

## Original Research Article

# Prophylactic effects of triptolide on colon cancer development in azoxymethane/dextran sulfate sodium-induced mouse model

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### Abstract

**Purpose:** To investigate effects of triptolide on colon cancer cell growth and its capacity to prevent tumor development in an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of colon cancer.

**Methods:** HCT116 cell viability and migration potential were assessed. Control and AOM/DSS-treated mice (with and without triptolide) were analyzed for tumor development. The animals were divided into five groups (n = 5). Normal control group was given saline, animals in the untreated control group received AOM and DSS while animals in the treatment groups received 10, 50 and 100 mg/kg doses of triptolide intraperitoneally alternately for 2 months after AOM and DSS injection.

**Results:** Triptolide enhanced nuclear material condensation, significantly ( $p < 0.05$ ) increased the levels of cleaved poly (ADP-ribose) polymerase, reduced the levels of pro-caspase-3 and pro-caspase-8 in HCT116 cells. Triptolide also significantly ( $p < 0.05$ ) decreased the expression of plkB $\alpha$ , activated peroxisome proliferator-activated receptor  $\gamma$ , and markedly reduced the activity of both metalloproteinase-2 and metalloproteinase-9. Treatment of AOM/DSS mice with triptolide significantly reduced adenocarcinoma multiplicity compared to the control group.

**Conclusion:** Triptolide administration suppresses growth of HCT116 cells and colon cancer development in mice by inhibiting inflammatory responses. Therefore, triptolide has potentials to be developed for colon cancer therapy.

**Keywords:** Cell viability, Condensation, Metalloproteinase, Apoptotic, Triptolide, Adenocarcinoma, Colon cancer

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## INTRODUCTION

Colon cancer is one of the most commonly diagnosed causes of premature mortality in people of developed countries [1-3]. Tumors present in the lining of the colon are the main cause of deaths associated with cancer in the United States [4]. Adoption of westernized dietary patterns has greatly increased the incidence of colon cancer in Asian people [5].

Cellular apoptosis maintains homeostasis in living organisms by destroying unwanted cells via both extrinsic and intrinsic pathways [6]. In the extrinsic apoptotic pathway, receptors on the cell surface activate caspase-8 [7], whereas in the intrinsic apoptotic pathway, various types of cellular stimuli change the expression levels of members of the Bcl-2 family, leading to the release of cytochrome c [7].

The process of cellular apoptosis is evident by changes in cellular morphology, such as altered membrane shape, cell shrinkage, lower mitochondrial membrane potential, nuclear material condensation, and fragmentation of double-stranded DNA [8,9]. In various types of carcinomas, such as of the colon, liver, and pancreas, the expression levels of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) increase [10]. Studies have shown that PPAR $\gamma$  is associated with the regulation of various processes associated with inflammation [11]. The inflammatory responses induced by nuclear factor- $\kappa$ B (NF- $\kappa$ B) are suppressed by PPAR $\gamma$  and its upstream activators. It has been reported that PPAR $\gamma$  activation, after exposure to punicic acid, inhibits the inflammation of intestinal tissues in mouse [12]. The expression of genes associated with the inhibition of inflammation is suppressed by PPAR $\gamma$  by the targeting of various factors such as NF- $\kappa$ B, p65, and p50 [13].

Natural products isolated during phytochemical investigations have demonstrated promising potential in inhibiting tumor development and progression by stimulating cellular apoptosis [14,15]. For example, triptolide, which was isolated during the phytochemical investigation of *Tripterygium wilfordii* Hook F, is a complex diterpenoid molecule [16-18]. Biological investigations of triptolide have revealed its potential as a suppressor of the immune system, an anticancer agent, and a contraceptive [16-18]. Triptolide treatment of cancer cells induces apoptosis via cytochrome *c* release, damages mitochondria, and suppresses antiapoptotic protein expression [19].

In the present study, the growth inhibitory effects of triptolide on colon cancer cells and on tumor development in an azoxymethane/dextran sulfate sodium (AOM/DSS) mouse model were investigated. The results demonstrate that triptolide treatment of colon cancer cells reduces viability, induces apoptosis, and suppresses tumor development by inhibiting inflammation.

## EXPERIMENTAL

### Chemicals

Triptolide obtained from Sigma-Aldrich Co. (St. Louis, MO) was dissolved in dimethylsulfoxide to prepare the stock solution. Various concentrations of triptolide were prepared at the time of use in experiment. A colonic carcinogen azoxymethane (AOM) was purchased from Sigma-Aldrich. Dextran sulfate sodium (DSS) with a molecular weight of 36, 000 - 50, 000 (cat.

no. 160110) was purchased from MP Biomedicals, LLC (Aurora, OH).

### Cell culture

HCT116 colon cancer cells were purchased from Shanghai Institute of Biochemistry and Cellular Biology Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) containing 10 % fetal bovine serum. Antibiotics such as penicillin (100 U/ml) and streptomycin (100 U/ml) were also added to the medium. Culture of cells was performed at 37 °C in an incubator with humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air.

### MTT assay

HCT116 and DLD-1 cells after culture in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) for 24 h were incubated with various doses of triptolide for 72 h. MTT (3-(4, 5-dimethylthiazol2-yl)-2, 5-diphenyltetrazolium bromide, 5 mg/ml) 20  $\mu$ l was put into each of the well of 96-well plates and then incubation was continued for 4 h more. Following decantation of medium 150  $\mu$ l of Write in full the first time with the abbreviation in brackets was put into each well of the plate. Absorbance was recorded for each well three times by multi-well reader at a wavelength of 490 nm to determine the cell viability.

### Flow cytometry

HCT116 cells ( $2 \times 10^5$  cells per ml) after incubation with various doses of triptolide were collected, washed with PBS three times and then treated with binding buffer. Then 5  $\mu$ l each of annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were added to cells and the cells were subjected to 15 min incubation under dark atmosphere at room temperature. FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used for the analysis of induction of apoptotic changes in the cells.

### Western blot analysis

HCT116 cells were subjected to incubation with various doses of triptolide for 72 h. The cells were then collected and lysed on treatment with lysis buffer followed by measurement of protein concentration in the supernatant (Pierce, Rockford, IL). The protein separation was achieved using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto the

nitrocellulose membrane and subsequently incubated with primary antibodies against NF- $\kappa$ B, PPAR $\gamma$ , pIKB $\alpha$ , iNOS, p50, p65, MMP-2, MMP-9 and  $\beta$ -actin (all Wuhan Boster Biological Technology, Ltd., Wuhan, China; 1: 1, 000). After incubation the membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Wuhan Boster Biological Technology, Ltd.; 1: 10, 000). The complexes formed by interaction of antigen and antibody were analyzed using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Pittsburgh, PA).

### Analysis of migration potential

For the measurement of HCT116 cell migration 24-well Boyden chamber (Corning, Tewksbury, MA) was used. Into the inserts of upper compartment of the Trans well chamber HCT116 cells were put at a density of  $5 \times 10^5$  cells per 100  $\mu$ l in the medium devoid of serum. The lower chamber contained 600  $\mu$ l RPMI 1640 supplemented with 10 % FBS. Migration of the cells was allowed for 72 h under conditions of 37 °C temperature and in an atmosphere of 5 % CO<sub>2</sub>. Gentle rubbing was performed to remove the cells that did not migrate using cotton swab. The migrated cells on the lower surface PBS washed and then fixed using methyl alcohol. The cells were then subjected to crystal violet staining followed by measurement of optical density three times independently at 570 nm wavelength.

### Animals

Institute of Cancer Research strain (ICR) of mice twenty five in number and seven week old were supplied by the Experimental Animal Centre of Zhejiang University (Hangzhou, China). In the animal facility center housing was done under diurnal lighting conditions with 12 h dark and light cycles and were provided free access to water and food. The temperature in the animal facility centre was maintained  $\sim 25$  °C and humidity adjusted to ( $55 \pm 10$  %). The animals were acclimatized to laboratory atmosphere for seven days before the start of the experiment.

### Ethical statement

The protocol for the study on animals was approved by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital of Academy of Military Medical Sciences (no. MMSA-102/2014). The study was performed according to the guidelines for Ethical Conduct in the Care and Use of Non-human Animals in

Research by the American Psychological Association [20].

### Animal studies

An aqueous solution of dextran sulfate sodium (1.5 % w/v) was prepared for inducing colitis in. The animals were divided into five groups of five mice each. The normal control group were given saline, animals in the untreated control group received AOM and DSS whereas animals in the treatment groups received 10, 50 and 100 mg/kg doses of triptolide intraperitoneally alternately for 2 months after AOM and DSS injection. After completion of treatment (2 months) the animals were sacrificed using sodium pentobarbital anesthesia to extract colon. The colon tissues were subjected to histopathological analysis after staining with hematoxylin and eosin (H&E).

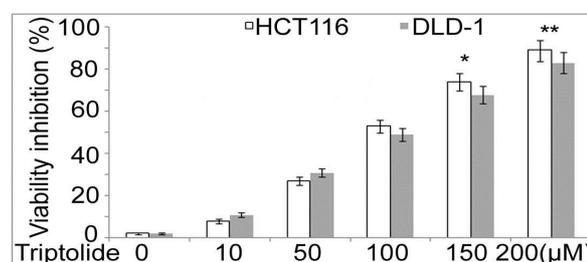
### Statistical analysis

The data obtained were processed using Statistical Package for Social Sciences for Windows, version 17.0 (SPSS, Inc, Chicago, IL, USA). Analysis of the data was performed using monofactorial analysis of variance. The data are presented as mean  $\pm$  standard deviation (SD).  $P < 0.05$  was taken as significant statistically.

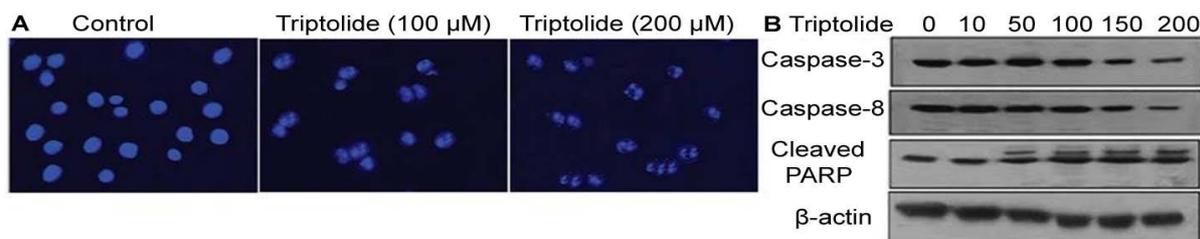
## RESULTS

### Viability of HCT116 cells is inhibited by triptolide

Triptolide decreased the viability of HCT116 and DLD-1 cells in a dose-dependent manner. At doses of 10, 50, 100, 150, and 200  $\mu$ M, triptolide decreased HCT116 cell viability by 8, 27, 53, 74, and 89 %, respectively, after 72 h of treatment (Figure 1). Additionally, the viability of DLD-1 cells was decreased by 11, 31, 49, 68, and 83 % after the addition of 10, 50, 100, 150, and 200  $\mu$ M triptolide, respectively (Figure 1).



**Figure 1:** HCT116 and DLD-1 cell viability is reduced by triptolide. Changes in cell viability were determined using an MTT assay and compared to control cultures. Measurements were made in triplicate. \* $p < 0.05$  and \*\* $p < 0.05$  compared with control cultures



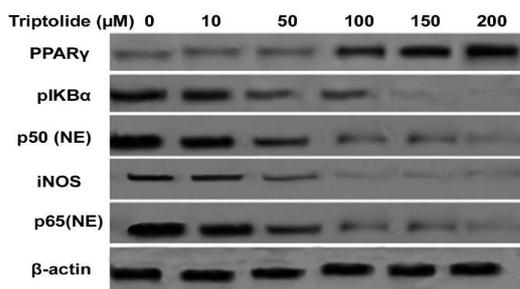
**Figure 2:** Triptolide induces apoptosis in HCT116 cells. (A) Cells were analyzed at a magnification of  $\times 320$  using a Hoechst 33342 stain and fluorescent microscope. (B) Western blot assay results show the expression levels of cleaved PARP, pro-caspase-3, and pro-caspase-8.  $\beta$ -actin was used as a loading control

### Triptolide stimulates apoptosis in HCT116 cells

Triptolide induced apoptotic changes in HCT116 cells. Increasing the triptolide dose from 10 to 200  $\mu\text{M}$  increased the population of HCT116 cells that underwent nuclear material condensation (from 6 to 72 %) after 72 h of treatment (Figure 2A). Western blot analysis showed a significant increase in the level of cleaved PARP and a reduction in the expression levels of pro-caspase-3 and pro-caspase-8 in HCT116 cells after the addition of triptolide (Figure 2B).

### Triptolide reduces NF- $\kappa\text{B}$ activity in HCT116 cells

The expression levels of NF- $\kappa\text{B}$  were analyzed in HCT116 cells by western blot analysis. After 72 h incubation with 200  $\mu\text{M}$  triptolide, the expression of pI $\kappa\text{B}\alpha$  was significantly decreased, and PPAR $\gamma$  activity was increased (Figure 3). No significant increase was observed in the activity of PPAR $\gamma$  at either the 10- or 50- $\mu\text{M}$  doses of triptolide. The levels of pI $\kappa\text{B}\alpha$ , inducible nitric oxide synthetase (iNOS), p50, and p65 were also decreased after treatment with triptolide.



**Figure 3:** Triptolide treatment of HCT116 cells activates PPAR $\gamma$  and downregulates pI $\kappa\text{B}\alpha$ . Levels of PPAR $\gamma$ , pI $\kappa\text{B}\alpha$ , iNOS, p50, and p65 were measured by western blot analysis.  $\beta$ -actin was used as a loading control

### Migration of HCT116 cells is reduced by triptolide

Triptolide reduced the migratory potential of HCT

116 cells after 72 h (Figure 4A). Compared to control cell cultures, the migratory potential of HCT116 cells was reduced significantly after a 72-h incubation with 100  $\mu\text{M}$  triptolide. In addition, HCT116 cells treated with 100  $\mu\text{M}$  triptolide for 72 h showed a marked reduction in the activity of both metalloproteinase-2 and metalloproteinase-9 (Figure 4B).

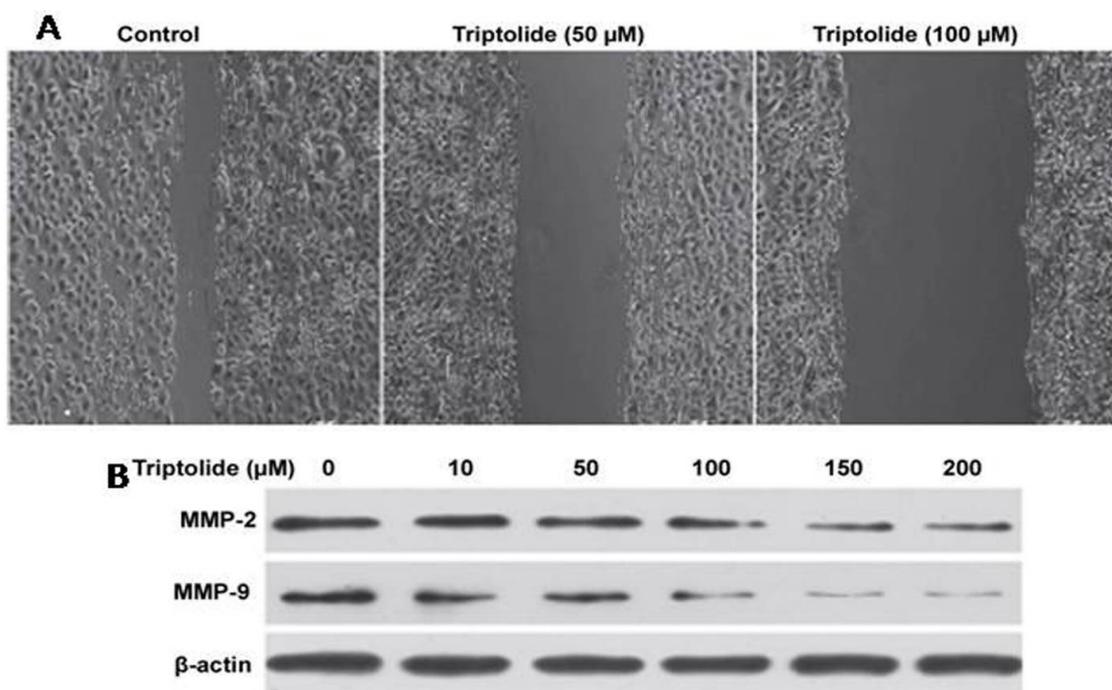
### Tumor growth is inhibited by triptolide in an AOM/DSS mouse model

Untreated control AOM/DSS mice showed markedly high tissue inflammation, as shown in hematoxylin- and eosin-stained sections. On the other hand, treatment of AOM/DSS mice with 100  $\mu\text{M}$  triptolide significantly prevented tissue inflammation (Figure 5). The incidence of tumors in the untreated control group was significantly higher than in the triptolide-treated groups. Adenocarcinoma multiplicity in the untreated control group was  $3.35 \pm 0.98$ , which was reduced to  $2.16 \pm 0.56$ ,  $1.87 \pm 0.46$ , and  $0.43 \pm 0.23$ , respectively, in mice treated with 10, 50, and 100 mg/kg triptolide.

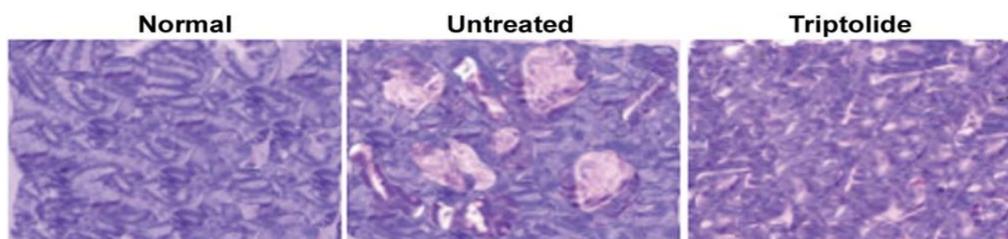
## DISCUSSION

The current study demonstrates the inhibitory effects of triptolide on colon cancer cell growth and on tumor growth in an AOM/DSS mice model. Triptolide reduced the viability of HCT116 cells via apoptotic cell death and prevented tumor growth in mice. Cell death can occur via an apoptotic or non-apoptotic pathway. Apoptosis, or programmed cell death, maintains homeostasis via either an intrinsic or extrinsic pathway [21].

Our study revealed that triptolide reduced HCT116 cell viability through an apoptotic pathway, which was evident by nuclear material condensation. In addition, triptolide promoted the expression of cleaved PARP and decreased the expression of pro-caspase-3 and pro-caspase-8 in HCT116 cells. It is known that agonists of PPAR $\gamma$  inhibit colorectal tumor progression via the upregulation of genes associated with tumor suppression [22].



**Figure 4:** Triptolide reduces migratory potential of HCT116 cells. (A) Cell cultures were scratched with a pipette tip and incubated for 72 h with either triptolide or solvent (control). The movement of cells into the wounded area was monitored, and photographs were taken. (B) Various doses of triptolide were then added, and the cells were incubated for 72 h prior to the analysis of metalloproteinase-2 and metalloproteinase-9 activity by zymography



**Figure 5:** Triptolide treatment of AOM/DSS mice prevents tumor growth. Mice were treated with 10, 50, and 100  $\mu\text{M}$  triptolide for 2 months and then sacrificed for analysis of tissue inflammation and tumor incidence

Such agonists are thus considered to be of great significance for the development of effective chemotherapeutic strategies for colon cancer. Active PPAR $\gamma$  inhibits cellular inflammatory processes by targeting gene transcription. Expression of various factors, such as iNOS, cyclooxygenase-2, etc., is associated with the upregulation of inflammatory processes that are regulated by NF- $\kappa$ B [23]. Colon cancer patients are reported to have high levels of active NF- $\kappa$ B, which stimulate tumor progression [24]. Results from the present study show that triptolide enhances PPAR $\gamma$  levels and reduces iNOS levels in HCT116 cells. Additionally, metalloproteinases have been shown to play important roles in promoting tumor migration and invasive potential [25].

In HCT116 cells, triptolide increases the expression levels of molecules that promote tumor migration and invasion. The levels of both metalloproteinase-2 and metalloproteinase-9

were markedly increased after incubation of HCT116 cells with triptolide. A previous study demonstrated that active PPAR $\gamma$  caused the inhibition of alimentary canal inflammation; thus, its expression is of significance for the treatment of colon cancer [26]. Our study demonstrated that triptolide treatment of AOM/DSS mice significantly prevented colon tissue inflammation. Moreover, tumor incidence was significantly reduced in these mice after a 2-month treatment with triptolide.

## CONCLUSION

The findings of this study show that triptolide reduces the viability of colon cancer cells via the induction of apoptosis and prevents tumor metastasis *in vivo*. Therefore, triptolide should be further evaluated as an effective chemotherapeutic agent for colon cancer.

## DECLARATIONS

### Acknowledgement

Financial assistance from Department of Oncology, Xuzhou Central Hospital, Xuzhou, China is acknowledged.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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