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Tanshinone I increases CYP1A2 protein expression and enzyme activity in primary rat hepatocytes

Wayne Y.W. Lee, Xuelin Zhou, Penelope M.Y. Or, Yiu Wa Kwan, John H.K. Yeung*

School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

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ABSTRACT

This study investigated the effects of Danshen and its active ingredients on the protein expression and enzymatic activity of CYP1A2 in primary rat hepatocytes. The ethanolic extract of Danshen roots (containing mainly tanshinones) inhibited CYP1A2-catalyzed phenacetin O-deethylation (IC_{50} = 24.6 μg/ml) in primary rat hepatocytes while the water extract containing mainly salvianolic acid B and danshensu had no effect. Individual tanshinones such as cryptotanshinone, dihydrotanshinone, tanshinone IIA inhibited the CYP1A2-mediated metabolism with IC_{50} values at 12.9, 17.4 and 31.9 μM, respectively. After 4-day treatment of the rat hepatocytes, the ethanolic extract of Danshen and tanshinone I increased rat CYP1A2 activity by 6.8- and 5.2-fold, respectively, with a concomitant up-regulation of CYP1A2 protein level by 13.5- and 6.5-fold, respectively. CYP1A2 induction correlated with the up-regulation of mRNA level of aryl hydrocarbon receptor (AhR), which suggested a positive feedback mechanism of tanshinone I-mediated CYP1A2 induction. A formulated Danshen pill (containing mainly danshensu and salvianolic acid B and the tanshinones) up-regulated CYP1A2 protein expression and enzyme activity, but danshensu and salvianolic acid B, when used individually, did not affect CYP1A2 activity. This study was the first report on the Janus action of the tanshinones on rat CYP1A2 activity.

Introduction

Natural products are commonly used by over 1.5 billion people all over the world as they are often considered safe (Hosbach et al. 2003), despite the risks of potential herb–drug interactions (Foster et al. 2005). Cytochrome P450 enzymes (CYPs) play a central role in drug–drug interactions and herb–drug interactions as CYP induction often leads to treatment failure and CYP inhibition leads to adverse drug reactions (Bachmann et al. 2003). The biological impact of CYP1A induction, apart from potential in reduction of the therapeutic effects of clinical drugs which are CYP1A substrate, is believed to impose additional risks of carcinogenesis (Ma and Lu 2007; Zhou et al. 2009). CYP1A2, a constitutive drug-metabolizing enzyme in liver, is of particular interest because of its crucial role in chemical carcinogenesis and its susceptibility to induction at transcriptional and translational levels. Induction of CYP1A2 is regulated by aryl hydrocarbon receptor (AhR) which mediates CYP1A2 gene transcription through dioxin response element.

Interactions between drugs and herbal medicines have been reported intermittently. A number of natural products have been demonstrated to elevate mRNA and protein levels of CYP1A2 in cultured human cell lines and in rodents (Jang et al. 2004; Brandin et al. 2007). *Salvia miltiorrhiza* Bunge (Danshen) is a popular Chinese herbal medicine used for the treatment of cardiovascular diseases, inflammatory diseases and chronic hepatitis. Despite its versatile medicinal purposes, Danshen extract is rarely reported to cause clinical problem when used concomitantly with orthodox drugs, except warfarin (Hu et al. 2005). However, as more pharmacological properties of Danshen and its major active ingredients, phenolic acids and tanshinones have been reported (Wang et al. 2007), the potential beneficial/harmful effects of concomitant use of these compounds and orthodox drugs must be taken into consideration. The effects of Danshen and its active ingredients on the activities of different CYP enzymes have been investigated. Previous studies have shown that major tanshinones competitively inhibited the metabolism of model CYP1A2 probe substrates *in vitro* and *in vivo* in the rat (Wang et al. 2009). The inhibitory effect of Danshen on CYP1A2 was mainly attributed to its lipid-soluble components, when tanshinone IIA, tanshinone I and cryptotanshinone have been shown to be competitive inhibitors to mice and human liver microsomes, while the water-soluble components were much less potent in term of CYP inhibition (Ueng et al. 2003; Qiu et al. 2008b; Wang and Yeung 2010). This discrepancy may partially explain why Danshen decoction, with the phenolic acids as major constituent, did not affect the catalytic activity of CYP1A2 in clinical study (Qiu et al. 2008a). However, a recent report showed that the up-regulatory effect of tanshinones on CYP1A1/2 in human HepG2 hepatoma cell line at both transcriptional and translational

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

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level (Zhang et al. 2011), suggesting a more complicated mode of herb–drug interaction by Danshen. CYP1A2 is highly conserved among species (Martignoni et al. 2006) and primary rat hepatocytes model represents a suitable alternative to primary human hepatocytes, which is limited by its scarce availability, and could give good predictive value for human herb–drug interaction involving CYP1A2. In this study, primary rat hepatocytes were used, both in suspension and in culture, to investigate the effects of different Danshen preparations and the individual active ingredients on rat CYP1A2.

Materials and methods

Animals

Male Sprague Dawley rats (260–280 g) were supplied by the Laboratory Animal Service Centre, The Chinese University of Hong Kong (CUHK). The rats were kept in a 12-h light–dark cycle holding room, and rat chow (Glen Forrest Stockfeeders, Australia) and tap water *ad libitum*. All the experiments 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.

Chemicals and reagent

Cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA, danshensu and salvianolic acid B were purchased from Chengdu Cogon Bio-tech Co. Ltd. (Sichuan, People's Republic of China). The purities of these compounds as determined by HPLC–MS and NMR by the supplier were all above 97%. Dried Danshen root was purchased from Eu Yan Sang Ltd. (HKSAR). Danshen pill was given by Winsor Health Products Ltd. (HKSAR). Acetonitrile (HPLC grade) was purchased from Labscan Analytical Science (Bangkok, Thailand). Methanol (HPLC grade) was from BDH Laboratory Supplies (Poole, UK), ethyl acetate (HPLC grade) was from Fisher Chemicals (Leicester, UK), and acetic acid (HPLC grade) was from Scharlau Chemie (Barcelona, Spain). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), antibiotics and antimycotics, and trypsin were purchased from Gibco (Grand Island, NY). Unless otherwise specified, all chemicals used in this study were purchased from Sigma (St. Louis, MO).

Preparation of aqueous and ethanolic extracts of Danshen root

For preparation of the 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 additional 250 ml distilled water and boiled for one more hour. The filtrate was combined with the previous filtrate and allowed to cool 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 the ethanolic fraction, 200 g Danshen root was minced and boiled in 250 ml 95% ethanol twice under reflex condition. Filtrate was collected and dried using a rotary evaporator with warming no more than 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 obtained finally represented the ethanolic fraction in which the percentage yield was about 1%. The content of individual tanshinones and phenolic acid in Danshen pill, ethanolic fraction and aqueous fraction was analyzed by HPLC and summarized in Table 1.

Table 1
Validation of HPLC analysis of the active ingredients of Danshen. Accuracy, intra- and inter-day precision, Lower limit of quantification (LLOQ) and limit of detection (LOD) of in-house HPLC-DAD on cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA, danshensu and salvianolic acid B ($n = 6$).

	Accuracy (% bias)	Intraday (% RSD)	Interday (% RSD)	LLOQ (μg/ml)	LOD (μg/ml)
Cryptotanshinone	0.413	0.5	3.09	1.48	0.037
Dihydrotanshinone	−0.128	0.4	2.66	1.39	0.035
Tanshinone I	−0.343	1.573	3.85	1.38	0.035
Tanshinone IIA	3.785	1.03	3.958	1.47	0.074
Danshensu	2.368	0.538	1.308	1.1	0.44
Salvianolic acid B	2.913	0.915	1.178	3.6	0.36

RSD, relative standard deviation.

Preparation of stock solution

Danshensu and salvianolic acid B were dissolved in distilled water to a concentration of 10 mM, and ground Danshen pill and aqueous fraction of Danshen were dissolved in PBS to a concentration of 50 mg/ml and filtered through sterile 0.22 μm syringe filter (Asahi Techno Glass Co., Japan). The tanshinones and 3-MC were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mM, and ethanolic fraction of Danshen was dissolved in DMSO to a concentration of 20 mg/ml. All the stock solution was freshly prepared for daily use. The final concentration of DMSO was 0.1% (v/v) throughout the experiments.

Isolation of rat hepatocytes

The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Alfasan, Woerden, Holland) (50 mg/kg) and immobilized on surgical board with their sternum facing upward. The abdomen was rinsed thoroughly with 75% ethanol before a midline incision from abdominal cavity to umbilicus was made to expose liver. Liver cells were isolated by a two-step collagenase perfusion technique described previously (Kreamer et al. 1986), with slight modification. Briefly, loose ligatures were placed around the right renal vein and artery, and bile duct. The thoracic cavity was opened, and a cannula (O.D. ≈ 1.7 mm) was then inserted into the inferior vena cava. The cannula was securing before initiating perfusion of the liver with Ca^{+}-free perfusion buffer (6.3 g/l NaCl, 0.32 g/l KCl, 0.13 g/l $MgSO_4$, 0.15 g/l KH_2PO_4, 1.81 g/l $NaHCO_3$, 3.58 g/l HEPES, 1.5 g/l glucose, 0.038 g/l EDTA, pH 7.2) at 37 °C, gassed with 95% O_2–5% CO_2. After 10 min perfusion, a cannula (O.D. ≈ 2.08 mm) was inserted into the hepatic portal vein to serve as an outlet. The loose ligatures placed previously were tightened to accomplish efflux. Once the liver became pale yellow in color, it was perfused with sterile collagenase (Type IV) (0.01%, w/v) containing-digestion buffer (6.3 g/l NaCl, 0.32 g/l KCl, 0.13 g/l $MgSO_4$, 0.15 g/l KH_2PO_4, 1.81 g/l $NaHCO_3$, 3.58 g/l HEPES, 1.5 g/l glucose, 0.58 g/l $CaCl_2 \cdot H_2O$, pH 7.2) at 37 °C, gassed with 95% O_2–5% CO_2 for further 20 min. Subsequently, the Glisson's capsule was opened in chilled DMEM supplemented with 1% (v/v) bovine serum albumin (BSA) (i.e. washing medium). The digested liver was agitated or combed to release the dissociated cells. The cell suspension was filtered through a stainless steel mesh, then a layer of sterile gauze and finally a 100 μm filter (Millipore, Billerica, MA). Hepatocytes were collected by low-speed centrifugation (50 g) in percoll (GE Healthcare, Uppsala, Sweden) containing washing medium in which heavier viable hepatocytes were separated from nonviable hepatocytes and nonparenchymal cells. Harvested hepatocytes were subjected to trypan blue exclusive assay. Batch of hepatocytes having 88% or above was used in subsequent experiments.

Culturing of rat hepatocytes

Freshly isolated rat hepatocytes (2×10^5 cells/cm^2) in a plating medium of DMEM supplemented with 10% (v/v) FBS, 1% (v/v) antibiotics and antimycotics (Gibco, Grand Island, NY), 100 nM dexamethasone, pH 7.4, for 3 h. This plating medium was replaced by culture medium thereafter (DMEM supplemented with 0.1% (v/v) BSA, 1% (v/v) antibiotics and antimycotics, 100 nM dexamethasone, pH 7.4) (i.e. culture medium) (LeCluyse et al. 2005).

Acute study using primary rat hepatocytes in suspension

The freshly isolated hepatocytes were resuspended in culture medium. Then, the hepatocytes (1×10^6 in 300 μl medium) were preincubated with modulators for 10 min at 37 °C with gentle shaking (800 rpm) in a thermomixer (Eppendorf, Barkhausenweg, Hamburg, Germany). 250 μl hepatocytes suspension was collected, mixed with 100 μM phenacetin and incubated for further 30 min at 37 °C with gentle shaking. The reaction was stopped by adding 250 μl acetonitrile, 250 μl ethyl acetate and 10 μl warfarin as internal standard (100 μg/ml in methanol). The mixture was shaked vigorously in a thermomixer at 25 °C, 1400 rpm for 30 min. A clear upper organic layer was collected after centrifugation at 13,000 rpm for 10 min and transferred to a test tube. The samples were dried with nitrogen gas and subjected to HPLC analysis.

4-Day treatment in cultured rat hepatocytes

In this study, a prolonged 4-day incubation period was selected to investigate CYP1A2 induction in the cultured rat hepatocytes. It has been suggested that at least 3-day and 2-day incubation period is required for the study of enzyme activities and mRNA expression level, respectively (Hewitt et al. 2007). Freshly isolated hepatocytes were seeded on 6-well plate pre-coated with rat tail collagen (BD Bioscience, Franklin Lakes, NJ). The hepatocytes were cultured in the presence of plating medium for 3 h at 37 °C in 5% CO_2. After incubation for 2 days, the differentiated hepatocytes were subjected to treatment of either Danshen extracts or tanshinones or phenolic acids or 3-MC or DMSO which served as vehicle control in culture medium at 37 °C in 5% CO_2. Throughout treatment, culture medium was replenished once for every 2 days. After 4 consecutive days of treatment, the culture medium was replaced by 500 μl fresh culture medium, which was supplemented with 100 μM phenacetin, and incubated for 4 h further at 37 °C in 5% CO_2. The reaction was stopped by adding 500 μl acetonitrile, 500 μl ethyl acetate and 10 μl warfarin (100 μg/ml in methanol) and same extraction procedure described previously was followed.

HPLC analysis of phenacetin and metabolite

CYP1A2 activity was assessed by formation of paracetamol from phenacetin by the method reported previously, with modifications (Wang and Yeung 2010; Wang et al. 2010a). HPLC analysis of phenacetin and paracetamol was performed on Agilent 1100 Series HPLC with DAD coupled. Phenacetin, paracetamol and warfarin (internal standard) were separated on an Alltech Allsphere ODS-2 C18 5 μm (4.6 mm × 250 mm) protected by XDB-C18 guard column. A gradient elution of A (1.5% acetic acid in water, pH 4.7) and B (acetonitrile) was used, commencing with 20% B, rising to 60% B on 5 min and then to 80% B on 7 min. The flow rate was 0.7 ml/min at 40 °C.

Reverse-transcriptase PCR

Rat hepatocytes were collected by trypsinization and washed twice with chilled PBS. Total RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's protocols and treated with DNase I to eliminate genomic DNA contamination. The concentration and purity of RNA was determined by SmartSpec™ Plus Spectrophotometer (Bio-rad, Hercules, CA). One microgram of total RNA was incubated in the presence of 1 μl oligo-d$(T)_{20}$ primer (0.5 μg/μl), 1 μl dNTPs (10 mM) (Invitrogen) at 65 °C for 5 min. Reverse transcription was performed using Superscript III with reverse transcription buffer, dithiothreitol and 1 μl RNaseOUT™ Recombinant Ribonuclease inhibitor (40 units/μl) (Invitrogen) at 50 °C for 50 min, and the reaction was inactivated by heating the mixture at 70 °C for 15 min. cDNA (1 μg for *GAPDH*, 3 μg for *CYP1A2*, 1 μg for *AhR*) was used for PCR amplification with FastStart High Fidelity PCR System (Roche). Primers (forward/reverse sequences) designed and utilized were as follows: CYP1A2 (5′-GAATCGGTGGCTAACGTCAT-3′/5′-CCAGAAGATGGCTGTTGTGA-3′); AhR (5′-GGGATCGATTTCGAAGACATCAG-3′/5′-AACGCCTGGGAGCCTGGAATCTC-3′) (Zhou et al. 2008) and GAPDH (5′-ACCACAGTCCATGCCATCAC-3′/5′-TCCACCACCCTGTTGCTGTA-3′) (Fossey et al. 2009). Annealing temperatures for CYP1A2, AhR and GAPDH were 53, 56, 55 °C, respectively. After amplification, the PCR products were subjected to electrophoresis through 1.5% agarose gels (Bio-rad) preloaded with GelRed (Biotium Inc., Hayward, CA) and visualized using Gel Doc XR system (Bio-Rad).

CYP1A2 protein expression

Total soluble protein in the rat hepatocytes was prepared as previously described (Lee et al. 2009). 40 μg proteins were subjected to 8% SDS-PAGE, and the membrane was incubated with 1:4000 primary antibody against CYP1A2 (Santa Cruz, Santa Cruz, CA), CYP3A4 (Affinity Bioreagents, Rockford, IL), CYP2C11, CYP2D6, CYP2E1 (Chemicon, Billerica, MA) overnight at 4 °C, followed by incubation in 1:2000 HRP-conjugated anti-mouse secondary antibody (Dako, Glostrup, Denmark) for 1 h. The membrane was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Chemicon, Billerica, MA) as loading reference. Densitometric analysis was performed with Scion software.

Cell viability

Hepatocytes after 4-day pretreatment vehicle, or 3-MC, or tanshinone I were incubated in the presence of 250 μM tacrine for additional 24 h. After that, the cells were incubated with 100 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT, 0.5 mg/ml) for another 3 h at 37 °C. Formazan crystals were then dissolved in DMSO and quantified spectrophotometrically at 570 nm.

Statistical analysis

All results were presented as the means ± standard error of mean. Statistical analysis of data having equal variance was carried out by one-way or two-way analysis of variance (ANOVA) followed by Tukey's Post Hoc test where appropriate. A value of $p < 0.05$ was considered statistically significant.

Results

Quantitative determination of tanshinones and phenolic acids

The contents of the tanshinones and the phenolic acids in Danshen pill, aqueous fraction and ethanolic fraction of crude Danshen were determined by HPLC–DAD. As summarized in Table 2, salvianolic acid B, among the six compounds of interest, is the most abundant ingredients in the Danshen extracts from different ways of preparation. Danshensu, another phenolic acid, was the second

Table 2
Major components in Danshen extract. The quantity and percentage of six active ingredients per gram of Danshen pill, aqueous fraction and ethanolic fraction of crude Danshen determined by HPLC-DAD was summarized. Data is expressed as mean ± SD ($n = 3$).

	Danshen pill	Aqueous fraction	Ethanolic fraction
Cryptotanshinone	45.6 ± 2.37 μg/g	34.6 ± 0.86 μg/g	36.8 ± 0.56 mg/g
	0.0046 ± 0.0002%	0.0035 ± 0.0000%	3.682 ± 0.0559%
Dihydrotanshinone	99.4 ± 2.03 μg/g	12.7 ± 0.46 μg/g	9.3 ± 0.12 mg/g
	0.0099 ± 0.0002%	0.0012 ± 0.0000%	0.9290 ± 0.0123%
Tanshinone I	26.6 ± 1.55 μg/g	10.4 ± 0.40 μg/g	17.9 ± 0.32 mg/g
	0.0027 ± 0.0002%	0.0010 ± 0.0000%	1.793 ± 0.0317%
Tanshinone IIA	17.5 ± 1.15 μg/g	22.6 ± 0.51 μg/g	118.4 ± 2.91 mg/g
	0.0017 ± 0.0001%	0.0023 ± 0.0000%	11.84 ± 0.291%
Danshensu	28.7 ± 0.32 mg/g	3.1 ± 0.03 mg/g	2.7 ± 0.08 mg/g
	2.871 ± 0.032%	0.312 ± 0.003%	0.268 ± 0.0075%
Salvianolic acid B	38.5 ± 0.73 mg/g	37.3 ± 0.50 mg/g	209.3 ± 5.48 mg/g
	3.846 ± 0.073%	3.731 ± 0.050%	20.93 ± 0.548%

%, Percentage per 1 g of crude Danshen.

predominant compound in Danshen pill, but was less abundant in the in-house prepared extracts of Danshen root. The content of the four tanshinones were concentrated by ethanolic extraction in which tanshinone IIA was concentrated by more than 5239-fold, and 1721-fold for tanshinone I, 1064-fold for cryptotanshinone, and 732-fold for dihydrotanshinone I.

Effects of acute treatments on CYP1A2 activity in primary rat hepatocytes

Fig. 1 showed the effects of the Danshen extracts and the compounds on CYP1A2-catalyzed phenacetin O-deethylation after 1 h treatment. Furafylline (a selective CYP1A2 inhibitor), cryptotanshinone, dihydrotanshinone and tanshinone IIA inhibited CYP1A2 activity in a concentration-dependent manner from 3.13 to 50 μM, and the calculated concentration at which 50% of CYP1A2 activity being inhibited (IC_{50}) was 17.8, 12.9, 17.4 and 31.9 μM, respectively. Dihydrotanshinone at 50 μM almost inhibited the activity completely. Tanshinone I was less effective in term of inhibition, but still inhibited the activity by 30% at 50 μM. While ethanolic fraction of crude Danshen (3–50 μg/ml) inhibited CYP1A2 activity concentration- dependently with the IC_{50} = 24.5 μg/ml. Except for danshensu (50 μM), the two phenolic acids, Danshen pill and aqueous extract of crude Danshen did not exhibit any inhibitory effect on the catalytic activity of CYP1A2 in primary rat hepatocytes.

Effects of 4-day treatment of tanshinones on the metabolic activity, mRNA and protein level of CYP1A2 in primary rat hepatocytes

Fig. 2a showed the change of CYP1A2 in transcriptional level after 4-day treatment. 3-MC up-regulated *CYP1A2* mRNA level by 2-fold significantly, while dihydrotanshinone, tanshinone I and the ethanolic fraction exhibited considerable upregulation. The protein expression level of CYP1A2 with 4-day treatment was shown in Fig. 2b. 3-MC induced 26-fold increment of CYP1A2 expression, while tanshinone I, Danshen pill and ethanolic fraction of crude Danshen apparently up-regulated the protein level by 13.5-, 9.2- and 6.5-fold, respectively, although not statistically significant. Fig. 2c showed the fold change in CYP1A2 catalytic activity relative to the control group after 4-day treatment with various agents. Treatment with 3-MC and tanshinone I augmented CYP1A2 activity significantly by 11.8- and 6.8-fold, respectively. Dihydrotanshinone, Danshen pill and ethanolic fraction of crude Danshen increased CYP1A2 activity but statistically insignificant. A correlation study was performed in which there was a moderate correlation between CYP1A2 activity and its mRNA level (r^2 = 0.745), while the correlation between the activity and the protein level was strong (r^2 = 0.952). This simply implied a better predictor power from protein expression to the CYP1A2 activity *in vitro* as shown in this study. Furthermore, it was found that tanshinone I and 3-MC up-regulated the transcriptional level of AhR by 2-fold or more, simultaneously and significantly (Fig. 2d).

Concentration-course study of tanshinone I in vitro

Three different concentrations (0.1–10 μM) of tanshinone I was applied to the cultured primary rat hepatocytes for four consecutive days. CYP1A2 metabolic activity and its protein level were then evaluated. 3-MC (10 μM) was used as positive control for CYP1A2 induction. There were significant increases in the CYP1A2 protein level and enzyme activity at 10 μM tanshinone I treatment, but not at 0.1 μM treatment, and there was slight increases in both metabolic activity and protein level of CYP1A2 at 1 μM tanshinone I treatment (Fig. 3a and b). A simultaneous analysis indicated a 10-fold increase in the protein level of Nrf2 at 10 μM of 3-MC treatment which was not noticed at any concentration of tanshinone I treatment (Fig. 3c and d).

Augmentation of tacrine cytotoxicity by tanshinone I

Tacrine, a CYP1A2-activated hepatocytotoxic agent (Meng et al. 2007), was used to evaluate the impact of tanshinone I-mediated CYP1A2 upregulation in primary rat hepatocytes. As shown in Fig. 4, the hepatocytes pretreated with 10 μM 3-MC or tanshinone I were more susceptible to tacrine-induced cell death as determined by trypan blue exclusion assay.

Discussion

Previous studies have shown that the tanshinones competitively inhibited CYP1A2 activity with varying effectiveness, with the IC_{50} of cryptotanshinone, dihydrotanshinone, tanshinone I, and tanshinone IIA on CYP1A2 activity at 4.07, 3.64, 22.6, and 23.8 μM, respectively (Wang et al. 2009). Unlike intact primary cell culture, the boundary-free rat liver microsomal system displayed a greater susceptibility for inhibition by the tanshinones. The discrepancy of this, and the previous study using enzyme preparations, may be due to the presence of cell membrane, membrane transporters, phase II metabolizing enzymes and so on in the cellular system. The sole and enriched CYP activity in microsomes has limited its quantitative estimations of *in vivo* drug metabolism (Brandon et al. 2003). The most unexpected finding was the low inhibitory effect of tanshinone I in primary cell culture. This implies the low availability of tanshinone I at microsomes which might be due to the binding to cytosolic AhR. Untill now, there remains a lack of *in vivo* study on the inhibitory effects of individual tanshinones on CYP1A2, which is probably because of the expensive cost that would be required.

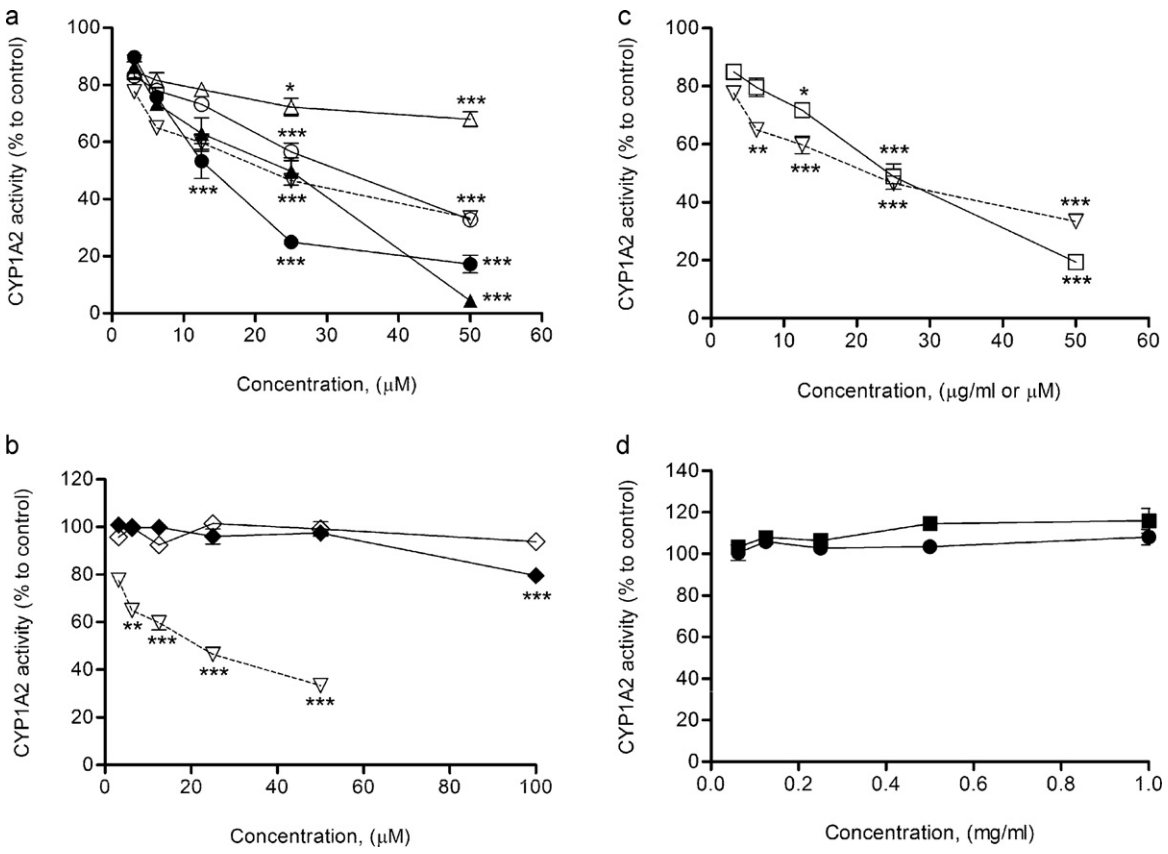

Fig. 1. Acute effects of test compounds on phenacetin O-deethylase (CYP1A2) activity. The hepatocytes in suspension were treated with cryptotanshinone (●), dihydrotanshinone (▲), tanshinone I (△), tanshinone IIA (○), furafylline (▽), danshensu (♦), salvianolic acid B (◊), Danshen pill (●), ethanolic fraction of Danshen (□) or aqueous fraction of Danshen (■) for 30 min. Data, relative to the control, are means ± SE of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significance compared with lowest concentration treatment group.

Thus, the inhibitory potency and order of the tanshinones obtained from this study may provide additional information on how the tanshinone–drug interaction may be estimated *in vivo*.

The result from the enzyme induction study was consistent with a recent study in which tanshinone I up-regulated CYP1A2 mRNA expression in a human hepatoma cell line (HepG2) (Zhang et al. 2011), although the primary rat hepatocytes were less responsive to tanshinone I in terms of CYP1A2 induction in this study. The use of HepG2 cell line for CYP1A induction study has been supported previously (Wilkening et al. 2003), although it is arguable that HepG2 cells behave differently from primary human hepatocytes (Zhang et al. 2006). Nevertheless, result in this study was the first piece of evidence showing the increase in tacrine-induced cytotoxicity in tanshinone I-pretreated rat hepatocytes, an issue not previously addressed.

The present study showed the biphasic effect of tanshinone I in acute and sub-chronic treatments on the catalytic activity of CYP1A2 at mRNA, protein and enzyme activity levels. However, without a pharmacokinetic profile of the individual component concerned, it will still be difficult to predict and justify *in vivo* CYP induction from *in vitro* potency. For instance, there is lack of clinical data in supporting the notions of omeprazole-enhanced detoxification of aflatoxin B_1 and omeprazole-induced toxicity of phenacetin (Ma and Lu 2007), which could be a matter of dosage because another study reported that omeprazole at therapeutic dose (40 mg) failed to induce CYP1A2, but caused induction at a higher dose (120 mg) (Rost et al. 1994). Generally, Danshen is given to patients in decoction form which is rich in phenolic acids, while lipid soluble compounds like tanshinones are often the minor components. Due to the relatively poor absorption of tanshinones (Zhou et al. 2005; Hao et al. 2006; Bi et al. 2007), the potential herb–drug interaction was overlooked in the past. However, with increasing evidence to indicate the potential therapeutic effect of tanshinone I on liver cancer (Lee et al. 2009, 2010), the manipulation of tanshinone I through direct administration via transcatheter arterial chemoembolization alone or concomitantly with other chemotherapeutics is reasonably expected in future study, and thus potential herb–drug interaction with tanshinone is a definite possibility.

Induction of CYP1A2 by tanshinone IIA via a typical AhR-mediated pathway in a C57BL/6J mouse model was reported previously (Ueng et al. 2004). However, the effects of the other tanshinones on CYP1A2 remains poorly understood. CYP1A induction was observed with polycyclic aromatic hydrocarbon (PAH) which enhanced its own metabolism (Conney 2003). The list of possible CYP1A2 inducers has been lengthened to involve molecules which are structurally distinct from PAH (Nagy et al. 2002). 3-MC, a potent CYP1A inducer, was capable of up-regulating CYP1A activity and expression in human and rat models (Kahn et al. 1987; Runge et al. 2000). Structurally, the tanshinones resemble PAH and it was not surprised to notice their capability of inducing CYP1A2 activity. Induction of CYP1A2 is mediated through the hetero-dimerization of ligand-activated AhR in cytosol and aryl hydrocarbon receptor nuclear translocator (ARNT) in nucleus. The activated AhR is vulnerable to degradation by ubiquitin-cytoplasmic proteasome pathway, thus sustained concentration of the AhR is believed to be a determinant factor to cellular response regarding ligand binding (Pascussi et al. 2004). The four tanshinones share a similar structure skeleton (Wang et al. 2007), but only dihydrotanshinone and tanshinone I induced considerable transcriptional up-regulation of CYP1A2, and tanshinone I-treated hepatoyctes were capable of

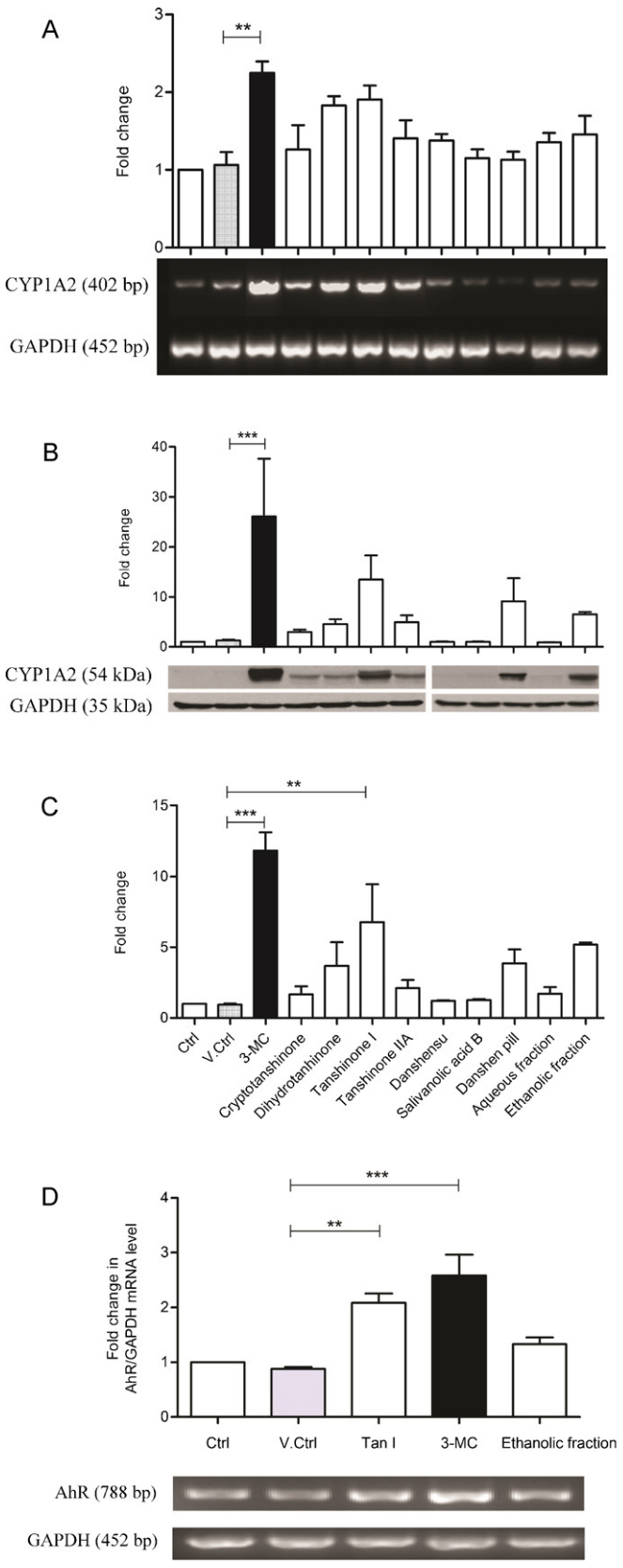

Fig. 2. Effects of tested compounds on the phenacetin O-deethylase (CYP1A2) activity at (a) mRNA level, (b) protein expression, and (c) CYP1A2 after 4-day treatment. The applied concentration was 10 μM for individual compound, 1 mg/ml for Danshen pill and aqueous fraction of Danshen, 50 μg/ml for ethanolic fraction of Danshen. A concomitant change of AhR mRNA level in treatment groups of tanshinone I, 3-MC and ethanolic fraction of Danshen was monitored (D). 0.1% (v/v) DMSO was used as vehicle control. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significance.

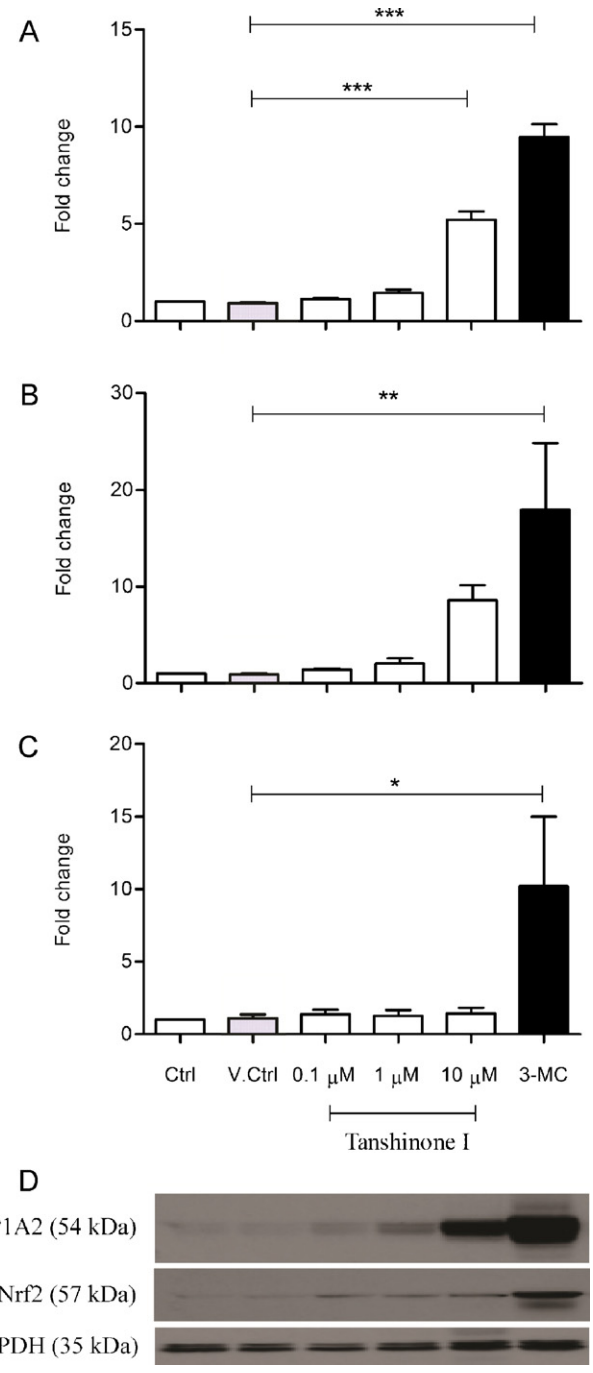

Fig. 3. Concentration-course study on the effects of tanshinone I on the phenacetin O-deethylation activity (a) and protein expression (b) of CYP1A2 after 4-day treatment. A concomitant measurement of total Nrf2 in the cultured hepatocytes was carried out (c and d). 0.1% (v/v) DMSO was used as vehicle control. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significance.

proceeding this epigenetic regulation although the degree is modest compared to 3-MC treatment. Indeed, the AhR pathway has been suggested to have cross-talk with other transcriptional factors and signal transduction pathways, including hypoxia-inducible factor 1α (Semenza 2001), AhR repressor (Mimura et al. 1999), Sp-1-like family of transcription factors (Kaczynski et al. 2002), nuclear factor 1 (NF1) (Zhang et al. 2000) (Le Ferrec et al. 2002). Diydrotanshinone was shown to be a stronger oxidative stress inducer than tanshinone I (Lee et al. 2008, 2009) and oxidative stress was speculated to down-regulate human CYP1A1 expression through NF1 (Morel and Barouki 1998; Morel et al. 2000). It is speculated that

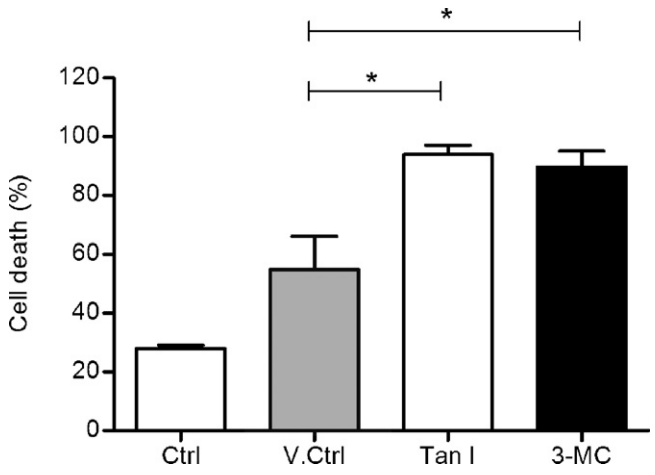

Fig. 4. Effects of pretreatment of tanshinone I and 3-MC at 10 μM on tacrine-induced cytotoxicity. 0.1% (v/v) DMSO was used as vehicle control. *$p < 0.05$ indicate significance.

the oxidative stress inducing property of dihydrotanshinone might compensate its induction potential on CYP1As, and thus unable to sustain the activation of AhR and subsequent up-regulation of CYP1A2. Furthermore, there was an increase in the mRNA level of AhR in cultured hepatocytes treated with tanshinone I and the ethanolic fraction of crude Danshen, which was comparable to previous report (Yanagiba et al. 2008) and thus implied the involvement and positive feedback regulatory role of AhR in tanshinone I-mediated CYP1A2 induction. Compared to other studies in which tanshinone IIA was shown to be CYP1A2 inducer in mice (Kuo et al. 2006), this study identified a more potent CYP1A2 inducer in the Danshen extract. It is understandable that the potent CYP1A2 induction effect of tanshinone was often omitted because of its low content in aqueous extract of Danshen root. Therefore, the different preparations of Danshen with different proportion of tanshinones were tested for their regulatory effects on CYP1A2 in this study. The ethanolic fraction of crude Danshen exhibited similar effect as tanshinone I on CYP1A2. 50 μg/ml ethanolic fraction consisted of 6.2 μM cryptotanshinone, 1.7 μM dihydrotanshinone, 3.3 μM tanshinone I, 20.1 μM tanshinone IIA, 0.6 μM danshensu and 14.6 μM salvianolic acid B as detected by in-house HPLC. The presence of cryptotanshinone, dihydrotanshinone and tanshinone IIA contributed to the inhibitory effect on CYP1A2 activity at acute treatment, while tanshinone I was believed to participate in up-regulating CYP1A2 expression and enzyme activity to a moderate extent when compared with 10 μM tanshinone I treatment. Interestingly, the formulated Danshen pill, which was rich in danshensu and salvianolic acid B, could enhance the catalytic activity and protein expression of CYP1A2 after 4-day treatment, which have not been reported previously. Although these effects were not statistically significant, it implied danshensu and salvianolic acid B as possible regulatory agents on the expression and catalytic activity of CYP1A2.

Like many Chinese medicinal herbs, Danshen has clearly been shown that the concentrations of the active ingredients present in ethanol (or solvent) extract and in water decoctions differ, such that the pharmacological effects of the solvent and water extracts depended on the method of extraction used (Lam et al. 2006a,b, 2007, 2008). The water extract of Danshen contains primarily salvianolic acid B, danshensu, rosmarinic acid and small traces of tanshinones (Wagner in press). However, the lipid soluble tanshinones such as cryptotanshinone, dihydrotanshinone, tanshinone I and IIA, are present in relatively higher concentrations in ethanol and other solvent extracts. These tanshinones isolated from Danshen are currently under investigation for their apoptotic effects and anticancer potential (Lee et al. 2008, 2009, 2010), thus pointing to a new pharmacological effect with significance, despite the fact that the tanshinones may be involved in potential herb–drug interactions (Wang et al. 2010b; Wu and Yeung 2010; Wang and Yeung 2010, 2011).

In this study, the *in vitro* regulatory effect of the tanshinones and Danshen extracts from different preparations on rat CYP1A2 expression and enzyme activity was studied in acute treatment and 4-day treatment in rat hepatocytes. Cryptotanshinone, dihydrotanshinone and tanshinone IIA inhibited CYP1A2 catalytic activity, but did not influence CYP1A2 expression, but vice versa for tanshinone I. The structural similarities among the four tanshinones do not imply a similar property on CYPs induction and inhibition. The structural difference between dihydrotanshinone and tanshinone I at C-15 position of furan ring have resulted in the different modes of inhibition of CYP3A activity (Wang and Yeung 2011). This information may be particularly important for those studying the therapeutics effect of natural active compounds with similar chemical structure. The CYP induction/inhibition properties of tanshinone I was partly revealed in this study. Further study on the pharmacokinetic profile of tanshinone I is necessary to calculate its concentration in plasma and in various organs, thus to predict its potential effects on CYP1A2. The protocol using rat hepatocytes may be used to investigate the herb–drug interaction potentials of other herbal/natural products with CYP1A2.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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