JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

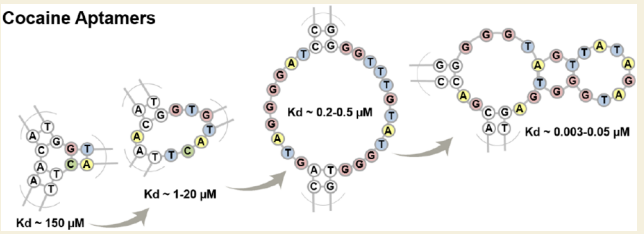
pubs.acs.org/jacsau
Article

Exploring the Landscape of Aptamers: From Cross-Reactive to Selective to Specific, High-Affinity Receptors for Cocaine

Kyungae Yang, Obtin Alkhamis, Juan Canoura, Alexandra Bryant, Edward M. Gong, Mihaela Barbu, Steven Taylor, Dragan Nikic, Saswata Banerjee, Yi Xiao, Milan N. Stojanovic,* and Donald W. Landry*

Cite This: JACS Au 2024, 4, 760−770
Read Online

ABSTRACT: We reported over 20 years ago MNS-4.1, the first DNA aptamer with a micromolar affinity for cocaine. MNS-4.1 is based on a structural motif that is very common in any random pool of oligonucleotides, and it is actually a nonspecific hydrophobic receptor with wide cross-reactivity with alkaloids and steroids. Despite such weaknesses preventing broad applications, this aptamer became widely used in proof-of-concept demonstrations of new formats of biosensors. We now report a series of progressively improved DNA aptamers recognizing cocaine, with the final optimized receptors having low nanomolar affinity and over a thousand-fold selectivity over the initial cross-reactants. In the process of optimization, we tested different methods to eliminate cross-reactivities and improve affinity, eventually achieving properties that are comparable to those of the reported monoclonal antibody candidates for the therapy of overdose. Multiple aptamers that we now report share structural motifs with the previously reported receptor for serotonin. Further mutagenesis studies revealed a palindromic, highly adaptable, broadly cross-reactive hydrophobic motif that could be rebuilt through mutagenesis, expansion of linker regions, and selections into receptors with exceptional affinities and varying specificities.

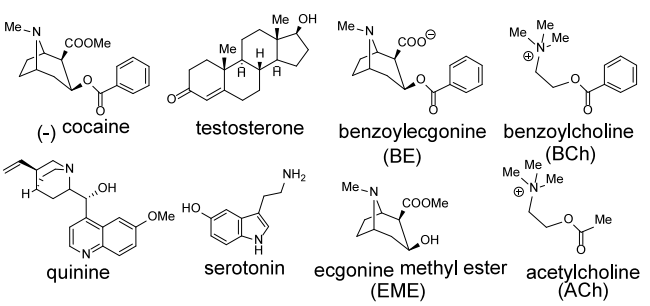


KEYWORDS: *cocaine, aptamer, specific, high-affinity, cross-reactive, hydrophobic receptor*

Aptamers that recognize small molecules are selected from vast random libraries of oligonucleotides through affinity-based enrichment-amplification cycles.[1−5] An important advantage of aptamers over antibodies is in our ability to adapt them to a variety of novel biosensor formats while relying on no more than partial knowledge of their presumed secondary structures.[5−8] For biosensors to be useful, however, the properties of the underlying aptamers need to match the practical requirements, which raises the issue of the selection of aptamers with affinities and selectivity that are adjusted to the intended applications.

Consider, for example, the interrelated MNS-series of DNA aptamers that bind cocaine (Scheme 1) in Figure 1 (parent, full length structure MNS-3.3): we reported these in the context of implementing early aptameric sensors.[9,10] The most often used analogs have micromolar affinities for cocaine and strong binding to a number of other alkaloids and steroids.[11−13] The minimal receptor motif in these aptamers is derived from a three-way junction through a single mismatch. Possibly because of this simple structure, verified binding, and an intriguing target, these aptamers have been extensively used to demonstrate new classes of biosensors,[14−18] including in the context of supporting law enforcement (interdiction) efforts.[17,18] Yet, due to their low-to-moderate affinity and the lack of selectivity of the underlying receptors, their real applications are limited. Clinical applications, e.g., emergency room point-of-care quantifications or passive immunization[19] would require substantial improvements in the affinity, with stoichiometric monoclonal antibodies providing a suitable benchmark.[20−23]

Scheme 1. Key Small Molecules Discussed in the Text



Received: December 7, 2023
Revised: January 25, 2024
Accepted: January 25, 2024
Published: February 13, 2024

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

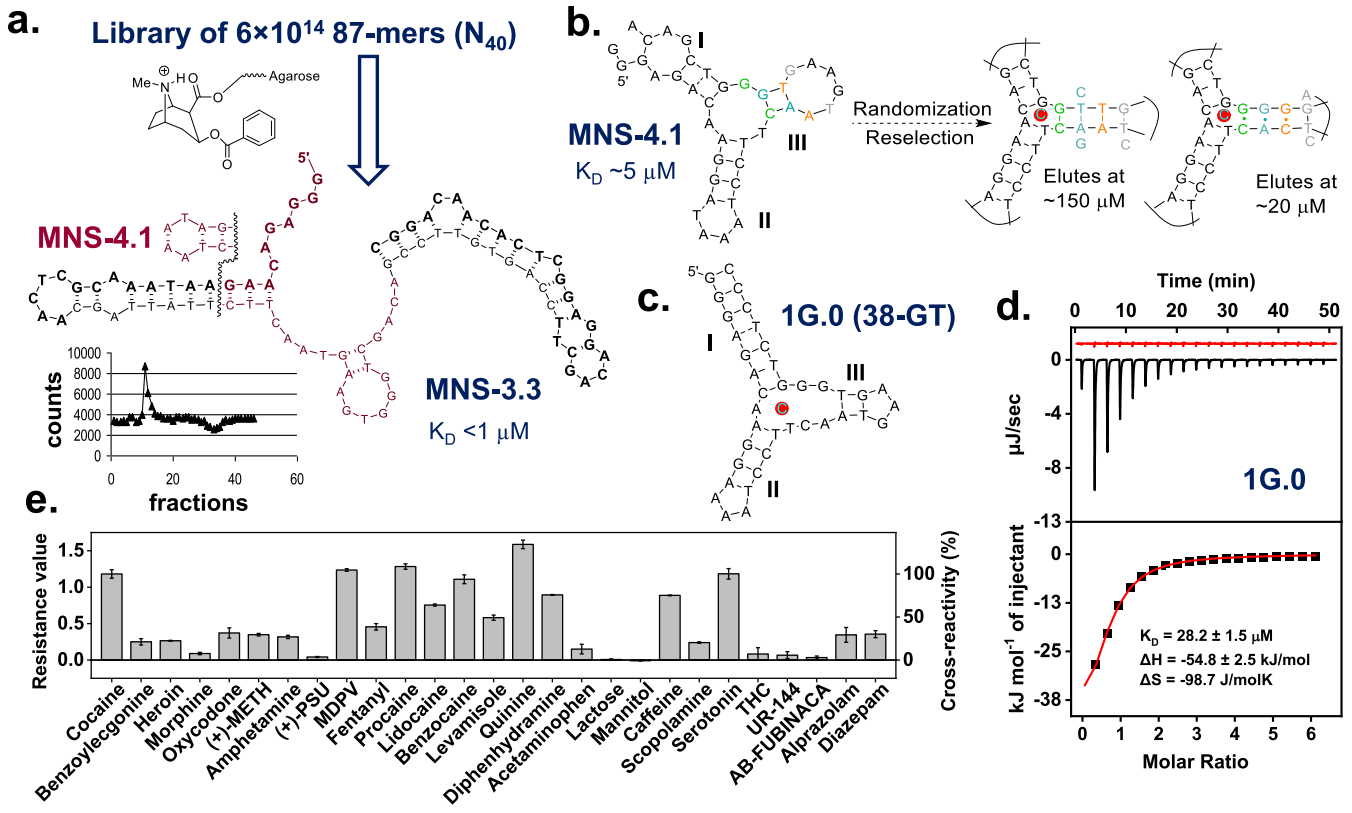


Figure 1. Isolation and characterization of the first-generation cocaine aptamers: (a) starting from a library of DNA 87-mers, comprising two primers (bold) and a randomized 40-mer region, we performed the affinity separation of cocaine binders on an agarose column displaying a cocaine analog. This protocol resulted in MNS-3.3 (the inset shows equilibrium gel filtration results using 10 μM radiolabeled cocaine (^{3}H at Me-*N*), showing full saturation of 1 nmol of receptor). Subsequent minimization identified the maroon sequence as sufficient for high affinity binding (below 10 μM), leading to the aptamer MNS-4.1[10]. (b) MNS-4.1 (secondary structure generated at the time) was rerandomized and subjected to reselection. The final pool (after four cycles) was subjected to affinity separation on a cocaine-displaying agarose column, with two peaks identified, one with a fully matched junction eluting at a calculated 150 μM affinity, and the other with a single point G*A mismatch in the junction, eluting at under 20 μM (the secondary structure shown is in an analogy to the fully matched junctions). (c) An example of a cocaine-binding sequence used in the literature, herein labeled as 1G.0 aptamer (alt. 38-GT), with three stems (**I**–**III**). (d) Determination of the affinity of 1G.0 with isothermal calorimetric titration (ITC), injection of 2400 μM cocaine to 80 μM 1G.0. (e) The cross-reactivity profile of 1G.0, showing interactions with different drugs, additives and metabolites (experimental section in Supporting Information).

Beyond this, purely practical perspective, we were interested in additional questions related to our general effort to optimize aptameric receptors:[24,25]

First, the initial selection was based on a capture on an affinity column with modified cocaine displayed on agarose (Figure 1a), which limits the exposure of functional groups for interactions with nucleic acids. The resulting aptamers were based on a three-way junction motif that is very abundant in any random pool of oligonucleotides, and we were interested to see whether we can channel the newer approaches with free ligands in solution toward different, less common receptors.

Second, and of major concern, stringent attempts to suppress these abundant motifs while increasing the affinity might lead to elimination of high-affinity cocaine binders with even minimal cross-reactivity with other targets.[26] Thus, cocaine is a testbed for our ability to focus selections away from common receptors through a combination of new approaches, stringent targeted counterselections, and classic considerations of the complexity aspects on the outcomes of selections[26−28] (for more recent examples of the efforts to increase affinity and expand targets see refs [29,30]).

Third, we observed that for planar compounds with aromatic fragments, the standard solution-phase selection protocols optimize the affinity in proportion to the number of nonhydrogen atoms.[25] Cocaine is not a planar molecule, and it would require a substantially more complex binding pocket for optimized binding; thus, we were intrigued to test whether this observation would still hold and, if not, what are the opportunities for the improvement in affinity against this benchmark. Relatedly, cocaine consists of two independent fragments, tropanyl and benzoyl, and we were interested in whether with an increase in the affinity we will start seeing more binding to these individual fragments with less overall selectivity.

Finally, we felt obliged to clarify the origins of the initial cocaine aptamer, because it has never been reported outside of the context of our biosensor work.[9,10]

RESULTS AND DISCUSSION

MNS-3.3 and Its Congeners

Aptamer MNS-3.3 (Figure 1a) was one of the three sequences (the other two are in Table S2), with confirmed binding from the cloned and sequenced pool that was isolated through capture on the agarose affinity column displaying a cocaine analogue at 1 mM concentration. During selections, all captured sequences were eluted with the matching concentration of free cocaine and subjected to polymerase chain

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

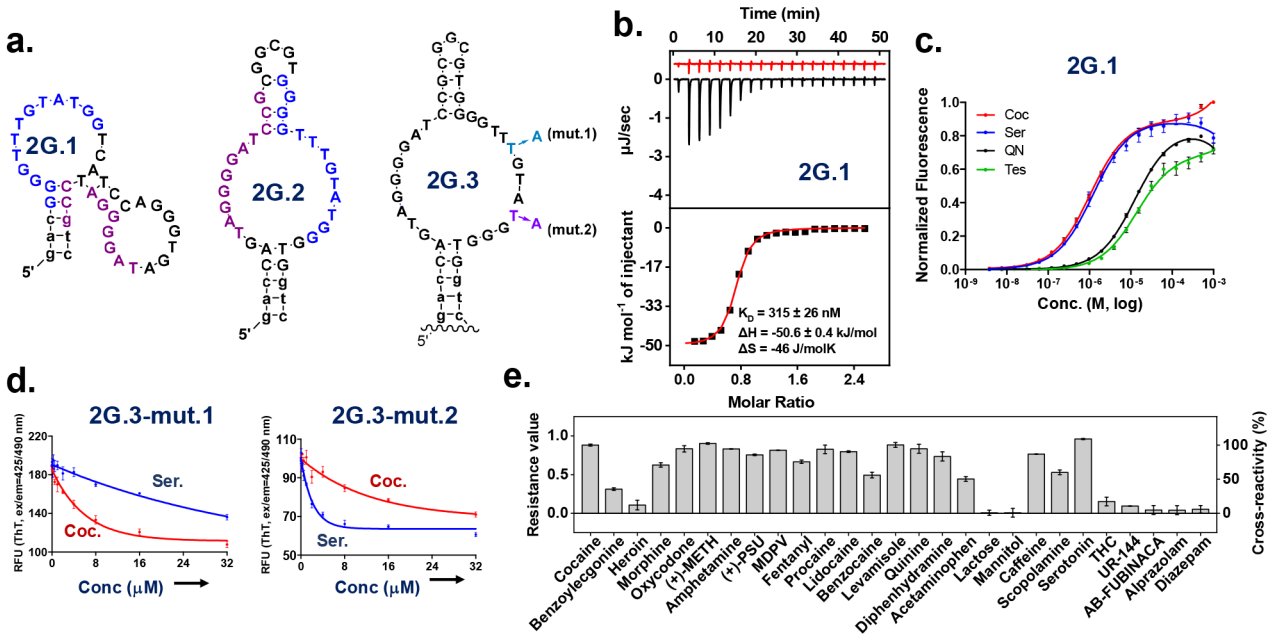


Figure 2. Characterization of the second-generation cocaine aptamers (lower font sequences are derived from the constant flanking region of library used in the initial selection): (a) the protocol with cocaine in solution-phase and N_{36} library displayed on a column with complementary oligonucleotide resulted predominantly in the aptamers 2G.1 and 2G.2. Two sequences were related through a circular permutation, despite substantially different predicted secondary structures. 2G.3 is an example of a consensus receptor engineered to represent their common binding pocket core. (b) Determination of K_D using ITC, 250 μM cocaine was injected to 20 μM 2G.1). (c) The 2G aptamers were highly selective over quinine and testosterone (Scheme 1), which were used in the counterselection, but bound equally well serotonin and cocaine. We show here the displacements by ligands of a quencher-labeled competitor oligonucleotide from fluorescently labeled 2G.1. (d) Single point mutations of 2G.3 yield aptamers selective for cocaine (2G.3-mut.1, cf. (a)) or serotonin (2G.3-mut.2, cf. (a)). The comparison of single point mutants was made using a ligand-induced displacement assay of Thioflavin T (ThT) dye. (e) Cross-reactivity of 2G.1 was assessed with the standard panel of cross-reactants using an exonuclease protection assay.

reaction (PCR) amplification. At the time, using tritiated cocaine as a ligand and equilibrium gel filtration, we obtained for the original clone the full saturation of receptors at 10 μM, which indicated a submicromolar K_D.

The initial minimization yielded 4.1 (Figures 1a,b and S1; $K_D \sim 5$ μM), which was partially randomized into a pool subjected to affinity chromatography on an agarose column displaying tethered cocaine (1 mM). Two elution peaks were noted by semiquantitative PCR, centering around affinities of ~150 and ~20 μM (Figure 1b). These pools were individually cloned and sequenced, revealing fully matched and mismatched junctions, respectively. In our hands, using at the time equilibrium gel filtration, the additional mismatches present in the original sequence at the 5′ and 3′ ends (Stem I) slightly improved binding ($K_D \sim 5$ μM vs >10 μM). While we now know that this observation was reproduced in detailed studies by Johnson's group,[31,32] at the time we felt that such differences could be also attributed to batch-to-batch variations in aptamer production. Therefore, while we initially used aptamers with a sequence as in Figure 1b, we later switched to a more commonly used variant with two fully complementary stems (Figure 1c).

Further minimization of Stem I provided the conformationally unstable aptamer 7.9 (Figure S1), which was the basis for a folding sensor, used in fluorescent[10] and, by others, electrochemical sensors[14,26] (often losing an extra A−T base pair in the Stem 3). The minimized stable sequence (1G.0, 1G: the first generation) has a K_D of 20−30 μM[31,32] (we confirmed this value using isothermal titration calorimetry, ITC, Figure 1d).

Aptamer 4.1 and related structures bound to both steroids and alkaloids while being selective for cocaine over its two main metabolites, benzoyl-ecgonine and ecgonine-methyl ester. We characterized a new cross-reactivity of 1G.0 (Figure 1e) using the broad panel of drugs and additives that has recently became standard for characterization of aptamers binding to narcotics.[33−36] This was also done to compare this early aptamer more systematically to the subsequently isolated species.

Eliminating Three-Way Junctions

For our initial approach toward improved cocaine receptors, we opted for solution-phase selection, which would maximize accessible surfaces of cocaine available for interactions with monomer residues. In that protocol, oligonucleotide libraries with internal random regions (here, 36-mers) were captured via flanking regions of the library on columns displaying complementary sequences. Potential aptamers were then preferentially released via interactions with the unmodified targets in solution if these stabilized the stem formation and the concomitant release of the capture strand.

Furthermore, in order to minimize the interference by the three-way junctions, fully matched or mismatched, we implemented a harsh counterselection with quinine and testosterone. The elimination of the cross-reactivity for steroids was necessary for any measurements in bodily fluids relying on the cocaine aptamers because of the presence of high-concentrations of steroid conjugates, both in circulation in urine. Quinine was used as a tight binder for all three-way junctions and could thus be applied to eliminate even weakly binding fully matched receptors.

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

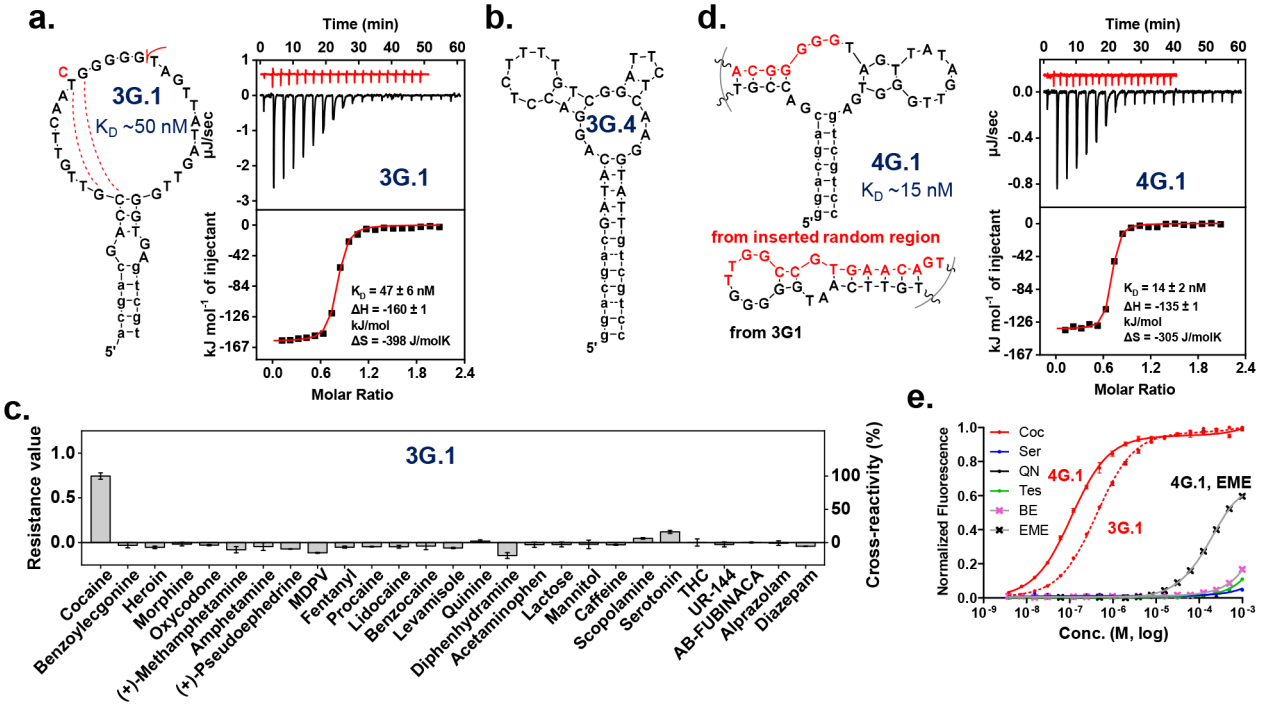


Figure 3. Third and fourth generations of cocaine aptamers (lower font sequences are derived from the flanking, constant regions used in the initial selection): (a) the highest affinity third-generation aptamer, 3G.1, obtained through a procedure that included counterselection with serotonin; C in the red font next to T shows the substitution that connects the mfold-predicted secondary structures of the 3G.1 and 4G aptamers (the red, dashed lines). We show the ITC data (injection of 100 μM cocaine to 10 μM aptamer). (b) 3G.4 was the most abundant aptamer isolated while using the counterselection with a complement; it was the only sequence that lacked more than three guanosines in a row. (c) The cross-reactivity of 3G.1 with a standard panel using an exonuclease protection assay. (d) Further attempts to improve the affinity led to the related 4G.1 aptamer (red font is used to stress the inserted random sequence, the insertion position is shown in (a) by a red mark); with its affinity characterized by ITC (injection of 50 μM cocaine to 5 μM 4G.1). (e) The 3G.1 and 4G.1 receptors both show over a 10,000-fold preference over the initial cross-reactants, quinine, testosterone, and serotonin (Scheme 1) in the fluorescence displacement assays, and a >2000-fold preference over ecgonine methyl ester (EME).

This first solution-phase selection, using in the 19th cycle a final concentration of 0.5 μM cocaine for elution of aptamers from the column, resulted in the fully converged pool with two identified aptamers, KY-2G.1 and 2G.2 (Figure 2a, 2G for the second generation). The subsequent next generation sequencing (NGS) showed these two sequences comprising over 98% of the pool (55% and 43%), with the rest being only their single point mutants, likely arising from the original sequence due to errors in amplification. The ITC measurement resulted in a K_D ~ 315 nM for 2G.1 (Figure 2b), while the displacement assay using fluorescent aptamers and quenching oligonucleotides (Figure 2c) revealed an about 10-fold selectivity over quinine and testosterone.

Two structures, 2G.1 and 2G.2, were related through a circular permutation,[37] indicative of a common secondary structure containing two stems, leading us to establish 2G.3 (Figure 2a) as the representative, consensus receptor. Further studies on 2G.3, including single-point mutations at each position, revealed that out of 11-mer and 14-mer sequences linking two stems, only 14 bases showed less than the estimated 10-fold decrease in affinity upon single-point mutations (Supporting Information, Figure S2).

The binding pocket of the 2G family contains two palindromes, in a linguistic sense: 5′-TAGG‖GGAT⋯GGTTT ‖G‖TATGG. If we focus only on their aromatic bases, each semipalindrome defines a mirror image hydrophobic surface, which may facilitate interactions with individual enantiotopic faces of flat molecules with low polarity. Furthermore, palindromic sequences are less likely to fold into stable structures with Watson−Crick base pairings, which could make them more likely to be displayed for binding during selections. Finally, the pairs of palindromic sequences are more likely to lead to multiple conformations with similar energies due to the conflicting implementation of interaction rules (so-called frustration[38,39]), thus leaving unpaired residues available to accept ligands.

Studies of Serotonin Cross-Reactivity

We were informed by Drs. Kevin Plaxco and Tod Kippin, from the University of California, Santa Barbara, that our initial shortened aptamer (7.9) bound to serotonin. This led us to establish that the 2G series bound equally well to cocaine and serotonin (Figure 2c).

Subsequent mutagenesis studies revealed that the common sequence, 2G.3, which bound cocaine and serotonin equally well, could be switched through single point mutations into the aptamers selective for each of the two targets (Figure 2a,d). These volumetric tuning experiments are consistent with the adaptable binding pocket restructuring itself to maximize interactions with the individual ligands.

An exonuclease protection assay on the full panel of drugs and additives on one analogue (Figure 2e) revealed its outstanding promiscuity, with 19 out of 27 of the tested molecules cross-reacting above 50%, which is more than double from the mismatched three-way junctions (Figure 1e). Our preferred strand displacement assays (Figure S3) provided

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

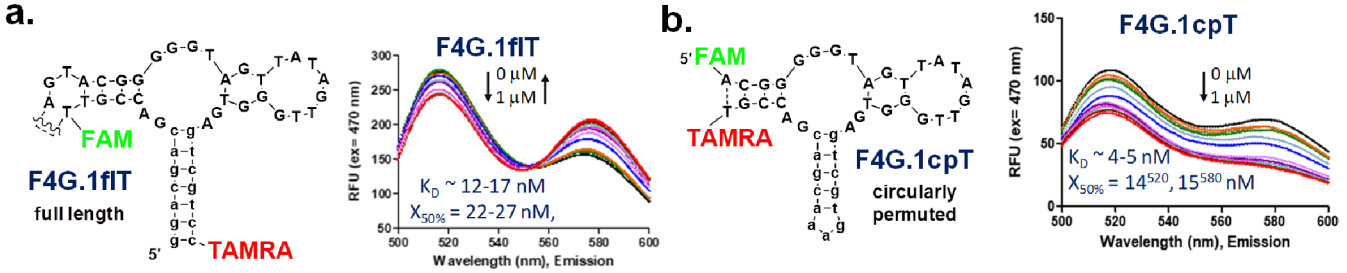


Figure 4. Two different types of fluorescent sensors derived from the 4G.1 receptors, labeled with the donor (FAM) and acceptor (TAMRA) fluorescent dyes: (a) this classical fluorescence energy resonance transfer (FRET) aptameric sensor,[40] F4G.1flT, is based on the full length 4G.1, with a K_D of 17 ± 3 nM matching, as predicted, the ITC values (14 ± 2 nM). (b) This sensor, F4G.1 cPT, is circularly permuted. It has a shortened new stem labeled to operate as the classical[10] proximity quenching sensor (half-points were calculated using 520 nm and 580 nM emissions). Unexpectedly, it showed an improvement over the affinity measured of the original 4.1, possibly due to the overhangs of dyes.

additional insights into the relative affinities of various hydrophobic molecules for these types of receptors.

Elimination of Cross-Reactivity

In order to eliminate this broad cross-reactivity, we decided to repeat our selection but with a counterselection with serotonin. We were again concerned that the high concentrations of serotonin combined with counterselections using quinine and testosterone would lead to suppression of all binders, favoring ligand-independent processes for release. We thus decided to test in parallel two different mechanisms for the elimination of serotonin binding on the seventh cycle from the selection that led to the 2G aptamers: (a) a traditional approach using elutions with high concentrations of serotonin and, as a novel alternative, (b) a passage through a column displaying complementary strands to 2G.1 and 2G.2 sequences (cross-reactive with serotonin). The outcomes of these two protocols were five new sequences, 3G.1−3G.5 (3G.1 and 3G.4, Figure 3a,b; 3G: the third generation, Figure S4a for other aptamers), all highly selective for cocaine. Using high-throughput sequencing, we then systematically compared pools that were emerging through individual cycles of two counterselections (Figure S4b):

First, we detected in our initial pool all of the sequences that were predominant in all of the final pools. Thus, a combination of NGS and expanded screening would have rendered an extended selection process unnecessary, at least when targeting cocaine. However, we would have missed the opportunity to study the nonspecific hydrophobic receptors based on their lower affinity (see discussion below).

Second, although by cycle #13 both methods suppressed efficiently the sequences that bound to serotonin, the outcomes were completely different: the traditional counter-selection with serotonin yielded 3G.1, 3G.2, and 3G3, in contrast to using complements, which resulted in 3G.4 (>90%; further 8% was 3G.3). 3G.4 had a smaller overall guanosine content (22%) than the receptors that were effectively suppressed and lacked three consecutive Gs, observations consistent with avoiding the elimination by complements. We initially conceived the counterselections using the complements to eliminate dominant short motifs that can overwhelm pools leading to a loss of binders; however, instead, we demonstrated that we can focus selections to specific subsections of oligonucleotide space (here, reducing the participation of G-rich sequences).

Third, once we removed the pressure of the complement counterselection, 2G aptamers rapidly reemerged. This observation is indicative of these sequences having properties that would lead to their preferred selection in the initial experiments, despite the substantial cross-reactivity with quinine and testosterone (Figure 2c) and weaker affinity than 3G.1. At least when it comes to the preferential selection over 3G.1, the poorer PCR amplification efficacy is an important factor behind this result (Figure S5).

Based on the lowest half-point concentration ($X_{50\%}$) in the ThT dye displacement assay by cocaine (note: in one case, 3G.4, we had to compare results of oligonucleotide-displacement assay, because the dye displacement was not possible, see Figure S4), we chose for further studies 3G.1, which had a K_D < 50 nM by ITC (Figure 3a). Studies of extended cross-reactivity using an exonuclease protection assay further confirmed the exceptional selectivity of the binding pocket (Figure 3c).

Further attempts to improve the cocaine aptamer through insertion-reselection and substitution-reselection[25] led to about a 3-fold improvement in affinity of 4G.1 (Figure 3d, a structure related to 3G.1 through a single point mutation, followed by stem extension). The final K_D value for this aptamer was about 15 nM, as established by ITC (Figure 3d), with near-absolute specificity (>10,000-fold) for cocaine over ligands used in the counterselections confirmed with the displacement assays (Figure 3e). Attempts to minimize the stable stem in 4G.1 led to about a 2-fold reduction of affinity, while attempts to rerandomize and reselect did not lead to any improvement.

Experiments to generate fluorescent sensors based on an adaptable binding of the stable aptamer[40] succeeded. For the full-length sequence (**F4G.1flT**, fl for full length, Figure 4a; Figure S6 for the suite of screened FRET sensors), we obtained a classical FRET sensor, with the K_D of the labeled aptamer matching that of the original receptor (both around 15 nM).

In contrast, the terminal labeling of a shorter circularly permuted sequence (**F4G.1 cpT**, cp for circularly permutted, Figure 4b) resulted in an unstable quenching sensor that, unexpectedly, had higher apparent affinity than 4G.1, despite this predicted structural destabilization. This is consistent with the stabilizing roles of fluorophores at the end of the unstable double-helix, similar to the role of overhangs at the end of the helix.[41,42] The lower initial fluorescence in comparison to **F4G.1flT**, as well as the predominantly contact quenching aspects of the spectra (i.e., the relative intensity of 520 and 580 nM peaks), is consistent with the moderate rearrangements of the structure that would be required to achieve the observed higher affinity.

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

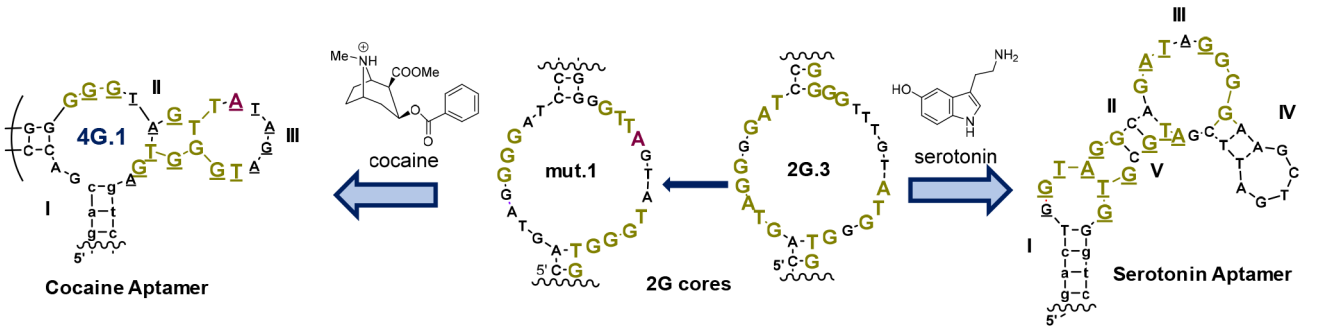


Figure 5. Similarities between families of cocaine receptors and the previously reported serotonin aptamer:[40] in the center, we show two members of the 2G family, showing overlaps with the high specificity cocaine and serotonin aptamers in golden font. The similarity between the high-specificity cocaine and serotonin aptamers is shown through underlining. The mutation in 2G core is the same as in Figure 2a.

The binding pocket of the 4G.1 aptamer has a substantial sequence overlap with the high affinity, high selectivity, serotonin aptamer[40] (15/26 bases in 4G.1 core, the overlap is underlined in Figure 5). Furthermore, we can also recognize in both aptamers the remnants of the 2G sequences (golden font, Figure 5), with 12/29 and 18/29 bases within the 2G.3 core conserved in the cocaine and serotonin aptamers, respectively. All three aptamers have strong G-content (>44%), thus, some aspects of this overlap are possibly products of chance. Interestingly, 3G.4, resulting from the counterselection using complement, had much lower G-content (~22%), but it also had a cluster of GG motifs concentrated together in an mfold[43]-generated secondary structure (Figure 3b).

Insights into Binding to 2G and 3/4G Aptamers

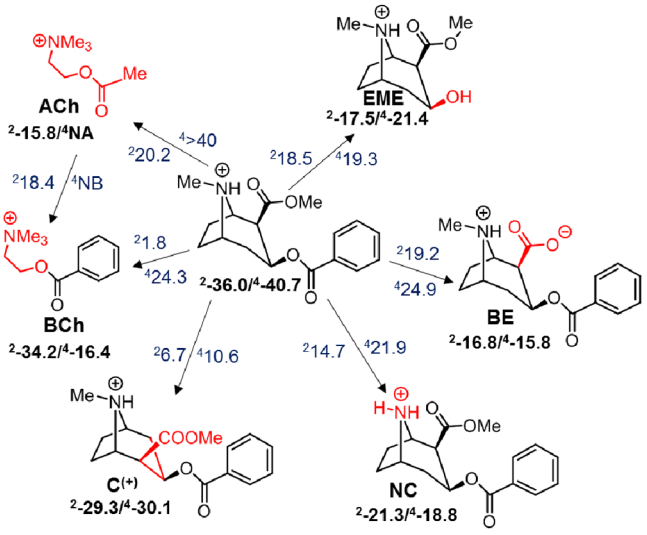
We next decided to characterize the relationship between two primary receptors, 2G.1 and 4G.1, in the context of binding to cocaine. For this purpose, we calculated $\Delta\Delta G_D$ values as follows (Scheme 2): first, we transformed $X_{50\%}$ values (Figure S7) into free energies of oligonucleotide displacement (ΔG_D) for cocaine and all available analogs, for both receptors. Second, we subtracted these values always using cocaine values as minuends and analog values as subtrahends. With this procedure, using the strand displacement assay, we isolated the impact of individual modifications on each receptor.

For example, we can calculate from the affinities of the cocaine and ecgonine methyl ester (EME) to the 2G.1 and 4G.1 receptors that the loss of the benzoyl group costs around 19 kJ/mol for either. In contrast, the substitution of the methyl group on the nitrogen with hydrogen, yielding norcocaine (NC), costs around 15 and 22 kJ/mol in the same receptors. Furthermore, norcocaine binds both receptors with a similar affinity. With the caveat that we should not commit to the final conclusions without structural studies, this loss of optimization benefits in norcocaine could be attributed in part to cation−π interactions[44] of the charged *N*-Me with hydrophobic base surfaces at the fixed distance.

We tested two further analogs that shared norcocaine the presence of both tropinyl and benzoyl fragments: enantiomeric cocaine ($C^{(+)}$) and benzoyl ecgonine (BE). Compared to cocaine, and unlike EME that lacked a benzoyl fragment, all three changed more their binding to 4G.1 than to 2G.1.

We can expand these considerations to two analogs with a quaternary amine and without bicyclic structures, benzoylcholine and acetylcholine. The former binds to both receptors, but it has a catastrophic collapse in affinity for 4G.1, while the latter has a much weaker, millimolar, affinity to 2G.1 and no specific interactions with 4G.1 in the strand displacement assay (acetylcholine seems to stabilize duplex formation with a complementary oligonucleotide, which we can use to separate the signatures of nonspecific interactions).[45] This relative preference of all analogs (i.e., a smaller loss in binding energy), except EME, for 2G.1, supports the hypothesis that 4G.1 has a significantly preformed arrangement of binding motifs that is less tolerant to structural changes in a ligand that was used in the optimization.

Scheme 2. Impacts of the Transformations (Stressed with Red Color) of Cocaine into Analogs on the Affinities of the 2G.1 and 4G.1 Aptamers[a]



[a]The superscripts 1 and 2 Refer to Values Obtained for the 2G.1 and 4G.1 Aptamers, Respectively. Under each structure, we provide ΔG_D values (kJ/mol). These values are impacts of a compound on the equilibrium between a fluorescently labeled aptamer and the competitor oligonucleotide used in the selection labeled with a quencher. These values were obtained directly from the displacement half-point ($X_{50\%}$) in these assays (Figure S7). At the arrows connecting the individual structures, we show the impact of each modification on this equilibrium ($\Delta\Delta G_D$, kJ/mol). We should not compare directly the ΔG_D between two aptamers without standardizing quenching levels because each aptamer has different interactions with its matching competitor oligonucleotide (Figure S3).

Comparison to Monoclonal Antibodies

It is instructive to compare the affinity and specificity of our 4G.1 aptamer to two well-characterized antibodies (with crystal structures with bound ligand), which were studied as

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

candidates for peripheral blocking of cocaine, h2E2 (K_D ~ 4 nM by ITC[21]) and GNC92H2 (K_D ~ 200 nM by equilibrium dialysis;[20] the reported affinity of GNC92H2 seems dependent on the method for determining the unbound fraction of antibodies; here, equilibrium dialysis would be a gold standard for small molecules). In contrast to aptamers, the gain in affinity for h2E2 came at the price of the increased affinity for BE (K_D ~ 20 nM). This observation was reproduced with mAb08,[23] another antibody with similarly low affinity (these antibodies were elicited using haptens similar to 1G aptamers, attached at modified carbomethoxy groups). Thus, 4G.1 is an outstanding reagent based on its specificity and affinity.

ASSESSING PROGRESS IN THE ISOLATION OF COCAINE APTAMERS

Thermodynamic Considerations

While progress when it comes to an increase in both affinity and specificity is obvious, cf. Table 1, there are additional insights that emerge upon more detailed considerations and that may yet prove useful in design of aptamers.

Table 1

TABLE 1	K_D (ITC, 290 K)	ΔH(kJ/mol)	−TΔS (J/molK)	K_D (FRET)
1G.0	28.2±1.5 μM	-54.8±1.5	28.7	
2G.1	315±26 nM	-50.6±0.4	13.3	
3G.1	47±6 nM	-160±1	115	
4G.1	14±2 nM	-135±1	88	17±3 nM
F4G.1flT				4±3 nM

We observed previously[25] a correlation between the number of nonhydrogen atoms (NHA) in hydrophobic fragments in planar primary amines and the free energy of binding to aptamers directly isolated in selections for these individual targets, with a slope of about −2.4 kJ/mol/NHA. The benzoyl group has eight NHAs, and its contribution to binding of cocaine to 2G.1 and 4G.1, and to binding of benzoylcholine to 2G.1, is about 18−19 kJ/mol, matching the contributions per NHA in hydrophobic fragments of aromatic amines.[25] This is consistent with this planar, hydrophobic fragment within a target (cocaine) being already optimized during selection with this specific library in both receptors.

We have also never achieved, in direct selections (i.e., without further optimization), with planar hydrophobic molecules as targets, affinities of aptamers higher than with serotonin and methylene blue, with free energy released upon binding below −45 kJ/mol. Thus, through multiple rounds of optimization, we exceeded the affinity of the best examples from the previous direct selections but did not meet the expectations when we account for the increase in the nonhydrogen atom count vs serotonin.

Cocaine shares with the previous targets the presentation of hydrophobic surfaces and a positive charge, but it has a large nonplanar, bicyclic fragment. This means that there are less accessible hydrophobic surfaces per nonhydrogen atoms, and that it is more challenging to orient monomeric residues to optimize hydrophobic contacts in a receptor. Furthermore, cocaine is a tertiary amine, which is unprecedented in our initial collection,[25] and it can introduce new types of contacts with nucleic acids.

The ITC data allow us to compare the intergenerational changes in enthalpy and entropy upon cocaine binding (Table 1), but we have to keep in mind the limitations of such analysis in the absence of detailed structural studies, more extensive sets of analogs, and the ability to account for conformational flexibility of aptamers.

The change in enthalpy (ΔH) upon binding of cocaine to 1G.0 and 2G.1 receptors is very similar, about −50 kJ/mol, consistent with the common mode of interactions with the same ligand shared by these negatively charged, primarily hydrophobic receptors. Next, there is a more than a 2-fold increase in the ΔH from the nonspecific 1G, 2G to the specific 3G, 4G receptors, with only partial enthalpy−entropy compensation.[46−48] Regarding this, at best we can say, without further structural details, that these changes are consistent with the expanded number of points of interactions between receptors and ligands. Finally, there is the significantly higher ΔH of binding of cocaine to 3G.1 than to 4G.1, despite these receptors likely having binding pockets with nearly identical contacts with the ligand. Because the binding of cocaine to 3G.1 is coupled to the additional conformational changes (cf. the secondary structures in Figure 3; here, we assume that the extended stem undergoes only minimal changes upon binding of cocaine), everything that is not identical between the two generations is, to the first approximation, attributable to this difference.

In the case of cocaine, we also observed that a more dramatic increase in affinity was matched by a substantial improvement in selectivity from 2G.1. This is actually expected because some of the improvement comes from the enhanced contacts with the hydrophobic surfaces, which, when combined with the preorganization of the binding pockets, should result in less contacts with unrelated hydrophobic ligands. Even the selectivity with more cognate ligands, EME and BS, was at least conserved (EME) and even substantially improved (BE and NC), being close to that previously attained for a polar molecule upon removal of hydrogen bonding networks[49] (theophylline → caffeine change).

Information Content Considerations

We can also analyze the correlations between the affinity of these aptamers and the information content of their sequences[27,50] (cf. Shannon's entropy).[51,52] This type of information content reflects the surprise of finding during a selection a structure in a pool with a particular function. Indeed, we observe that the weakest binding family of fully matched three-way junctions, with an estimated affinity of ~150 μM, is very common. In a series of receptors described here, from this fully matched three-way junction to 3G.1 and 4G.1, the ranking is straightforward because all of our examples have binding pockets within the internal loops, with differences in information content between any pair of aptamers reduced to a number of conserved bases connecting two stems. Indeed, the highest affinity aptamers are the most complex because they have the longest conserved sequence spanning two stems. Although a benefit to affinity upon single point mutation of 3G.1 is marginal (cf. Figure S7), fixing this base to transition to 4G families also leads to a marginal increase in information content. These results are fully intuitive because the higher affinity and selectivity require optimized contact of monomeric residues with cocaine. Optimal positioning requires longer sequences and, thus, by definition, higher information content and less common structures.

We are reluctant to pursue assigning a more precise number of bits to each sequence for several reasons: first, there is a

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

gradual degradation of binding upon mutations of hydrophobic molecules, which would make the assignment more challenging. Second, the palindromic relationship within sequences and preferences for the absence of C would, in theory, allow encoding in a smaller number of bits. Third, we expect that some pairwise motifs are more likely to appear together in the proximity of binding pockets, often for reasons of compatibility, which reduces the surprise of finding one if the other is present. Recognizing such motifs presents an opportunity to improve the chances of finding a rare receptor, *per* our previous efforts to isolate leucine aptamers;[25] however, the analysis of informational content in such cases may not be straightforward. Nevertheless, an increasing number of aptamers for a variety of ligands being reported together with full sequencing results may lead to a more comprehensive use of information content to improve library design[53] for specific ligands.[25]

CONCLUSIONS

The work described here was performed over the past 25 years, with the project restarted every several years due to realizations that previously isolated aptamers did not satisfy a particular practical requirement related to either cross-reactivity or affinity. Finally, however, we were simply curious to contribute to defining general principles of creating a hydrophobic pocket of a higher-complexity from a nucleic acid sequence, looking to build on our initial point of view that these are analogous to organic receptors,[54−57] but could be subjected to evolutionary protocols.

The most important outcome of our efforts, with immediate potential significance, is practical: we now have receptors with nanomolar affinity (sensors ∼5 nM) that could be used to measure concentrations of active cocaine (rather than its metabolites, which is useful for forensic reasons) in the blood of patients in the emergency room. Exquisite selectivity over metabolites of cocaine is a great advantage of the latest generation of aptamers over reported high-affinity monoclonal antibodies.

Furthermore, the affinities of our best aptamers are only slightly below that of the best reported monoclonal antibodies against cocaine.[22,23] Yet, the free energy of binding per nonhydrogen atom of cocaine (compared to serotonin and tryptamine), unimproved contacts with sections of cocaine, preliminary observations that stems could be used in optimization, and groove binding of the highest affinity antibodies,[21] all indicate that we might still have the space to increase affinity in both, for example, in our case by increasing contacts with the *endo* face of the *exo*-tropinyl fragment. Because we primarily pursued analytical applications, we also had to work on thermostabilizing any candidates for *in vivo* use. These improvements would depend on our ability to isolate even more complex receptors and present us with a challenge to pursue further generations of cocaine aptamers.

The broad cross-reactivity of receptors built around palindromic sequences, spanning from cocaine and serotonin to ephedrine, methamphetamine, and even caffeine, is indicative of adaptable binding interacting with all hydrophobic molecules partitioning to less polar environments. A self-organization (packing) of hydrophobic surfaces around a ligand then propagates from the individual faces of the targets, with a gradual transition to two stable double helical stems, all with the goal of minimized overall exposure of aromatic surfaces to water, which is balanced out with optimal hydrogen-bonding networks (to both solvent and within the aptamer).

Through our experiments, we also built on the classical work,[26−28] which described the relationship between informational content of aptamers, affinity, selectivity, and preorganization of originating libraries on an example of GTP. The comparison of 2G receptors with various cocaine and serotonin aptamers provides us with snapshots of interconnectivities between these simpler, promiscuous hosts and more complex and selective hydrophobic receptors. Here, the variants of palindromic hosts with broad cross-reactivity are at the intersections of individual recognition (fitness) landscapes,[58−63] each describing the interactions between sequence space and a specific ligand. While here we primarily focused on eliminating these promiscuous sequences through counter-selections, alternatively, we can learn to take advantage of them as the anchor points in selections.[25] From these intersections, we can perform volumetric tuning through mutagenesis and insertions in order to find more selective receptors for a variety of ligands, as proven through isolation of related receptors with selectivity for either serotonin or cocaine.

Our next challenge will be to harness these accumulated insights in a manner that would allow us to isolate aptamers with increasingly improved properties. Here, our focus on a target such as cocaine is useful as the proof-of-concept of pushing to extremes, binding to a small molecule with no particularly favorable interactions with nucleic acids. To make further progress, we will need to learn to scour through vast, interacting, multidimensional fitness landscapes using more formalized forms of intuition.[25]

METHODS

Detailed experimental materials and methods (selection procedures, sequence analysis, the assay procedure for fluorescence readout, isothermal titration calorimetry, and T5 exonuclease digestion) and general information regarding analytical instrumentation can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.3c00781.

Method description, oligonucleotide sequence information, and the corresponding supplementary figures associated with the manuscript (PDF)

AUTHOR INFORMATION

Corresponding Authors

Milan N. Stojanovic − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States; Departments of Biomedical Engineering and Systems Biology, Columbia University, New York, New York 10032, United States*; Email: mns18@columbia.edu

Donald W. Landry − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States*; Email: dwl1@columbia.edu

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

Authors

Kyungae Yang − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* orcid.org/0000-0001-5575-4107

Obtin Alkhamis − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Juan Canoura − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Alexandra Bryant − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Edward M. Gong − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* Present Address: Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University, Chicago, Illinois, United States

Mihaela Barbu − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* Present Address: Department of Biomedicine, University of Basel, Basel, Switzerland

Steven Taylor − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* orcid.org/0000-0002-9810-4531

Dragan Nikic − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* Present Address: no affiliation, College Station, Texas, United States

Saswata Banerjee − *Department of Medicine, Columbia University Irving Medical Center, New York, New York 10032, United States;* Present Address: Cactus Communications.

Yi Xiao − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0001-7278-9811

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.3c00781

Author Contributions

K.Y. isolated 2G, 3G, and 4G aptamers and performed their initial characterization (ThT and strand displacement assays and mutagenesis). NC State group performed ITC and exonuclease experiments. M.N.S. isolated and characterized 1G aptamers with the help of E.M.G., M.B., D.N., S.B., and S.T. The initial draft of the manuscript was written by M.N.S., with help of K.Y., and provision of data by Y.X. D.W.L. initiated research in cocaine peripheral blockers, obtained funding and provided general directions. All authors have given approval to the final version of the manuscript. **Kyungae Yang** conceptualization, data curation, formal analysis, investigation, validation, visualization, writing-review & editing; **Obtin Alkhamis** investigation, validation, visualization; **Juan Canoura** investigation, validation, visualization; **Alexandra Bryant** investigation, validation, visualization; **Edward M Gong** investigation; **Mihaela Barbu** investigation; **Steven Taylor** investigation, visualization, writing-review & editing; **Dragan Nikic** investigation ; **saswata banerjee** investigation; **Yi Xiao** data curation, supervision, review & editing; **Milan N. Stojanovic** conceptualization, data curation, funding acquisition, investigation, supervision, validation, visualization, writing-original draft, writing-review & editing; **Donald W. Landry** conceptualization, funding acquisition, review & editing.

Funding

NIH GM138843 (to M.N.S.), NSF CCF 1763632 (to M.N.S.), NSF CHE 2135005 (to Y.X.), the Counterdrug Technology Center of the Office of National Drug Control Policy (D.W.L.), and an F32 NIH postdoctoral fellowship (M.N.S.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

M.N.S. is thankful to Dr. Paloma de Prada for teaching him how to run gels, PCR, and to clone and to Dr. Shixian Deng for providing the initial procedures to make modified cocaine for affinity materials.

■ REFERENCES

(1) Ellington, A. D.; Szostak, J. W. In vitro selection of RNA molecules that bind specific ligands. *Nature* **1990**, *346*, 818−822.

(2) Tuerk, C.; Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **1990**, *249*, 505−510.

(3) Ruscito, A.; DeRosa, M. C. Small-molecule binding aptamers: Selection strategies, characterization, and applications. *Front. Chem.* **2016**, *4*, 14.

(4) Yu, H.; Alkhamis, O.; Canoura, J.; Liu, Y.; Xiao, Y. Advances and challenges in small-molecule DNA aptamer isolation, characterization, and sensor development. *Angew. Chem. Int. Ed.* **2021**, *60*, 16800−16823.

(5) Dunn, M. R.; Jimenez, R. M.; Chaput, J. C. Analysis of aptamer discovery and technology. *Nat. Rev. Chem.* **2017**, *1*, 0076.

(6) Song, S.; Wang, L.; Li, J.; Fan, C.; Zhao, J. Aptamer-based biosensors. *TrAC Trends Anal. Chem.* **2008**, *27*, 108−117.

(7) DeRosa, M. C.; Lin, A.; Mallikaratchy, P.; McConnell, E. M.; McKeague, M.; Patel, R.; Shigdar, S. In vitro selection of aptamers and their applications. *Nat. Rev. Methods Primers* **2023**, *3* (1), 54.

(8) Zhou, W.; Jimmy Huang, P.-J.; Ding, J.; Liu, J. Aptamer-based biosensors for biomedical diagnostics. *Analyst* **2014**, *139*, 2627−2640.

(9) Stojanovic, M. N.; de Prada, P.; Landry, D. W. Fluorescent sensors based on aptamer self-assembly. *J. Am. Chem. Soc.* **2000**, *122*, 11547−11548.

(10) Stojanovic, M. N.; de Prada, P.; Landry, D. W. Aptamer-based folding fluorescent sensor for cocaine. *J. Am. Chem. Soc.* **2001**, *123*, 4928−4931.

(11) Green, E.; Olah, M. J.; Abramova, T.; Williams, L. R.; Stefanovic, D.; Worgall, T.; Stojanovic, M. N. A rational approach to minimal high-resolution cross-reactive arrays. *J. Am. Chem. Soc.* **2006**, *128*, 15278−15282.

(12) Pei, R.; Shen, A.; Olah, M. J.; Stefanovic, D.; Worgall, T.; Stojanovic, M. N. High-resolution cross-reactive array for alkaloids. *Chem. Commun.* **2009**, *22*, 3193−3195.

(13) Taylor, S.; Pei, R.; Moon, B.; Damera, S.; Shen, A.; Stojanovic, M. N. Triggered release of an active peptide conjugate from a DNA device by an orally administrable small molecule. *Angew. Chem. Int. Ed.* **2009**, *48* (24), 4394−4397.

(14) Baker, B. R.; Lai, R. Y.; Wood, M. S.; Doctor, E. H.; Heeger, A. J.; Plaxco, K. W. An electronic, aptamer-based small-molecule sensor for the rapid, label-free detection of cocaine in adulterated samples and biological fluids. *J. Am. Chem. Soc.* **2006**, *128*, 3138−3139.

(15) Roncancio, D.; Yu, H.; Xu, X.; Wu, S.; Liu, R.; Debord, J.; Lou, X.; Xiao, Y. A label-free aptamer-fluorophore assembly for rapid and specific detection of cocaine in biofluids. *Anal. Chem.* **2014**, *86*, 11100−11106.

(16) Swensen, J. S.; Xiao, Y.; Ferguson, B. S.; Lubin, A. A.; Lai, R. Y.; Heege, R. A. J.; Plaxco, K. W.; Soh, H. T. Continuous, real-time

monitoring of cocaine in undiluted blood serum via a microfluidic, electrochemical aptamer-based sensor. *J. Am. Chem. Soc.* **2009**, *131* (12), 4262−4266.
(17) Soni, S.; Jain, U.; Burke, D. H.; Chauhan, N. Recent trends and emerging strategies for aptasensing technologies for illicit drugs detection. *J. Electroanal. Chem.* **2022**, *910*, 116128.
(18) Bilge, S.; Dogan-Topal, B.; Gürbüz, M. M.; Yücel, A.; Sınağ, A.; Ozkan, S. A. Recent advances in electrochemical sensing of cocaine: A review. *TrAC Trends Anal. Chem.* **2022**, *157*, 116768.
(19) Landry, D. W.; Zhao, K.; Yang, G. X.-Q.; Glickman, M.; Georgiadis, T. M. Antibody-catalyzed degradation of cocaine. *Science* **1993**, *259*, 1899−1901.
(20) Carrera, M. R. A.; Ashley, J. A.; Parsons, L. H.; Wirsching, P.; Koob, G. F.; Janda, K. D. Suppression of psychoactive effects of cocaine by active immunization. *Nature* **1995**, *378*, 727−730.
(21) Kirley, T. L.; Norman, A. B. Isothermal titration calorimetry determination of thermodynamics of binding of cocaine and its metabolites to humanized h2E2 anti-cocaine mAb. *Biochem. Biophys. Rep.* **2022**, *32*, 101354.
(22) Pozharski, E.; Moulin, A.; Hewagama, A.; Shanafelt, A. B.; Petsko, G. A.; Ringe, D. Diversity in hapten recognition: Structural study of an anti-cocaine antibody M82G2. *J. Mol. Bio.* **2005**, *10*, 570−582.
(23) Ramakrishnan, M.; De Melo, F. A.; Kinsey, B. M.; Ladbury, J. E.; Kosten, T. R.; Orson, F. M. Probing cocaine-antibody interactions in buffer and human serum. *PloS One* **2012**, *7* (7), No. e40518.
(24) Yang, K. A.; Barbu, M.; Halim, M.; Pallavi, P.; Kim, B.; Kolpashchikov, D. M.; Pecic, S.; Taylor, S.; Worgall, T. S.; Stojanovic, M. N. Recognition and sensing of low-epitope targets via ternary complexes with oligonucleotides and synthetic receptors. *Nat. Chem.* **2014**, *6*, 1003−1008.
(25) Yang, K.; Mitchell, N. M.; Banerjee, S.; Cheng, Z.; Taylor, S.; Kostic, A. M.; Wong, I.; Sajjath, S.; Zhang, Y.; Stevens, J.; Mohan, S.; Landry, D. W.; Worgall, T. S.; Andrews, A. M.; Stojanovic, M. N. A functional group−guided approach to aptamers for small molecules. *Science* **2023**, *380*, 942−948.
(26) Carothers, J. M.; Oestreich, S. C.; Szostak, J. W. Aptamers selected for higher-affinity binding are not more specific for the target ligand. *J. Am. Chem. Soc.* **2006**, *128*, 7929.
(27) Carothers, J. M.; Oestreich, S. C.; Davis, J. H.; Szostak, J. W. Informational complexity and functional activity of RNA structures. *J. Am. Chem. Soc.* **2004**, *126*, 5130−5137.
(28) Davis, J. H.; Szostak, J. W. Isolation of high-affinity GTP aptamers from partially structured RNA libraries. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (18), 11616−11621.
(29) Ding, Y.; Liu, J. Pushing adenosine and ATP SELEX for DNA aptamers with nanomolar affinity. *J. Am. Chem. Soc.* **2023**, *145*, 7540−7547.
(30) Huang, P.-J.-J.; Liu, J. Simultaneous detection of L-lactate and D-glucose using DNA aptamers in human blood serum. *Angew. Chem. Int. Ed.* **2023**, *62* (12), No. e202212879.
(31) Neves, M. A. D.; Reinstein, O.; Saad, M.; Johnson, P. E. Defining the secondary structural requirements of a cocaine-binding aptamer by a thermodynamic and mutation study. *Biophys. Chem.* **2010**, *153*, 9−16.
(32) Neves, M. A. D.; Reinstein, O.; Johnson, P. E. Defining a stem length-dependent binding mechanism for the cocaine-binding aptamer. A combined NMR and calorimetry study. *Biochemistry* **2010**, *49*, 8478−8487.
(33) Wang, Z.; Yu, H.; Canoura, J.; Liu, Y.; Alkhamis, O.; Fu, F.; Xiao, Y. Introducing structure-switching functionality into small-molecule-binding aptamers via nuclease-directed truncation. *Nucleic Acids Res.* **2018**, *46* (13), No. e81.
(34) Canoura, J.; Liu, Y.; Perry, J.; Willis, C.; Xiao, Y. Suite of aptamer-based sensors for the detection of fentanyl and its analogues. *ACS Sens.* **2023**, *8* (5), 1901−1911.
(35) Alkhamis, O.; Canoura, J.; Willis, C.; Wang, L.; Perry, J.; Xiao, Y. Comparison of aptamer signaling mechanisms reveals disparities in sensor response and strategies to eliminate false signals. *J. Am. Chem. Soc.* **2023**, *145*, 12407−12422.
(36) Alkhamis, O.; Canoura, J.; Ly, P. T.; Xiao, Y. Using exonucleases for aptamer characterization, engineering, and sensing. *Acc. Chem. Res.* **2023**, *13*, 1731−1743.
(37) Truong, J.; Hsieh, Y.-F.; Truong, L.; Jia, G.; Hammond, M. C. Designing fluorescent biosensors using circular permutations of riboswitches. *Methods* **2018**, *143*, 102−109.
(38) Gianni, S.; Freiberger, M. I.; Jemth, P.; Ferreiro, D. U.; Wolynes, P. G.; Fuxreiter, M. Fuzziness and frustration in the energy landscape of protein folding, function, and assembly. *Acc. Chem. Res.* **2021**, *54*, 1251−1259.
(39) Carrino, S.; Hennecker, C. D.; Murrieta, A. C.; Mittermaier, A. Frustrated folding of guanine quadruplexes in telomeric DNA. *Nucleic Acids Res.* **2021**, *49* (6), 3063−3076.
(40) Nakatsuka, N.; Yang, K. A.; Abendroth, J. M.; Cheung, K. M.; Xu, X.; Yang, H.; Zhao, C.; Zhu, B.; Rim, Y. S.; Yang, Y.; Weiss, P. S.; Stojanović, M. N.; Andrews, A. M. Aptamer-field-effect transistors overcome Debye length limitations for small-molecule sensing. *Science* **2018**, *362*, 319−324.
(41) Bommarito, S.; Peyret, N. Thermodynamic parameters for DNA sequences with dangling ends. *Nucleic Acids Res.* **2000**, *28* (9), 1929−1934.
(42) Moreira, B. G.; You, Y.; Behlke, M. A.; Owczarzy, R. Effects of fluorescent dyes, quenchers, and dangling ends on DNA duplex stability. *Biochem. Biophys. Res. Commun.* **2005**, *372* (2), 4730484.
(43) Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **2003**, *31*, 3406−3415.
(44) Dougherty, D. A. The cation-π interaction. *Acc. Chem. Res.* **2013**, *46*, 885−893.
(45) Frutiger, A.; Tanno, A.; Hwu, S.; Tiefenauer, R. F.; Vörös, J.; Nakatsuka, N. Nonspecific binding - fundamental concepts and consequences for biosensing applications. *Chem. Rev.* **2021**, *121*, 8095−8160.
(46) Chodera, J. D.; Mobley, D. L. Entropy-enthalpy compensation: Role and ramifications in biomolecular ligand recognition and design. *Annu. Rev. Biophys.* **2013**, *42*, 121−142.
(47) Fox, J. M.; Zhao, M.; Fink, M. J.; Kang, K.; Whitesides, G. M. The molecular origin of enthalpy/entropy compensation in biomolecular recognition. *Annu. Rev. Biophys.* **2018**, *47*, 223−250.
(48) Peccati, F.; Jiménez-Osés, G. Enthalpy-entropy compensation in biomolecular recognition: A computational perspective. *ACS Omega* **2021**, *6*, 11122−11130.
(49) Jenison, R. D.; Gill, S. C.; Pardi, A.; Polisky, B. High-resolution molecular discrimination by RNA. *Science* **1994**, *263*, 1425−1429.
(50) Warner, K. D.; Hajdin, C. E.; Weeks, K. M. Principles for targeting RNA with drug-like small molecules. *Nat. Rev. Drug Discovery* **2018**, *17*, 547−558.
(51) Shannon, C. E. A mathematical theory of communication. *Bell System Technol. J.* **1948**, *27*, 379−423.
(52) Adami, C. Information theory in molecular biology. *Physics Of Life Rev.* **2004**, *1*, 3−22.
(53) Ruff, K. M.; Snyder, T. M.; Liu, D. R. Enhanced functional potential of nucleic acid aptamer libraries patterned to increase secondary structure. *J. Am. Chem. Soc.* **2010**, *132* (27), 9453−9464.
(54) Del Valle, E. M. M. Cyclodextrins and their uses: A review. *Process Biochem.* **2004**, *39*, 1033−1046.
(55) Zhang, M.; Yan, X.; Huang, F.; Niu, Z.; Gibson, H. W. Stimuli-responsive host-guest systems based on the recognition of cryptands by organic guests. *Acc. Chem. Res.* **2014**, *47*, 1995−2005.
(56) Barrow, S. J.; Kasera, S.; Rowland, M. J.; Del Barrio, J.; Scherman, O. A. Cucurbituril-Based Molecular Recognition. *Chem. Rev.* **2015**, *115* (22), 12320−12406.
(57) Späth, A.; König, B. Molecular recognition of organic ammonium ions in solution using synthetic receptors. *Beilstein. J. Org. Chem.* **2010**, *6*, 32.
(58) Kun, Á.; Szathmáry, E. Fitness landscapes of functional RNAs. *Life* **2015**, *5*, 1497−1517.

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770

(59) Held, D. M.; Greathouse, S. T.; Agrawal, A.; Burke, D. H. Evolutionary landscapes for the acquisition of new ligand recognition by RNA aptamers. *J. Mol. Evol.* **2003**, *57* (3), 299−308.
(60) Jimenez, J. I.; Xulvi-Brunet, R.; Campbell, G. W.; Turk-MacLeod, R.; Chen, I. A. Comprehensive experimental fitness landscape and evolutionary network for small RNA. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 14984−14989.
(61) Pressman, A.; Moretti, J.; Campbell, G. W.; Muller, U.; Chen, I. A. Analysis of in vitro evolution reveals the underlying distribution of catalytic activity among random sequences. *Nucleic Acids Res.* **2017**, *45*, 8167−8179.
(62) Pressman, A. D.; Liu, Z.; Janzen, E.; Blanco, C.; Muller, U. F.; Joyce, G. F.; Pascal, R.; Chen, I. A. Mapping a systematic ribozyme fitness landscape reveals a frustrated evolutionary network for self-aminoacylating RNA. *J. Am. Chem. Soc.* **2019**, *141* (15), 6213−6223.
(63) Pitt, J. N.; Ferre-D'Amare, A. R. Rapid construction of empirical RNA fitness landscapes. *Science* **2010**, *330*, 376−379.

https://doi.org/10.1021/jacsau.3c00781
JACS Au 2024, 4, 760−770