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Modified DNA Aptamer That Binds the (*R*)-Isomer of a Thalidomide Derivative with High Enantioselectivity

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Abstract: A thalidomide-binding aptamer was produced by systematic evolution of ligands by exponential enrichment from a library of non-natural DNA in which thymidine had been replaced with a modified deoxyuridine bearing a cationic functional group via a hydrophobic methylene linker at the C5 position. The additional functional group in the modified DNA aptamer could improve stability against nucleases and increase the binding affinity to thalidomide. The selected aptamer could recognize thalidomide enantioselectively, although a racemic thalidomide-attached gel was used for the selection. Surface plasmon resonance and fluorescence titration studies revealed that the selected modified DNA aptamer and a truncated version bound with an (*R*)-thalidomide derivative with high enantioselectivity, but not with the (*S*)-form. The modified group in the DNA aptamer is indispensable for the interaction with thalidomide, as the corresponding natural type DNA bearing the same base sequence showed no binding affinity with (*R*)- nor (*S*)-thalidomide. Computational sequence analysis suggested that the selected apatamer (108mer) could fold into a three-way junction structure; however, truncation of this aptamer (31mer) revealed that the thalidomide-binding site is a hairpin-bulge region that is a component of one of the arms of the three-way junction structure. The K_d value of the truncated 31mer aptamer for binding with the (*R*)-thalidomide derivative was 1.0 μM estimated from fluorescence titration study. The aptamer that can recognize a single enantiomer of thalidomide will be useful as a biochemical tool for the analysis and study of the biological action of thalidomide enantiomers.

Single-stranded nucleic acids are capable of adopting highly ordered structures, which depending on base sequence bind various target molecules.[1−3] Such a nucleic acid is called an aptamer and can be artificially prepared by SELEX (systematic evolution of ligands by exponential enrichment) or in vitro selection. Various aptamers, recognizing target molecules from huge complex molecules like protein[4−7] to simple organic small molecules such as ATP,[8,9] dyes,[10,11] FMN,[12] or amino acids,[13,14] have been obtained so far. Some aptamers can even discriminate optical isomers of a target molecule such as L-arginine, L-tyrosinamide, and D-vasopressin.[13−16] Thus, aptamers behave similar to antibodies, but are more easily prepared and at a lower cost than an antibody composed of protein. Moreover, by conjugation of additional functions to an aptamer, it is possible to construct a variety of molecular tools, for example, an aptazyme, a conjugate of an aptamer and catalytic nucleic acid, or a molecular sensor, a conjugate of an aptamer and a reporter group.[17−19]

Through in vitro selection, both RNA and DNA aptamers that bind with various target molecules have been isolated. However, they are susceptible to nuclease attack under physiological conditions, and their binding affinity is not satisfactory. If a nonstandard substrate with an additional functional group can be incorporated into DNA enzymatically, application of in vitro selection to the resulting modified DNA could produce a modified DNA aptamer. The modified DNA aptamer, with additional functionality, would have increased stability against nuclease attack and improved binding affinity for its target molecule. However, an aptamer is rarely obtained from modified

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DNA because modified DNA produced by PCR is limited due to the low incorporation efficiency of modified bases.[20,21] To overcome this obstacle, various modified nucleotides have been synthesized, and their substrate specificities to several kinds of DNA polymerases have been studied by a number of groups.[22−28] We reported that some triphosphates of thymidine analogues bearing functional groups at the C5 position could be readily accepted as a substrate for PCR, forming the corresponding modified DNA when using KOD Dash DNA polymerase.[29−31] The modified DNA bears a cationic ammonium group via a hydrophobic hexamethylene linker at the C5 of the thymidine residue. We undertook in vitro selection of thalidomide-binding aptamers using the modified DNA to prepare an aptamer with high binding affinity and nuclease-resistance.

Thalidomide, which has significant physiological activity, has one chiral center. It was used widely as a hypnotic drug, but was withdrawn from the market in the 1960s because of its strong teratogenic activity.[32,33] Recently, thalidomide has come into the limelight again as a potential drug for various diseases such as autoimmune disease, AIDS, and some cancers.[34−40] It attracts great interest due to its unique biological activity and its potential as a lead compound to develop a new biological response modifier.[41,42] The difference in the biological activity between the enantiomers of thalidomide is not still clear, although it has been reported that the biological actions of thalidomide are different for the (*S*)- and (*R*)-isomers.[43−45] Thalidomide is reported to racemize easily under physiological conditions, and specification of the physiological activities of the enantiomers of thalidomide is difficult.[46−48] Thus, an aptamer that binds thalidomide with high enantioselectivity will be useful as a tool for the analysis and biological study of an enantiomer of thalidomide. Herein, we report the selection of a modified DNA aptamer that can recognize thalidomide with high enantioselectivity. Although the selection was carried out using a racemic thalidomide derivative, the selected aptamer clone showed high binding affinity for the (*R*)-form thalidomide but not for the (*S*)-form.

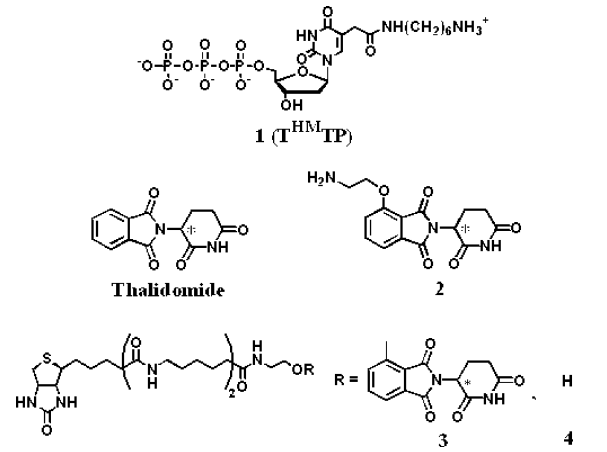

Figure 1. Modified dUTP (**1**), thalidomide, and thalidomide derivatives, **2**, **3**, and **4**, used for in vitro selection.

Results

In Vitro Selection. A DNA aptamer recognizing thalidomide was selected from a library of modified DNA in which a 60-nucleotide random region was flanked by 5′- and 3′-constant primer regions for PCR. The modified DNA library was prepared by PCR with 5-*N*-(6-aminohexyl)carbamoylmethyl-2′deoxyuridine triphosphate (T^{HM} triphosphate, **1**) in place of TTP and using KOD Dash DNA polymerase, as **1** was found to be a good substrate for this polymerase, but not for Taq DNA polymerase[29] (Figure 1).

The terminal protonated ammonium ion, hydrophobic hexamethylene linker, and amido linkage at the thymidine residues in a modified DNA aptamer could all contribute to form a thalidomide-binding site. In vitro selection of thalidomide-binding aptamer was carried out according to Scheme 1. A 110 mer DNA pool with a random region of 60 bases was amplified by PCR with natural nucleotides as substrates.

Each primer contains one ribonucleotide, and thus each strand of the amplified dsDNA has one ribonucleotide residue (step 1). One primer PCR was conducted to prepare modified DNA, using **1** instead of TTP, a forward primer without a ribonucleotide portion and the amplified dsDNA (step 2). The one-primer PCR mixture was treated with 100 mM NaOH at 95 °C for 5 min to hydrolyze the ribonucleotide part in the unmodified DNA (step 3). Modified DNA was purified by denaturing PAGE (step 4) and applied to in vitro selection with thalidomide (step 5). For positive selection, thalidomide bearing a linker (**2**) was conjugated with biotin (**3**) and immobilized on a streptavidin gel. Ethanolamine−biotin conjugate (**4**) immobilized on the gel was used for negative selection. The modified DNA pool was incubated with a negative selection gel to remove a fraction

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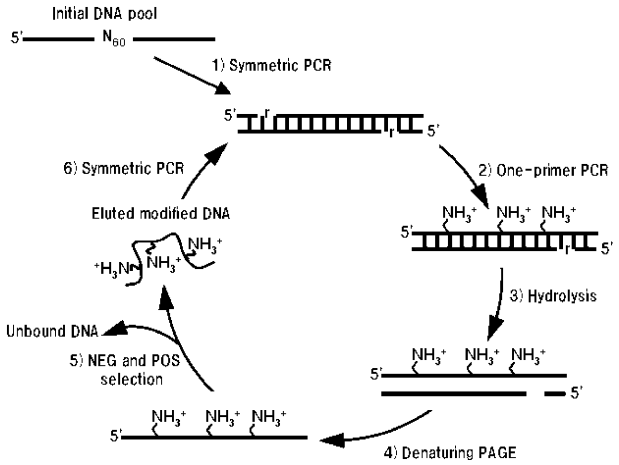
Scheme 1. Schematic Illustration of the in Vitro Selection of Thalidomide-Binding Aptamers[a]



[a] (1) 110mer initial DNA pool is amplified by PCR using chimera primers to synthesize DSDNA containing a ribonucleotide. (2) The modified DNA is synthesized from DSDNA by one primer PCR using **1** instead of TTP. (3) The resultant mixture is treated with NaOH to hydrolyze the ribonucleotide part. (4) The modified DNA is isolated by PAGE. (5) Purified modified DNA is incubated with selection gels. (6) The eluted modified DNA is amplified by PCR and used for the next round of selection.

that binds with streptavidin gel-bound **4**. The unbound modified DNA was then incubated with a positive selection gel and washed with selection buffer. The modified DNA bound to thalidomide on the gel was eluted with elution buffer, amplified by PCR as described above, and used for the next round of selection. During the first round to the ninth round, 7 M urea buffer was used for elution by denaturing the DNA. After the 10th round, modified DNA was eluted by a thalidomide buffer containing thalidomide derivative **2**.

The progress of the selection from the modified DNA pool is shown in Figure 2. No significant amounts of modified DNA were eluted in the initial two rounds of selection.

The eluted modified DNA was enriched from the third round to the sixth round, and the increase in elution percentage reduced after the seventh round. Therefore, elution of modified DNA after the 10th round was carried out with a buffer containing **2**, to elute thalidomide-binding DNA. Furthermore, the amount of **3** on the positive selection gel used was reduced to one-tenth in the last two rounds to make the selection conditions more severe. Finally, after the 15th round, the eluted modified DNA was cloned, and the selected 44 clones of modified DNA were subjected to binding assays and sequencing. Base sequence analysis showed no identical sequence among the 44 clones. However, several local homologous sequences, which could form a common secondary structure and may contribute to the binding with thalidomide, were found among the 44 clones. The local homologous regions will be described later in the section of thalidomide-binding site in the clone 5 (**T5**). The binding assay of the 44 clones was done by the method described in the Experimental Section, and each binding affinity was estimated from the percentage of thalidomide-bound DNA with respect to the total input of modified DNA. The binding ability of each clone is shown in Figure 3.

Among the 44 clones, relatively high binding affinity was observed for five clones, whose sequences are shown in Table 1. In all five clones, guanine content is rather high, exceeding 25%, while the content of T^{HM} varies from 11% to 27%. Clone

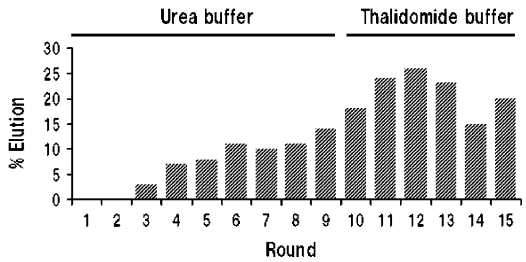


Figure 2. Progress of the selection from the modified DNA pool. The ordinate represents percentage of modified DNA from each pool that bound to the positive selection gel and eluted with elution buffer. The elution buffer used each round is indicated on the top of the graph. Eluted DNA after the 15th round was used for cloning.

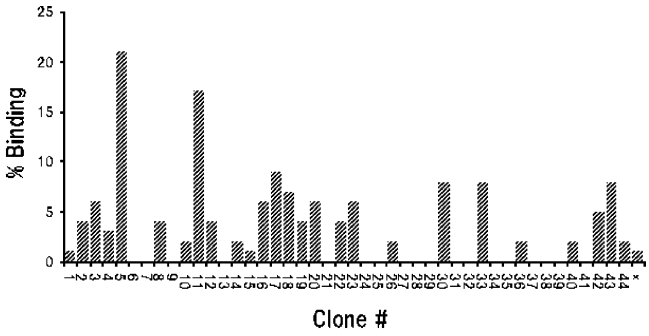


Figure 3. Binding ability of each clone. The ordinate indicates the binding ratio that is calculated by the following equation. Binding percentage = (NEG − POS)/ICD × 100, where the amount of unbound DNAs from positive and negative selection gel is indicated by POS and NEG, respectively, and ICD is the amount of DNA added to gels. The asterisk indicates the result using the initial DNA pool.

5 (**T5**) exhibited the highest binding affinity to thalidomide among all of the clones, and its properties and binding ability were studied in detail by spectroscopy and surface plasmon resonance (SPR).

To evaluate the effect of the modified group in the **T5** aptamer on binding with thalidomide, **T5N**, which has a sequence identical to that of **T5** but contains only natural T in place of T^{HM}, was chemically prepared and assayed for binding affinity. Essentially no binding was observed in case of **T5N**. The result clearly demonstrates that modified groups in the aptamer **T5** are indispensable for the binding of thalidomide. The modified groups in the DNA aptamer could aid in the formation of a highly ordered structure that supports binding with thalidomide. A primary amine in the modified group is protonated under neutral condition forming a positive charge. The positive charge could interact with the negatively charged phosphate backbone of DNA by electrostatic interaction, promoting the formation of the thalidomide-binding structure. The UV melting curve of **T5** was measured along with **T5N** to investigate the influence of the modified group on the structure formation of the DNA. The modified DNA aptamer **T5** was found to have a high thermal stability as compared to that of **T5N**, as shown in Figure 4. The melting point of **T5** was 61 °C, while that of **T5N** was 48 °C. The thermodynamic parameters for the structure formation are larger for **T5** than for **T5N** as shown in Table 2.

On the other hand, it is reported that introduction of the modified thymidine, T^{HM}, into DNA has little effect on duplex formation, and there is little difference between the T_m values

Table 1. Sequence of Selected DNA Aptamers[a]

no.	binding %	random sequence region	%T
5	21	*T*CGA*T*G*T*AGGAA*T*CGCGAAGGAAGCG*T*G*TT*CGAGC*T*CGG*T*CCGGC*T*AG*T*G*T*CC*T*GAC*T*G*T*	25
5N	3	TCGATGTAGGAATCGCGAAGGAAGCGTGTTCGAGCTCGGTCCGGCTAGTGTCCTGACTGT	0
11	17	GGCCGGGGGGAA*T*GCCGCGC*T*GAG*T*CGGG*T*GAGGGGGG*T*AC*T*GAGGGAGGGGC	11
17	9	G*T*CCCCGCCCACGGAA*T*CA*T*CAGGAC*T*CGGG*T*GA*T*C*T*CC*T*CCAGG*T*GCC*T*GCAC*T*GAGG*T*	20
32	8	AGCAGACG*T*GG*T*CGG*T*G*T*GGGGCGCG*T*AGC*T*ACCCGACGCAGGGGCG*T*GA*T*ACA*T*GA*T*CG	17
43	8	G*T*GGAGG*T*CG*T*GAGC*T*G*TA T*ACAGGCGG*T*GG*TT*ACG*TT*GG*T*CCGG*T*G*T*GC*T*CCC*T*CCACC	27
		5-ACCAGTCTGAATTCGCGATAGTACT-N_{60}-*A*TAGA*T*GGCA*T*GGA*T*CCAG*T*C*TA T*C-3	

[a] Five clones were chosen from the result of binding assay. The number of clones and binding rate are shown on the left side, and the frequency of occurrence of T^{HM} is shown on the right. The italic T indicates modified deoxyuridine, T^{HM}.

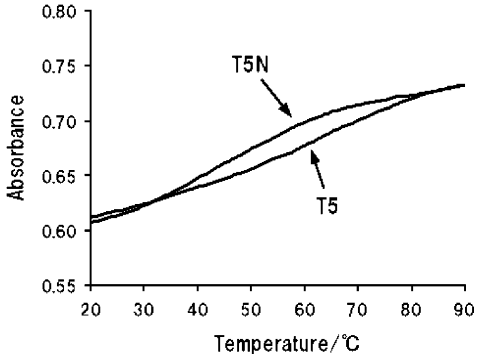


Figure 4. UV-melting curves of **T5** (black line) and **T5N** (gray line) measured in selection buffer (10 mM Tris-HCl, 250 mM NaCl, 5 mM $MgCl_2$).

Table 2. Melting Points and Thermodynamic Parameters of DNA Aptamers

	T_m (°C)	ΔH^0 (kcal mol^{-1})	ΔS^0 (kcal mol^{-1} K^{-1})	ΔG_{27} (kcal mol^{-1})
T5	61	−0.56	−167	−5.7
T5N	48	−0.38	−120	−2.5

of modified and unmodified DNA duplexes.[49] In cases of the single-stranded DNA demonstrating special property, this may be due to either the aminohexylcarbamoyl group in the modified DNA contributing to the formation of a higher ordered structure or the local stabilization of the B-form helix in the stem region of the modified DNA by cationic ammonium group in the T^{HM}.

Determination of the Binding Ability of T5 with 2 by SPR. We have examined the binding process of the aptamer **T5** with a thalidomide derivative by SPR-based assay. The biotinylated **T5** (**T5-B**) was immobilized on a sensor chip attached with streptavidin, and SPR measurement was performed using **2** as an analyte. The SPR signal increased significantly, as the analyte concentration was raised. However, the molecular weight of the analyte **2** was too small to get sufficiently large signals to determine a reliable dissociation constant (K_d) of the complex between the **T5** with **2**. Therefore, the thalidomide derivative **5**, in which **2** was conjugated with a PEG derivative as a weight tag, was used as an analyte. A substantial increase in the SPR signal was observed, as the concentration of the analyte **5** was raised as shown in Figure 5.

The SPR studies demonstrated that association and dissociation between **T5-B** and **5** were rapid processes. The sensorgram was a box type, and the association and the dissociation domains were very short. Estimation of kinetic parameters for the association and dissociation processes was difficult. Therefore,

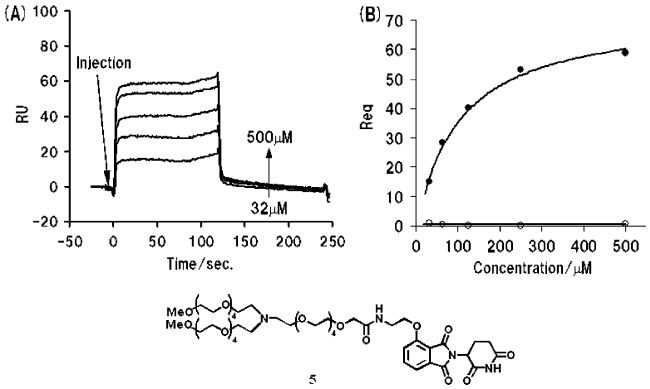


Figure 5. SPR analysis of the interaction between **T5** and thalidomide derivative **5**. (A) SPR response was taken when 32, 63, 125, 250, or 500 μM of **5** was injected onto the **T5** immobilized sensor surface. (B) The dissociation constant was calculated using the following equation, $R_{eq} = C \cdot R_{max}/(C + K_d)$, as described in the Experimental Section. **T5-B**, ●; **T5N-B**, ○.

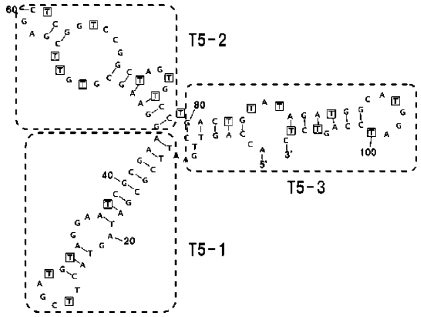


Figure 6. Secondary structure of **T5** predicted by the GENETYX software. "T" enclosed with square corresponds to T^{HM}. **T5** was divided into three fragments, **T5-1**, **-2**, and **-3**, surrounded by the dashed line.

the dissociation constant was determined by plotting an average response at equilibrium binding state (10−100 s) against analyte concentration (Figure 5B). The K_d value was estimated to be 113 μM, assuming a molar binding ratio of 1:1. SPR measurement was also performed using **T5N** with a biotin tag (**T5N-B**). However, no response signal was observed for **T5N-B**, even when the concentration of analyte **5** was raised up to 0.5 mM. The results of SPR indicate that modified groups in T5 are absolutely essential for binding to the target molecule.

Determination of Thalidomide-Binding Site in T5. The secondary structure of **T5** was estimated by the nucleic acid folding program, GENETYX. **T5** was predicted to have a three-way junction structure consisting of three stem-loop arms around the core as shown in Figure 6.

We divided **T5** into three fragments and studied the binding of each fragment to **5** by SPR, to explore the thalidominde-binding domain in the **T5** aptamer. Three biotinylated fragments,

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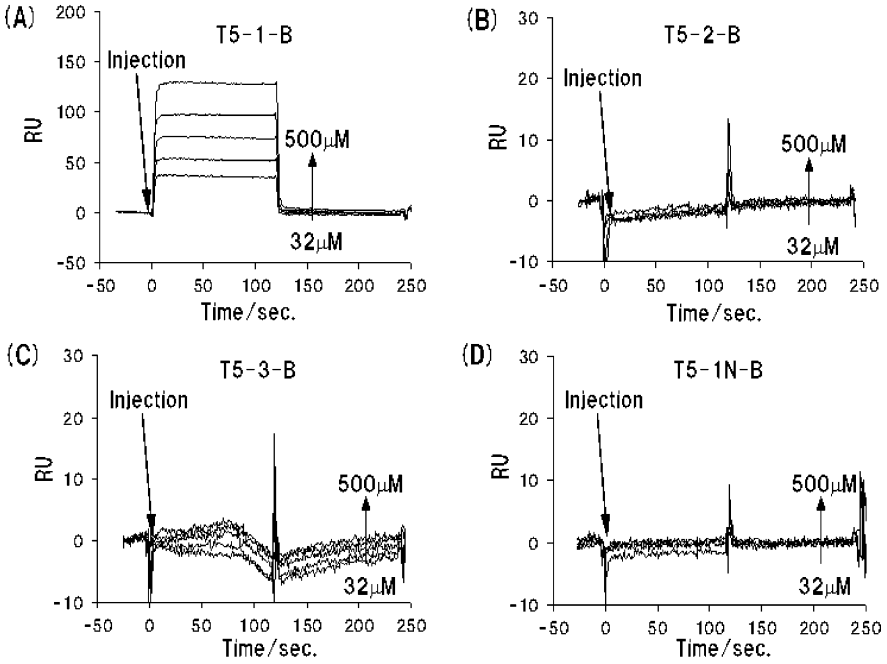

Figure 7. SPR analysis of the interaction between fragments of **T5** with a biotin tag and thalidomide derivative **5**: (A) **T5-1-B**, (B) **T5-2-B**, (C) **T5-3-B**, (D) **T5-1N-B**. Measurements were performed by the same procedure as for the parent aptamer **T5**.

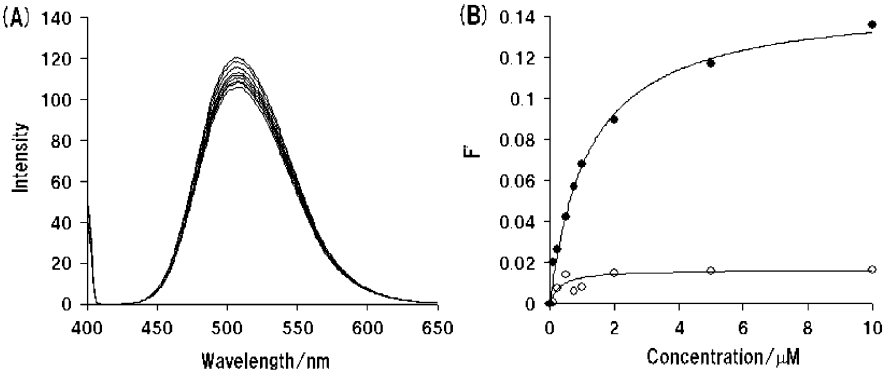

Figure 8. (A) Fluorescence emission profiles for the (*R*)-isomer of **2** at 1 μM concentration in the presence of increasing concentrations of **T5-1a** (0.1−10 μM) in the selection buffer (10 mM Tris-HCl, 250 mM NaCl, 5 mM $MgCl_2$). Excitation wavelength is 400 nm. (B) Plot of the F' against the concentration of **T5-1a**, using the (*R*)-isomer (●) or (*S*)-isomer (○) of **2**. $F' = (F - F_0)/F_0$. F is the fluorescence intensity of **2** at 506 nm in the presence of a given concentration of **T5-1a**, and F_0 is the fluorescence in the absence of the DNA.

T5-1-B, **T5-2-B**, and **T5-3-B**, were synthesized enzymatically and immobilized on a sensor chip in the same way as **T5-B**. The concentration of the analyte **5** was raised up to 0.5 mM in the SPR measurement. A substantial increase in SPR signal was observed when **T5-1-B** was used as shown in Figure 7.

However, no response signal was observed in the cases of **T5-2-B** and **T5-3-B**. The sensorgram of **T5-1-B** was similar to that of **T5-B**, and the dissociation constant of **T5-1-B** with **5** was determined by the same method as described above. The K_d value of **T5-1-B** was found to be 133 μM, which is almost the same as that of the parent aptamer, **T5-B**. Therefore, it is suggested that the **T5-1** region is the real binding site of the original aptamer, **T5**. SPR measurement was further performed using natural type DNA (**T5-1N-B**), which has the same sequence as that of **T5-1-B** but no T^{HM}. However, essentially no binding to the target molecule was observed in the case of **T5-1N-B**.

Enantioselective Binding of T5-1 with 2 Studied by Fluorescence Titration. Since we had shown that the **T5-1** fragment region may be the thalidomide-binding site of **T5**, the binding of the truncated aptamer, **T5-1a**, with the thalidomide derivative **2** was further investigated by fluorescence spectroscopy. **T5-1a**, in which the DNA tag, biotin, and one base-pair were deleted from **T5-1-B**, was prepared chemically. Thalidomide derivative **2** displayed fluorescence emission around 500 nm on excitation at 400 nm. The binding between **T5-1a** and thalidomide was studied by monitoring the change in fluorescence of **2** on addition of **T5-1a**. To examine whether the aptamer can recognize an optical isomer of thalidomide with high selectivity, enantiomers of **2** were resolved by HPLC on a chiral-resolving column, and the interaction of each enantiomer of **2** with **T5-1a** was examined by fluorescence titration. As shown in Figure 8, when **T5-1a** was added to the (*R*)-isomer of **2**, the fluorescence intensity of **2** increased with increasing concentration of **T5-1a**.

On the other hand, no significant increase in fluorescence intensity was observed when the (*S*)-isomer of **2** was used, even though up to 10 equiv of **T5-1a** was added. Fluorescence anisotropy study also demonstrated that the (*R*)-isomer of **2** binds with **T5-1a**, but the (*S*)-isomer of **2** does not (data in Supporting

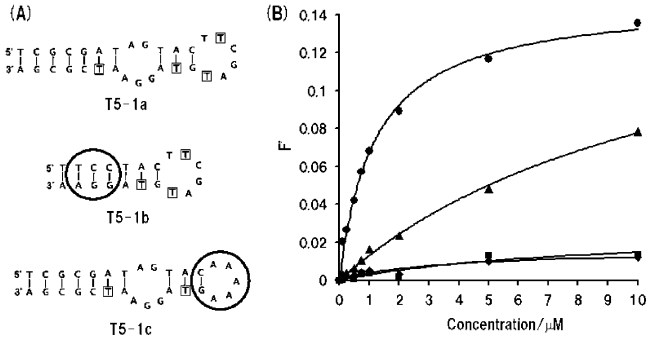

Figure 9. Fluorescence titration to determine the structural factors responsible for the binding ability of **T5-1a**. (A) Secondary structures of **T5-1a**, **T5-1b**, and **T5-1c**. (B) Plot of F' for the (*R*)-isomer of **2** against the concentration of **T5-1a** (●), **T5-1b** (▲), **T5-1c** (■), and **T5-1aN** (◆), respectively.

Information). These results indicate that **T5-1a** recognizes the (*R*)-isomer of thalidomide with high enantioselectivity. **T51-a** forms a stem-loop structure with a T*T*CGA*T* loop region, which could be a binding domain for (*R*)-thalidomide. We could identify a stem-loop structure similar to that in **T5-1a** in the five clones among the 44 sequenced clones, although none of the clones were identical at the full-length sequence level. Another five clones have also consensus CT*T*CGA*T*G region, although formation of the similar stem-loop structure is unlikely estimated from the secondary structure analysis by GENETYX.

To determine the structural factors responsible for the binding ability of **T5-1a**, fluorescence titration measurements were performed using two mini-variants of **T5-1a** (Figure 9).

One variant, **T5-1b**, in which the unpaired AG was converted to TCC to form base pairings with GGA, was studied to clarify the role of the internal loop in **T5-1a**. Another variant, **T5-1c**, in which TTCGAT was replaced with A_5, was prepared to shed light on the terminal loop with two T^{HM}'s. The increase in fluorescence intensity of the (*R*)-isomer of **2** by addition of the **T5-1a** variant was suppressed considerably in both cases. In particular, **T5-1c** resulted in very little increase in fluorescence intensity even at high concentrations of **T5-1c**. This result suggests that the base sequence of the end loop containing T^{HM} is necessary for binding between **T5-1a** and thalidomide. The internal loop portion also contributes to the binding ability of **T5-1a** to some extent. We have also confirmed that addition of the corresponding natural type DNA, **T5-1aN**, which has the same sequence as **T5-1a** but no T^{HM}, produced no increase in fluorescence of the (*R*)-isomer of **2**, indicating no interaction between **T5-1aN** and thalidomide. The K_d value of **T5-1a** was found to be 1.05 ± 0.59 μM from curve fitting of the fluorescence titration experiment.

Discussion

The K_d value of **T5-1a** estimated from fluorescence titration was 2 orders of magnitude lower than that of **T5-1-B** obtained from SPR measurement. This discrepancy of K_d values obtained by two methods could be derived from differences in measurements. A racemic mixture of thalidomide bearing a PEG weight tag, **5**, was used as an analyte in the SPR measurements, while the (*R*)-isomer of **2** was used in the fluorescent titration to determine the K_d value. The high molecular weight tag is likely to suppress interaction of the aptamer with **5**, resulting in the larger K_d value. Binding between **T5-1a** and the (*R*)-isomer of **2** was conducted in a homogeneous aqueous solution in the fluorescence titration study. On the other hand, **T5-1-B** bearing the DNA tag and biotin was immobilized on a sensor chip by biotin−streptavidin interactions, and binding between **T5-1-B** and **5** takes place on the surface of the sensor chip in SPR. Use of the solid chip in the SPR could also suppress binding of the aptamer with the analyte as compared to the homogeneous solution system used in the fluorescent titration.

Sequencing of five selected modified DNA aptamers revealed that the aptamers have a high G content, while modified deoxyuridine content in the aptamers is moderate to relatively high. Use of modified nucleotides in place of their corresponding natural substrates, for the preparation of a modified DNA by PCR, is problematic due to their low incorporation efficiency. Thus, DNA with a high modified nucleotide content will be rather difficult to amplify by PCR. We previously reported that **1** is a good substrate for KOD Dash DNA polymerase during PCR but not for Taq DNA polymerase.[29] We have found very recently that **1** is incorporated into DNA with over 80% efficiency as compared to the natural substrate, TTP, during the primer extension reaction when a primer bears a natural nucleotide at the growing 3′-terminus. However, the incorporation efficiency drops to less than 10% during the primer extension reaction when a primer bears one T^{HM} at the growing 3′-terminus. Incorporation of **1** or TTP was further suppressed when a primer has two-successive T^{HM}'s at the 3′-terminus.[50] These results suggest that a single T^{HM} is incorporated into DNA rather easily during PCR, but not multi-successive incorporation. On the other hand, T^{HM} in the template has little effect on the incorporation efficiency of the corresponding substrate, dATP, even if the template has successive T^{HM}.[50] Thus, the modified group has a greater effect on the polymerase reaction with KOD DNA polymerase when it is adjacent to the elongation terminus than when it is on the template. For screening of an aptamer by in vitro selection from a library of modified DNA, any bias except for the intended selection criteria is undesirable. Thus, the low incorporation efficiency of T^{HM} in case of successive T sequences during PCR could cause unfavorable bias for the selection. To construct a selection system that reduces such unfavorable biases, we performed a two-step PCR: symmetric PCR with natural substrates using the selected modified DNA as a template, forming the corresponding natural type DNA, followed by one primer PCR using the modified substrate to form the modified DNA library for the next round of selection. Nevertheless, few *TT*- and no *TTT*-successive sequences were found in the five selected and sequenced modified DNA aptamers. The absence of successive T^{HM} sequences in the aptamer could be caused either by selection pressure during PCR or by selection during affinity binding with the target. However, the result of the binding studies of the modified DNA aptamer with thalidomide demonstrates that the present selection system gives a desirable modified DNA aptamer, which has a high binding ability.

The aptamer **T5-1a**, which is obtained by truncation of the selected aptamer, binds with (*R*)-thalidomide, but not with (*S*)-thalidomide. The original aptamer, **T5**, could also recognize (*R*)-thalidomide. As we performed in vitro selection using a racemic

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thalidomide derivative on the gel, two species, (*R*)- and (*S*)-forms of thalidomide, were present on the gel in a 1:1 ratio. While **T5** would have bound with the (*R*)-form of thalidomide on the gel during the selection process, other aptamer clones may have bound with the (*S*)-form of thalidomide. As DNA is a chiral molecule, the binding properties of each aptamer with the enantiomers of thalidomide will be different. Further studies on binding behavior of the other clones will be the basis of future work.

The aptamer **T5**, which could form a three-way junction structure, was selected originally. However, the thalidomide-binding region of the aptamer is a fragment of **T5**, **T5-1**. **T5-1** is composed of a stem-loop structure in which a terminal 6-base loop structure with two T^{HM}'s, T*T*CAG*T*, is absolutely necessary for the binding with (*R*)-thalidomide. Four other clones also could form a similar stem-loop structure. The modified groups in **T5-1** are likely involved in direct interactions with thalidomide, although further investigations such as NMR study are needed to clarify the real binding mode. In addition, the introduction of the modified group into DNA not only improved affinity to the target molecule, but could also improve nuclease resistance as compared to natural type DNA. We have previously shown that oligodeoxyribonucleotides incorporating T^{HM} are stable against several nucleases.[51]

In addition to the TACTTCGATGT sequence in **T5-1**, essentially common sequences of GAGTCGGTG and C(T)-GACGCAGGGGCG are found in five and six clones, respectively, among the sequenced 44 clones by multiple sequence alignment program, DIALIGN.[52] These common local sequences may form another binding domain for thalidomide, although further studies on binding behavior of other clones are needed to clarify the role of the sequences.

Thalidomide has significant biological activity and has attracted widespread interest recently as a potential drug for several diseases such as autoimmune diseases and some cancers,[34−40] although it was discarded from the market in the 1960s because of its teratogenic activity.[32,33] It has one chiral center; however, it is used as a racemic form. The differences in biological activity between the enantiomers of thalidomide are still not clear. The obtained aptamer, which binds with thalidomide with high enantioselectivity, will be useful for the analysis and biochemical study of the enantiomers of thalidomide. Study on a combination of this aptamer and a fluorescence resonance energy transfer (FRET) system is in progress to develop an easy method to analyze thalidomide.

Experimental Section

Analytical Method. ^{1}H NMR spectra were measured on a JEOL JNM-AL 300 FT-NMR system, and ESI-mass spectra were measured by a MDS-Sciex API-100 mass spectrometer (Applied Biosystem). UV spectra were measured using a Shimadzu UV-1200 spectrophotometer. T_m measurements were performed with a Shimadzu UV-2550 spectrophotometer equipped with a TMSPC-8 temperature controller. Fluorescence spectra were measured with a RF-5300PC spectrofluorophotometer (Shimadzu). HPLC was performed using a Wakosil 5C18 reversed phase column (4 × 250 mm), using a linear gradient of 50 mM triethylammonium acetate (pH 7.6) with acetonitrile (2−37%) as an eluent and a flow rate of 1.0 mL/min. Detection and quantification of DNA on a gel electrophoresis were performed with a Molecular Imager FX PRO (BIO-RAD). Sequencing analysis was carried out using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). A BIACORE X was used for surface plasmon resonance (SPR) analysis.

Materials. The modified deoxyuridine derivative, **1**, was prepared according to the method described previously.[53,54] Thalidomide with a linker (**2**) was synthesized according to the procedure reported previously.[55] Streptavidin-Sepharose was purchased from Amersham Pharmacia, and 5-[5-(*N*-succinimidyloxycarbonyl)pentylamido]hexyl D-biotinamide (Biotin-$(AC_5)_2$-OSu) was from Dojindo Laboratories. Preparations of biotinylated thalidomide (**3**), thalidomide with a weight-tag (**5**), and biotinylated ethanolamine (**4**) used for the negative pre-selection, and resolution of enantiomers of the thalidomide analogue **2** by HPLC on a chiral-resolving column are described in the Supporting Information. All templates and primers used for PCR were purchased from Japan Bio Service and Hokkaido System Science. Short-versions of aptamers derived from the selected aptamer **T5** were synthesized by Japan Bio Service using a phosphoramidite of T^{HM}.[53] KOD Dash DNA polymerase was purchased from Toyobo, and Taq DNA polymerase was from Takara. For cloning and sequencing, the DNA Ligation Kit was purchased from Takara, TA Cloning Kit from Invitrogen, GenElute Plasmid Miniprep Kit from SIGMA, and Sequencing Kit from Applied Biosystems. All other chemicals were reagent grade and used without further purification.

Preparation of Modified DNA Pool. PCR was performed using 110 mer DNA containing a random region of 60 nucleotides flanked by primer regions as a template: 5′-ACCAGTCTGAATTCGCGAT-AGTACT-N_{60}-ATAGATGGCATGGATCCAGTCTATC-3′, a forward primer P-1R; 5′-ACCAGTCTGAATTCGCrGATAGTACT-3′, a reverse primer P-2R; and 5′-GATAGACTGGATCCATrGCCATCTAT-3′ (rG indicates ribonucleotide). 100 μL of the PCR mixture contained 1 nM template, 0.4 μM primers, 0.2 mM dNTPs, 0.5 U KOD Dash DNA polymerase, and buffer for KOD Dash. 20 cycles of PCR were carried out at 94 °C for 30 s, 60 °C for 45 s, and 74 °C for 60 s. After the amplification, the PCR mixture was subjected to ethanol precipitation to remove the unreacted dNTPs yielding the natural dsDNA. Next, we conducted asymmetric PCR to produce the modified ssDNA from the natural dsDNA using **1** in place of TTP and only one primer. The PCR mixture (1 mL) containing the above PCR amplified dsDNA as a template, 1.5 μM forward primer P-1N, 5′-ACCAGTCTGAATTCGC-GATAGTACT-3′, 0.2 mM dNTPs (**1** is included in place of natural TTP), 25 U KOD Dash DNA polymerase, and buffer for KOD Dash was divided into 20 portions, and each 50 μL was put into a reaction tube for PCR. 30 cycles of PCR were performed at 94 °C for 30 s, 60 °C for 60 s, and 74 °C for 120 s. After the amplification, the PCR reaction mixtures were combined and treated with 10 M NaOH solution (100 μL) and heated for 5 min at 95 °C to hydrolyze the ribonucleotide portion of nature DNA. After ethanol precipitation, the residue was purified by 10% denaturing PAGE. The resultant modified ssDNA (modified DNA) pool was used for the selection.

Preparation of Affinity Gel. 50 μL of Streptavidin-Sepharose gel was washed twice (400 μL × 2) with the selection buffer (50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM $MgCl_2$). 10 μg (13 nmol) of biotinylated thalidomide (**3**) in the selection buffer (400 μL) was added to the gel (50 μL of gel can bind with 15 nmol of biotin compound), and the suspension was agitated for 3 h at room temperature. Next, the supernatant fluid was removed, and the gel was washed twice (400 μL × 2) with selection buffer to form the positive selection gel. HPLC assay of **3** in the solution before and after the reaction by revealed that **3** bound with the gel quantitatively. Similarly, negative selection gel

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was obtained by binding of **4** (7 μg, 13 nmol) with 50 μL of Streptavidin-Sepharose gel.

In Vitro Selection. The prepared modified DNA pool (~0.5 nmol) in 400 μL of selection buffer (50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM $MgCl_2$) was denatured at 95 °C for 5 min and then allowed to cool at room temperature for 30 min. The annealed DNA solution was added to the negative selection gel (50 μL) and incubated at room temperature for 2 h. The solution containing modified DNA that did not bind with the gel was collected and was added to the positive selection gel (50 μL) and incubated for 2 h. The unbound modified DNA was removed, and the gel was washed five times (400 μL × 5) with selection buffer. The modified DNA bound with the thalidomide on the gel was eluted by denaturation with urea buffer (200 mM sodium citrate (pH 5.0), 3 mM EDTA, 7 M urea) for the first round to the ninth round or was eluted with buffer containing excessive thalidomide derivative (selection buffer and 5 mM of **2**) for the 10th round to the 15th round. In the case of elution with urea buffer, the urea buffer (500 μL) was added to the gel, the mixture was heated for 5 min at 95 °C, and then the modified DNA solution was collected. This procedure was repeated twice. The collected solution was dialyzed, and the obtained modified DNA was used as a template for PCR of the next round. In the case of elution with thalidomide buffer, thalidomide buffer (500 μL) was added to the gel, and the mixture was incubated at room temperature for 2 h. Next, the eluted solution was collected and dialyzed. The obtained modified DNA was used as the template of PCR of the next round. Using eluted modified DNA as a template, natural dsDNA was formed by the first symmetric PCR, and then a new modified DNA pool was prepared by the second asymmetric PCR using one primer as described above. This pool was used for the next selection. The amount of modified DNA in the input pool and that eluted from the positive selection gel was estimated from the band intensity on denatured PAGE after staining with ethidium bromide, and the ratio of the modified DNA that was bound to the gel was assumed from the ratio of the band intensities.

Cloning and Sequencing of Selected Modified DNA. After 15 rounds of selection and amplification, the modified DNA bound to the thalidomide gel was amplified by PCR using KOD Dash DNA polymerase, forward and reverse primers, and natural dNTPs. The resulting dsDNA was further amplified by PCR with Taq DNA polymerase. The amplified dsDNA was cloned into a TA vector by the TA cloning method according to the manufacturer's protocol. Plasmid DNAs were prepared from 44 different clones and isolated using the GenElute Plasmid Miniprep Kit. DNA sequence was carried out with an ABI PRISM 310 Genetic Analyzer using a BigDye-termination method. The secondary structure of the selected DNA aptamer was predicted from its sequence by GENETYX software.

Binding Assay. The modified DNA (0.065 OD), which was synthesized on each cloned plasmid DNA template in a way similar to that of the DNA pool preparation, was dissolved in selection buffer (65 μL). The solution was heated for 5 min at 95 °C and allowed to cool at room temperature. Each DNA solution (30 μL × 2) was added to the negative and the positive selection gels (10 μL), and incubated at room temperature for 2 h. The unbound DNAs from the positive and the negative selection gel were collected, and each 5 μL solution was applied to 10% denaturing PAGE, along with the same amount of input DNA. The DNA on the gel was stained with ethidium bromide and quantified by a Molecular Imager FX PRO. The percentage of bound modified DNA was estimated from the following equation: binding ratio (%) = (NEG − POS)/ICD × 100, where NEG, POS, and ICD are the band intensities of DNA obtained from the positive and negative gel and the input DNA, respectively.

T_m Measurements. UV absorbance of the DNA aptamer was measured with a UV-2550 spectrophotometer equipped with the TMSPC-8 temperature controller. Sample DNA was dissolved in selection buffer (150 μL) at a concentration of 0.5 μM. The rate of heating or cooling was 0.5 °C/min. T_m values were determined by a first differential method using T_m analysis software, LabSolutions.

SPR Measurements. The interaction of the modified DNA aptamer and thalidomide derivative was evaluated by surface plasmon resonance (SPR) using a Biacore X. The modified DNA aptamer for immobilization on a sensor chip was prepared by the same method as a DNA pool preparation using a biotinylated primer P-1B, 5′-biotin-ACCAGTCTGAATTCGCGATAGTACT-3′, and the selected plasmid (#5). For the control DNA on a reference cell, a biotinylated DNA that was amplified at the AT-rich region (110 mer) of pUC18 was also prepared using the corresponding biotinylated primer (Supporting Information). Each biotinylated DNA was dissolved in running buffer (50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM $MgCl_2$) at a concentration of 0.5 μM and injected onto a positive and a negative cell, respectively, at a flow rate of 5 μL/min (5 min × 2) to immobilize the biotinylated DNA on a sensor chip. The sensor chip surface was washed with wash buffer (50 mM NaOH, 1 M NaCl), and nonspecific adsorption was removed. A thalidomide derivative (**2** or **5**) as an analyte was injected at a flow rate of 20 μL/min in the range of 32−500 μM concentrations (120 s) for SPR measurement. Furthermore, three truncated DNAs of the selected aptamer (**T5**), **T5-1-B**, 5′-bio-ACCAGTCTGAATTCGCGATAGTACTTCGATGTAGGAATCGCGAA-3′; **T5-2-B**, 5′-bio-TTTTAGGAAGCGTGTTCGAGCTCGGTCCGGCTAGTGTCCT-3′; and **T5-3-B**, 5′-bio-TTTACCAGTCTGCTGACTGTATACATGGCATGGATCCAGTCTC-3′, were synthesized enzymatically (Supporting Information), immobilized on a sensor chip, and underwent SPR measurement to explore the thalidomide-binding region in the aptamer **T5**. The dissociation constant K_d was determined from the SPR data by curve fitting to the following equation, $R_{eq} = C \cdot R_{max}/(C + K_d)$, where R_{eq}, C, and R_{max} are SPR response, concentration of analyte, and maximum SPR response at high analyte concentrations, respectively.

Fluorescence Measurements. Changes in the fluorescence of the (*R*)- or (*S*)-form of thalidomide derivative **2**, by addition of the DNA aptamer, were measured on a RF-5300PC spectrofluorophotometer. The short-versions of the selected DNA aptamer, **T5-1a** (5′-TCGCGATAGTACTTCGATGTAGGAATCGCGA-3′), **T5-1b** (5′-TTCCTACTTCGATGTAGGAA-3′), and **T5-1c** (5′-TCGCGATAGTACAAAAAGTAGGAATCGCGA-3′), were synthesized chemically and used for fluorescence study. Optical isomers ((*R*)- and (*S*)-forms) of **2** were resolved by HPLC using a chiral-resolving column (Chiralcel OJ, Daicel Chemical Industry) and ethanol as a mobile phase at a flow rate of 1 mL/min and 4 °C.[56] Retention times of the (*R*)- and (*S*)-isomers of **2** were 16.3 and 22.2 min, respectively. Configuration of the isomers was assigned by comparison of their HPLC elution order on the Chiralcel OJ column[56] and CD spectra to those of enantiomers of thalidomide and its metabolites.[57]

Each enantiomer of **2** at a concentration of 1.0 μM and the aptamer DNA in the range of 0.1−10 μM were dissolved in selection buffer (80 μL). The fluorescence spectra were taken at the excitation wavelength of 400 nm. Dissociation constant K_d was determined from the change in peak fluorescence intensity at 506 nm by addition of the DNA aptamer using a curve fitting to the following equation, $F' = C \cdot F_{max}/(C + K_d)$, where F', C, and F_{max} are fluorescence intensity at the DNA aptamer concentration of C, concentration of the DNA aptamer, and maximum fluorescence intensity at high DNA concentrations, respectively.

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Supporting Information Available: Synthetic procedure and chiral resolution of thalidomide derivatives used for in vitro selection and SPR studies, enzymatic synthesis of truncated aptamers, **T5-1-B**, **T5-2-B**, and **T5-3-B**, data on fluorescence anisotropy using **T5-1a**, and complete ref 35. This material is available free of charge via the Internet at http://pubs.acs.org.

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