

A DNA Aptamer for Theophylline with Ultrahigh Selectivity Reminiscent of the Classic RNA Aptamer

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Abstract

Since the report of the RNA aptamer for theophylline, theophylline has become a key molecule in chemical biology for RNA switches and riboswitches. In addition, theophylline is an important drug for treating airway diseases including asthma. The classic RNA aptamer with excellent selectivity for theophylline has been used to design biosensors, although DNA aptamers are more desirable for stability and cost considerations. In this work, we selected DNA aptamers for theophylline and all the top sequences shared the same binding motifs. Binding was confirmed using isothermal titration calorimetry and a nuclease digestion assay, showing a dissociation constant (K_d) around 0.5 μ M theophylline. The Theo2201 aptamer can be truncated down to 23-mer while still has a K_d of 9.8 μ M. The selectivity for theophylline over caffeine is around 250,000-fold based on a strand-displacement assay, which was more than 20-fold higher compared to the classic RNA aptamer. For other analogs, the DNA aptamer also showed better selectivity. Using the structure-switching aptamer sensor design method, a detection limit of 17 nM theophylline was achieved in the selection buffer, and a detection limit of 31 nM was obtained in 10% serum.

Keywords: aptamers; biosensors; SELEX; fluorescence, caffeine

Introduction

Aptamers are single-stranded nucleic acids that can selectively bind target molecules.¹⁻³ Compared to antibodies, aptamers are smaller and possess programmable structures, making them attractive for developing biosensors.⁴⁻⁹ When the excellent specificity of aptamers is described, the most likely cited example is the RNA aptamer for theophylline reported in 1994 by Jenison et al.¹⁰ Its affinity for theophylline is 10,900-fold higher than caffeine, although these two molecules only differ by a methyl group. This RNA aptamer has since been widely used for designing RNA switches,¹¹ riboswitches¹² and biosensors for theophylline,¹³ and a review paper was recently published to highlight its applications.¹⁴

The success of this aptamer has made theophylline a model target for many related studies. For example, Zhou et al used *in silico* computational methods to search for aptamers for theophylline and the resulting aptamers had comparable K_d 's with the Jenison's aptamer.¹⁵ Page et al replaced the aptamer domain of a TPP-responsive riboswitch by a 40-nt random region to evolve in the presence of theophylline in *E. Coli*, although the final activation by theophylline was quite moderate.¹⁶

Theophylline exists naturally in tea and cocoa, and it is also a minor metabolic product of caffeine. Theophylline is among the most used bronchodilators and respiratory stimulators for the treatment of asthma.¹⁷ The therapeutic window of theophylline in plasma is between 20 μ M and 200 μ M.¹⁸ Thus, there is a need to detect theophylline in blood and the detection method should not respond to its close analogs such caffeine and theobromine that might exist in biological fluids. Over the last few decades, more than a dozen papers have been published using the RNA aptamer for the detection of theophylline.^{13, 19-21}

For sensing outside cells, DNA aptamers have the advantage of being more stable and more cost-effective.²²⁻²⁵ However, DNA aptamers for theophylline have yet to be isolated. In addition, it is interesting to compare the conserved sequences for these two types of aptamers for the same target.²⁶ We recently reported the selection of DNA aptamers for binding caffeine, where we used theophylline for counter selection.²⁷ None of the tested caffeine aptamers had affinity for theophylline. Herein, we switched the order and used theophylline as the target, and caffeine was used for counter selection.

The RNA aptamer was obtained by immobilization of theophylline.¹⁰ While this method has yielded a high quality aptamer, we were more interested in using the library immobilization method since the aptamer can access all parts of the target molecule, resulting in potentially better binding performance.²⁸⁻³⁷ In this work, we report our isolation of a high quality DNA aptamer for theophylline with sub-micromolar affinity and even better specificity than the RNA aptamer. The use of this aptamer-based biosensor for the detection of theophylline in serum was also demonstrated.

Materials and Methods

SELEX. The library design (Table S1) and selection method (Figure S1) are based on Stojanovic's previous publications with a few modifications.^{29, 38} The procedures including for DNA sequencing have been recently published.²⁷ The condition and the progress of the selection including the concentration of theophylline used is presented in Figure S2.

Isothermal titration calorimetry (ITC). The tested DNA sequences are shown in Table S2. MicroCal VP-ITC was used for the ITC experiments. DNA (9 μ M, 2 mL) and target molecules (1 mM, 2 mL) were dissolved in the SELEX buffer (500 mM NaCl, 10 mM MgCl₂, 50 mM HEPES pH7.5) and degassed for 10 min prior to measurement. In some experiments, 20 μ M and 100 μ M aptamers were also used. A volume of 1.4 mL aptamer was loaded into the cell chamber and 300 μ L target was loaded in the syringe. Except for an initial injection of 0.5 μ L, 10 μ L of target was titrated into the cell each time over 20 sec duration for a total of 20 to 28 injections at 25°C. The spacing was set for 360 sec between each injection. The thermodynamic values were obtained by fitting the titration curves to a one-site binding model using the Origin software.

Nuclease digestion assays. Theo2201 aptamer (1 μ L, 10 μ M) was first mixed with 8 μ L of various concentration of theophylline or other molecules in the reaction buffer (10 mM MgCl₂, 10 mM Tris-HCl pH7.5) in a 200 μ L PCR tube.³⁹ After incubating at 23°C for 10 min, 1 μ L of 5 U/ μ L exonuclease III (Exo III) or 1 μ L of 14 U/ μ L exonuclease I (Exo I), or their mixture was added to each sample and continue incubated at 23°C for another 30 min followed by 20 min at 80°C for enzyme deactivation. The sample was cooled and quenched with equal volume of 1 $\times$ gel loading dye containing 8 M urea. 5 μ L of mixture was loaded into each lane of 15% denaturing

polyacrylamide gel (dPAGE). Separation was carried out at 200 V for 100 min. The gel was stained with 1× SYBR Gold for 30 min and imaged using Bio-Rad ChemiDoc MP image system.

Sensing. The sensing experiments were performed in a 96-well plate using a SpectraMax M3 microplate reader ($Ex = 485\text{ nm}$; $Em = 525\text{ nm}$). A stock of 2 μM sensor complex was formed by annealing the 200 pmol of FAM-labeled caffeine aptamer (Theo2201-FAM) with 800 pmol of short quencher-labeled complementary strand (Theo2201-Q) in 100 μL SELEX buffer. In a typical sensing experiment, 100 μL sensor sample with 20 nM aptamer strand was used in the SELEX buffer. The background fluorescence of the sensor was monitored for 5 min before 2 μL of theophylline or other analytes was added. The signaling kinetics were continuously monitored for 15 min. Since some compounds have low solubility, the order of addition was altered for detection in high concentration sample, where 1 μL of 2 μM sensor was added to 100 μL of analyte solution. The experiments were run in triplicate and error bars indicate the standard deviations.

Sensing in 10% serum. A Tecan Sparks microplate reader was used for detecting theophylline in serum sample ($Ex = 485\text{ nm}$, $Em = 525\text{ nm}$). For this experiment, human serum was diluted to 10% using the SELEX buffer. In a typical experiment, each sample contained 100 μL of 20 nM of sensor complex in 10% serum. The background fluorescence of the sensor in serum was monitored for 5 min before 2 μL of theophylline was added. The signaling kinetics were then continuously monitored for another 15 min. For interference study, 1 μL of 2 μM sensor was added to 100 μL of 10% serum sample containing 50 μM theophylline or 50 μM theophylline with 50 μM analogue. After 15 min incubation at room temperature, the fluorescence was recorded.

Results and Discussion

Selection of DNA aptamers for theophylline

Our method of selection is described in Figure S1,^{27,29} where the DNA library with a 30-nucleotide random region (N_{30}) was immobilized via hybridization to a biotinylated DNA attached to a streptavidin column. The library was designed to fold into a hairpin structure, and the assumption was that some sequences could bind theophylline and induce the hairpin formation, releasing these

112 sequences from the column. The released DNA strands were then collected and amplified by PCR
113 to seed the next round of selection.

114 During the selection process, the concentration of theophylline was gradually decreased
115 from 100 mM to 0.05 mM from round 1 to 22. From round 11, we introduced counter selections
116 using caffeine, where the sequences that can bind caffeine were discarded (see Figure S2A for the
117 selection conditions). We then sequenced the round 22 library, and the sequences were highly
118 converged. Out of the ~60,000 sequences obtained, the most abundant 20 sequences were aligned
119 with the assistant of Clustal Omega⁴⁰ and they can be separated into two families (Figure 1).
120 Family 1 (61.7% of the total sequences) has 19 highly conserved nucleotides colored in
121 red/green/blue, and Theo2201 is a representative sequence. The rest sequences are classified into
122 Family 2. Interestingly, the red and blue regions were also found in Family 2, although their order
123 was switched. Therefore, we believe that Families 1 and 2 have the same binding mechanism for
124 theophylline, and just the orientation of the binding was flipped. The good sequence convergence
125 and alignment suggested a successful selection. The RNA aptamer selection also had two families
126 with two permutated regions, although the bases were different (AUACCA and CCUUGGAAG).¹⁰

Family 1 (61.7% of 60808 sequences)

Theo2201	<u>GACGACGATTGT</u> <u>GGTCTATTCATAGGCGTCCG</u> CTGA	<u>GTCGTC</u>
Theo2204	<u>GACGACAACGGT</u> <u>GGTCTATTCATAGGTGTCCG</u> ACTT	<u>GTCGTC</u>
Theo2219	<u>GACGAC</u> TGCT <u>GGTCTATTCATAGGCGTCCG</u> AGTTCAGTCGTC	
Theo2202	<u>GACGAC</u> GGTT <u>GGTCTATTCATAGGCGTCCG</u> GGTGA	<u>GTCGTC</u>
Theo2205	<u>GACGAC</u> GGTT <u>GGTCTATTCATAGGCGTCCG</u> GGTTA	<u>GTCGTC</u>
Theo2214	<u>GACGAC</u> GACAT <u>GGTCTATTCATAGGTGTCCG</u> CGGAA	<u>GTCGTC</u>
Theo2209	<u>GACGAC</u> CAT <u>GGTCTCTTTAGAGGTGTCCG</u> TTAG	<u>GTCGTC</u>
Theo2213	<u>GACGAC</u> GTTG <u>GGTCTATTCATAGGTGTCTG</u> CATC	<u>GTCGTC</u>
Theo2220	<u>GACGACGGTAC</u> <u>AGGTCTATTCATAGGTGTCCG</u> CAAC	<u>GTCGTC</u>
Theo2218	<u>GACGACCAGTG</u> <u>AGGTCTATTCATAGGTGTCCG</u> CCAG	<u>GTCGTC</u>
Theo2211	<u>GACGACGGGGG</u> <u>AGTCCATT</u> CATAGTTGTCTGCCGT	<u>GTCGTC</u>
Theo2212	<u>GACGAC</u> CAGAG <u>GGTCTATTTATAGG</u> GTCGTCCCCAGGTCGTC	
Theo2215	<u>GACGAC</u> TAGAG <u>GGTCTATTAATAGG</u> GCTGTCCCCTGGTCGTC	
Theo2206	<u>GACGACCTGATT</u> <u>GGTCTATTCATAGGC</u> CAGA GGGTCGTC	

Family 2 (12.3% of 60808 sequences)

Theo2203	<u>GACGACGCAGA</u> <u>AGGTGTCCATGGTTTAT</u> <u>GGTCTAT</u> CGTCGTC
Theo2217	<u>GACGACTCT</u> <u>AGGTGTCCACCGCTCGGAA</u> <u>GGTCTAT</u> AGTCGTC
Theo2207	<u>GACGAC</u> CT <u>AGGTGTCCCTGCATTTGCCGA</u> <u>GGTCTAT</u> GTCGTC
Theo2208	<u>GACGAC</u> CT <u>AGGTGTCCGGGCCTATTGCTT</u> <u>GGTCTAT</u> GTCGTC
Theo2210	<u>GACGAC</u> CT <u>AGGTGTCCGCACGTTCTGTGAT</u> <u>GGTCTAT</u> GTCGTC
Theo2216	<u>GACGAC</u> T <u>AGGTCTATGCACTTTGGATA</u> <u>GGTGTCCAGGTCGTC</u>

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Figure 1. The alignment of the 20 most abundant sequences from the round 22 library. These 20 sequences represent 74% of the total sequences. The red and blue highlighted sequences are present in both families indicating that they have the same binding mechanism. The green highlighted sequence is also conserved in Family 1 but does not appear in Family 2. The underlined nucleotides are from the primer binding regions. The boldface bases in Family 1 are immediately outside the conserved region and most of them are a G-T wobble pair.

Binding assay using ITC

The Mfold predicted secondary structure of Theo2201 is shown in Figure 2A.⁴¹ To confirm binding, we first used isothermal titration calorimetry (ITC). ITC is a label-free technique that measures the heat produced during binding.⁴²⁻⁴⁴ When theophylline was titrated, heat release indicative of aptamer binding was observed (Figure 2D). By fitting the integrated heat, a dissociation constant (K_d) of $0.51 \pm 0.13 \mu\text{M}$ was obtained. When caffeine, theobromine or paraxanthine was titrated, no heat was observed (Figure 2E-G), indicating the excellent selectivity of this aptamer. We also tested Theo2203 (Figure 2B), which is the most abundant sequence in Family 2, and binding was also observed using ITC ($K_d = 1.05 \pm 0.20 \mu\text{M}$, Figure S3). The thermodynamic value of these two aptamers are shown in Table 1. This binding affinity is comparable with that of the RNA aptamer (0.1 to 0.6 μM).^{10, 14} The main different between Theo2201 and Theo2203 is the relative position of their conserved sequences. Since Theo2201 displayed stronger binding affinity, our subsequent studies focused on this aptamer.

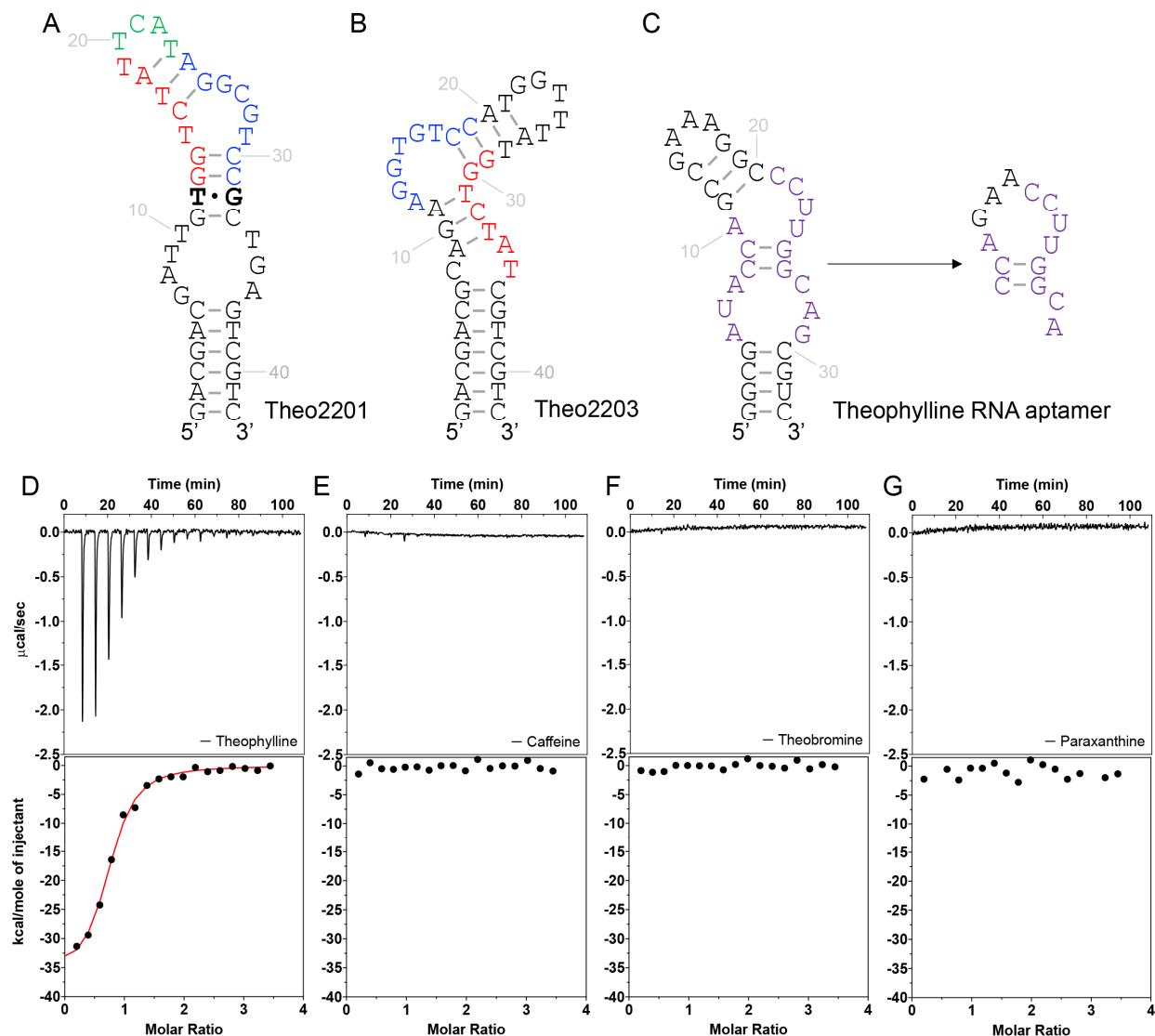


Figure 2. The predicted secondary structure of (A) Theo2201, (B) Theo2203 and (C) the RNA aptamer and the truncated minimal RNA aptamer. The highly conserved regions are color coded. ITC traces and integrated heat of titrating 0.25 mM (D) theophylline, (E) caffeine, (F) theobromine, and (G) paraxanthine into 9 μ M Theo2201 aptamer in the SELEX buffer.

Table 1. Binding thermodynamic values of the theophylline aptamers from ITC in the SELEX buffer at 25°C by fitting using a one-site binding model.

Aptamer	N	ΔH (kcal mol ⁻¹)	ΔS (cal K ⁻¹ mol ⁻¹)	ΔG (kcal mol ⁻¹)	K_d (μM)
Theo2201	0.75 ± 0.02	-34.6 ± 2.9	-87.3 ± 10.1	-8.6 ± 0.1	0.51 ± 0.13
Theo2203	0.73 ± 0.05	-31.3 ± 3.0	-77.6 ± 16.5	-8.2 ± 0.2	1.05 ± 0.20

Nuclease digestion assay

To further confirm binding, we then used a nuclease digestion assay developed by the Xiao group.^{39, 45} The principle of this reaction is shown in Figure 3A. Exonuclease III (Exo III) is a double-strand DNA exonuclease that removes mononucleotides stepwise from the 3'-end of a DNA. Its activity is sensitive to local structural changes induced by small-molecule binding. In the absence of theophylline, Exo III digested the aptamer into nucleotides. In the presence of theophylline, the digestion was halted a few bases before the binding site. After optimization, we picked 30 min as the incubation time (Figure S4A). Under the optimized conditions, we observed that the halted cleavage showed a nice dependence on the concentration of theophylline (Figure 3B). The fitted binding curve showed a K_d value of 0.50 μM, comparable to the 0.51 μM from ITC. The specificity of the protection by theophylline was also demonstrated (Figure S4B). As a control, when a mutant was tested (Theo2201-m1 in Table S2), no protection was observed with up to 200 μM theophylline (Figure S5).

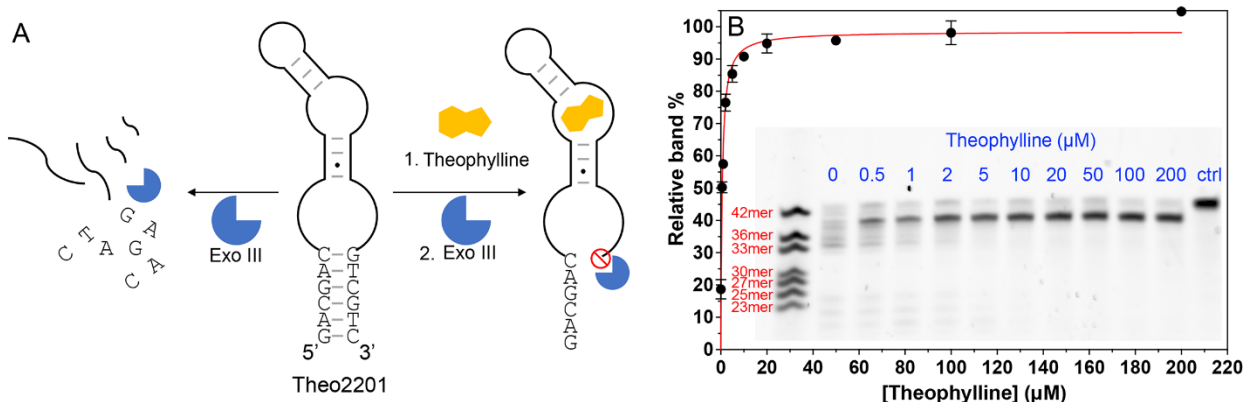


Figure 3. (A) Schematic of Exo III digestion of Theo2201 in the absence and presence of theophylline. (B) Theophylline concentration dependent digestion. Inset: a gel micrograph of Exo

III digestion of 1 μ M Theo2201 aptamer with increasing concentration of theophylline after 30 min of digestion.

Truncation and mutation studies.

After confirming binding of the aptamer, we then performed truncation studies to understand whether all the nucleotides were required for binding. The highly conserved nucleotides are color marked in Figure 4A and it appeared to form a hairpin. Therefore, a natural question is whether the rest of the sequences are important or not. We first full removed the six terminal base pairs, and the resulting Theo2201-t1 still showed binding to theophylline with a K_d of 3.0 μ M (Figure 4B). Its binding affinity dropped only about 5-fold, suggesting that the truncated pocket played a minor role in assisting the binding. When we fully removed the dangling nucleotides to generate Theo2201-t2, binding was still detectable (K_d of 9.8 μ M, Figure 4C). The fact that the few dangling nucleotides could promote binding confirmed the auxiliary role of the truncated loop. The result also correlated well with the nuclease digestion assay, where the digested product was 38 to 39-nt long in the presence of theophylline. Finally, we removed the second stem to generate Theo2201-t3, and this fully abolished the binding (Figure 4D). Anderson and Mecozzi truncated the RNA aptamer down to 13-nt (Figure 2C), which still exhibited a K_d of 13.1 μ M.⁴⁶ In that truncation, the first bulge was the key remaining structure.

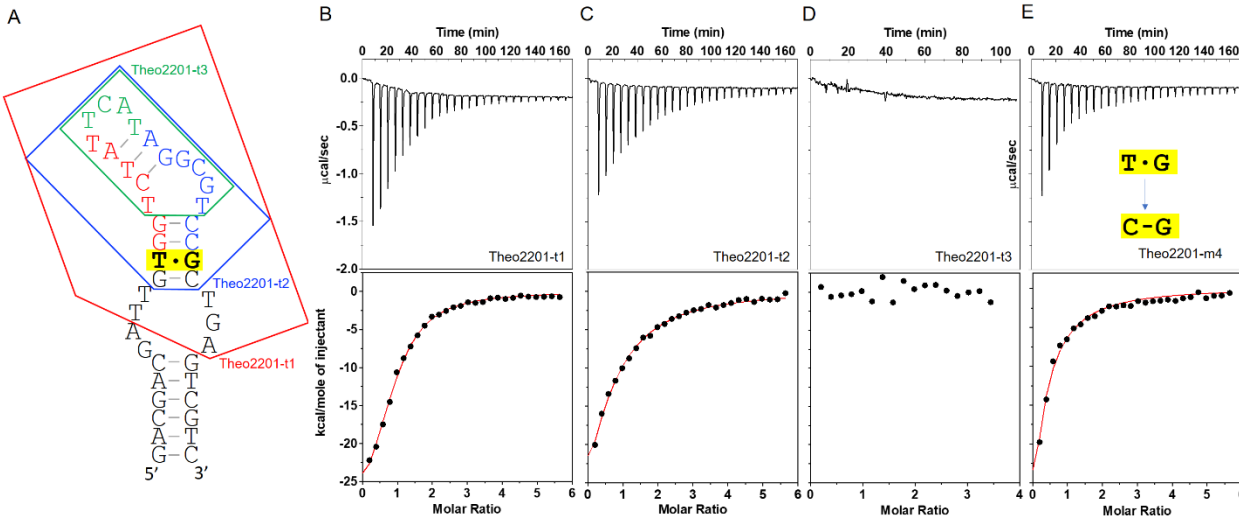


Figure 4. (A) The secondary structure of Theo2201 aptamer and its various truncated structures. The ITC traces and integrated heat of (B) Theo2201-t1, (C) Theo2201-t2, (D) Theo2201-t3 and (E) Theo2201-m4 in SELEX buffer at 25°C.

To confirm specific aptamer binding, we also performed mutation studies. First, we want to test whether the top stem-loop structure is just for structural role or involved in binding, we replace the TTCA loop by ACTT (Theo2201-m2), and this change abolished binding (Figure S6A, B). Thus, this loop is likely involved in binding. We further mutated a guanine to adenine and this mutation (Theo2201-m3) also abolished binding (Figure S6C).

A G-T wobble pair was predicted in Theo2201 (highlighted boldface in Figure 4A). This wobble pair is immediately outside the highly conserved sequence of the aptamer. When we examined the aligned sequences in Figure 1, this wobble pair was also highly conserved although it was replaced by a G-A mismatch in some sequences. Nevertheless, none of the sequences showed a Watson-Crick pair at this place. Since fixing mismatched bases in the stem region of the RNA aptamer improved the binding affinity, we also replaced this wobble pair to a G-C base pair (named Theo2201-m4). ITC showed that Theo2201-m4 had a K_d of 8.9 μ M (17-fold higher than the wild-type Theo2201). Thus, having a more stable stem here decreased the binding affinity, and this might explain why most of the aptamers had a wobble pair at this position (the nucleotides in boldface in Figure 1).

From the structural standpoint, the RNA aptamer is more similar to Theo2203, since both have a top stem-loop structure that can be replaced by other stem loops.¹⁰ In contrast, Theo2201 has a conserved stem-loop sequence. In the RNA aptamer, C22 was responsible for its excellent selectivity against caffeine.⁴⁷ We suspected that C16 in Theo2201 and C18 in Theo2203 might play a similar role, which will need to be confirmed by structural biology studies and further biochemical assays.⁴⁸

Fluorescent detection of theophylline

We then designed a biosensor for the detection of theophylline taking advantage of its structure-switching property.^{28, 49, 50} We extended the aptamer strand by 5-nt and labeled it with a fluorophore. A quencher-labeled 12-nt DNA was hybridized with the aptamer, resulting in

quenched fluorescence. Addition of theophylline is expected to induce binding, thus releasing the quencher-labeled strand to produce enhanced fluorescence (Figure 5A).

Without theophylline, the sensor had a low fluorescence reading of ~60 fluorescence units. After adding theophylline, fluorescence increase was observed and saturated fluorescence was achieved within 2 min (Figure 5B). The higher the theophylline concentration, the stronger the final fluorescence. We plotted the percent change in fluorescence intensity as a function of theophylline concentration to obtain a calibration curve (Figure 5C). Based on the initial linear region (inset of Figure 5C), the detection limit was calculated to be 17 nM theophylline ($3\sigma/\text{slope}$), and the sensor can quantify up to 1 μM theophylline.

Even better selectivity than the RNA aptamer

This fluorescent sensor allowed us to estimate the selectivity against other molecules.^{51, 52} We define Reaction 1 to be FAM-aptamer (F) and quencher-cDNA (Q) forming the FQ DNA complex: $F + Q = FQ$. Thus, The K_d of reaction 1 can be written as $K_{d1} = [F][Q]/[FQ]$.

We define Reaction 2 to be the sensing reaction, where FQ reacts with theophylline (L): $FQ + L = FL + Q$. The K_d of reaction 2 can be written as $K_{d2} = [FQ][L]/[FL][Q]$.

Therefore, for Reaction 3 (the aptamer binding reaction), $F + L = FL$, its K_d can be written as $K_{d3} = K_{d1}K_{d2} = [F][L]/[FL]$.

K_{d1} is a constant, and K_{d2} can be calculated based on the apparent K_d from the sensor calibration curve. When the theophylline (or other analyte) concentration is at the apparent K_d value, $[FQ]$ and $[FL]$ had the same concentration. Thus, $K_{d2} = (K_{d2 \text{ apparent}})/[Q]$. Based on Figure 5C, $K_{d2 \text{ apparent}} = 524 \text{ nM}$. Since we used 20 nM of the FAM-Aptamer and 80 nM Quencher-cDNA for the sensor, $[Q]$ was 70 nM when theophylline was equal to the apparent K_d . Thus, $K_{d2} = 524 \text{ nM}/70 \text{ nM} = 7.5$, and $K_{d3} = K_{d1}K_{d2} = 51.8 \text{ nM} \times 7.5 = 388 \text{ nM}$, which is comparable with the 510 nM K_d from ITC.

Therefore, K_{d3} is proportional to apparent K_{d2} measured from the sensor response. When an apparent K_{d2} cannot be obtained (which is the case for most of the compounds as shown in Figure S7), we took the highest response and extrapolated it to conservatively estimate the selectivity.

We used the sensor to study the selectivity of the aptamer at four analyte concentrations up to 100 μ M (Figure 5D). Consistent with the ITC results, caffeine, theobromine and paraxanthine did not show any signal. To push for the limit of selectivity, we further tested a few compounds at 1, 10 and even 25 mM, for which we had to add concentrated sensors to the analyte solutions due to solubility issues (Figure S7, S8). The response of 0.1 μ M theophylline was higher than 25 mM of caffeine, and the aptamer was at least 250,000-fold more selective for the former. The RNA aptamer was 10,900-fold more selective for theophylline over caffeine, and thus our DNA aptamer was 23-fold more selective than the RNA aptamer in this regard. The selectivity of the RNA aptamer was measured by using a competitive assay with ^{14} C-labeled theophylline.¹⁰ We also used a competitive assay, although a complementary DNA was used as a competitor. Although the selectivity comparison was made using different methods, the relative difference should still be comparable.

The sensor responses to xanthine, hypoxanthine, uric acid, glucose, dopamine, tryptophan and tyrosine were also minimal. A number of the compounds have solubility issues at above 1 mM and thus we could not test higher concentrations. The selectivity numbers listed in Figure 5E are a conservative estimation.

Among the tested compounds, only 3-methylxanthine showed a concentration-dependent fluorescence enhancement and the apparent K_d was $\sim$ 50 μ M. To confirm this result, we picked caffeine and 3-methylxanthine to further run the nuclease digestion assay. No protection was observed with up to 100 mM caffeine and all the aptamers were digested. Thus, the K_d for caffeine was higher than 100 mM, which was consistent with our fluorescent sensor data (Figure S9). We further ran ITC with 100 μ M Theo2201 aptamer and still could not see binding with caffeine or paraxanthine. Since 3-methylxanthine showed binding, we used ITC to obtain a K_d of 8.1 μ M, which was 16-fold higher than that of theophylline (Figure S10). For the RNA aptamer, the selectivity for theophylline over 3-methylxanthine was 6.3-fold. This similarity in the relative selectivity between theophylline and 3-methylxanthine also suggested that the binding of theophylline by both aptamers might follow a similar mechanism.

Overall, the DNA aptamer was more selective than the RNA aptamer. This could be related to the different selection methods, where our library immobilization method allows the target theophylline to bind more optimally. A rationally designed DNA aptamer for theophylline was

also reported by using a duplex DNA containing an abasic site. While it has showed selectivity for theophylline (the exact fold of selectivity not reported), the K_d was only around 10 μM .⁵³

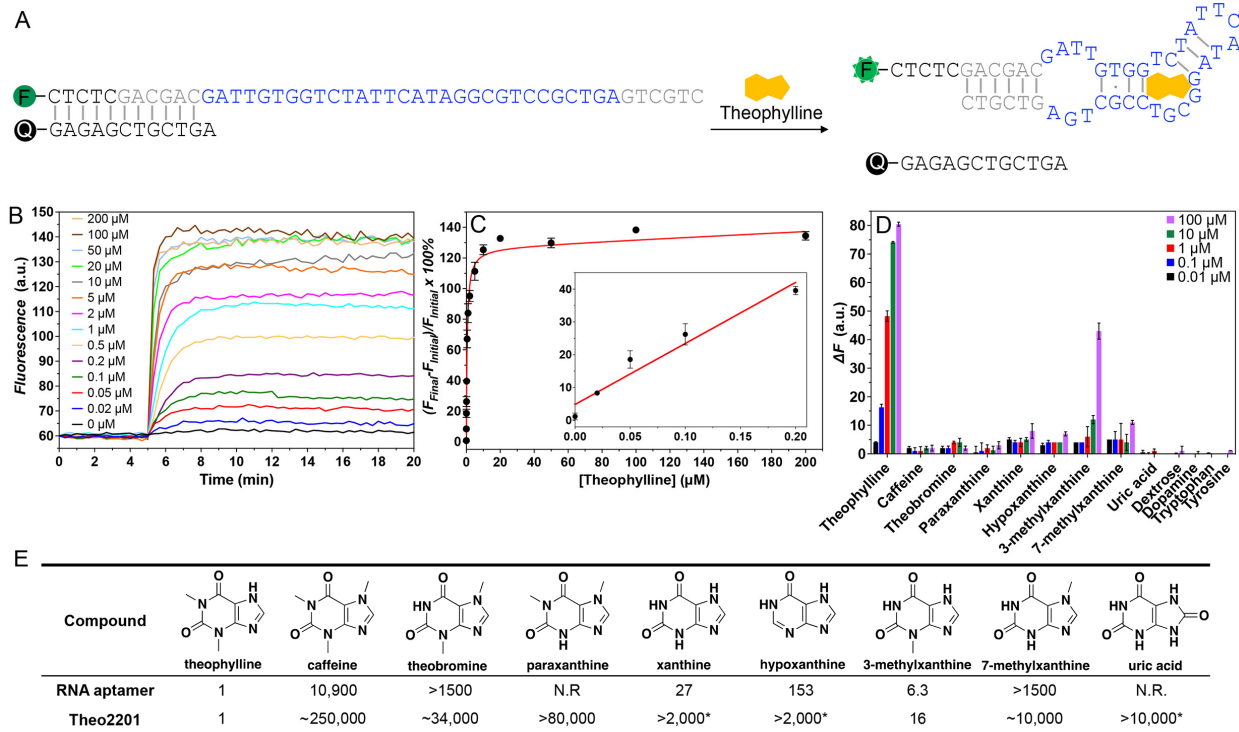


Figure 5. (A) Scheme of a structure-switching fluorescent signaling aptamer biosensor for theophylline detection. (B) Kinetic traces of 20 nM sensor in the presence of various concentration of theophylline in the SELEX buffer. Theophylline was added at the 5 min mark. (C) Percentage of fluorescence change as a function of theophylline concentration. Inset: Linear response at low theophylline concentrations. (D) Sensor selectivity with various type of theophylline analogues and biomolecules at different concentrations. (E) Selectivity comparison of the RNA and DNA aptamers for theophylline. The RNA aptamer data were from Jenison et al.¹⁰ The asterisks indicate low solubility of some compounds, where conservative estimations were made here.

Detection of theophylline in serum

Since a potential application of the theophylline sensor is to monitor its concentration in blood, we then tested whether this sensor works in serum.^{54, 55} When the serum concentration is too high, it strongly scatters light resulting in a high background. To avoid this effect, we diluted the serum to

10% and then measured the concentration-dependent responses (Figure 6A, B). The response was quite similar to that in buffer, and the detection limit was determined to be 31 nM. Therefore, the performance of the aptamer was not affected by serum. The high sensitivity of this sensor allows us to detect theophylline in serum, and the detection range is relevant to the therapeutic window of theophylline after 10-fold dilution (2 to 20 μ M). Similarly, the sensor performance was not affected by the additional presence of caffeine, theobromine, or paraxanthine in the serum (Figure 6C).

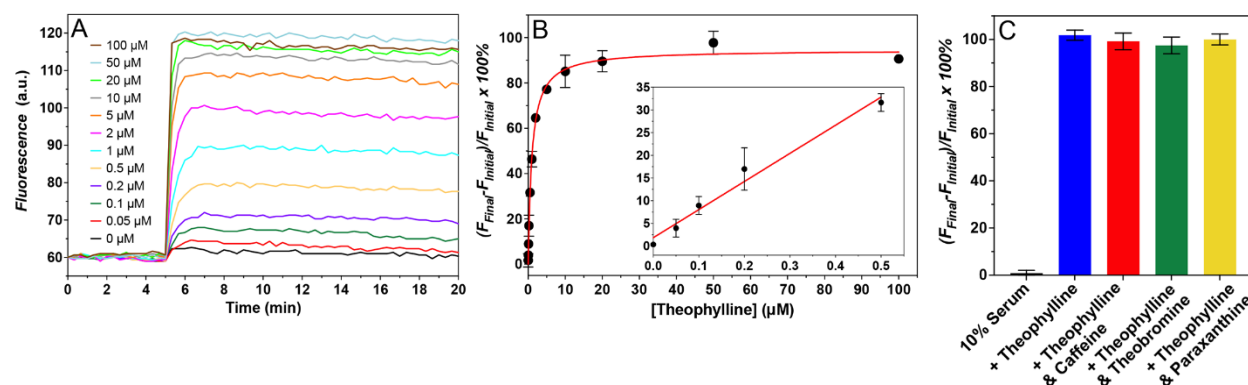


Figure 6. (A) Kinetics traces of 20 nM sensor in the presence of various concentrations of theophylline in 10% serum. (B) Percentage of fluorescence change as a function of theophylline concentration. Inset: linear response at low theophylline concentrations. (C) Sensor performance in the presence of 50 μ M theophylline alone or with the additional 50 μ M related analogue mixture.

Conclusions

In this work, we selected a high affinity DNA aptamer for theophylline using the library immobilization method. While extensive work has been done on the RNA aptamer, this is the first report of a high-affinity DNA aptamer for theophylline via SELEX. This aptamer had a K_d of around 0.5 μ M based on ITC and the nuclease digestion assay. While its affinity towards theophylline is comparable to the RNA aptamer, the selectivity against caffeine and other analogs was even better than the RNA aptamer. Truncation and mutation studies confirmed the core structure required for binding. The detection limit of theophylline reached 17 nM in buffer and 31 nM in 10% serum. This aptamer will likely find applications in the detection of theophylline, for fundamental aptamer studies and for DNA nanotechnology.

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324

325 **Supporting Information**

326 The Supporting Information is available free of charge at

327 <https://pubs.acs.org/doi/10.1021/acschembio.xxxxx>

328 Chemicals, aptamer selection scheme, conditions, and progress analyzed by real-time PCR,
329 additional ITC traces with Theo2203 aptamer and mutated aptamers, Exo III digestion gel
330 micrographs, original fluorescence traces of the sensor (PDF)

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