Journal of Ethnopharmacology 152 (2014) 372–376

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Cyto-/genotoxic effects of the ethanol extract of Chan Su, a traditional Chinese medicine, in human cancer cell lines

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ARTICLE INFO

Article history:
Received 13 August 2013
Received in revised form
6 December 2013
Accepted 21 January 2014
Available online 1 February 2014

Keywords:
Chan Su
Bufo bufo gargarizans Cantor
Cytotoxic effects
Genotoxic effects
Human cancer cell lines

Chemical compounds studied in this article:
Bufalin (PubChem CID: 9547215)
Cinobufagin (PubChem CID: 11969542)

ABSTRACT

Ethnopharmacological relevance: Chan Su, an ethanolic extract from skin and parotid venom glands of the *Bufo bufo gargarizans* Cantor, is widely used as a traditional Chinese medicine for cancer therapy. Although the anti-cancer properties of Chan Su have been investigated, no information exists regarding whether Chan Su has genotoxic effects in cancer cells. The aim of the present study was to examine the cyto-/genotoxic effect of Chan Su in human breast carcinoma (MCF-7 cells), human lung carcinoma (A-549 cells), human T cell leukemia (Jurkat T cells), and normal human lymphocytes.
Materials and methods: Effects on the viability of MCF-7, A-549, Jurkat T cells, and normal lymphocytes were evaluated by Trypan blue exclusion assays. The DNA content in the sub-G1 region was detected by propidium iodide (PI) staining and flow cytometry. The genotoxicity of Chan Su was assessed by single-cell gel electrophoresis (comet assay) and the cytokinesis-block micronucleus assay (CBMN assay).
Results: Chan Su significantly inhibited the viability of MCF-7, A-549, and Jurkat T cells dose dependently, but had no effect on normal human lymphocytes. Apoptotic death of the cancer cells was evident after treatment. Chan Su also induced genotoxicity in a dose-dependent manner, as indicated by the comet and cytokinesis-block micronucleus assays.
Conclusions: These findings suggest that Chan Su can induce apoptotic death of, and exert genotoxic effects on, MCF-7, A-549, and Jurkat T cells.

1. Introduction

Traditional Chinese medicine (TCM) consists of medicinal products from plants, animals, and minerals that are used for acupuncture and other practices (Efferth et al., 2007). TCM with anti-tumor activities have been considered candidate novel cancer therapeutics, and TCM has been practiced in China, Japan, Korea, and other Asian countries for many centuries (Morishita et al., 1992; Qi et al., 2010; Meng et al., 2012).

Chan Su, one of the major components of TCM, is an extract from skin and parotid venom glands of the Chinese toad (*Bufo bufo gargarizans* Cantor) (Krenn and Kopp, 1998). Chan Su is widely used in clinical therapy – for example, as an antipyretic, a detoxicant, a local anesthetic, a cardiotonic, and a diuretic – and has been used to treat various cancers, such as liver, lung, pancreatic, and colorectal cancer, at oncology clinics in China (Meng et al., 2009; Wang et al., 2011; Meng et al., 2012).

Previous studies have demonstrated that Chan Su has various biological effects, such as anti-neoplastic, antiangiogenesis, immunomodulatory, cardiotonic, anaesthetic activities (Lu et al., 2008; Wang et al., 2009). Numerous experimental studies have investigated the anti-cancer properties of Chan Su and the possible underlying mechanisms. Chan Su inhibits cell proliferation by inducing cell cycle arrest and apoptosis in various cancer cells, including hepatic, gastric, pancreatic, and lung cancer cells (Qi et al., 2010, 2011a). Several constituents of Chan Su have been identified, including bufodienolides (bufalin, cinobufagin, resibufogenin, and bufotalin), biogenic amines, alkaloids, peptides, and proteins. Bufodienolides are considered the major components of Chan Su (Xie et al., 2012). Bufalin and cinobufagin are the most bioactive components. Because bufalin and cinobufagin can induce apoptosis in various cancer cells, the anti-cancer effect of Chan Su may be attributable to these compounds (Yu et al., 2008; Qi et al., 2011b; Zhu et al., 2012).

Although many studies of the mechanisms of Chan Su-induced apoptosis have been conducted, no information exists concerning its genotoxic effects. Therefore, we examined the cyto-/genotoxic effects of the ethanol extract of Chan Su on breast cancer cells (MCF-7), lung cancer cells (A-549), human T cell leukemia cells (Jurkat T cells), and normal human lymphocytes.

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http://dx.doi.org/10.1016/j.jep.2014.01.023

2. Materials and methods

2.1. Crude extract preparation of Chan Su

The skin of *Bufo bufo gargarizans* Cantor was obtained from the Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China. The skin was converted to a powdered form by cold extraction using grain alcohol, and then sonicated at room temperature and ambient pressure. A biomolecular extract of 100 g of biomass was yielded in 400 mL of 70% grain alcohol (30% pure water). The concentration of Chan Su was 0.25 g/mL, and the extract of Chan Su was diluted in distilled water. Finally, we used 0.025, 0.05, 0.1, and 0.15 mg/mL Chan Su for cytotoxic tests and 0.025 and 0.05 mg/mL for genotoxic tests.

2.2. Quantitative analysis of bufalin and cinobufagin

The Chan Su extract comprised two major components, bufalin and cinobufagin, whose concentrations were determined by high performance liquid chromatography (HPLC) using individual standards. HPLC analysis was performed using an HP series 1100 HPLC instrument (Agilent, Santa Clara, CA, USA). The HPLC system was equipped with an Optima Pak C 18 column (250 × 4.6 mm, 5 μm), using a flow rate of 1 mL/min and an injection volume of 20 μL. Column effluents were monitored at 296 nm.

2.3. Cell culture and treatment

MCF-7, A-549, and Jurkat T cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Normal peripheral blood mononuclear cells (PBMCs) were isolated as described by M'Bemba-Meka et al. (2005). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/mL of penicillin (Gibco, Invitrogen, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Gibco) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were treated with various concentrations of Chan Su. PBMCs were stimulated with phytohemagglutinin (PHA) for 24 h, and Jurkat T cells were plated at a density of 1×10^6 cells/mL in 24-well plates. MCF-7 and A-549 cells (1×10^5 cells/mL) were plated in six-well plates and were allowed to attach for 24 h.

2.4. Evaluation of cell viability

The effects of Chan Su on the viability of cancer cells were examined using the Trypan blue exclusion assay. Because there is no standard concentration of Chan Su, we used several concentrations and multiple treatment times. Cells were treated with various concentrations of Chan Su (0.025, 0.05, 0.1, or 0.15 mg/mL) and incubated for 48 h. MCF-7 and A-549 cells, treated with diluted trypsin-EDTA (Gibco), as well as Jurkat T cells and PBMCs, were placed on a hemocytometer with Trypan blue. Cells that stained blue were considered to be dead.

2.5. Analysis of apoptosis

MCF-7, A-549, and Jurkat T cells were treated with Chan Su (0.025 or 0.05 mg/mL). After 48 h of incubation, cells were collected and fixed with 1 mL of ice-cold 70% ethanol in phosphate-buffered saline (PBS) at −20 °C overnight. The cell pellets were harvested after washing with PBS, followed by the addition of propidium iodide (50 μg/mL; Sigma) and RNase A. The mixture was incubated at room temperature in the dark for 30 min. Finally, the nuclei were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA). Apoptotic cells were identified by the sub-G1 DNA content. Data were analyzed using the Cell Quest Pro software (Becton–Dickinson).

2.6. Single-cell gel electrophoresis (comet assay)

Single-cell gel electrophoresis was used to assess DNA strand break and abasic sites, as described by Singh et al. (1988). Jurkat T cells and PBMCs were treated with Chan Su (0.025 or 0.05 mg/mL) for 4 h, and MCF-7 and A-549 were treated for 3 h, which are the optimal treatment durations for Chan Su-mediated DNA damage. Thereafter, cells were harvested and maintained at 4 °C. Cells were mixed with 100 μL of 0.5% low-melting agarose and placed on the agarose-layered slide. Another 100 μL of 0.5% low-melting agarose were layered on top. The slides were placed in lysis buffer (2.5 μM NaCl, 0.1 M Na_2-EDTA, 0.01 M Tris–HCl, 1% Triton X-100, and 10% dimethyl sulfoxide (DMSO) adjusted to pH 10) at 4 °C for 1 h. Next, the slides were rinsed with distilled water, placed in the electrophoresis buffer (300 mM NaOH and 1 mM Na_2-EDTA adjusted to pH 13) for 20 min, and electrophoresed for 25 min at 0.78 V/cm and 300 mA. The slides were neutralized with 0.4 M Tris–HCl buffer (pH 7.5) and fixed with absolute ethanol. Sixty randomly selected cells per slide were measured using the Olive tail moment as an indicator of the degree of DNA damage. The Olive tail moment was measured under a microscope (Nikon, Tokyo, Japan) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm, and analyzed using a KOMET 5.5 image analysis system (Kinetic imaging, Nottingham, UK).

2.7. Cytokinesis-block micronucleus assay (CBMN assay)

MCF-7, A-549, Jurkat T cells, and PBMCs were treated with Chan Su (0.025 or 0.05 mg/mL). Cytochalasin-B (4 μg/mL; Sigma) was added 20 h after the start of the culture, and the cells were incubated for a further 28 h. Harvested cells were treated twice with 0.075 M KCl hypotonic solution for 1 min and fixed in fixation solution (a mixture of acetic acid and methanol; 1:3). The samples were air-dried and Giemsa stained (5%). All slides were scored according to standard criteria (Fenech, 2000). A total of 1000 binucleated cells were scored.

2.8. Statistical analysis

Statistical analysis was performed using SPSS statistics ver. 17 (SPSS Inc., Chicago, IL, USA). All experiments were performed in triplicate, and the results were expressed as means ± standard deviation (SD). Differences in cell viability were analyzed using Student's *t*-test. Differences between the control and treatment groups of Olive tail moments and micronuclei (MN) formation were evaluated using the Mann–Whitney *U*-test. A P value < 0.05 was deemed to indicate statistical significance.

3. Results

3.1. Identification of bufalin and cinobufagin in Chan Su using the HPLC/ultraviolet technique

HPLC/UV analysis of Chan Su led to identification of the two major components, bufalin and cinobufagin (Fig. 1). There are 0.025 mM of cinobufagin and 0.023 mM of bufalin in Chansu (0.25 g/mL). The Chan Su was diluted with distilled water to 2.5 nM bufalin and 2.3 nM cinobufagin (0.025 mg/mL Chan Su).