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High-throughput Assay for Quantification of Aminoglycoside–Ribosome Interaction

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The serious side effects of aminoglycosides and the spread of aminoglycoside-resistant strains have restricted the clinical application of aminoglycosides. A compound with the identical mode of action with aminoglycosides and with different molecular skeleton would be an alternative drug for aminoglycosides. In this study, we constructed an SPR-based high-throughput screening system for the discovery of such compounds.

Keywords: Aminoglycoside | Antibiotics | Ribosome

Aminoglycosides are highly potent and broad-spectrum antibiotics, which are active against most Gram-negative aerobic bacteria such as *Pseudomonas*, *Acinetobacter*, and *Enterobacter* species.[1] The first aminoglycoside, streptomycin, was isolated from *Streptomyces griseus* in 1944, which was used as the first effective therapeutic agent for the treatment of tuberculosis.[2] So far, several aminoglycosides such as neomycin B, tobramycin, gentamicin, and paromomycin (PAR), have been identified and have been used to combat severe infectious diseases (Figure 1a).

Aminoglycosides bind to the decoding A-site, where the ribosome accurately selects aminoacyl tRNA (Figure 1b).[3] The binding of aminoglycosides to helix 44 of 16S-rRNA forces two adenine bases (A1492 and A1943) in the bulged-out conformation, which plays a critical role for codon–anticodon recognition.[4] Consequently, aminoglycosides perturb the protein synthesis by impairing the proofreading process, leading to the production of nonfunctional proteins.[5] The structure of the ribosome differs between prokaryotes and eukaryotes, especially in residues A1408 and G1491 of 16S rRNA.[6] The aminoglycosides that are less active to mammals than bacteria can be used as antibiotics.

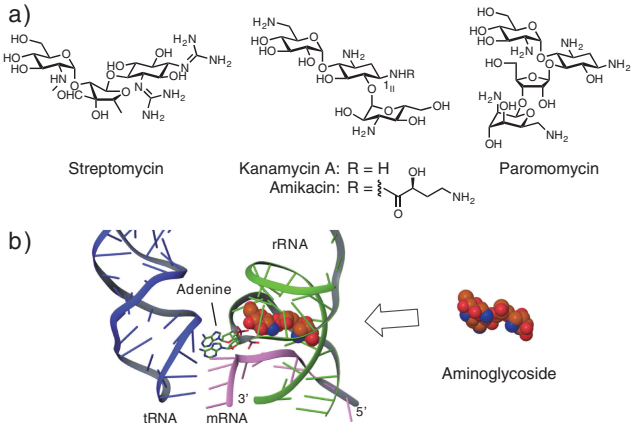


Figure 1. Aminoglycoside antibiotics. a) Structures of aminoglycoside antibiotics. b) The mode of action for aminoglycosides. Aminoglycosides binds to the A-site rRNA constituting decoding center leading to the production of inaccurately structured proteins.

The concerns with the clinical use of aminoglycosides are nephrotoxicity and ototoxicity, which are the major reasons for therapeutic limitation of aminoglycosides.[7] Diverse mechanisms are proposed for the exertion of side effects. For instance, mutations in the A-site rRNA of the mitochondrial ribosome induces hypersensitivity to aminoglycoside-derived side effects, indicating that the binding of aminoglycosides to the mitoribosome can be a cause of the side effects.[8] Despite their serious side effects, aminoglycosides have commonly been used to treat infectious diseases because the clinical efficacy of aminoglycoside is irreplaceable. In particular, aminoglycosides have been used to treat chronic lung infections associated with cystic-fibrosis.[9]

Another concern with aminoglycosides is the emergence of drug-resistant strains. The major mechanism of resistance is reduced drug uptake, accumulation in bacteria, and expression of aminoglycoside-modifying enzymes, such as acetyltransferases, phosphotransferases, and adenyltransferases.[10] To overcome these issues, various aminoglycoside derivatives have been synthesized and their antibiotic activities to the resistant bacteria have been tested.[11] For example, amikacin is a semi-synthetic aminoglycoside, of which the 1_{II}-position of kanamycin A is chemically modified, is used to treat multidrug-resistant tuberculosis. However, the newly developed aminoglycosides often cause new drug-resistant strains like other antibiotics. Especially, multidrug-resistant strains are causing serious problems for hospitalized patients.

One of the possible strategies to overcome the concerns with aminoglycosides is to develop non-aminoglycoside compounds that bind to the aminoglycoside-binding site in ribosome. Such compounds should be potent antibiotic drugs against existing aminoglycoside-resistant strains. In addition, a compound with a higher selectivity to the bacterial A-site should have fewer side effects than aminoglycosides. However, lack of an efficient method for evaluating the ribosome–ligand interaction has hampered the screening of such compounds. We herein describe a high-throughput screening system for ligands that bind to the 16S-rRNA A-site.

As shown in Figure 2a, we designed a competition assay for the evaluation of ribosome-binding molecules based on surface plasmon resonance (SPR). The injection of A-site model hairpin RNA (Figure 2b) to the aminoglycoside-immobilized SPR sensor should result in the increase of the response signal, whereas the co-injection of a competitive ligand to the A-site should impede the increase in response.[3,12] Since the SPR

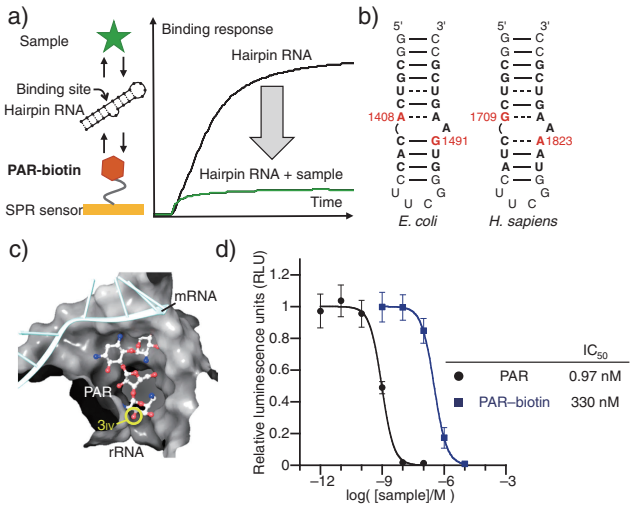
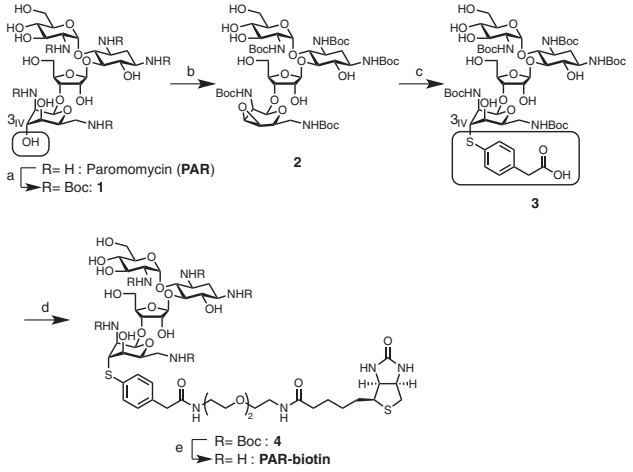


Figure 2. a) Illustration of competition assay for analyzing the binding of ligand to A-site hairpin RNA. Sample binding can be detected by the decrease in response. b) Secondary structures of hairpin RNAs. Solid lines during the strands represent Watson–Crick base pairs, while dashed lines represent mismatched base pairs. c) The binding structure of paromomycin to bacterial ribosome (PDBid: 4V51). d) Translation inhibition activity of paromomycin derivatives on *E. coli* ribosome. The results are the averages of at least three independent experiments and the mean ± s.d. is plotted.

response is proportional to the change in mass concentration on the surface, the competition assay should be more sensitive than direct detection of small molecular ligand with immobilized RNA. According to the crystal structure of paromomycin in complex with bacterial ribosome, we assumed that modification of the 3_{IV} position of paromomycin has less effect on the paromomycin–ribosome interaction compared to the other positions (Figure 2c).[3,12] Therefore, we introduced biotin to the 3_{IV} position of paromomycin. Synthesis of the compound was conducted by following the previously reported method for the modification of neomycin B (Scheme 1).[13] The *talo*-epoxide was synthesized by Mitsunobu reaction of Boc-protected paromomycin and was subjected to an addition reaction with 4-mercaptophenylacetic acid. After the condensation with the biotin containing an amino-linker, the compound was treated under acidic conditions to afford the biotinylated paromomycin (PAR-biotin). The inhibitory activity of PAR-biotin on translation was analyzed using bacterial ribosome. An mRNA-coding firefly luciferase was translated in the presence of paromomycin or PAR-biotin, and the luciferase expression was quantified by a standard luciferase assay.[14] The inhibitory activity of PAR-biotin on the ribosome was considerably weaker than that of paromomycin but still strong with an IC_{50} value below the micromolar range (Figure 2d). These data indicate that PAR-biotin retains strong affinity to the bacterial A-site.

PAR-biotin was then immobilized to the streptavidin-coated surface of an SPR sensor. A solution of hairpin RNA containing the aminoglycoside-binding site in *E. coli* was injected to the sensor. The response increased over 200 s and reached a plateau. The increase of response was dependent on the concentration of hairpin RNA, as shown in Figures 3a and 3b and the dissociation constant was calculated to be 37 nM, which is comparable to the previously reported value of 27 nM obtained



Scheme 1. Preparation of paromomycin derivative **PAR-biotin**. Reagents and conditions: a) Boc_2O, Et_3N, 1,4-dioxane/water, rt, 1 d, 58%; b) DIAD, PPh_3, 4-nitrobenzoic acid, THF/toluene, 0 °C to rt, 3 h; 28% NH_3 aq MeOH, rt, 5 h 75%; c) 4-mercaptophenylacetic acid, NaH, DMF, 0 °C to rt, 65%; d) NHS, EDC·HCl, DMF, rt, 30 min; EZ-Link™ Amine-PEG2-Biotin, DMF, rt, 15%; e) TFA/CH_2Cl_2, rt, 30 min., quant. Boc: *tert*-butoxycarbonyl; DIAD: diisopropyl azodicarboxylate; TFA: trifluoroacetic acid.

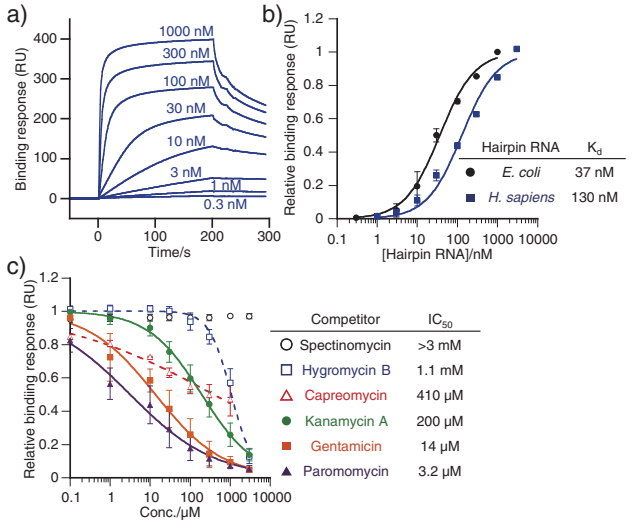


Figure 3. Evaluation of ligand binding to model hairpin RNA of ribosome A-site. a) Concentration dependency of the response. The binding response at 200 s was plotted against the concentration of hairpin RNA. The dissociation constant of each RNA to paromomycin was obtained by curve fitting. b) SPR sensorgram for the binding of model hairpin RNA to the paromomycin-immobilized SPR sensor surface. c) Results of competition assay for known A-site ligands as well as aminoglycosides that do not bind to A-site helix 44 of 16S-rRNA. Each compound (0–3 mM) was co-injected with hairpin RNA (50 nM). The response at 200 s was plotted against the concentration of hairpin RNA.

from isothermal titration calorimetry experiment.[6a] The binding of human ribosome hairpin RNA was evaluated as well, and turned out to be 130 nM, which is of the same order as the previously reported value of 420 nM. These data suggest that the SPR-based analysis of RNA–aminoglycoside interaction

correctly reflects the affinity in solution. The reduced activity of PAR-biotin on translational inhibition may indicate that the complex structure of ribosome hinders the binding of paromomycin derivative containing a large substituent.

The competition assay using this system was demonstrated with well-defined aminoglycosides. The hairpin RNA was co-injected with spectinomycin, hygromycin B, kanamycin A, gentamicin, or paromomycin and the response at 200 s was plotted against the concentration of aminoglycosides (Figure 3c). The known ligands to the A-site rRNA, such as paromomycin, gentamicin, and kanamycin A, reduced the response in a concentration-dependent manner. The non-aminoglycoside A-site ligands, capreomycin, also reduced the response. In contrast, spectinomycin and hygromycin B that bind to sites other than the helix 44 of 16S-rRNA barely affected the response. These data suggest that this system well-reflects the binding affinity of ligands to the ribosome A-site.

In summary, we have established an SPR-based method for evaluating ribosome–ligand interaction. Our method is applicable to the screening of A-site binding molecules, which may enable the discovery of novel antibiotic agents.

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