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A strategy for the design of selective RNA binding agents. Preparation and RRE RNA binding affinities of a neomycin-peptide nucleic acid heteroconjugate library

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Abstract—We have successfully developed a new strategy for RNA ligand design, which applies the antisense concept to enhance and make more specific loop region interactions while at the same time preserving stem region anchoring. The heteroconjugates, prepared in this effort, have proven to be the most specific small molecule ligands against RRE RNA that have been uncovered to date.

The strategy approach for lead discovery in the pharmaceutical industry focuses on substances that target key proteins. An alternative approach to address proteins that have failed to yield leads is to target RNAs.[1] In addition, novel structured RNAs serve as alternative targets for drug development.[2] Aminoglycosides are well-known natural products that have evolved as inhibitors and modulators of RNA function.[3] Binding of these substances to RNA is a consequence of mainly electrostatic interactions.[4] Owing to this, most aminoglycosides unselectively bind to a variety of RNA targets and, as a result, they often display severe toxicity.[5,6] Thus, efforts to uncover aminoglycoside derivatives that have increased RNA binding specificities are crucial to the development of new types of RNA binding drugs.

An approach that has been used to construct more diverse pharmacophores involves the addition of simple hydrophilic[7] or hydrophobic moieties[8] to a lead pharmacophore in order to promote additional interactions with RNA. Following this approach, we recently designed and synthesized neomycin–chloramphenicol, neomycin–linezolid,[9] and neomycin-dipeptide[10] heteroconjugates in order to introduce additional binding to loop regions of RNA. Chloramphenicol (or linezolid) and dipeptide moieties of the heteroconjugates directly interact with specific bases in the loop regions of RNA.

The greatly improved binding affinities and specificities observed for these heteroconjugates suggest that the incorporation of a combination of stem- and loop-binding moieties will lead to substances that interact with RNA targets in a highly specific manner. Peptide nucleic acid (PNA) appendages should be ideal in this regard. Owing to their high stabilities in biological fluids, PNAs have been widely used as gene targeting[11] agents and diagnostic tools.[12] Although the synthesis of an aminoglycoside-PNA conjugate and its intended use to form a DNA triplex[13] has been briefly described, no report exists describing the construction of aminoglycoside-PNA libraries and their screening against RNA targets. Below, we present the results of an effort in which a library of neomycin-PNA (neo-PNA, **4**) has been prepared (Scheme 1) and evaluated for selection against Rev Response Element (RRE) RNA. The results show that selected substances in this family have nanomolar binding affinities toward RRE RNA and that they display higher binding selectivities than any known RNA specific ligands (e.g., neomycin B or neo-acridine).

Since they are designed to bind to RNA loop region bases, the PNA groups incorporated into the heteroconjugates we have prepared have lengths that are shorter than 2 nt. Owing to its ubiquitous binding properties, neomycin B was selected as the RNA stem-binding moiety of the new conjugates. Four monomeric PNAs and

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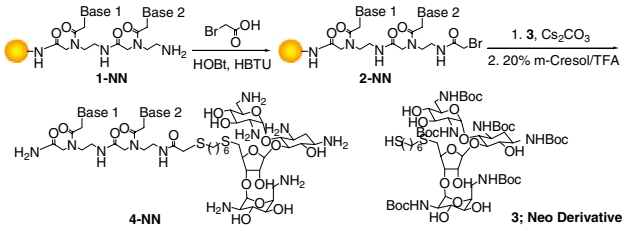

Scheme 1. Synthetic routes for preparation of neo-PNA (**4-N** and **4-NN**, **N** = A, T, G, or C) heteroconjugates and structures of related compounds.

16 dimeric PNAs were linked to neomycin B to generate the 20 heteroconjugates, **4-N** and **4-NN** (**N** = A, T, G, or C) shown in Scheme 1.[14] A six-carbon spacer link was employed to tether the two pharmacophores.[9] Preparation of each neo-PNA conjugate (**4**) required a total three steps starting with solid-immobilized PNA (**1**)[15] and **3**, a derivative of neomycin B.[16] The conjugates were purified by using HPLC (>95%) and characterized by using MALDI TOF mass spectrometry.[16]

RRE RNA was chosen as the target for the conjugates because it has a typical stem-loop structure and a naturally occurring specific ligand, the Rev Peptide. Binding affinities of the conjugates to RRE RNA (Table 1) were determined by using a fluorescence anisotropic method.[9] Six conjugates were found to have dissociation constants of <100 nM, which are as much as five times lower than that of neomycin B. The other conjugates have either equal or poorer affinities compared to neomycin B. Thus, it appears that the PNA-moieties in the conjugates add to interactions taking place between the ligands and RNA. In addition, the magnitude of the interactions is dependent on the PNA sequences, that is, the binding affinity of **4-GC** is not same as that of **4-CG**.

The target specificities of the six most strongly binding heteroconjugates listed in Table 2 were determined by comparing RRE RNA to tRNA, the most abundant natural RNA. Specificities of the conjugates are represented by discrimination factors. The data show that most of the selected heteroconjugates have higher discrimination factors than that of neomycin B, suggesting that both binding affinities and specificities are improved by incorporating PNA tethers.

Table 1. Binding affinities of Neo-PNA (**4**) to RRE RNA[a]

Base 1 Base 2	**A**	**T**	**G**	**C**
None	0.31	0.27	0.13	0.32
A	0.058	0.25	0.038	0.051
T	0.071	0.13	0.24	0.36
G	0.20	0.093	0.030	0.24
C	0.13	0.11	0.17	0.43

[a] Values are in μM. Binding affinities were measured at 20 °C by using a luminometer (Aminco-Bowman) and an anisotropy technique. Error boundaries are shown in supporting information.

Table 2. Comparison of binding affinities (K_d) of the selected neo-PNA conjugates to RRE RNA and tRNA[a]

Compound	RRE	tRNA	Discrimination factor[a]
4-AA	0.058	0.41	7.1
4-AT	0.071	0.23	3.2
4-CA	0.051	0.076	1.5
4-GA	0.038	0.17	4.5
4-GG	0.030	0.21	7.0
4-TG	0.093	0.22	2.4
Neomycin B	0.18	0.37	2.1

[a] Values are in μM. Same conditions were applied for Table 1. Discrimination factor equals K_d against tRNA/K_d against RRE.

The heteroconjugates **4-AA** and **4-GG** have the highest tRNA versus RRE RNA discrimination factors (7.1 and 7.0, respectively). As a result, the binding specificities of these substances, represented again as ratios, were evaluated by comparing their IC_{50} values in the absence and presence of genomic RNA library.[17] The IC_{50} values were determined by using back-titration of ^{32}P-labeled RRE RNA with Rev Peptide followed by employing a gel mobility shift assay.[18] As shown in Figure 1, **4-AA** and **4-GG** have nearly the same IC_{50} values (specificity ratios of 1.2 and 1.4 for **4-AA** and **4-GG**, respectively) in the absence and the presence of competitor RNA. In contrast, the specificity ratio of neo-acridine[8] is much higher even though it has a higher binding affinity than **4-AA** or **4-GG**. To our knowledge, **4-AA** and **4-GG** have the highest RRE RNA binding specificities among all of the small molecule ligands that have been prepared thus far. A comparison of the binding affinities and specificities of the heteroconjugates we have prepared demonstrates that the most selective binders do not necessarily possess the strongest binding affinities to the target RNA. This suggests that the general strategy of affinity-based selection might be altered to include specificity-based selection[19] at least when probing RNA targets.

Foot-printing of RRE in the presence of **4-AA** and **4-GG** was performed to demonstrate that these substances

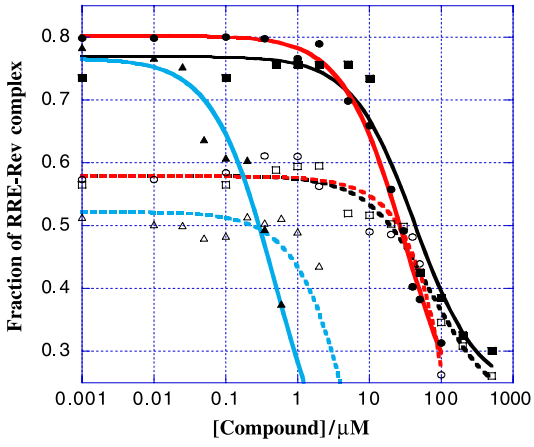

Figure 1. Inhibition of Rev-binding to RRE RNA by **4**-AA (□, ■), **4**-GG (○, ●) and neo-acridine (△, ▲) in the presence and absence of 100 nM of genomic RNA library. IC_{50} values are 68, 55 μM by **4**-AA, 45, 32 μM by **4**-GG, and 9.8, 0.5 μM by neo-acridine in the presence and absence of competitor RNA.

display conjugate binding to RRE RNA.[16] The results (supporting information) show that RNAse cleavage of RRE either increases or decreases in the presence of the selected heteroconjugates as compared to when neomycin B is present. This finding indicates that regions where the conjugates bind to RRE RNA extend beyond those where neomycin B binds. The large specificities seen with heteroconjugates **4** are likely due to interactions of their PNA moieties with a loop region to form base-pairs[20] or a stem region to form a triple stranded complex with RRE RNA.[21] This proposal was confirmed by the results of studies in which mutations of selected loop region of RRE RNA were carried out by using in vitro transcription in a manner to prevent alteration of the secondary structure.[9] The results of binding affinity measurements[16] show that binding of **4-AA** and **4-GG** to the mutants is significantly lower than to wild-type RRE RNA (supporting information). In contrast, binding of neomycin to the mutants is the same as to wild-type RNA. These observations indicate that some parts of heteroconjugates directly interact with bases in the loop region. However, a detailed structural study is needed in order to assign the exact binding orientation of heteroconjugates to the hairpin RNA.

In the investigation described above, we have successfully developed a new strategy for RNA ligand design, which applies the antisense concept to enhance and make more specific interactions while at the same time preserving anchoring. The heteroconjugates, prepared in this effort, have proven to be the most specific small molecule ligands against RRE RNA that have been uncovered to date. They have up to 5-fold increased binding affinities compared to that of the parent aminoglycoside. In addition, these substances display more specific binding than any natural or synthetic aminoglycosides, attributable to interactions between bases in the PNAs with those in RNA loop regions. Finally, the heteroconjugate strategy, relying on the use of short PNAs as loop binding moieties, may be generally applicable to targeting any stem-loop RNA molecule.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.06.094.

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