Chemistry & Biology, Vol. 9, 351–359, March, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00111-4

GnRH Binding RNA and DNA Spiegelmers: A Novel Approach toward GnRH Antagonism

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Summary

Mirror-image oligonucleotide ligands (Spiegelmers) that bind to the pharmacologically relevant target gonadotropin-releasing hormone I (GnRH) with high affinity and high specificity have been identified using the Spiegelmer technology. GnRH is a decapeptide that plays an important role in mammalian reproduction and sexual maturation and is associated with several benign and malignant diseases. First, aptamers that bind to D-GnRH with dissociation constants of 50–100 nM were isolated out of RNA and DNA libraries. The respective enantiomers of the DNA and RNA aptamers were synthesized, and their binding to L-GnRH was shown. These Spiegelmers bind to L-GnRH with similar affinity to that of the corresponding aptamers that bind to D-GnRH. We further demonstrated dose-dependent inhibition of GnRH-induced Ca^{2+} release in Chinese hamster ovary cells that were stably transfected with the human GnRH receptor.

Introduction

The technique of in vitro selection of combinatorial oligonucleotide libraries has been used to isolate nucleic acid ligands that recognize a wide range of target molecules with affinities and specificities comparable with or even excelling those of antibodies [1, 2]. Over the last years, tremendous progress has been made in the field, and it has been anticipated that these so-called aptamers will rival antibodies in their applications as therapeutic and diagnostic agents in the future [3].

However, the utility of oligonucleotide-based therapeutics is limited due to their susceptibility to nucleases present in biological fluids. The Spiegelmer technology provides an elegant way to circumvent this limitation using mirror-image L-DNA or L-RNA aptamers. Previously, it has been shown that the principles of reciprocal chiral substrate specificity, coupled to the in vitro selection process, make it possible to generate L-oligonucleotide ligands, so-called Spiegelmers, that are insensitive to enzymatic degradation. Using in vitro selection, an oligonucleotide sequence that binds the synthetic enantiomer of the target molecule, e.g., a D-peptide, is isolated. Subsequently, the selected nucleic acid ligand is chemically resynthesized in the enantiomeric L-configuration. Following the principle of chiral inversion, this Spiegelmer will bind to the physiological target with the same affinity and specificity as the aptamer to its mirror-image target. This strategy has been used to identify L-RNA ligands of arginine and adenosine and an L-DNA ligand of vasopressin [4–6].

We have identified DNA and RNA Spiegelmers that bind to the peptide hormone gonadoliberin (gonadotropin-releasing hormone, GnRH I) with high affinity and high specificity. GnRH is a decapeptide that is released from neurons of the hypothalamus in a pulsative manner [7–9], binding to receptors on gonadotrophic cells of the pituitary gland. GnRH triggers the secretion of the gonadotropin-luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [10]. The gonadotropins stimulate the production of sexual steroids (estrogens, progesterone, or testosterone), which regulate the maturation of reproductive and other target organs. In addition, sexual hormones are involved in pathological states like endometriosis [11, 12] and uterine fibrosis [13, 14] as well as in malignant tumors like breast and prostate cancer [15]. These tumors can be treated with GnRH analogs that lead to an inactivation of gonadal steroid production [16].

Using the Spiegelmer technology, we have been able to generate GnRH binding L-RNA and L-DNA ligands. Both Spiegelmers specifically antagonize the response to GnRH in cell culture and therefore demonstrate their activity in vitro. Since lowering the concentration of free GnRH by Spiegelmers would reduce the gonadotropin release and thus the production of the sexual hormones, GnRH binding Spiegelmers can be envisioned for the treatment of benign and malign hormone-responding tumors. Further possible applications include their use as contraceptives and as gonadotropin-lowering drugs in the context of in vitro fertilization [17].

Results

Isolation of D-GnRH Binding RNA and DNA Ligands

Using the method of in vitro selection, we sought to isolate both RNA and DNA ligands of D-GnRH from oligonucleotide libraries with an initial complexity of 10^{15} different RNA or ssDNA molecules, respectively. Each population contained a central core of 60 completely randomized positions. Affinity chromatography using D-GnRH immobilized on thiol-modified sepharose was employed to separate binding from nonbinding oligonu-

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Table 1. Sequence Analysis of the Clones Obtained by RNA and DNA In Vitro Selection

(A) RNA Sequence of the Random Region	
-G-CCGATG-CGTCTCCTAATTGATAGAAAGACTACTTGAGCCCTTAAATGAGGTTATGTGCA	A10 59x
----------G	A01 6x
---------C----------	A06 2x
----------------A----------	B05 1x
-----T----------	B06 2x
A----------	C12 1x
--C------C----------	C02 1x
-------C----------	D02 1x
----------Δ	E10 1x
----------------------------T----------	G02 1x
GTTGGGAAGGGGTATGACCGACAGTAGGCGTCCTGATCTCCTGCGGCCTACTCCAACGCC	D03 2x
TCGTCGCTTGGCATACATCTCGAGCAATGATTCGTTGCAGATAACTGTGAGAGCATGGTGC	B04 1x
GTCACGAATTTGGATTGTGGCTTGAGAGCGTCTGACTGCTTGTCTAGACAAACTGTCGCG	D12 1x
AATTCGTTAACGGGTCTAATTCTAACTTACGGTAAGCCGGAGTCGAAATTAGGTTACGCG	F01 1x
(B) DNA Sequence of the Random Region	
GGACGGCGTGGTAGGCTCAGGCGATATGCACGTGTGGATCACCTACACTTTGCTTGGGTG	S40 10x
TATGGGCTGGGCAAGGGCTGGACAACAGATCCGTAGCTGTGGTGGCTGGCATACTGTAGG	S15 4x
CGATCGCCACCACATGATAATAGCTTGTGTGAGGTACCAAGTGCATCTGTCCGTGGA	S09 15x
AAAAGGATATTCGGCGTGCACATGGCCTCTCGGAGAAGTGCAGGGGTGGGTGGGAGCGTT	S20 5x
CAAGACGACGGACGAGGGGTTAGGGGATTGATAGTAATCTCGGATATCCCCTCAAGCTCA	S03 10x
AGGGTGGGCTGGGGCTGGGCCGGGGGGCGTGAGCGGAAAGTGCGACACGTAGCCTTGCA	S42 1x
GCAGCTGCTGAGAAGCGAGCGTGGTGACTGGGTAATGTAATGGGGAAGGGCTGTATGGTT	S35 1x
TAGTGGGGACCCCAATCTGTAGATGCCCGTAACGGGCTGGGGNTNCTGACACGGCATGGT	S10 1x
GAAACCACGGGGACCCGCAATTGTGGGATAGCTACGTTCACATCACGTCAGCTATAGT	S04 1x

The triangle indicates a base deletion compared to clone A10, and the hyphens indicate base homology to clone A10. The numbers in the second column show the frequency with which the respective sequence was found.

cleotides. D-GnRH binding molecules were eluted from the column by affinity competition with D-GnRH in solution and amplified by either RT/PCR and transcription for the RNA selection or by PCR for the DNA selection, respectively. For the DNA selection experiment, positive DNA strands were obtained from double-stranded PCR products based on the use of a negative-strand PCR primer with an uncopyable tail [18]. The enriched oligonucleotide population formed the starting pool for the next round of selection. After the second round of selection, a negative selection on underivatized resin was introduced to prevent the accumulation of nonspecific column binding aptamers. The stringency of the selection experiments was progressively increased by decreasing the ratio of target to nucleic acid. Affinity elution with D-GnRH included long incubation times in order to encourage the isolation of high-affinity aptamers with long off rates (see the Experimental Procedures).

Only 0.01% of the input RNA and ssDNA populations was eluted with D-GnRH during the first selection cycle. The percentage of eluted RNA increased up to 20% in the sixth round of selection and up to 13% in the eighth round for ssDNA. Oligonucleotides recovered from the respective rounds were converted to dsDNA for cloning and sequencing.

Among the 80 clones of the RNA selection that were sequenced, one sequence was found 59 times (A10, see Table 1A). An additional 16 sequences differed from this parent sequence only by point mutations or by single base insertions or deletions. Another five sequences did not show any similarity to the parent sequence (see Table 1A).

Sequencing of 48 clones from round 8 of the ssDNA selection revealed 9 different sequences. One sequence was found 15 times, 2 sequences were found 10 times, and 2 sequences occurred 4 and 5 times, respectively. Four sequences just occurred once. Computer-assisted alignment of distinct sequences from the ssDNA selection did not reveal any common motif based on primary sequence conservation (see Table 1B).

Using equilibrium dialysis, we screened the affinities of individual sequences to D-GnRH from both RNA and DNA selections. Of the sequences identified from the RNA selection, sequence A10, which accounted for the majority of the selected population, showed the highest affinity to D-GnRH, with an equilibrium dissociation constant of $K_D = 92 \pm 12$ nM. Of the DNA ligands tested, clone S42, which is unique among the selected molecules, bound to GnRH with an affinity significantly higher than that of all other DNA binding sequences. The equilibrium dissociation constant was found to be $K_D = 55 \pm 7$ nM for D-GnRH binding (data not shown).

Identification of Minimal Binding Domains

Based on the results obtained from the affinity screening of individual RNA and DNA ligands, sequences A10 (RNA) and S42 (DNA) were chosen for further characterization.

In order to determine the minimal sequence requirements needed for D-GnRH binding, various truncated and mutated fragments of both sequences were generated, and their respective binding affinities to D-GnRH were determined by equilibrium dialysis. In addition, enzymatic and chemical probing was carried out to evaluate the secondary structure formed [19]. By combining the results of these experiments with secondary structure predictions using the Zuker *mfold* computer algorithm (*mfold* Zuker 2.3 [20, 21], *mfold* Zuker 3.0 [22]),

A

GGGAAUUCGAGCUCCUGACAGCCGAUGCGUCUCCU<u>AAUUGAUAGAAAGACUACUUGAGCCCUUAAAUGAGGUUAUGUGCA</u>**<u>AUU</u>CGAAGACGUCCAGCUGA**

CGGCC----------GGCCG

B

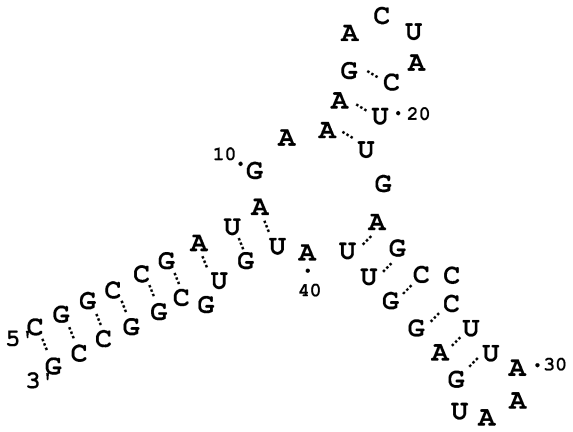

Figure 1. Sequence and Proposed Secondary Structure of the RNA Ligand

(A) Sequence of RNA clone A10. The defined primer binding sites are shown in bold. The 48-nt long binding core is underlined. Base substitutions introduced for thermal stabilization of the RNA oligonucleotide are indicated with respect to their position in the original molecule in the line below.

(B) Proposed secondary structure model of the 50-nt RNA oligonucleotide ligand.

we were able to effectively truncate the RNA and DNA aptamers to their minimal binding motifs.

The D-GnRH binding domain of the RNA aptamer consists of a 48 nucleotide-(nt) sequence formed by parts of the original random region including three nucleotides of the 3′ primer binding site (Figure 1A). The secondary structure model obtained from the RNA *mfold* program and the results from DMS and kethoxal probing and enzymatic probing with nucleases S1 and T2 (data not shown) suggest that the 48-nt D-GnRH binding ligand forms a three-way helix junction with four unpaired nucleotides at the branching point. To increase the thermal stability of the molecule, the A:U base pairs at the 5′- and 3′-terminal helical region of the 48-nt binding region were changed to G:C base pairs without affecting affinity. An additional G:C base pair was added to further stabilize the secondary structure (Figure 1). Any attempts to further truncate the molecule by either deleting base pairs from the proposed stems or single bases from unpaired regions (e.g., deletion of G44) resulted in a complete loss of binding.

The 99-nt sequence of the DNA ligand S42 could initially be truncated to a 68-mer oligonucleotide by removing parts of the original primer binding sites that were not essential for binding to D-GnRH (Figure 2A). To investigate the potential secondary structure elements of the 68-mer DNA ligand, we used chemical probing on the bases of G, A, and T as well as enzymatic probing with S1 nuclease (data not shown). The results suggest the presence of a loop between bases 42 and 48 (see Figure 2B). G probing disclosed long stretches of protected guanosines, interrupted by unprotected pyrimidines (T probing), indicating a possible G-quartet-like structure (data not shown). Combining these results with the data obtained by secondary structure prediction, we proposed the secondary structure model shown in Figure 2B.

For further truncation of sequence S42, we deleted the bases A39–A52 and introduced a thermodynamically stable GTAA loop closed by a G:C base pair. In addition, the base pairs T3:A66 and A6:T63 were substituted by G:C base pairs. In order to circumvent the problematic synthesis of four consecutive G nucleotides, the terminal C:G base pair was inverted. The substitutions and deletions led to a 60-nt aptamer (Figure 2C). We were not able to further truncate the DNA ligand without significant loss of binding affinity. The deletion of the unpaired nucleotides C53 and T54, for example, led to a complete loss of binding. In contrast to the thrombin aptamer [23, 24], a 15-nt DNA ligand that adopts a highly compact G-quartet structure that is sufficient for binding, we observed that the isolated G-quartet fold is not able to bind to D-GnRH.

Functional Characteristics of the RNA and DNA Spiegelmers

The mirror-image L-oligonucleotides corresponding to the 50-nt RNA ligand and the 60-nt DNA ligand were generated by standard solid phase synthesis using enantiomeric nucleotides.

The affinity of the DNA Spiegelmer to L-GnRH was determined by equilibrium dialysis and compared to the data obtained for the 60-nt D-DNA ligand to D-GnRH. As demonstrated in Figure 3A, both the DNA aptamer as

A

CCAAGCTTGCATGCCTGCAGAGGGTGGGCTGGGGCTGGGCCGGGGGGCGTGAGCGGAAAGTGCGACACGTAGCCTTGCA**GGTACCGAGCTCGAATTCCC**

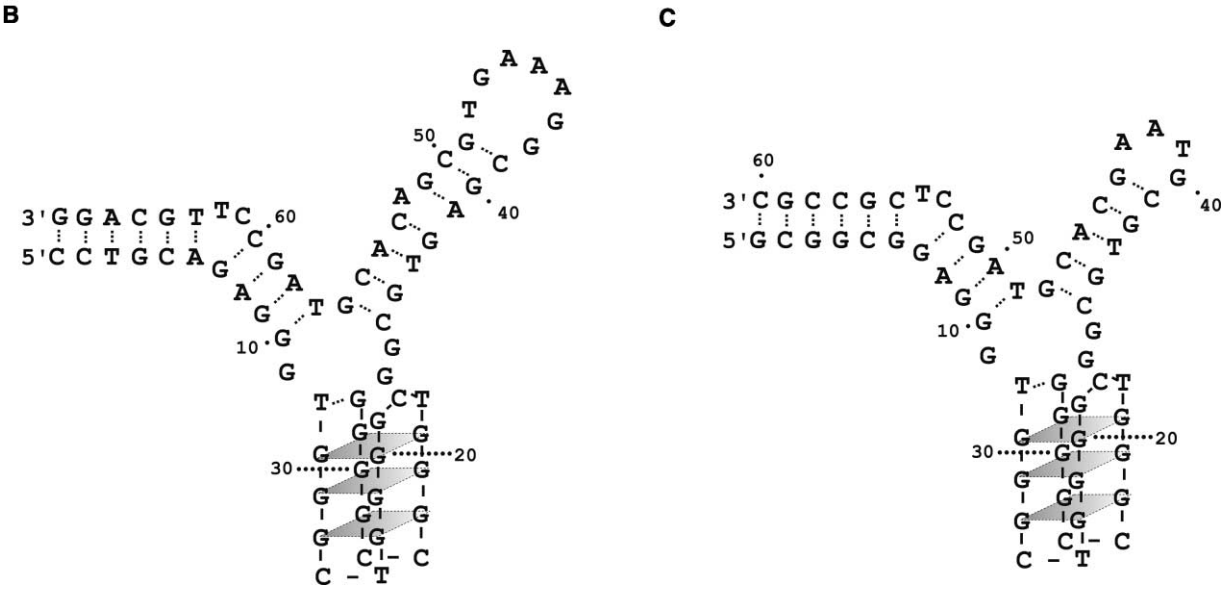

Figure 2. Sequence and Proposed Secondary Structure of the DNA Ligand

(A) Sequence of DNA clone S42. The defined primer binding sites are shown in bold. The 68-nt long binding core is underlined.

(B) Proposed secondary structure model of the truncated 68-nt DNA oligonucleotide ligand.

(C) Secondary structure model of the modified 60-nt DNA oligonucleotide ligand. Bases A39–A52 of the 68-nt long version of S42 were replaced by a thermodynamically stable GTAA loop closed by a G:C base pair. In addition, base pairs T3:A66 and A6:T63 were substituted by G:C base pairs. In order to simplify chemical synthesis, the terminal base pair C1:G68 was inverted.

well as the Spiegelmer bind to their respective peptide targets with an equilibrium dissociation constant of about 45 nM.

Since reproducable determination of the equilibrium dissociation constants of the 50-nt D- and L-RNA ligands could not be obtained by equilibrium dialysis, possibly because of diffusion of a fraction of the RNA through the membrane, isothermal calorimetry was used for determination. Figures 3B and 3C show typical calorimetric profiles of GnRH binding to the RNA oligonucleotide ligands. The data sets satisfactorily fit with a one-site model after subtraction of a constant peak area to correct for dilution effects. A total of 37.7 ± 0.2% of the D-RNA oligonucleotides have one binding site with an equilibrium constant of 97 ± 7 nM, while the remaining molecules do not bind significantly. This corresponds to a nominal equilibrium constant of 263 nM. The standard reaction enthalpy of the interaction is $\Delta H = -1.8$ kcal mol^{-1}.

For the L-RNA oligonucleotide, 46.1 ± 0.3% of the Spiegelmers have one binding site with an equilibrium constant of 87 ± 9 nM, while the remaining molecules do not bind significantly. This results in a nominal equilibrium constant of 190 nM. The standard reaction enthalpy of the interaction is $\Delta H = -1.8$ kcal mol^{-1}.

To examine the binding specificity of the RNA and DNA Spiegelmer, their affinity to various neuroactive peptides (see Table 2), both unrelated and related to GnRH, was determined by real-time kinetic measurements using a Biacore 2000. The respective Spiegelmer was biotinylated at its 5′ end and immobilized on the surface of a streptavidin-coated sensor chip. The interaction of bound Spiegelmers with increasing concentrations (0.1–1000 μM) of the neuropeptides was monitored in real-time with the change in the SPR response. For analysis, the responses are scaled to the molar mass of GnRH, and maximum binding of GnRH is set to 1 (100%). These responses are plotted versus the logarithm of the concentration of analyte. As shown in Figure 4 for both Spiegelmers, saturation of binding can only be observed in the case of GnRH at the peptide concentrations used in this assay. Both Spiegelmers are able to discriminate between GnRH and chicken LHRH, which differ only in one amino acid (Arg8 is changed to Gln) by an affinity difference of 50-fold or greater for the RNA Spiegelmer and a factor of 400 or greater for the DNA Spiegelmer. While the DNA Spiegelmer binds to the GnRH analog buserelin with at least 1000-fold reduced affinity, the RNA Spiegelmer shows an at least 5-fold reduced affinity to buserelin. No binding, even at high concentrations, can be observed for the completely unrelated peptides vasopressin and oxytocin.

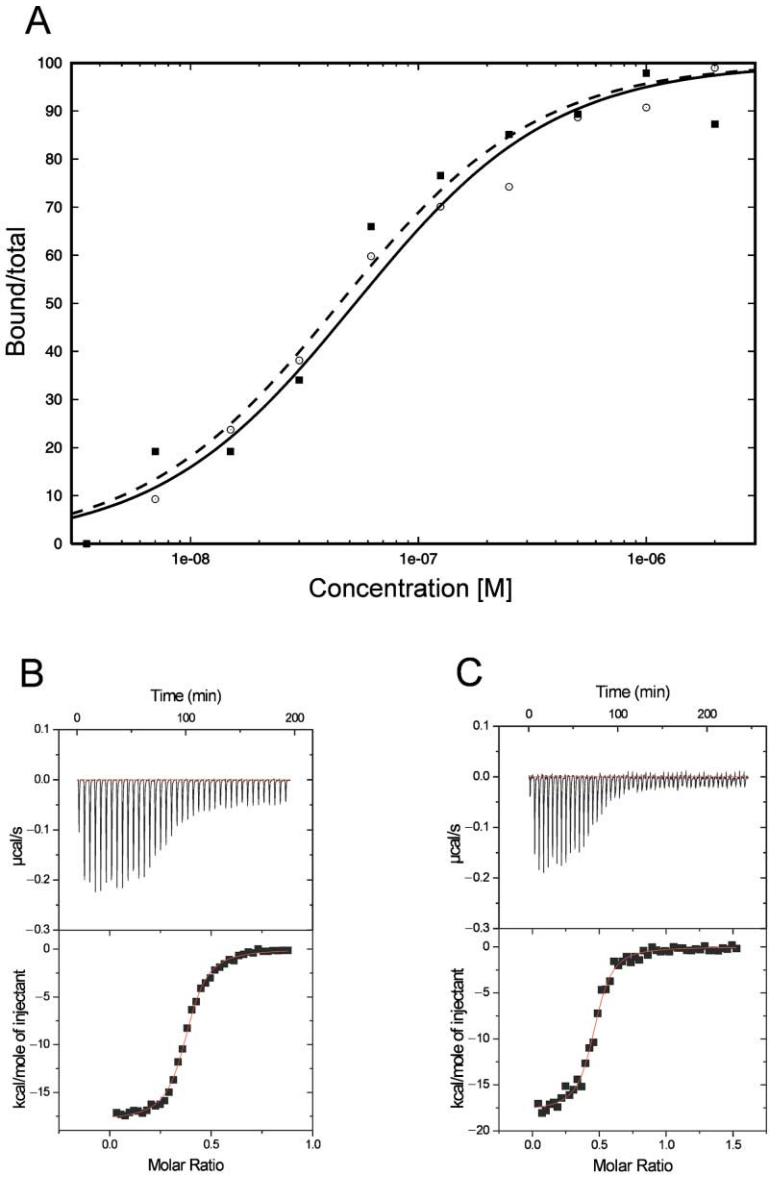

Figure 3. Affinity of the RNA and DNA Spiegelmers

(A) Equilibrium dissociation constants (K_D) of the DNA aptamer from D-GnRH (circles) and the corresponding Spiegelmer from L-GnRH (squares) determined by equilibrium dialysis. The data was fitted assuming a 1:1 binding process: 100% × [c]/(Kd + [c]).

(B) Calorimetric profile of the RNA aptamer binding to D-GnRH. A total of 37.7 ± 0.2% of the RNA molecules have one binding site with an equilibrium constant of 97 ± 7 nM; the remaining molecules do not bind significantly. This corresponds to a nominal equilibrium constant of 263 nM.

(C) Calorimetric profile of the RNA Spiegelmer binding to L-GnRH. A total of 46.1 ± 0.3% of the RNA molecules have one binding site with an equilibrium constant of 87 ± 9 nM; the other 53.9 % do not bind significantly. This corresponds to a nominal equilibrium constant of 190 nM.

Bioactivity of GnRH Spiegelmers

Chinese hamster ovary (CHO) cells that were stably transfected with the human GnRH receptor [25] were used to examine the ability of the candidate Spiegelmers to inhibit the binding of GnRH to its cell surface receptor.

Table 2. Sequence of the Neuroactive Peptides Used for Determining the Specificity of the RNA and DNA Spiegelmer

Peptide	Amino Acid Sequence
GnRH (LHRH)	PyrGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Buserelin	PyrGlu-His-Trp-Ser-Tyr-D-Ser-Leu-Arg-Pro-NHEt (Bu^+)
Chicken LHRH	PyrGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2
Oxytocin	H-Cys-Tyr-Ile-Glu-Asn-Cys-Pro-Leu-Gly-NH_2
Vasopressin	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

Stimulation of the receptor by GnRH leads to Ca^{2+} release [26], which was detected by the Ca^{2+}-sensitive fluorescent dye Fluo-III. It could be shown that increasing amounts of Spiegelmer prevent the docking of GnRH to its receptor, resulting in a decrease of Ca^{2+} release. Doses of 0.01–1.0 µM of the RNA or DNA Spiegelmer, respectively, resulted in a sigmoidal dose-response curve showing the antagonistic activity of both Spiegelmers (Figure 5). IC_{50} values were determined to be about 200 nM for the RNA Spiegelmer and 50 nM for the DNA Spiegelmer. The IC_{50} of both Spiegelmers correlated with the respective equilibrium dissociation constants to GnRH obtained by equilibrium dialysis and isothermic calorimetry.

Discussion

Using the Spiegelmer technology, we have identified D-GnRH binding oligonucleotide ligands from a combi-

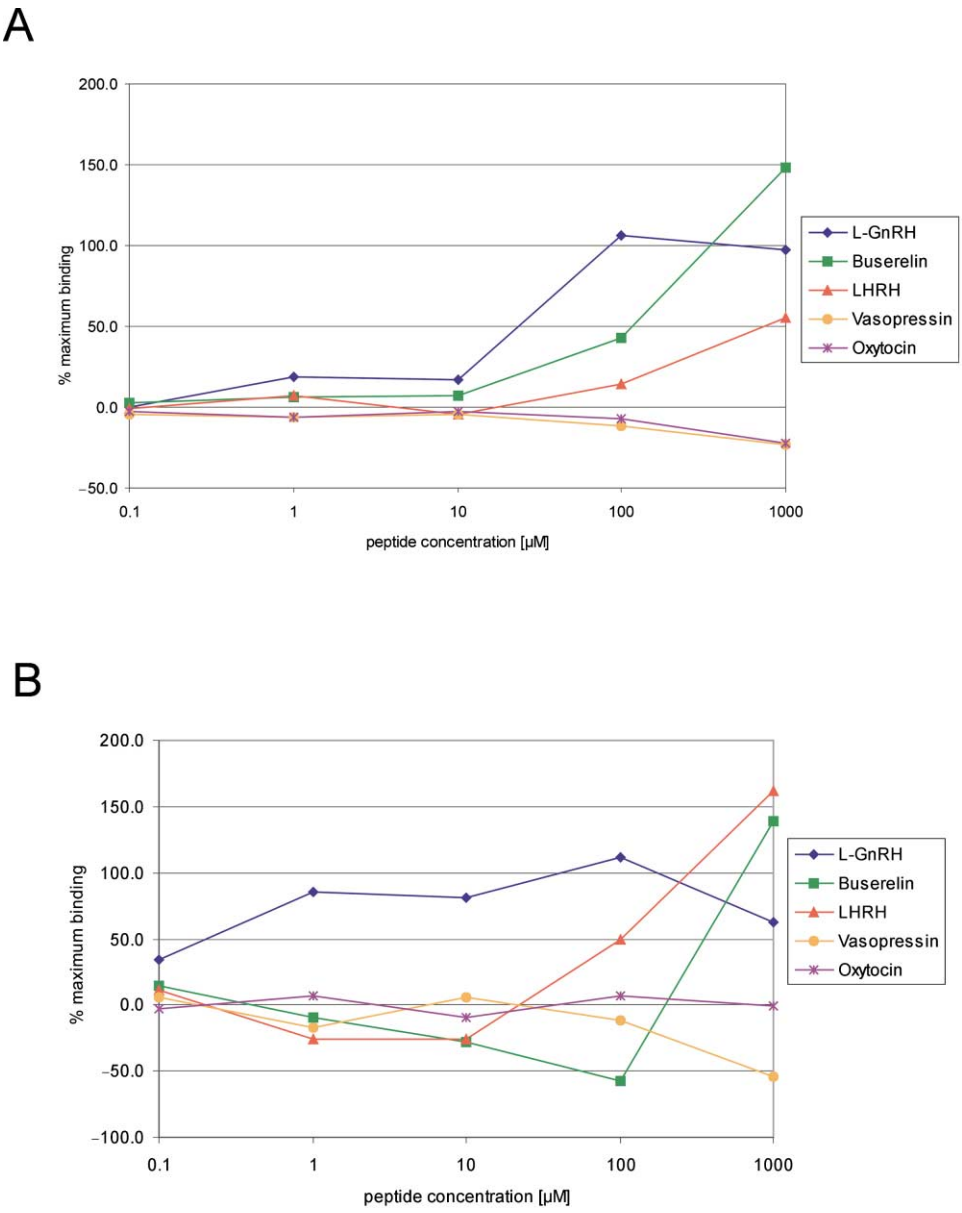

Figure 4. Specificity of the RNA and DNA Spiegelmers

Using a Biacore 2000, the interaction of the immobilized Spiegelmers with increasing amounts (0.1–1000 μM) of various neuroactive peptides was monitored in real-time by the change in the SPR response. For analysis, the responses are scaled to the molar mass of GnRH, and maximum binding of GnRH is set to 1 (100%). The responses are plotted versus the logarithm of the concentration of analyte.

(A) The results for the RNA Spiegelmer.

(B) The results for the DNA Spiegelmer.

natorial RNA library as well as from a combinatorial DNA library. Both starting populations had an initial complexity of about 10^{15} different molecules. While the RNA selection revealed only one major binding sequence, several sequence solutions without any similarity regarding primary and secondary structure elements were obtained from the DNA selection. As anticipated, the affinities to D-GnRH of individual sequences from both the RNA and the DNA selection are similar due to the relatively low selection pressure that was applied during the selection experiments. As found in previous selection experiments that identified RNA and DNA ligands for binding to the same target [27, 28], the selected RNA and DNA variants show no similarities with each other with respect to their primary sequence and proposed secondary structure. While it appears that the RNA and DNA variants do not differ much in the complexity of the structures needed for binding to GnRH, it seems that, at least in this case, DNA solutions are more common than RNA solutions. These results are consistent with findings from earlier publications [29].

The oligonucleotide ligands selected for binding to

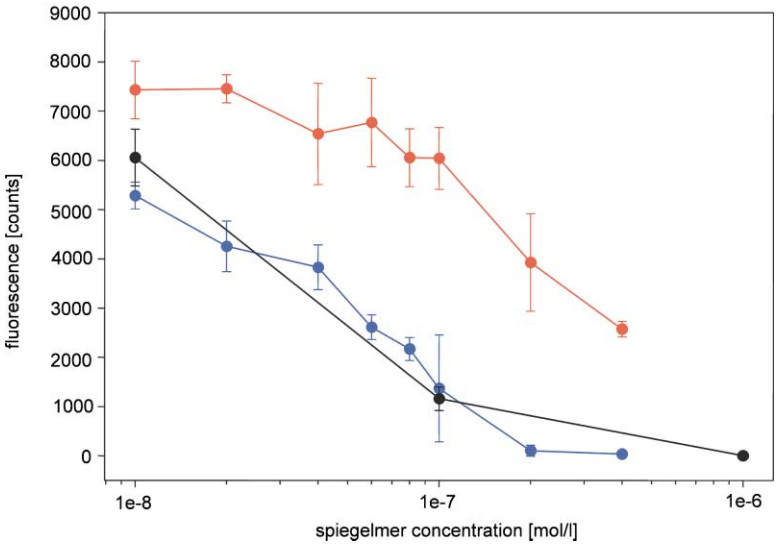

Figure 5. Bioactivity of the RNA and DNA Spiegelmers

Ca^{2+} release in response to GnRH in the presence of RNA (red circles) and DNA (blue circles) Spiegelmers. RNA or DNA was preincubated with GnRH for 20 min prior to addition to the cells. The resulting final GnRH concentration was 2×10^{-9} M. The sigmoidal dose-response curve showed antagonistic activity of both Spiegelmers, with an IC_{50} of 50 nM for the DNA Spiegelmer and 200 nM for the RNA Spiegelmer, respectively. The positive control used in the assay was the GnRH antagonist ZK 204859 (black circles).

D-GnRH display equilibrium dissocation constants in the range of 50–100 nM. While truncation of the RNA ligand to a 50-mer resulted in an approximately 2-fold decrease of binding affinity, truncation of the best GnRH binding DNA sequence to a 60-mer did not decrease binding affinity. These results clearly demonstrate that it is possible to isolate oligonucleotide ligands with high affinity for a small peptide, even though the structure of the peptide is flexible and presumably exists in an equilibrium of multiple conformers. It is conceivable that the Spiegelmers force the decapeptide into a stable conformation, as was observed for the binding of the HIV-1 Rev peptide by the Rev-responsive element and a related RNA aptamer obtained by in vitro selection [30]. With equilibrium dissociation constants of 45 and 180 nM, the Spiegelmers obtained in this study rank among the oligonucleotides with the highest affinities for small peptides selected so far. Dissociation constants of ligands to substance P were determined to be 190 nM [31], while the RNA ligands selected for binding to the 16-mer ARM of HIV-1 Rev protein showed K_D values of 19–36 nM [32]. In the case of the ARM peptide, the rather high affinity may partly be due to interactions between the positively charged peptide and the negatively charged RNA molecules.

Both Spiegelmers show high specificity for GnRH, since the exchange of a single amino acid (Arg8 → Gln) results in a dramatic loss of binding affinity, as was shown for chicken LHRH. Obviously, the interaction with Arg8 plays a major role for the binding of both Spiegelmers to GnRH. However, the RNA Spiegelmer also recognizes the GnRH analog buserelin, albeit with reduced affinity. In contrast, the DNA Spiegelmer shows significant binding to buserelin only at the highest concentration examined, corresponding to an at least 1000-fold reduced affinity. Therefore, one can conclude that, in the case of the DNA Spiegelmer, the structural and conformational differences caused by the introduction of unnatural amino acids have a much greater effect on binding than in the case of the RNA Spiegelmer. However, detailed information regarding how specific recognition is achieved can only be obtained by crystal structure analysis or NMR spectroscopic methods.

Cell culture experiments carried out to determine the ability of the RNA and DNA Spiegelmer to inhibit binding of L-GnRH to its cell surface receptor resulted in IC_{50} values of 200 nM for the RNA Spiegelmer and 50 nM for the DNA Spiegelmer. It was shown for the first time that both RNA and DNA Spiegelmers can be employed as inhibitors of a pharmacologically relevant target. Since the selected Spiegelmers are directed against the peptide hormone, they might circumvent the problem of side effects that are displayed by other known GnRH analogs that bind to the receptor. Agonists like buserelin stimulate hormone secretion in the initial treatment phase (flare) by increasing LH and FSH release from the pituitary gland, before they exert an inhibitory effect. The GnRH receptor/signaling complex will be desensitized only upon prolonged application. By contrast, the release of LH and FSH is expected to be suppressed immediately upon the application of Spiegelmers.

Significance

The Spiegelmer technology provides a unique approach to generate mirror-image oligonucleotide ligands for use in therapeutic and diagnostic applications, circumventing the limitations of natural nucleic acid ligands. We have identified mirror-image DNA and RNA oligonucleotide ligands (Spiegelmers) binding to the peptide hormone L-GnRH with high affinity and high specificity. By demonstrating the bioactivity of both the RNA and the DNA Spiegelmer as GnRH antagonists in cell culture, we show that DNA as well as RNA Spiegelmers can be envisioned for the application as therapeutic agents.

Experimental Procedures

Materials

All oligonucleotides were synthesized at NOXXON Pharma AG using standard phosphoramidite chemistry. D-GnRH (all D[pyrGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2] and D-GnRH-Cys (all D[pyrGlu-

His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Cys-NH_2]; PyrGlu = pyroglutamate = 5′-oxo-D-proline) that contained an additional cysteine at the C terminus were synthesized by Jerini Biotools. L-GnRH was purchased from Bachem. All peptides were analyzed by HPLC (220 nm) and electrospray ionization mass spectroscopy (ESI-MS). The peptide purity was greater than 95%. ^{3}H-labeling (at the tyrosin residue) was performed by Amersham Pharmacia.

Buserelin was purchased from Sigma Aldrich; chicken LHRH, oxytocin, and vasopressin were purchased from Bachem AG.

Sepharose

Thiolsepharose 4B (Pharmacia) was derivatized with 10–100 μM D-GnRH-Cys according to the manufacturer's protocol. The coupling efficiency was determined by reduction of the disulfide bonds with DTT, followed by quantification of the released D-GnRH-Cys by HPLC analysis. Thiopropylsepharose 6B (Pharmacia) was derivatized as described above and used for DNA selection.

In Vitro Selection Procedure

A combinatorial RNA library of approximately 10^{15} different molecules was obtained by the conversion of a synthetic ssDNA library (ssDNA pool: 3′-CCCTTAAGCTCGAGGACTGT-N_{60}-TAAGCTTCTG CAGGTCGACT-5′; forward-primer: 5′-TTCTAATACGACTCACTA TAGGGAATTCGAGCTCCTGACA-3′ [T7 promotor is underlined]; reverse primer: 5′-TCAGCTGGACGTCTTCGAAT-3′) into a double-stranded DNA product and further T7 transcription to an RNA library (3′-CCCTTAAGCTCGAGGACTGT-N_{60}-TAAGCTTCTGCAGGTCG ACT-5′). The copyable fraction of the pool was determined to be 23% by extension of trace-radiolabeled, negative-strand primer under conditions of a single PCR cycle. The starting pool was initially labeled with α^{32}P-UTP by T7 transcription.

The peptide-derivatized sepharose was equilibrated with 6 ml selection buffer (10 mM HEPES [pH 7.5], 137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.005% Triton X-100). The RNA was dissolved in ddH_2O, heated (5 min/95°C), and cooled down in selection buffer to room temperature for 15 min before being applied to the derivatized sepharose 4B column. For the first round, a D-GnRH to RNA ratio of 10:1 was chosen. For the following rounds, the selection pressure was raised by decreasing the ratio from 5:1, 4:1, 3:1, and 2:1 to 1:4 in the last round of selection. Nonbinding oligonucleotides were removed by washing with 12 volumes of selection buffer. GnRH binding RNA molecules were eluted with selection buffer containing free D-GnRH. The first volume was passed through the column and collected. During the following two elution steps, the affinity column was incubated end over end for 1 hr at RT with two volumes of binding buffer containing D-GnRH. Two more volumes were passed through and collected. The concentration of free peptide was five times higher than the concentration of D-GnRH immobilized on sepharose. After the second round of selection, a precolumn with underivatized sepharose was used to prevent the accumulation of nonspecific column binding aptamers. The precolumn was washed with three volumes of selection buffer; the flow-through was collected and loaded onto the D-GnRH-derivatized column.

After elution and collection of the D-GnRH binding oligonucleotides, each fraction was extracted with phenol/chloroform and further amplified by reverse transcription and PCR. The selected oligonucleotides formed the starting pool for the next cycle.

For the DNA selection, a ssDNA library (5′-d[CCAAGCTTGCATG CCTGCAG-N_{60}-GGTACCGAGCTCGAATTCCC]-3′) was generated using a negative-strand PCR primer with an uncopyable tail [18]. A ssDNA library generated by solid phase synthesis was amplified by PCR using forward primer 5′-$(T)_{20}$-XCGGAATTCGAGCTCGGTACC-3′ (X = Glen Research spacer phosphoramidite 18) and reverse primer 5′-CCAAGCTTGCATGCCTGCAG-3′. The smaller DNA template (100 nt) was separated from the longer template (120 nt) by 8% denaturing polyacrylamide gel electrophoresis. About 10^{15} different ssDNA molecules of ^{32}P-labeled ssDNA were used for the selection. D-GnRH-derivatized sepharose 6B was used for affinity chromatography. The column was equilibrated with 6 ml DNA-selection buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.005% Triton X-100). The selection procedure was carried out as described above. In the first round, a target to DNA ratio of 40:1 was used. In the following rounds, the ratio was decreased from 20:1, 10:1, and 5:1 (four times) to 1:1 for the last round of selection.

Equilibrium Dialysis

To determine the binding affinity of individual oligonucleotide ligands, equilibrium dialysis in microdialysis chambers was employed [33]. ^{3}H-labeled D- or L-GnRH (c ≅ 10 Ci/mmol) was equilibrated with increasing amounts of oligonucleotide (100 nM–5 μM) in selection buffer or DNA-selection buffer, respectively. The dialysis was performed through a Spectra/Por cellulose ester membrane (Spectrum) with a molecular weight cutoff of 8,000–10,000 Da. After 24 hr of dialysis at RT, aliquots were withdrawn from each compartment, and the radioactivity was measured by scintillation counting. The fraction of bound oligonucleotide was calculated from the amount of bound GnRH determined by the difference in GnRH concentration in the two chambers. The calculated relative activities were fitted with a nonlinear regression technique (GraFit, Version 4.0) by using a standard binding equation for a 1:1 stoichiometry: 100% × [c]/(Kd + [c]).

Isothermal Calorimetry

Calorimetry experiments were carried out in degased DNA selection buffer at 25°C with a stirring speed of 300 rpm. The measuring cell was loaded with 8.8 μM D- or L-RNA, respectively. The corresponding GnRH solution (0.06 mM) was injected (one 3-μl injection first, followed by 6-μl injections) from a 250-μl syringe over a time interval of 6 s, with 300 s between the injections to allow for complete equilibration and baseline recovery.

Biacore Analysis

For real-time kinetic measurements, a Biacore 2000 from Biacore AB was used. The interaction analysis was performed at 25°C. During the whole assay, 20 mM Tris (pH 7.4), 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.005% Triton X-100 was used as running buffer.

The 5′-biotinylated RNA and DNA Spiegelmer was immobilized on a Sensor Chip SA from Biacore AB. To ensure the highest sensitivity, the sensor surfaces were pretreated with three 1-min injections of 1 M NaCl in 50 mM NaOH at a flow rate of 20 μl/min. Subsequently, a 200 nM solution of the RNA Spiegelmer was manually injected with a flow rate of 5 μl/min through flow cell (fc) 2 until 1120 response units (RU) were captured by the streptavidin surface. Comparably, 1230 RU of the biotinylated DNA Spiegelmer were immobilized on fc 3. Fc 1 was left with streptavidin only, to be used as a reference surface. After Spiegelmer immobilization, 0.1% Triton X-100 in 10 mM sodiumacetat (pH 4.5) was injected in three 1-min pulses to wash off unbound oligonucleotides.

The respective peptides were injected in varying concentrations (0.1–1000 μM) in sequence over reference and Spiegelmer surfaces during 4 min at a flow rate of 5 μl/min. Each cycle consisted of a 2-min waiting period for the monitoring of baseline stability, a 4-min injection of peptide, a 5-min dissociation phase of bound peptide in running buffer, and a regeneration step of sensor surfaces by the injection of 1 M NaCl in 0.02% Triton X-100 for 1 min at a flow rate of 20 μl/min. Between each cycle, the system was idle for 3 min to maintain baseline stability. At the beginning and in a frequency of 15 cycles, an injection of water and running buffer was made to check for carryover effects.

Data obtained in the reference flow cell (fc 1) were subtracted from those obtained in the Spiegelmer flow cell (fc 2 and fc 3). For each sample, response values at 170 s after the end of injection were extracted. The responses are scaled to the molar mass of GnRH, and maximum binding of GnRH is set to 1 (100%). These responses are plotted versus the logarithm of the concentration of analyte.

Bioassay

The bioassay was performed at Schering AG. Chinese hamster ovary (CHO) cells expressing the human GnRH receptor were employed [25]. A total of 5 × 10^4 cells per well were grown overnight at 37°C on black, clear bottom well microtiter plates in Ham's F12 medium. The following day, dye loading was performed after removal of the

medium (Ham's F12 with 19.2 μM Fluo-III, 0.04% pluronic acid, 4.8 mM probenecid, 19 mM HEPES, 100 μl/well) followed by 1 hr of incubation at 37°C. The plate was washed three times with Hanks' solution (138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM Na_2HPO_4, 5.5 mM glucose, 20 mM HEPES [pH 7.5]) containing 5 mM probenecid and 0.2% BSA. The assay was performed by incubating DNA or RNA Spiegelmers at a concentration of 1×10^{-6}–10^{-8} M together with 2×10^{-9} M GnRH for 20 min before adding the mixture to CHO cells. Binding of GnRH to its receptor triggers the intracellular Ca^{2+} release. The release is detected by an intracellular fluorescence marker (Fluo-III) a few seconds after receptor stimulation. The signal is measured as a fluorescence peak in each individual well in a fluorescent imaging plate reader (FLIPR). The positive control used in the assay was the GnRH antagonist ZK 204859 (systematic name: 1-[7-Chloro-3-(3,5-dimethylphenyl)-2-oxo-4-2(2-piperidin-2-ylethoxy)-1,2-dihydroquinolin-6-yl]-3-pyridin-2-yl urea) proprietory to Merck.

Acknowledgments

We thank Stefan Vonhoff and the Noxxon oligonsynthesis team for synthesis of the D- and L-oligonucleotides, Jana Zenker and Kornelia Mika for excellent technical assistance, and Frank Kleinjung for many helpful discussions.

Received: October 22, 2001
Revised: December 7, 2001
Accepted: December 14, 2001

References

1. Wilson, D.S., and Szostak, J.W. (1999). In vitro selection of functional nucleic acids. Annu. Rev. Biochem. *68*, 611–647.
2. Brody, E.N., and Gold, L. (2000). Aptamers as therapeutic and diagnostic agents. J. Biotechnol. *74*, 5–13.
3. Jayasena, S.D. (1999). Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin. Chem. *45*, 1628–1650.
4. Klussmann, S., Nolte, A., Bald, R., Erdmann, V.A., and Furste, J.P. (1996). Mirror-image RNA that binds D-adenosine. Nat. Biotechnol. *14*, 1112–1115.
5. Nolte, A., Klussmann, S., Bald, R., Erdmann, V.A., and Furste, J.P. (1996). Mirror-design of L-oligonucleotide ligands binding to L-arginine. Nat. Biotechnol. *14*, 1116–1119.
6. Williams, K.P., Liu, X.H., Schumacher, T.N., Lin, H.Y., Ausiello, D.A., Kim, P.S., and Bartel, D.P. (1997). Bioactive and nuclease-resistant L-DNA ligand of vasopressin. Proc. Natl. Acad. Sci. USA *94*, 11285–11290.
7. Carolsfeld, J., Powell, J.F., Park, M., Fischer, W.H., Craig, A.G., Chang, J.P., Rivier, J.E., and Sherwood, N.M. (2000). Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology *141*, 505–512.
8. Belchetz, P.E., Plant, T.M., Nakai, Y., Keogh, E.J., and Knobil, E. (1978). Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science *202*, 631–633.
9. McCann, S.M. (1977). Luteinizing-hormone-releasing hormone. N. Engl. J. Med. *296*, 797–802.
10. Kiesel, L. (1993). Molecular mechanisms of gonadotrophin releasing hormone-stimulated gonadotrophin secretion. Hum. Reprod. *8* Suppl. *2*, 23–28.
11. van der Linden, P.J. (1996). Theories on the pathogenesis of endometriosis. Hum. Reprod. *11* Suppl. *3*, 53–65.
12. Bulletti, C., Flamigni, C., Polli, V., Giacomucci, E., Albonetti, A., Negrini, V., Galassi, A., and Morselli-Labate, A.M. (1996). The efficacy of drugs in the management of endometriosis. J. Am. Assoc. Gynecol. Laparosc. *3*, 495–501.
13. Cramer, S.F. (1998). Effects of gonadotropin-releasing hormone agonists on uterine leiomyomas. Arch. Pathol. Lab. Med. *122*, 1045–1046.
14. Morini, A., Aleandri, V., Cantonetti, G., and Benagiano, G. (1993). Directions of future research on GnGH analogs in the treatment of endometriosis and uterine fibromyoma. Minerva Ginecol. *45*, 455–465.
15. Cook, T., and Sheridan, W.P. (2000). Development of GnRH antagonists for prostate cancer: new approaches to treatment. Oncologist *5*, 162–168.
16. Leone, M., and Valenti, G. (1987). GnRH analogs: experimental and clinical review. Minerva Endocrinol. *12*, 305–316.
17. Jennings, J.C., Moreland, K., and Peterson, C.M. (1996). In vitro fertilisation. A review of drug therapy and clinical management. Drugs *52*, 313–343.
18. Williams, K.P., and Bartel, D.P. (1995). PCR product with strands of unequal length. Nucleic Acids Res. *23*, 4220–4221.
19. Ehresmann, C., Baudin, F., Mougel, M., Romby, P., Ebel, J.P., and Ehresmann, B. (1987). Probing the structure of RNAs in solution. Nucleic Acids Res. *15*, 9109–9128.
20. Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. *288*, 911–940.
21. Zuker, M., and Jacobson, A.B. (1998). Using reliability information to annotate RNA secondary structures. RNA *4*, 669–679.
22. SantaLucia, J. (1998). A unified view of polymer, dumbbell, and oligonucleotide DNA nearest- neighbor thermodynamics. Proc. Natl. Acad. Sci. USA *95*, 1460–1465.
23. Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H., and Toole, J.J. (1992). Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature *355*, 564–566.
24. Macaya, R.F., Schultze, P., Smith, F.W., Roe, J.A., and Feigon, J. (1993). Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. Proc. Natl. Acad. Sci. USA *90*, 3745–3749.
25. Grosse, R., Schmid, A., Schoneberg, T., Herrlich, A., Muhn, P., Schultz, G., and Gudermann, T. (2000). Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to G(q/11) proteins. J. Biol. Chem. *275*, 9193–9200.
26. Naor, Z., Harris, D., and Shacham, S. (1998). Mechanism of GnRH receptor signaling: combinatorial cross-talk of $Ca2^{+}$ and protein kinase C. Front. Neuroendocrinol. *19*, 1–19.
27. Kubik, M.F., Stephens, A.W., Schneider, D., Marlar, R.A., and Tasset, D. (1994). High-affinity RNA ligands to human alpha-thrombin. Nucleic Acids Res. *22*, 2619–2626.
28. Huizenga, D.E., and Szostak, J.W. (1995). A DNA aptamer that binds adenosine and ATP. Biochemistry *34*, 656–665.
29. Ellington, A.D., and Szostak, J.W. (1992). Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures. Nature *355*, 850–852.
30. Hermann, T., and Patel, D.J. (2000). Adaptive recognition by nucleic acid aptamers. Science *287*, 820–825.
31. Nieuwlandt, D., Wecker, M., and Gold, L. (1995). In vitro selection of RNA ligands to substance P. Biochemistry *34*, 5651–5659.
32. Xu, W., and Ellington, A.D. (1996). Anti-peptide aptamers recognize amino acid sequence and bind a protein epitope. Proc. Natl. Acad. Sci. USA *93*, 7475–7480.
33. Wrede, P., Pongs, O., and Erdmann, V.A. (1978). Binding oligonucleotides to *Escherichia coli* and *Bacillus stearothermophilus* 5 S RNA. J. Mol. Biol. *120*, 83–96.