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Sensitivity of apoptosis-resistant colon cancer cells to tanshinones is mediated by autophagic cell death and p53-independent cytotoxicity

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ABSTRACT

Background: Multidrug resistance (MDR) develops in nearly all patients with colon cancer. The reversal of MDR plays an important role in the success of colon cancer chemotherapy. One of the commonest mechanisms conferring MDR is the suppression of apoptosis in cancer cells.
Purpose: This study investigated the sensitivity of cryptotanshinone (CTS) and dihydrotanshinone (DTS), two lipophilic tanshinones from a traditional Chinese medicine *Salvia miltiorrhiza*, in apoptosis-resistant colon cancer cells.
Methods: Cell viability was measured by MTT assay. Cell cycle distribution and apoptosis were determined by flow cytometry. Protein levels were analyzed by western blot analysis. The formation of acidic vesicular organelles was visualized by acridine orange staining.
Results: Experimental results showed that multidrug-resistant colon cancer cells SW620 Ad300 were sensitive to both CTS and DTS in terms of cell death, but with less induction of apoptosis when compared with the parental cells SW620, suggesting that other types of cell death such as autophagy could occur. Indeed, the two tanshinones induced more LC3B-II accumulation in SW620 Ad300 cells with increased autophagic flux. More importantly, cell viability was increased after autophagy inhibition, indicating that autophagy induced by the two tanshinones was pro-cell death. Besides, the cytotoxic actions of the two tanshinones were p53-independent, which could be useful in inhibiting the growth of apoptosis-resistant cancer cells with p53 defects.
Conclusion: The current findings strongly indicate that both CTS and DTS could inhibit the growth of apoptosis-resistant colon cancer cells through induction of autophagic cell death and p53-independent cytotoxicity. They are promising candidates to be further developed as therapeutic agents in the adjuvant therapy for colon cancer, especially for the apoptosis-resistant cancer types.

Introduction

Colon cancer is the third most frequently diagnosed cancer in the world (Gill et al. 2003). The response rate to current chemotherapies for colon cancer is up to 50% due to the availability of various chemotherapy regimens. However, multidrug resistance (MDR) still develops in nearly all patients with colon cancer and leads to chemotherapy failure (Dallas et al. 2009).

The mechanisms conferring MDR include overexpression of drug efflux transporters, downregulation of drug influx transporters and suppression of apoptosis, etc. (Gottesman et al. 2002). Suppression of apoptosis is often associated with increased expression of anti-apoptotic proteins and decreased expression of pro-apoptotic proteins (Fulda 2009). For instance, anti-apoptotic protein Bcl-2 is overexpressed in various cancer cells, contributing to the inhibition of apoptosis (Fernald and Kurokawa 2013). Defects in the apoptotic signaling pathways cause MDR of cancer cells to chemotherapy and radiation therapy in clinics, as these two therapies kill target cells mainly through inducing apoptosis (Igney and Krammer 2002). Thus, the resistance to apoptosis is an important clinical problem in the treatment of cancers including colon cancer.

Autophagy, type II programmed cell death, is activated in response to stressful stimuli including starvation, hypoxia and high temperature or intracellular stress such as damaged organelles and mutant proteins. During the cellular process of autophagy, the redundant, damaged or aged organelles are sequestered, degraded and recycled

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(Kondo et al. 2005). One function of autophagy is to keep cellular homeostasis, however, excessive activation of autophagy can also lead to cell death by destroying major proportions of the cytoplasm (Martinet and De Meyer 2009). Thus, autophagic cell death, which may kill cells as an alternative pathway in addition to apoptosis, represents a novel approach to inhibit the growth of apoptosis-resistant cancer cells.

Besides, tumor suppressor p53 defects in cancer cells also contribute to the resistance to apoptosis. Indeed, the mutation of p53 has been found in more than 50% of all human cancers including colon cancer (Greenblatt et al. 1994). The p53 defects can facilitate the evasion of p53-mediated apoptosis and give rise to MDR (Reles et al. 2001). Accumulating evidences have demonstrated that p53 null cancer cells tend to be more resistant to various cytotoxic drugs especially to DNA-damaging agents (Lowe et al. 1993; O'Connor et al. 1997). Thus, cytotoxic agents which can kill cancer cells p53-independently should be promising candidates for the circumvention of MDR caused by p53 defects.

Cryptotanshinone (CTS) and dihydrotanshinone (DTS) are two lipophilic tanshinones from traditional Chinese medicine *Salvia miltiorrhiza*. Although the anticancer properties of tanshinones have been studied over the past years, the potential application of tanshinones especially CTS and DTS in reversing MDR in colon cancer has rarely been reported. Our previous findings have demonstrated the reversal of MDR mediated by overexpression of P-glycoprotein (P-gp), one of the most important drug efflux transporters, by CTS and DTS in P-gp overexpressing colon cancer cells SW620 Ad300 (Hu et al. 2014). In this study, the effects of CTS and DTS in inhibiting the growth of apoptosis-resistant colon cancer cells and their mechanisms of action were evaluated, with an aim to develop potential MDR-reversing agents which could be used in the adjuvant therapy for colon cancer, especially for the apoptosis-resistant colon cancer.

Materials and methods

Materials

CTS and DTS were purchased from Chengdu Cogon Bio-tech Co., Ltd. (Sichuan, China). Their purities were >98% as determined by HPLC–UV. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS) and penicillin–streptomycin were obtained from Gibco (Carlsbad, CA, USA). McCoy's 5A medium and all the chemicals were obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA) unless otherwise specified. P-gp antibody was purchased from Calbiochem. Primary antibodies of caspase-3, caspase-8, Bax, Bcl-2, PARP, LC3B, ATG5 and ATG7 were from Cell Signaling. p21 and p53 antibodies were from Santa Cruz. p62 antibody was from BD Laboratories. GAPDH antibody was from Millipore.

Cell culture

The resistant SW620 Ad300 subline was developed from its parental cell line SW620 by stepwise selection in increasing concentrations of doxorubicin. The cells were maintained in RPMI 1640 (HCT116, SW620 and SW620 Ad300), McCoy's 5A ($p53^{-/-}$ HCT116) or DMEM (MEFs and $ATG5^{-/-}$ MEFs) medium, supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability assay

Cell viability was evaluated by MTT assay. In brief, cells were seeded into 96-well plates at a density of 5000–8000 cells/well. After drug treatment, MTT at the final concentration of 0.5 mg/mL was added into each well, followed by incubation at 37 °C for another 4 h. Purple formazan product was dissolved in 100 μL DMSO and the absorbance was determined at 570 nm.

Western blot analysis

Cells were seeded into 60 mm dishes at a density of $1.0–1.5 \times 10^5$ cells/mL and were harvested following the drug treatment. Equal amounts of proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated at 4 °C overnight with different primary antibodies diluted in 5% bovine serum albumin in washing buffer. Afterward, the membranes were incubated with respective HRP-conjugated secondary antibodies at room temperature for 2 h. Chemiluminescent signals were then developed with LumiGLO reagent and peroxide (Cell Signaling) and detected by the ChemiDoc XRS gel documentation system (Bio-Rad, Hercules, CA, USA).

Measurement of apoptosis by flow cytometry

Cells were seeded into 12-well plates at a density of 1.0×10^5 cells/mL. After drug treatment, cells were harvested, washed and resuspended in annexin-binding buffer containing propidium iodide (PI) and annexin V. After incubation at room temperature in the dark for 15 min, the stained cells were subjected to a BD LSRFortessa Cell Analyzer (BD Biosciences, San Jose, CA, USA), measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation. Data were analyzed using Flow Jo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

Detection of acidic vesicular organelles (AVOs) by acridine orange staining

Cells were seeded into 6-well plates at a density of 1.0×10^5 cells/mL. After treatment with tanshinones, cells were washed twice with pre-warmed PBS, followed by incubation with 1 μg/mL acridine orange at 37 °C for 15 min. Fluorescence signal was detected and recorded using a fluorescence microscope (Nikon, TS-100-F) equipped with a digital camera (Nikon, DS-5Mc).

Soft agar assay for colony formation

In brief, 1.5 mL RPMI 1640 medium with 0.5% agar was added into each well of the 6-well plate. After the medium became solid, 5000 cells were suspended in 2 mL RPMI 1640 medium with 0.3% agar and seeded on the top of the basal gel. Cells were then treated with different drugs as designed and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for around 2 weeks. Afterward, cells were fixed with 4% formaldehyde and stained with 0.5 mL 0.1% crystal violet for 1 h. They were then rinsed with PBS. The number of colonies (larger than 50 μm in diameter) were finally assessed microscopically.

Statistical analysis

Statistical analysis of the data was carried out using Prism 5.0 (GraphPad Software, CA, USA). All the data were expressed as mean ± standard error of mean (S.E.M.). The significance of difference between groups was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. *P* value less than 0.05 indicated statistical significance. All the assays were performed in three independent experiments.

Results

P-gp overexpressing SW620 Ad300 cells showed MDR to different anticancer drugs, but were sensitive to CTS and DTS

The cytotoxicities of CTS, DTS and several anticancer drugs were compared in SW620 parental and P-gp overexpressing cells. As shown in Fig. 1A, when compared with SW620 cells, SW620 Ad300

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