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Research Article

Spectroscopic and thermodynamic characterization of the interaction of a new synthesized antitumor drug candidate *2H4MBBH* with human serum albumin

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Abstract

In the present work we studied the interactions of a newly synthesized drug candidate, 2-(2-hydroxy-4-methoxybenzylidene)-1-(1H-benzimidazol-2-yl)hydrazine (*2H4MBBH*), with human serum albumin (HSA) by fluorescence spectroscopy.

2H4MBBH-HSA binding parameters were assessed by fluorescence quenching strategy. As made clear by the concentration data, *2H4MBBH* unequivocally quenched the instrinsic HSA fluorescence. The calculated Stern-Volmer quenching constant Ksv, the Ka of *2H4MBBH*-HSA complexes, as well as the thermodynamic parameters ΔH°, ΔS° and ΔG°, showed that the H-bonding forces play major part in the interaction of *2H4MBBH* with HSA. These calculations point to a quenching component based on *2H4MBBH*-HSA static complex formation rather than energetic collisions.

Keywords

2H4MBBH, drug binding, fluorescence spectroscopy, human serum albumin, quenching

Introduction

The pharmacokinetics and pharmacodynamics of any medication will depend substantially on the interaction that it has with human serum albumin (HSA), the dominant inexhaustible plasma protein. HSA is the prevailing carrier of exogenous and endogenous molecules in human blood plasma. It has high affinity to numerous drugs, facilitating this way their transport by blood circulation (Raoufinia et al. 2016). The nature of the authoritative has coordinate results on sedate conveyance, pharmacokinetics, pharmacodynamics, and helpful adequacy. This work provides new knowledge on the mechanism of *2H4MBBH*-HSA interactions and contributes to a better understanding of the impact of modern orchestrated potential antitumor medications on the properties and basic alterations of the carrier blood plasma proteins. It was found that 1H-benzimidazole-2-yl hydrazones, containing

different fluoro-, hydroxy- and methoxy- substituted benzaldehyde residues (Fig. 1), exhibit remarkable in vitro anthelmintic activity against isolated muscle larvae of T. spiralis as well as potent antiproliferative activity against MCF-7 and AR-230 breast cancer cells (Anichina et al. 2021; Argirova et al. 2021, 2023). The compounds are also able to inhibit tubulin polymerization (Argirova et al. 2021). The 1H-benzimidazol-2-yl hydrazones containing hydroxyphenyl and methoxyphenyl moieties demonstrated potent antioxidant and radical scavenging properties and iron-induced oxidative damage in lecithin and deoxyribose model systems. Density functional theory calculations demonstrated that the 1H-benzimidazole-2-yl hydrazones possesses very versatile radical scavenging properties due to the existence of several reaction sites characterized by relatively low reaction enthalpies and possibility to act simultaneously through different reaction pathways: hydrogen atom transfer in nonpolar medium, sequential proton loss electron transfer in polar medium and radical adduct formation in both polar and nonpolar media (Argirova et al. 2021).

We have chosen to examine here the fluorescence profiles of HSA upon application of the compound *2H4MBBH* and to utilize these profiles for characterization of the *2H4MBBH*-HSA interaction parameters. The approach taken illustrated that there are noteworthy changes of the fluorescence parameters which will serve to assess the restorative effectivity of the syntesized anti-cancer sedate 2-(2-hydroxy-4-methoxybenzylidene)-1-(1H-benzimidazol-2-yl) hydrazine.

Materials and methods

Synthesis

The compound under study – 2-(2-hydroxy-4-methoxybenzylidene)-1-(1H-benzimidazol-2-yl) hydrazine, was synthesized in a four-step reaction pathway (Argirova et al. 2021). o-Phenylenediamine, potassium hydroxide and carbon disulfide were used to obtain benzimidazole-2-thione which was further oxidized by alkaline solution of $KMnO_4$ to afford the corresponding 1H-benzimidazol-2-yl-sulfonic acid. As a next step a nucleophilic substitution of the sulfo group was carried out with hydrazine hydrate and finally the hydrazine derivative was converted in the target compound by condensation with 2-hydroxy-4-methoxy-benzaldehyde. Detailed description of the synthesis and product identification is reported in (Argirova et al. 2023).

Fluorescence spectroscopy

A Scinco 2 South Korea spectrofluorimeter was used to measure fluorescence spectra. The slit widths were 2.5 nm for excitation and 10 nm for emission wavelengths in a 3 mL quartz cuvette with a 10 mm path length. *2H4MBBH*–HSA fluorescence measurements were carried out by keeping the concentration of HSA fixed at 4 μM and those of *2H4MBBH* were 10, 20, 30, 40, and 50 μM. Fluorescence spectra were recorded at two different temperatures of 15 and 25 °C in the spectral emission range 300–500 nm upon excitation at 283 nm (n = 5 replicates).

Results and discussion

Fluorescence spectroscopy

According to the literature, fluorescence spectroscopy is broadly utilized for exploring the interactions between drugs and proteins (Macii and Biver 2020). The inherent fluorescence of serum albumins shows up at 340 nm when excited at 280 nm, which is due to the presence of three aromatic l-amino residues (tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe)). The intrinsic fluorescence of serum albumins is primarily contributed by the Trp and Tyr buildups since phenylalanine has an extremely low quantum yield. The fluorescence characteristics are remarkably sensitive to the microenvironment of the fluorescent residues or changes within the neighborhood environment of serum albumins, such as conformational transitions, biomolecular binding and denaturation (Santhamani and Sambandam 2013).

Fluorescence quenching diminishes the quantum yield of fluorescence from a fluorophore caused by an assortment of atomic interactions, such as ground-state complex formation, excited-state reactions, molecular improvements, energy transfer and collisional quenching. The distinctive instruments of quenching are more often

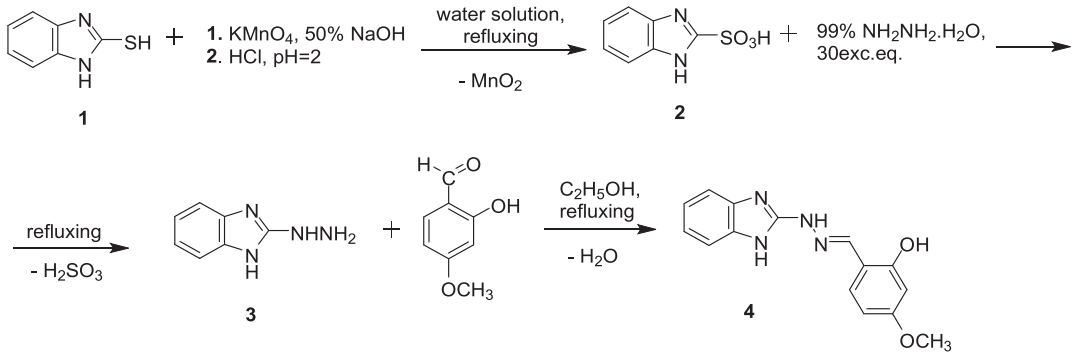

Figure 1. Synthesis of 2-(2-hydroxy-4-methoxybenzylidene)-1-(1H-benzimidazol-2-yl)hydrazine.

than not constructed as either static quenching, or dynamic quenching (Lakowicz 2006).

For the purpose of elimination of the inner-filter effects, a method proposed by Lakowicz is shown in eq 1 (Lakowicz 2006):

$$F_{cor}=F_{obs}\times e^{Aex+Aem2} \quad (1)$$

where F_{cor} and F_{obs} represent the corrected and observed fluorescence intensities, respectively, whereas Aex and Aem denote the absorbance values at excitation and emission wavelengths, respectively. The corrected fluorescence was used for further analysis related to HSA fluorescence quenching.

To characterize further the fluorescence quenching mechanism of the *2H4MBBH*-HSA system, the quenching experiments were conducted at two different temperatures, 15 and 25 °C. The fluorescence spectra of HSA in the presence of *2H4MBBH* at different concentrations are shown in Fig. 2.

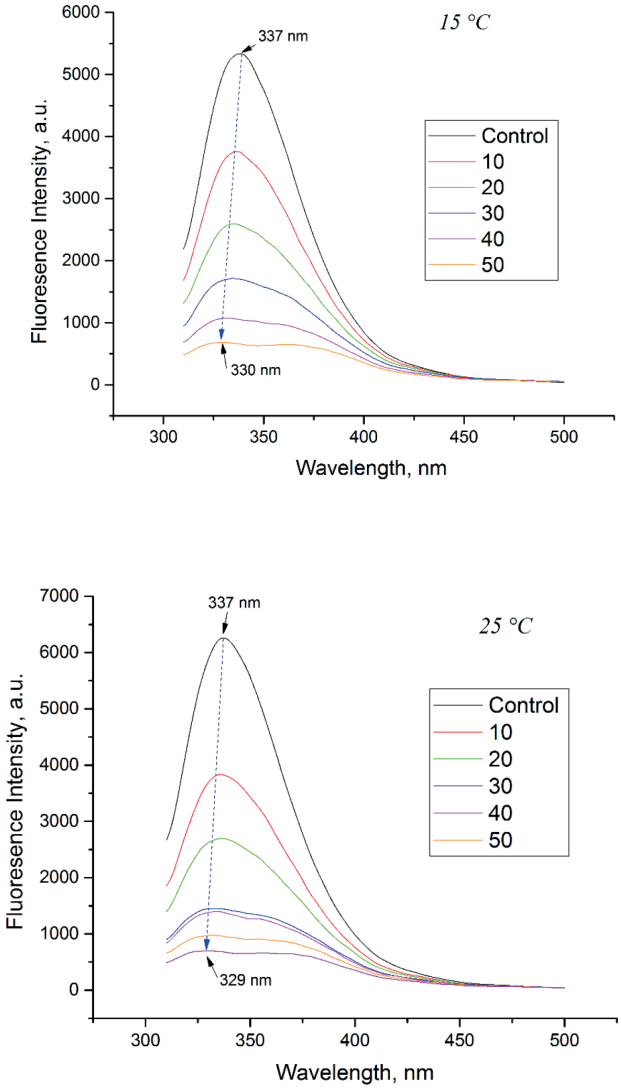

Figure 2. Fluorescence titration curve of HSA (4 μM) with the *2H4MBBH* in saline at 15 °C and 25 °C. Different curves correspond to *2H4MBBH* concentrations of 0, 10, 20, 30, 40, 50 μM, respectively.

Addition of *2H4MBBH* caused quenching of the HSA fluorescence at both temperatures. HSA exhibited a strong fluorescence emission peak at 338 nm upon excitation with wavelength of 283 nm. The fluorescence intensity of HSA showed a significant decrease with a well-expressed shift of the peak toward shorter wavelengths (from 338 to 329 nm), after the addition of *2H4MBBH*. This shows that *2H4MBBH* has apparently associated with HSA and quenched its intrinsic fluorescence, so that the micro-environment of the tryptophan residue in HSA has changed, producing an increment of hydrophobicity within the region of this residue. To resolve the fluorescence quenching mechanism, the well-known Stern-Volmer condition was utilized (Lakowicz 2006):

$$\frac{F_0}{F}=1+K_{SV}[Q]=1+K_q\tau_0[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, Q is the total concentration of the quencher (*2H4MBBH*), and K_{SV} is the Stern-Volmer quenching constant. Kq and τ_0 are quenching rate constant, and the average lifetime for the biomolecule without quencher, respectively. Since the fluorescence lifetime of the biopolymer was assume to be 10^{-8} s (Lakowicz 2006; Lakowicz 1983), the quenching rate constant, Kq can be calculated using the following equation:

$$K_q=\frac{K_{sv}}{\tau_0} \quad (3)$$

The values for K_{SV} and Kq at the two temperatures are given in Table 1.

Table 1. Stern-Volmer quenching constants K_{sv}, and quenching rate constant K_q for HSA complexes with *2H4MBBH*.

Temperature [°C]	K_{SV}, [10^4 M^{-1}]	Kq, [10^{12} $M^{-1}s^{-1}$]
15	7.99	7.99
25	7.57	7.57

It is known that linear Stern-Volmer plots indicate a single type of quenching mechanism as predominant, either static or dynamic (Lakowicz 2006). The intensity changes are given in Stern–Volmer representation in Fig. 3. From the plots of F_0/F vs. Q the type of quenching can be selected, i.e., these plots can either correspond to static or dynamic quenching, or display a upward bend for mixed quenching types (Lakowicz 2006). In order to obtain values for the fluorescent quenching constant we used the Nedler-Mead simplex algorithm to fit a linear regression model to F_0/F and Q for the values of *2H4MBBH* in the range 0–50 μM (Fig. 3).

The quenching constant Kq and the Stern–Volmer constant Ksv at different temperatures are usually used to identify the quenching mechanisms, static or dynamic. The former is caused by ground-state complex formation, the latter is due to the diffusion (Lakowicz 1983). For the static quenching mechanism, the quenching constant

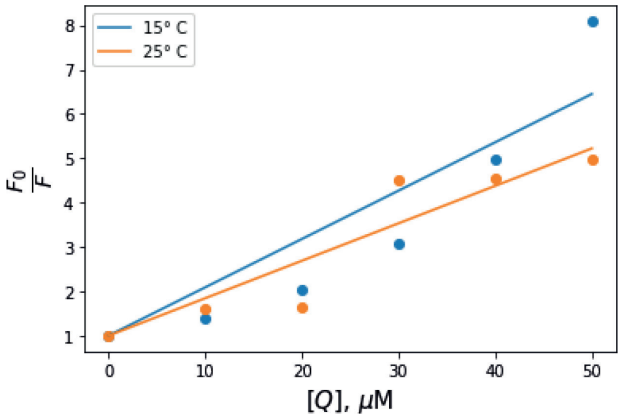

Figure 3. Stern-Volmer plots for quenching of different *2H4MBBH* concentrations (10–50 μM) to HSA (4 μM) in saline at 15 and 25 °C.

decreases with temperature increase (Lakowicz 2006). Moreover, in Table 1, the values of Kq at different temperatures were much higher than the limiting diffusion rate constant of the protein molecules (kd ≈ 2.0 × 1010 M−1·s−1) (Bhat et al. 2010), which revealed static quenching mechanism via forming protein-ligand complexes between *2H4MBBH* and HSA complex.

Binding constant K_a and the number of binding sites

For the static quenching process, the number of binding sites can be obtained by a double-logarithmic equation (Lakowicz 1973):

$$\lg[(F0 - F)/F] = \lg Ka + n\lg[Q] \quad (4)$$

where F0 and F are the fluorescence intensities without and with the ligand, and Ka and n are the binding constant and the number of binding sites, respectively.

Based on this equation, the slope of the log((F0F)/F) two fold logarithmic regression curve versus the log[complex] (Fig. 4) was utilized to assess the number of binding sites (n).

The slope of the lines is the n value. If the value of n is equal to 1, it means that a strong binding exists between the protein and the drug. Our results suggest that inside the temperature range considered, the estimated number n of the *2H4MBBH*-HSA complex is close to 1, demonstrating that HSA contains a single high affinity binding location for *2H4MBBH*.

Ka is calculated to be roughly 10^4, showing strong binding interactions between *2H4MBBH* and HSA (Table 2).

It is additionally found that, as the temperature increases, the Ka value diminishes, suggesting that the stability of

Table 2. Binding constants Ka of *2H4MBBH*–HSA at two different temperatures.

Temperature [°C]	Ka [10^4 M^{-1}]
15	2.71
25	1.37

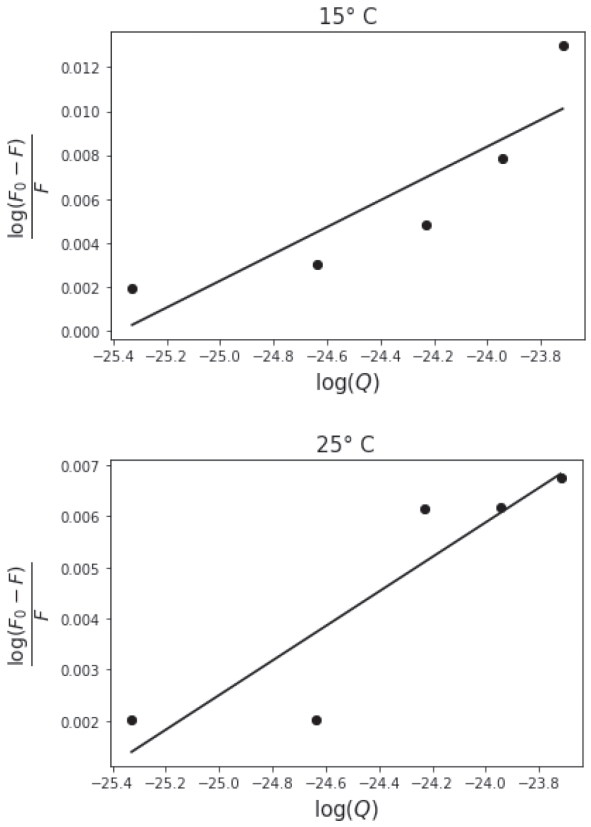

Figure 4. Modified Stern-Volmer plots for *2H4MBBH*-HSA complexes at 15 and 25 °C.

Mab–HSA complex diminishes with temperature increase. The estimate of Ka is critical to evaluate the attraction of the drug to plasma proteins. The binding of *2H4MBBH* to HSA is of significance, because it determines the pharmacological activity of the medication. It is known that protein-binding may modify drug action in two diverse ways: by changing the medication effective plasma concentration at its location of activity, or by changing the rate at which the drug is dispensed with, hence changing the period of time for which viable concentrations are kept up.

Thermodynamic parameters of the HSA-2H4MBBH complex formation

The thermodynamic parameters provide remarkable data when examining the interaction between biomolecules. These parameters provide important data on the leading target interaction due to the high sensitivity of the binding characteristics to intrinsic and external components.

Binding forces involved in the interaction between serum albumin and ligands can be determined by calculating the thermodynamic parameters enthalpy (ΔH°) and entropy (ΔS°). These parameters give rich data on the interaction due to the high sensitivity of the apparent binding characteristics to intrinsic and extrinsic factors.

To better understand the binding between HSA and *2H4MBBH*, the van't Hoff Eq. (4) was used to calculate the thermodynamic enthalpy Δ*H*° and entropy Δ*S*° of the HSA and *2H4MBBH* complex.

$$lnK_a = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{T} \quad (5)$$

where R is the universal gas constant (1.987 cal·K^{-1}·mol^{-1}) and T is the absolute temperature in degrees Kelvin.

To delineate the intermolecular forces existing between *2H4MBBH* and HSA, a thermodynamic system was utilized and assessed at 15 and 25 °C. The standard binding free energy ΔG° is related to the binding constant Ka by the Gibbs relationship:

$$\Delta G° = \Delta H° - T\Delta S° = -RTlnK_a \quad (6)$$

where K_a represent the binding constant at its corresponding temperature and R is the gas constant. Δ*G*° can be determined using van't Hoff plot, where Δ*H*° is the slope and Δ*S*° the intercept.

Our results revealed negative thermodynamic parameters for the enthalpy Δ*H*° = – 48.47 kJ/M and entropy ΔS° = – 237.5 J.M^{-1} of binding, and thus clearly emphasized that the interaction between *2H4MBBH* and serum albumin is exothermic. Concurrent with the Ross theory (Ross and Subramanian 1981), negative values of ΔH° and ΔS° show that the binding may be enthalpy driven, whereas non-covalent attractive forces such as hydrogen holding and van der Waals dispersion forces in low dielectric media and protonation are basically responsible for the formation of a stable complex. Moreover, the binding of *2H4MBBH* with HSA was spontaneous and enthalpy-driven process because |ΔH° |>|TΔS°|. On the other hand, if ΔG°>0, the process is spontaneous at low temperatures. The obtained results demonstrate a spontaneous binding of the drug to the protein molecule, represented by a positive sign of ΔG° = 19.67 kJ M^{-1} and ΔG°= 22.037 kJ M^{-1} at both temperatures, 15 and 25 °C, respectively [13].

Conclusions

The interaction between *2H4MBBH*and human serum albumin, the main blood plasma carrier protein, was studied at two different temperatures, 15 and 25 human serum albumin, the main blo.

Fluorescence quenching results revealed formation of static complexes between *2H4MBBH* and HSA. The binding was determined to be due to non-bonded (van der Waals) and/or hydrogen bonding interactions. The *2H4MBBH* binding to HSA is a spontaneous and enthalpy-driven process. It resulted in significant alterations of the HSA structure and conformation displayed in decreased protein stability and increase of the non-polar or accessible hydrophobic surface of HSA to solvent. This study helps to gain useful theory, into the significance of the binding of a newly syntesized anti-cancer drug with the most abundant plasma carrier protein, serum albumin, on the drug overall distribution and pharmacological activity.

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