ABSTRACT

Thrombin-inhibiting DNA aptamers have already been obtained through the systematic evolution of ligands by exponential enrichment (SELEX). However, SELEX is a method that screens DNA aptamers that bind to their target molecules, and it sometimes fails to screen good inhibitors. Therefore, it is necessary to develop a method of screening DNA aptamers based on their inhibitory effects on the target molecules. We developed a novel method of detecting aptamers using an evolution-mimicking algorithm, and we applied it to the search of new aptamers which inhibit thrombin. First, we randomly designed and synthesized ten 15mer oligonucleotides presumed to form G-quartet structures, and then measured their thrombin-inhibiting activities. The aptamers showing high inhibitory activity were selected, and we shuffled and mutated those sequences in silico to generate 10 new sequences of next-generation aptamers. After repeating the cycle five times, we successfully obtained the same aptamers reported previously, and they showed high inhibitory activity. In addition, we added 8mer oligonucleotides to both the 5’ and the 3’ end of the selected 15mer aptamers, and then repeated the evolution in silico. After two cycles, we were able to obtain aptamers with higher inhibitory activity than that of the 15mer aptamers.

INTRODUCTION

Many aptamers which recognize various molecules have been reported (1–4), and some of them showed very strong binding in the nanomoles range, especially to protein. Some aptamers with high enzyme-binding activity also showed high inhibitory activity, and can therefore be used as pharmaceuticals. Aptamers are usually screened by systematic evolution of ligands by exponential enrichment (SELEX) (5), but this type of selection is based on binding activity, whereas the aptamer showing the strongest binding activity is not always the best enzyme inhibitor. The aptamer might bind to the part of the enzyme which does not affect its activity, and therefore the aptamer as inhibitor is ideally screened according to its inhibitory activity.

Nevertheless, huge numbers of aptamers require screening, and there is no method of assaying the inhibitory activities of many aptamers simultaneously, which means that assays have to be carried out one by one. A method of limiting the number of aptamers to be assayed is necessary, and this could be achieved by using an evolution-mimicking method, such as a genetic algorithm (6).

We have already reported the screening of a trypsin-inhibiting hexapeptide using a genetic algorithm (GA) (7). In that case, 24 peptides consisting of six amino acids were synthesized, and the inhibitory effect of each aptamer on trypsin was assayed. The peptides showing high inhibitory activity were selected and their sequences were amplified, crossed-over and mutated using GA. After the GA operations were performed, a new set of 24-peptide sequences was obtained (the second generation) and the process was repeated. Consequently, we successfully obtained a hexapeptide that effectively inhibits trypsin activity. It was thus ascertained that an evolution-mimicking algorithm (EMA) such as GA is useful for searching functional peptide sequences effectively. We have also reported the screening of a 15mer peptide which has a high tendency to form an α helix, using GA (8). EMA methods such as GA enable us to screen aptamers according to characteristics other than target affinity, such as inhibitory activity and structural properties, by restricting the number aptamers to be assayed.

In this study, we chose the DNA aptamer that binds to thrombin as the model in applying EMA, since this 15mer...
aptamer forming a G-quartet for thrombin has already been screened by SELEX and has shown thrombin-inhibiting activity (9). This aptamer is one of the most well known; its structure has already been determined (10–12) and the effect of its loop sequence on the G-quartet structure has been investigated (13). Extensive work to determine the binding site has already been carried out, and the binding site has been determined (14–17). Additionally, the application of this thrombin-inhibiting aptamer as a drug has been investigated, such as in the inhibition of clot formation in vivo (18–21), and its stability in vitro (22) and in vivo (18) has been determined.

Moreover, Macaya et al. (23) have synthesized longer thrombin-inhibiting aptamers, which are essentially a 15mer thrombin-inhibiting aptamer bearing a duplex of different lengths at the 5′ and 3′ ends that forms the G-quartet structure, and have investigated the thrombin-inhibiting activity of such aptamers. The binding site of the 15mer thrombin-inhibiting aptamer is positively charged, so that the aptamer bearing the longer duplex was expected to show higher inhibitory activity, since it would bind to the thrombin more strongly because of the increased negative charge of the longer duplex. However, Macaya et al. have found no clear correlation between the inhibitory activity and the length of the aptamer duplex.

We presumed that the 3D position of the duplex relative to the G-quartet structure might play an important role in the binding of the aptamer to thrombin, and that there might be an optimal direction of the duplex reaching out from the G-quartet part. It is difficult to predict the direction of the duplex, but its sequence can be optimized using EMA. In this case, the G-quartet structure is indispensable for the inhibitory activity of the aptamer, and therefore the G-base formation should remain intact, without mutation. However, it is difficult to introduce crossovers of the sequence through GA while maintaining the G bases intact; hence we developed a new algorithm to shuffle the sequence of the loop part of the G-quartet structure among multiple parent aptamer sequences. This sequence shuffling might mix up the aptamer sequences more effectively than GA crossover, and it might result in an increase of the diversity of aptamer sequences, which would enable us to avoid local minima of the inhibitory activity of the aptamers.

Using the thrombin-inhibiting aptamer as a model, we investigated the effectiveness of applying EMA to the screening of aptamers as inhibitors. Some of the outcome of this study were presented at the Third International Symposium on Nucleic Acids Chemistry held at Sapporo, Japan in 2003 and it had already been published as an abstract (24).

### MATERIALS AND METHODS

#### Materials

Bovine thrombin was purchased from Fisher Scientific Japan, Ltd (Tokyo, Japan) and human thrombin was purchased from Sigma (Missouri, USA). Imidazole buffer was purchased from Fisher Scientific Japan, Ltd (Tokyo, Japan) and human plasma was purchased from DADE Behring, Inc. (Liederbach, Germany). All oligonucleotides were obtained from Sawady Technology Corporation (Wako, Japan).

#### Evolution process using EMA

In the case of the 15mer DNA oligonucleotides, a set of ten 15mer DNA sequences with the form GGNNGGNNNGGNNGG (N = A, G, C, T) (Figure 1A) was randomly generated by a computer and synthesized. The scheme of evolution in silico of these oligonucleotides using the EMA is illustrated in Figure 2. The inhibitory effect of each oligonucleotide on fibrin clot formation was measured, and the 10 oligonucleotides were ranked according to their thrombin times. The top five oligonucleotides showing extended thrombin times for clot formation were chosen, and the sequences of those oligonucleotides were shuffled by a computer to generate a new set of 10 sequences for the next generation. The three loops attached to the presumed G-quartet structure were divided into three blocks (Figure 2) and the 2- or 3-base sequences in each block were shuffled within the same block among the five oligonucleotide sequences. The appearance rate of each block sequence was changed depending on the rank of the oligonucleotide from which it was originated. After

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**Figure 1.** Scheme of the DNA oligonucleotides and the presumed form of the G-quartet structure. (A) 15mer oligonucleotide. (B) 31mer DNA oligonucleotide. N = A, G, C, T.
the generation of 10 new sequences through this shuffling process, one base mutation per sequence was introduced with variations in occurrence. The 10 new oligonucleotide sequences for the next generation were then determined, and they were synthesized for the next clot-inhibition assay. By repeating this cycle, the oligonucleotide showing the highest inhibitory effect on clot formation was found.

In the case of the -31mer DNA oligonucleotides, a set of ten 31mer DNA sequences with the form GGTTGGTGTGGTGG (N = A, G, C, T) was randomly generated by a computer and synthesized.

Clot-inhibition assay

In order to determine the inhibitory effect of the oligonucleotides on thrombin, a clot-inhibition assay was carried out. The thrombin time was measured in order to evaluate clot inhibition. Fifty microlitres of imidazole buffer containing 30 mM of each oligonucleotide were added to 100 µl of human plasma and incubated for 2 min at 37°C. Fifty microlitres of imidazole buffer containing 25 NIH units of thrombin were then added to the solution, and the thrombin time was measured.

Binding assay

The dissociation constant, $K_d$, was measured and calculated using a Biacore 3000 (Biacore International AB, Neuchatel, Switzerland).

RESULTS

Identification of the best 15mer oligonucleotide using EMA

We first synthesized a set of ten 15mer DNA oligonucleotides bearing the sequence form of GGNNGGNNGGNNGG (N = A, G, C, T), which is presumed to be in the form of the G-quartet (Figure 1A), and measured the clot-inhibition activity of each of the 10 oligonucleotides. We ranked the 10 oligonucleotides, and chose 5 of them to generate the sequences of the next 10 oligonucleotides, using EMA. The scheme of the evolution in silico of these oligonucleotides using this algorithm is illustrated in Figure 2. In order to evaluate the clot-inhibition activity, the extended thrombin times to the thrombin time of a solution without any oligonucleotide were calculated and compared. The cycle of oligonucleotide synthesis, clot-inhibition assay and evolution of the oligonucleotide sequences in silico using the EMA was repeated seven times, with one cycle corresponding to one generation when we assume this process to be the in silico evolution of the thrombin-inhibiting oligonucleotides.

The extended thrombin time for each oligonucleotide in each generation is shown in Figure 3, and each value is the average of three measurement values. The thrombin time of the negative control was 19.1 s. The first-generation oligonucleotides were randomly designed, leaving intact the G-quartet structure, and they showed a slight extension of the thrombin time. Those oligonucleotides were ranked according to their inhibitory activity, and the top five oligonucleotide sequences were selected for the production of the 10 next-generation oligonucleotide sequences, using EMA. The sequences of three different loops presumed to constitute G-quartet structures were divided into three blocks (Figure 1A), and the sequences in each block were shuffled among the five sequences selected (Figure 2). The appearance rate of the sequences at each block was changed depending on the ranking of the oligonucleotides, and the rank 1:rank 2:rank 3:rank 4:rank 5 ratio was 5:4:3:2:1 in the 1st generation, 2:2:1:1:1 in the 2nd and 3rd generations, and was 1:1:1:1:1 from the 4th to 7th generations. These appearance rates were changed depending on the extension of the thrombin time and the homology of the oligonucleotide sequences showing high thrombin-inhibition activity.

After 10 new oligonucleotide sequences were generated through the shuffling of the sequences in each of the three blocks, a mutation was introduced. We changed one base of the oligonucleotide sequence (leaving the region forming the G-quartet intact) to one of the other three bases. The rate of introduction of the mutation was changed thus: one base mutation per two oligonucleotides from the 1st to the 3rd generation and one base mutation per one oligonucleotide after the 4th generation. Ten of the sequences generated through the shuffling and mutation process were used as the 10 oligonucleotide sequences for the next generation.

The average extended thrombin time progressively increased until the 6th generation, and then decreased (Figure 3B). The oligonucleotide showing the highest clot-inhibition activity was obtained at the 5th generation, and the sequence, GGTGTGTGGTGGG, was the same as that of our model. The sequences of the top 10 oligonucleotides
showing high thrombin-inhibition activity are shown in Table 1, and their corresponding extended thrombin times are also shown. Each oligonucleotide was designated X-Y, where X is the number of the generation and Y is the identification number of the sequence. The best oligonucleotide, which was identical to the one reported by Bock et al. (9), had an extended thrombin time of 65.2 s and a total time of 84.3 s, which was outstandingly high compared to the performance of the other oligonucleotides. In order to further improve this inhibitory effect, we next attempted to find longer oligonucleotides, following the suggestion in the report by Macaya et al. (23).

Identification of the best 31mer oligonucleotide using EMA

We designed a 31mer oligonucleotide library with the sequence 5′-CACTGNNNNGTTGTTGTTGNNGGAGTG-3′, by adding 8mer oligonucleotides to both the 5′ and the 3′ ends of the prototype thrombin-inhibiting aptamer, as shown in Figure 1B. The five-base regions at the 5′ and 3′ ends were designed to form a duplex, and the two loops composed of the three bases connecting the duplex at the 5′ and 3′ ends to the G-quartet structure of the prototype thrombin-inhibiting aptamer were named Block 4 and Block 5, respectively. We generated ten 31mer oligonucleotide sequences by randomizing both Block 4 and Block 5, and evolved those sequences in the same manner as in the case of the 15mer oligonucleotide. The appearance rate of the sequences for each block was changed as in the case of the 15mer oligonucleotide, and the rank 1:rank 2:rank 3:rank 4:rank 5 ratio was 1:1:1:1:1 in all generations. A mutation of one base per one oligonucleotide was introduced after the sequence shuffling in each generation, and that mutation rate was not changed throughout the search. The extended thrombin times caused by the oligonucleotides are shown in Figure 4, and each value is the average of three measurement values. The thrombin time without the addition of any oligonucleotide was 19.1 s this time as well. The average of the extended thrombin times did not increase dramatically, but the oligonucleotides showing longer thrombin times than the prototype thrombin-inhibiting aptamer were obtained already in the 2nd and 4th generation. The sequences of the top five oligonucleotides are shown in Table 2. Each oligonucleotide was designated LX-Y, where X is the number of the generation and Y is the identification number of the sequence.

The binding properties of the selected oligonucleotides and a study of their mutations

An oligonucleotide which appeared in the 2nd generation, L2-3, and one which appeared in the 4th generation, L4-2, had thrombin times of 127 and 106.9 s, respectively, which represents a great improvement in thrombin-inhibition activity compared to that of the prototype 15mer thrombin-inhibiting aptamer, which had a thrombin time of 80.8 s. We measured the $K_d$ value of both oligonucleotides; L2-3 showed $3.43 \times 10^{-8}$ M and L4-2 showed $2.89 \times 10^{-8}$ M. Both of the selected oligonucleotides showed greater $K_d$ values than the prototype 15mer aptamer (which had a $K_d$ value of $1.87 \times 10^{-8}$ M) despite having higher inhibitory activities than the prototype 15mer aptamer. This result supports the hypothesis that the screening of oligonucleotides according to their inhibitory activity using EMA is more efficient than affinity selection, if the aim is to obtain a good inhibitor.

Both L2-3 and L4-2 have the GTA sequence at Block 4 and GGC or GGG, and so we made 12 sets of 1- or 2-base mutated

Table 1. The 15mer oligonucleotides showing high inhibition

| Oligonucleotide | Sequence                  | Extended thrombin time (s) |
|-----------------|---------------------------|----------------------------|
| 5-9             | GG TT GG TGT GG TT GG    | 62.6                       |
| 6-2             | GG TT GG GAT GG TG GG    | 36.2                       |
| 6-7             | GG TT GG GGC GG TA GG    | 36.1                       |
| 7-1             | GG TT GG GGC GG CT GG    | 32.6                       |
| 5-4             | GG TT GG GGT GG TT GG    | 31.4                       |
| 6-1             | GG TT GG TTG GG TT GG    | 29.9                       |
| 6-10            | GG TT GG AGT GG TT GG    | 19.8                       |
| 6-6             | GG TT GG GCT GG TT GG    | 18.9                       |
| 4-10            | GG TT GG GTC GG TT GG    | 18.7                       |
| 6-9             | GG TT GG TCT GG TT GG    | 17.7                       |

Figure 3. The extended thrombin time of the 15mer oligonucleotides in seven generations of EMA evolution. (A) The results for all oligonucleotides in the seven generations. (B) The average of the extended times for each generation. The thrombin time was measured by adding 50 μl of imidazole buffer containing 25 NIH units of thrombin to 150 μl of imidazole buffer containing 30 μM of each oligonucleotide and 100 μl of human plasma and incubating at 37°C.
oligonucleotides and measured their inhibitory activity. The results are shown in Table 3; interestingly, seven oligonucleotides showed higher inhibitory activity than the prototype thrombin-inhibiting aptamer, but did not exceed that of L2-3. More interestingly, five oligonucleotides showed much lower inhibitory activity than the prototype thrombin aptamer, which means that the sequences of these loops critically influence the capacity of the oligonucleotides to inhibit thrombin.

The measurements mentioned above were all performed using bovine thrombin, and we measured the inhibitory effect on human thrombin of L2-3, L4-2 and other oligonucleotides showing high inhibitory effects on bovine thrombin. The values were almost the same, and no significant difference was observed.

**DISCUSSION**

This is the first report of a screening of oligonucleotides according to their enzyme-inhibition function. This screening was achieved by the use of EMA; otherwise it is very difficult to search or screen oligonucleotides according to their function, since there is no method of selecting functional oligonucleotides simultaneously, except according to the binding. If the aim is to screen oligonucleotides according to their inhibitory properties, the inhibitory effect on the target enzyme of each oligonucleotide must be measured one by one. On the other hand, it is impossible to measure the inhibitory activities of all oligonucleotides in a library, and so the number of oligonucleotides to be measured must be somehow limited. For that purpose, EMA methods such as GA are very
effective, and we have already reported the screening of a trypsin-inhibiting hexapeptide and a 15mer peptide forming an α helix by GA (7,8).

First, we tried to screen the 15mer oligonucleotides and designed a library of sequences presumably forming the G-quartet, using the thrombin-inhibiting aptamer screened by Bock et al. (9) as a model. After the five cycles of EMA evolution, we successfully obtained the oligonucleotide bearing the same sequence as the thrombin-inhibiting aptamer. The designed library contained 4^4 (16384) sequence variations, and the prototype thrombin-inhibiting aptamer was obtained in the 5th generation. Fifty oligonucleotides were synthesized and screened before the aptamer was found, so that 0.3% of the total variations of the library was searched. We can say that this screening method using EMA is effective, and the usefulness of EMA was demonstrated in our screening of oligonucleotide enzyme inhibitors. After the 5th generation, the activity decreased since we removed the sequence that had appeared at the evolution stage.

Considering the relation between sequence homology and inhibitory effect shown in Figure 3 and Table 1, the TT sequence at both Loop 1 and Loop 3 is important for the inhibitory activity, and even a single mutation in the TGT sequence at Loop 2 causes a serious decrease in inhibitory effect. Therefore, the sequence dependency of thrombin inhibition seems to be very exclusive, and the search for a specific oligonucleotide in such a case would be rather difficult to undertake. We have already reported the search for a trypsin-inhibiting hexapeptide (7); its inhibitory effect was found to have broad sequence dependence. In that case, we used a genetic algorithm, and a mild mutation was enough to enable the identification of that hexapeptide whose inhibitory effect had broad sequence dependence, but it would be difficult to search through a sequence space bearing very sharp peaks with that method, as in the case of this thrombin-inhibiting aptamer.

Based on the results reported by Bock et al. (9), we expected that the oligonucleotide we were looking for had a narrow sequence dependence. We developed an algorithm of shuffling the sequences of the loop part of the DNA among several oligonucleotides while keeping the G-quartet structure intact, and this also resulted in an improvement of the crossover rate, and we succeeded in pinpointing the oligonucleotide identical to the prototype thrombin-inhibiting aptamer. The appearance rate of each sequence was changed in each generation depending on the inhibitory effect of the oligonucleotide. From the 1st to the 3rd generation, the appearance rates of the sequences in each block of the highly ranked oligonucleotides were raised in order to accelerate the search for the oligonucleotide having a sequence similar to the oligonucleotide showing the highest inhibition at the previous generation, but from 4th generation onward, the appearance rates of the sequences at each block were equalized, in order to search through oligonucleotides with various sequences and to avoid missing oligonucleotides with potential inhibitory activity.

In the search of the 31mer oligonucleotides, most of the aptamers showed lower inhibitory activity than the prototype 15mer aptamer. This means that the double helix connected to the G-quartet structure might be an obstacle to the inhibitory action of the oligonucleotide in many cases. However, 3 out of 50 oligonucleotides showed higher inhibitory activity; therefore, it is reasonable to assume that there are rare cases in which the double helix connected to the G-quartet actually boosts the inhibitory activity. Our screening using EMA did not fail to pick up those rare cases, which also prove that this is an effective method of screening oligonucleotides as inhibitors.

Tasset et al. (25) have reported a 29mer aptamer which binds to a thrombin site different from that bound by the 15mer prototype thrombin-inhibiting aptamer, but it also has a G-quartet structure and a duplex part. As such, it would have been possible for the 29mer aptamer to appear in our screening process, but it did not appear. This 29mer DNA aptamer binds more strongly to thrombin than the prototype 15mer thrombin-inhibiting aptamer, but has lower inhibitory activity. The fact that it failed to appear in our screening might be due to the fact that we screened the oligonucleotides according to their inhibitory activities.

In this study, we used an EMA screening method in searching through oligonucleotides according to their enzyme-inhibiting function not to their affinity. We emphasize that the essential architecture of the oligonucleotides was conserved throughout the procedure in this work, and this architecture was derived from information obtained from SELEX, so that our method described here is essentially a post-SELEX optimization procedure. As a procedure, this has a significant potential since it can be applied to the screening of oligonucleotides according to any other function than affinity.

Aptamers are currently drawing attention as molecular recognition elements, and their application to affinity chromatography (26), biosensing (27,28) and biosensors (29,30) is now of great interest. Selectivity is one of the most important properties of the molecular recognition element when considering practical application, and a screening method using EMA would be effective in improving the selectivity of aptamers. This method has the potential for vast applications in many fields, and a combination with SELEX or high throughput screening methods such as microarrays will in turn generate more applications.

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