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Exploring the interaction between *Salvia miltiorrhiza* and human serum albumin: Insights from herb–drug interaction reports, computational analysis and experimental studies

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ABSTRACT

Human serum albumin (HSA) binding is one of important pharmacokinetic properties of drug, which is closely related to in vivo distribution and may ultimately influence its clinical efficacy. Compared to conventional drug, limited information on this transportation process is available for medicinal herbs, which significantly hampers our understanding on their pharmacological effects, particularly when herbs and drug are co-administrated as polytherapy to the ailment. Several lines of evidence suggest the existence of *Salvia miltiorrhiza*–Warfarin interaction. Since Warfarin is highly HSA bound in the plasma with selectivity to site I, it is critical to evaluate the possibility of HSA-related herb–drug interaction. Herein an integrated approach was employed to analyze the binding of chemicals identified in *S. miltiorrhiza* to HSA. Molecular docking simulations revealed filtering criteria for HSA site I compounds that include docking score and key molecular determinants for binding. For eight representative ingredients from the herb, their affinity and specificity to HSA site I was measured and confirmed fluorometrically, which helps to improve the knowledge of interaction mechanisms between this herb and HSA. Our results indicated that several compounds in *S. miltiorrhiza* were capable of decreasing the binding constant of Warfarin to HSA site I significantly, which may increase free drug concentration in vivo, contributing to the herb–drug interaction observed clinically. Furthermore, the significance of HSA mediated herb–drug interactions was further implied by manual mining on the published literatures on *S. miltiorrhiza*.
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1. Introduction

In order to exert their therapeutic effects in vivo, drugs need to be distributed to potential action sites through circulation system after administrated through various routes. As the most abundant transporting protein in the blood, human serum albumin (HSA) plays a key role in the distribution of a wide variety of endogenous and exogenous ligands, including drugs [1]. Since efficacy of drug is directly related to free drug concentration in plasma, which is influenced by plasma proteins, binding ability towards plasma proteins is one of the important pharmacokinetic (PK) parameters in drug research [2]. Previous structural biological studies reveal that there are two high affinity ligand binding sites on HSA located in subdomains IIA and IIIA, also known as Sudlow's sites I and II, respectively [3]. For a drug with high plasma protein binding, such as cystic fibrosis drug Ivacaftor (>97%) [4], plasma protein drug–drug interactions may likely happen when co-administrated with other drugs competing for the same plasma protein binding site, thus leading to unexpected fluctuation of in vivo concentration of the first drug and drastic changes in its clinical outcomes may occur [5,6].

Salvia miltiorrhiza, known as “Danshen” in China, is a common traditional Chinese medicinal herb frequently used to treat cardiovascular diseases (CVD) for hundreds of years. Currently there are over 900 commercial Danshen-based drug preparations available in China [7]. As increasing utility of herbal medicine as a complementary/supplementary therapy to conventional pharmacotherapy in China and western countries [8], *S. miltiorrhiza* derived drug preparations often use simultaneously with other CVD drugs, including anti-coagulation drug Warfarin. Due to chemical complexity, it is not surprising to observe accumulating clinical reports on herb–drug interactions between *S. miltiorrhiza* and Warfarin [9,10]. Warfarin is a highly protein-bound drug with specific preference to site I of HSA [11]. HSA mediated drug interactions with *S. miltiorrhiza* remains to be exploited for this anticoagulant, which could induce alterations of its PK profile, change unbound Warfarin concentration, thus contributing to clinical observed herb–drug interactions.

A number of pharmacological researches have been performed on the extraction or individual compounds from *S. miltiorrhiza* and various mechanisms of action, such as anti-platelet aggregation [12], anti-coagulation [13] and anti-oxidative [14] properties, are suggested for its beneficial effects. However, compared to mechanistic elucidation, sparse information is available for in vivo distribution process of this medicinal plant. Through spectroscopic methods, salvianolic acid B

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[15] is found to bind to HSA. Rosmarinic acid, another compound in *S. miltiorrhiza*, is also shown to bind to bovine serum albumin [16]. However, majority of chemicals in *S. miltiorrhiza* still require to be carefully investigated on their HSA binding properties.

Trp214 positioned in HSA site I is a naturally fluorescent residue and ligand binding to this pocket would induce local conformation changes that lead to alterations on intensity and/or the shift in wavelength of the maximum peak of the protein in the corresponding fluorescence spectrum, which allows for a convenient, sensitive detection of ligand–protein interactions. In addition to experimental approaches, computational methods, such as molecular docking, are important technique for investigating the protein–drug system with high-throughput, which also provides detailed information on binding mode and molecular interactions with protein [17,18]. It is now a common practice to integrate molecular docking with in vitro experiments for studying ligand binding to HSA [19,20].

Herein, a comprehensive list of small molecule compounds found in *S. miltiorrhiza* was extracted from the literature and their affinities to HSA site I were investigated first by molecular docking. Subsequently, eight representative chemicals (6 active and 2 inactive compounds) from computational predictions were tested by fluorescence spectrometry to validate their interactions with HSA and their selectivity to site I were investigated by using site-specific fluorescent probe. Some characteristic data of the interaction, such as binding constant and the stoichiometry of binding, were determined accordingly. Moreover, we evaluated the effect of compounds from the medicinal herb on Warfarin–HSA binding system, which suggests *S. miltiorrhiza* may reduce HSA binding of Warfarin. Text mining on other drugs interacting with this medicinal herb suggested more comprehensive investigation on plasma protein herb–drug interactions is warranted.

2. Materials and methods

2.1. Chemicals and materials

Tanshinone IIA, lithospermic acid (LA), rosmarinic acid (RA), salvianolic acid A (SAA), salvianolic acid B (SAB), salvianolic acid C (SAC), ursolic acid (UA) and savianic acid A sodium (SAS) were purchased from Shanghai Winherb Medical Technology Co., Ltd (Shanghai, China).Human serum albumin (HSA) was obtained from Sigma-Aldrich Co. (St Louis, MO). Dansylamide (DA) that is a HSA IIA specific fluorescence probe and Warfarin were provided by TCI Development Co., Ltd (Shanghai, China). Ultra-pure water used in all experiments was obtained from Milli-Q gradient water purification system (Millipore, USA).

2.2. Molecular docking

The three-dimensional conformations of 69 ingredients in *S. miltiorrhiza* were generated and further energy optimized to local minimum with the MOE program (Chemical Computing Group, Canada). Atomic coordinates of HSA structure was downloaded from the Protein Data Bank (PDB) (PDB code: 1HA2) [21]. Before docking, water molecules, co-factors and the co-crystallized ligand (Warfarin) in the binding site I were removed and hydrogen atoms were added to receptor atoms. The docking pocket for site I of HSA was defined as residues that fell within 10 Å of the bound Warfarin in the crystal structure. The compounds were then docked into site I of HSA using the ligand–receptor docking program GOLD (Genetic Optimization of Ligand Docking) [22]. The intermolecular interactions between the two queries (Warfarin and Phenylbutazone) and HSA were employed as reference points for the docked natural compounds from *S. miltiorrhiza*. The top-ranked conformation of each docked compound was selected from 30 independent docking runs. To select promising compounds for fluospectrometric evaluation, the corresponding GOLD score was employed as the filter. Analysis on molecular interactions of ligand–protein complex was carried out using LigX module of MOE software.

2.3. Preparation of stock solutions

The appropriate amount of HSA protein was dissolved in 150 mM phosphate buffer (pH 7.4) at a concentration of 0.1 mM. Stock solutions of tanshinone IIA, LA, RA, SAA, SAB, SAC, UA, and SAS were prepared at a concentration of 4 mM in DMSO then diluted to 0.1 mM with the phosphate buffer for fluorescence experiments. Stock solution of Warfarin was prepared in the same manner and the final concentration of Warfarin was 1 mM. A stock solution of 0.1 mM DA was prepared in phosphate buffer and stored in dark for fluorescence displacement measurements.

2.4. Fluorescence spectroscopy

The fluorescence investigations were performed with a TECAN Infinite F200 Multi-Mode Microplate Reader (Shanghai, China). Tryptophan fluorescence of HSA was initiated by exciting the protein solution at 280 nm and the emission wavelength was set at 340 nm. The fluorescence measurements were recorded at a temperature of 310.15 K by keeping the concentration of HSA constant (50 μM) while ligands with a range of various concentrations (0–50 μM) were used. For fluorescence studies of the HSA–Warfarin system coexisting with ingredients in *S. miltiorrhiza*, the concentration ratio of HSA and ligands were kept to 1 with the presence of Warfarin at a concentration range of 0, 10, 20, 30, 40, and 50 μM.

Fluorescence displacement measurements with the site I-specific indicator DA were performed by varying the concentrations of ingredients in *S. miltiorrhiza* at 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM while keeping both the concentrations of protein and indicator constant at 0.25 mM. An excitation wavelength of 340 nm was used for DA and 485 nm was applied for emission spectrum.

There are two modes of fluorescence quenching, e.g. static and dynamic quenching. The static quenching suggests the formation of a stable complex between the protein and quencher, while the dynamic quenching is resulted from collision between the fluorophore and quencher. Both the static and dynamic quenching can be described using a well-known linear Stern–Volmer Eq. (1) as following:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \tag{1}$$

where F and F_0 represent the fluorescence intensities with and without the quencher, respectively. K_q, K_{sv}, τ_0 and $[Q]$ are the quenching rate constant, the Stern–Volmer quenching constant, the average life time of the biomolecule without the quencher (10^{-8} s used here) and the concentration of quencher, respectively.

The fluorescence emission intensities of HSA can also be analyzed the modified Scatchard Eq. (2) as following:

$$\log[(F_0 - F)/F] = \log K_b + n\log[Q] \tag{2}$$

where K_b and n represent the binding constant and the number of binding sites, respectively. The values of K_b and n were determined from the intercept and slope of the plot of $\log[(F_0 - F)/F]$ against $\log[Q]$, respectively.

2.5. Statistical analysis

Linear regression analyses were performed by the least-squares method with Sigmaplot (version 12.3). Statistical significance is indicated as * ($p < 0.05$) as determined by one-way analysis of variance followed by Dunnett's multiple comparison test.

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