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Selection of small peptides, inhibitors of translation

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Abstract

Identification of small molecular weight compounds targeting specific sites in the ribosome can accelerate development of new antibiotics and provide new tools for ribosomal research. We demonstrate here that antibiotic-size short peptides capable of inhibiting protein synthesis can be selected by using specific elements of ribosomal RNA as a target. The 'h18' pseudoknot encompassing residues 500–545 of the small ribosomal subunit RNA was used as a target in screening a heptapeptide phage display library. Two of the selected peptides could efficiently interfere with both bacterial and eukaryotic translation. One of these inhibitory peptides exhibited a high-affinity binding to the isolated small ribosomal subunit (K_d of 1.1 μ M). Identification of inhibitory peptides which likely target a specific rRNA structure may pave new ways for validating new antibiotic sites in the ribosome. The selected peptides can be used as a tool in search of novel site-specific inhibitors of translation.

Keywords

translation; inhibitory peptides; phage display; antibiotics; ribosome

The ribosome is an excellent antibiotic target. Many antibiotics discovered over the past 50 years inhibit cell growth by interfering with its functions (reviewed in refs 1–3). However, the initial clinical success of these antibiotics (as well as that of any other clinically-relevant antibacterial drug) is always closely followed by the appearance and rapid spread of the resistance strains 4. This race between the availability of medically useful antibacterials and the appearance of resistant strains fuels the perpetual quest for new antibiotics, including protein synthesis inhibitors, that can evade known resistance mechanisms. One intriguing approach in this crusade is to design drugs that would target principally new ribosomal sites.

Several techniques have been employed to identify new 'druggable' sites in the ribosome and some rRNA loci have been pinpointed as promising antibiotic targets 5–8. There is, however, a long way between knowing the site one wants to target and finding inhibitors which act specifically at such a site. The enormous size (ca. 2.5 million Da) and complexity (more than 50 different components) of the ribosome make it a daunting task to find ligands that would bind to a specific element in the ribosome structure. High-throughput screening (HTS) techniques that can be applied for identification of the general inhibitors of translation 9–12 cannot identify compounds that target a specific structural element of the ribosome. Although

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several computational 13;14 approaches have been tried in search of new site-specific protein synthesis inhibitors, their success has been fairly restricted because of the limitations of the algorithms for modeling interactions of small ligands with RNA targets.

One possible way to discover site-specific ligands that can serve as inhibitors of protein synthesis is through the use of phage display libraries in conjunction with isolated rRNA elements as targets. Short inhibitory peptides can validate the corresponding ribosomal site as an antibiotic target, and may be used in subsequent screens of chemical libraries or developed into peptidomimetic drug leads. In this approach, the confounding structural complexity of the ribosome is confronted with the combinatorial complexity of phage display peptide libraries.

Here we demonstrate the utility of this approach by identifying heptapeptides which inhibit protein synthesis by, likely, targeting a specific functional element in rRNA.

Phage display selection of peptides capable of recognizing h18

As a model antibiotic target in the ribosome we chose the idiosyncratic pseudoknot structure (h18) formed by the segment 500-545 of 16S rRNA (Figure 1A,B)¹⁵. This RNA pseudoknot plays a central role in functions of the ribosome decoding center and is directly involved in controlling the accuracy of aminoacyl-tRNA selection¹⁶. Many lethal mutations have been identified in h18^{7; 15; 17; 18}. Such clustering of deleterious mutations are the key signatures of 'good' antibiotic sites in the ribosome^{6-8; 19}. In the ribosome, h18 is located on the surface of the small subunit, at its interface side, and is engaged in fairly limited interactions with other structural elements (Figure 1A). Therefore, it is reasonable to expect that the structure of h18 in isolation would resemble its native fold in the 30S subunit. A synthetic 59-nt RNA-DNA hybrid which was composed of a 49-nt RNA segment representing h18 of *Bacillus anthracis* 16S rRNA followed by a 10-nt poly-dA tail with biotin attached at the 3' end was used as a target for phage display screening (Figure 1C). The selections were carried out using 2×10^{11} phage particles (representing 1.28×10^9 possible 7-residue sequences) of the commercially-available M13 phage library, Ph.D.-7 (New England Biolabs), which displays random-sequence heptapeptides fused to coat protein pIII. The library was initially counter-selected by pre-incubation with the streptavidin-coated solid carrier used in selection (streptavidin-coated microtiter plate wells or streptavidin-coated magnetic beads).

The library was then incubated with the h18 target present at 100 nM in the presence of 2 mg/ml of total *E. coli* tRNA. We carried out three independent selections, using three different formats for isolation of the phage-target complexes. In the first approach, target-bound phages were captured on streptavidin-coated paramagnetic beads (DynaL Biotech) and released by DNase treatment. In the second approach, the phages captured on the beads were eluted with a low-pH buffer. Finally, in the third approach, the phage-target complexes were captured in the wells of streptavidin-coated microtiter plate and were eluted with a low-pH buffer.

Sequencing individual phages selected on the basis of DNase elution showed that already after the second round of selection most of the phages in the population corresponded to a single species exhibiting the peptide with the sequence SILPYPY (Table 1). This was the only sequence found among 60 sequenced phages after the third round of selection. Such a rapid drop in library complexity was unexpected and might have reflected an unusual susceptibility of the poly-dA tail to DNase I when the RNA/DNA hybrid target was associated with the corresponding phage variant.

After four rounds of selection based on the use of paramagnetic beads in conjunction with a low-pH elution, 32% of the sequenced phages displayed the peptide AGAAMSH (Table 1). Several other peptide variants were also identified although they did not show any obvious sequence convergence.

The selection protocol based on the use of streptavidin-coated 96-well plates yielded the sequence AMSAPIP which was found in 94% of the phages sequenced after four rounds of selection. This peptide shared a motif AMS with the predominant peptide AGAAMSH from the bead-based selection (Table 1). Altogether, three selection strategies yielded 25 different peptide sequences.

The 'winner' peptides in the three selection formats, SILPYPY, AMSAPIP and AGAAMSH, are enriched in non-polar amino acid residues and do not contain any positively-charged amino acids which are commonly found in RNA binding peptides 20. Nevertheless, the prevalence of non-polar or polar uncharged amino acids in RNA binding peptides is not uncommon 21 and might indicate a preferential interaction of the selected peptides with the RNA bases, rather than the sugar-phosphate backbone.

Inhibition of protein synthesis by selected peptides

Several heptapeptides found in the selected phages were chemically synthesized and tested for their ability to inhibit protein synthesis in the bacterial (*E. coli*) cell-free transcription-translation system programmed with the plasmid (pBEST*luc*) DNA. Protein synthesis was monitored by following incorporation of [³⁵S]-methionine in the trichloroacetic acid-precipitable fraction. In the initial tests, the peptides were dissolved in water:ethanol:DMSO (1:1:1, v/v/v) and added to the reaction at 100 μ M concentration. Several of the tested peptides exhibited a well-pronounced inhibition of protein synthesis (Figure 2A). The strongest inhibitors were peptides AGAAMSH, GTMLAAV and AMSAPIP which reduced protein yield by 70-80%. Remarkably, two of these peptides, AGAAMSH and AMSAPIP, were the 'winners' in the beads- and plate-based selections (Table 1). Several other of the tested selected peptides were also able to inhibit activity of the cell-free system but to a lesser degree. The peptide SILPYPY identified in the DNase-based selection moderately stimulated protein synthesis. The unrelated control heptapeptide, LRRASLG 22, did not show any effect.

Because of the very poor solubility of the peptide GTMLAAV in water, only two other best inhibitory peptides, AGAAMSH and AMSAPIP were analyzed in more detail. Titrating these peptides in the *E. coli* cell-free transcription-translation system yielded IC₅₀ values of 15 μ M (AGAAMSH) and 137 μ M (AMSAPIP) (Figure 2B) revealing the first of them as a fairly potent inhibitor. (An unexpectedly high IC₅₀ of the AMSAPIP peptide - compare with Figure 2A - is likely related to its limited solubility in water which was used as a solvent for peptides in this experiment). The peptides also inhibited protein synthesis programmed by the bacteriophage MS2 RNA with IC₅₀ values of 30 μ M (AGAAMSH) and 111 μ M (AMSAPIP) (Figure 2C). This result confirmed translation as the target of the peptides' inhibitory activity.

In accordance with the high degree of conservation of h18 across the evolutionary domains, both peptides, AMSAPIP and AGAAMSH, inhibited protein synthesis catalyzed by the eukaryotic ribosome (Figure 2D). While the inhibitory activity of AGAAMSH in the rabbit reticulocyte cell-free translation system (IC₅₀ = 20 μ M) was comparable to that observed in the bacterial system, the peptide AMSAPIP showed a 20-fold higher potency in inhibiting eukaryotic translation (IC₅₀ = 6.3 μ M) compared to its effect upon bacterial translation.

Binding of the AGAAMSH peptide to the 30S subunit

In spite of several attempts, our efforts to directly map the binding site of the selected peptides in the ribosome using RNA probing have not yielded concluding evidence. No peptide-specific protections of 16S rRNA nucleotide residues from chemical modification with dimethyl sulfate, kethoxal, or carbodiimide was observed in h18 or in any other site in 16S rRNA (data not shown). Although disappointing, this result, however, does not argue against binding of

the peptide to the anticipated ribosomal target: many well-characterized ribosomal antibiotics do not produce any discernable RNA protections 23;24.

The small size of the heptapeptide ligands and the lack of an easily-traceable moiety in the peptide structure makes it difficult to use conventional techniques for analyzing binding of the peptide to the ribosome. Therefore, in order to investigate binding of the inhibitory peptide to its likely target, we used a recently developed method 25 which employs fluorescently-labeled 30S subunits. The ribosomal protein S12 was mutagenized to carry a single cysteine residue at position 44 and then conjugated with the environment-sensitive fluorophore 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS). The small subunits were then reconstituted *in vitro* from 16S rRNA and purified small subunit proteins 26 where wild type protein S12 was replaced with its fluorescently-modified variant. Incorporation of the modified protein S12 places the fluorophore in close vicinity of h18. We have previously shown that attachment of the IAANS moiety at position 44 of protein S12 does not prevent incorporation of the protein in the 30S subunit and that the fluorophore responds to binding of streptomycin and other aminoglycoside antibiotics which bind in the vicinity of the fluorescent probe 25. The assembled subunits were purified by sucrose gradient centrifugation and used in peptide binding experiments. The binding isotherm of the heptapeptide AGAAMSH to the isolated modified 30S subunit (Figure 3) yielded a dissociation constant (K_d) of 1.1 μ M. It is tempting to think that in the 30S subunit, the peptide binds to h18 which was used as a target in the phage display library screening. The limited available amounts of reconstituted fluorescently-labeled 30S subunits prevented us from testing binding of the AMSAPIP peptide.

In the experiments presented above, we described isolation of short peptides which are capable of inhibiting protein synthesis. These peptides were identified by using a phage display library which was screened using h18 of 16S rRNA as a target. Although peptides identified using different selection protocols did not show a clear sequence convergence, the winners of the two independent selections, peptides with the sequence AGAAMSH and AMSAPIP, shared a three amino acid-long stretch, AMS, which accounted for 45% of the entire peptide sequence. This sequence might be involved in interaction with the RNA target. Remarkably, these same 'winning' peptides showed the highest inhibitory activity in the cell-free translation system underscoring the utility of the approach for identifying peptides capable of inhibiting translation.

We have assumed that the 'amputated' h18 assumed its natural 'ribosomal' fold in isolation. We do not have any direct evidence whether this is the case. Furthermore, studies of the h18 variants suggest that its conformation may be prone to certain structural transitions (27 and D. Draper, personal communication). Therefore, it is generally possible that the selected peptides recognize a non-native conformation of h18. In this scenario, the inhibitory effect of the peptides observed in the *in vitro* translation experiments could result from stabilizing a non-functional conformation of h18 in the ribosome.

Although the bacterial version of h18 was used as the target in phage-display selection, the identified inhibitory peptides did not show selectivity against the bacterial versus the eukaryotic ribosome. In contrary, the peptide AMSAPIP was a more potent inhibitor of eukaryotic translation. The lack of selectivity is not surprising. The loop of h18 carries one of the most conserved sequences in the ribosome and if selected peptides recognize the conserved part of h18 they are expected to bind to (and possibly inhibit) ribosomes from various species. The stem of h18, however, shows considerable variability (Figure 1B) which might be exploited in development of selective antibiotics targeting this structure. Identifying such selective protein synthesis inhibitors might require counter-selection of a phage-display library with the RNA target representing h18 of the mammalian (human) ribosome.

We were unable to directly demonstrate binding of the selected peptides to h18 in the ribosome. However, several considerations argue that the identified peptides might inhibit translation by interacting with the desired target. Specifically: 1) phage-display selection was carried out in the presence of excess total *E. coli* tRNA which should exclude (or at least diminish) selection of phages with non-specific RNA-binding activity; 2) The peptide AGAAMSH directly tested in the binding experiments, showed high-affinity binding to a single site in the 30S subunit; 3) Binding of the AGAAMSH peptide to the 30S subunit elicits the response of an environment-sensitive fluorescent label attached in the immediate vicinity of h18. If our assertion that the selected inhibitory peptides interact with h18 in the ribosome is correct, then our data validate this ribosomal site as a legitimate antibiotic target and may provide incentive for more extensive searches for compounds targeting this ribosomal element.

The approach we described here can be used for isolation of small ligands (peptides) that target other ribosomal elements capable of maintaining their structure in isolation. Several such elements, originating from functionally-important ribosomal sites, have been characterized 28-32 but many more are awaiting exploration. The ribosome-targeting peptides acting upon these sites can be used for development of high-throughput displacement assays. Such assays, based on the displacement of the radioactive or fluorescently-labeled peptide from its ribosomal binding site, can be suitable for the screening of libraries of synthetic and natural organic compounds for drug leads. Some of the peptides may also be developed into drug leads using the peptidomimetic approach, which holds potential for improving the stability, bioavailability and cell uptake of physiologically-active natural peptides (reviewed in 33). Ribosome crystallography can be used to deduce the location of the peptide binding site(s) and the mode of binding of the peptide inhibitors. This information may provide the starting ground for developing new protein synthesis inhibitors using rational drug design.

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Figure 1.

A. The location of the h18 pseudoknot at the interface side of the bacterial (*E. coli*) small ribosomal subunit. 16S rRNA is shown in beige and ribosomal proteins are shown in green. h18 is highlighted in red. The pdb coordinates (2AVY) of the *E. coli* small subunit from the 70S ribosome structure 34 were used for rendering. B. Structure of h18. The nucleotide residues conserved between bacterial (*E. coli*) and human cytoplasmic ribosomes are shown in red; the non-conserved residues are shown in orange. The loop of protein S12 is shown in green and the alpha-carbon atom of the position modified with the IAANS fluorophore is shown as a sphere. C. The structure of the RNA/DNA hybrid used as a target in the phage-display library screening. The position numbers corresponding to the *E. coli* 16S rRNA are indicated.

Figure 2.

Inhibitory activity of the selected heptapeptides. A. Inhibition of protein synthesis in the *E. coli* cell-free transcription-translation system by peptides present at 100 μM concentration. Controls (no peptide or an unrelated heptapeptide) are represented by open bars. B. Concentration-dependent inhibition of the *E. coli* *in vitro* transcription-translation system programmed with plasmid (pBESTluc) DNA. C. Inhibition of the *E. coli* *in vitro* transcription-translation system programmed with phage MS2 RNA. D. Inhibition of rabbit reticulocyte cell-free translation system by peptides.

Synthetic heptapeptides used in the experiments were provided by SynPep or Sigma-Genosis. Peptides were C-terminally amidated and N-terminally acetylated. Peptides used in the titration

experiments were additionally purified by HPLC. Peptides were added to the reactions prior to addition of nucleic acid templates.

In A and B, the *E. coli* S30 cell-free transcription-translation system (Promega) was programmed with 0.8 µg of pBEST*luc* plasmid DNA. Reactions were carried out for 5 min at 25°C in a total volume of 10 µl. In B, reactions were programmed with 2.4 µg of MS2 RNA (Roche). In D, 10 µl of the rabbit reticulocyte cell-free translation system (Promega) was programmed with 0.3 µg of capped and polyadenylated luciferase mRNA and incubated for 20 min at 30°C. The reactions in bacterial and eukaryotic *in vitro* systems were carried out in the presence of [³⁵S] methionine. At the end of incubation, reactions were stopped by addition of 245 µl of 1 M NaOH and incubated at 37 °C for 10 min. Synthesized proteins were precipitated by addition of 1 ml ice-cold solution of 25% trichloroacetic acid (TCA)/2% casamino acids, and incubation on ice for 30 min. The samples were filtered through glass fiber filters, washed three times with 5% TCA and once with acetone, dried and subjected to scintillation counting. Data were processed using Prism software (GraphPad).

Figure 3.

Binding of the peptide AGAAMSH to the *E. coli* small ribosomal subunit. 30S subunits were reconstituted from 16S rRNA and from overexpressed and purified small subunit proteins as described 23. The mutant protein S12 carrying IAANS-modified single cysteine residue at position 44 was substituted for wild type S12 during reconstitution. Reconstituted subunits were purified by sucrose gradient centrifugation and used in the binding assay. Binding was carried out at 37°C in a 40 μl quartz cuvette in 50 mM HEPES-KOH pH 7.3, 20 mM MgCl₂, 100 mM NH₄Cl, 6 mM β-mercaptoethanol. 30S subunits were present at 100 nM. The aliquots of peptide solution (in 50 mM HEPES-KOH pH 7.3) were added to the cuvette and incubated at 37°C for 2 min prior to acquiring fluorescence readings. Measurements were done using a Varian Cary Eclipse Fluorescence Spectrophotometer (excitation - 327 nm, emission - 450 nm). The data were fitted into the hyperbolic one-site binding equation using Prism software (GraphPad).

Table 1
Selected peptides ¹⁾.

Peptide sequence	% of sequenced clones encoding the peptide
Beads, DNase I elution ²⁾, 2 rounds	
TMTPPTR	6.6
GNDWPHW	6.6
EHPYITV	6.6
SSLLPTT	6.6
NTNTLHL	6.6
SYPDLHL	6.6
SILPYPY	60
Beads, low pH elution ³⁾, 4 rounds	
FPGHSGP	2
VQSLPSP	2
EPLQLKM	2
TPHNTST	2
QWTWTQY	2
LTHPRWP	2
TKTDTWL	2
LSPKLPT	2
NTPQGMT	3
THPLLS	7
GHWEARE	7
AVPRASF	7
YHPMPVP	8
TPTTDGP	20
AGAAMSH	32
Microtiter plate, low pH elution ⁴⁾, 4 rounds	
MKHPPRI	2
GTMLAAV	4
AMSAPIP	94

¹⁾ The 59-nt RNA-DNA h18 target was obtained from Dharmacon and purified by gel electrophoresis. The target was renatured by incubating at 90 °C for 1 min and gradually cooling down to 20°C over 2 hrs in 80 mM HEPES-KOH pH 7.4. The heptapeptide M13 phage library, Ph.D.-7 (New England Biolabs) was counter-selected by incubation in binding buffer for 1 hr at 4°C in a streptavidin-coated well of a microtiter plate (80 mM HEPES-KOH pH 7.4, 150 mM KCl, 20 mM MgCl₂) or for 30 min at 25°C with 0.5 mg washed magnetic beads. The library (2×10^{11} phages in 25 µl binding buffer) was then transferred to a fresh tube. Following addition of 100 µg *E. coli* tRNA and 40 U RNase Inhibitor (Cat. No. 03335399001, Roche) the h18 target was added to 100 nM. The mixture was incubated at 25°C for 30 min.

²⁾ The library/target mixture (50 µl) was added to 0.5 mg of washed magnetic beads and incubation continued for another 30 min at 25°C. Biotin was added to a final concentration of 0.1 mM and reactions were incubated for 5 min at 25°C. The supernatant was removed, and the beads with the bound phages were washed five times with 200 µl of binding buffer supplemented with 0.1% Tween-20. The target-bound phages were eluted by incubating beads carrying the captured phages with 250 U of DNase I in 10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 0.1 mM CaCl₂ for 30 min at 37°C. The DNase treatment was quenched by adding 1 µl of 0.5 M EDTA pH 8.0. Individual phages were sequenced after the second and third rounds of selection.

³⁾ Capturing of the target-bound phages on the beads and washing was as in ²⁾. The target-bound phages were eluted by incubating beads with 100 μ l of 0.2 M glycine-HCl, pH 2.2, 1 mg/ml BSA, for 10 min at 25°C followed by addition of 15 μ l of 1 M Tris-HCl, pH 9.1. After the first round of selection, the stringency of washes was increased by raising the concentration of Tween-20 in the wash buffer from 0.1% to 0.5% (v/v).

⁴⁾ The library/target mixture in binding buffer was added to a well of the streptavidin coated microtiter plate (Pierce) and incubated at 4°C for 1 h. Biotin was added to a final concentration of 0.1 mM and the plate was incubated at 4°C for 5 min. The supernatant was removed and the wells were washed four times with 250 μ l of binding buffer containing 0.05% Tween-20, and twice with binding buffer. The target-bound phages were eluted by adding to the well 100 μ l of 0.2 M glycine-HCl, pH 2.2, 1 mg/ml BSA, incubating at 25°C for 10 min, and neutralizing solution with 15 μ l of 1M Tris-HCl, pH 9.1. The eluted phages were amplified and tittered following the manufacturer's protocol.