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# Single-stranded DNA aptamers specific for antibiotics tetracyclines

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ABSTRACT

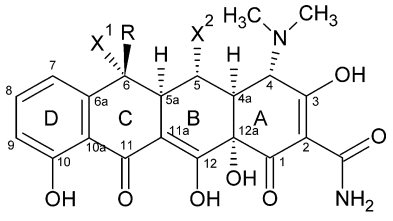
Tetracyclines (TCs) are a group of antibiotics comprising of a common tetracycline (TET) nucleus with variable $X_1$ and $X_2$ positions on 5 and 6 carbon atoms, such as oxytetracycline (OTC) and doxycycline (DOX). In this study, the tetracycline group specific (TGS) ssDNA aptamers were identified by modified SELEX method by employing tosylactivated magnetic beads (TMB) coated with OTC, TET, and DOX, respectively, as targets and counter targets. Twenty TGS-aptamers were selected, of which seven aptamers, designated as T7, T15, T19, T20, T22, T23, and T24, showed high affinity to the basic TET backbone ($K_d$ = 63–483 nM). The specificity of these TGS-aptamers to structural analogues followed the order in which the TCs was employed during SELEX process (OTC > TET > DOX) except aptamer T22, which was highly specific to TET than OTC or DOX. Aptamers that were specific to one target molecule but fail to bind the other structurally related TCs were eliminated during counter selection steps. Three aptamers, T7, T19, and T23 contained palindromic consensus sequence motif GGTGTGG. The remaining TGS-aptamers showed many consensus sequences that are truncated forms of this palindrome forming mirror image or inverted sequences. For example, GTGG or its inverted form, GGTG motif was found in all TGS-aptamers. A consensus sequence motif TGTGCT or its truncated terminal T-residue was found in most TGS-aptamers, which is predicted to be essential for high affinity and group specificity. These TGS-aptamers have potential applications such as target drug delivery, and detection of TCs in pharmaceutical preparations and contaminated food products.



## 1. Introduction

Tetracyclines (TCs) are group of antibiotics that comprise various derivatives of basic tetracycline (TET) nucleus. For example, oxytetracycline (OTC), doxycycline (DOX), and tetracycline (TET) differ in presence or absence of —H and/or —OH groups at $X_1$ and $X_2$positions on 5 and 6 carbon atoms on rings B and C, respectively, in a tetracycline backbone (Fig. 1). These are widely used in pure form or mixed with structurally related TCs for their medical applications as effective antibacterial agents. They have been extensively used in veterinary medicine as growth promoters for animals. Therefore, these have been detected in food products, such as meat,[1,2] milk,[3], and eggs/chicken,[4] which lead to a serious threat to human health.[5–7] The formation of 4- and 6-epimers of TET analogues in pharmaceutical preparations under abnormal condition such as heat, pH, and humidity is well documented.[8] Therefore, TCs may exist in different epimeric forms, which make it even more difficult for conventional detection methods to monitor these in pharmaceutical preparations and various food products. Researchers are now undertaking constant efforts to detect TCs in food products, pharmaceutical preparations, and in contaminated waters employing chromatographic methods, such as HPLC.[8,9] However, the existing methods might lead to false detection because of their conversion to different forms. Moreover, the existing conventional methods for detection are often time consuming and expensive that lack specificity and always rely on the authentic samples as reference standards.

Recently, pharmaceutical products, such as drugs and antibiotics, are considerable targets for development of aptamers for vari-



| Target | R | $X^1$ | $X^2$ |
|---|---|---|---|
| Tetracycline (TET) | $CH_3$ | OH | - |
| Oxytetracycline (OTC) | $CH_3$ | OH | OH |
| Doxycycline (DOX) | $CH_3$ | H | OH |

**Figure 1.** General structure of tetracyclines (TCs) known as 1,4,4a,5,5a,6,11,12a-octahydronaphthacene formed by four condensed rings consisting of six carbon atoms each. The letters A, B, C, and D, respectively, denote the rings from right to left. The $X_1$ and $X_2$ are variable functional groups involved in their structural analogues, and R represents the alkyl group.

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ous applications. For example, aptamers for antibiotics that have been found to have applications in targeting drug delivery[10,11] and purifying RNA-binding proteins.[12,13]

These aptamers are short, single-stranded oligonucleotides of DNA or RNA origin which can be selected in vitro to bind nearly any target molecules, from small molecules to proteins.[14–20] In most diagnostics and biomolecular sensing studies, the antibodies were used for their high affinity and specific molecular recognition, but there are some limitations, including animals or cell lines requirement with complicated purification steps for antibody production. Unlike antibodies, aptamers have many advantages over using antibodies because of the following: (a) advantages of aptamers to rival antibodies, (b) they can be selected by in vitro methods for their target molecules, (c) aptamers can be chemically synthesized and modified using well-established nucleic acid chemistries, (d) they are highly stable under elevated temperatures, unlike antibodies, and (e) they can be stably used as biorecognition elements in conjunction with a detection systems, such as surface plasmon resonance (SPR) and electrochemical methods.[18,21–24] Applications of DNA/RNA aptamers as analytical tools have now emerged because of high affinity, specificity, and selectivity for their target molecules.[25–27]

The sequence and three-dimensional structure of aptamers play an important role in recognition and binding of targets.[28] This also implies on the specific groups present on the target molecule, such as tetracycline antibiotics to which an aptamer binds.[20] Recently, an 108-mer ssDNA aptamer was selected from a random pool that can recognize thalidomide enantioselectively from the racemic mixture.[29] Detailed analysis of aptamer sequences enables understanding the secondary folding, binding sites, conserved, and consensus sequence motifs that determine the affinity, specificity and selectivity of aptamers to their target molecules. This information helps researchers to design and modify the variants of some selected aptamers for improved affinity or selectivity to their target molecule.[29–31]

In this paper, we have extended our previous studies with OTC-specific aptamers that failed to bind structurally related TCs such as TET and DOX. Here, we designed a modified SELEX method, which is an extended form of Toggle-SELEX[32] combined with Flu-Mag SELEX[33] by employing target and counter target molecules sequentially different to generate tetracycline group specific (TGS) aptamers. As a result, 20 TGS-aptamers were selected out of which seven showed high affinity to basic TET-backbone whose $K_d$ values were in nanomolar range. The order of the target molecules employed for selection of aptamer influenced the specificity of these aptamers to their target molecules. Our results showed that the variable region of aptamers contained many consensus sequences and most of them are mirror image sequences, and palindormic sequence motifs found in some high affinity aptamers.

## 2. Results

### 2.1. Selection of ssDNA aptamers that bind tetracycline analogues by modified SELEX

Tetracycline group specific (TGS) aptamers were selected from a random pool of $10^{15}$ ssDNA molecules by modified Toggle-SELEX[32] combined with Flu-Mag SELEX.[33] Aptamers were selected by SELEX process against two target molecules, such as oxytetracycline (OTC) and tetracycline (TET) in part-1 and -2, respectively, as shown in the schematic diagram (Fig. 2). SELEX part-1 was composed of four selection rounds against tosylactivated magnetic beads coated with OTC (OTC-TMB) to separate aptamers that are highly specific to OTC. A counter selection step using ethanolamine-coated TMB was introduced after second round to avoid non-specific binding.[33] After four selection rounds with OTC-TMB (part-1), a second counter selection was performed, but this time TET-coated TMB (TET-TMB) was employed. Those aptamers which fail to bind TET but only bind OTC were eliminated, and the TET-TMB bound fraction was subjected to further SELEX rounds (part-2) using TET-TMB (Figs. 2 and 3).

The ssDNA aptamers obtained after 4 rounds of enrichment were expected to have binding ability to more than one target molecule, such as OTC, TET, and other tetracyclines (TCs). After the seventh SELEX round, a third counter selection step using DOX-TMB was introduced to eliminate ssDNAs that are more specific to doxycycline (DOX) than OTC or TET. After the eighth SELEX round ~81% of the added ssDNA fraction was recovered, and this fraction can bind to OTC, TET, and DOX. Additional four selection rounds were performed using TET-TMB to further enrich the ssDNA pool to a total of 12 SELEX rounds. Recovery of no more than 81% of enriched fraction was achieved from the initial ssDNA pool probably because of the saturation of binding sites (Fig. 3). Thus obtained final fraction of ssDNAs tuned to bind all three tetracycline analogues (OTC/TET/DOX) by the modified SELEX method allowed the selection of TGS aptamers. The ssDNA fraction from the 12th round

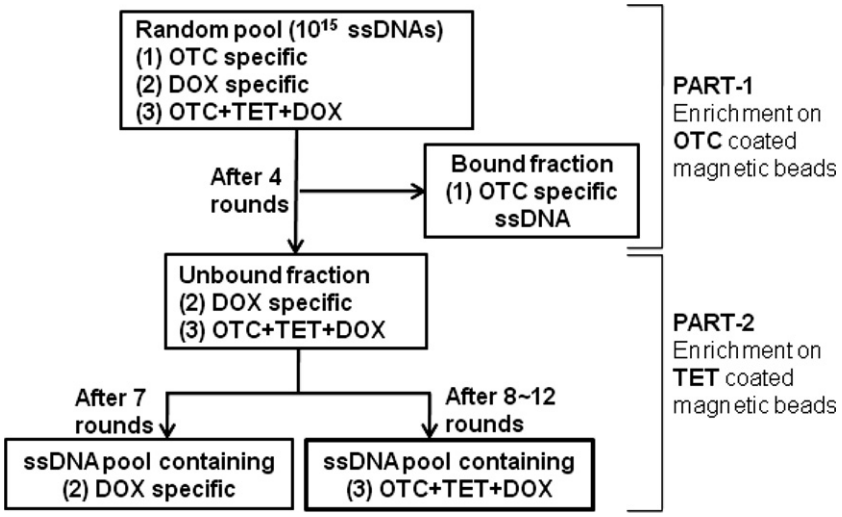



**Figure 2.** Schematic diagram of modified systematic evolution of ligands by exponential enrichment (SELEX): parts 1 and 2 represent the SELEX process performed using oxytetracycline (OTC) and tetracycline (TET)-coated tosylactivated magnetic beads (TMB). Fractionation of aptamers specific for their targets at different steps is shown in boxes.

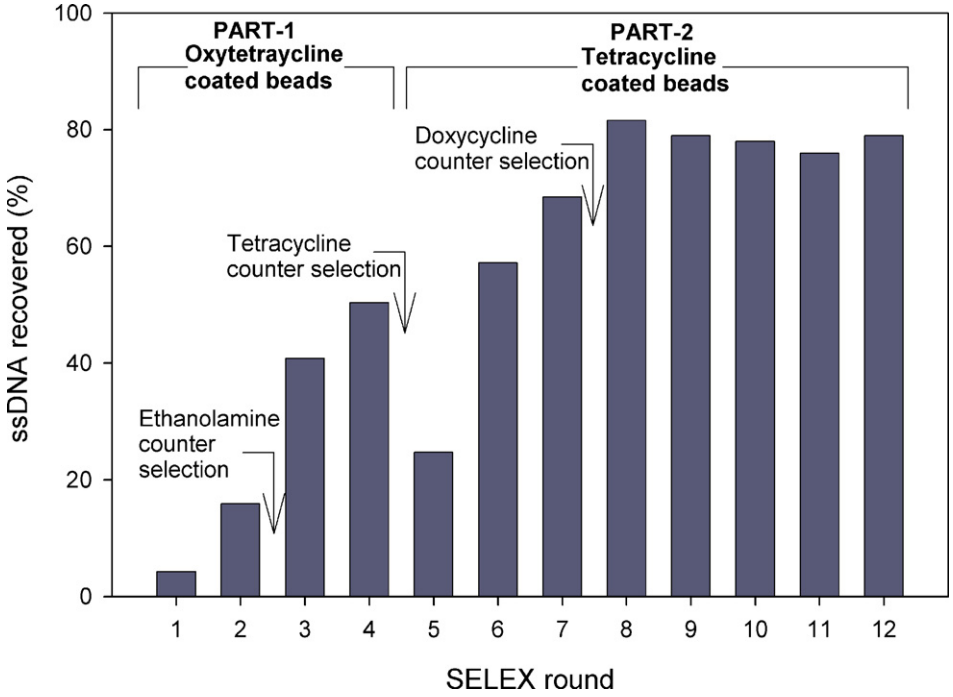



**Figure 3.** Selection of tetracyclines group specific (TGS) aptamers: recovery of ssDNA pool after each SELEX round, the arrows indicate the counter selections performed using tosylactivated magnetic beads coated with the following chemicals: ethanolamine, which was used for blocking of unoccupied groups on the magnetic beads; tetracycline and doxycycline are structural analogues of oxytetracycline.

was cloned, and a total of 20 potential clones were picked that carried aptamer fragments and were subjected to sequence analysis.

### 2.2. Dissociation constants and sequence analysis of group specific aptamers

Binding assays were conducted using TET as a model antibiotic because all TCs are derived from the parent TET backbone with variable 5 and 6 carbon atoms of TCs. Binding assays with all the 20 aptamers were conducted using constant number of TET-coated TMBs against varying concentrations of ssDNA aptamers (0–2 μM) (Fig. 4). The aptamers that had the capacity to bind strongly were selected based on the dissociation constants ($K_d$) as determined by non-linear regression analysis and Eq. (1) (see Section 5). Seven aptamers were selected whose $K_d$ values with TET were determined to be in nanomolar range, and these are designated as T7, T15, T19, T20, T22, T23, and T24 with $K_d$ values of 357.8, 197, 424.8, 63.6, 483.5, 100.6, and 70.7 nM, respectively. Among

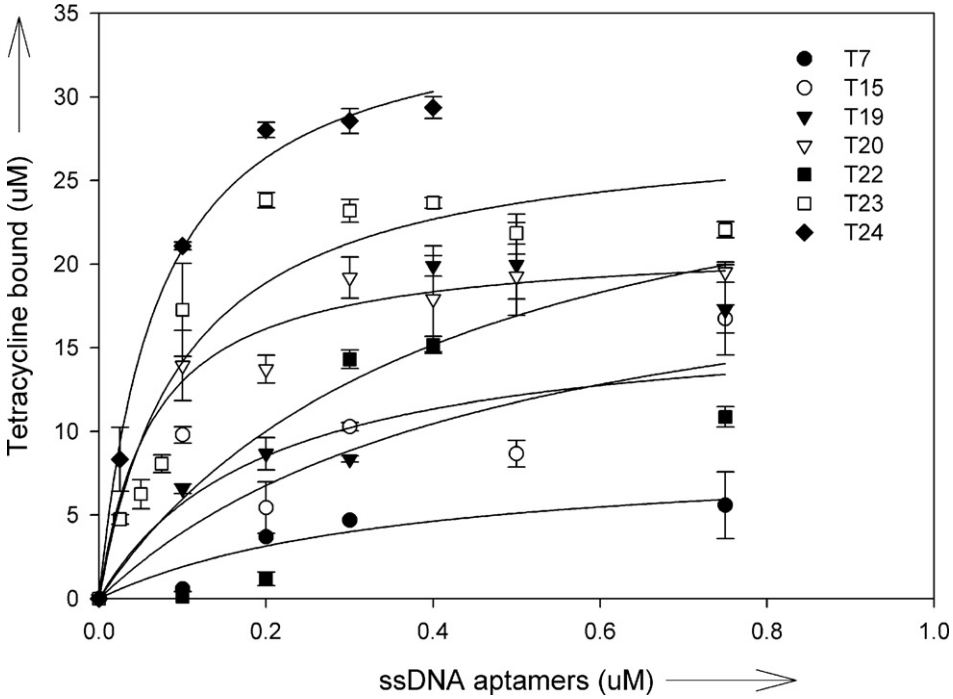



**Figure 4.** Binding assays with ssDNA aptamers to tetracycline (TET). The saturation curves were obtained by plotting the concentration of bound TET as a function of ssDNA concentration, and the dissociation constants ($K_d$) were calculated by non-linear regression analysis ($n = 3$).

these seven aptamers T20, T24, and T23 showed strong affinities compared to the remaining aptamers. These aptamers, along with those weakly bound to TET, and their sequences are shown in Table 1.

The variable 40-nucleotide ($N_{40}$) region of 76-mer aptamers showed a number of G-rich conserved sequences and eight consensus sequence motifs (Table 1). These consensus sequence motifs are (i) GGTGTGG—is a palindromic sequence and the corresponding consensus sequences appeared to be 1–3 nucleotide/s truncated forms of this palindrome that gave rise to asymmetric feature or mirror image sequences. These sequences are: (ii) GTGTGG, (iii) GGTGTG, (iv) GGTG, and (v) GTGG which are asymmetric sequences. Consensus sequence motifs GTGTGG and GTGG formed mirror image structures with GGTGTG and GGTG, respectively. These consensus motifs were frequently found located in the variable region or overlapped with constant region in the TET-group specific (TGS) aptamers. They are found to be located on different positions of the same aptamer and/or in the different aptamers (see Table 1). The frequency of occurrence of these consensus sequences in the variable regions appears to be significant for binding and recognition of TGS-aptamers to the target molecules. However, the variable $N_{40}$ region of T20 was highly diverse as compared with other aptamers, and it had high affinity for TET ($K_d$ = 63.6 nM) with only a GTGG consensus sequence motif overlapped with constant region, a distinct feature that was not seen in the rest of TGS-aptamers (Table 1).

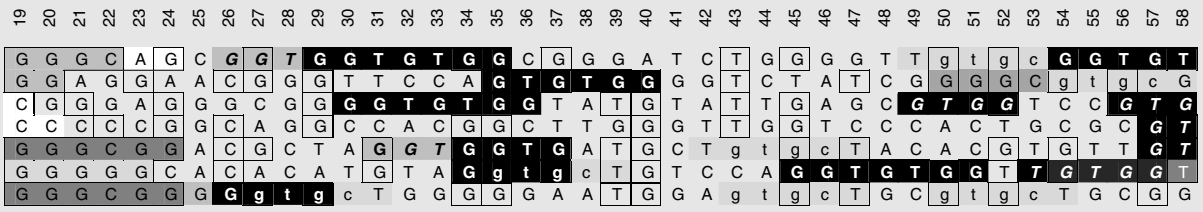
The remaining three out of eight consensus sequence motifs in TGS-aptamers are (vi) TGTGCT in aptamers T22, T3, and T13, but only T22 showed strong binding with $K_d$ = 483.5 nM. Two truncated T-residues on either terminal from TGTGCT motif were found. They are (vii) TGTGC in T7 and T25, but only T7 showed strong binding with $K_d$ = 357.8, and (viii) presence of GTGCT motifs repeatedly occurred, thrice in the variable region of T24 ($K_d$ = 70.7 nM) and once in T23 ($K_d$ = 100.6 nM) that showed strong affinity for TET than those aptamers with TGTGCT or TGTGC motifs. For example, TGTGCT motif in T3, T13, and T22 or TGTGC motif in T7 and T25 aptamers showed relatively higher $K_d$ values than T24 and T23 containing GTGCT consensus sequence motif. This means that presence of T residue at the beginning of GTGCT motif dramatically reduced-binding affinity of TGS-aptamers with TET. Therefore, GTGCT motif appears to be crucial for strong affinity of TGS-aptamers to their target molecule.

The secondary structure of selected seven aptamers (T7, T15, T20, T22, T23, and T24) as deduced by *m*-fold is depicted in Fig. 5. Typical stem and loop motifs were seen that are predicted to be the binding sites of the aptamers. A majority of the highly conserved nucleotides are located in the loop region as seen highlighted by arrows in Fig. 5. For example, it was found that a G-residue is strictly required at 35th position followed by 40 and 45 positions (5′ to 3′) in six out of seven aptamers within their variable $N_{40}$ regions (Table 1 and Fig. 5). Consensus sequence motifs mainly (i) GGTGTGG, (ii) GTGTGG, and (iii) GGTGTG are localized at the juncture of the stem and loop structures. Whereas (iv) GGTG in T23 and T24 and (v) GTGG in T19 and T22 localized strictly in the loop regions except in T20, the GTGG was located in the stem region. The remaining motifs, such as (vi) TGTGCT, (vii) TGTGC, and (viii) GTGCT, were also found located in the loop regions. The significance of specific locations of the above consensus sequences in the secondary structure of these aptamers was assumed to have contributed to strong binding and specificity of TET-TGS aptamers to their target molecules (TCs). Those aptamers that showed weak binding to TCs were having structurally distinct features although with few common consensus and conserved sequences (supplementary Figure S1).

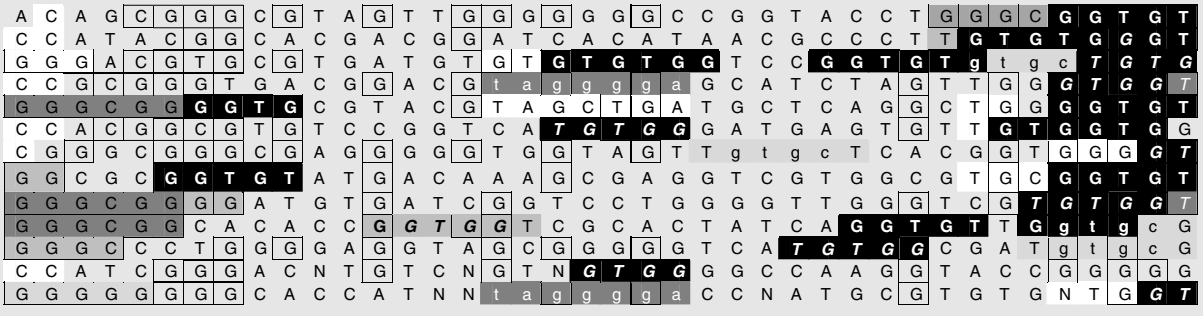
**Table 1**
Sequences shown in the table are the variable 40-nucleotide regions ($N_{40}$) that are flanked by 18-nucleotide constant regions in 76-mer ssDNA aptamers (5′-CGTACGGAATTCGCTAGC-**$N_{40}$**-GGATCCGAGCTCCACGTG-3′)

| Aptamer | Variable $N_{40}$ sequences from 19th to 58th position (5′ → 3′) in the 76-mer ssDNA aptamers[a] |
|---|---|

$N_{40}$ sequences of the aptamers that strongly bind tetracycline analogues




$N_{40}$ sequences of the aptamers that weakly bind tetracycline analogues




[a]Sequences shown in boxes are the conserved sequences matching to their positions; those highlighted in dark, gray, and dark-gray are the consensus sequences that are represented by their matching case letters located at different positions in the same aptamer and/or in different aptamers. Sequences shown in italics represent truncated asymmetric consensus sequences.

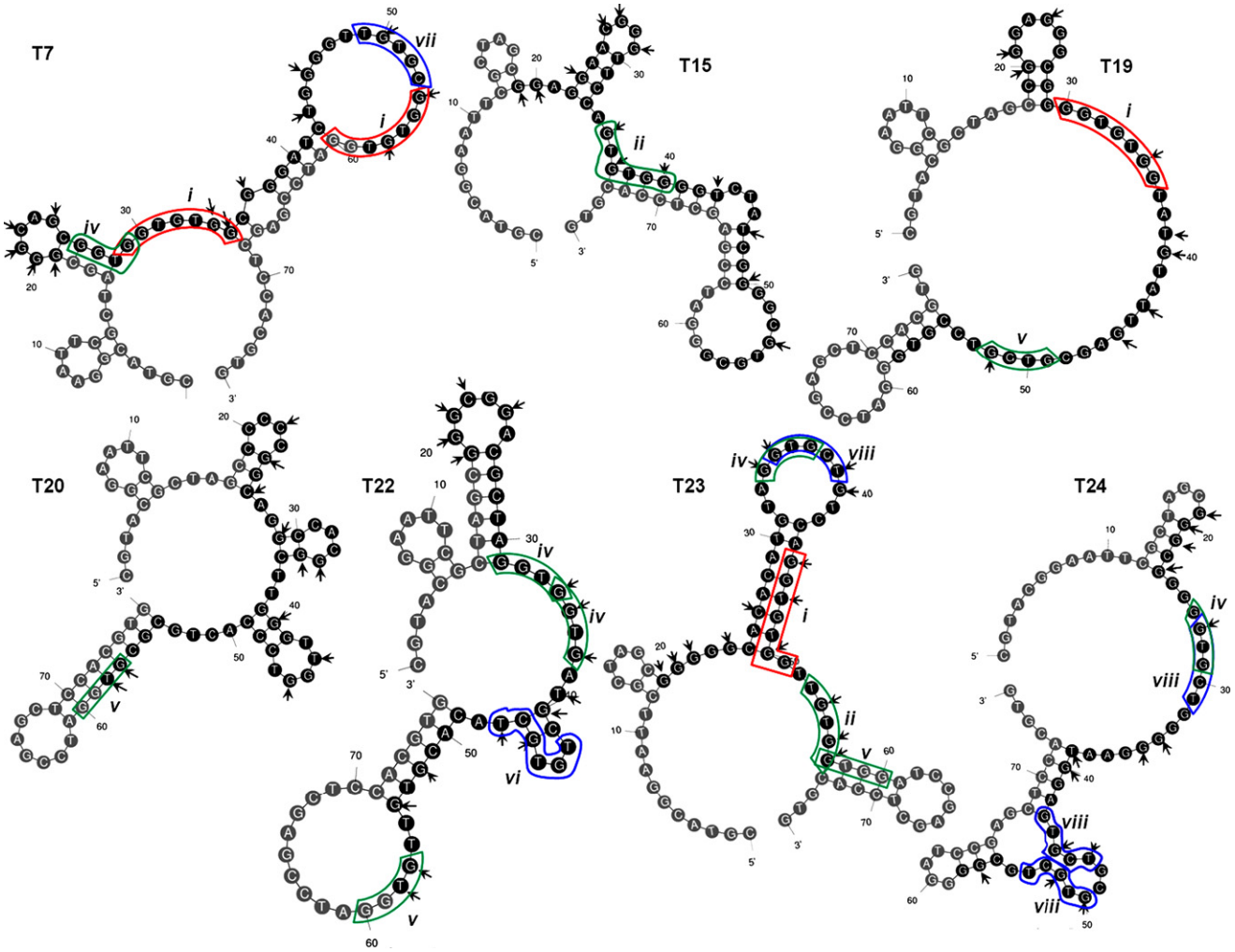



**Figure 5.** Secondary structure models of TGS-aptamers as predicted by *m*-fold. Aptamers composed of 76-mer size with central 40 nucleotides variable region (dark circles) flanked by 18 nucleotides each primer-binding constant region (gray circles). The arrows indicate the location of conserved nucleotides, and those shown in boxes represent the consensus sequence motifs (i–viii) found after the sequence analysis.

### 2.3. Specificity of ssDNA aptamers

The modified SELEX method designed in this study enabled the ssDNA aptamers to bind more than one type of TCs. This was because the aptamers were selected against two different target (OTC and TET) molecules and those which were specific to one target molecule were eliminated during counter selection stepwise in parts 1 and 2 of SELEX process as shown in Figures 2 and 3. The results of specificity tests showed that six out of seven selected aptamers (T7, T15, T19, T22, T23, and T24) showed high specificity toward binding to OTC than TET and DOX (Fig. 6). The specificity of these six aptamers followed the order of target molecules employed for selection processes (OTC > TET > DOX) (Figs. 2 and 3). However, T20 was highly specific to TET than OTC or DOX, which followed the order TET > OTC > DOX. This result is consistent with (a) the diverse sequences found in the $N_{40}$ region of T20 and (b) T20 showing high specificity and affinity to TET ($K_d$ = 63.6 nM) as compared with other six aptamers (T7, T15, T19, T22, T23, and T24) whose $K_d$ values were determined to be higher than that of T20 (70–483 nM) (Table 1 and Figs. 4 and 6). Further, none of the TGS-aptamers bind a structurally distinct chemical, diclofenac (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid), indicating that aptamers selected in this study are specific to TCs.

## 3. Discussion

We had reported in our previous study that the high affinity OTC-specific aptamers failed to bind other structurally related tetracycline group's of antibiotics.[34] In this paper, we report an extension of our previous work by a modified SELEX method, which enabled enrichment of the aptamers that bind structural analogues of TCs (Figs. 2 and 3). Pairwise alignment using Emboss::global (needle) program for the comparison of the sequences of OTC-specific aptamers with TET-group specific aptamers revealed that one of the OTC aptamers (No.4) shared ~75% of similar sequences with T22 within the variable $N_{40}$ regions (Table 2). These two aptamers (OTC-No.4 and T22) also showed similarities in their secondary structures except an additional stem and loop appendage found in T22 aptamer, which may have contributed to its group specificity. The $N_{40}$ variable region within the OTC-No.4 and T22 showed highest similarity (~90%) sequences corresponding to 29–58 region (5′ to 3′) except at four nucleotide positions that are predicted to have contributed to the specificity of the aptamers to OTC or TCs, respectively. Among these four mismatching nucleotide positions within 29–58 region (29 nucleotides), three positions, such as 30, 41, and 46 (5′ to 3′) in OTC-No.4 aptamer are occupied by T, G, and T residues, respectively[34] whereas similar positions in T22 aptamer are

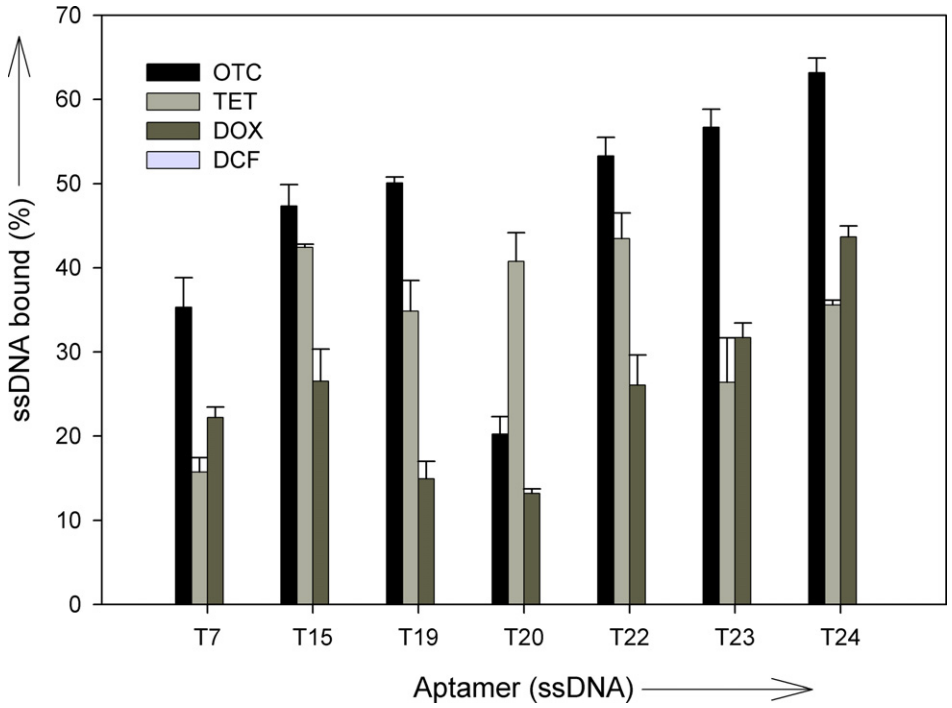



**Figure 6.** Binding affinities of the aptamers for structurally similar targets. One microgram of each ssDNA aptamer was incubated with constant number of tosylactivated magnetic beads ($2 \times 10^8$) coated with equimolar concentrations of oxytetracycline, tetracycline, doxycycline, and diclofenac.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

replaced by A, C, and C residues, respectively (Tables 1 and 2). Out of these three miss-matching nucleotide positions, two C-residues at 41 and 46 positions of T22 contributed to the formation of a minor stem and loop appendage which is missing in OTC-No.4 (Fig. 5 and Tables 1 and 2). Interestingly, a consensus sequence motif TGTGCT which is specific for TGS-aptamers was found in this extra loop appendage of T22 aptamer (Fig. 5). Therefore the minor stem and loop appendage (40–46 position) might be crucial for binding of T22 to TCs (OTC/TET/DOX) making it TGS-aptamer (Fig. 5).

Additionally, the structural similarities of OTC aptamers No.2 and No.4 and TGS-aptamers T19 and T22 (supplementary Figure S2) might imply very well that such a stem-loop structure is important for TCs binding, and the stem structure stabilizes the conformation for binding.[34] Furthermore, similarities of at least 50% within $N_{40}$ regions of OTC-specific and TGS-aptamers are shown in Table 2. The $K_d$ values for OTC-aptamers are relatively lower than those for TGS-aptamers with OTC and TET molecules, respectively. This can be correlated to the elimination of high affinity OTC-specific aptamers during Part-1 of SELEX process (Figs. 2 and 3). The conserved sequence motifs between the two groups of aptamers are highlighted in Table 2. A consensus sequence motif GGTGTGG was found common in some OTC-specific and TGS-aptamers indicating that this motif might be crucial for binding to common TET backbone (Fig. 1). Furthermore, the truncated forms of this motif, such as GGTGTG, GTGTGG, TGTGG, GGTGT, GTGG, and GGTG, were also found common to both OTC and TGS-aptamers suggesting that these motifs might be the recognition elements of common tetracycline backbone (Fig. 5 and Tables 1 and 2).

We previously showed that the conserved sequence motifs CGYTGGTG and GTKKTGT were specific for OTC, and are predicted to be essential for selectivity and affinity of aptamers to bind OTC.[34] Whereas the TGS-aptamers required GGTGTGG, a palindromic sequence, and its truncated versions, such as GTGTGG, GGTGTG, GGTG, and GTGG, that formed asymmetric or mirror images of respective sequences (Table 1). The GGTGTGG palindrome was found in T7, T19, and T23 located mainly in the major loop region precisely positioned at the juncture of the stem-loop structures (Fig. 5). Such palindromic sequences have also been reported in DNA aptamers for chitin and L-tyrosinamide target molecules, but the significance of palindromes in these aptamers is still unclear.[35,36] In this study, we speculate that the palindromic sequence and its truncated forms found in $N_{40}$ region of TGS-aptamers appears to be specific for the aromatic nucleus of TCs. This palindromic sequence is also found in two OTC-specific aptamers (No.2 and 4), while its truncated motifs were found frequently in all the OTC-specific aptamers, but these failed to bind other TCs, such as TET and DOX probably because of their strong affinities to OTC.[34] Therefore OTC-specific motifs CGYTGGTG and GTKKTGT are probably crucial for binding at 5 and 6 carbon atoms ($X_1$ and $X_2$ positions) on B and C aromatic rings at one face of the TET nucleus (Fig. 1). Whereas consensus sequence motifs found in TGS-aptamers GGTGTGG and/or TGTGCT and their truncated forms are probably required for binding functional groups associated with carbon atoms 10, 11, 12, and 2 at the other face of the TET nucleus which is common for tetracycline group antibiotics. Further, the TGTGCT motif was unique to TGS-aptamers which are not found in OTC-specific aptamers.[34] It is evident from the specificity tests that the TGS-aptamers can bind to all three TCs (OTC, TET, and DOX), but the preferential order of specificity followed the order in which the target molecules were employed (OTC > TET > DOX) for enrichment of aptamers except T20 which followed TET > OTC > DOX (Figs. 2 and 3). The differential order of specificity was well correlated to its high affinity to TET ($K_d$ = 63.6 nM), distinct sequence and structural features with only a GTGG consensus motif, whereas most other TGS-aptamers contained multiple consensus sequence motifs (Table 1 and Fig. 5).

**Table 2**
Pairwise alignment and sequence match performed by a web tool EMBOSS::needle (global) program

| OTC/TET aptamer | $N_{40}$ sequence match (5′ to 3′)[a,b] | Match (%) | $K_d$ (nM) | |
|---|---|---|---|---|
| | | | OTC[c] | TET[d] |
| No.4/T7 | CGACGCGCGTTGGTGGTGGATGGTGTGTTACACGTGTTGT<br>\|.\|\|...\|\|\|\|\|\|\|\|\|\|.\|.\|.\|\|\|\|\|<br>GGGCAGCGGTGGTG--TGGCGGGAT-CTGGGGTTGTGCGGTGT | 50 | 9.6 | 357 |
| No.4/T15 | CGACGCGCGTT--GGTGGTGGATGGTGTGTTAC---ACGTGTTGT<br>.\|...\|\|.\|\|\|.\|\|\|\|\|\|.\|..\|.\|\|\|\|..<br>GGAGGAACGGGTTCCAGTG----TGGGGTCTATCGGGGCGTGCG | 42 | 9.6 | 197 |
| No.4/T19 | CGACGCGCGTTGGTGGTGGATGG--TGTGTTACACGTGTT--GT<br>\|\|.\|..\|\|.\|\|.\|..\|\|\|\|\|\|.\|\|...\|\|\|.\|\|<br>CGGGAGGGCGGGGGTGTGGTATGTATTGAGCGTGGTCCGTG | 51 | 9.6 | 424 |
| No.4/T22 | C-GACGCGCGTTGGTGGTGGATGGTGTGTTACACGTGTTGT<br>\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|<br>GGGCGGACGC---TAGGTGGT-GATGCTGTGCTACACGTGTTGT | 75 | 9.6 | 483 |
| No.4/T23 | CGACGCGCGTTGGTGGTGG-ATGGTGTGTTACACGTGTTGT<br>\|\|\|.\|...\|\|.\|\|\|\|.\|\|..\|\|\|\|\|\|.\|\|\|.\|\|<br>GGGGGC-ACACATGTAGGTGCTGTCCAGGTGTGGT-----TGTGGT | 52 | 9.6 | 100 |
| No.4/T24 | CGACGCGCGTTGGTGGTGG----ATGGTGTGTTACACGTGTTGT<br>\|.\|\|\|..\|\|\|\|.\|\|\|\|\|\|\|\|.\|\|\|.\|\|\|\|.\|\|.<br>GGGCGGGGGGTGCTGGGGGGAATGGAGTGCT--GCGTGCTGCGG | 54 | 9.6 | 70 |
| No.5/T7 | ACGTTGACGCTGGTGC--CCGGTTGTGGTGCGAGTGTTGTGT<br>.\|....\|\|.\|\|\|\|.\|\|.\|.\|\|\|.\|.\|\|\|..\|\|\|\|<br>GGGCAGCGGTGGTGTGGCGGGATCTGGGGT-TGTGCGGTGT | 54 | 12.0 | 357 |
| No.5/T15 | ACGTTGACGCTGGTGCCCGGTTGTGGT--------GCGAGTGTTGTGT<br>\|..\|...\|.\|\|\|.\|\|.\|..\|\|.\|\|\|\|\|\|.\|.\|<br>GGAGGAACGGGTTCCAGTGTGGGGTCTATCGGGGCGTGCG | 40 | 12.0 | 197 |
| No.5/T19 | ACGTTGACGCTGGTGCCCGGT-TGTGGTGCGAGTGTTGTGT<br>\|\|.....\|\|\|\|.\|...\|\|\|\|\|\|..\|\|.\|\|\|.\|..\|\|<br>CGGGAGGGC-GGGGTGTGGTATGTATTGAGCGTGGTCCGTG | 52 | 12.0 | 424 |
| No.5/T20 | ACGTTGACGCTGGT--GCC-CGG-TTG---TGGTGCGAGTGTTGTGT<br>.\|.\|.\|\|.\|\|\|\|\|\|\|\|\|\|\|\|.\|.\|.\|\|.\|.\|\|<br>CCCCCGGCAGGCCACGGCTTGGGTTGGTCCCACTG-CGCGT | 51 | 12.0 | 63 |
| No.5/T22 | ACGTTGACGC----TGGTGCCCGGTTGTGGTGCGAGTGTTGTGT<br>.\|\|\|\|\|\|\|\|\|\|\|..\|.\|\|\|\|.\|.\|..\|\|\|\|\|\|\|<br>GGGCG--GACGCTAGGTGGTGAT--GCTGTGCTACACGTGTTGT | 57 | 12.0 | 483 |
| No.5/T23 | ACG-TTGACGCTGGTGCCCGGTTGTGGTGCGAGTGTTGTGT<br>\|\|.\|.\|\|..\|.\|\|.\|\|.\|\|.\|.\|\|\|\|\|\|\|\|.\|\|<br>GGGGGCACACATGTAGGTGCTGTCCAGGTGTGGT-----TGTGGT | 50 | 12.0 | 100 |
| No.5/T24 | ACGTTGACGCTGGTGCCCGGTTGTGGTGC-GAGTGTTGTGT<br>.\|\|..\|..\|\|\|\|.\|\|..\|\|..\|\|\|\|\|.\|\|\|.\|\|.\|.<br>GGGCGGGGGTGCTGGGG---GAATGGAGTGCTGCGTGCTGCGG | 53 | 12.0 | 70 |
| No.20/T15 | CGAGTTGAGCCGGGCGCGGTACGGGTACT---GGTATGTGTGG<br>.\|..\|\|.\|.\|\|...\|.\|\|..\|\|\|..\|\|\|\|...\|\|\|.\|<br>GGAGGAACGGGTTCCAGTGTGGGGTCTATCGGGGCGTGCG | 46 | 56.8 | 197 |
| No.20/T19 | CGAGTTGAGCCGGGCGCGGTACG---GGTAC-TGGTATGTGTGG<br>\|\|.\|.\|\|.\|\|\|.\|.\|\|\|\|.\|\|\|\|.\|..\|.\|\|\|\|..\|\|\|<br>CGGG-AGGGCGGGGTGTGGTATGTATTGAGCGTGGTCCGTG | 54 | 56.8 | 424 |
| No.20/T24 | CGAGTTGAGCCGGGCGCGGTACGG-GTACTGGTATGTGTGG<br>\|\|.\|.\|.\|\|.\|\|\|\|\|\|.\|.\|\|\|\|.\|\|\|\|\|.....\|\|.\|\|<br>GGGCGGG-GGTGCTGGG---GGAATGGAGTGCTGCGTGCTGCGG | 52 | 56.8 | 70 |

The table shows $N_{40}$ variable sequence match hit between oxytetracycline (OTC) specific aptamers (No.4, 5, and 20) and the aptamers (T7, T15, T19, T20, T22, T23, and T24) that bind tetracycline analogues (OTC/TET/DOX). Sequences highlighted in grey are conserved sequence motifs.

[a] Matching nucleotides are connected with a "I" symbol. Mismatches are connected with a "." (dot) symbol. A gap is represented with a "-" (hyphen) symbol.

[b] Shaded sequences are conserved and consensus sequence motifs.

[c] The $K_d$ values with [c] OTC-specific aptamers.

[d] TGS-aptamers that are indicated in the first column of the table.

## 4. Conclusions

In this paper, we report a modified Toggle-SELEX[32] combined with Flu-Mag SELEX[33] methods designed for selection of group specific ssDNA aptamers by employing different structural analogues of TCs as targets and counter targets during selection process. This enabled selection of 20 TET-group specific ssDNA aptamers from a random pool of $10^{15}$ ssDNA molecules. These aptamers bind to more than one analogue of tetracycline group of antibiotics. Seven of them (T7, T15, T19, T20, T22, T23, and T22) had high affinities for the basic tetracycline backbone with which the TET-analogues are derived. The dissociation constant ($K_d$) of the selected seven aptamers was in the range of 63–483 nM. The random $N_{40}$ region of these aptamers showed several conserved and consensus sequences. A G-residue was strictly required to be specifically located at 35th position (5′ to 3′) in all the high affinity aptamers. The presence of consensus palindormic sequence motif GGTGTGG in T7, T19, and T23 and its truncated

forms spread in all aptamers that are predicted to be essential for binding on the basic TET backbone because similar motifs were also found in OTC-specific aptamers. The T/GTGCT conserved motif was specifically found in TGS-aptamers, which is predicted to be crucial for group specificity because this motif was absent in aptamers specific for OTC as reported in our previous studies. Based on our current and previous studies on OTC-specific and TGS-aptamers, respectively, we propose that GGTGTGG and its truncated motifs that were found common in both OTC-specific and TGS-aptamers might be required for binding to the common TET backbone, that is , at carbon atoms 10, 11, 12, and 1. Whereas T/GTGCT found only in TGS-aptamers was probably required for binding on the other face of TET nucleus, such as carbon atoms 7, 6, 5, and 4.

Finally, with the help of modified selection process it was possible to decipher the ssDNA aptamers that bind to structurally related TCs, which find application in target drug-delivery and detection in pharmaceutical preparations, contaminated food products, such as meat, milk, and environmental samples.

## 5. Experimental

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

Tosylactivated magnetic beads (TMB) (M-280, Dynal Biotech ASA, Norway) were immobilized with OTC, TET, or DOX by covalent binding as described previously.[33,37] Briefly, covalent attachment of amino groups in OTC, TET, or DOX (10 μmol/mL) was allowed to react overnight with approximately $2 \times 10^9$ Tosylactivated beads in borate buffer (0.1 M, pH 9.5) at 37 °C by mild shaking. The target-coated beads were washed and the free/unoccupied groups were blocked by incubation of beads with ethanolamine (1 M, 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 phosphate buffered saline (PBS) buffer, pH 7.4, and stored at 4 °C until use. The unbound targets were estimated by HPLC (Waters, USA) as previously described[38] using the following solvent system: acetonitrile:methanol: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 was performed for counter selection.

### 5.2. In vitro selection

A random ssDNA library of 11.25 μg or $\sim 10^{15}$ molecules with the following sequence: 5′-CGTACGGAATTCGCTAGC-$N_{40}$-GGATCC-GAGCTCCACGTG-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-CGT ACGGAATTCGCTAGC-3′ and primer APTR: 5′-CACGTGGAGCT CGGATCC-3′. Selection of aptamer, elution, and amplification during SELEX process and separation of dsDNA to ssDNA was performed as described previously.[37] Briefly, the target chemical-coated magnetic beads were washed several times with binding buffer containing NaCl (100 mM), Tris–HCl (20 mM, pH 7.6), $MgCl_2$(2 mM), KCl (5 mM), $CaCl_2$(1 mM), and Tween 20 (0.02%) before each round of SELEX process.[33] The Random DNA library pool was dissolved in binding buffer and denatured 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 ($2 \times 10^8$ OTC-coated beads or 3 mg/100 μL) were suspended in 200 μL binding buffer, and $10^{15}$ molecules of denatured random ssDNA 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 tosylactivated magnetic beads, the beads-DNA complex was incubated in 200 μL elution buffer containing Tris–HCl (40 mM, pH 8.0), EDTA (10 mM), urea (3.5 M), and Tween 20 (0.02%) at 80 °C for 10 min with mild shaking as reported previously by Stoltenburg et al.[33] 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 ssDNA fraction was amplified, and the dsDNA was separated to ssDNA as described previously.[34] After the first selection round, the whole ssDNA fraction obtained from the previous round was applied in the next selection round as starting DNA pool for enrichment using the target-coated TMBs. Counter selection steps were introduced to avoid the undesired ssDNAs after every two SELEX rounds using naked magnetic beads, TMB coated with ethanolamine, TET, or DOX, respectively.

The SELEX process was modified by splitting into two parts: (a) part-1 composed of SELEX process using OTC as a primary target coated on TMB for initial four selection rounds during which counter selection was performed using naked beads and beads coated with ethanolamine. After the last round (after fourth SELEX round) with OTC-TMB, the ssDNA fraction was subjected to counter selection by incubating the ssDNA fraction with TET-TMB for 30 min at 25 °C and the unbound ssDNA fraction (specific to OTC but not TET) was eliminated. Only the TET-bound fraction was subjected to further SELEX rounds; (b) part-2 composed of SELEX process with TET-bound ssDNA fraction obtained from part-1 using TET-coated TMB. After two selection rounds on TET-TMB, a counter selection was performed using DOX-coated TMB to eliminate the ssDNA fraction that binds strongly to DOX than TET. The ssDNA fraction which only binds to TET was subjected to further selection rounds and the selection process continued till a maximum recovery of ssDNA fraction was achieved. Eluted ssDNA fraction after every selection round was calculated as the percent ssDNA recovered from the initial ssDNA pool added. The concentration of ssDNA or dsDNA was measured by using Nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies, Inc.).

### 5.3. Cloning and sequencing

The aptamer pool after eight rounds of SELEX was given four more SELEX rounds using TET-coated TMB to attain high recovery rate. The recovered ssDNA pool after the last SELEX round was amplified using unlabeled primers and cloned in *Escherichia coli* with a cloning kit (TOPO TA, Invitrogen).[33] Plasmid DNA from the selected positive clones was purified by miniprep kit (Qiagen), and the aptamer inserts was sequenced (Genotech Inc., Korea).[33] Analysis of secondary structure with several aptamers was performed by free energy minimization algorithm according to Zuker[39] using the internet-tool *m*-fold (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). Pairwise alignment and sequence match between TGS-aptamers and previously reported OTC-specific aptamers[34] were performed by a web tool Emboss::needle (global) program.[40]

### 5.4. Binding assays

Sequenced aptamer clones were tested for their individual-binding characteristic to free TET. Binding assays were performed by equilibrium filtration method as previously described.[41] A series of ssDNA aptamer concentrations ranging from 0 to 2 μM were dissolved in binding buffer (200 μL) reaction mixture and incubated at 25 °C for 30 min. The mixture was loaded onto YM10 Microcon filter columns (Amicon) and centrifuged for 8 min at 12,000*g*,

allowing approx. 100 μL of solution to flow through the membrane similar to equilibrium dialysis; the solution that remained above the molecular weight cutoff membrane contained free TET, free ssDNA, and ssDNA-bound TET, and the filtrate contained only TET, an equivalent concentration of the free TET in the initial solution. An 80 μL filtrate sample was subjected to spectrophotometric analysis and partially to HPLC analysis to determine the concentration of TET.

### 5.5. Determination of dissociation constants ($K_d$)

To calculate the dissociation constants, the percent of bound TET versus 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, and $K_d$ is the dissociation constant.[42]

### 5.6. Determination of specificity of aptamers

Selected aptamers were tested for their ability to bind OTC/TET/DOX. Specificity assays was conducted using fresh aliquots of $2 \times 10^8$ TMBs (3 mg/100 μL) coated with OTC/TET/DOX as described above. The chemical-coated beads were washed five times with binding buffer and suspended in 200 μL of the same buffer containing 1 μg of aptamers. The unbound ssDNA was washed three times with binding buffer and pooled. The target-bound ssDNA was eluted with 200 μL of elution buffer thrice after the 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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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.06.033.

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