

# Triptolide induces apoptosis in human anaplastic thyroid carcinoma cells by a p53-independent but NF- $\kappa$ B-related mechanism

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Received June 11, 2009; Accepted August 5, 2009

DOI: 10.3892/or\_00000580

**Abstract.** Triptolide, a traditional anti-inflammatory and anti-immunodepressive agent, has been reported to exert anti-neoplastic activity on several human tumor cell lines. This study investigates the pro-apoptotic function and the functional mechanism of triptolide on anaplastic thyroid carcinoma (ATC) cells. Experiments presented here demonstrated that triptolide had dose-dependent effects on cell viability of human ATC cell line TA-K cells through inducing cell apoptosis. In the molecular level, triptolide did not successfully initiate p53 signaling pathway, but downregulated the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway. Our studies suggest that triptolide functions as an effective apoptotic inducer in a p53-independent, but NF- $\kappa$ B-dependent mechanism, thus providing a promising agent for tumor types with p53 mutation/deletion.

## Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most aggressive and lethal human cancers (1). Most patients with ATC have an extremely poor prognosis due to its rapid and pathological growth (2). This malignancy is unsuitable for radical resection and seems completely refractory to the non-surgical therapies such as radiation therapy and chemotherapy (3,4). Efforts to elucidate the molecular mechanisms related to the tumor's aberrant growth and drug resistance are urgently needed in order to develop innovative and effective treatments.

Suppression of apoptosis, together with deregulated cell proliferation, are critical events in tumor progression (5). Commitment of cells to apoptosis is governed largely by

protein-protein interactions between members of the Bcl-2 protein family, which is categorized into two groups: pro-apoptotic oncoproteins such as Bax, Bad and anti-apoptotic ones such as Bcl-2, Bcl-X<sub>L</sub> (6). Bax homodimers promote apoptosis, while Bax/Bcl-2 heterodimers suppress apoptosis, leading to the idea that the relative level of these two proteins determines the destiny of cells to live or to proceed to apoptosis. In the case of tumors, the anti-apoptotic proteins have been found to be overexpressed, causing aberrant proportion and abnormal cell survival (7). Thus, an obvious strategy for cancer therapy is to induce apoptosis through increasing the relative ratio of pro- and anti-apoptotic proteins in tumor cells.

Resistance to apoptosis is a key factor preventing response to chemotherapy drugs in thyroid cancer and many other cancers. Cancer cells have several survival mechanisms that help them elude cell death induced by chemotherapeutic drugs, among which, mutant p53 is of particular importance (8). In human tumors, the outcome of p53 reactivation is to induce apoptosis of abnormally proliferating cells, which largely depends on its downstream effector genes such as cyclin-dependent kinase inhibitor p21, pro-apoptotic protein Bax and so on (9). To restore p53 expression and its transcriptional function, or to develop a p53-independent and pro-apoptotic target becomes a promising modality for good response to chemotherapy agents.

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is active in most tumor types, and regulates a series of pivotal events in the tumor progress including cell survival, cell proliferation, angiogenesis and invasion (10). For cell survival, NF- $\kappa$ B mainly suppresses cell apoptosis by increasing the transcription of genes encoding anti-apoptotic proteins, for instance Bcl-2 and Bcl-X<sub>L</sub> (11). Thus, suppression of NF- $\kappa$ B pathway should be effective to induce apoptosis of tumor cells.

Triptolide is a small molecule and a natural product, which is extracted from the Chinese medicinal herb *Tripterygium wilfordii* Hook F (TWHF). Since the 1960s, triptolide has been used as anti-inflammatory and anti-immunity agents, especially in the treatment of rheumatoid arthritis and systemic lupus erythematosus (12). With increasing research, triptolide has been demonstrated to have anti-neoplastic activity mainly via inducing apoptosis in various cancer types (13-16). We analyzed the pro-apoptotic effect of triptolide on

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**Key words:** triptolide, apoptosis, anaplastic thyroid carcinoma, p53, nuclear factor  $\kappa$ B

human anaplastic thyroid carcinoma TA-K cells and explored the possible mechanism involved.

## Materials and methods

**Cell culture and drug treatment.** Human ATC cell line TA-K cells were maintained in William's medium E with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY). Cells were cultured in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C. Triptolide (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO to obtain 1 mM stock solution and then was added in medium at indicated concentrations for indicated time.

**MTT assay.** Cells were incubated with various concentrations of triptolide for indicated time. Then 20  $\mu$ l 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (Sigma, 5 mg ml<sup>-1</sup> in PBS) was added to induce the production of formazan crystals. MTT solution was aspirated off after 4 h and 100  $\mu$ l dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The optical density (OD) was determined at 570 nm using an EXL800 microimmunoanalyser (BioTek, Burlington, VT). The cell viability rate = OD experiment/ODcontrol  $\times$  100%.

**Hoechst 33258 staining and transmission electron microscopy.** Hoechst 33258 staining was performed according to the method suggested by the manufacturer. In brief, cells were fixed with 4% paraformaldehyde for 10 min and then stained with Hoechst 33258 (Sigma, 5  $\mu$ g/ml) in the dark. The chromosomal DNA morphology was observed by fluorescence microscopy with a 340 nm excitation filter. Transmission electron microscopy (TEM) was conducted as described (17).

**Western blot analysis.** Cells treated with triptolide for indicated time were scraped and then resuspended in protein extraction reagent. The cell lysate was centrifuged at 140,000 g for 10 min at 4°C and the supernatant was collected and stored at -70°C. The concentration of protein was determined using BCA protein assay kit according to the manufacturer's instructions. Equal amounts of protein (60  $\mu$ g) was separated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membrane, blocked with 5% skimmed milk in TBS for 2 h, reacted with antibodies against p53 (1:1000, Cell Signaling Technology, Beverly, MA), p21 (1:1000, Cell Signaling Technology), Bax (1:1000, Cell Signaling Technology), p65 (1:200, Santa Cruz, CA), Bcl-2 (1:1000, Cell Signaling Technology), Bcl-X<sub>L</sub> (1:1000, Cell Signaling Technology),  $\beta$ -actin (1:1000, Thermo Scientific IHC, Fremont, CA) or tubulin (1:5000, Sigma) overnight. After reaction with horseradish peroxidase-labeled secondary antibody, the immune complexes were visualized using the ECL-detection reagents following the manufacturer's procedure.

**Statistical analysis.** Data are presented as mean  $\pm$  standard deviation (SD) of three separate experiments. Student's paired t-test was used to compare two sets of data from the same subjects. A result with a P-value of <0.05 was considered statistically significant.

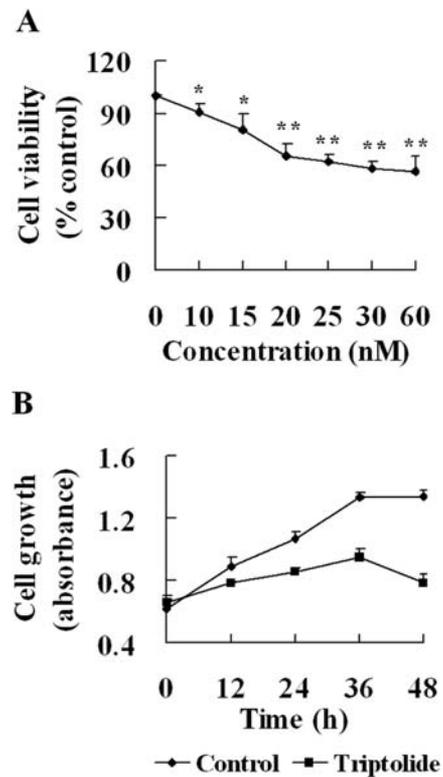


Figure 1. Effects of triptolide on the cell viability and cell growth of TA-K cells. (A) The concentration-dependent effect of triptolide on the cell viability. Cells were treated with various concentrations of triptolide and cell viability was determined using the MTT assay at 48 h. The cell viability rate = OD experiment/ODcontrol  $\times$  100%. \*P<0.05, \*\*P<0.01 compared with the control. (B) The effect of triptolide on the cell growth. Cells were treated with 30 nM triptolide for 12, 24, 36 and 48 h and cell growth was measured by MTT assay.

## Results

**Triptolide reduces cell viability dose-dependently and inhibits cell growth in TA-K cells.** Using MTT assay, we determine the cell viability and cell growth of TA-K cells. A dose-related inhibitory effect on cell viability was observed after exposure to triptolide in the 10-60 nM range for 48 h (Fig. 1A). From 20 nM on, this inhibitory effect of triptolide gradually leveled off. In addition, as shown in Fig. 1B, the cell growth of the 30 nM triptolide-treated group is significantly slower than control in the time range of 12 to 36 h. After 36 h, the amount of cells in the treated group was gradually reduced, indicating the existence of cell death induced by triptolide.

**Triptolide induces apoptosis of TA-K cells.** Cell death can contribute to either apoptosis or necrosis. To distinguish whether triptolide induces apoptosis or necrosis in TA-K cells, we stained the nuclear of TA-K cells cultured with 30 nM triptolide for 48 h with Hoechst 33258. Fig. 2A clearly shows that the nucleus of apoptotic cells display characteristics of morphological alterations such as condensed nuclei and cell shrinkage in triptolide-treated group. To further confirm this result, we fixed the untreated and treated cells and observed cell morphology using transmission electron microscopy. Typical apoptotic bodies confirmed apoptosis in cells treated with triptolide for 48 h (Fig. 2B).

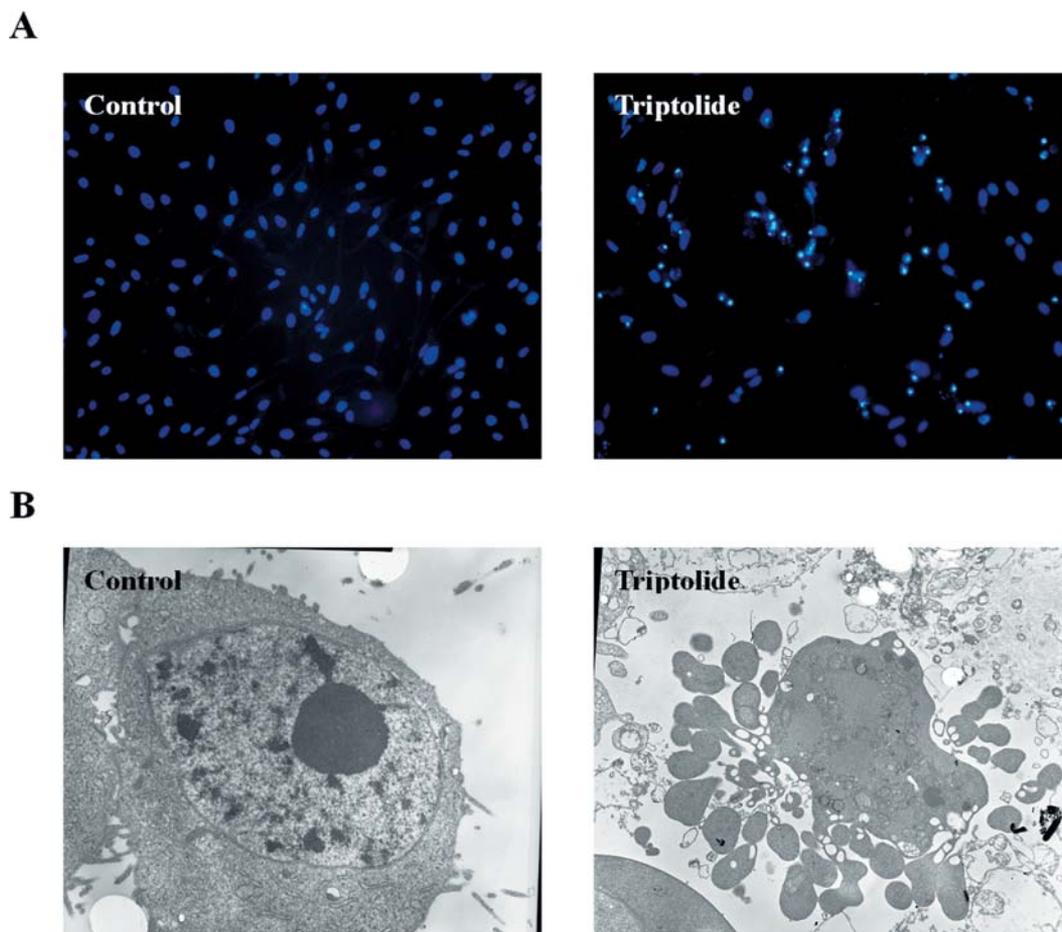


Figure 2. Hoechst 33258 staining (A) and transmission electron microscopy (B) of untreated and triptolide-treated TA-K cells. Cells were treated with 30 nM triptolide for 48 h.

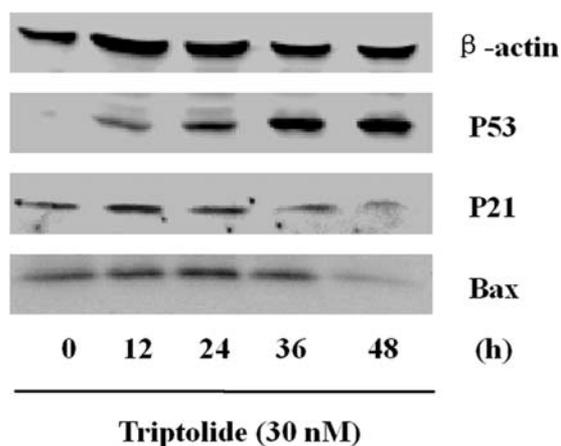


Figure 3. Effects of triptolide on the protein levels of p53, p21 and Bax in TA-K cells. Cells were treated with 30 nM triptolide for 12, 24, 36 and 48 h and then harvested for Western blot analysis.

*Triptolide increases p53 protein level, but not the protein level of p53 target genes p21 and Bax.* In response to the cellular stress such as DNA-damaging agents or radiation, p53 promotes apoptosis via regulating the transcription of cell cycle and apoptosis-related genes (9). To clarify whether

triptolide-induced apoptosis is mediated by p53 signaling pathway, the protein level of p53 and its regulated effectors such as p21 and Bax were examined. Fig. 3 shows that triptolide triggered a time-dependent increasing level of p53 protein, but no such tendency occurred in p21 and Bax protein levels. Instead, p21 and Bax proteins even slightly reduced at 48 h, accompanied with an obvious enhanced p53 protein expression. These data indicate that the p53 pathway was not successfully activated.

*Triptolide decreases the expression of p65 (the active subunit of NF- $\kappa$ B) and NF- $\kappa$ B downstream genes Bcl-2 and -X<sub>L</sub>.* Considering the predominant role of NF- $\kappa$ B pathway in cancer cell survival, we probed the alteration of NF- $\kappa$ B and its targeting apoptosis-associated genes in response to triptolide. Western blot analysis identified that the protein level of p65, the active subunit of NF- $\kappa$ B, was stable until 36 h and then markedly declined (Fig. 4A). The protein levels of Bcl-2 and Bcl-X<sub>L</sub> also began to decrease from 36 h, showing the same trend as p65 (Fig. 4A). Meanwhile, this effect of triptolide on p65, Bcl-2 and Bcl-X<sub>L</sub> protein expressions appears to be dose-dependent and was maximal at concentration of 30 nM (Fig. 4B). Our results support the notion that the NF- $\kappa$ B signal is definitely down-regulated in the process of cell apoptosis induced by triptolide.

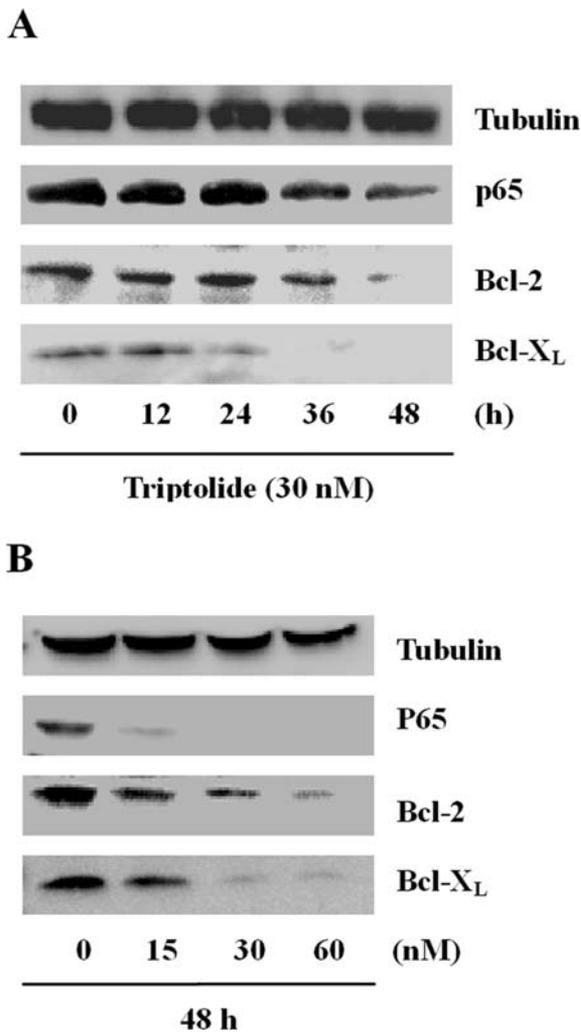


Figure 4. Effects of triptolide on the protein levels of p65, Bcl-2 and Bcl-X<sub>L</sub>. (A) The time-dependent effect of triptolide on the protein level of p65, Bcl-2 and Bcl-X<sub>L</sub>. Cells were treated with 30 nM triptolide for 12, 24, 36 and 48 h. (B) The concentration-dependent effect of triptolide on the protein levels of p65, Bcl-2 and Bcl-X<sub>L</sub>. Cells were treated with 0, 15, 30 and 60 nM triptolide for 48 h.

## Discussion

ATC is a highly aggressive malignant cancer and has a dismal prognosis because of its rapid growth and early invasion. Strategies for attacking uncontrolled tumor cells to induce apoptosis using small molecular drugs have emerged from an understanding of how tumor cells escape cell death. In our study, we show that triptolide, a natural small molecular product, significantly induces apoptosis of human anaplastic thyroid carcinoma cells. It is well known that p53 and NF- $\kappa$ B are common transcriptional factors which have the ability to modulate the expression of apoptosis relevant proteins (18). To clarify the mechanism by which triptolide induces cell apoptosis, we therefore probed its effects on two pathways and found that triptolide does not activate the p53 pathway, but indeed inhibits the NF- $\kappa$ B pathway. Taken together, triptolide promotes apoptosis of TA-K cells by down-regulating NF- $\kappa$ B and the targeting gene expression.

Wild-type p53 is functional to trigger cell apoptosis in the circumstance of cell stress (9). However, p53 gene is inactivated/unavailable in approximately half of tumors and p53 mutation/depletion results in the failure of most cancers to respond to radiotherapy and chemotherapy (19,20). There is evidence to support the notion that restoration of p53 function leads to the commitment of tumor cells to apoptosis (21). In this study, it is of interest that up-regulation of p53 downstream genes such as p21 and Bax is not seen in conjunction with up-regulation of p53 by triptolide in our anaplastic thyroid cells. This is in contrast to the up-regulation of these genes that generally accompanies p53-mediated apoptosis in response to DNA damaging drugs, but consistent with previous studies on triptolide-induced apoptosis (22). Increasing p53 protein accompanied with decreased cell cycle arrest/apoptosis regulators p21 and Bax expression suggest that apoptosis triggered by triptolide is not mediated by the p53 pathway. Additionally, it is tempting to speculate that triptolide recovers the protein level of p53, but not the transcriptional function or triptolide regulates p21 and Bax expression via a p53-independent mechanism, thus further investigation is required in additional experimental models.

The overactivation of NF- $\kappa$ B plays a major role in tumor pathogenesis, allowing neoplastic cells to survive and providing a promising target for the development of chemotherapy agents (11). Bcl-2 and Bcl-X<sub>L</sub> are NF- $\kappa$ B-induced members of the Bcl-2 family and exert their protecting effects on apoptosis through stabilization of the mitochondrion (6,23). In addition, basal level of Bcl-X<sub>L</sub> but not Bax and Bcl-2 shows a strong negative correlation with the sensitivity to cytotoxic agents (24), indicating Bcl-X<sub>L</sub>'s unique role in determining the survival of cancer cells exposed to chemotherapeutic agents. Accumulating research demonstrates that targeting NF- $\kappa$ B and its downstream genes Bcl-2 and Bcl-X<sub>L</sub> is effective in causing cytotoxicity and thus achieving the goal of tumor cell killing (25,26). Our results show that triptolide leads to reduction of NF- $\kappa$ B active unit p65 and anti-apoptotic protein Bcl-2 and Bcl-X<sub>L</sub> and the extent of reduction is stronger than Bax. These data reveal that the increased ratio of Bax:Bcl-2/-X<sub>L</sub> may have a role in the induction of apoptosis by triptolide, and Bcl-2 and Bcl-X<sub>L</sub> exert the principal efficiency.

In summary, our data reveal that triptolide induces human ATC cell apoptosis through downregulating p65 expression and anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> which are transcriptionally regulated by NF- $\kappa$ B, not through p53 signaling pathway. Considering the high mutation rate of p53, triptolide-induced apoptosis in a p53-independent pathway is beneficial to evade drug resistance, thus providing a promising new therapeutic agent for the treatment of ATC and even other tumors.

## Acknowledgements

This study was supported by Key program, National Natural Science Foundation of China (No. 30830111), National Natural Science Foundation of China (No. 330801408) and National Natural Science Foundation of Guangdong Province (No. 8451008901000297).

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