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Calorimetric and spectroscopic studies of the interaction between zidovudine and human serum albumin

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ABSTRACT

A quantitative analysis of the interaction between zidovudine (AZT) and human serum albumin (HSA) was achieved using Isothermal titration calorimetry (ITC) in combination with fluorescence and ^{1}H NMR spectroscopy. ITC directly measure the heat during a biomolecular binding event and gave us thermodynamic parameters and the characteristic association constant. By fluorescence quenching, the binding parameters of AZT-HSA interaction was determined and location to binding site I of HSA was confirmed. Via T_1 NMR selective relaxation time measurements the drug-protein binding extent was evaluated as dissociation constants K_d and the involvement of azido moiety of zidovudine in molecular complex formation was put in evidence. All three methods indicated a very weak binding interaction. The association constant determined by ITC (3.58×10^2 M^{-1}) is supported by fluorescence quenching data (2.74×10^2 M^{-1}). The thermodynamic signature indicates that at least hydrophobic and electrostatic type interactions played a main role in the binding process.

1. Introduction

To achieve a deeper understanding of the molecular recognition between a protein and its ligand, it is necessary to know the physicochemical mechanisms underlying the protein–ligand interaction. The binding forces involved in drug – plasma protein association is an important aspect that can significantly influence the different pharmacokinetics stages of the drugs reached in the bloodstream: distribution, availability on the target, metabolism and excretion and finally its therapeutic efficacy and toxicity. In this regard, a particular attention was given to human serum albumin, HSA, the most abundant plasma protein and the main carrier of endogenous (heme, fatty acids, metabolites) and exogenous (drugs) compounds into the bloodstream [1–3]. HSA contain three homologous domains (I, II and III) each of which is made up by two sub-domains, A and B. It is well accepted that the main areas of ligand binding to HSA are located in IIA and IIIA hydrophobic cavities, known as drug binding sites I and II or as Sudlow's sites I and II [Fasano, 2005] [4–7]. In subdomain IIA is also located Tryptophan residue (Trp 214) the main fluorophore of HSA molecule.

Zidovudine (ZDV), also known as azidothymidine (AZT) (see Scheme 1) belongs to the nucleoside reverse transcriptase inhibitors class of antiretroviral drugs. Both nucleoside and non-nucleoside reverse transcriptase inhibitors inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Zidovudine is used in combination with other medications to treat human immuno deficiency virus infection in patients with or without acquired immune deficiency syndrome.

The present study is focused on determining the binding and thermodynamic parameters that characterize AZT-HSA interaction by using modern, specific, and complementary techniques such as isothermal titration calorimetry (ITC), fluorescence spectroscopy and NMR. In the approach to this topic, ITC and NMR techniques are used for the first time. ITC have a unique capability to provide a complete thermodynamic description of a binding interaction, including explicit estimates of enthalpy and entropy, based on a single experiment conducted at a fixed temperature. ^{1}H NMR spectroscopic technique is specific for weak binding characterization. Also for quantitative determination of the affinity of AZT to subdomain IIA of HSA is used a correct approach of fluorescence quenching methodology.

2. Materials and Methods

2.1. Materials

Human serum albumin (HSA), fraction V (fatty acid free, 99%) and zidovudine (AZT) were purchased from Sigma-Aldrich Chemie GmbH. The solutions were prepared at room temperature using doubly distilled water 20 mM phosphate buffer for ITC, UV–Vis and Fluorescence

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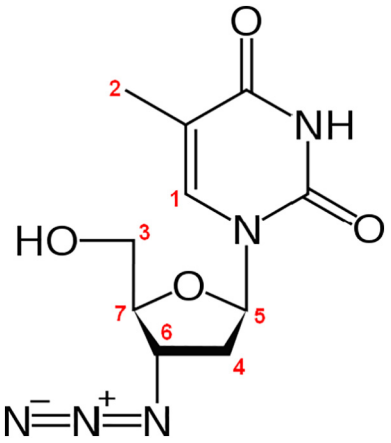

Scheme 1. Chemical structure of zidovudine.

measurements and D_2O (99.8% at. D) 50 mM buffer phosphate for NMR measurements, pH = 7.4.

2.2. Apparatus

The energetics of the binding of AZT to HSA was assessed using NanoITC^{2G} isothermal titration nanocalorimeter at constant temperature (TA Instruments, New Castle, DE, USA) of 25 °C next to the electrically and chemically calibration. The volume of the titration and reference cells was 1 ml. Small aliquots of a solution are injected under computer control into the titration cell at predefined time intervals. The recorded quantity is the rate of heating as a function of time. The individual pulses are integrated to obtain plots recording the ratio of heat to the amount of injected molecules.

All fluorescence measurements were carried out on a Jasco 6500 spectrofluorometer equipped with a xenon lamp source and 1.0 cm cell. HSA emission spectra were recorded in the wavelength range 300–500 nm, by exciting at λex = 280 nm or λex = 295 nm. The excitation/emission bandwidth was 3/5 nm.

The UV–Vis absorption spectra were recorded on a double-beam Jasco V-550 spectrophotometer using 1 cm path length quartz cuvettes.

The ^{1}H NMR relaxation measurements were carried out on a Bruker AVANCE III spectrometer operating at 500.13 MHz for proton and equipped with a broad-band observation probe. In all experiments the temperature was stabilized at 300 ± 0.1 K and all chemical shifts were measured relative to TMS.

2.3. Procedures

For ITC measurements, all solutions were thoroughly degassed before use. The reference cell was loaded with phosphate buffer and the sample cell was filled with 400 μM HSA solution. The titrations of 8 mM AZT into HSA were started after equilibrating the calorimeter to obtain baseline stability. From a computer controlled 250 μL Hamilton syringe, aliquots of 10 μL have been injected with a 500 s interval which was sufficiently long for the signal to return to the baseline and to ensure the equilibrium for the system. In order to ensure rapid and complete mixing of AZT-HSA solution, the contents in the sample cell were thoroughly stirred with a stirring speed of 250 rpm. By measuring the enthalpy change at each injection, the raw data, consisting of 25 peaks, were obtained as a plot of heat flow (kJ s^{-1}) against time (min). A plot of observed enthalpy changes per mole of injectant (kJ/mol^{-1}) against molar ratio was obtained after integration of raw data peaks using the NanoAnalyze software (TA Instruments, New Castle, DE, USA). Dilution heat of the titrant was measured under identical experimental conditions by injecting the AZT into buffer. The blank effects were subtracted in order to correct for dilution, mixing and injection effects.

The corrected calorimetric data were analyzed based on an independent binding model to determine the values of the association constant (K_a) and binding stoichiometry (n), as well as the enthalpy and entropy changes (ΔH and ΔS) of the reaction. The value of the free energy change (ΔG) was subsequently calculated with the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

For HSA fluorescence quenching experiments, a stock solution of 4×10^{-6} M HSA and a series of HSA/zidovudine assay solutions were prepared, keeping HSA concentration at 4×10^{-6} M and varying zidovudine concentration in the range of $4 \times 10^{-5} - 2 \times 10^{-3}$ M. Samples were incubated 1 h before measurement. The same set of samples was used to obtain UV–Vis spectra.

The overlapping of the exciting wavelength values with the ligand absorbance range requires inner-filter effect corrections for 1 cm cell fluorescence measurements, given by the relation [12]:

$$F_{corr} = F_{obs} 10^{\frac{A_{exc}+A_{em}}{2}} \quad (2)$$

where F_{corr} is the corrected fluorescence value, F_{obs} the measured fluorescence value, A_{exc} and A_{em} the measured change in absorbance value at the excitation and emission wavelength, respectively.

Changes in the conformation of albumin induced by the presence of zidovudine were determined by synchronous fluorescence method. The emission spectra were recorded in the 310–380 nm wavelength range for tryptophan (Trp) and 280–340 nm range for tyrosine (Tyr), varying the excitation wavelength with a difference (Δλ) of 60 nm and 15 nm respectively.

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 a ligand – macromolecule complex. In this work we used NMR as an alternative approach to fluorescence and ITC techniques, to study the low affinity interaction between zidovudine and HSA. We utilized an excess of zidovudine over the albumin in order to saturate the higher-affinity binding sites and to ensure that we investigate the weak low-affinity interaction, where the zidovudine molecules in free and bound states are in the fast exchange on the NMR time scale.

The NMR method used by us was to monitor AZT binding to HSA by measuring selective spin-lattice relaxation time of protons on the ligand. The AZT, H6 selective relaxation time observed in the presence of HSA is considerable shorter than its selective relaxation time in pure D_2O ($T_{1,free}$ = 2.803 s). For this reason, we followed the H6 selective spin-lattice relaxation as a function of AZT concentration in order to determine the extent of AZT bind to HSA, as dissociation constant K_d.

Selective spin-lattice relaxation times were measured by a 180° − τ − 90° inversion recovery method with a selective 180° - Gauss 1_180i_1000 soft pulse. With a length of the selective pulse between 11.2 ms and 18.35 ms and a power of 51 dB, the soft pulse was able to excite a frequency band between 65 Hz and 40 Hz. For τ we used 15 values ranging from τ_{min} = 0.01 s to τ_{max} = 20 s.

The relaxation times were fitted by means of an exponential regression analysis of the longitudinal magnetization components via the standard equation:

$$A(t) = A(0)\{1 - 2\ \exp(-\tau/T_1)\} \quad (3)$$