Systematically Modulating Aptamer Affinity and Specificity by Guanosine-to-Inosine Substitution

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ABSTRACT: Aptamers are widely used in small molecule detection applications due to their specificity, stability, and cost effectiveness. One key challenge in utilizing aptamers in sensors is matching the binding affinity of the aptamer to the desired concentration range for analyte detection. The most common methods for modulating affinity have inherent limitations, such as the likelihood of drastic changes in aptamer folding. Here, we propose that substituting guanine for inosine at specific locations in the aptamer sequence provides a less perturbative approach to modulating affinity. Inosine is a naturally occurring nucleotide that results from hydrolytic deamination of adenosine, and like guanine, it base pairs with cytosine. Using the well-studied cocaine binding aptamer, we systematically replaced guanosine with inosine and were able to generate sequences having a range of binding affinities from 230 nM to 80 μM. Interestingly, we found that these substitutions could also modulate the specificity of the aptamers, leading to a range of binding affinities for structurally-related analytes. Analysis of folding stability via melting temperature shows that, as expected, aptamer structure is impacted by guanosine-to-inosine substitutions. The ability to tune binding affinity and specificity through guanosine-to-inosine substitution provides a convenient and reliable approach for rapidly generating aptamers for diverse biosensing applications.

Aptamers are single-stranded oligonucleotides that selectively recognize and bind to a target analyte through non-covalent interactions. Their specificity, stability, and cost-effectiveness have made them attractive for use as affinity reagents in small-molecule detection applications, and a large number of DNA aptamer-based sensor designs have been described.[2-7] In particular, the cocaine aptamer has been extensively utilized in biosensor development for drug monitoring,[8] and significant effort has been directed toward improving aptamer affinity through truncations and base modifications. However, aptamer truncations and drastic base modifications often compromise stability, leading to diminished selectivity and binding affinity.[9-11] In addition to improving affinity, generating aptamers having a range of binding affinities can yield biosensors having a wide dynamic range. Altering aptamer structure can also lead to a change in selectivity,[12] and thus using combinations of aptamer analogues can enable more efficient detection or sequestration of analyte mixtures containing structurally related molecules.

We recognized that guanosine-to-inosine substitution could provide a convenient approach to modulating aptamer affinity and selectivity, as this substitution changes the number of hydrogen bonds involved in base pairing, but should not affect folding to the same extent as other non-synonymous mutations.[13, 14] While guanosine-to-inosine substitution has been explored in aptamers, these studies have focused on single mutations of key binding residues in order to study aptamer folding and binding as opposed to modulating affinity.[15] Additionally, these substitutions are predominantly studied in G-quadruplex aptamer sequences.[16-18]

As an initial model system, we chose the MNS-4.1 anti-cocaine aptamer reported by Stojanovic and coworkers.[19] Stojanovic and others have explored multiple modifications to this sequence, most of which result in decreased binding affinity.[1, 19, 20] Due to this perturbed binding, other efforts were sought after to improve binding by modifying the MNS-4.1 aptamer. Roncancio et al. tested the parent MNS-4.1 aptamer in their dye displacement cocaine biosensor.[1] Because improving the binding affinity improves the LOD of aptamer-based biosensors, they performed various base mutations, leading to both slight increases and decreases in cocaine binding affinity.[1] Like Stojanovic and coworkers, Sachan et al. reported that truncations of their modified MNS-4.1 sequence or substituting key adenosines in stems 1 and 2 with 2-aminopurine not only decreased the binding affinity for cocaine, but also increased the affinity to off-target analytes.[20] From these studies, they concluded that cocaine binding relies on structure as opposed to specific sequence.

Starting from the MNS-4.1 and related 38-GC and 38-GT parent aptamer sequences; we explore systematic replacement of guanine with inosine in strategic locations known to impact structure or target binding. Using microscale thermophoresis (MST) to quantify the binding affinity of these aptamers to cocaine and structurally similar analytes, we find that guanosine-to-inosine substitution can be used to dramatically alter both binding affinity and specificity. Whereas the parent aptamer has a K_d value of ~80 μM, we are able to generate aptamers having a range of K_d values of 230 nM–80 μM. Additionally, we found that guanosine-to-inosine substitution could have a surprisingly large impact on binding specificity. Together, these data demonstrate that guanosine-to-inosine substitution serves as a convenient method for rapidly generating aptamer analogues having varying binding properties, and thus is well-suited for use in diverse biosensing applications.

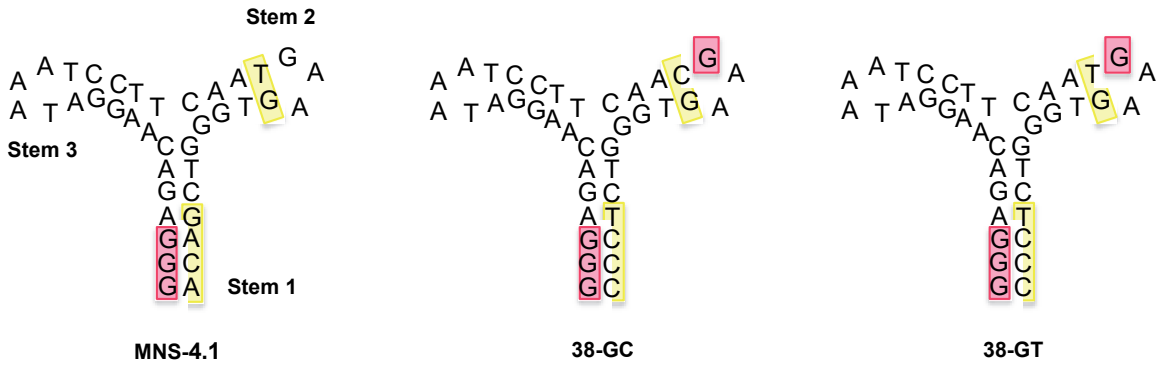

Figure 1. Structures of parent aptamers MNS-4.1, 38-GC, and 38-GT with stems 1-3 labeled for MNS-4.1. Stems 1-3 are the same for all aptamers. Magenta boxes indicate positions of guanosine-to-inosine substitutions. Yellow boxes indicate key differences between parent aptamers implemented by Roncancio et al. in an attempt to increase aptamer stability.[1] Inosine substitutions were formed at positions $G_{1\text{-}3}$ for all aptamers and G_{25} of 38-GT and 38-GC.

Experimental Section

Chemicals. Cocaine, benzoylecogonine, ecogonine methyl ester, cocaethylene, and norcocaine were purchased from Sigma-Aldrich. All stock solutions were prepared in water and stored at 4 °C. For biological samples, artificial saliva was purchased from Sigma-Aldrich.

Aptamer Synthesis. Oligonucleotides were purchased from the University of Utah DNA/Peptide Synthesis Core Facility or Integrated DNA Technologies (Table S1). All oligonucleotides were purified by 10% denaturing polyacrylamide gel electrophoresis prior to use. Gel bands were excised and incubated in 300 mM sodium acetate, 1 mM EDTA (pH 8.0) at 37 °C for 24 h. The DNA was then separated from the gel pieces using cellulose acetate membrane filters (ThermoFisher) and concentrated using 10K Amicon Ultra-0.5 centrifugal unit with Ultracel 10 membrane (EMD Millipore). DNA concentrations were measured on a NanoDrop 2000 (Thermo Scientific).

Microscale thermophoresis (MST). MST experiments were performed using a Monolith NT.115 (NanoTemper Technology). All measurements were performed in 10 mM Tris buffer (pH 7.4), 0.01 mM $MgCl_2$ and 5% DMSO or with with 2.5% artificial saliva at 25 °C. The aptamer concentration was kept constant at 5 nM, while the titrant (cocaine, EME, BE, cocaethylene, or norcocaine) concentrations ranged from 50 µM to 1.65 mM. We introduced 16 Monolith capillaries (NanoTemper) of 5 µL each at N=3 for cocaine, cocaethylene, and norcocaine and N=1 for EME and BE. Data were fitted using Prism 8 analysis software to determine the aptamer K_D values.

Melting temperature studies. Samples were prepared at a concentration of 3 µM. All measurements were performed in 10 mM Tris buffer (pH 7.4), 0.01 mM $MgCl_2$ and 5% DMSO at 25 °C in 8-well cuvettes and run on a Shimadzu UV-1800 spectrophotometer to monitor absorbance at N=3. Measurements were taken from 20-95 °C at a ramping rate of 0.5 °C per minute. Melting temperatures were determined by the first derivate method.

Structure-switching biosensor. Cy5-labeled aptamer stock solutions were diluted in 2X tris buffer as used in MST. Biosensors were prepared by combining Cy5-labeled aptamer (2.5 µM) and BHQ3-labeled capture strand (2.5 µM) in 2X tris buffer used in MST. This solution was heated to 95 °C and slow-cooled to 25 °C over 30 minutes in a thermal cycler. Biosensor solutions were stored at 4 °C until use. The biosensor was equilibrated to room temperature and 20 µL added to 384-well black plates (Corning, #3573). In triplicate, increasing concentrations of cocaine (30 µL) was added to the wells

Table 1. Binding affinity for cocaine and analogues and melting temperature of parent aptamers and inosine-substituted variants.

Aptamer	Positions	Cocaine K_d (µM)	Norcocaine K_d (µM)	Cocaethylene K_d (µM)	T_m (°C)
MNS-4.1	-	79.6 ± 14.0	15.1 ± 2.6	22.2 ± 4.9	41.5 ± 0.1
MNS-4.1 Inosine	$G_{1\text{-}3}$	4.5 ± 1.2	159 ± 69	3.8 ± 1.8	42.9 ± 0.1
38-GT	-	20.3 ± 2.8	15.1 ± 4.2	25.6 ± 2.5	45.6 ± 0.8
38-GT Inosine	$G_{1\text{-}3}$	6.2 ± 2.6	111 ± 24	1.4 ± 0.5	31.9 ± 0.2
38-IT	G_{25}	13.7 ± 3.8	23.2 ± 13.0	128 ± 33	45.0 ± 0.7
38-GC	-	14.5 ± 8.8	N.D.	15.5 ± 7.6	51.4 ± 0.7
38-GC Inosine	$G_{1\text{-}3}$	18.7 ± 3.9	N.D.	35 ± 14	41.8 ± 0.3
38-IC	G_{25}	0.23 ± 0.10	13.1 ± 3.8	15.2 ± 4.6	48.9 ± 0.1

and the solutions incubated for 40 minutes at 25 °C while protected from light. Displacement was quantified by measuring the fluorescence intensity on a Cytation 5 multi-mode plate reader (BioTek) using excitation at 650 nm and emission at 670 nm (bandwidth 9, read height 10.5 mm). All samples were normalized to wells containing Cy5-labeled aptamer alone. Percent displacement was calculated and plotted using GraphPad Prism.

Results and Discussion

The cocaine aptamer has been subjected to many sequence truncations and mutations, and we based our investigation on the parent aptamer sequences MNS-4.1, 38-GC, and 38-GT.[1] Because it has been shown that changes made to stem 1 affect binding affinity, we substituted $G_{1\text{-}3}$ of all aptamers to inosine to yield MNS-4.1 Inosine, 38-GC Inosine, and 38-GT Inosine.[1, 19, 20] In addition, we substituted G_{25} of 38-GC and 38-GT to inosine to yield 38-IC and 38-IT respectively (Figure 1). G_{25} is near, but not in, the binding pocket therefore we did not suspect that changing this base would severely alter the binding pocket.[20-23]

Using fluorescently labeled DNA strands, we employed microscale thermophoresis (MST) to measure the binding affinities of all eight aptamer sequences for cocaine. MST is a highly sensitive method based on changes in thermophoretic mobility upon target binding. Unlike surface plasmon resonance and many other analytical methods, MST does not require that the target or aptamer be immobilized on a surface. In addition, compared to isothermal titration calorimetry, MST requires only small amounts of sample. Thus, MST offers an accurate and convenient method for quantifying aptamer binding affinity.[24, 25] MST analyses indicated that most inosine-containing aptamers bound cocaine with a higher affinity than their unmodified counterparts (Table 1, Figure 2a). The K_d value of the MNS-4.1 Inosine aptamer having substitutions at $G_{1\text{-}3}$ improved 17-fold in comparison to the parent MNS-4.1 aptamer. In addition, 38-GT Inosine bound cocaine with a ~2.5-fold improvement in comparison to the parent 38-GT aptamer. However, 38-GC Inosine bound with a K_d of 18.7 µM, which is comparable to the 38-GC parent aptamer. In the case of the G_{25} substitution, 38-IT only bound with a ~1.5-fold improvement in comparison to the 38-GT aptamer. Finally, the 38-GC G_{25} substitution, 38-IC, showed an impressive 63-fold increase in affinity compared to the parent 38-GC aptamer. We were very excited about these results, as most modifications to the cocaine-binding aptamer have resulted in a decrease in binding affinity for the target, and achieving an increase in affinity is significantly more challenging.

To investigate the relationship between folding stability and cocaine binding affinity, we purchased non-labeled aptamer strands and used UV absorbance to determine the melting temperature (T_m) of each aptamer (Table 1, Figure S9). We found that substituting $G_{1\text{-}3}$ for inosine had a large effect on the T_m of the 38-GT and 38-GC parent sequences, decreasing T_m by 13.7 and 9.6 °C, respectively. This is not unexpected, as I-C base pairs have one fewer hydrogen bond than G-C base pairs.[26, 27] However, the $G_{1\text{-}3}$ substitutions interestingly had a slightly stabilizing effect on the MNS-4.1 aptamer. Given that where $G_{1\text{-}3}$ are located in the S_1 stem has been noted to be crucial for cocaine binding,[20-22] we found it interesting that we did not observe a distinct correlation between binding affinity and melting temperature. We also found that changing G_{25} of 38-GT and 38-GC only slightly affected the T_m values of the aptamers indicating that the changes in affinity for these inosine-substituted aptamers are likely a result of much more subtle changes in fold or structure as opposed to overall stability.

Next, we were curious to investigate the effect of inosine substitution on binding selectivity against structurally related analytes. Cocaine is primarily hydrolyzed to benzoyl ecgonine (BE) by human choline esterase 1 and to ecgonine methyl ester (EME) by butyryrlcholinesterase, human cholinesterase 2, and cocaine esterase.[28-30] Therefore, binding to these metabolites is of significant interest in biosensing applications. We performed MST with each of the parent and inosine-substituted aptamers and found no detectable binding of any of the aptamers to BE or EME (Figure S4 and S5). This indicates that the change in aptamer structure arising from inosine substitution does not decrease specificity against EME and BE.

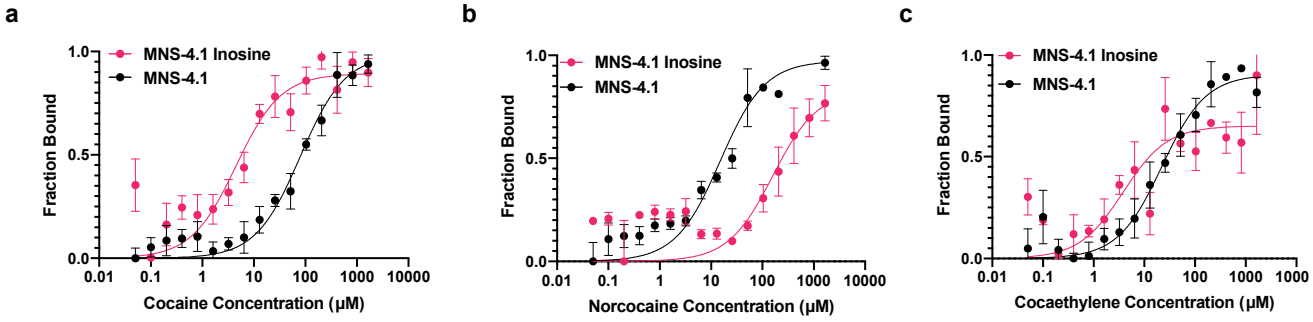

Figure 2. (a) Cocaine (b) norcocaine and (e) cocaethylene binding of parent MNS-4.1 aptamer in comparison to MNS-4.1 Inosine measured using MST. All MST experiments were performed in triplicate.

We were also curious to test the binding affinity of the aptamers with norcocaine and cocaethylene, as the parent MNS-4.1 aptamer has been reported to bind these metabolites.[20] Interestingly; norcocaine is also the only known metabolite of cocaine to be biologically active.[31] As expected, we found that the parent MNS-4.1 aptamer binds norcocaine (Figure 2b, Table 1). However, inosine substitution at $G_{1\text{-}3}$ resulted in 11-fold weaker binding, demonstrating that this substitution can dramatically impact selectivity. Similarly, we found that inosine substitution at $G_{1\text{-}3}$ of the 38-GT aptamer resulted in 7.4-fold weaker binding however, inosine substitution at G_{25} of the 38-GT aptamer did not have a significant impact on norcocaine binding affinity. Interestingly, the parent 38-GC aptamer shows no affinity for norcocaine, and this selectivity was re-

tained with inosine substitution at $G_{1\text{-}3}$. However, substitution of the 38-GC aptamer at the G_{25} position restored norcocaine binding, and in fact, this sequence had the highest affinity for norcocaine of all of those tested. In the case of cocaethylene, we also found that the parent MNS-4.1 aptamer binds with an affinity of 22 μM, which is not unexpected given previous reports and the similarity between cocaethylene and cocaine.[20] In a similar trend, our MNS-4.1 Inosine aptamer bound with a higher affinity to cocaethylene than MNS-4.1 and with a higher affinity to cocaethylene than cocaine (Figure 2c, Table 1). Inosine substitution at the $G_{1\text{-}3}$ positions of the 38-GT and 38-GC aptamers produced similar changes in cocaethylene binding as observed for cocaine. However, while 38-IC bound cocaethylene with the same affinity as its parent 38-GC aptamer, 38-IT bound with a 5-fold lower affinity than its parent 38-GT aptamer. Together, these data demonstrate that inosine substitution not only impacts binding for the target substrate, but can also dramatically impact selectivity by modulating the affinity of aptamers for off-target analytes. For cocaethylene, substitutions made to either $G_{1\text{-}3}$ and G_{25} resulted in an increase in affinity in most cases. However, norcocaine showed a nearly opposite trend, with the exception of 38-IC.

MNS-4.1 has been shown to have salt-dependent affinity for cocaine, and thus we sought to also test binding in a biological fluid.[12, 20] We performed MST analysis with MNS-4.1 and MNS-4.1 Inosine in 2.5% artificial saliva, which has higher KCl concentration than the standard binding buffer used for the aptamer. Interestingly, we observed a ~10-fold increase in binding affinity in the case of MNS-4.1, but observed no change in affinity in the case of MNS-4.1 Inosine (Figure S8).

Cocaine-binding aptamers have found wide use in structure-switching biosensors, and thus we sought to test MNS-4.1 and its inosine counterpart, MNS-4.1 Inosine in this context. We functionalized our aptamers with Cy5 and hybridized each to a BHQ3-functionalized complementary strand.[32, 33] In the absence of cocaine, the two sequences hybridize, resulting in quenching of the Cy5 fluorescence. In the presence of cocaine, however, the capture strand (CS) is displaced, resulting in an enhancement in fluorescence (Figure 3a). We observed dose-dependent curves with K_{sens} values of 11.4 ± 4.6 μM and 20.9 ± 6.6 μM for MNS-4.1 and MNS-4.1 Inosine, respectively (Figure 3b). Using a previously reported method, we combined these measurements with the K_d of each aptamer for the complementary strand in order to independently calculate the K_d of each aptamer for cocaine.[33, 34] This resulted in K_d values of 41.2 μM for MNS-4.1 and 13.9 μM for MNS-4.1 Inosine, which are consistent with our MST data. These data further support our earlier observations and demonstrate that inosine modification can also be used to modulate the dynamic range of aptamer sensors.

Conclusion

Here we explore guanosine-to-inosine substitution as a method to modulate the affinity and specificity of DNA aptamers. Using the well-studied cocaine-binding aptamer, we designed and analyzed five inosine-containing sequences based on the parent aptamers MNS-4.1 38-GT and 38-GC.[1, 19] Using MST, we found that inosine substitution at $G_{1\text{-}3}$ and G_{25} resulted in sequences having a wide range of binding affinities. Excitingly, in some cases, we observed improved affinity with one aptamer showing 63-fold stronger binding as a result of a single inosine substitution. Melting temperature studies showed that inosine substitution also dramatically impacts thermal stability, but we did not observe a strong correlation between changes in T_m value and changes in binding affinity for cocaine. Thus, we hypothesize that inosine impacts cocaine binding through more subtle structural changes to the aptamer that are not necessarily reflected in thermal stability.

A survey of aptamer binding to cocaine metabolites demonstrated that inosine substitution does not impact selectivity against EME and BE as no binding was detected for the parent aptamers or their inosine-containing analogues. However, we did find that selectivity for norcocaine and cocaethylene could be modulated by inosine substitution with $G_{1\text{-}3}$ modification resulting in up to 10-fold increase in selectivity compared to

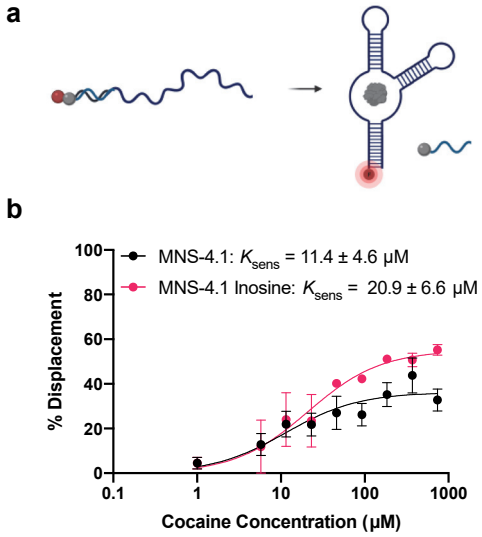

Figure 3. Biosensor characterization for MNS-4.1 and MNS-4.1 Inosine. (a) Schematic of our structure-switching biosensing method. The red circle represents our fluorophore, and the grey represents our quencher. (b) Dose-dependent curves for MNS-4.1 and MNS-4.1 Inosine biosensors with cocaine.

the parent aptamer. We also demonstrated that inosine substitution could be used to modulate the dynamic range of aptamer sensors.

While at times we observe dramatic changes to analyte binding upon inosine substitution, we have not yet been able to elucidate clear trends that would predict how inosine substitution could be used to strategically tune affinity. We envision that future studies harnessing a greater number of analogues in tandem with machine learning approaches could provide insight into this interesting question. We also look forward to exploring inosine substitution with other aptamer sequences. We envision that this approach will provide a facile, rapid, and cost-efficient method for obtaining aptamers having a range of binding affinities, not only for the desired target, but also for off-target analytes. We anticipate that this will significantly advance the use of DNA aptamers in biosensing applications by enabling researchers to carefully tune binding affinity to match the desired dynamic range for analyte detection.

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Notes

The authors declare no competing financial interest.

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