The Binding of Silibinin, the Main Constituent of Silymarin, to Site I on Human Serum Albumin

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Silibinin is the main constituent of silymarin, an extract from the seeds of milk thistle (Silybum marianum). Because silibinin has many pharmacological activities, extending its clinical use in the treatment of a wider variety of diseases would be desirable. In this study, we report on the binding of silibinin to plasma proteins, an issue that has not previously been extensively studied. The findings indicated that silibinin mainly binds to human serum albumin (HSA). Mutual displacement experiments using ligands that primarily bind to sites I and II clearly revealed that silibinin binds tightly and selectively to site I (subsites Ia and/or Ic) of HSA, which is located in subdomain IIA. Thermodynamic analyses suggested that hydrogen bonding and van der Waals interactions are major contributors to silibinin–HSA interactions. Furthermore, the binding of silibinin to HSA was found to be decreased with increasing ionic strength and detergent concentration of the media, suggesting that electrostatic and hydrophobic interactions are involved in the binding. Trp214 and Arg218 were identified as being involved in the binding of silibinin to site I, based on binding experiments using chemically modified- and mutant-HSAs. In conclusion, the available evidence indicates that silibinin binds to the region close to Trp214 and Arg218 in site I of HSA with assistance by multiple forces and can displace site I drugs (e.g., warfarin or iodipamide), but not site II drugs (e.g., ibuprofen).

Key words human serum albumin (HSA); silibinin; silymarin; binding site; site I

Silymarin is an extract from the seeds of the milk thistle (Silybum marianum), and is mainly composed of flavonolignans, including silibinin (Fig. 1), isosilybin, silychristin and silidanin. Because of its hepatoprotective effects, it has been used for centuries for treating liver disorders such as hepatitis, cirrhosis, alcoholic liver disease and amatoxin mushroom poisoning. Silymarin and its major constituent, silibinin, are considered to be safe because there are only few reports on adverse effects. Therefore, this extract is widely consumed as a dietary supplement for liver protection world-wide. The mechanisms responsible for these actions of silymarin and silibinin are known to involve the several events, including enhanced protein synthesis through the stimulation of polymerase I and ribosomal RNA transcription, protecting the cell membrane from radical-induced damage by antioxidant activity, and the blockage of the uptake of toxins by inhibiting their binding to hepatocytes. Furthermore, their anti-inflammatory and antifibrotic activities are also thought to contribute to their hepatoprotective effects.

Silibinin or silybinin has recently been reported to function as an antioxidant agent. There are numerous studies suggesting the broad-spectrum efficacy of these compounds in inhibiting cancer metastasis. The anticancer effects of these compounds are thought to involve cell cycle arrest at the G1/S-phase, the induction of cyclin-dependent kinase inhibitors, the down-regulation of anti-apoptotic gene products, the inhibition of cell-survival kinases, and the inhibition of inflammatory transcription factors. Silibinin and silibinin also down-regulate gene products that are associated with the proliferation of tumor cells, invasion, angiogenesis and metastasis. In addition, the preventive activities of silymarin and silibinin against gastrointestinal problems, nephropathy, cardio-plumonary problem have also been suggested. Taking the multiple pharmacological effects of silibinin into consideration, it is likely that it will be widely used in treating a wide variety of diseases not only as a dietary supplement but as a prescribed drug.

Human serum albumin (HSA) is a monomeric protein consisting of 585 amino acid residues, with a molecular weight (MW) of approximately 66500 Da. HSA is a major protein component of blood plasma and functions as a carrier for numerous endogenous and exogenous compounds. HSA contains two distinct ligand binding sites, which are referred to as sites I and II. X-Ray crystallographic analyses indicate that sites I and II are located in subdomains IIA and IIIA of HSA, respectively. The distribution and pharmacological actions of silibinin are predicted to be controlled by their

Fig. 1. Chemical Structure of Silibinin
binding to HSA in the systemic circulation, if HSA is the major binding protein for silibinin in human plasma. Therefore, clarification of the binding protein, binding site and binding mode of silibinin are essential in predicting its pharmacokinetics and therapeutic effects and its potential interactions with other pharmaceutical agents. Maiti et al. investigated the binding of silibinin to HSA using spectroscopic techniques aided by docking studies, and proposed that the silibinin molecule lies within hydrogen bonding distance of the Trp214 and Asp415 residues of subdomains IIA and IIIA.17) However, the issue of whether HSA is the major binding protein in plasma or whether silibinin interacts with drugs that bind to sites I and II remains unclear. Thus, in spite of the fact that silibinin is in widespread use and will likely be extended to variety of diseases, it is surprising that the state of our knowledge regarding its binding to plasma proteins is limited.

In present study, we report on a systematic study of the protein binding of silibinin. HSA was first identified as the main protein in human plasma that binds silibinin. We also determined the binding parameters for silibinin to HSA. The effects of temperature, ionic strength and detergent on silibinin–HSA interactions were also investigated. Based on these findings, we propose that multiple types of interactions contribute to silibinin–HSA binding. In addition, the binding site and the amino acid residues involved in silibinin binding were also identified using displacement of site marker ligands in conjunction with the binding of silibinin to chemically modified- and mutant–HSAs. We conclude that silibinin binds to the region close to Trp214 and Arg218 in site I and mainly interacts with sites I drugs, but not site II drugs.

MATERIALS AND METHODS

Materials Silibinin, HSA (fraction V, fatty acid free), α₅-acid glycoprotein (AAG), γ-globulins, warfarin, iopidamide, dansyl-1-asparagine (DNSA) and 2-hydroxy-5-nitrobenzyl bromide (HNB) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium chloride (NaCl) and polyoxyethylene lauryl ether (PLE, hydroxyl number: 40–60) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) and Cosmo Bio Co., Ltd. (Tokyo, Japan), respectively. Ibuprofen was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human plasma was obtained from Interstate Blood Bank, Inc. (Memphis, TN, U.S.A.). All other chemicals from commercial sources were of the highest grade. Sixty-seven micromole sodium phosphate buffer (pH 7.4) was used in the protein binding experiments.

Determination of Protein Binding of Silibinin The binding of silibinin to proteins was examined using ultrafiltration and equilibrium dialysis techniques. Ultrafiltration was carried out using Amicon® Ultra-0.5 mL centrifugal filter unit with an Ultracel®-30 membrane (Merck Millipore Co., MA, U.S.A.). Samples of 500 μL were centrifuged at 2500 × g at 25°C for 5 min. This procedure was repeated 5 times in order to minimize adsorption to the membrane. The samples that remained and the filtrates were discarded until the fourth centrifugation, and the concentration of free (unbound) ligand in the filtrates was quantified by HPLC after the fifth centrifugation step. Equilibrium dialysis experiments were performed using 2 mL Sanko plastic dialysis cells (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membranes. Aliquots (1.5 mL) of samples were dialyzed for 12 h against the same volume of buffer solution. After reaching equilibrium, the ligand concentrations in the buffer compartment (free ligand concentration) and in the protein compartment (free+bound ligand concentration) were determined by HPLC. The volume shift after equilibrium dialysis was corrected according to the method of Giacominii et al.18)

Quantitative Analysis of Binding Data Data regarding protein binding were analyzed quantitatively. The free (unbound) fractions were calculated using ultrafiltration data, as follows.

\[ \text{Free fraction (%)} = \frac{C_f}{C_f + C_b} \times 100 \]  

where \(C_f\) is the free ligand concentration determined by measurement of the filtrate. \(C_b\) is the bound ligand concentration and was calculated by subtracting \(C_f\) from the total ligand concentration (before ultrafiltration) \(C_t\). For equilibrium dialysis, \(C_b\) was calculated by subtracting the ligand concentration in the buffer compartment \(C_f\) from the ligand concentration in the protein compartment \(C_f + C_b\). Binding parameters of ligands to HSA were obtained with the data from ultrafiltration and equilibrium dialysis. The experimental data were fitted to the following equation using GraphPad PRISM® Version 7 (GraphPad Software, Inc., CA, U.S.A.).

\[ r = \frac{C_b}{C_t} = \frac{nKC_t}{1 + KC_t} \]  

where \(r\) is the number of moles of ligand bound per mole of protein. \(P_t\) is the protein concentration. Binding parameters, \(n\) and \(K\) are the number of binding sites and the association constant for the high-affinity binding site, respectively. All experiments and analyses were performed using the condition, \(r<0.4\), to minimize ligand binding to any low-affinity binding sites.

Interaction Mode of the Two Ligands at High-Affinity Binding Sites on HSA In order to simultaneously estimate the interaction mode between two ligands, \(A\) and \(B\), which are binding to each primary binding site of HSA, the data were treated according to the method of Kragh–Hansen.9,20) In this method, the number of moles of ligands \(A\) and \(B\) bound per mole of protein, \(r_A\) and \(r_B\) can be described as follows:

\[ r_A = \frac{A_b}{P_t} = \frac{A_b + A_k A_k A_b B_t}{1 + A_k A_t + A_k B_t + A_k A_k A_b A_b B_t} \]  

\[ r_B = \frac{B_b}{P_t} = \frac{B_b + B_k B_k B_t}{1 + B_k B_t + B_k A_t + B_k B_k A_k A_k B_t} \]  

where \(A_b\) and \(B_b\) are the concentration of bound ligands \(A\) and \(B\), respectively. \(A_k\) and \(B_k\) are the binding constants of ligand \(A\) and \(B\), respectively. \(A_t\) and \(B_t\) are the free concentration of ligands \(A\) and \(B\), respectively. \(\chi\) is the coupling constant. In these equations, the independent binding of the two ligands is characterized by \(\chi=1\), while a competitive interaction results in \(\chi>1\). In this work, \(0<\chi<1\) expresses cooperative- and anti-cooperative interaction between ligands \(A\) and \(B\) on the protein, respectively.

Thermodynamic Analysis of Silibinin Binding to HSA Thermodynamic parameters were calculated by the method of Pederson et al.21) using van’t Hoff plots taken at four specified temperatures, 18, 25, 30 and 35°C. From the tempera-
ture dependence of the association constants obtained in the equilibrium dialysis experiments, it is possible to calculate the values for the thermodynamic parameters involved in the binding process. If the enthalpy change, $\Delta H$ does not vary significantly over the temperature range studied, $\Delta H$ and the entropy change, $\Delta S$ can be both determined by the van’t Hoff equation below,

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}$$  \hspace{1cm} (5)

where $K$ is the association constant at temperature $T$ (absolute temperature) and $R$ is the gas constant. $\Delta H$ and $\Delta S$ were obtained by plotting the association constants determined using the equation, and the free energy change, $\Delta G$ was calculated from the following relation.

$$\Delta G = \Delta H - T \Delta S$$  \hspace{1cm} (6)

**HPLC Conditions** The HPLC system used in this study consisted of a Hitachi model D-2000 Elite HPLC system (Hitachi Co., Tokyo, Japan). YMC-PACK ODS AM-303 (5 $\mu$m particle size, 250×4.6mm i.d., YMC Co., Kyoto, Japan) was used as the stationary phase and was maintained at 40°C. Two solvents, solvent A (50 mM sodium dihydrogen phosphate) and solvent B (50 mM sodium dihydrogen phosphate and acetonitrile (30:70, v/v)) were used as the mobile phases. The following linear gradient elution of the solvents was programmed for the quantitation of silibinin, warfarin, ibuprofen, iodipamide and DNSA: 0–7 min (30–100% B), 7–10 min (100% B), 10–15 min (100–30% B). The flow rate of the mobile phase was maintained constant at 1.0 mL/min. The detection wavelength was fixed at 210 nm and the effluent was monitored during 15 min for each sample.

**Modification of Albumin** The lone tryptophan residue, Trp214, of HSA was modified according to the method of Fehske et al.\textsuperscript{22} In a typical experiment, 1 g of HSA was dissolved in 200 mL of a 10 mM urea solution and the pH was adjusted to 4.4 by adding acetic acid. HNB in 25 mL methanol was then added and the resulting solution was subjected to occasional shaking. After 2 h, the supernatant was dialyzed against water for 60 h, and the resulting solution was then lyophilized. The modification ratio, determined spectrophotometrically,\textsuperscript{22} indicated that 93% of the tryptophan residues had been modified, indicating that Trp214 in HSA was essentially completely modified. The selective modification of tyrosine residue, Tyr411, of HSA was carried out according to the method of Hagag et al.\textsuperscript{23} In a typical run, 375 $\mu$L of 20 mM $p$-nitrophenyl anthranilate in acetonitrile was added under continuous stirring to 60 mL of 100 $\mu$M HSA in 0.1 M sodium phosphate buffer (pH 8.0). The reaction was allowed to proceed for 7 h and the preparation was dialyzed against water for 60 h, and then lyophilized. It is known that, in this modified procedure, a single anthraniloyl moiety is selectively incorporated into Tyr411 of HSA.\textsuperscript{23} The modification ratio was determined spectrophotometrically,\textsuperscript{23} indicating that 5.7% of the tyrosine residues were modified. These data suggest that about one out of 18 tyrosine residues in HSA had been modified. No structural changes in these chemically modified HSAs were detected, as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and circular dichroism measurements.

**Results**

**Synthesis and Purification of Wild-Type HSA, W214A, R218A and Y411A Mutants** The recombinant DNA techniques used to produce recombinant wild-type HSA and single residue mutants, W214A, R218A and Y411A were essentially those described by Watanabe et al.\textsuperscript{24,25} SDS-PAGE and circular dichroism measurements indicated that there were no structural changes in the wild-type HSA and the mutant-HSAs. The wild-type HSA and the mutant-HSAs were stored at −20°C until used.

**Statistical Analysis** Statistical analyses were performed using Dunnett’s test for multiple comparisons after one-way ANOVA. A probability value of $p<0.05$ was considered to be significant.

**RESULTS**

**Identification of the Binding of Silibinin to Proteins in Human Plasma** To identify the proteins in human plasma that bind silibinin, the binding experiments were conducted using isolated HSA, AAG and $\gamma$-globulins, at concentrations corresponding to those in human plasma (Fig. 2). Here, for the binding experiments, we choose a clinically relevant silibinin concentration at which therapeutic doses are administered.\textsuperscript{1,4,26–28} Ultrafiltration experiments revealed that the free fraction of silibinin in the HSA solution was comparable to that in human plasma. On the other hand, much higher free fractions were observed for AAG and $\gamma$-globulins solutions. These results indicate that silibinin mainly binds to HSA in human plasma.

**Determination of the Binding Parameters of Silibinin for HSA** The binding parameters of silibinin for HSA were determined by means of ultrafiltration and equilibrium dialysis techniques. The binding parameters for a high-affinity binding site on HSA. No differences were observed between the parameters obtained from ultrafiltration and equilibrium dialysis techniques.

**Thermodynamic Analysis for Silibinin–HSA Interaction**
Thermodynamic parameters for silybinin–HSA interactions were calculated by analyzing the temperature dependency of the association constant obtained by equilibrium dialysis. The van’t Hoff lines obtained by plotting $\ln K$ against $1/T$ were linear (data not shown), indicating that the $\Delta H$ values are constant over the experimental temperature range studied. As shown in Table 2, the formation of silybinin–HSA complexes is a spontaneous process, as evidenced by negative $\Delta G$. Furthermore, the process is an exothermic reaction accompanied by a negative $\Delta H$ and $\Delta S$. Consequently, binding processes is mainly driven by enthalpy.

**Effects of Electrolyte and Detergent on Silybinin–HSA Interactions** To confirm the binding forces of silybinin to HSA, the effects of NaCl as an electrolyte and PLE as a nonionic detergent on the silybinin–HSA interaction were examined. PLE was added so that its concentration was below the critical micelle concentration (approx. 100 µM). The addition of NaCl and PLE to the medium increased the free fraction of silybinin in a concentration-dependent manner (Fig. 3).

**Interaction between Silybinin and Sites I or II Marker Ligands on HSA** To determine the site at which silybinin binds to HSA, mutual displacement between silybinin and typical site marker ligands (site I; warfarin, site II; ibuprofen) were analyzed using ultrafiltration techniques (Figs. 4A, B, a, b). The binding curve for silybinin in the presence of warfarin is close to the curve assuming competitive binding, and spontaneously, the binding curve of warfarin in the presence of silybinin is also close to the curve assuming competitive binding. In contrast, for silybinin–ibuprofen, almost no displacement was observed and the binding curve was close to the curve assuming independent binding. These data suggest that site I is the primary binding site of silybinin. Furthermore, mutual displacement between silybinin and other site I ligands, iodipamide and DNSA were also investigated (Figs. 4C, D, c, d). Silybinin was shown to interact with iodipamide in a competitive manner, but its binding to DNSA was in an independent manner.

**Binding of Silybinin to Wild-Type and Mutant HSAs** To estimate the specific amino acid(s) that is involved in the binding of silybinin, the binding of free fractions of silybinin to chemically modified HSAs obtained from ultrafiltration data were compared to that for HSA (Fig. 5). The binding of warfarin and ibuprofen were decreased by the chemical modification of tryptophan and tyrosine residues on HSA, respectively. Similar to warfarin binding, the binding of silybinin to tryptophan-modified HSA was inhibited, whereas no effect was observed in the case where the tyrosine residue was modified. These data suggest that silybinin binds to the region close to the tryptophan residue in site I.

**DISCUSSION**

Recent evidence suggests that silymarin and its major constituent, silybinin, has therapeutic potential for the treatment of liver diseases such as hepatitis, hepatic cirrhosis, alcoholic liver disease and amatoxin mushroom poisoning. Since Trp214 and Arg218 are known to be involved in the binding of site I ligands, silibinin binding was further characterized using mutant HSAs (W214A and R218A) (Fig. 6). The data obtained from ultrafiltration experiments showed that substituting Trp214 and Arg218 for a relatively small amino acid, alanine, caused a decrease in the binding of silybinin. In contrast, substituting Tyr411 which is located in the site II region for alanine (Y411A) had no effect on the binding of silybinin (Fig. 6). These data strongly suggest that silybinin binds to a region close to Trp214 and Arg218 in site I.

![Fig. 3. Binding of Silybinin to HSA in the Presence of NaCl (0.1 and 0.2 M) and PLE (10 and 20 µM) at pH 7.4 and 25°C](image)

The concentration of HSA and modified HSAs was 20 µM, and the silybinin concentration was 10 µM. Values are expressed as the mean±S.D. (N=3). **p<0.01 in comparison with the control (no NaCl and PLE are included).**

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**Table 1. Binding Parameters Obtained by Ultrafiltration and Equilibrium Dialysis for Binding of Silybinin to HSA at pH 7.4 and 25°C**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ultrafiltration</th>
<th>Equilibrium dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K\times10^5$ M$^{-1}$</td>
<td>4.81±0.22</td>
<td>4.45±0.17</td>
</tr>
<tr>
<td>$n$</td>
<td>1.02±0.05</td>
<td>0.98±0.07</td>
</tr>
</tbody>
</table>

The results are means±standard deviation (S.D.) (N=3).

**Table 2. Thermodynamic Parameters for Silybinin–HSA Interaction at pH 7.4**

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J·K$^{-1}$·mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>-32.67±0.02</td>
<td>-42.44±0.78</td>
<td>-33.91±2.74</td>
</tr>
<tr>
<td>298</td>
<td>-32.33±0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>303</td>
<td>-32.16±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>308</td>
<td>-31.96±0.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are means±S.D. (N=3).
limitations to its prospective clinical use. We carried out this study in order to address this issue, and have come to the following conclusions: 1) HSA is the main silibinin binding protein in human plasma; 2) The binding of silibinin to HSA appears to be assisted by multiple types of forces; 3) Silibinin and site I ligands, warfarin and iodipamide, displace each other; 4) Silibinin binds to the region close to Trp214 and Arg218 in site I of HSA. These findings led us to develop a detailed understanding of the protein binding of silibinin and a precise prediction of the pharmacokinetics and therapeutic effects of silibinin and co-administered drugs in patients.

Our results from ultrafiltration techniques using human plasma and its component proteins indicated, for the first time, that silibinin preferentially associates with HSA but not AAG or γ-globulins (Fig. 2). The binding parameters for silibinin to HSA showed that silibinin forms a strong 1:1 complex with HSA (Table 1). In order to characterize the binding mode of silibinin to HSA, a thermodynamic analysis was performed.

As shown in Table 2, all of the measured thermodynamic parameters ($\Delta G$, $\Delta H$, $\Delta S$) were negative. These results indicate that the formation of a silibinin–HSA complex is a spontaneous and exothermic process and that it is driven by enthalpy. Ross and Subramanian31) characterized the sign and magnitude of the thermodynamic parameters for various types of ligand–protein interactions. According to their characterization, the negative $\Delta H$ and $\Delta S$ values observed in silibinin–HSA interactions can largely be attributed to van der Waals forces and hydrogen bond formation. These are similar to the interactions of other polyphenols with albumins.32–35) Maiti et al.17) indicated an initial hydrophobic association followed by electrostatic interactions as well as van der Waals forces and/or hydrogen bond formation. These are similar to the interactions of other polyphenols with albumins.

Fig. 4. Binding of Silibinin to HSA in the Presence of Site Marker Ligands (A–D) and vice versa (a–d)

A–D indicate the binding of silibinin (1–10 $\mu$M) to HSA (20 $\mu$M) in the presence of warfarin (10 $\mu$M), ibuprofen (8 $\mu$M), iodipamide (6 $\mu$M) and DNSA (10 $\mu$M), respectively. a, b, c and d indicate the binding of warfarin (1–10 $\mu$M), ibuprofen (1–8 $\mu$M), iodipamide (1–6 $\mu$M) and DNSA (1–10 $\mu$M) to HSA (20 $\mu$M) in the presence of silibinin (8 $\mu$M). Solid and dashed lines are the theoretical curves assuming independent ($\chi=1$) and competitive ($\chi=0$) bindings, respectively. Closed circles are experimental values. All theoretical curves were constructed using the association constant for each ligand (silibinin, $4.81\times10^5$ M$^{-1}$, warfarin, $3.43\times10^5$ M$^{-1}$, ibuprofen, $3.16\times10^5$ M$^{-1}$, iodipamide, $5.24\times10^5$ M$^{-1}$, DNSA, $1.65\times10^5$ M$^{-1}$).

Fig. 5. Binding of Silibinin (A), Warfarin (B) and Ibuprofen (C) to Native, Trp- and Tyr-Modified HSAs at pH 7.4 and 25°C

The concentration of HSA and modified HSAs was 20 $\mu$M, and the concentrations of silibinin, warfarin and ibuprofen were 10 $\mu$M. Values are expressed as the mean±S.D. ($N=3$). **$p<0.01$ in comparison with native HSA.
The free fraction of silibinin was dependent on the ionic strength of HSAs. Respectively, based on binding studies using chemically modified tryptophan and tyrosine residues as part of sites I and II, to site II, where ibuprofen binds. Fehske et al. previously identified ibuprofen preferentially binds to site I where warfarin also binds, but not strongly suggest that, at therapeutic concentrations, silibinin binding of warfarin- and silibinin-binding regions in site I. These data may be involved within the dynamic process until complex formation between silibinin and HSA occurs. Indeed, the free fraction of silibinin was dependent on the ionic strength of the medium and the concentration of non-ionic detergent (Fig. 3), suggesting that electrostatic and hydrophobic interactions are also involved as a driving force for binding. Thus, not a single force but multiple forces, including hydrogen bonds, van der Waals forces, hydrophobic and electrostatic interactions appear to play important roles in the binding of silibinin to HSA.

The binding site of silibinin on HSA was determined by analyzing the interaction mode between silibinin and site-marker ligands (Fig. 4). Competitive interactions were observed between silibinin and warfarin on HSA (Fig. 4A, a), while the binding of silibinin to HSA is likely to be independent of ibuprofen binding (Fig. 4B, b). These findings strongly suggest that, at therapeutic concentrations, silibinin preferentially binds to site I where warfarin also binds, but not to site II, where ibuprofen binds. Fehske et al. previously identified tryptophan and tyrosine residues as parts of sites I and II, respectively, based on binding studies using chemically modified HSAs. In the present study, we examined the binding of silibinin to chemically modified HSAs to collect further evidence to show that silibinin binds to site I and to confirm whether Trp214, one of the residues in the site I region, is involved in silibinin binding. As described in Materials and Methods, Trp214 and Tyr411 can be selectively modified by HNB and p-nitrophenyl anthranilate, respectively. Therefore, the results for the binding of silibinin to these modified HSAs (Fig. 5) suggest that silibinin binding occurs in a region close to Trp214 in site I, but not Tyr411 in site II. Warfarin and silibinin binding were similarly decreased by chemical modification (Figs. 5A, B), suggesting the presence of overlapping of warfarin- and silibinin-binding regions in site I. These data were supported by the fact that the binding of silibinin to HSA was significantly decreased when Trp214 in site I was mutated to alanine (W214A, Fig. 6). In addition, similar to the binding of typical site I drugs, including warfarin, the binding of silibinin to HSA was also decreased by the mutation of Arg218 to alanine (R218A, Fig. 6). It therefore appears that Arg218 as well as Trp214 are located in close proximity to binding region of silibinin in site I.

Kragh-Hansen reported that site I is “a large and flexible region” based on the diversity of ligands that interact and the apparent ability to accommodate more than one of them at a time. Crystallographic studies also directed at HSA-site I drug complexes demonstrated that site I is larger than site II and that site I drugs occupy different regions of the binding pocket of subdomain IIA. The interior of site I is predominantly apolar, but contains two clusters of polar residues (Tyr150, His242, Arg257 as an inner cluster and Lys195, Lys199, Arg218, Arg222 as an outer cluster). Warfarin participates in hydrogen bonding with Tyr150 His242 and Arg222 in the center of the site I pocket, where iodipamide, a larger molecule (MW: 1140 Da), occupies the wider region from the back-end to front in site I with interacting with Arg257, Trp214, Lys198. We previously reported that site I is not a simple binding region but is rather complex and is comprised of three subsites, namely, Ia, Ib and Ic. Warfarin binds to subsite Ia, and iodipamide binds to a wider region, subsites Ia and Ic. Competitive interactions of silibinin–warfarin and silibinin–iodipamide and independent binding observed in the interaction between silibinin and DNSA, subsite Ib ligands, indicate that silibinin binds to subsite Ia and/or Ic in site I. Similar to iodipamide, silibinin which has relatively large molecular size (Fig. 1, MW: 482 Da) may occupy both Ia and Ic. Maiti et al., through molecular docking studies, suggested that silibinin lies within hydrogen bonding distance of Trp214 and Asp451 of subdomains IIA and IIIA, respectively. Although our results did not show an interaction of silibinin with subdomain IIA, the present data may also indicate that silibinin occupies the region at the entrance of the site I pocket, which faces subdomain IIA as iodipamide. Flavonoids including its glycoside substituents have been shown to bind site I. In flavonoid-site I interactions, hydrogen bonding between the 7-OH of the benzopyrone moiety and Arg222 or 5-OH and Arg257 have been proposed as warfarin-site I and iodipamide–site I interactions. Like the relatively large flavonoid, quercetin, the benzopyrone moiety of silibinin may be located within the pocket in site I, while the other parts may protrude toward the interface between subdomains IIA and IIIA. Further studies directed at identifying the exact silibinin binding site and the amino acids other than Trp214 and Arg218 that contribute to silibinin binding should be conducted by binding studies with the other mutants or a crystallographic study.

Silymarin and its main constituent, silibinin are generally considered to be safe because their adverse effects are rare and are limited to nausea, headache, joint pain, itching and mild laxative symptoms. Therefore, relatively high doses of silibinin (up to 480 mg/d) have been orally administered in clinical trials. Furthermore, dietary supplements that show a high bioavailability (e.g., Siliphos®, complex of silibinin with phosphatidylcholine) are currently being marketed worldwide.

As a dietary supplement, approximately 50–60 mg twice to three times daily is suggested. However, overdoses by patients who abuse such supplements can cause unexpected accumulations, thereby eliciting interactions with co-administered drugs. Furthermore, considering the selective binding of
silibinin to subsites Ia and/or Ic of site I, the pharmacokinetics and pharmacodynamics of drugs that bind to these regions (e.g., warfarin or iodipamide) may be modified by silibinin. Thus, changes in drug efficacy and the appearance of adverse effects should be monitored carefully when silibinin is administered in conjunction with site I drugs. Modification of the silibinin binding by co-administered drugs or endogenous compounds that accumulate at abnormally high levels in diseased states also should be considered in predicting its pharmacological action. Warfarin, iodipamide, furosemide or bucolome which all strongly bind to site I may displace silibinin. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMFP) which accumulates in patients with renal disease also exerts inhibitory effects on site I ligand bindings.44) The unbound fraction of the site I drug, phenytoin, in patients with hepatitis or cirrhosis is known to correlate with the plasma concentration of bilirubin that also binds to site I.45) Changes in the concentrations of plasma proteins can also affect silibinin binding, although these effects do not appear to be site-specific. In the diseased state, the plasma concentration of HSA is generally decreased, resulting in decreased silibinin binding. While AAG and γ-globulins are minor contributors to the binding of silibinin to proteins in plasma (Fig. 2), binding to these proteins might need to be considered in cases of inflammation or infection, since the plasma concentrations of AAG and γ-globulins typically increase significantly under these conditions, compared to normal.46–50) Thus, the findings regarding the binding of silibinin to plasma protein reported in this study will permit a more relevant assessment of the pharmacokinetics and pharmacodynamics of silibinin and co-administered drugs in various clinical situations.

CONCLUSION

In this study, we attempted to clarify the mechanism and the site at which silibinin binds to HSA. Silibinin interacts with subsites Ia and/or Ic of site I on HSA with assistance by multiple forces, including hydrogen bonds, van der Waals forces, hydrophobic and electrostatic interactions. Trp214 and Arg218 were identified as amino acid residues that are located close to the binding site for silibinin. Such a detailed analysis of silibinin–HSA interactions provides valuable information in terms of our understanding of the pharmacokinetics and the pharmacological effects of silibinin and related co-administered drugs. Furthermore, the findings presented herein will also be useful when the multiple pharmacological actions of silibinin are clinically applied for the treatment of a variety of diseases.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

(2000).


