Title Page

Inhibition of Human UGT1A1-Mediated Bilirubin Glucuronidation by the Popular Flavonoids Baicalein, Baicalin and Hyperoside is responsible for Herbs (Shuang-huang-lian) -Induced Jaundice

Xiaolei Yang^{a,#}, Guanghao Zhu^{b,#}, Ying Zhang^a, Xubo Wu^{c,d}, Bei Liu^{ae}, Ye Liu^a, Qing Yang^a,
Wandi Du^a, Jingru Liang^a, Jiarong Hu^a, Ping Yang^a, Guangbo Ge^b, Weimin Cai^a, Guo Ma^{a*}

^a School of Pharmacy, Fudan University, Shanghai 201203, PR China (X.Y., Y.Z., B.L., Y.L., Q.Y., W.D., J.L., J.H., P.Y., W. Ca, G.M.

^b Shanghai Frontiers Science Center of Chinese Medicine Chemical Biology (G.Z., G.G.);
Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China (G.Z., G.G.)

^c Department of Hepatobiliary and Pancreatic Surgery, Minhang Hospital, Fudan University, Shanghai 201199, P.R. China (X.W.)

^d Institute of Fudan Minhang Academic Health System, Minhang Hospital, Fudan University, Shanghai 201199, P.R. China (X.W.)

^e Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200000, P.R. China (B.L.)

Running Title Page

Running title: Inhibition of hUGT1A1-mediated bilirubin glucuronidation by flavonoids

[#]The two authors contributed equally to this paper.

* **Address correspondence to:** Guo Ma, Ph.D., School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai, 201203, P.R. China. Tel.: 86-21-5198-0025; E-mail: mg0328@fudan.edu.cn; mg0328@126.com

Numbers of text page: 42

Numbers of tables: 1

Numbers of figures: 11

Numbers of references: 56

Numbers of words in the Abstract: 248

Numbers of words in the Introduction: 748

Numbers of words in Discussion: 1171

Abbreviations

BAI, Baicalein; BA, Baicalin; HYP, Hyperoside; SHL, Shuang-huang-lian; BDG, bilirubin diglucuronide; BG, bilirubin glucuronides; BMG1, bilirubin monoglucuronide 1; BMG2, bilirubin monoglucuronide 2; TBG, total bilirubin glucuronides; UCB, unconjugated bilirubin; HILI, herb-induced liver injury; HPLC, high-performance liquid chromatography; hUGT1A1, human UDP-glucuronosyltransferases1A1; V , velocity; V_{max} , maximum velocity; IC_{50} , half maximal inhibitory concentration; K_i , inhibition constant; K_m , Michaelis–Menten constant.

Abstract

Bilirubin-related adverse drug reactions (ADRs) or malady (e.g., jaundice) induced by some herbs rich in certain flavonoids have been widely reported. However, the causes and mechanisms of the ADRs are not well understood. The aim of this paper was to explore the mechanism of Shuang-huang-lian injections (SHL) and its major constituents-induced jaundice *via* inhibiting human UDP-glucuronosyltransferases1A1 (hUGT1A1)-mediated bilirubin glucuronidation. The inhibitory effects of SHL and its major constituents in the herbal medicine including baicalein (BAI), baicalin (BA) and hyperoside (HYP) on bilirubin glucuronidation were investigated. This study indicated that the average formation rates of bilirubin glucuronides (i.e., BMG1, BMG2, BDG) displayed significant differences ($P < 0.05$), specially, the formation of mono-glucuronides (BMGs) was favored regardless whether an inhibitor was absent or presence. SHL, BAI, BA and HYP dose-dependently inhibit bilirubin glucuronidation, showing the IC_{50} values against total bilirubin glucuronidation (TBG) were in the range of $(7.69 \pm 0.94) - (37.09 \pm 2.03)$ $\mu\text{g/mL}$, $(4.51 \pm 0.27) - (20.84 \pm 1.99)$ μM , $(22.36 \pm 5.74) - (41.35 \pm 2.40)$ μM , and $(15.16 \pm 1.12) - (42.80 \pm 2.63)$ μM for SHL, BAI, BA, and HYP, respectively. Both inhibition kinetics assays and molecular docking simulations suggested that SHL, BAI, BA, and HYP significantly inhibited hUGT1A1-mediated bilirubin glucuronidation *via* a mixed-type inhibition. Collectively, some naturally occurring flavonoids (BAI, BA and HYP) in SHL have been identified as the inhibitors against

hUGT1A1-mediated bilirubin glucuronidation, which well-explains the bilirubin-related ADRs or malady triggered by SHL in clinical settings.

Significance Statement

Herbal products and their components (e.g., flavonoids), which been widely used in the whole world, may cause liver injury. As a commonly used herbal products rich in flavonoids, Shuang-huang-lian injections (SHL), easily lead to symptoms of liver injury (e.g., jaundice) owing to significant inhibition of hUGT1A1-mediated bilirubin glucuronidation by its flavonoid components (i.e., baicalein, baicalin, hyperoside). Herbs-induced bilirubin-related ADRs and its associated clinical significance should be seriously considered.

Introduction

Herbs have been widely used as drugs or dietary supplements in the world (Ballotin et al., 2021; He et al., 2019). About 80% of the world's population have consumed herbs for treatment of various diseases (Afolabi et al., 2012). Herbs were commonly thought to be safe. However, more and more reports have demonstrated that some herbs may cause liver injury (He et al., 2019). Herb-induced liver injury (HILI) (Danan and Teschke, 2015; Jing and Teschke, 2018; Teschke et al., 2020) easily lead to adverse events such as jaundice, hepatitis, hepatic fibrosis, hepatic failure, and even death. HILI often accompany jaundice with the elevated plasma concentration of bilirubin.

Bilirubin is primarily originated from the breakdown of erythrocyte hemoglobin (Vítek, 2012), which is an important basis for clinical determination of jaundice, index of liver function and biomarker of hepatotoxicity. As an important endogenous substance, bilirubin exerts strong antioxidant activity in physiological concentration, but cytotoxicity in high concentration leads to irreversible brain damage (e.g., kernicterus) (Fujiwara et al., 2018). Bilirubin exists in two forms *in vivo*, i.e., unconjugated bilirubin (UCB) and conjugated bilirubin (CB, namely, bilirubin glucuronides, BG). Normally, UCB is extensively metabolized to BG by UDP-glucuronosyltransferases1A1 (UGT1A1) (Bosma, 2003), and generates two bilirubin mono-glucuronides (BMGs) isomers in the liver, including bilirubin monoglucuronide 1 (BMG1) and bilirubin monoglucuronide 2 (BMG2) (Ma et al., 2014). Then BMGs

are metabolized into bilirubin diglucuronide (BDG) (Fig.1). Finally, BG are excreted into the bile by the canalicular efflux pump multidrug resistance protein 2 (MRP2) and passed on for excretion *via* feces and urine (Tátrai and Krajcsi, 2020). UGT1A1 is the sole enzyme responsible for bilirubin glucuronidation, playing an important role in bilirubin metabolism and detoxification (Bock, 2015). If UGT1A1 is inhibited by herbs or drugs, bilirubin metabolism will be disrupted, and bilirubin will accumulate in the body, leading to hepatotoxicity (e.g., jaundice) and neurotoxicity (e.g., kernicterus).

Naturally occurring flavonoids have been developed to drugs and dietary supplements for their favorable pharmacological effects (Lv et al., 2019). Many flavonoids (apigenin, chrysin, genistein, hesperetin, kaempferol, naringenin, phloretin, scutellarein, and wogonin) have displayed strong inhibitory effects on UGT1A1, which possibly lead to jaundice (Wang et al., 2015; Zhou et al., 2021). Some cases (Lin and Ho, 1994) reported that the patients taking flavonoids occurred jaundice, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were markedly increased, indicating that flavonoids may cause jaundice and liver injury.

Shuang-huang-lian (SHL), a well-known preparation of traditional Chinese medicine containing the extracts of *Lonicerae japonicae flos*, *Scutellariae radix* and *Forsythiae fructus*, shows good anti-bacterial, anti-viral and immunomodulation activity, and is

recorded in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2020). SHL injection is widely used to treat the patients with acute upper respiratory tract infection, acute bronchitis and mild pneumonia (Zhuang et al., 2020) for its rapid onset and effectiveness. According to the requirement of the Chinese Pharmacopoeia 2020, dosing regimens of SHL injection for patients with respiratory diseases were 60 mg/kg each time, intravenous drip, once a day, or following the doctor's advice. However, with its extensive use of SHL injections, high incidence of severe ADRs has been observed (Li and Tang, 1996; Tang et al., 2016; Wang et al., 2010; Chen et al., 2014; Zhang et al., 2012). In some case reports, the total bilirubin, the CB and UCB exceeded more than 3 times of the normal upper limit (NUL), even up to 13.8 times (289.5 $\mu\text{mol/L}$) and 12.2 times (166.9 $\mu\text{mol/L}$) of NUL (Chen et al., 2014). The National Medical Products Administration (NMPA) has suspended the sales and marketing provisions of SHL injections in 2009, and prohibited the use for children under 4 years old and pregnant women in 2018 (NMPA, 2018; NMPA, 2020). Unfortunately, the related causes and mechanism have not been disclosed up to now. We speculated that it was possibly due to the flavonoid components in SHL, e.g., baicalein (BAI), baicalin (BA), hyperoside (HYP) (Fig.2), are the potential UGT1A1 inhibitors (Teng et al., 2013), significantly inhibiting UGT1A1-mediated bilirubin glucuronidation, thus resulting in jaundice, and severe liver disease.

The major objective of the present study was to reveal the vital role of UGT1A1 in

herbs-induced jaundice by assessing the inhibitory effects of hUGT1A1-mediated bilirubin glucuronidation by components of SHL. The research will provide a novel understanding on the mechanisms of bilirubin-related ADRs caused by the certain flavonoids-enriched herbal products, shifting away from the idea that herbs are always safe, and help clinicians in deciding for whom to conduct or to avoid herbs in patients having a bilirubin-related ADR risk.

Materials and methods

Materials

BAI, BA and HYP (>98%; National Institutes for Food and Drug Control, Beijing, China); Shuang-huang-lian injection (prepared from the lyophilization of *Forsythia suspensa*, *Lonicera japonica* Thunb. and *Scutellaria baicalensis* Georgi.; the total herbal extracts 600 mg/bottle; Batch No.200603; Jiangsu Shenlong Pharmaceutical Co., Ltd, Jiangsu Province, China); bilirubin (bilirubin III- α 5.93%, IX- α 90.11%, and XIII- α 3.12%), uridine 5'-diphospho-glucuronic acid trisodium salt (UDPGA), recombinant hUGT1A1 enzyme (Biosciences-Discovery Labware, Woburn, MA, USA); KH_2PO_4 , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, formic acid, and NaCl (all of analytical grade; Sinopharm Chemical Reagent Co., Ltd, Shanghai, China); methanol and acetonitrile (HPLC grade; Fisher Scientific International, Inc., Fair Lawn, NJ, USA); argon (99.99%, Shanghai Lvmin Gas Co. Ltd, China). Purified water was filtered *via* a Millipore (Millipore Corp., Billerica, MA, USA). The other chemicals were analytical grade and obtained from standard sources.

Assay of UGT1A1 Activity.

The incubation procedures were processed under the dim light. Bilirubin was dissolved in dimethylsulfoxide (DMSO) (the final DMSO concentration: 1%). The incubation mixtures in the amber glass vials (full of argon) contained 0.2-5.0 μM bilirubin, 50 mM potassium phosphate buffer (pH 7.4), 0.88 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and

12.5 µg/mL hUGT1A1 Supersomes. The reaction was initiated by adding UDPGA (3.5 mM) after a pre-incubation in a shaking water bath (5 min, 37 °C); it was terminated after 15-30 min by adding 600 µL of ice-cold methanol with 200 mM L-Ascorbic acid. The proteins were precipitated by centrifugation at 12,000 rpm (10 min, 4 °C), and the supernatants were analyzed by high-performance liquid chromatography (HPLC) system (Ma et al., 2014; Ma et al., 2017) for assaying bilirubin and its glucuronides. The assays were conducted in triplicates respectively.

Inhibition of hUGT1A1-mediated Bilirubin Glucuronidation.

The Michaelis–Menten constant (K_m) values were determined by the samples as described above. Glucuronidation of bilirubin was evaluated at a series of concentrations of bilirubin and the potential inhibitors. For assays with inhibitors, the final concentration of bilirubin was 0.2-5.0 µM. The final concentrations of the potential inhibitors were as follows: SHL, 0-74.84 µg/mL; BAI, 0-50 µM; BA, 0-100 µM; and HYP, 0-100 µM. The concentrations of SHL were calculated based on the concentration of total herbal extract in the injections. The detailed reaction conditions were shown in Supplemental Table 1A and 1B.

HPLC Assay.

The chromatographic analyses were carried out using a LC-2010A HT HPLC system (Shimadzu, Kyoto, Japan) equipped with an automatic sampler, a quaternary pump, a

UV-Vis detector, a temperature control oven, a system controller, and a LabSolution workstation. Briefly, UCB and its metabolites were separated on a reverse phase Diamonsil™ C₁₈ column (200 mm × 4.6 mm, i.d., 5 μm, Dikma) with a guard column (Cartridge Guard Column E, Inertsil ODS-SP, 10 mm × 4 mm, i.d., 5 μm, GL Sciences Inc.), using the following mobile phase gradient program at a flow rate of 1 mL/min: mobile phase A (A) = H₂O with 0.1% formic acid; mobile phase B (B) = 100% acetonitrile; 0-9 min, 40-75% B; 9-18 min, 75-95% B; 18-26 min, 95% B; 26-28 min, 95-40% B; 28-30 min, 40% B. The column temperature was set at 45 °C. Detection of samples was set by wavelength of 450 nm, and an injection volume of 100 μL as which was done previously (Ma et al., 2014).

The calibration curves of UCB were used to estimate the concentrations of BMG1, BMG2 and BDG for lacking of commercial products of these bilirubin glucuronides. The representative chromatograms of UCB and its metabolites in hUGT1A1 incubation samples were shown in Supplemental Fig. 1, and were analyzed by LC-MS/MS systems which was the same as our recent publication (Ma et al., 2017). The validation parameters of the HPLC methods for assay of bilirubin and its glucuronides were shown in Supplemental Tables 2-7.

Determination of the Major Constituents in SHL.

HPLC system was the same as the above, and was used to analyze the major

constituents in the SHL injections, using the following mobile phase gradient program at a flow rate of 1 mL/min: The mobile phase A = 100% acetonitrile, mobile phase B (B) = H₂O with 0.1% formic acid; 0-15 min, 92% B; 15-25 min, 92-80% B; 25-28 min, 80-70% B; 28-30 min, 70-40% B; 30-40 min, 40-55% B; 40-45 min, 55-92% B. The column temperature was 40°C. The detection wavelength was set 327 nm for determination of chlorogenic acid, caffeic acid, BAI and BA, and was set 360 nm for determination of rutin and HYP in SHL, respectively. The sample injection volume was 10 µL. Representative chromatograms of the primary constituents in the SHL were shown in Supplemental Fig.2. The validation parameters of the HPLC methods for assay of main components in SHL injection were shown in Supplemental Tables 8-9.

Determination of Inhibitory Kinetics of hUGT1A1-mediated Bilirubin Glucuronidation.

The Kinetics profiles, Lineweaver–Burk (double-reciprocal) plots (1/V versus 1/[S]) and apparent kinetic parameters (e.g., V_{max} , K_m , and K_i) of hUGT1A1-mediated bilirubin glucuronidation in the presence of different concentrations of inhibitors were determined by fitting the reaction rates of different concentrations of inhibitors, substrate and initial velocities, using nonlinear regression analysis, respectively. The good fitting was assessed using square of correlation coefficient (R^2) and Akaike information criterion (AIC) values. The mechanism and type of hUGT1A1-mediated

bilirubin glucuronidation by SHL, BAI, BA, and HYP were determined in the light of changing trends of K_m and V_{max} as well as the double-reciprocals plots ($1/V$ versus $1/[S]$) in the present study. Kinetics of the mixed-type inhibition was described by the following equation (1)

$$V = \frac{V_{max}}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{\alpha K_i}\right)} \quad (1)$$

Where V_{max} is the maximum reaction velocity; K_m is a Michaelis–Menten constant; I is the inhibitor concentration; K_i is an inhibition constant; S is the substrate concentration; α is a modifying factor; and V is the reaction velocity. IC_{50} values were calculated by a decrease in the formation of the substrate glucuronide(s) in assays of UGT1A1 activity.

Molecular Docking Simulations.

To obtain more profound insights into the inhibition of hUGT1A1-mediated bilirubin glucuronidation by BAI, BA, and HYP, molecular docking simulations were performed by AutoDock Vina (1.1.2) (Trott and Olson, 2010). Firstly, the AlphaFold-predicted structure of hUGT1A1 (UniProt code: P22309) and the UDPGA-complexed structure of medicago truncatula UGT71G1 (PDB code: 2acw) were superimposed and aligned by PyMOL (The PyMOL Molecular Graphics System 2.4.0a0 Open-Source, Schrödinger LLC., New York, USA) so as to insert UDPGA correctly into hUGT1A1 (Jumper et al., 2021; Shao et al., 2005). Next, structures of hUGT1A1,

bilirubin and the inhibitor (i.e, BAI, BA, or HYP) were preprocessed by AutoDockTools 1.5.6, including adding polar hydrogens, adding Kollman charges and adjusting AD4 atom types. Then, bilirubin and the inhibitor were docked into the catalytic site of hUGT1A1, and the inhibitor was docked into the allosteric site of hUGT1A1 predicted by CavityPlus besides (Xu et al., 2018). Finally, the detailed receptor-ligand interactions analysis of the top docking poses was conducted by Discovery Studio Visualizer (BIOVIA Discovery Studio 2019, Dassault Systèmes, San Diego, USA).

Statistical Analysis.

The data were analyzed in GraphPad Prism 9.0 software for Windows (GraphPad Software, San Diego, CA). A two-way ANOVA and one-way ANOVA were used. $P < 0.05$ was considered to be significant.

Results

Bilirubin Glucuronidation Kinetics.

In order to reliably evaluate inhibitory effect of glucuronidation and accurately determine the IC_{50} values, K_m values of bilirubin glucuronidation were determined in this study. The K_m values for BMG1, BMG2, BDG and TBG were $(0.66 \pm 0.11) \mu M$, $(0.70 \pm 0.13) \mu M$, $(0.73 \pm 0.15) \mu M$ and $(0.69 \pm 0.12) \mu M$, respectively (Table 1 and Fig.3). In the selected substrate concentration range (0.2-5.0 μM), bilirubin glucuronidation showed substrate inhibition kinetics.

Inhibitory Effect of SHL and its Primary Constituents on hUGT1A1-Mediated Bilirubin Glucuronidation.

The contents of components in SHL injection (600 mg/bottle) were (9.77 ± 0.20) mg chlorogenic acid, (2.59 ± 0.09) mg caffeic acid, (178.92 ± 4.18) mg BA, (2.09 ± 0.11) mg BAI, (7.86 ± 0.44) mg rutin and (2.27 ± 0.12) mg HYP, respectively (Supplemental Table 10). In order to explore inhibitory effect of SHL and its primary constituents on hUGT1A1-mediated bilirubin glucuronidation, SHL extract, BAI, BA, HYP, rutin, chlorogenic acid and caffeic acid, were co-incubated with bilirubin and hUGT1A1, respectively. These results indicated SHL, BAI, BA, HYP exhibited strong inhibitory effects, but rutin, chlorogenic acid, and caffeic acid exhibited poor inhibitory effects on hUGT1A1-mediated bilirubin glucuronidation. IC_{50} values of SHL, BAI, BA, HYP against glucuronidation of bilirubin ($0.69 \mu M$) were $(22.57 \pm$

0.85) $\mu\text{g/mL}$, $(11.39 \pm 0.43) \mu\text{M}$, $(34.74 \pm 2.53) \mu\text{M}$, $(28.25 \pm 0.75) \mu\text{M}$ for TBG, respectively. IC_{50} values of BAI, BA and HYP against bilirubin glucuronidation for BMG1, BMG2, BDG, and TBG showed the same trend (Figs.4-5 and Supplemental Table 11). The inhibitory effects (IC_{50}) of BAI, BA and HYP on hUGT1A1-mediated bilirubin glucuronidation exhibited significant differences ($P < 0.05$), and the IC_{50} values was $\text{BA} > \text{HYP} > \text{BAI}$ at $0.69 \mu\text{M}$ bilirubin. Inhibitory percentage of rutin, chlorogenic acid, caffeic acid (all of them were $100 \mu\text{M}$) against bilirubin glucuronidation for TBG were only 17.40%, 4.50%, 2.12%, and their IC_{50} values were much higher than $100 \mu\text{M}$ (Supplemental Fig.3). Thus, it can be seen that, SHL, BAI, BA, and HYP were inhibitors of UGT1A1 owing to their relatively low IC_{50} values, but rutin, chlorogenic acid and caffeic acid were not inhibitors of UGT1A1 because of their poor inhibitory effect.

Rates of Bilirubin Glucuronidation in the Presence of SHL, BAI, BA, and HYP.

To reveal the underlying mechanism of SHL-induced jaundice, inhibition of hUGT1A1-mediated bilirubin glucuronidation by SHL and its major constituents BAI, BA and HYP was subsequently investigated. As shown in Fig.6-9, compared to the control group (without SHL, BAI, BA, and HYP), average rates of BMG1, BMG2, BDG, and TBG formation showed a significant decrease with elevated concentrations of SHL (0-74.84 $\mu\text{g/mL}$), BAI (0-50 μM), BA (0-100 μM), or HYP (0-100 μM). Meanwhile, no matter the SHL, BAI, BA, and HYP were present or not, the

average rates of BMG1, BMG2, BDG, and TBG formation showed a trend of first increase and then decrease, accompanied with the increasing bilirubin concentrations. It indicated that hUGT1A1-mediated bilirubin glucuronidation was inhibited by SHL, BAI, BA, or HYP. The glucuronidation reaction at a higher concentration of substrate ($>2 \mu\text{M}$) showed dual inhibition by both the inhibitor (i.e., SHL, BAI, BA, or HYP) and bilirubin itself. The average rates of bilirubin glucuronides were ranked as $\text{BMG2} > \text{BMG1} > \text{BDG}$, suggesting that hUGT1A1-mediated bilirubin glucuronidation displayed a regioselectivity.

Inhibitory extents of bilirubin glucuronidation were enhanced with increase of concentration of the SHL, BAI, BA or HYP, and all showed a gradually decreasing profile with the increase in concentrations of bilirubin. As a result, inhibition of hUGT1A1-mediated bilirubin glucuronidation by SHL, BAI, BA or HYP showed a concentration dependence of substrate and inhibitor (Figs.6–9 and Supplemental Figs.4-7). Compared to that of the control group, formation rates of TBG were inhibited in the range of $(60.50 \pm 1.44)\%$ to $(86.56 \pm 1.26)\%$ in the presence of $74.84 \mu\text{g/mL}$ SHL, $(67.11 \pm 1.61)\%$ to $(86.66 \pm 0.18)\%$ in the presence of $50 \mu\text{M}$ BAI, $(67.76 \pm 1.14)\%$ to $(74.42 \pm 3.25)\%$ in the presence of $100 \mu\text{M}$ BA, and $(78.70 \pm 1.05)\%$ to $(94.87 \pm 0.18)\%$ in the presence of $100 \mu\text{M}$ HYP, respectively. The inhibition of formation rates of BMG1, BMG2 and BDG displayed the same tendencies as that of TBG, compared with the controls (without SHL, BAI, BA, and

HYP).

IC₅₀ Values of SHL, BAI, BA and HYP as the Inhibitors of Glucuronidation of Bilirubin.

On the whole, average IC₅₀ values of SHL, BAI, BA and HYP against BMG1, BMG2, BDG, and TBG increased with the increasing concentrations of bilirubin (0.2-5.0 μM), showing a bilirubin concentration-dependent manner. Inhibition of SHL, BAI, BA and HYP on bilirubin glucuronidation decreased with the increasing concentrations of bilirubin ($P < 0.05$) (Fig.10). The IC₅₀ value ranges were distributed at $(7.69 \pm 0.94) \mu\text{g/mL}$ to $(37.09 \pm 2.03) \mu\text{g/mL}$, $(4.51 \pm 0.27) \mu\text{M}$ to $(20.84 \pm 1.99) \mu\text{M}$, $(22.36 \pm 5.74) \mu\text{M}$ to $(41.35 \pm 2.40) \mu\text{M}$, and $(15.16 \pm 1.12) \mu\text{M}$ to $(42.80 \pm 2.63) \mu\text{M}$ for TBG in the presence of SHL, BAI, BA and HYP, respectively. Average IC₅₀ values of SHL, BAI, BA and HYP against three glucuronides (BMG1, BMG2, and BDG) showed the same tendency at various bilirubin concentrations through various glucuronidation pathway. Compared with that on BMG1 and BMG2, these inhibitors showed a stronger inhibitory effect on BDG (Fig.10, Supplemental Tables 12-15). This means that the inhibition on formation of BDG is stronger than that of BMG1 and BMG2 by SHL, BAI, BA and HYP ($P < 0.05$). Namely, inhibition of glucuronidation of bilirubin by SHL and its component BAI, BA and HYP displayed regioselectivity. It can be speculated that, compared to UCB, it will be difficult for bilirubin mono-glucuronides (BMGs, i.e., BMG1 and BMG2) to bind with UGT1A1

when the inhibitor (SHL, BAI, BA or HYP) is already bound to UGT1A1, and where the inhibitor appears to favor binding to the UGT1A1-bilirubin complex (Storey, 2005). In other words, compared to that of UCB, the mono-glucuronidated bilirubin (BMG1, BMG2) binding to UGT1A1 is more easily inhibited by the inhibitor, and it is difficult for it to further form double-bound glucuronic acid conjugate (i.e., BDG) in the presence of the inhibitor.

Inhibition Type of Bilirubin Glucuronidation by SHL, BAI, BA and HYP.

As shown in the Table 1 and the supplemental Table 16-19, compared to the kinetic parameters of hUGT1A1-mediated bilirubin glucuronidation in the absence of inhibitors (SHL, BAI, BA and HYP), both the apparent K_m (K_m^{app}) and the apparent V_{max} (V_{max}^{app}) values of hUGT1A1-mediated bilirubin glucuronidation appear to decrease in the presence of inhibitors, which suggested that inhibition by SHL, BAI, BA or HYP obeyed the mixed-type inhibition. In accordance with the criteria of R^2 and AIC of the best fit kinetic parameters, inhibition of hUGT1A1-mediated bilirubin glucuronidation in the presence of SHL (0-74.84 $\mu\text{g/mL}$), BAI (0-50 μM), BA (0-100 μM), or HYP (0-100 μM) were fitted to the mixed-type inhibition best (Supplemental Tables 16-19). Identical with the kinetic analysis, the double reciprocal plots (Figs.6-9) also conformed to the defined features of the mixed-type inhibition (Segel, 1975).

K_i values of inhibition of TBG were found to be $(12.69 \pm 2.26) \mu\text{g/mL}$, (5.72 ± 1.24)

μM , $(32.43 \pm 8.47) \mu\text{M}$ and $(16.62 \pm 3.20) \mu\text{M}$ by SHL, BAI, BA, and HYP, respectively (Supplemental Tables 16-19). It is similar between the K_i values of inhibition of BMG1, BMG2, BDG and TBG by SHL (Supplemental Tables 16). The K_i for BMG1, BMG2, BDG and TBG by BAI, BA and HYP displayed the same as inhibitory trend through various glucuronidation pathway as the IC_{50} values, with a stronger inhibitory effect on BDG compared with that on BMG1 and BMG2, respectively. Among the three flavonoids in SHL, BAI showed the strongest ability to inhibit the bilirubin glucuronidation. In brief, these lower K_i and IC_{50} values meant that SHL, BAI, BA, and HYP showed the strong ability to inhibit the bilirubin glucuronidation mediated by hUGT1A1.

Molecular docking simulations.

As shown in Fig.11 and Supplemental Fig.8, the catalytic pocket of h-UGT1A1 was large, and was sufficient to accommodate both bilirubin and UDPGA (Ciotti and Owens, 1996; Locuson and Tracy, 2007). All of BAI, BA and HYP overlapped bilirubin in the catalytic site (Supplemental Fig.9). Either of bilirubin and BAI formed a robust hydrogen bond with UDPGA of 2.17 Å (Fig.11A, 11B). The hydrogen bonds connecting UDPGA with BA and HYP were 2.51 Å and 2.67 Å, respectively. The measurement of hydrogen bonds was exactly in accordance with the inhibitory activities of BAI, BA and HYP, as shorter hydrogen bonds meant stronger interactions, implying stronger inhibition. 2D interaction analysis showed that BAI

and HYP had partiality for binding Asp396 with hydrogen bonds (Supplemental Fig.10B, 10F). It was remarkable that natural variant G395V in patients found no residual bilirubin glucuronidation activity (Servedio et al., 2005). Phe92 and Leu95 formed strong interactions with BA and HYP *via* variety of hydrophobic bonds, including Pi-alkyl, Pi-Pi T-shaped, Pi-sigma, Pi-Pi stacked interactions (Supplemental Fig.10D, 10F). Solely HYP formed hydrogen bond and hydrophobic interaction with Ser38 and His39 (Supplemental Fig.10F), both of which were crucial for sugar donor binding and glucuronyl transfer (Locuson and Tracy, 2007). In the allosteric site, Cys177, Asn188, Phe190, Ala210 and Asn220 were well combined by BAI, BA and HYP (Supplemental Fig.10C, 10E, 10G). Impacting Cys177 was fatal, according to previous research and its location, which was next to the membrane-binding peptide (Kadakol et al., 2000; Locuson and Tracy, 2007). The above results of molecular docking simulation implied that BAI, BA and HYP could firmly occupy the catalytic site as well as the allosteric site of hUGT1A1, which were consistent with the mixed-typed inhibition mode.

Discussion

Recently, herbs, herbal products and their components (e.g., flavonoids, anthraquinones)-induced various degrees of liver injury with jaundice, were usually reported, challenging the widespread use in folk medicine (Larrey et al., 1992; Teschke et al., 2011).

SHL products are widely used in folk and clinical conditions. Case reports (Chen, 2004; Li and Tang, 1996; Li, 2008) have shown that SHL can lead to jaundice or hyperbilirubinemia. However, the cause and mechanism has not been disclosed up to now. For the first time, this study found that SHL and its major components BAI, BA and HYP, but not rutin, chlorogenic acid and caffeic acid, are the potent inhibitors of UGT1A1 (Fig.4, Fig.5, Fig.10 and Supplemental Tables 11-19). BAI, BA and HYP are primarily responsible for SHL-induced jaundice by inhibiting hUGT1A1-mediated bilirubin glucuronidation through a mixed-type inhibition mechanism.

BAI and BA are from *Scutellaria baicalensis* Georgi, and HYP is from *Lonicera japonica* Thunb. and *Forsythia suspense* (Thunb.) Vahl. BAI exhibited the most potent inhibitory effects on hUGT1A1-mediated bilirubin glucuronidation among the tested components in SHL. Although the content of BA is the highest in SHL, its inhibitory effect on UGT1A1 is not as strong as BAI. The ability of inhibition of BAI on bilirubin glucuronidation mediated by hUGT1A1 was about 3-5 folds higher than

that of BA with various concentrations of bilirubin (0.2-5.0 μM) and inhibitors (0-100 μM) (Fig.10, Supplemental Table 12, Table 13, Table 16 and Table 17). As aglycone of BA (i.e., baicalein 7-O- β -D-glucuronide), BAI displayed a stronger inhibitory potential towards UGT1A1 than that of BA. It is probably because BA adds one β -D-glucuronic acid group at its 7-hydroxyl (7-OH) site than that of BAI, which increases the steric hindrance of bilirubin binding to UGT1A1 (Fig.2). It can be predicted that, inhibitory effect of some flavonoid aglycone on UGT1A1 was stronger than that of its glycoside, which is much easier to inhibit hUGT1A1-mediated bilirubin glucuronidation, further leads to jaundice or hyperbilirubinemia. However, it cannot be ignored that BA can be transformed into BAI by gut microbiota (Noh et al., 2016; Zhang et al., 2007), meanwhile, enterohepatic circulation of BA and mutual transformation of BAI and BA in the liver increase greatly their exposure *in vivo* (Xing et al., 2005), which significantly strengthen inhibitory potential of BAI and BA towards UGT1A1.

As the key enzyme for bilirubin glucuronidation, UGT1A1 may accommodate multiple binding interaction modes since it has a large binding pocket (Öeren et al., 2021; Schirris et al., 2015). The fact is consistent with our results that in the presence of SHL, BAI, BA and HYP, bilirubin glucuronidation showed the characteristics of mixed-type inhibition (Figs.6-9). As the flavonoids, BAI, BA and HYP possessing multiple phenolic hydroxyl groups ($-\text{OH}$) undergo *O*-glucuronidation at various

positions can be metabolized by UGT1A1, meanwhile, they can also inhibit hUGT1A1-catalyzed glucuronidation of endogenous substances (e.g., bilirubin) and xenobiotics (e.g., polyphenol, flavonoids) (Ma et al., 2013; Zhou et al., 2011b). It can be speculated that BAI, BA and HYP act as both an inhibitor and substrate of UGT1A1, not only they can competitively bind to the active site of UGT1A1 with bilirubin *via* the competitive inhibition mode, but they can also bind to the allosteric site of UGT1A1 *via* the uncompetitive inhibition. It meant that inhibition of hUGT1A1-mediated bilirubin glucuronidation by SHL, BAI, BA and HYP obey the mixed-type inhibition, which is a “mixture” of competitive inhibition and uncompetitive inhibition.

In the present study, an increase in the apparent affinity of the UGT1A1 for the bilirubin where SHL, BAI, BA and HYP more favor binding to the UGT1A1-bilirubin complex (more closely mimics uncompetitive binding) than binding to the UGT1A1 ($K_m^{app} < K_m$, respectively. Meanwhile, the apparent maximum enzyme reaction rate of UGT1A1-mediated bilirubin glucuronidation decreased ($V_{max}^{app} < V_{max}$) in the present of SHL, BAI, BA and HYP. Obviously, the decrease of K_m and V_{max} indicated inhibitory kinetics of UGT1A1-mediated bilirubin glucuronidation by SHL, BAI, BA and HYP were fitted to the mixed-type inhibition (Wu et al., 2012) (Supplemental Tables 16-19).

As mentioned above, UGT1A1 plays an important role in herbs-induced bilirubin-related ADRs or liver injury. It has been known that the risk for treatment-related jaundice for the tyrosine kinase inhibitors (e.g., pazopanib, erlotinib, and vemurafenib) which inhibit UGT1A1 was higher in patients carrying loss-of-function polymorphisms in gene encoding UGT1A1 (e.g., UGT1A1*6 and UGT1A1*28), and different inhibitors exhibited different inhibitory effect on different UGT1A1 phenotypes (Abumiya et al., 2014; Henriksen et al., 2020; Qosa et al., 2018). It could be speculated that SHL and its different components (e.g., BAI and BA) possibly exhibited different inhibitory effect on different UGT1A1 phenotypes (UGT1A1*6 and UGT1A1*28) given the differences in glucuronidation activity. In addition, it could also be deduced from the molecular docking analysis, the interaction (i.e., affinity of binding) of the active and allosteric sites within the UGT1A1 enzyme towards the flavonoids in SHL was possibly different in patients carrying UGT1A1*6 variant allele compared to that in wild-type allele (UGT1A1*1 allele). Namely, BAI, BA, and HYP inhibited activity of UGT1A1 *via* direct ligand-receptor interactions upon catalytic and allosteric site of UGT1A1. In order to avoid an interruption of anti-infectious therapy using SHL products due to SHL-induced bilirubin-related ADRs, it may be advisable to reduce the initial dose of SHL for poor UGT1A1 metabolizers with UGT1A1*6 or UGT1A1*28 genotypes. However, these deductions need to be verified by the further experiments.

Besides UGT1A1-catalyzed glucuronidation of bilirubin, organic anion transporting polypeptides (OATPs, e.g., OATP1B1, OATP1B3), multidrug resistance-associated protein (MRPs, e.g., MRP1, MRP2, MRP3) and bile salt exporter pump (BSEP) at the sinusoidal and/or canalicular membrane of hepatocytes also possibly play important roles in herbs (e.g., SHL)-induced jaundice (Chang et al., 2013). Cases were reported (Chen and Zhang, 2007) that both plasma concentrations of CB and UCB were significantly elevated in the patients administrated SHL. For CB, the inhibition of MRP2-mediated efflux of bilirubin glucuronides by baicalin (BAI) has been confirmed in the previous study (Kalapos-Kovács et al., 2015), which explained the elevated plasma concentration of CB. For UCB, the inhibition of UGT1A1 and OATP1B1/1B3 should be considered comprehensively. But there is no solid evidence that the SHL and its major components inhibit OATP1B1/1B3-mediated uptake of bilirubin at present. Our study revealed that inhibition of UGT1A1 by SHL and its major components was one of main mechanism of SHL-induced jaundice, and explained the reason for the elevated plasma concentration of UCB. In the future, effect of these major ingredients as well as the other potential components (e.g., scutellarin, wogonin, secoxyloganin, luteolin-7-O- β -D glucoside, isochlorogenic acid A, B, C, neochlorogenic acid, cryptochlorogenic acid, phillyrin, forsythoside A, E) (Xue et al., 2021) in SHL on OATP1B1/3-mediated hepatocyte uptake of bilirubin and MRP2-mediated efflux of bilirubin should be investigated so as to comprehensively and systematically disclose the causes and mechanisms of herbs-induced jaundice.

In summary, our study disclosed that SHL-induced bilirubin-related ADRs or malady (e.g., jaundice) mainly due to its major components BAI, BA and HYP inhibited hUGT1A1-mediated bilirubin glucuronidation *via* a mixed-type inhibition mechanism. The potential risk of herbs-induced bilirubin-related ADRs should be seriously considered by medical personnel and the public.

Acknowledgments

The author would like to thank Dr. Ming Hu of Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston for his help in research design and writing of the manuscript.

Authorship Contributions

Participated in research design: Ma and X. Yang

Conducted the experiments: X. Yang, Zhang, Y. Liu, Q. Yang and Du

Contributed the new reagents or analytic tools: Ma, P. Yang and X. Wu

Molecular docking simulations: G. Ge and G. Zhu

Performed data analysis: X. Yang, Liu, Hu, and Liang

Wrote or contributed to the writing of the manuscript: X. Yang, Ma and G. Zhu

Reference:

- Abumiya M, Takahashi N, Niioka T, Kameoka Y, Fujishima N, Tagawa H, Sawada K, Miura M (2014). Influence of UGT1A1 6, 27, and 28 polymorphisms on nilotinib-induced hyperbilirubinemia in Japanese patients with chronic myeloid leukemia. *Drug Metab Pharmacokinet* **29**:449-454.
- Afolabi SO, Akindele AJ, Awodele O, Anunobi CC, and Adeyemi OO (2012). A 90 day chronic toxicity study of Nigerian herbal preparation DAS-77 in rats. *BMC Complement Altern Med* **12**:79.
- Ballotin VR, Bigarella LG, Brandão ABM, Balbinot RA, Balbinot SS, Soldera J (2021). Herb-induced liver injury: Systematic review and meta-analysis. *World J Clin Cases* **9**:5490-5513.
- Bock KW (2015). Roles of human UDP-glucuronosyltransferases in clearance and homeostasis of endogenous substrates, and functional implications. *Biochem Pharmacol* **96**:77-82.
- Bosma PJ (2003). Inherited disorders of bilirubin metabolism. *J Hepatol* **38**:107-117.
- Chang JH, Plise E, Cheong J, Ho Q, and Lin M (2013). Evaluating the in vitro inhibition of UGT1A1, OATP1B1, OATP1B3, MRP2, and BSEP in predicting drug-induced hyperbilirubinemia. *Mol Pharm* **10**:3067-3075.
- Chen H, Wen J, Zhao W, Li WR, Wang NS, Fu SQ (2014). The renal toxicity of Shuanghuanglian combined with cefradine for tail injection in rats. *Pharmacol Clin Chin Mater Med* **30**:95-98.
- Chen L (2004). Hemolytic anemia and drug-induced liver injury caused by Shuanghuanglian: a case report. *China's Naturopathy* **12**:50.
- Chen L, and Zhang ZZ (2007). Hemolytic anemia and drug-induced liver damage caused by Shuanghuanglian: a case report. *Chin J Ethn Ethn* **1**:61.
- Chinese Pharmacopoeia Commission (2020) Shuanghuanglian, The Pharmacopoeia of the People's Republic of China pp 773, China Medical Science Press, Beijing, China.
- Ciotti M, and Owens IS (1996). Evidence for overlapping active sites for 17 alpha-

ethynlestradiol and bilirubin in the human major bilirubin UDPglucuronosyltransferase. *Biochemistry* **35**:10119-10124.

Danan G, Teschke R (2015). RUCAM in Drug and Herb Induced Liver Injury: The Update. *Int J Mol Sci* **17**:14.

Fujiwara R, Haag M, Schaeffeler E, Nies AT, Zanger UM, and Schwab M (2018). Systemic regulation of bilirubin homeostasis: Potential benefits of hyperbilirubinemia. *Hepatology* **67**:1609-1619.

He S, Zhang C, Zhou P, Zhang X, Ye T, Wang R, Sun G, and Sun X (2019). Herb-Induced Liver Injury: Phylogenetic Relationship, Structure-Toxicity Relationship, and Herb-Ingredient Network Analysis. *Int J Mol Sci* **20**:3633.

Jing J, Teschke R (2018). Traditional Chinese Medicine and Herb-induced Liver Injury: Comparison with Drug-induced Liver Injury. *J Clin Transl Hepatol* **6**:57-68.

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* **596**:583-589.

Henriksen JN, Böttger P, Hermansen CK, Ladefoged SA, Nissen PH, Hamilton-Dutoit S, Fink TL, Donskov F (2020). Pazopanib-Induced Liver Toxicity in Patients With Metastatic Renal Cell Carcinoma: Effect of UGT1A1 Polymorphism on Pazopanib Dose Reduction, Safety, and Patient Outcomes. *Clin Genitourin Cancer* **18**:62-68.

Kadakol A, Ghosh SS, Sappal BS, Sharma G, Chowdhury JR, and Chowdhury NR (2000). Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. *Hum Mutat* **16**:297-306.

Kuzmic P, Cregar L, Millis SZ, and Goldman M (2006). Mixed-type noncompetitive

inhibition of anthrax lethal factor protease by aminoglycosides. *FEBS J* **273**:3054-3062.

Larrey D, Vial T, Pauwels A, Castot A, Biour M, David M, and Michel H (1992). Hepatitis after germander (*Teucrium chamaedrys*) administration: another instance of herbal medicine hepatotoxicity. *Ann Intern Med* **117**:129-132.

Li CY, Tang ZQ (1996). Two cases of anthrax caused by Shuanghuanglian [J]. *Chin J Rural Med* **4**:57.

Li Y (2008). Analysis of 327 cases of adverse reactions of Shuanghuanglian injection. *Practical Pharmacy and Clinical Remedies* **11**:35.

Lin JL, Ho YS (1994). Flavonoid-induced acute nephropathy. *Am J Kidney Dis* **23**:433-440.

Locuson CW, and Tracy TS (2007). Comparative modelling of the human UDP-glucuronosyltransferases: insights into structure and mechanism. *Xenobiotica* **37**:155-168.

Lv X, Xia Y, Finel M, Wu J, Ge G, and Yang L (2019). Recent progress and challenges in screening and characterization of UGT1A1 inhibitors. *Acta Pharm Sin B* **9**:258-278.

Ma G, Lin J, Cai W, Tan B, Xiang X, Zhang Y, and Zhang P (2014). Simultaneous determination of bilirubin and its glucuronides in liver microsomes and recombinant UGT1A1 enzyme incubation systems by HPLC method and its application to bilirubin glucuronidation studies. *J Pharm Biomed Anal* **92**:149-159.

Ma G, Wu B, Gao S, Yang Z, Ma Y, and Hu M (2013). Mutual regioselective inhibition of human UGT1A1-mediated glucuronidation of four flavonoids. *Mol Pharm* **10**:2891-2903.

Ma G, Zhang Y, Chen W, Tang Z, Xin X, Yang P, Liu X, Cai W, and Hu M (2017). Inhibition of Human UGT1A1-Mediated Bilirubin Glucuronidation by Polyphenolic Acids Impact Safety of Popular Salviaanolic Acid A/B-Containing Drugs and Herbal Products. *Mol Pharm* **14**:2952-2966.

NMPA (2018). Announcement of the National Medical Products Administration on the Revision of Instructions for Shuanghuanglian Injection. Available from: <https://www.nmpa.gov.cn/xxgk/ggtg/ypshmshxdgg/20180611164901887.html>

NMPA (2020). Notice of the National Medical Products Administration on Revising the Instructions for Prescription and Non-Prescription Oral Preparations of Shuanghuanglian Granules. Available from: <https://www.nmpa.gov.cn/xxgk/ggtg/ypshmshxdgg/20200325174501785.html>

Noh K, Kang Y, Nepal MR, Jeong KS, Oh DG, Kang MJ, Lee S, Kang W, Jeong HG, Jeong TC (2016). Role of Intestinal Microbiota in Baicalin-Induced Drug Interaction and Its Pharmacokinetics. *Molecules* **21**:337. Öeren M, Walton PJ, Hunt PA, Ponting DJ, and Segall MD (2021). Predicting reactivity to drug metabolism: beyond P450s- modelling FMOs and UGTs. *J Comput Aided Mol Des* **35**:541-555.

Qosa H, Avaritt BR, Hartman NR, Volpe DA (2018). In vitro UGT1A1 inhibition by tyrosine kinase inhibitors and association with drug-induced hyperbilirubinemia. *Cancer Chemother Pharmacol* **82**:795-802.

Schirris TJJ, Ritschel T, Bilos A, Smeitink JAM, and Russel FGM (2015). Statin Lactonization by Uridine 5'-Diphospho-glucuronosyltransferases (UGTs). *Mol Pharm* **12**:4048-4055.

Segel IH (1975). Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady-state enzyme systems. *Biochem Educ* **4**:16.

Servedio V, d'Apolito M, Maiorano N, Minuti B, Torricelli F, Ronchi F, Zancan L, Perrotta S, Vajro P, Boschetto L, Iolascon A. (2005). Spectrum of UGT1A1 mutations in Crigler-Najjar (CN) syndrome patients: identification of twelve novel alleles and genotype-phenotype correlation. *Hum Mutat* **25**:325.

Shao H, He X, Achnine L, Blount J W, Dixon R A, and Wang X (2005). Crystal structures of a multifunctional triterpene/flavonoid glycosyltransferase from *Medicago truncatula*. *Plant Cell* **17**:3141-3154.

Storey KB (2005). Functional Metabolism: Regulation and Adaptation. *E-STREAMS*:

Electronic Rev Sci Technol **148**:112-115.

Takeda S, Kitajima Y, Ishii Y, Nishimura Y, Mackenzie PI, Oguri K, and Yamada H (2006). Inhibition of UDP-glucuronosyltransferase 2b7-catalyzed morphine glucuronidation by ketoconazole: dual mechanisms involving a novel noncompetitive mode. *Drug Metab Dispos* **34**:1277-1282.

Tang W, Shi QP, Ma T, Jiang XD, Liu SX, and Wang YX (2016). Meta-analysis on incidence of adverse drug reaction induced by Shuanghuanglian injection. *Zhongguo Zhong Yao Za Zhi* **41**:2732-2742.

Tátrai P, and Krajcsi P (2020). Prediction of Drug-Induced Hyperbilirubinemia by In Vitro Testing. *Pharmaceutics* **12**:755.

Teng Y, Nian H, Zhao H, Chen P, and Wang G (2013). Biotransformation of baicalin to baicalein significantly strengthens the inhibition potential towards UDP-glucuronosyltransferases (UGTs) isoforms. *Pharmazie* **68**:763-767.

Teschke R, Qiu SX, Xuan TD, and Lebot V (2011). Kava and kava hepatotoxicity: requirements for novel experimental, ethnobotanical and clinical studies based on a review of the evidence. *Phytother Res* **25**:1263-1274.

Teschke R, Zhu Y, and Jing J (2020). Herb-induced Liver Injury in Asia and Current Role of RUCAM for Causality Assessment in 11,160 Published Cases. *J Clin Transl Hepatol* **8**:200-214.

Trott O, and Olson AJ (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* **31**:455-461.

Vitek L (2012). The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases. *Front Pharmacol* **3**:55.

Wang L, Cheng L, Yuan Q, Cui X, Shang H, Zhang B, and Li Y (2010). Adverse drug reactions of Shuanghuanglian injection: a systematic review of public literatures. *Evid Based Med* **3**:18-26.

Wang XX, Lv X, Li SY, Hou J, Ning J, Wang JY, Cao YF, Ge GB, Guo B, and Yang L

(2015). Identification and characterization of naturally occurring inhibitors against UDP-glucuronosyltransferase 1A1 in Fructus Psoraleae (Bu-gu-zhi). *Toxicol Appl Pharmacol* **289**:70-78.

Wu B, Ako R, and Ming H J P A A (2012). A Useful Microsoft Excel Add-in Program for Modeling Steady-state Enzyme Kinetics. *Pharmaceutica Analytica Acta* **1**:S1.

Xing J, Chen X, and Zhong D (2005). Absorption and enterohepatic circulation of baicalin in rats. *Life Sci* **78**:140-146.

Xu Y, Wang S, Hu Q, Gao S, Ma X, Zhang W, Shen Y, Chen F, Lai L, and Pei J (2018). CavityPlus: a web server for protein cavity detection with pharmacophore modelling, allosteric site identification and covalent ligand binding ability prediction. *Nucleic Acids Res* **46**:W374-W379.

Xue G, Zhu M, Meng N, Guan J, Zhang J, Yang J, Wang Y, Cui Y, and Chai X (2021). Integrating Study on Qualitative and Quantitative Characterization of the Major Constituents in Shuanghuanglian Injection with UHPLC/Q-Orbitrap-MS and UPLC-PDA. *J Anal Methods Chem* **2021**:9991363.

Zhang L, Lin G, and Zuo Z (2007). Involvement of UDP-glucuronosyltransferases in the extensive liver and intestinal first-pass metabolism of flavonoid baicalein. *Pharm Res* **24**:81-89.

Zhang R, Xu TZ, Liu QD, Fu SQ, and Wang NS. Study on toxicity of Shuanghuanglian injection and its combination with cefradine on human normal hepatocytes. *J New Chin Med* **44**:163-165.

Zhou QH, Zhu GH, Song YQ, Que YF, He QQ, Tu DZ, Zeng HR, Qin WW, Ai CZ, and Ge GB (2021). Methylophiopogonanone A is a naturally occurring broad-spectrum inhibitor against human UDP-glucuronosyltransferases: inhibition behaviours and implication in herb-drug interactions. *Basic Clin Pharmacol Toxicol* **129**:437-449.

Zhou W, Di LQ, Shan JJ, Bi XL, Chen LT, Wang LC, and Cai BC (2011b). In vitro metabolism in Sprague-Dawley rat liver microsomes of forsythoside A in different

compositions of Shuang-Huang-Lian. *Fitoterapia* **82**:1222-1230.

Zhuang Z, Wen J, Zhang L, Zhang M, Zhong X, Chen H, and Luo C (2020). Can network pharmacology identify the anti-virus and anti- inflammatory activities of Shuanghuanglian oral liquid used in Chinese medicine for respiratory tract infection? *Eur J Integr Med* 37:101139.

Footnotes.

This work was supported by the National Natural Science Funds of China [Grants 82074109, 81873078, 81374051 and 81773687]; the Science and Research Program of Shanghai Municipal Health Commission [Grant 201740094]; the Science and Research Program of Traditional Chinese Medicine of Shanghai Municipal Health Commission [Grant 2018YP001]; and MHHFDU-SPFDU Joint Research Fund [Grants RO-MY201707 and 2021MHJC11].

Conflicts of interest. Authors have no conflict of interest.

Legends for Figures

Fig. 1. The elimination pathways of bilirubin. UCB, unconjugated bilirubin; BG, bilirubin glucuronides; BAI, Baicalein; BA, Baicalin; HYP, Hyperoside.

Fig. 2. Chemical structures of baicalein, baicalin and hyperoside.

Fig. 3. Kinetics profiles of hUGT1A1-mediated bilirubin glucuronidation (red circles represented the observed rates, smooth lines represented the formation rates). Each data point presented as the average value in triplicates.

Fig. 4. Inhibitory effects (IC_{50}) of SHL, BAI, BA and HYP on hUGT1A1-mediated bilirubin glucuronidation at 0.69 μ M bilirubin. Data were presented as means $\pm$ S.D. in triplicates, $*P < 0.05$, compared with the BAI groups.

Fig. 5. Inhibitory effects (IC_{50}) of SHL, BAI, BA, and HYP on hUGT1A1-mediated bilirubin glucuronidation at 0.69 μ M bilirubin. The data were presented as means $\pm$ S.D. in triplicates.

Fig. 6. Kinetics profiles (A, C, E, G) and Lineweaver–Burk (double-reciprocal) plots ($1/V$ versus $1/[S]$) (B, D, F, H) of hUGT1A1-mediated glucuronidation of bilirubin (0.2–5.0 μ M) in the presence of different concentrations of SHL (0–74.84 μ g/mL).

Dots represented the measured values, and smooth lines represented the fitted values.

The data were presented as means $\pm$ SD in triplicates.

Fig. 7. Kinetics profiles (A, C, E, G) and Lineweaver–Burk (double-reciprocal) plots (1/V versus 1/[S]) (B, D, F, H) of hUGT1A1-mediated glucuronidation of bilirubin (0.2–5.0 μ M) in the presence of different concentrations of BAI (0–50 μ M). Dots represented the measured values, and smooth lines represented the fitted values. The data were presented as means $\pm$ SD in triplicates.

Fig. 8. Kinetics profiles (A, C, E, G) and Lineweaver–Burk (double-reciprocal) plots (1/V versus 1/[S]) (B, D, F, H) of hUGT1A1-mediated glucuronidation of bilirubin (0.2–5.0 μ M) in the presence of different concentrations of BA (0–100 μ M). Dots represented the measured values, and smooth lines represented the fitted values. The data were presented as means $\pm$ SD in triplicates.

Fig. 9. Kinetics profiles (A, C, E, G) and Lineweaver–Burk (double-reciprocal) plots (1/V versus 1/[S]) (B, D, F, H) of hUGT1A1-mediated glucuronidation of bilirubin (0.2–5.0 μ M) in the presence of different concentrations of HYP (0–100 μ M). Dots represented the measured values, and smooth lines represented the fitted values. The data were presented as means $\pm$ SD in triplicates.

Fig. 10. Inhibitory effects (IC_{50}) of SHL(A), BA, BAI, and HYP (B) on hUGT1A1-mediated bilirubin glucuronidation at a series of concentrations of substrate and inhibitor, respectively. Concentrations of bilirubin, SHL, BA, BAI, and HYP were 0.2-5.0 μ M, 0-74.84 μ g/mL, 0-50 μ M, 0-100 μ M and 0-100 μ M. The detailed IC_{50} values see the Supplemental Tables 12-15. The data were presented as means $\pm$ SD in triplicates.

Fig. 11. Docking simulations of bilirubin (A, green), baicalein (B, dark blue), baicalin (C, cyan) and hyperoside (D, magenta) in hUGT1A1 with UDPGA (yellow). The 3D-structure of hUGT1A1 is download from the AlphaFold Protein Structure Database (UniProt code: P22309).

Tables

Table 1 Enzyme kinetic parameters of hUGT1A1-mediated bilirubin glucuronidation ($n = 3$, $\bar{x} \pm SD$)

Kinetic parameters	BMG1	BMG2	BDG	TBG ^a
K_m (μM)	0.66 ± 0.11	0.70 ± 0.13	0.73 ± 0.15	0.69 ± 0.12
V_{max} (nmol/mg/min)	0.74 ± 0.07	0.90 ± 0.08	0.26 ± 0.03	1.90 ± 0.17
K_{si} (μM)	14.78 ± 4.27	14.03 ± 3.53	8.77 ± 2.05	13.35 ± 3.01

^a - TBG = BMG1 + BMG2 + BDG.

Figure 1

Baicalein

Baicalin

Hyperoside

Figure 3

Figure 4

Figure 5

A

B

C

D

E

F

G

H

Figure 6

Figure 7

Figure 8

Figure 9

A

B

Figure 10

A

B

C

D

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 26, 2023

Figure 11