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ORIGINAL PAPER

Study of β-lactam-based drug interaction with albumin protein using optical, sensing, and docking methods

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Abstract

The quality and strength of drug and albumin interaction affecting the drug-free concentration and physiological activity are important issues in pharmacokinetic research. In the present study, not only did we evaluate the binding strength of ceftriaxone and ceftizoxime to bovine serum albumin (BSA), but we also investigated the kinetic and thermodynamic parameters including KD, KA, ΔS, and ΔH. We applied in vitro optical fluorescence spectroscopy and surface plasmon resonance (SPR) sensing approaches as well as molecular docking analyses. The kinetic and thermodynamic investigations were done using different concentrations of drugs at three temperatures. Thermodynamic parameters visibly demonstrated that the binding was an exothermic and spontaneous process. The obtained negative values of both enthalpy change (ΔH) and entropy change (ΔS) in fluorescence and SPR and also molecular docking investigations showed that the major binding force involved in the complexation of drugs to BSA was hydrogen bonding. Static quenching was the foremost fluorescence quenching mechanism between them. Furthermore, the results of ΔG and K_D values proved that the interaction of ceftriaxone-BSA was stronger than ceftizoxime-BSA. Finally, molecular docking confirmed that the preferable binding sites of ceftizoxime and ceftriaxone were site IIA and site IB of albumin, respectively.

Keywords Fluorescence spectroscopy · Surface plasmon resonance biosensor · Molecular docking · Albumin-drug interaction

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1 Introduction

Ceftriaxone and ceftizoxime are third-generation hydrophilic cephalosporin antibiotics, active against an extensive range of both gram-positive and gram-negative bacteria and broadly applied to treat bacterial infections [1, 2]. These antibiotic drugs can bind plasma albumin protein as the carrier agent with different affinities [3, 4]. Albumin is the most plentiful protein in plasma, carrying various endogenous and exogenous molecules, and has been used as an ideal protein for investigating drug-protein binding [5]. BSA and HSA are frequently used in biology and biophysical studies since they have an equivalent folding, a well-known primary structure, and they are related to the binding of many different small biomolecules [6]. BSA is one of the most widely examined albumins in the investigations carried out on the drug albumin interaction due to its structural homology with HSA [7].

Generally, drug-protein interaction is an important aspect in pharmacokinetics and toxicological studies and the main goal of preclinical ADME (absorption, distribution, metabolism, and excretion) research [8–10]. In drug-protein interaction, the drugs with lower binding affinity have a short elimination half-life. In addition, protein conformation can change according to the drugs, probably affecting the serum albumin function [11]. Therefore, improving the drug efficacy requires a detailed understanding of drug-protein interaction. There are two forms of drugs circulating in the bloodstream: free ones and those bound to a protein. Although the non-protein-bound antibiotics are pharmacologically active, they bind to plasma proteins decreasing their activity [12]. In recent years, spectroscopic techniques with high sensitivity and high rapidity, such as fluorescence, have been widely used to study kinetics and thermodynamics [13]. Previous studies have shown that antibiotics can quench the intrinsic fluorescence of BSA strongly [14, 15]. Moreover, new methods such as SPR have been utilized to study the interaction of two ingredients [16, 17]. SPR is a label-free and real-time method with a wide range of uses in various fields of science, capable of sensing the changes in the refractive index of surfaces like gold [17]. By sensing the interactive properties between ligand (immobilized fraction on gold chips) and analyte (running sample on ligand surface), any small variation in mass and refractive index above the gold sensor surface can be monitored in a real-time manner by SPR sensor [18, 19]. Furthermore, this technique has been used in the investigation of kinetics binding constants such as association rate constant (K_a), dissociation rate constant (k_d), and affinity (K_D) [20]. The SPR-based methods not only were used in investigating the interaction between small molecules such as antibody-antigen, drug-protein, and DNA-RNA, but also they were applied for the detection of cell markers and cellular behaviors induced by some agents [21, 22]. In the preceding investigations, SPR has been confirmed to be a useful method for the evaluation of antibiotics-serum proteins interaction [23]. Traditionally, albumin-binding properties have been studied using titration calorimetry, fluorescence spectroscopy, liquid chromatography, or circular dichroism, consuming a large volume of samples and possessing a fairly low throughput [24]. So, gathering more information on a drug/albumin interaction with higher resolution and throughput methods is a vital need in drug discovery.

This study aimed at investigating and comparing the kinetic constants, thermodynamic, affinity, and quenching mechanism of the two most common antibiotics with various structures (ceftriaxone and ceftizoxime) to BSA via two experimental methods: fluorescence and real-time SPR and the in silico method of molecular docking. The calculated kinetic and thermodynamic parameters including K_a, K_d, K_D, K_A, affinity, ΔS, ΔH, and ΔG at

different temperatures obtained using these methods were compared, and also the effect of hydrophilic functional groups in the structure of ceftriaxone and ceftizoxime at the binding and kinetic constants was investigated. Moreover, the number of binding sites and the type of binding interaction in the formed drug/albumin complex were investigated.

2 Material and procedures

2.1 Materials

Ceftriaxone ($C_{18}H_{18}N_8O_7S_3$) and ceftizoxime ($C_{13}H_{13}N_5O_5S_2$) were all injection grade. Phosphate buffered saline (PBS) solution, ethanolamine-HCl, BSA, ammonia (NH_4OH, 30%), hydrogen peroxide (H_2O_2, 25%), and all other chemicals and reagents, analytical-reagent grade, were obtained from Sigma-Aldrich (Steinheim, Germany). The bare gold chip was supplied by Bionavis Company (Tampere region, Finland).

2.2 Procedures

2.2.1 Fluorescence quenching spectra of BSA

All fluorescence spectra of drug and BSA interactions were measured by Cytation 5 (BioTek) at three temperatures (304, 310, and 316 K) with different concentrations of ceftriaxone and ceftizoxime (0, 20, 40, 80, 100, 140, and 200 μM). BSA emission was carried out at the range of 300 to 450 nm ($\lambda_{em} = 300–450$ nm), and excitation wavelength was set at 280 nm ($\lambda_{ex} = 280$ nm). BSA and antibiotics stock solutions (1×10^{-2} M) were organized by dissolving their suitable amounts in aqueous solutions containing phosphate (pH 7.4).

2.2.2 SPR measurements

Cleaning the bare gold sensor Before the BSA immobilization step, gold chips were cleaned using a 30 ml boiling solution of 30% ammonia (NH_4OH), 30% hydrogen peroxide (H_2O_2), and distilled water at the ratio of 1:1:5 for 20 min at 100 °C. Then the gold substrates were rinsed with pure ethanol 3 times and dried using a stream of nitrogen.

Immobilization of BSA on a gold sensor surface The immobilization of BSA on the gold sensor surface was performed using a very simple method. First, we washed the sensors and inserted them on the SPR instrument. After 20 min, we reached a steady state of baseline shown by a straight-line form in the sensogram. During the baseline stage, only PBS running buffer flowed onto the bare gold sensor surface. After reaching a steady form, the BSA solution (0.50 mg/ml) in PBS solution at a flow rate of 15 μl/min was injected into the surface for 10 min. After the immobilization of BSA, post wait time for reaching a stable form was set to 10 min. Finally, an ethanolamine-HCl (1.0 M) solution was added for 10 min at a flow rate of 20 μl/min for the closing of the non-specific binding onto the sensor surface.

Kinetic evaluations by SPR K_a and K_d of ceftriaxone and ceftizoxime (as an analyte to BSA) were analyzed using SPR. To decrease the mass transport effect (which makes inaccurate data), BSA protein solution was injected at a low concentration, and the flow rate of the binding test was set at a higher rate of 30 µl/min [25]. Different concentrations of ceftriaxone and ceftizoxime solution (100–1000 µM) were prepared in PBS pH 7.4 and injected into both flow cells at a flow rate of 30 µL/min for 2 min. A control channel was established by using a non-immobilized BSA. The test flow channel was administered using BSA immobilization. BSA immobilization was done only in one flow channel, and the other one was given up without immobilization of BSA used as a reference channel. The regeneration step was not crucial because of the rapid dissociation of both beta-lactam antibiotics from the BSA surface. However, the BSA-modified gold surface was renewed through a 1-min injection of glycine–HCl (10 mM, pH = 2.0) before any temperature investigations of interactions. We used Trace Drawer TM for SPR NaviTM and SPR Navi™ Data viewer software to calculate the kinetic factors upon the interaction of BSA protein with antibiotics including evaluation of the association (k_a) and dissociation (k_d) rate constants, affinity, and the equilibrium rate constants (K_D) based on one to one fit model.

Thermodynamic analysis The effect of temperature on the ceftriaxone and ceftizoxime binding to BSA protein was monitored via obtaining the thermodynamic constants of complex structures. For this purpose, SPR analyses were carried out at three different temperatures (304 K, 310 K, 316 K), and drugs were injected into both flow cells at a flow rate of 30 µL/min for 2 min.

2.2.3 Molecular docking

In this study, molecular docking simulation between ceftizoxime and ceftriaxone with BSA was performed by Auto Dock Vina software. We used Auto Dock Vina for docking, while the calculation speed and precision were optimized via using a new scoring function in comparison to a classic AutoDock [26]. The docking simulation was performed to find the non-covalent interaction, calculate binding energy value, and recognize the appropriate binding site of ceftizoxime and ceftriaxone in the BSA structure. At first, the chemical structures of ceftizoxime and ceftriaxone were drawn by Chem3D-Ultra software. Afterward, we optimized the molecular geometry of the drugs' structure using Gaussian 09 software (Quantum Chemistry package). Specifically, we employed the density functional theory (DFT) method with Becke's three-parameter hybrid functional (B3LYP) through the 3-21G basis set [27]. The X-ray crystallography structure of BSA (PDB ID code 4F5S, 2.47 Å resolution) was selected for docking simulation. The additional compounds in the PDB structure like waters and hetero atoms were omitted. The PDB format files of drugs and BSA changed to PDBQT format by adding the polar hydrogens, Gasteiger charge, and Kollman charge in Auto Dock Tools 1.5.6. The PDBQT format files of ligands and BSA were inserted in grid boxes for two drugs separately. For scanning the whole surface of the BSA structure and finding the appropriate binding sites of drugs in BSA, we first used the blind docking method [28]. We performed the focus docking procedure in the defined sites after selecting the sufficient binding sites of ceftizoxime and ceftriaxone in the BSA structure using the blind docking process. In the focus docking method, the grid box's dimension size at grid points in x*y*z directions were set into 22.5*22.5*22.5 $Å^3$ with a default grid spacing value (1 Å) in Auto Dock Vina.

3 Results and discussion

3.1 Fluorescence analyses

3.1.1 Fluorescence quenching of BSA induced by ceftriaxone and ceftizoxime

Fluorescence quenching is an effective method, widely used to study the mechanism of ligand–protein interactions [29]. A protein's intrinsic fluorescence mainly originates from the tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues, and it decreases in the presence of ligand. This reduction depends on ligand concentration [30]. There are two important tryptophan residues (Trp134 and Trp213) in the BSA structure, responsible for its intrinsic fluorescence properties. Trp134 is placed in the surface of subdomain IB, and its environment is hydrophilic, while Trp213 is deeply buried in the hydrophobic binding site of subdomain IIA (Sudlow's site I) [31]. Fluorescence spectra changes during the addition of drugs provide suitable information about the micro-environment of the intrinsic fluorophores (especially tryptophan residues) [32]. However, the quenching of fluorescence spectra and their exchanges were used as a beneficial tool for understanding the interaction between antibiotics studied with BSA in this study. From Fig. 1a, b, the addition of various concentrations of ceftriaxone and ceftizoxime to the constant concentration of BSA led to the remarkable decrease in the FI (fluorescence intensity) of BSA. The maximum fluorescence wavelength of BSA in the presence of ceftriaxone shifted from 343 to 340 nm and also shifted from 343 to 339 nm after adding ceftizoxime to the solvent. Therefore, the slight blue shift of the BSA maximum emission wavelength was detected in both of the studied systems. It could be concluded that the fluorophore residue of protein was probably placed in a more hydrophobic environment after the addition of ceftriaxone and ceftizoxime [33]. Therefore, Trp213 was maybe a fluorophore residue of BSA influenced by ceftriaxone and ceftizoxime during the interaction.

The quenching mechanisms were categorized as static quenching (complex formation) and dynamic quenching (collisional processes) or combined static and dynamic quenching determined by their response to temperature. The quenching mechanism of the BSA-drug was analyzed using the Stern–Volmer equation [34], and the results were presented in Table 1.

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \tag{1}$$

where F_0 and F correspond to the FI of BSA in absence and presence quencher (ceftriaxone, ceftizoxime), respectively. K_{sv}, [Q], K_q, and τ_0 represent the Stern–Volmer constant, antibiotics total concentration, quenching rate factors, and the average lifetime of the fluorophore in the absence of the quencher (for BSA is 10^{-8} s), respectively. K_{sv} was determined from the slopes of Stern–Volmer plot (F_0/F vs. [Q]) (Fig. 1 and b). K_q was calculated by Eq. (2) [35].

$$K_{sv} = K_q \tau_0 \tag{2}$$

As shown in Fig. 1, the slope of the Stern–Volmer plots decreased with the increase in temperature, meaning that static quenching happened for both of the antibiotic-BSA interactions [36]. From Table 1, the K_{sv} (in both antibiotics) value was inversely

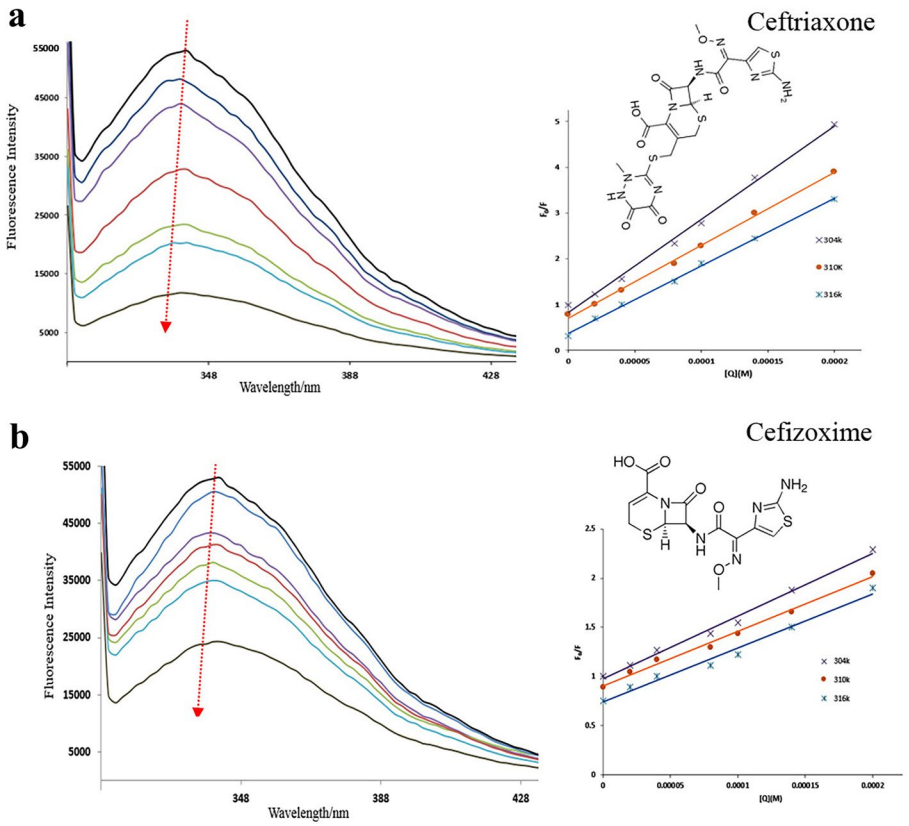

Fig. 1 Fluorescence spectra of BSA (1×10^{-2} M) in the presence of different concentrations of drug 1–7 (0, 20, 40, 80, 100, 140, and 200 uM). **a** Ceftriaxone, **b** ceftizoxime (insets: Stern–Volmer plots of drug-BSA complexes at 304, 310, and 316 K)

correlated with temperature, confirming previous studies about the quenching mechanism of drug-BSA interactions [3, 14, 37].

3.1.2 Determination of interaction constants and sites

The affinity of drugs and BSA protein binding is expressed by the binding constant (K_A). When a drug (like ceftriaxone or ceftizoxime) binds to a protein, K_A and the number of binding sites (n) for a drug-protein system can be calculated using the logarithmic relationship of Eq. (3) [34]:

$$log(F_0 - F/F) = logKA + nlog[Q] \tag{3}$$

where F_0 and F represent fluorescence intensities of the BSA in the absence and presence of quencher (ceftriaxone, ceftizoxime). K_A is the binding constant, and n is the number of binding sites. Besides, [Q] is defined as a quencher concentration (drug concentration). The values 304 K, 310 K, and 316 K were calculated from the intercept and slope of log [(F0eF)/F] vs. log [Q] plot, and the results are shown in Table 1. As seen, the binding

Table 1 The binding and thermodynamic parameters of the ceftizoxime, ceftriaxone, and BSA interaction

Temperature		K_{SV} ($mol\ L^{-1}$)	K_q ($mol\ L^{-1}\ s^{-1}$)	K_A ($mol\ L^{-1}$)	n	ΔH° ($kJ\ mol^{-1}$)	ΔS° ($kJ\ mol^{-1}\ K^{-1}$)	ΔG° ($kJ\ mol^{-1}$)	r
	304 K	6.08×10^{3}	6.075×10^{11}	1.48×10^{4}	1.14	−151.38	−0.415	−24.72	0.994
Ceftizoxim	**310 K**	5.33×10^{3}	5.334×10^{11}	7.56×10^{3}	1.09	−151.38	−0.415	−22.59	0.981
	316 K	4.82×10^{3}	4.818×10^{11}	1.65×10^{3}	0.891	−151.38	−0.415	−19.45	0.976
	304 K	16.84×10^{3}	16.844×10^{11}	58.3×10^{4}	1.45	−258.78	−0.739	−33.52	0.992
Ceftriaxone	**310 K**	15.58×10^{3}	15.584×10^{11}	6.7×10^{4}	1.19	−258.78	−0.739	−28.62	0.981
	316 K	14.90×10^{3}	14.898×10^{11}	1.3×10^{4}	1.00	−258.78	−0.739	−25.02	0.987

constant of ceftriaxone-BSA and ceftizoxime-BSA decreased with the rising temperature, related to the reduction of the drug-BSA complex stability at higher temperatures.

Furthermore, the results showed that the interaction of ceftriaxone-BSA is stronger than that of ceftizoxime-BSA because K_A of ceftriaxone-BSA (58.3×10^4) was near 40 times larger than that of ceftizoxime-BSA (1.48×10^4) at 304 K. In other words, the affinity of ceftriaxone to BSA was greater than that of ceftizoxime to BSA. The values of *n* for BSA were approximately equal to one, indicating that there was one binding site for ceftriaxone and ceftizoxime per BSA molecule.

3.1.3 Estimation of thermodynamic parameters

Not only do non-covalent forces such as hydrogen bonds and hydrophobic forces play main roles in drug-protein interactions, but also electrostatic and van der Waals forces have a significant role in the bidding processes. The type of forces between antibiotics and BSA was obtained from the thermodynamic parameters calculated by Van't Hoff plot and the Gibbs free energy equation (Eqs. 4 and 5) [38]. The results are summarized in Table 1.

$$logK_A = \frac{-\Delta H}{2.30RT} + \frac{\Delta S}{2.30R} \tag{4}$$

$$\Delta G = -RTLnK_A \tag{5}$$

where K_A is the binding constant, T is the temperature, and R is the gas constant (8.314 JK^{-1} mol^{-1}) [38]. The positive rate of ΔH and ΔS shows that the hydrophobic forces play main roles in the interaction of two molecules. In addition, the negative values of ΔH and ΔS are characteristics of hydrogen and van der Waals bonding interactions [39]. Furthermore, a negative value of ΔG is evidence of a spontaneous binding process. Usually, hydrogenic forces are very strong than other weak intramolecular interactions like hydrophobic and Van der Waals forces and have a dominant role in the stability of the BSA-drug complex [40]. The results shown in Table 1 indicated that the value of ΔH and ΔS was negative. Therefore, the interaction between ceftriaxone and ceftizoxime with BSA protein occurred through hydrogenic bond processes and Van der Waals forces, and they had a dominant role in the stability of the BSA-drug complex. Moreover, the greater number of hydrogen bond acceptors and donors in ceftriaxone could lead to more tight interaction with BSA in comparison to ceftizoxime.

3.2 SPR analysis

3.2.1 Albumin protein immobilization

In Fig. 2, a schematic illustration of BSA attachment using a simple coupling method is shown. In our previous SPR studies, we typically applied carboxylic-modified gold chips including carboxymethyl dextran hydrogel (CMD) or mercaptoundecanoic acid (MUA) modified chips for the immobilization of proteins after EDC/NHS activation [41, 42]. However, in the present study, we used the dative bond (a coordinate covalent bond) formation between the 6S empty orbital in Au atoms and free electron pairs of amine groups in albumin protein [43]. The dative bond energy is the same as the energy for any other covalent bond with the same two elements. Therefore, as shown in Fig. 2b, this type of immobilization using dative bond formation was strong enough for the observation of real-time SPR kinetics due to having a stable response unit (RU) in sensogram

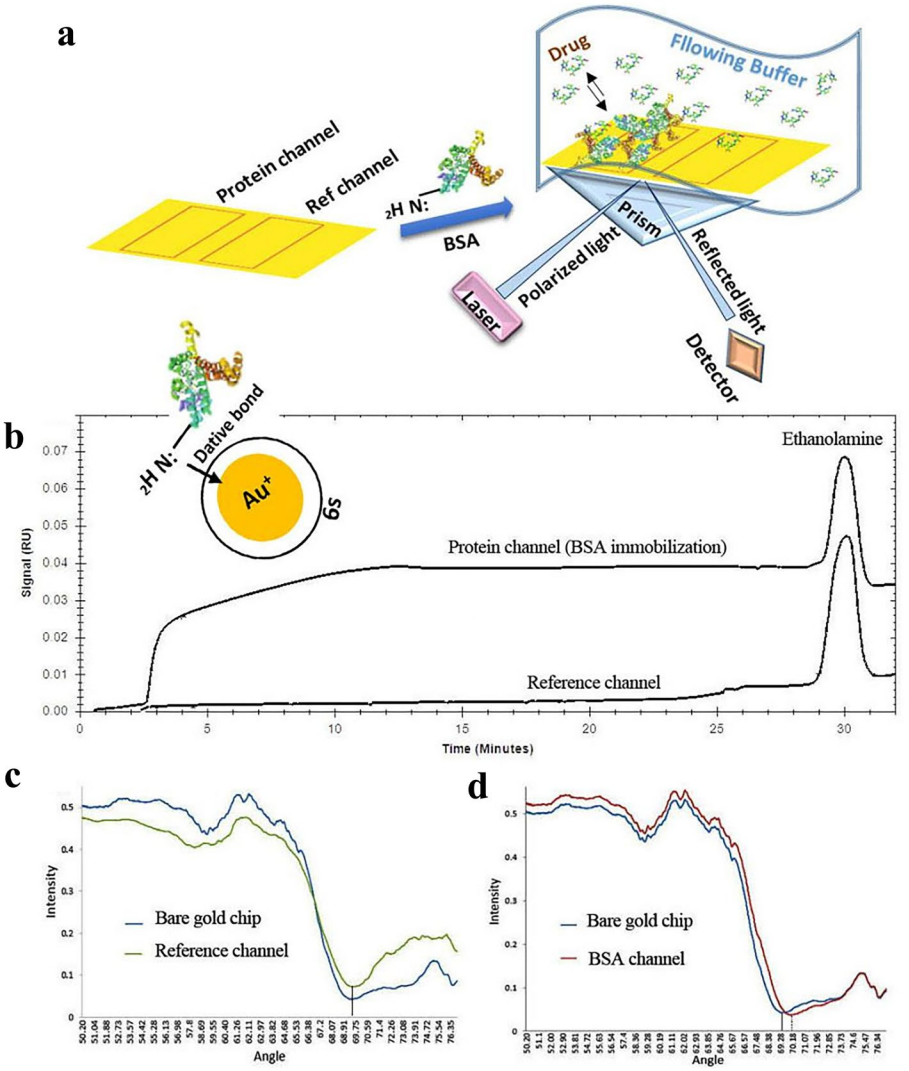

Fig. 2 **a** Schematic illustration of BSA immobilization on SPR gold sensor surface. **b** SPR curve of BSA attachment on a gold sensor surface. **c** SPR curve before BSA injection. **d** SPR curve after 0.50 mg/ml BSA injection

during 30 min. After BSA immobilization on the gold sensor surface, 350×10^{-4} RU was obtained (Fig. 2b). When the Au sensor slide surface was modified by a monolayer of molecules, due to the adding thickness on the gold surface, the resonance curves moved to higher incident angles and became wider. The SPR curve displays the steepest falling slope location during binding of analytes like drugs to the immobilized ligand surface and shift of total internal reflection (TIR) [44]. The shifted angle of SPR curve before and after BSA protein attachment from 69.28 to 70.18° shown in Fig. 2c, d completed the results obtained from the immobilization sensogram of Fig. 2b.

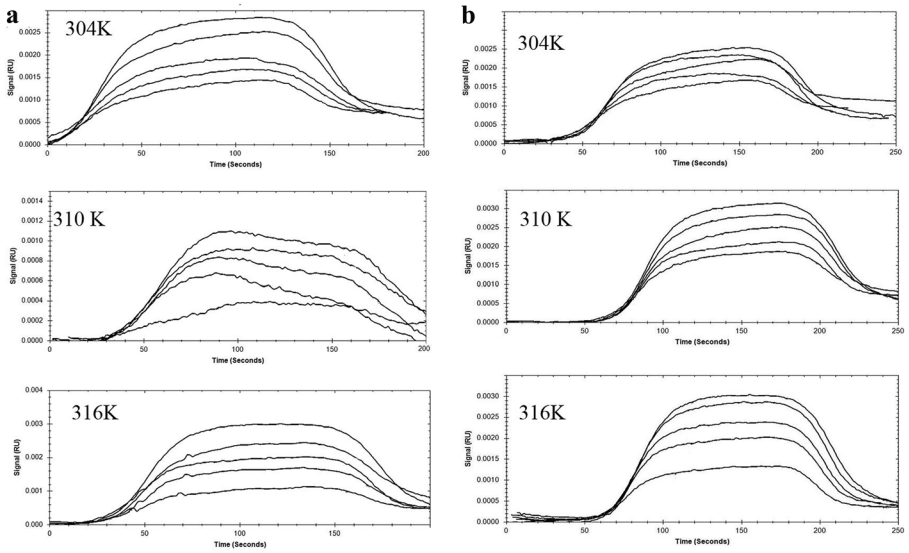

Fig. 3 The SPR curve of binding between BSA and different concentrations of **a** ceftizoxime (0.5–1.5 mM) and **b** ceftriaxone (0.1–1 mM) at three temperatures

3.2.2 Kinetic parameters

K_a (K association) and K_d (K dissociation) are the numbers of ligand-macromolecule complex (drug-BSA) formed per second and degenerated per second, respectively. The analysis of association and dissociation phases gives kinetic data while studying the steady-state levels as a function of drug concentration provides affinity values. K_a, K_d, and K_D or K_A (the equilibrium constants, defined as $K_D = K_d/K_a$ or $K_A = K_a/K_d$), were calculated for the binding of the drug to BSA as well as affinity to obtain the kinetic features [45]. Figure 3 depicts SPR sensograms of the ceftizoxime-BSA and ceftriaxone-BSA interactions and the reference subtraction at 3 temperatures (304 K, 310 K, 316 K), respectively. Kinetic parameters were obtained at three temperatures and are listed in Table 2. These results indicated that with an increase in the temperature, the reaction rate between drugs and BSA decreased. The K_D values for ceftizoxime-BSA and ceftriaxone-BSA decreased upon raising the temperature, confirming the decreasing affinity between drugs and BSA. Affinity

Table 2 Association rate (k_a), dissociation rate (k_d), and equilibrium constant (K_D) and thermodynamic values of ceftriaxone and ceftizoxime with BSA

Drug	Tem (K)	k_a (1/M × s)	k_d (1/s)	K_D (M)	Affinity	ΔH (kJ mol^{-1})	ΔS (J mol^{-1})
Ceftriaxone	304	4.94×10^{1}	8.86×10^{-3}	1.46×10^{-4}	1.91×10^{-4}	−13.319	−116.21
	310	1.23×10^{2}	2.05×10^{-2}	1.66×10^{-4}	1.01×10^{-4}	−13.319	−116.21
	316	9.8×10^{1}	1.43×10^{-2}	1.79×10^{-4}	6.53×10^{-5}	−13.319	−116.21
Ceftizoxime	304	1.02×102	9.98×10^{-2}	8.32×10^{-4}	3.61×10^{-3}	−10.092	−80
	310	1.02×102	9.42×10^{-2}	9.23×10^{-4}	3.29×10^{-3}	−10.092	−80
	316	1.00×102	8.32×10^{-2}	9.78×10^{-4}	2.52×10^{-3}	−10.092	−80

determines how many drug-albumin complexes are formed at equilibrium when the association is balanced with dissociation. Meanwhile, the smaller K_D is attributed to the higher affinity of drug and albumin interaction. The comparison of the K_D values of ceftriaxone-BSA (1.66×10^{-4}) and ceftizoxime-BSA (9.23×10^{-4}) at 310 K showed that the complex between the ceftriaxone-BSA was stronger than ceftizoxime-BSA, in line with the results obtained from fluorescence spectroscopy.

3.2.3 Thermodynamic parameter analyses

The interaction between BSA protein, as a ligand, and small drug molecules, as an analyte, usually consists of the following acting forces: electrostatic forces, hydrophobic interactions, hydrogen bonds, and Van der Waals forces. The thermodynamic values of interactions defined by enthalpy change (ΔH) and entropy change (ΔS) are important in the evaluation of the reaction feasibility at a defined temperature.

These parameters can be calculated through the Van't Hoff equation

$$LnkD = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{6}$$

where R and T are the universal gas constant and temperature, respectively. ΔH and ΔS are both negative in the equation of ΔH-TΔS, ΔH is negative, and TΔS is negative; so, -TΔS is positive. As temperature increases, -TΔS will be positive and will eventually outweigh the effect of ΔH. Besides, the positive rate of ΔH and ΔS confirms that the hydrophobic forces play main roles in the interaction of two molecules. The results shown in Table 2 indicated that the value of ΔH and ΔS was negative. Therefore, the interaction between ceftriaxone and ceftizoxime with BSA protein occurred through hydrogenic bond processes and Van der Waals forces.

3.3 Molecular docking result

The lowest binding energy of ceftizoxime and ceftriaxone to BSA calculated during the molecular docking simulation in this study were −7.6 Kcal mol^{-1} (−31.82 KJ mol^{-1}) and −9.0 Kcal mol^{-1} (−37.68 KJ mol^{-1}), respectively. In addition, the results showed that the binding energy value of ceftriaxone-BSA complex was lower than that of ceftizoxime-BSA complex, and the results of the fluorescence study confirmed it too ($\Delta G_{\text{Ceftizoxime -BSA}} = -22.59$ and $\Delta G_{\text{Ceftriaxone-BSA}} = -28.62$ at 310 K). In addition, according to the calculation of binding constant values by Gibbs free energy equation from Eq. (5) ($\Delta G = -RTLnK_A$), the binding constant value was measured for two systems by this equation in the 310 °K. The K_A values calculated by Eq. (5) in the ceftizoxime-BSA system and ceftriaxone-BSA system were 3.78×10^5 M^{-1} and 2.23×10^6 M^{-1}, respectively. The obtained results indicated the strong interaction of ceftriaxone with BSA rather than with ceftizoxime, confirming our obtained experimental results from fluorescence and SPR analyses (7.56×10^3 M^{-1} and 6.70×10^4 M^{-1}, in the ceftizoxime-BSA and ceftriaxone-BSA systems, respectively).

The lowest binding energy conformer of interacted drugs with BSA is presented in Fig. 4a. Accordingly, the preferable binding sites of ceftizoxime and ceftriaxone resulted in docking study were site IIA and site IB of albumin, respectively. This result was similar to the result obtained from the previous work done by Carter et al. investigating the binding site in HSA with drugs [46]. In the 4F5S PDB code, the resolution and R-value were 2.47 Å and 0.259, respectively. The calculated distances of the

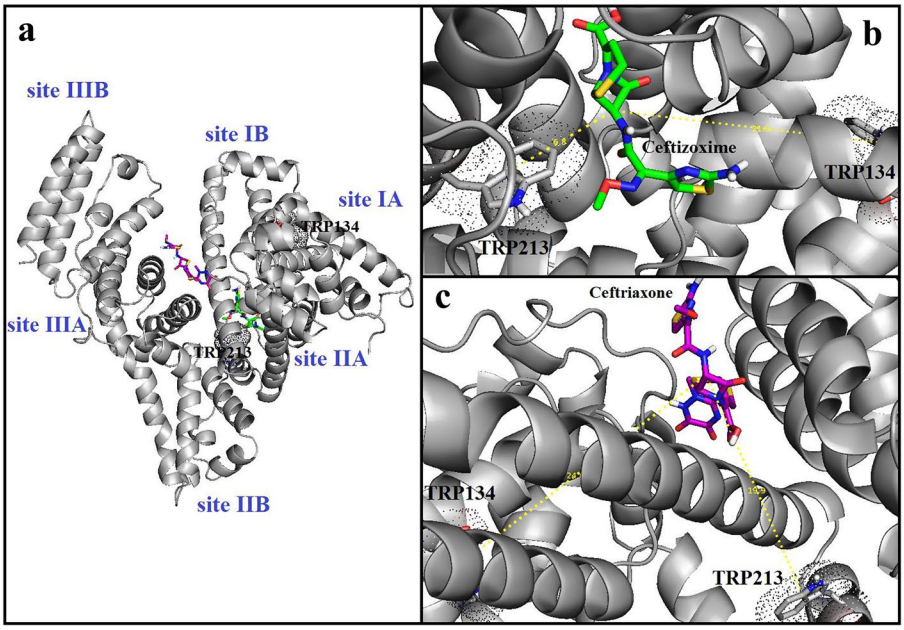

Fig. 4 Ceftizoxime and ceftriaxone interactions with BSA at the lowest binding energy conformer of docking. The calculated distance between fluorophore residues (TRP134 and TRP213) with drugs indicated by yellow dashed lines

best-docked conformer of ceftizoxime with Trp134 (embedded in site IB) and Trp213 (embedded in site IIA) as a fluorophore residue were 21.9 Å and 6.80 Å, respectively (Fig. 4b), while for ceftriaxone, they were 24.5 Å and 19.9 Å, respectively (Fig. 4c). The distances calculated from these conformers of ceftizoxime and ceftriaxone by docking indicated that drug distances with Trp213 were less than Trp134. Fluorescence quenching results showed a slight blue shift of BSA emission spectra in interaction with antibiotics. This suggested that a more hydrophobic environment was the place of fluorophore residue (Trp213). Therefore, molecular docking and the fluorescence quenching findings indicated that drugs were placed close to Trp213 in the hydrophobic binding site IIA. However, ceftriaxone was three times farther away from Trp213 in comparison to ceftizoxime. Obtained distances for both drugs from these amino acids were less than 80 Å (8 nm). For quenching protein fluorescence emission, the mentioned distance was adequate in the presence of ligand. Also, the obtained docking simulation results were confirmed by calculated energy transfer distance (r) between the donor (BSA) and the acceptor drugs via the Förster resonance energy transfer (FRET) method in this study (see Supplementary File) [47].

We examined the residues involved in the interaction between BSA and ceftizoxime and ceftriaxone in all conformations. Ceftizoxime interacted with the following residues: Tyr149, Glu152, Tyr156, Lys187, Thr190, Ser191, Arg194, Arg198, Trp213, Arg217, Leu218, Lys221, Phe222, Leu237, Val240, Arg256, Leu259, Ile263, His287, Ile289, Ala290, and Glu291. In addition, ceftriaxone and BSA interactions were analyzed for all conformations of docking. The residues participating in this binding

included the following: Lys114, Leu115, Ile141, Arg144, His145, Arg185, Leu189, Thr190, Ser192, Ala193, Pro420, Val423, Glu424, Arg427, Ser428, Lys431, Arg435, Tyr451, Leu454, Ile455, and Arg458.

Hydrogen bonds formed among BSA amino acids and drugs are indicated in Fig. 5. Polar amino acids like arginine are the main amino acids forming hydrogen bonds with both drugs. Furthermore, in the lowest energy conformer of docking conformers, ceftizoxime and ceftriaxone had hydrophobic interaction with Glu152, Ser191, Arg194, Arg198, Trp213, Arg217, Lys221, Leu237, His241, Leu259, Ile263, His287, Ile289, Ala290 residues, and Leu189, Ala193, Arg196, Glu424, Ser428, Lys431, Arg458, Ile 522 residues, respectively, presented by red color spoked arcs in Fig. 5a, b. Another type of non-covalent interaction, π–π interaction, was not seen between amino acids and drugs in our docking conformers. DruLiTo open-source software and VEGA ZZ software were used to extract and calculate the molecular properties of ceftizoxime and ceftriaxone interactions with BSA [48]. We calculated the number of hydrogen bond acceptors, hydrogen bond donors, and the drug's volume. In addition to van der Waals/surface, accessible solvent area (VdW/SAS) and the polar surface area (PSA) are represented in Fig. 5.

According to docking analyses, the binding ability of ceftriaxone to BSA was more than ceftizoxime. The greater number of hydrogen bond acceptors and donors caused the ceftriaxone to interact tightly with BSA rather than ceftizoxime. Furthermore, a greater number of hydrogen bonds formed by ceftriaxone led to a decrease in the ceftriaxone-BSA

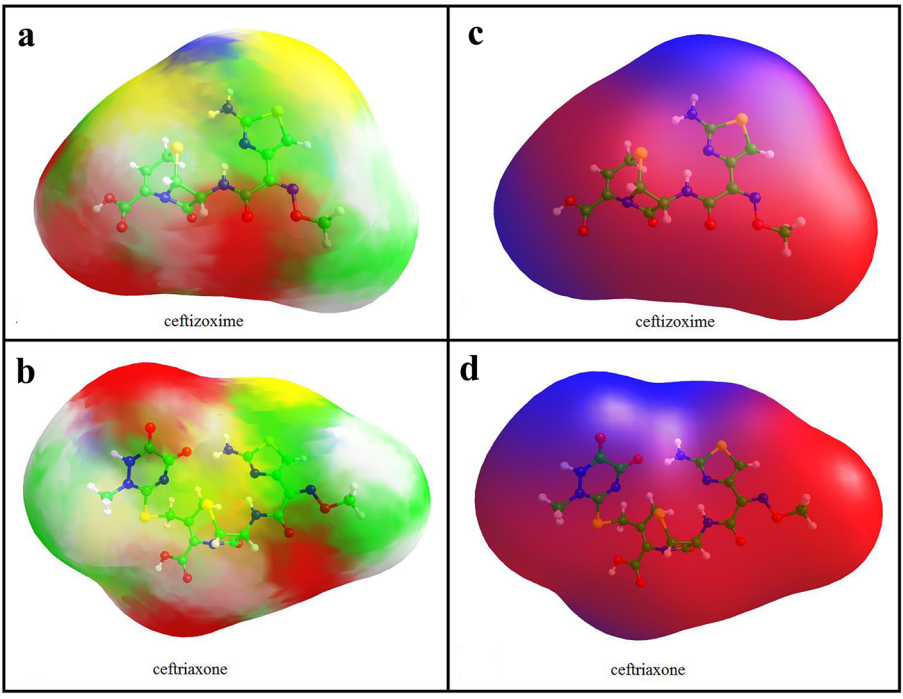

Fig. 5 The ball and stick models of ceftizoxime and ceftriaxone drugs embedded in their transparent space-filling models. The atoms are colored according to type with O red, N blue, S yellow, C green, and H white. The surface of the van der Waals area in **a**, **b**. The polar surface area (PSA) of both drugs in **c**, **d** (the polar surface area is presented by red color and a polar surface area presented by blue color)

complex energy, increasing the sustainability of this complex. Therefore, the binding energy value of this system was lower than the ceftizoxime-BSA complex. Additionally, the hydrogen bond effect in interaction, the large volume, and a bigger surface of VdW/SAS as well as polar surface areas were other factors directly influencing the interaction. The experimental results of this study indicated that the type of interaction was van der Waals, and both hydrogen and ceftriaxone binding to BSA were more stable than ceftizoxime. Different functional groups such as amine, amide, ketone, and carboxylic acid in drug structure facilitated the formation of hydrogen bonds with BSA residues. The molecular docking simulation results and calculated molecular properties of drugs confirmed our obtained experimental results.

3.4 Comparison of calculated kinetic values using various methods

In the SPR method, the comparison of K_D values in ceftriaxone-BSA (1.79×10^{-4}) and ceftizoxime-BSA (9.78×10^{-4}) at 316 K showed that the complex between the ceftriaxone-BSA was stronger than ceftizoxime-BSA, in line with the results obtained from fluorescence spectroscopy (ceftriaxone-BSA: 0.14×10^{-4}, ceftizoxime-BSA: 1.3×10^{-4}). The results obtained from the spectrofluorometry, and SPR biosensor showed that the K_D value of drugs binding to BSA increased with increasing temperature in SPR analyses, while the related K_A value in fluorescence decreased. Considering that the K_D value equals $1/K_A$, the obtained value in SPR and fluorescence were compatible. In the work done by Pan et al. the interaction of ceftriaxone sodium with the BSA was investigated using fluorescence spectra of Hitachi F-4500 fluorescence spectrophotometer equipped with a 1-cm quartz cell [37]. The obtained data for K_D, ΔH, ΔS, and ΔG were similar to those obtained in this work; however, they were different in terms of numerical values, related to different spectrophotometers used in fluorescence investigation. Besides, the negative rate of ΔH and ΔS in both SPR and fluorescence confirmed the hydrogenic bond processes and Van der Waals forces in the interaction between ceftriaxone and ceftizoxime with BSA protein. In addition, in silico molecular docking study showed that the binding energy value of ceftriaxone-BSA complex was lower than ceftizoxime-BSA complex, confirmed by fluorescence in vitro study ($\Delta G_{\text{Ceftriaxone-BSA}} = -28.62$ and $\Delta G_{\text{Ceftizoxime-BSA}} = -22.59$ at 310 k). The comparison of obtained kinetic values using various methods is summarized in Table 3.

Table 3 Comparison of obtained kinetics values using various methods

Method	$K_D = 1/K_A$ (M)	ΔH° (kJ mol^{-1})	ΔS° (kJ mol^{-1} K^{-1})	ΔG° (kJ mol^{-1})	Ref
Fluorescence* (301 K)	Cfr: 3×10^{-4}	−30.59	−0.052	−20.13	[45]
Fluorescence (310 K)**	Cfr: 0.14×10^{-4} Cfz: 1.3×10^{-4}	Cfr: − 258.78 Cfz: − 151.38	Cfr: − 0.739 Cfz: − 0.415	Cfr: − 28.62 Cfz: − 22.59	This work
SPR (310 K)	Cfr: 1.66×10^{-4} Cfz: 9.23×10^{-4}	Cfr: − 13.319 Cfz: − 10.092	Cfr: − 116.21 Cfz: − 80	-	This work
Molecular docking (310 K)	Cfr: 0.44×10^{-6} Cfz: 2.6×10^{-6}	-	-	Cfr: − 37.68 Cfz: − 31.82	This work

*Using Hitachi F-4500 fluorescence spectrophotometer with a 1-cm quartz cell; **Using Cytation 5 (BioTek) with 96-well plate; *Cfr* ceftriaxone, *Cfz* ceftizoxime

4 Conclusion

In the present study, we investigated BSA interaction with ceftriaxone and ceftizoxime by applying SPR and fluorescence spectral in vitro methods and also in silico molecular docking analysis for the first time. K_D value of drugs binding to BSA increased with increasing temperature in SPR analyses and the related K_A value (K_A value equals $1/K_D$) in fluorescence decreased. Static quenching was the notable fluorescence quenching mechanism between them. The calculated ΔH and ΔS thermodynamic parameters in SPR and fluorescence methods, which were negative, indicated that the main forces between ceftriaxone and ceftizoxime with BSA were most probably hydrogen bonds and van der Waals forces. The drug and BSA binding contribution were found to be characterized by an exothermic process ($\Delta H < 0$), and the thermodynamic possibility of the interaction was completed by an overall negative free energy change ($\Delta G < 0$), indicative of spontaneous and enthalpy-driven binding processes. In addition, molecular docking denoted that the preferable binding sites of ceftizoxime and ceftriaxone were site IIA and site IB of albumin, respectively. Besides, the comparison of absolute value for ΔG ($\Delta G_{\text{Ceftriaxone-BSA}} > \Delta G_{\text{Ceftizoxime-BSA}}$) showed that the interaction of ceftriaxone-BSA was stronger than ceftizoxime-BSA, approved by K_D value calculated using SPR and fluorescence methods. All these experimental and theoretical results can deliver valuable information to know the mechanistic way of drug delivery and to pharmacological behavior of β-lactam-based drugs.

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Author contribution F Fathi studied conception and design, performed SPR experiments, prepared draft manuscript, and co-wrote the paper; H Monirinasab performed spectral experiments and co-wrote the paper; M Zakariazadeh performed docking analyses; H Kohestani analyzed data and co-wrote the paper; and Morteza Kouhestani analyzed and interpreted the results.

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Availability of data and material The data that support the findings of this study are available on request from the corresponding author.

Code availability Trace Drawer TM for SPR NaviTM and Auto Dock Vina.

Declarations

Conflict of interest The authors declare no competing interests.

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