Spiegelmers: Biostable Aptamers

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1. Introduction: The SELEX Process and Aptamers

In the early 1980s the groups of Cech and Altman discovered catalytically active RNA molecules, the ribozymes.[1,2] This finding showed that RNA has more facets than just being the carrier and transporter of genetic information or serving as a structural element. From now on, nucleic acids could be regarded as molecules which have the potential to actively contribute to the catalytic and regulative processes that maintain cell life. The versatility of nucleic acids, which consist of only four different building blocks, is due to their ability to display distinct and stable 3D structures, thus being capable of forming binding pockets and catalytically active centres. In this respect nucleic acids are comparable to proteins.

Nucleic acid molecules have two properties that make them ideal candidates for the intelligent screening of vast combinatorial libraries: Not only are they highly structurally diverse molecules but they are also easily replicated carriers of their own structural information. The structural information of each molecule (the phenotype) is contained within its individual nucleotide sequence (the genotype) so that the amplification of molecules that survive a screening process for a desired phenotype is easily accomplished by standard enzymatic methods.

This property of nucleic acids combined with the possibility to generate the required immense structural diversity of up to 10^{15} different sequences by standard solid-phase oligonucleotide chemistry, allowed the establishment of an efficient evolutionary screening technology, the so-called SELEX process (systematic evolution of ligands by exponential enrichment). This technique, which is based on the principles of in vitro selection and in vitro evolution, was published independently by three research teams in 1990.[1]

Comparable to Darwinian evolution, nucleic acid libraries of RNA or DNA are subjected to a selection process in vitro. These libraries usually carry a central randomized region that is flanked by fixed sequences to facilitate enzymatic amplification through PCR. When molecules that bind to a given target structure are selected, the library is contacted with the immobilized target. Nonbinding molecules can be partitioned from binders by simple washing in an affinity chromatography-like process. Binding molecules that survive the selection procedure are then eluted and amplified. By executing this procedure iteratively, the complexity of the original library is depleted and target-binding candidates are enriched. As soon as the affinity of the enriched library cannot be further increased, generally after six to twenty selection rounds, the nucleotide sequences of individual binding molecules are determined by cloning and sequencing the enriched nucleic acid library. For target-binding nucleic acids that emerge from the process of in vitro selection, Ellington and Szostak coined the term 'aptamer' from the latin 'aptus', to fit.[3b] To date, the sequences and binding characteristics of more than one hundred aptamers against a plethora of different targets that range from small molecules, such as organic dyes, to membrane fragments and cells have been published. The reported dissociation constants are in the picomolar to micromolar range. For an excellent review on aptamers, see the book by James.[4]

2. Unmodified Aptamers Are Unstable In Vivo

The high specificity and affinity of aptamers selected against a given target molecule can be used to affect biological pathways by simply blocking molecular interactions. Because aptamers are biopolymers of natural origin, the omnipresence of degrading enzymes in virtually all biological fluids is a problem. In fact, nucleic acids are degraded by nucleases in blood with half-lives between seconds to a few minutes.[5] To allow use of RNA or DNA aptamers in a biological context, the stability problem has to be addressed.

A possibility to overcome this severe limitation, at least partially, is to introduce non-natural nucleotide analogues into the SELEX process (pre-SELEX modifications), which result in modified aptamers that should be less susceptible to nuclease action. Because the use of enzymes as amplification tools during in vitro selection is imperative, these nucleotide analogues have to be compatible with the amplification reactions, that is, they must allow for Watson–Crick base pairing, and they have to be substrates for the respective enzymes both as free NTPs and within a template strand.

In 1994, the first modified aptamers that contain 2′-NH_2-2′-deoxy-pyrimidines instead of 2′-OH-pyrimidines were selected.[6] RNA libraries containing these modified nucleotides could be successfully employed in selecting aptamers against several targets, including basic fibroblast growth factor (bFGF),[7] L-selectin,[8] human keratinocyte growth factor (hKGF),[9] and human thyroid stimulating hormone (hTSH).[10] The obtained 2′-NH_2-pyrimidine RNA aptamers exhibited a great increase in stability compared to 2′-OH-RNA. For the hKGF inhibitor, the half-life in human serum was extended from less than 8 seconds to 174 h, thus prolonging the life time approximately 80,000-fold;[9] for

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the bFGF inhibitor, only a 1000-fold increase in stability was reported.[7] The increased nuclease stability, however, came at the cost of weaker binding at physiological temperatures and longer binding sequences. Probably, the reason for this phenomenon is that the presence of 2′-NH_2-pyrimidines can decrease the stability of model DNA/DNA, RNA/RNA, and DNA/RNA duplexes.[11] In contrast, 2′-F-2′-deoxypyrimidine-based oligonucleotides, which can also be amplified enzymatically, show higher thermal stability. Aptamers raised against vascular endothelial growth factor (VEGF) by using 2′-F-pyrimidine-based RNA libraries, did in fact display higher affinities for VEGF than 2′-NH_2-pyrimidine-based aptamers.[12] One of the most affine 2′-F-pyrimidine RNA ligands was selected against hKGF with a dissociation constant in the low picomolar range.[9]

In addition to the introduction of 2′-modified pyrimidine nucleotides during the SELEX process itself, other non-natural nucleotide analogues can be introduced into already selected aptamers (post-SELEX modifications). Thus, the number of unmodified, nuclease susceptible positions can be further reduced. By stepwise exchange and subsequent experimental testing, all but four of the purine nucleotides in a VEGF ligand could be substituted by the respective 2′-*O*-methyl purine.[13] The resulting modified aptamer not only showed improved nuclease resistance but also a surprising increase in affinity, presumably because of higher thermal stability of the tertiary structure.

The introduction of non-natural nucleotide analogues involves the risk of unwanted side effects caused by the modified aptamers themselves or by breakdown of the products. In particular, the incorporation of nucleotide analogues into cellular RNA or DNA could lead to unforeseen consequences. Therefore, the potential toxicity of 2′-F-pyrimidines was assessed in experiments with rats and woodchucks. The results of these studies strongly suggest that the administration and degradation of 2′-F-pyrimidine RNA based therapeutics could lead to the incorporation of 2′-F-pyrimidines into cellular DNA. Nevertheless the studies also suggest that this is an event with marginal toxicological consequences.[14, 15]

Recently, two 2′-F-pyrimidine RNA aptamers with medical potential were developed in the group of Sullenger against the targets coagulation factor IXa and angiopoietin-2.[16, 17] However, to date, only one stabilized aptamer has entered clinical trials: the anti-VEGF 2′-F-pyrimidine RNA aptamer pegaptanib sodium (Macugen; NeXstar Pharmaceuticals Inc/Gliead Sciences Inc/Eyetech Pharmaceuticals Inc/Pfizer Inc).[12] This 27-nucleotide aptamer carries a 40 kDa polyethylene glycol moiety at its 5′-end to increase plasma half-life by reducing glomerular filtration. The biostability was further enhanced by coupling one 2′-deoxythimidine through a 3′-3′-linkage to the 3′-end. For Macugen, plasma half-lives of 9.3 h for intravenous and 12 h for subcutaneous administration were observed in rhesus monkeys.[18]

Much progress has been made over the past few years with the goal of developing aptamers as drug compounds, many efforts focusing on the improvement of in vivo stability.[19] Another, elegant concept to enhance biostability is to exploit the restricted substrate spectrum of nucleases not only by partly exchanging natural nucleotides by unnatural analogues as in the case of the 2′-NH_2- or 2′-F-pyrimidine aptamers (which still are substrates for many enzymes), but by the eversion of the whole 3D structure of the aptamer.

3. Mirror-Image Oligonucleotides: Biostable Aptamers

The omnipresent nucleases that account for the instability of aptamers consist of chiral building blocks, that is, L-amino acids. Consequently, the structure of nucleases is also inherently chiral, thus resulting in stereospecific substrate recognition. Hence, these enzymes only accept substrate molecules in the correct chiral configuration.[20] As naturally occurring nucleic acids are composed of D-nucleotides, an L-oligonucleotide should escape from enzymatic recognition and subsequent degradation. Unfortunately, in this case, due to the same principle nature developed no enzymatic activity to amplify such mirror-image nucleic acids. Accordingly, L-nucleic acid aptamers cannot be directly obtained by employing the SELEX process. However, the principles of stereochemistry reveal a detour that eventually leads to the desired functional L-nucleic acid aptamers.

If an in vitro selected (D-)aptamer binds its natural target, the structural mirror image of this aptamer binds with the same characteristics the mirror image of the natural target. Here, both interaction partners have the same (unnatural) chirality. Due to the homochirality of life and most biochemical compounds, such enantio-RNA ligands would be of limited practical use. If, on the other hand, the SELEX process is carried out against an (unnatural) mirror-image target, an aptamer recognizing this (unnatural) target will be obtained. The corresponding mirror-image configuration of said aptamer, the desired L-aptamer, in turn recognizes the natural target (Figure 1). This mirror-image selection process for the generation of biostable oligonucleotides was published first in 1996 by Fürste and co-workers[21,22] The approach couples fundamental chiral principles to the powerful screening technology of SELEX. This results in the

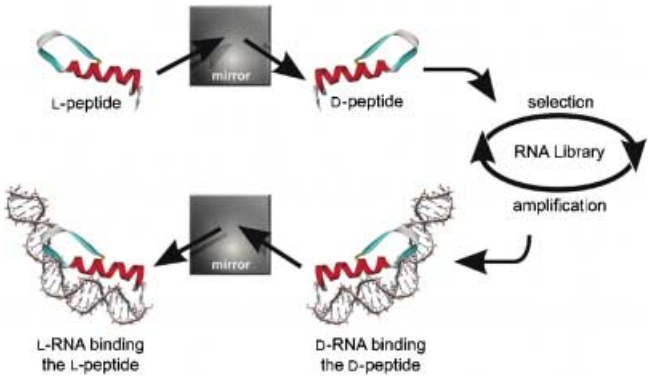

Figure 1. *The spiegelmer technology: Mirror-image in vitro selection. In a first mirroring step, the mirror-image of the L-peptide target of interest is chemically synthesized by using the unnatural D-amino acids. The SELEX process is then carried out with a conventional D-RNA library against the mirror-image (D-) peptide target. Individual (D-RNA) aptamers can finally be obtained by cloning and sequencing of the enriched library after several selection rounds. In a second mirroring step, the identified, mirror-image peptide-binding D-RNA sequences are synthesized by using the unnatural L-nucleotides. If the principles of chirality are followed, the resulting (L-RNA) spiegelmer binds to the natural target of interest.*

generation of functional mirror-image oligonucleotide ligands that display not only high affinity and specificity for a given target molecule but also biological stability. Such ligand-binding L-oligonucleotides were named 'spiegelmers' (from the German word 'Spiegel', mirror).

The first functional spiegelmers were designed to bind to the small molecules arginine and adenosine.[21, 22] They indeed exhibited the expected biostability, as demonstrated for the D-adenosine specific L-RNA spiegelmer. It showed no evidence of degradation in human serum, even after 60 h of incubation at 37 °C (Figure 2).[21]

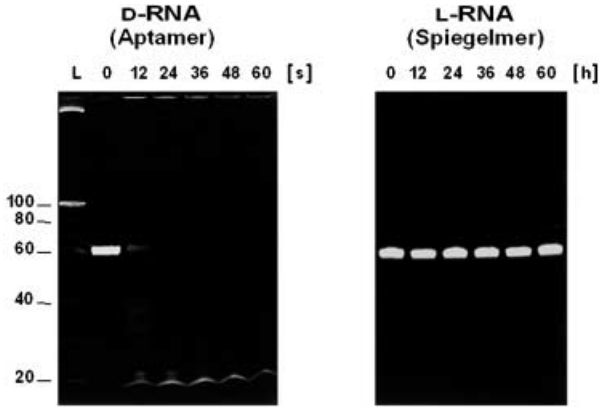

Figure 2. Biostability of natural D-RNA and L-RNA (spiegelmers). A 58-nt RNA aptamer and its corresponding spiegelmer were incubated at 37 °C in human serum. After indicated times, aliquots were taken and frozen in liquid nitrogen. Analysis was performed by denaturing PAGE with subsequent ethidium bromide staining. The timescale for D-RNA is in seconds, for L-RNA (spiegelmer) in hours. (Reprinted with permission from the Nature Publishing Group and S. Klussmann, A. Nolte, R. Bald, V. A. Erdmann, J. P. Fürste, Nat. Biotechnol. ***1996***, 14, *1112 – 1115).*

For the selection against adenosine, L-adenosine was used as the target. High affinity binding motifs could be identified that showed the expected reciprocal chiral specificity; the estimated dissociation constants for the heterochiral binding pairs (aptamer: L-adenosine; or speigelmer: D-adenosine) were in the range of 2 μM; the homochiral pairs (aptamer: D-adenosine; or spiegelmer: L-adenosine) exhibited dissociation constants of $>$ 20 mM. The mirror-inverted circular dichroism (CD) spectra of the adenosine-binding aptamer (D-RNA) and spiegelmer (L-RNA) indicate that the tertiary structures of both oligonucleotides are also mirror-inverted as well (Figure 3). Consequently, the chiral inversion is responsible for the reciprocal chiral specificity of the oligonucleotides.

The concept of coupling chiral principles to an evolutionary selection process is not limited to nucleic acids. In fact, the first pioneering work concerning mirror-image peptides was published in 1996 describing a modified phage display approach.[23] In this so-called "mirror-image phage display", a D-peptide target is used to select for peptides from a phage display library expressing random L-amino acid peptides. By using this technique, a D-peptide could be identified that interacts with the Src homology 3 domain of chicken Src.

An inherent complication of these mirror-image selection techniques is the synthesis of the target structure in the

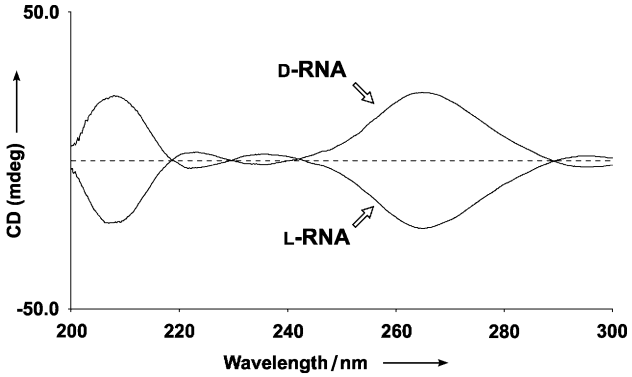

Figure 3. Circular dichroism (CD) spectra of D-RNA and L-RNA (spiegelmer). CD spectra of a 38-nt aptamer and its corresponding spiegelmer were recorded on a JASCO J-600 spectropolarimeter. (Reprinted with permission from the Nature Publishing Group and A. Nolte, S. Klussmann, R. Bald, V. A. Erdmann, J. P. Fürste, Nat. Biotechnol. ***1996***, 14, *1116 – 1119).*

appropriate non-natural configuration. Most pharmacologically relevant target structures are peptides and proteins. Peptides are readily synthesized in their mirror-image configuration by employing standard synthesis methods, but with D- instead of L-amino acid building blocks.[24] However, standard peptide synthesis is currently limited to sequences of approximately 100 amino acids; though by additionally using peptide-ligation techniques, even longer peptides and proteins can be obtained.[25] If the target protein is not accessible to chemical peptide synthesis, physically stable epitopes or domains have to be defined and synthesized. Such domains should be functional, or at least close to a functional site, to accomplish the inhibition of the targeted protein – protein interaction by spiegelmer binding. Furthermore, these domains should be located on the surface of the protein because a resulting spiegelmer must recognize the peptide segment in the context of the whole protein. By employing this so-called 'domain approach', a spiegelmer to staphylococcal Enterotoxin B could be identified by selecting aptamers against a 25 amino acid domain of the 28 kDa full-length protein.[26]

4. Spiegelmers with Biological Activity

The first biological activity of a DNA spiegelmer in the context of a cell-culture assay was reported in 1997 by the research groups of Kim and Bartel.[27] This spiegelmer, specific for the peptide hormone L-vasopressin, was obtained by performing an in vitro selection against the unnatural D-vasopressin. After several selection rounds, the enriched pool was again partly randomized and subjected to an additional selection scheme. As a result of this procedure, a 55 nt long vasopressin binder was identified, which was synthesized as aptamer (D-DNA) and spiegelmer (L-DNA). The dissociation constants for both binding complexes (aptamer: D-vasopressin; and spiegelmer: L-vasopressin) were determined to be around 1 μM by equilibrium dialysis. In a cell-based assay with cultured kidney cells expressing the V_2 vasopressin receptor, inhibition of the vasopressin response could be demonstrated for the spiegelmer with a calculated IC_{50}

value in the low micromolar range. An L-DNA control sequence with the same base composition and secondary structure as the aptamer was virtually inactive in the same assay.

More biological data for spiegelmers that bind to pharmacologically relevant targets was generated by Leva et al.,[28] who identified RNA- and DNA-spiegelmers against the peptide hormone gonadotropin-releasing hormone I (GnRH). GnRH, a key hormone of the mammalian reproductive cycle, is a decapeptide which is produced in the hypothalamus and binds to a single class of G-protein coupled receptors on gonadotrophic cells of the pituitary gland. This process triggers secretion of the gonadotropin-luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which, in turn, stimulates the production of sexual steroids.

Both RNA- and DNA-spiegelmers exhibited dissociation constants in the range between 50 and 100 nM for the speigelmer:L-GnRH complexes. The binding specificity was examined by using related peptides such as chicken luteinizing hormone-releasing hormone LHRH (one exchanged amino acid compared to GnRH) and the GnRH receptor agonist buserelin as well as the nonrelated peptides vasopressin and oxytocin; both spiegelmers did not recognize any of these peptides, thus underlining their high specificity.

A GnRH receptor-expressing cell line (Chinese-hamster ovary cells) was used to demonstrate the potential of the spiegelmers to inhibit GnRH binding to its receptor. By measuring Ca^{2+} release after receptor stimulation through GnRH, IC_{50} values were determined to be 50 nM for the DNA- and 200 nM for the RNA-spiegelmer.

5. Spiegelmers In Vivo

The in vitro characterization of anti-GnRH speigelmers was swiftly followed by animal studies to demonstrate in vivo activity of these molecules[28–30]. For these experiments, an improved anti-GnRH DNA-spiegelmer with increased affinity to GnRH was generated (NOX 1255). This molecule was further modified with a 40 kDa polyethylene glycol moiety attached to the 5′-end (NOX 1257) to prolong the residence time of that spiegelmer in the bloodstream to >12 h. The IC_{50} of NOX 1257 in GnRH receptor expressing CHO cells was calculated to be 20 nM. The in vivo activity of NOX 1255 and 1257 was analyzed in a widely used rat model for studying GnRH regulation. The efficacy parameter is the elevated LH concentration in the serum of orchidectomized (castrated) rats which can easily be determined by radioimmunoassays. If the GnRH activity is neutralized by an antagonist, the serum LH concentration returns to the basic level, which was determined in a control group of intact rats. When the elevated LH level had equilibrated eight days after orchidectomization, NOX 1255 was administered subcutaneously at 100 mg kg^{-1}, whereas PEGylated NOX 1257 was injected intravenously at 150 mg kg^{-1}. The receptor antagonist Cetrorelix, a peptide analogue, was used as positive control. The subcutaneoulsy administered NOX 1255 showed maximal GnRH antagonism 1.5 h after administration. This effect leveled off during the following hours, a finding which probably arose from fast renal clearance of the unmodified spiegelmer. After administration of the PEGylated spiegelmer, NOX 1257, the observed LH levels were very similar to the control group, which received Cetrorelix. In both groups, LH level suppression was maintained during the entire observation period of 24 h, thus demonstrating the pharmacological potency of spiegelmers.

When developing macromolecules into drugs, a major safety concern is their immunogenic potential. The elicitation of antibodies by administration of spiegelmers was examined in Zimmermann rabbits. Both PEGylated NOX 1257 and non-PEGylated NOX 1255 were repeatedly administered to animals in three parallel groups following a standard immunization protocol. The test substances were injected alone, together with an adjuvant, or conjugated to the immunogen bovine serum albumin (BSA) plus adjuvant. No titers of antibodies could be detected in serum from animals that had received spiegelmer with or without adjuvant. Only the BSA-spiegelmer conjugates had evoked a minuscule immune response against the spiegelmer part, whereas the BSA-specific antibody titer was very high. These results suggest that spiegelmers have only weak, if any, antigenic potential.[30]

6. Conclusion

The development of aptamers into drugs is severely limited by their in vivo stability. The concept of combining the principles of chirality with the powerful SELEX process has led to the development of highly biostable and extremely specific agents, spiegelmers, which are capable of inhibiting pharmacologically relevant targets.

During the last few years, spiegelmers have proved to be molecules with great therapeutic potential. Spiegelmers are macromolecules with very low immunogenicity, and their efficacy has been strikingly demonstrated in vivo. Further investigation into the potential of spiegelmers is in progress and it is expected that mirror-image oligonucleotides will have applications in miscellaneous medicinal disciplines.

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