




RESEARCH ARTICLE

REVISED Zuotai (β -HgS)-containing 70 Wei Zhen-Zhu-Wan differs from mercury chloride and methylmercury on hepatic cytochrome P450 in mice [version 2; peer review: 2 approved]

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Abstract

Background: Zuotai (mainly β -HgS)-containing 70 Wei-Zhen-Zhu-Wan (70W, *Rannasangpei*) is a famous Tibetan medicine for treating cardiovascular and gastrointestinal diseases. We have shown that 70W protected against CCl_4 hepatotoxicity. CCl_4 is metabolized via cytochrome P450 (CYP) to produce reactive metabolites. Whether 70W has any effect on CYPs is unknown and such effects should be compared with mercury compounds for safety evaluation.

Methods: Mice were given clinical doses of 70W (0.15-1.5 g/kg, po), Zuotai (30 mg/kg, po), and compared to HgCl_2 (33.6 mg/kg, po) and MeHg (3.1 mg/kg, po) for seven days. Liver RNA and protein were isolated for qPCR and Western-blot analysis.

Results: 70W and Zuotai had no effects on hepatic mRNA expression of Cyp1a2, Cyp2b10, Cyp3a11, Cyp4a10 and Cyp7a1, and corresponding nuclear receptors [aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor- α (PPAR α); farnesoid X receptor (FXR)]. In comparison, HgCl_2 and MeHg increased mRNA expression of Cyp1a2, Cyp2b10, Cyp4a10 and Cyp7a1 except for Cyp3a11, and corresponding nuclear receptors except for PXR. Western-blot confirmed mRNA results, showing increases in CYP1A2, CYP2B1, CYP2E1, CYP4A and CYP7A1 by HgCl_2 and MeHg only, and all treatments had no effects on CYP3A.

Conclusions: Zuotai and Zuotai-containing 70W at clinical doses had minimal influence on hepatic CYPs and corresponding nuclear receptors, while HgCl_2 and MeHg produced significant effects. Thus, the use of total Hg content to evaluate the safety of HgS-containing 70W is inappropriate.

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Reviewer Status 

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1

2

version 2

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report



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report



report

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Keywords

Zuotai, 70 Wei-Zhen-Zhu-Wan (Rannasangpei, Qishiwei), HgCl₂, MeHg, Cytochrome P450, Nuclear receptors.

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REVISED Amendments from Version 1

Figure 1 legend: "total" was added to "total RNA".

Introduction: "Pamda-28" is removed.

All other comments are minor and already shown in the prior work with references cited.

Any further responses from the reviewers can be found at the end of the article

Introduction

Tibetan Medicine is one of the important medical heritages of the world¹. Zuotai, a Tibetan medicine mixture containing β -HgS, has been included in many famous Tibetan medicines for the treatment of diseases²⁻⁴. A systematic review of available studies of Tibetan medicine, however, indicates that the literature in Western industrialized countries is scarce⁵. Traditional Tibetan medicines use polyherbo-metallic mixture recipes as opposed to a single ingredient in the treatment of diseases. For example, in a review of 193 herbo-metallic Tibetan medicine recipes for liver diseases, herbs/plants (181 kinds), animal products (7 kinds), and minerals (5 kinds) were frequently used⁶. Well-designed pharmacology and clinical studies are encouraged to elucidate the pharmacology, safety, and clinical efficacy of Tibetan medicines^{5,6}.

We have recently indicated that chemical compositions of minerals (metals) are a major determinant of their therapeutic effects and toxicity in Tibetan medicines⁷. 70W Zhen-Zhu Wan (70W, also called *Rannasangpei*, Qishiwei) is such an example⁸. 70W was developed in the middle of fifteenth century and is composed of herbo-metallic mixtures, mainly from pearl, Hong-sik, *Albergia odorifera*, Nine stone, Saffron, Bezoar, Musk and Zuotai (a mineral mixture) in the treatment of cardiovascular, gastrointestinal, and neurodegenerative diseases⁸, and is listed in the 2015 edition of Pharmacopoeia of China⁹. 70W is effective experimentally against vascular dementia in rats¹⁰, and protects cerebral ischemia-reperfusion injury via blood-brain barrier and metabolism with 18 identified active ingredients¹¹. We have recently demonstrated that 70W is effective in protecting against LPS plus MPTP-induced chronic neuroinflammation and dopaminergic neuron loss⁸ and could modulate gut microbiota as a means of protection^{8,12}. 70W dose-dependently protected against CCl₄-induced liver injury, probably by activation of the Nrf2 antioxidant pathway¹³.

CCl₄ is metabolized via cytochrome P450 (CYP450), particularly CYP2E1, to produce reactive metabolites¹⁴. Whether the protective effects of 70W against CCl₄ hepatotoxicity is related to CYP450 inhibition is not known. In addition, 70W might be used in combination with other medications since it has many beneficial effects because it contains many ingredients. It has the potential to cause herb-drug interactions, especially on the liver CYP450 gene, similar to other Chinese medicine formulae¹⁵. CYPs are the mixed function oxidase system mainly existing in the liver, and play roles in the metabolism of over 80% drugs¹⁶. Induction or inhibition of CYP450 is

implicated in traditional medicine-induced hepatoprotection and/or hepatotoxicity^{15,17,18}. CYP450 genes are regulated by corresponding nuclear receptors, their coordinated regulation affects hepatic phase I and phase II metabolisms¹⁹.

This study was therefore designed using 1–5 times clinical doses of 70W (0.15, 0.5 and 1.5 g/kg, po) for oral administration to mice for 7 days and comparing its effects with equivalent Hg contents of Zuotai, HgCl₂, and 1/10 Hg contents of MeHg, in an attempt to obtain information for the safe use of Zuotai-containing 70W in the clinic.

Methods

Reagents

70W and Zuotai was provided by Tibetan Medicine Manufacture Factory as described previously⁸, based on the 2015 edition of Pharmacopoeia of China for QA/QC control (Lot number Z20110561). 70W was prepared by grinding the pill into powder, adding distilled water to prepare the suspension for oral administration. Mercury chloride (HgCl₂ Cat# M1136) and methylmercury (MeHgCl Cat# 442534) were from Sigma (St. Louis, MO, USA). All other chemicals were commercially available reagents.

Animals

Male Kunming mice (20 ± 2 g) were purchased from Animal Experimental Center of the Third Military Medical University (Chongqing, China). Animals were maintained in the SPF-grade facilities at Zunyi Medical University, with a controlled environment (22 ± 1°C, 50 ± 2% humidity and a 12 h: 12 h light: dark cycle) and free access to purified water and standard laboratory feed. Efforts were made to ameliorate distress and harm to animals by daily monitoring and humane treatment of the animals. To reduce the use of animals, the minimal number of mice (n=5)/group according to the experiment requirement was used which are sufficient for statistical analysis. All animal care and experimental protocols are complied with the Animal Management Guidelines of the Chinese Ministry of Health and approved by Animal Use and Care Committee of Zunyi Medical University (2015-07).

Animal treatments

Mice were randomly divided into seven groups of five mice each (Total number n=35), respectively as the control, 70W (0.15, 0.5, 1.5g/kg), Zuotai (30 mg/kg, the amount contained in 70W), HgCl₂ (33.6 mg/kg, equivalent Hg as HgS) and MeHgCl (MeHg, 3.1 mg/kg, 1/10 of Hg). Mice were given oral administration for seven consecutive days. The dose regimen selection was based on our prior publications for 70W (at clinical dose)⁸ or for zuotai and mercury compounds²⁰. Twenty-four hours after the last dose, the animals were euthanized and the livers were collected and stored at 80°C prior to analysis.

Liver toxicity evaluation

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by commercial kits (Jaingcheng, Nanjing, China)²¹. Liver samples were fixed in 10% formalin prior to routine processing and paraffin embedding. Liver sections (4 μm) were dewaxed in xylene,

rehydrated in different concentrations of alcohol (100%, 95%, 80%, 75%) and stained with hematoxylin, followed by counterstaining with eosin. After rinsing, the slides were rehydrated with series of alcohol (75%, 95%, 100%) and mounted with cover glass slip. The slides were examined in nine random fields under a light microscope (Leica Microsystems Ltd., Wetzlar, Germany)^{21,22}.

Real-time PCR

Approximately 50–100 mg of tissue was homogenized in 1 ml TRIzol (TakaRa Biotechnology, Dalian, China) and the total RNA was extracted according to manufacturer's instructions. The quality and quantity of RNA were determined by the Nanodrop (Thermo Scientific, ND-2000, USA), with 260/280 ratio >1.8. Total RNA was reverse transcribed with a High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). The primers were designed with Primer3 software and listed in Table 1.

The 15 μ L PCR reaction mix contained 3 μ L of cDNA (10 ng/ μ L), 7.5 μ L of iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 μ L of primer mix (10 μ M each), and 4 μ L of ddH₂O. After 5 min denature at 95°C, 40 cycles were performed: annealing and extension at 60°C for 45 seconds and denature at 95°C for 10 seconds. Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. Relative expression of genes was calculated by the 2^{- $\Delta\Delta$ Ct} method and normalized to the house keeping gene β -actin or expressed as a percentage of controls^{8,21}.

Western blot analysis

Approximately 80 mg of liver tissue was homogenized with RIPA lysis buffer containing 1 mM PMSF and freshly prepared proteinase inhibitors. The homogenates were centrifuged at 12,000 g at 4°C for 10 min, and the protein concentration in the supernatants was determined by the BCA assay, and

denatures at 90°C for 10 min with Nupage loading buffer. Approximately 30 μ g proteins were separated in the 10% Nupage gel and transferred to the PVDF membrane. The membranes were blocked in 5% of the skim milk for 1 hour at room temperature, followed by incubation with primary antibodies (CYP1A2 (1:500), CYP2B1 (1:500), CYP2E1 (1:500), CYP3A4 (1:500), CYP4 (1:500), CYP7A1 (1:500), and GAPDH (1:2000)) at 4°C overnight. After washing the membranes with TBST four times, the secondary horseradish peroxidase (HRP) labelled anti-rabbit, or anti-mouse antibodies were added (1:5000) (Beyotime, Shanghai, China), and incubated at room temperature for 1 hour. The enhanced chemiluminescent reagents (ECL) were used to detect the intensity of protein-antibody complexes, and intensity was semi-quantified with Quantity One software (Bio-Rad, USA)¹⁸.

Statistical analysis

Data were expressed as mean and standard error. SPSS 19 was used for statistical analysis. Data were analyzed using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, and a *p* value < 0.05 was considered significant.

Results

Animal general conditions

At the doses of 70W and Zuotai used in the present study, animals were healthy, without body weight loss and no mortality occurred. No significant elevations of serum ALT and AST were evident, and histology did not reveal overt lesions^{12,20–22}. HgCl₂ and MeHg groups showed body weight loss and mild histology lesions, consistent with prior publications^{12,21,22}.

mRNA expression of nuclear receptors and cytochrome P450 genes

Figure 1 illustrates mRNA expression of nuclear receptors (left side) and cytochrome P450 isozyme genes (right side). The aryl hydrocarbon receptor (AhR) mainly mediates the expression

Table 1. Primer sequences for real-time RT-qPCR.

	Access#	Forward	Reverse
<i>AhR</i>	NM_013464	ACCAGAACTGTGAGGGTTGG	CTCCCATCGTATAGGGAGCA
<i>β-actin</i>	NM_007393	GATCTGGCACCACACCTTCT	GGGGTGTGAAGGTCTCAAA
<i>CAR</i>	NM_009803	CTCAAGGAAAGCAGGGTCAG	AGTTCCTCGGCCATATTCT
<i>Cyp1a2</i>	NM_009993	AATGTCACCTCAGGGAATGC	GCTCCTGGACAGTTTTCTGC
<i>Cyp2b10</i>	NM_009999	AAGGAGAAGTCCAACCAGCA	CTCTGCAACATGGGGTACT
<i>Cyp2e1</i>	NM_021282	GGACGCTGTAGTGCATGAGA	CAACTGTACCCTTGGGGATG
<i>Cyp3a11</i>	NM_007818	AGGGAAGCATTGAGGAGGAT	GGTAGAGGAGCACCAGCTG
<i>Cyp4a10</i>	NM_010011	CACACCCTGATACCAACAG	TCCTTGATGCACATTGTGGT
<i>Cyp7a1</i>	NM_007824	CAACGGGTTGATTCCATACC	ATTTCCCATCAGTTTGCAG
<i>FXR</i>	NM_001163700	TGGGTACCAGGGAGAGACTG	GTGAGCGCGTTGTAGTGGTA
<i>PPARα</i>	NM_011144	GTCCTCAGTGCTCCAGAGG	GGTCACCTACGAGTGGCATT
<i>PXR</i>	NM_010936	CCCATCAACGTAGAGGAGGA	TCTGAAAAACCCCTTGCATC

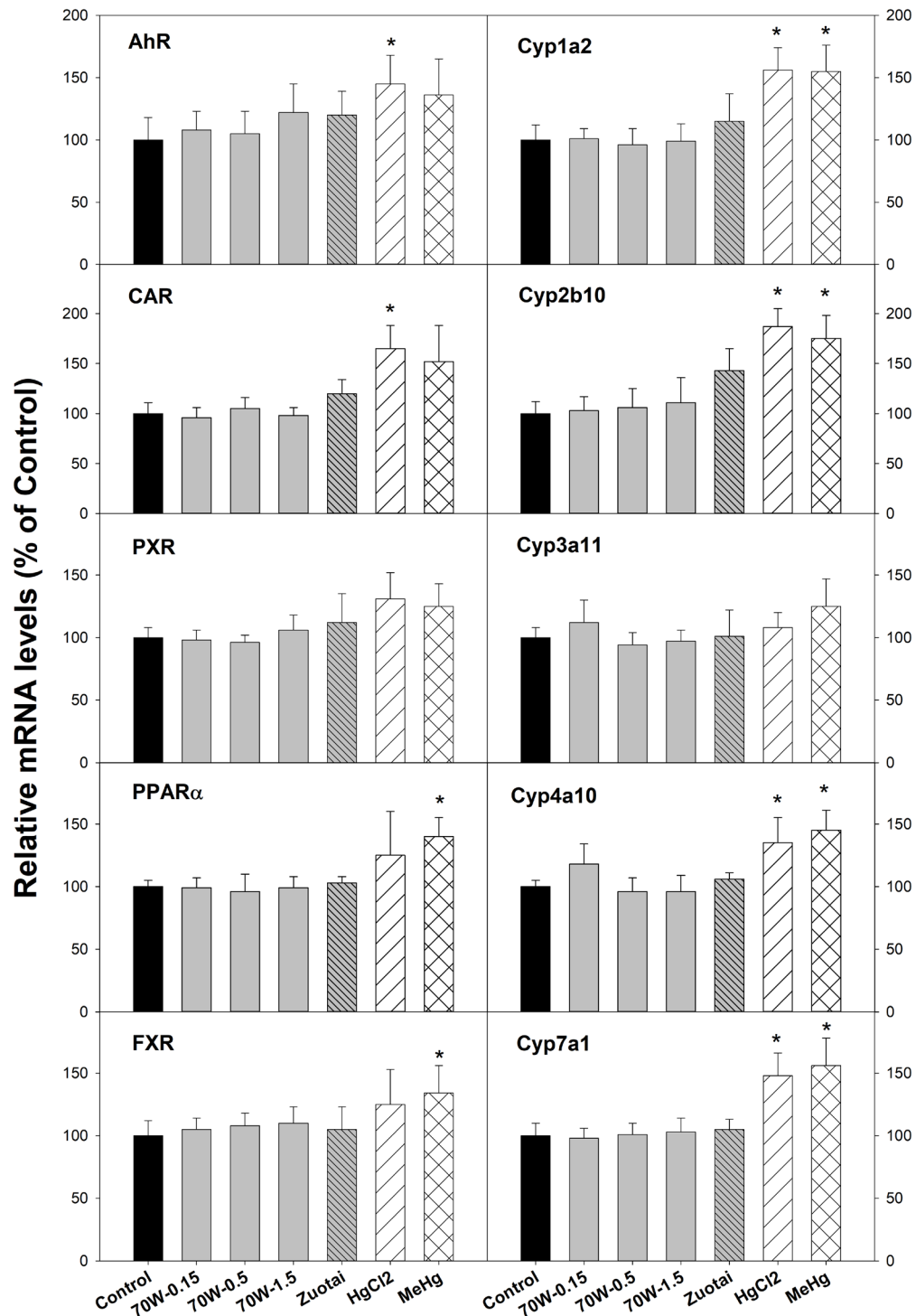


Figure 1. Effect of 70W and mercury compounds on nuclear receptor and corresponding CYP gene expression. Mice were given 70W 0.15, 0.5, and 1.5 g/kg, po. Zuotai (30 mg/kg, po), HgCl₂ (33.6 mg/kg, po), and MeHg (3.1 mg/kg, po) daily for seven days, and hepatic total RNA and protein were extracted for RT-PCR analysis. Data are mean \pm SE, n = 5. *Significantly different from control, $p < 0.05$.

of CYP1A enzymes such Cyp1a2; Constitutive androstane receptor (CAR) mediates CYP2 enzymes such as Cyp2b10; Pregnane X receptor (PXR) plays an important role in the regulation of CYP3A enzymes such as Cyp3a11; Peroxisome proliferator-activated receptor α (PPAR α) regulates induction of CYP4A enzymes such as Cyp4a10¹⁹. The Farnesoid X receptor (FXR) regulates cholesterol 7 α hydroxylase (Cyp7a1) induction²³. The results show that compared with the controls, 70W at 0.15, 0.5, and 1.5 g/kg doses and Zuotai (β -HgS, 30 mg/kg) had no effects on these nuclear receptor and CYP gene expressions. In contrast, HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) significantly increased AhR, Cyp1a2, CAR, Cyp2b10, PPAR α , Cyp4a10, FXR, and Cyp7a1, while having no significant effects on PXR, Cyp3a11 (Figure 1).

Protein expression of cytochrome P450 isozymes

Figure 2 illustrates protein expression of P450 isozymes. Figure 2A shows representative western-blot for CYP1A2, CYP2B1, CYP2E1, CYP3A, CYP4A, and CYP7A1; Figure 2B shows the statistical analysis of 3-5 replicates. Consistent with mRNA expression, 70W at 0.15, 0.5, and 1.5 g/kg doses and Zuotai (β -HgS, 30 mg/kg) had no apparent effects on cytochrome P450 isozyme protein expressions. In contrast, HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) significantly increased CYP1A2, CYP2B1, CYP4A, and CYP7A1, while having no effects on CYP3A (Figure 2).

Discussion

The potential efficacy and toxicity of minerals (metals) in traditional medicines is currently a matter of debate^{7,24}. In the present research, we examined the effects of β -HgS-containing Zuotai and Zuotai-containing 70W on hepatic CYP 1-4 and CYP-7 families, and their corresponding nuclear receptors, compared to HgCl₂ and MeHg at both mRNA and protein levels. Briefly, 70W at 1 to 5-times clinical doses and Zuotai (β -HgS, 30 mg/kg, po) administered for seven days did not produce significant effects on the liver CYP450 gene and protein expressions in mice. HgCl₂ and MeHg at 1/10 Hg dosing increased the expression of CYP1A, CYP2B, CYP2E1, and CYP7A at the mRNA and/or protein levels. These results further demonstrate that chemical forms of metals are a major determinant of their biological effects and that the use of HgCl₂ or MeHg for risk assessment on minerals in traditional medicines is inappropriate⁷.

70W and Zuotai in Tibetan Medicines

Tibetan medicine has thousands of years of history and is still used in the world today to treat a variety of diseases, including liver diseases^{1-4,6}. Herbal-metallic preparations are believed to assist the delivery of drugs to the target, contribute to therapeutic effects, and reduce toxicity⁷. 70W is a famous Tibetan medicine listed in the 2015 Edition of Chinese Pharmacopoeia for the treatment of various diseases^{3,10}. The major ingredients in 70W and the mode of the protection against cerebral ischemia-reperfusion injury has recently been demonstrated¹¹. We have shown that 70W is effective against CCl₄-induced liver injury, protected LPS plus MPTP-induced neurotoxicity⁸, and modulated gut microbiota^{8,12}. The present study further

demonstrated that the hepatoprotective effects of 70W is not due to the inhibition of CYP450 to reduce CCl₄ bioactivation, rather the activation of the Nrf2 antioxidant pathway¹³.

Zuotai is a mineral mixture, with 54% of β -HgS²⁵, and is included in a small amount to many valuable Tibetan medicines¹⁻³. Mercury (Hg) is a toxic metal; the safety of Hg-containing traditional medicines is of concern²⁴. The chemical speciation, spatial distribution of mercury from Zuotai are different from that of HgCl₂^{7,25}, resulting in differential toxicity. A recent human study revealed that Zuotai-containing Tibetan medicines are safe at clinical doses²⁶⁻²⁸, including 70W²⁹. Indeed, Zuotai differs from HgCl₂ and MeHg in producing hepatotoxicity²¹, nephrotoxicity³⁰, and intestinal toxicity with gut microbiome disruptions²⁰. The present study demonstrated that Zuotai-containing 70W at clinical doses had minimal effects on hepatic CYP450, supporting the notion that Zuotai and 70W at clinical doses are safe²⁶⁻²⁹.

Effects of mercury compounds on cytochrome P450

Cytochrome P450 1A1 (CYP1A1) is a hepatic and extrahepatic enzyme that is regulated by the AhR signaling pathway and is regarded as carcinogen activation CYP450 family³¹. CYP-1 family includes CYP1A1, CYP1A2, and CYP1B1, and CYP1A1/CYP1A2 has become a therapeutic tool for the bioactivation of prodrugs, particularly cytotoxic agents. Little is known about effects of 70W on CYP1A family. We have shown previously that oral Zuotai (β -HgS) and cinnabar (α -HgS) had minimal effects of hepatic P4501A family gene expression³². However, in rats, Zuotai at higher doses could decrease CYP1A2 activity³³. In comparison, the effects of HgCl₂ on CYP1A expression were more dramatic. In Zebra fish, a low dose (0.1 LC50) of HgCl₂ increased CYP1A1, but at higher doses (0.4 and 0.8 LC50), the expression of CYP1A1 was suppressed³⁴. In the mouse heart, kidney and lung, HgCl₂ (2.5 mg/kg, ip) increased CYP1A1, along with other CYP450 isoforms³⁵. In the present study, HgCl₂ at 33.6 mg/kg increased CYP1A2 at mRNA and protein levels, largely in agreement with the above literature³²⁻³⁵. In another study, mice that chronically (6 weeks) received HgCl₂ (32 mg/kg) and MeHg (2.6 mg/kg), had increased expressions of hepatic Cyp1a1 and Cyp1b1, while cinnabar (HgS, 300 mg/kg) and cinnabar-containing An-Gong-Niu-Huang Wan were ineffective³⁶. Thus, the effects of mercury compounds on CYP1 family are dependent on the mercury forms, the dose, route, and duration of administration.

The CYP-2 family is easily induced by many xenobiotics such as phenobarbital. CAR is shown to play a crucial role in the activation of CYP2B genes by xenobiotics¹⁹. The CYP-2 family mainly includes the CYP2B subfamily and CYP2E1. CYP2E1 metabolizes an extensive array of pollutants, drugs, and other small molecules, often resulting in bioactivation to reactive metabolites, which in turn damage mitochondria³⁷. HgCl₂-induced hepatotoxicity and oxidative stress is partially mediated through its effects on CYP2E1³⁸. HgCl₂ (2.5 mg/kg, ip) increased the expression of Cyp2b9 and Cyp2b10 in mice hearts³⁹ and HgCl₂ (33.6 mg/kg, po) increased Cyp2b10 expression in

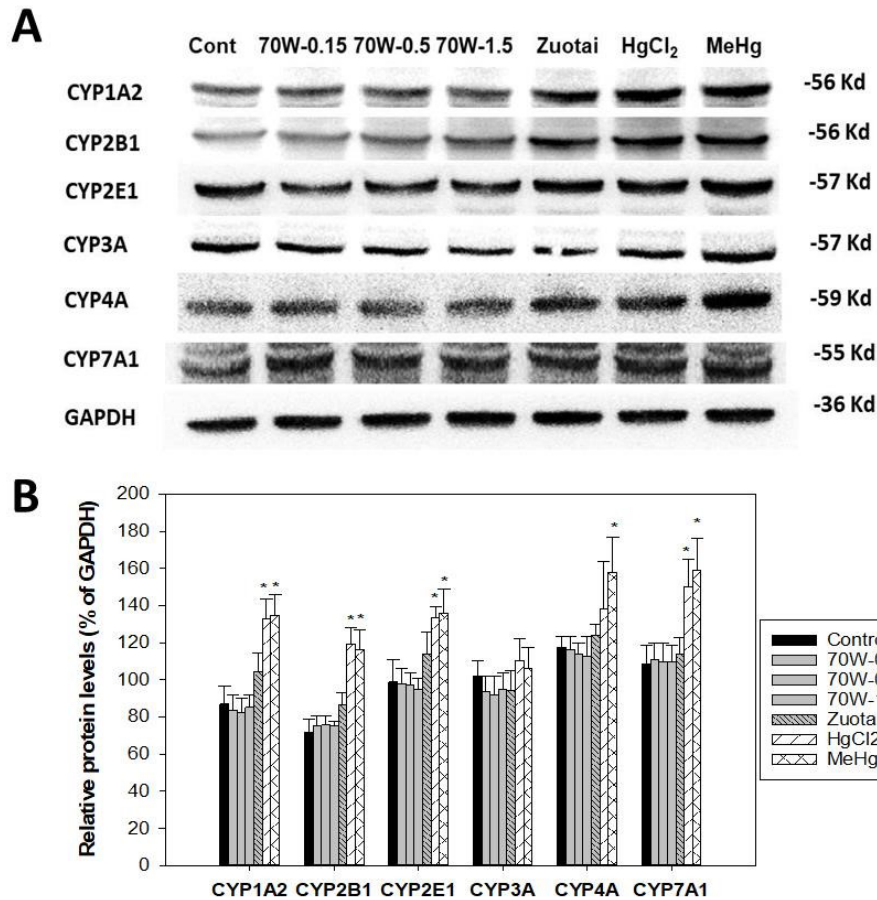


Figure 2. Effect of 70W and mercury compounds on cytochrome P450 isoenzyme protein expression. Mice were given 70W 0.15, 0.5, and 1.5 g/kg, po. Zuotai (30 mg/kg, po), HgCl₂ (33.6 mg/kg, po), and MeHg (3.1 mg/kg, po) daily for seven days, and hepatic proteins were extracted and pooled for Western-blot analysis. **A**, the representative western-blot; **B**, statistical analysis of P450 proteins. Data are mean \pm SE of 3-5 replicates. *Significantly different from control, $p < 0.05$.

the livers of mice³². Under the present experimental conditions, Cyp2b10 mRNA and CYP2B protein expression were increased by HgCl₂ and MeHg only.

CYP3A is the most abundant subfamily of CYP450, with the highest content in the liver and intestines, and is involved in the metabolism of clinical drugs^{17,18}. CYP3A can be induced or inhibited by a variety of substances. In the present study conditions, 70W and mercury compounds had minimal effects on Cyp3a11 mRNA and CYP3A protein expression. The length of Hg compound administration could make a difference as compared to the present study.

CYP4A is involved in lipid metabolism and is regulated by PPAR α , their dysregulations are implicated in xenobiotics induced adverse effects leading to various human diseases¹⁹. Researchers found that HgCl₂ exposure is associated with increased risk of cardiovascular disease and profound cardiotoxicity, and their results show that mercury treatment caused a significant induction of the cardiac hypertrophy markers, along

with CYP4A genes (Cyp4a10, Cyp4a12, Cyp4a14)³⁵. In the present study, 70W and Zuotai at 1–5 times clinical doses do not have appreciable effects on PPAR α and Cyp4a10 mRNA expression and CYP4A protein expression, while HgCl₂ and MeHg increased PPAR α and Cyp4a10 mRNA, as well as CYP4A protein, consistent with our prior observation that HgCl₂ increased PPAR α and Cyp4a10 in livers of mice after seven days of administration³². In mice chronically (6 weeks) dosed with HgCl₂ (32 mg/kg) and MeHg (2.6 mg/kg), the expression of Cyp4a10 was increased, but cinnabar (HgS, 300 mg/kg) and cinnabar-containing An-Gong-Niu-Huang Wan was ineffective³⁶. Increased expression of the CYP-4A family genes under the dose of HgCl₂ and MeHg used in the present study could impact lipid metabolism.

CYP7A1 is a rate-limiting enzyme for bile acid synthesis and is regulated by FXR²³. Little is known on the effects of mercury compounds on FXR and CYP7A1 expression. The present study showed that 70W and Zuotai did not affect CYP7A1, while HgCl₂ and MeHg increased Cyp7a1 mRNA and

CYP7A1 protein. The biological effects of CYP7A1 induction by HgCl₂ and MeHg warrant further investigation.

Conclusions

The present study showed β-HgS and β-HgS containing 70W (1–5-times of clinical dose) did not produce appreciable effects on hepatic CYP450 enzyme gene/protein expression compared to equal Hg content as HgCl₂ or 1/10 of Hg content as MeHg, suggesting that (1) the protection of 70W against CCl₄ hepatotoxicity is not due to inhibition of CYP450 (CYP2E1); (2) 70W appeared to be safe under recommended clinical doses; and (3) HgCl₂ and MeHg had significant effects on CYP450 expression, correlated with their potential toxic effects to the liver.

Abbreviations

70 Wei-Zhen-Zhu-Wan (70W, also called Rannasangpei; Qishiwei); Cytochrome P450 (CYP450); Aryl hydrocarbon receptor (AhR); Constitutive androstane receptor (CAR); Pregnane X receptor (PXR); Peroxisome proliferator-activated receptors (PPARs); farnesoid X receptor (FXR).

Data availability

Underlying data

Zenodo: Zuotai (β-HgS)-containing 70 Wei Zhen-Zhu-Wan differs from mercury chloride and methylmercury on hepatic cytochrome P450, <http://doi.org/10.5281/zenodo.440371740>

This project contains the following underlying data:

- PCR and WB figure data (PCR-WB)
- Raw western-blot data (CYP-WB). Please note that full blot images are not available

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](https://creativecommons.org/licenses/by/4.0/).

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A previous version of this article is available on Research Square: <https://doi.org/10.21203/rs.3.rs-32118/v1>

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Xingguo Cheng

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The authors successfully addressed my comments. No more comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 30 April 2021

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Xian-Ju Huang 

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The article was well designed to discuss the hepatotoxicity of Zuotai-containing 70W and the data persuasive. However, some errors should be corrected.

1. The dose of 70W (0.15-1.5 g/kg) should be 1-10 times clinical doses. The author described as 1-5 times.

2. The data of ALT and AST as well as pathological changes were not shown in the results. However, the methods have mentioned. Please check them.

3. The introduction section, the second paragraph, line 2, "Pamda-28" may not be true, please check it.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology and toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 30 March 2021

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Xingguo Cheng

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Because of their toxic components and potential drug-drug interaction, many traditional Asian Medicine, including Tibetan Medicine, have been raising health concerns. In this manuscript, Nie et al evaluated and compared the expression regulation of several P450s by Zuotai, mercury

chloride and methylmercury, given the same mercury content level. The authors suggested that it is not appropriate to simply use total Hg content to evaluate the safety of HgS-containing Zuotai. Overall, the study is straight-forward.

1. Figure legend of Figure 1: should be total RNA from mouse liver was extracted and processed for RT-PCR analysis.
2. In animal treatment section, the mice should be treated with Zuotai, mercury chloride and methylmercury by using the unit of mmole/kg or micromole/kg, but not mg/kg unit.
3. The authors have assessed Cyp7a1 and FXR expression. To make the studies more relevant, the authors may consider to measure total bile acids in mouse serum, a biomarker of liver injury.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Apr 2021

Jie Liu, Zunyi Medical University, Zunyi, China

Thanks for the comments. Some of the inquiries could be found in listed references Nie et al., 2018.

Competing Interests: I have no competing interests.

Author Response 30 Apr 2021

Jie Liu, Zunyi Medical University, Zunyi, China

1. Figure legend of Figure 1: should be total RNA from mouse liver was extracted and processed for RT-PCR analysis.
Answer: Yes
2. In animal treatment section, the mice should be treated with Zuotai, mercury chloride and methylmercury by using the unit of mmole/kg or micromole/kg, but not mg/kg unit. Answer: Since Zoutai is a mineral mixture, we can only use mg/kg. mercury chloride and MeHg were used based on mol basis as HgS
3. The authors have assessed Cyp7a1 and FXR expression. To make the studies more relevant, the authors may consider to measure total bile acids in mouse serum, a biomarker of liver injury. Answer: Good suggestion for future consideration. ALT and histology in prior publications verified the toxicity

Competing Interests: I have no competing interest.

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