Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 365–370

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Binding of Aminoglycoside Antibiotics with Modified A-site 16S rRNA Construct Containing Non-Nucleotide Linkers

Jeffrey B.-H. Tok,* Wilson Wong and Nyla Baboolal

Department of Chemistry & Natural Sciences, York College and Graduate Center of the City University of New York, 94-20 Guy R. Brewer Blvd., Jamaica, NY 11451, USA

Received 27 August 2001; accepted 9 November 2001

Abstract—The design and synthesis of synthetically modified cyclic A-site 16S rRNA construct is reported. The binding characteristics of several members of the aminoglycoside antibiotics with this novel class of synthetically modified A-site 16S rRNA constructs were subsequently investigated.

The potential in utilizing synthetic oligonucleotides as a therapeutic approach to control gene expression has been widely explored.[1–3] Modifications in the backbone, sugar and bases of oligonucleotides have all been well documented to result in higher binding affinities against their complementary target nucleic acid, and this approach was generally referred to as the 'antigene' approach.[4–11] In order to obtain more stable duplex (two single-stranded nucleic acid) molecules, modifications to the hairpin loop have also been reported. For example, instead of nucleotide-containing hairpin loop, synthetic linkers containing either the ethylene glycol or stilbenedicarboxamide functional moieties have been synthesized to replace the natural hairpin loop.[12–16] Such resulting constructs have been observed to form exceptionally stable duplex structures, with a marked increase in thermal stability (T_m) values.[13,17,18] Specifically, Ma and coworkers have utilized a series of synthetic linkers derived from hexaethylene glycol to generate synthetically modified double stranded TAR RNA construct.[12,13] They have proceeded to attach the synthetic linkers at both ends of the RNA duplex to create cyclic miniduplexes.[12] On a similar note, Pils and Micura have also utilized linkers containing repeating units of both tris- or tetrakis(ethylene glycol) phosphate and 3-hydroxy-propane-1-phosphate to generate non-nucleotide linkers as loop replacement of double helical RNA.[14] In addition, Letsinger and Wu have also investigated the utilization of stilbenedicarboxamide as a synthetic replacement for the natural nucleotide (nt) hairpin loop to create a mini-cyclic oligonucleotide conjugate.[17,18] The stilbene moiety has been strategically placed in-between two short pieces of oligonucleotide, to potentially create a proper alignment of the stilbene chromophore with the adjacent base pair, upon the hybridization of the two complementary RNA strands.

We have been interested in the design of high binding RNA decoys that could potentially be applied in controlling the in vivo translation process. RNA decoys, such as RNA aptamers, have traditionally been generated through the in vitro selection (SELEX) process. For example, it was recently demonstrated that the techniques of both chemical modification and in vitro selection can be jointly applied to enable RNA decoys that exhibit high binding affinities towards the Rev peptide of HIV-1.[19]

In this report, we have applied the synthetic non-nucleotide hairpin linkers in ethylene glycol (L_1 and L_2) or stilbenedicarboxamide (L_3) onto the duplex region of the 27 nt A-site 16S rRNA to create synthetically modified cyclic A-site 16S rRNA constructs (Fig. 1). This model RNA substrate is designed specifically to enable higher aminoglycoside binding through additional stabilization of the duplex at both its ends. The RNA duplex is joined at both ends to form a dumbbell-shaped construct with the two ends of the duplex covalently linked through the same synthetic linkers. It was generally hypothesized that, through reducing the number of nucleotides that are necessary for hairpin loop formation in the oligonucleotide, the potential in form-

*Corresponding author. Tel.: +1-718-262-2656; fax: +1-718-262-2652; e-mail: tok@york.cuny.edu

0960-894X/02/$ - see front matter
PII: S0960-894X(01)00760-0

ing multiple secondary structures could be minimized. In addition, the synthetic linkers have been demonstrated to exhibit nuclease-resistant properties, improved cellular uptake properties, offer specific manipulation of nucleic acid properties, and also reduce the synthetic cost of the nucleic acid construct.[20–23]

Synthetic linkers in both the ethylene glycol (L_1 and L_2) and stilbenedicarboxamide (L_3) moieties were prepared according to standard published procedures to render them for standard nucleotide coupling (synthetic approach is shown in Fig. 2).[13,16] It is noted that the polyethylene glycol linker L_1 (derived from triethylene glycol) is much shorter than linker L_2 as it contains only three ethylene groups (total of seven atoms), whereas the hexaethylene glycol L_2 (derived from hexaethylene glycol) contains six ethylene groups (total of 17 atoms). The stilbenedicarboxamide (L_3) was calculated to have a very similar length to the linker L_2 as it contains 20 atoms in its linker. The synthetically modified RNA constructs **A**–**D** were then synthesized via the standard phosphoamidite chemistry (Millipore Expedite Oligonucleotide Synthesizer) using both the controlled pore glass (CPG) support (Glenn Research, Sterling, VA, USA) and the derivatized linkers L_1, L_2 and L_3 according to previously published procedures,[13] and purified using RP-HPLC [Waters Spherisorb ODS-1 (C_{18}) Columns].[24] The cyclic RNA construct **B** was obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA), and showed the appropriate oligomeric mass-spectrum data.

The A-site decoding region of the 16S rRNA has been the primary site in which the aminoglycoside antibiotics bind.[25–27] Upon binding, it interferes with the protein synthesis process through the induction of codon misreading. The A-site 16S rRNA region was chosen as the subject of study as it had been well demonstrated to be able to be truncated to a much shortened 27 nt RNA construct, without any major resulting effect on the binding properties of the aminoglycoside antibiotics.[28,29] Briefly, aminoglycoside antibiotics (Fig. 3) are among the few known existing classes of natural RNA-binding molecules. Aminoglycosides are low molecular weight molecules capable of interacting with a wide range of RNAs,[30–32] also known to be able to

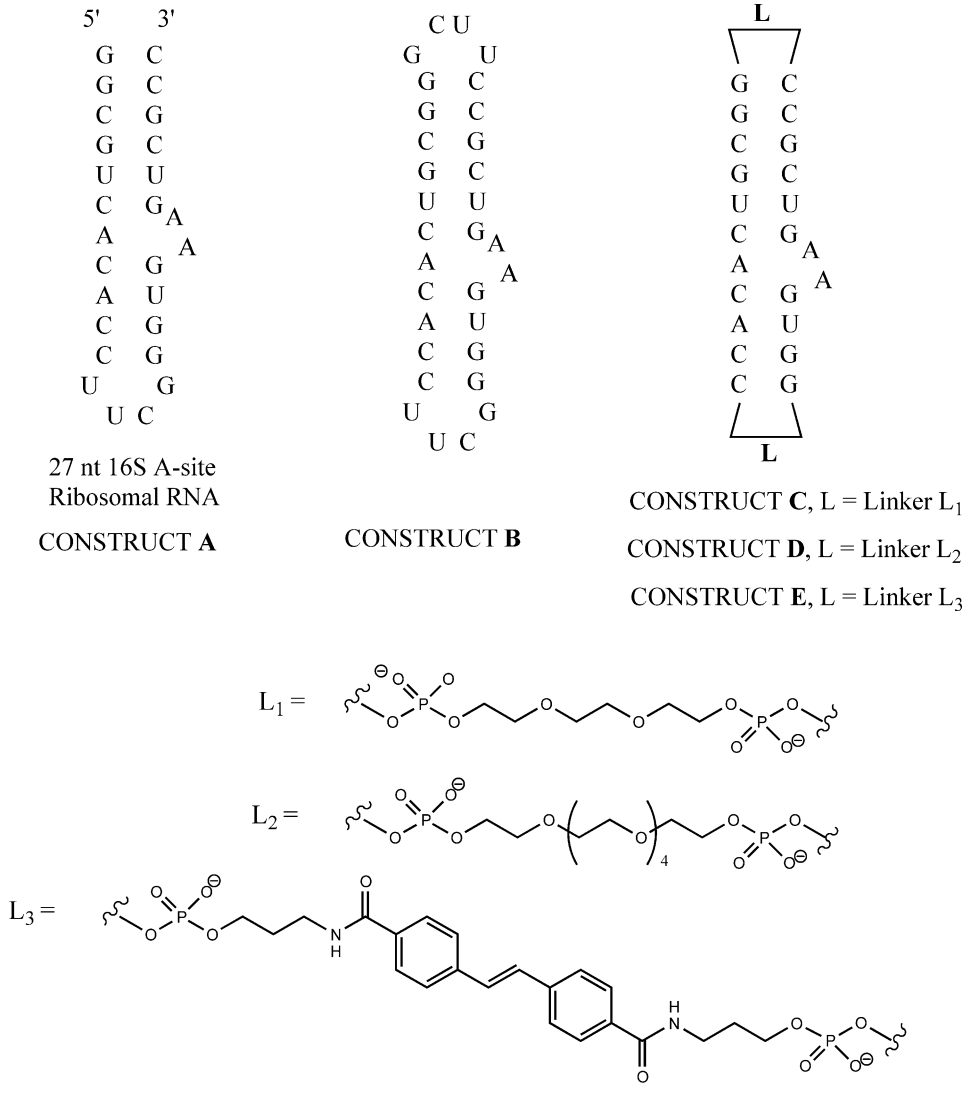
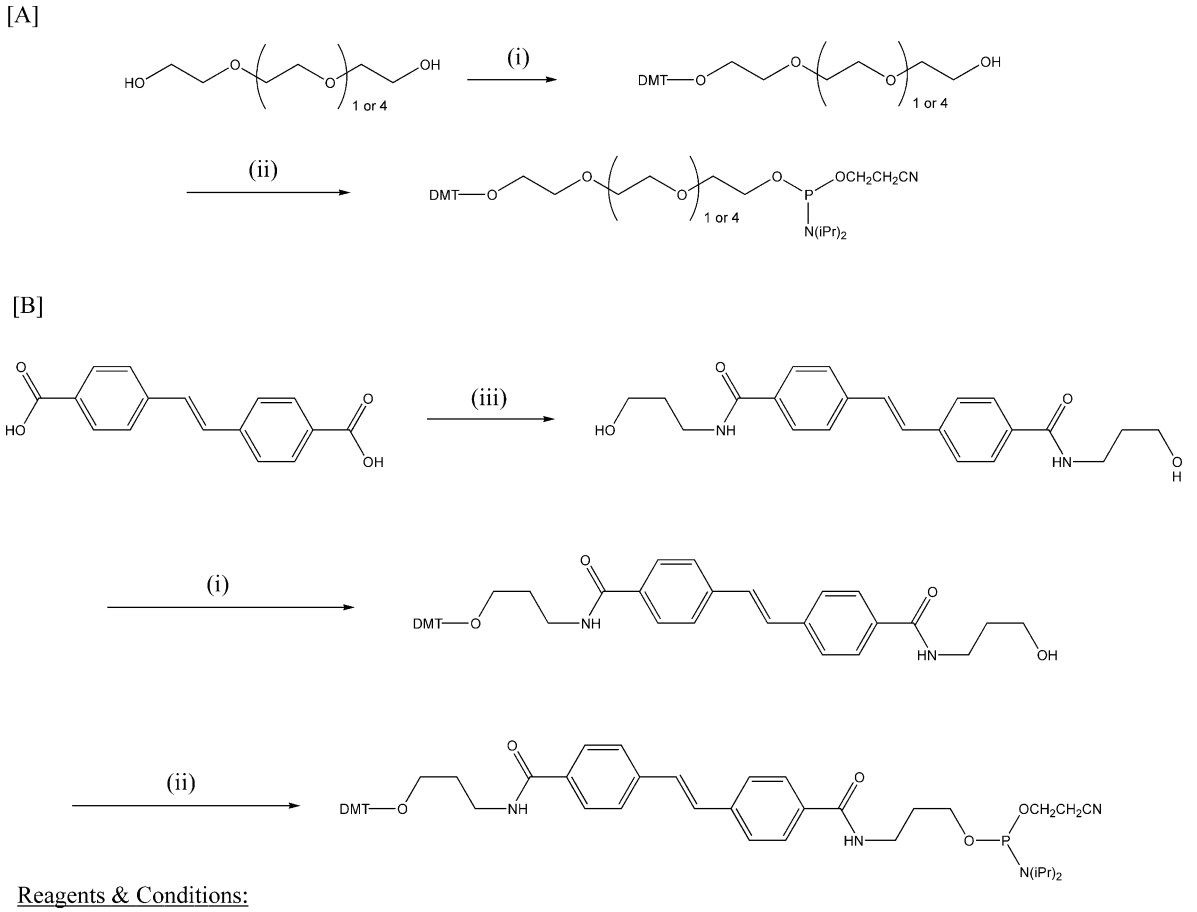


Figure 1. Secondary structures of the linear and cyclic A-site 16S rRNA constructs utilized in this study.

inhibit several catalytic RNAs (e.g., self-splicing group I introns, RNase P and small ribozymes), and are also very powerful inhibitors of prokaryotic cell growth.[30–32]

To quantify the K_d between the synthetically modified RNAs to the aminoglycoside antibiotics, a recently developed fluorescence method that enables direct and quantitative binding measurements between aminoglycoside–RNA interactions was employed.[33] It should be noted that the K_d values obtained for the RNA–aminoglycoside interaction via the fluorescence method are generally a few fold higher than values obtained via the Surface Plasmon Resonance (SPR) approach, first demonstrated by Wong and coworkers.[34,35] To enable the fluorescence methodology, the fluorescently conjugated paromomycin aminoglycoside was first employed. Paromomycin possesses a primary hydroxy group and was easily derivatized through conjugation with the rhodamine moiety to afford rhodamine-conjugated paromomycin (CRP; Fig. 3).[33] CRP was subsequently utilized in increasing concentration to bind the RNA constructs of interest, and its changes in fluorescence intensity measured (excitation at 550 nm, monitored at 580 nm).

In this experiment, it was hypothesized that CRP binds to the 27 nt and all the synthetically modified A-site 16S rRNA constructs in a 1:1 stoichiometry. The binding studies were performed 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). The fluorescence intensity curve was observed to increase when a corresponding increasing concentration of CRP was titrated with 5 nM of the 27 nt RNA construct **A**. The fluorescence intensity curve reached saturation at around 500 nM (Fig. 4), and upon application of a previously described curve-fitting equation,[33] the K_d of CRP can be calculated to be 0.33 ± 0.02 μM, a value which is in agreement with previously reported observations. Interestingly, when the same experiment was repeated with the various synthetically modified RNA constructs **B**–**E**, a comparable binding curve was observed in each of the three RNA constructs, in which saturation was reached around 480–520 nM (Fig. 4; only saturation curves for constructs **B** and **E** are shown). These results strongly suggest that the stoichiometry of binding for RNA constructs **B**–**E** is similar with that of the 27 nt RNA construct **A**. Applying the curve-fitting equation, the K_d of the interaction between CRP and RNA constructs **B**–**E** can be calculated to be 0.31 ± 0.02, 3.89 ± 0.19, 0.34 ± 0.03 and 0.081 ± 0.005 μM, respectively. First, the data indicate that creating a cyclic RNA construct **B**, through introducing a similar



Reagents & Conditions:

(i) 4,4'-Dimethoxytrityl chloride (DMT-Cl), pyridine, 0°C to r.t., 4 hrs, r.t.; (ii) DIPEA, dropwise over 5 mins, r.t., 2 hrs.

(iii) (a) $SOCl_2$, DMF, reflux, overnight, (b) 3-aminopropanol, Et_3N, CH_3OH, 0°C to r.t.

Figure 2. Synthetic schemes for the [A] ethylene glycol linker-derivatized phosphoamidite linkers L_1 and L_2, and [B] stilbenedicarboxamide linker-derivatized phosphoamidite L_3.[13,16]

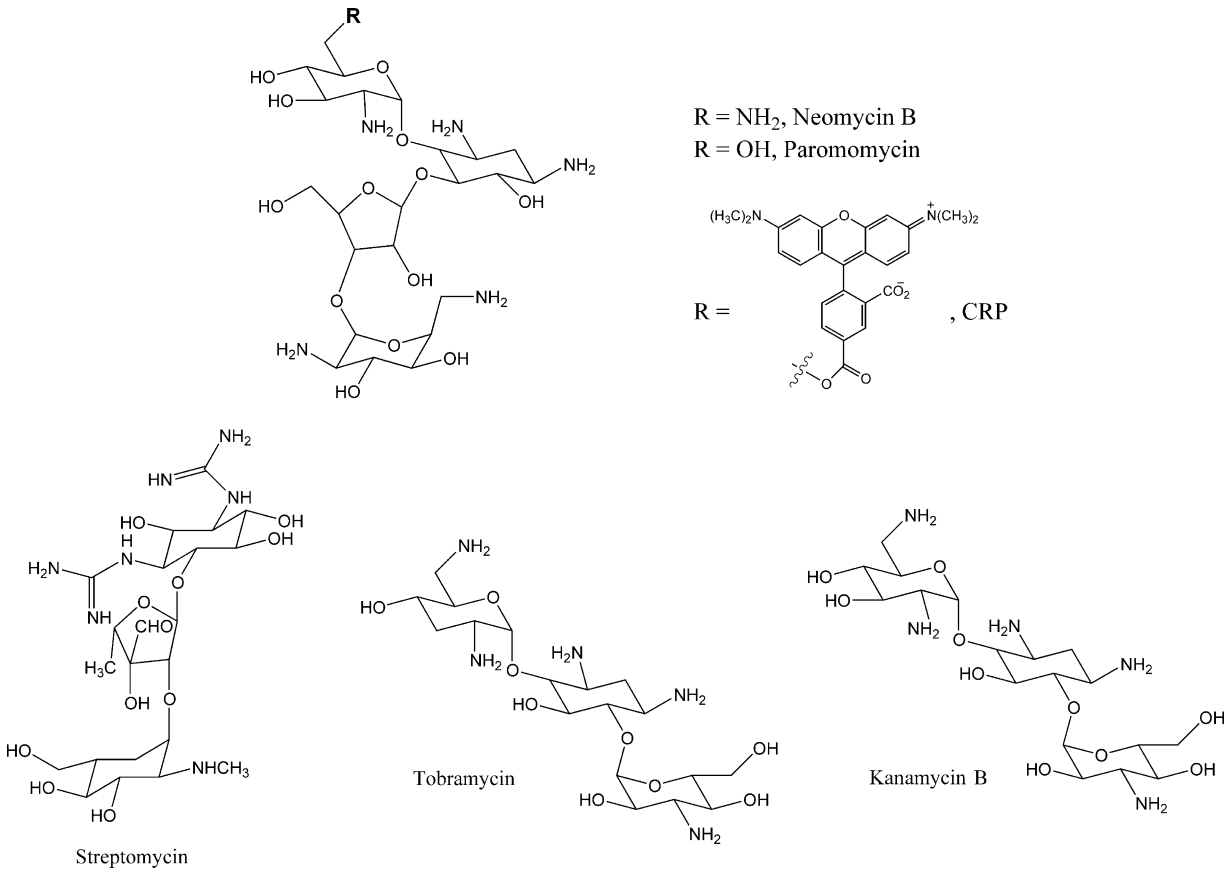


Figure 3. Structures of the various aminoglycoside antibiotics utilized in this study.

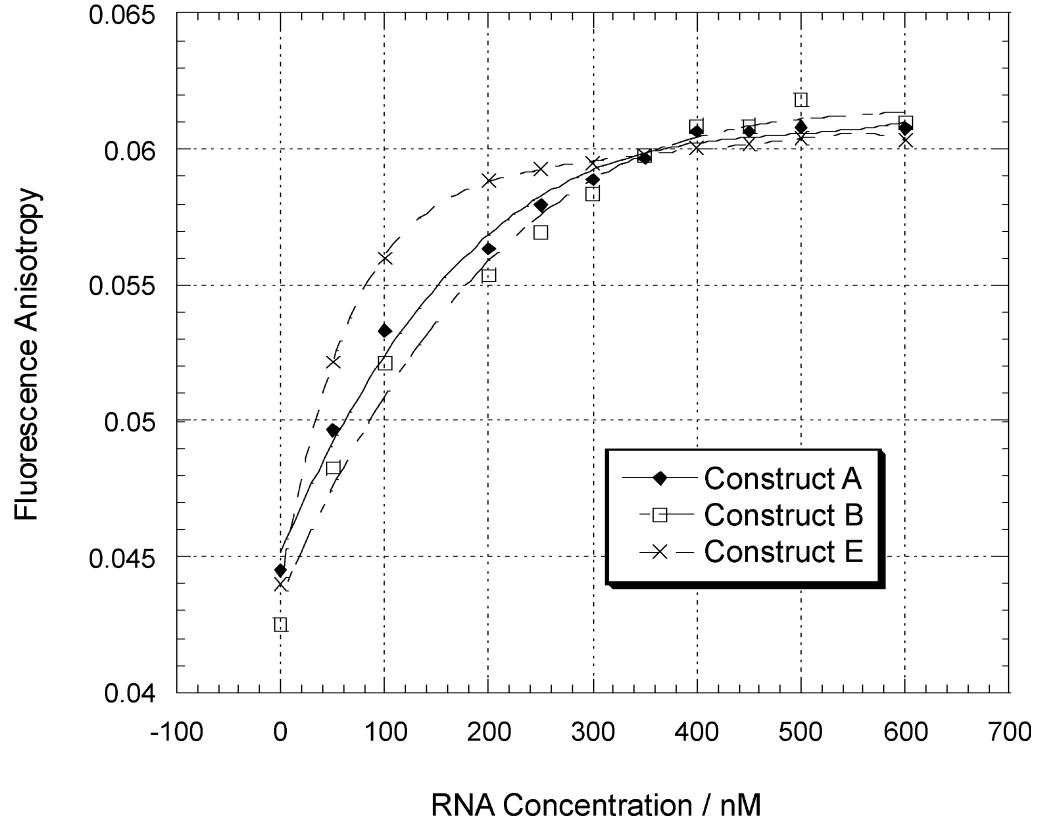


Figure 4. Representative fluorescence anisotropy plots of CRP (10 nM) as a function of concentration of [A] 27 nt A-site 16S rRNA construct **A**, [B] cyclized A-site 16S rRNA construct **B**, [C] cyclized A-site 16S rRNA construct **E**.

UUCG loop region onto the other end of the A-site RNA duplex region, does not exhibit any significant improvement in the K_d binding affinity toward CRP. Second, the usage of the short triethylene glycol linker (L_1) to replace the two UUCG loop-region of the A-site construct to generate the cyclic RNA construct **C** drastically decreases the binding affinity for CRP by nearly 12-fold, from 0.33 to 3.89 μM. This observation strongly suggests that linker L_1 does not provide sufficient length to enable the structural flexibility required by the RNA oligomers to adopt the optimized conformation for CRP aminoglycoside binding. It was previously predicted that the short linker L_1 would potentially lead to distortion of the terminal heterocyclic bases, and thus be unable to effectively maintain optimum Watson–Crick hydrogen bonding formation.[13] However, by increasing the length of the polyethylene glycol linker chain length (to L_2) restores the binding affinity back to approximately the value exhibited by the *wt* 27 nt A-site 16S rRNA construct.

Interestingly, the only visible improvement in the K_d binding affinity of CRP is observed towards cyclic RNA construct **E**, which consists of stilbenedicarboxamide linkers at both ends of the duplex RNA. The measured K_d of 0.081 μM of CRP interaction with cyclic RNA construct **E** is approximately 4-fold higher compared to that of CRP–RNA construct **A** interaction, for which the K_d is measured to be 0.33 μM (data summarized in Table 1A).

To further examine the binding characteristics of other aminoglycosides towards the cyclized RNA constructs **B**–**E**, a competition experiment was performed. Again, it was hypothesized that 1:1 stoichiometry binding exists between the various examined aminoglycosides (namely neomycin B, paromomycin, tobramycin, kanamycin B and streptomycin) with the RNA constructs **A**–**E**. In the competition experiment, we are interested in monitoring the changes in fluorescence anisotropy values through the displacement of RNA-bound CRP molecules when an increasing concentration of the various aminoglycoside ligands was titrated to the CRP–RNA complex.

To ensure that this method of approach is appropriate for this study, an increasing concentration of the well-studied neomycin B (0–50 μM) was added to the CRP construct **A** complex first, which comprised of 10 nM of CRP and 400 nM of RNA construct **A**. The fluorescence of the complex was observed to quenched gradually in a saturable fashion, and using the previously described curve-fitting equation, the K_d of neomycin B can be calculated. The K_d value was calculated to be 0.41 ± 0.03 μM, a value which is in agreement with the previous observations utilizing the same fluorescence techniques.[33,36]

Using the same experimental procedures and conditions, neomycin B was individually titrated against the various complexes of CRP (10 nM) and RNA constructs **B**–**E** (400 nM). Extrapolating the observed values in each of the four complexes afforded us K_d values of 0.38 ± 0.03, 4.34 ± 0.24, 0.47 ± 0.03 and 0.091 ± 0.008 μM for neomycin against RNA constructs **B**–**E**, respectively (data summarized in Table 1B). The results obtained again reconfirmed the previous observations that: (i) cyclizing the A-site 16S rRNA construct **A** to afford construct **B** does not exhibit any significant improvement in binding affinities towards the neomycin B aminoglycoside; and (ii) cyclic RNA constructs **C** and **D**, which comprised of both short and long ethylene glycol linkers, do not enhance the stability of the resulting RNA constructs as demonstrated by the K_d binding values towards neomycin B. Instead, RNA construct **C**, comprised of short triethylene glycol linker L_1, again demonstrated an overall 11-fold decrease in its K_d values towards neomycin B when compared with construct **A**. Again, the obtained K_d data for construct **D** showed a comparable K_d binding value when compared to the 27 nt 16S rRNA construct **A**. Likewise, an enhancement in K_d values is observed only with the cyclic RNA construct **E**. Taken together, these results strongly suggest that the stilbenedicarboxamide linker affords a much stabler resulting RNA construct when compared to either the natural nucleotides or the polyethylene glycol linkers L_2 and L_3, thus translating into a much tighter binding with the neomycin B aminoglycoside.

The same experimental approach was subsequently applied to the remaining aminoglycosides in paromomycin, tobramycin, kanamycin B and streptomycin. Their binding affinities were examined towards RNA constructs **B**–**D**. The K_d values obtained for these aminoglycosides all showed a similar trend in their affinities towards the various cyclic RNA constructs studied, with neomycin B being the tightest, while streptomycin

Table 1. (A) Summary of the K_d (μM) values of the CRP tracer molecule against RNA constructs **A**–**E**; (B) summary of K_d (μM) of the various tested aminoglycoside molecules against RNA constructs **A**–**E**

Molecule	RNA Construct **A**	RNA Construct **B**	RNA Construct **C**	RNA Construct **D**	RNA Construct **E**
(A)					
CRP	0.33 ± 0.02	0.31 ± 0.02	3.89 ± 0.19	0.34 ± 0.03	0.081 ± 0.005
(B)					
Neomycin B	0.41 ± 0.03	0.38 ± 0.03	4.34 ± 0.24	0.47 ± 0.03	0.091 ± 0.008
Paromomycin	1.64 ± 0.12	1.58 ± 0.11	5.33 ± 0.37	1.70 ± 0.09	0.40 ± 0.03
Tobramycin	2.07 ± 0.24	2.04 ± 0.22	n.b.[a]	2.12 ± 0.23	0.68 ± 0.07
Kanamycin B	2.95 ± 0.30	2.90 ± 0.18	n.b.	2.75 ± 0.25	0.91 ± 0.10
Streptomycin	n.b.	n.b.	n.b.	n.b.	n.b.

[a]n.b., no observable binding.

showed no visible binding properties (data summarized in Table 1B). Importantly, among all the cyclic RNA constructs studied, only the cyclic RNA construct **E**, which contained the stilbenedicarboxamide linker at both ends, again demonstrated the tightest overall binding characteristics towards the aminoglycosides (data summarized in Table 1).

In summary, the data presented indicate that cyclic RNA comprising of stilbenedicarboxamide linker at both ends to create a dumbbell-shaped construct is approximately four-fold more effective in the general binding of aminoglycosides when compared to its hairpin-shaped RNA counterpart. The binding characteristics of rhodamine-derivatized paromomycin tracer (CRP) ligand are used to bind the various cyclic RNA constructs, and its complex is subsequently utilized to afford K_d values of five more other aminoglycosides in paromomycin, neomycin B, tobramycin, kanamycin B and streptomycin. The data obtained are consistent with previous observations,[33] which also have indicated neomycin B demonstrated the tightest overall binding affinities to the A-site 16S rRNA molecular targets. The application of the stilbenedicarboxamide linker to replace the UUCG RNA loop in the A-site 16S rRNA construct had been shown to be both useful and feasible in further stabilizing the duplex region. It is hypothesized that the extra stabilization conferred to the overall RNA construct is through the π–π stacking interactions between the stilbenedicarboxamide moiety and the adjacent base pairs of the nucleotides at the end of the RNA duplex region.[16,37,38] Through molecular modeling (MM2 minimization using Hyperchem V5.01a), it was indeed demonstrated by Lewis and coworkers that the stilbenedicarboxamide moiety does adopt a coplanar structure with the adjacent guanine and cytosine base pairs.[37] Although a modest four-fold enhancement in K_d binding affinity was obtained, nevertheless these results have generated a possible approach towards our effort in designing and synthesizing RNA decoys that are capable of better regulating gene expression at the translation level.

Acknowledgements

J. B.-H. Tok gratefully acknowledges York College, the McNair Scholars Program and RF-CUNY for a Summer '01 Faculty Award. W. Wong and N. Baboolal are both McNair undergraduate scholars.

References and Notes

1. Agrawal, S. *Antisense Therapeutics*; Humana Press: Totowa, NJ, 1996.
2. Crooke, S. T.; LeBleu, B. *Antisense Research and Applications*; CRC Press: Boca Raton, FL, 1993.
3. Wickstrom, E. *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*; Wiley-Liss: New York, 1991.
4. Nielsen, P. E. *Curr. Opin. Mol. Ther.* **2000**, *2*, 282.
5. Micklefield, J. *Curr. Med. Chem.* **2001**, *8*, 1157.
6. Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Gutierrez, A. J.; Moulds, C.; Froehler, B. C. *Science* **1993**, *260*, 1510.
7. Wagner, R. W. *Nature* **1994**, *372*, 333.
8. Wagner, R. W.; Matteucci, M. D.; Grant, D.; Huang, T.; Froehler, B. C. *Nature Biotech.* **1996**, *14*, 840.
9. Stein, C. A. *Chem. Biol.* **1996**, *3*, 319.
10. Yamakawa, H.; Abe, T.; Saito, T.; Takai, K.; Yamamoto, N.; Takaku, H. *Bioorg. Med. Chem.* **1998**, *6*, 1025.
11. Riordan, M. L.; Martin, J. C. *Nature* **1991**, *350*, 442.
12. Ma, M. Y.-X.; McCallum, K.; Climie, S. C.; Kuperman, R.; Lin, W. C.; Sumner-Smith, M.; Barnett, R. W. *Nucleic Acid Res.* **1993**, *21*, 2585.
13. Ma, M. Y.-X.; Reid, L. S.; Climie, S. C.; Lin, W. C.; Kuperman, R.; Sumner-Smith, M.; Barnett, R. W. *Biochemistry* **1993**, *32*, 1751.
14. Pils, W.; Micura, R. *Nucleic Acid Res.* **2000**, *28*, 1859.
15. Letsinger, R. L.; Wu, T. *J. Am. Chem. Soc.* **1994**, *116*, 811.
16. Letsinger, R. L.; Wu, T. *J. Am. Chem. Soc.* **1995**, *117*, 7323.
17. Herrlein, M. K.; Nelson, J. S.; Letsinger, R. L. *J. Am. Chem. Soc.* **1995**, *117*, 10151.
18. Herrlein, M. K.; Letsinger, R. L. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 599.
19. Nelson, J. S.; Giver, L.; Ellington, A. D.; Letsinger, R. L. *Biochemistry* **1996**, *35*, 5339.
20. Bensenler, F.; Fu, D.; Ludwig, J.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1993**, *115*, 8483.
21. Wiederholt, K.; McLaughlin, L. W. *Nucleic Acid Res.* **1997**, *27*, 2487.
22. Wu, X.; Pitsch, S. *Nucleic Acid Res.* **1998**, *21*, 4315.
23. Steiz, O. *Angew. Chem., Int. Ed. Engl.* **1999**, *111*, 3466.
24. Coupling yields in using linkers L_1–L_3 to yield RNA constructs **C**, **D** and **E** were satisfactory (93, 90, 91%, respectively), in which the oligomers were isolated as trityl-on derivatives by RP-HPLC, and detritylated with 80% acetic acid. The concentrations of the oligomers were quantified by OD_{260} measurements.
25. Woodcock, J.; Moazed, D.; Cannon, M.; Davies, J.; Noller, H. F. *The EMBO J.* **1991**, *10*, 3099.
26. von Ahsen, U.; Noller, H. F. *Science* **1995**, *267*, 234.
27. von Ashen, U.; Davies, J.; Schroeder, R. *J. Mol. Biol.* **1992**, *226*, 935.
28. Fourmy, D.; Recht, M. I.; Blanchard, S. C.; Puglisi, J. D. *Science* **1996**, *274*, 1367.
29. Fourmy, D.; Yoshizawa, S.; Puglisi, J. D. *J. Mol. Biol.* **1998**, *277*, 333.
30. Sucheck, S. J.; Wong, C.-H. *Curr. Opin. Chem. Biol.* **2000**, *4*, 678.
31. Schroeder, R.; Waldsich, C.; Wank, H. *The EMBO J.* **2000**, *19*, 1.
32. Tor, Y.; Hermann, T.; Westhof, E. *Chem. Biol.* **1998**, *5*, R277.
33. Wang, Y.; Hamasaki, K.; Rando, R. R. *Biochemistry* **1997**, *36*, 768.
34. Hendrix, M.; Priestley, E. S.; Joyce, G. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 3641.
35. Park, W. K. C.; Aner, M.; Jaksche, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1996**, *118*, 10150.
36. Tok, J. B.-H.; Huffman, G. R. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1593.
37. Lewis, F. D.; Wu, T.; Liu, X.; Letsinger, R. L.; Greenfield, S. R.; Miller, S. E.; Wasielewski, M. R. *J. Am. Chem. Soc.* **2000**, *122*, 2889.
38. Lewis, F. D.; Liu, X.; Wu, Y.; Miller, S. E.; Wasielewski, M. R.; Letsinger, R. L.; Sanishvili, R.; Joachimiak, A.; Tereshko, V.; Elgi, M. *J. Am. Chem. Soc.* **1999**, *121*, 9905.