INSIGHTS INTO COUMARIN ANALOG BINDING WITH HUMAN SERUM ALBUMIN USING FLUORESCENCE SPECTROSCOPY AND MOLECULAR DOCKING

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Abstract : Coumarin isothiocyanate (CMITCN) is potential anticancer drug. It is an effective inhibitor against panel of human colon cancer cell lines including COX-2 positive HCA-7, HT-29 cells lines. Using fluorescence spectroscopy and molecular docking the binding characteristics of CMITCN with human serum albumin are investigated which reveal the binding constant being 1.73 x 10^5mol L^{-1} and number of CMITCN bound to HSA is 1.763. The thermodynamic study reveals spontaneous binding with involvement of hydrophobic interactions to stabilize CMITCN in the cavity of HSA. The competitive site binding study shows the binding site of CMITCN in HSA is located at site II situated in sub-domain IIIA.

I. INTRODUCTION

Albumin has emerged as a versatile carrier for therapeutic and diagnostic agents, primarily for diagnosing and treating diseases such as diabetes, cancer, rheumatoid arthritis and microbial diseases (Feng Yang 2014).Our group has been reporting on the anticancer activities of some of the promising phytochemicals and their analogs used in the treatment of cancers in the traditional medicinal systems such as Unani and Ayurveda (Misra et al. 2014;Prasad et al. 2009;Ronghe et al. 2014;Ronghe et al. 2016;Sahin et al. 2015;Zhang et al. 1994).Some of the common limitations in the use of phytochemicals from the traditional systems include their limited solubility in aqueous medium and rapid metabolic degradation in the biological system(Adsule et al. 2006;Kaur et al. 2015;Padhye et al. 2009). Such limitations can be overcome through preparation of their synthetic analogs and cyclodextrin or polymer conjugates (Dandawate et al. 2014;Dandawate et al. 2012;Dandriyal et al. 2016;Emami and Dadashpour 2015;Padhye, Yang, Jamadar, Cui, Chavan, Dominiak, McKinney, Banerjee, Dou, & Sarkar 2009;Prasad, Rath, Mathur, Bhatnagar, & Ralhan 2009). Coupling of low molecular weight anticancer drugs to antibodies, polymers or serum proteins also provides an effective method for improving the therapeutic index of established phytochemical (Gantert et al. 2009;He et al. 2016). The serum albumins (human and bovine) and the milk beta-lactoglobulin (beta-LG) have emerged as versatile agents for formulating such phytochemical analogs(Kim et al. 2017). In the present communication we have studied the interaction of human serum albumin and coumarin isothiocyanate with a focus on finding its binding characteristics.

Dietary phytochemicals are known to play a key role in the prevention of diseases like diabetes and cancer(Farzaei et al. 2016). Amongst the phytochemicals, coumarins constitute a class of compounds containing benzene and pyrone ring fused together which are present in oils of cinnamon, cassia, lavender oil and in fruits like bilberry, cloudberry etc (Puupponen-Pimia et al. 2013).Coumarin was first isolated in 1820 from tonka beans (*Dipteryxodorata* wild)(HASKINS and GORZ 1963) and since then it has been shown as an active pharmacophore against a number of diseases (Wozniakiewicz et al. 2017). Coumarins have been reported to show anti-inflammatory(Chougala et al. 2017), anti-oxidant(Filipsky et al. 2015), anti-coagulant(Daly 2013), anti-tuberculosis(Keri et al. 2015) and anti-cancer activity(Dandriyal, Singla, Kumar,

& Jaitak 2016;Emami & Dadashpour 2015;Farzaei, Bahramsoltani, & Rahimi 2016;Garro and Pungitore 2015;Kaur, Kohli, Sandhu, Bansal, & Bansal 2015).A conjugate of chalcone and coumarin possess potent cytotoxic activity against ovarian cancer(Rostom et al. 2009). A naturally occurring coumarin, viz. Osthole, inhibits the growth of lung cancer(Chen et al. 2015).The cinnamonyl-coumarin conjugate regulates the growth of breast and ovarian cancer(Sommer et al. 2016).

Colon cancer is a major cause of deaths in industrialized countries like US and studies have revealed the consumption of green leafy vegetables and cruciferous vegetables reduces its occurrence (Lin et al. 2002;Siegel et al. 2011;Steinmetz and Potter 1991a;Steinmetz and Potter 1991b).Isothiocyanate analogs like benzyl isothiocyanate, allyl isothiocyanate, phenyl ethyl isothiocyanate and sulforaphanes are present in cruciferous vegetables. Sulpharophane inhibits carcinogen induced mammary gland tumor(Zhang, Kensler, Cho, Posner, & Talalay 1994) and lung cancer(Hecht et al. 1995;Hecht et al. 2002).Phenyl ethyl isothiocyanate inhibits lung cancer caused due to smoking(Chung et al. 1992;Hecht, Chung, Richie, Jr., Akerkar, Borukhova, Skowronski, & Carmella 1995;Hecht, Kenney, Wang, & Upadhyaya 2002). It is reported to affect xenobiotic metabolizing enzymes in liver, lung and nasal mucosa in phase I and phase II and inhibit the activity of NNK and NDMAd in the liver(De et al. 2016;Guo et al. 1992).Looking at the therapeutic properties of coumarin and isothiocyanate motif we tried to investigate their combined effect. We synthesized coumarin isothiocyanate analog namely [(1E)-1-(1-(2-oxo-2H-chromen-3-yl)ethylidene) thio semicarbazide] (**CMITCN**) (Figure 1) and investigated its activity in HA/CD44v6 pathway. The results revealed **CMITCN** inhibits HA/CD44v6/COX2-5-LOX pathway in colon cancer(Misra, Ghatak, Vyas, O'Brien, Markwald, Khetmalas, Hascall, McCarthy, Karamanos, Tammi, Tammi, Prestwitch, & Padhye 2014). All the cell line experimental results explain the potential use of **CMITCN** as an anticancer agent and thus investigating its interaction with human proteins is essential.

The distribution of the drug in human body is regulated by number of factors including volume of blood and binding to serum proteins(Carter et al. 1989). Human serum albumin (HSA) is a protein present in human blood maintaining the osmotic pressure of the body. HSA performs vital functions in the body like transport of endogenous compounds, transport of drugs and is involved in calcium metabolism of the body. Since the binding of the drug to HSA alters its distribution and metabolism in the body(Curry 2009),it is essential to study its interaction with HSA. HSA is a macroscopic protein with 585 amino acids and 17 disulphide bridges(Carter, He, Munson, Twigg, Gernert, Broom, & Miller 1989). The heart shaped protein has seven binding sites for fatty acids and two sites for drug binding, viz. namely site I and site II respectively (Sudlow et al. 1975). A number of spectroscopic techniques are employed to study drug-HSA interactions like fluorescence spectroscopy, absorption spectroscopy, Fourier transform infra-red spectroscopy and circular dichroism respectively(Patra 2010;Pulla Reddy et al. 1999;Sengupta and Sengupta 2002;Sochacka and Baran 2012).

In the present investigation we have examined the interaction of **CMITCN** with HSA by fluorescence spectroscopy and molecular modeling in order to calculate the binding constant of such interaction, number of drug molecules binding to HSA, binding site and conformational changes in the protein and thermodynamic parameters of the binding interactions.

Materials and Methods

Materials

All the chemicals including human serum albumin (HSA) (fraction V), Tris hydrochloric acid buffer, bilirubin, acetyl coumarin and thiosemicarbazide were purchased from Sigma-Aldrich chemical company and were used without further purification. The **CMITCN** was prepared by condensation reaction as described in the earlier protocol(Adsule, Barve, Chen, Ahmed, Dou, Padhye, & Sarkar 2006;Scovill et al. 1982). The solution of HSA (fatty acid free) were made in Tris-hydrochloric acid buffer (pH=7.4) in order to maintain the physiological conditions. The stock solution of **CMITCN** was prepared in dimethylsulphoxide.

Fluorescence quenching measurements

The FP-8200 spectrofluorimeter (JASCO) was utilized for all fluorescence measurements. Quartz cuvette of 1cm path length and spectral bandwidth of 5nm were used in fluorescence detection. HSA was excited at the excitation wavelength of 285nm and the emission wavelength was scanned in the range 200-750nm. The fluorescence quenching spectra of HSA was monitored on successive addition of **CMITCN** to the solution of HSA. The thermodynamic parameters were evaluated by carrying out the quenching experiments at three different temperatures, viz. 298,308 and 318 K respectively. The binding constants were calculated using modified Stern-volmer equation(Samson et al. 1990) (Equation 1) while the thermodynamic parameters were obtained using Van't Hoff equation (Equation 2) (Cui et al. 2007).

Competitive site binding experiments

HSA has two sites for drug binding and there are selective drugs binding to specifc site popularly known as site markers. The competitive site binding experiments were carried out in order to evaluate the binding site of **CMITCN** in the cavity of HSA. Bilirubin and warfarin were used as site markers for site I and site II respectively. Equimolar solution containing HSA and the site marker were mixed and incubated (15 minutes) for complexation and thus the site marker occupies the specified site. The HSA-site marker solution was excited at 285 nm and the emission wavelength was scanned in the region 200-700nm. The fluorescence spectra of the complex (HSA-site marker) were monitored after each addition of the **CMITCN**. The change in the fluorescence intensity and emission wavelength indicated the site at which **CMITCN** binds to HSA(Ranjbar et al. 2013).

Docking Studies

The drug **CMITCN** was docked in the cavity of HSA in order to elucidate the binding site, binding energy and the amino acid residues stabilizing the drug in the cavity of the protein. The docking analysis were done by using Autodock4.2 program (Goodsell et al. 1996) and Autodocktools 1.5.4(Sanner 1999). The PDB file of the drug was obtained from 3D corina software. Three HSA PDB files are selected for the docking studies and their respective crystal structures were down-loaded from Protein Data Bank(PDB IDs: 1BM0; 2BXD and 2BXF)(Berman et al. 2000). The protein PDB files were cleaned and converted into PDBQT format for docking. The protein was kept rigid and the drug was allowed free rotations in the protein cavity. The grid space of 0.375Å was set for the binding of the drug in protein. At sub-domain IIA, the grid boxes were centered at x =35.26, y = 32.41 and z = 36.46 for 1BM0; (5.101, 213.346, 7.444) for 2BXD and (1.333, 210.093, 8.189) for 2BXF. On the other hand, for sub-domain IIIA, the grid boxes were centered at (14.42, 23.55,23.21), (15.226, 4.383, 27.693) and (5.276, 4.635, 210.078), for 1BM0, 2BXD and 2BXF respectively(Feroz et al. 2013). The drug and the protein docking are done using Autodock Vina and the result file is viewed in Pymol. The images of the docking results are analyzed and saved as PNG files.

Results and Discussion

Fluorescence spectroscopy

HSA is a macroscopic protein comprising of 585 amino acid residues. The crystal structure of HSA unveils heart shaped geometry containing three domains and each domain is further divided into sub-domains A and B respectively(Carter, He, Munson, Twigg, Gernert, Broom, & Miller 1989). Sudlow et al have classified the cavity of HSA into two major drug binding domains, viz. site I and site II respectively(Sudlow, Birkett, & Wade 1975), wherein site marker drugs such as Bilirubin and Warfarin bind specifically to site I and site II respectively(Dale and Nilsen 1984). The intrinsic fluorescence of HSA is mainly due to the tryptophan residue

which decreases in intensity upon addition of the drug and this decrease is known as fluorescence quenching. The quenching of fluorescence can take place due to number of molecular interactions between drug and protein.

The interactions between drug and protein can be predicted from extent of quenching caused upon addition of **CMITCN** to HSA (Figure 2a). The fluorescence intensity without addition of **CMITCN** to HSA and its quenching upon successive additions of the drug is monitored (Figure 2a) and the binding parameters are calculated using modified Stern-Volmer equation (Equation 1)(Samson, Morissette, & Popovic 1990).

$$Log(Fo - F)/F = LogK_a - nLog[Q] \quad (1)$$

where F_o is the fluorescence intensity without the drug, F is the intensity upon addition of the drug, K_a is the binding constant, n is number of drug molecules binding and Q is the concentration of the drug respectively. The binding parameters calculated therein help in predicting the drug-protein interactions and drug affinity towards the protein. The graph obtained by plotting the Stern-Volmer equation yields the binding constant as1.73×10^5mol $L^{-1}$and number of bound pigment molecules being 1.763 respectively (Figure 2b).Drugs binding within the range of 10^4 to 10^6 are reported to be high binding drugs (Deamer et al. 1967).The higher binding constant thus obtained indicates greater affinity of **CMITCN** towards HSA. The value of n (approximately equal to 2) shows that two drug molecules bind to a macromolecule of HSA.

The quenching of fluorescence is classified into two types i.e. static and dynamic quenching respectively. In the static quenching there is formation of the excited state complex among drug and protein resulting in increase in binding constant with rise in temperature. In dynamic quenching mechanism complexation between the drug and protein takes place and hence the binding constant decreases with increase in temperature. The higher binding affinity of **CMITCN** with HSA is found to alter with temperature and the binding constant increases with temperature as listed in **Table 1**. Primarily four intermolecular interactions i.e. hydrophobic force, van der waal's force, hydrogen bonding and electrostatic interactions are involved in stabilizing the drug in the protein cavity. The thermodynamic parameter like Gibb's free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) explains the type of interaction among drug and protein. The thermodynamic parameters can be elucidated from Van't Hoff equation (Equation 2)

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (2)$$

Where K is the binding constant at different temperatures (T) and R is the gas constant. The Gibbs free energy (ΔG°) was calculated using the following equation (Equation 3):

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (3)$$

The values of all the thermodynamic parameters are listed in Table 1. The negative value of free energy indicates spontaneous binding of **CMITCN** and HSA while the negative enthalpy and positive entropy reveal involvement of the hydrophobic interactions in stabilizing the **CMITCN** and HSA complex.

Binding Site analysis

HSA acts as a transport protein in the body carrying a number of endogenous compounds like bile, fatty acid etc. It has seven binding sites to carry fatty acids and two binding sites to transport drugs in the body. Sudlow et al have classified these sites into two classes, viz. site I and site II, respectively(Sudlow, Birkett, & Wade 1975). Drugs binding specifically to a particular site are known as site markers of that siterespectively. Bilirubin and Warfarin are drugs which bind selectively at site I and site II respectively. On addition of site marker to HSA results in the decrease in its fluorescence and on further addition of the drug fluorescence intensity changes. Such changes in fluorescence intensity and emission wavelength can be used to evaluate binding site of drug in HSA. The competitive site binding experiment showed that on addition of **CMITCN** to the solution of HSA-bilirubin complex causes gradual decrease in the fluorescence without any change in emission wavelength while such addition to the solution of HSA-ibuprofen complex exhibits a bathochromic shift indicating the binding of the drug **CMITCN** takes place at site II situated in sub-domain IIIA. Binding of **CMITCN** to HSA causes change in the conformation of the protein. Slight changes in conformation result in

shifting of the emission wavelength indicating the binding site. As the change in emission wavelength is observed at site II, **CMITCN** binds at Site II. These experimental results are verified by molecular docking and the visual images confirm the results.

Molecular Docking analysis

Molecular Docking analysis is used as a tool to investigate the amino acid residues involved in the binding and binding site of **CMITCN** with HSA. The binding energy, number of hydrogen bonds and the amino acids involved in the hydrogen bonding are listed in **Table 2**. Site markers diazepam and warfarin are used in the competitive site binding analysis. Uncomplexed HSA shows heart shaped conformation whereas site marker complexed HSA restricts the amino acid bound to the site marker from binding to CMITCN. As crystal structures of HSA complexed with diazepam avoids the site I binding similar to that of bilirubin and warfarin avoids binding at site II as ibuprofen. Diazepam and warfarin are selected for docking studies. On basis of availability crystal structures of uncomplexed HSA and site marker diazepam or warfarin complexed HSA have been selected for docking analysis. The average binding energy of **CMITCN**in the different HSA pdb files i.e 1BMO, 2BXD and 2BXF are -7.6, -6.85 and -7.1 kcal/mole respectively. The negative sign of binding energy indicates the sponateous binding among **CMITCN** and HSA. Binding energy of site II is similar to free HSA confirming the experimental results. Both the molecular docking and site binding experiments disclose binding site of **CMITCN** as site II situated in subdomain IIIA. The high binding energy suggests that the drug molecule is stabilized in protein cavity of heart shaped HSA comprising of Lys349, Lys541, Asp324, Arg222, Ala291, Glu292, Phe134, Try161 residues respectively. **CMITCN** is surrounded by amino acid residues forming hydrogen bond and completely encapsulating it in the cavity of HSA. Entropy and enthalpy of binding are in agreement of involvement of hydrogen bonding in stabilizing **CMITCN** in HSA.

Conclusions

The binding characteristics of the novel anticancer drug developed in our lab with human serum protein albumin reveal a fairly strong binding with binding constant of 1.73 x 10^5mol L^{-1} and number of bound drug molecules being 1.76 respectively. HSA carries two **CMITCN** molecules in the human body. As **CMITCN** is reported to have activity against colon cancer on binding to HSA its delivery may be facilitated due to high binding. The competitive site binding analysis reveals that **CMITCN** drug binds at Site II situated in sub-domain IIIA of HSA. Thermodynamic parameters and molecular docking reveal spontaneity in binding and hydrophobic interactions are involved in the **CMITCN**- HSA complex formation. The molecular modeling results support strong binding of the drug in the protein cavity through hydrogen bonding interactions with the protein residues.

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Figure captions

Figure 1. Structure of Coumarin isothiocyanate (CMITCN)

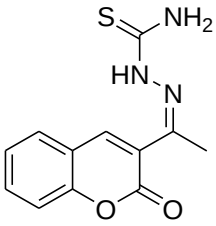
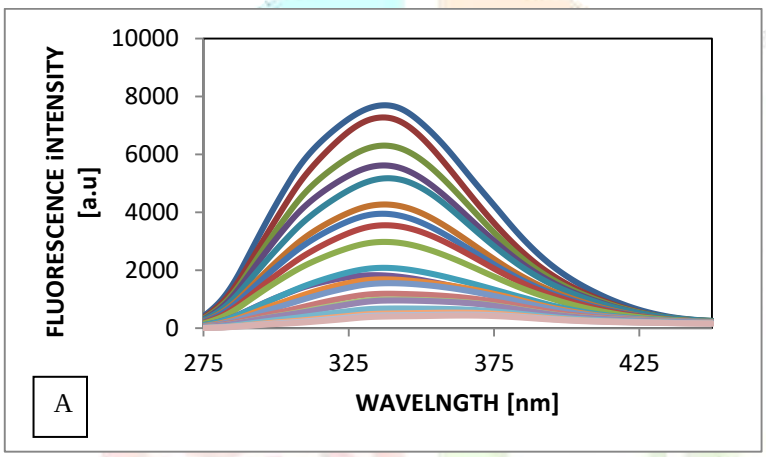


Figure 2: (a) Flourescence quenching spectra of HSA on successive addition of CMITCN ,b) Stern-volmer plot of Log[Fo/Fo-F] versus Log [Q]



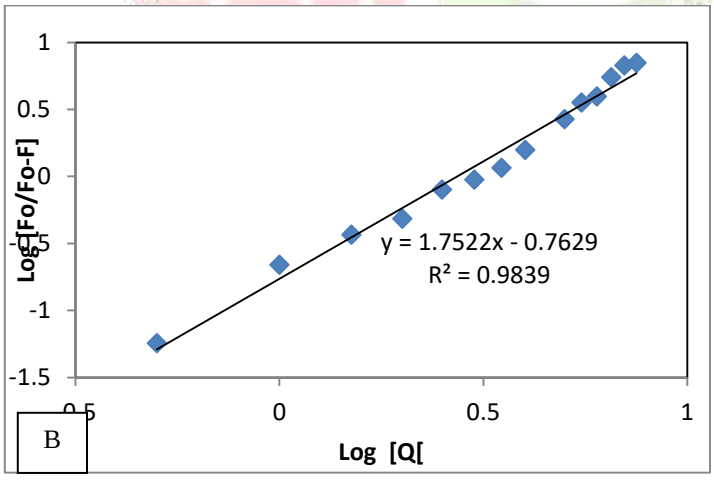


Figure 3 a) Fluorescence quenching spectra of HSA-bilirubin with increasing concentration of CMITCN b) Fluorescence spectra of HSA-Ibuprofen with increasing concentration of CMITCN

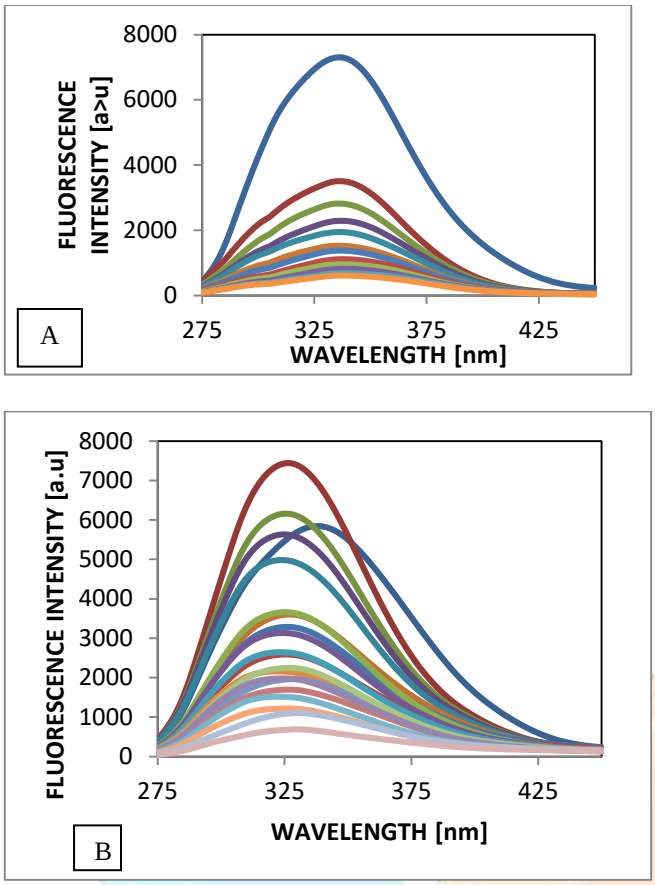


Figure 4: Docking Images of* CMITCN *in the cavity of HAS

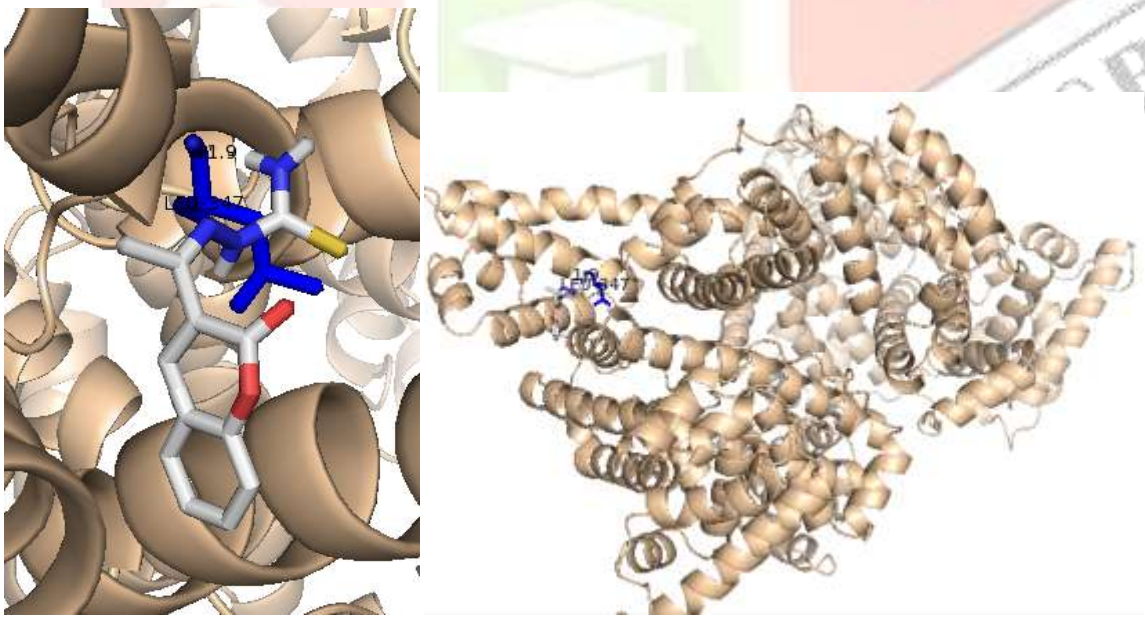


Table 1. Thermodynamic parameters at various temperatures

Temperature K	K ($x10^5$ mol L^{-1})	ΔG° (kJ mol^{-1} K^{-1})	ΔH° (kJ mol^{-1} K^{-1})	ΔS° (J mol^{-1})
308	1.73	-83.73	-4.81	256.23
318	2.78	-86.29		
328	5.50	-87.89		

Table 2.Docking results of CMITCN in HSA

Sr no.	Name of protein	PDB id	Domain	Binding Energy	No of Hydrogen bonds	Amino Acids to which drug is binding
1.	Crystal structure of HSA	1BMO	Subdomain II A	-7.7	1	Lys349
			Subdomain III A	-7.5	2	Lys541
2.	Crystal structure of HSA complexed with warfarin	2BXD	Subdomain II A	-6.4	1	Asp324
			Subdomain III A	-7.3	3	Arg222,ALA291,Glu292
3.	Crystal structure of HSA complexed with diazepam	2BXF	Subdomain II A	-7.3	3	Phe134,Try161
			Subdomain III A	-6.9	2	Leu203,Ser487

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