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The development of peptide ligands that target helix 69 rRNA of bacterial ribosomes

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Abstract

Antibiotic resistance prevents successful treatment of common bacterial infections, making it clear that new target locations and drugs are required to resolve this ongoing challenge. The bacterial ribosome is a common target for antibacterials due to its essential contribution to cell viability. The focus of this work is a region of the ribosome called helix 69 (H69), which was recently identified as a secondary target site for aminoglycoside antibiotics. H69 has key roles in essential ribosomal processes such as subunit association, ribosome recycling, and tRNA selection. Conserved across phylogeny, bacterial H69 also contains two pseudouridines and one 3-methylpseudouridine. Phage display revealed a heptameric peptide sequence that targeted H69. Using solid-phase synthesis, peptide variants with higher affinity and improved selectivity to modified H69 were generated. Electrospray ionization mass spectrometry was used to determine relative apparent dissociation constants of the RNA-peptide complexes.

Graphical Abstract

Keywords

peptide; ribosomal RNA; helix 69; ligand binding; pseudouridine

1. Introduction

A number of antibiotics in use today were derived from bacteria as defense against other pathogens.¹ Bacterial resistance to these defensive measures is a common challenge that the organisms and pharmaceutical industries alike face with the need to create unique inhibitors. Targeting biologically important regions within bacteria is difficult because a variety of

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resistance mechanisms involving these locations may already exist. The goal of our work has been to identify new functionally important locations for bacterial inhibition along with the discovery of compounds that can selectively target those regions. The bacterial ribosome is a common target for antibiotics due to its essential role in protein synthesis.² Many currently used antibiotics target key domains of the protein synthesis machinery³ such as the aminoacyl-tRNA binding site and peptidyl transferase center.^{3d, 4} In efforts to study a unique region for future antibiotic development, helix 69 (H69) of the 50S subunit of the bacterial ribosome was chosen. This 19-nucleotide stem-loop motif is located at the heart of the ribosome (Figure 1).^{3b-d, 4d, 5}

The high degree of sequence conservation⁵ as well as the presence of highly conserved modified nucleotides⁷ in H69 suggest that it plays key roles in ribosome function. Alterations to the sequence or modification status have been shown to be deleterious in bacteria as well as other organisms.⁸ H69 contains three modified nucleotides, two pseudouridines (Ψ)^{7b} and one 3-methylpseudouridine ($m^3\Psi$)^{7a} (Figure 2), which are important for ribosome function^{8a, 8b, 9} and modulation of the loop structure.^{9c, 9d, 10} H69 has also been implicated in a number of key ribosome functions such as ribosome recycling,¹¹ tRNA selection,¹² translational fidelity,¹³ and subunit association,^{8a, 9a} making it an attractive target for antibiotic drug design.

Peptides have been shown by several laboratories to have favorable antimicrobial properties and also target bacterial ribosomes.¹⁴ Previous work in our laboratory using phage display selection identified NQVANHQ, a heptameric peptide, as an H69 binder with moderate affinity (low μ M) and five-fold selectivity for the bacterial over human H69 or the unrelated A-site RNA.¹⁵ Although phage display has many advantages, one key disadvantage of using this method with H69 is that the chosen RNA target is important for bacterial vitality. Therefore, high-affinity ligands to H69 may not be readily identified if they inhibit bacterial growth and cannot be amplified by the phage-infected *E. coli* host strain in subsequent rounds of selection. The goal of the present work was to determine whether tighter-binding and more selective species could be identified through sequence variations in solid-phase peptide synthesis, while at the same time assessing the role of each individual amino acid in binding to H69.

A substitution scan of the original parent sequence was carried out, in which amidated variants of NQVANHQ were synthesized with replacement of each amino acid with alanine. The peptides were produced with an amidated C-terminus to simulate peptides expressed on the phage from the original selection. Since the parent sequence does not contain charged residues, likely due to preferences of the non-lytic phage used in the original experiment,¹⁶ an arginine scan was also carried out to determine whether introduction of a positive charge would enhance binding to the negatively charged RNA target. To study the binding interactions between the peptide ligands and RNA target, modified ($\Psi\Psi\Psi$) and unmodified (UUU) constructs of H69 rRNA (Figure 1) were employed. Both RNA constructs were used in order to determine the selectivity of the variant peptides to the pseudouridylated versus unmodified RNA target. Although targeting the modified H69 was the initial goal, the unmodified rRNA that gets post-transcriptionally modified *in vivo* or H69 mutants that can lead to antibiotic resistance are also potential targets of interest.

2. Materials and Methods

2.1 Materials

Materials used in the synthesis of the amidated alanine and arginine peptide variants included the following reagents from Novabiochem (Billerica, MA): Rink amide AM (200–400 mesh) resin, amino acids with fmoc temporary protective groups, and *O*-(1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU). Unless otherwise noted, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 Instrumentation

Binding studies with unmodified and modified H69 constructs with the peptide variants were performed using a Micromass Quattro LC triple quadrupole mass spectrometer with an electrospray/APCI source (Micromass, Manchester, UK), Waters Alliance 2685 LC, autosampler. and photodiode array UV detector.

2.3 RNA preparation

Unmodified H69 (UUU) (5'-GGCCGUAACUAUAACGGUC-3' ($\epsilon = 187,000 \text{ L mol}^{-1} \text{ cm}^{-1}$)), modified H69 ($\Psi\Psi\Psi$) (5'-GGCCG Ψ AAC Ψ A Ψ AACGGUC-3' ($\epsilon = 187,000 \text{ L mol}^{-1} \text{ cm}^{-1}$)), 5'-biotinylated modified H69 (5'-Bi- $\Psi\Psi\Psi$) (5'-biotin-GGCCG Ψ AAC Ψ A Ψ AACGGUC-3' ($\epsilon = 187,000 \text{ L mol}^{-1} \text{ cm}^{-1}$)) and 5'-biotinylated unmodified H69 (5'-Bi-UUU) (5'-biotin-GGCCGUAACUAUAACGGUC-3' ($\epsilon = 187,000 \text{ L mol}^{-1} \text{ cm}^{-1}$)) RNAs were purchased from GE Dharmacon, Inc. (Lafayette, CO) with 2' deprotection and desalting, and quantified using the calculated extinction coefficients.¹⁷ An ethanol precipitation with ammonium acetate was performed on each RNA. Pellets were washed with 70% ethanol, dried, and stored at -20°C until use. Pellets were dissolved in minimum amounts of double-deionized water (ddH₂O), pH 7.0. Concentrations were determined with diluted samples on a Beckman Coulter DU800 spectrophotometer (Beckman Coulter, Brea, CA).

2.4 Solid-phase peptide synthesis

Manual solid-phase peptide synthesis (SPPS) was performed using standard fluorenylmethyloxycarbonyl (fmoc) chemistry.¹⁸ Free peptides with C-terminal amidation were generated by using Rink amide AM resin (Novabiochem). Approximately 0.15–0.20 mmol of the resin was pre-swollen using dichloromethane (DCM) in a glass peptide reaction vessel mounted on wrist-action shaker. The resin was washed 3 times with *N,N'*-dimethylformamide (DMF) followed by fmoc deprotection with 20% piperidine in DMF (2 times, 10 min with shaking). The resin was washed 10 times with DMF prior to addition of the coupling reagents. Amino acids were added in order from the carboxy to the amino terminus.

The coupling reaction employed 3 eq. each of amino acid and HBTU (relative to resin). Six eq. of diisopropylethylamine (DIPEA) were added with 10 $\times$ resin vol DMF to the peptide vessel. The coupling reaction was carried out with shaking for a minimum of 2 h at RT. Protection and deprotection were monitored using a Kaiser test.¹⁹ Deprotection of the fmoc group was repeated using 20% piperidine in DMF followed by washing 10 times in DMF.

The coupling/deprotection steps were repeated until deprotection of the final amino acid. The resin was washed 10 times each with DMF, DCM, and anhydrous diethyl ether. The beads were dried overnight at RT. Cleavage of the peptide from resin and removal of the permanent protective groups were done in 7 mL of 94:2:2:2 trifluoroacetic acid (TFA): anisole: thioanisole: triisopropylsilane. The reaction was shaken at RT for 2–4 h. Peptide was drained into two 50 mL Falcon® tubes containing cold (–20 °C) anhydrous diethyl ether. The tubes were balanced, vortexed, and centrifuged at 6,000 RPM for 8 min at 4°C using an Eppendorf 5804R tabletop centrifuge. The diethyl ether was removed from the pellet and the wash was repeated 2 times. With removal of the diethyl ether, a minimal amount of ddH₂O with 0.1% TFA was added to dissolve the peptide pellet. The peptide solution frozen at –80 °C followed by lyophilization.

2.5 Purification and preparation of synthesized peptides

Crude lyophilized peptides were purified using high performance liquid chromatography (Waters, Milford, MA) on a Phenomenox® Luna C18 column (Torrance, CA) in buffer A (0.1% TFA in ddH₂O) with a gradient of 0–30% buffer B (0.1% TFA in acetonitrile) over 85 min. All peptides were characterized on a Bruker Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany).

2.6 Electrospray ionization mass spectrometry (ESI-MS)

Binding studies were done on a Micromass Quattro LC mass spectrometer (Manchester, UK) and analyzed with MassLynx® 4.0 software (Waters, Milford, MA). To smooth, the half-height value of the peak area of the (4–) charge state of free RNA was applied to the Savitzky-Golay method.²⁰ A 50 µL solution containing peptide (0 – 120 µM) was incubated with RNA ($C_f = 2.7 \mu\text{M}$) in 50% isopropanol in water and ammonium acetate ($C_f = 150 \text{ mM}$). The spectrum of the infused sample was obtained for the mass-to-charge ratio (m/z) range of 1100–2400 to reveal complexes of RNA and peptide, as well as the free RNA, at the (5–) and (4–) charge states. Each sample was infused with a Hamilton® 100 µL glass syringe at a rate of 6 µL/min. A cone voltage of 40 V was maintained for the modified and unmodified H69 RNAs. A cone voltage of 50 V was maintained for 5′-biotinylated modified H69 RNA (5′-BiΨΨΨ). Results were plotted using KaleidaGraph software.

3. Results and Discussion

3.1 Synthesis of alanine and arginine peptide variants

Phage display selection²¹ against a modified RNA construct ($\Psi m^3 \Psi \Psi$) representing H69 of *E. coli* 23S RNA was used to identify peptide binders.¹⁵ One of the resulting peptide sequences in the amidated form, NQVANHQ-NH₂ (**1**), binds to H69 with moderate affinity (apparent K_d value of 5 µM). This result demonstrates the ability to obtain a unique peptide sequence to target modified RNA with moderate (five-fold) selectivity over the unmodified construct (UUU), but also leads to a number of questions about the ideal heptamer composition. Of the seven amino acids in the selected peptide sequence, perhaps not all are necessary for binding to H69, whereas other residues in the sequence might play key roles in binding. Our goal was to determine which of these residues is important for H69 binding, while at the same time identifying even tighter binding peptide ligands. Thirteen peptide

variants and the parent sequence, as shown in Table 1, were successfully synthesized on RINK amide AM resin, purified by reverse-phase HPLC, and characterized by MALDI-TOF MS. The peptide variants contain an alanine substitution at positions 1–3 and 5–7 of NQVANHQ-NH₂ (peptides **2–7**) or an arginine substitution at each position (peptides **8–14**). The MALDI data are provided in the Supplementary Material (Table S1, Figures S1–S4).

3.2 Determination of apparent dissociation constants for peptide-RNA complexes

Unmodified (UUU) and modified (ΨΨΨ) H69 RNAs were used to compare binding affinities of the peptide variants to constructs with and without pseudouridine. Although the uridine and pseudouridine isomers are structurally very similar, they impact the conformation of H69 such that differences in the loop region occur.²² Therefore, different binding selectivities were expected to reflect interactions of the peptides with the H69 loop region. Previous studies employed a custom-synthesized wild-type H69 RNA construct (Ψm³ΨΨ) for selection and binding studies, but the more readily available ΨΨΨ construct without the methylated residue was shown previously to have a similar thermal stability and secondary structure²³ and in this work to have similar affinity to the parent peptide **1** (Table 1). In one case (peptide **10**), 5'-biotinylated ΨΨΨ and 5'-biotinylated UUU RNAs were employed because the peptide dimer and H69 RNA had the same mass. The biotinylated RNA displays the same apparent dissociation constant as the non-biotinylated RNA counterpart with peptide **1** (Supplementary Materials, Figures S5–S7). The same construct was used with peptide **14**. The UUU and ΨΨΨ RNAs were incubated with increasing concentrations of each peptide variant and subjected to binding analysis via ESI-MS. The details of using this method for RNA-peptide binding analysis were reported previously.¹⁵ To obtain relative dissociation constants, peak area intensity values correlating to the (4–) and (5–) charge states of free RNA (R) and RNA-peptide (P) complex, including salt adducts, for each titration spectra were used in Equation 1. Figure 3 shows representative ESI-MS data from one titration point of a peptide (RQVANHQ-NH₂) bound to modified H69 RNA (ΨΨΨ).

$$\sum RP^{n-} / (\sum R^{n-} + \sum RP^{n-}) = ([R]_0 + [P]_0 + K_d) - (([R]_0 + [P]_0 + K_d)^2 - (4[R]_0[P]_0)^{0.5}) / 2[R]_0 \quad (1)$$

A representative binding curve obtained by plotting the fraction of RNA bound versus the concentration of peptide is shown in Figure 4. A non-linear curve fit with the quadratic equation was used. Results obtained from duplicate sets of experiments with both UUU and ΨΨΨ are shown in Table 1. Salt adducts associated each binding complex as well as free RNA were incorporated into the dissociation constant calculations using the peak area intensity values from the (4–) and (5–) charge states unless otherwise noted. Due to lower ionization efficiencies of the RNA-peptide complexes compared to the free RNAs, saturation is not achieved with increasing concentrations of peptide. This behavior has been observed before for non-charged peptides.¹⁵ Therefore, relative dissociation constants are obtained

that can be compared when minor changes are made to the peptide sequence such as single amino acid substitutions.

3.3 Range of peptide binding affinities and selectivities for H69

Table 1 shows the results of peptide variants binding to the H69 RNAs. The peptides from the amidated alanine and arginine scans display a greater than 50-fold range of apparent dissociation constants, indicating that the amino acid side chains in the parent peptide, NQVANHQ-NH₂ (**1**), have varying degrees of importance with respect to affinity and selectivity for the modified H69. Some of the positional variants show enhanced binding to H69 compared to **1**, whereas others are observed to be important for selectivity of the modified over unmodified H69 RNAs. Furthermore, some of the peptide variants have a preference for the pseudouridylated H69 while others are selective for the unmodified RNA. Only five of the 13 peptide variants retain greater affinity to the modified construct over the unmodified construct, and only one (RQVANHQ-NH₂, **8**) displays higher affinity relative to peptide **1**. For five of the peptide variants, little or no binding to the modified H69 is observed, but three of those variants retain affinity for the unmodified construct, revealing a switch in specificity.

3.4 Peptide binding affinities for modified H69

As illustrated in Figure 5 and shown graphically in the Supplementary Material (Figure S8), positions 1 and 4–7 appear to be most important for binding affinity with ΨΨΨ. For position 1, the preference is R > N >> A (in combination with positions 2–7, QVANHQ), in which replacement of N with R improves binding two-fold and the presence of A abolishes binding. In contrast, replacement of A with R in position 4 causes a greater than five-fold reduction of binding to ΨΨΨ. Similarly, an N to A or R substitution at position 5 abolishes binding. An H or R is preferred equally at position 6 for ΨΨΨ binding (apparent K_d value of 4.5 μM for R and 4.5 μM for H), and absence of the charged residue in the alanine variant shows no observable binding to either H69 RNA. For position 7, the parent amino acid Q is preferred over A and R, with three- and seven-fold reductions, respectively, in relative binding affinity to ΨΨΨ for these substitutions.

3.5 Peptide binding selectivity for modified versus unmodified H69

In addition to analyzing binding affinity of the variant peptides to modified H69, selectivity over the unmodified RNA was determined. The ΨΨΨ and UUU RNAs, as well as H69 sequence variants, have varying solution conformations that can be altered through ligand binding. Since both modified and unmodified forms of H69 exist in nature, targeting either one or both is of interest. At the same time, differences in affinities to these constructs will reflect the level of selectivity of each peptide for closely related RNAs. The original peptide (**1**) obtained from phage display displays a five-fold preference for ΨΨΨ over UUU H69. The 13 peptide variants show various levels of selectivity, revealing the importance of certain amino acids in the parent sequence for recognition of the pseudouridylation in H69. For example, peptides **2**, **5**, and **12** only show binding to UUU (Supplementary Material, Figure S9). In one case, a single amino acid substitution of V to A at position 3 (peptide **4**)

changes the binding preference from $\Psi\Psi\Psi$ to UUU H69 with a concomitant >20-fold enhancement in affinity for UUU (apparent K_d value of 1 μM).

In contrast to the results obtained with peptide **4**, an R at position 3 (peptide **10**) leads to enhanced selectivity for $\Psi\Psi\Psi$ with only a slight reduction in affinity. Although having opposite selectivities, the position 3 variants both bind with a 2:1 stoichiometry to the H69 RNAs. Further work is necessary to understand the binding mode of these variants, but the results are of interest because of the fact that multiple copies of peptides are displayed on the phage surface in the selection experiment.¹⁵ We did not observe evidence for dimer binding with the other peptide variants. The binding data shown in Table 1 and summarized in Figure 5 suggest that histidine, or other functional groups with hydrogen-bonding potential as seen in the arginine scan, in combination with certain non-polar amino acids are important for the selectivity to modified H69 RNA.

3.6 Binding of histidine peptide variants to H69

To further explore the role of charge or varying hydrogen-bonding potential at positions 5, 6, and 7 (NHQ in peptide **1**), four additional peptides were produced with single or double substitutions. These variants have the following amino acid content at positions 5–7: NAH (**15**), HAQ (**16**), NHH (**17**), or HHQ (**18**), respectively (with the substitutions underlined). In particular, this analysis reveals whether histidine is important specifically at position 6 or if its presence at neighboring positions might also lead to favorable binding to modified H69. For this experiment, a single titration point (20 μM peptide) with $\Psi\Psi\Psi$ was compared by ESI-MS for the four variant peptides (**15**–**18**) and the parent (**1**) (Supplementary Material, Figure S10). No binding is observed for peptide **16**, and peptides **15** and **17** are also poor ligands (K_d values > 20 μM) (position 6 and 7 variants), consistent with the alanine scan results for modified H69. A titration with peptide **18** (NQVAHHQ-NH₂, from 0 to 60 μM) and $\Psi\Psi\Psi$ was also done, revealing a dissociation constant of 16.0 ± 3.5 μM (data not shown). This result indicates an approximately three-fold higher apparent dissociation constant relative to the parent K_d value, providing further support that the identity at position 5 is important for binding to $\Psi\Psi\Psi$, favoring N in the selected parent sequence.

3.7 Roles of individual amino acids in H69 peptide variants

The data shown in Table 1 and summarized in Figure 5 reveal two distinct binding profiles for the peptide variants and modified or unmodified H69 RNAs. The parent peptide (**1**) selected by phage display against pseudouridylated RNA contains a majority of non-polar residues. These residues appear to have distinct roles in binding and selectivity for the pseudouridylated H69 RNA. For example, positions 2 and 3 can be substituted with alanine with little or no impact on binding affinity, whereas selectivity for $\Psi\Psi\Psi$ can be improved by substituting Q with A in peptide **3** or V with R in peptide **10**. Although replacement of amino acids in peptide **1** with arginine leads to tighter binding in one case (peptide **8**) and improved selectivity for another (peptide **10**), most of the R variants have reduced selectivity for the modified RNA. Similarly, positions 4 and 5 show a strong preference for the phage-selected amino acid over alanine or arginine with respect to binding affinity to $\Psi\Psi\Psi$.

Analysis of peptide variants binding to the unmodified H69 construct (UUU) reveals different roles for the individual amino acids in binding and selectivity (summarized in Figure 5). Not surprisingly, none of the parent amino acids are preferred over alanine or arginine when combined with the other parent amino acids, likely due to the fact the phage selection was carried out with modified H69. In contrast, one specific peptide with a V to A substitution at position 3 (peptide **4**) shows a strong preference for UUU over ΨΨΨ with a 23-fold enhanced affinity. It should be noted that a combination of the 7 preferred amino acids (for UUU) at individual positions does not lead to a better ligand for the unmodified RNA, showing the importance of cooperative effects. Overall, these results suggest that other peptide variants may be produced that could have affinity for other RNA helices, including H69 sequence variants or modifications that could play roles in resistance to existing antibiotics.

Other positional information obtained from the alanine and arginine scans is the role of certain amino acids in regulating the stoichiometry of binding. Importantly, the ESI-MS method provides this information because of the ability to observe the various peptide-RNA complexes directly under soft ionization conditions. For peptides **4** and **10**, 1:1 and 2:1 peptide:RNA complexes were observed. For both of these peptides, substitution of valine at position 3 of the parent sequence leads to the higher stoichiometry.

Taken together, these results support the idea that peptide-ligands can be selected from phage libraries to target modified RNAs, and that these ligands can be further developed into RNA-binding species with similar affinities to natural RNA-targeting compounds such as aminoglycosides (low μM).²⁴ Despite its limitations, the phage selection identified a moderate-binding peptide ligand which uses amino acids that are preferred over alanine at most positions. Our alanine and arginine scanning results suggest that a few amino acids can be varied through solid-phase synthesis approaches to generate sequences with enhanced binding and selectivity for either modified or unmodified RNAs. The cooperative effects of the amino acids also suggest peptide flexibility and adaptive binding, such that optimization through rational design would be challenging.

3.8 Discussion

The peptide variants used in this study demonstrate different affinities for modified and unmodified H69 RNAs. This result supports the hypothesis that peptides can be designed to target various RNA structures that are dependent on the presence of modified nucleotides for function. Previous work revealed that certain aminoglycoside antibiotics have different structural impacts on the modified and unmodified H69 RNAs.^{24a} For example, neomycin binding to ΨΨΨ causes increased exposure of residue A1913, which is not observed upon binding to UUU. Therefore, the affinity and selectivity differences between the peptide variants suggest that regulation of H69 structure upon binding may be possible. A deeper conformational analysis of peptide variant binding to H69 will need to be carried out in order to determine the structural implications and to relate these changes to the function of H69.

Our results with UUU and certain peptide variants suggest that selective targeting of the unmodified H69 is also possible. Since Ψ is a post-transcriptional modification, it may be

possible to design peptides capable of targeting H69 prior to the pseudouridylation reaction. Since the modification status of H69 is important for subunit association,^{9a, 25} the presence of such ligands may negatively impact ribosome assembly. Similarly, other RNA helices may be potential targets of variants that are produced by small library peptide synthesis and screening.

In addition to serving as potential therapeutics, peptide ligands could also serve as basic research tools to examine ribosome function or to advance our understanding of RNA recognition by small molecules.²⁶ The peptides generated in this study compare well with respect to their size and affinities to other small molecules that have been identified as RNA ligands, including those that bind riboswitches and inhibit ribosomes.²⁷ For example, puromycin, which binds to the A-site of 50S subunit with a K_d value of 480 μ M, has served as a valuable tool for studying peptidyltransferase reactions.^{26, 28} Small peptide variants can be easily synthesized and functionalized with fluorophores and affinity labels for such applications.²⁹ In contrast, the design of small organic ligands, particularly those with high selectivity, is extremely challenging and the rules for RNA recognition are still largely unknown with just a few exceptions.²⁶ Furthermore, many of the naturally occurring ligands have unique structural advantages specific to their selected targets that may not be suitable when targeting other RNAs such as functional mutants or nucleotide modifications that can lead to drug resistance.

Peptides offer several advantages for targeting RNA. Reliable methods, including ESI-MS, to study their binding to RNA have already been developed.^{27a, 30} The issue of selectivity can be addressed through the combined use of selection and library synthesis. Phage selections with modified targets and appropriate counter selections can produce reasonable starting peptides (parent) that bind modified rRNA or specific secondary structure motifs.^{15, 30a, 30b} In this work, alanine and arginine scans produced several peptides with higher affinity and selectivity for modified or unmodified RNAs than the parent peptide obtained from phage display. At least one peptide displayed a 23-fold change in affinity and selectivity for unmodified H69, which has potential utility for targeting the transcribed rRNA before modification. Overall, the peptide variants displayed a greater than 50-fold range in binding affinities to the H69 RNAs.

In future applications, the production of a larger library with amino acid variations at specific positions in the original heptameric sequence could yield peptides or peptide-based compounds with greater affinity and selectivity to H69. Data from this study suggest variations at the following locations. Position 1 could be randomized to enhance binding to UUU, or semi-conservative changes could be made to optimize for $\Psi\Psi\Psi$ binding. Positions 2 or 3 can be modified to enhance binding and selectivity for modified H69, but perhaps favoring non-polar residues. Position 6 may also be modified, but in this case possibly favoring cationic side chains for selective interactions with $\Psi\Psi\Psi$. Lastly, our data suggest that positions 4 and 5 should probably be maintained as A and N, respectively, in order to maintain favorable binding and selectivity to $\Psi\Psi\Psi$.

4. Conclusions

Our findings validate the premise that small peptide ligands can be identified to target modified RNAs. In addition, these results also support the argument that moderate affinity ligands can be identified through phage display, despite some of its limitations. The generation of peptide variants of the selected parent sequence allowed for identification of the positions that are important for target RNA binding and selectivity. With several peptide variants having similar K_d values (low μM) for H69 as known antibiotics such as neomycin and preferences for pseudouridylation status, future work will involve examining the structural requirements for H69 binding and selectivity at a more detailed level. Not only is there the possibility of identifying potent binders to H69, it may be possible to develop chemical biology tools capable of further probing modified nucleotides, thereby providing clarity to some of the functional aspects benefited from their presence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A. The 70S *E. coli* bacterial ribosome (2AVY, 2AW4)⁶ is shown with H69 highlighted in cyan, aminoacyl-tRNA site (A site) in red, peptidyl transferase center (PTC) in purple, 30S subunit in light grey, and 50S subunit in yellow. **B.** Secondary structures of H69 are given (from left to right): human H69, wild-type H69 with *E. coli* numbering, modified H69 ($\Psi\Psi\Psi$), and unmodified H69 (UUU).

Figure 2.
The base structures of uridine (left), pseudouridine (middle), and 3-methylpseudouridine (right) are shown.

Figure 3.

The ESI mass spectrum shows RQVANHQ-NH₂ (35 μ M) bound to modified H69 ($\Psi\Psi\Psi$, 2.7 μ M) RNA at a single titration point.

Figure 4.

The titration curve for RQVANHQ-NH₂ bound to ΨΨΨ is shown. The apparent dissociation constant (K_d) value is given.

amino acid position	1	2	3	4	5	6	7
binding affinity to $\Psi\Psi\Psi$	R _N	Q _A	V _A _R	A	N	H _R	Q _A
selectivity for $\Psi\Psi\Psi$	N _R	A _Q	R _V	A	N	H _R	Q _A
binding affinity to UUU	A _R	Q	A _V	R _A	A _R _N	H _R	R _Q
selectivity for UUU	A	R	A	R	A R		R

Figure 5.

The relative binding affinities and selectivities of the peptide variants for $\Psi\Psi\Psi$ (upper) or UUU (lower) are illustrated. Each position contains either A, R, or the parent amino acid along with the remaining parent amino acid sequence, which is indicated in blue. The size of the letter indicates the relative affinity or selectivity of that amino acid variant compared to the other amino acids at that position, with larger letters indicating tighter binding or more selective species.

Table 1

Apparent dissociation constants of peptide ligands to H69 RNAs

Amidated Peptide	K_d $\Psi\Psi\Psi$, μM	K_d UUU, μM	Amidated Peptide	K_d $\Psi\Psi\Psi$, μM	K_d UUU, μM
2, Δ QVANHQ	NBO	9.9 ± 2.1	8, Δ QVANHQ	2.1 ± 0.6	10.0 ± 4.3
3, Δ AVANHQ	4.6 ± 1.8	52.8 ± 9.7	9, Δ RVANHQ	>40	35.5 ± 10.1
4, Δ QAANHQ	5.0 ± 1.0	1.0 ± 0.5	10, Δ QRANHQ	6.0 ± 2.2	§ NBO
1, Δ QVANHQ	4.6 ± 1.7	22.9 ± 5.6	11, Δ QVRNHNQ	>20	21.7 ± 5.7
5, Δ QVA Δ HNQ	NBO	14.3 ± 6.5	12, Δ QVARNHQ	NBO	16.5 ± 5.5
6, Δ QVANAQ	NBO	NBO	13, Δ QVANRQ	4.5 ± 1.8	14.4 ± 3.1
7, Δ QVANHA	13.4 ± 4.7	47.5 ± 9.9	14, Δ QVANHR	31.8 ± 7.3	20.5 ± 2.8

NBO: no binding observed;

^{*} 2:1 peptide to RNA stoichiometry observed;[†] 5'-biotinylated $\Psi\Psi\Psi$ used instead of $\Psi\Psi\Psi$;[§] 5'-biotinylated UUU used instead of UUU;[‡] only the (4-) charge state was observed