Dedicated to Professor Dr. Cozar Onuc on His 70th Anniversary

LIDOCAINE - HSA BINDING CHARACTERIZED BY FLUORESCENCE SPECTROSCOPY AND MOLECULAR DOCKING

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ABSTRACT. Quenching fluorescence and molecular docking methods were used to evaluate changes in the local environment of intrinsic fluorophores of HSA in the presence of lidocaine and to calculate the binding parameters that characterize drug-protein interaction. We show that lidocaine induces significant fluorescence quenching of tryptophan and changes in conformation of IIA domain of HSA. The bimolecular quenching rate constant calculated using Stern-Volmer equation indicates a direct binding as the cause of fluorescence quenching. The protein-ligand association constant determined from Trp fluorescence quenching data showed a weak binding of lidocaine to HSA. The molecular docking calculations indicates three docking sites for lidocaine, in IIIA and IIA and IB domains of HSA, with preference for cavities located in IIIA.

Keywords: fluorescence quenching spectroscopy; lidocaine-HSA interaction; molecular docking.

INTRODUCTION

A fundamental characteristic of serum albumin (HSA), the most abundant protein of plasma, is the high ability to bind reversibly and to transport a large variety of drugs. HSA plasma abundance makes it an important factor in the pharmacokinetic behavior of many drugs, affecting their efficacy, toxicity and rate

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of drug delivery [1]. Studies related to drugs - HSA interaction give us a better understanding about drug binding process, drug delivery, how competition equilibria could affect drug availability, metabolism and excretion rate, which are of great importance for drug pharmacology and pharmacodynamics [2]. From structural viewpoint the albumin molecule has three homologous domain (I, II and III), each containing two subdomain (A and B) stabilized by 17 disulfide bridges. Three specific drug binding sites were identified in HSA molecule: Sudlow's sites located in subdomain IIA (drug site I) and in subdomain IIIA (drug site II) and recently, subdomain IB which was defined as the third major drug binding region of HSA, based on circular dichroism spectroscopic approach [3].

Lidocaine is a local anesthetic used in antiarrhythmic medication that works by blocking sodium channels. Studies concerning lidocaine binding to HSA are related mainly to equilibrium dialysis, crystallographic analysis [4, 5] and to a homogenous enzyme immunoassay [6]. Based on crystallographic data, Hein et al. [5] assert that lidocaine has a unique binding site located in the subdomain IB of HSA and that tryptophan residue (Trp 214) is not directly involved in lidocaine binding; but the structural features at the residue could be modified as a result of the lidocaine binding. Additionally, fluorescence experiments put in evidence HSA fluorescence quenching and a blue shift of the spectra that suggests lidocaine-induced conformational changes in the Trp environment.

In the present report we used two complementary and alternative methods for investigation and characterization of HSA – lidocaine interaction: fluorescence spectroscopy and molecular docking. Our goal was to identify the binding sites of lidocaine and to quantitatively characterize the binding affinity.

METHODS

Fluorescence quenching spectroscopy

Fluorescence quenching is a phenomenon where the light emitted by a fluorophore is diminished by the action of ligand. The phenomenon depends on the availability of HSA fluorophores (tryptophan and tyrosine) to the ligand. Trp-214 residue is the main intrinsic fluorophore of human albumin located in drug site I of IIA subdomain and its fluorescence quenching was used to find the lidocaine-HSA binding parameters. Using a synchronous fluorescence method to record the emission spectra of Trp we were able to provide additional information about the molecular environment in the vicinity of HSA intrinsic fluorophores.

The analysis of the experimental data based on Stern-Volmer equation (Eq 1) allows us to identify the nature of the interaction between protein and ligand and respectively between a process of dynamic collisional interaction and a process based on the formation of molecular complexes:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \tag{1},$$

where F_0 and F are the fluorescence emission intensity of HSA without and with the addition of a known concentration of quencher [Q], K_{SV} is the Stern-Volmer quenching constant, k_q is the bimolecular quenching rate constant, and τ_0 is the fluorescence lifetime of the unquenched fluorophore. k_q has a great importance because it is directly related to the sterical and electrical availability of fluorophore. As the fluorophore is more exposed, the fluorescence quenching efficiency (measured by k_q) is higher. For a collisional process, k_q cannot be larger than $2 \times 10^{10}\,M^{-1}s^{-1}$ [7].

In order to determine the association constant of protein-ligand complex, the experimental data have been fitted with the function proposed by M. van de Weert [8] which assumes a 1:1 stoichiometry and in normalized version can be expressed as:

$$\frac{F_{corr}}{F_0} = 1 + \left(\frac{F_c}{F_0} - 1\right) \frac{[P]_t + [Q]_a + K_d - \sqrt{([P]_t + [Q]_a + K_d)^2 - 4[P]_t[Q]_a}}{2[P]_t} \tag{2}$$

where F_{corr} is the measured fluorescence corrected for the inner-filter effect, F_0 is the fluorescence in the absence of lidocaine, F_c is the fluorescence of the fully complexed HSA, K_d is the dissociation constant, $[P]_t$ is the concentration of HSA, and $[Q]_a$ is the concentration of added lidocaine.

The inner-filter effect corrections were done according to the formula:

$$F_{corr} = F_{obs} 10^{\frac{A_{exc} + A_{em}}{2}} \tag{3}$$

where F_{corr} is the corrected fluorescence value, F_{obs} the measured fluorescence value, A_{exc} and A_{em} the measured absorbance values at the excitation and emission wavelengths, respectively.

Molecular docking simulations

Crystallographic data for albumin were downloaded from RCSB PDB website [http://www.rcsb.org/pdb/explore/explore.do?structureId = 1AO6]. The data for lidocaine molecule used as ligand in this study was downloaded from [https://pubchem.ncbi.nlm.nih.gov/compound/lidocaine] NCBI Chemistry website. The

optimized 3D structures of molecules were obtained with Gaussian09. AutoDockTools was used to build the input files (ligand + HSA) [http://autodock.scripps.edu/resources/adt] while the docking calculations were performed by using AutodockVina [http: //autodock.scripps.edu] [9] (open source). Viewing the results and some structure calculations were performed with VMD software [http://www.ks.uiuc.edu/Research/vmd/] and with Chimera1.9 [https://www.cgl.ucsf.edu/chimera/].

VINA code combines two methods, Monte Carlo and Simulated Annealing respectively, into a Lamarkian genetic algorithm LGA. It takes into account a temperature of 298.15 K performing 10 independent runs and starting with 150 random conformations. The calculation involves a random conformation disturbance and a local optimization (algorithm Broyden-Fletcher-Goldfarb-Shanno) followed by and an assessment in which the result is accepted or not. Each local optimization involves several evaluations of the scoring function and of its coordinates in term of position-orientation-twist. The number of optimization steps is heuristically determined, depending on the size and flexibility of the ligand. Each simulation may produce more results, automatically grouped and sorted to produce the final result. The code output results in nine possible locations for docking. To clearly indicate the binding sites of lidocaine in HSA we thus calculated 100 possible locations by using a 126x126x126 Å region with a grid of 0.375 Å. All the calculations were made by explicitly taking into account the polar hydrogens of HSA and by treating the ligand as flexible.

EXPERIMENTAL

Chemicals

Serum albumin, fraction V (fatty acid free, 99%) was purchased from Sigma – Aldrich Chemie GmbH. Lidocaine hydrochloride was achieved as 40 mg/ml sterile isotonic saline solution. All other reagents used were of analytical grade and double distilled water was used throughout the experiments.

Fluorescence measurements

The fluorescence spectra were measured at room temperature on a JASCO – 6500 spectrofluorimeter equipped with a Xenon lamp and 1.0 cm quartz cell. The HSA fluorescence emission spectra were recorded in the wavelength range of 300 – 500 nm, upon excitation at λ_{ex} = 295 nm. Synchronous fluorescence measurements were used to put in evidence the influence of lidocaine at the level of HSA intrinsic fluorophores,

tyrosine (Tyr) and tryptophan (Trp). The emission spectra were recorded in the wavelength range 280-340 nm upon excitation shifted with $\Delta\lambda_{tyr}$ = 30 nm for Tyr and in the range 310-380 nm with $\Delta\lambda_{trp}$ = 60 nm for Trp.

The UV-vis absorption spectra were recorded on a double beam JASCO–550 spectrophotometer equipped with 1.0 cm quartz cell, in the range of 250 – 500 nm.

Solutions were prepared at room temperature using double distilled water and 50 mM Tris/HCl (pH 7.4) as buffer. The HSA concentration was kept fixed at 4 μM and the lidocaine concentration was varied from 0 to 20 mM (2, 4, 6, 8, 10, 15, 20 mM).

RESULTS AND DISCUSSION

Tryptophan fluorescence quenching by lidocaine

Fig. 1 illustrate changes in the emission spectra of HSA fluorescence at 295 nm excitation wavelength and Fig. 2 the fluorescence spectra of tyrosine and tryptophan in the presence of lidocaine, obtained by synchronous fluorescence measurements. When the concentration of albumin were fixed at 4μM and lidocaine concentration was gradually increased, HSA and Trp fluorescence emission intensity decreased significantly and is accompanied by a maximum shift that suggests conformation changes of HSA molecule.
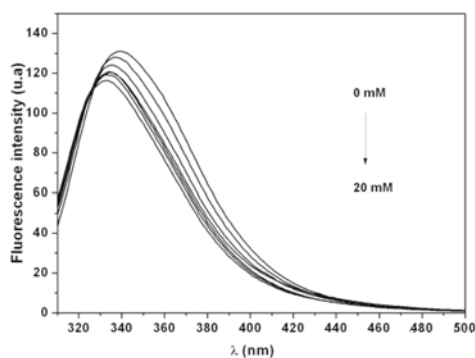

Fig. 1. Fluorescence emission spectra of HSA 4 μM, in the presence of lidocaine in the range of 2-20 mM λ_{ex} = 295 nm
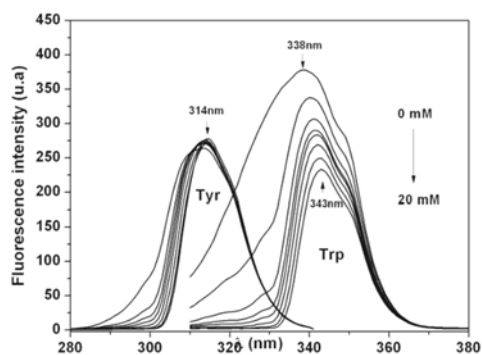
Fig. 2. The synchronous fluorescence spectra of tyrosine (Tyr) and tryptophan (Trp) in the presence of 0 – 20 mM lidocaine concentrations

Synchronous fluorescence measurements (Fig. 2) showed a reduction in the tyrosine emission spectra area with increasing lidocaine concentration but no fluorescence quenching.

Besides a significant quenching of Trp fluorescence, a 5 nm red shift of spectra maximum is observed which corresponds to changes of the polarity around the chromophore molecule. According to the accepted interpretation of changes in polarity of chromophore neighborhood [10], the red shift of Trp λ_{max} suggests a more polar environment for tryptophan residue as a result of HSA lidocaine interaction. The shift of the maximum intensity in the case of tyrosine was insignificant, reflecting little transformation around this fluorophore.

As can be seen from Stern-Volmer curve (Fig. 3), the plot of F_0/F_{corr} versus lidocaine concentration [Q] exhibits a good linearity (R = 0.8986). The values obtained for K_{SV} and k_q parameters were: K_{SV} = (2.31±0.088) x 10^5 M^{-1} and k_q= 3.3 x 10^{13} $M^{-1}s^{-1}$, using a typical HSA average lifetime without quencher of 7 ns [11].
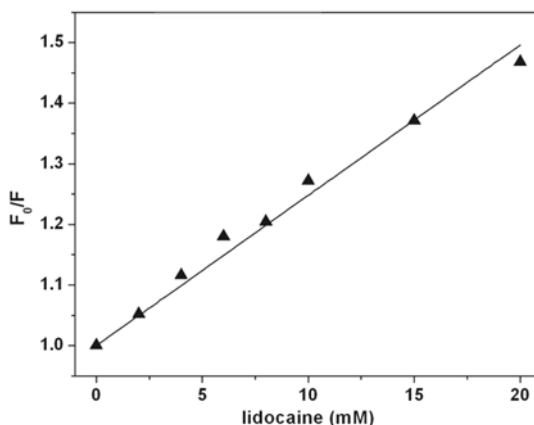

Fig. 3. The Stern-Volmer curve for lidocaine quenching
fluorescence of tryptophan

The calculated bimolecular quenching constant (k_q) is too large to be consistent with collisional quenching (>2x10^{10} $M^{-1}s^{-1}$) indicating that a binding event is the most likely cause of quenching.

Binding constant of lidocaine-HSA molecular complex

The dissociation constant K_d of drug-protein complex was determined from the decrease of Trp fluorescence intensity with the lidocaine concentration (Fig. 4) using the fitting function given by Ec. (2). The determined value was K_d = 1.51 x 10^{-2} M, corresponding to an association constant K_a = 0.66 x 10^2 M^{-1}. The small value of the binding constant reveals a weak lidocaine-HSA interaction, in agreement with equilibrium dialysis data (0.5 – 5 x 10^2 M^{-1}) previously reported [4,5,6].
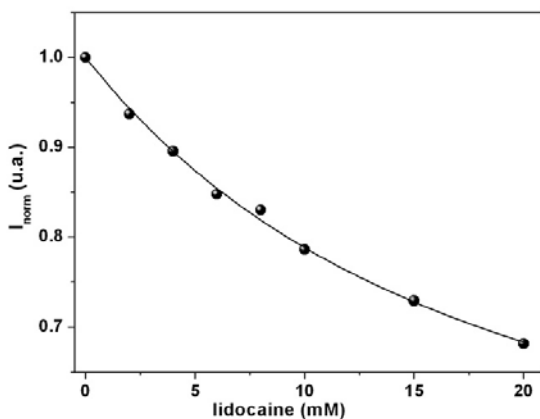
Fig. 4. Fitted curves of Trp fluorescence quenched by lidocaine

Molecular docking analysis of lidocaine – HSA binding

An alternative way to investigate lidocaine - HSA binding is by molecular docking calculations which besides the binding sites give also a qualitative assessment of the molecular interaction in terms of binding energy. The calculated energies of lidocaine - HSA interaction for 100 potential docking locations varies between - 6.7 kcal mol^{-1} (- 0.3 eV) and - 4.6 kcal mol^{-1} (- 0.2 eV). The energy difference between the conformers with the strongest and the smallest docking energy is only 1.61 kcal mol^{-1} (0.07eV). All these interaction energies are very small, indicating no chemical interaction. We may suppose that the lidocaine binds with HSA by weak physisorption, probably due to van der Waals interactions. Moreover, energy difference of about 1.6 kcal mol^{-1} (0.07 eV) between lidocaine conformers indicate that the molecule suffer no major deformations, another hint of relatively weak interaction with HSA.
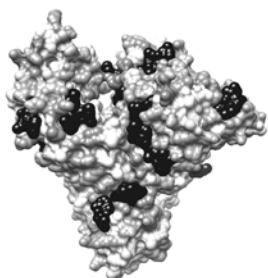

Fig. 5. 3D representation of HSA with binding sites depending on the likelihood of lidocaine docking.
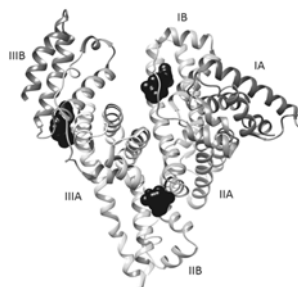

Fig. 6. Preferential binding sites of lidocaine in HSA molecule.

In terms of binding sites, the molecular docking also returns interesting results. We may see in Fig. 5 that lidocaine is able to dock in many region of HSA. It only seems to prefer the hydrophobic cavities.

A closer analysis revealed that lidocaine have three preferential docking locations placed in the segment IIIA, IIA and IB of HSA molecule (Fig. 6). The binding probabilities in each site are 18%, 15 % and 13% respectively. A detailed image is presented in Fig. 7a and b, where we may see the lidocaine molecule and its vicinity in segments IIA and IIIA.
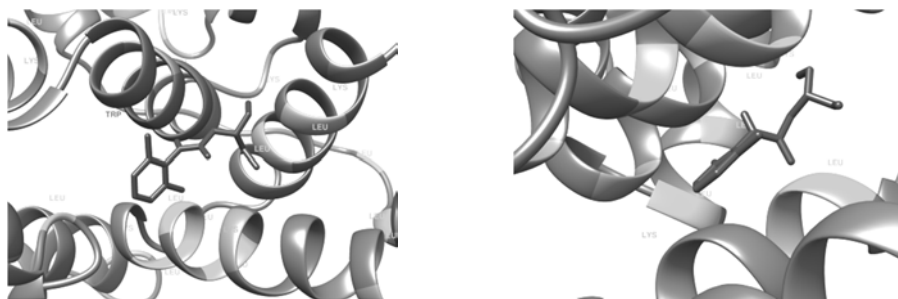

Fig. 7. Detail image of the lidocaine interaction with albumin.

We see that no direct interaction occurs between tryptophan and lidocaine, although they are quite close. In both segments IIA and IIIA the lidocaine is mainly surrounded by leucine and lysine.

CONCLUSIONS

Theoretical investigations based on molecular docking calculations points out three docking sites for lidocaine, located in IIIA, IIA and IB domains of HSA molecule, with highest affinity for the IIIA domain. This first result is in agreement with the three-site model of drug-HSA binding proposed recently in the literature [3]. Besides these particular sites there are many others with a very low binding affinity spread all around the HSA molecule surface. The binding energies obtained by docking calculations are relatively small indicating a weak physisorption mechanism, probably due to van der Waals interactions.

By fluorescence quenching spectroscopy applied on Trp 214 fluorophore we are able to find an association constant $K_a = 0.66 \times 10^2$ M^{-1} for lidocaine - HSA binding. This value characterizes in a quantitative manner the binding affinity of lidocaine to IIA domain which hosts the single tryptophan residue of the HSA molecule. The weak

interaction between lidocaine and the IIA domain of HSA signify that this binding site is only a minor carrier for this drug molecule. Additionally we were able to put into evidence conformational changes in the Trp 214 neighborhood as a result of lidocaine-HSA binding.

Previously reported values for the binding constant, determined from equilibrium dialysis (3.1×10^2 M^{-1}) [5] and homogeneous enzyme immunoassay (5.1×10^2 M^{-1}) [6] are several times higher as compared to the value obtained by us. The most probable explanation is that the above mentioned techniques are global methods taking into account all the binding sites of the HSA molecules.

As a final conclusion we emphasize that for a thoroughly characterization of the lidocaine-HAS association one need several complementary investigation techniques able to capture the complexity of the binding process.

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