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Library construction of neomycin–dipeptide heteroconjugates and selection against RRE RNA

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Abstract—An approach is described to the design of neomycin–dipeptide conjugates as ligands for Rev responsive element (RRE) RNA, which effectively inhibit Rev–RRE interaction. A library of 256 neomycin–dipeptide conjugates was constructed on TentaGel beads using a split-and-pool combinatorial synthesis. Five conjugates were selected after screening the library with fluorescence linked RRE RNA, and they were identified after sequencing by MALDI-TOF mass spectrometer. The heteroconjugates bind to RRE RNA with moderately improved affinities and highly improved specificity, compared to neomycin as determined by means of fluorescence anisotropy and surface plasmon resonance (SPR) experiments. This strategy, synthesis of the neomycin–peptide heteroconjugate library and selection against RNA target, could provide an efficient way to develop inhibitors against pathogenic RNA.

1. Introduction

Aminoglycosides are well known natural products that have evolved as inhibitors and modulators of RNA functions.[1] These substances take advantage of mostly electrostatic interactions between positively charged ammonium ions and negatively charged phosphate ions in RNA backbone.[2] However, most aminoglycosides bind to a variety of RNA targets with lack of selectivity, which often results in severe toxicity.[3] Consequently, efforts to generate various derivatives of aminoglycosides and to increase specificities of aminoglycosides are crucial components in the development of new types of RNA binding drugs.

To make more diverse pharmacophore, strategy involves addition of simple hydrophilic[4] or hydrophobic moieties[5] to the aminoglycoside pharmacophore in order to make heteroconjugates. The added moiety has a site(s) for additional interactions between the drug and RNA. Recently, we made a series of heteroconjugates of neomycin–chloramphenicol (or linezolid) for aiming both a stem and a loop region of RNA.[6] The improvement of binding affinity and specificity suggested that the combination of stem- and loop-binders may be a new strategy to generate specific ligands for various RNA targets. However, there are obstacles to generate more diverse heteroconjugates, either from stem- or loop-binding part. Especially for the loop-binding part, there are not many loop-specific chemicals known except chloramphenicol, macrolides, and linezolid. Furthermore, the diversity of these substances is not easily achieved.

To be a specific loop-binding molecule, a molecule should possess hydrogen bonding capabilities with bases in a loop region RNA. Peptides could be a plausible candidate, because they not only have hydrogen bonding donors and acceptors, but also they can be readily prepared and diversified by the conventional solid-phase synthesis. A specific amino acid, such as arginine or lysine, was used as a conjugate of aminoglycoside to increase inhibitory effect for protein synthesis or cellular uptake.[7] However; the diversity of the peptides has seldom been used to generate a library of the heteroconjugates as ligands for various RNA targets. In this letter, we describes (1) building up 'on bead' neomycin–dipeptide heteroconjugates library using a solid-phase synthesis, (2) screening the neomycin–dipeptide

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heteroconjugates against RRE RNA, which was fluorescein-labeled, and (3) measuring specificity and affinity of the selected heteroconjugates. Once again the selected heteroconjugates showed improved specificity against RRE RNA.

2. Results and discussion

2.1. Construction of neomycin–dipeptide library

For the loop-binding part of the heteroconjugates, dipeptide moiety was chosen to induce specific hydrogen bonding with target RNA. Furthermore, the solid-phase chemistry to buildup the diversity could be easily established by the conventional peptide chemistry methods. For the synthesis of the dipeptide, Fmoc-protected amino acids were used for the immobilization to TentaGel S NH_2 resin. A hexapeptide linker (Met-Arg-β-Ala-β-Ala-β-Ala-β-Ala) was first coupled to the resin to facilitate RNA binding to the solid supported candidate and to improve the quantity of MALDI mass analysis of the selected heteroconjugates.[8]

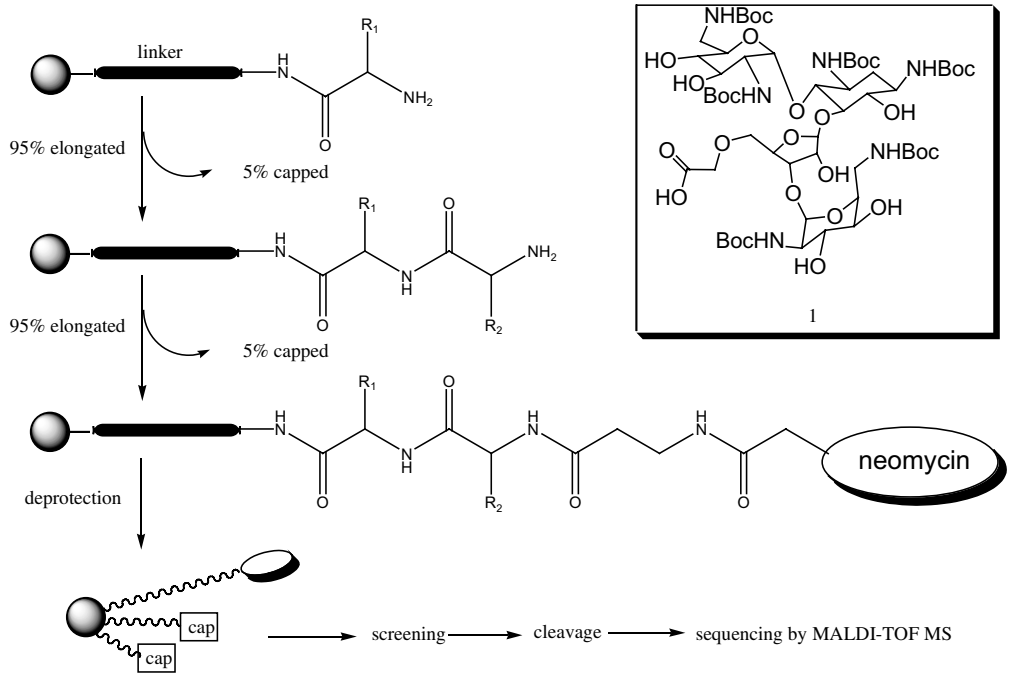
The library was then constructed by the 'split-and-pool' method in order to make the diversity as shown in Scheme 1. Sixteen amino acids (except Lys, Met, Ile, Cys) were used for the dipeptide synthesis to avoid the isomers of the same mass and to prohibit oxidation of the thiol group in the cleavage step. Theoretically, a total 256 dipeptides (16×16) could be obtained. For the partial termination of the coupled peptide for the identification,[8b] 10% of the Boc-protected Gly was used in the each amino acid coupling reaction. After two coupling reactions of the random dipeptide region, β-Ala was coupled, followed by the coupling of Boc-protected and acetic acid linked neomycin, **1** (Scheme 1).

There may be more diversity created if more than two amino acids were used for the loop-specific peptides. However, we thought that dipeptide is more than enough to make hydrogen bond(s) with base(s) in loop region of RNA, and dipeptide would more stably function than longer peptides in vivo when used as a drug. Previously, it was observed that two hydrogen bonds are formed between chloramphenicol and two loop region bases in 23S rRNA.[9] For chloramphenicol, there was eight-atom distance between the two hydrogen bonds. For the dipeptide, two hydrogen bonds are possibly formed in eight-atom distance to make specificity of the hetero conjugates.

2.2. Library screening and identification of the selected heteroconjugates

After completing the neomycin–dipeptide coupling to the resin, the conjugates were deprotected with a TFA cocktail containing thioanisole 2.5%, and water 2.5%, and washed with MeOH. After dried under vacuum, ca. 300 beads were incubated in 4 μM of fluorescein-labeled RRE RNA (F-RNA) in the buffered solution with shaking by inversion. After washing several times with the buffer, more intensely fluorescent beads than the control were monitored under a microscope (Fig. 1).

Six most brilliant fluorescent beads were selected under a low-magnification fluorescence microscopy and each of the beads was treated with CNBr solution to cleave the heteroconjugate from the resin. The cleaved heteroconjugates were then dried under vacuum. The resulting



Scheme 1. Construction of the library of partially elongated neomycin–dipeptide heteroconjugates. R_1 or R_2 = one of 16 amino acids used. Cap = Gly.

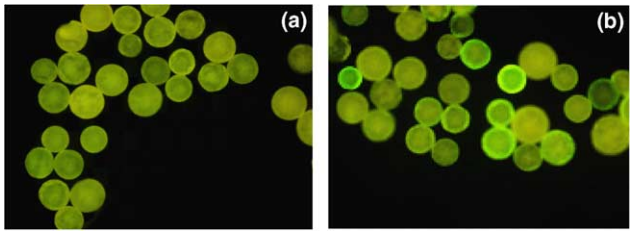


Figure 1. Fluorescence emitted from F-RRE bound beads: (a) without F-RRE (background); (b) with 4 μM of F-RRE.

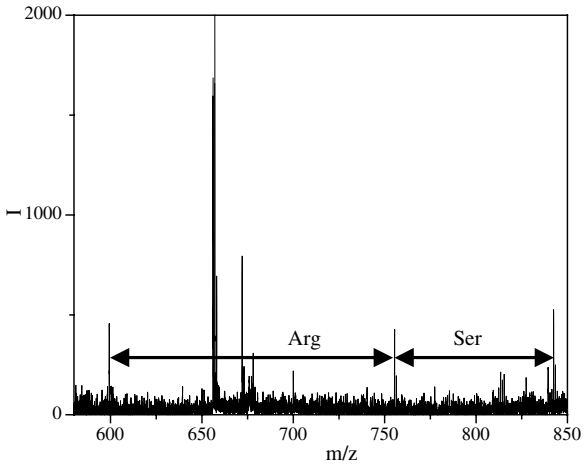


Figure 2. Sequencing by MALDI-TOF MS. Indication for Arg and Ser incorporated in a heteroconjugate. Peaks (656 and 672 Da) are from matrix.

each heteroconjugate was mixed with MS matrix, α-cyano-4-hydroxycinnamic acid, and analyzed by a MALDI-TOF spectroscopy. A typical MALDI-TOF spectrum of the cleaved heteroconjugate was shown in Figure 2. All of the molecular weights of the heteroconjugates and their identified amino acid sequences were listed in Table 1. Except two identical sequenced heteroconjugate (Neo-βAlaArgHis), amino acid sequences of six identified heteroconjugates were different. Even though there were no conserved amino acid sequences of the identified heteroconjugates, basic amino acids (Arg, His) was the most frequently found, suggesting that the side chains of these amino acids might form specific hydrogen bonding with bases in the loops. All of the identified heteroconjugates were individually synthesized as described in Section 4 to observe real binding affinities with RRE RNA.

Table 1. Characterization of synthesized heteroconjugates selected from the library

Heteroconjugate	Mass calcd [M+H]	Mass obsd [M+H]
Neo-βAlaAspLeu	993.0[a]	993.4
Neo-βAlaAspArg	1013.0	1013.2
Neo-βAlaArgHis	1036.1	1036.4
Neo-βAlaArgSer	986.0	986.3
Neo-βAlaArgAla	970.0	970.4

[a] [M+Na].

2.3. Evaluation of the selected heteroconjugates as specific RRE binders

The binding affinities of the six heteroconjugates to RRE RNA were then measured by several different methods using a surface plasmon resonance (SPR) and a fluorescence anisotropy (FA) techniques. In the SPR method, RRE RNA was immobilized to the surface of a sensor chip. Binding affinities (K_D) of heteroconjugates obtained by the SPR experiment are slightly lower (ca. 1.5–2 times) than that of neomycin (Table 2). It seems that conjugation with dipeptide does not significantly improve binding affinity. In order to determine an inhibitory effect of the heteroconjugates for RRE–Rev interaction, IC_{50} values of the heteroconjugates were measured using the FA method (Fig. 3). For the FA method, biotinylated RRE (B-RRE) was prepared and bound to antibiotin antibody (Ab). The B-RRE-antibiotin Ab complex was employed for FA method in order to have a larger anisotropic effect when the fluorescence-labeled Rev peptide (RevFl) binds to its target, RRE. Fluorescence anisotropy was measured upon titration of the heteroconjugates into the 1:1 mixture of RevFl and B-RRE-antibiotin Ab. Regarding the approximation that the change in fluorescence anisotropy is directly proportional to the fraction of RevFl bound to B-RRE-antibiotin Ab, IC_{50} values were calculated from the obtained fluorescence anisotropy data by using a formula that has previously been reported.[9] As consistent with the SPR results, the heteroconjugates show a similar level of IC_{50} values as neomycin (Table 2).

Although affinities of the heteroconjugates were not much improved compared to neomycin, some of them might show specific binding to RRE since the most specific binder to RRE could not be evaluated simply by the improvement of binding affinities.

In order to calculate the 'real' specificity, as recently Tor and co-workers devised,[9] the affinities of a heteroconjugate in presence and absence of general nucleic acids, such as tRNA were measured. Then, specificity is defined as the number resulting from division of the former value by the latter value as shown in Table 3. According to the calculation, Neo-βAlaArgHis was selected as the most specific binder to the RRE. The calculated RRE specificity ratio of Neo-βAlaArgHis was considerably improved compared to that of a native antibiotic neomycin, even though the binding affinity of Neo-βAlaArgHis is at the similar level as neomycin. These data might suggest that as amide bonds and functional groups in side chains of the dipeptide moiety can interact with bases of RRE RNA via hydrogen bonds they might contribute to specificity maintaining binding affinity.

For RNA targets, most of efforts to develop binders using a combinatorial library have been made at the level of peptides.[10] The library of neomycin–peptide conjugates have never been investigated. Moreover, the compounds selected from the library before were not systematically examined in terms of specificity. Since

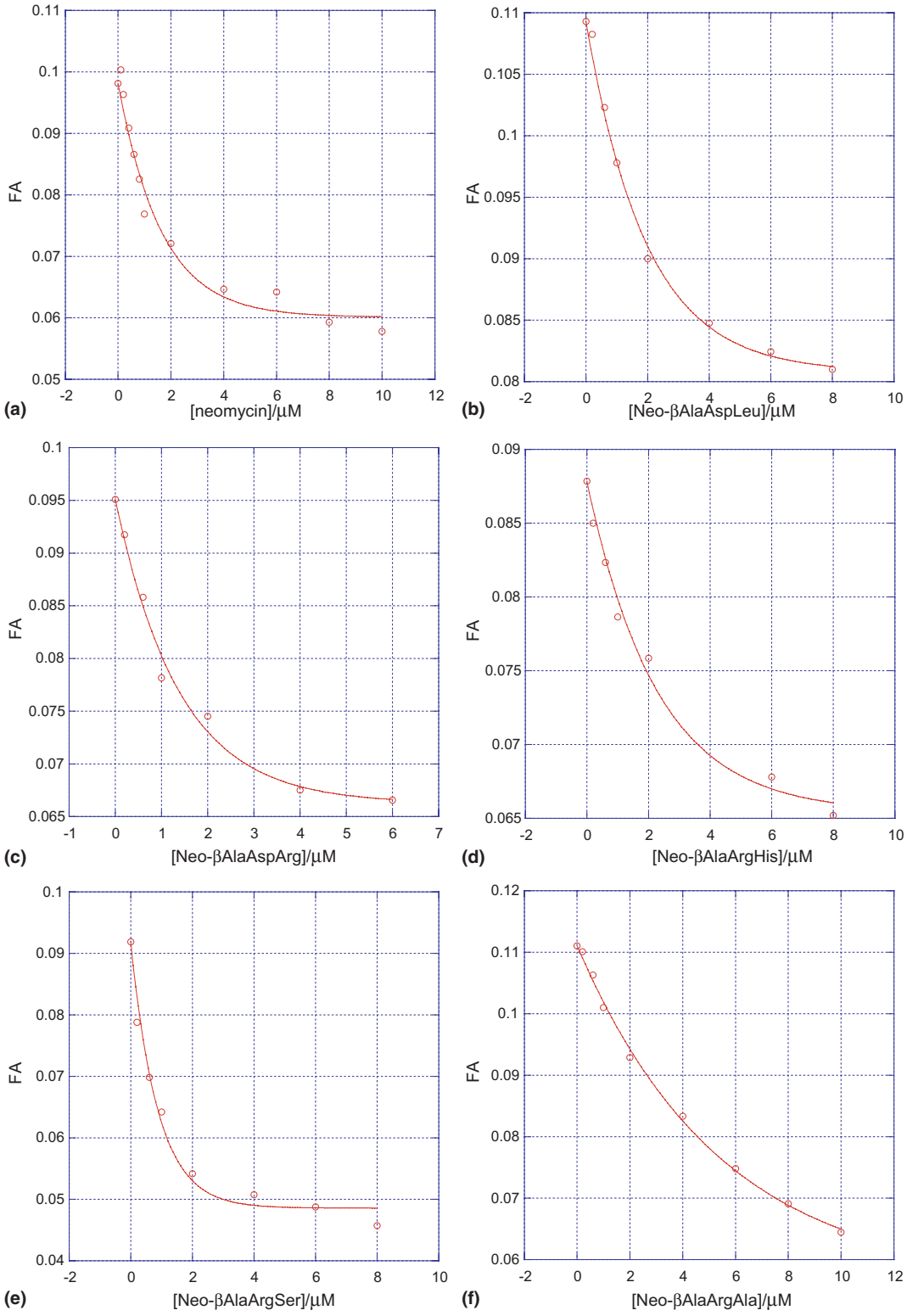


Figure 3. Fluorescence anisotropy data fit by KaleidaGraph™: (a) neomycin; (b) Neo-βAlaAspLeu; (c) Neo-βAlaAspArg; (d) Neo-βAlaArgHis; (e) Neo-βAlaArgSer; (f) Neo-βAlaArgAla.

there are many RNAs binding to protein and RNAs such as RRE and TAR having peptide ligands in the biological system, conjugation aminoglycosides, well known promiscuous antibiotics, with peptides could be a novel way to develop specific RNA targeted drugs.

3. Conclusion

We have designed and synthesized the neomycin–dipeptide heteroconjugate library for the facile screening against specific RRE RNA. The combinatorial synthesis

Table 2. K_D and IC_{50} values of the heteroconjugates determined by SPR and FA method relatively

Heteroconjugate	K_D (μM)	IC_{50} (μM)
Neo-βAlaAspLeu	0.64	1.35 (±0.13)[a]
Neo-βAlaAspArg	0.49	0.96 (±0.10)
Neo-βAlaArgHis	0.72	1.57 (±0.10)
Neo-βAlaArgSer	0.49	0.61 (±0.20)
Neo-βAlaArgAla	0.52	3.77 (±0.07)
Neomycin	0.88	1.12 (±0.29)

[a] Error range from curve fitting.

Table 3. Binding specificity of the heteroconjugates determined by SPR

Heteroconjugate	K_D (−tRNA, μM)	K_D (+tRNA, μM)	Specificity[a]
Neo-βAlaAspLeu	0.64	2.2	3.4
Neo-βAlaAspArg	0.49	1.2	2.4
Neo-βAlaArgHis	0.72	2.6	2.2
Neo-βAlaArgSer	0.49	1.4	4.9
Neo-βAlaArgAla	0.52	1.9	3.6
Neomycin	0.88	8.5	9.6

[a] K_D (+tRNA)/K_D (−tRNA).

of dipeptides with 16 different amino acids to the TentaGel S NH_2, followed by reaction with the acetylated neomycin gave approximately 300 different heteroconjugates to the resin. Selection and identification of the best binder using fluorescein-labeled RRE showed five heteroconjugates. The best heteroconjugates were then selected using affinity ratio against RRE/tRNA mixture. Even though the best selected heteroconjugates showed only sub-micromolar affinity to the RRE, it showed better specificity than neomycin. The experiments in here are applicable to any target RNA, which already exists or to come in near future in order to develop effective binders.

4. Experimental

4.1. General

All chemicals and solvents were purchased from *Sigma–Aldrich*. Fmoc-protected amino acids and *Rink Amide* resin (0.6 mmol/g) were purchased from *NorvaBiochem*. TentaGel S NH_2 resin (Ø = 130 μm, 0.27 mmol/g) was purchased from *Rapp Polymere*. RevFl peptide ($_{Succinyl}$-TRQARRNRRRRWRERQRAAAAK(FITC)-NH_2) was purchased from *AnyGen*. Column chromatography was performed using silica gel, and precoated TLC silica plate purchased from *Merck* was used for thin-layer chromatography. ^1H and ^{13}C NMR spectra were measured using 500 MHz *Varian Unity* spectrometer. Mass spectrum of **1** was obtained using LMS-700 Mstation™ (*Jeol Ltd*). Mass spectra of neomycin–dipeptide conjugates were measured using Voyager™ MALDI-TOF mass spectrometer (*Applied Biosystems*). All RNA sequences were synthesized by Gene Assembler Special™ (*Pharmacia*). All RNA monomeric phosphoramidites and CPG supports were purchased from either *Glen Research* or *Chemgene*. For all the solid-phase syntheses, funnels with sintered glass filter or Bio-Spin® disposable chromatography columns (Bio-Rad) were used. The buffer condition used for reverse-phase HPLC was as follows: for peptides, buffer A is 0.1% TFA in H_2O and buffer B is 0.1% TFA in CH_3CN; for RNA sequences, buffer A is 0.1 M TEAA (triethylammonium acetate, pH = 7.0) in H_2O and buffer B is 0.1 M TEAA in 80% CH_3CN.

4.2. Synthesis of the acid derivative of neomycin (1)

Synthesis of Boc-protected neomycin was carried out using a previously reported method.[11] To Boc-protected neomycin, the starting material (2.0 g) in DMF (10 mL) were added bromoacetic acid (350 mg, 1.5 equiv) and cesium carbonate (3.2 g, 6 equiv) at room temperature. The resulting mixture was stirred at room temperature overnight. After the mixture was concentrated by removing the solvent under a reduced pressure, the residue was purified by flash column chromatography (CH_2Cl_2/MeOH) = (5/1 → 4/1) to yield 250 mg (12%) of **1**. R_f: 0.21 (CH_2Cl_2/MeOH = 5/1); ^1H NMR (500 MHz, MeOD-d_3): δ 5.35 (s, 1H), 5.14 (br, 1H), 4.96 (s, 1H), 4.25–3.00 (m, 24H), 1.57–1.20 (m, 56H); ^{13}C NMR (125 MHz, MeOD-d_3): δ 177.78, 172.99, 158.92, 158.40, 158.28, 157.84, 109.12, 100.03, 99.41, 88.33, 83.79, 83.14, 80.18, 76.03, 74.88, 73.19, 72.91, 72.40, 71.63, 71.06, 69.14, 63.39, 58.73, 57.17, 53.50, 53.30, 51.81, 49.62, 49.44, 49.27, 49.10, 48.93, 42.59, 41.68, 35.86, 29.04, 28.98, 28.89, 28.79, 28.83; HRMS (FAB): $C_{55}H_{96}N_6O_{27}Na$, 1295.6221 (calcd), 1295.6210 (obsd).

4.3. Library construction

To construct the library, TentaGel S NH_2 (130 μm, 0.27 mmol/g) was used as a solid support. For the peptide synthesis, Fmoc coupling protocol was used. The solid-phase synthesis was carried out with 16 amino acids (except Met, Lys, Ile, and Cys). In order to sequence compounds with MALDI-TOF and give a distance between the solid support and the conjugates, a linker consisting of six amino acids (C-terminal-Met-Arg-βAla_4) was incorporated into the conjugates. Split-and-pool method and partial termination by capping with Boc-Gly (10%) were used to build the dipeptide library randomly having 16 amino acids in each position. Resin beads were split to 16 portions moved into each different container after finishing synthesis of the linker. Beads in each container were treated with 20% of piperidine in DMF for 20 min to deprotect Fmoc group, and washed with 5 × 1 mL of DMF. To the each portion were added Fmoc-protected amino acid (3 equiv), PyBop (3 equiv), HOBT (3 equiv), and DIPEA (6 equiv). Additionally, in order to introduce a partial termination 10% of Boc-protected glycine compared to the amount of the added amino acid was added to the mixture, and the coupling reaction was performed for 4 h at room temperature. Completion of the coupling was checked by Kaiser test. After the coupling, beads were gathered in one container, mixed well with shaking, and split into 16 portions again. After deprotection of

Fmoc with treatment of 20% of piperidine in DMF, another coupling reaction was carried out using the same coupling protocol. After synthesis of dipeptides on beads, all beads were collective in one container followed by coupling of β-Ala and **1** in a sequential manner. After the synthesis, beads were washed with DMF and MeOH, and dried under vacuum. To deprotect side chains of peptides, beads were treated with 1 mL of TFA/thioanisole/water (95/2.5/2/5) solution for 2 h at room temperature. Finally, beads were washed with MeOH, and dried under vacuum again.

4.4. Synthesis of fluorescein-labeled RRE RNA (F-RRE) and biotin-labeled RRE RNA (B-RRE)

For the RRE sequence, a truncated region, 5′-GGUGGGCGCAGCUUCGGCUGACGGUACACC-3′ was chosen for binding assay. 3′-Fluorescene linked RRE RNA (F-RNA) and 5′-biotinylated RRE RNA (B-RRE) were synthesized using commercially available FAM-dT CPG and biotin-dT phosphoramidite (*Glen Research*), respectively. All RNA sequences were synthesized on commercially available CPG (Core Pored Glass, *Glen Research*) solid supports with 1.0 μmol scale using a standard phosphoramidite protocol. Cleavage and deprotection were performed by treating CPGs with 1 mL of NH_4OH/EtOH (3:1) at 55 °C for 16 h. For the reaction, only screwed capped tubes should be used since the pressure inside of the tube is increased by NH_3 gas. The mixture was filtered using a syringe filter (0.45 micrometer), and solvent was removed under a reduced pressure. After treatment with 1 mL of 1.0 M tetrabutylammonium fluoride (TBAF) in THF, the residue was stirred at room temperature overnight. After removal of solvent, the residue was desalted through Sep-Pak C18 cartridge (*Waters*), and then purified by a reverse-phase HPLC using a C18 column and 0.1 M TEAA (triethylammonium acetate, pH = 7.0) buffer as a stationary phase and a mobile phase, respectively. All purified oligomers were dissolved in Milli-Q™ water, and stored in a freezer (−20 °C). Purity of oligomers was controlled by 15% denaturing polyacrylamide gel electrophoresis (PAGE). Quantification was performed by measuring UV absorption at 260 nm (at 492 nm for F-RRE).

4.5. Screening and sequencing

The library was screened at room temperature in a buffer containing 5 mM of KCl, 1 mM of $MgCl_2$, 140 mM of NaCl, and 20 mM of HEPES (pH = 7.4). About 300 beads from the library was mixed with 1 mL of F-RRE (4 μM) in the buffer. The suspension was incubated with shaking for 3 h in a dark place. The beads were then washed with 1 mL of the same buffer three times, and dried under air. Beads that showed high fluorescence intensity compared to the others were selected out manually under fluorescence microscopy using a twister.

The selected beads were washed with brine and deionized water. Each bead was then placed in an Eppendorf tube, and treated with 50 μL of CNBr solution (20 mg of CNBr dissolved in 70% aqueous TFA). Tubes were incubated in a dark place for 15 h, and solvent was removed by blowing nitrogen. The residue was dissolved in 10 μL of MALDI-TOF matrix (10 mg of α-cyano-4-hydroxycinnamic acid dissolved in 1 mL of 0.1% of TFA in 50% aqueous CH_3CN). Each sample (1 μL) was dropped on a MALDI-TOF plate, and then dried under air. After drying spots on the plate completely, MALDI-TOF MS spectra were measured.

4.6. Synthesis and identification of the selected neomycin–dipeptide heteroconjugates

Identified compounds were synthesized on Rink Amide™ resin (*NovaBiochem*) using a protocol for the solid-phase peptide synthesis. The synthesis was performed with 6 μmol scale using a standard protocol for Fmoc-protected amino acids. When synthesis of the peptide part was done, to the resin were added **1** (5 equiv), PyBop (3 equiv), HOBT (3 equiv), and DIPEA (6 equiv). The suspension was stirred at room temperature overnight. The resin was washed with DMF and MeOH, then, was treated with 1 mL of a deprotection solution (TFA/thioanisole = 60:1) for 2 h. The residue was filtered, and solvent of the filtrate were removed under a reduced pressure. The residue was dissolved in 0.5 mL of deionized water, and purified by means of HPLC using C18 column as the stationary phase. For the mobile phase, buffer A and buffer B were used. Usually the synthesized compounds were eluted at 10% of buffer B. The purity of separated compounds was monitored by another HPLC system using a C8 column. All compounds purified were characterized by MALDI-TOF mass spectrometer. Quantification of synthesized neomycin–dipeptide heteroconjugates were carried out as previously reported.[12]

4.7. Affinity measurement using SPR

A Biacore® 3000 instrument was used to determine dissociation constants of inhibitors assuming 1:1 binding stoichiometry between RRE and inhibitors. For immobilization of B-RRE, phosphate buffered saline (PBS) was used. The binding experiment was performed at 25 °C in a running buffer containing 30 mM of HEPES (pH = 7.5), 100 mM of KCl, 20 mM of NaCl, 20 mM of guanidinium HCl, 2 mM of $MgCl_2$, 20 mM of NH_4OAc, 10 mM of sodium phosphate, 0.5 mM of EDTA, and 0.001% of Tween-20. Streptavidin (20 μL of 50 mg/L in 10 mM (pH = 5.5) NaOAc) was precoated on two flow cells (one for reference and the other for analytes) in a CM5™ sensor chip (*Biacore Inc.*) at a flow rate of 5 μL/min using standard NHS/EDC chemistry. After blocking the remaining active sites with ethanolamine, B-RRE was immobilized on a flow cell by flowing 20 μL of 6 μM B-RRE in PBS buffer at a flow rate of 5 μL/min. The duration of injection was varied depending on the surface RRE density (about 500 RUs) that was monitored by Response Unit (RU) value.

Neomycin and neomycin–dipeptide conjugates in the running buffer were injected over the RNA surface through multiple rounds at a flow rate of 30 μL/min. Bound compounds were completely removed after each

round of injection by flowing 20–60 μL of regeneration buffer (1 M NaCl) at a flow rate of 30 μL/min over the sensor surface. In order to measure specificity neomycin–dipeptide conjugates were injected together with tRNA mixture (from *E. coli*). A steady-state 1:1 binding formula or 1:1 binding formula based on simultaneous association/dissociation were used to calculate K_Ds using BIAevaluation 2.1 software (*Biacore Inc.*).

4.8. Affinity measurement using fluorescence anisotropy

Fluorescence anisotropy experiments were conducted at 20 °C in a buffer containing 5 mM of KCl, 1 mM of $MgCl_2$, 140 mM of NaCl, and 20 mM of HEPES (pH = 7.4). K_D of the RevFl-(B-RRE-antibiotin Ab) interaction (5.59 ± 0.25 nM) was calculated by titration of the B-RRE-antibiotin Ab into a solution of 10 nM RevFl as previously reported.[13] For replacement experiments to calculate IC_{50} values of inhibitors, a RevFl-(B-RRE-antibiotin Ab) complex was formed by mixing 10 nM of RevFl with 10 nM of B-RRE-antibiotin Ab. Inhibitors were then titrated and a change of fluorescence anisotropy was monitored (Fluoromax-3, Horiba, slit widths 10 nm for both excitation and emission, excitation at 492 nm and emission at 530 nm, 5 s of integration time, 0.5% of error range, and 3–5 readings averaged per concentration). The IC_{50} values were calculated according to the following equation;[13]

$$A = \Delta A \exp((-0.69/\mathrm{IC}_{50}) * C) + A_F,$$

where A is the anisotropy value, ΔA is the difference between the initial anisotropy value and the final anisotropy value (A_F), C is the total concentration of inhibitor, and IC_{50} is the concentration of inhibitor where $A = 0.5\Delta A + A_F$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.11.021.

References and notes

1. Schroeder, R.; Waldsich, C.; Wank, H. *EMBO J.* **2000**, *19*, 1–9.
2. Leulliot, N.; Varani, G. *Biochemistry* **2001**, *40*, 7947–7956.
3. Kotra, L. P.; Haddad, J.; Mobashery, S. *Antimicrob. Agents Chemother.* **2000**, *44*, 3249–3256.
4. Wong, C.-H.; Hendrix, M.; Manning, D. D.; Rosenbohm, C.; Greenberg, W. A. *J. Am. Chem. Soc.* **1998**, *120*, 8319–8327.
5. Kirk, S. R.; Luedtke, N. W.; Tor, Y. *J. Am. Chem. Soc.* **2000**, *122*, 980–981.
6. Lee, J.; Kwon, M.; Lee, K. H.; Jeong, S.; Hyun, S.; Shin, K. J.; Yu, J. *J. Am. Chem. Soc.* **2004**, *126*, 1956–1957.
7. (a) Eubank, T. D.; Biswas, R.; Jowanovic, M.; Litovchick, A.; Lapidot, A.; Gopaian, V. *FEBS Lett.* **2002**, *511*, 107–112; (b) Carriere, M.; Vijayabaskar, V.; Applefield, D.; Harvey, I.; Garneau, P.; Lorsch, J.; Lapidot, A.; Pelletier, J. *RNA* **2002**, *8*, 1267–1279; (c) Litovchick, A.; Evdokimov, A. G.; Lapidot, A. *FEBS Lett.* **1999**, *445*, 73–79.
8. (a) Hilaire, P. M. St.; Lowary, T. L.; Meldal, M.; Block, K. *J. Am. Chem. Soc.* **1998**, *120*, 13312–13320; (b) Xian, M.; Fatima, Z.; Zhang, W.; Fang, J.; Li, H.; Pei, D.; Loo, J.; Stevenson, T.; Wang, P. G. *J. Comb. Chem.* **2004**, *6*, 126–134.
9. Luedtke, N. W.; Qi, L.; Tor, Y. *Biochemistry* **2003**, *42*, 11391–11403.
10. (a) Hwang, S.; Tamilarasu, N.; Huq, K. I.; Richter, S.; Still, W. C.; Rana, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12997–13002; (b) Hwang, S.; Tamilarasu, N.; Kibler, K.; Cao, H.; Ali, A.; Ping, Y.-H.; Jeang, K.-T.; Rana, T. R. *J. Biol. Chem.* **2003**, *278*, 39092–39103.
11. Michael, K.; Wang, H.; Tor, Y. *Bioorg. Med. Chem.* **1999**, *7*, 1361–1371.
12. Weigele, M.; DeBernardo, S. L.; Tengi, J. P.; Leimgruber, W. *J. Am. Chem. Soc.* **1972**, *94*, 5927–5928.
13. Luedtke, N. W.; Tor, Y. *Angew. Chem., Int. Ed.* **2000**, *39*, 1788–1790.