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Isolation and characterization of enantioselective DNA aptamers for ibuprofen

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ABSTRACT

Single stranded DNA aptamers that can bind to ibuprofen, a widely used anti-inflammation drug, were selected from random DNA library of 10^{15} nucleotides by FluMag-SELEX process. Five different sequences were selected and their enantioselectivity and affinity were characterized. Three out of five aptamer candidates did not show any affinity to (*S*)-ibuprofen, but only to racemic form of ibuprofen, suggesting that they are (*R*)-ibuprofen specific aptamers. Another two aptamer candidates showed affinity to both racemic form and (*S*)-ibuprofen, which were considered as (*S*)-ibuprofen specific aptamers. The affinity of five ssDNA aptamers isolated was in a range of 1.5–5.2 μM. In addition, all of these five aptamers did not show any affinity to analogues of ibuprofen in its profen's group (fenoprofen, flubiprofen, and naproxen) and the antibiotics of oxytetracycline, another control.

1. Introduction

The demands for separation of chiral compounds are continuously increased in fine chemical/biological and pharmaceutical industry. One of the main concerns in chiral separation is that each enantiomer of a racemic drug may have different pharmacokinetic and pharmacodynamic effects due to their differential metabolism. Consequently, only one enantiomer of a chiral drug shows therapeutic effect, whereas another enantiomer is inactive or toxic. Based on the different effects of chiral compounds on human health, the U.S. Food and Drug Administration in 1992, issued a guideline for chiral drugs in that the chiral drug have to be produced as single enantiomer having therapeutic effect or pass a stringent toxicology test. Therefore, the industries are in the need to develop new technologies for effective separation of chiral compounds.

High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are most commonly used techniques for the separation, purification, and analysis of chiral molecules. To date, various types of chiral selectors have been developed for chiral stationary phases (CSPs) in HPLC and CE. The commonly used chiral selectors for generation of CSPs are amino acids,[1] proteins,[2] crown ethers,[3] and oligo- and polysaccharides.[4] However, these conventional CSPs have a low resolution because they are not specifically generated for individual target chiral compound. To develop target specific chiral selectors, antibodies,[5,6] and molecular imprinted polymers[7,8] have been adapted. However, both of MIP and antibody-based CSPs still have not showed sufficient enantioselectivity and they are limited to only certain targets like non-immunogenic compounds.[9,10]

Aptamers are single stranded DNA or RNA that can bind to various targets such as proteins, peptides, amino acids, cells, and even small compounds with high affinity and specificity.[11,12] As a rival of antibodies, aptamers have been actively studied for various applications, that is, therapeutics, medical diagnostics, biosensors, and analytical systems. Especially, aptamers have a great potential as analytical tools for separation or purification of proteins and small molecules.[13] In compared to an antibody-based immunoaffinity system, which is the most popular format in chromatography devices, aptamers could have several merits over antibodies. Aptamers can be easily immobilized in column densely without loss of binding ability. They are stable against temperature, pH and other factors, and the conditions for elution are not harsh which is attractive for maintaining the columns properly. Moreover, aptamers can be chemically synthesized without a batch-to-batch variation. Aptamers can also discriminate specific target molecules from their derivatives, as demonstrated in previous reports such as a theophylline binding aptamer which showed a 10,000-fold higher binding capacity against caffeine (difference of only a methyl group),[14] by an L-arginine binding aptamer that has a 12,000-fold strong affinity compared to D-arginine.[15] With these advantages, several DNA or RNA aptamers have been incorporated in analytical instruments and used for the separation, purification, and quantification of proteins or small molecules.[16–18]

Since the high enantioselectivity of RNA aptamers against L-arginine was firstly reported by Geiger et al.,[15] DNA or RNA aptamers have been emerged as a new class of target specific chiral selectors in CSPs.[19] Peyrin and co-workers have firstly demonstrated that D-enantiomer of peptide could be separated using HPLC which was packed with beads coated with enantioselective

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aptamers.[20] Besides for peptides, resolutions of CSPs coated with aptamers were competitive or significantly higher than one of MIP based CSPs for D-adenosine and L-tyrosinamide, respectively.[21] In addition, aptamer-based CSPs for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD)[22] and D-histidine[23] were also reported. Recently, Sawai and co-workers firstly isolated the enantioselective DNA aptamers for chiral drug of (*R*)-thalidomide.[24]

Ibuprofen, one of the representative non-steroidal anti-inflammatory drugs (NSAIDs), is used for arthritis, primary dysmenorrhea, fever, and as an analgesic, especially where there is an inflammatory component. Due to its chirality, there are two enantiomers of ibuprofen which are showing different physiological effects. It was already known that only (*S*)-ibuprofen is the active. Over-the-counter (OTC) ibuprofen is currently produced as cost competitive racemate formulation in the market due to the existence of isomerase (2-arylpropionyl-CoA epimerase).[25] However, only (*R*)-ibuprofen is teratogenic (especially in the first trimester) and hence is not recommended for use during pregnancy.[26] Even though the pure (*S*)-isomer is not widely available due to the cost and difficulty of production, the use of single enantiomer can reduce the isomeric ballast of chiral drugs and is more consistent in terms of its content of active substance, considering that the effectiveness of the epimerase can vary.

In this study, therefore, we isolated DNA aptamers that could bind to (*R*)- or (*S*)-ibuprofen from random ssDNA library consisting about 10^{15} different sequenced nucleotides using magnetic beads coated with racemic ibuprofen in a FluMag-SELEX approach.[27] The enantioselectivity of selected aptamers were investigated with racemic and (*S*)-isomer of ibuprofen which are only commercially available, and the specificity for structurally similar other non-steroidal anti-inflammatory drugs (fenoprofen, flubiprofen, and naproxen) and antibiotics (oxytetracycline), another control, was also examined (Fig. 1).

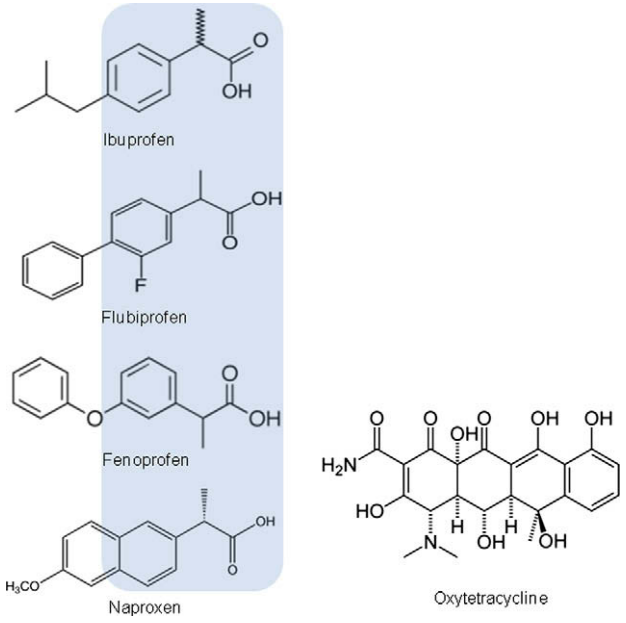

Figure 1. Structure formula of ibuprofen, its analogues in profen's group (fenoprofen, flubiprofen, and naproxen) and oxytetracyline.

2. Results and discussion

2.1. Selection of ibuprofen specific DNA aptamers

DNA aptamers binding to (*R*)- or (*S*)-ibuprofen were isolated simultaneously from a random ssDNA library consisted of 10^{15} nucleotides using magnetic beads immobilized with racemic form of ibuprofen. For each selection step, approximately 0.72 μmol of ibuprofen coated magnetic beads were incubated with ssDNA library. In the first round of selection, around 1.4% of the initial ssDNA library was selected via the magnetic separation after the selection step. The percentage of ssDNA binding to ibuprofen from DNA pool was significantly increased with increasing the SELEX round (Fig. 2). Before the fifth round of selection, the counter selection was performed by using bare magnetic beads. As a result, around 56 % of ssDNA, which might be adsorbed on the surface of magnetic beads, was excluded. After the eighth round of selection, one more counter selection with structurally similar anti-inflammation drugs of fenoprofen, flubiprofen and naproxen was performed to improve the specificity of aptamers for ibuprofen. In this counter selection, approximately 22%, 23% and 13% of ssDNA was eliminated by magnetic beads coated with fenoprofen, flubiprofen, and naproxen, respectively. After the ninth SELEX round, ssDNA showing high affinity to ibuprofen was dominated in DNA pool with 97 % of elution yield. After the tenth round of

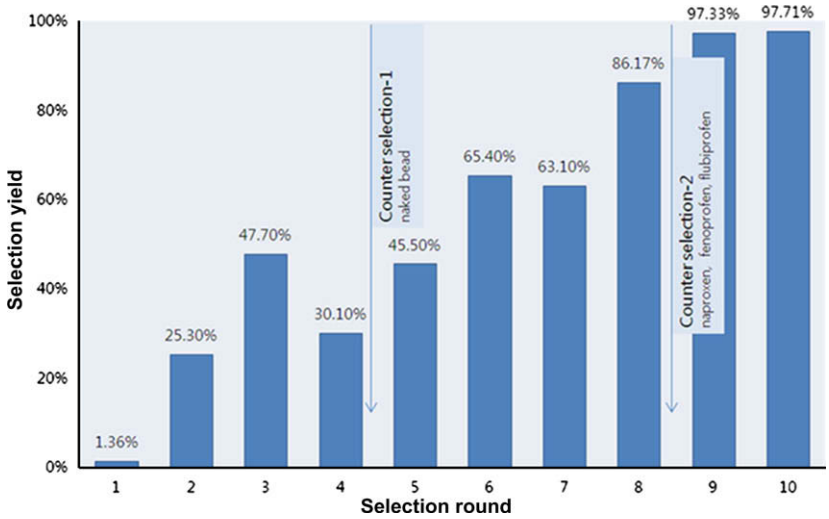

Figure 2. Recovery yield of eluted ssDNA from the DNA pool in each selection round. Counter selection was performed using non-coated magnetic and structurally similar non-steroidal anti-inflammatory drug (fenoprofen, flubiprofen, and naproxen) after fourth and eighth round of the selection, respectively.

selection, eluted ssDNA was amplified with non-modified primer set and cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit. Then, total 17 positive colonies were picked up and the plasmid DNA from individual colonies was purified, followed by selected ssDNA inserted in plasmids were sequenced. In the result of sequencing, 12 nucleotides had an identical sequence and one nucleotide contained 56mer which is relatively short compared to the length of normal selected ssDNA. Consequently, five nucleotides finally selected were further examined in the following experiments including binding assays.

Table 1
Sequence analysis of selected ssDNA

Aptamer	Sequences of N40 random region (5′ to 3′)
IBA2	ACAGTAG**TG**A**GG**GGTCCGTCGTGGGGTAGTTGG**GTCGT**GG
IBA8	GCGAACGACTTCATAAAATGC**TA**T**A**A**GG**TTGCCCTCTGTC
IBA12	GGATCGGCGACGTGGGT**GTCGTG**ATTCGGGGTG
IBA4	CC**ACAG**ACCCTTAGC**TTTCCTAT**TATTCTGCGCGACGCTG
IBA17	**ACAG**CGTGGGCGGTGTCGGAT**TTTCGTAT**GGATGGGGATG

The sequences of five different nucleotides were analyzed by multiple sequence alignment program DIALIGN. Sequences are divided into two groups, and bold characters are the conserved regions of each groups.

2.2. Sequence and structure analysis

Sequences of five different nucleotides were analyzed by multiple sequence alignment program DIALIGN and listed in Table 1. As a result, there are divided into two groups of sequence, IBA2, 8 and 12, and IBA4 and 17, based on the consensus sequences in central random region. They have different conserved regions bolded in the Table 1. This result is well correlated to binding assays for enantioselectivity analysis of selected ssDNAs following. It is expected that these conserved regions are considered to be the binding sites of aptamers to ibuprofen, based on in numerous previous reports.[28–30]

Secondary structures of these five sequences (IBA2, 4, 8, 12, and 17) were predicted by web-based MFOLD program (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi), which is based on free energy minimization algorithm. Typical stem and loop motifs, which are

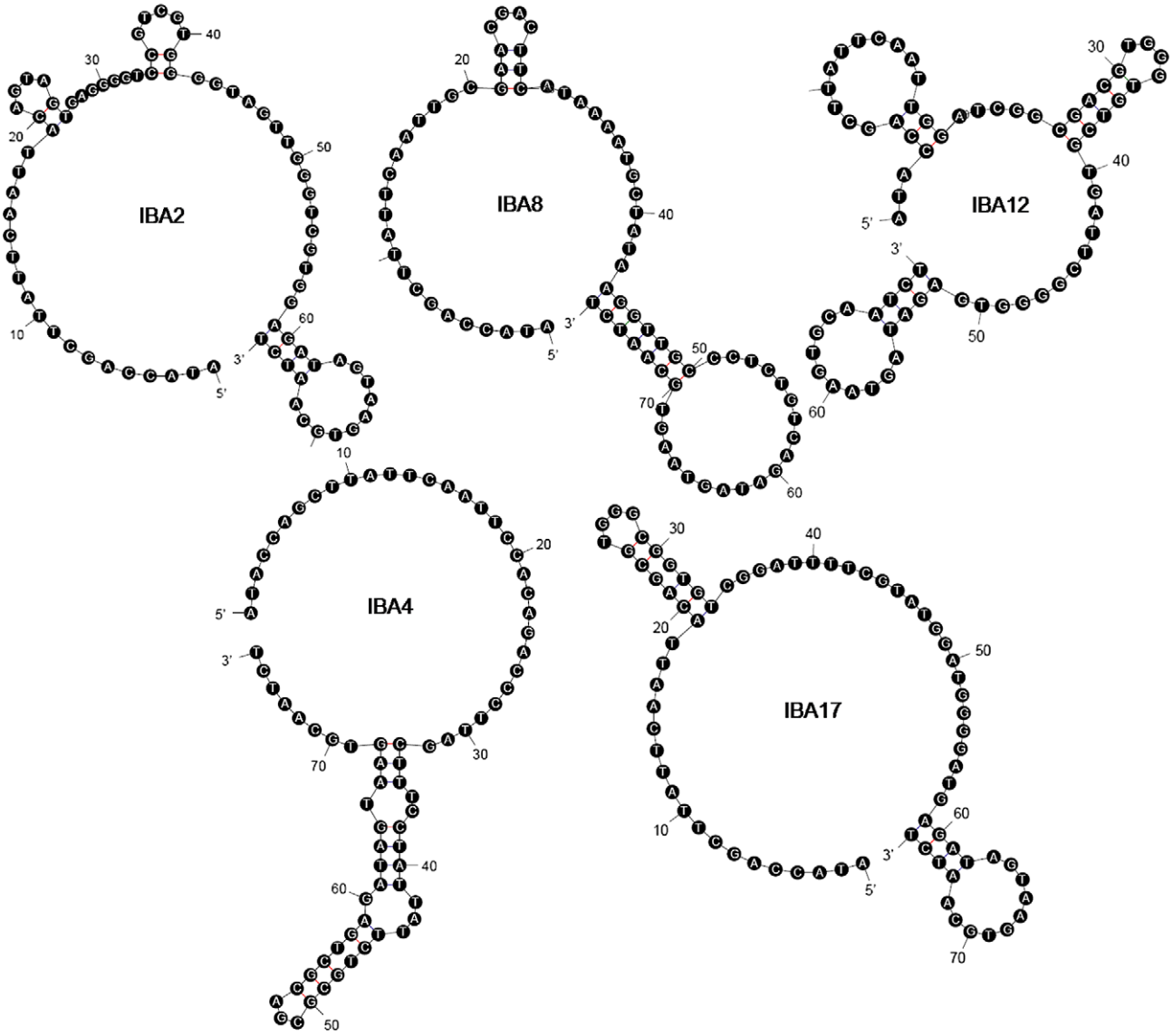

Figure 3. Secondary structures of selected aptamers. The secondary structures of these five sequences (Nos. 2, 4, 8, 12, and 17) were predicted by MFOLD program based on free energy minimization algorithm.

distinct in each sequence, can be seen in their secondary structures (Fig. 3). It was observed that two different consensus sequences formed significant stem-loop structures. These five individual ssDNA were used for further characterization studies.

2.3. Determination of enantioselectivity, specificity, and affinity

To determine enantioselectivity of five selected ssDNAs, the binding assay of individual ssDNA was performed with the both of racemic mixture and (*S*)-enantiomer of ibuprofen. Results of the binding assay were divided into two groups. One group including IBA2, 8, and 12 showed the affinity to only racemic mixture of ibuprofen, but not to (*S*)-ibuprofen at all (Fig. 4). This result means that three of IBA2, 8, and 12 ibuprofen specific aptamers are binding to only (*R*)-ibuprofen with high enantioselectivity. The dissociation constants and of IBA2, 8, and 12 were estimated to 3.0, 5.2, and 3.2 μM, respectively. However it presumes that the real affinity of them against (*R*)-ibuprofen is two times higher than the calculated K_d value for racemic ibuprofen, because only half concentration of (*R*)-ibuprofen is existed in racemic sample of ibuprofen. The other group of ssDNAs including IBA4 and 17 showed the binding ability to both of racemic mixture and (*S*)-enantiomer of ibuprofen, but their dissociation constants (K_d) for (*S*)-ibuprofen were twofold higher than the affinity to racemic ibuprofen (Fig. 5). This result, therefore, indicates that IBA4 and 17 ssDNAs are highly enantioselective DNA aptamers for (*S*)-ibuprofen. Considering the (*S*)-ibuprofen concentration in racemate sample, this can be explained how these results were obtained from the above conclusion. The dissociation constant of IBA4 and 17 for (*S*)-ibuprofen were estimated to 1.5 and 3.8 μM, respectively. The result of distinct enantioselectivity of two aptamer groups is well correlated with the sequence analysis data. IBA4 and 17, (*S*)-ibuprofen specific DNA aptamers, have their unique homologous sequences which were not found in the sequences of (*R*)-ibuprofen specific DNA aptamers, IBA 2, 8, and 12. In contrast, the conserved sequences in (*R*)-ibuprofen specific IBA 2, 8, and 12 were also not found in the sequences of (*S*)-ibuprofen specific IBA4 and 17.

The K_d value of five ibuprofen binding DNA aptamers are in a range of 1.5–5.2 μM. These values are similar or slightly higher than that of aptamers for other small molecular targets. One possible reason for the low affinities of five selected ibuprofen binding DNA aptamers is that a hydrophobic ibuprofen must not be attractive to interact with strong hydrophilic ssDNAs during SELEX procedures. Generally, the affinities of aptamers for hydrophobic chemicals are lower than that of hydrophilic molecules. To overcome this problem, the method of attaching non-polar functional groups to the oligonucleotides was suggested for the isolation of aptamers against hydrophobic targets.[9]

The specificity of five identified DNA aptamers was also examined with the racemic form of three structurally similar non-steroidal anti-inflammatory drugs (fenoprofen, flubiprofen, and naproxen), and one of representative antibiotics, oxytetracycline, as another control. Figures 4 and 5 showed the results of binding assays that verified the high specificity of both (*R*)- and

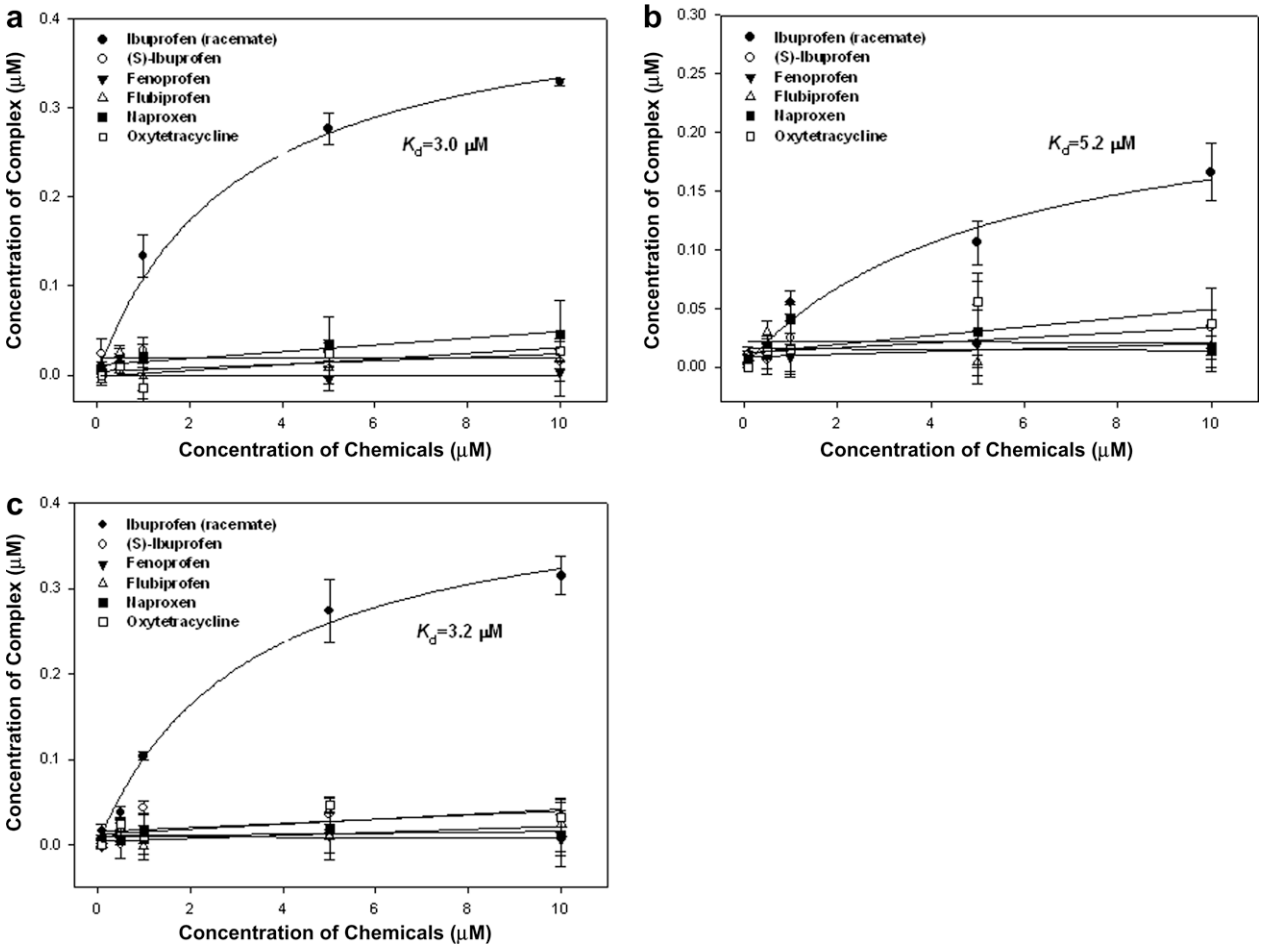

Figure 4. Binding assays for IBA2 (a), IBA8 (b) and IBA12 (c). These DNA aptamers showed the affinities only to racemic mixture of ibuprofen, but they did not bind to (*S*)-ibuprofen and other counter compounds.

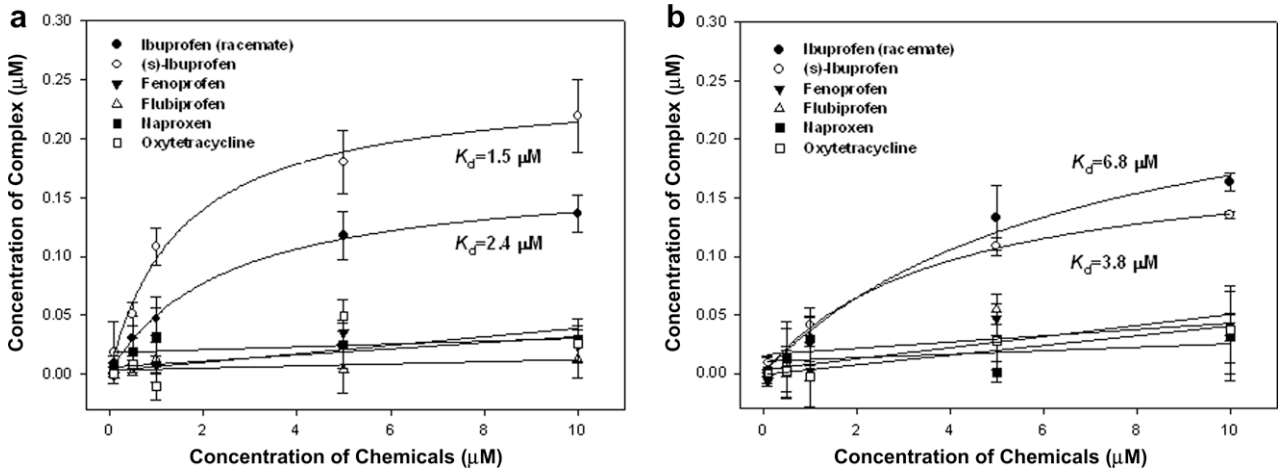

Figure 5. Binding assays for IBA4 (a) and IBA17 (b). These DNA aptamers showed the affinity to both racemic mixture and of (*S*)-enantiomer of ibuprofen, but the dissociation constant (K_d) of IBA4 and 17 for (*S*)-ibuprofen was twofold higher than the affinity for racemic ibuprofen.

(*S*)-ibuprofen enantioselective DNA aptamers. As a result, any meaningful interactions between all of aptamers and four reference chemicals were observed little. The enantioselective ibuprofen binding DNA aptamers are useful to develop the aptamer-based analysis system for the pharmacokinetic study of ibuprofen in the body. And these aptamers can also be applied to develop the model system of aptamer-based chiral stationary phases (CSPs) for the separation, purification, and analysis of any chiral compounds, because DNA or RNA aptamers have been reported for only a few chiral peptides or small compounds so far.

3. Conclusions

In this study, the five ibuprofen specific DNA aptamers, which have high enantioselectivity for both (*R*)- or (*S*)-ibuprofen, were successfully identified simultaneously. The three of them (IBA 2, 8, and 12) are the (*R*)-ibuprofen specific DNA aptamers and another two DNA aptamers (IBA4 and 17) can bind to only (*S*)-ibuprofen specifically. The affinities of five enantioselective DNA aptamers selected were ranged in a few micro-molar level and these DNA aptamers showed high specificity to both (*R*)- or (*S*)-enantiomer, but little affinities to three structurally similar non-steroidal anti-inflammatory drugs in profen's group (fenoprofen, flubiprofen, and naproxen), and a representative antibiotics of oxytetracycline.

4. Experimental

4.1. Materials and apparatus

The racemic- and (*S*)-ibuprofen as target molecules and racemic form of fenoprofen, flubiprofen and naproxen, structurally similar non-steroidal anti-inflammatory drugs (NSAIDs), and oxytetracycline were purchased from Sigma–Aldrich Co. Figure 1 showed the molecular structure of all these chemicals. The HPLC grade of acetonitrile, methanol and water were supplied for HPLC analysis from J. T. Baker Inc. A magnetic bead (Dynabeads, M-270 Amine) used for FluMag-SELEX process was purchased from Invitrogen Co. The 4.5 mm diameter of MBs has reactive primary amino-groups on surface for coupling with targets and the stock concentration of beads in an aqueous suspension is around 2×10^9 beads/ml (approx. 30 mg/ml).

Concentrations of all chemicals were analyzed using HPLC (Waters) under following conditions; 10 min of running time and 1 ml/min of flow rate using reverse phase C18 column (4.5 mm × 250 mm). Two different compositions of distilled water (pH 3)/acetonitrile = 34:66; methanol/acetonitrile/oxalic acid = 15:15:70 were used as a mobile phase for chemicals in profen's group and oxytetracycline, respectively. The excitation and emission wavelength of fluorescence detector for each chemical were as follows; ibuprofen: E_x/E_m = 225/290 nm, fenoprofen: E_x/E_m = 280/320 nm, flubiprofen: E_x/E_m = 250/315 nm, naproxen: E_x/E_m = 280/350 nm, oxytetracycline: E_x/E_m = 380/520.

4.2. Random DNA library and primer set

A random ssDNA library of 10^{15} nucleotides was chemically synthesized and purified by PAGE. This ssDNA pool consisted of a central random region of 40 nucleotides flanked by two different constant 18 nucleotides-sequences at 3′ and 5′ end which are primer binding regions for amplification: DNA library; 5′-ATACCAGCTTATTCAATT-N_{40}-AGATAGTAAGTGCAATCT-3′ (76mer).[27] The forward and reverse primers were modified with fluorescein and poly adenine tail, respectively, which are for the clear separation of single stranded DNA from amplified double stranded PCR products: forward primer (FP); 5′-fluorescein-ATACCAGCTTATTCAATT-3′ (18mer), reverse primer (RP); 5′-poly-dA_{20}-HEGL-AGATTGCACTTACTATCT-3′ (18mer), in which the hexaethyleneglycol ($C_{13}H_{28}O_7$, HEGL) in reverse primer was inserted as a spacer to block the extension of the amplification of poly adenine region. Unmodified forward and reverse primer was also used for PCR amplification and cloning after final round of the selection. All of initial DNA pool and primers were obtained from Genotech Inc., Korea and used after dissolving them in distilled water.

4.3. In vitro selection

Aliquots of 1×10^9 amine activated magnetic beads were washed three times with 500 μl of 0.1 M PBS buffer (pH 6.5) and 5 mg/ml of racemic ibuprofen in same buffer (500 μl) was mixed with beads solution. To make a covalent bonding between primary amine on bead surface and carboxylic group of ibuprofen, 200 μl of EDC (*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide) (10 mg/ml) was added and well mixed for 2 h by mild shaking at room temperature. After reaction, unbound ibuprofen was separated using magnet and the concentration of them was analyzed by HPLC (Waters). Then, 1 M of hydroxylamine (pH 8) was added to MB solution and incubated for 30 min at room temperature to block open activated amino group on magnetic beads. Finally, the

ibuprofen coated magnetic beads were washed and resuspended with 0.1 M PBS buffer (pH 6.5), and stored at 4 °C until use. Fenoprofen, flubiprofen, and naproxen were also immobilized on magnetic beads for further counter selection through same procedure. 2×10^8 of ibuprofen coated magnetic beads were washed five times with a binding buffer (100 mM NaCl, 20 mM Tris–HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02% Tween 20, pH 7.6) before each round of selection.[27] Each ssDNA pool was heated to 90 °C for 10 min and quickly cooled. After keeping the mixture at 4 °C for 15 min and the sample was incubated 8 min at room temperature before mixing it with magnetic beads. In the first selection round, $\sim 10^{15}$ sequences of initial random ssDNA library was added to the magnetic beads suspension and incubated for 30 min at room temperature with mild shaking. After the binding reaction, unbound oligonucleotides were removed by five washing steps in the binding buffer. To elute bound ssDNAs from ibuprofen coated magnetic beads, the magnetic beads suspension capturing oligonucleotides were resuspended in a 200 μl elution buffer (40 mM Tris–HCl, 10 mM EDTA, 3.5 M urea, 0.02% Tween 20, pH 8.0) and incubated at 80 °C for 10 min with mild shaking.[27] The ssDNA was purified from magnetic beads suspension by magnetic separation. This elution step was repeated three times because the stringent elution was required to recover ssDNAs which were strongly bound to ibuprofen. The eluted ssDNAs were purified by the ethanol precipitation in the presence of glycogen as a carrier and the purified ssDNAs were finally dissolved in a 10 μl of EB buffer (10 mM Tris–Cl, pH 8.5). Concentration of the eluted ssDNA was measured by ND 1000 spectrophotometer (Nanorop, Thermo Fisher Scientific Inc.).

The selected ssDNA was amplified by parallel PCR reactions. Each 50 μl of PCR mixtures contained 25 μl PCR master mix solution (HotStarTaq, Qiagen), 2.5 μl of modified forward and reverse primer (10 μM), and an appropriate amount of template ssDNA (100–200 ng). PCR conditions were initial heat activation at 94 °C for 5 min and 25 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. By this amplification, double stranded PCR products were labeled with a fluorescein at the 5′ end of the original strand and a poly-dA20 extension at the 5′ end of the complementary strand. After the enrichment of DNA, purity and size of the PCR product was confirmed by a gel electrophoresis using a 2.0% agarose gel. Then, all PCR products were purified by MinElute Kit (Qiagen) and were dissolved in EB buffer. It was necessary to separate relevant DNA strand from dsDNA PCR products after the amplification step for the next round of selection. For this purpose, denaturing PAGE containing 12% acrylamide, 7 M urea, 20% formamide in TBE buffer was performed as described previously.[31] Before loading the samples, the purified PCR products were denatured by heat treatment at 95 °C for 10 min and quickly cooled on ice. The fluorescence labeled strand was verified in the gel by observing fluorescence of the labeled fluorescein. Relevant DNA fragments (fluorescent band) were cut out and crushed with 1 ml Crush and Soak solution (500 mM NH_4OAc, 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 the ethanol precipitation. The concentrations of ssDNAs or dsDNAs were measured by using the Spectrophotometer. Then, fluorescein-labeled ssDNA pool was ready for the next round of selection.

To exclude non-specifically absorbed ssDNAs on the surface of magnetic beads and improve the specificity of the selected aptamers, two round of counter selection were performed using naked magnetic beads and fenoprofen, flubiprofen, naproxen, oxytetracycline coated magnetic beads after fourth and eighth selection rounds, respectively. These selection and amplification were repeated when the ibuprofen specific ssDNA were dominated in DNA pool.

4.4. Cloning and sequencing of selected DNA

After 10 SELEX rounds, when ssDNAs bound to ibuprofen were dominated in a DNA pool, selected ssDNAs were amplified with non-modified primer set and cloned into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen). Positive colonies were picked and plasmid DNAs were purified by miniprep kit (Qiagen), followed by the plasmids with aptamer inserts were sequenced (Genotech Inc., Korea). Sequences of selected ssDNAs were analyzed using multiple sequence alignment program DIALIGN.[32] Analysis of secondary structures with several aptamers was performed by free energy minimization algorithm using the Internet-tool MFOLD.[33]

4.5. Binding assays

The enantioselectivity, affinity and specificity of selected nucleotides for each isomers of ibuprofen were analyzed by equilibrium-filtration method using Microcon filtration column (YM10, Millipore Co.). Selected ssDNA samples (1 μM) were added to the racemic ibuprofen in the 50 μl of binding buffer at a final concentration of 0.1, 0.5, 1, 5, 10 μM. Each mixture was incubated for 30 min at room temperature. Then, the mixture was poured into a Microcon 10 filtration unit and centrifuged for 20 min at 13,000 rpm to separate the unbound ibuprofen from inbuprofen–aptamer complexes and free aptamers through the membrane. To compensate chemisorption of ibuprofen on the column wall and cellulose membrane, equivalent concentration of racemic ibuprofen solution without selected ssDNA was passed through the same procedure and then the difference between two samples was estimated as the amount of ibuprofen to only selected nucleotides. Affinities of isolated sequences against (*S*)-ibuprofen and other structurally similar NSAIDs (fenoprofen, flubiprofen and naproxen) and OTC were examined under the same process for the analysis of enantioselectivity and specificity, respectively. Concentrations of all unbound chemicals in individual filtrated solution were analyzed by HPLC. To estimate the affinity (K_d) of selected ssDNA for each chemicals, the concentration of aptamer–target complex versus added chemical concentration was plotted. These data showing saturation curve was fitted by a non-linear regression analysis and the dissociation constant was calculated by Sigmaplot 10.0 software.[34]

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