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Inhibitory effect of tanshinones on rat CYP3A2 and CYP2C11 activity and its structure-activity relationship

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ABSTRACT

This study investigated the effect of tanshinones on rat liver microsomal CYP3A2 and 2C11 activity and explored the structure-activity relationship of tanshinones with CYP3A activity. Cryptotanshinone, tanshinone I and tanshinone IIA were competitive CYP3A2 inhibitors (K_i = 199–243 μM) and CYP2C11 inhibitors (K_i = 91–118 μM). Dihydrotanshinone was not only a noncompetitive inhibitor of CYP3A2 (K_i = 110 μM), but also a competitive CYP2C11 inhibitor (K_i = 55 μM). The structural difference between dihydrotanshinone and tanshinone I at C-15 position of furan ring resulted in the different modes of inhibition on CYP3A activity.

1. Introduction

Danshen, the dried root of *Salvia miltiorrhiza Bunge*, has been widely used in China, Japan, the United States of America and European countries for the treatment of cardiovascular and cerebrovascular diseases [1,2]. Diterpenoid tanshinons are major bioactive constituents of Danshen with notable pharmacological activities and the potential as new drug candidates against some important human diseases [2].

Cytochrome P450 (CYP) represents the major mechanism for the enhanced or reduced bioavailability of drugs when herbal/botanical products are co-administered [3]. In recent years, some studies have been reported on the effect of Danshen on CYP enzymes [4–11]. Our previous studies have shown that tanshinones inhibited warfarin metabolism in rats, which may be a possible mechanism for the interaction of Danshen with warfarin [12]. Recently we reported a Danshen extract containing major tanshinones that prolonged the hypnotic effects of midazolam, decreased the clearance of midazolam, and decreased CYP3A expression in the rat [13]. These results and potential mechanisms require further exploration. In fact, one of the major obstacles in the identification of different CYP isoforms involved in the metabolism of a drug is the lack of specific and simple enzymatic assays to distinguish the individual isoforms. The stereospecific hydroxylation of the steroid nucleus is a sensitive fingerprint for the identification of a specific CYP isoform [14]. The metabolism of testosterone has been used to probe *in vitro* preparations of rat liver for CYP isoform activity, including CYP2C11 and CYP3A2 [14–16].

The aim of this study was to investigate the effects of major tanshinones (tanshinone I, tanshinone IIA, dihydrotanshinone, and cryptotanshinone) (Fig. 1) on rat CYP3A2 and CYP2C11 activity, the major CYP isoforms to metabolize testosterone to 6β-hydroxytestosterone and 2α-hydroxytestosterone in the male rat liver. At the same time the structure–activity relationship of tanshinones with CYP activity was also explored.

2. Experimental

2.1. Animals

Male Sprague–Dawley rats (250–300 g) were supplied by the Laboratory Animal Service Center, The Chinese University

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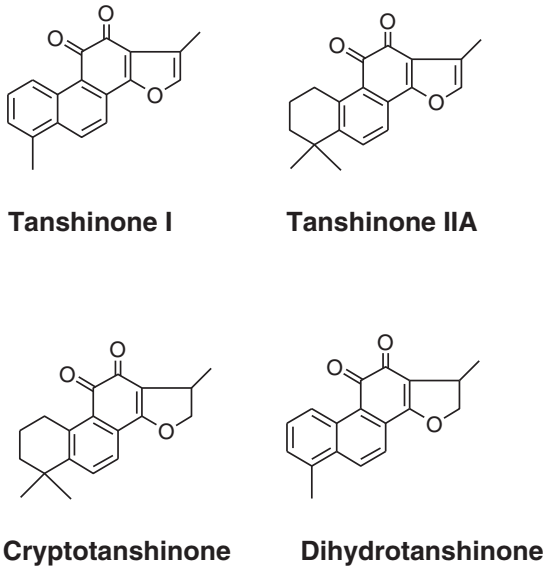

Fig. 1. Structures of the tanshinones from Danshen.

of Hong Kong. Animals were housed with alternating 12-hours light–dark cycles, 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.

2.2. Materials

Testosterone, 6β-hydroxytestosterone, corticosterone, dexamethasone, ketoconazole, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), β-nicotinamide adenine dinucleotide phosphate (NADP), and Tris–HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2α-hydroxytestosterone was purchased from Steraloids Inc. (Newport R.I., USA). Sulphaphenazole was from UFC Limited (Manchester, UK). Cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA (Fig. 1) were purchased from Chengdu Congon Bio-tech Co., Ltd. (China). Acetonitrile (HPLC grade) was purchased from Labscan Analytical Sciences (Bangkok, Thailand). Methanol (HPLC grade) was purchased from BDH Laboratory Supplies (Poole, England), ethyl acetate (HPLC grade) from Fisher Chemicals (Leicester, UK). Acetic acid glacial (HPLC grade) were from Scharlau Chemie (Barcelona, Spain). Carbon monoxide was supplied by Hong Kong Special Gas Co.

2.3. Preparation of rat liver microsomes

In this study, hepatic CYP3A2 activity was modulated by pre-treating the rats with the enzyme inducer dexamethasone (DEX) as reported previously [16]. Control- and DEX-treated rats were fasted overnight and killed by cervical dislocation before removal of the liver. The liver was excised, rinsed with ice-cold saline (0.9% NaCl w/v), weighed and homogenized in a 0.05 M Tris/KCl buffer (pH 7.4). The tissue homogenates were centrifuged at 10,000*g* at 4 °C for 25 min and the supernatants further ultracentrifuged at 105,000 *g* at 4 °C for 60 min. The final microsomal pellets were resuspended with 0.05 M Tris/KCl buffer (pH 7.4) and stored under liquid nitrogen until used. Total P450 content of the rat liver microsomes was determined from the sodium dithionite-reduced carbon monoxide difference spectrum, using a molar extinction coefficient of 91 cm^{-1} mM^{-1} [17]. Microsomal protein concentration was determined by a protein assay [18].

2.4. Testosterone hydroxylation assay

The effects of tanshinones on testosterone hydroxylation were evaluated by incubation with the individual tanshinones (6.25–100 μM) and testosterone (100 μM) in rat liver microsomes. Stock solution of tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone was dissolved in dimethyl sulfoxide (DMSO) and diluted with DMSO to prepare the final concentrations used (6.25–100 μM). The final concentration of DMSO in each incubation mixture was 0.4% (v/v). Control incubations for testosterone hydroxylation did not contain any tanshinones. Liver microsome (1 mg/ml) was incubated in 0.05 M Tris/KCl buffer (pH 7.4) with NADPH-regenerating system (1 mM NADP, 10 mM G6P, 2 U/ml G6PDH, and 5 mM magnesium chloride). The tubes were incubated in Eppendorf Themomixer at 800 rev/min, 37 °C. The reaction was initiated by adding NADP to the incubation mixture. After 10 min, the incubations were terminated by adding of 500 μl of ice-cold acetonitrile. The incubation tubes were then centrifuged at 13 000 *g* for 12 min to precipitate protein. The supernatant was collected and corticosterone (100 μg/ml, 10 μl) was added as the internal standard. The whole mixture was then extracted with 500 μl ethyl acetate at 1400 rev/min in Thermomixer for 30 min at 25 °C. The tubes were then centrifuged at 8000*g* for 8 min. The organic layer was dried under a gentle stream of nitrogen, resuspended in 120 μl methanol, with 50 μl used in HPLC analysis.

2.5. Inhibition kinetics studies of CYP3A2 (testosterone 6β-hydroxylase) and CYP2C11 (testosterone 2α-hydroxylase) activity

Tanshinones (tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone) (6.25–100 μM) and testosterone (50–400 μM) were used in the inhibition kinetics studies. Ketoconazole (1–10 μM), a selective CYP3A inhibitor, was used as positive control of testosterone 6β-hydroxylation. Sulphaphenazole (6.25–50 μM), a selective rat CYP2C11 inhibitor, was used as a positive control of testosterone 2α-hydroxylation. In all other assays, reactions were initiated by the addition of the NADPH generating system. The incubation reactions were performed as described in the previous section.

2.6. High-performance liquid chromatography (HPLC) analysis of testosterone and its metabolites

Testosterone and its metabolites were measured by HPLC as described previously with modifications [19]. The HPLC system consisted of a Hewlett-Packard 1050 Series instrument with UV detection at 245 nm. A 4.6 × 150 mm reversed-