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Molecular docking and enzyme kinetic studies of dihydrotanshinone on metabolism of a model CYP2D6 probe substrate in human liver microsomes

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ABSTRACT

The effects of Danshen and its active components (tanshinone I, tanshinone IIA, dihydrotanshinone and cryptotanshinone) on CYP2D6 activity was investigated by measuring the metabolism of a model CYP2D6 probe substrate, dextromethorphan to dextrorphan in human pooled liver microsomes. The ethanolic extract of crude Danshen (6.25–100 μg/ml) decreased dextromethorphan O-demethylation *in vitro* (IC_{50} = 23.3 μg/ml) and the water extract of crude Danshen (0.0625–1 mg/ml) showed no inhibition. A commercially available Danshen pill (31.25–500 μg/ml) also decreased CYP2D6 activity (IC_{50} = 265.8 μg/ml). Among the tanshinones, only dihydrotanshinone significantly inhibited CYP2D6 activity (IC_{50} = 35.4 μM), compared to quinidine, a specific CYP2D6 inhibitor (IC_{50} = 0.9 μM). Crytotanshinone, tanshinone I and tanshinone IIA produced weak inhibition, with IC_{20} of 40.8 μM, 16.5 μM and 61.4 μM, respectively. Water soluble components such as salvianolic acid B and danshensu did not affect CYP2D6-mediated metabolism. Enzyme kinetics studies showed that inhibition of CYP2D6 activity by the ethanolic extract of crude Danshen and dihydrotanshinone was concentration-dependent, with K_i values of 4.23 μg/ml and 2.53 μM, respectively, compared to quinidine, K_i = 0.41 μM. Molecular docking study confirmed that dihydrotanshinone and tanshinone I interacted with the Phe120 amino acid residue in the active cavity of CYP2D6 through Pi–Pi interaction, but did not interact with Glu216 and Asp301, the key residues for substrate binding. The logarithm of free binding energy of dihydrotanshinone (−7.6 kcal/mol) to Phe120 was comparable to quinidine (−7.0 kcal/mol) but greater than tanshinone I (−5.4 kcal/mol), indicating dihydrotanshinone has similar affinity to quinidine in binding to the catalytic site on CYP2D6.

Introduction

Danshen (*Salvia miltiorrhiza*) has been widely used in China and other countries for the treatment of cardiovascular and cerebrovascular diseases (Wang et al., 2007; Zhou et al., 2005). Both the water-soluble and lipophilic compounds of *Salvia miltiorrhiza* root extract appear to improve the infarction/reperfusion-induced vascular damage synergistically (Han et al., 2008). However, the use of Danshen has been associated with a number of clinically important herb–drug interactions leading to adverse outcome (Holbrook et al., 2005; Izzo et al., 2005; Yu et al., 1997). Interaction of Danshen with warfarin may be mediated *via* both pharmacodynamic and pharmacokinetic mechanisms (Lo et al., 1992). Danshen exaggerated the pharmacological effects of warfarin by prolonging the prothrombin time and increased the bioavailability and decreased the elimination of warfarin in the rat (Chan et al., 1995). The major tanshinones of Danshen inhibited warfarin hydroxylation and increased the steady-state plasma warfarin concentration (Wu and Yeung, 2010). The recent finding was in line with those from previous studies in which Danshen altered the metabolism of R- and S-warfarin (Chan et al., 1995), reactions widely accepted as being mediated through CYP isoforms such as 1A2, 2C9 and 3A4.

Advances in separation techniques have enabled isolation and characterization of the active components of Danshen (Liu et al., 2006; Ma et al., 2006; Shi et al., 2005; Zhang et al., 2005), followed by extensive research to investigate the pharmacology and therapeutic potential of the individual components of the herb. Tanshinone IIA, one of the major lipid soluble components of Danshen, has been reported to inhibit CYP1A2 activity in mouse, human and rat *in vitro* (He et al., 2007; Ueng et al., 2003; Wang et al., 2009b; Wang and Yeung, 2011c). Other major tanshinones such as tanshinone I, cryptotanshinone and dihydrotanshinone also exhibited different modes of inhibition on human CYPs 1A2, 2C9, 2E1 and 3A4 *in vitro* (Wang et al., 2010a), rat CYP2C11 (human CYP2C9 equivalent) and CYP3A2 *in vitro* and *in vivo* (Wang et al., 2010b, 2010c; Wang and Yeung, 2011a, 2011b). The aqueous extract from Danshen has also been shown to affect both human and rat CYP1A2 activity *in vitro* and in the rat after chronic treatment (Wang and Yeung, 2010, 2011c).

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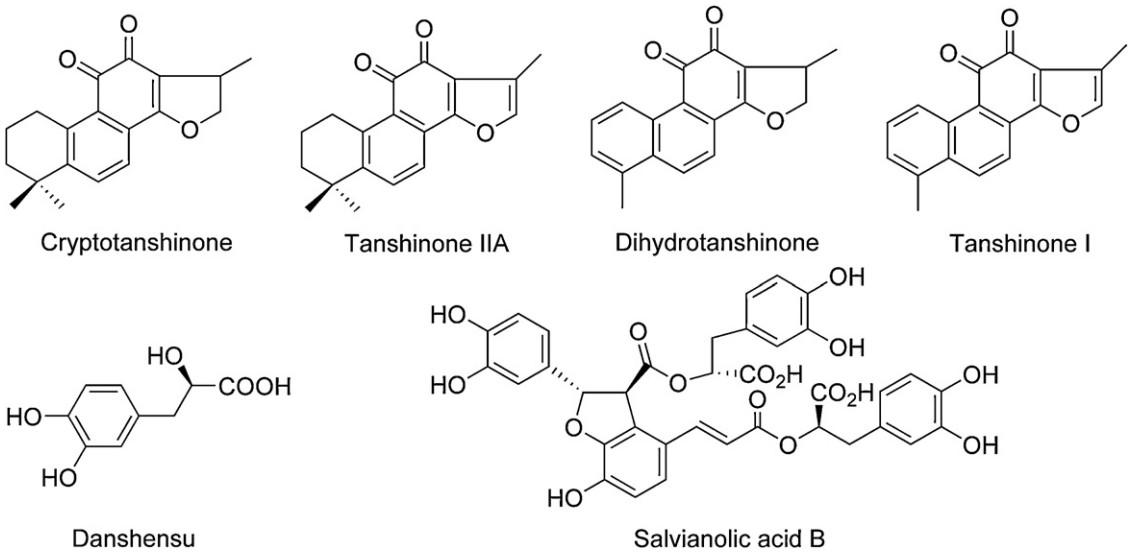

Fig. 1. Structures of the major constituents isolated from Danshen (*Salvia miltiorrhiza*).

CYP2D6 is involved in the metabolism of approximately 25–50% of clinically used drugs, including antidepressants, neuroleptics, tamoxifen, HMG-CoA reductase inhibitors, anti-emetics, anti-arrthymic drugs and beta-antagonists (De Gregori et al., 2010; Hsu, 2010; Prisant, 2008; Sideras et al., 2010; Vermes and Vermes, 2004; Wang et al., 2009a; Zhou, 2009). CYP2D6 is one of the highly polymorphic CYPs and the polymorphism can be translated into risk differences for drugs which are metabolised by these enzymes (Johansson and Ingelman-Sundberg, 2011). Approximately 5–14% of Caucasians, 0–5% Africans, and 0–1% of Asians lack CYP2D6 activity, and are known as poor metabolisers (Zhou et al., 2009). Given the widespread use of Danshen and Danshen-containing formulations, alone or in combination with many cardiovascular drugs which are CYP2D6 substrates, it would be of interest to study the effect of Danshen or its active components on CYP2D6 activity, especially when a fraction of the population being poor CYP2D6 metabolisers. The aim of this study was to investigate the effects of tanshinones (tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone), danshensu, and salvianolic acid B (Fig. 1) on rat CYP2D6 activity, using dextromethorphan as the model probe substrate *in vitro*. The study design was similar to interaction studies with other CYP isoforms in the rat (Wang et al., 2009b, 2010b, 2010c; Wang and Yeung, 2010). A molecular docking study was used to determine the free binding energy and binding simulations of the tanshinones on the binding to the catalytic site on CYP2D6, which could be helpful for the understanding of the interaction between the tanshinones and human CYP2D6.

Materials and methods

Materials

Pooled human liver microsomes (HLMs) were obtained from GenTest Corporation (Woburn, MA, USA) and stored at −80 °C until use. Danshen was supplied by Winsor Health Products Ltd. (Hong Kong). Dried Danshen root was purchased from Eu Yan Sang Limited (Hong Kong) where an initial screening test has been carried out that the batch of Danshen was free from other contaminants. Cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA, danshensu and salvianolic acid B were purchased from Chengdu Congon Bio-tech Co., Ltd. (China). Dextromethorphan, dextrorphan, quinidine, chlorpheniramine, β-nicotinamide adenine dinucleotide phosphate (NADP), D-glucose 6-phosphate, glucose 6-phosphate dehydrogenase, heparin sodium, urethane, and phenacetin were from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (HPLC Grade) was purchased from Labscan Analytical Sciences (Bangkok, Thailand). Methanol (HPLC Grade) was from BDH Laboratory Supplies (Poole, U.K.), ethyl acetate (HPLC grade) was from Fisher Chemicals (Leicester, U.K.). Acetic acid, glacial, (HPLC grade) was from Scharlau Chemie (Barcelona, Spain). Phenobarbitone sodium was obtained from Universal Pharmaceutical Lab. (Hong Kong). Carbon monoxide was supplied by Hong Kong Special Gas Co.

Preparation of aqueous and ethanolic extracts of Danshen root

Aqueous and ethanolic extract of crude Danshen were prepared as previously described (Lee et al., 2012). For aqueous extract, Danshen root (200 g) was cut into small pieces and boiled in 250 ml distilled water in reflux. After 1 h, the residue was mixed with distilled water (250 ml) and boiled for another hour. The filtrate was combined with the previous filtrate and cooled at room temperature. Water in the filtrate was removed by freeze-drying and about 35 g (17.5% of yield) of the aqueous extract powder was obtained. For ethanolic fraction, Danshen root (200 g) was minced and boiled in 95% ethanol (250 ml twice under reflex condition. The filtrate was collected and dried using a rotary evaporator with warming below 50 °C. The brown residue was re-dissolved in ethyl acetate. The ethyl acetate layer was collected and dried by a rotary evaporator. The reddish brown crystals finally obtained represented the ethanolic fraction in which the percentage yield was about 1%. The content of individual tanshinones and phenolic acids in Danshen pill, ethanolic fraction and aqueous fraction was analyzed by HPLC (Wang et al., 2010b, 2010c). The major active constituents present in extracts of Danshen pill, aqueous extract and ethanolic extract of crude Danshen are shown in Table 1.

Analysis of CYP2D6 (dextromethorphan O-demethylase) activity

Human liver microsome (1 mg/ml) was incubated in 0.05 M Tris/KCl buffer, pH 7.4 with NADPH-regenerating system (10 mM NADP, 5 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, and 5 mM Magnesium chloride). For inhibition study, 100 μM dextromethorphan was used. For kinetic study, dextromethorphan concentrations ranged from 50 μM to 400 μM. The concentrations of Danshen pill and water