Spectroscopic Studies on the Mode of Interaction of an Anticancer Drug with Bovine Serum Albumin

Jaldappa SEETHARAMAPPA* and Bhalchandra Purushottam KAMAT

Department of Studies in Chemistry, Karnatak University; Dharwad 580 003, India.
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The mechanism of interaction of vinblastin sulphate (VBS) with bovine serum albumin (BSA) has been reported. Association constant for VBS–BSA binding was found to be $3.146\pm0.06\times10^4$ M^{-1}. Stern–Volmer analysis of fluorescence quenching data showed that the fraction of fluorophore (protein) accessible to the quencher (drug) was close to unity indicating thereby that both tryptophan residues of BSA are involved in drug–protein interaction. The rate constant for quenching, greater than 10^{10} M^{-1} s^{-1}, indicated that the drug-binding site is in close proximity to tryptophan residues of BSA. Binding studies in the presence of an hydrophobic probe, 8-anilino-1-naphthalein-sulphonic acid, sodium salt (ANS) indicated that there is hydrophobic interaction between VBS and probe and they do not share common sites in BSA. Thermodynamic parameters obtained from data at different temperatures showed that the binding of VBS to BSA involves predominant hydrophobic forces. The effects of some additives and paracetamol on binding of VBS–BSA have also been investigated. The CD spectrum of BSA in presence of VBS shows that the binding of VBS leads to change in the helicity of BSA.

Key words anticancer drug; bovine serum albumin; interaction study

Drug–protein interactions are important since most of the administered drugs are extensively and reversibly bound to serum albumin and drug is transported mainly as a complex with protein. The nature and magnitude of drug–protein interaction significantly influences the biological activity of the drug.[1—3] The binding parameters are useful in studying the pharmacological response of drugs and design of dosage forms.[4,5] Serum albumin being the major binding protein for the drugs and other physiological substances is considered as a model for studying drug–protein interaction in *in vitro*.[6] Vinblastin sulphate (VBS) is an anticancer drug administered to patients with pre-existing neuromuscular disease and also when other drugs with neurotoxic potential are being used. It is also used in combination for treatment of other lymphomas, tumors and advanced breast cancer. Two common methods that have been used in evaluating the binding of drugs to albumin include equilibrium dialysis and ultrafiltration.[7] These methods are laborious and time consuming and the results, at times, are not reproducible. More over, these conventional methods are often inapplicable to the analyses of strongly bound drugs because of technical problems such as drug adsorption on the membrane and the leakage of bound drug through membrane. In view of this, we planned to carry out the detailed investigations on the interaction of VBS with BSA using spectrofluorometric and circular dichroism techniques, as this kind of study has not been reported so far.

Results and Discussion

The structure of VBS used in the present study is shown in Fig. 1. Fluorescence spectra of BSA were recorded in the presence of increasing amounts of VBS (Fig. 2). It was observed that on interaction with VBS, λ_{max} of tryptophan fluorescence in BSA shifted to longer wavelength (from 341 to 348 nm) on the addition of 20—120 μM VBS to 15 μM BSA. VBS did not show any fluorescence intensity during the wavelength observed but it quenched the fluorescence of albumin. Shift in λ_{max} towards longer wavelength and decrease in intensity of fluorescence is usually caused by increase in polarity of the solvent. The fraction of drug bound, θ, was determined according to Weber and Young,[8] and Maruyama *et al*.[9] using the equation,

$$\theta=(F_o-F)/F_o \quad (1)$$

where F and F_o denote the fluorescence intensities of protein in a solution with a given concentration of drug and without drug, respectively. Fluorescence data was analyzed using the method described by Ward.[10] It has been shown that for equivalent and independent binding sites

$$\frac{1}{(1-\theta)K}=\frac{[D_t]}{\theta}-n[P_T] \quad (2)$$

where, K is the association constant for drug–protein interac-

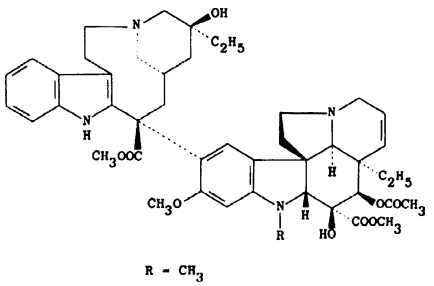

Fig. 1. Structure of Vinblastine Sulphate

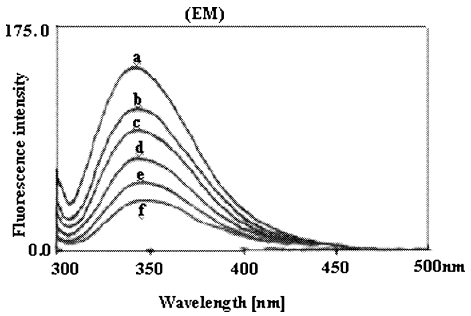

Fig. 2. Fluorescence Spectra of BSA (15 μM) in the Presence of VBS (a-0, b-20, c-40, d-60, e-90 and f-120 μM)

* To whom correspondence should be addressed. e-mail: jseetharam@yahoo.com

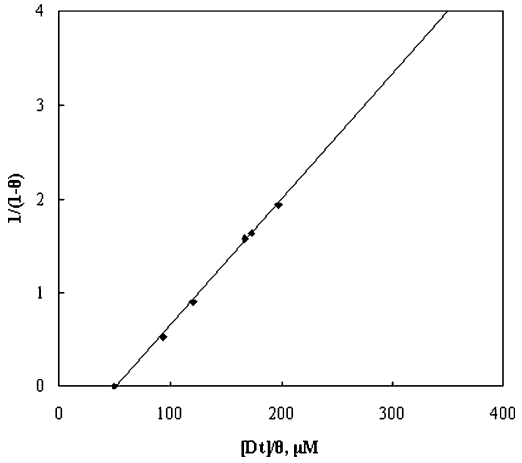

Fig. 3. $1/(1-\theta\Delta)$ *versus* $[D_t]/\theta$ Plot for the Binding of VBS to BSA

tion, n is the number of binding sites, $[D_t]$ is the total drug concentration and $[P_T]$ is the total protein concentration. A plot of $1/(1-\theta)$ *versus* $[D_t]/\theta$ is shown in Fig. 3. The values of K and n, obtained from slope and intercept were found to be $3.146\pm0.06\times10^4\,\mathrm{M}^{-1}$ and 3.77 ± 0.05. The order of K value (10^4) is consistent with non-covalent interactions.[11] Since the data fits equation 2 in all cases, it may be concluded that under the conditions of the experiment, all the binding sites are equivalent and independent.

Standard free energy change ($\Delta G°$) value was obtained from the relationship, $\Delta G° = -2.303\,RT\log K$ and was found to be $-25.70\,\mathrm{kJ\,mol}^{-1}$. Parachor, which is a measure of molar volume of drug, was calculated for VBS from the atomic parachors and other structural features[12] using the equation

$$\text{parachor}=\frac{\text{molecular weight of drug}\times[\text{surface tension of drug solution}]^{1/4}}{\text{density of drug solution}}$$

and the value was found to be $1872.5\pm0.2\ (\mathrm{N\,m}^{-1})^{1/4}\,\mathrm{m}^3$. It appears that the molecular size of the drug also plays a significant role in the binding of drug to BSA. The large size of the drug molecule probably has larger hydrophobic area, which can interact with hydrophobic surface of the protein molecule.

Fluorescence intensity data was also analyzed according to Stern–Volmer law,

$$F_o/F=1+K_q\,[Q] \tag{3}$$

by plotting F_o/F *versus* [Q], where F_o and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (drug) respectively and [Q] is the total drug concentration. The Stern–Volmer plot (Fig. 4) showed positive deviation from straight line, suggesting the presence of a static component in the quenching mechanism.[13] The static quenching is often observed if the fluorophore is adjacent to a quencher at the moment of excitation. A modified form of Stern–Volmer equation[14] that describes quenching data when both dynamic and static quenching is operative is

$$F_o/F=(1+K_q\,[Q])\,e^{V[Q]} \tag{4}$$

where, K_q is the collisional quenching constant or Stern Volmer quenching constant and V is the static quenching constant. This equation can be represented as

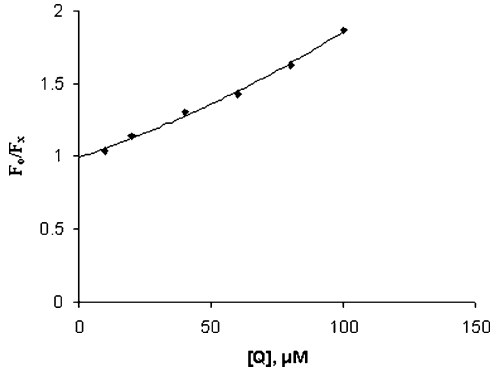

Fig. 4. Stern–Volmer Plot of F_o/F *versus* [Q] for the Binding of VBS

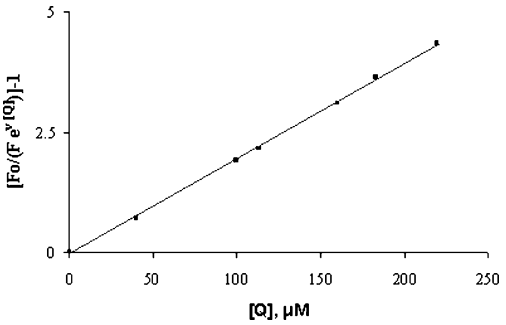

Fig. 5. Plot of $[F_o/F\,(e^{V[Q]})]-1$ *versus* [Q] for VBS

$[F_o/F\,e^{V[Q]}]=(1+K_q\,[Q])$, which follows the straight line, $Y=mX+C$ where $Y=[F_o/F\,e^{V[Q]}]$, $X=[Q]$, $m=K_q$ and $C=1$. This expression may also be shown as $[F_o/(F\,e^{V[Q]})]-1=K_q\,[Q]$. The value of V was obtained from equation 4 by plotting $[F_o/(F\,e^{V[Q]})]-1$ *versus* [Q] for varying V until a linear plot was obtained. The value of K_q was obtained from the slope of $[F_o/(F\,e^{V[Q]})]-1$ *versus* [Q] plot (Fig. 5). The values of V and K_q so obtained[15] are found to be $2.13\pm0.03\times10^3\,\mathrm{M}^{-1}$ and $2.0\pm0.07\times10^4\,\mathrm{M}^{-1}$ respectively.

According to Eftink and Ghiron[15] upward curvature in the Stern–Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue is nearly identical, while downward curvature indicates buried tryptophan residues. At a concentration of 120 μM drug, $72.26\pm0.05\%$ of the fluorescence intensity was quenched. The maximum quenching was obtained by extrapolating a plot of $(F_o-F)/F_o$ *versus* 1/[Q] to 1/[Q]=0, corresponding to infinite concentration of VBS. We have observed that at infinite concentration of VBS, fluorescence quenching was more than 90%. This again shows that both the tryptophan residues of BSA are accessible to drug molecule.

For a bimolecular quenching process, $K_q=k_q\,\tau_o$, where τ_o is the lifetime in the absence of quencher and k_q is the rate constant for quenching. As τ_o value for tryptophan fluorescence in proteins is known to be equal to $10^{-9}\,\mathrm{s}$[1,12] the rate constant, k_q, would be of the order of $10^{13}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$. The value of k_q depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that

$$k_q=4\pi aDN_a\times10^{-3} \tag{5}$$

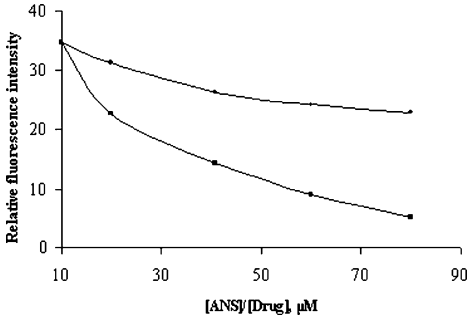

Fig. 6. The Effect of VB (—•—) and ANS (—▪—) on the Fluorescence Intensity of BSA (10 μM, Excitation Wave Length 296 nm and Emission Wave Length 344 nm)

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and N_a is the Avogadro's number. The upper limit of k_q expected for a diffusion-controlled bimolecular process[16] is $10^{10}\,M^{-1}\,s^{-1}$. The high magnitude of k_q in the present study ($10^{13}\,M^{-1}\,s^{-1}$) can probably be attributed to a specific long-range interaction between drug molecules and tryptophan residues on protein. Thus, the process of energy transfer occurs by intermolecular interaction forces between tryptophan and drug and this is possible only when the drug-binding site is in close proximity to tryptophan residues of BSA.

Fluorescence spectra of 10 μM BSA in the presence of increasing amounts of VBS/ANS (4 to 25 μM) were recorded. Both VBS and ANS quench the fluorescence of BSA, but the magnitude of decrease in fluorescence intensity was much larger for ANS as compared to that for VBS (Fig. 6). The ANS bound to BSA calculated from the fraction of occupied sites (θ) was found to be 79±0.08% where as the VBS bound to BSA was only 22±0.05%. It is known that excitation at 296 nm involves fluorescence due to only tryptophan residues of BSA. Further, under conditions of the experiment, tryptophan residues of BSA are partially exposed and their accessibility depends upon the nature of molecules of the interacting species.[17] It thus appears that whereas, tryptophan residues are fully accessible to the hydrophobic probe, ANS they are only partially accessible to the VBS which has partially hydrophilic character.

In another set of experiments, BSA–VBS interaction was studied in the presence of 5, 10, 15, 20 and 22 μM of ANS by monitoring ANS fluorescence after excitation at 370 nm. Fluorescence spectra were recorded in the range 390—550 nm (Fig. 7). It was observed that for a given concentration of BSA–VBS, fluorescence intensity increased when ANS was added to BSA–VBS system indicating that the VBS and ANS do not share common sites in BSA. It is known[15] that the hydrophobic probe, ANS shows greatly increased fluorescence as a result of hydrophobic interactions with proteins and other macromolecules due to transfer of probe from an aqueous to non-polar environment. Increase in fluorescence intensity of BSA–VBS system on addition of ANS can be explained as follows. When ANS is added to BSA–VBS system it can compete with VBS for hydrophobic sites on the surface of BSA. In that case it would inhibit the binding of VBS, *i.e.*, displace VBS from its binding site and fluorescence intensity should decrease. But fluorescence intensity actually increases. This shows that VBS and ANS do not share common sites in BSA.

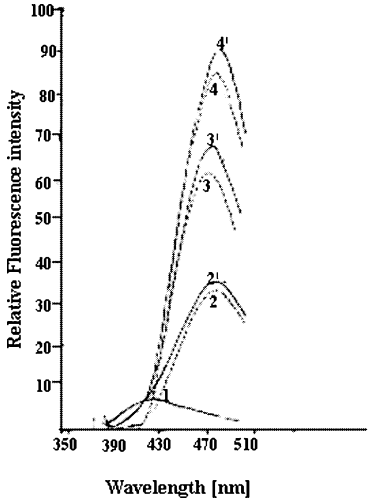

Fig. 7. Fluorescence Spectra of BSA–ANS in the Presence (———) and Absence (– – – – –) of VBS

BSA and VBS concentrations were fixed at 10 μM, and that of ANS was varied (1,1′-BSA+0 μM ANS; 2,2′ -BSA+5 μM ANS; 3,3′ -BSA+10 μM ANS; 4,4′- BSA+20 μM ANS.

Thermodynamic studies were studied by carrying out the interaction of drug–protein at three different temperatures using Vant Hoff's reaction,

$$\text{Log}\,K = -\Delta H^\circ/2.303RT + \Delta S^\circ/2.303R \quad (6)$$

The K values were found to be $6.92\pm0.03\times10^3$ (at 14 °C), $3.146\pm0.06\times10^4$ (at 26 °C) and $2.83\pm0.05\times10^4$ (at 35 °C). Log K *versus* $1/T$ plot enabled the determination of standard enthalpy change, ΔH° and standard entropy change, ΔS° for the binding process. The ΔH°, ΔS° and ΔG° values were found to be $+58.39\,kJ\,mol^{-1}$, $+274.18\,J\,K^{-1}\,mol^{-1}$ and $-23.56\,kJ\,mol^{-1}$, respectively. The positive ΔH° and ΔS° values indicate the predominant hydrophobic character of binding[18] because of the increased freedom of the water molecules, which were in the neighborhood of the non-polar groups before association. Therefore, the binding of VBS to serum albumin involves the formation of predominant hydrophobic forces.

Surface tension data was expressed as surface activity, which in turn expressed as surface pressure, π, which is the difference between surface tension of the solvent and that of the solution. Thus, surface activity value, expressed as surface pressure, $\pi=\lambda_{solvent}-\lambda_{soln}$, was found to be $3.92\times10^{-3}\,N\,m^{-1}$. Reduction in surface tension of solvent or increase in surface pressure is attributed to hydrophobicity of the VBS molecule. However, the order of π value suggests that the VBS has less hydrophobic character.

To understand further the nature of interaction involved, fluorescence spectra of VBS–BSA were recorded in the presence of each of 20 μM urea, dextrose, magnesium stearate, starch, and gum-acacia solution. The increase in fluorescence intensity of albumin–VBS mixture in the presence of urea shows that urea inhibits the VBS–BSA binding while, the decrease in fluorescence intensity values in case of magnesium-sterate, dextrose, starch and gum-acacia indicate that they facilitates VBS–BSA binding. Thus, urea, dextrose, magnesium-stearate, starch and gum-acacia alter the microenvironment of the binding sites by affecting the iceberg structure of

Table 1. Fluorescence Intensity of VBS–BSA System in the Presence of Additives

Sample	Fluorescence intensity at 344 nm
Only BSA	31.58
BSA+drug (VBS)	28.82
BSA+urea	30.12
BSA+drug+urea	33.92
BSA+magnesium-stearate	31.24
BSA+drug+magnesium-stearate	27.03
BSA+dextrose	31.12
BSA+drug+dextrose	27.12
BSA+starch	31.35
BSA+drug+starch	27.67
BSA+gum-acacia	31.18
BSA+drug+gum-acacia	27.50

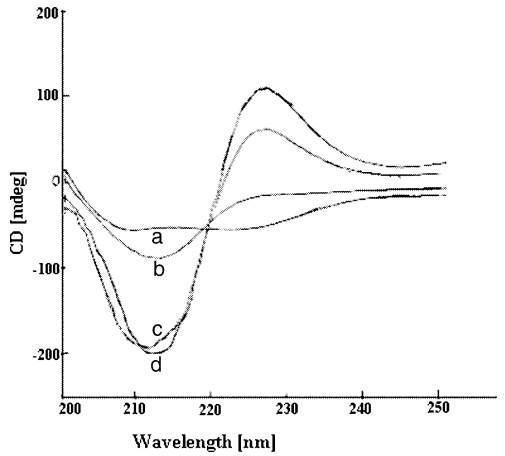

Fig. 8. Circular Dichroism Spectra in the 200—250 nm Range
(a) BSA, 0.1 μM; [BSA]: VBS=1 : 1(b) ; 1 : 3 (c) and 1 : 5 (d).

water. It may thus be concluded that hydrophobic interaction plays a significant role in the interaction of VBS to serum albumin. The results of analysis are given in the Table 1.

The simultaneous administration of two or more strongly bound drugs can compete with one another for the binding sites on albumin and so result in displacement interactions.[19,20] Although paracetamol is not strongly bound at therapeutic concentrations it can still affect the protein binding behavior of other drugs either by blocking an active site or by causing conformational changes in the protein molecule. Thus, the presence of paracetamol can significantly alter the pharmacological response of other drugs by altering the concentration of free drug in plasma. It was observed that the association constant of VBS decreased in the presence of paracetamol from $3.146\pm0.06\times10^4$ to $3.62\pm0.05\times10^2\,\mathrm{M}^{-1}$. In other words the availability of free drug in plasma gets increased in the presence of paracetamol. Once the interference of the paracetamol in the protein binding of drug is established one can anticipate the need for an adjustment in dosage in the presence of paracetamol. The relative ability of paracetamol to interfere in the binding of other drugs can be quantitatively determined from K_{ratio}, the ratio of association constant in the presence and absence of paracetamol. K_{ratio} can, therefore, be a guide to the modified design of dosage forms in the presence of paracetamol.

Typical CD spectra of VBS with BSA are shown in Fig. 8. The CD spectra of 0.1 μM BSA in buffer (a) and varied ratios of BSA–VBS 1 : 1 (b); 1 : 3 (c) and 1 : 5 (d) showed marked changes. As expected the α-helices of protein show a strong double minimum at 222 and 209 nm.[21] The intensities of this double minimum reflected the amount of helicity of BSA and further this indicated that BSA contains more than 50% of α-helical structure. On increased addition of VBS to BSA (1 : 1, 1 : 3 and 1 : 5) the second minimum (negative peak) at 222 nm vanished but a new positive peak was observed at 222—224 nm and the intensity of the first minimum started increasing with a red shift of 4 nm (209 to 213 nm). This is indicative of increase in helicity when VBS is completely bound to protein.[21]

Experimental

Bovine Serum albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) and 8-anilino-1-naphthalein-sulphonic acid, sodium salt were obtained from Sigma Chemical Company, St Louis, U.S.A. Vinblastin sulphate was obtained from Cipla Ltd India. All other materials were of analytical reagent grade. The solutions of VBS and BSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on molecular weight of 65000. For CD measurements, solutions were prepared in 0.01 M phosphate buffer of pH 7.4 containing 0.15 M NaCl.

VBS–BSA Interaction Some preliminary studies were carried out to select optimum protein and VBS concentrations for VBS–protein interaction. On the result of preliminary experiments, BSA concentration was kept fixed at 15 μM and VBS concentration was varied from 20 to 120 μM. Fluorescence measurements were performed on a Hitachi spectrofluorometer Model F-2000 equipped with a 150W Xenon lamp and slit width of 10 nm, and using a 1.00 cm quartz cell. Fluorescence spectra were recorded at room temperature (26 °C) in the range 300—500 nm after excitation at 296 nm. The absorbances of VBS–BSA mixtures in the concentration range employed for the experiment did not exceed 0.05 at the excitation wavelength in order to avoid inner filter effect.

Binding Studies in the Presence of Hydrophobic Probe Experiments were also carried out in the presence of an hydrophobic probe, ANS. In the first set of experiments, the interaction of VBS and ANS with BSA was studied. BSA concentration was kept fixed at 10 μM and VBS/ANS concentration was varied (4—25 μM). Fluorescence spectra were recorded in the range of 300—500 nm after excitation at 296 nm. In the second set of experiments, BSA–VBS interaction was studied in the presence of 5, 10, 15, 20, and 22 μM of ANS. BSA and VBS concentration was kept fixed at 10 μM. Fluorescence spectra were recorded in the range 390 to 550 nm after excitation at 370 nm.

Effect of Additives The fluorescence spectra of VBS–BSA were recorded in presence and absence of various additives at 344 nm after excitation at 296 nm. The concentration of BSA and VBS was fixed at 10 μM and 20 μM, respectively and that of each additive was maintained at 20 μM.

Thermodynamics of VBS–BSA Interaction Thermodynamic parameters for the binding of VBS to BSA were determined by carrying out the binding studies at three different temperatures, 14 °, 26 ° and 35 °C by fluorometric method. BSA concentration was kept fixed at 15 μM and VBS concentration was varied from 20 to 120 μM.

Effect of Paracetamol on VBS–Protein Interaction Protein–VBS binding was studied in presence and absence of paracetamol using fluorescence spectroscopy. Emission spectra were recorded in the range of 300—500 nm.

Surface Tension Measurements Surface tension of VBS solution (0.8%) prepared in phosphate buffer of pH 7.4 containing 0.15 M NaCl at 29 °C was determined by drop weight and drop number method using a stalagmometer.

Circular Dichroism (CD) Studies CD measurements of BSA in the presence and absence of VBS were made. The BSA to VBS concentration was varied (1 : 1, 1 : 3 and 1 : 5) and the CD spectrum was recorded. CD measurements were made on a JASCO-810 spectropolarimeter using a 1.00 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200—250 nm.

Conclusions

This work is an example of rarely encountered study

wherein the interaction of BSA with an anticancer drug, VBS has been investigated by employing fluorometry and CD methods. The obtained results suggest that the VBS bind to BSA possibly by predominant hydrophobic interactions. The shape and intensity of negative CD bands of BSA at 209 and 222 nm show major differences in the presence of VBS due to change in the chemical environment of a-helices lying at the surface of the protein. Complete binding of VBS to BSA is found to induce conformational changes in protein leading to increase in the helicity of protein.

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