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Structure–affinity relationship of the cocaine-binding aptamer with quinine derivatives

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ABSTRACT

In addition to binding its target molecule, cocaine, the cocaine-binding aptamer tightly binds the alkaloid quinine. In order to understand better how the cocaine-binding aptamer interacts with quinine we have used isothermal titration calorimetry-based binding experiments to study the interaction of the cocaine-binding aptamer to a series of structural analogs of quinine. As a basis for comparison we also investigated the binding of the cocaine-binding aptamer to a set of cocaine metabolites. The bicyclic aromatic ring on quinine is essential for tight affinity by the cocaine-binding aptamer with 6-methoxyquinoline alone being sufficient for tight binding while the aliphatic portion of quinine, quinuclidine, does not show detectable binding. Compounds with three fused aromatic rings are not bound by the aptamer. Having a methoxy group at the 6-position of the bicyclic ring is important for binding as substituting it with a hydrogen, an alcohol or an amino group all result in lower binding affinity. For all ligands that bind, association is driven by a negative enthalpy compensated by unfavorable binding entropy.

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1. Introduction

Aptamers are nucleic acid molecules with the ability to bind a variety of different ligands ranging from small molecules to whole cells.[1] Ever since they were first developed, aptamers have been widely investigated and are used in numerous applications including medical treatments, pharmaceuticals and biosensors.[2] The process of selecting aptamers is called SELEX and involves screening for desired aptamers from a large library of oligonucleotides with the selected aptamers amplified.[3,4] However, it is not well understood how aptamers work. To better understand aptamer structure and how they interact with other molecules we are using the cocaine-binding aptamer as a model system. This aptamer has been used as a model system for many biosensor applications.[5–14] An important reason why the cocaine-binding aptamer has gained such wide usage is that it can be engineered to follow a structural switching or ligand-induced folding mechanism.[6,15–17]

The cocaine-binding aptamer is a DNA aptamer that contains 3 stems built around a 3-way junction containing a dinucleotide TC bulge with an adjacent pair of non-canonical GA base pairs (Fig. 1). When stem 1 is shortened to three base pairs, the free aptamer is loosely folded or unfolded and becomes more tightly structured when it binds its target ligand.[18] However, when stem 1 is longer, the aptamer has its secondary structure formed in both the free and bound form.[18] In addition, the aptamer can be split into two or three separate DNA strands with the annealing of the strands coupled with ligand binding.[9,19] Of the different variations of the cocaine-binding aptamer that have been studied, we have chosen MN4 (Fig. 1) for detailed study as it displays excellent NMR spectra and binds cocaine slightly tighter than the originally reported aptamer.[18]

Despite being selected for cocaine affinity, the cocaine-binding aptamer binds alternate molecules, including other alkaloids as well as steroids. Studies where the identity of the nucleotides at the three-way junction have been changed have taken advantage of this changed binding selectivity to build a sensor array.[20,21] Most significantly amongst the binding promiscuity shown by this aptamer, is that the cocaine-binding aptamer exhibits an almost 30-fold stronger affinity for quinine than cocaine.[22,23] This adaptability in ligand specificity is not common among aptamers and prompted our investigation to determine what regions in the structure of the quinine are important for aptamer binding. To achieve this, we are using isothermal titration calorimetry (ITC) techniques to compare the binding affinity and thermodynamics of thirteen quinine analogs (Fig. 2) for the MN4 cocaine-binding aptamer to that of quinine and cocaine. We also assay the affinity

Abbreviations: DMSO, dimethyl sulfoxide; ITC, isothermal titration calorimetry; SELEX, systematic evolution of ligands by exponential enrichment; TRIS, 2-amino-2-hydroxymethyl-propane-1,3-diol.

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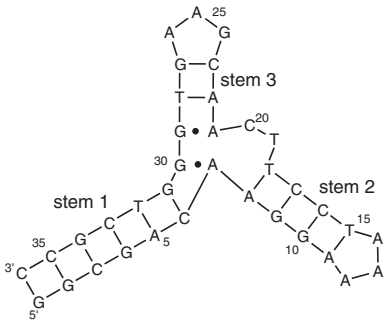

Figure 1. Secondary structure of the MN4 cocaine-binding aptamer. Dashes between nucleotides indicate Watson–Crick base pairs while dots indicate non-Watson–Crick base pairs.

of a set of cocaine metabolites and the alkaloid atropine to the cocaine-binding aptamer (Fig. 2). Our results suggest that the presence of the fused aromatic rings and methoxy group in the quinine structure play an important role in the tight binding of quinine by the cocaine-binding aptamer.

2. Material and methods

2.1. Materials

Aptamer samples were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) and used without further purification. The identity of the DNA was confirmed by electrospray ionization mass spectrometry by the manufacturer. DNA samples were dissolved in distilled, deionized water and then exchanged three times in a 3 kDa molecular weight cutoff concentrator with 1 M NaCl and washed three times with distilled deionized water. All DNA samples were exchanged with buffer A (20 mM TRIS (pH 7.4), 140 mM NaCl, 5 mM KCl) three times before use. Aptamer concentrations were determined by absorbance spectroscopy using the calculated extinction coefficients. All small molecule ligands were obtained from Sigma Aldrich. Stock solutions of compounds for binding experiments were prepared by dissolving the appropriate weight of each analog in buffer A. Stock solutions of quinoline, 6-methoxyquinoline, 6-hydroxyquinoline, 6-aminoiquinoline, acridine and benzo(h)quinoline were prepared by dissolving the appropriate weight of each analog in buffer B (buffer A with 1% DMSO).

2.2. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC instrument and the data were analyzed using Origin 5 software fitting to a one-site binding model. Samples were degassed before analysis with a MicroCal Thermo Vac unit. All experiments were corrected for the heat of dilution of the titrant. Binding experiments were performed at 15 °C with the aptamer solution concentration set at 20 μM and the small molecule concentration at 0.312 mM. This temperature was used in order to better compare this data with data acquired for the short stem 1 version of the cocaine-binding aptamer (MN19[23]). Titrations were performed with the aptamer samples in the cell and with the ligand as the titrant, in the needle. All aptamer samples were heated in a boiling water bath for 3 min and cooled in an ice water bath prior to use in a binding experiment to allow the DNA

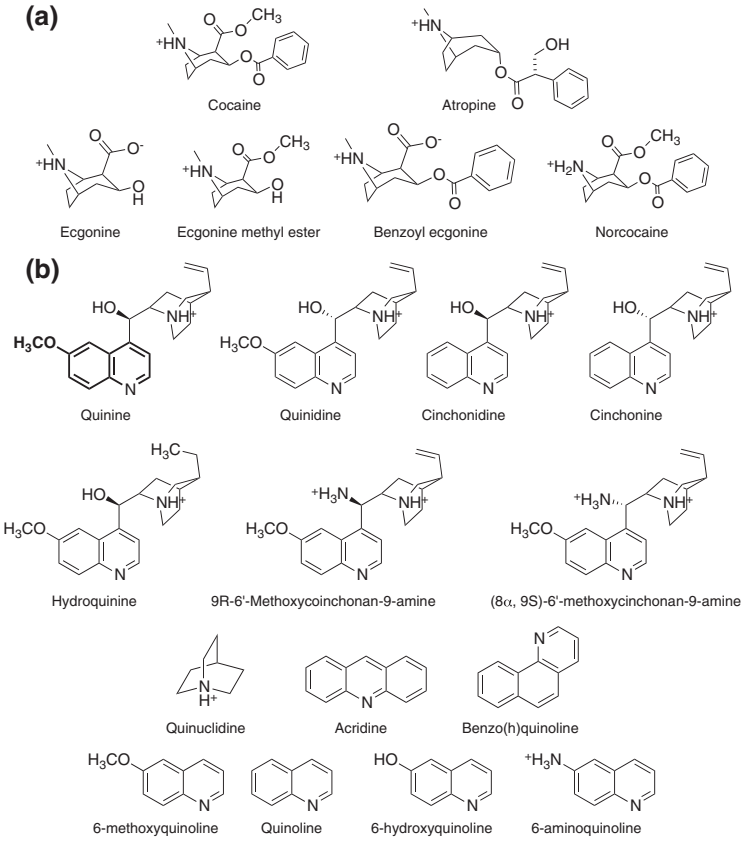

Figure 2. Structures of the molecules used in this study. In (a) are cocaine, atropine and the cocaine metabolites investigated. In (b) are quinine and the different analogs of quinine studied. The chemical groups on quinine that are most important for aptamer binding are in bold and are drawn with thicker bonds.

aptamer to anneal in an intramolecular fashion. The experimental concentration for the MN4 construct was established using a *c* value of approximately 50.[24] A typical binding experiment consisted of 35 successive injections spaced every 300 s where first injection was 2 μL and subsequent 34 injections were 8 μL. For the data fitting of the binding experiments, the stoichiometry of the interaction (*n*) was set to 1.

3. Results

3.1. Binding affinity and thermodynamics of quinine and cocaine

We used ITC methods to determine which region(s) of the cocaine-binding aptamer ligands are important for high-affinity binding. To achieve this, we compared the binding affinity and thermodynamics of thirteen quinine analogs along with four cocaine metabolites for the cocaine-binding aptamer MN4 to that of quinine and cocaine. Figure 3 provides a sample ITC thermogram of the MN4 aptamer binding to quinine as well as an example of a case, for benzo(h)quinoline, where no detectable binding by MN4 was observed. The binding affinity and the thermodynamic properties of all ligands used in this study for the MN4 aptamer are shown in Table 1. The data presented here for the binding of quinine and cocaine by MN4 agree with the results published previously.[19,23]

Both cocaine and quinine consist of an aromatic and an aliphatic region (Fig. 2). As a first guess as to what may be required for binding by the cocaine-binding aptamer we assayed the alkaloid atropine (Fig. 2a) for binding to MN4. Despite containing both aromatic and aliphatic regions, atropine is not bound by the MN4 aptamer (Table 1).

3.2. Cocaine metabolites

In order to determine which regions of cocaine are important for aptamer recognition we used ITC to study the binding of four cocaine metabolites: ecgonine, benzoyl ecgonine, ecgonine methyl ester and norcocaine (Fig. 2). Our results show that all metabolites, except for norcocaine, do not exhibit affinity to the cocaine-binding aptamer MN4. In contrast, norcocaine exhibits slightly tighter binding to the MN4 aptamer than cocaine (Table 1).

3.3. Quinine analogs—the importance of fused ring

We probed the importance that the aromatic ring plays in quinine binding by MN4 by studying the affinity of structural analogs where the aliphatic region is separated from the aromatic portion. Initially, quinine was divided roughly in half with a molecule representing the aliphatic region, quinuclidine (Fig. 2b), showing no detectable binding. Surprisingly, a molecule representing the aromatic portion of quinine, 6-methoxyquinoline (Fig. 2b), was bound by MN4 with significant affinity. The affinity of MN4 for 6-methoxyquinoline was (0.5 ± 0.1) μM (Table 1), only 5 times weaker than for intact quinine. We further tested the affinity of fused aromatic rings by testing the binding affinity of the MN4 aptamer for aromatic ligands with three fused six-membered rings. Neither acridine or benzo(h)quinoline (Fig. 2b) are bound by MN4 (Table 1).

3.4. Quinine analogs—methoxy variants

We investigated the importance of the methoxy group on the quinoline ring of quinine for aptamer binding by measuring the affinity of compounds with the methoxy group replaced by a hydrogen an alcohol group and an amino group. In each case studied, changing the methoxy group reduced ligand affinity. We replaced the methoxy group with hydrogen in intact quinine by studying the binding of cinchonidine (Fig. 2b). The affinity of MN4 for cinchonidine is ~10 fold weaker than MN4 for quinine. Additionally, we looked at the binding of quinoline (Fig. 2b) as a comparison for the binding of 6-methoxyquinoline. For this pair of ligands, replacing the methoxy with a hydrogen atom reduces the affinity seven fold (Table 1). Next, we replaced the methoxy group on 6-methoxyquinoline with an alcohol and an amino group. For both 6-hydroxyquinoline and 6-aminoquinoline (Fig. 2b) the affinity of the MN4 aptamer for the ligand is reduced by 3 and 5 fold, respectively, when compared with the affinity for 6-methoxyquinoline (Table 1).

3.5. Quinine analogs—isomerism

We next studied the effect of changing the position around the stereogenic carbon-9 connecting the aliphatic and aromatic portions of quinine by studying three pairs of optical isomers. In the first pair, quinine and quinidine (Fig. 2b), changing the stereochemistry at position 2 from *R* in quinine to *S* in quinidine results in a 2.3 fold reduction in MN4 binding affinity (Table 1). Similarly, changing the stereochemistry at the same location from *R* in cinchonidine to *S* in cinchonine reduces the ligand affinity of MN4 by 2.8 fold (Table 1).

We also analyzed the binding by MN4 to another pair of optical isomers, (9*R*)-6′-methoxycinchonan-9-amine and (8α,9*S*)-6′-Methoxycinchonan-9-amine (Fig. 2b). This pair of molecules has an amino group instead of an alcohol group at carbon-9 between the aliphatic and aromatic parts of quinine. At the pH studied, 7.4, this amino should be protonated. The switch from an –OH to the $-NH_3^+$ reduces the affinity of MN4 for the ligand by approximately 10 fold (Table 1) with binding by the 9*S* isomer 1.3 fold weaker than for the 9*R* isomer.

3.6. Effect of saturating the vinyl group

The final quinine analog tested has a change in the aliphatic portion of the molecule. Hydroquinine differs from quinine in that the double bond in quinine has been saturated to a methyl group in hydroquinine (Fig. 2b). This change has no significant effect on the affinity of the ligand by MN4 (Table 1).

4. Discussion

4.1. Binding thermodynamics

All the compounds studied here that are bound by MN4 have binding driven by a favorable enthalpy contribution that is balanced by an unfavorable binding entropy (Table 1). When the enthalpy is plotted against entropy, the data follow a straight line (Fig. 4). What an observed linearity of enthalpy–entropy compensation means is a contentious issue with different views on whether the effect is real and if so, what is the origin of the correlation.[25–28] Whatever the origin of the correlation is, having an enthalpy–entropy correlation does show that the correlated ligands follow a similar binding mechanism. We expect the different ligands studied here that are bound by MN4 follow a similar binding mechanism, this is consistent with our previous finding that both quinine and cocaine compete for the same binding site in MN4.[23]

4.2. Analysis of the binding results of the different compounds

The ability of the cocaine-binding aptamer to bind quinine tighter than the molecule it was originally selected for is unusual. A clear similarity between these ligands is that there is an aromatic portion of the molecule and an aliphatic region that contains a basic nitrogen. In order to see if having these two components is

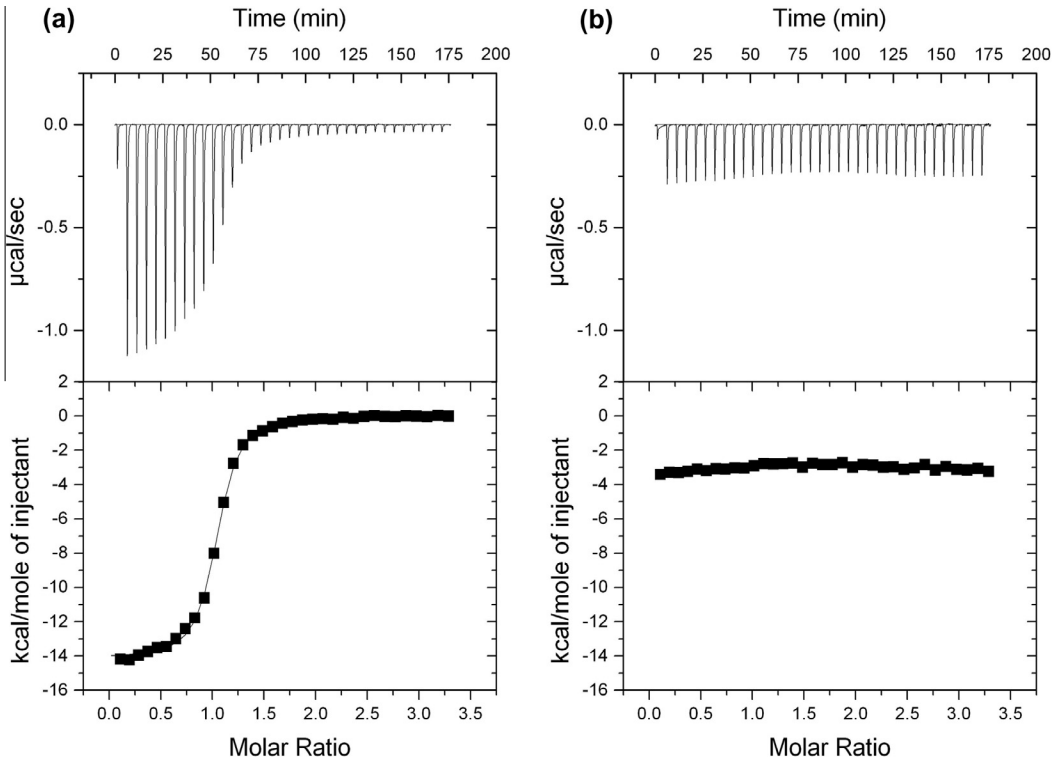

Figure 3. Sample of the ITC data showing the interaction of MN4 with (a) quinine and (b) benzo(h)quinoline. On top is the raw titration data showing the heat resulting from each injection of into the aptamer solution. On the bottom is the integrated heat after correcting for the heat of dilution. In (a) the binding experiment was performed at 15 °C in a buffer of 20 mM Tris (pH 7.4), 140 mM NaCl, 5 mM KCl. In (b) the binding experiment was performed at 15 °C in the same buffer as (a) that also contained 1% DMSO.

Table 1
Affinity and thermodynamic parameters of ligands used in this study for binding to the MN4 cocaine-binding aptamer[a]

Ligand	K_d (μM)	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
Cocaine	5.5 ± 0.4	−11 ± 1	4 ± 1
Norcocaine	4 ± 1	−11.3 ± 0.3	4.3 ± 0.4
Ecgonine		NB	
Benzoyl ecgonine		NB	
Ecgonine methyl ester		NB	
Quinine	0.11 ± 0.04	−13.6 ± 0.5	4.4 ± 0.6
Atropine		NB	
Quinidine	0.25 ± 0.04	−23.5 ± 0.1	14.7 ± 0.1
Hydroquinine	0.10 ± 0.04	−13 ± 2	4 ± 2
Cinchonidine	1.2 ± 0.5	−9 ± 2	1 ± 1
Cinchonine	3.3 ± 0.1	−7.9 ± 0.2	0.7 ± 0.2
(9*R*)-6′-Methoxycinchonan-9-amine	1.3 ± 0.1	−11.7 ± 0.1	3.9 ± 0.1
(8α,9*S*)-6′-Methoxycinchonan-9-amine	1.7 ± 0.1	−11.2 ± 0.4	3.6 ± 0.4
Quinuclidine		NB	
Quinine (1% DMSO)[b]	0.16 ± 0.04	−12.7 ± 1.3	4.7 ± 0.9
6-Methoxyquinoline[b]	0.5 ± 0.1	−16.72 ± 0.01	8.5 ± 0.1
Quinoline[b]	3.5 ± 0.1	−10.2 ± 0.7	3 ± 1
6-Hydroxyquinoline[b]	1.4 ± 0.1	−12.1 ± 0.7	4.4 ± 0.8
6-Aminoquinoline[b]	2.6 ± 0.5	−11 ± 2	4 ± 1
Acridine[b]		NB	
Benzo(h)quinoline[b]		NB	

The values reported are averages of 2–6 individual experiments. NB denotes no binding.
[a] Data acquired at 15 °C in buffer A (20 mM TRIS, 140 mM NaCl, 5 mM KCl, pH 7.4) except where marked.
[b] Where the data was acquired at 15 °C in buffer B (buffer A plus 1% DMSO).

the sole requirement for aptamer binding we tested the ability of MN4 to bind atropine (Fig. 2a). Like quinine and cocaine, atropine has an aromatic and aliphatic region that contains a basic nitrogen. Unlike cocaine and quinine, atropine is not bound by MN4 (Table 1). This indicates that there are some other features in ligands for MN4 than simply possessing an aromatic and a nitrogen-containing aliphatic region.

We also studied the binding of different metabolites of cocaine to MN4. Binding of these metabolites has previously been studied, though not using ITC methods. Each of ecgonine, ecgonine methyl ester and benzoyl ecgonine are not bound by MN4 and norcocaine is bound by MN4 with the same affinity as cocaine (Table 1). These results are consistent with previously published studies.[5–7,9,10,12,29] We will note that even the minor change of removing a methyl group from cocaine to give benzoyl ecgonine (Fig. 2a) results in the loss of binding. The removal of a methyl group results in creating a negatively charged carboxylate that likely interferes with binding by the negatively charged DNA aptamer. Nevertheless, this

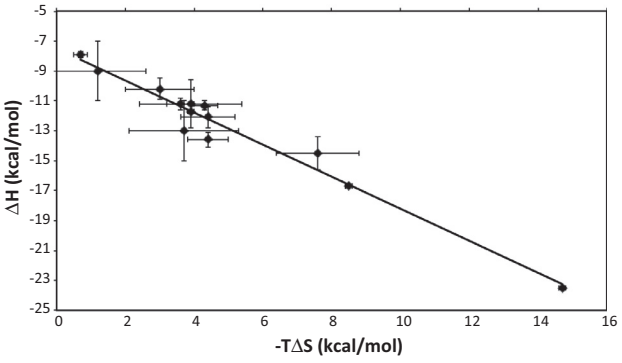

Figure 4. Plot of the binding enthalpy versus entropy (ΔH vs $-T\Delta S$) for the ligands in this study that are bound by the MN4 cocaine-binding aptamer. Error bars in both axes are shown.

subtle change reflects the high specificity of the MN4 aptamer for cocaine over modified cocaine molecules, and is striking in light of the high affinity MN4 has for quinine and related compounds.

The ability of the cocaine-binding aptamer to bind a molecule tighter than the one it has been selected for is an unusual feature for an aptamer. The tight binding of quinine by MN4 has been reported previously.[22,23,30,31] Here, we aim to determine what structural and chemical features of the ligand are important for recognition. From the analysis of the binding results for the different compounds investigated (Fig. 2; Table 1) we can make a number of conclusions about what regions of quinine are important for high affinity binding.

Firstly, the aromatic region of the ligand is key for tight binding. This is shown by the nonbinding of quinuclidine, the aliphatic region of quinine, and our observation that the change in the aliphatic portion of quinine to hydroquinine does not result in any significant change in affinity. The importance of the aromatic ring portion is emphasized by the fact that 6-methoxyquinoline alone is bound at an affinity of 0.5 ± 0.1 μM, a level still 10 fold tighter than MN4 has for cocaine. This demonstrates that the bicyclic aromatic portion of quinine alone can be bound by MN4. However, when the ligand was expanded to 3 fused rings, such as in acridine and benzo(h)quinoline, there was no detectable binding, likely because the ligand is now too large to fit into the binding site. This importance of the aromatic region of an MN4 ligand is consistent with the thermodynamic signature of binding falling into the intercalating region as defined by Chaires.[32] These data also indicates that the optimum ring size for MN4 binding is two fused six-membered rings as the aptamer does not bind ligands with three fused aromatic rings.

The substituents on the aromatic rings are also important for recognition. Having a methoxy group at the 6-position, as found in quinine and 6-methoxyquinoline, appears to be the optimum configuration. Removing the methoxy group reduces affinity as seen in cinchonidine and quinoline when compared to quinine and 6-methoxyquinoline, respectively (Table 1). Additionally, substitutions of a hydroxyl and an amino group at the 6 position in quinoline reduce affinity compared to having a methoxy group, but do not reduce affinity as much as having a hydrogen only in this position. This indicates that retaining some sort of hydrogen bond acceptor is important at this position.

Finally, we investigated the importance of the stereogenic center at carbon 9; the carbon that connects the aromatic and aliphatic rings in quinine. In all cases studied, the 9*R* configuration, as found in quinine, results in the tightest binding. This is shown when comparing the pairs quinine and quinidine, cinchonidine and cinchonine and (9*R*)-6′-methoxycinchonan-9-amine and (8α,9*S*)-6′-methoxycinchonan-9-amine (Table 1). It is interesting to also note in this last pair, that when an amino group is introduced into the molecule, and this amino should be protonated at the pH studied, that binding is reduced 10 fold from that of quinine. This indicates that adding an extra positive charge at position 9 does not add to affinity and that removing the hydrogen bond acceptor results in a decreased affinity.

5. Conclusion

In summary, we have shown that the bicyclic aromatic ring in quinine is essential for tight affinity by the cocaine-binding aptamer. 6-Methoxyquinoline on its own is bound tightly by the MN4 aptamer while the aliphatic portion of quinine, represented by quinuclidine, does not show detectable binding. Larger aromatic molecules that contain three fused rings are also not bound by the aptamer. The presence of a methoxy group at the 6-position of the quinoline ring is important for binding as changing it to a hydrogen, an alcohol or an amino group all result in lower binding affinity. For all ligands that bind, association is driven by a negative enthalpy compensated by an unfavorable binding entropy.

Acknowledgements

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References and notes

1. Cho, E. J.; Lee, J.-W.; Ellington, A. D. *Annu. Rev. Anal. Chem.* **2009**, *2*, 241.
2. Bunka, D. H. J.; Stockley, P. G. *Nat. Rev. Microbiol.* **2006**, *4*, 588.
3. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818.
4. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.
5. Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2000**, *122*, 11547.
6. Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2001**, *123*, 4928.
7. Stojanovic, M. N.; Landry, D. W. *J. Am. Chem. Soc.* **2002**, *124*, 9678.
8. Kawano, R.; Osaki, T.; Sasaki, H.; Takinoue, M.; Yoshizawa, S.; Takeuchi, S. *J. Am. Chem. Soc.* **2011**, *133*, 8474.
9. Sharma, A. K.; Heemstra, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 12426.
10. Sharma, A. K.; Kent, A. D.; Heemstra, J. M. *Anal. Chem.* **2012**, *84*, 6104.
11. Das, J.; Cederquist, K. B.; Zaragoza, A. A.; Lee, P. E.; Sargent, E. H.; Kelley, S. O. *Nat. Chem.* **2012**, *4*, 642.
12. Zou, R.; Lou, X.; Ou, H.; Zhang, Y.; Wang, W.; Yuan, M.; Guan, M.; Luo, Z.; Liu, Y. *RSC Adv.* **2012**, *2*, 4636.
13. Kent, A. D.; Spiropulos, N. G.; Heemstra, J. M. *Anal. Chem.* **2013**, *85*, 9916.
14. Malile, B.; Chen, J. I. L. *J. Am. Chem. Soc.* **2013**, *135*, 16042.
15. Reinstein, O.; Neves, M. A. D.; Saad, M.; Boodram, S. N.; Lombardo, S.; Beckham, S. A.; Brouwer, J.; Audette, G. F.; Groves, P.; Wilce, M. C. J.; Johnson, P. E. *Biochemistry* **2011**, *50*, 9368.
16. Li, D.; Song, S.; Fan, C. *Acc. Chem. Res.* **2010**, *43*, 631.
17. Plaxco, K. W.; Soh, H. T. *Trends Biotechnol.* **2011**, *29*, 1.
18. Neves, M. A. D.; Reinstein, O.; Johnson, P. E. *Biochemistry* **2010**, *49*, 8478.
19. Neves, M. A. D.; Reinstein, O.; Saad, M.; Johnson, P. E. *Biophys. Chem.* **2010**, *153*, 9.
20. Stojanovic, M. N.; Green, E. G.; Semova, S.; Nikic, D. B.; Landry, D. W. *J. Am. Chem. Soc.* **2003**, *125*, 6085.
21. Green, E.; Olah, M. J.; Abramova, T.; Williams, L. R.; Stefanovic, D.; Worgall, T.; Stojanovic, M. N. *J. Am. Chem. Soc.* **2006**, *128*, 15278.
22. Pei, R.; Shen, A.; Olah, M. J.; Stefanovic, D.; Worgall, T.; Stojanovic, M. N. *Chem. Commun.* **2009**, 3193.
23. Reinstein, O.; Yoo, M.; Han, C.; Palmo, T.; Beckham, S. A.; Wilce, M. C. J.; Johnson, P. E. *Biochemistry* **2013**, *52*, 8652.
24. Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131.
25. Sharp, K. *Protein Sci.* **2001**, *10*, 661.
26. Chodera, J. D.; Mobley, D. L. *Annu. Rev. Biophys.* **2013**, *42*, 121.
27. Olsson, T. S. G.; Ladbury, J. E.; Pitt, W. R.; Williams, M. A. *Protein Sci.* **2011**, *20*, 1607.
28. Starikov, E. B.; Norden, B. *J. Phys. Chem. B* **2007**, *111*, 14431.
29. Smith, J. E.; Griffin, D. K.; Leny, J. K.; Hagen, J. A.; Chavez, J. L.; Kelley-Loughnane, N. *Talanta* **2014**, *121*, 247.
30. Bao, J.; Krylova, S. M.; Reinstein, O.; Johnson, P. E.; Krylov, S. N. *Anal. Chem.* **2011**, *83*, 8387.
31. Spiropulos, N. G.; Heemstra, J. M. *Artif. DNA: RNA XNA* **2012**, *3*, 123.
32. Chaires, J. B. *Annu. Rev. Biophys.* **2008**, *37*, 135.