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Binding of Dimeric Aminoglycosides to the HIV-1 Rev Responsive Element (RRE) RNA Construct

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Abstract—Through a series of elegant fluorescence measurements, particularly through stopped-flow kinetic measurements, it was recently demonstrated that aminoglycoside antibiotics are able to bind to the HIV-1 Rev responsive element (RRE) RNA construct in more than a 1:1 stoichiometry (Lacourciere, K. A.; Stivers, J. T.; Marino, J. P. *Biochemistry* **2000**, *39*, 5630). Here, we present the binding study results of dimeric neomycin ligands through fluorescence anisotropy studies, to the HIV-1 RRE RNA construct. The dimeric neomycin molecules are observed to be able to bind the HIV-1 RRE RNA construct approximately 17-fold higher when compared to the monomeric neomycin, lending evidence that there are indeed two or more neomycin binding sites within the HIV-1 RRE construct.

Understanding the mechanistic actions of aminoglycoside antibiotics has recently been of significant interest. Aminoglycosides 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.[1,2] Since RNAs are able to achieve intricate tertiary structures, many interesting and important functions are conferred. Thus, it is of prime importance to understand the rules that govern the recognition of RNAs by the aminoglycoside antibiotics. Besides 16S rRNA,[3,4] aminoglycoside antibiotics have also been reported to bind to a variety of other naturally occurring RNA species, which include the group I introns,[5,6] the hammerhead ribozyme,[7] the site 1 mRNA of thymidylate synthase,[8] and the Rev responsive element (RRE) of HIV-1.[9,10]

The HIV-1 RRE region of RNA is an essential region for the successful transcription of the HIV-1 genome.[11–14] It is also on the RRE region that the arginine-rich Rev peptide binds, whereupon the complex is then subsequently transported either through the nuclear membrane or the nuclear pore complex into the cytoplasm. At this point, the RNA is eventually translated into the viral protein.[13,15] It has been shown that the Rev–RRE interaction is modular, as these constructs have been successfully demonstrated to undergo dissection into smaller fragments.[15,16] These fragments have since been meticulously studied, and the relevant information for understanding the Rev peptide–RRE RNA construct interaction can be minimized into a very manageable 16 amino acid peptide ($Rev_{34–50}$) and a 37 nucleotide (nt) RNA fragment, respectively (Fig. 1).[17–20] With the ever increasing concern of the treatment of AIDS disease to the human population, the Rev–RRE interactions have attracted immense attention as understanding and developing inhibitors to circumvent this interaction could potentially lead to the development of drugs that are effective in treating the disease.[9]

Aminoglycoside antibiotics have been examined intensively as potential small molecules that may prevent the Rev–RRE interaction, and have been shown to do so successfully in the micromolar (μM) range.[21–24] The only exception to this class of aminoglycoside binding is neomycin B, in which the binding affinity is in the nanomolar (nM) range. However, understanding the exact mechanism of how the aminoglycosides bind to the RRE construct and prevent the binding of the Rev peptide is still under intensive investigation. It was recently suggested that the aminoglycoside–RNA interaction has been a rather nonspecific event, and the

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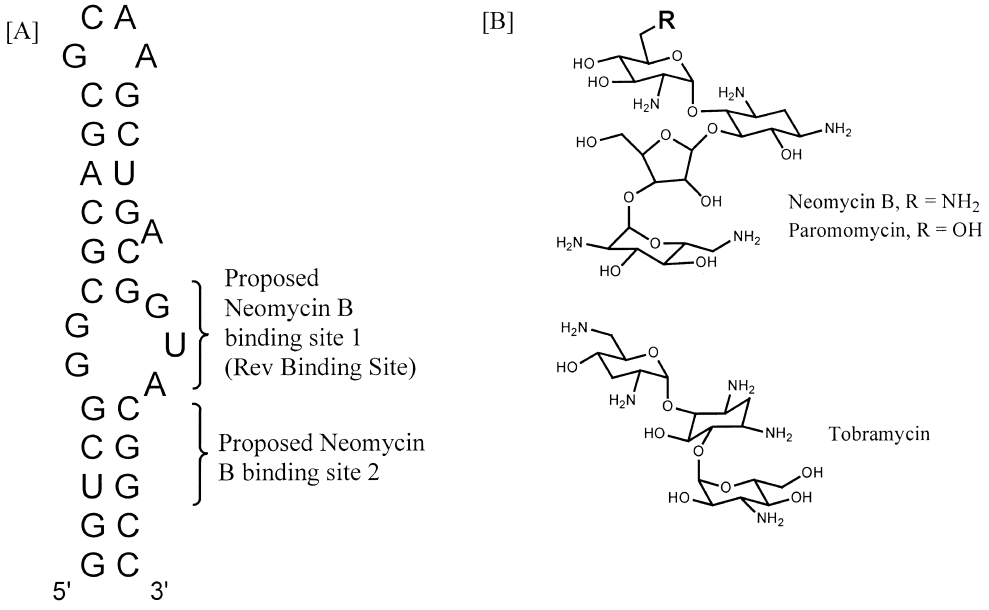
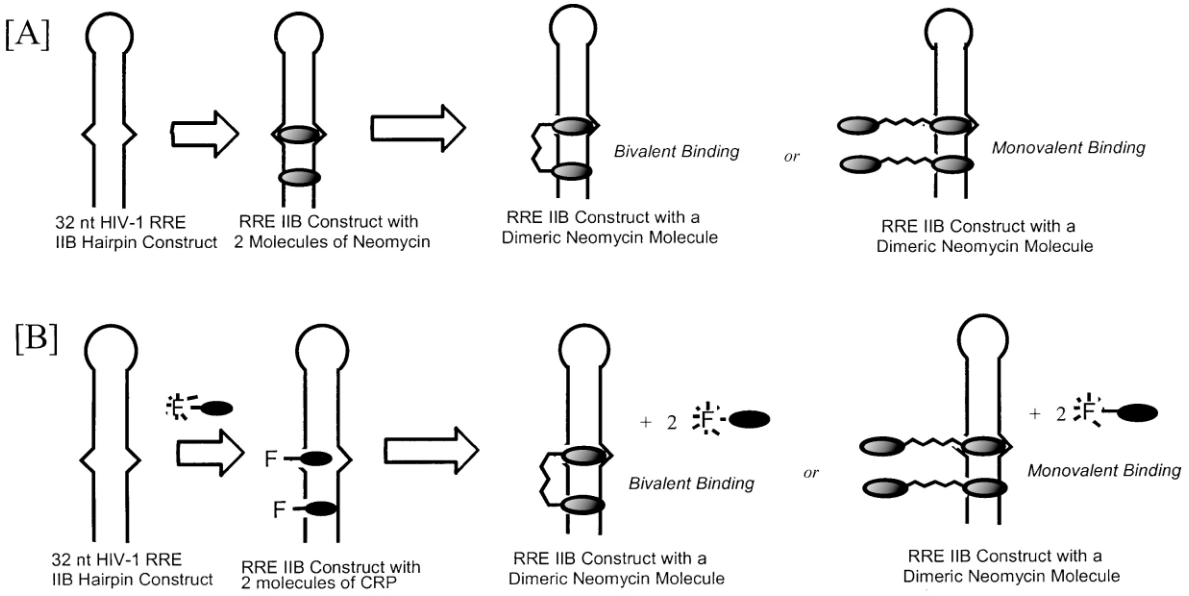
recognition of the aminoglycosides may be occurring through the secondary folding structure of the RNA rather than its sequences (Scheme 1).[23,25]

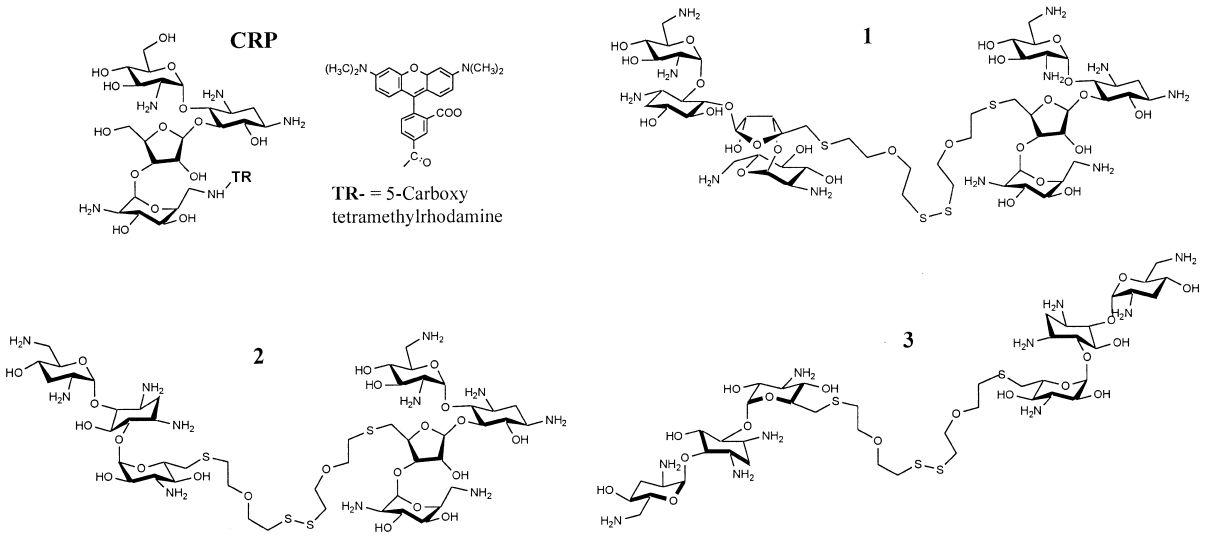
To address the binding characteristics of aminoglycosides to the RRE construct, Wong and co-workers have recently reported through the usage of surface plasmon resonance (SPR) method, that a stoichiometry of three molecules of neomycin is able to bind to a single RNA construct of RRE.[21,22] It was also previously observed by Rando and Cho that the binding of neomycin does not involve the bases in the bulge directly, which highly suggests that there may be additional binding sites within the RRE construct.[23] These observations were further substantiated recently though a series of elegant stopped-flow fluorescence measurements by Marino and co-workers.[24] The data obtained unequivocally showed that there were a total of three neomycin-binding sites. The first site is noninhibitory to the Rev peptide binding, the second site inhibited the Rev peptide binding, and the third site is attributed to nonspecific binding. It was noted that the first two binding sites are able to bind the neomycin aminoglycoside in the nM range (Scheme 2A), but more importantly, it was also observed that the second neomycin-binding site is at the lower duplex stem region of the RRE RNA construct.



Scheme 1. (A) Secondary structure of the HIV-1 RRE–RNA construct as predicted by the Mulfold Program[26] and the two hypothesized high binding sites of neomycin B as predicted by Marino and coworkers,[24] and (B) chemical structure of the aminoglycoside antibiotics used in this study.



Scheme 2. (A) Hypothesized model for binding the HIV-1 RRE construct with dimeric neomycin through either a bivalent or monovalent binding, and (B) schematic illustration of the competitive binding assay with the dimeric neomycin molecules against the CRP–RRE complex.



Scheme 3. The structures of CRP, neomycin–neomycin (neo–neo, **1**), neomycin–tobramycin (neo–tob, **2**), and tobramycin–tobramycin (tob–tob, **3**), aminoglycoside dimers.

These observations suggest that neomycin may not be strictly restricted to only recognizing RNA secondary folds.

To further probe these observations that more than one neomycin-binding sites are indeed available within the RRE construct, we chose to study the RRE RNA construct with dimeric aminoglycoside molecules[25,27–30] through fluorescence anisotropy studies. We have previously studied such dimeric molecules towards the A-site 16S rRNA construct, and have observed that the dimeric molecules do not have any significant improvement in binding affinities towards the simplified 27 nt A-site 16S rRNA construct. However in the presence of a novel 47 nt RNA construct that contains two 16S A-site bulges, an enhancement of dissociation constants (K_d) up to 19-fold can be achieved.[31] These results highly suggest that the A-site 16S rRNA construct bound the neomycin molecule in a 1:1 stoichiometry. Hence in the case of the 34 nt RRE RNA hairpin construct, if there are more than one neomycin binding sites within it, the dimeric neomycin molecules, when compared to the monomeric neomycin, would subsequently be expected to have a significantly improved binding affinity towards the RRE construct (should the molecules bind in a bivalent manner; Scheme 2).

In this work, we continue to explore the binding properties of the dimeric aminoglycosides (Scheme 3), namely dimeric neomycin–neomycin (**1**), dimeric neomycin–tobramycin (**2**), and dimeric tobramycin–tobramycin (**3**) molecules towards the RRE RNA construct of HIV-1. Through the fluorescence competition binding assay, the K_d of dimeric aminoglycoside **1** towards the RRE construct was observed to be approximately 17-fold higher, when compared to its monomeric form.

To quantify the K_d between the monomeric and dimeric aminoglycosides to both RNA constructs, we employed the recently developed fluorescence method that enables direct and quantitative binding measurements between aminoglycoside–RNA interactions.[23,32] 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, room temperature). First, the rhodamine-conjugated paromomycin molecule (CRP,[32] Scheme 3) at 10 nM was titrated with an increasing concentration of RRE construct.

In this experiment, we hypothesized that rhodamine-conjugated paromomycin (CRP) molecule binds to the RRE construct in a similar fashion to the neomycin aminoglycoside (i.e., at more than one site; Scheme 2B). It was previously reported that paromomycin, although structurally similar to neomycin, binds the RRE construct at a much lower affinity with only one of the aminogroups in neomycin being substituted for a hydroxyl group.[32] For this competition experiment, we are primarily interested in the manner of displacement of CRP through the dimeric aminoglycosides. It was of interest to note that during the titration process, a slow and gradual addition of CRP to the RRE construct was performed in the hope of detecting additional saturation curves that would enable us to study the stoichiometry event. However, we are unable to accurately quantify the stoichiometry of CRP binding, as an additional saturation curve was not detected. Instead, it was observed that the fluorescence curve gradually increased in a continuous fashion, reaching saturation at around 600 nM (Fig. 1B). Using a previously described curve fitting equation,[23,32] the K_d of CRP was found to be $0.50 \pm 0.04\,\mu M$, and is in agreement with the observations previously reported.[32] Subsequently, when an increasing concentration of the neomycin molecule was added to the CRP–RRE complex, the fluorescence of the complex began to gradually quench in a saturable fashion (Fig. 1B). Again using a previously described curve fitting equation, the K_d of the neomycin molecule was calculated to be $0.18 \pm 0.013\,\mu M$. Using the same experiment with tobramycin molecule (figure not

Table 1. Summary of K_d (μM) of various tested aminoglycoside molecules with HIV-1 RRE–RNA

CRP	Neomycin (Neo)	Tobramycin (Tob)	Neo–Neo dimer **1**	Neo–Tob dimer **2**	Tob–Tob dimer **3**
0.50±0.04	0.18±0.01	4.11±0.21	0.01±0.001	0.16±0.002	3.69±0.02

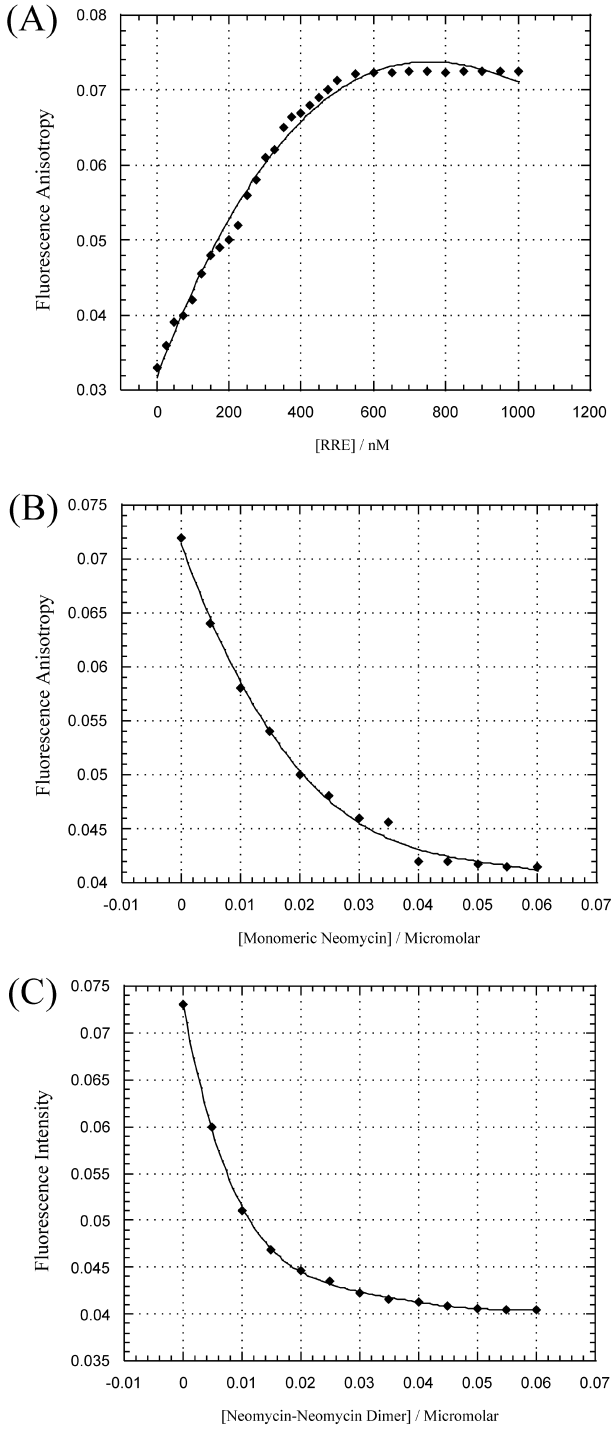


Figure 1. Fluorescence binding isotherms for (A) CRP with increasing concentrations of RRE construct, (B) competition of CRP–RRE complex with monomeric neomycin molecules, and (C) competition of CRP–RRE complex with dimeric neomycin aminoglycoside molecules.

shown), it was calculated that tobramycin has a K_d that is drastically lowered, which is 4.11±0.21 μM against the RRE–RNA construct.

The dimeric aminoglycosides **1**–**3** were subsequently studied with respect to their abilities to compete with CRP for binding to the RRE construct. Using a similar approach for the monomeric aminoglycosides, dimer **1** in neomycin–neomycin was observed to exhibit much improvement in binding affinity with a K_d of 0.01±0.001 μM, and this obtained K_d value corresponds to approximately 17-fold enhancement when compared to neomcyin (Fig. 1C). On the other hand, dimer **2** in neomycin–tobramycin was observed to have a K_d of 0.16±0.002 μM, and the dimer **3** in tobramycin–tobramycin has a K_d of 3.69±0.02 μM (data summarized in Table 1).

In summary, the use of fluorescence anisotropy has shown that dimeric neomycin aminoglycoside antibiotics are able to bind the HIV-1 RRE–RNA construct with an approximately 17-fold increase in K_d as compared to their monomeric counterparts. The binding results attained with the dimeric aminoglycosides support the notion that there are indeed more than one neomycin-binding sites within the RRE–RNA construct, and the most likely mode of binding is through a bivalent mechanistic approach. However, the limitation with this approach is that we are unable to probe the exact number of neomycin binding sites. We are currently synthesizing trimeric and tetrameric aminoglycoside molecules to further understand this issue. Systematic mutational studies and chemical footprinting studies on the lower stem of the HIV-1 RRE–RNA construct are also in progress, and will be reported in due course.

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