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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].

2.4. Characterization of selected E2-binding aptamers

The affinity and the specificity of the selected aptamers for specific functional groups on E2 were elucidated using a Biacore T200 according to the SPR principle [13,14].

Streptavidin (SA) sensor chips were immobilized by a standard immobilization procedure with filtered 1 × HBS-EP$^+$ (10 mM HEPES, 500 mM NaCl, 3 mM EDTA and 0.05% v/v surfactant P20) as running buffer. Using SA-biotin coupling, flow cell (FC) 1 and 3 were immobilized with biotinylated random DNA (5′-biotin-TTTTT-TAGCAGCACAGAGGTCAGTTCGCCTGTAAGGTGGTCGGTGTGG CGAGTGTGTTAGGAGAGATTGCCCTATGCGTGCTACCGTGAA-3′) as a reference and FC 2 and 4 with biotinylated aptamer. Both the random DNA and the aptamers were enlarged using 5T's at the 5′ biotin binding site to give them more flexibility for target-binding.

To determine the binding affinity of the selected aptamers, different concentrations of E2 ranging from 0.1 μg/ml to 50 μg/ml were passed over the immobilized FCs via a standard kinetics/affinity procedure with filtered 1 × PBS and 10% ethanol as running buffer. Sensorgrams, depicting the response in function of the time, were double referenced. This includes subtraction of blanks (buffer without E2) from the datasets once the contributions from the reference FC are substracted ($[FC\,2 - FC\,1]_{concX} - [FC\,2 - FC\,1]_{buffer}$; $[FC\,4 - FC\,3]_{concX} - [FC\,4 - FC\,3]_{buffer}$). The concentration that led to half the maximum response was defined as the dissociation constant (K_D) of the aptamer.

To define the epitope specificity of the selected aptamers, fixed concentrations of E2 and 6 additional steroids were injected over the immobilized FCs via a standard binding procedure with filtered 1 × PBS and 10% ethanol as running buffer. Sensorgrams were double referenced. Subsequently, binding responses were corrected for molecular weight (MW) and normalized with respect to the MW adapted response of E2, which was set at 100%. Negative values were set at zero. All reactions were conducted in triplicate at 25 °C.

3. Results

3.1. Selecting two sets of E2-binding aptamers: sequences and secondary structures

Following SELEX A, 19 clones were sequenced after 5 selection cycles. All clones showed a unique sequence, whereas 9 of them expressed a similar motif of 8 nucleotides (CTCTGCAT). Following SELEX B, 24 clones were sequenced after 10 selection cycles. Results revealed a strong enrichment of 2 G-rich sequences in 18 and 5 clones, respectively. The remaining clone Apta 24B had the same nucleotide composition as clones Apta 19B to Apta 23B except for a C to T transition at position 22 (Table 1).

Secondary structures of the 19 selected sequences of SELEX A and their free energies were predicted using Mfold software. In 68% of all sequences, results revealed the presence of a three-way junction structure consisting of three double helical arms linked via a common cross point. An example of a predicted secondary structure containing a three-way junction is given in Fig. 1. An overview of all predicted secondary structures can be found in the supplementary information (Fig. S3). Predicted ΔG's of the 19 aptamers varied from −10.8 to −3.7 kcal/mol (Table 1).

QGRS mapper was used to predict the formation of G-quadruplexes by the G-rich aptamers of SELEX B. Two possible 4-plane G-quadruplexes were predicted for Apta 1B and 1 potential 4-plane G-quadruplex for Apta 19B and Apta 24B (Table 1). The suggested G-quadruplexes for Apta 19B and Apta 24B were composed of 3 G-repeats forming the G-tetrad planes, whereas the G-quadruplexes of Apta 1B were only composed of 2 G-repeats. This caused the potential quadruplexes of Apta 19B and Apta 24B to be more stable than those of Apta 1B, which is reflected in their higher G-scores. Apta 5A, 6A, 8A, 9A, 11A, 13A, 1B and 19B were chosen for further characterization.

Table 1
Sequences, ΔG values and G-scores of selected E2 aptamers.

Clone ID (SELEX A)	Initially random sequence 5′ → 3′	ΔG (kcal/mol)
Apta 1A	TCTGTATAGGTCATTCTATCGGCGATTCTCGAATAAATGT	−8.23
Apta 2A	**CTCTGCAT**TCGTGGCTGACTATCTTTGGGAATATCAAGCC	−8.3
Apta 3A	GTTGTATTTTGGTGCATTTCAATGTTTTGTTATATGGTGT	−4.74
Apta 4A	CTGATATATTTATTGTAGTGCTATATTTGCTACATGTGTT	−3.69
Apta 5A	GTCCATTATTCTGGTAGCGTTGAACAACATTCAACACGCC	−10.76
Apta 6A	GTCGAATCAGCAC**CTCTGCAT**AGGTTACGTTTATACTGCG	−9.95
Apta 7A	TCCTT**ACTCTGCAT**AGGATGCATACCCTGTCTTGCTAATG	−7.36
Apta 8A	AGCGTC**CTCTGCAT**AGTGATTTACGCTATGTGTACAGTGC	−9.5
Apta 9A	CATGA**CTCTGCATA**GGTCTAATGAAACTAGTTCTTATTTA	−10.9
Apta 10A	CAGACTATACCCTCCACCGCTCCGATAAATAGGTGTTGGC	−6.42
Apta 11A	CAAGTCGTACGACACAGGGTAATCCTGTTTGGTAAAACGA	−9.89
Apta 12A	GGGACGTTCGCAACTGTAAAT**CTGCAT**AAGGTCTTTCGAT	−5.99
Apta 13A	TTATGTCGAACTAAATTTGGCCTGACCAAGCCGAATTTGA	−10.67
Apta 14A	CTCTAATATTCAGTTTAATATTGTGTTGCCAGGCGCCATT	−9.11
Apta 15A	GTAAGGACTGGTGCACATGGATGTAATAATAGTGCAGCGA	−9.8
Apta 16A	TGTTGTACTGGGTCCAACGATTTATATGAGTTATGGGCCA	−5.52
Apta 17A	TCTACTCTATACCCCACTTTCTTACTTCAAC**CTCTGCAT**T	−5.7
Apta 18A	TCGGGCCCCGTATTTACTTTAATTATGTAC**CTCTGCAT**TA	−6.03
Apta 19A	ATTATCACCATGGAGTCGATTTGAAATC**CTCTGCAT**ATTA	−7.16

Clone ID (SELEX B)	Initially random sequence 5′ → 3′	G-score
Apta 1 → 18B	GGCGAT**GG**GGTAGGG**GG**TGTGGAG**GG**GCCGGAC**GG**AGGGG	21
	(*TGTGTGTGAGACTTCGTTCCGGCGATGGGGTAGGGGGTGTGGAGGGGCCGGACGGA**GGGG**CAGCAAGGCATCAGAGGTAT*)	−14
Apta 19B → 23B	CCCGGTCGGTG**GGG**TAGG**GGG**CGTGGAGTCACC**GGG**G**GGG**	31
Apta 24B	CCCGGTCGGTG**GGG**TAGG**GGG**TGTGGAGTCACC**GGG**G**GGG**	31

Sequences of clones obtained after 5 (SELEX A) and 10 (SELEX B) selection cycles. Fixed primer binding regions are omitted.
SELEX A: a common motif is highlighted. ΔG values (kcal/mol) of predicted secondary structures were determined using Mfold.
SELEX B: nucleotides involved in G-quadruplex formation are highlighted. G-scores, denoting the likelihood to form a stable G-quadruplex, were predicted by QGRS mapper. For Apta 1B, two potential G-quadruplexes were predicted. One of them includes a fixed primer binding region. Therefore, fixed primer binding regions for this sequences were indicated in italic.

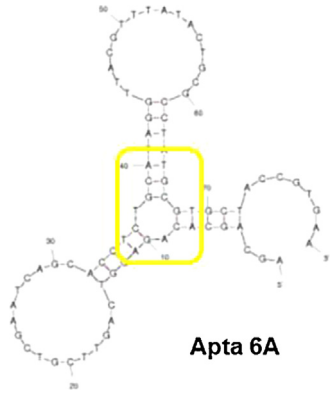

Fig. 1. An illustration of a secondary structure containing a three-way junction as predicted by Mfold. The three-way junction is highlighted by a squared box.

3.2. Characterizing two sets of E2-binding aptamers: affinity and specificity toward E2 and additional steroids

Rapid assessment of the binding affinities of the selected aptamers was obtained using novel SPR methodology. Therefore, binding characteristics were examined toward different concentrations of E2. Results showed a fast association and dissociation pattern (Fig. S4). Due to this specific binding pattern, general K_D values were calculated rather than thermodynamic equilibrium constants k_{on} (association) and k_{off} (dissociation) [15]. The K_D values of the six different ssDNA aptamers of SELEX A were in the range of 27–124 μM (Table 2). Among them, the K_D of Apta 8A (124 μM) was remarkably higher than the other aptamers. Therefore, this aptamer was not further analyzed. The binding affinities of the two aptamers from SELEX B were 10–100 times higher than those of SELEX A, which is represented by their K_D values of 0.9 and 6 μM (Table 2).

Table 2
K_D values of selected E2-binding aptamers as determined by SPR methodology.

Clone ID	K_D value (μM)	Clone ID	K_D value (μM)
Apta 5A	27	Apta 1B	0.9
Apta 6A	36	Apta 19B	6
Apta 8A	124		
Apta 9A	52		
Apta 11A	51		
Apta 13A	63		

In order to assess the specificity of the selected aptamers and to elucidate the most important interacting functional groups of E2 with each of these aptamers, six steroid hormones (α-ethinylestradiol, androstenedione, cortisone, deoxycholic acid, estrone and testosterone) were selected based on their structural similarity to E2 (Fig. S2). By means of SPR methodology, binding characteristics of each aptamer toward these hormones were examined. Results showed that the aptamers of SELEX A behave differently regarding the tested steroids in comparison to those of SELEX B. The 5 aptamers of SELEX A show various binding patterns (Fig. 2). Besides E2, they were all able to bind α-ethinylestradiol and testosterone to a greater or lesser degree. Regarding other steroids, however, aptamer–target interaction was dependent on the

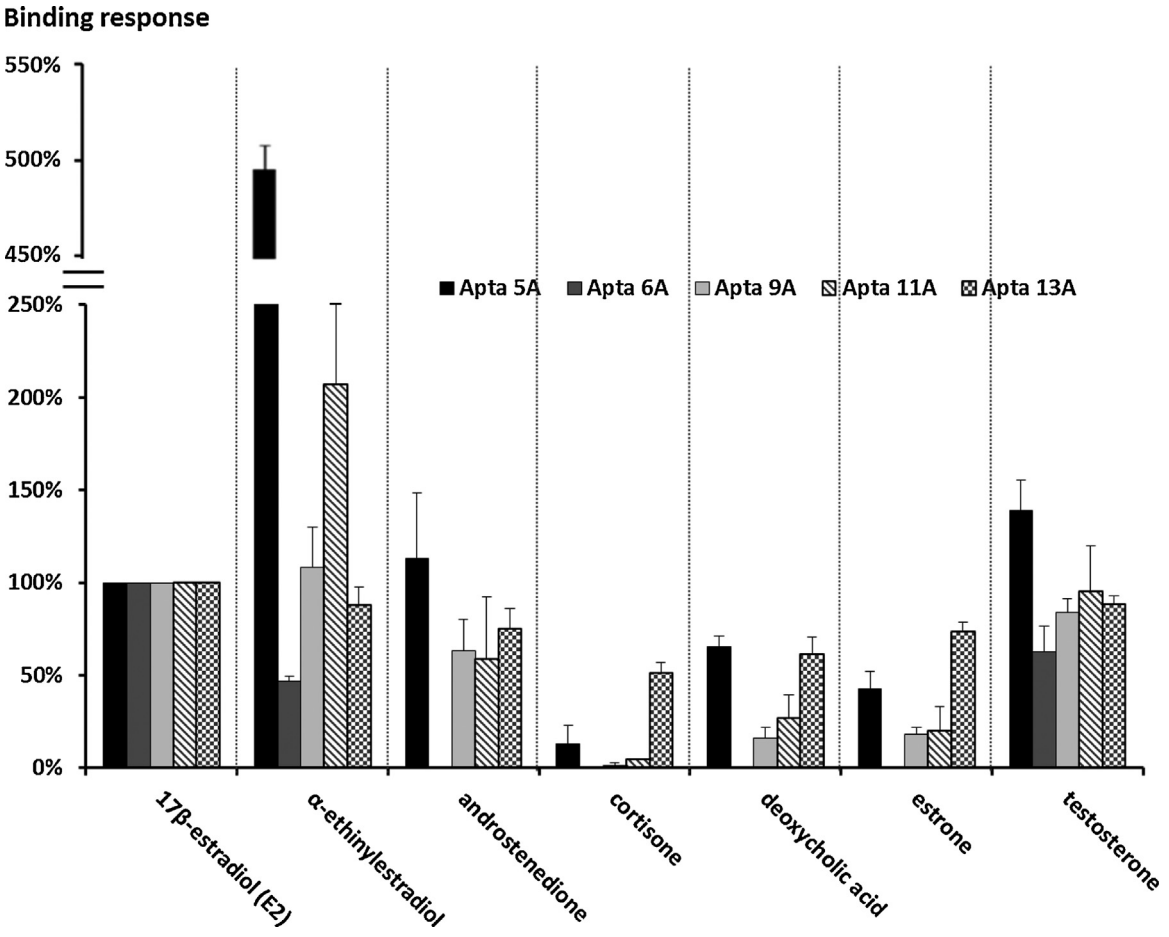

Fig. 2. Binding responses of selected E2-binding aptamers of SELEX A toward several steroid hormones determined by SPR methodology. The average binding responses are presented as a percentage of the E2 binding response (which was set at 100%) ± standard deviation ($N = 3$).

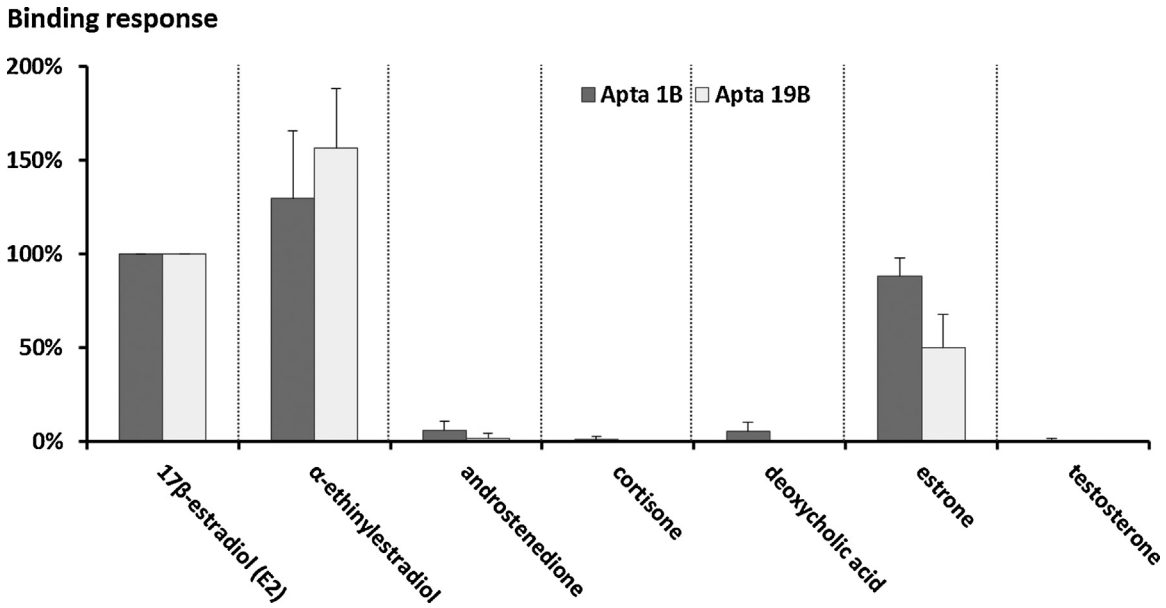

Fig. 3. Binding responses of selected E2-binding aptamers of SELEX B toward several steroid hormones determined by SPR methodology. The average binding responses are presented as a percentage of the E2 binding response (which was set at 100%) ± standard deviation ($N = 3$).

binding characteristics of the particular aptamer. Apta 6A showed a clear distinction between binding steroids E2, α-ethinylestradiol and testosterone and non-binding steroids androstenedione, cortisone, deoxycholic acid and estrone. The other aptamers of SELEX A were able to bind E2, α-ethinylestradiol and testosterone. Their binding responses toward other steroids, however, differed dependent on the particular aptamer.

Conversely, both aptamers of SELEX B are similar in their binding pattern (Fig. 3). Both were binding E2 as well as α-ethinylestradiol and estrone. They were not able to bind androstenedione, cortisone, deoxycholic acid and testosterone. Small differences in binding responses toward the three binding steroids can be seen. α-ethinylestradiol shows a slightly higher binding response and estrone a lower binding response compared with E2.

4. Discussion

In order to select aptamers which target different functional groups of E2, two independent SELEX procedures were performed using E2 as target molecule during positive selection steps and dexamethasone or nortestosterone during counterselection steps. E2 and dexamethasone exhibit several differences in functional groups on their cyclopentanoperhydrophenanthrene ring. In contrast, the only structural difference between E2 and nortestosterone is the A ring of their cyclopentanoperhydrophenanthrene ring. E2 contains an aromatized A ring with a hydroxyl group whereas nortestosterone shows a ketone group on its A ring [16]. Therefore, the selected aptamers in both procedures were foreseen to target different functional groups of E2. Aptamers are mainly targeting functional groups that are expressed on the molecule applied during positive selection steps which are not present on the molecule used during counterselection steps [17].

Both SELEX procedures successfully resulted in the enrichment of originally random DNA pools (Table 1). All clones obtained by SELEX A displayed a unique sequence, but a common motif was detected in 47% of the clones. Formation of this motif is a clear indication of transition of a random library into an enriched library. However, aptamers without this motif showed good binding affinity for E2. Probably, this motif may only contribute to the formation of specific secondary structures. Several aptamers with and without motif converged to three-way junction structures. Since random DNA oligonucleotides are able to form all kinds of secondary structures without preference for a specific structure, the increased presence of this three-way junction structure further supports the success of SELEX selection. A three-way junction structure for DNA aptamers was previously described by Kato et al. [18]. The authors selected several cholic acid-binding aptamers which could all fold into three-way junction structures. In these structures, cholic acid was suggested to interact with a hydrophobic cavity formed by three stems linked to a common cross-point [18]. Later, Yang et al. [19] used a structurally biased DNA library to generate a series of 4 three-way junction aptamers that bind with varying affinity and selectivity to several steroids. In contrast with SELEX A, SELEX B resulted in the clear enrichment of two sequences. Both sequences tended to fold into G-quadruplexes [12]. G-quadruplex structures couple a common scaffold to varying loop motifs that enable the recognition of target molecules [20]. These structures are not only biologically relevant since they frequently occur in eukaryotic genomes [21], also a significant proportion of aptamers described in literature display this type of architecture [22–24]. For SELEX B, the aptamers were selected in 1 × PBS with 10% ethanol containing K^+ and Na^+ ions. K^+ and Na^+ are known to thermodynamically and kinetically stabilize G-quadruplexes by fixing themselves into the assembled quadruplex structure [25]. The formation of these structures can thus be assumed for aptamers obtained by SELEX B. In SELEX A, on the other hand, a K^+ and Na^+ free Hepes buffer was used. Stabilization of potential G-quadruplexes could not occur here, resulting in the absence of this kind of structures for aptamers of SELEX A. Besides, the difference in library design between the two SELEX procedures could have an important impact on the final structures of the selected aptamers. As expected, stronger enrichment appeared after SELEX B in comparison with SELEX A. Since E2 and dexamethasone are structurally different, aptamers obtained by SELEX A cannot be directed against one particular epitope. This caused a heterogeneous population of DNA strands to bind to E2. On the other hand, E2 and nortestosterone are structurally more similar. Hence, this caused SELEX B to be a more stringent selection procedure than SELEX A. Aptamers obtained by SELEX B can be

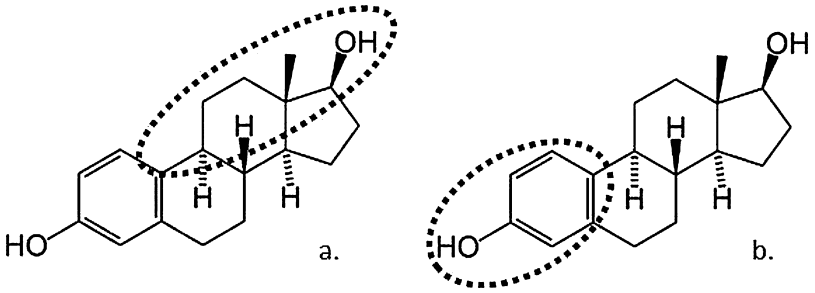

Fig. 4. Schematic illustration of the chemical structure of E2 with the most crucial epitopes for binding of Apta 6A (a) and Apta 1B/Apta 19B (b).

directed against one particular functional group of E2. This way, few different DNA strands were able to bind. However, selecting these few different strands in SELEX B required more cycles than selecting a heterogeneous pool of aptamers in SELEX A. The heterogeneity in functional active aptamers also clearly illustrates that molecular recognition of E2 can be achieved by different kinds of ssDNA sequences.

All selected aptamers showed affinities in the low μM range as determined by SPR methodology (Table 2). In general, K_D values of aptamers for small molecules such as diclofenac, tryptophan and ATP are typically in the high nM to low μM range [26], which is markedly higher than aptamers for proteins and peptides such as thrombin and IgE [8]. Moreover, aptamers for hydrophobic molecules typically show lower affinities than aptamers for hydrophilic molecules, since hydrophobic molecules are less attractive for strongly hydrophilic ssDNA [27]. E2 is a hydrophobic compound containing two polar groups [16]. Therefore, detecting K_D values in the low μM range in this study was reasonable. The higher affinities of the aptamers of SELEX B in comparison with SELEX A are due to the more stringent selection character of SELEX B.

Ultimately, both sets of selected E2-binding aptamers have shown to be oriented toward different functional groups of E2. Their binding behavior toward several steroids was different during measurement by SPR methodology (Figs. 2 and 3). The 5 tested aptamers of SELEX A showed various binding patterns. Besides E2, they were all able to bind α-ethinylestradiol and testosterone. Regarding other steroids, however, aptamer–target interaction was dependent on the binding characteristics of the particular aptamer. Apta 6A showed a clear distinction between binding steroids E2, α-ethinylestradiol and testosterone and non-binding steroids androstenedione, cortisone, deoxycholic acid and estrone. Therefore, the epitope for this aptamer could be defined as the upper part of the B, C and D ring of the cyclopentanoperhydrophenanthrene ring of steroids (Fig. 4a). This epitope is present on the binding steroids and absent on the non-binding steroids. The other aptamers of SELEX A were also able to bind E2, α-ethinylestradiol and testosterone. Their binding responses toward other steroids, however, were different dependent on the particular aptamer. Various binding behaviors suggest that these aptamers are all oriented toward slightly different epitopes of E2. Based on our current information, the epitopes of these aptamers cannot be defined. Epitope heterogeneity may be caused by the large structural difference between E2 and dexamethasone, the countermolecule used during SELEX A. Conversely, both aptamers of SELEX B were similar to each other in binding pattern. Both were binding E2 as well as α-ethinylestradiol and estrone. They were not able to bind androstenedione, cortisone, deoxycholic acid and testosterone. These results indicate that both aptamers are directed toward the hydroxylated aromatic A ring (Fig. 4b). Small differences in binding responses toward the three binding steroids could be seen. α-ethinylestradiol showed a slightly higher binding response and estrone a lower binding response compared with E2. This is probably due to structural differences surrounding the defined epitope which among others leads to different torsion angles between the A ring and rings B, C and D as well as to different charge distributions over the entire molecules [16]. This suggests that not only a specific epitope, but also the context surrounding an epitope is crucial for aptamer–target interaction.

To the best of our knowledge, this study is the first one to select aptamer sets against different functional groups of E2. In the future, the selected E2-binding aptamers can be used to develop a cross-reactive array for in-field monitoring of a broad range of steroids present in food and environmental samples. Depending on which epitopes are present or absent in a certain steroid hormone, the hormone will exhibit different binding responses toward the different aptamers of the sensor. So each type of steroid hormone will show its own characteristic binding pattern when analyzed, making identification possible. Furthermore, on the fundamental research front, in-depth knowledge about how to design a SELEX procedure in order to direct aptamers toward predefined epitopes may help the future selection of new aptamers.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.10.013.

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