Design of Bifunctional Antibiotics that Target Bacterial rRNA and Inhibit Resistance-Causing Enzymes

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Deoxystreptamine-based aminoglycosides are a clinically important class of antibiotics that are effective against a broad range of microorganisms.[1] It is believed that aminoglycosides exert their therapeutic effect by interfering with translational fidelity during protein synthesis via interaction with the A-site rRNA on the 16S domain of the ribosome.[2] Unfortunately, the high toxicity and rapid emergence of high-level aminoglycoside resistance have severely limited the usefulness of this class of antibiotics. Numerous aminoglycoside resistance mechanisms have been identified, and enzymatic acetylation, phosphorylation and ribosylation are the primary causes of high level resistance in most clinical isolates.[3] Of the modifying enzymes, the acetyl- and phosphotransferases (AAC and APH) have been extensively studied with respect to their specificity.[3,4] To tackle the problem of antibiotic resistance, we are developing novel bifunctional aminoglycosides that can resist or inhibit aminoglycoside-modifying enzymes while simultaneously targeting ribosomal RNA.

The dissociation constant (K_d) and binding stoichiometry were determined using surface plasmon resonance (SPR) against an immobilized rRNA sequence modeling the A-site of prokaryotic rRNA (Figure 1).[5c,d] The dissociation constants were obtained from equilibrium binding curves through nonlinear curve-fitting and were comparable to those obtained using Scatchard analysis. We focused on neamine as it represents the simplest effective aminoglycoside antibiotic and contains the key β-hydroxyamine motif for interaction with the phosphodiester group and the Hoogsteen face of guanine residues in RNA (Figure 1b).[6] Neamine was found to bind biotinylated AS-wt in a 2:1 complex with a K_d of 10 μM for each binding site (Figure 1c). Various dimers of neamine were therefore constructed to identify a bivalent aminoglycoside that would bind AS-wt with high affinity (Figure 1d), and at the same time resist or inhibit the modifying enzymes due to its unnatural structure.[7]

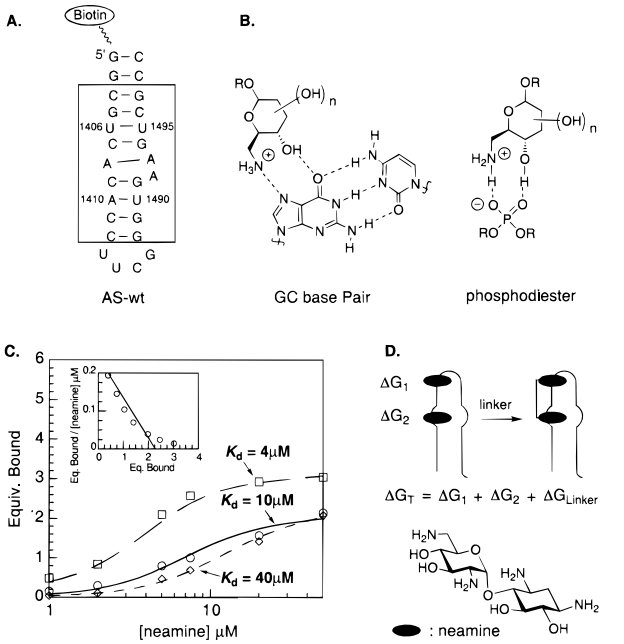

Figure 1. (A) Biotinylated *E. coli* 16S rRNA A-site (AS-wt) rRNA sequence, (B) The mode of action of β-hydroxyamine commonly found in aminoglycosides antibiotics, (C) Binding isotherm and Scatchard plot (inset) of neamine binding to AS-wt (circles) and control mutants (U1406A, squares; U1485A, diamonds) for determination of dissociation constants (K_d = inverse slope) and binding stoichiometry (x-intercept). The binding is sequence selective. (D) Energetic analysis of a bivalent neamine.

Neamine dimers were prepared starting from perbenzyl perazido 5-*O*-carboxyethylneamine[8] (see Scheme 1), which was prepared from the 5-*O*-allyl precursor.[5e] Carboxyethylneamine was distributed into a Quest 210 parallel synthesizer and was activated using a cyclohexylcarbodiimide bound to macroporous polystyrene resin. Resin (2 equiv), acid (1 equiv), and various diamines (0.4 equiv each) were utilized to synthesize a library of neamine dimers of variable linker length. The intermediate amides were isolated by filtration and were >95% pure, as determined by NMR. The resulting dimers were first reduced under Staudinger conditions to convert the azides to amines, which were captured from solution using the resin-bound sulfonic acid scavenger MP-TsOH (Argonaut). The resin was washed, and the free amine was released from the resin by elution with 2 M NH_3 in methanol. The resulting amines were debenzylated by hydrogenolysis in the presence of 2 equiv of acetic acid per amine. The reaction mixture was filtered, concentrated, and purified by silica gel chromatography using 8:2:4:5 $NH_4OH-CHCl_3-n$-BuOH−EtOH, followed by cation exchange chromatography to give the pure aminoglycoside dimers **4**−**13**. The amide-linked dimers could also be prepared via Ugi reactions, for example, dimer **14**, starting from the same perbenzyl perazido 5-*O*-carboxyethylneamine. This procedure is also directly applicable to parallel synthesis and could be used to increase the molecular diversity of the library.

The dimers with the highest affinity for AS-wt determined by SPR were also the most potent antibiotics, as determined by the

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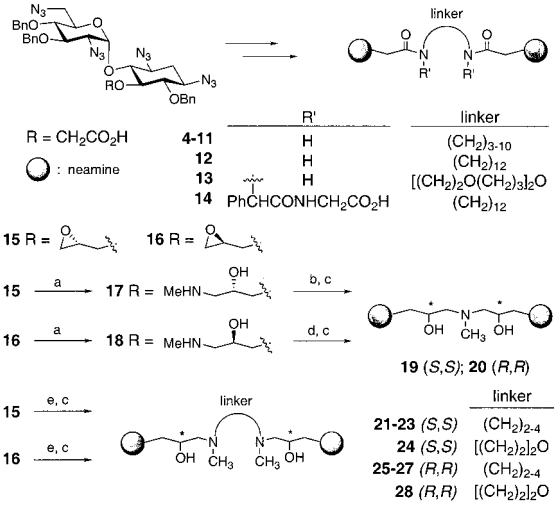
Scheme 1. Neamine Dimers Linked via Amides and 1,2-Hydroxyamines[a]



[a] (a) CH_3NH_2; (b) **15**, EtOH, 95 °C, 16 h; (c) i. $P(CH_3)_3$, THF, H_2O; ii. 20% $Pd(OH)_2$/C (Degussa), H_2, H_2O, AcOH; (d) **16**, EtOH, 95 °C, 16 h; (e) diamine, EtOH, 95 °C, 16 h.

antimicrobial assays[5e,9] and by IC_{50} of in vitro translation.[5e] Of this series, the dimers with the highest antibiotic activity, **4** and **6**, showed a K_d of 1.1 and 0.8 μM on AS-wt, respectively, 10-fold greater than neamine. Dimers with longer linker lengths had weaker affinities for AS-wt, a trend that correlated with antibiotic activity. Interestingly, all of the dimers continued to display a 2:1 binding stoichiometry, indicating that the increase in affinity is most likely due to an additional favorable (not dimeric) yet weak interaction with AS-wt. Antibiotic activities of dimers **4** and **6** were comparable to neamine, MIC = 31 and 125 μM, respectively, against the *Escherichia coli* reference strain (See Supporting Information for antibiotic testing data).

The relatively weak antibiotic activity of these dimers led us to design a flexible and hydrophilic linker by opening the 1,2-propyloxirane with an amine as shown in Scheme 1. The triflate of (*S*)-(−)- and (*R*)-(+)- glycidol[10] was used to alkylate perbenzyl perazido neamine to form epoxides **15** and **16**, respectively. Epoxides **15** and **16** were heated for 16 h in a sealed tube with excess methylamine to form 1,2-hydroxy amines **17** and **18**, respectively. These hydroxy amines could then be used in an addition reaction with another equivalent of epoxide **15** or **16** to form dimers **19** and **20**, respectively, after deprotection. Epoxides **15** and **16** were also opened with 0.5 equiv of a *N*,*N*′-methyldiamines to afford protected dimers **21**−**28**. *N*,*N*′-methyldiamines that were not commercially available were readily prepared by a one-pot synthesis via imine formation with a primary diamine and benzaldehyde, alkylation of the intermediate imime with dimethyl sulfate followed by hydrolysis of the alkylimine afforded *N*,*N*′-methyldiamines in high yield.[11] The resulting dimers were deprotected as previously described to afford dimers **21**−**28**. These dimers possessed significantly increased antibiotic activity compared to the amide-linked dimers. Antibiotic activity was greatest with the diaminobutane linker in dimer **27**, which showed a MIC = 6.25, μM against *E. coli* and K_d = 40 nM (AS-wt) with 1-to-1 stoichiometry.[12]

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(12) Compound **27** is also effective against other strains, including *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa*, PAO-1, *S. aureus* ATCC 29213 and ATCC 33591-MRSA, and *Enterococcus faecalis* ATCC 29212 and is 3 times more effective than tobramycin against the tobramycin-resistant strain *of P. aeruginosa* from cystic fibrosis patients.

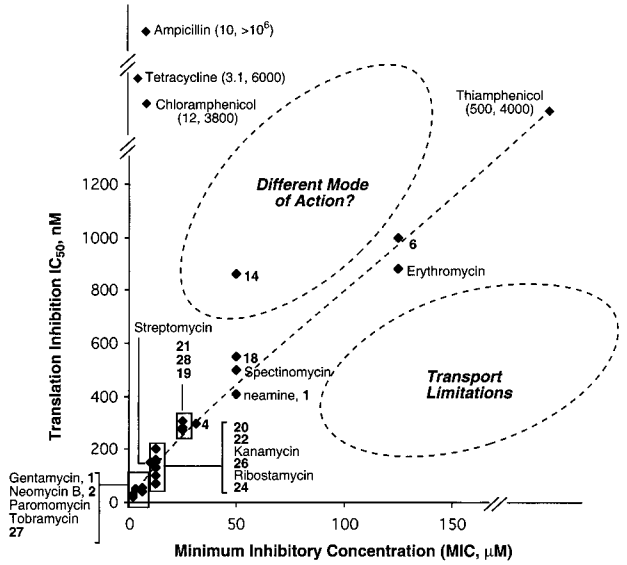

Figure 2. The relationship between antibiotic activity (MIC, minimum inhibitory concentration) and translation inhibition (IC_{50}). The compounds above the line do not target RNA and have different modes of antibiotic action, while those to the right of the line exhibit transport limitations.

In an attempt to better understand the relationship between RNA binding and antibiotic activity, inhibition of in vitro translation of luciferase gene[5e] was measured as a function of MIC, Figure 2. This analysis was used to validate the target and characterize potential transport limitations for the aminoglycosides, and in vitro translation inhibition is expected to be a better indicator of aminoglycoside selectivity for 16S rRNA compared to binding affinity measurements with the A-site sequences.[5c−e] A nearly linear relationship between the IC_{50} of translation inhibition and the MIC was observed. This analysis should be useful for analyzing structure activity relationships within a similar series of compounds. Compounds falling below the line in Figure 2 may suffer from transport limitation while compounds above the line may act via a fundamentally different mode of action than compounds at or near the line.

Further study of neamime dimers **4**, **6**, and **27** using several aminoglycoside-modifying enzymes revealed that the dimers were poor substrates for AAC(6′)-Ii and APH(3′)-IIIa, responsible for 6′- and 3′-*N*-acetylation and *O*-phosphorylation, respectively.[3] In addition, dimers **4**, **6**, and **27** were poor substrates for the AAC(6′) activity of the bifunctional aminoglycoside modifying-enzyme AAC(6′)−APH(2″),[3,4] and not substrates for the APH(2″) activity of AAC(6′)−APH(2″). They were in fact potent competitive inhibitors of the APH(2″) activity, K_{is} = 0.8 μM for dimer **4**, 0.1 μM for **6**, and 0.7 μM for **27**.

In summary, the neamine dimers prepared in this study represent a new class of aminoglycoside antibiotics that are functionally simpler than previously known aminoglycosides. In addition, they are potent inhibitors of the APH(2″) activity of the bifunctional AAC(6′)−APH(2″) enzyme, one of the most clinically significant of the aminoglycoside-modifying enzymes. This research thus provides a new direction for the development of novel antibiotics that target bacterial RNA and resistance-causing enzymes.

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Supporting Information Available: Detailed procedures for the synthesis of compounds **4**−**28** as well as their characterization and functional analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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