Notes

Enhanced Binding Affinity of Neomycin-Chloramphenicol (or Linezolid) Conjugates to A-Site Model of 16S Ribosomal RNA

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Since aminoglycosides are well known natural products that inhibit or modulate RNA function,[1] a large number of synthetic analogues of natural aminoglycosides have been designed and synthesized as potential antibiotics.[2] Aminoglycosides form mostly electrostatic interactions to promote induced fit and conformational capture of ribosomal RNA (rRNA).[3] However, most aminoglycosides bind to rRNA with poor selectivity,[4] which often results in severe toxicity.[5,6]

A few strategies to improve specificity have been reported.[7,8] Another new strategy involves the addition of simple chemicals to aminoglycosides to make hetero-conjugates.[9] These heteroconjugates have sites for additional interactions, affording more specific affinities to RNA targets.[10] Encouraged by the last strategy, we recently designed and synthesized heteroconjugates, which contain both stem- and loop-loving groups to expand regions of interactions.[11] The conjugates were comprised of neomycin B (**Neo**, **N**), a stem-binding component and chloramphenicol (**Cam**, **C**) or linezolid (**Lnz**, **L**), a loop-binding moiety (Figure 1). Significant increases in specificity and affinity by heteroconjugates were observed against RNA stem-loop motifs.[11]

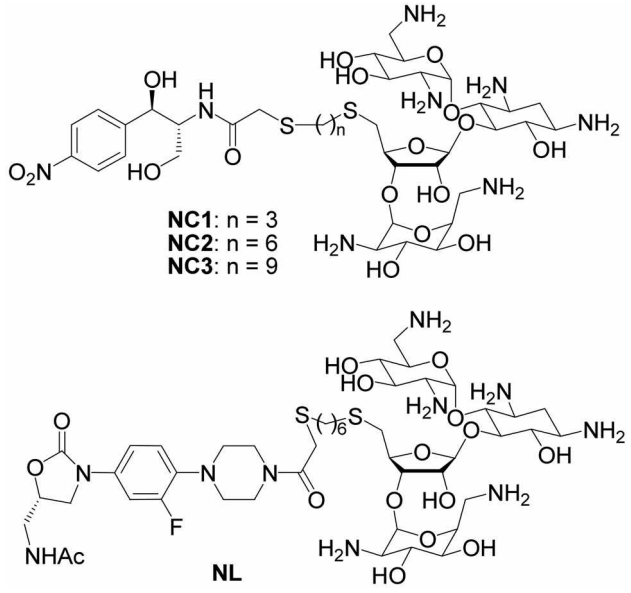

Figure 1. Structures of heteroconjugates.

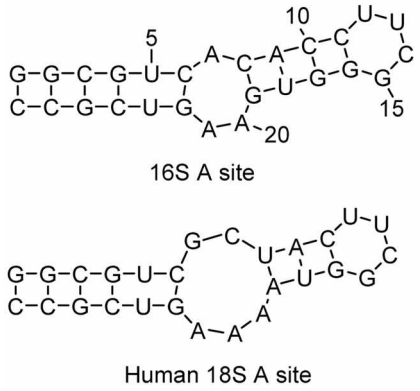

Figure 2. Secondary structures of 16S ribosomal RNA A-site model sequences for E.coli and human.

Since stem-loops are typical target RNA motifs by the heteroconjugates, a stem with a small bulge might be another possible target. In order to test this idea, a model structure of 16S rRNA A-site was chosen. This target RNA is composed of a stem with two bulges and an artificial short terminal loop (Figure 2). Since the binding site of neomycin B was defined in the upper stem region, heteroconjugates would make another interaction with either a bulge or a loop region RNA, resulting in enhanced affinities relative to neomycin B. In this report, we describe the binding affinities of **NC** (or **NL**) heteroconjugates to models of 16S rRNA A-site. More than 20 times of affinity enhancement by selected heteroconjugates was observed.

Model RNAs of 16S rRNA A-site of bacteria and of 18S rRNA A-site, a human analogue[12] were *in vitro* transcribed and were purified as described.[13] A fluorescence anisotropic technique was used to measure the binding affinities of the conjugates to targets (Table 1).[14] For 16S rRNA A-site, binding affinities of the heteroconjugates were enhanced compared that of neomycin B. **NL** and **NC2** showed two largest affinity enhancements, respectively, suggesting that a six-carbon tether is suitable. Only **NL** showed a significant enhanced binding affinity against 18S rRNA A-site for human, while other heteroconjugates showed no enhancement

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

RNA	Neo	NC1	NC2	NC3	NL
16S A-site	0.45	0.20	0.051	0.14	0.034
18S A-site	0.27	0.24	0.22	0.39	0.071

[a]Values are in μM.

Table 2. Selected results of antimicrobial assay using **NC2** and **NL** heteroconjugates

Strain	Neo	NC2	NL
Streptococcus pyogenes 308A	25	> 50	12.5
Streptococcus pyogenes 77A	12.5	25	12.5
Streptococcus faecium MD 8b	25	50	50
Staphylococcus aureus SG 511	0.391	6.25	6.25
Staphylococcus aureus 285	0.391	6.25	6.25
Staphylococcus aureus 503	0.195	3.125	6.25
Escherichia coli 078	1.563	25	> 50
Escherichia coli DC O	1.563	50	> 50
Escherichia coli DC 2	3.125	12.5	25
Escherichia coli TEM	1.563	50	> 50
Escherichia coli 1507 E	3.125	50	> 50
Pseudomonas aeruginosa 9027	3.125	50	> 50
Pseudomonas aeruginosa 1592E	12.5	> 50	> 50
Pseudomonas aeruginosa 1771	12.5	> 50	> 50
Pseudomonas aeruginosa 1771M	6.25	> 50	> 50
Salmonella typimurium	0.781	25	> 50
Klebsiella oxytoca 1082 E	0.781	50	> 50
Klebsiella aerogenes 1522 E	0.781	> 50	> 50
Enterobacter cloacae P 99	0.781	25	> 50
Enterobacter cloacae 1321 E	0.781	25	> 50

compared with neomycin B. Therefore, **NC2** showed the highest discrimination factor between the bacterial 16S rRNA A-site from human 18S rRNA A-site. This discrimination might be owing to the fact that the binding region of **NC2** is an anticipated stem with A-rich bulge motif.

Encouraged by the enhanced affinity of heteroconjugates, antimicrobial assay was carried out with 20 standard pathogenic bacteria. As shown in Table 2, however, antibiotic activities of **NC2** and **NL** did not correlate well with K_d values to the model RNA. In spite of their superior K_d values compared to neomycin B, heteroconjugates were less potent than neomycin in all strains. One of reasons for this discrepancy might be poor cell permeability of **NL** and **NC2**. In order to test this idea, an *in vitro* translation inhibition assay was carried out with a luciferase reporter.[15] As shown in Figure 3, **NL** and **NC2** showed weaker IC_{50} values than neomycin B, even though they showed much improved values than chloramphenicol or linezolid. This result correlates with those of the antimicrobial assay and removes the possibility of poor cell-wall permeability of heteroconjugates.

Next, we tried to obtain information about binding orientation of the heteroconjugates to the RNA target. There might be two major binding modes possible: the anticipated stem with bulges and the stem with the artificial terminal loop. In order to test this idea, mutants of the terminal loop in the 16S

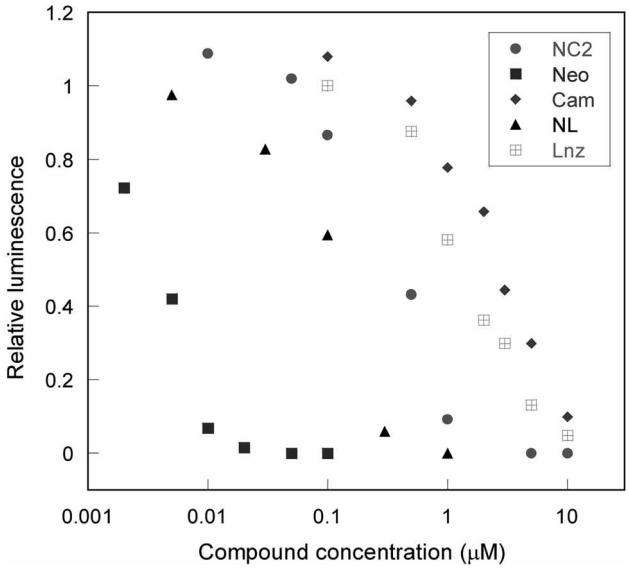

Figure 3. In vitro translation inhibition of **NC2** and **NL** using luciferase reporter gene assay.

Table 3. Binding Affinities (K_d) of Conjugates to Mutant RNA of 16S rRNA A-site[a]

Mutation	NC2	NL
Wild type	0.082 (1.0)	0.037 (1.0)
U12A	0.28 (3.4)	0.11 (3.7)
U13C & C14U	0.16 (2.0)	N.C.[b]

[a]Conditions are same as in Table 1. Mutation sites are shown in Figure 2. Values in parenthesis are ratios of binding affinities compared with that of wild type RNA ($K_{d\text{-}m}/K_{d\text{-}wt}$). [b]The low binding affinity caused widely varying results.

rRNA A-site model were *in vitro* transcribed in a manner to prevent alteration of the secondary structure of the model RNA. Binding affinities of **NL** and **NC2** to each mutant were then measured (Table 3). Binding affinities by the conjugates to mutant RNA are significantly weaker than to the wild-type RNA. Data strongly suggest that the major binding region by the heteroconjugates is not the anticipated stem-bulge region, but the terminal stem-loop region, which is not present in wild type A-site RNA. Thus, the discrepancy between *in vitro* K_d values and antimicrobial assay or *in vitro* translation assays can be explained by use of an inadequate model RNA for the 16S rRNA A-site.

Conclusion

NC2 and **NL** conjugates display enhanced site selective binding to the 16S rRNA model RNA. One of these conjugates, **NC2**, has a low nanomolar binding affinity to the target RNA, which is 20 times higher than that of neomycin B. Results of antimicrobial and *in vitro* translation inhibition assay, however, did not correlate well with the improved K_d values. Mutation studies demonstrate that the drug is not binding to the anticipated stem-bulge region but instead to the terminal loop region, where the heteroconjugates interact with specific base(s).

Experimental Section

Fluorescence Anisotropy. Binding affinities were measured at 20 °C by using a luminometer (Aminco-Bowman) and an anisotropy technique with < 10% error boundaries.[11] The binding affinities are measured at 20 °C. Cam and Lnz binding affinities showed > 10 μM to any RNA target.

***In vitro* Inhibition Assay.** Reactions were carried out with *E. coli* S30 extract system for circular DNA (Promega) as specified by the manufacturer. To measure translated lucifierase activity, reaction mixture was added to the luciferase assay reagent (Promega) into a luminometer tube. For every single point, three independent measurements were made and averaged.

Minimum Inhibitory Concentration Assay. Assay was performed with a modified agar micro-dilution technique in a 96-well microplate.[16]

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