Original Article

Fluorometric and Docking Analysis of the Complex Formation between an Anti-cancer Drug, Chlorambucil and Bovine Serum Albumin

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ABSTRACT

Background: To characterize the interaction between chlorambucil (CHB) and the carrier protein, bovine serum albumin (BSA) in order to understand the transport of this drug in blood circulation. **Methods:** Fluorescence quenching titration method was used to examine the interaction of CHB with BSA by determining its binding constant and binding stoichiometry. The binding site identification was probed with molecular docking techniques. **Results:** Values of the Stern-Volmer constant (K_{SV}), bimolecular quenching rate constant (k_q) and binding constant (K_a) for CHB-BSA system were determined as 3.57×10^4 M^{-1}, 5.67×10^{12} M^{-1} s^{-1} and 5.58×10^4 M^{-1}, respectively. Binding stoichiometry was found to be ~1.0, as obtained from the double logarithmic plot. The molecular docking results revealed that CHB binds to both Site I and Site II of BSA, however Site II was predicted to be the preferred binding site. **Conclusion:** The value of K_a suggested intermediate binding affinity between CHB and BSA with the binding stoichiometry of 1:1. CHB was found to have the binding preference at Site II of BSA due to formation of greater contacts.

Key words: Bovine serum albumin, Chlorambucil, Drug-protein interaction, Fluorescence quenching, Molecular docking.

INTRODUCTION

Chlorambucil (CHB) (Figure 1) is an FDA-approved anticancer agent, which is used to treat chronic lymphocytic leukemia and some other types of cancers like Hodgkin's and Non-Hodgkin's lymphoma.[1] Pharmacokinetic and pharmacodynamic properties of a drug are affected by several factors such as its transport in the blood circulation, bioavailability, distribution and metabolism, which depend on its association with plasma proteins.[2,3] Improvement of the drug solubility, efficacy and half-life in plasma are also influenced by its binding to the plasma proteins. Therefore, molecular action of these drugs in human body can be well understood by studying the drug binding to the carrier proteins.[4-8]

The major carrier protein in blood circulation is serum albumin.[9] Bovine serum albumin (BSA) has been widely selected as a prototype for drug binding research due to its availability, low cost and being homologous (76% homologous sequences) to human serum albumin.[9-12] BSA consists of 583 amino acid residues in a single chain with a molecular mass of 66430 Da. It is assemble of domains I, II and III, which are homologous to each other and are comprised of two sub-domains, A and B.[9-12] Subdomains IIA and IIIA house the

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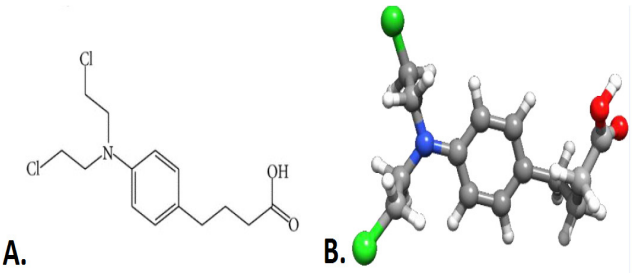

Figure 1: Structural representations of CHB. (A) Chemical structure and (B) ball-and-stick model.

well-characterized ligand binding sites *i.e.* Sudlow's sites I and II, respectively.[9-12] Due to the absence of any published report on the transport of CHB in blood circulation, it is valuable to get insight about the CHB-BSA binding mechanism by determining the binding affinity and binding stoichiometry along with binding site identification. Fluorescence spectroscopy and molecular docking approaches were employed to characterize CHB-BSA interaction.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), Fraction V, essentially γ-globulin free (Lot 087K0675) was supplied by Sigma-Aldrich Inc., St. Louis, MO, USA. Chlorambucil (CHB) was purchased from Selleck Chemicals, USA. Analytical grade samples of other chemicals were used. The deionized water was secured from Milli-Q® Integral Water Purification System for Ultrapure Water (Merck KGaA, Darmstadt, Germany).

Analytical Procedures

A constant volume of sodium phosphate buffer (60 mM, pH 7.0) was added to the desired amount of BSA to make the protein stock solution (30 μM). The actual concentration of the prepared protein stock solution was determined using the specific extinction coefficient of 6.67 at 279 nm.[13] The drug stock solution was made by dissolving 5 mg of CHB in 5 mL of dimethylsulfoxide (DMSO). The working CHB solution (100 μM) was made from the stock solution by diluting it with the same buffer to be used in titration experiment. DMSO concentration in all experiments was <1%.

Fluorescence Spectroscopy

The fluorescence spectra of BSA (3 μM) were attained in the wavelength range, 300–400 nm after exciting the protein sample at 295 nm on a Jasco FP-6500 spectrofluorometer. Various parameters were fixed as: excitation and emission slits - 10 nm each, data pitch - 1 nm, scan speed - 300 nm min^{-1} and response time - 0.08 s.

CHB-BSA Interaction Studies

The fluorescence quenching titration method was employed to study the binding of CHB to BSA.[7] Increasing CHB concentrations in the range of 0–18 μM with 3 μM intervals were added to a fixed concentration (3 μM) of BSA solution in the total volume of 3.0 mL. After incubating the mixture for 1 h at 25°C for equilibrium establishment, fluorescence spectra were recorded. The fluorescence spectra were amended due to inner filter effect contribution with the help of the following equation[14]

$$F_{cor} = F_{obs}\, 10^{\left(\frac{Aex+Aem}{2}\right)} \quad (1)$$

The corrected fluorescence intensity values at 344 nm were then changed to relative fluorescence intensity following the published procedure.[7]

Fluorescence Data Analysis

The Stern-Volmer quenching constant (K_{SV}) of CHB–BSA system was determined with the help of the following equation:

$$\frac{F_0}{F} = 1 + K_{sv}\,[Q] = 1 + k_q \tau_0 [Q] \quad (2)$$

where each term has its usual meaning.[14] Value of the bimolecular quenching rate constant (k_q) was obtained by dividing the K_{sv} value with τ_o, the biomolecule's (BSA) average lifetime in the absence of any quencher (6.3×10^{-9} s).[15]

Values of the binding constant, K_a and the binding stoichiometry, n of CHB–BSA interaction were acquired after fitting the data according to double logarithmic equation[16]

$$\log\frac{(F_0 - F)}{F} = \log K_a + n \log[Q] \quad (3)$$

Free energy change value, $\Delta G°$ for CHB-BSA interaction was quantified after substitution of the values of R (8.314 J mol^{-1} K^{-1}) and T (=273+ °C) in the following equation[17]

$$\Delta G° = -RT \ln K_a \quad (4)$$

Molecular Docking

The docking of CHB to BSA was executed with AutoDock4 by employing the Lamarckian genetic algorithm search engine.[18] The three-dimensional (3D)

structure of CHB was built and optimized with the MMF94 force field[19] using Avogadro software.[20] Crystallized structure of BSA was obtained from the Protein Data Bank (PDB) with the resolution of 2.47 Å (PDB ID: 4F5S). Preparation steps for the BSA structure included the deletion of crystallized water, addition of polar hydrogens and computing its Kollman charges by AutoDockTools4[18] prior to the docking simulation. Gasteiger charges were applied on CHB and its rotatable bond was defined as part of its preparation. Independent docking simulation for the two main drug binding sites on BSA, namely subdomain IIA (Site I) and subdomain IIIA (Site II) was conducted by specifying each of their grid coordinates. The grid box dimension was 70 × 70 × 70 points with a spacing of 0.375 Å and was centred at: x = −4.49, y = 32.71, z = 100.41 for Site I and x = 10.04, y = 18.22, z = 122.13 for Site II, separately. The docking simulation search run was set to 100, with a population size of 150 with 27,000 generations and 250,000 energy evaluations for both docking procedures. Furthermore, the operator weights for crossover, mutation and elitism were set at 0.8, 0.02 and 1, respectively. Assessment of the docking results was performed by clustering them based on the root-mean-squared deviation (RMSD) tolerance of 2.0 Å and the generated interactions were visualised and captured using UCSF Chimera.[21] In addition, LigPlot+analysis was also performed to investigate the interaction between CHB and the binding sites I and II of BSA.[22]

RESULTS AND DISCUSSION

Fluorescence Data Analysis of CHB-BSA System

Drug-protein interactions have been widely investigated using fluorescence spectroscopy. Trp residues of the protein (BSA) are excited at 295 nm and contribute to its intrinsic fluorescence spectrum.[23] Variation of the protein's fluorescence spectrum may reflect changes in protein conformation, denaturation, ligand binding or association of subunits.[24] The addition of a drug to BSA generally leads to the quenching of its intrinsic fluorescence spectrum, which can be caused by several molecular interactions, such as the formation of ground state complex, molecular rearrangements, energy transfer, excited-state reactions and collisional quenching.[25] Figure 2 depicts the BSA fluorescence spectra for both with and without CHB at increasing concentrations in the wavelength range, 300–400 nm. BSA's fluorescence spectrum showed an emission maximum at 344 nm due to Trp residues in the protein molecules. The fluorescence intensity gradually reduced upon CHB addition. About 39% reduction in the fluorescence intensity (Figure 2 inset) along with 4 nm red shift were evident in the presence of 18 µM CHB. The reduction in the fluorescence intensity of BSA after addition of CHB might be resulted due to microenvironmental alteration (reduced hydrophobicity) around Trp residues of BSA (Trp-134 and Trp-213). Similar fluorescence properties of BSA have been reported earlier upon drug binding.[4,16,26]

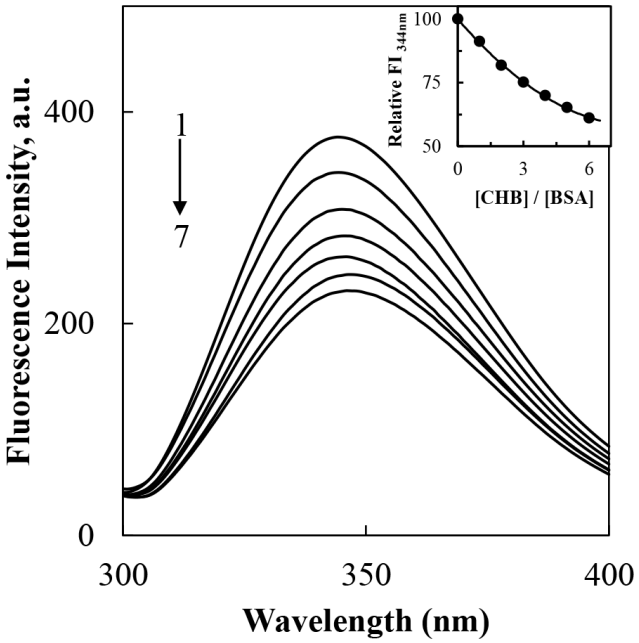

Figure 2: Spectra showing fluorescence quenching titration results of BSA (3 µM) with increasing CHB concentrations upon excitation at 295 nm, as studied in 60 mM sodium phosphate buffer, pH 7.0 at 25°C. Spectrum 1 shows the fluorescence spectrum of BSA whereas spectra 2-7 were obtained upon addition of 3, 6, 9, 12, 15 and 18 µM CHB, respectively. Decrease in the relative fluorescence intensity of BSA at 344 nm (Relative $FI_{344\ nm}$) with increasing CHB concentrations is shown in the inset.

The fluorescence data were treated with the Stern-Volmer equation (2) and the results are depicted in Figure 3A. A linear Stern-Volmer plot generally indicates single type fluorescence quenching, which might represent either static or dynamic quenching.[27] The Stern-Volmer plot was found to be linear for CHB-BSA system. The linear regression analysis of the plot yielded the value of the Stern-Volmer constant, K_{SV} as 3.57×10^4 M^{-1} (Table 1). This value of K_{SV} was used to calculate the k_q value (5.67×10^{12} M^{-1} s^{-1}, Table 1), which was found higher than the maximum dynamic quenching rate constant (2.0×10^{10} M^{-1} s^{-1}), reported for a number of quencher–fluorophore systems[27-29] and suggested complexation between CHB and BSA.

CHB-BSA interaction was characterized by determining the binding parameters such as binding constant (K_a) and number of binding sites (n), which were obtained from the double log plot (Figure 3B). The K_a value

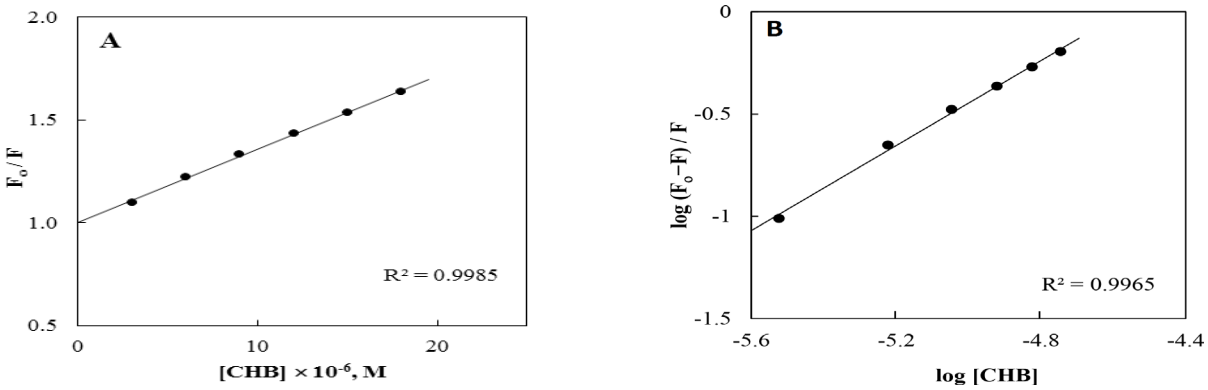

Figure 3: (A) Stern-Volmer plot and (B) Double logarithmic plot of log (F_0–F) / F against log [CHB] for the fluorescence quenching data, shown in Figure 2.

Table 1: Binding and thermodynamic parameters of the CHB–BSA interaction, studied at 25°C, pH 7.0.

Parameter	Value
K_{SV}	3.57 × 10^4 M^{-1}
k_q	5.67 × 10^{12} M^{-1} s^{-1}
K_a	5.58 × 10^4 M^{-1}
n	1.0
Δ*G*	−25.95 kJ mol^{-1}

was determined to be 5.58 × 10^4 M^{-1} (Table 1), which indicated moderate binding affinity for CBH-BSA system. This value was similar to earlier reports for several ligand-BSA interactions.[5,30] Moderate binding affinity is beneficial for the transportation of the drug through blood circulation, as it is easy to release the drug at the target sites from the blood stream.[6,30] Value of n=1.0 suggested single binding site of CHB on BSA. Furthermore, the value of Δ*G*° (−25.95 kJ mol^{-1}) for CHB-BSA interaction suggested that the binding process was spontaneous.[31]

Molecular Docking Analysis

Molecular docking simulation allowed the prediction of favourable binding interactions and orientation of CHB on the main hydrophobic cavities (Site I and Site II) of BSA. Evaluation of cluster analysis (Figure 4) indicated that a total of 4 and 21 multimember conformational clusters were generated for Site I and Site II of BSA, respectively. The highest populated cluster on Site I was comprised of 94 members with a mean binding energy of −25.82 kJ mol^{-1}. Meanwhile, the highest populated cluster on Site II had a mean binding energy of −18.54 kJ mol^{-1} with 21 members. Moreover, the lowest binding energy calculated on Site I and Site II was −27.99 kJ mol^{-1} and −30.75 kJ mol^{-1}, respectively. This result indicated that CHB had a binding preference for Site II since stronger energy was generated. Further inspection of the generated binding interactions

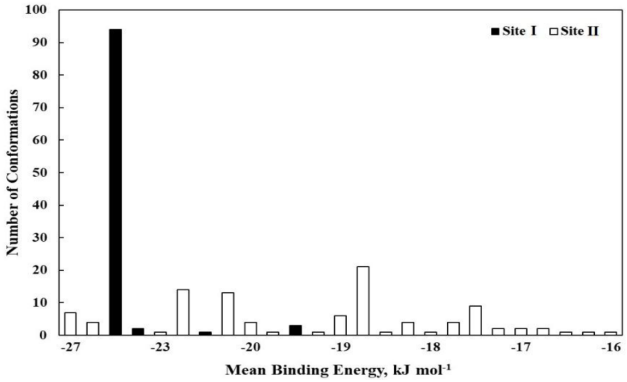

Figure 4: Cluster analysis of the molecular docking simulation of CHB binding to Site I (subdomain IIA) and Site II (subdomain IIIA) of BSA. A total of 100 runs were made for each biding site.

between CHB and BSA was made using the predicted model with the lowest binding energy as visualised in Figure 5. As can be seen from the figure, three hydrogen bonds were formed at Site II compared to two hydrogen bonds, formed at Site I in the complex (Table 2). The docked position of CHB on Site II was lined by 18 amino acid residues within 5 Å distance: Leu-386, Ile-387, Asn-390, Cys-391, Phe-394, Phe-402, Leu-406, Arg-409, Tyr-410, Lys-413, Leu-429, Val-432, Gly-433, Cys-437, Thr-448, Leu-452, Arg-484 and Ser-488. Around 50% of these residues were hydrophobic in nature while remaining 50% also contributed towards hydrophobicity of the binding site. Meanwhile, formation of two hydrogen bonds at Site I (Table 2) represented a weaker interaction between CHB and BSA. Overall, CHB appeared to form a more stable complex with BSA at Site II than Site I. In addition, both binding sites I and II of BSA were found to be surrounded by hydrophobic amino acid residues which might stabilize CHB-BSA complex through hydrophobic interactions (Figure 6). Thus, the molecular forces *i.e.*, hydrogen bonds and hydrophobic forces are supposed to be the

Table 2: Predicted hydrogen bonds formed between atoms of the BSA amino acid residues and CHB at Site I and Site II, as obtained from docking evaluation.

BSA binding site	BSA atom	CHB atom	Distance (Å)
Site I (subdomain IIA)	Arg-198: HH11	O	2.22
	Arg-217: HE	O	1.78
Site II (subdomain IIIA)	Asn-390: HD22	O	1.70
	Arg-409: HE	O	1.78
	Arg-409: HH21	O	2.13

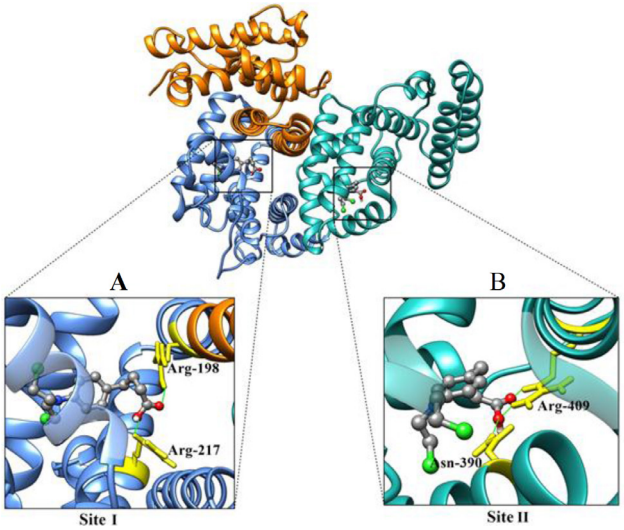

Figure 5: Predicted orientation of CHB (rendered in ball and stick) on (A) Site I and (B) Site II of BSA (PDB ID: 4F5S) based on the lowest binding energy. The three domains of BSA are coloured in orange (domain I), blue (domain II) and green (domain III). The zoomed-in image shows the hydrogen bonds (green lines), formed between the amino acid residues of BSA (rendered in yellow stick) and CHB at the binding site, located in the subdomain IIA (Site I) and IIIA (Site II), respectively.

major forces for stabilizing the CHB-BSA complex. These results were in accordance to the previously published reports, where the ligand binding has been shown to involve both hydrophobic interactions and hydrogen bonding.[7,8,26,32]

CONCLUSION

The results of CHB–BSA interaction study revealed moderate binding affinity between CHB and BSA and the complexation was found to be spontaneous. Interaction of CHB with BSA involved both hydrophobic interactions and hydrogen bonds. Besides, the preferred binding site for CHB was identified in subdomain IIIA which was Sudlow's Site II of BSA. Therefore, these results offer valuable information about the pharmacokinetics of CHB in mammalian circulation.

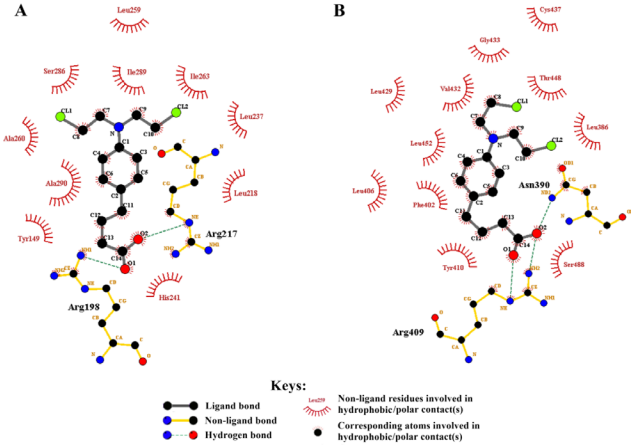

Figure 6: The ligand-protein interaction between CHB and BSA at Site I (A) and Site II (B) showing hydrophobic cavities of BSA using LigPlot+.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BSA: Bovine serum albumin; **CHB**: Chlorambucil; K_{sv}: Stern-Volmer constant; k_q: Bimolecular quenching rate constant; K_a: Binding constant.

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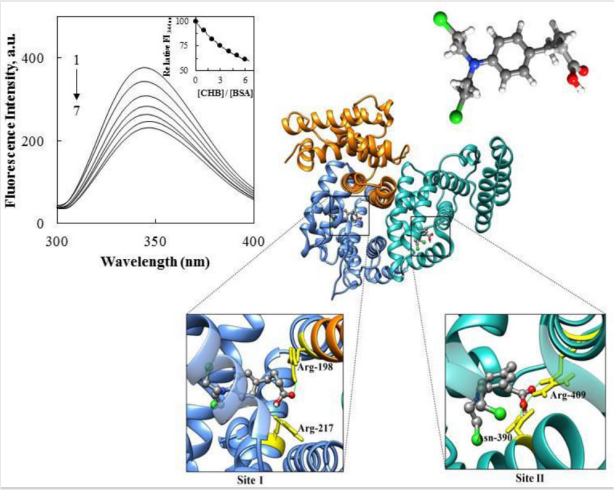
PICTORIAL ABSTRACT



SUMMARY

Chlorambucil, an anticancer drug binds to serum albumin with moderate binding affinity and has binding preference for Site II in the protein.

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