

# Antibacterial New Target Discovery: Sentinel Examples, Strategies, and Surveying Success

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**Abstract** Antibiotics are the bedrock of modern medicine but their efficacy is rapidly eroding due to the alarming emergence of multi-drug resistant bacteria. To begin to address this crisis, novel antibacterial agents that inhibit bacterial-specific cellular functions essential for growth, viability, and/or pathogenesis are urgently needed. Although the genomics era has contributed greatly to identifying novel antibacterial targets, it has failed to appropriately characterize, prioritize, and ultimately exploit such targets to significantly impact antibiotic discovery. Here we describe a contemporary view of new antibacterial target discovery; one which complements existing genomics strategies with a deeply rooted and fundamental understanding of target biology in the context of genetic networks and environmental conditions to rigorously identify high potential targets, and cognate inhibitors, for consideration as antibacterial leads.

**Keywords** Antibiotic, Antibiotic resistance, Conditional essential, Drug target, Outer membrane biogenesis, Synthetic lethality, Wall teichoic acid

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## 1 Introduction: An Evolving View of New and Legitimate Antibacterial Targets

Antibiotics are extraordinarily valuable therapeutic agents whose widespread use has transformed human health since the early twentieth century, largely relegating historically uncontrollable and deadly bacterial infections to mild and conveniently treatable illnesses due to the high efficacy, wide availability, and relatively low cost of these antibiotics [1]. Their remarkable success is tempered by the increasing rise of multi-drug resistant bacteria that are recalcitrant to our existing repertoire of chemotherapies [2, 3], principally due to the lack of stewardship in health care and overuse in livestock for food production [4, 5]. Alarming, the rate at which drug-resistance is emerging is in stark contrast to the abrupt decline in the discovery of novel antimicrobials with which to treat them [3, 6]. Existing antibiotics in clinical use target a surprisingly small subset of essential processes [7], and the pipeline in recent years has been awash in “me-too” inhibitors of similar classes that are incremental modifications of existing compounds [8]. There is an obligation among the research community to identify inhibitors from compound collections that interdict novel targets in pathways essential for bacterial growth or infection for which resistance has not yet been widely disseminated. Despite this clarion call and the herculean efforts of many, success in the discovery of clinically relevant antimicrobials to novel targets has remained elusive in recent decades despite the dawn of the genomics era that has provided researchers detailed blueprints of promising targets in countless bacterial organisms. The causes for this failure are likely multi-faceted and overcoming stagnation may require (1) a paradigm shift that will integrate modern approaches with lessons from the past; (2) a broader definition of druggable targets to include those involved throughout the course of a bacterial infection in the host-pathogen context rather than relying on targets that disrupt growth in artificial environments *in vitro*; and (3) a shift away from the expectations of a novel broad-spectrum panacea to a more narrow spectrum-focused effort to find treatments for multi-drug resistant bacterial infections of high-priority.

In contrast to the scarcity of antibacterials with new mechanisms of action (MOA) that meet or exceed standard of care antibiotic treatments in recent years, there is no lack in the literature of the discovery of new and exciting antibacterial targets of potential utility [8, 9]. However, defining the quality of any particular drug target and its relative prioritization versus literally 1000s of other potential targets is difficult, and is often considered from an antiquated and subjective perspective rooted in the idea that any gene required for microbial growth and/or viability is considered a plausible drug target. In fact, the genomes of most bacterial pathogens typically comprise hundreds of essential genes (for example, *E. coli* contains ~300 essential genes) [10, 11] required to facilitate fundamental cellular functions; fungal genomes contain even more, typically approaching as many as ~1000 essential genes [12–14]. These numbers can be whittled down considerably by introducing additional sensible drug target prioritization criteria, such as conservation of the protein target amongst medically significant microbial pathogens (i.e., genetically predicted achievable “spectrum” for the activity and efficacy of the cognate drug to the selected target) and

absence of the target in the human genome, hence mitigating the possibility of target-based cytotoxicity. This view, although seemingly necessary, is certainly not sufficient and the last 20 years of antibacterial discovery efforts only underscores the frailty of these simplistic considerations [15, 16]. Furthermore, such an approach neglects many valuable targets that are conserved in humans and yet are selectively inhibited by clinically successful antibiotics such as the ribosome, RNA polymerase, type II topoisomerase, dihydrofolate reductase, and the tRNA synthetases. Instead, antibiotic targets should be defined more rigorously and according to a continuum of validation criteria that describes their likelihood to deliver new therapeutics. Identifying and leveraging “high value” novel targets to discover new antibacterial leads requires a much greater level of biological insight and innovation to efficiently and unequivocally discover cognate small-molecule inhibitors. Here, we provide a contemporary perspective on the topic of new antibacterial targets; one streamlined to empirically identify and validate “druggable” targets and cognate inhibitors as antibiotic chemical starting points with demonstrated efficacy in a disease model of infection.

A central dogma driving the definition of a novel antibacterial target is that it is essential for the growth and/or viability of the pathogen(s) for which novel therapeutics are needed. Accordingly, cognate inhibitors of such targets are predicted to disrupt fundamental aspects of bacterial physiology and lead to cell death (i.e., bactericidal) or a growth arrest (bacteriostatic). Indeed, all successful antibiotics past and present meet this fundamental criterion. However, such successes whether pioneered by Fleming and Waxman or later by large pharmaceutical companies were almost entirely based on empiric screening of chemical collections (largely natural product extracts) displaying intrinsic antimicrobial activity [17] with target and MOA elucidation typically only achieved many years after their discovery and clinical use [7]. Decades later, success derived from the continued application of this strategy has fallen precipitously; whether resulting from (1) a diminishing return in discovering new leads versus the inefficient and time consuming rediscovery of known natural product compounds [18], (2) the perceived “undesirable” chemical space in which synthetic compound libraries tend to exist versus the physicochemical properties of natural products [16, 19, 20], and/or (3) the high therapeutic bar that clinically non-inferior new agents must achieve versus >70 years of standard of care antibiotics to which they are compared [21]. Consequently, a target-centric approach – fueled by the genomic era – has emerged where targets are first selected to screen and/or rationally design small-molecule inhibitors whose potency, spectrum, and safety can be later chemically optimized.

Defining robust validation criteria of a new antibiotic target spans three basic levels (Level-1, -2, and -3) in their broad continuum of characterization, where Level-3 targets are the most extensively substantiated. We propose defining Level-1 targets as having (1) genetic evidence under *in vitro* conditions that inactivation/inhibition of their function impairs growth and/or viability of the pathogen and (2) ideally, satisfy basic bioinformatics criteria pertaining to their spectrum and absence from man. In addition to these criteria, Level-2 targets also possess genetic verification that (1) abolishing target function impairs pathogenesis in a relevant animal model of disease and (2) that the target has been confirmed to be druggable by identifying whole-cell bioactive target-selective inhibitor(s) supported with (3) unambiguous MOA evidence

**Table 1** Comparative list of relevant new antibacterial targets and their respective cognate inhibitors

	Target	Potential spectrum	Compound	Whole cell activity	Frequency of resistance	Discovery method	Target validation	Reference
Outer membrane	LptD <sup>a</sup>	Broad G-ve	POL7080	Yes <sup>b</sup>	< 1E-10 <sup>c</sup>	Optimization based on the antimicrobial peptide protegrin I	Genetic and biochemical <sup>d</sup>	[22]
	BamD <sup>e</sup>	Broad G-ve	BamaA peptide	No	ND	In vitro	Biochemical <sup>d</sup>	[23]
Plasma membrane	LtaS <sup>f</sup>	Broad G +ve	Compound 1771	Yes <sup>b</sup>	< 2E-9 <sup>c</sup>	TS conditional empiric screening	Biochemical <sup>d</sup>	[24]
	LpxH <sup>e</sup>	Broad G-ve	Compound 1	Yes	2E-8 <sup>c</sup>	Phenotypic screen; AmpC cell wall reporter	Genetic	[25]
	LoiCDE <sup>e</sup>	Broad G-ve	Compound 2	Yes	7E-7	Phenotypic screen; AmpC cell wall reporter	Genetic and biochemical	[25]
	LoiCDE <sup>e</sup>	Broad G-ve	Compound 1,2	Yes	1E-6	Empiric screen	Genetic and biochemical	[26]
	MraY <sup>f</sup>	Broad G-ve/+ve	Tunicamycin	Yes	ND	Genetic	Biophysical and biochemical	[27]
	MurG <sup>f</sup>	Broad G-ve/+ve	Murgocil	Yes	1E-7	$\beta$ -Lactam potentiation screen	Genetic and biochemical	[28]
	MurJ <sup>f</sup>	Broad G-ve/+ve	DMPI, CDFI, compound D	Yes	< 1E-8 <sup>c</sup>	$\beta$ -Lactam potentiation screen	Genetic and biochemical	[29, 30]
	Pbp2A <sup>f</sup>	Broad G +ve	Antibiotic 2	Yes	ND	Allosteric inhibition	Genetic and biochemical <sup>d</sup>	[31]
	TarO <sup>f</sup>	Broad G +ve	Tarocin A, B + tunicamycin	No <sup>b</sup>	1E-8 <sup>c</sup>	Phenotypic screen; chemical suppression	Genetic and biochemical	[32]
	TarG <sup>f</sup>	Broad G +ve	Targocil + L275, L640, 638, L555, and L524	Yes <sup>b</sup>	1E-6 to 1E-g <sup>c</sup>	Phenotypic screen; conditional essentiality	Genetic and biochemical	[33, 34]
Lipid II and III <sup>f,g</sup>	WTA-AAC <sup>f</sup>	Broad G-ve/+ve	Teixobactin	Yes <sup>b</sup>	None detected	Empiric NP screen; iChip technology	Biochemical	[35]
	LspA <sup>a</sup>	Broad G-ve/+ve	TA, Globomycin	Yes	1E-8 <sup>c</sup>	Empiric NP screen	Genetic and biochemical <sup>d</sup>	[37-40]
						Design	Biochemical <sup>d</sup>	[36]

Soluble, cytosol	CoaD <sup>f,g</sup>	Broad G-ve/+ve	Compound A-D	Yes <sup>b</sup>	1E-7 to 1E-9 <sup>c</sup>	HTS in vitro biochemical screen	Genetic and biochemical <sup>d</sup>	[41]
	ClpP activation <sup>f</sup>	Broad G-ve/+ve	ADEP4	Yes <sup>b</sup>	1E-6	Empiric NP screen	Biochemical <sup>d</sup>	[42, 43]
	MurD, MurE <sup>f</sup>	Broad G-ve/+ve	Compound 9	Modest	ND	In silico screening	Biochemical <sup>d</sup>	[44]
	DitB <sup>f</sup>	Broad G-ve/+ve	Amsacrine	No	1E-7	Synthetic lethal screen	Genetic and biochemical	[45]
	FtsZ <sup>f</sup>	Broad G-ve/+ve	PC190723	Yes <sup>b</sup>	3e-8 <sup>c</sup>	Phenotypic screen	Genetic and biochemical <sup>d</sup>	[46, 47]
	MnaA <sup>f</sup>	Broad G-ve/+ve	Tunicamycin	NA	ND	Phenotypic screen	Biophysical and biochemical	[48]
	UppS <sup>f</sup>	Broad G-ve/+ve	Compounds 5-21, clomiphene	Yes <sup>b</sup>	ND	Phenotypic screen/in silico	Biochemical	[49, 50]
	UppS <sup>f</sup>	Broad G-ve/+ve	MAC-0547630	Yes, B, subtilis	1E-7	Phenotypic screen	Genetic and biochemical	[51]
	FMN riboswitch <sup>e</sup>	Broad G-ve/+ve	Ribocil	Yes <sup>b</sup>	1E-6 to 1E-8 <sup>c</sup>	Phenotypic screen, conditional essentiality	Genetic, biochemical <sup>d</sup> , and biophysical	[52, 53]

NA not applicable

ND not determined

<sup>a</sup>Target pathogen: *P. aeruginosa*

<sup>b</sup>Demonstrated in vivo efficacy

<sup>c</sup>Acceptable starting point for frequency of resistance. Note, however, that a high frequency of resistance in vitro does not always translate equivalently in vivo (see Sect. 5)

<sup>d</sup>Co-crystal structure available

<sup>e</sup>Target pathogen: *E. coli*

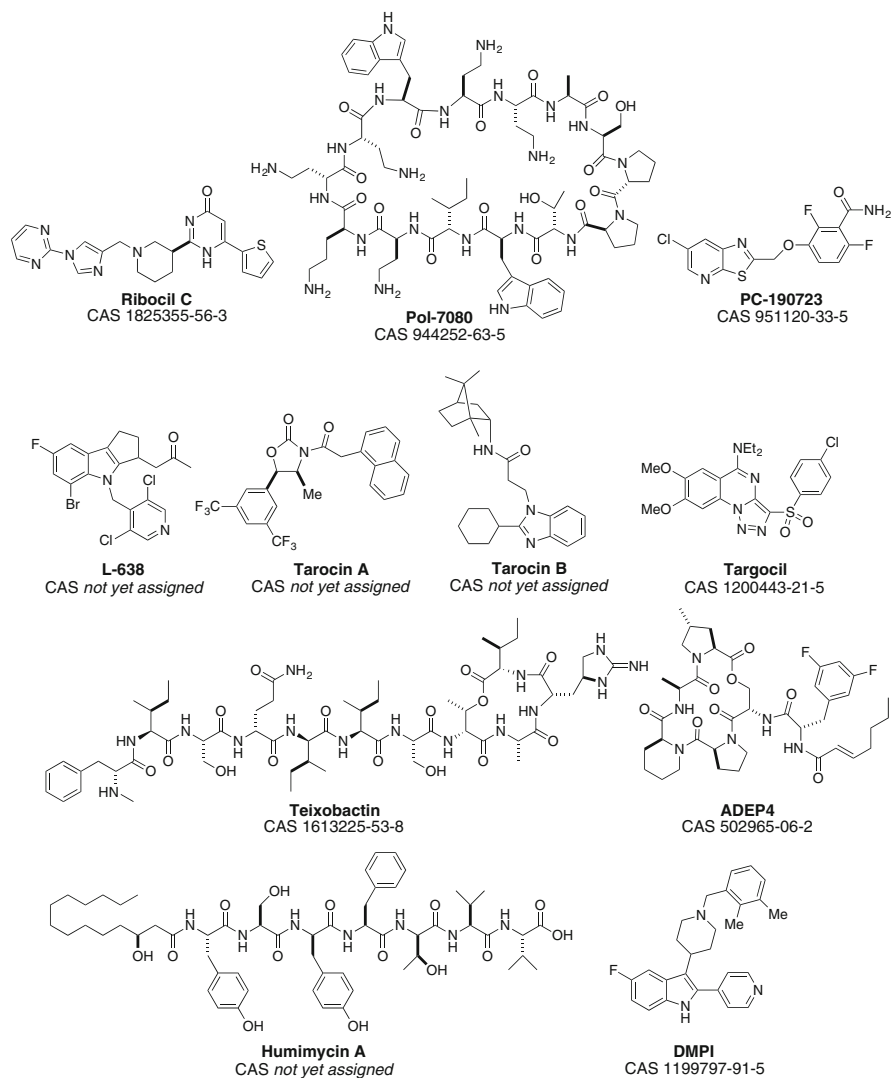
<sup>f</sup>Target pathogen: *S. aureus*

<sup>g</sup>Target pathogen: *S. pneumoniae*

(Table 1) [22–53]. Finally, Level-3 targets are those previously validated in a clinical setting by currently marketed antibacterial therapeutics. Such benchmarks clearly emphasize the enormous objectives sought within and between each level. Considering the sheer number of Level-1 targets that can easily be identified by surveying the scientific literature and perusing publically available databases, as well as the Level-3 targets which facilitate the developing of improved versions of existing agents (i.e., best in class agents [54]) rather than entirely new classes of antibiotics, our review will instead focus largely on new targets approaching or meeting Level-2 objectives.

## 2 Conditional Essentiality: Providing Novel Screens and Cognate Inhibitors to Validate New Druggable Targets

Although the importance of essential gene products serving as antibacterial drug targets is undisputed, the identification of novel antibacterial targets can be significantly expanded from this strict historical definition. Most important is to broaden the conditional context in which gene essentiality is defined. Routinely, gene essentiality is determined under rich nutritional conditions highly optimized for the growth of the pathogen in a laboratory setting but which does not reflect the more extreme conditions a pathogen must overcome during infection. To emphasize this point, large scale gene disruption experiments in *E. coli* identify ~300 genes required for growth on rich medium, whereas >100 additional genes are identified to be essential for growth strictly on minimal growth media [11]. Similar conclusions are drawn in yeast [55]. Indeed, anti-folates such as the early sulfa drugs and later, sulfamethoxazole and trimethoprim target the conditionally essential proteins *folP* and *folA*, respectively, and their activity is suppressed in vitro by exogenous addition of *p*-aminobenzoic acid (PABA) and thymidine [7, 56]. Therapeutic efficacy of these agents is nonetheless achieved because such metabolites are insufficiently low in an infectious setting to suppress the antibiotic effects of these agents. Conversely, exogenous fatty acids are present at sufficient concentrations in the host to support growth of type II fatty acid synthesis null mutant bacteria of the order *Lactobacillales*, including *Streptococcus agalactiae* and *Streptococcus pneumoniae*, illustrating that a precise understanding of the host environment is paramount when selecting metabolite-suppressed targets [57]. Considering the extent of additional biosynthetic pathways in which metabolite suppression is achieved, a robust chemical genetic strategy to identify new antibacterial inhibitors and empirically identify new druggable targets is certainly achievable [56]. One notable example of this approach relates to the discovery of ribocil, a synthetic mimic of the natural metabolite, flavin mononucleotide (FMN), which selectively targets a non-coding mRNA structural element (termed a FMN riboswitch) responsible for gene regulation within the riboflavin biosynthetic pathway [52, 53]. The structure of ribocil C (and other representative compounds discussed in this chapter) are given in Fig. 1. Here, ribocil and its cognate target, the FMN



**Fig. 1** Structures of representative compounds cited in this review

riboswitch, were identified by screening a bioactive compound collection for inhibitors whose bioactivity was specifically suppressed in the presence of exogenous riboflavin supplemented to the growth medium. Despite a conditional essentiality for de novo riboflavin biosynthesis by *E. coli* under in vitro conditions, genetic evidence demonstrates an absolute essential requirement for this metabolic pathway in a murine model of *E. coli* infection which is pharmacologically validated by demonstrating ribocil C provides dose-dependent efficacy in this model [52].

Temperature sensitive (TS) growth phenotypes of gene depletion mutants also offer a simple strategy to consider both a wider array of drug targets and rapidly identify cognate inhibitors. For example, *ltaS* encodes a lipoteichoic acid (LTA) synthase responsible for the biogenesis of this basic cell-wall polymer common to Gram-positive pathogens [58, 59]. Genetic studies in *S. aureus* reveal that *ltaS* is dispensable at 30°C, albeit resulting in severe cell division and morphological defects. However, at elevated temperatures such as 37°C (the physiologically relevant temperature of infection), *ltaS* depletion mutants are not viable [58]. Taking advantage of this TS phenotype, Richter et al. screened for compounds that phenocopy the *ltaS* phenotype and thus inhibit *S. aureus* growth only at the elevated temperature. One such compound resulting from this screen, compound 1771, is proposed to inhibit LtaS by structurally mimicking the phosphatidylglycerol substrate of the synthase [24]. Whereas the above examples of conditional essentiality are straightforward, more innovative strategies to exploit this phenomenon are also possible. One particularly intriguing opportunity relates to bacterial gene essentiality in the context of host innate immunity. For over 50 years, it has been known that the extracellular capsule and diverse *O*-antigen types that coat the surface of Gram-negative bacteria protect these pathogens from the lethal effects of human serum [60–64]. Recently, the Schembri lab has revisited this biology. Using a genome-wide transposon mutagenesis strategy in clinical isolates of *E. coli* they uncovered multiple non-essential genes involved in *O*-antigen biosynthesis and in outer membrane (OM) biogenesis which when genetically inactivated, profoundly sensitize the bacterium to the killing effects of serum [65–67]. The clever exploitation of these (and other) phenotypes that are relevant to the infectious disease setting offers the design of robust cellular screens to identify cognate inhibitors, and so to expand the diversity of new antibacterial targets.

Beyond any particular growth condition and/or environmental context in which gene function is essential, conditional essentiality may also manifest in a unique genetic context. Synthetic lethality (SL) describes such a context in which a gene is dispensable in a wild-type genetic background, but not in a particular mutant background in which another gene has been inactivated [68]. Typically, this phenomenon applies to genes either involved in a common biological process or distinct but interdependent biological processes which partially compensate or “buffer” the loss of the other [54]. The most extensive demonstration of the myriad of intrinsic synthetic lethal genetic interactions within a microbial genome undoubtedly has been characterized in the bakers’ yeast, *Saccharomyces cerevisiae* [68–70]. However, SL is also emerging as an important approach to identifying new antibacterial targets [45, 71–74] as well as mapping genetic interaction networks between a known target (for example, a clinically validated antibiotic drug target) and new targets that if inactivated, enhance the activity of the clinically used antibiotic. Such chemical genetic interaction networks are highly analogous to SL and provide a powerful means to rationally identify cognate inhibitors that are chemically synergistic with the clinical antibiotic, thus offering a compelling combination agent strategy to improve existing antibiotics [54, 75]. An elegant implementation of this strategy has been applied rigorously to methicillin resistant



*S. aureus* (MRSA) and methicillin resistant *S. epidermidis* (MRSE) as a means of restoring potent  $\beta$ -lactam efficacy against otherwise  $\beta$ -lactam resistant *Staphylococci* [47, 75, 76]. Here, a  $\beta$ -lactam genetic interaction network was first identified using antisense interference methodology [77, 78] to genetically deplete gene expression of ~250 possible targets and identify 24 distinct genes, which if partially inactivated render MRSA and MRSE specifically susceptible to  $\beta$ -lactam antibiotics [76]. Interestingly, many of these  $\beta$ -lactam potentiation targets contribute to various aspects of cell-wall peptidoglycan (PG) and wall teichoic acid (WTA) biosynthesis, offering a clear mechanistic basis for their SL when genetically knocked down in expression specifically in the context of sub MIC levels of  $\beta$ -lactams. Additionally, targets involved in other biologically significant processes, most notably cell division (e.g., FtsA, FtsZ, and FtsW), secretion (SpsB), and PG lipid II amidation were also revealed [76, 79]. Finally, the genetic prediction of  $\beta$ -lactam potentiation provided by this genetic interaction network was robustly verified by evaluating the effects of PC190723, a potent and highly selective inhibitor of FtsZ [46] in combination with diverse  $\beta$ -lactam antibiotics and demonstrating striking chemical synergy between these agents in vitro as well as in a murine deep thigh infection model of MRSA [47]. Consequently, the therapeutic context of PC190723 as a single-agent antibacterial lead targeting FtsZ [46] could be expanded into a role as a validated adjuvant, with analogy to  $\beta$ -lactamase inhibitors [80], as a result of its ability to restore the efficacy of  $\beta$ -lactams against methicillin resistant *Staphylococci* albeit through an entirely novel mechanism [47]. Subsequent examples reinforce this view, as demonstrated by the identification of target-specific inhibitors of MurG and MurJ-mediated PG biosynthesis [28, 29] and WTA-mediated biogenesis (see below).

Perhaps the most remarkable genetic context in which conditional essentiality was exploited to identify new antibacterial targets and screening opportunities for cognate inhibitors relates to the phenomenon of an “essential gene paradox.” First identified by Eric Brown and colleagues in both *S. aureus* and *Bacillus subtilis*, inactivation of genes involved in WTA biogenesis displays paradoxical growth phenotypes [81, 82]. Whereas early genes in WTA polymer synthesis are dispensable for growth in vitro, later stage enzymes in the pathway are indispensable for growth and result in a bacteriostatic terminal phenotype [83]. Remarkably, double deletion mutant analysis revealed that genetic inactivation of early stage WTA enzymes suppressed the essentiality of disrupting late stage enzymes in the pathway, perhaps by preventing sequestration of the essential bactoprenyl phosphate lipid carrier which otherwise accumulates in late stage mutants and which is also a shared lipid carrier essential for PG synthesis [50]. Regardless, the unique gene dispensability pattern within WTA biogenesis offers powerful whole-cell based phenotypic screens to identify early and late stage inhibitors of discrete biochemical enzymes within the pathway and their corresponding druggable targets [84]. Whole-cell screens designed to phenocopy the conditional essentiality of late stage lesions in WTA synthesis led to the discovery of targocil [33], targeting the membrane-associated subunit (TarG) of the WTA “flippase” responsible for transporting newly synthesized cytosolic WTA polymer to the cell surface

[85]. Accordingly, targocil is bioactive against wild-type *S. aureus* (including MRSA) but its bioactivity is dramatically suppressed when assayed against *S. aureus* strains deleted of early stage enzymes, such as TarO. Underscoring the robustness of TarG as a druggable target, similar screens have identified multiple new chemotypes with broader Gram-positive bacterial spectrum targeting the WTA transporter [34].

Recently, we have described a chemical suppression-based screen that similarly relies on the opposing gene dispensability pattern of WTA genes to identify inhibitors of early stage WTA enzymes [32]. Here, the entire Merck corporate library was screened for compounds that restored growth of *S. aureus* bacteria that were growth arrested due to the bacteriostatic effect of a TarG inhibitor. Compounds that enable bacterial growth in this context phenocopy the restored growth of WTA double mutants defective in both early and late polymer synthesis and are predicted to target one of the early non-essential WTA biosynthetic enzymes. Two structurally distinct, synthetically derived chemicals named tarocin A and B were identified [32] and demonstrated to inhibit TarO, a glucosyltransferase responsible for the initial step in WTA polymer synthesis and previously demonstrated to be inhibited by the natural product, tunicamycin [27]. Thus TarO is uniquely druggable by both synthetic chemistry and natural products. As TarO is not essential for growth in a wild-type strain background, tarocins are non-bioactive (MIC values  $>256$   $\mu\text{g/mL}$ ). Moreover, tarocins resensitize MRSA and MRSE to a broad diversity of  $\beta$ -lactams in vitro below the clinical breakpoint drug concentration defining  $\beta$ -lactam resistance and provide synergistic efficacy when paired with  $\beta$ -lactams in a murine infection model of MRSA infection [32]. Consequently, tarocins serve as novel and extensively validated non-bioactive adjuvants to pair with such antibiotics that are conceptually highly analogous to  $\beta$ -lactamase inhibitors used to restore  $\beta$ -lactam efficacy against Gram-negative pathogens [80].

### 3 Alternative Approaches to New Target Discovery

Historically, phenotypic screens have been enormously successful in identifying new classes of antibiotics. Best illustrative of this success is the discovery of thienamycin (the progenitor of imipenem and the entire carbapenem class of  $\beta$ -lactams), which was identified over 40 years ago from a natural product screen using a fluorescence-based readout of cell lysis indicative of cell-wall inhibitors [86]. Our reliance on phenotypic screens remains today. Recently, AstraZeneca researchers employed a high-throughput phenotypic screen utilizing the *Citrobacter freundii* AmpC  $\beta$ -lactamase, which when induced in *E. coli* serves as a sensor for inhibition of cell-wall biosynthesis. Screening over 1.2 million compounds against this reporter assay ultimately yielded specific whole-cell active inhibitors targeting LpxH (catalyzing the fourth step in lipopolysaccharide (LPS) biosynthesis) and the lipoprotein outer membrane localization (Lol) complex, LolCDE [25]. Both LPS and bacterial lipoproteins contribute greatly to the

composition of the Gram-negative OM, and the discovery by this approach underscores the functional interrelationship (think synthetic lethality!) between the OM and PG synthesis. Importantly, this work also provides the first reported inhibitors of these essential enzymes [25, 26]. Additional examples of phenotypic screening campaigns discussed above and which similarly identify novel druggable targets and cognate inhibitors emphasize the continued success of this approach [32, 33, 52].

Repurposing existing antibacterial leads in a new therapeutic context breathes new life into old compounds (and targets) but requires novel biological insights to either enhance the activity of the agent or circumvent previously perceived limitations of the antibiotic. The discovery of PC190723 as a  $\beta$ -lactam potentiation adjuvant that restores  $\beta$ -lactam efficacy against MRSA is one example [47]. Another clever example is the repurposing of ClpP inhibitors in the context of chronic *S. aureus* infections mediated by persister cells. Persister cells reflect a small minority of planktonic cells in a bacterial community which are metabolically inactive or dormant [87–89] and consequently resistant to antibiotics whose mode of action is dependent on cell growth. In an elegant series of experiments, Lewis and colleagues demonstrated that the semi-synthetic acyldepsipeptide, ADEP4, which was previously shown to activate ClpP-mediated proteolysis by the bacterial proteasome [43, 90], effectively kills *S. aureus* persister cells within planktonic communities as well as biofilms [42]. To circumvent the unacceptably high frequency of resistance ADEP4 exhibits as a single agent (*clpP* null mutants are highly resistant) it was paired with rifampicin. Remarkably, the ADEP4-rifampicin combination demonstrated complete sterilization of both planktonic and persister cells in a murine chronic infection model of *S. aureus* [42]. Conceptually, activating – rather than inhibiting – the proteasome (or other proteases) provides a compelling new target and general strategy to treat chronic infections refractory to standard antibiotics.

Innovative strategies to optimize chemical libraries for antibacterial activity have also demonstrated significant success. Starting with the host defense antimicrobial peptide protegrin I, researchers at Polyphor have performed iterative synthesis of this starting point for the design and optimization of a library of peptidomimetics with improved antibacterial potency and reduced hemolytic activity [22]. One optimized macrocyclic compound, POL7080, was demonstrated in *Pseudomonas aeruginosa* to inhibit LPS biogenesis by targeting LptD, which functions in the final step of LPS transport to the outer leaflet of the OM [91]. Based on drug resistant mutant mapping studies to the target and significant protein sequence differences between *P. aeruginosa* LptD and orthologs across other Gram-negative pathogens, POL7080 is predicted to display a narrow antibiotic spectrum. However, a clear unmet clinical need for novel and effective narrow spectrum anti-*Pseudomonas* agents undoubtedly exists. Moreover, POL7080 displays impressive nanomolar anti-*Pseudomonas* activity in vitro and robust efficacy against *P. aeruginosa* in a lethal septicemia model of infection, achieving a median effective dose in the range of 0.25–0.55  $\mu\text{g}/\text{mL}$  [22].

Revisiting natural product libraries as a source of new antibacterial leads involves clever methods to growing previously “unculturable” microorganisms,

thereby potentially overcoming the asymptotic inefficiencies of natural product rediscovery currently faced by conventional means [18, 92]. Domesticating such microbes has recently been achieved utilizing a multichannel device (named an iChip) where soil microorganisms are diluted into separate channels and enclosed in a semi-permeable membrane to support diffusion of nutrients and growth when incubated in a soil environment [35]. Screening ~10,000 natural products using this method led to the discovery of teixobactin, an unusual depsipeptide demonstrated to target both lipid II and lipid III precursors of PG and WTA biosynthesis, respectively. Teixobactin displays potent Gram-positive activity, dramatic efficacy in multiple murine infection models, and a highly favorable resistance profile achieved by its dual targeting mechanism. Consistent with teixobactin's unique mechanism, it is structurally distinct from vancomycin and other glycopeptides, lantibiotics, and defensins which solely target lipid II [93]. Unlike proteins encoding an essential enzyme activity, however, lipid II, lipid III, and the FMN riboswitch [52, 53] constitute non-conventional antibiotic targets. Whereas lipid II and III are essentially immutable lipid substrates, substantial mutation-based plasticity likely exists in non-coding RNA structural elements. Other successful antibiotics that interdict non-conventional targets include daptomycin and colistin, which disrupt membrane lipids, and bacitracin, which binds and sequesters the undecaprenylpyrophosphate lipid carrier from which PG and WTA are synthesized and translocated to the cell surface. One cannot help but think other classes of non-conventional antibiotic targets remain to be discovered.

In parallel to these efforts exploiting previously "unculturable" microbes, *in silico* methods have been developed to facilitate the design of new natural products for use as antimicrobials [94]. This approach utilizes bioinformatics to predict natural product structures from primary genomic sequence data and chemical synthesis to create these synthetic-bioinformatic natural products (syn-BNPs), which can then be assayed for antibacterial activity. A major problem with natural product drug discovery is the inability to access all biologically relevant chemical diversity through typical laboratory growth conditions, and this recently discovered method provides one potential solution to this issue. As a test case, Chu et al. show that, using sequence data from human commensals and pathogens, they are able to predict and synthesize a novel class of molecules, dubbed the humimycins, that inhibit the *S. aureus* lipid II flippase [94].

Notwithstanding the current view that *in vitro*-based biochemical high throughput screening (HTS) and downstream optimization of such synthetic chemistry hits has been largely unsuccessful in the search for new antibacterial leads with whole-cell potency [15, 16], there are quite compelling exceptions to this general rule, particularly as it applies to new Gram-positive targets with cognate inhibitors. *In vitro* HTS efforts against multiple isoforms of the CoaD enzyme, involved in the synthesis of the essential cofactor, coenzyme A (CoA), combined with structure-based optimization efforts has recently led to the discovery of a highly potent series of antibacterials with broad Gram-positive spectrum, *in vivo* efficacy across multiple models of infection, and an acceptably low frequency of resistance [41]. Although the further drug optimization of these compounds addressing

solubility and tissue penetration was not achieved, both CoaD and the CoA biosynthetic pathway were rigorously validated and offer the potential for the discovery of new series with superior physicochemical properties.

## 4 Structural Biology Advances Driving Target Discovery

Targeting OM biogenesis factors of Gram-negative bacteria remains a highly attractive, yet underexploited approach in novel antibacterial drug discovery. The OM is an asymmetric bilayer composed of phospholipids in the inner leaflet, LPS in the outer leaflet, OM beta-barrel proteins (OMPs) integrated within the bilayer, and lipoproteins anchored to the inner leaflet [95]. Because the OM is essential, inhibiting its assembly by intervening in lipoprotein,  $\beta$ -barrel protein, or LPS biogenesis will compromise the viability of the cell. Recent structural data for proteins involved in OM biogenesis helps to prioritize such targets. A recently solved *P. aeruginosa* co-crystal structure of LspA, the signal II peptidase responsible for processing of lipoproteins, with its cognate inhibitor globomycin [40] serves as a significant starting point for rational drug design against this target. The  $\beta$ -barrel assembly machine (BamABCDE) and the LPS-transport subcomplex located at the OM (LptDE) are particularly attractive targets because they are not only druggable enzymes [22, 23] but also contain surface-accessible essential proteins. The principal difficulty with discovering Gram-negative antibacterial leads is identifying compounds that can cross the robust barrier created by the LPS layer and avoid efflux once inside the cell; targeting a surface-exposed protein would circumvent these issues. Additionally, targeting the LPS assembly machine (LptDE) would not only kill the cell, but also permeabilize the OM to other agents that normally have a difficult time traversing the membrane [22].

Bam complex structural data now provide significant insight into the mechanics behind  $\beta$ -barrel protein assembly into the OM in Gram-negative bacteria. The recently solved BamACDE crystal structure overlaid with a previously solved BamAB subcomplex crystal structure permitted the first structural model of a fully assembled Bam complex from *E. coli* [96]. This BamABCDE structure confirmed previously reported interactions amongst the Bam components as well as revealed new interactions and Bam protein conformations to allow for speculation of a mechanism of  $\beta$ -barrel assembly. Of note, Bakelar et al. found that when the lipoprotein subcomplex BamCDE binds, the essential  $\beta$ -barrel component BamA undergoes a conformational change opening the exit pore and lateral gate in the barrel. This opening may serve to destabilize the membrane locally (near the lateral gate) to allow for OMP insertion through reduction of the kinetic barrier, rather than the threading of nascent OMPs through the lumen of the barrel and out the lateral gate [97], since the BamA N-terminal soluble POTRA domains occlude the lumen of the barrel when the exit pore and lateral gate are open in this crystal structure. Recent genetic studies demonstrating that periplasmic components of the assembly process interact with substrate after much of the  $\beta$ -barrel has formed also

support this mechanism of OMP insertion [98]. Another recent study has demonstrated that the only essential lipoprotein in the Bam complex, BamD, can be targeted with a peptide that mimics a substrate protein to which BamD normally binds in the assembly process, validating the druggability of this complex [23]. Understanding the critical points of interaction amongst the Bam components and movement of the Bam machine should enable the discovery of additional inhibitors of OM  $\beta$ -barrel protein assembly.

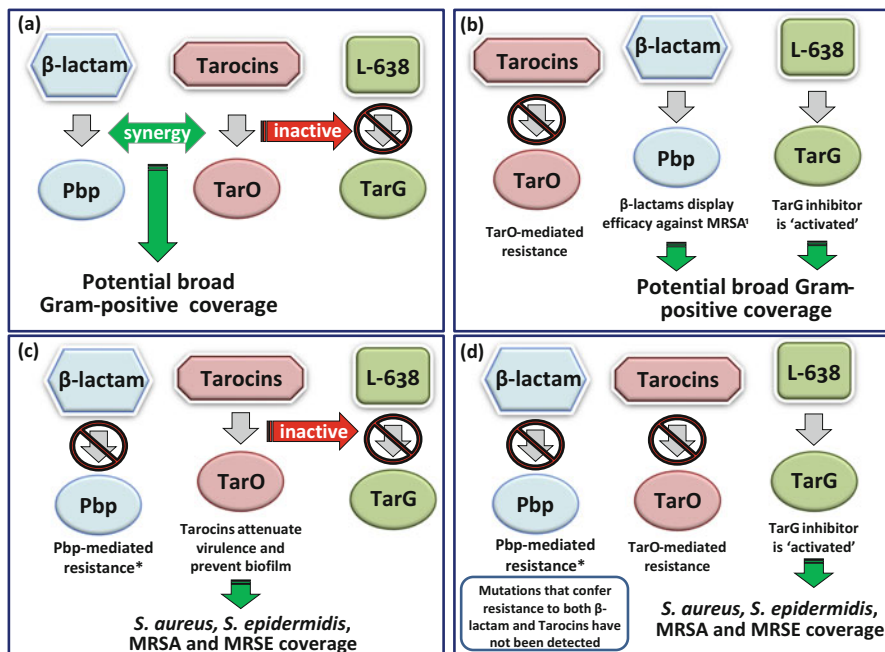
Like recent advances in the Bam complex structure dataset, the first crystal structures of the LPS-assembly subcomplex LptDE have shed light on a mechanism of LPS insertion into the outer leaflet of the OM [99, 100]. The crystal structures revealed a  $\beta$ -jellyroll N-terminal domain of LptD and an enormous 26- $\beta$ -stranded C-terminal barrel domain, the largest  $\beta$ -barrel discovered to date. The barrel contains two lobes, one adjacent to the N-terminal domain and one occupied by the essential lipoprotein LptE. LptE not only acts as a plug in the barrel, but also plays a role in LptD assembly as well as LPS assembly [101, 102]. Based on crystallographic and genetic data, the authors speculate that the hydrophilic portion of LPS (*O*-antigen and core sugars) traverse the lumen of the open lobe of the barrel, while that the lipid component is shielded from the aqueous periplasm by the N-terminal domain of LptD and shuttled through a lateral gate opening between the first and last  $\beta$ -strands of LptD, ensuring specific insertion into the outer leaflet of the OM [99]. Blocking this lateral gate with a peptide or small molecule may be one way to disrupt the function of this essential LptDE translocon. Validation of this hypothesis would highlight how these structural advances can facilitate design of novel antimicrobials.

## 5 Considering Antibacterial Drug Resistance as Contextual

A general theme to most new targets and cognate antibacterial leads highlighted in this review is their propensity for target-based drug resistance (Table 1). Often, this resistance likely reflects their single-target mode of action [103, 104]. We are also mindful of the disastrous impact drug resistance can have on antibacterial clinical development [105]. The likelihood that acceptable resistance profiles for Level-2 targets described here are achievable, however, either by structure-based design to improve and/or change drug-target binding contacts and/or increase potency should be considered on a case by case basis. One such example is that of antibiotic 2, a non- $\beta$ -lactam inhibitor which not only inhibits the classic targets of penicillin, PBP1, but also allosterically inhibits the target responsible for  $\beta$ -lactam resistance in MRSA and MRSE, PBP2a [31]. It is also appropriate to be mindful that in vitro-based resistance studies may not always reflect the prevalence of resistance in an infectious setting. The broad-spectrum  $\beta$ -lactam mecillinam serves as an important example of the potential paradoxical resistance profiles of an antibiotic observed by in vitro testing versus that encountered in a clinical setting. Multiple different mecillinam resistance mechanisms in *E. coli* are commonly identified in vitro,

ranging from target-based (*pbpB*) mutations to other processes including cell-wall synthesis, cell division, tRNA synthetases, and the ppGpp stringent response pathway [106]. Conversely, mecillinam-resistant *E. coli* from patients treated for a urinary tract infection are very rarely identified and reflect a single type of mutation: inactivation of *cysB*, a gene involved in cysteine biosynthesis [106]. Whereas all mutant classes selected in vitro share similar fitness costs, the *cysB* mutations uniquely lack a fitness cost in a more relevant urine-rich growth condition. Thus amongst a broad set of mutations that can confer mecillinam resistance under standard in vitro growth conditions, only *cysB* mutants are sufficiently fit to potentially persist in the urinary tract. Moreover, considering the high exposure level of the drug in the urine, few of these mutants cause resistance to the antibiotic in a clinical setting, likely because high mecillinam levels sufficiently impact the fitness of the pathogen in an environment where robust growth of the pathogen is required to offset their natural expulsion from the urinary tract. Therefore, understanding drug resistance in a more therapeutically relevant context is critical to avoid the risk of potentially deprioritizing new targets and antimicrobial leads solely based on their in vitro resistance profile.

It is also interesting to consider how a fundamental understanding of the genetic interactions within a single biological process combined with the discovery of multiple inhibitors to distinct targets within such a process can be leveraged in a systems biology-based combination agent strategy to mitigate drug resistance. Consider the WTA biosynthetic pathway (Fig. 2) [34, 48, 107, 108]. Tarocins restore the efficacy of  $\beta$ -lactams against MRSA with target-based resistance mapping to *tarO* [32]. Addition of a TarG inhibitor as a third component to this combination substantially reduced *tarO*-mediated resistance [32]. However, mechanistically this is not achieved by simply adding another antibiotic since the growth inhibitory activity of the TarG inhibitor is robustly suppressed by TarO inhibitors (Fig. 2a). Instead, in this three-way combination context the TarG inhibitor is inactive against the bacterial population sensitive to a tarocin-dicloxacillin combination (where TarO is inhibited) and only bioactive against tarocin-resistant *tarO* mutants (i.e., target-based mutations) (Fig. 2b) in the population that maintain TarO functional activity [33, 34]. Conversely, pre-existing *tarO* loss-of-function mutations which would suppress the activity of the TarG inhibitor are broadly and highly sensitive to  $\beta$ -lactams [27, 34] as well as strikingly attenuated in virulence across diverse animal infection studies [34, 108–110] (Fig. 2c). Consequently, such a three component combination therapeutic elegantly exploits a circuitry of genetic interactions and antibiotic hypersensitivities within the cell-wall network as well as avirulent phenotypes of *tarO* mutants to provide an integrated and interdependent means of mitigating target-based resistance of the  $\beta$ -lactam potentiator.

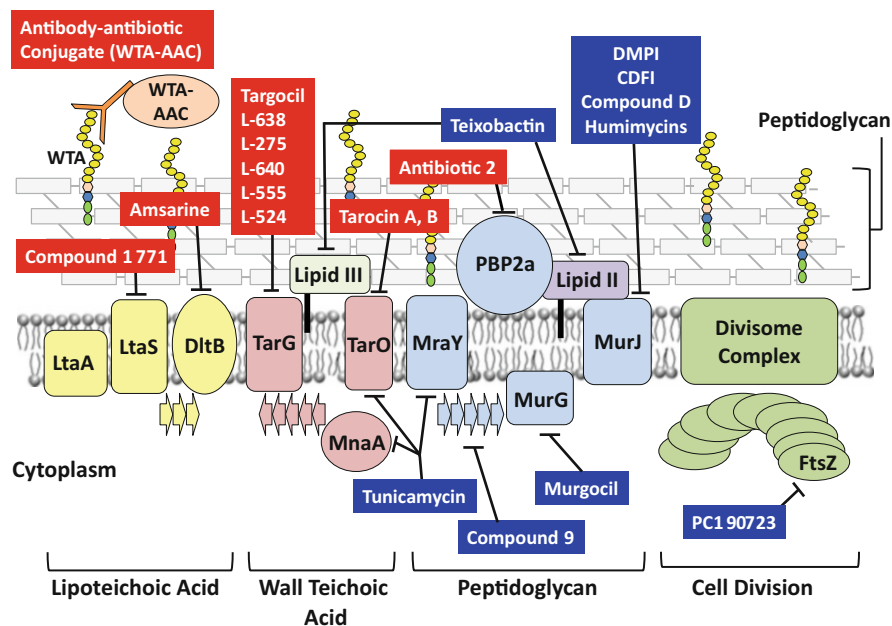


**Fig. 2** Triple combination strategy provides an interdependent mechanism of mitigating target-based resistance. The illustration depicts multiple scenarios in which of a triple combination of TarO and TarG inhibitors paired with a β-lactam antibiotic overcomes various potential mechanisms of resistance. **(a)** Synergistic activity of tarocin and β-lactam re-sensitizes β-lactam-resistant Gram-positive bacterial pathogens to provide broad Gram-positive antibacterial coverage; simultaneously inactivating L-638 such that it is non-bioactive in this context. **(b)** In this scenario, acquisition of TarO target-mediated mutations will confer resistance to tarocin but consequently “activate” L-638 antibacterial activity to re-establish broad Gram-positive coverage by the combination cocktail. In addition, Sakoulas et al. have demonstrated that β-lactams (e.g., nafcillin) enhance innate-immune mediated killing of MRSA despite its elevated MIC to the antibiotic [107]. **(c)** In this scenario, acquisition of Pbp target-mediated mutations that confer resistance to the β-lactam (a very rare event\*) may occur but still allows for tarocin to inhibit WTA synthesis, which has been demonstrated to reduce virulence and biofilm formation of methicillin-sensitive *S. aureus* (MSSA), MRSA, and MRSE during infection [34, 48, 108]. **(d)** In this final scenario, resistance that may arise due to mutations to both Pbp and TarO targets (an extremely rare event) also activates L-638 antibacterial activity MSSA, MRSA, and MRSE as well as potentially providing broader Gram-positive coverage

## 6 Target Discovery Parallels Between Gram-positive WTA and Gram-negative OM Biogenesis

Recent progress made in the discovery of Level-2 targets participating in Gram-positive WTA biosynthesis also serves as an instructive example for how new targets may similarly be discovered in other critical biological processes, particularly OM biogenesis amongst Gram-negative pathogens (Fig. 3) [111–113]. Central





**Fig. 3** (a) Select Gram-positive cell-wall associated targets and cognate inhibitors. Representative diagram of a prototypical *S. aureus* bacterial cell surface displaying color-coded biologically relevant biosynthetic pathways: lipoteichoic acid (yellow); wall teichoic acid (salmon); peptidoglycan (blue); cell division (green). New antibacterial targets and cognate inhibitors described in the main text and Table 1 are highlighted. The potential antimicrobial spectrum for the reported inhibitors is designated: potential broad Gram-positive spectrum (red box); potential broad Gram-positive and Gram-negative spectrum (blue box). (b) Select Gram-negative OM-associated targets and cognate inhibitors. Representative diagram of Gram-negative bacteria and their relevant biosynthetic pathways: The Lpt pathway (in salmon), Lipoprotein processing and assembly (yellow), and OMP assembly (purple). Much of the machinery used in the biosynthesis of the peptidoglycan is conserved among Gram-negatives and Gram-positives (see (a) for potential targets). In Gram-negatives, tunicamycin has been demonstrated to additionally target WecA, an IM protein involved in the biosynthesis of *O*-antigen [111]. Potential Gram-negative spectrum targets (green boxes) and potential broad Gram-positive and Gram-negative spectrum (blue boxes) are indicated [112, 113]. *OM* outer membrane, *IM* inner membrane, *PG* peptidoglycan, *PL* phospholipid, *LPS* lipopolysaccharide, *OMP* OM  $\beta$ -barrel protein

to this success is a deep functional understanding of WTA biogenesis and cell surface assembly from a genetic, biochemical, structural, and pathogenesis perspective [83, 108, 114] and from which an integrated systems biology mindset can be applied. Discovery of robust WTA Level-2 targets such as TarG and TarO, whether “essential” or “non-essential” are actually conditionally essential in the context of an unorthodox gene dispensability pattern and  $\beta$ -lactam exposure [115]. Exploiting WTA genetics provides elegant whole-cell target-based screens to efficiently identify target-specific inhibitors of the pathway [32–34] as well as other biochemical pathways impinging on WTA biogenesis (UppS) [50, 51] or by leveraging synthetic lethal interactions within the WTA genetic interaction network

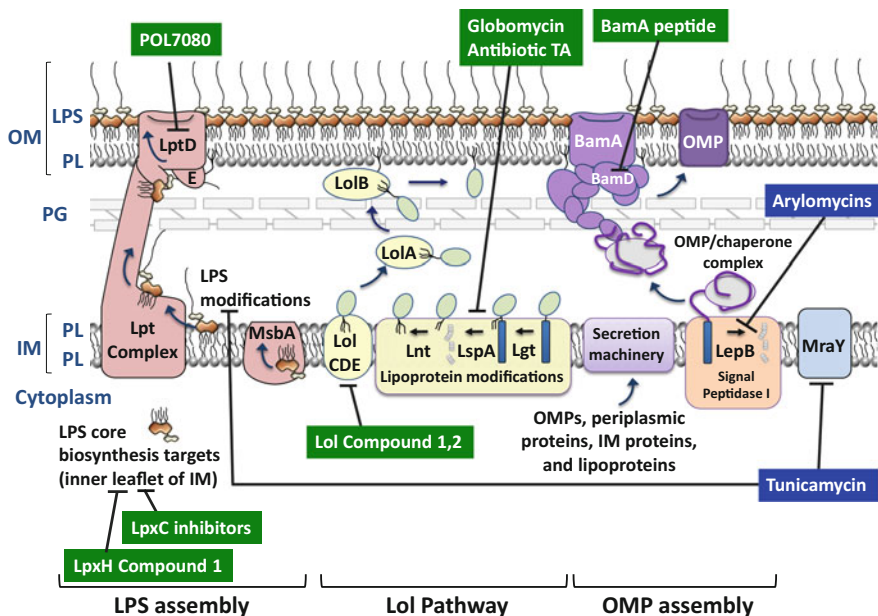


Fig. 3 (continued)

(DltB) [45]. Such targets and cognate inhibitors can also be considered as adjuvants for developing synergistic antibiotic combination agents from a rational biology-based perspective [32, 47] and mindful of virulence phenotypes that may augment efficacy. Finally, entirely new anti-infective approaches may be derived from such a fundamental understanding of WTA biology, as elegantly shown by Lehar et al. at Genentech who report an efficacious WTA antibody-antibiotic conjugate to target intracellular reservoirs of *S. aureus* associated with chronic infections [36].

Similarly remarkable advances in our understanding of Gram-negative OM biogenesis have also emerged over the last decade. Beyond the fundamental architecture and composition of the OM, we are gaining a deep functional understanding of the distinct biological assembly processes (i.e., Bam, Lpt, Lol, LPS, PG, capsule, and stress response signaling pathways) [116–126] contributing to its biogenesis and homeostasis as well as their functional interconnectivity [127, 128]. Synthetic lethal-based genetic strategies are also being employed to identify new OM targets such as LpoA and LpoB, two PBP accessory proteins central to PG biogenesis [71, 129], as well as to map genetic interactions within and between these biological processes [72, 102, 130, 131]. Such synthetic lethal interactions could be exploited to develop whole-cell pathway-based screens for novel OM biogenesis inhibitors. Parallels between the WTA essential gene paradox and analogous genetic dispensability patterns in *O*-antigen biogenesis also exist [132], suggesting similar whole-cell screening opportunities to identify inhibitors of *O*-antigen assembly are possible. Finally, recent work re-emphasizing the

importance of *O*-antigen and other aspects of the OM in protecting *E. coli* from the lytic effects of human serum provide exciting new avenues of conditionally essential targets and screens to impair Gram-negative virulence [66, 67].

## 7 Conclusions

Table 1 summarizes multiple new antibacterial targets discovered in recent years that approach or satisfy Level-2 criteria of (1) bioinformatics-based pathogen spectrum and target-based cytotoxicity predictions; (2) druggable with cognate inhibitor(s) identified with compelling MOA validation; and (3) pharmacological and/or genetic demonstration that target inactivation provides efficacy in a relevant animal model of infection. Surveying this list illustrates a number of emerging trends. For example, many of the druggable targets are multi-spanning membrane proteins localized to the cytoplasmic membrane in Gram-positive bacteria, or resident in the periplasm or OM of Gram-negatives where they functionally serve as biosynthetic enzymes or transporters involved in cell-surface biogenesis. In part, their druggable nature likely reflects their cell-surface location and ability of small molecules to engage such targets without confronting cell permeability and/or efflux issues. The highly hydrophobic nature of such druggable targets does however “select” for cognate inhibitors with high cLogP values and physicochemical properties incompatible with high solubility and drug-like properties [20]. Multi-spanning membrane proteins are also highly challenging from the perspective of target X-ray crystal structure determination, compound co-crystallization, and hence structure-based design and compound optimization. In this way, bacterial druggable targets resemble the majority of known therapeutic targets in human disease (e.g., G-protein coupled receptors, ion channels, and other cell-surface targets), emphasizing the need for technical improvements and greater focus towards X-ray crystallography of complex bacterial membrane proteins. It is also evident that the level of small molecule MOA validation in many of these studies can vary considerably and mechanistic evidence in a whole-cell context is often overly weighted by phenomenological evidence rather than direct target engagement within the cellular milieu. We suggest that in addition to in vitro-based biochemical studies and structural biology evidence, isolation and characterization of causal drug resistant mutations are critically needed to unambiguously validate the MOA of cognate inhibitors of such privileged antibacterial drug targets. Evident also in Table 1 are Level-2 targets with often an unattractive frequency of resistance observed by cognate inhibitors. However, with few exceptions [22] these are lead candidate molecules, not pre-clinical or clinical candidate therapeutics. Considerable medicinal chemistry optimization is required and substantial attrition is certain. Identifying new series with more favorable resistance profiles is also possible and often warranted considering the importance of such targets. Identifying more dual-target opportunities would also likely mitigate resistance development [35, 44, 103, 104]. A greater understanding of drug resistance in a relevant infectious setting

as well as potential genetic interaction circuits that can be pharmacologically interdicted to mitigate drug resistance also deserves greater consideration. Finally, a survey of Table 1 emphasizes a strong bias towards Gram-positive targets meeting Level-2 criteria despite the urgent need for new Gram-negative antibacterials. Perhaps in small part this reflects a lag time required to catch up to a growing government, industry and (and importantly) clinical perspective collectively shifting focus to addressing Gram-negative pathogens in recent years. In large part, however, this asymmetry is based on the OM barrier and extensive efflux pumps shared by Gram-negative bacteria that thwart the entry and concentration of potent and selective inhibitors; hence compounding the difficulty to identify and validate druggable targets [20, 133]. The recent commitments made by the Pew Charitable Trust, Wellcome Trust, BARDA, NIAID, and most recently CARB-X to fund research centered around OM biogenesis, small molecule permeability, and drug efflux is timely and much needed to address this fundamental issue.

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