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High-Affinity Nucleic-Acid-Based Receptors for Steroids

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Abstract

Artificial receptors for hydrophobic molecules usually have moderate affinities and limited selectivities. We describe three new classes of high affinity hydrophobic receptors for non-aromatic steroids based on deoxyribonucleotides, obtained through five high stringency selections coupled with tailored counter-selections. The isolation of multiple classes of high affinity steroid receptors demonstrates the surprising breadth of moderately sized hydrophobic binding motifs (<40 nucleotides) available to natural nucleic acids. Studies of interactions with analogs indicate that two classes, four-way junctions and 4xG_N motifs, comprise receptors with shapes that prevent binding of specific steroid conjugates used in counter-selections. Furthermore, they strongly prefer non-hydroxylated steroid cores, which is typical for hydrophobic receptors. The third new class accommodates hydroxyl groups in high-affinity, high-selectivity binding pockets, thus reversing the preferences of the first two classes. The high-affinity binding of aptamers to targets efficiently inhibits double-helix formation in the presence of the complementary oligonucleotides. The high affinity of some of these receptors and tailored elimination of binding through counter-selections ensures that these new aptamers will enable clinical chemistry applications.

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The SELEX protocol by rounds (Table S1), sequences of aptameric sensors (Table S2), G-quadruplex predictions (Table S3), additional steroid aptameric sensors and their characterization results (Supplementary Fig. S2–10), and graphic illustration of the aptameric sensor fluorescence assay (Fig. S11)

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INTRODUCTION

Steroid hormones play important roles in biological signaling.[1–4] We were primarily interested in developing artificial oligonucleotide-based receptors or aptamers[5, 6] for steroids[7–11] as biosensor components in rapid mix- or dip-and-measure assays. On the one hand, an aptamer-based approach to recognizing steroids is counterintuitive, because, in contrast to protein-based receptors for steroids, unmodified oligonucleotides do not have a variety of hydrophobic groups to enable and to fine-tune hydrophobic cavities.[8–14] In fact, the nucleic acid hydrophobic binding motifs generated thus far have modest affinities and marginal selectivities, thereby limiting the resolution of cross-reactive arrays,[7–11] which were our primary initially intended applications. For example, our best previously reported aptameric sensors had dissociation constants >500 nM,[11] a result consistent with previous reports.[12, 14]

On the other hand, the unique advantages of oligonucleotide-based receptors over other types of receptors is that they can be readily isolated by streamlined *in vitro* selection and amplification procedures,[15] and tailored by rational adjustments in stringencies and focused counter-selections.[11, 16] However, we were concerned that aptamer receptors defined by shorter sequences, *e.g.*, < 40 nucleotides, would have only limited affinities for purely hydrophobic targets, because the free energy benefits of hydrophobic interactions are directly proportional to contact surface areas.[17, 18] For hydrophilic steroids, such as those containing hydroxyl groups, selectivity may be easier to achieve due to more oriented polar interactions[19], but these may interfere with our goal of achieving high-affinity receptors with limited size. We were further concerned that concomitant increases in binding pocket complexity would make aptamers even less likely to be successfully isolated in selections.

As an additional consideration, we were intrigued by a report that modified steroids could be used to control gene expression through interactions with structural motifs based on single-stranded DNA.[20] In this context, any isolation of simple yet high-affinity receptors for natural steroids would indicate possible natural roles for interactions of steroids and short oligonucleotides whenever single-stranded DNA is generated in cellular processes.

BACKGROUND

Our previous work on hydrophobic receptors[8–11] focused on three-way junctions (TWJs).[13] Two complementary oligonucleotides form a duplex that terminates with an unstacked base pair, a hydrophobic surface exposed to water.[7, 13] Three such surfaces exist in three-way junctions, defining hydrophobic pockets that can recognize a variety of steroids (Figure 1A).[7] Previously, we tested modified TWJs[9–11], including those isolated through *in vitro* selection and amplification (also known as Systematic Evolution of Ligands by Exponential Enrichment or SELEX). Mutations in three-way junctions led to more complex hydrophobic pockets with differential cross-reactivities.[8–10, 21] We screened collections of three-way junctions with randomized N_8 regions with 4^8 full-length members.[11] While this collection guaranteed full coverage of the theoretical sequence space during selection and reproducible results of repeated selections, its disadvantage was the limited affinity and selectivity of the resulting receptors (Figure 1).

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The specific and quantitative goals of the current study were to overcome these limitations of the current three-way junctions and to isolate high affinity receptors based on oligomers with no available aliphatic side chains (examples of these are proprietary SOMAmers reported to be outstanding ligands for proteins[22]) in *routine* selections, *i.e.*, executable in any laboratory, without additives and modifications, while also using standard Taq polymerase. In the process, we also planned to assess the potential of these receptors for applications in minimal cross-reactive arrays focusing on minor steroid components, instead of dominant components, to compare affinities and selectivities with other hydrophobic receptors, and to create increased understanding of the scope, limitations, and success rates of solution-phase selection protocols that we recently optimized.[15]

RESULTS AND DISCUSSION

Deoxycorticosterone 21-glucoside *vs*. Dehydroisoandrosterone 3-sulfate.

In previous selections,[11] we used two soluble steroid conjugates, deoxycorticosterone 21-glucoside (DOG, [**1**]) and dehydroisoandrosterone 3-sulfate (DIS, [**2**] also, DHEA-S) (Scheme 1). These two steroids have almost identical hydrophobic shapes and volumes and thus, they represent a significant challenge for oligonucleotide-based receptors (and other artificial receptors) to achieve selectivity, particularly in light of adaptive binding of many hydrophobic pockets.[5] Past selections with three-way junction variants (Supplementary Fig. 1A) resulted in aptameric receptors and related sensors such as DOGS.1 and DISS.1 (Figure 1B, C) with estimated K_d's of 50 μM and 600 nM respectively, and only moderate (DISS.1), if any (DOGS.1), selectivities for one over the other. Further high-stringency selections with focused counter-selections did not lead to improvements in selectivity with this collection of oligonucleotides. These results suggested that the complexity of the binding pocket needed to be increased. Instead of receptors focusing on the α and β faces of these steroids, we isolated motifs that accommodated the edges of either ring A or ring D (Scheme 1), where the structures of these two steroids diverge. Therefore, to improve selectivity, we needed to eliminate simple pockets based on three-way junctions efficiently, allowing us to focus selections on more rare and complex pockets with additional hydrophobic surfaces in desired orientations.

Accordingly, we started with selection protocols for these two steroids, DOG [**1**] and DIS [**2**], as described before, but with three modifications: (1) We increased the complexity and size of the available binding pockets by using oligonucleotide libraries with larger (N_{22}) randomized regions and one constant stem (Supplementary Fig. 1B). With these libraries, we have reasonable coverage of DNA-based receptor-space in individual selections. By fixing two stems, we biased selections towards the junctions, while simultaneously leaving sufficiently long random regions to allow alternative folds, if favored by evolutionary pressures. (2) We introduced stringent counter-selections with non-targets at high-concentrations in every cycle of selection to minimize receptors recognizing both steroids (*e.g.*, TWJs). Nevertheless, due to the stochastic nature of binding, the abundance of junction motifs, and existence of slowly equilibrating alternative conformations that may kinetically protect TWJs from initial counter-selections, it may not be impossible to eliminate cross-reactivity completely. (3) We introduced gradual decreases in target concentrations as

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selections progressed. Because more complex binding pockets capture larger hydrophobic areas, we expected hydrophobic targets to be more tightly bound and thus, aptamers with higher affinities would be selected. These three rational modifications of the selection protocols recapitulate best practices of past solid-state selections now applied to solution-phase selections.

From the selection with DOG [**1**] as a target, we isolated an aptamer DOGS.2 (Figure 2A) (*cf.*, related DOGS.3 and DOGS.4, Supplementary Fig. 2) with a calculated dissociation constant of ~30 nM (Figure 2, Supplementary Fig. 3), which is approximately two orders of magnitude below previous aptamers targeting this steroid. Programs for secondary structure prediction (e.g., *Mfold*[23]) suggest a four-way junction, consistent with an increase in hydrophobic interactions leading to higher affinity; these programs are not sufficiently reliable for us to exclude a three-way junction with longer spacers that optimize orientations of the stems. The observed selectivity of this aptamer for DOG [**1**] over DIS [**2**] (its counter-target) was excellent, with minimal response to **2** observed (Figure 2D).

After unsatisfactory attempts to identify high affinity aptamers with opposite selectivities, (*i.e.*, for DIS [**2**] over DOG [**1**]) using N_{22} libraries, we performed selections using N_{30} libraries with DIS [**2**] as the target and DOG [**1**] as the counter-target. (Further counter-selections to minimize the presence of three-way junctions are described in Table S1). These selections led to DISS.2 (Figure 2), DISS.3, and DISS.4 (Supplementary Fig. 4, 5). These three aptameric sensors showed strong-to-moderate preferences for DIS over DOG, but could not be assigned any obvious junction motifs. All three aptamers shared a common pattern of at least four G-pairs or -triplets that might be induced by target binding to form G-quadruplexes (Table S3). As such, we named this motif $4XG_N$ (with N being from two to five G's), although we cannot exclude that more than four pairs or triplets are needed. Stable G-quadruplexes were previously identified to interact with steroids in a high-throughput screening (as well as *i*-motifs paired with G-quadruplexes).[20, 24] Moreover, a recent solid-state toggle-selection of aptamers for aromatic steroids, *i.e.*, binding to two targets,[14] reported similar sequences with moderate affinities (the lowest K_d was ~0.9 μM)[14]. Thus, our hypothesis that the non-aromatic steroid core is interacting with stacked G-quadruplex-like structures is consistent with earlier work.

We used responses of DOGS.2 and DISS.2 to other non-target steroids to understand the properties of the aptamer binding sites. The DOGS.2 aptamer is insensitive to modifications of a group that is displayed at the β-face at C.17 in the D ring; it binds with almost equal strength to a series of analogs (DC [**3**], PRO [**4**], TES [**5**]). Thus, we propose that substituents at the C.17 β-position are protruding outside of the binding pocket. The counter-target DIA [**6**], an unconjugated analog of DIS [**2**], shows residual binding to DOGS.2, in contrast to DIS [**2**] itself. Low-affinity binding of DIA [**6**] is also consistent with a hydrophobic pocket, with the sulfate being more difficult to accommodate than a hydroxyl group at the 3β site in the A ring (Figure 2).

The hydrophobic pocket of the DISS.2 aptamer was significantly more permissive than that of DOGS.2 and was able to accommodate all analogs **2–6** that did not have a glucoronate conjugated at C.21-OH, *e.g.*, DIA [**6**] and PRO [**4**], which had similar affinities for this

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sequence. Introduction of additional hydroxyl groups, as in DC [**3**] and TES [**5**], led to results consistent with selectivity based on a hydrophobic pocket encompassing C.17 substituents. Using cholesterol analogs with increased solubility, we investigated whether larger hydrophobic groups can be accommodated by the three DIS aptamer sensors identified here. The analog DHC [**7**] with an additional 25-hydroxyl group was barely soluble. Nonetheless, we could clearly observe reproducible fluorescence only with DISS.3 at low concentrations (<500 nM, with a LOD of ~10 nM) of DHC [**7**], presumably below critical micellar concentrations. Concentration-dependent responses rose faster than with other steroids indicating tight binding and a K_d below 100 nM. These findings also showed that while the DHC analog [**7**] had similar binding to DIA [**6**], the DHC alkyl chain was also accommodated in a hydrophobic pocket of this particular sensor, but not other sensors of this series (Supplementary Fig. 4G–H).

Finally, DOGS.2 and DISS.2–4 were sensitive to the introduction of a hydroxyl group at all tested positions in the steroid core (7β, 11β, and 17α in THC [**8**], 11-DC [**9**], CCS [**10**], and CS [**11**]), leading to a decrease in binding, consistent with a hydrophobic pocket engulfing all these positions.

Thus, with these two selections, we successfully isolated two motifs with properties that fit our search criteria, that is, high-stringency selections yielded higher-affinity hydrophobic pockets and high-stringency counter-selections minimized interactions to particular conjugates. The most unexpected aspect of the selection for DOG [**1**] was the exceptionally high aptamer affinities, indicative of greater hydrophobic contacts with each receptor. Moreover, the DOG aptamers showed selectivity against DIS [**2**], which was indicative of an additional steric element preventing DIS binding. Thus, these hydrophobic binding pockets were more functionally complex than three-way junctions.

The generation of aptamers with imperfect selectivity for DIS [**2**] *vs.* DOG [**1**] does not mean that even higher-affinity specific receptors do not exist. The nature of selections using libraries with larger randomized sequences is that the capture of rare motifs cannot be guaranteed, even after repeated selections. There are other potential obstacles. It is possible that the sulfate in DIS [**2**] is a better epitope for counter-selection leading to the highly selective DOGS.2 sensor. This may be in contrast to the neutral C.17 side chain in DOG [**1**], part of which may be easier to accommodate in hydrophobic pockets. The lower affinity of the DISS.2 sensor compared to the DOGS.2 [**1**] sensor can be attributed to a five-membered D ring being a weaker epitope for hydrophobic binding than a six-membered A ring, due to the smaller surface area of the former. The presence of a double bond in the B ring minimizing continuous availability of α face hydrogens for C-H···π interactions may also contribute to differences in affinities. Some of these hypotheses could be tested through additional selections, so, as our next step, we decided to design the counter-selections with bulkier substituents at the C.17 position, in part because of easy access to commercial sets of targets and counter-targets.

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Testosterone *vs*. Deoxycorticosterone 21-glucoside.

Neither selection for binding to DOG [**1**] or DIS [**2**] nor previous synthetic approaches[9] provided aptamers having both high-affinity and exceptional selectivity for DOG [**1**] over TES [**5**] or *vice versa*. To test directly whether a side chain at C.17 in the D ring is a good counter-selection epitope eliciting a steric hindrance with a hydrophobic surface closer to the edge of D ring, we performed a selection with TES [**5**] as a target and DOG [**1**] as a counter-target. Selectivity for the former over the latter would be useful in high-resolution cross-reactive arrays because it would circumvent a "dominant component effect" in which high concentrations of one component (*e.g.*, DOG [**1**]) prevent quantification of another component (*e.g.*, TES [**5**]).

The results of this experiment were outstanding *vis-à-vis* selectivity for target *vs*. counter-target. The TESS.1 sensor showed no measurable responses to DOG [**1**] (Figure 3B) with a K_d for TES [**5**] of ~80 nM, (Supplementary Fig. 3). The sensor had a strong response to progesterone (PRO, [**4**]), and a weaker, but measurable, response to DC [**3**], indicating that a hydrophobic surface responsible for selectivity over DOG [**1**] was able to accommodate side chains without the glucuronide group. Interestingly, the sensor showed responses to DIA [**6**], and had a series of G-pairs and one triplet, consistent with a G-quadruplex based receptor ($4XG_N$ motif, *vide supra*); in light of this last cross-reactivity, we note that in some antibody-based assays, similar cross-reactivities, for example, of DIS [**2**] with testosterone antibodies[25], were observed.

Other sensors (TESS.2–3) were also highly selective for TES over DOG, but had significantly lower affinities (1.7 μM and 0.7 μM, Supplementary Fig. 3) and different selectivity profiles from each other and from the TESS.1 sensor. The TESS.2 aptamer can tentatively be assigned as having an unstable three-way junction structure, while the TESS.3 sequence is consistent with a potential to form a G-quadruplex (Supplementary Fig. 6, Table S3).

Up to this point, our evolutionary experiments demonstrated that it was possible to achieve selectivity based on substituents on rings A or D. The new receptors help us to increase classification powers over a range of concentrations in cross-reactive arrays. However, in all cases, introduction of additional hydroxyl groups led to reduced affinities, as expected for receptors that are primarily hydrophobic in nature. To identify and to quantify a steroid with hydroxyl groups at low concentrations and in the presence of dominant hydrophobic components, we needed receptors that could accommodate hydroxyl groups, but also prevent binding of hydrophobic analogs. From work on hydrophobic recognition by proteins,[26, 27] we have an understanding that hydrophobic surfaces will most likely be the primary drivers controlling affinity, while substituents like hydroxyl groups are less likely to contribute to affinity, but may have greater impact on specificity.

Aptamers for Hydrocortisone (Cortisol).

Our first target for high-affinity, high-selectivity receptors recognizing a hydroxyl group was hydrocortisone (cortisol or CS [**11**]). None of our previous sensors had appreciable affinity for CS [**11**], thus, if successful here, we expected unprecedented binding motifs. We

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hypothesized that the decrease in availability of hydrophobic surfaces could be counterbalanced, under proper selection pressure, by improved fits in the hydrophobic pockets. This could lead to an increase in selectivity, but would likely mean more complex binding pockets that may be rarer and more difficult to isolate. From a practical perspective, receptors for cortisol with nanomolar affinity would have practical applications in clinical chemistry, particularly since the best reported aptamers only have K_d's of ~6–10 μM.[28, 29]

Compared to deoxycorticosterone (DC, [**3**]), hydrocortisone (CS, [**11**]) has two additional hydroxyl groups at C.11 in the C ring and C.17α in the D ring. This leads to one whole edge along the C and D rings of the steroid becoming more hydrophilic. However, the α face of the steroid core, except the C.17 hydrogen, is still available for CH···π binding, with some increase in positive charge on key hydrogens (*i.e.*, at C.11).

As a result of a high-stringency selection with an N_{30} library, CS [**11**] as a target, and TES [**5**] as one of the counter-targets (the other was aldosterone, to maximize recognition beyond the α-face of the steroid), we obtained a number of high affinity (K_d's 30–140 nM, Supplementary Fig. 3) aptameric sensors CSS.1–3 (Figure 4A–B, and Supplementary Fig. 7) with variable selectivities over DOG [**1**]). All three sensors had very different secondary structures predicted by folding programs, yet they all had convergent sequence motifs (*cf.* Supplementary Fig. 7E) and displayed similar selectivities, responding strongly to CS [**11**] and analogs with keto- or no functional group at position C.11 (corticosterone, **CCS** [**10**] or 11-deoxycortisol, **11-DC** [**9**]). In contrast, removal of the C.17 hydroxyl group led to a drastic reduction in binding, indicating that this group is important for recognition and was incorporated as an epitope in a binding pocket (Figure 4). The convergent sequences and similar selectivities are consistent with a new high-affinity motif incorporating a hydroxyl group. We were intrigued by the possibility that this result could be generalized to other steroids with hydroxyl groups and decided to test one more steroid with a hydroxyl group at a different position in the core.

Aptamers for Aldosterone.

Aldosterone (ALD, [**12**]) was our next choice of target because it has a different hydrophobic shape from all of the other steroids tested. Aldosterone (ALD, [**12**]) has an additional furanose ring formed between the C.11-OH group in the C ring and the aldehyde at the oxidized C.18 methyl group (shared by the C and D rings). Of all the sensors isolated herein, only DOGS.2 recognized ALD [**12**] with an affinity similar to the other mono-hydroxyl steroids (11-DC [**9**] and CCS [**10**]), indicating that DOGS.2 does not incorporate the C.18 methyl group into its binding pocket. Selection for an ALD [**12**] sensor using the same N_{30} library and both DOG [**1**] and TES [**5**] as counter-targets (to eliminate motifs similar to DOGS.2) resulted in ALDS.1 (Figure 4C–D) having an affinity of 30 nM and excellent specificity. No measurable cross-reactivity with any other steroid was consistent with a binding pocket incorporating the C.18-OH group, a role similar to that of the C.17-OH group in hydrocortisone. Intriguingly, the sequence of ALDS.1 showed substantial convergence with the CSS.1 sensor (color-coded in Figures 4A and 4C). While folding programs failed to suggest a common secondary structure, this observation allows us to

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propose that we discovered another more general hydrophobic motif, but one that is dependent on hydroxyl groups for high affinity.

We characterized from the random pool one more G-rich aptameric sensor ALDS.2 (Supplementary Fig. 3, 8) with a dissociation constant of 300–400 nM that was cross-reactive, but with reduced affinities for other steroids. This is a potentially new sub-motif of more general $4XG_N$ type receptors that did not show interactions with other steroids with a hydroxylated core. This receptor had cross-reactivity with steroids lacking hydroxyl groups and the highest affinity for ALD [**12**]. Thus, we propose that this aptamer either has a hydrophobic interaction with at least part of the furanose ring or it has beneficial, but not absolutely necessary, more directed interactions with the C.18-OH.

Characterization of Binding Motifs with CD Spectroscopy:

Multiple sensors, *e.g.*, DISS.2, TESS.1, and ALDS.2, had sequence patterns implying formation of G-quadruplexes with calculated G-scores above 20.[30] Because some G-quadruplexes can be identified by their unique circular dichroism (CD) spectra,[31–33] we investigated these three aptamers with and without their targets *via* CD spectroscopy. Non-aromatic steroids are not UV active above 220 nm, thus, the spectra fully reflect structural DNA motifs and how they change upon interactions with targets (Figure 5). As controls, we investigated CD spectra of other aptamers listed in Figure 5C, for which we did not have sequence-based indications of G-quadruplex formation.

The CD spectra of DISS.2 and ALDS.2 gave unambiguous indications of structures containing an antiparallel topology of a G-quadruplex (Figure 5B), characterized by maxima at 290–300 nm, minima at 260 nm, and a maxima or shoulder at ~245 nm.[31–33] The third aptamer, TESS.1, had a CD spectrum consistent with a parallel topology of a G-quadruplex, (*i.e.*, a maximum at ~269–270 nm and a minimum at 240 nm). This assignment is more tentative than with anti-parallel topologies (Figure 5C) because it is based solely on hypsochromic shifts of maxima and minima when compared with maxima observed for control aptamers with typical characteristics of hybrid hairpin and double helical structures.

We tested all presumed $4xG_N$ sensors for the impact of K^+ on their affinities and CD spectra (Supplementary Fig. 9), because K^+ is known to stabilize G-quadruplex structures better than Na^+. The DISS.2 and TESS.1 aptamers showed only minimal K^+ dependence, while with the ALDS.2 sensor, K^+ at higher concentrations competitively displaced ALD [**12**] (Supplementary Fig. 9C). Furthermore, Na^+ was necessary and sufficient for target recognition by ALDS.2, could be substituted by K^+ in DISS.2, and neither of the two monovalent cations was needed for TESS.1. In the last case, Mg^{2+} was sufficient. The CD spectra in the presence of K^+ with and without targets demonstrated some inhibition of the formation of higher-order structures in the presence of K^+ for the TESS.1 and ALDS.2 sensors (Supplementary Fig. 9G–I). Both CD and ion dependence studies with ALDS.2 sensor are consistent with a G-quadruplex structure stabilized either by steroid or K^+ ion, with the two negatively regulating each other's binding.

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Steroids Inhibit Formation of Double Helices Between Aptamers and Their Full Complements.

We next characterized the behavior of our highest-affinity aptamers (K_d<100 nM) derived from DOGS.2, TESS.1, CSS.1, and ALDS.1 sensors (ALDS.1 aptamer in Figure 5D–E, others in Supplementary Fig. 10) in the presence of fully complementary oligonucleotides (*i.e.*, their full complements). These experiments were designed to test the hypothesis that due to stem-loop structures and high affinity for ligands, the fully complementary sequences will be blocked from interacting with aptamers and forming double helices; we were intrigued by this hypothesis because it was relevant to one possible mechanism through which small molecules such as steroids could impact the lifetime of single-stranded DNA *in vivo*. We labeled aptamers with fluorescein and their complements with a quencher (Iowa Black FQ), and observed that targets indeed hindered formation of double helices (Figure 5D). At the highest target concentrations tested (*e.g.*, >50 μM), three out of four aptamers were *fully* prevented from hybridizing with their complementary sequences. After hours of incubation, even nanomolar concentrations of steroids had measurable impact on hybridization (Figure 5E). In control experiments, in which we heated and cooled pre-mixed aptamers, fully complementary strands, and targets, we observed immediate duplex formation, indicating that a pre-formed binding pocket bound to ligand was necessary for inhibitory effects, and there was no reversible formation of aptamers from complementary duplexes under physiological conditions. The results of these control experiments are consistent with a purely kinetic inhibition of duplex formation, (*i.e.*, a process in which a target blocks a single-stranded oligonucleotide stretch preventing exposure of an internal "toe-hold" needed to initiate complementary binding interactions) in an analogy to strand-displacement reactions.[34–36]

CONCLUSIONS

In this work, we describe high-stringency *in vitro* selection-and-amplification protocols for five steroid targets and counter-targets selected to impose particular preferences and leading to high-affinity steroid receptors based on non-modified nucleic acids. These receptors have high specificity (*e.g.*, for aldosterone), or low likelihood of clinically problematic cross-reactivity (*e.g.*, for cortisol[37]), or have selectivity over counter-targets that can be used to suppress responses to dominant components in cross-reactive arrays (*e.g.*, all others).[8–11] They are now available for practical applications in clinical chemistry and due to their stem-loop structures, can be immediately used to construct electrochemical aptamer-based sensors.[39]

We isolated all our aptamers from libraries with limited sizes of randomized regions (up to N_{30}). Some of these aptamers have affinities within an order of magnitude of monoclonal antibody-based receptors for similar molecules[40], which is an unexpected result for short oligomers that are traditionally viewed as hydrophilic (when without modification[22]) and do not have protein-like variability in structures of monomers (even with modifications[22]).

One possible explanation for our results is that the limited availability of hydrophobic surfaces available in receptors to create tight 'sandwich-like' pockets for steroids[41], is compensated by a significantly smaller penalty than when exposing large and uniformly

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hydrophobic surfaces of non-polar (aromatic) amino acid side chains to water. With the limited size of our receptors, this moderates requirements for gradated transitions between hydrophobic pockets and hydrophilic surfaces of the receptor, leaving more flexibility to arrange optimally smaller numbers of residues in binding sites.

Despite limitations in the conclusions regarding the aptamer structures we currently offer, we have effectively demonstrated that by varying structures used in selection and counter-selection protocols, a broad variety of hydrophobic receptors based on DNA can be isolated. This is a counterintuitive outcome for oligomers not usually considered rich in hydrophobic character (except in the context of π-stacking). The number of motifs that could be isolated from even longer libraries ($>N_{30}$), as well as their available affinities and specificities, could approach those present in proteins that interact with steroids, even without the more sophisticated side chains present in amino acids (as used in, *e.g.*, SOMAmers[22]). This is, however, a brute force approach limited by our ability to examine efficiently, the expanded sequence space of potential receptors theoretically available to larger libraries.

In each of these selections, we used counter-targets, that is, molecules that we did *not* want our receptors to bind to, and we were able to isolate receptors that were selective, often fully specific, for targets over counter-targets. In each case, selectivity could be explained by simple models, *e.g.*, steric hindrance, an inability to bury a hydrophobic surface in the receptor, or incorporation of a hydroxyl group into the binding site. We observed, for selections in which the target preserved hydrophobic edges of the steroid core (*i.e.*, lacked hydroxyl groups beyond C.17), that selectivity over analogs that were not specifically taken into account during counter-selections varied. These results could be explained by similarities in shapes and adaptive binding of both receptor and orientation of its ligand, and are consistent with observations of promiscuous antibody-based receptors[37–38]. With targets purely hydrophobic in the B and C rings, selectivity could be correlated with the numbers of additional hydroxyl groups, as is the case with other hydrophobic receptors (*cf.*, cyclodextranes[42]). Along these lines, the steroids with two hydroxyl groups showed little binding to three- and four-way junctions, and $4XG_N$ motifs, all proposed to be primarily hydrophobic receptors.

Two selections where we added hydroxyl groups to hydrophobic edges, for CS [**11**] and ALD [**12**], and performed stringent counter-selections to isolate binding pockets incorporating these hydroxyls, resulted in yet another motif that was, unexpectedly, common for both selections, albeit it in different order. While a more precise structural description of this new motif awaits further characterization, our inability to find a common secondary structural motif through folding programs or manual searches, as well as the necessary incorporation of a hydroxyl group, suggest a motif that is more idiosyncratic than junctions and stacked quadruplexes.

We initiated this work to improve our ability to assess changes in steroid concentrations in urine using arrays of cross-reactive receptors.[8, 11] In this context, our results unequivocally demonstrate that evolutionary approaches can be used to address fundamental limitations of cross-reactive arrays (*i.e.*, limitations associated with electronic nose approaches): (1) We isolated sensors with new selectivities, improving our ability to project targeted steroids in

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different regions of *N*-dimensional sensor spaces and to minimize the impact of dominant components (*i.e.*, we were able to focus on minor constituents); (2) We identified higher-affinity receptors that will enable analyses at higher-dilutions of urine, thereby minimizing the impact of matrix variations; and (3) We expanded arrays to steroids that have cores festooned by hydroxyl groups, thus enabling analyses beyond the most hydrophobic analogs.

Single-stranded DNA exists in cells during replication and transcription, in the synthesis of chromosome ends, following DNA damage, and there is even a possibility of the spontaneous unwinding of DNA[43, 44]. Richness of interactions between steroids and single-stranded nucleic acids that we demonstrated here suggests that systematic searches for natural interactions between biogenic steroids and hydrophobic nucleic acid motifs might be fruitful, as well as that steroid analogs represent a class of potential drugs that has been undervalued in targeting natural nucleic acid motifs.[20]

MATERIALS AND METHODS

Preparation of Buffer and Target Solutions.

All of the steroids tested were available from Sigma Aldrich ([**1**] was discontinued after we performed selections). The steroid stock solutions were prepared at the following concentrations: 50 mM deoxycorticosterone 21-glucoside (DOG, [**1**]) in DMSO, 50 mM dehydroisonadrosterone 3-sulfate (DIS, [**2**]) in DMSO, 25 mM deoxycortisone (DC, [**3**]) in methanol, 10 mM progesterone (PRO, [**4**]) in ethanol, 50 mM testosterone (TES, [**5**]) in ethanol, 25 mM dehydroisoandrosterone (DIA, [**6**]) in DMSO, 25 mM Reichstein's substance S (11-deoxycortisol, 11-DC, [**9**]) in DMSO, 25 mM corticosterone (CCS, [**10**]) in DMSO, 30 mM cortisol (hydrocorticosteone, CS, [**11**]) in ethanol, and 30 mM aldosterone (ALD, [**12**]) in ethanol. Stock solutions were stored tightly sealed at room temperature for several weeks or at −20 °C for longer periods. For the cholesterol derivatives, we used 10 mM stock solutions of 25-hydroxycholesterol (DHC, [**7**]) in ethanol and 7α,25-dihydroxycholesterol (THC, [**8**]) in DMSO. These stock solutions were stored at −20 °C. The concentrations of 1× target solutions for elution and 2× analyte solutions for fluorescence measurements were always freshly prepared in selection buffers from stock solutions. (2× was two times relative to final concentrations). Three different buffer solutions were used. (1) SELEX reaction buffer: 20 mM HEPES, 1 M NaCl, 10 mM $MgCl_2$, 5 mM KCl, pH 7.5. (2) 2× SELEX reaction buffer where all components of the SELEX reaction buffer were doubled. (3) No $MgCl_2$ strand separation buffer: 20 mM HEPES, 300 mM NaCl, pH 7.5. Detailed protocols for buffer preparation can be found in our previous publication.[15]

In Vitro Selection and Amplification Protocols.

The SELEX experimental procedures were modified from those described in our previous publication.[15] The oligonucleotides used for SELEX were as follows:

(1) A random (N_{22}) library: 5'-GGAGGCTCTCGGGACGAC **(N_2)** GGATTTTCC **(N_{20})** GTCGTCCCGATGC TGCAATCGTAAGAAT-3', (2) a random (N_{30}) library: 5'–GGAGGCTCTCGGGACGAC **(N_{30})** GTCGTCC CGCCTTTAGGATTTACAG -3', (3) forward-primer for the N_{22} and N_{30} libraries: 5'-GGAGGCTCTCGGGA CGAC-3', (4)

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reverser-primer for the N_{22} library: 5'-ATTCTTACGATTGCAGCATCGGGAC-3', (5) biotinylated reverse primer for the N_{22} library: 5'- biotin-ATTCTTACGATTGCAGCATCGGGAC-3', (6) reverse-primer for the N_{30} library: 5'-CTGTAAATCCTAAA GGCGGGACGAC -3', (7) biotinylated reverse-primer for the N_{30} library: 5'-biotin- CTGTAAATCCTAAAGGCGGGACGAC-3', (8) biotinylated column immobilizing capture strand: 5'-GTCGTCCCGAGAGCCATA-BioTEG-3'. Specific positive/counter-selection conditions are described in Table S1.

Assays for Responses of Aptameric Sensors to Ligands.

Selected sequences after cloning were synthesized with fluorescent modifications at their 5' ends. The sequences of the sensors and the optimized sequences of the complementary oligonucleotides are shown in Figure 5 and Table S2. All measurements were performed in triplicate in SELEX buffer with ligand concentrations indicated on the graphs. All sensors were tested multiple times to ensure reproducibility of conclusions regarding selectivities and affinities. The mixtures of sensor (with fluorescein) and competitor (with DABCYL quencher) strands were incubated at 95 °C for 5 min then cooled down slowly to room temperature (~24 °C, >40 min). Separately, the steroid target solutions were prepared, and mixtures of sensor, competitor, and target were mixed 1:1:1 for binding assays (Supplementary Fig. 11). The fluorescence measurement conditions were as described by Yang *et al.*,[15, 16] while the K_d's were determined following Hu and Easley's method.[45]

Time-Course Monitoring of Interactions between Aptamers and Their Complementary Strands.

Four hundred nM FAM-conjugated ALDS.1-derived aptamer solution and 400 nM Iowa Black conjugated complementary strand solution were prepared separately in buffer (20 mM HEPES, 1 M NaCl, 10 mM $MgCl_2$, 5 mM KCl, pH 7.5). These solutions were denatured by heating in a boiling water bath for 5 min. They were then cooled to room temperature for ~30 min. Meanwhile, two times concentrated target solutions for each aptamer were prepared. After a 30-min preincubation of aptamer and its fully complementary strand, 18.75 μl of aptamer solution was added to the wells of a 384-well plate. Next, 37.5 μl of target solution was added to each well. Finally, 18.75 μl of complementary strand solution was added to each well (time=0 min). Fluorescence measurements were recorded every 5 min thereafter at room temperature for >8 h using a fluorescence plate reader (Victor II microplate reader, PerkinElmer). Sequences used for this assay are in Table S2.

Circular Dichroism Spectroscopy.

Aptamer and target concentrations were 2 μM in 20 mM HEPES, 1 M NaCl, 10 mM $MgCl_2$, 5 mM KCl, pH 7.5. Aptamers were heated at 95 °C for 5 min and cooled to room temperature slowly. Spectra were collected on a JASCO J-715 circular dichroism spectrophotometer at room temperature. Four scans were acquired per sample with 0.5 nm resolution, 1.0 nm bandwidth, 4 s response times, and a 20 nm/min scan rate. Scans in the figures are averages with baseline scans collected in HEPES buffer alone and subtracted as background. To investigate the impact of K^+ on CD spectra, HEPES buffer with an increased concentration of KCl (140 mM) was used.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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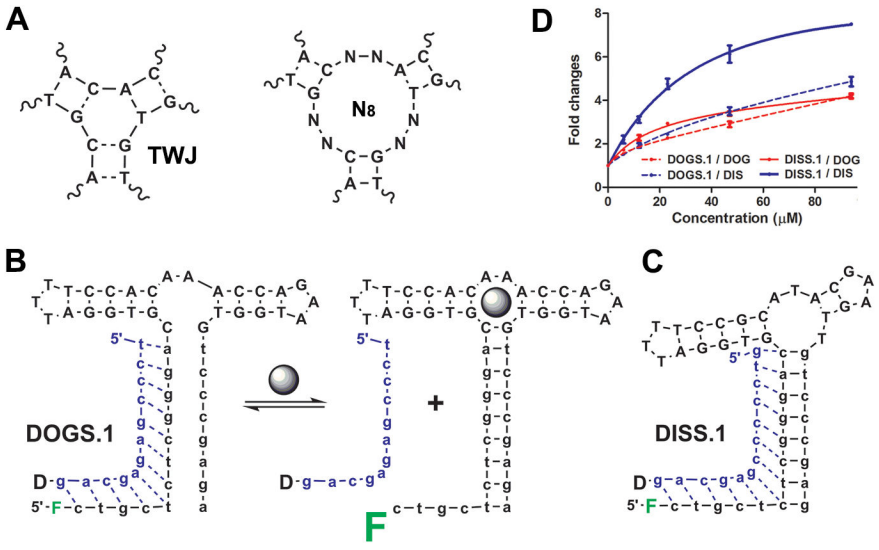

Figure 1.

Two non-specific aptamers for steroids previously isolated from libraries of three-way junctions (TWJs)[11] shown in their structure-switching forms: (**A**) Typical fully matched three-way junction that binds steroids and N8 library used to isolate modified junctions with different selectivities; (**B**-**C**) Sensors DOGS.1 and DISS.1 (**'F'** indicates fluorescein) are shown together with complementary oligonucleotides carrying a quencher (**D**, dabcyl). As an example for **DOGS.1** in (**B**) we show a competitive assay in which concentrations of ligands were measured by displacing complementary oligonucleotide complexed to the 5' ends of aptamers. (**D**) Fluorescence *vs.* concentration curves for DOG [**1**] (red lines) or DIS [**2**] (blue lines) with DISS.1 (solid lines) or DOGS.1 (dashed lines) aptamer sensors.

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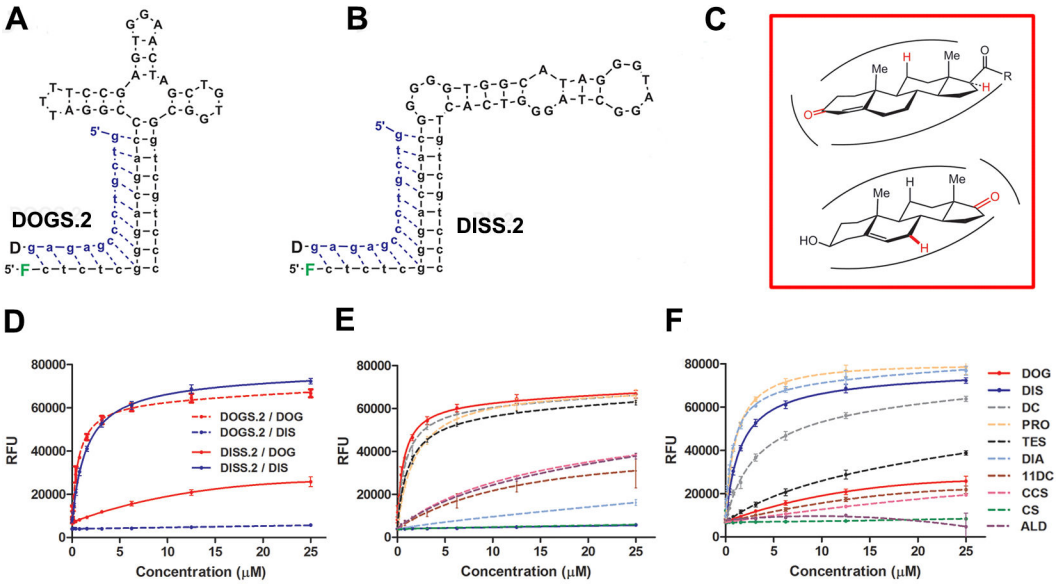

Figure 2.

Aptameric sensors isolated from more complex libraries under stringent selection conditions using deoxycorticosterone-21-glucoside (DOG [**1**]) and dehydroisoandrosterone 3-sulfate (DIS [**2**]) as targets: (**A-B**) Predicted secondary structures (Mfold[23]; http://mfold.rna.albany.edu/) of sensors selected to respond to DOG (**A**) or DIS (**B**). **(C)** Proposed shapes of binding pockets based on their cross-reactivities with other steroids and selection conditions. Lines represent presumed hydrophobic walls; functionalities where substitutions cause dramatic decreases in affinities are marked red. (**D**) Fluorescence *vs.* target concentration curves for DOGS.2 (dashed lines) and DISS.2 (solid lines) with DOG *vs.* DIS as targets. **(E-F)** Fluorescence *vs.* target concentration curves to include additional nontarget steroids for DOGS.2 **(E)** and DISS.2 (**F**). Legend for dose-response curves: DOG [**1**] (●), DIS [**2**] (●) DC [**3**] (●), PRP [**4**] (●), TES [**5**] (●), DIA [**6**] (●), 11-DC [**9**] (●), CCS [**10**] (●),CS [**11**] (●), and ALD [**12**] (●).

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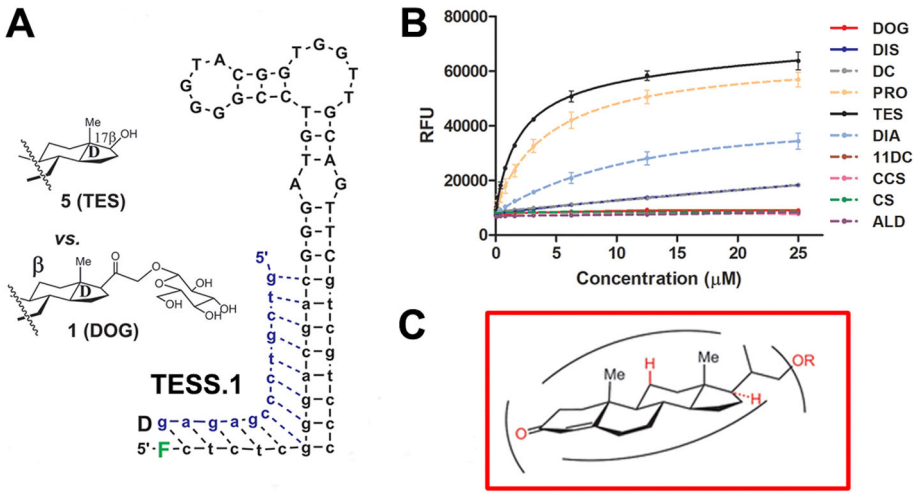

Figure 3.

Aptamer sensor isolated for selectivity for testosterone (TES [**5**]) *vs.* DOG **[1]**. (**A**) Predicted secondary structure of TESS.1 and D ring as the focus of selection. (**B**) Fluorescence *vs.* steroid concentration curves for TESS.1. Legend is the same as in Figure 2. (**C**) Proposed binding pocket based on interactions with analogs.

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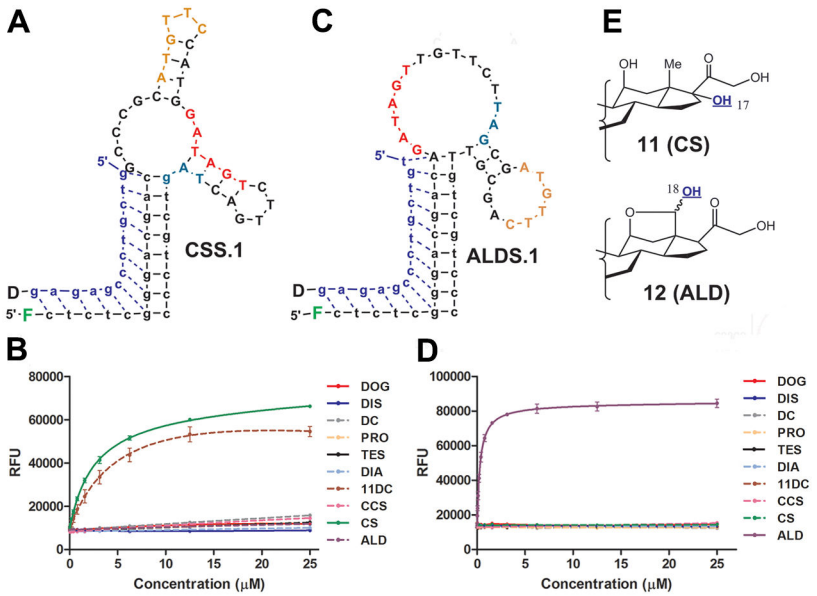

Figure 4.

Aptameric sensors isolated from selections using hydrocortisone (cortisol, CS [**11**]) or aldosterone (ALD [**12**]) as targets. (**A**) Predicted secondary structure of CSS.1. Common sequences with ALDS.1 are marked by color-matched letters. (**B**) Fluorescence *vs.* steroid concentration response curves for ten steroids with CSS.1. This sensor only responds to target and corticosterone (CCS [**10**]), which is an analog having an 11-keto group. Legends for fluorescence response curves are the same as in Figure 2. (**C**) Predicted secondary structure of ALDS.1. The sequences in common (albeit present in different order) with CSSS.1 are denoted in color. (**D**) Fluorescence *vs.* steroid concentration response curves for ten steroids with ALDS.1. This sensor only responds to ALD [**12**]. Legends for fluorescence response curves are the same as in Figure 2. (**E**) Common sequences indicate similar binding pocket motifs with adaptations selective for the different steroid analogs. The C.17-OH is absolutely necessary for CSS.1 binding and presumably substituted by C.18-OH in the target of ALDS.1.

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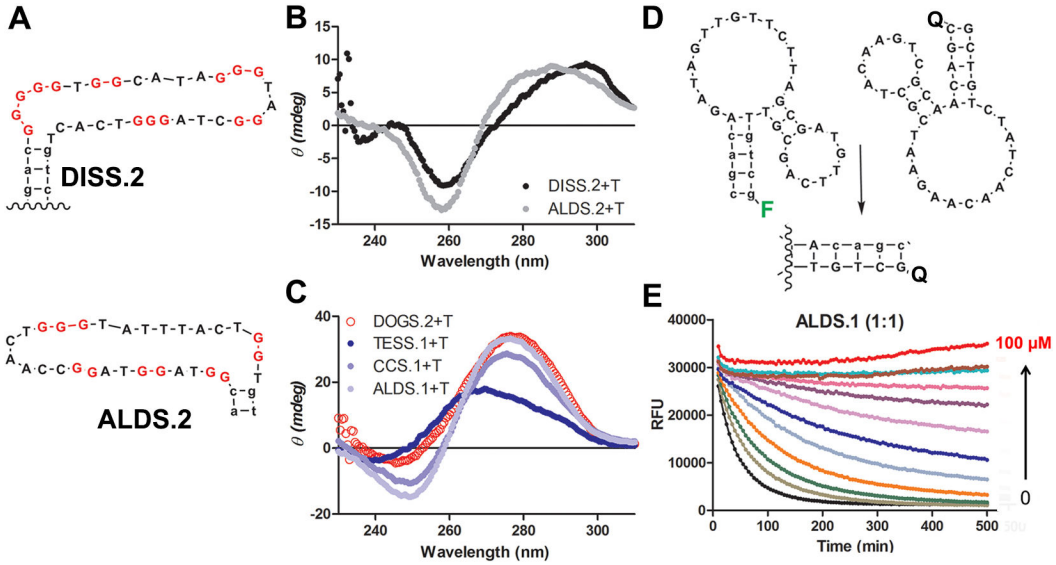

Figure 5.

Further characterization of aptamers. (**A**) Two G-rich aptamers with calculated G-scores >20 and (**B**) circular dichroism (CD) spectra in the presence of targets indicating a partial antiparallel topology of the G-quadruplexes. (**C**) The CD spectra of other aptamers in the presence of their targets. The spectra for TESS.1 are consistent with a parallel topology (supported by a hypsochromic shift from the spectra of hairpin/double helix standards, *cf.*, other aptamers in (**C**). (**D**) The proposed secondary structures of the minimal stable aptamer derived from the ALDS.1 sensor and its fully complementary strand. The aptamer is conjugated with fluorescein ('F') and its complement is conjugated with a quencher (Iowa Black FQ), thus formation of a double helix results in fluorescence quenching. (**E**) Time-course monitoring of aptamer and anti-aptamer hybridization in the presence of increasing concentrations of aldosterone (ALD **[12]**), with concentrations >25 mM completely inhibiting interactions over >8 h.

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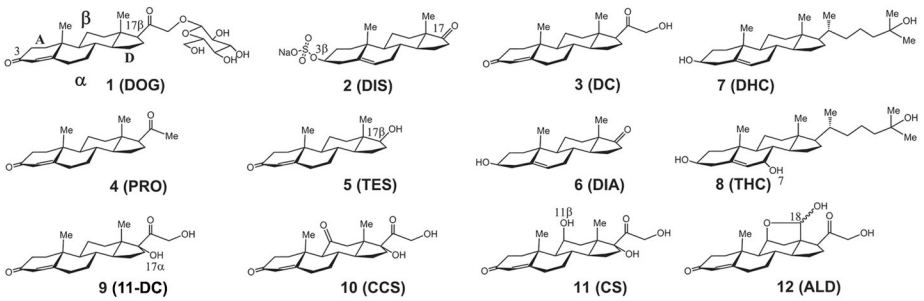

Scheme 1.

Structures of steroids used to select aptamers or to test cross-reactivities of aptamer sensors. Deoxycorticosterone 21-glucoside (**DOG** [**1**]), dehydroisoandrosterone 3-sulfate (**DIS** [**2**]), deoxycorticosterone (**DC** [**3**]), progesterone (**PRO** [**4**]), testosterone (**TES** [**5**]), dehydroepiandrosterone (**DIA** [**6**]), 25-hydroxycholesterol (**DHC** [**7**]), 7α,25-dihydroxycholesterol (**THC** [**8**]), 11-deoxycortisol (**11-DC** [**9**]), corticosterone (**CCS** [**10**]), cortisol (**CS** [**11**]), and aldosterone (**ALD** [**12**]).