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 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.

Open Peer Review

Approval Status

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1. Xingguo Cheng, St. John’s University, Queens, New York, USA
2. Xian-Ju Huang, South-Central University for Nationalities, Wuhan, China

Any reports and responses or comments on the article can be found at the end of the article.
Keywords
Zuotai, 70 Wei-Zhen-Zhu-Wan (Rannasangpei, Qishiwei), HgCl2, MeHg, Cytochrome P450, Nuclear receptors.

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Author roles: Nie Y: Conceptualization, Data Curation, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; Xu SF: Conceptualization, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Lu YL: Data Curation, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Zhao XR: Data Curation, Investigation, Writing – Review & Editing; Li C: Conceptualization, Validation, Writing – Review & Editing; Wei LX: Conceptualization, Project Administration, Supervision, Writing – Review & Editing; Liu J: Conceptualization, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

Tibetan Medicine is one of the important medical heritages of the world. Zuotai, a Tibetan medicine mixture containing β-HgS, has been included in many famous Tibetan medicines for the treatment of diseases. A systematic review of available studies of Tibetan medicine, however, indicates that the literature in Western industrialized countries is scarce. 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 used. Well-designed pharmacology and clinical studies are encouraged to elucidate the pharmacology, safety, and clinical efficacy of Tibetan medicines.

We have recently indicated that chemical compositions of minerals (metals) are a major determinant of their therapeutic effects and toxicity in Tibetan medicines. 70W Zhen-Zhu Wan (70W, also called Ramnasangpei, Qishiwei) is such an example. 70W was developed in the middle of the 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 diseases, and is listed in the 2015 edition of Pharmacopoeia of China. 70W is effective experimentally against vascular dementia in rats, and protects cerebral ischemia-reperfusion injury via blood-brain barrier and metabolomics with 18 identified active ingredients. We have recently demonstrated that 70W is effective in protecting against LPS plus MPTP-induced chronic neuroinflammation and dopaminergic neuron loss and could modulate gut microbiota as a means of protection. 70W dose-dependently protected against CCl4-induced liver injury, probably by activation of the Nrf2 antioxidant pathway.

CCl4 is metabolized via cytochrome P450 (CYP450), particularly CYP2E1, to produce reactive metabolites. 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 formulae. CYPs are the mixed function oxidase system mainly existing in the liver, and play roles in the metabolism of over 80% drugs. Induction or inhibition of CYP450 is implicated in traditional medicine-induced hepatoprotection and/or hepatotoxicity. CYP450 genes are regulated by corresponding nuclear receptors, their coordinated regulation affects hepatic phase I and phase II metabolisms.

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 previously, 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) or for zuotai and mercury compounds. 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). 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).

**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 µL PCR reaction mix contained 3 µL of cDNA (10 ng/µL), 7.5 µL of iQ™ 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 house keeping 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), 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).

**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

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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 total RNA and protein were extracted for RT-PCR analysis. Data are mean ± SE, n = 5. *Significantly different from control, p< 0.05.
of CYP1A enzymes such as Cyp1a2; Constitutive androstane receptor (CAR) mediates CYP2 enzymes such as Cyp2b10; Pregnan 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 Cyp4a10β. The Farnesoid X receptor (FXR) regulates cholesterol 7α hydroxylase (Cyp7a1) induction11. 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, 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.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 CYP1A1, 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.5

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.12-14. Herbal-metallic preparations are believed to assist the delivery of drugs to the target, contribute to therapeutic effects, and reduce toxicity.25. 70W is a famous Tibetan medicine listed in the 2015 Edition of Chinese Pharmacopoeia for the treatment of various diseases.13,14. The major ingredients in 70W and the mode of the protection against cerebral ischemia-reperfusion injury has recently been demonstrated.15. We have shown that 70W is effective against CCl₄-induced liver injury, protected LPS plus MPTP-induced neurotoxicity17, and modulated gut microbiota.18-21. 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.13.

Zuotai is a mineral mixture, with 54% of β-HgS22, and is included in a small amount to many valuable Tibetan medicines.23-25. Mercury (Hg) is a toxic metal; the safety of Hg-containing traditional medicines is of concern.26. The chemical speciation, spatial distribution of mercury from Zuotai are different from that of HgCl₂,27, resulting in differential toxicity. A recent human study revealed that Zuotai-containing Tibetan medicines are safe at clinical doses26-28, including 70W.29. Indeed, Zuotai differs from HgCl₂ and MeHg in producing hepatotoxicity,28, nephrotoxicity,39, and intestinal toxicity with gut microbiome disruptions.30. 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.26-29.

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 family.31. 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.32. However, in rats, Zuotai at higher doses could decrease CYP1A2 activity.33. In comparison, the effects of HgCl₂ on CYP1A1 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.34. In the mouse heart, kidney and lung, HgCl₂ (2.5 mg/kg, ip) increased CYP1A1, along with other CYP450 isoforms.35. In the present study, HgCl₂ at 33.6 mg/kg increased CYP1A2 at mRNA and protein levels, largely in agreement with the above literature.32-35. 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 ineffective.40. 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.36. The CYP-2 family mainly includes the CYP2B2 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.37. HgCl₂-induced hepatotoxicity and oxidative stress is partially mediated through its effects on CYP2E1.38. HgCl₂ (2.5 mg/kg, ip) increased the expression of Cyp2b9 and Cyp2b10 in mice hearts9, and HgCl₂ (33.6 mg/kg, po) increased Cyp2b10 expression in
the livers of mice\textsuperscript{32}. Under the present experimental conditions, Cyp2b10 mRNA and CYP2B protein expression were increased by HgCl\textsubscript{2} 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\textsuperscript{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\textalpha, their dysregulations are implicated in xenobiotics induced adverse effects leading to various human diseases\textsuperscript{19}. Researchers found that HgCl\textsubscript{2} 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)\textsuperscript{35}. In the present study, 70W and Zuotai at 1–5 times clinical doses do not have appreciable effects on PPAR\textalpha and Cyp4a10 mRNA expression and CYP4A protein expression, while HgCl\textsubscript{2} and MeHg increased PPAR\textalpha and Cyp4a10 mRNA, as well as CYP4A protein, consistent with our prior observation that HgCl\textsubscript{2} increased PPAR\textalpha and Cyp4a10 in livers of mice after seven days of administration\textsuperscript{32}. In mice chronically (6 weeks) dosed with HgCl\textsubscript{2} (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\textsuperscript{36}. Increased expression of the CYP-4A family genes under the dose of HgCl\textsubscript{2} 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 FXR\textsuperscript{23}. 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 HgCl\textsubscript{2} and MeHg increased Cyp7a1 mRNA and

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**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), HgCl\textsubscript{2} (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 ± SE of 3-5 replicates. *Significantly different from control, p< 0.05.
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 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 Rannasarangpe; 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

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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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.

Xian-Ju Huang
College of Pharmaceutical Science, South-Central University for Nationalities, Wuhan, China

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

https://doi.org/10.5256/f1000research.43732.r81779

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Xingguo Cheng
Department of Pharmaceutical Sciences, St. John's University, Queens, New York, NY, USA

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.

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**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 Zuotai 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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**Comments on this article**

**Version 1**

Reviewer Response 03 Jul 2021

**Zhang Wang,** College of Ethnomedicine, Chengdu University of Traditional Chinese Medicine, China

This manuscript studies the effects of Zuotai (β-HgS) and its Tibetan compound preparation, 70 Wei Zhen-Zhu-Wan, on hepatic cytochrome P450. The results show that the effects are less than HgCl2 and MeHg. These results are helpful to support the continued use of these Tibetan medicines in clinical practice. Therefore, this manuscript is valuable and will attract the attention of traditional medicine scholars and benefit from it.

However, some suggestions were put forward in the manuscript. For example, in describing the effects of Zuotai and 70 Wei Zhen-Zhu-Wan on animal weight, ALT and AST, and liver histopathology, the manuscript only quoted the links of published articles. However, we suggest that the relevant data should be written appropriately, which is beneficial for the author to read, because this part of data is actually the most important and basic.

**Competing Interests:** I have no conflict of interest with this article.
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