tion encoded in the PNA-encoded inhibitor/substrate enables the design of small-molecule inhibitors, which can then serve as tools for cellular assays and as a further basis for drug design. Indeed, the same group of researchers has developed PNA-encoded small-molecule libraries for irreversible protease inhibitors [13]. The use of PNA-encoded libraries should significantly reduce the steps required to identify the relevant protease(s) and their substrate(s) for a phenotype of interest. It will be exciting to see if these strategies can also be extended to small molecules that bind proteases reversibly as well as to other enzyme families. Finally, many changes in protein function cannot be detected in cellular lysates, and therefore future experiments will require the development of cell-permeable probes to monitor changes in vivo.

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A Suppression Strategy for Antibiotic Discovery

High-throughput phenotype screening and target identification have been combined in an effort to isolate antimicrobial, small-molecule therapeutics [1]. This approach, developed by Brown and colleagues and reported in this issue, is a major technological advance for antimicrobial drug discovery.

The discovery and development of novel antimicrobials via target-based approaches has historically been plagued by difficulties associated with optimizing small molecule leads out of biochemical screens while preserving or improving upon antimicrobial activity. This is due in large part because the factors governing small-molecule permeability and substrate selection criteria for efflux pumps in bacterial cells are poorly understood phenomena. However, high-throughput, phenotype-based screening methods offer a new promising strategy for identifying compounds from high-throughput screens that elicit a specific biological response. Unlike target-based screening of biochemical activities, phenotype-based screening selects for compound candidates that can penetrate cells, remain relatively unaffected by efflux pumps, and function properly in vivo. Thus, many of the former problematic issues affecting target-based screening are circumvented.

For example, by using a phenotype-based screen, Mitchison, Schreiber, and colleagues [2] identified an inhibitor of mitosis in mammalian cells with monopolar spindles, out of a library of 16,320 compounds. The inhibitor discovered, monastrol, attacks the motility of the mitotic kinesin Eg5, preventing normal spindle bipolarity and thereby validating it as a potential anticancer drug. At the time of this study, the only other previously known inhibitors of kinesin were cell impermeable. This work clearly demonstrates the advantages of employing phenotype screens in finding compounds that have novel activities within a biological system.

However, there is a slight problem. Although phenotype screens allow the rapid and selective identification of compounds that elicit a specific biological response, the mode of action of active compounds cannot be effectively and clearly deduced given the inherent complexity resulting from the large number of possible targets whose function is altered by the presence of the biological modifier. The success rate of finding a specific mechanism of action hinges on the stringency afforded by the phenotype screen as well as the level of knowledge of the possible targets impacted by the small-molecule effector. In the aforementioned example, Mitchison and Schreiber's search for a target was facilitated by the fact that the small molecule caused a mitotic

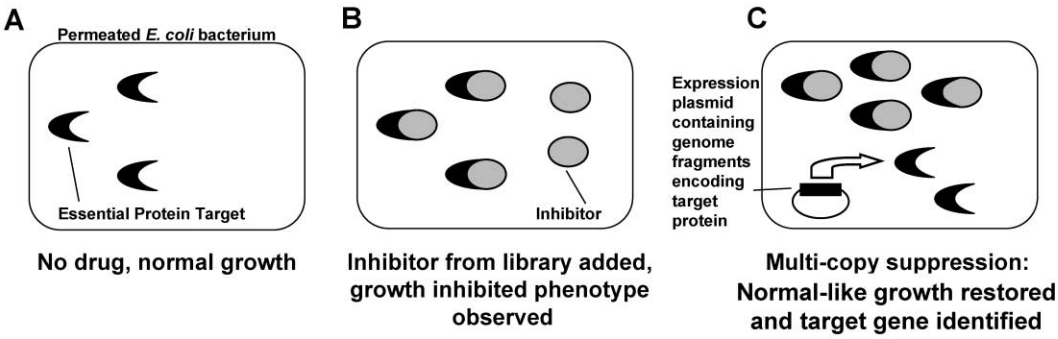

Figure 1. Multi-Copy Suppression in High-Throughput Antimicrobial Discovery

(A) Control: An illustration of a bacterial cell containing within its genome a potential target protein essential for cell growth or viability.

(B) High-throughput phenotype screening for antimicrobial leads: Upon addition of a small molecule from a high-throughput screening (HTS) library, growth is inhibited and the lead compound is identified based on inhibited-growth phenotypes.

(C) High-throughput multi-copy suppression target identification. In the presence of the small-molecule antimicrobial candidate, bacteria containing multiple copies of overexpression plasmids containing random genome fragments from the parent bacterial genome are induced, with the overexpressed target protein identified from colonies exhibiting a restoration of the normal growth phenotype. The overexpression plasmid containing the expressed genome fragment is subsequently isolated and sequenced for purposes of target identification. By this method, a candidate antimicrobial lead compound from HTS library screening might be rapidly paired to a target protein identified by a genome-wide analysis using high-throughput, phenotype-directed screening.

disturbance, narrowing the number of suspected targets [2]. However, for more general screens that select for bioactive compounds based upon cell or colony growth, inhibition or lysis can have many possible mechanisms of action. For the search of novel antimicrobials, what is needed is the combination of the power of high-throughput phenotype screening with a mechanism of rapid target discovery and validation.

In this issue of *Chemistry & Biology*, Brown, Wright, and coworkers from the Antimicrobial Research Centre at McMaster University have devised the first integrated technology for rapid high-throughput phenotype-based antimicrobial discovery and concomitant target identification and validation by using multi-copy suppression techniques [1]. Multi-copy or high-copy suppression is a forward chemical genetics-based technique that allows the specific identification of target proteins impacted by small-molecule effectors by providing multiple copies of the target proteins by using high-copy expression plasmids (Figure 1). This technique accelerates the validation of targets, elucidation of resistance mechanisms to established drugs, and the selection of compounds biologically compatible with the host system [3–5]. Typically, multi-copy suppression techniques are primarily utilized as a confirmatory step in the final stages of in vivo target evaluation of a single gene product or small subset of gene product target candidates. For example, Burger and colleagues [6] employed multi-copy suppression to identify resistance genes to the anticancer drug cisplatin. More closely related to antimicrobial discovery, Li and colleagues have employed multi-copy suppression in order to identify the targets of bacterial growth inhibitors [7]. In this report, Brown and coworkers have developed a significant technological advance for antimicrobial discovery by combining the power of high-throughput phenotype-based screening with a novel high-throughput library-based approach to multi-copy suppression-based target identification and validation [1]. In effect, the technology allows one to work from both ends of the problem simultaneously.

First, by using a hyperpermeable rough lipopolysaccharide mutant strain of *E.coli* (MC1061) as the small-molecule permeable reporter strain, Brown and coworkers screened a library of 8640 compounds, discovering 196 lead compounds that altered cell growth [1]. After determining the minimum inhibitory concentrations (MIC) of each substance, they narrowed the antimicrobial leads to 49 candidates by selecting representatives of similar chemical structures. An innovative improvement on traditional multi-copy suppression was then applied for the evaluation of the mechanism of action of these 49 candidates. Instead of using a single target gene for multi-copy suppression, Brown and colleagues screened each lead compound against cells that overexpressed 3–4 kb random genomic fragments from an *E. coli* genomic library. Cultures that grew despite compound levels exceeding MIC values were thus identified as containing multiple copies of the suppressing target protein. Clones with phenotypes possessing resistance above wild-type MIC values reduced the pool of leads from 49 to 33. Of these 33 clones, 31 clones acquired resistance because they overexpress *acrB*, the membrane-spanning subunit of the acridine efflux transporter. The remaining 2,4-diaminopyrimidine- and 2,4-diaminoquinazoline-containing molecules, were paired to clones that overexpressed the gene *folA*, encoding dihydrofolate reductase (DHFR). Inhibition of DHFR by these compounds was subsequently confirmed in vitro and by paralleling protein expression levels with compound MIC value changes. Importantly, this work also lead to the discovery of a novel inhibitor of DHFR with no structural relationship to the well-known DHFR inhibitor, methotrexate.

This approach elegantly tackles two prime obstacles associated with antimicrobial discovery with high-throughput screening techniques: specific identification

of the target protein and mode of action, and selection against compounds influenced by permeability restrictions or efflux mechanisms in vivo. This approach is highly innovative, for the first time the process of lead discovery and target evaluation are integrated and both high-throughput.

Another especially useful aspect of this technology is to not only identify the targets of bioactive leads, but to rapidly identify those compounds that are susceptible to drug efflux, a common obstacle encountered in the development of antibiotics for Gram-negative bacteria [7–9]. In this report, since the large-scale selection of compounds yielded leads that were matched to a specific efflux pump for resistance, one can now comparatively examine these efflux pump substrates in order to probe structure-activity relationships. Such studies will undoubtedly better our understanding of the substrate requirements of this defense mechanism in bacteria as well as in other eukaryotic systems. Lastly, this technology opens up this type of work to academic-caliber resources, broadening the number of compounds and targets that can be explored in this post-genomic age.

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