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Optimizing the bioavailability of small molecular optical imaging probes by conjugation to an albumin affinity tag

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ABSTRACT

Small molecular imaging probes are often found to be rapidly cleared from the circulation. In order to improve signal to noise ratio (SNR) by high probe accumulation in the target tissue we intended to prolong the presence of the probes in the circulation by exploiting inherent transport mechanisms. Human serum albumin (HSA) is playing an increasingly important role as a drug carrier in clinical settings and drugs directly bound to albumin or attached to albumin binding moieties have been successfully developed for treatment approaches. To optimize the bioavailability of existing fluorescent probes, a hydrophobic affinity tag is installed, which enhances albumin binding. In a first experiment an endothelin-A receptor (ET_AR) probe is modified by inserting a trivalent linker, attaching an albumin affinity tag and labeling the conjugate with the fluorescent dye Cy 5.5. The spectroscopic properties of the conjugate are examined by photometer- and fluorometer measurements in comparison to a probe without albumin binding tag. Albumin binding was proven by agarose gel electrophoresis. The affinity towards ET_AR was confirmed *in vitro* by cell binding assays on human fibrosarcoma cells (HT-1080) and *in vivo* by murine xenograft imaging studies. *In vitro*, the modified probe retains high target binding in the absence and presence of albumin. Binding could be blocked by predosing with ET_AR antagonist atrasentan, proving specificity. The *in vivo* examinations in comparison to the established probe showed a reduced renal elimination and a prolonged circulation of the tracer resulting in significantly higher signal intensity (SI) at the target and a higher signal-to-noise ratio (SNR) between 3 h and 96 h after injection. In summary, we designed a small molecular, non-peptidic fluorescent probe which targets ET_AR and reversibly binds to serum albumins. The reversible binding to albumin enhances the biological half-life of the probe substantially and enables near infrared optical imaging of subcutaneous tumors for several days. This approach of reversibly attaching probes to serum albumin may serve as a tool to optimize tracer distribution for more precise target characterization in molecular imaging experiments.

1. Introduction

In the past decades, human serum albumin (HSA) has emerged as a versatile tool for the *in vivo* transport of therapeutic and diagnostic agents. HSA is by far the most abundant protein in plasma, where it reaches a concentration of up to 700 μM and makes up 60% of total plasma protein [1,2]. But only 40% of total HSA is present in plasma; the remaining 60% are distributed in muscle, skin, extracellular space and the lymphatic system. Structurally, HSA is composed of three similar globular domains, each of which containing two subdomains, denominated IA, IB, IIA, IIB, IIIA, and IIIB (Fig. 1). Its (small) net mass of 66.5 kDa, its high cystein-content and its rather acidic character make it a very soluble and highly robust protein, stable over a wide range of pH and temperature [3]. In plasma it has two main functions: 1) the maintenance of oncotic pressure and 2) the binding and transport of molecules and ions. Due to its unique properties, HSA is capable of non-covalently binding a large variety of endogenous and exogenous substances. It is able to bind seven equivalents of long-chain fatty acids at multiple binding sites; one of those sites, located in subdomain IB, also acts as a heme/bilirubin binding site. Additionally, it is able to bind a large number of therapeutic drugs, steroid hormones, amino acids and metal ions (e.g. Fe^{3+} and Cu^{2+}). In 1975, *Sudlow et al.* identified two distinct binding sites, now referred to as *Sudlow site I* (located in subdomain IIA, where bulky heterocyclic anions bind) and *Sudlow site II* (located in subdomain IIIA, which is preferred by aromatic compounds), respectively (Fig. 1), which are responsible for the binding of the majority of pharmaceutical drugs [4]. *Warfarin*, an anti-coagulant drug and typical rodenticide, and *Ibuprofen*, a non-steroidal anti-inflammatory compound, are typical ligands for *Sudlow site I* and *site II*, respectively [5].

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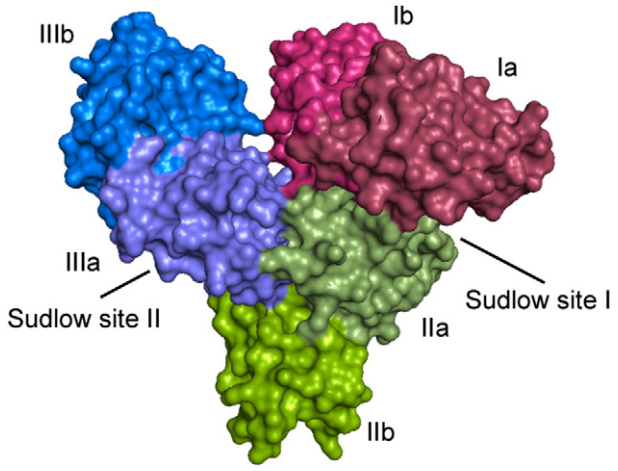

Fig. 1. X-ray crystal structure of human serum albumin (*pdb-code* 3TDL) showing the heartshaped form with the three domains I–III, each of which consists of two subdomains, and the location of the two drug-binding sites *Sudlow site* I and II.

The unique potential of drug-binding offers a number of advantages, including prolongation of biological half-life and reduced toxicity and immunogenicity. Clinically, it has been utilized in e.g. the treatment of diabetes [6] and cancer [7,8] and the diagnosis of rheumatoid arthritis [5,9]. *Levemir®*, a fatty acid derivative of insulin, binds to circulating albumin and has been approved for the treatment of diabetes. Abraxane®, an albumin-paclitaxel nanoparticle, has been approved by the FDA in 2005 for the treatment of metastatic breast cancer. Molecular imaging approaches utilize the albumin-binding properties of probes as a possibility to improve signal-to-noise ratios (SNRs), especially for cancer imaging. The attachment of molecular imaging probes to albumin can be achieved either covalently or non-covalently [10]. Covalent labeling of albumin is facilitated by using amine- or thiol-reactive probes. These bind to either one or more of the 58 lysine residues or the single free cys-34 residue in its reduced form. The major drawback of covalent binding to albumin is the lack of site specificity. The single cys-34 site might not be the optimal place for the conjugated ligand to exert its effects and the conjugation to any of the lysine residues abolishes any specificity. Non-covalent binding offers transient, reversible attachment of probes to albumin with a specific equilibrium between bound and free state. This route was chosen by *Lauffer et al.*, who designed MS-325, an HSA-targeted MRI contrast agent based on Gd-DTPA and an albumin affinity tag [11,12]. A fraction of 88% of MS-325 is bound to HSA at 37 °C and it has an association constant to HSA of $K_i = 11.0$ mM [13]. In typical applications MS-325 is used as an intravascular imaging agent for MRI, especially in MR angiography [14].

The endothelin (ET) axis is a prominent target for cardiovascular and inflammatory diseases and in cancer. While in normal physiological conditions endothelin-1 (ET-1) is responsible for the maintenance of vascular tone, in pathophysiology a dysregulation results in irregular vascular tension, and triggers cell proliferation, angiogenic spread of tumor vessels, inflammation and metastasis [15–17]. We report here the pharmacokinetic optimization of a recently designed fluorescent endothelin-A receptor (ET_AR) probe [18] to enhance contrast-to-noise ratios (CNR) in preclinical *in vivo* experiments, by reversibly attaching our probe to serum albumins via a small albumin-binding tag.

2. Materials and methods

2.1. General

All chemicals, reagents and solvents for the synthesis of the compounds were analytical grade and purchased from commercial sources. Bovine serum albumin (BSA) and recombinant human serum albumin (rHSA) were from *Sigma Aldrich* (St. Louis, MI); recombinant mouse serum albumin (rMSA) was from *Albumin Bioscience* (Huntsville, AL). Fluorescent ET_AR tracer **1** and Benzo [1,3]dioxol-5-yl-3-(2-{2-[2-(2-aminoethoxy)-ethoxy]ethoxy}ethoxy)-4,5-dimethoxy-benzyl-5-hydroxy-5-(4-methoxy-phenyl)-5H-furan-2-one, **2** (Fig. 2) were synthesized as described before [18]. 4,4-Diphenylcyclohexanol (**3**) was synthesized as described by Amedio [19].

The fluorescent probes **9** and **10** are synthesized as described in the supplemental material. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker ARX 300 or an AMX 400 (*Bruker BioSpin GmbH*, Rheinstetten, Germany), respectively. Major and minor signals from rotational isomers are given if an assignment was possible. Mass spectrometry was performed using a Waters QUATTRO LCZ (*Waters Micromass*, Manchester, UK) or an Orbitrap LTQ XL (*Thermo Scientific*, Dreieich, Germany) spectrometer with nanospray capillary inlets. HPLC-purification was performed on a gradient RP-HPLC using a Knauer system with two K-1800 pumps, an S-2500 UV detector (*Herbert Knauer GmbH*, Berlin, Germany) and a RP-HPLC Nucleosil 100-5 C18 column (250 mm × 8.0 mm). The recorded data was processed by the *ChromGate* HPLC software (*Knauer*).

2.2. Photo- and fluorometer measurements

The influence of BSA concentration on the absorption and emission spectra was determined by photometric and fluorometric measurements using a Hitachi U-3010 spectrophotometer and a Hitachi F-4500 spectrofluorometer (*Hitachi High Technologies Europe GmbH*, Mannheim, Germany). The fluorescent probes (equal amounts) were dissolved in 1 mL of either phosphate buffered saline (PBS) or PBS containing different amounts of BSA (0.001%–4% *w*/*v*). Also, the influence of sodium dodecyl sulfate (SDS, 2% *w*/*v*) and acetonitrile (50% *v*/*v*) was recorded. The absorption and emission spectra (excitation wavelength $\lambda_{ex.} = 630$ nm) were recorded at room temperature with the manufacturers' software (*UV-Solutions*/*FL-Solutions*) and analyzed with the *GraphPad Prism 4.0* software.

2.3. Agarose gel electrophoresis

Reconstituted solutions of the three fluorescent tracers **1**, **9** and **10** (0.5 nmol) were mixed with PBS or PBS containing 2% (*w*/*v*) of BSA, rMSA or rHSA, respectively, to give a final volume of 30 μL. After incubation at 37 °C for 1 h 5 μL of gel loading buffer (*R*0631, *Thermo Scientific*, Schwerte, Germany) was added. Analysis was performed by agarose gel electrophoresis (1.5%, in 0.5% Tris, acetic acid, EDTA puffer/TAE, pH 8) with a voltage of 120 V for approximately 1 h. Visualization of the fluorescent bands was possible by fluorescence reflectance imaging (FRI, see below). Coomassie blue staining of the protein bands was then perfomed to visualize the albumins.

2.4. Xenografts

Animal experiments were carried out according to approved protocols of the animal ethics committee of the University of Münster, Germany. Athymic 7- to 9-week-old female nude mice were obtained from Charles River (Sulzfeld, Germany) and maintained in a pathogen-free animal facility with food and water available *ad libitum*. Human fibrosarcoma cell line HT-1080 was purchased from DSMZ (Braunschweig, Germany) and were cultivated in DMEM supplemented with 10% FCS and 2 mM glutamine. Cells were grown routinely in T75 flasks, incubated at 37 °C in a 5% CO_2 humidified air atmosphere until the cultures were sub-confluent (70–80%); medium was changed every 3–4 days. Before injection the cells were incubated with trypsin/EDTA and resuspended in PBS at appropriate concentrations. For injection, approximately 3×10^6 cells were subcutaneously implanted in the right hemithorax of 9- to 11-week-old nude mice through 26-gauge needles and grown for 3–4 weeks until tumors reached a diameter of 4–5 mm.