COMMENTARY

Going on offense against the gram-negative defense

Bryon S. Drown[a,b] and Paul J. Hergenrother[a,b,1]

Bacterial infections resistant to antibiotics are becoming more common, posing a significant health risk (1). The CDC and WHO have identified gram-negative drug-resistant pathogens as being particularly dangerous, and such infections are resistant to nearly all available antibiotics in some cases (2, 3). The most concerning of these infections are three classes of nonfastidious gram-negative pathogens, Enterobacteriaceae (including *Escherichia coli* and *Klebsiella pneumonia*), *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. As only nine classes of antibiotics (that engage five targets) are available for the treatment of these infections (4), the need for new classes of antibiotics effective against gram-negative bacteria is self-evident. In PNAS, Zhang et al. (5) present an innovative approach for discovering antibiotics that exploit previously untargeted components of lipopolysaccharide (LPS) biogenesis and use this screening platform to identify a lead compound that inhibits LPS biosynthetic machinery.

Historically, there has been a heavy reliance on whole-cell toxicity assays for identification of new antibiotic classes, and, in fact, all classes of US Food and Drug Administration-approved antibiotics were initially discovered in these types of cell culture phenotypic screens (1). Unfortunately, this approach has rarely provided compelling lead compounds with activity against gram-negative bacteria; the quinolones are the most recently introduced class of broad-spectrum antibiotics, over 50 y ago (in 1968) (4). AstraZeneca, GlaxoSmithKline, and others have all published detailed accounts of their independent screening of a combined >8 million compounds in bacterial cell culture death assays; it is sobering to note that these experiments failed to produce any compounds with sufficient gram-negative activity to merit advancement (6–9). While biochemical assays against essential protein targets can provide compounds with high in vitro potency, these compounds rarely can be translated into activity against whole organisms, especially for gram-negative infections. It is encouraging to see compounds that target FabI (10, 11), discovered in a biochemical screen, advancing through clinical trials for gram-positive infections, but this success will be difficult to replicate for the nonfastidious gram-negative pathogens listed above. Cell culture screens remain the gold standard for antibiotic lead generation, and still represent the best hope for identification of new chemical matter for the most problematic gram-negative pathogens.

While the challenges that most compounds face in traversing the outer membrane of gram-negative bacteria chiefly account for the failure of large screening campaigns (12, 13), paradoxically, this additional membrane represents a vulnerability for gram-negative bacteria. The outer membrane is a unique asymmetrical barrier that displays LPS molecules on the outer leaflet. These LPS molecules feature shorter, less fluid lipid tails and tightly packed, negatively charged sugars. Gram-negative bacteria rely on this barrier to provide structure and exclude toxins. In particular, targeting LPS biogenesis (which includes LPS synthesis and transport) could be a particularly attractive strategy, as most gram-negative bacteria suffer significant fitness costs from even partial inhibition of LPS synthetic pathways (14). Additionally, as LPS decorating the bacterial outer membrane provides a protective shell that excludes most antibiotics effective against gram-positive species, compounds that disrupt LPS integrity (e.g., colistin and derivatives) often potentiate other antibiotics that have poor penetrance (15). The renal toxicity of colistin and other polymyxins limits their broad clinical use, so there is a great clinical opportunity for compounds that inhibit LPS formation, either as potentiators or single-agent antibiotics.

Many components of LPS synthesis are membrane-bound, making their reconstitution in a biochemical activity assay extremely challenging. Additionally, LPS biogenesis involves hundreds of components; thus, systematic exploration would be time- and labor-intensive. Writing in PNAS, Zhang et al. (5) now demonstrate a different approach to exploiting LPS biogenesis for discovery of novel antibiotics. Through a

[a]Department of Chemistry, University of Illinois, Urbana, IL 61801; and [b]Institute for Genomic Biology, University of Illinois, Urbana, IL 61801

Author contributions: B.S.D. and P.J.H. wrote the paper.

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[1]To whom correspondence should be addressed. Email: hergenro@illinois.edu.

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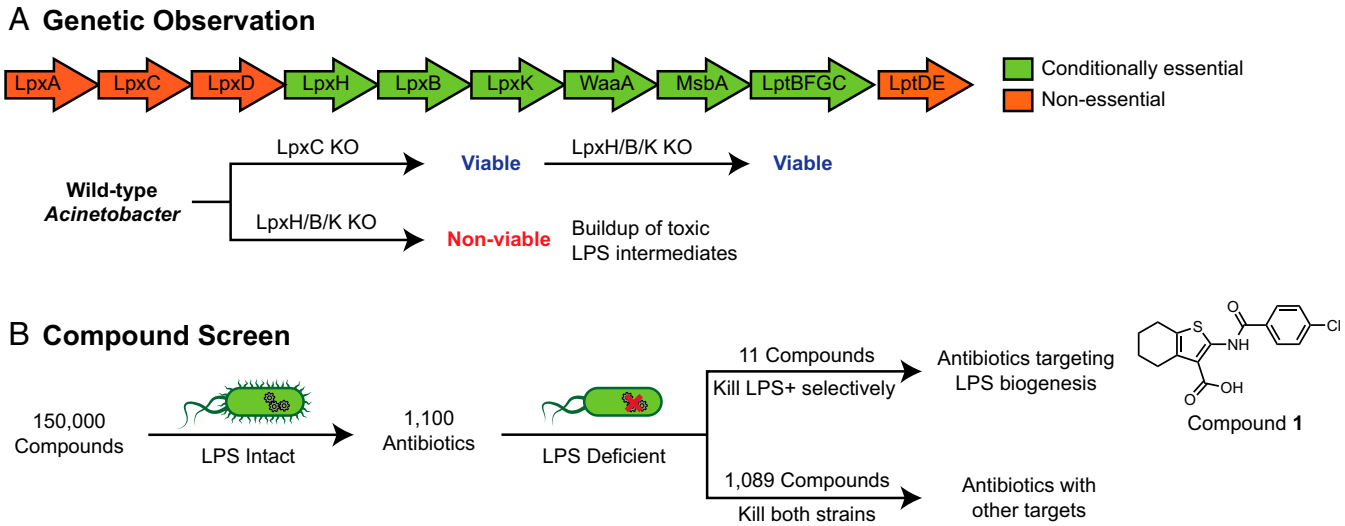

Fig. 1. (*A*) **Identification of genes that are essential for bacteria with active LPS synthetic flux. Intermediate genes could be knocked out only when LpxC, which performs the first committed step in LPS synthesis, was also knocked out.** (*B*) **Screen for compounds that target the LPS synthetic pathway, based on screening and counterscreening of isogenic strains.**

series of elegantly simple genetic knockout experiments, the authors first identified an *Acinetobacter* species susceptible to interruptions midway through LPS synthesis, but that can be rescued by complete pathway shutdown. As shown in Fig. 1*A*, these genetic experiments demonstrate that a knockout early in the biosynthetic pathway (deletion of LpxC) is tolerated but that knockouts later in the pathway (Fig. 1*A*, genes in green) lead to toxic buildup of LPS intermediates and nonviability. This interesting finding led to the development of isogenic strains with either active or inactive LPS biogenesis. Compound screening in the LPS^+ strain identified 1,100 compounds that kill bacteria; counterscreening in the LPS^- strain then separated these compounds into antibiotics that act through disruption of LPS biogenesis (11 compounds) and those that indiscriminately kill both strains and thus have other (non-LPS) antibacterial targets (Fig. 1*B*). This clever system allows for the best of both worlds: the identification of compounds that inhibit a specific pathway, but in a cell culture screen. Indeed, a series of tetrahydrobenzothiophenes, exemplified by compound **1**, were identified with interesting potency and selectivity for antibiotic activity via disruption of LPS biogenesis.

A challenge with this approach is in the downstream target identification, that is, the determination of exactly which step of LPS biogenesis is being disrupted. Since components of LPS synthesis are only conditionally essential, several routes to resistance can be envisioned. Indeed, Zhang et al. (5) observe a relatively high resistance frequency to compound **1** via mutations in nonessential genes early in the LPS pathway. Since the antibacterial activity of colistin relies on active LPS synthesis, bacteria resistant to compound **1** with LPS biogenesis still active could be identified by replica plating onto colistin. Whole-genome sequencing of colonies resistant to compound **1** revealed mutations on the periplasmic face of MsbA. While this technique was successful in determining the target of compound **1**, this complication highlights the need for existing compounds (in this case, colistin) that can report on the status of the pathway of interest. MsbA is an essential ABC transporter responsible for shuttling LPS molecules across the bacterial inner membrane. Recent structural biology reports indicate that allosteric binders to MsbA can decouple ATP hydrolysis from transport (16), and the tetrahydrobenzothiophene series reported by Zhang et al. (5) appears to act through a similar mechanism. Although the hit compounds described only demonstrate activity against engineered strains that lack efflux pumps, this article most importantly describes a new paradigm for discovery of novel LPS biogenesis inhibitors, a long-time goal in antimicrobial development.

Here, Zhang et al. (5) present a strategy for combining whole-cell and pathway-specific screening, and this strategy can be extended to the hundreds of genes that are known to be essential, yet are currently not targeted. Whole-organism screens and counterscreens have been successfully implemented to discover antibiotic leads in the past (17), but rarely focused on entire pathways. The key criterion is identification of a resistance mechanism that does not interfere with potential compound target engagement (i.e., not binding site mutation or increased efflux). In the case at hand, this was accomplished by shutting down LPS synthetic flux, but it also could be achieved by introduction of genes that eliminated the toxic LPS intermediates or redundant pathways from other species. This strategy offers an opportunity for creative design of isogenic pairs that exploit underlying conditionally essential genes. In contrast to a gene reporter assay that might be used to probe a pathway's activity, this approach draws a direct line to cell viability; compounds that inhibit components in a pathway but, ultimately, do not affect viability are disregarded. Ultimately, such creative methods that allow discovery of new chemical matter and validate novel targets are needed to help restock the pipeline of antibiotics active against gram-negative pathogens.

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