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Research paper

Discovery and characterization of naturally occurring chalcones as potent inhibitors against bile salt hydrolases

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Abstract

Bile salt hydrolases (BSHs) play crucial roles in deconjugation of the conjugated bile acids, have been recognized as key targets to modulate bile acid metabolism. This study aims to find efficacious BSH inhibitors from a natural compound library and to characterize the inhibitory mechanism of identified BSH inhibitors. Following assaying of the inhibition potentials of more than 100 natural compounds against BSH produced by *Lactobacillus salivarius* (lsBSH), several chalcones were found with strong or moderate lsBSH inhibition activity. Of all tested chalcones, licochalcone C and isobavachalcone displayed the most potent lsBSH inhibition activity (IC_{50} < 1 μM). Inhibition kinetic analyses demonstrated that both licochalcone C and isobavachalcone reversibly inhibited lsBSH-catalyzed CA-AMCA hydrolysis *via* a mixed inhibition manner. Docking simulations suggested that they could bind on lsBSH at two distinct sites mainly *via* hydrogen bonding and hydrophobic interactions. Additionally, licochalcone C and isobavachalcone can inhibit various BSHs and reduce the total BSH activities in mice feces, suggesting that these agents are broad-spectrum BSH inhibitors. Collectively, our findings reveal that licochalcone C and isobavachalcone are naturally occurring inhibitors of BSH, and these two agents can be used as promising lead compounds to develop more efficacious BSH inhibitors for modulating bile acid metabolism.

Keywords: Bile salt hydrolases (BSHs); Inhibitor; licochalcone C; isobavachalcone; Inhibitory mechanism

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1. Introduction

The gut microbiome encodes a large number of enzymes that play vital roles in the biotransformation of a huge variety of xenobiotics and endogenous substances [1]. Among all reported gut microbiota-produced enzymes in mammals, bile salt hydrolase (BSH, E.C.3.5.1.24), a group of cysteine-hydrolases, play crucial roles in deconjugation of the glycine- or taurine- conjugated bile acids (BAs) [2, 3], *via* catalyzing the hydrolytic reactions of the C24 amide bond of conjugated BAs into unconjugated BAs and free glycine or taurine. It is well-known that BAs are key endogenous substances in humans, which play a panel of important biological functions, such as facilitating the digestion and absorption of dietary lipids, as well as acting as hormones or signaling molecules targeting on distinct receptors [4-6]. Parts of BAs have been reported as potent ligands for a range of host nuclear receptors (such as farnesoid X receptor and pregnane X receptor) [7] and G protein-coupled receptors [8], thereby strongly influence host lipid and glucose metabolic and immunomodulatory processes [9, 10]. Accumulating evidence has demonstrated that BSH abundance and BSH activity are tightly associated with BAs homeostasis and a variety of human diseases including inflammatory bowel disease (IBD), obesity, type 2 diabetes, liver and cardiovascular diseases [11-14], suggesting that BSH are key targets for treating metabolic diseases.

Although some natural and synthetic compounds have been found with BSH inhibition activity in the past few decades, very few compounds have been reported with potent inhibition potency and good safety profiles [15, 16]. Therefore, it is an urgent need to discover more effective BSHs inhibitors. Considering that natural products are still the major source for the

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identification of drug leading compounds, we initiated a large-scale screening to assess the inhibition potentials of natural products on lsBSH. Following preliminary assessment of six classes of natural products (more than 100 natural products), some naturally occurring chalcones was found with strong to moderate lsBSH inhibition activities (**Fig. 1**). It is a well-known fact that chalcones are widely distributed in many medicinal plants and herbs [17, 18], which have been reported with a wide variety of biological activities, such as anti-inflammatory effects and inhibitory activities against a panel of enzymes including tyrosine kinase, aldose reductase, cyclooxygenase, carboxylesterases and pancreatic lipase [19-23]. However, the BSH inhibition activities of naturally occurring chalcones and their inhibitory mechanisms have not been reported and investigated yet.

The objectives of this study were to determine the inhibitory activities of naturally occurring chalcones on BSH in *vitro* and to investigate the inhibitory mechanisms of two newly identified potent inhibitors against lsBSH. To this end, series of inhibition assays were firstly performed to assess the inhibition potentials of chalcones on lsBSH, while the inhibitory mechanisms of licochalcone C and isobavachalcone (two most potent inhibitors against lsBSH) were seriously investigated using a range of inhibition kinetic assays, fluorescence quenching assays and docking simulations. All these findings are very helpful for the medicinal chemists to deep understand the inhibitory mechanisms of chalcones against bacterial BSHs and to develop more efficacious BSH inhibitors for modulating bile acid metabolism.

2. Materials and methods

2.1 Chemicals and reagents

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Licochalcone A, licochalcone B, licochalcone C and licochalcone D were purchased from Vikeqi Biology Technology Co., Ltd. (Sichuan, China). Naringenin chalcone, flavokawain A, 4'-*O*-methylbroussochalcone B and taurocholic acid (TCA) were ordered from yuanye Bio-Technology Co., Ltd. (Shanghai, China). Butein and dithiothreitol (DTT) were ordered from J&K Chemical Ltd. (Beijing, China). Caffeic acid phenethyl ester (CAPE) was ordered from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Isoliquiritigenin, echinatin, isobavachalcone, bavachalcone, isoliquiritin, neoisoliquiritin and other natural compounds used in BSH inhibition assay were obtained from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone gained from Mr. Wei Xing of East China University of Science and Technology. The purities of all tested natural compounds were higher than 98%. Glycerol, imidazole, sodium acetate, NaCl, lysozyme, and phenylmethylsulfonyl fluoride (PMSF) were ordered from Sinopharm Chemical Reagent Co., Ltd. SuperNuclease was obtained from Sino Biological Inc. Cholic acid-AMCA probe (CA-AMCA) was synthesized by one of the authors (Peng-chao Huo), while its hydrolytic metabolite 7-amino-4-methyl-3-coumarinylacetic acid (AMCA) was gained from Energy Chemical Ltd. (Shanghai, China). LC grade methanol, acetonitrile, DMSO and formic acid were order from Tedia (Fairfield, OH, USA) and used thoroughly, while 100 mM phosphate buffered saline (PBS, pH 6.0) was prepared by using ultrapure water.

2.2 Expression and purification of BSHs

A construct of amino-terminally fused BSHs (lsBSH, btBSH or efBSH) in a pET29a (+) vector was overexpressed in Escherichia coli (*E. coli*) [14, 24, 25]. The expression plasmid was transformed into BL21 (DE3) cells, and then expression was induced in auto-induction method

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at 18 °C. After 36 h of induction, cells were harvested by centrifugation and resuspended in 40 mL lysis buffer (25 mM Tris-HCl, pH 8.0, mixed with 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 0.1 mg/mL lysozyme and 25 U/mL SuperNuclease). After 30 min at room temperature, the cells were lysed by sonicating on ice and centrifuged at 18000 rpm at 4 °C for 30 min. 2 mL Ni-NTA agarose were mixed with supernatant and the lsBSHs was eluted with elute buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT and 100-200 mM imidazole). The eluted protein was purified on a Superdex 200 10/300 GL column equilibrated in protein storage buffer (10 mM sodium acetate, pH 5.5, 400 mM NaCl, 1 mM DTT) (**Fig. S1**).

2.3 Chemical synthesis of CA-AMCA

Cholic acid-AMCA probe (CA-AMCA) was synthesized using a published method (**Fig. S2**) [26]. Both ^{1}H- and ^{13}C-NMR spectra were used for structural characterization of CA-AMCA, which were acquired on a Bruker AVANCE instrument, by using DMSO-d6 as the solvent. Chemical shifts were recorded by using tetramethylsilane (TMS) as the reference. ^{1}H NMR (600 MHz, DMSO-d6) δ (ppm): 10.34 (s, 1H), 7.91-7.64 (m, 2H), 7.49 (dd, J = 8.8, 2.1 Hz, 1H), 4.33 (s, 1H), 4.13 (s, 1H), 4.06-3.95 (m, 1H), 3.80 (s, 1H), 3.62 (s, 1H), 3.53 (d, J = 18.0 Hz, 2H), 3.19 (dt, J = 11.0, 6.3 Hz, 1H), 2.41 (ddd, J = 14.7, 10.1, 4.6 Hz, 1H), 2.36 (s, 3H), 2.32-2.11 (m, 3H), 2.05-1.94 (m, 1H), 1.91 (s, 1H), 1.89-1.71 (m, 5H), 1.71-1.60 (m, 2H), 1.51-1.41 (m, 3H), 1.41-1.15 (m, 7H), 1.03-0.93 (m, 4H), 0.81 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6) δ (ppm): 173.02, 161.43, 152.76, 148.82, 142.53, 126.41, 115.61, 115.59, 105.64, 71.47, 70.90, 66.70, 46.55, 46.22, 41.98, 41.85, 40.52, 35.78, 35.62, 35.37, 34.86, 34.04, 31.70, 30.87, 29.05, 27.75, 26.69, 23.29, 23.10, 21.64, 17.64, 15.37, 12.83. ESI-MS m/z: 624.35 (M)+ (**Fig. S3, S4, S5**).

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2.4 LC-FD analysis of CA-AMCA and its hydrolytic product AMCA

Both CA-AMCA and AMCA were analyzed by a LC system equipped with a RF-20A fluorescence detector (Shimadzu, Kyoto, Japan) (**Fig. S6**). Chromatographic separation was performed on a Shim-pack VP-ODS C18 column (4.6 mm × 150 mm, 5 mm, Shimadzu, Japan) and the column temperature was kept at 40 ℃. The fluorescence signals of AMCA and CA-AMCA were recorded with the excitation wavelength at 345 nm and the emission wavelength at 455 nm. The mobile phase was a mixture of acetonitrile (A) and 0.2% formic acid water (B) with the following gradient conditions: 0-2 min, 75%-55% B; 2-3 min, 55%-20% B; 3-5 min, 20%-10% B; 5-5.1 min, 10%-75% B; and 5.1-7 min, balance to 75% B. And the flow rate was 0.5 mL/min. The standard curve of AMCA and enzymatic kinetic plot of lsBSH-catalyzed CA-AMCA hydrolysis were shown in **Fig. S7** and **Fig. S8**, respectively.

2.5 BSHs inhibition assays using CA-AMCA as substrate

Briefly, a mixture (190 μL) containing lsBSH (2 μg/mL, final concentration), DTT (1 mM, final concentration) and different inhibitors was pre-incubated in 100 mM PBS (pH 6.0) at 37°C for 3 min. Subsequently, 10 μL of reaction buffer containing 0.2 mM CA-AMCA was added to start the biotransformation. The reaction was terminated with ice-cold acetonitrile (200 μL) after incubation at 37°C for 30 min. After centrifugation at 20000 × g for 30 min, 100 μL of the supernatant was transferred into a LC vial and analyzed by LC-FD. The residual activity of BSH was calculated by (peak area of AMCA in the presence of inhibitor)/peak area of AMCA in negative control (DMSO only) × 100%.

2.6 Inhibition of TCA hydrolysis by licochalcone C and isobavachalcone

In brief, a mixture (190 μL) containing lsBSH (2 μg/mL), DTT (1 mM) and different

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inhibitors was pre-incubated in 100 mM PBS (pH 6.0) at 37°C for 3 min. Subsequently, 10 μL of PBS containing 1 mM TCA was added to start the biotransformation. The reaction was terminated with ice-cold acetonitrile (200 μL) containing internal standard diclofenac after incubation at 37°C for 30 min. Then the mixtures were centrifugation at 20000 × g for 30 min and 100 μL of the supernatant was diluted with 100 μL ultrapure water. The samples were analyzed by LC-MS/MS using a published method after minor modification [27].

2.7 Inhibition kinetic analyses of licochalcone C and isobavachalcone

To explore the inhibition mechanism of the two natural chalones against lsBSH, series of inhibition assays were performed with different concentrations of the fluorescent substrate (CA-AMCA) and increasing concentrations of inhibitors. The types of inhibition kinetic were determined by the intersection point in the Lineweaver-Burk plot. The slopes of the lines in the Lineweaver-Burk plot were then used to depict the second plot and to determine the inhibition constant (K_i) value [28-30]. The following equations for competitive (a), non-competitive (b) or mixed inhibition (c) were used.

$$V=(V_{max}S)/\ [K_m\,(1+I/K_i)+S] \quad \text{(a)}$$

$$V=(V_{max}S)/\ [(K_m+S)\times(1+I/K_i)] \quad \text{(b)}$$

$$V=(V_{max}S)/\ [(K_m+S)\times(1+I/\alpha K_i)] \quad \text{(c)}$$

Here V is the velocity of CA-AMCA hydrolyzed by lsBSH and V_{max} is the maximum velocity. S and I are substrate (CA-AMCA) and inhibitors concentrations, respectively. K_i is the inhibition constant of the inhibitors towards lsBSH; K_m is the Michaelis constant for CA-AMCA hydrolyzed by lsBSH.

2.8 Fluorescence quenching assay

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Fluorescence spectra of lsBSH with or without inhibitor were recorded by using a Duetta fluorescence and absorption spectrometer (HORIBA Scientific). In brief, 1 mL of 100 mM PBS (pH 6) contained lsBSH (20 μg/mL), TCEP (0.25 mm) and inhibitors with increased concentrations (0, 0.25, 0.5, 1, 1.5, 2, 3 and 4 μM). After incubation at 25 ℃ for 10 min, the mixture was transferred to a cuvette and was scanned at an excitation wavelength of 274 nm. The fluorescence quenching mechanism was described by the Stern-Volmer equation

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (D)$$

Here, F_0 and F were the fluorescence intensity in the absence and presence of inhibitors, respectively. K_{SV} was the Stern-Volmer dynamic quenching constant. K_q was the bimolecular fluorescence quenching rate constant. τ_0 was the fluorescence lifetime of fluorescent group without quencher, and [Q] was the concentration of inhibitors. For biological macromolecules, the average value of τ_0 was about 10^{-8} s.

2.9 Molecular docking simulations

Docking simulations were performed by using AutoDock Vina (Version 1.1.2). Firstly, the crystal structure of lsBSH (PDB ID: 5YP7) was obtained from Protein Data Bank (https://www.rcsb.org/). Then the hydrogens were added and the Kollman charges were assigned. The search space was set to 70×70×70 $Å^3$ centered on the catalytic residue Cys2 with the spacing of 0.375 Å. The docking modes with the lowest binding energy were displayed and further analyzed in this study.

2.10 Inhibition of BSHs in mice feces by licochalcone C and isobavachalcone

Fecal pellets collecting form six healthy ICR mice were suspended in PBS respectively and then broken into fine particles to obtain a final concentration of 50 mg/mL. The mixtures were

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centrifuged at 9000 × g for 30 min at 4°C, and the supernatants were mixed in equal volumes. The reaction solutions (100 μL) contained the fecal solution (1 mg/mL, final concentration), DTT (1 mM, final concentration), different inhibitors with increasing concentrations and CA-AMCA (10 μM, final concentration). The reaction was quenched with ice-cold acetonitrile (300 μL) after incubation at 37°C for 30 min. After centrifugation at 20000 × g for 30 min, 100 μL of the supernatant was transferred into a LC vial and analyzed by LC-FD.

2.11 Statistical analysis

In this study, all inhibition assays were conducted in triplicate. All data were shown as mean ± SD of triplicate determinations. The inhibition data (IC_{50} and K_i values) were evaluated by GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, USA).

3. Results and discussion

3.1 Screening of lsBSH inhibitors from a natural product library

In order to identify strong BSH inhibitors from natural products, the inhibitory potentials of 110 natural compounds against lsBSH was measured at three inhibitor concentrations (1 μM, 10 μM and 100 μM). As presented in **Fig. 1** and **Table S1**, some naturally occurring chalcones strongly inhibited lsBSH, while licochalcone C and isobavachalcone showed the most significant inhibition, with more than 50% activity loss at the dose of 1 μM. By contrast, the residual activities of lsBSH in the presence of the other tested compounds including the positive lsBSH inhibitor (CAPE), were higher than 50% at the dose of 1 μM. This finding encourages us to carefully study the inhibitory effects of naturally occurring chalcones against lsBSH and other bacterial BSHs.

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3.2 Inhibition of chalones against lsBSH-catalyzed CA-AMCA hydrolysis

Among all tested chalcones, two chalcone glycosides (isoliquiritin and neoisoliquiritin) and flavokawain A (a polymethoxylated chalcone) showed very weak lsBSH inhibition activity even at high inhibitor concentration (100 μM). To quantify the inhibition potency of other 12 natural chalcones, the dose-response curves were plotted by using increasing inhibitor concentrations. As shown in **Fig. 2** and **Fig. S9**, all tested chalcones dose-dependently inhibited lsBSH-catalyzed CA-AMCA hydrolysis. The chemical structures and IC_{50} values of all tested chalcones were listed in **Table 1**. Of these, licochalcone C and isobavachalcone showed the most effective lsBSH inhibitory activity, showing the IC_{50} values of less than 1 μM. Meanwhile, the inhibitory effects of bavachalcone, 4'-*O*-methylbroussochalcone B, licochalcone D, licochalcone A and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone were also stronger than the positive inhibitor CAPE (IC_{50} = 13.64 μM, **Fig. S10**). Other tested chalcones displayed moderate inhibition, with IC_{50} values ranging from 10 μM to 100 μM. These results clearly suggest that parts of naturally occurring chalcones potently inhibit lsBSH, some of which are more potent than the reported positive lsBSH inhibitor.

After carefully analyzing the inhibitory effect of these natural chalcones on lsBSH, we summarized the structural-inhibition relationships of chalcones as lsBSH inhibitors. Firstly, addition of a glucosyl group on the chalcone skeleton is unbeneficial for lsBSH inhibition. By contrast, the chalones with multiple hydroxyl groups is seemed good for lsBSH inhibition. When the hydroxyl groups at C-4', C-6' and C-4 were replaced by methoxy groups, the inhibition effect will be weakened, evidenced by naringenin chalcone (IC_{50} = 22.42 μM) *VS* flavokawain A (IC_{50} > 100 μM). Moreover, introduction of unsaturated alkyl chain in the A or

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B ring is beneficial for lsBSH inhibition, evidenced by isobavachalcone (IC_{50} = 0.92 μM), bavachalcone (IC_{50} = 1.15 μM) *VS* isoliquiritigenin (IC_{50} = 14.90 μM), as well as licochalcone C (IC_{50} = 0.78 μM), Licochalcone A (IC_{50} = 3.73 μM) *VS* echinatin (IC_{50} = 33.17 μM). These findings are very useful for the medicinal chemists to design and develop more efficacious chalcone-type lsBSH inhibitors.

3.3 Inhibition on lsBSH catalyzed TCA hydrolysis by *licochalcone C and isobavachalcone*

Considering that conjugated bile acids were the physiological substrates of lsBSH, the inhibitory effects of two chalcones on lsBSH-catalyzed TCA hydrolysis were also investigated. As showed in **Fig. S11**, both licochalcone C and isobavachalcone could strongly inhibit lsBSH in dose-dependent manner, with the IC_{50} values of 2.57 μM and 5.84 μM, respectively. These results clearly show that licochalcone C and isobavachalcone can inhibit the hydrolysis of both the optical substrates and physiological substrates catalyzed by lsBSH, indicating that these agents may modulate bile acid metabolism in gastrointestinal system.

3.4 Inhibitory mechanisms of licochalcone C and isobavachalcone agianst lsBSH

Next, a series of inhibition kinetic analyses were performed to explore the inhibitory mechanisms of licochalcone C and isobavachalcone against lsBSH catalyzed *CA-AMCA hydrolysis*. First, time-dependent inhibition assays showed that the inhibition tendency and IC_{50} values of licochalcone C and isobavachalcone were not time-dependent, suggesting that these two agents were reversible inhibitors of lsBSH (**Fig. S12**). Then, we determined the inhibition modes and the inhibition constants (K_i) of these two natural compounds against lsBSH. As shown in **Fig. 3**, the Lineweaver-Burk plots of licochalcone C and isobavachalcone against lsBSH-catalyzed CA-AMCA hydrolysis showed that these two compounds strongly inhibited

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lsBSH *via* a mixed inhibition manner, with the K_i value of 0.75 μM and 1.00 μM, for licochalcone C and isobavachalcone, respectively. These findings clearly demonstrate that both licochalcone C and isobavachalcone can effectively and reversibly inhibit lsBSH-catalyzed hydrolytic reaction.

3.5 Fluorescence spectroscopy analysis

The fluorescence quenching assays were also carried out to study the interaction between licochalcone C and isobavachalcone with lsBSH. As depicted in **Fig. 4A** and **Fig. 4B**, the maximum inherent fluorescence emission of lsBSH was observed around 322 nm, while the fluorescence intensity of lsBSH could be significantly decreased with adding of increasing concentrations of licochalcone C or isobavachalcone. The quenching mechanism was further analyzed according to Sterne-Volmer plot. As shown in **Fig. 4C** and **Fig. 4D**, a good linear relationship between the F_0/F and the concentration of inhibitors was obtained, suggesting that both licochalcone C and isobavachalcone could quench the intrinsic fluorescence of lsBSH by a single quenching mechanism. The K_{sv} values of licochalcone C and isobavachalcone were calculated as 0.95×10^6 and 0.82×10^6 L/mol, respectively, while the K_q values were calculated as 0.95×10^{14} and 0.82×10^{14} L/mol/s, respectively. And the K_q values of these two agents were much greater than the maximum scatter collision quenching constant of each type of enzyme quencher (2.0×10^{10} L/mol/s) [31]. These data indicate that licochalcone C and isobavachalcone quench the intrinsic fluorescence of lsBSH by a static mechanism caused by the formation of ligand-protein complexes, which can affect the microenvironment of some important fluorescent groups (such as Trp and Tyr) in lsBSH surrounding the ligand-binding sites.

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3.6 Molecular docking simulations

To further investigate the potential ligand-binding sites and the interaction modes between two newly identified lsBSH inhibitors and the target enzyme, molecular docking simulations of either licochalcone C or isobavachalcone were performed by using a reported 3D structure of lsBSH (PDB ID: 5Y7P). Prior to docking, the potential ligand-binding cavities of lsBSH were predicted using CavityPlus and the top 2 potential sites were selected based on ligandability and druggability scores (**Fig. S13**). One was located at the catalytic site where was relatively conserved and contained the key nucleophilic residue Cys2, while another was the back site locating below the back of the catalytic site. As shown in **Fig. 5**, both licochalcone C and isobavachalcone could well-docked in to the catalytic site or the back site. The spatial conformations of licochalcone C and isobavachalcone superimposed at the catalytic site or the back site were shown in **Fig. 5A**. As a result, the predicted binding energy of both licochalcone C and isobavachalcone on the catalytic site were calculated as -9.3 kcal/mol and -9.4 kcal/mol, respectively, and on the back site were -6.7 kcal/mol and -7.0 kcal/mol, respectively, implying that both two agents could tightly bound on the catalytic site of lsBSH.

At the catalytic site of lsBSH, the benzene rings of the two chalcones formed strong π-π interactions with Phe65 and Phe100, respectively. In addition, licochalcone C strongly interacted with Gly77 and Leu134 *via* hydrogen bonding, while isobavachalcone formed a hydrogen bond with Phe130 of lsBSH (**Fig. 5B, 5C, Fig. S14**). In the back site, two benzene rings of the chalcones formed strong hydrophobic interactions with Leu182 and Phe230, respectively. Meanwhile, a hydrogen bonding interaction between isobavachalcone and Tyr-185 was additionally observed (**Fig. 5D, 5E, Fig. S15**). These findings clearly indicate that

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licochalcone C and isobavachalcone can bind stably on lsBSH at two different ligand-binding sites, which well-explains the mixed inhibition modes of these two agents against lsBSH-catalyzed CA-AMCA hydrolysis. In addition, the interaction of these two agents with Phe and Tyr at each ligand-binding site can partly-explains the fluorescence quench effects on the endogenous fluorescence of lsBSH, as shown in the results of the fluorescence quenching assays.

3.7 Inhibition of licochalcone C and isobavachalcone on other bacterial BSHs

Next, the inhibitory effects of licochalcone C and isobavachalcone on other bacterial BSHs (including btBSH and efBSH) were tested. As shown in **Fig. 6**, both licochalcone C and isobavachalcone could inhibit btBSH and efBSH-catalyzed CA-AMCA hydrolysis in a dose-dependent manner. In btBSH, the IC_{50} values of licochalcone C and isobavachalcone were 7.46 μM and 5.17 μM, respectively. In efBSH, the IC_{50} values of these two agents were 14.01 μM and 7.91 μM, respectively. These results demonstrate that these two naturally occurring chalcones inhibit three various bacterial BSHs, suggesting that some chalcones can serve as broad-spectrum BSH inhibitors.

3.8 Inhibitory effects of licochalcone C and isobavachalcone against BSHs in mice feces

Finally, the inhibitory effects of licochalcone C and isobavachalcone on the total activity of bacterial BSHs in mice feces were evaluated. As shown in **Fig. 7,** both licochalcone C and isobavachalcone could block the total activity of bacterial BSHs in mice feces *via* a dose-dependent manner. The apparent IC_{50} values of these two compounds on the total activity of bacterial BSHs in mice feces were determined as 44.66 μM and 21.24 μM, for licochalcone C and isobavachalcone, respectively.

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As an important classes of signal molecules in the mammals, bile acids play a critical role in regulating host metabolism, including energy consumption, glucose and lipid homeostasis, as well as innate and adaptive immune responses [4, 32-34]. BSHs catalyze the critical first step in the metabolism of BAs by microorganisms in the intestinal tract, and their activity will significantly influence bile acid characteristics and many physiological processes in the host [11, 35]. In recent studies, it had been found that theabrownin from Pu-erh tea reduced serum and liver cholesterol levels and the mechanism was related to suppressed the BSHs related microbes and BSHs activity [36]. In addition, berberine inhibited the activity of BSH in the intestinal flora and significantly increased the level of tauro-cholic acid in the intestine, thereby activating the intestinal FXR pathway and reducing serum lipid level [37]. In spite of these important implications of BSHs activity, the lack of small-molecule inhibitors has limited the delving deeper, which inspired us to find efficacious BSHs inhibitors from natural products [38].

Chalcones, as intermediates in the biosynthesis of flavonoids, are widely found in medicinal plants such as *Glycyrrhiza inflata* Bat., *Psoralea corylifolia* L. and *Carthamus inctorius* L., and contribute significantly to the medicinal value of the herbs [39-42]. Meanwhile, these herbs have been shown to modulate bile acid metabolism [43-45]. In this study, natural chalcones were found to inhibit BSHs strongly or moderately, and these components could easily contact with BSHs after oral administration of the herbs. Considering the low oral bioavailability and rapid hepatic metabolism of natural chalcones, it is highly likely that these herbal medicines exert their effects on regulating bile acid metabolism partly by inhibiting BSHs in intestinal bacteria. Additionally, as a simple scaffold, chalcones can be easily synthesized in a number of

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ways and various of chalcone derivatives have been synthesized and shown enhanced biological activity [19, 46]. All these data and findings suggest that the medicinal chemists can design and develop more powerful and innovative BSHs inhibitors by using chalcones as the scaffold, aiming to get ideal drug candidates for modulating bile acid metabolism and for treating BA-associated diseases.

4. Conclusion

In summary, this study screened the inhibition potentials of more than 100 natural compounds against lsBSH (a key gut microbiota-produced cysteine-hydrolase in deconjugation of conjugated bile acids) and reported that parts of naturally occurring chalcones are strong lsBSH inhibitors. Of all tested compounds, licochalcone C and isobavachalcone showed potent inhibitory effects against lsBSH. Further investigation demonstrated that these two naturally occurring chalcones strongly inhibited lsBSH-catalyzed hydrolytic reaction in a mixed inhibition manner, with K_i values of 0.73 μM and 1.00 μM, respectively. Fluorescence quenching assays suggested that both licochalcone C and isobavachalcone quenched the intrinsic fluorescence of lsBSH by a static mechanism caused by the formation of stable ligand-protein complexes. Molecular docking simulations showed that both licochalcone C and isobavachalcone could stably bind on lsBSH at two distinct ligand-binding sites, mainly *via* the formation of hydrogen bonds and hydrophobic interactions. Following studies found that these two agents inhibited various bacterial BSHs and could inhibit the total activity of bacterial BSHs in mice feces. These findings demonstrate that licochalcone C and isobavachalcone are strong broad-spectrum BSH inhibitors and suggest that chalcones can be used as leading

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compounds for developing more efficacious BSH inhibitors.

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Conflicts of interest

No conflicts of interest.

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