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Binding thermodynamics of Diclofenac and Naproxen with human and bovine serum albumins: A calorimetric and spectroscopic study

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ABSTRACT

Serum albumins are ubiquitous proteins able to bind a variety of exogenous and endogenous ligands including hydrophobic pharmaceuticals. Most drugs bind to two very active binding regions located within sub-domains IIA and IIIA of the protein, also known as Sudlow's sites. The drug binding mode of serum albumin provides important pharmacological information and influences drug solubility, efficacy, biological distribution, and excretion. Here, the binding thermodynamics of Diclofenac and Naproxen, two non-steroidal anti-inflammatory drugs (NSAIDs) to bovine and human serum albumins (BSA and HSA, respectively) were studied by isothermal titration calorimetry (ITC), fluorescence spectroscopy and differential scanning calorimetry (DSC). The ITC data show that the binding affinity (K) of Diclofenac to BSA and HSA is on the order of 10^4 M^{-1} with a binding stoichiometry (n) of 2 drug molecules per protein. Naproxen binding to the two proteins exhibits a different profile with K and n values on the order of 10^6 M^{-1} and 0.75 for BSA, and 10^5 M^{-1} and 3 for HSA, respectively. The binding of the two drugs to HSA is found to be both enthalpically and entropically favored suggesting the formation of hydrogen bonds and van der Waals hydrophobic effects. Binding of the two drugs to BSA is only enthalpically favored with an unfavorable entropy term. Significant enthalpy–entropy compensation phenomena were reported for Diclofenac and Naproxen binding to BSA but not to HSA. Fluorescence quenching data between Diclofenac and the two proteins suggest static collisions and the formation of ground-state protein-drug complexes. The DSC data corroborate the ITC findings and show multiple sequential unfolding events and a strong drug stabilization effect at high drug to protein ratios. Overall, the calorimetric and spectroscopic data provided insights into the nature of these protein-drugs interactions and might offer useful information in future drug discovery studies.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are powerful analgesic, antipyretic, and anti-inflammatory agents that are amongst the most commonly prescribed medications worldwide [1]. Unlike steroidal drugs, NSAIDs are non-narcotics and operate by essentially blocking the COX enzymes with relatively minor side effects [2,3]. While common NSAIDs are derivatives of organic acids such as salicylic, propionic, acetic, enolic, and anthranilic, other NSAID categories consist of sulfonanilides, derivatives of popular antibiotic sulfa drugs. For instance, Naproxen (trade names Aleve, Anaprox, Apronax, Naprelan, Naprosyn) belongs to the phenylacetic acid class of NSAIDs (2-(6-methoxy-2-naphthalen)propanoic acid), Diclofenac (trade names Aclonac, Cataflam, Voltaren) is a propionic acid derivative inferred from the chemical name 2-(2,6-**dich**l**o**ranilino) **phen**ylacetic **ac**id, and Celecoxib (trade names Celebrex and Celebra) is a sulfonamide-based type drug (4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide) which in 2015 received strong warnings from the FDA and the American Heart Association that included an increased risk of major cardiovascular problems. Interestingly, the S-enantiomer of Naproxen is 28-fold more active as an anti-inflammatory drug than the R-isomer which is reported to be a liver toxin and to cause gastrointestinal disorders [4,5]. The differences in the chemical structures and half-lives of these drugs and their binding to serum albumin influence several important pharmacological properties such as efficacy, solubility, bioavailability, toxicity, dosage, distribution, and excretion. In the circulatory system, both Diclofenac and Naproxen bind to a large extent to serum albumin (>99%) with minimal accumulation in tissues. However, Diclofenac's half-life is at least 6 times shorter than Naproxen and is thus excreted much faster [6].

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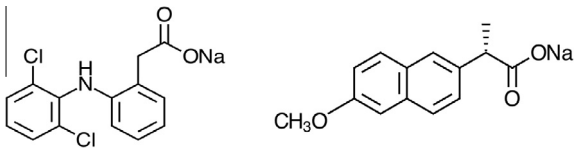

Fig. 1. Molecular structures of Diclofenac (-2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt (left) and Naproxen (-6-Methoxy-α-methyl-2-naphthaleneacetic) acid sodium salt S-enantiomer.

Mammalian serum albumin is the most abundant protein of the circulatory system (40–50 g/L). It plays an important role in the regulation of osmotic blood pressure, blood pH, and the transport of numerous endogenous and exogenous compounds such as fatty acids, amino acids, hormones, ions, metals, drugs and their byproducts [7]. It is a 67 kDa globular monomeric, hydrophilic, and non-glycosylated protein. It is arranged in a globular heart-shaped conformation and is composed of three α-helical domains (I, II and III), each with two subdomains (IA, IB, IIA, IIB, IIIA, IIIB) and several folded disulfide bridged loops with up to five different potential binding sites [8–11]. Two so-called Sudlow sites (site I and site II) are major binding regions within subdomains IIA and IIIA that allow the protein to bind a variety of hydrophobic, heterocyclic and anionic compounds including 70% of man-made drugs [12–17]. Due to its versatile binding and transport properties, serum albumins are one of the best studied protein models that are well known for their involvement in the reversible binding and transport of free drugs in the plasma [8]. Because NSAIDs are normally insoluble in blood plasma, the two Sudlow sites have been proposed to decrease drug toxicity and increase drug solubility and transport [17]. Sudlow's site I has been shown to bind mostly bulky heterocyclic compounds such as coumarin compounds, sulfonamides, and salicylates while Sudlow's site II binds aromatic carboxylic acids and profens such as ibuprofen and ketoprofen [15–18]. The distribution, metabolism, and free concentration of administered drugs in the blood stream is strongly affected by the nature of protein-drug interactions, which can influence drug stability and toxicity. Therefore, the quantitative characterization of serum albumin-drug binding thermodynamics is essential to improving our knowledge of biomolecular recognition and drug pharmacokinetics. Human serum albumin (HSA) has been shown to protect the drugs it binds to from oxidative damage as well as change their pharmacokinetic and pharmacodynamic properties [7,9,19–22].

Two non-steroidal anti-inflammatory drugs, Diclofenac and Naproxen (Fig. 1) were chosen for this study owing to their wide use as over-the-counter pain and anti-inflammatory medications. Diclofenac has pKa values of 3.80–4.19 while Naproxen's pKa ranges between 4.26 and 4.76 [23]. At neutral pH, the two drugs are in their anionic forms and bind preferably to Sudlow site II of serum albumin as previously described [10,16,24]. Despite their high sequence and structural similarities (76%), the distribution and binding ability of serum albumin domains is not the same as evidenced in the different binding thermodynamics of several non-steroidal anti-inflammatory drugs with bovine (BSA) and human serum albumins [10 and references therein]. Whereas some agreement regarding the first strong NSAID-albumin binding site is reported in the literature, many inconsistencies exist for the second set of weaker, non-specific binding sites with stoichiometric ratios upward of 6 drug molecules per protein and a wide range of low and high affinity constants spanning five orders of magnitude [11 and Refs. therein]. These discrepancies are likely due to different experimental conditions and investigative techniques used to carry out the binding interactions.

The purpose of this study is to evaluate the interaction of Diclofenac and Naproxen with BSA and HSA using isothermal titration calorimetry (ITC), fluorescence spectroscopy, and differential scanning calorimetry (DSC). ITC and fluorescence spectroscopy are amongst the most commonly used techniques to evaluate the thermodynamics of biomolecular interactions and in particular the binding of NSAIDs to serum albumins. All three techniques employed here will allow a more in-depth characterization of the mechanism underlying drugs-serum protein interactions. As a universal calorimetric tool, ITC is able to provide in a single experiment all of the interaction thermodynamics including the binding affinity (K_a), the stoichiometry of binding (n), the enthalpy change (ΔH), the entropy change (ΔS), and the free energy change (ΔG) of the reaction allowing a complete thermodynamic description of the molecular interaction to be generated. The partitioning of the Gibbs free energy change into enthalpic and entropic contributions provides new insights into structure function relationships and a deeper understanding of the nature of these interactions and is particularly useful in drug discovery studies. Fluorescence spectroscopy relies on the quenching of the intrinsic fluorescence spectra of the protein following drug binding and allows the characterization of the type of binding interaction (static *vs.* dynamic) between the protein and the drug molecules. DSC can complement these measurements and assist in characterizing protein folding dynamics and determining the strength and stability of the protein-drug complexes by evaluating the temperature of denaturation (T_m), the heat capacity change (ΔC_p) and the enthalpy of thermal denaturation (ΔH).

2. Materials and methods

Human serum albumin (HSA, ⩾99% purity, essentially globulin free), bovine serum albumin (BSA, ⩾98% purity, essentially protease free), high purity Diclofenac sodium salt and S-Naproxen sodium salt, were purchased from Sigma-Aldrich chemical company (St. Louis, USA) as lyophilized powders and used without further purification. Purified HSA and BSA were isolated from blood plasma and thus contain fatty acids. The two protein solutions were freshly prepared in 50 mM phosphate buffer, 50 mM NaCl, pH 7.40 using DI water and their concentrations checked at 280 nm using a Varian Cary 50-Bio UV–vis spectrometer and a molar absorptivity (ε) value of 42,684 M^{-1} cm^{-1} for HSA and 43,824 M^{-1} cm^{-1} for BSA, respectively. Dibasic anhydrous sodium phosphate (Na_2HPO_4) was purchased from Fisher Scientific.

2.1. Specification of chemical samples

Chemical name	Source	Initial mole fraction purity	Purification method	Final mole fraction purity	Analysis method*
Human serum albumin (HSA) – Prod# A8763; Lot# SLBK6136V	Sigma-Aldrich	⩾0.99	Agarose electrophoresis	1	Agarose electrophoresis
Bovine serum albumin (BSA) – Prod# A4737; Lot# SLBD1578V	Sigma-Aldrich	⩾0.98	Agarose electrophoresis	1	Agarose electrophoresis