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Chemical and biological properties of a supramolecular complex of tuftsin and cucurbit[7]uril

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ABSTRACT

Cucurbit[7]uril (CB7) is an uncharged and water-soluble macrocyclic host. CB7 binds to doubly protonated tuftsin, which is the tetrapeptide Thr-Lys-Pro-Arg, with moderate affinity ($K_a = 2.1 \times 10^3\ M^{-1}$). In this study, the host–guest complexation was confirmed by fluorescence titration. This affinity would allow for easy release of the peptide under physiological conditions. According to density functional theory calculations, the structural binding motif involves hydrogen bonding. The most energetically stable form had the Arg side chain inside the CB7 cavity. The effects of the tuftsin–CB7 complex on the proliferation and cytokine activity of immune cells were studied. The complex had broader spectrum immunomodulation than free peptides, and caused statistically significant ($p < 0,05$) changes in cytokine production (tumor necrosis factor-α, interleukin-2, interferon-γ, and interleukin-10) by mononuclear cells. By contrast, the free peptide only activated tumor necrosis factor-α production.

1. Introduction

Tuftsin is an immunomodulator tetrapeptide (Thr-Lys-Pro-Arg) that is produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G. It was discovered in 1970 by Najjar and Nishioka [1] and named after Tufts University. Its biological activity is related to the immune system function of immunoglobulin G, which stimulates phagocytosis by neutrophils. Tuftsin deficiency can be hereditary or occur following splenectomy, and results in increased susceptibility to certain infections caused by capsulated bacteria such as *Haemophilus influenza*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Salmonella typhi* [2,3]. Binding of the peptide to a specific receptor, neuropilin-1, leads to activation of phagocytes, which stimulates their migration, phagocytic, bactericidal, and antitumor activity. It also enhances antibody production.

Macrocyclic host molecules have the ability to encapsulate biologically relevant guest molecules and to act as drug carriers, drug solubilizers, drug stabilizers, and drug bioavailability enhancers. This strategy has been extensively explored both for naturally occurring hosts such as cyclodextrins [4–8], and for synthetic molecular receptors such as calixarenes and crown ethers [8–11].

Cucurbit[*n*]urils (CB[*n*], $C_{6n}H_{6n}N_{4n}O_{2n}$), which were first discovered by Behrend et al. in 1905, are promising macrocyclic nanoscale containers that are formed by acid-catalyzed condensation of *n* glycoluril units with formaldehyde [12]. According to recent reports, CB[*n*] and its derivatives are non-toxic, which has increased interest in the use of CB[*n*] as a drug-delivery vehicle [13]. The pumpkin-shaped CB[*n*]s are cage compounds, with the *n* glycoluril units linked by pairs of methylene groups, and have outer diameters ranging from 4.5 Å for CB [5] to 12.5 Å for CB [10]. These diameters allow for guest molecules of various sizes to be included in the cavity [14–19]. The internal hydrophobic cavity is responsible for complexation of small guest molecules through hydrophobic interactions, whereas the two portals, which are lined with urea carbonyl groups, are responsible for complexation with positively charged molecules through ion–dipole interactions [14,15,19,20]. These interactions enhance fluorescence and shift the pK_a [20–27]. Moreover, this kind of host-assisted pK_a shift has also been observed for other hosts, such as calixarenes [28].

Cucurbit[7]uril (CB7, Scheme 1) [29,30]. has a hydrophobic cavity and hydrophilic portals, and is capable of forming a range of host–guest

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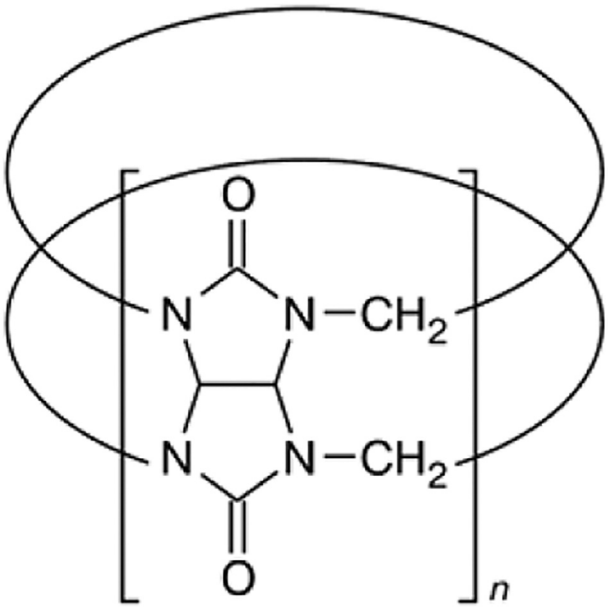

Scheme 1. Molecular structure of the CB7 macrocycle.

complexes with organic and inorganic compounds. CB7 is largely non-toxic in vitro and in vivo [13,31–33], and it has the best water solubility among the CB[*n*] homologues. It shows extraordinary binding affinities with various guest molecules in aqueous media and has an ideal cavity size for drug storage and release. Because of these factors, CB7 has great potential as a drug delivery vehicle, a biodiagnostic agent, and a modulator of bioregulation [34–36]. Partial or full encapsulation by CB7 can have a number of effects on a drug including the following: increasing its physical stability against degradation and changes from the solid phase during manufacturing, formulation, and storage [37,38]; prevention of in vivo detoxification/degradation and reduction of drug side-effects [39,40]; modulation of cellular drug uptake [32]; and targeted delivery [41]. In addition, further delivery enhancements are possible and include masking of taste and altering of drug absorption. It has been suggested that the excellent binding affinity of CB7 with guest molecules occurs through a combination of electrostatic interactions of its carbonyl groups at its portals and hydrophobic interactions between its cavity and the guest [42–51]. Alternatively, it could be caused by impairment of the hydrogen bonding networks of the large number of water molecules contained in the cavity [52]. To date, the origin of differences among the binding affinities of amino acids toward CB7 has not been studied in detail [42,50,51,53–65]. Investigation of this would reveal the contributions of various factors to the host − guest interactions and provide insight to optimize these interactions and assist with the rational design of supramolecular systems with CB7 and proteins/peptides. CB7 could bind with either peptide substrates [66] or inhibitors [67], and in this way, it affects the activity of enzymes. CB7 has also been used to immobilize proteins on surfaces by noncovalent interactions [68].

This work presents a systematic study of the host–guest interactions between the macrocyclic host molecule CB7 and the tetrapeptide tuftsin (Thr-Lys-Pro-Arg). Complex formation between CB7 and tuftsin was examined in solution by fluorescence titration, and the results were further evaluated using computational investigations. Immunological properties were measured, and the in vitro biological activities of the complexes were studied by investigation the effects on proliferation and cytokine-producing ability of immune cells from somatic healthy donors.

2. Materials and methods

2.1. Materials

CB7 was synthesized at the Nikolaev Institute of Inorganic Chemistry (Novosibirsk, Russia). Tuftsin was purchased from Verta NPF (Saint Petersburg, Russia). RPMI 1640 Medium, phosphate buffered saline, and L-glutamine were obtained from Biolot (Saint Petersburg, Russia). Mercaptoethanol and HEPES buffer were purchased from Sigma-Aldrich (St. Louis, MO). Dapoxyl was obtained from Invitrogen (Carlsbad, CA). HyClone fetal calf serum was obtained from GE Healthcare (Chicago, IL). Concanavalin A (ConA) was purchased from Merck Millipore (Darmstadt, Germany). Ficoll was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Urografin was obtained from Schering AG (Berlin, Germany). [^{3}H]-Thymidine was purchased from the V. G. Khlopin Radium Institute (Saint Petersburg, Russia). Gentamicin was obtained from Krka (Novo Mesto, Slovenia).

2.2. Competitive fluorescence titration

The stability constant (K_a) of the tuftsin–CB7 complex was determined by competitive florescence titration using a Varian Eclipse spectrofluorimeter (Varian, Palo Alto, CA) at 25 °C using a 100-μL quartz cuvette. Studies were carried out in 10 mM acetate buffer at pH 6.0. The method is based on the properties of the Dapoxyl–CB7 complex [69–72]. Complexation with CB7 alters the fluorescence spectrum of Dapoxyl, which can be used to distinguish between the free dye in solution and the Dapoxyl–CB7 complex. Therefore, the first step was to obtain the Dapoxyl–CB7 complex, and to select the initial concentrations for the competitive fluorescence titration. The second step was the competitive fluorescence titration of Dapoxyl–CB7 with tuftsin. When Dapoxyl was displaced from the CB7 cavity with a competing substance, in this case a peptide, the spectrum became characteristic of that of free Dapoxyl.

2.3. Density functional theory calculations

All calculations were performed using the PRIRODA program package [73] using generalized gradient approximation PBE functional [74]. For all the atoms under consideration, Ahlrichs' triple zeta split-valence atomic basis set VTZ was used [75]. Functional PBE was effective in our earlier theoretical studies with cucurbituril [76–79]. Full gas-phase geometry optimization of all systems was carried out without any restrictions on the symmetry. After geometry optimization, vibrational frequencies of the macromolecules were calculated. The absence of imaginary modes in the vibrational spectrum indicated that the optimized structures corresponded to minima on the potential energy surface. On the basis of thermochemical analysis, the total entropy of each system was obtained, as well as the total enthalpy and Gibbs free energy. We noticed that the computed energies for the externally bound CB7:amino acid arrangements were very close to those of the inclusion complexes, and it was not possible to establish definitively which one prevailed in the gas phase based on the DFT calculations. As stated by Kalenius and Nau [80], the ability to distinguish between inclusion versus externally bound arrangements is strongly dependent on the calculation method.

2.4. Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy individuals using a standard Ficoll-Urografin density gradient method (ρ = 1.077 g/cm^3) [81]. PBMCs (1×10^6/mL) were cultured in 24- and 96-well flat bottom plates (CoStar, Washington, DC, USA) in the absence or presence of ConA (final concentration 10 μg/mL). Cells were cultured in RPMI 1640 Medium containing 10% fetal