RESEARCH ARTICLE

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In Vitro Characterization of the Interactions of Human Serum Albumin with Rosuvastatin and Atorvastatin Using Fluorescence Spectroscopy and Molecular Modeling

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ABSTRACT

Human serum albumin (HSA) is one of the most important transporters for drugs in the systemic circulation. In this study, we investigated the interaction of rosuvastatin (ROS) and atorvastatin (ATO) with HSA. Binding of a drug molecule to HSA significantly affects the pharmacokinetics of the drug as it increases drug solubility in plasma, decreases toxicity and protects molecules from oxidation. This study was made using fluorescence spectroscopy and molecular modeling approach. Fluorescence spectra were recorded for two different statins brands at seven different concentrations. The results revealed that both statins (ROS and ATO) cause the fluorescence quenching of the HSA solution. ROS and ATO binds strongly to HSA with the binding constant (K_b) of 1.0246×10^6 and $0{,}9018\times10^6$, respectively. In addition, it was observed that high concentrations of ATO cause a shift of the emission maximum towards longer wavelengths (red-shift), which may be due to the unfolding of protein chains or denaturation. Furthermore, it was calculated that HSA possesses one binding site for ROS and ATO. Results from molecular docking showed that ROS has a higher affinity for Sudlow site I compared to Sudlow site II and the main binding forces are hydrogen bonds. ATO has nearly equal affinity for both binding sites on HSA, and the main binding forces are hydrophobic interactions.

Keywords: Atorvastatin, Fluorescence quenching, Human serum albumin, Rosuvastatin.

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I. Introduction

Human serum albumin (HSA) is the most abundant plasma protein, accounting for 60% of all plasma proteins. HSA is a small globular non-glycosylated protein, consisting of a single polypeptide chain of 585 amino acids organized into three repeating homologous domains (I, II and III), each of which has two sub-domains (A and B) [1]–[3].

It is produced in liver by hepatocytes and has a half-life of approximately 20 days [3], [4]. Albumins have many physiological and pharmacological functions: it transports endogenous and exogenous compounds such as drugs, fatty acids, vitamins, bilirubin, metabolites, and amino acids in blood; it has an important role in maintaining colloid-osmotic pressure, coordinating vascular endothelial integrity,

antioxidant activity, maintaining acid-base status, and also exhibits an antithrombotic effect [1], [3], [4]. HSA is one of the most important transporters for drugs in the systemic circulation. Most orally administered drugs are hydrophobic and poorly soluble in plasma, and binding to HSA increases their solubility and thus bioavailability. Research on the binding of drugs to albumin can be important in the study of their transport, distribution, and metabolism [5]. Binding of a drug molecule to HSA significantly affects the pharmacokinetics of the drug as it increases drug solubility in plasma, decreases toxicity and protects molecules from oxidation [6]. Stronger binding of the drug to albumin results in a lower concentration of the free drug in the plasma, and vice versa, a weak interaction of the drug with albumin results in a higher concentration of the free drug in the plasma, which can lead to a reduced half-life of the drug or poor distribution [2].

Statins are one of the most commonly prescribed groups of drugs in the world. They act as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which reduces the level of atherogenic lipoproteins. Currently, there are six statin drugs available on the market: pitavastatin, atorvastatin, rosuvastatin, pravastatin, simvastatin and fluvastatin [7], [8].

The aim of this research is to investigate and confirm the molecular interaction between statins and HSA using fluorescence spectrometry and molecular modeling approach. In our research, we will examine the binding capacity of two selected statins, rosuvastatin (ROS) and atorvastatin (ATO) to HSA.

II. Materials and Methods

A. Fluorescence Spectra Measurement

The exact concentration of HSA was determined spectrophotometrically (UV-1240 Shimadzu Spectrophotometer) using molar extinction coefficient of 35.700 M^{-1} cm^{-1} [9]. HSA (≥99%, recombinant) was purchased from Sigma-Aldrich. Stock solution of HSA was prepared in phosphate buffer solution (10 mM phospate buffer, 100 mM NaCl, pH 7.4) and stored at 0-4°C. Stock solutions of rosuvastatin calcium and atorvastatin calcium tryhidrate were prepared in DMSO (Honeywell International Inc.). All the other chemicals were of analytical grade and used as supplied without further purification.

Fluorescence was measured using a Shimadzu model RF-5301 Spectrofluorometer equipped with 1 cm quartz cells. Fluorescence emission spectra of the HSA were measured in the absence and presence of the various concentrations of rosuvastatin calcium and atorvastatin calcium tryhidrate (2.0; 4,0; 6.0; 8.0; 20.0 and 42.0 μM). The intrinsic fluorescence was excited at 292 nm and emission spectra were recorded from 300 to 500 nm. The enterance and exit slits were both 1.5 nm.

B. Molecular Docking

To stimulate the binding interaction of HSA with statins, we used the free-of-cost molecular docking software AutoDock Vina [7], [10], [11]. The crystal structure of HSA (PDB ID: 4LB2) was obtained from Protein Data Bank (http://www.pdb.org/pdb/home/home.do). 3D structures of ROS (Pub Chem CID: 446157) and ATO (PubChem CID: 60823) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). To prepare the protein (HSA) and ligand (ROS and ATO) structure, we used AutoDock Tools by removing water molecules, adding polar hydrogen atoms and adding Kollman's charge. Both, protein and ligand structures were saved as PDBQT file. Then, we created a grid-box with dimensions of 50 Å × 50 Å × 50 Å and a resolution of 0.375 Å. We placed the grid box on two binding sites (Sudlow site I and II) on the HSA structure. The results were obtained in the form of binding energy values. BIOVIA Discovery Studio 2021 Cilent software was used to visualize the interaction between statins and HSA.

III. Results and Discussion

A. Fluorescence Spectra

The intrinsic fluorescence of HSA originates from tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues. These endogenous fluorophores are often used to study the conformational changes of HSA, as well as the interaction of HSA with a ligand. Trp residue shows the strognest fluorescence intensity and HSA contains one Trp residue (Trp-214) [2], [10], [12]. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching [13]. Therefore, fluorescence quenching of Trp residues can serve as a powerful tool to study the interaction of various molecules (e.g., drugs) with HSA [14].

In our study, we treated HSA solutions with increasing concentrations (from 2.0 to 42.0 μM) of selected statins (ROS and ATO). The results show that at an excitation wavelength of 292 nm HSA shows a strong fluorescence emission peak at 341 nm, which mainly originates from Trp residues, as highlighted earlier. The results revealed that both statins (ROS and ATO) cause the fluorescence quenching of the HSA solution (Fig. 1).

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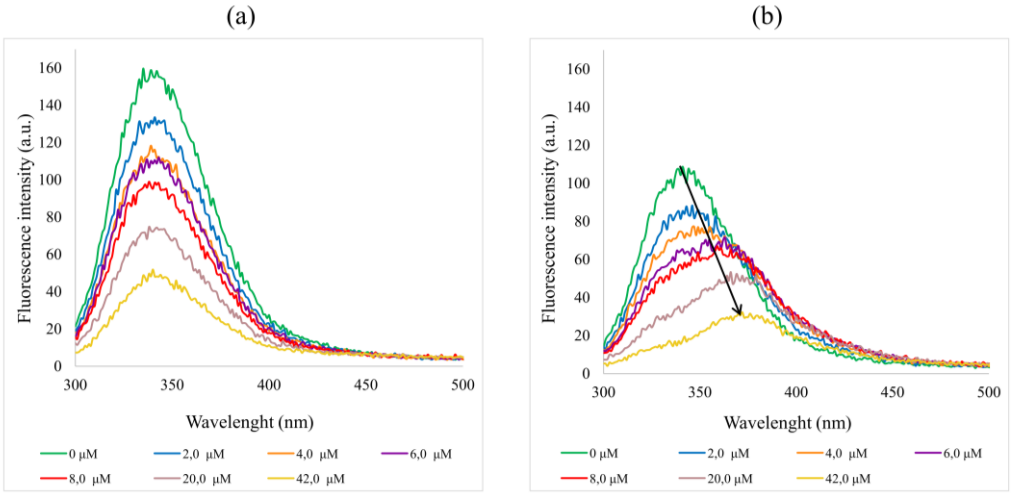

Fig. 1. Fluorescence emission spectra of HSA in the presence of different concentration of rosuvastatin (a) and atorvastatin (b).

As shown in Fig. 1a HSA fluorescence intensity gradually decreases with increasing concentration of ROS. Our results confirm the results reported by Sheida Afkham *et al.* who propose that ROS binds to HSA forming a ROS-HSA complex. Thermodynamic studies of their research showed that the HSA-ROS complex is stabilized by hydrogen bonds and van der Waals interactions [7].

Atorvastatin has also been shown to act as a fluorescence quencher of HSA (Fig. 1b). The fluorescence intensity of HSA gradually decreased in the presence of increasing concentrations of ATO. This finding confirms the results previously reported by Hossein Haghaei *et al* [11]. According to their study, the interaction between ATO and HSA is carried out through combined dynamic and static quenching mechanisms [11]. In addition, a red-shift was observed at high concentrations of ATO. The shift in the position of fluorescence emission maximum corresponds to changes of the polarity around the chromophore molecule. A blue shift of λ_{max} means that the amino acid residues are located in a more hydrophobic environment, and are less exposed to the solvent, while a red shift of λ_{max} implies that the amino acid residues are in a polar environment and are more exposed to the solvent due to unfolding of the protein [15], [16]. The spectra evidence a shift from 341 to 375 nm showing that there are changes in conformation near tryptophan. The red shifts of the maximum emission wavelength of the tryptophan residue suggest that the interaction of HSA with ATO resulted in a more polar environment for tryptophan residue [15].

Fluorescence quenching of a protein in the presence of a quencher can occur through either a dynamic or static mechanism. The static quenching is caused by forming ground–state complex of protein with quenchers, while the dynamic quenching is caused by the collision of protein and quenchers [10], [17].

In order to clarify the mechanism of HSA fluorescence quenching by statins, in our study the quenching experiments were carried out at 298 K. The fluorescence spectra were recorded at λ_{exc} = 292 nm and λ_{em} from 300 to 500 nm. The intensity at 341 nm was used to calculate the binding constant (K_b).

To analyze the interaction of statins with HSA we used the well-known Stern-Volmer equation [18]–[20]:

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

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. [Q] is the concentration of quencher (i.e., drug), and K_{SV} is the Stern-Volmer quenching constant, which indicates the strength of the interaction between the drug and protein [14], [20], [21].

To calculate the binding constant (K_b) and the number of binding sites (n) in the interaction of statins with HSA, we used the modified Stern-Volmer equation, also known as the Hills equation [11], [22]:

$$\log(F_0 - F)/F = \log Kb + n\log[Q] \qquad (2)$$

The values of K_b and n were obtained from the intercept and slope of the modified Stern-Volmer plot, respectively [2], [14]. The Hill plot is linear for both statins that acts as quenchers (ROS and ATO) (Fig. 2 c, d). Table I shows the calculated values of the binding constant (K_b) and the number of binding sites (n).

TABLE I: STERN-VOLMER QUENCHING CONSTANTS (K_{SV}), CORRELATION COEFFICIENT (R^2), BINDING CONSTANT (K_B) AND NUMBER OF BINDING SITES (N) FOR STATINS BINDING INTO HSA

Statin	Ksv (M^{-1})	$R^2_{Stern\text{-}Volmer\ plot}$	Kb (M^{-1})	n	$R^2_{Hill\ plot}$
Rosuvastatin	0.502×10^5	0.9976	1.0246×10^6	0.8401	0.9947
Atorvastatin	1.244×10^5	0.9991	0.9018×10^6	0.9844	0.9961

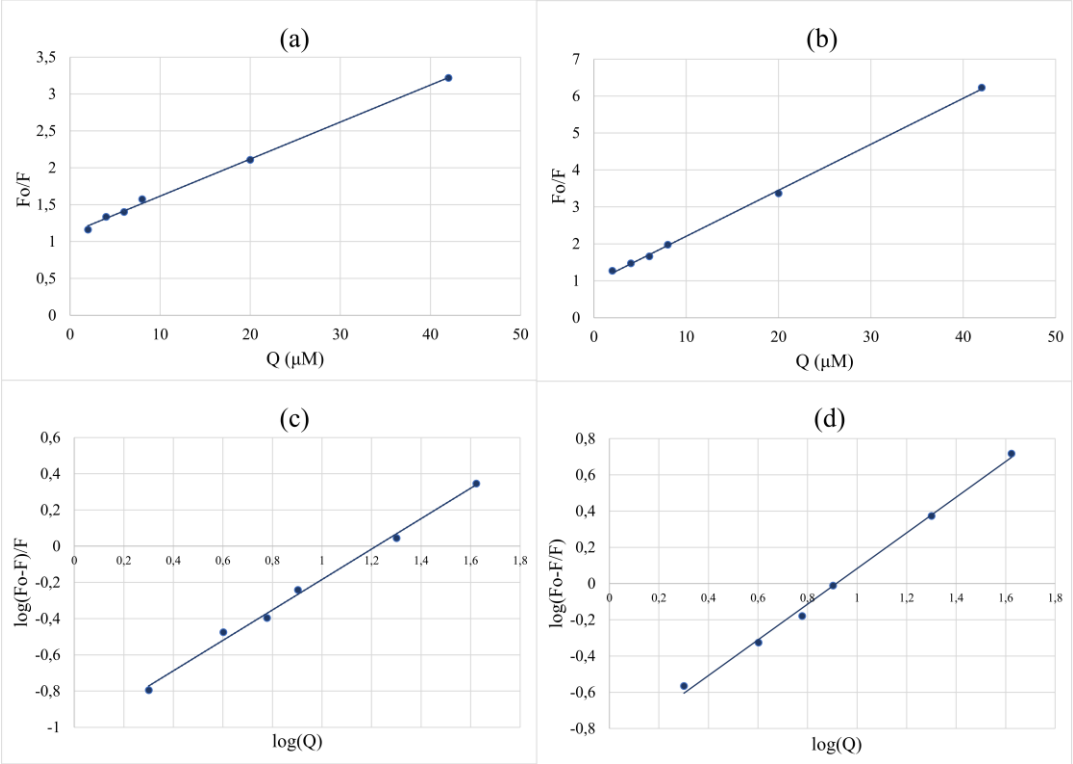

Fig. 2. The Stern-Volmer plots for HSA in presence of rosvastatin (a) and atorvastatin (b). The Hill-plots for HSA in presence of rosuvastatin (c) and atorvastatin (d).

The calculated K_b values for ROS and ATO differ slightly, with ROS showing higher affinity for HSA compared to ATO. Both K_b values are of the order of 10^6, which indicates a strong binding interaction of the mentioned statins with HSA.

The results showed that the value of n for the ROS-HSA system is close to 1, which indicates that HSA has one binding site for ROS, i.e., they bind in a stoichiometric ratio of 1:1. The obtained results correlate with the findings reported by Sheida Afkham *et al.* [7]. Furthermore, the calculated value of n from the Hill plot for the ATO-HSA system is about 1, indicating that HSA also possesses one binding site for ATO. Hossein Haghaei *et al.* [11] reported similar findings.

C. Molecular Docking Studies

Molecular docking is a powerful tool that can predict the preferred orientation of a small molecule (ligand) bound to a biomacromolecule (protein) and the strength of association or binding affinity between them [10], [11]. Using molecular docking, we examined the binding energies, binding sites and amino acid residues involved in the interaction between statins (ROS and ATO) and HSA. It has been reported that HSA possess two major binding sites located in the hydrophobic pockets of sub-domains IIA and IIIA, often referred to as Sudlow site I and Sudlow site II [10], [23], [24]. Using AutoDock Vina, we docked ROS and ATO onto above binding sites of HSA, separately. The most probable conformer is the one with the lowest binding energy [17]. The docking results in the form of free binding energies are presented in Table II.

TABLE II: ENERGIES OF BINDING COMPLEXES (KCAL/MOL) OBTAINED FROM MOLECULAR DOCKING

Statin	Binding energy (Site I)	Binding energy (Site II)
Rosuvastatin	-7.7	-6.5
Atorvastatin	-8.0	-7.9

In order to determine the amino acid residues directly involved in the binding as well as the type of interactions, the obtained results were visualized in 2-D (Fig. 3 and Fig. 4) and 3-D form (Fig. 5).

As shown in Fig. 3a and Fig. 5a, ROS binds to Sudlow site I mainly via hydrogen bonds with Val116, Arg117, Tyr138, Tyr 161 and Gly189. Also, with Arg186, it forms an electrostatic interaction (pi-cation). In addition, Arg117 and Arg186 form hydrophobic interactions (pi-alkyl) with the benzene ring of ROS molecule. It was observed that ROS binds to Sudlow site II (Fig. 3b and Fig. 5b) through hydrophobic interactions (Ala406, Val409, Arg410), hydrogen bonds (Gln390 and Lys545) and electrostatic interaction (Lys541).

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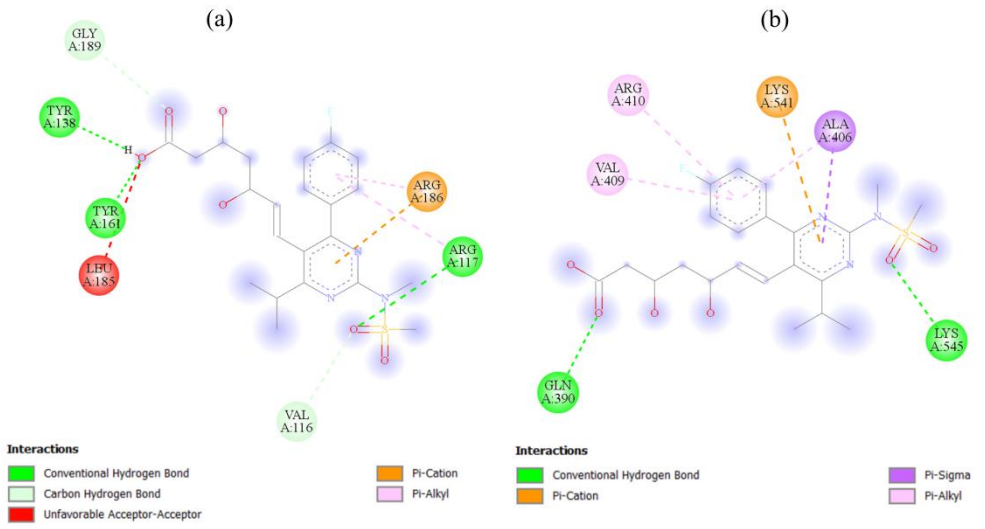

Fig. 3. 2-D representation of ROS in the Sudlow's binding sites I (a) and II (b).

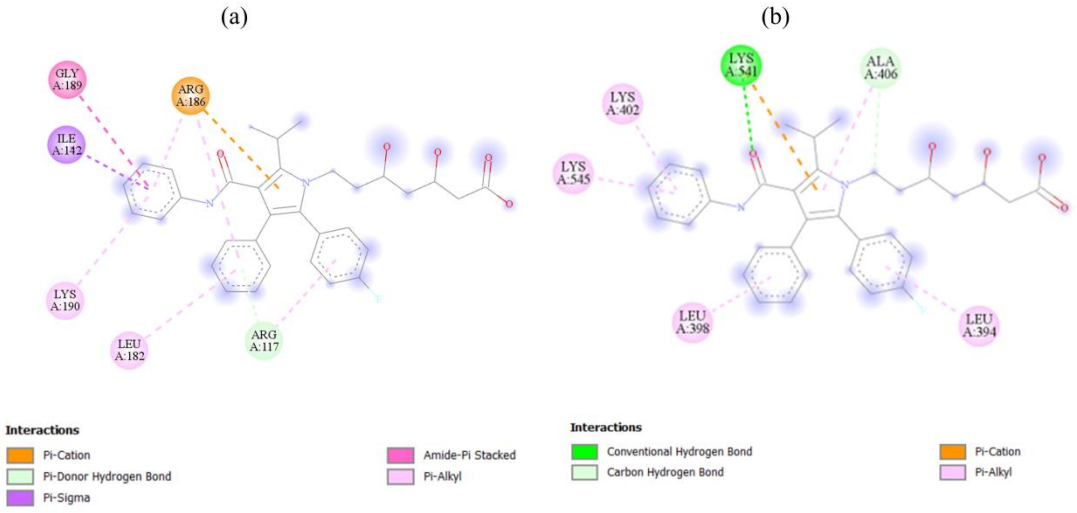

Fig. 4. 2-D representation of ATO in the Sudlow's binding sites I (a) and II (b).

The results of the docking study show that ATO in Sudlow site I (Fig. 4a and Fig. 5c) binds predominantly through hydrophobic interactions (Ile142, Leu182, Gly189 and Lys190). In addition, it was observed that ATO form one hydrogen bond with Arg117 and one electrostatic interaction (pi-cation) with Arg186. Furthermore, it was observed that ATO forms five hydrophobic interactions (Leu394, Leu 398, Lys402, Ala406 and Lys545) in Sudlow site II (Fig. 4b and Fig. 5d). These interactions mainly occur between the benzene rings of ATO and the aliphatic chains of the above-mentioned amino acid residues (pi-alkyl interactions). Two hydrogen bonds with Ala406 and Lys541 were also observed. In addition, the guanidino group of Lys541 forms an electrostatic interaction (pi-cation) with the pyrrole ring of ATO.

The results of the molecular docking study shows that the free binding energy of ROS bound to Sudlow site I is more negative compared Sudlow site II (Table II), indicating that ROS has a higher affinity for Sudlow site I. Similar results were obtained by Sheida Afkam *et al.* who examined the binding of ROS to all six domains of HSA molecule [7]. Analyzing the amino acid residues that forms interactions with ROS, it can be observed that ROS in Sudlow site I forms mainly hydrogen interactions, while in Sudlow site II it forms mainly hydrophobic interactions. Considering that ROS has hydrophilic properties due to its structural characteristics, it can be assumed that stronger binding is achieved in Sudlow site I.

Furthermore, the obtained results of molecular docking for ATO show that the values of free binding energy for Sudlow sites I and II are similar (Table II). More precisely, the free binding energy of ATO on Sudlow site I is more negative by 0.1 kcal/mol, compared to Sudlow site II. This suggests that ATO probably binds to both binding sites with equal affinity but prefers Sudlow site I (which is more energetically favorable). In a study by Hossein Haghaei *et al.*, the lowest binding energy for ATO was at domain IIA, where Sudlow site I is located [11]. The results of molecular modeling showed that ATO binds to Sudlow site I and II predominantly by hydrophobic interactions, which is expected considering that ATO possesses hydrophobic properties. In addition, hydrogen bonds and electrostatic interactions are also involved in the binding of ATO to HSA.

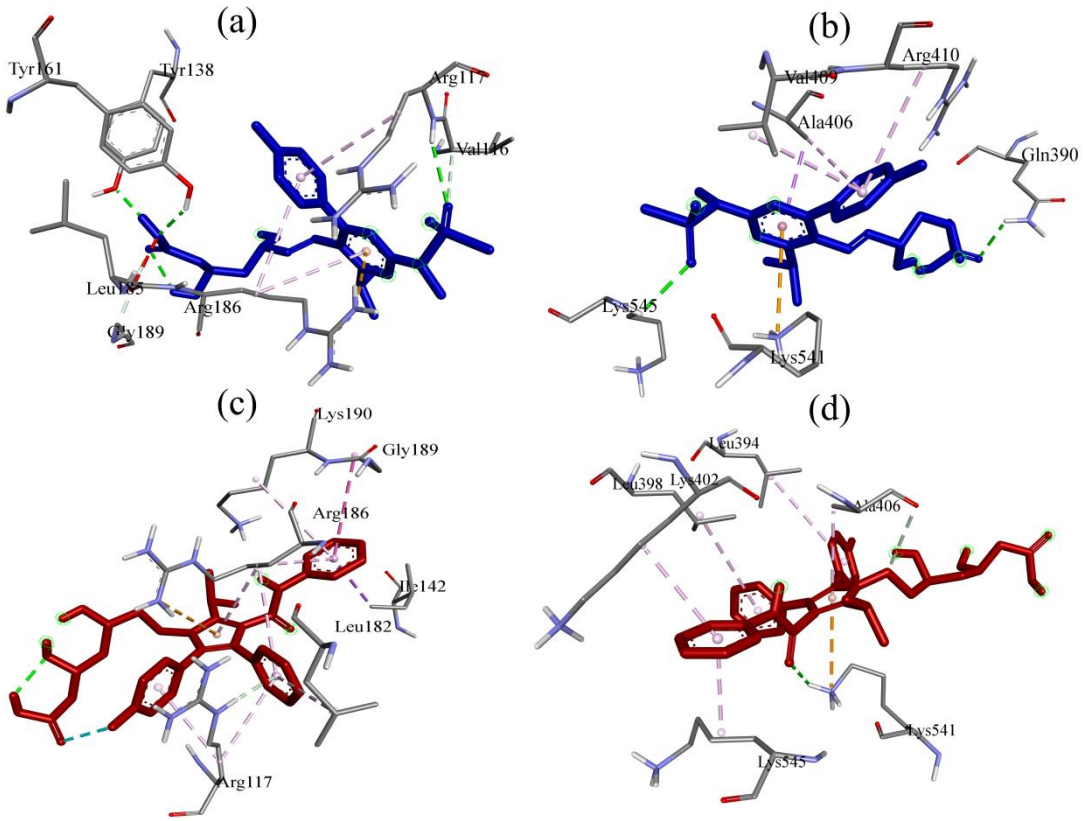

Fig. 5. 3-D representation of ROS (a,b) and ATO (c,d) in the Sudlow's binding sites I (a,c) and II (b,d).

IV. CONCLUSION

The results revealed that both statins (ROS and ATO) quench the intrinsic fluorescence of the HSA solution, suggesting that binding occurs. The calculated binding constants (K_b) were of the order of 10^6, indicating strong binding of the statin to the protein. Also, it was calculated that the mentioned statins bind to HSA in a stoichiometric ratio of 1:1, indicating that the HSA molecule possesses one binding site for the statin. In addition, it was observed that high concentrations of ATO cause a shift of the emission maximum towards longer wavelengths (red-shift), which may be due to the unfolding of protein chains or denaturation.

Molecular docking results showed that ROS has a higher affinity for Sudlow site I on HSA molecule. In the stabilization of the ROS-HSA complex, the main binding forces are hydrogen bonds, but hydrophobic and electrostatic interactions are also involved. ATO has nearly equal affinity for both binding sites on HSA, and the main binding forces are hydrophobic interactions combined with hydrogen bonds and electrostatic interactions.

The results of our research could help in better understanding the nature and mechanism of interactions between drugs and proteins, transport of drugs in the systemic circulation and therefore the pharmacokinetics of the drugs.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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