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Interaction of inosine with human serum albumin as determined by NMR relaxation data and fluorescence methodology

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ABSTRACT

Understanding the binding mechanisms of drug–protein interactions is a crucial factor in determining the pharmacokinetics and pharmacodynamics of a drug.

In this study, the binding affinity of inosine toward the HSA, as well as the dynamic properties of HSA-inosine complexes was obtained from applying NMR methodology. The changes in proton selective and non-selective relaxation rates suggested that inosine interacts with HSA. The values of normalized affinity indexes and equilibrium constant *K* provided information about the binding strength, which were in accordance with fluorescence result. The detailed binding mechanisms were obtained through fluorescence method, and the binding mode was displayed using Discovery Studio. Circular dichroism data further confirmed the secondary structure changes in HSA upon inosine addition. This study provided insights on the interactions between inosine and HSA.

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1. Introduction

Studies on the binding mechanism of drugs to plasma proteins are the primary interests in therapeutic drug monitoring because most drugs are transported as complexes with plasma proteins in the circulatory system [1]. Understanding the mechanisms and related parameters of interaction between drugs and proteins is helpful in elucidating the transport and distribution of drugs in the body and further clarifying the action mechanism, pharmacokinetics, and drug toxicity [2].

Inosine (Fig. 1), a nucleoside formed by hypoxanthine and ribose, is an important bioactive compound involved in energy metabolism and protein synthesis of body in vivo. Inosine has a potential function in increasing the level of 2,3 diphosphoglycerate in red blood cells, which results in the easy release of oxygen from the blood cells to the tissues, thereby improving overall energy production and exercise performance [3]. Therefore, athletes are usually supplemented with inosine because of its numerous functions, such as improving endurance and performance and enhancing ATP production. Inosine also induces axon outgrowth from primary neurons in culture through a direct intracellular mechanism [4,5], which resulted in the improvement of various heart conditions by promoting the generation of ATP, the energy substance that enables muscles to contract [6,7]. Given that inosine base pairs with cytosine, inosine is read as guanine by most cellular processes. Therefore, adenosine-to-inosine (A-to-I) editing can create a codon for a different amino acid, a stop codon, or even a new splice-site that contributes to the diversification of protein created from a single gene [8,9]. This work mainly aims to explore the binding behavior of such a biologically important molecule with protein in the simulative in vivo environment.

Human serum albumin (HSA) was selected as the model of plasma proteins because it is the most abundant protein in plasma and possesses a well-known primary structure. HSA is a monomeric protein that contains 585 amino acids with three homologous α-helical domains (I to III), each with two subdomains (A and B) [10]. The two primary drug-binding sites in HSA are located in the subdomains IIA and IIIA, which respond to sites I and II, respectively [11]. Numerous drugs and other small bioactive molecules bind to albumin reversibly, which implicates the role of HSA as carriers [12].

Nuclear magnetic resonance (NMR) spectroscopy is a well-known technique that has been widely used for studying the interactions between small molecules and macromolecules. The proton spin relaxation rate of the small molecule has been proved to provide useful information about the dynamic properties and binding affinity of ligand–macromolecule complex. In this article, to study the interaction of inosine with HSA, the NMR methodology based on the comparison of selective (R^{se}) and non-selective (R^{ns}) proton spin-lattice relaxation rate of inosine alone and after HSA addition was performed. The normalized affinity index, $[A_i^N]_L^T$ and the complex equilibrium constant *K* were calculated from the NMR data, providing key information about the interaction process. Complementarily, as fluorescence is a sensitive method, it was applied to investigate the binding mechanism thoroughly and obtain detailed binding parameters. The results were in line with the NMR analysis. Competitive experiments showed that inosine mainly binds to site I, thus molecular docking, as a powerful computer-aided drug design approach, was chosen to conduct further

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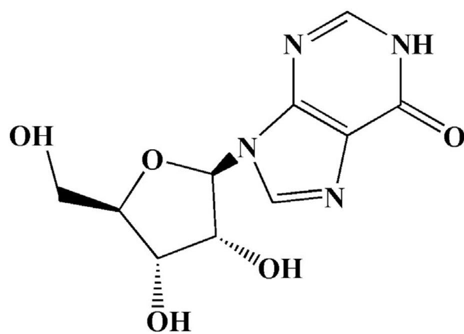

Fig. 1. Molecular structure of inosine.

in-depth study on this binding. Furthermore, to analyze whether the secondary structure of HSA changed after binding with inosine, circular dichroism (CD) spectroscopy was employed.

2. Materials and methods

2.1. Reagents and chemicals

Essential fatty acid free HSA was purchased from Sigma-Aldrich (Milwaukee, USA) and used without further purification. Deuterium oxide with 99.9% purity was provided by Cambridge Isotope Lab (Germany). Inosine, warfarin, and ibuprofen were obtained from Aladdin Chemical Reagent (Shanghai, China). All other reagents and chemicals were of molecular and analytical grade. Ultrapure water was purified using the Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. NMR measurements

The solutions used for the NMR experiments were obtained by dissolving appropriate amounts of inosine and HSA in D_2O and 50 mM phosphate buffer (pH 7.4), respectively. All data were acquired on a Bruker Avance 400 MHz spectrometer operating at 400.13 MHz for hydrogen at 298 K. Bruker Software Topspin (version 2.1) was employed to process all spectra.

The spin–lattice relaxation rates were measured using the $(180° - \tau - 90° - t)_n$ sequence. The τ values applied for the selective and non-selective experiments were 0.0001, 0.01, 0.1, 0.2, 0.3, 0.5, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 10 s. All spin–lattice relaxation rates, including selective and non-selective, refer to the H-4 and H-11 protons of inosine. The delay time t in this case is 10 s. The 180° selective inversion of the proton spin population was obtained by a selective soft Gaussian perturbation pulse (width: 20 ms, power: 60 dB) corresponding to an excitation width of approximately 45 Hz. In all experiments, the ligand inosine concentration was constant at 5.0 mM. The HSA concentrations used in calculating the affinity index were 0, 5, 10, 15, 20 and 25 μM. The enhancements in observed selective spin–lattice relaxation rates can be directly related to the formation of the HSA–ligand complex, given that the addition of HSA did not change the viscosity of the system as reported previously [13]. The affinity index was calculated by linear regression analysis of the experimental data.

In addition, the concentration of HSA used in calculating the equilibrium constant K was 25 μM constantly. The ligand inosine concentrations were varied from 3.0 mM to 15 mM. The NMR data were recorded under the same experimental conditions described above.

2.3. Fluorescence spectroscopy

The stock solution of HSA was prepared at 2.0×10^{-5} mol·L^{-1} concentration in phosphate buffer at pH 7.4. Inosine was dissolved in ultrapure water to obtain a 2.0×10^{-3} mol·L^{-1} stock solution. Warfarin and ibuprofen were dissolved in anhydrous ethanol to obtain 2.0×10^{-4} mol·L^{-1} stock solutions. All fluorescence measurements were recorded on a Cary Eclipse fluorescence spectrophotometer (Varian, USA) equipped with 1.0-cm quartz cells. An appropriate volume of inosine solution was added to HSA (2.0×10^{-6} mol·L^{-1}) that ranged from 0 mol·L^{-1} to 4.8×10^{-5} mol·L^{-1} and mixed for 10 min at room temperature. The fluorescence spectra from 200 nm to 500 nm were scanned using 5/10 nm (excitation/emission) slit widths at 298, 305, and 310 K. The excitation wavelength was fixed at 280 nm.

Site marker competition experiments were conducted with the use of warfarin, which binds to site I, and ibuprofen, which primarily binds to site II. The HSA–inosine mixture solution was added to volumetric flasks, and the ratio of inosine to HSA was maintained at 4:1 (C_{HSA}: 2.0×10^{-6} mol·L^{-1}). After 1 h of incubation of the mixtures, warfarin and ibuprofen were gradually added to the binary systems separately and then incubated for another 1 h. The fluorescence spectra were recorded under the same experimental conditions described above.

Moreover, the fluorescence intensities were corrected for the absorption of excited light and the reabsorption of emitted light to decrease the inner filter based on the formula described in the literature during monitoring of UV–vis and fluorescence spectra. UV spectra were obtained using a TU-1901 UV–vis spectrophotometer (Persee, China).

2.4. Molecular modeling

The available crystal structures of HSA (PDB ID: 2BXD and 2BXG) were obtained from Protein Data Bank for docking simulations. A 3D form of inosine was generated with ChemBioOffice 2010. Molecular modeling of HSA-inosine interaction was performed using the CDOCKER docking program, implemented in DS 3.1 (State Key Laboratory of Biotherapy, Sichuan University, China). The receptor (HSA) and ligand (inosine) were pretreated before docking simulation. For receptor preparation, Chain B was removed from 2BXD and 2BXG. Missing bond orders, hybridization states, charges, and angles were assigned to the protein structure. Explicit hydrogen was added at a desired pH of 7.40 before docking [14]. For ligand preparation, the 3D structure of inosine was optimized with DS 3.1 and subjected to CHARMm force field [15]. Subsequently, the docking was performed, and the best results with the lowest docking energy were used for further analysis.

2.5. Circular dichroism method

Circular dichroism (CD) spectra were obtained on a CD spectrometer (Model 400; AVIV, USA) in a 2-mm quartz cell from 250 nm to 200 nm at room temperature. Dry nitrogen gas was used to purge the machine before and during the measurements. The band width was 1 nm, and an average time of 0.5 s was used. The samples for CD were prepared with a fixed concentration of HSA (2.0×10^{-6} mol·L^{-1}), whereas complex concentration varied from 0 mol·L^{-1} to 2.0×10^{-5} mol·L^{-1} [ri = (inosine)/(HSA) = 0:1, 10:1].

3. Results and discussion

3.1. NMR analysis

The relaxation rate investigation implemented in the NMR methodology is a powerful approach to investigate the binding affinity of the ligand to the receptor and the dynamic properties of ligand–protein complexes. NMR is based on the comparison between non-selective (R^{ns}) and selective (R^{se}) spin–lattice relaxation rates in the presence

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