Binding of the alkaloid aristololactam-β-D-glucoside and daunomycin to human hemoglobin: spectroscopy and calorimetry studies

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The interaction of the plant alkaloid aristololactam-β-D-glucoside (ADG) and the anticancer agent daunomycin (DAN) with human hemoglobin was studied by different spectroscopic and calorimetric methods. The binding affinity values of ADG and DAN, estimated from spectroscopic experiments, were $3.79 \times 10^4$ and $6.68 \times 10^4$ M$^{-1}$, respectively. From circular dichroism, 3D fluorescence, and FTIR studies it was observed that, DAN induced stronger conformational changes than ADG in the protein. From synchronous fluorescence spectroscopy results, a pronounced shift in the maximum emission wavelength of tyrosine residues was observed in both cases suggesting that the drugs changed the polarity around tyrosine residues with marginal change around the tryptophan residues. The thermodynamics of the binding interaction analyzed using microcalorimetry presented single binding events that were exothermic in nature in both cases. Negative heat capacity changes in both cases are correlated to the involvement of significant hydrophobic forces in the complexation process. The affinity of DAN to Hb was higher than that of ADG.

Keywords: aristololactam-β-D-glucoside; daunomycin; human hemoglobin; spectroscopy; thermodynamics

Introduction

Proteins play a fundamental role in living cells, and targeting them by small molecules may provide information about the pharmacodynamics, pharmacokinetics, toxicity, etc. The majority of bioactive small molecules of interest manifest their selective biological effects through binding to and interfering with the functions of proteins. Therefore, detection and target identification of the interactions between small molecules and proteins are of vital interest to contemporary chemical biologists. Hemoglobin (Hb) is the major respiratory blood protein, which efficiently carries oxygen from the lungs to the tissues of the body. Structurally, Hb is a tetramer composed of four globin molecules, two identical α globins, and two identical β globins non-covalently bound among themselves. The α and β globin chains are composed of 141 and 146 amino acids residues, respectively (Perutz, 1963, 1978). Both chains exhibit almost similar secondary and tertiary structural features. Each globin chain is bound to a non-protein heme group composed of a porphyrin ring, which contains four pyrrole molecules cyclically linked together, and an iron–ion ligand bound in the center (Perutz, 1963, 1978). Apart from the transport of oxygen from lungs to the rest of the body, Hb also plays an important role in maintaining the pH of the blood (Berg, Tymoczko, & Stryer, 2002; Bunn & Forget, 1986; Edsall, 1986; Epstein & Hsia, 1998). As Hb is an important functional protein of the blood and can bind with a number of exogenous and endogenous molecules (Jang, Liu, Chen, & Zou, 2009; Mandal, Kalke, & Li, 2004; Messori et al., 2006; Shanmugaraj, Anandakumar, & Ilanchelian, 2015; Wang, Zhang, & Zhou, 2009) it may be a potential target for drugs and other therapeutic small molecules for delivering to required physiological sites. In this context, it needs to be mentioned that a large number of studies on the interaction of bioactive compounds with the most abundant plasma protein serum albumin are recently undertaken as it is the first step of new drug design (Chakrabarty, Mallick, Haldar, Das, & Chattopadhyay, 2007; Chamani, Vahedian-Movahed, & Saberi, 2011; Iranfar, Rajabi, Salari, & Chamani, 2012; Kuznetsova, Sulatskaya, Povarova, & Turoverov, 2012; Perry et al., 2003; Sarzehi & Chamani, 2010; Sattar et al., 2012; Zhang et al., 2007). Like serum albumin studies of small molecules binding with Hb also have significance in chemistry, life sciences, and clinical medicine.

Aristololactam-β-D-glucoside (Figure 1(A)) and daunomycin (Figure 1(B)) are two sugar containing natural compounds possessing potential drug value. Aristololactam-β-D-glucoside, isolated from the plant

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**Aristolochia indica**, has clinical importance as antitumor agent (Balachandran, Wei, Lin, Khan, & Pasco, 2005; Cassady, Baird, & Chang, 1990; Chen & Zhu, 1987). ADG has proven DNA binding property intercalating preferentially with the guanine–cytosine base pairs (Chakraborty, Nandi, & Maiti, 1990; Chakraborty et al., 1989; Nandi, Chakraborty, & Maiti, 1991). On the other hand, daunomycin, the anthracycline derivative originally isolated from *Streptomyces peucetius*, is a potent anticancer agent used in clinical cancer therapy to treat acute myelogenous leukemia and acute lymphoblastic leukemia (Crooke & Reich, 1980). It is also a well-known DNA intercalator (Chaires, 1990, 1997; Chaires, Fox, Herrera, Britt, & Waring, 1987; Gianni, Corden, & Meyer, 1983; Marco & Arcamone, 1975; Rabbani, Abdosamadi, & Sari-Saraf, 2007; Weiss, 1992; Zunino, Gambetta, Di Marco, & Zaccara, 1972). Apart from the common DNA binding property both molecules have significant binding affinity toward various RNA molecules such as poly(A), tRNA, single stranded RNAs, and double stranded RNAs, which has been extensively studied recently (Das, Bhadra, Achari, Chakraborty, & Kumar, 2011; Das, Bhadra, & Suresh Kumar, 2013; Das & Suresh Kumar, 2012). However, their binding to protein molecules has not been well studied. Our recent studies have revealed that both DAN and ADG bind to human serum albumin though the affinity was found to be much lower than those to nucleic acids (Das & Kumar, 2014). But the binding of both compounds induced strong structural perturbation in serum albumins. Except to HSA, ADG is not known to bind to any other proteins. Here, we have studied the interaction of ADG and DAN with human hemoglobin to understand the structural and thermodynamics aspects of the binding. The results may be helpful to determine the pharmaceutical effectiveness of ADG in comparison to DAN.

**Experimental**

**Apparatus**

Absorbance spectral measurements were performed on a Jasco V660 spectrophotometer (Jasco International Co., Ltd., Hachioji, Japan) equipped with a Peltier controlled device. For steady state fluorescence measurements Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) was used. Temperature dependent fluorescence spectral studies were conducted on a Hitachi F4010 unit (Hitachi Ltd., Tokyo, Japan) equipped with a Lab Companion water bath (model RW-2025G) for controlling the sample temperature. The temperature was monitored by the electronic devise Sensortek, model BAT-12 (Sensortek Inc., NJ, USA). Three-dimensional (3D) fluorescence spectroscopy experiments were performed on a PerkinElmer LS55 fluorescence spectrometer (PerkinElmer Inc., USA). Circular dichroism spectroscopy was performed in a Jasco J815 spectropolarimeter (Jasco International Co., Ltd.). A VP-ITC unit (MicroCal LLC, Northampton, MA, USA) was used for calorimetry studies.

**Materials**

Human methemoglobin (Cat No. H 7379, M = 64,500 Da) and daunomycin hydrochloride (>90% pure) were purchased from Sigma–Aldrich LLC (St. Louis, MO, USA). Aristololactam-β-D-glucoside was extracted from *Aristolochia indica* as reported earlier, and its purity was checked by various analytical techniques (Achari, Bandyopadhyay, Chakravarty, & Pakrashi, 1984; Pakrashi, Ghosh-Dastidar, Basu, & Achari, 1977). The stock solution of ADG was prepared in dimethyl sulfoxide (DMSO). The concentration of hemoglobin was determined using molar absorption coefficient values of 179 mM$^{-1}$ cm$^{-1}$ at 405 nm (Antonini & Brunori, 1971). ADG and DAN (drugs in general hereafter) concentrations were determined using molar absorption coefficients of 10,930 M$^{-1}$ cm$^{-1}$ at 398 nm for ADG and 11,500 M$^{-1}$ cm$^{-1}$ at 480 nm for DAN, respectively (Das & Kumar, 2013). No deviation from Beer’s law was observed for the drugs in the concentration range used in this study. Deionised and triple distilled water was used for preparing the buffer solution. All experiments were conducted in citrate–phosphate (CP) buffer (10 mM [Na$^+$]) of pH 7.2, containing 5 mM...
Na₂HPO₄. Additional 240 mM DMSO was present in the buffer in experiments with ADG. The pH was adjusted using citric acid. pH measurements were made on a Sartorius PB-11 high precision bench pH meter (Sartorius GmbH, Germany) with an accuracy ± 0.01. All chemicals and reagents used were of analytical grade and obtained from Sigma–Aldrich. The buffer solution was filtered through millipore filters (Millipore India Pvt. Ltd, Bangalore, India) of 0.22 μm pore size.

**Methods**

**UV–vis absorption spectroscopy**

Absorbance spectral measurements were performed at 298.15 ± 0.5 K. For titration, matched quartz cuvettes (Hellma, Germany) of 1 cm path length were used. Briefly, a known concentration of the protein solution was kept in the sample cuvette and small aliquots of a known concentration of the drug solution were titrated into the sample and reference cuvettes. After each addition, the solution was thoroughly mixed and allowed to re-equilibrate for at least 10 min before noting the absorbance at the desired wavelength maxima. The absorbance data were analyzed by Benesi–Hildebrand plots to determine the equilibrium constant using the equation

\[
\frac{1}{\Delta A} = \frac{1}{\Delta A_{\text{max}}} + \frac{1}{K_{\text{BH}}(\Delta A_{\text{max}})} \times \frac{1}{[M]}
\]

where \(K_{\text{BH}}\) is the binding constant and \([M]\) is the concentration of drugs (Benesi & Hildebrand, 1949).

**Fluorescence spectroscopy**

All steady state fluorescence measurements were performed keeping excitation and emission band passes of 5 nm. The sample temperature was maintained at 298.15 ± 0.5 K. For measurement of intrinsic fluorescence of Hb in the presence of drugs the protein sample was excited at 295 nm, the excitation maximum of tryptophan, and emission spectra were scanned from 300 to 400 nm. The fluorescence emission intensity of ADG and DAN were monitored at 483 and 556 nm, when excited around 400 and 480 nms, respectively. Temperature dependent fluorescence spectral studies were conducted at three different temperatures viz., 288.15, 298.15 and 308.12 K respectively.

The synchronous and asynchronous fluorescence spectra were obtained through the simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval (Δλ) between them. Synchronous fluorescence spectra were measured in the excitation range of 220–380 nm keeping Δλ set at 15 and 60 nm, respectively.

The fluorescence emission intensities of Hb, and Hb–complexes were corrected for inner filter effect due to the strong absorption of heme protein and both drugs in the UV–vis region. The correction for the inner filter effect was performed as described by MacDonald, Lvin, and Patterson (1997) using the relation

\[
F_{\text{ideal}} = F_{\text{obs}} \times CF_p \times CF_s
\]

as described previously by Hazra and Suresh Kumar (2014). In Equation (2), \(F_{\text{ideal}}\) and \(F_{\text{obs}}\) are the ideal and observed fluorescence intensities, and \(CF_p\) and \(CF_s\) are the correction factors for the primary and secondary inner filter effects, respectively. These primary and secondary correction factors were modified for the standard one cm square cuvette as \(CF_p \times CF_s \approx 10^{(A_{\text{ex}}+A_{\text{em}})/2}\) where \(A_{\text{ex}}\) and \(A_{\text{em}}\) are the values of absorbance of the solution at the excitation and emission wavelengths (fluorophore and other absorbers). The actual fluorescence emission intensity of the Hb-drug complexes was obtained by multiplying this correction factor and the observed fluorescence intensity.

**Three-dimensional spectroscopy**

Three-dimensional (3D) fluorescence spectroscopy experiments were performed at 298.15 ± 0.5 K. The fluorescence emission spectrum of Hb was measured in the 270–500 nm range. The excitation wavelength was initially kept at 200 nm, and 15 scans were performed up to 340 nm in increments of 10 nm.

**Circular dichroism spectroscopy**

Secondary and tertiary structural changes in Hb on interaction with ADG and DAN were monitored by circular dichroism spectroscopy. Cuvettes of path length 0.1 and 1 cm, respectively, were used for far UV CD and Soret band CD spectral measurements. A Peltier cell holder and temperature controller PFD 425 L/15 maintained the cuvette temperature at 298.15 ± 0.5 K. A scan speed of 20 nm/min, bandwidth of 1.0 nm and sensitivity of 100 milli degrees were applied. Five successive scans were performed and averaged to improve the signal-to-noise ratio and the spectra were smoothed within permissible limits by the software. The molar ellipticity values were expressed in terms of the mean residue molar ellipticity [θ], in units of deg cm² dmol⁻¹.

**Infrared spectroscopy studies**

In infrared spectral studies for each spectrum 512 scans were performed at a resolution of 4 cm⁻¹. The spectra were measured in triplicate (three individual samples of the same Hb and drug concentrations).

**Isothermal titration calorimetry**

For isothermal titration calorimetry experiments Hb and drug solutions were degassed on the MicroCal’s
Absorption spectral study

The characteristic absorption spectrum of Hb shows two major peaks in the 190–500 nm region with maxima at 195 nm and 405 nm, respectively (Curve 1, Figure 2(A)). The Soret peak at 405 nm was used to monitor the change in the absorbance spectrum of Hb in the presence of ADG and DAN. Typical absorbance spectral changes in the Hb spectrum in the presence of ADG and DAN are presented in Figure 2(A) and (B). A gradual decrease in the absorption band was observed in the presence of increasing concentration of both ADG and DAN, which suggests the formation of a complex of both the molecules with Hb. The hypochromic effect in the band was more pronounced in the case of DAN. The absorbance data were analyzed by Benesi–Hildebrand plots (Figure 2(C) and (D)) (Benesi & Hildebrand, 1949) and the binding constants evaluated yielded values of (3.79 ± 0.52) × 10^4 and (6.68 ± 0.28) × 10^4 M^-1 for ADG and DAN, respectively.

Fluorescence spectral study

The intrinsic fluorescence property of proteins is mainly due to the tryptophan, tyrosine, and phenylalanine residues and is highly sensitive to the local environment. Hb has six tryptophan residues; each αβ dimers contains three Trp residues as α-Trp14, β-Trp15, and β-Trp37 (Venkateshrao & Manoharan, 2004). There are also five tyrosine residues in each αβ dimer as α-Tyr24, α-Tyr42, α-Tyr140, β-Tyr34, and β-Tyr144 (Mueser, Rogers, & Arnone, 2000). It is known that the primary fluorescence emission of Hb essentially originates from the Trp-37 residue located at the αβ2 interface of the protein (Alpert, Jameson, & Weber, 1980; Burstein, Vedenkina, & Ivkova, 1973). Thus, the Trp can be used as an intrinsic fluorophore to study the quaternary state change of the protein upon binding with ligands. Hb exhibits a fluorescence emission band at 329 nm on excitation at 295 nm. When a fixed concentration of Hb was titrated with different amounts of ADG and DAN, fluorescence intensity was found to quench gradually (Figure 3(A) and (B)) till saturation was achieved. ADG binding decreased the fluorescence of Hb by 67% at saturation and the emission maximum (329 nm) was blue shifted to 327 nm. On the other hand, DAN induced significantly higher quenching of the fluorescence of Hb (88% at saturation) with concomitant red shift of the emission maximum to 333 nm. The observed fluorescence quenching in both cases revealed the complexation between the drugs and Hb. The blue shift in fluorescence maximum indicates the displacement of the Trp residues to a less polar environment in the presence of ADG. The red shift on binding of DAN suggested a microenvironmental change and decrease in hydrophobicity around the Trp residues.

The effect of Hb on the fluorescence of ADG and DAN was also studied and the results are presented in Figure 3(C) and (D). Quenching of the fluorescence intensity of both drugs was observed confirming their binding to Hb.

In order to determine the fluorescence quenching mechanism, quenching experiments were performed at three different temperatures viz., 288.15, 298.15 and 308.15 K and the data were analyzed by the classical Stern–Volmer equation (Lakowicz, 1983) given below

\[
F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q]
\]

where \(F_0\) and \(F\) denote the fluorescence intensities at the emission maxima of Hb alone and in the presence of the drugs, \(K_q\) is the apparent bimolecular quenching rate constant, \(K_{sv}\) is the Stern–Volmer quenching constant, \(\tau_0\) is the average fluorescence lifetime of free Hb (usually taken to be 10^8 s^-1), and \([Q]\) is the quencher concentration (Lakowicz, 1983). The values of \(K_q\) and \(K_{sv}\) at three different temperatures are depicted in Table 1. From the

Results and discussions

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temperature-dependent fluorescence study it was observed that the $K_{sv}$ and $K_q$ values for both ADG–Hb and DAN–Hb complexation decreased with increasing temperature (Figure 4(A) and (B)) and the magnitudes of the $k_q$ values obtained by analysis using the classical Stern–Volmer equation were greater than $2.0 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$. This observation suggests that the quenching followed a static mechanism due to complex formation and dynamic collision effects, if any, may be negligible.
In the case of small molecules that bind independently to a set of equivalent sites on the protein, the apparent binding constant and the number of binding sites can be determined from the following equation (Abou-Zied & Al-Shihi, 2008; Khan, Hossain, & Suresh Kumar, 2012)

\[
\log \left( \frac{F_o - F}{F} \right) = \log K_a + n \log [Q]
\]

(6)

Here \( K_a \) is the binding constant to a site and \( n \) is the number of binding sites per protein. The binding affinity values of ADG and DAN complexing with Hb obtained from the linear plots of \( \log (F_o - F/F) \) vs. \( \log [Q] \) at the three temperatures (not shown) are presented in Table 1.

The fluorescence quenching data were further analyzed by the double reciprocal equation (Cui, Fan, Li, & Hu, 2004; Zhang, Huang, Mei, Li, & Yan, 2006) as given below

\[
\frac{1}{(F_o - F)} = \frac{1}{F_o} + \frac{1}{K_{DR} F_o [Q]}
\]

(7)

Here static quenching constants (\( K_{DR} \)) were obtained from the ratio of the intercept to the slope of the double reciprocal plot, describing the efficiency of quenching at the ground state. The data are presented in Table 1.

**Synchronous fluorescence study**

Synchronous fluorescence technique is an important method to study the conformational changes occurring in a protein on binding of a ligand (Lloyd, 1971; Lloyd & Evett, 1977). When the scanning intervals between the excitation and emissions wavelength are fixed at 15 and 60 nm, respectively, synchronous fluorescence of the protein is the characteristic of Tyr and Trp residues and the quenching caused by the ligand then implies alteration of the polarity around these amino acid residues. The effect of ADG and DAN on the synchronous fluorescence of Hb with \( \Delta \lambda = 60 \text{nm} \) revealed that the fluorescence intensity diminished systematically with a red shift of the emission maximum by 2–3 nm for both drugs (Figure 5(A) and (C)), whereas, a higher red shift of about 6 nm for ADG and 10 nm for DAN occurred with simultaneous decrease in fluorescence intensity when \( \Delta \lambda \) was fixed at 15 nm (Figure 5(B) and (D)). The significant bathochromic shift in the latter cases is indicative of a decrease in hydrophobicity around Tyr residues. The complexation of ADG and DAN with Hb may lead to the exposure of Tyr residues buried inside the nonpolar hydrophobic cavities to a more hydrophilic environment. This observation also suggests that the microenvironment around the Trp residues was less significantly changed. The results imply the possibility of greater participation of the Tyr residues of Hb in the complexation process. In this context, it is noteworthy to mention that several small molecules such as paraquat, berberine, sanguinarine have been reported to bind preferably close to the Trp residues and induce a polarity change around the \( \beta \)-Trp37 residue of Hb (Hazra, Hossain, & Suresh Kumar, 2013; Hazra & Suresh Kumar, 2014; Wang et al., 2007). On the other hand the binding of the cationic phenothiazinium dye thionine and the broad spectrum antibiotic oxytetracycline with bovine Hb was reported to cause changes in the microenvironment around the Tyr residues (Chi, Liu, Yang, & Zhang, 2010; Shanmugaraj, Anandakumar, & Ilanchelian, 2014). Similarly, both the drugs under study here alter the microenvironment around the Tyr residues leading to their exposure to a more hydrophilic environment.

**Three-dimensional fluorescence study**

3D fluorescence is another useful tool to study the conformational and microenvironmental changes in the protein on ligand binding. The representative 3D fluorescence spectra and the contour maps of Hb in the absence and presence of ADG and DAN are presented in Figure S1 and the corresponding parameters are listed in Table 2. In the figure, the peaks ‘a’ and ‘b’ arise due to first-order Rayleigh scattering (\( \lambda_{ex} = \lambda_{em} \)) and second-order Rayleigh scattering peak (\( \lambda_{ex} = 2 \lambda_{em} \)), respectively. The binding of ADG and DAN leads to decrease in the fluorescence intensity of Hb at peak ‘a’ because the scattering effect was weakened by formation of the complex. Peak 1 (\( \lambda_{ex} = 280 \text{nm} \) and \( \lambda_{em} = 334 \text{nm} \)) is the characteristic fluorescence spectral behavior of Trp residues.

| Drug | Temperature (K) | \( K_{sv} \times 10^{-3}/\text{M}^{-1} \) | \( K_{q} \times 10^{-12}/\text{M}^{-1} \text{s}^{-1} \) | \( K_{b} \times 10^{-4}/\text{M}^{-1} \) | \( K_{DR} \times 10^{-4}/\text{M}^{-1} \) | \( n \) |
|------|----------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|------|
| ADG  | 288.15         | 1.60 ± 0.09                     | 1.60 ± 0.09                     | 3.62 ± 0.20                     | 3.54 ± 0.22                     | 1.05 ± 0.05 |
|      | 298.15         | 1.41 ± 0.05                     | 1.41 ± 0.05                     | 3.16 ± 0.08                     | 3.05 ± 0.10                     | 1.09 ± 0.10 |
|      | 308.15         | 1.11 ± 0.05                     | 1.11 ± 0.05                     | 2.90 ± 0.05                     | 2.86 ± 0.05                     | 1.12 ± 0.08 |
|      | 288.15         | 2.16 ± 0.15                     | 2.16 ± 0.15                     | 5.52 ± 0.15                     | 5.50 ± 0.21                     | 1.01 ± 0.10 |
|      | 298.15         | 1.70 ± 0.18                     | 1.70 ± 0.18                     | 5.01 ± 0.10                     | 4.80 ± 0.15                     | 1.04 ± 0.05 |
|      | 308.15         | 1.36 ± 0.08                     | 1.36 ± 0.08                     | 4.68 ± 0.10                     | 4.04 ± 0.14                     | 1.07 ± 0.05 |

*The data presented are averages of four determinations. \( K_{sv} \) is the Stern–Volmer quenching constants, \( k_q \) is the apparent bimolecular quenching rate constant, \( K_{qav} \) is the static quenching constant from double reciprocal plot analysis, and \( K_b \) is the binding constant and \( n \) is the number of binding sites.*
It was observed that on binding with ADG the fluorescence intensity of peak 1 of Hb was decreased and the Stokes shift was increased by ~2 nm. In DAN–Hb complexation similar decrease in emission intensity of peak 1 with an increase in Stokes shift of ~4 nm was observed. The increase in Stokes shift in both the cases suggests a change in polarity with concomitant decrease in hydrophobicity around the Trp residue. Peak 2,

| System      | Peaks | Peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm) | Stokes shift | Intensity ($F$) |
|-------------|-------|-----------------------------------------------|--------------|-----------------|
| Hb          | Peak 1| 280/334                                      | 54           | 115.92          |
|             | Peak 2| 230/334                                      | 104          | 113.42          |
| Hb + ADG    | Peak 1| 280/336                                      | 56           | 63.95           |
|             | Peak 2| 230/335                                      | 105          | 37.50           |
| Hb + DAN    | Peak 1| 280/338                                      | 58           | 43.20           |
|             | Peak 2| 230/337.5                                    | 107.5        | 30.92           |

Figure 4. Stern–Volmer plots of (A) ADG and (B) DAN at 288.15, 298.15, and 308.15 K, respectively.

Figure 5. Synchronous fluorescence ($\Delta \lambda = 60$ and 15 nm) spectra of Hb (5 µM) in presence of different concentrations of ADG (A, B) and DAN (C, D). Panel (A) curves (1–10) denote 0, 10, 25, 50, 75, 100, 125, 150, 175, and 200 µM of ADG, Panel (B) curves (1–10) denote 0, 10, 25, 50, 75, 100, 125, 150, 175, and 200 µM of ADG, Panel (C) curves (1–10) denote 0, 10, 20, 30, 50, 70, 90, 110, 130, and 170 µM of DAN and Panel (D) curves (1–10) denote 0, 10, 20, 30, 50, 70, 90, 110, 130, 160 µM of DAN.
another strong fluorescence peak ($\lambda_{ex} = 230$ nm and $\lambda_{em} = 334$ nm) was obtained due to the $\pi-\pi^*$ transition of the polypeptide backbone of the protein. Fluorescence intensity of peak 2 also decreased significantly on binding of ADG and DAN with the protein. The results showed that, in both the cases, the 3D fluorescence contour map of native Hb was clearly different from that of drug bound Hb. Further, the fluorescence intensity of the peaks 1 and 2 obviously decreased with different degree in the presence of ADG and DAN, which is summarized in Table 2. The increase in Stokes shift along with the decrease in emission intensity of the peaks 1 and 2 is ascribed to the changes in the microenvironment of Trp or Tyr residues and ADG and DAN induced conformational changes in the secondary structure of Hb, which is consistent with the static and synchronous fluorescence data. Overall the results from 3D fluorescence emission here suggest the occurrence of conformational changes in the polypeptide chain, due to slight unfolding, leading to the exposure of some hydrophobic regions of the protein which is in full agreement with the absorption spectra, static quenching, synchronous fluorescence data, and CD spectral data.

Circular dichroism study

Circular dichroism spectroscopy is a quantitative technique to investigate the drug-induced conformational changes in proteins. The far ultraviolet CD spectrum of native Hb contained two negative minima at 208 and 222 nm, respectively, which are characteristic of the $\alpha$-helical structure (curve 1 of Figure 6(A)). The 208 nm band corresponds to $\pi-\pi^*$ transition of the $\alpha$-helix, and the 222 nm band is due to $n-\pi^*$ transition for both the $\alpha$-helix and random coil conformations (Woody, 1995, 1996). From the far UV CD spectral data, the $\alpha$-helical content of Hb was calculated according to the equation given in the literature (Hazra et al., 2013) and the value obtained was 39%, which was in full agreement with the previous reports (Chen, Yang, & Martinez, 1972; Woody, 1995, 1996). As DAN was titrated to the Hb sample, the molar ellipticity of Hb displayed a gradual reduction with the occurrence of a sharp isodichroic point at 203 nm (Figure 6(A)). The existence of the isodichroic point indicates a local two-state (alpha-helix, random coil) population. The mean residue ellipticity at the isodichroic point, of course, independent of helix content which may represent equilibrium among the native and DAN bound conformation of Hb. Complexation of Hb with DAN leads to a reduction of the $\alpha$-helical content from 39 to 5%. Therefore, DAN-induced unfolding and loss of a large part of the helical stability of Hb resulting in extended polypeptide chains exposing the hydrophobic cavities with concomitant exposure of the aromatic amino acid residues. The far-UV spectral changes of Hb in the presence of ADG could not be recorded because of the presence of DMSO in the buffer that absorbs in the 250–200 nm region. The Soret band CD spectrum of Hb (curve 1 of Figure 6(B)) has one positive maximum centered at 413 nm and a negative minimum around 395 nm (Chen et al., 1972). The positive peak was affected in the presence of both drugs. The molar ellipticity of Hb at 413 nm was 85,045 deg cm$^2$ dmol$^{-1}$, which reduced by 9% in the presence of ADG (Figure 6(B)) and by 13% in the presence of DAN (Figure 6(C)). This result implicates the interference of both ADG and DAN with the secondary structure and results in conformational changes around the heme part of Hb subunit.

Fourier transform infrared spectral study

Fourier transform infrared (FTIR) has been widely applied for qualitative determination of the average
secondary structure of proteins (Liu, Shaw, Man, Demibinski, & Mantsch, 2002; Shaw, Kotowich, Leroux, & Mantsch, 1998). The most intense absorption band is the amide I band (1700–1600 cm\(^{-1}\)), associated with the C=O and C–N stretching vibrations of the peptide linkage related to the backbone conformation (Mahato et al., 2010; Shang, Wang, Jiang, & Dong, 2007). For ADG–Hb complexation, the amide I band of Hb shifted from 1651 cm\(^{-1}\) to 1646 cm\(^{-1}\) and for DAN–Hb complexation to 1640 cm\(^{-1}\) (Figure 7). The addition of both the drugs results in the shifting of the amide-I band toward lower wave number, which indicates the microenvironmental changes around the peptide linkage probably caused by hydrogen bonding, hydrophobic, and hydrophilic interactions. The complexation process may induce conformational change of Hb from more helical structure to slightly unfolded structures, and thus the changes in associated forces involved in peptide backbone conformation has been reflected from the shifting of amide-I band.

**Hydrophobic probe ANS displacement assay**

The fluorescent dye 8-anilino-1-naphthalenesulfonic acid (ANS) was used to characterize the preferred binding region of ADG and DAN on Hb. ANS is known to bind to the hydrophobic region of the central cavity of Hb (Syakhovich, Parul, Ruta, Bushuk, & Bokut, 2004). Both the drugs were separately added to Hb solution and a mixture of ANS–Hb in 1 : 1 and 5 : 1 ratios. The relative fluorescence intensity \(F/F_o\), where \(F\) and \(F_o\) are the fluorescence intensity of Hb in the presence and absence of the drugs vs. concentration of drugs were plotted (Figure S2). The result revealed that in comparison to ADG, DAN can more effectively displace the bound ANS from the central cavity of the protein. The binding of ADG only marginally affected the ANS–Hb complex formation. This result suggested that DAN has greater affinity to the hydrophobic region of the Hb central cavity compared to ADG. In this context the structurally similar doxorubicin has been suggested to interact in the proximity of the central oxygen binding pocket of Hb (Khan et al., 2008).
Table 3. Temperature-dependent isothermal titration calorimetry data for the binding of ADG and DAN to Hb. a

| Drug | \( T \) (K) | \( K \times 10^2 \text{M}^{-1} \) | \( \Delta H^o \) kcal/mol | \( T\Delta S^o \) kcal/mol | \( \Delta G^o \) kcal/mol | \( \Delta C_p^o \) cal/mol/°C |
|------|-------------|----------------|----------------|----------------|----------------|----------------|
| ADG  | 288.15      | 4.18 ± 0.05    | −0.45 ± 0.01   | 5.64 ± 0.05   | −6.09 ± 0.04  | −21.0 ± 0.52  |
|      | 298.15      | 3.46 ± 0.08    | −0.51 ± 0.02   | 5.66 ± 0.04   | −6.23 ± 0.08  | −21.0 ± 0.52  |
|      | 308.15      | 2.75 ± 0.04    | −0.88 ± 0.02   | 5.33 ± 0.08   | −6.21 ± 0.05  | −21.0 ± 0.52  |
| DAN  | 288.15      | 6.80 ± 0.10    | −1.05 ± 0.01   | 5.45 ± 0.05   | −6.50 ± 0.04  | −21.0 ± 0.52  |
|      | 298.15      | 5.81 ± 0.08    | −1.66 ± 0.02   | 4.80 ± 0.05   | −6.46 ± 0.04  | −62.5 ± 1.05  |
|      | 308.15      | 4.25 ± 0.05    | −2.30 ± 0.01   | 4.03 ± 0.06   | −6.33 ± 0.02  | −21.0 ± 0.52  |

a All the data in this table are derived from ITC experiments conducted in CP buffer of 10 mM [Na⁺], pH 7.2, and are averages of four determinations. \( K \) and \( \Delta H^o \) values were determined from ITC profiles fitting to Origin 7 software as described in the text.

**Isothermal titration calorimetric study**

The binding of ADG and DAN to Hb was investigated by isothermal titration calorimetry (ITC) study to derive insights into the thermodynamics of the complexation. The ITC profiles of the binding of ADG and DAN to Hb presented in Figure 8(A) and (B) revealed the exothermic binding with one binding event in both cases. The thermodynamic parameters, standard molar Gibbs energy change (\( \Delta G^o \)), enthalpy of binding (\( \Delta H^o \)), entropy contribution (\( T\Delta S^o \)), binding affinity (\( K \)), and stoichiometry (\( N \)) were evaluated from the profiles using a model of “one set of binding sites” and these are presented in Table 3. The binding constant of ADG to Hb at 298.15 K was evaluated to be \((3.46 ± 0.08) \times 10^4 \text{M}^{-1}\) with a \( \Delta H^o \) value of \((-0.51 ± 0.02) \text{ kcal/mol}\), a \( T\Delta S^o \) value of \((5.66 ± 0.04) \text{ kcal/mol}\), and a \( \Delta G^o \) value of \((-6.23 ± 0.08) \text{ kcal/mol}\), respectively. The binding of ADG was thus essentially entropy driven with small favorable enthalpy contribution. The binding affinity of DAN, on the other hand, was higher with a \( K \) value of \((5.81 ± 0.08) \times 10^4 \text{M}^{-1}\). A \( \Delta H^o \) value of \(-1.66 ± 0.05 \text{ kcal/mol}\), \( T\Delta S^o \) of \(4.80 ± 0.05 \text{ kcal/mol}\), and \( \Delta G^o \) of \(-6.46 ± 0.04 \text{ kcal/mol}\), characterized the binding. The binding stoichiometry near unity, in both cases, confirmed the 1:1 binding of ADG and DAN to Hb.

Optical melting and DSC experiments showed that the thermal melting temperature of Hb was unaffected upon binding with ADG and DAN. From the data on the variation of the enthalpy with temperature obtained in ITC information on the standard molar heat capacity changes (\( \Delta C_p^o \)) of the binding reaction can be obtained (Chaires, 2008).

ITC experiments were performed at three different temperatures viz., 288.15, 298.15, and 308.15 K. It was observed that the binding affinity of ADG and DAN to Hb gradually decreased with increase in temperature. The heat capacity change deduced from the plot of the variation of \( \Delta H^o \) with temperature (Figure 9(A)) was around −21.0 and −62.5 cal/(mol K), respectively, for ADG and DAN–Hb complexation. This negative \( \Delta C_p^o \) value may be associated with changes in hydrophobicity during the interaction of ADG and DAN with Hb leading to the removal of water from hydrophobic interfaces and is indicative of the involvement of a strong hydrophobic component in the binding process. Strong enthalpy–entropy compensation was also observed making Gibbs energy of the binding nearly independent of the temperature (Figure 9(B)). The thermodynamic parameters at the temperatures studied for ADG and DAN complexing with Hb are depicted in Table 3.

Salt dependent ITC experiment was also performed to gather information about the nature of the forces involved in the complexation. The salt dependence of the binding studies was performed at three different [Na⁺] viz. 10, 20, and 50 mM Na⁺. The binding affinity values for both drugs decreased with increase in [Na⁺]. The results of the dependence of \( K \) on [Na⁺] were used to partition the \( \Delta G^o \) into the polyelectrolytic (\( \Delta G_{pe}^o \)) and the non-polyelectrolytic (\( \Delta G_{t}^o \)) contributions from the equations 
\[
\frac{\partial \ln(K)}{\partial \ln([\text{Na}^+]^+)} = -Z\phi, \Delta G_{pe}^o = -Z\phi R T \ln([\text{Na}^+]^+) \quad \text{and} \quad \Delta G^o = \left( \Delta G_{pe}^o + \Delta G_{t}^o \right)
\]
(Record, 1995).
Anderson, & Lohman, 1978). These parameters are presented in Table 4. At 10 mM [Na+] the $\Delta G^o$ values for ADG and DAN were $-6.23$ and $-6.54$ kcal/mol, respectively. On partitioning these Gibbs energy values, it was found that at 10 mM [Na+], only 12 and 14% of the $\Delta G^o$ were found to be from polyelectrolytic contribution for ADG–Hb and DAN–Hb complexations (Figure 10). As the salt concentration was increased the values of the polyelectrolytic contribution ($D G_{pe}$) decreased to about 8–9%, respectively, though non-polyelectrolytic contribution ($D G_t$) remained invariant, irrespective of the salt concentration (Figure 10). The major contribution to the binding Gibbs energy comes from non-polyelectrolytic forces. The results clearly suggest the dominant role of hydrophobic forces such as $\pi-\pi$ stacking, H-bonding, and van der Waals in the complexation of both ADG and DAN.

**Conclusions**

Both ADG and DAN can effectively interact with hemoglobin, but DAN has better affinity than ADG. The binding affinity values evaluated from the spectroscopic results were found to be of the order of $10^4$ M$^{-1}$ in both cases, but were higher for DAN than ADG. The quenching mechanism involved was of static type in both the cases and was due to the formation of complexes at the ground state. From circular dichroism, 3D fluorescence study, and FTIR results it was revealed that both ADG and DAN induced conformational changes in Hb. From synchronous fluorescence study, a pronounced shift in the maximum emission wavelength for Tyr residues was observed in both cases. The calorimetric study revealed exothermic binding of both ADG and DAN to Hb that was predominantly entropy driven. The binding appears to involve strong hydrophobic interactions along with small polyelectrolytic contribution. Overall, this study provides important biophysical insights into the differential binding affinity of ADG and DAN with hemoglobin. The results may be helpful in the pharmacological application of these molecules.

**Supplementary material**

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2015.1055304.

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