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In vitro selection using a dual RNA library that allows primerless selection

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ABSTRACT

High affinity target-binding aptamers are identified from random oligonucleotide libraries by an *in vitro* selection process called Systematic Evolution of Ligands by EXponential enrichment (SELEX). Since the SELEX process includes a PCR amplification step the randomized region of the oligonucleotide libraries need to be flanked by two fixed primer binding sequences. These primer binding sites are often difficult to truncate because they may be necessary to maintain the structure of the aptamer or may even be part of the target binding motif. We designed a novel type of RNA library that carries fixed sequences which constrain the oligonucleotides into a partly double-stranded structure, thereby minimizing the risk that the primer binding sequences become part of the target-binding motif. Moreover, the specific design of the library including the use of tandem RNA Polymerase promoters allows the selection of oligonucleotides without any primer binding sequences. The library was used to select aptamers to the mirror-image peptide of ghrelin. Ghrelin is a potent stimulator of growth-hormone release and food intake. After selection, the identified aptamer sequences were directly synthesized in their mirror-image configuration. The final 44 nt-Spiegelmer, named NOX-B11-3, blocks ghrelin action in a cell culture assay displaying an IC_{50} of 4.5 nM at 37°C.

INTRODUCTION

Aptamers are highly structured oligonucleotides that can bind to targets with affinities comparable to antibodies. They are identified through an evolutionary selection process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) (1,2). The SELEX process comprises alternate selection and amplification steps. Using SELEX, aptamers have been identified to a plethora of different target molecules that comprise proteins, peptides or small molecules. Due to the ability to block strong protein–protein interactions, aptamers have considerable potential as therapeutics (3,4). However, in order to use aptamers therapeutically, they have to be stable *in vivo* and inexpensive in production.

A widely used approach to stabilize aptamers against nucleolytic attacks involves the successive introduction of chemically modified nucleotides into the sequences (5–7). An alternative strategy to achieve full biostability at once is to use oligonucleotides that are composed of mirror-image nucleotides. Such functional mirror-image oligonucleotides (L-RNA or L-DNA) are named Spiegelmers (from the German word '*Spiegel*' meaning mirror). Spiegelmers can be identified through introducing chiral principles into the SELEX process (8): First, aptamers are selected to bind to the mirror-image configuration of a given target (D-polypeptide). The aptamer sequences are then synthesized in their respective mirror-image configuration to give Spiegelmers, which consequently bind to the natural configuration of said target molecule (L-polypeptide). Based on this technique, high affinity Spiegelmers to a variety of targets have been generated (9).

Besides biological (in)stability, the length of aptamer sequences is a major issue for further development and commercial application. This is due to the fact that manufacturing of oligonucleotides by standard solid phase synthesis becomes increasingly inefficient and costly with growing length of the oligonucleotides.

Conventional nucleic acid libraries consist of a central randomized region flanked by primer binding sites that are needed for PCR amplification. These fixed sequences comprise up to 50% of the total available nucleotides. Therefore, first attempts to truncate the isolated aptamers routinely encompass cutting off the primer binding sequences. However, the primer binding sites or segments of them are often an 'unwanted' part of the target-binding domain or they stabilize the active structure of the aptamer. This can especially be an issue if aptamers are selected to bind to flexible targets, such as peptides (10–13) or if very high stringencies within the selection process are applied so that even the fixed primer binding sites may be 'directly included' into the active functional sequences. In such cases, truncation of an aptamer to a reasonable size may be difficult or even impossible. Therefore, new or improved methods to facilitate the truncation of aptamers are desirable.

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The first ideas in this direction were introduced into the patent literature by Toole *et al*. (14), but experimental results are still lacking. Toole *et al*. suggested to use a primerless library and up to 512 different double-stranded DNA molecules that contain the primer binding sequences. This pool of double-stranded DNA molecules can hybridize up to four 5′- and 3′-terminal, undefined nucleotides of the respective library. Contrary to the contemplated method of Toole *et al*., the Tailored-SELEX process (15) envisions four and six fixed nucleotides at the 5′ and 3′ end of a primerless RNA library, respectively. The primer binding sequences are ligated to the library using three double-stranded molecules (adapters) and removed within the amplification procedure without the need of purification steps. Tailored-SELEX has been successfully applied to identify high affinity oligonucleotides to calcitonin gene-related peptide (15).

In order to combine the benefits of an RNA library without primer binding sites with the speed of an automated selection protocol (16), a new type of RNA library was designed. This dual library can be either used as a full-length library or a short library without primer binding sites. The single or repeated use of the short library allows direct identification of short aptamers.

The practicality of the new library design was validated through an *in vitro* selection against the mirror-image peptide target D-ghrelin. Ghrelin is a peripheral hormone that stimulates appetite as well as growth hormone release (17,18). It plays an important role in the regulation of energy homeostasis and has therefore attracted substantial attention as potential target in obesity (19) and the Prader–Willi Syndrome (20).

MATERIALS AND METHODS

Oligonucleotides and peptides

All oligonucleotides were synthesized at NOXXON Pharma AG using standard phosphoramidite chemistry. Human L-ghrelin as well as human D-ghrelin modified with a biotin group linked via D-lysine and two amino-ethyloxy-ethyloxy-acetyl groups (ghrelin-D-Lys-AEEAc-AEEAc-biotinyl-OH) at the C-terminus were purchased from Bachem (Bubendorf, Switzerland).

In vitro selection

A combinatorial RNA library of $\sim 10^{15}$ different molecules was obtained by enzymatic conversion of 3 nmols of a synthetic DNA library (ssDNA pool that contains a T7 RNA polymerase (bold) promoter sequence, 5′-CTTAGTGA-TACGCTCGTCGATGTGTGCTGC-N_{34}-GCAGCACCC**TA-TAGTGAGTCGTATTA**CTCCC-3′; forward primer with T3 RNA polymerase (underlined) and T7 RNA polymerase (bold) promoter sequence: 5′-TCAATTAACCCTCACT-AAAGGGAG**TAATACGACTCACTATA**GGGT-3′) into dsDNA which was then *in vitro* transcribed [80 mM HEPES/KOH (pH 7.5), 22 mM $MgCl_2$, 1 mM spermidine, 10 mM DTT, 0.12 μg/μl BSA, 4 mM NTPs (Larova, Teltow, Germany), 1 μl RNaseOUT (Invitrogen, Karlsruhe, Germany), 0.1 U/μl T3 RNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) at 37°C for 6–12 h] into RNA (5′-GGGAGUAAUACGACUCACUAUAGGGUGCUGC-N34-GCAGCACACAUCGACGAGCGUAUCACUAAG-3′). This starting pool was initially labelled with [α-^{32}P]GTP (Hartmann, Braunschweig, Germany) during *in vitro* transcription.

The first three selection rounds were performed manually at 10 μM biotinylated D-Ghrelin as described previously (21) using the T3 RNA polymerase for *in vitro* transcription. The next six rounds were carried out using an automation system (RoboAmp 4200 E, MWG Biotech, Ebersberg, Germany) according to the protocol of Eulberg *et al*. (16), again using the T3 RNA polymerase for *in vitro* transcription to result in the full-length library. The RNA was gel-purified (22) after every second selection round. Over the six rounds of automated *in vitro* selection the peptide concentration was decreased down to 33 nM.

From round 10 on, the enriched library was employed as a short library. Therefore the primer binding sites were removed before the selection step. After the selection step the primer binding sites were added to the isolated oligonucleotides by ligation. Within the following five manual selection rounds the peptide concentration was reduced to 10 pM. Similar to the previous selection rounds the selection process was carried out at 37°C and the binding reaction was done in solution for 12 h. The complexes of RNA and biotinylated human D-ghrelin were immobilized for 30 min on the selection matrix (Neutravidin Agarose or Streptavidin Ultralink beads; Pierce, Rockford). Non-binding molecules were removed by washing the matrix with 10 to 25 vol of selection buffer (16). Bound RNA oligonucleotides were eluted twice (8 M urea, 10 mM EDTA, 65°C for 10 min and 95°C for 10 min), extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and dissolved in water.

Ligation and amplification

The ligation reaction was carried out at 25°C for 12 h [not more than 71.4 nM RNA and 36 U T4 ligase/$pmol_{RNA}$ (Fermentas, St. Leon-Rot, Germany), 1× ligation buffer (Fermentas), 5% PEG 4000, 0.5 μl RNaseOUT (Invitrogen, Karlsruhe, Germany), 20-fold excess of forward adapter and 10-fold excess of reverse adapter 1 and 2 each]. The adapters are double-stranded oligonucleotides: forward adapter [forward ligate, (RNA: 5′-GGGAGUAAUACGACUCA-CUAUA) plus forward bridge (DNA: 5′-GCACCCTATAGT-GAGTCGddT-3′) with 2′,3′-dideoxythymidine at the 3′ end], reverse adapter 1 [reverse ligate 1 (DNA: 5′-pACATCGAC-GAGCGTATCACTAAddG-3′, 5′-phosphorylated and a 2′,3′-dideoxyguanosine at the 3′ end) plus reverse bridge 1 (DNA: 5′-CTTAGTGAUACGCTCGTCGATGUGTGCTG-3′ with two incorporated uridines] and reverse adapter 2 [reverse ligate 2 (DNA: 5′-pCATCGACGAGCGTATCACTAAddG-3′, 5′-phosphorylated and a 2′,3′-dideoxyguanosine at the 3′ end) plus reverse bridge 2 (DNA: 5′-CTTAGTGAUACGC-TCGTCGAT-GIGTGCTddG-3′) with the two incorporated ribonucleotides uridine and inosine, respectively].

The reverse transcription reaction was carried out at a final RNA concentration of not more than 30 nM for 20 min at 51°C and 10 min at 54°C [8 μM reverse primer (5′-CTTAGTGAUACGCTCGTCGATGUGT) with two incorporated uridines, 1× first strand buffer (Invitrogen), 0.5 M betain (Sigma–Aldrich, Steinheim, Germany),

0.5 mM dNTPs (Larova, Teltow, Germany), 2 U/µl Superscript reverse transcriptase II (Invitrogen), 10 mM DTT]. The cDNA was directly transferred into the PCR [not more than 5 nM cDNA, 1× PCR-buffer (Roche), 3 µM forward primer, 3 µM reverse primer, 0.2 mM dNTPs (Larova), 0.05 U/µl *Taq* DNA Polymerase (Roche, Mannheim, Germany), 2.5 mM Mg^{2+}, 0.5 M betain, annealing temperature at 65°C, 7–17 cycles]. The cleavage of the PCR reverse strand was done by alkaline fission of the ribonucleotides [310 mM (final) NaOH, 10 min at 95°C, neutralization with HCl, buffered with 0.1 mM (final) sodium acetate].

The PCR product was ethanol precipitated before *in vitro* transcription in 80 mM HEPES/KOH (pH 7.5), 22 mM $MgCl_2$, 1 mM spermidine, 10 mM DTT, 0.12 µg/µl BSA, 4 mM NTPs (Larova), 32 mM 5′-GMP (Sigma, Taufkirchen, Germany), 1 µl RNaseOUT (Invitrogen), 0.1 U/µl T7 RNA polymerase (Stratagene, La Jolla) at 37°C for 6–12 h. The transcribed RNA was gel-purified (22) and radioactively labelled [2 µM RNA, 1× exchange buffer (Invitrogen), [γ-^{32}P]ATP (Hartmann, Braunschweig, Germany), 1 U/µl T4 Polynucleotide Kinase (Invitrogen)]. The enriched library from round 16 was ligated and PCR-amplified before cloning and sequencing at AGOWA (Berlin, Germany).

Competition assay

Radiolabeled aptamer NOX-B11 (2 pmols) was denatured for 3 min at 95°C in selection buffer [20 mM Tris, 150 mM NaCl, 5 mM KCl (pH 7.4) was adjusted at 37°C] without Ca^{2+} and Mg^{2+}, folded by addition of these ions to a final concentration of 1 mM at 37°C. A total of 20 nM radiolabelled NOX-B11 and 40 or 200 nM of the unlabelled aptamers were incubated with 20 nM biotinylated human D-ghrelin for 1 h at 37°C. A constant amount of neutravidin agarose was added for 30 min. The neutravidin agarose was sedimented, the supernatant removed, the matrix washed and the ratio of bound radio-labelled NOX-B11 was determined by measuring the radioactivity using a szintillation counter (Beckman Coulter LS6500).

Bioassay

Stably transfected CHO-cells expressing the human ghrelin receptor GHS-R1a (Euroscreen, Gosselies, Belgium) were seeded with 5–7 × 10^4 cells per well in a black 96-well plate with clear bottom (Greiner, Frickenhausen, Germany) and grown overnight at 37°C and 5% CO_2 in UltraCHO medium (Cambrex, Verviers, Belgium) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml geneticin and 2.5 µg/ml fungizone. Spiegelmers were incubated for 15 to 60 min with bioactive human ghrelin in UltraCHO medium containing 5 mM probenecid and 20 mM HEPES (CHO-U+) at 37°C in a 0.2 ml low profile 96-well plates. Cells were washed once with 200 µl CHO-U+, loaded with 50 µl 0.08% pluronic 127, 10 µM fluo-4 indicator dye solution (Molecular Probes, Leiden, The Netherlands) in CHO-U+, and incubated for 60 min at 37°C. Thereafter, cells were washed three times with 180 µl CHO-U+. A total of 90 µl CHO-U+ were added per well and cells stimulated with 10 µl of the preincubated Spiegelmer-ghrelin mixture. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a Fluostar Optima multidetection plate reader (BMG, Offenburg, Germany). For each well the difference between the maximum fluorescence and the base line value was determined and plotted against ghrelin concentration or, in Spiegelmer inhibition experiments, against concentration of Spiegelmer. The half maximum effective concentration (EC_{50}) or inhibition constants IC_{50} were read from the graphs.

RESULTS

Library design for short and full-length libraries with a shared PCR product

The dual library that can be either used as full-length library or as short library was constructed with a randomized region of 34 nt flanked by seven fixed nucleotides on each side which can form a double stranded structure (Figure 1). The primer binding sequences are designed to partially hybridize with each other. Both structural pre-settings have the function to minimize potential interactions of the primer binding sites with the randomized region. In addition to the seven fixed nucleotides at the 5′ end of the short library, two fixed dangling nucleotides are added to improve the yield of the ligation reaction (after the selection step) while selecting with the short library.

The forward (or 'plus') strand of the PCR product includes a tandem of two different RNA polymerase promoters at the 5′ end, the T3 and T7 RNA polymerase promoter, respectively. Both promoters encompass part of the sequence of the forward primer. The annealing sequence of the reverse primer is located at the 3′ end of the forward strand.

In the reverse (or 'minus') strand of the PCR product, two ribonucleotide cleavage sites are incorporated by the reverse primer: one is located directly upstream of the fixed sequence (7 nt) and the other in the middle of the reverse primer sequence. Two cleavage sites ensure that potential re-hybridization of the fragments or read-through of the RNA polymerase is avoided. Finally, the 3′-region of the reverse strand constitutes the annealing site for the forward primer.

In order to conduct selection with the short library, at first the main part of the reverse primer is removed from the reverse strand of the PCR product by alkaline hydrolysis at the ribonucleotides. Secondly, *in vitro* transcription is carried out using T7 RNA polymerase. The resulting RNA transcripts start directly after the T7 promoter sequence and end with the 7 nt fixed sequence of the 3′ end. An 8-fold excess of the initiation nucleotide guanosine monophosphate in the transcription reaction leads nearly quantitatively to a 5′-terminal monophosphate which is essential for the ligation reaction afterwards.

On the other hand, selection with the full-length library only requires transcription of the PCR product with T3 RNA polymerase. Here, the transcripts begin directly after the T3 promoter and end with the reverse primer binding site.

The T3 as well as the T7 RNA polymerases are known to add non-templated nucleotides to up to 50% of the synthesized RNA transcripts in a random fashion (23). These so-called 3′-microheterogeneities consist mainly of one random nucleotide. Since the amount of these modified transcripts is significant (~50%) and the added nucleotides complicate

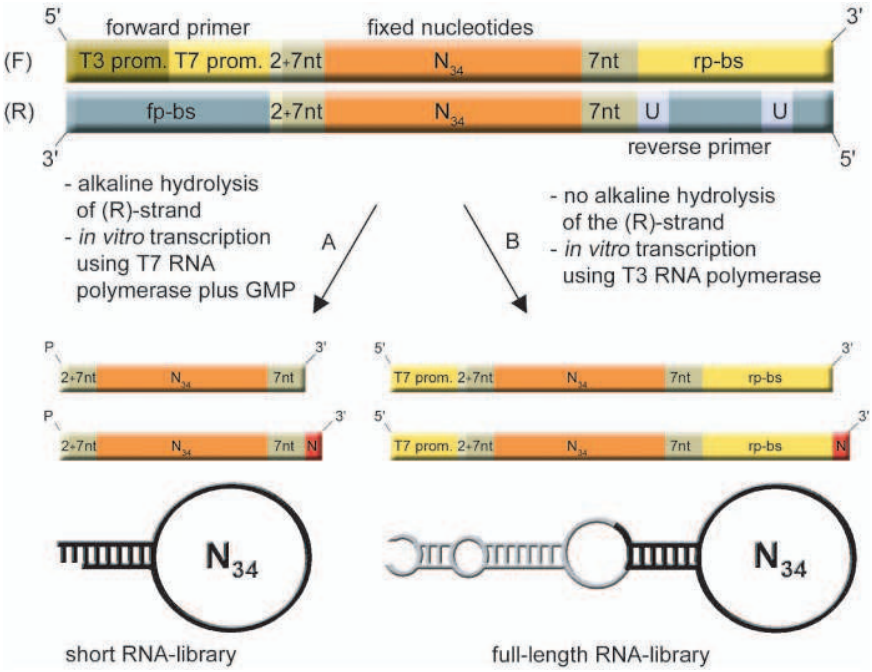

Figure 1. Design of the shared PCR product that leads to the short (**A**) and full-length (**B**) RNA libraries. The PCR product serves as a shared template for generating both the short and the full-length library. Tandem promoter sequences for the T3 and T7 RNA polymerase, respectively, are located at the 5′ end of the forward strand (F) and are part of the forward primer at the same time. The integration of the reverse primer that contains two ribonucleotides, introduces the (necessary) cleavage sites for alkaline hydrolysis of the template for synthesis of the short library. (A) To employ the short RNA library for the selection step, the reverse strand of the PCR product is cleaved by alkaline hydrolysis at the positions of the ribonucleotides so that the main part of the reverse primer is removed from the transcription template. The use of the T7 RNA polymerase ensures that transcription starts directly after the T7 promoter to generate the short RNA library. An excess of guanosine monophosphate in the transcription reaction establishes a monophosphate at the 5′ end that is essential for the template-directed ligation of the forward primer after the selection step. (B) To employ the full-length RNA library for the selection step, the *in vitro* transcription is carried out utilizing T3 RNA polymerase. As a consequence, the library contains the full sequence of the T7 RNA promoter at the 5′ end. Both polymerases add to their respective transcripts partially non-templated single nucleotides (3′-microheterogeneities) so that the transcribed libraries result in N- and N+1 mixtures, indicated by an additional 'N'. prom.: promoter; nt: nucleotides; fp-bs: forward primer binding site; rp-bs: reverse primer binding site.

subsequent ligation reactions, they are also depicted as products in Figure 1.

Workflow for *in vitro* selection

Both libraries can be used for *in vitro* selection. The selected binding molecules of the full-length library can be directly transcribed into cDNA, transferred to PCR, and transcribed into the enriched RNA library by T3 RNA polymerase (Figure 2). Selection with the short library requires a ligation step to add the primer sequences for subsequent reverse transcription and PCR. The PCR product is then subjected to an alkaline hydrolysis step to cleave off the primer binding site of the reverse strand before transcription with T7 RNA polymerase generates the enriched short library for the next round of selection (Figure 2).

The design of the libraries also allows to switch very easily on the level of the PCR between the short and full-length library and vice versa (Figure 2). Employing the short library within the selection scheme in at least one selection round should make sure that the binding characteristics of the selected aptamers are independent of the primer sequences.

Ligation of the short library

In order to conduct the selection with the short library the primer binding sequences need to be added for subsequent PCR amplification. Based on a technique already published for Tailored-SELEX (15), a ligation strategy with adapter molecules and highly concentrated T4 DNA ligase was developed (Figure 3).

Three different adapter molecules for the ligation reactions were designed. The 'forward adapter' for the 5′-ligation consists of the 'forward ligate' and the 'forward bridge'. The 3′-ligation requires the 'reverse adapter 1' which consists of the 'reverse ligate 1' and the 'reverse bridge 1' for the ligation of N-transcripts (Figure 3A). To accommodate the problem of 3′-microheterogeneities the 'reverse adapter 2', consisting of the 'reverse ligate 2' and the 'reverse bridge 2', permits the 3′-ligation of N+1-transcripts (Figure 3B).

During the 5′-ligation reaction the forward ligate of the forward adapter is added to the 5′ end of the short library. The forward ligate contains the T7 RNA polymerase promoters upstream of the ligation site. Six nucleotides at the 5′ end of the forward bridge are complementary to the first 6 nt of the 5′-fixed region (9 nt) of the short library so that a directed ligation of the forward adapter can be achieved. To reduce the risk that the forward bridge interferes with cDNA synthesis, it was kept to a minimal but sufficient length of 19 nt. Moreover, it is blocked at its 3′ end by the introduction of a terminal 2′,3′-dideoxynucleotide.

The reverse ligates of the reverse adapters restore the reverse primer binding sequence in the 3′-ligation reaction.

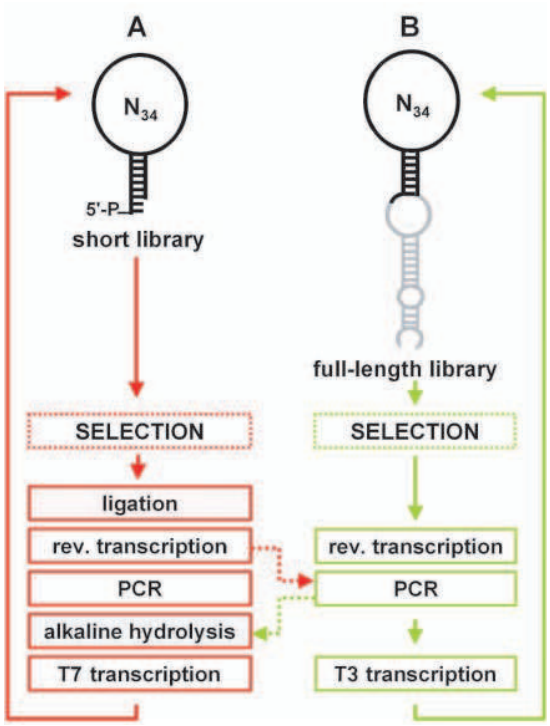

Figure 2. The short (**A**) and full-length (**B**) library and the workflow for *in vitro* selection. Both variants of the dual RNA library differ in the absence [(A); short library] or presence [(B); full-length library] of the primer binding sites. The random region, depicted as a huge loop structure, is clamped by seven terminal base pairs. (A) In order to amplify the short library after the selection step, the primer binding sites are added by a template-directed ligation strategy. The two dangling nucleotides at the 5′ end improve the ligation yield; the 5′-terminal nucleotide is monophosphorylated. Subsequent to ligation the RNA is reverse transcribed and amplified by PCR. The PCR product is a shared template between both libraries and can be switched to (B). Ribonucleotides within the reverse primer permit the cleavage of one strand of the PCR product by alkaline hydrolysis. The following transcription with T7 RNA polymerase regenerates the (fully truncated) short library. (B) In order to amplify the full-length library, the isolated RNA sequences are reverse transcribed and amplified by PCR. As described under (A), the PCR product is a shared template that can be switched to (A). To transcribe the RNA for the next round of selection with the full-length RNA library, the dsDNA is transcribed using T3 RNA polymerase.

For transcripts of regular length, i.e. N-transcripts, the reverse ligate 1 is added to the 3′-fixed nucleotides of the short library. Since the reverse ligate 1 is the phosphate donor in the ligation reaction, it carries a monophosphate at its 5′ end; the 3′ end is blocked by a 2′,3′-dideoxynucleotide to avoid unwanted extension reactions. The reverse bridge 1 is the template for the directed ligation, whereby 6 nt are complementary to the 3′-fixed region. Additionally, the reverse bridge 1 also serves as reverse primer during reverse transcription and PCR. Thus, comparable to the reverse primer, two ribonucleotides are incorporated into the reverse bridge 1 to allow site-directed cleavage of the PCR product.

The reverse adapter 2 is necessary for the ligation of the N+1-transcripts. Due to the additional nucleotide at the 3′ end of the N+1 transcripts, the reverse ligate 2 lacks the first 5′-nucleotide compared to reverse ligate 1. At the same time the reverse bridge 2 carries at the complementary position inosine instead. Inosine is a universal nucleobase analogue that can form a base pair with any nucleobase of the N+1-position of the N+1-transcript. Using this design of reverse adapter 2, the reverse primer binding site can be successfully ligated to N+1-transcripts as well. Similar to the reverse ligate 1 the reverse ligate 2 was blocked with a 2′,3′-dideoxynucleotide to prevent potential extension of the 3′ end.

The functional design of the adapter molecules and the optimized reaction conditions permit to directly add the ligation products to the reverse transcription reaction without any further purification. Therefore, the reverse transcription and the following PCR can be carried out under the same conditions as the reactions with the full-length library. Indeed, the resulting PCR products of the short as well as the full-length library are indistinguishable, so that the PCR product can be used either to continue with a short library or a full-length library selection.

The efficiencies of the respective ligation reactions was determined using radioactively labelled RNA transcripts (Figure 3C). As expected, the 5′-ligation of the forward ligate usually produces acceptable ligation yields in the range of ~80% due to the two 5′-dangling nucleotides (Figure 3C, lane a). On the other side, the sterically less accessible 3′ end only generates ~30% (Figure 3C, lane b) of the 3′-ligation product. However, if the ligation reactions are carried out in parallel so that both forward and reverse adapters are present in equimolar concentrations, the overall ligation yield is markedly improved (Figure 3C, lane c).

In vitro selection against D-ghrelin

Overall, 18 rounds of *in vitro* selection against human D-ghrelin were carried out. The selection step was continuously performed at 37°C. During the first nine rounds, the full-length library was used; the initial three rounds were conducted manually, whereas the other six rounds were executed using a robotic system as described by Eulberg *et al.* (16). In order to increase the selection pressure, the initial peptide concentration of 1 μM was decreased to 33 nM in round 9 (see Supplementary Data).

Starting with round 10, *in vitro* selection was carried out by employing the short library. Due to the restriction of limited reaction volumes to be handled by the robotic system (up to 100 μl), the selection rounds were performed manually again in a reaction volume of up to 50 ml (starting with round 10 at a peptide concentration of 1 nM). The selection pressure was further increased by decreasing the peptide concentrations to 10 pM in rounds 17 and 18 (Figure 4). Since the signal to noise ratio in round 18 started to deteriorate, the PCR product resulting from selection round 16 was cloned and sequenced.

In 19 out of 23 clones, an extension of 6 nt compared to the length of the starting library was observed (Figure 5). Three conserved motifs are indicated by the background colours (blue, yellow and red). By sequence alignment, two families with one common motif (red) were identified. The red motif is already described as a sequence involved in ghrelin-recognition as reported for the Spiegelmer NOX-B11 (21). Firstly, all new sequences were synthesized as aptamers and ranked in a competition assay with NOX-B11 for their ghrelin binding potency. The clones showed similar or improved binding characteristics (data not shown). The best clones

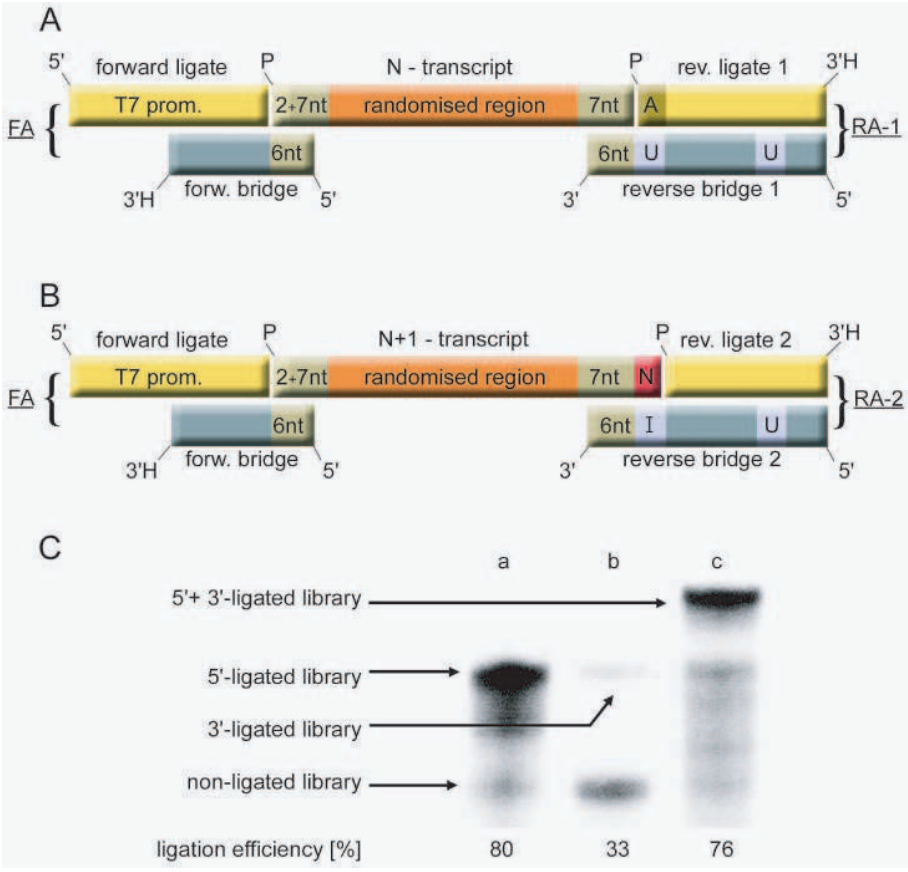

Figure 3. Template-directed ligation of the short RNA library. The cartoon (**A** and **B**) presents the structural designs of the short RNA library (N- and N+1-transcripts) and the double-stranded adapters which consist each of a ligate and a bridge. The randomized region (34 nt) is flanked by seven nucleotides that can form base pairs (see Figure 1), where upon the 5′ end carries two additional fixed nucleotides. Six nucleotides at both ends of the library serve as hybridization sites for the forward bridge and the two reverse bridges. The uridines (U) and the inosine (I) in the reverse bridge 1 (A) and 2 (B), respectively, introduce the alkaline fission sites into the primer binding site. The forward bridge, the reverse ligates 1 and 2 additionally contain a 3′-terminal 2′,3′-dideoxynucleotide (3′-H) to prevent mispriming during PCR. Up to 50% of all transcripts are N+1-transcripts (B) that contain a non-coded nucleotide at the 3′ end. In order to ligate primer binding sites to these molecules as well, the reverse adapter 2 was designed. It comprises the reverse ligate 2 i.e. 1 nt shorter at its 5′ end than ligate 1, and reverse bridge 2 that offers the universal base inosine (I) for hybridization to the non-coded nucleotide in the N+1 position of the transcript. The autoradiogram (**C**) exemplarily shows the results of the template-directed ligation. While ligation yield of the forward adapter to the 5′ end of the RNA molecules was acceptable (lane a), the ligation of the two reverse adapters 1 and 2 to the 3′ end of the library was with ~33% quite insufficient (lane b). However, in the case that both forward and reverse adapters are present in equimolar amounts at the same time, the overall ligation yield was markedly improved (lane c). FA: forward adapter; RA-1: reverse adapter 1; RA-2: reverse adapter 2; forw: forward; rev: reverse; prom: promoter; nt: nucleotides.

were synthesized as Spiegelmers and their IC_{50}-values were determined in cell culture experiments (Figure 5). Sequence NOX-F1 turned out to be the best binding molecule and was therefore subjected to further modifications. First, NOX-F1 could be truncated at the 5′ end by 6 nt; in addition, a hexa-loop of a potential hairpin structure (within the yellow motif) was bridged with a hexaethylene glycol linker (L) (24). Both modifications did not compromise the binding potency.

Ghrelin inhibitory characteristics of Spiegelmer NOX-B11-3

NOX-F1-trunc was synthesized as a mirror-image RNA oligonucleotide, and the Spiegelmer was named NOX-B11-3. To determine its potency to inhibit ghrelin action, NOX-B11-3 was tested in a cell culture system using CHO cells that stably express the GHS-R1a (growth hormone secretagogue receptor 1a). Different concentrations of NOX-B11-3 were analyzed to inhibit activation of the GHS-R1a by 2 nM ghrelin at 37°C. The readout parameter was the mobilization of intracellular Ca^{2+}. NOX-B11-3 inhibits ghrelin-mediated activation of the GHS-R1a resulting in a half-maximum inhibitory concentration (IC_{50}) of 4.5 nM (Figure 6). Thus, the potency of NOX-B11-3 under 37°C is ~5-fold higher than the potency of NOX-B11 which was analyzed under the same assay conditions (Figure 6).

DISCUSSION

Standard *in vitro* selection experiments are carried out with oligonucleotide libraries that contain a central randomized region, flanked by primer binding sites (25). To facilitate chemical synthesis and to reduce synthesis costs, the resulting aptamers are usually truncated to minimal target binding

motifs in a trial and error fashion. Since the primer binding sites are fixed sequences and ought to be without any further function, these parts of the oligonucleotides are the preferred choice to be cut off. However, the primer binding sites or segments of themselves may often be necessary to stabilize the overall structure of the aptamers or even become integral part of the target binding motif (10,26). This phenomenon especially appears if aptamers (and Spiegelmers) are directed to bind to structurally flexible targets that usually require a rigid binding partner (27). Thus, it is not surprising that only a limited number of oligonucleotides that were generated by conventional *in vitro* selection techniques to bind to small peptides comprise <50 nt (10–13,15,16,21,28).

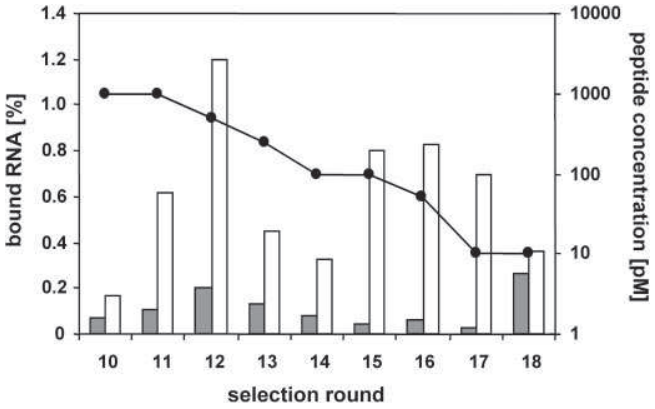

Figure 4. Progress of manual selection rounds (10–18) using the short library. The histogram shows the progress of the manual *in vitro* selection carried out with the short library starting from round 10. The black circles indicate the peptide target concentration offered in the respective selection round. In each selection round equal amounts of radio-labelled RNA were incubated with the respective peptide concentrations and with pure selection matrix as a control in order to verify the specific enrichment of binding sequences versus unspecific binding. The fraction of RNA specifically binding to the target peptide (white columns) is compared to the fraction of RNA that unspecifically binds to the selection matrix (grey columns).

Only few technical approaches address the problem to implement steps or techniques within the SELEX process that lead to readily truncatable aptamers. They are mainly described in the patent literature and include the blocking of primer binding sites by hybridization of complementary oligonucleotides during the selection step (29), ligation strategies that restore primer binding sites of primerless libraries before each selection step (14,15,29), and 'fishing' of primerless sequences using full-length (primer-bearing) complementary strands (29). The idea of 'fishing' was also used by Boiziau & Toulmé to identify fully 2′-*O*-methyl-aptamers (30).

The Tailored-SELEX approach is based on a ligation strategy for the primer binding site to an RNA library carrying a small number of fixed nucleotides on both sides of the randomized region. Compared to a manual selection, only two additional reaction steps are needed (15). Nevertheless, an automation protocol for the so-called Tailored SELEX-process is possible (16) but one selection round requires approximately one day, which is significantly slower than automation processes that employ conventional protocols (16,31,32).

We have developed an RNA library design that can be used in two different constitutions: as a (conventional) full-length library with primer binding sites or as a short library without primer binding sites. The library carries seven complementary (fixed) nucleotides which clamp the randomized region and constrain the oligonucleotides into a partly

Clone	Sequence	F	IC_{50} [nM]
NOX-B11	CGUGUGAGGCAAUAAAACUUAAGUCCGAAGGUAACCAAUCCUACACG		20.0
NOX-A2	GGGUGCUGUGAGGCAA-AAAA-GUAAGUCCGAAGGUAACCAAUCCUACAGCAC	1	7.5
NOX-H3	GGGUGCUGUGAGGCAAUG---CGUAAGUCCGAAGGUAUCCAAUCCUGCAGCAC	1	3.5
NOX-D1	GGGUGUUGUGAGGCAAUAA---GUAAGUCCGAAGGUAACCAAUCCUGCAGCAC	2	n.t.
NOX-E3	GGGUAAGCGUAAGACCGAAAGUAACCAAUCCUACCGUAUAUACGGUGAGGCAGCAC	1	n.t.
NOX-G2	GGGUAAGCGUAAGACCGAAGGUAACCAAUCCUACCGUAUCUACAGUGAGGCAGCAC	1	12.0
NOX-D2	GGGUAAGCGUAAGACCGAAGGUAACCAAUCCUACCGUAUCUACGGUGAGGCAGCAC	4	4.5
NOX-A3	GGGUAAGCGUAAGACCGAAGGUAACCAAUCCUAUCGUAUCUAUGGUGAGGCAGCAC	1	n.t.
NOX-E1	GGGUAUGCAUAAGACCGAAGGUAACCAAUCCUACCGUAUCUACGGUGAGGCAGCAC	1	n.t.
NOX-B1	GGGUAUGCGUAAGACCGAAGGUAACCAAUCCUACCGUAUCUACGGUGAGGCAGCAC	4	n.t.
NOX-F1	GGGUGAGCGUAAGACCGAAGGUAACCAAUCCUACCGUAUCUACGGUGAGGCAGCAC	2	4.5
NOX-C3	GGGUGUAUGUAAGACCGAAGGUAACCAAUCCUACCAUAUCUACGGUGAGGCAGCAC	1	n.t.
NOX-C2	GGGUGUGCGUAAGACCGAAGGUAACCAAUCCUACCAUAUCUACGGUGAGGCAGCAC	1	4.5
NOX-H2	GGGUGUGCGUAAGACCGAAGGUAACCAAUCCUAUCAUAUCUACGGUGAGGCAGCAC	1	n.t
NOX-A4	GGGUGUGCGUAAGACCGAAGGUACCCAAUCCUACCUACUAACUGGUGAGGCAGCAC	1	8.0
NOX-B2	GGGUGACG-UAAGACCGAAGGUAUCCAAUCCUACCUUUCCUGAGGUGAGGCAGCAC	1	6.5
NOX-F1-trunc	GCGUAAGACCGAAGGUAACCAAUCCUACCG---L--CGGUGAGGCAGCAC		4.5

Figure 5. Ghrelin-binding sequences. After 16 selection rounds, 23 clones were sequenced. The sequences comprise the fixed nucleotides (nucleotides are underlined) and the former randomized region. Column (F) indicates the frequency of occurrence of each individual sequence. The obtained sequences were grouped into two families and compared to the already published ghrelin-binding sequence NOX-B11 (21). Three conserved motifs are indicated by the background colours (blue, red and yellow). All sequences comprise of a 25 nt motif (red), which was already described as a sequence involved in ghrelin-binding (21). Firstly, the new sequences were synthesized as aptamers and ranked in a competition assay for their ghrelin binding potency in comparison to aptamer NOX-B11. All clones showed similar or improved binding characteristics (data not shown). An assortment of clones were synthesized as Spiegelmers and their IC_{50}-values were determined in cell culture experiments. NOX-F1 turned out to be the strongest binder and this oligonucleotide was further truncated and modified with a hexaethylene glycol linker (L) to give the final sequence NOX-F1-trunc. n.t. = not tested.

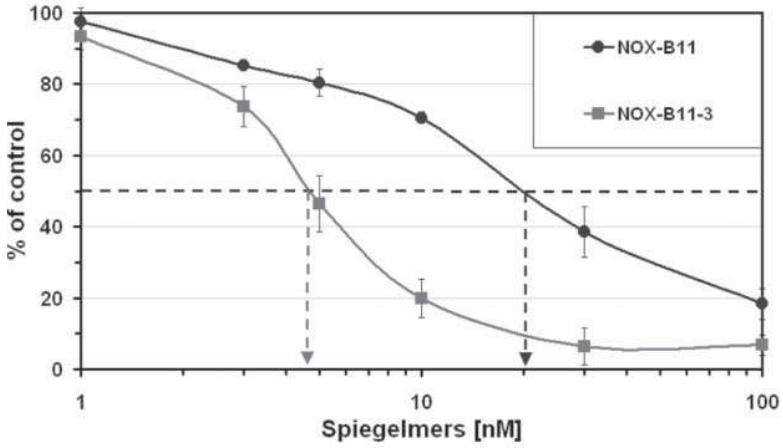

Figure 6. Inhibition of ghrelin-mediated activation of GHS-R1a by Spiegelmers NOX-B11-3 (= NOX-F1-trunc) and NOX-B11. CHO cells expressing GHS-R1a were stimulated with 2 nM ghrelin at 37°C in the presence of the indicated concentrations of the respective Spiegelmers, and the resulting Ca^{2+}-mobilization was determined. The ghrelin action is suppressed by both Spiegelmers in a dose-dependent manner, but the sequence of NOX-B11-3 shows an ~5-fold better IC_{50} than NOX-B11.

double-stranded structure. This design already minimizes the risk that the primer binding sites become part of the target-binding motif. However, the use of primer binding sites forming a double-stranded structure does not guarantee their truncation without reduction or loss of binding to the target (F. Jarosch, unpublished data). Therefore, moreover, the sequence arrangement of the dual library also allows to carry out the selection step with the short library. In this case the flanking primer binding sites are removed before the selection step and added subsequently.

The structural constraints of the RNA library and the specific use of the short library within the selection step facilitate the direct identification of short aptamer sequences without the need of time-consuming or unsuccessful truncation experiments. However, in order to limit potential losses of valuable target binding sequences due to additional enzymatic steps, in early selection rounds only the full-length library is employed. Furthermore, the full-length library allows the use of a faster (automated) selection protocol (16). Therefore, the RNA library described herein is primarily used as full-length library for automated *in vitro* selection. After a solid basis of target-binding (full-length) sequences is achieved (here after nine selection rounds), the selection protocol is switched to the short library. This switch avoids enrichment of sequences which functions are potentially dependent on the existence of primer binding sites.

In contrast to the length of the starting (short) library, most of the enriched oligonucleotides were extended by 6 nt. Since the additional nucleotides were integrated into the original random region, this result may be a consequence of the stringent selection conditions rather than an unwanted side-effect of the design of the dual library. Nevertheless, all sequences share a common sequence (shaded in red) that was already described as a ghrelin-binding motif (21). Ranking experiments revealed that the aptamer sequence NOX-F1 showed the best potency. The corresponding Spiegelmer of NOX-F1 was further truncated by cleaving off the six 5′-terminal nucleotides and substituting a potential loop region of 6 nt by a hexaethyleneglycol linker. The resulting Spiegelmer NOX-F1-trunc (NOX-B11-3) inhibits activation of the GHS-R1a receptor displaying an inhibition constant (IC_{50}) of 4.5 nM at 37°C. Thus, the IC_{50} of NOX-B11-3 is ~5-fold lower than the IC_{50} of Spiegelmer NOX-B11 if compared at 37°C.

This data underlines that our devised process using a dual RNA library delivers high affinity aptamers that can easily be truncated by cleaving off at least the primer binding sites. Furthermore, the library design permits an easy integration into an automated selection protocol. Right now, ghrelin-inhibitor NOX-B11-3 serves as new lead compound and is currently being evaluated in preclinical studies for the indication obesity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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