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Spectral elucidation with molecular docking study between isatin analogous and bovine serum albumin

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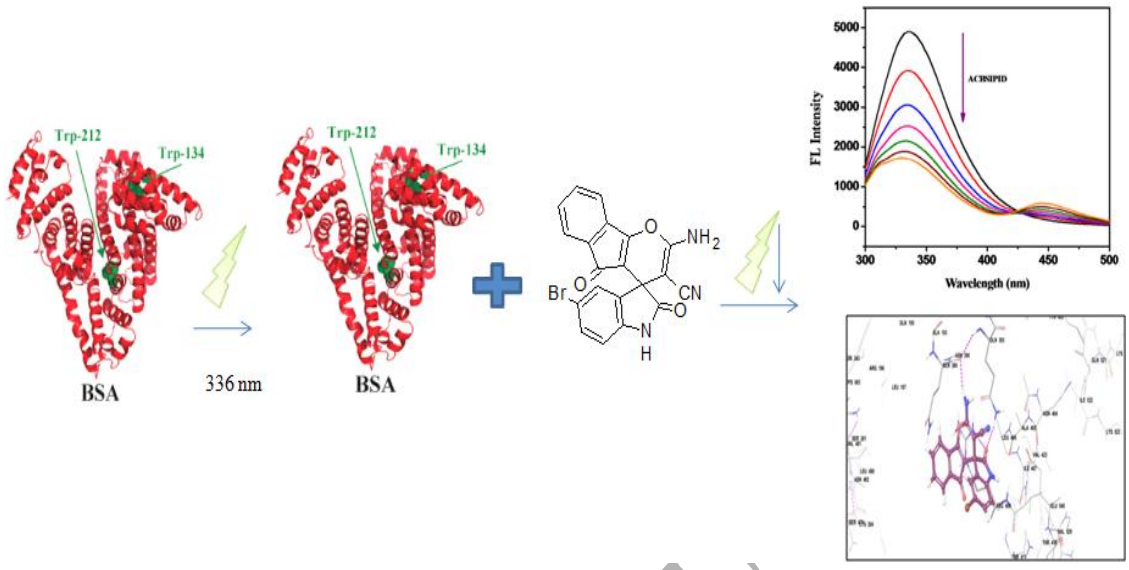
Abstract The interaction between isatin moiety 2-amino-3-cyano-5-bromospiro (5H-indeno [1,2-b] pyran-4,3'indoline)-2'5,-dione (ACBSIPID) and bovine serum albumin (BSA) has been interpret under physiological conditions using fluorescence, circular dichroism and molecular docking studies. The formation of ACBSIPID-BSA complex leading to the fluorescence quenching of BSAwas evaluated by spectrofluorimetry. The mechanism for fluorescence diminishing and mode of binding was investigated by temperature effect. Furthermore, the quenching assessed by time-resolved fluorescence study, result confirmed the static nature of quenching. Whereas the binding distance (r) between ACBSIPID and BSA was found to be 3.12 nm by Forster's theory of nonradiative energy transfers. Based on CD spectra, it was observed that the presence of ACBSIPID decreased the α-helical content of BSA and induced unfolding of a polypeptide chain of the protein. The molecular docking study indicates ACBSIPID drug having plausible binding affinity towards the BSA complex enzyme.

Graphical abstract



Keywords: ACBSIPID; BSA; Circular Dichroism; Fluorescence; Molecular docking study.

Specifications Table

Subject area	*Spectroscopy, Physical Chemistry, etc.*
Compounds	*2-amino-3-cyano-5-bromospiro (5H-indeno [1,2-b] pyran-4,3'indoline)-2'5,-dione (ACBSIPID)*
Data category	*Spectral, physicochemical ,molecular dynamics, etc.*
Data acquisition format	*Fluorescence, circular dichroism and molecular docking, etc.*
Data type	*Analyzed.*
Procedure	*Interaction analysis between bovine serum albumin and 2-amino-3-cyano-5-bromospiro (5H-indeno [1,2-b] pyran-4,3'indoline)-2'5,-dione by spectroscopic techniques*
Data accessibility	*Manuscript and supplementary data enclosed with this article*

1. Rationale

The cancer is major cause of death across the world [1]. Among the various types of cancer, the breast cancer is a disease of great concern where as it is the second important cause of death in women [2]. Beginning past few decades, there is a rising attention on searching the novel chemotherapeutic agents based on biologically active substances found in nature [3]. For example, an indole derivative which obtained first in 1841 by Erdmann [4] and Laurent [5] is 1H-indole-2,3-dione(Isatin). It is investigated for their pharmaceutical properties in the form of Schiff bases [6], which involved during synthesis of range of heterocyclic compounds such as quinolines, indoles and as raw material for medicinally important drugs. Isatin displayed various biological activities such as anticonvulsant, sedative, hypnotic, monoamine oxides inhibitors, antianxiety, antipsycho activity etc. Its derivatives indicated as potent anticonvulsant in a large variety of preclinical anticonvulsant models [7]. It is well known as pharmacological agent having a seriesof action in the brain and protective against certain types of infections. Many derivatives displayed anti-bacterial, anti-fungal, anti-viral, anti-HIV, anti-protozoal, anti-allergic and anti-inflammatory activities [8]. A literature survey reveals that there has been a use of isatin for organic synthesis during the last twenty-five years, and importantly of its biological properties [9]. Therefore, we have selected anticancer isatin derivative i.e. 2-Amino-3-cyano-5-bromospiro (5H-indeno [1,2-b] pyran-4,3'-indoline)-2'5,-dione [ACBSIPID] for its interaction study with BSA.

Serum albumin is the main constituent present in blood plasma which responsible to maintain osmotic pressure of the bloodvessels. It serves as a carrier protein for a variety of molecules, including metabolites of both endogenous and exogenous origin, hormones, and drugs [10]. The protein binds a number of relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport throughout the circulation [11]. For therapeutic purposes the binding of drug-protein can be useful because variation in protein binding may change the volume of distribution, clearance, and elimination of a drug and may adapt its therapeutic effect [12]. BSA (Figure 1) has been one of the most comprehensively studied proteins, mainly because of its structural homology with human serum albumin (HSA). BSA has two tryptophan residues; Trp-134 and Trp-212 in the first and second domains that possess intrinsic fluorescence [1]. Trp-212 is located within a hydrophobic and Trp-134 is located on the surface of molecule [13]. Here we proposed to study the binding affinity of isatin derivative (ACBSIPID) especially for BSA molecule with the help of steady state fluorescence, circular dichroism and molecular docking techniques. Here we study the Forster resonance energy transfer (FRET) and molecular docking. The inner filter effect (IFE) is a competitive phenomenon of FRET

and observed by absorption of light by analyte in the excitation and/or emission region of probe hence directly related with absorbance of analyte and fluorescence intensity [14]. This study is carried out to understand the carrier role of serum albumin in blood. The isatin derivative i.e. 2-Amino-3-cyano-5-bromospiro (5H-indeno [1,2-b] pyran-4,3'-indoline)-2'5,-dione [ACBSIPID] prepared by Dr. A. A. Patravale and et al via following procedure [15] (Figure 2) acts as anticancer agent against breast cancer cell lines MCF7, MDA-MB 435. This compound was found appreciably cytotoxic against the estrogen negative cell line MDA-MB-435 and referred it against the standard drug, i.e., doxorubicin (ADR) and tamoxifen (TAM). The GI_{50}% value of ACBSIPID is 1.8 μM respectively. The percent control growth of compound with respect to concentrations (10^{-7}M to 10^{-4}M) is 2.93±02.25%, −8.9±04.04%, −17.26±02.05 % and –20.7±04.07 %. Thus, it is clearly concluded that test compound shows dose dependent growth control activity over estrogen negative cell line MDA-MB-435 and substantial potency toward the estrogen negative cancer cell line [15]. Due to these result it is important to study the binding of this drug compound with serum albumin for understanding absorption, transportation, and elimination in body, which will be useful guideline for further new drug design.

2. Experimental

2.1. Materials

0.5 x 10^{-5} M solutions of ACBSIPID were prepared in DMSO-water (1: 9) mixture. BSA was purchased from Himedia Chemical Company and its 1 x 10^{-5} M solutions were prepared in doubly distilled water. Potassium phosphate buffer (0.1 M) containing NaCl (0.1 M) solutions were prepared at pH 7.4. It is used for keeping the pH of solution at 7.4. All chemicals were of AR grade and used as such. Double distilled water was used throughout all experimental work.

2.2. Equipments and spectral measurements

All quenching spectra were recorded on fluorescence spectrometer (JASCO Japan FP-8300) equipped with a xenon lamp and 1.0 cm quartz cell at three different temperatures. An excitation wavelength of 280 nm was chosen. All pH values were measured by a digital pH meter (GLOBEL DPH 500). Synchronous fluorescence measurements were carried out at excitation range 260 – 360 nm and Δλ was set at 15 as well as 60 nm. Circular dichroism spectra were recorded on Jasco J-815 spectropolarimeter using a quartz cuvette of 0.1 cm path length at 25^0 C, under constant nitrogen flush. The three dimensional fluorescence spectra were also recorded. Horiba's JobinYvon IBH time resolved fluorescence spectrometer (Model: Delta Flex TCSPC) was used for measurement of

fluorescence decay.Studies of Molecular Docking were performed in Maestro 9.1 using Glide v6.8 (Schrodinger, LLC,New York, NY, USA).

3. Data, value and validation

3.1.Study of fluorescence quenching

Tryptophan, tyrosine and phenylalanine are three intrinsic fluorophores exhibited by BSA that are susceptible to quenching by interaction with other molecules. While phenylalanine has a very low quantum yield and fluorescence of tyrosin is approximately totally quenched if it is ionized or near an amino group, carbonyl group, but the intrinsic fluorescence of BSA is almost contributed by tryptophan only [16]. For interpretation of the binding interactions of BSA with ACBSIPID, fluorescence emission spectra of BSA only as a blank and with rising addition of drug molecules were recorded upon excitation at 280 nm at 301 K. It is clearly seen (Figure 3) that fluorescence intensity of BSA decreased with increasing concentration of ACBSIPID, having a blue shift of wavelength emission maximum λ_{max} (336 to 327 nm). This suggests the formation of drug-protein complex [17].

Binding of ACBSIPID to BSA quenches the intrinsic fluorescence of tryptophan. The quenching occurs by dynamic and static mechanisms. This quenching data was analyzed by using Stern-Volmer equation,

$$F_0/F = 1 + Kq\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

Where Kq = bimolecular quenching constant, τ_0 = fluorescence lifetime of biopolymer is equal to 10^{-8} s, K_{sv} = the Stern-Volmer quenching constant, $[Q]$ = the concentration of quencher and F_0 and F = fluorescence intensities of protein in absence and presence of quencher respectively. The formation of complex was again confirmed from values of quenching rate constants, Kq

$$Kq = K_{sv} / \tau_0 \quad (2)$$

Figure 4 displayed the Stern-Volmer curves, F_0/F vs $[Q]$ at three different temperatures showing linearity of all plots. The calculated K_{sv} and Kq values (Table 1) showing inverse proportionality of K_{sv} to temperatures. It indicates that fluorescence quenching was taking place by the formation of ground-state complex i.e. static type quenching is dominant. The maximum scattered collisional quenching constant Kq of various quenchers with the biopolymer is $10x10^{10}$ $Lmol^{-1}s^{-1}$ [18]. Here values of Kq for quenching of protein by ACBSIPID were found to be greater than scattered

process. This indicates that fluorescence is not initiated by dynamic but from formation of complex [19].

The fluorescence data was examined using modified Stern-Volmer equation,

$$F_0/\Delta F = 1/f_a K_a \, 1/[Q] + 1/f_a \quad (3)$$

Where, f_a = fraction of initial fluorescence that is accessible to quencher, K_a = Stern-Volmer quenching constant of the accessible fraction. The modified Stern-Volmer plots (Figure 5) showing results detected as linearity, within investigated concentrations. This depicts that the binding between BSA and ACBSIPID is remarkable and the effect of temperature is small. Thus, ACBSIPID can be stored and removed by protein in body [20].

3.2. The binding constant and number of binding sites

For static quenching the relationship between fluorescence intensity and concentration of a quencher can be described by the equation 4, [21]

$$\log (F_0 - F) / F = \log K + n \log [Q] \quad (4)$$

Where K = binding constant and n = number of binding stoichiometry for BSA [22]. The values of K and n are evaluated from above equation (Table 2). Plot of log (F_0 - F) / F vs log [Q] gives a straight line, whose slopes equal to n and intercept on Y-axis equal to log K (Figure 6). The larger values of K observed in the present study indicated strong binding between drug and protein. Further, the binding constant decreased with increasing temperature suggesting the reduction in stability of drug-BSA complex [23]. The values of (n) were calculated as 1.10, 1.14 and 1.09 for BSA, indicating there is approximately one binding stoichiometry in BSA for ACBSIPID.

3.3. Time Resolved Fluorescence Spectra

The time-resolved fluorescence has been studied, for the confirmation of static quenching and its measurements were carried out in absence and presence of quencher. There isno significant change in lifetime of BSA after addition of ACBSIPID (Figure S1). Thus, there is no possibility of dynamic quenching; therefore, indicating possibility of static mechanism of quenching [16].

3.4. Nature of interactingforces between BSA and ACBSIPID

Usually, the binding between most of drugs with proteins occurs in a reversible methodi.e. weak chemical interactions such as ionic, van der Waal's, hydrogen bond and hydrophobic interactions with the hydroxyl, carboxyl or other sites available on amino acids residues that come together to constitute drug binding sites on proteins. For protein–drug

interaction, the signs and magnitude of thermodynamic parameters can be exploited to estimate main forces contributing in formation of protein–drug complex [24, 25]. The thermodynamic parameters such as enthalpy change (*ΔH*) and entropy change (*ΔS*) of binding reaction are the main evidences to confirm binding modes [23]. Therefore, temperature dependent thermodynamic parameters were studied for understanding the nature of binding forces. The values of *ΔH* and *ΔS* for ACBSIPID and BSA interaction were estimated by Van't Hoff equation 5:

$$\ln K_T = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (5)$$

Where, K_T = binding constant at temperature T, R = gas constant. The change in enthalpy (*ΔH*) was obtained from slope of the Van't Hoff relationship. The change in free energy (*ΔG*) was investigated with the help of following relationship:

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

The study was carried out at 300, 310 and 320 K at which BSA did not undergo any structural degradation. Based on linearity of Vant Hoff plot (Figure 7) we determined the thermodynamic parameters (Table 3). Briefly the basis of thermodynamic data is [26]

a) *ΔH* > 0, *ΔS* > 0 corresponds to hydrophobic forces, van der Waals interaction,
b) *ΔH* < 0, *ΔS*< 0 corresponds to van der Waals interaction, hydrogen bond formation.
c) *ΔH* < 0, *ΔS* > 0 corresponds to electrostatic / ionic interactions.

The negative values of ΔH and positive values of ΔS at studied temperatures indicate the involvement of hydrogen bond and hydrophobic interaction in the formation of the BSA–ACBSIPID complex [22].

3.5. FRET study between BSA and ACBSIPID

Forster theory of dipole-dipole energy transfer was used to determine the average distance between donor and acceptor molecule. According to theory of FRET, the energy transfer will take place under certain conditions such as 1) donor can produce fluorescence light, 2) emission spectrum of the donor should overlap with UV-visible absorption spectrum of the acceptor, 3) distance between donor and acceptor is lower than 7 nm. We have recorded emission spectrum of donor and excitation spectrum of acceptor (Figure 8).

By using FRET, the distance r between ACBSIPID and BSA could be calculated by using the equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6} \quad (7)$$

Where R_0 = critical distance when the efficiency of excitation energy transferred to the acceptor is 50 %, r = binding distance between donor and acceptor. It can be calculated by using Forster formula:

$$R^6_0 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J \quad (8)$$

Where n = refractive index of medium, K^2 = spatial orientation factor of the dipole, ϕ = fluorescence quantum yield of the donor, J = overlap integral between the fluorescence emission spectrum of donor and excitation spectrum of the acceptor, which can be calculated by using the equation:

$$J = \frac{\int_0^\infty F(\lambda)\, \varepsilon(\lambda)\, \lambda 4\, d\lambda}{\int_0^\infty F(\lambda)\, d\lambda} \quad (9)$$

Where ε (λ) = the molar absorption coefficient of acceptor at wavelength λ, F (λ) = fluorescence intensity of donor at wavelength λ to $\lambda + \Delta\lambda$. In this case K^2 = 2/3, n = 1.336 and ϕ = 0.15. Hence from equations (7) – (9) we could calculate that J = 20.76 x 10^{-15} cm^3M^{-1}, R_0 = 2.88 nm, E = 0.4806 and r = 3.12 nm. The binding distance r is smaller than 8 nm and 0.5 R_0<r< 1.5 R_0. This indicates that there is high probability of energy transfer from BSA to ACBSIPID [27,28].

3.6. Analysis of conformational changes in protein

The possible influence of binding between ACBSIPID and BSA on the secondary structure of protein that affects physiological functions of serum albumin was investigated by synchronous fluorescence, circular dichroism (CD) and three-dimensional (3D) fluorescence spectroscopy.

3.6.1. Synchronous fluorescence spectra

For confirmation of alteration in protein structure, we carried out synchronous fluorescence spectral study. This spectrum gives information about the molecular microenvironment of amino acid residues of protein in the vicinity of chromosphere molecules by measuring the possible shift in wavelength emission maximum λ_{max}. The shift in position of emission peak wavelength is a sign of change in polarity around Trp and Tyr residues and shift may be attributed to selective binding and consequent quenching of Trp and Tyr emission. While $\Delta\lambda$ is fixed at 60 nm, the synchronous fluorescence of protein represents characteristic of Trp residues. When $\Delta\lambda$ is set at 15 nm, a spectrum characteristic of Tyr residues is obtained [29]. The effect of ACBSIPID on synchronous fluorescence spectra of BSA is depicted in Figure 9. There is no shift when $\Delta\lambda$= 15 nm, while a small red shift

(338 to 341 nm) was observed at $\Delta\lambda$ = 60 nm. This shift effect indicates change in conformation of BSA and the polarity around Trp-212 residue was increased and hydrophobicity was decreased [30].

3.6.2. Circular dichroism spectra

It is a sensitive technique to validate the conformational changes in protein. The far-UV CD spectrum of protein at pH 7.4 exhibited two minima at 208 and 222 nm which show the protein has α-helical structure [31]. In this study, circular dichroism (CD) spectra of BSA were recorded in presence and in absence of ACBSIPID (Figure 10). The spectra indicated as two negative bands in the UV region at 208 and 220 nm, which is a characteristic of α-helical structure of protein [32]. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg.cm^{-1}.d mol^{-1} according to following equation:

$$MRE = \frac{ObservedCD\ (mdeg)}{Cp \times n \times l \times 10} \qquad (10)$$

Where *n* = number of amino acid residues and *l* = path length, *Cp* = molar concentration of protein.

The α-helical content of free BSA and its complex with drug were calculated from MRE values at 208 nm using the equation:

$$\text{-helix (\%)} = \frac{\text{MRE}_{208} - 4000}{33000 - 4000} \times 100 \qquad (11)$$

MRE value at 208 nm, 4000 is MRE value of β-form and random coil conformation cross at α-helix at 208 nm. From above equations the α-helicity in the secondary structure of BSA was determined. This differed from that of 43.27 % in free BSA to 40.23 % in its complex. This result indicates that there is loss of α-helicity upon protein-drug interaction. Further the CD spectra of BSA in presence and absence of ACBSIPID are similar, indicating that the structure of BSA is also predominantly α-helical [33,34].

3.6.3. Three-dimensional fluorescence spectra

3-D fluorescence spectra giveinformation of understanding theconformational changes in the protein due to the simultaneous observation of excitation/emission wavelengths and fluorescence intensity [35]. To study the conformational changes of BSA induced by ACBSIPID, three-dimensional fluorescence spectra were recorded (Figure S2). From two spectra it is clearly seen that, the intensity of peak for BSA and its complex was different. So it again confirms there were interactions between drug-protein and conformational, microenvironmental changes in protein.

3.7. Molecular Docking Study

Molecular Docking Studies were performed in Maestro 9.1 using Glide v6.8 (Schrodinger, LLC, New York, NY, USA). The docking study is carried out on the Bovine serum albumin complex enzyme (PDB ID: 4OR0) to explore the binding affinity of most active bromospiro derivative (ACBSIPID). The compound interacts well through hydrogen bonding interaction with the amino acid residues Gln 393 and Gln 389 respectively **(**Fig. 11**)**. The standard drug taken was Sunitinib for the docking study. The compound ACBSIPID resembles well with the docking interaction of the standard Sunitinib. So, on the basis of docking interpretation, it can be suggested that the synthesized compound could have probable binding affinity towards the Bovine serum albumin complex enzyme.

The amino acid residues ASN 390, LEU 429 and ARG 409 are most importantly involved in the binding of the drug on the Bovine serum albumin complex enzyme. The criterion is the drug under study should fit in the pocket binding with the above amino acids smoothly to show its biological action.

The volume enclosed by the force field grid may have different x, y, and z extents. All receptor atoms are included in the calculation whether or not they fall within the grid volume. The force field grid may be positioned either by direct specification of its coordinates or by centering within a sphere cluster; in this manner, one can define a box that efficiently encloses the space that docked molecules are likely to occupy [36-38].

The protein-ligand binding free energy ΔG_{bind} is calculated as $\Delta G_{bind} = G(PL) - G(P) - G(L)$, where $G(PL)$, $G(P)$, and $G(L)$ are free energies of the protein-ligand complex, the free protein, and the free ligand, respectively. Proteins are considered as rigid and free energies of protein-ligand complexes and free ligands are calculated in the multiwell approximation which is similar to the "mining minima" method of Chen et al [39]. The potential energy of a molecular system is approximated by a set of independent parabolic wells in these methods. The multiwell approximation differs from the "mining minima" method mainly by more uniform and exhaustive low-energy local minima search by the FLM program instead of configuration space exploration along a combination of low-frequency modes as it was made by the "mining minima" method; also we used the Cartesian coordinates instead of the bond-angle-torsion coordinates. The configuration integral of a molecular system Z (thus, the free energy $G = -kT\ln(Z)$) is approximated by a sum of contributions from different energy wells Z^i:

$$Z=\sum iZi=\sum i\exp(-E_0{}^i kT)Z_v{}^i Z_t{}^i Z_r{}^i$$

Where $E_0{}^i$ is the potential energy value in the minimum of the ith energy well, $Z_v{}^i$ corresponds to the vibrational degrees of freedom of the ligand in the ith energy well, and $Z_t{}^i$ and $Z_r{}^i$ correspond to the

translation and rotation of a molecular system as a whole, respectively [40]. The Potential energy of standard Sunitinib is 175.177 and isatin derivative is 191.70

4. Conclusions

The binding between BSA and isatin derivative ACBSIPID have been investigated by fluorescence, time resolved fluorescence, CD spectroscopic and molecular docking techniques for the first time. The results of the current study highlight the credentials of binding interactions between drugs and serum albumin particularly in terms of their pharmacological and pharmacodynamics importance [9]. The obtained results implied that the intrinsic fluorescence of BSA is quenched by ACBSIPID due to complex formation between protein and drug. Temperature effect study was carried out for understanding the binding mechanism. The inversely proportionality of K_{sv} values with temperature indicates that static quenching mechanism is predominant. Time resolved fluorescence spectra confirmed the nature of emission is static. There is one binding stoichiometry of protein for drug. Thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) has negative value which indicates that van der Walls interaction and hydrogen bonds played a major role in the binding mechanism. FRET study shows that the binding distance 'r' between donor and acceptor was calculated to be 4.58 nm. The conformational changes in BSA were confirmed by synchronous, circular dichroism and three-dimensional fluorescence studies. The molecular docking study indicated plausible binding affinity of ACBSIPID drug towards the Bovine serum albumin complex enzyme and compound interact well through hydrogen bonding interaction with the amino acid residues Gln 393 and Gln 389 respectively. All these experimental results and calculated data reveals that ACBSIPID could bind to BSA and effectively transported and eliminated in body, which is a useful guideline for further drug design.

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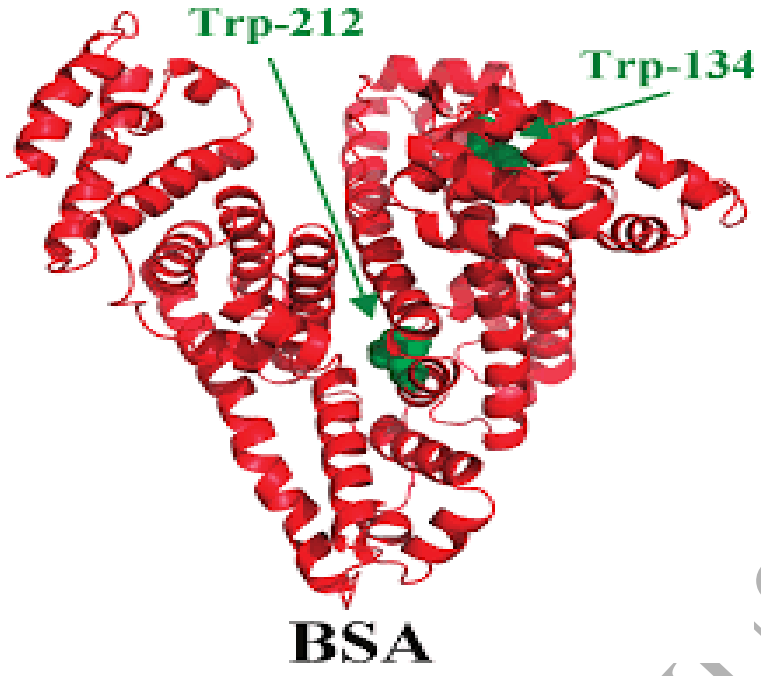
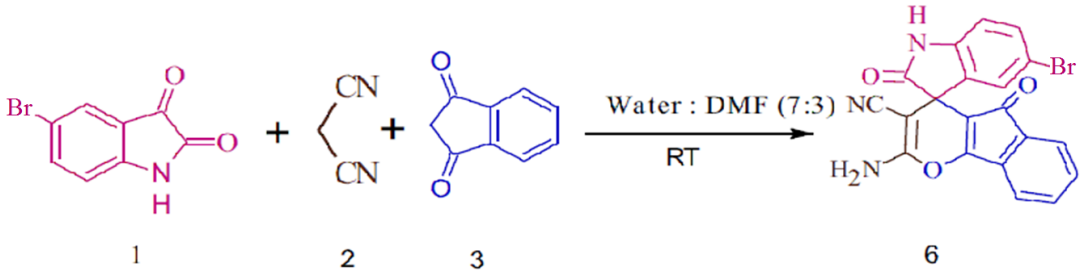
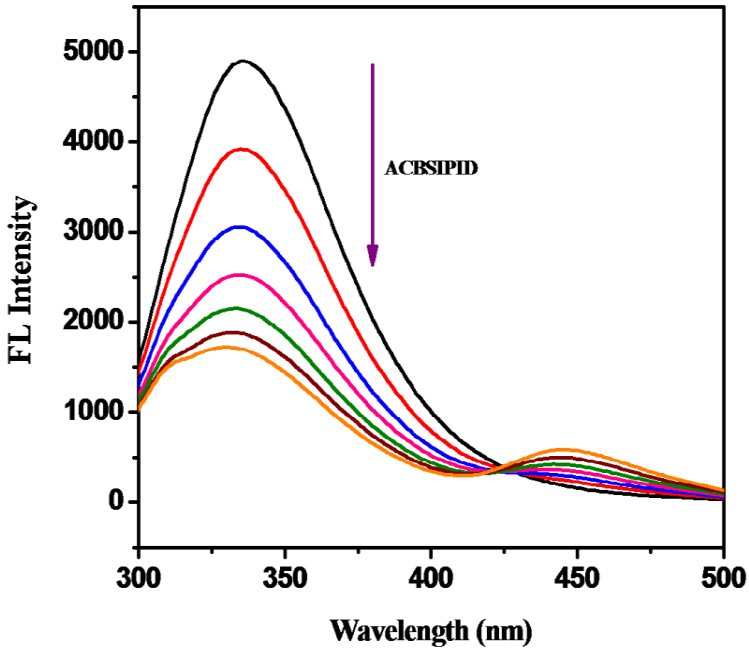


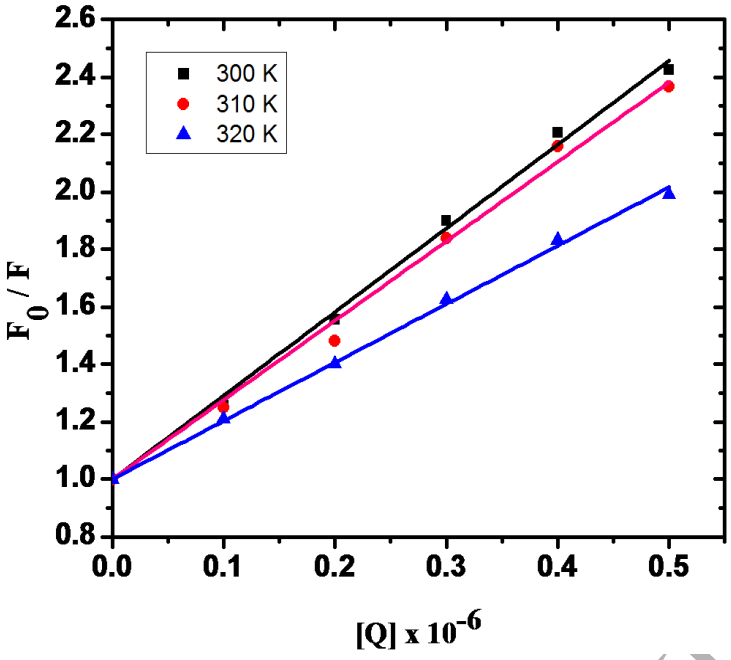
1) Structure of Bovine Serum Albumin showing the tryptophan residues.



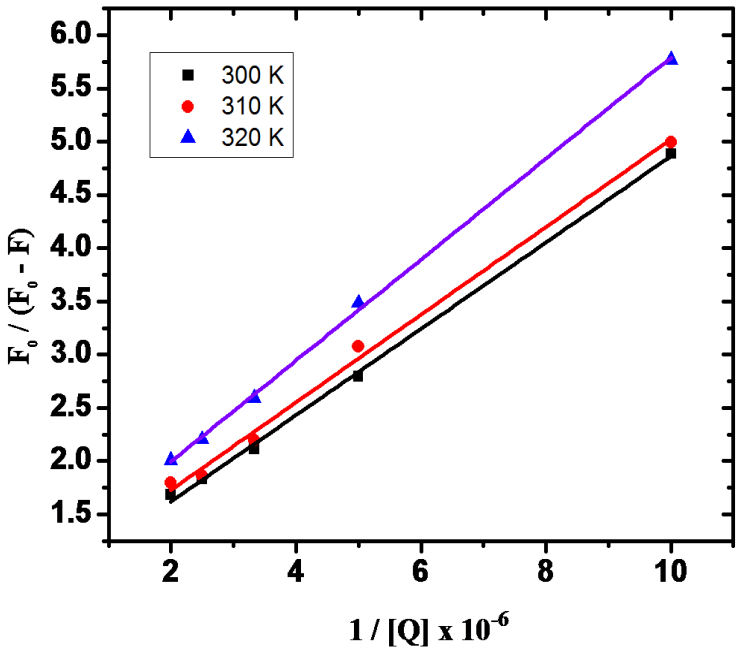
2) Structure and synthesis scheme of isatin derivative (ACBSIPID), (2-Amino-3-cyano-5-bromospiro(5H-indeno[1,2-b]pyran-4,3'-indoline)-2'5,-dione).



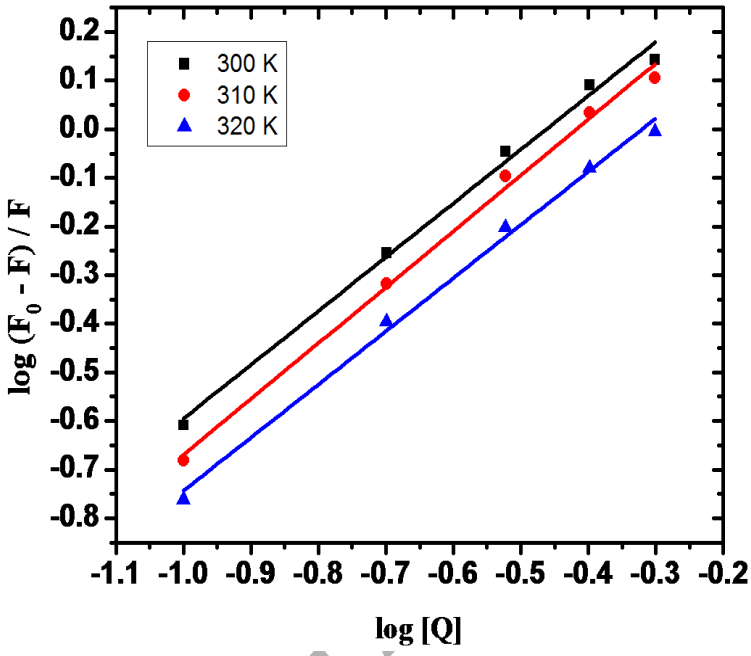
3) The fluorescence emission spectra of BSA with increasing concentration of ACBSIPID, CBSA = 1.0 x10-6 mol L-1, CACBSIPID /(10-6 mol L-1) a-g: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 (T = 300 K, pH = 7.4, λ = 280 nm).



4) Stern-volmer plots at three different temperatures. CBSA and CACBSIPID are same as those in Figure 3.

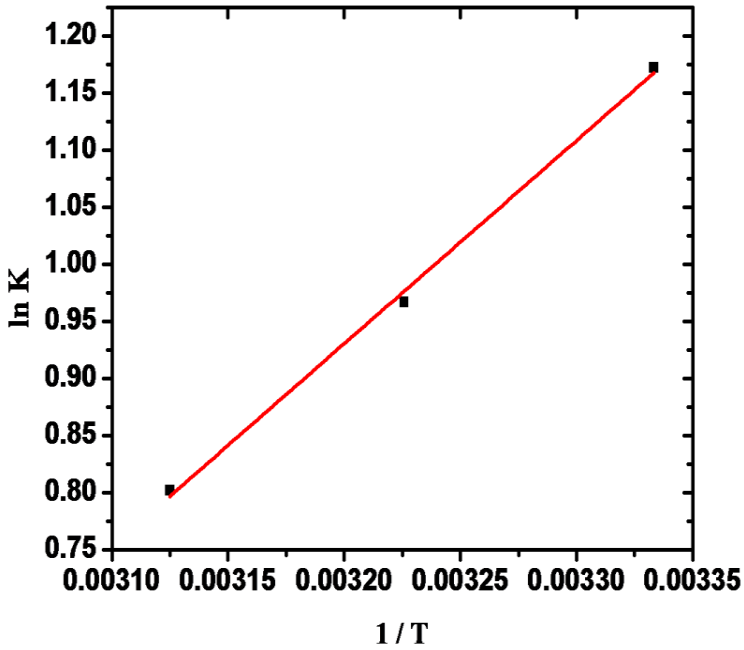


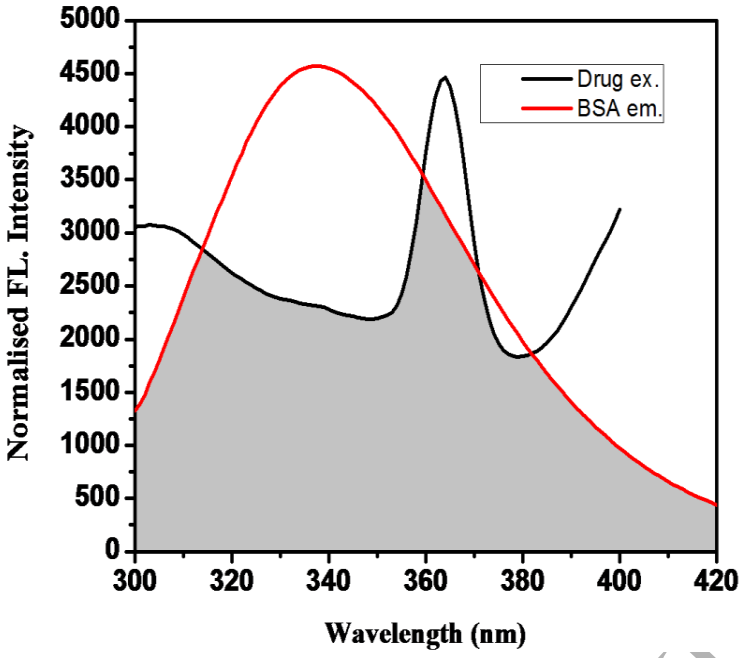
5) Modified Stern-Volmer Plot for the binding of BSA-ACBSIPID.



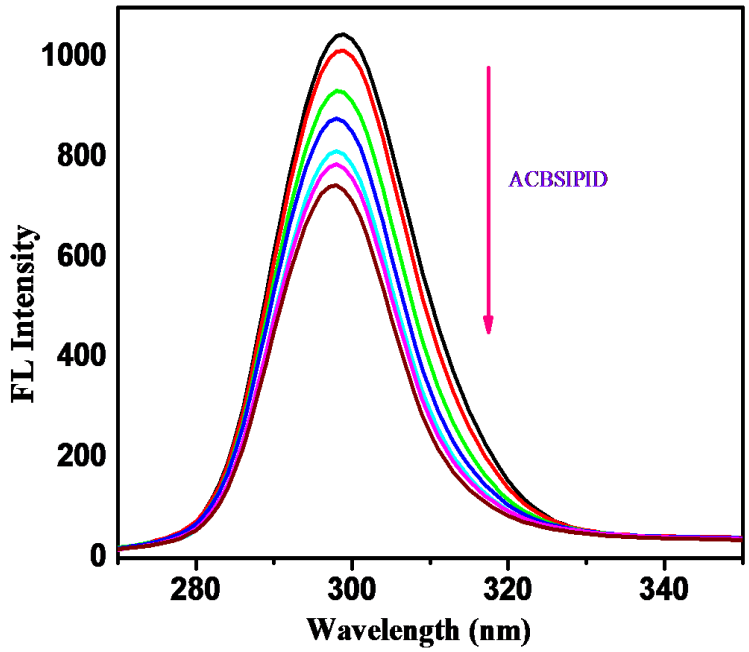
6) The plots of log (F0 – F) / F versus log [Q] at three different temperatures CBSA and CACBSIPID are the same as those in Figure 3

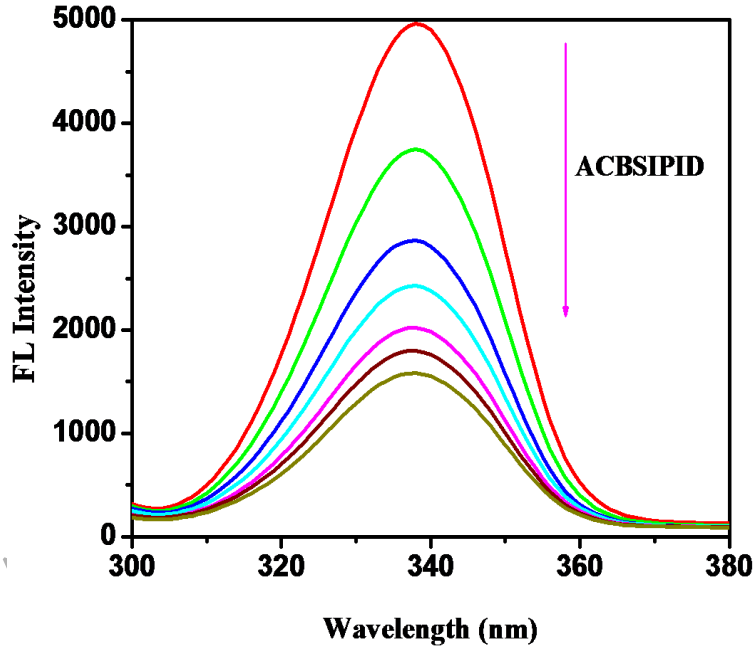
.7) Van't Hoff plot for the binding of BSA to ACBSIPID.





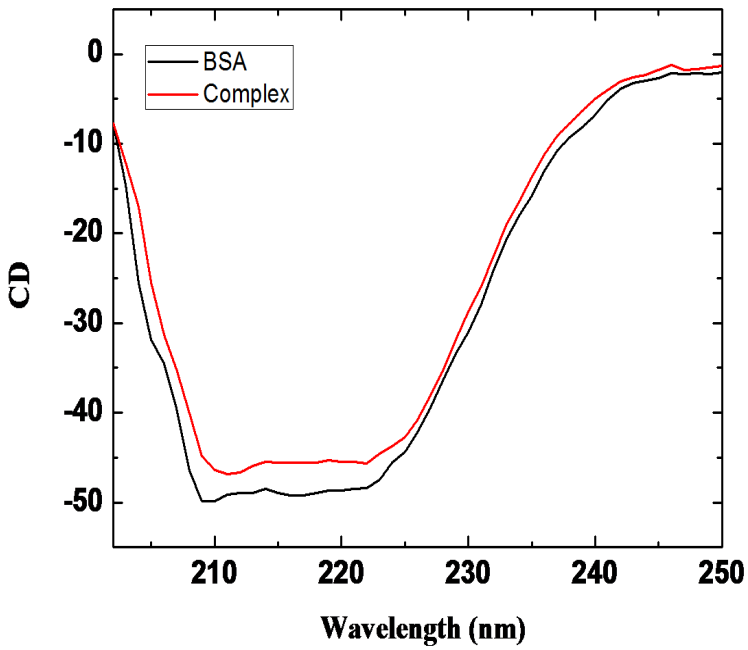
8) Spectral overlap of ACBSIPID excitation with BSA emission; T = 300 K, CBSA = 1x10-5 mol L-1 and CACBSIPID = 0.5 x 10-3 mol L-1.



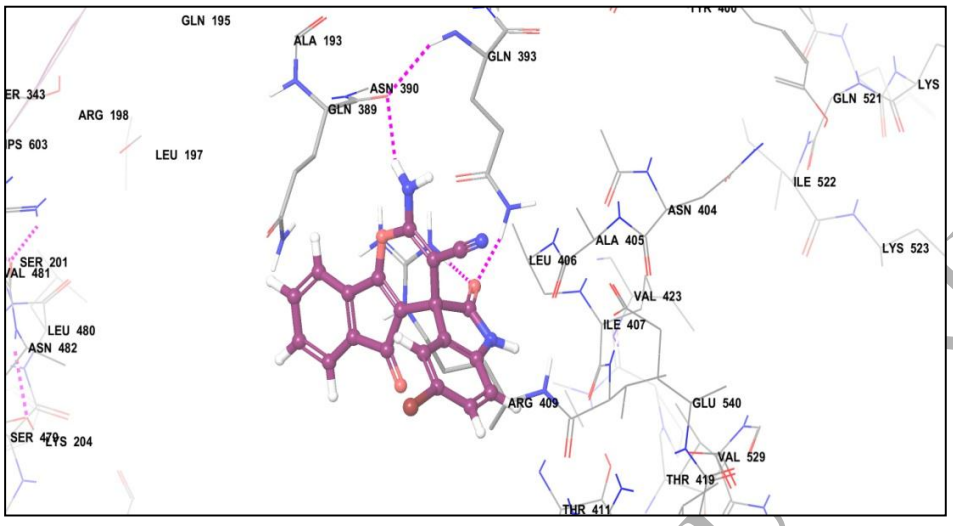


9) Synchronous fluorescence spectrum of BSA (T = 300 K, pH = 7.4), CBSA and CACBSIPID are the same as those in figure 3, (a) $\Delta\lambda$ = 15 nm and (b) $\Delta\lambda$ = 60 nm.

10) Circular dichroism (CD) spectra of free BSA and its ACBSIPID-BSA complex.



11) Docking pose of compound ACBSIPID.



1) Stern-Volmer quenching constants and bio-molecular quenching rate constants for BSA-ACBSIPID at various temperatures.

Tables:1)

pH	T (K)	106 Ksv /(Lmol-1)	1014Kq/(Lmol-1s-1)	R
7.4	300	2.91	2.91	0.9996
	310	2.76	2.76	0.9994
	320	2.03	2.03	0.9998

R = correlation constant

2) Binding constants (K) and number of binding stoichiometry (n) of competitive experiment of BSA-ACBSIPID system.

2)

T(K)	104K/(Lmol-1)	n	R
300	3.23	1.10	0.9944
310	3.09	1.14	0.9917
320	2.24	1.09	0.9938

R = correlation constant

3)

T (K)	ΔH (kJ/mol-1)	ΔG (kJ/mol-1)	ΔS (kJ/mol-1)	R
300				
-4.82	-29.32	9.76		
9.39				
6.71				
0.9963				
310		-29.15		
320		-21.52		

R = correlation constant