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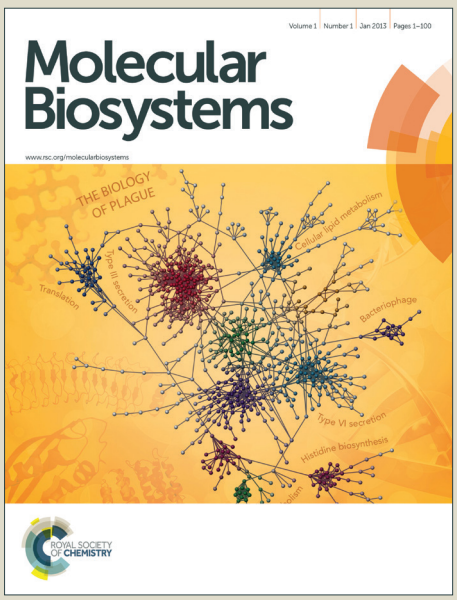

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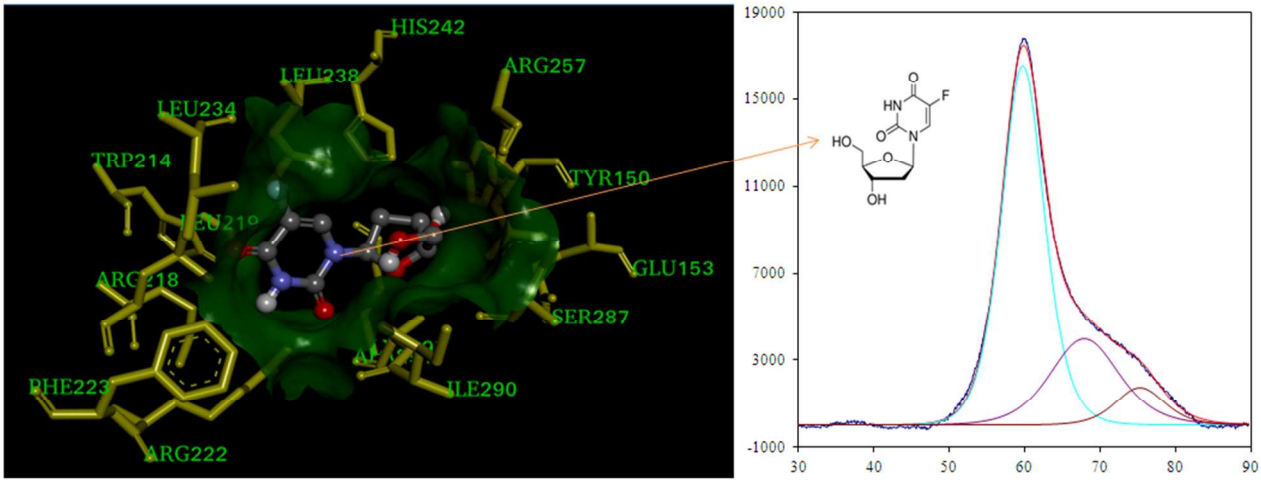

5-Fluoro-2'-Deoxyuridine an analog of 5-Fluorouracil have high binding affinity and thermal stability toward N-isoform in compare to B-isoform of HSA.

Research Article

Interaction of 5-Fluorouracil analog 5-Fluoro-2'-Deoxyuridine with 'N' and 'B' Isoform of Human Serum Albumin: A Spectroscopic and Calorimetric Study

Mohd Ishtikhar[1], Shawez Khan[2] Gamal Badr[3], Amany Osama[4] and Rizwan Hasan Khan[1]*

[1] *Protein Biophysics Laboratory, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh – 202002, India*
[2] *Department of Computer Science, Jamia Millia Islamia, Jamia Nagar, New Delhi-110025, India*
[3]*Laboratory of Immunology and Molecular Biology, Zoology Department, Faculty of Science Assiut University, Assiut, Egypt*
[4]*Department of Biochemistry, Faculty of Medicine, Assiut University, Assiut, Egypt*

*To whom correspondence should be addressed
Rizwan Hasan Khan
Interdisciplinary Biotechnology Unit,
Aligarh Muslim University,
Aligarh-202002
E-mail: rizwanhkhan1@gmail.com
Phone: +91-571-2727388
Fax: +91-571-2721776

Abstract

Drugs and metabolites are transported in the blood by plasma proteins, such as human serum albumin (HSA). The uridine analog 2'dFUrd, which is a cytotoxic prodrug metabolite of capecitabine, has remarkable activity against solid tumors when administered orally. We report the results of an *in vitro* experimental study on the interactions of 2'-dFUrd with the N-isoform (at pH 7.4) and B-isoform (at pH 9.0) of HSA, investigated using fluorescence spectroscopy, circular dichroism (CD), isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and molecular docking. The binding constant (K_b) was higher for the N-isoform than for the B-isoform. Thermodynamic parameters, such as enthalpy change (ΔH°), entropy change (ΔS°), and Gibbs free energy change (ΔG°), were also calculated for both interactions using calorimetric techniques. The thermostabilities of HSA and the HSA-2'dFUrd complex were found to be higher for the N-isoform. The interaction of 2'dFUrd with HSA was also explored in molecular docking studies, which revealed that 2'dFUrd was bound to Sudlow site I in subdomain IIA through multiple modes of interaction, such as hydrophobic interactions and hydrogen bonding. These results suggest that the binding affinity of 2'dFUrd for HSA is favors the N-isoform of HSA.

Keywords: 5-Fluoro-2'-deoxyuridine, Circular dichroism, Differential scanning calorimetry, Human serum albumin, Isothermal titration calorimetry, Capecitabine, Cancer

Abbreviations: 2'dFUrd, 5-Fluoro-2'-deoxyuridine; HSA, Human serum albumin; CD, Circular dichroism; DSC, Differential scanning calorimetry; ITC, Isothermal titration calorimetry.

Introduction

5-Fluoro-2'-deoxyuridine (2'dFUrd) is an antineoplastic agent that acts as an anti-metabolite in a fashion similar to 5-flurouracil (5-FU). 2'dFUrd (a cytotoxic prodrug metabolite of capecitabine) is commonly used in the treatment of advanced human cancers, especially colorectal and breast cancers[1]. Capecitabine is an oral systemic prodrug that is enzymatically converted to 5-FU. Strongly proliferating and tumor cells metabolize 5-FU to 5-fluoro-2-deoxyuridine monophosphate (FdUMP) and 5-fluorouridine triphosphate (FUTP). The cell injury caused by these metabolites follows two different mechanisms. First, they inhibit the formation of thymidine triphosphate, which is necessary for DNA synthesis. 2'dFUrd inhibits DNA synthesis by blocking thymidylic acid synthetase (TS)[2]. Second, during the synthesis of RNA, nuclear transcriptional enzymes may aberrantly integrate FUTP into the RNA. These metabolic errors can interfere with RNA processing and protein synthesis, and the levels of TS expression in cancer tissues are potential predictors of the response to 5-FU-based chemotherapy[3]. Therefore, it has been suggested that this compound can be used to target thymidylate synthase in cancer chemotherapy[4].

Human serum albumin (HSA), which is the most important carrier of drugs and other small molecules among the plasma proteins[5, 6], can bind different types of amphiphilic biological molecules and is believed to play an important role in determining the physiological functions of these molecules. Serum albumin is a convenient model to study the molecular basis of specific ligand–protein interactions due to the low binding stoichiometry of the protein and its receptor-like properties[7]. Estimations of the extent of drug–protein binding are crucial for clinical drug development. HSA contains 585 amino acids of known sequence[8] and has a molecular mass of 66.5 kDa[9]. Crystal structure analysis shows that the drug binding sites I and II are located in subdomains II A and III A, respectively. A large hydrophobic cavity is present in the II A

subdomain. In general, the binding of most drugs with proteins occurs in a reversible manner though weak chemical interactions, such as ionic, van der Waal's, hydrogen bonding, and hydrophobic interactions with the hydroxyl, carboxyl, and other sites available on the amino acids residues that constitute the binding sites on the protein[10]. The binding of drugs to the plasma and tissue proteins is considered to be an important factor in determining the pharmacokinetics and pharmacodynamics of these drugs. To understand the pharmacology of a specific drug and the association between its structure and function, the various modes of binding of the drug to proteins under different pH conditions should be considered[11]. Given that HSA is known to undergo different pH-dependent conformational transitions, it is a promising model for studying protein-drug interactions[12]. At pH 7.4, HSA assumes the native form (the N-isoform), which dramatically changes to an extremely charged form (the B-isoform) at pH 9.0. While the pH of the blood is normally stable, there are variations in pH among the blood, intracellular, and extracellular compartments in which the drug-HSA interactions take place. Therefore, the conversion states of HSA can affect the interactive properties of the drug[13].

In the present study, we investigated the interactions between 2'dFUrd and HSA by utilizing several spectroscopic and calorimetric techniques, as well as thermodynamic and molecular docking experiments to examine the mode and type of forces responsible for the interactions and the drug-induced conformational changes in HSA that are responsible for of the different interactions seen for the N-isoform and B-isoform. An additional aim of the present study was to investigate as a function of temperature whether the binding is dynamic or static. The binding of a drug to a protein may affect the conformation as well as the stability of that protein. Thus, using fluorescence spectroscopy, the thermodynamic and calorimetric effects on the N-isoform and B-isoform of HSA were monitored. Furthermore, the impacts on the HSA-2'dFUrd

interaction of using different temperatures and concentration ratios were determined. The strands of information derived from the molecular-interaction data using multiple techniques are dissected to understand and explore the intricacies of protein-ligand interactions.

MATERIALS AND METHODS

Materials

Fatty acid-free human serum albumin (A1887), 5-fluoro-2'-deoxyuridine (F0503), and glycine (G8898) buffer were procured from Sigma Aldrich. MOPS (134894) buffer was procured from SRL.

Sample preparation

First, 20 mM MOPS (pH 7.4) and glycine-NaOH buffer (pH 9.0) were prepared using a Mettler-Toledo pH meter. These buffers were filtered through a 0.45-µm syringe filter. For the 20308 µM stock, 10 mg of 2'dFUrd were dissolved in the respective buffers and volume was adjusted to 2 ml. Protein concentrations were determined spectrophotometrically using an $E_{1\,cm}^{1\%}$ value of 5.30 at 280 nm[14] in a Perkin-Elmer Lambda 25 spectrophotometer.

Fluorescence quenching measurements

All fluorescence measurements were made on a Shimadzu (5301PC) spectrofluorophotometer equipped with a constant temperature holder attached to a Neslab RTE-110 waterbath, which had a temperature accuracy of ±0.1°C. Intrinsic fluorescence was measured by exciting HSA at 295 nm, as tryptophan (Trp214) fluorescence is used as a probe of the local environment in a protein for the determination of protein structure and dynamics, as well as ligand binding. The decrease in fluorescence intensity at 340 nm was analyzed according to the Stern-Volmer equation[15]:

$$\frac{F_o}{F} = Ksv\,[Q] + 1 \quad (1)$$

where $F_\circ$ and F are the fluorescence intensities in the absence and presence of quencher (2'dFUrd), respectively, K_{sv} is the Stern-Volmer quenching constant and:

$$Ksv = k_q \tau_o \quad (2)$$

where k_q is the bimolecular rate constant of the quenching reaction, and $\tau_\circ$ is the average integral fluorescence lifetime of tryptophan, which is ~5.7×10^{-9} sec[16]. Binding constants and binding sites were obtained from[17]:

$$\log\left(\frac{F_o}{F} - 1\right) = \log K_b + n \log[Q] \quad (3)$$

where, K_b is the binding constant and n is the binding stoichiometry.

The changes in enthalpy, entropy, and free energy at different temperatures were derived from:

$$\ln K_b = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} \quad (4)$$

and

$$\Delta G^o = -RT\ln K \quad (5)$$

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

where K_b is the binding constant, ΔH^o is the enthalpy change, ΔG^o is the Gibbs free energy change, ΔS^o is the entropy change, and R is the gas constant (1.987 cal mol^{-1} K^{-1}).

Three-dimensional fluorescence spectra were created from measurements made in the F-4500 Hitachi fluorescence spectroscope under the following conditions: the emission wavelength was recorded between 200 nm and 600 nm; the initial excitation wavelength was set at 200 nm with an increment of 5 nm; and the excitation and emission slit widths were set at 10 nm, respectively.

Competitive binding experiments

In the displacement experiments, a solution of 2'dFUrd was gradually titrated to a pre-incubated solution that contained HSA (2 μM) and site marker (diazepam and warfarin), in which the ratio of the concentration of HSA to site marker was 1:1. The excitation and emission wavelengths used for HSA and the site marker system were 295 nm and 340 nm, respectively.

Circular dichroism measurements

Isothermal wavelength scans of HSA in the absence and presence of 2'dFUrd were carried out in a JASCO-J-815 spectropolarimeter equipped with a Peltier-type temperature controller. All the CD spectra were collected in cells of 1-mm path length, and a protein concentration of 2 μM was used for the far-UV CD measurements. The scan speed was 100 nm/min, and a response time of 1 s was used for all the measurements. The raw data obtained (in millidegrees) was converted into mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$, which is given by:

$$\text{MRE} = \frac{\theta_{\text{obs}}\,(m\,\text{deg})}{10 \times n \times C \times l} \tag{7}$$

where θ_{obs} is the observed ellipticity in millidegrees, C is the protein concentration in molar terms, n is the number of amino acid residues, and l is the length of the light path in centimeters. All the spectra were smoothed by the Savitzky–Golay method with 15-convolution width, and the helical content was calculated from the MRE values at 222 nm using the following equation[18]:

$$\%\alpha\text{-helix} = \left(\frac{\text{MRE}_{222\,\text{nm}} - 2{,}340}{30{,}300}\right) \times 100 \tag{8}$$

Isothermal titration calorimetric measurements

The energetics of the binding of 2'dFUrd to HSA at 15°C, 25°C, and 37°C were measured using a VP-ITC titration microcalorimeter (MicroCal Inc., Northampton, MA). Prior to the titration experiment, all samples were degassed in a thermovac. The sample and reference cells of the

calorimeter were loaded with HSA solution (25 μM) and 20 mM MOPS buffer (pH 7.4) and glycine buffer (pH 9.0), respectively. Multiple injections of 10 μl of 2'dFUrd solution (2.0 mM) were made into the sample cell that contained HSA. Each injection was made over 20 s, with an interval of 180 s between successive injections. The reference power and stirring speed were set at 16 μcal s^{-1} and 307 rpm, respectively. The heats of dilution for the ligands were determined in control experiments, and these values were subtracted from the integrated data before curve fitting.

The first derivative of temperature dependence of the enthalpy change was used for the calculation of experimental heat capacity change, as calculated from[19]:

$$\Delta C_P^{exp} = \frac{d\Delta H}{dT} \tag{9}$$

The temperature-dependent van't Hoff enthalpy (ΔH_{vH}) at each temperature was calculated by the equation:

$$\Delta H_{vH} = \left[\frac{\left\{ \ln \frac{K(T_2)}{K(T_1)} - \frac{\Delta C_P}{R} \ln \frac{T_2}{T_1} + \frac{\Delta C_P T_1}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \right\} \times R}{\left(\frac{1}{T_1} - \frac{1}{T_2} \right)} \right] \tag{10}$$

where, T_1 and T_2 are the maximum and minimum experimental temperatures, respectively, and $K(T_1)$ and $K(T_2)$ are the values of the binding constants at these respective temperatures.

Differential scanning calorimetry

Thermal denaturation experiments were conducted in a VP-DSC microcalorimeter (MicroCal). The DSC scans spanned 20°–90°C at a rate of 1°C min^{-1}. The experiments were performed using 15 μM HSA. The respective reference scans were run under identical DSC set-up conditions and the obtained values were subtracted from each sample scan. The heat capacity curves, midpoint

temperature (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_{vH}) values were analyzed using the Origin ver. 7.0 software.

Molecular modeling and docking

The three-dimensional structure of HSA was downloaded from the RCSB Protein Data Bank [PDB: 1AO6]. All the water molecules were removed and hydrogen atoms were added. The three-dimensional structure of 2'dFUrd was downloaded from PubChem [CID: 5790]. The docking simulation for HSA and 2'dFUrd, was performed using the AutoDock ver. 4.0 software, which was also used to evaluate the ligand binding energy over the conformational search space using the Lamarckian genetic algorithm. The size of the grid box was set at 60×60×60 Å, and the spacing between grid-points was 0.37 Å. Amino acid residues that fell within 5 Å of the binding site of HSA were extracted and combined to define the residues constituting the binding site. All the other parameters used were set at the default values. Graphical representations were prepared using the Pymol Molecular viewer.

RESULTS AND DISCUSSION

Tryptophan fluorescence quenching by 2'dFUrd

Analyses of tryptophan fluorescence quenching for determination of the interactions between 2'dFUrd and the N-isoform and B-isoform of HSA were performed by titrations of 2'dFUrd against the protein at 15°C, 25°C, and 37°C. Figure 1 (A-I and B-I) shows that HSA has a strong fluorescence emission peak at ~340 nm upon excitation at 295 nm, and that following addition of the drug (0–100 μM) there is a decrease in the emission spectral intensity of HSA without a significant change in the wavelength of the maximal fluorescence emission (λ_{max}). The changes in the emission intensity values at 340 nm during titration were used to measure the drug-binding affinity. The fluorescence intensity of Trp214 decreased continuously, although at higher

concentrations of 2'dFUrd (in the range of 90–100 μM), the pattern of decreasing fluorescence intensity displayed a mutual overlap, indicating saturation of binding of 2'dFUrd to a specific binding site on HSA. In Figure 1, the saturation pattern is much more evident for the N-isoform than for the B-isoform of HSA. The same experiments were performed at 15ºC and 37ºC. We found as the temperature increased, the value of K_{sv} decreased. The decrease in fluorescence intensity upon addition of drug was analyzed according to the Stern-Volmer equation. The finding (Figure 1, A-II and B-II) that the Stern-Volmer quenching constant K_{sv} was inversely correlated with temperature and that k_q was greater than 2×10^{10} M^{-1} s^{-1}, which indicate the specific binding of the ligand to the protein, reveals a 'static quenching' mechanism[15, 20, 21]. The Ksv values for 2'dFUrd at different temperatures and for different values of pH are listed in Table 1.

Determination of binding constant and binding stoichiometry

For the determinations of the binding constant and binding stoichiometry, $\log[(F_o/F) -1]$ was plotted against log[2'dFUrd], as shown in Figure 1, A-III and B-III. The data (Table 1), which were calculated from Eq. 3 show that there was a decrease in K_{sv} or an increase in K_b after an increase of temperature for both the N- and B-isoforms of HSA. In addition, the K_{sv} values were higher for the N-isoform (3.17×10^3 M^{-1}) than for the B-isoform (2.38×10^3 M^{-1}) at 25ºC. This implies that for the B-isoform of HSA, binding to 2'dFUrd is reduced. These pH-induced changes in the microenvironment of the protein at the drug-binding site affect the mode and mechanism of quenching and ultimately the drug-binding ability, which in turn influence the bioavailability of the drug and its distribution in the bloodstream.

Thermodynamics of HSA-2'dFUrd complex formation

The thermodynamic parameters, i.e., enthalpy change (ΔH°) and entropy change (ΔS°), associated with the reaction are very important for confirming binding modes. The temperature-dependencies of the binding constants were investigated for all three temperatures, considering that HSA could not undergo any structural changes.

The thermodynamic parameters were determined from the linear van't Hoff plot (Figure 1, A-III and B-III) (Table 1). For determination of the enthalpy-entropy relationship of the protein-drug interactions, we considered three temperatures (15°C, 25°C, and 37 °C) based on the assumption that during the binding process the structure of the protein is unaltered, given that major conformational changes result in false readings of the thermodynamic parameters in interaction studies. In other words, the obtained enthalpy-entropy changes are assumed to be linked to the binding of the 2'dFUrd molecule to HSA through rigid-body docking. The negative value of ΔG° (Table 1) reveals that the interaction process is spontaneous. Hydrogen bonds and van der Waal's interactions can play major roles in protein-ligand interactions[22]. In the case of the N-isoform and B-isoform of HSA, the ΔH° and ΔS° values for complex formation between 2'dFUrd and HSA were found to be 4.59 and 11.15 kcal mol^{-1} and 30.55 and 52.81 cal $mol^{-1}K^{-1}$, respectively. Thus, formation of the 2'dFUrd-HSA complex is an endothermic reaction that is accompanied by a positive ΔS° value (Figure 2B). The role of water bound to the protein molecule in or in close proximity to the binding pockets could be important, since we observed a positive value for TΔSº, which may indicate that the water molecules were removed upon binding.

Competitive binding of 2'dFUrd to HSA in the presence of site markers at 37°C

To facilitate the identification of the precise binding site, we used site-specific probes that are known to bind to a recognized region of HSA[23]. It has been already been established by X–ray crystallography that warfarin is a suitable probe for subdomain IIA or Sudlow site I and that

diazepam acts as a probe for subdomain IIIA or Sudlow site II[24]. To compare the effects of site markers on the HSA-2'dFUrd complexes, the changes in fluorescence intensity (FI; at 340 nm) in the absence and presence of site-specific probes were plotted using the Stern-Volmer equation (Figure 2A). The slight decrease in fluorescence intensity observed for 2'dFUrd in the presence of diazepam reveals that 2'dFUrd competes with diazepam. This suggests that 2'dFUrd binds weakly to Sudlow site II is located in subdomain IIIA. However, the presence of warfarin resulted in positive heterotropic cooperativity or allosteric activation of binding to 2'dFUrd. This implies that when HSA interacts with this probe it acquires a higher affinity for binding to 2'dFUrd.

Three-dimensional conformational investigation of 2'dFUrd binding to HSA using fluorescence spectroscopy

To examine further the conformational alterations induced in HSA by the addition of 2'dFUrd, we examined the three-dimensional fluorescence spectra of HSA and the HSA-2'dFUrd complex. According to Figure 3 and Table 2, which displays the three-dimensional spectra, two representative fluorescence peaks, peak 1 and peak 2, are clearly observed, while peak 3 and peak 4 represent a Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) and a second-order scattering peak ($\lambda_{em} = 2\lambda_{ex}$), respectively[25]. These peaks indicate that binding causes a conformational change in HSA, such that the fluorescence of HSA is quenched by 2'dFUrd. HSA was excited at 290 nm; the intrinsic fluorescence of aromatic amino acid residues that shows negligible fluorescence [26] therefore we presume that peak 1 represents the polypeptide backbone structures and that peak 2 is characteristic for tryptophan and tyrosine residues.

Circular dichroism measurements

To obtain an insight into the secondary structural changes of HSA, the far-UV CD spectra were recorded in the presence and absence of drug. CD spectra of HSA in the presence of different concentrations of 2'dFUrd at pH 7.4 (4A-I) and pH 9.0 (4A-II). The binding of ligands to globular proteins affects the intermolecular forces that are responsible for maintaining the secondary and tertiary structures, which if altered result in conformational changes to the proteins[27]. The CD spectra of serum albumins reveal two negative bands in the far-UV region at 208 nm and 222 nm, which are characteristic of the α-helical form/structure of the protein[28]. The interaction of 2'dFUrd with HSA induced an increase in the helical content of the protein. For the N-isoform of HSA, the % α-helicity, which was calculated using the method of Chen et al.[18],was 58.10%, and after the addition of 2-, 5-, and 10-fold molar excesses of 2'dFUrd, the % α-helicity increased to 61.08%, 64.42%, and 69.34%, respectively. For the B-isoform of HSA, the % α-helicity was 53.90%, and this increased after the addition of 2-, 5-, and 10-fold molar excesses of 2'dFUrd to 57.18%, 60.57%, and 63.70%, respectively. However, the change in helicity was less pronounced for the B-isoform of HSA, which appeared to be more stable in the presence of 2'dFUrd.

Isothermal titration calorimetric measurements

ITC is used to measure the affinity and associated thermodynamics of the interaction between 2'dFUrd and HSA. Thermodynamic properties, i.e., enthalpy (ΔH°) and entropy (ΔS°) changes, were determined for the N-isoform and B-isoform of HSA. Representative calorimetric titration profiles of the interactions between 2'dFUrd and HSA at 37°C are shown in Figure 4, B-I and B-II. In the upper panels of the figure, each peak in the binding isotherm represents a single injection of the drug into the protein solution. The lower panels show integrated plots of the amount of heat liberated per injection as a function of the molar ratio of the drug to the protein.

The association constant (K_b) and enthalpy change ($\Delta H°$) were directly obtained after fitting with the non-linear, least-square, regression single-set binding site that had the lowest χ^2 value. The binding of 2'dFUrd shows exothermic characteristics and the best-fitted data are indicated by the solid line in the profile. The Gibbs free energy and entropy changes were calculated from Eqs. 5 and 6, respectively.

The thermodynamics parameters of the 2'dFUrd-HSA complexes are summarized in Table 3. The titration of 2'dFUrd to HSA showed negative heat deflection, which indicates that the binding of 2'dFUrd to HSA is an exothermic process for both isoforms. According to the results, the binding affinity is in the order of 10^3, so the affinity for binding sites appears to be pH-dependent. To elucidate the forces that drive the interaction of 2'dFUrd with HSA, the effect on complex formation at 37°C was examined. A comparison of the ITC data in terms of the fitted curves is shown in Table 3; these results suggest that the mode and mechanism of binding are dependent upon the pH, which may influence drug availability and distribution within the bloodstream. The thermodynamics of the HSA-2'dFUrd interactions, i.e., the negative values for $\Delta H°$ and $\Delta S°$ at both of the studied pH levels, suggest the involvement of hydrogen bonding and conformational changes during the formation of the HSA-2'dFUrd complex[29]. The binding stoichiometry obtained by spectroscopy and calorimetry showed a minor discrepancy, which may have been due to a photophysical phenomenon related to the presence of a single Trp214 residue in the HSA[23] used in these studies. The discrepancies in enthalpic changes indicate a linkage between the contribution of the shift in solvent ions and the conformational changes that occur during the binding process, which may be induced by either drug binding or an increase in temperature in both isoforms of HSA used in these studies[30]. That the values shown in Tables 1 and 3, which were obtained by fluorescence spectroscopy and ITC, respectively, are different

from each other may be due to the locations of the fluorophore and quencher[30-32]. Not only the binding affinities, but also the thermodynamic values obtained using both techniques showed variability, which is assumed to reflect temperature-independency, as reported in the literature, which means that the values obtained from ITC are more plausible[16, 29, 33, 34]. However, in the literature, non-calorimetric determination of thermodynamic parameters from fluorescence quenching data collected at different temperatures has been used to generate an estimated result[16, 23, 29, 34] .

Thermostability measurements by differential scanning calorimetry

Figure 5, A-1, A-II, A-III and B-I, B-II, B-III show the archetypal intemperance heat capacity curves for HSA-2'dFUrd complexes in the molar ratios of 1:0, 1:50, and 1:100 and the thermodynamic parameters associated with the thermodynamic denaturation of the N-isoform and B-isoform of HSA. Under both conditions, the HSA:drug molar ratios exhibit higher T_m values with the thermogram shifted towards a higher temperature, as compared with the peaks for the native HSA. After the addition of 2'dFUrd, a positive change in heat capacity was observed, suggesting an increase in the hydrophobic surface area of the protein, which accords with the results our fluorescence studies. By reheating the sample after cooling just after the first run, it could be shown that the thermal unfolding of HSA was irreversible in the absence or presence of 2'dFUrd. Thus, to diminish the kinetic factors, a slower scanning rate was selected.

The changes in Tm and ΔH^o of the protein in the presence of ligand are the most obvious demonstration of ligand binding effects, which can be estimated by DSC[35]. Thus, to confirm the binding of 5'dFUrd to HSA, changes in the Tm and ΔH^o were monitored by DSC. The denaturation of HSA yielded more than one endothermic peak, reflecting the domain denaturation mechanism[36]. Thus, it was deconvoluted with the assumption of three sub-

transitions, each of which might be related to the linkages between the three structural domains of HSA. Furthermore, it has been established that domain III melts prior to domain II, so T_{m1} may correspond to domain III[37]. As shown in Table 4, as the molar ratio increased to 1:100, theT_{m1} increased appreciably and T_{m2} changed slightly, whereas T_{m3} did not change at all. Moreover, the increases in T_{m1} and T_{m2} were accompanied by increases in the values of the enthalpy of unfolding, albeit only slightly in the latter case. Thus, 2'dFUrd preferentially binds to the folded or native form of HSA to stabilize the folded state, which means that unfolding of HSA becomes progressively less favorable as the concentration of 2'dFUrd is increased[38].

Molecular docking

The molecular docking analyses indicate that the most important regions of the ligand binding sites in HSA are located in the hydrophobic cavity of subdomain IIA, which is consistent with Sudlow site I[39, 40]. The molecular docking model of the HSA-2'dFUrd complex was examined on the basis of the crystal structure of HSA, to determine the binding residues and their positions. The best energy ranking is shown in Figure 6, and the geometry exhibits the lowest free energy. The docking result shows that 2'dFUrd binds within the binding pocket of subdomain IIA with a binding energy of -6.21 kcal mol^{-1}. The inside wall of the pocket of subdomain IIA consists of hydrophobic amino acids, whereas the entrance to the pocket is surrounded by positively charged residues, consisting of Lys195, Arg218, Arg222, Phe223, Arg257, and Ala291[41], as shown in Figure 6A. Therefore, we conclude that 2'dFUrd is able to fit well within the hydrophobic cavity of subdomain IIA (Figure 6B). The 2'dFUrd molecule moiety was located within the binding pocket and was adjacent to hydrophobic residues Tyr150, Trp214, Leu219, Leu234, Leu238, His242, Ser287 and Ile290 of Sudlow site I in subdomain IIA of HSA[42] in Figure 6A. Therefore, the nature of the 2'dFUrd-binding site on HSA is in good agreement with the results of our

fluorescence and displacement experiments. The drug binds to the site essentially *via* hydrophobic interactions and hydrogen bonding.

Conclusions

In this study, we have evaluated the effect of pH on protein-drug interactions. The observed results indicate that the quenching mechanism of Trp214 fluorescence of HSA, upon binding of 2'dFUrd is a spontaneous and static event involving both hydrogen bonding and hydrophobic interactions. The significance of intrinsic fluorescence quenching of HSA by 2'dFUrd demonstrates that the drug bound in the vicinity of the single Trp214 which is situated in the binding cavity of subdomain IIA. This fact was further supported by competitive binding experiments with warfarin and diazepam, where the strong binding of 2'dFUrd to Sudlow's Site I of HSA could be clearly seen. The circular dichroism study suggests that there were more secondary structural changes upon addition of 2'dFUrd under 'N' isoform in comparison to 'B' isoform of HSA. These results also recommend stability of secondary structure that exists higher in 'N' isoform. Interaction of 2'dFUrd with HSA was furthermore demonstrated by ITC experiments, which supported our other spectroscopic results, with the thermodynamic parameters being accurately measured through the ITC experiments. Thermal stability was observed by DSC, thermogram in both conditions shifted progressively towards higher temperatures in a similar manner upon addition of different molar ratio of 2'dFUrd to HSA. Molecular docking studies also recommended that the 2'dFUrd was mainly bound at Sudlow site I in subdomain IIA of HSA through hydrogen-bonding and hydrophobic interactions. Overall the binding and thermal stability property of "N" isoform was higher in presence of 2'dFUrd in

compare to "B" isoform of HSA, that have a great significance in the field of drug development and the protein interaction research community.

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Table 1. Binding isotherms for the HSA-2'dFUrd complexes at different temperatures

pH/HSA isoform	**Tempe** (°C)	**K_{sv}** $\times10^3$ (M^{-1})	**k_q** $\times10^{11}$ ($M^{-1}s^{-1}$)	**n**	**K_b** $\times10^3$ (M^{-1})	**ΔG** (kcal mol^{-1})	**ΔH** (kcal mol^{-1})	**ΔS** (cal mol^{-1} K^{-1})	**Dominant interactions forces involved** (Inferred)
7.4/N	15	3.39±0.04	5.95±0.02	0.925	1.62±0.02	–4.23±0.017			
	25	3.17±0.06	5.57±0.03	0.985	2.72±0.05	–4.68±0.024	4.599	30.55	Hydrophobic
	37	2.92±0.07	5.13±0.02	0.999	2.91±0.04	–5.91±0.032			
9.0/B	15	2.53±0.08	4.44±0.02	0.924	1.20±0.03	–4.05±0.075			
	25	2.38±0.05	4.18±0.05	1.002	2.44±0.05	–4.62±0.039	11.151	52.81	Hydrophobic
	37	2.12±0.04	3.73±0.03	0.998	4.89±0.04	–5.23±0.023			

Table 2. Three-dimensional fluorescence spectra characteristics of 2'dFUrd complexed with the N-isoform or B-isoform of HSA at pH 7.4 and pH 9.0, respectively

pH/HSA isoform	System	Peak 1 ($\lambda_{ex}/\lambda_{em}$) (nm/nm)	Δλ (*F*)	Intensity	Peak 2 (nm)	Δλ (*F*)	Intensity ($\lambda_{ex}/\lambda_{em}$) (nm/nm)	Peak 3 (nm)	Δλ (*F*)	Intensity
7.4/N	HSA	280/335	55	210.8	230/330	100	168.2	280/280→ 350/350	0	114.6→ 172.4
	HSA + 2'dFUrd (1:1)	280/335	55	183.3	230/325	95	160.4	286/286→ 350/350	0	130.7→ 211.3
9.0/B	HSA	280/330	50	207.5	225/335	110	173.0	280/280→ 350/350	0	138.6→ 226.5
	HSA + 2'dFUrd (1:1)	280/335	50	205.1	230/335	105	180.2	285/285→ 350/350	0	146.4→ 228.9

Table 3. Thermodynamic parameters of 2'dFUrd binding to HSA, as calculated from ITC data at pH 7.4 and pH 9.0

pH/HSA isoform	**Temperature** (°C)	**n**	**K_b** (M^{-1}) $\times 10^3$	**ΔG°** (kcal mol^{-1})	**ΔH°** (kcal mol^{-1})	**TΔS°** (kcal $mol^{-1}deg^{-1}$)
7.4/N	37	0.969	7.24±0.837	-5.277	-4.958 ±0.368	0.319
9.0/B	37	0.920	5.43±0.956	-5.168	-5.710±0.598	-0.542

Table 4. Thermodynamic parameters of the thermal unfolding of has, as measured by DSC at pH 7.4 and pH 9.0

pH/ HSA isoform	HSA: 2'dFUrd	T_m^1 (°C)	T_m^2 (°C)	T_m^3 (°C)	ΔH^1	ΔH^2	ΔH^3
7.4/N	1:00	57.92±0.02	65.70±0.17	74.45±0.22	91.86±2.54	38.00±4.85	16.76±2.40
	1:50	58.32±0.02	66.84±0.20	74.55±0.27	107.40±2.05	36.48±5.01	-15.37±3.06
	1:100	59.84±0.01	68.08±0.21	75.39±0.30	125.60±2.23	48.57±6.07	14.98±3.92
9.0/B	1:00	52.06±0.14	60.23±0.23	72.52±0.21	37.70±2.85	44.66±3.77	12.09±1.05
	1:50	52.54±0.05	61.63±0.14	72.03±0.16	43.62±1.28	26.29±2.05	09.57±0.86
	1:100	53.11±0.04	63.00±0.13	72.29±0.21	72.24±0.92	25.82±1.92	16.75±1.19

T_m is expressed in °C.
ΔH is expressed in kcal/mole

Figure Captions

Figure 1: (A-I) and (B-I): Fluorescence emission spectra of HSA (2 μM) in the presence of 2'dFUrd (0–100 μM). (A-II) and (B-II): Plot of log [(Fo/F)-1] *vs* log[Q] for the determination of the binding constants and binding sites for the HSA- 2'dFUrd complexes at 15°C, 25°C, and 37°C and at pH 7.4 and pH 9.0, respectively.

Figure 2: (A) Competitive binding of 2'dFUrd to HSA in the presence of site markers at 37ºC [HSA at 2 μM; site markers at 4 μM; 2'dFUrd at 2 μM]. (B) van't Hoff plot and thermodynamic signatures (inset) for the HSA- 2'dFUrd complexes involving the N- and B-isoforms.

Figure 3: Three-dimensional fluorescence spectra (3A and 3B) of HSA (2 μM) (A-I and B-I) and the HSA-2'dFUrd complex (ratio of 1:1) (A-II and B-II) at pH 7.4 and at pH 9.0.

Figure 4: A: CD spectra of HSA in the presence of different concentrations of 2'dFUrd at pH 7.4 (A-I) and pH 9.0 (A-II), respectively. **B:** ITC profiles of the HSA-2'dFUrd system for pH 7.4 (B-I) and pH 9.0 (B-II) at 37ºC. MRE, mean residue ellipticity.

Figure 5: DSC profiles of native HSA and modified HSA at molar ratios (HSA to 2'dFUrd) of 1:0, 1:50 and 1:100 at pH 7.4 **(**A-I to A-III, respectively) and at pH 9.0 (B-I to B-III, respectively.

Figure 6: Molecular docking results for HSA complexed with 2'dFUrd. (A) 2'dFUrd is shown in a ball-and-stick representation in the binding pocket of HSA; the yellow sticks represent different amino acids in the grid box. (B) Detailed view of the docking poses of the HSA-2'dFUrd complex; selected protein side-chains are shown as ribbons.

Illustration:

Figure 1.

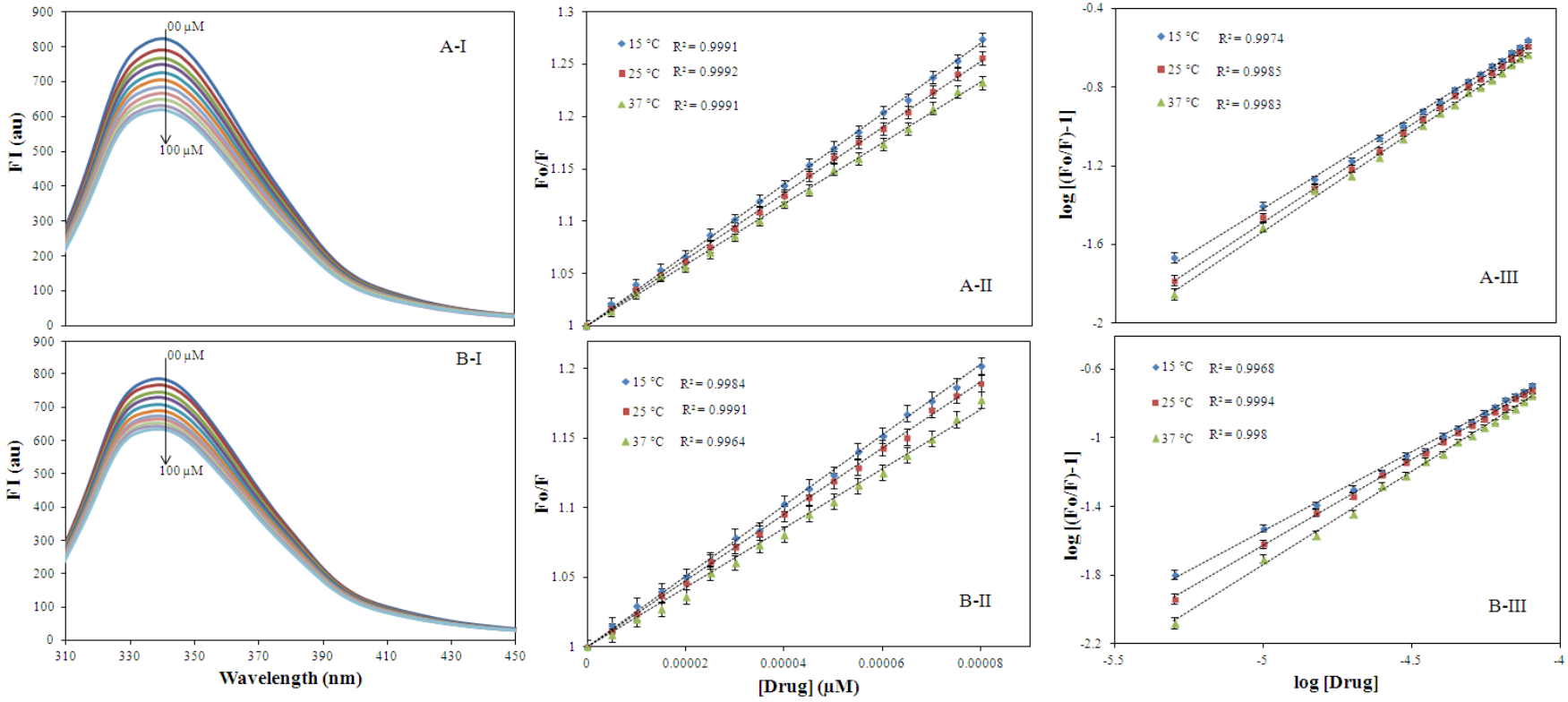

Figure 2.

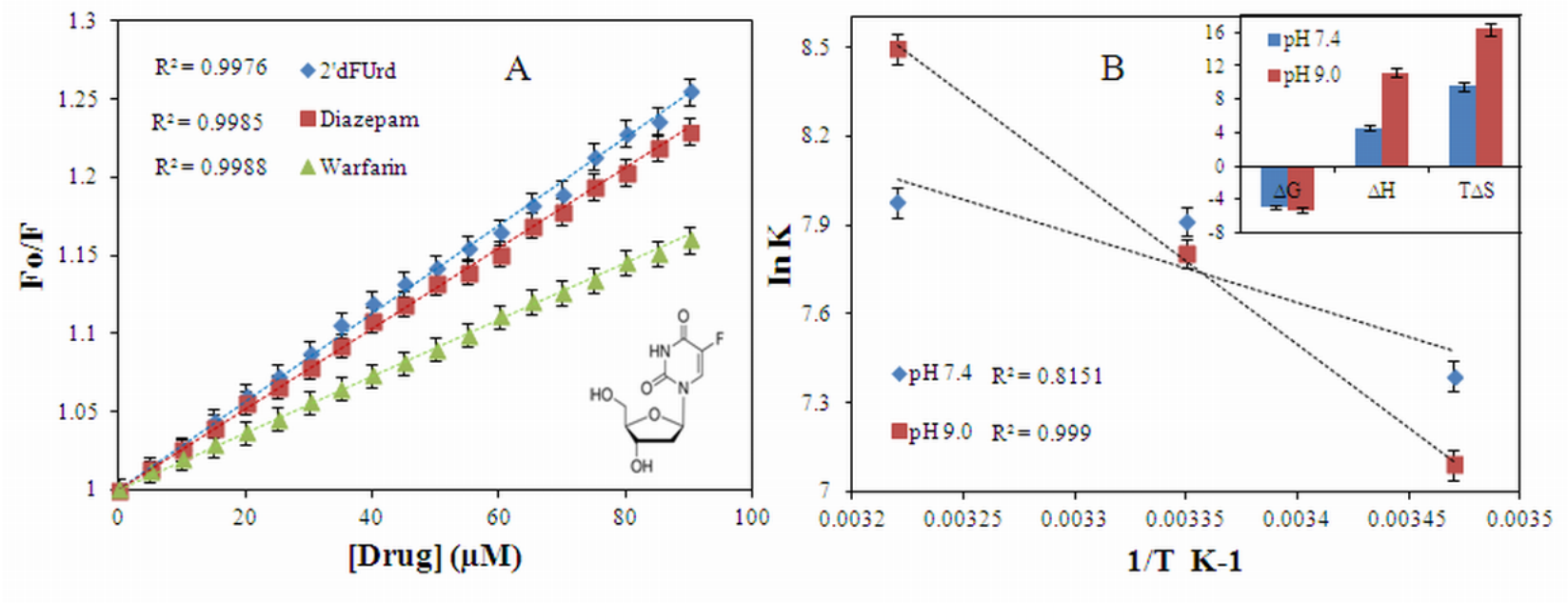

Figure 3A.

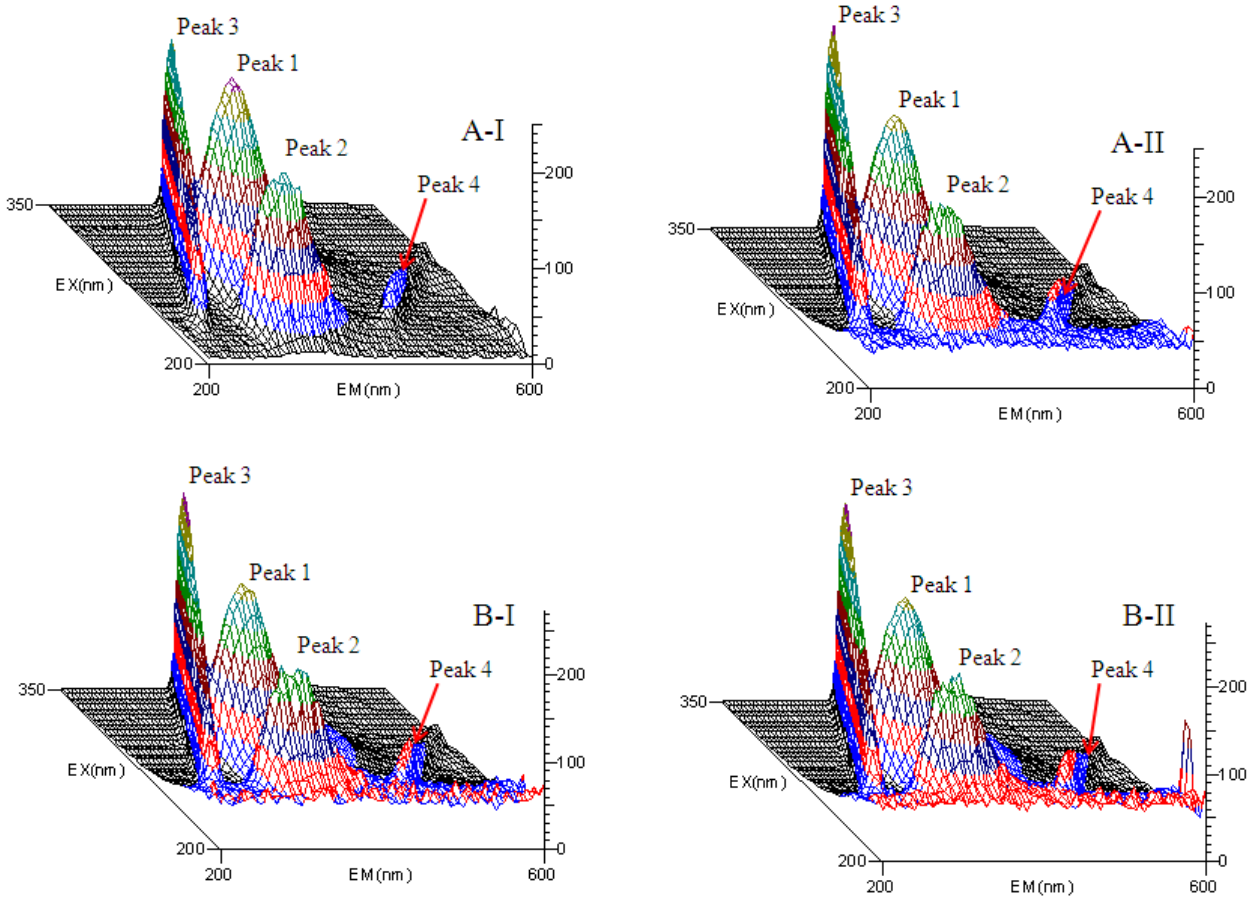

Figure 3B.

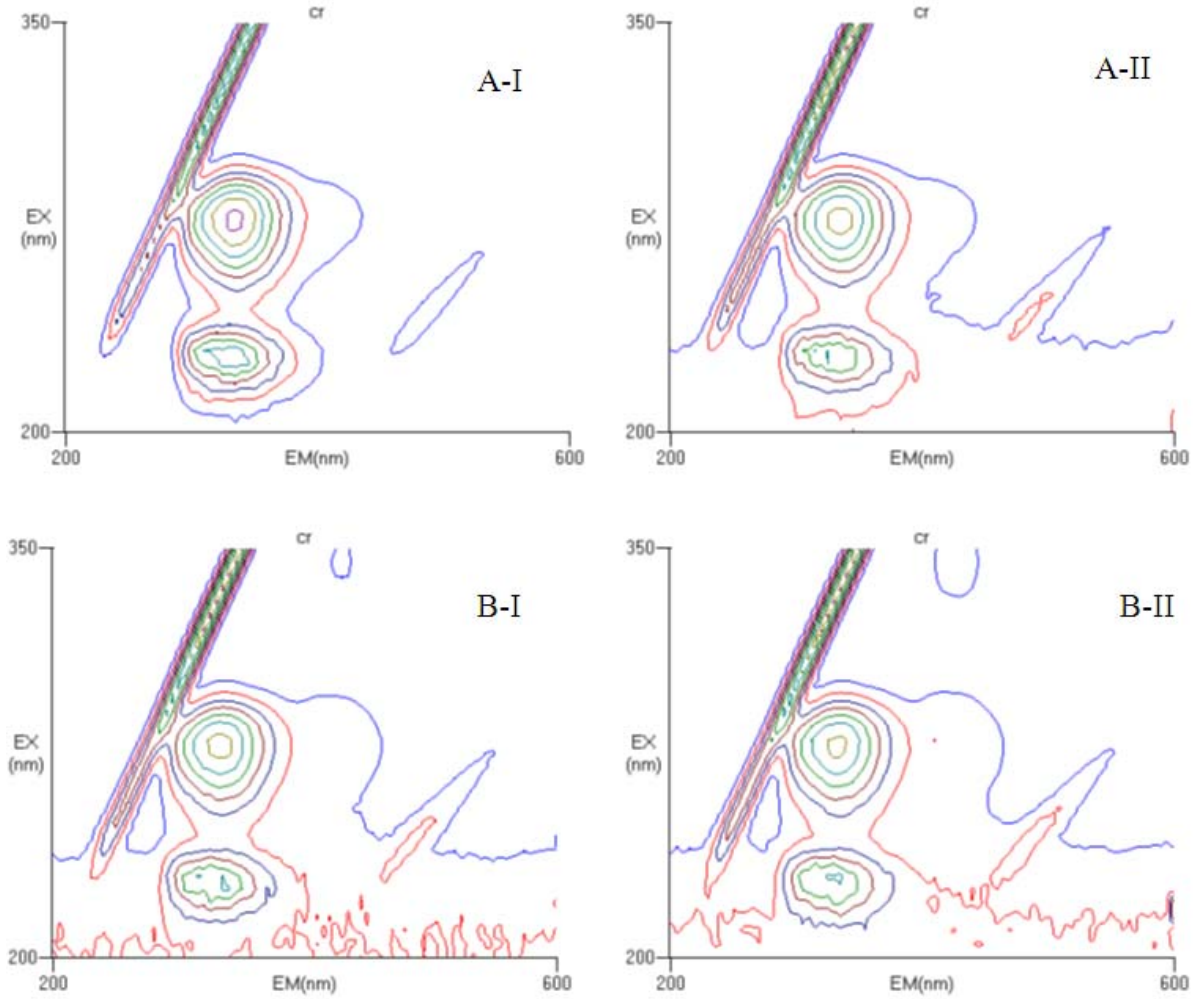

Figure 4A.

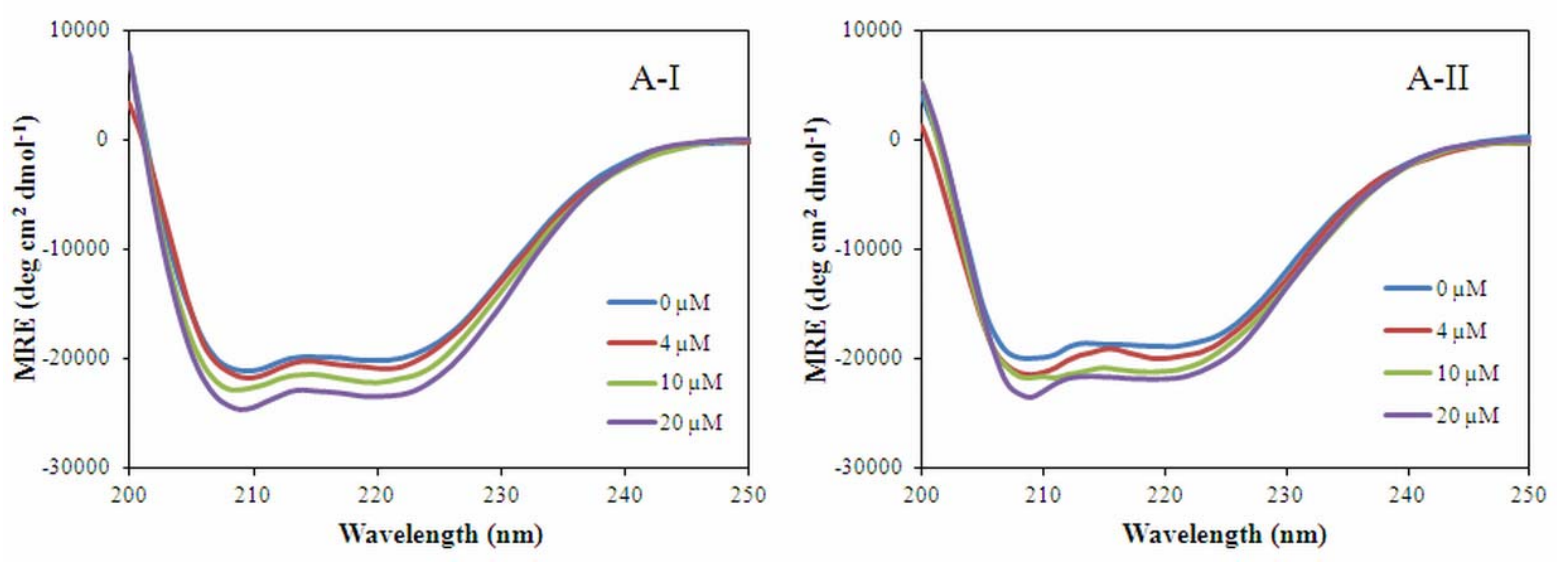

Figure 4B.

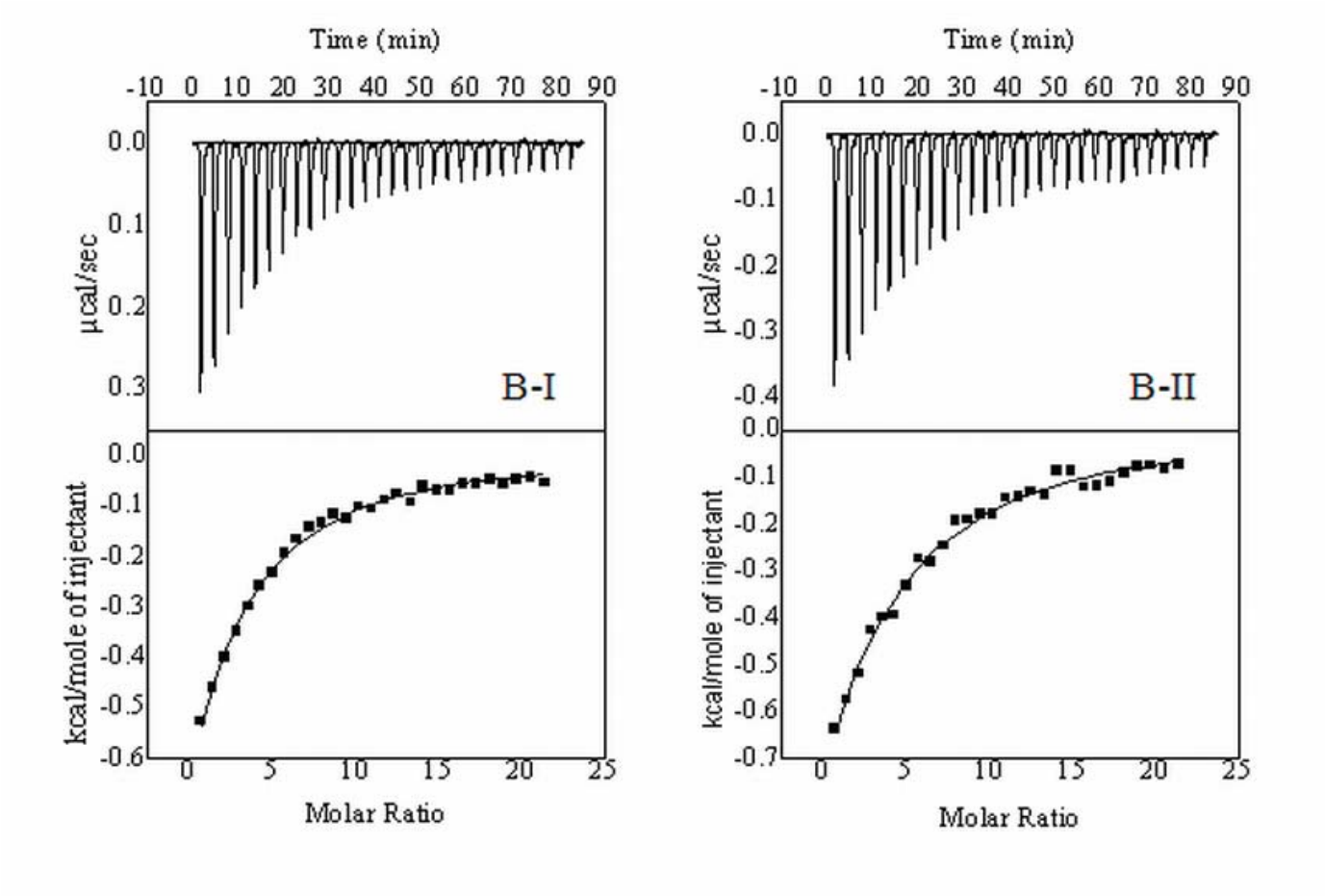

Figure 5

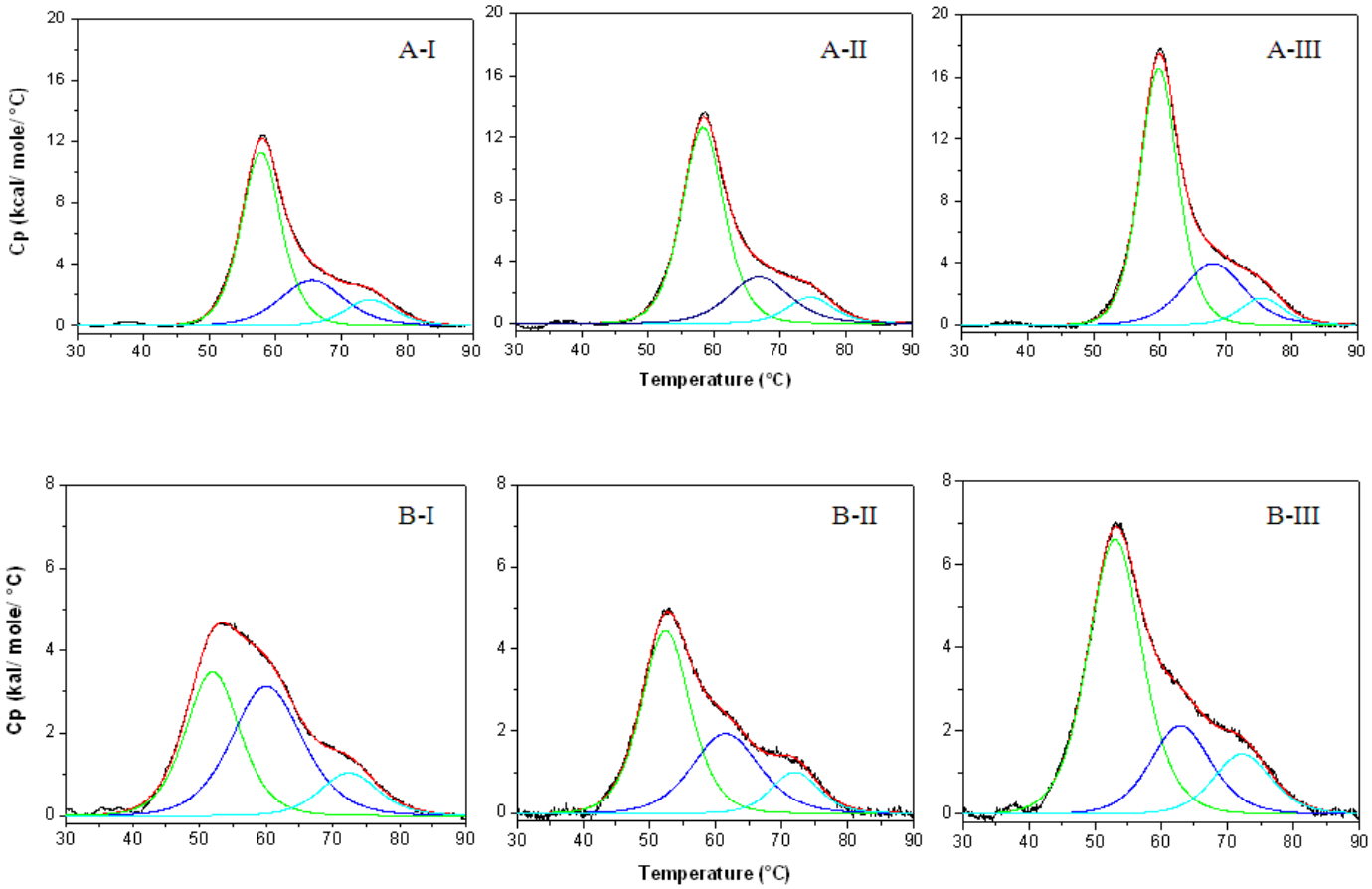

Figure 6