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ORIGINAL ARTICLE

Investigation on the differences of four flavonoids with similar structure binding to human serum albumin

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Abstract Flavonoids are structurally diverse and the most ubiquitous groups of polyphenols distributed in the various plants, which possess intensive biological activities. In this study, the interaction mechanisms between four flavonoids containing one glucose unit with similar molecular weight isolated from the Tibetan medicinal herb *Pyrethrum tatsienense*, namely, apigenin-7-*O*-*β*-D-glucoside(**1**), luteolin-7-*O*-*β*-D-glucoside(**2**), quercetin-7-*O*-*β*-D-glucoside(**3**), quercetin-3-*O*-*β*-D-glycoside(**4**), and human serum albumin(HSA), were investigated by fluorescence, UV–vis absorbance, circular dichroism, and molecular modeling. The effects of biological metal ions Mg^{2+}, Zn^{2+}, and Cu^{2+} on the binding affinity between flavonoids and HSA were further examined. Structure–activity relationships of four flavonoids binding to HSA were discussed in depth and some meaningful conclusions have been drawn by the experiment data and theoretical simulation. In addition, an interesting phenomenon was observed that the microenvironment of the binding site I in HSA has hardly changed in the presence of **4** differentiating from the other three flavonoids on the basis of conformation investigations.

1. Introduction

Human serum albumin (HSA), the most abundant protein in blood serum with a concentration of 0.63 mM, is the major soluble transport protein in the circulatory system for reversibly binding a large diverse of metabolites and organic compounds such as unesterified fatty acids, hormones, and metal ions [1]. HSA not only plays a significant role in absorption, distribution, metabolism and excretion of drug, but also influences the drug toxicity and stability during the chemotherapeutic process in the circulatory systems [2]. It is well known that the functions of various drugs are strongly affected by the protein–drug interactions in blood plasma. The deep knowledge of the mechanism of the drug–HSA interaction is of great importance in understanding the process of drug transportation, the prediction of the free drug concentrations, and the clinical application. Consequently, the study on the binding of drug to HSA may provide some useful information of structure

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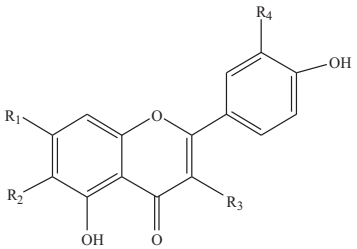

	R_1	R_2	R_3	R_4
1	*O*-glu	H	H	H
2	*O*-glu	H	H	OH
3	*O*-glu	OH	H	OH
4	OH	H	*O*-glu	OH

Fig. 1 The chemical structures of the four flavonoids.

features that determine the therapeutic effect of drug, and has become an important research field in life sciences [3].

Flavonoids are widely distributed in plants and are also present in considerable amounts in *Pyrethrum tatsienense*, which could be regarded as the most representatively secondary metabolites of this Tibetan medicinal herb in China [4]. Research on flavonoids has witnessed a remarkable interest during the last decades, due to high efficacy and low toxicity of these compounds that make them viable alternatives to conventional medicines [5]. Among therapeutic properties of flavonoids, we mention antioxidant [6], anti-cancer, anti-AIDS, anti-inflammatory, anti-neoplastic, as well as neuro-protective activities [7,8]. It can be predicted that a huge number of biochemical signaling pathways and, therefore, physiological and pathological processes, can be affected by flavonoids [9]. Thus, the structure–activity relationship of flavonoids binding to HSA is especially important in further evaluating the transportation and distribution of flavonoids in blood.

Spectral methods are powerful tools for the study of drug binding to proteins since they allow nonintrusive measurements of substances in low concentrations [3]. In this work, four flavonoids (Fig. 1), namely, apigenin-7-*O*-*β*-D-glucoside (**1**), luteolin-7-*O*-*β*-D-glucoside(**2**), quercetin-7-*O*-*β*-D-glucoside(**3**), and quercetin-3-*O*-*β*-D-glycoside(**4**), were isolated from *P. tatsienense* by our group [10]. The mechanism and characteristics of interaction between the above flavonoids (containing one glucose unit with similar molecular weight) and HSA were first investigated systematically by spectroscopic and molecular modeling methods. The effects of some metal ions, the number of phenolic hydroxyl and location of glycosylation in flavonoids on the binding affinity between the flavonoids and HSA were discussed in depth. We hope that this study will be helpful for realizing the transportation and distribution of flavonoids in *vivo* at the molecular level, which is also in some degree beneficial to the in-depth understanding of therapeutic effects of *P. tatsienense* herb.

2. Experimental

2.1. Materials and apparatus

HSA was obtained from Sigma Chemical Company(USA). Four flavonoids were isolated from *P. tatsienense* (Bur. et Franch.) Ling. Their purities were over 98% by normalization of the peak areas detected by HPLC-UV. Steady-state fluorescence measurements were carried out through a F2500 spectrophotometer (Hitachi, Japan). UV–vis and CD measurements were performed with a UV1000 UV–vis spectrophotometer (Techcomp, China) and Chirascan spectropolarimeter (Applied Photophysics Ltd., England), respectively.

2.2. Spectroscopic measurement

Four flavonoids were dissolved in ethanol to obtain 1.20×10^{-3} M stock solution, respectively. A Tris–HCl buffer (0.10 M, pH = 7.4) containing 0.10 M NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. Fluorescence measurements were carried out keeping the concentration of HSA fixed at 4.0×10^{-7} M and that of drugs varied from 0 to 2.88×10^{-5} M. The excitation wavelength was 280 nm and the intrinsic fluorescence emission spectra of HSA were recorded at three different temperatures (25, 31 and 37 °C). Absorption spectra were recorded at 0.5 nm intervals keeping the concentration of HSA fixed at 1.0×10^{-5} M and that of drugs varied from 0 to 4.0×10^{-5} M. CD spectra were recorded at 0.5 nm intervals under constant nitrogen flush keeping the concentration of HSA fixed at 2.00×10^{-6} M and the mole ratio of the drugs to HSA varied from 0:1 to 20:1.

The fluorescence spectra of HSA were also recorded in the presence of some metal ions, which contained Zn^{2+}, Mg^{2+}, and Cu^{2+} at 25 °C in the range of 280–600 nm at excitation wavelength of 280 nm. In the system, the overall concentrations of HSA and the metal ions were fixed at 6.0×10^{-4} M.

The crystal structure of HSA was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential 3D structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structure of all the molecules was generated by software Sybyl-X1.1. Geometries of the ligands were optimized using the Tripos force field with Gasteiger–Hückel charges. Surflex program was applied to calculate the possible conformations of the ligand binding to protein in which the lattice water remained.

3. Results and discussion

3.1. Fluorescence quenching of HSA by the four flavonoids

As shown in Fig. 2A, upon addition of the flavonoids into HSA solution, the fluorescence intensity of HSA at around 347 nm regularly decreased and the fluorescence intensity decreased tardily in each titration curve, indicating that the flavonoids could interact with HSA and that HSA binding site was gradually saturated. Furthermore, the maximum wavelength of HSA shifted about from 347 to 366 nm after the addition of flavonoids **1**, **2** and **3**, but the shift of maximum emission wavelength of HSA after addition of flavonoid **4** was not observed. A red shift of the emission peak could be deduced that the Trp-214 residue of HSA was placed in a more hydrophilic environment [11], namely, the polarity of microenvironment around Trp-214 of HSA was increased after addition of flavonoids **1**, **2** and **3**.

In order to further confirm the possible quenching mechanism of the four flavonoids binding to HSA, the fluorescence quenching constants were usually analyzed by the Stern–Volmer equation [12] and the results are listed in Table 1. The values of K_q decreased with rising temperature, and were larger than the limiting diffusion constant K_{diff} (2.0×10^{10} M^{-1} s^{-1}) [13], which suggested that the possible quenching mechanism was a static quenching process accompanied with the formation of HSA–flavonoid complexes, while dynamic collision could be negligible.

Because the concentrations of flavonoids were far greater than those of HSA, the logarithm equation [14,15] used to calculate the binding constant K_a and the number of binding site n was reasonable for a static quenching process. The values of K_a and n were obtained from the intercept and slope of the plots (shown in the inset of Fig. 2A), and are listed in Table 1, respectively. At 25 °C, K_a was found as 6.56×10^5, 7.74×10^5, 2.85×10^6, 5.97×10^5 M^{-1}, and n