Zuotai (β-HgS)-containing 70 Wei Zhen-Zhu-Wan differs from mercury chloride and methylmercury on hepatic cytochrome P450 in mice [version 1; peer review: 1 approved with reservations]

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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 CCl₄ hepatotoxicity. CCl₄ 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 HgCl₂ (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 (PPARα); farnesoid X receptor (FXR)]. In comparison, HgCl₂ 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 HgCl₂ 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 HgCl₂ and MeHg produced significant effects. Thus,
the use of total Hg content to evaluate the safety of HgS-containing 70W is inappropriate.

Keywords
Zuotai, 70 Wei-Zhen-Zhu-Wan (Rannasangpei, Qishiwei), HgCl2, MeHg, Cytochrome P450, Nuclear receptors.
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 diseases2–5. A systematic review of available studies of Tibetan medicine, however, indicates that the literature in Western industrialized countries is scarce6. 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 medicines6,7.

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, Pamda-28, Qishwei) is such an example 8. 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 neuro-degenerative diseases9, and is listed in the 2015 edition of Pharmacopoeia of China10. 70W is effectivellyy used for treatment of liver and kidney diseases in the clinic9–11. Zuotai, a Tibetan medicine mixture containing HgCl₂, has been included in many famous Tibetan medicines for the treatment of diseases2–5. A systematic review of available studies of Tibetan medicine, however, indicates that the literature in Western industrialized countries is scarce6. 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 medicines6,7.

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Methods

Reagents

70W and Zuotai was provided by Tibetan Medicine Manufactory (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, 1.5 g/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) or for zuotai and mercury compounds10. 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 alamine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by commercial kits (Jiaingcheng, Nanjing, China)11. 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%, 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)12,13.

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 extraction was followed by DNase treatment and RNA was precipitated in 95% alcohol. Single-stranded cDNA was synthesized with the modified 5′-end RT Primer and PrimeScript High Fidelity cDNA Synthesis Kit (TakaRa Biotechnology, Dalian, China). TaqMan qPCR was performed according to the manufacturer’s instructions. The qPCR was performed with iQSYBR GREEN Supermix (Bio-rad, Hercules, CA) and run on the iCyclerIQ (Bio-rad, Hercules, CA). The primer sequences are listed in Table 1. The relative expression levels of each gene were calculated using the comparative Ct method (ΔΔCT).
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 µL PCR reaction mix contained 3 µL of cDNA (10 ng/µL), 7.5 µL of iTaq™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 µL of primer mix (10 µM each), and 4 µL 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^ΔΔCt method and normalized to the housekeeping gene β-actin or expressed as a percentage of controls.

Western blot analysis
Approximately 80 mg of liver tissue was homogenized with RIPA lysis buffer containing 1 mM PMSF and freshly prepared protease 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 µg 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), 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).

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. HgCl₂ and MeHg groups showed body weight loss and mild histology lesions, consistent with prior publications.

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 of CYP1A enzymes such as 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 (PPARα) regulates induction of CYP4A enzymes such as Cyp4a10. The Farnesoid X receptor (FXR) regulates cholesterol 7α-hydroxylase (Cyp7a1). 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 genes.

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

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<tr>
<th>Access#</th>
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<th>Reverse</th>
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<td>AhR</td>
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<td>CTCCCATCGTATAGGGAGCA</td>
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<td>β-actin</td>
<td>GATCTGGCACCACACCTTCT</td>
<td>GGGGTGTTGAAGGTCTCAA</td>
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<td>CAR</td>
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<td>Cyp2b10</td>
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<td>CTCTGCAACATGGGGGTACT</td>
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<td>Cyp2e1</td>
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<tr>
<td>Cyp7a1</td>
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<td>ATTTCCCACATGTTGCAG</td>
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<tr>
<td>FXR</td>
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<tr>
<td>PPARα</td>
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<td>PXR</td>
<td>CCCATCAACGTAGAGGAGGA</td>
<td>TCTGAAAACCCCTTGCATC</td>
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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), HgCl$_2$ (33.6 mg/kg, po), and MeHg (3.1 mg/kg, po) daily for seven days, and hepatic RNA and protein were extracted for RT-PCR analysis. Data are mean ± SE, n = 5. *Significantly different from control, $p<0.05$. 
gene expressions. In contrast, HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) significantly increased AhR, Cyp1a2, CAR, Cyp2b10, PPARα, 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.

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 inappropriate.

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. Herbal-metallic preparations are believed to assist the delivery of drugs to the target, contribute to
therapeutic effects, and reduce toxicity. 70W is a famous Tibetan medicine listed in the 2015 Edition of Chinese Pharmacopoeia for the treatment of various diseases. The major ingredients in 70W and the mode of the protection against cerebral ischemia-reperfusion injury has recently been demonstrated. We have shown that 70W is effective against CCl4-induced liver injury, protected LPS plus MPTP-induced neurotoxicity, and modulated gut microbiota. 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 pathway.

Zuotai is a mineral mixture, with 54% of β-HgS, and is included in a small amount to many valuable Tibetan medicines. Mercury (Hg) is a toxic metal; the safety of Hg-containing traditional medicines is of concern. The chemical speciation, spatial distribution of mercury from Zuotai are different from that of HgCl2, resulting in differential toxicity. A recent human study revealed that Zuotai-containing Tibetan medicines are safe at clinical doses, including 70W. Indeed, Zuotai differs from HgCl2 and MeHg in producing hepatotoxicity, nephrotoxicity, and intestinal toxicity with gut microbiome disruptions. 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 safe.

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 family. 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 expression. However, in rats, Zuotai at higher doses could decrease CYP1A2 activity. 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 suppressed. In the mouse heart, kidney and lung, HgCl2 (2.5 mg/kg, ip) increased CYP1A1, along with other CYP450 isoforms. In the present study, HgCl2 at 33.6 mg/kg increased CYP1A2 at mRNA and protein levels, largely in agreement with the above literature. 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 ineffective. 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 xenobiotics. 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 mitochondria. HgCl2-induced hepatotoxicity and oxidative stress is partially mediated through its effects on CYP2E1. HgCl2 (2.5 mg/kg, ip) increased the expression of Cyp2b9 and Cyp2b10 in mice hearts and HgCl2 (33.6 mg/kg, po) increased Cyp2b10 expression in the livers of mice. 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. 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 PPARα, their dysregulations are implicated in xenobiotics induced adverse effects leading to various human diseases. 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). In the present study, 70W and Zuotai at 1–5 times clinical doses do not have appreciable effects on PPARα and Cyp4a10 mRNA expression and CYP4A protein expression, while HgCl2 and MeHg increased PPARα and Cyp4a10 mRNA, as well as CYP4A protein, consistent with our prior observation that HgCl2 increased PPARα and Cyp4a10 in livers of mice after seven days of administration. 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 ineffective. The present study demonstrated that 70W and Mercury compounds had minimal effects on hepatic CYP450, supporting the notion that Zuotai and 70W at clinical doses are safe.

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 hepatoxicity 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; Qishiwai); Cytochrome P450 (CYP450); Aryl hydrocarbon receptor (AhR); Constitutive androstane receptor (CAR); Pregnen X receptor (PXR); Peroxisome proliferator-activated receptors (PPARs); farnesoid X receptor (FXR).

**Data availability**

**Underlying data**


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).

**Acknowledgements**

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

**References**


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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.

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