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Research Article

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Unravelling Interaction of an Anticancer drug Ifosfamide with Bovine Serum Albumin

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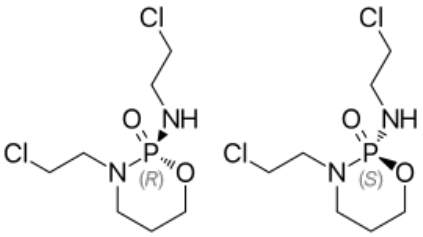
Abstract

The interaction between Ifosfamide(IFO) and bovine serum albumin (BSA) has been studied. The studies were carried out in a buffer of medium at pH 7.4 using fluorescence spectroscopy, UV-vis spectroscopy, and Viscometric methods. The results of fluorescence quenching and UV-vis absorption spectra experiments indicated the formation of the complex of BSA-IFO. Binding parameters were determined using the Stern-Volmer equation. From fluorescence and UV-vis spectroscopic data, the binding constant between IFO and BSA was calculated to be 4.275 x 10^3 L mol^{-1} and 8.173 x 10^3 L mol^{-1} respectively. The results of thermodynamic parameters ΔG°, ΔH° and ΔS° at different temperature indicate that the electrostatic interactions and also hydrogen bonds play a major role for IFO-BSA association.

General Drug Profile:

General name	N,3-bis(2-chloroethyl)-2-oxo-1,3,2$l^{5}-oxazaphosphinan-2-amine
Structure	
Molecular formula	$C_7H_{15}Cl_2N_2O_2P$
Molecular weight	261.083 g/mol
Melting point	39-41 °C
Description	Belongs to the group of medicines called alkylating agents
Solubility	Soluble in water
Category	Anticancer drug

Introduction

The drug–protein binding constant is a physicochemical parameter that help us to understand the absorption, transport, and the target molecules of the drugs at the cellular level[1,2]. Serum albumin is one of the main extracellular proteins, with a high concentration in blood plasma, present in 6.0 x 10^{-4}M, contributes to about 80 % of the blood osmotic pressure[3,4]. Bovine serum albumin (BSA) is homologous, having ~88 % sequence homology, with human serum albumin and is the major soluble protein component of the blood serum of cow. The remarkable binding properties of serum albumin account for the central role in both the efficacy and rate of delivery of drugs[5–7]. Therefore, the studies on the binding of drugs to serum albumin become an important research field in chemistry, life science and clinical medicine[8-10]. Plenty of studies on the interactions between serum albumin with internal compounds and pharmaceutical molecules have been carried out[11–13], and are considered to further broaden the perspective on the scientific research of drug in interdisciplinary fields.

Ifosfamide (IFO) is used in the treatment of a variety of paediatric tumours, especially sarcomas, and is usually combined with a number of different agents such as vincristine, actinomycin D or doxorubicin[14]. Although it is considered to be an analogue of cyclophosphamide, IFO appears to have specific activity in some tumour types, for example, rhabdomyosarcoma[15]. Like cyclophosphamide, IFO requires metabolic activation, mediated by cytochrome *P*450 enzymes[16], initially forming a 4-hydroxy metabolite, which spontaneously releases the active form – isophosphoramide mustard (IPM). Competing pathways for IFO metabolism result in inactive, dechloroethylated metabolites (2-dechloroethylifosfamide (2DCI) and 3-dechloroethylifosfamide (3DCI))[17]. In addition, up to 20% of a dose of IFO can be recovered unchanged in the urine. So, in contrast to cyclophosphamide where 90% of a dose is activated, as much as 70% of a dose of IFO may be eliminated by inactivating pathways. An intermediate on the activation pathway is also subject to metabolic inactivation, aldoifosfamide being further oxidised to an inactive carboxy form by aldehyde dehydrogenases[18].

In spite of these broad pharmacological uses of IFO mentioned above, its effects on plasma protein and the mechanism of action has seldom been reported. In the present work, spectroscopic and viscometric approaches were performed in order to elucidate the site selective binding of IFO to BSA. The interaction information regarding quenching mechanisms, binding parameters, thermodynamic parameters, binding modes, site-selective binding site, and conformation investigation is reported here.

Experimental

Standard drug and reagents:

Bovine serum albumin (BSA) was purchased from Sigma Chemical Company, St.Louis, USA and used without purification. Ifosfamide(IFO) was obtained from Sigma Aldrich, India. The solutions of IFO and BSA were prepared in 0.1M phosphate buffer of pH 7.4 with respect to their molecular weight and stored at 4˚C. All other chemicals were of analytical reagent grade and Millipore water was used throughout the work.

Instrumentation:

All of the fluorescence measurements were carried out on a F-2700 recording spectrofluorometer (Hitachi, Japan) equipped with a150W Xenon lamp source and 1.0cm quartz cells. The excitation and emission band widths were both 5nm. An Ellico UV-visible spectrophotometer equipped with a 1.0 cm cuvette was used to scan the UV spectrum. All of the pH measurements were made with Scott Gerate pH meter CG 804. The viscosity measurements were made with viscometer which was immersed in a thermostat water-bath at room temperature.

Recommended procedures

The following procedures were recommended for the study of binding characterization of IFO with BSA after a detailed and systematic study of the various parameters involved in the study of binding characterization.

UV/Vis Spectra measurements:

The UV measurements of IFO in the presence and absence of BSA were made in the range of 210-300nm. IFO concentration was fixed at1.5 x 10^{-4} M L^{-1} while the BSA concentration was varied from 0 to 30x 10^{-6} M L^{-1} in presence of phosphate buffer of pH 7.4 at 298K.

Fluorescence Spectra measurements:

A solution of BSA (8.0 x 10^{-5} M L^{-1}) was titrated by successive additions of a stock solution of IFO (1.5 x 10^{-4} M). Each solution was allowed to reach equilibrium for 5 min. The fluorescence spectra of the mixtures were then recorded in the wavelength range of 350–550 nm when excited with λ_{ex} = 290 nm. The emission spectra were recorded at three different temperatures, i.e., 293, 303 and 310 K.

Viscosity measurements:

Viscometric titrations were made using a viscometer, which was immersed in a thermostatic water-bath at 25°C. The experiments were conducted by adding appropriate amounts of IFO into the viscometer to give a certain *r* (=[IFO]/[BSA]) value while keeping the BSA concentration constant. The flow time of the solution through the capillary was measured with an accuracy of ± 0.20 s by using a digital stop watch. The mean values of three replicate measurements were used to evaluate the average relative viscosity of the sample. The data were presented as $(\eta/\eta_0)^{1/3}$ versus r[12], where η and η_0 are the viscosities of BSA in the presence and absence of IFO, respectively. Viscosity values were calculated from the observed flow time of BSA - containing solutions (t) and corrected for buffer solution (t_0), $\eta = (t - t_0)/t_0$.

Results and Discussion

UV/Vis Absorption Spectroscopy:

Figure 2(a) showed the UV/Vis absorption spectral study of IFO with BSA. It was observed that on the addition of IFO, BSA showed a decrease in molar absorptivity with a red shift of 1-5 nm. This hypochromic effect is thought to be due to the interaction between the electronic states of the intercalating chromophore and those of the BSA bases[19].Generally, the blue shift (or red shift), hyperchromic (or hypochromic) effects are the properties of BSA-drug interaction which are closely related with double helix structure[20]. The BSA solution exhibited peculiar hypochromic effect and bathochromic shift in UV/Vis spectra upon binding to IFO, a typical characteristic of an intercalating mode[19].

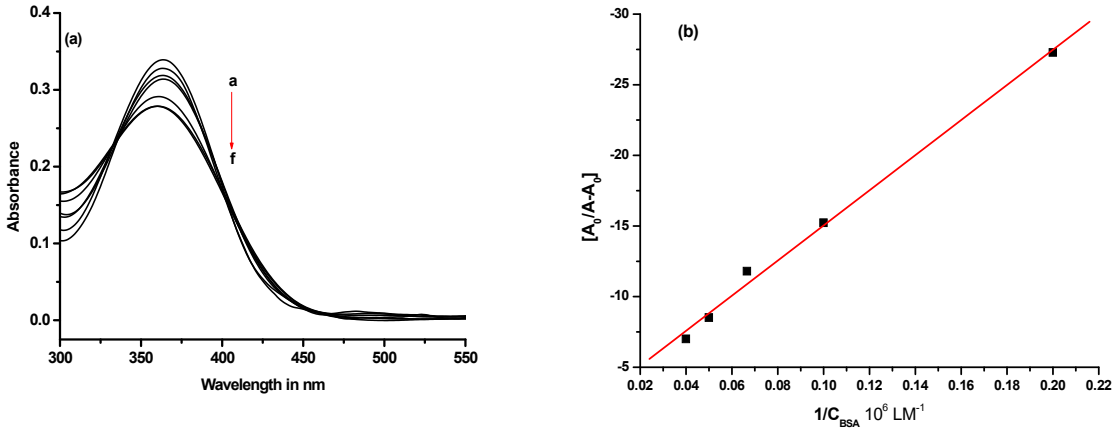


Fig.2 (a) UV-visible spectra of 1.5 x 10^{-4} M IFO in the presence ofC_{BSA}= 0, 5, 10, 15, 20, 25 and 30 µM L^{-1} BSA (a to f) in buffer of pH-7.4 and (b) Plot of (A_0 / (A-A_0) *versus* 1/ [BSA] for IFO - BSA system

Based on the variations in absorbance spectra of IFO upon binding to BSA, the binding constant (K) was calculated according to the following equation[21].

$$\frac{A_0}{A-A_0} = \frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G} \times \frac{1}{K\,[\mathrm{BSA}]}$$

Where, A_0 and A are the absorbance of drug in the absence and presence of BSA, ε_G and ε_{H-G} are the absorption coefficients of drug and its complex with BSA, respectively. The plot of $A_0/(A-A_0)$ *versus* 1/[BSA] was constructed (Fig. 2b) using the data from the absorbance titrations and a linear fitting of the data yielded the binding constants (K) (8.173 x 10^3 LM^{-1}) for IFO-BSA. These results are close to that from spectrofluorimetry.

Fluorescence quenching spectra

The binding of IFO to BSA was also examined by fluorescence titration measurement. Quenching of the intrinsic fluorescence of bovine serum albumin (BSA) was observed by selectively exciting tryptophan residues at 290 nm. Emission spectra were recorded in the range from 350 to 550 nm for each quencher addition. An obvious decrease of the fluorescence intensity of BSA was observed with increasing of IFO concentration (Fig. 3). This shows that BSA fluorescence is efficiently quenched upon binding to BSA.

Quenching mechanism and Binding Constants

A quenching process can be usually induced by a collisional process which is dynamic quenching or a

formation of a complex between quencher and fluorophore which is static quenching. Dymnamic quenching depends upon diffusion. Since higher temperatures results in larger diffusion coefficients, the biomolecular quenching constants are expected to increase with increasing temperature. In contrast, an increase in temperature is likely to result in a decrease in stability of complexes, and thus lower values of the static quenching constants[22]. In order to confirm the quenching mechanism, the fluorescence quenching was analysed according to the Stern-Volmer equation[23].

$$F_0 / F = 1 + kq\tau_o [Q] = 1 + Ksv [Q] \quad (1)$$

Where, F_0 and F represent the steady-state fluorescence intensities in the absence and presence of quencher, respectively. [Q] is the concentration of quencher. k_q is the quenching rate constant of biomolecule. τ_o is life time of biomolecule without the quencher and its value is 10^{-8} s[24], and K_{SV} is the Stern-Volmer dynamic quenching constant, which was determined by linear regression of a plot of F_0/F

against [Q]. According to Eq. 2, the quenching constant k_q was calculated to be about 10^{11} L mol^{-1} as listed in Table 1. However, the maximum scatter collision quenching constant kq of various quenchers with the biopolymer is 2.0 x 10^{10} L mol^{-1} s^{-1} [25], which suggests that the fluorescence quenching process may be mainly controlled by a static quenching mechanism rather than dynamic. From Table 1 we can also clearly see that K_{SV} is inversely correlated with temperature which indicates again that the quenching is not caused by dynamic collision but comes from the formation of a complex. So fluorescence quenching mechanism of IFO by BSA is a static quenching type.

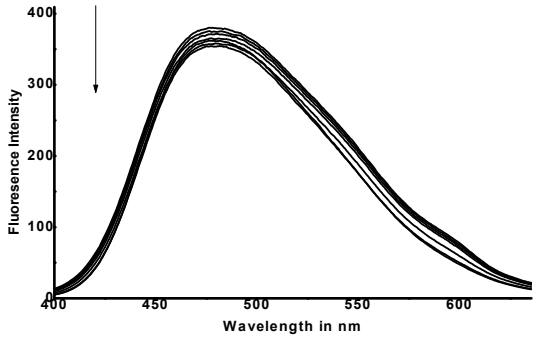


Fig. 3. Fluorescence spectra of 8.0 x 10^{-5} M BSA in the presence ofC_{IFO}= 0, 5, 10, 15, 20, 25.0, 30, 35 µM L^{-1} BSA in phosphate buffer solution of pH-7.4

The binding constant K and the number of binding sites n of IFO with BSA are calculated by the following equation using the data from fluorescence titration:

$$\text{Log } (F_0 - F) / F = \log K_b + n\log [Q] \quad (2)$$

where in the present case, K_b is the binding constant and n is the number of the binding sites, which can be determined by the ordinate and slope of double logarithm regression curve (Fig. 4) of log $(F_0 - F)$ versus log [Q] based on the eq.2, respectively. The values of K_b and n are evaluated and presented in Table 1. From Table 1, it can be found that IFO may effectively bind to BSA with high affinity, and the ratio of binding of BSA to IFO is about 1:3. Additionally, we can also see that the values of K_b decrease with the increase in temperature, which is in good agreement with the trend of K_{SV} as mentioned above. It implies that an unstable complex may be formed in the binding reaction and the complex would possibly be dissociated partly when the temperature increases.

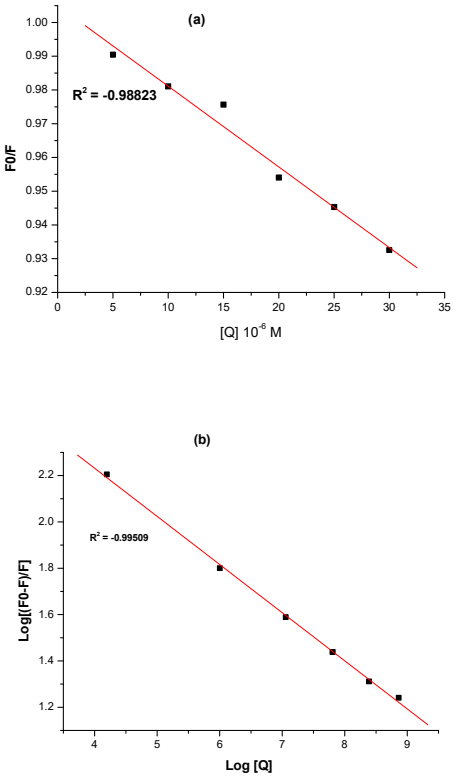


Fig. 4 (a) Stern-volmer plot of (F / F_0) *vs.* [Q] for IFO-BSA system and (b) Plot of log [(F_0 - F)/F] *vs.* log [Q] for IFO-BSA system

Table 1 The dynamic quenching constants between IFO and BSA at different temperatures

Table 1 The dynamic quenching constants between IFO and BSA at different temperatures

T (K)	K_{sv} (L mol^{-1})	k_q (L mol^{-1}s^{-1})	K_b (L mol^{-1})	n	R^a
293	3.338 x 10^3	3.3375 x 10^{11}	4.275 x 10^3	1.579	0.9882
303	2.956 x 10^3	3.341 x 10^{11}	6.714 x 10^3	1.497	0.9803
310	2.617 x 10^3	2.623 x 10^{11}	5.128 x 10^3	1.441	0.9871

R^a is the correlation coefficient

Binding Mode

The acting forces between a small molecular substance and BSA mainly include hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force and so on. The signs and magnitudes of thermodynamic parameters for BSA interactions can account for the main forces contributing to BSA stability[26,27]. If the enthalpy changes (ΔH^0) do not vary significantly over the temperature range studied, then its value could be determined from Van't Hoff equation[28]:

$$\ln K = - \Delta H^0 /RT + \Delta S^0 /R \quad (3)$$

The free energy change ΔG^0 of the binding reaction at different temperature was estimated from the eq. 5:

$$\Delta G^0 = \Delta H^0 - T \Delta S^0 \quad (4)$$

From the linear relationship between ln*K* and 1/T, the value of ΔH^0 and ΔS^0 could be obtained (Fig.5). The ΔG^0 at different temperatures were calculated using eq. 4, the results were presented in Table 3.

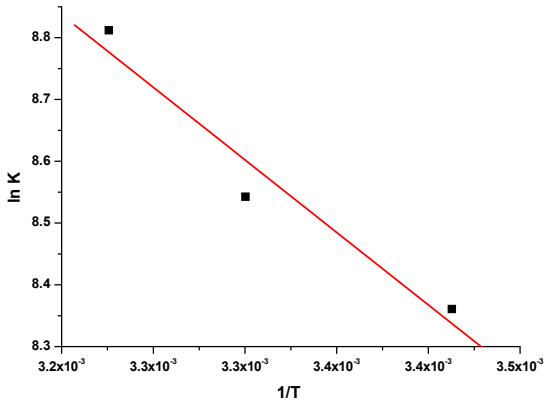


Fig.5. Van't Hoff plots of *ln*K vs. 1/T

Many references have reported the characteristic sign of the thermodynamic parameter associated with the various individual kinds of interaction that may take place in macromolecules association process[29]. The thermodynamic parameters (ΔH, ΔS) before and after reaction can be used to determine the type of interaction: when ΔH > 0, ΔS > 0, the acting force was hydrophobic force; when ΔH < 0, ΔS < 0 it was van der Waals' force and hydrogen bond; and when ΔH < 0, ΔS > 0, it was electrostatic force[30,31]. It was displayed in Table 2 that the fact ΔG < 0 proved that the reaction was spontaneous, and that ΔH > 0, ΔS > 0 proved the acting force type was hydrophobic force.

Table 2 The thermodynamic parameters for the IFO binding to BSA at different temperatures

T (K)	ΔG^0 (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	ΔS^0 (J $mol^{-1} K^{-1}$)
293	-20.36		125.31
303	-21.51		124.98
310	-22.56	16.35	126.00

Viscosity Measurements

Viscosity experiment is an effective tool to study the binding mode of small molecules to BSA. The interaction between IFO and BSA, we carried out viscosity measurements at room temperature. A classical intercalation binding demands the space adjacent base pairs to be large enough to accommodate the bound ligand and elongate the double helix, resulting in an increase of BSA viscosity while a non-classical intercalation or a groove mode would reduce the BSA viscosity[32]. The viscosity measurements were taken by varying the concentration ratio of BSA and IFO. The values of relative specific viscosity $(\eta/\eta_0)^{1/3}$ vs. [IFO]/[BSA] were plotted in the absence and presence of BSA.

As it was observed from figure, the relative specific viscosities of BSA exhibited a dependence on the concentration of IFO, which increases with the value of [IFO]/[BSA]. The behaviour indicates that non-classical intercalation mode of binding and possibly a groove binding *via* hydrophobic interaction between IFO with BSA.

Conclusion

The interaction of IFO with BSA was studied by UV/Vis in combination with fluorescence spectroscopy and viscometric techniques under the physiological condition. We have investigated that fluorescence quenching mechanism of BSA by IFO is a static quenching mechanism; K_{sv}= 3.338 x 10^3 L mol^{-1}, k_q = 3.3375 x 10^{11} L $mol^{-1}s^{-1}$ and K_b = 4.275 x 10^3 L mol^{-1}. The results of thermodynamic parameters obtained indicated that hydrophobic force and hydrogen bond were predominant forces to be stable the IFO – BSA

complex. In order obtained from UV-vis absorption spectrum suggested that the conformation of BSA changed when combining with IFO.

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