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A spectroscopic and molecular modeling study of sinomenine binding to transferrin

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Abstract—Sinomenine, an herbal ingredient isolated from *Sinomenium acutum*, is used for the amelioration of arthritis. It has been found that this molecule could bind to human serum transferrin (Tf), the iron (III) transport protein in the blood, by using fluorescence, circular dichroism (CD) spectroscopy, and molecular modeling methods. The results provide possible usage of transferrin to transport sinomenine.

Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) (Fig. 1) is one of alkaloids extracted from Chinese medical plant, *Sinomenium acutum*.[1] Sinomenine (SIN) is considered as a major content of *S. acutum* and exerts some of its pharmacological actions. Sinomenine has been widely used clinically for the treatment of rheumatoid arthritis (RA) because of its anti-rheumatic and anti-inflammatory properties.[2–5] At the cellular level, sinomenine reduces the production of prostaglandin (PG) E_2, leukotriene C_4, nitric oxide (NO), and tumor necrosis factor-α (TNF-α) from macrophage.[6–9] Additionally, sinomenine could reduce inflammatory parameters and attenuates proliferation of synovial fibroblasts in rat adjuvant arthritis models.[2] Furthermore, it has been recently reported that acute and chronic cardiac allograft rejection could be blocked by the immunomodulatory actions of sinomenine.[10]

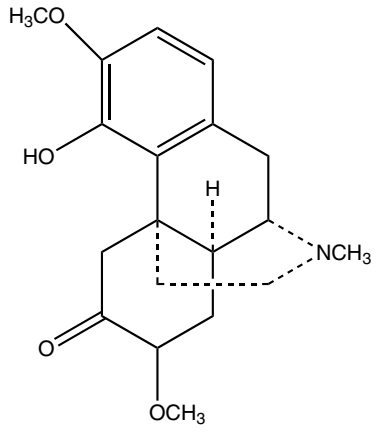

Figure 1. Chemical structure of sinomenine.

Due to the wide usage of phytomedicine, including natural products from traditional herbal medicines for medical and health fortifying purposes, sinomenine has gained international popularity. However, little is known about their active principles and even less about their mechanisms of action, which may affect the future application of this compound. Furthermore, we found recently that sinomenine binds to proteins in vitro,[11] however, the action mode of sinomenine complexes is relatively poorly understood. More importantly, previous work[12] found that sinomenine could markedly inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis in rat by utilizing in vivo Matrigel plug assay and ex vivo aortic ring assay. As anti-angiogenic strategies are now being used to treat cancers, the anti-angiogenic properties of sinomenine may make it a prime candidate for studies of possible anti-neoplastic agents. The binding of sinomenine to the blood plasma protein transferrin (Tf) has been selected for the first time since this protein has been implicated in the transport of drug

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molecules,[13,14] which may help understanding the interaction of sinomenine with protein molecules in the blood. In addition, transferrin exhibits a significant uptake in tumor tissue, due to high amount of specific transferrin receptors on the cell surface of tumor cells.[15,16]

Commercial sinomenine hydrochloride was purified by recrystallization in ethanol. The purity has been confirmed by MS and ^{1}H NMR. Transferrin was purchased from Sigma–Aldrich Co (Catalog No.T3309), and used without further purification. The reaction of transferrin and sinomenine was carried out in phosphate buffer solution (pH 7.4, containing 3 mM NaH_2PO_4, 7 mM Na_2HPO_4, and 5 mM NaCl) at room temperature for 12 h.

When excited at 270 nm, Tf gives rise to emission spectra in the range of 280–450 nm with λ_{max} at 330 nm. Under the similar condition, little fluorescence has been observed for sinomenine alone. Further addition of sinomenine into Tf solution resulted in the decrease of the Tf fluorescence intensity, shown in Figure 2. The fluorescence quenching indicated the binding of sinomenine to Tf resulted in non-radiative energy (Föster) capture and microenvironment change of tryptophan and tyrosine residues. Thus, the fluorescence quenching could be utilized to calculate the association constant (K_A) and then probe the binding site of sinomenine on Tf by combination with molecular modeling method.

The fluorescence quenching behavior in the system of sinomenine (S) and protein (P) could be analyzed by the Stern–Volmer equation.[17]

$$\frac{F_0}{F} = 1 + k_Q\tau_0[Q] = 1 + K_D[Q] \quad (1)$$

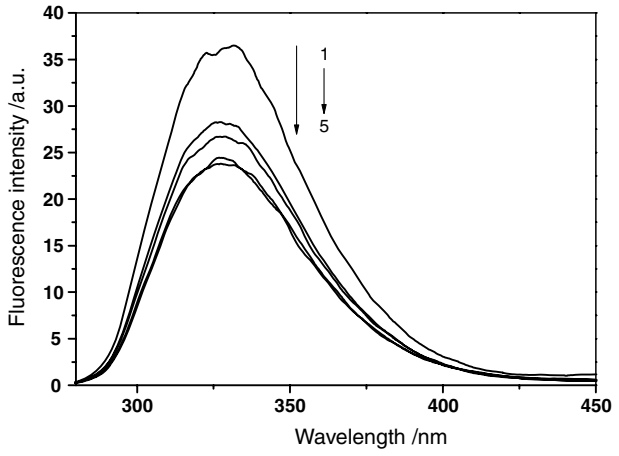

Figure 2. The fluorescence spectra of 2.5×10^{-6} M Tf in the presence of sinomenine at λ_{ex} 270 nm. The concentration of sinomenine is: (1) 0.0, (2) 15.0, (3) 20.0, (4) 30.0, and (5) 35.0×10^{-6} M. All fluorescence spectra were recorded on Hitachi F-4500 spectrofluorometer at room temperature. The emission spectra were obtained using the emission slit widths of 10 nm with a quartz cuvette of 1 cm pathlength.

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the quencher concentration, and K_D is the Stern–Volmer quenching constant, which can be written as $K_D = k_Q\tau_0$, where k_Q is the bimolecular quenching rate constant and τ_0 is the lifetime of the fluorophore in the absence of quencher.

Since biomacromolecules generally have more than one binding site for small organic molecules, one may expect the binding of sinomenine to Tf could be expressed by the following equation.

$$nQ + P = Q_nP \quad (2)$$

where n is the number of sinomenine molecules bound to Tf forming static complexes. Thus, the binding constant, K_A, can be calculated as Eq. 3

$$K_A = \frac{[Q_nP]}{[Q]^n[P]} \quad (3)$$

where [P], [Q], and [Q_nP] are the concentrations of Tf, sinomenine, and non-fluorescent Tf–sinomenine complex. Given the total protein concentration is [P_0], then [Q_nP] = [P_0] − [P] and then Eq. 3 could be rewritten as

$$K_A = \frac{[P_0] - [P]}{[Q]^n[P]} \quad (4)$$

The observed fluorescence intensity is proportional to the concentration of free protein, which is described as Eq. 5

$$\frac{P}{P_0} = \frac{F}{F_0} \quad (5)$$

From Eqs. 4 and 5, it follows that

$$\lg\frac{(F_0 - F)}{F} = \lg K_A + n\lg[Q] \quad (6)$$

Then K_A and the number of binding sites, n, can be calculated from Eq. 6.Indeed, the linear relationship based on Eq. 6 (see Fig. 3) provides the determination of the binding constant and the number of the binding sites. The calculated binding constant (K_A) and the number

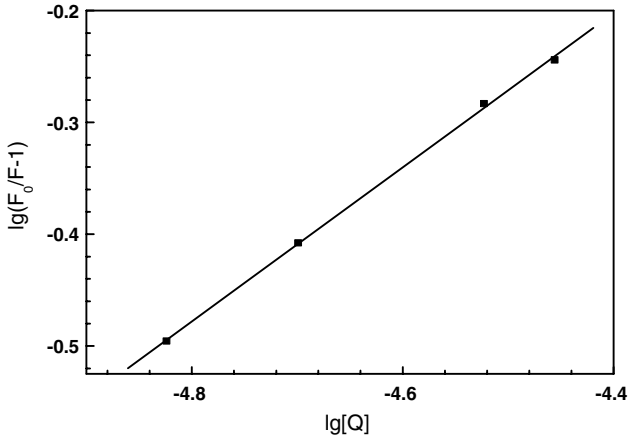

Figure 3. The plots of lg [Q] versus lg(($F_0 - F$)/F) in Tf–sinomenine system.

of the binding sites (n) are $0.68 \times 10^3\ M^{-1}$ and 0.69, respectively, indicating that the binding capacity of sinomenine to Tf is much weaker compared with Fe(III) bound to protein,[18,19] which is assigned to the fact that the interaction between sinomenine and Tf is via non-covalent bonds, rather than via ionic bonds.

The circular dichroism (CD) technique is particularly suited to monitor the conformational state of protein in solution: specifically CD provides direct information on the secondary structure of protein.[20,21] CD spectrum in the 200–300 nm regions has been widely used to investigate the conformation of protein and their changes. However, subtle conformational changes caused by protein–drug binding, intercalation, etc. are difficult to be analyzed, particularly when the absorption band of drug overlaps with those of protein.[22] Figure 4 shows the CD data of sinomenine–Tf complexes (A) and sinomenine (B) alone. Clearly, the spectral change of induced CD signal is too small to be distinguished directly. Therefore, we attempted to describe the results of using the sinomenine concentration-dependent magnitude of negative sign at 270.5 nm for sinomenine–transferrin solution and sinomenine alone to show the binding of sinomenine to transferrin as illustrated in Figure 5. We can see that the slopes are different slightly in these two cases, indicating the interaction between sinomenine and transferrin, consistent with the fluorescence results.

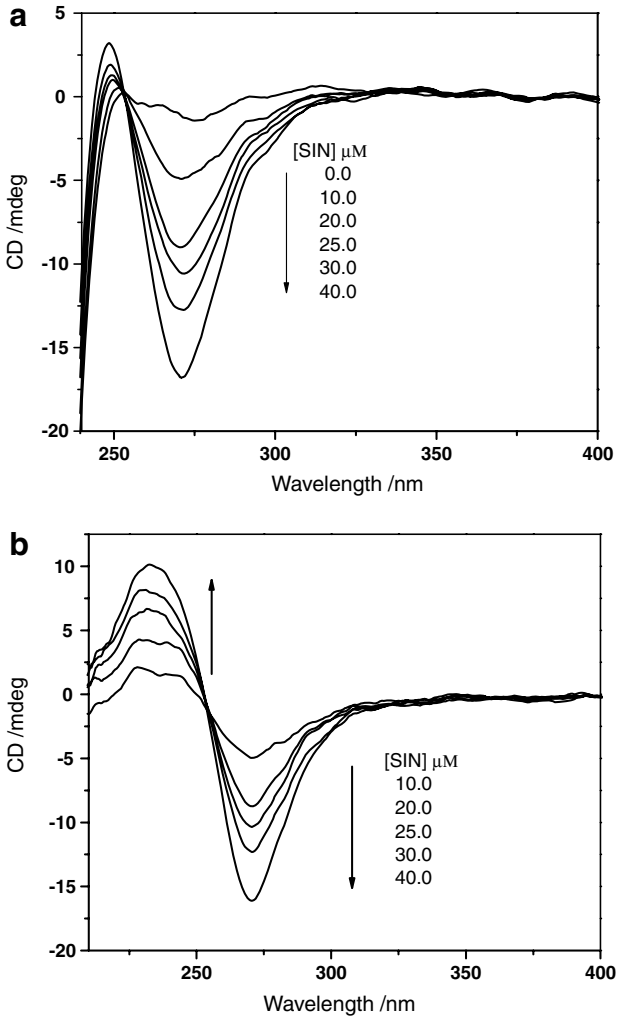

Figure 4. CD measurements were performed with a 1 cm pathlength cuvette between 200 and 400 nm at room temperature on a Jasco 810 spectropolarimeter. Data were collected at 500 nm/min speed, 1 nm band width, 1 s response time, and 1 nm resolution. CD spectra of sinomenine–Tf complexes (a) and sinomenine (b) in phosphate buffer with pH 7.4. The Tf concentration is 2.5 μM.

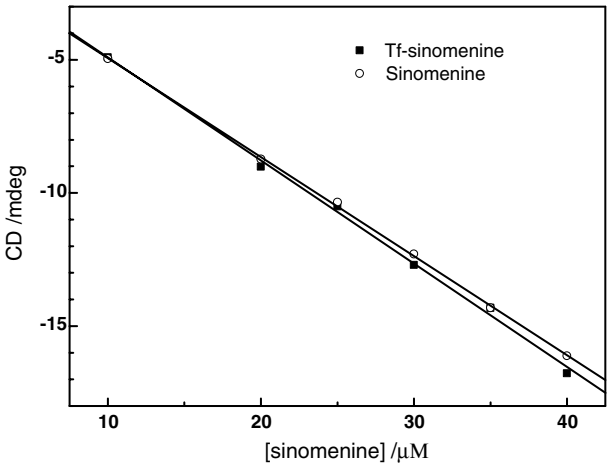

Figure 5. Comparison of the linear dependence of the sinomenine concentration versus CD signal intensity at 270.5 nm in sinomenine–Tf and sinomenine only.

With the aim to study structure–function relationship as well as explore the mechanism of conformational change while binding sinomenine to transferrin, an interaction model has been generated by using molecular modeling method. All computational studies were performed on a computer cluster consisting of SGI silicon graphics fuel workstation using Insight-II 2005 software (Accelrys Inc., San Diego, CA), with Builder, Biopolymer, Binding Site Analysis, Docking, and Affinity software packages. The crystal structure of Tf taken from the Protein Data Bank (PDB entry code 1D3K) is used to find the binding site of sinomenine to Tf. The model shows the best conformation ranked by the energy. One can see that sinomenine coordinates with distorted octahedral geometry to Asp292 in the N-lobe of Tf via non-covalent bond with 1.38 Å in length. The total binding energy is −94.426 kcal/mol (a.u.) including −60.4567 (van der Waals force) and −33.9693 (electrostatic force) kcal/mol. Clearly, all experimental results including fluorescence and CD measurements together with theoretical calculation provide a clear message that the binding of sinomenine to Tf is weaker via non-covalent bonds than that of metal. However, it is possible for the transport of sinomenine through Tf when sinomenine is fed by human.

The conformational change and non-covalent binding have an important role in the life process at the molecular level, which are implicated in many biological processes to regulate some important cellular processes.

In summary, we have demonstrated the binding of sinomenine to human serum transferrin at pH 7.4. The

association constant calculated from the fluorescence spectra is $K_A = 0.68 \times 10^3\ M^{-1}$. The sinomenine binding properties of Tf were also described by CD and molecular modeling. These results lead to a probability of the transport of sinomenine by Tf. Present studies have shown that sinomenine has much more potential protein binding ability in the plasma of human serum transferrin.[23] Further studies are ongoing in our laboratory to further shed light on the binding mechanism of sinomenine to Tf so that transferrin could transport drug molecule, sinomenine to targeted sites. Additionally, biological investigation of sinomenine–transferrrin would therefore appear to be warranted.

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