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Proapoptotic and TRAIL-sensitizing constituents isolated from *Salvia militiorrhiza* (Danshen)

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Natural compounds isolated from medicinal plants are invaluable resources for drug discovery. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent unique by its cancer cell-specific proapoptotic action, but its potential is heavily curbed by acquired resistance. We herein reported for the first time the identification of cytotoxic and TRAIL-sensitizing components of *Salvia miltiorrhiza* (Danshen), a traditional medicinal plant effective for treating cardiovascular disorders. Specifically, we found that the ethanol extract and its group 5 fraction of *S. miltiorrhiza* showed evident cytotoxicity against the human lung adenocarcinoma cell line A549 and ovarian adenocarcinoma cell line TOV-21G in a concentration-dependent manner. Likewise, a dose-dependent cytotoxicity was exerted by the standard solutions of cryptotanshinone, tanshinone I and tanshinone IIA, the major components of the group 5 fraction, where tanshinone IIA were most potent and displayed an IC_{50} of 2.00 ± 0.36 μM and 2.75 ± 0.23 μM for A549 and TOV-21G, respectively. Induction of apoptosis represents an essential mechanism underlying tanshinone IIA-mediated cytotoxic action, as evidenced by the proteolytic processing of PARP upon tanshinone IIA stimulation and, importantly, a marked rescue of the viability of tanshinone IIA-treated cells when co-treatment with the pan-caspase inhibitor z-VAD-fmk. Noteworthy, stimulation with cryptotanshinone, tanshinone I or tanshinone IIA all effectively potentiated TRAIL to reduce viability and inhibit the colony formation capacity of TRAIL-resistant TOV-21G and SKOV3. Collectively, we revealed the proapoptotic and TRAIL-sensitizing components of *S. miltiorrhiza* and further implicated the potential of developing these active compounds as monotherapeutic agent or TRAIL-based therapy for cancer chemoprevention or chemotherapy.

[**Key words:** Tanshinone IIA; Cryptotanshinone; Tanshinone I; Tumor necrosis factor-related apoptosis-inducing ligand; Ovarian cancer; Danshen]

Apoptosis plays a central role in the regulation of normal tissue homeostasis and participates in the elimination of potentially dangerous cells including the precursors of tumor cells. Most cancer chemotherapeutic agents act primarily by causing apoptotic cell death in susceptible cancer cells. Therefore, apoptosis-inducing compounds are generally regarded as candidate anticancer agents (1,2). Current efforts are focused on seeking new therapeutics that elicit apoptosis more selectively in cancer cells while sparing normal cells. In line with this, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) represents a promising anticancer agent due to its proapoptotic activity selectively against malignant cells, and recombinant human TRAIL monotherapy actually demonstrated encouraging results in early clinical trials (3). Nonetheless, the potential of TRAIL as a single anticancer therapeutic is profoundly limited given a number of human tumors are resistant to TRAIL-induced apoptosis or through acquired TRAIL resistance, raising the necessity of combinatorial strategy that employs therapaurics to circumvent the resistance mechanisms for TRAIL sensitization (3,4). Searching for TRAIL-sensitizing agents is therefore in urgent demand in order to achieve efficacious TRAIL-based cancer therapy.

The dried roots of the medicinal plant *Salvia miltiorrhiza*, named Danshen in Chinese, is a traditional herbal medicine with low toxicity and few side effects, and is commonly prescribed for treating cardiovascular diseases (5). In general, components of *S. miltiorrhiza* can be grouped into two major classes: the lipid-soluble diterpene quinones and the water-soluble phenolic acids.

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Abbreviations: PARP, poly(ADP-ribose) polymerase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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The lipid-solubles, normally obtained by extraction with alcohol solvents, are rich in abietanoids and diterpene quinone pigments. More than 30 diterpenoid tanshinones have been isolated and identified from *S. miltiorrhiza*. Among them, cryptotanshinone, tanshinone I, and tanshinone IIA are the three representative bioactive components. As to the water-soluble class of *S. miltiorrhiza*, the major active ingredients include many plant phenolic acids which are mostly caffeic acid derivatives. Salvianolic acid B is the most abundant member of salvianolic acids and has been assigned as the biochemical marker for *S. miltiorrhiza* species (6).

The aim of this study was to investigate the anticancer and TRAIL-sensitizing effects of the ethanol extracts of *S. miltiorrhiza* and identify its active components using HPLC analysis-based activity profiling. We herein report for the first time that cryptotanshinone, tanshinone I, and tanshinone IIA all exert potent *in vitro* cytotoxicity as a single agent but also evidently facilitate TRAIL sensitization in a number of TRAIL-resistant cancer cell lines. Our results further implicate the potential of developing these active compounds as monotherapeutic agent or in combination with TRAIL for cancer chemoprevention or chemotherapy.

MATERIALS AND METHODS

Plant materials Herbal plants of *S. miltiorrhiza* Bunge (Danshen) used in this study was provided by the biotechnology division of Taiwan Agricultural Research Institute. After growth for 10 months, the plants were harvested from agricultural practice farms (Wufen, Taichung). The roots of plants were subjected to preliminary treatments according to traditional procedures such as cleaning, cutting and dry heating at 70°C for 48 h (Fig. 1A). The dried plants were grinded using electric miller and sieved (0.75 mm filter) and then were kept at 4°C until use.

Preparation of *S miltiorrhiza* ethanol extract (SME) Twenty grams of *S. miltiorrhiza* dry slice were extracted with 220 mL of 95% ethanol. Soxhlet extraction was then performed using Büchi Extraction System B-811 for 5 h. The resultant *S. miltiorrhiza* ethanol extracts (SME) were then dried under reduced pressure and resuspended in 100% methanol to make up the 50 mg/mL sample solution for subsequent chromatography-based fractionation. The elution gradient consisted of methanol and H_2O solvent system from 20% methanol to 100% methanol. Elution was performed in 30 min at the flow rate of 35 mL/min and each fraction was collected every 6 min, consequently yielding five fractions (group 1 to group 5). Next, each SME fraction was dried under reduced pressure and then redissolved in dimethyl sulfoxide (DMSO) to achieve an indicated concentration with the highest concentration of DMSO less than 0.1%.

Activity profiling by HPLC analysis A Waters alliance 2695 High performance liquid chromatography (HPLC) system equipped with a Waters 2489 UV/VIS detector was used to analyze the content of different fractions of SME. Chromatographic separations were carried out on a X-Bridge Shield RP 18 column (4.6 × 250 mm, 5 μm; Waters). The column temperature was set at 35°C, and eluted compounds were detected by monitoring UV absorbance at 254 nm. The gradient condition was as follows: MeOH: 0.02% H_3PO_4: CAN = 0:75:25 → 10:45:45 (20 min) → 30:10:60 (50 min) → 0:75:25 (55 min) → 0:75:25 (70 min). The injection volume was 10 μL.

Preparation of standard solutions and calibration Salvianolic acid B, tanshinone I, tanshinone IIA and cryptotanshinone were purchased from Kingstone Bioproducts Co., Ltd. Salvianolic acid B, tanshinone I, tanshinone IIA and cryptotanshinone were dissolved with methanol to prepare the mixed standard stock solution. Working standards at the concentration of the calibration range (20, 40, 60, 80, and 100 μg/mL) were prepared by stepwise dilution with methanol. The calibration curve was obtained by peak area/corresponding concentration of the injected standard solutions, and the concentrations of target compounds in the samples were calculated based on the calibration curve.

Cell culture Human lung cancer cell line (A549), human ovarian cancer cell lines (OVCAR3, SKOV3, TOV-21G, and TOV-112D), and human leukemia cell lines (K562, Kasumi-1, Raji, THP-1, and U937) were used in this study. A549 cells were grown in a medium consisting of 90% RPMI 1640 with 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. OVCAR3 cells were grown in RPMI 1640 medium with 20% FBS supplemented with 0.01 mg/mL insulin, TOV-21G and TOV-112D cells were grown in 1:1 mixture of MCDB 105 and medium 199 with 15% FBS, and SKOV3 cells were grown in McCoy's 5α medium with 10% FBS supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. The media and supplements were purchased from Invitrogen (Carlsbad, CA). All cell lines were cultured at 37°C and 5% CO_2. Recombinant human TRAIL (Gibco, USA) was prepared as 100 μg/mL stock solution and stored in aliquots at −20°C before use.

Cell viability assay Cell viability was determined by MTS assay in accordance with our established protocol (7). All experiments were repeated for at least three times with triplicated samples in each experiment.

Western blotting analysis Immunoblotting was performed by following the established protocol (7). The antibody against poly (ADP-ribose) polymerase (PARP) was purchased from Cell Signal (Beverly, MA, USA). β-tubulin antibody was purchased from Sigma–Aldrich. The signals were detected with an enhanced superSignal West Pico chemiluminescence (Pierce, USA).

Colony formation assay TOV-21G cells were plated onto 60-mm dishes at a density of 2×10^6 cells/dish for overnight and were then treated with the indicated doses of drugs for 24 h. Control cells were exposed to 1% (v/v) solvent instead. At the end of drug treatments, cells were washed twice with phosphate-buffered saline (PBS) and then trypsinized to determine cell numbers. The drug-treated cells were then seeded at a density of 200 cells per 60 mm-dish in triplicate for each treatment. Cells were then allowed to form colonies by incubation in drug-free medium for 2 weeks. To count the numbers of colonies, the cell monolayer was rinsed twice with PBS, followed by staining cells with 1% crystal violet solution in 30% ethanol. The colonies composed of 50 or more cells were counted under microscopy. The plating efficiency was calculated as the ratio of the numbers of colonies counted to that of cells seeded, and a plating efficiency of 50–60% was routinely achieved. The same procedure was repeated for at least three times.

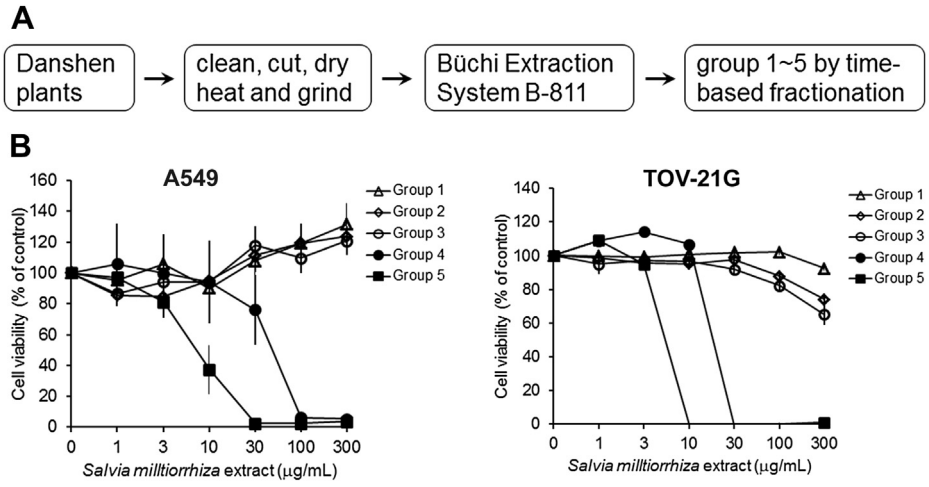

FIG. 1. Cytotoxic effect of *S. miltiorrhiza* ethanol extracts (SME). (A) Flow chart for the preparation of the crude ethanol extract of *S. miltiorrhiza* (Danshen) and its distinct fractions (group 1 to group 5) as described in the Materials and Methods section. (B) A549 human lung adenocarcinoma cell line and TOV-21G human ovarian adenocarcinoma cell line were treated without (DMSO control) or with increasing doses (1, 3, 10, 30, 100 and 300 μg/mL) of the distinct fractions (groups 1–5) of SME for 48 h, followed by MTS assay for cell viability determination.