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Comparative Studies on the Interaction of Genistein, 8-Chlorogenistein, and 3′,8-Dichlorogenistein with Bovine Serum Albumin

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ABSTRACT: Chlorination can significantly enhance the antioxidant and antitumor activity of genistein. In this paper, genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein were selected to investigate the binding to bovine serum albumin (BSA) using fluorescence spectroscopy and circular dichroism (CD). The results showed that chlorination, especially at position 3′, had significant effects on the binding constant value of chlorinated genistein derivatives to BSA; however, the binding site and the binding number were slightly affected. The thermodynamic parameters indicated that hydrophobic and electrostatic forces played important roles in the binding process and the enhanced binding affinity mainly associated with the increase of the hydrophobicity caused by the chlorine atom substitution. Furthermore, the CD data demonstrated that the conformation of BSA was slightly altered in the presence of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein, with different reduced α-helix contents. The results obtained herein will be of biological significance in toxicology investigation and genistein derivative drug design.

KEYWORDS: Genistein, 8-chlorogenistein, 3′,8-dichlorogenistein, bovine serum albumin, fluorescence quenching, circular dichroism

INTRODUCTION

Serum albumin is the major soluble protein in the circulatory system. It plays important roles in the transport and disposition of various endogenous and exogenous compounds, such as metabolites, drugs, and other biologically active substances, mostly through the formation of noncovalent complexes at specific binding sites. The absorption, distribution, metabolism, and excretion (ADME) profiles and, consequently, the levels of activity and toxicity can be significantly influenced as a result of their binding to serum albumins.[1] Moreover, the conformational changes of serum albumin induced by its interaction with low-molecular-weight drugs may affect the biological function of serum albumin as the carrier protein. Consequently, investigation of the binding of drugs and their derivatives to serum albumin has great toxicological and medical importance, and it may provide key information to rational drug design.[2−6] As a kind of serum albumin, bovine serum albumin (BSA) has the advantages of medical importance, low cost, ready availability, and unusual ligand-binding properties. Therefore, BSA has been studied extensively, owing to its structural homology with human serum albumin.[2−5,7−11]

Genistein, 4′,5,7-trihydroxyisoflavone (Figure 1), is a phytoestrogen with a wide variety of pharmacological effects in animal cells. Dietary genistein ingestion has been linked with a range of potential beneficial health effects, especially the pronounced effect on breast cancer attributed to its moderate binding affinities to estrogen on several tissues. However, there are conflicting results in the literature regarding the protective versus adverse effects of genistein. Several *in vitro* and *in vivo* studies have demonstrated that genistein at very low concentrations stimulates the proliferation of human breast cancer cells (MCF-7) and human uterine leiomyoma cells and enhances the growth of MCF-7 cell tumors in ovariectomized athymic mice presumably through the activation of estrogen receptors.[12−14] At higher concentrations, genistein inhibits the proliferation of several breast cancer cell lines and human uterine leiomyoma cells.[15,16] The most commonly proposed mechanisms of the inhibitory effects of genistein include the induction of cell-cycle arrest at the G_2/M phase, reduction of invasiveness, inhibition of angiogenesis, induction of apoptosis, and induction of differentiation. The suggested intracellular mechanisms of genistein include inhibition of tyrosine-specific protein kinases and topoisomerase II and scavenging O_2^- and H_2O_2 production.[17,18] However, because of the complexity of biological systems, the relevance of these findings is still unclear and needs further clarification. Inspired by the versatility and the controversial effects of genistein, the development of new derivatives using isoflavone nucleus as a potential privileged structure has been investigated.[18−21] Recently, novel genistein derivatives, such as 3′,8-dichlorogenistein and 8-chlorogenistein (Figure 1), with increased antioxidant and antitumor activities have been isolated from *Actinoplanes* sp. HBDN08,[22] suggesting that chlorinated genistein could be a promising candidate for pharmaceutical use or as a food additive. To further investigate the mechanism of chlorinated genistein derivatives and their roles in pathological processes, the reactivity toward different biomolecules should be characterized.

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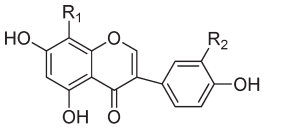

Genistein	R_1 = H	R_2 = H
8-Chlorogenistein	R_1 = Cl	R_2 = H
3',8-Dichlorogenistein	R_1 = Cl	R_2 = Cl

Figure 1. Structures of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein.

There have been several studies on the influence of hydroxylation, sulfatation, and glucuronidation of isoflavones or flavones on the interaction with serum albumin;[23−27] however, the influence of chlorination on their binding characteristics with serum albumin has not yet been reported. In the present study, the fluorescence spectroscopy and circular dichroism (CD) techniques were employed to explore the influence of chlorination on the interactions of chlorinated genistein derivatives with BSA under simulative physiological conditions using 8-chlorogenistein and 3′,8-dichlorogenistein (Figure 1) as reference compounds.[22] The study provides a quantitative understanding of the effect of chlorinated genistein derivatives on the structure of BSA, which could be a useful guideline for the further design of much more suitable genistein derivatives with structural variants.

MATERIALS AND METHODS

Reagents. BSA (fatty-acid-free and electrophoresis-grade reagents), genistein, and daidzein were purchased from Sigma (St. Louis, MO). 8-Chlorogenistein and 3′,8-dichlorogenistein were obtained as previously described.[22] The concentration of BSA was determined spectrophotometrically using an extinction coefficient (ε = 280) of 44 000 M^{-1} cm^{-1}. A stock solution of BSA was prepared by dissolving it in Tris-HCl buffer solution (0.1 M Tris base and 0.02 M NaCl at pH 7.4) and kept in the dark at 277 K. Genistein, daidzein, 8-chlorogenistein, and 3′,8-dichlorogenistein were initially dissolved in a minimum amount of methanol and then diluted with distilled water. Other materials were of analytical reagent grade, and distilled water was used throughout the experiments.

Fluorescence Measurements. All fluorescence spectra were measured on a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan) equipped with a 5.0 mm quartz cell and a thermostat bath. In a typical fluorescence measurement, 1.0 mL of BSA solution (pH 7.4) with the concentration of 1×10^{-6} M was added accurately to the quartz cell and then titrated by successive additions of drug solutions using a 2 μL trace syringe to attain a series of final concentrations. Titrations operated manually and mixed moderately. The fluorescence emission spectra were measured at 300, 310, and 320 K with the width of the excitation and emission slit both adjusted at 5.0 nm. The excitation wavelength was 280 nm, and the emission spectrum was recorded from 300 to 500 nm. The scan speed was 240 nm/min. The photomultiplier tube (PMT) voltage was 450 V.

Site Marker Competitive Experiments. Binding location studies between chlorinated genistein derivatives and BSA in the presence of three site markers (daidzein, ibuprofen, and digitoxin) were measured using the fluorescence titration methods. The concentration of BSA and site markers were all stabilized at 2.0×10^{-6} M (pH 7.4). 8-Chlorogenistein or 3′,8-dichlorogenistein was then gradually added to the BSA−daidzein, BSA−digitoxin, or BSA−ibuprofen mixtures. An excitation wavelength of 280 nm was selected, and the fluorescence spectra were recorded over a wavelength range of 300−500 nm.

CD Measurements. CD measurements were performed at 0.2 nm intervals with a MOS-450 spectrometer (BioLogic, France) equipped with a 0.1 cm path-length cell at 310 K. Three scans were averaged for each CD spectrum in the range of 190−250 nm. Spectra were corrected for buffer absorbance and recorded at the molar ratio of BSA/drugs of 1:5.

RESULTS AND DISCUSSION

Fluorescence Quenching Spectra of BSA. For macromolecules, fluorescence measurements can give some information about the binding properties of small-molecule substances to protein, such as the binding mechanism, binding mode, binding constant, binding sites, and intermolecular distances. There are only three intrinsic fluorophores: tryptophan, tyrosine, and phenylalanine in BSA.[3,7] Actually, the intrinsic fluorescence of BSA is almost contributed by tryptophan alone, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan. That is, the change of intrinsic fluorescence intensity of BSA is that of fluorescence intensity of the tryptophan residue when small-molecule substances are added to BSA.[28]

BSA contains two tryptophan residues: Trp-212 buried inside and Trp-134 located on the surface of BSA.[10] The intrinsic fluorophones in BSA show significant advantages, because of the fact that tryptophan is highly sensitive to the local environment and also displays a substantial spectral shift. As a result, the position of the spectra maximum depends upon the properties of the environment of the tryptophanyl residues, and the fluorescence spectra depend upon the degree of exposure of the tryptophanyl side chain to the polar aqueous solvent and its proximity to specific quenching groups.[3] In plasma, serum albumin, the most abundant protein, reaches a blood concentration of about 7.0×10^{-4} M.[26] Because the fluorescence intensity of serum albumin is proportional to the concentration over only a limited range of optical densities,[29] the concentration of BSA in our study was fixed at 1.0×10^{-6} M, which is at the same order of magnitude as the literature described.[23−27] For genistein, the total concentration in plasma after intake of soy products ranges currently from 0.5 to 5 μM[30,31] and there are three molecular forms, including freely circulating genistein, glucuronide, and sulfate conjugates in the plasma. However, only half of the total genistein in plasma could be available for BSA binding, because approximately 48 and 8% of genistein are present as glucuronide and sulfate conjugates, respectively.[32] Therefore, the fluorescence intensity could not be effectively quenched by free genistein at the plasma concentration. To better understand the impact of the chlorination pattern of genistein on the interactions between genistein and BSA, the concentrations of genistein and its chlorinated derivatives were fixed at $2-16 \times 10^{-6}$ M, which are relatively higher than the physiological concentration but equal to the concentrations of flavonoids and isoflavonoids used during the investigation of the interaction of these compounds with bovine serum in previous literature.[23−27]

The fluorescence spectra of BSA at a series of concentrations (0, 2, 4, 6, 8, 10, 12, and 16×10^{-6} M) of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein at 310 K are shown in panels a−c of Figure 2. It is obvious that BSA has a strong fluorescence emission peaked at 338 nm after being excited with a wavelength of 280 nm. When a fixed concentration of BSA is titrated with different amounts of genistein and its chlorinated derivatives, a remarkable decreased fluorescence and the obvious

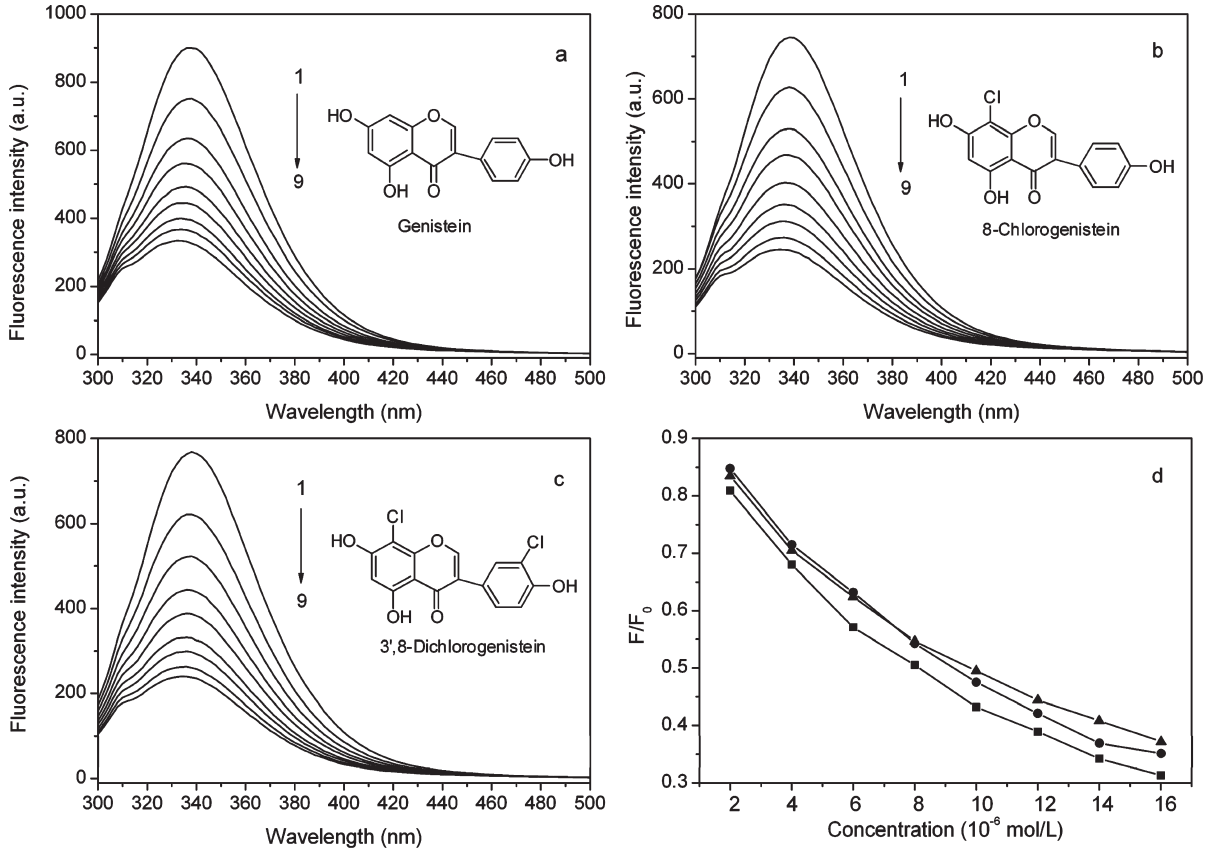

Figure 2. Effect of (a) genistein, (b) 8-chlorogenistein, and (c) 3′,8-dichlorogenistein on the fluorescence intensity of BSA and (d) normalized fluorescence intensity of BSA with (▲) genistein, (●) 8-chlorogenistein, and (■) 3′,8-dichlorogenistein concentrations. From curves 1−9, the BSA concentration was at 1×10^{-6} M and drug concentrations were at 0, 2, 4, 6, 8, 10, 12, 14, and 16×10^{-6} M, respectively. All data were obtained at pH 7.4 and 310 K.

blue shift at the maximum wavelength of BSA are observed. This suggests that the fluorescence intensity of the tryptophan residue (Trp-212 or Trp-134) was quenched and the microenvironment around BSA is changed after the addition of the three compounds.[18−21] To make the trends more clear, the normalized fluorescence F/F_0 of the spectra is plotted in Figure 2d, where F and F_0 are the fluorescence intensities of BSA before and after the addition of compounds, respectively. As shown in Figure 2d, genistein and 8-chlorogenistein are found to lead to a similar fluorescence quenching when the concentrations of these compounds are fixed in the range of $0-8 \times 10^{-6}$ M. However, the extinction of BSA tryptophans by 3′,8-dichlorogenistein decreases more rapidly than those caused by genistein and 8-chlorogenistein. At the concentration of 16×10^{-6} M, genistein is found to lead to 60.1% quenching, while 8-chlorogenistein quenches 63.9%. 3′,8-Dichlorogenistein quenches 69.7% of BSA fluorescence. From the slopes of the curves, it can be concluded that the binding affinity of 3′,8-dichlorogenistein or 8-chlorogenistein to BSA is much more stronger than that of genistein.

Mechanisms of Fluorescence Quenching. Quenching can occur by different mechanisms, which are usually classified as dynamic and static quenching. Dynamic and static quenching can be distinguished by their different dependence upon the temperature of binding constants and viscosity or, preferably, by lifetime measurements.[33] Generally, the quenching constants decrease with an increasing temperature for static quenching, but the reverse effect is for dynamic quenching.[33] To investigate the quenching mechanism, the fluorescence quenching data were analyzed according to the Stern−Volmer equation[33]

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

where F_0 and F are the fluorescence intensities of BSA in the absence and presence of the quencher, respectively, K_{SV} is the Stern−Volmer quenching constant, k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule without the quencher ($\tau_0 = 10^{-8}$ s^{-1} [34]), and $[Q]$ is the concentration of the quencher.

The Stern−Volmer plots for the quenching of BSA by genistein, 8-chlorogenistin, and 3′,8-dichlorogenistein at 310 K are shown in Figure 3. The curves are linear with high R values, and the calculated quenching constants at the corresponding temperatures are listed in Table 1. It is known that linear Stern−Volmer plots represent a single quenching mechanism, either static or dynamic.[35] As shown in Table 1, K_{SV} decreases with a rising temperature, which indicates that the fluorescence quenching mechanism may be static. The maximum scatter collision quenching constant of various quenchers with biopolymer is 2.0×10^{10} M^{-1} s^{-1}.[36] In the present study, the values of the rate constant k_q (K_{SV}/τ_0) for the quenching of BSA caused by the three compounds are all much larger than the maximum diffusion collision quenching rate constant for the scatter mechanism. Hence, we propose that the quenching is not initiated

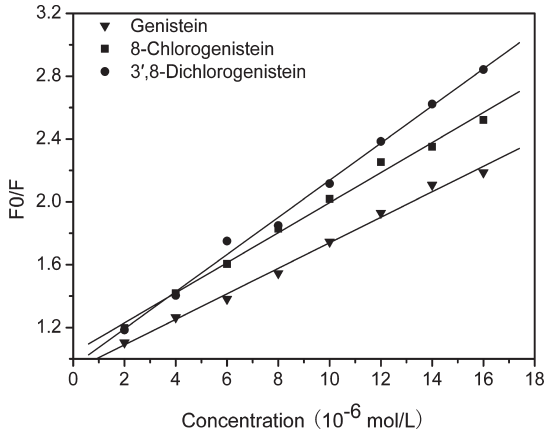

Figure 3. Stern−Volmer plots for genistein (▼), 8-chlorogenistein (■), and 3′,8-dichlorogenistein (●) with BSA at 310 K (pH 7.4). The concentration of BSA = 1×10^{-6} mol/L.

Table 1. Stern−Volmer Quenching Constants for Genistein−BSA, 8-Chlorogenistein−BSA, and 3′,8-Dichlorogenistein−BSA Systems at Different Temperatures

	T (K)	K_{SV} ($\times 10^5$, M^{-1})	R^a
genistein	300	1.05	0.99172
	310	0.81	0.99473
	320	0.76	0.9921
8-chlorogenistein	300	1.21	0.99835
	310	0.98	0.99969
	320	0.74	0.99646
3′,8-dichlorogenistein	300	1.56	0.99289
	310	1.18	0.99596
	320	1.01	0.99514

[a] R is the correlation coefficient.

by dynamic collision but originates from the formation of a complex.[3,37]

Binding Constant and Binding Sites. For the static quenching interaction, the binding constant and the number of binding sites can be determined according to the method described by Chipman et al.,[38] using the following equation:

$$\log[(F_0 - F)/F] = \log K_A + n \log[Q] \quad (2)$$

where F_0 and F are the fluorescence intensity without and with the ligand, respectively. K_A is the binding constant, and n is the number of binding sites per BSA. Figure 4 shows the plot of $\log[(F_0 - F)/F]$ versus $\log[Q]$, and the calculated values of K_A and n for genistein−BSA, 8-chlorogenistein−BSA, and 3′,8-dichlorogenistein−BSA are summarized in Table 2. The values of K_A suggest that there is a strong binding force between genistein or its chlorinated derivatives and BSA. The values of n approximately equal to 1 indicate that only a single binding site exists in BSA for genistein and its chlorinated derivatives. Recently, there have been several studies on the influence of hydroxylation, sulfatation, and glucuronidation patterns in flavone and isoflavone on the binding characteristics with serum albumin.[23−27] The incorporation of chlorine in biological molecules can enhance their antibiotic properties.[22] However, the influence of chlorination of drugs on the binding with serum

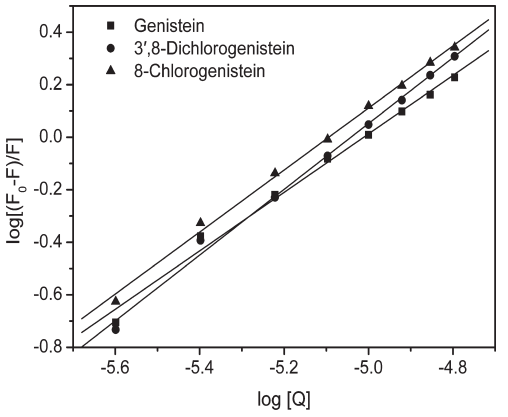

Figure 4. Double-log plots of (■) genistein, (▲) 8-chlorogenistein, and (●) 3′,8-dichlorogenistein binding to BSA at 310 K (pH 7.4).

albumin has not been reported. Comparing the binding constants of 8-chlorogenistein and 3′,8-dichlorogenistein to that of genistein indicates that chlorination that occurred at position 3′ and/or 8 can increase the binding affinity to BSA and the binding affinity follows the order: 3′,8-dichlorogenistein > 8-chlorogenistein > genistein. The result is in agreement with the previous study that the hydroxyl substitution at the positions 8 and 3′ can significantly affect the binding affinity of daidzein to human serum albumin[26] and further confirms that the groups at positions 8 and 3′ play important roles in the binding affinity. The larger K_A implies the increased stability of the BSA−drug complex, which suggests that the addition of chlorine has effects on the interactions between genistein and BSA. It is known that the hydrophobic force plays an important role in the binding of genistein to serum albumin,[25,39] while the chlorine substituted for hydrogen increases the hydrophobicity of genistein,[22] which subsequently increases the stability of the drug−BSA complex.

Identification of Binding Sites on BSA. Similar to HSA, BSA consists of amino acid chains forming a single polypeptide, which consists of three homologous α-helix domains (I−III). Each domain contains two subdomains (A and B). The binding sites of BSA for endogenous and exogenous ligands may be in these domains, and the principal regions of binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA. Many ligands can bind specifically to serum albumin, for example, warfarin and phenylbutazone for site I, flufenamic acid and ibuprofen for site II, and digitoxin for site III.[37] It has been reported that genistein and warfarin can bind simultaneously to the subdomain IIA of serum albumin and in the near vicinity of one another, while genistein can be replaced by daidzein on its serum albumin binding site.[10,11,39] Therefore, the site marker competitive experiments using daidzein, ibuprofen, and digitoxin were carried out to identify the binding sites of chlorinated genistein derivatives on BSA. The fluorescence quenching data with the presence of site markers are analyzed using the Stern−Volmer equation, and the values of the quenching constant are listed in Table 3. The binding constants of chlorinated genistein derivatives are remarkably decreased after the addition of daidzein (DA), while the addition of ibuprofen (IB) and digitoxin (DIG) results in only a small difference. The results indicate that the binding sites of 8-chlorogenistein and 3′,8-dichlorogenistein to BSA mainly locate within subdomain IIA and share the same binding site with genistein,[26,39] suggesting the involvement of hydrophobic interactions in the binding of

Table 2. Binding Constants and Relative Thermodynamic Parameters of the Genistein−BSA, 8-Chlorogenistein−BSA, and 3′,8-Dichlorogenistein−BSA Systems at 310 K

	K_A ($\times 10^6$, M^{-1})	n	R^a	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})
genistein	0.38	1.11	0.99547	11.64	144.32	−33.10
8-chlorogenistein	1.04	1.18	0.99847	13.57	158.91	−35.69
3′,8-dichlorogenistein	2.06	1.25	0.99757	16.34	173.58	−37.47

[a] R is the correlation coefficient.

Table 3. Effects of the Site Probe on the Binding Constants of 8-Chlorogenistein and 3′,8-Dichlorogenistein to BSA

	K_A ($\times 10^6$, M^{-1})	K_A (with DA) ($\times 10^6$, M^{-1})	K_A (with IB) ($\times 10^6$, M^{-1})	K_A (with DIG) ($\times 10^6$, M^{-1})
8-chlorogenistein	1.04	0.65	1.01	0.94
3′,8-dichlorogenistein	2.06	1.32	1.93	1.98

8-chlorogenistein and 3′,8-dichlorogenistein to BSA. It is worth noting that Trp-212 is located in an internal part of domain IIA; therefore, we can conclude that the fluorescence quenching of BSA may mostly arise from the interaction between Trp-212 and drugs. Resonance energy-transfer studies from the tryptophan donor site to genistein or chlorinated genistein derivatives as an acceptor (discussed below) also point to a similar conclusion.

Thermodynamic Parameters and Binding Forces. The interaction forces between ligands and biological macromolecules may include hydrophobic force, hydrogen bond, van der Waals force, and electrostatic interactions. The signs and magnitudes of the thermodynamic parameters enthalpy change (ΔH) and entropy change (ΔS) can account for the main forces involved in the binding process. For this reason, the temperature-dependent binding constant was studied. The thermodynamic parameters were evaluated using the van't Hoff equations[40]

$$\ln K = -\Delta H/RT + \Delta S/R \quad (3)$$

where R is the gas constant, T is the experimental temperature, and K is the binding constant at the corresponding T. The plot of $\ln K$ versus $1/T$ enabled the determination of the values of ΔH and ΔS.

If the temperature does not vary significantly, ΔH can be regarded as a constant. ΔG can be estimated from the following equation based on the binding constants at different temperatures:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K \quad (4)$$

The thermodynamic parameters for the interaction of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein with BSA are shown in Table 2. Ross and Subramanian[41] have characterized the sign and magnitude of the thermodynamic parameters associated with various individual kinds of interaction that may take place in protein association processes. From the point of view, the negative sign for ΔG indicates the spontaneity of the binding of the three compounds with BSA. The signs for ΔH and ΔS of the binding reaction are both found to be positive, which indicates that the binding is mainly entropy-derived and the enthalpy is unfavorable for it. The hydrophobic forces play a major role in the binding process of genistein or chlorinated genistein derivatives to BSA.[3,37,41] However, the electrostatic interaction can also not be excluded, because the main source of ΔG is derived from a large contribution of the ΔS term with a little contribution from the ΔH factor.[24,41] The results above are consistent with the suggestion that hydrophobic and ionic interactions involve the binding of genistein to human serum albumin[10,11,39] and also further confirm that the binding sites of 8-chlorogenistein and 3′,8-dichlorogenistein are mainly located within subdomain IIA of BSA. The chlorination can influence the hydrophobic characteristics of genistein. As described in our previous literature,[22] 3′,8-dichlorogenistein has the longest retention time, followed by 8-chlorogenistein and genistein, on reverse-phase high-performance liquid chromatography (HPLC), which implies that the hydrophobicity of 3′,8-dichlorogenistein is the highest. Hence, it can be concluded that 3′,8-dichlorogenistein has the strongest affinity to BSA and the binding of genistein to BSA was weaker than that of 8-chlorogenistein, because the hydrophobic interaction plays the main role in the binding process. This conclusion is compatible with the results obtained by comparing the binding constant values of the three compounds to BSA.

Energy Transfer between BSA and 3′,8-Dichlorogenistein. Förster's non-radioactive energy-transfer theory is often used to determine the drug binding site distance between the site and amino acid residues.[42] The extent of energy transfer depends upon the extent of overlap and the distance between the donor and acceptor. The efficiency of energy transfer, E, was determined according to Förster's energy-transfer theory[42]

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6) \quad (5)$$

where F and F_0 are the fluorescence intensities of BSA in the presence and absence of 3′,8-dichlorogenistein, r is the distance between the acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%. The value of R_0 was evaluated using the equation

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \quad (6)$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, and J expresses the degree of spectral overlap between the donor emission and the acceptor absorption. J could be calculated by the equation

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (7)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, $k^2 = {}^2/_3$, $N = 1.336$, and $\Phi = 0.15$ for BSA. The binding distance r between genistein and the tryptophan residue in BSA is found to be 4.31 nm, while the values of r for 8-chlorogenistein and 3′,8-dichlorogenistein are 4.37 and 4.69 nm, respectively. The values of r for the three compounds are less than the academic value (7 nm), which indicates that the fluorescence quenching of BSA

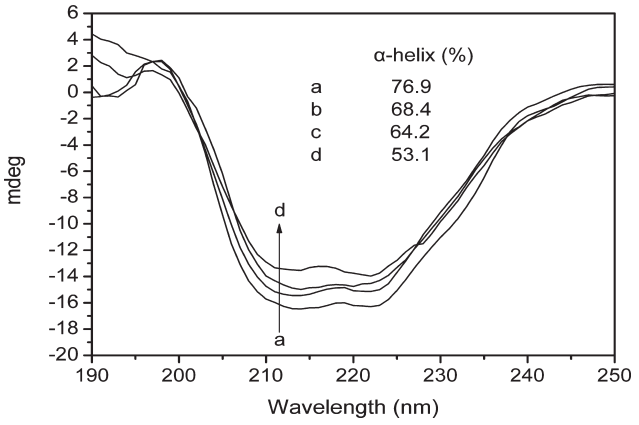

Figure 5. CD spectra of BSA in the (a) absence and presence of (b) genistein, (c) 8-chlorogenistein, and (d) 3′,8-dichlorogenistein. The concentration of BSA was 1×10^{-6} M, and the concentrations of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein were all 5×10^{-6} M, with pH 7.4 and $T = 310$ K.

is also a nonradiation transfer process.[4,5,9] The different values of r imply the different degrees of conformational changes of BSA induced by the three compounds, although they bind the similar site on BSA.

Secondary Structural Changes of BSA Shown by CD. CD spectroscopy is a sensitive technique to monitor the secondary structural change of protein upon interaction with ligands. The CD spectra of BSA in the presence of drugs are shown in Figure 5. As can be seen from Figure 5, BSA exhibits two negative bands at 208 and 222 nm in the ultraviolet region, which are characteristic of the typical α-helix structure of protein.[3,37,43] The reasonable explanation is that the negative peaks of 208 and 222 nm both contribute to the $n \rightarrow \pi^*$ transfer for the peptide bond of α helix. With an increasing addition of drugs, the CD signals of BSA decrease regularly, indicating a decrease of the α-helical secondary structure content. The α-helix contents of free and combined BSA can be calculated from mean residue ellipticity (MRE) values at 208 nm using the following equation:[3,37,44]

$$\mathrm{MRE} = \mathrm{CD}\ (\mathrm{mdeg})/10nlC_\mathrm{p} \quad (8)$$

$$\alpha\ \mathrm{helix}\ (\%) = [(-\mathrm{MRE}_{208} - 4000)/(33000 - 4000)] \times 100 \quad (9)$$

where n is the number of residues in the protein, l is the path length, C_p is the concentration of protein, MRE_{208} is the observed MRE at 208 nm, 4000 is the MER value of the β-form and random-coil conformation cross at 208 nm, and 33 000 is the MRE value of a pure α helix at 208 nm. As shown in Figure 5, the α-helix content of BSA decreases with the additions of drugs when the molar ratio of drug/BSA is 5:1 and the changes induced by the compounds follow the order: 3′,8-dichlorogenistein > 8-chlorogenistein > genistein. The different effects of these compounds on the α-helix content of BSA may associate with the binding affinity of the compounds to BSA, which further confirms that the interaction between genistein and BSA can be influenced by chlorination at 8 and 3′ positions. The larger protein unfolding of chlorinated compounds than genistein is mostly derived from the increased hydrophobicity, because of the fact that hydrophobic interaction plays an important role in the binding process, which is compatible with the results of competitive experiments and thermodynamic parameters. Furthermore, the shape of CD spectra and the α-helix contents of BSA in the presence of genistein and two chlorinated genistein derivatives demonstrate that the chlorination at position 8 has limited effects on the conformational change of BSA induced by genistein, while the chlorination that simultaneously occurred at position 3′ can result in significant influences. The decreased contents of the α-helical structure indicate that drugs bind to the amino acid residues of the main polypeptide chain of BSA and destroy the hydrogen-bonding networks.[8,45] However, the CD spectra of BSA in the presence and absence of drugs are observed to be similar in shape, thereby indicating that the structure of BSA is predominantly α helix even after binding.[9,46]

The metabolism of genistein has been studied extensively.[31,32,47] Most genistein can be absorbed and metabolized as glucuronide conjugates in enterocytes or sulfate and glucuronide conjugates in liver, which lead to genistein circulating in several molecular forms in the blood plasma, including free genistein and glucuronide and sulfate conjugates. Glucuronidation and sulfation can increase the hydrophilicity of genistein and then decrease the binding to serum albumin.[26,27] Therefore, the bioavailability of genistein can be influenced by the modes of drug administration.[48] Chlorinated genistein possesses a higher hydrophobic property than genistein, which may facilitate the intestinal absorption and, subsequently, increase the total concentration of chlorinated genistein in plasma. Although half of the total genistein is metabolized as glucuronide and sulfate conjugates, it is interesting to note that the chlorination positions 3′ and 8 at the vicinity of the natural conjugation sites 4′ and 7 are likely to prevent these glucuronidation and sulfatation reactions. Therefore, the concentration of glucuronide and sulfate conjugates of chlorinated genistein may be lower than that of glucuronidated and sulfated genistein in plasma when these compounds are administrated orally or via intravenous injections at the same concentration. During the intestinal absorption, chlorinated genistein can be metabolized by bacteria in the intestinal tract or uridine 5′-diphospho (UDP)-glucuronyltransferase in enterocytes,[31,32,47] resulting in different contents of glucuronide and sulfate conjugates in the plasma when the compound is administrated by different modes. There are reports suggesting that the conjugates could be a source of free cellular genistein after enzymatic hydrolysis in target tissues,[32] but these more water-soluble compounds can facilitate their urinary and biliary excretion[49] and decrease the binding affinity to serum albumin,[26,27] which both lead to their rapid elimination and lower bioavailability. On the contrary, chlorination may prevent the glucuronidation and sulfatation of genistein and can enhance the binding affinity to serum albumin, which can increase its bioavailability and decrease the elimination in plasma. The higher bioavailability and bioactivity of chlorinated genistein implies that chlorinated genistein derivatives, especially 3′,8-dichlorogenistein, possess notable potential in clinical therapy or use as food additives. However, detailed pharmacokinetics need further investigations.

In conclusion, the binding characteristics of genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein to BSA were investigated by spectroscopic methods. The results reveal that chlorination at positions 3′ or 8 of genistein can influence the binding characteristics of chlorinated genistein derivatives to BSA and the binding affinity follows the order: 3′,8-dichlorogenistein > 8-chlorogenistein > genistein. Although the conformational change of BSA induced by 3′,8-dichlorogenistein is more

significant than that of 8-chlorogenistein and genistein, the tertiary structure remains intact, which suggests that the addition of chlorine has limited effects on the function of serum albumin. Protein−drug binding greatly influences the absorption, distribution, metabolism, and excretion properties of drugs. Also, serum albumins are known to increase the apparent solubility of hydrophobic drugs in plasma and modulate their delivery to cells. They can play a dominant role in drug disposition and efficacy. Our results demonstrate that chlorination enhance the interaction between genistein and serum albumin, implying a longer elimination half-time and different pharmacologies and pharmacodynamics. These data may be important for deciding the dosage in therapeutics as well as designing the new antitumor agents.

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