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An Approach to the Construction of Tailor-Made Amphiphilic Peptides That Strongly and Selectively Bind to Hairpin RNA Targets

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Abstract: The hairpin RNA motif is one of the most frequently observed secondary structures and is often targeted by therapeutic agents. An amphiphilic peptide with seven lysine and eight leucine residues and its derivatives were designed for use as ligands against RNA hairpin motifs. We hypothesized that variations in both the hydrophobic leucine-rich and hydrophilic lysine-rich spheres of these amphiphilic peptides would create extra attractive interactions with hairpin RNA targets. A series of alanine-scanned peptides were probed to identify the most influential lysine residues in the hydrophilic sphere. The binding affinities of these modified peptides with several hairpins, such as RRE, TAR from HIV, a short hairpin from IRES of HCV, and a hairpin from the 16S A-site stem from rRNA, were determined. Since the hairpin from IRES of HCV was the most susceptible to the initial series of alanine-scanned peptides, studies investigating how further variations in the peptides effect binding employed the IRES hairpin. Next, the important Lys residues were substituted by shorter chain amines, such as ornithine, to place the peptide deeper into the hairpin groove. In a few cases, a 70-fold improved binding was observed for peptides that contained the specifically located shorter amine side chains. To further explore changes in binding affinities brought about by alterations in the hydrophobic sphere, tryptophan residues were introduced in place of leucine. A few peptides with tryptophan in specific positions also displayed 70-fold improved binding affinities. Finally, double mutant peptides incorporating both specifically located shorter amine side chains in the hydrophilic region and tryptophan residues in the hydrophobic region were synthesized. The binding affinities of peptides containing the simple double modification were observed to be 80 times lower, and their binding specificities were increased 40-fold. The results of this effort provide important information about strategies that can be used to prepare peptides that both strongly and selectively target hairpin RNAs. Specifically, the findings indicate that tailor-made amphiphilic peptide ligands against certain hairpin RNAs can be obtained if the RNA target possesses a deep groove in which both the hydrophobic and hydrophilic spheres of the peptide interact.

Introduction

RNA molecules, ranging from prokaryotic pathogen's rRNA to eukaryotic pre-miRNA, are potential therapeutic targets.[1,2] This is especially true of hairpin RNAs that have major grooves that are much deeper than those of DNA and, thus, provide pockets in which small molecules can specifically bind.[3] A variety of small molecules that bind to these RNA hairpin structures have been synthesized.[4] However, the relatively weak affinities and poor specificities displayed by these substances suggest that small molecules are not ideal RNA ligands.

Peptides, which have high propensities of α-helicity to form structured motifs, also serve as ligands of hairpin RNA.[5] Natural peptides, such as the Rev and Tat peptides, are known to be ligands of the respective RRE or TAR hairpin RNA of an HIV-1 target.[6] For example, the Rev peptide with a high α-helical content specifically interacts with its natural partner, RRE, a typical hairpin RNA.

Encouraged by the observations made in studies of natural peptides, we have designed a new family of Lys- and Leu-rich amphiphilic peptides as ligands against RNA hairpins.[7] At first glance, hairpin RNA targets seem to share great similarities making selective targeting by peptides difficult. In addition, certain hairpins are more promiscuous than others, adding to the difficulty in designing specifically binding ligands. For

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example, as a consequence of its structural flexibility and ability to undergo ready conformational changes,[8] TAR RNA is a highly promiscuous hairpin.[9] Moreover, it is difficult to predict the promiscuity of a hairpin RNA until its detailed structural information is obtained. Since a promiscuous hairpin is able to change its conformation according to the nature of the ligand it confronts, we believed binding of amphiphilic α-helical peptides could not differentiate between promiscuous hairpin(s). However, this should not be the case for nonpromiscuous hairpins. As a result, a series of peptides, such as those in an Ala-scanned peptide library, can be used to elucidate the relative degree of promiscuity of hairpin targets and thus reveal specific RNA hairpins that can be targeted by drugs.

Once a peptide is found to target a specific binding hairpin, modifications of its α-helical region can be carried out. Since peptides can be bound very deeply inside the grooves of specific hairpins, it is likely that both the hydrophilic and hydrophobic spheres of the peptide interact with complimentary components of the RNA hairpins. To promote greater interactions between the peptide and the hairpin, both the hydrophilic and hydrophobic spheres of the peptide can be altered. In the study described below, we have prepared an amphiphilic peptide that contains a leucine-rich hydrophobic sphere and a lysine-rich hydrophilic sphere. Two major modifications were made to this peptide.[10] First, important Lys residue(s) in the hydrophilic sphere were replaced by ornithine (Orn) or amino acids containing side chain amine groups tethered by shorter chains to bring about more deep binding in the RNA hairpin groove. Second, Leu residues in the hydrophobic sphere were replaced by Trp to enhance interactions with base(s) in the RNA hairpins.[11] In this way, the modified peptides will be located deeper inside of the groove and will be capable of having greater interactions with the RNA. By using this simple strategy, we were able to design a peptide that displays an 80-fold lower binding affinity and more than a 40-fold improved specificity against a specific hairpin in IRES of HCV compared to those of the original amphiphilic peptide.[12]

Experimental Section

Peptide Synthesis. The peptides used in this study were synthesized manually by using the standard Fmoc solid-phase methodology. Briefly, Rink AmideTM resins (0.064 mmol/g loading, 25 mg, 16 μmol) were deprotected with 20% piperidine in DMF. A solution of glycine, the first C-terminal Fmoc protected amino acid (6 equiv), benzotriazole-1-yl-oxy-tris-pyrridino-phosphonium hexafluoro-phosphate (6 equiv), and *N*,*N*-diisopropylethylamine (6 equiv) was stirred at room temperature for 1.5 h. The progress of the reaction was monitored by using the 2,4,6-trinitrobenzenesulfonic acid test. The resin was separated by filtration and washed with DMF and methylene chloride. The coupling reaction described above was repeated with different protected amino acids until the last amino acid was added. The N-terminus was then acetylated by adding a solution of acetic anhydride (6 equiv) and *N*-hydroxybenzotriazole (6 equiv) in 1 mL of DMF. The resin was then treated with a cleavage cocktail (1 mL, TFA/triisopropylsilane/water = 95:2.5:2.5) for 2 h at room temperature. The cleaved resin was then separated by filtration and washed with TFA (2 × 1.0 mL). The combined filtrates were concentrated by using a stream of nitrogen. To the resulting residue, a 15 mL mixture of diethyl ether and *n*-hexane (v/v = 50/50) was added to achieve precipitation. The resulting suspension was centrifuged at 1500 rpm for 10 min at room temperature. After the supernatant was carefully decanted, the pellet was dissolved in DMSO. All of the peptides obtained in this manner were purified by using HPLC (C_{18}, 5 μm, 4.6 mm × 15 cm, for the stationary phase; Buffer A, H_2O containing 0.1% TFA, Buffer B, CH_3CN containing 0.1% TFA for the mobile phase). The structures of the peptides were confirmed by using an Auto Flex II MALDI-TOF/TOF mass spectrometer equipped with a 337 nm nitrogen laser and 1.2 m flight tube. **1a**: MS $[M+H]^+$: 1876.6 (calcd), 1877.2 (obsd). Rev-N-rhodamine: MS $[M+H]^+$: 3260.8 (calcd), 3260.7 (obsd). **1b**: MS $[M+H]^+$: 1819.3 (calcd), 1819.0 (obsd). **1c**: MS $[M+H]^+$: 1819.3 (calcd), 1819.0 (obsd). **1d**: MS $[M+H]^+$: 1819.3 (calcd), 1819.3 (obsd). **1e**: MS $[M+H]^+$: 1819.3 (calcd), 1819.1 (obsd). **1f**: MS $[M+H]^+$: 1819.3 (calcd), 1819.2 (obsd). **1g**: MS $[M+H]^+$: 1819.3 (calcd), 1819.1 (obsd). **1h**: MS $[M+H]^+$: 1819.3 (calcd), 1819.1 (obsd). **2a**: MS $[M+H]^+$: 1847.3 (calcd), 1847.0 (obsd). **2b**: MS $[M+H]^+$: 1833.3 (calcd), 1833.4 (obsd). **2c**: MS $[M+H]^+$: 1819.3 (calcd), 1819.4 (obsd). **2d**: MS $[M+H]^+$: 1847.3 (calcd), 1847.5 (obsd). **2e**: MS $[M+H]^+$: 1833.3 (calcd), 1833.8 (obsd). **2f**: MS $[M+H]^+$: 1819.3 (calcd), 1820.3 (obsd). **2g**: MS $[M+H]^+$: 1847.3 (calcd), 1847.5 (obsd). **2h**: MS $[M+H]^+$: 1833.3 (calcd), 1833.4 (obsd). **2i**: MS $[M+H]^+$: 1819.3 (calcd), 1819.4 (obsd). **2j**: MS $[M+H]^+$: 1819.3 (calcd), 1820.0 (obsd). **2k**: MS $[M+H]^+$: 1805.3 (calcd), 1805.4 (obsd). **2l**: MS $[M+H]^+$: 1791.3 (calcd), 1791.3 (obsd). **2m**: MS $[M+H]^+$: 1777.2 (calcd), 1777.6 (obsd). **2n**: MS $[M+H]^+$: 1934.3 (calcd), 1934.3 (obsd). **2o**: MS $[M+H]^+$: 1934.3 (calcd), 1934.4 (obsd). **2p**: MS $[M+H]^+$: 1934.3 (calcd), 1934.4 (obsd). **2q**: MS $[M+H]^+$: 1934.3 (calcd), 1934.3 (obsd). **2r**: MS $[M+H]^+$: 1934.3 (calcd), 1934.5 (obsd). **2s**: MS $[M+H]^+$: 1934.3 (calcd), 1934.6 (obsd). **2t**: MS $[M+H]^+$: 1934.3 (calcd), 1934.1 (obsd). **2u**: MS $[M+H]^+$: 1934.3 (calcd), 1934.1 (obsd). **3a**: MS $[M+H]^+$: 1906.3 (calcd), 1906.4 (obsd). **3b**: MS $[M+H]^+$: 1920.3 (calcd), 1920.4 (obsd). **3c**: MS $[M+H]^+$: 1892.3 (calcd), 1892.4 (obsd). **3d**: MS $[M+H]^+$: 1878.3 (calcd), 1878.3 (obsd). **3e**: MS $[M+H]^+$: 1906.3 (calcd), 1906.6 (obsd). **3f**: MS $[M+H]^+$: 1920.3 (calcd), 1920.5 (obsd). **3g**: MS $[M+H]^+$: 1892.3 (calcd), 1892.8 (obsd). **3h**: MS $[M+H]^+$: 1878.3 (calcd), 1878.3 (obsd).

RNA Preparation. IRES, RRE, TAR, and 16S A-site RNAs were prepared by using the described procedures.[13] $tRNA^{mix}$ from baker's yeast was purchased from Sigma. Secondary structures of the RNAs, prediction by using the M-fold program,[14] are shown in Figure 1. For all RNA-related applications, the water used was autoclaved and treated with 0.1% diethylpyrocarbonate.

Fluorescence Anisotropy. Fluorescence anisotropy measurements were performed using a fluorometer equipped with a thermocontrolled water circulator. Rev-N-rhodamine was used as the fluorescence probe. A probe solution (200 nM) was excited at 550 nm and monitored at 580 nm (band paths 10 nm). The integration time was 10 s. The average results of five scans were used for each data point. Measurements were performed in a buffer containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethansulfonic acid (HEPES), 1 mM $MgCl_2$, 5 mM KCl, and 140 mM NaCl at pH 7.4. Competitive binding experiments were performed by using aliquots of concentrated samples of peptides. Dissociation constants

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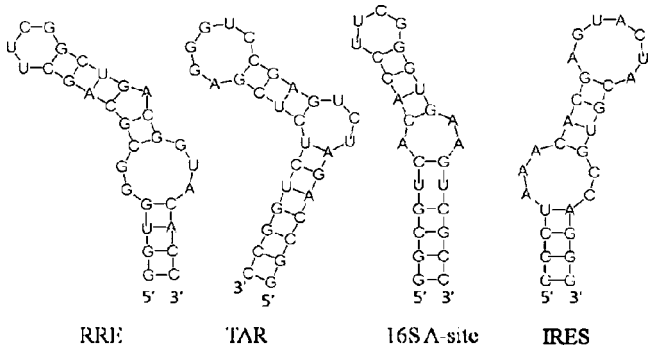

Figure 1. Secondary structures of hairpin RNA targets used in this study.

between RNA and Rev-N-rhodamine (K_d) were determined by using the method previously described.[15]

Fluorescence Intensity. Fluorescence intensity measurements were performed on a fluorometer equipped with a thermocontrolled water circulator. A solution of peptide which contains tryptophan (1 μM) was excited at 285 nm, and the emission was scanned from 300 to 420 nm (band path 6 nm) with a scan speed of 60 nm/min. Measurements were performed in a buffer containing 20 mM HEPES, 1 mM $MgCl_2$, 5 mM KCl, and 140 mM NaCl at pH 7.4. Intensities of fluorescence emissions were measured by adding aliquots of concentrated sample of IRES RNA to a solution of the peptide. Figures containing the results of these experiments are presented in the Supporting Information.

Circular Dichroism Spectroscopy. Circular dichroism (CD) measurements were performed at 20 °C with a spectropolarimeter equipped with a temperature controller. Spectra were acquired from 190 to 250 nm using a bandwidth of 1 nm and data pitch of 0.5 nm at a scan speed of 20 nm/min, step resolution 1.0 nm, response time 16 s, sensitivity 50 mdeg, using a 0.1 cm path length cuvette with three accumulations per sample. Calculations of α-helical contents of each peptide along with the representative CD spectra are presented in the Supporting Information.

Solid-Phase Pull down Assay. An IRES probe (~50 pmol) was radioactively labeled at the 5′-end. For each binding reaction, a Reacti-Bind Streptavidin High Binding Capacity Coated 96-well plate (Pierce) was washed with 0.1% Bovine Serum Albumin containing binding buffer to reduce nonspecific binding to the plastic. The indicated concentrations of substances were immobilized on each well of the streptavidin-coated plates. 5 μL of the labeled IRES stock solution were added to each well to make a final concentration of 500 pM of labeled IRES in the absence or in the presence of 5 nM of $tRNA^{mix}$. The plate was incubated for 30 min at 0 °C. Unbound IRES RNA was removed by washing with binding buffer twice. 150 μL of the elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) were added to each well and incubated for 1 h at 37 °C. The eluted RNA was separated by precipitation (ethanol/3 M NaOAc/10 mg/mL glycogen = 300 μL/15 μL/2 μL). The resulting precipitates were loaded on a 15% native polyacrylamide gel in 7 M urea and then subjected to 230 V for 20 min. The gel was exposed to a phosphorimager screen, and individual bands were quantified by using a FLA-3000 and analyzed with Multi Gauge Ver. 3.0 software (Fuji Photo).

Results and Discussion

Construction and Usage of the Ala-Scanned Peptide Library. To investigate the RNA binding properties of a series of peptides that could be selectively altered, we synthesized an Ala-scanned amphiphilic library (**1b**−**1h**), in which Lys at each position of the hydrophilic sphere of the original peptide (**1a**) was replaced by Ala. The original peptide, **1a**, is a de novo designed amphiphilic peptide originally aimed toward calmodulin,[16] which turned out to bind to hairpin RNA.[7a] The syntheses of these peptides was carried out using standard solid phase chemistry. The binding affinities of the resulting peptides against several hairpin RNA targets were measured by using a fluorescence anisotropic technique. Four in vitro transcribed (Figure 1) hairpin RNAs were used for this purpose, including the well-known RRE[17] and TAR[18] from HIV-1 and 16S rRNA A-site from *E. coli* (16S A-site)[19] and a novel hairpin from IRES domain IV of HCV (IRES).[20]

Large differences were observed in the average K_d values of the first generation peptides (**1b**−**1h**) against these hairpins (Table 1). For example, the average K_d against TAR was the highest (70 nM) with a narrow standard deviation (50 nM). The K_d value against the 16S A-site was the lowest (130 nM, standard deviation 60 nM), but that against the IRES hairpin had the largest standard deviation (70 nM). The results are surprising, considering the fact that the K_d value of the original peptide (**1a**) against TAR RNA is much higher (66 ± 5 nM) than that of IRES hairpin (45 ± 3 nM). TAR RNA possesses the greatest conformational flexibility, and therefore, it can form appropriate conformations to bind to each peptide.[21]

The results show that four out of the seven peptides studied, each of which lack one lysine amine group, have lower K_d values in contrast to the original peptide against the TAR hairpin. Only a single peptide that lacks one amine group has a lower K_d value against the IRES hairpin. The IRES hairpin, therefore, could be the most specific hairpin, because it gave the most deviated average K_d value. Since amine groups in the peptides are essential for complimentary interactions with the IRES hairpin target, it is understandable that a loss of an amine group in specific positions (eg., 6, 9, and 13) affords a reduced affinity and highly deviated average K_d values. Alterations of amine groups at four positions in the peptide give rise to significant reductions in binding against the 16S A-site, affording the highest average K_d values but with narrow deviations relative to IRES RNA. Therefore, among the four hairpins probed, the most specific binder seems to be IRES.

Another important finding, arising from studies with the Ala-scanned peptides, concerns the Lys residue(s) that mostly influence binding to hairpin RNA targets. An analysis of the number(s) of influential Lys residues against various hairpins (Table 1) demonstrates that only one Ala-scanned peptide (**1d**) gave a significantly reduced (3-fold) affinity against TAR. In contrast, at least three Ala-scanned peptides (**1d**, **1e**, **1g**) showed a significant reduction (>3-fold) against the IRES hairpin. The data also suggest that several specific amine functionalities in the peptide are important in governing binding to the specific IRES hairpin, while only a few lysine position(s) are important

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Table 1. Sequences of Ala Scanned Peptides and Their α-Helicities and K_d Values against Various Hairpin RNA Targets[a]

			K_d [nM][c]			
peptide	sequences	helicity (%)[b]	RRE	TAR	16S A-site	IRES[d]
1a	LKKLLKLLKKLLKLKG	26/57	22 ± 2	66 ± 5	100 ± 4	45 ± 3 (1.4)
1b	LAKLLKLLKKLLKLKG	9/44	140 ± 10	16 ± 3	160 ± 10	53 ± 9 (2.0)
1c	LKALLKLLKKLLKLKG	10/42	56 ± 6	87 ± 10	92 ± 6	62 ± 8 (1.3)
1d	LKKLLALLKKLLKLKG	12/47	150 ± 10	150 ± 9	160 ± 12	150 ± 10 (1.0)
1e	LKKLLKLLAKLLKLKG	12/43	130 ± 10	74 ± 10	180 ± 15	120 ± 20 (1.1)
1f	LKKLLKLLKALLKLKG	17/45	92 ± 10	25 ± 5	64 ± 3	33 ± 4 (1.8)
1g	LKKLLKLLKKLLALKG	8/44	90 ± 8	39 ± 5	200 ± 9	210 ± 10 (0.52)
1h	LKKLLKLLKKLLKLAG	9/43	25 ± 2	63 ± 9	53 ± 2	49 ± 2 (0.96)
	average K_d [nM][e]		100	70	130	100
	standard deviation		50	50	60	70

[a] Affinities were measured at 20 °C using a fluorescence anisotropic technique and rhodamine-Rev peptide as a probe. [b] α-Helicities of peptides alone were measured in 10 mM H_3PO_4 (first value) and in 50% TFE in the same buffer (second value). [c] Measurements of binding affinity were triplicated and averaged. [d] Discriminaion ratios[24] against IRES RNA are written in parentheses. [e] K_d of **1a** is not included.

in binding to the promiscuous TAR hairpin target. Thus, it appears that position-specific amine-rich peptides, such as those in the Ala-scanned library studied here, can be conveniently used to evaluate the promiscuity (or specificity) of hairpin targets. Accordingly, more specific hairpin RNA targets will show larger standard deviations of averaged K_d values.

Variations in the Hydrophilic Sphere. Since the main goal of this study was to establish a method to generate specific peptide ligands against specific hairpin RNA targets, further investigations were carried out using the IRES hairpin target. The results of studies of Ala-scanned peptides show that three Lys positions (6, 9, 13) contain amine groups that most profoundly affect binding. As a result, second generation peptides were constructed by replacing Lys at these positions with shorter side chain amino acids, such as ornithine (Orn), 1,4-diaminobutyric acid (Dab), and 1,3-diaminopropionic acid (Dap). The hypothesis used for selection of these substitutions was that binding affinities could be enhanced by reducing or shrinking the outer diameter of the helical peptide while maintaining important amine functionalities to interact with RNA counterparts.[22] Thus, it was felt that the second generation peptides might be located deeper inside of the RNA groove, thereby affording tighter binding. Actually, shortening the length of the carbon chain connecting the amine group might result in an enhancement of other van der Waals interactions owing to more intimate contacts between the peptide and RNA.[23]

As the results in Table 2 show, the second generation peptides have significantly improved K_d values against the IRES hairpin in comparison to their mother peptide (**1a**). Interestingly, the improvements in K_d values reach a lower limit when one or two carbon-deficient amino acids are employed as lysine replacements. For example, the peptide with Dab at position 6 has a 3-fold improved affinity, and the peptide with Orn at position 9 has a 3-fold improved affinity relative to the respective mother peptides.

Peptides containing multiple mutations were constructed by replacements of lysines by shorter-chain amine containing amino acids at two or more of the three potential sites. Selections of the positions altered were made based on the results of the single mutant experiments. Since **2b** (K6Dab), **2d** (K9Orn), and **2i** (K13Dap) have the greatest improved affinities against the IRES hairpin, double- and triple-mutated peptides altered at these sites were synthesized and their binding affinities were measured by using the same technique described above (Table 2). Only one double mutant peptide, **2k** (K9OrnK13Dap), had a significantly improved binding affinity (K_d = 680 ± 60 pM) relative to its single mutant counterparts. Importantly, the triple mutant peptide **2m** (K6DabK9OrnK13Dap) did not have an improved binding affinity.

Table 2. Sequences of Second Generation Peptides Using Orn, Dab, and Dap and Their K_d Values against IRES[a]

peptide	sequences[b]	helicity(%)[c]	K_d [nM][d,e]
2a	LKKLLOrnLLKKLLKLAG	13/82	6.1 ± 0.3 (2.5)
2b	LKKLLDabLLKKLLKLAG	23/63	1.6 ± 0.2 (6.3)
2c	LKKLLDapLLKKLLKLAG	26/61	3.8 ± 0.2 (2.4)
2d	LKKLLKLLOrnKLLKLAG	28/57	1.3 ± 0.2 (9.1)
2e	LKKLLKLLDabKLLKLAG	43/57	3.5 ± 0.2 (6.5)
2f	LKKLLKLLDapKLLKLAG	25/57	20 ± 1 (0.6)
2g	LKKLLKLLKKLLOrnLAG	36/53	4.4 ± 0.5 (3.3)
2h	LKKLLKLLKKLLDabLAG	24/34	8.7 ± 0.3 (2.0)
2i	LKKLLKLLKKLLDapLAG	39/54	1.4 ± 0.2 (12)
2j	LKKLLDabLLOrnKLLKLAG	49/57	2.4 ± 0.3 (6.3)
2k	LKKLLKLLOrnKLLDapLAG	48/61	0.68 ± 0.06 (25)
2l	LKKLLDabLLKKLLDapLAG	40/50	1.1 ± 0.1 (13)
2m	LKKLLDabLLOrnKLLDapLAG	49/69	4.3 ± 0.2 (4.9)

[a] Affinities were measured at 20 °C using a fluorescence anisotropic technique and rhodamine-Rev peptide as a probe. [b] Orn = Ornithine, Dab = 1,4-diaminobutyric acid, Dap = 1,3-diaminopropionic acid. [c] α-Helicities of peptides alone were measured in 10 mM H_3PO_4 (first value) and in 50% TFE in the same buffer (second value). [d] Discrimination ratios[24] against IRES RNA are written in parentheses. [e] Measurements of binding affinity were triplicated and averaged.

Owing to the fact that the second generation peptides were chosen by using the biased results obtained from studies with members of the first generation Ala-scanned peptide library, the double mutant peptide **2k** with improved affinity had a greatly increased discrimination ratio (Table 2).[24] Specifically, the discrimination ratio of the double mutant peptide **2k** is 25 (Figure 2), a value much higher than alanine-scanned peptides as well as single mutated (**2b**, **2d**, and **2i**) peptides.

Variations in the Hydrophobic Sphere. The hydrophobic sphere of the peptide might be involved in specific interactions with bases of RNA especially when the hairpin groove is deep. Previous RNA−protein binding data suggest that interactions of aliphatic amino acids with RNA are much less important

(22) (a) Hermann, T.; Westhof, E. *Biopolymers* **1998**, *48*, 155–165. (b) Corzana, F; Cuesta, I.; Freire, F.; Revuelta, J.; Torrado, M.; Bastida, A.; Jiménez-Barbero, J.; Asensio, J. L. *J. Am. Chem. Soc.* **2007**, *129*, 2849–2865. (c) Lowton, G. R.; Appella, D. H. *J. Am. Chem. Soc.* **2004**, *126*, 12762–12763.

(23) Kim, Y.; Geiger, J. H.; Hahn, S.; Sigler, P. B. *Nature* **1993**, *365*, 512–520.

(24) Discrimination ratio is defined as average $K_{d\ \text{against other hairpins (RRE, TAR, 16S A-site)}}/K_{d\ \text{against a specific hairpin (IRES)}}$. The bigger the discrimination ratio, the more specific the hairpin.

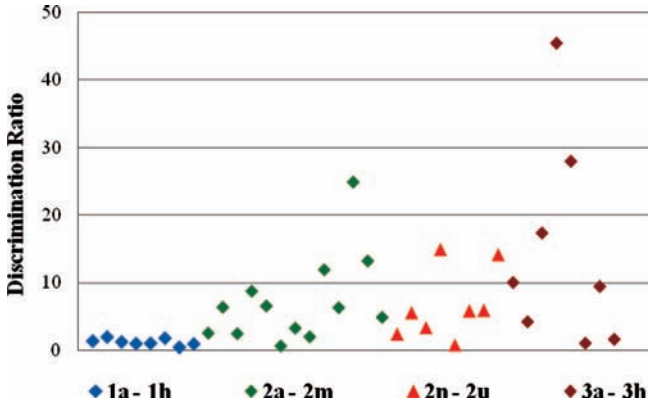

Figure 2. A comparison of discrimination ratios of the peptides against the IRES hairpin.

than with aromatic amino acids,[25] showing that stacking interactions between aromatic rings and bases could be significant.[26,27] To probe this feature, a library of Trp scanned peptides (**2n**−**2u**) were synthesized by sequentially replacing each Leu (Table 3) of the original peptide (**1a**). An average K_d value of 12 nM was observed for the Trp scanned peptides **2n**−**2u** which reflects a 4-fold improved binding against the IRES hairpin relative to that of **1a**. The data suggest that the groove in IRES is sufficiently deep to enable entry and specific RNA base interactions of aromatic rings present in the hydrophobic sphere of the amphiphilic peptides.

Interestingly, improvement of the binding affinities against the IRES hairpin caused by Trp replacement is position-dependent. For example, **2n** (L1W), **2q** (L7W), and **2u** (L14W) have the largest improved affinities, affording subnanomolar K_d values. Owing to its high α-helicity,[28] the **2n** (L1W) mutant generally has a higher binding affinity against IRES as well as other RNA hairpins.[28] In contrast, the **2q** (L7W) and **2u** (L14W) mutants do not have a dramatically high α-helicity, yet they have the most improved binding affinities against the IRES hairpin.

The results of these experiments suggest that indole moieties in two mutants can make specific interactions with specific bases in RNA. In accord with this proposal is the observation that the fluorescence intensity of the indole group in **2u** (L14W) was strongly decreased (1.6-fold) relative to **2q** (L7W) and its emission maximum was blue-shifted in the presence of the IRES hairpin.[28,29] This finding suggests that a significant interaction exists between the indole in **2u** (L14W) and base(s) in the IRES hairpin. In spite of the unbiased nature of the Trp-scanned library, discrimination ratios of members that are strong binders against the IRES hairpin increased significantly. For example, discrimination ratios of **2u** and **2q** against the IRES hairpin are 14 and 15, respectively (Table 3 and Figure 2). These high discrimination ratios also provide evidence for specific interactions between the indole and bases moieties.

Double mutants, in which two out of the three most influential Trp positions were mutated, have K_d values for binding to IRES that are similar to those of the single mutants.[28]

Variations in Both Hydrophilic and Hydrophobic Spheres. We have observed that replacement of lysines by shorter chain amine group(s) in the hydrophilic sphere and introduction of tryptophans in place of leucines in the hydrophobic sphere of an amphiphilic peptide promotes a significant increase in binding affinities to hairpin RNA. To determine if a combination of these two effects results in a further enhancement in binding affinities, both types of mutations were incorporated into one peptide. Based on the results of the single mutant experiments, third generation peptides comprised of eight double mutant peptides (**3a**−**3h**), which contain shortened amine residues in the hydrophilic sphere and one Trp mutation in the hydrophobic sphere, were combinatorial synthesized and their binding affinities against IRES and other hairpins were measured (Table 4). Although most of the third generation peptides were expected to possess subnanomolar affinities against the IRES hairpin, the observed variations seen in their affinities were enormous. Only a few of these peptides had significantly improved affinities while the others showed only slightly improved binding to the RNAs. The results suggest that additive effects caused by variations in both the hydrophilic and hydrophobic spheres will take place only if they are complimentary. Accordingly, the strongest binders in this series are **3d** ($K_d = 0.55 \pm 0.05$ nM) and **3e** ($K_d = 0.62 \pm 0.05$ nM), both of which have two variations in hydrophilic and hydrophobic spheres that do not share the same positions.

The fluorescence intensity of the Trp residue at position 7 in **3d** undergoes a decrease (2.3-fold), and its emission is more blue-shifted than that at position 14 in **3h** in the presence of the IRES hairpin.[28] Among the single Trp mutant peptides, the most influential Trp against the IRES hairpin was found to be located at position 14 (Table 3). The reversal observed for **3d** suggests that small changes in the hydrophilic sphere can affect interactions in the hydrophobic sphere.[30] Two additional mutant peptides, **3i** (K9OrnK13DapL8Y) and **3j** (K9OrnK13DapL8F), were also synthesized by replacing the Phe or Tyr residue with Trp. These mutants have much higher K_d values against IRES relative to **3d**.[31] This finding shows that a significant amount of the improved binding of **3d** is associated with specific interactions between the indole moiety and base(s) in the IRES hairpin. The data suggest that interactions between the indole ring in the peptide and base(s) in the IRES hairpin are not a consequence of simple van der Waals, hydrophobic, or π-stacking forces.[32] The specificity observed for the best ligand was also confirmed by using a solid-phase pull down assay that showed that the peptide has similar EC_{50} values in both the absence and presence of $tRNA^{mix}$ (Figure 3 and Table 5).[33]

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(26) Sinha, R.; Hossain, M.; Kumar, G. S. *Biochim. Biophys. Acta* **2007**, *1770*, 1636–1650.
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(28) See Supporting Information for details.
(29) Maraboeuf, F.; Voloshin, O.; Camerini-Otero, R. D.; Takahashi, M. *J. Biol. Chem.* **1995**, *270*, 30927–30932.
(30) Trp in other positions also can be influenced significantly by small changes in the hydrophilic sphere. Trp-scanned peptides with one short mutation (K13Dap) were synthesized (**3k**−**3r**), and their binding affinities were measured against the IRES hairpin. The two most influential sites (Trp-7 and Trp-14) remained the same as those in the Trp-scanned peptides. See Supporting Information for mass data and binding affinities of these peptides.
(31) **3i** MS ($M+H^+$): 1855.2 (calcd), 1855.4 (obsd). **3j**: MS ($M+H^+$): 1839.2 (calcd), 1839.3 (obsd). See Supporting Information for binding affinities of these peptides.
(32) (a) Jones, S.; Daley, D. T.; Luscombe, N. M.; Berman, H. M.; Thornton, J. M. *Nucleic Acids Res.* **2001**, *29*, 943–954. (b) Ellis, J. J.; Broom, M.; Jones, S. *Proteins* **2007**, *66*, 903–911. (c) Allers, J.; Shamoo, Y. *J. Mol. Biol.* **2001**, *311*, 75–86. (d) Morozova, N.; Allers, J.; Myers, J.; Shamoo, Y. *Bioinformatics* **2006**, *22*, 2746–2752. (e) Patel, D. J.; Ma, J. B.; Yuan, Y. R.; Ye, K.; Pei, Y.; Kuryavyi, V.; Malinina, L.; Meister, G.; Tuschl, T. *Cold Spring Harb. Symp. Quant. Biol.* **2006**, *71*, 81–93.

Table 3. Sequences of Trp Scanned Peptides and Their α-Helicities and K_d Values against Various Hairpin RNA Targets[a]

peptide	sequences	helicity (%)[b]	K_d [nM][c] RRE	TAR	16S A-site	IRES[d]
2n	WKKLLKLLKKLLKLAG	48/65	2.5 ± 0.3	2.0 ± 0.3	0.76 ± 0.05	0.74 ± 0.07 (2.4)
2o	LKKWLKLLKKLLKLAG	4.6/52	5.9 ± 0.4	34 ± 0.9	15 ± 1	3.3 ± 0.3 (5.5)
2p	LKKLWKLLKKLLKLAG	20/56	6.2 ± 0.5	9.8 ± 0.7	12 ± 1	2.8 ± 0.2 (3.3)
2q	LKKLLKWLKKLLKLAG	19/71	1.2 ± 0.07	21 ± 1	17 ± 2	0.87 ± 0.05 (15)
2r	LKKLLKLWKKLLKLAG	14/59	37 ± 0.2	82 ± 10	65 ± 8	92 ± 10 (0.67)
2s	LKKLLKLLKKWLKLAG	27/60	9.5 ± 1	43 ± 7	24 ± 1	4.4 ± 0.3 (5.8)
2t	LKKLLKLLKKLWKLAG	12/58	13 ± 1	10 ± 1	17 ± 1	2.3 ± 0.02 (5.8)
2u	LKKLLKLLKKLLKWAG	20/52	18 ± 2	7.8 ± 0.6	3.3 ± 0.3	0.69 ± 0.06 (14)

[a] Footnote same as that in Table 1. [b] Footnote same as that in Table 1. [c] Footnote same as that in Table 1. [d] Footnote same as that in Table 1.

Table 4. Sequences of Third Generation Peptides and Their α-Helicities and K_d Values against IRES[a]

peptide	sequences[b]	helicity (%)[c]	K_d [nM][d,e]
3a	LKKLLDabWLKKLLKLAG	29/55	1.8 ± 0.2 (10)
3b	LKKLLKWLOrnKLLKLAG	34/52	5.1 ± 0.4 (4.2)
3c	LKKLLKWLKKLLDapLAG	29/36	0.75 ± 0.08 (17)
3d	LKKLLKWLOrnKLLDapLAG	32/38	0.55 ± 0.05 (45)
3e	LKKLLDabLLKKLLKWAG	39/45	0.62 ± 0.05 (28)
3f	LKKLLKLLOrnKLLKWAG	36/58	6.1 ± 0.8 (1.0)
3g	LKKLLKLLKKLLDapWAG	41/63	1.2 ± 0.02 (9.4)
3h	LKKLLKLLOrnKLLDapWAG	12/56	13 ± 0.9 (1.6)

[a] Footnote same as that in Table 2 [b] Footnote same as that in Table 2 [c] Footnote same as that in Table 2 [d] Footnote same as that in Table 2 [e] Footnote same as that in Table 2

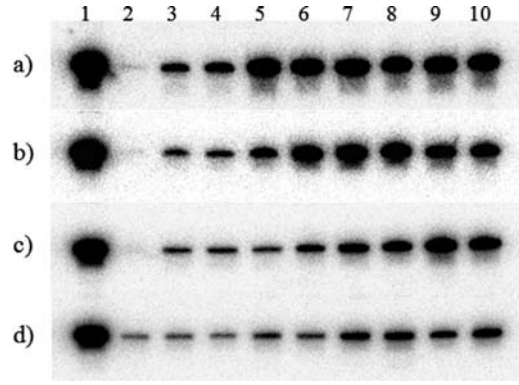

Figure 3. Autoradiogram of solid-phase pull-down assay. Lane 1, input IRES 500 pM; lanes 2−10, 0, 10, 100, 200, 400, 600, 800, 1000, and 1250 nM of peptide described. (a) **Biotin-3d** w/ IRES; (b) **biotin-3d** w/tRNA^{mix}; (c) **biotin-1h** w/IRES; (d) **biotin-1h** w/tRNA^{mix}.

Table 5. EC_{50} Values and Specificity Ratios of Peptides Determined by Using a Solid-Phase Pull Assay Method in the Presence and Absence of tRNA^{mix} [a]

peptide	EC_{50} (nM) wo/ tRNA^{mix}	EC_{50} (nM) w/ tRNA^{mix}	specificity ratio
biotin-3d	100 ± 12	140 ± 38	1.4
biotin-1h	660 ± 310	1700 ± 210	2.6

[a] For a solid-phase pull down assay, all peptides were biotinylated. 500 pM of IRES RNA was mixed w/ 5 nM of tRNA^{mix} (10 equiv) as a competitor. Specificity Ratio (SR) = EC_{50} w/ tRNA^{mix}/EC_{50} wo/ tRNA^{mix}. Each value indicates the average and 1 SD of more than three independent experiments.

Despite the fact that only a relatively small number of the doubly mutated amphiphilic peptides displayed higher affinities for RNAs, the trends observed in the discrimination ratios against the IRES hairpin (Figure 2) demonstrate that the double mutation strategy represents a viable tactic to increase the selectivities of peptide binding to specific hairpin targets. The most specific peptides derived by a single modification (second generation peptides) show at most a discrimination ratio of 25. Among those tested, the peptide with the highest discrimination ratio is **3d** (K9OrnK13DapL7W), a member of the third generation peptides that contains mutations in both spheres. This peptide has a discrimination ratio of 45, which is 2-fold higher than those of members of the third generation peptides in spite of having only a 1.2-fold improved affinity. To the best of our knowledge, this high discrimination ratio against hairpin RNA targets has not been achieved by any other ligands. Even though some peptides have been synthesized that have better binding affinities than **3d**,[34] none have as high a discrimination ratio.

A diagram between the α-helicity of all peptides and binding affinities against the IRES hairpin (Figure S3) showed no significant correlation. Data suggest that induced fit conformational changes when the peptides bind to RNA are major factors in being the best peptides.[35]

Conclusion

Since all RNAs share highly common structural features, specificity is the most important challenge in developing efficacious RNA ligands. This is especially true when the fact that hairpins are composed of an A-form helix and more flexible loop regions is taken into account. As a result, it is a difficult task to devise ligands that are selective against hairpins. This study has shown that two main strategies can be employed to prepare amphiphilic peptides that are specifically recognized by hairpin RNA. The first strategy involves specific positioning of short chain amine containing amino acid residues in the hydrophilic sphere of amphiphilic peptides. This leads to binding that takes place more deeply inside of the groove of the hairpin RNA target and allows for the maximization of interactions between the ligand and target. The second strategy makes use of insertions of Trp indole moieties in the hydrophobic sphere of amphiphilic peptides to bring about interactions with RNA bases present at the entrance of the groove. Finally, a suggestion that the two strategies work in a complimentary fashion comes from the finding that they can be combined to generate peptides that are more specific against a certain specific hairpin, such as the IRES hairpin. This has led to a peptide that has a discrimination ratio for the IRES hairpin that is more than 40.

(33) The known structure of tRNA is a pseudo-knot that gives compact structure and may not be suitable as a reference sample for hairpin RNA. Conformations of tRNA^{mix}, however, are surely complex and diverse and can be used for reference RNA.

(34) For example, intercalating agents, such as acridine, can be added to the RNA-specific peptides. (a) Gooch, B. D.; Beal, P. A *J. Am. Chem. Soc.* **2004**, *126*, 10603–10610. (b) Krishnamurthy, M.; Simon, K.; Orendt, A. M.; Beal, P. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 7044–7047.

(35) Calabro, V.; Daugherty, M. D.; Frankel, A. D *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 6849–6854.

When discrimination ratios are used to measure the degree of specificity of certain peptides against a specific RNA target, the increasing trends seen in the discrimination ratios caused by the kinds and degree of mutations are interesting (Figure 2). The results of this effort show that Ala-scanned peptides that are not biased against certain targets display discrimination ratios of less than 2. Selected peptides containing mutations in the hydrophilic sphere caused by replacing Lys residues by shorter chain amine analogues have discrimination ratios up to 25. Addition of Trp residues at specific positions of hydrophobic sphere led to similar increases (up to 15) in the discrimination ratios, and a few of the double mutant peptides showed amazingly high discrimination ratios (>40). This finding suggests that in these cases the two types of mutations work in a cooperative manner. However, only a small number of the double mutant peptides have high discrimination ratios. This is likely due to the fact that a variation in one sphere of an amphiphilic peptide can significantly affect interactions taking place between groups in the other sphere with RNA. It is important to note that interactions with RNA hairpins take place on irregular and flexible structures that permit use of all of the RNA functional groups. Therefore, the flexible conformations of RNA hairpins make it difficult to predict interactions with both spheres of amphiphilic peptides. However, this investigation has demonstrated that a combination of two independent mutations represents the basis for a workable and plausible strategy to obtain specific ligands against hairpin RNA targets.

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Supporting Information Available: Experimental procedures for preparation of α-helical peptides were described. This material is available free of charge via the Internet at http://pubs.acs.org.

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