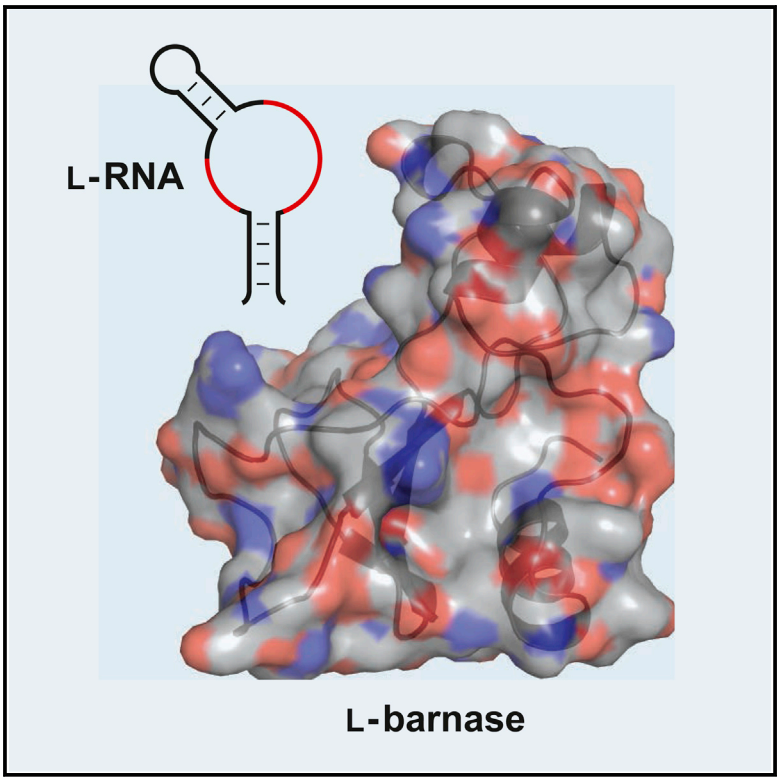
Brief Communication

Chemistry & Biology

An L-RNA Aptamer that Binds and Inhibits RNase

Graphical Abstract



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In Brief

Olea et al. select mirror-image RNA molecules, composed of the non-natural L-isomer, for their ability to bind and inhibit RNase, an enzyme that rapidly degrades natural RNA. This was accomplished by selecting natural RNAs that bind the full-length, non-natural isomer of RNase then reversing the mirror to enable L-RNA to protect its natural counterpart.

Highlights

- L-RNA aptamers were selected that bind and inhibit barnase RNase
- This required synthesizing the full-length synthetic D-enantiomer of barnase
- The L-RNA aptamers are competitive inhibitors of degradation of D-RNA substrates
- The first time a mirror-image aptamer has been raised against an entire enzyme

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An L-RNA Aptamer that Binds and Inhibits RNase

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SUMMARY

L-RNA aptamers were developed that bind to barnase RNase and thereby inhibit the function of the enzyme. These aptamers were obtained by first carrying out in vitro selection of D-RNAs that bind to the full-length synthetic D-enantiomer of barnase, then reversing the mirror and preparing L-RNAs of identical sequence that similarly bind to natural L-barnase. The resulting L-aptamers bind L-barnase with an affinity of ~100 nM and function as competitive inhibitors of enzyme cleavage of D-RNA substrates. L-RNA aptamers are resistant to degradation by ribonucleases, thus enabling them to function in biological samples, most notably for applications in molecular diagnostics and therapeutics. In addition to the irony of using RNA to inhibit RNase, L-RNA aptamers such as those described here could be used to measure the concentration or inhibit the function of RNase in the laboratory or in biological systems.

INTRODUCTION

Numerous RNA aptamers have been obtained that bind small-molecule and protein targets with high affinity and high specificity (Gold et al., 1995; Klussmann, 2006). Unmodified RNAs are rapidly degraded by ribonucleases that are ubiquitous in biological samples. The half-life of RNA in human serum, for example, is only a few seconds (Tsui et al., 2002). Thus, if RNA aptamers are to be applied in a biological context they must be modified with nuclease-resistant nucleotide analogs, such as 2′-*O*-methyl nucleotides (Keefe and Cload, 2008; Wang et al., 2011). These modifications, unless built in from the start of an aptamer selection, can potentially alter binding affinity, thus requiring further optimization to accommodate the modifications.

An alternative approach utilizes L-RNA, the enantiomeric counterpart of natural D-RNA. Unlike D-RNA, L-RNA is not degraded by biological ribonucleases. L-RNA aptamers, termed Spiegelmers, are generated by selecting natural D-RNAs against the enantiomer of the desired target (Klussmann et al., 1996; Nolte et al., 1996). Once a D-aptamer is obtained, an L-RNA aptamer of the same sequence can be prepared and used to bind the natural target in a mirror-identical fashion. Several L-RNA aptamers are currently in phase II human clinical trials (Schwoebel et al., 2013; Hoellenriegel et al., 2014), and an L-aptamer has been engineered for the quantitative analysis of ligand concentrations in human serum (Olea et al., 2012).

To date, L-RNA aptamers only have been selected against small molecules (Klussmann et al., 1996; Nolte et al., 1996), peptides (Maasch et al., 2010), chemokines (Eulberg et al., 2008), and small structured D-RNAs (Sczepanski and Joyce, 2013). None have been selected against a complete functional enzyme, largely because of the difficulty in synthesizing the full-length enantiomeric protein. Proteins are instead targeted for selection by preparing a small peptide that corresponds to an epitope within the overall protein. However, there are two advantages in carrying out an aptamer selection using the entire protein. First, it ensures that the aptamer will bind to the intact protein as well as the chosen epitope. Second, it increases the likelihood that any inhibition of protein function that occurs as a consequence of binding will apply in the fully native context.

The goal of the present study was to generate an L-RNA aptamer against an intact protein enzyme. Barnase, the well-studied RNase from *Bacillus amyloliquefaciens*, was chosen as the target because it provides a case whereby a D-RNA aptamer would be ineffective due to degradation of D-RNA by the target enzyme. The complete D-enantiomer of barnase was prepared by chemical synthesis and allowed to fold into its active conformation. The D-form of the enzyme was used as the target to select D-RNA aptamers with high affinity for barnase. As is inevitably the case, an L-RNA version of these aptamers binds to natural L-barnase in the same manner. Aptamer binding to the enzyme results in corresponding inhibition of enzyme function, thereby enabling the L-aptamer to block the ability of RNase to degrade D-RNA.

RESULTS

Full-length D-barnase, containing 110 amino acids, was prepared by solid-phase synthesis. Two half-length polypeptides were prepared, then joined by native chemical ligation (Dawson et al., 1994, 1997) to yield the complete protein. The lysine at position 49 was replaced by cysteine to provide a site for derivatization with biotin, which enabled immobilization of the protein on streptavidin. The site of biotinylation is on the opposite face of the enzyme relative to the RNA binding cleft, thus presenting the active site of the enzyme as a potential target for aptamer binding (Figure 1). The full-length D-barnase was analyzed by electrospray ionization mass spectrometry (ESI-MS), which gave an observed mass of 12,357 ± 1 Da, compared with a calculated mass of 12,357.8 Da (Figure S1A).

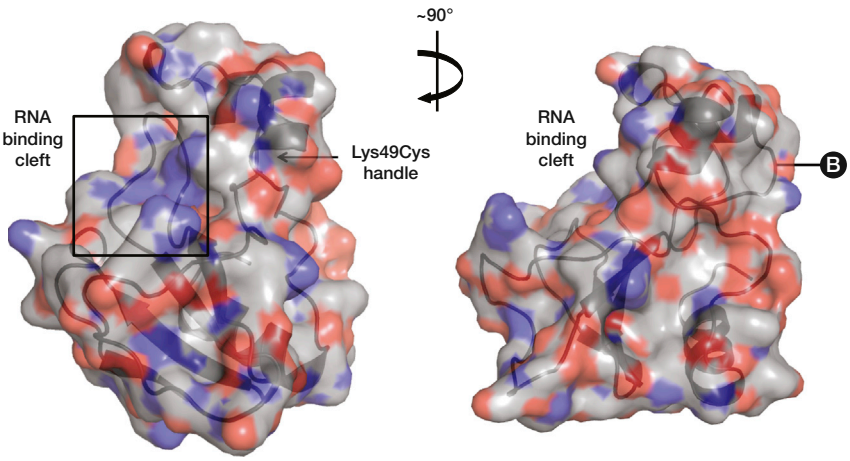

Figure 1. Structure of Barnase

X-Ray crystal structure of barnase (Buckle et al., 1993) showing the site of lysine to cysteine substitution that enables derivatization with biotin (encircled B). The RNA binding cleft is on the opposite face (see also Figure S1).

D-Barnase was folded by dilution from 6 M guanidine hydrochloride and assayed for activity using an L-RNA oligonucleotide substrate. The synthetic D-enzyme maintains the natural preference for cleavage at the 3′-side of guanylate residues, but with reciprocal chiral specificity for L-RNA (Figure 2A). Contrary to a recently published report (Vinogradov et al., 2015), there was no detectable cleavage of D-RNA by D-barnase, which made it possible to carry out in vitro selection of D-RNAs that bind D-barnase and are then amplified using biological polymerases. To enable this selection procedure we conjugated a biotin moiety, linked via polyethylene glycol to maleimide, at position Cys-49 of D-barnase. The composition of the resulting biotinylated enzyme was confirmed by ESI-MS (Figure S1B).

Aptamers for D-barnase were obtained by in vitro selection starting from a library of 5×10^{14} D-RNA molecules, each containing a central region of 35 random-sequence nucleotides, flanked by fixed primer binding sites (Figure S2A). Selection for D-barnase aptamers was carried out in the presence of 25 mM $MgCl_2$ and 100 mM NaCl at pH 8.0 and 23°C. The RNA pool (1 μM) was first incubated with streptavidin-coated magnetic beads for 30 min to capture materials that bound to the beads in the absence of barnase. Then the supernatant was withdrawn and mixed with 1 μM D-barnase, incubated for 30 min, and applied to fresh streptavidin-coated beads to capture barnase and any RNAs that were bound to the protein. After 15 min the beads were washed four times with 1 ml of the same binding solution. Then the bound RNAs were eluted with 25 mM NaOH and 1 mM EDTA, reversed transcribed, and PCR amplified. The resulting double-stranded DNAs were transcribed to generate an enriched pool of selected RNAs. This procedure was repeated for seven rounds. Individual clones were isolated from the population following the fifth and seventh rounds and assayed for their ability to inhibit the activity of D-barnase (Figure S2B).

Two clones, referred to as **5-4** and **7-3**, were chosen for further analysis based on their frequency of occurrence (after rounds five and seven, respectively) and the ability of the corresponding L-RNAs to inhibit L-barnase (Figure 2B). **D-5-4** RNA was truncated at both its 5′ and 3′ ends and engineered to contain a stable GUAA tetraloop within the distal stem, resulting in the minimized construct **D-5-4t** (Figure 3). **D-5-4t** contains 45 nucleotides and consists of two stem regions joined by a double internal bulge loop. The full-length **D-7-3** RNA has greater ability to inhibit D-barnase compared with various truncation versions that were tested, and therefore was left intact (Figure 3). It contains 72 nucleotides and also consists of two stem regions joined by a double internal bulge loop.

The binding affinity of **D-5-4t** and **D-7-3** for D-barnase was determined using an electrophoretic mobility shift assay, which gave apparent K_D values of 150 and 73 nM, respectively (Figure 4A). The corresponding L-RNA aptamers were prepared by chemical synthesis and gel purified. The recombinant wild-type L-barnase was expressed and purified from *Escherichia coli* as described previously (Hartley and Rogerson, 1972; see also Supplemental Experimental Procedures), then analyzed by ESI-MS (Figure S1C).

In-line probing, based on partial hydrolysis under nondenaturing conditions (Soukup and Breaker, 1999), was carried out for both L-RNA aptamers in either the absence or presence of saturating concentrations (12 μM) of L-barnase (see Supplemental Experimental Procedures). This was done to identify nucleotides that interact with the protein, resulting in their relative protection from hydrolysis (Figure 3). For **L-5-4t**, both sides of the internal bulge loop were substantially protected from hydrolysis in the presence of barnase, but there was no significant protection within the two flanking stems. For **L-7-3**, there was substantial protection within the proximal portion of the distal stem and adjoining 3′-portion of the internal bulge loop, but no significant protection within the proximal stem or the distal portion of the distal stem.

In the absence of the L-RNA aptamers, the observed rate of cleavage by L-barnase of a short, unstructured D-RNA substrate was 0.33 min^{-1}. Under the conditions of the assay, which employed 0.125 nM barnase and 0.5 μM RNA at pH 8.0 and 23°C, the RNA substrate was completely degraded within 15 min. Various concentrations of **L-5-4t** and **L-7-3** were added to the mixture to determine an IC_{50} value for their inhibition of L-barnase activity (see Supplemental Experimental Procedures). Both aptamers were found to be effective inhibitors, with IC_{50} values of 190 and 160 nM, respectively (Figure 4B). This pales in comparison, however, with the subpicomolar inhibition exhibited by barstar (Hartley, 1993), a bacterial protein that coevolved with barnase. At a saturating concentration of 2 μM L-RNA aptamer, the D-RNA substrate was not fully degraded until ~12 hr, corresponding to a 40- to 50-fold increase in substrate half-life compared with the absence of the aptamer (Figure S4A). Neither L-RNA aptamer, even at 20 μM concentration, exhibited detectable inhibition of either RNase A or RNase T1 (Figure S3), demonstrating the specificity of these aptamers for L-barnase.

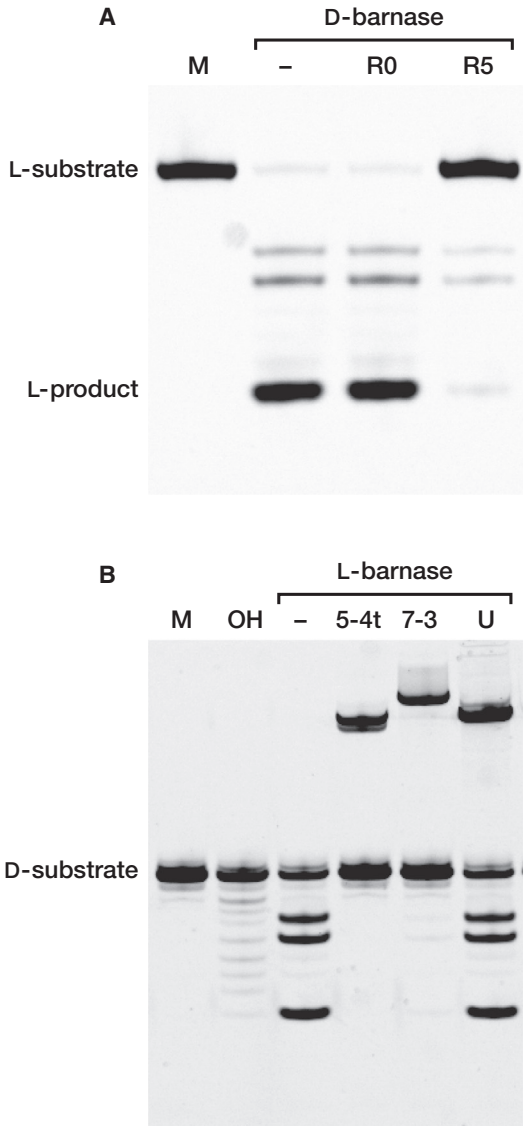

Figure 2. Inhibition of Barnase Activity by the Selected RNA Aptamers

(A) D-Barnase cleavage of 5′-fluorescein-labeled L-RNA substrate, having the sequence 5′-GAUAGGUAG-3′, is inhibited by the population of D-RNAs obtained after the fifth round of selection (R5), but not by the starting population (R0). Cleavage occurs at the 3′ side of G residues, resulting in 5′-labeled products corresponding to GAUAGG, GAUAG, and G. Reaction conditions: either none (–) or 20 μM D-RNA population, 25 nM D-barnase, 0.5 μM substrate, 25 mM $MgCl_2$, 100 mM NaCl, 50 mM EPPS (pH 8.0), and 0.1% Triton, incubated at 23°C for 1 min. Unreacted substrate (M) is shown on the left.

(B) L-Barnase cleavage of 5′-fluorescein-labeled D-RNA substrate, having the same sequence as above, is inhibited by **L-5-4t** and **L-7-3**, but not by a 55mer control L-RNA of unrelated sequence (U) (see also Figure S3). Reaction conditions as above, but with either none (–) or 10 μM 5′-hexachloro-fluorescein-labeled L-RNA (seen as the slow-migrating bands in the denaturing 15% polyacrylamide gel) and 0.25 nM L-barnase, incubated for 5 min. Unreacted substrate (M) and substrate that was subject to partial alkaline hydrolysis (OH) are shown on the left.

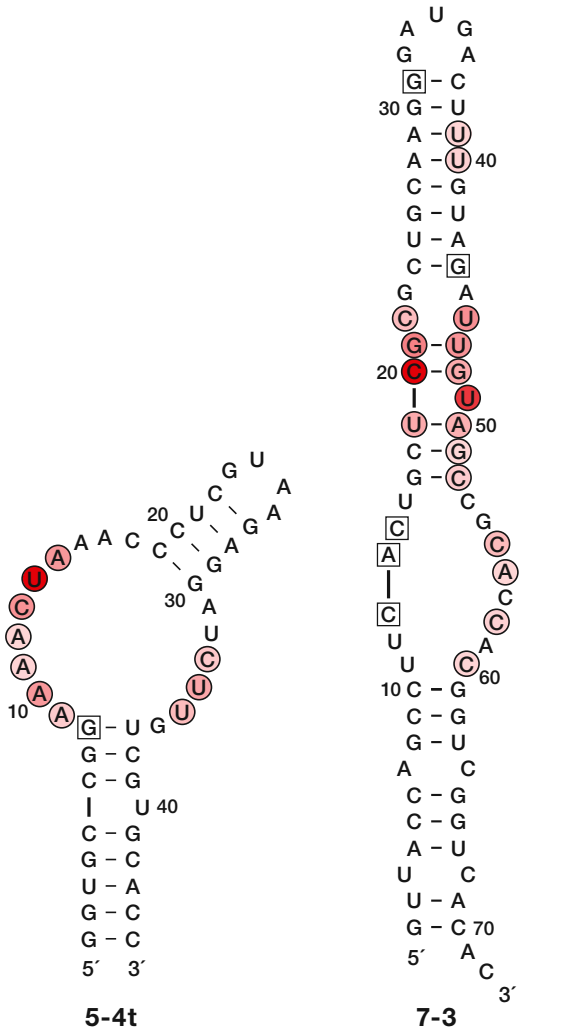

Figure 3. Sequence and Predicted Secondary Structure of Barnase Aptamers

Red circles indicate nucleotide positions that are relatively protected against hydrolytic cleavage in the aptamer-barnase complex compared with the aptamer in isolation. Increasing color intensity corresponds to increasing levels of protection. Boxes indicate nucleotide positions with enhanced susceptibility to hydrolysis in the aptamer-barnase complex.

See also Figure S2.

All of the clones that were isolated following the in vitro selection process were found to inhibit barnase activity (Figure S2B). This may be a result of electrostatic interactions between the anionic RNA aptamer and the cationic substrate binding cleft of barnase (Figure 1). The active site of barnase is dominated by positive charges, with three basic residues that guide the interaction with the negatively charged RNA substrate (Mauguen et al., 1982; Baudet and Janin, 1991). In support of this hypothesis, both the **L-5-4t** and **L-7-3** aptamers were found to exhibit strictly competitive inhibition kinetics in the barnase-catalyzed RNA-cleavage reaction (Figures S4B and S4C). While these results do not necessarily exclude binding of the aptamers at an allosteric site on the free enzyme that prevents substrate binding, it seems more likely that the L-RNA aptamers and D-RNA substrate compete for a common site. It would have been difficult to capture this interaction if the selection had been carried out using a peptide fragment rather than the intact protein,

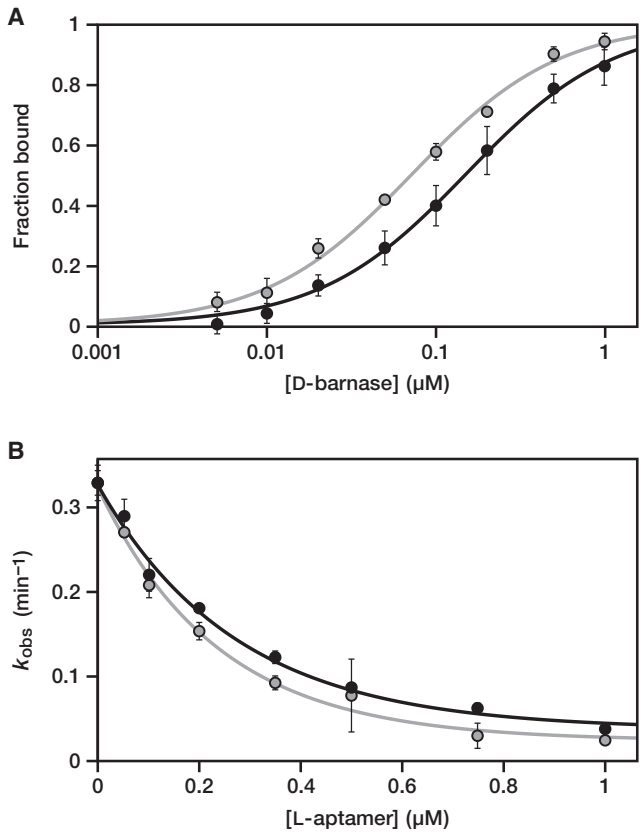

Figure 4. Binding and Inhibition of Barnase by Aptamers 5-4t and 7-3

Aptamer **5-4t** is shown in black and aptamer and **7-3** in gray.

(A) Saturation plot for binding of the D-aptamers to D-barnase, as determined by an electrophoretic mobility shift assay. Data were obtained in triplicate and fitted to the equation $F_{bound} = F_{bound\text{-}max}$ {[barnase]/(K_D + [barnase])}.

(B) Rate of L-barnase cleavage of 0.2 μM D-RNA substrate, having the sequence 5′-UUGAUAGGUAGU-3′, measured in the presence of various concentrations of L-aptamer (see also Figure S4). Data were obtained in triplicate and fitted to the equation $k_{obs} = \{k_{obs\text{-}max}\ (e^{-a\cdot[aptamer]})\} + k_{obs\text{-}min}$, where $k_{obs\text{-}max}$ is the observed rate in the absence of aptamer and $k_{obs\text{-}min}$ is the calculated rate at infinite aptamer concentration. Error bars represent the standard deviation about the mean.

especially because the active site of barnase is formed by multiple epitopes that come together to form the substrate binding cleft (Buckle et al., 1993).

DISCUSSION

The generation of L-RNA aptamers that bind biological proteins requires selecting against a synthetic enantiomer of the desired target. Previous efforts to develop such aptamers have employed peptides or peptide epitopes within the larger protein. However, intact folded proteins present functionally relevant tertiary epitopes that may not be captured by protein fragments, and therefore provide a greater opportunity to generate aptamers that can modulate protein function.

Despite having identical chemical composition as its natural biological counterpart, L-RNA is orthogonal to biological systems. This point was emphatically demonstrated here by choosing biological RNase as the target for in vitro selection of enantiomeric RNA aptamers. The inhibition of specific ribonucleases may be useful in deducing their function in both normal and diseased tissues, most notably in cancer and inflammatory conditions (Kim and Lee, 2009; Sun et al., 2013). Ongoing efforts to push the limits of the chemical synthesis of D proteins, most recently to 312 residues (Weinstock et al., 2014), will make available additional protein targets for the in vitro selection of L-RNA aptamers. Such molecules may have broader applications in molecular diagnostics and therapeutics.

SIGNIFICANCE

Although biology is overwhelmingly homochiral, chemical biologists are free to play on both sides of the mirror. Previous studies have led to the synthesis of functional proteins and nucleic acids of opposite handedness compared with their biological counterparts. Mirror-image aptamers that bind to biological macromolecules in a cross-chiral fashion have been obtained by in vitro selection. Here, for the first time, a mirror-image aptamer is raised against an entire enzyme, which necessitated the total synthesis of the non-biological enantiomer of that enzyme. Barnase RNase was chosen as the target because of the impossibility of targeting this molecule with biological D-RNA. By employing the entire enzyme in its properly folded state, there was a high likelihood that the selected L-RNA aptamers would inhibit RNase function, which turned out to be the case. Two distinct aptamer motifs were identified, each with an affinity of ~100 nM for barnase and a corresponding ability to inhibit the enzyme's catalytic activity. Although RNase wins the battle against biological D-RNA, L-RNA aptamers can turn the tables and protect their enantiomeric counterparts from RNase degradation.

EXPERIMENTAL PROCEDURES

Preparation of D-Barnase

The D-enantiomer of barnase K49C was synthesized using in situ neutralization cycles for Boc-protected solid-phase peptide synthesis (Schnölzer et al., 1992) and native chemical ligation (Dawson et al., 1994) of two polypeptides, as described previously (Dawson et al., 1997). See Supplemental Experimental Procedures for synthetic methods and protein purification protocol.

In Vitro Selection

The starting library was prepared by synthesizing a DNA template containing 35 random-sequence nucleotides flanked by fixed primer binding sites (Figure S2A), which was made double stranded by primer extension, then transcribed to generate a pool of 5 × 10^{14} corresponding RNAs. One nmol of pool RNAs was incubated in a 1-ml volume containing 100 mM NaCl, 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propane sulfonic acid (EPPS) (pH 8.0), and 0.1% Triton at 70°C for 2 min, then cooled on ice for 2 min. Then a 200-μl volume containing 2 mg of streptavidin resin, which had been pre-blocked with 1 mg/ml tRNA and 25 mM $MgCl_2$, was added and allowed to incubate at 23°C for 30 min. The resin was captured magnetically, removing any RNAs that had bound to it directly. Next 1 μM biotinylated D-barnase was added, the solution was incubated at 23°C for 15 min, and the protein and any associated RNAs were captured on 8 mg (800 μl) of streptavidin resin, as above. The resin was washed with 4 × 1-ml volumes of 25 mM $MgCl_2$, 100 mM NaCl, 50 mM EPPS (pH 8.0), and 0.1% Triton, then the RNA was eluted with 25 mM NaOH and 1 mM EDTA, neutralized with 0.5 M Tris-HCl, reverse transcribed, and amplified by PCR. The amplified DNA was

transcribed using T7 RNA polymerase, and the resulting RNAs were gel purified and used to begin the next round of in vitro selection. The volume of incubation was reduced 4-fold in the subsequent rounds.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.09.017.

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