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Accelerating the discovery of antibacterial compounds using pathway-directed whole cell screening

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ABSTRACT

Since the introduction of penicillin into the clinic in 1942, antibiotics have saved the lives of millions of people around the world. While penicillin and other traditional broad spectrum antibiotics were effective as monotherapies, the inexorable spread of antibiotic resistance has made alternative therapeutic approaches necessary. Compound combinations are increasingly seen as attractive options. Such combinations may include: lethal compounds; synthetically lethal compounds; or administering a lethal compound with a nonlethal compound that targets a virulence factor or a resistance factor. Regardless of the therapeutic strategy, high throughput screening is a key approach to discover potential leads. Unfortunately, the discovery of biologically active compounds that inhibit a desired pathway can be a very slow process, and an inordinate amount of time is often spent following up on compounds that do not have the desired biological activity. Here we describe a pathway-directed high throughput screening paradigm that combines the advantages of target-based and whole cell screens while minimizing the disadvantages. By exploiting this paradigm, it is possible to rapidly identify biologically active compounds that inhibit a pathway of interest. We describe some previous successful applications of this paradigm and report the discovery of a new class of D-alanylation inhibitors that may be useful as components of compound combinations to treat methicillin-resistant *Staphylococcus aureus* (MRSA).

1. Introduction

The incidence of antibiotic-resistant infections is rising worldwide and these infections are increasingly difficult to treat. In the USA alone, antibiotic-resistant bacteria cause at least two million infections and 23,000 deaths annually, and nearly half of those deaths are due to MRSA.[1] The burden of drug resistant infections on healthcare systems is extremely costly and despite the effort of many academic and industrial teams, antibiotic discovery has not kept pace with the rise in antibiotic resistance. The paucity of new antibiotics has been the subject of much debate and scrutiny over the years, with the lack of success in bringing compounds to market attributed to: poor quality compounds in screening libraries; poor financial incentives; unreasonable regulatory barriers; and the changing landscape of resistant microorganisms.[2] It is clear that solutions to the antibiotic resistance crisis must come from multiple sources and directions at once. In this paper we address one aspect of the problem: improving the efficiency of bioactive compound discovery.

For the past two decades, high throughput screening has served as the most common approach to identify antibacterial compounds for further development, whether for use alone or in combination with other compounds.[3,4] High throughput screening approaches have generally been classified into two categories: target-based screens, in which an enzyme is screened in vitro for direct binding and inhibition, and whole cell screens, in which growth inhibition is the usual readout (Table 1). In a much-discussed paper from 2007, Pompliano and co-workers described the results of 67 high throughput screening campaigns carried out over a period of seven years at GlaxoSmithKline against a wide range of antibacterial targets.[5] Only 16 of those screens, each involving approximately 250,000–500,000 compounds, resulted in hits, defined as chemically tractable, low-micromolar inhibitors of a given target, and only five of those hits progressed to leads, defined as compounds with biological activity and some evidence for target engagement. As the paper made abundantly clear, target-based screening is problematic because the likelihood that a hit can be developed into a useful lead is low. While improving the quality of compounds in a library may partially address this problem, the screening process is

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Table 1
Pros and cons of different screening approaches

	Target-based	Cell-based	Pathway-directed whole cell
Pros	• Predefined target	• Screens performed in relevant organisms • Assay format is simple • Biological activity is guaranteed	• Predefined pathway(s) • Screens performed in relevant organisms • Assay format is simple • Biological activity is guaranteed • On-target activity expected
Cons	• Limited to enzymes that can be expressed • Extensive assay development required • Achieving biological activity for hits can be difficult • Demonstrating on-target biological activity for leads can be challenging	• Many hits; sorting good hits from non-specific toxic compounds is difficult • Target identification can be time consuming	• Assays require appropriate genetic or pharmacological tools

Pathway-directed whole cell screens attempt to merge the advantages of target-based and whole cell screens while circumventing major disadvantages.

inherently inefficient. Additionally, target-based screens can only be applied to well-behaved targets, which excludes most membrane proteins and overlooks the possibility that the best-behaved targets for an in vitro screen may not be the most druggable targets in a given pathway. Whole cell screens have a major advantage over target-based screens because biological activity is guaranteed and bacterial growth/inhibition assays are simple to implement. However, target identification is more difficult and it can also be difficult to prioritize hits for follow-up. Because nuisance compounds with non-specific activities may represent a large fraction of the hits and can be difficult to recognize, considerable time and effort may be spent sorting through the hits to identify the more promising compounds.

To improve the efficiency of high throughput screening for discovery of biologically active compounds, the field has turned to screening strategies that combine the advantages of target-based and whole cell screens while minimizing the disadvantages. There are different ways to accomplish this. One way is through target depletion. For example, Merck developed an antisense platform to reduce expression of 245 essential genes in *Staphylococcus aureus*.[6] Antisense strains were pooled based on growth rates and then the pools were screened against compound libraries to identify agents that resulted in depletion of particular antisense strains from the pools. The pathway targeted by a given compound could be deduced from the strains that were most sensitive to it. This strategy not only guarantees the discovery of biologically active compounds, but increases the likelihood that hits will have a desirable mechanism of action.[7] We developed an alternative approach to accomplish the same goal, which involves screening a chemical library against a wildtype and a mutant bacterial strain to identify compounds that differentially affect growth of one of the strains.[8,9] This approach can be used to discover compounds that inhibit essential targets as well as compounds that inhibit non-essential targets involved in antibiotic resistance or virulence. Below we describe the application of this approach to discover compounds that inhibit cell envelope targets in *Staphylococcus aureus*. Using the same wildtype/mutant strain pair, we have identified multiple biologically active scaffolds for each of two different targets. In a testament to the efficiency of the approach, we report here the discovery of a new class of teichoic acid D-alanylation inhibitors based on following up only two hits from a screen of 230,000 small molecules.

2. Teichoic acids in *Staphylococcus aureus* as antibacterial targets

Teichoic acids are anionic polymers that are major constituents of the *S. aureus* cell envelope.[10–13] There are two types: lipoteichoic acids, which are embedded in the cell membrane, and wall teichoic acids, which are covalently attached to peptidoglycan (Fig. 1). Both types of teichoic acids play important roles in cell growth and division and are required for survival in a host, making them targets for antibacterials.[14] Lipoteichoic acids are composed of a poly(glycerol phosphate) chain attached to a diglucosyl-diacylglycerol anchor.[15,16] LTAs continue to be produced when synthesis or export of diglucosyl-DAG is prevented, but strain growth is compromised and polymer length is altered.[17] Wall teichoic acids are composed of a disaccharide sugar linked through the reducing end to PG and through the non-reducing end to a poly(ribitol-phosphate) chain.[16,18,19] Both lipo- and wall teichoic acids are functionalized with D-alanine esters; wall teichoic acids are also heavily glycosylated with *N*-acetyl-D-glucosamine.[10,20,21] D-Alanine ester levels are regulated by at least one multicomponent sensory system, the GraRS/VraFG system, and increase under various stress conditions.[22–24] The D-alanine esters on lipoteichoic acids are installed by the four protein Dlt pathway (DltABCD) and are then transferred to wall teichoic acids in a process that remains unknown.[25] Strains in which Dlt pathway genes have been removed are highly susceptible to host immune defenses and are also sensitive to cationic antibiotics such as aminoglycosides.[26–29] Therefore, compounds that inhibit teichoic acid D-alanylation may be useful as potentiators of aminoglycosides, which have dose-limiting toxicities, and may also attenuate *S. aureus* virulence.

3. Exploiting suppression of growth inhibitory activity to target wall teichoic acid biosynthesis

Although WTAs are not essential for survival in vitro, genes that act late in the pathway cannot be deleted unless flux into the pathway is prevented.[31,32] This behavior is due to the fact that blocking a late step in WTA biosynthesis depletes Lipid II, the peptidoglycan precursor, which is synthesized on the same undecaprenyl phosphate carrier lipid as the WTA precursor.[33,34] Therefore, it is possible to identify compounds that inhibit a late step in WTA biosynthesis by monitoring growth of a wildtype *S. aureus* strain and a Δ*tarO* mutant in which the first gene in the pathway has been deleted. From a screen of ~55,000 compounds, we identified three compounds that inhibited growth of the wildtype strain but not the mutant (Fig. 2A, red hits).[8] We raised resistant mutants and performed targeted sequencing of genes in the WTA pathway based on the expectation that the screen was pathway specific. Only two types of mutations were found: null mutations in *tarO* or *tarA*, the first two genes in the WTA pathway, and missense mutations in *tarG*, which encodes the transmembrane component of the two component ABC transporter that exports WTA precursors from the cytoplasmic surface to the extracellular surface of the membrane.[8] Replacing wildtype *tarG* with the mutant alleles conferred resistance to the compound, establishing TarG as the target. Compound potency was improved ten-fold through medicinal chemistry to produce targocil, which has been used as a probe in a