Spectroscopic Studies on the Interaction Between Novel Antiviral Drug Favipiravir and Serum Albumins

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Under physiological conditions, *in vitro* interaction between favipiravir (FAV) and serum albumins (BSA/HSA) was investigated at excitation wavelength 280 nm and at different temperatures (298 K, 313 K) by fluorescence emission spectroscopy. The hydrogen bond, van der Waals forces and electrostatic interaction plays a major role in stabilizing the complex; the binding constants K_A at different temperatures were calculated. The distance r between donor (BSA/HSA) and acceptor (FAV) was obtained according to fluorescence resonance energy transfer (1.55/1.90 nm for BSA/HSA-FAV systems). The effect of FAV on the conformation of BSA/HSA was analyzed using synchronous fluorescence spectroscopy and UV/vis absorption spectroscopy.

Introduction

A novel coronavirus, SARS-CoV-2, emerged in December 2019 in Wuhan, China. As the scale of the ongoing COVID-19 outbreak has reached pandemic proportions, significant interest exists in repurposing existing antiviral agents for use against COVID-19 [1]. Favipiravir (FAV) is an oral antiviral approved for the treatment of influenza in Japan. This drug is a purine nucleoside analogue, which acts as a competitive inhibitor of RNA polymerase, which

is necessary for viral replication [2]. It has activity against influenza A and B, Ebola virus and SARS-CoV-2 *in vitro* [3].

Serum albumins are the most abundant protein in plasma, and participate in the binding and transportation of various drugs. Among albumins, bovine serum albumin (BSA) and human serum albumin (HSA) have been widely used as models for studying the interaction of drug with protein *in vitro* [4, 5]. It is a convenient protein for intrinsic fluorescence measurement

due to the presence of tryptophan (Trp) residues which is highly sensitive to its local environment, and can be used to observe changes in the fluorescence emission spectra due to protein conformational changes and binding to substrates [6]. Therefore, study of the interaction between protein and drug molecules will help provide basic information on the pharmacological actions, bio-transformation, bio-distribution of drugs.

There are a number of reports in the literature, where binding of metabolites, drugs, dyes, fatty acids, bio-active-substances have been studied in detail. So the nature of binding of a ligand with HSA (BSA) is different for different ligands [7].

In recent years, a few research groups [8 - 22] reported the interactions between various antiviral drugs and serum albumins by fluorescence, UV-vis spectra, synchronous fluorescence, circular dichroism, and 3D fluorescence spectra.

In the present work, the interaction between FAV and BSA/HAS has been investigated *in vitro* using fluorescence, UV-vis and synchronous fluorescence spectra techniques under physiological conditions. The association constants, the thermodynamic parameters, the number of binding sites, and the energy transfer distance of FAV-BSA/HSA complexes were estimated. At the same time, the effect of FAV on the microenvironment and conformation of proteins was discussed. In addition, the binding

mechanism of FAV with BSA/HSA was explored. These studies will be useful to design and synthesis of FAV derivatives with high antiviral activity.

Experimental part

Materials

Bovine serum albumin (BSA, 98% purity, $M_r = 68000$), and human serum albumin (HSA, 98% purity, $M_r = 66000$) purchased from Sigma-Aldrich, were used without further purification. They were both dissolved in the Tris-HCl buffer solution (pH 7.4) to form a solution of 1×10^{-4} mol L^{-1} and then stored in the dark at 4 °C.

Favipiravir (FAV, 99% purity) was obtained from Cangzhou Wisdom Pharma Co., Ltd (China). FAV stock solution (1×10^{-2} mol L^{-1}) was prepared by dissolving 0.157 g of substance in 100.0 mL of water. The working solution (1×10^{-4} mol L^{-1}) was prepared daily by diluting the stock solution with water.

Scheme 1. The structure of FAV

Tris (hydroxymethyl) aminomethane (purity $\geq 99\%$) and sodium chloride (purity $\geq 99.5\%$) were purchased from Sigma-Aldrich. Tris-HCl buffer solution (0.05 mol L^{-1} , containing 0.15 mol L^{-1} NaCl) was used to maintain the pH of the solutions at 7.40.

All of the used chemicals were of analytical reagent grade and also used without further purification. Doubly distilled water was used throughout the experiment.

Apparatus

Absorption spectra were recorded at room temperature on a UV-2401 PC spectrophotometer (Shimadzu) equipped with 1.0 cm quartz cells.

A Cary Eclipse spectrofluorimeter (Varian) equipped with a 1.0 cm quartz cell was applied to monitor all fluorescence spectra. The excitation wavelength was 280 nm, and the fluorescence emission spectra were recorded in the wavelength range of 285–550 nm. The slit width of $\lambda_{\text{ex}}/\lambda_{\text{em}}$ was set at 5/5 nm for steady-state fluorescence spectroscopy, and synchronous fluorescence spectroscopy. Fluorescence emission spectra were measured at 298 and 313 K.

To obtain different temperatures a waterbath (WNB 14 Memmert GmbH, Germany) was used. The pH values of solutions were measured on Seven Easy pH meter (Mettler Toledo) with a glass electrode.

Procedures

For steady-state quenching studies, the BSA/HSA solution with the fixed concentration of $1 \times 10^{-5} \text{ mol L}^{-1}$ was titrated by successive additions of FAV ($1 \times 10^{-6} \text{ mol L}^{-1}$ – $5 \times 10^{-4} \text{ mol L}^{-1}$) under physiological conditions (pH 7.4) at two different temperature (298, and 313 K). All solutions were mixed thoroughly and kept 5 min before measurements.

FAV has intrinsic fluorescence with a maximum at 430 nm which does not interfere with protein quenching.

The UV-vis absorbance spectra were recorded at room temperature. A fixed concentration of BSA/HSA ($5 \times 10^{-6} \text{ mol L}^{-1}$) with various concentration of the FAV solution (5×10^{-5} ; 1×10^{-4} ; $2 \times 10^{-4} \text{ mol L}^{-1}$), 1 mL Tris-HCl buffer were added and the solutions were diluted to 10 mL with water. The corresponding solution of the FAV was used as the reference solution.

In the present work, the inner-filter effect can be considered negligible due to the very low absorbance value of FAV at the excitation wavelength of BSA/HSA. For example, all figures are for BSA.

Results and discussion

UV-vis absorption spectra experiments

UV-vis absorption measurement is a simple method that is used to investigate structural changes and to explore complex formation [23]. FAV exhibited three peaks at 228 nm, 323 nm, and 363 nm (**Figure 1, a**).

To initially verify the quenching mechanism, the UV absorption spectra of (BSA/HSA) and ((BSA/HSA + FAV)- FAV) were recorded. As shown in **Figure 1, b**, BSA possessed two absorption peaks at 220 nm and 280 nm. The strong absorption peak at around 220 nm reflected the absorption of the backbone of BSA, while the weak absorption peak at around 280 nm resulted from the aromatic amino acids (Trp, Tyr, and Phe). With varying concentration of FAV, a noticeable decrease in the absorbance intensity of the peak at 220 nm coupled with a red shift of the maximum wavelength (about 2 nm) was

observed, which indicated that there was a variation in the framework conformation of BSA due to the formation of FAV-BSA complex. These changes further reconfirmed that static quenching was dominant in the interaction process.

Fluorescence quenching spectra

Any process, which decreases the fluorescence intensity of a sample, is called fluorescence quenching. Fluorescence detection is a highly sensitive method for exploring the change in the fluorophore environment upon quencher interaction, hence resulting of considerable information regarding the binding mechanism.

The interaction of FAV with BSA was evaluated by monitoring the intrinsic fluorescence intensity changes of proteins upon addition of FAV (**Figure 2**). The emission of BSA/HSA was characterized by a broad emission band at 348 nm (BSA) and 330 nm (HSA). BSA/HSA fluorescence intensity decreased remarkably as FAV concentrations increased. FAV produced ~10% quenching in BSA/HSA fluorescence intensity, which was accompanied by a 28 nm blue shift in the emission maximum at the same ligand concentration. The shift in the emission maximum of BSA/HSA towards shorter wavelength suggested increased hydrophobicity in the microenvironment of the protein fluorophores upon interaction with this compound [24]. This indicated that FAV could

interact with BSA/HSA.

Analysis of the emission change of BSA/HSA with various amounts of FAV was carried out with the Stern–Volmer equation:

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

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher, respectively, Q the quencher concentration, and k_q the quenching rate constant for a biomolecular reaction, K_{SV} the Stern–Volmer quenching constant and τ_0 is the average lifetime for fluorophore in the absence of quencher evaluated at 10^{-8} s [25].

Figure 3 shows plots of F_0/F versus FAV concentrations at different temperatures. They exhibit good linear correlations. It is clear that plots in Fig. 3 are approximately consistent with the Stern–Volmer equation, from which the Stern–Volmer quenching constant K_{SV} are derived and are listed in Table 1. A quenching process can be usually induced by a collisional process and/or a formation of a complex between quencher and fluorophore. The values for quenching rate constants in Table 1 are of the magnitude of 10^{12} L mol⁻¹ s⁻¹. Obviously, the quenching rate constant k_q for FAV–BSA/HSA is greater than that of a scatter process.

Figure 1. Absorption spectrum of FAV ($c_{\text{FAV}} = 1 \times 10^{-4} \text{ mol L}^{-1}$) (a); effect of FAV on UV absorption spectra of BSA (b) ($T = 298 \text{ K}$, $\text{pH } 7.4$, $c_{\text{BSA}} = 5 \times 10^{-6} \text{ mol L}^{-1}$, $c_{\text{FAV}} \times 10^{-4} \text{ mol L}^{-1}$, 1-4: 0, 0.5, 1.0, 2.0 (2-4 - difference absorption spectrum between FAV - BSA and FAV).

It may be attributed a quenching process initiated by bioactive substances. So it shows that the above quenching was not initiated by dynamic collision but forms compound, it was static quenching. Furthermore, linear Stern–Volmer plots may either reveal the occurrence of just a binding site for quencher in the proximity of the fluorophore or indicate the existence of a single type of quenching [26].

When small molecules are bound independently to a set of equivalent sites on a macromolecule, the binding constant (K_A) and sites (n) can be derived from the Eq. (2):

$$\log \frac{F_0 - F}{F} = \log K_A + n \log [Q] \quad (2)$$

where F_0 and F are the fluorescence intensity in the absence and presence of bioactive substance, respectively. There is a good linear correlations for the plots obtained by Eq. (2).

Figure 2. Fluorescence emission spectra of BSA in the presence of FAV various amounts ($c_{\text{FAV}} \times 10^{-4} \text{ mol L}^{-1}$, curves (1-13): 0, 0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0, 3.0, 5.0; $\text{pH } 7.4$, $\lambda_{\text{ex}} = 280 \text{ nm}$, $c_{\text{BSA}} = 1 \times 10^{-5} \text{ mol L}^{-1}$, $T = 298 \text{ K}$).

The values for K_A and n derived from according Eq. (2) and listed in Table 2.

They indicate that the amount of FAV will affect the pattern of FAV binding to BSA/HSA. The influence of temperatures on the FAV binding to BSA/HSA is significant. It is obvious that higher temperature will lead to a rapid collision, whereas results in a decrease in the

binding strength. As a result, K_A and n will decrease with raising temperature.

Furthermore, the value for n close to one, we may infer that there exists only one kind of binding site on BSA/HSA for FAV binding.

Binding mode

There are several intermolecular forces causing small drug molecules binding to proteins, such as hydrogen bonding, van der Waals, ionic, electrostatic and hydrophobic interactions, etc.

Figure 3. Stern-Volmer plots for quenching of BSA fluorescence by FAV at different temperatures (pH 7.4, λ_{ex} = 280 nm, $c_{\text{BSA}} = 1 \times 10^{-5} \text{ mol L}^{-1}$; $\blacklozenge$ - 298 K; $\blacksquare$ - 313 K).

Table 1. Stern-Volmer quenching constant K_{SV} , bimolecular quenching rate constant k_q of the BSA/HSA-FAV system at different temperatures, R represents the correlation coefficients of the plots

T(K)	Albumin	$K_{\text{SV}} \times 10^{-4} (\text{L mol}^{-1})$	R	SD	$k_q \times 10^{-12} (\text{L mol}^{-1} \text{s}^{-1})$
298	BSA	2.73	0.9821	0.85	2.728
313		1.76	0.9981	0.18	1.760
298	HSA	5.38	0.9976	0.06	5.384
313		4.94	0.9974	0.06	4.944

Table 2. Binding constant K_A , the number of binding sites n and distance between acceptor and donor r at different temperatures and thermodynamic parameters of FAV-BSA/HSA interaction, R represents the correlation coefficients of the plots

T(K)	Albumin	$K_A \times 10^{-3} (\text{L mol}^{-1})$	n	R	r	$\Delta H^\circ (\text{kJ mol}^{-1})$	$\Delta G^\circ (\text{kJ mol}^{-1})$	$\Delta S^\circ (\text{J mol}^{-1} \text{K}^{-1})$
298	BSA	56.24	1.102	0.9852	1.55	-10.02	-27.10	57.30
313		46.33	1.100	0.9809	1.62		-27.96	
298	HSA	3.61	0.941	0.9916	1.90	-104.59	-20.29	-282.88
313		0.48	0.727	0.9914	1.92		-16.05	

The signs and magnitudes of thermodynamic parameters for protein reactions can be accounted for the main forces contributing to protein stability. The values for the enthalpy change (ΔH°) and entropy change (ΔS°) will be derived from the van't Hoff equation by

considering ΔH° not varying significantly over the experimental temperature range:

$$\ln K_A = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (3)$$

where K_A is the binding constant at a definite temperature T and R is the gas constant. Consequently, the amount of free energy change ΔG° required for the binding is estimated from the following equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (4)$$

The data for ΔH° and ΔS° of FAV binding to BSA/HSA are summarized in Table 2. With respect to recognized literature data on analogous systems [27], the positive ΔS° and negative ΔH° values suggested that there was specific electrostatic interactions between FAV and BSA, the negative values of ΔH° and ΔS° can be attributed in part to van der Waals forces and in part to the formation of hydrogen bonds for HSA-FAV.

Energy transfer from BSA/HSA to FAV

Energy transfer between drugs and proteins usually can be divided into two kinds: radiative energy transfer and non-radiative energy transfer. The latter can be explained and determined by Foster's energy transfer theory - fluorescence resonance energy transfer (FRET).

In this interaction, excitation energy is transferred from one molecule (donor) to another (acceptor) through direct electrodynamic interaction, without emission of a photon from the former molecular system [28]. Energy transfer may occur under the following conditions: when the donor can produce

fluorescent light; when there is an overlap between the fluorescence emission spectrum of the donor and the absorbance spectrum of the acceptor; and when the distance between the donor and the acceptor is less than 8 nm. The efficiency of energy transfer between FAV and the BSA/HSA Trp-residue could be used to evaluate the distance between the two using FRET. The overlap of the absorbance spectrum of FAV with the fluorescence emission spectrum of BSA has been shown in **Figure 4**.

Figure 4. Overlap of the absorption spectrum of FAV (1) with the fluorescence emission spectrum of BSA (2) ($c_{BSA}/c_{FAV} = 1:1$, 1×10^{-4} mol L $^{-1}$; $T = 298$ K; pH 7.4; $\lambda_{ex} = 280$ nm).

According to Forster's non-radiative resonance energy transfer theory [29], energy transfer efficiency E is related not only to the distance (r) between the bound bio-active molecule (acceptor) and the protein residue (donor), but also to the critical energy transfer distance (R_0). By Forster's theory, the efficiency of energy transfer (E) can be calculated according to the following equation:

$$E = 1 - F / F_0 = R_0^6 / (R_0^6 + r^6) \quad (5)$$

where r is the distance between acceptor (FAV) and donor (BSA/HSA), and R_0 is the critical

distance when the transfer efficiency is 50%. The value of R_0 is calculated using the following equation:

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J \quad (6)$$

where K^2 is the spatial orientation factor of the dipole; n is the refractive index of the medium; ϕ is the fluorescence quantum yield of donor; and J is the spectral overlap between the emission spectrum of donor and the absorption spectrum of acceptor (Figure 4), given by:

$$J = \frac{\int_0^\infty F(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda} \quad (7)$$

where $F(\lambda)$ is the normalized fluorescence intensity of the donor at wavelength λ , and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ . In the present case, $K^2 = 2/3$, $n = 1.336$, and $\phi = 0.15$ (BSA), $\phi = 0.118$ (HSA) [30]. Hence, from equations (5)–(7), we could calculate the following parameters are listed in Table 3. In conclusion, the distance (r) between BSA/HSA Trp residues and bound FAV was much less than 8 nm, and accorded with the relationship $0.5R_0 < r < 1.5R_0$. This implied that the non-radiative energy transfer from BSA/HSA to FAV occurred with high possibility, which was in accordance with the occurrence of a static quenching mechanism. This result indicated that the binding obeyed the conditions of Forster's energy transfer. A comparison of the J , R_0 and r values of different ligands bound to proteins is given in Table 3. As can be seen from this table, the values of these parameters are comparable.

Synchronous Fluorescence Spectroscopy

The possible shift of the maximum emission wavelength is related to the change in polarity around the fluorophore molecule. When the scanning interval ($\Delta\lambda$) between the excitation and the emission wavelength is fixed at 15 and 60 nm, this provides the characteristic information on the Tyr and Trp residues of the protein, respectively [31]. The results of the synchronous fluorescence spectra of BSA with increasing concentration of FAV were represented in Fig. 5 (a) and (b). Synchronous fluorescence spectra at $\Delta\lambda = 60$ nm and $\Delta\lambda = 15$ nm show a hypsochromic shift (from 282 nm to 269 nm and from 286 nm to 282 nm, respectively), which indicates the presence of changes in protein conformation near tryptophan and tyrosine residues. However, the pitch of quenching of Trp residues was more obvious than that of Tyr residues, which revealed that FAV was closer to Trp residues compared to Tyr residues.

Conclusions

In this work, the interaction between FAV and serum albumins (BSA/HSA) have been investigated by fluorescence method combined with UV–vis spectroscopy techniques under simulative physiological condition.

The experiment results indicate that FAV quenches the intrinsic fluorescence of proteins through static quenching mode of

Table 3. Comparative assessment of the FAV (ligand) distance to proteins (BSA/HSA) measured by Forster's nonradiative energy transfer with other ligands bound to proteins

Ligand	Protein	$J \text{ (cm}^3 \text{ L mol}^{-1}\text{)}$	$R_0 \text{ (nm)}$	$r \text{ (nm)}$	$K_A \text{ (L} \cdot \text{mol}^{-1}\text{)}$	Ref
amodiaquine	HSA	2.09×10^{-14}	2.68	4.59	1.38×10^3	[8]
darunavir	BSA	4.25×10^{-15}	2.13	3.13	1.26×10^4	[9]
ribavirin	BSA	1.65×10^{-13}	4.26	5.43	1.58×10^4	[10]
ritonavir	BSA	3.25×10^{-15}	2.12	2.91	5.08×10^3	[11]
sofosbuvir	HSA	-	-	-	5.43×10^3	[12]
	HSA	3.51×10^{-16}	1.41	1.04	5.35×10^3	[13]
oseltamivir phosphate (tamiflu)	HSA	-	-	-	3.86×10^3	[14]
telaprevir	HSA	-	-	-	2.56×10^4	[15]
tenofovir	HSA	-	-	-	1.03×10^4	[16]
MIQ	HSA	7.35×10^{-14}	2.83	2.37	2.55×10^4	[17]
zidovudine	HSA	-	-	-	2.74×10^2	[18]
atazanvir	BSA	-	-	-	1.48×10^3	[19]
tilorone	HSA	7.88×10^{-16}	1.67	1.63	7.19×10^4	[20]
dolutegravir sodium	HSA	3.16×10^{-15}	2.03	2.14	9.82×10^3	[21]
daclatasvir dihydrochloride	HSA	1.04×10^{-15}	1.69	1.42	8.13×10^4	[22]
favipiravir	BSA,	7.33×10^{-15}	2.43	1.55	5.62×10^4	this work
	HSA	5.98×10^{-15}	2.25	1.90	3.61×10^3

Figure 5. Synchronous fluorescence spectra of BSA with increasing concentration of FAV ($\Delta\lambda = 60 \text{ nm}$ (a) and $\Delta\lambda = 15 \text{ nm}$ (b); $c_{\text{BSA}} = 1 \times 10^{-5} \text{ mol L}^{-1}$; ($c_{\text{FAV}} \times 10^{-4} \text{ mol L}^{-1}$, curves (1-13): 0, 0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0, 3.0, 5.0).

FAV-BSA/HSA system, which supported the BSA/HSA UV spectral shifts and the decreasing binding constant for FAV and BSA/HSA complex with increasing temperature indicates the decomposition of the system.

The values of binding constant and the number of binding sites of the FAV-BSA/HSA system are determined using the Stern-Volmer equation. The average binding distance between donor and acceptor molecules was found from the Forster non-radioactive resonance energy transfer theory 1.55/1.90 nm for BSA/HSA-FAV systems.

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