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To cite this article: Md. Zahirul Kabir, Shevin R. Feroz, Abdul Kadir Mukarram, Zazali Alias, Saharuddin B. Mohamad & Saad Tayyab (2016) Interaction of a tyrosine kinase inhibitor, vandetanib with human serum albumin as studied by fluorescence quenching and molecular docking, Journal of Biomolecular Structure and Dynamics, 34:8, 1693-1704, DOI: 10.1080/07391102.2015.1089187

To link to this article: http://dx.doi.org/10.1080/07391102.2015.1089187
Interaction of a tyrosine kinase inhibitor, vandetanib with human serum albumin as studied by fluorescence quenching and molecular docking

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Communicated by Ramaswamy H. Sarma

(Received 9 May 2015; accepted 28 August 2015)

Interactions of a tyrosine kinase inhibitor, vandetanib (VDB), with the major transport protein in the human blood circulation, human serum albumin (HSA), was investigated using fluorescence spectroscopy, circular dichroism (CD) spectroscopy, and molecular docking analysis. The binding constant of the VDB--HSA system, as determined by fluorescence quenching titration method was found in the range, 8.92--6.89 × 10\textsuperscript{3} M\textsuperscript{−1} at three different temperatures, suggesting moderate binding affinity. Furthermore, decrease in the binding constant with increasing temperature revealed involvement of static quenching mechanism, thus affirming the formation of the VDB--HSA complex. Thermodynamic analysis of the binding reaction between VDB and HSA yielded positive Δ$H$ (52.76 J mol\textsuperscript{−1} K\textsuperscript{−1}) and negative Δ$S$ (−6.57 kJ mol\textsuperscript{−1}) values, which suggested involvement of hydrophobic interactions and hydrogen bonding in stabilizing the VDB--HSA complex. Far-UV and near-UV CD spectral results suggested alterations in both secondary and tertiary structures of HSA upon VDB-binding. Three-dimensional fluorescence spectral results also showed significant microenvironmental changes around the Trp residue of HSA consequent to the complex formation. Use of site-specific marker ligands, such as phenylbutazone (site I marker) and diazepam (site II marker) in competitive ligand displacement experiments indicated location of the VDB binding site on HSA as Sudlow’s site I (subdomain IIA), which was further established by molecular docking results. Presence of some common metal ions, such as Ca\textsuperscript{2+}, Zn\textsuperscript{2+}, Cu\textsuperscript{2+}, Ba\textsuperscript{2+}, Mg\textsuperscript{2+}, and Mn\textsuperscript{2+} in the reaction mixture produced smaller but significant alterations in the binding affinity of VDB to HSA.

\textbf{Keywords:} vandetanib; human serum albumin; ligand–protein interaction; fluorescence quenching; fluorescence spectroscopy; molecular docking

1. Introduction

Tyrosine kinase inhibitors are small and hydrophobic compounds, which can inhibit the abnormal activities (e.g. cell proliferation, differentiation) of tyrosine kinases (Bennasroune, Gardin, Aunis, Crémel, & Hubert, 2004). Hence, they are considered as the key promising molecules in anticancer drug development (Fabbro, Parkinson, & Matter, 2002). These inhibitor molecules can quickly reach their specific target sites in signaling pathways and are able to reduce or block the abnormal proliferation of various carcinomas (Brassard & Rondeau, 2012; Fabbro et al., 2002). Vandetanib (VDB) is a potential and selective tyrosine kinase inhibitor exhibiting inhibition activity against the vascular endothelial growth factor receptor 2 (VEGFR2) and epidermal growth factor receptor (EGFR) (Brassard & Rondeau, 2012; Sano et al., 2011). It is an orally administered drug used for the treatment of metastatic medullary thyroid cancer (Wedge et al., 2002), which has been approved by Food and Drug Administration, USA for clinical applications (Brassard & Rondeau, 2012; Solomon & Rischin, 2012). Many studies have shown effective responses of VDB (Figure 1A and B) on cancer cell inhibition (Arora & Scholar, 2005; Lin et al., 2002; Yoshikawa et al., 2009).

Interactions between various biologically active compounds (e.g. pharmaceutical ligands, drugs) and the plasma proteins are of pharmacological importance, as they directly affect their pharmacokinetic and pharmacodynamic properties (Colmenarejo, 2003; Fasano et al., 2005). Such interactions may influence the drugs’ bioavailability, distribution, metabolism, and elimination processes in the body and may also produce alterations in the microenvironment around the carrier proteins as well as their physiological action (Ebrahim et al., 2015; Kragh-Hansen, Chuang, & Otagiri, 2002; Petitpas, Bhattacharya, Twine, East, & Curry, 2001). The weak
binding might lead to a short lifetime or low distribution of drugs whereas strong binding can reduce the concentration of free drug in plasma. Therefore, drug–protein interactions are important for the safe distribution and increased half-life of the drug in the plasma (Kragh-Hansen et al., 2002; Moreno & González-Jiménez, 1999). Thus, it is important to investigate the characteristics of ligand–protein interaction for understanding the physiological action of a pharmaceutical compound at the molecular level in the human body.

Human serum albumin (HSA) is the most abundant extracellular protein, present in the blood plasma, which functions as the main transporter for a large number of molecules, both endogenous and exogenous in nature (Peters, 1996). It has pharmacological importance to carry out different drugs into the bloodstream and deliver them to the specific target sites directly, thus increasing their efficacy (Kratz, 2008; Pal & Saha, 2014; Yamasaki, Chuang, Maruyama, & Otagiri, 2013; Yeggoni, Rachamallu, Kallubai, & Subramanyam, 2015). It is a single polypeptide chain, comprised of 585 amino acid residues with a molecular weight of 66.5 kDa (Peters, 1996). The crystallographic studies on HSA have revealed a heart-shaped structure formed from three homologous domains I, II, and III, which are further divided into two subdomains A and B (Carter & Ho, 1994; Peters, 1996). Sudlow’s sites I and II, commonly referred to as sites I and II are located in subdomains IIA and IIIA and are two major recognized sites available for high-affinity binding of various ligands to HSA (Kragh-Hansen et al., 2002). Both sites I and II are characterized as hydrophobic ligand-binding pockets, surrounded by positively charged residues at the entrance, thus favoring the binding of heterocyclic molecules possessing negatively charged groups (Kragh-Hansen et al., 2002). Binding site II is less flexible, narrower, and smaller compared to site I, thus, the larger molecules generally prefer to bind to site I (Kragh-Hansen et al., 2002).

Fluorescence spectroscopy is widely used to investigate the binding of ligands to serum albumin. Presence of a single tryptophan (Trp-214) residue in subdomain IIA of HSA offers greater advantage in ligand-binding studies using fluorescence spectroscopy (Peters, 1996). In view of this, interaction of VDB with HSA was investigated using fluorescence spectroscopy supported by molecular docking methods. The results obtained from such study would be beneficial in understanding VDB pharmacokinetics in blood plasma.

2. Materials and methods
2.1. Materials
Essentially fatty acid-free human serum albumin (HSA) (Lot # 068K7538V) and phenylbutazone (PBZ) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Diazepam (DZM) was the product of Lipomed AG, Arlesheim, Switzerland. Vandetanib (VDB) was procured from Cayman Chemical Company, Michigan, USA. All other chemicals used were of analytical grade purity.

2.2. Analytical procedures
The stock solution of HSA was prepared by dissolving an appropriate amount of HSA in 60 mM sodium phosphate buffer at pH 7.4 and its concentration was determined spectrophotometrically using a molar extinction coefficient of 36 500 M⁻¹ cm⁻¹ at 280 nm (Painter, Harding, & Beeby, 1998).

Stock solutions of VDB, PBZ, and DZM were prepared by dissolving 10 mg of their crystals in 10 ml of dimethylsulfoxide (DMSO) while the final working solutions were made by diluting the stock solutions with the phosphate buffer. The final concentration of DMSO was less than 1% in all experiments. Stock solutions were filtered through 0.45-μm Millipore filters and stored at 4°C.

2.3. Fluorescence spectroscopy
Fluorescence measurements were carried out on a Jasco FP-6500 spectrofluorometer using a quartz cuvette of 1-cm path length, positioned in a thermostatically controlled water-jacketed cell holder. The fluorescence spectra of HSA (3 μM) were obtained in the absence and presence of VDB (0–60 μM with 5-μM intervals) in the wavelength range of 310–380 nm using an excitation wavelength of 295 nm. The excitation and emission bandwidths were manually fixed at 10 nm each, whereas the data pitch and the scan speed were set at 1 nm and 500 nm min⁻¹, respectively.

Three-dimensional (3-D) fluorescence spectra of HSA (3 μM) and VDB–HSA complexes (5:1 and 10:1 molar ratios) were recorded using the excitation wavelength range of 220–350 nm with 5 nm increments and the emission wavelength range of 220–500 nm.

2.4. VDB–HSA interaction
Fluorescence spectroscopy was used to investigate the binding of VDB to HSA. Titration experiments were carried out at three different temperatures, i.e. 288, 303, and 318 K in order to study the effect of temperature on VDB–HSA interaction. In these experiments, HSA concentration was fixed at 3 μM while VDB concentration was varied in the range of 0–60 μM in a total volume of 3 ml. These solutions were incubated for 1 h at 25°C and the fluorescence spectra were recorded after an equilibration time of 6 min at each temperature. The inner filter effect corrections in the fluorescence data were made using the following equation (Lakowicz, 2006):
\[ F_{\text{cor}} = F_{\text{obs}} 10^{(A_{\text{ex}} + A_{\text{em}})/2} \]  

where \( F_{\text{cor}} \) and \( F_{\text{obs}} \) are the corrected and observed fluorescence intensity of ligand–protein complex, \( A_{\text{ex}} \) and \( A_{\text{em}} \) are the differences in the absorbance values of the protein at the excitation (295 nm) and emission wavelengths (300–400 nm), respectively, produced by the addition of the ligand.

In order to determine the mechanism of HSA fluorescence quenching by VDB, the fluorescence data were analyzed using the Stern–Volmer equation:

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \]

where \( F_0 \) and \( F \) are the fluorescence intensity values of the protein in the absence and presence of quencher, respectively, the \( K_{SV} \) is the Stern–Volmer constant, and \([Q]\) is the concentration of the quencher (Lakowicz, 2006). The bimolecular rate constant \( (k_q) \) of the fluorescence quenching process was calculated using the following equation:

\[ k_q = \frac{K_{SV}}{\tau_0} \]

where \( \tau_0 \) is the average lifetime of the biomolecule in the absence of the quencher and its value was taken as 6.38 \times 10^{-9} \text{ s} \) for HSA (Abou-Zied & Al-Shihi, 2008).

The binding constant \( (K_b) \) for VDB–HSA interaction was determined using the following equation (Bi et al., 2004):

\[
\log\left(\frac{F_0 - F}{F}\right) / F = n \log K_b - n \log \left[ \left( \frac{[L_T]}{(F_0 - F)} \frac{[P_T]}{[F_0]} \right) \right]
\]

where \( n \) is the Hill coefficient, while \([L_T]\) and \([P_T]\) are the total concentrations of the ligand (VDB) and the protein (HSA), respectively. \( F_0 \) and \( F \) represent the fluorescence intensity values of the protein (HSA) in the absence and presence of quencher (VDB), respectively.

In order to characterize the binding forces involved in VDB–HSA complex formation, thermodynamic parameters were analyzed using the van’t Hoff Equation (4). The enthalpy change \((\Delta H)\) and entropy change \((\Delta S)\) data for the binding reaction were acquired from the slope and intercept of the linear van’t Hoff plot between \( \ln K_a \) and \( 1/T \), respectively.

\[
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

where \( K_a \) is the binding constant, \( R \) is the gas constant \((8.31 \text{ J mol}^{-1} \text{K}^{-1})\), and \( T \) is the absolute temperature. The free energy change \((\Delta G)\) of the reaction was obtained from the following equation (5):

\[
\Delta G = \Delta H - T \Delta S
\]

The influence of some common metal ions, i.e. \( \text{Ca}^{2+}, \text{Zn}^{2+}, \text{Cu}^{2+}, \text{Ba}^{2+}, \text{Mg}^{2+}, \) and \( \text{Mn}^{2+} \) on the binding of VDB to HSA was investigated using fluorescence spectroscopy. The solution containing HSA (3 \( \mu \text{M} \)) and different metal ions (30 \( \mu \text{M} \)) were incubated in 60 mM sodium phosphate buffer at pH 7.4 for 12 h prior to titration with VDB at 25°C. Fluorescence spectra of VDB–HSA system were recorded in the absence and presence of different metal ions in the wavelength range of 300–400 nm upon excitation at 295 nm.

2.5. Circular dichroism spectroscopy

In order to investigate the influence of VDB binding on the secondary and tertiary structures of HSA, the far- and the near-UV CD spectral measurements were performed on a Jasco spectropolarimeter (model J–815), equipped with a thermostatically controlled cell holder attached to a water bath. CD spectra were recorded at 25°C with a scan speed of 100 nm min\(^{-1}\) and a response time of 1 s. Far-UV CD spectral measurements were made using 1 \( \mu \text{M} \) protein concentration in 1-mm path length cuvette, while 5 \( \mu \text{M} \) protein concentration in 10-mm path length cuvette were used for near-UV CD spectral measurements both in the absence and the presence of VDB. Each spectrum was the average of four scans. The observed ellipticity values were expressed as mean residue ellipticity (MRE) in deg.cm\(^2\).dmol\(^{-1}\), which is defined as:

\[
\text{MRE} = \frac{[\theta_{\text{obs}} \times \text{MRW}]}{10 \times I \times C_p}
\]

where \( \theta_{\text{obs}} \) is the ellipticity in millidegree, MRW is the mean residue weight (molecular weight of protein, 66,500 divided by total number of amino acids, 585), \( I \) is the path length of the cuvette, and \( C_p \) is the protein concentration in mg ml\(^{-1}\) (Chen, Yang, & Martinez, 1972).

2.6. Competitive displacement experiments with site markers

In order to elucidate the specificity of VDB binding to HSA, competitive displacement experiments for the two ligand binding sites of HSA were performed with different site markers viz. PBZ for site I and DZM for site II. In these experiments, a fixed concentration of HSA (3 \( \mu \text{M} \)) or VDB–HSA complex (mixed in a molar ratio of 5:1 and pre-incubated for 1 h) were titrated with increasing concentrations (0–30 \( \mu \text{M} \) with 5-\( \mu \text{M} \) intervals) of site marker ligands. After incubating the solutions for another 1 h, the fluorescence spectra were recorded in the wavelength range of 300–400 nm upon excitation at 295 nm. All experiments were performed in 60 mM sodium phosphate buffer, pH 7.4, and at 25°C.

2.7. Molecular docking

The structure of VDB was constructed and its geometry was optimized with MMFF94 force field (Halgren, 1996).
using Avogadro Software (Hanwell et al., 2012). This was exported as a mol2 file. Docking, visualization, and rendering simulation were performed using AutoDock 4.2 (Goodsell, Morris, & Olson, 1996) and AutoDockTools 1.5.6 at the Academic Grid Malaysia Infrastructure (Sanner, 1999). The crystal structure of HSA (PDB code 1BM0, 2.5 Å resolution) was downloaded from the Protein Data Bank (Berman et al., 2000). Water molecules were removed and the atomic coordinates of chain A of 1BM0 were stored in a separate file and were used as input for AutoDockTools, where polar hydrogens, Kollman charges, and solvation parameters were added. In the case of the ligand VDB, nonpolar hydrogens were merged and rotatable bonds were defined. The two binding sites (subdomains IIA and IIIA) were defined using two grids of 70 × 70 × 70 points each with a grid space of 0.375 Å, centered at coordinates x = 35.26, y = 32.41, and z = 36.46 for site I and x = 14.42, y = 23.55, and z = 23.31 for site II, respectively. Lamarckian genetic algorithm with local search was used as the search engine, with a total of 100 runs for each binding site. In each run, a population of 150 individuals with 27 000 generations and 250 000 energy evaluations were employed. Operator weights for crossover, mutation, and elitism were set at 0.8, 0.02, and 1.0, respectively. For the local search, default parameters were used. Cluster analysis was performed on docked results using a root-mean-square deviation (RMSD) tolerance of 2.0 Å. The ligand–protein complex was visualized and analyzed using AutoDockTools.

2.8. Statistical analysis
The curve plotting and statistical data processing were made using the OriginPro 8.5 software (OriginLab Corp., Northampton, MA).

3. Results and discussion

3.1. VDB–HSA interaction

3.1.1. Fluorescence spectra
Figure 2(A) shows the fluorescence spectra of HSA in the absence and presence of increasing concentrations of VDB. The fluorescence spectrum of HSA was characterized by the presence of an emission maximum at 342 nm, which was indicative of the presence of tryptophan (Trp) in HSA. A progressive decrease in the fluorescence intensity and red shift in the emission maximum were observed upon addition of VDB to HSA. About 35% decrease in the fluorescence intensity at 342 and 8 nm red shift were noticed at the highest concentration (60 μM) of VDB. Alteration in the fluorescence characteristics (fluorescence intensity and emission maximum) of HSA upon addition of VDB were suggestive of VDB binding to HSA. Similar changes in the fluorescence characteristics have been shown in many ligand-binding studies (Cheng, Liu, & Jiang, 2013; Feroz, Mohamad, Bakri, Malek, & Tayyab, 2013; Li, Zhu, Jin, & Yao, 2007). It seems probable that VDB binds to a site near the single Trp residue (Trp-214) of HSA, which may account for the significant decrease in the fluorescence intensity and red shift in the emission maximum upon VDB binding. In view of the unique sensitivity of the emission maximum to hydrophobic
changes in the microenvironment around fluorophores (Lakowicz, 2006), red shift in the emission maximum observed upon VDB addition may point toward increased polarity of the microenvironment around Trp residue. This was also supported by the decrease in the fluorescence intensity of HSA in the presence of VDB, as fluorescence intensity is known to be affected by hydrophobic changes and movement of charged groups in the vicinity of the Trp residue (Lakowicz, 2006).

3.1.2. Mechanism of fluorescence quenching
The mechanism of fluorescence quenching of a protein can be classified either as static or dynamic quenching process. These two mechanisms can be distinguished by their response to temperature (Lakowicz, 2006). Higher temperatures lead to faster diffusion, thus producing a large amount of collisional quenching between the fluorophore and the quencher. Contrary to it, higher temperatures contribute to the dissociation of weakly bound complexes, resulting in a decrease in static quenching (Lakowicz, 2006). Therefore, the quenching constant increases with temperature for dynamic quenching process and shows a decrease for static quenching (Maiti, Ghosh, Samanta, & Dasgupta, 2008; Tunç, Çetinkaya, & Duman, 2013; Xu et al., 2013). In order to investigate the fluorescence quenching mechanism involved in VDB–HSA system, the titration experiments were carried out at three different temperatures, i.e. 288, 303, and 318 K and the fluorescence quenching data were analyzed according to the Stern–Volmer Equation (2). The Stern–Volmer plots shown in Figure 2(B) indicate linear relationship between $F_0/F$ and [VDB]. Regression analysis of these plots yielded the values of the Stern–Volmer constant, $K_{SV}$ at different temperatures, which are listed in Table 1. A gradual decrease in the $K_{SV}$ value with increasing temperatures (Figure 2(B), Table 1) clearly demonstrated that the fluorescence quenching observed was due to the complex formation between VDB and HSA and therefore, can be characterized as static quenching. This seems understandable as increase in temperature would have weakened the forces involved in VDB–HSA complex formation. Furthermore, the calculated $k_q$ values ($8.77 \times 10^{11}$, $7.70 \times 10^{11}$ and $6.90 \times 10^{11}$ M$^{-1}$s$^{-1}$ at 288, 303, and 318 K, respectively) of VDB–HSA system were found higher than the maximum dynamic quenching constant ($2 \times 10^{10}$ M$^{-1}$s$^{-1}$), reported for various quenchers of biomolecules (Ware, 1962). This has further supported the involvement of static quenching mechanism in the interaction between VDB and HSA.

3.1.3. Binding parameters
To determine the binding constant ($K_a$) of VDB–HSA system at different temperatures, fluorescence quenching data were analyzed using Equation (3) and were plotted as $\log(F_0 - F)/F$ vs. $\log([L/V] - ([F_0 - F]/[P]/F_0))$, as shown in Figure 3. The values of $K_a$ at three different temperatures were obtained from the y-axis intercept of the plots and are listed in Table 1. The $K_a$ values (8.92–6.89 $\times 10^{3}$ M$^{-1}$) reflected moderate binding affinity between VDB and HSA, which is useful for efficient transport and release of VDB at the target site. Several reports have suggested the values of $K_a$ falling in the range, 3.35 $\times 10^{1}$–1.3 $\times 10^{4}$ M$^{-1}$ (Afrin, Riyazudddeen, Rabbani, & Khan, 2014; Li & Wang, 2015; Liu, Zheng, Yang, Wang, & Wang, 2009). The decreasing trend of $K_a$ values with temperature indicated reduction in the VBD-binding capacity of HSA, thus leading to less stable VDB–HSA complex (Feroz, Mohamad, Bujang, Malek, & Tayyab, 2012; Trnková, Boušová, Staňková, & Dršata, 2011).

3.1.4. Acting forces
The ligand–protein complex formation may involve various noncovalent forces such as, hydrogen bonds, hydrophobic interactions, van der Waals forces, and electrostatic forces. Thermodynamic parameters such as the enthalpy change ($\Delta H$), the entropy change ($\Delta S$), and the free energy change ($\Delta G$) of the binding reaction can establish the binding mode between a ligand and the protein (Li et al., 2007; Olsson, Williams, Pitt, & Ladbury, 2008; Ross & Subramanian, 1981). In order to examine the acting forces involved in VDB–HSA interaction, thermodynamic parameters ($\Delta H$ and $\Delta S$) were assessed using the van’t Hoff plot between ln $K_a$ and $1/T$, as shown in the inset of Figure 3. Values of $\Delta H$ and $\Delta S$ along with $\Delta G$ (obtained by using Equation 5) are summarized in Table 1. The negative values of $\Delta G$ clearly demonstrated that the binding between VDB and HSA was spontaneous at all temperatures. The negative sign of $\Delta H$ revealed exothermic nature of the binding reaction. The water molecules that were otherwise arranged in an orderly fashion around the ligand (VDB) and the protein (HSA) molecules, must have acquired a more random conformation upon VDB–HSA complex formation, thus resulting in the positive entropy change ($\Delta S$). A positive value of $\Delta S$ obtained for ligand–protein interaction is frequently taken as an evidence for the involvement of hydrophobic interactions (Ross & Subramanian, 1981). Meanwhile, $\Delta S$ value seems to play the major role in making the $\Delta G$ value negative for VDB–HSA interaction. (Li et al., 2007; Zhang, Dai, Zhang, Yang, & Liu, 2008). On the other hand, the large negative value of $\Delta H$, obtained for VDB–HSA system can account for the involvement of hydrogen bonding and/or van der Waals forces (Ross & Subramanian, 1981). Involvement of electrostatic interactions in VDB–HSA complex formation can be ruled out as the value of $\Delta H$ should be
either very small or close to zero for typical electrostatic interactions (Rahman, Maruyama, Okada, Yamasaki, & Otagiri, 1993; Ross & Subramanian, 1981; Zhang et al., 2008). Since VDB lacks any ionizable/charged group, it is inconceivable to suggest the involvement of electrostatic interactions in the VDB–HSA binding process.

It is not reasonable to account for the observed changes in the thermodynamic parameters using only a single binding force, as these changes reflect several intermolecular phenomena between the ligand and the protein (Li et al., 2007; Zhang et al., 2008). Since VDB lacks any ionizable/charged group, it is inconceivable to suggest the involvement of electrostatic interactions in the VDB–HSA binding process.

3.2. Ligand-induced conformational changes

Figures 4 (A) and (B) show the effect of VDB binding on the secondary and tertiary structures of HSA, respectively, at different ligand/protein molar ratios. The far-UV CD spectrum of HSA (Figure 4(A)) was characterized by the presence of two minima at 208 and 222 nm, characteristics of the α-helical structure of the protein (Reed, Feldhoff, Clute, & Peters, 1975). Presence of VDB in the system produced significant alterations in the far-UV CD spectra of HSA, showing 7.5 and 14.5% decrease in the MRE222nm value at 1:1 and 2:1 VDB/HSA molar ratios, respectively. Such a decrease in the MRE222nm value was indicative of the secondary structure alteration of HSA in the presence of VDB, thus implying complex formation between VDB and HSA. The near-UV CD spectra of HSA in the absence and presence of VDB are displayed in Figure 4(B). Presence of two minima at around 263 nm and 270 nm and shoulders around 272 nm and 284 nm characterized the near-UV CD spectrum of HSA. These spectral features were similar to those reported earlier and characterized the presence of the disulfide bonds and aromatic chromophores in the protein (Lee & Hirose, 1992; Uversky, Narizhneva, Ivanova, & Tomashevski, 1997). A significant reduction in the CD spectral signals was observed in the presence of VDB, suggesting perturbations around

![Figure 3. Double-logarithmic plots \(\log(F_0 - F)/F\) vs. \(\log[1/([L] - ([F_0 - F][P]_L)/F_0)]\) for fluorescence quenching data of the VDB–HSA system at three different temperatures, i.e. 288, 303, and 318 K. Inset shows the van’t Hoff plot for VDB–HSA interaction at pH 7.4.](image)

![Figure 4. Far-UV (A) and near-UV (B) CD spectra of HSA, 1:1 VDB–HSA, and 2:1 VDB–HSA systems. The CD spectra were recorded using a protein concentration of 1 and 5 \(\mu\)M in the far-UV and the near-UV regions, respectively, in 60 mM sodium phosphate buffer at pH 7.4 and 25°C.](image)

### Table 1. Binding and thermodynamic parameters of the VDB–HSA system, studied at three different temperatures, pH 7.4.

<table>
<thead>
<tr>
<th>(T (K))</th>
<th>(K_w (M^{-1}))</th>
<th>(K_a (M^{-1}))</th>
<th>(\Delta S (J\text{ mol}^{-1}\text{ K}^{-1}))</th>
<th>(\Delta H (kJ\text{ mol}^{-1}))</th>
<th>(\Delta G (kJ\text{ mol}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>288</td>
<td>((8.77 \pm 0.15) \times 10^3)</td>
<td>((8.92 \pm 0.05) \times 10^3)</td>
<td>52.76</td>
<td>−6.57</td>
<td>−21.77</td>
</tr>
<tr>
<td>303</td>
<td>((7.70 \pm 0.10) \times 10^3)</td>
<td>((7.63 \pm 0.17) \times 10^3)</td>
<td>52.76</td>
<td>−6.57</td>
<td>−22.56</td>
</tr>
<tr>
<td>318</td>
<td>((6.90 \pm 0.17) \times 10^3)</td>
<td>((6.89 \pm 0.10) \times 10^3)</td>
<td>52.76</td>
<td>−6.57</td>
<td>−23.35</td>
</tr>
</tbody>
</table>
the Trp residue and disulfide bonds due to tertiary structural change. In view of the above, it can be concluded that VDB binding to HSA produced both secondary and tertiary structural alterations in HSA. Similar CD spectral changes of HSA have been reported in the presence of various other ligands (Chatterjee, Pal, Dey, Chatterjee, & Chakrabarti, 2012; Dockal, Carter, & Ruker, 1999; Lee & Hirose, 1992).

Ligand-induced microenvironmental changes around aromatic fluorophores (Trp and Tyr) can be easily monitored by three-dimensional (3-D) fluorescence spectroscopy. This is particularly important as one of the ligand binding sites i.e. Sudlow’s site I is located in the vicinity of Trp-214 of HSA (Sudlow, Birkett, & Wade, 1975). Three-dimensional fluorescence spectra and corresponding contour maps of free HSA (A) and VDB–HSA complexes (B and C) are shown in Figure 5. The fluorescence characteristics, such as peak position (\(\lambda_{ex}/\lambda_{em}\)) and intensity of the peaks are listed in Table 2. As shown in Figure 5, peaks ‘a’ and ‘b’, which are known as Rayleigh scattering peak (\(\lambda_{ex} = \lambda_{em}\)) and the second-order scattering peak (\(2\lambda_{ex} = \lambda_{em}\)), respectively, are common in 3-D fluorescence spectra (Cheng et al., 2013; Feroz et al., 2013; Lu, Fan, Liu, & Hou, 2009). In addition to these peaks, two other fluorescence peaks, namely ‘1’ (\(\lambda_{ex} = 280 \text{ nm}\)) and ‘2’ (\(\lambda_{ex} = 230 \text{ nm}\)) were also observed, which were due to the presence of Trp residue in the protein (Feroz, Teoh et al., 2015). Presence of VDB in the incubation mixture produced significant change in the spectral characteristics. Whereas peak 1 showed a red shift of 11 nm and 36% decrease in the fluorescence intensity, 13 nm red shift and 80% decrease in the fluorescence intensity were noticed in peak 2 upon VDB/HSA (5:1) addition to HSA (Table 2). These changes in the spectral characteristics became more pronounced on increasing VDB/HSA molar ratio to 10:1 (Table 2). These results suggested microenvironmental perturbation around Trp-214 upon VDB binding. Due to the presence of the lone tryptophan (Trp-214) in domain II of HSA (Peters, 1996), it seems that domain II might have undergone significant structural perturbation upon VDB binding.

### 3.3. Location of VDB-binding site

Identification of VDB-binding site on HSA was made using competitive displacement experiments with site markers as well as molecular docking.

#### 3.3.1. Site marker-induced VDB displacement

According to Sudlow et al. (1975), a large number of common ligands as well as drugs interact with HSA at two different binding sites, namely site I and site II, that are resided in subdomains IIA and IIIA, respectively (Peters, 1996). Site I shows affinity for several specific markers such as warfarin, phenylbutazone, and indomethacin, whereas diazepam, ketoprofen, and ibuprofen are well-known markers for site II (Kragh-Hansen et al., 2002). In order to identify the location of the VDB binding site on HSA, competitive displacement experiments were performed with PBZ (site I marker) and DZM (site II marker) using fluorescence spectroscopy. Figures 6(A)
and (B) show titration results of HSA and VDB–HSA complex with increasing concentrations of PBZ and DZM, respectively. As can be seen from the figure, a progressive decrease in the fluorescence intensity at 342 nm of HSA was noticed upon addition of PBZ or DZM, which was indicative of their binding to HSA. Interestingly, presence of VDB on HSA (VDB–HSA complex) offered significant protection against PBZ-induced decrease in the spectral signal, but lesser effect was noticed on DZM binding. These results clearly suggested preference of site I of HSA for VDB binding compared to site II. Furthermore, these results were in line with our three-dimensional (3-D) fluorescence spectral results, suggesting structural perturbation around protein fluorophores upon VDB binding. Since site I contains Trp-214 in it, its microenvironment might have been changed upon VDB binding to this site to produce spectral shift and decrease in fluorescence intensity.

Table 2. Three-dimensional fluorescence spectral characteristics of HSA and VDB–HSA systems at 25°C, pH 7.4.

<table>
<thead>
<tr>
<th>System</th>
<th>Peak</th>
<th>Peak position [λ&lt;sub&gt;ex&lt;/sub&gt;/λ&lt;sub&gt;em&lt;/sub&gt; (nm/nm)]</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>a</td>
<td>230/230→350/350</td>
<td>17.91→113.91</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>250/500</td>
<td>93.97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/338</td>
<td>342.62</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>230/335</td>
<td>161.63</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>230/230→350/350</td>
<td>14.53→95.82</td>
</tr>
<tr>
<td>[VDB]:[HSA] = 5:1</td>
<td>b</td>
<td>250/500</td>
<td>32.87</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/349</td>
<td>221.10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>230/348</td>
<td>31.64</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>230/230→350/350</td>
<td>14.10→76.00</td>
</tr>
<tr>
<td>[VDB]:[HSA] = 10:1</td>
<td>b</td>
<td>250/500</td>
<td>16.35</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/355</td>
<td>157.04</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>230/355</td>
<td>19.14</td>
</tr>
</tbody>
</table>

3.3.2. Molecular docking analysis

A molecular docking study was made to predict the binding site of VDB in HSA and to support the results of the displacement experiments described above. Blind docking with 500 production runs was performed prior to the site-specific docking studies and the results are shown in the Supplementary Figure 1. Preferred orientation based on energetics of the blind docking conformation of VDB to HSA showed scattered distribution of VDB throughout the HSA structure. Presence of clefts in the HSA structure might have allowed nonspecific binding of VDB, when it was docked to HSA. Cluster analysis for the blind docking yielded four structural conformations with the lowest binding energy (Supplementary Figure 2). The first cluster consisted of three conformational members, with the lowest mean binding energy of −23.86 kJ mol<sup>−1</sup>. The three members of this cluster had the lowest binding energy of −28.05, −22.64, and −20.87 kJ mol<sup>−1</sup>, respectively. The second cluster consisted of only one member, which had the lowest binding energy of −24.89 kJ mol<sup>−1</sup>. The locations of these four conformations are shown in the Supplementary Figures 3–6. As can be seen from these figures, the locations of the highest-ranked structures in the blind docking were indeed not placed in the well-defined drug-binding sites, i.e. Sudlow’s site I or site II. Since the drug concentration in the blood plasma is very low compared to HSA concentration, the drug is more likely to bind at the specific binding sites. Site marker-induced VDB displacement results (Section 3.3.1) suggested Sudlow’s site I (subdomain IIA) as the preferred binding site of VDB on HSA. Therefore, we selected site-specific areas (Sudlow’s site IIA and site II of HSA) for docking studies to confirm the experimental results. The ligand (VDB) was constructed, geometrically optimized, and docked to the X-ray structure of HSA with high-resolution (PDB code 1BM0) to predict the binding mode of VDB for the two main drug-binding sites I and II (Sudlow’s nomenclature; at subdomains IIA and IIA of
HSA, respectively) (Sudlow et al., 1975). Cluster analysis for the binding site I using RMSD tolerance of 2.0 Å yielded a total of 10 multimember conformational clusters from 100 docking runs with the lowest mean binding energy of $-36.11 \text{kJ mol}^{-1}$. The highest populated cluster had 27 out of 100 conformations. However, the conformation with the lowest binding energy ($-37.24 \text{kJ mol}^{-1}$) was not a member of the highest populated cluster (Figure 7). On the other hand, at the binding site II, 12 multimember conformational clusters, possessing the lowest mean binding energy of $-23.53 \text{kJ mol}^{-1}$ were identified, with the highest populated cluster possessing 26 members out of 100 conformations (Figure 7). As the docking energy of the most favorable docking conformation in site II was higher than the one in site I, it suggested the binding preference of VDB for the drug-binding site I (subdomain IIA) of HSA.

The results of these docking studies were in good agreement with the displacement experiments discussed above. The predicted binding model with the lowest docking energy ($-37.24 \text{kJ mol}^{-1}$) was then used for binding orientation analysis (Figure 8). The binding site (defined as amino acids within a 5 Å distance to the ligand) was found to be deep inside the protein structure and mostly located in a hydrophobic cleft lined by the following amino acid residues: Tyr-150, Glu-153, Lys-195, Gln-196, Leu-198, Lys-199, Trp-214, Arg-218, Leu-219, Arg-222, Leu-238, His-242, Arg-257, Leu-260, Ala-261, Ile-264, Ser-287, His-288, Ile-290, Ala-291, Glu-292, Val-343, Asp-451, Ser-454, and Val-455. Presence of hydrophobic amino acid residues at the binding site of HSA might have contributed toward the stability of the VDB–HSA complex through hydrophobic interactions, as illustrated in red in the LigPlot+ diagram shown in Figure 9. Several reports have shown the involvement of hydrophobic interactions and hydrogen bonding in the stabilization of the drug–serum albumin complexes based on LigPlot+ diagram (Ebrahimi et al., 2015; Taghavi et al., 2014; Yeggoni et al., 2015). As can also be seen from the figure, VDB orientates itself at site I such that its electronegative atoms (fluorine and bromine) are facing the Trp-214 residue of HSA and thus create a more polar environment around the Trp residue. However, presence of several polar amino acid residues within the proximity of the bound ligand indicated that the interaction between VDB and HSA at site I (Figure 9) cannot be exclusively hydrophobic. Furthermore, in the VDB–HSA complex docking conformation at the site I, one
Table 3. $K_a$ values of VDB–HSA interaction in the absence and the presence of different metal ions (30 μM) at 25°C, pH 7.4.

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>$K_a$ ($\times 10^3$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>8.09 ± 0.15</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>7.87 ± 0.21</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>7.36 ± 0.20</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>7.27 ± 0.07</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>7.22 ± 0.16</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>6.82 ± 0.03</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>9.00 ± 0.15</td>
</tr>
</tbody>
</table>

hydrogen bond was predicted involving the hydrogen atom of Arg-257 and the ethereal oxygen atom of VDB. Thus, results obtained from the docking analysis also suggested that both hydrophobic interactions and hydrogen bonding collectively contribute toward VDB–HSA complex formation. These results were in line with our thermodynamic analysis of the binding reaction, suggesting involvement of both hydrophobic interactions and hydrogen bonding in VDB–HSA complex formation.

3.4. Influence of metal ions on VDB–HSA interaction

Metal ions are vital for various biochemical processes and some of these are present in low concentrations in blood plasma (Guidotti, McNamara, & Moses, 2008). The presence of some common metal ions such as Ca$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ on VDB–HSA interaction was investigated at pH 7.4, 25°C by determining the binding constant ($K_a$) of VDB–HSA complex in the absence and presence of these metal ions. As can be seen from Table 3, the $K_a$ value of VDB–HSA complex showed smaller but significant decrease in the presence of Mg$^{2+}$, Zn$^{2+}$, Ba$^{2+}$, and Cu$^{2+}$, while a smaller but significant increase in $K_a$ was noticed in the presence of Mn$^{2+}$. These results suggested metal ions interference with VDB–HSA complex formation. Such interference may shorten or prolong the storage time of drug in blood plasma that could weaken or enhance the effectiveness of the drug (Cheng et al., 2013; Cui, Fan, Li, & Hu, 2004). Therefore, presence of metal ions in blood plasma could affect the pharmacokinetics of VDB in blood circulation.

4. Conclusions

In summary, VDB quenched the intrinsic fluorescence of HSA through static quenching mechanism, thus suggesting formation of VDB–HSA complex. Thermodynamic and molecular docking data suggested the involvement of both hydrophobic interactions and hydrogen bonding in VDB–HSA complex formation at site I of HSA, located in subdomain IIA. VDB-induced alteration in both secondary and tertiary structures of HSA was evident from the far-UV and the near-UV CD spectral results. Metal ions were found to affect the binding affinity of VDB–HSA to a smaller extent.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2015.1089187

Acknowledgments

The financial assistance from the University of Malaya to Md. Zahirul Kabir in the form of doctoral fellowship under the Bright Sparks Program (BSP/APP/1892/2013) is highly appreciated. We thank the Dean, Faculty of Science and the Head, Institute of Biological Sciences, University of Malaya for providing the necessary facilities.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Ministry of Education, Government of Malaysia [grant number UM.C/625/1/HIR/MOE/SE/02] and the University of Malaya [grant number BSP/APP/1892/2013].

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