca²⁺)/calmodulin-dependent protein kinase kinase β (CaMKK β)/AMP-activated protein kinase (AMPK) signaling pathway by regulating the intracellular Ca²⁺ concentration levels, which increased the phosphorylation levels of AMPK and reduced the phosphorylation levels of AKT, ultimately leading to apoptosis. The CaMKK β blocker STO-609 and the AMPK blocker Compound C significantly inhibited the apoptosis-promoting effect of triptolide. In conclusion, the results of the present study suggested that triptolide may induce apoptosis through the CaMKK β -AMPK

signaling pathway and may be a promising drug for the treatment of NSCLC.

Introduction

Lung cancer is one of the most common types of malignancy, with an estimated 1.8 million new lung cancer cases reported in 2012, accounting for ~13% of total cancer diagnoses worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for >80% of lung cancer cases and its overall 5-year survival rate is <15% (2). Surgery is currently the standard treatment for patients with stage I NSCLC (2); however, NSCLC has no typical symptoms and lacks specificity in clinical signs at an early stage (3); therefore, the majority of patients who are diagnosed with lung cancer are already at the advanced stage and are not suitable for surgical treatment (1). Even so, among the patients who do undergo surgery, the rate of recurrence and metastasis remains high; for example, in a prospective study of 1,361 patients with NSCLC undergoing surgical resection, Sugimura *et al* (4) reported that 41% of the patients developed recurrence following surgery, and the overall 2- and 5-year survival within this cohort was found to be 17 and 12%, respectively. However, NSCLC is not very sensitive to chemotherapeutic drugs; thus, the therapeutic treatment of the disease requires further improvements. Therefore, it is of great practical significance to study effective drugs and therapeutic approaches.

Triptolide, a diterpenoid epoxide, is the major active compound extracted from a traditional Chinese medicinal herb named thunder god vine (*Tripterygium wilfordii*) (5), which is predominantly used to treat autoimmune diseases, including systemic lupus erythematosus (6), rheumatoid arthritis (7) and asthma (8). In addition to its immunosuppressive effects, triptolide has demonstrated numerous other pharmacological effects, such as anti-inflammatory, anti-fertility and cyst reduction activities (9). However, the most important feature of triptolide is considered to be its anticancer effect. Previous

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studies have reported that triptolide exerted an efficient, broad-spectrum antitumor effect; for example, triptolide demonstrated a strong anticancer effect on various types of cancer cells *in vitro* and *in vivo*, including lung cancer (10), prostate cancer (11), human breast cancer (12), oral cancer (13) and mouse leukemia cells (14,15). In addition, triptolide inhibited the growth of tumor cells by inhibiting angiogenesis in prostate cancer (16). It was also discovered to act synergistically with tumor necrosis factor (TNF)- α in tumor cells and inhibit the upregulation of NF- κ B expression caused by the latter, thereby enhancing the sensitivity of cancer cells to TNF- α , which killed the tumor cells (17). In addition, a previous study indicated that triptolide reduced the levels of total RNA in cells (18), thus it may be considered a universal transcriptional inhibitor (19), which exerts an anticancer effect. Previous studies have revealed that triptolide also inhibited the proliferation of NSCLC and promoted cell apoptosis *in vitro* and *in vivo* (10,20). However, the anticancer mechanism of triptolide in NSCLC remains unclear.

Calcium (Ca²⁺) ions are very important, ubiquitous intracellular second messenger molecules that are involved in numerous signal transduction pathways, including cell cycle progression, apoptosis, differentiation and proliferation (21). Calmodulin (CaM) is a central mediator of Ca²⁺-dependent signaling; it participates in the regulation of Ca²⁺ signaling synergistically with various CaM-binding proteins (22). Important CaM-binding proteins are Ca²⁺/CaM-dependent protein kinases (CaMKs), which are a family of structurally related serine/threonine protein kinases, including CaMKI, CaMKIV and Ca²⁺/CaM-dependent protein kinase kinase (CaMKK) (23). CaMKK has two isoforms: CaMKK α and CaMKK β (also named CaMKK1 or CaMKK2, respectively) (24). CaMKK β is predominantly expressed at higher levels in central nervous tissues and at lower levels in the thymus, testis, spleen and lung (25). CaMKK β is known to activate CaMKI, CaMKIV and AMP-activated protein kinase (AMPK) (25), and it has been discovered to be involved in the regulation of several important physiological and pathophysiological processes, including energy balance, obesity, glucose homeostasis, hematopoiesis, inflammation and cancer (26). AMPK serves as a protein kinase downstream of CaMKK β , and CaMKK β forms a functional complex with AMPK to activate AMPK (27). AMPK is an important energy receptor in cells, serving a crucial role in regulating energy metabolism in cells and organisms (28). Activated AMPK was reported to promote catabolism, inhibit anabolism, respond to the external and internal stimuli of cells and affect basic biological processes, such as cell growth, proliferation and apoptosis (28-30). In addition, AMPK and its related signaling molecules have been discovered to closely regulate the biological behaviors of NSCLC, including proliferation, apoptosis and autophagy (31,32). The activated CaMKK β /AMPK signaling pathway was revealed to inhibit mTOR, which is downstream of AMPK, thereby exerting a tumor-suppressive effect (33,34). Therefore, the identification of compounds that target CaMKK β /AMPK signaling may be a novel strategy for NSCLC treatment.

The present study investigated the antitumor effects and mechanism of action of triptolide in NSCLC cells *in vitro*. The results revealed that triptolide inhibited cell

viability and promoted apoptosis through activating the CaMKK β /AMPK signaling pathway in NSCLC cells. In addition, the results discovered that the CaMKK β blocker STO-609 and the AMPK blocker Compound C significantly inhibited the apoptosis-promoting effect of triptolide.

Materials and methods

Materials. Triptolide powder, the CaMKK β blocker STO-609, the AMPK blocker Compound C and BAPTA-AM were purchased from Sigma Aldrich; Merck KGaA. Primary antibodies against β -actin (cat. no. 4970), GAPDH (cat. no. 5174), total AKT (cat. no. 4691), phosphorylated (p)-AKT (cat. no. 4060), total AMPK (cat. no. 2603), p-AMPK (cat. no. 4188) and poly [ADP-ribose] polymerase 1 (PARP; cat. no. 9542) were purchased from Cell Signaling Technology, Inc. The FITC-conjugated goat anti-rabbit (cat. no. F1262) IgG secondary antibody was purchased from Sigma-Aldrich; Merck KGaA. The Annexin V-FITC/propidium iodide (PI) double staining kit was purchased from BD Biosciences. The Apo-ONE[®] Homogeneous Caspase-3/7 assay kit (cat. no. G7792) was purchased from Promega Corporation.

Cell lines and culture. NSCLC cell lines A549 and H1395 and the human bronchial epithelial cell line 16-HBE were purchased from the American Type Culture Collection. A549 and H1395 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and 16-HBE cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.). All cells were supplemented with 100 μ g/ml streptomycin, 100 U/ml penicillin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were all maintained at 37°C in a 5% CO₂ incubator.

MTT assay. A549, H1395 and 16-HBE cells were plated at a density of 4x10³ cells/well into 96-well plates at 37°C for 24 h. The cells were treated with a wide range of concentrations of triptolide (0, 50, 100, 150 and 200 nM) or the vehicle control (0.1% DMSO), and incubated at 37°C for 24 h. In view of the low sensitivity of 16-HBE cells to triptolide, doses of 250 nM and 300 nM were added for 16-HBE cells. In another experiment, the cells were treated with 200 nM triptolide in the presence or absence of 5 μ M BAPTA-AM for 24 h at 37°C. Each condition was plated in triplicate. Subsequently, 10 μ l MTT dye solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and incubated at 37°C for 4 h. Following the incubation, the culture medium was removed and 150 μ l DMSO was added/well to stop the reaction. Finally, the absorbance of the plate was measured using wavelengths of 570 nm (absorbance) and 650 nm (reference) using a Multimode Microplate reader (Tecan Group, Ltd.).

The half maximal inhibitory concentration (IC₅₀) was calculated using CalcuSyn version 2.1 software (Biosoft). The cell viability was calculated as the percentage change of the absorbance of treated cells/the absorbance of untreated cells.

Flow cytometric analysis of apoptosis. H1395 cells were treated with 0, 50, 100 or 200 nM triptolide at 37°C for 24 h. In another experiment, H1395 cells were treated with 200 nM triptolide, 10 μ M STO-609, 10 μ M Compound C or combinations at 37°C for 24 h. H1395 cells were harvested

by centrifugation for 5 min at 200 x g at room temperature and the cells were washed in PBS and then suspended in 1X binding buffer (100 μ l). A dye solution consisting of 5 μ l PI and 5 μ l Annexin V-FITC was added and after mixing, the cells were incubated for 15 min at room temperature in the dark. Apoptotic cells were then analyzed using a BD FACSAria III flow cytometer (BD Biosciences) and analyzed using FlowJo version 7.6.1 software (FlowJo LLC). Single Annexin V-FITC positive cells were considered as early apoptotic, while both PI and Annexin V-FITC positive cells were considered as late apoptotic. Meanwhile, single PI positive cells indicated necrosis. The apoptotic rate was determined as the percentage of early and late apoptotic cells.

Western blotting. H1395 cells were treated with 0, 25, 50, 100 or 200 nM triptolide at 37°C for 24 h. In another experiment, H1395 cells were treated with 200 nM triptolide, 10 μ M STO-609, 10 μ M Compound C or combinations at 37°C for 24 h. Total protein was extracted from cells using RIPA lysis buffer (Cell Signaling Technology, Inc.) and freshly PMSF (1 M; Sigma-Aldrich; Merck KGaA). Total protein was quantified using a DC protein assay kit (Bio-Rad Laboratories, Inc.) and 30 μ g protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane (EMD Millipore) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated at 4°C with primary antibodies (1:1,000) overnight. Following the primary antibody incubation, the membranes were washed three times with TBS-0.05% Tween-20 and incubated with a FITC-conjugated secondary antibody (1:10,000) at room temperature for 1.5 h. GAPDH or β -actin were used as the loading controls and for normalization. The membranes were then scanned using the Odyssey Infrared Imaging system (Li-Cor Biosciences). Image Lab version 5.0 software (Bio-Rad Laboratories, Inc.) was used for image acquisition and densitometric analysis.

Intracellular Ca^{2+} measurement. A total of 1×10^5 H1395 cells/ml were cultured and treated with 0, 50, 100 or 200 nM triptolide at 37°C for 24 h. Cells were washed with PBS three times and stained with 1 μ M Fluo-3 AM (cat no. S1056; Beyotime Institute of Biotechnology) for 30 min at 37°C in the dark. Fluo-3 AM is cleaved by intracellular esterases to form Fluo-3 after entering the cells, which emits green fluorescence upon binding to Ca^{2+} (35). Flow cytometric analysis was performed to analyze the intracellular Ca^{2+} concentration using a BD BDFACSAria™ III flow cytometer (BD Biosciences), an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The data was analyzed using FlowJo 7.6.1 software (FlowJo LLC).

Caspase-3/7 activity apoptosis assay. Caspase-3/7 activity was measured using an Apo-ONE® Homogeneous Caspase-3/7 assay kit, according to the manufacturer's protocol. Briefly, H1395 cells were seeded in 96-well plates at a density of 4×10^3 cells/well. The cells were treated with different concentrations (0, 50, 100, 150, 200 or 250 nM) of triptolide at 37°C and 5% CO_2 for 24 h. Caspase-3/7 assay loading solution was composed by adding 50 μ l caspase-3/7

substrate (Component A) to 10 ml assay buffer (Component B) and mixing well. Then, 100 μ l/well caspase-3/7 assay loading solution was added and incubated at room temperature for >1 h in the dark. The luminescence intensity was measured using a Multimode microplate reader (BioTek Instruments, Inc.) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Cell morphology detection. A total of 1×10^5 H1395 cells were treated with 200 nM triptolide, 10 μ M STO-609, 10 μ M Compound C or combinations at 37°C for 24 h and visualized using an inverted light microscope (Leica Microsystems GmbH; magnification, x100).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, Inc.) and SPSS 22.0 software (IBM Corp.). The data from three independent experiments are presented as the mean \pm SD, unless otherwise noted. Statistically significant differences were determined using a one-way ANOVA, followed by a Bonferroni's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Triptolide effectively inhibits the cell viability of NSCLC cells. The cytotoxicity of triptolide was determined using an MTT assay; briefly, the effects of triptolide on two NSCLC cell lines, H1395 and A549 were determined following the incubation of cells with a range of different concentrations of triptolide (0, 50, 100, 150 or 200 nM) for 24 h. The cell viability of both NSCLC cell lines was significantly inhibited by triptolide in a dose-dependent manner (Fig. 1A). The IC_{50} values of triptolide in A549 and H1395 cell lines at 24 h was determined to be 140.3 and 143.2 nM, respectively. In addition, the effect of triptolide in 16-HBE cells was investigated; it was identified that triptolide had the weakest inhibitory effect on 16-HBE cells, demonstrating an IC_{50} value of 286.4 nM (Fig. 1B). These results indicated that triptolide may exert a cytotoxic effect in both H1395 and A549 NSCLC cell lines.

Triptolide induces apoptosis in NSCLC cells. To determine whether triptolide induced apoptosis in NSCLC cells, H1395 cells were treated with the indicated concentrations (0, 50, 100, 150 or 200 nM) of triptolide at 37°C for 24 h and stained with Annexin V-FITC/PI. Annexin V-FITC-stained cells and double-stained cells were considered to be in the early and late stages of apoptosis, respectively. The results revealed that the percentage of apoptotic cells significantly increased in a dose-dependent manner following the treatment with triptolide compared with the untreated cells (Fig. 1C). These results indicated that triptolide may be a potent inducer of apoptosis.

The activation of PARP and caspase-3/7, which are the most commonly used indicators of apoptosis (36) was subsequently analyzed. The protein expression levels of p-AKT, p-AKT and cleaved PARP were detected using western blotting. As the triptolide concentration increased, the expression levels of p-AKT/AKT were increasingly downregulated compared

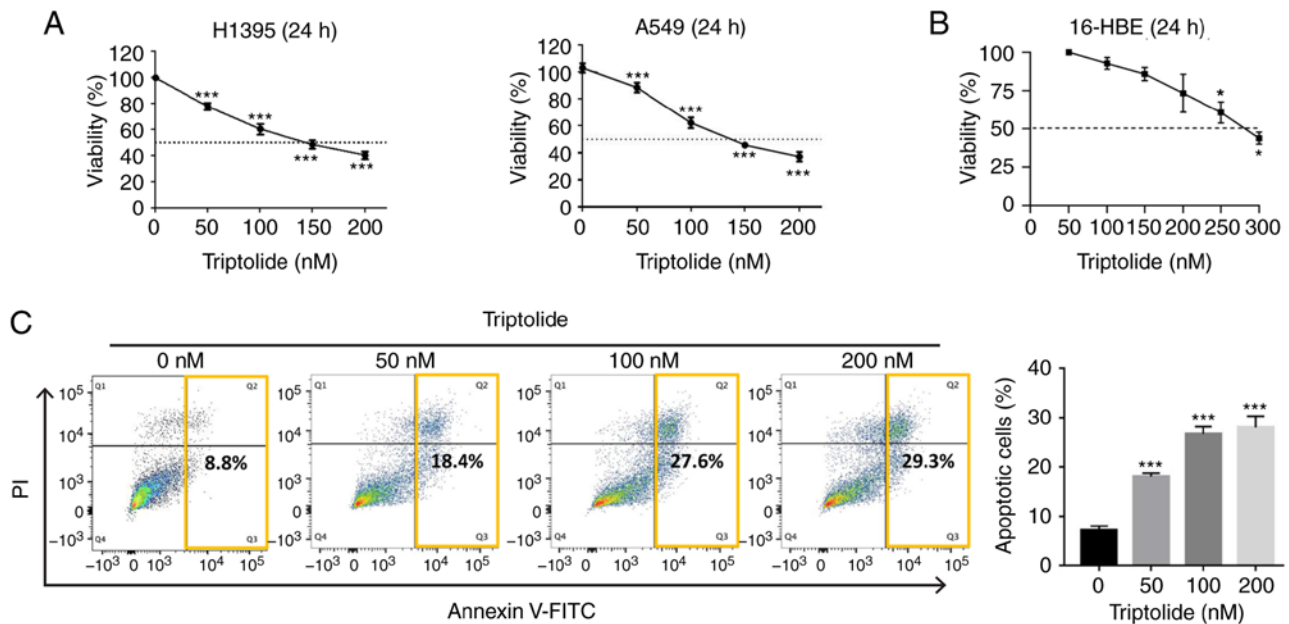


Figure 1. Triptolide effectively inhibits the cell viability of NSCLC cells. (A) MTT assay results demonstrated that triptolide decreased the cell viability of H1395 and A549 cells following 24 h of treatment. (B) MTT assay was used to analyze the inhibitory effect of 24-h triptolide treatment in 16-HBE cells. (C) Triptolide induced apoptosis in H1395 cells at different concentrations, as determined using flow cytometry. * $P < 0.05$, *** $P < 0.001$ vs. 0 nM triptolide. PI, propidium iodide.

with the untreated cells, while the expression levels of cleaved PARP protein were gradually upregulated and the expression levels of total AKT protein remained unchanged (Fig. 2A).

To determine the effect of triptolide on caspase-3/7 protein activity in the NSCLC cell line, H1395, the cells were treated with 0, 50, 100, 150 or 200 nM triptolide at 37°C for 24 h and the luminescence intensity was measured to determine the degree of caspase-3/7 protein activation. As the concentration of triptolide increased, the activity of caspase-3/7 protein significantly increased in a dose-dependent manner compared with the control cells (Fig. 2B).

Triptolide induces apoptosis through promoting Ca^{2+} influx. Since it has been previously demonstrated that triptolide is able to regulate Ca^{2+} signaling (37) it was investigated whether triptolide was involved in apoptosis induction by analyzing the concentration of Ca^{2+} in NSCLC cells using flow cytometry. H1395 cells were treated with 0, 50, 100 or 200 nM triptolide at 37°C for 24 h and the fluorescence intensity of Ca^{2+} in the cytoplasm was detected. As the concentration of triptolide increased, the cytoplasmic Ca^{2+} levels were observed to significantly increase in a dose-dependent manner (9.58, 26.1, 40.9 and 61.7% for 0, 50, 100 and 200 nM triptolide, respectively; Fig. 3C and D). These results suggested that triptolide may increase intracellular Ca^{2+} levels in NSCLC in a dose-dependent manner.

Subsequently, the association between triptolide-induced apoptosis and Ca^{2+} influx was investigated. BAPTA-AM, a selective calcium chelator, was used to reduce the activation of Ca^{2+} signaling. Compared with the triptolide treatment alone group, the cell viability was partially reversed in the BAPTA-AM treatment and BAPTA-AM+ triptolide groups (Fig. 2E). These results indicated that BAPTA-AM may effectively reduce the apoptosis induced by triptolide by decreasing intracellular Ca^{2+} concentrations.

Triptolide-induced apoptosis may require the activation of the CaMKK β /AMPK signaling pathway. As CaMKK β /AMPK signaling is one of the main signaling pathways downstream of Ca^{2+} signaling (38) the effect of triptolide on the activation of CaMKK β /AMPK signaling was investigated. Following the increases in triptolide concentration, the expression levels of p-AMPK/AMPK were significantly upregulated compared with the untreated cells in a dose-dependent manner (Fig. 3A). These results indicated that triptolide may promote the phosphorylation of AMPK. Next, the specific blockers of CaMKK β and AMPK, STO-609 (10 μ M) and Compound C (10 μ M), were used to inhibit the anticancer effect of triptolide (200 nM). Both STO-609 and compound C effectively blocked the decrease of p-AKT and the cleavage of PARP induced by triptolide (Fig. 3B). The cleavage of PARP is the ultimate characteristic of apoptosis (39). Thus, the above results indicated that triptolide may induce apoptosis through CaMKK β /AMPK signaling. In the caspase-3/7 activity assay, compared with the triptolide treatment group, the activation of caspase-3/7 was partially decreased in the co-treated groups (STO-609/Compound C + triptolide groups; Fig. 3C). Moreover, the effects of STO-609 and Compound C on cell viability were verified; compared with the triptolide treatment alone group, the cell viability was increased in the co-treated groups (STO-609/Compound C + triptolide groups; Fig. 3D). Thus, these two treatments partially reversed the effects of triptolide. The changes of cell morphology were consistent with the results of the viability assay (Fig. 4A).

Finally, the effect of STO-609 and Compound C on cell apoptosis was analyzed. Following treatment, cells were stained with Annexin V-FITC/PI. Both inhibitors in the co-treatment groups (STO-609/Compound C + triptolide) significantly decreased the apoptotic rate compared with triptolide treatment alone (Fig. 4B). These results indicated that STO-609

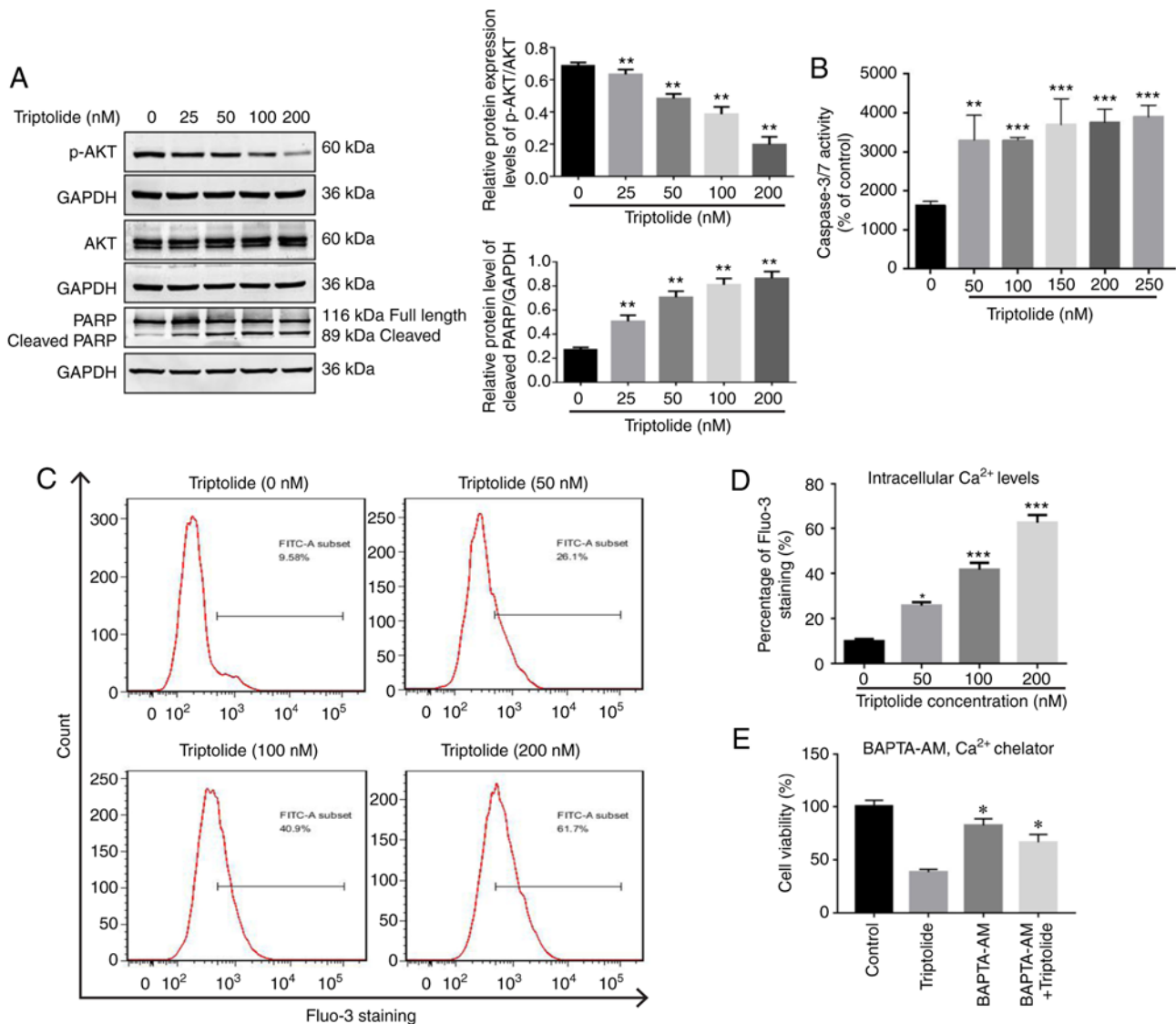


Figure 2. Triptolide induces apoptosis in NSCLC cells. (A) Following treatment with triptolide (0, 25, 50, 100 or 200 nM) for 24 h, the protein expression levels of AKT, p-AKT, PARP and cleaved PARP in H1395 cells were determined using western blotting. The phosphorylated and total proteins detected on the different membranes were from the same extract and used the same loading concentration/mass. ** $P < 0.01$ vs. 0 nM triptolide. (B) Activation level of caspase-3/7 protein in each group was detected. ** $P < 0.01$, *** $P < 0.001$ vs. 0 nM triptolide. (C) Levels of Ca^{2+} in H1395 cells were analyzed following the treatment with triptolide for 24 h using flow cytometry and Fluo-3 staining. (D) Quantification of the Ca^{2+} levels from part (C). * $P < 0.05$, *** $P < 0.001$ vs. 0 nM triptolide. (E) Compared with the triptolide treatment group, BAPTA-AM partially attenuated the triptolide-mediated effects on the cell viability of H1395 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Triptolide. p-, phosphorylated; PARP, poly[ADP-ribose] polymerase 1; Ca^{2+} , calcium.

and Compound C may significantly attenuate the anticancer effect of triptolide, which suggested that triptolide-induced apoptosis may require the activation of the CaMKK β /AMPK signaling pathway.

Overall, the findings of the present study suggested that triptolide may induce endoplasmic reticulum (ER) stress (11,40) which subsequently promotes Ca^{2+} to be released from the ER into the cytoplasm (11). The cytoplasmic free Ca^{2+} is then able to induce apoptosis by inhibiting mTOR via the CaMKK β -mediated activation of AMPK in H1395 cells (Fig. 5).

Discussion

In previous years, the anticancer activity of triptolide has become a research hotspot that has attracted increasing

attention from researchers; for example, within certain types of cancers such as pancreatic (41), breast (12) and lung cancer (10). The broad-spectrum anticancer effect of triptolide has been discovered to mainly promote the induction of apoptosis, and the inhibition of proliferation and metastasis through various molecules and signaling pathways (42,43). The results of the present study suggested that triptolide may be a potent inducer of apoptosis and exert a significant cytotoxic effect over NSCLC cell lines. However, although triptolide has a variety of pharmacological effects, its clinical application is greatly limited due to its toxicity (44). In view of its broad-spectrum anticancer activity against a variety of types of tumor, it is suggested that low doses of triptolide in combination with other drugs may exert an anticancer effect, while reducing its toxicity and side effects.

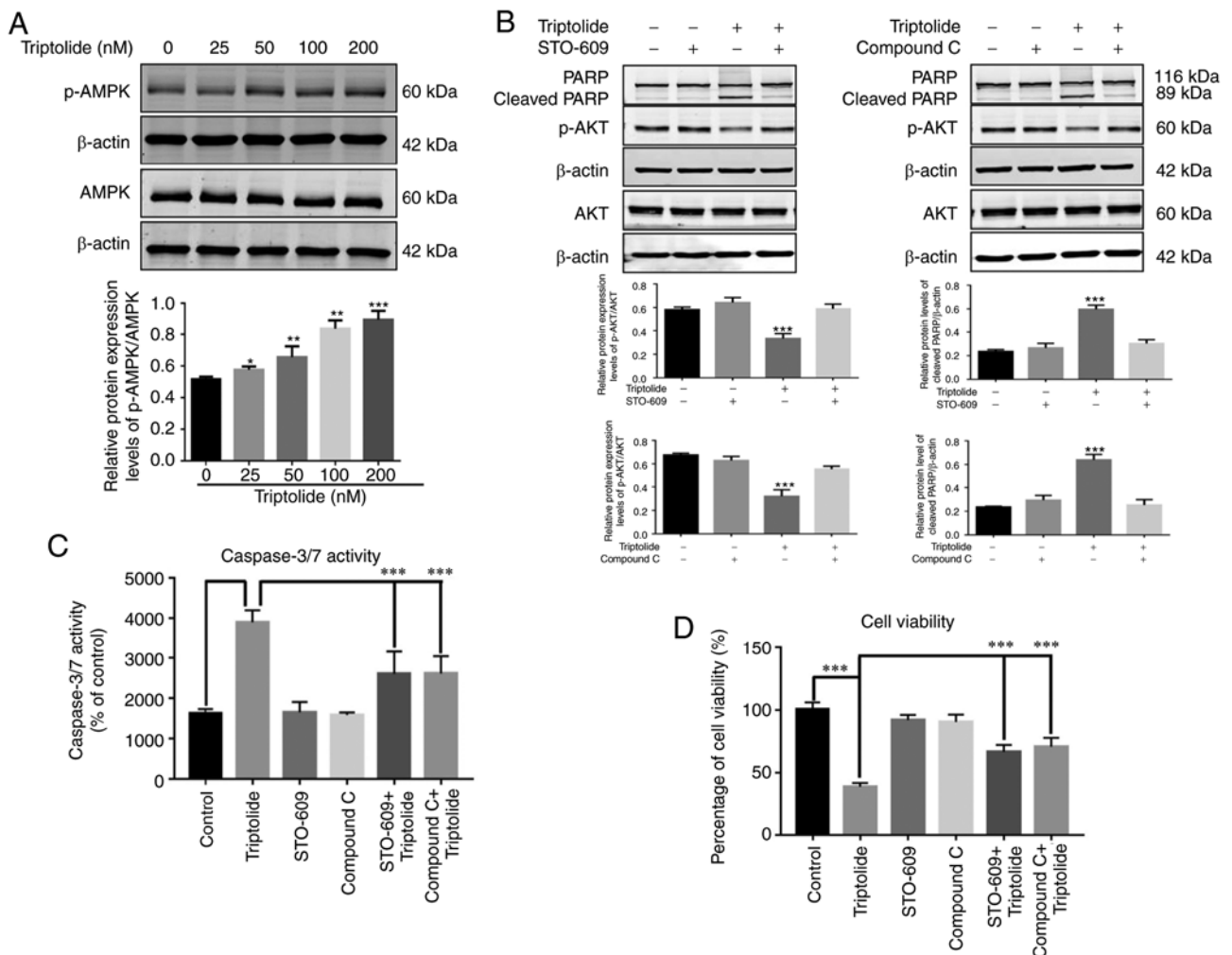


Figure 3. Triptolide induces apoptosis through promoting Ca^{2+} influx. (A) Following the treatment with triptolide (0, 25, 50, 100 or 200 nM) for 24 h, the protein expression levels of AMPK and p-AMPK in H1395 cells were determined by western blotting. The phosphorylated and total proteins detected on the different membranes were from the same extract and used the same loading concentration/mass. (B) Effects of STO-609 and Compound C on the activation of p-AKT and PARP were detected at western blotting following the treatment with 200 nM triptolide. The left panel demonstrates the western blot results for STO-609 and the right panel shows the western blot results for Compound C. The phosphorylated and total proteins detected on the different membranes were from the same extract and used the same loading concentration/mass. (C) STO-609 and Compound C partially attenuated the activation of caspase-3/7 induced by 200 nM triptolide in H1395 cells. (D) Compared with the triptolide treatment group, STO-609 and Compound C partially reversed the reduced cell viability induced by triptolide. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control/0 nM triptolide. p-, phosphorylated; PARP, poly[ADP-ribose] polymerase 1.

CaMKK β is a Ca^{2+} -dependent kinase, whose activity is mediated by intracellular Ca^{2+} signaling (26). In the present study, it was demonstrated that triptolide significantly increased Ca^{2+} levels in the cytoplasm, while the calcium chelator BAPTA-AM partially attenuated the cytotoxic effect of triptolide. Therefore, these findings suggested that triptolide may induce elevations in intracellular Ca^{2+} levels and activate CaMKK β .

AMPK, a heterotrimeric complex comprising a catalytic α subunit, and regulatory β and γ subunits, has been primarily studied as a major regulator of energy balance in both single cells and the whole organism (45). Activated AMPK has been discovered to enhance catabolism, inhibit anabolism, respond to the external and internal stimulation of cells and affect basic biological processes, such as cell growth, proliferation and apoptosis (28-30). Shao *et al.* (46) reported that the activation of the AMPK signaling pathway promoted growth inhibition and apoptosis in A549 lung cancer cells. The predominant upstream kinases of AMPK are liver kinase B1 (LKB1)

and CaMKK β (47). Previous studies have reported that in LKB1-deficient cells, AMPK was activated by the phosphorylation of the CaMKK β signaling pathway when Ca^{2+} levels were elevated (38). Other studies have identified that the LKB1 gene was mutated and/or functionally inactivated in ~30% of NSCLCs (48-50). Therefore, it was hypothesized that the Ca^{2+} -mediated CaMKK β /AMPK signaling pathway may serve an important role in the occurrence and development of lung cancer. In the present study, the expression levels of p-AMPK protein were upregulated in a dose-dependent manner following the treatment with triptolide, which suggested that the treatment with triptolide may activate the CaMKK β /AMPK signaling pathway. AMPK and mTOR are crucial molecules for the regulation of bioenergy metabolism and biosynthesis, and their negative regulatory relationship can promote apoptosis (51). Apoptosis is a major mechanism of cell death; thus, the induction of cell death may represent the most promising anticancer treatment (52). Activated AMPK inhibits downstream mTOR complex 1 activity, leading

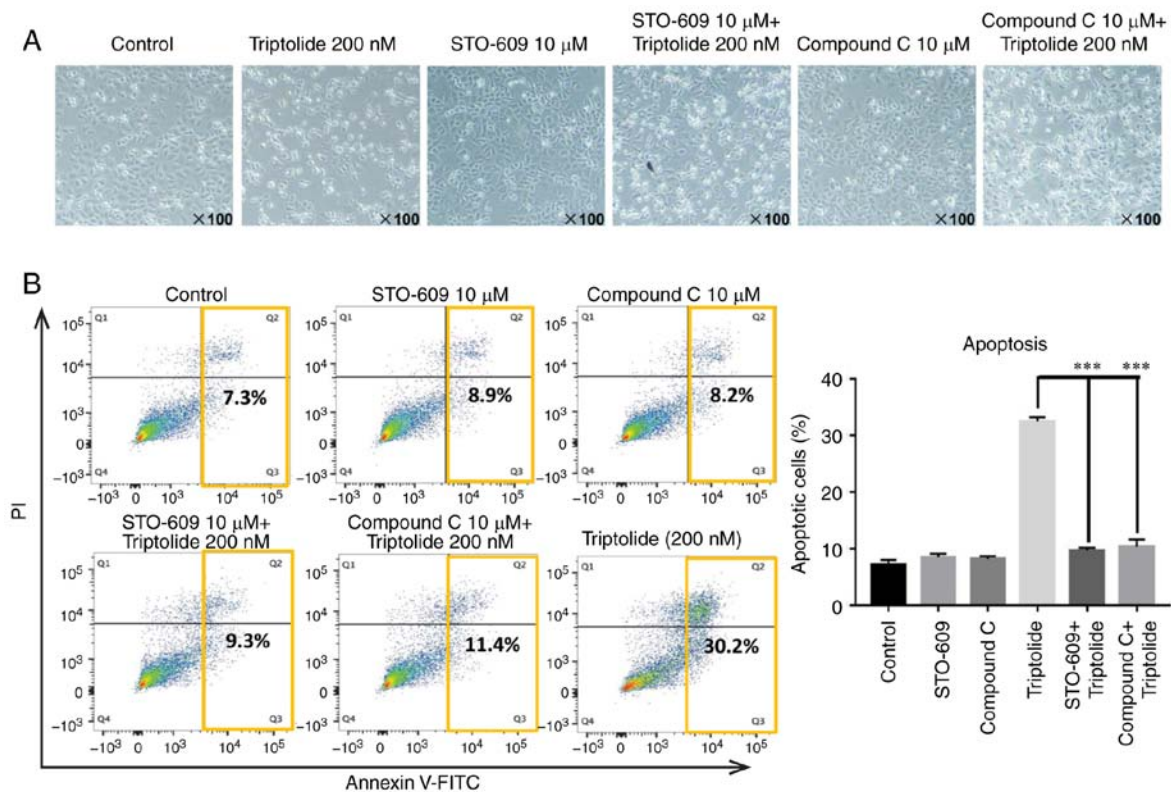


Figure 4. Triptolide-induced apoptosis may require the activation of the CaMKK β /AMPK signaling pathway. (A) Compared with the triptolide treatment group, STO-609 and Compound C significantly attenuated the changes of cell morphology induced by triptolide. Magnification, x100. (B) Flow cytometric analysis of the apoptotic effect of different treatments in H1395 cells. ***P<0.001. PI, propidium iodide.

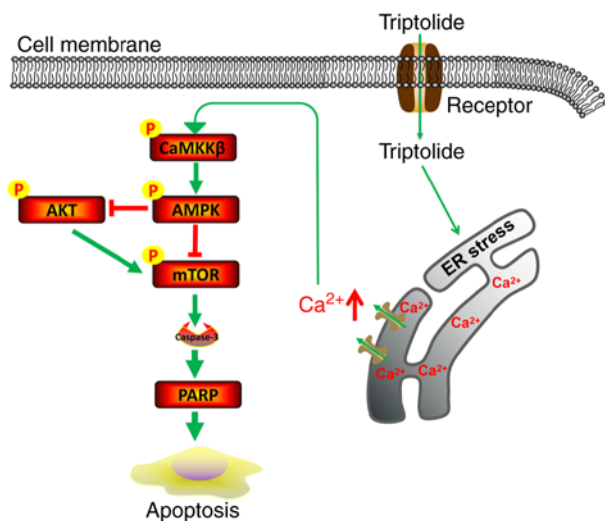


Figure 5. Schematic diagram of the anticancer mechanism of action of triptolide. Triptolide induces ER stress, which causes Ca^{2+} to be released from the ER into the cytoplasm. Cytoplasmic free Ca^{2+} induces apoptosis by inhibiting mTOR via the CaMKK β -mediated activation of AMPK in H1395 cells. PARP, poly[ADP-ribose] polymerase I; CaMKK β , calmodulin-dependent protein kinase kinase β ; AMPK, AMP-activated protein kinase; Ca^{2+} , calcium; P, phosphorylated; ER, endoplasmic reticulum.

to the inhibition of caspase-3 phosphorylation, hindering protein synthesis, arresting the cell cycle in the G0/G1 phase, arresting cell growth and proliferation and inducing apoptosis (53). In the present study, it was revealed that triptolide induced apoptosis in a dose-dependent manner. In addition,

it was also discovered that the activation of caspase-3/7 and cleaved-PARP proteins was significantly increased in NSCLC cells treated with triptolide. Thus, to further validate the critical role of the CaMKK β /AMPK signaling pathway in triptolide-induced apoptosis, triptolide-treated NSCLC cells were co-cultured with the CaMKK β inhibitor STO-609 or the AMPK inhibitor Compound C. Compared with the triptolide treatment alone, STO-609 and Compound C significantly attenuated triptolide-induced caspase-3/7 and cleaved PARP protein activation and increased the phosphorylation of AKT. In addition, STO-609 and Compound C partially reversed the cytotoxic and apoptotic effects induced by triptolide. Zhao *et al* (11) previously reported that triptolide promoted protective autophagy in prostate cancer by inducing CaMKK β /AMPK signaling activation. AMPK signaling is also an important signal for the activation of autophagy (54). In certain types of cancer, such as ovarian, breast and prostate cancer, following the induction of apoptosis by drugs, cells often end up undergoing autophagy, that is, autophagic death (55). In the future, one must investigate whether triptolide induces autophagy and apoptosis in lung cancer cells through CaMKK/AMPK signaling. This may be an effective strategy for cancer drug discovery. The present study indicated that triptolide may induce apoptosis through CaMKK β /AMPK signaling in lung cancer cells. Unfortunately, the current study neither determined the toxicological effects of triptolide *in vivo*, nor verified the mechanism of action of triptolide *in vivo*. Therefore, a complete explanation of its mechanism of action is lacking. In the future, the aim is to further investigate the mechanism of triptolide in NSCLC in depth.

In conclusion, the findings of the present study suggested that triptolide may promote the elevation of Ca²⁺ levels in the cytoplasm of NSCLC, induce apoptosis and inhibit cell viability through activating the CaMKK β /AMPK signaling pathway. These findings revealed a novel mechanism by which triptolide may induce apoptosis in NSCLC cells. Thus, triptolide may represent a promising drug for the treatment of patients with NSCLC in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TR, YJT and MWC conceived and designed the study; TR, HSW, YL, XQ performed the laboratory experiments; MFW and CC analyzed the data; TR wrote the draft of the manuscript; and MWC, YJT and MFW reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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