A Chalcone-Based Potential Therapeutic Small Molecule That Binds to Subdomain IIA in HSA Precisely Controls the Rotamerization of Trp-214

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Supporting Information

ABSTRACT: The principal intent of this work is to explore whether the site-specific binding of a newly synthesized quinoline-appended anthracenyl chalcone, (E)-3-(anthracen-10-yl)-1-(6,8-dibromo-2-methylquinolin-3-yl)prop-2-en-1-one (ADMQ), with an extracellular protein of the human circulatory system, human serum albumin (HSA), can control the rotamerization of its sole tryptophan residue, Trp-214. With this aim, we have systematically studied the binding affinity, interactions, and localization pattern of the title compound inside the specific binding domain of the transport protein and any conformation alteration caused therein. Multiple spectroscopic experiments substantiated by an in silico molecular modeling exercise provide evidence for the binding of the guest ADMQ in the hydrophobic domain of HSA, which is primarily constituted by residues Trp-214, Arg-218, Arg-222, Asp-451, and Tyr-452. Rotationally restricted ADMQ prefers to reside in Sudlow site I (subdomain IIA) of HSA in close proximity (2.45 nm) to the intrinsic fluorophore Trp-214 and is interestingly found to control its vital rotamerization process. The driving force for this rotational interconversion is predominantly found to be governed by the direct interaction of ADMQ with Trp-214. However, the role of induced conformational perturbation in the biomacromolecule itself upon ADMQ adoption cannot be ruled out completely, as indicated by circular dichroism, 3D fluorescence, root-mean-square deviation, root-mean-square fluctuation, and secondary structure element observations. The comprehensive spectroscopic study outlined herein provides important information on the biophysical interaction of a chalcone-based potential therapeutic candidate with a carrier protein, exemplifying its utility in having a regulatory effect on the microconformations of Trp-214.

1. INTRODUCTION

Chalcones and their heterocyclic derivatives are recognized for their plethora of promising pharmacological activities due to their DNA-targeting properties, proven gametocytocidal activity in the life cycle of Plasmodium falciparum, superior vasodilative properties in treating hypertension, and usefulness for the treatment of inflammatory diseases. Investigations of the binding mechanisms of several bioactive compounds with the transport protein human serum albumin (HSA) present in the human circulatory system is an important course to understand the behavior of drugs in terms of therapeutics as well as toxicity. Simultaneously it is also significant to apprehend their transport and disposition under physiological conditions. In this context, HSA, a well-structured major circulatory system protein, binds such ligands and also acts as an important determinant for the study of the pharmacokinetics of the drug molecules.

The fluorescence of HSA is predominantly governed by the sole Trp residue along with minor contributions from few tyrosines. Out of these, Trp is the most investigated fluorescent probe for protein conformation and dynamics. The presence of the highly sensitive indole side chain in Trp

Received: May 22, 2018
Accepted: August 20, 2018
Published: August 29, 2018

DOI: 10.1021/acsomega.8b01079
ACS Omega 2018, 3, 10114–10128

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makes it more capable for exploring the conformational ensembles of proteins in solution. The fluorescence emission profile of Trp is sensitive to slight transitions in the protein quaternary structure, ligand binding, or any other subunit association. The governing fluorophore Trp displays strong emission in the 310–350 nm region (when excited at its absorption maximum) and also exhibits multieponential decay with fluorescence lifetimes ranging from 50 ps to 8 ns. This multieponential fluorescence decay may be attributed to various ground-state conformers. Upon interaction with a ligand, the populations of Trp rotamers display variation in their percentage distribution. More precisely, the Trp rotamerization is governed by the orientation of the aromatic heterocyclic indole group, whose changes in geometry are so fast that they can only be captured on a shorter time scale. The different deactivation pathways and rates of depopulation of the excited state account for the existence of different decay times for each rotamer. The decay profile of the host HSA is sensitive to the ligand, which may have a direct influence on the microenvironment of the sole Trp residue. The extent of interaction between the Trp residue and ligand/quinone is governed by their distance and relative orientation.

Hence, the different rotamer populations of Trp in HSA provide key information on the binding mode of the ligand. In this context, the interaction of quinoline-appended chalcone derivatives (which are highly bioactive and characterized by rich photophysical properties) with human plasma proteins may be of great importance as it can alter the pharmacodynamics and pharmacokinetics (including distribution, metabolism, and elimination) of chalcone-based pharmaceutically relevant molecules.

In this article, we demonstrate the site-specific interaction and mode of binding of a newly synthesized multitherapeutic quinoline-appended chalcone derivative, (E)-3-(anthracen-10-yl)-1-(6,8-dibromo-2-methylquinolin-3-yl)prop-2-en-1-one (ADMQ), with the model plasma protein HSA and its consequences using optical spectroscopic methodologies. The spectroscopic results obtained herein are vindicated by computational molecular modeling studies. Besides, the understanding of the Trp emissive characteristics by virtue of the population distribution of its rotamers evokes interest as to whether the vital Trp rotamerization process is controlled by the protein binding enhances the solubility of the ligand in human plasma. Thus, it is necessary to get acquainted with protein–drug binding studies in order to determine the biochemical consequences of the synthesized/design drug inside the human body, which may further facilitate formulation efficacy. Hence, it is necessary to understand the exact mode and affinity of binding of ADMQ with HSA. The intrinsic fluorescence of plasma protein can be monitored using steady-state fluorescence spectroscopy. This emission is attributed to the sole Trp-214 (in subdomain IIA) and 18 tyrosine residues. For the ADMQ–HSA system, a wavelength of 295 nm was chosen for excitation to selectively monitor the emission of Trp-214 (exclusively at 340 nm) at three different temperatures: 298, 303, and 308 K. When the ligand ADMQ was gradually introduced into the aqueous HSA solution at pH 7.4, a concomitant diminution in the protein fluorescence along with a 4 nm hypsochromic shift (340 to 336 nm) in the emission maximum was observed (Figure 1A).

This progressive diminution of the tryptophan fluorescence by ADMQ suggests its binding interaction with the host protein molecule, whereas the increased hydrophobicity around the fluorophore is manifested by the hypsochromic shift. The above-said fluorescence quenching phenomenon is quantified by the Stern–Volmer (SV) equation:

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \] (1)

where \( F \) and \( F_0 \) are the steady-state fluorescence intensities in the presence and absence of the quencher ADMQ, respectively, \( K_{SV} \) is the SV quenching constant, \([Q]\) is the total concentration of quencher (ADMQ), and \( \tau_0 \) is the average lifetime of the protein in the absence of ADMQ, and here is considered to be \( 1 \times 10^{-8} \) s.

The SV plots thus obtained (Figure 1B) exhibit an upward deviation at higher ADMQ concentrations (for all three temperatures), as reported earlier for many other drug–protein interactions. The calculated \( K_{SV} \) is on the order of \( 10^7 \), and \( k_q \) is on the order of \( 10^{12} \) (much higher than the value for a diffusion-controlled process, where \( k_q = 2.0 \times 10^{10} \) M\(^{-1}\) s\(^{-1}\)), indicating the interaction of ADMQ with HSA via formation of a ground-state complex. This was further confirmed by the destabilization of the complex formed by increasing the temperature to 308 K (Table 1). Overall, the biphasic nature of the SV plot in the steady-state fluorescence quenching experiment suggested that a dual static and dynamic quenching mechanism is operative, which was further confirmed by time-resolved fluorescence (TRF) studies (discussed later).

**Determination of the Binding Affinity.** The quenching data were further analyzed in order to determine the binding affinity of ADMQ with HSA using the following modified SV (MSV) equation:

\[ \frac{F_0}{\Delta F} = \frac{1}{f_a}K_i[Q] + \frac{1}{f_s} \] (2)

where \( \Delta F \) is the change in fluorescence intensity, \( F_0 \) is the initial fluorescence intensity, \( f_a \) and \( f_s \) are the quantum yields of donating and quenching species, respectively, and \( K_i \) is the affinity constant. The affinity constants \( K_i \) were obtained with correlation coefficients of 0.99 (Figure 1C) and are listed in Table 1. The magnitude of \( K_i \) was found to be on the order of \( 10^{7} \), which implies moderate binding affinity of ADMQ toward HSA.

TRF measurements were also employed to ascertain the simultaneous involvement of dynamic quenching (if any, along with static quenching) in the binding process. In this context, the mean fluorescence lifetime (\( \tau_m \)) was obtained using the following expression:

\[ \tau_m = \sum a_i\tau_i/\sum a_i \]

where \( a_i \) is the relative percentage contribution of the decay component possessing lifetime \( \tau_i \). From the TRF results for HSA (shown in Table 4), it can be seen that \( \tau_0 \) significantly decreases from 4.51 to 0.74 ns upon gradual addition of...
ADMQ to the protein solution. The SV plot obtained from the mean fluorescence lifetimes, which is depicted in Figure 2, follows linearity only up to [ADMQ] = 40 μM. Intriguingly, an upward curvature is seen at higher concentrations of ADMQ, which indicates possibilities such as (i) the presence of a sphere of action around the fluorophore and (ii) the operation of simultaneous static and dynamic quenching. However, these possibilities may come into play individually or simultaneously.

**Thermodynamics of the Binding Interaction.** Generally, four kinds of non-covalent interactions govern the binding of the ligand to the protein that eventually help to extract the thermodynamic parameters. These includes van der Waals interactions, H-bond formation, electrostatic interactions, and hydrophobic interactions. Ross and Subramanian reported that the signs and magnitudes of the thermodynamic parameters so obtained indicate the predominant forces involved in binding of the ligand to the protein. The enthalpy (ΔH) and entropy (ΔS) of formation of the ADMQ–HSA complex were determined on the basis of the van’t Hoff equation (eq 3):

$$
\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
$$

where K is in the present case the association constant Kₐ, R is the gas constant, and T is the absolute temperature. The data are pooled in Table 2.

The slope of the van’t Hoff plot is related to ΔH, while the intercept of the plot indicates ΔS (Figure 3). The value of ΔG is calculated using the expression

Table 1. Quenching Variables for the ADMQ–HSA Interaction at 298, 303, and 308 K

| T (K) | Kᵥ (10⁴ L mol⁻¹) | kᵣ (10¹¹ L mol⁻¹ s⁻¹) | Kₛ (10⁴ L mol⁻¹) |
|------|----------------|---------------------|----------------|
| 298  | 12.21 ± 0.88    | 12.21 ± 0.88        | 4.85 ± 0.35    |
| 303  | 11.74 ± 0.83    | 11.74 ± 0.83        | 4.45 ± 0.41    |
| 308  | 8.75 ± 0.42     | 8.75 ± 0.42         | 4.11 ± 0.17    |

Table 2. Thermodynamic Variables for the ADMQ–HSA System at Various Temperatures

| T (K) | ΔH (kJ mol⁻¹) | ΔG (kJ mol⁻¹) | ΔS (J K⁻¹ mol⁻¹) | Rᵃ | SDᵇ |
|------|--------------|--------------|------------------|----|-----|
| 308  | 5.84         | -33.25       | 108.0            | 0.99| 0.19|
| 303  | -32.71       |              |                  |    |     |
| 298  | -32.17       |              |                  |    |     |

ᵃR is the correlation coefficient. ᵇSD is the standard deviation.
The negative value of $\Delta G$ obtained indicates that the formation of the complex between ADMQ and HSA is spontaneous. The obtained values of $\Delta S$ and $\Delta H$ are positive, indicating that the binding process mainly involves hydrophobic interactions. It is assumed that whenever the ligand binds to the protein in water-accessible area,38 the protein releases the excess solvent from its surface, thus driving the entropy to a positive value. From this perspective, it can be concluded that the nonpolar quinoline and anthracene groups can effectively interact with amino acids present in the hydrophobic region. Hence, we can presume the involvement of hydrophobic interactions to be predominant in securing the ligand ADMQ into the protein scaffold.

### 2.2. Protein-Induced Rotational Confinement

The principle of fluorescence anisotropy measurements lies in photoselective excitation of a fluorescent molecule using polarized light that ultimately results in polarized emission. It is important to note that transition dipole moments for absorption and emission lie in specific directions within the fluorophore structure. Such anisotropic measurements provide an outlook for protein conformational dynamics. Fluorescence anisotropy strongly depends on various factors such as solvent viscosity, fluorophore shape, and protein flexibility. A higher value of the anisotropy is observed as the environment around fluorophore becomes restricted and hinders its rotational diffusion. The anisotropy value in fluids gradually decreases because of the ease of rotation of the fluorophore molecule, but rotation can be restricted in different matrices such as micelles, reverse micelles, dextrin, etc.39 In the present experiment, variation in the anisotropy of ADMQ was recorded with increasing concentration of HSA by monitoring the emission of excited ADMQ only. It is relevant to mention here that the synthesized molecule ADMQ exhibits two distinct absorption bands at 259 and 434 nm in water and shows a remarkable emission with a maximum at around 550 nm ($\lambda_{\text{em}} = 434$ nm) in water.40

The plot (Figure 4) shows that as the concentration of HSA was increased, the fluorescence anisotropy ($r$) increased to a value of $r \approx 0.232$ at 260 $\mu$M and then leveled off. This observation is attributed to restricted motion of ADMQ somewhere inside the protein scaffold and not in the aqueous phase.

### 2.3. Identification of the ADMQ Binding Site in HSA

#### Site-Specific Interaction Studies with Site Markers

To discern the location of ADMQ in HSA, site-marker competitive experiments were conducted. According to Sudlow’s nomenclature, warfarin (War) and ibuprofen (Ibu) occupy the hydrophobic pockets31,42 and show affinity for binding site I (subdomain IIA) and binding site II (subdomain IIIA), respectively.53 War binds in a stable fashion within subdomain IIA of SA,54,45 which is reflected by its enhanced fluorescence that arises as a result of the close proximity of Trp-214 of HSA and its benzyl moiety.56,47

In this experiment, ADMQ was gradually added to a solution with a fixed [HSA];[site marker] concentration ratio of 1:1, and the changes in the emission spectrum were monitored upon excitation at 295 nm. A decrease in the fluorescence intensity of HSA was observed, accompanied by a hypsochromic shift from 345 to 340 nm (Figure S1). This observation reveals that there is an increased nonpolar region in vicinity of Trp-214 and that ADMQ addition somewhat perturbs the site in which War is bound to HSA. On the other hand, with Ibu there is just a decrease in fluorescence intensity without any shift, indicating that Ibu is incapable of averting ADMQ binding.

In order to quantify the extent of the binding interaction of the ADMQ–HSA complex in the absence and presence of the stereotypical site markers, eq 238,49 was used, and the affinity constant ($K_a$) for ADMQ–HSA was found to be $(4.85 \pm 0.35) \times 10^4$ at 298 K and pH 7.4, whereas the values of $K_a$ for ADMQ–HSA in the presence of Ibu and War were $(4.75 \pm 0.29) \times 10^4$ and $(4.20 \pm 0.34) \times 10^4$, respectively (Figure S1). It is prudent that the $K_a$ dwindled upon War addition but remained similar to the original value upon introduction of Ibu. The lower $K_a$ of ADMQ toward HSA in the presence of War indicates that ADMQ competes with War for the same site, i.e., site I of the protein. Hence, this site-marker experiment indicates that the ligand ADMQ is localized in subdomain IIA of serum albumin.

### 2.4. In Silico Investigation of the ADMQ Binding Site in HSA

In order to substantiate the detailed in vitro spectroscopic observations, a three-in-one molecular modeling exercise was employed, involving regular extra-precision (XP)
molecular docking, induced-fit docking, and molecular dynamics (MD) to decipher the site-specific interaction between the transport protein (HSA) and ADMQ.

**Molecular Docking.** The arrangement and configuration of ligands inside carrier proteins have a significant influence on their conformational change and bioactivity under physiological conditions. The globular protein HSA is characterized primarily by two sites I and II, each divided into two subdomains A and B. The key factors that hold importance for any ligand to bind to HSA are its affinity, site specificity, and binding pose within the protein pocket. Therefore, a complete picture of HSA and its interaction with ADMQ can be better understood through computational studies.

ADMQ exists in different structural forms in different environments, as shown in Scheme 1. In this scenario, a molecular docking exercise was carried out with the β-hydroxy keto form of ADMQ. Docking of the β-hydroxy keto form of ADMQ with HSA was performed and compared with that of the site-specific marker warfarin (which prefers to bind in subdomain IIA) by running the docking program Glide to uncover their respective binding modes.

Glide searches for favorable interactions between the ligand and the receptor molecule, usually a protein, using the Optimized Potentials for Liquid Simulations (OPLS) force field. The molecular docking study of the ADMQ−HSA system suggests that the ligand prefers to occupy Sudlow binding site I (subdomain IIA) of HSA where warfarin resides, near Trp-214 (Figure 6). The hydrophobic, electrostatic, and H-bonding interactions secure the ligand in the binding cleft of HSA in subdomain IIA. The hydrophobic residues Trp-214, Leu-198, Ala-291, Val-343, Val-344, Tyr-452, and Val-455 facilitate the binding inside the protein pocket. A comparative picture of the docking scores and binding energies of ADMQ and warfarin is documented in Table 3, indicating that ADMQ is stable while accommodating itself in binding site I (BS I), in good agreement with site-specific interaction studies in the presence of site markers.

**Induced-Fit Docking Study.** This preliminary research prompted further that a more sophisticated docking method like induced-fit docking (IFD) had to be adopted to shed light on the binding site, binding affinity, orientation, and binding pose of the ligand and receptor. IFD uses Glide and the refinement module in Prime to induce adjustments in the receptor structure so that it can accommodate the ligand as per its geometry and orientation. The receptor here also is untrimmed but a softened one compared with the standard virtual docking study, in which the receptor is kept rigid.

From the IFD studies, it can be judged that the minimum-energy conformation of ADMQ prefers to acquire an arclike shape with a dihedral angle of 113.8° within the hydrophobic pocket with slight structural perturbations in the receptor site and is characterized by an IFD score of −1283.42 kcal mol⁻¹. Trp-214 is also found to lie in close proximity to ADMQ (estimated distance of ~1.97 nm), as depicted in Figure 7 left. The IFD-generated pose depicts that the hydroxyl group of ADMQ forms H-bonds with Trp-214 and Asp-451, whereas the nitrogen atom of the quinoline moiety forms a H-bond with Arg-222. The two-dimensional 2D ligand interaction pattern (Figure 7 right) depicts the predominance of hydrophobic amino acids (apple-green balls) in proximity to the ADMQ molecule along with a few positively charged residues like Arg-222, Arg-218, and Lys-195. Thus, several non-covalent interactions shelter ADMQ within HSA.

**MD Simulations.** In order to analyze various features of ADMQ−HSA binding (e.g., stability, types of interactions, etc.) in explicit solvent under physiological conditions, MD simulations were employed. Here the pose-scoring aspect of IFD was taken into consideration, and out of the 12 poses that were generated, the ADMQ−HSA conjugate pose with the highest IFD score (−1283.42 kcal mol⁻¹) was taken for the next level of 15 ns MD simulation by Desmond, an explicit-solvent program with emphasis on accuracy, speed, and scalability. MD simulations were used to gain an idea regarding

![Figure 5. Modified SV plots for the ADMQ−HSA binding interaction in the absence or presence of the site markers ibuprofen and warfarin at 298 K. [HSA]:[site marker] = 1:1; λ₁ = 295 nm; T = 298 K.]

**Scheme 1. Structural Changes of ADMQ in Different Environments**

![Scheme 1. Structural Changes of ADMQ in Different Environments](image-url)
the comparative stability of ADMQ and warfarin inside the same binding pocket, as represented in terms of root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), secondary structure elements (SSEs), and protein–ligand contacts. The RMSD for ADMQ–HSA is shown in Figure 8 while that for the warfarin–HSA system is presented in Figure S2.

The RMSD plot illustrates that ADMQ stays in its primary binding pocket and remains in contact with the protein chain without diffusing away into the solvent. The RMSD change during the complete 15 ns run lies within the 1–3 Å range, which shows that the system has equilibrated and the RMSD has stabilized around fixed values in both the case of ADMQ and warfarin.

The RMSF plot presents peaks that are indicative of those regions of the protein that have undergone maximum fluctuations. The RMSF changes throughout the run were found to be similar in the cases of ADMQ (Figure 9) and warfarin (Figure S3). Inspection of the peak fluctuations reveals how similar side chains of the protein have undergone fluctuations with both ADMQ and warfarin. The agreement between the protein RMSD and protein RMSF is found to be

Table 3. Comparison of the Energy Parameters of Warfarin and ADMQ Located Inside BS I of HSA

| ligand  | docking score (kcal mol$^{-1}$) | MM-GBSA (kcal mol$^{-1}$) |
|---------|---------------------------------|---------------------------|
| warfarin| −6.0                            | −49.0                     |
| ADMQ    | −7.2                            | −74.8                     |

Figure 6. (a) Energetically best docked pose of ADMQ inside site I of HSA (PDB ID 1AO6). (b) Three-dimensional illustration of various amino acids in close proximity to ADMQ.

Figure 7. (left) Distance (in nm) between ADMQ and Trp-214. (right) Ligand interaction diagram depicting the amino acid residues and non-covalent interactions.

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excellent, which concretizes our observation that the probable binding site of ADMQ is site IIA (the warfarin binding site). Moreover, the SSE distributions by residue index for ADMQ (Figure 10) and warfarin (Figure S4) throughout HSA can be attributed to similar changes in \( \alpha \)-helix percentage to the tune of 57.24\% for ADMQ and 57.72\% for warfarin.

The SSE compositions for each trajectory frame for ADMQ on HSA (Figure 11) and warfarin on HSA (Figure S5) were also found to be similar, along with analogous changes in \( \alpha \)-helix. The findings signify that subdomain IIA undergoes similar changes when ADMQ and warfarin are incorporated at the same site. Hence, this comprehensive in silico exercise provides insight into the binding location, affinity, stability, and orientation pattern of the bioactive ligand ADMQ inside the HSA scaffold.

2.5. Precise Control over Rotamerization of Trp-214. Modulation of the Excited-State Dynamics of Trp-214 by ADMQ. HSA is prone to continuous wobbling motion, and so is its sole Trp residue, Trp-214. In its natural habitat, HSA exhibits three lifetime components, each representing a specific ground-state conformer of Trp with lifetimes of 1.1 ns \((r_1)\), 3.96 ns \((r_2)\), and 7.32 ns \((r_3)\). These three Trp rotamers...
are denoted as conformer I, conformer II, and conformer III, respectively. The disparity in the lifetimes of the three conformers in native HSA itself signifies their relative exposure to the microenvironment. The shortest and intermediate lifetimes reflect a degree of quenching for certain Trp conformers due to the rigidity of the Trp microenvironment, whereas the longest lifetime represents a “free” conformer of Trp. Hence, a longer lifetime (conformer III) corresponds to greater exposure of the conformer to the aqueous microenvironment, and a shorter lifetime (conformers I and II) indicates exposure of the conformer toward a less polar environment and its burial deep inside, away from the influence of bulk water. When HSA is subjected to increasing ADMQ concentrations (Figure 12), the lifetimes of the conformers are affected differently (the data are pooled in Table 4). \(\tau_3\) remains almost unaltered throughout the entire concentration range, indicating that it is unlikely that ADMQ interacts with conformer III. For conformer II having an intermediate lifetime, \(\tau_2\) shows a marginal variation and decreases from 3.96 to 3.56 ns. The lifetime of conformer I, \(\tau_1\), decreases markedly from 1.1 to 0.52 ns, which is nearly a 50% reduction relative to its initial value.

Another interesting observation is the profound transformation in the percentage contributions of these three rotamers in HSA with increasing ADMQ concentration. The percentage contributions dwindle radically for both conformer II and III from 34.64 to 3.82% and 38.82 to 1.50%, respectively. However, for conformer I the reverse situation applies, as the percentage contribution mounts from 26.54% to a very large value of 94.68% (Figure 13). The change triggered in the relative percentage contributions and fluorescence decays of the three rotamers/microconformations of Trp (in concentration-dependent ADMQ binding to HSA) points toward ADMQ-induced perturbations of the Trp-214 neighborhood. This disposition also points out the fact that ADMQ does exhibit precise control over the rotamerization of Trp-214. This control over the rotamerization process may be...
due to conformational alteration in HSA, direct interaction between ADMQ and Trp-214, or both.

To understand the effect of changing pH on the conformational transformation of HSA, the fluorescence lifetime parameters (lifetime and pre-exponential value) were monitored carefully as the ADMQ concentration was increased in the HSA environment at different pH at 298 K. The changes in lifetime values as well as in pre-exponential factors throughout the pH range under study (Figures S7–S10) are very similar to those reported at physiological pH.

### 2.6. Verification of Conformational Alteration in HSA upon ADMQ Adoption

Binding of any exogenous ligand to serum albumin may induce an alteration in the conformation and three-dimensional (3D) structure of the host macromolecule. The drug-induced conformational change, if major, may affect the biochemical/biological activity of the protein itself because maintaining the active site in the proper configuration is the cardinal function of the protein structure, and thus, a minimal conformational change may be highly appreciated. The conformational flexibility and adaptability exhibited by a protein to interact with a drug molecule is known to influence the transport mechanism of any drug in the physiological microenvironment. Hence, the ADMQ-induced conformational alteration in HSA was further investigated by circular dichroism (CD) and 3D fluorescence spectroscopy.

#### Verification by CD Spectroscopy

Far-UV circular dichroism in the range of 200–260 nm was measured to observe alterations (if any) in the secondary structure of the host protein to accommodate the newly synthesized ADMQ molecule at increasing concentration. The dichroic absorption bands for $\alpha$-helix, $\beta$-sheet (parallel and antiparallel), $\beta$-turn, and random coil content were documented for the addition of ADMQ to HSA. The CD spectrum of HSA manifests itself with two signature absorption bands at 208 and 222 nm, characteristic of $\alpha$-helices.55 Upon gradual addition of ADMQ,
the decrease in the ellipticities at 208 and 222 nm (Figure 14) indicates an increase in the negative Cotton effect, which reveals slight changes in the secondary structure of HSA.

![Figure 14. CD spectra of HSA alone and the ADMQ−HSA complex at 298 K and pH 7.4. [HSA] = 2.5 μM; curves A through F correspond to [ADMQ] = 0, 2.85, 7.32, 21.19, 39.27, and 58.01 μM.](image)

The CD results also indicate that upon addition of ADMQ to free HSA, the antiparallel β-sheet structure rises from 2.30% to 3.30%, the parallel β-sheet arrangement from 2.70% to 3.50%, the β-turn structure from 11.10 to 12.50%, and the random coil structure from 13.00 to 15.50%. Moreover, the α-helical content shows a reduction from 73% (free HSA) to 63.70% (ADMQ−HSA complex), as shown in Figure 14. This diminution in the percentage of α-helices (~10%) upon introduction of ADMQ is indicative of minor conformational alterations in HSA. The observation that the CD spectrum of free HSA overlapped with the spectrum of the ADMQ−HSA complex suggests that HSA in complex with ADMQ is predominantly α-helical with very little change in the relative quantities of each component, as mentioned above. This indicates that binding of ADMQ may have caused the polypeptide chain to become slightly more tender in order to accommodate the ligand inside the protein pocket. This meager decrement in the percentage of α-helices may not render HSA inactive but may have some effect on the rotamerization of Trp-214.

**Verification by 3D Fluorescence Spectroscopy.** To gain more insight into the ADMQ-rendered alteration in the polypeptide backbone of HSA, 3D fluorescence measurements were also performed on free HSA and in presence of increasing concentrations of ADMQ. The 3D fluorescence spectra and contour diagrams for native HSA and the ADMQ−HSA complex are presented in Figure 15.

In the 3D spectra, peak a (λex = λem) corresponds to Rayleigh scattering. Additionally, two peaks designated as peak 1 (λex = 295 nm) and peak 2 (λex = 235 nm) are also observed.56,57 Peak 1 is characterized as the fluorescent tryptophan residue in HSA and reflects changes in the tertiary structure of the protein, whereas peak 2 is indicative of changes in the secondary structure of the protein upon addition of ADMQ. The decreasing trends in the fluorescence intensity for both peak 1 (27%) and peak 2 (25%) individually with increasing ADMQ concentration point toward microenvironmental changes along with slight alterations of the peptide strand (secondary structure) and overall tertiary structure of the host protein. Hence, both CD and 3D fluorescence spectroscopy establish minimal conformational alteration of the host HSA to accommodate the potential bioactive ADMQ molecule. Hence, at this juncture it may be inferred that this minor conformational alteration may also be conducive to Trp-214 rotamerization.

### 2.7. Verification of a Direct Interaction between ADMQ and Trp-214

The relative distribution of the Trp microconformers in HSA can be regulated by ADMQ only when a direct interaction is possible between them. Hence, it was necessary to determine whether these two partners are close enough to each other to achieve an effective dynamic interaction.

**Determination of the Proximity between Trp-214 and ADMQ.** In connection with the possibility of the direct interaction of ADMQ with Trp-214, Förster resonance energy transfer (FRET) measurements were performed for the ADMQ−HSA system to investigate the distance-dependent energy transfer between the donor (HSA) and acceptor (ADMQ) in the biological microenvironment.58 Energy transfer occurs when the absorption spectrum of the acceptor molecule overlaps with the emission spectrum of the donor.59 This spectral overlap between the interacting partners is depicted in Figure 16. The efficiency of energy transfer between the donor and acceptor can be estimated from the photoluminescence quenching using the Förster equation,60

\[
E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6}
\]

where \(E\) is the efficiency, \(r\) is the distance between the donor and acceptor, and \(R_0\) is the distance at which the energy transfer becomes 50%, which can be calculated as

\[
R_0^6 = 8.79 \times 10^{-25} \times [K^2n^{-4}q_0(J)]
\]

where \(K^2\) is the dipole orientation factor for the donor and acceptor, \(q_0\) is the fluorescence quantum yield of the donor in the absence of acceptor, \(n\) is the refractive index, and \(J\) is the spectral overlap integral, expressed as

\[
J(\lambda) = \frac{\int F_0(\lambda) \varepsilon(\lambda) \lambda^4 \, d\lambda}{\int F_0(\lambda) \, d\lambda}
\]

For ADMQ and HSA, \(\varepsilon_0 = 7.49 \times 10^1\) M\(^{-1}\) cm\(^{-1}\) (at 430 nm), \(n = 1.37\), and \(q_0 = 0.15\). From eqs 5–7, the evaluated values are \(J = 4.80 \times 10^{-3}\) M\(^{-1}\) cm\(^{-1}\) nm\(^4\), \(R_0 = 2.21\) nm, \(E = 0.3574\), and \(r = 2.45\) nm. The obtained value of \(r\) lies within 2–8 nm scale and satisfies the condition 0.5\(R_0 < r < 1.5R_0\), which implies the existence of energy transfer between HSA and ADMQ. The distance between Trp-214 and ADMQ (\(r = 2.45\) nm) is analogous with the result of the IFD study (Figure 7a).

### 2.8. Control Experiment with Warfarin and Ibuprofen

ADMQ occupies a similar protein scaffold as the site marker warfarin, i.e., subdomain IIA of BS I in proximity to Trp-214 of HSA. Thus, a control experiment was also performed with same anticoagulant warfarin to see its effect on the three conformers of tryptophan. The addition of warfarin to HSA resulted in an increase in the percentage of the short-lifetime rotamer (conformer I) from 26.19 to 72.69% and precisely points toward similar changes in the Trp-214 microenvironment (Figure 17).

This observation inclines the fact that ADMQ and warfarin exhibit similar effects on the carrier protein in...
subdomain IIA (BS I) and have similar control over the rotamerization of Trp-214. Interestingly, performing the same experiment with another stereotypical ligand, ibuprofen (a non-steroidal anti-inflammatory drug), which binds to subdomain IIIA (BS II) in the cleft/pocket, remote from vicinity of Trp-214, did not bring out the same picture. Upon gradual addition of ibuprofen to HSA at physiological pH, the percentage contribution of conformer I varied meagerly from 31.91 to 40.5%, which indicates that ibuprofen does not interfere with the microconformer (conformer I) that is buried deep inside the pocket of subdomain IIA and does not control rotamerization of Trp-214, as observed in case of ADMQ and warfarin. Hence, from the proximity determination and control experiments in presence of site markers, it may be concluded that although ADMQ exerts a minor conformational perturbation on the polypeptide backbone, the change in rotamerization of Trp-214 is mainly influenced by direct interaction of ADMQ with Trp-214, as they reside in close proximity to each other (2.45 nm).

3. CONCLUSION

The present comprehensive study enlightens a coherent flow of the binding affinity, location, and spatial structural changes induced in HSA by a newly synthesized quinoline-appended anthracenyl chalcone, ADMQ, as shown by several in vitro spectroscopic techniques combined with molecular modeling exercises. The site-marker competitive assay in conjunction with the three-in-one molecular modeling approach generated comparable profiles in terms of binding location for ADMQ and the standard site marker warfarin in the HSA scaffold. ADMQ is enveloped inside the hydrophobic domain (BS I, subdomain IIA) of HSA via hydrophobic interactions with Trp-214, Arg-218, Arg222, Asp451, Tyr452, etc. Our quest provides salutary quantitative data not only on how the dynamic behavior of ADMQ with Trp-214 is manifested via
Figure 16. Spectral overlap of ADMQ absorption (black) and HSA fluorescence (blue). The shaded region depicts the actual overlap of the two spectra.

Figure 17. Change in percentage contribution of conformer 1 in native HSA with increasing ADMQ (■), warfarin (○), and ibuprofen (▲) at 298 K. λ\text{ex} = 280 nm; λ\text{em} = 340 nm.

A concentrated stock solution of HSA and warfarin was prepared in HEPES buffer (10 mM, pH 7.4). A stock solution of ADMQ and ibuprofen was prepared in 1,4-dioxane (Fisher Scientific, spectroscopic grade).

4.2. Instrumentation. 4.2.1. Absorption Spectroscopy. UV absorption spectra were recorded at room temperature using a V-630 UV/vis spectrophotometer from JASCO Deutschland GmbH and quartz cuvettes with a path length of 1 cm.

4.2.2. Steady-State Fluorescence Spectroscopy. A JASCO FP-8300 spectrofluorimeter with a spectral resolution of 2.5 nm was used for steady-state excitation and emission measurement with 1 cm path length cuvettes. The excitation wavelength of 295 nm was chosen to measure the emission from the sole Trp residue in HSA. Spectral corrections were carried out by subtracting appropriate blanks from the sample spectra run under the same conditions. Samples were equilibrated for 10 min at a particular temperature before data acquisition. All of the fluorescence intensities were corrected for the inner-filter effect using the following expression:

\[ F_c = F_0 \frac{A_{ex} + A_{em}}{2} \]

where \( F_o \) and \( F_c \) are the observed and corrected fluorescence intensities, respectively, and \( A_{ex} \) and \( A_{em} \) are the absorbances at the wavelength of emission and wavelength of excitation, respectively. In the site-marker competitive experiments using the fluorescence titration method, backgrounds of warfarin and ibuprofen were subtracted before data analysis.

4.2.3. Steady-State Fluorescence Anisotropy. The steady-state fluorescence anisotropy measurements were carried out on a JASCO FP-8300 spectrofluorimeter at 298 K with manual control of the parallel and perpendicular polarizers. The fluorescence anisotropy \( r \) is expressed as:

\[ r = \frac{(Ex_{90}/Em_{90}) - G(Ex_{90}/Em_{90})}{(Ex_{90}/Em_{90}) + 2G(Ex_{90}/Em_{90})} \]

where \( Ex_{90}/Em_{90} \) and \( Ex_{0}/Em_{0} \) are vertical and horizontal excitation/emission polarizers, respectively. The value of \( G \) is formulated as:

\[ G = \frac{(Ex_{90}/Em_{90})}{(Ex_{0}/Em_{0})} \]

4.2.4. Circular Dichroism Measurements. A JASCO J-815 CD spectropolarimeter (Jasco Inc., Easton, MI) was used to record the spectra at 298 K with a rectangular quartz cuvette with a path length of 1 cm. An average of three successive scans was made for each CD spectrum at a rate of 20 nm/min over the wavelength range from 200 to 260 nm. The HSA concentration was kept constant (2.5 \( \mu \)M) while the ADMQ concentration was varied. The CD results were expressed as mean residual ellipticity (MRE) in units of deg cm\(^2\) dmol\(^{-1}\):

\[ \text{MRE} = \frac{\text{observed CD (in mdeg)}}{C_p n I \times 10} \]

where \( C_p \) is the molar concentration of protein, \( n \) is the number of amino acid residues (585 for HSA), and \( I \) is the path length (1 cm). The \( \alpha \)-helical content was estimated from the MRE value at 208 nm using the following expression:

\[ \% \alpha = \frac{MRE_{208} - MRE_{190}}{MRE_{208} - MRE_{185}} 	imes 100 \]

4. EXPERIMENTAL DETAILS

4.1. Materials. The ligand ADMQ used for the present study was synthesized previously by our group as described elsewhere. HSA and warfarin were procured from Sigma-Aldrich (St. Louis, MO, U.S.). Ibuprofen was purchased from Bangalore Fine Chemicals (Bangalore, India). HEPES buffer (extra-pure 99%) was procured from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India).

Figure 18. Change in percentage contribution of conformer 1 in native HSA with increasing ADMQ (■), warfarin (○), and ibuprofen (▲) at 298 K. λ\text{ex} = 280 nm; λ\text{em} = 340 nm.
%α-helix = \left(\frac{-\text{MRE}_{208} - 4000}{33000 - 4000}\right) \times 100

where \text{MRE}_{208} is the observed MRE value at 208 nm. The MRE value of 4000 corresponds to the β-form and random coils, whereas the MRE value of 33000 is for pure α-helix at 208 nm.

4.2.5. Fluorescence Lifetime Measurements. Time-resolved fluorescence decays were measured using the time-correlated single-photon counting (TCSPC) technique with a PTI Pico Master instrument. A pulsed diode with a wavelength of 280 nm was used as the excitation source for HSA. The instrument response function (IRF) was acquired using sodium dodecyl sulfate as a scatterer. The decays and pre-exponential values obtained at an emission wavelength of 340 nm was used as the excitation source for HSA. The decays and pre-exponential values obtained at an emission wavelength of 340 nm were analyzed using FelixGX 4.1.2 software. The reduced χ2 value and Durbin–Watson parameter were used to authenticate the goodness of fit. The lifetime data were acquired with similar parameters at pH 2, 4, 9, and 11 using sodium acetate and disodium hydrogen orthophosphate buffers with increasing ADMQ concentration in the HSA environment.

4.3. In Silico Studies. The molecular modeling studies were conducted using an Intel core i5~2500 CPU running at a clock speed of 3.3 GHz. The 64-bit processors were coupled with 4 GB of high-speed RAM while using Maestro version 9.9 (Schrödinger LLC, New York, 2014). The machines were operated by the Linux Centos 6 operating system.

4.3.1. Ligand and Protein Preparation. The synthesized ligand ADMQ was prepared in Maestro 9.9 over the LigPrep module63~65 using the OPLS-2005 force field66,67 for rectification of molecular geometries and subsequent ionization at a physiological pH of 7.4, thereby enabling specific chirality retention in order to obtain a conformation with minimum energy. HSA (PDB ID 1AO6)68,69 was prepared using the Protein Preparation Wizard (Schrödinger).70 The HSA model prepared in this fashion may be considered reliable as it closely resembles the native 3D structure. Finally, the conformational stability of the ligand complex

HSA in water/buffer at pH 7.0 at 298 K (Figure S6); percentage contributions of three rotamers in native HSA with increasing ADMQ at pH 2, 4, 9, and 11 (Figures S7–S10) (PDF)

4.3.2. Regular XP Molecular Docking and Induced-Fit Docking. The site map module was used to identify possible binding sites in the fully processed protein. The main purpose of the docking exercise was to generate a list of feasible binding sites in the fully processed protein. The main purpose of the docking exercise was to generate a list of feasible binding sites in the fully processed protein. The main purpose of the docking exercise was to generate a list of feasible binding sites in the fully processed protein. The main purpose of the docking exercise was to generate a list of feasible binding sites in the fully processed protein.

4.3.3. MD Simulations. Desmond MD System v.2.2 (D. E. Shaw Research, Schrödinger)66,72 was used to perform MD simulations. The TIP3P solvation model was configured to an orthorhombic box shape. Neutralization of the ions was carried out by introducing Na+ salt. To minimize the solvated ligand–protein complex, 2000 iterations were carried out. The simulation was carried out for 15 ns. The overall method for MD simulation was adopted as reported earlier for the ADMQ–BSA system.

Effect of the selected site marker (warfarin) on the ADMQ–HSA system in HEPES buffer at pH 7.0 (Figure S1); HSA and warfarin RMSD plot during 15 ns simulation (Figure S2); HSA and warfarin RMSF plot during 15 ns simulation (Figure S3); SSE distribution by residue index of warfarin throughout HSA during the course of 15 ns simulation (Figure S4); SSE composition for each trajectory frame over the course of simulation and SSE assignments of each residue over time for warfarin on HSA (Figure S5); absorption and emission spectra of ADMQ in water/buffer at pH 7.0 at 298 K (Figure S6); percentage contributions of three rotamers in native HSA with increasing ADMQ at pH 2, 4, 9, and 11 (Figures S7–S10) (PDF)

ACKNOWLEDGMENTS

CSIR Financial Support Scheme 37 (1493)11/EMR-II is thankfully acknowledged. Thanks to Dr. P. Bhavana (BITS Pilani, K. K. Birla Goa Campus) for her kind support.

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