Unraveling the Stability of Plasma Proteins upon Interaction of Synthesized Androstenedione and Its Derivatives—A Biophysical and Computational Approach

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ABSTRACT: 4-Androstene-3-17-dione (4A), also known as androstenedione, is the key intermediate of steroid metabolism. S/β-Androstane-3-17-dione (5A) and (+)-6-methyl-S/β-androstane-3-17-dione (6M) are the steroid derivatives of androstenedione. The interactions of androstenedione and its derivatives with plasma proteins are important in understanding the distribution and bioavailability of these molecules. In our present study, we have studied the binding affinity of androstenedione and its derivatives with plasma proteins such as human serum albumin (HSA) and α1-acid glycoprotein (AGP). Our results showed that the 4A, 5A, and 6M steroid molecules can form stable complexes with HSA and AGP. The affinity of the studied steroid molecules with HSA is high compared to that with AGP, and the binding constants obtained for 4A, 5A, and 6M with HSA are \(5.3 \pm 2 \times 10^4\), \(5.3 \pm 1 \times 10^4\), and \(9.5 \pm 0.2 \times 10^4\) M\(^{-1}\), respectively. Further, binding sites of these steroid molecules in HSA are identified using molecular displacement and docking studies: it is found that 4A and 5A bind to domain III while 6M binds to domain II of HSA. Furthermore, the circular dichroism data revealed that there is a partial unfolding of the protein while interacting with androstenedione and its derivatives. Also, molecular dynamics simulations were carried out for HSA–androstenedione and its derivative complexes to understand their stability; hence, these results yielded that HSA–androstenedione and its derivative complexes were stabilized after 15 ns and maintained their stable structures.

1. INTRODUCTION

Plasma proteins bind reversibly to drugs, hormones, and metabolites, which are hydrophobic in nature and circulate in the bloodstream. The binding affinity of plasma proteins determines free and effective concentrations, thereby regulating the pharmacokinetics and pharmacodynamics of the drugs. Hence, understanding the interaction mechanisms of various drugs and metabolites with these plasma proteins is of clinical significance. The major carrier proteins in plasma include human serum albumin (HSA), α1-acid glycoprotein (AGP), and lipoproteins.1 Among the aforementioned plasma proteins, HSA is one of the most abundant proteins in human plasma, and it acts as a carrier for most drugs. Drugs that interact with HSA are acidic/neutral in nature, but they are basic in the case of AGP.2

Steroids get transported in the bloodstream with the aid of specific transporter proteins. Even though specific carrier proteins are available for the transport of steroids, they can also effectively interact with HSA.3,4 Earlier reports on steroid hormones such as testosterone and progesterone show that they can interact with HSA.5,6 Natural variants of HSA such as Niigata and Tagliacozzo show a high affinity toward prostaglandin and progesterone, respectively.6 Besides HSA, another plasma protein AGP, an acute protein, also interacts with steroids and their derivatives. AGP can interact strongly with progesterone,7 and the binding site for the progesterone is located in the N-terminus. Consequently, the binding site for progesterone is near to carbohydrate residue, demonstrated by using calcofluor white as a probe.8

4-Androstene-3-17-dione (4A) is a naturally occurring steroidal hormone, which is produced in gonads and adrenal glands, and is found to be the key metabolic intermediate in the metabolism of steroids such as testosterone, estradiol, progesterone, cortisone, and cortisol.9 Synthetically, this compound is also used as a precursor molecule for the synthesis of various steroids. It possesses both androgenic and anabolic properties,10 and thus, the level of 4A in blood plasma is significant. Also, it is an immediate precursor for testosterone, and the enzyme 17-β-hydroxysteroid dehydrogenase converts 4A to testosterone. Further, the enzyme aromatase converts 4A...
to estrone and estradiol.\textsuperscript{11} In this line, we have synthesized $\beta$-androstane-3-17-dione (5A) through an organocatalysis method by taking 4A as the starting material.\textsuperscript{12} Physiologically, 4A gets converted to 5A in the presence of 3-oxo-$\beta$-steroid-4-dehydrogenase. Another derivative is (+)-6-methyl-$\beta$-androstane-3-17-dione (6M), in which the methyl group is attached at the C6 position of $\beta$-androstane-3-17-dione.

Structurally, HSA is a nonglycosylated 585-amino-acid polypeptide chain, predominantly made up of $\alpha$-helix (67%).\textsuperscript{13,14} It possess multidomain organization with three homologous domains, domain I (1−195), II (196−383), and III (384−585), forming a three-dimensional (3D) heart-shaped globular molecule. It is a negatively charged protein and has 17 highly cross-linked disulfide bonds.\textsuperscript{15,16} It contains a single tryptophan residue at position 214. The protein mainly functions as a regulator of plasma colloid oncotic pressure, and it also acts as a carrier for many exogenous and endogenous metabolites and drugs.\textsuperscript{17} In addition to drug-carrying properties, this protein possesses several other properties such as antioxidant and (pseudo)enzymatic properties.\textsuperscript{18,19} It has a wide range of biochemical properties and interactions with several kinds of molecules such as fatty acids, porphyrins, bilirubin, thyroxin, and metal ions;\textsuperscript{20−24} hence, this protein can be exploited for various applications. There are two major binding sites in HSA, site I and site II, located in domain IIA and IIIA, respectively.\textsuperscript{25} Further, domain IB is also named a drug-binding site.\textsuperscript{26} It has an important role as drugs/ligands compete for the same binding site. Also, it is an allosteric protein and can accommodate more than one molecule at a time. Thus, understanding of these complex mechanisms of ligand interactions can help to understand the clinical outcome.

Perhaps, AGP also known as orosomucoid and acute phase protein plays an important role in binding of drugs. It has a normal range of 0.6−1.2 mg/mL (1−3% of plasma proteins),\textsuperscript{26} during inflammation, the plasma concentration increases to 2−5 folds. Moreover, this protein has antiinflammatory and immunomodulatory roles. Surprisingly, the levels of AGP elevated in acute inflammation and show systemic response to local inflammation.\textsuperscript{27} It is a glycoprotein of 183 amino acid residues with 41 kDa molecular weight, consists of 45% carbohydrate attached in the form of N-linked glycans, and confines to the N-terminus of the protein. This protein is negatively charged at physiological pH because of 16 sialic acid residues.\textsuperscript{28} Also, it has three tryptophan residues, where tryptophan 160 is present on the surface of the protein and rest two are located at the inner side of the protein.\textsuperscript{29} Predominantly, there is only a single high-affinity basic drug-binding site in AGP.\textsuperscript{30} Recently, our group reported that there are several molecules specifically bind to AGP.\textsuperscript{31,32} Competition for the single basic drug-binding site can control the therapeutically effective plasma levels of basic drugs. Because of the single binding site, the drugs binding to AGP can attain saturation and are competitively displaceable. Stereo selectivity also exists for the single binding site.

In the present study, we have aimed to understand the following observations such as interaction mechanism of HSA and AGP with the synthesized steroid molecules, 4-androstene-3-17-dione (4A), $\beta$-androstane-3-17-dione (5A), and (+)-6-methyl-$\beta$-androstane-3-17-dione (6M) (Scheme 1). Further, the conformational changes and stability of the complexes were emphasized using biophysical and computational approaches.

Scheme 1. Structures of 4A, 5A, and 6M\textsuperscript{a}

\textsuperscript{a}The molecular weights of 4A, 5A, and 6M are 286.41, 288.42, and 302.45 Da, respectively.
the microenvironment around tryptophan has been altered because of the formation of HSA–4A (androstenedione), HSA–5A, and HSA–6M complexes.

Another important factor that could lead to a decrease in emission intensity is the inner filter effect. The inner filter effect refers to the absorbance of light at the excitation or emission wavelength by the ligands present in the solution. The absorption and emission spectra of 4A, 5A, and 6M at 10 μM concentration are given in Figure S1. The emission intensities were corrected for the inner filter effect using the following relationship:

$$F_{\text{Cor}} = F_{\text{Obs}} \times e^{\left(A_{\text{exc}} + A_{\text{em}}\right)/2}$$

where $F_{\text{Cor}}$ is the corrected fluorescence intensity, $A_{\text{exc}}$ and $A_{\text{em}}$ represent the absorbance at the fluorescence excitation (285 nm) and emission wavelengths, respectively, and $F_{\text{Obs}}$ is the observed fluorescence.

Two common mechanisms of quenching are well-known: dynamic (or) collisional and static quenching, which can be distinguished by temperature, viscosity dependence, and the difference of their fluorescence lifetimes. The relation between the quencher concentration and the fluorescence intensity can be well-explained using the Stern–Volmer equation.

$$F_0/F = 1 + K_q [Q] = 1 + K_{SV}[Q]$$

where $F_0$ represents the fluorescence intensity in the absence of quencher, $F$ represents the fluorescence intensity in the presence of quencher, $[Q]$ is the quencher concentration, $K_q$ is the bimolecular quenching rate constant, $\tau_0$ is the lifetime of the fluorophore in the absence of quencher and its value is around $10^{-8}$ s for most of the biomolecules, and $K_{SV}$ is the Stern–Volmer quenching constant, which can be written as

$$K_q = K_{SV}/\tau_0$$

The emission quenching data of HSA were plotted as $F_0/F$ against the concentration of ligands, which is shown in Figure S2. It shows that within the investigated concentrations, the Stern–Volmer plot exhibited a good linear relationship. Further, the values of the Stern–Volmer quenching constant $K_{SV}$ were obtained from the slope, by keeping the intercept at 1. The average lifetime ($\tau_0$) of the biopolymer without any quencher has already been reported as $10^{-8}$ s$^{37}$. From the above equation, bimolecular quenching rate constant was calculated and was far greater than the maximum scatter collision quenching constant $2.0 \times 10^{10}$ M$^{-1}$ s$^{-1}$, showing the formation of a ground-state complex between the protein and the ligands. It has been well-established that for a static quenching process, the quenching constant $K_q$ should be greater than the maximum scatter collision quenching constant. The calculated $K_q$ values are $9.0 \pm 0.2 \times 10^{12}$, $9.8 \pm 3 \times 10^{12}$, and $1.0 \pm 1.0 \times 10^{13}$ M$^{-1}$ s$^{-1}$, respectively, for 4A, 5A, and 6M with HSA, which are greater than the maximum scatter collision quenching constant. This identification shows that there is a static ground-state complex formation between HSA and the ligands. In a similar way, the interaction between AGP and the

Figure 1. Fluorescence emission spectra of HSA in the presence of increasing concentrations of (A) 4A, (B) 5A, and (C) 6M from 1 to 9 μM. Fluorescence quenching was shown with increasing concentrations of the molecules. There was no fluorescence emission for buffer and the ligands in the given wavelength ranging from 300 to 500 nm. Modified Stern–Volmer plots for (a) 4A, (b) 5A, and (c) 6M obtained by plotting log [Q] values on the X-axis and log [dF/F] values on the Y-axis. The slope of the plot gives the number of binding sites. The Y intercept value gives log K values, from which the binding constant values were calculated. All three molecules have only one binding site in HSA. The binding constant values are $5.3 \pm 2 \times 10^4$, $5.3 \pm 1 \times 10^4$, and $9.5 \pm 0.2 \times 10^4$ M$^{-1}$, respectively, for 4A, 5A, and 6M.
ligands was also identified as the ground-state complex formation. The binding constants and the number of binding sites were calculated using the modified Stern−Volmer equation.

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log [Q]
\]

where \( n \) corresponds to the number of binding sites, \( K \) is the binding constant, and \([Q]\) is the quencher concentration.

\( \log K \) can be calculated from the intercept of the graph of \( \log(\frac{F_0 - F}{F}) \) versus \( \log [Q] \), and the slope of the graph gives the number of binding sites. The \( n \) values of 4A (0.818), 5A (0.911), and 6M (0.963) are nearer to 1, showing the one-to-one interaction of the three molecules with HSA. The binding constants were calculated, and they are \( 5.3 \pm 2 \times 10^4 \), \( 5.3 \pm 1 \times 10^5 \), and \( 9.5 \pm 0.2 \times 10^5 \) M\(^{-1}\), respectively, for 4A, 5A, and 6M (Figure 1). In a similar way, the binding constants for 4A, 5A, and 6M with AGP were calculated as \( 7.4 \pm 4 \times 10^3 \), \( 2.6 \pm 0.6 \times 10^3 \), and \( 2.1 \pm 0.7 \times 10^3 \) M\(^{-1}\), respectively (Figure 2). HSA is showing much higher affinity compared to AGP with these three molecules; however, binding of these three compounds with AGP is also significant. Comparing to 4A and 5A, the methyl derivative of androstane (6M) is showing higher affinity toward HSA and AGP. These results indicate that both HSA and AGP play a major role in binding which in turn acts as major transporters in the blood system.

Because the binding constant (or) association constant value is known, \( K_d \) can be calculated from the reciprocal of \( K \), and from that, binding free energies can be calculated using the following equation:

\[
\Delta G^0 = -RT \ln K_d
\]

where \( \Delta G^0 \) is the free energy change, \( R \) is the gas constant at room temperature, and \( K_d \) is the dissociation constant which is given by

\[
K_d = 1/K
\]

The calculated free energies are \(-6.59\), \(-6.51\), and \(-6.83\) kcal/mol, respectively, for 4A, 5A, and 6M with HSA, whereas with AGP, the binding free energies are calculated as \(-5.18\), \(-4.64\), and \(-5.85\) kcal/mol, respectively, for 4A, 5A, and 6M. These results are in agreement with those from our laboratory work on different ligands binding with HSA and AGP.\(^{31,32,34,36}\) These data infer that 4A (androstenedione), 5A, and 6M bind strongly with plasma proteins. Binding of these molecules to AGP is of additional importance because it also acts as a transporter in some disease conditions such as renal failure, pregnancy, burns, and so forth. Differential binding of these ligands is due to different functional groups attached to the original molecules.

### 2.2. Locating the Binding Site Using Site-Specific Probes.

Several attempts have been made to map the binding sites on HSA, as it can bind to several exogenous and endogenous ligands. Sudlow showed the presence of two specific drug-binding sites, namely, site I (also called the warfarin binding site) and site II (the benzodiazepine binding site) using a fluorescent probe displacement method.\(^{39}\) Even though there are two binding sites located in subdomains IIA and IIIA, other secondary binding site is also located at subdomain IB in HSA.\(^{25}\) Among the available list of site-specific markers, we selected phenylbutazone and ibuprofen as site probes for IIA and IIIA, respectively. An equimolar ratio of HSA and site markers was titrated with 4A, 5A, and 6M in three
different experiments. The binding constant values were calculated as mentioned above (Figure 3). The binding constant for 4A in the presence of ibuprofen is $4.6 \pm 0.1 \times 10^4$ M$^{-1}$. Compared to only HSA, the binding constant value is decreased in the presence of site markers and is lower in the case of ibuprofen, showing that the ligand is competing with ibuprofen to bind to IIIA. Similarly, 5A is also competing with ibuprofen; however, 6M is competing with phenylbutazone, with binding constant values $4.0 \pm 0.6 \times 10^4$ and $3.5 \pm 1 \times 10^4$ M$^{-1}$, respectively. Figure 3 shows the fluorescence emission spectra of HSA in the presence of ibuprofen and phenylbutazone while titrating with increasing concentrations of 4A, 5A, and 6M. Thus, molecular displacement results in turn determined the specific binding pockets of 4A, 5A, and 6M.

2.3. Secondary Structure Analysis from Circular Dichroism Data. To understand the influence of ligand binding on the conformation of secondary structure of HSA, circular dichroism (CD) spectroscopy measurements were performed in the presence of increasing concentrations of 4A, 5A, and 6M. The room-temperature CD spectra measured in the presence of 4A, 5A, and 6M are shown in Figure 4. The characteristic CD spectra of HSA measured in the far-UV region show two minima at 222 and 208 nm, which are the contributions of $n \rightarrow \pi^*$ transfer of the peptide bonds from $\alpha$-helix. As shown in Figure 4, CD spectra of free HSA exhibit two negative bands in the ultraviolet region at 208 and 222 nm. It was observed that in the presence of steroid molecules, the CD signal of HSA got decreased. The decrease of the CD signal indicates a decrease of helical secondary structure content. However, the CD spectra of HSA in the presence or absence of ligands are similar in shape, indicating that there are very minute changes occurred in HSA due to ligand binding. Using CDNN software, the percentage of secondary structures was quantified for both free HSA and HSA in the presence of ligands. The percentage of $\alpha$-helix differed from 66.05% in free HSA to 58.1% in 4A−HSA at pH 7.4 while $\beta$-turns and random coils were increased from 17.43 to 20.1% and from 16.51 to 21.7%, respectively. In the cases of 5A and 6M, the $\alpha$-helix content was decreased to 59.1 and 53.6%, respectively. There is a gradual decrease in the $\alpha$-helical percentage with increasing concentrations of ligands. The percentage changes of secondary structural elements were plotted against the concentration of the ligands, which is shown in the inset of Figure 4. The marginal changes in the percentage of secondary structural elements indicate that 4A, 5A, and 6M are interacting with HSA; hence, there is a partial unfolding of the protein. Structural changes of the protein indicate the changes in the microenvironment around the binding pocket residues upon binding of these molecules. Similar results were observed from our laboratory work that upon binding of ligands, HSA undergoes conformational changes. To understand the stability of HSA in various temperatures, CD spectra were recorded for HSA with increasing temperature from 25 to 85 °C. The temperature influence is not significant up to 65 °C, consistent with the fact that HSA alone is stable up to 65 °C (data not shown).

2.4. Mode of Ligand Binding to HSA Using the Molecular Docking Approach. Molecular docking studies were further used to understand the interaction between HSA and the ligands. The 3D structure (taken from PDB ID: 1AO6) of HSA consists of three homologous domains, denoted as I, II, and III. Each domain contains six-helix and four-helix subdomains. Each domain further subdivided into A and B subdomains that assemble to form a heart-shaped tertiary
structure. HSA has two major drug-binding sites known as site I and II. Site I is a large, flexible region, has poor stereoselectivity, and is located in subdomain IIA. The side chains of Tyr150, His242, and Arg257 are located at the bottom of the pocket, and Lys195, Lys199, Arg218, and Arg222 are located on an outer cluster at the pocket entrance. On the other hand, site II is a smaller or narrower site than site I, shows stereo selectivity, and is located in subdomain IIIA. Site II is a largely apolar cavity formed by Leu387, Ser489, Leu453, and a single dominant polar patch at the pocket entrance, having Tyr411 and Arg410.

The docking studies of HSA with 4A (androstenedione), 5A, and 6M give the information about the binding site of these steroid molecules in HSA and the interacting residues that form the binding groove (Figure 5). 4A binds in the groove formed by the residues of domain III. The residues forming this groove are different from those of site I. However, the groove is located immediately next to site II, having some of the residues from site I. The electronegative oxygen atom of 4A is forming a hydrogen bond with hydrogen from the ε-amino group of Lys413, with a bond length of 2.79 Å. Further, Thr540, Lys541, Glu542, Lys545, Leu529, Leu544, and Met548 from subdomain IIIB and Asn405, Ala406, Val409, and Lys413 from subdomain IIIA are forming the binding groove. Val409, Ala406, Leu544, and Lys545 are having hydrophobic interactions, and the rest of the residues forming the binding groove are interacting with van der Waals interactions, thereby holding 4A. Also, site II is interacting with the residues of domain III. However, the binding groove is distinct from that of 4A. Further, the binding site of 5A is also overlapping with site II. In this, Phe206, Arg209, Ala210, Ala213, Leu331, Ala350, Lys351, Glu354, Leu347, Ser480, Leu481, and Val482 are the amino acid residues that form a binding groove for 5A. Moreover, Leu481 is forming a hydrogen bond with the electronegative oxygen of SA, and the bond length is 2.91 Å. Further, Ala210, Arg209, Leu347, Val482, Ala213, and Lys351 are forming hydrophobic interactions with 5A, and the rest are interacting with van der Waals interactions. Distinct from 4A and 5A, 6M is interacting with the residues of domain II. Hence, 6M is interacting with Leu203, Thr243, Cys246, His247, Gln204, Gly207, Arg209, and Glu208 of subdomain IIA of HSA. Additionally, Glu208 is forming a hydrogen bond with 6M, and the bond length is 2.72 Å. From docking results, the binding free energies were calculated to be $-7.36$, $-6.42$, and $-6.99$ kcal/mol, respectively, for 4A, 5A, and 6M. These values are nearer to the experimental values that are obtained from fluorescence emission studies.

2.5. Molecular Dynamics Simulation Studies. HSA-ligand complex formation, complex stability, and the effect of ligands on HSA conformation with respect to time were analyzed using molecular dynamics and simulation studies. The properties such as root-mean-square deviation (rmsd) of HSA and complexes with respect to their initial structures, root-mean-square fluctuations (RMSFs), and radius of gyration (Rg) of the protein were obtained from molecular dynamics simulation (MDS) analysis. For the MDS analysis, the best conformer was taken from the docking studies for all of these molecules (4A, 5A, and 6M).

The rmsd values of HSA are different from those of 4A, 5A, and 6M complexes with respect to their initial structures were calculated along 100 ns trajectories in GROMACS. The rmsd data show that HSA, HSA−4A, HSA−5A, and HSA−6M reach equilibration after 15 ns and later after the complexes got stabilized (Figure 6). Rg values of free HSA and complexes are also shown in Figure 6. The initial Rg value of free HSA is 2.68 Å and got stabilized at 2.55 Å. Experimentally defined Rg value of HSA using small-angle neutron scattering is 2.74 ± 0.035 nm, nearer to the value obtained in the present study.

There is not much difference in the Rg values of HSA−4A and HSA−5A, with respect to that of HSA alone, up to 60 ns. After 60 ns, there is a decrease in the Rg value of HSA−4A. In the case of HSA−6M complex, the Rg value increased slightly, with respect to that of HSA alone, and the variation started from 5 ns. Our experimental analysis with CD spectroscopy to understand the influence of ligands on protein conformation also showed that there is a marginal conformational change in the protein during 4A, 5A, and 6M binding (Figure 4) and it is high in the case of 6M. The CD data are further supporting the Rg data.

Local protein mobility was analyzed by plotting the RMSFs of HSA and the ligands against the atom number. Figure 7 shows the RMSF data of HSA and HSA−4A, where the RMSF value of HSA is 0.21 nm with 0.09 nm standard deviation and is

![Figure 4. CD spectra of HSA alone (red line) and with increasing concentrations of (A) 4A, (B) 5A, and (C) 6M. The characteristic spectrum of protein shows spectral minima at 208 and 222 nm, indicating that HSA is an α-helical protein. The inset shows the percentage of secondary structural elements of HSA, obtained by deconvoluting the CD spectra using CDNN software.](image-url)
$0.18 \pm 0.09$ nm for HSA−4A. Comparative RMSF in the 4A binding region, that is, IIIB shows that the RMSF values of HSA−4A are similar to those of HSA alone with little fluctuations, showing the rigidity of the residues because of ligand binding. The RMSF values of HSA−5A and HSA−6M are $0.17 \pm 0.08$ and $0.18 \pm 0.08$, respectively. The IIIA region
and IIA region of HSA show less RMSFs in the cases of 5A and 6M binding, respectively, compared to HSA protein alone. Further, the interactions of 4A, 5A, and 6M to HSA were visualized at every 3, 6, and 10 ns time point from the 100 ns MDS data to get better insights into the complex stability. The snapshots for HSA−4A, HSA−5A, and HSA−6M at 3 ns time intervals are shown in Figure 8. Initially, 4A is interacting to Lys413 by a hydrogen bond (Figure 5), in time course, reoriented toward Asn405 in the binding groove itself, and form a hydrogen bond with Asn405. From 3 ns time point, 4A is interacting with Asn405 by a hydrogen bond, and this is stable up to 10 ns (Figure S3). The molecule is held by van der Waals interactions and hydrophobic interactions given by the binding site residues (Figures 5, 8, and S3). In a similar way, the stabilities of 5A and 6M were shown by plotting the interactions at 3 ns (Figure 8), 6, and 10 ns. Interactions at 6 and 10 ns are shown for 5A and 6M in Figures S4 and S5, respectively.

3. CONCLUSIONS

The present study gives the detailed description about the binding mechanism of androstenedione and its derivatives to HSA and AGP with various biophysical techniques. Fluorescence studies show that the synthesized compounds (4A, 5A, and 6M) can interact with both HSA and AGP, however, with more affinity toward HSA. The association constants and free energies were found to be $5.3 \pm 2 \times 10^4$, $5.3 \pm 1 \times 10^4$, and $9.5 \pm 0.2 \times 10^4 \text{ M}^{-1}$ and $-6.59$, $-6.51$, and $-6.83 \text{ kcal/mol}$, respectively, with HSA. Among 4A, 5A, and 6M, the methyl derivative (6M) has high affinity toward HSA and AGP. Competitive binding studies by site-specific markers indicate that the compounds 4A and 5A are binding to domain III of HSA, whereas 6M is binding to domain II of HSA. Conformational changes in the protein upon ligand binding were observed by CD spectroscopy, which indicates that there is a partial unfolding of the protein upon binding of 4A, 5A, and 6M. Further, these studies were corroborated by in silico studies wherein hydrogen bonding, hydrophobic interactions, and van der Waals interactions are major driving forces toward
high affinities to HSA. Furthermore, the rmsd values from MDS data show that the complexes are stabilized after 15 ns. The Rg data calculated using MDS further support the partial unfolding of the protein during ligand binding as observed in CD spectroscopy.

The synthesized molecule 6M is a methyl derivative of 5A and shows approximately 2-fold and 10-fold increased affinity toward HSA and AGP, respectively, because of the presence of additional methyl group wherein hydrophobic interactions slightly dominate over other forces. The present study reiterates the importance of synthetic molecules in the drug discovery that enhance the drug distribution at the target sites without sacrificing the drug activity.

4. MATERIALS AND METHODS

4.1. Preparation of Stock Solutions. Pure fat-free HSA and AGP (purchased from Sigma-Aldrich) were dissolved at a concentration of 1.0 mM in an aqueous solution of 0.1 M phosphate buffer at a physiological pH 7.4. Stock solutions of 5 mM 4A, 5A, and 6M (Scheme 1) were prepared by dissolving appropriate amounts of 4A, 5A, and 6M in dimethyl sulfoxide (DMSO). The compounds 4A, 5A, and 6M were synthesized through organocatalysis, and these compounds were pure as reported earlier.12 The site-specific markers were prepared as 1 mM stock solution. Final working concentrations were maintained at 0.1 mM for all ligands and site-specific probes. All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

4.2. Measurements of Fluorescence Emission. Fluorescence emission spectra were recorded at 25 °C from a wavelength range of 300–500 nm with an excitation wavelength of 285 nm and a slit width of 8.0 nm for both excitation and emission using a PerkinElmer LS55 fluorescence spectrophotometer. Final concentration of HSA and AGP were fixed at 1 × 10−6 M, and the increasing concentrations of compounds varied from 1 to 9 × 10−6 M13 were suspended in 0.1 M phosphate buffer with pH 7.4. Three independent experiments were performed, and each time, identical spectra were obtained. The binding constant was calculated by taking the maximum fluorescence emission at 350 nm for HSA and 340 nm for AGP using the Stern−Volmer equation.

4.3. Molecular Displacement Experiment with Site-Specific Markers. For competitive binding studies, the site-specific probes were used. The site markers phenylbutazone and ibuprofen specifically bind to subdomains IIA and IIIA of HSA. The solution having the protein and the site marker in a ratio of 1:1 was titrated with increasing concentrations of 4A, 5A, and 6M from 1:1 to 1:10 to HSA. The fluorescence emission spectra were recorded as mentioned above. The binding constant values were evaluated using the Stern−Volmer equation.

4.4. CD Spectroscopy Measurements. The CD measurements of HSA in the presence and absence of 4A, 5A, and 6M were made in the range of 195–260 nm using a JASCO J-815 CD spectropolarimeter and a quartz cell with a path length of 0.2 cm. For all spectra, the baseline was subtracted using buffer. HSA was made into a solution using 10 mM phosphate buffer at pH 7.4. The HSA-to-ligand concentration was varied in a ratio of 1:1 to 1:5, and the CD spectra were recorded. Three scans were accumulated at a scan speed of 100 nm min−1, and the bandwidth was kept at 2 nm. Finally, the CD spectra data collected were deconvoluted using CDNN 2.1 software to get the percentages of secondary structural elements. The JASCO J-815 CD spectropolarimeter was attached to a Peltier, which facilitated the measurement of secondary structural changes with increasing temperatures from 25 to 85 °C.

4.5. Molecular Docking. To find out the binding site, interacting residues, and the type of interactions involved in the complex formation, in silico docking was performed using AutoDock 4.2.3 program.46 AutoDock 4.2.3 uses the Lamarckian genetic algorithm (GA).47 The known crystal structure of HSA was taken from the protein data bank (PDB), and its PDB ID is 1AO6. The protein was edited in AutoDock. Before using it, water molecules and ions were removed. Hydrogen atoms were added to the functional groups and ionized as required at physiological pH. Further, Kollman united atom partial charges were assigned to HSA. The ligand structures (4A, 5A, and 6M) were designed using Discovery Studio 3.5. The two-dimensional (2D) structures of the ligands were drawn, and the geometry was optimized. The energy minimization was performed to relieve the steric effects. Both protein and ligand files were saved as the PDBQT format. PDBQT is the readable format for AutoDock. HSA was held rigid, all torsion bonds of the ligands were considered as free, and there was no consideration regarding the effect of solvent on the interactions. Blind docking was performed to locate the binding site of the ligands. The size of the grid box was set to a maximum with 0.0637 nm grid spacing. The center of the grid was set to 2.95, 3.18, and 2.35 nm. The docking parameters used were as follows: GA population size: 150 and maximum number of energy evolutions: 250 000. During docking, a maximum number of top 30 conformers were considered, and the rms cluster tolerance was set to 0.2 nm. A conformer with the least free energy and whose binding energy was close to the experimentally determined free energy values was considered. The conformer was visualized using PyMOL software.48

4.6. Molecular Dynamics Simulations. GROMACS 4.0 package was used for molecular dynamics studies, using GROMOS96 43a1 force field.49 The protein conformation with lowest binding energy, which is close to the experimentally obtained value, was taken as an initial structure. The protein topology was prepared using pdb2gmx, which can read PDB files and generate coordinate and topology in the GROMACS format. The topology parameters of the ligands were created using PRODRG2.5. The box type used is dodecahedron, which defines the unit cell. The protein was placed at 1.0 nm from the box edge. The simulation was done in the aqueous system by taking a simple point charge water model. HSA is a negatively charged protein, and the neutralization was done by adding 15 Na+ ions. Energy minimization was done for the entire system having proteins, ligands, ions, and water molecules to release conflicting contacts. The minimization was performed, which optimizes the geometry and solvent orientation, using the steepest descent method of 1000 steps, followed by another 1000 steps of conjugate gradient energy minimization. Before proceeding to production, MD step equilibration was done. Equilibration of the system brings it to proper temperature and pressure conditions. The equilibration was performed in two phases. In the first phase, NVT, position restrain of the solute was done at 300 K for 100 ps, followed by NPT at 1 bar pressure. Berendsen thermostat and Parrinello-Rahman pressure coupling were used for temperature coupling and pressure coupling, respectively.52 Then, the system was subjected to 100 ns MD at 300 K temperature and 1 bar pressure. The
motion equations were integrated using the leap-frog algorithm. The trajectories were recorded at every 10 ps interval.

**ASSOCIATED CONTENT**

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00577.

Fluorescence emission and absorption spectra of the selected ligands; Stern–Volmer plots for the interaction of HSA to the selected androstenedione derivatives; and interaction of the selected androstenedione derivatives to HSA at 6 and 10 ns from 100 ns MDS (PDF)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by UPE-2 University of Hyderabad, Science and Engineering Research Board (SB/EMEQ-064/2014 dated 14-07-2015 and DST-FIST) and UGC-SAP. We thank BIF for Bioinformatics facilities. A.N. acknowledges CSIR (2014 dated 14-07-2015) and DST-FIST) and UGC-SAP. We thank BIF for Bioinformatics facilities. A.N. acknowledges CSIR for fellowship.

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