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Inhibition of warfarin hydroxylation by major tanshinones of Danshen (*Salvia miltiorrhiza*) in the rat *in vitro* and *in vivo*

Windy W.P. Wu, John H.K. Yeung *

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China

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ABSTRACT

Danshen (*Salvia miltiorrhiza*) is commonly used in the treatment of cardiovascular and cerebrovascular diseases. In this study, the effects of a Danshen ethyl acetate extract containing the major tanshinones, an aqueous extract containing salvianolic acid B and danshensu, and individual tanshinones (tanshinone I, tanshinone IIA and cryptotanshinone) on warfarin hydroxylation was investigated. In rat liver microsomes study, the ethyl acetate extract of Danshen, tanshinone I, tanshinone IIA and cryptotanshinone decreased the formation of 4′-, 6- and 7-hydroxy-warfarin, mediated by CYP1A1, CYP2C6 and CYP2C11 activities, respectively. The aqueous extract of Danshen had no effect on warfarin hydroxylation. Both acute and 3-day Danshen treatment significantly decreased Cmax and prolonged Tmax of warfarin in the rats. The formation of 4′- and 7-hydroxywarfarin *in vivo* was decreased significantly after 3-day danshen treatment. In steady state study *in vivo*, the steady state plasma warfarin concentration was increased by 23% when Danshen was co-administered. The results suggest that tanshinones inhibited CYP1A1, CYP2C6 and CYP2C11-mediated warfarin metabolism both *in vitro* and *in vivo* in the rats. The timing of Danshen intake relative to warfarin contributed to different pharmacokinetics of the free warfarin concentration.

Introduction

Danshen (*Salvia miltiorrhiza*) has been widely used in China, Japan, the United States of America and other European countries for the treatment of cardiovascular and cerebrovascular diseases (Zhou et al. 2005). The use of Danshen has been implicated in a number of clinically important herb-drug interactions, leading to adverse outcome (Holbrook et al. 2005; Hu et al. 2005). Suspected cases of Danshen-warfarin interactions have been reported in patients on warfarin therapy (Yu et al. 1997; Izzat et al. 1998; Chan 2001; Izzo et al. 2005). Despite the lack of large scale controlled studies to show unequivocally the importance of the Danshen-warfarin interaction clinically, it has become common practice to warn patients not to consume Danshen and/or other Chinese herbal medicine when they are prescribed warfarin.

Danshen has been one of the most investigated herbal medicine in recent years and the chemistry of the herb has been reviewed (Wang et al. 2007; Li et al. 2009). Advances in separation techniques have enabled studies to investigate the pharmacology and therapeutic potential of the isolated, chemically characterised components of Danshen (Shi et al. 2005; Zhang et al. 2005; Liu et al. 2006; Ma et al. 2006; Zhu et al. 2007). The Danshen-warfarin interaction may be mediated via both pharmacodynamic and pharmacokinetic mechanisms. The effects produced by Danshen or its tanshinone ingredients (Lam et al. 2006a, 2006b, 2007, 2008a, 2008b) or salvianolic acids (Tang et al. 2002) may have contributed to the observed pharmacodynamic changes when Danshen was co-administered with warfarin. Previous studies in our laboratory have shown that Danshen prolonged the prothrombin time of warfarin, an indicator of anti-coagulation in the rat (Lo et al. 1992); increased the bioavailability and decreased the elimination of warfarin in the rat (Chan et al. 1995); inhibited the metabolism of model CYP1A2 probe substrates in the rat (Wang et al. 2009). Reports by Li et al. (2006) and Sun et al. (2007) showed that tanshinones were metabolised mainly to hydroxy metabolites, although the exact CYPs involved have not been elucidated. Tanshinone IIA, one of the major components of Danshen, had been shown to inhibit human and mouse CYP1A2 activities *in vitro* (Ueng et al. 2003; He et al. 2007). These findings were consistent with our recent study in which rat CYP1A2-mediated metabolism of model CYP1A2 probe substrates were inhibited by the tanshinones *in vitro* and *in vivo* (Wang et al. 2009).

In this study, the effects of Danshen extract and some of the major tanshinone ingredients, tanshinone I, tanshinone IIA and cryptotanshinone, on warfarin metabolism were investigated. In rat, R-warfarin is metabolized by CYP1A1 to 6- and 8-hydroxywarfarin, CYP2B1 to 4′-hydroxywarfarin, CYP2C6 to

* Corresponding author. Tel.: +852 26096864; fax: +852 26035139.
E-mail address: johnyeung@cuhk.edu.hk (J.H.K. Yeung).

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7-hydroxywarfarin, CYP2C11 to 4′- and 6-hydroxywarfarin, and CYP3A2 to 10-hydroxywarfarin. S-warfarin is metabolized by CYP1A1 to 6-hydroxywarfarin, CYP2C6 to 7-hydroxywarfarin and CYP2C11 to 4′- and 6-hydroxywarfarin. Quantitation of the hydroxy-metabolites of warfarin (4′-, 6-, 7-, 8- and 10-hydroxywarfarin) thus provides information on how Danshen may affect the activities of CYP isoforms. The effects of a Danshen extract, containing the major tanshinones, on the pharmacokinetics of warfarin were studied *in vivo*, to investigate the effects of Danshen on single dose or steady state warfarin administration.

Materials and methods

Animals

Male Sprague Dawley rats (250–300 g) were supplied by the Laboratory Animal Service Center, The Chinese University of Hong Kong (CUHK). The rats were kept in animal holding room under standard conditions with 12-hour light-dark cycle, with free access to rodent cubes (Glen Forrest Stockfeeders, Australia) and tap water. All the experimental procedures had been approved by the Animal Experimentation Ethics Committee (CUHK) in accordance to the Department of Health (HKSAR) guidelines in Care and Use of Animals.

Materials

Danshen was obtained from Winsor Health Products Limited (Hong Kong). Tanshinone I, tanshinone IIA, cryptotanshinone were purchased from Chengdu Congon Bio-tech Co., Ltd (China). Warfarin, 4′-, 6-, 7-, 8-, 10-hydroxywarfarin, 7-ethoxycoumarin, β-nicotinamide adenine dinucleotide phosphate (NADP), D-glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase, cimetidine, sodium potassium tartrate, Folin and Ciocalteau's phenol reagent and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ammonia, magnesium chloride, sodium dihydrogen phosphate monohydrate, potassium chloride and potassium hydroxide were supplied by Merck (Darmstadt, FRG). Acetic acid was obtained from Schartau Chemie (USA). Sodium hydroxide was purchased from BDH Laboratory Supplies (Poole, England). Phenobarbitone sodium B.P. was obtained from Universal Pharmaceutical Lab., Limited (Hong Kong). Acetonitrile and methanol (both HPLC grade) were supplied by Mallinckrodt Baker Inc. (Paris, Kentucky, USA). Ethyl acetate was purchased from Fluka Chemicals (Buchs, Switzerland).

Preparation of Danshen extracts

Danshen was supplied and characterized by Winsor Health Products Ltd. (Hong Kong) where an initial screening test has been carried out that the batch of Danshen was free from contaminants. Danshen was extracted with ethyl acetate to obtain the ethyl acetate fraction containing the major tanshinones and an aqueous fraction containing hydrophilic components such as salvianolic acid and danshensu. The ethyl acetate fractions were combined and dried by a rotary evaporator. The aqueous fraction was then freeze-dried under vacuum. The contents of the ethyl acetate fraction of Danshen were measured by HPLC analysis.

HPLC analysis of tanshinones

The tanshinones (cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA) were separated on an Agilent Zorbax Eclipse XDB-C18 5 μm (4.6 × 150 mm) column with XDB-C18 guard column in a gradient mobile phase containing acetonitrile and water. Typical conditions for elution were as follows: 45 to 60% acetonitrile (0 to 3 min); 60 to 80% acetonitrile (3 to 19 min), using a flow rate of 1.0 ml/min. Dihydrotanshinone and tanshinone I were detected by UV absorbance at 250 nm. Cryptotanshinone and tanshinone IIA were detected by UV absorbance at 260 nm. Standard curves for cryptotanshinone, tanshinone I and tanshinone IIA were linear between 5.0 to 100 μM (0.74 to 14.80 μg for cryptotanshinone, 0.735 to 14.70 μg for tanshinone I and 0.69 to 13.80 μg for tanshinone IIA). The calibration curves had a minimum coefficient of determination (r^2) 0.99. The intra-assay coefficients of variation of cryptotanshinone, tanshinone I and tanshinone IIA were 1.49, 2.53 and 3.17%, respectively. The inter-assay coefficients of variation of cryptotanshinone, tanshinone I and tanshinone IIA were 1.77, 2.39 and 2.54%, respectively. The accuracy of cryptotanshinone, tanshinone I and tanshinone IIA were 108, 103 and 91.3%, respectively. Fig. 1 showed the structures of the major tanshinones and the water-soluble ingredients, danshensu and salvianolic acid B, present in the Danshen used in this study.

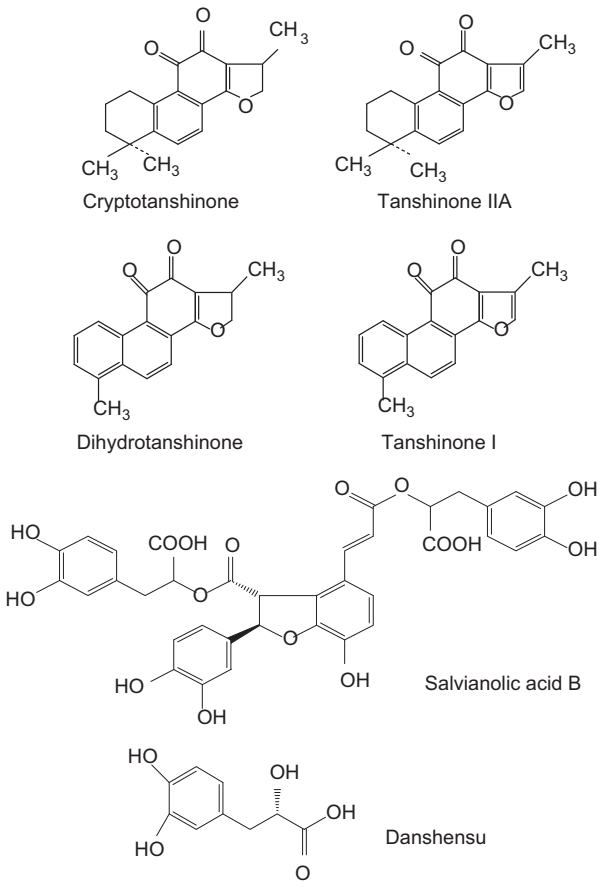

Fig. 1. Structures of the lipid soluble ingredients (cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA) and water soluble components (salvianolic acid B, danshensu) isolated from Danshen (*Salvia miltiorrhiza*).

Preparation of rat liver microsomes

Sprague Dawley Rats (male, 250–300 g) were killed by exsanguinations. The liver was excised, rinsed with ice-cold 0.9% NaCl solution, weighed and homogenised in a 0.1 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 10,000*g* at 4 °C for 30 min. the supernatant was