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Surface plasmon resonance and circular dichroism characterization of cucurbitacins binding to serum albumins for early pharmacokinetic profiling

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ABSTRACT

Cucurbitacins are a group of tetracyclic triterpenoids, known for centuries for their anti-cancer and anti-inflammatory properties, which are being actively investigated over the past decades in order to elucidate their mechanism of action. In perspective of being used as therapeutic molecules, a pharmacokinetic characterization is crucial to assess the affinity toward blood carrier proteins and extrapolate distribution volumes. Usually, pharmacokinetic data are first collected on animal models and later translated to humans; therefore, an early characterization of the interaction with carrier proteins from different species is highly desirable. In the present study, the interactions of cucurbitacins E and I with human and rat serum albumins (HSA and RSA) were investigated by means of surface plasmon resonance (SPR)-based optical biosensing and circular dichroism (CD) spectroscopy. Active HSA and RSA sensor chip surfaces were prepared through an amine coupling reaction protocol, and the equilibrium dissociation constants (K_d) for the different cucurbitacins-serum albumins complexes were then determined by SPR analysis. Further information on the binding of cucurbitacins to serum albumins was obtained by CD competition experiments with biliverdin, a specific marker binding to subdomain IB of HSA. SPR data unveiled a previously unreported binding event between CucI and HSA; the determined binding affinities of both compounds were slightly higher for RSA with respect to HSA, even though all the compounds can be ranked as high-affinity binders for both carriers. CD analysis showed that the two cucurbitacins modify the binding of biliverdin to serum albumins through opposite allosteric modulation (positive for HSA, negative for RSA), confirming the need for caution in the translation of pharmacokinetic data across species.

1. Introduction

Cucurbitacins (Cuc) are a class of highly oxygenated, tetracyclic triterpenoids primarily isolated from the plant family of Cucurbitacee. The cytotoxicity of cucurbitacins has been known for centuries and the elucidation of their mechanism of action is an active topic of research; several biological and pharmacological activities have been reported, proposing cucurbitacins as potential anti-cancer, anti-inflammatory and anti-microbial agents [1–4]. For instance, cucurbitacin E (CucE, Fig. 1) disrupts the F-actin cytoskeleton, inhibiting the growth of human prostate carcinoma PC-3 cells [5], and displays anti-angiogenesis activity in human umbilical vascular endothelial cells by suppressing the VEGFR2-mediated Jak2–STAT3 signaling pathway [6]; cucurbitacin I (CucI, Fig. 1), the deacetylated form of CucE, induces apoptosis in Sézary syndrome cells through the inhibition of the same pathway [7] and displays cardioprotective properties through the suppression of the tissue growth factor-mediated signaling pathways in hypertrophic cardiomyocytes [8]. In view of the potential use of cucurbitacins as therapeutic agents, binding studies with carrier proteins, and in particular with serum albumins, are highly desirable in order to better characterize their distribution and bioavailability for their primary targets, and therefore understand their pharmacological behavior.

Human serum albumin (HSA) is the most abundant protein carrier in blood serum [9]. HSA possesses the ability to bind to several

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different classes of compounds, either endogenous and exogenous, modulating their properties such as solubility, availability, toxicity and stability [9]. Several techniques have been successfully applied to characterize the binding properties of HSA, e.g. equilibrium dialysis, high-performance affinity chromatography and fluorescence spectroscopy to name a few [10–15]. Among these, circular dichroism (CD) spectroscopy [16,17] offers the analytical advantages given by its trademark sensitivity to chirality, allowing the investigation of binding events through the phenomenon of induced circular dichroism (ICD). The occurrence of ICD is the result of strong, stereospecific binding phenomena between interacting molecules, whose chiroptical properties are consequently modified through conformational restraint and electronic coupling between the chromophores of both guest and host molecules [18]; the ICD signals of specific high-affinity markers for the binding sites of HSA can therefore be exploited to characterize the binding of other analytes by means of competition studies in solution [19,20].

Optical biosensors based on surface plasmon resonance (SPR) have also been employed during the last years in order to study the interaction between small molecules and proteins [21–24]. In a typical SPR experiment, one of the two binding partners is chemically attached to the surface of a sensor chip (ligand), while the other is free to flow in solution (analyte) and is thus able to interact with the ligand. The response (*R*), given in arbitrary resonance units (RU), is dependent on the refractive index of the chip surface, which varies proportionally to the change in mass due to the association and/or dissociation of the binding complex between ligand and analyte. This technique offers advantages over the conventional solution-based techniques:

(a) The interaction between two molecules can be monitored without the need of any labeling, obtaining binding information about unmodified reactants and avoiding the possibility to alter the binding properties [25].
(b) Sensor chips can be used for several cycles of analysis without compromising their functionality, therefore increasing the throughput of the technique [26].
(c) Unlike other equilibrium-based techniques, the interaction can be monitored in real time, providing more detailed information about the binding process [27].

The latter feature is of particular interest when monitoring biological systems, especially when dealing with carrier proteins, since the kinetics of binding greatly influences the pharmacokinetic properties of the bound molecules [28].

During pre-clinical and clinical trials, animal models are routinely used to test the binding properties and the consequent bloodstream distribution of new drug candidates in order to extrapolate pharmacokinetic data for the human body [29]. Though HSA and rat serum albumin (RSA) share a high degree of homology in their amino acid sequences, differences in their binding properties has already been demonstrated [30]. Therefore, the investigation of the binding differences among serum albumins from different species becomes of great importance before extrapolating data for clinical studies in humans. Exploiting the versatility of the experimental setup given by SPR-based biosensors, evaluations on the binding properties of different compounds toward immobilized serum albumins from different species can be achieved and compared with data obtained from other solution-based affinity techniques [21].

In the present study, cucurbitacins–serum albumins (Cuc–SA) binding complexes were investigated by means of a combination of SPR-based optical biosensing and CD spectroscopic analysis, with particular focus on the binding of CucE and CucI with HSA and RSA.

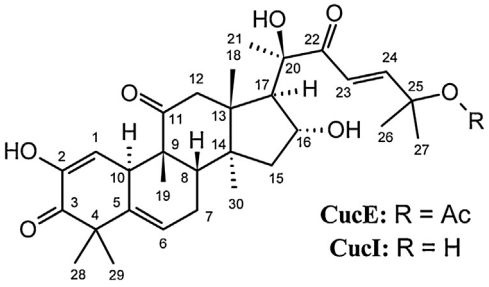

Fig. 1. Chemical structures of CucE and CucI.

2. Materials and methods

2.1. Materials

Cucurbitacin E (CucE; molecular weight, MW: 556.76 Da) and cucurbitacin I (CucI; MW: 514.16 Da) were purchased from Extrasynthese (Genay, France).

HSA (essentially fatty acid free, ≥96%, product code A1887; MW: 66.4 kDa), RSA (essentially fatty acid free, essentially globulin free, ≥99%, product code A6414; MW: 64.6 kDa), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), dimethyl sulfoxide (DMSO), sodium naproxen (MW: 252.24 Da), (*S*)-warfarin (MW: 308.33), biliverdin hydrochloride (BV; MW: 619.12), sodium chloride (NaCl) and sodium acetate were all purchased from Sigma–Aldrich (Milan, Italy).

Research-grade CM 5 sensor chips, 10X phosphate buffer saline (PBS) and the amine coupling kit, consisting in *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine hydrochloride (pH 8.5; 1 M), were all purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden).

Water was bi-distillated, de-ionized, filtered and degassed by Millipore Elix 10. Phosphate buffer (PB) (20 mM) + NaCl (150 mM) (pH 7.4) (running buffer A) was used for the immobilization procedure, while 1X PBS–DMSO (95:5, v/v) (pH* 7.4) (running buffer B) was used for the Cuc–SA interaction studies. All buffer solutions were freshly prepared prior to the analysis and filtered through a 0.22-μm pore size membrane made of a mixture of cellulose nitrate and acetate.

2.2. SPR analysis

2.2.1. Instrumentation

SPR measurements were performed with a Biacore™ X100 system (GE Healthcare Bio-Sciences, Uppsala, Sweden) equipped with an in-line degasser and thermostated at 25 °C. Data were analyzed and processed using the Biacore™ X100 2.0 evaluation software.

2.2.2. Surface preparation and validation

Stock solutions of HSA at a 30 $\mu g\,mL^{-1}$ concentration were prepared in sodium acetate buffers (10 mM) at various pH values (pH 4.0, 4.5, 5.0 and 5.5), and the electrostatic pre-concentration at the chip surface was evaluated by injecting each stock solution for 180 s at a 5 $\mu L\,min^{-1}$ flow rate, after which the baseline was re-established by injecting a NaOH solution (50 mM). The best condition was achieved using the pH 5.0 stock solution.

Consequently, HSA was immobilized on two CM 5 sensor chips via amine coupling according to the standard Biacore procedure, using the pH 5.0 stock solution. Briefly, the chips were equilibrated at room temperature for 30 min before docking, then the system was primed three consecutive times with running buffer A. The carboxymethyl dextran matrix was activated on both channels of the sensor chip by injecting a freshly prepared mixture of