In Vitro Selection of a DNA Ligand that Selectively Binds to the Anticancer Agent Methotrexate

Yoshihiro Ito,* Akinori Suzuki, Naoki Kawazoe, and Yukio Imanishi

Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-01

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A DNA ligand that selectively binds to the anticancer agent methotrexate was identified using the *in vitro* selection method. A pool of single-stranded DNAs consisting of random sequences was incubated with a methotrexate-immobilized agarose gel; bound DNAs were then collected and amplified using the polymerase chain reaction (PCR). This selection process was repeated six times. The selected DNAs were cloned and were found to contain two common sequences. One of these selected DNA clones selectively bound to the methotrexate-immobilized gel and showed a high level of discrimination against the gel immobilized with folic acid, which has a similar chemical structure to methotrexate. In the selection process, a buffer solution was used for DNA binding to the methotrexate-immobilized gel and water was used for elution from the gel. Ultraviolet absorption measurement showed that the conformation of the DNA ligand in the binding buffer was different from that in elution water.

Oligonucleotide ligands that exhibit high-affinity specific binding to target molecules have been reported.[1,2] The isolation methodology of the ligands is termed as systematic evolution of ligands by exponential enrichment (SELEX), *in vitro* selection, or *in vitro* evolution. RNA ligands that bind to various target molecules such as amino acids, bases, nucleotides, cofactors, and proteins have been identified.[3—5] Single-stranded (ss) DNA ligands that bind to thrombin,[6] organic dyes,[7] and ATP[8] have been isolated using the *in vitro* selection. In addition, we previously used *in vitro* selection to obtain DNA ligands suitable for the patterned staining of material surfaces.[9]

Nucleic acid ligands offer several potential advantages over traditional antibody-based reagents, because the ligands are not derived from living organisms and can be accurately synthesized and reproduced in a short time by automated processes. In the present study, a DNA ligand that selectively binds to the anticancer drug methotrexate was isolated using the *in vitro* selection. Since antibodies to methotrexate are conventionally used as a diagnostic to evaluate the concentration of methotrexate in patient's blood streams, the DNA ligand may potentially eliminate the need for antibodies and the associated disadvantages.

Experimental

Materials: Deoxyribonucleoside triphosphate (dNTP) was obtained from Pharmacia Biotech. Co. (Uppsala, Sweden). AmpliTaq DNA polymerase was purchased from Perkin–Elmer Co. (Foster City, CA). Custom synthesis of DNA was performed by Sawady Technology Co. (Tokyo, Japan).

Gels Immobilized with Methotrexate or Folic Acid: Methotrexate-immobilized agarose gel was purchased from Pierce Co. (Rockford, IL) and used without further treatment. Using absorption data at 380 nm, the amount of immobilized methotrexate was determined to be 4 μmol mL^{-1} gel.

The immobilization of folic acid onto agarose gel was carried out according to Salter et al.[10] Folic acid (2.5 mg) in 0.05 M $NaHCO_3$ (5 mL, 1 M=1 mol dm^{-3}) was mixed with EAH-Spharose 4B (Pharmacia) in the presence of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC, 25 mg). The mixture was stirred at room temperature for 2 h. The unreacted amino groups were then blocked by stirring with sodium acetate (10 mg) in the presence of EDC at room temperature for 2 h. Using 282-nm absorption data, the amount of immobilized folic acid was determined to be 0.7 μmol mL^{-1} gel.

***In vitro* Selection:** *In vitro* selection of the DNA oligomers specific for methotrexate was carried out according to the method of Szostak and coworkers.[7,8] The procedure is shown schematically in Fig. 1. For the initial selection cycle, synthetic 104-mer oligonucleotides with a random insert of 60 nucleotides, 5′-TAGGGAATTCGACGGATCC-N_{60}-CTGCAGGTC-GACGCATGCGC-3′, was amplified using the primers, 5′-TAAT-ACGACTCAACTATAGGGAATTCGTCGACGGAT-3′ (P1) and 3′-GTCCAGCTGCGTACGCGCC-5′ (P2). The synthetic ssDNA (5 μg) was amplified using 30 polymerase chain reaction (PCR) cycles (one cycle: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) in 100 μL of PCR solution [25 U mL^{-1} AmpliTaq DNA polymerase; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 0.02 mM dNTPs; primers P1 (0.5 μM) and P2 (0.5 μM)]. The amplified double-stranded (ds) DNA was purified using a 3% low-melting agarose gel [NuSieve GTG, FMC BioProducts (Rockland, ME)] in order to remove unreacted primers. ssDNA was then obtained from the dsDNA by 45 additional PCR cycles using only the P1 primer. The PCR-ssDNA was purified using the NuSieve GTG and precipitated with ethanol. Five hundred microlitres of the ssDNA pool (6 μg) in a binding buffer (0.5 M LiCl; 10 mM Tris-HCl, pH 7.6; 1 mM $MgCl_2$) was loaded onto the methotrexate-immobilized gel column, incubated for 20 min at room temperature, and subsequently rinsed with 5 mL of the binding buffer (5 column volumes). The bound DNA was eluted using three column volumes of distilled water. The eluted ssDNA was precipitated with ethanol in the presence of 40 μg glycogen, washed with 70% ethanol, and

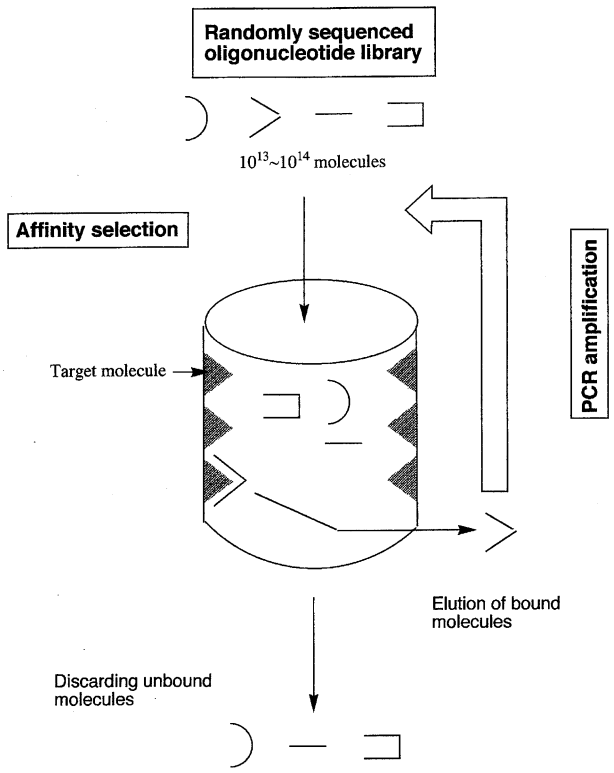

Fig. 1. Schematic illustration of *in vitro* selection. A pool of single stranded DNAs containing random sequences was loaded on column with target molecule-immobilized gel. After binding unbound DNA was discarded. Bound DNA was recovered by elution for subsequent PCR amplification. This process was repeated 6 times.

dissolved in 40 μL of the binding buffer. The ssDNA was amplified using PCR and used as the input DNA for the next selection cycle. This process was repeated six times.

The ability of the DNA strands to bind to the gels immobilized with folic acid or methotrexate was determined by comparing the amount of DNA loaded onto the column with that eluted from the column by the binding buffer. The amount of DNA was monitored by measuring ultraviolet absorption at 260 nm. UV spectra were measured using a UV-vis spectrophotometer Ubest-50 (JASCO, Tokyo, Japan).

Characterization of Selected DNA: The primer regions of the selected DNA strands were digested using the restriction enzymes *Eco*RI and *Bam*HI, and ligated into the pGem3Z vector. The DNA sequences were determined using the dideoxy method. The secondary structure of the ssDNA was predicted using Zuker's method.[11]

Results and Discussion

The percentage of DNA bound to immobilized methotrexate is shown in Table 1. The amount of bound DNA increased with increasing cycles of the selection process.

From the pool of selected DNAs, thirty-five clones were sequenced. Two common sequences were found within the N_{60} region. Among the 35 clones, 12 clones contained either one or both of the common regions. These sequences are shown in Fig. 2.

The binding affinity for methotrexate and folic acid of the DNA strand, hereafter called M1, consisting of both the mtx-01 strand and the primer-binding regions was examined. The M1 DNA ligand was found to selectively bind to methotrexate and showed a high level of discrimination against folic acid, which has a very similar chemical structure to methotrexate (Fig. 3). In a related investigation, Jenison et al.[11] selected an RNA ligand that binds with high affinity and specificity to theophlline, while showing a much lower binding affinity for other chemicals with similar structures. They reported that substitution of methyl groups of theophilline altered the dissociation constant from 10 to 10^3 times. Similarly the M1 DNA ligand is thought to have a complex three-dimensional structure that forms a molecular pocket that recognizes the functional groups such as methyl and amino groups of methotrexate but not those of folic acid.

The secondary structure of the M1 DNA was predicted as is seen in Fig. 4. The regions of common sequences form a

Table 1. Percentage of Bound DNA at Each Cycle

Cycle	DNA bound (%)
1	N.D.
2	N.D.
3	N.D.
4	21
5	—
6	41

N.D. means not detected.

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mtx-01:            AGCGAGCAGCGTGTGGCGAGGTCCTGTGCTTAAGGTGGTCCGCTGAGGGGG
mtx-02:          GTCACGGGCAAC--GTGGTGGACTCATGACCAGGGAGAGAGTGTGTGATGGGTCTTGGGGTC
mtx-03:                                                 TGAGGAGGGGGCACTGGGTTAGAAGCGGGACATGGCCAAGCTAAAGTATTGCT
mtx-04:                                              AGGATGATGGGTGTGATTGTGAATCAGGGTCAGTCGCGGTTAAAGCATGAATGGATGAGA
mtx-05:                                AGGCGTCAGGATGGAGGAACCGGGACGTGGAGCGGTTTTGGGTTATCGGGATGT
mtx-06:                                     AGGTCCGATCCAAATGGCCTAGGGGGTTGGGAGCGTTGGCAGTTTGTGCTG
mtx-07:                                   AGTGGGAGTGAGTGTGGACGGTGAGCGGGGAAGGCACGCGGACCACGGCATTGTATATC
mtx-08:                                                 ATGCCAGGGGAAGGGACGGCAGCGTCAGGCTTGTCTAGTGTGGGCAC
mtx-09: ACCTATGTGCGTAGGTAGCAGCTGGTAGCTGGATACGTTTCGGATCGTGTACCA
mtx-10:            TGCGCGCAGCGGGTGTTGCGGGACGCCGGTGACAATCAGACGGTGGCCAGTGGGTACGAT
mtx-11:              CGTGTAGTGCGTCTGGAGGTTCCGCTCGCGTGGTAAAGCAGTTCGGGTAGCGTGGATCAG
mtx-12:       GTGCTGAAGCACCAGC-GGTGGGGATGTGACGTAGGTAGTGTGGGGAGGAGCCA

TAATACGACTCAACTATAGGGAATTCGTCGACGGATCC-AGCGAGCAGCGTGTGGCGAGGTCCTGTGCTTAAGGTGGTCCGCTGAGGGGG-CTGCAGGTCGACGCATGCGCCG
(M1)
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Fig. 2. Sequences of cloned DNAs having common sequences. Common sequences are underlined. The M1 DNA ligand which consists of mtx-01 and primer regions and which was used for the binding experiment (Fig. 3) is also shown.

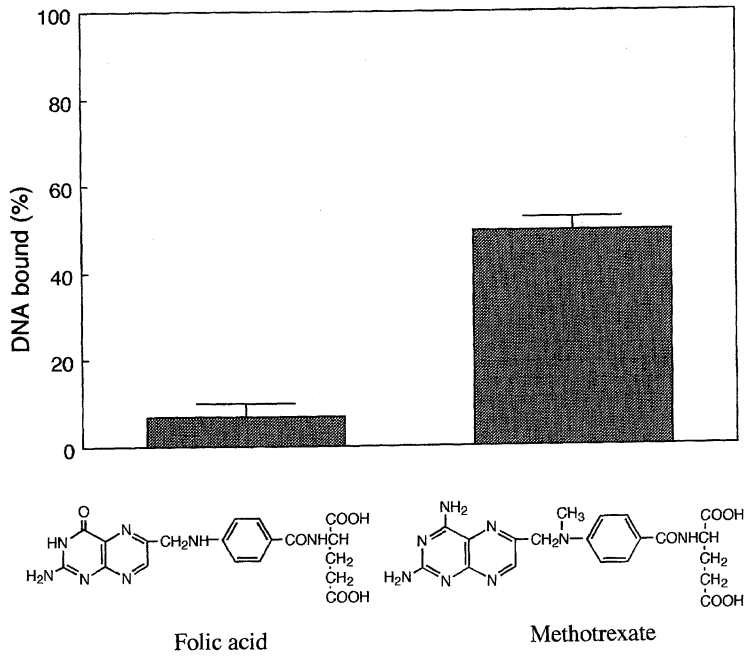

Fig. 3. Binding of DNA ligand M1 to methotrexate- and folic acid-immobilized gels. The amounts of immobilized methotrexate and folic acid in each column are normalized.

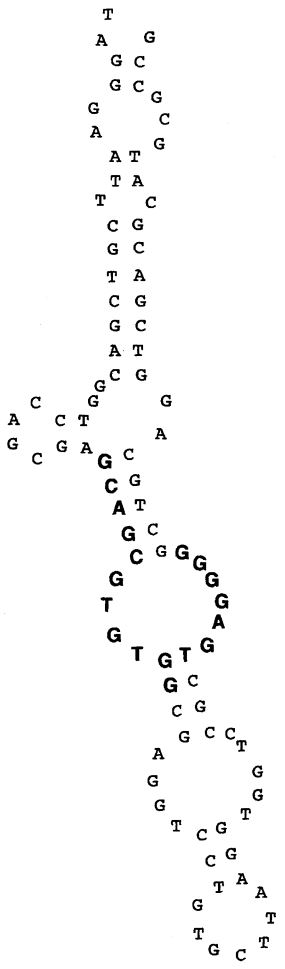

Fig. 4. Possible secondary structure of DNA M1 sequence. Free energy to form the structure was -11.8 kcal mol^{-1}. Bold letters represent common sequences.

bulge loop, that is thought to be a binding pocket that recognizes methotrexate. Other studies have also demonstrated the importance of the bulge loop structure. Sassanfar and Szostak,[13] Giver, et al.,[14] and Famulok and Huttenhofer[15] found bulge loop structures of RNA ligands for ATP, Rev protein of HIV-1, and neomycin, respectively. The structure of an RNA ligand interacting with its target molecule AMP was recently investigated.[16] Although prediction of the three-dimensional structure of RNA or ssDNA is generally difficult,[17] loop structures formed by a single stranded nucleic acid molecule most likely were involved in the formation of molecular recognition sites for target molecules than the rigid helix structure of double-stranded nucleic acid molecules.

In the present investigation, the DNA was bound in binding buffer and eluted using water. Ultraviolet spectra of the M1 DNA ligand shows that the conformation of the ligand depends on the solution that the DNA is dissolved in (Fig. 5a). The absorbance decreased by transferring DNA from water to the binding buffer. Mg^{2+} (1 mM) and Li^{+} (500 mM) had a greater effect on the conformation of M1 DNA than 10 mM Tris-HCl. A high concentration of monovalent ions had the same effect on DNA conformation as the low concentration of divalent ions. The metal ions most likely induced the formation of ordered structure with an increase in base–base stacking.[18,19] A similar spectral change was observed when the ordered structure in the binding buffer was disordered by raising the temperature, as shown in Fig. 5b.

In the present study, a DNA ligand that selectively binds to the anticancer drug was isolated using *in vitro* selection method. Considering the convenient preparation of oligonucleotides, the procedures using the ligand will replace the traditional techniques using antibodies.

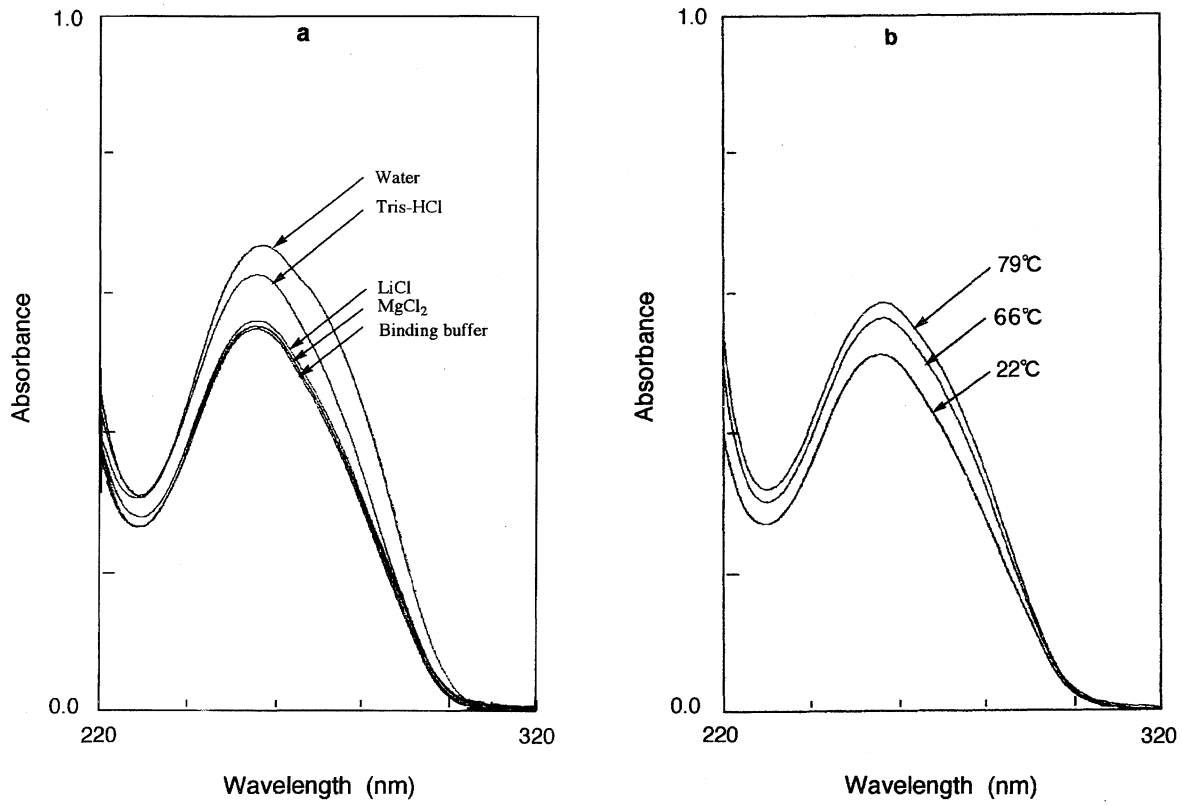

Fig. 5. (a) Ultraviolet spectra of DNA ligand M1 (0.75 μM) in binding buffer [1 mM $MgCl_2$, 500 mM LiCl, 10 mM Tris-HCl (pH 7.6)], in 1 mM $MgCl_2$, in 500 mM LiCl, in 10 mM Tris-HCl (pH 7.6), or in water used for recovery elution. (b) Temperature dependence of ultraviolet spectra of DNA ligand M1 (0.57 μM) in binding buffer.

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