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Enhanced Binding of Aminoglycoside Dimers to a "Dimerized" A-Site 16S rRNA Construct

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Abstract—In this work, we investigated the binding of a series of dimeric aminoglycoside molecules to (i) a 27 nt A-site 16S rRNA construct, and (ii) an artificially grafted 46 nt 'dimerized' A-site 16S rRNA construct. It was observed that the dissociation constants of dimeric aminoglycosides to the dimerized A-site 16S rRNA construct can achieve up to approximately 19-fold enhancement compared to the monomeric aminoglycoside molecules.

Significant attention has been devoted to the understanding of the mechanistic actions of aminoglycoside antibiotics since the discovery of their usage in the treatment of Gram negative infections.[1,2] These aminoglycoside antibiotics are thought to function primarily by binding to the decoding region of bacterial 16S rRNA, thus causing premature termination and mistranslation of proteins and consequently, bacterial death. Besides 16S rRNA, aminoglycoside antibiotics have also been reported to bind to a variety of naturally occurring RNA species, which include ribosomal RNA,[3,4] group I introns,[5,6], the hammerhead ribozyme,[7] the RRE transcriptional activator region from HIV,[8,9] and the site 1 mRNA of thymidylate synthase.[10] However, the binding of aminoglycoside antibiotics to these constructs has been observed to be primarily in the micromolar (μM) range, with neomycin being the only exception in the high nanomolar (nM) range.[11] Since RNAs are able to achieve intricate tertiary structures which confer many interesting and important functions, it is of prime importance to understand the rules that govern the recognition of RNAs by the amino-glycoside antibiotics.[12–14] Hence it would be extremely useful to design tighter binding ligands towards RNA molecules.

Wang and Tor have recently reported that electrostatic interactions between aminoglycoside antibiotics and their RNAs are critical in the recognition event,[15] and they have also proceeded to show that dimeric aminoglycoside antibiotics are able to show cooperative interaction to the hammerhead ribozyme, thus enhancing their inhibitory capability.[16] In this work, we report on utilizing such dimeric aminoglycosides,[17,21] namely dimeric neomycin-neomycin (**1**), dimeric neomycin-tobramycin (**2**), and dimeric tobramycin-tobramycin (**3**) molecules, to investigate their binding properties against the wild type (*wt*) 27 nt A-site 16S rRNA construct (construct **A**) and (II) a 46 nt 'dimerized' A-site 16S rRNA construct (construct **B**, see Scheme 1). The "dimerized" A-site 16S rRNA construct **B** was created by artificially grafting two A-site 16S rRNA constructs.[18] It was observed that the dissociation constant (K_d) of the dimeric aminoglycosides towards the "dimerized" construct **B** could achieve an approximately 19-fold of enhanced binding when compared to the monomeric aminoglycoside molecule.

To quantify the K_d between the monomeric and dimeric aminoglycosides to both RNA constructs, we employed a recently developed fluorescence method that enables direct and quantitative binding measurements between aminoglycoside-RNA interactions.[11,19] The basis of this methodology involves the use of fluorescent aminoglycoside conjugates to bind the RNA molecules in an incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.40). First, the fluorescently conjugated paromomycin molecule (CRP, Scheme 2) at 10 nM was titrated with an increasing concentration of *wt* construct **A**. It was observed that the fluorescence curve gradually increased and saturated at around 450 nM. Using a previously described curve fitting equation,[27,28] the K_d of CRP was found to be 0.31 ± 0.03 μM. Subsequently when an

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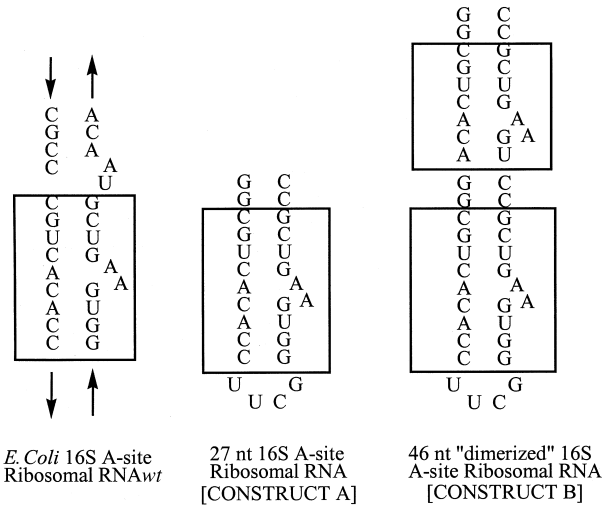
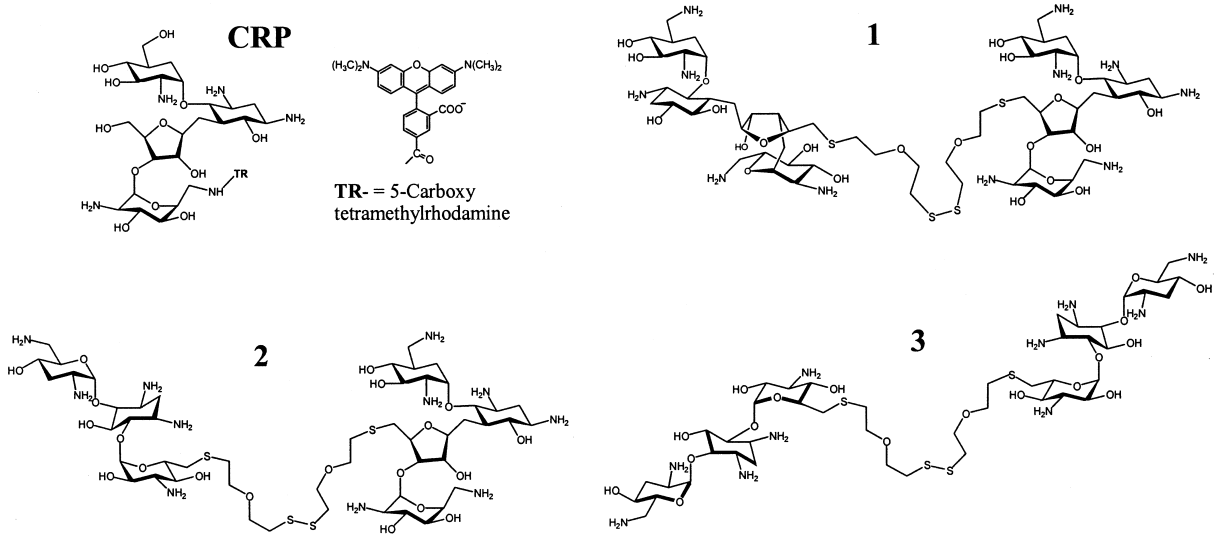


Scheme 1. Secondary structure of the (a) *wt* A-site 16S rRNA; (b) a minimized 27 nt A-site 16S rRNA construct; (c) a 46 nt "dimerized" A-site 16S rRNA construct.

increasing concentration of the neomycin molecule was added to the CRP-construct **A** complex, the fluorescence of the complex began to gradually quench in a saturable fashion. Again using a previously described curve fitting equation,[11,19] the K_d of the neomycin molecule was calculated to be 0.44 ± 0.03 μM. When the same experiment was repeated with the tobramycin molecule, it was calculated that tobramycin has a K_d of 2.11 ± 0.12 μM towards construct **A**. When the dimeric aminoglycosides **1** to **3** were studied with respect to their abilities to compete with CRP for binding to the *wt* construct **A**, it was observed that dimer **1** in neomycin-neomycin has only a moderate increase in binding with a K_d of 0.32 ± 0.02 μM. Similarly, dimer **2** in neomycin–tobramycin was observed to have a K_d of 0.41 ± 0.04 μM, and the dimer **3** in tobramycin–tobramycin has a K_d of 1.78 ± 0.11 μM (data summarized in Table 1).

The same fluorescence binding experiment was repeated with construct **B**. Again, CRP (10 nM) was titrated with an increasing concentration of construct **B**, the K_d of which was found to be 0.29 ± 0.02 μM. With increasing concentration of neomycin and tobramycin being added to the CRP-construct **B** complex, the fluorescence of the complex was again observed to gradually quench in a saturable fashion. As summarized in Table 1, the K_d of neomycin and tobramycin to construct **B** was calculated to be 0.38 ± 0.03 μM and 1.68 ± 0.14 μM respectively. When the dimer molecules **1**, **2** and **3** were studied with respect to their abilities to compete with CRP for binding to construct **B**, it was observed that dimer **1** had a very much enhanced increase in binding with a K_d of 0.02 ± 0.00 μM, which corresponds to approximately 19-fold enhancement when compared to neomcyin. Dimer **2**, however, showed much less enhancement with a K_d of only 0.29 ± 0.02 μM, while dimer **3** showed a K_d of only 1.81 ± 0.16 μM.

In summary, the use of appropriate dimeric aminoglycoside antibiotics to bind artificially grafted dimeric A-site 16S rRNA constructs shows an approximately 19-fold increase in K_d as compared to their monomeric counterparts. This study shows that covalently linking two aminoglycoside antibiotics as an attempt to enhance the binding to RNA is feasible, and is in agreement with



Scheme 2. The structures of CRP, Neomycin–Neomycin (**1**), Neomycin–Tobramycin (**2**), and Tobramycin–Tobramycin (**3**), aminoglycoside dimers.

Table 1. Summary of K_d (μM) of various tested aminoglycoside molecules with RNA construct **A** and **B**

Molecules	CRP	Neomycin [Neo]	Tobramycin [Tob]	Dimer **1** [Neo–Neo]	Dimer **2** [Neo–Tob]	Dimer **3** [Tob–Tob]
Construct **A**	0.31 ± 0.03	0.44 ± 0.03	2.11 ± 0.12	0.32 ± 0.02	0.41 ± 0.04	1.78 ± 0.11
Construct **B**	0.29 ± 0.02	0.38 ± 0.03	1.68 ± 0.14	0.02 ± 0.00	0.29 ± 0.02	1.81 ± 0.16

the recent work of Tor and co-workers, in which they observed that certain dimeric aminoglycosides were able to (1) achieve 20-fold enhancement in inhibiting Pb^{2+}-mediated cleavage,[20] and (2) inhibit ribozyme function to 1.2×10^3 fold more effectively, than their natural parent compounds.[21] These studies should facilitate studies exploring further permutations of conjugations to existing RNA binders to improve the binding efficiency.

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