Amphiphilic Peptides

DOI: 10.1002/anie.200703090

Amphiphilic Helical Peptides Containing Two Acridine Moieties Display Picomolar Affinity toward HIV-1 RRE and TAR**

*Yeongran Lee, Soonsil Hyun, Hyun Jin Kim, and Jaehoon Yu**

Knowledge is increasing about how endogenous RNA, which plays many functional roles in living systems, might serve as a potential drug target.[1,2] However, in contrast to the abundance of drugs which act on protein targets, only a limited number of pharmacophores with relatively poor binding affinities and specificities[3] have been developed that target RNA.[4]

Multivalency is a strategy that is widely used in nature and mimicry systems[5] to enhance binding affinity and specificity when only a limited number of ligands exist. This general approach should be applicable to the design of conjugated ligands which have affinities for different motifs of RNA (i.e. stem and loop).[6] Another strategy focuses on conjugated ligands that utilize different binding modes (i.e. ionic, hydrogen bonding, and intercalation) against RNA targets. Examples of conjugated ligand pairs that could be used in this regard are acridine/neomycin[6] and acridine/amino acids,[7] whereby the acridine moiety intercalates into and electrostatically interacts with the negatively charged phosphate backbone of RNA and the other targets RNA stem regions. Importantly, acridine has been selected as a pharmacophore both for in vitro screening[8] and virtual screening[9] against RNA targets.

We recently demonstrated that methylated α-helical amphiphilic peptides specifically bind to RNA stem regions.[10] In addition, we hypothesized that the introduction of an alkyl or aryl group larger than methyl might give rise to a diverse set of α-helical peptides that specifically bind to the stem regions of RNA. We hypothesized that incorporation of the intercalator[11] acridine into α-helical peptides might result in additional π–π interactions with bases in the target RNA. Below, we describe the synthesis of a library of amphiphilic α-helical peptides that contain acridinylated lysine moieties and an evaluation of their binding affinities to hairpin RNA. The results of this study show that members of this group have binding affinities and selectivities of less than 1 nM against RRE and TAR RNA.

Sequences of the amphiphilic peptide (peptide **0**) were adapted from a peptide originally aimed at calmodulin.[12] Introduction of acridine into the peptides through the ε-amino groups of Lys[13] leads to positioning of this moiety at the hydrophilic phase of the amphiphilic α-helical peptide. By using standard Fmoc protection chemistry,[14] six peptides (peptides **1**–**6** in Table 1) were prepared by acridinylation of the N^{ε} atoms of the Lys amino groups on peptide **0**.[14] Analysis of circular dichroism (CD) spectra of these peptides in phosphate buffer at pH 7.4 showed that they have different α-helical contents (Table 1), which suggests that the position of the acridine moiety significantly affects the conformational rigidity.

Table 1: Sequences of the synthesized peptides and their α helicity and binding affinities against RRE, TAR, and $tRNA^{mix}$.[a]

Peptide	Sequence (position of K*)[b]	α Helicity [%][c]	K_d [nM] against RRE	K_d [nM] against TAR	K_d [nM] against $tRNA^{mix}$
0	LKKLLKLLKKLLKLKG	26, 57	22	62	55
1	LK*KLLKLLKKLLKLKG (2)	14, 52	3.2 (4.4)	2.5 (5.6)	14
2	LKK*LLKLLKKLLKLKG (3)	18, 57	11	17	16
3	LKKLLK*LLKKLLKLKG (6)	18, 55	14	8.3	19
4	LKKLLKLLK*KLLKLKG (9)	36, 62	4.3 (3.0)	1.3 (10)	13
5	LKKLLKLLKK*LLKLKG (10)	25, 63	6.8 (2.1)	1.5 (9.3)	14
6	LKKLLKLLKKLLK*LKG (13)	15, 65	13	16	9.3

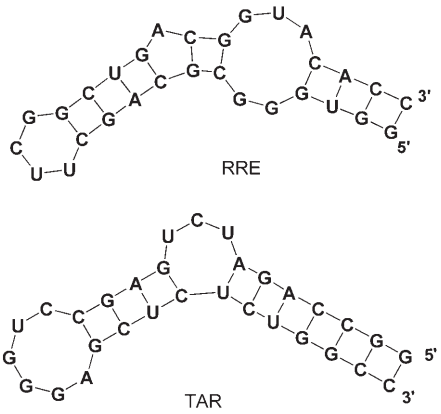
[a] Affinities were measured at 20°C using a fluorescence anisotropy technique and rhodamine-Rev peptide as a probe. [b] K* = N^{ε}-acridinyl-Lys. [c] α Helicities of peptides alone were measured in 10 mM H_3PO_4 (first value) and in 50% TFE in 10 mM H_3PO_4 (second value). Discrimination ratio (K_d against $tRNA^{mix}$ to K_d against RRE or TAR) is written in parentheses.

Owing to their typical stem–loop structures, RRE and TAR RNA from HIV-1 were chosen as initial targets (Scheme 1). Binding affinities of members of the initial peptide library were determined by using a fluorescence anisotropy technique with rhodamine-labeled Rev as a probe.[14] The binding affinities of the mono-acridinylated peptides were two- to sevenfold higher against RRE and from four- to 50-fold higher against TAR than those of peptide **0** (Table 1).[15] Even though peptide **1** (3.2 nM) and peptide **4** (1.3 nM) bind most strongly to RRE and TAR, respectively, peptides **1**, **4**, and **5** have approximately the same binding affinities to both targets. Thus, it appears that even though

[*] Y. Lee, S. Hyun, H. J. Kim, Prof. J. Yu
Department of Chemistry & Education
Seoul National University, Seoul 151-742 (Korea)
Fax: (+82) 2880-7761
E-mail: jhoonyu@snu.ac.kr
Homepage: http://bcncb.snu.ac.kr/

[**] Y.L. and S.H. equally contributed to this work. Financial support for this work was provided by the 21st Frontier-CBM (M107KH010010-07K0801-01020) and KOSEF (2007-01247).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. Predicted structures of RRE and TAR RNA.

their binding affinities are high, the mono-acridinylated peptides do not display specificity in binding hairpin RNA.

The observed dependence of the binding affinities to hairpin RNA on the position of the acridine moiety in the peptide backbone suggests that the acridine groups might be positioned differently in the peptide–RNA complexes and, if so, that the placement of two or more acridine groups into the peptide might bring about a "multivalency" effect. This proposal was confirmed in studies with three bis-acridinylated peptides (peptides **14**, **15**, and **45**) designed by selecting two out of the three positions that led to the highest binding affinities of the mono-acridinylated peptides. As expected, binding affinities of all bis-acridinylated peptides were higher than those of monofunctionalized counterparts (Table 2).

Table 2: Sequences of the second-generation peptides and their α helicity and binding affinities against RRE, TAR, and $tRNA^{mix}$.[a]

Peptide	Sequence (positions of K*)[b]	α Helicity [%][c]	K_d [nM] against RRE	K_d [nM] against TAR	K_d [nM] against $tRNA^{mix}$
14	LK*KLLKLLK*KLLKLKG (2, 9)	35, 57	0.61 (8.0)	0.55 (8.9)	4.9
15	LK*KLLKLLKK*LLKLKG (2, 10)	15, 48	0.72 (8.5)	0.64 (9.5)	6.1
45	LKKLLKLLK*K*LLKLKG (9, 10)	12, 54	0.92 (7.5)	0.37 (18)	6.9
145	LK*KLLKLLK*K*LLKLKG (2, 9, 10)	15, 49	0.25 (0.92)	0.20 (1.1)	0.23

[a], [b], [c] Footnotes are the same as in Table 1.

Notably, incorporation of acridine at the first- and second-best positions affords a peptide (peptide **14**) with a binding affinity of 610 pM to RRE. CD studies showed that the α helicity of peptide **14** is the same as the mono-acridinylated substances, while those of other bis-acridinylated or tris-acridinylated peptides are reduced.[14] These data agree with the proposal that specific binding of Rev peptide to RRE RNA correlates with α-helix formation.

The tightest binding peptide against TAR is peptide **45**, which was also designed by considering acridinylation sites that led to the best two TAR binders in the mono-acridinylated peptides. However, the α-helical content of peptide **45** is greatly lowered compared to that of peptide **4**, suggesting that the presence of two acridine moieties reduces conformational rigidity. Moreover, the α helicity of peptide **45** does not increase much (12 to 16%) in the presence of TAR RNA (0.1 equiv),[16] suggesting that at least the best fit is not forming an α-helical conformation in the groove of TAR.[17,18] Finally, the binding affinity of tris-acridinylated peptide **145** is less than 250 pM against all RNA targets, and it displays poor specificity.

To evaluate binding specificities of the mono- and bis-acridinylated peptides, their affinities against $tRNA^{mix}$ were determined and compared to those against RRE and TAR. The discrimination ratio (DR; ratio of K_d against $tRNA^{mix}$ to K_d against hairpin RNA) of peptide **1** for RRE was about 4.4, showing that this peptide is not only the strongest but also the most specific binder among mono-acridinylated peptides studied. The observation that the DR of peptide **14** against RRE is 8.0 suggests that the acridine moieties in this peptide are located at spatially different positions than those in peptide **1**. The binding specificity of peptide **45** against TAR, reflected in a DR of 18, is much greater than that of the mono-acridinylated peptide **4** (DR = 10).

In another approach to assessing specificities, solid-phase assays of C-terminal biotinylated peptide **14** and peptide **1** were carried out against RRE in competition with $tRNA^{mix}$.[14] The affinities (EC_{50}) of these peptides were measured in the absence and presence of 100 equivalents of $tRNA^{mix}$ (ca. 250 equiv in bases) as shown in Figure 1. The specificity ratios (EC_{50} with $tRNA^{mix}$ to EC_{50} without $tRNA^{mix}$; Table 3) of these peptides were found to be 6.7 and 8.2, respectively. The fact that the specificity ratio of peptide **14** is much better than that of the mono-acridinylated peptide suggests that incorporation of two acridine moieties as intercalators into peptides might be a general strategy for the design of targets

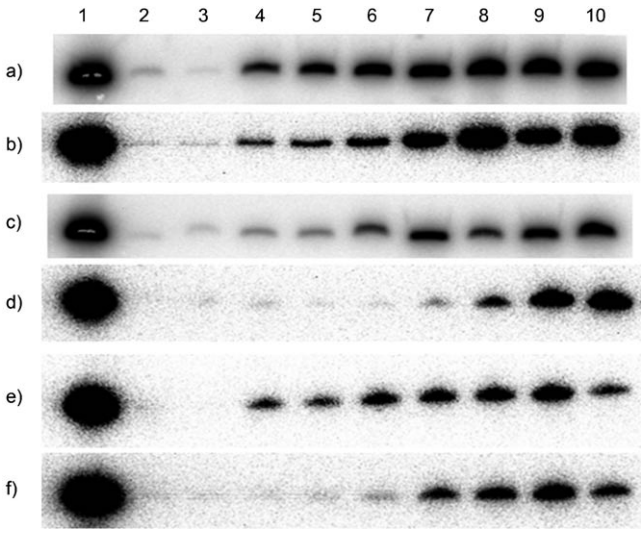


Figure 1. Autoradiogram of solid-phase pull-down assay; lane 1: input RRE 500 pM; lanes 2–10: 0, 1, 10, 20, 40, 100, 200, 400 nM, and 1 mM of chemicals described. a) Rev; b) Rev with tRNA; c) peptide **1**; d) peptide **1** with tRNA; e) peptide **14**; f) peptide **14** with tRNA.

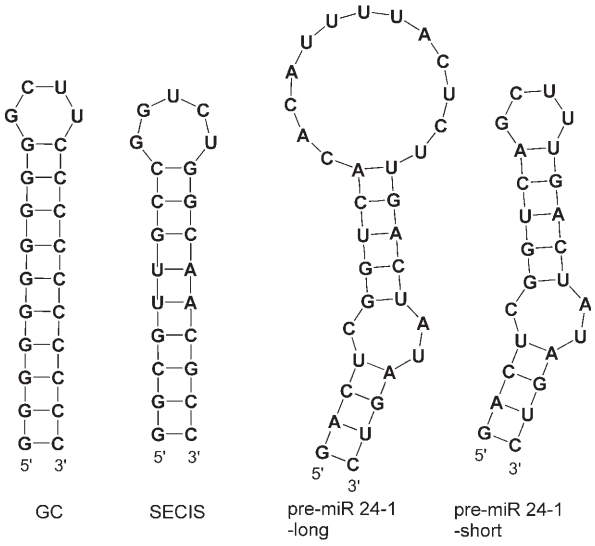
Table 3: EC_{50} values and specificity ratios of peptides determined by using a solid-phase assay method[a] in the presence and absence of $tRNA^{mix}$.

Peptide	EC_{50} [nM] without $tRNA^{mix}$	EC_{50} [nM] with $tRNA^{mix}$	Specificity ratio
1	89 ± 3.1	730 ± 24	8.2
14	51 ± 17	340 ± 66	6.7
Rev_{34-50}[b]	36 ± 3.3	140 ± 18	3.9

[a] For the solid-phase assay all peptides were biotinylated. 500 pM of RRE RNA was mixed with 50 nM of $tRNA^{mix}$ (100 equiv) as a competitor. Specificity ratio: EC_{50} with $tRNA^{mix}$ divided by EC_{50} without $tRNA^{mix}$. Each value indicates the average and one standard deviation of three independent experiments. [b] suc TRQ ARR NRR RRW RER QRA AAA R.

with increased affinity and specificity against hairpin RNA targets.[19]

To prove the minimal structural requirements of hairpin RNAs favored by acridinylated peptides, a variety of RNAs (Scheme 2) were prepared and their K_d values were measured



Scheme 2. Predicted structures of other hairpin RNAs.

(see the Supporting Information). Firstly, the peptides did not show any significant differences in binding affinities against long- and short-loop RNA (pre-miR 24-1-long, pre-miR 24-1-short),[23] suggesting that the loop region of the RNA contributes negligibly. Secondly, the affinities of the peptides against a DNA-like RNA helix (GC RNA[14] and SECIS RNA[24]) are about an order of magnitude lower than those of typical stem–loop RNA with bulges and kinks. Furthermore, the K_d values between mono-acridinylated peptides are similar to these kinds of RNA (only two- to threefold difference in K_d values, compared with 4–11-fold differences in typical hairpin RNAs). The data suggest that bases in the bulges and kinks are significantly involved in interactions with acridinylated peptides.

The dramatic increases in binding affinities (40-fold for RRE, 170-fold for TAR) that result from addition of acridine group(s) to amphiphilic peptides demonstrate that conjugation of intercalators that operate by different binding modes (ionic or hydrogen bonding) leads to one of the most tightly binding pharmacophores against RNA targets.[6]

Experimental Section

Syntheses of N^{α}-Fmoc-N^{ε}-acridinyl-lysine: Phenol (4.0 g) was heated to 60 °C. Upon melting of the phenol, N^{α}-Fmoc-lysine (0.92 g, 2.5 mmol), 9-chloroacridine (2.0 equiv), and *N*,*N*-diisopropylethylamine (2 equiv) were added. The reaction mixture was stirred at 60–80 °C for 30 min. The product was purified by flash column chromatography, affording a yellow solid (75% yield). ^{1}H NMR and ^{13}C NMR spectra were identical to the previously reported data.[20]

Synthesis of peptides: Peptides were synthesized on Rink Amide resins using a standard solid-phase protocol,[21] purified by HPLC, and identified by MS.

Fluorescence anisotropy (FA): FA measurements were performed as previously described.[22] The solid-phase pull-down assay is described in reference [14].

Received: July 11, 2007
Revised: September 6, 2007
Published online: November 6, 2007

Keywords: acridine · hairpin RNA · intercalations · peptides · RNA

[1] a) B. J. Tucker, R. R. Breaker, *Curr. Opin. Struct. Biol.* **2005**, *15*, 342–348; b) N. Sudarsan, M. C. Hammond, K. F. Block, R. Welz, J. E. Barrick, A. Roth, R. R. Breaker, *Science* **2006**, *314*, 300–304; c) K. F. Blount, J. X. Wang, J. Lim, N. Sudarsan, R. R. Breaker, *Nat. Chem. Biol.* **2007**, *3*, 44–49.

[2] a) G. A. Calin, C. M. Crose, *Nat. Rev. Cancer* **2006**, *6*, 857–866; b) T. Dalmay, D. R. Edwards, *Oncogene* **2006**, *25*, 6170–6175; c) L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, J. M. Johnson, *Nature* **2005**, *433*, 769–773; d) C. Arenz, *Angew. Chem.* **2006**, *118*, 5170–5172; *Angew. Chem. Int. Ed.* **2006**, *45*, 5048–5050.

[3] M. T. Hyvonen, A. Uimari, T. A. Keinanen, S. Heikkinen, R. Pellinen, T. Wahlfors, A. Korhonen, A. Narvanen, J. Wahlfors, L. Alhonen, J. Janne, *RNA* **2006**, *12*, 1569–1582.

[4] a) S. Magnet, J. S. Blanchard, *Chem. Rev.* **2005**, *105*, 477–498; b) Q. Vicens, E. Westhof, *ChemBioChem* **2003**, *4*, 1018–1023; c) Y. Tor, *ChemBioChem* **2003**, *4*, 998–1007; d) G. R. Lawton, D. H. Appella, *J. Am. Chem. Soc.* **2004**, *126*, 12762–12763.

[5] M. Mammen, S. K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908–2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754–2794, and references therein.

[6] S. R. Kirk, N. W. Luedtke, Y. Tor, *J. Am. Chem. Soc.* **2000**, *122*, 980–981.

[7] B. D. Gooch, P. A. Beal, *J. Am. Chem. Soc.* **2004**, *126*, 10603–10610.

[8] E. S. DeJong, C. E. Chang, M. K. Gilson, J. P. Marino, *Biochemistry* **2003**, *42*, 8035–8046.

[9] S. Renner, V. Ludwig, P. Boden, U. Scheffer, M. Göbel, G Schneider, *ChemBioChem* **2005**, *6*, 1119–1125.

[10] S. Hyun, H. J. Kim, N. J. Lee, K. H. Lee, Y. Lee, D. R. Ahn, K. Kim, S. Jeong, J. Yu, *J. Am. Chem. Soc.* **2007**, *129*, 4514–4515.

[11] a) A. Kuzuya, K. Machida, R. Mizoguchi, M. Komiyama, *Bioconjugate Chem.* **2002**, *13*, 365–369; b) K. Fukui, K. Tanaka, *Nucleic Acids Res.* **1996**, *24*, 3962–3967; c) P. S. Nelson, M. Kent, S. Muthani, *Nucleic Acids Res.* **1992**, *20*, 6253–6259.

[12] J. A. Cox, M. Comte, J. E. Fitton, W. F. DeGrado, *J. Biol. Chem.* **1985**, *260*, 2527 – 2534.
[13] Synthesis of N^{α}-Fmoc-N^{ε}-acridinyl-Lys was carried out by an adapted method described in N. W. Luedtke, Q. Liu, Y. Tor, *Biochemistry* **2003**, *42*, 11391 – 11403.
[14] See the Supporting Information.
[15] 1:1 stoichiometric ratio of peptide/RNA was obtained by minimum fluorescence intensity value of the peptide molecule when it was 0.5 mole fraction with RRE RNA; see the Supporting Information.
[16] For reference, the α helicity of peptide **1**, which has the same low value as peptide **45**, increases to 21 % and 28 % when 0.05 and 0.1 equiv of RRE are present; see the Supporting Information.
[17] J. D. Puglisi, L. Chen, S. Blanchard, A. D. Frankel, *Science* **1995**, *270*, 1200 – 1203.
[18] C. Faber, H. Sticht, K. Schweimer, P. Rösch, *J. Biol. Chem.* **2000**, *275*, 20660 – 20666.
[19] Y. Gosser, T. Hermann, A. Majumdar, W. Hu, R. Frederick, F. Jiang, W. Xu, D. J. Patel, *Nat. Struct. Biol.* **2001**, *8*, 146 – 150.
[20] C. K. Francis, T. C. Robert, R. Don, *Aust. J. Chem.* **1993**, *46*, 1207 – 1212.
[21] W. C. Chan, P. D. White, *Fmoc Solid Phase Peptide Synthesis; A Practical Approach*, Oxford University Press, Oxford, UK, **2000**.
[22] J. Lee, M. Kwon, K. H. Lee, S. Jeong, S. Hyun, K. J. Shin, J. Yu, *J. Am. Chem. Soc.* **2004**, *126*, 1956 – 1957.
[23] M. Otsuka, Q. Jing, P. Georgel, L. New, J. Chen, J. Mols, Y. J. Kang, Z. Jiang, X, Du, R. Cook, S. C. Das, A. K. Pattnaik, B. Beutler, J. Han, *Immunity* **2007**, *27*, 123 – 134.
[24] S. Yoshizawa, L. Rasubala, T. Ose, D. Kohda, D. Fourmy, K. Maenaka, *Nat. Struct. Mol. Biol.* **2005**, *12*, 198 – 203.

