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EXPERIMENTAL STUDY

Diterpenoid Tanshinones, the extract from Danshen (*Radix Salviae Miltiorrhizae*) induced apoptosis in nine human cancer cell lines

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Abstract

OBJECTIVE: To identify the active anti-tumor constituents in the extract from Danshen (*Radix Salviae Miltiorrhizae*) and investigate the mechanisms underlying the actions.

METHODS: First, we introduced a two-step counter-current chromatography to extract the therapeutically active diterpenoid, tanshinone from Danshen (*Radix Salviae Miltiorrhizae*). The cholecystokinin (CCK-8) method was used to evaluate the inhibitory effect of diterpenoid tanshinone in liver cancer QGY-7703, lung cancer PC9, lung cancer A549, gastric cancer MKN-45, gastric cancer HGC-27, colon cancer HCT116, myeloma cellU266/RPMI8226, and human breast cancer MCF-7 *in vitro*. Fluorescence staining was used to observe the cytotoxicity ofditerpenoid tanshinone on PC9 cells. The Western blot was used to detect apoptosis-related protein poly ADP-ribose polymerase (PARP), cysteinyl aspartate specific proteinase3/9 (caspase3/9), and cleaved-cysteinyl aspartate specific proteinase3/9 (cleaved-caspase3/9). The endoplasmic reticulum stress-related activating transcription factor 4 (ATF4), phosphorylated eukaryotic initiation factor 2α (p-eIF2α), and phosphorylated jun amino-terminal kinase (p-JNK), and caspase-12 were also analyzed using the Western blot.

RESULTS: Diterpenoid tanshinone inhibited the nine human tumor cell lines, with an IC50 of 4.37-29 μg/mL, with the PC9 and MCF-7 displaying the lowest values. Fluorescence staining showed a lethal effect of diterpenoid tanshinone on PC9 cells. The Western blot showed that the expression of caspase3/9 protein and ATF-4 protein decreased gradually. However, the PARP, cleaved-caspase 3/9 and the expression of p-eIF2 α, P-JNK, and caspase-12 increased gradually, in a dose-dependent fashion.

CONCLUSION: We successfully introduced a two-step counter-current chromatography method to extract diterpenoid tanshinone, and demonstrated its antitumor activity. Diterpenoid tanshinone can induce apoptosis in nine human cancer cell lines.

Key words: Tanshinone; Salviae Miltiorrhiza; Antineoplastic agents; Apoptosis; Cell line, tumor

INTRODUCTION

Cancer is one of the fatal diseases worldwide. In the treatment of many cancers, even with radiation and chemotherapy, 5-year-survival rates are as low as 5% to 10%. We focused on herbal Traditional Chinese Medicines (TCMs). Danshen (*Radix Salviae Miltiorrhizae*), is a well-known traditional Chinese herbal medicine. In terms of TCM, it can promote blood circulation, regulate menstruation, with pain relief, and promote vascular circulation for enhanced tissue regeneration. It is mainly composed of fat-soluble diterpenoids and water-soluble phenolic components, in addition to flavonoids, triterpenes, and sterols. According to TCM literature, its extract has often been obtained by processing with bitter wine and swine fat (lard). The product, known as the "red lamb", has mainly been used to treat cutaneous diseases. "Red lamb", technically known as Danshen (*Radix Salviae Miltiorrhizae*) diterpenoid quinones, is a collection of secondary metabolites of Danshen (*Radix Salviae Miltiorrhizae*) containing constituents including etanshinone ⅡA (Tan ⅡA), cryptotanshinone, dihydrotanshinone, Danshen (*Radix Salviae Miltiorrhizae*) new ketone, and others. To date, this preparation has primarily been used for the treatment of cardiovascular and cerebrovascular diseases.

The use of diterpenoid quinone in treatment of tumors is still in its infancy. Over 90 known forms of diterpenoid tanshinone were derived from Danshen (*Radix Salviae Miltiorrhizae*), nearly half of which are classified as lipophilic[1,2] Of these components, Tan IIA in particular, has been shown to prohibit growth and trigger apoptosis in a variety of cancer cells and cell lines.[3-9] Many studies have also shown anti-cancer effects of dihydrotanshinone.[10-12] Most of them investigated the individual effects of diterpenoid tanshinone on cancer cell. As with many modern Western medicines, isolated active components are the central focus of the pharmaceutical industry and elevated doses of individual components may lead to undesired side effects commonly seen in cancer therapeutics. In this study, we investigated the overall anti-cancer effects of the diterpenoid quinone on proliferation and apoptosis in nine human cancer cell lines and the possible mechanisms underpinning the actions.

MATERIALS AND METHODS

Reagents

Gibco fetal bovine serum was obtained from Shanghai Biological Technology Ltd., (Shanghai, China). Rabbit monoclonal antibodies against poly (ADP-ribose) polymerase (PARP), caspases-3/9, cleaved-caspases-3/9, caspase-12, activating transcription factor 4 (ATF4), phosphorylated eukaryotic initiation factor 2α (p-eIF2α), and phosphorylated jun amino-terminal kinase (p-JNK) were purchased from Cell Signaling Technologies (CST, Danvers, MA, USA). Mouse monoclonal antibody against β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 cell culture medium and trypsin were purchased from Hangzhou Branch Yi Biotechnology (Hangzhou, China). The purity of the active constituent was more than 95%, as isolated and verified by the College of Pharmaceutical Sciences Zhejiang Chinese Medical University. The final working concentration was 10 mg/mL. The reserves were stored at −20 ℃ away from light until use.

All solvents used for the preparative isolation ofditerpenoid tanshinone by counter-current chromatography were of analytical grade. These solvents were purchased from Huadong Medicine Co., Ltd. Equipment Chemical Reagent Branch (Hangzhou, China), and pure water was made by Milli-Q equipment.

Equipment

The preparation of high-speed countercurrent chromatography (HSCCC) involved use of aCO_2 cell incubator (Thermo Scientific, Waltham, MA USA), super clean bench (Suzhou Essence Equipment Company, Wujiang, China), microscope (Olympus Co., Shanghai, China), multifunctional full wavelength microplate reader (Thermo Scientific, Waltham, MA USA), AE2405 electronic analytical balance, 96-well cell culture plate, and cell culture flask (BD Biosciences, San Jose, CA, USA).

Cryptotanshinone, dihydrotanshinone I, tanshinone Ⅰ, tanshinone ⅡA

The counter-current chromatography to obtain therapeutically effective constituents of Danshen (*Radix Salviae Miltiorrhizae*) includes two steps: (a) extraction and (b) separation.

(a) Preparation of crude diterpenoid extract from Danshen (*Radix Salviae Miltiorrhizae*) extract. For extraction, raw Danshen (*Radix Salviae Miltiorrhizae*) were ground into powder, sieved and set aside. We used su-