Elucidation of binding mechanism of stigmasterol with human serum albumin: a biophysical and molecular dynamics simulation approach

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ABSTRACT

In the present study, we have analyzed the interaction of a phytochemical, stigmasterol (Stig), with human serum albumin (HSA) under physiological conditions using fluorescence quenching, circular dichroism and molecular modeling methods. Cytotoxic studies with Stig in mouse macrophages (RAW264.7) and HeLa cell lines showed anti-inflammatory and anti-cancer properties. Further, the intrinsic fluorescence of HSA was quenched by Stig, which was considered a static quenching mechanism. The site-specific marker experiments revealed that Stig binds to the IIIA subdomain of HSA with a binding constant of $K_{d} = 1.8 \pm 0.03 \times 10^{5} M^{-1}$ and free energy of $-7.26 \pm 0.031$ Kcal/mol. The secondary structure of HSA was partially unfolded after binding of Stig, which indicates an alteration in the microenvironment of the protein binding site. Molecular docking experiments found that Stig binds strongly with HSA at the IIIA domain of the hydrophobic pocket with one hydrogen bond. The rigidity of the protein-Stig complex and free energies were analyzed by molecular dynamic simulation (MDS) for 100 ns, where the HSA-Stig was stabilized after 40 ns. MDS studies revealed that HSA does not significantly change the secondary structure when it binds with Stig, which is in agreement with the circular dichroism data. Overall, the results obtained gave qualitative and quantitative insight into the binding interaction between HSA and Stig, which is essential in understanding the latter as a therapeutic molecule.

Introduction

Stigmasterol (Stig) is an unsaturated plant sterol occurring in the plant fats of soybean rapeseeds, Calabar and medicinal herbs, and the four o'clock flower Mirabilis jalapa (Siddiqui et al., 1990). Stig is also found in various vegetables, legumes, seed oils and unpasteurized milk. Edible oils contain a higher amount than vegetables (Jun-Hua et al., 2008). It is used as a precursor in manufacturing semisynthetic progesterone to rebuild the mechanism related to estrogen effects and acts as an intermediate in the synthesis of androgens and corticoids. It is also used as a precursor of vitamin D3 (Kametani & Furuyma, 1987; Sundararaman & Djerassi, 1977). Stig may be useful in the prevention of certain types of cancers like ovarian, prostate, breast and colon. It also possesses potent antioxidant, hypoglycemic and thyroid inhibiting properties (Panda et al., 2009). In addition, Stig has potential anti-osteoarthritic and anti-inflammatory properties (Gabay et al., 2010).

One major factor affecting the pharmacokinetics of administered ligands is binding affinity for human serum albumin (HSA) (Ghuman et al., 2005). HSA is the most abundant protein in the circulatory system and a carrier of exogenous ligands such as drugs through blood circulation to specific target sites (He & Carter, 1992; Mokaberi et al., 2021; Shahri et al., 2018). HSA has extraordinary acceptor capabilities with a wide range of molecules, making it an important protein to consider in the development of novel therapeutic agents as it is a critical player in drug metabolism (Jahanban-Esfahan et al., 2017). The crystallographic structure of HSA has revealed that the protein has 585 amino acid residues and contains three similar domains (I, II and III), with each domain consisting of two subdomains (A and B). Further, the tertiary organization of HSA is maintained by 17 disulfide bridges (He & Carter, 1992). As a carrier protein, HSA possesses several binding sites for structurally different ligands (Varshney et al., 2010). The presence of these multiple binding sites enables HSA to bind with many organic and inorganic compounds. Thus, HSA plays a vital role in the pharmacokinetics of ligands such as Stig (Tajmir-Riahi, 2007). However, the interaction between HSA and Stig has yet to be fully characterized. Among the pharmacokinetic parameters to be measured is the affinity of Stig for plasma proteins and the distribution of drug in-vivo. Therefore, unraveling...
the interaction of drugs, exogenous molecules, and biological molecules with albumin is very important, and in recent years a large number of these studies have been reported in the literature (Jahanban-Esfahlan et al., 2017; Roudini et al., 2020; Sadeghzadeh et al., 2020; Sohrabi et al., 2018). Previously, our group did some studies on natural products like pentacyclic triterpenoid betulinic acid and feruloyl-maslinic acid, which were isolated from Tephrosia calophylla and Tetracera asiatica. We found that these compounds bind strongly to HSA. Furthermore, we found that other natural compounds like trimethoxy flavone and coumaroyltiramine isolated from Anogeissus vicosula and Physalis minimashow good binding to HSA at IIIA and IIA sub-domains. The steroid molecules of androstenedione and its derivatives, 5α-dihydrocortisol and 5α-dihydrocortisol acetate molecules strongly bind to HSA (Nerusu et al., 2017). However, detailed and systematic investigation of the interaction between HSA and Stig at the molecular level have not yet been reported. In this paper, we characterize the interaction of HSA with Stig using fluorescence based approaches, circular dichroism (CD) spectroscopy, and in silico experiments. We investigated the flexibility at the HSA-Stig binding site using molecular docking and molecular dynamic (MD) simulation, which thoroughly illustrates the structural modification within HSA upon binding of Stig. The binding characteristics of binding mode, binding constants, location of the binding site, secondary structural changes of HSA upon Stig binding are reported.

Materials and methods

Preparation of stock solutions

Human serum albumin (HSA, essentially fatty acid-free and globulin free, purity 99%) was obtained from Sigma-Aldrich Company, and Stig was purchased from Natural remedies Pvt., Ltd, Bangalore, India, with a purity of 99.2%. Its stock solution (1 mM) was prepared in DMSO and used as received. All solutions were prepared with double-distilled water. Phosphate buffer (PBS, 100 mM, pH 7.4) was made up of K2HPO4 and NaH2PO4. The solution of HSA was prepared in PBS (pH 7.4). Later, we have also optimized the incubation time of Stig binding to HSA by fluorescence emission, and the maximum binding time was found to be 5 min. Thus, for the rest of the parameters, we have considered incubation of Stig with HSA as 5 min. Protein precipitation was not observed upon titration of Stig with HSA, which indicates that the mixture is transparent.

Cell response assay (MTT assay)

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). The cell response was carried out by the MTT assay in mouse macrophage (Raw 264.7) and pretreated with Lipopolysaccharide at 1 μg/mL for 4 h to induce inflammation. Human cervical cancer cells (HeLa) were subcultured and were seeded in 96 well plates at a density of 5 × 10^3 cells. After a while, the cells were treated with Stig in increasing concentration of (5 μM to 100 μM) for 48 h in a volume of 100 μL. The cells were grown in the same media, without Stig, and taken as the control. In the end, 20 μL of MTT (5 mg/mL in PBS) was added, and cells were incubated for 4 h. About 100 μL of DMSO was added to each well and mixed with repeated pipetting to dissolve the MTT crystals. Finally, cell response was measured at an absorbance of 570 nm using a microplate reader (μ QuantTM from Bio Tek Instruments, Inc.). Experiments were carried out in triplicates. It was taking control as the 100% reference cell response was calculated. The mean±SE was calculated and reported as the cell response (%) vs concentration (μM).

UV/visible and fluorescence spectral measurements

The UV/Visible spectra were recorded on PerkinElmer lambda 35. For these experiments, free HSA (25 μM) was titrated with increasing concentrations of Stig (10, 50 and 100 μM). In addition, a quantitative analysis of the potential interaction between Stig with HSA was performed by LS-55 spectrofluorimeter (PerkinElmer, USA) with 1.0 cm quartz cells. Fluorescence emission spectra were recorded at 25 °C with a 300–500 nm wavelength range and excited at 280 nm. The bandwidth was fixed to 5.0 nm for both excitation and emission. The sample temperature was maintained at 25 °C. HSA concentration was 1 × 10^-6 M, and the concentrations of Stig were 1–9 μM in 0.1 M phosphate buffer with pH 7.2 (physiological pH). Temperature was controlled at the set values (±0.5 °C) using a peltier (Jasco FP-8500 DP WRE) and water jacket cell holder equipped with a stirrer. The excitation wavelength was set 280 nm at 3 different temperatures (15 °C, 25 °C and 37 °C). Three independent experiments were performed, and each time identical spectra were obtained. All protein fluorescence spectra were corrected by subtraction of buffer. All protein fluorescence spectra were corrected by subtraction of buffer. Furthermore, the spectra were corrected to avoid the inner filter effect using the below equation.

\[
F_{\text{cor}} = F_{\text{obs}}[A_{\text{exc}} + A_{\text{em}}]/2
\]

Where the intensity of fluorescence observed \(F_{\text{obs}}\) was corrected for inner filter effect \(F_{\text{cor}}\) using the absorption of HSA at the excitation wavelength of 280 nm \(A_{\text{exc}}\) and absorption at the emission wavelength of 360 nm \(A_{\text{em}}\) (Lakowicz, 2009; Zohoorian-Abbootarbi et al., 2012). The corrected values allow for evaluation of fluorescence quenching produced solely from interaction of the drug with albumin (Van de Weert & Stella, 2011).

Site-specific experiment with sites I (phenylbutazone) and II (ibuprofen) and IB (lidocaine) markers

Site marker competitive experiments were carried out using three known site-specific markers, phenylbutazone for site I (IIA), ibuprofen (IBU) for site II (IIIA) (Kragh-Hansen et al., 2002) and lidocaine for site III (IB) (Hein et al., 2010). The
concentration of HSA and the site probe were held constant at 1 μM, and titrated against a Stig gradient of 1 to 9 μM. The fluorescence quenching data was analyzed using the Stern-Volmer equation (Zsila et al., 2003).

**CD spectra measurements**

The CD spectra were recorded on a Jasco J-815 spectropolarimeter under a constant nitrogen flush. For measurements in the far-UV region (190–300 nm), a quartz cell with a path length of 0.2 cm was used. Three scans were accumulated with continuous scan mode and a scan speed of 200 nm min⁻¹, with data being collected at 0.2 nm and a response time of 2 s. The protein HSA final concentration was 0.001 mM and concentrations of the Stig were 0.001, 0.005 and 0.009 mM. The CDNN2.1 web-based software was used to calculate the secondary structural changes in the protein—all the spectra were recorded at room temperature.

**Molecular docking**

The docking study was conducted to explore possible positions between Stig and HSA. The discovery studio generated the initial structures of all the molecules. Autodock 4.2.3 program is used for molecular docking (Morris et al., 2009). Water molecules were removed, and hydrogen atoms were added to the proteins; Kollman Uni charges were also assigned to HSA (Morris et al., 1996, 1998). All other parameters were maintained at their default setting. The known crystal structure of HSA (PDB ID: 1AQ6) was obtained from Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The Lamarckian genetic algorithm was applied in the docking, and 100 genetic algorithm runs were performed with a population size of 150 and the maximum number of 2.5 × 10⁶ energy evaluations. The resulting conformations were clustered using a root-mean-square-deviation of 2.0 Å and the number of GA run; 30. Finally, the least energy conformation was selected as the final bioactive conformation.

**Molecular dynamics simulations**

Molecular dynamics simulation is an important parameter to find out the stability of the protein-ligand complex. Here a 100 ns MD simulation of the complex was carried out with the GROMACS 4.5.6 Package by using a GROMOS96 43A1 force field (Berendsen et al., 1995; Van Gunsteren et al., 1998). A 10 ns simulation was more than enough for this study as the HSA+Stig were stabilized within 5 ns; but we even extend it to 100 ns in order to understand the stability of unliganded HSA, HSA-Stig complexes. The topology parameters of Stig were built by using PRODRG (Schuttelkopf & van Aalten, 2004). Then the complex was immersed in a cubic box of extended single point charge (SPC) water model. The solvated system was neutralized by adding 14 Na⁺ counter ions and 23 341 solvent water molecules. To release conflicting contacts, energy minimization was performed using the steepest descent method with 2000 steps. The periodic boundary condition was used (pbc = xyz, 3-D PBC), and the motion equations were integrated by applying the leaf-frog algorithm with a time step of 2fs. The atomic coordinates were recorded at every 2ps during the simulations. For later analysis electrostatics were followed as coulomb type = PME, Particle Mesh Ewald Trynda-Lemiesz (2004) for long-range electrostatics, where pmeorder was kept to be 4 and cubic interpolation was there with Fourier spacing of 0.16, grid spacing for FFT. The short-range vdW cutoff was kept to be 1.0 in nm. Finally, the entire system was subjected to 10 ns MD at 0k temperature and 1 bar pressure Modified Brendensen Thermostat where time constant was 0.1 ps and pressure coupling was Parrinello-Rahman, where uniform scaling of box vectors was kept with isothermal compressibility of water, was bar⁻¹. The MD simulation and results analysis were performed on the OSCAR Linux cluster with 36 nodes (dual Xeon processor) at the BIF facility, University of Hyderabad. Furthermore, the stability of the HSA-Stig complexes was determined in 100 ns timescale simulations. Our main aim was first to understand the binding capacity of the Stig with the plasma protein HSA and as it is the major carrier protein for all the endogenous and exogenous ligands. For further information see previous articles (Yeggoni et al., 2014, 2016).

**Free energy by MMPBSA**

To calculate the binding free energy of the HSA-Stig complex, MM-PBSA for GROMACS was used (Baker et al., 2001; Kumari et al., 2014). It estimates the free energy using the ensemble average of the HSA-Stig complex trajectories. GMXPBSA 2.1 is a user-friendly suite of Bash/Perl scripts for streamlining MM/PBSA calculations on structural ensembles derived from GROMACS trajectories, which calculates binding free energies for ligand-protein or protein-protein complexes (Bradshaw et al., 2011). GMXPBSA 2.1 performs various comparative analyses, including a posteriori generation of alanine mutants of the wild-type complex, calculation of the mutant complexes binding free energy values, and comparing the results with the wild-type system (Roy et al., 2015). Moreover, it compares the binding free energy of different complex trajectories, allowing the study of the effects of non-alanine mutations, post-translational modifications or unnatural amino acids on the binding free energy of the system under investigation (Païsoni et al., 2015). Finally, it can calculate and rank relative affinity to the same receptor utilizing MD simulations of proteins in complex with different ligands. To dissect the different MM/PBSA energy contributions, including molecular mechanic (MM), electrostatic contribution to solvation (PB), and nonpolar contribution to solvation (SA), the tool combines two freely available programs: the MD simulations software GROMACS ( Pronk et al., 2013) and the Poisson–Boltzmann equation solver APBS (Baker et al., 2001). In conventional terms, these are the following equations used to calculate the binding free energy.

\[
\Delta G_{\text{binding}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}}) \\
G_x = E_{\text{MM}} + G_{\text{solvation}} \\
E_{\text{MM}} = E_{\text{bonded}} + E_{\text{non-bonded}} = E_{\text{bonded}} + (E_{\text{vdW}} + E_{\text{elec}}) \\
G_{\text{solvation}} = G_{\text{polar}} + G_{\text{non-polar}}
\]
Where $\Delta G_{\text{binding}}$ is the binding free energy; $G_{\text{complex}}$ is the total free energy of the docked protein–ligand complex; $G_{\text{protein}}$ and $G_{\text{ligand}}$ are total free energy of the separated protein–ligand in the solvent, $x$ is protein, ligand or docked complex, $E_{\text{MM}}$ is the average molecular mechanics potential energy in vacuum and $G_{\text{solvation}}$ is free energy of solvation, $E_{\text{bonded}}$ has bonded interactions of the unsuitable interactions, angle, bond and dihedral, $E_{\text{non-bonded}}$ is nonbonded interactions including electrostatic ($E_{\text{elec}}$) and van der Waals ($E_{\text{vdw}}$) interactions, $G_{\text{polar}}$ accounts for the electrostatic contribution to solvation from the Poisson–Boltzmann (PB) equation and $G_{\text{non-polar}}$ accounts for the non-polar contribution to solvation estimated from the solvent-accessible surface area (SASA) as equation following:

$$G_{\text{non-polar}} = \gamma \text{SASA} + b \quad (6)$$

where $\gamma$ is a coefficient of surface tension of the solvent and $b$ is a fitting parameter. The generated output contains average and standard deviations of all energetic components including the binding energy and the values of energetic terms as a function of time (Kumari & Subbarao, 2021). All calculations were performed in a single or distributed automated fashion on a cluster facility to increase the calculation by dividing frames across the available processors.

**Results and discussion**

**Cell response assay**

To understand the therapeutic activity of Stig, we employed this ligand in an MTT assay using mouse macrophages (RAW 264.7). To induce inflammation in mouse macrophages, we treated cells with lipopolysaccharides (LPS) 1 $\mu$g/mL. Stig has been shown to possess anti-inflammatory and antioxidant properties (Lee et al., 1984). Indeed, our results indicated that Stig could reduce inflammation in mouse macrophages with an IC$_{50}$ of 45 $\mu$M (Figure 1A). The results obtained indicate that Stig showed an increased inhibition in the growth of inhaled macrophages in a dose-dependent manner (Ying et al., 2013). We have also tested the anti-cancer property on human cervical cancer cell lines (HeLa) and examined the effects of Stig on the viability of these cells by using the above method. A decrease in cell viability with Stig in a dose-dependent manner was observed, with an IC$_{50}$ value of 5.9 $\mu$M. (Figure 1B) which indicates strong activity against HeLa cell lines.

Nevertheless, we tested Stig with other cell lines like HepG2 (hepatic carcinoma) and HT-29 (human colon adenocarcinoma cell line). However, Stig was specific to macrophages, which indicates that Stig can act categorically against inflammatory diseases. Thus, the phytochemical Stig can be used as a potential therapeutic agent.

**UV/visible spectroscopy analysis**

The absorption band observed at 280 nm is the product of aromatic amino acids within HSA (Zhao et al., 2010), and signal intensity at this wavelength is inversely proportional to the concentration of bound Stig. We used this UV-absorption assay to determine the type of quenching mechanism involved in the HSA:Stig interaction. So, the data indicates the UV data of free HSA and its complexes with various concentrations of Stig (10 to 100 $\mu$M) after incubation for 5 min (Supplementary Figure S1). The decrease in the intensity was seen as a result of complexation with Stig. The type of quenching mechanism involved in the interaction was identified by analyzing UV-absorption spectra. At the mentioned wavelength, it is well known that the binding to HSA can be seen quickly by the change in the absorbance (Ghuman et al., 2005; Petitpas et al., 2001). In the case of dynamic quenching, only the excited state of the fluorophore molecule is involved; hence, the absorption spectra should remain unchanged with a change in ligand concentration. In the case of static quenching, a complex formation occurs between the protein and ligand and hence the absorption spectra is altered upon changes in the ligand concentration (Lakowicz, 2009). So, absorption intensity showed an increase at 280 nm, which the wavelength at which HSA absorbs increased the Stig concentrations (Supplementary Figure S1). Based on the decreased absorption intensity of HSA:Stig compared to HSA alone, a complex formation between HSA and Stig is predicted, and the static quenching mechanism is established for HSA-Stig interaction as shown earlier (Kandagal et al., 2006). Similar results have been observed for HSA and drug-like compounds (Ahmad et al., 2006).

**Quenching, binding number and binding affinity**

The intrinsic fluorescence quenching of HSA has been used to analyze many drug-protein interaction mechanisms. When the excitation wavelength is 280 nm, the tryptophan residues
in HSA contribute to fluorescence emission. The fluorescence emission decreases with a slight change in the local microenvironment of HSA, such as the transition in protein conformation, binding, and denaturation. The effect of Stig on HSA fluorescence intensity is shown in Figure 2A. The intensity of HSA fluorescence quenched with the increasing concentrations of Stig, suggesting the formation of complex HSA and Stig. It can be interpreted that the microenvironment in protein around the fluorophore binding site has changed after the addition of Stig. We have plotted $F_0/F$ against $Q$ to determine the static and dynamic quenching of HSA. The $K_q$ was estimated according to the Stern-Volmer equation:

$$F_0/F = 1 + \frac{K_q}{F_0} Q = 1 + K_{SV} Q$$  \hspace{1cm} (7)$$

Where $K_{SV}$ is the Stern-Volmer quenching constant ($K_{SV} = K_q t_0$), $K_q$ is the bimolecular quenching rate constant, $t_0$ is the lifetime of the fluorophore in the absence of quencher, $[Q]$ is the quencher concentration, $F_0$ and $F$ are the fluorescence intensities in presence and absence of quencher (Tayeh et al., 2009; Zsila et al., 2003). From the above equation, we have calculated the static quenching constants, $K_q$ (Stig) = $5.8 \pm 0.03 \times 10^{13}$ M$^{-1}$s$^{-1}$ (Figure 2B). According to the standard procedures, if $K_q$ is more than the maximum collisional quenching constant, then static quenching is dominant. We found that the dynamic quenching constant is $2.06 \pm 0.03 \times 10^{10}$ M$^{-1}$s$^{-1}$ (Agudelo et al., 2012; Lakowicz, 2009; Yeggoni et al., 2014); hence the binding of Stig to HSA was static as $K_q$ is three orders of magnitude greater than the colloidal constant. In other words, protein fluorescence was quenched as a result of specific complex formation as a ground state. This indicates the formation of the HSA:Stig complex. The HSA:Stig interaction could be stabilized by hydrogen bonding, hydrophobic interaction, ionic interaction.

The Stern-Volmer equation allows us to explore only the intramolecular quenching process, and it cannot be used to calculate binding constants, number of binding sites and free energy. To find the binding constant of the HSA:Stig interaction, we used a modified Stern-Volmer equation.
penicillin bind HSA with lower affinity (2.6 
approved drugs like furosenamide, imipramine, and benzyl-
interaction with HSA; however, most of the known FDA
temic circulation and has a fundamental role in the transpor-

Kb is the binding constant, and [Q] is the drug concentration.

between HSA and Stig at 25

is a one-to-one binding site for Stig with HSA.

tive system. When the value of n is almost equal to 1 there

are hydrogen bonds, Vander Waals forces, electrostatic, and

hydrophobic interactions, which can be confirmed by

The binding constant calculated from the above equation

was Kstig=1.8 ± 0.03 × 105 M⁻¹ upon binding of Stig to HSA

at 25 °C. These data are very much correlated with the data

found in silico, i.e. 4.1 ± 0.03 × 105 M⁻¹. The fluorescence

spectral data and the calculated binding affinity Kstig =

1.8 ± 0.03 × 105 M⁻¹ value indicate a strong interaction

between HSA and Stig at 25 °C. These results indicated that

Stig could be carried by HSA through blood circulation to

various organs in vivo. HSA is the core protein in the sys-
temic circulation and has a fundamental role in the transpor-
tation and distribution of ligands in vivo. Stig has a strong
interaction with HSA; however, most of the known FDA
approved drugs like furosenamide, imipramine, and benzyl-
penicillin bind HSA with lower affinity (2.6 × 10⁻⁴, 2.5 × 10⁻⁴,
and 1.2 × 10⁻³ M⁻¹ respectively) (Chamani, 2010; Varshney
et al., 2010). Since Stig possesses binding constants in the
range of 10⁻³–10⁻⁴ M⁻¹, it represents a stronger binder of
HSA. Among the significant pharmacokinetic characteristics
are to be measured are binding of Stig to plasma proteins and
drug distribution in vivo. The fluorescence spectral data and
the calculated binding affinity Kstig = 1.8 ± 0.03 × 105 M⁻¹
value indicate a strong interaction between HSA and Stig.
Further, with different temperatures 15 and 37 °C the bind-
ing constants were found to be Kstig= 3.5 ± 0.12 × 10⁴ and
0.3 ± 0.09 × 10⁴ M⁻¹, respectively. This indicated that Stig
could be carried by HSA and carried through blood circula-
tion to various organs in vivo.

### Calculation of free energy change

There are essentially four types of noncovalent interactions
that could play a role in ligand binding to proteins. These
are hydrogen bonds, Vander Waals forces, electrostatic, and
hydrophobic interactions, which can be confirmed by

Calculating thermodynamic parameters. We know that the
free energy change (ΔG°) can be calculated from the follow-
ing equation:

\[
\Delta G° = -RT\ln K
\]  (9)

Where ΔG° is the free energy change, R is the gas con-
stant at room temperature, and K is the binding constant.
From the above equation, the free energy change for Stig
has been calculated and found to be –7.26 ± 0.031 Kcal/mol
at 25 °C when binding with HSA. Interestingly, this data also
supports the in silico analysis of the HSA-Stig complex, where
ΔG°Stig = –7.1 ± 0.031 Kcal/mol. Similar results were found
with other natural-compounds (trans-feruloyl maslinic acid,
betulinic acid, β-sitosterol, lupeol, L-dopa, 7-hydroxycou-
marin, tri methoxy flavone corilagin, embelin etc.) (Yeggoni
et al., 2014, 2016).

### Thermodynamic parameter for the binding of stigmasterol to HSA

The binding forces between the ligands and biomolecules
include 4 types of interactions: hydrophobic, hydrogen bond-
ing, van der Waals interactions, and electrostatic forces
(Leckband, 2000). To calculate the thermodynamic param-
eters, the van’t Hoff equation was used as represented below:

\[
\ln K = -\Delta H°/RT + \Delta S°/R
\]  (10)

\[
\Delta G° = \Delta H° - T\Delta S°
\]  (11)

In the above equation, the association constant, K, is the
effective quenching constants Ks at the corresponding tem-
perature, and R is the gas constant. ΔH and ΔS are the

| Parameter | 15 °C | 25 °C | 37 °C |
|-----------|-------|-------|-------|
| Kₘ (binding constant, M⁻¹) | 3.5 ± 0.12 × 10⁴ | 1.8 ± 0.03 × 10⁴ | 0.3 ± 0.09 × 10⁴ |
| Kₘ (bimolecular quenching rate constant, M⁻¹ s⁻¹) | 5.0 ± 0.02 × 10¹¹ | 5.8 ± 0.03 × 10¹¹ | 3.9 ± 0.06 × 10¹³ |
| ΔH° (binding enthalpy, kcal mol⁻¹) | –25.25 | –25.25 | –20.10 |
| ΔS° (entropy change, kcal mol⁻¹) | –18.67 | –18.99 | –18.99 |
| ΔG° (Gibbs free energy change, kcal mol⁻¹) | –6.18 ± 0.01 | –7.26 ± 0.031 | –4.74 ± 0.024 |

Figure 3. Displacement of ibuprofen from HSA-Ibuprofen complex by stig. Fluorescence emission spectra were recorded on Perkin Elmer LS55 fluorescence spectrometer for stig with HSA in the presence of site probe markers. Ibuprofen is referred to as IIIA site-specific probe. The concentrations of HSA and ibuprofen were maintained constant at a concentration of 1 μM whereas, the stig was varied from 1 to 9 μM. Inserts: Modified Stern-Volmer plot. Plot of log (dF/F) against log [Q] λex = 280 nm, λem = 360 nm.
enthusiasm and entropy changes, respectively. From the temperature dependence of the binding constants (Supplementary Figures S2 and S3), the thermodynamic functions involved in the binding process were calculated in Table 1. The negative sign for Gibb’s free energy ΔG indicates that the interaction process was spontaneous between HSA and Stig. Table 1, ΔH and ΔS for the binding interactions were calculated to be -25.25 kcal mol⁻¹ and -18.99 kcal mol⁻¹ for Stig-HSA. According to Ross and Subramanian (1981), the negative values of ΔH and ΔS shows that binding is mainly enthalpy driven, whereas entropy was unfavorable. The magnitude and signs of thermodynamic parameters ΔH and ΔS for HSA reactions can account for the main forces contributing to the stability of HSA. From a thermodynamic viewpoint, ΔH > 0 and ΔS > 0 implies hydrophobic interaction, ΔH < 0 and ΔS < 0 indicates either Van der Waal forces or hydrogen bonding, and ΔH < 0 and ΔS > 0 reflects electrostatic interactions. From this we see that the negative values obtained for the interaction of Stig with HSA is mainly driven by hydrogen bond formation and Van der Waal’s interactions. Similar observations are represented between the interaction of pambagin and juglone with serum albumin (Jahanban-Esfahan et al., 2017; Li et al., 2011). With regard to the thermal stability of HSA and HSA-ligand complexes, we have done temperature-dependent circular dichroism for HSA and HSA with bioactive compounds (trimethoxy flavone and corilagin), from 25-85 °C in previous publications. We have observed that the secondary structural conformation of the protein is not significantly changed up to 60 °C in HSA-ligand complexes. An earlier report showed that the Tm of the HSA alone was around 65 °C, which shows that the unfolding of the protein occurs only after this point (Yeggoni et al., 2016). Therefore, we assume that at 60 °C the HSA-Stig complex is stable; thus, the protein complex is thermodynamically and conformationally stable.

**Table 2. The secondary structural study of the HSA alone and its interaction with Stig.**

|          | HSA       | HSA + 0.001 mM | HSA + 0.005 mM | HSA + 0.009 mM |
|----------|-----------|---------------|---------------|---------------|
| α-Helix  | 58 ± 1.0  | 56.70 ± 2.02  | 56.20 ± 2.1   | 55.6 ± 1.5    |
| β-sheet  | 23 ± 0.72 | 23.80 ± 0.5   | 24.00 ± 0.7   | 24.70 ± 0.2   |
| Random   | 19 ± 1.0  | 19.10 ± 0.7   | 19.40 ± 0.41  | 19.70 ± 0.6   |

The data analyzed by web-based software CDNN 2.1.

However, there is a site-specific marker for IB, IIA, and IIIA subdomain of HSA; but Stig displaces only ibuprofen from subdomain IIA. Therefore, from this study, we can report that Stig binds independently at the IIA sub-domain of HSA.

**Protein conformational study from circular dichroism**

Circular dichroism spectroscopy is a powerful tool to study the structural changes in the protein. The amendments in the structure of the HSA polypeptide backbone can be studied with the help of far UV-CD spectra. Secondary structural elements like α-helix, β-sheets and turns give rise to peculiar CD signals; thus, it can study well with the help of CD. To understand the changes of HSA secondary structure induced by the binding of Stig to HSA, the CD spectra were measured. CD spectrum of HSA shows two minima at 208 and 222 nm, characteristic of the α-helical protein. The negative absorption band at 208 nm is due to the π→π* transition of carbonyl groups in polypeptide chains, and the other band at around 222 nm is due to the n→π* transition of the carbonyl group. CD spectrum of HSA shows a gradual loss in intensities of the 222 and 208 nm bands, which indicates the loss of α-helicity with a concomitant increase of β-sheet structure. This phenomenon is predominately exhibited by considerable changes in the secondary structure of a protein. Consequently, the loss of helical stability may result from the formation of a complex between HSA and Stig. Here, the secondary structural changes are calculated by using the CDNN program. The secondary structure of free HSA consists of ~58% α-helix, ~23% β-sheets and ~19% random coils, which is in agreement with previous reports (Kanakis et al., 2007; Yeggoni et al., 2014). From this method, it was found that upon complexation of HSA with different concentrations of Stig (0.001, 0.005, 0.009 mM), the α-helical content of the protein decreased from 58 ± 1.0 to 55.6 ± 1.5% with an increased β-sheets from 23 ± 0.72 to 24.7 ± 0.2%, and random coils 19 ± 1.0 to 21 ± 0.6%, respectively (Table 2). Therefore, the results suggest that changes in the secondary structural...
elements arise from the marginal unfolding of HSA upon binding of Stig (Figure 4). Earlier reports also indicate that conformational changes occur in HSA upon complexation with ligand (Beauchemin et al., 2007; Jiang, 2008; Kanakis et al., 2007; Zsila et al., 2003). Thus, the protein conformation in our experiment arises due to change in the local structural changes in the secondary structural elements. Stig complexation did not alter the significant conformational change in HSA, indicating partial structural changes in the HSA.

Likewise, to understand the stability, we performed molecular dynamics studies on Stig to corroborate the HSA:Stig complex conformers with our docking studies (Figure 5). The conformational changes observed were consistent with the RMSD and Rg data (Figure 6A,B).

**Stigmasterol binding site with HSA by docking**

Molecular docking is an important method for understanding the binding properties (binding energy and binding site) of HSA and Stig at the atomic level. Based on the binding energy calculation, we noticed that Van der Waals interactions and hydrogen bonds are the major force in the binding model. The presence of hydrogen bond with Thr478 would decrease the hydrophilicity of Stig and increased their hydrophobicity, which made the HSA-Stig complexes more stable. Schematic representation of Stig in Sudlow’s site II is shown in Figure 5A,B. The main residues involved in the interaction were generated using LigPlot v.4.5.3 and depicted in Figure 5C. The corresponding binding constant and free energy value of HSA-Stig were obtained from the in silico docking orientation analysis and found to be $4.1 \times 10^5$ M$^{-1}$ and $-7.1$ kcal mol$^{-1}$. In our study, the thermodynamic parameters for the binding interaction had provided significant evidence about the nature of the intermolecular forces, especially parameters like free energy, describing the type of interactions involved in the formation of the HSA:Stig complex. The calculated free energy change for the formation of the HSA-Stig complex is negative which indicates that the interaction is spontaneous (Roudini et al., 2020). The negative
free energy and the docking data suggest the type of interactions within the HSA:Stig complex include hydrogen bonds and hydrophobic interactions. The sorting of conformers in docking is energy-based, which is in descending order of binding energy. Thus, the best conformer falls in the top 10 (which is the default threshold value in the auto dock), choosing the conformer that has binding energy equal/near to the experimental Gibbs free energy based on the best binding. The binding constants and free energy were almost close to that of the above mentioned experimental values i.e. 1.8 \times 10^5 M^{-1} and –7.26 kcal mol^{-1} and a slight difference was due to the docking process which was conducted in a simulated vacuum state, whereas the interaction between HSA and Stig happened in aqueous solution state. The difference in binding energy was within acceptable limits. These results correspond with those obtained by the experimental methods mentioned above.

Overall, the HSA-Stig docking results validated the experimental results by finding a preferred binding region of Stig in the subdomain IIIA (site II) of HSA. Among the top conformers, the least binding energy conformer was considered for the binding site on the HSA using LigPlot v.4.5.3, as there are two major ligand-binding sites on HSA, namely site I and site II, which are represented as subdomains IIA and IIIA, respectively (He & Carter, 1992; Sudlow et al., 1976). In the binding pocket, Stig is surrounded by the following residues Glu479, Ser480, The206, Ala 213, Lys351, Ala210, Leu347, Arg209, Ala350 and Leu327. Docking results showed that Stig bind within the binding pocket of the subdomain IIIA of HSA stabilized through H-bonding (with Thr478) at a distance of 3.06 Å and hydrophobic interactions with various amino acid residues (Figure 5C). Therefore, it can conclude that the interaction between the Stig and HSA is mainly hydrogen bonding and van der Waals interactions. The docking results were consistent with Figure 3, suggesting that hydrogen bonds were the most important interaction forces information of the HSA-Stig complex, and the binding site was in site II. These results further support the adequate fluorescence quenching of HSA in the presence of Stig.

**Characteristics of molecular dynamics simulation**

The structural features of HSA-Stig interaction were further analyzed based on the molecular trajectory of MD simulation. Root mean square deviation of HSA and Stig was examined to investigate the system’s stability (protein, ligand, ions, water etc.). RMSD values for free HSA and HSA-Stig complexes during 100 ns MD simulations are shown in Figure 6A. It is observed that the total energy for all molecular systems remains stable with some fluctuations after 40 ns of simulation, so the following analysis was carried out based on the stability of simulation after 40 ns of equilibration. The stability of the whole HSA structure makes it possible to obtain reliable analysis on the ligand binding and its effects on HSA-Stig interactions through MD simulations. The relative decrease in RMSD value of the complex to free HSA also

| Table 3. Free energy values evaluated by MMPBSA. |
|-----------------------------------------------|
| HSA-Stig (binding energy) | –123.075 ± 0.232 kJ/mol |
| Electrostatic energy | –29.158 ± 0.165 kJ/mol |
| Polar solvation energy | 92.385 ± 0.273 kJ/mol |
| SASA energy | 0.014 ± 0.000 kJ/mol |
| Vander Waals energy | –186.316 ± 0.206 kJ/mol |
indicates the conformational changes, increased rigidity and stability of the protein HSA upon binding of Stig. The time evolution of radius of gyration (Rg) in the course of 100 ns of MD simulation of free HSA and the HSA-Stig complex and the result is shown in Figure 6B. As seen, the initial Rg values of both HSA and HSA-Stig complex is 2.65 nm, while they are finally stabilized at 2.6 ± 0.009 nm and 2.576 ± 0.018 nm, respectively. Earlier work reported that the free HSA Rg value, as determined experimentally by neutron scattering in aqueous solution, is 2.74 ± 0.035 nm, which agrees with that obtained from our MD simulations. Thus based on the RMSD and Rg values obtained in this work, it can be concluded that the HSA molecule exhibits a slight conformational change when it combines with Stig. Thus, both theoretical and experimental results suggest a slight conformation change was observed due to the binding of Stig at the binding site of the HSA. The mobility of the protein was also analyzed by calculating the time-averaged root mean square fluctuations (RMSF) of free HSA and HSA-Stig complexes based on the 100 ns trajectory data. The RMSF values of HSA–Stig overlap with the RMSF values of unliganded HSA, and these are plotted against residue numbers. The general profiles of atomic fluctuations were found to be very similar to each other. The comparison of RMSF for HSA-Stig in subdomain IIIA, where Stig is binding (Figure 6C), the results indicate fewer atomic fluctuations at ligand binding sites I and II to other domain regions as shown in Figure 6C. It show that RMSF value of HSA-Stig in subdomain IIIA was much more than free HSA, with very little fluctuation. Many of the residues at the less and rigid fluctuations suggest that amino acids that can affect the binding affinity in HSA molecules have smaller atomic fluctuations; that means the amino
acid residues are rigid compared with the rest of the residues at a different domain. Even docking result harmonized with the MDS that indicates structural changes of the HSA domain shows different modes of binding site properties, which can help the Stig be apt to the different binding modes, as viewed in the docking result (Figure 5).

Thus, both experimental and computational results suggest a slight conformational change probably around the binding pocket of the HSA. In silico interaction studies showed the excellent stability of Stig with HSA. RMSF of HSA-Stig showed less fluctuations after binding with Stig indicates that this stig formed a rigid structure with HSA. Also, they have good hydrogen bond interaction throughout the simulation, which explains that the HSA is stabilized upon binding of Stig. Rg and RMSD values observed the stability of Stig after complex formation with HSA. The complexes were stable from initial ns to 100 ns simulations. The rigidity of residues where the Stig binds to the HSA was shown through the root mean square fluctuation. Thus, our in silico studies explain that Stig was interacting with the HSA in the hydrophobic core through hydrogen bonding and was stable after the interaction. The stability of the complexes was analyzed and shown in the form of Rg and RMSD of the complexes.

**Free energy by MMPBSA**

To better understand the strength of these interactions, binding free energies between HSA and Stig were calculated using the MM/PBSA approach (Fujiiwara & Amisaki, 2008; Genheden & Ryde, 2015). G_mmpbsa has been proved to be an effective tool that can be used to estimate the relative binding free energy and provide a decomposition of the residue contribution to the binding. In our study, coordinates were saved every 20 ps from 80 to 100 ns stable frames. Based on these MD trajectories, binding free energies were calculated using the MM/PBSA method (Table 3). After adopting a stable pose, Stig and HSA were analyzed, and they have energies from $-123.075 \pm 0.232$ kJ/mol. However, docked complex of HSA-Stig the electrostatic energy ($-29.158$ kJ/mol) contributes to the total binding energy. The VDW interaction energy term ($-186.316$ kJ/mol) plays substantial role for the stability of docked HSA+Stig. The $\Delta G$ for binding affinity of HSA+stig complex are driven by VDW interaction energy and electrostatic interaction. Based on the molecular dynamic's trajectory, binding free energy analysis and its corresponding components were obtained from the MM/PBSA calculation (Table 3). Here, the value of Stig is looking very energetically stable.

To illustrate interactions between binding residues of HSA with Stig, time frames were analyzed at different nanoseconds (10, 20, 30, 40 50, 60, 70, 80, 90 and 100 ns) (Figure 7). Similar results were observed with HSA-Stig complex, the Stig showed hydrogen bond interaction with Glu 354, Lys351, and later in a further increase in MD simulations above 40 ns, there is a conformational change and decline in the structure of HSA, which is also confirmed through RMSD fluctuations (Figure 6). Furthermore, the visual inspection of HSA-Stig complex conformations during MD showed interactions in the hydrophobic region indicate the presence of hydrophobic interactions between Stig and residues of HSA as this region is the prime hydrophobic core (Figure 8).

**Conclusion**

In conclusion, the interaction between Stig and HSA has been probed by fluorescence, molecular docking and molecular dynamics simulations. The pharmacological effect of the bioactive compound Stig showed a decrease in the viability of inflammatory macrophages and human cervical cancer cells. As per the experimental results, Stig can bind with HSA during transport and metabolic processes in vitro. This provides crucial insight into the interactions of HSA, which are physiologically significant. Experimental results show that fluorescence quenching results confirmed the static nature of the quenching mechanism as the values obtained for the quenching constant ($5.8 \pm 0.03 \times 10^{13}$ M$^{-1}$S$^{-1}$) were more significant than scatter collision ($\sim 10^{10}$). The binding constant value ($1.8 \pm 0.03 \times 10^5$ M$^{-1}$), demonstrated a mild and equitable binding of Stig to HSA, which is the range of known FDA approved drug values ($10^3 - 10^6$ M$^{-1}$). The results obtained from CD confirmed that there are Stig induced partial conformational changes in the secondary structure of HSA upon interaction. Besides, site-specific marker experiments show Stig is competing with ibuprofen, thereby replacing it for the binding site of subdomain IIIA, further supported by molecular docking results.

Further, molecular docking studies indicated that that Stig binds with robust affinity to HSA at the Sudlow site IIIA and the forces involved for complexation were hydrogen bonds and hydrophobic interactions. The result showed that binding of Stig induces marginal changes in HSA, which was further proved by the in silico molecular dynamics simulation study. Furthermore, molecular dynamics simulation studies indicated that the docked conformer is stable. In a time-dependent simulation study, Stig binds at the IIIA subdomain of HSA within 40 ns and is stabilized. The MMPBSA method was further used to investigate the binding free energies. The least binding energy for the HSA-Stig complex indicated that Stig showed higher binding affinity, which is mainly due to the stronger hydrogen bonding interactions. The outcomes of this study contribute to the understanding of the HSA:Stig interaction at the molecular level, which may lead to the future designing of Stigmasterol based therapeutical agents.

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