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An Approach To Enhance Specificity against RNA Targets Using Heteroconjugates of Aminoglycosides and Chloramphenicol (or Linezolid)

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The importance of small molecules that specifically bind to RNA has increased because newly structured RNA motifs have been implicated in disease states.[1] Aminoglycosides are well-known natural products that have evolved as inhibitors and modulators of RNA functions.[2] These substances take advantage of electrostatic and hydrogen-bonding interactions to promote induced fit and conformational capture of many RNA targets.[3] However, most aminoglycosides bind to a variety of RNA targets with moderate affinity due to the nonspecific electrostatic interactions. This lack of selectivity often results in severe toxicity.[4] Consequently, efforts to improve specificities of aminoglycosides are crucial components in the development of new types of RNA binding drugs.

One effort to mimic the pharmacophore of naturally occurring aminoglycoside antibiotics uses a combinatorial approach to construct derivatives with one-ring or two-ring amino sugars that mimic the pseudotetrasaccharide skeleton of neomycin.[5] In another approach, the pharmacophore is dimerically attached by using the same or different aminoglycosides.[6] A third strategy involves the addition of amino acids[7] or a simple hydrophilic[8] or hydrophobic moieties[9] to the pharmacophore to make heterodimeric conjugates, which have sites for additional interactions between the drug and RNA. For example, conjugates containing acridine were found to have an extra interaction with a specific bulged base, thus expanding the binding region to include a non-Watson−Crick (WC) duplex RNA.[9]

Importantly, the results of these efforts suggest that the introduction of new types of interactions between the conjugates and RNA could lead to new sequence-specific RNA binding agents. Guided by the result by Tor and co-workers,[9] we hypothesize that heteroaminoglycoside conjugates, which possess both anchoring groups (stem-loving) and sequence-recognizing groups (loop-loving), may have expanded regions of interaction with RNA. As a result, they should display more specific and stronger RNA stem-loop binding affinities.

In this Communication, we describe the design and synthesis of new heteroconjugates, which are comprised of a neomycin B (Neo) stem-binding component and a chloramphenicol (Cam) or linezolid (Lnz) loop-binding component. Neo was chosen as the RNA stem-binding component because it has a well-defined binding region to both RNA targets[10] and Neo aptamers.[11] Cam was selected as the loop-binding moiety because mutation[12] and X-ray crystallographic[13] studies have shown that this substance specifically binds to the central multibranched loop of 23S rRNA. Aptamers against this drug possess consensus A-rich loops.[14] Even though its exact binding mode is still unknown, we chose Lnz as another loop-binding molecule because it promotes the same translational in accuracy[15] and has the same binding site as does Cam.[16] The studies

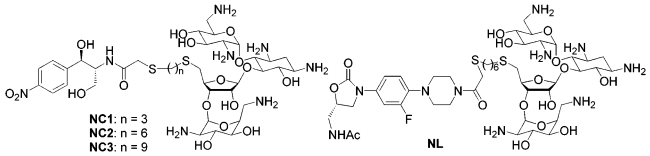

Figure 1. Structures of heterodimeric conjugates.

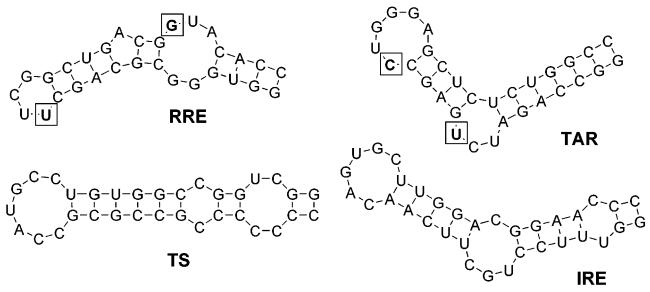

Figure 2. Secondary structures of truncated RRE, TAR, TS, and IRE.

described below have shown that some of the heteroconjugates, designed by using this strategy, display enhanced affinities to RNA and that binding occurs in both stem and loop regions of the RNA targets. In addition, the results of foot-printing and mutation studies suggest that the enhanced binding affinity of the conjugates is RNA sequence-specific.

In designing the conjugates, QSAR analyses of Neo,[17,18] Cam,[19] and Lnz[20] have facilitated the selection of sites for placement of tethers that have minimal effects on binding (Figure 1). Neo and Cam (or Lnz) were conjugated with spacers of different lengths to form Neo-Cam-1, -2, -3 (**NC1**, **NC2**, **NC3**) and Neo-Lnz (**NL**). Preparation of each of these conjugates requires a total of six steps (yields >5%) from commercially available neomycin sulfate.[21] Rev Response Element (RRE), trans-activating region (TAR) of HIV-1, thymidine synthase mRNA (TS), and Iron Responsive Element (IRE) were chosen as RNA targets to test binding of these conjugates (Figure 2). All of the targets possess well-established stem-loop structures, and they are responsible for moderate affinity Neo binding. The RNA targets were synthesized and purified as was previously described.[22]

Fluorescence anisotropy was used to measure the binding affinities of the conjugates to various targets. As seen by viewing the results tabulated in Table 1, binding affinities are dependent upon the structures of the conjugates, and, in some cases, they reach values that are at least 10 times greater than that of Neo. Thus, it appears that the non-Neo components of the conjugates contribute cooperatively to binding. In addition, the binding affinities to most of the RNA targets are dramatically dependent on the spacer length of the tether connecting the Neo and Cam components. For example, **NC1** has the highest binding affinity to TS and IRE, while **NC2**

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Table 1. Binding Affinities (K_d) of Conjugates to Various RNA Targets[a]

RNA	Neo	NC1	NC2	NC3	NL
RRE	0.18	0.063	0.022	0.031	0.54
TAR	0.18	0.40	0.041	0.19	0.047
TS	0.33	0.049	0.22	2.1	0.33
IRE	0.31	0.079	0.50	>4.0	0.12

[a] The values are in micromolar. The binding affinities are measured at 20 °C by using an LS 50B luminometer (Perkin-Elmer). Cam and Lnz binding affinities showed >10 μM to any RNA target. See Supporting Information for error boundaries.

Table 2. Binding Affinities (K_d) of Conjugates to Mutant RNA Targets[a]

mutation in RRE	Neo	NC2	NC3
wild type	0.18 (1.0)	0.022 (1.0)	0.031 (1.0)
U13A	0.18 (1.0)	0.22 (10)	0.14 (4.5)
G24A	0.22 (0.80)	0.023 (1.1)	0.087 (2.8)
mutation in TAR	**Neo**	**NC2**	**NL**
wild type	0.16 (1.0)	0.041 (1.0)	0.047 (1.0)
U10C	0.16 (1.0)	1.1 (22)	1.1 (25)
C15A	0.27 (0.70)	0.033 (0.70)	0.40 (8.0)

[a] Conditions are the same as those in Table 1. Mutation sites are shown in Figure 2. Values in parentheses are ratios of binding constants as compared to that of wild-type RNA (K_{d-m}/K_{d-wt}). See Supporting Information for error boundaries.

binds most tightly to TAR. These results suggest that the Cam moiety is responsible for specific interactions with these targets. Finally, **NC2** and **NL** show dramatic differences in their binding affinities to many RNA targets even though these conjugates have the same spacer lengths. Thus, it appears that the loop-specific moiety is the key determinant for enhanced binding.

RRE and TAR were chosen as RNA targets for more detailed studies because of their well-described binding mode to Neo and their high binding affinities to the conjugates. Foot-printing experiments were carried out to elucidate the nature of the conjugate binding to these targets.[21] The results show that RNAse cleavage of RRE and TAR in the presence of the conjugates is either enhanced or attenuated as compared to when Neo is present (data in Supporting Information). The results suggest that the stronger binders induce larger conformational changes when they bind to RNA targets. Also, the regions where the strong binders bind to the RNA targets extend beyond those of Neo to include internal or terminal loop sites. For example, while the binding regions of Neo are known to be down-stem in RRE and TAR, those of **NC2** and **NL** extend to top-loop regions. Therefore, the results of the foot-printing experiments show that the anticipated stem-loop binding by the conjugates is possible even when the stem and loop regions are not adjacent.

Mutations of the selected loop region bases in RRE and TAR by using in vitro transcription were executed in a manner to prevent alteration of the secondary structures of the wild-type RNAs. Binding affinities of the two strongest binding conjugates to each mutant were measured by using fluorescence anisotropy (Table 2). The data show that binding affinities by the conjugates to most of the mutants are significantly less than to the wild-type RNAs, whereas Neo binding affinities to the mutant and wild-type RNAs are similar. Thus, each conjugate interacts with specific base(s) in loop regions. This differs from Neo, which is known to bind mainly to stem regions. Also, the reduced binding affinity by **NC2** and **NL** to some mutants is not dependent on tether length. The observation described above suggests that binding of Cam (Lnz) occurs at specific base(s) sites in loops of its RNA targets.

In summary, Neo−Cam and Neo−Lnz conjugates prepared in this study display enhanced, site-selective binding to several RNA targets. One of the Neo−Cam conjugates, **NC2**, has a nanomolar binding constant to the RNA target RRE, a value which is 10 times higher than that of Neo, and binding by the related conjugates, **NC2** and **NL**, to TAR is 10-fold greater than that of Neo. The results of foot-printing and mutation studies demonstrate that their binding extends into loop regions where specific interactions take place between base(s) in the RNA loop and the non-Neo part of the heteroconjugates. Perhaps the most significant observation made in this investigation is that nonspecific RNA binders, such as Neo and Cam (Lnz), can be transformed into specific binding agents by their incorporation into conjugates. This strategy should be generally applicable to the development of substances that specifically bind to RNAs.

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Supporting Information Available: Procedures for the preparation of the conjugates and the foot-printing study (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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