

## RESEARCH COMMUNICATION

# Triptolide Inhibits Proliferation and Induces Apoptosis of Human Melanoma A375 Cells

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### Abstract

Triptolide, a diterpenoid obtained from *Tripterygium wilfordii* Hook.f, has attracted interest for its anti-tumor activities against human tumor cell lines in recent years. This report focuses on anti-proliferative and pro-apoptotic activities in human melanoma A375 cells assessed by CCK8 assay, Hoechst 33258 staining and flow cytometry. In addition, triptolide-induced arrest in the S phase was also observed. Caspase assays showed the apoptosis induced by triptolide was caspase-dependent and probably through intrinsic apoptotic pathways. Furthermore, expression of NF- $\kappa$ B (p65) and its downstream factors such as Bcl-2, Bcl-X<sub>L</sub> was down-regulated. Taken together, the data indicate that triptolide inhibits A375 cells proliferation and induces apoptosis by a caspase-dependent pathway and through a NF- $\kappa$ B-mediated mechanism.

**Keywords:** Triptolide - melanoma A375 cells - apoptosis - caspase - NF- $\kappa$ B

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### Introduction

As the most aggressive form of skin cancer, cutaneous malignant melanoma (CMM) frequently affects young individuals, with a mean age of 50 years (Ferrari et al., 2008). Because of the resistance to current conventional therapeutic methods of CMM, approximately 13,000 deaths of CMM happen every year, but this has not been paralleled by the development of new therapeutic agents with an ideal effect (Finn et al., 2012). Therefore, efforts to develop new therapeutic approaches are in urgent need. Triptolide is a diterpenoid triepoxide and the principal active ingredient of *Tripterygium wilfordii* Hook. f. (Leigongteng) (He et al., 2009). It has been reported to have pharmacological and biochemical properties in the treatment of rheumatoid arthritis and several other autoimmune and inflammatory diseases, including immune complex nephritis and systemic lupus erythematosus (Lu et al., 2011). Besides, triptolide has been shown to have antitumor properties in a variety of human tumor cells via inhibiting cell proliferation and inducing apoptosis (Mujumdar et al., 2010; Meng et al., 2011; Wu et al., 2011). Despite the recognized potent antitumor activity of triptolide, our knowledge regarding its mechanisms of action is still limited. However, the effect of triptolide on human melanoma A375 cells has not yet been investigated. Thus, we managed to identify the mechanism of triptolide-induced apoptosis in human melanoma cells *in vitro*.

As cancer is a disease of deregulated cell proliferation and survival, deregulated cell cycle and inhibition of cell

apoptosis are critical for the progression of carcinogenesis (Evan and Vousden, 2001). Therefore, suppression of cell proliferation and induction of cell apoptosis are potent strategies for tumor intervention. Cell apoptosis can be divided into two pathways: the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway (Carter et al., 2006). When the final steps of consequential signaling cascades take place, apoptotic cells can display a series of morphological alterations such as condensed nuclei and cell shrinkage (Thornberry, 1998).

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is an important gene transcriptional factor involved in the mediation of a series of critical events in the tumor progress including cell cycle, proliferation, differentiation and invasion (Aggarwal, 2004). Moreover, it also modulates the expression of a plethora of survival factors that interfere with mitochondrial and death receptor-mediated apoptosis (Karin and Lin, 2002). NF- $\kappa$ B is thought to be constitutively activated in human melanoma cells, and its downregulation may sensitize cell to apoptosis by increasing the transcription of genes encoding anti-apoptotic proteins, for instance Bcl-2 and Bcl-X<sub>L</sub> (Karin, 2006).

In this study, we investigated the anti-proliferative and pro-apoptotic effects of triptolide on human melanoma cell line A375. We also postulated that triptolide mediated its effects through multiple mechanisms, including activating cell cycle arrest and caspase-dependent pathway, as well as blocking NF- $\kappa$ B activation.

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## Materials and Methods

### Cell culture and drug

Human CMM cell line A375 cells were provided by Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College. Cells were cultured in DMEM medium (Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) and 1% penicillin-streptomycin. All cultures were maintained in a tissue incubator at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Triptolide (purity> 99.0%, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China) was prepared in dimethyl sulfoxide (DMSO) to obtain 1 mM stock solution and then was added in medium at required concentrations for a certain period of time.

### Cell counting kit-8 (CCK8) assay

Cells grown in 96-well culture plates were treated as required. Next, cells in each well were incubated with 10 µl of CCK8 at 37 °C for 2 h. Then the optical density (OD) for each well was measured at 450 nm using a microplate reader (Bio-Rad Model 550, CA). The cell inhibitory rate was calculated according to the following equation:  
The cell inhibitory rate = [1-(OD experiment - OD blank)/(OD control - OD blank)] × 100%

### Flow cytometry analysis of cell cycle distribution

Approximately 1×10<sup>6</sup> A375 cells were plated in 25 cm<sup>2</sup> flasks with 10 ml medium for 24 h. The cells were then treated for a further 48 h with triptolide at various concentrations. Both the floating and adherent cells were collected together for the analysis. Cells were washed with PBS and centrifuged at 800 r.p.m., then resuspended in 100 µl of PBS. The resuspended cells were stained according to the protocol for the Coulter DNA-Prep Reagents Kit (Beckman Coulter, UK): 100 µl DNA-prep LPR (Lyse) was added to the tube and the cell suspension was vortexed for 7 s, and then 1 ml DNA-prep stain (propidium iodide . RNase) was added. While staining, cells were placed in dark for 30 min at room temperature. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) within 1 h of staining using Cellquest and ModFit software.

### Hoechst 33258 staining

The method was carried out according to the method suggested by the manufacturer. In brief, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min. Then cells were washed three times with PBS before stained with Hoechest 33258 (Sigma, 5 µg/ml) for 20 min at room temperature in dark. The chromosomal DNA morphology was observed by fluorescence microscopy (Olympus, Japan) with a 340 nm excitation filter and captured.

### Flow cytometry analysis of cell apoptosis

An immunofluorescent flow cytometry assay was performed according to the method suggested by the manufacturer. In brief, cells were harvested, washed twice with cold PBS and were resuspended in Binding Buffer

at a concentration of 1×10<sup>6</sup> cells/ml. After being stained with 5 µl Annexin V-FITC and 5 µl propidium iodide, cells were gently vortexed and incubated in dark for 15 min at room temperature. 400 µl binding buffer was added to each tube before cells were analyzed on a flow cytometer.

### Cell caspase activity assay

Cells were incubated in opaque 96-well plates at 1×10<sup>4</sup> cells/well in 100 µl volumes for 48 h with the final triptolide concentrations of 0, 10, 20 and 30 nM. A fluorogenic viability and cytotoxicity reagent were prepared by adding 10 µl GF-AFC to 2 ml Assay Buffer; 20 µl of the reagent was delivered to each well, mixed by orbital shaking, and returned to a 37 °C incubator for 30 min. The resulting fluorescence was measured using a fluorometer (Synergy 2, BioTek). Caspase-Glo® 3/7 and Caspase-Glo® 9 reagents (Promega, Madison, WI) were prepared as directed and added in equal volumes to the well 2 h later. For Caspase-Glo® 9, MG132 inhibitor was added for 15 min and incubated for 45 min at room temperature in the dark. Luminescence was measured by a Luminometer (Luminoskan Ascent, Thermo).

### Western blot analysis

After cells were treated with varying concentrations of triptolide for 48 h, total protein was extracted. The concentration of protein was determined by using Bradford protein assay kit according to the manufacturer's instructions. Equal amounts of total proteins were separated by SDS-PAGE and transferred to 0.22 µm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST (Tris-buffered solution, pH 7.6, 0.05% Tween-20) with 5% non-fat dry milk for 2 h. The membranes were further incubated with specific antibodies, including p65 (1:200), Bcl-2 (1:1000), Bcl-X<sub>L</sub> (1:1000) and β-actin (1:1000) overnight. After three washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and then developed in ECL-detection reagents following the manufacturer's procedure. Expression levels of the proteins were compared to the control based on the relative intensities of the bands.

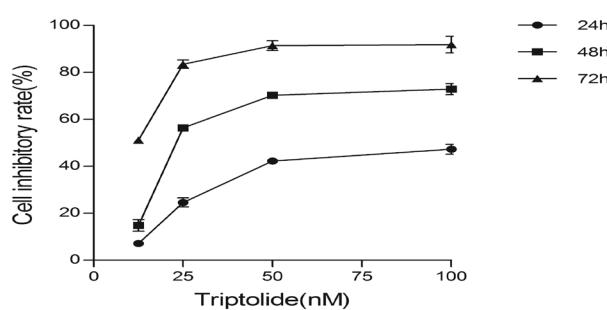
### Statistical analysis

Data were presented as mean ± standard deviation (SD) of three separate experiments. The Statistical Package for Social Sciences (SPSS) 13.0 software was used for Student's t-test. A P-value of < 0.05 was considered as statistically significant.

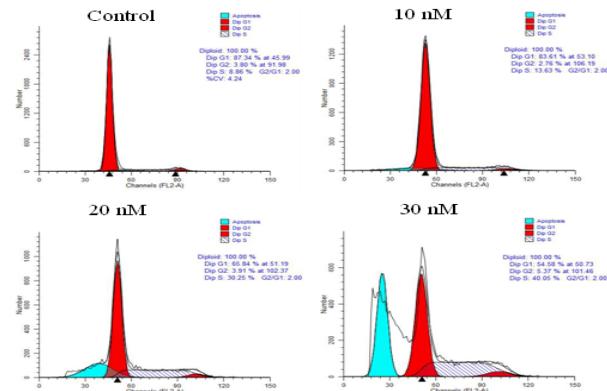
## Results

### Inhibition of proliferation by triptolide in A375 cells

To investigate the inhibitory effect of triptolide on growth and survival of melanoma cells, we treated human melanoma A375 cells with the triptolide concentrations of 12.5, 25, 50, 100 nM for 24, 48 or 72 h, respectively. As shown in Figure 1, triptolide brought about a dose-dependent inhibitory effect of cell proliferation with statistical significance (P < 0.05) on A375 cell line. Besides, with the prolonged reaction time, the inhibition



**Figure 1. Treatment with Triptolide Significantly Inhibited the Proliferation of A375 Cells.** A375 cells were treated with triptolide at indicated concentrations (12.5, 25, 50, 100 nM) for 24, 48 or 72 h. Cell viability was tested by CCK8 assay as described in the Methods. The absorption value of the untreated group at each time point was control. Data represent mean  $\pm$  SD of three separate experiments



**Figure 2. Triptolide Induced Cell Cycle Arrest at S Phase in A375 Cells.** A375 cells were treated with triptolide at indicated concentrations (0, 10, 20, 30 nM) for 48 h. The distribution of cell cycle was detected by flow cytometry. The figures were representative of three separate experiments

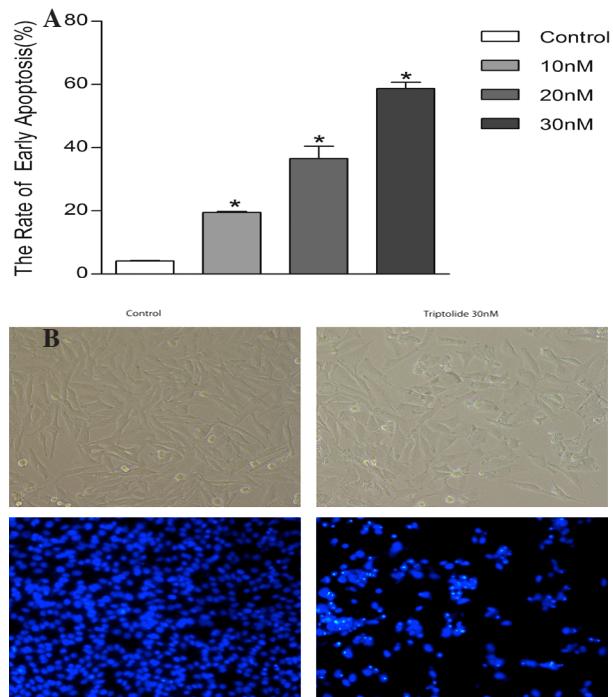
rate of cell proliferation presented a time-dependent trend. The IC<sub>50</sub> of triptolide on A375 cells at 24, 48, 72 h were 84.46, 33.00 and 8.53 nM.

#### *Triptolide induces cell cycle arrest at S phase in A375 cells*

In order to further determine whether triptolide-induced apoptosis is related to the alteration of cell cycle, we detected the dose-dependent effect of triptolide on cell cycle distribution. After A375 cells were treated with triptolide at 10, 20 and 30 nM for 48 h, the cell population of S phase was 13.2 $\pm$ 0.62%, 30.5 $\pm$ 0.39% and 40.8 $\pm$ 1.07%, respectively, which was statistically different from the control (10.1 $\pm$ 1.89%). Meanwhile, the fraction of cells in G<sub>0</sub>/G<sub>1</sub> phase decreased from 87.0 $\pm$ 0.56% (control), 83.5 $\pm$ 0.13%, 66.1 $\pm$ 0.33% to 54.6 $\pm$ 0.05% accordingly. Our data indicated that triptolide-induced apoptosis is cell cycle-dependent and could result in enhanced accumulation in S phase. The results presented in Figure 2 described a representative profile of cell cycle distribution in cells.

#### *Triptolide induces apoptosis in A375 cells*

Apoptosis induction by triptolide was observed in A375 cells using Hoechst 33258 staining and flow cytometry analysis. First, the degree of apoptosis is quantitatively expressed as the Annexin V-positive in



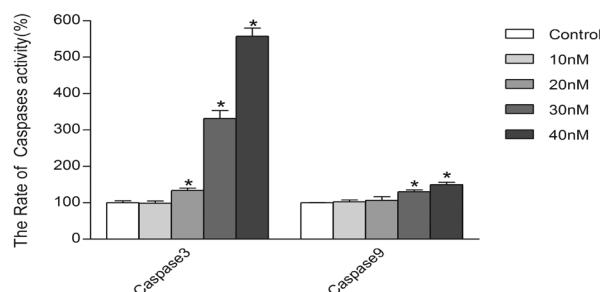
**Figure 3. Triptolide Treatment Induced Apoptosis in A375 Cells.** (A) Apoptosis of A375 cells treated by 0, 10, 20, 30 nM triptolide for 48 h were examined by Annexin V-FITC/PI double-staining assay. Data represented the mean  $\pm$  SD of at least three independent experiments. \*P < 0.05 vs. control. (B) Morphological changes and Hoechst 33258 staining of A375 were observed in the cells cultured with or without triptolide (30 nM) for 48 h (100 $\times$ )

the presence of triptolide. In the control group, cells without drug exposure presented 4.20% population in early apoptosis rate. However, the rate was 19.5% when cells were treated with 10 nM triptolide, and with the treatment of 20 and 30 nM triptolide, the percentage of apoptotic cells rose up to 36.6% and 58.7%, respectively. As shown in Figure 3A, the apoptosis rate of A375 cells raised with the concentration of triptolide increasing. This dose-dependent trend was in accordance with the effect of triptolide on cell cycle.

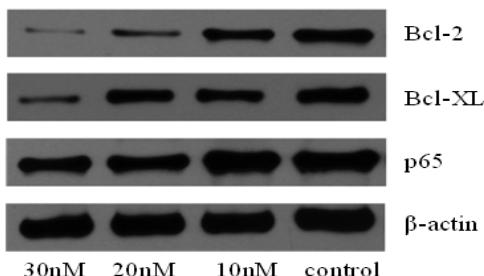
In addition, we observed the effects of triptolide on apoptosis in A375 cells by visualizing the morphological changes using light microscope and fluorescence microscope. Figure 3B clearly shows that the nuclei in the control cells are in regular shape, while the cells became shrunken and dark after treatment with 30 nM triptolide, and display characteristics of morphological alterations such as nuclear fragmentation, cellular disintegration, and even typical apoptotic bodies. And an increased percentage of apoptotic cells was seen compared with untreated cells in the mean time. Along with the concentration of triptolide going higher, a more salient trend was manifested.

#### *Effect of triptolide on caspase activation*

Next, we evaluated the activity of caspase-3 and caspase-9 to investigate the possible mechanisms underlying the pro-apoptotic action of triptolide in A375 cells. Caspase-3 and caspase-9 activity was measured at 48 h after treatment with 10, 20, 30 nM of triptolide. Increasing levels of caspase-3 and caspase-9 activity



**Figure 4. Effects of Triptolide on Caspase Activity of A375 Cells.** A375 cells were treated with triptolide of 0, 10, 20, 30 nM for 48 h. Caspase-3 and caspase-9 activity was detected by Caspase-Glo 3/7 and Caspase-Glo 9 Assay Kit. Data represented the mean  $\pm$  SD of at least three independent experiments. \*P < 0.05 vs. control



**Figure 5. Effects on the Protein Expression of p65 (the active subunit of NF- $\kappa$ B), Bcl-2 and Bcl-X<sub>L</sub>.** A375 cells were treated with various concentrations (10, 20, 30 nM) triptolide for 48 h, then cell lysates were subjected to SDS-PAGE followed by Western blotting.  $\beta$ -actin was used as a loading control

were observed at 48 h, as shown in Figure 4. Caspase-9 and caspase-3 play important roles in apoptosis through a mitochondrial-dependent pathway. Since caspase-3 and caspase-9 activity was leveled up by triptolide in a dose-dependent manner, it is suggested that triptolide may induce apoptosis through mitochondrial-mediated apoptotic pathway in a caspase-dependent way.

#### *Triptolide decreases p65 (the active subunit of NF- $\kappa$ B), Bcl-2 and Bcl-X<sub>L</sub> expression*

To determine the role of NF- $\kappa$ B in the process of triptolide-induced cell apoptosis in melanoma A375 cells, we probed the alteration of NF- $\kappa$ B and its targeting apoptosis-associated genes in response to triptolide. A375 cells were incubated with triptolide concentration of 10, 20, or 30 nM for 48 h. After triptolide treatment, the levels of p65, the active subunit of NF- $\kappa$ B, and its downstream genes were measured by Western blot analysis. The levels of p65 significantly declined after cells were exposed to triptolide. We also found that the expression of Bcl-2 and Bcl-X<sub>L</sub> decreased following treatment with increasing concentrations of triptolide, showing the similar trend to p65. The effect of triptolide on p65, Bcl-2 and Bcl-X<sub>L</sub> protein expressions appeared to be in a dose-dependent manner.

## Discussion

Currently there is no effective chemotherapy against cutaneous malignant melanoma despite only two FDA therapies, dacarbazine and high dose interleukin 2 (HD IL-2), were approved. However, neither of them increases median overall survival (Finn et al., 2012). Therefore, a search for novel biological therapeutic agents against this tumor is in urgent need. Recently, several investigators demonstrated that triptolide have anticancer activity in a variety of tumor cell lines (Liu et al., 2011; Wong et al., 2012). In our study, triptolide induced apoptosis in melanoma A375 cells. Our data suggested that the apoptotic effect of triptolide on human melanoma cells may result from the modulation of activities and/or the expression of multiple cellular factors, including caspases and NF- $\kappa$ B.

The results of cell viability assay demonstrated that cell growth inhibitory effect of triptolide was time- and concentration-dependent during a 0-100 nM range in A375 cells. The cell inhibitory rate was increased over gradually increasing time and concentration. Cell cycle and apoptosis detected by flow cytometry analysis showed that triptolide arrested the A375 cells at S phase and induced apoptosis in a dose-dependent manner. With the increasing dose of triptolide, the early apoptosis rate increased from 19.5 $\pm$ 0.32%, 36.6 $\pm$ 3.85% and 58.7 $\pm$ 2.03% to the exposure of 10, 20 and 30 nM of triptolide, respectively. It was found that triptolide drastically increased apoptosis in A375 cells. Meanwhile, marked morphological changes such as cellular shrinkage, chromatin condensation and marginalization, nuclear beading or even apoptotic bodies were clearly observed when cells were treated with 30 nM triptolide for 48 h. Based on these findings, caspase acitivity assay and Western blot analysis were performed to further investigate the potential pathway and regulatory mechanism in A375 cells treated by triptolide.

Caspases are essential proteins for the execution of cell death induced by apoptotic stimuli (Kim et al., 2011). The activity of proteolytic caspase-9 and downstream effector caspase-3 promoted subsequent cellular destruction. After treatment for 48 h, the elevation of two effector caspases (caspase-3 and caspase-9) was detected in A375. Both caspases were significantly activated after cells were treated with triptolide for 48 h, which was in accordance with the trend of apoptosis induced by triptolide with the same concentration. It is well-known that apoptosis can be divided into two pathways such as the mitochondrial and the death receptor pathways, which also called intrinsic and extrinsic caspase pathways (Banjerpongchai and Kongtawelert, 2011). In intrinsic caspase pathway, cytochrome c released from mitochondria activates procaspase-9 and results in release of mature caspase-9 with the help of the adapter protein Apaf-1. Caspase-9 then activates effector caspases such as caspase-3, which once activated, cleaves many substrate proteins and structural proteins to generate the characteristic apoptotic morphology (Kothakota et al., 1997). Our results indicate that triptolide induces apoptosis of A375 cells in a caspase-dependent way, of which intrinsic caspase pathway is probably involved.

NF- $\kappa$ B is a transcription factor considered “at the crossroads of life and death” by its function as a modulator of inflammation, angiogenesis, cell cycle, differentiation, adhesion, migration and survival (Karin M and Lin, 2002). Recently, researches have shown that triptolide is able to

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induce cell apoptosis by inhibiting NF- $\kappa$ B activation in some tumor cell lines (Geng et al., 2011; Zhu et al., 2009). To investigate whether this regulatory mechanism is also involved in the process of apoptosis of A375 cells, we evaluated the protein level of the p65 and some of their downregulating genes.

NF- $\kappa$ B can modulate the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion and suppression of apoptosis (Lugman and Pezzuto, 2010). Researches have shown that disregulation of NF- $\kappa$ B transcription machinery is one of the factors thought to be common early events in malignant tumor progression (Meng et al., 2012). Thus, it is becoming increasingly clear that compounds that block NF- $\kappa$ B activation could be highly useful for the treatment of cancers (Singh et al., 2011). Bcl-2 and Bcl-X<sub>L</sub> are members of the Bcl-2 family regulated by NF- $\kappa$ B. Bcl-2 is an integral membrane protein, whereas Bcl-X<sub>L</sub> only becomes tightly associated with the membrane after a cytotoxic signal by an induced conformational change. These Bcl-2 family proteins can prevent cytochrome c release, and hence activation of caspases (Liang, 2011). Both of them are highly expressed in many tumors and exert its protecting effects on apoptosis through stabilization of the mitochondrion (Burlacu, 2003). Our results showed that triptolide reduced p65, most abundant and active unit of NF- $\kappa$ B and its downstream gene, Bcl-2 and Bcl-X<sub>L</sub>. That is to say, triptolide is enabled to induce cell apoptosis by means of inhibiting NF- $\kappa$ B through downregulation of the Bcl-2 and Bcl-X<sub>L</sub> gene. Our study supported the opinion that the NF- $\kappa$ B pathways took part in the process of melanoma cell apoptosis induced by triptolide.

In conclusion, the present study demonstrated that human melanoma A375 cells are susceptible to triptolide-induced apoptosis and inhibition of proliferation. Our results indicate that triptolide exert anticancer activity against A375 cells by inducing S cell cycle rest, thereby causing apoptosis, which is mediated in part by inhibition of NF- $\kappa$ B expression and caspases activation. In view of these data, triptolide may be considered as a potential chemotherapy agent to deal with refractory human malignant melanoma. However, further studies are needed to explore more detailed mechanisms of triptolide on human melanoma cells.

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