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Content decline of SERCA inhibitors saikosaponin a and d attenuates cardiotoxicity and hepatotoxicity of vinegar-baked *Radix bupleuri*

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ABSTRACT

Improper usage of unprocessed *Radix bupleuri* root (chaihu) may cause cardiotoxicity and liver injury. Baking herb with vinegar is believed to attenuate the adverse responses. However, the chemical and molecular basis involved remained unclear. To this end, we investigated the *in vitro* toxicity of saikosaponin a, c, d, and their hydrolysates saikosaponin b1 and b2. Results showed that SSa and SSd possessed higher affinity with sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) by molecular docking, and exhibited stronger toxic responses on cardiomyocytes and hepatocytes than the other three saikosaponins in equivalent concentrations. Further, SSa and SSd induced LC3 puncta formation in U2OS-mCherry-EGFP-LC3 cells. Blockage of autophagy by 3-methyladenine did not abrogate the cytotoxicities induced by SSa and SSd. In parallel, none of SSc, SSb1, or SSb2 caused cell injury. Our study reveals how changes in chemical ingredients are connected to the toxicity of Chaihu during vinegar baking process and also provides a guidance for structure optimization to reduce drug induced toxicity.

1. Introduction

Radix Bupleuri root is a favorite herbal medicine that known as the term of "chaihu in Chinese, which is derived from the roots of *Bupleurum chinense DC.* or *B. scorzonerifolium Willd.* Chaihu plays decisive roles in many herbal remedies, examples include Chaihushugansan and Xiaochaihutang for depression (Qiu et al., 2014; Zhang et al., 2015b), and Shengxian decoction for the treatment of heart failure (Zhang et al., 2014). Saikosaponin derivatives are principal components in Chaihu (Bao et al., 2004), SSa, SSc, and SSd share a parental chemical bone, while SSb1 and SSb2 are their derivatives. According to Chinese Pharmacopeia 2015, the total content of SSa and SSd should occupy more than 0.3% in dried chaihu products. In recent decades, increasing toxicity incidents occurred from improper usage of chaihu. A case in point is Xiao-chai-hu-tang induced toxicity (syo-saiko-to in Japanese) that used for respiratory, hepatobiliary, gastrointestinal disorders, and chronic liver diseases (Liming Hsu et al., 2006).

It should be noted that chaihu is generally baked with vinegar prior to make up a decoction for the treatment of chronic diseases. Processing of herbs is a distinguished characteristic of traditional Chinese medicines, and this technique has been used for more than one thousand and five hundred years. An herb could meet the demands of various therapeutic applications through diverse processing procedures. Importantly, baking herbs with auxiliary material is considered to be able to improve their bioactivities and impair the toxicities (Su et al., 2016). A series of studies suggest that chemical compositions of chaihu are changed after the vinegar-baking process. Interestingly, percentages of saikosaponin a and d reduced, while total contents of saikosaponin b1, b2, and c elevated after being processed (Li et al., 2015). The implications of contents alteration on the toxicity impairment of chaihu

Abbreviations: 3-MA, 3-methyl adenine; LC3, microtubule-associated protein light chain 3; *Cmax*, maximum plasma concentration; GFP, green fluorescent protein; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; SSa, Saikosaponin a; SSb1, saikosaponin b1; SSb2, saikosaponin b2; SSc, saikosaponin c; SSd, saikosaponin d; STSP, staurosporine; DILI, drug induced liver injury

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was still not answered.

Vinegar-baked chaihu has been used for more than 2000 years in China, toxic responses were reported merely in recent years, mainly due to inappropriate processing procedures or overdose usage of chaihu. We suspected that vinegar could provide acidic condition during baking process and promote the hydrolysis of some certain saikosaponins, which may be responsible for impairment of chaihu induced toxicity. Therefore, the current study aimed to reveal the chemical-toxicity relationship between saikosaponin a, c, d, and their hydrolysates saikosaponin b1 and b2.

2. Materials and methods

2.1. Chemicals and reagents

Five saikosaponin derivatives (saikosaponin a, b1, b2, c, and d) were purchased from Jiangxi Bencao Tiangong Technology Co., Ltd. (Nanchang, Jiangxi, China). Stock solutions (10 mM) of saikosaponins were prepared with dimethyl sulphoxide (DMSO) and stored at −20 °C as stock solutions for testing. Puromycin was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and was dissolved in PBS by 5 mg/mL. Rapamycin was obtained from LC laboratories (MA, United States) and dissolved in DMSO. The white powder, 3-methyl adenine (3-MA), was purchased from Tocris (Bristol, UK), and was dissolved in DMEM complete culture medium when it was used. Purity of all the chemicals exceeded 98%. 96 well micro-plates for cell culture were purchased from Grenier bio-one (Frickenhausen, Germany). Other chemicals were obtained from Sigma-Aldrich (Saint Louis, Mo, USA) if not stated otherwise.

2.2. Molecular docking

Ligand docking of five saikosaponins with SERCA were performed with Discovery Studio, version 4.0 (Accelrys Software Inc., San Diego, California, USA). The protein crystal structure of SERCA (Clausen et al., 2016) that bounded with thapsigargin (Protein code: 5A3Q) was downloaded from the Protein Data Bank. Protein structure was prepared by removing all non-protein entities, adding hydrogen atoms, and conducting an energy minimization with the CHARMm field. Active binding site was defined according to the binding mode of TNP-AMPPCP with 5A3Q. Molecular docking was done with LibDock. The binding pose with top K-score value was selected for calculation of interaction energy.

2.3. Autophagic influx assay

U2OS-mCherry-EGFP-LC3 stable cell line was cultured in DMEM complete medium supplemented with 2 μg/mL puromycin. For drug induced autophagy evaluation, 6,000 cells/well were seeded into 96 well micro-plates and maintained in the incubator overnight. On the following day, compounds were added to the cells and co-incubated for 4 h. Subsequently, fixed the cells with 4% paraformaldehyde for 20 min at room temperature, and then stained the nuclei with Hoechst 33342 (10 μg/mL) for 15 min. Finally, capture the signals of mCherry-LC3 and EGFP-LC3 on HCS Reader using a 20 × magnification ArrayScan VTI Reader (Thermo Fisher Scientific, Waltham, MA, USA). Green and red puncta were quantified using spot detector protocol.

2.4. Cardiotoxicity assay

Cardiotoxicity was performed according to the procedures we reported previously (Yu et al., 2016). In brief, neonatal rat Cardiomyocytes were isolated from 24-h-old Sprague-Dawley rats (Beijing, China) including both sexes. This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee of Beijing University of Chinese Medicine, Beijing, China. All efforts were made to ensure the minimal number of animals necessary to produce convincing scientific data. Cardiomyocytes were plated into matrigel-coated E-Plate 96. The cells were maintained in complete DMEM medium at 37 °C with 5% CO_2. Signals of cardiomyocytes contractility and impedance were monitored every 15 min using xCELLigence RTCA cardio system (Roche, Mannheim, Germany). When stable cell index, beating rate, and amplitude signals were achieved, the cells were subjected to test compounds. Signals were acquired once per minute during the first hour when compound was added, and then the reading frequency decreased to once per 15 min for the rest time. Captured signals were normalized to vehicle control by xCELLigence Cardio Software.

2.5. H9c2 cytotoxicity assay

H9c2 cells were incubated with DMEM medium supplemented with 10% FBS. For apoptotic assay, the cells were seeded into 96-well microplate at a density of 3,000 cells per well and incubated overnight to allow cells to recover. On the following day, saikosaponins were added to cells pretreated with 3-MA (5 mM) or vehicle control. After treated for 24 h, the cells were subjected to TMRM (25 nM) and Calcein-AM (100 nM) for 30 min in dark condition. Fluorescent signals of TMRM and Calcein-AM staining were acquired by 10 × objective on ArrayScan VTI Reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Drug induced liver injury assay

HepG2 (ATCC, Manassas, VA, USA) were kept under humidified condition with 5% CO_2 at 37 °C. The cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For drug induced liver injury assay, cells were seeded into 96-well plates and incubated overnight to ensure cells to recover. On the following day, the cells were administrated with test compounds or vehicle control for 24 h. Subsequently, the cells were monitored with ToxInsight™ DILI Cartridge (Thermo Fisher Scientific, Waltham, MA, USA) in dark conditions for 45 min at 37 °C, 5% CO_2. Fluorescence signals of nuclei, GSH, ROS, and MMP were collected on the ArrayScan VTI Reader. The images were analyzed using the Cellomics Compartmental Analysis protocol.

2.7. YP/PI double-staining

HepG2 cells were treated with tested compounds in the presence or absence of 3-MA, respectively. After incubated for 8 h with compounds, YP/PI staining were performed with each fluorescent dye (1 μg/mL) at 37 °C for 30 min. Images were acquired using 10 × objective on ImageXpress Micro XLS (MD, Sunnyvale, CA, USA). YP/PI positive rates were acquired by using multi-parameter analysis.

2.8. Data analysis and statistics

All data were presented by GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Data represented mean ± SEM of at least three independent experiments. $p < 0.05$ indicated statistically significant by One-way ANOVA.

3. Results

3.1. Saikosaponin a and d bound with SERCA by molecular docking

Previous study suggested that SSd inhibited Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) activity and induced autophagic cell death (Wong et al., 2013), whether its analogues existed similar toxic trends remained unclear. Thus comparative molecular docking analysis of five chemicals with the protein crystal of SERCA was conducted. Ninety binding poses were obtained for thapsigargin with SERCA with top k-score