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Binding of an anticancer drug, axitinib to human serum albumin: Fluorescence quenching and molecular docking study

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ABSTRACT

Binding characteristics of a promising anticancer drug, axitinib (AXT) to human serum albumin (HSA), the major transport protein in human blood circulation, were studied using fluorescence, UV–vis absorption and circular dichroism (CD) spectroscopy as well as molecular docking analysis. A gradual decrease in the Stern-Volmer quenching constant with increasing temperature revealed the static mode of the protein fluorescence quenching upon AXT addition, thus confirmed AXT–HSA complex formation. This was also confirmed from alteration in the UV–vis spectrum of HSA upon AXT addition. Fluorescence quenching titration results demonstrated moderately strong binding affinity between AXT and HSA based on the binding constant value ($1.08 \pm 0.06 \times 10^5\ M^{-1}$), obtained in 10 mM sodium phosphate buffer, pH 7.4 at 25 °C. The sign and magnitude of the enthalpy change ($\Delta H = -8.38$ kJ mol^{-1}) as well as the entropy change ($\Delta S = +68.21$ J mol^{-1} K^{-1}) clearly suggested involvement of both hydrophobic interactions and hydrogen bonding in AXT–HSA complex formation. These results were well supported by molecular docking results. Three-dimensional fluorescence spectral results indicated significant microenvironmental changes around Trp and Tyr residues of HSA upon complexation with AXT. AXT binding to the protein produced significant alterations in both secondary and tertiary structures of HSA, as revealed from the far-UV and the near-UV CD spectral results. Competitive drug displacement results obtained with phenylbutazone (site I marker), ketoprofen (site II marker) and hemin (site III marker) along with molecular docking results suggested Sudlow's site I, located in subdomain IIA of HSA, as the preferred binding site of AXT.

1. Introduction

Tyrosine kinase is an important mediator of the signaling pathways as well as known to be responsible for triggering various cancers, *viz.*, breast, lung, thyroid and kidney cancers [1,2]. Due to exhibiting inhibition properties against tyrosine kinases, various key promising drug molecules, known as tyrosine kinase inhibitors, have been developed as therapeutic agents to treat various cancers [3]. Axitinib (AXT) is a Food and Drug Administration (FDA) approved drug molecule, which is currently being used for the treatment of advanced renal cell carcinoma [3]. AXT has already shown favorable safety profiles and is being used as a supplementary therapy along with chemotherapy and radiation therapy [4].

AXT (Fig. 1) is a strong and selective inhibitor of vascular endothelial growth factor receptor (VEGFR) tyrosine kinases 1, 2 and 3, which are involved in the development of various cancers [4,5]. AXT competes with ATP for the ATP binding site and subsequently fits inside the tunnel of the catalytic domain of VEGFR. By doing so, AXT inhibits activation of VEGFR and thus prevents the signaling cascade of the kinases, which can lead to the growth and migration of cancers [3,6]. Owing to the anticancer properties of AXT, several researches are currently in progress to develop AXT as the potential drug in the treatment of other cancers, such as thyroid and lung cancers [4,7,8].

Binding of a drug to the plasma proteins improves its pharmacokinetic properties [9]. For instance, the *in vivo* half-life of a therapeutic drug is increased upon protein binding, which can elongate the duration of the drug's efficacy [10,11]. Drug-protein interaction also improves the drug solubility, decreases its toxicity and protects the drug's elimination from the human body [10,12]. Human serum albumin (HSA) is unique as a major transport protein of human circulation due to its ability to bind reversibly a wide variety of drugs with higher affinity compared to other transport proteins [13]. Three well-characterized high affinity

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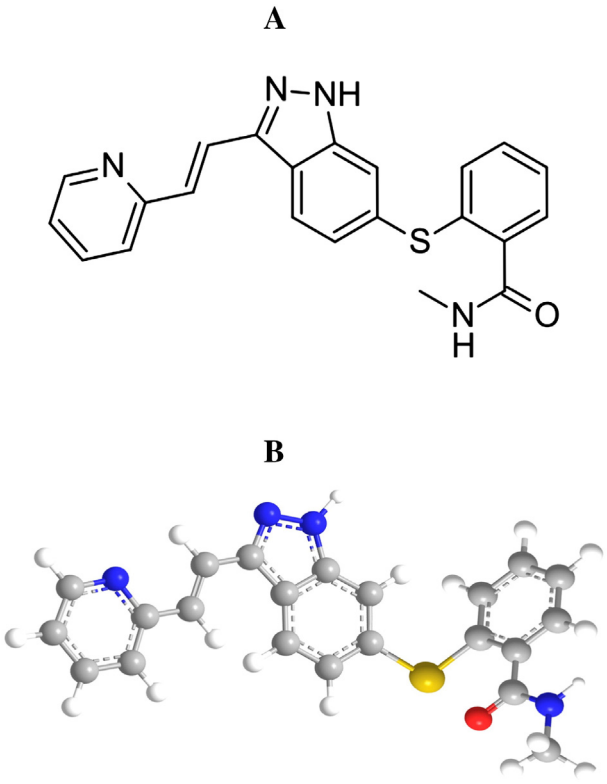

Fig. 1. Structural representation of axitinib. (A) 2-D structure and (B) 3-D structure.

drug binding sites I, II and III, located in the hydrophobic cavities of subdomains IIA, IIIA and IB of HSA, respectively, assist the drugs in their effective transport in the blood circulation [10,14]. Therefore, it is important to understand the binding characteristics of a drug to HSA in the body.

UV–vis [15], fluorescence [16] and circular dichroism (CD) [17] spectroscopy, equilibrium dialysis [18] and potentiometry [19] are commonly employed in the study of ligand-protein interactions. Of these, equilibrium dialysis is the most widely used method, however, it requires the analysis of free and bound ligand concentrations and is time consuming. Potentiometric method involves the use of ion selective electrodes, which may lack the selectivity for many ligands [19]. On the other hand, fluorescence technique is convenient in drug-protein interactions due to high sensitivity, rapidity and ease of implementation. The fluorescence measurements can provide some useful information about the binding of small molecules to proteins such as binding mechanism, binding mode, binding constants and binding numbers [20].

Despite several reports, highlighting the pharmacological significance of AXT, its interaction with HSA need to be explored. In view of the importance of the drug-protein interaction, this study aims to reveal the characteristics of AXT–HSA interaction in terms of the binding affinity, intermolecular forces involved, location of the binding site and effect on HSA fluorophore microenvironment using fluorescence spectroscopy and molecular docking analysis.

2. Materials and Methods

2.1. Materials

Essentially fatty acid free human serum albumin (Lot #068K7538), phenylbutazone (Lot #124K1625), ketoprofen (Lot #BCBG9546V) and hemin (Lot #015K0872) were purchased from Sigma-Aldrich Co. (St Louis, MO). Axitinib (Batch #044620005) was supplied by Cayman Chemical Co. (Ann Arbor, MI). All other chemicals used were of analytical grade purity.

2.2. Analytical Procedures

Human serum albumin (HSA) stock solution was prepared by dissolving a fixed amount of HSA crystals in 10 ml of 10 mM sodium phosphate buffer, pH 7.4 and it's concentration was determined spectrophotometrically using a molar extinction coefficient of 36,500 M^{-1} cm^{-1} at 280 nm [21].

The stock solutions of axitinib (AXT), phenylbutazone (PBZ), ketoprofen (KTN) and hemin (HMN) were prepared by dissolving 10 mg of these drugs in 10 ml of dimethyl sulphoxide (DMSO). The working solutions of these drugs were made by diluting their stock solutions to the desired concentration with above buffer.

All experiments were performed in 10 mM sodium phosphate buffer, pH 7.4 at 25 °C. In all experiments, the final concentration of DMSO was less than 1%.

2.3. UV–vis Absorption Spectroscopy

The absorption spectral measurements were carried out on a UV–vis spectrophotometer (Perkin-Elmer Lambda 25) using 10 mm path length quartz cuvettes. The absorption spectra of 20 μM HSA in the absence and presence of increasing concentrations (2–20 μM with 2 μM intervals) of AXT were recorded in the wavelength range, 240–400 nm. Absorption spectra of pure AXT solutions were also obtained in the same wavelength range at the studied concentrations.

2.4. Fluorescence Spectroscopy

Fluorescence measurements were carried out on a Jasco FP-6500 spectrofluorometer using a quartz cuvette of 1 cm path length, placed in a thermostatically-controlled water-jacketed cell holder, which was connected to a Protech 632D circulating water bath. The emission and the excitation bandwidths were set at 10 nm each, while a data pitch of 1 nm and a scan speed of 500 nm min^{-1} were used for spectral measurements.

The three-dimensional fluorescence spectra of HSA (3 μM) were recorded both in the absence and the presence of AXT (AXT/HSA molar ratio of 4:1) in the wavelength range, 220–500 nm, while the excitation wavelength range was set as 220–350 nm with 5 nm intervals.

2.5. Fluorescence Quenching Titration of HSA with AXT

Following the published procedure [22], the fluorescence spectra of HSA both in the absence and the presence of increasing AXT concentrations were recorded in the wavelength range of 310–390 nm upon excitation at 295 nm. HSA concentration was kept constant at 3 μM, while the concentration of AXT was varied in the range, 0–18 μM (with 2 μM intervals) in a total volume of 3 ml. An incubation time of 1 h was used to allow the equilibrium to be established at a fixed temperature before fluorescence measurements.

To evaluate the effect of temperature on AXT–HSA interaction, the fluorescence quenching titration experiments were carried out at four different temperatures, *i.e.*, 288, 298, 308 and 318 K.

2.6. Data Analysis of the Fluorescence Quenching Titration

The inner filter effect correction in the fluorescence data was made using the following equation, as described by Lakowicz [23]:

$$F_{cor} = F_{obs} 10^{(A_{ex} + A_{em})/2} \quad (1)$$

where F_{cor} and F_{obs} are the corrected and the measured fluorescence intensities, respectively. A_{ex} and A_{em} represent the differences in the