



**Trana Discovery, Inc.**

***Staphylococcus aureus* 201  
High-Throughput Screening (HTS)  
Drug Discovery Assay**

**White Paper**

**June 2011**

Support for *S. aureus* assay from North Carolina Biotechnology Center through a SRL loan.

## Executive Summary

The need for new effective antibiotics to treat infections caused by resistant bacteria has never been more evident. While a number of Gram-negative pathogens have presented real treatment challenges over the past 30 years, now what were once considered relatively benign community associated bacteria such as *Staphylococcus aureus* are resistant to second- and third-line treatment options. Since the 1960s, only a limited number of new antibiotics truly represented novel classes. The vast majority of new antibiotics, on the other hand, were merely structurally modified derivatives of existing scaffolds, which still worked by the same mechanisms of action and whose avoidance of resistance by troublesome pathogens was often short-lived. Accordingly, the real solution to discovery and development of highly active and durable treatments for these resistant bacteria is to exploit novel targets. Companies like Trana Discovery, whose technology involves the inhibition of uniquely modified tRNAs used by specific pathogens, are developing high throughput screening assays to identify compounds that work at blocking these novel sites of action.

Trana Discovery, Inc. is a North Carolina based drug discovery technology company whose mission is to help its partners identify drug candidates that work by a novel mechanism of action – the direct inhibition of biochemical processes that involve transfer RNA (tRNA) – for treatment of serious bacterial and viral infectious diseases. Trana currently has a validated high-throughput capable assay for identification of compounds that interfere with the use of tRNA<sup>Lys3</sup> by the human immunodeficiency virus (HIV), the cause of AIDS, and this assay is available for licensing.

Our second assay, and first for a bacterial target, is designed to identify compounds that inhibit the essential use of a *S. aureus* unique tRNA<sup>Arg</sup> that is required for protein synthesis. The assay identifies compounds that interfere with the interaction of a fluorescein-labeled oligonucleotide mimic of the ASL loop of tRNA<sup>Arg</sup> with a programmed ribosome. The validated assay was used to screen a 60,000 compound library from which 283 compounds were initially identified. These compounds were retested in a dose response curve and 89 compounds were confirmed as biochemically active. Thirty-eight of these compounds were selected based on an acceptable IC50 concentration and tested in a bacterial assay where 8 compounds demonstrated activity against 2 or more strains of *S. aureus*. The assay is now fully HTS functional and is immediately available for licensing to the pharmaceutical industry for the discovery, development, and market availability of critically needed new anti-infectives.

## Tackling Resistant Bacteria: the Need for New Targets

The need to discover new classes of antibiotic compounds and/or antibiotics with different target sites is being reiterated frequently with the threat of drug resistant pathogens, reemerging pathogens and/or bio-terrorism concerns. With each passing decade, strains of virtually all important bacterial pathogens of humans have arisen that are resistant to at least one class of antibiotics, and strains resistant to multiple classes of antibiotics have become increasingly widespread. In fact, according to the Centers for Disease Control and Prevention (FDA.gov 2009), virtually all significant bacterial infections in the world are becoming resistant to the antibiotic treatment of choice. This rise is generally attributed to pathogens that have become resistant to commonly used antibiotics which focus on a limited number of target sites.

Some pathogens that were generally considered historical disease causing agents are reemerging either due to genetic modifications making the organism more virulent and/or exposure to a larger portion of the world population. Related to the naturally occurring genetic modifications are intentional genetic modifications conducted by groups with bio-terrorist desires. Frequently, these intentional genetic modifications will focus on making an otherwise susceptible disease pathogen resistant to the current antibiotics with known target sites.

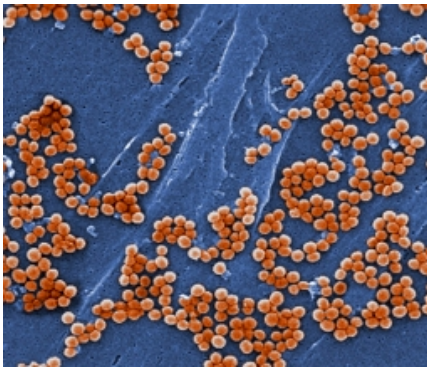


Figure 1. *Staphylococcus aureus* (Source: CDC)

For *Staphylococcus aureus* in particular, evolving resistance mechanisms have created significant treatment challenges over the years. Beginning with penicillinase-producing strains that were resistant to conventional penicillins, the need for newer, effective antibiotics against this organism has ensued. Most recently, with the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA), traditional first-line antibiotics are once again ineffective, and unfortunately the prevalence of CA-MRSA is already high, accounting for well over 60% of all cases of community-associated *S. aureus* infections. Even worse is the recent discovery of multi-drug resistant strains of CA-MRSA that are capable of rapidly acquiring resistance to most all available agents via a plasmid-mediated mechanism. In the face of this threat, a number of new antibiotics

targeted against *S. aureus* are under development. However, of the new antibiotics known to be in clinical development, none has a novel mechanism of action. For these reasons the need for totally new treatments for *S. aureus* infections is evident.

## Assay Background

Over the years many antimicrobial drugs have been developed that target various biological processes such as enzymatic reactions and signal receptors. However, one class of underutilized therapeutic targets is the RNAs involved in bacterial protein synthesis; more specifically, the protein synthesis processes that use post-transcriptional modified RNA nucleotides as substrates. Post-transcriptional nucleotide modifications can be as simple as the addition of a methyl group to a standard nucleotide or complex multi-step addition of amino acid like side chains (Soll 1995, Grosjean 1998). While 1 to 2% of all RNA bases are modified, the nucleotides in the active sites of the ribosome and the transfer RNA (tRNA) that interact with the ribosome are modified at 10-fold the rate of modification outside these active sites. The function of these modifications is to enhance the selectivity and specificity of the RNA:ribosome interactions that occur during translation.

Significant knowledge on the structure and function of these modifications has come from the development by this research team of core technology that provides an approach to synthesize these modified nucleotides in vitro. In addition to answering fundamental questions on the RNA:ribosome interaction this technology provides the potential to discover small compounds that inhibit protein synthesis at the translation level by interfering with the interaction of the RNA and the ribosome. These interactions have not been extensively exploited for development of antimicrobial compounds due to the inability to produce the necessary substrates required for the conduct of HTS assays.

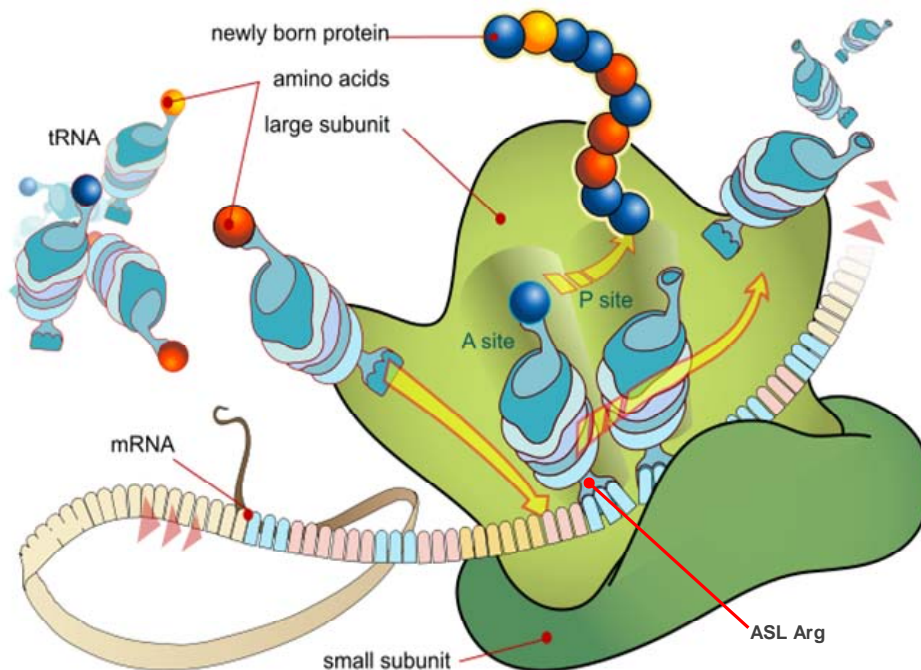
The use of synthetic mimics (fragments of tRNA) of the anticodon stem loops (ASL) that include the modified nucleotide bases in place of unmodified bases has led to the discovery that for certain tRNAs their natural, post-transcriptional nucleotide modifications are essential for proper ribosomal binding (Yarian et. al. 2002). A survey of tRNA sequences of the *E. coli* genome reveals at least 45 unique ASL sequences and since there are only 20 amino acids some sequences must code for the same amino acid. These duplicate ASL sequences are called iso-acceptors. Of the 45 ASL sequences 31 contain at least one modified base. While it was known that not all of the 31 modified ASL require modifications for ribosome binding, the number of those that do require modification is unknown.

As an initial survey to determine which tRNA require anticodon modifications for binding a series of unmodified ASLs were synthesized and tested for ribosomal binding using the single reaction assay. Two criteria were used for the selection

of ASL to test. First, only ASL sequences where all iso-acceptors are modified were determined. Second, the first list was reduced to those for which modified phosphoramidites had been previously synthesized. This reduced the initial series of ASL to 12 which were tested for their ability to bind to programmed ribosomes.

These initial experiments had two outcomes. For four of the unmodified sequences, sub-micromolar ribosome binding was observed indicating that those ASLs modifications were not required. For eight of the unmodified sequences no ribosomal binding was observed suggesting modifications may play a significant role in their binding.

As additional support that modifications are required for some ASL binding, sequences containing modified bases were able to restore binding for two additional ASLs. These screening experiments have demonstrated that: i) the RNA mimics are active in ribosome; ii) that these assays can be used to select the proper RNA oligomer for transferring amino acid residues in the ribosome assay; and, iii) that the investigators and their collaborators can synthesize the modified bases and subsequent RNA oligomers necessary to conduct these experiments.



*Figure 2. Cartoon of protein synthesis. (Source: Wikimedia Commons) Trana Discovery's S.aureus assay consists of a ribosome complex programmed with a short oligomer representing mRNA with a codon for fMet at the P site and a codon for a S. aureus specific tRNA at the A site. A full length fMet tRNA is used to stabilize the complex prior to the addition of the RNA mimic for ASL Arg. Compounds are then added that will interfere with the release of ASL Arg during protein synthesis.*

The synthetic mimic model system developed by Trana scientists and their collaborators can be used as a screening assay to identify compounds that inhibit protein translation by blocking tRNA ASL Arg binding to the ribosome.

The overall results of this research developed a novel high-throughput screening assay that identifies inhibitors of protein translation at the ribosome. Specifically, this assay detects compounds that interfere with the binding of the tRNA coding for the amino acid, arginine, during protein synthesis to ribosomes isolated from *S. aureus*.

## ***S. aureus* 201 HTS Assay Validation**

The assay was successfully optimized and robustness was validated producing an average Z-factor of 0.53 for EDTA (complex inhibitors) and 0.54 for neomycin (complex agonist) when compared to buffer controls. Following the acceptable validation with 4,000 compounds, an additional 56,000 compounds were screened with this validated assay resulting in the selection of 283 additional compounds that were more than 3 standard deviations away from the buffer control. We need to point out that this assay will identify both inhibitors and agonists; thus, the reason for indicating selection of compounds away from the buffer control (see Figure 1). These 283 compounds were further tested with this assay to confirm assay activity and to determine the concentration which inhibited or agonized the assay complex by 50% (IC<sub>50</sub>).

Biochemical activity was confirmed for 89 compounds and 38 were selected for biological assay testing based on a determined IC<sub>50</sub> concentration and/or a very promising curve shape. Initially, the 38 compounds were tested to determine which compounds would inhibit 7 different *S. aureus* strains using 2 fold dilutions starting at a nominal concentration of 128 μM.

Eight compounds were selected based on inhibiting 2 or more strains at a concentration less than 128 μM. Secondary testing was performed with these 8 compounds to determine activity against gram positive and gram negative bacteria as well as toxicity to 3 mammalian cell lines. All 8 compounds were active against the gram positive organisms and only 1 compound demonstrated weak activity against gram negative organisms indicating a lack of selectivity for *S. aureus*. In addition, all 8 compounds were toxic to the mammalian cell lines.

The results of this screening campaign failed to identify any compounds that would selectively inhibit *S. aureus*. However, the project was successful in that an assay was developed, optimized, validated, and used to screen a small compound library.

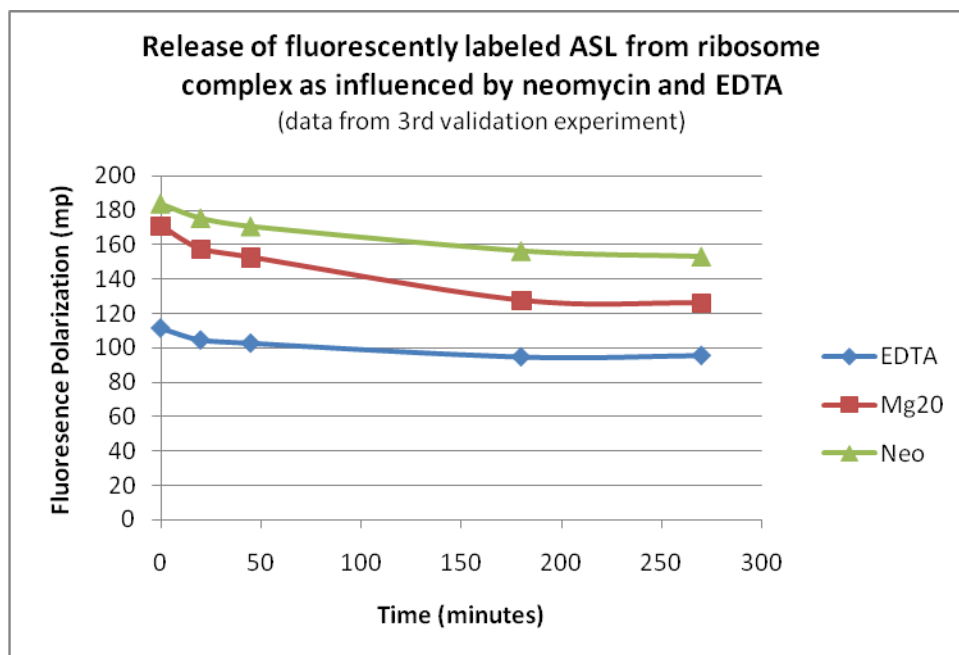


Figure 1. Example of complex dissociation over time. Mg 20 represents buffer control.

## Summary

Just as with HIV, *S. aureus* is a major public health concern and accordingly is of tremendous interest in the pharmaceutical industry for pursuit of new effective treatments. Entirely different targets are likely to be necessary to overcome ongoing resistance issues that make infections due to this pathogen so difficult to treat.

Until recently, tRNA could not be efficiently exploited as a potential drug target because of the lack of sufficient quantities of tRNA substrate to conduct research and the lack of a cost-effective method of screening thousands of compounds for inhibitors of *S. aureus*. Scientists at Trana Discovery successfully overcame that barrier by devising ways to synthesize exact mimics of the highly conserved anticodon stem loop that is critical for proper binding to the programmed ribosome for use in a high-throughput screening assay.

Trana Discovery technology and intellectual property offers prospective partners the opportunity for exclusive licensing of the assay and, in turn, full ownership of new classes of anti-infectives discovered through its application.

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