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ARTICLE INFO

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.

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that pyranmycin has the same antibacterial mode of action as pyrankacin, have antibacterial profile similar to traditional aminoglycosides, such as, neomycin. In contrast, the lead amphiphilic aminoglycoside, NEOF004 (R = C₆H₁₃), 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). The activity of NEOF004 against VRE is peculiar since facultative enterococci are intrinsically resistant against traditional aminoglycosides, including neomycin, kanamycin or chemically synthesized pyramycin, due to the lack of aminoglycoside-uptake mechanisms. Schweizer and co-workers have also reported that amphiphilic aminoglycosides exert broad spectrum antibacterial activities. Mingeot-Leclercq and co-workers have showed the membrane distortion of *Pseudomonas aeruginosa* due to the presence of different amphiphilic aminoglycosides (Figure 2). Nevertheless, our in vitro enzymatic studies reveal that NEOF004, like other synthetic neomycin derivatives, has similar $K_u/k_\text{cat}$ toward an aminoglycoside modifying enzyme (AME), APH(3’)-I and, therefore, should not be active against aminoglycoside resistant bacteria. The discrepancy between antibacterial profile and enzyme kinetic prompts us to investigate two questions: 1) does pyramycin 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 pyramycin 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.

The structures of the selected aminoglycosides, which include eight members of the pyramycin class and two members of the neomycin class, are shown in Figure 3. The minimum inhibitory concentrations (MICs) against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), have been determined previously (Table 1). Two model RNA constructs were confirmed in our earlier study to have N-1 position of ring II further increases the antibacterial activity.

Two model RNA constructs (RNA1 and RNA3) were used for the binding study (Figure 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. 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 Dharmaco PACE-purified, 2’,5’-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. 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 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 Sigmamart to obtain $K_d$ values for each aminoglycosides. 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

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**Figure 5. Stated Pyramycin for RNA Binding Study**

**Figure 4. RNA Constructs for Binding Affinity Study**

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shown in Figure 5. The binding affinities and the MIC's are shown in Table 1.

**Table 1. Binding Affinity ($K_a$) and Minimum inhibitory concentrations (MIC) $^a$**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity</th>
<th>MIC E. coli (ATCC 25922)</th>
<th>MIC S. aureus (ATCC 25923)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA1</td>
<td>RNA3</td>
<td></td>
</tr>
<tr>
<td>Neomycin</td>
<td>1.148</td>
<td>1.270</td>
<td>4.4</td>
</tr>
<tr>
<td>TC001</td>
<td>9.667</td>
<td>0.608</td>
<td>92.4</td>
</tr>
<tr>
<td>TC002</td>
<td>26.05</td>
<td>0.845</td>
<td>35.1</td>
</tr>
<tr>
<td>TC003</td>
<td>3.720</td>
<td>0.261</td>
<td>41.8</td>
</tr>
<tr>
<td>TC005</td>
<td>NB $^b$</td>
<td>0.556</td>
<td>19.8</td>
</tr>
<tr>
<td>TC007</td>
<td>NB</td>
<td>1.152</td>
<td>57.2</td>
</tr>
<tr>
<td>TC012</td>
<td>5.675</td>
<td>2.827</td>
<td>44.0</td>
</tr>
<tr>
<td>RR501</td>
<td>1.331</td>
<td>1.203</td>
<td>26.0</td>
</tr>
<tr>
<td>Pyranksin</td>
<td>0.650</td>
<td>1.357</td>
<td>5.59</td>
</tr>
<tr>
<td>NEOF004</td>
<td>1.269</td>
<td>0.737</td>
<td>4.69</td>
</tr>
<tr>
<td>Neokacin</td>
<td>NB</td>
<td>1.642</td>
<td>8.59</td>
</tr>
</tbody>
</table>

$^a$ Unit: µM, $^b$ No binding affinity can be measured.

From the binding affinity investigation, several interesting discoveries were noted. For example, similar to what has been reported previously, binding affinity toward the RNA targeted by aminoglycosides cannot be correlated with the antibacterial activity of aminoglycosides. In general, aminoglycosides with more hydrophilic groups (NH$_2$ or OH) will have higher binding affinity (lower $K_a$) toward the targeted RNA's. For instance, TC003 that has a total of six amino groups manifests the highest binding affinity among other pyranmycin members. However, TC003 only displays modest or even less antibacterial activity as compared to other members. On the other hand, RR501 that has two less hydroxyl groups at 3' and 4' positions and consequently lower binding affinity shows superior antibacterial activity. Similar discrepancy between $K_a$'s and MIC's has also been reported previously.

Interestingly, initial binding study from three pyranmycin members like (TC005, TC007 and neokacin) demonstrated abnormal binding patterns toward RNA1, which mimics the targeted bacterial RNA sequences (Figure 4a). They demonstrated increase in fluorescence reading as the concentration of each aminoglycoside was increased while other pyranmycin members and neomycin demonstrated sigmoidal binding curves. This means that these three pyranmycin members may interact differently in the region of WT RNA potentially by non-specific interactions, probably due to the presence of non-natural 2-AP. However, by using structurally similar RNA3, normal binding mode was observed for all the tested aminoglycosides, including TC005, TC007 and neokacin. Since neomycin showed very similar binding affinities toward both RNA1 and RNA3, we believe that the abnormal binding of TC005, TC007 and neokacin toward RNA1 is due to the presence of 2-AP. Thus, these synthetic aminoglycosides are all capable of binding to the A-site RNA but may interact differently in the region via non-specific interactions, probably due to the presence of non-natural 2-AP. Similar to the reported result, the attachment of AHB group at the N-1 as in the case of pyranksin, a synthetic aminoglycoside, can increase the binding affinity. Pyranksin that has the highest antibacterial activity also has the best binding affinity.

Surprisingly, when comparing neokacin that has an AHB group at the N-1 position and NEOF004 that has a much larger hexadecanoyl (C16) group at the 5" position, we noted that NEOF004 has a related stronger binding affinity than several other pyranmycin members and even neomycin. Since the high lipophilicity of the hexadecanoyl group is not expected to interact closely with the highly charged RNA's, these results imply: 1) the RNA constructs and, possibly, the WT RNA's contain several that these three aminoglycosides, including TC005, TC007 and neokacin, for instance, that has a much larger lipophilic group at the 5" position, we noted that NEOF004 has a related stronger binding affinity than several other pyranmycin members and even neomycin. Since the high lipophilicity of the hexadecanoyl group is not expected to interact closely with the highly charged RNA's, these results imply: 1) the RNA constructs and, possibly, the WT RNA's contain several that these three aminoglycosides, including TC005, TC007 and neokacin, for instance, that has a much larger lipophilic group at the 5" position, we noted that NEOF004 has a related stronger binding affinity than several other pyranmycin members and even neomycin. Since the high lipophilicity of the hexadecanoyl group is not expected to interact closely with the highly charged RNA's, these results imply: 1) the RNA constructs and, possibly, the WT RNA's contain several

From our previous study, we have reported that NEOF004 has prominent antibacterial activity against aminoglycoside resistant bacteria MRSA and VRE. Pyranksin, including TC005 and pyranksin, acts like traditional aminoglycosides (neomycin, amikacin and gentamicin), and are much less active or even inactive against MRSA, which is equipped with various AME's, including APH(3'), ANT(4'), and AAC(6')-APH(2')

Pyranksin also behaves like traditional aminoglycosides that are inactive (MIC $\geq$ 250 μg/mL) against VRE, which does not import aminoglycosides. Combining with the binding affinity study, it strongly suggests that NEOF004 must exert its antibacterial activity outside the bacteria and the bacterial
membrane, as reported by Mingeot-Leclercq and co-workers, is the most likely target.

For investigating the membrane disruption, we used a fluorogenic dye, SYTOX. SYTOX dye cannot penetrate the intact bacterial membrane. However, if the membrane is damaged by membrane disrupting agents, SYTOX dye can bind to nucleic acid and emit strong fluorescence. The experiment was conducted with procedure modified from the literature using *E. coli* and *S. aureus* as the tested strains. PI pictures are taken under phase mode (optical view) (Figures 6a, 6c and 6e) and fluorescence mode (Figures 6b, 6d and 6f). Since the membrane of Gram positive *S. aureus* is more prone to the action of membrane disrupting agents, we tested only *S. aureus* against neomycin. Following the treatment of NEOF004, many cells show significant green fluorescence (Figures 6b and 6d). In contrast, cells (S. aureus) treated with neomycin show no sign of membrane disruption which is expected from a traditional aminoglycoside (Figure 6f). The result from using SYTOX confirms that NEOF004 causes damage to the bacterial membrane. The observed binding of NEOF004 is likely due to the absence of complete ribosome which contains both ribosomal RNA's and proteins.

**Figure 6.** Mode of Action Investigation Using SYTOX Dye

<table>
<thead>
<tr>
<th>phase modes</th>
<th>fluorescence mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) <em>E. coli</em> treated with NEOF004 (2hrs)</td>
<td>(b) <em>E. coli</em> treated with NEOF004 (2hrs)</td>
</tr>
<tr>
<td>(c) <em>S. aureus</em> treated with NEOF004 (2hrs)</td>
<td>(d) <em>S. aureus</em> treated with NEOF004 (2hrs)</td>
</tr>
<tr>
<td>(e) <em>S. aureus</em> treated with neomycin (2hrs)</td>
<td>(f) <em>S. aureus</em> treated with neomycin (2 hrs)</td>
</tr>
</tbody>
</table>

In conclusion, the binding affinity investigation confirms that pyramycin has the same antibacterial mode of action as neomycin by binding toward the A-site of 16S RNA. Together with the enzyme kinetic study and whole cell-based antibacterial assay, these pyramycin members behave like traditional aminoglycosides. The binding affinity of NEOF004 also suggests that the antibacterial activity of aminoglycosides cannot be correlated with the binding affinity of aminoglycosides. The reason for the observed discrepancy is that factors, such as, cellular uptake and salvation of aminoglycosides simply cannot be evaluated via *in vitro* binding affinity measurement. It is, therefore, essential to employ whole cell-based antibacterial assay to obtain actual antibacterial activity for compounds of interest.

Finally, we have shown that chemical modification of neomycin, as in the case of NEOF004, can lead to the production of new aminoglycoside with novel antibacterial mode of action. Our group has previously reported that similar modification on karnamycin, a class of aminoglycoside with 4,6-disubstituted 2-deoxystreptamine core, will turn the modified aminoglycoside into antifungal agent. Together with the results from Mingeot-Leclercq and co-workers, it demonstrates that simple chemical modifications on an old drug can serve as a potentially useful method for new drug development.

**Acknowledgments**

We thank the financial support from USTAR TCG grant. We also thank Professor Takemoto from Department of Biology at USU for his valuable suggestion and discussion in using the fluorescence dyes. We thank the technical support from Mr. Sanjib Shrestha at Biology Department, and Prof. Dong Chen at the USTAR Synthetic Bio Manufacturing Center (SBC), USU for allowing the access of fluorescence microscope.

**References and notes**

Procedure for SYTOX Dye Assay. The bacterial cells were washed with HEPES buffer (1M, pH 6.5, KOH). After removal of HEPES and re-suspension in ddH$_2$O, the bacterial cells were mixed with the tested aminoglycosides at 4 times the individual MIC's and incubated at 30°C with agitation. After the incubation for 10 min and 2 hrs, the bacterial solution was stained with 0.5 uM of SYTOX dye (10 mM) for 10 min in the agitated incubator (30°C). Pictures were taken under the phase mode and fluorescence mode. The slides were stored at 4°C in dark prior to microscope examination.