

ssDNA aptamers that selectively bind oxytetracycline

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Abstract—Single stranded DNA aptamers that bind with high affinity and specificity to the oxytetracycline (OTC) were identified by selection from an oligonucleotide library of 10^{15} molecules. The binding affinities of four aptamers were in nanomolar range. The aptamers were highly selective in that, lack of –OH group at 5-position in tetracycline and –H group in place of –OH at 6-position in doxycycline determined the specificity of these aptamers to bind OTC. Three aptamers designated as No. 4, 5, and 20 shared strong affinities with $K_d = 9.61$, 12.08 , and 56.84 nM, respectively, as well as selectivity to bind OTC (72–76%). Aptamer No. 4 had strong affinity among all with high selectivity, whereas No. 2 had relatively weak affinity ($K_d = 121.1$ nM) and moderate selectivity (52%). Our results indicated that the aptamers No. 4, 5, and 20 with variable 40-base oligonucleotides can be good candidates for selectively binding to OTC with high molecular discrimination over its analogs such as tetracycline and doxycycline.
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1. Introduction

Aptamers are ssDNA or RNA oligonucleotides with very high affinity for their target. They bind to the target with high selectivity and specificity because of their three-dimensional structure. Systematic evolution of ligands by exponential enrichment (SELEX) is a technique widely used for the in-vitro selection of aptamers that can bind to desired targets.¹ Several studies on selection of aptamers for small organic molecules have been reported.^{2,3} Antibiotics belonging to the class of tetracyclines are considerable targets to develop aptamers for a variety of applications. Aptamers for such antibiotics can be useful for the development of a biosensing system, which enables detection of tetracyclines found in many food products such as meat,^{4,5} milk,⁶ and eggs/chicken.⁷ Aptamers for antibiotics have also been found to have other applications such as targeting drug delivery⁸ and tools to control gene expression or to purify RNA-binding proteins.^{9,10}

Tetracycline antibiotics comprise a class of broad spectrum antimicrobial agents, namely oxytetracycline (OTC), finding application in human therapy, animal husbandry, aquaculture and fruit crop production. They are widely used for the therapy of infectious diseases of

animals. They are designed to act very effectively at low doses and to be completely excreted from the body after a short time of residence. Only a fraction of the ingested antibiotics is metabolized in the animals, hence a large percentage of the antibiotics is either accumulated in tissues or excreted and released into the environment via manure and sludge used as fertilizer on fields or effluent from aquaculture.¹¹

OTC is one of the most frequently used tetracyclines for its application as growth promoter supplied with livestock feed.^{12,13} This study describes the in-vitro selection of ssDNA aptamers that bind to OTC with high affinity, and characterization of their specificity and selectivity. These aptamers were also tested with structurally similar small molecules for their ability to distinguish tetracycline group of antibiotics, which differ in minor groups like –H or –OH. The aptamers developed can be good candidates for development of biosensors for detection of OTC, which may be present as contaminant in meat, milk, and other food products.

2. Results

An in-vitro selection of ssDNA that binds to oxytetracycline (OTC) based on tosylactivated magnetic beads as immobilization matrix by SELEX process was conducted (Figs. 1 and 2). A total of seven rounds of SELEX process was performed till the recovery of OTC-bound ssDNA reached to approx. 90% of the

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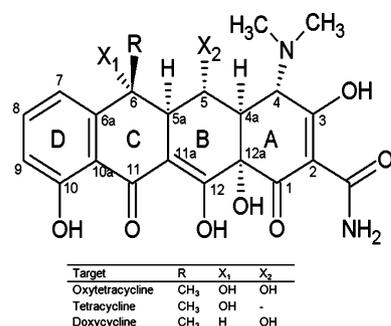


Figure 1. General structure of tetracyclines. The X₁ and X₂ are variable functional groups involved in their structural analogs and R represents the alkyl group.

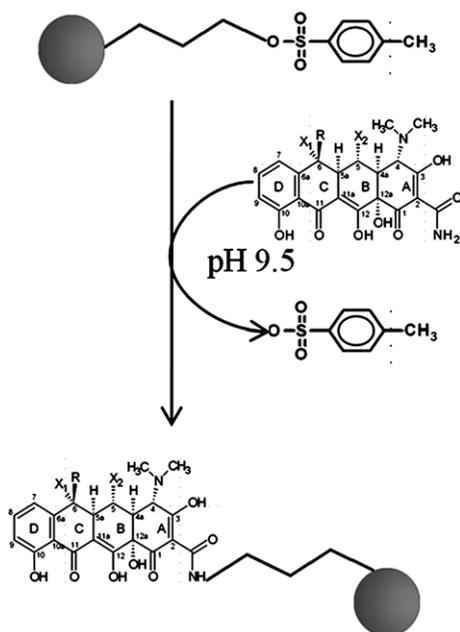


Figure 2. Schematic diagram of covalent binding of the targets (oxytetracycline/tetracycline/doxycycline) to tosylactivated magnetic beads. Covalent attachment of the terminal –NH₂ groups in the target molecules was allowed to react overnight with appropriate amount of tosylactivated magnetic beads under alkaline conditions (pH 9.5) at 37 °C. The target-coated beads were finally washed and capped the unoccupied groups by ethanolamine as described in methods to avoid non-specific binding.

ssDNA pool added. Enrichment of OTC-specific aptamers was obtained after counter selection at every two rounds of SELEX process using magnetic beads coated with (a) ethanolamine, (b) tetracycline, and (c) doxycycline (Figs. 1 and 3).

The amount of OTC-bound ssDNA recovered in round 8 remained same as in round 7 possibly because of saturation of binding sites on the beads. Non-specific enrichment of ssDNA to the bead matrix was not detected after the first counter selection using naked tosyl-activated beads coated with ethanolamine. After eight rounds of selection, the selected pool of ssDNA was cloned and characterized. Five different types of sequences were selected among the total 12 clones for

characterization and the sequences of all 12 clones are shown in Table 1. The secondary structure models of the selected five clones (No. 2, 4, 5, 8, and 20) as predicted with the help of *Mfold* are shown in Figure 4. Typical stem and loop motifs, which are distinct in each aptamers, can be seen in their secondary structures (Fig. 4). A search for the sequence-match among 12 clones revealed several conserved regions and consensus sequences highlighted in Table 1. Out of 12 clones, only the sequence of aptamer No. 5 matched with No. 14. Four consensus sequence motifs were identified within N40-regions of 76-mer size ssDNA aptamers that are unique to No. 2, 4, 5, and 20. Aptamer No. 8 sequence had no matching consensus sequences but contained only few conserved bases. The conserved regions in the selected four out of five aptamers (No. 2, 4, 5, and 20) are unique and did not match the rest of the six aptamers (No. 3, 11–13, 17, and 18) that showed no binding to OTC in nanomolar range (Table 1). The conserved regions are frequently found located in the stem and loop regions in the secondary structures of the aptamers. Positions 52–58 (5' to 3') in aptamer No. 2, 4, 5, and 20 are conserved sequence motifs that form major portion of the main stem and loop in their secondary structures (Fig. 4). The possibility of G-quartet confirmation can be seen one each in aptamers No. 20 and 13 and two in No. 12. However, only aptamer No. 20 which is predicted to form one G-quartet structure showed high affinity to OTC.

Binding reactions were performed using free OTC and a concentration series of each ssDNA aptamers (0–1 μM). From Eq. 1 and the results in Figure 5, the dissociation constants of the three aptamers, that is, No. 4, 5, and 20, were found to be in the low nanomolar range except aptamer No. 2. The lowest K_d of 9.61 ± 0.3 nM was obtained with aptamer No. 4. The K_d values of the other three aptamers were relatively higher: 12.08 ± 2.25 nM for aptamer No. 5, 56.84 ± 3.62 nM for aptamer No. 20, and the highest being 121.1 ± 5.3 nM for aptamer No. 2, whereas binding of aptamer No. 8 was not distinguishable from the analysis of student's *t*-test for the determination of binding constant because of its low selectivity. The aptamers which had K_d values in μM range are considered to have weak binding capacity (data not shown).

Further experiments were performed with all five aptamers to investigate the specificity and binding affinity to OTC. Structurally similar chemicals such as tetracycline and doxycycline, which were also used as targets for counter selection, and the non-specific oligonucleotides or those which are specific to tetracycline or doxycycline were eliminated during counter selection steps. These structural analogs of OTC were tested again for the specificity or selectivity of OTC-aptamers. It is evident that the aptamers No. 2, 4, 5, and 20 are specific to OTC (Fig. 6). The OTC-bound ssDNA recovered was 52%, 73%, 74%, and 76%, respectively, suggesting that these aptamers are highly selective for OTC over its analogs (Fig. 1). Aptamer No. 8 did not show efficient binding capacity with OTC. Among the five aptamers, No. 20 was highly specific followed by No. 5 and

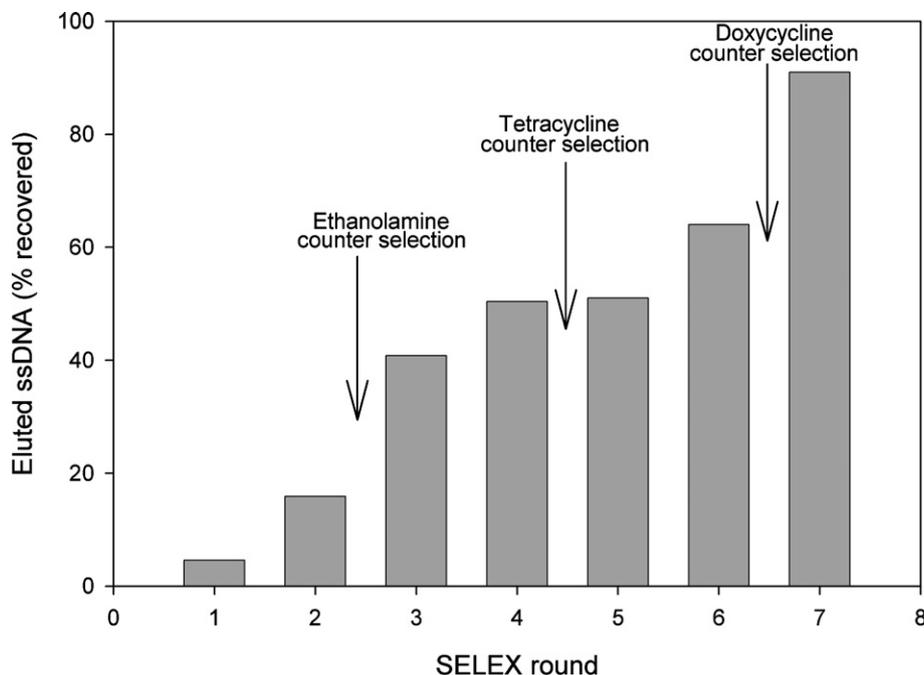


Figure 3. Selection of aptamers for oxytetracycline: recovery of ssDNA pool after each SELEX round, the arrows indicate the counter selections performed using tosylactivated magnetic beads coated with the following three chemicals: ethanolamine, which was used for blocking of unoccupied groups on the magnetic beads; tetracycline and doxycycline are the analogs of oxytetracycline used for counter selection.

Table 1. Sequences shown in the table are the 40-nucleotides variable regions (N40) in a total 76-mer size of each ssDNA aptamers that are flanked by 18-nucleotides each constant primer binding regions as follows: 5'-CGTACGGAATTCGCTAGC-N40-GGATCCGAGCTCCACGTG-3'

Aptamer ^a	Variable sequences of N40 region from 19 th to 58 th position in 76-bp aptamers (5' to 3') ^b
Sequences of N40 region in the aptamers that strongly bind to oxytetracycline	
No.2	C G A C C G C A G G T G C A C T G G G C G A C G T C T C T G G G T G T G G T G T
No.4	C G A C G C G C G T T G G T G G T G T G G A T G G T G T G T T A C A C G T G T T G T
No.5	A C G T T G A C G C T G G T G C C C G G T T G T G G T G C G A G T G T T G T G T
No.20	C G A G T T G A G C C G G G C G C G G T A C G G G T A C T G G T A T G T G T G G
No.14	A C G T T G A C G C T G G T G C C C G G T T G T G G T G C G A G T G T T G T G T
Sequences of N40 region in the aptamers that weakly bind to oxytetracycline	
No.8	G G G C G G G C G T G G A C T A C A T G C T T G G G T G T C A T T G G G T G G C G
No.3	G G G C G G G C A A A T G C G A T A C T G T G G G C A G A G G G T G G T G G C G
No.11	G G G C G G A G C G T G G G T G G G T G G A G T T G G C T G G G C G C G G T G C T
No.12	G G G G G A G C G G T G T A C A T G A C G G G A T G T C G C G T T C G G T G G
No.13	G G G G C G C G T G T C G T A G G A T G C G G T G T T G C G T G G C C G T G G T
No.17	C C A C G A A C G G T A T C G C A T G T T C C C G G T G G T C A C G G T G T G T
No.18	G G G C G G G A G G A T G T C C A G G T G G C G A T G G T C T G T C C G T G G T

Sequences highlighted in grey are the conserved sequences. The sequences shown in boxes represent consensus sequences and those highlighted bold indicates possible G-quartets. The aptamers shown in the table were selected from the pool of 10¹⁵ random ssDNA molecules after the enrichment using tosylactivated magnetic beads immobilized with oxytetracycline.

^aAptamers that bind to oxytetracycline were selected by Systematic Evolution of Ligands by Exponential enrichment (SELEX) method as described in methods and the enriched aptamers were sequenced.

^bSecondary structures of the aptamers are shown in Figure 4.

4. Although aptamers No. 2 and 4 are shown to possess binding capacity of about 3–5% with tetracycline and doxycycline, they also showed considerably higher binding preference to OTC. In addition, aptamer No. 8 did not bind to tetracycline but it did show binding capacity of about 2% to doxycycline. Finally, aptamer No. 20 showed high selectivity among all the aptamers tested with no or insignificant binding to tetracycline and doxycycline (Fig. 6).

3. Discussion

Oxytetracycline (OTC) is one of the most frequently used tetracyclines, which is used as a growth promoter supplied with livestock feed and veterinary medicine. It has been detected in food products such as meat,^{4,5} milk,⁶ and eggs/chicken⁷ and therefore causes serious threat to human health. This study aims to develop ssDNA aptamers that bind to OTC with high affinity,

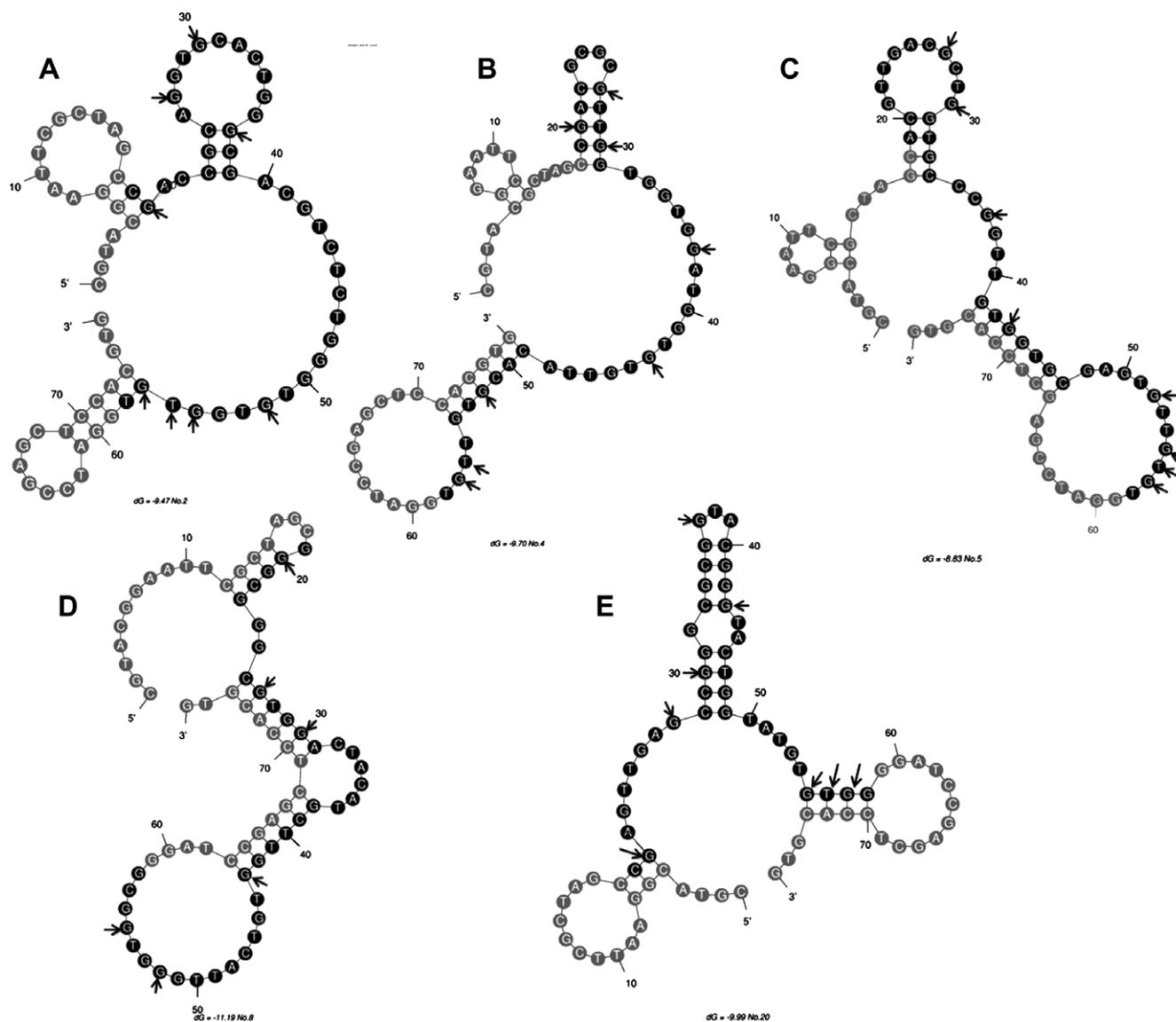


Figure 4. Secondary structure model of oxytetracycline aptamers as predicted by *Mfold*: aptamers (A) No. 2, (B) No. 4, (C) No. 5, (D) No. 8, and (E) No. 20, composed of 76-mer size with central 40-nucleotide variable region (highlighted in dark circles) flanked by 18-nucleotides each constant primer binding region (highlighted in grey circles). The arrows indicate the conserved bases found after the sequence analysis.

specificity, and selectivity, which enables its detection at low concentrations. Here we report in-vitro selection of aptamers that selectively bind to oxytetracycline (OTC) by FluMag-SELEX method aiming for the aptamer which have a low nanomolar K_d . Five aptamers have been developed, out of which four aptamers bind to OTC at low nanomolar range (9–121 nM). These aptamers did not show significant binding to the structurally similar small organic molecules, which differ in minor groups like –H or –OH, making them more chemo-selective and highly specific for binding to OTC. Structurally similar antibiotics such as tetracycline and doxycycline did not bind significantly to the aptamers but are specific to bind OTC (Fig. 6). Aptamers No. 4, 5, and 20 have both strong binding affinities and selectivity. Contrastingly, the aptamer No. 2 had strong selectivity over other analogs (52%) but relatively low affinity ($K_d = 121.1$ nM), as against aptamer No. 8 which showed binding of only 7% to OTC with no distinguish-

able affinity. This difference in their binding characteristics could be related to their differing sequences, ionic interactions, and their three-dimensional structures (Table 1 and Fig. 4).

Analysis of sequences from 12 clones revealed that the five selected aptamers (No. 2, 4, 5, 8, and 20) are different from other clones except aptamer No. 14 which was similar to No. 5 (Table 1). Sequences at 52–58-nucleotide regions from 5' to 3' end are conserved motifs in each 76-mer size aptamers No. 2, 4, 5, and 20, which showed strong selectivity to OTC, suggesting that these conserved sequences are required for the selectivity of the aptamers (Table 1 and Fig. 6). Similarly, the 26–33-nucleotide region from 5' to 3' end in aptamers No. 4 and 5 is a conserved consensus sequence motif (CGYTGGTG) which may be required for the strong affinity of the aptamers to bind OTC, as evidenced by their $K_d = 9.61 \pm 0.3$ nM and 12.08 ± 2.25 nM, respec-

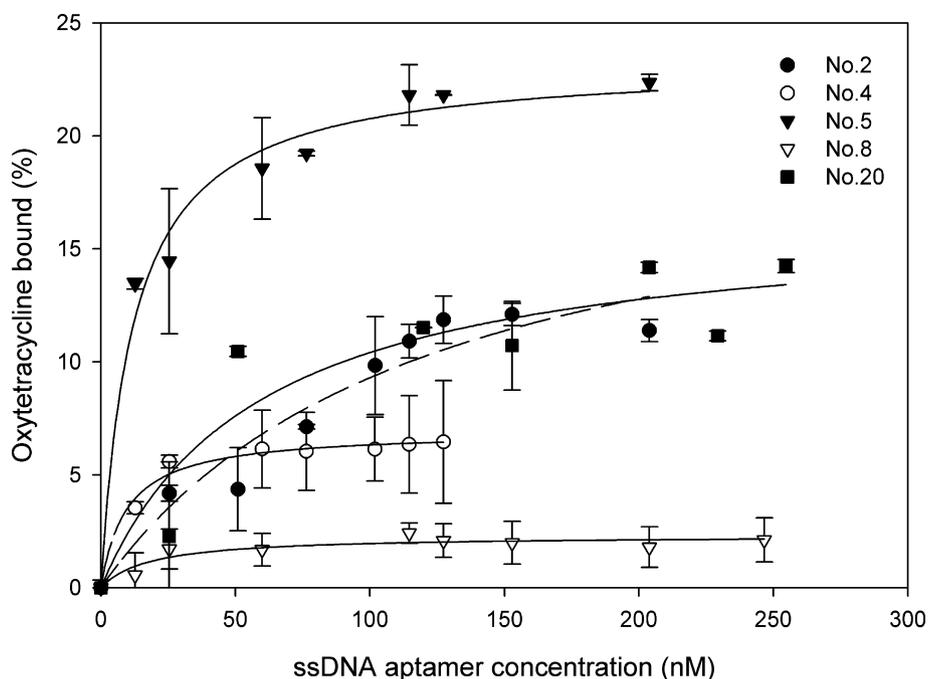


Figure 5. Binding of ssDNA aptamers to oxytetracycline (OTC) was performed by equilibrium dialysis as described in methods. The saturation curves were obtained by plotting the concentration of bound OTC (%) as a function of total ssDNA concentration using average values from three independent determinations and the dissociation constants (K_d) were calculated by non-linear regression analysis.

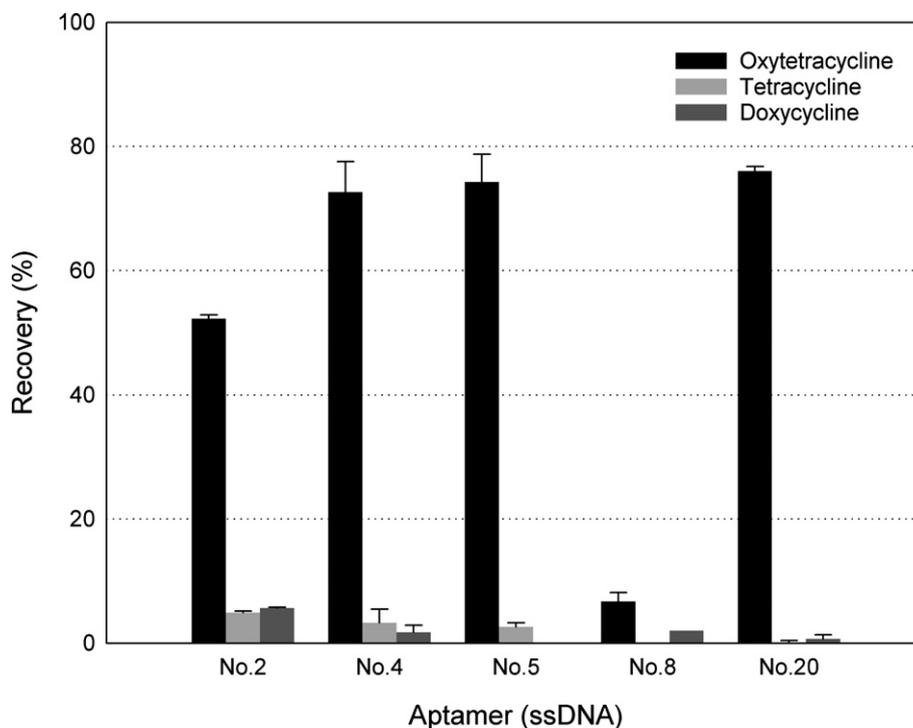


Figure 6. Binding affinities of the aptamers to structurally similar targets. One microgram of each ssDNA aptamer was incubated with constant number of beads (2×10^8) coated with equimolar concentrations of oxytetracycline, tetracycline, and doxycycline. Aptamers bound to the target-coated beads were eluted by heat treatment and the concentration of eluted ssDNA was calculated as % ssDNA recovered.

tively (Fig. 5). In addition, several conserved bases were identified whose locations are highlighted with arrows in Figure 4 and shaded in Table 1. These conserved regions are located mainly in the stem and loop regions. The conserved 53–58-nucleotide regions composed of

GTKKTGT are localized in the major loops of aptamer No. 2, 4, 5, and 20. The 26–33-nucleotide and 53–58-nucleotide regions from 5' to 3' end could be predicted to stabilize the structure of the aptamers, which facilitates strong binding and selectivity to OTC. Despite dis-

tinct sequences in the variable N40 region of the aptamers, there exists a typical structural similarity in aptamers No. 2, 4, 5, and 20 but not in No. 8 (Fig. 4). It is clear by the fact that the distinct sequence and structure of aptamer No. 8 from other aptamers may have influenced distinct binding characteristics with OTC (Fig. 6).

RNA aptamers for tetracycline have been shown to have ionic interactions with one face of poly-cyclic molecule while the other face allows either hydrophobic or stacking interactions.^{14–16} The aromatic part of tetracycline is predicted to interact by stacking with nucleotide bases of aptamers particularly of RNA origin.¹⁴ A similar interaction is expected to be taking place with OTC and ssDNA aptamers, although, the ssDNA sequences did not match with any of the previously reported RNA aptamers, because the ssDNA aptamers selected in this work are specific to OTC but not tetracycline. Moreover, ssDNA aptamers are stabler than the RNA aptamers, which are susceptible to attack by nucleases. Our study was designed to identify short ssDNA oligonucleotides (76-mer), with 40-nucleotides random region that bind with externally high affinity to the small molecule target OTC, and also have high levels of molecular discrimination against tetracycline and doxycycline, which differ in 5- and 6-positions in the tetracycline nucleus, respectively (Fig. 1). The variation in X₁ position of doxycycline and X₂ position for tetracycline determined the binding specificity of ssDNA aptamers with OTC (Figs. 1 and 6). Studies with tetracycline derivatives revealed that the hydroxyl group at position 6 of tetracycline is essential for recognition, while functional groups at positions 4, 5, and 7 do not interfere with RNA binding.¹⁶ We found similar characteristic binding of ssDNA aptamers with OTC and its structural analogs such as tetracycline and doxycycline. These structural analogs have common 4 and 7 positions, but varying 5 and 6 positions, which make the ssDNA aptamers to be highly specific. A similar interaction with other group of structurally similar small molecule targets such as theophylline and caffeine with RNA aptamers has been previously reported.¹⁷ To our knowledge there are no reports on ssDNA aptamers that bind to oxytetracycline and we report here the ssDNA aptamers and present ssDNA sequences that specifically bind oxytetracycline with high molecular recognition. Such aptamers find applications as biosensors in conjunction with electrochemical detection system for detection and monitoring small organic molecules.^{18,19}

4. Conclusions

We have selected five ssDNA aptamers that bind to oxytetracycline (OTC) with the help of OTC-coated magnetic beads as immobilization matrix by Systematic Evolution of Ligands by Exponential enrichment (SELEX) method. These aptamers are composed of 76-nucleotides in size with 40-nucleotides variable region flanked by 18-nucleotides each primer binding constant regions needed for PCR. The five aptamers selected in this paper differ in 40-nucleotides variable region and

are thus shown to have differing binding strengths. These aptamers can strongly bind to OTC and discriminate structurally similar chemicals based on the variations in minor functional groups such as –H or –OH at 5 and 6 positions as in tetracycline and doxycycline. The positions 5 and 6 in OTC are thus essential for the aptamers' specificity to bind OTC with high affinities ($K_d = 9–121$ nM). The aptamers which can bind at common positions in tetracycline nucleus were eliminated during counter selection steps to avoid non-specificity to OTC.

5. Experimental

5.1. Immobilization of oxytetracycline (OTC), tetracycline (TET), and doxycycline (DOX)

Aliquots of M-280 tosylactivated magnetic beads (DynaL Biotech ASA, Norway) were used for immobilization of OTC, TET, or DOX by covalent binding. Covalent attachment of amino groups in OTC, TET, or DOX (10 μ mol/mL) was allowed to react overnight with approximately 2×10^9 tosylactivated beads in 0.1 M borate buffer, pH 9.5, at 37 °C by mild shaking. The coating process is the covalent coupling of the *p*-toluenesulfonyl (tosyl) groups on the surface of the magnetic beads with the primary amino groups (NH₂).^{20,21} The target-coated beads were washed as described by the manufacturer and blocked the free/unoccupied groups during immobilization procedure by incubation of coated beads with 1 M ethanolamine (pH 8.0) for 6 h at 37 °C with mild shaking. The beads coated with the targets were washed and re-suspended in original volume of PBS buffer, pH 7.4, and stored at 4 °C until use. The unbound targets were estimated by HPLC (Waters) as described previously²² using the following solvent system: acetonitrile/ethanol/0.1 M potassium phosphate buffer, pH 7.6, in the ratio of 12.5:12.5:75. The bound target concentration was calculated as the difference of initial and unbound concentrations. A separate aliquot of naked beads coated with only ethanolamine, TET, and DOX was also performed for further counter selection steps.

5.2. In-vitro selection

A random ssDNA library of 11.25 μ g or 10^{15} molecules with the following sequence: 5'-CGTACGGAATTCGTAGC-N₄₀-GGATCCGAGCTCCACGTG-3' chemically synthesized and purified by PAGE (Genotech Inc., Korea) was used as the initial pool. The following modified primers that anneal to the 5' and 3' ends of the library were used for amplification of the selected oligonucleotides during the aptamer selection process: primer APTFF: 5' fluorescein-CGTACGGAATTCGTAGC-3' and primer APTR: 5'-CACGTGGAGCTCCGATCC-3'. Selection of aptamer and amplification during SELEX process and separation of dsDNA to ssDNA were performed as described previously.²³ The SELEX process was performed similar to previously reported FluMag-SELEX method.¹ Briefly, the OTC-coated magnetic beads were washed several times with binding buffer (100 mM NaCl,

20 mM Tris–HCl, 2 mM MgCl₂, 5 mM KCl, 1 mM CaCl₂, 0.02 Tween 20, pH 7.6) before each round of SELEX process. The random DNA library pool in binding buffer was heated at 90 °C for 10 min, quickly cooled at 4 °C for 15 min and incubated at 25 °C for 7 min. In the first selection round, the washed OTC-coated magnetic beads (2 × 10⁸ OTC-coated beads or 3 mg/100 μL) were suspended in 200 μL binding buffer and 10¹⁵ molecules of denatured random DNA pool were added. This mixture was incubated at room temperature for 30 min with mild shaking and the unbound oligonucleotides were removed by five washing steps with binding buffer. For elution of the bound oligonucleotides from the OTC-coated magnetic beads, the beads-DNA complex was incubated in 200 μL elution buffer (40 mM Tris–HCl, 10 mM EDTA, 3.5 M urea, 0.02% Tween 20, pH 8.0) at 80 °C for 10 min with mild shaking followed by magnetic separation of beads and ssDNA was recovered, the process was repeated five times to elute all traces of bound ssDNA.

The eluted oligonucleotides were precipitated by ethanol precipitation in the presence of 5 μL glycogen (20%) as a carrier and the ssDNA was finally dissolved in 10 μL of EB buffer (10 mM Tris–Cl, pH 8.5). The bound ssDNA after the elution was amplified by PCR. Each 50 μL parallel PCR mixtures contained 25 μL PCR master mix solution (HotStarTaq, Qiagen), 10 μM of each primer (APTFf and APTR), and an appropriate amount of template ssDNA (100–500 ng). PCR conditions used are as follows: an initial heat activation step at 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, and an extension step of 10 min at 72 °C. After the PCR, the purity and size of the PCR product was confirmed by resolving on 2.0% agarose gel by electrophoresis. All PCR products were purified by MinElute Kit (Qiagen) and were pooled in EB buffer.

It was required to separate dsDNA to the proper ssDNA strand after the amplification step for the next round of selection process. For this purpose, denaturing PAGE containing 12% acrylamide, 7 M urea, 20% formamide in TBE buffer was performed as described previously with slight modification when needed.¹ Before loading the samples, the purified PCR products were denatured by heat treatment at 95 °C for 10 min and immediately cooled on ice. The fluorescence labeled strand was verified on the gel with the help of an UV transilluminator. Desired DNA fragments (fluorescent band) were cut out and crushed with 1 mL Crush and Soak solution (500 mM NH₄OAc, 0.1% SDS, 0.1 mM EDTA). The ssDNA fragments were eluted from the gel after incubation at 37 °C for 12 h with mild shaking and subjected to ethanol precipitation. The concentration of ssDNA or dsDNA was measured by using Nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies, Inc.).

After the first selection round, or rather the whole ssDNA from previous round was used in the next selection round as starting DNA pool for enrichment using the target immobilized magnetic beads. Counter selec-

tion steps were introduced to avoid the non-specific ssDNA after every two SELEX rounds using naked magnetic beads, tosylactivated beads coated with ethanolamine, TET, and DOX, respectively.

5.3. Cloning and sequencing

The aptamer pool after seven rounds of SELEX was given a final eighth SELEX round using OTC-coated beads to attain high recovery rate. After the last round, the recovered ssDNA was amplified with unlabeled primers and cloned using TOPO TA Cloning Kit (Invitrogen). Positive clones were picked and plasmid DNA was purified by miniprep kit (Qiagen) and the clones with aptamer inserts were sequenced (Genotech Inc., Korea).

Analysis of secondary structure with several aptamers was performed by free energy minimization algorithm according to Zuker²⁴ using the Internet-tool *Mfold* (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi>).

5.4. Binding assays

Sequenced aptamer clones were tested for their individual binding characteristic to free OTC. Binding assays were performed by equilibrium filtration method as previously described.¹⁷ A series of ssDNA aptamer concentrations ranging from 0 to 1 μM were dissolved in binding buffer (20 mM Tris/HCl, pH 7.6; 100 mM NaCl; 2 mM MgCl₂; 5 mM KCl; 1 mM CaCl₂; 0.02% Tween 20) containing 50 μM OTC in 200 μL reaction and incubated at 25 °C for 30 min. The mixture was loaded onto YM10 Microcon filter columns (Amicon) and centrifuged for 8 min at 12,000g, allowing 100 μL of solution to flow through the membrane similar to equilibrium dialysis; the solution that remained above the molecular weight cutoff membrane contains free OTC, free ssDNA, and ssDNA-bound OTC, and the filtrate contained only OTC, an equivalent concentration of the free OTC in the initial solution. An 80 μL filtrate sample was subjected to HPLC analysis to determine the concentration of OTC.

5.4.1. Determination of dissociation constants (K_d). To calculate the dissociation constants, the percent of bound OTC verses ssDNA concentrations was plotted and the data points were fitted by the non-linear regression analysis with the help of the following equation using Sigmaplot 8.0 software:

$$y = B_{\max} \cdot \text{free ssDNA} / K_d + \text{free ssDNA} \quad (1)$$

where y is degree of saturation, B_{\max} is the number of maximum binding sites, K_d is the dissociation constant.¹⁴

5.5. Determination of specificity of aptamers by affinity elution

Selected aptamers were tested for their ability to bind with OTC/TET/DOX. Assays using immobilized targets were conducted using fresh aliquots of 2 × 10⁸ beads

(3 mg/100 μ L) coated with OTC/TET/DOX as described above. The chemical-coated beads were washed five times with binding buffer and suspended in 200 μ L of the same buffer containing 1 μ g of aptamers. The unbound ssDNA was removed by washing three times with binding buffer and pooled. The target-bound ssDNA was eluted with 200 μ L of elution buffer (40 mM Tris/HCL, pH 8; 10 mM EDTA; 3.5 M Urea; 0.02% Tween 20) thrice after heat treatment for 5 min at 80 °C. The pooled ssDNA (unbound and bound) was purified and measured using Nanodrop (ND-1000) and the % recovery of bound aptamer with each target was determined.

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