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Characterization of the binding of a novel antitumor drug ibrutinib with human serum albumin: Insights from spectroscopic, calorimetric and docking studies

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ABSTRACT

Ibrutinib (IBR) is a novel Bruton's tyrosine kinase inhibitor and shows good efficacy for several B-cell malignancies. In the current study, the molecular mechanism of the interaction between IBR and the transport protein human serum albumin (HSA) was ascertained by spectroscopic, calorimetric, and docking studies. Detailed investigations on affinity parameter, binding model, conformational change, and site selectivity were implemented by receptor-based and ligand-based analysis. An unusual fluorescence co-quenching (mutual quenching) was observed in the binding of IBR to HSA, followed by a static mechanism. Fluorescence spectroscopy and isothermal titration calorimetry indicated that the binding affinity was at $10^4\,M^{-1}$ level and electrostatic interactions and hydrophobic forces contributed the interaction. UV–vis and 3D fluorescence spectroscopy suggested the conformational changes of HSA after binding with IBR. Fourier transform infrared and circular dichroism spectroscopy further verified the variation in the secondary structure of HSA. Site-markers competition and molecular docking confirmed that IBR preferably binds to HSA at the cysteine-rich region of Sudlow's site I (subdomain IIA). This study systematically clarified the binding process of the novel antitumor drug with the functional biomacromolecule for the first time. The findings are helpful for IBR pharmacological assessment and can provide valuable reference for other tinib-type drugs.

1. Introduction

Ibrutinib (IBR, Fig. 1) is a novel inhibitor of Bruton's tyrosine kinase (BTK) that has demonstrated considerable efficacy in several B-cell malignancies [1]. This compound binds covalently to Cys-481 on BTK, leading to an inhibition on kinase activity and prevention of malignant B-cell proliferation [2]. The FDA firstly approved IBR as a breakthrough therapy for treating mantle cell lymphoma in 2013 and for the treatment of chronic lymphocytic leukemia in 2014 [3]. IBR also shows efficacy in patients with Waldenström's macroglobulinemia [4]. IBR has a high plasma protein binding rate of 97.3% which strongly affects drug distribution and pharmacokinetic behavior [5], indicating that IBR exhibits relatively strong affinity toward proteins in the plasma. Nevertheless, the binding mechanism at molecular level is still unclear.

In the blood circulatory system, most drugs reversibly bind to plasma proteins and are transported in the plasma by the formation of protein–drug complex. Human serum albumin (HSA) is the most predominant carrier protein in the plasma (up to 60%) and the most extensively studied biomacromolecule because of its significance in the pharmacological field. HSA is comprised of 585 amino acid residues, which organizes to form a heart-shaped protein. HSA contains three structurally similar α-helical domains (I–III) and each domain can be divided into subdomains A and B [6]. Most of the drugs bind to HSA at two major active regions, *i.e.* subdomains IIA and IIIA, which are also named as Sudlow's sites I and II [7]. A wide range of endogenous and exogenous molecules, such as fatty acids, nutrients, steroids, metal ions, hormones, and drugs, can bind with HSA, and the HSA–drug interaction may vitally affect the pharmacokinetics and pharmacodynamics of drugs including bioavailability, distribution, metabolism, excretion, stability, and toxicity [8,9]. Therefore, studies on the HSA–drug interaction will aid in the interpretation of the metabolism and transport mechanism of drugs.

Tinib-series drugs are the fastest growing antitumor drugs in the world today and greatly attract researcher's attention due to the prominent efficacies. In recent years, the interactions of many tinib-series drugs, such as axitinib [10], crizotinib [11], gefitinib [12–14], imatinib [15], lapatinib [13,16], nilotinib [17], sunitinib [13,18], and tofacitinib [19] with serum albumin (human or bovine) have been probed by various methods and showed a certain difference in affinity level (10^3~$10^5\,M^{-1}$). So far, there was no specific study on the binding of

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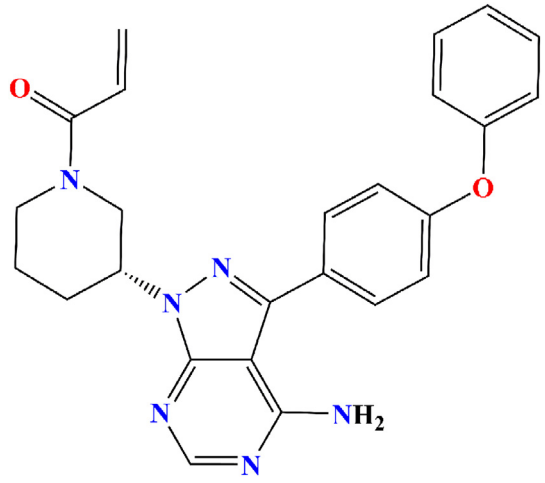

Fig. 1. Chemical structure of ibrutinib (IBR).

the newly approved IBR to serum albumin. Lack of albumin-binding information may impede the development of this promising drug such as the design of new dosage forms [16,20]. Therefore, it is necessary and urgent to explore the HSA–IBR interaction to provide more reference for pharmacological assessment of the drug.

This work is designed to determine the interaction between IBR and HSA to elucidate the molecular mechanism of the binding process. Particularly, combination of receptor-based (HSA) and ligand-based (IBR) analysis was used to ascertain the affinity and protein conformational changes. Multi-spectroscopic methods including fluorescence, ultraviolet-visible (UV–vis), Fourier transform infrared (FT-IR), and circular dichroism (CD) spectroscopy offer a clear insight into the HSA–IBR interaction. Isothermal titration calorimetry (ITC) directly gives specific thermodynamic information. Computational docking further examines the binding process. This study investigates the binding of IBR to HSA for the first time, and is beneficial for understanding the transport of BTK inhibitors and the pharmacokinetics of tinib-type drugs.

2. Materials and Methods

2.1. Reagents and Chemicals

Fatty-acid-free HSA (A1887-5G) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was directly used without further purification. A stock solution with 20 μM HSA was prepared with 0.01 M phosphate-buffered saline (PBS: 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl) at pH 7.4. Different protein–ligand interactions were determined using a fresh albumin stock solution prepared on the same day.

IBR (99.5%) was purchased from Meilun Biotechnology (Dalian, China), and was identified by the Analysis and Testing Center of Sichuan University. Phenylbutazone (99.0%), ibuprofen (98.5%), and lidocaine (99.2%) were purchased from J&K Chemicals (Beijing, China). These small molecules were dissolved in 2 mL methanol, and diluted to 5 mL with PBS to obtain 1.0 mM stock solutions. Dansyl-sarcosine (99.0%) was obtained from Heowns Biochemical Technology Co., Ltd. (Tianjin, China), and dissolved in ethanol to final concentration of 2.0 mM. Triple-distilled water was used in all the experiments. Other unlisted regents were of analytical grade and purchased from Kelong Reagents (Chengdu, China).

2.2. Fluorescence Measurements

All fluorescence experiments were performed on a Cary Eclipse Fluorescence Spectrophotometer (Varian, CA, USA). Steady-state fluorescence spectra (300–550 nm) were scanned by setting the excitation wavelength (λ_{ex}) to 280 nm and slit widths to 2.5/10 nm (excitation/emission). Concentrations of both HSA and IBR samples increased from 0 μM to 8 μM. The observed fluorescence were further corrected to avoid the influence of inner filter effect by using the following equation [21].

$$F_{cor} = F_{obs} \times \exp\left(\frac{A_{ex} + A_{em}}{2}\right) \quad (1)$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence signal, respectively; A_{ex} and A_{em} are the absorbance of the system at the excitation and emission wavelengths, respectively. All fluorescence intensities used in this study were the corrected values.

3D fluorescence spectra of free IBR and HSA of 8 μM and their mixture at 8:3 molar ratio were obtained with λ_{ex} ranging from 200 nm to 400 nm with an increment of 10 nm. Emission spectra between 200 and 600 nm were successively recorded.

Site-marker displacement experiments were conducted by adding the probes, phenylbutazone, ibuprofen, and lidocaine, into the mixture of HSA (8 μM) with IBR (8 μM). Probe concentrations increased from 0 μM to 28 μM. Other parameters were the same as steady-state fluorescence tests. The effect of dansyl-sarcosine was estimated by measuring the fluorescence of dansyl-sarcosine at λ_{ex} = 350 nm. In the tested samples, the concentrations of HSA and dansyl-sarcosine were kept at 2 and 20 μM, and the concentration of IBR increased from 0 to 30 μM.

Time-resolved fluorescence spectra were obtained using a FluoroLog TCSPC spectrofluorometer (Horiba Scientific, France). In HSA-based tests, λ_{ex} and λ_{em} were set at 280 nm and 345 nm; In IBR-based tests, λ_{ex} and λ_{em} were set at 290 and 420 nm. Free IBR and HSA at 2 μM and their mixture at 1:5 molar ratio were measured in sequence.

2.3. Isothermal Titration Calorimetry

The heat changes during the titration of IBR into HSA were recorded on a MicroCal iTC200 system (Malvern Instruments, UK) at 298 K. Solutions of HSA and IBR were prepared by dissolving them in a specialized PBS at 0.01 M, containing 5% v/v dimethyl sulfoxide (IBR has a poor solubility in water). The sample cell was filled with freshly prepared HSA solution at 0.02 mM. IBR solution of 0.36 mM was introduced into the syringe and then titrated into the sample cell with 20 individual injections of 2 μL per injection. Other operational parameters were set as follows: injection spacing of 120 s, duration of each injection at 4 s, and syringe rotation rate of 750 rpm. The measured data was processed and analyzed by Origin software (version 7.0) and the first data point was removed and OneSites was selected as the fitting model.

2.4. Ultraviolet-visible Absorption Spectroscopy

Absorption spectra of samples with different protein or ligand content were performed using a TU1901 spectrophotometer (Purkinje General, Beijing, China). In IBR-based tests, the drug was kept at 4 μM and HSA increased from 0 μM to 4 μM. In HSA-based tests, on the other hand, the protein was kept at 4 μM and IBR increased from 0 μM to 20 μM. A series of UV–vis spectra of 200–320 nm were recorded with 0.01 M PBS as the reference solution.

2.5. Fourier Transform Infrared Spectroscopy

FT-IR measurements were performed on a Nicolet 6700 FT-IR spectrometer (ThermoScientific, WI, USA) and a specialized accessory named Smart OMNI-Sampler was used for scanning liquid samples. The broad spectra (500–4000 cm^{-1}) were collected with a resolution of 4 cm^{-1} and scanning times of 256. Four samples including PBS (0.01 M), free HSA (0.1 mM), free IBR (0.1 mM) and the mixture of HSA and IBR (1:1 molar ratio), were successively scanned. HSA differential