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# Reflections on a Novel Therapeutic Candidate

Minireview

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**Aptamers composed of L-nucleic acids, Spiegelmers, were selected to specifically bind GnRH. Spiegelmer inhibition of GnRH activity was demonstrated in both cellular and animal models. Rabbit studies showed minimal immunogenic response to the agents.**

Aptamers are selected nucleic acid species that typically bind their targets with high affinities and specificities, rivaling those of antibodies. They are usually isolated from a random sequence nucleic acid library of up to $10^{15}$ species via several rounds of affinity partitioning and in vitro amplification (see Figure 1). Unfortunately, aptamers composed of natural nucleotides are subject to degradation by organismal or environmental nucleases.

To obviate problems with stability, Fürste developed a variation on the "reflection selection" pioneered by Peter Kim and coworkers [3, 4, 6]. These researchers selected natural L-amino acid peptides that bound to D-peptide targets; synthesis of the selected sequences as D-peptides resulted in compounds that bound to the natural L-peptide targets. Similarly, D-deoxyribose aptamers selected against synthetic D-peptide targets can be resynthesized using L-nucleotides. The resultant L-oligonucleotide "Spiegelmers" (from the German "spiegel," for mirror) will again bind the naturally occurring L-peptide targets with similar specificity and affinity. This was demonstrated by Fürste in his selections against mirror-image adenosine and arginine molecules [3, 4] and later by Bartel in a selection against D-vasopressin, a nine-residue cyclic peptide [5]. Spiegelmers are nuclease resistant because the mirror-image L-oligonucleotides are not found in nature and most nucleases have instead evolved to recognize at least some portion of the natural D-sugars. The combination of low $K_d$ values, high specificity, and nuclease resistance is potentially attractive for therapeutic development, yet the path from academic discovery to clinical application is fraught with difficulties, as most biotechnology companies eventually realize.

Thus, it is all the more remarkable that Klussmann and his coworkers at NOXXON have managed to shepherd the scientific invention to the point of being a potential pharmaceutical candidate [1, 2]. As with other biotechnology companies, one of the first problems NOXXON faced was choosing an interesting target for pharmaceutical development. In this respect, GnRH is a decapeptide that binds gonadotrophic cell receptors on the pituitary gland. Binding results in secretion of gonadotropin-lutenizing hormone (LH) and follicle-stimulating hormone (FSH). These, in turn, stimulate the production of sexual hormones, a process that is linked to certain diseases, including malignant breast and prostate cancers. It is therefore possible that anti-GnRH therapy may prove useful for the treatment of these cancers and other sexual-hormone-dependent proliferative disorders. Cetrorelix is a current clinical treatment that acts as a GnRH receptor antagonist. It is noteworthy that NOXXON chose to target the receptor ligand instead of directly targeting the receptor; the ability to interdict the function of either small hormones or their larger receptors may be a novel advantage of aptamers as therapeutics.

In order to generate anti-GnRH Spiegelmers for further development, Klussmann and coworkers began with both RNA and ssDNA libraries that contained $10^{15}$ different species [1]. Each library consisted of D-oligonucleotides with a central randomized region of 60 bases. Figure 1 illustrates the selection process. Following several rounds of selection, the anti-GnRH aptamers were sequenced and assayed for their binding ability. From the RNA selection, clone A10 demonstrated the best binding with a $K_d$ value of 92 $\pm$ 12 nM; DNA aptamer clone S42 demonstrated the best binding with a $K_d$ value of 55 $\pm$ 7 nM. The Spiegelmers have binding affinities that are similar to those of other aptamers [9] and that approach those of antibodies, which typically have a $K_d$ value less than 10 nM.

The specificities of the Spiegelmers also proved to be suitable for further development. Chicken LHRH, which differs from GnRH by only one amino acid, bound 50-fold worse to the RNA Spiegelmer [1]. The DNA Spiegelmer bound the chicken protein over 400-fold more poorly than the human protein. Similarly, buserelin, a GnRH analog, showed an affinity difference of 1000-fold for the DNA Spiegelmer. No binding to the unrelated proteins vasopressin or oxytocin could be detected.

While aptamers are frequently overlooked as drug candidates, the process by which they can be optimized for pharmaceutical applications is in fact very flexible when compared with the laborious medicinal chemistry procedures common to small-molecule drug design. For example, to minimize the amount of material that would have to be synthesized for further studies (and eventually for applications), the deletion constructs of the aptamers were generated, and the RNA aptamer was readily truncated to 50 nucleotides and the DNA aptamer to 60 nucleotides [1]. The aptamers were then directly synthesized using L-nucleotide phosphoramidites. The binding affinities of the truncated species rose slightly: the DNA Spiegelmer had a $K_d$ value of 263 nM, while the RNA Spiegelmer had a $K_d$ value of 190 nM. Even after inversion, the Spiegelmers maintained their binding specificities.

These proof-of-principle demonstrations of the Spiegelmer technology were quickly followed by attempts to move the molecules from mere characterizations to efficacy studies. The ability of the Spiegelmers to inhibit

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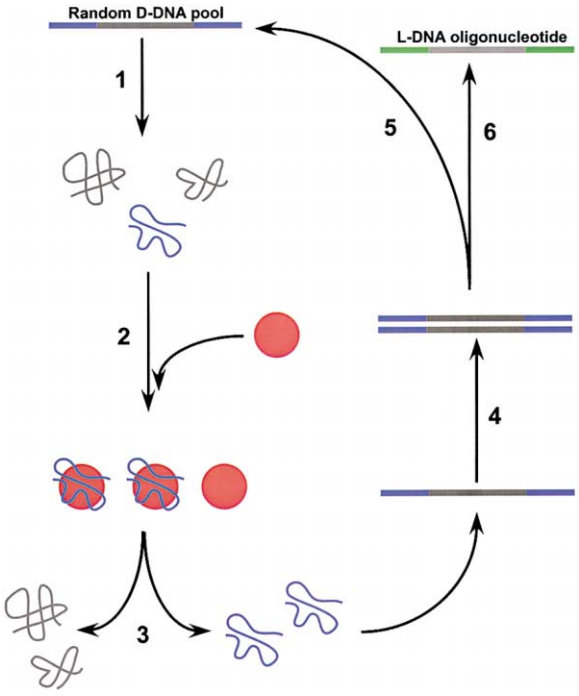


Figure 1. In Vitro Selection Process for DNA Aptamers and Spiegelmers

(1), Single-stranded, randomized D-DNA pool containing approximately $10^{15}$ different species; (2), DNA pool is incubated with target molecules and allowed to bind; (3), nonbinding species are partitioned away from binding species; (4), binding species are PCR amplified; (5), PCR product is single-strand separated, and the leading strand is subjected to further rounds of selection; (6), after several rounds of selection, binding species are cloned, sequenced, and assayed for activity. Successful binders are resynthesized using L-nucleotides.

GnRH response was first examined in Chinese hamster ovary (CHO) cells expressing the human GnRH receptor [1]. The RNA Spiegelmer inhibited GnRH response with an $IC_{50}$ of 200 nM, and DNA Spiegelmers inhibited with an $IC_{50}$ of approximately 20 nM. These low values are surprising and gratifying, given the slight hits in affinity the truncated molecules had taken. Not only were the Spiegelmers effective in cellular assays, but their specificities were maintained and no decreases in cell viability were observed.

One potential concern with a biopolymer drug is its immunogenicity. To study whether Spiegelmers would elicit antibodies, Zimmermann rabbits were inoculated with NOX 1255 and NOX 1257 over a six-week period [2]. Rabbits receiving Spiegelmer or Spiegelmer with adjuvant produced no immune response, while those receiving NOX conjugated to cBSA produced low serum titers in the range of 1:1000–1:3000 (much less than the immune response observed with a true antigen). These are some of the first immunogenicity studies with selected nucleic acids, and the results are therefore especially gratifying for the field as a whole.

A number of groups have previously demonstrated the efficacies of aptamers in tissue culture [5, 7], but there have been few attempts to apply these results to animal studies. One notable exception is an RNA aptamer to VEGF165. Wiles assayed for activity in tissue culture [7], and Floege conducted studies in rats [8]. The NOXXON team bridged this gap using a well-studied animal model of GnRH regulation, castrated male Wistar Shoe rats [2]. In these animals, LH levels are high as a result of low testosterone levels. However, the LH level in plasma can be returned to normal by inhibiting GnRH activity (for example, by the peptide drug mentioned earlier, Cetrorelix, a GnRH receptor antagonist). Just as the lengths of the Spiegelmers were handily manipulated, their pharmacokinetics could be rationally engineered by the addition of a 40 kDa polyethylene glycol (PEG) moiety to the 5′ end of NOX 1255, creating NOX 1257. The drug candidate NOX 1255 was administered subcutaneously, while the modified NOX 1257 was administered intravenously. The unmodified Spiegelmer maximally suppressed serum LH levels 1.5 hr after administration and maintained suppression for 4.5 hr. The modified Spiegelmer reduced LH levels to that of intact rats for 48 hr. These effects were similar to those observed with Cetrorelix. While this was an amazing demonstration of the viability of Spiegelmers as therapeutic agents, the fact remains that the affinities and/or systemic availabilities of the Spiegelmers may yet limit their applicability. The nucleic acid drugs had to be administered at molar amounts 30-fold higher than the peptide Cetrorelix to get equivalent effects, yet were on the order of 20 times larger. Given the likely difficulties and costs of synthesizing Spiegelmers relative to small organics, this might ultimately limit their viability as drug candidates.

### Selected Reading

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