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Aptamers targeting different functional groups of 17β-estradiol

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ABSTRACT

Aptamers, short synthetic ssDNA or RNA molecules with a specific three-dimensional structure, are promising recognition elements in biosensor technology. In vitro generation of aptamers with high sensitivity and specificity toward a broad range of analytes has been achieved using the systematic evolution of ligands by exponential enrichment (SELEX) process. This iterative pathway of aptamer generation consists of sequential positive and counterselection steps.

The present research aimed to select two sets of ssDNA aptamers which both are able to bind to different functional groups on the cyclopentanoperhydrophenanthrene ring of 17β-estradiol (E2). By repetitively switching between positive selection steps using E2 as target molecule and counterselection steps with nortestosterone as countermolecule, aptamers were successfully selected against the hydroxylated aromatic A ring of E2. Additionally, an aptamer which binds the upper segments of the B, C and D ring of the cyclopentanoperhydrophenanthrene ring of E2 was generated after repetitively swapping between positive selection steps with E2 as target molecule and counterselection steps with dexamethasone as countermolecule. Epitope specificity of the aptamers was demonstrated by evaluating their binding responses toward a number of steroid hormones structurally related to E2.

The selected aptamers with affinities for different functional groups of E2 can potentially be applied to develop a cross-reactive aptasensor. This aptasensor introduces a promising tool for the future of in-field real-time monitoring of a wide range of steroid hormones.

1. Introduction

The release of steroid hormones such as estrogens and androgens into the environment has become an issue of great concern, given their interference with the endocrine system of humans and wildlife [1,2]. A strong demand for efficient monitoring methods has led to the exploration of several new strategies.

Currently, biosensors are offering outstanding benefits for the detection of steroid hormones such as 17β-estradiol (E2). So far, human estrogen receptors have proven their effectiveness in molecular recognition of E2, but their specificity is rather limited due to their binding affinity for xeno-endocrines other than E2 [3]. E2 antibodies are offering a valuable alternative as recognition elements because of their structure-specific binding, but applying them as receptor molecules has some functional limitations, including relative instability and complex production and purification steps from animals or cell lines. In addition, production of antibodies against target molecules that are inherently less immunogenic (e.g., small molecules) is difficult to achieve [4]. Therefore, there is a significant need to establish a biosensor that incorporates receptor molecules which are able to overcome these problems. In this context, aptamers have been emerged as extremely promising recognition elements for detecting small molecules such as E2 [5].

An aptamer is a short synthetic ssDNA or RNA oligonucleotide with a high affinity and specificity toward a certain target due to its characteristic base sequence and steric configuration [6]. Since aptamers are selected in vitro, they can be reproducibly synthesized without any target restrictions. Furthermore, experimental parameters such as temperature, ionic strength and pH can easily be adjusted according to the use of the selected aptamers in future

Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; E2, 17β-estradiol; SPR, surface plasmon resonance; FC, flow cell; RU, response units; SA, streptavidin.

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applications [4]. Moreover, aptamers are ideal molecular recognition probes for small molecules because of their high degree of epitope selectivity [7].

Aptamer selection is usually carried out via an in vitro process named systematic evolution of ligands by exponential enrichment (SELEX) starting from a random oligonucleotide library via iterative positive selection steps of target-binding, separation, amplification and conditioning. These steps are repeated until the random library is transformed into an enriched library mainly consisting of target-binding oligonucleotides. To eliminate cross-recognition of structurally related non-target molecules or to direct aptamers toward a predefined functional group on the target, positive selection steps need to be alternated with counterselection steps. During counterselection steps, oligonucleotides showing binding affinity for a particular compound structurally related to the target of interest get removed from the library [8].

This study aimed to identify two sets of ssDNA aptamers, both showing high affinity toward different functional groups on the cyclopentanoperhydrophenanthrene ring of E2. Therefore, two independent SELEX procedures (SELEX A and B) were performed. E2 was used as target molecule during positive selection steps of both procedures, ensuring that both sets are able to bind to E2. During counterselection steps, dexamethasone (SELEX A) or nortestosterone (SELEX B) were applied in order to direct both aptamer sets to different functional groups of E2.

Selection of aptamers against different functional groups on the cyclopentanoperhydrophenanthrene ring of steroids can open up new avenues in the development of a cross-reactive aptasensor for the detection of a broad range of steroid hormones. To our knowledge, the present study is the first research to select aptamer sets against two different functional groups of E2.

2. Materials and methods

2.1. Materials

DNA was purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). E2 Sepharose® 6B affinity chromatography beads, Dexamethasone Sepharose® 6B Novel Immobilized Steroid Beads and Nortestosterone Sepharose® 6B Novel Immobilized Steroid Beads (Fig. S1) were supplied by Polysciences Inc. (Eppelheim, Germany). Steroids E2, α-ethinylestradiol, androstenedione, cortisone, deoxycholic acid, estrone and testosterone (Fig. S2) and blocking solutions Bovine Serum Albumin (BSA) and marvel were obtained from Sigma (Diegem, Belgium). Synthetic blocker NB3025 was purchased from NOF corporation (Tokyo, Japan). Sonicated salmon sperm ssDNA, the TOPO® TA Cloning® Kit (including TOP 10 chemically competent *E. Coli* cells and pCR® 2.1-TOPO vector system), Lennox L agar, the ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM® 310 Genetic Analyzer were received from Life Technologies (Carlsbad, US). A Biacore T200 surface plasmon resonance (SPR) was supplied by GE Healthcare (Uppsala, Sweden).

2.2. In vitro *selection of E2-binding aptamers*

Selecting two sets of ssDNA aptamers toward different functional groups on the cyclopentanoperhydrophenanthrene ring of E2 was performed by means of two independent SELEX procedures comprising positive and counterselection steps.

Starting material for both procedures were a random oligonucleotide library and target/counter molecules immobilized onto sepharose beads. Two analog libraries (SELEX A and SELEX B) consisting of 10^{15} molecules of ssDNA were used. These libraries were comprised of a central random region of 40 nucleotides (40N) flanked on either side by a 20-nucleotide-long primer binding region which is necessary for amplification and cloning purposes. 5′-AGCAGCACAGAGGTCAGTTC-40N-CCTATGCGTGCTACCGTGAA-3′ (SELEX A) 5'-TGTGTGTGAGACTTCGTTCC-40N-CAGCAAGGCATCA-GAGGTAT-3' (SELEX B). E2 Sepharose® 6B affinity chromatography beads were used for positive selection in both SELEX A and B. Dexamethasone Sepharose® 6B Novel Immobilized Steroid Beads and Nortestosterone Sepharose® 6B Novel Immobilized Steroid Beads were used for counterselection in SELEX A and B, respectively. All beads were blocked using a blocking solution containing 1% BSA, 1% marvel, 4% synthetic blocker NB3025 and an excess of sonicated salmon sperm ssDNA.

Each selection cycle of the iterative SELEX process consisted of a counterselection step, a positive selection step and another counterselection step prior to PCR amplification and conditioning. For each positive selection step, a homogeneous suspension of target-linked and blocked beads was washed 3 times with 1 × PBS. The oligonucleotide pool was dissolved in binding buffer (10 mM HEPES, pH 74 (SELEX A) or 1 × phosphate buffered saline (PBS) with 10% ethanol, pH 74 (SELEX B)), denatured at 90 °C for 5 min and cooled at ice for 5 min. Thereafter, the oligonucleotide pool was added to the target-linked beads and incubated at room temperature for 1 h with rotation. The unbound oligonucleotides were removed by 5 washing steps using washing buffer [1 × tris buffered saline (TBS)]. The bound oligonucleotides were eluted from the target by incubating the target-DNA complex in elution buffer (tris-EDTA (TE) buffer + 3 M urea, pH 8) at 80 °C for 5 min. Finally, the eluted oligonucleotides were purified by means of phenol–chloroform extraction and sephadex purification before using them for the next selection step. For each counterselection step, counter molecule-linked and blocked beads were exposed to the oligonucleotide pool as described for positive selection steps. However, after the incubation period unbound oligonucleotides were collected and purified before further use. The conditioning step following PCR amplification included the removal of PCR by-products on agarose gel and the transformation of dsDNA into ssDNA by means of enzymatic digestion. After conditioning, the oligonucleotide pool was ready to start a new selection cycle.

Enrichment during the SELEX process was monitored by means of remelting curve analysis using qPCR according to Vanbrabant et al. [9]. In short, after each selection cycle a fraction of the DNA pool was collected. Thereafter, amplification of this fraction in the presence of SYBR green was followed by a first melting curve analysis, a short reannealing phase at 72 °C and a second melting curve analysis. The SELEX process was terminated when a clear shift in second melting curve, an indication of enrichment, was detected.

2.3. Cloning and sequence analysis of selected E2-binding aptamers

The enriched oligonucleotide pools containing the collected dsDNA products of the 5th (SELEX A) and 10th (SELEX B) selection cycle were transformed into a pCR® 2.1-TOPO vector system and cloned by means of a TOPO® TA Cloning® Kit for subcloning with TOP 10 chemically competent *E. Coli* cells [10]. *E. Coli* cells were grown overnight on Lennox L agar plates. DNA isolation and amplification of individual clones was followed by highly specific amplification and sequencing using the ABI Prism® BigDye® Terminator v3.1Cycle Sequencing Kit on a ABI PRISM® 310 Genetic Analyzer.

The secondary structure analysis of aptamers was performed by free energy minimization algorithms according to Zuker using Mfold software [11]. Predictions of G-quadruplex formations were done by means of the internet tool QGRS Mapper [12].