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Cryptotanshinone induces ROS-dependent autophagy in multidrug-resistant colon cancer cells

Zhenyu Xu [a], Hui Jiang [a], Yanhong Zhu [a], Huifang Wang [a], Jia Jiang [a], Lu Chen [a], Wenke Xu [a, *], Tao Hu [b, **], Chi Hin Cho [b, c]

[a] Department of Pharmacy, Yijishan Affiliated Hospital of Wannan Medical College, Wuhu, Anhui Province, China
[b] School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China
[c] Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, China

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ABSTRACT

The development of novel chemotherapeutic agents is highly desired for colon cancer treatment, in particular for the multidrug-resistant cancer types. Cryptotanshinone (CTS), an active quinoid diterpene isolated from *Salvia miltiorrhiza* Bunge, was previously reported to induce autophagy in various colon cancer cell lines. However, its mechanisms of action have not been fully understood. The current study aims to explore the mechanisms by which CTS induces autophagy in a multidrug-resistant human colon cancer cell line SW620 Ad300. Using MTT assay, CTS at 10 μM exhibited no significant cytotoxicity on human normal colon fibroblasts CCD-18Co, but induced 45.67% and 48.35% cell death in SW620 and SW620 Ad300 cells, respectively. Further studies revealed that CTS induced weak apoptosis (9.37%) and significant caspase-independent cell death in SW620 Ad300 cells. In the same cell line, CTS also induced significant autophagy, which was found to promote cell death and to mediate the cytotoxicity of CTS in these multidrug-resistant cells. Moreover, activation of ROS-p38 MAPK-NF-κB signaling pathway was involved in autophagic cell death induced by CTS in SW620 Ad300 cells. Interestingly, our results also demonstrated a complementary relationship between CTS-induced apoptosis and autophagic cell death in SW620 Ad300 cells. Taken together, CTS induces autophagic cell death in SW620 Ad300 cells via the ROS-p38 MAPK-NF-κB signaling pathway, and it might be a potential candidate as a chemotherapeutic agent for the treatment of multidrug-resistant colon cancer.

1. Introduction

Colon cancer is the third most commonly diagnosed cancer worldwide [1,2]. Despite the development of novel targeted therapies, chemotherapy, which uses chemicals to inhibit and damage cancer cells, is still one of the main treatment options for colon cancer. However, chemotherapy is usually used for the treatment of advanced colon cancer, in which cancer cells often acquire multidrug resistance upon exposure to stressful circumstances and cytotoxic drugs. In this regard, multidrug resistance is an obstacle causing chemotherapy failure [3]. Hence, development of safe and effective agents targeting multidrug-resistant cancer types is highly desired for colon cancer treatment.

The seeking of promising candidates from natural products and their derivatives provides efficient approach to develop novel anticancer drugs. It has been reported that natural products including triptolide, thymoquinone, crocetin, α-iso-cubebenol, epigallocatechic-3-gallate, β-sitosterol,7-hydroxyfrullanolide, betulinic acid and ursolic acid could inhibit cell proliferation and induce apoptosis in different cancer cell lines especially in those p53-deficient cancer cells [4–6]. In addition, phytochemicals that can induce autophagic cell death may overcome the resistance of cancer cells to apoptosis. Indeed, a variety of structurally different natural products such as curcumin, resveratrol, oridonin, quercetin, genistein, securinine, evodiamine, helenalin, piperovatine and piperlonguminine have been shown to activate autophagic signaling pathways and cause cell death in various cancer cell lines, including those of the colon, gastric, breast, ovarian, lung, prostate

* Corresponding author.
** Corresponding author. School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China.
E-mail addresses: 1447486817@qq.com (W. Xu), taohu1985@hotmail.com (T. Hu).

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and liver [7,8].

Danshen (*Salvia miltiorrhiza*) is a Traditional Chinese Medicine widely used in China for the prevention and treatment of cardiovascular, cerebrovascular and liver diseases [9–12]. Cryptotanshinone (CTS), a natural quinoid diterpene isolated from *Salvia miltiorrhiza* Bunge, has exhibited versatile anticancer actions against different cancers including inhibition on cell proliferation, induction of cell cycle arrest, activation of apoptosis and deduction of angiogenesis [13,14]. Using P-glycoprotein (P-gp)-overexpressing colon cancer SW620 Ad300 cells and its parental cell line SW620, our previous findings showed that CTS sensitized the multidrug-resistant cells to anticancer drugs doxorubicin and irinotecan via its inhibition on the efflux function of P-gp [15]. In the same *in vitro* system, CTS produced similar cytotoxicity on multidrug-resistant SW620 Ad300 cells as on the parental SW620 cells [16]. Interestingly, apoptosis and autophagic cell death were found to mainly mediate the cytotoxic action of CTS in SW620 and SW620 Ad300 cells, respectively [16]. Thus, CTS might be a potential candidate for the development of chemotherapeutic agent for multidrug-resistant colon cancer. However, the underlying mechanisms by which CTS induces autophagy in multidrug-resistant cells are largely undefined.

As known, reactive oxygen species (ROS) generated in cells are one of the driving forces of cell death including apoptosis, autophagy and necrosis. In fact, the study done by our group showed that dihydrotanshinone, another tanshinone with extremely similar chemical structure as CTS, was able to cause ROS generation in colon cancer HCT116 cells, which subsequently mediated its induction of apoptosis [17]. It is therefore speculated that ROS generation was also involved in the cytotoxic actions of CTS in cancer cells. In this regard, the current study aims to investigate the cellular events related to autophagy induced by CTS in multidrug-resistant colon cancer cells SW620 Ad300, in particular the relationship between ROS generation and the induction of autophagy. Such investigation would provide potential molecular targets in the modulation of the therapeutic effect of CTS against multidrug-resistant colon cancer cells.

2. Materials and methods

2.1. Materials

CTS (purity > 98%) was purchased from Chengdu Cogon Bio-tech Co., Ltd. (Sichuan, China). RPMI 1640 medium, Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS) 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). GAPDH antibody was from Millipore. Other primary antibodies were from Cell Signaling Technology unless otherwise specified.

2.2. Cell culture

Human colon cancer cell line SW620 and its multidrug-resistant subline SW620 Ad300 were kindly provided by Dr. Susan Bates (National Cancer Institute, Bethesda, MD, USA). CCD-18Co and HCT116 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). $p53^{-/-}$ HCT116 cell line was a generous gift from Prof. Bert Vogelstein at Johns Hopkins University. Cells were maintained in RPMI 1640 (HCT116, SW620 and SW620 Ad300), EMEM (CCD-18Co) or McCoy's 5A ($p53^{-/-}$ HCT116) medium, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell viability assay

The viability of the cells was measured by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 5000–8000 cells/well and allowed to grow overnight. After exposure to different compounds as designated for 24 h, 0.5 mg/mL MTT was added into each well and incubated with cells at 37 °C for another 4 h. Purple formazan product was dissolved in 100 μL DMSO and the absorbance was determined at 570 nm.

2.4. Western blot analysis

Cells were seeded into 60 mm dishes at a density of 1.0×10^5 cells/mL and were harvested after drug treatment as designed. Equal amounts of proteins (30 μg) were resolved by SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with different primary antibodies diluted in 5% bovine serum albumin (BSA) in washing buffer at 4 °C overnight. Then the membranes were incubated with respective HRP-conjugated secondary antibodies at room temperature for 2 h. Chemiluminescent signals were developed with LumiGLO reagent and Peroxide (Cell Signaling Technology) and detected by the ChemiDoc XRS gel documentation system (Bio-Rad, Hercules, CA, USA).

2.5. Measurement of apoptosis by flow cytometry

Cells were seeded into 12-well plates at a density of 1.0×10^5 cells/mL. After exposure to drugs as designed, cells were harvested, washed with ice-cold PBS and resuspended in annexin-binding buffer containing annexin V and PI. After incubation at room temperature in the dark for 15 min, cells were analyzed by a BDLSRFortessa 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).

2.6. Detection of intracellular ROS generation

Cells were seeded into 12-well plates at a density of 1.0×10^5 cells/mL and allowed to incubate overnight. After pre-treatment with a ROS-sensitive fluorescent probe 2′,7'-dichlorodihydrofluorescein diacetate (H2DCFDA, 10 μM) for 30 min, the cells were treated with CTS for 1 h. Finally, intracellular ROS level was determined by flow cytometry using a 488 nm argon laser and a 530 nm bandpass filter. Data were analyzed using Flow Jo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

2.7. 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 CTS for 24 h, cells were washed with PBS and incubated with 1 μg/mL acridine orange at 37 °C for 15 min. Fluorescence signal was detected using a fluorescence microscope (Nikon, TS-100-F).

2.8. 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 *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. *P* value less than 0.05 indicated statistical significance.