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Cytotoxicity of major tanshinones isolated from Danshen (*Salvia miltiorrhiza*) on HepG2 cells in relation to glutathione perturbation

W.Y.W. Lee [a], L.C.M. Chiu [b], J.H.K. Yeung [a,*]

[a] *Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR, China*
[b] *Department of Biology, Faculty of Science, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR, China*

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Abstract

Tanshinones are abietane type-diterpene quinones isolated from the roots of *Radix Salvia miltiorrhiza* (Danshen), a well-known traditional Chinese medicine in the treatment of cardiovascular diseases. Among the major diterpenes isolated, including cryptotanshinone, tanshinone I, tanshinone IIA and dihydrotanshinone, tanshinone IIA had been shown to posses various pharmacological activities including antioxidant, protection/prevention from angina pectoris and myocardial infarction, and anticancer properties. Tanshinone IIA, usually the most abundant tanshinone present in the herb, has been the focus of studies in its clinical potential, among which its ability to inhibit the proliferation of cancer cell lines. The aim of this study was to study the cytotoxicity of the tanshinones on human HepG2 cells *in vitro* in relation to intracellular glutathione perturbation (reduced glutathione, GSH and oxidized glutathione, GSSG). Studies using MTT assay showed that all tanshinones decreased cell viability of HepG2 cells in a concentration-dependent manner, with the cell viability decreased to 60% and 35% after 24 h and 48 h treatment, respectively. Assessment of apoptotic cells with fragmented DNA by flow cytometry indicated that only tanshinone IIA (12.5 and 25 μM) induced apoptosis in the cancer cells. Tanshinone IIA and cryptotanshinone caused significant decreases in G_1 cells by 23% and 13%, respectively, after 24 h treatment. The declines in G_1 cells were compensated by increases in G_2/M (15% for tanshinone IIA) and S cells (8% and 13% for tanshinone IIA and cryptotanshinone, respectively). All the tanshinones studied, except tanshinone IIA, elevated GSH/GSSG ratio at low concentrations (1.56 and 3.13 μM), but the ratio decreased, indicating oxidative stress at high concentrations (6.25–25 μM). Taken together, tanshinone IIA caused HepG2 cytotoxicity through apoptosis without influencing oxidative stress, while the other tanshinones showed lower efficacy in inducing apoptosis in the HepG2 cells.
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Keywords: Danshen; *Salvia miltiorrhiza*; Glutathione; Cytotoxicity; HepG2 cells; Cryptotanshinone; Tanshinone I; Tanshinone IIA; Dihydrotanshinone; GSH/GSSG ratio

1. Introduction

Danshen (*Salvia miltiorrhiza* Bunge) is a herb that has been widely used in traditional Chinese medicine (TCM) for treating coronary heart diseases such as angina pectoris and myocardial infarction. Along with more than 20 phenolic acids, about 30 diterpene compounds have been isolated from Danshen, including tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone which are the relatively abundant tanshinones (Zhou et al., 2005). Among the major diterpenes isolated, including cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA, tanshinone IIA has been shown to possess pharmacological activities including antioxidant (Cao et al., 1996), protecting and/or preventing angina pectoris and myocardial infarction (Zhao et al., 1996). A recent report has shown that tanshinone IIA, one of the most abundant

* Corresponding author. Tel.: +852 2609 6864; fax: +852 2603 5139.
E-mail address: johnyeung@cuhk.edu.hk (J.H.K. Yeung).

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diterpenes isolated from Danshen organic extract, inhibited the proliferation of human breast cancer (Wang et al., 2005) and caused cytotoxic effects on cell lines derived from various human carcinomas of colon, ovary, lung and mouth (Wu et al., 1991; Ryu et al., 1997).

Previous studies have shown that tanshinone IIA induced apoptosis in human leukemia cells through the activation of caspase-3 (Sung et al., 1999; Liu et al., 2006). Tanshinone IIA up-regulated the expression of Fas, p53 and Bax but down-regulated Bcl-2 and c-Myc in human hepatocarcinoma cell line (SMMC-7721) (Yuan et al., 2004). The down-regulation of Bcl-2 and Bcl-XL and the up-regulation of Bax in leukemia cells were reported by Liu et al. (2006). However, the upstream regulatory factor of tanshinone IIA in causing apoptosis remains unclear. It has been suggested that the planar phenanthrene quinone structure of tanshinones may be important in its cytotoxic effects (Wu et al., 1991). Other tanshinones such as tanshinone I, cryptotanshinone and dihydrotanshinone are structurally similar to tanshinone IIA (Fig. 1) and may also possess similar cytotoxic effects to tumor cells.

In aerobic organisms, reactive oxygen species (ROS) are continuously generated as a by-product of respiratory reaction in mitochondria. ROS such as superoxide anion radical is converted to hydrogen peroxide by superoxide dismutase (SOD), while glutathione peroxidase (GPx), another predominant defense system, reduces the hydrogen peroxide to water at the expense of glutathione (GSH). As a consequence, GSH is oxidized to disulfide (GSSG), which is in turn reduced back into GSH by GSSG reductase in the presence of NADPH and a closed redox system (redox cycle) is therefore formed. Although GSH is particularly important in controlling these energetic enemies ROS within mitochondria, it is originated from the cytosol because mitochondria are incapable of *de novo* GSH synthesis (Griffith and Meister, 1985). With a diminishing supply of GSH, excessive amounts of ROS are accumulated and the cell is under a state of oxidative stress in which there is an increase in intracellular, steady-state concentration of ROS (Sies, 1993). Numerous laboratory studies have demonstrated that glutathione depletion is associated with mitochondrial dysfunction and induction of apoptosis (Macho et al., 1997; Yang et al., 2000), with the general agreement that GSH depletion may be related to an increase in ROS generation in cell. Therefore, the balance of GSH and GSSG has been considered as a dynamic indicator of oxidative stress (Hultbery et al., 1999; Jones, 2002). Relevant to the study on Danshen and its major components, there has been only one report that has demonstrated that the aqueous extract of Danshen induced intracellular thiol depletion to cause apoptotic cell death (Liu et al., 2001), while the effects of the more lipid soluble tanshinones on GSH balance in cancer cells have not been studied. The aim of this study was to investigate the antitumor potential of other tanshinones, including cryptotanshinone, dihydrotanshinone and tanshinone I by studying their cytotoxic effects on HepG2 cells in parallel with the effects of these tanshinones on glutathione balance.

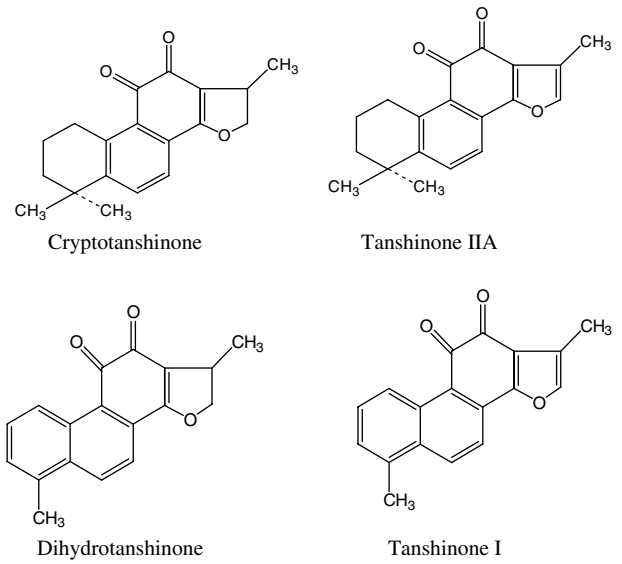

Fig. 1. Structures of the major tanshinones isolated from Danshen (*Salvia miltiorrhiza*).

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). Cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA were purchased from Chengdu Congon Bio-tech Co., Ltd. (Sichuan, People's Republic of China). Proof of purity was provided by the company in the form of LC–MS spectrum for each individual tanshinone. According to the "Certificate of Analysis" provided by the Chengdu Congon Bio-tach Co., Ltd., the purities of the four tanshinones are all above 98%. The purity of the individual tanshinones extracted from Danshen roots was further confirmed by HPLC–MS. Briefly, the stocks of the four tanshinones were freshly prepared daily, with their purities of monitored by our in-house HPLC. HPLC was performed with the danshen ethanol extract to quantify and confirm the presence of the tanshinones. The danshen ethanol extract was dissolved in methanol at a concentration of 500 μg/ml. HPLC analysis was performed in triplicate; each with 50 μl of the sample. Tanshinone 1, tanshinone IIA, cryptotanshinone and dihydrotanshinone were separated on an Agilent Zorbex Eclipse XDB-C18 5 μm (4.6 × 150 mm) with a XDB-C18 guard column. A gradient elution of A (1.5% acetic acid in water, pH 4.7) and B (acetonitrile) was used at a flow rate of 1 ml/min, commencing with 55% A and 45% B, rising to 60% B on 3 min, then to 80% B on 19 min. Detection was by an Agilent 1100 Series HPLC with diode-array detector at 260 nm. Standard curves for the tanshinones were linear between 5 and 100 μM (0.74–14.8 μg). A typical chromatogram of the four tanshinones is shown in Fig. 2 with α-naphthoflavone as internal standard. The stocks of the tanshinones were freshly-prepared daily, with their stability confirmed by HPLC. The tanshinones were dissolved in dimethylsulfoxide (DMSO) prior to use. The final concentration of DMSO was 1% (v/v) throughout the experiments.

2.2. Cell line and culture

The human hepatoma cell line (HepG2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in complete Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 50 units/ml

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