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A fluorescence-based screen for ribosome binding antibiotics

Derrick Watkins [a], F.A. Norris [a,b,1], Sunil Kumar [b], Dev P. Arya [b,*]

[a] NUBAD, LLC., Greenville, SC 29605, USA
[b] Department of Chemistry, Clemson University, Clemson, SC 29634, USA

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ABSTRACT

The development of new antibacterial agents has become necessary to treat the large number of emerging bacterial strains resistant to current antibiotics. Despite the different methods of resistance developed by these new strains, the A-site of the bacterial ribosome remains an attractive target for new antibiotics. To develop new drugs that target the ribosomal A-site, a high-throughput screen is necessary to identify compounds that bind to the target with high affinity. To this end, we present an assay that uses a novel fluorescein-conjugated neomycin (F-neo) molecule as a binding probe to determine the relative binding affinity of a drug library. We show here that the binding of F-neo to a model *Escherichia coli* ribosomal A-site results in a large decrease in the fluorescence of the molecule. Furthermore, we have determined that the change in fluorescence is due to the relative change in the pK_a of the probe resulting from the change in the electrostatic environment that occurs when the probe is taken from the solvent and localized into the negative potential of the A-site major groove. Finally, we demonstrate that F-neo can be used in a robust, highly reproducible assay, determined by a Z'-factor greater than 0.80 for 3 consecutive days. The assay is capable of rapidly determining the relative binding affinity of a compound library in a 96-well plate format using a single channel electronic pipette. The current assay format will be easily adaptable to a high-throughput format with the use of a liquid handling robot for large drug libraries currently available and under development.

Aminoglycoside antibiotics are bactericidal agents composed of two or more amino sugars joined in glycosidic linkage to a hexose nucleus [1,2]. This family of antibiotics has been highly successful in the treatment of many bacterial diseases [3–5]. The target of many aminoglycosides, such as neomycin, is the 16S RNA of the bacterial ribosome. The binding of the aminoglycosides to the bacterial ribosome results in an inhibition of protein synthesis and translational errors that ultimately kill bacterial cells [6]. However, during recent years the emergence of resistant strains of bacteria to aminoglycosides has limited their effectiveness as antibiotics [5,7,8]. The emergence of these resistant strains has necessitated the need for new drugs and next generation aminoglycoside-based compounds to treat these pathogens.

There are numerous mechanisms by which bacteria can become resistant to aminoglycosides, including reduced uptake, changes to the ribosomal binding site, and modifications to the aminoglycosides by bacterial enzymes [9–11]. However, the primary characteristic of compounds with antibiotic mechanisms similar to aminoglycosides remains their ability to bind to the ribosomal A-site with high affinity. Following the identification of compounds that bind to A-site RNA, antibacterial activity can be subsequently screened against resistant bacteria cell lines. Therefore, a large number of compounds and derivatives will need to be generated and screened in order to develop drugs that are not susceptible to the mechanisms of resistance of these pathogenic bacteria. To address this need, the number of compounds for the targeting of the ribosomal A-site is rapidly increasing due in part to the derivations of natural aminoglycosides and synthetic aminoglycoside mimics as well as the development of novel compounds that act by a similar mechanism [5,12–14].

As the number of compounds grows, it becomes more urgent to develop a high-throughput screen for the identification of compounds that bind to the A-site RNA as a first approximation of antibacterial activity. Multiple methods have been used in the identification of potential antibiotics. Nuclear magnetic resonance (NMR)[2] screening methods have been used previously to identify lead compounds that bind to the A-site using competition assays and other techniques [15]. However, these methods require large amounts of RNA and are not adaptable for high- throughput

* Corresponding author.
E-mail address: dparya@clemson.edu (D.P. Arya).
[1] Current address: Department of Biology, Anderson University, Anderson, SC 29621, USA.

[2] *Abbreviations used:* NMR, nuclear magnetic resonance; F-neo, fluorescein–neomycin conjugate; ESP, electrostatic potential; DEPC, diethylpyrocarbonate; EDTA, ethylenediaminetetraacetic acid.

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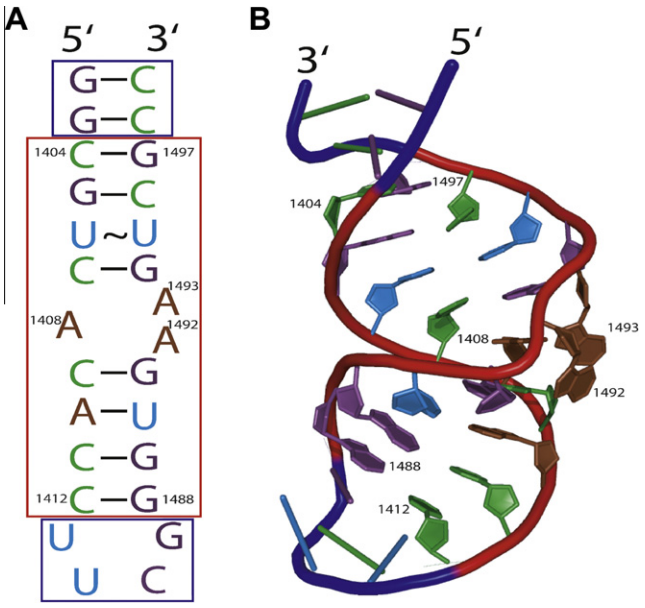

Fig.1. *E. coli* ribosomal A-site. (A) Secondary structure of the model A-site used in the development of the high-throughput screen for drugs that bind to the ribosomal A-site. The red box indicates the residues that are present in the *E. coli* 16S ribosomal RNA (rRNA). (B) Three-dimensional representation of panel A modeled from the NMR structure of the model A-site (PDB ID: 1PBR). Residues present in the *E. coli* 16S rRNA are shown as cartoon bases and are color-coded purple (guanine), green (cytosine), blue (uracil), and brown (adenine) and numbered according *E. coli* ribosome in both panels A and B. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

techniques. Fluorescently labeled RNA has been used in previous screens as a method for monitoring drug–RNA interactions. Although these derivatives have been useful tools in the detection of drug interactions with the A-site, each contains deficiencies for large-scale use in a high-throughput format.

We present here a fluorescence-based competition assay using a 27-base RNA model of the ribosomal A-site (Fig. 1) and a novel fluorescent reporter molecule. The model A-site contains the structural features of the *Escherichia coli* 16S RNA A-site and has been used to chemically and structurally characterize the interactions of aminoglycosides with the A-site [16–19]. In addition, we report the properties of a fluorescein derivative of neomycin (Fig. 2) that shows a robust decrease in fluorescence intensity on binding to the model ribosomal A-site. The fluorescein–neomycin conjugate (F-neo) allows the direct measurement of drug binding to the A-site. In addition, the fluorescein is attached via a thiourea linkage to neomycin at a position that is not predicted to be involved in contacts with the RNA bases in the absence of the fluorescein. We also demonstrate that the intensity change in the fluorescence is a result of a shift in the pK_a for the fluorescein moiety due to the electrostatic potential (ESP) of the RNA. Finally, we show that F-neo can be used as a probe in a competition assay for the screening of compound libraries for A-site binding. This assay is readily adaptable to a high-throughput format as larger compound libraries are established.

Materials and methods

The *E. coli* A-site model RNA oligonucleotide (5′-GGCGUCACAC-CUUCGGGUAAGUCGCC-3′) was synthesized using standard phosphoramidite solid phase synthesis with a 2′ ACE protecting group (Thermo Scientific). All RNA oligos were deprotected before use according to the manufacturer's protocol, and the deprotection buffer was removed by evaporation using a SpeedVac (GeneVac). The RNA oligos were resuspended in diethylpyrocarbonate (DEPC-treated water (OmniPur) to the desired concentration, and the concentrations of oligonucleotides were determined by absorbance at 260-nm and 10-mm pathlengths with a Nanodrop 2000c (Thermo Scientific) using an extinction coefficient provided by the manufacturer. Neomycin (Fisher), paromomycin (MP Biomedicals), gentamycin (Fisher), streptomycin (Fisher), and ribostamycin (MP Biomedicals) were suspended in DEPC-treated water to the stock concentration. Neamine was prepared as described previously [20] and suspended in DEPC-treated water. The neomycin–fluorescein conjugate (F-neo) was synthesized and purified as described previously [21]. Briefly, F-neo was prepared by coupling an activated fluorescein ester with neomycin amine followed by deprotection of Boc groups using trifluoroacetic acid (TFA).

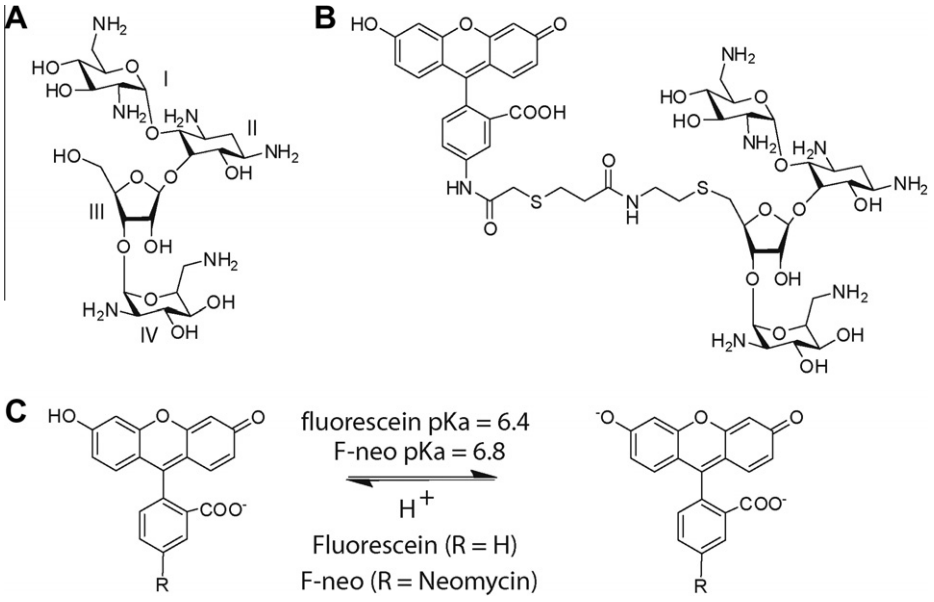

Fig.2. (A and B) Chemical structures of neomycin (A) and fluorescein–neomycin conjugate (F-neo) (B). (C) Equilibrium between the monoanion and dianion of fluorescein or F-neo. Only the dianion has a large fluorescence peak at 517 nm when excited at 490 nm.