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Probing the Interaction of Potent Anticancer Compound Plumbagin Isoniazid with Human Serum Albumin: Fluorescence Spectroscopy and Molecular Modelling Study

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Abstract: ***Human serum albumin is the most abundant drug carrier protein in the human body. It plays a vital role in pharmacokinetics and Pharmacodynamics of the drug. Plumbagin, isolated from Plumbago zeylanica is reported to show anticancer activity. Plumabgin Isonaizid is a synthetic analog of plumbagin having better properties than parent compound. The interaction of Plumbagin isoniazid with human serum albumin reveils dynamic quenching mechanism along with spontaneous binding. The binding site of PLINH is sudlow's site I located in subdomain IIA.***

Keywords: Plumbagin, Isoniazid, Human serum albumin, Bilirubin, Ibuprofen

Abbreviations: Plumbagin-isoniazid PLINH, Human Serum Albumin HSA

1. Introduction

Plumbagin is a hydroxynaphthoquinone compound isolated from the roots of Indian medicinal plant *Plumbago zeylanica* which is referred in Indian traditional medicine Ayurveda as 'Chitraka'. The roots of this plant have been used for therapeutic treatments of dyspepsia, piles, diarrhea and skin diseases in Ayurvedic medicine (Chopra RN, 1956, KM, 1954, UC, 1877). Plumbagin is known to exhibit antioxidant (Tilak et al., 2004), anti- inflammatory (Checker et al., 2009, Ahmad et al., 2008), and antibacterial activities (Wang et al., 1998). The anticancer activity of Plumbagin against different cancers has attracted attention of researchers (Ahmad et al., 2008, Powolny and Singh, 2008, Chen et al., 2009, Aziz et al., 2008, Shih et al., 2009, Wang et al., 2008, Sandur et al., 2006, Ding et al., 2005, Kawiak et al., 2007, Thasni et al., 2008, Kuo YH, 1997, Gomathinayagam et al., 2008, Sandur et al., 2010). A few synthetic analogs such as C-3 substituted Plumbagin with cyano, chloro, bromo and the N-acetyl amino acids substitutes have been prepared in order to improve its bioavailability and pharmacokinetic properties. (Salmon-Chemin et al., 2001, Adikaram, 2002, Kazuhito OGiHARA, 1997, Hazra et al., 2002). Similarly metal complexes of Plumbagin and some of its analogs have also been reported (Chen et al., 2009, Chen et al., 2011). Recently we have summarized the properties and anticancer activities of Plumbagin (Padhye et al., 2012, Dandawate et al., 2014b).

In our lab we have synthesized Plumbagin analogs with different aryl and heterocyclic hydrazones involving C-4 quinone carbonyl group (Dandawate et al., 2014a). Among these Plumbagin-isoniazid (PLINH) (Figure 1) analog exhibited highest cytotoxicity against breast cancer cell lines. The compound was also found to be highly active against multi-drug resistant *Mycobacterium tuberculosis*. It has been a common observation with drug discovery groups that most of the phytochemicals and their analogs tend to be highly lipophilic and which affects their bioavailability and metabolism in humans. It has also been well-established that any drug molecule entering the blood stream is known to bind with serum proteins and hence distribution of drugs in the human body is affected by its ability to bind to the plasma proteins (Curry, 2009). Serum proteins also help in maintaining the osmotic pressure of the body.

Human serum albumin (HSA) is a major carrier protein in the blood plasma and HSA is known to transport number of drugs in the blood stream and thus help target delivery of drugs to specific site (Elsadek and Kratz, 2012). It is also involved in maintaining the osmotic pressure(Peters, 1995) and is acting as a reservoir for number of signalling molecules including nitric acid (Ishima et al., 2008). HSA is known to transport a variety of endogenous compounds like fatty acids, hormones and excretory products like bile. In recent years many crystal structures have been published including free HSA and its complexes with various bio-ligands as well as drug molecules (Sugio et al., 1999, Curry et al., 1998, Zunszain et al., 2008). Their PDB structures have provided useful information on the binding sites in HSA structure.

Since the binding affinity of the drug with HSA alters its distribution and site specific action in the body, it is imperative to study the interaction of any novel and promising drug with HSA and to investigate its binding site and thermodynamic properties. The binding site explains the

amino acid residues involved in drug-HSA binding while the thermodynamic property unveils the strength and drug release characteristics under physiological conditions. Many spectroscopic methods including fluorescence spectroscopy, circular dichroism, Infra-red spectroscopy, ultraviolet spectroscopy as well as in-situ molecular modelling have been employed to predict the nature of HSA-drug binding. In the present investigations we have examined the binding constant, number of binding sites and thermodynamic parameters like free energy, enthalpy and entropy in case of PLINH with HSA by fluorescence spectroscopy and molecular modelling.

2. Material and methods

Materials

The protein HSA (fraction V) was purchased from Sigma-Aldrich Chemical Company along with Plumbagin, Isoniazid, Trifluroacetic-acid, TRIS buffer, Bilirubin and Ibuprofen. All chemicals were used without further purification. PLINH was synthesized according to procedure reported from our lab (Dandawate et al., 2014b). Its stock solution was prepared in dimethyl sulphoxide solvent. Tris hydrochloric acid 50 mM was prepared in double-distilled water. HSA solution was prepared in 50mM tris hydrochloric acid buffer (pH = 7.4).

Fluorescence spectroscopy:

The fluorescence quenching experiments were carried out on JASCO FP-8200 spectrofluorimeter at emission mode and by using quartz cuvette. Both the excitation and emission band widths were kept at 5nm having a response time of 0.5 sec with data interval of 1nm. The experiments were performed using a scan speed of 200 nm/min. The excitation wavelength of 285 nm (Tan et al., 2005) were used to excite the HSA. Fluorescence quenching titrations were carried out by keeping the concentration of HSA constant while varying the concentration of PLINH (Yue et al., 2009). All the solutions were stirred well and incubated for allowing proper mixing and interaction between HSA and PLINH. Fluorescence spectra were recorded on successive addition of 0.5 µL PLINH to HSA. The effect of emission intensity on increasing concentration was monitored to predict the HSA-PLINH interactions.

The thermodynamic parameters were determined by varying the temperature of HSA-PLINH system. The fluorescence quenching experiments were carried out at temperatures of 298, 303 and 313 K and parameters like free energy, enthalpy and entropy were calculated from the fluorescence emission intensity and concentrations. Stern-Volmer equation was used to calculate the binding and thermodynamic parameters of HSA-PLINH system.

Competitive site binding experiments are known to help reveal the binding site of PLINH in HSA. HSA has two binding sites and hence two site markers, viz. Bilirubin for site I and Ibuprofen for site II, which were used to predict the binding site of PLINH in the HSA cavity. The concentrations employed for HSA, site markers and PLINH were 100 µM respectively PLINH was added to HSA-site marker solutions and changes in fluorescence intensity with increasing concentration of PLINH were monitored to reveal the binding site (Ranjbar et al., 2013a).

Docking Studies

The binding site of PLINH in HSA cavity was predicted from molecular docking studies. Autodock tools 1.5.4 and Autodock 4.2 were used for docking and visualisations. The structure of PLINH was drawn using ChemDraw and its PDB file was obtained using 3D CORINA. The pdb file of PLINH was processed in Autodock tools and converted into PDBQT. Three crystal structures of the HSA i.e. BM0, 2BXD and 2BXF were obtained from the protein data bank. All the hetero atoms, water molecules and charges were removed and the cleaned protein structure was stored for further docking. The protein molecule was held rigid while PLINH molecule was allowed to attain different conformations in the protein cavity. The grid space was adjusted at 0.375 Å and the grid boxes were adjusted 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 centred 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. The docking of PLINH in HSA was carried out using Autodock Vina software and 10 resulting conformations of PLINH in HSA were visualised. The conformation with the highest binding energy was viewed using PYMOL (Feroz et al., 2013).

3. Results and Discussion

a) Fluorescence Quenching Analysis of HSA by PLINH:

HSA is a single polypeptide containing 585 amino acids along with three intrinsic fluorophores, viz. tryptophan, tyrosine and phenylalanine residues. The major contributor to the fluorescence is tryptophan residue as the quantum yield of phenylalanine is low and the fluorescence of tyrosine is quenched due to ionization by amino groups, carboxyl groups and tryptophan (Sharma, 1999). Any changes in the protein conformation, association, binding and denaturation are anticipated to result in changes in the fluorescence of the protein (Kratochwil et al., 2002). Thus, the change in fluorescence intensity provides information about the protein conformation, its binding properties and dynamics of binding. The reduction of the quantum yield and fluorescence of the protein occurring due to its interaction with any quencher drug molecule are known as fluorescence quenching. HSA exhibits fluorescence at 335 nm when excited at 280 nm. **Figure 2** shows the quenching of HSA fluorescence on addition of PLINH. On increasing the concentration of PLINH the quenching goes on increasing resulting in decrease in the fluorescence. The ratio of fluorescence intensity of HSA with and without the drug PLINH (Fo/F) shows linear increase with increasing concentration of PLINH (**Figure 3**). The binding constant of HSA-PLINH complex was calculated using by the Stern-Volmer equation:

$$\frac{\mathbf{Log\ (Fo - F)}}{\mathbf{F}} = \mathbf{LogK_a - nLog\ [Q]}$$

Where F_o and F are the fluorescence intensities of the protein without and with PLINH drug. K_a, n and Q represent the binding constant, number of binding sites and concentration of PLINH respectively. The graph of

$\frac{\text{Log } (Fo-F)}{F}$ versus log [Q] yields the binding constant and number of binding sites as $1.98x10^5$ $Lmol^{-1}$ and 0.50 respectively(**Figure 4**). PLINH exhibits higher binding constant than the parent Plumbagin molecule. The reported binding constant of the parent Plumbagin molecule by Li et al is 4.43 x 10^4 $Lmol^{-1}$(Shuang Li, 2011).

b) Thermodynamic studies

The quenching of fluorescence has been classified into two types, viz. dynamic and static respectively. Both quenching mechanisms can be distinguished by temperature and viscosity dependence of the protein-drug interactions. In dynamic quenching, increasing temperature accelerates the rate of collisions resulting in higher binding constants. On the other hand increasing temperature reduces the stability of the complex and hence binding constant in case of static quenching (Ranjbar et al., 2013b). In our studies the interaction of PLINH-HSA was studied at three different temperatures including 298, 303 and 313 K respectively while their corresponding Stern-Volmer plots are depicted in **Figure 5**. These plots show a straight line passing through origin while the slope of this line increases with increase in temperature indicating that binding among PLINH and HSA obeys dynamic quenching mechanism. It is interesting to note that while PLINH obeys dynamic quenching whereas the parent Plumbagin molecule exhibits static quenching mechanism. The values of binding constant and number of binding sites at different temperatures are listed in **Table 1**. The binding constant between PLINH-HSA is found to be high which reflects that PLINH is strongly bound to the blood plasma and shall be adequately circulated in the body.

The interaction of the drug with HSA are known to receive contributions from various other interactions including hydrogen bonding, Van der waal's forces and hydrophobic as well as electrostatic (Lakowicz, 1999). In order to determine the nature of such interactions the thermodynamic properties were calculated using the Van't Hoff equation.

$$\mathbf{Ln\ K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}}$$

Where K is the binding constant, R is gas constant; ΔH° and ΔS° are enthalpy and entropy at different temperatures. Substituting the values of enthalpy and entropy, free energy is calculated using the following equation:

$$\mathbf{\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ}$$

The free energy, entropy and enthalpy are listed in **Table 1.** Ross and Subramanian have studied the relation between sign and magnitude of the thermodynamic properties and the interaction of protein with drugs(Ross and Subramanian, 1981). The negative value of enthalpy and positive value of entropy indicates hydrogen bonding and hydrophobic interactions are present in the PLINH-HSA system. The type of hydrogen bonding interactions and their bond distances can be observed in the molecular docking results which are in agreement with the thermodynamics results.

c) Site binding studies

HSA belongs to globular proteins containing 585 amino acids and 17 disulphide bridges (Surewicz et al., 1993). It consists of three domains (I, II and III) where each domain is further divided into two sub-domains (A and B). Since the major function of HSA is the transport of drugs wherein the sub-domain IIA and sub-domain IIIA are mainly involved in the drug binding. Sudlow et al have classified sub-domain IIA as the binding site I and sub-domain IIA as the binding site II. The site at which PLINH binds to HSA was evaluated by competitive site binding experiments. HSA is primarily bound to the site marker of specific sites, followed by addition of drug. On monitoring the changes in fluorescence intensity and emission wavelength the binding site can be elucidated.

In order to study the binding site of PLINH in HSA protein the corresponding site markers, viz. Bilirubin for site I and Ibuprofen for site II were used. The fluorescence intensity of HSA was found to decrease on addition of Bilirubin due to its preferential binding at site I in HSA along with a red shift (**Figure 6**). On further addition of drug there is a decrease in fluorescence intensity indicating that the binding of PLINH to HSA takes place at the Bilirubin site. The fluorescence intensity of HSA decreased on addition of Ibuprofen followed by further decrease on addition of PLINH (**Figure 7**). These competitive site binding experiments indicate that binding of PLINH at site I is situated in subdomain IIA. PLINH is structurally similar of Plumbagin which binds to site I of HSA(Shuang Li, 2011).

d) Molecular Docking studies

Molecular docking studies reveal the binding site of PLINH in the protein cavity of HSA and the amino acids involved in the bonding which stabilises the drug in HSA cavity. The Crystal structure of HSA reveals mainly two binding sites i.e. site I and site II, for the delivery of the drug to the specific site (Sudlow et al., 1975, Sudlow et al., 1976). The binding energy involved in PLINH-HSA binding suggests strong binding between HSA and PLINH which is in agreement with the fluorescence quenching constant. Negative value of binding energy is attributed to the bound state of the drug in protein. For comparative study three crystal structures were employed by us including un-complexed HSA, HSA-complexed with Warfarin (site marker for I) and HSA-complexed with Diazepam(site marker for II) respectively which were docked with PLINH and the results are summarized in **Table 2.** In un-complexed HSA docking with PLINH has the freedom to bind at either sites whereas in the complexed HSA docking one of the sites is complexed with the site marker. The amino acids in vicinity of PLINH are Leu-103,Try-148,Arg-197,Lys-106,Leu-103,Try-148,Arg-197,Gln-459, Asp-108, Glu-425, His-146, Arg-117, Lys-212, His-510, Lys-524 respectively. It can be observed that PLINH complexes with HSA with binding energy of 8 Kcal/mol having an average of 2.5 hydrogen bonds which clearly indicates that hydrogen bonding probably plays a major role in stabilization of the complex.

4. Conclusion

In the present work we have studied the interaction between potent anticancer analog of Plumbagin with the most abundant protein HSA in the body. The fluorescence quenching results indicate higher binding constant for new analog than parent Plumbagin molecule. The thermodynamic studies reveal that PLINH obeys dynamic quenching mechanism and its binding with HSA is stabilized

by hydrogen bonding and hydrophobic interactions. Competitive site binding experiments indicate that the binding site of PLINH in the macroscopic protein HSA is situated in sub-domain IIA at site I. Molecular docking of PLINH in the cavity of HSA and complexed HSA reveal high binding energy in agreement with its high binding constant while docking results reveal that binding of PLINH and HSA is stabilized by hydrogen bonds with tryptophan. These studies clearly reveal that higher binding energy and binding constant contribute to the better activity of PLINH than its parent plumbagin compound.

5. Acknowledgement

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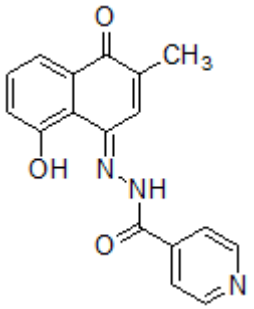

Figure 1: Structure of Plumbagin isoniazid

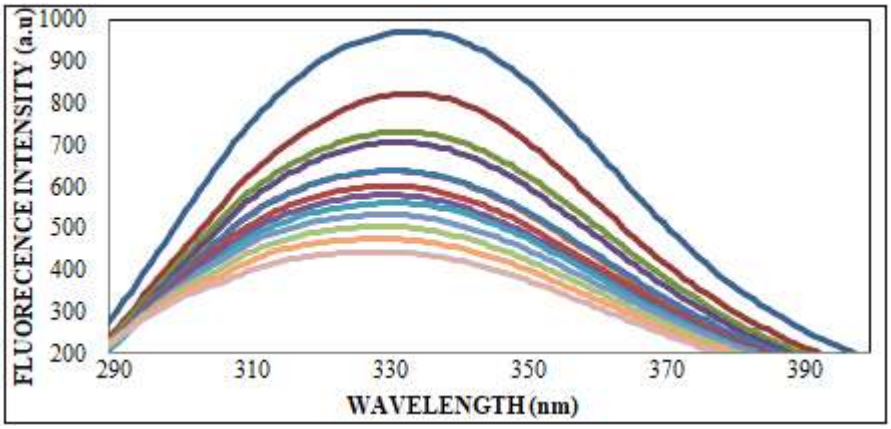

Figure 2: Fluorescence spectra of HSA with varying concentration of PLINH

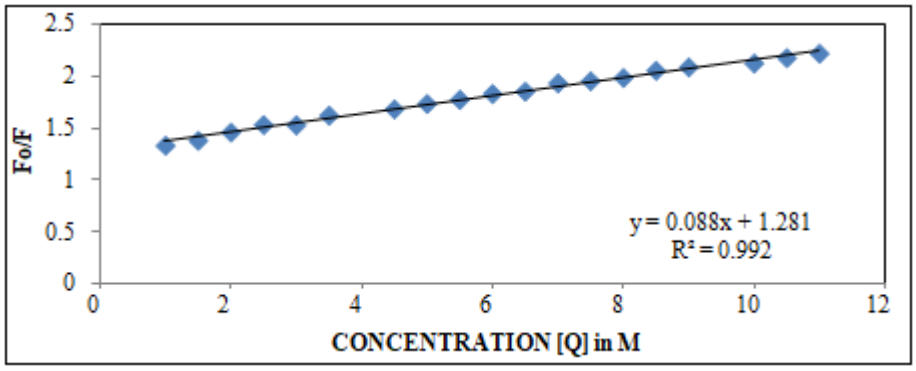

Figure 3: Stern-Volmer plot of Fo/F versus concentration

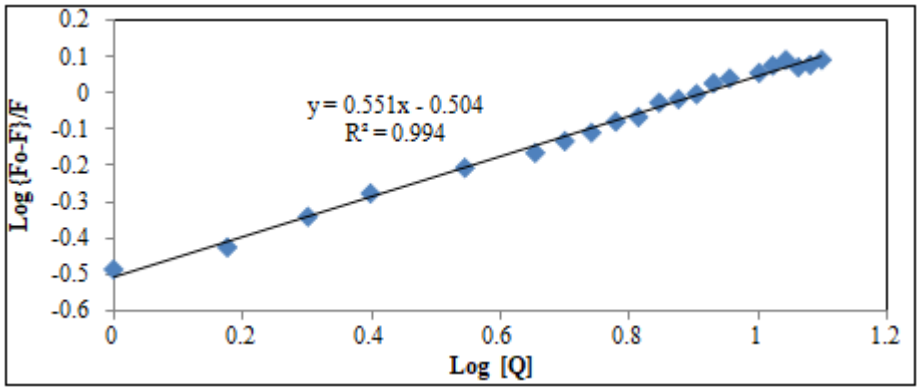

Figure 4: Stern-Volmer plot of Log {Fo-F}/F versus Log[Q]

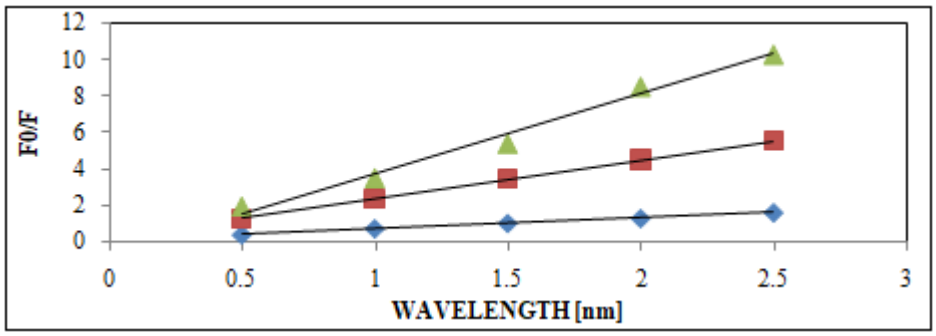

Figure 5: Stern-Volmer plots of fluorescence quenching of HSA by PLINH at different temperatures.

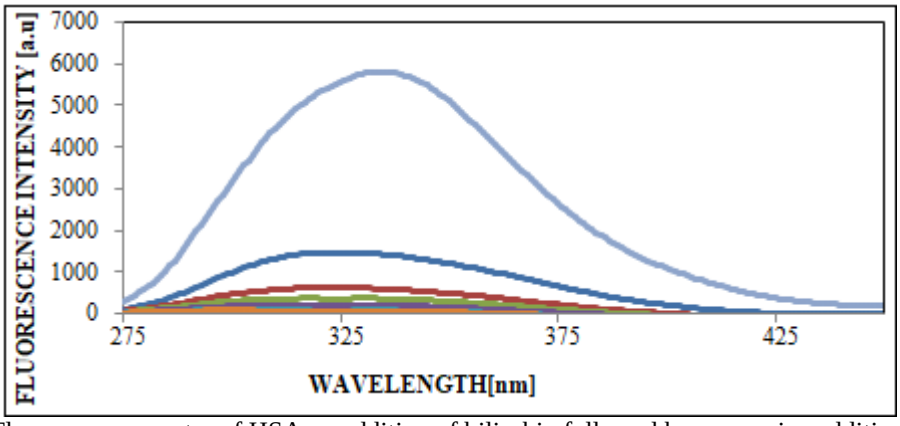

Figure 6: Fluorescence spectra of HSA on addition of bilirubin followed by successive addition of PLINH

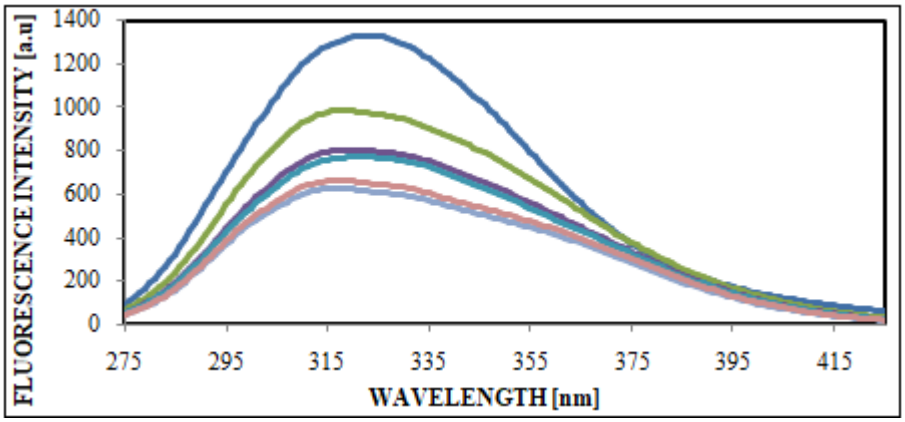

Figure 7: Fluorescence spectra of HSA on addition of ibuprofen followed by successive addition of PLINH

Table 1: Thermodynamic parameters of PLINH in HAS

T (K)	K ($Lmol^{-1}$) x10^5	ΔG° (kJ mol^{-1})	ΔH°(kJ mol^{-1})	ΔS° (Jmol K^{-1})
298	1.98	-87.09	-28.35	197.12
303	2.85	-88.07		
313	3.55	-90.05		

Table 2: Docking results of PLINH in different crystal structures of HSA

Sr no.	Name of protein	PDB id	Domain	Binding Energy{Kcal/mol}	No of Hydrogen bonds	Amino Acids to which drug is binding
1.	Crystal structure of HSA	1BMO	Subdomain II A	-8.0	3	LEU-103,TRY-148,ARG-197
			Subdomain III A	-8.0	4	LYS-106,LEU-103,TRY-148,ARG-197
2.	Crystal structure of HSA complexed with warfarin	2BXD	Subdomain II A	-8.0	3	GLN-459,ASP-108,GLU-425
			Subdomain III A	-8.0	2	HIS-146,ARG-117
3.	Crystal structure of HSA complexed with diazepam	2BXF	Subdomain II A	-7.7	1	LYS-212
			Subdomain III A	-8.4	2	HIS-510,LYS-524

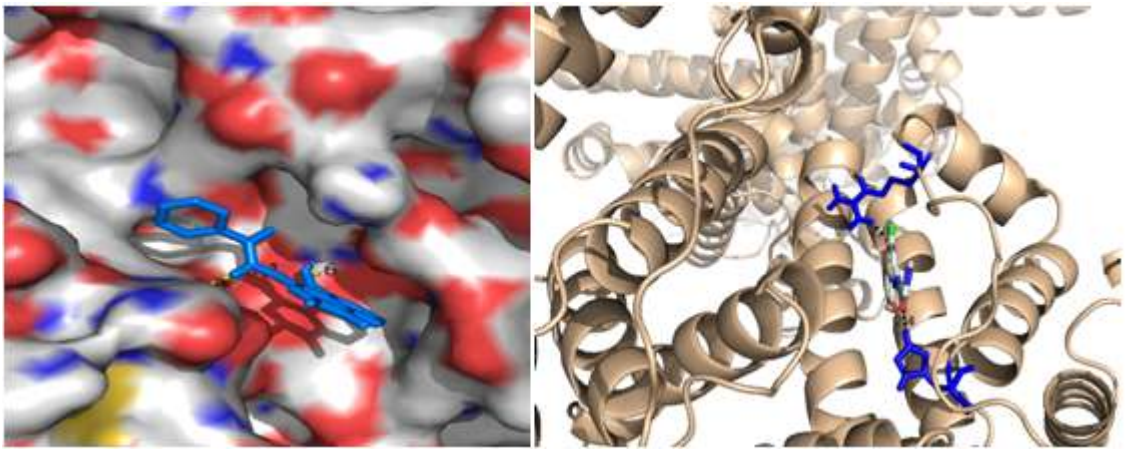

Figure 8: Docking Images of PLINH in HSA a) Surface view b) Ribbon view

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