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# Cryptotanshinone induces melanoma cancer cells apoptosis *via* ROS-mitochondrial apoptotic pathway and impairs cell migration and invasion

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ABSTRACT

Melanoma is the most serious type of skin cancer because it is highly frequency of drug resistance and can spread earlier and more quickly than other skin cancers. The objective of this research was to investigate the anticancer effects of cryptotanshinone on human melanoma cells in vitro, and explored its mechanisms of action. Our results have shown that cryptotanshinone could inhibit cell proliferation in human melanoma cell lines A2058, A375, and A875 in a dose- and time-dependent manner. In addition, flow cytometry assay showed that cryptotanshinone inhibited the proliferation of human melanoma cell line A375 by blocking cell cycle progression in G2/M phase and inducing apoptosis in a concentration-dependent manner. Moreover, western blot analysis indicated that the occurrence of its apoptosis was associated with upregulation of cleaved caspases-3 and pro-apoptotic protein Bax while downregulation of anti-apoptotic protein Bcl-2. Meanwhile, cryptotanshinone could decrease the levels of reactive oxygen species (ROS). Furthermore, cryptotanshinone also blocked A375 cell migration and invasion in vitro which was associated with the downregulation with MMP-9. Taken together, these results suggested that cryptotanshinone might be a potential drug in human melanoma treatment by inhibiting proliferation, inducing apoptosis *via* ROS-mitochondrial apoptotic pathway and blocking cell migration and invasion.



## 1. Introduction

The most serious form of skin cancers is melanoma, for which its incidence rate continues to increase worldwide over the past several decades [1]. It is estimated that 76,380 people will be diagnosed with melanoma, and about 10,000 people will die from this disease in the United States alone in 2016 [2]. Although significant progress has been made in melanoma detection and treatment, high-metastatic potential and a notoriously high resistance to conventional therapies have result in poor prognosis [3,4]. Thus, new candidates against melanoma that exert potential anti-tumor activity and exhibit low toxicity are urgently needed.

Drug development is an expensive, lengthy and incremental process, so finding new use(s) for existing drugs is more economical and much faster than a new drug [5,6]. Moreover, increasing evidences demonstrated that natural compounds present in the in the human diet or as supplements could alter the natural history of cancer [7,8]. Meanwhile, there has been growing interest in the role of nutrition in melanoma chemoprevention [9]. Cryptotanshinone (Fig. 1) is one of the major active

*Abbreviations:* MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; DMSO, Dimethyl sulfoxide; PI, Propidium iodide; MMP-9, Matrix metallopeptidase-9; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; FBS, Fetal bovine serum; FCM, Fow cytometry; PBS, Phosphate-buffered saline; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamid gel electrophoresis; PVDF, Polyvinylidene difluoride; ROS, reactive oxygen species.

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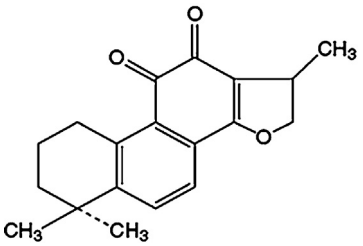


**Fig. 1.** The chemical structure of cryptotanshinone.

components isolated from *Salvia miltiorrhiza Bunge* (*Danshen*) [10], which is widely used in traditional Chinese medicine for the treatment of many diseases, such as cardiovascular diseases, inflammatory, Alzheimer's disease and circulation problems [11–13]. The pharmacokinetic profiles and therapeutic potential of cryptotanshinone have been studied particularly [14,15], and cryptotanshinone also showed no obvious adverse effects. Therefore, more and more researchers pay attention to the functions of cryptotanshinone in recent years. Recent studies have also shown that cryptotanshinone is a potential anticancer agent, and could inhibit cell proliferation in variety of cancer cell lines, including prostate, melanoma, chronic myeloid leukemia, breast cancer cells and so on [16–20]. However, the precise antitumor mechanism of cryptotanshinone on malignant melanoma has not been elucidated.

Thus, this study was aimed to evaluate the activities of cryptotanshinone in melanoma in vitro. Our results provided that cryptotanshinone could inhibit proliferation, induce cell apoptosis and suppress cell migration in human melanoma cell line. These data suggested that cryptotanshinone might be developed as a novel approach to treat melanoma.

## 2. Materials and methods

### 2.1. Materials

Cryptotanshinone was purchased from (Xiyashiji Chemical Co., LTD, ChengDu, SiChuan, China). Purity (98%) was measured by high-performance liquid chromatography (HPLC) analysis. 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI), 2′7′-dichlorofluorescein diacetate (DCFH-DA) and Triton X-100 were purchased from Sigma (St Louis, MO). Hoechst 33358 was obtained from Beyotime (Beijing, China). The Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (San Diego, CA). The antibodies against Cleaved caspase-3, Bcl-2, Bax, MMP-9 and GAPDH were acquired from Cell Signaling Technology Company (Beverly, MA). For in vitro studies, cryptotanshinone was prepared in DMSO at a stock concentration of 20 mM and stored at −20 °C, then was diluted to appropriate concentrations with the relevant medium at final DMSO concentration of 0.1% (V/V) when used.

### 2.2. Cell culture

Human melanoma A2058, A375 and A875 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were propagated in DMEM or RPIM 1640 media containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, Auckland, N.Z.) and 1% antibiotics (penicillin and streptomycin). Cells were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$.

### 2.3. Cell proliferation assay

The MTT assay was used to determine the respective cytotoxic effects of cryptotanshinone on A2058, A375 and A875 cell lines. Cells ($3–5 \times 10^3$) were seeded in 96-well culture plates, and treated with various concentrations of cryptotanshinone or vehicle control (0.1% DMSO) after cultured for 24 h. After 24, 48 or 72 h of treatment, 20 μL of a 5 mg/mL MTT solution was added to each well respectively and incubation for another 2–4 h at 37 °C. The medium was subsequently discarded, and the formazan crystal formed by living cells was dissolved with 150 μL of DMSO. After 15 min, the absorbance was read at 570 nm by a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA) [21]. Each experiment was replicated at least 3 times.

### 2.4. Colony formation assay

Briefly, 400–1000 cells were seeded in 6-well culture plates, and treated with various concentrations of cryptotanshinone (0–0.63 μM) after 24 h. Then, the cells were incubated once every 3 days for additional 13 days. Finally, the cells were washed by phosphate-buffered saline (PBS), then the colonies were fixed with methanol and stained with a 0.5% crystal violet solution, and the colonies (>50 cells) were counted under microscope.

### 2.5. Morphological analysis by Hoechst staining

To detect the apoptosis induction effects of cryptotanshinone, we analyzed the morphological changes associated with apoptosis by Hoechst 33358 staining. After cryptotanshinone treatment with various concentrations for 24 h, cells were washed with PBS twice and fixed with paraformaldehyde for 15 min, then stained with Hoechst 33358 solutions (5 μg/mL). The nuclear morphology of cells was observed under a fluorescence microscopy (Zeiss, Axiovert 200, Germany).

### 2.6. Cell cycle and apoptotic assays by flow cytometry (FCM)

The cycle distribution was analyzed by flow cytometry (FCM) after staining with propidium iodide (PI) solution. Briefly, A375 cells were treated with cryptotanshinone for 48 h, and fixed with 75% ethanol. Next, the cells were incubated with 500 μL of a solution containing 50 μg/mL PI and 0.1% Triton X-100 in the dark and analyzed by FCM (BD Biosciences). To further analyze the apoptosis induction effects of cryptotanshinone, the cell apoptosis was detected as described previously with slight modifications [22]. After treatment with cryptotanshinone for 48 h as described above, both attached cells and floating cells were harvested and washed with cold PBS twice. Harvested cells were stained with PI staining or Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions by FCM. Finally, the data was analyzed by FlowJo solfware.

### 2.7. Scratch-healing migration assay

Scratch-healing migration assay was performed as described previously [21]. When A375 cells were grew to 80% confluence, cell monolayers were wounded by a sterile 0.2 mL pipette tips, and fresh medium containing different concentrations of cryptotanshinone were added. After 24 h incubation, cells were fixed and photographed. Images were acquired with a microscope (Zeiss, Axiovert 200, Germany). The percentage inhibition rate of migrated cells was expressed compared with untreated cells [21].