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# Investigation of antibacterial mode of action for traditional and amphiphilic aminoglycosides

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ABSTRACT

Aminoglycoside represents a class of versatile and broad spectrum antibacterial agents. In an effort to revive the antibacterial activity against aminoglycoside resistant bacteria, our laboratory has developed two new classes of aminoglycoside, pyranmycin and amphiphilic neomycin (**NEOF004**). The former resembles the traditional aminoglycoside, neomycin. The latter, albeit derived from neomycin, appears to exert antibacterial action via a different mode of action. In order to discern that these aminoglycoside derivatives have distinct antibacterial mode of action, RNA-binding affinity and fluorogenic dye were employed. These studies, together with our previous investigation, confirm that pyranmycin exhibit the traditional antibacterial mode of action of aminoglycosides by binding toward the bacterial rRNA. On the other hand, the amphiphilic neomycin, **NEOF004** disrupts the bacterial cell wall. In a broader perspective, it verifies that structurally modified neomycin can exert different antibacterial mode of action leading to the revival of activity against aminoglycoside resistant bacteria.



Aminoglycosides are structurally diverse antibiotics that exert broad spectrum activity against both gram positive and negative bacteria.[1,2] During the initial stage of antibacterial mode of action, aminoglycosides are imported by aerobic bacteria via a membrane-associated ATP-driven transportation system, and then bind selectively toward the A-site decoding region of 16S RNA leading to the disruption of protein synthesis and the eventual cell death. In an effort to provide new aminoglycosides, our group has synthesized two new classes of aminoglycosides that show prominent antibacterial activity.[3–5] One of the new classes of aminoglycosides, pyranmycin, is structurally related to the neomycin class bearing a 4,5-disubstituted 2-deoxystreptamine (DOS, ring II) core (Fig. 1).[6] However, pyranmycin contains a pyranose as the ring III component while neomycin has a furanose. The other class, which can be synthesized from commercially available neomycin, has the characteristic amphiphilic structural scaffold with a combination of polar aminoglycoside core and hydrophobic lipid chains (Fig. 2).

From our previous study,[3,7] we have observed that pyranmycin class of aminoglycosides, including the lead compound pyrankacin, have antibacterial profile similar to traditional aminoglycosides, such as, neomycin. In contrast, the lead amphiphilic aminoglycoside, **NEOF004** ($R = C_{15}H_{31}$), not only has prominent antibacterial activity against typical aminoglycoside resistant bacteria but also displays extraordinary activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[5a] The activity of **NEOF004** against VRE is peculiar since facultative enterococci are intrinsically resistant against traditional aminoglycosides, including neomycin, kanamycin or chemically synthesized pyranmycin, due to the lack of aminoglycoside-uptake mechanisms. Schweizer and co-workers have also reported that amphiphilic aminoglycosides exert broad spectrum antibacterial activities.[8] Mingeot-Leclercq and co-workers have showed the membrane distortion of *Pseudomonas aeruginosa* due to the presence of different amphiphilic aminoglycosides (Fig. 2).[9] Nevertheless, our in vitro enzymatic studies reveal that **NEOF004**, like other synthetic neomycin derivatives, has similar $K_M/k_{cat}$ toward an aminoglycoside modifying enzyme (AME), APH(3′)-I and, therefore, should not be active against aminoglycoside resistant bacteria.[5] The discrepancy between antibacterial profile and enzyme kinetic prompts us to investigate two questions: (1) does pyranmycin have the same binding site as the traditional aminoglycosides? (2) What is the actual antibacterial mode of action of **NEOF004**? In order to confirm that pyranmycin has the same antibacterial mode of action as neomycin, we decided to employ the binding affinity study using fluorogenic RNA constructs that mimic the bacterial rRNA targeted by neomycin. To answer the second question, we used a fluorogenic dye, SYTOX, which offers direct evaluation for the membrane disruption of bacteria.[10]

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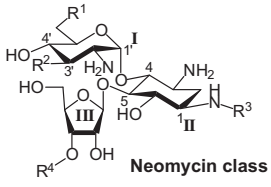


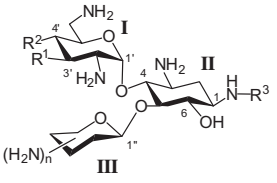


| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| Neomycin B | $NH_2$ | OH | H | DAG |
| Paromomycin I | OH | OH | H | DAG |
| Lividomycin B | OH | H | H | DAG |
| Butirosin B | $NH_2$ | OH | AHB | H |
| Ribostamycin | $NH_2$ | OH | H | H |

$R^1$, $R^2$ = H or OH

$R^3$ = H or AHB

DAG: 2,6-diamino-2,6-dideoxy–α-L-glucopyranosyl

AHB: (*S*)-4-amino-2-hydroxybutanoyl

**Figure 1.** Structure of neomycin and pyranmycin classes aminoglycosides.

The structures of the selected aminoglycosides, which include eight members of the pyranmycin class and two members of the neomycin class, are shown in Figure 3. The minimum inhibitory concentrations (MIC's) against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), have been determined previously (Table 1).[3–6] **TC005**, **RR501**,[11] and pyrankancin,[12] which all bear a 6-deoxy-4-aminoglucopyranose as the ring III component, and **NEOF004** were found to be the most active against *E. coli* and *S. aureus*. In addition, the attachment of (*S*)-4-amino-2-hydroxybutanoyl (AHB) group at N-1 position of ring II further increases the antibacterial activity.

Two model RNA constructs (RNA1 and RNA3) were used for the binding study (Fig. 4). The binding of aminoglycosides will trigger the conformational 'flipping out' of A1492 and A1493. Both model constructs have A1493 substituted with 2-aminopurine (2AP), a fluorescent analogue of adenine, to monitor interactions with aminoglycoside ligands. Upon the binding of aminoglycosides, 2-AP undergoes conformational flipping and results in decreases in fluorescence response. Both model RNA constructs were confirmed in our earlier study to demonstrate a sigmoidal binding curve with neomycin with comparable $K_d$ values reported for wt RNA target.[13] RNA1 has the pairing of G1405–C1496 that is identical to the WT RNA whereas RNA3 has reversed pairing at the same positions. These RNA constructs were purchased from Dharmacon PAGE-purified, 2′-deprotected, & desalted. They were used directly without further purification. Concentration of the RNA constructs was determined using extinction coefficient values of 244,200 L/mol cm and 242,200 L/mol cm at 260 nm, respectively for RNA1 and RNA3.

The binding affinity ($K_d$) was measured using the reported fluorescence assay protocol.[13] To RNA constructs labeled with 2AP (0.5 μM) were added small aliquots of aminoglycosides (0.9 μL of 0.05 μM–5 mM). After each titration, fluorescence was measured

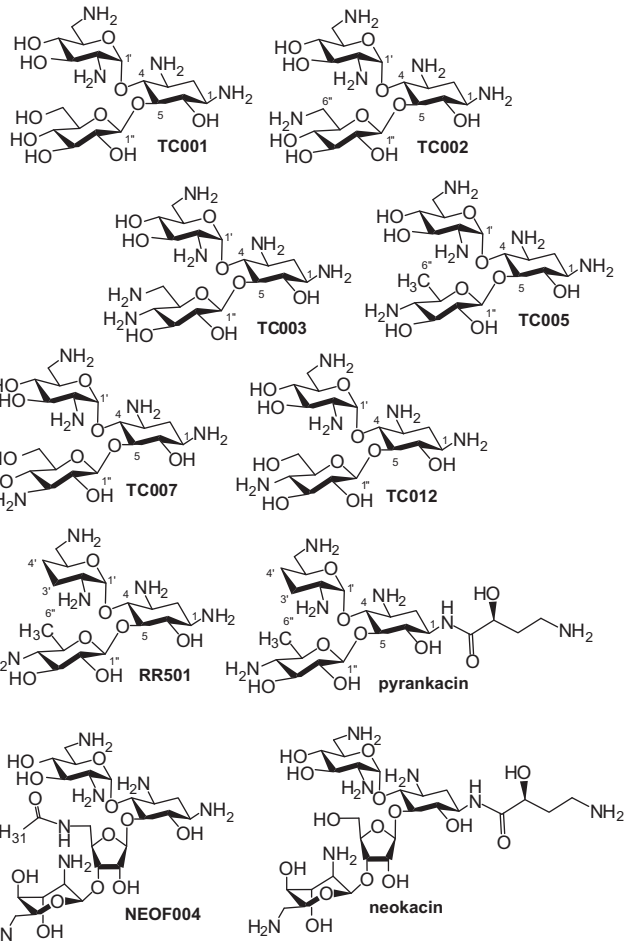


**Figure 3.** Selected pyranmycin for RNA binding study.

at 330–420 nm with its max wavelength at 370 nm while excited at 300 nm. Throughout the titration, total volume change was kept less than 7% of the initial volume, of which was factored in the calculation. Each titration curve was fitted using Sigmaplot to obtain $K_d$ values for each aminoglycoside. Neomycin that was used as a control demonstrated a sigmoidal binding curve with its dissociation constant at 1.148 μM, which is in good agreement with the previously reported $K_d$ values. A typical binding profile for several selected aminoglycosides is shown in Figure 5. The binding affinities and the MIC's are shown in Table 1.

From the binding affinity investigation, several interesting discoveries were noted. For example, similar to what have been reported previously,[10] binding affinity toward the RNA targeted by aminoglycosides cannot be correlated with the antibacterial

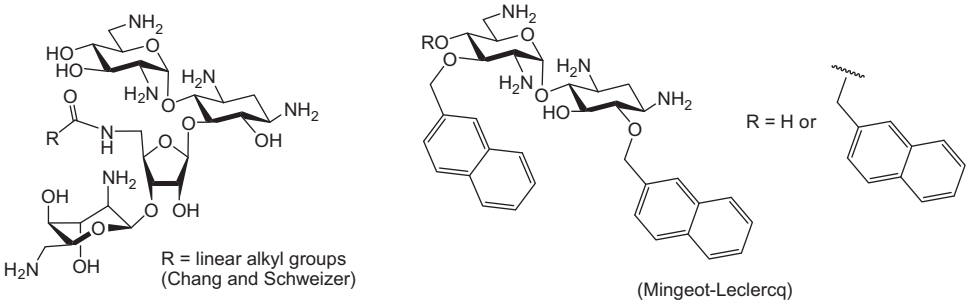


**Figure 2.** Amphiphilic aminoglycosides.