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In Vitro Selection of DNA Aptamers That Bind L-Tyrosinamide

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Abstract—We have applied SELEX (Systematic Evolution of Ligands by EXponential enrichment), a combinatorial method that employs biopolymers for drug discovery, to identify single stranded DNA sequences able to bind L-Tyrosinamide, a simple mimic of Tyrosine, an amino acid essential to the catalytic activity of several enzymes of pharmaceutical interest. After 15 SELEX cycles using L-Tyrosinamide immobilized on an affinity chromatography column, the percentage of aptamers specifically eluted from the affinity column with free L-Tyrosinamide was 55% of the total. Aptamers were subcloned and sequenced, allowing the identification of a highly conserved consensus sequence, and showed a K_d value for L-Tyrosinamide of 45 μM. The identified aptamer sequence will constitute the basis for further in vitro evolution protocols and structure-based drug design.

Introduction

DNA-topoisomerases are enzymes which relieve the torsional stress created in DNA by processes like replication, transcription and cell division.[1,2] They have been isolated from the most diverse organisms, and, based on structural and biochemical features, are grouped into three distinct subfamilies.[2,3] Despite their different structure and physiological significance, topoisomerases share a common molecular mechanism of action, characterized by the nucleophilic attack of an activated tyrosine residue onto the DNA phosphodiester backbone. The enzyme thus remains covalently bound to the DNA within the 'cleavage complex', leaving a free hydroxyl at the other end of the broken strand. After changes in the nucleic acid topology, the cleavage is resealed by the enzyme itself.[4,5]

Given the significance of these enzymes, it is not surprising that they are the target of many useful chemotherapeutics. Mammalian topoisomerase I and topoisomerase II are in fact poisoned by several clinically useful antitumor agents, whereas quinolones, widely prescribed antibiotics, act on the bacterial topoisomerase II, gyrase.[6] The mechanism of action of these drugs is linked to the induced stabilization of the cleavage complex, so that the enzyme remains bound to DNA through its active site tyrosyl residue. The 'mitotic catastrophe' resulting from the collision of the complex with an advancing replication fork, as well as from increased recombination rate and chromosomal deletions/rearrangements, eventually leads to cell death through apoptosis.[7]

The interest of research in novel and safer topoisomerases inhibitors is still high, due to the inherent toxicity and lack of specificity of today's drugs, and to the rapid emergence of resistant strains. However, despite the solution of the tridimensional structure of several topoisomerases, alone or complexed with the nucleic acid,[8] little is known about the molecular details of the drug–DNA–enzyme interaction at the cleavage complex. Hence, screening programs and structure–activity relationship studies still direct the design of new topoisomerase-targeted drugs.

Combinatorial chemistry applied to the discovery of novel topoisomerase inhibitors was recently employed to elucidate the mechanism of action of the enzyme. One paper reports an in vitro evolution scheme to identify topoisomerase II preferred cleavage sites on double stranded DNA,[9] while a hexapeptide able to inhibit tyrosine recombinase integrase (Int) and topoisomerase I from vaccinia virus and *E. coli* was identified from a peptide combinatorial library.[10] Following this line of research, we focused our attention to the tyrosine residue, which is the essential active site element common to all topoisomerases subtypes. We decided to

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apply a SELEX protocol for the identification of single stranded DNA aptamers able to bind specifically to tyrosinamide; we chose to mask the carboxylate residue of tyrosine to better mimic the situation of the amino acid in the polypeptide chain. Once identified, the sequence of the aptamer recognizing tyrosinamide can be used as a cassette and evolved against more complex targets, such as peptides mimicking the active sites of the different topoisomerases subtypes, or against other enzymes whose activity is strictly dependent on tyrosine. Besides being enzyme inhibitors per se, the identified aptamer(s) structure would constitute the frame for the rational drug design of new non-nucleotide compounds.

Results and Discussion

Selection of L-Tyrosinamide aptamers

We derived a protocol for the selection of DNA aptamers able to bind L-Tyrosinamide, the simplest mimic of tyrosine in the polypeptide chain of topoisomerases and other tyrosine dependent enzymes. The starting ssDNA pool contained a central region of 60 randomized nucleotides flanked by constant sequences at each end, necessary for PCR amplification. Aptamers were selected by affinity chromatography using a Sepharose 6B matrix derivatized with L-Tyrosinamide as shown in Figure 1. The ssDNA pool was first incubated with a resin blocked with ethanolamine to discard the molecules with affinity for the resin: 97% of the initial pool was not retained by Sepharose and applied to the L-Tyrosinamide resin. Following extensive washes to remove unbound and weakly bound oligodeoxyribonucleotides, DNA aptamers were eluted by free L-Tyrosinamide and amplified by PCR using one primer biotinylated at its 5′ end. The double stranded DNA was size purified by PAGE, incubated with streptavidin resin and denatured to regenerate the ssDNA pool for each successive SELEX cycle. We increased the selection stringency after the first two cycles by employing different resins, each derivatized with lower concentrations of L-Tyrosinamide: the first two cycles were done with a 29 mM resin, followed by 13 mM at cycles 3–6 and 9 mM till cycle 9. The effect of increased stringency is reflected by the yields of each cycle, shown in Figure 2: after the boost in yield obtained at cycle 2 (5.5 %), the stronger competition of aptamers for their binding site brought about a reduction in retained molecules (1.8% at cycle 3), which was kept constant by increasing the number of buffer washes throughout the successive three cycles at the same resin concentration. After cycle 6 the population of aptamers retained by the column increased exponentially, till a yield of 40% was obtained. Since the K_d of this pool for L-Tyrosinamide was in the millimolar range, we decided to counter select our DNA aptamers with L-Phenylalaninamide. Our goal was to retain only those molecules whose recognition site was specific for the phenolic moiety of the amino acid. The negative selection performed once at cycle 10 did indeed remove most of the aptamers, since the yield of molecules eluted by L-Tyrosinamide fell to 3%. We performed 5 more SELEX cycles with L-Tyrosinamide, modulating the enrichment with lower resin concentration and increased buffer washes, till the last two cycles, whose yields were 43 and 55% respectively. After cycle 15 the yield in aptamers did not increase further, and we proceeded with the identification of the selected DNA sequences.

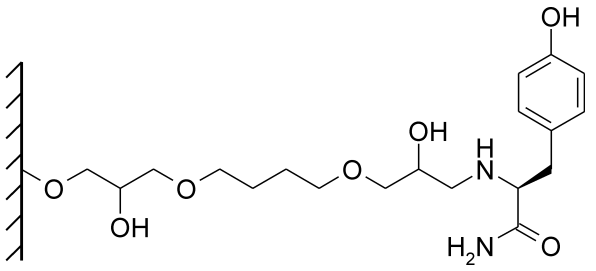

Figure 1. L-Tyrosinamide resin. L-Tyrosinamide was immobilized to the Sepharose 6B matrix as described in Experimental to obtain affinity chromatography matrices at different aminoacid concentration, namely 21 μmoles/g (6 mM), 31 μmoles/g (9 mM), 45 μmoles/g (13 mM) and 101 μmoles/g (29 mM).

Sequences of selected aptamers

PCR products obtained in the last cycles did not give products homogenous in size, since we repeatedly observed the appearance of a shorter population which was eluted by the streptavidin resin. However, to avoid possible loss of information contained in these shorter molecules we did not purify the DNA from the last two cycles. Aptamers from cycle 15 were subcloned into pUC19, and 22 clones positive at the blue/white screening were picked and sequenced. It was immediately apparent from the examination of the aptamer primary sequences the conservation of at least two motifs. Indeed, multiple alignment of all the sequences performed with CLUSTAL W allowed the classification of aptamers into three families, as shown in Figure 3. The first family (Fig. 3A) is characterized by a 38-base repeated sequence which is almost identical in all clones,

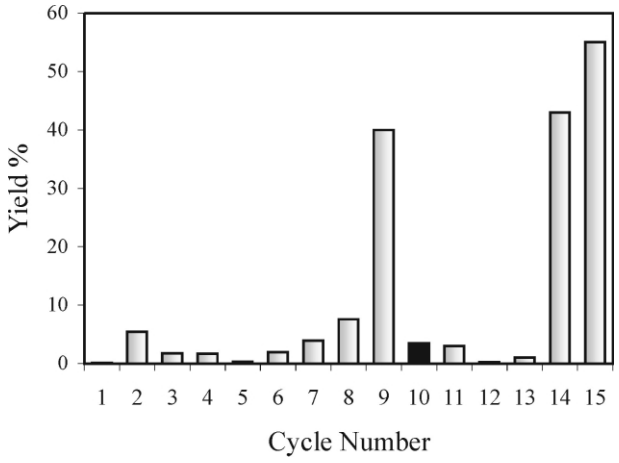

Figure 2. Yield of the selection of single stranded DNA aptamers for L-Tyrosinamide. The yield of each cycle was calculated as percentage of radioactivity eluted from the affinity column by free L-Tyrosinamide. The concentration of immobilized L-Tyrosinamide was 29 mM at cycle 1 and 2; 13 mM at cycles 3–6; 9 mM at cycles 7–9 and 10–11; and 6 mM at cycles 12–15. The bar in dark grey represents the cycle at which aptamers were counter selected with free L-Phenylalaninamide.

including the two which were much longer than expected. The length of the inserts is different, and 5 out of 9 clones match exactly the length of the consensus sequence (38 nucleotides), although they do not appear to derive from the same parental molecule. The longer insert of clone pe37 is characterized by a palindrome just upstream of the consensus sequence. This 22-nucleotide inverted repeat derives from primer-dimer formation and primer mispairing during PCR, allowing part of the 5′ primer sequence to be inserted into the amplified product.[11]

A longer repeated sequence of about 50 bases characterizes the second family of aptamers (Fig. 3B). The clone pe10 contains the sequence repeated twice as a dimer, fused at its 3′ end to the same 11-base motif identified in the palindrome of family 1 (not shown). The third set of inserts (Fig. 3C) is not significant since all sequences are palindromes deriving from the primer-dimer artifacts of the PCR, which were not purified before cloning.

A closer analysis at the two families of conserved sequences evolved in our selection shows a clear distinction of GG repeats in family 1 and CC repeats in family 2. Indeed, the sequence of family 2 reveals to be the exact complementary form of the family sequence 1 fused to the 11-nucleotide motif of the inverted repeat derived from the 5′ primer. Since the insert was subcloned using two different restriction sites, we believe that this inversion had taken place by reciprocal recombination of the inverted repeats during cloning. Figure 4 shows the alignment of the complementary sequences of family 2 to the sequences of family 1: the

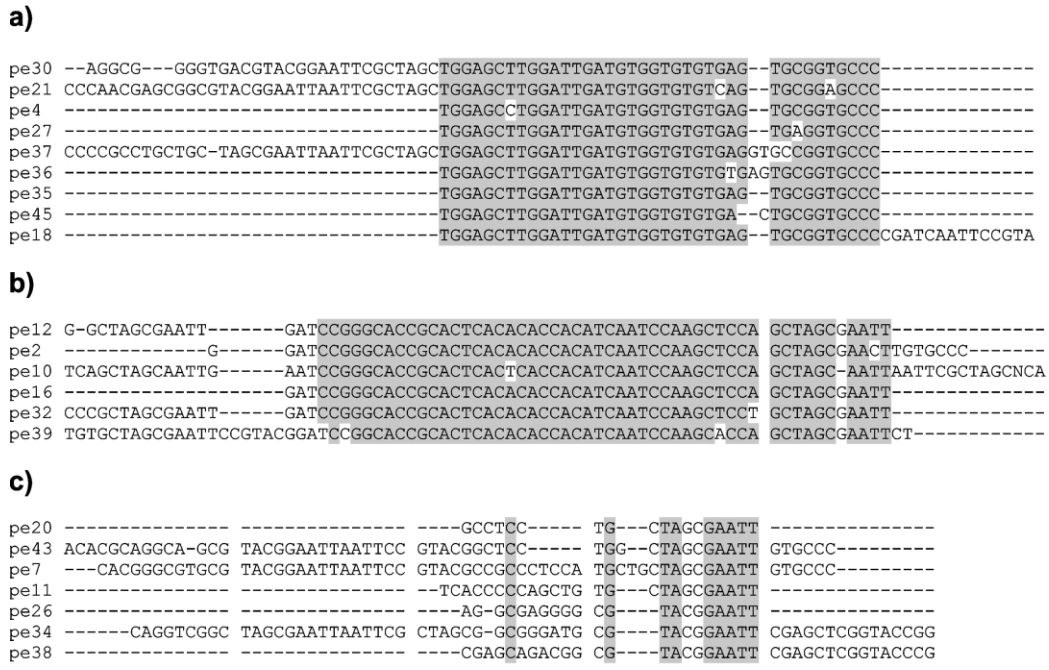

Figure 3. Multiple sequence alignment of the selected aptamers. Sequences identified from 22 clones were aligned with CLUSTAL W to reveal the presence of a consensus sequence (in grey) for each family. Clone identification numbers are written at the left of each sequence. The non-randomized part of the selected aptamer was not aligned, and is not shown. (a) Sequences of clones belonging to family 1. For longer clones (pe21 and 37), only the region around the consensus is shown; (b) Sequences of clones belonging to family 2. pe10 contains the consensus sequence repeated as a dimer (not shown in the figure); (c) Sequences of clones belonging to family 3.

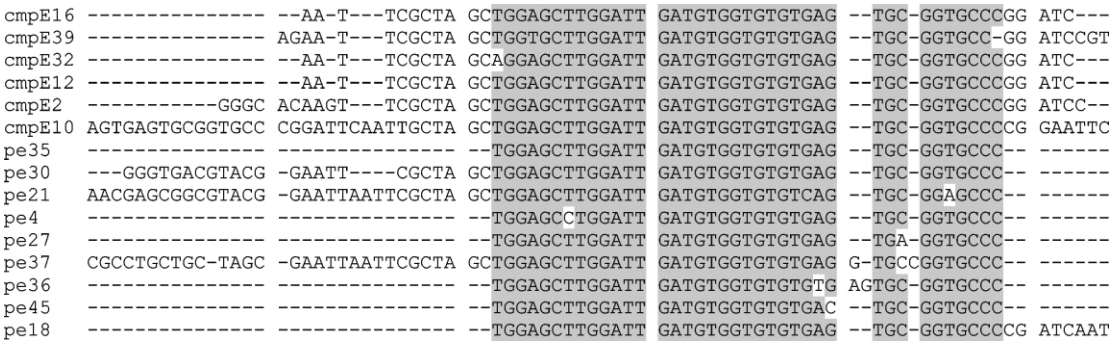

Figure 4. The consensus sequence identified is unique. The complementary sequence of all the clones belonging to family 2 (cmp*x* in the figure) were aligned with CLUSTAL W to the clones of family 1. The area shaded in grey shows the unique consensus sequence identified for L-Tyrosinamide.

consensus of 38 nucleotides identified is the same, and can be considered a unique structural theme.

To identify the hypothetical secondary structure of this consensus sequence we used the algorithm by Zuker[12] to generate the thermodynamically optimal structures for the 38 nucleotides consensus and for a representative aptamer (pe35). The structures generated were all unsatisfactory, since base pairing was minimal and insufficient to guarantee aptamer stability. However, the consensus sequence reveals the presence of several Guanines (45% of the total nucleotides) which are spaced in a regular pattern as G doublets: TGG-AGC-TTG-GAT-TGA-TGT-GGT-GTG-TGA-GTG-CGG-TGC-CC. This sequence can theoretically fold into a tertiary structure held by G quartets. The buffer we used for our selection did not contain potassium ions, a known stabilizer of guanine quartets.[13] However, G quartets stabilized by Na^+ ions have been described[14] and we cannot exclude the possibility of the sequence folding into this motif. Resolution of the tridimensional structure of the L-Tyrosinamide aptamer is warranted to reveal its spatial arrangement.

Interaction with tyrosinamide

The affinity of the selected aptamers for L-Tyrosinamide was determined by equilibrium dialysis,[15] and the corresponding K_d values, calculated assuming a 1:1 complex formation between the nucleic acid and the ligand, are shown in Table 1. Sequence from clone pe35 (Fig. 3A), chosen as the most representative of the selected aptamers, has a K_d of 45 μM for L-Tyrosinamide, well comparable to the value reported for one RNA aptamer selected for L-Tyrosine.[16] Binding of the nucleic acid is dependent on the presence of the consensus: pe38 (Fig. 3C), which presumably originates from PCR artifact, exhibits a much higher K_d value for the target, as well as the sequence complementary to the identified consensus, CMP, which does not bind L-Tyrosinamide. Since the aptamer is characterized by several GTs, a short poly[dG-dT] was included in our measures as a further negative control.

We then checked the aptamer selectivity for tyrosinamide by performing equilibrium dialysis with L-Phenylalaninamide. Surprisingly, the K_d value for L-Phenylalaninamide, 80 μM, is only twice the value for L-Tyrosinamide. This was unexpected, since we had counter selected our pool with L-Phenylalaninamide, reducing drastically the pool population. Hence, the phenolic hydroxyl seems not to be able to direct specificity to a large extent, ruling out a specific hydrogen bonding contribution to complex stability. Rather, the binding pocket of the aptamer is likely to accommodate the planar benzene moiety regardless of the hydroxyl group, a situation resembling that of lexitropsins,[17] minor groove binders tailor designed to be GC specific, that instead showed to accommodate equally well AT and GC sequences.

Table 1. Binding affinity to L-Tyrosinamide and related compounds[a]

	K_d (μM)		
	$TyrNH_2$	$PheNH_2$	*Tyr*
pe35	45±4	80±7	520±40
pe38	760±21	No binding[b]	ND[c]
CMP	No binding[b]	ND	No binding[b]
$Poly(GT)_{30}$	No binding[b]	ND	ND

[a]Binding affinities of the selected aptamer (pE35) and of controls (see text) to L-Tyrosinamide, L-Phenylalaninamide and L-Tyrosine are expressed as dissociation constant values (K_d), determined by equilibrium dialysis as detailed in Experimental.
[b]No binding as appreciated by the sensitivity of the detection method.
[c]Not determined.

In conclusion, stabilization of the aptamer-amino acid complex seems to rest on stacking interactions between the aromatic rings, as inferred by the specificities shown by the RNA aptamers recognizing phenylalanine and tryptophan[18] and by the RNA aptamer for tyrosine.[16] A significant contribution to binding could be also due to electrostatic interactions between the positively charged amino group of tyrosinamide and phenylalaninamide and the polyanionic nucleic acid backbone. Indeed, L-Tyrosine, which is a zwitterion at the pH of the selection buffer, has a dissociation constant value for the aptamer pe35 one order of magnitude higher than those of the two amido-derivatives.

Conclusions

We have identified a highly conserved single stranded DNA sequence which binds L-Tyrosinamide with a dissociation constant in the micromolar range. Our aim was to select from a combinatorial pool, a new recognition motif for an amino acid which is essential to the catalytic activity of several distinct enzymes. This aptamer constitutes a cassette for further in vitro evolution protocols against topoisomerases and other tyrosine dependent enzymes which are the target of many useful drugs.

The sequence identified is, to the best of our knowledge, the first single stranded DNA aptamer selected for an aromatic amino acid, and allows the comparison of the different flexibilities and versatility of RNA and DNA in recognizing this kind of target.[19] In fact, the RNA SELEX had identified more than one sequence and configuration with affinity for tyrosine,[16] while our SELEX allowed the identification of a unique conserved sequence.

The folding of this short single stranded DNA motif is presumably dictated by stable G quartets: hence the recognition elements for tyrosine could reside into one of the extruded loops, similarly to the arrangement of the single stranded DNA aptamer for thrombin.[20,21] The resolution of the tridimensional aptamer configuration is necessary to elucidate the elements involved in tyrosine-DNA recognition: this simple short sequence in fact would constitute the scaffold for modeling these recognition elements into the enzyme active site, thus directing the synthesis of new non-nucleotide inhibitors.

Experimental

Materials

L-Tyrosinamide and L-Phenylalaninamide were purchased from Sigma, as well as buffer components. All oligodeoxyribonucleotides were obtained from Eurogentec Bel SA (Belgium) and purified by PAGE before use. Taq polymerase and dNTPs were from Pharmacia Amersham Biotech, while T4-polynucleotide kinase, ligase and the restriction enzymes were from Gibco Life Technologies. Qiagen kits were used for plasmid miniprep purification, and sequencing was performed using T7 Sequenase (Pharmacia Amersham Biotech) and [α-^{33}P]dATP (Nen Life Sciences).

Resin derivatization

Sepharose 6B (Pharmacia Amersham Biotech) was derivatized with L-Tyrosinamide essentially as described by the manufacturer. However, in order to avoid competition between the phenate ion and the primary aminogroup of tyrosine for the reaction with the epoxide group of the resin, we chose reaction conditions characterized by milder pH than suggested. The swollen resin was reacted with tyrosinamide solutions at 45 °C for 20 h in 0.1 M HCO_3^-/CO_3^{2-} buffer, pH 9.0. After extensive washes, the unreacted epoxide groups were blocked with ethanolamine 1M pH 8.0. The titer of each resin was determined through differential absorbance of the input and output L-Tyrosinamide solutions, using the experimentally determined molar extintion coefficient of 2860 M^{-1} cm^{-1} (293 nm, pH 14). The resin concentration was calculated assuming that 1 gram of freeze-dried resin yields 3.5 mL of swollen resin. Besides the tyrosinamide resins (29 mM or 101 μmol/g, 13 mM or 45 μmol/g, 9 mM or 31 μmol/g, and 6 mM or 21 μmol/g), we prepared a resin derivatized with ethanolamine for our counter-selection cycles.

Generation of ssDNA library

The library of synthetic DNA oligodeoxyribonucleotides, whose sequence is CGTACGGAATTCGC-TAGC-N_{60}-GGATCCGAGCTCCACGTG is 96 bases long, and contains a central insert of 60 random nucleotides flanked by two constant regions used for PCR amplification and cloning. The primers used are respectively: Up, CGTACGGAATTCGCTAGC, which contains the EcoRI restriction site, and Down-B, Biot-CACGTGGAGCTCGGATCC, which has one biotin at its 5′ end and the site for BamHI cleavage.

The ssDNA pool was PCR amplified with Taq polymerase and the resulting double stranded DNA was gel-purified through an 8% native PAGE (Tris-borate 89 mM, EDTA 2 mM). To regenerate the ssDNA pool, the biotinylated PCR product was incubated for 30 min at room temperature with Streptavidin resin (Pierce), washed and the non-biotinylated strand collected after alkaline denaturation.[22] After precipitation, DNA concentration was measured by UV spectroscopy, and SELEX cycles were repeated adding one aliquot of aptamers radiolabeled at 5′ end with T4 polynucleotide kinase and [γ-^{32}P] ATP (Nen Life Sciences) as tracer. Cycle yield was calculated as percentage of radioactivity eluted by free L-Tyrosinamide on the total radioactivity.

Selection protocol

The selection of DNA aptamers for L-Tyrosinamide was performed by affinity chromatography. The ssDNA pool (20 μM and with a complexity of about 10^{14} different molecules) was incubated with L-Tyrosinamide resin in the binding buffer Tris 20 mM, pH 7.6, NaCl 300 mM and $MgCl_2$ 5 mM at room temperature. After 120 min of incubation, the column was washed with buffer and then eluted with free L-Tyrosinamide (30 mM). The eluted ssDNA was precipitated and PCR amplified as described above. PCR volume was 100 μl, and the number of cycles never exceeded 12. Employing these conditions, we could avoid the amplification of high molecular weight products which became apparent using an higher number of cycles. PCR purification was performed at all cycles, except the last two, through native PAGE electrophoresis (8%). After regeneration of the ssDNA pool through the streptavidin resin, the DNA was precipitated, quantitated, labeled and used as input for each successive cycle of selection. The resin concentrations were 29 mM (cycles 1, 2), 13 mM (cycles 3–6), 9 mM (cycles 7–9). The negative selection with L-Phenylalaninamide was done at cycle 10 incubating for 30 min a 13 mM Tyrosinamide resin with aptamers from cycle 9. After 10 washes with buffer, the aptamers immobilized on the resin were incubated with a solution of L-Phenylalaninamide 30 mM for 2 h. Sequences with affinity for L-Phenylalaninamide were eluted, and the remaining bound aptamers removed with free L-Tyrosinamide 30 mM. Affinity chromatography from this cycle on was done using L-Tyrosinamide resin 13 mM (cycle 11), and 6 mM for all other successive cycles (12–15). Counter selections with Sepharose 6B resin were done to eliminate aptamers with affinity for the matrix before cycles 1, 6, 9, and 15.

Cloning and sequencing

Aptamers from cycle 15 were PCR amplified using primer Up and the non-biotinylated analogue of Down-B. PCR products were direcly subcloned through their EcoRI and BamHI site into pUC19 vector and the ligation products were transformed by electroporation into *E. coli* (electrocompetent Sure cells from Stratagene). White colonies were picked and plasmid DNA was sequenced by the Sanger method using the two primers EleA457 ACGCCAAGCTTGCAT and EleS357 GGGTTTTCCCAGTCACGA.

Sequence analysis

Alignment of the cloned insert sequence was done using CLUSTAL W multiple sequence alignment. The program uses the CLUSTAL W algorithm[23] to progressively align multiple protein or DNA sequences. The basic multiple alignment algorithm consists of three main stages: (i) all pairs of sequences are aligned separately in order to calculate a distance matrix giving the

divergence of each pair of sequences; (ii) a guide tree is calculated from the distance matrix, (iii) the sequences are progressively aligned according to the branching order in the guide tree.

Prediction of folding for the ssDNA aptamer pe35 and for the consensus sequence TGGAGCTTGGATTGATGTGGGTGTGAGTGCGGTGCCC was done using the Zuker algorithm[12] as described.[22]

K_d determination

Determination of dissociation constants (K_d) of the selected aptamers was performed by equilibrium dialysis.[15,24] The oligodeoxyribonucleotide sequences were pe35: AATTCGCTAGCTGGAGCTTGGATTGATGTGGTGTGTGAGTGCGGTGCCC, pe38: AATTCGCTAGCCGAGCAGACGGCGTACGGAATTCGAGCTCGGTACCCGGGGATCC, CMP: GGGCACCGCACTCACACACCACATCAATCCAAGCTCCA, and $(GT)_{30}$.

The equilibrium dialysis experiments were performed in duplicates using 600 μL dialysis chambers and cellulose acetate membranes with a molecular weight cutoff of 3500 Dalton (Spectrapore). 5 or 10 μM oligodeoxyribonucleotide in the SELEX buffer was equilibrated overnight at 25 °C with the different ligands (L-Tyrosinamide, L-Phenylalaninamide and L-Tyrosine) at three concentrations (1, 4 and 10 times DNA). After dialysis, the ligand concentration was determined by reaction with fluorescamine.[25] Briefly, 3 vol of the dialysis solution adjusted at pH 8.0 or 9.5 (Tyrosine) was added to 1 vol of 0.1 mM fluorescamine (Sigma) dissolved in acetonitrile, and mixed. The fluorescence of the complex formed was measured ($\lambda_{exc} = 390$ nm; $\lambda_{em} = 480$ nm) and corrected for the blank. To avoid competition for the reaction with the fluorophore between the primary amino group of Tris and the aminoacid, dialysis was performed in a SELEX buffer containing Hepes instead of Tris.

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